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Published in:
Neurosurgery

DOI:
10.1227/NEU.0b013e318232dedd

2012

Link to publication

Citation for published version (APA):
Bexell, D., Gunnarsson, S., Svensson, A., Tormin, A., Henriques-Oliveira, C., Siesjö, P., Paul-Visse, G., Salford, L., Scheding, S., & Bengzon, J. (2012). Rat Multipotent Mesenchymal Stromal Cells Lack Long-Distance Tropism to 3 Different Rat Glioma Models. *Neurosurgery*, *70*(3), 731-739. https://doi.org/10.1227/NEU.0b013e318232dedd

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10

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Rat multipotent mesenchymal stromal cells lack long-distance tropism to three different rat glioma models

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ACKNOWLEDGMENTS

We thank professor Olle Lindvall for generously providing laboratory facilities. Dr. Cecilia Lundberg is thanked for valuable comments on the manuscript. This work was supported by

the Swedish Cancer Society, the Swedish Childhood Cancer Foundation, Hans and Märit Rausing Charitable Trust, the Segerfalk, the Crafoord, Elsa Schmitz, Magnus Bergvall and Lund University Hospital Foundations, the Gunnar Nilsson Cancer Foundation and the Royal Physiographic Society in Lund.

ABSTRACT

Background: Viral gene therapy of malignant brain tumors has been restricted due to the limited vector distribution within the tumors. Multipotent mesenchymal stromal cells (MSCs) and other precursor cells have shown tropism for gliomas and these cells are currently being explored as potential vehicles for gene delivery in glioma gene therapy.

Objective: To investigate MSC migration in detail following intratumoral and extratumoral implantation using syngeneic and orthotopic glioma models.

Methods: Adult rat bone marrow-derived MSCs were transduced to express enhanced green fluorescent protein and implanted either directly into or at a distance to rat gliomas.

Results: We found no evidence of long-distance MSC migration through the intact striatum towards syngeneic D74(RG2), N32 and N29 gliomas in the ipsilateral hemisphere, or across the corpus callosum to gliomas located in the contralateral hemisphere. Following intratumoral injection, MSCs migrated extensively, and specifically, within N32 gliomas. MSCs did not proliferate within tumors, suggesting a low risk of malignant transformation of *in vivo* grafted cell vectors. Using a model for surgical glioma resection, we found that intratumorally grafted MSCs migrate efficiently within glioma remnants following partial surgical resection.

Conclusion: The findings point to limitations for the use of MSCs as vectors in glioma gene therapy although intratumoral MSC implantation provides a dense and tumor specific vector distribution.

Keywords: glioma, tumor, mesenchymal stem cell, multipotent mesenchymal stromal cell, gene delivery

Short title: MSCs for glioma gene therapy

BACKGROUND

Glioblastoma multiforme (GBM) is an aggressive and invasive neoplasm in the brain and this tumor remains one of the most lethal forms of human cancer. Less than 3% of the 77,000 patients diagnosed each year with GBM in the USA and Europe will survive more than 5 years. GBM infiltrates crucial structures in the brain, preventing curative surgical resection. Radiation and chemotherapy offer only modest benefits and remain essentially palliative [1]. Gene therapy using viral vectors to target malignant gliomas is a potentially promising approach to improve GBM treatment. However, clinical trials have had only very limited success so far. This is mainly due to inefficient spread of viral vectors *in vivo*, and effective and sustained gene delivery into brain tumors still presents a major obstacle [2].

Implanted stem and precursor cells have emerged as an alternative vector system for gene delivery to gliomas and other tumors. Malignant gliomas have been reported to attract implanted and injected multipotent mesenchymal stromal cells (MSCs), neural precursor cells (NPCs), endothelial cells, and hematopoietic stem and progenitor cells [3-7]. The previously reported capacity of these cells to track and home to malignant brain tumors would make these cells potentially more efficient than viral vectors for local delivery of therapeutic tumoricidal substances [8].

MSCs have been reported to migrate throughout normal brain parenchyma towards brain tumors following implantation at a distance from tumors in the ipsi- or contralateral hemisphere [3, 6]. However, a detailed analysis of the migratory capacity of adult MSCs, syngeneic to both glioma and to host tissue, has not yet been reported, but is certainly critical because the concept of using cells as gene vectors to tumors raises and falls with their tumor-tropic migratory capacity.

Therefore, we implanted adult rat bone marrow-derived MSCs either directly into or at a distance to orthotopic and syngeneic rat gliomas and investigated MSC

intratumoral infiltration as well as MSC migration toward three different glioma models. We provide striking alternative findings compared to previous reports using MSCs as therapeutic delivery vehicles.

