CHARACTERIZATION OF IGF-1 EXPESSING RG3.6 CELLS FOR TRANSPLANTATION FOLLOWING TRAUMATIC BRAIN INJURY

by

SWETA SINGH

Thesis submitted to the

Graduate School-Newark

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Biology

written under the direction of

Radek Dobrowolski, Ph.D.

and approved by

_____

_____

_____

Newark, New Jersey
October, 2016

ABSTRACT OF MASTER THESIS

CHARACTERIZATION OF IGF-1 EXPESSING RG3.6 CELLS FOR TRANSPLANTATION FOLLOWING TRAUMATIC BRAIN INJURY

By SWETA SINGH

Master Thesis Director:

Radek Dobrowolski, Ph.D.

Traumatic brain injury is one of the leading cause of neuronal damage which affects the cognitive function of the brain. Traumatic brain injury (TBI) also causes damage to the neuronal stem cells pool. IGF-1 is an important trophic factor that increases cell survival by inducing neuroprotection and anti-apoptotic activity. Therapeutic strategy to protect the neurons/neuronal stem cell pool and restoration of damage neuron and induce their ability to produce multipotent, proliferative cell that can adopt the neuronal phenotype could have beneficial effect on functional recovery of the brain after TBI. Here, we characterized and used genetically engineered rat neural stem cells, RG3.6/IGF-1^{+} cells, which conditionally express the insulin growth factor-1 (IGF-1). Our results demonstrate that IGF-1 induces neurogenesis in a culture system and in damaged area of the rat brain after transplantation and could have important implication in amelioration of brain damage after TBI.

Dedication

This thesis is dedicated to my adorable husband Mr. Abhishek Singh without whom this work would not have been possible. He has always been a source of inspiration and overall encouragement to carry on my research work. With his support and love I was able to achieve my goals.

Dr. Sweta Singh

Table of Contents

List of Illustrations

Introduction

Recently much attention has been dedicated to understanding the molecular underpinnings of traumatic brain injury (TBI), a major cause of disabilities with an approximated 1.7 million cases each year (1). TBI, in many instances, leads to impairment of overall cognitive and motor functions. These permanent consequences of TBI are due to neuronal loss (2-4). Neuronal death is observed immediately and long after injury. Interestingly, these long-term consequences mostly lead to seizure (5, 6), endocrine disorder (7, 8), reconstitute the pathophysiology of Alzheimer's disease, and develop most of its molecular hallmarks, like the neurotoxic phosphoTau (pTau) proteins (9, 10).

Postmortem pathological studies and experimental brain injury using murine model systems have provided several lines of evidence that hippocampal lesion and loss of the stem cell pool are the contributing factors to cognitive dysfunction, like the hippocampus-driven learning and memory, after TBI (4, 11-12). Neurogenesis, the differentiation of stem cells into neurons, is an ongoing process throughout the adult life and after injury, takes place in subventricular zone (SVZ) and the dentate gyrus region of the hippocampus. During this process, neural stem cells expand and migrate towards the injured site of the brain to minimize the damage (13, 14). Newly born neurons play important roles to re-establish proper network connectivity to restore cognitive function. Any impairment or depletion of these neural stem cell pools leads to learning and memory deficits (15, 16). Controlled cortical impact (CCI) is an established and widely used model for the study of neurobehavioral dysfunction and neurons death following TBI, a technique facilitated in the presented studies (17-20). Recent findings suggest that

CCI leads to rapid death of newly born neurons in dentate gyrus and alteration in hippocampal neurogenesis (21). This process, subsequently leads to loss in number of newborn neurons. Restoration of the number of newborn neurons in the subgranular zone of the hippocampus has been observed weeks following CCI and is thought to be due to post-traumatic neurogenesis (22, 23). However, only a small population of the newly born neurons lives on and grows into functionally active mature neurons that incorporate subsequently to the neuronal network (24). TBI induced neurogenic responses show the potential of multipotent cell in ameliorating the damage and integrating into neuronal network, however, this response is not sufficient for complete functional recovery (25).

Neurotrophic factors play a pivotal role in proper development of the CNS including several aspects of neuronal development crucial for their normal functioning such as growth, survival, and migration. Impairment of these crucial developmental processes can have debilitating effects on overall brain development and function which may lead to CNS disorders later in life. Recently, growth factors have gained scientific attention in understanding their role in the disease etiology and potential treatment for many neurodegenerative disorders has expanded. One of these growth/trophic factors is the Insulin-like Growth Factor-1(IGF-1), a polypeptide of 70 amino acids which has an important implication on brain development. IGF-1 has also been used to treat several neurodegenerative disorders such as Parkinson's disease and amyotrophic lateral sclerosis (ALS) (26-28). Given the importance of IGF-1 in neuronal homeostasis and neurogenesis, it is crucial to understand the molecular pathway triggered in response to IGF-1 stimulation.

IGF-1 Signaling Cascade

The IGF signaling cascade encompasses IGF-1, IGF-2, and insulin and their receptors IGF-1R, IGF-2R, and IR respectively, and many other associated proteins such as insulin receptor substrate (IRS) and Src homology domain. In extracellular circulation, most of the IGF-1 (99%) is found in complexed form with IGFBP3 or IGFBP5 and to a glycoprotein which is an acid labile subunit. IGFBPs help in IGF-1 stabilization, regulation of concentration and bioavailability. Apart from this, IGFBPs also play an important role in the regulation of IGF-1 binding with its receptors (IGF-1R). After binding with IGF-1R, IGF-1 triggers a conformational change and leads autophosphorylation of tyrosine residue of the receptor which in turn enhances tyrosine kinase activity (29). Further, it recruits IRS, CRK, and SHC which lead to the activation of the entire major pathways i.e. the phosphatidylinositol-3-kinase/AKT/mTOR (PI3K/AKT) pathway, the MAPK/Ras-Raf-Erk pathway, and the Janus kinase/signal pathway (30). The pathways converge to activate the mechanistic target of Rapamycin (mTOR) to initiate protein synthesis and controls cell survival, cell cycle, and cytoskeleton remodeling. Activated mTOR also regulates activity of the cell death antagonist Bcl-2 to inhibit programmed cell death (PDC). Up-regulation of AKT activity also leads to inhibition of c-Jun N-terminal kinase (JNK) pathway that ultimately inhibits cell death. IGF-1 induced serine–threonine mitogen-activated protein (MAP) kinases pathway also regulates programmed cell death in different kind of cells. Upon activation, IGF-1 receptors get phosphorylated at tyrosine residue and recruit Shc protein which in turn recruits an adaptor protein Grb2 and leads to activation of the Ras-ERK pathway.

MAPK/ERK pathway of IGF-1 signaling, mostly controls cell differentiation, gene expression, migration and in some case apoptosis (31).

Role of IGF-1 in CNS/CNS Disorder

IGF-1 is primarily produced by liver under the control of growth hormone (GH) and affects early CNS and neuronal development. It is also produced locally by many other types of cells under different physiological/pathophysiological conditions. Expression of IGF-1 is spatially and temporally regulated during the early development of central nervous system (CNS) (32). IGF-1 is strongly expressed in different parts of CNS/PNS such as cerebral cortex, hippocampus, midbrain, olfactory bulb, and spinal cord during embryonic development. In the adult, however, IGF-1 expression is restricted to distinct areas of the CNS. High IGF-1 protein levels demonstrate context-dependent function which leads to proliferation and differentiation of neurons (33, 34).

As mentioned above, IGF-1 is predominantly produced by the liver under the regulation of GH in adults; however, it can also be secreted by several other tissues. IGF-1 can work through endocrine, paracrine and autocrine mechanism. IGF-1 receptors are ubiquitously present in all cell types but they are mainly expressed by cells of mesenchymal origin, like fibroblasts, chondrocytes, and osteoblasts. In the human brain, IGF-1 receptors are present mostly in the hippocampus and parahippocampal areas, whereas amygdala, cerebellum, and cortex also show low expression levels of these receptors (34, 36). The IGF-1 receptors and the low-density lipoprotein receptor-related protein 1 (LRP1) facilitate the uptake of circulating IGFs into the brain (35). There are several research reports which demonstrated that IGF-1 is mainly transported across the BBB through

transcytosis. However, several other studies demonstrated that a significant quantity of IGF-1 is definitely produced in the CNS as IGF-1 mRNA expression has been seen especially in the adult rat's cerebellum and brain stem (36, 37).

