Hepatocyte Growth Factor-Preconditioned Neural Progenitor Cells Attenuate Astrocyte Reactivity and Promote Neurite Outgrowth

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Abstract

The astroglial scar is a defining hallmark of secondary pathology following central nervous system (CNS) injury that significantly obstructs neuroregeneration. Neural progenitor cell (NPC) therapies for repair and regeneration have demonstrated favourable outcomes not only via cell replacement, but trophic support. In an effort to strategically enhance this secretory potential, we utilized the regenerative aptitude of hepatocyte growth factor (HGF) as a cellular preconditioning agent and assessed the capacity of modified secretome to attenuate astrocyte reactivity \textit{in vitro}. HGF-preconditioned NPCs demonstrated increased levels of tissue inhibitor of metalloproteinases-1 and reduced vascular endothelial growth factor compared to untreated NPCs. In reactive astrocytes, HGF-enhanced NPC secretome reduced glial fibrillary acidic protein expression and chondroitin sulfate proteoglycan deposition to a greater extent than either treatment alone, and enhanced neurite outgrowth of co-cultured neurons. These findings have important translational implications for modification of the post-injury milieu and optimization of cell-based strategies for CNS injury.
Acknowledgments

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List of Abbreviations

AKT: RAC-alpha serine/threonine-protein kinase

ALS: amyotrophic lateral sclerosis

BBB/BSCB: blood-brain-barrier/blood-spinal cord-barrier

Bcl-2: B-cell lymphoma 2

BDNF: brain derived neurotrophic factor

cAMP: cyclic adenoside monophosphate

CCR: chemokine receptor

ChABC: chondroitinase ABC

c-Met: tyrosine-protein kinase Met

CM: conditioned media

CNS: central nervous system

CNTF: ciliary neurotrophic factor

CSPG: chondroitin sulfate proteoglycans

CST: corticospinal tract

DAPI: (4',6-diamidino-2-phenylindole)

DMEM: Dulbecco's Modified Eagle's Medium

ECM: extracellular matrix

EGF: epidermal growth factor
ERK: extracellular signal-regulated kinase
ESC: embryonic stem cell
FBS: fetal bovine serum
FGF: fibroblast growth factor
GAG: glycosaminoglycan
GFAP: glial fibrillary acidic protein
GDNF: glial derived neurotrophic factor
H$_2$O$_2$: hydrogen peroxide
HIF-1$\alpha$: hypoxia-inducible factor 1
hiPSC: human-induced pluripotent stem cell
HGF: hepatocyte growth factor
ICC: immunocytochemistry
IGF-1: insulin-like growth factor 1
LIF: leukemia inhibitory factor
MAG: myelin-associated glycoprotein
MAI: myelin associated inhibitor
MAPK: mitogen-activated protein kinase
MAP2: microtubule-associated protein 2
MSC: mesenchymal stem cell
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBM: neurobasal medium

NDM: neural differentiation medium

NG2: neural/glial antigen 2

NGF: nerve growth factor

NPC: neural progenitor cell

NSC: neural stem cell

NT: neurotrophin

OMGP: oligodendrocyte myelin glycoprotein

OPC: oligodendrocyte progenitor cell

PBS: phosphate buffered saline

PDGF: platelet-derived growth factor

PI3K: phosphoinositide 3-kinase

PirB: paired immunoglobulin-like receptor B

PLCγ: phospholipase Cγ

PNS: peripheral nervous system

SCI: spinal cord injury

SDF-1: stromal cell-derived factor 1

SDS: sodium dodecyl sulfate
SEM: standard error of the mean

SGZ: subgranular zone

STAT: signal transducer and activator of transcription proteins

SVZ: subventricular zone

TBI: traumatic brain injury

TGFβ: transforming growth factor beta

TIMP-1: metalloproteinase inhibitor 1

VEGF: vascular endothelial growth factor

WB: western blot
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Chapter 1
Literature Review

1 Literature Review

1.1 Classification and Pathophysiology of Traumatic Injury in the Central Nervous System

Traumatic injury to the brain and spinal cord are among the most serious public health problems worldwide. Traumatic brain (TBI) and spinal cord injuries (SCI) comprise approximately half of all trauma-related injuries and are the leading causes of death and disability globally, carrying significant personal and socioeconomic costs (Hyder et al. 2007; Najem et al. 2018; Kraus 1996). The brain and spinal cord are the main constituents of the central nervous system (CNS), with the brain serving to coordinate higher-level functions and the spinal cord relaying information to and from the periphery (Najem et al. 2018).

Traumatic brain or spinal cord injuries typically arise when an external mechanical force causes neurological damage. The degree of subsequent functional deficit is largely dependent on the location and severity of insult (Kraus 1996). CNS injury is a continuous and bi-phasic process stemming from the initial mechanical insult or ‘primary injury,’ and progressing through a cascade of cellular and molecular processes termed the ‘secondary injury,’ ultimately resulting in exacerbated tissue damage and functional impairment (Kraus 1996).

1.1.1 Traumatic Brain Injury

It is estimated that approximately 1.4 million new cases of TBI are diagnosed in the United States annually, resulting in 50,000 deaths and 80,000 disabilities (Hyder et al. 2007). Most injuries are incurred by young adults, with the majority attributed to falls, strikes to the head, or motor vehicle crashes (Hyder et al. 2007). TBI is largely heterogeneous and dependent on
type, direction, and duration of force. Most cases involve a combination of impact and acceleration or deceleration of the head, and injuries are subsequently categorized as either focal or diffuse injuries, stemming from contact or noncontact forces, respectively (Hyder et al. 2007; Najem et al. 2018).

The CNS is comprised of grey and white matter, with the former containing mostly neuronal cell bodies and the latter containing myelinated axon tracts. As grey matter is widely distributed at the surface of the cerebral hemispheres and cerebellum, primary brain injury often results in widespread neuronal death and damage (Mouzon et al. 2014). High-speed impacts, however, may also result in axonal damage in deeper regions. A large number of patients that succumb to TBI do not die on impact, rather days to weeks afterward (Hyder et al. 2007). This effect is largely attributed to the secondary injury process which dramatically worsens the initial damage. Secondary injury events include damage to the blood-brain barrier and axonal tracts, neuroinflammation, excitotoxicity, progressive tissue necrosis, ischemia, cerebral hypoxia, edema, and raised intracranial pressure (Hyder et al. 2007).

The damage sustained from TBI often results in motor and cognitive deficits, the magnitude of which are dependent on injury severity. Damage can be either focal (confined to certain areas), diffuse (widespread), or both. Focal injuries are often the result of direct loading, causing macroscopic damage that is visible at the site of impact. Approximately half of all severe TBIs are focal injuries, carrying the highest incidence of mortality (Mouzon et al. 2014). Clinical presentation of symptoms is typically associated with the function of the anatomical area that sustained injury. The Glasgow coma score is a conventional tool utilized to assess the general level of consciousness in patients with TBI, with scores of 13-15 classified as mild injury, 9-12 as moderate, and 3-8 as severe (Najem et al. 2018). While clinical management of TBI is predominately geared toward patient stabilization, these actions do not effectively target progressive tissue damage nor promote repair and regeneration. Experimental therapies have traditionally consisted of pharmacological agents designed to reduce secondary tissue damage, yielding modest improvements in clinical outcomes (Mouzon et al. 2014).
1.1.2 Spinal Cord Injury

The global prevalence of SCI has been estimated to be between 236 and 1,009 per million, the majority of which are accredited to motor vehicle accidents, falls, and violence (Cripps et al. 2011). Mortality is typically highest in the first year after injury and largely dependent on injury level and severity, as well as access to timely and quality medical intervention (Cripps et al. 2011). Alongside direct physical health consequences, SCI carries substantial personal and socioeconomic costs. According to Krueger et al, the estimated lifetime economic burden accompanying SCI in Canada ranges from approximately CAD $1.5 million for an individual with incomplete paraplegia to $3 million for an individual with complete tetraplegia. Annually, the estimated economic burden associated with SCI in Canada is approximately CAD $2.5 billion (Krueger et al. 2013).

Similar to TBI, SCI is a biphasic and dynamic injury process characterized by an initial mechanical insult to the cord, followed by a cascade of intracellular and pathological proceedings ultimately comprising the secondary injury process. The primary injury phase, or mechanical trauma to the cord, largely results in widespread tissue necrosis and vascular disruption. Most cases of SCI are characterized by a contusion and subsequent compression. Very rarely is the cord fully transected, and, as such, the damaged but spared circuitry has become a target of interest for regenerative therapies striving to unlock or manipulate this endogenous potential (A. J. Mothe and Tator 2012).

While the primary injury phase occurs directly at impact, the secondary injury phase commences hours after insult, resulting in a surge of cellular and molecular changes both in and surrounding the lesion epicenter. As in TBI, these changes include exacerbation of cellular apoptosis and vascular disruption, oxidative stress, neuroinflammation, reactive astrogliosis, and formation of the glial scar. Apoptosis of resident oligodendrocytes manifests in axonal demyelination and further degeneration of white and grey matter surrounding the lesion center, peaking at approximately 1 week after injury and persisting until 3 weeks thereafter (Kraus 1996). In contrast, disruption of the blood-spinal cord barrier peaks at approximately 24 hours after injury, persisting to 2 weeks thereafter. During this time,
activated macrophages and resident microglia infiltrate the lesion site and contribute to further tissue damage (Kraus 1996).

A largely heterogeneous injury, SCI can result in symptoms ranging from chronic pain to complete loss of motor and sensory functions below the level of injury. The American Spinal Injury Association impairment scale is typically used to rank the severity of injury on a gradient from A to E, with A classifying complete lack of motor and sensory function below the level of injury and E classifying the return of all neurological function (Kraus 1996).

While traditional management of SCI has focused on treating secondary complications and providing rehabilitative therapy, further investigation and understanding of underlying cellular and molecular mechanisms have allowed for the evolution of novel treatment paradigms.

1.2 Barriers to Regeneration

Injury to the CNS can significantly compromise the functional circuitry of neurons and culminate in severe functional impairments. Traumatic brain and spinal cord injuries result in complex cellular and molecular interactions within the CNS. While these processes may arise in an effort to limit further tissue damage, they can also significantly obstruct neuroregeneration and functional recovery. One key contributing reason for the devastating and persistent disabilities after CNS injury is the failure of injured but spared axons to regenerate and re-build functional circuits (Adams and Gallo 2018). Thus, long-standing therapeutic goals have focused on unlocking this regenerative capacity in an effort to promote axon regeneration and functional restoration. Neuronal regeneration after SCI is influenced by a number of factors including inflammatory cell infiltration, glial scarring, and upregulation of inhibitory molecules (Table I) (Michael T Fitch and Silver 2008). Elucidating why injured axons cannot regenerate after injury has been a major challenge both pre-clinically and clinically; as such, studies have investigated the molecular and functional mechanisms of regenerative constraints within the CNS for targeted therapeutic intervention (F. Sun and He 2010)
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Description and Inhibitory Properties</th>
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| **Myelin-associated glycoprotein (MAG)** | - Type-1 transmembrane protein expressed by myelinating glia, Schwann cells, and oligodendrocytes  
- Enriched in periaxonal membrane of myelin sheaths allowing for formation of receptor complexes on axonal surface (Trapp et al., 1989)  
- MAG knockouts demonstrate delayed oligodendrocyte differentiation and transient hypomyelination (Li et al., 1994; Montag et al., 1994; Pernet et al., 2008) |
| **Nogo-A** | - Membrane-associated protein belonging to reticulon family  
- Nogo-66 and Nogo-20 identified as growth inhibitory motifs (Fournier et al., 2000; Oertle et al., 2003)  
- Highly expressed in oligodendrocytes; important regulator of myelin development  
- Reduced OPC differentiation and aberrant myelination observed in NogoA-null mice (Chong et al., 2012) |
| **Oligodendrocyte myelin glycoprotein (OMgp)** | - Glycosylphosphatidylinositol-anchored protein expressed by oligodendrocytes and neurons in the CNS  
- Enriched in CNS myelin; OMgp blockade or knockout demonstrates impaired differentiation of POCs into mature oligodendrocytes as well as hypomyelination (Huang et al., 2012; Lee et al., 2011) |
| **Semaphorin-3a** | - Member of the semaphorin family; critical in neuronal pattern development  
- Highly expressed in scar tissue following CNS injury, resulting in inhibition of neuronal regeneration and myelination (Mecollari et al., 2014)  
- Shown to inhibit OPC differentiation and contribute to remyelination failure (Syed et al., 2011) |
| **Chondroitin Sulfate Proteoglycans (CSPGs)** | - ECM proteoglycans comprised of a protein core and covalently attached GAG side chain  
- Secreted by astrocytes, neurons, and oligodendrocytes  
- Highly enriched in reactive astroglial scar after injury, constraining regenerative growth and restricting plasticity (Bradbury et al., 2002; Morgenstern et al., 2002; Silver and Miller, 2004) |
1.2.1 Limited Regenerative Capacity of Spared Axons

Contrary to earlier claims that axons in the adult CNS are unable to regenerate following injury, several studies later demonstrated the capacity of spared axons to spontaneously sprout both within and proximal to the lesion site (Tom et al. 2004). This regenerative capacity, however, is limited as newly formed growth cones quickly become dystrophic and are unable to form long-distance synaptic connections. Studies have demonstrated conversion of actively extending axons to dystrophic states when exposed to a gradient of inhibitory extracellular matrix (ECM) molecules (Kerschensteiner et al. 2005; Tom et al. 2004). These dystrophic growth cones, which exhibit dynamic properties in vitro and in vivo, remain stalled in an inactive state unable to produce long-distance regeneration (Kerschensteiner et al. 2005; Tom et al. 2004). Interestingly, studies have demonstrated the capacity of CNS axons to produce long-distance connections when exposed to the peripheral nervous system (PNS) environment, although regeneration failure ensues upon re-exposure to the CNS environment (David and Aguayo 1981; Richardson, Issa, and Aguayo 1984). Other classical studies have further highlighted the distinct contrast between PNS and CNS regenerative capacity, with sensory dorsal root ganglion neurons exhibiting long-distance regeneration after injury in the former, but not in the latter (Golding, Shewan, and Cohen 1997; Kliot et al. 1990). These findings attest to the inhospitable and growth inhibitory environment of the injured CNS. As such, studies have focused on classifying the molecular mediators of growth inhibition for targeted therapeutic intervention.

The identification of growth-inhibitory factors in the CNS have allowed for better targeted therapeutics to foster regeneration in the injured CNS. Two major classes of regenerative inhibitors in the CNS are myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs). Consequently, targeted interference of either of their functions have conferred some degree of axonal growth (Bomze et al. 2001; Neumann and Woolf 1999).

MAIs are proteins comprising myelin within the CNS that are largely expressed by oligodendrocytes. MAIs have been shown to impair neurite outgrowth in vitro and are thought to limit axon growth in vivo following CNS injury. Some commonly known MAIs
include Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (Chen, Bailey, and Fernandez-Valle 2000; GrandPré et al. 2000; McKerracher et al. 1994; Benson et al. 2005; Moreau-Fauvarque et al. 2003). These MAIs interact with Nogo-66 receptor 1, consequently limiting axon growth. These ligands also demonstrate an affinity for the axon growth-inhibiting receptor paired immunoglobulin-like receptor B (Atwal et al. 2008). Studies that have genetically deleted Nogo-A have demonstrated increased corticospinal (CST) and raphespinal tract growth as well as functional improvements after SCI (Simonen et al. 2003; B. Zheng et al. 2003; Dimou et al. 2006). Moreover, antibodies that target and inhibit Nogo-A have demonstrated increased axonal growth and functional recovery after CNS injury, even progressing to clinical trials in SCI (Seymour et al. 2005).

CSPGs are among the main inhibitory molecules found in the astroglial scar that significantly impede axonal growth and neuroregeneration following injury to the CNS. Reactive astrocytes comprising the glial scar markedly upregulate CSPGs that are both membrane bound and secreted into the extracellular space (Morgenstern, Asher, and Fawcett 2002; Silver and Miller 2004b). Members of this class include neurocan, versican, brevican, phosphacan, aggrecan, and neuron-glial antigen 2 (NG2) (Asher et al. 2000; Schmalfeldt et al. 2000; Inatani et al. 2001; Dou and Levine 1994). As such, understanding these molecules has been a major focus for therapeutic intervention in recent decades. Strategic attenuation of CSPGs from specific cell populations in the glial scar may prove beneficial in stimulating the intrinsic growth machinery of spared axons, promoting long distance regeneration through the scar, and improving functional recovery.

Chondroitinase ABC (ChABC) is a well-known enzyme that is capable of cleaving CSPG glycosaminoglycan (GAG) side chains, effectively reducing inhibitory activity (Groves et al. 2005; García-Alfás et al. 2009). Regeneration of both descending CST and ascending sensory fibers as well enhanced locomotor and proprioceptive function have been demonstrated following ChABC administration in a rodent model of SCI (Bradbury et al. 2002). Several other studies have demonstrated axonal growth following ChABC administration after CNS

While regeneration of the injured CNS was once thought to be unachievable, advances in our understanding of growth-inhibitory factors have allowed for the development of targeted therapeutics that have facilitated some degree of functional recovery.

1.2.2 The Glial Scar

In otherwise normal or uninjured tissue, astrocytes play a critical homeostatic role in regulating ion and neurotransmitter levels and the blood-brain barrier as well as maintaining the basal lamina and perineural net via production of ECM molecules (McGraw, Hiebert, and Steeves 2001). In the absence of neurological injury, astrocyte processes occupy connecting and non-overlapping realms. Following injury, however, astrocytes serve as the major glial cell type responsible for walling off sites of damage in an effort to preserve healthy neural tissue, undergoing a process termed ‘reactive astrogliosis.’ This process is largely characterized by distinct changes in cellular morphology and molecular expression (Sofroniew 2005).

Following injury, the lesion core is largely populated with fibroblasts alongside ECM molecules such as fibronectin, collagen, and laminin (Adams and Gallo 2018). The core is comprised of a mixture of cells, including fibroblasts, pericytes, ependymal cells, and phagocytic macrophages. This lesion core is consequently referred to as the ‘fibrotic scar,’ which contains an array of ECM proteins that can inhibit axonal growth and remyelination (Adams and Gallo 2018). Fibroblasts work with astrocytes to assemble a continuous basal lamina on the outer astrocytic surface, culminating in the ‘glia limitans’ (Sherer and Fawcett, 2001). These cellular components have been associated with the expression of repulsive axon guidance molecules and astrocyte reactivity (Pasterkampe et al., 1999; Wanner et al., 2008). More recently, type-A pericytes have been shown to contribute to the fibrotic scar. Following injury to the CNS, the perivascular niche is disrupted, causing type-A pericytes to delaminate from blood vessels, proliferate, and produce stromal cells which deposit ECM. This
subpopulation of pericytes line the blood vessels in the uninjured spinal cord parenchyma, and, following SCI, are implicated in forming the stromal, non-glial core of the scar (Goritz et al., 2011). These cells also express fibronectin and α-semaphorin, suggesting their contribution to connective tissue elements within the scar. (Goritz et al., 2011). The fibrotic core of the scar reaches maturation by approximately 2 weeks after injury.

The glial scar refers to the glial cell border surrounding the lesion core. Beginning several days after SCI, astrocytes at the lesion perimeter undergo hypertrophy and proliferation, with progressive extension of overlapping processes, ultimately forming a dense network of gap and tight junctions. New astrocytes, the origins of which are still not fully known, also contribute to this process, culminating in a mature glial scar at approximately 2 weeks post-SCI (McGraw, Hiebert, and Steeves 2001). Although the source of these newly divided astrocytes is still poorly understood, studies have postulated cell proliferation following cell cycle re-entry, or derivation from various progenitor cells; these may include NG2 oligodendrocyte progenitors cells in the surrounding parenchyma, ependymal cell progenitors, or multipotent progenitors in the subependymal tissue that express GFAP and migratory potential (Hughes et al., 2013). NG2 oligodendrocyte progenitors have been shown to rapidly extend processes and migrate toward the lesion site, acting to stabilize dystrophic axons at the injury site (Hughes et al., 2013). The structural changes accompanying the glial scar persist long after injury, posing a significant barrier to regenerative efforts (Adams and Gallo 2018; Ribotta, Menet, and Privat 2004).

Therapeutic Benefit of the Glial Scar in Limiting Secondary Tissue Damage

Formation of the glial scar is a defining hallmark of secondary injury following CNS trauma and has traditionally been considered a homogenously negative process resulting in neurotoxicity, inflammation, neuronal growth inhibition, and ultimately regeneration failure (Michael T Fitch and Silver 2008). A substantial amount of literature has since deemed this viewpoint to be overly simplistic, revealing the glial scar to be far more than a physical barrier of astrocytes and connective tissue elements, rather a dynamic and fundamental
structure involving complex cellular and molecular interactions. Moreover, studies have demonstrated the neuroprotective and wound healing capacity of the glial scar, particularly within the acute post-injury phase (Sofroniew 2005).

Reactive astrogliosis exerts a number of beneficial effects in response to CNS injury by isolating the lesion site, limiting inflammatory cell infiltration, restoring the blood-brain/blood-spinal cord barrier, and preventing further tissue necrosis. In response to CNS injury, astrocytes are induced to an activated and hypertrophic state, migrating to the lesion site and secluding it from uninjured tissue by compacting infiltrated inflammatory cells, resulting in spared neuronal components and ultimately better functional recovery (Okada et al. 2006). Hypertrophic astrocytes are essentially rearranged into a network of intertwined filamentous processes, culminating in a dense scar that protects intact neural networks from further damage (Bush et al., 1999; Faulkner et al., 1994).