OBJECTIVE:

(1) To describe MSC migration in detail following intratumoral and extratumoral implantation to experimental syngeneic and orthotopic gliomas; (2) to investigate proliferation rates of implanted MSCs within gliomas, and (3) to elucidate MSC migratory pattern within glioma tissue following partial surgical tumor resection.

METHODS:

Rat glioma cell lines

The rat glioma cell lines D74(RG2), N32 and N29, syngeneic with the Fischer 344 rat were originally induced by transplacental injection of ethyl-*N*-nitrosourea to a pregnant rat whose offspring developed malignant brain tumors [9, 10]. N29 and N32 tumor cells were maintained in R10 medium, consisting of: RPMI 1640 medium (1X) with L-glutamine supplemented with 10% fetal bovine serum (FBS) (VWR, West Grove, PA), 10 mM HEPES buffer solution, 1 mM sodium pyruvate and 50 mg/ml gentamicin. D74 cells were maintained in D-MEM medium with 10% FBS and 50 mg/ml gentamicin (all chemicals except FBS from GIBCO, Invitrogen, Carlsbad, CA). Cells were detached using trypsin-EDTA (0.25% trypsin with EDTA 4Na) 1X (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere containing 6.0% CO_2. Before inoculation *in vivo,* cells were washed and resuspended in medium without FBS and gentamicin (referred to as R0 medium).

Establishment and culture of bone marrow-derived rat MSCs

MSC cultures were derived from bone marrow of a Fischer 344 male rat (8 weeks old) as previously described [11]. Briefly, MSCs were generated by adherent culture of Ficoll-isolated nucleated bone marrow cells in NH expansion medium (Miltenyi Biotech, Bergisch Gladbach, Germany) or minimum essential medium-alpha supplemented with 10% fetal bovine serum and 1% Antibiotic-Antimycotic-Solution (Sigma-Aldrich, St. Louis, MO). Non-adherent cells were removed after three days and culture medium was changed weekly thereafter.

eGFP retroviral production and transduction of MSCs

In order to visualize the MSCs, cells were genetically modified to express eGFP. MSCs were transduced with a Moloney leukemia based-retroviral vector, which has the characteristic of infecting dividing cells. The Moloney leukemia retroviral vector pCMMP-IRES2eEGFP-WPRE used in this study has been described elsewhere [12]. The viral particles were produced using the producer cell line 293VSVG [13]. Concentrated particles were resuspended into 0.5 ml of DMEM medium (Sigma). The titer was measured by FACSCalibur analysis, based on eGFP reporter gene expression, 3 days after infection of the HT1080 cells and varied from $0.7x10^9$ to $1.2x10^9$ TU/ml depending on the batches. When at 60-70% confluency, MSCs were transduced at a multiplicity of infection of 5. To increase transduction efficiency, protamine sulfate was added to the medium at a final concentration of 1 mg/ml (Sigma). More than 90% of the cells expressed eGFP as assessed in an inverted microscope 4 days following transduction.

Animal procedure and experimental design

Adult male Fischer 344 rats (8-9 weeks old, from Scanbur, Stockholm, Sweden) were used. Animal procedures were approved by the Ethical Committee for Use of Laboratory Animals at Lund University, Sweden. Rats were anaesthetized with Isoflurane (2.5% in O_2, Forene) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The following coordinates were used for tumor cell inoculation into the right striatum (relative to bregma): anterior-posterior (AP): +1.7, medial-lateral (ML) ī 2.5 and dorso-ventral to dura (DV) ī 5.0. The tooth bar was set at ī 3.3 mm. Coordinates for tumor cell inoculation into the corpus callosum were: anterior-posterior (AP): +0.7, medial-lateral (ML) ī 2.0 and dorso-ventral to dura (DV) ī 3.0. The tooth bar at ī 3.3 mm. Tumor cells were inoculated at 1 ε l/min, using a 10 ε l Hamilton syringe. MSCs were grafted at 0.5 ε l/min, using a 10 ε l Hamilton syringe with a glass micropipette attached to the needle tip. After cell inoculation, the micropipette was kept in place for 5 minutes before being slowly retracted.

For studying MSC intratumoral infiltration, 3000 N32wt tumor cells were inoculated into the right striatum of male rats (*n* = 14) on day 1. eGFP+ MSCs (2.5 x 10^5 cells) suspended as single cells in 5 µl cell medium, were grafted on day 14 using the same coordinates as for tumor cell inoculation. Animals were sacrificed on day 15, 18 and 22 following tumor cell inoculation which correlates to day 1, 4 and 8 following MSC grafting (*n* = 4-5 each time-point).