Besides playing a pivotal role in brain development and amelioration of neurodegenerative diseases, IGF-1 also plays an important role in recovery from traumatic brain injuries (38). Several recent reports have demonstrated the evidence in support of IGF-1 for its role in wound healing and damage repair of the brain after injury as IGF-1 can promote differentiation of neural stem cells into different phenotype (i. e. neurons, astrocytes, and oligodendrocytes) in vitro and in vivo (37). A significant increase in IGF-1 expression has been observed soon after TBI and remained elevated throughout the week after, in contrast to the control group (39-41). Another finding has shown a significant increase of IGF-1 mRNA, with a peak at 24 h after the impact in rat TBI model. It has also been demonstrated that there is both spatial and temporal change in IGF-1 expression which was suggested to act as an autocrine/paracrine regulator of neuronal survival after TBI in rats (42).

Several in vivo and in vitro experimental studies have authenticated that IGF-1 potentiate overall neurogenesis due to increased neuroprotection, cell proliferation and neuronal differentiation (43-45). Recently, several reports have shown that long-term administration of IGF-1 into rats significantly improved neurogenesis, cognitive and motor function after TBI (46, 47). It has been also documented that IGF-1 is actively involved in the regulation of astrogliosis, which is one of the earliest manifestations of the damage repair response in the brain, typically occurring delayed in the areas of injury as well as surrounding area and also leads to the formation of glial scar (48). Several in

vitro and in vivo experimental models have demonstrated that IGF-1 enhances neurogenesis by inducing differentiation of neural stem cells, as well as induces growth of dendritic processes in newly formed neurons (49-51).

Insulin and IGF are also known to involve in maintaining stemness and promoting self-renewal of human embryonic stem cells (ESCs). Inhibition of IGF-signaling leads in differentiation of ESCs, however, it's still elusive if fate of a particular cell is favored under such circumstances. Studies of blocking downstream Insulin/IGF-1 signaling at a various level have provided a significant amount of evidence that confirms that blocking of Insulin and IGF-1 induce differentiation (52, 53). These studies also revealed that context-dependent role of Insulin or IGF-1, are based on PI3K/AKT signaling and cross-talk with other signaling pathways, such as mTOR. These revelations led to the use of growth factors to treat brain injury and have shown promising effects on neurogenesis and recovery of cognitive function (54, 55). Induction of post-traumatic neurogenesis/neuroprotection has a potential to overcome from debilitating effect of TBI on learning disability and memory deficits.

Considering these properties, IGF-1 has been used extensively to treat several neurodegenerative diseases such as ALS and CNS trauma (26-28). However, delivery of IGF-1 remains challenging and limited due to short half-life and poor pharmacokinetic property (56). Availability of IGF-1 to CNS is also limited due to its inactivation, fast clearance and sequestration by binding proteins. Gene transfer-based approach with viral vector has opened plethora of possibility in gene therapy. As presented in our study, transplantation of genetically modified neuronal stem cells has promising potential in regeneration of damaged brain.

In this study, we sought to provide exogenous IGF-1 and genetically engineered neural stem cells (NSCs) and investigate their role in (1) amelioration of TBI-induced damage and (2) induction of differentiation of neural progenitors into neurons. We hypothesize that the transplanted progenitor cells will secrete IGF-1 that can induce damage repair and neurogenesis of the exogenously introduced as well as host NSCs. We have used a well characterized rat NSC line (RG3.6) to generate stable lines containing lentiviral constructs pSLIK-Neo-IGF-1-HA programmed for conditional expression of IGF-1. The resulting RG3.6/IGF^{+} cell line has been used for all studies presented in this thesis.

Material and Methods

RG3.6 cell line

RG3.6 cell line has been immortalized by stably transfecting the v-myc gene into RG cells harvested from embryonic cortex of GFP positive (GFP+) rats from Dr. Martin Grumet, Rutgers, New Brunswick. Culture of the RG3.6 cells was carried out on matrigel (BD Biosc., USA) coated plates. FGF-2 (10µg/ml), heparin sulphate (1ng/ml) and 1 µl/ml B-27 were supplemented to RGM to prepare RGM-FGF for culturing the RG 3.6 cells. Cells were provided equal volume of fresh RGM-FGF every day. Further, we generated stable cell lines containing the conditional lentiviral constructs pSLIK-Neo-IGF-1-HA (Figure 1). We have followed established viral transfection protocols to introduce these vectors into these NSCs and selected successfully transduced cells with the antibiotic hygromycin to establish stable cell lines.

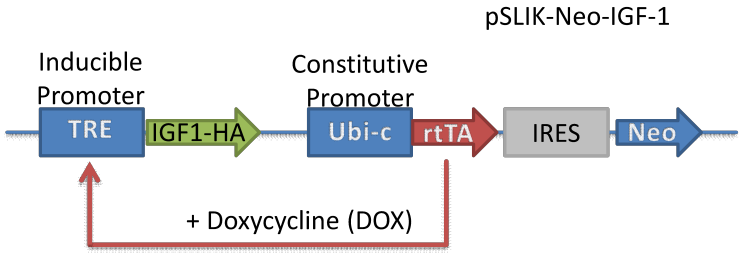


Figure 1: The conditional pSLIK vector system has been used for expression of IGF1-HA in RG3.6 cells. Schematics show the composition of the vector: Doxycyline (Dox) activates the reverse tetracycline transactivator (rtTA) that binds to the tetracycline response element (TRE) to drive expression of IGF-1-HA.

Immunohistochemistry

Cells were cultured on coverslips coated with matrigel, rinsed in DMEM/PBS once, and fixed in 4% paraformaldehyde (PFA). Cultures were washed in PBS (3X, 10min each) and blocked by blocking buffer (5% normal goat serum, 0.5% BSA and 0.5% TritonX-

100 in PBS) for 1h. Further, culture were placed in primary antibody solutions in blocking buffer and incubated overnight at 4°C. Cultures were rinsed with PBS (3X, 10min each) and placed in secondary antibody for 1h at RT and mounted after washing with ProLong® Gold Antifade Mountant with DAPI. Confocal images were obtained using a 63x objective on a ZEISS spinning disc microscope, taken randomly from different fields (n=10-15), and three independent experiments.

Protein Extraction and Western blot analyses

Cells were cultured on matrigel-coated 6 wells plate with and without doxycycline in RGM-FGF medium. To assess the expression and secretion of IGF-1, cells were lysed using lysis buffer (10% glycerol, 1% NP40, 20mM Tris (pH7.4), 2.5 mM EDTA (pH8), 2.5mM EGTA (pH8), including Roche Protease inhibitor cocktail (#04693116001) at 2 different time points (18h and 24h) with / without Dox treatment. Media was also collected at corresponding time point (18hr and 24hr). BCA assay was carried out to determine protein concentration (ThermoScientific, Rockford, IL, USA) before loading the protein samples on SDS-PAGE gels. Twenty-five µg of protein sample was used and run on SDS-PAGE gels. Approximately, 3µl of Precision Plus Protein WesternC Standards (161-0376 BIO-RAD, USA) was used for standard molecular weight markers. Gels were run for 3h at 70mV and further transferred to PDVF (Immobilon) membrane for 2h. The PDVF membranes were blocked by using 5% Milk, 0.1% Triton X-100 in TBS 1h and incubated with primary antibody such as rabbit monoclonal anti-HA for IGF-1 at 1:1000 (H9608 Sigma, USA) and anti-pAKT (phospho-Threonine 308), a mouse monoclonal antibody against βIII -tubulin (1:1000, Covance, Princeton, NJ, USA) in 5% BSA in PSB-Tween at 4°C overnight on shaker. Next day, membranes were

washed (3X, 15 min each) with PBS-Tween followed by secondary antibody incubation (rabbit and mouse horseradish peroxidase (DARHRP)-conjugated, 1:1000; 711-165-152-Jackson ImmunoResearch) for 1 h at room temperature. Further, the membrane was washed in PBS-Tween and signal developed with SuperSignal West Dura Extended Duration Substrates (34076, ThermoScintific, USA) as per instruction.

