Enhanced Stem Cell Repair of Nervous Tissue: Feasibility of a PLGA Microsphere SDF-1 Dosing Transdermal Microneedle Patch

Enhanced Stem Cell Repair of Nervous Tissue:

Feasibility of a PLGA Microsphere SDF-1 Dosing Transdermal Microneedle Patch

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Abstract

Here is evaluated a strategy for stem cell repair of nervous tissue with a potential therapeutic application of a transdermal microneedle stromal-derived-factor 1 (SDF-1) chemokine delivery system. The central question of this research focuses on whether enhanced SDF-1 signaling to mesenchymal stem cells (MSCs) at injury sites promotes more effective regeneration of damaged nerves. SDF-1/CXCL12 is a potent chemokine protein known to bind to ligands CXCR4 and CXCR7, both of which are proven to drive processes involved with nervous tissue regeneration (Carbajal 2010). While it is shown that SDF-1 delivery systems can facilitate repair of nerves, many of the mechanisms behind this are still unclear (Purcell 2012). In all studies evaluated in this work, significant neuro-regenerative improvements were seen as: improved MSC chemotaxis, greater volumes of repairing cells at injury sites, and an improved quality of injury repair. Many factors and cell types are also involved in nerve repair and each injury involves a coordination of signaling and checkpoints (Jiang 2017, Sullivan 2016). This study looks closely at specific nervous tissue damage and repair systems, finding that, in all cases, SDF-1 supplementation improves neural regeneration. This study concludes that further investigation of timed-release SDF-1 in microspheres via microneedle patch, using the tested approaches described here, is warranted to understand diffusion rates, cell signaling, inflammation response, fibrosis, and bio-compatibility of such a repair system.

Dedication

This study is dedicated to those in the field of nerve regeneration and those who may experience a need for this research.

Table of Contents

Chapter I.

Introduction

This case study aims at evaluating the regenerative capability of enhanced SDF-1 signaling, and clarifying the optimal effective signaling of SDF-1 administered transdermally in PLGA microspheres. In exploring the central molecular signaling aspects involved in nerve repair, the specific aim is to evaluate the state of other known and tested methods of repair to formulate a multifaceted SDF-1 delivery strategy that will enhance function of both native and exogenous nerve repair, while avoiding harm to existing systems (Carbajal 2010).

It is shown that mesenchymal stem cells (MSC) are powerful contributors to nerve repair and do so by multiple means, both natively and through external manipulation and administration. These cells may be expanded from easily accessible tissue sources such as fat tissue and delivered back to sites of injury, either directly or after directed differentiation into other cell types, including Schwann cells and oligodendrocytes (Cai 2017, Clements 2017). It is shown that MSC can natively differentiate into neurons, repair cells via partial fusion and organelle transfer, and modulate surrounding immune cells, including the macrophages involved in debris clearance (Acquistapace 2011, Giordano-Santini 2016).

Another strategy for enhanced signaling includes low-intensity pulsed ultrasound, which has been shown to increase targeted SDF-1 stem cell homing (Wang 2018). In the

current study, the goal is to increase targeted SDF-1 stem cell homing without the complexities of added electronics, surgery, or scaffolds, attempting to mimic and enhance the natural stem cell calling process.

By understanding how nerve tissue regenerates naturally, this study might better assess the feasibility of a repair strategy for each injury type. In the case of spinal cord injury (SCI) and peripheral nerve injuries (PNI), scaffolds and gap bridges have been employed (Zhao 2017). In one specific scaffold type, easily obtainable pluripotent hair follicle stem cells were seeded on thin bio films, rolled into tubes, and inserted at the site of an induced sciatic nerve injury in a mouse model. These bridges effectively formed differentiated neurons which joined and repaired the severed nerve in mice (Yamazaki 2017). It remains to be examined if perhaps the current transdermal or a hydrogel SDF-1 delivery system at the injury site might support these invasive methods by increasing supporting factors, immunomodulation, and cell differentiation. (Cross 2011, Purcell 2012).

This study will also discuss how these same signaling systems have been implicated in cancers and how to avoid instantiating them by establishing dose limits and optimal administration protocols. Future testing with animals predisposed to cancers or with an existing condition may elucidate the process, as many studies are based only on in vitro observations (Dai 2013). Nerve signaling damage at the DNA level may not be repairable and, in fact, may be one driving force behind the tenacity of certain cancers. While the enhancement of these signals is a positive force for tissue repair in some cases, the suppression of the same signals is a requirement in cases of cancer development, and

a requirement for exhaustive testing of these new types of therapies (Boilly 2017).

This study will also show that the response to signaling repair therapies is dose dependent in generating the stem cell signaling gradient and requires tuning of time-release delivery systems (Sopko 2016). Few studies have established optimal signaling gradients for nerve repair. This study will consider functionalized PLGA microparticles of varying sizes and composition to accomplish this (Purcell 2012). Here PLGA containing SDF-1 is conjugated to site-specific antibodies to bind to cells or tissue at the site of injury and release the chemoattractant SDF-1. This approach may be combined with other stem cell, factors, scaffolds and manipulation strategies at the site of injury to enhance repair.

