Co-Delivery of Chondroitinase ABC and Pre-Differentiated Progeny of Human Neuroepithelial Cells in Hydrogels to the Injured Spinal Cord

by

Priya Nivashini Anandakumaran

A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science

Institute of Biomaterials and Biomedical Engineering
University of Toronto

© Copyright by Priya Nivashini Anandakumaran 2016
Co-Delivery of Chondroitinase ABC and Pre-Differentiated Progeny of Human Neuroepithelial Cells in Hydrogels to the Injured Spinal Cord

Priya Nivashini Anandakumaran
Masters of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto
2016

Abstract

The inability of injured axons to regenerate across the lesion that forms following a spinal cord injury is largely attributed to their reduced growth capacity, and the inhibitory microenvironment surrounding the lesion. To overcome these obstacles, a combinatorial strategy was designed involving the delivery of the enzyme chondroitinase ABC (chABC), from a methylcellulose hydrogel to degrade inhibitory molecules, and the transplantation of neuronal precursor cells in a hyaluronan-methylcellulose hydrogel to bridge the lesion. Although motor and sensory functionality was not recovered through this strategy in rat models of spinal cord injury, high levels of transplanted cells remained 8 weeks after transplantation, the cells retained a neuronal phenotype and migrated toward the lesion, despite being injected 1 mm away from the injury site. Future work involves determining the effect that chABC had in this system, and what modifications can be made to achieve functional recovery through this type of combinatorial strategy.
Acknowledgments

First and foremost I would like to thank my supervisor Professor Molly Shoichet for her constant motivation and support over the last two years, and for always being excited to hear about my results and progress. She encouraged me to challenge myself, and because of that I learned far more than I expected to learn, for which I will always be grateful.

I would also like to thank my committee members Prof. Milica Radisic and Prof. Cindi Morshead for their help in guiding this project, my independent examiner Prof. Penney Gilbert for her willingness to learn more about this project, Dr. Balazs Varga and Prof. Andras Nagy for providing us with the cNECs, as well as Dr. Tator and members of his laboratory for always being willing to provide in vivo support.

They say it takes a village to raise a child, and it definitely took a village to complete this project. Huge thanks to Dr. Tobias Fuehrmann for spending countless hours doing surgeries and behavioural testing, for always providing advice and support, and also for continuing this project. Many thanks to Samantha Payne, Dr. Malgosia Pakulska and Dr. Roger Tam for numerous discussions and for lending their expertise to the different parts of this project. Also, this project would not have been possible without Peter Poon who performed many of the surgeries.

To the rest of the Shoichet Lab – Thanks for being an amazing group of people, for always sharing your knowledge, and for making the last few years so enjoyable. Special thanks to Jenn Logie and Steph Fisher for bringing me into the lab as an undergrad, Alex Baker for answering all of my chemistry questions and for always having a fun story to share, Nick Mitrousis for convincing everyone that my name is not actually my name, and Jessica Ngai for being a great friend and for always providing moral support.

Thank you to the rest of my friends for their support and encouragement and for being so understanding during that period of time when I saw my rats more often than I saw them.

To JP, thank you for loving pizza as much as I do, and thank you for everything. I honestly don’t know what I did to deserve having you in my corner.

The biggest thanks goes to my parents and my sister Abi for always being supportive of my dreams, for bringing me food every week, and for always being there.

Finally, thank you to my funding sources QEII-GSST and CIHR.
Table of Contents

Acknowledgments ................................................................................................................. iii

Table of Contents .................................................................................................................... iv

List of Tables ............................................................................................................................ vii

List of Figures ........................................................................................................................... viii

List of Appendices ..................................................................................................................... ix

1 Introduction ........................................................................................................................... 1

1.1 Healthy Spinal Cord ........................................................................................................... 1

1.2 Spinal Cord Injury Pathophysiology ............................................................................... 1

1.3 Hypothesis & Objectives ............................................................................................... 2

1.4 Inhibitory Microenvironment .......................................................................................... 3

  1.4.1 Myelin Associated Inhibitors (MAIs) ........................................................................ 3

  1.4.2 Treatments to overcome MAI inhibition: .................................................................. 5

  1.4.3 Chondroitin Sulphate Proteoglycans (CSPGs) ......................................................... 5

  1.4.4 Chondroitinase ABC (chABC) ................................................................................... 7

  1.4.5 Biomaterials for sustained release of chABC ........................................................... 8

  1.4.6 Affinity Based Release of ChABC from Crosslinked Methyl Cellulose Hydrogel .... 8

1.5 Cell transplantation to SCI ............................................................................................... 9

  1.5.1 Stem Cells .................................................................................................................. 10

  1.5.2 Lineage Committed Cells .......................................................................................... 10

  1.5.3 Human iPSC-derived cortically-specified neuroepithelial cells (cNECs) ................. 12

1.6 Biomaterials for cell transplantation for SCI Repair: .................................................. 12

  1.6.1 HAMC + Potential Modifications to HAMC ............................................................. 13

1.7 Combination treatments of Cells + chABC to SCI ....................................................... 13

2 Methods ............................................................................................................................... 18

2.1 Cell Culture ....................................................................................................................... 18

2.2 HAMC Preparation ......................................................................................................... 18

2.3 Preparation of 0.5/0.5 HAMC and 0.75/0.75 HAMC for In Vitro Assays ...................... 19
2.4 Cell Survival Assay: Cells in HAMC Stored in Ice .......................................................... 19
2.5 Cell Survival Assay: Cells in HAMC injected through a 32 Gauge (GA) needle .......... 20
2.6 Mechanical Testing of Gelatin and Agarose Gels ......................................................... 20
  2.6.1 Preparing gelatin gels ................................................................................................. 20
  2.6.2 Preparing agarose gels ............................................................................................. 20
  2.6.3 Mechanical Testing (compressive testing) ............................................................... 21
2.7 Cell distribution assay: Cells in HAMC injected into gelatin gels .......................... 21
2.8 chABC Expression & Purification ................................................................................ 21
2.9 Endotoxin removal & detection .................................................................................... 22
2.10 chABC Kinetic Assay .................................................................................................... 23
2.11 Synthesis of Carboxylated Methyl Cellulose (MC-COOH) .................................. 23
2.12 Synthesis of Thiolated Methyl Cellulose (MC-SH) ............................................... 23
2.13 MC-SH Lyophilization and Sterilization for xMC Gel ............................................ 24
2.14 Weak binding peptide synthesis, modifications and purification ......................... 24
  2.14.1 Peptide Synthesis: ................................................................................................. 24
  2.14.2 3-maleimidopropionic acid modification of the peptide: ...................................... 24
  2.14.3 Resin Cleavage ....................................................................................................... 25
  2.14.4 Peptide Purification ............................................................................................... 25
2.15 MC-WBP Synthesis ....................................................................................................... 25
2.16 xMC-chABC-SH3 Preparation .................................................................................... 25
2.17 In Vitro chABC Release Profile .................................................................................. 26
2.18 Cell preparation for pilot in vivo study & combinatorial in vivo study .................... 27
2.19 In Vivo Surgeries ......................................................................................................... 27
  2.19.1 Pilot In Vivo Study ................................................................................................. 28
  2.19.2 Combinatorial In Vivo Study ................................................................................ 28
2.20 Housing and post-operative care ............................................................................... 29
2.21 Functional Assessment ................................................................................................. 30
  2.21.1 Handling & Training .............................................................................................. 30
  2.21.2 BBB Score ............................................................................................................. 30
  2.21.3 Motor Subscore ..................................................................................................... 30
  2.21.4 Ladder Walk .......................................................................................................... 30
  2.21.5 Tail Flick ................................................................................................................ 31
2.22 Immunohistochemistry/Tissue Analysis .................................................................... 31
2.23 Statistical Analysis ........................................................................................................ 33

3 Results ................................................................................................................................ 34
  3.1 Pilot in vivo study to determine whether cNECs remain in the injured spinal cord ......... 34
  3.2 Physical modifications of HAMC to enhance cell survival ............................................. 36
     3.2.1 Cell survival on ice ........................................................................................................ 36
     3.2.2 Cell survival through a fine gauge needle ........................................................................ 37
     3.2.3 Cell distribution following injection into a gelatin gel ..................................................... 37
  3.3 Synthesis of materials for the chABC-SH3 delivery hydrogel (xMC-chABC-SH3) .......... 39
  3.4 Co-delivery of cNECs and chABC into rat models of SCI ............................................... 41
     3.4.1 Functional Recovery ...................................................................................................... 42
     3.4.2 Quantification of transplanted cells 1 week post-transplantation ................................. 46
     3.4.3 Quantification & characterization of transplanted cells 8 weeks post-transplantation .... 47
     3.4.4 Lesion Area & Rostrocaudal Spread of Transplanted Cells ........................................... 50

4 Discussion ............................................................................................................................... 52
  4.1 In Vitro Work ...................................................................................................................... 52
  4.2 In Vivo Behaviour & Lack of Functional Recovery ............................................................ 53
  4.3 In Vivo Tissue Analysis ..................................................................................................... 55
  4.4 Future Work ....................................................................................................................... 57
     4.4.1 Overcome flaws with behavioural testing ......................................................................... 57
     4.4.2 Cells ................................................................................................................................ 58
     4.4.3 chABC ............................................................................................................................ 59

5 Conclusions ............................................................................................................................. 61

References ..................................................................................................................................... 62

Appendix A: Comparison of BBB scores between studies which used the clip compression injury ................................................................................................................................. 72

Appendix B: List of Abbreviations ............................................................................................. 73

Appendix C: Contributions to this Project .................................................................................. 75
List of Tables

Table 1.1: Literature review of studies that have delivered chABC and cells

Table 3.1: Groups in the pilot \textit{in vivo} study

Table 3.2: Groups in the combinatorial \textit{in vivo} study

Table A.1: BBB Scores 1 week post-clip compression injury in injury alone groups
List of Figures

Figure 1.1: Schematic of events that occurs following spinal cord injury

Figure 1.2: In vitro differentiation profile of cNECs

Figure 2.1: Method to determine association between transplanted cells and βIII Tubulin staining

Figure 3.1: Timeline of pilot in vivo study to assess undifferentiated cNEC survival in the injured spinal cord.

Figure 3.2: In vivo pilot study to determine whether the cNECs are able to survive in the injured spinal cord one-week post transplantation.

Figure 3.3: Chemical structure of (a) Hyaluronan (HA) and Methyl Cellulose (MC)

Figure 3.4: Cell survival in aCSF, 0.5/0.5 and 0.75/0.75 HAMC on ice and after being injected through a 32-gauge needle.

Figure 3.5: Compressive modulus of different hydrogels

Figure 3.6: cNEC distribution following injection into a 6% gelatin gel in aCSF, 0.5/0.5 and 0.75/0.75 HAMC

Figure 3.7: Schematic of the components of the xMC-chABC-SH3 hydrogel.

Figure 3.8: Synthesis and characterization of components of the xMC-chABC-SH3 hydrogel.

Figure 3.9: Timeline for combinatorial in vivo study.

Figure 3.10: Motor recovery assessed through the BBB scoring system.

Figure 3.11: Motor recovery assessed through the motor subscore and ladder walk.

Figure 3.12: Sensory recovery assessed through the tail flick.

Figure 3.13: Representative images of transplanted cells one week post transplantation.

Figure 3.14: Quantification of transplanted cells one week post transplantation.

Figure 3.15: Representative images of transplanted cells eight weeks post transplantation.

Figure 3.16: Quantification of transplanted cells eight weeks post transplantation.

Figure 3.17: Proliferative capacity of transplanted cells eight weeks post transplantation.

Figure 3.18: βIII Tubulin Immunostaining to determine if transplanted cells have a neuronal phenotype eight weeks post transplantation.

Figure 3.19: Lesion area nine weeks post injury (8 weeks post cell transplantation).

Figure 3.20: Rostrocaudal spread nine weeks post-injury (8 weeks post cell transplantation).

Figure A.1: Events that occur following a spinal cord injury.

Figure B.1: Method to analyze level of association between transplanted cells and βIII tubulin positive cells in tissue.
List of Appendices

Appendix A: Comparison of BBB scores between studies which used the clip compression injury

Appendix B: List of Abbreviations

Appendix C: Contributions to this Project
1 Introduction

1.1 Healthy Spinal Cord

The spinal cord extends from the base of the brain to the lumbar vertebrae and enables communication between the brain and peripheral nerves. The spinal cord is composed of grey matter, which contains mainly interneurons, as well as neuronal cell bodies and dendrites, and the surrounding white matter, which contains mainly longitudinally arranged, myelinated axons. These myelinated axons ascend and descend through the cord to transmit sensory and motor information between the periphery and the brain. The spinal cord is segmentally arranged into 4 major divisions: cervical, thoracic, lumbar, and sacral, where motor and sensory information is transmitted to and from specific regions of the body at each of the four segments. The spinal cord has many forms of protection, including three layers of surrounding connective tissue membranes: the dura mater, arachnoid mater, and pia mater, in addition to being enclosed by the vertebral column.

1.2 Spinal Cord Injury Pathophysiology

Following a traumatic spinal cord injury (SCI), a series of events occurs (shown in Figure 1.1) which can ultimately result in kidney and bladder infections, bowel problems, cardiac and respiratory dysfunction, and long term neurological deficits such as a loss of motor and sensory function below the level of trauma [1]. The initial mechanical injury, such as a contusion or compression by a blunt force, is called the “primary injury” which causes necrosis, edemas,

Figure 1.1: Schematic showing the various events that occur following a spinal cord injury [2]. Reproduced with permission.
hemorrhaging and vasospasms at the injury site [2]. The mechanical injury is followed by a series of events, which comprise the “secondary injury”, and can take minutes to weeks to months to manifest themselves. Secondary events include ischemia, breakdown of the blood brain barrier which allows infiltration of inflammatory cells, free radical formation and lipid peroxidation, glutamate excitotoxicity, apoptosis of neurons, oligodendrocytes and microglia, and inflammation, which causes swelling and blood flow reduction, thereby causing further neurological damage [1], [2]. The secondary injury is followed by the ”chronic phase” which persists for years after the injury, and consists of white matter demyelination, gray matter dissolution, and reactive gliosis, which results in the formation of the glial scar around the fluid-filled cavity. This glial scar contributes substantially to the inhibitory microenvironment surrounding the injury site (explained in greater detail below).

Long term neural dysfunction occurs because the spinal cord has limited regenerative capacity following injury, due to an imbalance of axonal growth-promoting, and growth-inhibiting molecules. Although Ramon Y Cajal previously described injured axons as being “dystrophic endballs” that are incapable of regeneration [3], it has since been shown that these dystrophic growth cones are actually highly active structures that are unable to regenerate through the inhibitory microenvironment of the lesion site. Furthermore, the lack of neurotropic stimulation and the reduced growth capacity of adult axons of the central nervous system further prevents injured axons from repairing the wound by extending through the lesion [4]. Therefore, our strategy to recover motor and sensory function following a SCI through is twofold – (1) to promote a more permissive environment within the spinal cord by degrading inhibitory molecules (specifically chondroitin sulphate proteoglycans) which surround the lesion and prevent axonal growth across the lesion, and (2) to transplant a neuronal cell population which can integrate into the neuronal circuitry to bridge the cavity between injured axons.

1.3 Hypothesis & Objectives

It is hypothesized that transplanted progeny of human induced pluripotent stem cells will integrate into the neural circuitry of the injured spinal cord to promote functional recovery in a rat model of spinal cord injury. The objectives for this project include: (1) promoting cell survival following transplantation into the injured spinal cord by delivering the cells in a protective hyaluronan-methyl cellulose hydrogel vehicle; and (2) investigating the co-delivery of
pre-differentiated progeny of human induced pluripotent stem cell derived cortically specified-neuroepithelial cells and the enzyme chondroitinase ABC into the injured spinal cord as a function of regeneration and repair.

