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# Subacute Transplantation of Native and Genetically Engineered Neural Progenitors Seeded on Microsphere Scaffolds Promote Repair and Functional Recovery After Traumatic Brain Injury

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## Abstract

There is intense interest and effort toward regenerating the brain after severe injury. Stem cell transplantation after insult to the central nervous system has been regarded as the most promising approach for repair; however, engrafting cells alone might not be sufficient for effective regeneration. In this study, we have compared neural progenitors (NPs) from the fetal ventricular zone (VZ), the postnatal subventricular zone, and an immortalized radial glia (RG) cell line engineered to conditionally secrete the trophic factor insulin-like growth factor 1 (IGF-1). Upon differentiation *in vitro*, the VZ cells were able to generate a greater number of neurons than subventricular zone cells. Furthermore, differentiated VZ cells generated pyramidal neurons. *In vitro*, doxycycline-driven secretion of IGF-1 strongly promoted neuronal differentiation of cells with hippocampal, interneuron and cortical specificity. Accordingly, VZ and engineered RG-IGF-1-hemagglutinin (HA) cells were selected for subsequent *in vivo* experiments. To increase cell survival, we delivered the NPs attached to a multifunctional chitosan-based scaffold. The microspheres containing adherent NPs were injected subacutely into the lesion cavity of adult rat brains that had sustained controlled cortical impact injury. At 2 weeks posttransplantation, the exogenously introduced cells showed a reduction in stem cell or progenitor markers and acquired mature neuronal and glial markers. In beam walking tests assessing sensorimotor recovery, transplanted RG cells secreting IGF-1 contributed significantly to functional improvement while native VZ or RG cells did not promote significant recovery. Altogether, these results support the therapeutic potential of chitosan-based multifunctional microsphere scaffolds seeded with genetically modified NPs expressing IGF-1 to promote repair and functional recovery after traumatic brain injuries.

## Keywords

neurotrauma, regenerative medicine, radial glia, multifunctional scaffold, fibroblast growth factor, fibronectin, cell transplantation, neural stem cells, insulin-like growth factor 1, regeneration

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## Introduction

Stem cell transplantation is an exciting area of central nervous system (CNS) regeneration research. Stem cell transplantations are being tested for therapeutic use after CNS injury because mature neurons are postmitotic and there are inadequate numbers of neural progenitors (NPs) present in the mature brain to sufficiently replace all the neurons lost after trauma. While the adult brain maintains pools of NPs in the subventricular zone (SVZ) and in the subgranular zone (SGZ) of the dentate gyrus, the proliferative and migratory responses of these progenitors after injury are ineffective in repairing a severely damaged cortex, especially since these pools diminish with age (Salman et al., 2004; Molofsky et al., 2006; Encinas et al., 2011). Furthermore, these NPs are developmentally restricted and thus unable to regenerate the variety of neurons that were damaged (Niimi and Levison, 2017). Exogenous NP transplants can be used to supply the damaged brain with therapeutic NPs.

Prior studies have obtained promising but limited results using rodent neural stem cell lines to treat traumatic brain injuries (TBIs) in mice and rats (Riess et al., 2002; Bakshi et al., 2005; Boockvar et al., 2005; Bakshi et al., 2006). However, a concern is that engrafted stem cell lines can produce tumors after transplantation (Riess et al., 2007). Thus, the likelihood that these cell lines can be used to treat patients is low. Therefore, researchers have explored the efficacy of grafting primary NPs generated from fetal and early postnatal brains into the adult rat CNS. Two sources of primary NPs have been transplanted into the brain after TBI. NPs harvested from the SVZ of newborn animals (postnatal days 1–5 in rat) and from the ventricular zone (VZ), of embryonic fetuses (approximately Day 13.5 in rat). SVZ NPs are the more commonly used NP population. These cells are tripotential progenitors that can be expanded *in vitro* and upon mitogen removal, differentiate into three neural subpopulations: neurons, astrocytes, and oligodendrocytes. NPs from the VZ, also known as radial glial cells, are more primitive as they are direct descendants of neuroepithelial cells. These cells also are tripotential but have the advantage of being able to generate large numbers of projection neurons.

However, it is difficult to obtain primary NPs, especially for transplantation studies into patients; therefore, several preclinical studies have evaluated the potential of mesenchymal stem cells (MSCs) to reduce damage and to promote repair after brain injury. The rationale for using MSCs is that: They are easy to harvest, can be easily expanded *in vitro*, can be manipulated while *in vitro* to enhance their supportive capacities (e.g., by exposing them to hypoxia), do not elicit an immune response, and can be infused intravenously. Studies in which MSCs have been infused intravenously in experimental models of TBI have shown that they reduce neuronal apoptosis (Azari et al., 2010; Chuang et al., 2012; Chang et al., 2013) likely because they are a rich source of a number of neurotrophic factors that include vascular endothelial growth factor-A, BDNF, NGF and HGF. They also reduce the extent of edema (R. Zhang et al., 2013), reduce oxidative stress (Torrente et al., 2014), and reduce the extent of astrogliosis. In a study using the controlled cortical impact (CCI) model of TBI, intravenous administration of human umbilical cord MSC overall improved neuronal function and, therefore, recovery following TBI (Zanier et al., 2011). These and other encouraging preclinical results supported the use MSCs in human clinical trials. To date, three small clinical trials have been performed, with two showing some modest improvement in the patients treated with MSCs (Z. X. Zhang et al., 2008; Cox et al., 2011; Tian et al., 2013). A major limitation of MSCs is that there is very little evidence that MSCs can replace any of the cells damaged by a TBI.

A major hurdle preventing successful integration and functionality of transplanted cells lies in the fact that so few of the transplanted stem cells survive. It has been reported that less than 2% of the cells that are transplanted after TBI survive (Shindo et al., 2006; Harting et al., 2009; Wallenquist et al., 2009). Studies on stem cell transplantation have shown that a biomaterial matrix improves cell survival (M. C. Tate et al., 2002; C. C. Tate et al., 2009). However, transplantation paradigms using biomaterials require improvements in graft retention that can be achieved by providing growth and neurotrophic factors (Bible et al., 2012). The insulin-like growth factors (IGFs) promote neurogenesis and have been shown to improve functional recovery after brain injury (Aberg et al., 2000; O'Kusky et al., 2000; Kazanis et al., 2004; Lin et al., 2009), and several preclinical studies, as well as some clinical trials, have tested the therapeutic benefits of IGF-1 for neurodegenerative diseases, stroke, and CNS trauma (Saatman et al., 1997; Liu et al., 2001; Vincent et al., 2004; Bianchi et al., 2017). However, native IGF-1 has a short half-life, is rapidly cleared, and has poor pharmacokinetic properties (Clemmons, 1997). Thus, an alternative to providing the growth factor is to deliver it directly using viral particles or transplanting neural stem cells that have been genetically modified to secrete IGF-1.

For the studies described here, we compared the engraftment of secondary NPs derived from fetal and neonatal VZ and SVZ, respectively, either administered directly into the parenchyma or adhered to a multifunctional biomaterial scaffold. We also generated a radial glial NP cell line to conditionally secrete IGF-1; these genetically engineered NPs were attached to a multifunctional scaffold and injected during the subacute period after a moderate cortical contusion. We evaluated the

capacity of transplanted NPs to produce neurons and glia, to ameliorate TBI-induced damage and to improve neurological function. Our hypothesis was that the scaffold would improve the engraftment of the transplanted NPs and that the conditional secretion of IGF-1 would further improve recovery of the ailing brain.

## Methods

### Reagents

Chitosan (low molecular weight ~50 kDa), heparin sodium salt from bovine intestinal mucosa, MTT (3-[4,5-dimetylthiazol-2-yl]-2,5-dipheniltetrazolium), 4′, 6′-diamidino-2-phenylindole, and rhodamine-conjugated phalloidin were purchased from Sigma (St Louis, MO). Genipin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Recombinant human-fibroblast growth factor-2 (FGF-2) was purchased from Peprotech (Rocky Hill, NJ). NT-3 and brain-derived neurotrophic factor (BDNF) were purchased from Alomone Labs (Jerusalem, Israel). Nitrocellulose and NuPAGE 4% to 12% Bis-Tris Gels were purchased from Life Technologies (Carlsbad, CA).

### Chitosan Scaffold

Chitosan powder (1.5 g) was dispersed in 50 mL of 2.0% acetic acid to create a 3% chitosan solution. The chitosan solution was mechanically stirred at 700 rpm until completely dissolved. The resulting solution was collected and centrifuged at 2,000 rpm for 10 min. The supernatant was collected, and the pelleted impurities were discarded.

### Chitosan Microsphere

Microspheres were formed by extruding a 3% acid chitosan solution through a syringe with a 30-gauge needle into a basic coagulation bath, consisting of 2.5 M sodium hydroxide: methanol: water (20:30:50 v/v). To create smaller spheres, the electrospray method was implemented as previously described (Skop et al., 2016). The spheres were removed from the ionic solution and rinsed four times in distilled water, sterilized in 70% ethanol for 30 min, rinsed three times in distilled water, and once with phosphate-buffered saline.

### Chitosan Surface Chemistry Modification

Chitosan films or microspheres were rinsed in distilled water. To cross-link the heparin to the scaffolds, chitosan was incubated overnight (ON) with 0.5 mg/ml heparin and 0.45 mM genipin in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma) buffered solution containing 0.9% NaCl pH 7.4 HEPES buffered saline (HBS), as previously described (Skop et al., 2013). The following day the heparin cross-linked chitosan films or spheres were rinsed three times 10 min in HBS and incubated for 4 h with 10 μg/ml fibronectin (BD Biosciences, San Jose, CA) and for 2 h in 1 μg/ml FGF-2 in 1 mg/ml bovine serum albumin (Thermo Fisher, Waltham, MA) solution. Spheres were then centrifuged at 730 *g*, the supernatant discarded, and spheres were rinsed in HBS to remove any unbound FGF-2. They were resuspended in Neural Progenitor Media (NPM) containing DMEM/F12 media supplemented with B27 (Gibco, Grand Island NY), 50 μg/ml gentamycin (Gibco), and 50 μg/ml apo-transferrin (Sigma). Chitosan films were rinsed once in HBS to remove unbound FGF-2.

### Establishing NPs for Transplantation

VZ NPs were harvested from embryonic day 13.5 enhanced green fluorescent protein (Sprague-Dawley-Tg (green fluorescent protein [GFP])Bal/2Rrrc [RRRC:0065] rat neocortex, [Missouri Research Animal Diagnostics Laboratory, IDEXX RADIL, Iowa City, IA]). Cells were grown as neurospheres or as monolayers on fibronectin-coated petri dishes with NPM supplemented with 10 ng/ml FGF-2 and 1 ng/ml heparan sulfate (NPM-F). Ten percent of the medium was changed every day and replaced with equal volume of 10X FGF-2 containing media (100 ng/ml). When seeding VZ NPs onto modified chitosan microspheres, cells were seeded at a 20:1 ratio cells over spheres (1,000,000 cells for 50,000 microspheres) and incubated at 37°C ON.

