ROLE OF MATRIX MICROENVIRONMENT ON NEURAL STEM CELL PHENOTYPE AND DIFFERENTIATION UNDER HEALTHY AND INFLAMMATORY CONDITIONS

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Dan Simon, Ph.D., Doctoral Program Director
This thesis is dedicated to my mother Colleen Farrell, my father, William Farrell, my brother, Kyle Farrell, my close friends, and my partner Emma Knoth.
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ROLE OF MATRIX MICROENVIRONMENT ON NEURAL STEM CELL PHENOTYPE AND DIFFERENTIATION UNDER HEALTHY AND INFLAMMATORY CONDITIONS

KURT W. FARRELL

ABSTRACT

Localized host inflammatory microenvironment resulting from several neuropathologies (e.g., trauma, amyotrophic lateral sclerosis (ALS), glioblastomas) leads to progressive degeneration of neuronal tissue and destruction of axonal tracts in the adult central nervous system (CNS). Failure to reinstate healthy cells and axonal connections under these conditions can severely compromise locomotion and cognitive function, resulting in muscle atrophy, paralysis and even death. Annually, thousands of people are diagnosed with various neuropathologies and a majority of them succumb to these conditions soon after. The adult CNS has a limited ability for self-repair, which necessitates repair strategies focused on ameliorating secondary cellular degeneration, promoting endogenous repair mechanisms, and exogenous cell replacement therapy. Currently, pharmacological and surgical treatments options are limited in their outcomes for these types of ailments. Neural stem cells (NSCs) isolated from the embryonic and adult striatum have the capacity to divide and differentiate into various neuronal and glial lineages, thus demonstrating their utility in regenerating lost neuronal populations. To further investigate their clinical potential, in this work, we first developed and tested the utility of uncrosslinked 3D biological hydrogels (compressive strength < 600 Pa) for their ability to promote murine NSC survival, differentiation into desired
lineages and neurite outgrowth, in the presence (or absence) of exogenous cues such as retinoic acid. In the second step, the influence of an activated murine microglia in regulating the phenotype and genotype of murine NSCs within a localized 3D coculture microenvironment was investigated, and the key cytokines and chemokines which regulate NSC survival, differentiation and neurite outgrowth were identified. Finally, in the third step, the effects of paracrine-signaling between adult human NSCs and human pediatric glioblastoma cells within a coculture microenvironment, in regulating their survival, proliferation, migration, differentiation and axonal outgrowth were investigated. Results from this work provide insights into neural stem cell interactions with 3D biological matrices, inflammatory microenvironment, and glioblastoma cells, which could lead to development of strategies for CNS repair and regeneration utilizing NSC transplantation techniques.
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CHAPTER I

INTRODUCTION

Genetic or acquired diseases and trauma contribute to changes in normal motor, sensory or autonomic functions of neuronal pathways. Inflammatory environment resulting from an injury or disease leads to progressive degeneration of various neuron populations and destruction of neuronal tracts in the adult central nervous system (CNS) [1]. CNS abnormalities in the United States are widespread: at least 250,000 individuals suffer from spinal cord injuries, 30,000 individuals are diagnosed annually with ALS, and conditions including Parkinson’s disease, Multiple Sclerosis, and various forms of brain cancer affect hundreds of thousands more annually [2-5]. The combination of the adult CNS limited ability for self-repair paired with the lack of clinically-effective pharmacological, radiological, and surgical treatments has prompted researchers to explore tissue-engineering approaches for a wide spectrum of CNS anomalies [2, 6-8].
In 1992, cells isolated from the adult mouse striatum were shown to have the capacity to divide and differentiate into neurons and astrocytes, thus opening the possibility of utilizing neural stem cells (NSCs) and neural precursor cells (NPCs) to regenerate populations of lost neurons [9]. Factors driving NSC and NPC differentiation include the surrounding extracellular matrix microenvironment, the presence of biochemical markers in their milieu (i.e., growth factors, cytokines, signaling molecules) and various biomechanical forces [10]. Of these growth factors, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) maintain NSC and NPC “stemness”, where upon their removal the cells are able to commit to various neural and glial lineages [9]. To enhance differentiation into a specific lineage, researchers have supplemented a plethora of signaling molecules, including but not limited to, pro- and anti-inflammatory cytokines and chemokines (e.g., interleukins, TNF-α), neurotransmitters (e.g., gamma-aminobutyric acid, dopamine), neurotrophins (e.g., nerve growth factor, brain-derived neurotrophic factor) and several other biological proteins, resulting in a mixed progeny of cells dependent on exposure time and concentration [11]. One aspect of this study continues to further understand how exposure to biochemical signaling molecules influences NSC fate.

As mentioned earlier, the surrounding substrate and subsequent mechanical forces experienced by the cells play an equal role in influencing NSC or NPC fate. It has been well documented that CNS matrix microenvironment can direct differentiation of these cells into specific neural and glial lineages [12]. To date, several groups have engineered scaffolds from naturally-derived matrix
proteins, including collagen, hyaluronic acid, agarose, alginate, chitosan, methyl cellulose, xyloglucan, Matrigel®, fibrin, and various peptides, as well as synthetic hydrogels including such as graphene, polyacrylamide, polyethylene glycol, and several other synthetic polymers [12]. Of these matrices, scaffolds designed with a softer mechanical modulus (< 1000 Pa) has shown promise in directing NSCs to a neural lineage, whereas stiffer scaffolds appear to influence these cells into glial lineages [13]. However, it is still unclear what compositions and concentrations of extracellular matrix (ECM) proteins provide the most suitable environment for stimulating precise NSC or NPC differentiation into specific neural lineages, and enhance axonal outgrowth and targeting [2, 12]. In addition, numerous other questions remain: How does CNS ECM composition vary under inflammatory conditions? Does this altered ECM composition influence NSC and NPC phenotype and genotype? How can we study NSC and NPC response to such conditions in vitro under controlled culture conditions? A detailed understanding of the inflammatory microenvironment created by trauma or disease is needed for long-term restoration and repair of the damaged CNS tissue.

Although current work in regenerative medicine has demonstrated the utility of 3D ECM-based cell-laden scaffolds for integumentary, skeletal, respiratory and circulatory system repair, using this type of repair strategy in the CNS is relatively unexplored [14-18]. CNS microenvironment is relatively soft (modulus < 1 kPa), protected by the blood-brain barrier, consists of billions of neurons in constant communication with each other, and aided by numerous
other glial cell types which maintain homeostasis under healthy conditions. Hydrogels made of various biological ECM components including proteins, proteoglycans, glycosaminoglycan and lecticans, interact with NSCs and NPCs through native cell-substrate adhesion transmembrane proteins including selectins, integrins, syndecans, and cadherins which consequently can trigger a number of signal transduction pathways to influence NSCs and NPCs differentiation, viability, and axonal outgrowth [12, 19]. A critical review of literature suggest that scaffolds containing synthetic polymers or chemical crosslinkers might elicit undesirable effects on NSC and NPC viability and fate, compromise host integration, and increase the likelihood of immuno-rejection. Such scaffolds might not only lack requisite integrin-mediated binding sites for NSCs and NPCs to attach and home in, but also lack matching mechanical properties to be precisely injected into damaged regions of CNS tissue. To overcome some of these limitations, biological scaffolds developed in this study were intended to be injected in situ and allow for NSC integration into host tissue, with the understanding that their composition would allow for rapid clearance and reduced immunogenicity. The first aim of this study shows the utility of injectable, uncrosslinked, 3D biomimetic ECM-based scaffolds for their suitability to NSC homing and differentiation in the presence of retinoic acid.

In addition to engineering scaffolds that foster specific NSC and NPC lineages and enhance viability, these scaffolds should also possess neuro-protective properties to shield these cells from the complex inflammatory microenvironment created after CNS injury or disease. Upon in situ injection,
NSCs and NPCs will be exposed to variety of cytokines and chemokines (termed analytes), released by host inflammatory glial cells, which will alter the native behavior of these transplanted cells. Previous work in this area has focused on single analyte exposure via direct addition to culture media, or full panel exposure within *in vivo* animal models. It is still not clear from these studies as to how these analytes detrimentally influence NSC behavior, or how they can be modulated to allow NSCs to partially awaken lost functionality. Thus, in the second component of this study, NSC behavior in the presence of an activated phenotype of a spontaneously-immortalized microglial cell induced inflammatory environment was investigated, using a customized coculture setup. In 2D and 3D microenvironments, both by themselves and in the presence of these activated cells, NSC genotype, phenotype, viability, and axonal outgrowth was quantified and results compared to the stand-alone exposure to several such analytes. Results from these experiments (second aim of this study) would increase our understanding of the specific role played by various analytes in a localized inflammatory microenvironment, and expand on our first aim by providing better strategies for *in situ* delivery of NSCs towards regenerative medicine applications.

Recent discoveries in genetic analogies within stem cell biology and oncology have motivated researchers to investigate the influence cancerous cells have on stem cell fate and vice versa [20]. These studies have led to the identification of a small population of cancer cells termed “cancer stem cells” (CSCs), which share characteristics observed in healthy stem cells, (i.e. maintain
proliferation, self-renewal and differentiation) [21]. It is unclear where these cells originate from, although it has been suggested it is likely from NSCs, NPCs or differentiated subpopulations of these cells. It has been shown that CSCs possess the ability to initiate tumor formation while concurrently exhibiting NSC and NPC properties, which fuels the speculation that these stem cells play a major role in tumor development [22, 23]. Furthermore, *in vivo* studies have shown that NSCs migrate from the subventricular zone (SVZ) toward a tumor site, which suggests a link between tumor growth and NSC or NPC presence [24]. The parallelisms between these two cell populations have raised several questions: Is the microenvironment of the SVZ critical in controlling and regulating NSC or NPC self-renewal properties? Do glioblastomas produce analytes that drive transformation and tumor growth from NSCs or NPCs? Can NSCs or NPCs be used to repair tissue lost upon tumor removal, or will they drive tumor relapse?

To date, cancer research has focused on impeding disease progression by altering glioblastoma metabolic processes (i.e. angiogenesis, cellular metasisis, etc.) through radiotherapy or pharmaceuticals (i.e temozolomide, procarbazine) however these techniques carry undesirable side-effects (communications deficit, motor dysfunction and physical functioning) that reduce patient quality of life regardless of disease progression status [25]. Furthermore, while neurosurgery can potentially rid the patient’s tumor there is no current options to replace the lost tissue, and no guarantee the tumor will not relapse [26]. Therefore, in the final aim of this study, we investigated how NPCs and cancer cells interact with
each other in a coculture microenvironment to influence their morphology, migration, proliferation and differentiation. We believe that the outcomes from this study might help us in designing better strategies for delivering NPCs to replace the excised tumor lesions or deploy NPCs to deliver therapeutic cargo to the site of tumors for inhibiting tumor growth.

The third aim of this study expands upon the ideas presented in aims 1 and 2 and continues the exploration of how inflammatory microenvironments can influence NSC or NPC survival, differentiation, axonal outgrowth, and cellular homing. The novelty of work is highlighted by the utilization of NPCs in cocultures with pediatric glioblastomas to understand paracrine cell signaling as it relates to cancer milieu.

In summary, this study will first demonstrate the feasibly of designing 3D ECM based biological scaffolds which could be injected in situ at the site of damaged host tissue for delivering NSCs. It then describes the interactions between NPCs/ NSCs with two distinct cell types in the unhealthy CNS - microglial and pediatric glioblastoma cells - with which the delivered stem cells are expected to interact in vivo. Taken together, outcomes from our study will not only detail the precise architecture required to design tissue-engineered based strategies but also enable the development of protocols for neural stem cell transplantation in vivo to treat neurological disorders. Results from this study will also enable development of optimized scaffolds to overcome the surrounding inflammatory environment caused by both barrier and trophic factors leading to the eventual restoration of axonal connections across regions of injury or disease.
1.2 Specific aims

**Aim 1)** Develop and characterize the physical and mechanical properties of 3D biomimetic ECM-based scaffolds composed of hyaluronic acid, laminin, glycosaminoglycans, and collagen and evaluate their suitability for neural stem cell (NSC) homing, differentiation, neurite outgrowth, viability in the presence or absence of retinoic acid.

**Rationale:** Direct host injection of NSCs typically results in a lack of cell survival, attachment, homing in the native tissue, directed differentiation, and axonal elongation. *In situ* injection of NSCs laden in a scaffold composed of biological constituents will not only overcome the above obstacles but also provide the requisite signals that will enhance CNS repair strategies.

**Hypotheses:** (1) Scaffold selection influences NSC survival, attachment to matrix proteins, differentiation, morphology, axonal outgrowth, and genotype. (2) The addition of RA further promotes these outcomes.

**Aim 2)** Investigate the role of induced-inflammatory microenvironment (altered 3D matrix composition, with the presence of cytokines, chemokines & interleukins) on NSC survival, homing, and differentiation.

**Rationale:** The current inability to employ pharmaceutical-based therapeutic options in the CNS has prompted demand to provide cell-based therapies that might overcome inflammatory and ECM based obstacles. Investigating the
analytes released by activated microglial and their subsequent effects on NSCs behavior will aid in formulating repair strategies.

**Hypotheses:**
(1) Cocultures with activated microglia mimics inflammatory microenvironment in vivo. (2) NSC Interactions with activated microglia influence their survival, differentiation patterns, phenotype, genotype, and axonal outgrowth. (3) The types and amounts of cytokines and chemokines released by activated microglia in vitro mimic in vivo conditions.

**Aim 3)** Investigate the role of a pediatric cancerous microenvironment (cytokines, chemokines, cancer specific proteins) on human NPC survival, homing, and differentiation.

**Rationale:** The results obtained from aims 1 and 2 will provide information on NSC behavior under healthy and inflammatory microenvironments, respectively, which will aid in understand how these behaviors change in a cancerous environment. Results will further the therapeutic potential of NPCs for cancer research which has only recently gained traction.

**Hypotheses:**
(1) GBM cells influence NSC survival, morphology, axonal outgrowth, differentiation, and migration in a coculture setup. (2) NSC could be influencing similar outcomes in GBM cells as well. (3) A transwell coculture setup is a reproducible biomimetic in vivo culture model to test these hypotheses.
CHAPTER II

BACKGROUND AND LITERATURE

2.1 Neurological disorders: pathology, etiology, and prevalence

The origin of abnormal motor, sensory or autonomic function of neuronal tracts can be traced to two sources: diseases (acquired or inherited) or injury (trauma, accidents). These pathological conditions promote localization of an inflammatory environment surrounding neural tracts, resulting in the degeneration and destruction of healthy axonal connections [27]. The eventual death of these neurons supposedly plays an important role in the clinical manifestation of numerous neurological disorders, typically facilitated by changes in upstream effectors, mitochondrial alterations, and caspase activation [28].
Immunological repair responses in the CNS are limited due to the blood-brain barrier's unique involvement in limiting antigens into the system. This, coupled with the reduced expression of major histocompatibility complex antigens (classes I and II) on certain CNS cells creates an immunosuppressive microenvironment [29-31] which shields against the much-desired remodeling of the dynamic and complex CNS neural network after trauma [31]. During a CNS injury or disease, the surrounding ECM is transformed in a manner (glial scar formation) that prevents nerve fibers from extending axons and re-establishing...
neural networks, resulting in the death of the corresponding cell bodies due to apoptosis or necrosis (Fig. 1) [32]. In addition, the death of these primary cell bodies initiates a sequence of events in which undamaged fibers in the vicinity of the injury site also become affected and undergo secondary degeneration, unless promptly treated [31, 33, 34]. Secondary degeneration is signaled to the surrounding tissue by glutamate, free radicals or additional apoptotic biomolecules, some of which are associated exclusively with CNS axonal injury rather than a direct injury [35-38]. Trauma or disease affecting the CNS causes irreversible tissue damage by three broad mechanisms: mechanical disruption of neurons and/or their projections, direct or secondary biochemical changes that are initiated by the trauma, and reactive inflammatory or gliotic changes [33]. The following table summarizes the etiology and prevalence of several major CNS diseases (Table 1).

Table 1 Summary of CNS diseases. Summary of the etiology and prevalence of several CNS diseases and disorders.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Etiology</th>
<th>Prevalence</th>
<th>Treatment options</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyotrophic Lateral Sclerosis (ALS)</td>
<td>Late-onset, progressive, neurodegenerative disease, life expectancy of &lt; 5 years</td>
<td>~30,000 North Americans</td>
<td>Riluzole® reduces the body's natural production of the neurotransmitter glutamate, but only extends life by a few months, with no major relief in symptoms.</td>
<td>[39-41]</td>
</tr>
<tr>
<td>Spinal Muscular Atrophy (SMA)</td>
<td>Autosomal recessive neurodegenerative disease characterized by the degeneration of spinal cord motor neurons. Life expectancy varies form SMA type (Type 0/1 &lt; 10 years Type III full life)</td>
<td>one in 10,000 live births</td>
<td>Valproic acid, aclarubicin, C5-quinazoline derivatives, and sodium butyrate have been shown to elevate the transcription expression of SMN2, and restore the correct splicing expression of SR proteins. Benefits are yet to be seen in human clinical trials.</td>
<td>[4, 42-45]</td>
</tr>
<tr>
<td>Disorder</td>
<td>Description</td>
<td>Prevalence</td>
<td>Treatment Notes</td>
<td>References</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Hereditary Spastic Paraplegia (HSP)</td>
<td>A large group of inherited neurologic disorders which are broadly characterized by the presence of lower extremity spastic weakness</td>
<td>~200,000 people in the US</td>
<td>Restricted by the limitations in our current understanding of the chromosomal loci which contribute to the symptoms, and thus lacks a pharmacological treatment option</td>
<td>[46-48]</td>
</tr>
<tr>
<td>Parkinson's Disease (PD)</td>
<td>Idiopathic results from the death of dopamine-generating cells</td>
<td>At least a million US citizens</td>
<td>Dopaminergic, anti-glutamatergic and anticholinergic based drugs, with each working only to alleviate a specific symptom with limited effectiveness over time. Fetal mesencephalic cell transplantation or deep brain stimulation shows promise when however there is a paucity of data to understand the mechanism by which this treatment works.</td>
<td>[5, 49-55]</td>
</tr>
<tr>
<td>Multiple Sclerosis (MS)</td>
<td>Idiopathic, results from the destruction of myelin sheaths of neurons.</td>
<td>~2 million people globally</td>
<td>Eight disease-modifying treatments have been approved for relapsing-remitting multiple sclerosis (RRMS), and all are modestly effective at decreasing attacks.</td>
<td>[5, 49].</td>
</tr>
<tr>
<td>Glioblastoma multiforme (GBM)</td>
<td>Unknown, could result from a combination of environmental and genetic factors. Effects both adult and pediatric populations</td>
<td>Fewer than 200,000 US cases per year</td>
<td>Surgical removal paired with radiation and/or chemotherapy</td>
<td>[56]</td>
</tr>
<tr>
<td>Spinal cord injuries (SCI), Traumatic brain injury (TBI)</td>
<td>Blunt trauma to brain or spinal cord</td>
<td>~3.2 million (TBI), quarter million (SCI)</td>
<td>Reduce intracranial and intraspinal pressure in an effort to minimize neural tissue damage caused by swelling. Mannitol and various barbiturates, only have a modest effect on acute CNS damage and subsequent death of cellular pathways leading to functional impairment. For SCI injury, methylprednisolone, an anti-inflammatory steroid, is the most widely used treatment. Treatments are limited 24-72 h timeframe following traumatic injury.</td>
<td>[57-62]</td>
</tr>
</tbody>
</table>
Annually thousands of new patients are diagnosed with various neurological related injuries and diseases in the United States, and a majority of them succumb to this condition within 3-5 years post-diagnosis [63]. Given these incidence rates, it is extremely important to restore the lost neuronal tracts in the CNS on a priority basis. The loss or destruction of CNS tissue as a result of disease or trauma normally results in reduced patient quality of life. If CNS injuries are afflicted within a younger populace, they will require a lifetime of rehabilitation [64, 65], with a major economic impact (>$70 billion) on taxpayers [58, 66]. The complexity of the CNS, its inability for self-repair, and variability in the clinical onset of various pathological conditions make pharmaceutical and rehabilitative repair strategies particularly challenging. This prompts the demand for novel and clinically-effective treatment options, such as tissue-engineering strategies and exogenous cellular supplementation, which utilize biomimetic scaffolds that guide and reconnect damaged axons while simultaneously overcoming the surrounding apoptotic environment [67-69].

2.2 Composition of the healthy CNS ECM

An understanding of the native CNS ECM composition is crucial in formulating any repair and/or regeneration strategies in diseased or injured CNS tissues. Previous *in vitro* and *in vivo* studies have demonstrated evidence suggesting that the CNS ECM affects virtually all aspects of nervous system development and function [70, 71]. The CNS ECM is composed of glycosaminoglycans, proteoglycans, and other link proteins (*Fig. 2*) including laminin, nidogen, reelin, and tenascins - C & R,[70, 72, 73]. These components
contribute to only 20% of the total tissue volume, creating pores with an average size of 120 µm, and a soft elastic modulus of approximately 500 Pa [73-75]. Other structures unique to the CNS ECM are perineuronal nets, present during embryogenesis and fetal maturation [76-78]. These structures are composed of condensed layers of pericellular matrices, which encapsulate cell bodies and axons [79]. These nets contain hyaluronan, chondroitin sulfate proteoglycans, and tenascin-R, which aid in neuronal differentiation as well as the stabilization of axons and their subsequent connections to surrounding cell bodies [76, 80].

![Figure 2 CNS ECM constituents](image)

**Figure 2 CNS ECM constituents.** Schematic of major CNS ECM constituents. Volpato et al. [2]

The components of the CNS ECM regulate cellular function through the transduction of mechanical signals and integrin -activated signaling pathways [13, 81]. However, each molecule has been recognized for being multivalent, meaning it possesses the ability to bind to several other ECM ligands, which in turn can either inhibit or promote cellular function. HA has the ability to bind to
several other large glycosaminoglycans, proteoglycans, and specific cell-surface receptors via specific HA-protein interactions [82, 83]. However, only HA hydrogels with high molecular weight (> $10^6$ Da) have been shown to inhibit astrocyte activation and CSPG deposition, which reinforces specific multivalent properties of CNS ECM molecules and its effect on cellular behavior [84, 85]. Laminin is said to play a role in cellular differentiation, migration and adhesion specifically through interactions with the α, β-integrins on the neuronal surface. Similar to HA, laminin has a complex binding domain that directs a variety of cellular functions, which are also concentration-dependent. For example, neurons have been shown to have approximately a two-fold increase in neurite outgrowth and a two-fold decrease in branching, for a corresponding 100-fold increase in laminin concentration [70, 86].

The utility of various types of collagen based scaffolds for CNS regeneration has been widely studied despite its low presence in native CNS tissue. This is because, collagen gels can be easily manipulated to control their mechanical properties and physicochemical characteristics by varying concentration, density and pH [87]. It should be noted that depending on the cell-specific binding domain, both collagen type and concentration may elicit either adhesive or anti-adhesive effects that can modulate axonal outgrowth and neuronal adhesion, hence the need to further investigate this ECM protein for tissue regeneration purposes [88-90]. Furthermore, several studies have shown that changing the isoform or concentration of tenascin-C, HSPGs, CSPGs, and other ECM molecules has paradoxical effects (i.e., promote or hinder neurite
extension) on cellular behavior, which highlights the specificity and complexity of each ECM constituent’s function as it relates to cellular phenotype.