1.4 Inhibitory Microenvironment

The inhibitory extracellular environment that forms following a spinal cord injury has been largely implicated in the inability for injured axons to regenerate through the lesion/site of injury. Following a spinal cord injury, a number of cell types (microglia, oligodendrocyte precursors, meningeal cells, and astrocytes) are recruited to the injury site during reactive gliosis, and eventually form a glial scar surrounding the lesion. The formation of the glial scar has both beneficial and deleterious effects. Some benefits of the glial scar include isolation of the injury site by sealing the wound to minimize the area of inflammation and cellular degradation and closing the blood brain barrier to prevent the infiltration of non-CNS (central nervous system) cells. Anderson et al. recently demonstrated that preventing astrocytic scar formation either by selectively killing scar-forming astrocytes, or by deleting STAT3 signaling from astrocytes in mouse models of spinal cord injury did not result in regrowth of damaged axons through the lesion site [5]. Rather, they found that animals in which astrocytes were selectively killed, showed increased retraction of axons from the injury site in comparison to wild-type animals, thereby demonstrating beneficial effects of the astrocytes [5]. The deleterious effects of the glial scar are largely due to the secretion of inhibitory molecules by cells within the glial scar that inhibit growth by acting through axonal receptors. As such, a substantial area of research in developing treatments for spinal cord injury is focused on developing and delivering anti-inhibitory molecules to promote a more permissive microenvironment for axon growth and regeneration. It is widely accepted that there are two main components within the injured microenvironment that are responsible for inhibition of axonal growth: myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs), however this project focuses on degrading the CSPGs specifically.

1.4.1 Myelin Associated Inhibitors (MAIs)

The disruption of myelinated axons and oligodendrocytes result in the release of myelin debris and myelin-associated inhibitors at the site of injury which contribute substantially to the inhibition of axonal regeneration and sprouting following SCI [1], [6]. MAIs, which have been
found to contribute to axon inhibition in the CNS, include Nogo-A [7], [8], myelin associated
glycoproteins [9], oligodendrocyte myelin glycoprotein (Omgp) [10], and some semaphorins

Nogo-A is a member of the reticulon family, and has two isoforms: Nogo B and C, although only
Nogo-A is found naturally in myelin. Nogo-A has two inhibitory domains: amino-Nogo and
Nogo-66, which is a 66 amino acid sequence that is also found in Nogo-B and C. Another MAI
is myelin associated glycoprotein (MAG), which is a member of the immunoglobulin (Ig)
family. A more recently identified MAI is Omgp, which is a glycosylphosphatidylinositol (GPI)-
linked protein expressed by both oligodendrocytes and neurons.

Although Nogo-A, MAG and Omgp are neither structurally similar, nor are their sequences or
domains similar, they induce inhibition of neurite outgrowth and growth cone collapse by acting
through the same neuronal receptor, Nogo receptor 1 (Ngr1). In terms of the two inhibitory
domains of Nogo-A, the Nogo-66 domain binds through Ngr1, while the Amino-Nogo domain
disrupts neuronal integrin function through the sphingosine-1-phosphate receptor (S1PR2) [13].
MAG binds to both Ngr1 and Ngr2, however it has a higher affinity for Ngr1 [14]. Since Ngr1 is
a glycosylphosphatidylinositol (GPI)-linked membrane receptor, it lacks a transmembrane or
cytoplasmic domain to facilitate signal transduction. As such, Ngr1 interacts with co-receptors
LINGO-1 and p75NTR of the TNF (tumor necrosis factor) family, or with the co-receptors
TAJ/TROY to transmit signals across the membrane to regulate axon growth. Furthermore, as
well as Ngr1, a second common receptor that exists for these three extensively studied MAIs is
paired immunoglobulin-like receptor B [15]. Activation of NgR1 and its co-receptors following
interactions with MAIs activates the Rho signaling pathway, in which the small GTPase Rho
sequentially activates Rho associated kinase (ROCK) and other downstream effector proteins
which leads to the destabilization of the actin cytoskeleton, and consequently retraction of
growth cones and inhibition of neurite outgrowth [3], [16], [17]. Another less studied mechanism
that could result in retraction of growth cones is through a rise in intracellular calcium following
activation of NgR1 and its co-receptors, which could activate epidermal growth factor receptor
(EGFR) [18] or protein kinase C (PKC) [3], [19], [20].
1.4.2 Treatments to overcome MAI inhibition:

Neutralization of inhibitory myelin associated inhibitors involve either blocking the inhibitor itself, blocking the receptors through which the inhibitors act, or disrupting the pathway through which the inhibitors cause growth inhibition. Strategies to neutralize the inhibitors include the Anti-Nogo-A antibodies, IN-1, 11C7 and 7B12, which are antagonists for Nogo-A and have been shown to consistently reduce myelin inhibition and subsequently enhance axon sprouting and outgrowth as well as improve locomotor function (Reviewed in [21]). A phase I clinical trial involving the delivery of the humanized Anti-Nogo-A antibody ATI355 was carried out to assess tolerance, pharmacokinetics, safety and dosage of the antibody. The antibody was administered into the lumbar cerebral fluid space either by continuous intrathecal injection for up to 28 days, or in 6 intrathecal bolus injections over 4 weeks [22]. Although the antibody did not have any serious adverse effects, the bolus injections were found to be safer than the catheter-mediated continuous intrathecal infusion from external pumps [22].

Rather than only block inhibition from Nogo-A, a potentially more comprehensive strategy is to block the NgR1 receptor through which all of the MAIs act, or to block intracellular Rho signaling pathways, which eventually causes cytoskeleton destabilization and growth inhibition. Strategies to block NgR1 include the NgR1 competitive agonist peptide NEP1-40 which blocks Nogo66 action at NgR1 [23], [24] and soluble Ngr-Fc which is a soluble form of extracellular NgR1 that is fused to human Fc [25]. Strategies to disrupt the Rho signaling pathway which is activated by MAIs, include the pyridine derivative Y-27632 which inhibits ROCK [26], clostridium botulinum-derived Rho antagonist (C3 ribosyltransferase) [27], and VX210 (formerly Cethrin) which is a recombinant fusion protein consisting of C3 ribosyltransferase and a membrane transport sequence to enable cell membrane crossing where it inactivates RhoA activity. BioAxone completed a Phase I/IIa clinical trial to assess pharmacokinetics, safety, tolerability and neurologic status of patients following single extradural application of Cethrin [28].

1.4.3 Chondroitin Sulphate Proteoglycans (CSPGs)

CSPGs are a family of molecules which have a protein core and covalently linked glycosaminoglycan (GAG) branches. The number of GAG chains, as well as the degree and position of sulfation of the GAG branches govern the specific type of CSPG, as there are many
different types of CSPGs, such as aggrecan, versican, neurocan and brevican. Although not all CSPGs are growth inhibitory (such as CSPG4 and CSPG5), the potent inhibitory nature of other CSPGs has been demonstrated in vitro, where different types of neurons preferentially grew on growth promoting substances such as laminin, and avoided areas rich in CSPGs [29], [30] and in vivo, where adult DRG neurons transplanted into the injured spinal cord extended long axons until they reached increased levels of CSPGs [31], [32]. Following a spinal cord injury, CSPGs are secreted by many cells within the glial scar, including reactive astrocytes, pericytes, microglia, margraphages, pericytes and fibroblast lineage cells [5], [33], [34].

While the inhibitory nature of CSPGs has been well demonstrated, the axonal receptors through which CSPGs elicit growth-inhibition has been unknown until recently. These axonal receptors include the transmembrane protein leukocyte common antigen-related phosphatase (LAR), the protein tyrosine phosphatase PTPσ, and the Nogo receptors NgR1 and NgR3. Fisher et al showed that systemic delivery of LAR-targeting peptides promoted locomotor function as well as significant growth of descending serotenergic fibres [35]. Similarly, ablation of the receptor PTPσ promoted growth of sensory axons [36] and of corticospinal tract axons[37], while the delivery of a peptide mimetic of the PTPσ wedge domain which binds to PTPσ restored serotonergic innervation below the level of spinal cord injury, and also restored both locomotor and urinary function [38]. Ngr1 and Ngr3 have also been implicated in mediating the inhibitory effects of CSPGs, as ablation of these receptors resulted in enhanced axonal regeneration following retro-orbital optic nerve crush injury [39]. At the intracellular levels, downstream activation of the Rho/ROCK pathway, phosphorylation of EGFR, and inhibition of Akt and Erk1/2 phosphorylation have been implicated in CSPG mediated growth cone collapse and inhibition [18], [40]. Since specific receptors through which CSPGs mediate growth inhibition of axons have only recently been better understood, no clinical trials have been carried out which involve specifically blocking the axonal receptors to overcome the inhibitory effect of the CSPGs.

CSPGs are also a major component of perineuronal nets (PNNs), which are aggregates of extracellular matrix molecules that surround neuronal cell bodies and neurites to stabilize neuronal structures in the healthy spinal cord. However, following SCI, CSPGs in PNNs act in an inhibitory manner to prevent axonal sprouting and restrict neuroplasticity.
1.4.4 Chondroitinase ABC (chABC)

While the NgR1, NgR3, LAR and PTPσ are receptors on axonal growth cones which bind to CSPGs to induce growth inhibition, there is substantial evidence which shows that the inhibitory portion of CSPGs which bind to those receptors, are the glycosaminoglycan (GAG) chains. The bacterial enzyme chondroitinase ABC is the most extensively studied method for overcoming CSPG inhibition, and it does so by degrading the GAG chains on CSPGs. ChABC treatments have made inhibitory regions growth permissive in vitro [41][42], and numerous studies have shown the beneficial effects of chABC in terms of promoting recovery as well as sprouting/plasticity of injured axons in vivo (Reviewed in [43]). ChABC degrades the inhibitory GAG chains on CSPGs by cleaving glycosidic bonds between GAG chains. There are three sub-families of chondroitinases: chondroitinase ABC, chondroitinase AC, and chondroitinase B. Chondroitinase AC depolymerizes chondroitin-4-sulfate and chondroitin-6-sulfate, chondroitinase B specifically degrades dermatan sulphate, and chondroitinase ABC, which is the most commonly used, degrades chondroitin sulphate, dermatan sulphate and hyaluronan [44], [45]. A potential issue with using chondroitinase ABC as a treatment strategy is that its lack of specificity for specific CSPGs means that it not only degrades inhibitory CSPGs such as brevican and aggrecan, but it also degrades growth-promoting CSPGs such as CSPG4 and CSPG5, all of which are upregulated post-SCI [5].

Many studies have seen beneficial effects by delivering chABC to treat spinal cord injuries in animal models; however, many details regarding chABC delivery are not fully understood. As such, no clinical trials involving chABC delivery to treat SCI have been carried out. One such detail regarding chABC delivery that is not fully understood is the optimal window for chABC delivery. Since CSPGs reach peak levels two weeks after injury, and remain upregulated for over a month after injury, longer-term delivery of chABC may be necessary in order to achieve recovery [46], [47]. Long-term delivery of chABC can be achieved through the use of an intrathecal catheter device [48], however, there are a number of issues associated with using such devices, such as the potential for infection, gliosis and scar formation at the tip of the catheter, and damage to the spinal cord itself. Achieving long term delivery of the enzyme following a single injection is made complicated by the instability of chABC at body temperature [49], [50]. Furthermore, diffusion throughout the cord at the site of delivery is difficult following intrathecal delivery, largely due to the enzyme being carried away by the CSF in the intrathecal space.
1.4.5 Biomaterials for sustained release of chABC

In order to achieve local, long-term delivery of bioactive chABC without requiring multiple injections or the insertion of osmotic pump/catheter systems, biomaterials have been employed as reservoirs from which chABC can be released locally in a controlled and prolonged manner. Hyatt et al. developed a fibrin-chABC gel from which chABC was released for up to 15 days in vitro, and was also delivered in vivo into rats with a C4 SCI (forceps injury) [51]. Sustained release of chABC from the fibrin gel was achieved by increasing the concentration of the fibrinogen within the fibrin to increase the crosslink density of the gel, resulting in both slowed diffusion of the enzyme from the gel, and also slowed degradation of the enzyme itself. Three weeks after implantation of the chABC-fibrin, animals that received the chABC-fibrin system contained approximately six times more chABC, and significantly lower levels of inhibitory GAG chains when compared to animals that received chABC intraspinally. Although the optimal time and length of chABC delivery is unknown, the general consensus is that long-term delivery of the enzyme is required in order to mitigate the inhibitory CSPGs, which are upregulated for weeks after the injury.

1.4.6 Affinity Based Release of ChABC from Crosslinked Methyl Cellulose Hydrogel

The Shoichet lab previously achieved sustained release of chABC through an affinity-based interaction from a methyl cellulose hydrogel vehicle [52], [53]. ChABC was expressed as a fusion protein with an Src homology domain-3 (chABC-SH3), and methyl cellulose was modified with an SH-3 binding peptide such that a reversible interaction between the chABC-SH3 and the SH3 binding peptide resulted in sustained, controlled release from the hydrogel [52]. The release rate of chABC-SH3 from the MC gel can be altered by varying the binding strength of the SH-3 protein/peptide pair, and by altering the SH-3 peptide to protein ratio. This affinity-based release system of chABC was incorporated into a physically and chemically crosslinked methyl cellulose (xMC) hydrogel from which sustained release of chABC could be achieved for up to 1 week [58]. The xMC hydrogel is comprised of thiol modified methyl cellulose (MC-SH), poly(ethylene glycol)-bismaleimide, methyl cellulose modified with an SH3 binding peptide and unmodified methyl cellulose. Chemical crosslinks were formed between MC-SH and poly(ethylene glycol)-bismaleimide through a Michael type addition and physical crosslinks between MC chains. By varying MC content, thiol content, and the maleimide to thiol
ratio, an injectable, *in situ* gelling, minimally swelling, and long-lasting hydrogel was developed as a delivery vehicle [58]. The biocompatibility of the xMC hydrogel vehicle was tested *in vivo* through intrathecal injections into healthy rat spinal cords, where the gel was found to remain localized to the site of injection despite cerebrospinal fluid (CSF) flowing around it. It was also found to be safe since it neither negatively affected locomotor function nor GFAP (astrocytes) nor ED-1 (macrophage and monocyte) levels in comparison to control animals that received artificial CSF (aCSF) intrathecally. Since this affinity-based hydrogel delivery system of chABC has been shown to release chABC for 7 days *in vitro*, it was employed in this co-delivery strategy to degrade inhibitory molecules prior to cell transplantation.

### 1.5 Cell transplantation to SCI

Although overcoming the inhibitory microenvironment is necessary to enhance axon regeneration, many studies have shown that removal of inhibitory molecules mainly result in limited sprouting and rarely in long distance axonal regeneration. This can be partially attributed to the reduced growth capacity of adult CNS axons, due to limited intrinsic mechanisms that initiate growth [54]–[56]. Regrowth of injured axons requires extension by growth-cone like tips, which is fundamentally similar to developing axons in embryonic stages. However as neurons mature from embryonic to adult neurons, a number of age-dependent genetic and epigenetic changes occur, such as down-regulation of intracellular cyclic AMP levels, down-regulation of mTOR (mechanistic target of rapamycin), and changes in KLF (Kruppel-like factor) expressions that prevent regeneration of adult axons either by reducing the intrinsic regenerative capacity of the injured axons, or by making injured axons more susceptible/responsive to inhibitory molecules [55], [57], [58]. While reactivation of intrinsic growth mechanisms is one mechanism to promote regeneration of these injured axons and complete the neuronal circuitry, another mechanism is cell replacement. Exogeneous neuronal cells can extend axons, which can integrate with host injured neurons to re-establish the neuronal circuitry

Cell-based therapies are promising treatments for spinal cord injury, and generally aim to promote neuroprotection by providing trophic support for endogenous cells, and neuroregeneration by replacing injured cells in order to regain functionality. A variety of cell types have shown promise in animal studies, and have been translated into clinical trials. For example, in 2013 Asterias Biotherapeutics Inc. completed the Phase I safety trial initially carried
out by Geron Corp in 2010 with the human embryonic stem cell derived oligodendrocyte precursor cells, and are currently undergoing a Phase 1/2a trial to assess safety and dose escalation [59]. Furthermore, Neuralstem Inc. completed a Phase I safety trial involving human fetal derived neural precursor cells in patients with chronic SCI [60], Stem Cells Inc. is carrying out a Phase II efficacy clinical trial involving neural stem cells in patients with chronic SCI [60], and the Miami Project carried out a Phase I clinical trial involving Schwann cells to remyelinate axons [61].