### Primary SVZ Progenitors

SVZ NPs were harvested from postnatal day 2 to 4 enhanced green fluorescent protein rat neocortex. Cells were grown as neurospheres in NPM supplemented with 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rockhill, NJ; NPM-E+F). Fifty percent of the medium was changed every other day and replaced with equal volume of NPM-E+F.

### RG3.6 Cell Line

The RG3.6 cell line, which was generously provided by Dr. Martin Grumet (Rutgers University, Piscataway), was immortalized by stably transfecting the v-myc gene into RG cells harvested from embryonic cortex of GFP-positive rats (Hasegawa et al., 2005). They were propagated on matrigel-coated plates in DMEM/F12 supplemented to 10 μg/ml FGF-2, 1 ng/ml heparin sulfate, and 1X B-27 (RGM-FGF). A stable RG3.6-IGF-1 cell line was generated by transducing the conditional lentiviral construct pSLIK-Hygro-IGF-1-HA and selecting clones with hygromycin. Eighty-two clones were picked, expanded, and cultured in doxycycline (DOX)

**Table 1.** Primary Antibodies Used in Immunofluorescent Staining.

| Antibody | Immunogen | Supplier | Species | Dilution | Research resource identifiers |
|---|---|---|---|---|---|
| anti-Neuronal Class III β-Tubulin | Raised against microtubules derived from rat brain, clone TUJ1 | Covance | Mouse monoclonal IgG2a | 1:500 | AB_2313773 |
| Anti-O4 | Supernatant collected from B cells fused with immortalized O4 hybridoma cells | Developed in-house | Mouse monoclonal IgM | 1:4 | AB_2619717 |
| Anti-GFAP | Isolated from cow spinal cord | DAKO | Rabbit polyclonal IgG | 1:500 | AB_10013382 |
| Anti-GFAP | Recombinant peptide of human GFAP protein | Cell Signal. Tech. | Rabbit monoclonal IgG | 1:500 | AB_2631098 |
| Anti-Parvalbumin | Purified from frog muscle | Millipore | Mouse monoclonal IgG1 | 1:1000 | AB_2174013 |
| Anti-Somatostatin | Synthetic 1-14 cyclic somatostatin conjugated to bovine thyroglobulin using carbodiimide | Millipore | Rat monoclonal IgG2b | 1:50 | AB_2255365 |
| Anti-Calbindin | Recombinant mouse calbindin | Millipore | Rabbit polyclonal IgG | 1:1000 | AB_2068336 |
| Anti-Calretinin | Recombinant rat calretinin | Millipore | Rabbit polyclonal IgG | 1:500 | AB_2068506 |
| Anti-FoxP2 | Synthetic peptide conjugated to KLH within residues 700 to C-terminus of human FOXP2 | Abcam | Rabbit polyclonal IgG | 1:4000 | AB_2107107 |
| Anti-Tle4 | Synthetic peptide derived from an internal sequence of human TLE4 | Abcam | Rabbit polyclonal IgG | 1:200 | AB_2203850 |
| Anti-Cutl1 | Recombinant fragment corresponding to amino acids 521–621 of human CUTL1 | Abcam | Mouse monoclonal IgG1 | 5μg/1mL | AB_941209 |
| Anti-Reelin | Recombinant fusion protein, corresponding to amino acids 164-496 of mouse Reelin | Abcam | Mouse monoclonal IgG2 | 1:1000 | AB_444539 |
| Anti-Nestin | homogenized Sprague-Dawley rat spinal cord embryonic day 15 (Rat 401) | DSHB | Mouse monoclonal IgG1 | 5μg/2mL | AB_2235915 |
| Anti-BLBP | Synthetic peptide conjugated to KLH derived from within residues 1–100 of Mouse BLBP | Abcam | Rabbit polyclonal IgG | 1:1000 | AB_10711451 |
| Anti-Ki67 | Recombinant fusion protein corresponding to a 1086bp cDNA fragment containing Ki67 motif | Vector Labs | Rabbit polyclonal IgG | 1:1000 | AB_2336545 |
| Anti-DCX | C-terminus of human doublecortin | Santa Cruz | Goat polyclonal IgG | 1:100 | AB_2088491 |
| anti-Olig2 | Recombinant mouse Olig-2 | Millipore | Rabbit polyclonal IgG | 1:500 | AB_2299035 |
| Anti-Vimentin | Mouse hybridoma(clone v 9) | Millipore | Mouse monoclonal IgG1 | 10μg/ml | AB_94843 |
| Anti-NeuN | Purified cell nuclei from mouse brain | Millipore | Mouse monoclonal IgG1 | 1:400 | AB_2314889 |
| Anti-S100B | Mouse ascites fluid | Sigma | Mouse monoclonal IgG1 | 1:1000 | AB_1856538 |
| Anti-MAP2 | Recombinant human MAP2 | Abcam | Chicken polyclonal IgY | 1:5000 | AB_2138153 |
| Anti-HA | Recombinant peptide (YPYDVPDYA) of human Influenza virus hemagglutinin (HA) | Sigma | Rabbit polyclonal IgG | 1:1500 | AB_260070 |
| Anti-β-Actin | Cytoskeletal preparation from Gizzard lysate | DSHB | Mouse monoclonal IgM | 1:1500 | AB_528068 |
| Anti-Phospho-Akt (Thr308) | Recombinant phosphopeptide of mouse pThr308-Akt | Cell Signal. Tech. | Rabbit monoclonal IgG | 1:1000 | AB_10695743 |
| Anti-Akt (pan) | Recombinant carboxy-terminal peptide of mouse Akt | Cell Signal. Tech. | Rabbit monoclonal IgG | 1:1000 | AB_915783 |

medium ON to stimulate expression of the IGF-1-HA transgene. Clones that tested positive for transgene expression were expanded and used for differentiation and transplantation experiments.

### *Cell Differentiation*

Primary VZ, SVZ NPs, or engineered RG3.6 cells were seeded onto separate poly-L-lysine and laminin-coated dishes and maintained for 24 h in NPM-F or

NPM-E+F, respectively. Cells were differentiated by removing the mitogens FGF-2 and EGF from the media. Seven days later, cells were fixed with 3% paraformaldehyde (PFA) and stained for tripotentiality using mouse monoclonal class III beta-tubulin (βIIITub) antibody, rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antiserum, and mouse O4 monoclonal supernatant (Table 1) to identify neurons, astrocytes, and oligodendrocytes. VZ and SVZ NPs also were differentiated *in vitro* in NPM supplemented with neurotrophic factors NT-3 (30 ng/ml) and brain-derived neurotrophic factor (20 ng/ml) to examine neuronal subclasses. Cells were fixed 4 days later in 3% PFA and stained with cortical laminar specific markers FoxP2, Tle4, Cutl1, and Reelin (Table 1). They were also analyzed for interneuron markers calbindin, calretinin, parvalbumin, and somatostatin (Table 1). Neurons, astrocytes, and oligodendrocytes generated *in vitro* from SVZ NPs and VZ NPs were manually counted in three separate experiments with four fields per well for each condition. Images were acquired at 20X with an Olympus Provis AX70 microscope.

For quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses, differentiated RG cells were lysed and their total RNA isolated using TRIZOL reagent (Thermofisher, Waltham, MA). Two μg of total RNA was used for cDNA synthesis using the single strand cDNA Synthesis kit (Thermofisher). cDNA was diluted 10-fold and used for quantitative PCR analyses using SYBR-green (Thermofisher) in a Roche Lightcycler 480. Primer sequences used for RT-PCR analyses are listed in Table 2.

## Controlled Cortical Impact

Two-month old adult wild-type Sprague Dawley male rats were anesthetized by intraperitoneal injection of ketamine or xylazine mixture (90 mg/kg and 10 mg/kg). The head was shaved, and a midline incision made through the scalp using a scalpel. The skin was deflected, and a craniectomy was made using a 5-mm diameter trephine. The trephine was placed midway between Bregma and Lambda, with the edge of the trephine adjacent to midline. Cold phosphate-buffered saline was suffused onto the surface of the skull during the craniotomy to reduce the generation of heat that could cause damage to the underlying dura mater and neocortex. The skull flap was removed, and the animal placed into a stereotactic apparatus under the controlled cortical impactor device (eCCI 6.3 device built by Custom Design and Fabrication, Richmond, VA). A focal brain injury was produced using a 3.5-mm diameter anvil that impacted the exposed dura mater at a velocity of 4.0 m/s, to a depth of 1.5 mm, with a duration of deformation of 150 msec. After impact, the integrity of the dura mater was confirmed, and the scalp incision sutured with 4-0 nylon thread. Buprenorphine (0.05 mg/kg, subcutaneously [SC]) was administered postoperatively, and the rats were placed on heating pads at 37° and monitored continuously for 2 h after surgery. In addition, immediately

**Table 2.** RT-PCR Primer Sequences Used.

| Function | Primer set or gene name | Forward sequence | Reverse sequence |
|---|---|---|---|
| Housekeeping | β-Actin | CTACAATGAGCTGCGTGTGGC | CAGGTCCAGACGCAGGATGGC |
| | GAPDH | TGCACCACCAACTGCTTAGC | GGCATGGACTGTGGTCATGAG |
| Lineage specification | Nestin | CAGCGTTGGAACAGAGGTTGG | TGGCACAGGTGTCTCAAGGGTAG |
| | GFAP | GTACCAGGACCTGCTCAAT | CAACTATCCTGCTTCTGCTC |
| | MAG | TCTGGATTATGATTTCAGCC | GCTCTGAGAAGGTGTACTGG |
| | Tuj1 | ATGAGGGAGATCGTGCACAT | GCCCCTGAGCGGACACTGT |
| | MAP2 | CCACCTGAGATTAAGGATCA | GGCTTACTTTGCTTCTCTGA |
| Interneuronal specification | Calbindin | GCAAACAAGACTGTTGATGACAC | AAGAGCAAGATCCGTTCGGTAC |
| | Calreticulin | TGATCCCACAGACTCCAAGC | TCAGCGTATGCCTCATCGTT |
| | Parvalbumin | GTGGGGCCTGAAGAAAAAGAGTG | GTCCTTGTCTCCAGCAGCCATC |
| | GABA or GAD1 | GTCGAGGACTCTGGACAGTA | GGAAGCAGATCTCTAGCAAA |
| Cortical specification | Reelin | AAGCTTTGGCAGTGCCAGACT | AAAGGACGTGATTAGCTGCCG |
| | Cutl1 | GGTTTAAATTGATGTTTTATG | CTCGTTCAAGAGTAAAGC |
| | CTIP2 | CAGAGCAGCAAGCTCACG | GGTGCTGTAGACGCTGAA |
| | FoxP2 | CAACAGCAGCAGCAGCAAC | GAGGCCCCAGTCTCCTCA |
| | Cux1 | ATTGATGTTCCAGATCCCGTAC | CTCGTTCAAGGTCAGTCATAATCA |
| Hippocampal specification | PROX1 | GACTTTGAGGTTCCAGAGAG | GTAGGCAGTTCGGGGATTTG |
| | TBR1 | GGAGCTTCAAATAACAATGG | GAGTCTCAGGGAAAGTGAAC |

after surgery, all subjects received 3% body weight of 0.9% saline SC to prevent dehydration.