Significance of Nerve Repair and the Problems with Regeneration

Peripheral nerve injury (PNI) affects approximately 13 to 23 per 100,000 persons per year while spinal cord injuries (SCI) are statistically fewer at approximately 5.4 per 100,000 persons per year (Sullivan 2016). These injuries are typically permanent and devastating to those affected. In half a century of increasingly advanced surgical repairs of injured nerves, outcomes remain limited (Cooney 2016). As understanding of endogenous repair mechanisms of nervous tissue grows, much treatment focus is on enhanced regeneration with stem and other relevant cells, and also related neurotrophic, growth, and immunomodulatory factors. In terms of monetary significance, as of 2015, sales of stem-cell products were projected to surpass $600M (Hofer 2016). This funding will drive further advancements and applications to regeneration as proposed in this and

other studies. This case study will explore the theory that extended release of SDF-1 loaded in PLGA microspheres, functionalized to neuronal tissue, will enhance stem cell mediated repair of damaged nerve tissue.

The first aim is to confirm that there is potential for an increase in regenerative capability with the outlined approach. The second aim is to clarify the effective signaling times of SDF-1 administered in this way. Time is limited with endogenous repair of nerve tissue in the window of response prior to fibrosis and other limiters. In multiple studies it has been shown that the optimal window for angiogenic therapy in SCI is approximately 3 to 7 days after injury (Yu 2016). Furthermore, in SCI, it is shown that inflammation, reactive gliosis, axonal demyelination, neuronal death, and cysts formation drive a secondary injury (Cizkova 2018). Here this study seeks evidence of extended or even reactivated repair activity by maintaining a repair signal at the site of injury. It was shown in multiple studies that diffusion of SDF-1 loaded in PLGA (or hydrogel) will establish a homing gradient to bone marrow derived cells (BMC) including mesenchymal stem cells (MSC) involved in repair at the site of deposition of SDF-1 (Cross 2011, Purcell 2012).

This case study attempts to clarify issues regarding the use of PLGA in this manner. Firstly, we seek to resolve questions regarding systemic clearance and site-efficacy of PLGA. It was shown that some PLGA nanoparticles are removed from circulation rapidly and may be found accumulating in liver, bone marrow, lymph nodes, spleen and peritoneal macrophages (Sah 2013, Makadia 2011). Conversely, it is claimed that other administrations of PLGA, in larger diameters, increasing molecular weights,

and sites of lesser clearance will sustain delivery for weeks and even months (Yu 2016, Makadia 2011). Secondly, we seek to establish the efficacy of localized timed-release delivery of growth and regeneration promoting substances that would diffuse from PLGA adhered directly to the surface of cells. Ultimately, we are seeking evidence of increased potency of nerve tissue regeneration therapies by extending repair signaling time, increasing signaling strength, and improving the accuracy of repair while minimizing toxicity and any adverse or possibly carcinogenic effects.

View of Prior Studies

This case study compares numerous investigations of stem cell repair of nerve tissue. It was shown in both PNI/SCI that MSC and MSC conditioned medium/trophic factors support improved regeneration of both nerve injury types. In a direct approach MSCs are injected locally at the site of PNI in a sciatic nerve and hindlimb transplant model showing improved regeneration in both cases (Cooney 2016). Intrathecal delivery of MSC conditioned medium is also shown to improve regeneration in a rat SCI model (Cizkova 2018). However, the goal of this case study differs with its focus primarily on recruiting endogenous cells. It does not exclude data on exogenous applications, and may benefit from such studies, but the goal is to support an enhancement of endogenous repair systems via the signaling molecule SDF-1. Using endogenous cells may prove superior to external culturing as the cells are already in a naturally balanced and activated state and protected from genetic drift and other altered expression caused by extraction, culturing, expansion, storage, and reincorporation.

Enhanced repair signaling to stem cells is accomplished by creating an SDF-1 signaling gradient, as proven in works demonstrating this with both PLGA and hydrogel as a carrier. Stem cell repair of heart tissue was the primary focus in these prior studies, with systemically infused BMC counts in the mouse heart increased by 8.5 fold in the hydrogel study (Purcell 2012). Furthermore, it was shown that controlled release of SDF-1 in PLGA microspheres caused significant in vitro migration of MSC's during the diffusion period and it was claimed that such timed release of SDF-1 in PLGA can be maintained for over 50 days (Cross 2011). Again, the Cross and other studies are making claims from in vitro observations, and we will attempt to support that further time extension and tissue containment is possible by functionalizing the PLGA spheres for cell-specific targeting (Sah 2013).

There are primarily three categories of supportive studies referenced for this work: stem cell/SDF-1 modulated repair, PLGA as SDF-1/cytokine carrier, and studies that link these two categories. Numerous stem cell studies support improved regeneration by increasing activity of MSC at the site of varied tissue damage (Acquistapace 2011, Cai 2017). MSCs are known to differentiate into Schwann cells supporting myelination and axon sprouting, and MSCs modulate inflammatory conditions to promote regeneration rather than scar formation (Cooney 2016). The secretomes of MSC comprising a soluble fraction of neurotrophic factors, cytokines and other proteins, and also a vesicular fraction of microvesicles and exosomes are considered to be of potentially greater benefit on repair than the MSC cell differentiating (Cizkova 2018). This was further supported in dosing SCI rats with angiogenic PLGA microspheres

containing vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1) and basic fibroblast growth factor (bFGF) that promoted neural regeneration and motor function. Other MSC generated factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell derived neurotrophic factor (GDNF) were shown also to stimulate neurite outgrowth in vitro and nerve extension after injury in vivo (Hofer 2016). Most studies do not focus on the direct advantage of increasing MSC derived factors by increasing focused MSC numbers, but in this case study it is sought to evaluate this advantage. A recent finding demonstrates that MSC exosomes contain fibroblast stimulating factors involved in cutaneous wound healing. It may be possible to encapsulate and deliver MSC exosomes derived from cell culturing as an alternative to SDF-1. A staged or simultaneous delivery of PLGA loaded microspheres may also be warranted, as shown with BDNF and VEGF applications to promote neuronal survival (Hofer 2016).