A number of studies have investigated attenuation of the scar itself as a potential avenue to fostering increased axonal growth and remyelination (Bush et al. 1999; Faulkner et al. 2004). While the glial scar has demonstrated growth inhibitory effects on injured axons within the CNS, studies have demonstrated regeneration failure in the absence of glial scar formation. Astrocytic ablation proximal to the lesion during the first week post-injury has demonstrated widespread inflammation, neuronal degeneration, demyelination, and vascular disruption (Bush et al. 1999; Faulkner et al. 2004). Transgenic animal models have further demonstrated that complete genetic ablation or severe attenuation of the glial scar results in increased lesion size, neuronal loss, demyelination, and significantly impaired function after injury (Anderson et al. 2016). Moreover, ablation or attenuation of reactive scar-forming astrocytes have been shown to exacerbate the spread of inflammatory cells during both the innate local inflammatory response and the peripheral adaptive immune response. A number of studies by the Sofroniew lab have further demonstrated that preventing formation of the astroglial scar does not result in enhanced regeneration. Selective ablation of proliferating astrocytes has been shown to increase axonal dieback, a mechanism postulated to arise from altered inflammatory response (Anderson et al. 2016).
Reactive astrocytes play critical roles in restricting the inflammatory response to the damaged CNS and protecting healthy tissue from damage. These protective influences include sequestration of blood-derived macrophages and repair of the BBB/BSCB (Michael T Fitch and Silver 2008). In addition to exacerbating the inflammatory response post-injury, ablation of scar-forming astrocytes has also been shown to exacerbate neuronal cell death and demyelination following injury, due to the influx of blood-derived macrophages and fibrotic cells. The astroglial scar is critical to preservation of tissue integrity and mitigation of further inflammatory damage to healthy surrounding tissue (Adams and Gallo 2018; Faulkner et al. 2004; Anderson et al. 2016; Sofroniew 2005). Thus, total inhibition of the scar is no longer regarded as an optimal therapeutic strategy for CNS injury.

Detrimental Effect of the Glial Scar on Regeneration and Functional Recovery

A number of studies have implicated the molecular composition of the scar and production of inhibitory ECM molecules, instead, as key contributing factors to regeneration failure (Busch and Silver 2007; Fawcett 2006; M.-Z. Zhao et al. 2006; McGraw, Hiebert, and Steeves 2001; Silver and Miller 2004a; Yiu and He 2006). Davies et al. demonstrated that, following transplantation of DRG neurons into undamaged white matter tracts, axons displayed long-distance rapid regeneration. However, after encountering areas of CNS damage containing inflammatory infiltrate and inhibitory molecules, extending axons were converted to a dystrophic state and ceased regeneration (Davies et al. 1999). In addition to morphological changes, reactive astrocytes undergo molecular changes, most notably an increase in expression of the cytoskeletal intermediate filaments, glial fibrillary acidic protein (GFAP), Vimentin, and Nestin, as well as deposition of inhibitory proteins such as chondroitin sulfate proteoglycans (CSPGs) (Lu et al. 2003; McKeon, Höke, and Silver 1995; Rhodes and Fawcett 2004; Hagino et al. 2003; Apostolova, Irintchev, and Schachner 2006).

As previously highlighted, CSPGs are one such class of inhibitory molecules produced by reactive astrocytes in the glial scar that largely inhibit neurite outgrowth (Morgenstern,
Asher, and Fawcett 2002). Under normal physiologic conditions, CSPGs are structural components of various tissues that exert functional roles in neural development. CSPGs are a complex family of macromolecules consisting of a core protein and one or more covalently attached glycosaminoglycan (GAG) side chains. The chains vary in number and composition, undergoing extensive modification which ultimately dictates resultant biological activity (Morgenstern, Asher, and Fawcett 2002). Although secreted by almost all cell types present at the injury site, CSPGs are most predominately secreted by reactive astrocytes within the glial scar (M T Fitch and Silver 1997). CSPGs have indeed been found in the brain and spinal cord following injury, with a particularly rapid and prolonged upregulation within the glial scar (M T Fitch and Silver 1997; Pindzola, Doller, and Silver 1993; Lu et al. 2003). Reactive astrocytes begin producing and depositing CSPGs into the ECM approximately 24 hours after injury, with relatively high concentrations persisting at the injury site for months thereafter (Jones et al., 2003; McKeon et al., 1999; Tang et al., 2003). Proteoglycans upregulated by reactive astrocytes have been shown to inhibit neurite outgrowth in vitro while demonstrating similar effects in vivo; their modification has been shown to unlock endogenous regenerative potential (Dou and Levine 1994; McKeon et al. 1991; Tom et al. 2004; Bradbury et al. 2002; Houle et al. 2006; Steinmetz et al. 2005). In vitro, CSPGs have been shown to repel regenerating axons as well as prevent oligodendrocyte maturation and remyelination. As such, the glial scar not only impedes axonal growth but presents an inhibitory environment for remyelination, contributing to the limited regenerative capacity of the injured CNS.

The nature and extent of astrocytic changes is largely dependent on complex intra and intercellular cross-talk between multiple cell types in a context and time-dependent manner. While the scar is predominately comprised of astrocytes, other cell types including microglia, macrophages, and pericytes also contribute to this complex framework (Adams and Gallo 2018). Interestingly, extensive crosstalk occurs between reactive astrocytes and inflammatory cells, namely activated microglia, both of which have the capacity to exert pro or anti-inflammatory regulatory functions via secretion of various cytokines (Liddelow and Barres 2017). Furthermore, deletion or knockdown of particular secretory molecules has been
associated with a reduction in inflammation after injury. Identification of the initial molecular inducers of inhibitory gliosis still remains largely unknown.

Although several inflammatory cytokines have been implicated in the induction of this process, transforming growth factor beta (TGFβ) is one such molecule of interest. TGFβ1 expression is upregulated immediately following both traumatic brain and SCI, and is considered a key component in the inductive astrogliotic process (Jeong et al. 2012; Buss et al. 2008; Logan et al. 1994). TGFβ1 has also been shown to induce CSPG expression (Asher et al. 2000; Smith and Strunz 2005). TGFβ2 expression, on the other hand, has been shown to increase more slowly and in close proximity to the injury site in a number of cells including astrocytes, macrophages, and endothelial cells (Buss et al. 2008). Experimental attenuation of both TGFβ1 and TGFβ2 has demonstrated reduced glial scarring, suggesting potential therapeutic targets of interest after SCI (Logan et al. 1999; Jeong et al. 2012). Other molecular mediators have also been implicated as key contributors to reactive astrogliosis in vivo, including epidermal growth factor (EGF), fibroblast growth factor (FGF), endothelin-1, and ATP (Schachtrup et al. 2010).

While classically considered a detriment to axonal regeneration, the astroglial scar plays a critical role in protecting healthy neural tissue at the lesion site from further damage (Adams and Gallo 2018). While it is important to consider the multitude of neuroprotective and repair-mediated functions exerted by the glial scar after SCI, it is equally imperative to acknowledge the physical and chemical barrier that the scar presents to regenerative processes. The growth-inhibitory nature of the glial scar is considered to be a major obstacle to neuroregeneration, and a key contributing factor to inefficient recovery after traumatic brain and spinal cord injury. As such, therapeutic interventions have shifted from complete ablation of the scar to strategic modification of inhibitory components to promote endogenous axonal regeneration and remyelination.
1.3 Cellular Transplantation Strategies for CNS Injury

Given the complexity of the CNS alongside the heterogenous nature of traumatic brain and spinal cord injury, strategies to repair damaged tissue and restore function have proved challenging. Historically, experimental therapies involving pharmacological agents have been developed to reduce secondary damage, but with limited ability to improve clinical outcomes (Marklund et al., 2006; Royo et al., 2003). Neurosteroids are another widely examined therapeutic for the treatment of CNS injury after CNS injury. Neurosteroids are endogenous molecules synthesized from cholesterol or steroid hormone precursors that are produced in the brain after local synthesis or conversion of adrenal or gonadal steroids. Neurosteroids exert a pleiotropic effects on neural function, including neural plasticity, learning and memory, as well as behaviour (Benarroch et al., 2007; Vallee et al., 2001; Engel et al. 2001; King et al., 2008). The neurosteroid progesterone, in particular, has drawn notable attention as a potential therapeutic for CNS injury primarily for its neuroprotective and myelinating capacity. In preclinical models of SCI, progesterone treatment has been shown to restore the expression of motoneuron molecular markers and promote the proliferation and differentiation of oligodendrocyte progenitors into myelin-producing cells (De Nicola et al., 2009). In an experimental model of TBI, acute administration of progesterone and its metabolite, allopregnanolone, demonstrated reduced expression of pro-apoptotic proteins caspase-3 and Bax, as well as apoptotic DNA fragmentation within 24 hours after injury (Djebaili et al., 2005). A reduction in GFAP-positive astrocytes was also observed at the lesion site as well as cognitive improvements (Djebaili et al., 2005). The neuroprotective capacity of progesterone has also been investigated in models of stroke, demonstrating significant reductions in infarct volume and functional deficit (Sayeed et la., 2005). Clinical trials have also demonstrated improved outcomes in patients with TBI following progesterone administration (Wright et al., 2007; Xiao et al., 2008).

In recent decades, progress has been made in the development of novel experimental therapies focusing on cell replacement, stimulation of axonal outgrowth, and neutralization of inhibitory cues to modify regenerative barriers within the injury site. In addition to
pharmacological agents and endogenous neurosteroids, stem-cell based therapies have become an increasingly attractive and promising approach for addressing the dynamic and multifactorial nature of secondary injury following CNS trauma.

1.3.1 Rationale for Cellular Transplantation

Stem cell-based therapies have been increasingly explored as a promising treatment strategy for traumatic brain and spinal cord injury, and are particularly attractive in their ability to replace and promote the survival of damaged cells, remyelinate axons, and alter the inhospitable environment of the damaged CNS to one more conducive for regeneration.

Stem cells are characterized by their intrinsic ability to self-renew and differentiate into committed progenitors that give rise to mature cells (Bongso and Richards 2004). Stem cells are further classified by their developmental potential, and characterized as totipotent (able to generate all embryonic and extra-embryonic cell types), pluripotent (able to generate all cell types within the body), or multipotent (able to generate limited cell types) (Bongso and Richards 2004).

Cell-based therapies are largely aimed at facilitating neuroregeneration either directly via cell replacement or repair of damaged cells themselves, or indirectly via secretion of beneficial factors that can alter the inflammatory environment to one more permissive for regeneration (Miller and Gauthier-Fisher 2009; Bliss, Andres, and Steinberg 2010; A. J. Mothe and Tator 2012). Mesenchymal stem cells (MSCs), neural stem/progenitor cells (NSPCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and their differentiated progeny, have all been investigated as treatment strategies in the injured CNS, yielding modest but promising functional benefits (Willerth 2011; English 2013; A. J. Mothe and Tator 2012; Fehlings and Vawda 2011).

1.3.2 Neural Stem Cell Therapy

While a number of stem cell types have been investigated in the treatment of CNS injury, neural stem/progenitor cells (NSPCs) are particularly attractive for transplantation, most
notably for their ability to provide tissue-specific replacement of lost or damaged cells, as well as trophic support.

NSPCs are multipotent, self-renewing cells that have the capacity to differentiate into the main cellular lineages of the CNS: neurons, astrocytes, and oligodendrocytes. In the brain, NSPCs are located in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. In the spinal cord, NSPCs reside within the periventricular zone of the central canal (Reynolds and Weiss 1992; Clarke and van der Kooy 2011; Rampon et al. 2008a).

NSPCs can be derived from a number of sources for therapeutic application. One such source is embryonic stem cells (ESCs), which are pluripotent cells originating from the inner cell mass of the developing blastocyst. Given their high propensity to form teratomas, ESCs are typically induced towards a neural lineage prior to grafting. Human induced pluripotent stem cells (hiPSCs), on the other hand, are adult somatic cells that have been reprogrammed to a pluripotent state by a combination of transcription factors, and have the capacity to differentiate into any cell type, including NPCs (Angelos and Kaufman 2015). HiPSCs are a clinically attractive cellular source because they can be derived from patient-specific somatic cells, enabling autologous transplantation while circumventing ethical constraints traditionally associated with ESC derivation (Angelos and Kaufman 2015). As such, hiPSCs constitute a promising avenue and source of NPCs for preclinical and clinical studies.

Given their attributes, NPCs may confer a distinct advantage over single-target drug therapies in providing multifaceted therapeutic potential to patients with brain or spinal cord injury. The clinical benefit of NPC transplantation, is, however, significantly hampered by the inflammatory and inhibitory nature of the post-injury glial environment, which poses a substantial physical and chemical barrier to stem-cell mediated regeneration (Michael T Fitch and Silver 2008). Furthermore, the inflammatory cytotoxic milieu of the injured CNS contributes to relatively poor cell survival and engraftment post-transplantation, and consequently reduced levels of available trophic support. Despite these pressing challenges, studies have demonstrated tissue repair, regeneration, modulation of inflammatory cell
activity, reduced glial scarring, and neurobehavioural improvements following NPC transplantation (Jin et al. 2016; Wilcox et al. 2014a; Andres et al. 2011).

1.3.2.1 Mechanisms of NPC-mediated neuroprotection and repair

While the processes involved in NPC-mediated tissue repair are still poorly understood, their inherent ability to not only home to areas of injury and serve as a reservoir for cell replacement but secrete an array of neuroprotective and immune-modulatory factors, highlights their multimodal therapeutic value in CNS trauma (Figure 1) (Bliss, Andres, and Steinberg 2010). As such, studies have demonstrated the capacity of transplanted NPCs to modify the injured CNS milieu, ultimately rescuing endogenous glial and neuronal cells that have survived primary damage (Ziv et al. 2006; Lindvall and Kokaia 2010). Thus, transplanted NPCs facilitate tissue remodeling, aiding in regenerative efforts and functional recovery. Moreover, functional benefit has been demonstrated with only small numbers of transplanted NPCs undergoing terminal differentiation in vivo (Domeniconi et al. 2002; Chu et al. 2004; Dai and Liu 2004; Fujiwara et al. 2004).

NPSCs provide a direct reservoir of neurons and glial cells for tissue replacement. Several studies have demonstrated successful integration and differentiation of rodent and human-derived NSPCs following transplantation into the injured CNS in vivo into functional neurons, oligodendrocytes, and astrocytes (Fujiwara et al. 2004). While studies have highlighted the marked differentiation of transplanted NSCs into remyelinating oligodendrocytes in the CNS, the functional extent of remyelination achieved by these transplants is limited. Transplantation of human and mouse-derived NPCs into rodent models of SCI and TBI have also demonstrated angiogenesis, increased tissue sparing, reduced glial scarring, and functional improvements (Wilcox et al. 2014b). These studies not only attest to the dynamic cross-talk between NPCs and their cellular environment, but highlight their therapeutic potential in limiting cellular damage and reconfiguring the inhibitory post-injury environment to better facilitate regeneration. Moreover, manipulation of endogenous or transplanted NPCs has also been investigated as a means of improving therapeutic efficacy in neurodegenerative disease and CNS injury (Cao, Benton, and Whittemore 2002).
NPCs secrete an array of cytokines, growth factors, neurotrophins, and immune-modulatory molecules that have the capacity to not only modify the molecular composition of the post-injury microenvironment to one more permissive for regeneration, but also evoke beneficial responses from resident cells through transient paracrine actions (Ziv et al. 2006).

Neurotrophins contribute to the functional integrity of the CNS in a number of ways including regulation of neuronal survival, differentiation, neurite outgrowth, and synaptic plasticity (Chao 2003). Transplanted NPCs have the unique and multifaceted capacity to integrate within existing host circuitry, provide and provoke trophic support, and modulate host immune responses in the injured CNS (Cossetti et al. 2012). Moreover, NPC secreted factors have the capacity to assemble endogenous stem cells and enhance neuroregenerative responses, such as neurogenesis within the injured CNS (Shetty 2014).
1.3.2.3 Changes in Trophin Expression and Associated Benefits following NPC Transplantation in the Injured CNS

The limited intrinsic growth capacity of the adult CNS in conjunction with the inhospitable and cytotoxic injury microenvironment are significant challenges for axonal regeneration and functional recovery (Michael T Fitch and Silver 2008). Trophin secretion by transplanted NPCs has been implicated in repair and regeneration of the CNS following injury or disease (Liang et al. 2014a; Cheng et al. 2017; Doeppner et al. 2017). While stem cell-based therapies for CNS repair and regeneration have largely centered on directed differentiation to intended cell phenotypes, it has since become apparent that functional improvements are not solely ascribed to cell differentiation and repopulation, but paracrine actions of cellular secreted factors in host tissue. As such, research has since shifted to elucidate and modify the paracrine actions underlying tissue repair and regeneration following NPC transplantation to enhance overall therapeutic efficacy.

Neurotrophins are a family of growth factors that play key roles in the development and homeostatic maintenance of the CNS, exerting a number of effects including neurogenesis, neuronal survival, axonal growth, and synaptogenesis (Chao 2003; Bregman et al. 1997; Lu et al. 2003). Neurotrophic factors have proven to be attractive candidates for facilitating neuronal repair and establishing functional circuitry through the activation of several growth signaling mechanisms (Bregman et al. 1997; Lu et al. 2003). As such, research endeavours have been aimed at elucidating the specific roles of neurotrophins in promote neuroregeneration and functional recovery following traumatic brain and spinal cord injury.

Our lab has previously investigated the environmental interaction between transplanted NPCs and the host tissue in a rodent model of SCI (Hawryluk, Mothe, Wang, et al. 2012). In the injured cord, a number of growth factors were shown to be upregulated, including nerve growth factor (NGF), leukemia inhibitory factor, insulin-like growth factor-1, and TGF-β1. A reduction in vascular endothelial growth factor (VEGF)-isoform A and platelet-derived growth factor-A was also observed. Moreover, while NPC transplantation led to modest changes in trophin expression, isolation and assessment after transplantation in the injured
cord revealed increased levels of ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) relative to host levels (Hawryluk, Mothe, Wang, et al. 2012).

Other studies have demonstrated an increase in factors such as NGF, BDNF, CNTF, VEGF, and GDNF in vivo after NPC transplantation, indicating a plausible source of neuroprotection following CNS injury (Madhaven et al., 2009; Schmidt et al., 2009; Satake et al., 2000). Madhaven et al., demonstrated that after exposure to an oxidative stress-like environment in vitro, NPCs displayed enhanced secretion of BDNF, CNTF, and VEGF, effectively stimulating anti-apoptotic effects on neurons (Madhavan et al. 2009). NGF, GDNF, BDNF, and GDNF have also been implicated in axonal pathfinding and neuronal survival, demonstrating modest improvements in neurobehavioural recovery follow CNS injury.

Secretion of VEGF by NPCs was shown to elicit expression of guidance molecules thrombospondin 1 and 2, that in turn regulated dendritic sprouting and axonal plasticity in a model of ischemic stroke (Schmidt et al. 2009). Furthermore, transplantation of human NPCs in vivo demonstrated neurobehavioural recovery, with NPC-secreted VEGF responsible for angiogenesis, improved blood-brain barrier integrity, and reduced microglial infiltration. In a murine model of chronic experimental autoimmune encephalomyelitis, NPC transplantation demonstrated proliferation and remyelinating capability of host oligodendrocyte progenitor cells (OPCs), the effects of which were mediated by platelet-derived growth factor and fibroblast growth factor secretion (Proescholdt et al. 2002). Another study demonstrated that, in aged mice, grafted NPCs migrated to tissue lesions and released GDNF, preventing cell death and facilitating regeneration of targeted cell populations (Satake et al. 2000). Thus, trophin expression in the spinal cord is influenced not only by injury, but cell transplantation, suggesting contributions to some of the observed benefits associated with cell-based repair strategies for CNS injury.

Growing literary evidence suggests that the biomolecules produced by NSCs may be just as important, if not more so, than cell replacement in promoting recovery after CNS injury. Given the ischemic and inflammatory composition of the injury milieu, NPC-mediated
trophic support may prove advantageous in enhancing repair and regeneration through anti-apoptotic, angiogenic, or immunomodulatory mechanisms (Bregman et al. 1997; Lu et al. 2003). Thus, current research avenues have expanded to elucidate, harness, and modify NPC secreted factors to enhance overall therapeutic efficacy and functional benefit.

A number of studies have utilized NPC conditioned media, or secretome, as a therapeutic strategy in vitro and in vivo. Intravenous administration of NPC conditioned media in a rodent model of cerebral ischemia demonstrated long-term post-ischemic neuroprotection, neurogenesis, and angiogenesis, and functional improvements. Neuroprotective effects were associated with increased levels of GDNF and VEGF (Doeppner et al. 2017). Another study demonstrated that, in a rodent model of SCI, continuous administration of human NSC conditioned media resulted in long-term neuroprotection, extensive growth of the cervical corticospinal tract, propriospinal neural circuit reconnection, and functional improvements (Liang et al. 2014b). More recently, Cheng et al. demonstrated that systemic administration of NSC conditioned media in a rodent model of SCI significantly reduced inflammatory cytokine expression in activated macrophages and injured tissue, while also reducing expression of inducible nitric oxide synthase in the spleen, suggesting a regulatory role in attenuating systemic inflammation (Cheng et al. 2017).

These studies attest to the capacity of NPC secreted factors to foster neuroprotective, immunomodulatory, and pro-regenerative effects in the injured CNS, and highlight a potential alternative to direct stem cell engraftment.

1.4 The Therapeutic Potential of Hepatocyte Growth Factor in CNS Injury

1.4.1 Developmental and Homeostatic Function of the HGF/c-Met Signaling Pathway

HGF is a pleiotropic cytokine that exhibits proliferative, motogenic, and morphogenic activity in a number of cell types (K Matsumoto and Nakamura 1992; Toshikazu Nakamura
et al. 1989). Initially identified as a motility or ‘scatter’ factor for hepatocytes, HGF is secreted as a single-chain, biologically inactive precursor that is proteolytically converted to its active form, which is comprised of an alpha and beta chain connected by a disulphide bond. The alpha chain is comprised of an N-terminus hairpin loop followed by four kringle domains connected by internal disulphide bridges. The beta chain is homologous in structure to serine protease, but lacks enzymatic functionality (Basilico et al. 2008; Birchmeier et al. 2003; Weidner et al. 1993).