### Subacute NP Transplantation

*Cells only.* At 7 days postinjury, the animals were reanesthetized with isoflurane, the scalp incision reopened, and a cell suspension containing $5 \times 10^5$ cells/μl with 0.1 μm rhodamine fluorospheres was stereotactically injected into the ipsilateral peri-injured cortex at three sites around the perimeter of the injury. One microliter of cell suspension was injected at each site. The skull was sutured closed, and the animals were allowed to recover on a heating pad until they could ambulate independently. All subjects received 3% body weight of 0.9% saline SC to prevent dehydration.

*Cells on microspheres.* Transplantations were performed 7 days after CCI injury. The animals were anesthetized by intraperitoneal injection of ketamine or xylazine mixture, and the sutures were removed to expose the skull. Cell-microsphere complexes were collected from culture dishes and resuspended in phenol-red free culture medium without supplements. A cell or sphere suspension containing approximately $1 \times 10^4$ cells adhered to ~500 microspheres was loaded into a 10 μl Hamilton syringe with a 21 gauge needle. The needle was stereotactically lowered into the center of the injury cavity at three different depths: 1.5, 1.0, and 0.5 mm below the dura mater. One microliter was injected at each depth more than 5 min, with 5-min intervals between each injection. The needle was withdrawn 10 min after the last injection. The scalp incision was sutured with 4-0 nylon thread, and the animals placed onto a 37°C heating pad until they were fully awake. All subjects received 3% body weight of 0.9% saline SC to prevent dehydration. For experiments to assess whether NPs genetically engineered to conditionally produce IGF-1 would improve neurological function, 1 mg/ml DOX dissolved in 5% sucrose was provided to the rats in their drinking water for beginning the day after the CCI and continuously for the following 28 days.

### Immunofluorescence on Brain Sections

Rats were perfused at 3 days, 2 weeks, and 4 weeks post-transplantation using phosphate buffered 4% PFA and then postfixed by immersion ON. Brains were cryoprotected by immersion in 30% sucrose in $dH_2O$. After one change of sucrose solution, the brains were placed into plastic cryomolds and frozen in optical curing temperature embedding medium in a dry-ice-ethanol slush. The brains were cryosectioned coronally at 40 μm thickness and incubated in blocking solution containing either normal donkey serum or normal goat serum. Sections were stained using a variety of primary antibodies as summarized in Table 1. The sections were then extensively rinsed and incubated in appropriate secondary antibodies 1:300 (Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature. All secondary antibody combinations were carefully examined to ensure that there was no bleed through between fluorescent dyes or cross-reactivity between secondary antibodies. No signal above background was obtained when the primary antibodies were replaced with preimmune sera. After secondary antibody incubation, the sections were washed, counterstained with 1 μg/ml 4′, 6′-diamidino-2-phenylindole for 5 to 10 min, and coverslipped with GelMount (Biomeda, Foster City, CA). Sections were imaged on a Nikon A1R laser scanning confocal microscope. A total of five brains were analyzed for each time point. Exposure times for capturing negative controls were identical to the exposure times for the images. Images were assembled into montages using Photoshop CS5.1.

### Behavioral Analysis

At 7, 14, and 28 days postinjury and transplantation, rats were subjected to beam walking test to assess their sensorimotor function. Rats were acclimatized for 30 min prior to placing them individually at the end of wooden beams. Three beams of 80 cm in length and 50 cm above the ground with different widths were used: 5 (wide), 2.5 (medium), and 2 cm (small). A dark box with food at the other end of the beam was put to serve as a target and increase animal's motivation. The time to cross the beams as well as the number of foot slips (both hind legs and front legs were recorded and analyzed).

### Statistical Analysis

Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test to detect significant differences between the means with $p < .05$. Analyses were performed using GraphPad Prism. Statistical significance was set at $p$ values of $< .05$.

## Results

### Tripotentiality of VZ Versus SVZ NPs

Prior to initiating cell transplantation studies, we set out to define several parameters, such as whether one NP population was biased toward generating neurons or glia, which NPs would generate the greatest number of neurons and whether their regenerative capacity was affected by expansion *in vitro*. To evaluate which NP subtype could generate the greatest number and variety of neurons, VZ, SVZ NPs, and RG3.6 cells were differentiated by growth factor withdrawal *in vitro*. Primary and tertiary VZ and SVZ NPs were compared to assess

the effect of passaging these NP populations. Equal numbers of cells were transplanted for each cell type. Primary VZ NPs generated greater numbers of neurons than primary SVZ NPs. However, upon passaging, the ability of the VZ NPs to generate neurons decreased. However, even third passage VZ NPs produced more neurons than the SVZ NPs. As shown in Figure 1, primary VZ NPs generated ~60% neurons and ~40% astrocytes upon differentiation, whereas tertiary passaged VZ NPs generated ~8% neurons, 80% astrocytes, and 12% oligodendrocytes. When primary SVZ NPs were differentiated *in vitro*, they generated ~13% neurons, ~78% astrocytes, and 9% oligodendrocytes. The numbers of SVZ NP-derived neurons decreased during passaging to a much lower percentage than passaged VZ NPs (to 3% from tertiary cultures). Concomitantly, there was a larger production of oligodendrocytes (7%) while maintaining a very high percentage (90%) of astrocytes. Altogether, these studies recommended VZ NPs as the more appropriate cell type for neocortical cell

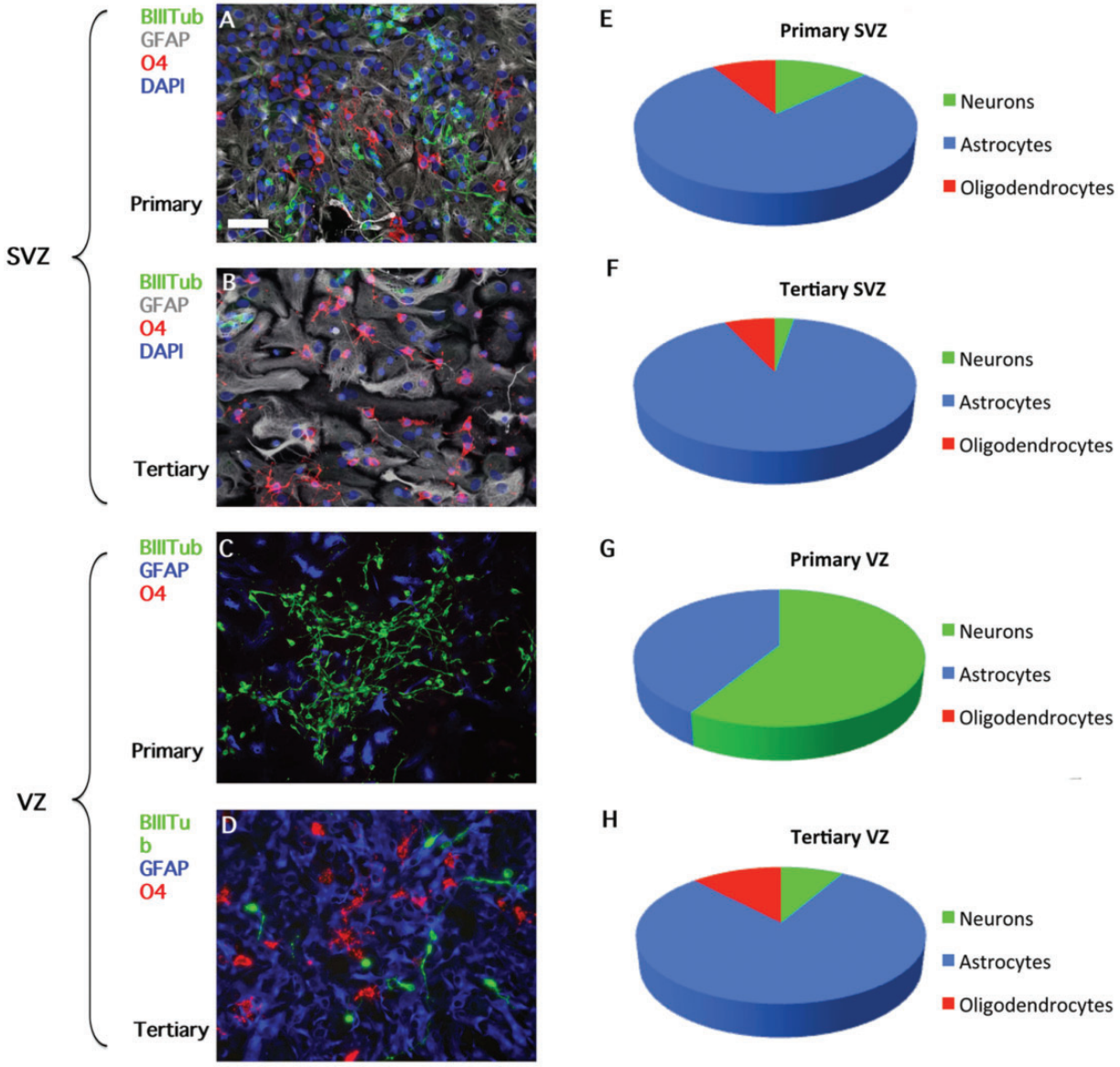


**Figure 1.** Potentiality of SVZ- and VZ-derived neural progenitors differs and becomes restricted with passaging. Multipotentiality of (a) primary and (b) tertiary SVZ NPS. Cells were differentiated *in vitro* for 7 days and stained for βIII-Tubulin (βIII-Tub, green), GFAP (white), and O4 (red). Multipotentiality of primary (c) and (d) tertiary VZ NPs. Cells were differentiated *in vitro* for 7 days and stained for βIIITub (green), GFAP (blue or magenta), and O4 (red). (e–h) Percentages of neurons (βIIITub) astrocytes (GFAP) and oligodendrocytes (O4) in VZ NPs and SVZ NPS cultures after serial passaging. Images were acquired at 20× magnification. Assay was performed in triplicate in three independent experiments. SVZ = subventricular zone; GFAP = glial fibrillary acidic protein; VZ = ventricular zone.

replacement for TBIs where the goal is to replace damaged neurons and glia.

