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Notch-Induced Rat and Human Bone Marrow Stromal Cell Grafts Reduce Ischemic Cell Loss and Ameliorate Behavioral Deficits in Chronic Stroke Animals

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Gene transfection with Notch 1 intracellular domain and subsequent growth factor treatment stimulate neuron-like differentiation of bone marrow stromal cells (BMSCs). Here, we examined the potential of transplanting Notch-induced BMSCs to exert therapeutic effects in a rat model of chronic ischemic stroke. In experiment 1, Notch-induced rat BMSCs were intrastriatally transplanted in rats at 1 month after being subjected to transient occlusion of middle cerebral artery (MCAo). Compared to post-stroke/pretransplantation level, significant improvements in locomotor and neurological function were detected in stroke rats that received 100 k and 200 k BMSCs, but not in those that received 40 k BMSCs. Histological results revealed 9%–15% graft survival, which dose-dependently correlated with behavioral recovery. At 5 weeks post-transplantation, some grafted BMSCs were positive for the glial marker GFAP (about 5%), but only a few cells (2–5 cells per brain) were positive for the neuronal marker NeuN. However, at 12 weeks post-transplantation, where the number of GFAP-positive BMSCs was maintained (5%), there was a dramatic increase in NeuN-positive BMSCs (23%). In experiment 2, Notch-induced human BMSCs were intrastriatally transplanted in rats at 1 month following the same MCAo model. Improvements in both locomotor and neurological function were observed from day 7 to day 28 post-transplantation, with the high dose (180 k) displaying significantly better behavioral recovery than the low dose (90 k) or vehicle. There were no observable adverse behavioral effects during this study period that also involved chronic immunosuppression of all animals. Histological analyses revealed a modest 5%–7% graft survival, with few (<1%) cells expressing an intermediate MAP2 neuronal marker, but not glial or oligodendroglial markers. In addition, striatal peri-infarct cell loss was significantly reduced in transplanted stroke animals compared to vehicle-treated stroke animals. The present study demonstrates the potential of Notch-induced BMSC cell therapy for patients presenting with fixed ischemic stroke.

Introduction

Transplantation of embryonic- and adult-derived stem cells has been shown to ameliorate behavioral deficits in animal models of neurological disorders and has reached clinical trials in Parkinson's disease, Huntington's disease, and stroke [1–3]. Two schools of thought have embraced the notion that cell transplantation affords therapeutic benefits by either directly replacing the injured host brain cells or acting as a bystander secreting growth factors thereby retarding the neurodegeneration and/or enhancing the endogenous repair mechanisms (eg, neurogenesis, angiogenesis, synaptogenesis) [4,5]. The ultimate goal of cell replacement therapy is to deliver viable cells into the injured brain, with the hope that these grafted cells will re-establish the damaged host neural connections, either by forming new networks or by reconstructing the old pathways. Until recently, the yardstick for evaluating a successful transplantation outcome is visualization of graft survival in the brain.

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Indeed, absence of surviving grafts correlates with continued display of neurological deficits in transplant recipients, in both animal models of CNS disorders and patients [6,7]. However, with accumulating evidence supporting the concept of bystander effects [4,5], a shift in transplant functional index from detecting graft survival to characterization of graft-mediated host neuroprotective and neurorestorative processes has been recognized. Arguably, the advent of cell therapy has challenged the dogma of a "non-regenerative central nervous system." Although endogenous regeneration alone may not fully reverse the disease or aging process, enhancing the host repair mechanism with exogenous cell therapy may improve the neuroprotective or neuroregenerative outcome [8–14].

A primary disease target for cell therapy has been the chronically ill patient; thus, it is not surprising that stroke has become a major focus of attention for this treatment. Stroke is the third leading cause of death and affects over a half-million people each year in the United States alone, with about 3 million stroke survivors left with debilitating behavioral deficits [9–14]. Current stroke treatments are typically limited to supportive care and secondary stroke prevention, and despite rigorous rehabilitation therapy many stroke patients still suffer from permanent loss of independent function, resulting in a substantial economic burden on society estimated at $30 billion each year [9–14]. Although >300 experimental stroke treatments have reached clinical trials, to date only intravenous tissue plasminogen activator (tPA) administration has been effective in ameliorating the neurological deficits arising from acute stroke [15]. However, tPA treatment has an extremely limited window of efficacy, requiring drug injection within 3 h of stroke onset, thereby only benefiting <3% of ischemic stroke patients [9–14]. These statistics represent a significant unmet clinical need that warrants investigations of novel treatments, such as cell therapy, for treating stroke.

The use of neuroteratocarcinoma cells for transplantation in stroke is one such cell therapy approach that has transitioned from the laboratory to the clinic [6,16–18]. Following retinoic acid and mitotic inhibitor treatment, the cancerous NT2 cells are coaxed to differentiate into post-mitotic NT2N cells displaying the morphological features and expressing the phenotypic markers reminiscent of neural progenitor cells [19,20]. Intrastriatal transplantation of these cells in chronic stroke rats resulted in behavioral recovery [6]. Similarly, intracerebral NT2N grafts in patients with fixed stroke were demonstrated to be feasible and safe in both Phase I and Phase II clinical trials [16–18]. However, alternative noncarcinoma cell sources have been proposed as safer transplantable cells than NT2N cells. Recent studies demonstrate that bone marrow is a good source of neural progenitor cells [14,21]. In our desire to transplant neuron-like cells resembling NT2N cells (sans their cancerous origin), since positive outcome from laboratory and clinical transplant studies in stroke was generated from such cell population, we postulate that if cells resembling neural progenitors could be harvested from bone marrow, then transplantation of these cells should likely promote therapeutic benefits in stroke. To this end, a recent study has shown that gene transfection with Notch 1 intracellular domain and subsequent treatment with basic fibroblast growth factor, forskolin, and ciliary neurotrophic factor stimulate neuron-like differentiation of bone marrow stromal cells (hereafter referred to as BMSCs) [22]. Accordingly, the present study was designed to explore the therapeutic efficacy of Notch-induced bone marrow neural progenitor cells in an experimental model of ischemic stroke.

Materials and Methods

Animals

This study was approved by MCG IACUC and followed the NIH guidelines for use of animals in research. Adult, male Sprague-Dawley rats (Harlan Inc., IN) weighing about 250 g (±20 g) served as subjects and were housed singly under standard conditions. Two experiments were performed to reveal the efficacy of transplanting rat and human BMSCs in a chronic ischemic stroke model. All surgical procedures were conducted under aseptic conditions. In both experiments, a baseline behavioral testing was initially conducted to confirm normal motor and neurological function in animals that were subsequently subjected to the transient ischemic stroke surgery. At 1 month postinjury, animals reaching the criteria for behavioral deficits (75% biased swing activity and a score of 2.5 in neurological examination) were randomly assigned to a specific transplant condition, namely: 40 k, 100 k, or 200 k rat BMSCs for experiment 1 ($n = 8$ per group), and medium alone, 90 k, or 180 k human BMSCs for experiment 2 ($n = 10$ per group). All animals were immunosuppressed (10 mg/kg CsA, i.p., daily) throughout the study. Animals again underwent behavioral tests weekly or monthly after transplantation, then at the conclusion of the test period were euthanized for histological examination.