HGF is the only known ligand for the tyrosine kinase receptor c-mesenchymal-epithelial transition factor (c-Met). HGF binds to its tyrosine kinase receptor c-Met, subsequently activating a number of regulatory signaling pathways involved in cellular proliferation, motility, and migration (Bladt et al. 1995). Physiologically, c-Met exerts cell-scattering activity via disruption of cadherin-based cell-to-cell contact and motility. This function is critical to developmental embryogenesis and wound repair in allowing for long-range skeletal muscle progenitor migration (Bladt et al. 1995). HGF binds to c-Met resulting in receptor homodimerization and phosphorylation of the catalytic loop-localized tyrosine residues Y1234 and Y1235. Further downstream, a number of adaptor proteins are recruited, as well as the effector molecules phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ), and the transcription factor signal transducer and activator of transcription (STAT-3) (Y.-W. Zhang et al. 2002; Boccaccio et al. 1994).

The HGF/c-Met signaling pathway evokes dynamic and context-dependent biological responses that regulate cellular survival, morphogenesis, and regeneration. In otherwise normal physiological circumstances, the HGF/c-Met signaling pathway regulates developmental cellular function and tissue homeostasis (Maina and Klein 1999). During development, the HGF/c-Met signaling pathway is instrumental in promoting survival and proliferation of hepatocytes and placental trophoblasts and has also been shown to exert chemoattractant properties, promote embryonic neuronal survival, and the differentiation and proliferation of neural stem cells (Hamanoue et al. 1996; Maina and Klein 1999; Kokuzawa et al. 2003; W. Sun, Funakoshi, and Nakamura 2002).
NSC proliferation, self-renewal, differentiation, and migration is largely dependent on the stem cell niche, which serves as a specialized milieu of secreted regulatory factors. HGF has been implicated as a key player in subventricular zone (SVZ) NSC proliferation, the effect of which has been associated with activation of downstream MAPK/ERK1/2 signal transduction (Nicoleau et al. 2009). Nicoleau et al demonstrated neurosphere formation and self-renewal with HGF treatment. SVZ cell cultures were shown to produce and secrete HGF, and immature cells also expressed c-Met. Furthermore, while in vivo intracerebroventricular injection of HGF promoted SVZ cell proliferation, HGF-neutralizing antibodies greatly reduced this effect (Nicoleau et al. 2009). Moreover, HGF has been shown to promote the proliferation and neuronal differentiation of mouse ES-derived neural stem cells.

1.4.2 Regenerative Capacity of HGF in the Injured CNS

Both HGF and its c-Met receptor are expressed in the developing and adult CNS (Hamanoue et al. 1996). Deficits in HGF expression have been implicated in neurodegenerative disease, prompting its investigation as a therapeutic agent following CNS injury. HGF and c-Met have been shown to gradually decline in anterior horn cells with progression of sporadic amyotrophic lateral sclerosis (Kato et al. 2003; W. Sun, Funakoshi, and Nakamura 2002). In a rodent model of SCI, HGF was found to be significantly expressed and peaked at 7 days post-injury, while c-Met was upregulated at one day, peaking at 2 weeks thereafter (Munehisa Shimamura et al. 2007). This level of increased expression persisted to 8 weeks after injury. Interestingly HGF protein levels were significantly elevated in the injured region, with elevated expression in reactive astrocytes in the subacute and chronic stages of injury (Munehisa Shimamura et al. 2007). Provided the neurotrophic capacity of HGF, activation of the HGF/c-Met signaling pathway has been implicated in regeneration (Munehisa Shimamura et al. 2007).

Developmentally, HGF has also been recognized for its role in enhancing neuronal survival and fostering neurite outgrowth. Outside of the CNS, HGF has been shown to enhance wound healing processes and suppress excessive scar formation after injury (Bevan et al. 2004; K Matsumoto and Nakamura 1992). Following injury to the CNS, a number of studies
have highlighted the capacity of HGF to promote neurogenesis, motor neuron neurite outgrowth, oligogenesis, and synaptogenesis, process that are known to foster neuroplasticity and functional recovery (Ebens et al. 1996; Maina and Klein 1999; Shang et al. 2011; Yan and Rivkees 2002). HGF activates c-Met and subsequent downstream pathways including the protein kinase-B (PKB/Akt) cascade, the MAP-kinase pathway (ERK1/2 and p38MAPK), and transcriptional STAT3 signaling (Shang et al. 2011; Birchmeier et al. 2003). Although PI3K/Akt and ERK1/2MAPK pathways stimulate varying trophic effects, the former is most implicated in cellular survival with the latter implicated in neuronal differentiation. Furthermore, phosphorylation of Akt and ERK have been shown to promote synaptic plasticity (Shang et al. 2011).

There has been a wealth of evidence demonstrating the pro-regenerative effect of HGF after CNS injury. These effects may be circumscribed directly through stimulation of neurogenesis and neurite outgrowth, and indirectly through modulation of inhibitory components of the glial scar. In cultured NPCs, HGF has been shown to promote neuronal differentiation and neurite outgrowth (W. Sun, Funakoshi, and Nakamura 2002). Moreover, in serum-deprived retinal ganglion cell cultures, exogenous application of HGF demonstrated increased survival and stimulation of neurite outgrowth (Tönges et al. 2011). In vivo, treatment with HGF after optic nerve axotomy significantly improved survival of axotomized retinal ganglion cells and enhanced axonal regeneration (Tönges et al. 2011). In a rodent model of SCI, transplantation of HGF-expressing MSCs were shown to reduce levels of TGFβ and CSPG deposition while promoting neurobehavioural improvement (Jeong et al. 2012). Administration of HGF-expressing MSCs also demonstrated increased serotonergic axonal density below the caudal lesion boundary, suggesting regeneration through the lesion or increased axonal sprouting from the uninjured contralateral side (Jeong et al. 2012). These findings implicate the role of HGF in modifying inhibitory components of the glial scar as a means of enhancing regeneration in the injured CNS.

In addition to its role in post-traumatic regeneration within the CNS, HGF has been shown to promote tissue repair, further highlighting its multifunctional therapeutic capacity.
Exogenous administration of HGF has also been shown to enhance angiogenesis, improve microcirculation, and inhibit destruction of the blood-brain barrier in rodent models of cerebral ischemia and ALS. Moreover, in a stroke model of ischemia-induced neuronal death, HGF reduced infarction area (M.-Z. Zhao et al. 2006). In a model of motor neuron degeneration, HGF overexpression delayed degeneration and prolonged motor neuron survival (Kadoyama et al. 2007; W. Sun, Funakoshi, and Nakamura 2002). A study by Kitamura et al demonstrated that pre-injury administration of HGF in a rodent model of thoracic SCI demonstrated increased neuronal and oligodendrocyte survival as well as enhanced angiogenesis around the lesion epicenter. These effects culminated in an overall reduction in post-injury secondary damage and provided a better scaffold for axonal regeneration, promoting functional recovery. A subsequent study by this group examined the effect of continuous 4-week intrathecal HGF administration in a primate model of cervical SCI, demonstrating preservation of corticospinal fibers, myelinated regions, and spinal cord parenchyma, all of which corresponded with enhanced neurobehavioural recovery (Kitamura et al. 2011).

Although several growth factors have been shown to promote neuronal survival and regeneration after CNS injury, few have demonstrated multifunctional therapeutic value. Several studies have attested to the distinct advantages conferred by HGF in supporting cell survival and axonal regeneration, among other processes, when compared to other well-known neurotrophic factors. In an *in vivo* model of optic nerve injury, intravitreal administration of HGF proved to be equally potent as BDNF in promoting short-term retinal ganglion cell survival, yet promoted long-term cell survival, in contrast to BDNF or CNTF administration (Wong et al. 2014). In a rodent model of cerebral ischemia, both HGF and GDNF demonstrated equally significant neurotrophic effects in reducing infarct size and promoting tissue preservation, but only HGF was shown to promote neurogenesis, angiogenesis, synaptogenesis, and inhibition of fibrosis after injury (Shang et al. 2010). These findings further highlight the multimodal reparative potential of HGF in addressing the dynamic and multifactorial nature of CNS injury and promoting repair and recovery (Figure 2).
Growth factors have the capacity to stimulate resident stem cells and promote recruitment to the injury site. Interestingly, in the injured spinal cord, HGF has been identified as one of the key chemo-attractants secreted by the lesion site that facilitates NSC migration post-transplantation (Takeuchi et al. 2007). In the study, NSC migration to the spinal cord lesion was highest at 1-week post-injury which also paralleled the peak of HGF expression at the lesion site. Grafted NSCs differentiated into neuronal and glial subpopulations 3 weeks post-transplant. This could suggest that exogenous administration of growth factors such as HGF might also stimulate resident NSCs and promote their recruitment to the injury site, contributing to increased tissue sparing and remyelination of damaged spared axons (Takeuchi et al. 2007). Moreover, a recent study by Takano et al. implicated HGF in promoting better NSC graft survival and subsequent functional recovery in old versus young mice with SCI. HGF was also shown to enhance synapse formation between grafted NSC-derived neurons and descending corticospinal fibers. Similar to previous findings, increased HGF expression was reported at the injury site and localized predominately to reactive astrocytes and microglia. Furthermore, functional inhibition of HGF via neutralizing antibodies resulted in reduced NSC survival, neuronal differentiation, spinal cord atrophy, and demyelination (Takano et al. 2017).

Given its multimodal trophic and regenerative effects within the injured CNS, HGF may be in a unique and advantageous position for utilization as a combinatorial agent alongside cellular therapies.
1.5 Combinatorial Strategies to Promote Regeneration

Therapeutic goals for the management of patients with traumatic brain or spinal cord injury have largely centered on reducing cell death and minimizing the extent of secondary injury while facilitating regeneration to repair damaged tissue. Given the heterogenous and multifactorial nature of TBI and SCI, it has become increasingly apparent that the most promising treatment for CNS injury may involve a number of therapies, that, while modestly effective individually, garner enhanced effects when used together. Such combinatorial therapies would aim to create a neuroprotective environment, foster regeneration, and counter growth-inhibitory factors released after CNS injury, mechanisms which are critical to inducing robust regeneration following injury.

While cell-based therapies to treat CNS injury have yielded promising preliminary findings, a number of challenges still remain. Clinical translation of cell transplantation techniques is limited by poor cell survival and ineffective integration into host tissue, largely due to the inhospitable and cytotoxic microenvironment within and proximal to the injury site. Glial scar formation, upregulation of growth-inhibitory ECM molecules, and neuroinflammation all contribute to this limited therapeutic efficacy (A. J. Mothe and Tator 2013).

Strategies are needed to effectively address the complexity of the injured CNS and improve the survival and function of grafted stem cells. This has prompted further investigation into combinatorial treatment tactics. Given the dynamic and inhibitory nature of the post-injury CNS environment, it has become increasingly ostensible that the most promising treatment strategies may not be relegated to singular, rather multiple efforts that may exert a heightened effect in combination. Strategies utilizing a combination of stem cells grafts, biomaterial scaffolding, growth factors, and CSPG degrading enzymes, have shown beneficial effects in regulating the balance between inhibitory and growth-promoting factors implicated in neuroregeneration.
1.5.1 Trophic Factors and Cellular Transplantation Paradigms

The therapeutic potential of growth factors in modifying the inhospitable post-injury environment has been increasingly recognized and demonstrated in traumatic CNS injury, ischemic stroke, and neurodegenerative disease. Studies have shown that specific growth factor administration may induce a more favourable outcome for regeneration (A.-R. Liu et al. 2013). In rodent models of SCI, BDNF has shown promise in increasing motor functioning, possibly via recruitment of oligodendrocytes and subsequent myelination of damaged and growing axons. Studies have also demonstrated the capacity of NT-3 to promote modest corticospinal axon regrowth and functional improvements in rodent models of SCI (Bregman et al. 1997). Furthermore, administration of neurotrophic factors BDNF, CNTF, NT-3, and NT-4 have demonstrated increased axonal growth of injured mature CNS neurons (Bregman et al. 1997). These studies attest to the utility of growth factors in limiting tissue damage and promoting neural regeneration at the injury site, mediating improvements in locomotor function.

Combinatorial strategies that harness the specific benefits of cellular and growth factor therapies in particular, may target multiple facets of the secondary injury process, effectively modifying the inhibitory lesion environment and promoting robust regeneration to a greater degree than individual treatments alone. There are a number of strategies that can be utilized to facilitate this goal, including co-transplantation, cell grafts that are genetically modified to secrete neurotrophins, biomaterial substrates that facilitate regulated growth factor release alongside cell transplantation, as well as cellular preconditioning, as demonstrated in the current study (S. Karimi-Abdolrezaee et al. 2010; Drago et al. 2013).

Efficient and timely delivery of diffusible proteins such as neurotrophins into the injured CNS is particularly challenging given the blood-brain/spinal cord-barrier, unless administered at an earlier time point of vascular permeability. NPC-mediated delivery has been achieved by both focal or systemic cell injection, via the blood stream, lymph, or the cerebrospinal fluid circulation. Transplanted cells are then able to follow a gradient of chemoattractant cytokines and chemokines at the site of damage (Müller, Snyder, and Loring...
In a model of experimental autoimmune encephalomyelitis, injected murine NPCs have been shown to express functional cell adhesion molecules such as CD44, integrins, and chemokine receptors CCR1/2/5 and CXCR3/4, which effectually regulate adhesion to inflamed endothelial and ependymal cells and promote migration towards the inflamed injury site. These findings have also been confirmed in rodent and human NPCs (Pluchino et al. 2003; Ziv et al. 2006; Rampon et al. 2008b; Pluchino et al. 2009). In models of SCI, direct intraspinal transplantation also provides a more direct approach for delivery of cells and cell secreted factors to the injury site. Moreover, stem cells can be genetically engineered to deliver desired factors at the site of injury.

Combinatorial approaches utilizing cell grafts that are either transduced to secrete growth factors or transplanted with exogenous growth factor administration haven shown increased efficacy relative to grafts alone (S. Karimi-Abdolrezaee et al. 2010). Studies have investigated viral transduction of NSPCs with a number of neurotrophic factors; among the most widely studied are BDNF, CDNF, GDNF, NGF, VEGF, and NT-3, all of which have been shown to improve cell survival and functional recovery. GDNF has demonstrated potent neuroprotective effects in a number of neuronal injury models such as stroke and Parkinson’s disease (Zhang et al. 2002; Kobayashi et al. 2006). Typically, however, trophic effects are transient and consecutive administrations are needed to sustain substantial results. NPCs over-expressing GDNF have been utilized in animal models of Parkinson’s disease demonstrating neuroprotective effects (Behrstock et al. 2006). Following striatal transplantation, NPCs were shown to release physiologically relevant levels of GDNF up to 3 months after transplantation, resulting in increased dopaminergic neuron survival and neurite outgrowth (Lin et al. 1993). Other neurotrophic factors, such as BDNF and VEGF, have also been investigated in NPCs, demonstrating increased cell survival and improved functional recovery after neurotrauma (Bonner et al. 2009; Andres et al. 2011). Furthermore, NPCs modified to overproduce NT-3 have been shown to promote axon extension after injury. (Schmidt et al. 2009; Nesic et al. 2010a). NPCs have also been investigated as vehicles for growth factor overexpression and drug delivery to promote tissue regeneration in the injured CNS. Genetically manipulating NPCs with a number of transgenes can be done via viral or
non-viral delivery of plasmids and switches for conditional gene expression (Lu et al. 2003; Gossen and Bujard 1992).

In addition to combinatorial stem cell therapies, biomaterial scaffolding has frequently been used in conjunction with cell grafts and growth factor administration. Teng et al. demonstrated significant axonal growth and functional improvement using a PLGA scaffold seeded with NSCs in a rodent model of SCI. The authors hypothesized this effect to be attributed to NSC trophic support, as the majority of grafted cells remained undifferentiated (Teng et al. 2002). Significant hurdles associated with growth factor treatment for CNS injury include the establishment and maintenance of optimal concentrations following administration as well as subsequent gradients for directed neural outgrowth. Scaffolding strategies offer promising solutions to these problems, offering sustained cellular release of growth factors; moreover, studies have implemented growth factors into scaffolds designed to guide axonal growth (Teng et al. 2002).

Altogether, these studies highlight the strategic benefit of combinatorial treatment paradigms employing the benefits of both cellular and growth factor therapies to foster enhanced neuroprotection while limiting regenerative constraints to promote regeneration and recovery after injury. Given its neurotrophic and regenerative aptitude, HGF may serve as a promising preconditioning or combinatorial agent alongside NPCs in enhancing overall trophic support and facilitating these therapeutic goals (Takano et al. 2017).

1.5.2 Neurotrophins and Inhibitory Molecule-degrading Enzymes

As previously highlighted, CSPGs are a major class of growth-inhibitory molecules that are up-regulated after CNS trauma, and key contributing factors to regenerative failure in the injured mammalian CNS. Reactive astrocytes proliferate at the lesion site approximately 24 hours after injury, depositing high amounts of CSPG and ultimately establishing an inhibitory zone that significantly hampers the growth of CNS axons (Lu et al. 2003; Silver and Miller 2004a).
Chondroitinase ABC (ChABC) is a well-known bacterial enzyme with the capacity to digest CSPG glycosaminoglycan chains and overcome CSPG-mediated inhibition. A number of studies have demonstrated enhanced sprouting and axonal regeneration following administration of ChABC (H. Lee, McKeon, and Bellamkonda 2010; Bradbury et al. 2002). Even without cellular grafts or trophic provision, ChABC treatment alone and in combination with rehabilitative training, has been shown to promote regeneration of axonal tracts in SCI (Massey et al. 2008; Bradbury et al. 2002; García-Alfás et al. 2009). Administration alongside a cellular graft at the lesion site, however, has demonstrated increased numbers of axons that regenerated across the lesion site (CHAU et al. 2004; Fouad et al. n.d.; Houle et al. 2006). The utilization of degrading enzymes such as ChABC are, however, not without limitations. Instability at physiological temperatures consequently results in a relatively quick rate of inactivation, limiting ChABC-mediated effects on functional recovery. Thermostabilized versions have since been developed to combat this challenge, resulting in attenuated levels of chondroitin sulfate GAG chains for approximately 6 weeks after SCI (Hyunjung Lee, McKeon, and Bellamkonda 2010).

ChABC has also been investigated in combination with cellular grafts and growth factor administration. In combination with delivery of GDNF at and around the lesion site, ChABC and a peripheral nerve graft were applied one month after injury and shown to promote regeneration of descending axons into and through the graft, establishing new connections (Tom et al. 2009).

Moreover, when ChABC was administered in conjunction with NT-3 in a gel scaffold at the injury site, a significant increase in serotonergic axon sprouting was observed proximal to the lesion (Hyunjung Lee, McKeon, and Bellamkonda 2010). Similarly, injection of ChABC alongside lentiviral delivery of NT-3 demonstrated substantial growth of transplanted neurons into CSPG-dense domains relative to either treatment alone. Further, in a rat model of SCI, intrathecal administration of ChABC alongside EGF, FGF, and PDGF for a week prior to NPC transplantation demonstrated enhanced oligodendroglial differentiation,
generation of new vascular endothelial cells, and reduced levels of inflammatory cell proliferation (Soheila Karimi-Abdolrezaee et al. 2012).

These findings demonstrate the therapeutic benefit in utilizing CSPG-degrading enzymes such as ChABC alongside neurotrophin delivery to modify the inhospitable CNS milieu. Efficient removal of growth inhibitory ECM components within the glial scar can ultimately facilitate enhanced neuroregeneration and functional recovery.

1.5.3 Modified Cell Secretome Strategies

While stem cell-based strategies to treat CNS injury and neurological disease have yielded promising preliminary findings, there are a number of challenges that remain to be considered with respect to feasibility of clinical implementation. One palpable concern regarding cell-based therapies stemming from pluripotent sources, is the potential to form teratomas post-transplantation (Angelos and Kaufman 2015). While the use of lineage-restricted stem and progenitor cells such as NPCs may circumvent this issue and offer a safer alternative, they are also limited by restricted plasticity and senescence upon in vitro expansion (Hachem, Mothe, and Tator 2015). Learning to modulate and harness the paracrine actions of stem cell populations may provide an alternative treatment option for CNS injury that is currently limited by cell sourcing issues.

While stem cell therapies for tissue regeneration are conventionally thought to have been primarily driven by cellular replacement of damaged tissues, advances in the field have demonstrated the beneficial paracrine actions of these cells. The use of stem cells to modify tissue microenvironments is one current focus of stem cell research. Application of NPC therapies for traumatic brain and spinal cord injury have yielded modest but promising results for tissue regeneration, with trophic and immunomodulatory effects mediated by cell secreted factors (Lu et al. 2003). Research endeavours have since expanded to consider alternatives to cell transplantation that involve harnessing and enhancing stem cell-secreted factors for tissue repair and regeneration in the injured CNS. As a result, optimal genetic
engineering and preconditioning regimens of cells to augment trophic factor secretion for intended application have become widely investigated.

A number of approaches have been investigated to modify and enhance the secretory capacity of stem cells, including NSPCs, for therapeutic use in CNS injury. The majority of these approaches have been centered on genetic manipulation or preconditioning as a means of increasing cell survival post-transplantation or directly enhancing biomolecule production in order to maximize tissue repair and regeneration to facilitate functional recovery (Drago et al. 2013).

NPCs have been genetically engineered with a number of transgenes to more effectively engraft in hostile environments and secrete increased amounts of trophic factors. Studies have shown that NPCs virally transduced with NT-3, BDNF, or GDNF demonstrated prolonged survival after transplantation and improved neurobehavioural outcomes (Yick et al. 2000; Bonner et al. 2009; Behrstock et al. 2006). While genetic manipulation of NPCs demonstrate increased cell survival in the post-injury hostile environment via enhanced secretion of proteins, there are several considerations to be taken into account regarding clinical application. Such considerations include optimal methods for genetic modification, unexpected or unwanted effects post-transplantation, and maintenance of desired trophic secretion. While these methods may demonstrate improvements in therapeutic efficacy post-transplantation, an equally beneficial, simpler, and safer approach might be needed for future clinical application.