As will be discussed in further detail, this study hypothesizes that recruiting MSC via SDF-1 gradient from PLGA microspheres, and maintaining them at an injury site for extended periods, will improve nerve repair. Additionally, the study suspects that MSC will maintain a balance of factors optimal for regeneration by amplifying the endogenous repair system milieu.

Hypotheses of This Case Study

The primary hypothesis of this paper is that continuously released SDF-1 loaded PLGA microspheres delivered transdermally via microneedle patch, in a cell-targeted

manner, will increase the duration of SDF-1 signaling to MSC at the site of nerve injury, increasing repair signaling duration and strength, and thus improve the outcome of repair. We expect that maintaining MSC mediated repair at an injury site for extended periods of weeks or months will contribute to the long term repair of serious nerve injuries. A secondary hypothesis of the case study is that MSC will orchestrate a continuous balance of appropriate factors for optimal regeneration, and that this balance will prove superior to external estimation and dosing of other factors at the damage site.

We are seeking supporting study data showing that cell-surface-adherent released factors over time might produce enhanced regenerative signaling, suggesting that maintaining a continuous controlled rate of diffusion through the skin at the site of an injury will provide superior repair results over one-time injections. Additionally, such results indicate that injured patients could sustain the damage repair signal through microneedle self-administration.

Injury of Nervous Tissue and its Endogenous Repair

For the purposes of this case study, focus is mainly on traumatic type injury to peripheral nerves. This type of injury typically results in transection of a myelinated axon and it surrounding Schwann cells. In these types of injuries superior regenerative outcomes are observed when the axon's basal lamina remains slightly intact, as the axon is able to follow this trail to regain connectivity (Brushart 2011). Peripheral nerve damage can lead to complete loss of an affected limb and transected nerves will undergo Wallerian degeneration, which is a breakdown and recycling of axon and myelin

(Matthes 2013). Wallerian degeneration is a complex series of events that stimulates the upregulation of growth factors considered to enhance regeneration of the axon. Twenty minutes after transection in axonally GFP-labeled mice, 200-300 micrometers of both proximal and distal stumps fragment abruptly, in a process termed acute axonal degeneration (AAD). Following this, and a cascade of other degeneration steps, there is a breakdown and clearance of myelin from the Schwann cell tube involving toll-like receptors and macrophages similar to intrinsic Schwann cell myelin maintenance (Brushart 2011).

Axonal transection regeneration response time is critical to the the outcome of repair (Matthes 2013); also, vascular injury may result in nerve ischemia and further injury. Endogenous repair is limited to 12-18 months due to loss of neuromuscular junction endplates and fibrosis. Repair is limited and proportional to the extent of damage, unlike other injuries that fully heal. After a year, there is a low chance of recovery and the result is typically loss of function and/or chronic pain.

Both Schwann cells and MSC play a crucial role in axonal repair and share a link in that both secrete factors involved in axonal growth. Schwann cells dedifferentiate toward a more primitive stem like cell and upregulate growth related genes for neurotrophic and transcription factors for axonal growth. This factor-rich environment can induce axonal sprouting normally at a rate of 1 to 4 mm per day (Sullivan 2016). Additionally, the MSC cell may differentiate into Schwann-like cells and fully functional myelinating Schwann cells with axon sprouting support, contributing further PNI repair effects. Despite these proven effects, MSCs have only recently been explored in a

preclinical model for PNI repair in primates (Cooney 2016).

The Nature of SDF-1

Stromal-derived-factor 1 (SDF-1) is an 8-kDa protein that provides a signal to mobilize a variety of cell types such as lymphocytes, monocytes, hematopoetic progentors, and stem cells. It is implicated in neo-angiogenesis and cardiogenesis, and considered neuroprotective in Parkinson's disease. To improve homing and engraftment of stem cells, pre-treating tissue with a local injection of SDF-1 has shown to recruit stem cells to the heart. (Purcell, 2012) Although beneficial, a problem with this approach is that the effects of such injections are quickly undone by systemic clearance (Cross 2011). To address this persistence issue approaches have involved genetic modification of MSC to over-express SDF-1, resulting in increased MSC homing and improved cardiac function in heart regeneration models. Additionally, in vivo inhibition of SDF-1 proteolysis has improved cardiac function. (Cross 2011) In this case study is an attempt to evaluate SDF-1 as a controlled, targeted, timed-release diffusion factor.

PLGA Package Delivery

Polylactide-co-glycolide (PLGA) is approved by the US Food and Drug Administration (FDA) and has a long history of safety and approval by regulatory agencies worldwide. Spherical assemblies, due to their amphiphilic nature, will self-assemble into micellular forms encapsulating substances for delivery. Surfaces of these particles may be conjugated with biotin-PEG-NH2, and targeting ligands such as

antibodies may be used to functionalize particle surfaces for tissue and cell-specific targeting (Sah 2013).

