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Scholarly Report submitted in partial fulfillment of the MD Degree at Harvard Medical School

Date: 01 March 2016

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Scholarly Report Title: Use of Glycosyltransferase-Programmed Stereosubstitution (GPS) to improve targeted cell therapies in Traumatic Brain Injury

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Abstract

Use of Glycosyltransferase-Programmed Stereosubstitution (GPS) to improve targeted cell therapies in Traumatic Brain Injury

Manuel Ozambela Jr, Thomas Morgan Jr., Ali Darkazalli, Megan Muroski, Geoffrey Strouse, Cathy W. Levenson, Robert Sackstein

Purpose: Traumatic Brain Injury (TBI) is the leading cause of injury-related death and disability in the US. Given the complex pathophysiological mechanisms that ensue following a TBI, solutions that involve mesenchymal stem cell-based therapies are particularly appealing. To date, successful implementation of stem cell therapies has been largely hindered by the inability to efficiently deliver therapeutic doses of cells to the CNS via a vascular route. The goal of this study was to assess whether modification of cell surface glycoproteins using Glycosyltransferase-Programmed Stereosubstitution (GPS), could improve cell delivery and cell integration at sites of brain injury.

Methods: We surgically induced controlled brain injuries in rats, and at either 6 hours or 5 days following the injury, administered a tail vein injection with 200,000 GPS-modified or unmodified stem cells. All rats were sacrificed 24 hours following injection and brain tissue was fixed, sectioned and later inspected by fluorescent microscopy for quantitative analysis. Quantitative western blots of brain tissue following controlled injury were conducted at each of the above time point to assess E-selectin expression.

Results: E-selectin expression was significantly higher in the rat brain 5 days post injury compared to 6 hours post injury. An increased number of cells were observed at the site of injury at 5 days compared to 6 hours post TBI ($p=0.004$). GPS modification did not seem to statistically increase cell homing to the site of injury. However, there was a trend, with a 20 % increase in the average number of modified cells reaching the injury site.

Conclusion: These findings serve as persuasive logic to further pursue the use of cell surface ligand modification to enhance delivery of stem cells to the CNS.

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Section I

Introduction

In the last decade, especially in light of football-related injuries, there has been increasing awareness and recognition of traumatic brain injury (TBI) as a major public health concern. TBI refers to the injury that is caused by a mechanical blow (blunt trauma) or biomechanical force (blast injury) to the head that results in neuronal injury.[1] A major problem in managing and treating TBI is the multifactorial nature of the condition. Immediately after the primary injury, a secondary injury occurs due to local inflammation and a build-up of excitotoxic chemicals as neuronal cells lyse and spill their contents. This leads to a wave of neuronal cell death, which is often more extensive than the primary injury.[2] Investigators actively seek strategies to limit the extent of this secondary injury by reducing inflammation, preventing apoptosis, and promoting neurogenesis.

Several preclinical studies using bone marrow-derived mesenchymal stem cells have shown promise for treating central nervous system disorders and injuries including TBI.[3,4] To date, however, researchers have been unable to direct sufficient numbers of stem cells into the site of injury via an intravenous route. Thus, ideal outcomes have not been realized. Mesenchymal stem cells lack the expression of molecules that direct endothelial adhesion requisite to extravasate into tissues ("homing receptors") making it more difficult for them to migrate and engraft into sites of inflammation in their native form. [5]

In the present study, I sought to utilize a novel stem cell glycoengineering technology known as Glycosyltransferase-Programmed Stereosubstitution (GPS), developed by the Sackstein Laboratory at Harvard Medical School to tackle the current problem facing the implementation of targeted stem cell therapies for traumatic brain injury. Specifically, I aimed to assess whether GPS could be used to improve stem cell delivery to sites of traumatic brain injury. GPS works by selectively modifying cell surface glycoproteins to enforce expression of adhesion molecules required in the most important steps of cell trafficking and migration through the vasculature. The long-term goal of my project is to determine whether improving delivery of bone marrow-derived mesenchymal stem cells to sites of neuronal injury will yield a meaningful biological response. I hypothesized that exogenous intravenous administration of GPS-engineered human mesenchymal stem cells (hMSC) to overexpress the potent E-selectin ligand

HCELL (Hematopoietic Cell E/L Selectin Ligand) would significantly improve cell migration and integration to the site of traumatic brain injury in a an established rodent model for TBI.

The Problem and the Approach

TBI is the leading cause of injury related death and disability in the United States, affecting 1.7 million people annually. In addition, it is a source of major economic loss, accounting for more than 76 billion dollars in direct medical cost and indirect cost due to lost productivity.[6] Typical clinical symptoms associated with a mild TBI include depression, anxiety, post-traumatic stress disorder (PTSD), as well as problems with learning, memory and attention. More severe TBI can lead to significant and irreversible neurological deficits and even death. Despite the great potential for regenerative medicine using stem cell-based therapies to target central nervous diseases/conditions, we have achieved less than optimal results to date. Successful implementation has been largely hindered by our inability to efficiently deliver cells to sites of ischemia, inflammation, and even cancer, via a targeted intravenous route.[5]

Within the last decade, MSCs have garnered significant attention emerging as a potential tool for treating neurodegenerative disorders and central nervous system injuries.[7, 19, 20] Animal models of TBI have demonstrated that systemically-infused MSCs have some tropism for injured and inflammatory sites, and through paracrine signaling can lead to increased expression of neuro-protective growth factors.[8, 21] MSCs have been shown both in vitro and in vivo to express as well as induce intrinsic parenchymal cells to express multiple growth factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). [22] In addition, MSC have been observed to have potent anti-inflammatory properties which could improve the cerebral microenvironment and promote endogenous neurogenesis.[9] However, current methods of systemic intravenous infusion have seen many drawbacks including low rates of engraftment to the injury site, as well as migration of MSCs in high volumes to non-target organs such as the lungs.[10] Several other delivery approaches have been attempted including direct intra-cerebral injection, intrathecal injection and intra-arterial injection.[11,12] While some of these methods have been shown to get more stem cells to the desired site of injury, they are also much more invasive and are associated with more risks for patients.[23] Therefore, it is necessary to improve our ability to specifically target MSCs to sites of brain injury via an intravenous route to maximize therapeutic benefit

while minimizing collateral damage. To date there has not been any attempts to modify cell surface molecules on hMSCs to induce more selective homing to the CNS via the intravenous route.