For examination of proliferation rates of grafted MSCs, eGFP+ cells were grafted intra-tumorally into established N32 intracerebral tumors (*n* = 3). Animals were sacrificed on day 8 or on day 16 following MSC grafting.

In the experiment studying MSC migration throughout the normal brain parenchyma toward a distant malignant brain tumor, we established D74 (RG2; 3000 cells), N32 (1000 cells) and N29 tumors (3000 cells) in the right striatum. Seven days later, eGFP+

MSCs (2.5 x 10^6 cells, n = 5 in each group) were grafted 4.0 mm caudally and 2.0 mm lateral to the inoculation coordinates of the tumor. Animals were sacrificed on day 22 following tumor cell inoculation corresponding to day 14 following MSC grafting. In a parallel experiment, D74 (RG2; 3000 cells), N32 (1000 cells) and N29 tumors (3000 cells) were injected into the right medio-frontal corpus callosum. Seven days later, eGFP+ MSCs (2.5 x 10^6 cells, n = 5 in each group) were grafted into the corpus callosum at corresponding coordinates on the contralateral (left) side. Animals were sacrificed on 21 days following tumor cell inoculation.

To study long-time survival and migration in the adult normal brain, eGFP+ MSCs were grafted into either the striatum, (n=4), or the corpus callosum, (n=4), of non-tumor bearing animals. Animals were sacrificed on day 118 following MSC grafting (Fig. 1B). The brain, the liver, the spleen and the cervical lymph nodes were analyzed for presence of eGFP+ MSCs.

In order to study migration of grafted MSCs in gliomas following surgical resection, 3000 N32 glioma cells were inoculated into the striatum on day 1 (n=5). Fourteen days later, established gliomas were partially resected. Subsequently, 2,5 x 10^5 GFP+ MSCs were inoculated directly into the remaining tumor mass. Animals were sacrificed 7 days later and the brains were analyzed for presence of eGFP+ MSCs.

Immunohistochemistry

The rats were deeply anaesthetized and perfused through the ascending aorta with PBS, pH 7.4, followed by cold 4% paraformaldehyde (PFA) in PBS. The brains were removed and postfixed in cold 4% PFA overnight and then transferred to 20% sucrose solution in PBS. Transversal sectioning of the brains was performed on a freezing microtome (40 µm) and the sections were put in anti-freeze solution. Free-floating sections were rinsed three times in

KPBS (potassium phosphate-buffered saline). Sections were blocked with 5% normal goat serum (NGS) and 5% normal donkey serum in 0.25% Triton X-100 solution and then incubated with the primary antibodies chicken anti-GFP (1:1500, Chemicon, Temecula, CA), mouse anti-Ki67 (1:50, Novocastra, Newcastle upon Tyne, UK), mouse anti-NG2 (1:500, Chemicon), rabbit anti-Ŭ-sma (1:400, Abcam) at 4°C over night. The next day the sections were rinsed three times in appropriate sera in KPBS and incubated for 2 hours with one or two of the following secondary antibodies: Alexa488 goat anti-chicken (1:400, Molecular Probes), Alexa594 goat anti-rabbit (1:400, Probes) or Cy3 donkey anti-mouse (1:400, Jackson). The sections were counterstained with Hoechst nuclear staining to visualize tumors, mounted onto glass slides and cover-slipped with DABCO mounting medium.

Image analysis

Sections were analyzed with an Olympus TX60 light microscope (Olympus, Tokyo, Japan), an Olympus BX60 epifluorescence microscope or by confocal laser scanning microscopy (Leica Microsystems, Mannheim, Germany).

Statistical analysis

Studentôs unpaired *t*-test was used for comparison between groups. Data are presented as means ± standard error of the mean (SEM) and data are considered significant at $p<0.05$.

RESULTS

Extensive and tumor-specific intratumoral MSC migration

We have previously characterized the MSCs used in the present study by their adherent growth, surface marker profile and by their differentiation capacity. Using FACS we have shown that the MSCs display the mesenchymal markers CD73, CD90 and CD105 but not the

hematopoietic markers CD34 and CD45. Thus, the MSCs express MSC-associated markers and no HSC-associated markers. Furthermore, the MSCs possess the capacity to differentiate into osteoblasts and adipocytes upon exposure to conditions promoting differentiation [11]. In the present study, adult rat bone marrow-derived MSCs were found to be positive for the mesenchymal markers desmin, CD73 and CD90 by immunocytochemistry (**Fig 1a**).