### *Cortical Marker Expression in Differentiated NPs*

Since a large percentage of the cortical neurons lost after TBI are pyramidal neurons, we evaluated the potential of the VZ NPs versus the SVZ NPs to differentiate into pyramidal versus nonpyramidal type neurons. Primary VZ NPs and SVZ NPs were differentiated after 4 days *in vitro* and stained for markers of neocortical pyramidal neurons and interneurons. SVZ NPs were negative for almost all the cortical laminar markers that included Cutl1, Tle4, and FoxP2 (Supplementary Figure 1(a) to (d)). By contrast, differentiated primary VZ NPs stained positively for markers of specific cortical layer neurons: Reelin, Layer 1 (Supplementary Figure 1(e)), Cutl1, Layer 2/3 (Supplementary Figure 1(f)), and Tle4 and FoxP2, Layers 5/6 (Supplementary Figure 1(g) and (h)).

Since the medial ganglionic eminence (MGE) produces interneurons during development and the SVZ is a remnant of the ganglionic eminences, we hypothesized that the majority of SVZ NPs generated neurons would be interneurons. Indeed, most SVZ-derived neurons were positive for the interneuron markers calbindin and calretinin (Supplementary Figure 1(j) and (k)); however, they were negative for somatostatin and parvalbumin (data not shown).

### *VZ NPs Are More Primitive and Neurogenic*

To further determine which NP types would be most appropriate for transplantation procedures, we analyzed the transcription factors and progenitor cell protein levels as well as gene profiles from NPs isolated from the VZ and SVZ. RT-qPCR analyses, shown in Figure 2(a) to (d), revealed that secondary VZ NPs were more primitive than secondary SVZ NPs. In particular, VZ NPs expressed twofold higher levels of FABP7 or brain lipid-binding protein (BLBP) and SOX 2. Corresponding with the immunostaining results in Figure 1, VZ NPs were also more neurogenic than SVZ NPs, expressing greater levels of early progenitor cell marker, Mash1 (ASCL1) and significantly lower levels of the early glial progenitor cell marker, Olig 2. Western blot analyses also revealed VZ NPs to have higher protein levels of BLBP, the cell proliferation protein PCNA, and neuronal cytoskeletal protein βIII-Tubulin, while SVZ NPs expressed 5- to 10-fold higher levels of the glial markers GFAP and Olig2 (Figure 2(e)).

### *Greater Survival of VZ NPs Following Subacute Transplantation After TBI*

We sought to determine whether NPs derived from the VZ could withstand the caustic conditions of the injured brain better than SVZ NPs. VZ and SVZ NPs were prepared from GFP-expressing transgenic rats and cultured for 1 week. They were transplanted into the brains of rats 7 days after CCI and evaluated 1 month following transplantation (Figure 3(a) and (b)). We chose to transplant the NPs at 7 days after the CCI as prior to 7 days, there is significant neuroinflammation, and previous transplantation studies have shown that transplanted cells do not survive well because of the presence of cytotoxic cytokines, free radicals, and phagocytic microglia. Beyond 7 days, the glial scar becomes well ensconced and this scar will impair neurite extension, which is necessary for synaptogenesis and ultimately the survival of the engrafted neurons (Skop et al., 2014). Thus, the 7-day time point seemed an appropriate time point. At 1 month after transplantation, very few $GFP^+$ SVZ NPs were observed and those $GFP^+$ cells that were seen were located near the injury site. By contrast, numerous $GFP^+$ VZ NPs engrafted and survived for this 1-month interval. However, they tended not to disperse into the adjacent host brain tissue (Figure 3(c) and (d)). When volumes of the grafts were evaluated, the VZ NPs produced greater than threefold the numbers of new cells compared with the SVZ NPs (Figure 3(e)).

### *SVZ NPs Generate Glia or Remain Undifferentiated Posttransplantation After TBI*

One month-posttransplantation brains engrafted with $GFP^+$ SVZ NPs (Figure 3(f) to (i)) immunostained positively for the immature marker Nestin (Figure 3(f)) suggesting that few of the transplanted cells had differentiated; however, a few cells were positive for GFAP (Figure 3(g)) or NG2 (data not shown). Cells descended from the $GFP^+$ SVZ NPs were negative for the neuronal markers doublecortin and neuronal nuclei (NeuN), and they did not express the mature oligodendrocyte marker GSTπ. Primary, secondary, and tertiary SVZ NPs all produced similar *in vivo* differentiation and survival results.

### *VZ NPs Generate Neurons or Glia Posttransplantation After TBI*

One month-posttransplantation brains engrafted with $GFP^+$ VZ NPs (Figure 3(j) to (m)) were immunostained for markers of progenitors, neurons, and glia. Consistent with the *in vitro* data reported in Figure 1, the NPs that had been passaged fewer times produced greater numbers of neurons. $NeuN^+$ cells comprised most of the cells obtained from the primary $GFP^+$ VZ cultures (Figure 3 (j)). Tertiary VZ NPs (Figure 3(r) to (u)) produced predominantly $Nestin^+$ and $GFAP^+$ cells (Figure 3(r)). Although much less common, analysis of tertiary $GFP^+$ VZ NPs also revealed differentiation into some $NG2^+$

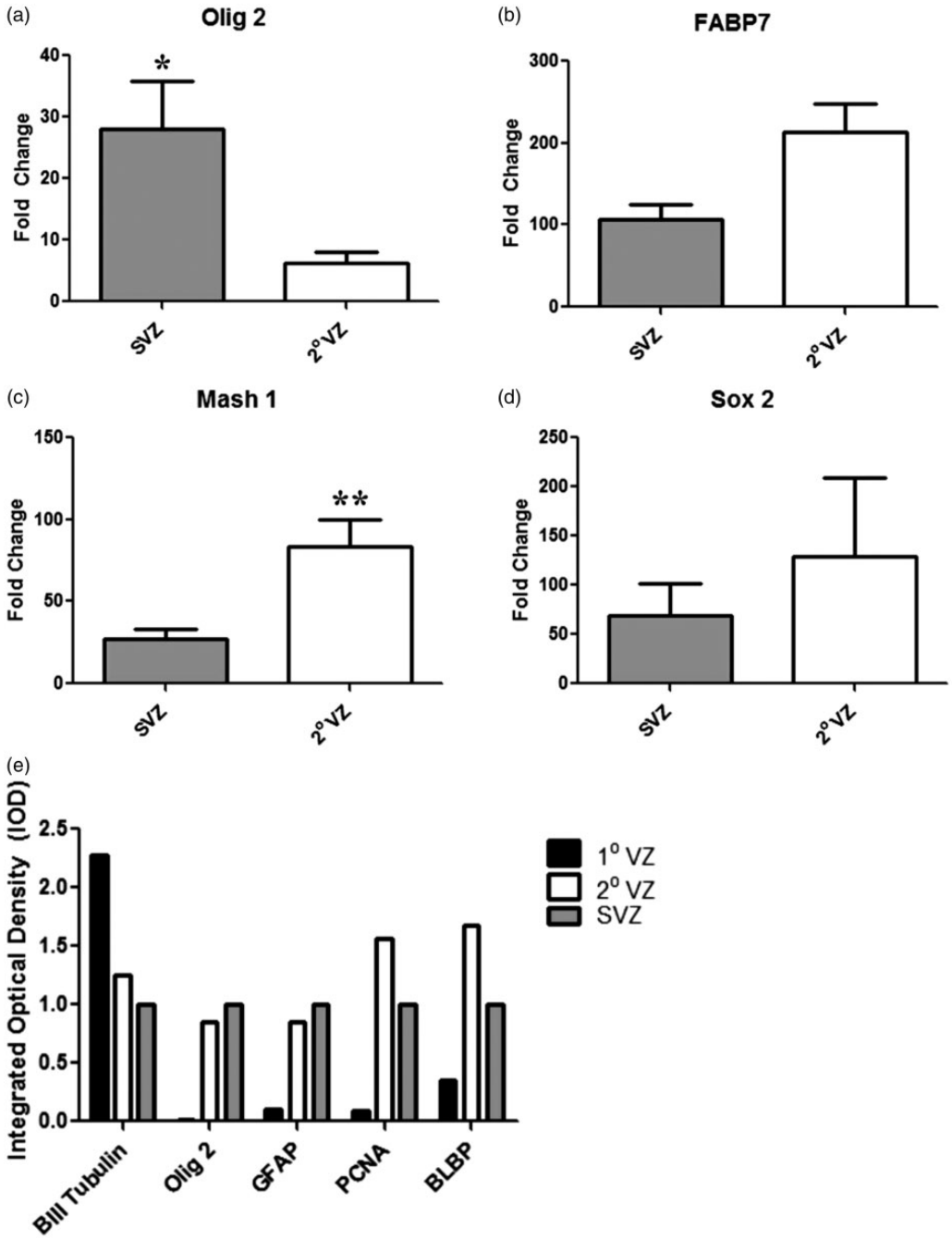


**Figure 2.** Differences between SVZ and VZ cells *in vitro*. Secondary SVZ cells and VZ cells were collected for mRNA (a–d) and protein (e) analysis of stem and progenitor cell genes. Expression levels of RNAs were analyzed by q-PCR. (a) Olig2 and Mash1 (b) are expressed by multipotential progenitors, while FABP7 (c) and Sox2 (d) are markers of more primitive cells. (e) Quantitation of protein levels by Western blot comparing SVZ cultures to primary and 2° passaged VZ cultures. Levels of βIII Tubulin (neurons), GFAP (astrocytes), BLBP (FABP7; radial cells), and PCNA (proliferation). Each assay was performed in triplicate from five independent experiments. *$p<.05$ SVZ compared with VZ in Olig2 and **$p<.01$ VZ compared with SVZ in Mash1. GFAP = glial fibrillary acidic protein; VZ = ventricular zone; SVZ = subventricular zone; PCNA = proliferation cell nuclear protein; BLBP = brain lipid-binding protein.

cells and a few $NeuN^+$ cells. While $NeuN^+$ cells were rare, they were never observed in the transplanted SVZ NP group. Neither NP produced mature oligodendrocytes expressing GSTπ or Olig2.