Case Study Evaluations

One intention of this case study is to evaluate the feasibility of maintaining a concentration of target-functionalized FBS-PLGA spheres at the site of cell growth that might cause enhanced cell growth and add support to the enhanced regeneration model outlined. Case study data will investigate in vitro studies of MSC homing to a signaling gradient. Furthermore, rodent studies have will be evaluated that have been effective in demonstrating the efficacy of SDF-1 mediated repair of a number of tissue damage models (Purcell 2012, Carbajal 2010).

Other case study considerations will seek to answer a number of questions regarding the limitations of this system. A goal is to prove that SDF-1 alone is sufficient to generate an optimal repair environment against other strategies involving MSC related factors and staged delivery of alternating or complementary substances within the patch or multiple unique patches. This has been demonstrated with BDNF and VEGF applications promoting neuronal survival (Wang 2011). This may clarify multifactor strategies in comparison to the more common single factor approaches observed.

Further case study evaluations will consider the stability of the prepared functionalized SDF-1-PLGA within microneedles for stability over time. We will investigate the appropriate dimensions and composition of the PLGA spheres and the ideal carrier. We will seek to establish reliability of dosing based on skin surface factors

such as temperature, pH, pressure and other skin and environmental conditions.

Other case study data may stem from multifaceted approaches involving the various tested implanted physical structures, the addition of pulsed ultrasound, or electrical stimulation (Zhao 2017, Wang 2018). Investigation of the combined effects of these strategies is warranted with potential for significant amplification of regenerative effects.

In seeking an optimal dosing and timed release strategy of PLGA, a consderation is the potential for excessive or carcinogenic signaling with this system. Studies of excessive and varied dosing may demonstrate runaway development, teratomas, or other malignancy. Timing of release rates may be an important part of dosing, as it is shown that the CXCR4 receptor is implicated in certain cancers (Dai 2013).

We may also consider if SDF-1 signaling results in inferior tissue regeneration or interferes with normal repair. It is shown that, with some age dependence, the attenuation in aged animals of the SDF-1 signal results in reduced fibrosis and improved full-thickness healing of skin (Nishiguchi 2018). This observation contradicts the basis of this study in enhanced nerve regeneration but is limited to skin tissue and may not be relevant to the nerve tissue regeneration process.

Limitations

There are a number of limitations with this study, and foremost among them are the complexities of in vivo studies and the time involved in testing them. Nerves are particularly slow to regenerate, if there is even regeneration at all. It is also not clear if

the process will interfere with or diminish the quality of endogenous repair. Targeting cells or tissue at the damage sites may prove to interfere with normal signaling processes. Studies have proven the efficacy of PLGA bound SDF-1, yet determination of a specific site-targeting antibody is not obvious (Cross 2011) . Literature analysis suggests targeting Schwann cell surface markers with functionalized PLGA but it is not known if binding to receptors proves disruptive for this application. Schwann cells are chosen as a target based on knowledge that the CNS astrocyte, another glial cell, is known to produce SDF-1 in a similar model of neural stem cell homing (Carbajal 2010). Various other surface targets affiliated with the repair process would be valuable to assay.. The end product would also need testing under real world patch delivery conditions of temperature, skin surface, and duration, and may require further material selection based on these criteria. Materials used for the patch would require testing for safety over extended use for allergic reactions, toxicity, and discomfort.

The system may require supplementary therapies including MSC supplementation or scaffolding structures. If it is found that signaling via SDF-1 delivery alone is inadequate in applications of severe nerve injury, further testing steps might involve implanted physical structures, pulsed ultrasound, electrical stimulation, and heat, all of which adds to the complexity (Zhao 2017, Wang 2018).

Lastly, there may be issues with malignant or defective cell development. Due to pushing the repair system beyond its normal limitations, unexpected results may be seen. Close observation of the newly formed structures, sequencing of regenerated cells for mutations, and long term observations of the repair areas affected will help understand

the longer term outcomes in future experiments.

Chapter II.

Case Study Results

In a CXCR4-EGFP transgenic mouse model, Dutta et al. show that exogenous intracortical administration of soluble SDF-1 promotes local endogenous SDF-1 expression leading to activation of CXCR4 for three days after administration, diminishing entirely after 7 days. However, sustaining release of SDF-1 from PLGA nanoparticles (NP) induced an extended SDF-1 expression response that persisted for seven days after administration. This enhanced SDF-1 production increased CXCR4+ cell concentrations locally and distally to the injection site for three days and adjacent to the injection site for seven days. Immunohistochemical staining demonstrated a complex response over time to cortical injections with most trends related to the injection itself and not specific AlexaFluoro-647nm SDF-1 (AFSDF-1) administration.

Studying AFSDF-1 delivery and diffusion in the Cortex, Dutta et al. use this fluorescently labeled SDF-1 with AlexaFluor-647 conjugated to its C-terminus. This tagging allowed for a useful measure of spatial distribution of the exogenous protein distinguished from endogenous SDF-1. In the case of cortical distribution, much of the labeled SDF-1 experienced short diffusion distances of approximately 400um from the site of injection due to known limited diffusion in the brain parenchyma. Because cortical extracellular matrix is comprised of negatively charged substances such as hyaluronic acid, heparan sulfate and chondroitin sulfate proteoglycans and others, the diffusion of

the highly basic SDF-1 and other such proteins (SF-1 isoelectric point ~9.6 for the a-isoform), is impeded by electrostatic interactions. This type of limited diffusion of AFSDF-1 is seen in this study, and presents a useful model of SDF-1-focusing for other areas in the body, including areas of peripheral nervous tissue damage.