In order to determine the capacity of MSCs to migrate within gliomas following intratumoral implantation, we established N32 gliomas in the striatum *(n* = 14). MSCs, transduced to express eGFP, were grafted into tumors and the migration pattern was assessed at three time-points (*n* = 4-5 for each time-point) following grafting. On *day 1 post grafting*, eGFP+ MSCs were found within a well-defined elongated cluster at the inoculation site adjacent to or surrounding the tumor. Single MSCs were located in the outer periphery of the tumor, or in the normal brain parenchyma, but the absolute majority of the grafted cells were seen at the inoculation site (**Fig 1b**). Already on *day 4 post grafting*, numerous MSCs were found within the peripheral zone of the tumor (**Fig 1b**). Single MSCs were also found in the core of the tumor. On *day 8 post grafting*, abundant numbers of MSCs were located in both tumor periphery and core (**Fig 1b**). The vast majority of MSCs were located within the graft or within the tumor, and only a few numbers of MSCs were found in the normal brain parenchyma at any time-point.

No evidence of ongoing proliferation of MSCs *in vivo*

Confocal microscopy analysis of eGFP and Ki67 expression was used to investigate cell-cycle state of MSCs grafted into N32 malignant brain tumors. Ki67 is a marker of cells in G1, S, M and G2 cell-cycle phases, i.e. dividing cells. MSCs were grafted into established N32 tumors and analyzed on day eight and day 16 following grafting. One hundred eGFP+ cells in each tumor model were randomly chosen and analyzed by confocal microscopy for expression of

Ki67. Grafted eGFP+ cells located within the core of the graft as well as migratory eGFP+ cells located intra-tumorally but far away from the graft core were analyzed. Proliferating tumor cells expressing Ki67 were used as positive controls. We did not find a single eGFP+ MSC that expressed Ki67. Representative examples of non-Ki67 expressing eGFP+ MSCs and within highly proliferative tumors are shown in **Fig 2a and b**. We conclude that the absolute majority of intratumorally grafted MSCs are in a non-cycling state 8 and 16 days following MSC grafting, respectively.

No long-distance MSC migration following grafting into the normal brain parenchyma

We investigated whether MSCs, grafted at an ipsilateral but distant site to established N32, N29 and D74 tumors, would migrate through the normal brain parenchyma toward tumors. eGFP+ MSCs were grafted 4 mm behind and 2 mm lateral to striatal tumors established 7 days earlier. eGFP+ cell distribution was assessed 14 days following MSC and NPC grafting. In contrast to the MSC distribution pattern following intratumoral grafting, grafting into the normal brain parenchyma did not result in directed MSC migration toward any of the tumor types (**Fig 3**). MSCs were seen in a coherent cluster of cells at the inoculation site. Scattered cells were also observed in the immediate vicinity outside of the cluster (**Fig 3**). MSC grafting was also performed into the frontal corpus callosum contra-lateral to N32, N29 and D74 tumors. No tumor-tropic MSC migration along the corpus callosum was observed (**Fig 4**). In addition to the analysis of eGFP+ cells, we analyzed the expression of endogenous markers expressed by grafted MSCs (NG2 and Ŭ-sma). We found no evidence of spindle-shaped cells expressing NG2 or Ŭ-sma that migrated from the MSC graft toward tumors (data not shown). These results confirm the absence of MSC migration throughout normal brain tissue towards tumors.

Long-term MSC migration in the intact brain

To study long-time survival and migration in the adult normal brain, eGFP+ MSCs were grafted into either the striatum or the corpus callosum of non-tumor bearing animals. Animals were sacrificed 118 days following intracranial MSC grafting. Low numbers of MSCs were seen preferentially at the site of the inoculation coordinate. We did not find any MSCs at any other location of the analyzed sections. To elucidate tropism to other organs, the liver, spleen and the cervical lymph nodes were analyzed for presence of eGFP+ MSCs. Using immunofluorescence microscopic analysis, we did not find any eGFP+ MSCs in sections from the liver, spleen or the cervical lymph nodes.

Grafted MSCs migrate following surgical resection

In order to examine grafted MSC migration in a glioma surgical treatment model, we first established a glioma model which includes partial surgical resection of established N32 gliomas. **(Fig 5a)**. We then utilized this model to investigate whether grafted MSCs migrate within tumor remnants following partial surgical resection. Brains were analyzed for presence of eGFP+ MSCs seven days following tumor resection and MSC implantation. We found extensive MSC survival and migration within the remaining glioma tissue and only minimal numbers of MSCs were found in the surrounding normal brain tissue **(Fig 5b)**.