Behavioral testing

Animals were assessed in elevated body swing test (EBST) and Bederson test at baseline, 1 month after stroke surgery, and at different post-transplantation time points. The EBST provided a motor asymmetry parameter and involved handling the animal by its tail and recording the direction of the biased body swings [23]. The EBST consisted of 20 trials with the number of swings ipsilateral and contralateral to the ischemic hemisphere recorded and expressed in percentage to determine the biased swing activity. About 1 h after the EBST, the Bederson test was performed to determine neurological function. Neurologic score for each rat was obtained using four tests, which include (1) observation of spontaneous ipsilateral circling, graded from 0 (no circling) to 3 (continuous circling); (2) contralateral hindlimb retraction, which measures the ability of the animal to replace the hindlimb after it is displaced laterally by 2 to 3 cm, graded from 0 (immediate replacement) to 3 (replacement after minutes or no replacement); (3) beam walking ability, graded 0 for a rat that readily traverses a 2.4-cm wide, 80-cm long beam to 3 for a rat unable to stay on the beam for 10 s; and (4) bilateral forepaw grasp, which measures the ability to hold onto a 2-mm diameter steel rod, graded 0 for a rat with normal forepaw grasping behavior to 3 for a rat unable to grasp with the forepaws. The scores from all four tests were added to give a neurologic deficit score (maximum possible score of 12 points from the four component tests). An investigator blinded to the treatment condition conducted both behavioral tests.

Stroke surgery

Rats were anesthetized with gas inhalation composed of 30% oxygen (0.3 L/min) and 70% nitrous oxide (0.7 L/min) mixture. The gas was passed through an isoflurane vaporizer set to deliver 3% to 4% isoflurane during initial induction and 1.5% to 2% during surgery. Transient (1 h) unilateral focal ischemia was produced using a well-established middle cerebral artery occlusion (MCAo) using the intraluminal suture model as previously described [8]. Physiological parameters, via blood gases assays, and laser Doppler (placed at the level of the dura directly above the expected infarcted striatal region, AP: +2.0, ML: ±2.0, and DV: −4.0 mm) revealed successful occlusion (at least 80% reduction in regional cerebral blood flow) and reperfusion levels that did not significantly differ across all stroke treatment groups. The body temperature of all animals was maintained at 37°C during the surgery until they recovered from anesthesia.

Cell preparation

The Notch-activated BMSCs used in this study (provided by SanBio, Inc., Mountain View, CA) were previously characterized by Dezawa and colleagues [22,24]. In brief, BMSCs subcultured two times were transfected with a vector (pCI neo-NICD) containing the Notch 1 intracellular domain (NICD) using Lipofectamine 2000™ (Invitrogen Corp., Carlsbad, CA). Cells were selected by G418 treatment for 7 days. The cells were allowed to recover from selection and expanded through two additional passages in medium without G418. The cells were then cryopreserved in CryoStore (BioLife Solutions, Bothell, WA). In order to delineate rat BMSCs from host rat transplant recipient, the rat BMSCs were labeled with green fluorescent protein (GFP) by lentiviral infection using procedures as described elsewhere [25]. GFP labeling for human BMSCs was not necessary due to availability of specific human antigens. Pilot studies revealed no detectable differences in cell viability, morphology, neuronal differentiation, proliferation, among other phenotypic features between nonlabeled and GFP-labeled BMSCs. Cryopreserved BMSCs were thawed and washed at 37°C just prior to transplantation surgery. Viability was determined using the Trypan blue dye exclusion method and cell concentration was adjusted to 20,000/μL for transplantation. A minimum of 85% viability conducted prior to and immediately after the transplantation on the last animal recipient was used as a criterion for further inclusion of animals in the study.

BMSC injection

Between 4 weeks and 6 weeks post-injury, randomly selected animals were anesthetized (equithesin 300 mg/kg i.p.), fixed in stereotaxic apparatus (Kopf instruments), and implanted with BMSCs or vehicle directly into the striatum (0.5 mm anterior to bregma, 2.8 mm lateral to midline, and 5.0, 4.5, and 4.0 mm below the dural surface) using a 28-gauge implantation cannula [6]. The predetermined cell dosages were based on pilot studies demonstrating that these dosages are within therapeutically effective dose range. Transplantation surgery was carried out within 2 h after thawing the cells. Infusion rate was 1 μL of cell solution per minute. Following each infusion, a 3-min absorption period was allowed before the needle was retracted. One needle pass was used, but there were three dorsoventral deposits (5.0, 4.5, and 4.0 mm), with each site receiving a 3-μL cell solution. A heating pad and a rectal thermometer allowed maintenance of body temperature at about 37°C throughout surgery and following recovery from anesthesia.

Immunohistochemical and histological analyses

At the conclusion of behavioral testing, animals were euthanized (equithesin 500 mg/kg i.p.) and perfused through the ascending aorta with 150 mL of cold PBS, followed by 150 mL of 4% PFA in PBS. Brains were removed and post-fixed in 4% PFA in PBS for 3 days followed by 30% sucrose in phosphate buffer (PB) for 1 week. Thereafter, 20 μm cryostat-sectioned tissues were obtained. Free-floating sections were incubated overnight at 4°C with anti-MAP2 antibody (1:500, rabbit polyclonal; Chemicon, Temecula, CA), anti-NeuN antibody (1:500, mouse monoclonal; Chemicon, CA), anti-GFAP antibody (1:500, rabbit polyclonal; DAKO, CA), anti-04 antibody (1:50, mouse polyclonal; Chemicon, CA), and anti-HuNu antibody (1:200, mouse anti-human nuclei monoclonal antibody; Chemicon, CA) with 10% normal horse serum. After several rinses, sections were incubated for 1 h in goat anti-mouse IgG Alexa Fluor 488 conjugate (1:1,000; Molecular Probes, CA) and goat anti-rabbit IgG Alexa Fluor 594 conjugate (1:1,000) with Hoechst33342 (1:1,000, Sigma, St. Louis, MO). The sections were then washed, mounted on Superfrost® Plus glass slides (Erie Scientific, NH), and embedded with mounting medium (Biomeda, CA). Control studies included exclusion of primary antibody substituted with 10% normal goat serum in PBS. No immunoreactivity was observed in these controls. Sections were counterstained with Hoechst (Sigma, St. Louis, MO). For estimation of graft survival, GFP- or HuNu-positive cells were counted in all the striatal area of every six sectioned slices and summed up. For estimation of host neuronal cell viability within the ischemic striatal region, Nissl staining was performed using cresyl violet solution (Sigma, St. Louis, MO), and randomly selected visual fields of the striatal region and corresponding contralateral intact striatum in three sections were photographically captured (Carl Zeiss, Axiophot2), and cells were quantified by counting per high power field view selected in random (28,800 μm^2). The percentages of preserved neurons in damaged striatum relative to the intact side were calculated and used for statistical analyses. Brain sections were blind-coded and the total numbers of counted stained cells were corrected by Abercrombie formula. Additionally, confocal analysis was performed using Zeiss LSM 510 confocal Laser scanning microscope.

Statistical analyses

The behavioral data from EBST were analyzed using repeated measures of ANOVA and single ANOVA, while raw ordinal scores from Bederson were analyzed using nonparametric Kruskal–Wallis rank test. The evaluation of cell loss was analyzed using single ANOVA. The level of significance was set at $P < 0.05$. Post-hoc *t*-tests were performed for pairwise comparisons between treatment conditions. In addition, a simple regression analysis, via coefficient of correlation *r*, was performed to reveal interaction between cell dose and behavioral improvement.

Results

In experiment 1, an allogeneic (ie, rat BMSCs into stroke rats) transplant regimen envisioned for clinical application was pursued, whereas in experiment 2, the efficacy of the proposed clinical product (ie, human BMSCs) was evaluated.

