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Optimizing a Multifunctional Microsphere Scaffold to Improve Neural Precursor Cell Transplantation for Traumatic Brain Injury Repair

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Abstract

Tissue engineering using stem cells is widely used to repair damaged tissues in diverse biological systems; however, this approach has met with less success in regenerating the central nervous system (CNS). In this study we optimized and characterized the surface chemistry of chitosan-based scaffolds for CNS repair. To maintain radial glial cell (RGC) character of primitive neural precursors, fibronectin was adsorbed to chitosan. The chitosan was further modified by covalently linking heparin using genipin, which then served as a linker to immobilize fibroblast growth factor-2 (FGF-2), creating a multifunctional film. Fetal rat neural precursors plated onto this multifunctional film proliferated and remained multipotent for at least 3 days without providing soluble FGF-2. Moreover, they remained less mature and more highly proliferative than cells maintained on fibronectin-coated substrates in culture medium supplemented with soluble FGF-2. To create a vehicle for cell transplantation, a 3% chitosan solution was electrosprayed into a coagulation bath to generate microspheres (range: 30-100µm, mean: 64µm) that were subsequently modified. RGC seeded onto these multifunctional microspheres proliferated for at least 7 days in culture and the microspheres containing cells were small enough to be injected using 23 Gauge Hamilton syringes into the brains of adult rats having previously sustained cortical contusion injuries. When analyzed 3 days later, the transplanted RGCs were positive for the stem cell/progenitor marker Nestin. Altogether, these results demonstrate that this multifunctional scaffold can be used as a cellular and growth factor delivery vehicle for the use in developing cell transplantation therapies for traumatic brain injuries.

Keywords: Neurotrauma; Regenerative Medicine; Radial Glia; Multifunctional scaffold; immobilized growth factors; fibroblast growth factors; Fibronectin, Chitosan

1. Introduction

Stem cell therapeutics is a promising field for tissue regeneration but it has shown limited success in repairing the brain after severe injury. Brain injuries often cause extensive tissue damage characterized by neuronal and glial cell death where there is virtually no functional replacement of cells from the endogenous neural stem cells (NSCs). In an animal model of stroke, Arvidsson et al. (2002) reported that less than 1% of the destroyed neurons are replaced from the endogenous neural precursors of the subventricular zone (SVZ) (Arvidsson, Collin et al. 2002). Similar results have been obtained in animal models of traumatic brain injury (TBI) (Salman, Ghosh et al. 2004). Salman et al. (2004) observed neural precursors (NPs) from the SVZ repopulated a mechanically injured cortex. The SVZ cells proximal to the injured area produced a very small percentage of new neurons (not quantified), with the majority of the transplanted cells becoming astrocytes. Direct transplantation of NPs into the penumbra of brain lesions has yielded minor advancements (Richardson, Singh et al. 2010, Sanberg, Eve et al. 2012). Most of the transplanted cells either do not survive (Shindo, Matsumoto et al. 2006, Harting, Sloan et al. 2009, Wallenquist, Brannvall et al. 2009) or differentiate into glial cells instead of neurons (Shear, Tate et al. 2004, Boockvar, Schouten et al. 2005, Ma, Yu et al. 2011, Sun, Gugliotta et al. 2011). Shear et al. (2004) and Boockvar et al. (2005) found that NG2 positive glial cells were produced upon transplanting NPs and Sun et al. (2011) observed that the majority of the precursors they transplanted became Olig2 positive cells (presumably glia). Ma et al. (2011) reported that only four percent of NPs that they transplanted were NSCs, whereby only 11% differentiated into cells expressing a neuronal marker. Transplanting stem cells attached to a supportive matrix directly into the lesion site may be more effective in promoting regeneration. Tate et al. (2009) showed improvement in the long-term survival of NPs that were transplanted within a supportive fibronectin and laminin matrix after TBI. Animals receiving these

transplants also showed improved performance in spatial learning tasks compared to injured mice that did not receive NPs (Tate, Shear et al. 2002, Tate, Shear et al. 2009).

Using principles from material engineering and molecular biology tissue engineers are developing organic substitutes to support or replace portions of malfunctioning tissues or organs to create substitutes (Langer and Vacanti 1993). The common approach to create these substitutes is to use living cells, scaffolding and signaling molecules (Kim, Seo et al. 2008). Evans et al., (2000) identified four components necessary for nervous tissue scaffolds: growth factors, extracellular matrix (ECM), support cells and molecules that will promote axonal regeneration (Evans 2000). Traumatic brain injuries are appropriate for the application of biomaterial scaffolds because there is extensive and localized loss of cells and ECM. A scaffold can serve as an artificial matrix and supportive network for engrafted cells as well as for the host tissue. Furthermore, it serves as both a physical and chemical barrier against glial scarring, which is well known to inhibit axonal regeneration (Hou, Xu et al. 2005, Cui, Tian et al. 2006, Li, Yang et al. 2009, Bozkurt, Mothe et al. 2010, Jurga, Dainiak et al. 2011, Martinez-Ramos, Valles-Lluch et al. 2012). The ECM is also an important regulator of cell function. ECM-integrin interactions govern cellular processes such as proliferation, survival, migration and differentiation (Mooney, Hansen et al. 1992) (Ingber 1992, Meredith, Fazeli et al. 1993, Aplin, Howe et al. 1999, Aplin and Juliano 1999). Accordingly, biomaterial systems that mimic the native ECM should be considered when designing regenerative therapies for neural tissue.

Chitosan is a naturally occurring biodegradable polysaccharide formed from the deacetylation of chitin, which is the major structural component of the exoskeletons of animals such as crabs and insects. Chitosan has been shown to be a suitable biomaterial for neural tissue engineering applications (Haipeng, Yinghui et al. 2000), eliciting high cellular compatibility with low toxicity. The abundance of side chains in chitosan allows for easy modification and addition

of other peptides or molecules. Hydrophilic polymers, such as polylysine, allow for a more polar and wettable material that increases the affinity of cells when added to chitosan (Khor and Lim 2003). Peptide sequences such as arginine-glycine-aspartic acid "RGD" (found in fibronectin, collagen or gelatin) or isoleucine-lysine-valine-alanine-valine "IKVAV" (found in laminin) can be immobilized easily to chitosan to aid in cell adhesion, migration, proliferation and differentiation (Muzzarelli 1977, Gupta and Ravi Kumar 2000, Kumar 2000). The RGD peptide sequence can bind to integrin receptors found on stem cells, activating integrin-signaling pathways to prevent differentiation and increase proliferation (Comisar, Kazmers et al. 2007, Dellatore, Garcia et al. 2008, Martino, Mochizuki et al. 2009). Heparin is a highly sulfated glycosaminoglycan, having high binding affinity for growth factors such as fibroblast growth factors (FGF), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (Ashikari-Hada, Habuchi et al. 2004). The binding of heparin to these growth factors protects them from proteolytic degradation (Sommer and Rifkin 1989).

In this study, we engineered a chitosan based biomaterial scaffold to promote CNS regeneration from primitive neural precursors. We optimized a method to manufacture an injectable multifunctional microsphere scaffold and subsequently modified the surface chemistry of the biomaterial in order to achieve a scaffold highly suitable as a vehicle for cell transplantation to repair traumatic brain injuries.

2. Methods:

2.1 *Reagents*

Chitosan (low molecular weight ~50kDa), heparin sodium salt from bovine intestinal mucosa, MTT (3-[4,5-dimetylthiazol-2-yl]-2,5-dipheniltetrazolium), 4',6'-diamidino-2-phenylindole

(DAPI) and phalloidin conjugated rhodamine 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). Nitrocellulose and NuPAGE 4-12% Bis-Tris Gels were purchased from Life Technologies (Carlsbad, CA). Fibronectin, laminin and collagen were purchased from BD BioSciences (Franklin Lakes, NJ). Bovine serum albumin, gelatin, poly-L-lysine (PLL), acetic acid, sodium hydroxide and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Common laboratory chemicals were purchased from either Sigma or VWR International, Radnor, PA).