In addition to the aforementioned ECM molecules, several growth factors integrated within the ECM direct neural function. The table below highlights several major neurotrophins found within the CNS ECM (Table 2). Of particular interest to this work is the binding of FGF-2 to heparin sulfate proteoglycans, which are necessary for maintenance of the NSC phenotype [91]. In general, a plethora of studies have shown that neurotrophic factors influence neural differentiation, survival, and axonal extension and guidance, under both healthy and inflammatory (disease, injury) phenotypes [92-101]. Results from the studies outlined in Table 2 suggest that post-injury, developing neurons could be guided by a mixture of diffusible biomolecular gradients through varying ECM environments, either at a close range or over a distance, to induce robust axonal outgrowth and target identification. Since considerable functional integration in vivo is evident, these studies strongly attest to the role of growth factors, either secreted by cells or supplied exogenously, to influence pathogenesis of the inflammatory CNS and promote growth of injured axons, which is fundamentally vital for awakening neural functionality.

### Table 2 Selected growth of the CNS. Selected growth and soluble factors and their effect on neural tissue. *indicates soluble factor. Portions of the table adopted from Farrell K., Kothapalli CR [192].

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Observed effects</th>
<th>Cell/tissue type</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve Growth Factor (NGF)</td>
<td>Promote survival and outgrowth of neurons</td>
<td>Primarily sensory neurons</td>
<td>[102-104]</td>
</tr>
<tr>
<td>Neurotrophin-3 (NT-3)</td>
<td>Motor neuron survival and outgrowth, as well as sensory axon growth</td>
<td>Motor and sensory neurons in the dorsal root ganglia</td>
<td>[105, 106]</td>
</tr>
<tr>
<td><strong>Ciliary neurotrophic factor (CNTF)</strong></td>
<td>Architecture, organization</td>
<td>Spinal cord motor neurons and nerves of the PNS</td>
<td>[107, 108]</td>
</tr>
<tr>
<td><strong>Bone morphogenetic protein (BMP)</strong></td>
<td>Maintain and increase the number of neurons</td>
<td>Subventricular neurons, spinal cord motor neurons, as well as neural tissue in the hindbrain</td>
<td>[109-111]</td>
</tr>
<tr>
<td><strong>Basic fibroblast growth factor (bFGF)</strong></td>
<td>Maintenance of the generation of astrocytes, involved in cellular migration, maintain stemness in neural stem cells</td>
<td>Wide variety of tissue types thought brain and spinal cord, also plays a strong role in the wound healing and the maintenance of multiple tissue types</td>
<td>[112-114]</td>
</tr>
<tr>
<td><strong>Vascular derived endothelial growth factor (VEGF)</strong></td>
<td>Neurogenesis, neuronal migration, neuronal survival and axon guidance, however further research is needed to understand if this is a direct or indirect effect.</td>
<td>Superior cervical ganglia (SCG), dorsal root ganglia (DRG) from adult mice, as well as embryonic cell types</td>
<td>[115, 116]</td>
</tr>
<tr>
<td><strong>Brain-derived neurotrophic factor (BDNF)</strong></td>
<td>Neuronal survival and plasticity during development and after injury.</td>
<td>Rat embryo septal cholinergic neurons as well as hippocampal neurons</td>
<td>[117-120]</td>
</tr>
<tr>
<td><strong>Retinoic acid</strong> (RA)</td>
<td>Differentiation of stem cells and extension and organization of axons</td>
<td>Dorsal root crest, additionally in the dorsal root ganglia</td>
<td>[121-124]</td>
</tr>
<tr>
<td><strong>Sonic hedgehog</strong> (SHH)</td>
<td>Neuron guidance, protection, and proliferation.</td>
<td>During development, and midline structure of brain and spinal tissue</td>
<td>[125-128]</td>
</tr>
</tbody>
</table>

### 2.3 Composition of the unhealthy CNS ECM

Upon injury or trauma, the CNS ECM transforms into a glial scar which contains both physical and biochemical barriers, which limit axonal outgrowth, cellular differentiation and survival. The nature of the exact composition of the glial scar is dependent on the specific disease or trauma acting upon the environment, however several specific ECM molecules and biochemical factors are frequently found within glial scars at various concentrations and can be
broadly categorized as barrier factors or trophic factors (Fig. 3) [129]. Tenascin, heparan sulfate proteoglycan (HSPG), chondroitin sulfate proteoglycan (CSPG), and keratin sulfate proteoglycans (KSPG), which are found in healthy CNS ECM, are highly up-regulated during gliosis, causing inhibition of neurite outgrowth [130-132]. The source of up-regulation of these ECM molecules that inhibit neural regeneration is linked to reactive microglial cells that are overexpressed within the glial scar [133-136]. It has been suggested that the mechanism for outgrowth inhibition and cellular neurosis is likely due to negatively-charged sulfate groups on GAG molecules or via binding to growth-promoting ECM molecules such and laminin and tenascin, essentially blocking their functions [137-139]. Specifically, axon inhibition is a function of CSPG binding to receptor proteins on the cellular membrane (tyrosine phosphatase receptor and leukocyte antigen-related phosphates receptor) [140, 141]. Currently, it is suggested that axonal inhibition is the product of glial scar constituents’ interactions with cellular receptor proteins [142, 143]. Aside for CSPGs, the ECM surrounding injury or disease has an increased meshwork of dense collagen, which not only serves as a mechanical barrier but also as a binding site for other ECM components and inhibitory molecules like proteoglycans and semaphorins [144, 145]. Thus, axonal inhibition in a glial scar is a product of the isoform, the combination and concentration of ECM molecules, and the type and age of neuron [2]. The constituents of the CNS ECM can be broadly classified as “growth-promoting” and “growth inhibiting”; however the reality is that the molecules are dependent
on the interactions with one another which ultimately dictate how it will affect neuronal behavior.

Figure 3 Factors that effect neurite outgrowth. In a glial scar neurite outgrowth is inhibited by several mechanisms, both trophic (yellow) and mechanical (red) factors. Factors that disrupt the function of cell-matrix receptors or directly induce neurite collapse will prevent migration by reducing traction (blue). ECM molecules and receptors that increase neurite adhesion will prevent migration by disabling release for the substratum (orange). [145]

In addition to the alterations in the ECM constituents upon injury or disease, there is also an influx of inflammatory markers, which prohibit the restoration of neuronal circuits. Immune reaction in any biological system is broadly characterized as a dynamic equilibrium of both inflammatory responses (interleukins and interferons) and anti-inflammatory responses (various cytokines...
and chemokines as well as transforming growth factors), with a majority of these factors being produced by surrounding microglial cells [146]. Within the CNS, surrounding microglial cells can express proteolytic enzymes which break down ECM proteins, specifically cytokines and chemokines [tumor necrosis factor (TNF-α), interleukin (IL-α), interferon (INF-γ)] reactive oxygen species, oxidative enzymes, and metalloproteinases, which amplify secondary tissue damage and the formation of an inhibitory glial scar [147, 148]. It has been suggested that TNF-α might act together with glutamate, which induces neural and oligodendrocyte cell necrosis [149]. Interleukins have been reported to cause the differentiation of NSCs into reactive microglial cells by activating the JAK-STAT signaling pathway immediately following injury [150]. As mentioned previously these reactive microglial cells account for the production of ECM constituents which inhibit axonal growth. The site of injury or trauma can also cause unique cytokinetic responses. For instance, following IL-1β microinjection, lymphocytes infiltrated the spinal cord but never in deep brain tissue, whereas TNF-α microinjections in deep brain tissue elicited a macrophagic response, while identical injections into the spinal cord elicited both neutrophilic and macrophagic responses [151, 152]. The immune response may also contribute to tissue repair by clearance of necrotic tissue, reduction in lesion size, and the release of trophic factors (interleukin (IL-10), transforming growth factor (TGF-β), which can be neuroprotective and promote axonal outgrowth [147, 153]. Broadly, pro-inflammatory cytokines, such as interleukin-1, can mediate a diverse range of effects through activation of T cells, induction of expression of acute-phase
proteins and direct effects on the nervous tissue [154]. Despite providing some regenerative properties, the primary consequence of the inflammatory response in the CNS is the loss of myelin and cell apoptosis, which is dependent on the location of injury or disease and type of cytokines or chemokines released.

2.4 Composition of a cancerous microenvironment in the CNS

The transformation of healthy cells to a cancerous phenotype creates a unique microenvironment with altered levels of biochemical analytes which can be produced by the cancerous cells themselves or those cells in close proximity [155]. A hallmark feature of any type of cancer cell is the inherent self-sufficiency in releasing analytes into the surrounding microenvironment required to further their own growth and proliferation. These cells also have a limited response to growth-inhibitory analytes, inability to become apoptotic, undergo unlimited self-replication, promote sustained angiogenesis, and can rapidly invade surrounding tissue and metastasize, all of which modifies the surrounding microenvironment and influences surrounding cellular fate [156]. This has prompted research that aims to understand the origins and transformation of surrounding cells to aid in developing new therapies which impede cancer metastasis.

Because there are several genetic similarities between NSCs and glioblastomas (GBMs), research has begun exploring the possibility that the origins of GBMs could be linked to NSC differentiation (Fig. 4) [20, 157, 158]. NSC differentiated astrocytes have been proven experimentally in murine models.
to dedifferentiate into GBMs via deletion the INK4A/Arf gene, which is said to increase the potential activation of these cell’s oncogenes [159]. Direct transformation of NSCs to GBMs was initially based on the histological similarities between the two cell types, specifically that each contains populations of cells which exhibit multipotentiality, the ability to self-renew, and the formation of neurospheres [22]. This information then prompted the notion that germinal regions the cells (e.g., in the SVZ) are able to provide the requisite cues which keep these cells from deviating into a cancerous phenotype [160]. More recently, it has been shown in several animal models that the regions of the brain containing large populations of NSCs are very sensitive to chemical or viral oncogenesis than the areas where NSCs do not reside [161]. Genetic analysis of this type of transformation revealed the p53, Rb, and RTK signaling pathways to be potentially responsible for NSC GBM transformation [162]. In summary, further studies including clinical trials are necessary to understand the origins of this unique pathology [20].
Figure 4 Origins of GBMs. Relationship between NSCs’ differentiation and cancer initiation. NSCs (purple) are able to differentiate to glial (grey) and neural progenitors (yellow). Neural progenitors give rise to neurons (pink) whereas glial progenitors are committed to oligodendrocytes (red), ependymal cells (light blue) and astrocytes (dark blue). Brain cancer formation could arise from the transformation of NSCs into GICs (Brain Cancer Initiating Cells, light purple) which in turn give rise to a more differentiated cancer cell population (green). In the same line, glial progenitors could induce tumor development after malignant transformation of normal progenitor cells (Brain Tumor Progenitor-like Cells, orange). Taken from Goffart et al. 2013 [20]

After tumor formation, GBMs produce analytes which influence themselves as well as the surrounding cells (i.e. vascular cells, microglia, and NSCs) which subsequently plays a crucial role in controlling the course of the pathology (Fig. 5) [20]. GBMs have been shown both in vitro and in vivo to produce several interferons, interleukins, and other cancer specific analytes during the progression of the disease, in similar concentrations to those produced by activated microglial cells [163, 164]. Specifically, protein-3 (MCP-3),
colony-stimulating factor 1 (CSF-1) and granulocyte-colony stimulating factor (G-CSF) recruit surrounding microglia, which then produce various MMPs and other factors that can degrade the surrounding ECM [165, 166]. Production of CCL2, CCL22 and TGF-β by GBMs has been correlated with the recruitment of regulatory T-cells to the tumor site [167]. Other studies have shown NSC tumor site recruitment is mediated by increased production of CXC [168], and consequently migrating NSCs are thought to produce anti-tumorigenic analytes, however it is unclear what specific analytes are causing this phenomena and studies have been limited to murine models [169, 170]. GBMs like most other cancer types also have been found to produce VEGF which increases vascularization network within the surrounding microenvironment [171]. In summary, it is understood that the release of these analytes by GBMs or the surrounding cells into the microenvironment can directly influences cellular behavior [172, 173].
2.5 Neural stem cells

Several cell sources have been explored for the regeneration of neural tissue; here we will briefly describe the currently employed stem cell types and their limitations. Embryonic stem cells (ESCs), the most pristine form of stem cells, remain totipotent in humans up to the 8-cell stage (3-4 days) after fertilization [176]. Although a promising cell source for tissue-engineered repair strategies, there are currently ethical concerns related to sourcing, necessity for immunosuppression, risk of tumor formation and possible chromosomal instability, which dampen the enthusiasm generated by ESCs for potential clinical applications [177].
Neonatal stem cells, also referred to as umbilical-cord derived stem cells, are isolated from the umbilical cord immediately after birth [178]. These cell types are immunologically-immature and may still be rejected by the body when transplanted, unless the patient’s immune system is strongly suppressed or has been ablated before transplantation [179]. Mesenchymal stem cells (MSCs) are multi-potent cells derived primarily from bone marrow that can differentiate into various cell types or lineages under tightly-controlled microenvironments, consisting of ECM proteins and growth factors [180]. Although early pre-clinical and clinical data demonstrate the safety and effectiveness of MSC therapy, specifically its potential in cardiac tissue repair, there are still many questions to be answered surrounding the mechanisms of neural differentiation and the efficiency of this process, thus they are not currently a strong candidate to provide clinically translatable neural repair strategies [181]. However, more research on MSC differentiation into neural lineages might promote their applicability in vivo in the near future. Besides, clinical trials over the past two decades resulted in no single FDA-approved MSC-based treatment for any disease in the body.

Neural stem cells (NSCs) are self-renewing, multi-potent cells, capable of differentiating primarily into neuronal and glial lineages. Unlike many other cells in the body, neurons do not divide within the CNS, which necessitates replacement of lost or injured neurons with these CNS resident cells. NSCs typically assume a quiescent phenotype until they receive exogenous signals from their microenvironment to proliferate, migrate or differentiate into a specific
lineage [182]. Many researchers observed that adult NSCs are not restricted to specific lineage dictated by their developmental origin, rather they are capable of differentiating into regionally specific neuronal subtypes in vivo when exposed to the appropriate environmental cues (Fig. 6) [183, 184]. NSCs have been shown to expand and differentiate into both neurons and glia in the presence of epidermal growth factor (EGF) and bFGF, and via genetic modification, and could be explored as a promising therapeutic treatment for transplantation into those afflicted with the injuries and diseases outlined in Table 1 [185]. NSCs exhibit great potential for the repair and regeneration of damaged neural tissue neurons, however given the infancy of the field there is a lack of awareness of the signals involved in their differentiation and integration within a given neural network. One of the goals of this project is to investigate how ECM signals as well as soluble factors affect NSC phenotypes, thus expanding the breadth of knowledge within the field of neural tissue engineering.
2.6 Synthetic hydrogel substrates

Synthetic scaffolds can be custom-designed to have precise mechanical and biochemical properties, as well as a high level of reproducibility. Direct surgical insertion of 3D scaffolds, seeded with cells and laden with growth factors, into a damaged lesion of the CNS or PNS is perhaps the most common approach employed when demonstrating the feasibility of such scaffolds [187-189]. However, studies have shown that even slight changes in the composition of synthetic materials, such as the addition of poly (lactic acid) to a PEG scaffold, can affect the functional use of that material as the lactic acid leaching during gel
degradation severely impacts the function of encapsulated neural cells [190-192]. Despite current understanding and progress, further research is required to hone synthetic polymeric scaffolds designed to support specific neural and glial cell types, as well as to the specific region of the nervous system into which the polymer is being incorporated. Thus, given the information highlighted in Table 3 alluding to the superiority of biologically based scaffolds, the research proposed here will focus on the 3D hydrogels composed only of biological polymers.

Table 3 Pros and cons of CNS scaffolds. Pros and Cons of synthetic and biological (italicized) scaffolds used for culturing NSCs, portions adapted from Li et al.[12]

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyacrylamide</td>
<td>+Adjustable mechanical properties</td>
<td>-Non-biodegradable</td>
</tr>
<tr>
<td></td>
<td>+Easy control of architecture and chemical composition</td>
<td>-Non-neuronal permissive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Neurotoxic</td>
</tr>
<tr>
<td>Polyethylene-glycol</td>
<td>+Highly biocompatible</td>
<td>-Very hydrophilic</td>
</tr>
<tr>
<td></td>
<td>+Non-immunogenic</td>
<td>-Non-biodegradable</td>
</tr>
<tr>
<td>Polycaprolactone</td>
<td>+ Tunable mechanical properties</td>
<td>- Possibly immunogenic</td>
</tr>
<tr>
<td></td>
<td>+Biodegradable</td>
<td>- Low quality axonal outgrowth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Possibly immunogenic</td>
</tr>
<tr>
<td>Polylactic acid</td>
<td>+ Biodegradable</td>
<td>- Low quality axonal outgrowth</td>
</tr>
<tr>
<td></td>
<td>+ Tunable mechanical properties</td>
<td>- Possibly immunogenic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Possibly immunogenic</td>
</tr>
<tr>
<td>Agarose</td>
<td>+Injectable</td>
<td>- Does not degrade naturally</td>
</tr>
<tr>
<td></td>
<td>-Hinders neural growth</td>
<td>- Does not degrade naturally</td>
</tr>
<tr>
<td>Alginate</td>
<td>+ Easy to crosslink</td>
<td>-Net negative charge inhibits protein absorption</td>
</tr>
<tr>
<td></td>
<td>+Injectable</td>
<td>-Net negative charge inhibits protein absorption</td>
</tr>
<tr>
<td>Collagen</td>
<td>+Highly biocompatible</td>
<td>-Possible immunogenic</td>
</tr>
<tr>
<td></td>
<td>+Injectable</td>
<td>-Can inhibit neurite growth</td>
</tr>
<tr>
<td>Chitosan</td>
<td>+Highly biocompatible</td>
<td>-Only soluble in dilute acid</td>
</tr>
<tr>
<td></td>
<td>+Easy modification</td>
<td>-Inflammatory response</td>
</tr>
<tr>
<td></td>
<td>+Injectable</td>
<td>-Inflammatory response</td>
</tr>
<tr>
<td>Fibrin</td>
<td>+Highly compatible</td>
<td>- Only soluble in dilute acid</td>
</tr>
<tr>
<td></td>
<td>+Injectable</td>
<td>-Inflammatory response</td>
</tr>
<tr>
<td></td>
<td>+Chemical modification</td>
<td>-Inflammatory response</td>
</tr>
<tr>
<td></td>
<td>+Enzymatic degradation</td>
<td>-Inflammatory response</td>
</tr>
</tbody>
</table>
### 2.7 Biological hydrogel substrates

As the name suggests, biological substrates are made from naturally available connective tissue components, conserved across species within the mammalian system. As discussed previously, the CNS ECM is a unique tissue when compared other mammalian organs and tissues, thus many different scaffold systems have been evaluated for CNS applications, with a majority attempting to mimic the specific biochemical and mechanical properties unique to the CNS ECM. These properties, including mechanical modulus, morphology, structure, and biological moderations, greatly affect NSC behaviors including viability, attachment, differentiation, neurite outgrowth, and migration [193]. The following table highlights the research and results conducted in the last 12 years, limited to neural stem cells and their behavior in 3D cultures. This table reveals a paucity of data quantifying each scaffold’s physical and mechanical properties as well as specific lineage commitment and neurite outgrowth, while most studies have provided qualitative observations. By quantifying NSC’s ability to differentiate into several neural subtypes along with neurite outgrowth, and relating this behavior to the mechanical and physical properties of altered

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Properties</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>+Highly biocompatible</td>
<td>-Highly hydrophilic</td>
</tr>
<tr>
<td></td>
<td>+Injectable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+Non-immunogenic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+Chemically modifiable</td>
<td></td>
</tr>
<tr>
<td>Matrigel</td>
<td>+Injectable</td>
<td>-Immunogenic</td>
</tr>
<tr>
<td></td>
<td>+Highly biocompatible</td>
<td>-Unknown exact composition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-From mouse tumor cells</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>+Injectable</td>
<td>-Limited protein absorption</td>
</tr>
<tr>
<td>Peptide Hydrogel</td>
<td>+Injectable</td>
<td>-Low mechanical properties</td>
</tr>
<tr>
<td></td>
<td>+Highly biocompatible</td>
<td></td>
</tr>
</tbody>
</table>
concentrations of biological polymers, the work proposed here will broaden the current understanding of neural engineering by going beyond the studies discussed in Table 4 which have only broadly investigated NSC viability/single neural subtype commitment.

**Table 4 Scaffolds used in other studies.** 3D scaffold environments direct NSC neurite outgrowth, differentiation and survival.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Cell type</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate, various concentrations</td>
<td>Rat NSCs from hippocampus</td>
<td>Rate of proliferation was hindered with higher scaffold moduli. TUJ1 expression is greatest with softer hydrogels (180-20,000 Pa)</td>
<td>[194]</td>
</tr>
<tr>
<td>Collagen-(1)</td>
<td>Rat cortical NSCs cortical harvested from subcortical epithelium</td>
<td>Patch clamp electrical signal analysis revealed an appearance of both GABAergic and glutamatergic synaptic activities. Approximately 40% of cells expressed TUJ1 marker</td>
<td>[195-197]</td>
</tr>
<tr>
<td>Collagen(1)-HA crosslinked blended scaffolds</td>
<td>Rat NSCs</td>
<td>Cells attached to the culture surface, and neurite extensions were observed. MAP2 expression was much higher in the HA–Coll samples than in the HA scaffolds. There was little expression of MAP2 by cells in the collagen scaffolds. TUJ1 was found in all the groups.</td>
<td>[198]</td>
</tr>
<tr>
<td>Collagen (1) sponges immobilized with bFGF</td>
<td>Rat NSC</td>
<td>Increased proliferation of NSCs in collagen sponges incorporating engineered bFGF than in those with native bFGF or control after 7 days in culture. Mechanical properties of sponge better mimic CSN ECM then collagen alone</td>
<td>[199]</td>
</tr>
<tr>
<td>Collagen (1) supplemented with sonic hedgehog</td>
<td>Mouse dorsal midbrain NSC</td>
<td>Endogenous Shh regulates NSC proliferation.</td>
<td>[200]</td>
</tr>
<tr>
<td>Collagen (1) electrospun nanofibers using acetic acid</td>
<td>Mouse NSCs</td>
<td>Increase in cell viability, but not significantly higher then tissue culture plastic alone, cells had increased projections and were hypothesized to be differentiated at day 7 but no quantitative supporting data.</td>
<td>[201]</td>
</tr>
<tr>
<td>Blended gels made from fibronectin, laminin type 1 collagen and HA.</td>
<td>Rat Dorsal root ganglia</td>
<td>Increased laminin concentrations affected axonal outgrowth. Fibronectin inhibited outgrowth. Collagen and HA concentrations didn’t effect neuronal extensions. It was hypothesized the effects of all four blended components were not syngestic.</td>
<td>[202]</td>
</tr>
<tr>
<td>HA, high</td>
<td>Mouse NSCs</td>
<td>Gels with a moduli higher then 5.1 kPa</td>
<td>[203]</td>
</tr>
<tr>
<td>Group Description</td>
<td>Sample Source</td>
<td>Observation</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------</td>
<td>---------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Molecular weight photocross-linked with methacrylate</td>
<td>From ventral midbrain</td>
<td>Were unable to support NSC colulture for periods greater than 21 days. NSC expressed the TUJ1 marker in the 3.0 kPa hydrogels</td>
<td></td>
</tr>
<tr>
<td>HA, with BDNF</td>
<td>Rat fetus stratum</td>
<td>Promoted survival of NSCs</td>
<td>[204]</td>
</tr>
<tr>
<td>HA and methyl cellulose blends</td>
<td>Rat NSC from subventricular zone</td>
<td>Increased oligodendrocyte and neural lineage commitment and cell survival</td>
<td>[205]</td>
</tr>
<tr>
<td>HA, thiol-modified sodium and gelatin</td>
<td>Mouse NSCs stably expressing LacZ</td>
<td>Cell and scaffolds were injected mouse spinal cord grey matter whereas a neuroprotective effect was observed by the scaffold, however two weeks post injection a mild inflammatory response was observed</td>
<td>[206]</td>
</tr>
<tr>
<td>HA binded to BDNF and VEGF microspheres</td>
<td>Rat forebrain NSCs</td>
<td>Increased neurite length and cell survival in scaffolds with the growth factor compared to control</td>
<td>[207]</td>
</tr>
<tr>
<td>HA and Poly-L-lysine blended scaffolds</td>
<td>Rat cortical NSCs</td>
<td>Cell survival was increased and differentiate was observed into astrocyte and pre-neural lineages. Increased attachment and axonal outgrowth on scaffolds contacting poly-L-lysine</td>
<td>[208, 209]</td>
</tr>
<tr>
<td>HA crosslinked (ADH) with Nogo receptor antibody or poly-L-lysine</td>
<td>Rat Forebrain cortical neuroepithelium NSCs</td>
<td>Fewer TUJ1 and antineurofilament antibody-positive cells were found on HA-PLL hydrogel compared with HA or HA NgR-Ab hydrogels. PLL-modified HA hydrogels reduce differentiation of NSCs. NgR-Ab modification had no such effect.</td>
<td>[210]</td>
</tr>
<tr>
<td>Matrigel, various concentrations</td>
<td>Human NSCs</td>
<td>Cell survival in 10% Matrigel was significantly reduced vs. controls whereas cells in 50% Matrigel had increased survival. Cells primarily differentiated into astrocyte lineages.</td>
<td>[211]</td>
</tr>
<tr>
<td>Peptide hydrogels (tryptophan and proline repeats)</td>
<td>Mouse NSCs</td>
<td>Weaker hydrogels support proliferation, differentiation, and increased axonal outgrowth</td>
<td>[212]</td>
</tr>
</tbody>
</table>
CHAPTER III

INJECTABLE UNCROSSLINKED BIOMIMETIC HYDROGELS AS CANDIDATE SCAFFOLDS FOR NEURAL STEM CELL DELIVERY

3.1 Introduction

A traumatic injury or disease in the CNS initiates an inflammatory microenvironment, which subsequently compromises the survival and functionality of specialized CNS populations. Given the limited ability of the adult mammalian CNS for self-repair and regeneration under these conditions, such cell loss could ultimately result in muscle atrophy, paralysis and even death. Existing pharmaceutical and surgical options for treating various CNS injuries and diseases are limited to slowing down the progression of symptoms, and therefore a majority of patients succumb to these conditions within 3-5 years post-diagnosis [213]. Exogenous cell-based therapies offer promise for the treatment of such neurodegenerative CNS conditions by enabling cell infiltration into host tissue, ameliorating inflammation, and promoting targeted differentiation to replace lost cell populations [67], towards restoration of a healthy microenvironment. However, pluripotent stem cell phenotypes such as embryonic
stem cells are currently limited by ethical and technical concerns related to sourcing, necessity for immunosuppression, risk of tumor formation, and possible chromosomal instability, which hinder their potential clinical applications. In this regard, the utility of autologous NSCs has been gaining attention towards re-establishing and reconnecting lost neural networks [182].