Allogeneic rat BMSCs ameliorate behavioral deficits in chronic stroke rats

This initial experiment was designed to provide efficacious cell dose range of Notch-induced rat-derived BMSCs in a rodent stroke model. Such approach of rat-to-rat allogeneic (ie, same species) transplantation mimics the clinical setting, in that we plan to transplant human-derived BMSCs in stroke patients. We characterized weekly locomotor and neurologic performance of transplanted rats over a period of 4 weeks post-transplantation, and again once at 12 weeks post-transplantation. For weekly testing, overall ANOVA revealed significant main treatment effects for EBST ($F_{2,21} = 57.06$, $P < 0.0001$) and Kruskal–Wallis rank test similarly revealed significant effects in Bederson test ($P < 0.0001$) (Fig. 1). For EBST, the motor asymmetry was significantly reduced in each of the 4 weeks post-transplantation compared to post-stroke level (ie, prior to transplantation) (P's < 0.0001), with the most robust recovery seen at 1 week post-transplantation (P's < 0.0001), and with stable recovery displayed for the subsequent 3 weeks post-transplantation (Fig. 1A). Post-hoc tests revealed that the significant reduction in motor asymmetry at 1 week post-transplantation did not differ across the three cell doses, but with dose-dependent effects (high dose 200 k > medium dose 100 k > low dose 40 k) and with the low dose 40 k reverting to post-stroke level at 2, 3, and 4 weeks post-transplantation (P's < 0.05). For Bederson test, the improvements in neurologic deficit scores were significantly reduced in each of the 4 weeks post-transplantation compared to post-stroke level (P's < 0.0001), with a trend toward better improvement over time in that transplanted animals performed better at 2, 3, and 4 weeks post-transplantation compared to 1 week post-transplantation (P's < 0.0001) (Fig. 1B). Post-hoc tests revealed that the significant reduction in neurologic deficit scores at 1 week post-transplantation did not differ across the three cells doses, but the higher doses 100 k and 200 k produced better recovery than the low dose 40 k cells at 2 weeks (P's < 0.05), and dose-dependent effects (200 k > 100 k > 40 k) were seen at 3 and 4 weeks post-transplantation (P's < 0.05). At 12 weeks post-transplantation, significant main treatment effects were detected for both EBST ($F_{2,9} = 11.84$, $P < 0.005$) and Bederson test ($P < 0.001$) (Figs. 1B and 1C). Post-hoc tests further revealed significantly reduced motor asymmetry and neurological deficits compared to post-stroke level, with the two higher doses of 100 k and 200 k promoting better recovery than 40 k in both tests (P's < 0.001).

Allogeneic rat BMSCs survive in ischemic rat striatum

In order to delineate transplanted rat BMSCs from host rat tissue, the donor cells were transfected with lentiviral GFP. Epifluorescent microscopy revealed at least 90% of cultured rat BMSCs expressed GFP (Fig. 2). Results of GFP epifluorescence revealed dose-dependent graft survival at 5 weeks (200 k > 100 k > 40 k) ($F_{8,32} = 33.9$, $P < 0.0001$) and partially at 12 weeks post-transplantation (200 k = 100 k > 40 k) ($F_{8,27} = 14.88$, $P < 0.0001$). However, when percentages for each cell dose were calculated, no significant differences ($F_{8,32} = 1.67$, $P > 0.05$ at 5 weeks; $F_{8,27} = 1.37$, $P > 0.05$ at 12 weeks) in the percent graft survival (9%–15% at 5 weeks; 10%–15% at 12 weeks) were obtained across the three doses in both post-transplantation periods. Regression analyses revealed that the higher the cell dose (200 k > 100 k > 40 k), the better the functional improvement. At both post-transplantation periods, GFP epifluorescence revealed that majority of the transplants (72% at 5 weeks; 62% at 12 weeks) remained within the original transplant site (Fig. 2). When migration was observed, the grafted cells remained within the general target area, in that graft migration was observed within the ischemic striatum, characterized by grafted cells lining the ischemic peri-infarct area in the striatum. Furthermore, a medial to lateral (2.0 mm at 5 weeks; 1.8 mm at 12 weeks) and dorsal to ventral (2.5 mm at 5 weeks; 2.3 mm at 12 weeks) migration of cells along the striatal ischemic peri-infarct area was observed. At 5 weeks post-transplantation, grafted BMSCs were positive for GFAP (about 5%) and a very few cells (2–5 cells per brain) were also positive for NeuN. Both these markers co-localized with GFP. These observations were consistent for all doses. However, at 12 weeks post-transplantation, about 60% of cells that have migrated away from the transplant site were NeuN-positive (ie, since 38% BMSCs migrated, the adjusted percent of NeuN-positive cells from total surviving grafted cells equates to only 23%). These cells display neuronal morphology, characterized by elaborate and long processes. Some GFP-positive BMSCs (about 5%) exhibited the morphology of glial cells, which is confirmed by GFAP staining. Most, if not all, GFAP-positive cells were found near or within blood vessels.

Xenogeneic human BMSCs reduce behavioral impairments in chronic stroke rats

Next, we examined the efficacy and safety of Notch-induced human-derived BMSCs, also supplied by SanBio, Inc. when transplanted at 4 weeks after MCAo stroke surgery in rats. The 1-h MCAo stroke surgery produced consistent behavioral impairments at 1 month post-stroke as revealed by significant biased swing activity and neurological deficits in EBST and Bederson test, respectively, compared to pre-stroke performance of the animals in both tests (Fig. 3). Pairwise comparisons between pre-stroke and post-stroke performance of the animals revealed significant impairments in both tests (P's < 0.0001) in all stroke animals included in this study. Following random assignments of the stroke animals to either vehicle, low dose 90 k BMSCs, or high dose 180 k BMSCs, ANOVA revealed significant treatment effects for both tests (EBST: $F_{2,27} = 210.35$, $P < 0.0001$; Bederson: $P < 0.0001$). Pairwise comparisons between treatment groups revealed that as early as day 7 post-transplantation, stroke animals that were transplanted with BMSCs, regardless of the dose, exhibited significant amelioration of behavioral deficits compared to vehicle-treated stroke animals (P's < 0.05). This behavioral recovery by BMSCs-transplanted stroke animals was