A key finding by Dutta et al., showing considerable support for the current study, is that sustained release of SDF-1 induces SDF-1 expression. To clarify the effects of exogenous SDF-1 protein application, immunostaining of SDF-1 was employed to track expression across the cortex at 1,3 and 7 days after injections. Results of this tracking show AFSDF-1 NPs inducing significantly higher total SDF-1 levels vs controls. Within a day after exposure to exogenous AFSDF-1, surrounding cells initiate autocrine/paracrine signaling which increases endogenous SDF-1 expression. The half-life of SDF-1 in vivo has been reported as approximately 26 minutes when administered systemically, therefore, the significant increase in total SDF-1 levels particularly at 3 and 7 days most likely consists of endogenously produced SDF-1. This data along with the lack of positive SDF-1 immunostaining in vehicle and blank NP groups supports the concept of exogenous SDF-1 kick starting autocrine/paracrine signaling to induce endogenous SDF-1 production.

It is also shown that sustained SDF-1 release induces transient distributed CXCR4 activation and recruitment. A transgenic CXCR4-EGFP mouse enabled tracking of cellular CXCR4 activation in response to SDF-1 administration and sustained release of AFSDF-1. Quantifying the CXCR4+ cell density across the entire cortical ROI revealed a significant increase in the AFSDF-1 NP group compared to all other groups at days 1

and 3, but not at day 7. At day 3, the blank NP control initiated a significant increase in total CXCR4+ cell density over vehicle and AFSDF-1 groups. Looking closer at the distribution of the CXCR4+ cells revealed some trends in cellular response. At day 1, the AFSDF-1 NPs induced a strong and distributed CXCR4 expression compared to all other injection groups uniformly across the entire cortical region. This trend continued at day 3, however, by day 7, the only significant observation was at the most proximal region within 400 μm of the injection tract. Both the AFSDF-1 bolus and blank NP injections induced a CXCR4 response within 400 μm of the injection compared to vehicle control at days 1 and 3. However, at day 7 the blank NP group remained higher than vehicle and AFSDF injections.

Heavily populated CXCR4+ cells were associated with the needle tract at day 1 post injection suggesting that exogenous AFSDF-1 induced CXCR4 activation and strong CXCR4+ cell recruitment, agreeing with other studies. Sustained release of AFSDF-1 induced greater total CXCR4+ cell populations compared to injected AFSDF-1 that was more distributed across the cortex. Regarding how exogenous SDF-1, with minimal direct diffusion into the brain, affected cellular activation at such large distances, it is thought that slow release of exogenous SDF-1 in the NP group propagated the endogenous signaling axis much further than SDF-1 can physically diffuse.

These results of Dutta et al. and previous studies with mesenchymal stem cells (MSCs) support this idea as MSCs treated with SDF-1 upregulated SDF-1 mRNA. As opposed to AFSDF-1 injection, where the entire dose is bioavailable immediately, and subject to degradation proteolytically, the sustained release group is expected to release

AFSDF-1 in a more controlled manner even in the first 24 hours.

Apart from autocrine/paracrine signaling it is also possible that other soluble factors such as vascular endothelial growth factor, VEGF, basic fibroblast growth factor, bFGF, immune modulators, and other factors are known to influence the SDF-1/CXCR4 signaling axis. The VEGF involvement with SDF-1/CXCR4 signaling shows that SDF-1 treatment of MSCs increases VEGF secretion in vitro, and studies show that VEGF upregulates SDF-1 and CXCR4. VEGF and SDF-1 overlap in their gene regulation through hypoxia-inducible factor-1 and endothelial cells express both proteins simultaneously in a hypoxic environment. Regarding bFGF, it is also shown that CXCR4 and SDF-1 expression increases in CXCR4+ endothelial cells after bFGF exposure.

Both VEGF and bFGF appear to increase CXCR4 expression only, and do not alter expression of CXC or CC chemokine receptors, suggesting that SDF-1, VEGF and bFGF are involved in a positive feedback loop promoting SDF-1/CXCR4 signaling. The immunomodulators tumor necrosis factor-a (TNF-a) and interleukin 1-ß (IL-1ß) also modify the SDF-1/CXCR4 signaling axis by inducing release of VEGF and/or bFGF. TNF-a has also been shown to have two-phase effect on CXCR4 expression as CXCR4 expression is downregulated first within 3 hrs, and then upregulated after 24 hrs. Therefore, it seems that secondary signaling may play a significant role in the propagation of SDF-1/CXCR4 signaling to the distal regions of the cortex. An additional activation process of distal cells may rely on exogenous SDF-1 bypassing diffusion through the brain in favor of the cerebrovasculature. Although unclear whether intracortical injections lead to breakage of the blood-brain barrier (BBB), nervous system

injuries such as focal TBI have been shown to cause BBB dysfunction. In the Dutta et al. study, distribution of AFSDF-1 and/or NPs systemically into circulation due to a leaky vasculature may permit an alternate means of distribution and interaction with endothelial cells of the BBB in distal areas of the brain. As endothelial cells densely express both SDF-1 and CXCR4 they may generate signals with a directly or indirect affect on endogenous SDF-1 expression in distal regions of the brain.