NSCs are self-renewing, multi-potent cells, capable of differentiating into specific neuronal and glial lineages [9], making them an attractive option for cell-based therapies. Within healthy adult CNS, they typically assume a quiescent phenotype, unless stimulated by exogenous signals to proliferate, migrate, or differentiate into a specific lineage [182]. Adult NSCs can be found very specific regions of the brain in small populations, are not restricted to to their developmental origin, and can differentiate into neural and glial subtypes in vivo when exposed to a specific set of environmental cues [184]. Because CNS ECM affects a majority of neuronal development and function [214], it is expected that NSCs could be delivered (via bolus delivery or grafting) in situ for effective treatment and regeneration of diseased or damaged CNS tissues, and awaken their lost functionality. However, the cues released by activated microglial cells in an inflammatory CNS could contribute to further deterioration of the damaged neurons in the CNS, compromise the survival and functionality (e.g., attachment, migration, proliferation, homing into native ECM) of exogenously-delivered NSCs in that region, and promote differentiation of injected NSCs into undesirable lineages [215]. To overcome this, an appropriate carrier vehicle is essential to deliver NSCs at the desired site, shield them from hostile inflammatory
microenvironment in vivo, and enable full functionality of NSCs post-transplantation.

An ideal delivery vehicle should be easily injectable (i.e., have smooth flow with least resistance), broadly mimic host microenvironment composition, possess physical and mechanical characteristics representative of native CNS tissue (to minimize mechanical mismatch), present binding sites for cell attachment, and hold cells for required durations without affecting their survival. Conventional approaches of delivering cell suspensions in saline medium are undesirable because cells will diffuse away from the delivery site, and get exposed to inflammatory microenvironment which compromises their survival and functionality. Cell attachment, survival, and functionality are highly contingent on the choice of substrates which could evoke native integrin-ECM interactions while preserving the native cell phenotype. Numerous substrates have been explored to deliver mammalian NSCs for grafting and transplantation purposes [193, 216, 217]. A majority of these substrates are synthetic in nature and have been crosslinked to achieve scaffold integrity. Other studies have explored the effects of physical and mechanical cues provided by 2D or 3D biological substrates to NSC survival and differentiation, in the presence or absence of signaling cues such as sonic hedgehog and brain-derived neurotrophic factors [194, 203, 218-222].

Since NSC cultures on 2D monolayers or within 3D crosslinked (synthetic or biological) scaffolds do not accurately mimic NSC microenvironment in vivo, the outcomes from such experimental setup might not be truly physiologically-
relevant. Besides, variations in homing patterns of NSCs within these scaffolds and the adverse effects of chemical crosslinking agents and mechanisms (e.g., genipin, divinyl sulfone, carbodiimide, UV light) might also significantly alter NSC phenotype [219, 223]. We hypothesize that uncrosslinked 3D biomimetic hydrogels reconstituted from CNS ECM components might provide a sustainable microenvironment to deliver NSCs in the region of interest, maximize cell survival and proliferation, provide biophysical substrate for cell attachment and differentiation, facilitate geometrical guidance for cell migration and growing neurites, and allow diffusion of growth factors and their gradients to mediate cellular processes. Therefore, in this study, we have developed and characterized the properties of such injectable gels, and investigated both qualitatively and quantitatively, the utility of such uncrosslinked gels in guiding NSC differentiation and functionality.

3.2 Materials and methods

*Preparation of hydrogels:* Three stand-alone gels were prepared with the following components (*Table 5*): 1.2 mg/mL type I collagen (termed C-1.2), or 1% or 2% hyaluronic acid (termed HA 1% and HA 2%, respectively). Sodium hyaluronan gels (HA; 1.2 - 1.8 MDa; Lifecore Biomedical, Chaska, MN) were prepared by dissolving as-received HA powders in NSC media at a concentration of 1% or 2% (w/v), stirred vigorously for 20 min, and stored overnight at 4 °C in sterile 10 mL syringes (BD biosciences). Collagen gels were prepared using type-I rat tail tendon-derived collagen (8.94 mg/mL; BD biosciences, San Jose, CA), diluted with 10× PBS and deionized water to a final concentration of 1.2
mg/mL. The pH of collagen gels was adjusted to ~7.4 using 1 N NaOH. All samples were maintained on ice to prevent gelation prior to cell seeding.

In addition, two different blended gels were prepared (termed, Case 1 & Case 2) with each containing the following components: type I collagen, sodium hyaluronan, laminin (Sigma) and chondroitin sulfate proteoglycan sodium salt (CSPG; Spectrum, Gardena, CA). Each gel contained varied concentrations of both HA and collagen, but all contained equal amounts of laminin and CSPG. Laminin, CSPG, collagen and HA were added to NSC media to result in the gel compositions shown in Table 1. All gels were prepared in 10 mL syringes and maintained on ice to prevent gelation prior to cell seeding. The role of HA concentration in these gels was tested on the basis that each of these scaffolds would have varying densities, porosities and related mechanical structures.

**Table 5 The composition of the scaffolds in this study.** Composition and notation used for various ECM based gels developed in this study

<table>
<thead>
<tr>
<th>Standalone gels</th>
<th>Blended gels</th>
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<tr>
<td>1.2 mg/mL type-1 collagen</td>
<td><strong>Case 1</strong></td>
</tr>
<tr>
<td>1% (w/v) Hyaluronic acid (&gt; 1.2 Mda)</td>
<td>2% (w/v) Hyaluronic acid (&gt; 1.2 Mda)</td>
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**Gel integrity:** Qualitative analysis of the gel integrity was performed after 10 days to ensure the stability of the hydrogel structure. Using a protocol similar to Hopkins et al., 400 µL of each hydrogel sample was pipetted into a 2 mL micro-centrifuge tube and red food dye was added for enhanced visualization [224]. Hydrogels were incubated at 37 °C (5% CO\(_2\) and 95% humidity) for 10 days, equivalent to the duration of time the cell cultures were performed. After 10 days, the tubes were inverted, digital photographs obtained, and compared to those taken before incubation.

**Scanning electron microscopy (SEM) analysis:** The Case 1, Case 2, HA 1% and HA 2% scaffolds were dehydrated (Labconco model number 7751020) over 48 h, samples freeze-fractured via liquid nitrogen immersion for 30 sec, and cut using an Exacto™ knife, exposing the internal structure. Samples were then mounted on carbon tape, sputter-coated (SPI sputter model 13131) with gold for one min (350 angstroms, 40 milliamps, 170 micro pressure), and imaged using a SEM (Amray 1820, IXRF 500 digital processor) at 15 KeV electron source. Images were obtained at multiple magnifications. To prevent the structures from collapsing, pure collagen (C-1.2) scaffold was prepared by immersing in 4% paraformaldehyde (PFA) and subsequently dehydrating using graded-alcohol baths. Such collagen samples were dried in a desiccator (2 h), sputter-coated and imaged as described above.

**Water swelling capacity:** All samples were lyophilized using a freeze drier (Labconco) for 24 h and the dry weight recorded (Ohaus Adventurer; NJ) accurate to 4 decimals. Samples immersed in PBS (5 mL, 5 min) were blotted on
Kimwipe™ tissues and their weight recorded. Previous experiments run with these samples had demonstrated their ability to reach saturation within 4 min, thus the 5 min soaking period was deemed sufficient to analyze their maximum swelling ratio. The percent mass increase due to water absorption was calculated from the expression: 

\[ M_i = \frac{(M_w - M_d) \times 100}{M_d}, \]

where \( M_i \) is the mass percent increase of sample, and \( M_w \) and \( M_d \) represent the wet and dry mass, respectively.

**Gel porosity:** Scaffold porosities were determined using ethanol displacement method as explained previously. Samples were weighed (\( W \)) using a bench-top scale (Ohaus Adventurer) and immersed in a graduated microcentrifuge tube containing a known volume (\( V_1 \)) of ethanol. Immediately upon immersion in ethanol, the volume increase was recorded as \( V_2 \) in microcentrifuge tube. The volume difference (\( V_2 - V_1 \)) typically represents the volume of the scaffold material. After an hour, the ethanol-impregnated scaffolds were removed from the microcentrifuge tubes and the residual ethanol volume was recorded as \( V_3 \), and a final weight of sample was recorded (\( W_f \)). Taking the density of ethanol (0.789 g/cm\(^3\)) into account, the porosity of each scaffold was calculated using the equation: 

\[ \frac{(W_f - W_i)}{(\rho_{ethanol} \times (V_2 - V_3))}. \]

**Rheological characterization of gels:** The viscoelastic nature of these gels was evaluated using a Paar Physica MCR 301 rheometer (Ashland, VA, USA). A 50 mm cone-and-plate geometry was selected to provide an even shear field. The machine was set up to leave a 0.1-mm gap between the cone and the plate, and the angled geometry (\( \delta \)) between the cone and plate was 0.981°. The linear viscoelastic region (LVR) of the gels was determined by performing amplitude
sweeps using the following constraints: 0.01-100% strain, $\omega = 6$ rad/s at 37 °C ($n = 3$). To compare the relative stiffness and viscosity of the gels, frequency sweeps were run on each gel using the following constraints: 5% strain, 0.1-100 mrad, 37 °C ($n = 3$). Temperature was maintained constant (± 0.1°C) using a controlled water bath. Results were plotted as loss and storage modulus (Pa) vs. angular frequency (rad/s), and viscosity (Pa.s) vs. shear rate (s$^{-1}$), to describe the elastic, viscous and shear behaviors of the gels. Gels were further tested for creep and stress-relaxation using the same plate geometry. Creep-compliance was ran at $\tau = 10$ Pa, for 15 min to understand the material response to small deformations. Finally, a stress-relaxation test was run at strain ($\gamma$) of 100%, for 15 min intervals, to evaluate the material response to induced stresses. All data were collected and evaluated using Rheoplus software (Rheoplus/32 V.3.62 Ashland VA, USA).

The methodology used to load the gel on the rheometer was carried out in a manner designed to simulate the mechanical forces experienced by the gel immediately post-injection. Approximately 1 mL of each sample was ejected from a 10 mL syringe on to the top of the peltier plate, and immediately the cone measuring plate was lowered leaving a 0.1 mm gap spacing. The gel was trimmed to the recommended size and allowed to equilibrate for a period of 10 min on the peltier plate which was set to 37 °C, before any tests (loss, storage, viscosity, creep, stress-relaxation) were performed. To avoid gel dehydration, which can be a common problem when using temperature-maintained cone-and-
plate geometries, a hood attachment was placed around the sample to ensure proper humidity for the duration of the tests [226, 227].

**Gel compressive strength:** Gel compressive moduli were obtained using dynamic mechanical analysis (TA instruments; DMA Q-800). Given the low mechanical properties of the gels, a compression clamp was fitted to the DMA as specified by the manufacturer. To load the DMA, samples were first placed on the top of a glass cover slip using a similar methodology described above and incubated (37 °C, 15 min) before placing on the flat lower fixed surface of the clamp. After incubation, samples were trimmed with an Exacto™ knife to a standard size and shape as specified by the manufacturer before testing. Immediately after loading the scaffold and glass coverslip in the DMA, the upper oscillating plate was gently lowered to begin sample testing. Tests were run for a period of 30 min, at 37 °C, with no preload force and a ramp strain of 5% to 100%. Stress and strain curves were generated using Universal Analysis™ software.

**Gel Injectability:** To test the injectability of these gels, a modified protocol described by Tunesi et al [228] was used. Briefly, 1 mL of each gel was loaded into a 10 mL syringe (BD biosciences), fitted with a 26G needle, and placed into a custom-made jig which held the syringe beneath the loading cell of an Instron™ MTS 5655 (Norwood, MA, n = 3/ gel). The jig was constructed by boring a hole into a polycarbonate sheet that could accommodate the circumference of the syringe, and the sheet was attached with four legs which were leveled to ensure that a uniform force would be applied to the plunger (Fig. 2A). The entire jig was designed to fit within the designated space below the
Instron™ load cell. Once the jig was mounted in the Instron™, the load cell was lowered until it came in contact with the plunger of the syringe, and the force value was zeroed. The machine was then set to induce force needed to eject the gel from the Instron™ at a rate of 10 mm/s. The approximate time to eject the entire gel was found to be less than a minute for each scaffold, and the results were reported as displacement vs. force. To validate the procedure, we successfully reproduced the force required to extrude an empty syringe (~ 2 N) as reported by Tunesi et al.

**Murine NSC cultures:** Embryonic mouse brain (E11.5) derived dissociated cells were obtained from NeuraCell (Rensselaer, NY), where they were harvested and characterized using cell sorting techniques to ensure the highest quality. NSC culture media components were purchased from Life technologies (Grand Island, NY) unless otherwise noted. Passaging media was prepared by adding DMEM containing Na-pyruvate with 100× L-glutamate, 100× N₂ supplement, 100× B-27 supplement, and 100× N-acetyl-L-cysteine (Sigma). NSCs were maintained in an undifferentiated state by culturing on poly-L-lysine coated T-25 flasks in the presence of 100× basic fibroblast growth factor (bFGF). Passaging media was changed every 24 h, and the cells were seeded within respective scaffolds when the size of the neural rosettes exceeded 500 µM. All experiments were performed with cells harvested from passages 2-3, and performed in triplicate.

**Neural rosette formation and differentiation:** NSCs were subjected to a 2-stage induction procedure based on techniques optimized by NeuraCell. As-received cells were initially seeded on poly-L-lysine-coated T-25 flasks and
allowed to proliferate for 7 days (37 °C, 5% CO₂) in the presence of fresh bFGF. During this time the cells expanded and formed neural rosettes. Cell density per flask was approximately 2 × 10⁵ cells. At day 7, neural rosettes reached a critical size of approximately 500-700 µM [218]. Cells were gently detached using StemPro Accutase® (Life Technologies, Carlsbad CA), spun down, suspended in media lacking bFGF, and seeded in 96-well plates containing a specified gel. Cells cultured for 10 days (37 °C, 5% CO₂).

Cell viability: Cell viability in each gel was measured using a LIVE/DEAD® viability and cytotoxicity kit (Life Technologies). After a 10-day culture period, cells were stained as per recommended protocol. After a 30 min incubation period with both dyes, cells were imaged in respective color channels (red and green filters) using an AxioVert A1 fluorescent microscope (Carl Zeiss Microimaging, NY) and quantified.

Immunofluorescence imaging: After 10 days of culture, NSC differentiation into neural and glial lineages was quantified using protocols detailed elsewhere [229]. The following primary antibodies were used: rabbit monoclonal anti-beta III tubulin (TUJ1, early developmental state neuron marker, Abcam), rat monoclonal anti-myelin basic protein (MBP, oligodendrocyte marker, Abcam), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, astrocyte marker, Abcam), integrins α6 and β1 (transmembrane receptors, Santa Cruz Bio), and CD44 (cell-surface glycoprotein, Millipore). Cells were washed three times in PBS and incubated with appropriate secondary anti-bodies (Santa Cruz Bio) at room temperature for 2 h. Cells were washed three times (5 min each), and
counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). Cells were imaged with an AxioVert A1 fluorescent microscope under both phase contrast and fluorescent channels, using an Axiocam C1 digital camera and Axiovision data acquisition software. At least 10 images were taken in each well at random locations. From these images, the total number of cells (DAPI signal) and the cell density displaying signal to a specific marker were quantified.

**Neurite outgrowth analysis:** Neurite outgrowth was quantified using Alexa 488 Phalloidin actin staining (Life Technologies), as detailed earlier [229]. After the 10-day incubation period, cells were washed twice with warm PBS and fixed with 4% PFA for 30 min at room temperature. Cells were washed twice with PBS, blocked with 0.1% triton X-100 (30 min), washed twice with PBS, and incubated for 2 h in 1% BSA solution. Phalloidin 488 (diluted 1:20 in PBS) was then directly added to the cells and incubated for 45 min. Cells were then washed three times with PBS and imaged as described above. Images were analyzed via the NeuronJ plugin in NIH ImageJ software to trace and calculate neurite length.

**Effect of RA:** The influence of 1 µM all-trans retinoic acid (RA; Sigma) on NSC differentiation, viability, and neurite outgrowth was quantified using procedures described above and based on our previous studies [229]. RA was added to the cultures after bFGF was removed from the media.

**Statistical analysis:** Three independent tests were performed with n = 6/gel type/test, unless otherwise stated. Data acquired was pooled from all the runs and expressed as mean ± SD. Comparison between groups was performed by
the non-parametric Bonferroni-corrected Mann-Whitney U test. Statistically significant data was marked at $p < 0.05$.

3.3 Results and discussion

Physical characterization of gels

The morphology of the pure and blended scaffolds was obtained from SEM imaging and shown in Fig. 7A. Pure HA scaffolds appear to be porous sheet-like in nature, in contrast to their highly crosslinked counterparts [209, 230]. On the other hand, C-1.2 scaffold is fibrillar in nature, and such dense fibrous appearance of collagen gels was noted by Yamamura et al., who determined the porosity and pore-size to be highly pH-dependent [231]. In comparison, the blended scaffolds appear to be highly-porous with thin strut walls, and had 3D inter-connected structured network similar to the cross-linked laminin-HA structure reported by Hou et al [232].

Results from porosity measurements (Fig. 7B) showed ~38% porosity for C-1.2 gels, while uncrosslinked 1% HA gels had the highest porosity of all the scaffolds tested (~75%; $p < 0.05$ for 1% HA vs. other gels), similar to that of native brain tissue (~76%) [233]. The porosities of the remaining gels ranged between 38-75%. In contrast, crosslinked type-1 collagen scaffolds have been reported with as high as 80% porosity [234]. Given the small variability between the two blended scaffolds, it appears that HA concentration does not significant effect overall scaffold porosity.
In agreement with the literature that HA is highly hydrophilic, our results show that pure HA scaffolds gained 8 to 9-fold in weight due to water absorption, the highest among the scaffolds tested (Fig. 7C). Previous studies reported the ability of HA to uptake water within the range of 8 to 60-fold [233], depending on the concentration, molecular weight, and the presence and type of crosslinker. Our results suggest that HA concentration does not significantly affect its ability to absorb water. Uncrosslinked type-1 collagen gels of similar concentration absorbed around 3.7-fold of water by weight [235], similar to that noted in this study. The blended cases appear to have the same affinity for water uptake, each showing 6 to 8-fold increase in swelling ratio with no significant differences among the two cases. Thus, all the tested gels appear to possess the ability to adequately allow for the exchange of liquids within the surrounding environment. A visual examination of all scaffolds post 10-day incubation period confirmed their ability to maintain structural integrity with insignificant gel degradation (Fig. 7D).
Figure 7 Physical properties of scaffolds. (A) SEM photomicrographs of the scaffolds fabricated in this study, (B) the porosity of gels as measured by ethanol displacement (n=3 ± S.D.), (C) the swelling ratio measured via scaffold submersion in PBS for a period of 5 m (n=3 ± S.D.). and, (D) digital photographs of the dyed scaffolds after a 10 day incubation period demonstrating their structural integrity.

Gel injectability

One crucial clinical application of these hydrogels is their ability to be precisely injected into brain or spinal cord for cell transplantation purposes. Seamless injection of these gels minimizes damage and reduces inflammation to the surrounding tissue, which could aid in the preservation of the remaining healthy neural tissue. Our results suggest that the Case 1 gels required the highest force (60 N) to extrude all material (p < 0.01 for Case 1 vs. all other gels), whereas C-1.2 and PBS required the least amount of force (~ 2.2 N, Fig. 8B). Although gels made of 2% HA (Case 1 and pure HA 2%) had similar porosity levels, Case 1 gels required significantly higher force to squeeze the gel from nozzle possibly
due to interactions between collagen fibers and high molecular weight long chain HA. Oliveria et al. performed similar studies using microsphere/alginate gels which required significantly higher extrusion forces than the ones investigated in this study [236]. However they noted similar force requirements to extrude an empty syringe, as well as similar values when displacement rate was varied, confirming that displacement rate does not significantly affect required extrusion force. Another noteworthy aspect is that the force values in our study are below 100 N, which apparently was the maximum acceptable force required to inject a product manually in a clinical setting [237].
Figure 8 Injectability of scaffolds. (A) A schematic and (B) digital photograph of the fabricated jig used to hold syringes containing scaffolds where were compressed in an Instron™. Outputs displaced (C) were plotted as force vs. distance of the plunger (n=3).

**Mechanical analysis**

NSC lineage commitment is strongly influenced by the mechanical forces exerted by the cellular microenvironment [13, 194]. Specifically, softer substrates appear to elicit a stronger pre-neural lineage commitment, while stiffer moduli (< 1 kPa) typically elicit astrocyte lineage commitment. One goal of our study was to design injectable gels which possess moduli within the range of native brain tissue (0.1–
1 kPa) [192]. Given the viscoelastic nature of these gels, a wide range of moduli has been reported for HA or collagen gels, using different measurement tools and quantification techniques (Table 6). For instance, uncrosslinked 2 mg/mL collagen gel has a modulus of 890 Pa at a low strain rate weight, whereas the moduli increased 3-fold if this strain is decreased by a factor of a thousand [238]. Given the dynamic nature of the elastic and viscous components of these gels, we used both DMA and rheological analysis to investigate loss modulus, storage modulus, viscosity, and compressive modulus.