In general, cell migration is regulated by a series of sequential molecular interactions that are initiated when circulating cells are captured by its target endothelium. This "Step 1 interaction" acts as a physiological brake bringing the circulating cell to a slow roll, well below the velocity of the prevailing fluid stream, and allowing it to sample the local chemical environment. If the right signals are present the cell proceeds to extravasate and take residence within the tissue parenchyma. The most potent mediators of the Step 1 interaction are the calcium-dependent lectins known as selectins (CD62E, CD62P and CD62L).[13,14] These selectins bind to sialofucosylated glycans displayed on relevant cell surface ligands. HCELL, which has come to be known as the "bone marrow homing receptor", is the most potent E-selectin ligand known and directs cell migration to sites where E-selectin is expressed on endothelial cells.[13] HCELL was discovered on hematopoietic stem cells to be a specifically glycosylated form of the membrane protein CD44. The glycosylation pattern responsible for selectin binding activity is a canonical tetra-saccharide known as Sialyl Lewis X (sLex). HCELL is thus the standard CD44 isoform decorated with N-linked sLeX decorations.[13]

Throughout the body, E-selectin is constitutively expressed only in the skin and bone microvasculature, but its expression is induced at all other endothelial beds by inflammatory cytokines such as tumor necrosis factor alpha (TNF). MSCs lack any potent E-selectin ligands on their cell surface, thus hindering their ability to home to sites of inflammation in higher amounts following exogenous administration.[13] However, MSC do contain the standard isoform of CD44.[15] Sackstein's group was able to show that using GPS, CD44 could be glycoengineered *in vitro* to express HCELL and, in a human-NOD/SCID xenotransplant model, they observed significantly improved E-selectin-dependent bone marrow engraftment by human mesenchymal stem cells (hMSCs).[15] These findings open the possibility of applying this technology to a host of other inflammatory conditions such as TBI where stem cell therapy has not had great success to date.

Recently, the Sackstein laboratory has used GPS to target mouse MSC's to the pancreas of nonobese diabetic mice (NOD). Following IV infusion of allogeneic GPS-engineered ($HCELL^{+}$) mouse MSCs in NOD mice, investigators observed a three-fold increase in peri-islet

infiltrates of MSCs compared to that of buffer-treated MSCs (control) with a distribution in proximity to E-selectin-expressing microvessels. They found that HCELL^{+} MCSs resulted in durable reversal of hyperglycemia in these animals out to 90 days compared to only transient reversal with control (HCELL^{-}) MSCs. Glycoengineering the cell surface to enforce HCELL expression boosted both trafficking to the target site and efficacy of the treatment in this animal model.[18]

What makes my study unique is that GPS has never been used to study cell migration to the central nervous system. Others have used a variety of routes in an attempt to improve cell delivery to sites of brain injury however there remains a gap in the literate as it relates to the optimization of stem cell delivery to CNS trauma via the intravenous route. The vascular route is the most ideal route as it is associated with the fewest risks and allows the possibility to have cells reach all anatomical sites of interest.

Section II

Student Role

I played a significant and active role in the completion of this scholarly project. Dr. Sackstein conceived the use of GPS-mediated cell surface glycoengineering to target cell trafficking to sites of tissue injury, and his lab created all the requisite reagents and biochemical manipulations to achieve this goal. However, as pertains to the application of GPS in the TBI model employed in my scholarly project, I conceived the study and developed and maintained the collaboration between the Sackstein laboratory at Harvard Medical School and the Levenson laboratory at the Florida State University College of Medicine that specialized in TBI models in rat. I thus chose to bring together two preeminent labs to perform this kind of study. I performed all of the *in vitro* experiments and biochemical techniques required to successfully enforce HCELL on hMSC surfaces. I also performed the rat surgeries and collected all brain tissue samples required for analysis. I was responsible for writing this scholarly report in its entirety. Our collaborators provided data regarding western blots of brain tissue and histologic evaluations of brain sections.

Section III

Methods

Utilization of GPS to enforce HCELL expression on bone marrow-derived hMSC

Bone marrow-derived hMSC's were obtained from the Texas A&M Health Science Center Institute for Regenerative Medicine, cultured and expanded in Dulbecco Modified Eagle medium with 10% fetal bovine serum according to established protocols. Only cells between 4 and 6 passages were used for experimentation. This was important in order to limit variability in cell lines as stem cells begin to acquire and accumulate genetic changes with increasing passages.

GPS reaction via Fucosyltransferase VI Modification

hMSCs were suspended in 60 mU/mL of alpha (1,3) fucosyltransferase (FTVI) in Ca^{2+}- and Mg^{2+}-free Hank's buffered salt solution (HBSS) containing 20 mM HEPES, 0.1% human serum albumin, and 1 mM GDP-fucose for 90 minutes at 37 degrees Celsius. These are the necessary conditions and reagents to achieve modification of sugar groups on CD44 to generate sLex. The use of Ca^{2+}- and Mg^{2+}- free HBSS is required to maintain cell viability after the enzymatic reaction occurs. Trypan blue exclusion and propidium iodide staining were conducted to assess cell viability after the reaction.

E-selectin binding determination

To determine if cell surface glycan modification had occurred after exofucosylation and to ensure that the modification had generated E-selectin binding activity, flow cytometery and western blot assays were conducted.

Flow cytometry

Following GPS modification of hMSCs, 2e5 cell aliquots were washed with 2% fetal bovine serum (FBS) in Phosphate buffered saline (PBS) and incubated with primary antibodies (recombinant mouse E-selectin-Ig chimera (comprised of mouse E-selectin linked to human IgG1 Fc region; commercially available from Bio-Techne, Minneapolis, MN) and HECA 452 monoclonal antibodies (mAb)) or isotype control mAb (antibodies available from BD

Pharmingen). Samples were then washed with buffer to remove unbound antibodies and stained with anti-human IgG FitC and anti-rat IgM FitC secondary antibodies, respectively (these are the appropriate secondary fluorochrome-conjugated antibodies required for completion of the indirect immunofluorescence). In this assay, recombinant E-selectin-Ig chimera binds to E-selectin ligands and, therefore, is used to provide evidence for E-selectin binding activity in GPS-modified and -unmodified cells. HECA 452 mAb binds to the canonical sialofucosylated E-selectin binding determinant sLex. This assay was used to assess for modification of CD44 with sLex, creating HCELL, and for the generation of E-selectin binding activity.