Cell differentiation

The RG3.6 cells were plated into 24 wells plate containing coverslip coated with matrigel, and cultured for 24 h in RGM-FGF. Further, cells were allowed to differentiate for 6 days by withdrawing the bFGF-2 from the media into two conditions, with Dox and without Dox. After 6 days, cells were washed and fixed with 4% PFA and washed again in PBS (3X,10min each) and blocked with blocking solution (5% normal goat serum, 0.5% BSA and 0.5% Triton X-100 in PBS) for 1h followed by overnight incubation at 4° C with rabbit monoclonal anti-HA for IGF-1 (1:1000, H9608 Sigma, USA), βIII-tubulin (Tuj1) mouse monoclonal antibody (1:1000, Covance, Princeton, NJ, USA), rabbit monoclonal anti- microtubule associated protein-2 (MAP2, 1:500 , Sigma), mouse monoclonal anti-Nestin (1: 1000, DSHB, #401) to identify glia and neurons. Next day, cultures were washed with PBS (3X, 10min each) and incubated with corresponding secondary antibody for 1h at RT followed by washing and mounting with ProLong® Gold Antifade Mountant with DAPI.

Chemical modification of Chitosan microsphere surface

Chemical modification of chitosan microspheres was done as per previously reported protocol (57). Before seeding with RG 3.6 cells, chitosan microspheres were washed in

deionized water (DI) water and incubated overnight with 50mM HEPES buffer having 0.9% NaCl (pH 7.4) (HBS) and supplemented with 0.5 mg/ml heparin and 0.45 mM Genipin, to facilitate the cross-linking of the heparin to the Chitosan scaffolds as per previous protocol . Next day, heparin cross-linked chitosan microspheres were rinsed (3X, 10 min each) in HBS followed by incubation with fibronectin (10 μg/mL) for 4 h and FGF-2 (1 μg/mL) for 2 h in BSA solution (1mg/ml). Further, spheres were centrifuged at 730*g* and rinsed with HBS to remove any excess of FGF-2 followed by resuspension in Neural Precursor Media (NPM) with DMEM/F12 containing B27, gentamycin (50 μg/ml) and apo-transferrin (50 μg/ml). Chitosan microspheres were washed again in HBS followed by seeding with RG 3.6 cells (20:1::cells to microspheres) and incubation at 37°C overnight. Three microliter of spheres/cells carrying approximately, 10,000 cells/ μl as injected into the lesioned somatosensory cortex.

Controlled Cortical Impact

CCI in rat brain was carried out as per previously established protocol (57). For transplantation, twelve, two months old adult male rats (Sprague Dawley) were anesthetized by ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture intraperitoneally. The fur surrounding the head was cleared and a midline scalp cut was carried out using a scalpel. The skin was deflected to open up the skull to carry out craniotomy midway between Bregma and Lambda with a drill (5 mm diameter trephine) as per earlier protocol (57). To avoid heat production and damage of underneath dura mater and neocortex, PBS was spread on the skull while performing craniotomy. CCI injury was performed using a stereotactic apparatus which was controlled by cortical impactor device with anvil tip of 3.5 mm diameter. To perform the CCI injury, the impact was

done with 4.0 ± 0.2 m/s velocity and penetration depth of impactor was set at 1.5 mm while the period of deformation was 150 msec. Following the CCI injury, dura mater integrity was checked and the scalp cut was stitched with 3-0 nylon thread. All subjects received Buprenorphine (0.05 mg/kg, SC) and 0.9% saline solution (3% body weight, SC) after injury to alleviate any pain and avoid dehydration. Animals were put on hot pads (at 37°) and supervised for 2-3h after craniotomy.

RGC-sphere transplantation

RGC-sphere transplantation was carried out as per previous protocol (57). RGC-microspheres were transplanted into the injured cavity of rats 7 days post CCI. All rats were anesthetized by ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture followed by incision to open up the skull. Microspheres coated with RGCs were suspended again in phenol-free media devoid of all supplements. 3 μL cell-microspheres complex suspension was loaded into a 10 μL Hamilton syringe and implanted into the cortex at the center of the injured cavity below the dura mater at three different depths (1.5, 1.0 and 0.5 mm) over 5 min. Cells were Injected to the injured cavity at 5 minute intervals. The syringe was removed after 5 minutes of the last injection. The cut on scalp was stitched with 3-0 nylon thread and the rats were placed on a hot pad (37 °C) and supervise for next 2-3hrs (56).

After cell transplantation, rats were further divided into two groups; one group was supplemented with Dox in drinking water (1mg/ml) to induce the IGF-1secretion while other received normal water. Third group only received microspheres.

Behavioral analysis

At 21 days after transplantation, between 11 a.m. and 1 p.m., all animals were subjected to a set of behavioral test by a researcher who was unaware about the different experimental groups of rats. All rats were allowed to accustom with the experimental environment 30 min prior to any tests. The following behavioral tests were performed: **Sticky label test**: This test examines somatosensory asymmetry. Each animal received a training trial and a test trial 21 days post transplantation; the latency to remove the adhesive labels attached to both forelimbs was recorded for the test trial. **Beam walking test**: This test was performed to assess the motor function to evaluate the efficacy of IGF-1 in amelioration of damage after TBI. The rats were placed at the end of wooden beams of 80 cm in length placed 50 cm above the ground. Two different beam widths were used (5 and 2cm). A dark box with fruit loop at the other end of the beam was put to serve as a target for the rat to reach. For the inclined beam-walking test, a wooden beam of 80 cm in length and 2 cm in width was elevated at a 30˚angle. The number of foot slips (both hind legs and front legs) and the time to cross the beam was recorded and analyzed.

Immunohistochemistry of brain sections

Animals were anesthetized by a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg) and perfusion (3% PFA) was carried out 21 days post RGCs-microspheres transplantation. Further, rats were euthanized and brains were collected and post-fixed in 4% PFA overnight. Following day, brains were washed in PBS to remove excess PFA and cryoprotected by immersing in sucrose (30% in PBS) for 3 days. Brain specimens were frozen OCT in cryo-embedding media (CA95057-838, VWR Clear Frozen Section

Compound). 20 μm thick coronal tissue slices were cut, washed and permeabilized with PBS-TritonX-100, followed by blocking with 10% BSA in PBS, and incubation with primary antibodies overnight at 4°C. The following antibodies were used for the presented studies: anti-HA antibody for IGF 1:500 (Sigma, H9608), mouse monoclonal anti-βIII-tubulin antibody (1:1000, Covance MMS-435P), and anti-GFP antibody 1: 2,500 (AVES, Tigard, Oregon). Following day, brain sections were washed and incubated with corresponding secondary antibodies (1:1000) for 1h at RT. After washing, specimens were embedded with mounting media (ProLong® Gold Antifade Mountant with DAPI). Confocal images were acquired using a ZEISS spinning disc microscope near injury cavity in cortex.

Results

Characterization of R.G.3.6 Cell for IGF-1 Expression and Secretion

In order to provide exogenous IGF-1 and additional NSCs into the injured cavity, we used lentiviral-based system and genetically modified NSCs (RG 3.6 cells) for conditional expression of IGF-1. To characterize the capability of the RG3.6 cells to induce expression of IGF1-HA, cells were cultured in RGM-FGF with and without Dox for 24h. After 24h, cells were fixed and probed for HA (IGF-1) (**Figure 2**).

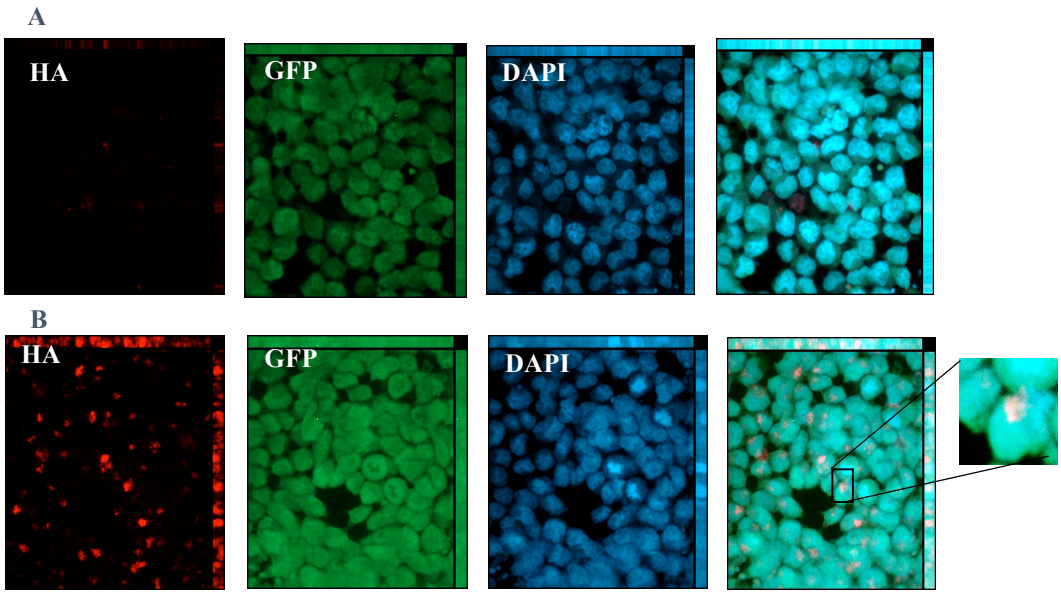


Figure 2: Neuronal Progenitor Cells **(**RG 3.6 Cells) induce the express of IGF-1-HA upon Doxycycline (Dox) treatment. (A) RG3.6/IGF-1^{+} cells before Dox and (B) after Dox treatment (24h incubation). Dox treatment induces expression of HA tagged IGF-1 in RG3.6 cells (red).