CONCLUSION:

Here we describe in detail the migratory patterns of grafted rat MSCs using three different rat orthotopic glioma models syngeneic to the Fischer 344 rat. Grafting was performed either into or at a distance from gliomas. In contrast to previous data [5, 6], we found no evidence of long-distance MSC migration throughout the corpus callosum towards gliomas located in the

contralateral hemisphere. In addition, there were no signs of long-distance MSC migration through normal brain tissue towards distant gliomas located in the ipsilateral hemisphere. In contrast, MSCs migrate efficiently and specifically within gliomas following intratumoral grafting. Our results imply that MSC migration towards tumors in normal brain tissue is severely restricted, and that MSCs should be implanted by intratumoral injection for efficient distribution within tumors. Furthermore, using a glioma surgical treatment model, we found that intratumorally grafted MSCs migrate efficiently within glioma remnants following partial surgical resection.

The discrepancy between our results and previous studies [5, 6] might be due to species-specific interactions (i.e. between human MSCs and human glioma xenografts in mice) and/or to different tumor-tropic migratory properties between different subpopulations of MSCs. Tumor model specific factors, such as the production of growth factors and molecules involved in angiogenesis [5, 14-17] as well as the levels of chemo- and cytokines [15, 18-21] in the vicinity of the tumor could also play a decisive role on the attraction of grafted MSCs. The discrepant results might also, possibly, be due to differences in graft labeling techniques. Interpretation of survival and migratory behavior of grafted cells is critically dependent on the sensitivity and specificity of graft labeling. Non-genetical labeling techniques, such as fluorescent dye or iron labeling, may result in unspecific labeling following the death of grafted cells and the uptake of dye in resident host microglia, macrophages and rapidly dividing tumor cells. Importantly, we have previously shown that the MSC eGFP expression pattern *in vivo* correlates with findings from FISH analysis where Y chromosome carrying male MSCs were implanted into female hosts [11]. Thus, our results are derived from two independent analyses of implanted MSCs.

It has been reported that human MSCs can be found in human glioma xenografts in immunocomprised mice following intracarotid injections [5]. In contrast, we previously

found no evidence of i.v. injected MSCs within intracranial gliomas following a single injection [11], though using a rat syngeneic transplantation model. However, as shown in the present study, intratumoral injections of MSCs result in substantial tumor-specific migration throughout the entire tumor. Furthermore, keeping in mind that systemic vascular administration of stem- and progenitor cells carries a risk for serious systemic side-effects, such as pulmonary embolism at high frequency [22, 23], our data suggest that the best administration route for MSCs in glioma therapy may be by intratumoral implantation rather than by systemic injections.

A large number of soluble and membrane-bound factors produced by tumor cells, tumor vasculature and inflammatory cells can attract stem and progenitor cells to gliomas (see [24] for review). The substantial intratumoral MSC migration and virtual absence of MSC migration in normal brain tissue suggest that the tumor micro-environment, e.g. tumor vasculature and inflammatory cells, is permissive for migration of grafted MSCs. Active neoangiogenesis and/or inflammation is presumably required for MSC attraction to gliomas. A major safety issue in the development of stem cell therapies for neurological disorders is the risk of tumor formation of grafted stem cells [25]. Bone marrow-derived mouse MSCs have been implicated in the development of Ewing´s Sarcoma [26]. In our experiments, we found no indication of MSC or NPC proliferation *in vivo* eight and 16 days following grafting into the highly proliferative N32 tumor. These results point to a low risk for development of secondary malignancies from grafted MSCs. Noteworthy in terms of safety, no infiltration of cervical lymph nodes, liver or spleen was seen in the present study following grafting of MSCs intratumorally.

In summary, we show herein that rat MSCs effectively spread out in experimental rat glioma tumor tissues following intratumoral implantation. In contrast to previous reports, we found no evidence of long-distance MSC migration across the corpus

callosum or through the striatum towards malignant gliomas. Our results indicate that intratumoral implantation may be the method of choice for MSC-based treatment approaches of malignant brain tumors.

FIGURE LEGENDS

Fig. 1

MSC phenotype *in vitro* and migratory pattern after implantation to gliomas. **(A)** Adult rat bone marrow-derived MSCs express mesenchymal markers desmin, CD73 and CD90 *in vitro*. Cells are counterstained with DAPI (blue). Scale bar is 50 µm. **(B)** Migration patterns of MSCs following grafting into malignant brain tumors in the striatum. eGFP+ MSCs (green) grafted into malignant N32 brain tumors (depicted by Hoechst nuclear staining, light blue). One day following grafting, eGFP+ MSCs are mainly located within the elongated graft. On day 4, MSCs are predominantly found in tumor periphery. High numbers of MSCs are found in both tumor periphery and tumor core on day 8. Only single eGFP+ MSCs are seen in the normal brain parenchyma. Scale bar is 200 µm.