1.5.1 Stem Cells

A variety of stem cells such as ESCs, induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), neural stem/progenitor stem cells (NSPCs) have been developed as SCI treatments, and have been shown to either provide neurotrophic support to endogenous cells or to replace injured cells by differentiating into neural cell types such as neurons, oligodendrocytes or astrocytes. Although pluripotent cell types such as ESCs have been shown to differentiate into neural precursors, as well as specific neuronal and glial lineages (Reviewed in [2]), a major concern with delivering pluripotent cells is tumor formation due to incomplete or uncontrolled differentiation into non-neural cells. Multipotent NSPCs on the other hand are committed to the neural lineage and can differentiate into neurons, oligodendrocytes and astrocytes in vitro. However following transplantation in vivo, depending on the source as well as the region from which the cells are isolated, NSPCs have been shown to differentiate into astrocytes [62], oligodendrocytes [63], [64], or a combination of astrocytes, oligodendrocytes and neurons [65]. In order to commit mouse forebrain-derived NSPCs specifically towards the neuronal lineage following transplantation, Abematsu et al transplanted embryonic forebrain derived NSCs into the injured spinal cord in combination with valproic acid to induce in situ neuronal differentiation, which resulted in increased motor control [66].

1.5.2 Lineage Committed Cells

In order to overcome tumorigenicity that is associated with pluripotent cells, and the propensity for multipotent NSPCs to differentiate into glial cells rather than neurons, an increasing number of publications have reported that a more committed population, such as neuronal precursor cells, exhibit greater survival and integration into the CNS in comparison to less committed cells. For example, Kim et al. demonstrated that NSPCs that were pre-differentiated with dibutryl
cyclic AMP in vitro into βIII tubulin positive immature neurons experienced significantly greater levels of cell survival, in comparison to undifferentiated NSPCs both 2 and 6 weeks after transplantation into rat models of SCI [67]. Furthermore, Abeysinghe et al. demonstrated that transplantation of pre-differentiated GABAergic neurons into stroke injured rat brains resulted in accelerated motor recovery in comparison to control groups [68]. Immunohistological analysis showed that 28 days post transplantation, the pre-differentiated cells maintained a GABAergic neuronal phenotype, while the undifferentiated hNSCs primarily differentiated into astrocytes and were incorporated into the glial scar [68]. Lepore et al. compared the transplantation of multipotent neuroepithelial (NEP) stem cells and lineage-restricted precursors for neurons (NRPs) and glia (GRPs) into the adult rat hippocampus, stratum and spinal cord [69]. Three days after transplantation, no NEPs were found in either of those three CNS regions, whereas both three and five weeks after transplantation, the lineage-restricted precursors exhibited robust survival in all three CNS regions, and expressed mature neuronal, astrocytic, and oligodendrocytic markers, as well as the synaptic marker synaptophysin to demonstrate integration into the host tissue [69]. Therefore, lineage committed cells have been shown to both promote motor recovery, and maintain a specific phenotype following transplantation into the CNS.

![Figure 1.2: In vitro differentiation profile of cNECs from immature neuroepithelial cells (Sox2 positive) and neural stem cells (Nestin positive) to neuronal-restricted cells (DCX positive) to immature neurons (Beta III tubulin positive) and mature neurons (MAP2 positive). cNECs that were pre-differentiated for 16 days in vitro and sorted for cell surface receptor NCAM-PSA were transplanted in vivo [71].](image-url)
1.5.3 Human iPSC-derived cortically-specified neuroepithelial cells (cNECs)

The Nagy Lab previously generated human iPSC-derived, cortically-specified neuroepithelial cells (cNECs) [70]. When the cNECs are cultured in the presence of inhibitory factors they maintain an immature state and express stem cell markers Sox2 and Nestin. However when the inhibitory factors are removed, the cNECs differentiate into a neuronally-enriched population of cortically-specified, inhibitory GABAergic and excitatory glutamatergic interneurons over 32 days in vitro. As seen in Figure 1.2, over this 32 day period, as the cells differentiate from immature neuroepithelial cells and neural stem cells to neuronally-restricted cells to immature/mature neurons, there is a decrease in expression of immature markers such as Sox2 and Nestin, and an increase in the expression of neuronal markers such as βIII tubulin and MAP2 [71].

Neuron-committed cell types have shown increasing levels of survival and integration following transplantation into the CNS in comparison to immature cell types. Therefore, cNECs which had been pre-differentiated for 16 days in vitro, and are a population of neuronal precursor cells and immature neurons, were transplanted. As seen in Figure 1.2 the day 16-pre-differentiated cNECs are a heterogeneous population in which the majority of the cells are neuronal precursor cells that express double cortin X (DCX), approximately 40% of which are βIII tubulin positive immature neurons, 20% are MAP2 positive mature neurons, and 20% express the multipotent marker Nestin [71]. Although there were no Sox2 positive cells found in the day 16-pre-differentiated cNECs through immunohistochemistry, in order to further ensure that there were no potentially pluripotent or Sox2 positive cells within the transplanted cell population, the cells were magnetically sorted for the cells that express the cell surface receptor NCAM-PSA (polysialylated form of the neural cell adhesion molecule), which is a known marker of neuronally restricted cells [72]–[75]. Although the cells were not stained following NCAM-PSA sorting, it is likely that the sorted cells have a similar differentiation profile as the pre-sorted cells, because nestin, DCX and βIII tubulin positive cells can express NCAM-PSA [76].

1.6 Biomaterials for cell transplantation for SCI Repair:

Cell transplantation to replace damaged cells is a promising strategy to promote regeneration following a SCI, but there are three main issues associated with cell transplantation which must be overcome in order to further improve recovery: 1) cell survival; 2) cell aggregation due to
uneven cell distribution; and 3) cell integration into host tissue [77]. In order to overcome the aforementioned issues, hydrogels have been developed as cell delivery vehicles. Hydrogels can enhance cell survival by providing a scaffold for cell retention at the transplantation site and by preventing cell death due to anoikis. Hydrogels can also be modified with guidance channels to guide cell or axonal growth, and can also be modified with peptides or growth factors to further enhance cell survival, integration or differentiation [77]. A number of biomaterials (fibrin, chitosan, gelatin, collagen, hyaluronan, agarose) have been employed as cell (i.e MSCs, ESCs, NSPCs, Schwann cells) delivery vehicles to the injured spinal cord, as reviewed thoroughly in [78]. Depending on the injury type and the scaffold used, the encapsulated scaffold can either be injected into the cord, or implanted into the injury site. For example, Itosakl et al. implanted fibrin-based hydrogels with MSCs into acute hemisection injury models, and found improved cell survival as well as functional recovery in animals that received MSCs in the fibrin hydrogel, in comparison to cells in saline [79].

1.6.1 HAMC + Potential Modifications to HAMC

In order to enhance the survival of transplanted cells, the Shoichet lab previously developed an injectable hyaluronan-methyl cellulose (HAMC) hydrogel delivery vehicle. Hyaluronan is shear thinning and can be delivered in a minimally invasive manner through a fine gauged needle, while methyl cellulose has inverse thermal gelling properties, enabling it to form a gel at 37°C to provide localized cell delivery. HAMC hydrogels as cell delivery vehicles have been previously shown to enhance cell survival and integration into the retina [80], [81] and stroke-injured mouse brain [84]. These hydrogels can be physically modified by altering the concentrations of the hyaluronan and methyl cellulose to generate gels with varying mechanical properties and gelation times [82]. Alternatively, the gels can be chemically modified with peptides to enhance survival and integration. It has been previously shown that modification of HAMC with PDGF-A (platelet derived growth factor) promoted oligodendrocytic differentiation of rat NSPCs from the subventricular zone in vivo [83]. Therefore, the HAMC hydrogel was studied to promote survival of the cNECs following transplantation in vivo.

1.7 Combination treatments of Cells + chABC to SCI

While cell transplantation has shown promising therapeutic potential following SCI, combination strategies involving the delivery of both cells and chondroitinase ABC is especially effective,
because the presence of CSPGs post-injury can hinder the migration and integration of exogenously transplanted cells; however, preconditioning of the lesion site by chABC delivery prior to cell delivery may mitigate these issues [46], [84]. Ikegami et al. delivered NSPCs to a T10 contusion rat model of SCI and found that the transplanted cells adhered to the cavity wall and failed to migrate across into the lesion site [46]. In order to overcome this, chABC was continuously infused into the intrathecal space for one week post injury with the use of an osmotic pump and catheter which was inserted into the intrathecal space, after which NSPCs were transplanted intraspinally on day 7 post-injury. Pre-treatment with chABC promoted migration of the transplanted cells, and resulted in increased outgrowth of GAP-43 (growth associated protein-43) positive fibers at the lesion epicenter in comparison to animals that solely received cells. Other groups who delivered cells in combination with chABC are summarized in Table 1.1.

Similar to Karimi-Abdolrezaee et al. [84], Ikegami et al. [46], and Zhang et al. [85], all of which delivered chABC prior to cell transplantation, in this combinatorial project, the xMC-chABC-SH3 was delivered immediately after the injury on day 0 in order to pre-condition the lesion before introducing the cells into the system. Furthermore, xMC-chABC-SH3 was delivered on day 7 to extend the length of chABC release from the gel to 14 days post-injury, which is when CSPGs reach peak levels. The cells were delivered in the 0.75/0.75 HAMC on day 7 post-injury because it has been previously shown that sub-acute transplantation results in higher levels of cell survival in comparison to acute transplantations due to the immune response immediately after the injury [86].
Table 1.1: Summary of Combination Strategies of Chondroitinase ABC and cells to the injured spinal cord

<table>
<thead>
<tr>
<th>Cell Type (# of cells, location of transplantation)</th>
<th>chABC delivery method</th>
<th>Order of Delivery</th>
<th>Animal &amp; Injury Model</th>
<th>Functional Recovery &amp; Histology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Neurospheres were grown from NSPCs collected from rat embryonic striatum -1 million cells (in neurospheres) in 5μL culture medium was injected into injury epicentre</td>
<td>-chABC loaded into osmotic pump and a catheter attached to the osmotic pump was advanced into the intrathecal space at T11 (caudal to the injury) -chABC was continually infused for 1 week</td>
<td>-1 wk after SCI: chABC continuous intrathecal infusion for 7 days -2 wks after SCI, cells injected into injury epicentre</td>
<td>Rat T10 Contusion injury</td>
<td>-No behavioral tests were reported - ChABC pretreatment promoted migration of transplanted cells -Combination treatment induced outgrowth of greater number of growth associated protein-43 (GAP-43) positive fibers at the lesion epicenter</td>
<td>[46]</td>
</tr>
<tr>
<td>-Adult mouse neural precursor cells (NPCs) were isolated from subventricular zone of mouse forebrain -Intraspinally injected 50,000 cells/μL at 4 sites around the lesion, and at each site 2μL was injected (total of 400,000 cells injected)</td>
<td>-chABC loaded into osmotic pump and a tube attached to the osmotic pump was advanced into the intrathecal space near the injury site -chABC was continually infused for 1 week</td>
<td>-6 wks after SCI: chABC continuous infusion for 7 days -7wks after SCI: NPCs + continuous intrathecal infusion of growth factor cocktail (EGF, bFGF, PDGF-AA)</td>
<td>Rat T7 Compression injury</td>
<td>-Motor recovery in combination group from weeks 13-15 post injury (assessed by BBB, Grid walk) -No exacerbation of neuropathic pain (assessed by Von Frey testing for mechanical allodynia, and tail flick for thermal hyperalgesia) -Increased surviving NPCs in combination group (28.27%) vs. cells in vehicle group (4.91%). -9 weeks post transplantation, increased NPC survival and migration in NPC+chABC+GF group compared to NPC+GF group</td>
<td>[84]</td>
</tr>
<tr>
<td>-Rat bone marrow mesenchymal stem cells (MSCs)</td>
<td>-chABC was injected 1mm from the edge of the injury</td>
<td>-Cells differentiated primarily into oligodendrocytes</td>
<td>[85]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telecephalon tissue from human fetal brain was isolated to generated human NSCs</td>
<td>-chABC loaded into osmotic pump and a tube attached to the osmotic pump was inserted into the intrathecal space one level rostral to the lesion, and advanced until tube opening was over the scaffold</td>
<td>-Motor recovery in combination group 2 weeks post injury (assessed by BBB)</td>
<td>[87]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Human NSCs were transduced to express NT-3</td>
<td>-PCL scaffold seeded with human NSCs was implanted into the lesion site, and then chABC was continually infused for 28 days</td>
<td>-Reduced necrotic area and cavity formation, and increased number of GAP43+ fibres crossing the injury site in combination group 2 weeks post injury</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caprolactone scaffold at least 3 days before implantation</td>
<td>28 days</td>
<td>combination treatment had almost completely abolished CS-56 staining around PCL scaffold</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scaffold with cells were implanted into hemisection injury site</td>
<td></td>
<td>-Increased axonal growth into the scaffold and rostrocaudal migration of transplanted cells in the combination group seven weeks after injury</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| ESC-derived progenitor motor neurons (pMNs) fibrin scaffolds with embryoid bodies containing pMNS was implanted into hemisection injury site | -Lipid microtubes encapsulated in chABC was encapsulated in fibrin scaffolds | -Immediately after injury: Fibrin scaffold with cells, lipid microtube encapsulated chABC (+PLGA microsphere encapsulated NEP1-40 + growth factors) were implanted into the lesion site |
| | | -A second fibrin gel was polymerized in situ to secure the scaffold inside the lesion |
| | | T8 hemisection |
| | | 2 weeks after implantation/injury: Decrease in GFP+ area when cells were delivered in combination with anti-inhibitory molecules (AIMs) or with AIMs and growth factors |
| | | -Groups that had decrease in GFP+ area also had increase in ED1+ staining |
| | | -Same level of NeuN+ and GFP+ per GFP+ area for all groups analyzed |
| | | -Increased CSPG staining in groups that received cells + AIMs, indicating a decrease in chABC activity when cells and chABC were delivered together |

[88]
2 Methods

2.1 Cell Culture

Human induced pluripotent stem cell derived cortically-specified neuroepithelial cells (cNECs) were characterized and generated by Professor Andras Nagy’s Laboratory (Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital) [70]. One day before cell passaging, 6-well tissue culture plates were coated with laminin at a concentration of 5µg/µL for around 14 hours. The next day, cells were dissociated from the tissue-culture well using 1mL of cold Accutase and then placed in the 37 °C incubator for 3-5 minutes. Once all cells were dissociated from the plate, 1mL of basal media (DMEM) was added to the well to dilute down the Accutase. The entire volume was transferred to a 15mL Falcon tube, then another 1mL of basal media was added to the well to ensure all cells were removed from the well, and transferred to the Falcon tube again. The cell suspension was topped up to 10mL of basal media and centrifuged at 1400 rpm for 4 mins. The supernatant was aspirated, 1mL of media was added to the cell pellet, and the cell pellet was broken up by gentle pipetting. The cells were then seeded into the laminin-coated wells at a density of 1 million cells in 2mL of media. The cells were cultured in a media that contains specific inhibitors to maintain the cells in their undifferentiated state. Media was completely replaced every 48 hours.

To differentiate the cNECs, the cells were dissociated and centrifuged as above, however the cells were seeded onto 12-well plates that had been coated with laminin for 14 hours (5 µg/mL), and with poly-d-lysine (20uL/mL) for 4-8 hours. The cells were plated at a density of 150,000 cells/well. These cells were cultured in serum-free media containing 25 mL 10x DMEM/F12 with glutamine and HEPES, 25 mL Neurobasal (Gibco), 1 mL penicillin/streptomycin, 1 mL B27, and 50 µL BSA fraction V (Thermo Fischer) for 16 days, with half media changes every 48 hours [71].

2.2 HAMC Preparation

A blend of hyaluronan (HA, 1400-1800 kDa, Novamatrix, Drammen, Norway) and methylcellulose (MC, 300kDa, Shin-Etsu, Tokyo, Japan) was used to prepare HAMC. Sterile-filtered HA and sterile-filtered MC were dissolved in artificial cerebrospinal fluid (aCSF) at a concentration of 1% HA (w/v) and 1% MC (w/v) (1/1 HAMC). This 1/1 HAMC was mixed in a
SpeedMixer (DAC 150 FVZ, Siemens) for 30 seconds at 3,500 RPM, centrifuged at maximum speed for 1.5 minutes, and allowed to fully dissolve on the shaker at 4 °C for 14 h. The next day, the gel was mixed again using a SpeedMixer for 30 seconds at 3,500 RPM, centrifuged at maximum speed for 5 minutes, and stored on ice for 20 minutes. The last 2 steps (centrifugation for 5 minutes at maximum speed and storage on ice for 20 minutes) were repeated until no bubbles remained. The 1/1 HAMC was then stored on ice until needed.