Immunoprecipitation

Whole cell lysates of modified (FTVI treated) and unmodified (buffer treated) hMSCs were pre-cleared with protein G-agarose beads and then incubated with antibody against CD44. After a 2-hour incubation, antibody-lysate mixture was added to protein G-agarose beads and placed on a rotator overnight at 4 degrees Celsius. Immunoprecipitates were separated via SDS-PAGE, transferred to a PVDF membrane, and immune-stained using E-selectin Ig or anti-CD44 antibodies to assess for HCELL expression. This step is important because it provides evidence that the membrane protein that is being modified is indeed CD44 and not another protein scaffold.

Western blot

FTVI-treated and buffer-treated hMSC whole cell lysates were prepared and western blots were conducted under reducing conditions. Blots were visualized with chemiluinescence Lumi-light Western Blotting substrate. I again used the recombinant human E-selectin-Ig chimera to assess E-selectin binding activity and HECA452 staining to assess sLeX expression.

Animal Model and Injection of hMSCs

Traumatic Brain Injury

Two groups of six male Sprague-Dawley rats (n=12) underwent a unilateral brain injury of the right medial frontal cortex using a controlled cortical impact (CCI) device as previously described. [25] To this end, rats were anesthetized using isoflurane gas. Aseptic surgical technique

was employed to prevent animal infection. Rat body temperature was maintained by using a homeothermic blanket. Following prone stereotaxic placement of the rat in the surgical field, a midline scalp incision was made to expose the calvaria. The bregma (the point or area of the skull where the sagittal and coronal sutures join) was identified, and a 6 mm craniotomy was made, 3 mm rostral, and 2 mm lateral towards the animals right from the bregma. Next, a steel piston, 5 mm in diameter, affixed to a pneumatic CCI device (MyNeuroLab, Inc, Richmond, Illinois) was placed upon the exposed cortex and activated. This device delivered a 3.0 mm deep contusion using an impact velocity of 2.25 m/s and an impact time of 500 ms. Following TBI, the incision was sutured upon attaining hemostasis.

hMSC transplantation and experimental groups

Four groups of rats received a tail vein injection of modified or unmodified hMSCs labeled with a fluorescent peptide at 6 hours or 5 days following TBI. Each animal received a tail vein injection of approximately 200,000 cells.

Group A (n=3): TBI + injection of $HCELL^-$ hMSC 6 hours post injury

Group B (n=3): TBI + injection of $HCELL^+$ hMSC 6 hours post injury

Group C (n=3): TBI + injection of $HCELL^-$ hMSC 5 days post injury

Group D (n=3): TBI + injection of $HCELL^+$ hMSC 5 days post injury

Cell Labeling

Prior to modification, stem cells were labeled using a delivery platform based on a solid gold nanoparticle, a technology developed by the Strouse laboratory at Florida State University. The particle is modified to house a cell penetrating peptide for cell transfection and enhance cellular uptake as well as a linearized expression vector to induce expression of a red fluorescent protein, permitting visualization of cells under florescent microscopy. Particle uptake was performed according to previously published protocol[17]. Cells were plated to 80% confluence and incubated for 24 hours in a 6.6 pmol concentration of nanoparticle complex in Dulbecco's modified Eagle's medium (DMEM) supplemented with an addition of 10% fetal bovine serum and 20 mM glutathione monoester. The media was exchanged after 24 hours of transfection to remove any nontransfected nanoparticle complex remaining.

Tissue section preparation and cell counting

24 hours following hMSC transplant, the rats were sacrificed and transcardially perfused with 0.9% saline followed by 4%PFA in 1X PBS. Whole brains were excised and post-fixed in 4%PFA for 24h followed by 48-72h sucrose embedding. 40μm coronal sections were taken using a freezing microtome. Fluorescence images of the injury boundary were acquired. Labeled hMSC were counted manually per slice. Engraftment was measured by counting fluorescent-labeled cells. Only cells that were determined to be in the parenchyma were counted. Vascular intraluminal cells were ignored, as they did not complete diapedesis into the tissue. Three peri-lesional slices in approximately the same location per rat were used for visual quantification.

Determining the kinetics of E-selectin expression in brain tissue following TBI

Three groups of three male Sprague-Dawley rats underwent a unilateral controlled cortical impact as described above. Different groups of animals were then sacrificed at 6 hours and at 5 days following injury to evaluate the timing of E-selectin expression in the brain lesion and in the injury boundary region following TBI. Animals were euthanized using carbon dioxide per approved animal protocol. The whole brain of each rat was then collected and placed in a stereotaxic device (known as a brain matrix) that is used for brain tissue sampling. The first 5mm of frontal cortex in each animal (which encompassed the lesion and injury boundary region) was excised for further processing for quantitative western blot as described below.

Quantitative western blot analysis

Brain tissue samples were immersed and homogenized in 2mL lysis buffer containing 50mM Tris, 7M Urea, 2M Thiourea, 2.5% CHAPS, 65mM DTT and protease inhibitor cocktail. Homogenized samples were centrifuged at 10,000g for 10min at 4ºC, and supernatants were removed and subjected to western blot analysis. Quantitative infrared westerns were performed to determine the relative expression of E-selectin on rat brain endothelial cells at 6 hours and 5 days following a TBI using the Odyssey® imaging system. Mean fluorescent intensities for each band of interests were quantified relative to background noise per standard protocol. The antibody used against E-selectin in the westerns was the Rabbit polyclonal anti-CD62E from Abcam (ab18981) at a 1:500 dilution. The secondary antibody used for visualization on the

Odyssey blot scanner was the IRDye 680LT Goat anti-rabbit from Li-Cor (926-68021) at a 1:20,000 dilution.