Stem cell preconditioning, or exposure to a particular stimulus for a defined period of time, is another method for inducing increased or desired trophic factor production, further augmenting functional and cell paracrine actions. In vitro preconditioning by hypoxic exposure is one such approach for enhancing paracrine actions, primarily through the induction of HIF-1α, a transcription factor which binds to hypoxia response elements in a number of target genes, including angiogenic growth factors. HIF-1α overexpression has been shown to promote NSC proliferation and differentiation after intracerebral hemorrhage and hypoxic/ischemic injury (Sharp et al. 2004).
Cytokine preconditioning methodologies have also demonstrated utility in modulating host immune responses through enhanced paracrine actions. Contrast to genetic engineering, which modifies a sole target gene, preconditioning can be used to provoke a more global cell response to a single stimulus (Drago et al. 2013). Cellular preconditioning also bypasses inflammatory issues associated with implanted pumps or invasive catheters for co-administration of growth factors, as well as mutational and neoplasmic risks associated with genetic manipulation. Non-genetic strategies are an appealing alternative for enhancing cellular survival and trophic support without further compromising tissue integrity. Moreover, preconditioning strategies offer a relatively straightforward and safer alternative to genetic modification, from a clinical perspective. The cost effectiveness and easy adoptability of this approach make it highly attractive for intended application in the injured CNS.

1.6 Study Overview

Reactive astrogliosis is a dynamic, but critical response mechanism to CNS trauma. While formation of the glial scar has been traditionally viewed as a strictly negative process resulting in neurotoxicity, inflammation, and neuronal growth inhibition, several studies have highlighted its utility in limiting secondary tissue damage by walling off the lesion site (Adams and Gallo 2018). Nevertheless, the molecular composition of the scar, and more specifically the production of growth-inhibitory ECM molecules, have been identified as major obstacles to the regenerative capacity of the CNS, prompting the development of targeted therapeutics (Michael T Fitch and Silver 2008).

Stem cell-based therapies are a rising and clinically attractive approach for the treatment of CNS injury, predominately because of the ability to repair and regenerate damaged tissue. While numerous cell types have been investigated for use in traumatic brain and spinal cord injury, NPCs are particularly attractive given their migratory capacity and ability to differentiate into the neuronal and glial lineages of the CNS (Takeuchi et al. 2007). While NPCs are widely known for their cell replacement capacity, they are also a substantial source of trophic support. NPCs secrete an array of beneficial cytokines, growth factors, and ECM
molecules that have been shown to aid in modifying the inhospitable post-injury environment to one more conducive for tissue repair and regeneration (Lu et al. 2003).

The therapeutic capacity of NPC secretome following CNS injury has been increasingly investigated, demonstrating reduced tissue damage, enhanced neural regeneration at the injury site, and improvements in locomotor function (Cheng et al. 2017; Doeppner et al. 2017; Liang et al. 2014b). Following NPC transplantation, several studies have demonstrated tissue repair, remyelination, and reduced glial scarring, the effects of which are mediated not only though cell replacement but trophic support (Lu et al. 2003). Following transplantation at or proximal to the injury site, however, NPCs are introduced to an inflammatory ischemic environment, and, as a result, undergo apoptosis, significantly limiting their therapeutic efficacy. Moreover, therapeutic efficacy of surviving NPCs is further limited by reactive astrogliosis and regenerative barriers such as the glial scar. As such, research avenues have expanded to elucidate, harness, and enhance the secretory capacity of NPCs to target the inhibitory post-glial injury environment as a means of increasing both endogenous and stem cell-mediated regenerative processes (Cheng et al. 2017; Doeppner et al. 2017; Drago et al. 2013). Combinatorial approaches in which stem cells are either preconditioned, engineered to secrete growth factors, or co-administered alongside growth factors, may enhance overall therapeutic efficacy and facilitate regeneration of the injured CNS.

HGF is a multifunctional cytokine that regulates a number of cellular processes including growth, motility, and morphogenesis (Hamanoue et al. 1996). HGF and its tyrosine kinase receptor, c-Met, have been found to be expressed in the CNS, and, following injury, stimulate a number of neuroprotective and regenerative effects through MAPK and PI3-kinase/Akt signaling pathways (Kunio Matsumoto et al. 2014). Given its neurotrophic and regenerative aptitude following CNS injury, we assessed the effect of HGF as a preconditioning agent for enhancing NPC secretory capacity, attenuating inhibitory facets of astrocyte reactivity, and promoting neurite outgrowth in vitro.

In the current study, adult rat spinal cord-derived NPCs were preconditioned with human recombinant HGF. First, we assessed the effect of HGF preconditioning on levels of NPC
cytokine production. To establish the most optimal pre-conditioning concentration, we assessed varying concentrations of HGF on NPC viability during oxidative stress exposure. Next, we evaluated the efficacy of HGF-enhanced NPC secretome in reducing TGFβ-induced astrocyte reactivity and promoting neurite outgrowth of hiPSC-neurons in an in vitro co-culture system. We demonstrate a significant increase in TIMP-1 and decrease in VEGF levels by HGF-preconditioned NPCs relative to untreated controls. Furthermore, in reactive astrocytes, treatment with HGF-enhanced NPC secretome resulted in a significant reduction in intermediate filament protein expression and chondroitin sulfate deposition relative to either treatment option alone. Moreover, treatment with HGF-enhanced NPC secretome demonstrated enhanced neurite outgrowth of hiPSC-neurons grown on reactive astrocyte substrates, relative to either treatment alone.

Capitalizing on the distinct benefits of cellular and growth factor therapies via combinatorial paradigms may not only inform but optimize overall therapeutic efficacy in CNS injury. Tactically enhanced neurotrophic support within the hostile post-injury CNS milieu could not only substantially facilitate tissue repair but promote endogenous and stem cell-mediated repair and regeneration. Utilizing HGF as a preconditioning or combinatorial agent alongside NPCs may serve as an enhanced and suitable therapy for modifying the complex astroglial environment post-injury to one more permissive for tissue repair and regeneration. Here, we show that application of this non-viral approach can effectively attenuate inhibitory facets of astrocyte reactivity, ultimately promoting neurite outgrowth. These effects may be mediated by distinct changes in cellular cytokine production following preconditioning. Our findings have important implications for the optimization of current cell-based strategies and functional recovery after CNS injury. Together, these data largely inform and warrant further mechanistic and in vivo investigation into the reparative potential of HGF-enhanced NPCs as a clinically translatable therapy for traumatic brain and spinal injury.
2 Hypothesis and Research Aims

In the current study, we assess the therapeutic potential of HGF-preconditioned NPCs as a combinatorial strategy for attenuating astrocyte reactivity and promoting concurrent neurite outgrowth of hiPSC-neurons \textit{in vitro}. To our knowledge, this is the first study to assess the astroglial-modifying and neurite growth-promoting effect of HGF-enhanced NPC secretome. From a clinical perspective, tactical modification of the post-injury astroglial scar may facilitate endogenous and stem cell-mediated regeneration, enhancing overall therapeutic efficacy and functional recovery. These findings have implications for the optimization of current cell-based paradigms for CNS injury.

2.1 Overarching hypothesis

Neural progenitor cells preconditioned with hepatocyte growth factor will exhibit distinct changes in cytokine levels that may aid in attenuation of growth-inhibitory astroglial constituents and enhanced neurite outgrowth.

2.2 Specific Aims

\textbf{Aim 1: Characterize the secretory cytokine profile of HGF-preconditioned NPCs}

To assess the effect of HGF on NPC cytokine production, adult rat spinal cord-derived NPCs were grown in cell-specific medium and preconditioned with recombinant human HGF (20 ng/mL). Conditioned media was then collected after 7 days, concentrated, and characterized via cytokine/vascular multiplex arrays.
Aim 2: Assess the effect of HGF-enhanced NPC secretome on intermediate filament protein expression and chondroitin sulfate proteoglycan deposition in reactive astrocytes

To assess the effect of HGF-enhanced NPC secretome on astrocyte reactivity, glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycan (CSPG) production were assessed. Primary rat cortical-derived astrocytes were activated using TGFβ1, then treated with HGF, NPC conditioned media, or a combination thereof. Western/Slot blot and immunocytochemistry were used to quantify expression of these astroglial markers.

Aim 3: Assess the effect of HGF-enhanced NPC secretome on neurite outgrowth in reactive astrocyte co-culture system

To assess the effect of HGF-enhanced NPC secretome on neurite outgrowth in an inhibitory astroglial environment, hiPSC-derived neurons were suspended over a feeder layer of TGFβ-activated astrocytes and subsequently treated with HGF, NPC-CM, or a combination thereof. Neurons were labelled for immunofluorescence staining and neurite length was assessed.
3 Materials and Methods

3.1 MTT Cell Viability Assay

50,000 rat spinal cord-derived NPCs per well were seeded onto a Matrigel-coated 96-well plate and subjected to oxidative stress via 500 μM of hydrogen peroxide treatment for 24 hrs alongside recombinant human HGF at different concentrations (10, 20, and 50 ng/mL), according to a previously published oxidative stress model (Hachem et al., 2015). MTT reagent (Sigma-Aldrich) was applied in accordance with Manufacturer’s instructions and cells were incubated for 4 hours at 37°C, ensuring light-sensitive conditions. 100 μL/well of DMSO was applied and the plate was incubated at 37°C until cells were lysed and purple crystals were dissolved. Absorbance was subsequently measured at 570 nm. Resultant readings were expressed as the total number of viable cells compared to hydrogen peroxide-treated NPCs controls, with an n=3 per treatment group.

3.2 Isolation and expansion of primary rodent NPCs

NPCs were isolated from the adult periventricular region of the spinal cord in female Wistar rats, in accordance with a previously published protocol (A. Mothe and Tator 2015). The spinal cord was excised and washed in Dulbecco’s phosphate buffered saline supplemented with 30% glucose and 1% penicillin/streptomycin (Sigma-Aldrich, Oakville, ON). Tissue from the periventricular region was harvested and enzymatically dissociated in a solution containing 0.01% papain and 0.01% DNase I for 1 hr at 37°C. Next, tissue was mechanically dissociated into cell suspension and further centrifuged using a discontinuous density gradient to remove cell membrane fragments. Cells were resuspended in Neurobasal-A medium supplemented with B27 (Gibco/Invitrogen), GlutaMax (Gibco/Invitrogen),
penicillin/streptomycin (Gibco/Invitrogen), 20 ng/ml EGF and FGF2 (Sigma Aldrich), 2 µg/mL heparin (Sigma-Aldrich), and hormone mix. Cells were cultured in uncoated Nunc T25 culture flasks and incubated at 37°C, 5% CO2, and 90% humidity. The resultant neurospheres were passaged weekly via trituration-induced mechanical dissociation in fresh medium described above. When grown as adherent cultures in monolayer, cells expressed the precursor marker Nestin (Figure 4a).

### 3.3 Preparation and collection of NPC conditioned media

At passage 4, neurospheres were collected, mechanically dissociated via trituration, and cultured at a density of 1,000 cells/µL in uncoated Nunc T25 culture flasks at 37°C, 5% CO2, and 90% humidity in fresh medium conditions described above. Viable cell calculation was assessed using Trypan blue staining, and cell density was calculated via hemocytometer. Resultant neurosphere-containing media was collected 7 days later, centrifuged at 16000 rpm for 5 minutes, and filtered using a syringe filter unit (0.44 µm; Millipore, Darmstadt, Germany) to remove any residual cells. The media was then aliquoted and stored at -80°C until further use in experimental assays.

To generate HGF-preconditioned NPC conditioned media, 20 ug/µL of recombinant HGF protein (#100-39, PeproTech, Canada) was added to free-floating neurospheres grown in fresh growth medium (1,000 cells/µL). This concentration was selected based on findings from the NPC oxidative stress assay mentioned previously. Conditioned media was collected 7 days later as formerly described.

### 3.4 NPC secretory cytokine analysis

Pre-conditioned and untreated NPC conditioned media (NPC-CM) were concentrated 25-fold using Vivaspin-20 3 kDa MWCO centrifugal concentrators (GE Healthcare Biosciences; Mississauga, Canada). Protein concentration was calculated using the microBCA assay (Thermo Scientific, Burlington, Canada) according to manufacturer’s instructions and 100 uL of sample (n=6) protein was diluted at a 1:1 ratio with 1x PBS (200 uL total).
Samples were sent to Eve Technologies (https://www.evetechnologies.com) for high-throughput ELISA profiling using their 27-plex rat cytokine /chemokine array (RD27) and rat vascular injury panel P1. All proteins that contained any interpolated or out-of-range signal were removed from the analyses. The proteins were then normalized using their calculated dilution factors and protein concentrations. The concentration of the proteins were either expressed in pg/ml derived from the company’s fluorescence standard curve (cytokine panel) or fluorescence intensity (vascular panel), and a one-way ANOVA with Tukey’s post hoc analysis (alpha = 0.05) was used to compute the p-values.

### 3.5 Culture and expansion of primary rat astrocytes

Primary rat cortical-derived astrocytes (#N7745100; Gibco, Thermo Scientific, Burlington, Canada), were thawed and expanded according to the manufacturer’s protocol. At passage 4, astrocytes were seeded in monolayer on 10-cm plates for Western and Slot blots. Conversely, a 24 well plate with glass coverslips was used to grow astrocytes for immunocytochemistry (VWR International, Mississauga, Ontario). In both cases, plates and coverslips were coated with Matrigel (Thermo Scientific, Burlington, Canada) and astrocytes were grown in medium containing 89% Dulbecco’s Modified Eagle Medium/F12 containing no phenol red (DMEM; Gibco/Invitrogen), 10% fetal bovine serum (FBS) and 1% 100 U/mL penicillin/100 μg/mL streptomycin (Gibco/Invitrogen). Cells were incubated at 37°C, 5% CO2, and 90% humidity, with growth medium changed every 3-4 days.

### 3.6 Astrocyte Activation and Treatment

Astrocytes were grown to ~80% confluency as described above and activated according to a previously published protocol by Yu et al (Yu et al. 2012). Astrocytes were treated with active human TGF-β1 (Abcam, Cambridge, MA) for 5 days in serum-free DMEM/F12 containing no phenol red. Cells were then washed 3 times with 1x PBS to remove any residual TGF-β1. Treatments were then applied for 24 hours (Table 1), after which cells and media were collected and stored at -80°C for future experiments.
3.7 Preparation of astrocyte conditioned medium and cell lysate

Conditioned medium: Following astrocyte activation and subsequent treatment, conditioned medium (CM) was collected, transferred to 15 mL conical tubes on ice, and centrifuged at 4°C for 10 minutes at 16,000 rpm. CM was subsequently filtered using a 0.44 μm syringe filter unit to remove any residual cells. Protein quantification was assessed using the BCA assay according to manufacturer’s protocol. CM was aliquoted into new tubes and stored at -80°C until further use in experimental assays.

Cell lysate: Following activation and subsequent treatment, astrocytes were washed 3 times in ice-cold 1x PBS and gently collected using a cell scraper. The cell suspension was transferred to Eppendorf tubes and centrifuged at 4°C, 16,000 rpm for 10 minutes. Media-containing supernatant was carefully extracted and discarded. Cells were then lysed using ice-cold radio-immunoprecipitation assay buffer (RIPA; Thermo Scientific) supplemented with protease inhibitors according to the manufacturer’s protocol. Samples were passed through a 25-gauge needle 5 times until sufficiently homogenized, and then centrifuged at 14,000 rpm for 15 minutes. The supernatant from each sample was carefully removed ensuring that the underlying pellet was not disturbed, and then transferred to a new Eppendorf protein LoBind
tube. Protein quantification was conducted using the BCA assay. Samples were stored at -80°C until further use in experimental assays.

3.8 Quantification of GFAP production in astrocyte cell lysate

3.7.1 Western Blot

15 μg cell lysate was mixed with 5x sample buffer and heated at 100°C in water bath for 5 minutes, then centrifuged at 15,000 rpm for 10 minutes. After samples were cooled, equal amounts of protein were loaded into the wells of an 8% sodium dodecyl sulfate (SDS) acrylamide gel and subsequently run for approximately 2 hours at 100 V. Protein from the gel was transferred to a nitrocellulose membrane and run for 1 hour at 100 V. The gels were then carefully removed and membranes were stained with Ponceau S solution to confirm protein transfer and assess total protein load. The membranes were blocked for 1 hour at room temperature in blocking solution containing 5% nonfat skim milk in 0.1% Tween20 phosphate buffered saline (PBST).

The membranes were incubated at 4°C overnight with the designated primary antibodies (Table II) in blocking solution: rabbit anti-GFAP (1:1000; Millipore) or mouse anti-αTubulin (1:200; Santa Cruz Biotechnology) in PBST containing 5% nonfat skim milk. Next, the membranes were washed 3 times in PBST (5 minutes each) and incubated for 1 hour at room temperature with the corresponding HRP-conjugated secondary antibody in blocking solution: anti-rabbit IgG (1:10,000, Thermo Scientific) or anti-mouse IgG (1:10,000, Thermo Scientific). Pierce ECL Western Blotting Substrate (ThermoFisher Scientific) was used according to manufacturer’s protocol for signal development. The membranes were exposed to film and developed. Experiments were performed in triplicate with n=3 and densitometric analysis was conducted using ImageJ software (NIH). Data were normalized to loading control.
3.7.2 Immunocytochemistry

As described previously, astrocytes were grown on Matrigel-coated glass coverslips in a 24 well plate. The cells were then fixed in 4% phosphate buffered paraformaldehyde for 10 minutes at room temperature. The cells were gently washed in 1x PBS 3 times for 5 minutes each. Next, cells were incubated for 30 minutes at room temperature in blocking solution containing 3% bovine serum albumin, 3% goat serum, and 0.1% Triton X 100 in 1x PBS.

The cells were incubated at 4°C overnight with the designated primary antibody (Table I) in blocking solution: rabbit anti-GFAP (1:500; Millipore). The cells were then washed 3 times in 1x PBS (5 minutes each) and incubated for 1 hour at room temperature with the corresponding secondary antibody in blocking solution: goat anti-rabbit Alexa 488 (1:200; Thermo Scientific), and DAPI nuclear stain (1:1000; Thermo Scientific). The cells were washed 3 times in 1x PBS and coverslips were carefully mounted to Superfrost Plus slides (Thermo Scientific) with Mowiol mounting medium (Sigma-Aldrich). Coverslips (n=3 per sample) were imaged using an epifluorescence microscope (Zeiss Axioplan 2, Carl Zeiss, Toronto, ON) using standardized camera settings with 3 random fields per coverslip.

Staining was quantified using a custom script in Image J and presented as total area of staining per cell relative to TGFβ-activated astrocyte controls. 20x images were converted from 16-bit color to 8-bit greyscale. Noise removal was performed to remove fluorescent artefacts using the “Despeckle” function in ImageJ (removes specks of signal that are 1 px² in area). A signal threshold of 15-255 greyscale was applied for staining detection. This was held constant across all images. An area fraction (% area) of staining was then calculated and converted to an actual area by taking the product of the area fraction to total image area. To normalize for the total number of cells in each field of view, the “Find Maxima” function with a noise tolerance of 25 was used to find nuclei foci, and the number (in counts) was outputted. The final metric that was computed was expressed as positive staining area per cell in inches².
3.9 Quantification of CSPG production in astrocyte conditioned medium

3.9.1 Slot Blot

Following protein quantification, 15 μg of CM sample in 200 μL of double distilled water were loaded into the wells of a Bio-Dot SF microfiltration apparatus (Bio-Rad, Mississauga, Ontario, Canada). The sample was allowed to dry before being subjected to the slot blot analysis.
Canada) and transferred to an underlying nitrocellulose membrane, following the manufacturer’s protocol. The membranes were then immunoprobed as described above for mouse anti-CSPG (C56S; 1:1000; Sigma) and corresponding anti-mouse IgG (1:10,000, Thermo Scientific). The membranes were exposed to film and developed as described previously. Experiments were performed in triplicate with n=3 and densitometric analysis was conducted using ImageJ software (NIH). Data were normalized to ponceau total protein load.

3.9.2 Immunocytochemistry

Astrocytes were grown on Matrigel-coated glass coverslips in a 24 well plate, fixed, and prepared as previously described. Next, cells were incubated for 30 minutes at room temperature in blocking solution and incubated at 4°C overnight with the designated primary antibody (Table II) in blocking solution: mouse anti-CSPG (C56S; 1:1000; Sigma). The cells were then washed 3 times in 1x PBS (5 minutes each) and incubated for 1 hour at room temperature with the corresponding secondary antibody in blocking solution: goat anti-mouse Alexa 568 (1:200; Thermo Scientific) and DAPI nuclear stain (1:1000; Thermo Scientific). The cells were washed 3 times in 1x PBS and coverslips were carefully mounted to slides with Mowiol mounting medium (Sigma-Aldrich). Coverslips (n=3 per sample) were imaged using an epifluorescence microscope (Zeiss Axioplan 2, Carl Zeiss, Toronto, ON) using standardized camera settings with 3 random fields per coverslip. Staining was quantified using a semi-automated ImageJ macros as previously described, and presented as total area of staining per cell relative.

3.10 Neurite Outgrowth Assay

3.10.1 Maintenance and expansion of hiPSC line

The human induced pluripotent stem cell (hiPSC) line, BC1, derived from human adult bone marrow hematopoietic cells, was adapted to serum and feeder-free conditions using E8
medium (Essential 8 medium, Life Technologies, CA) and expanded on Matrigel in mTeSR1.

3.10.2 Generation of NSCs from hiPSCs

Dual-SMAD inhibition using SB431542 and Noggin in monolayer was used to differentiate hiPSCs to neural stem cells (NSCs), as previously described by Chambers et al (Chambers et al. 2009). A monoclonal line of NSCs was established by two cycles of single cell-neurosphere formation in Ultra-Low Adhesion Plates (Corning, Tewksbury, MA) with initial plating at 10,000 cells/mL. NSCs were maintained in NSC expansion media consisting of DMEM/F12 + Glutamax (Gibco) + B27 Supplement without Vitamin A (Gibco) + bFGF (20ng/mL; Peprotech) + EGF (20ng/mL; Peprotech) + 1% Penicillin/Streptomycin (Invitrogen).