2.2. Chitosan scaffold: Chitosan powder (1.5g) was dispersed in 50 mL of water containing 2.0% v/v 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 minutes. Subsequently, the supernatant was collected and the remaining impurities at the bottom were discarded.

Chitosan Films: 3% acid chitosan solution was pipetted into two-well glass chamberslides (NUNC, Rochester, NY) to coat the bottom of chamber. The remaining solution was removed and the slides were set to dry for 2-3 h at room temperature. Chitosan coatings were neutralized in 0.5 M NaOH for 10 minutes and then rinsed 3 times in sterile distilled water for 5 minutes each. For substrate cell adhesion, chitosan was subsequently adsorbed with solutions of fibronectin 10 µg/mL, 20 µg/mL laminin, 0.1% gelatin, 0.1 mg/mL collagen type I, or 0.05 mg/mL PLL, all prepared in distilled water. For two-dimensional multifunctional scaffolds, chitosan films were prepared and chemically modified as described below.

Chitosan microspheres: were formed by extruding 3% acid chitosan solution through a syringe with a 30 gauge needle (30 Gauge Needle method) into a basic coagulation bath, consisting of 2.5 M sodium hydroxide: methanol: water (20:30:50 v/v). To create smaller spheres, two modifications were devised: the Coaxial Airflow method that consisted of adding a coaxial air pressure around the 30 gauge needle to reduce surface tension on the end of the needle as described previously (Skop, Calderon et al. 2013) and the Electrospray method. The Electrospray method reduces needle surface tension greater than the coaxial airflow technique and thus further reducing the size of the microspheres. A 25kV electric current was applied to the tip of a 23 gauge needle while the chitosan solution was extruded through a syringe. This method is adapted from the electrospinning technique, which is commonly used to form material fiber meshes. The electric current applies a high voltage to the chitosan solution droplet contained within the syringe. The charge on the liquid solution is enough to overcome the surface tension at the opening of the needle where the droplet is released quicker. By reducing the surface tension, the droplet is also expelled at a smaller size, producing microspheres of reduced diameter. Next, the spheres were removed from the ionic solution and rinsed four times in distilled water to eliminate any residual sodium hydroxide and methanol. Microspheres were then sterilized in 70% ethanol for 30 minutes. Microspheres were photographed with phase contrast in an inverted microscope (Nikon Ti-S) and camera (Nikon DS-Ril). The microsphere size was determined by measuring the microsphere diameter using Sigma Scan Pro 5 software.

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 HEPES Buffered Solution containing 0.9% NaCl pH 7.4 (HBS), as previously described (Skop, Calderon et al. 2013). The following day the heparin cross-linked chitosan films or spheres were rinsed 3 times for 10 minutes each in HBS

and incubated for ON with 10 µg/mL fibronectin in dH_20 and for 2 h in HBS containing 1 µg/mL FGF-2 in 1 mg/mL BSA solution. Spheres were then centrifuged at 730 x *g*, the supernatant discarded. The spheres were rinsed in HBS to remove any unbound FGF-2 and then resuspended in Radial Glia Media (RGM) containing DMEM/F12 media supplemented with B27, 50 µg/ml gentamycin and 50 µg/ml apo-transferrin. Chitosan films were rinsed once in HBS to remove unbound FGF-2. For the MTT reduction assay, chitosan films were modified with only one or more of the surface ingredients as indicated in the Results section.

2.3 Cell Culture

RG3.6 cell line: is an immortalized cell line obtained by introducing *v-myc* to radial glial cells from embryonic cortex day 13.5 of green fluorescent protein positive (GFP^+) rats (donated by Dr. Martin Grumet, Rutgers, New Brunswick). RG3.6 cells were grown as neurospheres for culture propagation or as adherent monolayers on coated petri dishes in RGM media containing 10 ng/ml FGF-2 prepared with heparan sulfate at 1 ng/ml final concentration (RGM-FGF). Ten percent of the medium was changed every day and replaced with equal volume of 10X FGF-2 containing media (100 ng/mL). When growing RG3.6 cells on modified chitosan microspheres, cells were seeded at a 20:1 ratio cells over spheres (1,000,000 cells for 50,000 spheres) and incubated at 37°C ON. The RG3.6 cell line was used instead of primary cells for specific experiments to eliminate several variables seen with heterogenous primary cell cultures and to achieve greater consistency during substrate optimization.

Primary radial glial cells (RGC): were harvested from embryonic day 13.5 EGFP (Sprague-Dawley-Tg(GFP)Bal/2Rrrc (RRRC:0065) rat neocortex, provided by Missouri Research Animal Diagnostics Laboratory (RADIL). Cells were grown as neurospheres for culture propagation or as adherent monolayers on coated petri dishes in RGM-FGF. Ten percent of the medium was

changed every day and replaced with equal volume of 10X FGF-2 containing media (100 ng/mL).

To obtain secondary RGC, neurospheres were collected by centrifugation at 200 x *g*. The pellet was then resuspended in 70% accutase (Millipore, Billerica, MA) for 5 minutes at 37° C to dissociate the cells. Then an equal volume of conditioned RGM was added and cells were centrifuged at 200 x *g*. The supernatant was aspirated and cells were resuspended in fresh RGM. The suspension was centrifuged for a final time at 240 x *g* and the pellet was resuspended in RGM-FGF. RGC were triturated gently to ensure cell dissociation. Lastly cells were counted and seeded into petri dishes in RGM-FGF.

Cell differentiation; RG3.6 or RGC were seeded onto PLL and laminin-coated dishes, and maintained for 24 h in RGM-FGF. Cells were differentiated by removing the mitogen FGF-2 from the media. Seven days later cells were fixed with 3% paraformaldehyde and stained with a mouse monoclonal antibody against class III beta-tubulin (βIIITub) 1:500 (Covance, Princeton, NJ), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antiserum 1:500 (Sigma, St. Louis, MO) and mouse monoclonal antibody supernatant O4 (1:4) (produced in-house) to identify neurons, astrocytes and oligodendrocytes, respectively.

Process length and number: Cells were fixed in 3% paraformaldehyde and stained for F-actin with phalloidin conjugated to rhodamine, at 0.1mg/ml (Sigma, St. Louis MO). Fluorescence photomicrographs were taken at 20x in four fields per well in triplicates. Cell process lengths were measured using Sigma Scan Pro 5 software and the number of processes extending from each cell was manually calculated. A total of 400-500 cells per condition were evaluated. Statistical analyses were performed using an ANOVA with Tukey post-hoc. Data are expressed as the mean ± standard error (SEM).

Ki67 Staining: Cells were grown in chamber slides and stained using a rabbit polyclonal anti-Ki67 antibody at 1:1000 (Vector Laboratories, Burlingame CA) and counterstained with 1 µg/ml DAPI. Positive cells were counted from photomicrographs taken at 20x in four fields/well in triplicate. A total of 200-300 cells per condition were analyzed. The percentage of proliferative cells was calculated as the number of $Ki67^+$ cells over the total number of cells stained with DAPI.

2.4. MTT reduction assay

The MTT assay is a colorimetric assay that measures the reduction of a yellow substrate 3-[4,5-dimetylthiazol-2-yl]-2,5-dipheniltetrazolium (MTT) in the cell into an insoluble purple formazan product (Mosmann 1983). The assay was performed in 96-well plates previously coated with 50 µl of 3% chitosan films. Cells were seeded at $5x10^4$ cells/well in 100 µl of media. Two days later, 10 µl of a 5 mg/ml MTT solution in PBS was added to each well and incubated on the cells for 2–4 h at 37° C. The reaction was stopped by adding 100 µl of a solution containing 50% (w/v) N,N dimethylformamide and 20% SDS (pH 4.8). The plates were maintained ON in the incubator at 37° C and absorption at 560–690 nm was determined using a microtiter plate reader (PowerWave 200, Bio-tek Instruments).