Cells treated with Dox for 24h showed perinuclear expression of IGF-1 suggesting IGF-1-HA in the secretory pathway, while cells without Dox were unable to express IGF-1 (Figure 2A and B). To evaluate the ability of the established neural stem cell line to secrete the IGF-1, cells were cultured in RGM-FGF with and without Dox. Both, cell lysate and conditioned medium were used in western blot analyses. Expression of IGF1-

HA was evident in cell (lysate but not in collected medium after 18h incubation with Dox. Further, expression and secretion of IGF-1-HA could be detected after 24h incubation with Dox as indicated by IGF-HA positive bands in cell lysate and collected medium. RG3.6/IGF^{+} cells not exposed to Dox treatment did not show expression of IGF-1-HA (Figure 3).

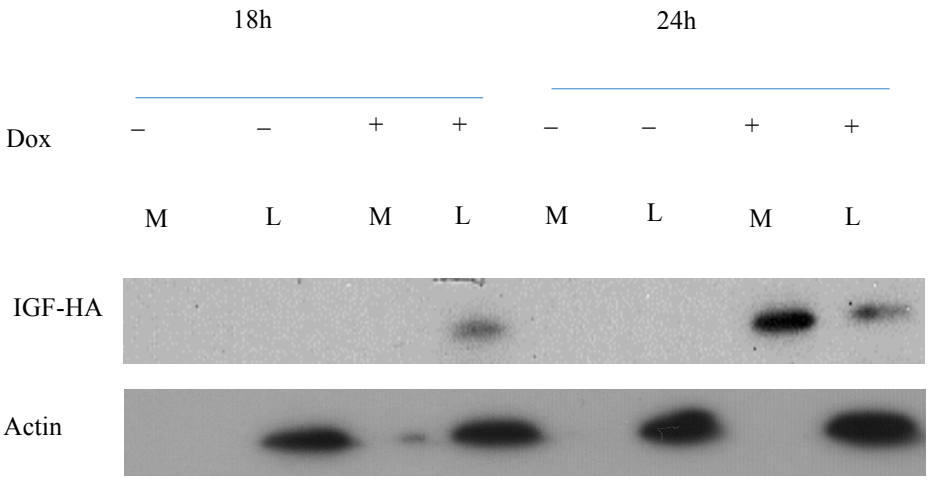


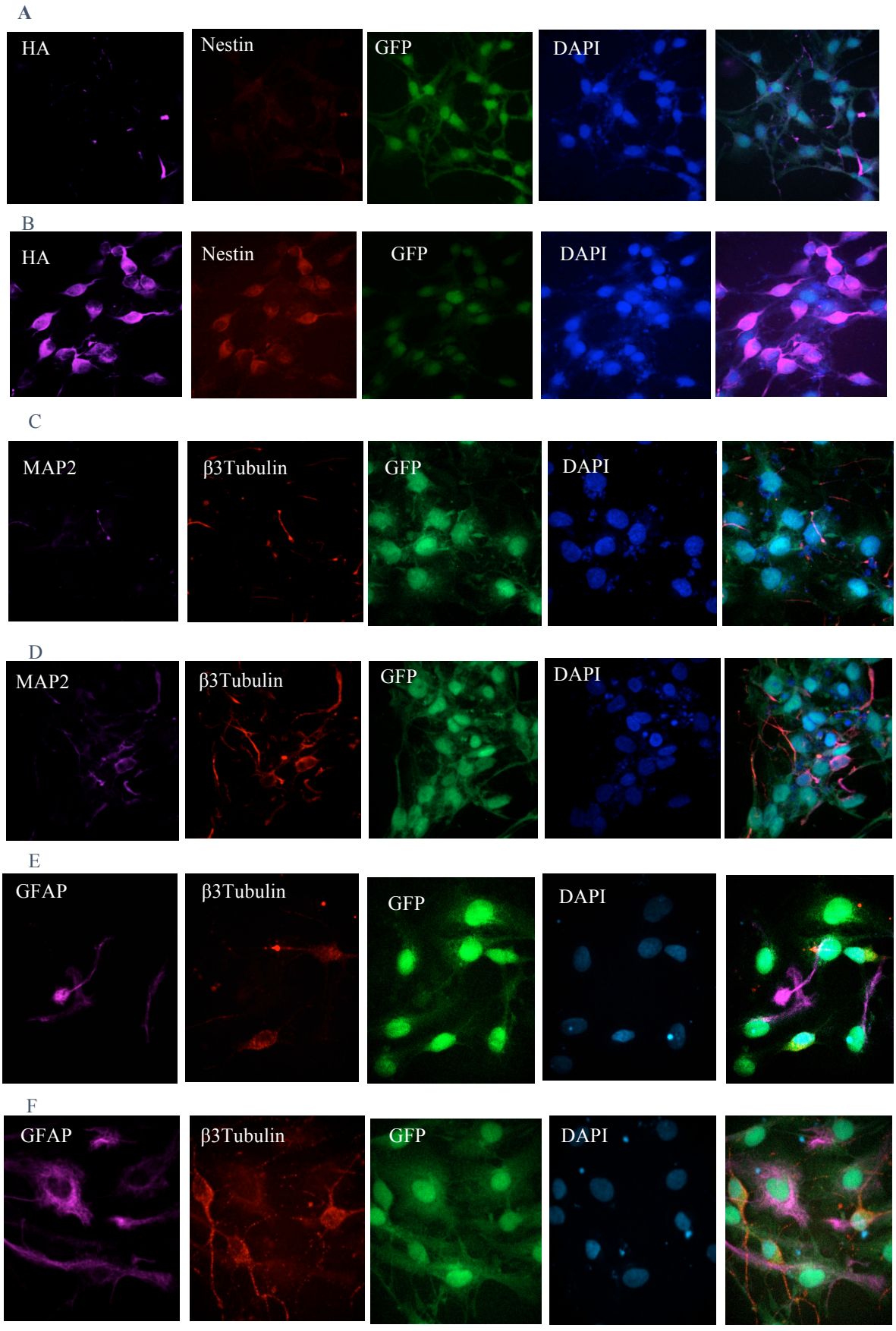
Figure 3. RG 3.6 cells secrete IGF-1-HA into medium after 24h of Doxycycline treatment. Representative western blot analysis of cell lysate (L) and medium (M) collected after an 18h and 24h Dox treatment shows induction and secretion of IGF-1-HA.

IGF-1 induces neuronal differentiation of RG cells

RG-IGF-1 cells were cultured on matrigel-coated plates for 24h with and without Dox treatment. After 24h, FGF was removed from culture medium to induce differentiation in both conditions (with and without Dox). Three days after differentiation, cells took spherical shape and started showing small processes. Six days after induction, cells morphology was dramatically changed, having small soma and extended processes, or showing clear glial morphology. Cells were fixed after 6 days in differentiation culture

and used to determine the number of astrocytic and neuronal cells (Figure 4). RG3.6/IGF^{+} differentiating in presence of Dox expressed IGF1-HA and showed higher expression of Nestin, an early marker of neurogenesis, MAP2, and βIII-tubulin, a marker for mature neurons. Numbers of βIII-tubulin positive cells and numbers of processes were counted manually to assess the effect of IGF-1 on differentiation (Figure 4G and H). Upon induction of IGF-1, the percentage of βIII-tubulin positive processes increased significantly (~52%) as compared to control cells (~34%, Figure 4G). Further analyses confirmed that IGF-1-HA induction increases neurogenesis as indicated by an increase of βIII-tubulin positive cells (~21%) as compared to controls (~11%) (Figure 4H). A similar result was seen in western blot analyses of differentiated cells (6 days) showing increased βIII-tubulin expression after IGF-1 induction (Figure 4J). Thus, these results indicate that IGF-1 increases neuronal differentiation of RG/IGF^{+} NSC.