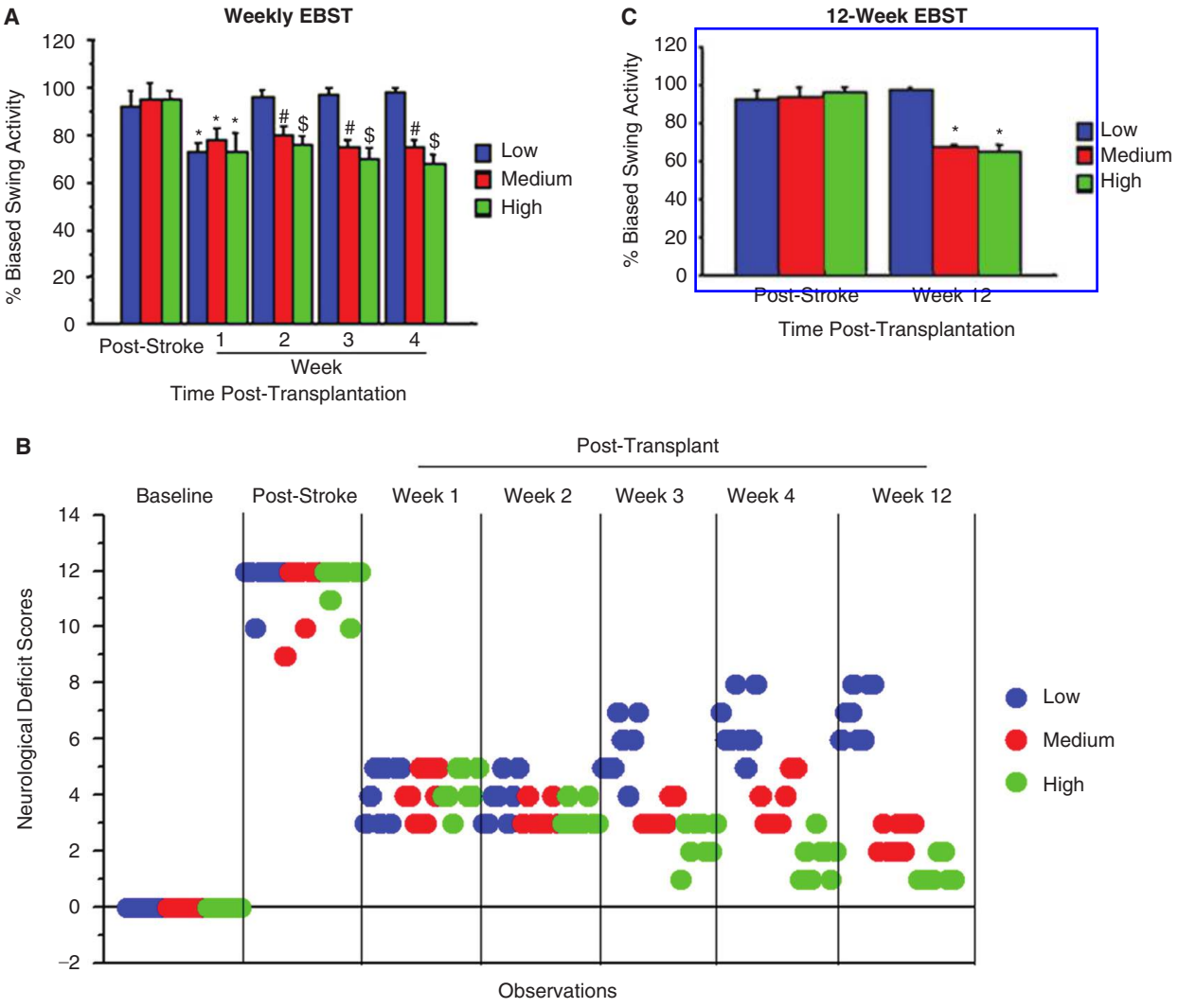

FIG. 1. Allogeneic rat bone marrow stromal cells (BMSCs) ameliorate stroke-induced behavioral deficits. Stroke animals transplanted with BMSCs displayed significant improvements in both locomotor and neurologic functions compared to their post-stroke (ie, prior to transplantation) performance. A significant reduction in motor asymmetry (elevated body swing test, EBST; **A**) was detected at 1 week post-transplantation, which did not differ across the three cell doses, but dose-dependent effects (high dose 200 k > medium dose 100 k > low dose 40 k) were seen at 2, 3, and 4 weeks post-transplantation (P's < 0.05), and with the low dose 40 k reverting to post-stroke level. Similarly, a significant reduction in neurologic deficit scores (Bederson test; **B**) was recognized at 1 week post-transplantation, which did not differ across the three cells doses, but the higher doses 100 k and 200 k produced better recovery than the low dose 40 k cells at 2 weeks (P's < 0.05), and dose-dependent effects (200 k > 100 k > 40 k) were seen at 3 and 4 weeks post-transplantation (P's < 0.05). At 12 weeks post-transplantation, again significant reductions in motor asymmetry (**C**) and neurological impairment (**B**) were observed compared to post-stroke performance, with the two higher doses of 100 k and 200 k promoting better recovery than 40 k in both tests (P's < 0.001). *Versus post-stroke; #versus post-stroke or low dose; $versus post-stroke, low dose, or medium dose.

sustained at days 14 and 28 post-transplantation, in that BMSC cell grafts again regardless of the dose promoted significant attenuation of both motor and neurological impairments compared to vehicle treatment (P's < 0.0001). Closer examination of the two BMSC dose revealed that the high dose 180 k produced significantly better amelioration of behavioral deficits compared to the low dose 90 k across all post-transplantation test days for EBST (P's < 0.01), and at days 14 and 28 post-transplantation for Bederson test (P's < 0.0005).

Xenogeneic human BMSCs survive in ischemic rat striatum at 4 weeks post-transplant

Graft survival of human-derived BMSCs was assessed using monoclonal human-specific antibody HuNu, which do not cross react with rodent cell surface markers or other rodent proteins. Graft survival rates of 7% and 5% were detected in low dose 90 k BMSCs and high dose 180 k BMSCs, respectively, and there was no statistically significant difference in graft survival between the two groups. To evaluate the

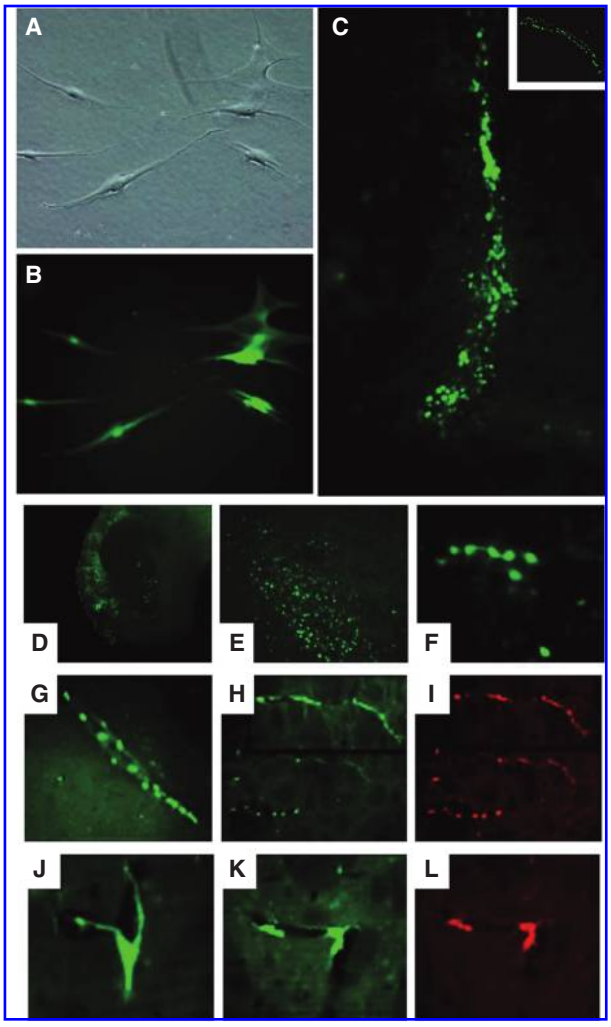

FIG. 2. Allogeneic rat bone marrow stromal cells (BMSCs) survive in the ischemic rat striatum. Lentiviral GFP labeling of rat BMSCs was confirmed by epifluorescent microscopy showing at least 90% of cultured rat BMSCs expressed GFP (**A**: phase contrast; **B**: GFP). Histological examination of graft survival was carried out in randomly selected animals at 5 weeks and 12 weeks post-transplantation. At 5 weeks post-transplantation, most of the cells (72%) that were found in the original transplant site exhibited an immature and beady appearance (**C**, **D**, and **E**), while a few cells that can be seen migrating along the ischemic peri-infarct area (*see inset* in **C**) displayed neuron-like morphology (**F**). At 12 weeks post-transplantation, a majority of BMSCs (62%) (**G**) remained in the original transplant site, but about 23% of those that migrated away from the transplant site (**H**: GFP) were NeuN-positive (**I**). These cells display neuronal morphology, characterized by elaborate and long processes (**J**). Some GFP-positive BMSCs (**K**) co-labeled with the glial marker GFAP (**L**), which were found near or within blood vessels. These observations were consistent for all doses.

expression of neuronal phenotype in cell grafts, immunofluorescence and confocal microscopy focused on demonstrating cells positive for MAP2 and double-labeled with HuNu (Figs. 4 and 5). Results revealed a few grafted BMSCs (<1%), regardless of dose, were MAP2-positive. Confocal images revealed merged images of HuNu- and MAP2-positive cells. HuNu immunolabeling also revealed the lack of extensive migration of grafted BMSCs, in that the cells remained close to the original implantation site (ie, striatum). In addition, HuNu-positive BMSC cells were detected along the needle track through the cortex and corpus callosum, which likely got deposited in these regions during the needle retraction (Fig. 6). Further immunohistochemical analyses of the grafts using specific markers for glial (GFAP) and oligodendroglial (O4) were performed. Immunofluorescent and confocal microscopy revealed that there were no detectable BMSCs that express either GFAP (Fig. 7) or O4 phenotypes (data not shown). These immunohistochemical results, taken together, suggest that the majority of BMSCs did not retain their neuronal phenotypes, with only a few cells expressing the immature neuronal marker MAP2, but lacking the glial GFAP or oligodendroglial O4 phenotypes.