2.5 Western Blot Analyses: Cells were scraped from 6-well plates in lysis buffer containing: phosphate buffered saline (PBS), 1% Triton-X 100, 0.1% SDS, 1% 0.1M sodium orthovanadate and 1% protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined using the BCA assay (ThermoScientific, Rockford, IL). Ten µg of protein per well was loaded onto NuPAGE 4-12% Bis-Tris Gels and electrophoresed. Approximately, 2uL of MagicMark XP (Invitrogen) was loaded for standard molecular weight markers. Gels were

transferred to nitrocellulose. The blots were blocked with 5% milk in PBS-Tween followed by incubation with primary antibody in 5% BSA in PSB-Tween overnight at 4°C with gentle rocking. The primary antibodies used were: rabbit polyclonal anti-brain lipid binding protein (BLBP) antiserum at 1:1000 (Abcam, Cambridge MA); rabbit polyclonal anti-sex determining region Y-box 2 (Sox2) at 1:200 (Chemicon, Temecula, CA); mouse monoclonal βIIITub at 1:1000 (Covance, Princeton, NJ); rabbit polyclonal anti- microtubule associated protein-2 (MAP2) at 1:200 (Sigma, St. Louis, MO); rabbit polyclonal anti-glial fibrillary acid protein (GFAP) at 1:500 (DAKO, Carpinteria, CA) and mouse monoclonal anti-proliferating cells nuclear antigen (PCNA) at 1:1000 (Cell Signaling (Beverly, MA). The Ki67 antibody was inadequate for Western blot. Following 3 rinses with PBS-Tween the following day, the blots were incubated with corresponding secondary antibodies such as DAR-HRP conjugated (1:10000, Jackson ImmunoResearch) or DAM-HRP conjugated (1:5000, Jackson ImmunoResearch) for 2h at room temperature. The blots were washed and signal developed with Western Lightning chemiluminescence reagent (PerkinElmer, Wellesley, MA) as per manufacturer guidelines. The bands were visualized using a UVP EpiChem^3 and processed with Labworks 4.0 digital quantification software (UVP, Upland, CA).

2.6. *Controlled Cortical Impact*: Two month old adult Sprague Dawley male rats were anesthetized by intraperitoneal injection (i.p.) of ketamine/xylazine mixture (90 mg/kg and 10 mg/kg). The fur covering the head was removed using an electric razor and a midline incision made through the scalp using a scalpel. The skin was deflected and a craniectomy was made using a drill with a 5 mm diameter trephine. The trephine was placed midway between Bregma and Lambda, with the edge of the trephine adjacent to midline. Cold PBS 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). The anvil tip of 3.5 mm diameter was zeroed by bringing it into contact with the exposed Dura mater. The velocity of the impactor was set at 4.0 ± 0.2 m/s, depth of penetration was 1.5 mm and the duration of deformation was 150 msec. After impact, the integrity of the Dura mater was confirmed and the scalp incision sutured with 3-0 nylon thread. Buprenorphine (0.05 mg/kg, SC) was administered post-operatively and the rats were placed on heating pads at 37° and monitored continuously for 2 h after surgery. In addition, immediately after surgery, all subjects received 3% body weight of 0.9% saline subcutaneously (SC) to prevent dehydration.

2.7 Cell transplantation: Subacute transplantations were performed 7 days after CCI injury. The animals were anesthetized by i.p. injection of ketamine/xylazine mixture and the sutures were removed to expose the skull. Cell-microsphere complexes were collected from culture dishes and resuspended in phenol-free media without supplements. A 23 Gauge Hamilton syringe (inner diameter of 260 μm) was used to inject the scaffold at three different depths: 1.5, 1.0 and 0.5 mm below the Dura mater. One uL was injected at each depth over 5 minutes, with 5 minute intervals between each injection. The needle was withdrawn 10 minutes after the last injection. The scalp incision was sutured with 3-0 nylon thread and the animals placed onto a 37 °C heating pad until they were fully awake. All animal procedures performed in this report were approved by the New Jersey Medical School IACUC under animal protocol #08056.

*2.8 Immunofluorescence of brain sections: R*ats were deeply anesthetized with ketamine/xylazine mixture perfused 3 days post transplantation using 4% paraformaldehyde (PFA). The brains were collected and kept in 4% PFA ON. Next day the brains were rinsed with PBS and 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 (OCT) embedding media (Sakura Finetek, Torrance, CA) on a dry-ice-ethanol slush. The brains were cryosectioned at 40 or 15µm thickness and stained with primary antibodies ON at 4°C using mouse monoclonal anti-Nestin antibody 1:5 (Developmental Studies Hybridoma Bank, Iowa) and chicken polyclonal anti-green fluorescent protein (GFP) antibody 1:2,500 (AVES, Tigard, Oregon). Sections were incubated in secondary antibodies 1:200 (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 pre-immune sera. After secondary antibody incubation the sections were washed, counterstained with 1 µg/ml DAPI for 5-10 minutes, and coverslipped with GelMount (Biomeda, Foster City, CA).

3. Results:

3.1 Effect of Chitosan Films Adsorbed with Adhesion proteins on Morphology and Proliferation of RGCs:

To establish which surface modifiers best maintained the morphology and proliferation of RGCs, chitosan films were adsorbed with different extracellular matrix (ECM) proteins that mediate cell adhesion, and used as substrate for the adhesion and growth of RGCs. RG3.6 cells, a cell line derived from embryonic RGC, were seeded onto chitosan films adsorbed with

fibronectin, laminin, gelatin, type 1 collagen, or the polymer poly-L-lysine and maintained in growth media for 4 days. Cell morphology was visualized by staining of F-actin, which clearly revealed cell processes. RGCs are morphologically distinct cells that project a few long processes, typically non-branched and in opposing directions. We observed that the RG3.6 cells adopted strikingly different morphologies (especially the length and number of processes) when grown on chitosan films absorbed with varying adhesive proteins. Chitosan films coated with fibronectin best preserved the RGC morphology (**Figure 1 A-C**). They had a small cell body and 1-3 straight processes that lacked branches and that on occasion were very long (49 ± 5 μm). RG3.6 cells grown on chitosan films coated with laminin also possessed few processes but they were shorter (35 ± 3 μm) than the processes of cells grown on fibronectin-chitosan films. Cells grown on gelatin had processes with significantly fewer branches than the control condition (unmodified chitosan films) but they were much shorter than the fibronectin condition with an average of 31 ± 2 μm. Cells grown on poly-L-lysine had numerous, very short processes (17 ± 1 μm) that branched frequently, very similar to the control condition. Based on these observations, fibronectin was the most appropriate ECM protein to use as surface modifier to maintain the RGC morphology.

These substrates did not affect the proliferation of RGCs, as determined by immuostaining for Ki67. Ki67 is a marker of cells in all active phases of the cell cycle G_1, S, G_2 and mitosis, but not resting phase G_0. Ki67 is, therefore, and indicator of proliferating cells. RG3.6 cells were grown in log phase on chitosan films adsorbed with different ECM proteins for 5 days and stained for Ki67. Figure 1D shows that the rate of RG3.6 cell proliferation was high in all conditions. Fibronectin- and gelatin-chitosan substrates produced a slightly higher proliferation rate than the other conditions 92 ± 1% and 93 ± 2%, respectively (**Figure 1D**). The Ki67 indices of cells grown on the other substrates were: laminin, 89 ± 2%; collagen, 87 ± 4% ; poly-(L-

lysine) 77 ± 5%; and unmodified chitosan 82 ± 1%; however the differences were not statistically significant using ANOVA.