2.3 Preparation of 0.5/0.5 HAMC and 0.75/0.75 HAMC for In Vitro Assays

The 1/1 HAMC was diluted 50% with the addition of cells in aCSF to have a final concentration of 0.5/0.5 HAMC, while the 1/1 HAMC was diluted 25% with the addition of cells in aCSF to have a final concentration of 0.75/0.75 HAMC. For example, the following steps were taken to make 100µL of a 0.75/0.75 HAMC hydrogel at a cell density of 20,000 cells/µL: 75 µL of 1/1 HAMC was prepared, 2 million cells were suspended in 25µL of aCSF, the cells suspension was slowly added to the 1/1 HAMC and the resultant 0.75/0.75 HAMC was mixed by slowly pipetting up and down using a wide orifice pipette tip. The cells in HAMC were then stored on ice.

2.4 Cell Survival Assay: Cells in HAMC Stored in Ice

Immature (day 0) cNECs were suspended in 120µL of aCSF, 0.5/0.5 HAMC or 0.75/0.75 HAMC at a cell density of 102K cells/120µL and stored in an ice box which was kept at 4°C. After 0, 6 and 24 hours on ice, a PrestoBlue assay was carried out to assess cell viability. At each time point, 20µL of cells were removed from each sample and pipetted into a 96-well plate. Each well also contained 70µL of aCSF and 10µL of the PrestoBlue reagent (ThermoFisher). Blank/control wells were also included which contained 70µL of aCSF, 10µL of the PrestoBlue reagent, and 20µL of either aCSF, 0.5/0.5 HAMC or 0.75/0.75 HAMC. The plate was stored in a 37°C incubator for three hours, and then fluorescence at 590nm was read on a Tecan plate reader.
2.5 Cell Survival Assay: Cells in HAMC injected through a 32 Gauge (GA) needle

Immature (day 0) cNECs were suspended in 60µL of aCSF, 0.5/0.5 HAMC or 0.75/0.75 HAMC at a cell density of 20,000 cells/µL and stored on ice. 10µL of each cell suspension was pipetted into Eppendorf tubes with 90µL of aCSF, and were kept aside as cells that were not injected through a needle/syringe. 10µL of each original cell suspension was then loaded into a 10µL Hamilton Syringe with a 32 GA needle. However, the 0.75/0.75 HAMC was too viscous to be pulled into the syringe using the 32 GA needle, so a 26 GA needle was used to load the syringe, and then it was replaced with a 32GA needle. The cells were ejected through the 32 GA needle at a rate of 1uL/minute for a total of 2 minutes into 18µL of aCSF. Note: The needles and injection rate that are used in in vivo studies were chosen for this in vitro study. The 20µL suspension of cells post-injection, and the 20µL suspension of cells that were not injected, were both mixed with 20µL of a live/dead staining solution (Hoechst (1:250), Calcein AM (1:500), and Ethidium Homodimer (1:250)). The stained cells were incubated in a 37°C incubator for 20 minutes, and then 10µL of each sample was pipetted into a haemocytometer and imaged on an Olympus fluorescent microscope. The number of live and dead cells in two squares in the hemocytometer were counted in order to assess the viability of cells that had and had not been injected through the syringe/needle.

2.6 Mechanical Testing of Gelatin and Agarose Gels

2.6.1 Preparing gelatin gels

6%, 12% and 15% gelatin gels were prepared the day before mechanical testing. Gelatin was mixed into PBS that had been heated to 55°C, and was stirred at 60 rev/min for 20 mins. 5mL of the gelatin solution was pipetted into a small Petri dish, which was refrigerated at 4°C overnight to set.

2.6.2 Preparing agarose gels

0.6% and 1.2% agarose gels were prepared the day of mechanical testing. Agarose was mixed in PBS and microwaved in 20 second intervals until the agarose was completely dissolved. The agarose was let to cool down for a few minutes, 5mL of the agarose solution was then pipetted into a small Petri dish, and the agarose gel was kept at room temperature to set.
2.6.3 Mechanical Testing (compressive testing)

The Young’s moduli of the agarose and gelatin hydrogels were measured using cylindrical samples with a diameter of 5 mm as explained in [89]. The hydrogels were placed between two impermeable platens connected to a DAQ-Nano17 force transducer (ATI Industrial Automation) attached to a Mach-1 micromechanical system (Biomomentum). An initial force of 0.01 N was applied, and the distance between the two platens was considered the initial sample height. The samples underwent uniaxial unconfined compression at a rate of 10 µm s⁻¹ until an applied strain of 20% was reached. The Young’s modulus was determined from the resultant stress–strain curve.

2.7 Cell distribution assay: Cells in HAMC injected into gelatin gels

6% gelatin gels were prepared as described above, however 0.2mL gelatin was pipetted into 8-well chamber slides and stored overnight at 4°C to set. The next day, cells were suspended aCSF, 0.5/0.5 HAMC or 0.75/0.75 HAMC at a cell density of 20,000 cells/µL. Cells were loaded into a 10µL Hamilton syringe and injected in the gelatin gels through a 32 GA needle at a rate of 1µL/minute for a total of two minutes. A total of 4 injections were made into the gelatin gels for all three sample (cells in aCSF, cells in 0.5/0.5 HAMC, and cells in 0.75/0.75 HAMC). The injected gelatin gels were then stained with Hoechst (1:250), Calcein AM (1:500), and Ethidium Homodimer (1:250), and incubated at 37°C for 30 minutes. The gelatin gels were imaged on an Olympus confocal microscope to qualitatively assess distribution of cells in either aCSF, 0.5/0.5 HAMC or 0.75/0.75 HAMC following injection into the 6% gelatin gel.

2.8 chABC Expression & Purification

Chemically competent BL21(DE3) Escherichia coli cells were previously transformed with plasmids containing the His-SH3-chABC-FLAG DNA vector. Clones were grown as explained in [52]. Essentially, clones were grown in a starter culture of 20 mL of Luria-Bertani (LB) broth and 50 µg/mL kanamycin overnight on a shaker at 37 °C. The next day, starter cultures were inoculated into 1.8 L of previously autoclaved Terrific Broth (TB) supplemented with 0.8% glycerol, 50 µg/mL kanamycin, and 10 drops of Anti-foam 204. Cells were grown in a 37°C water bath with air sparging until an OD600 of 0.6–0.8 was reached, at which point cells were induced with a final concentration of 0.8 mM IPTG and grown overnight in a 22 °C water bath.
The next day, a cell pellet was obtained from the cultures through centrifugation for 15 min at 6000 rpm and 4 °C (Beckman Coulter centrifuge Avanti J-26 with rotor JLA-8.1000). The pellet was collected and resuspended in 60 mL of binding buffer (50 mM Tris pH 7.5, 500 mM NaCl, 5 mM imidazole) by vortexing. The cell suspension was sonicated on ice for 5 minutes at 30% amplitude with a pulse of 10 s (Misonix S-4000 Sonicator Ultrasonic Processor equipped with a Dandual Horn probe), and then the sonicated cell solution was centrifuged at 45,000 g for 15 min at 4°C (Beckman Coulter centrifuge Avanti J-26 with rotor JA-25.50). The supernatant was carefully separated from the cell debris sludge, and the Ni-NTA resin (Qiagen) was added to the supernatant (at a concentration of 1mL Ni-NTA resin/1L culture) for 15 minutes at 4°C. After the supernatant was incubated with the resin, the total solution was collected in a column, where it was washed 10 x 10mL with wash buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 30 mM imidazole) to wash off any protein that was non-specifically and loosely bound to the resin. Elution buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 250 mM imidazole) was then added 1mL at a time to remove the protein of interest from the resin. Each 1mL fraction was collected separately and tested immediately for protein detection through the Bradford assay (90µL of Bradford reagent was mixed with 10µL of the protein solution). The fractions in which the Bradford reagent turned blue upon addition of the protein solution were collected, combined, and concentrated down to around 1 mL using a Vivaspin 10,000 kDa cutoff centrifugal filter (Sartorius). The protein was further purified by size-exclusion chromatography (SEC) in 10 mM phosphate buffer (pH 8.0, 50 mM sodium acetate) using fast protein liquid chromatography (FPLC, Hi-load 16/60 Superdex 200 prep grade column, AKTA Explorer 10, Amersham Pharmacia). Protein concentrations were determined by absorbance at 280 nm using an ND-1000 Nanodrop spectrophotometer.

2.9 Endotoxin removal & detection

Endotoxins were removed by using the ToxinEraser Endotoxin Removal Kit (GenScript L00338), and the level of endotoxins were subsequently quantified by using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript L00350).
2.10 chABC Kinetic Assay

The kinetic ChABC activity assay tracks the absorbance at 232 nm of the double bond formed after ChABC digestion of chondroitin sulfate A (CS-A) [52]. 10 µL of ChABC–SH3 was placed into a Costar 96-well UV transparent plate (Corning Inc., Corning, USA) and 90 µL of 10 mg/mL chondroitin sulfate A (CS-A) was then placed into each well using a multi-channel pipette. Control wells with 100 µL of CS-A were also included. The plate was immediately placed inside a TECAN Infinite M200 Pro spectrophotometer and measured every 20 s at 232 nm for 20 min at room temperature with 5 s of orbital shaking between each measurement. The values from the control wells were considered blank measurements and were subtracted from each value. chABC activity was determined measuring the rate at which chABC degraded CS-A, which was obtained by plotting average absorbance values versus time, and calculating the slope of the linear portion of the curve.

2.11 Synthesis of Carboxylated Methyl Cellulose (MC-COOH)

1.5 g of methyl cellulose (MC, 300kDa, Shin-Etsu, Tokyo, Japan) was slowly added to 150mL of cool (4°C) water and stirred at 4°C overnight until the MC was completely dissolved. 150mL of 3N NaOH was added to the solution for 1 hour at 4°C, to allow the NaOH and MC to equilibrate and to enable the NaOH to deprotonate the hydroxyl groups on MC. 14 mg of bromoacetic acid (around 100mmol) was mixed into the MC solution, and the solution was stirred overnight at 4°C. The next day 1g of Na$_2$HPO$_4$ was added to buffer the solution, and then the solution was brought to neutral pH with the addition of concentrated hydrochloric acid. The polymer was then transferred to 12-14kDa MWCO dialysis tubing and dialyzed against 0.1N NaCl (changed twice) and DI water (changed 5 times).

2.12 Synthesis of Thiolated Methyl Cellulose (MC-SH)

For 9mL of carboxylated methyl cellulose (MC-COOH) (at a concentration of 3mg MC-COOH/mL), 1mL of 0.1 M Phosphate buffer, pH 7.4 was added to buffer the solution. 81 mg of 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) was added to the solution, and was stirred for 30 minutes. 81 mg of 3,3’-dithiobis(propionic dihydrazide (DTP) was then added and stirred overnight at room temperature. The next day, the polymer was transferred to 12-14kDa MWCO dialysis tubing, and dialyzed against DI water (changed twice)
at room temperature to remove DMTMM byproducts and unreacted DTP. The dialyzed MC-DTP was buffered with the addition of 0.1 M phosphate buffer at pH 7.4, and then 50 molar equivalence of the DTT (dithiothreitol) reducing agent was added (where it was assumed that 1eq = 150nmol SH/mg MC), to reduce the MC-DTP to MC-SH. The solution was mixed overnight at room temperature. The next day, the polymer was transferred to 12-14kDa MWCO dialysis tubing, and dialyzed against water with 0.1N NaCl, pH 4 at 4°C (once), and then against water, pH 2 (four times with at least 2 overnights) at 4°C. Following dialysis, the pH was measured to ensure it was around pH 1-2, and then the MC-SH was stored at 4°C under N₂. An Ellmans assay was then performed to quantify thiol substitution of MC-SH.

2.13 MC-SH Lyophilization and Sterilization for xMC Gel

Prior to lyophilization, the pH of the MC-SH was adjusted from pH 2 to pH 4 through dialysis. MC-SH was transferred into 12kDa – 14kDa MWCO dialysis tubing and dialyzed against water with 0.1N NaCl, pH 4 (changed three times on the first day and left overnight), and then against DI water, pH 4 (changed three times). The MC-SH was then sterile filtered in the Biosafety Cabinet (BSC) and lyophilized. Following lyophilization, an Ellman’s assay was performed to determine the thiol substitution. The lyophilized MC-SH was stored at 4°C under sterile N₂.

2.14 Weak binding peptide synthesis, modifications and purification

2.14.1 Peptide Synthesis:

The SH3-weak binding peptide (sequence: GGGKPPVVKKPHYS) was synthesized through solid phase peptide synthesis on a Liberty 1 microwave peptide synthesizer (CEM). Following synthesis, the resin-bound peptide was thoroughly washed with dichloromethane (DCM) in an ISOLUTE column.

2.14.2 3-maleimidopropionic acid modification of the peptide:

3-maleimidopropionic acid was dissolved thoroughly in 20mL DCM with 2mL N-methyl-2-pyrrolidone (NMP). N,N’diisopropylcarbodiimide (DIC) was then added to the solution, and stirred for 30 minutes. The solution was then added to the resin inside the ISOLUTE column and mixed overnight.
2.14.3 Resin Cleavage

The following day, the resin-bound peptide was thoroughly washed with dichloromethane (DCM). The peptide was cleaved from the resin, and remaining protective groups were removed by adding a solution with trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and DIH₂O to the resin inside the ISOLUTE column and mixed for 6 hours. After 6 hours, the peptide was precipitated dropwise into cold ethyl ether. The peptide was then centrifuged to the bottom of the tube and the ethyl ether was poured off. Any remaining TFA and ethyl ether in the peptide was evaporated in the fume hood, after which the peptide was stored at -20°C.

2.14.4 Peptide Purification

Crude peptide was dissolved in trifluoroacetic acid, acetonitrile and water, filtered through a 0.2um filter, and then purified via reverse-phase high pressure liquid chromatography (HPLC). Any organic solvents (ie acetonitrile) in the fractions were evaporated in the fume hood, and the remaining fractions were lyophilized. Peptide purity as evaluated through mass spectrometry.

2.15 MC-WBP Synthesis

100 mg of thiolated methylcellulose was dissolved in 10mL PBS at pH 6.8 for 4 hours at 4 °C. The round bottom flask was covered with aluminum foil to prevent light oxidation of thiol groups. The WBP-maldeimide was added at a 1:5 molar equivalence between thiol groups on MC-SH to WBP-maleimide, and the reaction was carried out for 24 hours at 4 °C. The polymer was then transferred to 8kDa MWCO dialysis tubes and dialyzed against PBS for 48 hours at 4°C. The polymer was sterile filtered, lyophilized, and WBP substitution on MC-SH was quantified via amino acid analysis, which was performed at the SPARC Biocentre at Sick Kids Hospital.

2.16 xMC-chABC-SH3 Preparation

The relative amounts of MC-SH, MC-WBP, unmodified MC, chABC-SH3, crosslinker and phosphate buffer are dependent on the desired concentrations in the final xMC hydrogel formulation. The amount of MC-SH was calculated such that there was a final concentration of 0.1 µmol thiols/100µL of gel. The amount of chABC-SH3 was determined such that there was a final concentration of 0.3 units of chABC-SH3 in 5µL gel. The amount of MC-WBP was
calculated such that there was a 1:100 molar ratio of chABC-SH3:WBP. The final xMC hydrogel was comprised of 5 weight % MC, where the remaining MC (other than MC-WBP and MC-SH) was unmodified MC. The amount of poly(ethylene glycol)- bismaleimide (PEGMI₂, 3000 Da, Rapp Polymere, Tuebingen, Germany) crosslinker was calculated such that there was a 0.75 maleimide: 1 thiol ratio. The volume of PEG crosslinker that was added was 10% of the final xMC hydrogel volume.

In terms of preparing the gel, the MC, MC-SH, MC-WBP were weighed out, and the correct volume of phosphate buffer (minus the volume of the crosslinker and minus the volume of the chABC-SH3) was added. The solution was speed mixed for 2 minutes at maximum speed and centrifuged for 5 minutes in the cold room at maximum speed. The speed mixing and centrifugation steps were repeated 2-3 times until the polymers were fully dissolved in the buffer. The chABC-SH3 was then added, speed mixed for 30 seconds at maximum speed, and centrifuged at maximum speed for 1 minute in the cold room. The filter sterilized crosslinker solution (PEGMI₂ dissolved in phosphate buffer) was then added, and the solution was mixed carefully for around 1 minute. The xMC hydrogel was centrifuged for 1 minute at maximum speed in the cold room and then stored at 4 °C overnight.