Xenogeneic human BMSCs reduce ischemic cell loss

Despite only a modest graft survival and with low number of BMSC grafts that expressed neuronal markers, we found significant rescue of the ischemic peri-infarct area (Fig. 8). Cell loss along the striatal peri-infarct area was significantly reduced by transplantation of BMSCs, regardless of dose, compared to vehicle-treated stroke animals. Nissl staining revealed significant cell loss (about 45% reduction) along the striatal ischemic peri-infarct area of stroke animals that received vehicle alone. In contrast, stroke animals that received human BMSCs, regardless of dose, had a significantly lower cell loss (about 20%) in the ischemic peri-infarct area. ANOVA revealed significant treatment effects ($P < 0.01$) and post-hoc *t*-tests revealed statistically significant reduction in cell loss in BMSC-transplanted stroke animals compared to those that received vehicle only (P's < 0.05).

Allogeneic and xenogeneic BMSCs are well-tolerated by ischemic brain

Gross histological examination of Notch-induced rat- and human-derived BMSCs conducted separately by our laboratory and a contract research organization revealed no incidence of any donor-derived tumor or ectopic tissue formation. All transplanted stroke animals looked healthy and there were no observable overt adverse effects during the study period.

Discussion

The present results demonstrate that Notch-induced allogeneic rat and xenogeneic human BMSC grafts promote behavioral and histological benefits in MCAo ischemic stroke rats, with no observable deleterious side effects. The neurorestorative effects afforded by allogeneic and xenogeneic grafts did not differ in stability (4–12 weeks post-stroke), degree of behavioral recovery (about 25% better than controls), and reduction in ischemic cell loss (at least 20%). These observations indicate that human BMSCs, which are the envisioned clinical product, equally achieved the therapeutic benefits afforded by an allogeneic transplant regimen,

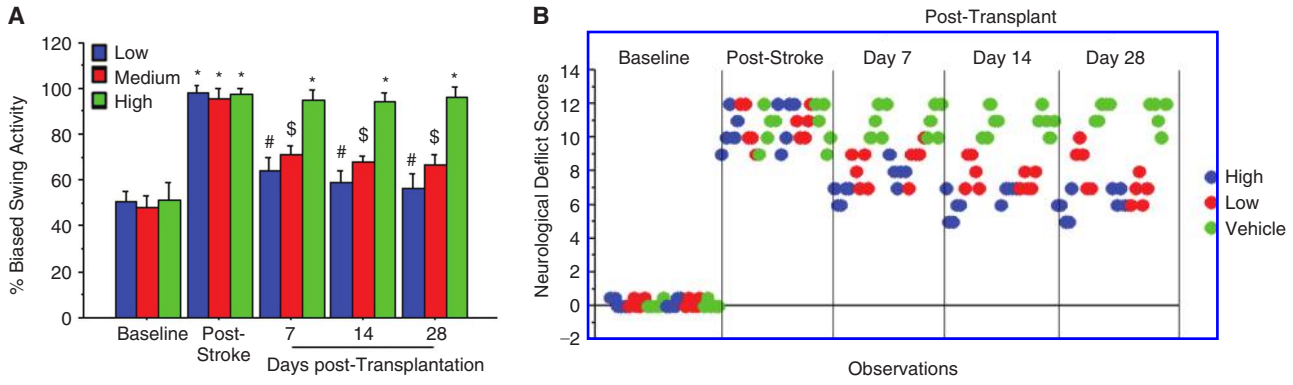

FIG. 3. Xenogeneic human bone marrow stromal cells (BMSCs) reverse stroke-induced behavioral deficits. Prior to stroke surgery, all animals included in this study exhibited no detectable motor or neurological impairment (Baseline), but displayed significant deficits (*P's < 0.0001 vs. Baseline) in both elevated body swing test (EBST) (**A**) and Bederson test (**B**) at 1 month postinjury (ie, prior to transplantation; Post-Stroke). Human-derived BMSCs promoted robust therapeutic benefits in transplanted stroke animals, characterized by behavioral recovery as early as day 7 post-transplantation and remained stable up to day 28 post-transplantation (the study cutoff period). A dose-dependent amelioration of motor and neurologic dysfunction (180 k > 90 k > vehicle) was observed across all post-transplantation test days for EBST (#P's < 0.05 vs. vehicle, low dose or Post-Stroke), and at days 14 and 28 post-transplantation for Bederson test ($$P$'s < 0.05 vs. vehicle or Post-Stroke). In contrast, vehicle-treated stroke animals continued to exhibit significant impairments in both tasks throughout the post-transplantation period (*P's < 0.0001 vs. Baseline).

thereby advancing the status of this cell population as a formidable donor cell graft for chronic ischemic stroke.

The target population is the chronically ill stroke patient, which clinically for a number of reasons is a good candidate for cell therapy. First, the prolonged interval between stroke onset and transplantation allows the disease to stabilize. Second, because acute stroke patients would be initially screened as candidates for tPA or other interventional therapies (eg, MCA recanalization), the introduction of cell therapy at such early phase may confound the outcome.