Statistics: Differences in lesion integration between the two animal groups and data from other experimental assays were determined using students t-test. P value less than 0.05 was considered significant.

Section IV

Results

GPS modification using FTVI enforces HCELL expression on hMSCs

hMSCs (passage 2-4) were exofucosylated using a-(1,3) fucosylransferase VI (FTVI) according to the the GPS protocol reviewed above. I tested the binding affinity of HECA-452 mAb (specific for sLex-like structures) and E-selectin-Ig chimera (E-Ig, known to bind in static conditions to E-selectin ligands) to hMSC to analyze cell surface selectin ligands before and after FTVI-treatment. Flow cytometric analysis in **Figure 1** shows almost no reactivity of hMSCs to HECA-452 mAb and very little binding to E-Ig before treatment with FTVI. Exofucosylation led to a significant increase in the binding of both probes to hMSCs, indicating an increase in cell surface expression of E-selectin ligands.

To determine which protein scaffolds express selectin ligands on hMSC after treatment with FTVI, I performed a western blot analysis of cell lysates from buffer treated and FTVI treated hMSC. **Figure 2A** shows staining of whole cell lysate with recombinant E-Ig. While no proteins have reactivity with E-Ig in buffer-treated hMSC, FTVI treatment enforces E-selectin binding on a protein at a molecular weight of ~80 kDa. As this protein has previously described by Sackstein et al as HCELL (E-selectin binding glycoform of CD44),[15] I immunoprecipitated the FTVI treated hMSC lysate with mAb against CD44 (**Figure 2B**). As shown, E selectin reactivity can only be seen in the "total" and "IP" lanes of the CD44 pull-down, and not the supernatant, confirming that the only selectin ligand present on FTVI-treated hMSC is HCELL.

Low hMSC viability following GPS reaction at outside laboratory

The *in vivo* portion of our experiment was conducted at the Florida State University College of Medicine. Therefore, the hMSCs used in our experimental animal model were cultured, modified and transfused into our animals at this outside location. I performed the *ex-vivo* hMSC fucosylation reaction using the exact reagents that I utilized in the Sackstein laboratory, and simulated the reaction conditions as closely as possible. Following the FTVI and buffer treatment of the hMSCs, I observed a significant increase in HECA-452 binding activity on flow cytometric analysis of the FTVI-treated sample compared to the untreated controls (**Figure 3**). This observation provides evidence that I was able to successfully fucosylate hMSC surface glycoproteins.

However, I also observed unusually low cell viability in my samples on flow cytometric analysis. Live cells were defined based on forward and side scatter parameters in flow cytometer. I observed 18.1% viability in the buffer treated samples and 17.5% viability in the FTVI-treated samples (**Figure 4**). This observation was highly unusual given that under similar conditions I was able to achieve greater than 95% viability following GPS in the Sackstein laboratory. The only difference between the two samples was that these samples had been already transfected for labeling with nanoparticles.

Location and distribution of hMSCs in the rat brain 24 hours following stem cell injection for TBI

All four groups of rats received one tail-vein injection of approximately 200,000 modified or unmodified hMSCs per animal (but, my original intention was to inject 1 million cells per animal). The groups were defined based on the time after TBI that injection was given (6 hours or 5 days) and the type of hMSC that was administered (FTVI treated or buffer treated). Rats were then sacrificed 24 hours following injections and 40μm coronal tissue sections of peri-injury rat brain were prepared for fluorescent microscopy analysis. **Figure 5** is a representative sample of the types of images I was able to obtain. Red spheres indicate an individual hMSC. Since cells were transfected with a gene for a fluorescent protein using a gold-based nano particle delivery platform, only live cells are visualized on each section.

I observed differences in the location and distribution of the injected cells among the four animal groups. I first noticed that, in groups A and B (the two 6-hour groups), there was a very

non-specific distribution of transplanted cells throughout the sections compared to that in groups C and D (the two 5-day groups). The cells seen on group A and B were also mostly restricted to the vasculature and very few cells were found to be located in the tissue parenchyma. In addition, there was very little difference between group A and B in terms of cell distribution and tissue integration.

On initial inspection I observed far more visible cells in Group C and D (the two 5-day groups) compared to Group A and B. In the 5-day groups I observed more targeted integration of transplanted cells near the brain injury site and the sub-ventricular zones in the hemisphere ipsilateral to the brain injury, consistent with what others have observed in the literature. [24] There were also fewer groups of cells restricted to the vasculature indicating that cells had completed migration and taken up residence in the tissue.

Quantitative analysis of hMSC homing and integration to brain injury site following TBI

Prepared tissue sections were inspected and hMSC tissue homing and integration near the site of injury was assessed by manually counting the number of cells visualized per section. Intraluminal cells (those restricted to the vasculature, and thus failed to complete extravasation into the tissue parenchyma) were excluded. I observed a significant difference in cell homing and integration to the site of injury in Groups A and B compared to Groups C and D **(Figure 6)**. The 5-day modified and control groups showed an average of 48 and 40 cells per tissue section respectively compared to an average of 2.7 and 1.3 cells per section seen in the 6 hour modified and control groups ($p=0.004$).

While there was a 20% increase in the average number of cells seen per tissue section in the 5-day FTVI treated group (48) compared to the 5-day buffer treated group (40), this difference was not statistically significant ($p>0.05$) given the small sample size and large variation seen within each group. However, there does seem to be a trend towards improved cell homing following GPS modification at both time points.

E-selectin expression in rat brain tissue is higher 5 days following TBI compared to 6 hours following TBI

Quantitative infrared westerns were performed with the help of our collaborators to assess the relative expression of E-selectin on rat brain endothelial cells at 6 hours and 5 days following a TBI. Immunohistochemistry was initially pursued in order to visualize the density of E-selectin molecules on rat brain endothelial cells following TBI, however, we had difficulty obtaining appropriate positive controls with our available assays. Instead, we chose to conduct a quantitative western blot using a commercially-available anti-rat E-selectin antibody (Rabbit polyclonal anti-CD62E from Abcam) that has been reported to be useful in quantifying E-selectin levels in western blots.