2.17 In Vitro chABC Release Profile

xMC-chABC hydrogels, and xMC (vehicle alone) were prepared as described above and stored at 4 °C overnight. 100μL of the xMC-chABC and xMC gels were injected into Eppendorf tubes. The tubes were gently centrifuged to ensure that all of the material was at the bottom of the tube, and were then placed into the 37 °C incubator to gel for 10 minutes, and then 400μL of warm aCSF was added on top of the gel. For the 0 day time point, the aCSF was removed immediately, stored in an Eppendorf tube, and another 400μL of fresh aCSF was placed on top of the gel. The gels were then put back into the 37 °C incubator, and the aCSF was removed and replaced on day 1, 2, 5 and 7. The release samples at each time point were stored at -80°C.

In order to quantify the amount of chABC released at each time point, an ELISA was carried out. All samples were diluted to an appropriate concentration, and a standard curve ranging from 1-0.06μg/mL of chABC was prepared. 200μL of the standard curve, samples, and a blank well were pipetted into a 96 well Ni-NTA plate. The plate was subsequently covered and incubated at 37°C on a shaker for 2 hours. The plates were washed with 250μL PBS three times. 200μL of the
anti-FLAG antibody was added to each well, the plates were covered and incubated at 30°C on a shaker for 2 hours. The plates were then washed again as above, 200µL of the secondary antibody was added, and the plates were covered and incubated at room temperature on a shaker for 45 minutes. The plates were washed as above, 100µL of ABTS was added to each well, and the plates were covered and incubated at room temperature on a shaker for 10 minutes. The absorbance levels were read on a TECAN plate reader at 405nm and 650nm.

2.18 Cell preparation for pilot in vivo study & combinatorial in vivo study

For the pilot study, day 0 undifferentiated cNECs were prepared in a 0.5/0.5 HAMC concentration at cell densities of 20,000 cells/µL, 50,000 cells/µL, and stored on ice until transplantation.

For the combinatorial in vivo study, cNECs that had been pre-differentiated in vitro for 16 days were magnetically sorted for the cell surface marker NCAM-PSA using the anti PSA-NCAM MicroBeads (Miltenyi Biotec). The cells were centrifuged at 1400 rpm for 4 minutes, resuspended in 60µL of buffer (PBS pH 7.2, 0.5% BSA, 2mM EDTA), and stored at 4°C for 10 minutes. 20µL of the MicroBeads were then mixed into the cell suspension, and stored at 4°C for 15 minutes. During this period, the LS column (Miltenyi Biotec) was placed in the magnetic separator (Miltenyi Biotec) and rinsed with 3mL buffer. The cells were then transferred to the column, and washed three times with 3mL of buffer. The column was removed from the magnetic separator, 5mL buffer was put into the column, and then cells were flushed out of the column using a plunger. Cells were then counted, resuspended in an appropriate volume of aCSF, and added to a 1/1 HAMC hydrogel, to dilute it to 25% and achieve a final 0.75/0.75 HAMC hydrogel with a cell density of 20,000 cells/µL. The cells were then stored on ice until transplantation.

2.19 In Vivo Surgeries

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and protocols were approved by the Animal Care Committee of the Research Institute of the University Health Network.
2.19.1 Pilot In Vivo Study

On day 0, adult female Sprague Dawley rats were anesthetized by inhalation of a general anesthetic, subjected to a laminectomy at the T1/2 vertebral level, and then the spinal cord was compressed with a 26g modified aneurysm clip for 1 minute, resulting in a moderate SCI. Immediately after the spinal cord was compressed, the overlying muscles, fascia and skin were closed, rats were placed under a heating lamp and allowed to recover. Six days after injury, rats received subcutaneous injections of the immunosuppressant cyclosporine A (cyclosporine A Sandimmune, Novartis Pharma) (10 mg/kg/day). The next day (seven days after the injury), the rats were anesthetized by inhalation of a general anesthetic, and the previously operated on site was re-exposed. Cells in HAMC were then stereotactically injected intraspinally 1mm below the dorsal surface of the cord through a 32 GA needle, using a syringe pump such that the cells were injected at a rate of 1 uL/minute, for a total of 2 minutes. A total of 4 injections were made, where 2 injections were made 1mm rostral to the injury, where each injection was 1mm lateral from the midline, and another 2 injections were made 1mm caudal from the lesion site, where each injection was also 1mm lateral from the midline. After each injection the needle was left in place for an additional 2 minutes to prevent backflow of cells. Animals received one of the following treatments: (1) 20K cells/µL in 0.5/0.5 HAMC for a total of 160K cells (n=4) and (2) 50K cells/µL in 0.5/0.5 HAMC for a total of 400K cells (n=3) (Table 3.1). After closing the overlying muscles, an osmotic pump loaded with the immunosuppressant cyclosporine A was subcutaneously implanted. The 2ML1 osmotic pump was used, which delivered the immunosuppressant at a rate of 10 µL/hour for 1 week. A final concentration of 10mg/kg/day of cyclosporine-A was delivered. The skin was then closed and the rats were placed under a heating lamp and allowed to recover.

2.19.2 Combinatorial In Vivo Study

On day 0, adult female Sprague Dawley rats were anesthetized by inhalation of a general anesthetic, subjected to a laminectomy at the T1/2 vertebral level, and then the spinal cord was compressed with a 26g modified aneurysm clip for 1 minute, resulting in a moderate SCI. Immediately after the injury, 5µL of chABC-xMC (or control solutions) was intrathecally injected through a 30 GA angled blunt-tipped needle. Upon injection, the needle was kept in place for 1 minute to prevent backflow. After closing the overlying muscles, fascia and skin, rats were placed under a heating lamp and allowed to recover.
Six days after injury, rats received subcutaneous injections of the immunosuppressant cyclosporine A (cyclosporine A Sandimmune, Novartis Pharma) (10 mg/kg/day). The next day (seven days after the injury), the rats were anesthetized by inhalation of a general anesthetic, and the previously operated on site was re-exposed. Cells in HAMC (or control solutions) were stereotactically injected (concentration of 20K cells/µL) as described above in Section 2.19.1. Immediately after the intraspinal injections, 5µL of chABC-xMC (or control solutions) was intrathecally injected through a 30 gauge angled blunt-tipped needle. Table 3.2 describes which groups received the treatment or the vehicle. Upon injection, the needle was held in place for 1 minute to prevent backflow. After closing the overlying muscles, an osmotic pump loaded with the immunosuppressant cyclosporine A was subcutaneously implanted. Animals that were sacrificed 1 week after cell transplantation received the 2ML1 osmotic pump, which delivered cyclosporine A at a rate of 10 µL/hour for 1 week. Animals that were sacrificed 8 weeks after cell transplantation received the 2ML4 osmotic pump, which delivered cyclosporine A at a rate of 2.5 mL/hour for 4 weeks, and the pumps were replaced after 4 weeks. The pumps delivered a final concentration of 10mg/kg/day of cyclosporine A. Lastly, the skin was closed and the rats were placed under a heating lamp and allowed to recover.

The groups for the behavioral tests for this study were as follows: Injury alone (n=10), vehicle alone (n=9, one died during the study), chABC alone (n=10 from week 1-7 but n=9 from week 8-9 because one had to be sacrificed), cells alone (n=10), cells + chABC (n=12, one was removed from the study because it was dehydrated/sick throughout the entire study). 1 week after transplantation the following animals were sacrificed from each group: cells alone (n=4), cells + chABC (n=4).

2.20 Housing and post-operative care

As explained in [90] buprenorphine (0.05 mg/kg) was administered twice daily every 12 h for 48 h after surgery. One animal was housed per cage in a temperature-controlled room with a 12 h light/dark cycle. Clavamox was added to the water for 2 days prior to surgery, and for 5 days post-surgery to prevent prevent urinary tract infections. Their bladders were manually expressed 3 times per day until bladder function returned, water and food were provided ad libitum, and skin clips were removed 14 d post-operatively [90].
2.21 Functional Assessment

2.21.1 Handling & Training

Animals were handled for 2 days sixteen days prior to injury, and animals were trained on the ladder walk daily for 14 days prior to the injury.

2.21.2 BBB Score

Motor behavior was evaluated weekly by two blinded observers using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale by placing the animals in an open area and evaluating them for 4 minutes. BBB scores on the left and right foot were scored individually and then averaged. Healthy animals have a BBB score of 21, while animals with no hindlimb function have a BBB score of 0.

2.21.3 Motor Subscore

For 7, 8, 9 weeks after injury, animals which had a BBB score of greater than or equal to 11 were concurrently evaluated using the BBB scoring system and the motor subscore system. Motor subscore was calculated by assessing toe clearance, paw placement, tail placement, and overall stability. Animals which had a BBB score less than 11 were given a motor subscore of 0.

2.21.4 Ladder Walk

Ladder walk testing occurred 2 days prior to injury to obtain baseline values, and then occurred weekly starting 2 weeks after injury (1 week after cell transplantation) for animals with a BBB score greater than or equal to 11. Briefly, animals were placed on a horizontal ladder, where they were recorded crossing the ladder three times. The number of gaps in the ladder were kept consistent every week, however the locations of the gaps were changed to prevent learning of the task. Videos were analyzed by counting the number of foot faults made by the left and right hindlimb through the gaps in the ladder, and the total number of foot faults were summed. Animals which had a BBB score less than 11 were given a maximum score of 10 because on average the animals took 10 steps to cross the ladder.
2.21.5 Tail Flick

Tail flick testing occurred weekly starting 2 weeks after injury (1 week after cell transplantation). During testing, the animals were held in place and their tail was placed in a channel such that the light was focused on a certain part of the tail. The amount of time taken for the tail to flick in response to the heat stimulus was recorded as the withdrawal latency. At every time point, each animal was tested on the tail flick three times, with at least 5 minutes between each run, and then the three runs for each animal were averaged. The light shone on the tail for a maximum of 20 seconds to prevent overheating/burning. The light intensity was kept consistent through the entire study.

2.22 Immunohistochemistry/Tissue Analysis

Animals were sacrificed and transcardially perfused with 4% PFA in 0.1 M phosphate buffer. Spinal cords were then removed, a 1.5 cm section centered around the lesion was dissected, the section was post-fixed in 4% PFA overnight, and cryopreserved in 30% sucrose in PBS for at least 1 week. The tissue was mounted in Cryomatrix (Thermo Fisher Scientific) and cryosectioned parasagitally in 20um sections.

The following animals were sectioned for the pilot study: 20K cells/µL in 0.5/0.5 HAMC (n=4), 50K cells/µL in 0.5/0.5 HAMC (n=3).

In the combinatorial *in vivo* study, the following animals were sectioned 1 week post-transplantation: cells alone (n=4), cells + chABC (n=4).

In the combinatorial *in vivo* study, the following animals were sectioned 8 weeks post-transplantation: cells + chABC (n=8), cells alone (n=8), injury alone (n=4), vehicle alone (n=4), chABC alone (n=4).

*Immunostaining:* For the pilot study and for the animals sacrificed 1 week post-transplantation in the combinatorial *in vivo* study, every 4th section was stained, however for the animals sacrificed 8 weeks post-transplantation in the combinatorial *in vivo* study, every 8th section was stained. Sections were equilibrated to room temperature for 15 minutes, outlined with a hydrophobic pen, hydrated with PBS-Tween (PBS-T) for 5 mins, then primary antibodies were added (GFAP 1:2000, hNUC 1:500, βIII Tubulin 1:2000, Ki67 1:500), and sections were stored at 4 °C.
overnight. The next day the primary antibody was removed, sections were washed 3 times for five minutes with PBS-T, then secondary antibodies were added (1:500 dilution) with DAPI (1:1000 dilution). Secondary antibody was added for 3 hours at room temperature and then washed 3 times with PBS-T. Sections were dried for 15 mins at room temperature, then ProLong Gold antifade (ThermoFisher Scientific) was added dropwise, coverslips were mounted on the sections, and the sections were left to dry at room temperature overnight. All sections were imaged on an Olympus confocal microscope, except for hNUC staining of animals which were sacrificed 1-week post-transplantation, and GFAP staining of animals that were sacrificed 8 weeks post-transplantation, which were imaged on a Zeiss slide scanner (Microscope Imaging Facility, University of Toronto).

**Quantification of number of cells (hNUC+ cells):** In the pilot study and for the animals sacrificed 8 weeks post-transplantation from the combinatorial *in vivo* study, the number of hNUC+ cells was quantified using Fiji by measuring the number of hNUC+ pixels and dividing this by the average number of pixels per cell. However for the animals sacrificed 1 week post-transplantation from the combinatorial *in vivo* study, the number of cells was determined by manually counting them.

**Quantification of Ki67 and hNUC positive cells:** A region of interest comprised of only the hNUC+ cells was created, and a region of interest comprised of only the Ki67+ cells was created. A third region of interest which contained the area of overlap between the two aforementioned regions of interest was created. In order to remove pixels which were not from cells, but rather debris, particles below a certain threshold area were excluded. Pixel count of the Ki67+/hNUC+ cells was measured, and this was divided by the average number of pixels per cell. The total number of Ki67+/hNUC+ cells was divided by the total number of hNUC+ cells per animal in order to determine the percentage of hNUC+ cells that are also Ki67+.

**βIII Tubulin association with hNUC positive cells:** As seen in Figure 2.1, a region of interest comprised of only the hNUC+ cells was created. Since βIII tubulin is not a nuclear stain, but is rather a cytoplasmic and axonal stain, in order to estimate the level of association between βIII tubulin with hNUC, the ROI of the hNUC+ cells was enlarged by 11.32um in every direction. Another ROI of only the βIII tubulin+ staining was created, and similar to above, the number of pixels which are colocalized between these 2 ROIs was measured. This value is then divided by
the total number of hNUC+ cells in order to determine the percentage of hNUC+ cells which are associating with βIII tubulin positive staining.

**Lesion Area:** The fluid-filled region (without DAPI) within the GFAP+ region was outlined and the area was measured.

**Rostrocaudal Spread:** The y-position (rostrocaudal position) of each cell was determined, and the distance from the most rostral and most caudal spread was measured. In order to remove cells which were very far from the majority of the cells, cells which are greater than 3 or greater than 4 standard deviations away from the mean were removed.

---

**Figure 2.1:** Steps to measure association between hNUC+ cells and surrounding βIII Tubulin positive staining using Fiji (DAPI = Blue, Red = Human Nuclear Antigen (Transplanted cells), Green = βIII Tubulin (Immature neurons), Scale bar = 500um)

---

### 2.23 Statistical Analysis

All data are reported as mean ± standard error unless reported otherwise. All statistical analysis was performed using GraphPad Prism 6. Statistical analysis for the *in vitro* assays are explained in figure captions. For the *in vivo* behavioral tests a 2-way ANOVA with Bonferroni’s *post-hoc* test was used. For the *in vivo* tissue analysis, a t-test was performed to assess differences between the cells alone and cells + chABC group. 1-way ANOVA with Bonferroni’s *post-hoc* was used to assess differences in terms of lesion size. Significance was set at p<0.05. Statistical outliers were removed as per Grubbs’ test for outliers.
3 Results

3.1 Pilot *in vivo* study to determine whether cNECs remain in the injured spinal cord

In our first set of experiments, we sought to determine whether the cNECs can survive in vivo in the injured spinal cord. This is because the cNECs have been previously shown to express forebrain markers such as Pax 6 and not caudal markers that are specific to the spinal cord. As such, a pilot *in vivo* study was performed in order to assess whether the cNECs (or their differentiated progeny) could survive in the injured spinal cord for up to one week post-transplantation (*In vivo* timeline shown in Figure 3.1)

Table 3.1: Groups in the pilot *in vivo* study

<table>
<thead>
<tr>
<th>Number of Animals</th>
<th>Cell Density Injected</th>
<th>Total # Cells Injected</th>
<th>Number of animals with surviving cells after 1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>20K/µL in 0.5/0.5 HAMC</td>
<td>160K</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>50K/µL in 0.5/0.5 HAMC</td>
<td>400K</td>
<td>2</td>
</tr>
</tbody>
</table>

The undifferentiated cNECs were suspended in a 0.5 weight percent HA and 0.5 weight percent MC (0.5/0.5 HAMC) hydrogel, and as shown in Table 3.1, two different cell densities were transplanted: 20K cells/µL, and 50K cells/µL. The purpose of this was to determine whether there was a correlation between cell survival and the number of cells transplanted. Transplanted
cNECs were found in 3 out of the 4 animals that received cells at a density of 20K cells/µL and in 2 out of the 3 animals that received cells at a density of 50K cells/µL. Transplanted cNECs can be seen in Figures 3.2a-c, which show a region of localized cells. As seen in Figure 3.2d, there is a trend towards increased cell numbers in the animals that received the higher cell density, however when cell number was expressed as a percentage of the number of cells that was initially transplanted on day 0, there is a trend towards an increased percentage of cells in the group that received the lower cell density (Fig 3.2e). Although the cNECs do remain in the injured spinal cord for up to 1 week post transplantation, the percentage of cells remaining is low. In order to improve cell survival following transplantation, the hyaluronan-methyl cellulose (HAMC) hydrogel vehicle in which the cells were delivered was modified (Figure 3.3).