Recently, it has been shown that IGF-1 induces proliferation of cerebral cortical precursors through the activation of (PI3K)/Akt pathway (56). To assess, IGF-1 mitogen effect in NPCs, we determine the AKT phosphorylation by western blot analysis. We found that Dox-driven expression of IGF-1-HA indeed increased AKT phosphorylation at Threonine (308) (Figure 4I). These results indicate that in NCPs, the PI3K/Akt pathway appeared to mediate neurogenesis-related effects of IGF-1 partially, if not completely.


A
HA
Nestin
GFP
DAPI
B
HA
Nestin
GFP
DAPI
C
MAP2
β3Tubulin
GFP
DAPI
D
MAP2
β3Tubulin
GFP
DAPI
E
GFAP
β3Tubulin
GFP
DAPI
F
GFAP
β3Tubulin
GFP
DAPI

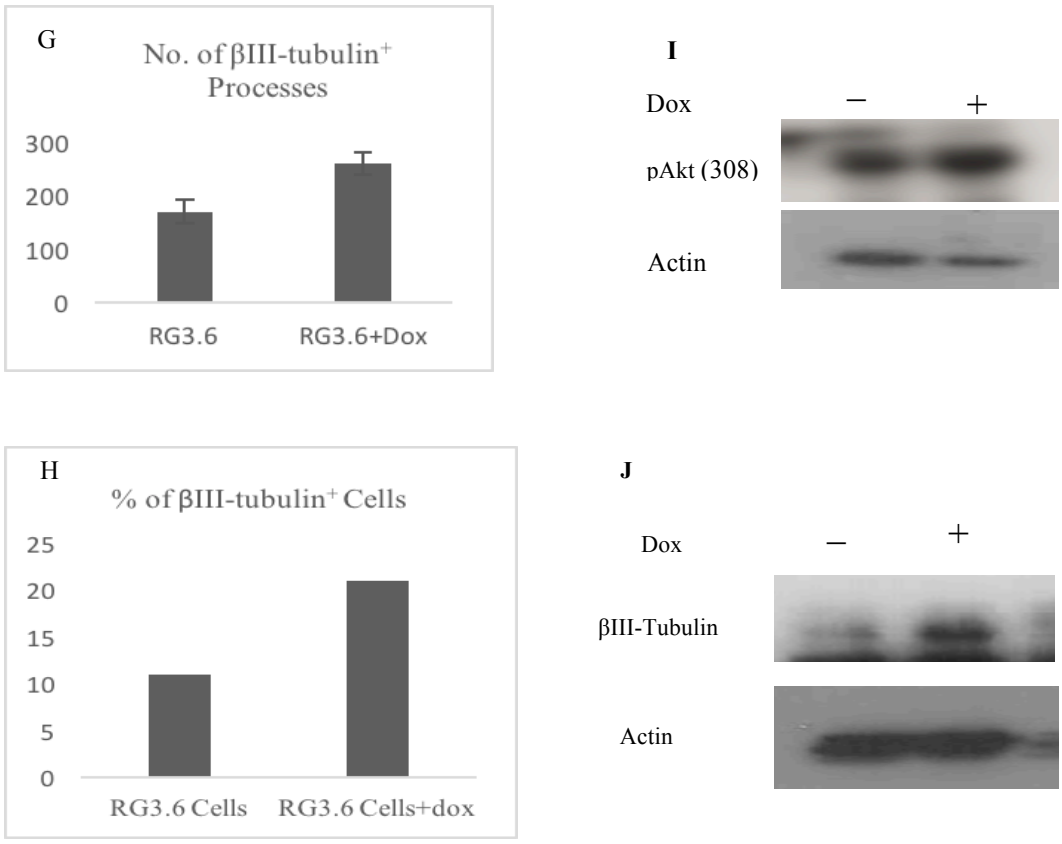


Figure 4: Insulin-like growth factor-1 (IGF-1) induces neurogenesis in Radial-glial cells (RGCs). Assessment of effect of Insulin-like growth factor-1 (IGF-1) on neurogenesis of neuronal progenitor cells (NCPs) in differentiation media (bFGF withdrawal). Phenotypic and quantitative analysis of RG 3.6 cells, 6 days after differentiation**.** Representative images of HA (IGF-1), Nestin (red), βIII-tubulin (red), MAP2 (purple). Immunoreactivity after 6 days differentiation (**A** (HA and Nestin**), C** (MAP2 and βIII-tubulin), **E** (GFAP AND βIII-tubulin) without Dox and (**B** (HA and Nestin**), D** (MAP2 and βIII-tubulin) and **F** (GFAP AND βIII-tubulin)) with Dox**.** Quantification showing of number of processes, (**G**) and number of βIII-tubulin^{+} Cells with/without Dox (**H**). Induction and secretion of IGF-1 enhances the βIII-tubulin expression and phosphorylation of Akt at Thr (308) (**I** and **J**).

Chitosan surface chemistry modification

The purpose of this experiment was to provide exogenous trophic factor and healthy stem cells population by transplanting genetically engineered neuronal progenitor cells (RG3.6 Cells) that can conditionally secrete IGF-1 and potentially differentiate into neurons. To deliver the engineered RG3.6 cells to the injury cavity, Chitosan microsphere were used

as a scaffold. Chitosan microspheres were soaked in FGF before seeding the cells onto them. The so-prepared microspheres provide a stable scaffold for the RG cells, and present an essential tool for stem cell transplantation studies. RG3.6 cells were seeded on the chitosan microspheres 12h before transplantation (Figure 5). RG3.6 cells coated on the surface of chitosan microsphere were grafted into a neocortical injured cavity 7 days after control cortical impact. The transplanted RG3.6 cells can easily be distinguished from the host brain cells tissue with the help of fluorescence microscopy as they express the green fluorescent protein (GFP).

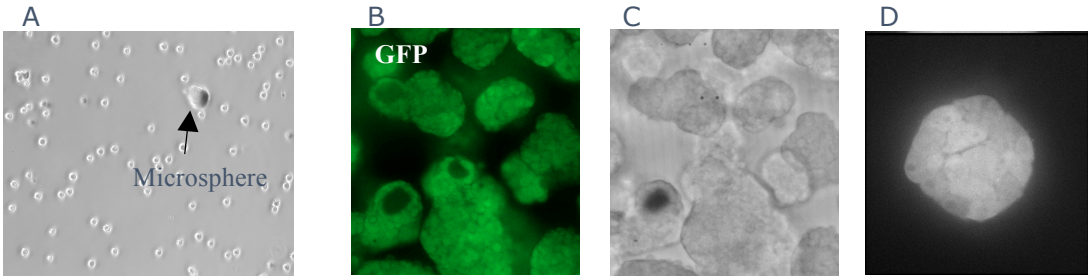
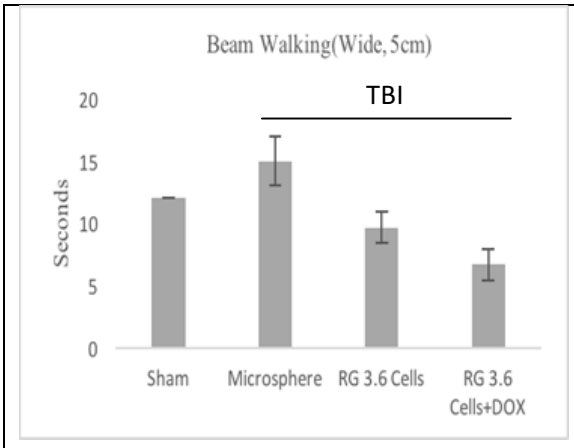


Figure 5. Immunocytochemical analyses of RG3.6 cells on chitosan microspheres. Images showing RG3.6-IGF+ cells seeded on microsphere scaffolds, instantly after seeding (**A**), cells coated on microspheres after an overnight incubation (GFP fluorescence in **B,** bright-field image in **C**) and single microsphere coated with cells (GFP fluorescence in **D**)

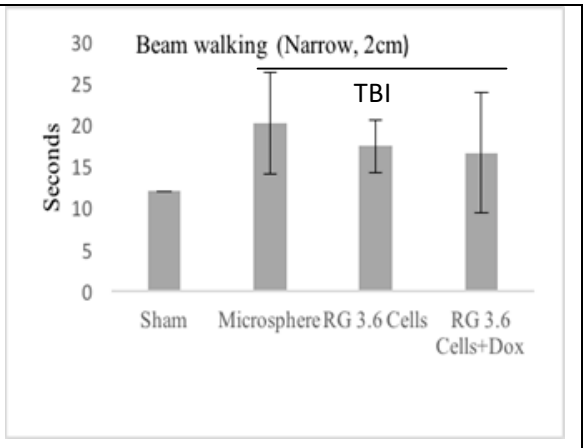
Behavioral Test

To evaluate the efficacy of IGF-1-HA secreting NSCs in preserving the motor/sensorimotor function, several sets of test were conducted. Animals in all groups were walking straight with upright tail position.