We observed minimal expression of CD62 (E-selectin) in brain tissue at 6 hours post-TBI (**Figure 7**). In contrast, E-selectin expression was approximately nine times higher at 5 days post-TBI. Those data were consistent with our in vivo observations that more cells integrate to the site of injury 5 days post TBI.

Section V

Discussion

While our data did not show a statistically significant increase in hMSC migration and integration to sites of brain injury following cell surface glycoprotein modification via GPS, it did demonstrate a trend towards improved cell homing following GPS modification. In addition, we found that the ability for stem cells to reach traumatized brain tissue may depend on the expression of E-selectin in the brain following TBI. We found that hMSCs injected 5 days following a traumatic brain injury were significantly more likely to reach the site of injury and take up residence in the cerebral parenchyma compared to those administered 6 hours following injury. This was true independent of any modifications to the cell surface adhesion molecules. This observation was consistent with our finding that of increased E-selectin expression 5 days post-TBI compared to 6 hours post-TBI in the rat brain.

Given the correlation of hMSC homing to the site of injury with E-selectin expression, one would have expected a statistically-significant increase in cell migration among GPS-modified cells (i.e., expressing HCELL) compared to that of buffer-treated ($HCELL^-$) controls. Of the known E-selectin ligands, HCELL has been demonstrated to be the most potent. [5] While

the average and median number of stem cells identified at the site of injury per rat brain section after injection were higher for $HCELL^+$ cells compared to controls, the difference was not statistically significant. The absence of the expected finding may be explained by the small samples size studied in this experiment. In addition, it is possible that $HCELL^+$ cells were preferentially recruited to competing anatomical sites with high E-selectin expression such as bone or skin, where E-selectin is constitutively expressed. If this is the case, we might expect to see improved homing of $HCELL^+$ cells to TBI sites with higher injection doses or with delivery of cells directly into vessels perfusing the brain (i.e., carotid artery).

A major limitation in this study was the unusually high hMSC death that was observed in our in vivo trial. All previous studies in the literature have used a minimum of 1 million cells per animal. Here I was only able to inject ~200,000 cells per animal given the 80% death rate. Previous studies have also shown cell integration to the site of injury to be dose dependent. As the number of cells injected intravenously increases, so does the number of cells found in the cerebrum. [24]

The most likely reason for the low cell viability was the use of the nanoparticle platform for cell labeling. The nanoparticles used for labeling were first made available to us, and thus first introduced as a variable, at the time of the experimental trial and were not tested earlier. It is possible that these nanoparticles introduced changes to the cell that render them unable to survive the GPS reaction conditions.

Compared to this study, most other investigators have utilized 24 hours post-TBI as the time point to inject stem cells. Prior experience in the Levenson lab using 24 hours as an injection time point for administration of hMSCs had not shown cell infiltrates. Therefore, we chose to use 6 hours and 5 days following injury as the time points for injection to assess how more acute and delayed changes to the cerebral microenvironment might affect stem cell therapies.

Another variable to consider is injection of cells via an intra-arterial route compared to an intravenous route. There have been a handful of animal studies that suggest that intra-arterial stem cell injections (via the carotid artery ipsilateral to injury) might lead to improved stem cell integration into cerebral parenchyma over intravenous administration by avoiding entrapment in the lung microvasculature.[23]

The present study does have some other limitations that are worth noting. In vivo models are always subject to variability that could bias data especially when working with a small sample size. To minimize this problem, all animals used in this study were in-bred and have been previously established for use in this specific TBI model. In addition, the method used for visually (manually) counting cells is subject to bias. Stereological cell counting would have been preferred but was unavailable at the time of this study. To minimize counting bias, I was blinded to the data during counting. Notably, results of western blot staining for E-selectin expression in brain tissue was not ideal, and these studies must also be repeated: in particular, there was no beta actin loading control employed in the blots, and, as a result, there is the possibility that observed differences in expression of E-selectin could reflect variations in protein concentration from well to well.

Unfortunately, overall, my results must be viewed as very preliminary as sample sizes are low and many studies could not be repeated for logistical reasons, not the least of which is that our collaborators suffered a critical loss in personnel and resources prohibiting them from further pursuing this work. As a result, I was unable to repeat more studies of this type. Still, my findings in this study have provided persuasive logic to further pursue the use of cell surface glycan modification to enhance delivery of stem cells to the CNS. Future studies should look to further assess reasons for loss of MSC viability following GPS treatment of nanoparticle-loaded MSCs, and then repeat this experiment with improved MSC viability. In addition, intra-arterial injections of modified MSCs should be studied and compared to intravenous injections.

A review of the literature implicates several neurotrophic factors and many other small molecules as being neuro-protective after brain injury. Besides native production of several key neurotrophic factors, MSC can be easily transfected to act as small reactors to deliver therapeutic doses of other desired neurotrophic molecules to sites of injury.[16] Achieving a way to target inflammatory sites in brain tissue following TBI will lay the foundation for the future study of possible neurotropic factors to induce cell survival and neurogenesis in order to limit, and possibly reverse, neuronal damage associated with TBI. There is great need for stem cell-based therapies to treat CNS trauma in patients, and my studies lead me to believe that the key to achieving the delivery of cells to the sites where they are needed lies in glycan modification of the stem cell surface.

Section VI

Acknowledgement

First, I would like to acknowledge Dr. Robert Sackstein for the countless hours of teaching and mentorship he has enthusiastically provided me during my time at HMS and during the completion of this scholarly project. Beyond academic mentorship, Dr. Sackstein has also been there for me and provided much needed support during some difficult personal times over the last several years. For that I am very grateful.