3.2 Multifunctional Scaffold Effect on Cell Proliferation and maintenance of stemness:

Each component of the modified chitosan scaffold has specific functions. Heparin was chosen because of its high affinity to bind to FGF-2. Genipin, as a natural cross-linker, keeps heparin in place and firmly attached to chitosan. As a result, FGF-2 is stably tethered to the scaffold (Skop, Calderon et al. 2013). FGF-2 is an essential growth factor for RGC cells to sustain their survival, proliferation and stemness (Lewis 1996, Yoon, Nery et al. 2004). In culture, FGF-2 loses over 80% of its biological activity within 24 h, making daily addition of FGF-2 an absolute requirement to maintain stemness of RCGs (Rifkin and Moscatelli 1989, Caldwell, Garcion et al. 2004). On the other hand, removing FGF-2 from the medium induces RGC differentiation. We evaluated whether the CHG-FN-FGF was capable of sustaining survival and proliferation in the absence of soluble FGF-2. Control cells were grown on chitosan films coated with fibronectin, and soluble FGF-2 was added daily to the medium (C-FN+sFGF). As negative control for growth, cells were seeded on chitosan films modified with genipin- heparin and fibronectin (CHG-FN) lacking FGF-2, and they did not receive FGF-2 in the medium. RGC were seeded on these substrates in media lacking FGF-2 for three days, whereupon the cells were photographed and assayed for MTT reduction. As expected, cells grown in the absence of FGF-2 did not proliferate and cell death was observed (**Figure 2A, B**: CHG-FN) due to the absence of FGF-2 at all times during culture, including seeding. By contrast, the control condition (Figure 2A, B: C-FN+sFGF) promoted survival and proliferation. Surprisingly, the multifunctional scaffold was even more efficient at promoting survival and proliferation (Figure 2A, B: CHG-FN-FGF). In this condition, cells had higher MTT reduction levels than the control condition where cells

received FGF-2 daily in the media (**Figure 2 B**). The higher % MTT reduction could be due to higher proliferation or decreased cell death in the CHG-FN-FGF condition. In either case, FGF-2 was more efficient in sustaining survival/proliferation of RGCs when immobilized to the scaffold than when soluble in the media. The same effect of immobilized FGF-2 was obtained using the immortalized RGCs (Skop, Calderon et al. 2013).

Another desired feature of the multifunctional scaffold is the capacity to maintain neural stem cells in undifferentiated state or stemness. Radial glia are multipotential progenitors that generate neurons, astrocytes and oligodendrocytes. *In vitro*, RGCs begin to differentiate 24 h after FGF-2 is removed to generate neurons, astrocytes and oligodendrocytes (**Figure 3B**). Therefore, we aimed to establish whether the multifunctional scaffold maintains the stemness of RGCs. Secondary RGCs were grown on CHG-FN-FGF multifunctional scaffolds for 4 days (**Figure 3A**) and the expression of stem cell and differentiation markers was compared with differentiated RGCs and proliferative RGCs. Differentiated RGCs were produced by seeding secondary RGCs on poly-D-lysine-Laminin coated substrate (Diff condition) and withdrawing FGF-2 (Figure 3 A). Proliferative cells were produced by growing RGCs in standard culture conditions namely poly-ornithine-fibronectin coated substrates that received soluble FGF-2 daily (Non-Diff condition) (Figure 3A). Cells survived well in all conditions, as expected. The expression of stem cell and differentiation markers was evaluated by Western blot analyses (**Figure 3C, D**). RGCs grown on CHG-FN-FGF scaffold expressed high levels of the stem cell and progenitor markers BLBP and SOX2 (Figure 3 C, D) comparable to RGCs maintained under standard culture conditions (Figure 3 C, D) revealing that the multifunctional scaffold maintained their stemness. Furthermore, the astrocytic marker GFAP was barely expressed by cells grown on the multifunctional scaffold condition compared to the standard condition (Figure 3D). In contrast, differentiated RGCs showed an increase in neuronal markers, ßIIITub and

MAP-2 and an increase in GFAP. RGCs grown on the multifunctional scaffold had similar levels of the proliferation marker PCNA (Figure 3 C, D) than the standard condition confirming our previous result that the multifunctional scaffold maintains the proliferative status. Oligodendrocyte markers, were not evaluated, as RGC rarely produce oligodendrocytes within four days. These data support the conclusion that the multifunctional scaffold promotes the stemness and proliferation of RGCs. In particular, our studies indicate that the immobilized FGF-2 was superior to soluble FGF-2 in maintaining their stemness, limiting their differentiation and even delaying the formation of astrocytes.

3.3 Optimization of chitosan microsphere size and cell attachment:

The goal of our research has been to manufacture a vehicle to facilitate the delivery of neural precursors into brain injuries with the cells adhered to the surface of the microspheres. A schematic for the vehicle design is presented in **Figure 4A**. We had previously generated microspheres that ranged between 200-500 µM in diameter using a coaxial airflow method (Skop, Calderon et al. 2013). Such spheres are too large to pass through the needles that we use for transplantation, which are 23 gauge needles. As the optimal sphere size to pass through a 23 gauge Hamilton syringe is less than100 µm, we reduced the size of the microspheres using the Electrospray method, which we designed for this purpose. Using this method microspheres were formed that ranged between 30-100µm with an average of 64µm in diameter (**Figure 4B, C**). The majority of the spheres fell within the 40-50 µm range as seen in the frequency distribution graph (**Figure 4D**).

The multifunctionality of the chitosan microspheres was enhanced when modified with heparin-genipin, fibronectin and FGF-2. Staining the microspheres using toluidine blue revealed the presence of bound heparin (**Figure 4E**). To establish whether RGCs would adhere and grow

on these microspheres, a single cell suspension of RG3.6 cells was mixed with microspheres and incubated for 18 h or up to 10 days. Cells were well attached to the spheres after 18 h (not shown), proliferated and sustained excellent survival/proliferation after 10 days in culture as observed by phase contrast microscopy (**Figure 4F**).

3.4 Transplantation:

Numerous studies have encapsulated cells inside spheres or other delivery vehicles to enable the cells to produce soluble growth and trophic factors [56-59]. However, the goal of our research has been to manufacture a delivery vehicle with the cells adhered to the surface of the microspheres. This configuration will enable the progeny of the stem cells to migrate off of the scaffold into the adjacent tissue, which will be crucial to reconstruct a damaged brain. To test our approach, multifunctional microspheres containing RG3.6 cells attached to the surface were transplanted into a neocortical lesion cavity at 7 days after a controlled cortical impact (CCI)**.** As the RG3.6 cells express GFP, they could be distinguished from the host cells using fluorescence microscopy. RG3.6 cells are pluripotential and can generate neurons, astrocytes and oligodendrocytes when they were differentiated in vitro (**Figure 5A**). At 3 days after transplantation, microspheres could be recognized within the wound cavity adjacent to the host tissue (**Figure 5B-D**). The RG3.6 cells, were largely still adhered to the microspheres **(**Figure 5 B-D**)** and many of them were positive for the stem cell/progenitor marker Nestin **(Figure 5 E-G:** magnified boxed insert in Figure B and arrows**)**. Some of the transplanted cells, or their progeny, were seen far from the beads in the adjacent tissue. These data support the conclusion that the cells thrive on the spheres and withstand the mechanical forces of the syringe all the while surviving three days post transplantation.