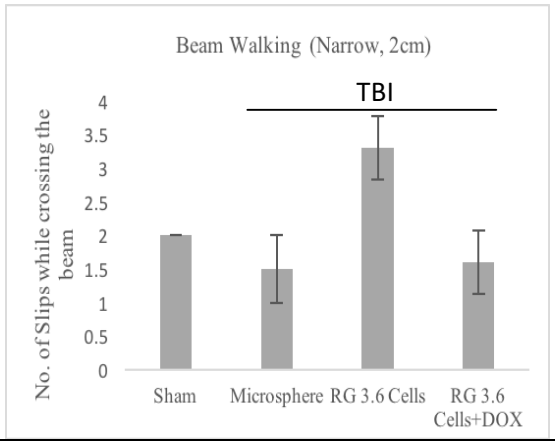
A



B



C



D

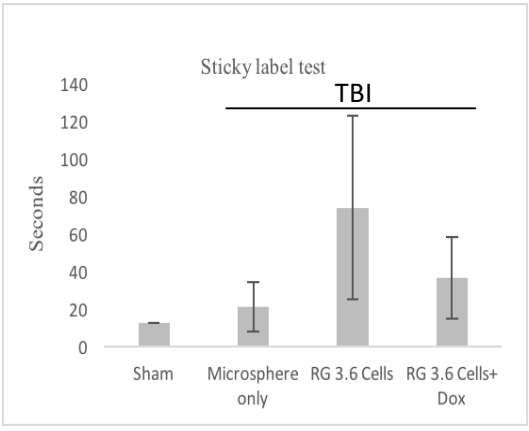


Figure 6: Assessment of sensorimotor activities after engineered RGCs-Microspheres transplantation after TBI. Beam Walking (A) wide beam (B) Narrow beam (C) Total slips while crossing the beam (D) Sticky Tape Removal have been used to determine the sensorimotor function.

Importantly, no signs of hemiparesis were seen in our experiments. In beam walk test, animals after RG3.6/IGF^{+} transplantation and Dox treatment (inducing exogenous IGF-1-HA expression) were walking faster and were better balanced on the beam as compared to the group that received cells but no Dox (no IGF-1), or microspheres alone (Figure 6A and B). In narrow beam test, we again evaluated the time to cross the beam and numbers of slips. The group of animal that received RG3.6/IGF^{+} cells and Dox was faster to cross the beam. They slipped fewer times while crossing the beam, which was similar to the control group. Surprisingly, the group of animal that only received the cell was even less balanced than animals that received microspheres only (Figure 6C). Sticky paper tests were performed twice to evaluate the role of IGF-1 in preservation sensory motor function after damage. IGF-1 induction showed improvement in sensory motor activity as compared to control rats. Our preliminary data in these tests also showed that rats which received microspheres alone had improved balance (6C) and better performance in sticky label removal test comparable to controls (Figure 6D).

Immunohistochemistry

To assess the ability of transplanted cells to secrete the IGF-1-HA in vivo upon Dox treatment, we carried out immunohistochemical analysis of brain sections of all groups of animals 21 days post transplantation near the injured zone of the cortex. We found that the group that received cells and Dox was able to express IGF-1-HA as seen in HA staining and those cells were also found positive for βIII-Tubulin (Figure, 7C, panel 2). Furthermore, βIII-tubulin staining showed the reduction of neuronal damage as neuronal morphology was looking significantly better (Figure 10C, panel 2) and comparable to sham brains (7A, panel 2). This effect was however not evident in the case of the animal

group that received cells without the Dox stimulation to express IGF1-HA (7C, panel 2). We could not find any microsphere in the brain section at the injury site and nearby area which indicates a possible degradation of chitosan over 21 days and induction of migration of transplanted cells from injury cavity to neighboring areas.

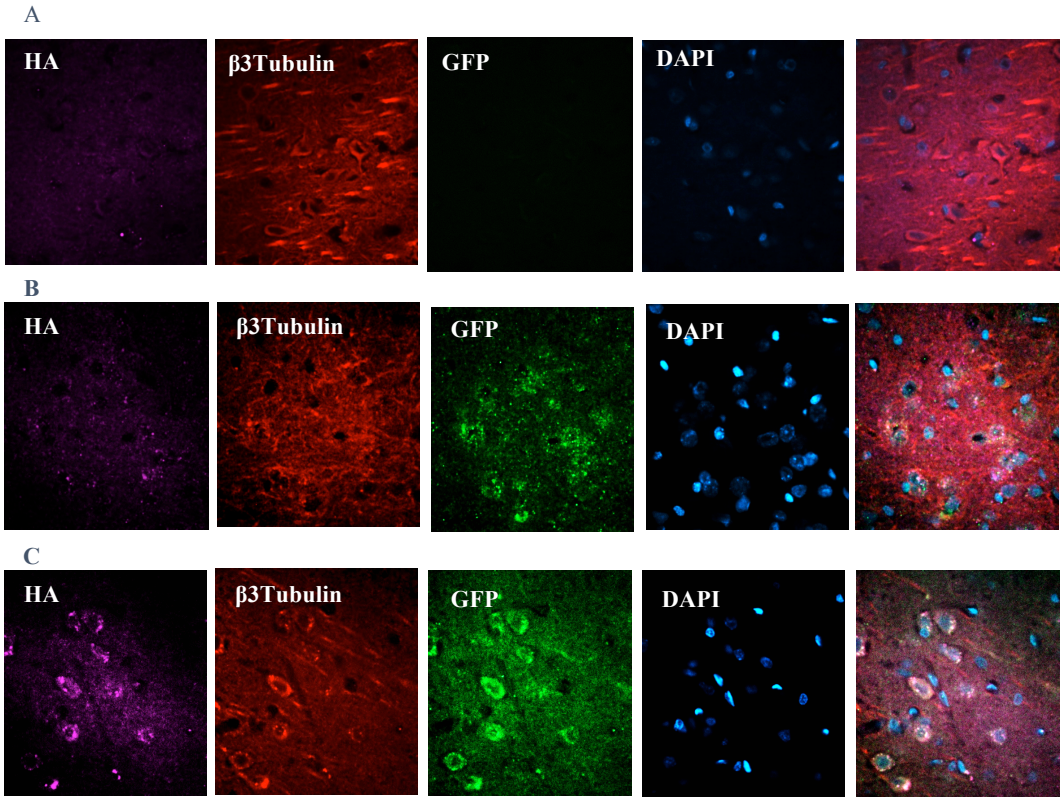


Figure 7: Immunohistochemistry of brain sections from Rats to assess the ability of GFP^{+} genetically engineered RG 3.6 cells to secrete IGF-1 in vivo (A) Sham/no injury (B) transplanted with RG3.6 cells along with chitosan scaffolds 7 days after traumatic brain injury, (C) transplanted with RG3.6 cells along with chitosan scaffolds and received Dox for 21days 7 days after traumatic brain injury. Sections were stained for HA (purple), βIII-Tubulin (red), GFP (green) and DAPI.

These results indicate that cells were able to secrete IGF1-HA which was indeed having a positive effect on neuronal regeneration. However, we were not able to find a large number of the initially 30,000 transplanted cells (GFP+). This may attribute to the technical issue while transplanting the cells. At the time of transplantation, technical

issues delayed the procedure by 4-5 hrs which possibly negatively impacted the overall health of RG-IGF^{+} NSCs. To gain more reliable data, these experiments are currently repeated avoiding long incubation periods on ice.