Dutta et al. established an array of immunohistological staining to elucidate the important cellular participants in the SDF-1/CXR4 signaling axis with certain phenotypic markers probed such as astrocytes (glial fibrillary acidic protein; GFAP), neuronal committed progenitor cells/neuroblasts (doublecortin; Dcx), immature neural progenitors (nestin), and microglia/macrophages (ionized calcium-binding adapter molecule 1; Iba-1). Although Dutta et al. probed for several specific cellular phenotypes, they did not observe significant colocalization of phenotype markers with CXCR4+ cells, and did not record such data. Dutta et al. did perform staining that revealed a complex series of cellular responses that suggested further observation beyond the scope of their study. A rough initial approach involved a discussion of immunostain images focused on their injections signaling the involvement of NPSCs from the SVZ. They stained for Dcx to probe for committed neuronal progenitors/migrating neuroblasts. Evidence of Dcx+ staining was visually recorded near the SVZ at day 1 following injection for all available groups. These results suggested that the injury sustained from the injection may have impacted neuroblast migration, but it is unclear based on the lack of controls used.

Recent studies recorded robust Dcx+ cellular recruitment following AFSDF-1

bolus or NP injections but it was observed that very limited or no Dcx+ cells focused near the injection tract by day 7 across all groups. Extreme observational differences in results of the Dutta et al. study versus two other studies may have been attributed to administration of SDF-1 in conjunction with a traumatic brain injury model and that the amount of SDF-1 delivered in the bolus injection was two orders of magnitude larger in the other studies. Also the stroke and brain injury literature observe that the presence of Dcx+ cells to a site of neural injury is nearly 2-4 weeks post injury.

Dutta et al. further qualified the activity of neural progenitor cells using nestin staining, knowing that nestin expression is not limited to neural progenitors implicated in neurogenesis, but also astrogliogenesis. With a focus on the most outstanding response at day 7, nestin+ cells appear adjacent and within the injection tract in all test cases, and no co-localization of nestin with CXCR4+ cells was recorded in any of the groups. Furthermore, they probed for the astrocytic marker GFAP against the nestin staining pattern. GFAP staining showed a distinct pattern over the 7-day period for all groups. At day 1, GFAP+ stain was observed clearly in the cortical area and corpus callosum below the injection site associated with the SVZ. By day 3 and 7, the GFAP+ signal grew to encompass the cortical region around the injection. The sequence of GFAP staining suggests that both local resident astrocytes in addition to newly instantiated astrocytes originating from the SVZ are continuously recruited in response to the injury created by the injection independent of injection group. Additionally, while they couldn't rule out a possible subset of cells co-expressing GFAP/nestin, the GFAP+ staining at day 7 was notably adjacent to the injection tract while nestin+ cells were also seen within the

injection tract. These results all support the notion that
cortical injury solely by the injection stimulates a strong astrogliogenesis and slight neurogenesis.

Finally, Dutta et al. probed inflammation activity with Iba-1 to characterize the activity of activated microglial and systemic macrophages. The most significant observation here was that the injection initiated substantial microglial/macrophage activity localized to the injection site by day 7. Subtle differences within the NP groups suggest further study as to the contribution of NP activating an inflammatory response versus the SDF-1. Prior studies have shown that PLGA microparticles implanted in the striatum are engulfed by activated microglia/macrophages as soon as 1 day post-injection. Since activated microglia and infiltrating macrophages utilize the SDF-1/CXCR4 signaling cascade, the EGFPCXCR4+ signal seen near the NP implants is a potential indication of a systemic host response.

Other types of CNS resident cells such as mature neurons, oligodendrocytes, and endothelial cells of the BBB are not only CXCR4+, but have been shown to regulate CXCR4 expression when exposed to SDF-1. Dutta et al. suggest that further studies should include probing of these phenotypes to characterize their involvement with this SDF-1 signaling environment. Further, they suggest that supportive in vitro studies will help clarify how SDF-1, CXCR4 and possibly CXCR7 expression relates to sustained signaling of SDF-1. Additionally, the authors suggest investigating whether desensitizing to SDF-1 plays a significant role as a result of sustained release. It remains to be shown if other release profiles such as non-linear or pulsatile are a better model for regulating the

SDF-1/CXCR4 signaling axis in the weeklong period tested, or other periods that might be employed (Dutta 2017).

In the Thevenot et al. study, homing assays were made of the capability of SDF-1a to initiate chemotaxis in vitro, showing that SDF-1a release from a PLGA scaffold caused cell migration across a transwell membrane. It was found that the increase and interval of SDF-1a correlated to the quantity of MSC migrating. Additionally, to measure this response the SDF-1a releasing scaffolds were monitored for cell adherence using CFDA-SE cell tracing. MSC were observed to cross the membrane, engrafting to the PLGA salt-leached scaffolds and establishing an adherent morphology to the scaffold.

The effects of SDF-1a on stem cell recruitment in vivo were also measured to determine whether scaffold treatment with SDF-1-a could result in changes in stem cell response to subcutaneous scaffold implants. The authors show that, after implantation for one week, SDF-1a loaded scaffolds were shown to attract a significantly larger number of MSC compared to control scaffolds. The density of MSC around SDF-1 loaded scaffolds was about 3 times greater than the density of MSC around control scaffolds at both 3 and 7 day time intervals. It was also found that, in addition to surface density, there was a 2 times greater density of MSC within the matrix of SDF-1a loaded scaffolds versus control scaffolds at day 7.