4. Discussion:

The brain is arguably the most difficult organ to repair after an injury due to the complexity of the central nervous system and its limited capacity to regenerate on its own. Neurons do not undergo mitosis and endogenous neural stem cells (NSC) are unable to replace the quantity of neurons lost after a typical injury. The transplantation of NSC has become an effective tool for studying the mechanisms of CNS regeneration. Primary fetal neural cells have shown success upon transplantation in Parkinson's and Huntington's diseases with notable symptomatic relief (Dunnett, Nathwani et al. 2000, Kendall, Hantraye et al. 2000, Bjorklund, Dunnett et al. 2003, Lindvall and Bjorklund 2004). Their application to spinal cord injury shows their ability to survive, integrate and improve functional outcome while also reducing lesion volume (Hasegawa, Chang et al. 2005, Bonner, Connors et al. 2011). Published data have suggested that engrafting rat or mouse stem/progenitor cells improves outcome following experimental traumatic brain injury (TBI). Cognitive and sensorimotor recovery has been observed, and in some cases neuronal differentiation has been obtained (Hoane, Becerra et al. 2004, Shear, Tate et al. 2004, Boockvar, Schouten et al. 2005, Bakshi, Shimizu et al. 2006, Gao, Prough et al. 2006). Many studies have shown that transplanted cells can survive in the host brain for up to a year; however, only a small percentage, typically less than 2% of the donor cells, engraft (Harting, Sloan et al. 2009, Wallenquist, Brannvall et al. 2009). Furthermore, most of the cells that do engraft differentiate into glia, not neurons.(Shear, Tate et al. 2004, Boockvar, Schouten et al. 2005, Ma, Yu et al. 2011, Sun, Gugliotta et al. 2011) The brain also has endogenous NSC located in distinct regions such as in the dentate gyrus and subventricular zone (Snyder 1994, Shear, Tate et al. 2004, Kulbatski, Mothe et al. 2005, Longhi, Zanier et al. 2005). However, these cells are limited in the types of neurons they can produce. More primitive cells, the RGCs that are found

in the fetal ventricular zone have the potential to differentiate into a wider variety of neurons and glia (Reid and Walsh 2002, Merkle, Mirzadeh et al. 2007)

One possible reason that the survival rate of transplanted cells is low is that the cystic cavity formed by the injury creates a harsh, non-permissive environment that lacks nutrients, survival factors and most importantly, a habitable substrate (Tate, Shear et al. 2002, Crompton, Goud et al. 2007, Tate, Shear et al. 2009, Mo, Yang et al. 2010). A scaffold would serve as a structural and functional support for the cells. Stem cells, when transplanted within a biomaterial matrix, have shown promise in addressing this issue for various brain injuries. Park et al., 2002 demonstrated greater engraftment using a polymer scaffold in a stroke model (Park, Teng et al. 2002). They used a fibrous poly(glycolic acid) scaffold seeded with the C17.2 NSC cell line and showed that these cells could differentiate and reduce the extent of inflammation and glial scarring. Tate et al., 2009 employed a collagen gel containing laminin and fibronectin to improve neural precursor cell engraftment and survival after TBI. The study extended up to eight weeks in which there was a significant difference in outcome when the NPs were seeded within a matrix versus control. Many cells were positive for NG2 and the animals showed improvements in spatial memory tasks (Tate, Shear et al. 2002, Tate, Shear et al. 2009).

Brain injuries are not uniform in shape or size; therefore a scaffold that is injectable and will mold to the injured tissue will be necessary. We have selected chitosan as the bulk material for our scaffolds. The chemical structure of chitosan allows it to be easily modified, making it a very attractive and versatile material. Chitosan has been previously demonstrated as a suitable material for nerve cell affinity (Haipeng, Yinghui et al. 2000). We also evaluated chitosan's biocompatibility as seen in Figure 1. Although the chitosan was not toxic (data not shown), RGCs require adhesive peptides to grow, therefore ECM proteins were incorporated into the scaffold to enhance the efficiency of growth on the scaffold. Proliferative rates were high on all

chitosan substrates, however they were noticeably higher when the chitosan was coated with fibronectin or gelatin.

Microspheres comprised of poly(lactic-co-glycolide) (PLGA) have been successfully transplanted into the brain (Nicholas, McInnis et al. 2002). However, synthetic polymers can degrade into caustic by-products. For example, Kou et al., 1997 showed that PLGA degraded into acidic by-products when transplanted into the brain, exacerbating the inflammation and damage produced by TBI (Kou JH and Cain G 1997). By contrast, chitosan degrades into non-toxic metabolites, making it appealing for CNS applications. The utility of chitosan has been demonstrated in studies using microcapsules containing the neurotrophic factor NT-3. These neurotrophins containing microcapsules increased neuronal regeneration of the rat hippocampus, demonstrating that chitosan is safe for in vivo applications (Mo, Yang et al. 2010).

In our studies the chitosan microspheres were modified by the addition of fibronectin to aid cell adherence, proliferation and stemness. Arginine-glycine-aspartic acid (RGD), first identified in fibronectin, is contained within other ECM proteins such as collagen, vitronectin, thrombospondin, von Willebrand factor, fibrinogen, gelatin and some laminins (Ruoslahti and Pierschbacher 1986, Ruoslahti 1996). Consistent with a role for the RGD peptide in enhancing the proliferation of the RGCs, RG3.6 cells grown on fibronectin-coated substrates exhibited a higher Ki67 index (Figure 1). Collagen also possesses an RGD (Ruoslahti 1996) sequence, but the RG3.6 cells didn't proliferate as significantly when attached to collagen. This may be due to collagen's negative charge and the resulting gel-like coating formed when bound to chitosan. Importantly the RGD sequence binds to the α5β1 integrin receptor whose intracellular amino terminus influences cellular migration (Hocking, Sottile et al. 1998), proliferation, self-renewal and differentiation (Campos, Leone et al. 2004, Leone, Relvas et al. 2005). Yoshida et al., 2003 observed that reducing α5β1 expression in cortical progenitors increased their differentiation

(Kimura, Miyazaki et al. 2003). Cui et al., 2006 applied this concept to an in vivo application using a hyaluronic acid based hydrogel with immobilized RGD for brain tissue engineering (Cui, Tian et al. 2006). Transplantations after cortical damage using hyaluronic acid-RGD scaffolds enhanced cell infiltration and angiogenesis into the matrix, while simultaneously inhibiting glial scar formation. An increase in neurite extension was also observed.

The modified chitosan microspheres used in this study were designed to allow FGF-2 to be tethered to the surface of the scaffold, which differs from many studies that have used microspheres to encapsulate growth factors. FGF-2 is a known survival factor for RGCs and maintains them in a primitive state. FGF-2 also has been shown to increase the numbers of stem/progenitor cells in the SVZ following TBI (Yoshimura, Teramoto et al. 2003, Sun, Bullock et al. 2009). Soluble FGF-2 has been reported to have a half-life of 24 hours at 32°C (Rifkin 1989) and less than 5 hours at 37°C (Shiba, Nishimura et al. 2003), whereas it's stability increases when affixed to heparan sulfate proteoglycans (Sommer and Rifkin 1989). Caldwell reported over an 80% reduction in FGF-2 stability when incubated at 37°C for 24 hours, whereas less than 20% was degraded with the addition of heparin under the same conditions. There was also an observed increase in striatal precursors grown in culture with the addition of heparin. Interestingly neither BSA, fetal calf serum, nor other proteoglycans such as chondroitin sulfate, dermatan sulfate, keratin sulfate and hyaluronic acid, prevented FGF-2 denaturation like heparin at 37°C (Caldwell, Garcion et al. 2004). By immobilizing FGF-2 to the surface of the chitosan, it is presented to the cells in a more biologically active form (due to heparin binding).