Fig. 2

Implanted MSCs are non-dividing within gliomas. **(A)** eGFP+ MSCs (green) grafted into the highly proliferative N32wt malignant brain tumor. No co-expression of eGFP and Ki67 (red) was evident 16 days post grafting. **(B)** Confocal microscopy analysis demonstrating single eGFP+ MSCs not co-expressing Ki67 but located in close association to Ki67+ cells. Scale bar is 60 µm in A and 30 µm in B.

Fig. 3

MSCs lack long-distance migratory capacity throughout the striatum towards distant glioma. Enhanced green fluorescent protein (eGFP)-expressing rat MSCs (green) grafted into rat malignant brain tumors (T, Hoechst, blue). MSCs were grafted 4 mm behind and 2 mm lateral of the brain tumor inoculation coordinates. MSCs remain at the injection site 14 days following implantation. There are no signs of eGFP-MSCs migrating towards previously

established **(A)** RG2 tumor, **(B)** N32 tumor or **(C)** the infiltrative N29 tumor in the striatum (str). The lower panels show eGFP-MSCs at the injection site. Scale bar is 700 µm in upper panels and 200 µm in lower panels.

Fig. 4

MSCs lack long-distance migratory capacity towards glioma in the contralateral hemisphere. No tumor-specific migration of implanted enhanced green fluorescent protein (eGFP)-expressing rat MSCs (green) through the corpus callosum (cc) towards rat malignant brain tumor (T, Hoechst, blue). MSCs are implanted in the cc, contralateral to a previously established brain tumor. Most MSCs remain at the injection site 14 days following implantation. A few eGFP+ MSCs are found randomly dispersed within the cc. There are no signs of eGFP-MSCs migrating specifically towards **(A)** RG2 tumor, **(B)** N32 tumor or **(C)** the infiltrative N29 tumor in the corpus callosum. The right panels show eGFP-MSCs at the injection site. Scale bar is 700 µm in left panels and 200 µm in right panels.

Fig. 5

MSC migration within tumor remnants following partial surgical resection. **(A)** Section of a brain following partial surgical resection of the tumor bulk (Hoechst, blue). **(B)** Proof-of-principle that grafted eGFP+ MSCs (green) migrate efficiently within tumor remnants, but not substantially into normal brain tissue, following partial tumor resection. LV, left ventricle; N, normal brain tissue; T = tumor tissue; R = resection cavity; G = site of MSC graft. Scale bar is 400 µm.

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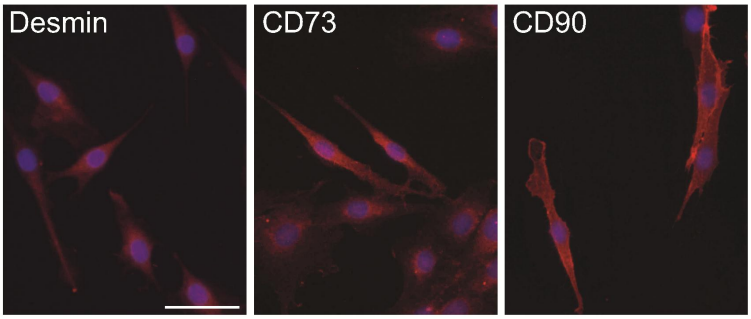

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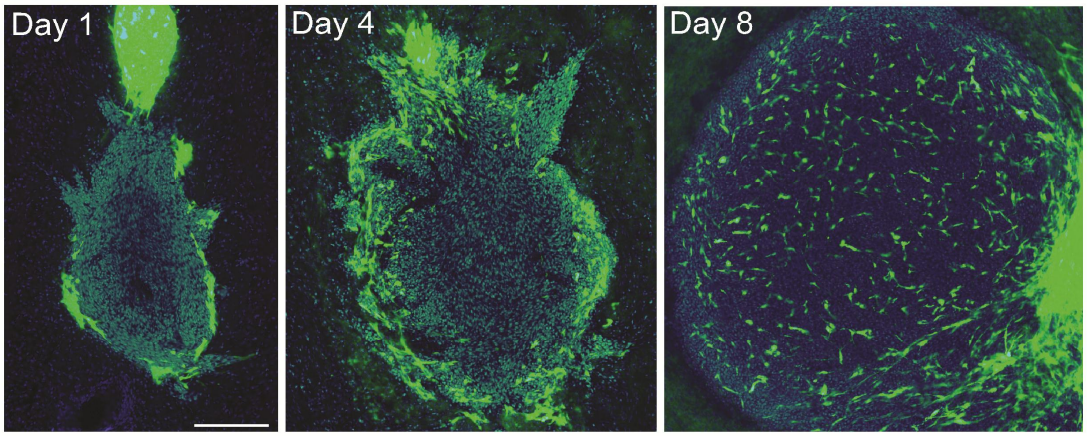