**Figure 3.2:** In vivo pilot study to determine whether the cNECs remain in the injured spinal cord one week post transplantation. (a) Immunostained section showing overview of spinal cord that received cells at a density of 20K cells/µL (Scale bar = 500um) (b) Enlarged region outlined in (a) showing transplanted cells (Scale bar = 250um) (c) Enlarged region outlined in (a) showing both DAPI and transplanted cells (Scale bar = 250um) (d) There is a trend towards increased cell numbers in the animals that received the higher cell density (d) However when the cell number is expressed as a percentage of the number of cells that were transplanted on day 0, there is a trend towards increased percentage of transplanted cells in the group that received the lower cell density (blue = DAPI, red= transplanted cells (human nuclear antigen), 20K/µL group (n=4), 50K/µL group (n=3), mean ± standard error)
3.2 Physical modifications of HAMC to enhance cell survival

The HAMC hydrogel was physically modified by altering the individual polymeric concentration of the HA and MC. Although the 0.5/0.5 HAMC has been previously used for cell transplantation strategies, a series of in vitro assays were carried out to determine whether the mechanically stiffer 0.75/0.75 HAMC hydrogel [82] which could potentially remain at the site of injury for longer, would better promote cell survival. Each in vitro assay was representative of a step in the cell transplantation process where cell survival is compromised.

![Chemical structure of (a) Hyaluronan (HA) and (b) Methyl Cellulose (MC). The HAMC hydrogel can be physically modified by altering the polymeric concentrations of HA and MC such that there is a (c) lower or (d) higher weight percentage of HA and MC in the gels.](image)

**Figure 3.3:** Chemical structure of (a) Hyaluronan (HA) and (b) Methyl Cellulose (MC). The HAMC hydrogel can be physically modified by altering the polymeric concentrations of HA and MC such that there is a (c) lower or (d) higher weight percentage of HA and MC in the gels.

3.2.1 Cell survival on ice

Initially, a pre-transplantation survival assay was carried out in which the purpose was to determine whether embedding the cNECs in either HAMC concentration would limit cell loss on ice in comparison to cNECs in media. This is considered a pre-transplantation assay because cells are typically kept on ice for a number of hours prior to transplantation, which could reduce their viability. As seen in Figure 3.4a, after 6 hours on ice there is a trend towards increased cell survival as a result of encapsulation in the 0.75/0.75 HAMC hydrogel, and after 24 hours on ice, there is significantly greater cell survival in the 0.75/0.75 HAMC in comparison to cells in aCSF.
3.2.2 Cell survival through a fine gauge needle

The second assay consisted of assessing cell survival as a result of being injected through a fine gauge needle, since cell viability is also severely reduced during cell injection. In order to assess whether encapsulation in HAMC limits cell loss as a result of injection, in comparison to cells dispersed in aCSF, encapsulated cells were injected through a 32 gauge needle as they would be in an *in vivo* study. As seen in Figure 3.4b, cell viability following injection is significantly lower after injection for cells that are dispersed in aCSF, whereas amongst cells that are encapsulated in either of the two HAMC hydrogels, there is no significant difference in the percentage of live cells before and after injection. Although this does not provide any insight into which of the 0.5/0.5 or 0.75/0.75 HAMC hydrogel should be pursued, it does demonstrate the benefits of delivering the cells in the HAMC hydrogel in comparison to in aCSF.

![Cell survival through a fine gauge needle](image)

**Figure 3.4:** Cell survival in aCSF, 0.5/0.5 and 0.75/0.75 HAMC (a) after 6 and 24 hours on ice (normalized to the initial 0 hour survival) as assessed through a PrestoBlue cell viability assay (n=3, one way ANOVA at each timepoint with Dunnett’s multiple comparison test, mean ± standard error) and (b) after being injected through a 32 gauge needle (normalized to live cells prior to injection), as assessed through a live/dead cell stain (n=4, one way ANOVA with Bonferroni’s *post-hoc* test, mean ± standard error)

3.2.3 Cell distribution following injection into a gelatin gel

Another issue with cell transplantations is uneven cell distribution following transplantation, which results in aggregated cells, thereby reducing cell survival [77]. In order to determine whether encapsulating the cNECs in the 0.5/0.5 HAMC, 0.75/0.75 HAMC or aCSF could promote a more even distribution of the cells following transplantation, the cells were again loaded into a 10μL Hamilton syringe, and then injected into a gelatin gel. While the elastic
modulus of the human spinal cord ranges from around 90-230kPa [91], [92], we aimed to use a material with a modulus similar to that of the rat spinal cord (around 8.1 ± 1.1 kPa [93]) since these cells would eventually be transplanted into rat models of SCI. As seen in Figure 3.5, although both the 6% gelatin gel and the 0.6% agarose gel had average compressive moduli that were similar to that of the rat spinal cord, the 6% gelatin gel was selected because its error was much lower than the latter.

As seen in Figure 3.6, cNEC encapsulation in either of the two HAMC hydrogels promoted a more even cell distribution following injection into the 6% gelatin gel, in comparison to cells dispersed in aCSF. This is could be due to cells in the HAMC hydrogels remaining dispersed throughout the hydrogels prior to injection, whereas the cells in aCSF may have aggregated even prior to injection. Alternatively, this could also be due to the HAMC hydrogel (and cells within the hydrogel) remaining localized to the site of injection, whereas when the cells in aCSF are injected into the gelatin, the aCSF may be dispersing through the gelatin gel, thereby leaving only the cells, which are then clumped together.

Based on these three in vitro assays to assess which of the 0.5/0.5 or 0.75/0.75 HAMC to move forward with in vivo, a difference between the two HAMC concentrations was only seen in the survival on ice assay, in which the 0.75/0.75 HAMC had a higher cell viability in comparison to
aCSF after 24 hours on ice. Therefore, the 0.75/0.75 HAMC hydrogel was selected for the combinatorial in vivo study described below.

![Image](image.png)

**Figure 3.6:** Compressed Z-stacks of cNECs following injection into a 6% gelatin gel, where the cells were dispersed in (a) aCSF, (b) 0.5/0.5 HAMC, (c) 0.75/0.75 HAMC (Green = Live cells (Calcein AM), red = dead cells (Ethidium homodimer), blue = DAPI, Scale bars = 100um)

### 3.3 Synthesis of materials for the chABC-SH3 delivery hydrogel (xMC-chABC-SH3)

The xMC-chABC-SH3 hydrogel is comprised of different components: physically crossinked unmodified MC (Figure 3.7a), MC modified with thiols (MC-SH) chemically crosslinked with PEG-Bismaleimide (Figure 3.7b) and MC modified with an SH3 Binding Peptide which has an affinity for the SH3-modified chABC (Figure 3.7c). ChABC-SH3 was bacterially expressed and subsequently purified through a Nickel column and through fast pressure liquid chromatography (FPLC). Figure 3.8a shows the absorbance spectrum following chABC-SH3 purification through FPLC, which shows a distinct absorbance peak at 280nm corresponding to the protein’s UV absorption peak. The fractions corresponding to this peak were collected, concentrated down, and further purified to remove any endotoxins. ChABC-SH3 activity was measured through a kinetic assay which tracks UV absorbance at 232nm of the double bond that forms as a result of chABC digestion of chondroitin sulfate-A. As shown in Figure 3.8b, the absorbance measurements increase linearly as time progresses, and as chABC digests more of the chondroitin sulfate-A. The slope of the line of best fit is then used to determine the activity of the enzyme. The activity of the enzyme synthesized for the in vivo study described below was 114 units/mL, where 1 unit is defined as being the amount of enzyme that catalyzes the formation of 1 umol of unsaturated disaccharide from chondroitin sulphate-A in 1 minute [52]. Another component of the xMC-chABC-SH3 hydrogel is MC-thiol (MC-SH), which was synthesized from MC. The MC-SH synthesized for this study had a thiol substitution of 62 umol SH/g MC-
SH. SH3-binding peptide modified MC was synthesized by first making the SH3-binding peptide (weak binding peptide (WBP)) through solid phase peptide synthesis. The HPLC spectrum in Figure 3.8c shows the purity of the maleimide-modified WBP, and also shows that the theoretical and experimental molecular weights of the maleimide-modified WBP are identical (1713.92 g/mol). MC-SH was reacted with the WBP-maleimide to form MC-WBP, which had a peptide substitution of 154 umol WBP/g MC-WBP. Following synthesis of the individual components of the xMC-chABC-SH3 hydrogel, an in vitro release assay was performed to determine the rate at which chABC-SH3 was released from the hydrogel. A double tag ELISA was used to quantify the amount of chABC-SH3 released at each time point, and as seen in Figure 3.8d, there was a substantial release of chABC-SH3 within the first 2 days, followed by a much smaller quantity until day 7.

Figure 3.7: Schematic of the individual components of the xMC-chABC-SH3 hydrogel. (a) Methyl cellulose polymers form physical crosslinks (b) MC-SH is chemically crosslinked with PEG-Bismaleimide through a Michael type reaction (c) MC-SH3 binding peptide reversibly interacts with SH3-chABC to enable sustained release of the enzyme.
Co-delivery of cNECs and chABC into rat models of SCI

After determining in which HAMC hydrogel to deliver the pre-differentiated (d16) cNECs, and after verifying that the chABC-SH3 was released from the xMC-chABC-SH3 hydrogel over 1 week *in vitro*, an *in vivo* study was carried out to assess motor and sensory recovery as well as tissue recovery following transplantation into rat models of SCI (T1/2 clip compression injury). The *in vivo* paradigm is shown in Figure 3.9, and the 5 groups included in the study are shown in Table 3.2.

Figure 3.8: Synthesis and characterization of components of the xMC-chABC-SH3 hydrogel. (a) FPLC spectra (absorbance measured at 280nm) following chABC purification (arrow shows chABC peak) (b) Kinetic assay to measure chABC-SH3 activity by measuring absorbance at 232nm as chABC-SH3 digests chondroitin Sulfate-A. The slope of this line is used to determine the activity of the chABC-SH3 (c) Spectra following HPLC purification of maleimide-modified weak binding peptide shows that the product is pure, and its molecular weight matches the theoretical molecular weight of WBP-maleimide (d) Release of chABC-SH3 from xMC hydrogel over 7 days (n=3, mean ± standard deviation)
Table 3.2: Groups in combinatorial in vivo study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of Animals per Group</th>
<th>Day 0 (Injury + Intrathecal Injection)</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury Alone</td>
<td>10</td>
<td>Phosphate buffer (Intrathecal)</td>
<td>aCSF (intraspinal) + Phosphate buffer (Intrathecal)</td>
</tr>
<tr>
<td>Vehicle Alone</td>
<td>9</td>
<td>xMC (Intrathecal)</td>
<td>HAMC (intraspinal) + xMC (Intrathecal)</td>
</tr>
<tr>
<td>chABC Alone</td>
<td>9</td>
<td>chABC in xMC (Intrathecal)</td>
<td>HAMC (intraspinal) + chABC in xMC (Intrathecal)</td>
</tr>
<tr>
<td>Cells Alone</td>
<td>14 (n=4 sacrificed 1 week post cell transplantation)</td>
<td>xMC (Intrathecal)</td>
<td>Cells in HAMC (intraspinal) + xMC (Intrathecal)</td>
</tr>
<tr>
<td>chABC + Cells</td>
<td>16 (n=4 sacrificed 1 week post cell transplantation)</td>
<td>chABC in xMC (Intrathecal)</td>
<td>Cells in HAMC (intraspinal) + chABC in xMC (Intrathecal)</td>
</tr>
</tbody>
</table>

3.4.1 Functional Recovery

Behavioural improvements of the combinatorial treatment was assessed through the BBB scoring system, BBB motor subscore and ladder walk for motor recovery, as well as the tail flick for sensory recovery. Unfortunately, none of the treatment groups (chABC alone, cells alone, or cells + chABC together) promoted motor recovery, as evaluated through the BBB score (Figure 3.10a). There is a trend towards the injury alone group having the highest BBB scores at all time points, including the first time point. This could suggest that the injury alone group was less injured than the other groups even one week post-injury because that was the only group that did not receive an intrathecal injection of the xMC gel. However as shows in Fig 3.10b, most of the groups have a similar percentage of animals with a BBB score greater than 11 (are able to take...
frequent to consistent weight supported steps), making it unlikely that the xMC gel had a deleterious effect. Motor recovery was also assessed through the BBB motor subscore (Figure 3.11a), and the ladder walk (Figure 3.11b), both of which demonstrated that the treatment groups did not achieve motor recovery in comparison to either of the control groups (injury alone and vehicle alone) at any of the time points. In all three of the behavioural assays which assess motor recovery, the same trends were seen; namely, animals which received only the spinal cord injury consistently scored the best (highest BBB score, highest motor subscore, least number of foot faults), and the cells alone group consistently scored the worst (lowest BBB score, lowest motor subscore, most number of foot faults). Although the injury alone group scored significantly higher than the cells alone group, this was only at a few early time points, and not throughout the whole study.

**Figure 3.10** – Motor recovery assessed through the BBB scoring system. (a) The combination delivery of chABC and cells neither impairs nor improves motor recovery in comparison to control groups, as assessed through the BBB scoring system (Mean ± standard error, 2-Way ANOVA, *p<0.05 between injury and cell groups). (b) The percentage of animals which have a BBB score greater than or equal to 11 (ability to take frequent to consistent weight supported plantar steps) (c) The percentage of animals which have a BBB score of greater than or equal to 13 (frequent forelimb-hindlimb coordination) (The following statistical outliers were removed: 1 in injury group, 2 in vehicle group, 1 in cells + chABC group)
The tail flick assay was used to evaluate sensory recovery. As seen in Figure 3.12a, there are also no significant differences in sensory recovery between the 5 groups at each time point. Rather than comparing all of the groups at each time point, when looking specifically at how each individual group recovers over time, the vehicle, cells and cells + chABC groups all have significantly lower withdrawal times from week 2 to week 9 post-injury while the injury and chABC alone groups do not (Figure 3.12b-f). 9 weeks after the injury, the former three groups are more sensitive to the thermal stimulus compared to their initial sensitivity levels, while the other two groups (vehicle and chABC alone) are not. The increased sensitivity in the injury alone group and the two groups that received cells from week 2 to week 9 is concerning, as those groups are developing thermal hyperalgesia faster than the others. However, all of the groups appear to have a decrease in withdrawal latency from week 8 to week 9, therefore it is unknown as to whether this decrease is due to the development of thermal hyperalgesia, or due to a change in the testing environment, which caused the decrease in withdrawal latency. In order to verify whether those groups were developing allodynia, or whether the decrease in withdrawal time was due to the environment, the study should have been carried out until at least week 10.