A key finding of this study, which has implications for the broader field of stem cell research is that cells maintained on the multifunctional film did not need to be fed for at least 3 days after plating, and yet the proportion of proliferating and undifferentiated cells was significantly greater than cells propagated under standard growth conditions. Stem cells normally

require feeding on a daily basis; however, with this matrix, the cells can clearly be left untended for at least 3 days. We believe that stem cell researchers studying multiple types of stem and progenitor cells would benefit from using this platform. Kang et al., 2011 obtained similar results in their studies on the effects of a recombinant FGF-2 protein on human adipose stem cells. In their study, FGF-2 was linked to a maltose-binding protein, which was then immobilized to polystyrene dishes. Interestingly, the bound FGF-2 promoted the specification of adipocytes while inhibiting the production of osteoblasts. They also noted that heparin inhibited the cells from binding to the immobilized FGF-2, thus demonstrating the strong interaction that heparin has for FGF-2.

Ideally, while the FGF-2 is present, the cells will begin to proliferate and form processes that extend to the pial surface, mimicking embryonic neurogenesis. Neuroblasts and other progeny would migrate along their processes, ultimately generating neurons appropriate to each cortical layer and supportive glia as the FGF-2 dissipates. Concurrently, the scaffold will degrade over time revealing a regenerated, and ordered cortex. A schematic depicting a method for CNS repair using stem cells delivered upon a multifunctional scaffold after TBI is provided as Figure 6.

5. Conclusion

In this study, we optimized multifunctional microspheres using natural biopolymers for cell replacement therapies for CNS injury. The combination of fibronectin and heparin-immobilized FGF-2 provides neural stem cells with a niche that resembles in many ways the region that they normally inhabit. We show that fetal rat RGCs, plated onto this multifunctional film, remain in a primitive, multipotent and proliferative state for at least 3 days. We show that the electrospray

method can be used to produce microspheres that are small enough to be conveniently injected and that RGCs attached to these multifunctional microspheres can be delivered into the core lesion produced by a focal TBI. When analyzed 4 days later, the transplanted RGCs are positive for the stem cell/progenitor marker Nestin. Altogether these results demonstrate that this approach can be used as a cellular and growth factor delivery vehicle to promote the regeneration of nervous tissue after brain injuries. While this application of brain tissue engineering shows promise, more detailed in vivo studies are required to assess survival and differentiation of transplanted neural precursors as well as detailing the extent of anatomical and functional recovery from TBI.

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Figure Legends

Figure 1: Substrate effects on morphology and proliferation of RGCs. RG3.6 cells were seeded on different substrates and propagated for five days. **(A)** Representative fluorescence photomicrographs of cells immunostained for F-actin (red) and DAPI (blue). Scale bar = 100 μm. LN: Laminin, FN: Fibronectin, Gel: Gelatin, PLL: Poly-L-lysine, Col: Collagen, C: Chitosan only) (**B)** Process length was measured using Sigma Scan Pro 5 (**C)** Process number quantification was determined manually. Values represent mean ± SEM from 3 independent experiments. Statistical significance was determined by ANOVA with Tukey's post hoc (process length *** = p<0.001 vs. control and ♦♦♦ = p<0.001 vs laminin and gelatin; process number *** p<0.001 vs. PLL and C, * = p<0.05 vs. PLL and C, and ♦♦ = p<0.01 vs LN, COL and GEL). (**D)** Cell proliferation was determined by immunostaining for Ki67 and expressed as percentage of $KI67^{+}$ cells over total number of cells DAPI stained. Data are averaged from 3 independent experiments. No statistical difference was found using ANOVA.

Figure 2: Effect of immobilized FGF-2 on survival/proliferation of RGCs. RGCs were passaged twice, then seeded and grown for 4 days in 96 well plates coated with chitosan-fibronectin receiving daily soluble FGF-2 in the media (C-FN+sFGF), chitosan with heparin-genipin, fibronectin and immobilized FGF-2 (CHG-FN-FGF multifunctional scaffold) or chitosan with heparin-genipin and fibronectin lacking FGF-2 (CHG-FN) during the culture. (**A)** Phase contrast images of RGCs grown for 4 days on the indicated substrates. Scale bar = 100 C: Chitosan, H: Heparin, G: Genipin, FN: Fibronectin, FGF: Fibroblast Growth Factor 2. (**B)** %MTT reduction of RGCs cultured on different substrates. Assay was performed in triplicate in 3 independent experiments. Values represent mean ± SEM. Statistical significance was determined by ANOVA with Tukey's post hoc. (*** p<0.001 compared to CHG-FN and ### p<0.001 compared to C-FN+sFGF).

Figure 3: Effect of immobilized FGF-2 on maintaining the stemness of RGCs. **(A)** Phase contrast images of RGCs seeded and grown for 4 days in culture dishes coated with different substrates: poly-D-lysine-Laminin and removal of FGF-2 after seeding (differentiating condition, Diff), chitosan with heparin-genipin, fibronectin and bound FGF-2 (CHG-FN-FGF/scaffold) or poly-ornithin-FN receiving daily soluble FGF-2 in the media (proliferative condition, Non-Diff). **(B)** Tripotentiality of RGCs. RGCs were passaged 2 times, cultured on poly-D-Lysine-Laminin for 7 days after FGF-2 removal and assessed for GFAP (blue), βIIITub (green) and O4 (red) by immunofluorescence **(C,D)** Western blots of RGCs grown on the indicated substrates for 4 days**.** **(D)** Densitometry of western blots. For each protein, optical density of the bands was normalized to actin and results from 3 independent experiments were averaged. IOD = Integrated Optical Density. βIIITub: Beta III-tubulin, GFAP: Glial Fibrillary Acidic Protein, BLBP: Brain Lipid Binding Protein, SOX2: SRY-box 2, PCNA: Proliferating Cell Nuclear Antigen, MAP-2: Microtubule-Associated Protein 2.

Figure 4: Characterization of transplantable multifunctional microsphere scaffolds. **(A)** Schematic representation of chemically modified microsphere scaffolds with heparin-genipin, fibronectin and FGF-2. **(B)** Phase contrast image of electrospray formed chitosan microsphere **(C)** Comparison of chitosan microspheres size formed by different methods, namely: 30 Gauge needle, Coaxial Airflow and Electrospray (see Methods for details) Values represent mean ±

SEM. Statistical significance was determined by ANOVA with Tukey's post hoc. (*** $p<0.001$ compared to 30 Gauge needle and ### $p<0.001$ compared to coaxial air flow)

(D) Representative frequency distribution of microsphere sizes obtained in one out of 3 microsphere preparations by the electrosprayed method. **(E)** Toluidine blue stain of heparin covalently cross-linked to chitosan microsphere. **(F)** Phase contrast microphotograph of RG3.6 cells seeded on electrosprayed microspheres modified with heparin-genipin-FN-FGF-2 incubated O/N.

Figure 5: Multifunctional microsphere scaffolds for cell replacement therapies after CNS injury. **(A)** Multipotentiality of RG3.6 cells differentiated *in vitro* for 7 days and stained for βIIITub (red), GFAP (blue) and GFP (green). **(B-D)** Immunofluorescence of brain sections from animals that received transplants of multifunctional microsphere scaffolds with adhered RG3.6 cells 7 days after traumatic brain injury. Sections were stained for GFP (green) (**C**), nestin (red) (**D**) and DAPI (blue). White arrows illustrate co-localization. Inset was magnified to show co-localization of GFP (green) and nestin (red) in panels E-G. (**A**) was taken at 20X, (**B-D**) at 10X magnifications. Spheres were injected at 3 depths 1.5mm, 1.0mm and 0.5m at 1 μl each.

Figure 6. Schematic representation of the intended use of the multifunctional scaffold in TBI. **(A)** Coronal illustrations depicting model for brain injury repair. **(B)** Magnified illustrations of boxed red inserts from (A). ***Top:*** Day 0 GFP+ VZ cells injected into lesion cavity on modified chitosan microspheres 7 days post CCI. ***Middle***: Recapitulation of neurogenesis. GFP+ VZ cells proliferate and produce neocortical cells. Spheres degrade. ***Bottom***: Microspheres completely degraded; GFP^+ neurons replace neurons lost from injury.