Table 6 Mechanical modulus of selected scaffolds. Modulus of 3D biological scaffolds tested to culture NSCs. Data collected from literature was compared to outcomes from this study.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Modulus</th>
<th>Test</th>
<th>Ref</th>
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<tbody>
<tr>
<td>Native rat brain tissue</td>
<td>500 Pa</td>
<td>AFM</td>
<td>[33]</td>
</tr>
<tr>
<td>1%, 2% (w/v) HA, 1.2 mg/mL type-1 Collagen, Case 1, and Case 2, respectively</td>
<td>See figure 9A</td>
<td>DMA (compression) Rheometer</td>
<td>Current study</td>
</tr>
<tr>
<td>Sodium alginate (MW 200–300 kDa), CaCl₂ added to change modulus</td>
<td>100, 1000, 10000 Pa</td>
<td>Rheometer</td>
<td>[194]</td>
</tr>
<tr>
<td>Blended ratios of HA and collagen I, II crosslinked with carbodiimide</td>
<td>67-315 Pa</td>
<td>Custom-fabricated device</td>
<td>[233]</td>
</tr>
<tr>
<td>HA (MW 1500 kDa, 1% w/v) crosslinked with methacrylic-anhydride</td>
<td>1000-8000 Pa</td>
<td>AFM</td>
<td>[203]</td>
</tr>
<tr>
<td>Collagen-1 and HA (1000 kDa)</td>
<td>110 Pa</td>
<td>Rheometer</td>
<td>[222]</td>
</tr>
<tr>
<td>Peptide gel with and without laminin motif</td>
<td>180 Pa</td>
<td>AFM</td>
<td>[239]</td>
</tr>
<tr>
<td>Photopolymerizable methacrylamide chitosan with laminin, (pseudo 3D test)</td>
<td>&lt; 1000 Pa, 3500 Pa, 7000 Pa</td>
<td>Tested in uniaxial, unconfined compression using a Mach-1 micromechanical testing system</td>
<td>[193]</td>
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Compressive loading on a modified DMA was used to investigate the compressive modulus of gels (Fig. 9A). Table 2 compares our results from this study to that reported in literature for similar gels. As expected, Case 1 (containing 2% HA) had the highest gel modulus ranging between 350-500 Pa, while Case 2 containing 1% HA had a significantly lower modulus (185-245 Pa; \( p < 0.01 \) for Case 2 vs. Case 1). Pure HA 1% gels had the lowest modulus at ~150 Pa of all the gels tested (\( p < 0.05 \) for 1% HA vs. other gels), which probably was due to the high porosity of that gel. These values are significantly higher than those observed for the same 1% and 2% HA, as tested by an atomic force microscope (AFM) [229]. It should be noted the HA scaffolds tested here were derived from hyaluronic acid sodium salt with an order of magnitude larger molecular weight, which is likely contributing to higher mechanical properties. C-1.2 gels recorded compressive modulus of 310 ± 55 Pa in this study, which is in the expected range based our previous studies on 2 mg/mL collagen gels (511 ± 142 Pa; pH ~ 7.4; tested with AFM) [229].

To further quantify the viscoelastic behavior of these gels, oscillatory rheological characterization was performed. The amplitude and frequency of the applied strain was varied to study a wide range of time scales and behaviors. Experiments were performed at small amplitudes within the linear viscoelastic regimes to obtain gel response without destroying the gel structure. Storage modulus was relatively linear for all scaffolds at the \( 1 \leq \omega \leq 100 \) rad/s range (Fig. 9C), but varied widely for \( \omega < 1 \) rad/s. Given that storage modulus can be used as an estimation of gel modulus [13], it is interesting to note that 1% HA recorded
the lowest modulus and Case 1 the highest modulus, and the other three gels displaying intermediate storage moduli values. This trend was strikingly similar to the pattern observed from compressive modulus analysis by DMA (Fig. 3A). Previous studies on rheological characterization of collagen-HA scaffolds reported storage moduli in similar ranges [240].

Loss modulus and viscosity were reported as a function of angular frequency and shear rate respectively. The loss modulus follows the same linear trend as the storage modulus for $\omega \geq 1$ rad/s (Fig. 9D). Blended gels had loss moduli values similar to that of storage moduli (180-220 Pa), whereas the standalone scaffolds (except 2% HA) exhibited lower loss modulus values. Results suggest a synergistic effect of blending HA and collagen in increasing the loss moduli value. Viscosity measurements indicate that all the gels tested in this study are exhibiting shear-thinning behavior, i.e., viscosity decreases as shear rate increases (Fig. 9B). Similar rheological characterization studies of pure collagen [226] or HA solutions [227] support the data we observed in this study. Interestingly, blending collagen and HA did not affect the shear-thinning behavior of these gels. Given the differences in the HA viscosity curves, it is likely the concentration and not the molecular weight of the HA is governing the shear rate [241]. It should be noted that the storage and loss modulus can crudely estimate compressive modulus (Fig. 9A) using the following equation

$$E = \sqrt{(G' + G")^2}.$$  

A comparison of the two experimental techniques demonstrate in some cases the error between the two techniques is as little as 13% (HA 2%) and as high as 80% (Col 2.0). We hypothesize some error could
be due to the uncertainty of the poisson’s ratio of the materials, the averaging of data ranges contained in the loss and storage moduli, or inherent instrumentation error given the low moduli of these materials.

Stress relaxation was reported as a function of time over a period of five minutes (Fig. 9F). Stress relaxation describes how these biological polymers relieve stress under a constant strain (100%). Although tests were run for a total of 15 min, no changes in the material behavior were observed beyond the first 5 min, and hence the curves were truncated. In general, all gels exhibited a nonlinear response that is expected of most viscoelastic polymers, as well as the native brain ECM [242]. Pure HA scaffolds required longer durations (~ 120 sec) to fully relax, whereas pure collagen and blended scaffolds relaxed in ~ 40 sec, indicating the presence of collagen adds additional shear strength to the materials. Our results are in excellent agreement with that reported by Zahalak et al., which demonstrated fast relaxation time for 1 mg/mL collagen gels seeded with fibroblasts and strained at 20% [243]. The relaxation times of the blended scaffolds resemble that of the native brain ECM, which had shown increased initial stress, and then an expedited relaxation period (> 10 sec) [244].

Similar to stress-relaxation, creep compliance ($J$, Pa$^{-1}$) reported as function of time (Fig. 9E) demonstrates the tendency of the biological polymers to deform under the influence of a low constant mechanical stress (10 Pa). The blended scaffolds were most resistant to deformation, whereas pure collagen was highly susceptible to the force applied, and pure HA scaffolds exhibited intermediate values. These results are in agreement with similar studies
conducted on vitreous (a naturally delivered HA-collagen ECM) gels, which demonstrated a sharp increase in compliance within the first 10 sec followed by a plateau [245]. In conclusion, the gels developed here exhibited predictable viscoelastic characteristics, which are a product of the loose polymerization of the collagen, HA, or both.
Figure 9 Mechanical properties of scaffolds. (A) Young’s modulus of compressed gels as extrapolated from the curves produced from dynamic mechanical analysis. The elastic, viscous and shear behaviors of the gels are shown as (B) viscosity (Pa.s) vs. shear rate (s⁻¹), (C) storage modulus (Pa) vs. angular frequency (rad/s), (D) loss modulus (Pa) vs. angular frequency (rad/s), (E) Creep-compliance (Pa⁻¹) vs. time (s) and, (F) Stress relaxation (Pa) vs. time (s). For all samples n=3, S.D omitted for graphical clarity.
**Growth rate and doubling time of neural rosettes**

NSC stemness during neural rosette formation was maintained by daily evaluation of the size and doubling rate of the cultures (Fig. 10). Representative images (Fig. 10A) suggest that neural rosettes almost doubled in size every 24 h, and expanded from ~17 µM to ~660 µM over seven days (Fig. 10B) with an exponential growth rate, in agreement with previous studies [222]. Culture periods greater than 7 days could result in overlap of individual neural rosettes and the potential loss of the cell stemness due to confluent flask conditions. From the data shown in Fig. 10B, assuming that the growth phase mimics a first-order chemical reaction analogous to that in bacteria, we calculated the specific growth rate $k$, given as $\left[\frac{\ln N_2}{N_1}\right]/(t_2 - t_1)$, where $N_1$ and $N_2$ are neural rosette sizes at times $t_1$ and $t_2$, respectively. Similarly, the doubling time $g$ of the neural rosettes in 2D cultures within that specific non-differentiating medium was calculated using, $g = \ln(2)/k$, where $k$ is the specific growth rate calculated earlier. From our results, the values of $k$ and $g$ were calculated to be 0.025 h$^{-1}$ and 27.48 h, respectively, which were significantly higher than that noted in human cell lines [246].
Figure 10 Doubling rate of NSCs. (A) Bright field images of NSCs cultured on 2D tissue culture polystyrene in expansion media containing bFGF at various time points demonstrating their expansion and, (B) growth rate of the Neural rosette size (µm) vs. time (days). Scale bar: 200 µm (n=3, error not shown).
**Role of 3D ECM-based gels on NSCs**

Immunofluorescence images of NSC differentiation within 3D gels revealed unique patterns ([Fig. 11A](#)). NSCs agglomerated in HA based gels forming clusters of 5-10 cells with significant cellular extensions (most notable in pure HA 1% gels), which was not seen in C-1.2 gels. Since NSCs do not proliferate within a 3D milieu in the absence of bFGF [218], we hypothesize that the expansion of NSC clusters in HA-based gels is not a product of cellular proliferation but rather clustering arising from cell migration. NSC differentiation patterns within 3D scaffolds appeared dependent on gel composition ([Fig. 11B](#)). Among the three pure gels tested, HA gels promoted the highest pre-neural differentiation ($p < 0.005$ vs. C-1.2; $p > 0.7$ for 1% HA vs. 2% HA). Surprisingly, Case 1 gels containing 2% HA promoted significantly higher pre-neural differentiation than that seen in Case 2 gels ($p < 0.05$ for Case 1 vs. Case 2; $p < 0.001$ for Case 1 vs. C-1.2; $p = 0.04$ for Case 2 vs. C-1.2). **Table 8** compares NSC differentiation patterns noted in this study to that in literature. It could be seen that the fraction of pre-neural or glial lineages formed highly depends on the type of the 3D gels and the harvest location of NSCs.

Interestingly, NSC differentiation levels into the three lineages remained similar within pure collagen (C-1.2) and Case 2 gels. On the other hand, astrocyte and oligodendrocyte lineage commitment in pure HA and Case 1 gels was significantly lower compared to pre-neural commitment. Astrocyte
Differentiation was the lowest in Case 1 among all gels tested ($p < 0.05$ for Case 1 vs. all other gels). Oligodendrocyte lineage commitment in pure HA and Case 1 gels was statistically similar, and significantly lower than that noted in Case 2 gels. The data presented here suggests that HA in some form plays a key role in neural commitment, based on its role as a major extracellular component during fetal development [203, 232]. Lastly, since Case 2 gels induced more oligodendrocyte formation than pure HA gels, they would be highly beneficial for applications in multiple sclerosis and leukodystrophies research.

Live/Dead™ assay revealed significant differences in cell survival after a 10-day culture period (Fig. 11C). Although C-1.2 and 1% HA gels supported $>75\%$ cell survival ($p < 0.01$ vs. other gels), $\sim 45-55\%$ cell survival was noted in other three gels. This result is in line with that observed by Wang et al. using HA scaffolds loaded with BDNF and VEGF over a 48 h culture period [247]. Table 7 compares cell survival data from various relevant studies in literature. We hypothesize that highly dense and viscous microenvironments induce morphological and survival changes within NSCs by limiting their ability to spread out and adhere to surfaces [248]. In general, 3D gels with a lower concentration of high molecular weight HA contribute to high NSC viability with potential in tissue engineering applications [203].

It is crucial that fabricated scaffolds enhance neuronal extensions and branching which ultimately can aid in restoring lost axonal connections. We observed branching of neurites within pure HA gels (Fig. 11D). While the average neurite length within 1% HA gels was significantly higher than that
observed within all other gels \((p < 0.001)\), C-1.2 gels had the lowest neurite outgrowth of all the gels \((p < 0.001)\). While limited data exists on NSC branching in 3D microenvironments, Wong et al. reported average neurite outgrowth on 2D surfaces as \(~ 48 \mu m\) [249]. We hypothesize that the presence of CSPGs could be contributing to the stifled neurite outgrowth within blended gels, as CSPG was shown to either promote or hinder neurite outgrowth depending on its concentration and/or exposure pattern during development [250]. Furthermore, the data from the stress relaxation tests suggest that the softer nature of HA gels could be creating a microenvironment which accommodates neurite extension (Fig. 11D). In summary, results point to the benefits of pure HA scaffolds over pure type-1 collagen or blended scaffolds in stimulating NSC differentiation to pre-neural lineages, neurite outgrowth, and cell survival.
Figure 11 Differentiation, viability, and neurite outgrowth of NSCs. (A) Representative immunofluorescence images of NSC cultures at day 10, differentiating into neural (TUJ1 staining) and glial (GFAP or MBP staining) lineages cultured within various 3D scaffolds. Cultures were counterstained with DAPI for cell identification. Scale bar: 50 µm. Blue staining indicates nuclei, green staining indicates immature neurons (TUJ1), yellow staining indicates astrocyte (GFAP), and pink staining indicates oligodendrocyte (MBP). (B) Quantification of NSCs for each lineage was performed on day 10 and is percentage of the total cells divided by the number cells stained by the respective marker. (C) The viability of the cells via LIVE/DEAD™ staining and, (D) The average length of the neurites extended by NSCs in each 3D scaffold measured via NeuronJ (n=3 for all cases and tests ± SEM).
Table 7: Viability of NSCs in selected scaffolds. NSC viability in various 3D biopolymeric substrates observed in this study was compared to data collected from literature.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Time (days)</th>
<th>Viability</th>
<th>Test</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 mg/mL type I collagen, 1% HA, 2% HA (type I collagen) and type I collagen</td>
<td>10</td>
<td>81%, 78%, 55%, 60%, 50%, respectively</td>
<td>Live/Dead™</td>
<td>[21]</td>
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<tr>
<td>0.3 - 3.0 mg/ml type I collagen</td>
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<td>99% alive after 14 days</td>
<td>Live/Dead™</td>
<td>[195]</td>
</tr>
<tr>
<td>Sodium alginate (MW 200-300 kDa), Pluronic F127 (PF127), Matrigel and PuraMatrix</td>
<td>7</td>
<td>Substantial cell death in stiffer gels</td>
<td>Observation</td>
<td>[203]</td>
</tr>
<tr>
<td>HA and methylcellulose blend</td>
<td>7</td>
<td>60% survival in both adult and embryonic NSCs</td>
<td>TUNEL assay</td>
<td>[205]</td>
</tr>
<tr>
<td>HA (1000 kDa) and type I collagen</td>
<td>11</td>
<td>60% viability in HA alone, 75% viable in modified scaffolds</td>
<td>MTT assay</td>
<td>[220]</td>
</tr>
<tr>
<td>HA and methylcellulose blend</td>
<td>7</td>
<td>60% (in vitro)</td>
<td>Live/Dead™</td>
<td>[221]</td>
</tr>
<tr>
<td>HA and methylcellulose blend</td>
<td>7</td>
<td>70% survival in scaffolds containing laminin, compared to 50% without laminin</td>
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<td>[252]</td>
</tr>
<tr>
<td>1% HA (MW 1500 kDa)</td>
<td>7</td>
<td>30% viability in HA alone, 75% viable in modified scaffolds with ADH</td>
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<td>[222]</td>
</tr>
<tr>
<td>Methylcellulose modified with laminin, compared to 50% without laminin</td>
<td>7</td>
<td>90% survival in both adult and embryonic NSCs</td>
<td>MTT assay</td>
<td>[221]</td>
</tr>
<tr>
<td>HA hydrogels (1% w/v) crosslinked with ADH</td>
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<td>90% survival in both adult and embryonic NSCs</td>
<td>TUNEL assay</td>
<td>[221]</td>
</tr>
<tr>
<td>HA and methylcellulose blend</td>
<td>7</td>
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<td>Substantial cell death in stiffer gels</td>
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<td>[221]</td>
</tr>
<tr>
<td>3 mg/ml type I collagen</td>
<td>14</td>
<td>95% at all concentrations of PuraMatrix</td>
<td>Live/Dead™</td>
<td>[253]</td>
</tr>
<tr>
<td>Sodium alginate (MW 200-300 kDa), Pluronic F127 (PF127), Matrigel and PuraMatrix</td>
<td>7</td>
<td>Substantial cell death in stiffer gels</td>
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<tr>
<td>3 mg/ml type I collagen</td>
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<td>95% at all concentrations of PuraMatrix</td>
<td>Live/Dead™</td>
<td>[253]</td>
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<td>14</td>
<td>90%</td>
<td>Live/Dead™</td>
<td>[221]</td>
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</table>

Data collected from literature.
Table 8 Differentiation patterns no RA

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<th>Ref</th>
<th>Scaffold</th>
<th>Time (days)</th>
<th>Harvest Location</th>
<th>Pattern</th>
<th>Ref</th>
<th>Time (days)</th>
<th>Harvest Location</th>
<th>Pattern</th>
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<td>Collagen, 1% HA, 2% HA, Case 1, and Case 2</td>
<td>10</td>
<td>Embryonic mouse,</td>
<td>NCS remained in aggregates with both PNS and MNS; astrocyte: 30%, oligodendrocyte: 25%, pre-neuronal: 45%, 1.2 mg/mL</td>
<td>[205]</td>
<td>7</td>
<td>Adult Rat SVZ</td>
<td>Oligodendrocyte: 43.3%, astrocyte: 22%, pre-neuronal: 17%</td>
</tr>
<tr>
<td>[222]</td>
<td>HA and methylcellulose blend</td>
<td>14</td>
<td>Embryonic mouse,</td>
<td>Oligodendrocyte: 40%, astrocyte: 20%, pre-neuronal: 20%, embryonic cell type: pre-neural: 45%</td>
<td>[245]</td>
<td>7</td>
<td>Mouse ventral</td>
<td>S/V: E13.5 Midbrain, E13.5 Midbrain, E13.5 Midbrain,</td>
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<tr>
<td>[195]</td>
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<td>Unspecified</td>
<td>Pre-neuronal reduced expression of culture becomes apparent</td>
<td>[224]</td>
<td>7</td>
<td>Rat location</td>
<td>Pre-neuronal: 25%, astrocyte: 55%, oligodendrocyte: 20%</td>
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<tr>
<td>[207]</td>
<td>HA (1000 KDa)</td>
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<td>Oligodendrocyte: 43.3%, astrocyte: 22%, pre-neuronal: 17%</td>
<td>Current study</td>
<td>[251]</td>
<td>7</td>
<td>Embryonic mouse,</td>
<td>Oligodendrocyte: 28%, astrocyte: 17%, pre-neuronal: 51%,</td>
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<tr>
<td>[203]</td>
<td>Collagen, 1% HA, 2% HA, Case 1, and Case 2</td>
<td>10</td>
<td>Embryonic mouse,</td>
<td>NCS remained in aggregates with both PNS and MNS; astrocyte: 30%, oligodendrocyte: 25%, pre-neuronal: 45%, 1.2 mg/mL</td>
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<td>7</td>
<td>Embryonic mouse,</td>
<td>Oligodendrocyte: 28%, astrocyte: 17%, pre-neuronal: 51%,</td>
</tr>
</tbody>
</table>

Table 8 Differentiation patterns no RA: Differentiation patterns of NSCs within various gels in the absence of RA (data collected from literature).
**Cell surface-receptor mediated interactions**

In the current study, while cells cultured within C-1.2 scaffolds exhibited faint immunofluorescence labeling (Fig. 12) for all the three surface-receptor markers tested, positive staining in pure HA and blended scaffolds attest to the strong interactions between these scaffolds and cells via these receptors. Selection of ECM constituents for the scaffolds used in this study was based on the ability of HA and collagen to interact with NSCs via various surface-receptors. Besides, these components are found in the native CNS ECM and play strong roles in cellular differentiation, migration and adhesion. HA is present in both the adult CNS and during embryogenesis, and binds to several large GAGs, proteoglycans, and cell surface-receptors via specific HA-protein interactions. Collagens and CSPGs regulate axonal outgrowth via adhesive and anti-adhesive binding domains recognized by different cell receptors, while laminin interacts with α and β integrins on the neuronal surface to influence differentiation and migration [255, 256]. It has been documented that CD44 is expressed in undifferentiated neural progenitor cells at embryonic stages [257], which allows the cells to readily bind to HA via the Rho/Rac pathways [258].
Figure 12 Integrin staining of NSCs. (A) Representative immunofluorescence images of NSC cultures in 3D scaffolds at day 10, expressing integrins (β1, α6, and CD44). Cultures were counterstained with DAPI for cell identification and green staining represents subsequent integrin staining. Scale bar: 50 µm (n=3 for all cases)
Effect of RA on cell survival and differentiation

Inducing NSC differentiation by exposure to RA has been investigated because of its ability to signal stem cells during both development and maturation to form neuronal and glial lineages [121, 124]. Compared to untreated groups, no significant effect of RA on cell viability was noted in all scaffolds tested (Fig. 13B), in line with the observations by Takahashi et al [121]. However, there were significant differences in the NSC differentiation patterns, when RA was added to the culture media (Fig. 13A). Qualitatively, NSCs still formed clusters within HA scaffolds as noted within cultures not exposed to RA (Fig. 5A). However, except within HA-2% scaffolds, a significant increase (1.3 to 2.2-fold) in astrocyte (GFAP) commitment was noted in all scaffolds in the presence of RA. Although pre-neuronal lineage commitment within pure HA and blended scaffolds remained at similar levels to that within non-RA controls, a 2.3-fold increase in TUJ1-positive cells were noted within C-1.2 scaffolds in the presence of RA ($p < 0.01$ for RA-treated vs. non-treated cultures in C-1.2 gels). Addition of RA significantly suppressed oligodendrocyte formation in C-1.2 and Case-1 scaffolds compared to their non-treated counterparts. Oligodendrocyte formation in Case 2 scaffolds in RA presence were significantly higher among all the scaffolds tested (~42%). Taken together, results suggest that (i) RA promoted formation of neurons and astrocytes at the expense of oligodendrocytes within C-1.2 scaffolds, (ii) addition of RA has no effect on lineages formation in pure HA scaffolds, irrespective of HA composition, and (iii) significantly higher astrocytes formed in blended scaffolds compared to their control counterparts. This suggests the
possibility that the combination of RA and type-1 collagen is synergistically signaling the NSCs lineage commitment patterns. For comparison, Table 9 shows the summary of literature on RA-induced differentiation of NSCs within various biological scaffolds.

In pure collagen and HA scaffolds, addition of RA did not significantly increase neurite outgrowth in any of the cases compared to their no-RA counterparts (Fig. 13C). However, in the two blended scaffolds, RA exposure significantly increased (almost doubled) neurite outgrowth relative to that noted in no-RA cultures ($p < 0.05$). Given the presence of CSPGs in these blended scaffolds which could potentially stifle neurite outgrowth, the addition of RA helped overcome that inhibition to encourage neurite outgrowth, which holds promise as a potential therapeutic agent in restoring damaged neuronal tracts within an inflammatory environment. Further research could provide better insights into the concentration gradients of RA required to enhance neurite branching and growth.
Figure 13 Differentiation viability and neurite outgrowth of NSCs with RA. The differentiation, neurite outgrowth and survival of NSCs within 3D scaffolds cultured in the presence of retinoic acid (RA). (A) Quantification of cell lineage performed on day 10 is expressed as the percentage of the total cells divided by the number cells stained by the respective marker. (C) The viability of the cells via LIVE/DEAD™ staining and, (D) the average length of the neurites extended by the NSCs in each 3D scaffold measured via NeuronJ (n=3 for all cases and tests ± SEM).
**Table 9 Differentiation patterns with RA.** A summary of the recent studies on NSC differentiation in the presence of RA.