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Section VII

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Section VIII

Figures

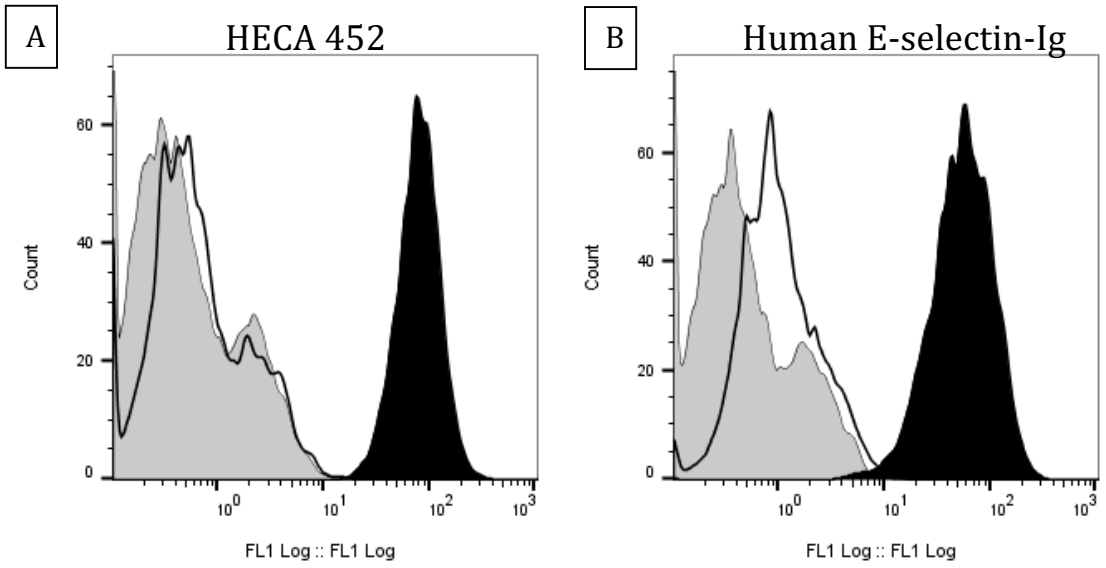


Figure 1: Flow cytometry analysis of hMSC stained with (**A**) HECA-452 mAb and (**B**) E-selectin-Ig chimera before and after treatment with FTVI. Grey shaded area represents Isotype control. Unfilled black line represents buffer-treated hMSCs. Black-filled lines represent FTVI-treated hMSC.

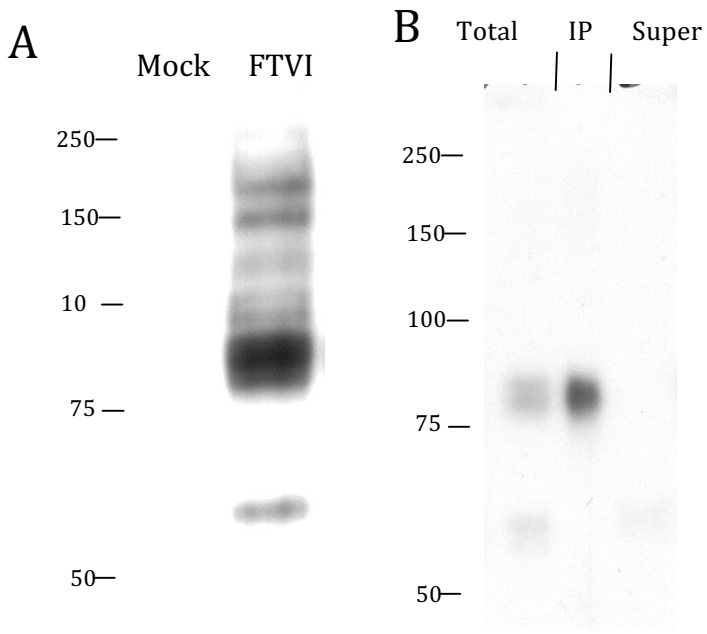


Figure 2: (A) Western blot analysis of buffer-treated ("Mock") and FTVI-treated ("FTVI") hMSCs stained with human E-selectin-Ig chimera to assess E-selectin binding activity. **(B)** Western blot analysis of E-selectin-Ig staining of whole cell lysate ("total), lysate immunoprecipitated with anti-CD44 mAb ("IP"), and CD44-cleared lysate supernatant ("super"), to assess expression of HCELL.

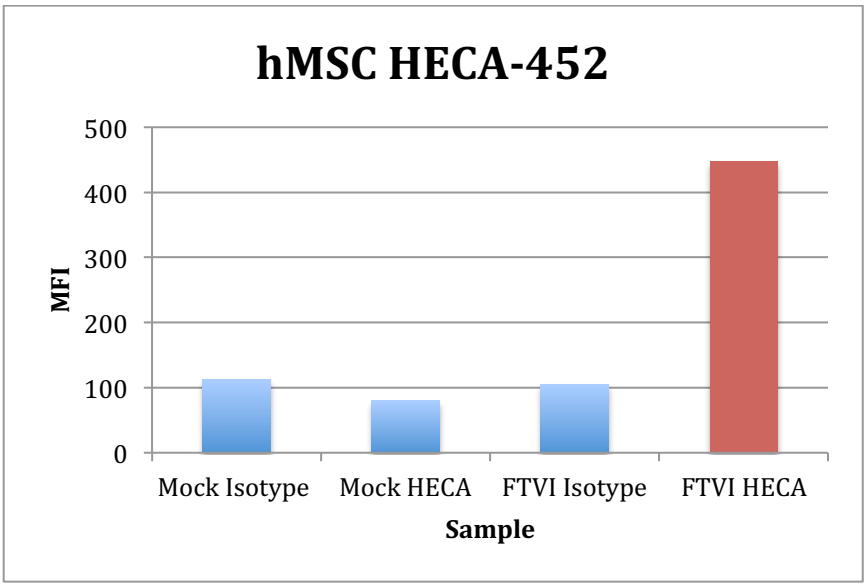


Figure 3: Flow cytometryc analysis of GPS-modified (FTVI) and buffer-treated ("mock") hMSCs. HECA-452 and isotype control antibodies were used to assess fucosylation. These data correspond to the cells used in our *in vivo* experiment. (MFI: mean fluorescence intensity)