Figure 1

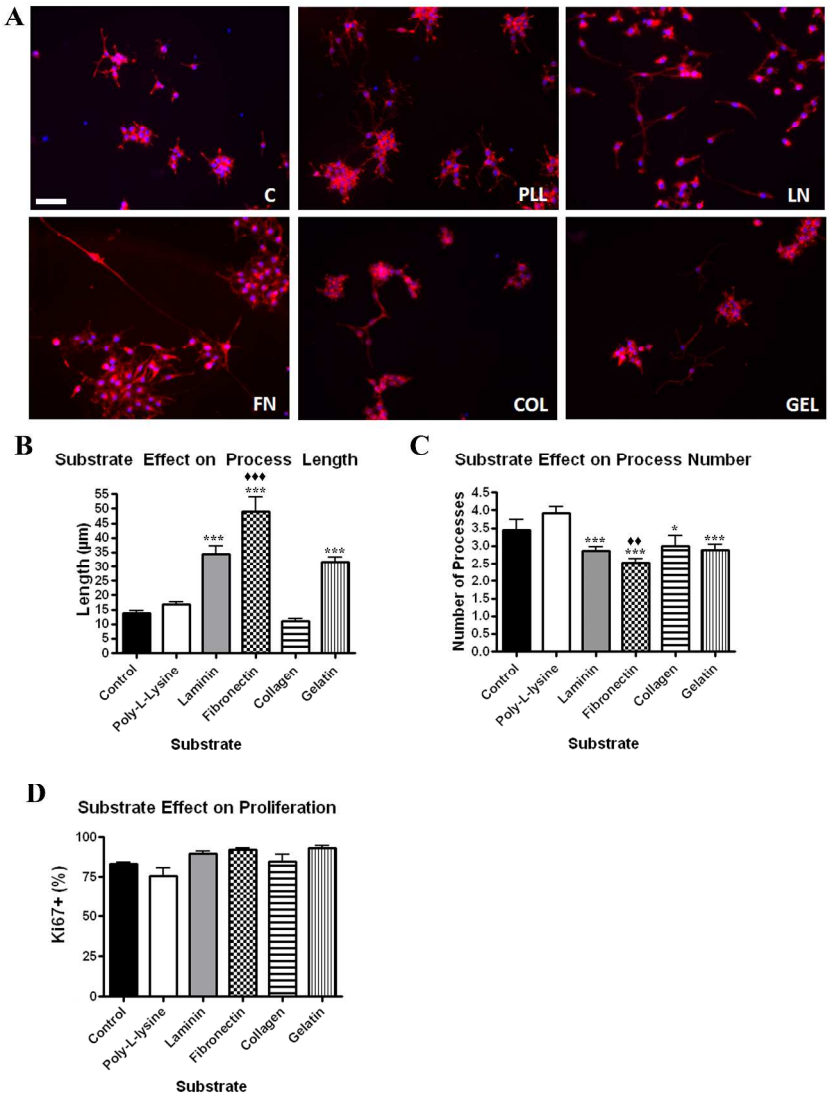

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Figure 2

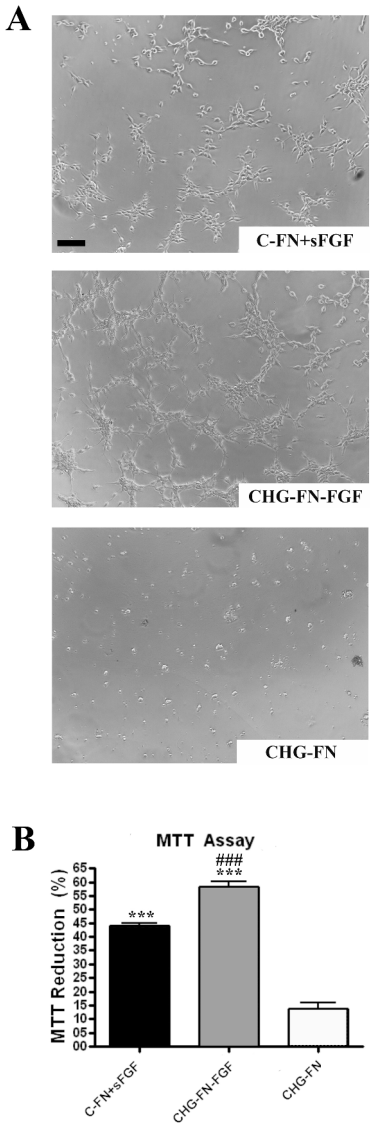

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Figure 3

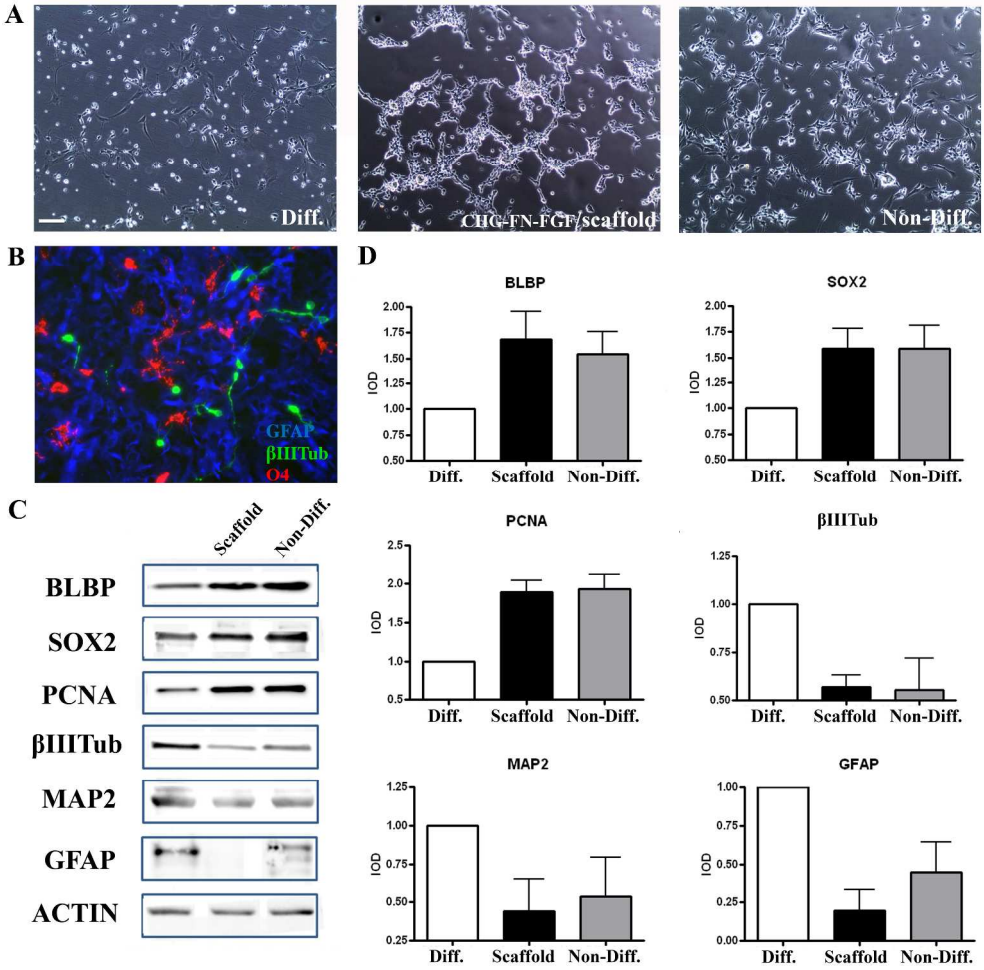

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Figure 4