<table>
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<tr>
<th>Scaffold</th>
<th>Time (days)</th>
<th>Harvest location</th>
<th>Pattern</th>
<th>Ref</th>
</tr>
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<tbody>
<tr>
<td>1.2 mg/mL Collagen, 1%, 2% (w/v) HA, and blended scaffolds</td>
<td>10</td>
<td>Mouse, embryonic E 11.5</td>
<td>Pre-neural: 55%,55%,60%49%,32% Astrocyte: 55%,30%,22%,39%,40% Oligodendrocyte: 10%, 19%, 22%, 16%,45%</td>
<td>Current study</td>
</tr>
<tr>
<td>Blended ratios of HA and Collagen types I and II, crosslinked with carbodiimide</td>
<td>14</td>
<td>Rat, location unspecified</td>
<td>Pre-neural: observed in all scaffold types (percentages not reported)</td>
<td>[198]</td>
</tr>
<tr>
<td>HA hydrogels (1% w/v) crosslinked with ADH</td>
<td>3</td>
<td>Rat, fetal forebrain</td>
<td>Pre-neural: 35-50% Astrocytes: 5-20%</td>
<td>[210]</td>
</tr>
<tr>
<td>Peptide gel with and within laminin motif</td>
<td>14</td>
<td>Rat, location unspecified</td>
<td>Pre-neural, astrocyte, and oligodendrocyte qualitatively observed in scaffold</td>
<td>[239]</td>
</tr>
<tr>
<td>poly-l-ornithine chamber slides</td>
<td>12</td>
<td>Rat, adult hippocampal</td>
<td>Pre-neural: 11% Astrocyte: 1%</td>
<td>[121]</td>
</tr>
<tr>
<td>Direct injection into rat retinal explants</td>
<td>6</td>
<td>Rat, hippocampal</td>
<td>Pre-neural:12% Astrocyte: 12%</td>
<td>[259]</td>
</tr>
</tbody>
</table>

### 3.4 Conclusions of this study

NSC differentiation is controlled by a complex set of cues provided by ECM components and signaling molecules. Previous studies have shown that the phenotype, neural and glial differentiation, and neurite outgrowth patterns of...
murine embryonic stem cells are strongly influenced by the matrix microenvironment. Here, we reported on the development and extensive characterization of CNS ECM-based uncrosslinked soft gels for neural stem cell delivery purposes. The gel integrity, pore characteristics, injectability and mechanical properties of these gels demonstrate their utility for clinical transplantation purposes. Furthermore, we noted that murine NSC differentiation, survival, and neurite outgrowth are regulated by the composition and concentration of ECM proteins within the gel, the physical and mechanical properties of the scaffolds, and the presence or absence of RA. Despite similarities in the architectural and mechanical properties of these uncrosslinked scaffolds to that of the native CNS ECM, minor fluctuations in the gel characteristics appear to induce significant alterations in neuronal/ glial differentiation and survival patterns. The data confirms our hypothesis that such uncrosslinked scaffolds do allow for easy injectability into the host tissue, as well as maintain higher cell viability by eliminating residual chemical crosslinker effects. RA addition appears to rescue the inhibitory effects of CSPGs in the blended scaffolds. The cellular processes seem to have been facilitated by the activation and involvement of cell surface integrins and CD44 receptors. Results from chapter 3 point to the utility of these CNS ECM mimicking gels for NSC delivery purposes and in evaluating similar tissue engineered and regenerative medicine therapies.
CHAPTER IV

ACTIVATED MICROGLIA DIFFERENTIALLY REGULATES NEURAL STEM CELL PHENOTYPE AND GENOTYPE WITHIN COCULTURES

4.1 Introduction

The loss of specialized neuronal populations and axonal networks is the hallmark attribute of a majority of neurological disorders and trauma [260]. Over the past decade, approaches based on activating endogenous neural precursor cells (e.g., NSCs) or their exogenously transplantation, is being explored to replace the lost neuronal populations and restore the degenerated axonal network. Adult NSCs are self-renewing phenotype and could be induced to differentiate into regionally-specific neuronal lineages based on the signals they receive from their resident microenvironment [9]. However, not much is known regarding the interactions between the NSCs and the inflammatory host cells (e.g., microglial) residing in the extracellular (ECM) microenvironment of the diseased or injured central nervous system (CNS) [261]. The molecular mechanisms underlying these interactions also remain categorically unexplored.
Microglia are the resident macrophages in the CNS, comprise 10-15% of total cell density, and modulate immunological responses to various diseases and trauma [262]. It has been well-documented that upon activation under pathological conditions, microglia release a variety of cytokines and chemokines including IFN-γ, interleukins, interferons, nitric oxide, and TNF-α which could regulate neuroprotection and neurogenesis [249, 263]. Such cues released by activated microglial cells could lead to further deterioration of the damaged neurons in the CNS and hinder cell survival and functionality, while simultaneously promoting NSC differentiation into undesirable glial lineages [215]. Given their prominent role in initiating and controlling inflammation cascade within CNS, studying the effects of various pro- and anti-inflammatory markers released by activated microglia on NSC homing, survival, and differentiation might enable formulation of better strategies for NSC transplantation and suppression of inflammation for better clinical outcomes [264].

Investigating NSC-microglia interactions under inflammatory conditions in vivo is difficult due to lack of proper experimental controls and the complexities inherent to in vivo environment [265]. Prior studies have shown that primary microglia and NSCs could be isolated and expanded in vitro for performing co-cultures [266, 267]. Several microglial lines have been established via viral vector transfection or by other genetic inductions, including, murine BV-2, N9, MG5, and human HMO6 lines [268]. Similarly, induced pluripotent stem cells and retroviral oncogene immortalized NSC lines have been used as models for studying NSCs in vitro. Although these cell lines provide an effective option for in...
vitro testing, their genetic manipulation would have altered the native cellular phenotype and genotype compared to primary lines [269]. The types and levels of cytokines and chemokines released by genetically-modified microglia might not be physiologically-representative, while immortalization of NSCs alters their viability and differentiation patterns. Thus, outcomes from cocultures of such cell lines would not provide a glimpse of their native interactions, which is necessary for formulating effective repair strategies [261, 270].

The objective of this study is to investigate the influence of paracrine signaling by freshly-isolated and immortalized microglia on NSC phenotype, viability, and genes related to differentiation patterns. The first spontaneously-immortalized microglial cell line from mouse cerebral tissue (SIM-A9) was isolated by Nagamoto-Combs et al. at the University of North Dakota, School of Medicine and Health Sciences [271]. These cells were cocultured in a transwell system with freshly-harvested primary embryonic murine derived NSCs, in both 2D and 3D cultures. Such cultures offer the advantages of reduced immunogenicity, no direct cell-cell contact, paracrine signaling between cell types mimicking the native CNS milieu, and physiologically-relevant 3D microenvironment [272]. Such an experimental design and outcomes provide a practical approach to understanding how inflammatory microenvironments regulate NSC behavior [273].
4.2 Materials and methods

NSC and Microglial Expansion: Embryonic mouse (E11.5) brain-derived NSCs were harvested at NeuraCell (Rensselaer, NY) and characterized using cell sorting techniques to ensure the highest population and quality of NSCs per vial. Media components for culturing NSCs and microglial cells were purchased from Life Technologies (Grand Island, NY), unless stated otherwise. NSC passaging media was prepared by adding 100× L-glutamate, 100× N2 supplement, 100× B-27 supplement and 100× N-acetyl-L-cysteine (Sigma) to DMEM w/ Na-pyruvate. NSCs were maintained in an undifferentiated state by culturing on poly-L-lysine coated T-25 flasks in the presence of 100× bFGF [261]. Passaging media was changed every 24 h, and after 7 days, NSCs were gently detached using Accutase. Cells were then allowed to attach on poly-L-lysine coated 24 well plates or in 3D Corgel® hydrogels (Lifecore Biomedical, Chaska MN). Experiments were performed in triplicate under serum-free conditions from primary NSCs (passage 1; Fig. 14B).

Murine microglial cells were obtained from the University of North Dakota School of Medicine and Health Sciences. These cells, harvested from the cerebral cortex of C57BL/6 mice and spontaneously immortalized, aggressively proliferate to confluency (Fig. 14B). To minimize bias and ensure purity of cell line in these spontaneously immortalized microglial cells (SIM), clonal cells were cultured separately in several wells, allowed to expand in DMEM/F12 medium containing serum, and clone A9 was arbitrarily selected for shipment. These cells, hitherto referred as SIM-A9 cells, have been extensively characterized for both
morphology and release of cytokines/chemokines as it relates to native primary microglial cells. Upon receiving the SIM-A9 cell line, they were passaged in uncoated T-75 flasks in DMEM/F-12 containing 10% heat-inactivated fetal bovine serum, 5% heat-inactivated horse serum, and 0.15% penicillin/streptomycin/neomycin. Cell passages were detached with a solution of EDTA, EGTA and glucose, and frozen in liquid N<sub>2</sub> till further use. For the coculture experiments described below, media containing lipopolysaccharide (LPS, 2.5 ng/mL) was added to induce inflammation, and the horse and fetal bovine serum were removed (i.e., serum-free conditions).

**Figure 14 Schematic of transwell coculture.** (A) Schematic of the representative well from a 24-well plate, containing a transwell permeable cell culture insert for coculturing NSCs and microglia. This system was used for 2D or 3D cultures. To accommodate 3D scaffolds within these wells, a plastic bumper (100 µm height) was designed to accommodate necessary height increase, ensuring no cell-to-cell contact. SIM-A9 microglia was cultured within the inserts, as 2D layers or within 3D gels, in the presence of LPS to induce inflammation and release cytokines and chemokines. E11.5 NSCs were cultured on tissue culture plastic or within 3D gels in a 24-well plate. (B) Representative images of microglia and NSCs used in this study.
**Preparation of 3D hydrogels:** Corgel®, a hyaluronan hydrogel based on di-hydroxyphenyl linkages of tyramine-substituted hyaluronan, was used to encapsulate NSCs for 3D cultures. The range of physical properties of the gel is based on the percentage of the tyramine-substituted hyaluronan (TS-NaHy) concentration. For this study, 5 mg/mL (the lowest recommended concentration) was chosen to mimic the modulus of brain tissue. Briefly, horseradish peroxidase in PBS (10 U/mL) was added to the as-received 1.5% tyramine-substituted sodium hyaluronan at the prescribed concentration. NSCs (1×10⁶ million) were then added to the solution, and aliquoted to each individual well of a 24 well plate. Finally, 1% H₂O₂ was diluted in distilled water and added to the cells containing hyaluronan solution at a volume ratio of 1:25 to initiate crosslinking. The final solution was gently pipetted to ensure proper mixing and gelation occurs almost instantaneously.

**NSC/SIM-A9 cocultures:** The following cultures were performed for 10 days. NSCs were cultured alone on 2D surfaces (2D NSC) or within 3D hydrogels (3D NSC). In parallel, cocultures of NSCs and SIM-A9 cells were performed by seeding NSCs directly in the 24-well plates and placing the SIM-A9 cells on TC-coated transwell cell culture inserts (PET membrane, Flacon/Corning, Durham, NC). Cells were placed either directly on 2D surfaces (2D NSC/SIM-A9), or within 3D hydrogels (3D NSC/SIM-A9), as shown in Fig. 14A. NSCs were first seeded in 24-well plates at a density of 30,000 cells/well with 200 µL of NSC media without bFGF. After 3 h, transwell inserts were placed and ~1.75×10⁵ SIM-A9
cells were seeded along with media containing LPS. Respective media for both cell types was replaced daily.

**Cytokine/chemokine analysis:** The following cell culture conditions were tested in duplicate: NSC cultures in 2D, NSC cultures in 3D, microglia cultures in 2D, NSC and microglia cocultures in 2D, and NSC and microglia cocultures in 3D. All NSC cultures were performed in differentiating media (no bFGF). Cytokine and chemokine analyses were performed and analyzed using Discovery Assays® (Eve Technologies, Alberta Canada) according to manufacturer’s protocol. Briefly, cell culture supernatants (150 µL) were collected from each well after 24 h, spun down at 3000 g for 5 min, and stored at -20 °C. The supernatants were then processed using multiplexing LASER bead technology and processed on a dual-laser flow-cytometry system (Bio-Plex 200). The technology works by utilizing different combinations of red and infrared fluorophore beads conjugated to specific antibodies targeted to the cytokine or chemokine of choice, and are read using a flow-cytometry based system. The quantity of the specific analyte generated was based off a series of standards set forth by the company. The media for culturing NSCs and microglia were also tested under similar conditions, but showed no detectable levels of analytes. The following 32 analytes were tested: Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, VEGF and LIX.
**Cell viability:** NSC viability was measured using the LIVE/DEAD® viability and cytotoxicity kit (Life Technologies). NSCs were cultured alone or cocultured with SIM-A9 cells, in both 2D and 3D formats, and stained on days 1, 5, and 10. Cells were incubated with calcein AM and ethidium homodimer-1 for 30 min and immediately imaged using an AxioVert.A1 fluorescent microscope (Carl Zeiss Microimaging). NSC viability was expressed as a percentage of the number of live cells counted over the total number of cells (n=3 wells/condition; at least 200 cells/ well). On day 10 microglial cells were also stained for viability and counted using the same procedure.

**NSC differentiation and neurite outgrowth:** After 10 days, NSC cultures were processed to identify the formation of neural or glial lineages, resulting from the differentiation process induced by the cytokines and chemokines produced by the microglial cells in conjunction with the removal of bFGF. Briefly, as detailed elsewhere [229], cell cultures were fixed with 4% paraformaldehyde, blocked with buffer (0.5% Triton-X, 5% serum, 1× PBS), incubated with respective primary (overnight, 4 °C) and secondary (2 h, RT) antibodies, and counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). Cultures were imaged using an AxioVert A1 fluorescent microscope under both phase-contrast and fluorescence channels using a digital camera (Axiocam C1, Carl Zeiss) and multiple images collected using Axiovision data acquisition software. Negative controls for antibody specificity were processed similarly by omitting primary antibody in each case. The total number of cells per well were quantified by individually counting DAPI-expressing cells in each well, and comparing that to the number of cells.
expressing specific neural/glial marker in that same well (n=3 wells/condition; at least 400 cells/well).

Axonal outgrowth was quantified, using a previously detailed protocol [229], from the fluorescent images acquired from the TUJ1 antibody staining. Images from each respective condition were imported into ImageJ (NIH, version 1.49) and analyzed via the NeuronJ plugin [274]. At least 75 axons per condition were quantified and results were analyzed for statistical significance in differences.

**Effect of individual cytokines/chemokines on NSC phenotype:** Based on the results from cytokine/chemokine analysis (discussed above), the most abundantly released analytes by microglial cells were identified and their individual effects on NSCs were investigated. The seven analytes identified were TNF-α, G-CSF, MIP-2, MIP-1β, MIP-1α, IL-6, and MCP-1, which were purchased in lyophilized form (Mouse recombinant, Peprotech, Princeton, NJ). NSCs were cultured on 2D PLL-coated substrates with each of these analytes separately for 5 or 10 days, with the media changed daily. The concentration of each analyte in culture media was similar to that released by activated microglial cells. Several endpoints including cell viability, neurite outgrowth, and differentiation lineages were quantified as detailed above.
Real-time PCR studies: Total RNA was extracted from NSC cultures at various time-points (days 1, 5 and 10) using RNAqueous®-micro total RNA isolation kit (Life Technologies) according to the manufacturer’s instructions. RNA concentration of each sample was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit with RNase inhibitor (Life Technologies) according to manufacturer’s instructions, and if the total RNA yield was deemed low (< 10 ng/µL), MessageBOOSTER™ cDNA synthesis kit for qPCR (Illumina Madison, WI) was used in conjunction with SuperScript® III reverse transcriptase and RNeasy® MinElute® cleanup kit according to the manufacturer’s specifications. Gene expression in NSCs was quantified using TaqMan® gene expression assays (Applied Biosystems), and TaqMan® gene expression master mix for each target using the ABI 7500 Real-Time PCR System (Applied Biosystems, Forest City, CA), and limited to only 40 cycles. The following genes were analyzed: beta class-III tubulin (TUJ1, Assay ID: Mm00727586_s1), nestin (NES, Assay ID: Mm00450205_m1), myelin-associated glycoprotein (MBP, Assay ID: Mm00487538_m1), glial fibrillary acidic protein (GFAP, Assay ID: Mm01253033_m1), choline acetyltransferase (ChAT, Assay ID: Mm01221882_m1), tyrosine hydroxylase (TH, Assay ID: Mm00447557_m1), and motor neuron and pancreas homeobox 1 (HB9, Assay ID: Mm01222622_m1). The levels of the target genes were normalized to the endogenous reference gene (18S, Assay ID: 4319413E), in conjunction with freshly harvested murine brain tissue and relative differences in target gene
expression were determined using ΔΔCt method. The Cleveland Clinic Institutional animal care and use committee approved the use of animals in this study.

**Scanning electron microscopy:** NSC attachment in 2D cultures, either standalone or coculture conditions, was investigated using a scanning electron microscope (SEM). After 10 days in culture, media was removed, and the cell layers were washed three times with PBS and fixed with 4% paraformaldehyde for 1 h. The bottom of the 24 plate culture wells was cut out and the cell layers were dehydrated with a graded ethanol series. Samples were allowed to air dry for 30 min and mounted on carbon tape and placed in sputter machine (SPI sputter model 13131) for gold-coating (2 min; 350 A; 40 mA). Samples were imaged using an Inspect F50 SEM (FEI Hillsboro, Oregon) using a 10 keV electron source.

**Statistical analysis:** The data acquired was expressed as mean ± standard error. Statistical analysis was performed with Graphpad Prism 6 software (La Jolla, CA) and plotted using Sigmaplot™ software (San Jose, CA). Statistical significance between varying experimental conditions were tested using a student’s t-test (two-tailed, unequal distribution) or a two-way ANOVA with multiple comparisons (Fishers least significant difference). Statistically significant data was marked by a $p$ value < 0.05.
4.3 Results from this study

*Microglial production of cytokines and chemokines*

Exposure of the SIM-A9 cell line to LPS encouraged the production and release of several cytokines and chemokines (**Fig. 15**), with the release amounts ranging over several orders of magnitude. Among the analytes tested, the following were below the assay sensitivity (< 1 pg/mL) in all the cases: Eotaxin, IFN-γ, IL-1β, IL-3, IL-4, IL-5, IL-7, IL-12 (p40), IL-12 (p70), IL-15, IL-17A, IP-10, KC, LIF, LIX, MIG, VEGF and LIX. With the exception of GM-CSF, no quantifiable analyte release by NSCs was noted within 2D and 3D cultures, in the absence of cocultures. Within cocultures, significant upregulation in the release of analytes was noted, as shown in **Fig. 15B-C**, compared to that within standalone cultures of NSCs or microglia. Besides, 3D cultures promoted significantly higher amounts of cytokines and chemokines compared to their 2D counterparts, in at least 50% of the cases (i.e., IL-2, IL-13, IL-6, TNF-a, MIP-2, G-CSF, MIP-1α). It is not clear from these results if the presence of activated microglia is influencing the NSCs to release cytokines and chemokines, or the autocrine signaling by activated microglia is enhanced by the presence of NSCs.
Figure 15 NSC/microglia analyte production. Cytokine and chemokine production by microglia over 10 days was measured by a laser bead immunoassay. Analytes released at low (A), medium (B), and high (C) concentrations were plotted separately. Analyte release by NSCs either in 2D or 3D cultures, in the absence of external stimulation by microglia, was insignificant to quantify in most of the cases. Release of numerous other analytes (IFN-γ, IL-1β, IL-3, IL-4, IL-5, IL-7, IL-10, IL-12(p40), IL-12(p70), IL-15, IL-17, LIF, LIX, MIG, Eotaxin, KC, VEGF) across all cases was too low to be quantified (n=2 for all cases and tests ± SEM).
**Effect of activated microglia on NSC survival**

NSC survival in differentiation media using the transwell coculture system was evaluated at various time points, i.e., days 1, 5 and 10 (Fig. 16). Over the 10-day culture period, NSC survival within both 2D and 3D cultures, in the absence of microglia cocultures, was ~65%. During this 10 day period, microglia viability was ~95%. There was no significant difference in NSC survival between 2D and 3D cultures over the first 5 days, compared to day 1. However, a significant decrease in NSC viability was noted by day 10 within both 2D and 3D conditions, compared to the day 1 or day 5. Stand-alone NSCs (not shown) were cultured for the full day period and had no significant differences between day 5 and day 10. These results suggest that the presence of microglia induces a long-term effect on NSC viability.
Figure 16 NSC/microglia viability. (A) Representative images from the Live/Dead® assay of NSCs and microglia in 2D cocultures, on day 5. Live cells were stained in green and dead cells in red. (B) NSC survival was quantified in both 2D and 3D coculture conditions over a 10 day period. Significant differences ($p < 0.05$) in cell survival for day 10 vs. day 1 was denoted by *, and for day 10 vs day 5 by §, under respective conditions (n=3 for all cases and tests ± SEM).
**Effect of activated microglia on NSC differentiation**

The effect of microglial cocultures on NSC phenotype was evaluated on day 10 using immunofluorescence staining. MBP expression was almost absent in 2D cultures regardless of whether the NSC were cultured alone or cocultured with microglial cells (Fig. 17A). TUJ1 staining was observed in all culture conditions (it should be noted that the population of TUJ1 positive cells in cocultures was reduced, see Fig. 17B,C), however, a more distinct cellular morphology was evident in coculture conditions. Although GFAP staining was seen in all culture conditions, it was more pronounced within coculture conditions (Fig. 17A). In general, qualitative image analysis suggested distinct differences in the cellular morphologies and staining patterns within coculture vs. standalone conditions, as well as the effects of 2D vs. 3D culture conditions.

The number of cells expressing each particular marker was quantified within all the culture conditions. In 2D cultures (Fig. 17B), neural lineage differentiation was significantly reduced in cocultures, compared to standalone NSC cultures ($p < 0.05$). Conversely, within these 2D cultures, astrocyte lineage commitment was significantly enhanced (4.5-fold) in cocultures compared to standalone cultures ($p < 0.05$). However, no significant differences in oligodendrocyte commitment were noted between cocultures and standalone conditions in 2D cultures. Within 3D cultures (Fig. 17C), slightly different patterns were observed. Similar to that noted within 2D cultures, coculturing NSCs with microglia significantly increased GFAP lineage commitment compared to standalone NSC cultures ($p < 0.05$). NSC differentiation into oligodendrocyte
lineage was very low in general, and no significant difference was noted between coculture and standalone cultures. Even within 3D cultures, a significant decrease in neural differentiation was evident within cocultures compared to NSC standalone cultures. For both the standalone and coculture conditions, 3D environment significantly enhanced TUJ1 lineage commitment compared to 2D substrates. Similar patterns were observed in GFAP lineage commitment, where 3D scaffolds significantly increased the number of positively stained cells compared to 2D. Taken together, results suggest that NSC differentiation was not only affected by the presence of activated microglial cells, but also by the surrounding microenvironment.