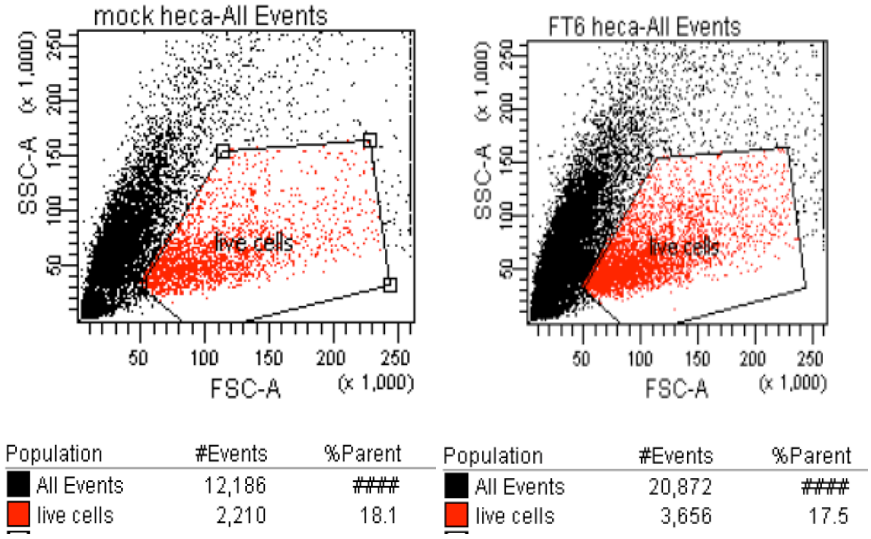


Figure 4: Flow cytometry analysis of modified (FTVI) and buffer-treated ("mock") hMSC to assess viability of cell samples prior to transplantation. Live cells were defined based on forward and side scatter.

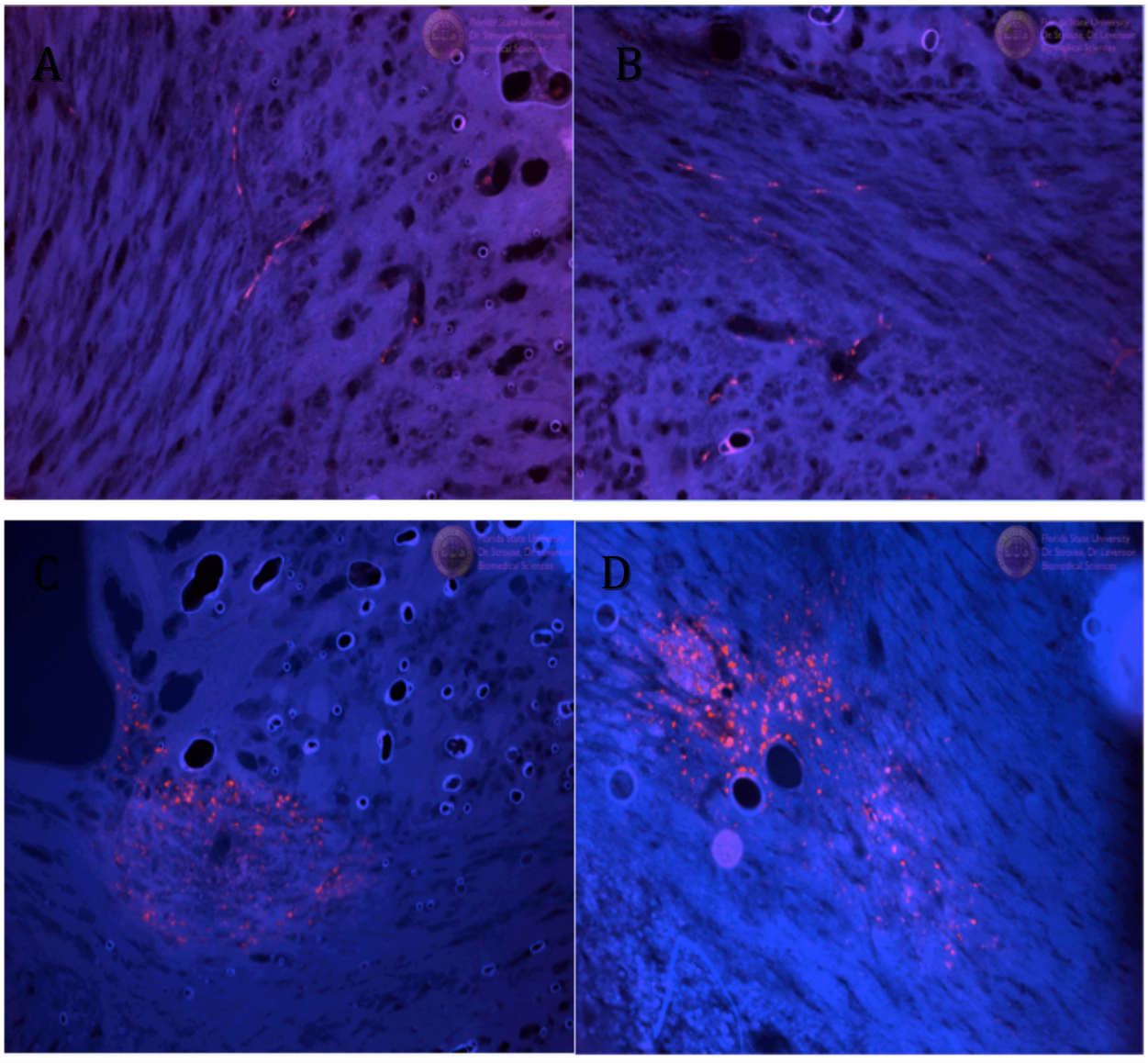


Figure 5: Fluorescent microscopy imaging of prepared rat peri-lesion brain slices following TBI and administration of GPS-modified and unmodified hMSCs. hMSCs were transfected to overexpress TDT fluorescent protein and appear red in the images. **(A)** 6 hours post-TBI section, rat injected with unmodified (buffer treated) hMSCs **(B)** 6 hours post TBI section, rat injected with GPS-modified (FTVI Treated) hMSCs **(C)** 5 days post-TBI section, rat injected with unmodified (buffer treated) hMSCs **(D)** 5 days post-TBI section, rat injected with GPS-modified (FTVI-treated) hMSCs.