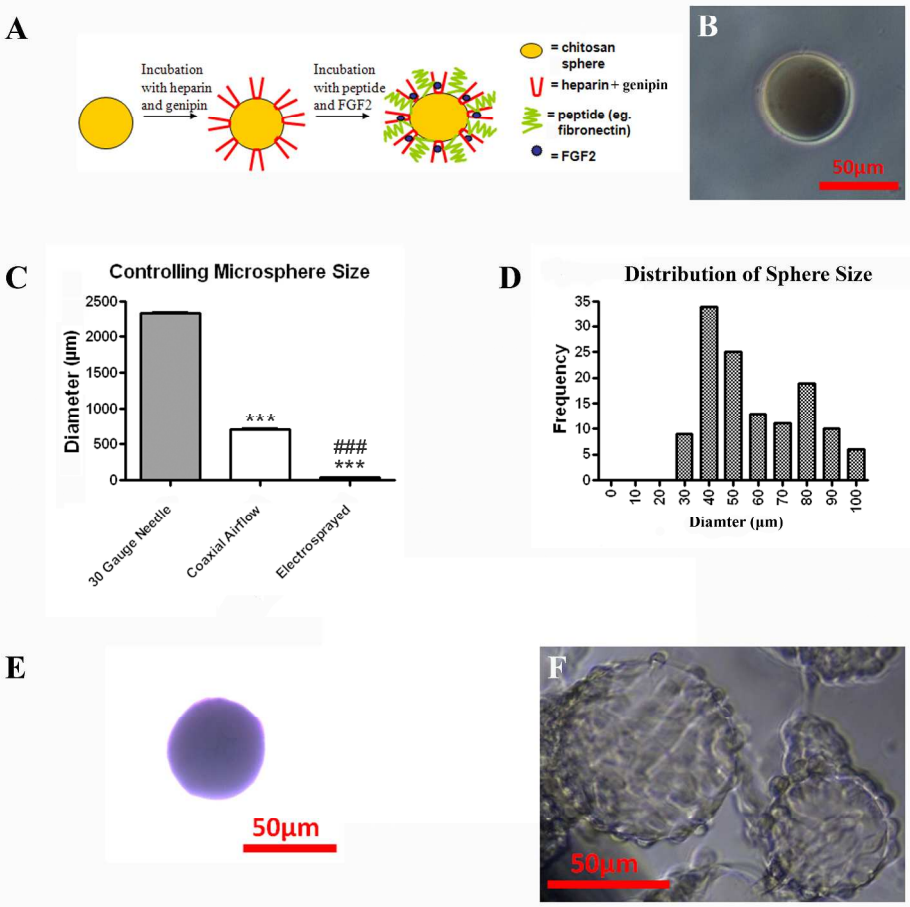

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Figure 5

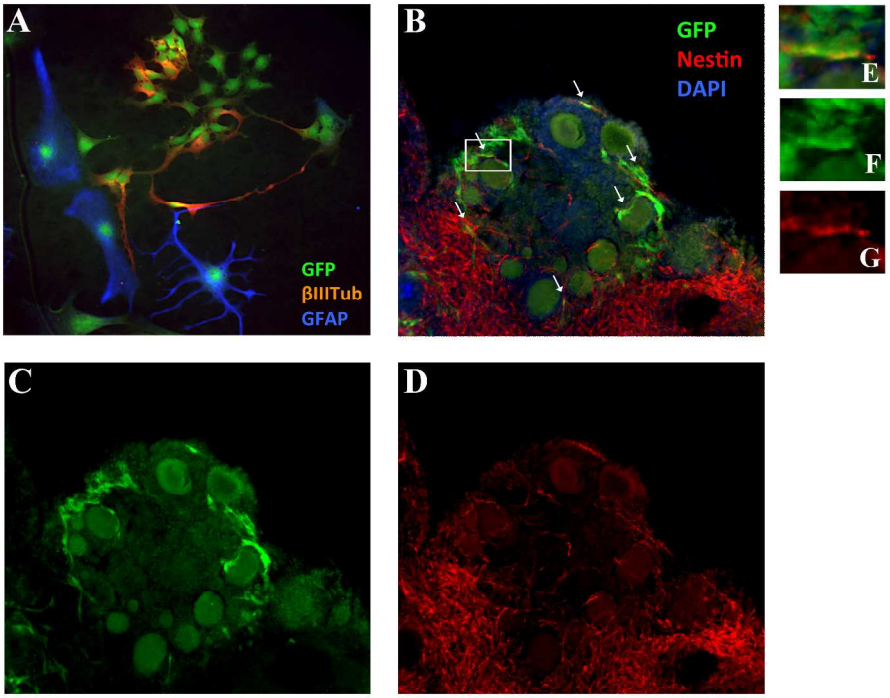

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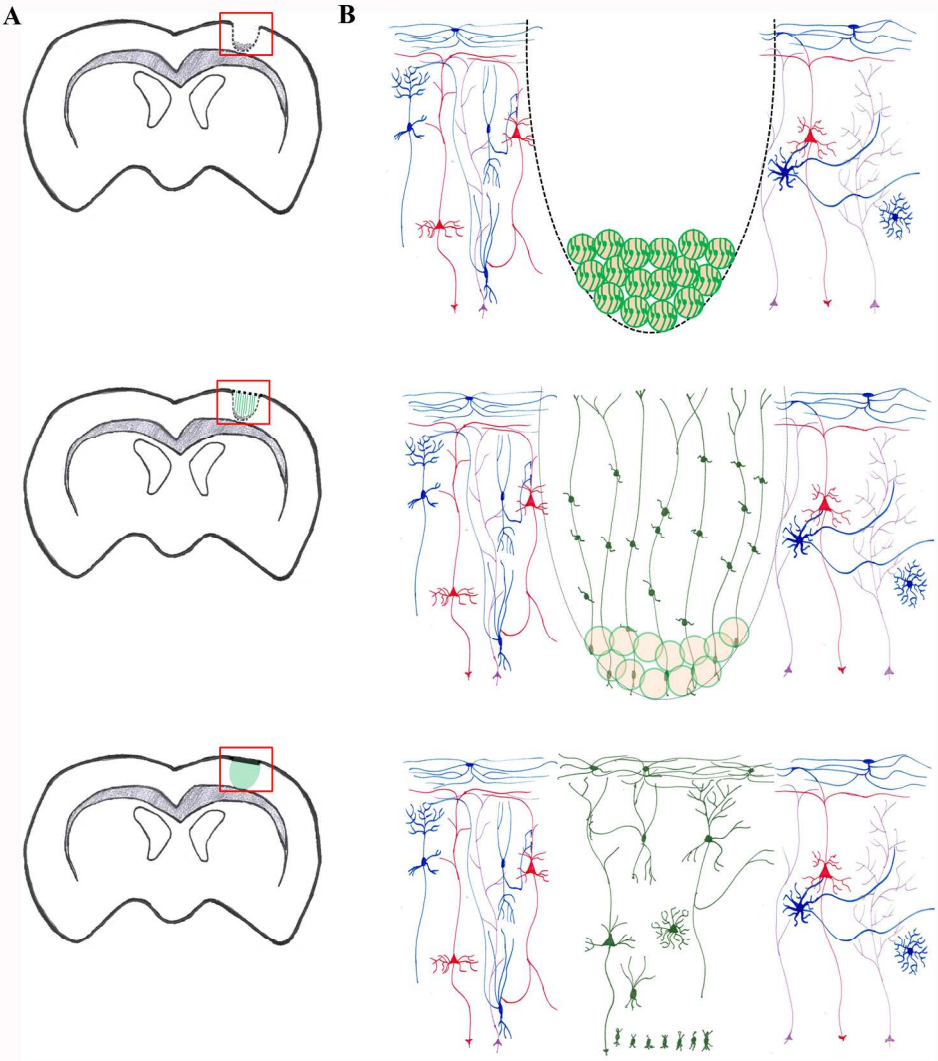
Figure 6



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