An in vivo imaging study with NIR labeled MSC was also employed to observe whether SDF-1a release was efficient at recruiting MSC from circulation. At 48 hours post injection (3 days post implantation), SDF-1-1a loaded scaffolds attracted and engrafted MSCs supplemented via tail vein injection as seen from the co-localization of

NIR fluorescent signal at the scaffold implant site.

Since the SDF-1-a supplementation enhanced MSC response, and knowing the potential for an SDF-1a related HSC chemotaxis response, there were also assays done of the recruitment of c-kit+ HSC to the scaffold implantation site using fluorescent IHC. As with the MSC response, Thevenot et al., observed a progressive increase in c-kit+ cell density through the progression of the implantation study. Measurement of the c-kit+ responses based on cell density shows a marked increase at timings at 3 days and 7 days in comparison to unloaded PLGA control scaffolds. Furthermore, the density of typical HSC markers CD34 and Sca-1 were evaluated between treatment groups and control groups. In agreement with the c-kit+ results, the authors observed a substantial increase in week 1 of of the density of both CD34 and Sca-1 positive cells compared to controls. Assays revealed more than double the increase of c-kit+ cells at identical time points to controls.

Thevenot et al. further examined interactions related to inflammatory responses. Due to the current understanding that mast cells are involved in the inflammatory response triggered by foreign body reactions, it was considered by the authors that mast cell reactions may be affected by SDF-1a supplementation. To test this, the authors measured the densities of mast cells surrounding both scaffold groups at day 3 which is the point at which the first significant increase in MSCs occurs. It was found that control scaffolds initiated the recruiting and degranulating of a significant quantity of mast cells. It was also found that SDF-1a loaded scaffolds recruited substantially lesser numbers of mast cells of a mostly non-activated nature compared with controls. An assay of the

density in the tissue reaction showed an approximately 84% decrease in mast cell density at the SDF-1a treated implants. Further evaluation of mast cell responses in the short-term release implants showed significant differences in the mast cell response for up to 6 weeks, though less significant than at day 3.

According to Thevenot et al., inflammatory cells are a major part of the fibrotic capsule surrounding biomaterial implants and their involvement is likely connected to mast cell activation and therefore it would seem that the stem cell and mast cell responses might reduce the accumulation of CD11b+ inflammatory cells at the implant site. They found that control scaffold implants had a thickly populated band of CD11b+ cells, while SDF-1a loaded scaffold implants had a significantly reduced density of inflammatory cells. By measuring cell density, it was found that SDF-1a loaded scaffold implants had a very low influence on inflammatory cell recruitment at day 3. However, it was found that SDF-1a supplementation greatly reduced inflammatory cell density by approximately 3-fold less than control scaffold implants after one week of implantation.

Inflammatory cells such as cd11b+ macrophages are involved in the granulation of tissue and subsequent fibroblast activity, and therefore Thevenot et al. examined the effect of SDF-1a on tissue responses at the scaffold implant at 1 week. It was found that SDF-1a loaded scaffolds induce significantly less inflammatory cell infiltration and granulation tissue formation than controls at day 7. These SDF-1a loaded scaffolds reduce the thickness of the inflammatory infiltrate by about 2-fold less and decrease the cell density by about approximately 1-fold compared to control implants. Cell density measurements from imaging quantified the composition of cells at the interface between

the treatment groups. The scaffold interface of control scaffolds at week 1 was composed of approximately 75% inflammatory CD11b+ cells and fibroblasts, which were identified by by their spindle shape and lack of positive stain for specific markers. Conversely, scaffold implants treated with SDF-1a prior to implantation resulted in an interface with 75% MSC and HSC composition at week 1.

An assessment of alternative SDF-1a supplementation strategies included downstream delivery during the period just prior to elevated stem cell numbers at the implantation site. It was hypothesised that physical adsorption leads to early burst release, thus untreated scaffolds were implanted for 3 days followed by a 100uL SDF-1a solution at 100ng/mL injected at the center of the scaffold in vivo.

Analysis of explants at day 7 showed unexpectedly that delayed delivery leads to an intermediate response compared to control and scaffolds treated with SDF-1a before implantation. SDF-1a supplementation at day 0 and day 3 showed a significant increase in MSC engraftment at the scaffold interface versus non-treated, but differences between SDF-1a treatment intervals was not significant. However,the delayed chemokine administration until day 3 resulted in an intermediate inflammatory response by CD11b+ inflammatory cells, substantially less than controls but greater than treatment prior to implantation.

Thevenot et al. further examined the effects of sustained delivery of SDF-1a using mini-osmotic pumps with either saline as control or delivering SDF-1a at 100ng/mL for 2 weeks. As with pre-implantation loading there was an altered mast cell response seen at the scaffold implants after 2 weeks. Control scaffolds without SDF-1a were surrounded

by degranulated mast cells, however, the SDF-1a scaffold implants had a reduction of total mast cells and a greatly reduced number of degranulated mast cells. Such differences were quantified as a 59% decrease in mast cell response and a 75% decrease in degranulated mast cells at day 14 measured against control scaffold implants.