Figure 3.11 – Motor recovery assessed through the (a) Motor subscore, showed no significant improvements in terms of fine motor skills between any of the groups (Injury: n=10 Vehicle: n=9, chABC: n=10, Cells: n=10, Cells + chABC: n=12, mean ± standard error, no statistical outliers were removed for this test) and (b) ladder walk, where the number of foot faults were quantified as the animal crossed a ladder with a set number of gaps, showed no improvement in fine motor skills in the combination treatment. (Injury: n=9, Vehicle: n=8, chABC: n=9, Cells: n=9, Cells + chABC: n=12, mean ± standard error. The following statistical outliers were removed from this test: 1 in injury group, 1 in vehicle group, 2-way ANOVA, *p<0.05 between injury and cell groups)
Figure 3.12 – Sensory recovery assessed through the tail flick, in which the time to withdraw the tail following exposure to thermal stimulus is used to assess thermal hyperalgesia. (a) There were no significant differences in withdrawal times between any of the groups at any of the time points. (b)–(f) shows the withdrawal latency for each individual group (b) injury (c) vehicle (d) chABC (e) cells (f) chABC + cells. There is a significant decrease in withdrawal time from week 2 to week 9 in the (c) injury, (e), cells and (f) cells + chABC groups, which means that those animals are more sensitive to the thermal stimulus at week 9 than they were initially (Injury: n=10 Vehicle: n=8, chABC: n=10, Cells: n=9, Cells + chABC: n=11, mean ± standard error. The following statistical outliers were removed: 1 in vehicle group, 1 in cells group, 1 in cells + chABC group, one-way ANOVA *p<0.05, **p<0.01, ***p<0.001)
3.4.2 Quantification of transplanted cells 1 week post-transplantation

The number of transplanted cells that remained in the spinal cord was determined through immunohistochemical analysis, where the transplanted cells were stained for the human nuclear antigen antibody, since the cNECs are derived from human iPS cells. As seen in Figure 3.13, the pre-differentiated (d16) cNECs (delivered in 0.75/0.75 HAMC hydrogel) remain in the cord for one week after transplantation, and are found amongst host cells. Furthermore, the cells that remain were found in clusters dispersed throughout regions of the spinal cord, rather than in one larger clump of cells, suggesting that the cells are migrating. As shown in Figure 3.14, the number of transplanted cells as well as the percentage of transplanted cells that remain (relative to the number of cells that were transplanted on day 0) is quite low, as only around 3-5% of the cells that were transplanted remained one week after transplantation. There is a trend towards increased cell number in the animals that received cells + chABC, but the difference is not statistically significant.

![Figure 3.13: Transplanted cells remain in the spinal cord 1 week post-transplantation.](image)

*Representative sections of animals that received (a) cells alone (scale bar=150um) and (b) cells + chABC. White arrows point to transplanted cells, indicating that the cells are dispersed in small cell clusters (c) Enlarged region outlined in (b) to show that each cell cluster contains both transplanted and host cells (scale bar = 25um, red = transplanted cells (human nuclear antigen), blue = DAPI)*

![Figure 3.14: Quantification of number of transplanted cells remaining one week post-transplantation.](image)

*In terms of (a) the number of hNUC positive cells, and (b) the number of hNUC positive cells expressed as a percentage of the number of cells that were initially transplanted (160,000 cells), there is a trend towards higher cell numbers in the group that received both chABC and cells (n=4 in both groups, mean ± standard error)*
3.4.3 Quantification & characterization of transplanted cells 8 weeks post-transplantation

While the number of transplanted cells that remained in the spinal cord 1 week after transplantation was quite low in both groups that received cells (cells alone, cells + chABC), the average number of transplanted cells that remained in the spinal cord 8 weeks post-transplantation was substantially greater. However, as seen in Figures 3.15 and 3.16, in both groups, there were some animals which had very few transplanted cells, while other animals in the same group had a very high number of transplanted cells. Some animals had greater than 100% of the number of cells that were initially transplanted indicating that these cells proliferated. While the variability within each of those two groups is very high, similar to the short term cell numbers, there is a trend towards an increased percentage of transplanted cells (relative to the number of cells transplanted initially) remaining in the spinal cord 8 weeks after transplantation in the group that received both cells + chABC, in comparison to just cells alone.

Fig 3.15: Transplanted cells remain in the spinal cord 8 weeks post-transplantation. Representative sections of animals that received (a,b) cells only (scale bar = 250um) and (c,d) Cells + chABC (scale bar = 250um). (e) Enlarged region outlined in (d) to show that the transplanted cells are dispersed amongst host cells (Scale bar = 75um) (Blue = DAPI, Red = Transplanted cells (Human Nuclear Antigen))
Since the percentage of cells remaining 8 weeks after transplantation was substantially higher than the percentage of cells remaining 1 week after transplantation, and since the percentage of cells remaining 8 weeks after transplantation was quite disparate between animals in the same group with some animals having more than two times the number of cells than were initially transplanted, the proliferative capacity of these cells was assessed. In order to assess the proliferative capacity of the cells, the sections were stained for the proliferation marker Ki67, with representative images given in Figure 3.17a, b. As seen in Figure 3.17c, there is a trend towards more hNUC positive and Ki67 positive cells in the cells alone group. However, when this is expressed as a percentage of the total number of hNUC positive cells per animal (Figure 3.17d), this difference is reduced. In both groups around 13-15% of hNUC positive cells are also positive for Ki67. There are no significant differences between the cells alone and cells + chABC group in terms of Ki67 expression of the cells, however, this was not unexpected, as the purpose of staining for Ki67 was to simply to determine whether the cells are still proliferative 8 weeks after transplantation.

**Figure 3.16: Quantification of number of transplanted cells remaining eight weeks post-transplantation.** The number of hNUC positive cells expressed as a percentage of the number of cells initially transplanted (160K cells), varies amongst animals in the same group. However, there is a trend towards greater cell numbers in the animals that received both chABC + cells (n=8 in both groups, mean ± standard error)

---

Since the percentage of cells remaining 8 weeks after transplantation was substantially higher than the percentage of cells remaining 1 week after transplantation, and since the percentage of cells remaining 8 weeks after transplantation was quite disparate between animals in the same group with some animals having more than two times the number of cells than were initially transplanted, the proliferative capacity of these cells was assessed. In order to assess the proliferative capacity of the cells, the sections were stained for the proliferation marker Ki67, with representative images given in Figure 3.17a, b. As seen in Figure 3.17c, there is a trend towards more hNUC positive and Ki67 positive cells in the cells alone group. However, when this is expressed as a percentage of the total number of hNUC positive cells per animal (Figure 3.17d), this difference is reduced. In both groups around 13-15% of hNUC positive cells are also positive for Ki67. There are no significant differences between the cells alone and cells + chABC group in terms of Ki67 expression of the cells, however, this was not unexpected, as the purpose of staining for Ki67 was to simply to determine whether the cells are still proliferative 8 weeks after transplantation.
Further characterization of the transplanted cells involved determining whether the cells maintained a neuronal phenotype 8 weeks after transplantation. In order to assess whether the transplanted cells are neuronal cells, they were immunostained for the immature neuronal marker βIII Tubulin. As seen in Figure 3.18a and 3.18b, βIII tubulin stains both axons and the cytoplasm, which is why βIII tubulin staining surrounds hNUC positive nuclei. The degree of hNUC positive cells corresponding to βIII tubulin positive tissue was quantified as explained in Section 2.23 and in Figure B.1. As shown in Figures 3.18c and 3.18d, the pixel count of βIII tubulin associated with hNUC positive cells, and that value expressed as a percentage of the total number of hNUC positive cells per animal are not significantly different between animals that received cells alone and cells+ chABC. As such, both groups have roughly similar levels of hNUC positive cells that closely associate with βIII tubulin, from which it can be inferred that in both groups, a similar level of transplanted cells are βIII tubulin positive immature neurons.
Lesion area was assessed by measuring the DAPI-free area within the GFAP-outlined lesion. As seen in Figure 3.19, there are trends towards animals which did not receive cells (injury, vehicle, chABC alone groups) having larger lesion areas than animals that received cells (cells, cells + chABC groups). This is likely because animals which did not receive cells have large fluid-filled cavities (Figure 3.19a), while animals which received cells generally have lesions which are largely filled with DAPI positive cells (Figure 3.19b). Since these sections were only stained for GFAP (not co-stained for both GFAP and hNUC), it is not definitively known whether the DAPI positive cells are the transplanted cells. However, as seen above in Figure 3.15a, c, d, since many of the hNUC positive cells are either lining the lesion, or located in the centre of the tissue (where the injury occurred), it can be inferred that at least some of the DAPI positive cells in Figure 3.19b are transplanted cells. This indicates the migratory capabilities of the transplanted cells. However, it is also possible that these DAPI positive cells could rather blood cells or immune cells that are largely present following a SCI.

Since cells were transplanted both rostral and caudal to the lesion, and it appears as though most of the transplanted cells migrated towards the lesion, it is not surprising that the rostrocaudal spread of the transplanted cells (distance between the most rostral to the most caudal cell) is not significantly different between animals that received cells and animals that received cells + chABC. As seen in Figure 3.20, the rostrocaudal spread between the two groups that received...
cells remains similar even when cells that are 3 or 4 standard deviations away from the mean are removed.

Figure 3.19 Lesion area 9 weeks post injury: Representative sections stained with DAPI (blue) and GFAP (astrocytes in red) to outline the glial scar in (a) injury alone and (b) cells + chABC groups. (c) Quantification of lesion area shows a decrease in lesion area in groups that received cells, in comparison to groups that did not receive cells (Scale bar = 500um, mean ± standard error, Injury: n=4 Vehicle: n=4, chABC: n=4, Cells: n=3, Cells + chABC: n=4. The following statistical outliers were removed: 1 in cells group, 1 in cells + chABC group)

Figure 3.20 Rostrocaudal spread 9 weeks post-injury (a) Quantification of the maximum rostrocaudal spread in animals that received cells alone, and animals that received cells + chABC shows no difference when (a) all cells are included (b) cells located 3 standard deviations away from the mean (in terms of rostrocaudal position) are excluded or (c) cells located 4 standard deviations away from the mean (in terms of rostrocaudal position) are excluded (mean ± standard error, Cells: n=5, Cells + chABC: n=6, (excluded animals which do not have any hNUC+ cells remaining), Scale bar = 500 um, Black = transplanted cells


4 Discussion

The initial pilot study was carried out to verify that the undifferentiated cNECs would remain viable in the spinal cord, despite their cortical specification. Although the percentage of cells remaining one week after transplantation was low, viable cells were found in the injured spinal cord. Other researchers have similarly shown that forebrain derived cells can survive in other parts of the CNS. For example, Braz et al. showed that when inhibitory interneurons of the cerebral cortex were transplanted into the spinal cord, the cells not only survived but also expressed molecular and morphological properties of cortical interneurons [94]. Delivery of these inhibitory cortical interneurons to different parts of the CNS such as the striatum and hippocampus and to various disease models is further reviewed in [95].

In addition, the pilot study was carried out to determine whether cell density/dose was correlated with the number of cells remaining in the cord. Although in rat models of Parkinson’s disease increasing the number of transplanted human NPCs from 200K to 2M cells increased cell survival/engraftment, it has been shown in spinal cord injury [96] [97] and stroke [98] models that increasing the number of transplanted cells past a certain threshold does not result in increased cell numbers, but rather a plateau is reached, and the number of cells remains uniform. This is potentially due to increased cell aggregation and higher cell death when large numbers of cells are transplanted, since there is limited space for engraftment at the site of injection. In this study, there was no significant difference in terms of the number of cells remaining between animals that received two different cell doses (160K vs. 400K cells total). Therefore, the animals that received the higher cell dosage would have had substantially more dead cells in the in vivo system. Since Modo et al. previously showed that delivering dead cells to the CNS further exacerbates the injury [99], we decided to transplant cells at a density of 20K cells/μL in the subsequent combinatorial in vivo study.

4.1 In Vitro Work

Upon verifying that the cNECs are capable of surviving in the injured spinal cord, the subsequent step was to improve cell survival by modifying the hyaluronan-methyl cellulose hydrogel in which the cells were delivered. The increase in cell viability following encapsulation in HAMC in comparison to cells dispersed in aCSF after storing the cells on ice has been previously demonstrated by Ballios et al [81]. The increase in cell viability in cells in the 0.75/0.75 HAMC
in comparison to cells in aCSF after 24 hours on ice could be attributed to cryoprotective properties of hyaluronan [100], [101]. Ujihira et al. [100] previously demonstrated that increasing concentrations of HA improved membrane integrity following a freeze-thaw cycle. Mechanistically, cryoprotectants exert their protective effects either intracellularly, through inhibition of ice crystal formation or inhibition of high solute concentrations, or extracellularly, through stabilization of the cell membrane or other means [100]. Although Ujihira et al. [100] believes that the HA mediates cryoprotection intracellularly since HA was found to be endocytosed into the cells, the exact intracellular mechanism remains unknown.

In addition, during this study it was observed that the cells in the 0.75/0.75 HAMC seemed to remain dispersed throughout the HAMC for at least 6 hours on ice, while the cells in the 0.5/0.5 HAMC and cells in aCSF had fallen to the bottom of the tube after 6 hours on ice, which also may have contributed to the improved viability found in the 0.75/0.75 HAMC hydrogel.

In terms of cell survival following injection through the 32 gauge needle, the significant decrease in cell viability following injection of cells dispersed in aCSF, but not following injection following encapsulation in either the 0.5/0.5 or 0.75/0.75 HAMC hydrogel further demonstrates the benefits of delivering the cells within the hydrogel vehicle. Aguado et al. demonstrated that HUVEC (human umbilical vein endothelial cell) encapsulation in shear thinning alginate hydrogels resulted in significantly greater levels of cell survival following injection through a syringe and needle in comparison to cells dispersed in Newtonian solutions such as media. They demonstrated that extensional flow - which causes a drastic increase in velocity due to an abrupt change in flow geometry, from a syringe with a wide diameter to a needle with a small diameter - was likely responsible for cell death following injection through a needle. The alginate hydrogel protected the cells from these extensional flows by forming a layer of shear thinned fluid along the walls of the needle, thereby acting as a lubricant to enable the rest of the hydrogel to slip through the needle through a process called “plug flow” [102]. Since hyaluronan is a shear thinning material, it is possible that the hyaluronan component of HAMC protected the cells through “plug flow”.

4.2 In Vivo Behaviour & Lack of Functional Recovery

The purpose of delivering the cells in combination with chondroitinase ABC was to provide both a neuroprotective and neuroregenerative treatment strategy to overcome functional impairments
that occur as a result of a spinal cord injury. In this study, no statistically significant functional improvements (motor or sensory) were found in animals that received cells + chABC for up to 9 weeks post-injury in comparison to control groups. In fact, there were trends towards the injury alone group having the highest BBB score in comparison to the other groups from week 1 to week 9 post-injury (Note: the injury alone group only has a significantly higher BBB score in comparison to the cells alone group 2 weeks post-injury). It is unknown as to whether this trend towards the injury alone group performing better than the other groups 1 week post-injury (as assessed via the BBB score) exists because the animals in the other 4 groups received a more severe injury due to the precarious nature of the clip compression injury, or whether it is because the animals in those 4 groups received an intrathecal injection of the xMC (with or without chABC) hydrogel which further injured those animals in comparison to the injury alone group. It has been previously shown that intrathecal injections of xMC into the healthy spinal cord does not cause motor impairments [53]. Although the safety of the xMC hydrogel was not tested in the injured spinal cord, the rheological properties (shear storage modulus) as well as the swelling ratio (maximum swelling ratio of around 1.4 times the initial mass) of the xMC gel are both comparable to other HAMC formulations which have been shown to be safe for intrathecal injections into the injured spinal cord [53], [103]–[105]. Therefore, the xMC gel should not be further compressing and injuring the spinal cord.

Additionally, the xMC gel (without chABC) in [53] was prepared in aCSF at pH 7.4, while the xMC-chABC-SH3 gel used here was prepared in a phosphate buffer at pH 8, since the majority of the volume within the hydrogel was contributed by the chABC-SH3 which is prepared in a phosphate buffer at pH 8 to prevent reduction of bioactivity. Since others have demonstrated that lower pH levels in localized regions due to degradation byproducts can cause tissue damage, an increase in pH in a localized region might also cause tissue damage. Jalalvand et al. [106] found that the spinal cord has pH sensors at the central canal that provide feedback that can inhibit motor activity in response to either slightly acidic or alkaline pH. Although for this injury model, the central canal at the site of the injury is mostly damaged, so it is unknown as to whether those pH sensors would detect the change in pH. Alternatively, the increase in pH may have increased the swelling ratio of the xMC gel, which could have contributed to the compressing of the spinal cord and caused further tissue damage.
Although it is possible that the xMC-chABC-SH3 hydrogel compressed the spinal cord, the differences in BBB score after 1 week between the groups could also be attributed to inconsistent injury severities as a result of the clip compression injury model. Furthermore, since the injury, chABC, cells, and chABC + cells groups have similar percentages of animals with a BBB score of greater than or equal to 11 one week post-injury, it seems unlikely that the intrathecal xMC injection had a deleterious effect on motor function, and more likely that there were simply a few animals with a lower BBB in some groups due to some animals receiving a more severe injury. The inconsistencies of this clip compression injury model is further demonstrated by differences in BBB scores between independent studies that that used clips of similar strengths to injure the animals. For example, as shown in Table A.1, 1 week after injury, Elliott Donaghue et al., found that animals in the injury alone group had a BBB score of less than 5, while in this study, the animals in the injury alone group had a BBB score of around 9 at the same time point, where the same clip strength was used.