3.10.3 hiPSC-NSC neuronal differentiation

The hiPSC-NSC line was plated on Poly-L-Ornithine/laminin coated dishes for further differentiation to neurons using neuron differentiation media (NDM) as described by Palm et al (Palm et al., 2015), consisting of neurobasal media (Gibco) + N2 Supplement (Gibco) + B27 Supplement (Gibco) + brain derived neurotrophic factor (BDNF; 10ng/mL; Peprotech) + glial derived neurotrophic factor (GDNF; 10ng/mL; Peprotech) + TGF-β3 (1ng/mL; Peprotech) + cyclic adenosine monophosphate (cAMP; 500µM; Sigma Aldrich). NDM was changed every 2 days for 6 weeks prior to harvesting of neurons.

3.10.4 Reactive Astrocyte-Neuron Co-Culture

5 x 10⁴ primary rat cortical derived-astrocytes per well were plated onto Matrigel-coated 12mm glass coverslips in a 24-well plate. Once cells were grown to ~80% confluency, they were activated and treated (Table I) as previously described. Following treatment, residual media was removed and 1x10⁴ hiPSC-derived neurons per well were plated onto the confluent monolayer of astrocytes and co-cultured for 4 days.
3.10.5 Immunofluorescence Staining

Neurons and astrocytes were labelled by immunofluorescence staining. The cells were fixed in 4% phosphate buffered paraformaldehyde for 10 minutes at room temperature and gently washed in 1x PBS 3 times for 5 minutes each. Next, cells were incubated for 1 hour at room temperature in blocking solution containing 5% bovine serum albumin, 3% goat serum, and 0.1% Triton X 100 in 1x PBS.

The cells were then incubated at 4°C overnight with the designated primary antibodies (Table II) in blocking solution: mouse anti-MAP2 (1:500; Millipore) or rabbit anti-GFAP (1:500; Millipore), to label neurons and astrocytes, respectively. The cells were then washed 3 times in 1x PBS (5 minutes each) and incubated for 1 hour at room temperature with the corresponding secondary antibody in blocking solution: goat anti-mouse Alexa 488 (1:500; Thermo Scientific) or goat anti-rabbit Alexa 568 (1:500; Thermo Scientific), and DAPI nuclear stain (1:1000; Thermo Scientific). The cells were washed 3 times in 1x PBS and coverslips were carefully mounted to Superfrost Plus slides (Thermo Scientific) with Mowiol mounting medium (Sigma-Aldrich). Coverslips (n=3 per sample) were imaged at 20x magnification using a Nikon Eclipse Ti microscope using standardized camera settings with 4 randomly selected fields per coverslip. Quantification of total neurite length per cell was performed using the NeuronJ plug-in available in ImageJ software (NIH).

3.11 Statistical Analysis

GraphPad Prism (La Jolla, CA) was utilized for all statistical analyses. Quantitative data were expressed as the ± standard error of the mean (SEM) with differences among groups assessed by one-way ANOVA with Tukey’s post-hoc test. Statistical significance was set at p≤0.05.
Chapter 4
Results

4 Results

4.1 HGF enhances NPC viability during oxidative stress exposure

Oxidative stress is a well-known pathogenic process in CNS injury resulting in cellular apoptosis. Here, we utilized hydrogen peroxide to induce oxidative stress in adult rat spinal cord-derived NPCs and investigated the effect of concurrent HGF treatment on cell viability. Following treatment and application of MTT reagent in accordance with Manufacturer’s instructions, absorbance was measured at 570nm.

Exposure of NPCs to 500 uM of H$_2$O$_2$ for 24 hours significantly decreased the viability of these cells, a finding that is congruent with previous studies utilizing the same cell type and source (Hachem et al., 2015). Relative to untreated NPCs, hydrogen peroxide treated cells demonstrated approximately 40% cell viability (-50,458 ± 4959 cells; p<0.0001; Figure 3) confirming the utility of this model as an apoptosis-inducing environment in vitro.

We further investigated whether concurrent treatment of NPCs with HGF conferred protection against H$_2$O$_2$-induced apoptosis. NPCs exposed to H$_2$O$_2$ were also treated with varying concentrations of HGF (10, 20, or 50 ng/mL) for 24 hours (Hu et al., 2010). HGF treatment demonstrated significantly increased cell viability relative to hydrogen peroxide-treated controls. HGF at concentrations of 20 and 50 ng/mL demonstrated the most notable effects on cell viability (with a trending increase in the latter), relative to hydrogen peroxide-treated NPC controls (26993 ± 4959 cells; p<0.0041 and 24501 ± 4959 cells; p<0.0020, respectively; Figure 3). Based on these findings, a concentration of 20 ng/mL HGF was used for subsequent NPC preconditioning experiments.
Figure 3: HGF enhances NPC viability during oxidative stress exposure. Plated rat spinal cord-derived NPCs were subjected to oxidative stress via hydrogen peroxide and treated for 24 hours with recombinant human HGF at different concentrations. MTT reagent was applied in accordance with Manufacturer’s instructions and absorbance was measured at 570 nm. A reduction of approximately 40% was observed in viable cell number of hydrogen peroxide-treated NPCs relative to untreated cells. Subsequent HGF application to hydrogen peroxide-treated NPCs demonstrated significantly increased cell viability relative to hydrogen peroxide-treated controls, with higher concentrations of HGF (20 ng/mL and 50 ng/mL) exhibiting the most notable effects. Data are expressed as mean ±SEM and presented as the total number of viable cells compared to hydrogen peroxide-treated NPC controls. One-way ANOVA statistical analysis with Tukey’s post-hoc test was performed; ns (p>0.05), *(p<0.05); **(p<0.01); ****(p<0.001).
4.2 HGF-preconditioned NPCs demonstrate reduced VEGF and increased TIMP-1 production.

In the current study, conditioned medium was utilized from adult rat NPCs derived from the periventricular region of the spinal cord. When grown as adherent cultures in monolayer, cells expressed the precursor marker Nestin (Figure 4a).

To characterize the secretory cytokine profile of HGF-preconditioned NPCs, proteomic analysis was conducted on resultant conditioned medium using Rat Cytokine Array/Chemokine Array 27 Plex and Rat Vascular Injury P1-4-Plex kits (Eve Technologies). Out of the 31 cytokines evaluated (Figure 4b), differences were observed in the levels of TIMP-1 and VEGF (one-way ANOVA/Tukey’s post-hoc; Figure 4b).

HGF-preconditioned NPCs demonstrated a notable increase (2341 ± 852.8 AU) in TIMP-1 production relative to untreated NPC-CM controls (p<0.0001). While both groups demonstrated a marked increase in VEGF levels relative to the other cytokines in the kits, HGF-preconditioned NPCs exhibited 2166 ± 309.5 AU less production in comparison to untreated NPC controls (p<0.05).
Figure 4: HGF-preconditioned NPCs demonstrate reduced VEGF and increased TIMP-1 production. A) Characterization of rat spinal cord-derived NPCs grown in monolayer. Immunocytochemistry revealed cellular expression of the progenitor cell marker Nestin. Representative image taken at 20x using a Zeiss Axioplan 2 epifluorescence microscope. (B) Secretory cytokine changes in HGF-preconditioned NPCs were assessed using the Rat Cytokine Array/Chemokine Array 27 Plex (RD27) and Rat Vascular Injury P1-4-Plex kits, comparing the relative levels of 27 and 4 cytokines, respectively. Protein levels were normalized to untreated NPC conditioned media control (n=6). Preconditioning of NPCs with HGF (20ng/mL) demonstrated reduced VEGF and increased TIMP-1 production relative to untreated NPCs. Data are expressed as mean ±SEM. One-way ANOVA statistical analysis with Tukey’s post hoc test was performed; ns (p>0.05), *(p≤0.05), ****(p≤0.0001).
4.3 Treatment of reactive astrocytes with HGF-enhanced NPC-CM demonstrates reduced GFAP expression.

Upregulation of the intermediate filament protein GFAP, constitutes a characteristic marker of astrocyte reactivity. Area of GFAP+ staining analysis in non-reactive astrocytes demonstrated a significant reduction in expression (0.37 ± 0.08; p=0.0023) relative to TGFβ1-activated astrocyte controls (figure 5a), confirming the utility of the astrocyte activation protocol. Subsequent treatment of reactive astrocytes with high concentrations of HGF (50 ng/mL) alone (p=0.0173) as well as combinatorial treatments, demonstrated significant reductions in GFAP expression (figure 5a). High dose combinatorial (50 ng/mL HGF + NPC-CM) treatment (0.39 ± 0.08; p=0.0008) and HGF-preconditioned NPC-CM (0.41 ± 0.08; p=0.0004) demonstrated the most notable reductions relative to reactive astrocyte controls (figure 5a).

Western blot densitometric analysis yielded a significant increase in reactive astrocyte GFAP production relative to non-activated astrocytes (0.35 ± 0.06; p=0.0005; figure 5b), confirming its utility as an appropriate activation method. Post-treatment, findings were similar to that observed via immunocytochemistry. Treatment of reactive astrocytes with NPC-CM or HGF protein at low concentrations demonstrated trending reductions in GFAP expression but did not result in any statistical difference relative to reactive astrocyte controls. High-dose HGF treatment (50 ng/mL) as well as combinatorial treatments, however, resulted in statistically significant reductions in GFAP production relative to reactive astrocyte controls (figure 5b). HGF-preconditioned NPC-CM (0.65 ± 0.06; p<0.0001) and combinatorial treatments demonstrated the most notable reductions in GFAP production not only in comparison to activated, but non-activated astrocytes (figure 5b).

Thus, combinatorial treatments, whether delivered via preconditioning or concurrent means, effectively reduced astrocyte reactivity as measured by expression of the astrocyte reactivity marker GFAP.
Figure 5: Treatment of reactive astrocytes with HGF-enhanced NPC-CM demonstrates reduced GFAP expression. A) Immunostaining of non-activated and TGFβ-induced reactive astrocytes with reactivity marker GFAP (green) and DAPI (blue). Non-reactive astrocytes demonstrated a significant reduction in GFAP expression relative to reactive astrocyte controls. Treatment of reactive astrocytes with high concentrations of HGF treatment as well as combinatorial treatments (preconditioned and concurrently applied) also demonstrated a significant reduction in GFAP expression relative to reactive astrocyte controls. Data are expressed as mean ±SEM; one-way ANOVA with Tukey’s post-hoc test; ns (p>0.05), *(p≤0.05), ***(p≤0.001). B) Western blot of cell lysate probed with GFAP (bands ranging from ~40-55 kDa) and α-actin loading control (42 kDa). Densitometric analyses were presented as a ratio to TGFβ reactive astrocyte controls; multiple comparisons were shown between treatment groups with 20ng/mL HGF concentrations (pretreated or concurrent application). While treatment with HGF alone demonstrate a significant reduction in GFAP, combinatorial treatments and HGF-preconditioned NPC-CM demonstrated the most notable effects. Data are expressed as mean ±SEM, normalized to α-tubulin loading control, and presented as a ratio to TGFβ activated control. Multiple comparisons were shown between treatment groups with 20ng/mL concentrations. One-way ANOVA statistical analysis with Tukey’s post-hoc test was performed; ns (p>0.05), ***(p≤0.001), *****(p≤0.0001).
4.4 Treatment of reactive astrocytes with HGF-enhanced NPC-CM demonstrates reduced CSPG deposition.

Upregulated expression and deposition of CSPGs is a key hallmark of reactive astrogliosis and a major target for therapeutic intervention in CNS injury.

Area of CSPG+ staining analysis in non-reactive astrocytes demonstrated a significant reduction in expression (0.04 ± 0.007; p<0.0001) relative to TGFβ1-activated astrocyte controls (figure 6a), confirming the utility of the astrocyte activation protocol. Subsequent treatment of reactive astrocytes demonstrated significant reductions in CSPG+ staining for all groups; NPC-CM demonstrated slightly less reduction (0.03 ± 0.007; p=0.0036) while all other treatment groups demonstrated significantly notable reductions in CSPG+ staining relative to reactive astrocyte controls (p<0.0001; figure 6a). Combinatorial concurrent (20 ng/mL HGF + NPC-CM) treatment (0.03 ± 0.007; p=0.0227) and HGF-preconditioned NPC-CM (0.03 ± 0.007; p=0.0146) demonstrated notable reductions relative to NPC-CM treatment alone (figure 6a).

Slot blot densitometric analysis yielded a significant increase in reactive astrocyte CSPG production relative to non-activated astrocytes (0.51 ± 0.09; p=0.0013; figure 6b), as expected. Treatment of reactive astrocytes with NPC-CM or HGF protein at 50 ng/mL demonstrated trending reductions in CSPG production but did not result in any statistical difference relative to reactive astrocyte controls. Notable reductions in CSPG production, however, were demonstrated with HGF-preconditioned NPC-CM (0.83 ± 0.09; p<0.0001) as well as high-dose (50 ng/mL HGF + NPC-CM) combinatorial (0.81 ± 0.09; p<0.0001) treatments (figure 6b).

Taken together, combinatorial treatment, whether delivered via preconditioning or concurrent means, effectively reduce astrocyte reactivity as demonstrated by CSPG deposition.
Figure 6: Treatment of reactive astrocytes with HGF-enhanced NPC-CM demonstrates reduced CSPG deposition. A) Immunostaining of non-activated and TGFβ-induced reactive astrocytes with reactivity marker CSPG (green) and DAPI (blue). Non-activated astrocytes demonstrate a significant reduction in CSPG expression relative to reactive astrocyte controls. All treatment groups demonstrated a significant reduction in CSPG expression relative to reactive astrocyte controls; Combinatorial treatment (preconditioned and concurrently applied) demonstrated a significant reduction relative to NPC-CM alone. Data are expressed as mean ±SEM; One-way ANOVA statistical analysis with Tukey’s post-hoc test; ns (p>0.05), *(p≤0.05), ***(p≤0.01), ****(p≤0.0001). B) Slot blot analysis of astrocyte conditioned media probed with CSPG. Densitometric analyses were presented as a ratio to TGFβ reactive astrocyte control; multiple comparisons were shown between treatment groups with 20ng/mL HGF concentrations (pretreated or concurrent application). While treatment with NPC-CM and HGF alone revealed trending reductions in CSPG expression, combinatory treatments and HGF-preconditioned NPC-CM demonstrated the most notable effects. Data are expressed as mean ±SEM, normalized to ponceau total protein load, and presented as a ratio to TGFβ activated control. Multiple comparisons were shown between treatment groups with 20ng/mL concentrations. One-way ANOVA statistical analysis with Tukey’s post-hoc test was performed; ns (p>0.05), **(p≤0.01), ***(p≤0.001), ****(p≤0.0001).
4.5 HGF-enhanced NPC-CM enhances neurite outgrowth of hiPSC-neurons in reactive astrocyte co-culture

A key consequence of astrocyte reactivity and glial scarring is inhibition of neurite outgrowth, the effect of which is largely mediated by the production of CSPGs and other inhibitory ECM molecules. Here, we mimic a growth-inhibitory environment through TGF-β1-induced astrocyte reactivity, which results in increased CSPG deposition (Figure 6a,b). HiPSC-derived neurons were co-cultured atop this feeder layer of reactive astrocytes and singular or combinatorial treatments were then administered. After 4 days, total mean neurite length per cell was assessed after 4 days.

HiPSC-derived neurons are indicated by the neuronal and dendritic marker MAP2 (Figure 7a), while reactive astrocytes and hiPSC-derived neurons are indicated in co-culture via GFAP and MAP2 staining, respectively (Figure 7b). TGFβ1-activated astrocytes demonstrated a significant reduction in neurite length, approximately two times less than that of non-activated astrocytes (-214.14 ± 33.81 µm; p<0.0001; figure 7c), confirming the utility of this activation protocol as largely inhibitory to neurite outgrowth. Treatment of co-cultures with NPC-CM or HGF protein alone did not demonstrate statistically significant differences in neurite length relative to reactive astrocyte-neuron controls, although higher concentrations of HGF did exhibit trending increases. Co-cultures that were subjected to HGF-preconditioned NPC-CM as well as high-dose (20 and 50 ng/mL HGF + NPC-CM) combinatorial treatments demonstrated a significant increase in total mean neurite length relative to TGFβ1-activated controls (Figure 7c). Interestingly, HGF-preconditioned NPC-CM demonstrated the most notable effect, with a total mean neurite length comparable to that of non-activated co-cultures; hiPSC-neurons exhibited approximately twice the neurite length of TGFβ1-activated controls (214.9 ± 33.81 µm; p<0.0001; Figure 7c). Taken together, combinatorial treatments effectively promoted neurite outgrowth of hiPSC-neurons in the presence of reactive astrocyte-induced CSPG deposition to a greater extent than either treatment alone.
Figure 7: HGF-enhanced NPC-CM treatment increases neurite outgrowth of hiPSC-neurons in reactive astrocyte co-culture. A) Immunostaining of hiPSC-derived neurons with neuronal and dendritic marker MAP2 (green) and DAPI (blue). Representative image taken at 20x. (B) hiPSC-derived neurons were plated onto a confluent monolayer of TGFβ-induced reactive astrocytes. Immunostaining of neuronal and dendritic marker MAP2 (green), astrocyte reactivity marker GFAP (red), and DAPI (blue). (C) Treatments were applied to reactive astrocyte-neuron co-culture for a span of 4 days and imaged at 20x thereafter. There was a decrease of approximately 50% in total neurite length of cells plated on reactive astrocyte substrate relative to non-activated astrocytes. Combinatorial treatments applied to reactive-neuron co-cultures demonstrated a significant increase in neurite length compared to reactive astrocyte controls. HGF-preconditioned NPC-CM (HGF-NPC-CM) also demonstrated a substantial increase in neurite length to an extent comparable to non-activated astrocytes (2 times the length of reactive astrocyte controls). Data are expressed as mean ±SEM and presented in comparison to reactive astrocyte controls. Multiple comparisons were shown between treatment groups with 20ng/mL concentrations. One-way ANOVA statistical analysis with Tukey’s post-hoc test; ns (p>0.05), **(p≤0.01), ****(p≤0.001), *****(p≤0.0001).
Chapter 5
Discussion

5 Discussion

5.1 Summary of Findings

Traumatic brain and spinal cord injuries constitute approximately half of all trauma-related injuries and are among the foremost causes of death and disability worldwide, carrying devastating and enduring personal and socioeconomic ramifications (Cripps et al. 2011; Hyder et al. 2007). CNS injury is a biphasic and progressive process with dynamic underlying molecular and cellular mechanisms that are not fully understood. The primary injury phase, or mechanical insult incurred during injury, is followed by a secondary cascade of cellular and molecular processes that further amplify the initial damage (Kraus 1996). Current treatments are largely aimed at limiting tissue damage and promoting regeneration within the injured CNS.

Stem-cell based therapies are a promising and transformative approach for addressing the dynamic and multifactorial nature of secondary injury following CNS trauma. NPCs are particularly appealing for transplantation given their migratory capacity and ability to differentiate into the cellular lineages of the CNS, providing a direct reservoir for tissue repair (A. J. Mothe and Tator 2013). In addition, the array of reparative biomolecules secreted by transplanted NPCs further act to facilitate repair and regeneration via trophic support (Lu et al. 2003). Despite these beneficial attributes, studies have yielded modest functional improvements following transplantation, as both tissue repair and regenerative processes are hampered by the hostile and inhibitory nature of the post-injury environment.

Reactive astrogliosis and formation of the glial scar are defining hallmarks of the secondary injury process and, while initially beneficial in limiting further tissue damage and inflammatory infiltration, culminate in a complex physical and chemical barrier to axonal regeneration (Yiu and He 2006; Adams and Gallo 2018; McKeon et al. 1991). While it is
important to recognize the critical protective role of the glial scar early after injury, it is also imperative to acknowledge the regenerative constraints poised by its physical and chemical constituents chronically. Increased expression and deposition of ECM molecules such as CSPGs constitute a particularly potent regenerative barrier to neurite outgrowth (McKeon et al. 1991; McKeon, Jurynev, and Buck 1999; Lu et al. 2003). As such, a less complex glial scar, concomitant with reduced CSPG deposition may foster a more permissive environment to better support regeneration and functional recovery after injury. Understanding the functional duality of the astrocytic glial scar may aid in the development of therapies that effectively address these regenerative barriers and promote endogenous and stem-cell mediated neuroregeneration. The overarching therapeutic goal would therefore not aim to eliminate, rather modify inhibitory astroglial components to foster improved recovery and regeneration. As such, efforts have expanded to harness and enhance the trophic capacity of NPCs as a means of modifying the post-injury glial environment to one that is more conducive to regenerative processes (Cheng et al. 2017; Doeppner et al. 2017; Drago et al. 2013).

There has been a wealth of increasing evidence demonstrating the neurotrophic and regenerative effects of HGF in various models of CNS injury and neurodegenerative disease (Takano et al. 2017; C. Liu et al. 2011; Yamane et al. 2018; W. Sun, Funakoshi, and Nakamura 2002; T Nakamura et al. 2000; Wong et al. 2014). In the current study, we assessed the therapeutic capacity of HGF as a combinatorial and priming agent alongside NPCs in attenuating astrocyte reactivity and promoting neurite outgrowth in vitro. We show that HGF preconditioning alters the level of secretory cytokine production in NPCs, with a significant increase in TIMP-1 and reduction in VEGF relative to untreated controls. While treatment of reactive astrocytes with HGF at increasing concentrations demonstrated reductions in GFAP and CSPG deposition, HGF-enhanced NPC secretome (whether via preconditioning or concurrent administration), demonstrated the most substantial effects. Furthermore, in a reactive astrocyte-neuron co-culture, HGF-enhanced NPC secretome significantly promoted neurite outgrowth of hiPSC-neurons to an extent comparable to untreated controls, or approximately twice that of activated astrocytes. These results provide
evidence that concurrent administration or preconditioning of NPCs with HGF may mitigate regenerative barriers poised by reactive astrocytes and promote regeneration to a greater extent than either treatment option alone. Furthermore, differences in NPC secretory cytokine production with HGF preconditioning may indicate a beneficial modification of cellular secretome and enhanced regenerative potential. From a clinical standpoint, these findings would have important implications for tactical manipulation of the post-injury glial environment to enhance endogenous and stem-cell mediated regeneration as well as functional recovery after CNS injury.