Discussion

Our recent studies underline the importance of IGF-1 in restoration of regenerative processes, such as neuroprotection and regeneration, after brain injury. IGF-1 is an important trophic factor which induces the differentiation of neurons, astrocytes, and oligodendrocytes (37). The brain has remarkable ability to repair the damage by itself. One of the very early events that happen in the brain after injury is induction of IGF-1 expression to recover brain damage (58). However, complete restoration of function is a rare phenomenon. Brain injury is also known to associate with depletion IGF-1 which mainly affects neuronal survival, regeneration and migration. Depletion of IGF-1 leads to severe neuronal dysfunction. Neuronal stem cell transplantation therapy has recently gained immense attention in the area of neurodegeneration. However, only a small fraction of transplanted cells is able to engraft themselves into the environment of host brain tissue and major fraction of transplanted cells which are unable to engraft themselves, differentiate into glial phenotype but not neuronal phenotype (59). Endogenous neuronal stem cell pool located in the dentate gyrus and sub ventricular zone has limited ability to produce neurons to fully repair damaged brain areas (24). Based on these reports several attempts have been done to provide the exogenous IGF-1 to reverse the TBI-induced brain damage, however, delivery of IGF-1 into the CNS remained a challenge (55). Using genetically engineered neural stem cells to administer exogenous IGF-1 could be a promising strategy.

In this study, we aimed to provide the exogenous IGF-1 through engineered NSCs capable of secreting trophic factors into the injury cavity and to determine whether engineered NSCs able to ameliorate the long-term effects of neurodegeneration after TBI.

An additional advantage of transplanting engineered stem cell is to provide healthy stem cells population to the injured cavity that could differentiate into neurons and glia cells and help in the recovery of the brain.

We characterized murine NSC line (RG3.6) to generate stable lines containing the conditional lentiviral constructs pSLIK-Neo-IGF-1-HA. We have followed the established viral transfection protocols to introduce these vectors into the RG3.6 cells and selected successfully transduced cells with the antibiotic Hygromycin. The conditional expression of IGF-1 was confirmed in immunocytochemical analyses. Further, secretion of IGF-1 was confirmed by immunoblot analyses of the cell lysate or supernatant before and after doxycycline administration. We confirmed secretion of IGF-1 after doxycycline treatment. Molecular tagging of IGF-1 with the human influenza hemagglutinin (HA), we were able to distinguish the exogenous IGF-1-HA from the endogenous proteins. The RG3.6 cells stably expresses the green fluorescent proteins (GFP) making them readily identifiable from surrounding brain tissues after transplantations.

The role of IGF-1 in proliferation has been well documented as well as its importance in stem cell differentiation (60, 61). To assess the effect of exogenous IGF-1-HA on RG3.6 cell differentiation, we removed bFGF from the culture medium containing or lacking doxycycline. Nestin, a marker for early neurogenesis is predominantly expressed in neural stem cells (7). During differentiation, expression of Nestin increased drastically (Figure 3B) and immunofluorescence staining showed that all HA positive cells (IGF-1 expressing cells) were positive for Nestin. Further, higher expression of βIII-tubulin in IGF-1 expressing cells as compared to the cells without IGF-1, suggests that IGF-1 promoted larger population of newly proliferated cells to adopt neuronal phenotype. These signalling pathways positively affect cell cycles progression and activate anti-

apoptotic downstream signalling. A major drawback in growth factor administration for therapeutic application is the potential risk of tumorigenesis. Our findings suggest that IGF-1 promotes neuronal differentiation in the absence of bFGF, this way largely reducing the oncogenic potential of the transplanted neural stem cells.

Recent research has been focused on improving the outcomes of cell transplantation into the brain in case of CNS disorders/TBI as only small population of cell is able to survive and engraft itself into the host environment. Application of biodegradable, non-immunogenic and non-tumorigenic biomaterial has gained focus as it protects the neural grafts from the host immune response and also guides graft growth and integration. Biomaterial scaffold promotes survival of the transplanted cells by establishing them into harsh and nutrient deficient injured tissues environment (62). In this study, we have used chitosan microsphere as a scaffold for grafting cells into the injured brain. Chitosan is a glucosamine-based polymer which mainly metabolizes into non-toxic by product and hence makes it attractive vehicle for cell transplantation (62). In immunohistochemical analyses, we were able to detect some of the transplanted cells in injured brain tissue. We also observed that the GFP-positive cells differentiated into mature neurons as indicated by expression of βIII-tubulin. We are currently repeating these analyses to increase experimental numbers, largely avoiding previous technical issues during the transplantation procedure attributed to long incubation periods on ice. We expect that these new experiments will increase survival of transplanted RG3.6/IGF^{+} cells, up-regulate neurogenesis and further improve regeneration. Altogether, our current results are promising and underline the potential of a combined therapy using IGF-1^{+} neural stem cells to ameliorate brain damage.

References

1. Summers CR, Ivins B, Schwab KA (2009) Traumatic brain injury in the United States: an epidemiologic overview. Mt Sinai J Med 76: 105–110.

2. Lundin A, de Boussard C, Edman G, Borg J (2006) Symptoms and disability until 3 months after mild TBI. Brain Inj 20: 799–806.

3. Levin HS (1998) Cognitive function outcomes after traumatic brain injury. Curr Opin Neurol 11: 643–646.

4. Lynch DR, Dawson TM. Secondary mechanisms in neuronal trauma (1994) Curr Opin Neurol 7: 510–516.

5. McKinney RA, Debanne D, Gähwiler BH, Thompson SM (1997) Lesion-induced axonal sprouting and hyperexcitability in the hippocampus in vitro: implications for the genesis of posttraumatic epilepsy. Nat Med 3: 990–996

6. Pitkänen A, McIntosh TK (2006) Animal models of post-traumatic epilepsy. J Neurotrauma 23: 241–261.

7. Taylor AN, Rahman SU, Tio DL, Sanders MJ, Bando JK, Truong AH, Prolo P (2006) Lasting neuroendocrine-immune effects of traumatic brain injury in rats. J Neurotrauma 23: 1802–1813.

8. Behan LA, Phillips J, Thompson CJ, Agha A (2008) Neuroendocrine disorders after traumatic brain injury. J Neurol Neurosurg Psychiatry 79: 753–759.

9. Johnson VE, Stewart W, Smith DH (2013) Axonal pathology in traumatic brain injury. Exp Neurol 246: 35–43.

10. McKee AC, Cantu RC, Nowinski CJ, Hedley-Whyte ET, Gavett BE, Budson AE, Santini VE, Lee HS, Kubilus CA, Stern RA (2009) Chronic traumatic encephalopathy in athletes: progressive tauopathy after repetitive head injury. J Neuropathol Exp Neurol 68: 709–735.

11. Bigler ED, Blatter DD, Anderson CV, et al (1997) Hippocampal volume in normal aging and traumatic brain injury. AJNR Am J Neuroradiol. 18: 11–23. [PubMed: 9010515]

12. Serra-Grabulosa JM, Junque C, Verger K, et al (2005) Cerebral correlates of declarative memory dysfunctions in early traumatic brain injury. J Neurol Neurosurg Psychiatry. 76: 129–31. [PubMed: 15608014]

13. Chen XH, Iwata A, Nonaka M, Browne KD, Smith DH (2003) Neurogenesis and glial proliferation persist for at least one year in the subventricular zone following brain trauma in rats. J Neurotrauma 20; 623–631.

14. Eriksson PS, Perfilieva E, Bjork-Eriksson T, et al (1998) Neurogenesis in the adult human hippocampus. Nat Med 4: 1313–1317.

15. Deng W, Saxe MD, Gallina IS, et al (2009) Adult-born hippocampal dentate granule cells undergoing maturation modulate learning and memory in the brain. J Neurosci 29: 13532–13542.

16. Jessberger S, Clark RE, Broadbent NJ, et al (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. Learn Mem 16: 147–154.

17. Dixon CE, Clifton GL, Lighthall JW, Yaghmai AA, Hayes RL (1991) A controlled cortical impact model of traumatic brain injury in the rat. J Neurosci Methods 39: 253–262.

18. Lighthall JW (1988) Controlled cortical impact: a new experimental brain injury model. J Neurotrauma 5: 1–15.

19. Dixon CE et al (1999) One-year study of spatial memory performance, brain. morphology, and cholinergic markers after moderate controlled cortical impact in rats. J Neurotrauma 16: 109–122

20. Washington PM et al (2012) The effect of injury severity on behavior: a phenotypic study of cognitive and emotional deficits after mild, moderate and severe controlled cortical impact injury in mice. J Neurotrauma 29: 2283–2296.

21. Rola R, Mizumatsu S, Otsuka S, et al. Alterations in hippocampal neurogenesis following traumatic brain injury in mice. Exp Neurol. 2006; 202:189–99.

22. Gao X, Deng-Bryant Y, Cho W, et al (2008) Selective death of newborn neurons in hippocampal dentate gyrus following moderate experimental traumatic brain injury. J Neurosci Res 86: 2258–2270.