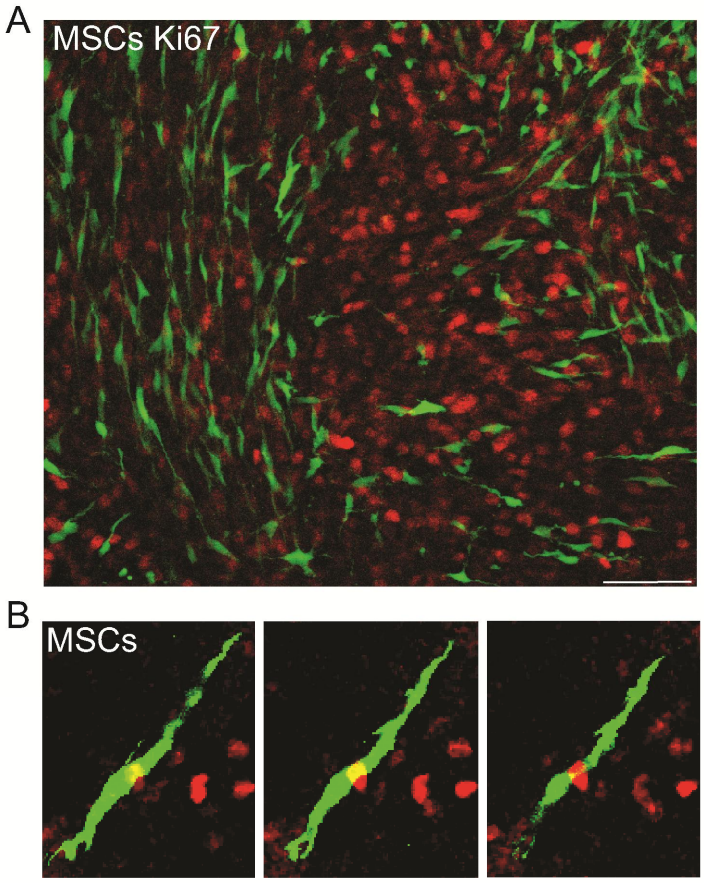
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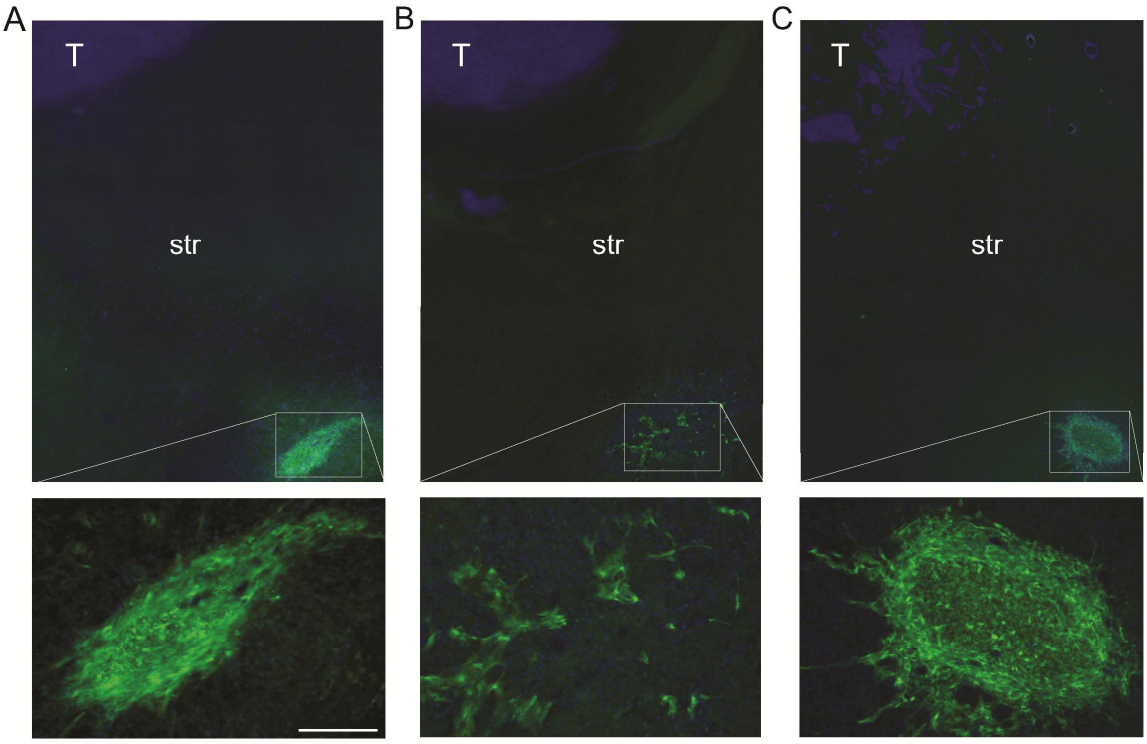
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