5.2 HGF-preconditioned NPCs demonstrate reduced VEGF and increased TIMP-1 production

5.2.1 Effect of HGF on NPC proliferation and differentiation

The capacity of HGF to promote neural stem cell proliferation and self-renewal have been documented in the literature. In cultures derived from the subventricular zone, nestin-expressing cells have been shown to produce HGF and c-Met (Nicoleau et al. 2009). Nicoleau et al also reported neurosphere formation and self-renewal of NSCs following HGF treatment, the effect of which was mediated in a MAPK-ERK1/2-dependent manner. Similarly, Kokuzawa et al. also demonstrated the proliferation of ESC-derived neurospheres with HGF treatment. Resultant neurospheres were largely comprised of progenitor cells (Kokuzawa et al. 2003). These findings attest to the proliferative and neurogenic-inducing capacity of HGF in NPC cultures.

5.2.2 Migrational properties of HGF and VEGF on endogenous and transplanted NPCs

NPCs demonstrate exceptional migratory capacity within the CNS, making them an attractive cell-based strategy for repair and regeneration (Takeuchi et al. 2007). While most transplantation paradigms are conducted via direct cellular administration at the injury site, a recent study by Takeuchi et al investigated minimally invasive intravenous NSC delivery in a
rodent model of SCI, the effect of which mediated by distinct chemo-attractants secreted by the lesion. Interestingly, the migration of NSCs to the lesion site was highest at 1 week after injury, correlating not with disruption of the blood-spinal cord-barrier, rather a peak in HGF and stromal cell-derived factor-1 expression (Takeuchi et al. 2007). These findings attest to the chemotactic properties of HGF not only on endogenous NSCs, but transplanted cells.

The availability of NSCs is a key consideration for increasing therapeutic efficacy in CNS injury and neurological disease. While exogenous transplantation of NSCs has demonstrated favourable outcomes and functional benefit, a key limitation and challenge associated with this approach is cell survival upon entry into the cytotoxic injury milieu, and consequent number of cells available to migrate to the site of injury. Activation and migration of endogenous NSCs following injury may prove advantageous in facilitating cell rescue and tissue repair. There is ample evidence demonstrating that endogenous neurogenesis occurs in the SVZ after stroke, with stem and progenitor cells migrating and integrating into damaged tissue, expressing neuronal markers and acquiring morphological neuronal phenotypes (Arvidsson et al. 2002; Kuge et al. 2009; Yagita et al. 2001). As such, another consideration for the treatment of CNS injury is the capitalization of endogenous NSC reserves as a source for further tissue repair and regeneration.

Chemotactic factors that are produced during injury often serve as homing or migratory cues that recruit NSCs to the injury site (Ekdahl, Kokaia, and Lindvall 2009). A number of growth factors are known to promote repair and regeneration by facilitating progenitor cell recruitment to injured tissue. HGF has been identified as a strong chemoattractant and proliferative NSC factor (Lan et al. 2008; Takeuchi et al. 2007). Indeed, use of a hydrogel enabling extended release of HGF in vitro demonstrated increased recruitment of NSCs to the site of application (J. Zhao et al. 2008). In hESC-derived NSCs, HGF also demonstrated a chemotactic effect facilitating cell migration via concentration gradient (Cacci et al. 2003; Lan et al. 2008). The chemotactic effect of HGF has also been shown in olfactory bulb-derived NPCs, suggesting a role in the guidance of neuroblasts migrating to the olfactory bulb (Garzotto et al. 2008). Thus, an optimized gradient of HGF concentrations could
potentially be used to direct migration of NSCs to the site of injury within the CNS, to further facilitate directed repair and regeneration.

VEGF is also another important chemotactic and angiogenic factor, the expression of which has been shown to increase in neurons, glia, and macrophages surrounding the injury site shortly after ischemia (Cross et al. 2003; Lambrechts and Carmeliet 2006; Munehisa Shimamura et al. 2006). VEGF-mediated NSC recruitment has been demonstrated through activation of VEGF receptor 2. Moreover, in the rodent brain, VEGF infusion into the contralateral hemisphere demonstrated migration of transplanted NSCs across the corpus callosum to the site of administration; VEGF-neutralizing antibodies reduced this migratory effect substantially (Schmidt et al. 2009). These studies attest to the role of VEGF in proliferation, homing, and recruitment of NSCs to the injury site.

Cell-to-cell interactions and cell-to-ECM interactions are critical to the proliferation, differentiation and migration of endogenous and exogenously administered NSCs in the uninjured and injured CNS. Growth factors and cytokines can be key contributing factors to the proliferation, homing, and recruitment of NSCs. As such, factors like HGF may not only promote regeneration of spared axons through modification of the glial scar, but also enhance the migration and homing of endogenous or exogenous NSPCs to the site of injury to further promote repair and regeneration.

5.2.3 Cross-talk between VEGF and HGF signaling pathways

The secretory cytokine profile of both preconditioned and untreated NPCs in the current study demonstrated a marked increase in VEGF production relative to all other cytokines examined. These findings are consistent with that of other studies. Largely known for its regulatory role in vessel growth during development, VEGF is also a well-known growth factor that is upregulated following CNS injury. In the injured spinal cord, an increase in VEGF expression has been postulated to promote neovascularization in an effort to attenuate inflammatory distress (Sköld et al. 2005).
While the angiogenic capacity of VEGF is well known, its multifaceted trophic effects have also been highlighted in the CNS. VEGF has been shown to promote neuronal and glial survival, and enhance neurite outgrowth (Park et al. 2010; Hongyu Wang et al. 2015; Rosenstein et al. 2003; Widenfalk et al. 2003). Moreover, pre-clinical studies of traumatic SCI and other neurological disorders have implicated VEGF in the improvement of functional outcomes. Park et al reported increased axonal outgrowth after exogenous VEGF therapy, the effect of which was mediated via VEGF receptors 1 and 2. Receptor upregulation was localized primarily to neurons and glia, and effects were attenuated with MAPK and PI3K inhibition, emphasizing the important role of these downstream signaling pathways (Park et al. 2010; Hongyu Wang et al. 2015; Rosenstein et al. 2003; Widenfalk et al. 2003).

In addition to regenerative aptitude, HGF is also known to induce angiogenic effects in the injured CNS (Jiang et al. 2000; Xin et al. 2001; Sengupta et al. 2003; M. Shimamura et al. 2004). Interestingly, HGF and VEGF appear to act synergistically on vasculature, demonstrating more vigorous endothelial proliferation and chemotactic effects than either factor alone. HGF has been shown to promote angiogenesis after CNS injury not only directly, but indirectly through induction of VEGF expression via MAKP, PI3K, PKC and Sp1, a modulator of the VEGF promoter (Jiang et al. 2000; Xin et al. 2001; Sengupta et al. 2003; M. Shimamura et al. 2004). The most biologically active form of VEGF, VEGFA-165, and HGF have been shown to activate comparable sets of MAPKs. When endothelial cells were stimulated by both factors, synergistic activation of ERK1/2 and p38 kinases were shown to be induced. VEGF and HGF also regulate distinct facets of cytoskeletal remodeling that are associated with differential activation of the GTPases Rho and Rac respectively. Given these angiogenic attributes, studies have postulated HGF and VEGF to promote enhanced neovascularization through enhanced intracellular signaling and regulation of cytoskeletal and migratory processes (Sulpice et al. 2009). In another study assessing ischemic skeletal muscle, VEGF and HGF were shown to collectively induce robust angiogenic effects (Makarevich et al. 2012).
VEGF and HGF have also been shown to exert synergistic effects on endothelial cell survival through enhanced expression of the anti-apoptotic genes Bcl-2 and A1 (Xin et al. 2001; Sulpice et al. 2009). These findings further highlight the therapeutic capacity of HGF and factors within NPC secretome to target multiple facets of the secondary injury process following damage to the CNS.

Significant VEGF upregulation has been identified in the injured spinal cord, particularly in activated astrocytes and microglia around the injury site (Nesic et al. 2010b). Similarly, in TBI, an upregulation of VEGF has been observed and localized predominately to reactive astrocytes (Salhia et al. 2000a). Despite its angiogenic effects, VEGF is also a potent edemogenic agent, resulting in endothelial permeability and immune cell infiltration, hence the synonymous name, Vascular Permeability Factor (Argaw et al. 2009; Proescholdt et al. 2002). Thus, upregulated expression of VEGF by reactive astrocytes not only modulates neoangiogenesis, but also the edema and breakdown of the BBB/BSCB associated with astrogliosis (Salhia et al. 2000b).

Whether HGF exerts direct regulatory effects on reactive astrocyte-specific VEGF expression remains elusive and a focus for investigation in future studies. In the broader context of CNS injury, enhanced neovascularization and endothelial cell survival following HGF-NPC treatment could serve as yet another mechanism for enhanced and strategic tissue repair, attesting to the therapeutic efficacy of this treatment paradigm.

5.2.4 Regulatory effect of TIMP-1 on glial scarring

Although multiple mechanisms are likely implicated in the regulation of astrocyte reactivity observed in the current study, we observed a significant increase in the endogenous matrix metalloproteinase (MMP) inhibitor, TIMP-1, by NPCs following HGF preconditioning. A study by Hsu et al revealed MMP-9 to be integral to the formation of the inhibitory glial scar and cytoskeleton-mediated astrocyte migration. The study further showed that, in the injured
spinal cord, MMP-9 null mice demonstrated less severe glial scarring and CSPG deposition (J.-Y. C. Hsu et al. 2008).

The family of TIMPs are endogenous regulators of matrix metalloproteinases (MMPs). MMPs are synthesized by a number of cell types including epithelial, stromal and inflammatory cells that, following injury, promote leukocyte migration, ECM remodeling, and collagen degradation of damaged tissue. Following CNS injury, MMPs are significantly upregulated in expression. Local inflammation is an immediate result of CNS injury that ultimately induces progressive cavitation and exacerbation of the lesion cavity. Activated macrophages and microglia significantly increase the expression on MMPs which contribute to vascular permeability and inflammatory cell infiltration at the lesion site, reaching a peak at approximately 1 month after injury. While the presence of activated macrophages and microglial cells is functionally critical in the debridement of injured tissue, secondary damage via inflammation is also a consequence. Thus, while an increase in MMP production has been implicated in promoting regeneration, an imbalance in MMP and TIMP expression can contribute to the pathogenesis of CNS disease and also prove detrimental to tissue repair after injury (Bellayr, Mu, and Li 2009). As such, MMP/TIMP regulation is imperative to maintaining homeostatic physiological function.

TIMP-1 and TIMP-2 are natural inhibitors of MMP-9 and MMP-2, respectively, and are highly upregulated in reactive astrocytes following CNS injury (Rivera et al., 1997; Pagenstecher et al., 1998; Jaworski, 2000). Formation of the glial scar after SCI involves not only proliferation, but migration of astrocytes toward the lesion (McGraw, Hiebert, and Steeves 2001). This migration is dependent on a number of mechanisms including actin cytoskeletal processes mediated by the GTPases Rho and Rac1, intermediate filaments such as GFAP and Vimentin, cell adhesion, and ECM degradation via MMP activity (Noble et al. 2002; Jung-Yu C Hsu et al. 2006; Menet et al. 2003; Okada et al. 2006). Several studies have demonstrated the capacity of MMP-9 to not only facilitate astrocyte migration in vitro, but also the development of a more extensive glial scar post-SCI. This effect was mitigated with MMP-9 inhibition via either genetic ablation or pharmacological means (Takenaga and
Alongside the upregulation of their endogenous inhibitors, MMP-9 and MMP-2 are both upregulated in the injured cord following SCI, acutely and subacutely, respectively, ultimately contributing to formation of the glial scar (Noble et al., 2002; Wells et al., 2003; Goussev et al., 2003). MMP-9 upregulation is predominately localized to reactive astrocytes and infiltrating leukocytes, suggesting a role in the early inflammatory response and blood-spinal cord barrier disruption (Noble et al., 2002). Given its expression in reactive astrocytes early after SCI, MMP-9 may induce astrocyte migration to the lesion site in establishing an immature astrogliotic structure.

MMP-9 has also been shown to induce inflammatory cell infiltration, increasing the production of cytokines such as interferon-γ and TGFβ which also induce glial scar formation (Silver and Miller 2004a). As such, MMP-9 null mice demonstrate a reduction in mature glial scar size as well as increased locomoter recovery following SCI. MMP-9 null mice have also demonstrated reduced CSPG immunoreactivity in the lesion site chronically. Moreover, in the acute phase post-SCI, MMP-9 null mice demonstrated less inflammation and vascular disruption, and improved behavioural recovery relative to wild-types (Ferguson and Muir 2000; J.-Y. C. Hsu et al. 2008; Noble et al. 2002).

In reference to these studies, the unfavorable effects of MMP-9 on tissue damage may overshadow associated benefits on astrocyte migration and inflammatory infiltration early after injury. Elucidating the complex and time-dependent relationship between MMP/TIMP expression may enable manipulation of the astroglial scar to better facilitate regeneration.

While the exact role of TIMP-1 production by HGF-preconditioned NPCs in the current study is unclear, its upregulation may be one such contributing factor to the notable attenuation in GFAP and CSPG production we observed following treatment in reactive astrocytes. While we did not assess the levels of TIMP-1 in reactive astrocyte cultures following treatment, such should be the focus of future mechanistic studies.
5.3 Treatment of reactive astrocytes with HGF-enhanced NPC-CM demonstrates reduced GFAP and CSPG deposition

5.3.1 Upregulation of HGF in reactive astrocytes after CNS injury

A number of studies have investigated the endogenous expression of HGF and its receptor c-Met in the injured CNS. In a rodent model of SCI, Shimamura et al noted a significant increase and peak of HGF expression at 1 week post-injury, contrast to c-Met, which was upregulated at 1 day post-injury, peaking at 1 week thereafter. Although both HGF and c-Met expression gradually decreased, elevated expression persisted into the chronic phase, up to 8 weeks after injury (Munehisa Shimamura et al. 2007). This reported delay in HGF overexpression is in contrast to growth factors such as BDNF, GDNF, and VEGF which have been reported to increase within 24 hours after SCI (Satake et al. 2000; Vaquero et al. 1999).

Interestingly, these studies have demonstrated the upregulation of HGF/c-Met to be localized predominately to reactive astrocytes around the injury site at the sub-acute and chronic phase following injury, a finding that is consistent with that of other SCI and CNS injury models, including ALS and focal cerebral ischemia (Jeong et al. 2012; Kato et al. 2003; W. Sun, Funakoshi, and Nakamura 2002; Nagayama et al. 2004). Shimamura et al. further noted that astrocytes distant from the lesion site did not prove immunoreactive for HGF, a finding that is also consistent with previous studies demonstrating an absence of HGF immunoreactivity in the uninjured spinal cord and brain (Munehisa Shimamura et al. 2006; Kato et al. 2003; Nagayama et al. 2004). Similarly, in a model of cerebral ischemia, HGF-expressing cells were primarily found in reactive astrocytes in the per-infarct region beginning acutely at 4 days after occlusion and peaking at approximately 2-4 weeks thereafter (Nagayama et al. 2004). C-Met expressing cells were also exclusive to reactive astrocytes in the peri-infarct region. Moreover, in a transgenic murine model of ALS, c-Met expression was localized to remaining neurons and GFAP-positive reactive astrocytes (Nagayama et al. 2004; W. Sun, Funakoshi, and Nakamura 2002). Shimamura et al demonstrated that few HGF/c-Met
expressing-reactive astrocytes in the injured cord were co-localized with BrdU, a marker used to detect cellular proliferation, in the chronic and subacute stages of injury.

As such, considering that HGF does not stimulate the proliferation of cultured astrocytes but induces astrocyte chemokinesis and neurite outgrowth, increased HGF secretion at and proximal to the injury site may promote astrocytic motogenesis or neuritogenesis rather than mitogenesis (W. Sun, Funakoshi, and Nakamura 2002; Machide, Kamitori, and Kohsaka 2000; Hamanoue et al. 1996). These findings further highlight the regenerative potential of endogenously upregulated HGF within the injured CNS, which, although beneficial, may be insufficient to generate an appreciable reparative effect.

5.3.2 Regulatory effect of HGF on glial scarring

Although the glial scar initially exerts a beneficial role in minimizing the extent of secondary damage incurred after traumatic CNS injury, this comes at the expense of limited regenerative capacity. GFAP upregulation is a key hallmark of astrocyte reactivity, and, as such, we assessed levels of this marker following HGF-NPC-CM treatment (Ribotta, Menet, and Privat 2004). Given the inhibitory role of astroglial-secreted ECM molecules such as CSPGs on neurite growth following injury, we also assessed total CSPG deposition using CS-56, which recognizes a mixture of sulfated disaccharide residues (Ito et al. 2005). CSPG expression and deposition is significantly upregulated by astrocytic scars after CNS injury and is regarded as a key inhibitor of axon regeneration. In the current study, given the array of CSPG subtypes and secreted isoforms with varying molecular weights, we assessed the total level of CSPG production by reactive astrocytes both pre- and post-treatment using a Slot blot assay.

Our results show that, following 24 hours of treatment, HGF at increasing concentrations, reduces both GFAP and CSPG, however combinatorial treatment (whether via preconditioning or concurrent administration), proved more efficacious in attenuating markers of astrocyte reactivity. NPC-CM alone, in contrast, demonstrated a modest therapeutic effect. The results suggest that HGF-enhanced NPCs could significantly modify
inhibitory components of the astroglial scar *in vivo*, and such should be the focus of future *in vivo* studies.

GFAP is a cytoskeletal intermediate filament protein expressed in mature astrocytes with a molecular weight of 50 kDa (Z. Zhang et al. 2014a). In the current study, we assessed levels of GFAP expression in reactive astrocytes following treatment; Western blots displayed both a 50 kDa band and a range of intermediate bands. Zhang et al demonstrated a similar pattern of band clustering ranging between 38-50 kDa in both human brain and primary rat astrocyte lysate. The study further confirmed the bands as GFAP (50 kDa) and GFAP mediated breakdown products attributed to calpain-mediated proteolytic processing (Z. Zhang et al. 2014a). The 38 kDa fragment and intermediate bands were attributed to calpain-induced removal of the amino and carboxyl termini of GFAP, while increases in 42-43 kDa bands indicated caspase-3 digestion. CNS injury results in the activation of calpains as well as caspases, which contribute to apoptotic and necrotic cell death (Z. Zhang et al. 2014b). Zhang et al demonstrated that GFAP is degraded readily via caspases *in vitro*, which may be a plausible explanation for the distinct clustering pattern of GFAP in our study.

A number of studies have demonstrated the effect of HGF on attenuation of scar formation both *in vitro* and *in vivo*. In a model of transient middle cerebral artery occlusion, Shang et al. demonstrated a reduction in glial scar formation and thickness of brain pia mater following HGF administration (Shang et al. 2011). Another study demonstrated significantly reduced GFAP expression following HGF adenoviral administration. This effect was found to be mediated through suppression of the sphingosine-1-phosphate pathway, a key player in cell proliferation. Interestingly, HGF enhanced pathway activity at lower concentrations but decreased activity at higher concentrations (Shang et al. 2011). Our study also demonstrated a gradual reduction in astroglial makers with increasing HGF concentration, although the underlying mechanism of which remain to be elucidated.

Several factors have been implicated in the formation of the glial scar. Due to disruption of the BBB/BSCB and infiltration of blood and serum components into the CNS parenchyma, a number of blood and inflammatory molecules have been implicated as
potential inducers of scar formation; such factors include interleukin-1, TGFβ isoforms, and fibrinogen (Giulian et al., 1998; Asher et al., 2000; Schachtrup et al., 2010). TGF-β has been shown to significantly upregulate the production of proteoglycans by reactive astrocytes, culminating in astrogliosis (Yin et al. 2009; Schachtrup et al. 2010). Inhibition of TGFβ has demonstrated reduced glial scarring in SCI and other CNS injuries (Hellal et al. 2011; Logan et al. 1999). TGFβ acts through a TGFβ receptor/Smad2 signaling pathway in astrocytes, ultimately inducing gliosis; inhibition of Smad2 translocation have demonstrated reduced scarring at the lesion site (Hellal et al., 2011).

While numerous studies have demonstrated the beneficial role of HGF in reducing astrocytic gliosis and promoting axonal growth after CNS injury, the mechanisms underlying these anti-gliotic effects are still poorly understood (Kadoyama et al. 2007; W. Sun, Funakoshi, and Nakamura 2002; Mizuno et al. 2000; Jeong et al. 2012). Interestingly, HGF has been implicated in the attenuation of TGFβ isoforms (Y. Liu and Yang 2006; T Nakamura et al. 2000). Studies have reported that TGFβ1 is upregulated immediately after CNS injury, peaking approximately one week thereafter and persisting to 10 days (Semple-Rowland et al. 1995; Streit et al. 1998). Jeong et al. demonstrated that HGF-expressing MSCs almost completely attenuated TGFβ1 production at 2 weeks post-SCI, corresponding to its maximal upregulation.

In vitro, TGFβ induces a reactive phenotype in astrocytes, facilitating the production of an inhibitory ECM comprised largely of CSPGs (Asher et al. 2000; Hamel, Mayer, and Gottschall 2005; Gris et al. 2007; H. Wang et al. 2008). In the current study, TGFβ activation of astrocytes resulted in upregulated expression of the intermediate filament protein GFAP, as well as CSPG deposition, a finding substantiated in previous studies (Yu et al. 2012). Moreover, this activation model demonstrated significant attenuated neurite outgrowth of cocultured hiPSC-neurons. Qualitative and quantitative assessment of astrocyte activation in our study confirm the utility of this model in assessing changes in intermediate filament expression and CSPG production following HGF-NPC-CM treatment.
CSPGs constitute a substantial proportion of growth-inhibiting molecules that are produced by the glial scar and significantly impede axonal regeneration and functional recovery after injury. Reactive astrocytes secrete a number of CSPGs which are closely related to formation of the glial scar, among them neurocan, aggrecan, brevican, and versican (McKeon, Jury nec, and Buck 1999). Studies have also attested to the antifibrotic role of HGF in regulating proteoglycan synthesis. In a rodent model of SCI, Jeong et al demonstrated the capacity of HGF-expressing mesenchymal stromal cells to reduce TGFβ isoform levels as well as neurocan expression and CSPG deposition at the lesion site, facilitating axonal growth and functional improvements (Jeong et al. 2012).