### *Neovascularization Does Not Affect Survival*

Immunostaining for Collagen V was used to determine whether an increase in neovascularization was

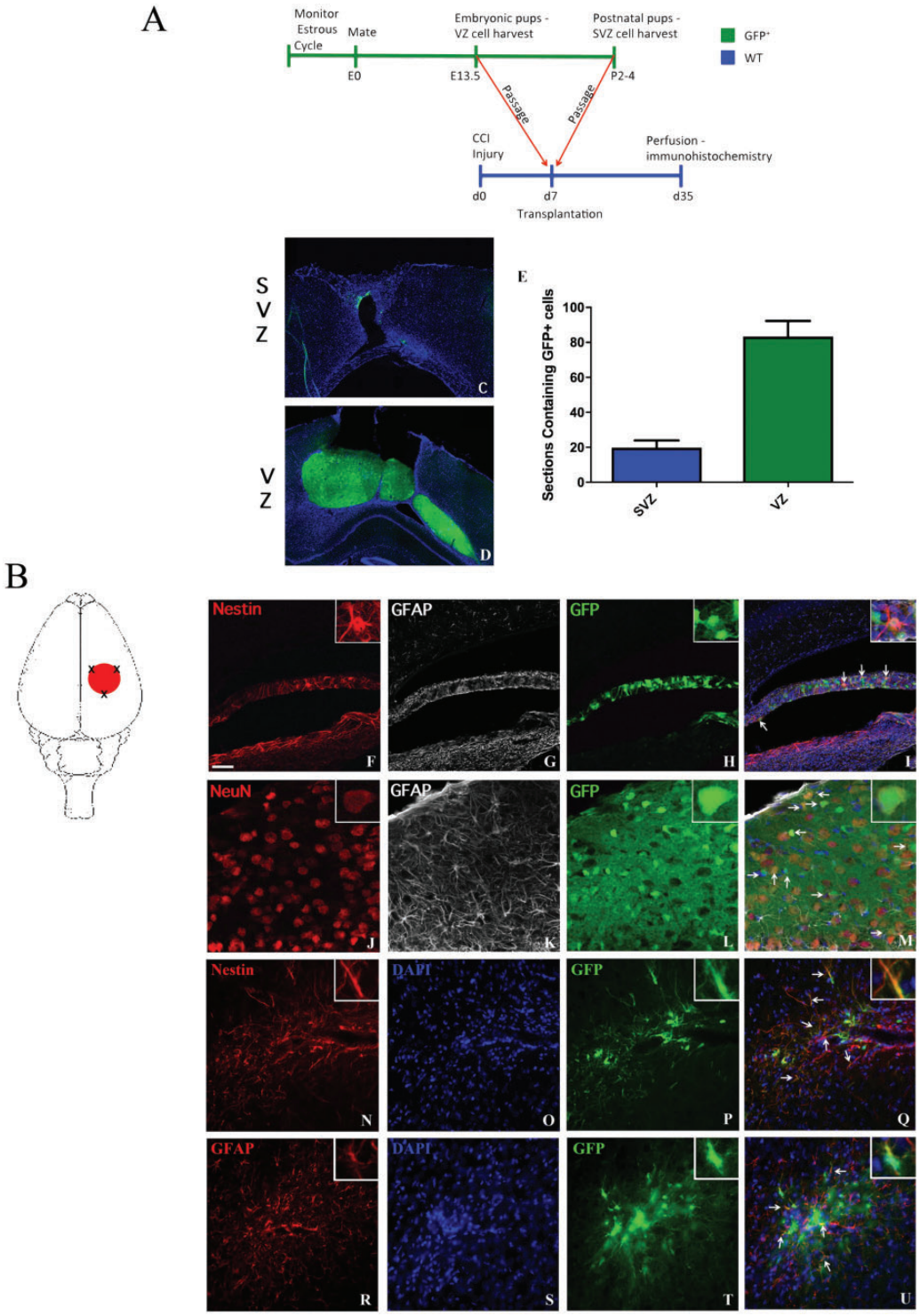


**Figure 3.** One-month posttransplantation of SVZ- and VZ-derived neural progenitors. Cells were culture for 7 days *in vitro*, dissociated and stereotaxically transplanted into CCI-injured rats 7 days posttrauma. Schematic representation of experimental design (a). A total of $1.5 \times 10^6$ $GFP^+$ NPs were injected at three locations around the penumbra as shown in (b). $GFP^+$ SVZ (c) and VZ NPs (d; green) were present within and surrounding the lesion cavity. Quantification of the tissue volume positive for $GFP^+$ after transplantations (e). SVZ NP-derived cells were found positive for Nestin (f–i), and VZ cells were found positive for the neuronal marker NeuN (j–m). $GFP^+$ tertiary VZ cells were positive for Nestin (n–q) and GFAP (r–u). Images were acquired at 10× (c–d) and 20× (f–u) magnification on epifluorescence and confocal microscopes. Scale bar represents 50 μm. $n = 5$ animals or group. VZ = ventricular zone; SVZ = subventricular zone; GFP = green fluorescent protein; CCI = controlled cortical impact; GFAP = glial fibrillary acidic protein.

responsible for the differences in survival between transplanted VZ versus SVZ NPs. Vessel formation was observed within the region surrounding the injured cavity containing GFP+ SVZ NPs (Supplementary Figure 2(a) to (c)). Blood vessels also were present throughout the parenchymal tissue containing transplanted $GFP^+$ NPs (Supplementary Figure 2(d) to (f)). The vessels were approximately the same thickness in each condition. The tubules were, however, longer in the VZ NP transplanted group (Supplementary Figure 2(g)). Western blot analysis revealed that there were no significant differences in vascular endothelial growth factor-A production by secondary SVZ versus VZ NPs *in vitro* (not shown).

### *Generation of Stable Rat NPs (RG3.6 NPs) Conditionally Expressing IGF-1-HA*

To enhance trophic support into the injured brain, we introduced a conditional vector containing the cDNA for a HA-tagged IGF-1 into the RG3.6 NP line. The lentiviral pSLIK-Hygro vector allows for constitutive expression of the reverse tetracycline transactivator that activates a tetracycline response element in response to DOX to drive expression of IGF-1-HA (Figure 4(a)). Upon infecting and selecting RG3.6 NPs with a pSLIK-Hygro-IGF-1-HA lentivirus and inducing expression with DOX, ~96±4% of all cells conditionally expressed IGF-1-HA (Figure 4(b)). Engineered RG3.6 NPs treated with DOX for 24 h efficiently secreted IGF-1-HA to their culture medium, activating protein kinase B (Akt) in an autocrine and dose-dependent manner (Figure 4(c) and (d)).

### *Induced Expression of IGF-1 Increased Neuronal Differentiation From RG3.6 Progenitors*

To assess the effect of DOX-driven expression of IGF-1 on NPs differentiation, engineered RG3.6 NPs were plated onto precoated glass bottom dishes and their differentiation was induced the following day. On the fourth day, significantly more small processes were visible in cells exposed to DOX (to drive IGF-1-HA expression), while on Day 7, more complex neuronal and glial morphologies were observed as well as more robust expression of the respective molecular markers MAP2, βIII Tubulin, O4, or GFAP (Figure 5(a) to 5(d)). Expression of IGF-1 nearly tripled the number of neurons (from 16±4% to 46±7%) simultaneously decreasing the numbers of astrocytes (from 73±8% to 45±6%), while the number of oligodendrocytes seemed unaffected (11±2% vs. 9±3%; Figure 5(e)). Quantitative RT-PCR analyses confirmed that expression of IGF-1 for up to 20 days promoted neurogenesis as indicated by a greater than threefold increase of βIII Tubulin (3.6±0.7 fold) and MAP2 (3.9±0.8 fold) transcripts, with decreases in the astroglial marker GFAP (0.3±0.1 fold) and the oligodendrocytic marker MAG (0.6±0.1; Figure 5(f)). Furthermore, as shown in this figure, IGF-1 expression promoted a modest (between 1.6- and 2.2-fold), but significant increase in interneuron markers (Calbindin, Calretinin, Parvalbumin, and GABA), and more prominent expression (between 2.7- and 3.6-fold over control) of tested hippocampal (PROX1 and TBR1) and cortical markers (Reelin, Cutl1, FoxP2, Cux1, and CTIP2). We conclude that conditional expression of IGF-1 promotes neurogenesis of a variety of neuronal subtypes with cortical neurons being the most prominent group.

### *VZ NPs Proliferate and Express Neural Stem Cell Markers at 3 Days Posttransplantation*

Since engraftment and retention of transplanted NPs was less effective than expected, we attached VZ NPs to multifunctional microspheres, which we have previously demonstrated improved the engraftment of transplanted RG3.6 cells (Skop et al., 2016). For these studies, approximately $3 \times 10^4$ VZ NPs attached to ~500 microspheres (each ~50 μm in diameter) were injected directly into the lesion cavity at 7 days of recovery from CCI (Supplementary Figure 3(a)). As the VZ NPs expressed GFP, they could be readily distinguished from the host cells using fluorescence microscopy. At 3 days after transplantation, microspheres could be recognized by their autofluorescence within the wound cavity adjacent to the host tissue (they auto-fluoresced). Using the optical defractionator and spectral analysis on a confocal microscope, we were able to separate the autofluorescence from the GFP fluorescence. While most VZ NPs were located in the tissue surrounding the microspheres, some remained adhered to the beads at 3 days after transplantation (Supplementary Figure 3(b)). These cells (on and around the spheres) were positive for the stem cell or progenitor markers Nestin (Supplementary Figure 3(d, ii) and (e, ii)) and BLBP (Supplementary Figure 3(f, ii) and (g, ii)). Many $GFP^+$ cells also were positive for the proliferative marker Ki67 (Supplementary Figure 3(h, ii) and (j, ii)).