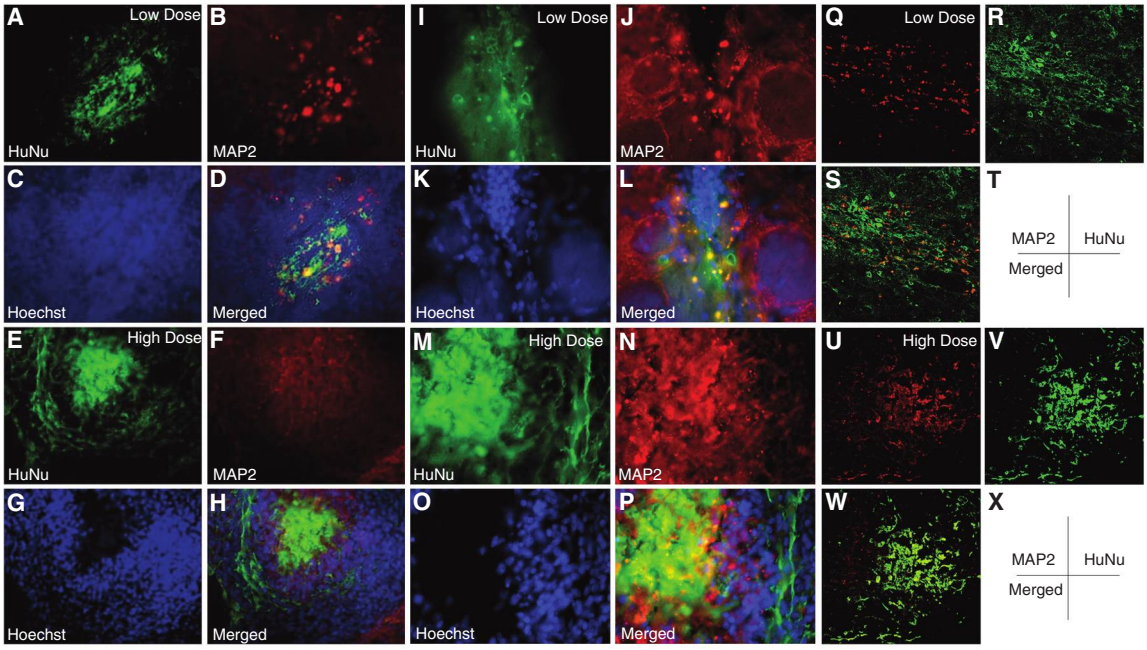

FIG. 4. Xenogeneic human bone marrow stromal cells (BMSCs) survive in the ischemic rat striatum. Immunofluorescent (**A–P**) and confocal (**Q–X**) microscopy at 4 weeks post-transplantation revealed surviving human BMSCs in the ischemic rat striatum. The monoclonal human-specific antibody HuNu showed a few BMSC grafts co-labeled with the neuronal marker MAP2-positive in low dose 90k (**A–D**: 40×; magnified in **E–H**: 1,000×) and high dose 180 k BMSCs (**I–L**: 40×; magnified in **M–P**: 1,000×). Confocal images revealed merged images of HuNu- and MAP2-positive cells positive in low dose 90 k (**Q–T**: 400×) and high dose 180 k BMSCs (**U–X**: 400×).

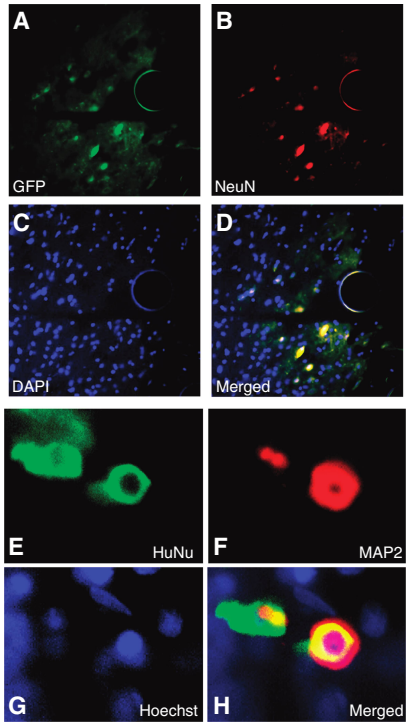

FIG. 5. Allogeneic and xenogeneic bone marrow stromal cell (BMSC) grafts partially express neuronal phenotype. Neuronal markers NeuN and MAP2 were used to reveal neuronal expression in grafted BMSCs. High magnification images (**A–D**, 400×; **E–H**, 1,000×) show grafted cells double-labeled with GFP and NeuN (rat BMSCs) or HuNu and MAP2 (human BMSCs).

Third, it is well documented that spontaneous recovery occurs in stroke in the absence of any intervention [26–28]. Accordingly, allowing an ample period for stroke to be properly diagnosed as candidate for more conventional therapies, as well as to provide an opportunity for spontaneous recovery to exert its potential benefits, prompted us to target the chronically ill stroke patients.

In view of a chronic, fixed stroke as our target disease, we contemplated on the logical route of cell delivery. At such late phase, the stroke brain would likely present with demarcated necrotic core and ischemic tissue regions. In order to maximize the potential of cell therapy to exert bystander neurotrophic secreting effects and/or cell replacement properties [4,5], we thought that BMSCs needed to be deposited accurately to the injured, but viable ischemic area. This prompted us to pursue the stereotaxic transplantation approach. The alternative peripheral delivery was considered; however, at best only a small number of grafted cells have been shown to reach the ischemic area [29–31]. Moreover, for the exogenous cells to penetrate the brain from the periphery, a compromised blood brain barrier (BBB) appears as a prerequisite and such BBB breakdown after stroke only persists over a few weeks necessitating the delivery of cells acutely post-injury [30,32]. To date, only a handful of studies [33,34] has demonstrated cell migration into the stroke brain when peripherally delivered at 1 month after stroke and only at very large cell doses, and even these studies detected only few surviving grafts, suggesting that optimal timing for peripheral transplantation corresponds to a few days or a week after stroke onset [35,36]. In contrast, our series of laboratory studies with NT2N cells provides evidence of safety and efficacy of intracerebral transplantation for chronic stroke [6,37]. Furthermore, the stereotaxic transplantation of the neural progenitor NT2N cells has been examined in the clinic [16–18], but amid concerns about the cells' cancerous origin and cessation of the biomedical company to operate thereby limiting the supply of clinical grade cells, additional clinical trials have not been pursued. Nonetheless, Phase I and Phase II trials of NT2N cells demonstrate that direct intracerebral transplantation for chronic stroke is feasible and well-tolerated by the patients [16–18]. Peripheral transplantation of BMSCs has also been shown to be practical and safe in stroke patients, but requiring two separate intravenous dosing once at 4–5 weeks and another at 7–9 weeks after stroke symptom onset [38]. Of note, clinical cell doses markedly differ between intracerebral and peripheral transplantation with 2–10 million cells employed in the stereotaxic NT2N cell approach [16–18] compared to a fairly high 50 million autologous BMSCs intravenously delivered per dosing [38]. The availability of an ample supply of donor cells obviously will reduce the number of patients who will benefit from cell therapy. Autologous transplantation presents with a logistical obstacle of harvesting a large number of viable and functional cells from an aged or diseased patient [39,40]. Healthy young donors appear as the optimal source for BMSCs, but this remains to be fully determined [41,42]. Equally challenging for autologous grafting is the use of freshly cultured cells compared to cryopreserved cells, with the former requiring a short period of time to screen the cells for homogeneity, infections, genetic mutations, and other risk factors prior to transplantation. On the other hand, autologous BMSCs may lower the risk for host immune response [43–45]. Furthermore, if autologous cells were to be delivered peripherally, then such approach also circumvents the relative trauma associated with intracerebral transplantation. However, the use of immunosuppressants and the long track record of stereotaxic surgical approach for cell therapy in neurological disorders, especially in Parkinson's disease [1,2,3], should allow a safe and efficacious clinical application of intracerebral transplantation of allogeneic cells in stroke. Accordingly, in our desire to transplant allogeneic cells with neuronal progenitor cell properties reminiscent of NT2N cells sans their cancerous origin, we opted for the Notch-induced BMSCs. We further maintain our position that based on the dynamics of cell migration (eg, temporal up-regulation of chemoattractant signals) [46,47] and transient BBB permeation following disease onset [30,32], peripheral transplantation may be more suitable for acute stroke, whereas direct stereotaxic cell administration seems appropriate for fixed, chronic stroke.