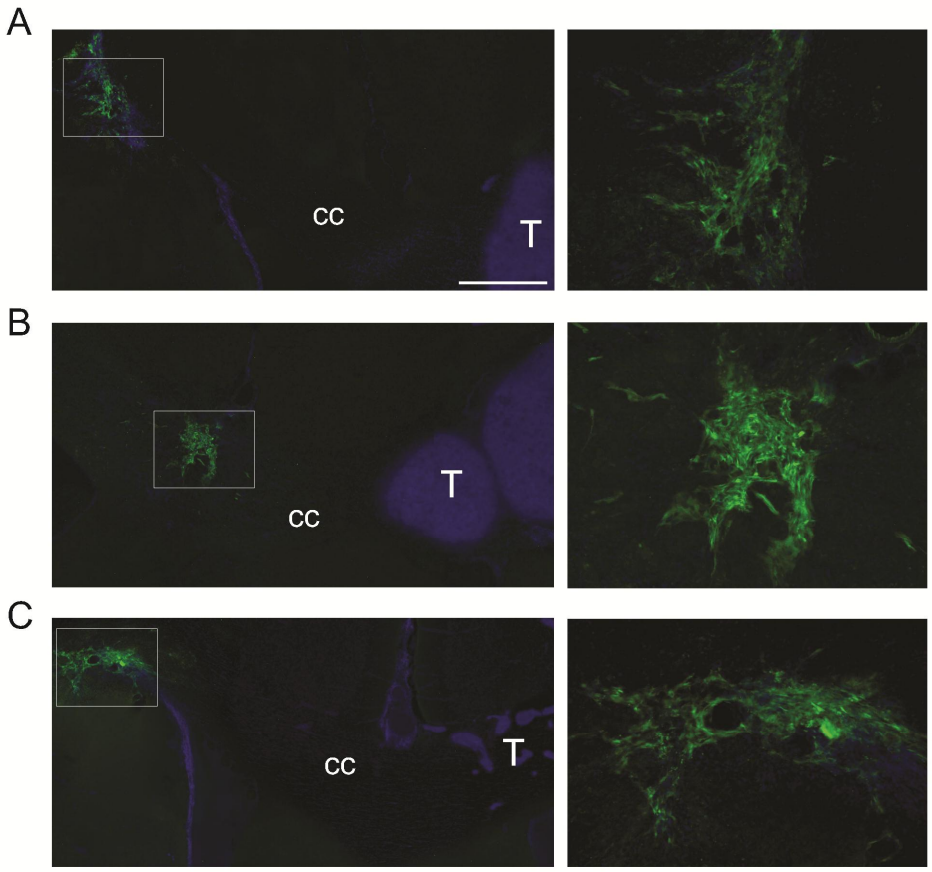
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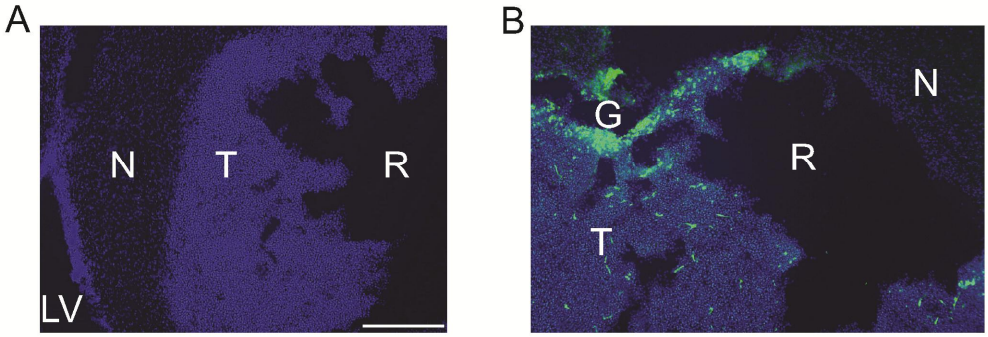
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