### *VZ NPs Seeded Microspheres Express Differentiated Markers at 2 Weeks Posttransplantation*

At 2 weeks after transplantation, there was evidence of significant wound repair for those rats that had received microspheres with cells at both the gross level (Figure 6 (a)) and at the microscopic level (Figure 6(c)). Microspheres could be found within the lesion cavity adjacent to the host tissue but there appeared to be fewer spheres than observed at 3 days. $GFP^+$ VZ NPs had now dispersed into the tissue and away from the

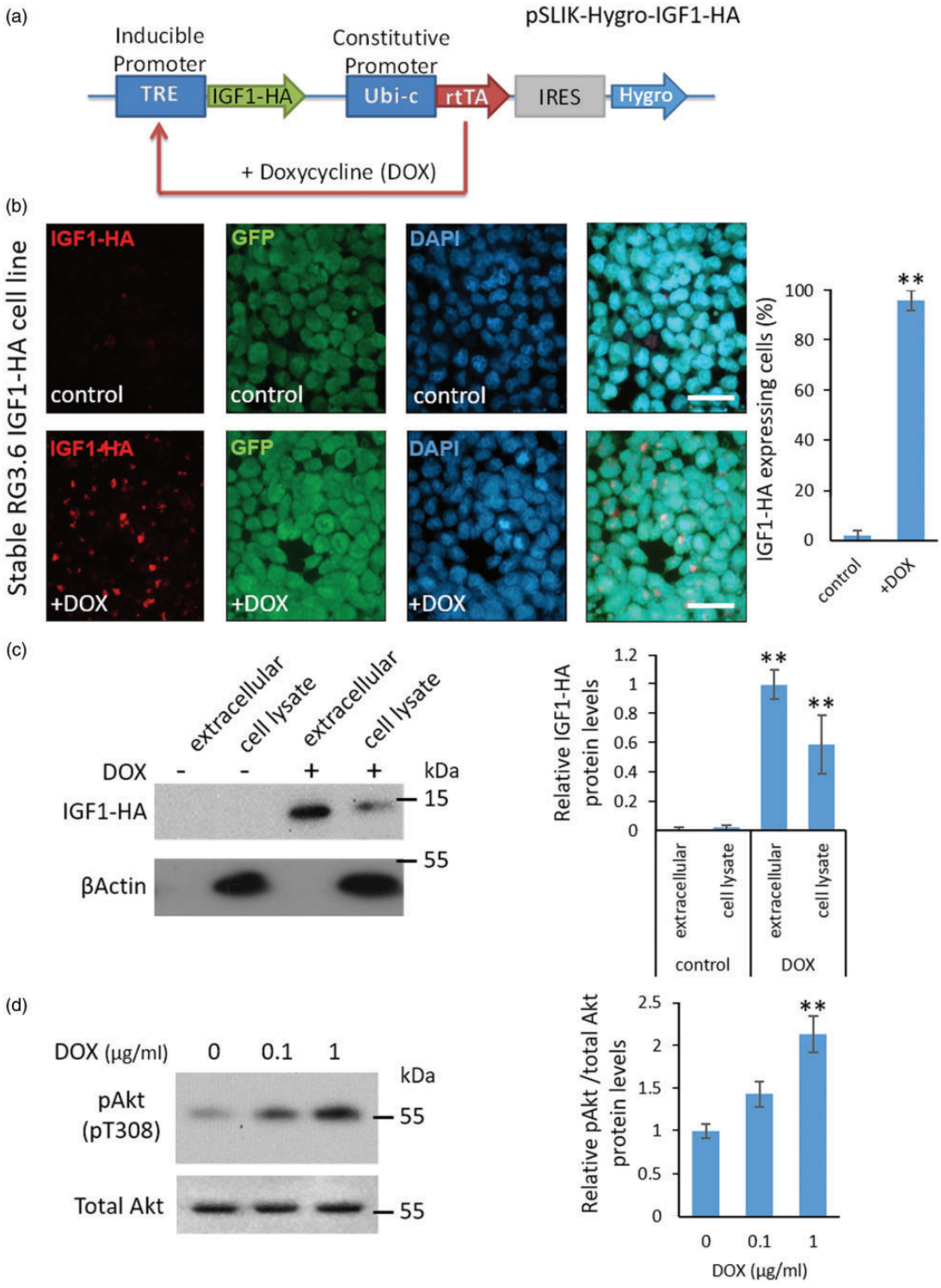


**Figure 4.** Generating stable rat neural progenitor cells (RG GFP+) conditionally expressing IGF-1-HA. (a) Schematics of the conditional pSLIK vector system in which doxycycline (DOX) activates the reverse tetracycline transactivator (rtTA) that binds to the tetracycline response element (TRE) to drive expression of IGF-1-HA. (b) Representative images of lentivirally infected cells versus controls (scale bar = 20 μm). (c) Western blot of cell lysate and of culture medium from pSLIK-IGF-1-HA infected RG3.6 cells probed for HA tag. Cells were untreated or incubated with × μM DOX for 24 h. Adjacent bar graph displays quantitative analysis of the Western blot. (d) Western blot for pAkt (pT308) and total Akt from untreated and DOX-treated cells. Bar graph displays quantitative analysis of the Western blot. Cells were untreated or incubated with 1 μg/ml DOX for 24 h. Scale bars represent 20 μm. Values represent means ± *SEM*. *$p < .05$. **$p < .01$. TRE = tetracycline response element; DOX = doxycycline; IGF-1 = insulin-like growth factor 1; GFP = green fluorescent protein.

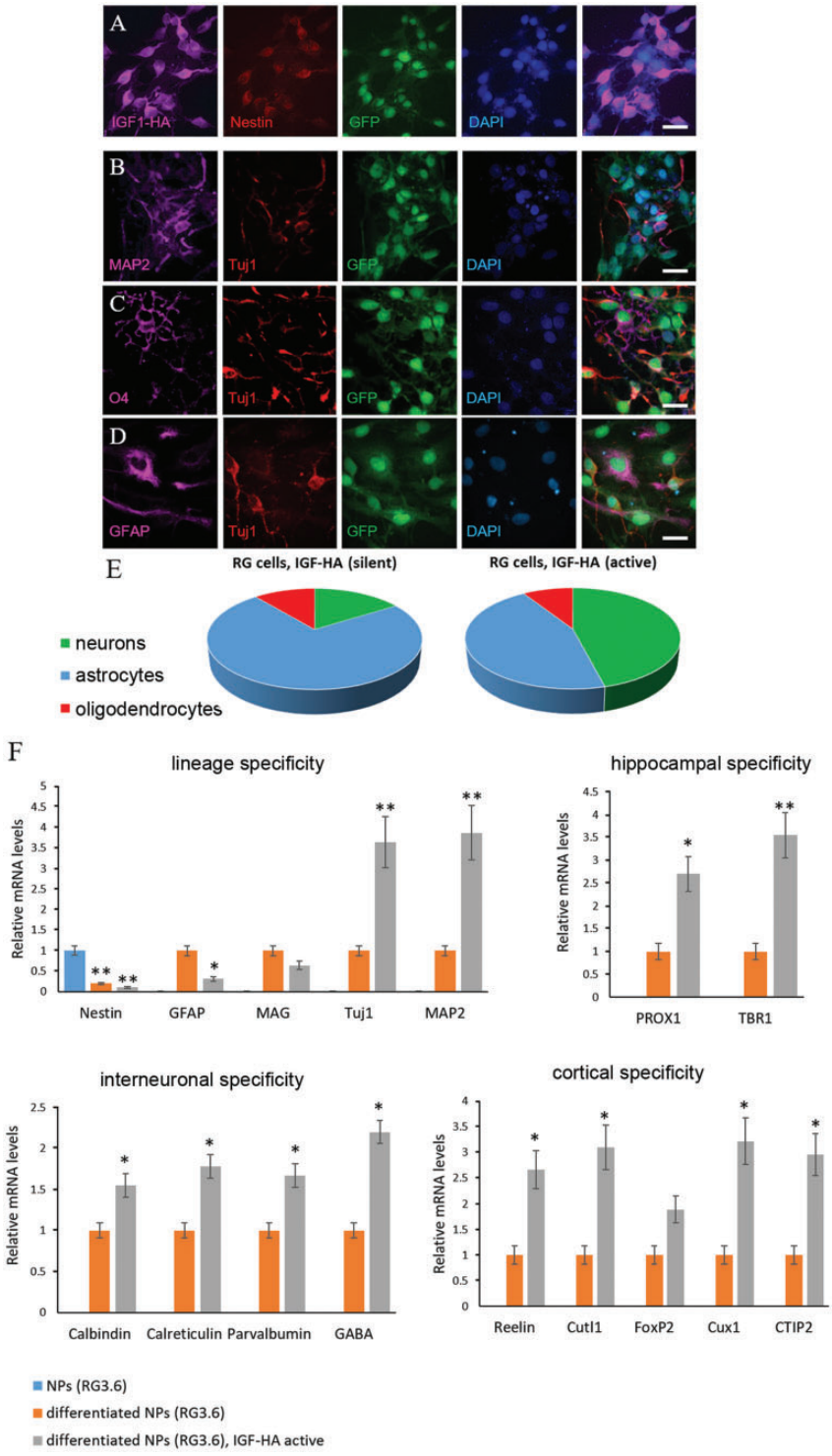


**Figure 5.** Expression of insulin-like growth factor 1 (IGF-1)-HA increases neuronal differentiation from RG3.6 cells. Representative images of cell expressing IGF-1-HA and Nestin (a), MAP2 and βIII Tubulin (b), O4 (c), and GFAP. Cells shown in panels (a–d) were treated with DOX-containing medium to stimulate expression and secretion of IGF-1-HA. (e) Quantification of neurons (Tuj1), astrocytes (GFAP), and oligodendrocytes (O4) after differentiation of pSLIK-IGF-1-HA infected RG3.6 cells in control or DOX-containing differentiation medium (scale bar = 20 μm). (f) IGF-1-HA effects on lineage marker expression after 20 days of differentiation. Doxycycline-mediated expression of IGF-1-HA significantly increased cDNA levels of interneuron, hippocampal, and neocortical neuronal markers. Scale bars represent 20 μm. Values represent means ± *SEM*. *$p < .05$. **$p < .01$. NP = neural progenitors; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein.