Bone marrow stromal cells pose as a good transplantable donor cell population since they are easily isolated and can be expanded from patients without serious ethical or technical problems. Dezawa and colleagues [22] recently developed a new method focusing on Notch 1 intracellular domain gene transfer and administration of certain trophic factors for highly efficient and specific induction of functional neurons and skeletal muscle cells from BMSCs [22,24,48,49]. These Notch-induced BMSCs have been transplanted into animal models of CNS disorders including Parkinson's disease and muscle degeneration, demonstrating safe and

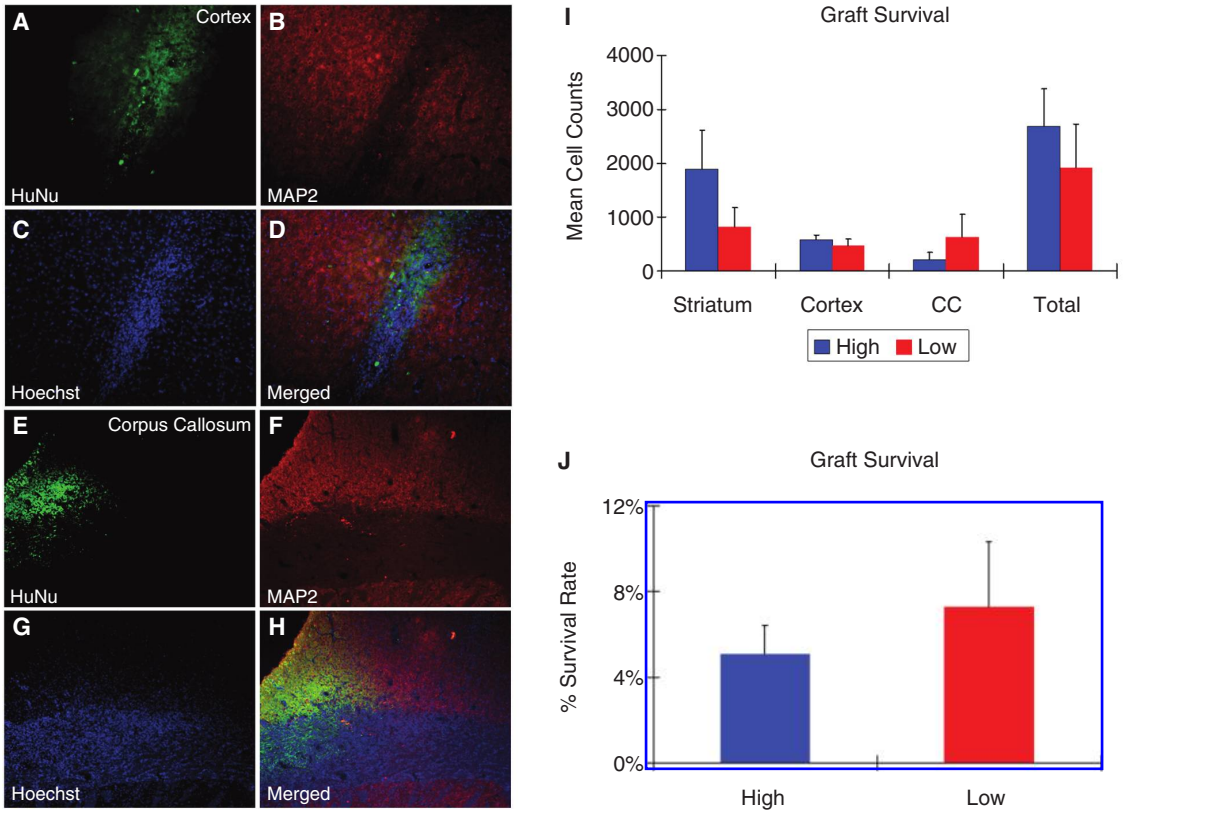

FIG. 6. Trace deposits of xenogeneic human bone marrow stromal cells (BMSCs) along the needle tract. Additional immunohistochemical examination of BMSC graft survival outside the target striatum transplant site revealed the lack of extensive migration of grafted BMSCs, in that the cells remained close to the needle passage. HuNu-positive cells detected in the cortex (**A**–**D**: 20×) and corpus callosum (**E**–**H**: 10×) were along the needle track injection and got deposited in these regions during the needle retraction. Quantitative analyses of graft survival, using HuNu and Hoechst double-labeling, are provided as mean cell counts (**I**) in striatum, cortex, and corpus callosum (CC), as well as percentage of surviving rate (**J**) based on the original implantation cell doses of 90 k and 180 k.

efficacious functional outcome characterized by successful integration of transplanted cells and improvement in the behavior of the transplanted animals [22,48]. The same Notch-induced rat BMSCs have also been examined in an acute ischemic stroke model, with the cells transplanted at 7 days after injury, showing good cell engraftment (45% survival) with majority of the grafted cells retaining their neuron-like phenotype (84% MAP2-positive) and accompanied by robust improvement in motor and cognitive function [22]. As discussed earlier, based on scientific and clinical considerations, the present study explored the efficacy of BMSCs in a chronic stroke paradigm, and further extended the clinical potential by transplanting the envisioned clinical product of human-derived BMSCs in addition to the rat BMSCs. Here, we observed a much lower engraftment (15% and 5%–7% for allogeneic and xenogeneic grafts, respectively) and neuronal expression profile for BMSCs, characterized by NeuN or MAP2 immunoreactivity in only 1% or less at 4 weeks post-transplantation for both type of grafts, and 23% at 12 weeks post-transplantation for allogeneic grafts. Compared to the acute ischemic brain, the chronically injured brain might present with diminished chemoattractant cues [46,47], an increasingly evolving necrotic tissue or a narrowing ischemic penumbra [50,51] succumbing to a peri-infarct area, which singly or in combination creates a host microenvironment that may be less conducive for survival and integration of the grafted cells. Such a suboptimal milieu may be exacerbated by inflammatory and immune factors [43,44], thereby further hindering BMSC fate. Interestingly, despite the modest graft survival and low percentage of neuronal markers observed in both types of grafts, allogeneic and xenogeneic BMSCs produced the same degree of behavioral recovery, which seems to favor a bystander-type effect as opposed to cell replacement mechanism. Furthermore, the robust amelioration of stroke-induced motor and neurologic deficits as early as day 7 after transplantation implicates the key role of BMSC bystander effects, in that the secretion of growth factors by the grafted cells, as previously reported by our group and others [4,5,30,52], likely contributed to the functional outcome at least in the early phase of recovery. However, the long-term and stable behavioral recovery, which lasted at least up to 12 weeks post-transplantation, might have been aided by an endogenous cell replacement mechanism, in that rather than the grafted BMSCs surviving and functioning as new neuron-like cells, the host cells might have been stimulated by the grafts to undergo endogenous neurogenesis or angiogenesis. These speculative neurorestorative

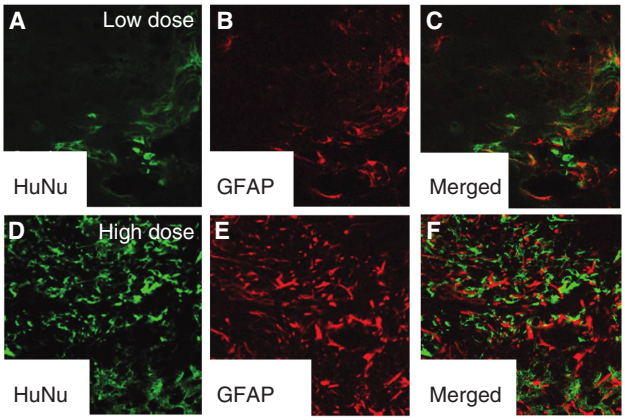

FIG. 7. Xenogeneic human bone marrow stromal cells (BMSCs) lack the glial GFAP phenotype. Confocal microscopy of BMSC grafts using the specific glial marker GFAP revealed that there were no detectable co-labeling of HuNu-positive BMSCs and GFAP. Such absence of GFAP expression by BMSCs was found for both low (**A**–**C**) and high (**D**–**F**) cell doses. Magnification is at 400×. A similar pattern characterized by lack of double-labeling was seen with the oligodendroglial marker O4 and HuNu-positive BMSCs (data not shown).

modes of action underlying the therapeutic benefits of BMSC transplantation warrant further examination.