NSC morphology in cocultures, acquired from the immunofluorescence staining (Fig. 17A), was used to further quantify neurite outgrowth (Fig. 17D, E). The distribution of the neurite length in cocultures reveals that a significantly larger population of NSCs extended axons greater than 20 µm, whereas a majority of the axons were under 10-15 µm within NSC standalone cultures. In general, the average neurite outgrowth in cocultures is significantly higher in both 2D and 3D compared to standalone cultures.
A

GFAP/DAPI  MBP/DAPI  TUJ1/DAPI

2D coculture  2D NSC alone

2D coculture  2D NSC alone

3D coculture  3D NSC alone

3D NSC alone

% of NSCs differentiated

% of axons

*
Figure 17 NSC/microglia differentiation and neurite outgrowth. (A) Representative immunofluorescence images of NSC cultures on day 10, differentiating into neural (TUJ1 staining) and glial (GFAP or MBP staining) lineages, when cultured on 2D substrates or within 3D scaffolds. NSCs were cultured standalone or as cocultures with microglia. Cultures were counterstained with DAPI for cell identification. Blue staining indicates nuclei, green indicates immature neurons (TUJ1), yellow indicates astrocyte (GFAP), and red indicates oligodendrocyte (MBP). NSC differentiation into each lineage was quantified in 2D (B) and 3D (C) cultures, and expressed as a percentage of the number cells stained with the respective marker to the total cells. * indicates statistically significant differences (p < 0.05) for results between NSCs cultured alone vs cocultures, and § indicates p < 0.05 for 2D vs. 3D cultures. Neurite outgrowth distribution within NSCs cultured alone (D) and within cocultures (E), was shown in both 2D and 3D culture conditions ((n=3 for all cases and tests ± SEM)).
In addition to the changes in phenotype, real-time PCR was used to analyze changes in NSC genotype across various culture conditions (Fig. 18). To establish that each of the selected primer was efficiently binding to its target gene, RNA was isolated from a whole mouse brain, converted to cDNA and cycled, and results show that all the seven aforementioned genes were expressed. This cDNA was then used to generate the standard curve for analyzing the 2D and 3D cultures of NSCs under standalone and coculture conditions. NSCs cultured in the presence of bFGF for 24 h showed a thousand-fold increase in the expression of both TUJ1 (preneuronal marker) and a hundred-fold increase in Nestin expression (NSC stemness marker), after normalization to housekeeping gene 18S and further to respective expressions in whole mouse brain tissue (ΔΔCt method; Fig. 18A). A low, but significant increase (20-fold) in TH gene expression was detected, although GFAP expression was slightly suppressed. Within 5 day and 10 day cultures, expressions of various genes were tested within 2D and 3D cultures, in the presence or absence of microglia cocultures. It was noted that GFAP, MBP, TH, HB9, and ChAT primers were not detectable within the first 40 cycles. When NSCs were cultured alone on 2D substrates, there was a significant increase in TUJ1 expression from day 5 to day 10 (Fig. 18B). However, coculture with activated microglia inhibited such time-dependent increase in TUJ1 expression within 2D cultures. Also, on day 5, there was no difference in TUJ1 expression between standalone vs. cocultures. Within 3D cultures, a significant downregulation of TUJ1 gene expression was noted between day 5 and day 10, in both the standalone and coculture conditions.
Figure 18 NSC/microglia genotypic analysis. (A) Fold-differences in gene expression of various neural and glial markers at 24 h time point, within 2D NSC cultures in the presence of bFGF. NSCs were cultured in the absence of microglia cocultures. Data was normalized to the expression of house-keeping gene 18S, and further normalized to the expression of these genes in brain tissue derived from mouse (ΔΔCt method). (B) Fold-differences in TUJ1 gene (pre-neural) expression within 2D or 3D NSC cultures, in the presence or absence of microglia cocultures, at 5 day and 10 day time points. TUJ1 gene expression was first normalized to 18S expression within respective cultures, and further to TUJ1 expression in mouse brain tissue (ΔΔCt method). Results were the average of at least two repeats unless noted otherwise, bars represent SEM.

Individual effects of selected cytokines & chemokines on NSC phenotype

Based on the findings from the transwell coculture system, the seven cytokines and chemokines abundantly released by activated microglia (Fig. 15) were selected to investigate which among them contributed significantly to the observed effect of cocultures on NSC viability, phenotype and genotype. NSCs were cultured on 2D PLL-coated substrates and exposed to each of these seven analytes separately. Our future studies will test the combinatorial effects of these cytokines and chemokines, when NSCs are exposed to multiple analytes simultaneously. NSC survival, average neurite outgrowth, and differentiation into various lineages were quantified at day 5 and day 10. Except in the presence of MCP-1 and MIP-1β, the average neurite outgrowth increased significantly by day 5 in the presence of other five analytes, compared to controls (p < 0.05; Fig. 19A). On day 10, with the exception of TNF-α and MIP-2, the remaining five analytes significantly increased axonal outgrowth compared to controls (p < 0.05). Among these analytes, MIP-1β, IL-6 and MCP-1 contributed to multi-fold increases in neurite outgrowth from day 5 to day 10. Compared to controls on day 5, all the analytes except G-CSF had no effect on NSC survival (Fig. 19B). However by day 10, all the seven analytes significantly suppressed NSC survival compared to controls, as well as day 5 (p < 0.05).
NSC differentiation was also affected by the presence of these individual cytokines and chemokines, at both day 5 and day 10 time points. Oligodendrocyte formation was barely visible within these cultures, irrespective of the culture conditions. During the first 5 days of culture, pre-neuronal lineage formation was significantly affected in the presence of MIP-1β, IL-6 and MCP-1, compared to controls \((p < 0.05; \text{Fig. 19C})\). However, by day 10, TUJ1 commitment recovered significantly within cultures receiving these three analytes. In general, by day 10, no significant differences in TUJ1 staining were noted in any of the cultures receiving analytes, compared to controls. GFAP staining was significantly lower in all the cases compared to TUJ1 staining. Only MCP-1 receiving cultures had significantly higher GFAP commitment on day 5 compared to controls. By day 10, GFAP stained cell density increased in cultures receiving MCP-1, IL-6, MIP-2, TNF-α and G-CSF, compared to controls \((\text{Fig. 19D} ~ p < 0.05)\). Furthermore, it should be noted that astrocyte lineage commitment in cultures exposed to individual analytes was ~20% lower than that found in coculture system, on day 10.
Figure 19 NSC neurite outgrowth, viability, and differentiation with individual analytes. NSCs were cultured on 2D substrates in media containing one of these cues (TNF-α, G-CSF, MIP-2, MIP-1β, MIP-1α, IL-6, MCP-1) at concentrations identified earlier. Neurite outgrowth (A), cell survival (B), and differentiation into neural (C) and glial (D) lineages was quantified at day 5 or day 10, in the presence of such cues. Control cultures at respective time points received no exogenous cues. § indicates $p < 0.05$ for test cases vs. controls at each time point, and * represents $p < 0.05$ for day 5 vs. day 10. (n=3 for all cases and tests ± SEM).

Effect of microglial coculture on NSC morphology

To further investigate NSC morphology after the 10 day culture period, 2D NSC alone and microglial culture were imaged using scanning electron microscopy to better display the unique differences in the cellular morphology across the two groups (Fig. 20A-C). When cultured with microglial cells, NSCs appear to exhibit the native neuronal morphology (Fig. 20A), with significant
axonal outgrowth and spreading across the field of view. When NSCs are cultured alone, NSCs do show some axonal outgrowth, but the morphology appears rounded and balled up, with limited attachment to the cellular surface. When magnification was increased (Fig. 20B) the integrin binding sites are clearly visible when NSCs are cultured in the presence of microglial cells, whereas this is not as pronounced in NSCs cultured alone.

Figure 20 SEM of NSC/microglia. Representative SEM images depicting extensive axonal outgrowth (A) and increased attachment (B) of NSCs under coculture conditions, while cells maintained a rounded morphology with relatively shorter neurite outgrowth (C). These images support our prior results from immunofluorescence imaging and quantification.

4.4 Discussion

SIM-A9 cells secrete physiologically-relevant cytokines and chemokines

The microglial SIM-A9 cell line was recently isolated, characterized and expanded by Kumi Nagamoto-Combs, and ELISA-based assays suggest that LPS activation induced SIM-A9 cells to produce ~150 pg/mL of TNF-α in a 24 h
period, a 1.7-fold increase compared to LPS-free control group [271]. The TNF-α production levels in 2D cocultures upon LPS stimulation was found to be in similar range in our current study. Although the fundamental immune response of the SIM-A9 cells is being preserved, the degree of the response may decrease with increasing passage number. Besides, TNF-α production could also be regulated by the downstream signaling pathways induced by the presence of other cytokines and chemokines (i.e., IL-6, IL-10 G-CSF). TNF-α levels after an injury or under disease are expressed over a broad range [275]. For example, post brain injury, TNF-α serum levels in humans was reported to be within 65-7500 pg/mL range [276], while it was <500 pg/mL in rats [277]. Although BV-2 immortalized microglia produced similar levels of TNF-α in vitro, other studies have contradicted the amounts of this cytokine [277, 278]. Nevertheless, it has been established that primary microglial cell lines harvested from human or animal tissue do produce TNF-α when activated by LPS [279].

In addition to TNF-α, activated SIM-A9 cells produced several physiologically-relevant biomolecules. For example, G-CSF, produced in vivo by microglia, has shown promise in Alzheimer’s patients as it can provide neuro-protection via mobilization of marrow-derived microglia and subsequent modulation of the immune system [280]. Similarly, pro-inflammatory cytokine IL-6 (similar to TNF-α) has been observed in Alzheimer’s as well as Multiple Sclerosis patients, although a few studies using primary microglia reported IL-6 release levels multi-fold higher than we report here [281, 282]. In addition to these, activated microglia from both primary and immortalized lines release cytokines
including IL-1α, IL-2, IL-9 and IL-13, which provide neurodegenerative and neuroprotective properties to the surrounding environment [283]. Although SIM-A9 cells studied here released a majority of the cytokines native to inflammatory environment in vivo, we hypothesize that the absence (undetectable levels) of IFN-γ and IL-1β might be due to higher passage number [284]. In general, the expression of a majority of the pro- and anti-inflammatory cytokines observed in this study have been linked to several disease states (e.g., Parkinson’s, ALS) and traumatic brain injury [285].

Activated microglial cells also produced several chemokines, many of them at concentrations higher than the cytokine levels. While MCP-1 concentration was similar to that in the cerebrospinal fluid of ALS patients (~900 pg/mL), MIP-1α levels (4000-8000 pg/mL) noted in our study were multi-fold higher than that in the same patients (~5 pg/mL) [286]. Post-mortem analysis of brain tissue from patients diagnosed with MS revealed that the gene expression of MIP-1β, RANTES, IP-10 significantly elevated compared to normal brain tissue [287]. Similarly, gene expression for IL-10, MCP-1, MIP-1α, MIP-1β and RANTES were all upregulated in rodent brain within 3 hours of traumatic brain injury [288]. In general, the blood-brain barrier's ability to limit lymphatic antigens from entering the central nervous system (CNS), coupled with the reduced expression of major histocompatibility complex antigens (classes I and II) on certain CNS cells creates an overall immunosuppressive microenvironment, further necessitating the need for increased understanding of the role of microglia in neural inflammation [30, 31]. Here we demonstrate that the panel of
chemokines produced by the SIM-A9 appears to mimic the physiological conditions of several neurodegenerative disorders which suggest that the SIM-A9 cell line could be an efficient model cell line to further understand these types of neurological conditions.

**NSC viability is compromised within microglial cocultures**

It has been well-documented that exposure to various inflammatory mediators compromises NSC viability *in vitro* [267]. However, these studies are qualitative in nature, limited by short culture periods (< 3 days), or lack quantification. Here we demonstrate that the inflammatory microenvironment has a chronic affect (5 and 10 day periods, both in 2D and 3D) rather than an acute effect. Furthermore, standalone NSC cultures of the 7 most abundantly expressed analytes appear to individually follow this trend as well, indicating that this need not be a synergistic effect of combination of cytokines and chemokines. Previous studies have shown that analytes such as IL-1α, IL-2 do not have effect NSC viability [289]. In the current study, we observed these analytes to be in lower concentrations (> 50 pg/mL) within cocultures, suggesting that their presence had no significant effect on NSC viability. NSC survival during the initial 5 days of culture might have been enhanced by an interplay of analytes (i.e., RANTES, G-CSF, MCP-1). However, dramatic decrease in NSC survival from day 5 to day 10 within cultures containing individual analytes suggests a strong correlation between NSC death and the type of analyte. We hypothesize that
NSC survival might have been affected by the activation of the NF-κB pathway, which has been implicated in the mechanism by which several analytes (e.g., TNF-α) induce apoptosis [290].

**Pre-neural lineage commitment is down-regulated within cocultures**

NSCs have been shown to expand and maintain an undifferentiated phenotype in the presence of epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF), and upon their removal, they differentiate into both neuronal and glial lineages [182]. Both phenotypic (immunofluorescence staining) and genotypic (RT-PCR) analyses have revealed distinct changes in NSC differentiation across a majority of the cases tested. Within 3D controls, NSCs appear to differentiate in patterns similar to those reported by Ge et al. in 3D collagen scaffolds [291]. When cultured under inflammatory conditions, several groups have noted that GFAP lineage commitment by NSCs is upregulated, likely do to the presence of IL-1α and IL-6 which can act on the gp130 receptor and trigger the JAK/STAT pathway [266, 292]. This is in agreement with our own results on the standalone exposure of NSCs to IL-6. However, comparing data from cocultures to NSC cultures receiving one analyte at a time, it is apparent that many of these cytokines and chemokines could be acting synergistically to influence GFAP lineage commitment. It has been shown that G-CSF induces neuronal differentiation in NSCs *in vitro* [293], while GM-CSF suppresses lineage-specific NSC differentiation [294]. Taken together, our results
support the hypothesis that these analytes could be working together to influence NSC fate.

The 3D culture environment appears to offer some protection for TUJ1 lineage commitment, which was not significantly different within cocultures compared to standalone cultures. This offers the possibility of designing scaffolds which could both influence NSC differentiation patterns and offer increased neuro-protection in hostile inflammatory environments such as SCI or TBI. Furthermore, the 3D environment better mimics in vivo conditions and thus likely can better leverage NSC differentiation [272, 291]. It is also possible that NSCs committed to TUJ1 lineage are less resistant to inflammatory environment within cocultures compared to their GFAP-counterparts. To summarize, the differentiation patterns in standalone NSC cultures are in agreement with literature, whereas GFAP is unregulated in coculture conditions, and 3D environments are beneficial from TUJ1 commitment standpoint.

In addition to the qualitative and quantitative phenotypic analysis, genotypic analysis was performed using RT-PCR. Although the expression levels of several genes were investigated, the expression levels for a majority of them were below the generated standard curve. When cultured with non-differentiation media, NSCs showed high levels of Nestin expression within the first 24 h indicating their stemness, similar to that reported by other groups [295, 296]. However, cells began to express TUJ1 gene expression as well as small amounts of TH and GFAP, indicating the onset of the differentiation process during the first 24 h. It is also possible that the same NSCs might be expressing
a combination of these genes without differentiating, depending on their maturity level. Previous genetic studies had limited their analysis to a single time-point and a majority focused on gene expression within the microglia [267, 297]. Our results indicate TUJ1 gene expression to significantly downregulate over the 10 day period, when cocultured in 3D with microglial cells.

**Microglial cells encourage NSC axonal outgrowth and surface attachment**

A unique observation in this study was the distinct morphological features within NSC cultures in both 2D and 3D, in the presence of microglia. The presence of various cytokines and chemokines is expected to contribute to glial scarring *in vivo*, which ultimately acts as a barrier for axonal regeneration after injury [298]. However, the role of these cytokines and chemokines could be context-dependent and at times be beneficial [299], as noted in the case of axonal outgrowth. Studies conducted *in vivo* have shown that NSC transplantation after SCI or TBI trauma could promote extensive axonal outgrowth and sprouting [300, 301]. Our results are also in agreement with other studies that demonstrate the stand-alone effects of IFN-γ, IL-1 and IL-6 in regulating axonal outgrowth within NSC cultures, demonstrating the therapeutic potential of these cytokines and chemokines [249, 302]. This is further supported by the addition of IL-6 alone to NSC cultures, which increased neuronal length by ~20 µm. We hypothesize that the enhanced axonal growth could be due to increased NSC expression of cell adhesion molecules in the presence of the
microglia [303], or via increased expression of metalloproteinases from the microglia which [304]. In summary, the 3D microglia coculture system described in this study provides a biomimetic microenvironment that could be used to efficiently investigate how the right combination of cytokines and chemokines, which could elicit enhanced axonal outgrowth within endogenous NSCs, aid in the repair of severed axons during disease or trauma [305]. Given the significance of outgrowth from day 5 to day 10 elicited by the individual addition of MIP-1β, IL-6, and MCP-1, we suggest further exploration of these analytes as a tool for axonal regeneration.

4.5 Conclusions of this study

The environment created after trauma or progression of neurological disorders results in the release of several key cytokines and chemokines by activated microglial cells, which impede the ability of NSCs to successfully repair lost or damaged tissue. We demonstrated that using transwell coculture set-up creates a simple but effective in vitro microenvironment, which could aid in a thorough understanding of the changes in NSC fate within an inflammatory niche. We not only compared outcomes from conventional 2D cell culture approaches to a 3D milieu, but also the differences and similarities in the individual action of numerous prominently released analytes to the full panel of analytes produced by activated microglial cells. The presence of a number of these analytes by themselves or in combination decreased NSC survival and modified NSC
phenotypic and genotypic lineage commitment, and in some cases simultaneously increasing neurite outgrowth in those surviving cells. In conclusion, results from this study would not only aid in increasing our understanding of the specific role played by analytes in a localized inflammatory microenvironment, and in the in situ delivery of NSCs for regenerative medicine applications. Results attest to the impact both individually and together these analytes have reducing NSC viability, as well as the ability to modulate differentiation patterns including the increased gliogenesis, which is amplified when NSCs are exposed to the full panel of analytes. The data also suggests that exposure to MIP-1β, IL-6, or MCP-1 is able to enhance neurite outgrowth almost three fold after 10 day days of exposure. These findings suggest the potential utility of some of these cytokines and chemokines in aiding in the restoration of lost axonal connections.
CHAPTER V

COCULTURES WITH PEDIATRIC GLIOBLASTOMA DERIVED CELLS MODULATE HUMAN NEURAL PROGENITOR CELL PHENOTYPE, MIGRATION, AND DIFFERENTIATION

5.1 Introduction

Glioblastoma multiforme (GBM) is a highly malignant form of cancer found within the central nervous system (CNS) which, when diagnosed, has a median patient survival time of less than a year [306]. GBM affects populations of all ages, although the pediatric form of the disease is understudied due to the histopathological diversity of the ailment. Pediatric GBM is also more challenging to treat due to intrinsic drug resistance [307]. Although the cellular origins of pediatric GBM are unclear, it has been suggested that they could arise from the transformation of proliferating neural progenitor cells (NPCs) during embryogenesis [158]. Therefore, in addition to cancer stem cell markers (e.g., CD133) [308, 309], GBM also cells express a variety of lineage markers including pre-neural (TUJ1) and astrocyte (GFAP), as well as a variety of mature neuronal markers including GABA and GalC [23].
NPCs respond to various spatio-temporal cues to determine their progeny, and their maturation is driven by a combination of intrinsic temporal factors as well as extracellular signals from the developing brain microenvironment. For example, at mid-gestation, young neurons migrate above the germinal ventricular zone (VZ) and eventually to the subventricular zone (SVZ). By postnatal stage, radial glia transform into astrocytes and the VZ disappears, but the SVZ remains into adulthood where NPCs continue to proliferate [310]. After birth, NPCs respond to a variety of growth factors mimicking responses seen during embryogenesis. When cultured in vitro, NPCs have been shown to proliferate and maintain their “stemness” in the presence of exogenous basic fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF) and will differentiate into both neuronal and glial subtypes upon removal of these mitogens [182]. NPCs have been shown to migrate and integrate into the surrounding tumor microenvironment, and therefore offer potential to impede GBM progression by delivering therapeutically-relevant molecules to the tumor site [24]. For example, studies have shown that GBM cells have a BMP cell-cycle exit pathway similar to that of NPCs, which if manipulated could impede tumor progression [157].

Such therapeutic potential of NPCs in a cancerous microenvironment is currently limited by the potential transformation of NPCs to tumorigenic phenotype, which is thought to be triggered by a variety of molecular mechanisms. Holland et al. reported that NPCs can produce similar histological characteristics as GBM in a rodent model, via transduction proteins such as Akt and KRas [311]. In another study, genetic analysis suggested that Olig2
regulates both NPC and GBM lineages and is critical for cell proliferation in both populations [312]. Dai et al. found that mature mouse astrocytes transfected with the platelet-derived growth factor appeared to be more susceptible to GBM transformation in vivo [313]. Other studies suggest that the mechanisms driving GBM transformation are based on increased production of the glycoprotein CD133, which is also a NPC marker. Thus, the origin(s) of GBM has been hypothesized to be either a mutation of CD-133 cell type which are normally not present in the mature brain, or from ependymal cells which can be CD133-positive and are also found in the mature brain [314]. In general, NPCs are at risk for malignant transformation based on activated pro-mitotic genes, telomerase activity, and anti-apoptotic genes, which can be triggered by a combination of less than seven mutations [156, 161].

Currently, limited information exists pertaining to the influence of GBMs on NPCs and vice versa. NSCs injected in vivo to mouse tumor sites have been shown to aggressively migrate into tumor sites and overexpress several cytokines and chemokines, which has the potential to trigger antitumor NPC-mediated immunity [24, 315]. GBMs also appear to transmit signals to the surrounding environment that cause the co-expression of several unique neuronal markers in both themselves as well as any surrounding stem cells [158, 316]. Investigating the paracrine signaling between GBM cells and NPCs in a controlled microenvironment (e.g., cocultures) might help in direct quantification of the influence of each cell type on the other, identification of molecules which arrest GBM growth and metastasis and suppress tumor formation, and
evaluating the potential of NPC transplantation in restoring lost cell populations at the lesions site after surgical removal of CNS tumor. Using a commercially-available Transwell® coculture system, limitations associated with in vivo studies could be overcome and direct cell-cell contact could be eliminated, while effectively exposing GBM cells and NPCs to the signaling molecules released by the other. This study is based on the hypothesis that the biochemical signals released by pediatric GBM-derived cells influences the normal phenotype of NPCs by altering their morphology, survival, migration, differentiation patterns, and release of various cytokines and chemokines. Given the similarities in the cellular pathways which regulate both NPC and GBM cell differentiation and proliferation [20], such coculture studies could also provide key insights into the effect NPCs have on GBM cells. The outcomes from such studies might help understand the conditions leading to the onset and progression of pediatric GBMs, identify the target molecules and pathways which might help impede GBM progression, and unlock the therapeutic potential of NPCs in this tumor microenvironment [21].

5.2 Materials and methods

**NPC and GBM expansion:** Human NPCs (ReNcell VM Human Neural Progenitor Cell Line, Billerica Massachusetts) and all media components were purchased from EMD Millipore unless otherwise noted. ReNcell non-differentiating media was prepared by adding 20 ng/mL of freshly-thawed bFGF
and EGF to ReNcell maintenance media. ReNcells were maintained in an undifferentiated state by culturing on laminin-coated T-75 flasks. Passaging media was changed every 24 h, and after 7 days, ReNcells were gently detached using Accutase (Life Technologies, Carlsbad, CA) and frozen in ReNcell freezing medium. Differentiation media was prepared using ReNcell maintenance media without any bFGF or EGF. All cells used in this study were before passage 10.