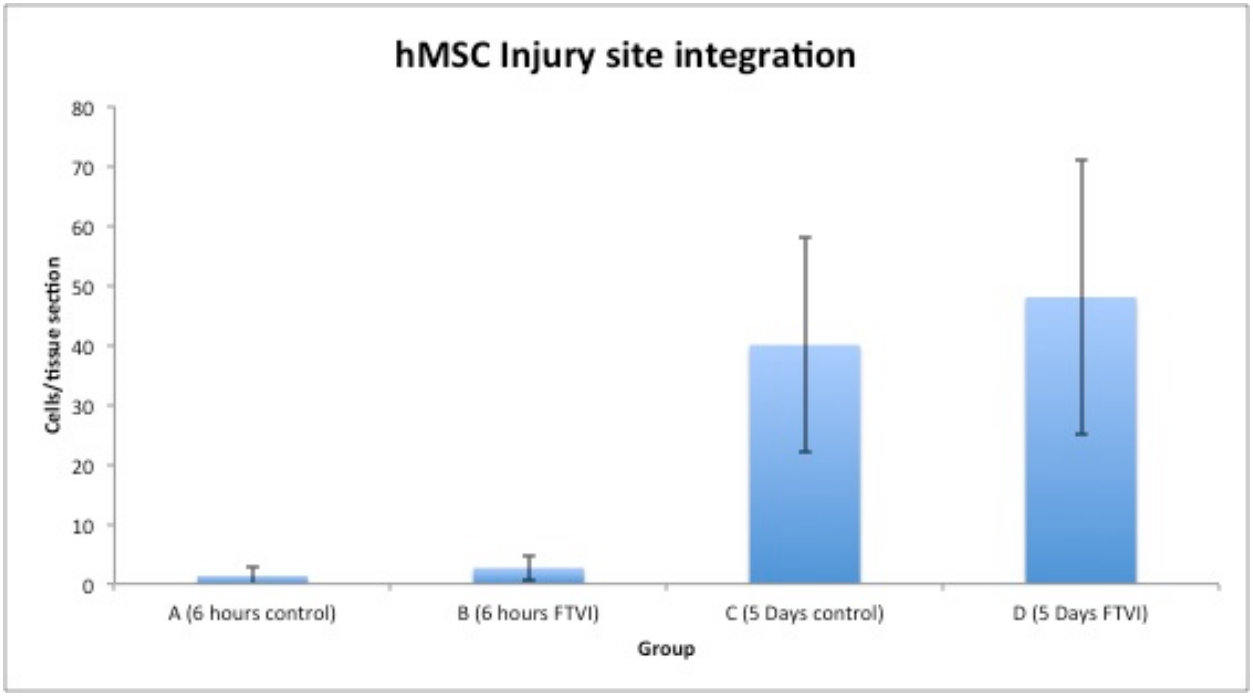


Figure 6: Bar graph showing density of transfused hMSCs at the site of injury in our animal groups. Cells were manually counted and numbers were reported as the mean number of cells identified in each peri-lesional section in the rat brain tissue. Group A (n=3) include rats that underwent TBI + injection of unmodified hMSC 6 hours post-injury. Group B (n=3) includes rats that underwent TBI + injection of FTVI-modified hMSC 6 hours post-injury. Group C (n=3) includes rats that underwent TBI + injection of unmodified hMSC 5 days post-injury. Group D (n=3) includes rats that underwent TBI + injection of FTVI-treated hMSC 5 days post-injury.

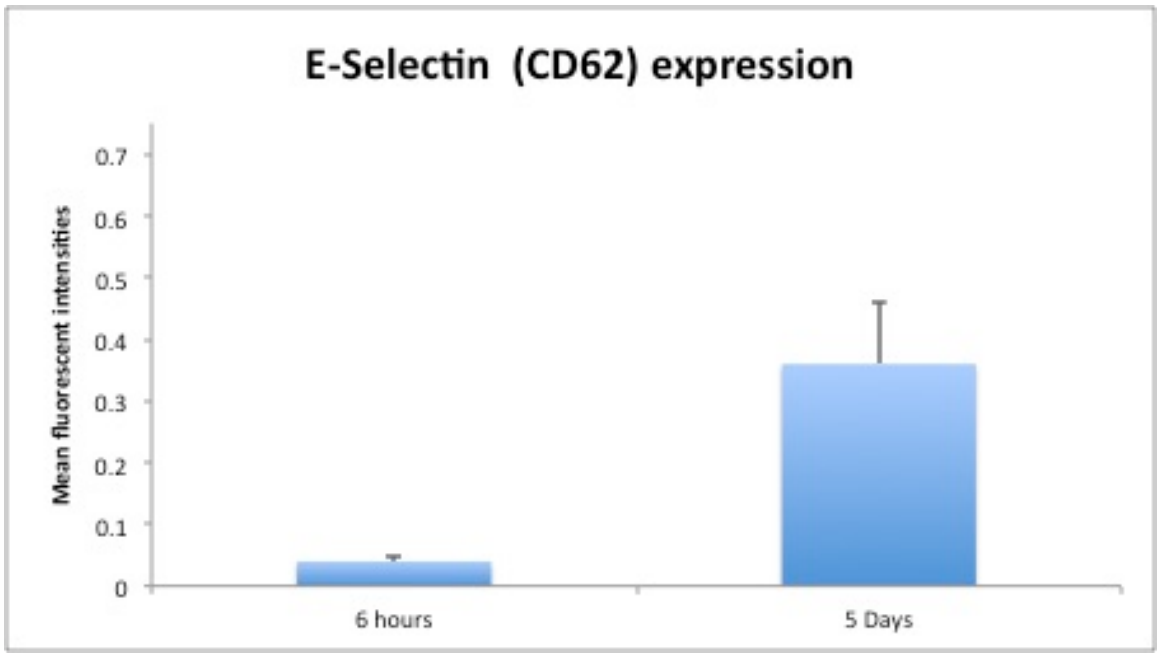


Figure 7: Quantitative infrared imaging of western blots performed to determine the relative expression of E-selectin on rat brain endothelial cells at 6 hrs and 5 days following TBI. The bar graph illustrates the relative intensity of E-selectin bands at the respective time points normalized to background using the Odyssey® imaging system.