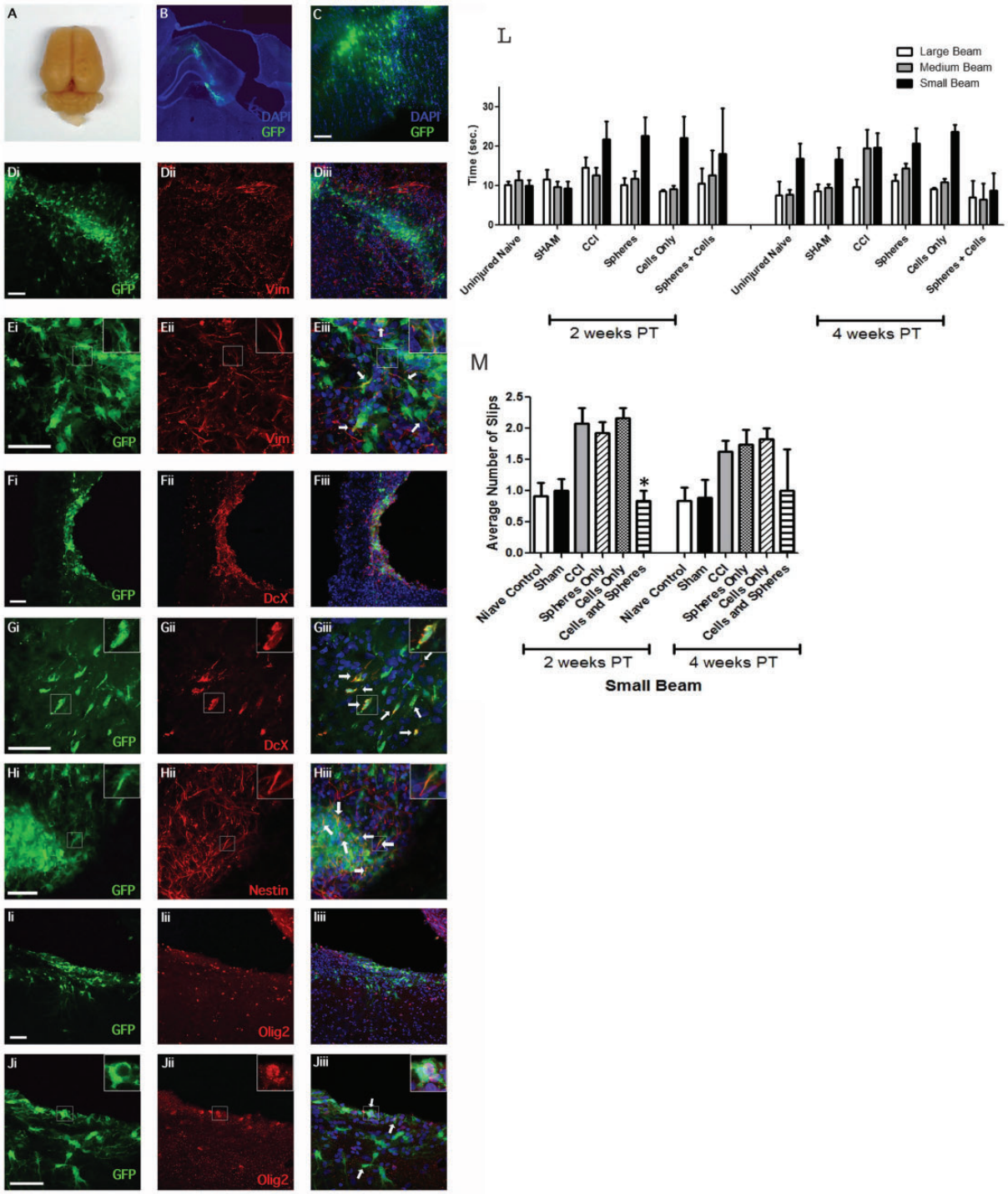


**Figure 6.** NPs transplanted on a multifunctional scaffold develop into neurons, astrocytes, and oligodendrocytes and improve sensorimotor performance. NPs seeded on modified chitosan microspheres were transplanted into CCI-injured rat somatosensory cortices at 7 days post trauma. (a) Appearance of a brain taken 2 weeks posttransplantation. (b–k) confocal images of NPs stained for markers at 2 weeks posttransplantation. VZ NPs were found in tissue sections spanning the injury (b–c). VZ NPs (green) were positive for astrocytic marker vimentin (d, ii to e, ii), neuronal marker DCX (f, ii to g, ii), progenitor marker Nestin (h, ii to i, ii), and oligodendrocyte marker Olig2 (j, ii to k, ii). Five animals were analyzed in this experiment. Scale bars represent 50 μm. Insets depict high power single optical plane confocal images. (l, m) Bar graphs show assessment of sensorimotor function in the beam walking test in which wide medium (5 cm) and small (2.5 cm) beams were used. Recovery after TBI was assessed in animals at 14 and 28 days posttransplantation (PT). Groups included, naïve, sham injured, CCI alone, CCI + spheres, CCI + cells alone, and CCI + spheres with cells. Values represent means ± *SEM*. *$p < .05$. $n = 4$ to 6 animals per group. GFP = green fluorescent protein; CCI = controlled cortical impact.

microspheres compared with the 3-day time point. Observationally, many of the cells spanned several layers of the neocortex, and the lesion cavity was smaller in recipients that had received the VZ NPs on microspheres versus controls that had received cells only, or microspheres only (Figure 6(b) and (c)). $GFP^+$ cells at this time point showed features of differentiation toward mature neural cell types. They began to lose their proliferative stem cell phenotype and tested negative for the Ki67 and the stem cell or progenitor marker BLBP (data not shown). $Nestin^+$ or $GFP^+$ cells were found in areas with higher cell clustering, but they were not as prevalent as the 3-day time point (Figure 6(h, ii)). $GFP^+$ cells were also positive for the neuroblast marker doublecortin (Figure 6(f, ii) and (g, ii)). However, they were negative for NeuN (not shown) suggesting that 2 weeks were insufficient for the cells to fully mature. While many $GFP^+$ cells expressed the immature astrocytic marker, Vimentin (Figure 6(d, ii) and (e, ii)), very few were positive for the more mature astrocytic marker GFAP (data not shown). This pattern held true as the $GFP^+$ cells expressed the oligodendrocyte progenitor marker Olig2 (Figure 6(i, ii) and (j, ii)), while there was no observable expression of the more mature oligodendrocyte marker GSTπ (data not shown). Behavioral studies of rats that received spheres alone, versus cells alone versus microspheres with seeded with VZ NPs showed improved recovery on the beam walking task using the VZ NPs on the spheres versus cells alone (Figure 6(l) and (m)), but the behavioral recovery was transient, as it was no longer evident at 28 days posttransplantation (although there was a trend for behavioral improvement out to 28 days).

## *DOX-Driven Expression of IGF-1-HA in Transplanted RG3.6 NPs Improves Sensorimotor Recovery After TBI*

To evaluate the efficacy of transplanted NPs in preserving the sensorimotor function, several sets of tests were conducted. General behavioral assessments were conducted first. All studied animals showed no symptoms of hemiparesis, and no obvious signs of pain were seen. Since these animals had received an injury to the somatomotor neocortex, we used tests of sensory and motor function. We used three different beam widths, where the task becomes more difficult as the beam width narrows. On the beam walk test, the group of animals that received cells seeded on microspheres and had received DOX in the drinking water (to drive IGF-1-HA expression) crossed the smallest width beam in 15 s, whereas injured animals that had received only spheres required 25 s to cross the beam. Moreover, the improvement in beam walking time was seen at all three time points evaluated (7, 14, and 28 days posttransplantation), and the improvement was statistically different ($p < .05$) compared with the groups that received only cells or microspheres (Figure 7). This was most evident on the smallest beam, which was the most difficult task. Animals in this experimental group also had fewer foot-slips crossing the beam (data not shown) and were closer in performance to the control group in all conducted beam walk tests. Behavioral studies of rats that received microspheres seeded with RG3.6 NPs that had not been genetically engineered showed improved recovery after injury versus controls; however, a statistical significance was not

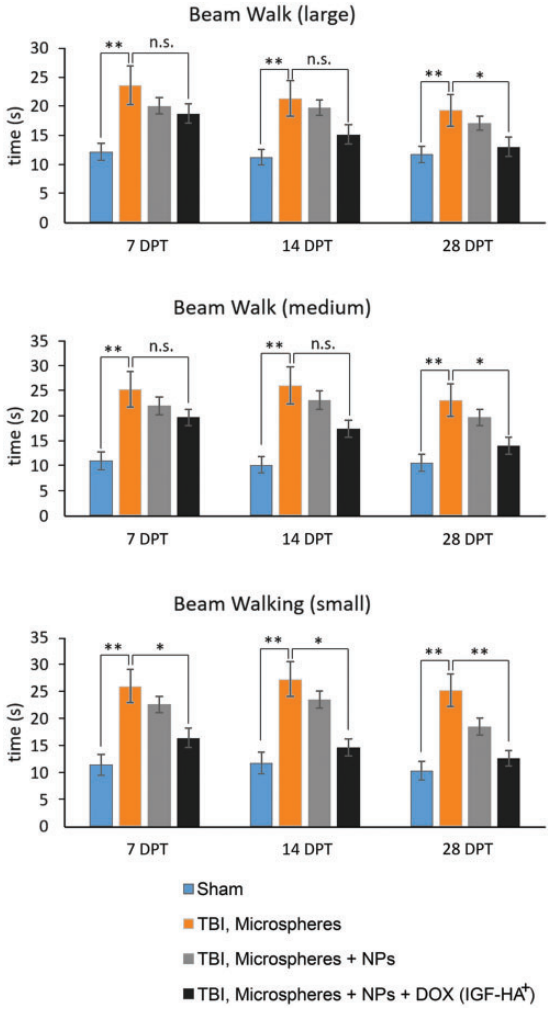


**Figure 7.** Exogenous IGF-1 provided by genetically engineered transplanted RG3.6 NPs on multifunctional microspheres improves sensorimotor recovery. Bar graphs show assessment of sensorimotor function in the beam walking test in which wide (10 cm), medium (5 cm), or narrow (2.5 cm) beams were used. Recovery after TBI was assessed in animals at 7, 14, and 28 days after transplanting days post-transplantation (DPT) microspheres, microspheres with NPs, or microspheres with NPs where the rats were provided doxycycline in their drinking water to drive exogenous IGF-1-HA expression. Values represent means ± *SEM*. $^*p < .05$. $^{**}p < .01$. $n = 3$ to 6 animals per group. NP = neural progenitors; TBI = traumatic brain injury; DOX = doxycycline; DPT = days post-transplantation.

reached. We performed immunofluorescence to detect the GFP+ RG3.6 cells upon completing the behavioral analyses; however, GFP+ cells were extraordinarily rare. We conclude that induction of IGF-1-HA expression improves sensorimotor recovery after brain injury.

### *Neutrophil Activation After Transplantation*

At 3 days posttransplantation, we observed cells surrounding the spheres that did not express markers of neural cells. After testing many markers of immune cells, we determined they were neutrophils based on positive staining for Ly-6G staining and negative staining for Iba-1 (Supplementary Figure 4(a) and (b)). By 2 weeks posttransplantation, there were fewer Ly-6G$^+$ cells (Supplementary Figure 4(c)). Increased phagocytosis of GFP$^+$ VZ NPs or increased immunogenicity was not observed at either time point as evidenced by ionized calcium-binding adapter molecule 1 (Iba-1) staining for macrophages.

## Discussion

The mammalian neocortex is arguably the most complex region of the most complex organ of the body. Consequently, the neocortex will be the most difficult tissue in the body to repair after an injury. Its limited capacity to regenerate is complicated by the inability of mature neurons to undergo mitosis. While the mature CNS harbors stem cells, these adult stem cells have a limited repertoire; thus, stem cell transplantation after injury to the CNS has been regarded as them promising approach for repair. However, empirical studies have shown that engrafting new cells is not sufficient for effective regeneration. Therefore, we have been working to optimize transplantation methods for CNS regeneration. In the important studies reported here, we compared the regenerative potential of NPs from the fetal VZ, the postnatal SVZ. To promote the proliferation and survival of the NPs, we delivered them attached to a multifunctional chitosan-based scaffold developed by our team. Furthermore, to improve neuronal differentiation and survival, we implanted an immortalized radial glia cell line engineered to conditionally secrete IGF-1. Our results support the therapeutic utility of chitosan-based multifunctional microsphere scaffolds seeded with genetically modified NPs expressing IGF-1 to promote repair and functional recovery after TBIs.