Human pediatric glioblastoma multiforme cells (GBM) were obtained from the Children’s Oncology Group (COG) Cell Culture and Xenograft Repository at Texas Tech University Health Sciences Center School of Medicine. Cells were harvested from a 5 year old female and are labeled by the repository as the “SJ-GBM2” cell line [317]. The SJ-GBM2 cell line was expanded in uncoated T-75 flasks. All media products were purchased from Life Technologies unless otherwise noted. Media was prepared using Iscove’s modified Dulbecco’s medium containing 20% fetal bovine serum, 4mM L-glutamine, and ITS supplement (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid). Media was replaced every 3 days, and after 7 days, cells were detached using Pucks EDTA (140 nM NaCl, 5 mM KCl, 5.5 mM glucose, 4mM NaHCO3, 13 µm phenol red, 0.8 mM EDTA, and 9 mM HEPES) and frozen in a solution containing 50% fetal bovine serum and 7.5% DMSO in Iscove’s modified Dulbecco’s medium. Cells were used prior to passage 20. Henceforth, SJ-GBM2 cells will be referred to as GBM, and undifferentiated ReNcells as ReNcell-bFGF. All cultures in this study were performed on 2D tissue culture grade plastic, and ReNcells were always plated on laminin-coated dishes.
**Transwell® coculture conditions:** After the initial expansion of ReNcells and GBMs, cultures were run for a period of 5 or 10 days under the following conditions. The following conditions were tested in parallel: ReNcells cultured alone in differentiation media (*ReNcells alone*), GBMs cultured alone in media (*GBM alone*), ReNcells cultured alone in non-differentiating media (*ReNcell bFGF*) and cocultures of ReNcells (in differentiating media) with GBMs (*GBM cocultures* and *ReNcell cocultures*). To ensure no direct cell-cell interactions, 1 µm PET membrane Transwell® cell culture inserts (Flacon/Corning, Durham, NC) were used. NPCs were first seeded in 24-well plates at a density of 40,000 cells per well and cultured for 3 h with 200 µL of differentiation media or non-differentiating media. For coculture conditions, after a 3 h period, Transwell® inserts were placed and 40,000 GBMs were seeded within the inserts. Respective media for both cell types was replaced daily.

**Cytokine/chemokine analysis:** Cytokine and chemokine analyses were performed using Discovery Assays® (Eve Technologies, Alberta, Canada) according to manufacturer’s protocol. Briefly, cell culture supernatants (150 µL) were collected from each well after 24 h, spun down at 3000 g for 5 min, and stored at -20 °C. The supernatants were then processed using multiplexing LASER bead technology and processed on a dual-laser flow-cytometry system (Bio-Plex 200). The technology works by utilizing different combinations of red
and infrared fluorophore beads conjugated to specific antibodies targeted to the cytokine or chemokine of choice and are read using a flow-cytometry based system. The quantity of the specific analyte generated was based off of a series of standards set forth by the company. The following cases were tested: ReNcells alone, GBM alone, ReNcell bFGF, ReNcell cocultures, GBM cocultures. The following 42 cytokines and chemokines were tested: EGF, Eotaxin-1, FGF-2, Flt-3L, Fractalkine, G-CSF, GM-CSF, GRO, IFNα2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-18, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGF-α, TNF-α, TNF-β, and VEGF. The following 3 cancer markers were tested: Galectin-3, MPO, and FAP.

**Cell viability:** NSC and GBM viability was measured using the LIVE/DEAD® viability and cytotoxicity kit (Life Technologies) on days 5 and 10, for all the culture conditions. Cells were incubated with calcein AM and ethidium homodimer-1 for 30 min and immediately underwent fluorescent imaging using an Axio Vert.A1 Fluorescent microscope (Carl Zeiss Microimaging; NY). For coculture conditions, the Transwell® inserts containing the GBMs were removed and placed in a separate well, stained using the aforementioned procedure and imaged. Viability of ReNcells and GBMs was expressed as a percentage of the number of live cells counted over the total number of cells, using a custom-written macro in ImageJ® [NIH, version 1.49] (n=3; > 500 cells counted per well).
**Cellular morphology:** Cell shape index (CSI) was assessed using the images acquired from all of the aforementioned LIVE/DEAD® staining cases. Specifically only the living cells (FITC-channel) were considered. Images were batch processed using the particle analyzer function feature in ImageJ (n= 3; > 500 cells counted). The projected area (A) and perimeter (P) for each cell was measured and the average cell morphology was quantified using the formula: CSI = $4\pi A/P^2$. CSI values typically range from 0 (infinitely elongated polygon) to 1 (perfect circle).

**Immunofluorescence imaging:** On days 5 and 10 of culture, the following cultures were processed to identify and quantify the distinct neural and glial lineages: ReNcells alone, GBM alone, ReNcell cocultures, and GBM cocultures. Cells were washed once in sterile 1× PBS, fixed with 4% PFA (4 °C, 2 h), washed once with 1× PBS (5 min), and incubated with blocking buffer (0.5% Triton-X, 5% serum, 1× PBS) for 2 h at room temperature. Serum selection was based on primary and secondary antibody host species. After removing blocking buffer, cells were incubated with respective primary antibodies (4 °C, 24 h): rabbit monoclonal anti-beta III tubulin (TUJ1; early developmental state neuron marker; Abcam), mouse monoclonal anti-Nestin (stemness marker; Millipore), mouse monoclonal anti-CD133 (Prominin-1; glioblastoma and stem cell glycoprotein; Millipore), and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; astrocyte
maker; Abcam). Cells were washed three times in PBS, incubated with appropriate secondary antibodies (Santa Cruz Biotechnology, Dallas, TX) at room temperature for 2 h, washed again three times with PBS, and then counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). For coculture conditions, the Transwell® inserts containing GBMs were removed, bottom mesh was cutout and placed in separate wells, and stained using the above procedure. All cells were imaged using an Axio Vert.A1 fluorescent microscope (Carl Zeiss Microimaging; NY) under both phase contrast and fluorescent channels using a digital camera (Axiocam C1, Carl Zeiss) and Axiovision data acquisition software. The total number of cells per well was quantified using batch processing in ImageJ which quantified all the cells in the well emitting DAPI signal, and comparing that number to the total number of cells in that same well with positive staining for the aforementioned anti-body markers (n =3, > 1000 cells counted per well).

**Axonal outgrowth:** Axonal outgrowth was quantified using the fluorescent images acquired from the TUJ1 antibody staining of ReNcell cultures: ReNcells alone and ReNcell cocultures. Images from each respective condition were then imported into ImageJ and analyzed via the NeuronJ plugin [274]. Briefly, this program traces the length of neurite branching from the soma to the end of axon using the bright intensity of the stained pixels (n=3; >100 axons per condition counted).
**Cell proliferation assay:** Cell proliferation was assessed using a BrdU cell proliferation assay (EMD Millipore). Assays for this experiment were run in 96-well plates as recommended by the manufacturer. The assay was first run on cells cultured for only 2 h to validate that each cell type was able to uptake the BrdU reagent and the assay would be able to provide reliable readings. ReNcells and GBMs were serially-diluted and seeded independently at the following densities per well: 1, 2, 5, 10, 20, 40, 80, 100, 150, and 300 thousand cells. After 2 h culture, the assay was run as instructed by the vendor, the plates were read at 450/550 nm using a spectrophotometer (Biotek Synergy Winooski, VT) and analyzed using Gen 5 software provided by the company. The data acquired followed a linear trend of cell population size vs. optical density, which was in agreement with the manufacturer’s recommendations, and thus the test was deemed valid to use for each specific case. The data collected from the serial dilution of the cell populations for 2 h was called “initial seeding” and was only performed on stand-alone GBMs and NSCs in differentiating media. Given that there is no commercially-available Transwell® insert for 96-well plates, ReNcell coculture conditions with GBM were achieved by taking 100 µL of spent media from the GBM cultures every 24 h and adding it to the ReNcell culture. Similarly, 100 µL of spent ReNcell media was added to GBM cultures. At this time, 100 µL of fresh media was replaced in each respective culture condition, and a full media change (200 µL) was performed on stand-alone culture conditions for consistency. All cultures were seeded at an initial density of 20,000 cells per well,
which was measured electronically using a Moxi-z mini-automated cell counter (Orflo, Ketchum, ID). The proliferation outcomes were tested at 3 different time points: days 1, 5 and 10.

*Migration assay:* To understand chemotaxis of GBM and ReNcells, a scratch test assay was performed to quantify cellular migration in their respective microenvironments. Cells were seeded in 24 well plates and allowed to expand to confluence for a period of 3-5 days, at which point a channel ~500 µm wide was scratched in the center of the confluent cellular monolayer within each well. Time-lapse imaging in bright-field mode was immediately started using 10× objective to ensure that the entire scratch was in the field of view. The scratch was always performed on cell layers within the 24-well plate and not the transwell. Thus, to quantify GBM migration within cocultures, they were seeded in the lower well and ReNcells were seeded in the Transwell® insert, and vice versa for quantifying ReNcell migration within cocultures.

*Statistical analysis:* Data acquired was expressed as mean ± standard error. Statistical analysis was performed with Graphpad Prism 6 (La Jolla, CA) and plotted in Sigmaplot™ (San Jose, CA). Statistical significance between varying experimental conditions was tested using a student’s t-test (two-tailed, unequal distribution) or a two-way ANOVA with multiple comparisons (Fishers least significant difference). Statistically significant data was marked by a $p < 0.05$. 
Except the cytokine/chemokine analysis, all other tests were performed in triplicate.

5.3 Results

Cytokine and chemokine production

The biochemical analytes released by ReNcells and GBMs within standalone and cocultures was plotted based on quantified analyte concentrations (Fig 21A: 0 - 2.5 pg/mL, Fig. 21B: 0 - 14 pg/mL, Fig. 21C: 0 - 1400 pg/mL). When cultured alone, all analytes except IL-7 and IL-8 were expressed by ReNcells in non-differentiating media (presence of bFGF). However, once bFGF was removed and differentiation induced, ReNcells released significantly lower amounts of IL-1α, IL-27, PDGF-AA, MCP-1 and Fractalkine, and significantly higher amounts of MCP-3 and Galectin-3, but no detectable levels of IL-13, IL-12, G-CSF and VEGF-α. Compared to ReNcells within standalone cultures, GBMs released significantly higher amounts of IL-2, IL-6, IL-7, IL-13, IL-18, G-CSF and IL-12, and significantly lower amounts of IL-1Ra, IL-27a, MCP-3, Galectin-3, PDGF-AA, and MCP-1. GBMs didn’t release any Eotaxin-1, IFN-γ, IP-10, and Fractalkine. In cocultures, IL-7, IL-13, Eotaxin-1 and IFN-γ were not detectable, although they were quantified in standalone cultures. In general, the analyte amounts in cocultures were within the ranges measured in standalone cultures of ReNcells or GBMs (except IL-1B), suggesting that the levels of these analytes in cocultures were not simply a
combination of their individual levels in standalone cultures. Galectin-3 was the most abundantly released analyte in the pooled media for all the cases tested, as well as the only cancer-specific marker found; MPO and FAP were undetectable in all cases. Counter-intuitively, ReNcells released larger amounts of Galectin-3 than GBMs.
**Figure 21 NPC/GBM analyte production.** Cytokine and chemokine production by ReNcells alone (presence or absence of bFGF), GBMs alone, and in cocultures, as measured via laser bead immunoassay. The analyte levels were plotted based on their concentration levels: low (A) low, medium (B), and high (C). No detectable levels of IL-10, IL-3, MIP1a, MIP1b, TNF-β, Myeloperoxidase, and Fibroblast Activation Protein were found (n=1).

**Cell viability and proliferation**

Representative images from the Live/Dead® assay were shown in **Fig. 22A**. Although cell viability in both the standalone and cocultures was high (> 80%; **Fig. 22B**), viability in cocultures was marginally higher at both the time points. However, no differences in cell survival between day 5 and day 10 were evident. Cell proliferation, on the other hand, appears to be significantly influenced both by the duration of culture as well as cocultures (**Fig. 23**).
Expectedly, when ReNcells were cultured alone in non-differentiation media (containing bFGF), the number of actively dividing cells increased in an almost linear fashion from days 1 to 10. Conversely, when ReNcells were cultured in differentiation media (absence of bFGF), no active cell proliferation was noted over the ten day cultures. In both standalone and cocultures, GBM cells aggressively multiplied as demonstrated by the almost 3-fold change in optical density between day 1 and 5 in both culture conditions. The significant decrease in O.D. within these GBM cultures by day 10 could be attributed to cell confluence within culture dishes, as evident from light microscopy images. Finally, the presence of GBM cells (cocultures) appears to slightly stimulate ReNcell proliferation by day 10 compared to their standalone culture in differentiation media ($p < 0.05$).
Figure 22 NPC/GBM viability. Live/Dead® analysis of ReNcells and GBMs, in standalone and cocultures, on days 5 and 10. Representative images of the stained cells for all conditions on day 10 were shown (A). Green indicates live cells and red indicates dead cells. The images were quantified and the cell survival in respective cases plotted (B). (n=3 for all cases and tests ± SEM).
Figure 23 NPC/GBM proliferation. Cellular proliferation was measured via BrdU uptake and expressed as optical density (O.D.) in various culture conditions. Measurements were taken immediately after seeding (4 h), and at days 1, 5 and 10. (n=3 for all cases and tests ± SEM).

Cell phenotype and differentiation within standalone and cocultures

NPCs have been shown to differentiate and commit to distinct neural and glial lineages upon removal of EGF and bFGF from the culture media. Immunofluorescence imaging (Fig. 24) qualitatively revealed the lineage
commitment of ReNcells and GBM cells within standalone and coculture conditions after a ten day culture. The native phenotype of as-received undifferentiated ReNcells (cultured with bFGF) at 24 h time point was also shown for comparison. It could be seen that as-received ReNcells stained 100% positive for both Nestin and GFAP but not for TUJ1 and CD133, after 24 hours in culture. However, after differentiation was induced by bFGF removal, significant morphological changes in ReNcells were noted by the end of ten day culture. Significant TUJ1 staining was evident, with axonal outgrowth in both TUJ1 and Nestin positive images. Astrocyte phenotype was also remarkably different in differentiated cultures compared to their undifferentiated counterparts. However, no CD133 staining was noted in both undifferentiated and differentiated ReNcell standalone cultures. On the other hand, GBM cells strongly stained for CD133, Nestin and GFAP, within ten-day standalone cultures.

Similar to their standalone counterparts, both ReNcells and GBMs stained positive for Nestin within cocultures. In contrast to their standalone counterparts, within cocultures, (a) TUJ1 staining in a majority of ReNcells was suppressed, while some GBM cells stained positive for TUJ1 marker, (b) ReNcells exhibited strong and mature GFAP-staining indicative of astrocyte lineage commitment, while GBM cells completely lacked GFAP staining, (c) numerous ReNcells stained positive for CD133, while CD133 stain in GBM cells was slightly suppressed, and (d) axonal outgrowth in ReNcell cultures was significantly compromised. These results are important and highly relevant from numerous standpoints, as elaborated in the discussion section.
In general, the trends in quantitative data closely matched the observations from qualitative imaging. Quantification of images indicated no time-dependent differences in CD133 expressing cells (Fig. 25A). GBM cultures, standalone or cocultures, had the highest number of cells marked for CD133. CD133 expression in ReNcells was higher within cocultures compared to standalone counterparts. Of all the markers stained for, Nestin was most abundantly found across all cases (Fig. 25B, > 40% for all cases). Significant differences were not observed when comparing stand-alone cases to coculture cases on the same day, however a significant decrease in Nestin marker was observed from day 5 to day 10 within GBM cultures ($p < 0.05$). Surprisingly, ReNcells expressed significant Nestin marker even after a 10 day differentiation induction. A significant reduction in GFAP expression was seen from day 5 to 10 in GBM cultures (Fig. 25C, $p < 0.05$) but not in ReNcell cultures. ReNcells had significant GFAP expression in both standalone and cocultures. GFAP expression was significantly suppressed within GBM cocultures compared to their standalone counterparts, on both day 5 and day 10 ($p < 0.05$). While TUJ1 expressing cells were the highest in standalone ReNcell cultures, coculturing with GBMs significantly reduced TUJ1 expression in these cultures. In both cocultures and standalone cultures, several cells appeared to be expressing “stemness” markers (CD-133 or Nestin) in conjunction with specific lineage markers (neural or glial).
Representative immunofluorescence images of ReNcells and GBMs, in standalone and cocultures, on day 10. For comparison, ReNcells cultures in the presence of bFGF were also processed after 24 h. Cultures were counterstained with DAPI for cell identification. Blue staining indicates nuclei, green indicates immature neurons (TUJ1), yellow indicates astrocytes (GFAP), red indicates CD133 (stem cell marker), and magenta indicates Nestin (neural stem cell marker). Scale bar: 50 µm.

Figure 24 NPC/GBM Immunofluorescence Images. Representative Immunofluorescence images of ReNcells and GBMs, in standalone and cocultures.
Figure 25 NPC/GBM phenotype. Quantification of ReNcell and GBM phenotype in standalone and cocultures on day 5 and day 10, staining for (A) CD-133, (B) Nestin, (C) GFAP and (D) TUJ1. Values are expressed as a percentage of the number of cells stained positively for the respective marker divided by the total number cells staining positive for DAPI. * indicates significant differences (p < 0.05) between day 5 vs. day 10, while # indicates significance in differences (p < 0.05) between standalone and cocultures of ReNcells or GBMs, at a given time point. Error bars represent mean ± SEM for all cases (n=3 for all cases and tests ± SEM).
**Axonal outgrowth and cell morphology**

Significant time-dependent and culture condition dependent effects on cell shape index (CSI) were noted (Fig. 26A). Stand-alone ReNcells had a more elongated shape highlighted by robust axonal extensions on both day 5 and day 10. When ReNcells were cocultured with GBMs they became significantly more rounded at both the time points tested ($p < 0.05$). Stand-alone GBMs were more rounded on day 5, but became significantly more elongated by day 10, a pattern that was also observed in ReNcell cocultures ($p < 0.05$ for both cases). Although no significant changes in CSI were observed in GBM cocultures for day 5 vs. day 10, GBMs in cocultures on day 5 were significantly more elongated than their standalone counterparts. Lastly GBM cocultures by day 10 were significantly more rounded than GBM cultures alone.

From the immunostaining images, axonal outgrowth within TUJ1-stained ReNcell cultures was quantified at days 5 and 10 (Fig. 26B). In general, at both the time points, axons within standalone cultures were 7-fold longer than that in cocultures ($p < 0.01$).
Figure 26 NPC/GBM CSI and neurite outgrowth. (A) Cell shape index (CSI) was measured using a particle analyzer plugin in ImageJ software, from images captured on days 5 and 10. CSI equal to one indicates infinitely elongated cell shape and a value of zero indicates perfectly rounded shape. (B) Average neurite outgrowth (µm) on days 5 and 10 was measured from TUJ1-stained cells within ReNcell cultures, in both standalone and coculture conditions. A NeuronJ plugin in ImageJ software was used for measuring average outgrowth. * indicates significant differences ($p < 0.05$) between day 5 vs. day 10, while # indicates significant differences ($p < 0.05$) between coculture vs. standalone cultures at a given time point. Error bars represent mean ± SEM for all cases (n=3 for all cases and tests ± SEM).

**Cell migration, speed and persistence**

From a scratch test assay, the number of migrating cells in standalone and cocultures were assessed over a 80 h duration using time-lapse imaging, along with the cumulative distance migrated and their average speed. The total width of the scratch was 600 µm, however given that cells could migrate from both sides of the scratch, the maximum distance the cells could travel was ≈ 300 µm. Representative images of ReNcells and GBM cells at various time points were shown in Fig. 27. The dotted line in each case indicates the demarcation of scratch from cell monolayers. Compared to that in standalone cultures, ReNcells
migrated faster and in more numbers within cocultures, although such clear distinction could not be made in GBM cell cultures.

The number of migrating cells in standalone and cocultures were quantified from these images (Fig. 28A). The number of cells migrating within standalone ReNcell and GBM cultures were low (~ 15-30) and remained constant for the duration of the study. However, among all the four test cases, significantly higher number of ReNcells migrated within cocultures at any time point, suggesting the influence of paracrine signaling from GBM cells. Cell speed and cumulative distance were also found to be time-dependent in each culture condition. The cumulative distance migrated by ReNcells in cocultures was also the highest among the four cases at any given time point (Fig. 28B). A majority of cells migrated aggressively over the first 20 hours covering almost 60 - 250 µm, after which they significantly slowed down. ReNcells remained dormant in standalone cultures for the remainder of the culture period, which is strikingly different from the additional 150 µm they traveled in cocultures. Although there were no significant differences in the total distance traveled by GBMs in standalone vs. cocultures, ReNcells in cocultures traveled significantly more distance than their standalone counterparts (254.5 µm vs. 69.7 µm, respectively, p < 0.05). For the first 8 hours of study, cells in all conditions moved rapidly at an average speed of 8 µm/h or higher (Fig. 28C). After that, GBMs in standalone and cocultures, and ReNcells in cocultures, moved at an average speed of 4 µm/h.
**Figure 27 NPC/GBM migration images.** Representative bright-field images at selected time points, obtained from a scratch test assay. ReNcells or GBMs were cultured in the 24-well plates, either standalone or in coculture (Transwell® setup) with the other cell type. Images were taken at regular intervals over an 80 h period to visualize cellular migration. Yellow dotted lines in each image represent the starting position of the cells (at t = 0) when the scratch was initially made. Scale bar is 100 µm.
Figure 28 NPC/GBM migration, distance, and average speed. (A) The number of ReNcells or GBMs, in standalone or cocultures, which migrated from their initial starting point into the cell-free zone created by scratch, was quantified at various time points. * indicates significant differences in coculture vs. standalone cultures. (B) The average cumulative distance (µm) covered by the cells was quantified at various time points. (C) The average cell speed (µm/h) was also quantified in these cultures at various time points (n=3 for all cases and tests ± SEM).
5.4 Discussion

Brain tumors are the second most common malignancy in younger populations and treated routinely by surgery in conjunction with chemotherapy and/or radiation therapies. Such removal of malignant brain tissue could result in loss of cognitive function as well as the unintentional death of surrounding healthy neurons [318]. Given that surgical or radiological intervention is necessary for a majority of these patients, researchers have begun exploring the utility of stem cells to restore the lost neurons and their functionality and improve the patient quality of life [56]. On the other hand, NPCs have been shown to be capable of tracking tumors which has led to the notion that these cells could also be engineered as delivery vehicles for targeting therapeutic agents to tumor sites [319]. Therefore, towards developing an expedient in vitro model that mimics tumor microenvironment, the current study describes a coculture model of human NPCs and pediatric GBM cells to ascertain the effects of paracrine signaling on their phenotype and functionality.

Cytokines and chemokines influence the development of primary tumors in malignant diseases, which has prompted further research in identifying the types, amounts and effects of such markers in a tumor microenvironment [172, 173]. The concentrations of several cytokines and chemokines produced by GBMs in this study (e.g., MCP-1, interleukins, Galactin-3) and the absence of some markers (e.g., IFN-γ) is in agreement with prior studies in this regard [163, 164, 320, 321]. We also noted that ReNcells expressed high levels of several analytes (e.g., MCP-1, PDGF-AA) and the cancer marker Galectin-3, even when
cultured in non-differentiating media. Unfortunately, limited information exists in literature on the cytokine and chemokine production levels by NPCs, as it is assumed that microglial cells are the primary contributors of these analytes in the CNS. In agreement with the data shown here, Sheng et al. reported that when NPCs were exposed to inflammatory stimuli such as TNF-α or IL-1β, they produce significant concentrations of MCP-1 and IP-10 [322]. Altering the types and amounts of these analytes in a microenvironment could regulate tumor cell phenotype (invasion, proliferation, migration, neo-angiogenesis, immune cell infiltration), as well as influence NPC development and behavior, presumably via activation of similar biochemical pathways (i.e. JAK/STAT or NF-κB) [323, 324].

Our results indicate that coculturing with GBMs and the presence of various cytokines and chemokines significantly influenced the patterns of NPC differentiation, morphology, and migration.