Secondary neuronal cell death associated with stroke occurs well beyond the initial ischemic injury (4–8 weeks post-stroke). The present detection of delayed neurodegeneration following MCAo agrees with previous reports demonstrating neuronal cell death persists at least up to 3–10 weeks post-stroke [53–55] as revealed by magnetic resonance imaging and immunohistochemical analyses [55]. The chronic cell death in the present stroke animals was recognized in the control group (stroke animals infused with vehicle alone), which clearly has exhibited a much greater host cell loss in the peri-infarct region than stroke animals that received Notch-transfected BMSC grafts. Altogether, these findings demonstrate that neuronal cell death does occur at the late phase of ischemic stroke, thereby extending the therapeutic window for rescuing this specific peri-infarct region.

Our initial hypothesis was that BMSCs differentiate into neural cells following transplantation, thus we focused on neural markers. The observed neuronal phenotype expression in a few grafted BMSCs raises the possibility of fusion as contributing to the overlap of these phenotypic markers, as well as the delay in the number of NeuN cells. Because graft survival is low, thus supporting the notion that functional recovery was not closely associated with BMSC graft survival or neural differentiation per se, we posit that further exploration of revealing a few BMSCs expressing a neural phenotype may not add to the mechanistic pathway underlying the observed therapeutic effects of BMSC transplants. Along the same vein of low graft survival, either as nondifferentiated BMSCs (as revealed by GFP or HuNu labeling, but negative for neural markers) or as neurally differentiated BMSCs, we did not pursue revealing the status of these few surviving grafted cells, in particular whether they retained their CD133 phenotype.

Despite low graft survival and neural differentiation, one can ascertain obvious species differences between donor cells, which was characterized by initial glial phenotype preference (5%) than over time a commitment toward neuronal lineage differentiation (23%) for rat BMSCs, whereas only a small percentage (1%) of neuronally labeled human BMSCs was detected. Despite these species differences in their eventual fate, overall graft survival in both rat and human BMSCs is modest (9%–15% in rat and 5%–7% in

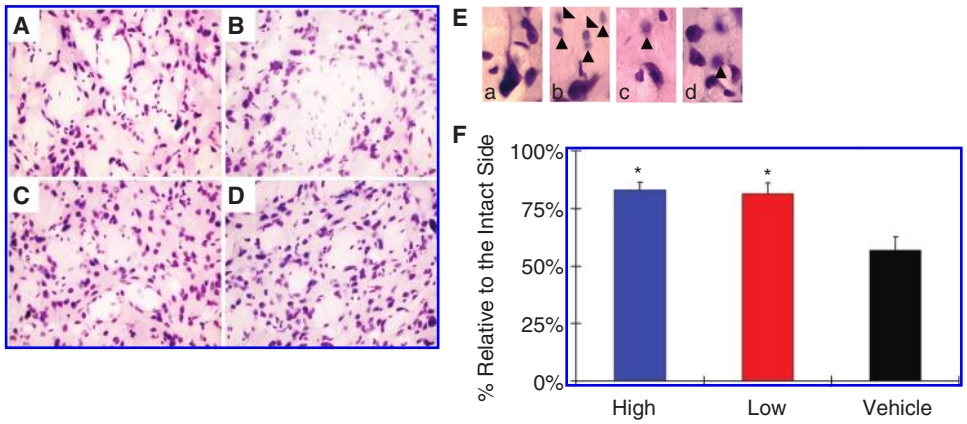

FIG. 8. Xenogeneic human bone marrow stromal cell (BMSC) grafts reduce ischemic cell loss. Nissl-positive cells in the host striatum adjacent to the transplant site and the corresponding intact striatum on the contralateral, nonischemic hemisphere (**A**) were identified via cresyl violet staining. Chronic stroke revealed obvious cell loss in the ipsilateral striatum of vehicle-treated animals (**B**). In contrast, cell loss in the ischemic striatal peri-infarct area was reduced by transplantation of both low dose (90 k) (**C**) and high dose (180 k) (**D**) human BMSCs. Cell loss/survival is further revealed in high magnification (1,000×; **E**) with panels **a–d** corresponding to intact, vehicle-infused, low dose transplanted and high dose transplanted ischemic striatum, respectively. Arrowheads point to ischemic cells. Quantitative analyses of host striatal cell survival revealed statistically significant (*P's < 0.05 vs. vehicle) rescue of the ischemic peri-infarct are by either low or high dose BMSC grafts compared to vehicle treatment (**F**).

human). Although it appears that rat BMSCs exhibited better glial and neuronal differentiation than human BMSCs (but note different graft maturation periods: 12 weeks vs. 4 weeks post-transplantation in rat and human BMSCs, respectively), the resulting behavioral improvements did not significantly differ between these cell grafts. These data further corroborate our claim that BMSC differentiation is not pivotal to the observed therapeutic benefits as mentioned earlier.

The major limitation of this study is the lack of vis-à-vis comparison between Notch-induced BMSCs and naive, non-Notch-transfected BMSCs, prohibiting determination of the therapeutic advantage of one cell type over another. However, in terms of clinical relevance, Notch-transfected BMSCs represent a characterized set of cells at least in vitro, in that these cells differentiate into a neuronal lineage [22]. As highlighted in the guidelines of Stem cell Therapeutics as an Emerging Paradigm in Stroke (STEPS), the characterization of donor cells is a prerequisite for cell therapy, so that assurance is provided for the same cell type to be transplanted [56,57]. From the Food and Drug Administration (FDA) standpoint, this donor cell characterization, which can be easily achieved if a homogeneous cell population is identified, such as in the case of Notch-induced BMSCs, should facilitate a critical assessment of safety and efficacy of the cell product. Indeed, the FDA has been consistent in its guidance on the use of a single lot of cells for preclinical studies and clinical trials. In contrast, the heterogeneous naive BMSCs hinder their full characterization, thus our position is that their clinical use is likely limited to autologous transplantation as opposed to the current allogeneic transplantation of Notch-transfected BMSCs. Accordingly, a defined set of BMSCs stands as a critical milestone in advancing cell therapy to the clinic and the Notch-transfected BMSCs satisfy this criterion. We, however, realize that while cultured Notch-transfected BMSCs display a neuronal phenotype, the present data reveal that such Notch transfection is not able to sustain a robust neuronal expression in BMSCs following transplantation. Nevertheless, these transplantation results do not defeat the purpose of having a characterized donor cell type as graft source. Notch transfection, regardless of the fact that majority of transfected BMSCs did not express a neuronal phenotype, may be necessary for promoting therapeutic benefits (ie, via growth factor secretion).

The use of BMSCs in the clinic has been examined in cardiac patients, but long-term efficacy and safety outcomes have yet to be fully examined [58–60]. As for BMSC entry into the clinic for stroke patients, a few clinical trials are currently being planned targeting the acute phase of ischemic injury with cell delivery via the peripheral route. The present set of preclinical data is unique as it targets chronic stroke, thus requiring the neurosurgical stereotaxic route of cell transplantation.

An equally important finding accompanying the observed efficacy in the present study is the recognition of the safety profile of Notch-induced BMSCs. The absence of overt behavioral averse side effects, tumor or ectopic formation in both allogeneic and xenogeneic grafts indicates that BMSCs are safe. Additional immunohistochemical studies reveal no aberrant proliferation of the grafts (data not shown).

In summary, these results collectively demonstrate the therapeutic potential of intracerebral transplantation of Notch-induced BMSCs in ischemic stroke. Dose-dependent behavioral recovery and histological rescue of the ischemic peri-infarct area in transplanted stroke animals reveal the therapeutic dose necessary to promote robust and stable functional improvement. The delayed timing of transplantation allows enrollment of chronically ill stroke patients. The present laboratory findings support proceeding with limited clinical trials of stereotaxic allogeneic BMSC transplantation in patients with fixed cortical and subcortical infarcts.

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