The promise of NP transplantation for TBI repair has been substantiated through studies by several groups who have demonstrated the ability of engrafted NPs to differentiate. Hoane et al. (2004) and Gao et al. (2006) reported retention of neurons derived from rodent NPs after CCI and fluid percussion injuries 1 year posttransplantation into the brain (Hoane et al., 2004; Gao et al., 2006). While no significant improvement in cognition was observed, they noted sensorimotor recovery. Alvarez-Dolado et al. (2006) transplanted cells from the MGEs and documented migration and differentiation into cortical GABAergic interneurons. Electrophysiological recordings from local pyramidal neurons indicated functional integration of the transplanted MGE cells into host tissue (Alvarez-Dolado et al., 2006). Some data also suggest that engrafting rodent stem or progenitor cells (Hoane et al., 2004; Shear et al., 2004; Boockvar et al., 2005; Bakshi et al., 2006; Gao et al., 2006) and even human stem cells (Hagan et al., 2003; Al Nimer et al., 2004; Wennersten et al., 2004, 2006) can improve outcome after experimental TBI, albeit the improvement was limited (Longhi et al., 2005; Richardson et al., 2010). Human neurons have even been successfully engrafted into brain-injured rats and have survived for up to 1 year. However, these grafted cells had no impact on behavioral recovery (Watson et al., 2003; C. Zhang et al., 2005). A general aspect of all these studies is that improvements in behavioral tests are rarely dramatic.

A major determinant in producing new neurons from transplanted NPs is their intrinsic bias. Whereas many ongoing clinical trials for TBI and for stroke are using MSCs, the goal of those studies is not to replace the cells that have been damaged or deleted, but to prevent the further demise of cells during the subacute period of recovery. This is because MSCs rarely produce neurons during normal development and thus have a very limited capacity to make functional CNS neurons upon transplantation after CNS injury. Many of the presumptive new neurons seen after MSC transplantation are likely due to fusion rather than to transdifferentiation (Wurmser and Gage, 2002). Therefore, we have pursued a complementary strategy of intraparenchymal transplantation for cell replacement.

Here we have confirmed the intrinsic biases of two forebrain NP populations: those from the embryonic VZ and those from the postnatal SVZ. These two NP populations are clearly distinct in the cell types formed. Primary VZ NPs preferentially generated neurons *in vitro* which was expected as they are direct descendants of the primitive neuroepithelium that initially produces large numbers of neurons during development. Many of the primary cultures generated large number of neurons, which could be a result of these cultures containing committed neuronal progenitors instead of neurons produced directly from the VZ NPs. Even so, the VZ NPs still proved to be more neurogenic than SVZ NPs over several passages, as they produced higher numbers of neurons following *in vitro* differentiation. Both sets of NPs became biased toward gliogenesis with successive passages, but at different rates. VZ NPs being more primitive took longer to become glial restricted than the SVZ NPs. This phenomenon recapitulates embryonic

neurogenesis where neurons are the first mature cells to form from the neuroepithelium, followed by astrocytes and eventually oligodendrocytes. Therefore, the time spent in culture works like a clock (Alvarez-Buylla and Temple, 1998; Temple and Alvarez-Buylla, 1999). The greater the number of divisions that these neuroepithelial progenitors complete in culture, the more they lose their neurogenic properties, paralleling embryonic neurogenesis. Despite the effectiveness of FGF-2 in maintaining stem cell self-renewal, no current growth factor combination can completely prevent their maturation. As investigators move toward using induced pluripotential stem cell-derived NPs for transplantation studies, our data indicate that cell passage needs to be carefully monitored if neuronal replacement is a desired outcome.

Another difference between VZ NPs and SVZ NPs developmentally is that VZ NPs initially produce pyramidal neurons (and then proceed to make interneurons and then glia), whereas SVZ NPs are restricted to producing nonpyramidal neurons (interneurons). A large percentage of cells lost after cortical contusions are pyramidal neurons that are not replaced. While some studies have shown that a very low number of neurons derived from endogenous NPs can *repopulate* a region of the lesion cavity, all of these new neurons are interneurons (Alvarez-Dolado et al., 2006; Covey et al., 2010; Goodus et al., 2015; Jinnou et al., 2018). Thus, we chose to use VZ NPs in our studies to reconstruct the neocortex after TBI because they have the greatest potential to form pyramidal neurons, nonpyramidal neurons, and eventually glia.

Transplanting the NPs on a biomaterial scaffold has several advantages over parenchymal transplantation. The scaffold we designed provides the cells with structural support that enhances their engraftment. The scaffold also contains fibronectin that increases cell attachment and induces proliferation and self-renewal through the RGD sequence found on fibronectin. RGD binds to the αVβ1 integrin receptor found on stem cells. An advantage to introducing the cells directly into the brain lesion is that this will reduce the migration necessary for cells to repopulate the injured cortex. At 3 days posttransplantation, most of the $GFP^+$ donor VZ NPs were adhered to the microspheres within the lesion cavity. Most of these GFP+NPs at this time point expressed the primitive stem cell or progenitor markers Nestin, and BLBP. Furthermore, these transplanted VZ NPs expressed the proliferative marker Ki67, validating a goal for the scaffold, which was to maintain the cells in a primitive state. However, it was important that these cells did not proliferate indefinitely. At 2 weeks following the transplantation of VZ NP seeded microspheres, we noticed Ki67 was no longer expressed in GFP+ cells, negating concerns that these cells might be tumorigenic. Moreover, the GFP+ NPs lost most of their stem or progenitor cell markers and acquired more mature features. Two weeks are still early to expect differentiation into fully mature neurons, astrocytes, and oligodendrocytes, and the continuous supply of FGF-2 from the scaffold also may have delayed their maturation. At this time point, the spheres were surrounded by neutrophils which we regard as adaptive as neutrophils are the primary cell that degrades chitosan via lysozyme. Fewer chitosan microspheres were seen at 2 weeks than at 3 days, consistent with the interpretation that they had been degraded and cleared.

Our findings are notable in that they demonstrate that when an appropriate NP is transplanted into the injured brain, it can generate five major neural cell types that comprise brain tissue (pyramidal and nonpyramidal neurons, astrocytes, NG2 cells, and oligodendrocytes). As we and others have shown, transplanted primary NPs have the tendency to produce large numbers of glial cells and few neurons (Shear et al., 2004; Boockvar et al., 2005; Ma et al., 2011; Sun et al., 2011). When transplanted with the scaffold, they exhibited complex morphologies and were distributed across cortical regions. Notably, the VZ NPs produced large numbers of GFP+ cells that repopulated portions of the injured cavity. Without the scaffold they often remained clustered or clumped. It is possible that the FGF-2 delivered by the scaffold increased their migration (Vergano-Vera et al., 2009).

A key barrier to CNS cell replacement therapy is the delay between when a neuron differentiates from a transplanted NP and the time that it takes for that neuron to synapse on an appropriate target cell, whereupon it will receive trophic factor support. IGF-1 is a multifunctional growth promoting protein that enhances the proliferation, differentiation, and survival of NPs, depending upon their state of maturation (Ziegler et al., 2015). Due to its potent biological activity, IGF-1 has been provided exogenously after TBI-induced brain damage where it has improved outcome (Saatman et al., 1997). However, delivering IGF-1 into the CNS remains challenging. Therefore, here we set out to test the hypothesis that genetically engineered NPs that can be conditionally stimulated to secrete IGF-1 would improve the outcome of cell replacement. Doxycycline-driven expression of IGF-1 induced differentiation of the RG3.6 NPs into a variety of neurons with cortical specificity. Supporting our hypothesis, genetically engineered NPs improved sensorimotor recovery with their efficacy persisting to 28 days postinjury. Interestingly, behavioral improvements were achieved within 1 week of transplanting the IGF-1 producing NPs. However, the exogenous IGF-1 did not improve the retention of the RG3.6 cells; therefore, the improvements in sensorimotor function cannot be attributed to cell replacement. As IGF-1 is a multifunctional growth factor, it is difficult at this time to establish with any confidence how it is functioning

however it likely reduced bystander cell death. We would caution against concluding that this approach is not worthy of further studies as the failure of the IGF-1 to improve retention of the RG3.6 cells is reflecting a limitation of using the RG3.6 cell line, as we previously reported that few of the neurons generated from the RG3.6 cell line persist long term (Skop et al., 2016). Moving forward, this experiment will need to be repeated using primary VZ NPs or with iPSC-derived VZ NPs.

Our studies were motivated by data that we and others had collected showing that transplanting NPs directly into an already densely populated parenchyma surrounding a lesion cavity is ineffective resulting in poor dispersion, low cell survival, and predominantly glial differentiation, especially when postnatal SVZ NPs are used (Richardson et al., 2010; Sanberg et al., 2012). Moreover, these transplanted cells rarely repopulate the lesion cavity. In this study, we show that using VZ NPs attached to multifunctional microsphere scaffolds results in a larger number of engrafted $GFP^+$ cells, while remaining proliferative for up to 3 days following implantation. These cells then began to differentiate toward all three neural cell types: neurons, astrocytes, and oligodendrocytes. Furthermore, the genetically engineered cells that conditionally secreted IGF-1 improved their capacity for neuronal differentiation, and, upon transplantation into the injured brain, these modified NPs improved recovery of somatosensory function. We confirmed that IGF-1-HA secreted by the engineered NPs triggers intracellular responses, increasing relative levels of pAkt (pT308). Many downstream effectors of IGF-1, including the mechanistic target of rapamycin complexes and extracellular-regulated kinase, likely regulate the expression of regeneration associated genes, driving neuroprotective processes. However, how IGF-1 promotes functional recovery, which downstream effectors and genes are involved remains to be addressed in future studies. Altogether our data support the conclusion that multifunctional microspheres carrying VZ NPs that can conditionally deliver trophic factors represent the more appropriate approach for regenerating the injured neocortex.

## Acknowledgments

All animal procedures were approved by the New Jersey Medical School IACUC under animal protocol #08056, and all procedures were performed in accordance with institutional guidelines. All efforts were made to minimize both the number of animals used and their suffering. We thank Iain Fraser for the pSLIK-Hygro plasmid (Addgene plasmid # 25737) and the members of our laboratories for their helpful comments.

## Author Contributions

N. B. S., S. S., H. A., C. S., and F. C. performed and analyzed experiments, discussed data, and contributed to writing the manuscript; D. E. R. placed injuries and transplanted progenitor cells; C. H. C., N. B. S., C. D. G., and S. W. L. developed the multifunctional scaffolds for cell transplantations; S. S., C. S., and R. D. engineered neural progenitor cells to conditionally secrete trophic factors; S. W. L. and R. D. designed the study, conducted experiments, analyzed data, and wrote the manuscript.

## Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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