Previous reports have suggested that the release of cytokines (e.g., TNF-α) and interleukins by activated microglial cells after injury (both in vivo and in vitro) could be detrimental to NPC survival [289]. Other studies involving in vitro cocultures or in vivo glioblastoma injection have reported high NSC survival rates in a cancerous microenvironment [315, 325], which is in agreement with the results from our current study. Our data suggests that the panel of cytokines and chemokines produced by the GBM/NPC cocultures did not suppress their viability; in fact, cocultures marginally promoted cell survival. SJ-GBM2 cells in this study might have released a panel of analytes unique to the cancer milieu, which helps maintain ReNcell viability. In agreement with literature [326], we
noted significant viable and rapidly proliferating GBM cells in standalone and cocultures during the initial five days of culture. A probable contributing factor for the decrease in O.D. readings from day 5 to day 10 in GBM cultures might be the lack of available space for cells to expand in the culture wells, which was confirmed by examination under the light microscope. As reported in literature [327, 328], NPCs continued to proliferate in non-differentiation media, although the number of actively-dividing cells (BrdU-incorporated) was significantly reduced in differentiation media containing wells. A few ReNcells in cocultures were actively dividing even on day 10, possibly due to dysregulation of the STAT3 or Wnt pathway from signals elicited from the surrounding GBMs, suggesting these NPCs could be exhibiting a cancerous phenotype [306, 329].

NPCs respond to a variety of biochemical and spatiotemporal cues to induce differentiation into specific neural and glial lineages [328]. In vivo studies demonstrated the feasibility of NPC transplantation to repair diseased tissues in the brain, and coupled with the evidence that NPCs could survive in a cancerous microenvironment, it could be inferred that similar strategies could be used for regenerative purposes during or after cancer treatment [330, 331]. However, research within the cancer milieu is limited to studies focusing on the potential integration and delivery of anti-cancer drugs to the lesion site [315, 332]. Glass et al. observed in NSC-GBM explant cocultures that approximately 30% of NSCs stained positive for Nestin ("stemness" marker), and within that Nestin-positive subpopulation, 60% co-expressed GFAP and another 30% co-expressed the pre-neural markers (PSA-NCAM and DCx) [316]. Here we see similar patterns in co-
expression of specific neuronal or glial lineages coupled with markers for stemness. It has also been documented that pediatric glioblastomas express the GFAP marker, as well as the stemness markers Nestin and CD133 [307]. Hemmati et al. isolated cells from pediatric GBMs which displayed qualities more commonly observed in healthy NSC cultures, including the ability to form neurospheres and express neural markers [158]. Given that CD133-expression is not normally present in the adult brain, rather only in GBMs and NSCs, cells expressing these markers may indicate a potential transformation of a NSC to a cancer phenotype [314]. Furthermore, the cytokine/chemokine production in coculture conditions appears to influence cell fate [333]: downregulation of TUJ1 commitment in NPCs, reduced expression of GFAP in GBMs, as well as increased co-expression of CD-133 [334]. However, more studies are required to understand the precise mechanisms driving these changes, so that it could lead to integration of NSCs into this microenvironment without transformation to undesired lineages.

In standalone cultures, NPCs displayed an elongated shape highlighted by axonal and dendrite extensions [248], whereas confluent GBMs were generally small and rounded with star like filopodia [335]. However, in cocultures, their morphologies weren’t so different, as GBMs appeared elongated and NSCs became more rounded. This suggests that NPCs were forced to differentiate into glial lineage, initiated by the presence of cytokines via a notch signaling pathway [336]. Given the similarities in the GBM cell proliferation within standalone and cocultures, and the lack of elevated staining for specific neuronal markers, the
morphological data gathered from GBM cocultures is not enough evidence to suggest these cells are losing their cancer phenotype.

Alterations in axonal outgrowth have been attributed to the Rho signaling pathway [337], and could be differentially regulated by the presence of several cytokines and chemokines [338]. Since ReNcells were found to have a more rounded morphology in cocultures, axons from TUJ1-positive cells were measured and compared, which revealed reduced neurite outgrowth in coculture conditions. This suggests the role of cancer cell microenvironment in inhibiting the axonal elongation, which is a crucial obstacle that must be overcome to regenerate any lost neuronal network *in vivo*. We hypothesize that a strategic combination of growth factors [339] and biological scaffolds [340] might help in shielding and promoting robust axonal outgrowth to overcome this obstacle.

Research quantifying NPC migration in a cancerous microenvironment has recently gained momentum as NPCs could act as a delivery vehicle for chemotherapeutics [24, 315, 319]. Aboody *et al.* observed the ability of NPCs to surround, track and target glioblastoma cells, both *in vivo* and *in vitro*, likely due to a complex mixture of biochemical signals acting as attractants, adhesion and substrate molecules, and chemokines [24]. Although they did not do further analysis, our cytokine and chemokine data supports their notion that a complex cocktail of signaling molecules were released in this coculture microenvironment. Using a similar scratch test, Natarajan et al. observed comparable number of migrating GBM cells, which they attributed to changes in focal adhesion kinases (FAK) modulated by several key analytes in the microenvironment [341]. Using
Boyden chambers, Heese et al. observed an increase in NSC migration when exposed to several GBM cell lines, or under exposure to analytes such as TGF-α and PDGF-AA [342]. Debray et al. noted that glioblastoma motility on laminin-coated surfaces was directly affected by the presence of Galectin-3, which has the ability to modulate both the α6 and β1 integrin expression and cause cellular locomotion [343]. Our results on GBM speed were strikingly similar to that of Chicoine et al. [344], however the calculated GBM motility coefficient in this study is much larger than previously reported values [345] which we believe is due to more robust modeling and the ambiguity of persistence time in mammalian cells. However, future studies calculating individual cell persistence time will negate or verify these values. In summary the data presented here demonstrated an increased NPC migration in the presence of GBMs, in terms of cell speed and number of cells migrating, which could have practical applications in targeted cell therapy and enhanced regeneration of lost tissue.

Individual cell motion can be described mathematically in terms of random motility coefficient ($\sigma$), cell speed ($s$) and persistence time ($p$) [defined as the length of time that the cell moves without changing its direction significantly]. For sufficiently long times ($t \gg p$), the random motility coefficient in a two-dimensional geometry is related to these parameters as $\sigma = (1/2)s^2p$. Assuming our model follows a persistence time of 9 h and the front of the cells is considered as one unit, cell speed can be extracted from that data point. The assumption of 9 hours was calculated via qualitative analysis of the individual images acquired, however as mentioned previously future studies will take a more robust approach in
tracing individual cell motion to verify or negate this value. Table 10 describes the random motility coefficient under each condition. It should be noted that in coculture cases, motion is likely no longer random, as cells are under the chemotactic influence of the corresponding cell cultures with them.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Speed (µm/h)</th>
<th>Motility coefficient (cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ReNcells alone</td>
<td>5.33</td>
<td>$1.27 \times 10^{-6}$</td>
</tr>
<tr>
<td>GBMs alone</td>
<td>12.1</td>
<td>$6.5 \times 10^{-6}$</td>
</tr>
<tr>
<td>ReNcells alone + bFGF</td>
<td>6.5</td>
<td>$1.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>NPC coculture</td>
<td>9.45</td>
<td>$4.01 \times 10^{-6}$</td>
</tr>
<tr>
<td>GBM coculture</td>
<td>17.87</td>
<td>$9.95 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

5.5 Conclusions of this study

Alternate therapeutic strategies to disrupt the progression of pediatric glioblastomas could lead to increased patient survival rates. Given the similarities in the molecular pathways driving NPC and GBM differentiation, survival, and proliferation, studies like this provide insight into the ideal microenvironment needed for NPCs to perform their intended functions. Our results suggest that within cocultures, a unique panel of analytes is produced which was regulating NPC migration, lineage commitment, and neurite outgrowth. Given that NPC survival and migration was not compromised in this coculture microenvironment, they still offer a suitable platform to deliver anti-tumor drugs. Given the
unpredictability of NPC differentiation in this microenvironment, coupled with the lack of axonal outgrowth, further analysis of the biochemical markers produced by both cell types in cocultures, as well as an increased understanding of the molecular pathways involved, could provide the requisite details needed to fully utilize NPCs for regenerative medicine applications in brain cancer.
CHAPTER VI

CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Conclusions

The emerging field of regenerative medicine is exploring therapies for conditions that in the past were considered untreatable (e.g., cancers, neurological conditions, heart disorders, etc.). Specific to the neural milieu, regeneration via an exogenous cell source is a promising treatment option, given the CNS’s lack of self-repair capabilities. As detailed in this dissertation, tissue engineering approaches explored by researchers and clinicians can provide treatment options tailored to meet individual patient needs. In this study, we systematically identified and quantified several of the components in the surrounding microenvironment (healthy and inflammatory) directing cellular fate, which could be further explored for their efficacy and potential in vivo. Furthermore, here we show the potential NSC or NPCs have as an effective exogenous cell sources, which when exposed to the appropriate cues, may help regenerate what the body itself cannot.
The work presented here is united by the feasibly of translating the work from the bench to the clinic. For example, the composition of the scaffolds (aim 1) were created from compounds which could be easily injected into the human body and facilitate cues that would allow for rapid host integration as well as tailored differentiation. This idea was continued in the second aim, with the understanding upon injection, cell-laden scaffold would be exposed to an inflammatory environment that would differentially regulate cell viability and fate, specifically increase neurite outgrowth and GFAP lineage commitment, while simultaneously decrease TUJ1 lineage commitment and viability. In the final aim, experiments were performed to understand how the delivered NPCs would react in a tumor microenvironment or how NSCs interact with glioblastoma cells in vivo. The outcomes from all the aims could be compared and integrated to further develop repair strategies. Furthermore, this study also addresses some long-standing issues in tissue engineering approaches: Is 3D culture superior to 2D? Are cell responses similar when exposed to individual analytes vs. full panel addition? In summary, the work presented here describes a tissue-engineered approach to utilize NSC or NPC laden injectable scaffolds to regenerate neural tissue lost after injury or disease. Table 11 summarizes the specific results of each studying, addressing these questions.
Table 11 Summary of the results from each individual aim. The aims, hypothesis, and outcomes of the work in this dissertation

<table>
<thead>
<tr>
<th>Aim 1</th>
<th>Aim 2</th>
<th>Aim 3</th>
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<tbody>
<tr>
<td>Scaffold selection influences NSC phenotype and genotype</td>
<td>Inflammatory microenvironment created by activated microglial cells influences NSC fate</td>
<td>Altered microenvironment created by pediatric glioblastomas in influences NPC fate</td>
</tr>
<tr>
<td>• Increased NSC viability</td>
<td>• Production of a panel of cytokines and chemokines</td>
<td>• Production of both cytokines, chemokines, and cancer analytes</td>
</tr>
<tr>
<td>• Predictable lineage differentiation</td>
<td>• Reduced NSC viability</td>
<td>• No changes NPC viability</td>
</tr>
<tr>
<td>• Enhanced axonal outgrowth to repair lost circuitry</td>
<td>• Altered differentiation</td>
<td>• Changes in NPC morphology</td>
</tr>
<tr>
<td>• Physical and mechanical properties similar to native ECM</td>
<td>• NSC fate Different in 2D and 3D microenvironments</td>
<td>• Modulation of differentiation patterns</td>
</tr>
<tr>
<td>• RA is able to rescue NSC functionality</td>
<td>• Increased NSC axonal outgrowth</td>
<td>• Decreased NSC axonal outgrowth</td>
</tr>
<tr>
<td>• Injectable</td>
<td></td>
<td>• No changes in proliferation of both cell types</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Extensive migration of both cell types</td>
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</tbody>
</table>

6.2 Future recommendations

1) Qualitative immunofluorescence images from aims 1 and 2, using murine embryonic NSCs, were inferior to that from aim 3 using ReNcell NPCs. If ReNcells were used for the entire study, the quality of images would have been relatively better; however the behavioral patterns would likely significantly change, and the data would be significantly different. Although ReNcells act as a model cell line and the outcomes are crucial to understand developmental biology aspects as well as design tissue engineering approaches, they might not be as
clinically applicable as freshly harvested murine NSCs, which have already shown some promise in clinical trials.

2) Mechanical testing of scaffolds using a rheometer or DMA requires a large sample size, whereas an atomic force microscope (AFM) would use less material. Furthermore, AFM would enable simultaneous probing, imaging and mechanical testing at sub-micron level which may be better suited and more accurate for this type of work. Unfortunately technical limitations at the time of this study prevented reliable data acquisition.

3) Performing RT-PCR analysis beyond cycle 40 should be considered for checking the expression of some genes which weren’t detected below cycle 40. Besides, using primary cell lines such as murine NSCs had some technical limitations with regard to the available cell density, which could be overcome using immortal cell lines such as ReNcells.

4) Scaffold design in the first aim of this study was limited to include just 4 biological components, however based on the results, it may be worthwhile testing more compositions of scaffolds by varying the components and their concentrations. This type of study would be well-suited for a high-throughput platform, such as that present in Dr. Lee’s group here at CSU, which at the time of the study was not available yet.

5) The cell migration data observed from the experiments in aim 3 could be further analyzed. Software systems such as “celltrack” (open source software available from The Ohio State University) may be applied to measure the
individual movement of each cell, and provide more precise and accurate data on motility and persistence length and time.

6) The specific analytes produced in high levels in cancer cell – ReNcell cocultures (aim 3) could be supplemented individually to the culture media, as performed in aim 2, to better understand which analyte is specifically influencing NPC differentiation patterns. It may also be beneficial to perform mixed additions of pre-conditioned culture media from each cell type and vice versa which could provide a more clear picture of which cells are producing what analytes. This type of work would be more suited for a high-throughput platform and may be explored in the future.

7) To further expand on the specific signaling pathways regulating differentiation and axonal growth, Western blots or ELISA based assays should be considered. This type of analysis was implemented in projects beyond that presented here (e.g., NSC toxicology studies), but not performed in this work.

8) In vivo studies should be performed to follow up the outcomes in aim 1 of this study. Specifically, the amount of time the scaffolds would remain intact in the body, as well as an understanding of the immunological response from these scaffolds should be investigated.

9) In addition to quantifying the NSC phenotype and genotype, it would be interesting to use a patch clamp or some form of electrophysiology studies to check if differentiated NSCs or NPCs in our cultures are transmitting electrical
signals. This would aid in understanding their level of maturity. While this idea was explored, we were unable to find facilities locally to aid in this work.

10) To expand on the objectives of aim 3, it would be beneficial to design a microfluidic device to quantify the migration patterns of ReNcell chemotaxis under gradients of analytes produced GBM cells, and vice versa. A scratch test is a simple and effective way to understand migration, however, it lacks directionality and induction of gradient of biochemical cues. These limitations could be overcome using a microfluidic platform.

11) While the visual of representation of the scaffold integrity after 10 days of information provides qualitative data support its ability to main its structure for a prolonged period of time, quantitative data demonstrating the degradation of the scaffolds in the presence of biologically relevant enzymes would further support the data presented in aim 1.

12) Further rheological analysis which measured changes in G’ and G” while increasing temperature would aid in the understanding of the specific point of crosslinking of the gels, and at what point they transition for a polymer solution to a crosslinked gel.

13) The addition of positive and negative controls to the 3D cellular staining images would aid in insuring the scaffolds were not creating artifacts or representing a phantom staining signal. Negative controls (the addition of stains with no cells) were used but not mentioned in the paper. Positive controls (cells
known to stain for the marker) were not used in this study. In the future both of these controls should be run and mentioned to verify the data presented.
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A.1 Mechanical data on additional scaffolds

Table 12 lists the original scaffolds tested in this study (it should be noted their differences in nomenclature here the what was presented in aim 1 of this thesis). Furthermore, one additional stand-alone scaffold was tested, collagen 2.0 mg/mL.

Table 12 Composition of additional scaffolds. The composition and naming scheme for the blended scaffolds tested in this dissertation. Given the low vitality in scaffolds contacting 2.0 mg/mL collagen scaffolds, case 2 and 4 were excluded from the write-up, and case Case 1 and 3 were re-named case 1 and 2 respectively.

<table>
<thead>
<tr>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Hyaluronic acid (&gt; 1.2 MDa) 2% w/v</td>
<td>50% Hyaluronic acid (&gt; 1.2 MDa) 2% w/v</td>
<td>50% Hyaluronic acid (&gt; 1.2 MDa) 1% w/v</td>
<td>50% Hyaluronic acid (&gt;1.2 MDa) 1% w/v</td>
</tr>
<tr>
<td>20% Type I Collagen (1.2 mg/mL)</td>
<td>20% Type I Collagen (2 mg/mL)</td>
<td>20% Type I Collagen (1.2 mg/ml)</td>
<td>20% Type I Collagen (2 mg/ml)</td>
</tr>
<tr>
<td>20% CSPG</td>
<td>20% CSPG</td>
<td>20% CSPG</td>
<td>20% CSPG</td>
</tr>
<tr>
<td>10% Laminin</td>
<td>10% Laminin</td>
<td>10% Laminin</td>
<td>10% Laminin</td>
</tr>
</tbody>
</table>

Mechanical properties of scaffolds were measured using DMA compression testing as outlined in aims 1 (Fig. 29D). Specifically, 2 mg/mL collagen has the highest modulus (528.8 Pa), followed by the blended collagen 2 mg/ml 2% HA scaffold. Blended scaffolds containing 1% HA seem to have a similar modulus regardless of the concentration of collagen added. Stand-alone HA 1% scaffolds have the lowest modulus. In general it appears that concentration of collagen contained in the scaffold accounts for an increased mechanical modulus.
The interconnecting pore morphology of all the scaffolds from aim 1, (including the excluded scaffolds) was revealed by scanning electron microscopy (Fig. 29A). As observed in aim 1 of this study, the walls of the stand-alone HA scaffolds are sheet-like in appearance whereas the stand-alone collagen scaffolds are fiber-like. In comparison, the blended scaffolds appear to be highly porous and are lacking the sheet-like appearance seen in the stand-alone HA. The addition of HA to the collagen scaffolds increased the water uptake capability, because of the hygroscopic nature of HA (Fig. 29B). Stand-alone collagen scaffolds had the lowest water uptake, whereas HA and the HA blended scaffolds appear to have a much greater fold increase in weight (after 5 minutes, ~3-fold increase for stand-alone collagens, whereas composites and HA stand alone exhibited 6, as high as 9 fold increase). Besides the ability to uptake water, overall scaffold porosity was examined (Fig. 29C). The data suggests that HA 1% scaffolds have the highest level of porosity, followed by HA 2. Blended scaffolds as expected have porosity in between the stand-alone collagen and HA scaffolds.
A.2 NSC differentiation, viability and outgrowth on additional scaffolds

Differentiation patterns in the blended scaffolds not presented in aim 1 followed trends similar to the blended scaffolds discussed in chapter 3 (Fig. 30A).

In general, NSCs cultured in HA based scaffolds resulted in a 10-15% higher neural differentiation rate than those cultured in collagen based scaffolds. Both collagen concentrations resulted in ~20% astrocyte lineage commitment with no
significant difference across concentration (p > 0.05). When HA (2% w/v) was blended with collagens of either concentration there was no significant effect on neuronal lineage commitment. However, low concentration HA with high concentration collagen-1 (case 4) produced the highest level of astrocyte lineage commitment, which is also significantly greater than any of the stand-alone constructs (p < 0.05). In general, HA based scaffolds appear to elicit a greater astrocyte lineage commitment (20-30%) than collagen scaffolds (p > 0.05), with the exception of HA 1% compared to collagen 1.2 mg/mg which produced statistically similar values. Oligodendrocyte lineage commitment in collagen 2 mg/ml, HA 1% and HA 2% based scaffolds all produced statistically similar values (~20% lineage commitment p > 0.05).

Varying concentrations of collagen-1 did not significantly modulate Neurite outgrowth (Fig. 30B,C). As described in aim 1, HA 1% produced a four-fold increase in NSC neurite length and HA 2% produced a two-fold increase in neurite outgrowth compared to both concentrations of collagen-1 scaffolds (Fig. 11C). In fact case 2 (high concentration of both HA and collagen) was significantly similar to stand-alone collagen scaffolds, which in general did not promote axonal outgrowth. This data points to an overall superiority of HA scaffolds over collagen-1 scaffolds in simulating the physicochemical ECM of native CNS tissue. Live/Dead™ assay revealed no significant differences in the cell survival over the 10 day culture period for the 4 samples (p > 0.05). It should be noted that < 75% of cells survived in all cases (Fig. 30D, E). The low viability of NSCs in case 2 and 3 shown in Fig. 30 lead to the decision to discontinue the
study of cells in this micro environment. This notion was supported by the literature suggesting this microenvironment is likely mimicking the harsh conditions contained in a glial scar (see chapter 2).

A

![Graph showing the percentage of NSCs differentiated in different conditions.](image)

B

![Graph showing neurite outgrowth in different conditions.](image)

C

![Graph showing neurite outgrowth in different cases.](image)
Figure 30 NSC fate in additional scaffolds. Comparison of the (A) differentiation, (B, D) stand-alone scaffold neurite outgrowth and survival, (C, E) blended scaffold neurite outgrowth and survival of the scaffolds described in chapter 3 as well as scaffolds not deemed suitable for NSC growth (n=3 for all cases and tests ± SEM).

A.3 NSC differentiation and outgrowth on additional scaffolds containing RA

In general, the addition of RA seemed to aid in the survival and neurite outgrowth of NSCs (Fig. 31A). The addition of RA elicited a higher NSC commitment to the astrocyte (GFAP) and decreased oligodendrocyte (MBP) in case 2, which would not be advantageous for tissue-engineered applications. However, RA did effect on neurite outgrowth in all of the blended scaffolds, as demonstrated by the nearly two-fold increase in neurite length in cases 1,2,4 (Fig. 31B). Thus the data suggests that RA is able to rescue neurite outgrowth even in the blended scaffolds not used, however it didn't significantly alter viability (data
not shown) which again lead to the choice to only focus on scaffolds containing 1.2 mg/mL concentrations of collagen.

![Graph A](image)

**Figure 31 NSC fate in additional scaffolds containing RA.** Comparison of the (A) differentiation and (B) neurite outgrowth in the scaffolds described in chapter 3 and the scaffolds deemed not suitable in the presence of RA (n=3 for all cases and tests ± SEM).
A.4 NSC viability in the presence of chemical cross-linkers

NSCs were cultured in the presence of 3 commonly used chemical cross-linkers, Genipin (Sigma), Divinyl sulfone (DVS; Sigma), and N-(3-Dimethylaminopropyl)-N’-ethyicybodiimide hydrochloride (EDC; Sigma) for a period of 24 h to evaluate their residual effect on cellular viability. Culture media was added containing varying dosages of a given cross-linker as well as controls. Upon completion of the study cell viability was quantified using a LIVE/DEAD® viability and cytotoxicity kit (Life Technologies, Carlsbad CA). Both qualitative and quantitative suggest that residual amounts of chemical cross-linker decrease NSC viability (Fig. 32). Here we demonstrated exposure nanomolar concentrations of crosslinking agents significantly effects cell viability (p < 0.05 for all concentrations vs. control). The toxic nature of the chemical requires repeated washing after initial crosslinking to reduce the residual molar concentration within the scaffold. The data here suggested that all concentrations of residual crosslinker tested significantly reduced cell viability (p < 0.05 for all concentrations vs. control). Chemical crosslinkers are widely used to enhance scaffold properties, and while our data does prove residual amounts left within the scaffold do in fact decrease NSC viability, the counterpoint would be that groups using this technique ensure that all of the crosslinker would be fully removed from the scaffold before cell seeding, and the scaffolds do not leach the crosslinker into the microenvironment. Thus the choice was made to exclude this data, given it assumes those groups using these chemicals do not properly
remove all residual crosslinker, and we did not test chemically crosslinked scaffolds (found in literature) leech the crosslinker.

Figure 32 Effect of residual crosslinker on NSC fate. The effect of residual chemical crosslinkers on NSC viability (n=3 for all cases and tests ± SEM).
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