23. Rola R, Mizumatsu S, Otsuka S, et al (2006) Alterations in hippocampal neurogenesis following traumatic brain injury in mice. Exp Neurol. 202: 189–199.

24. Emery DL, Fulp CT, Saatman KE, et al (2005) Newly born granule cells in the dentate gyrus rapidly extend axons into the hippocampal CA3 region following experimental brain injury. J Neurotrauma 22: 978–988.

25. Yu TS, Zhang G, Liebl DJ, Kernie SG (2008) Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors. J Neurosci 28: 12901–12912.

26. Dore S., Kar S. and Quirion R. (1997) Rediscovering an old friend, IGF- I: potential use in the treatment of neurodegenerative diseases. Trends Neurosci. 20, 326–331.

27. Ebert A. D., Beres A. J., Barber A. E. and Svendsen C. N. (2008) Human neural progenitor cells over-expressing IGF-1 protect dopamine neurons and restore function in a rat model of Parkinson's disease. Exp. Neurol. 209, 213–223.

28. Kaspar B. K., Llado J., Sherkat N., Rothstein J. D. and Gage F. H. (2003) Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. Science 301, 839–842.

29. Favelyukis S, Till JH, Hubbard SR, Miller WT (2001) Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nature Structural Biology 8(12): 1058–1063.

30. Arnaldez FI, Helman LJ (2012) Targeting the insulin growth factor receptor 1. Hematology/Oncology Clinics of North America 26(3): 527–542.

31. Andrea M, Vincent, Eva LF (2002) Control of cell survival by IGF signaling pathways Growth Hormone & IGF Research 12: 193–197.

32. Anlar B, Sullivan KA, Feldman EL (1999) Insulin-like growth factor-I and central nervous system development. Hormone and Metabolic Research 31(2-3): 120–125.

33. Bach MA, Shen-Orr Z, Lowe WL, Jr Roberts CT, LeRoith D (1991) Insulin-like growth factor I mRNA levels are developmentally regulated in specific regions of the rat brain. Brain Res Mol Brain Res 10(1): 43-48.

34. Baker J, Liu JP, Robertson EJ, Efstratiadis A (1993) Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75(1): 73-82.

35. Werner H, LeRoith D (2014) Insulin and insulin-like growth factor receptors in the brain: physiological and pathological aspects. European Neuropsychopharmacology 24(12): 1947–1953.

36. Favelyukis S, Till JH, Hubbard SR, Miller WT (2001) Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nature Structural Biology 8(12): 1058–1063.

37. Bondy C, Werner H, Roberts Jr CT, LeRoith D (1992) Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II. Neuroscience 46(4): 909–923.

38. Saatman KE, Contreras PC, Smith DH, Raghupathi R, McDermott KL, Fernandez SC, Sanderson KL, Voddi M, McIntosh TK. (1997). Insulin-like growth factor-1 (IGF-1) improves both neurological motor and cognitive outcome following experimental brain injury. Exp Neurol. 147:418-27.

39. Kazanis I, Giannakopoulou M, Philippidis H, Stylianopoulou F (2004) Alterations in IGF-I, BDNF and NT-3 levels following experimental brain trauma and the effect of IGF-I administration. Experimental Neurology 186(2): 221–234.

40. Li XS, Williams M, Bartlett WP (1998) Induction of IGF-1 mRNA expression following traumatic injury to the postnatal brain. Molecular Brain Research 57(1): 92–96.

41. Sandberg Nordqvista AC, von Holst H, Holmin S, Sara VR, Bellander BM, Schalling M (1996) Increase of insulin-like growth factor (IGF)-1, IGF binding protein-2 and -4 mRNAs following cerebral contusion. Molecular Brain Research 38(2): 285–293.

42. Walter HJ, Berry M, Hill DJ, Logan A (1997) Spatial and temporal changes in the insulin-like growth factor (IGF) axis indicate autocrine/paracrine actions of IGF-I within wounds of the rat brain. Endocrinology 138(7): 3024–3034.

43. Aberg MA, Aberg ND, Hedbacker H, et al (2000) Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. J Neurosci 20: 2896–2903.

44. Cheng CM, Mervis RF, Niu SL, et al (2003) Insulin-like growth factor 1 is essential for normal dendritic growth. J Neurosci Res 73: 1–9.

45. Liquitaya-Montiel A, Aguilar-Arredondo A, Arias C, et al (2012) Insulin growth factor-I promotes functional recovery after a focal lesion in the dentate gyrus. CNS Neurol Disord Drug Targets 11: 818–828.

46. Saatman KE, Contreras PC, Smith DH, Raghupathi R, McDermott KL, Fernandez SC, Sanderson KL, Voddi M, McIntosh TK (1997). Insulin-like growth factor-1 (IGF-1) improves both neurological motor and cognitive outcome following experimental brain injury. Exp Neurol 147: 418-427.

47. Carlson SW, Madathil SK, Sama DM, Gao X, Chen J, Saatman KE (2014) Conditional Overexpression of Insulin-Like Growth Factor-1 Enhances Hippocampal Neurogenesis and Restores Immature Neuron Dendritic Processes After Traumatic Brain Injury. J Neuropathol Exp Neurol. 73(8): 734–746.

48. Fitch MT, Silver J. 1997. Glial cell extracellular matrix: boundaries for axon growth in development and regeneration. Cell Tissue Res 290:379 –384.

49. Niblock MM, Brunso-Bechtold JK, Riddle DR (2000) Insulin-like growth factor I stimulates dendritic growth in primary somatosensory cortex. J Neurosci 20: 4165–4176.

50. Brooker GJ, Kalloniatis M, Russo VC, et al (2000) Endogenous IGF-1 regulates the neuronal differentiation of adult stem cells. J Neurosci Res 59: 332–341.

51. Aberg MA, Aberg ND, Hedbacker H, et al (2000) Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. J Neurosci 20: 2896–2903.

52. Sch¨abitz WR, Hoffmann TT, Heiland S et al (2001) Delayed neuroprotective effect of insulin-like growth factor-I after experimental transient focal cerebral ischemia monitored with MRI. Stroke 32(5): 1226–1233.

53. Guan J, Bennet L, Gluckman PD, Gunn AJ (2003) Insulinlikegrowth factor-1 and post-ischemic brain injury. Progress in Neurobiology 70(6): 443–462.

54. Dixon CE. et al (1999) Amantadine improves water maze performance without affecting motor behavior following traumatic brain injury in rats. Restor Neurol Neurosci 14: 285–294.

55. Marklund N, Hillered L (2011) Animal modelling of traumatic brain injury in \ preclinical drug development: where do we go from here? Br J Pharmacol 164: 1207–1229

56. Thorne RG and Frey 2nd WH (2001) Delivery of neurotrophic factors to the central nervous system: pharmacokinetic consider- ations. Clin. Pharmacokinet. 40, 907–946.

57. Skop NB, Calderon F, Cho CH, Gandhi CD, Levison SW (2014) Improvements in biomaterial matrices for neural precursor cell transplantation. Molecular and Cellular Therapies 2:19.

58. Li XS, Williams M, and Bartlett WP (1998), "Induction of IGF-1 mRNA expression following traumatic injury to the postnatal brain," Molecular Brain Research, 57, 1, 92–96.

59. Boockvar JA, Schouten J, Royo N et al. 2005; Experimental traumatic brain injury mod- ulates the survival, migration, and termi- nal phenotype of transplanted epidermal growth factor receptor-activated neural stem cells. Neurosurgery 56: 163–171

60. Benito M, Valverde AM and Lorenzo M, (1996) IGF-I: a mitogen also involved in differentiation processes in mammalian cells. Int. J. Biochem. Cell Biol. 28, 499–510

61. Lunn JS, Pacut C, Backus C, Hong Y, Johe K, Hefferan M, Feld- man EL and Marsala M, (2010) The Pleotrophic Effects of IGF-I on Human Spinal Cord Neural Progenitor Cells. Stem Cells Dev. 19, 439–452

62. Immunohistochemistry of brain sections from Rats to assess the ability of GFP^{+} genetically engineered RG 3.6 cells to secrete IGF-1 in vivo neural stem cells supported by scaffolds to reconstitute lost tissue. Nat Biotechnol 20: 1111.

63. Mo L, Yang Z, Zhang A et al. (2010). The repair of the injured adult rat hippocampus with NT-3- chitosan carriers. Biomaterials 31: 2184–2192.