Although there were no significant differences or trends towards improved motor recovery in the combination group in comparison to the control groups, the analysis of the motor recovery in this study was complicated by the animals starting off with different BBB scores at the earliest time point. However, others have observed motor recovery through the co-delivery of chABC and various other cell types to different models and severities of spinal cord injury (Table 1.1). In terms of thermal hyperalgesia, Karimi-Abdolrezaee et al. [84] similarly did not observe increased or decreased sensitivities to the thermal stimulus between any of the groups, therefore concluding that thermal hyperalgesia was not exacerbated in any of the treatment groups in comparison to the control groups.

4.3 In Vivo Tissue Analysis

At both 1- and 8- weeks post-transplantation, there was a trend towards increased number of transplanted cells in animals that received cells + chABC in comparison to animals that received cells alone, although these differences were not significant. There are conflicting reports in the literature as to how chABC delivery and subsequent CSPG degradation affects survival and proliferation of transplanted cells; some have reported increases in cell numbers [84], no differences in cell numbers [87], and decreases in cell numbers [88].
However, in this study there was a substantial increase in cell number for both groups from 1 week to 8 weeks after transplantation, likely due to proliferation of transplanted cells. Surprisingly, in both the cells alone and the cells + chABC group, 8 weeks post-transplantation there were some animals that had zero cells or substantially fewer cells remaining in comparison to other animals in the same group. This was similarly seen in the pilot study, where 2 animals (out of 7) had no cells remaining one-week post-transplantation. A potential reason for this variation could be due to inconsistencies with the cell delivery, in that for an unknown reason, some animals did not receive any cells, or received substantially fewer cells initially. Alternatively, the cyclosporine-A immunosuppression could have been insufficient for some animals, or some animals could have had an increased immune response, which reduced survival of transplanted cells. Wilems et al. [88] reported that groups with reduced cell survival two weeks after transplantation (4 weeks post-injury) also had increased levels of macrophages and monocytes in comparison to groups with increased cell survival, indicating that reduced cell survival was associated with an increased immune response.

In terms of characterization of the transplanted cells 8 weeks post-transplantation, in both groups that received cells (cells alone, cells + chABC), the transplanted cells had similar percentages of Ki67 positive cells (proliferating cells), and also had similar levels of ßIII tubulin positive cells (immature neurons). This indicates that chABC did not have an effect on either the proliferative capacity of the cells, or on the cells’ differentiation into immature neurons. Wilems et al. [88] similarly demonstrated that the delivery of anti-inhibitory molecules (including chABC) did not affect progenitor motor neuron differentiation into NeuN positive cells, and Karimi-Abdolrezaee et al. [84] also showed that chABC delivery did not alter the differentiation profile of NPCs in vivo. Furthermore, in terms of the proliferative capacity of the transplanted cells in this study, although the cells seems to be proliferating quite substantially in some animals, on average 13-15% of the transplanted cells are also Ki67 positive 8 weeks after transplantation. This is similar to other studies, which have shown that 10% of the transplanted cells are Ki67 positive cells 89 days post transplantation, without any tumor formation [107]. Furthermore, many other studies have moved towards transplanting cells that are partly lineage committed and therefore have some proliferative capacity – rather than terminally differentiated, post-mitotic replacement cells – without tumor formation (Reviewed in [108]). Thus, the fact that these cells are still proliferative may not be a major concern.
Co-delivery of chABC and cells also did not affect maximum rostrocaudal spread or lesion area in comparison to animals that received cells alone. However, both groups which received cells showed a trend towards having a smaller lesion area due to propensity of DAPI positive cells (likely the transplanted cells, although they could potentially be immune cells or blood cells) to migrate towards, and into the GFAP+ lesion site. A decrease in lesion area due to the presence of transplanted cells inside lesion has been demonstrated previously [109]. Although other groups [46], [84] have shown that chABC delivery increased the maximum rostrocaudal spread in comparison to animals that did not received chABC, since cells were injected both rostral and caudal to the lesion in this study and the cells seem to be migrating towards the lesion, then it is unlikely that the cells + chABC group would have a larger rostrocaudal spread than the cells alone group. Alternatively, another method of analyzing spread which takes into consideration ventral-dorsal spread as well as rostro-caudal spread may more accurately represent the spread of the cells throughout the cord, thereby making it more likely to see a difference in migration between animals that received cell alone and animals that received cells + chABC.

4.4 Future Work

Although there were flaws in the behavioral results which complicated its analysis, the lack of differences in functional recovery between the groups can be strongly attributed to the ineffectiveness of the cells and/or chondroitinase ABC to provide neuroprotection to, or promote neuroregeneration of the injured tissue.

4.4.1 Overcome flaws with behavioural testing

A major question that arose from the behavioural data, was whether the xMC or xMC-chABC-SH3 gel was compressing the injured spinal cord, thereby causing a reduction in the BBB score 1 week post-transplantation. Prior to moving forward with the xMC hydrogel, it is recommended to intrathecally inject the hydrogel either acutely (immediately after the injury) or sub-acutely (1 week after the injury), and assess the BBB scores, as well as the tissue one week post injection to determine whether the dorsal side of spinal cord is substantially damaged in comparison to cords that receive only aCSF intrathecally. If the xMC gel is compressing the spinal cord, then the degree to which the hydrogel swells can be reduced by decreasing the crosslinker ratio because PEG is highly hygroscopic. Alternatively, the degree of compression by the hydrogel can also be reduced by decreasing the shear modulus of the hydrogel by decreasing the concentration of
methyl cellulose in the gel [53]. Furthermore, simply injecting a smaller volume of gel into the intrathecal space might relieve some of the compression, or performing a laminectomy at the adjacent vertebral levels would enable the hydrogel to spread along a longer rostrocaudal distance, which should decrease the amount of gel localized to one area, thereby reducing compression of that localized region.

Alternatively, if it is found that the xMC gel is not compressing the spinal cord, then it is recommended to refrain from treating the animals acutely (on day 0). Although there were justifications for treating the animals on day 0, the analysis of functional recovery is complicated if the animals receive injuries with varying severities. By not treating animals on day 0, the animals can be sorted into groups prior to receiving treatments, so all of the groups would have similar BBB scores at the earliest time point.

4.4.2 Cells

Despite not seeing any behavioural recovery following cell transplantation, there were some promising outcomes from the tissue analysis, such as high levels of viable cells in both of the groups that received cells, the cells have the ability to migrate towards the lesion, and the cells differentiate into neurons. There may be a number of reasons as to why the cells did not promote functional recovery: the cells did not differentiate fully into mature neurons which could integrate into host tissue to cause functional recovery, or these cells are not the ideal cell type to achieve functional recovery in spinal cord injury models potentially because they are not spinal cord-specified cells. In order to verify these hypotheses, the hNUC+ cells at the 8 week timepoint could be stained with mature neuronal markers such as MAP2 or NeuN, or more specifically for glutamatergic markers such as vGlut to determine exactly what type of neurons they differentiate into. If the cells are not yet MAP2, NeuN or vGLUT positive then in the future, it might be necessary to take the study out for longer than 8 weeks post-transplantation, and sacrifice the animals at a time point by which the cells would be fully mature. In terms of synapse formation to assess integration, the tissue can be stained with synaptophysin to assess whether synapses are being made between transplanted and host tissue. Another potential issue with delivering progeny of the cNECs, is that the cNECs differentiate primarily into excitatory glutamatergic interneurons in vitro. This could be an issue because if they differentiate into glutamatergic interneurons in vivo, they could contribute to glutamate excitotoxicity, which is an
event in the secondary injury following SCI, and results in further cell death. If glutamate excitotoxicity is an issue, then delivering inhibitory GABAergic interneurons may be a better option.

One major issue with the number of cells remaining 8 weeks after transplantation is the high variation within both of the groups. It would be beneficial to determine why there was such a stark difference in the number of cells amongst animals within the same group. In order to determine whether the animals with fewer cells is related to an increased immune response, the tissue from those animals could be stained for ED1, and all of the tissue from the 2 week timepoint could be stained for ED1 to determine whether some animals have higher levels of macrophages and monocytes even at the early timepoint, which might suggest that those animals would have a lower cell number at the later time points.

4.4.3 chABC

In terms of chABC delivery, since the level of CSPGs at 2 and 9 weeks after injury was not quantified, it is unknown as to whether chABC successfully degraded CSPGs, and whether there are less CSPGs in animals that received chABC in comparison to animals that did not receive chABC. If there is no difference in CSPG levels between animals that did and did not receive chABC in the animals that were sacrificed 2 weeks after injury, that would suggest that either not enough chABC-SH3 was delivered, or that chABC-SH3 activity was reduced following injection in vivo. Wilems et al. (in Table 1.1) found that animals that received cells + anti-inhibitory molecules (including chABC) had increased CSPG levels in comparison to animals that did not receive both cells and anti-inhibitory molecules (including chABC) together [88]. They hypothesized that either delivering the cells in combination with the anti-inhibitory molecules reduced the activity of chABC, or that the increased macrophage and monocytes in those groups reduced chABC activity, however none of these hypotheses were tested.

If there are no differences in CSPG levels between animals that did and did not receive chABC-SH3, this would mean that the chABC-SH3 was not active in vivo, which could justify the lack of functional recovery that was observed through these treatments. Although chABC-SH3 activity was verified through the kinetic assay described above, it is possible that its activity was reduced after being injected in vivo due to various cell types and molecules present following SCI. In order to determine whether chABC-SH3 activity is reduced as a result of being exposed
either to the transplanted cells or to immune cells, an *in vitro* glial scar model such as the one described by Tom et al. [110] could be developed, in which the axons are grown on laminin, until reaching an inhibitory CSPG region which they are not able to extend through, and then assessing whether chABC remains active in this environment and is able to degrade the CSPGs to promote axonal growth. This system could be made more complex by co-culturing the pre-differentiated cNECs with immune cells to determine whether the immune cells have an effect on chABC activity, as hypothesized by Wilems et al [88].

Alternatively, chABC-SH3 simply may not be diffusing through the spinal cord to the injury site. In order to test this, an *in vivo* release can be performed in which animals are sacrificed at certain time points post-chABC delivery, and ELISAs are performed on different sections to quantify the amount of chABC at certain depths throughout the cord.

However, if there are differences in CSPG levels between groups that did and did not receive chABC, that could imply that chABC activity was maintained *in vivo*, but not enough CSPGs were degraded to cause functional recovery. Rather than deliver the xMC-chABC-SH3 intrathecally, an alternative could be to inject the hydrogel directly into the injury epicentre such that the chABC-SH3 is injected directly into the environment that it needs to act upon. Otherwise, the formulation of the xMC-chABC-SH3 hydrogel could be altered such that more than 60% of the enzyme is released, as was demonstrated in the *in vitro* release in Figure 3.8.
5 Conclusions

Spinal cord injury is a debilitating injury for which there are no effective treatments. This thesis explored the sustained delivery of chondroitinase ABC from a crosslinked methyl cellulose hydrogel to degrade inhibitory CSPGs, in combination with the transplantation of a population of hIPSC derived-neuronal precursor cells (specifically pre-differentiated progeny cortically specific neuroepithelial cells which are positive for NCAM-PSA) in a 0.75/0.75 hyaluronan-methyl cellulose hydrogel into rat models of spinal cord injury. No motor or sensory functionality was recovered through delivery of this combination treatment despite high levels of transplanted cells 8 weeks post-transplantation. Characterization of the transplanted cells demonstrated that chABC delivery neither affected the proliferative capacity of the cells, nor their association with βIII tubulin positive immature neurons 8 weeks after transplantation. However, there was a trend towards decreased lesion area in the animals that received cells or cells + chABC in comparison to control animals, due to an influx of DAPI positive cells inside the lesion in those two groups. Future work involves determining why functional recovery was not achieved by delivering the cells and chABC either in combination or alone, and determining what changes can be made to achieve functional recovery in the future with this chABC and neuronal cell combination strategy.
References


[70] Varga, B.V. and Nagy, A, “Multiple signals control the full neocortical potential of human neuroepithelial stem cells,” *Press*.


**Appendix A: Comparison of BBB scores between studies which used the clip compression injury**

Table A.1: BBB Scores 1 week post-injury in the injury alone group

<table>
<thead>
<tr>
<th>Clip Strength</th>
<th>BBB Score 1 week post-injury</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>24g</td>
<td>&lt;5</td>
<td>[90]</td>
</tr>
<tr>
<td>26g</td>
<td>&lt;5</td>
<td>[111]</td>
</tr>
<tr>
<td>26g</td>
<td>9</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Appendix B: List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF</td>
<td>Artificial Cerebrospinal Fluid</td>
</tr>
<tr>
<td>AIMs</td>
<td>Anti Inhibitory Molecules</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BBB</td>
<td>Basso Beattie Bresnahan</td>
</tr>
<tr>
<td>chABC</td>
<td>Chondroitinase ABC</td>
</tr>
<tr>
<td>chABC-SH3</td>
<td>Fusion protein of Src homology 3 with chondroitinase ABC</td>
</tr>
<tr>
<td>cNEC</td>
<td>Cortically Specified Neuroepithelial cell</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CS-A</td>
<td>Chondroitin Sulfate-A</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin Sulfate Proteoglycan</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DCX</td>
<td>Double Cortin X</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N’-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DMTMM</td>
<td>4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride</td>
</tr>
<tr>
<td>DTP</td>
<td>3,3’-dithiobis(propionic dihydrazide)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinases</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth Associated Protein-43</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GRP</td>
<td>Glial Restricted Progenitor</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HAMC</td>
<td>Hyaluronan Methyl Cellulose</td>
</tr>
<tr>
<td>hIPSC</td>
<td>Human Induced Pluripotent Stem Cell</td>
</tr>
<tr>
<td>hNUC</td>
<td>Human Nuclear Antigen</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>LAR</td>
<td>Leukocyte Common Antigen-Related</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin Associated Glycoprotein</td>
</tr>
<tr>
<td>MAI</td>
<td>Myelin Associated Inhibitor</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule Associated Protein 2</td>
</tr>
<tr>
<td>MC</td>
<td>Methyl Cellulose</td>
</tr>
<tr>
<td>MC-COOH</td>
<td>Carboxylated Methyl Cellulose</td>
</tr>
<tr>
<td>MC-DTP</td>
<td>3,3’-dithiobis(propionic dihydrazide) modified methyl cellulose</td>
</tr>
<tr>
<td>MC-SH</td>
<td>Thiolated Methyl Cellulose</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>MC-WBP</td>
<td>Methylcellulose modified with weak binding peptide</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>NCAM-PSA</td>
<td>Neural Cell Adhesion Molecule-Polysialic Acid</td>
</tr>
<tr>
<td>NEP</td>
<td>Neuroepithelial cells</td>
</tr>
<tr>
<td>Ngr</td>
<td>Nogo Receptor</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-Nitritriacetic acid</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural Precursor Cell</td>
</tr>
<tr>
<td>NRP</td>
<td>Neuron Restricted Progenitors</td>
</tr>
<tr>
<td>NSPC</td>
<td>Neural Stem/Progenitor Cell</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>OPCs</td>
<td>Oligodendrocyte Precursor Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS-Tween</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(e-Caprolactone)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>pMN</td>
<td>Progenitor Motor Neuron</td>
</tr>
<tr>
<td>PNNS</td>
<td>Perineuronal Nets</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein Tyrosine Phosphatase</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho Associated Kinase</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SH</td>
<td>Thiol</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology Domain-3</td>
</tr>
<tr>
<td>T1/2</td>
<td>Thoracic 1/2 vertebral levels</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>TIPS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WBP</td>
<td>Weak binding peptide</td>
</tr>
<tr>
<td>xMC</td>
<td>Crosslinked MC</td>
</tr>
<tr>
<td>xMC-chABC-SH3</td>
<td>Crosslinked MC modified with chABC-SH3</td>
</tr>
</tbody>
</table>
**Appendix C: Contributions to this Project**

- All behavioural assays were conducted by Priya Anandakumaran, Dr. Tobias Fuehrmann and Peter Poon
- All *in vivo* surgeries were conducted by Peter Poon and Dr. Tobias Fuehrmann
- Dr. Malgosia Pakulska assisted with chABC-SH3 expression and purification
- James Parker provided the MC-WBP used for the combination *in vivo* study
- All *in vitro* cell characterization (Figure 1.2) was conducted by Samantha Payne
- All other experiments were conducted by Priya Anandakumaran