Growing literary evidence has shown that HGF may play a role in the antagonization of TGFβ1-mediated profibrotic actions via interception of Smad signal transduction. Smad proteins are critical for TGF-β-mediated induction of CSPG core proteins and enzymes in astrocytes (Susarla et al. 2011). While both Smad 2 and 3 induce CSPG production in response to TGF-β, the latter facilitates enzymatic activity of chondroitin-4-sulfotransferase-1, an enzyme which induces GAG chain sulfation. Genetic knockdown of either Smad2 or Smad3 has demonstrated enhanced neurite outgrowth, with the latter inducing a greater effect likely due to its role in modifying the production of particular CSPG constituents (Susarla et al. 2011). Another study also demonstrated that Smad3 null mice exhibited reduced scar formation after cortical injury (Y. Wang et al. 2007). This inhibition may ultimately induce a more permissive environment for neuronal growth.

A number of studies have attested to the anti-fibrotic capacity of exogenous HGF for the treatment of chronic fibrotic disorders in the kidneys and lung (T Nakamura et al. 2000; Dworkin et al. 2004; Dai and Liu 2004; Herrero-Fresneda et al. 2006; Y. Liu and Yang 2006; Yanagita et al. 1993). Smad co-repressors bind to activated Smad-2/3, ultimately inhibiting transcriptional activation of TGF-β target genes. In interstitial fibroblasts, HGF has been shown to block activated Smad-2/3 nuclear translocation, while upregulating the expression of Smad transcriptional co-repressor, SnoN (Shukla et al. 2009). Furthermore, in glomerular mesangial cells, HGF has been shown to prevent degradation of the Smad co-repressor,
TGIF. In a model of idiopathic pulmonary fibrosis, HGF induced expression of the inhibitor Smad7, in a MAPK-dependent manner, ultimately limiting myofibroblast phenotypes in alveolar epithelial cells (Dai and Liu 2004; Crosby and Waters 2010; Panganiban and Day 2011).

While many of the TGFβ glial scar-forming actions are mediated through the TGFβ-Smad signaling pathway, it is important to recognize that reactive astrogliosis and concurrent CSPG deposition after injury relies on multiple signals to several cell types. Nonetheless, HGF may allow for targeted inhibition of individual Smads which may modify the production and composition of the astrocytic ECM molecules and thus, biological activity. The astroglial modifying effect observed via HGF treatment in the current study may be in part attributed to alterations in the Smad 2/3 signaling pathway and would be an insightful focus for future mechanistic investigation.

5.3.3 Effect of transplanted NPCs on glial scarring

In the current study we also observed a trending reduction in GFAP and CSPG deposition, two defining hallmarks of astrocyte reactivity, following application of untreated NPC conditioned media alone. Our lab has demonstrated reduced astrogliosis and glial scarring in vivo following NPC transplantation in a rodent model of SCI (Wilcox et al. 2014b). Transplanted cells demonstrated successful engraftment in areas of astrogliaosis and glial scarring at and proximal to the lesion site. Astrocytes at these sites demonstrated reduced expression of GFAP and CSPG deposition, an effect that was concurrent with white matter preservation and neurobehavioural improvements. Similarly, in TBI, NSC transplantation significantly reduced astrogliosis in the corpus callosum. Intraventricular NSC transplantation in rodent models of TBI have proved beneficial in eliciting immunomodulatory effects that ultimately reduced astrogliosis and inflammatory cell activation in the corpus callosum (Sullivan and Armstrong 2017). While the mechanisms of these effects are not fully understood, they attest to the reparative, but perhaps limited, glial-modifying potential of NPC-secreted factors.
Collectively, our findings indicate that HGF-preconditioned NPCs can effectively modify inhibitory components of reactive astrocytes to a greater extent than either treatment option alone. To the best of our knowledge, this is the first study to investigate HGF as a combinatorial and priming agent alongside NPCs as an effective astroglial-modifying therapy. These findings have important implications for endogenous and stem-cell mediated repair and regeneration.

5.4 HGF-enhanced NPC-CM treatment demonstrates increased neurite outgrowth of hiPSC-neurons in reactive astrocyte co-culture

Following CNS injury, the inflammatory and reactive astrocytic lesion site subjects neurons to axonal dieback towards the reactive astrocytic penumbra where they persist in a dystrophic state. Given the necessity of the glial scar in limiting secondary damage, therapeutic interventions have shifted from complete ablation of the scar to alteration of physical and chemical constituents to an extent that would facilitate axonal regeneration and functional recovery. Indeed, studies have shown that ablation of immature and mature glial scars have resulted in a larger lesion core with no endogenous axonal regeneration (Anderson et al. 2016).

While the glial scar is largely comprised of reactive astrocytes, it is important to acknowledge the presence of other cells within the lesion core and penumbra. While activated macrophages and microglia can directly inhibit neurite outgrowth and induce extensive retraction of axons, NG2 cells may also serve to shield severed axons from infiltrating macrophages, allowing them to persist in a dystrophic state (Filous et al. 2014). The glial scar, thus, houses a number of cell types, whose ablation would compromise endogenous axonal regrowth after injury. CSPGs inhibit axonal outgrowth via repulsion or excessive adhesion, in a context-dependent manner (McTigue, Tripathi, and Wei 2006; Filous et al. 2014). The inhibitory impact of proteoglycans on axonal growth have been highlighted in numerous studies (McKeon, Höke, and Silver 1995). When exposed to a CSPG-
dense area for an extended period of time, growth cones have been observed to be tightly stabilized to an overly adhered and dystrophic state (Lang et al. 2015). Thus, therapeutic interventions like the one presented in the current study, that can attenuate specific regenerative constraints like CSPG deposition, hold promise in unlocking the regenerative capacity of endogenous spared axons within the injured CNS while also facilitating endogenous or exogenous stem cell-mediated repair and regeneration.

Neurons contain two types of processes, axons and dendrites (collectively termed neurites), which are structurally and functionally distinct and are critical determinants of neuronal connectivity (Yamamoto et al. 2012). Neurons initially establish several apparently identical, short processes; with culture time, one of these processes begins to grow rapidly, eventually becoming an axon while the other processes become dendrites (Yamamoto et al. 2012). The capacity of neurons to project membrane extensions from their cell bodies is indicative of cell function and health (Yamamoto et al. 2012). Here, we examine the effects of HGF-enhanced NPC-CM on neurite outgrowth in mature dissociated hiPSC-neurons in a reactive astrocyte co-culture system; mean total neurite length per cell was measured following treatment.

HiPSCs are a clinically attractive source for cell replacement therapies, and circumvent a number of ethical constraints arising from embryonic stem cell sources (Angelos and Kaufman 2015). HiPSCs are genetically reprogrammed adult somatic cells that exhibit pluripotent stem cell-like states similar to ESCs (McKinney 2017). HiPSCs are generated through the delivery of specific reprogramming genes, Oct4, Sox2, c-Myc, and Kl4 (McKinney 2017). Moreover, hiPSCs can be easily derived from individual patients which allows for the development of customized stem cell therapies for autologous transplantation. HiPSCs have the capacity to differentiate into all cell types, including NSCs, neurons and glia. Furthermore, in models of SCI, transplantation of hiPSC-derived NPCs have demonstrated survival, migration, and differentiation toward all CNS cellular lineages. Indeed, the ability to utilize hiPSCs to model CNS injury and neurological disease, is a powerful tool for not only investigating underlying pathogenic mechanisms
but also testing the efficacy of modified cell and drug-based therapeutics. HiPSCs can serve as an unlimited cell source for injury modelling and can also be used to test the efficacy of regenerative therapies. Following reprogramming of adult somatic cells, hiPSC clones can then be differentiated to various cell types by adding the necessary growth/differentiation proteins and co-factors to cell culture medium (McKinney 2017). While primary rodent cultures and neuronal cell lines have been widely employed in *in vitro* studies, they may not fully recapitulate human biology. Utilization of hiPSC-derived neurons may help overcome this challenge and serve as an amenable source for neurite outgrowth assays (Druwe et al. 2016).

In the current study, characterization of hiPSC-neurons via immunocytochemistry revealed a largely pure population of mature post-mitotic neurons that were scaled according to our research needs. The data demonstrate that, following activation of co-cultured rodent astrocytes, neurite outgrowth of hiPSC-neuron is enhanced following treatment with HGF-NPC secretome, irrespective of species-specific cellular differences.

5.4.1 Effect of HGF on axonal regeneration following injury

HGF has been shown to promote dendrite growth in mature hippocampal neurons, stimulate neurite outgrowth in dorsal root ganglion, neocortical, and spinal motor neurons, and chemotactically guide extending axons to targets. HGF and c-Met have been shown to regulate the early stages of dendritogenesis and dendritic arborization in mature neurons, promote axonal growth, and alter the expression and distribution of synaptic proteins (Lim et al., 2008). HGF and c-Met are clustered at excitatory synapses and have been shown to enhance NMDA receptor current, facilitating the formation of neurites. Tyndall et al demonstrated that HGF administration induced a dose-dependent increase in dendritic length, number, and complexity in mature hippocampal neurons via enhanced dendritic calcium influx through the NDMA receptor (Tyndall et al., 2007).
HGF is well documented as a neurite growth-promoting factor for neurons (Maina and Klein 1999; Thompson et al. 2004). In maturing sympathetic neurons, HGF has been shown to increase survival and growth via PI3 kinase and MAP kinase signaling (Thompson et al. 2004). Moreover, several studies have reported the capacity of HGF to act as an axonal chemoattractant and promote axonal growth of motoneurons and cortical neurons (Ebens et al. 1996; Caton et al. 2000; Yamagata et al. 1995). Following optic nerve injury, HGF administration has also been shown to promote long-term survival and axonal regeneration (Wong et al. 2014). Overexpression of HGF in the chronic stage following cerebral infarction demonstrated enhanced neurite extension and increased synapse number, leading to cognitive improvements (Munehisa Shimamura et al. 2006). Kitamura et al also demonstrated significant outgrowth of corticospinal serotonergic fibers in a model of SCI following HGF treatment, the effect of which was associated with locomotor improvements (Bregman et al. 1997; Chu et al. 2004; Kitamura et al. 2007).

In the current study we observed a trending increase in neurite outgrowth of hiPSC-neurons that were co-cultured in an inhibitory reactive astrocyte co-culture system, following treatment with HGF alone. Given the propensity of HGF to attenuate glial scarring and CSPG deposition alongside inherent neurite growth-promoting effects, this finding is expected. Moreover, while not statistically significant, the observed trend suggested a greater increase in neurite outgrowth with higher concentrations of HGF treatment.

5.4.2 CSPG attenuation as possible mechanism for HGF-NPC-mediated neurite outgrowth

Numerous experimental approaches have been employed to limit tissue damage and enhance axonal growth after SCI. Reactive astrocytes present significant physical and chemical barriers to growing axons. Attenuation of intermediate filament proteins such as GFAP and Vimentin have resulted in enhanced axonal regeneration after SCI (Ribotta, Menet, and Privat 2004). Furthermore, therapeutic strategies utilizing the bacterial enzyme chABC, have gained considerable success in degrading chondroitin sulfates and reducing the neurite-
inhibitory environment, ultimately promoting plasticity and functional recovery after CNS injuries (Massey et al. 2008; Bradbury et al. 2002; Caggiano et al. 2005; Tom et al. 2009).

Interventions directed at CSPG inhibition have indeed demonstrated conversion of dystrophic neurons to functionally active states that are capable of forming long-distance regenerating axons *in vitro* and *in vivo*. We observed significant reductions in astrocytic CSPG production following combinatorial treatment; as such, it may be reasonable to attribute the observed neurite outgrowth to the lack of this key regenerative constraint.

### 5.4.3 The growth-promoting effect of NPC secreted factors

While we did not observe any significant increases in neurite outgrowth following NPC-CM treatment, numerous studies have demonstrated an upregulation of neurotrophic factors such as BDNF, CNTF, GDNF, and NGF following NPC transplantation, all of which may mediate attenuation of glial scar formation and growth of host axons (Chu et al. 2004; Lu et al. 2003; Ziv et al. 2006). In a model of ischaemic stroke, transplanted NPCs not only secreted trophic factors such as VEGF, but induced expression of molecules that regulated dendritic sprouting and axonal plasticity (Andres et al. 2011). More recently, the growth-enhancing potential of NPC-conditioned media has also been observed in injury-conditioned neurons (Merianda et al. 2017). Subsequent axonal growth was correlated with enhanced transcription of growth-promoting genes as well as increased axonal localization of regeneration-associated mRNA (Merianda et al. 2017).

In the current study, while trending increases in neurite outgrowth were observed with HGF treatment alone, significant increases were demonstrated following HGF-preconditioned NPC-CM and high-dose combinatorial treatments. Whether this effect was attributed to direct or indirect actions on neuritogenesis or neuron-glia interactions, respectively, remains elusive. These findings, however, are in accordance with notable reductions in GFAP and CSPG deposition that we observed in reactive astrocytes following combinatorial HGF-NPC-CM treatment.
While axons are able to migrate through areas of reactive astrocytes, they are halted at CSPG motifs. While the underlying mechanisms remain to be elucidated, our findings suggest that a reduction in growth inhibitory CSPGs by reactive astrocytes may mediate the observed neurite outgrowth of hiPSC-neurons. While HGF may also likely contribute to the observed findings, further studies are necessary to elucidate the underlying mechanisms mediating neurite growth and corresponding effects in vivo. Nonetheless, our findings illustrate the capacity of HGF to enhance neurite outgrowth of hiPSC-neurons in a reactive astrocyte coculture system, which, to the best of our knowledge, has not been previously described.

These findings further highlight the therapeutic aptitude of HGF as a priming or exogenously applied agent alongside NPCs in attenuating growth inhibitory molecules within the injured CNS.

Taken together, our findings demonstrate the capacity of HGF-enhanced NPC-CM to effectively attenuate astrocyte reactivity and associated inhibitory molecules, while promoting concurrent neurite outgrowth. In the injured CNS, this may translate to strategic attenuation of the inhibitory nature of the glial scar, potentially unlocking the regenerative capacity of host axons. Modification of regenerative barriers poised by the post-injury astroglial environment may also prove beneficial in not only facilitating endogenous but exogenously-mediated stem cell regeneration and tissue repair.

5.5 HGF reduces oxidative stress-induced NPC death

5.5.1 Neuroprotective effect of HGF on NPCs and implications for cell transplantation

Considering the potent anti-apoptotic capacity of HGF, we initially investigated its utility in protecting NPCs from hydrogen peroxide-induced oxidative stress, an event that significantly affects the survival of host and transplanted cells in the injured CNS. We observed enhanced cellular viability in NPCs treated with increasing levels of HGF compared to that of untreated
NPCs. We utilized these findings to inform preconditioning concentrations in all other experiments.

Oxidative stress is characterized as an imbalance between the production of reactive oxygen species (ROS) and the ability to detoxify these ROS in order to repair damage and restore homeostatic function injury (Chan 2001; Valko et al. 2007). Oxidative stress is a primary source of ROS such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide, all of which are neurotoxic and consequently induce damage to neuronal cells and NSCs. Oxidative stress plays a critical role not only in the pathogenic mechanisms of neurological disease, but CNS injury (Chan 2001; Valko et al. 2007). As such, a number of studies have focused on the development of antioxidants to mitigate these adverse effects and promote repair and recovery.

Although a number of factors mediate oxidative stress-induced cell death, hydrogen peroxide is one such oxidative molecule that is universally present in the biological system, has a relatively long half-life, and acts as a paracrine mediator of apoptosis (Dröge 2002; Pletjushkina et al. 2006). Hydrogen peroxide has been shown to induce apoptosis through a number of mechanisms including Bax/Bad mitochondrial translocation, upregulation of p53, mitochondrial cytochrome c release, caspase-3 activation, and DNA fragmentation (Cook, Sugden, and Clerk 1999).

While transplanted NPCs for CNS injury have demonstrated a number of notable effects including tissue sparing, reduced cavity size, trophic support, and improved neurobehavioural recovery, poor survival in the cytotoxic milieu means a major limitation to therapeutic efficacy (Michael T Fitch and Silver 2008). Oxidative stress plays a critical role in the pathogenesis of CNS injury and NSCs are susceptible to oxidative stress-induced injury. A marked increase in ROS production following injury results in lipid peroxidation, oxidative chain reactions, and cellular damage, all of which induce neuronal and glial death injury (Chan 2001; Valko et al. 2007). Moreover, high levels of ROS such as those found after injury to the CNS, can also result in NSC death, making cellular transplantation particularly susceptible to injury (Hachem, Mothe, and Tator 2015).
While endogenous and exogenously administrated NSCs contribute to tissue repair and regeneration in the injured CNS, they are also subject to various forms of stress, including oxidative stress. Identifying methods to protect and rescue NSCs from oxidative stress may thus prove important for the treatment of CNS injury by enhancing regeneration and recovery of neurologic functions.

In the current study, we induced severe oxidative stress in adult rat NPCs via 24 hour treatment with 500 µm hydrogen peroxide. In previous studies, this protocol was found to cause widespread cell death and membrane destruction (Hachem, Mothe, and Tator 2015). Following H₂O₂ exposure, NPCs demonstrated approximately 50% cell viability relative to untreated controls. We assessed the capacity of HGF to protect NPCs from oxidative stress induced injury via concurrent application alongside H₂O₂ exposure. HGF administration significantly increased NPC survival as indicated by increased cell viability via MTT assay. Whether this effect was indeed mediated predominately by a reduction in the number of injured or apoptotic NPCs or via increased proliferative effects, remains elusive. Taken together, these preliminary findings suggest enhanced resilience of NPCs against in vitro oxidative stress following co-administration of HGF. Thus, HGF-enhanced NPCs of their secretome might prove advantageous as a therapeutic strategy for CNS injury given the propensity to withstand oxidative stressors in the cytotoxic lesion microenvironment at the time of transplantation. Thus, HGF preconditioning could induce protective effects on NPCs and other cell types from apoptosis induced by various damage sustained during CNS injury, including oxidative stress.

The anti-apoptotic effects of HGF have been well documented in a number of cell types. The protective effect of HGF on NPCs has also been demonstrated. A study by Hu et al demonstrated that pre-treatment of ESC-derived NPCs with HGF conferred a protective effect against oxidative stress-induced apoptosis. HGF pretreatment increased both PI3K/Akt and ERK1/2 phosphorylation, enabling increased NPC survival (Hu et al. 2009, 2010). The study further showed that HGF treatment promoted the expression of pro-survival protein B-cell lymphoma 2 (Bcl-2), which was attenuated by H₂O₂ treatment. HGF pretreatment also
regulated facets of the H$_2$O$_2$-induced mitochondrial apoptotic pathway, particularly cytochrome c and caspase-3 activity. These effects were reversed by inhibition of the PI3K/Akt and ERKs pathways, indicating their involvement in the observed anti-apoptotic effects (Hu et al. 2009, 2010). This neuroprotective effect may be useful in stem cell-based therapies for CNS injury and disease. Another study by the same group demonstrated the protective effect of HGF on rat cortical neurons subjected to H$_2$O$_2$-induced oxidative stress. The effects were similar, with HGF causing a downregulation of mitochondrial cytochrome C and caspase-5 activity (Hu et al. 2009, 2010).

Primary mechanical damage and secondary damage sustained after CNS injury also result in subsequent inflammatory responses. Infiltration of macrophages and microglial activation can further exacerbate tissue damage through the release of lysosomal enzymes, proteases, and reactive oxygen species (Michael T Fitch and Silver 2008). HGF has been shown to exert anti-inflammatory effects through disruption of the nuclear factor NF-κB signaling pathway and subsequent expression of NF-κB-dependent pro-inflammatory mediators (Yamane et al. 2018). Yamane et al. further demonstrated that neutrophil and macrophage infiltration were reduced via administration of collagen-binded HGF. In addition to anti-inflammatory effects, the study also demonstrated significantly enhanced axonal growth and reduced scar formation following treatment (Yamane et al. 2018). Utilizing HGF as a conditioning or exogenously applied combinatorial agent alongside NPCs may allow for a more resistant and effective population of transplanted cells for therapeutic utilization in CNS injury.

Key challenges to overcome with respect to NPC transplantation are poor survival of cells in the cytotoxic inflammatory milieu as well as the presence of regenerative barriers such as the glial scar that largely impedes regeneration. HGF shows promise in tackling both of those issues. Our findings demonstrate not only a reduction in astrocyte reactivity and enhanced neurite outgrowth observed with HGF-NPC-CM treatment, but increased cell viability of NPCs subjected to oxidative stress. Our study further illustrates the importance of tackling these issues in order to maximize the therapeutic potential of host and transplanted NPCs for CNS injury.
Findings in the present study suggest that HGF, at increasing concentrations, can rescue NPCs from oxidative stress, which is a critical pathophysiological mechanism of traumatic brain and spinal cord injury. In this way, HGF might contribute to overall therapeutic efficacy through protection of endogenous and transplanted NPCs against oxidative stress, enabling a greater number of cells for repair and regeneration, as well as trophic factor production.

5.6 Technical Considerations and Challenges

While the current study has highlighted the efficacy of HGF-enhanced NPCs in attenuating astrocyte reactivity and promoting neurite outgrowth in vitro, it is important to acknowledge some technical considerations and challenges. As is the case with most in vitro work, we must be cautious in our interpretation of findings as they relate to an in vivo setting. Studies utilizing cell conditioned media as a therapy for CNS injury have demonstrated the potent paracrine effects exerted by secreted factors on other cell types and tissues in vitro and in vivo. The exact molecules or group of pathways mediating the beneficial effects of these cells, however, are still poorly understood.

Several studies have demonstrated the use of stem cell-conditioned medium as a therapeutic strategy for CNS injury and neurologic disease, in lieu of cell transplantation (Drago et al. 2013). While therapeutic use of conditioned media enables valuable insight into the biology and therapeutic application of cells, limitations do exist regarding therapeutic use. Contamination by animal products in vitro, half-lives of secreted molecules, relative amounts of secreted factors and effective dosage necessary to elicit substantial functional responses in vivo, all need to be taken into consideration for the development and optimization of stem cell-conditioned media as a therapeutic alternative.

Culture preparation has been shown to influence cellular biology and biological effects. Our lab has previously shown that undifferentiated rat and mouse NPCs express relatively similar expression levels of growth factors (IGF, GDNF, NT-3, and NT-4/5) irrespective of cell source (spinal cord vs. brain) and time spent in culture (Hawryluk, Mothe, Chamankhah, et
al. 2012). However, culture conditions influenced trophic expression, demonstrating a modest decrease in expression with passaging over time. This may suggest that cells from an earlier passage may prove more beneficial in maximizing the overall trophic support supplied by NPCs post-transplant. In the current study, NPCs were characterized and utilized in experimental assays at P4, an early passage that our lab typically utilizes for pre-clinical transplantation. Previous work in our lab has also demonstrated significant upregulation of NPC trophins when cultured in differentiation medium. This may suggest that NPCs upregulate trophin production after transplantation into the injured cord (Hawryluk, Mothe, Chamankhah, et al. 2012). Thus, while NPCs are well-suited to supply trophic support after CNS injury, it is important to consider the impact of environmental cues on trophin expression and the unpredictability of cell behaviour after transplantation into the injured CNS milieu. It is therefore important to be cautious in extrapolating trophin levels measured in vitro to that of an in vivo setting, particularly in the heterogeneous and dynamic post-injury microenvironment. Thus, the secretory cytokine profile presented in the current study may likely reflect cytokine production at the time of transplant only; further in vivo work is necessary to confirm changes in trophin expression post-transplant.

NSPCs secrete cytokines, growth factors, and ECM molecules that can function in autocrine or paracrine roles. Thus, developing a better understanding of biomolecule production may yield new insight into the functional role of stem cells in tissue repair processes. NPC secretome characterization has typically included genomic and proteomic methods to analyze secreted factors. Transcriptomic analyses of mRNA expression have undoubtedly contributed to our understanding of neural stem cell biology and aided in the identification of functional regulators (Drago et al. 2013).

Proteomic studies, however, are advantageous in providing a representation of cell state relative to mRNA profiling alone, especially considering that gene expression does not always correlate with protein production (Drago et al. 2013). Thus, proteomic studies are particularly well-suited to identify not only markers of NSCs but to identify secreted proteins that endogenous and transplanted neural stem cells may produce. Determining the expression
of multiple cytokines or growth factors in cell supernatant is both expensive and time consuming; moreover, performing multiple Western blots may not be ideal when dealing with valuable and limited samples. The use of multiplex antibody arrays to detect a range of cytokines in a single sample, offers a sample and cost-effective approach, while allowing for high sensitivity quantification. However, it is important to acknowledge that our secretory cytokine analysis is limited to those included in the kit. Proteomic analysis of HGF-preconditioned NPC-CM in the current study further highlights the intricate reciprocal interaction that may exist between different cytokine signal transduction pathways in NPCs. Indeed, a study by Lu et al demonstrated reduced expression of neurotrophins in NPCs that were modified to overexpress neurotrophin-3 (Lu et al. 2003). This reciprocity should be taken into account when investigating combinatorial strategies involving cellular and growth factor therapies to enable optimal therapeutic efficacy. Nonetheless, the cellular secretome characterization in our current study provides important insight into key cytokines and growth factors that are modified in NPCs following HGF priming.

Preconditioning strategies aimed at increasing or modifying the production of desired factors can include exposure to cytokines, as in our study, as well as cell-to-cell interactions or hypoxic exposure (Drago et al. 2013). It is critical to attain a better understanding of how cytokines that are expressed in the inflammatory and ischemic milieu of the injured CNS modify NPC-mediated trophic effects, in order to develop more effective pre-conditioning approaches. It would be informative to assess not only the effect of HGF-preconditioned NPCs themselves on astrocyte reactivity, but changes in secretory cytokine levels following co-culture of these cell types. Pre-conditioning strategies may offer less control for cell secretory manipulation than other approaches like genetic engineering, and can evoke transient effects due to self-regulatory mechanisms. As such, genetic engineering to evoke sustained trophic action might be an attractive option. Nonetheless, in the current study, preconditioning the NPCs for 7 days with HGF induced distinct changes in the level of cytokine production which were sufficient to induce a substantial effect on astrocyte reactivity and neurite outgrowth after only 24 hours of treatment. A number of working concentrations of recombinant HGF were utilized in the current study based on previous
studies (Jeong et al. 2012; Takano et al. 2017). Further in vivo investigation is necessary to confirm appropriate dosing and timing of administration for maximal therapeutic efficacy.

While the strength of in vitro systems may lie in the ability to model CNS injury or disease, the act of culturing cells can alter their qualities and characteristics by removing crucial in vivo spatial and temporal cues. TGFβ was used to induce astrocyte reactivity in this study. Several studies have found TGFβ to be notably upregulated after SCI, serving as a potent inducer of reactive astrogliosis. Furthermore, inhibition of the TGFβ signaling pathway has resulted in suppression of glial scarring in the CNS (Logan et al. 1994, 1999; Jeong et al. 2012). Despite the significant role of TGFβ in inducing astrogliosis, it is one of several key players in this complex and dynamic process. In this respect, our in vitro model is limited in its capacity to fully represent the post-injury glial environment and incorporate the complex cross-talk between cells comprising the glial scar. In recent years there has been a surge in studies highlighting the dynamic interplay between activated microglia and astrocytes, both of which have the capacity to secrete an array of pro and anti-inflammatory cytokines as well as growth factors at different time points after CNS injury (Liddelow and Barres 2017). It would be beneficial to assess the impact of our therapy on different activation models or co-culture systems that would best mimic the inflammatory lesion environment.

5.7 Future Directions

Here, we demonstrate the efficacy of HGF-enhanced NPC conditioned media in attenuating inhibitory constituents of reactive astrocytes, effectively promoting neurite outgrowth to a greater extent than either treatment alone. Whether these effects are mediated by changes in the observed NPC secretory cytokine levels, remains elusive; further mechanistic studies are necessary to elucidate the role that these factors play, if any, in regulating inhibitory facets of reactive astrocytes. Such should be the focus of future studies. Moreover, while candidate molecules such as TIMP-1 may be of investigative interest, further studies are also necessary to define other factors that may be responsible for these secretome-mediated neuroprotective
and regenerative properties, in order to best capitalize on this therapy. Elucidating the molecular pathways mediating our observed findings is a critical step towards improving our understanding of the reparative NPC secretory profile and its clinical utility, enabling us to effectively tailor and maximize therapeutic efficacy.

The scope of future in vitro studies should focus on identification of the key factors involved in the reduction of astrocyte reactivity and promotion of neurite outgrowth that we observed following treatment. Given our secretory cytokine analysis of modified NPCs, TIMP-1 and MMP-9 are prime candidates, the levels of which should be assessed in reactive astrocytes before and following treatment. Moreover, inhibition studies of TIMP-9 in reactive astrocytes can be used to confirm the role, if any, that this factor plays in resultant levels of CSPG deposition.

Future studies should also assess whether the enhanced neurite outgrowth observed in reactive astrocyte-neuron co-cultures is mediated via direct effects of our treatment on neuritogenesis or cell-to-cell crosstalk between neurons and glia. The effect of HGF-preconditioned NPC secretome solely on neurons should also be assessed. Furthermore, following treatment of reactive astrocyte-neuron co-cultures with HGF-NPC secretome, resultant conditioned media can be collected and applied to plated neurons to then assess neurite outgrowth. Astrocyte conditioned media can also be profiled before and after treatment to identify any growth-promoting factors or molecules. This would demonstrate whether our combinatorial treatment has beneficial changes not only on reactive astrocyte-mediated CSPG deposition, but secretion of growth-promoting factors.

A recent study by Liddelow et al. highlighted the distinct properties of ‘A1’ and ‘A2’ polarized astrocytes. A1 astrocytes upregulate several complement cascade genes that inhibit a number of processes including neuronal survival, outgrowth, and synaptogenesis, deeming them largely detrimental to repair and regeneration after CNS injury. Conversely, A2 astrocytes upregulate neurotrophic factors that are conducive to neurite outgrowth and recovery after injury. The study further demonstrated that the A1 morphology is induced by a number of cytokines including IL-1α, TNFα, and C1q (Liddelow et al., 2017). It would
be informative to assess the molecular and morphological phenotype of reactive astrocytes following HGF-NPC-CM treatment, and if a reduction in any of these cytokines are indeed observed.

Our findings in the current study also demonstrated significantly increased cell viability of NPCs that were exposed to oxidative stress and HGF at high concentrations, concurrently. Further investigation is necessary to determine whether this effect if mediated primarily through a reduction in NPC death, or conversely, increased cell proliferation, and to what extent. This can be assessed via Ki67+ cell staining. Moreover, while we assessed the concurrent effect of HGF on NPC viability following exposure to H$_2$O$_2$-induced oxidative stress, it would be beneficial to assess this effect on HGF-preconditioned NPCs themselves. This would largely inform the capacity of these cells to withstand the hypoxic and cytotoxic milieu of the injured CNS and foster increased therapeutic efficacy in vivo. Nonetheless, this experiment was critical in optimizing preconditioning concentrations and informing the capacity of HGF to aid in cellular survival during oxidative stress, a point to consider for future in vivo delivery paradigms.

In the current study, we assessed neurite outgrowth of hiPSC-neurons in a rodent astrocyte co-culture system (Johnson et al. 2007; Tang et al. 2013; Odawara et al. 2014). Other studies have indeed utilized co-culture systems with human neurons and rodent astrocytes, demonstrating improved functional neuronal maturation. Nonetheless, rodent astrocytes differ significantly from human astrocytes (Y. Zhang et al. 2016; Oberheim et al. 2009). Due to species-specific differences and the inability to fully simulate all features of human injury or disease, the translational value of mixed co-culture systems are limited (Y. Zhang et al. 2016; Oberheim et al. 2009). As such, fully human-derived models hold much value for effectively studying CNS injury, and the effect of cell or drug-based therapies. Utilizing a co-culture model of human iPS-derived neurons with human primary astrocytes or hiPSC-derived astrocytes would be beneficial and clinically relevant in assessing functional interactions following treatment with HGF-enhanced NPC secretome. Nonetheless, our findings provide important insight into these cellular interactions.
following treatment, and serve as a reference point for future investigation. While we assessed mean total neurite length in the current study, future work should also assess and confirm polarity establishment as it related to axon growth; this can be achieved via Tau-1 immunostaining or live-cell imaging of constitutively active kinesin-1, an early axonal marker (Yamamoto et al. 2012). Future studies should also assess differences in dendritic branching and number between groups. Moreover, while the number of plated neurons was kept consistent, cell survival should also be assessed both with and without HGF-NPC treatment.

Reactive astrogliosis begins days after injury, with the astrocytic glial scar reaching maturity at approximately 2 weeks post-injury (Adams and Gallo 2018). As such, the ideal window for therapeutic intervention must be taken into consideration. Future in vivo studies are necessary to elucidate not only the mechanisms underlying observed effects, but the optimal timing of HGF and NPC (or modified secretome) delivery. Moreover, the optimal method of administration should be investigated in vivo, whether via HGF-preconditioned NPCs themselves or enhanced conditioned medium. Should co-administration of HGF alongside NPCs be investigated as a pre-clinical combinatorial strategy, optimal dosing and timing of administration should be assessed and regulated to maximize regeneration and functional recovery.

The current study utilized HGF preconditioning as a means of enhancing NPC-secreted factors for therapeutic use. Given that our study demonstrates notable effects with HGF-preconditioned NPC-CM as well as concurrent HGF application alongside NPC-CM, direct administration of modified secretome could serve as a plausible therapeutic strategy in vivo. However, given the tissue-specific cell replacement capacity of NPCs, combinatorial paradigms utilizing not only enhanced cell secretome, but the cells themselves, may prove advantageous on multiple levels.

Direct administration of HGF is typically hindered by a number of issues including the short serum half-life as well as poor access to the CNS by systemic route due to the BBB/BSCB. Despite its potent neurotrophic and regenerative effects after injury, HGF is highly unstable
in systemic circulation with a half-life of less than 15 minutes. As a result, clinical application is limited given the inability to sustain a consistently high level of exogenous HGF in circulation, even with repeated injections at short intervals of time. As such, gene transfer strategies have served as alternatives to overcome this challenge and allow continuous expression of HGF in vivo. Continuous intrathecal delivery via pump infusion can also be used as an alternative method for selective and less invasive administration to the injury site. Gene therapies have been investigated as a solution to the issue of degradation, however safety and efficiency of the gene carrier are challenges to be considered. Transfecting stem cells with a vector encoding the HGF gene may circumvent immunity reactions and neoplastic risks associated with viral vectors. Subsequent transplantation of these modified cells into the injury site may serve as a plausible option for combinatorial cellular and growth factor delivery.

Utilization of a tightly regulated system to enable conditional expression of a gene of interest like HGF, is also an attractive option to consider moving forward. As is the case with growth factor treatments either alone or in combination with stem cell therapies, in vivo work must take into careful consideration the optimal administration route and dosing of HGF to best inform clinical application needs. Moreover, given the reparative anti-apoptotic and pro-angiogenic effects exerted by HGF, administration must be tightly regulated to avoid tumorigenesis and other toxic or adverse effects. One such strategy for combating this issue is tetracycline-controlled transcriptional activation or transducible gene expression via Tet-technology (Gossen and Bujard 1992). In this system, a tetracycline-regulated transcription factor binds DNA to a Tet-op promotor, resulting in either expression or inhibition of a specific gene (Gossen and Bujard 1992). Controlled protein expression would enable the assessment of optimal HGF levels and timing of release. The Tet system is a particularly attractive method of delivery as it would enable rapid and reversible HGF expression post-NPC engraftment. Alternatively, given comparable findings between both preconditioned media and concurrently applied HGF and NPC-CM, NPCs and HGF can be co-administered
separately. As such, NPCs can be transplanted via intrapsinal injection while HGF can be administered via intrathecal injection or osmotic pump.

While growth factors have been applied as therapeutic strategies for traumatic brain and spinal cord injury, lack of efficacy and adverse side effects remain significant challenges. To mitigate these constraints, an approach is needed to limit the diffusion of factors into non-target issues. Indeed gene therapy would serve as one plausible solution for this purpose. Tissue engineering strategies are another approach to delivering growth factors in desired and controlled quantities.

A significant challenge with the use of growth factors, is not only the establishment of optimal concentration gradients, but maintenance of these gradients (Bonner et al. 2009). Biomaterial scaffolding strategies offer a solution through sustained mechanisms of cell-mediated growth factor release. Studies have demonstrated the utility of neurotrophin-secretng grafts in promoting axonal regeneration via ascending concentration gradients (Bonner et al. 2009; Yamane et al. 2018). In keeping with this rationale, biomaterial scaffolds containing HGF concentration gradients can be used to target the glial scar and also guide neurons through the lesion center. Tissue engineering approaches and utilization of scaffolds for cellular or growth factor delivery have shown much promise and become increasingly attractive for use in CNS injury. Scaffolding methods provide a conducive environment for cell attachment, proliferation, and differentiation an can be utilized to achieve effective cellular or growth factor delivery at designated sites (Teng et al. 2002). Hydrogels in particular, are prime candidates for engineered tissue scaffolds due to their unique compositional and structural similarities to the natural ECM (Bonner et al. 2009; Teng et al. 2002; Yamane et al. 2018).

In a recent study in rodents with spinal cord compression, enhanced recovery was observed following a single administration of HGF fused to a collagen-binding domain (Yamane et al. 2018). Moreover, collagen-bound HGF combined with a gelatin hydrogel scaffold promoted endogenous repair and recovery to a significantly greater extent than either option alone in a severe model of SCI. This method of administration enabled delivery of HGF to the injury
site by direct injection, ultimately prolonging retention and extending the functional half-life of this factor. (Yamane et al. 2018).

Scaffolds aid in promoting tissue continuity across the lesion site, and act as bridging structures for tissue remodeling, providing a conducive environment for regeneration. Ideal scaffolds are biocompatible and can be designed to incorporate molecules such as growth factors (Yamane et al. 2018). Takano et al recently demonstrated the critical role of HGF in enhancing functional recovery after NSC transplantation in aged mice with SCI (Takano et al. 2017). As such, a combinatory strategy encompassing NPCs alongside HGF in hydrogel could prove efficacious in promoting functional recovery after injury. Further studies are necessary to assess this possibility and determine the most effective delivery strategy to achieve maximal therapeutic efficacy.

The *in vitro* model used in the current study serves as a necessary first step in optimizing our HGF preconditioning strategy, assessing changes in cytokine production, and identifying potential factors involved in astroglial modification and neurite outgrowth. Moreover, our findings serve to inform future pre-clinical studies as well as dosing and administration needs. Based on our results, one such approach would entail administration of HGF-preconditioned NPC secretome in the subacute injury phase, to target the glial scar and modify its inhibitory components, while also promoting axonal outgrowth through the lesion site. Given that we demonstrated similarly advantageous effects following concurrent HGF and NPC-CM administration, an alternative approach could involve intraspinal NPC transplantation in the subacute injury phase (1-2 weeks after injury) alongside HGF via osmotic pump or scaffold release. The latter approach would allow not only for secretome and growth factor-induced astroglial modification, but also direct cell replacement, ultimately fostering both tissue repair and regeneration.
5.8 Novelty and Significance

In the current study, we demonstrate the capacity of HGF-enhanced NPC secretome (both preconditioned and concurrent administration) to attenuate inhibitory hallmarks of reactive astrocytes. We also demonstrate the capacity of HGF-enhanced NPC-CM to promote neurite outgrowth of hiPSC-derived neurons grown in reactive astrocyte co-cultures. While these effects were observed in a mixed species co-culture system, our findings are a promising and translationally relevant first step in informing fully human in vitro models. To our current knowledge, this is the first study to assess the astrogial-modifying and growth promoting effect of HGF-enhanced NPCs.

While HGF has been investigated as a therapeutic agent alone as well as in combination with stem cells in CNS injury and neurodegenerative disease, for the first time, we have highlighted its therapeutic potential alongside NPCs. This practical, non-viral treatment paradigm may serve as a feasible strategy for modifying the inhibitory glial post-injury environment to one more conducive for neuroregeneration, while optimizing current cell-based therapies. Combinatorial cellular and growth factor therapies that collectively minimize the inhibitory properties of the astrogial scar while maximizing the intrinsic growth capacity of spared neurons, hold much promise for the treatment of CNS injuries and neurodegenerative diseases.
Chapter 6
Conclusions

6 Conclusions

6.1 Conclusions

Our views on regeneration after CNS injury have evolved considerably alongside our understanding of the glial scar as a complex and dynamic structure. Reactive astrocytes are dynamic cells with growth-supportive and inhibitory functions that are mediated by context-dependent environmental signaling (Davies et al. 1999; Tom et al. 2004). Regenerative constraints posed by the glial scar, particularly the upregulated expression and deposition of growth-inhibitory CSPGs, have been identified as major obstacles to neuroregeneration and functional recovery and, as such, been deemed key targets for therapeutic intervention (Busch and Silver 2007; Silver and Miller 2004b). In recognition of this functional duality, therapeutic goals have shifted from attenuation of the glial scar to modification of physical and chemical attributes to foster optimal regeneration of spared axons. Tactics that strategically manipulate the inhibitory facets of the astroglial scar must be further investigated to facilitate regeneration of axons into and beyond the lesion site.

Given the largely cytotoxic and inhibitory glial environment post-CNS injury, it is critical to design strategies that permit the greatest therapeutic efficacy of NPCs possible. Combinatorial therapies that harness the distinct benefits of cellular and growth factor therapies may enhance overall therapeutic benefit via not only cell replacement, but modification of the post-injury environment, ultimately enhancing repair and regeneration of the injured CNS. HGF may be in a unique position as a combinatorial agent for enhancing the reparative secretory potential of NPCs, given its anti-apoptotic, anti-fibrotic, and pro-regenerative capabilities in CNS injuries.
This the first study to investigate the astroglial-modifying efficacy of HGF-preconditioned NPCs. Here, we show that application of this enhanced secretome can effectively attenuate astrocyte reactivity and promote neurite outgrowth in vitro to a greater extent than either treatment alone. These effects may be mediated in part by distinct changes in NPC secretory cytokine levels. This treatment strategy may offer a multifaceted approach for addressing the complex and dynamic post-injury glial environment to better facilitate regeneration in vivo. These data suggest that enhancing the neurotrophic and regenerative capacity of NPC secreted factors with HGF may facilitate strategic attenuation of reactive astrogliosis and serve as a suitable therapy for modification of the inhospitable post-injury environment to one more permissive for repair and regeneration.

Overall, these findings have important implications for the optimization of current cell-based strategies and functional recovery after CNS injury. Taken together, these data warrant further mechanistic and in vivo investigation into the reparative potential of HGF-enhanced NPCs as a potentially clinically translatable therapy for traumatic brain and spinal cord injury.
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Appendices

I. Contributions

1) Rachel Dragas: Study inception and experimental design, data collection and assembly, analysis, interpretation, and manuscript writing.

2) James Hong: Data collection, assembly (proteomic work and quantitative staining analysis; Figures 4-6).

3) Christopher Ahuja: Data collection, assembly (reactive astrocyte-neuron co-culture work; Figure 7).

4) Michael G. Fehlings: Study inception and experimental design, data interpretation, manuscript editing and approval.

II. List of publications related to thesis