Due to current theories on biomaterial-related inflammatory responses suggesting the long term effect of macrophages reacting on foreign substances, Thevenot et al. used antibodies specific to mouse macrophages as an added comparison to the more generic CD11b+ marker which might include other cell types, possibly neutrophils. As shown in other mini-osmotioc pump experiments focused on inflammatory cell quantifications, the density of the CD11b+ cells at the implant interfaces is much greater in the control scaffold than with the SDF-1a mini-osmotic pumps. As was expected, the same trend is seen with macrophages, as control scaffolds have a greater density of macrophages focused at the implants compared to SDF-1a scaffolds, which have a lower density and a macrophage cell distribution limited to smaller pockets along the skin side of the scaffold interface.

Further examinations involved the relationship between MSC responses and CD11b+ inflammatory cell responses. It was seen that the engraftment of MSC was substantially higher in SDF-1a treated scaffolds versus controls and the same stem cell to inflammatory cell relationship was seen in the mini-osmotic pump implant experiments in which MSC density is increased while inflammatory cell density is decreased in comparison with controls at 2 weeks. Additionally, a comparison of the inflammatory cell density between saline control and SDF-1a mini-osmotic pumps shows a substantial

reduction in comparison to untreated controls.

Granulation tissue in control implants was observed histologically by Thevenot et al., showing a multi-cell thick granulocyte and fibroblast layer forming at the junction of implants and surrounding tissue. Unlike controls, the SDF-1a mini-osmotic pump implants had a reduced cell density and less structured tissue capsule around the implants. The authors theorized that the modified inflammatory cell processes at the interface might have affected the deposition of collagen around the implants at 2 weeks as part of the initialization of the fibrotic response. Control scaffolds showed a familiar 2 week fibrotic response with a thick collagen layer deposited around the scaffold implants. Unlike controls, SDF-1a treated scaffolds had a very thin collagen layer formation and it was found that a 35% reduced capsule thickness formed and a 60% reduced capsular cell density formed in SDF-1a mini-osmotic pump connected implants.

At 2 weeks, control implants showed a thick encapsulating layer of cells as a component of developing fibrotic tissue around the scaffold implants. As opposed to this, the SDF-1a mini-osmotic pump implants had none of the multi-cell thick granulation tissue, yet had many vessels organized through the interface of native tissue and the scaffold. This formation of vessels at the interface at 2 weeks was less apparent in control scaffolds. In the field of tissue engineering, one aim in biomaterial scaffold integration is the inducement of vessel formation beyond just the interface but also within the scaffold to promote cell migration and mineralization. To quantify this ability of intra-scaffold agiogenesis, some staining experiments were employed. Sections of tissue were stained with CD31 to verify endothelial cell phenotype, and SDF-1a pump samples were

compared to controls. The SDF-1a mini-osmotic pump samples were shown to have over a 3-fold increase in the number of vessels within the matrix of the scaffold as well as deeper penetration of vessels compared to control scaffolds which had an uneven vessel distribution that formed mostly at the edges of the scaffold.

The differences in CD11b+ cell responses and macrophage responses are possibly due to a variance in macrophage activation state, causing macrophage activity in differing aspects of wound regeneration. Evidence has shown recently that marrow-derived macrophages contribute to angiogenesis and lymphogenesis at the sites of inflammation. Therefore, studies looked at the possible role of marrow-derived macrophages in scaffold-related tissue responses with co-expression of CD11b+ VEGFR-1+ mononuclear myeloid cells having the ability to induce angiogensis, and CD11b+ LYVE-1+ macrophages that contribute to lymphangiogensis in regenerating tissue. It was observed that there was minimal contribution from CD11b+ VEGFR-1+ mononuclear myeloid cells in control implants, but these cells were identified in SDF-1a treated implants in the location of budding vessels. Also, few CD11b+ LYVE-1+ macrophages were seen in control scaffolds, but were seen expressed at specific locations throughout the SDF-1a treated implant interface. This would suggest that inflammatory cells have been activated at integrating host tissue with the treated implant to a much greater degree than what is seen in untreated implants.

With the pluripotent cell marker expression of the host stem cells engrafted at the interface it was theorized that these pluripotent cells might be involved in the budding vasculature observed at the interface and with the scaffold implants. SDF-1a pump

implants at 2 weeks demonstrated superior angiogensis in histological observations as well as improved endothelial progenitor cell engraftment. Within both a tissue cross section and at the interface of SDF-1a scaffolds the authors observed vessel-like structures with cells positive for CD34+CD133+ which indicates the phenotype of endothelial progenitor cells.

Lastly, Thevenot et al. examined the effects of SDF-1a on the inflammatory cytokine profile. As numerous other studies suggest a complex interplay between inflammatory cells, fibroblasts, granulation tissue and fibrosis responses, and SDF-1a has been observed to modify these processes, the authors used protein micro-arrays to characterize the local ctyokine/chemokine environment. In support of their histological observations, it was found that the presence of SDF-1a reduced the generation of various inflammatory cytokines and chemokines such as the interleukins, leptin, selectin, lymphoactin and TNF-a. The downregulation of some specific interleukins was found to support altered mast cell responses. SDF-1a was also shown to substantially increase certain inflammatory cytokines including GCSF and GM-CSF. SDF-1a had a marked influence on angiogenic factors including IGFBP-3, VEGF, and GM-CSF.

Supplementation of SDF-1a was also shown to lower production of pro-fibrotic cytokines including IL-13 and CXCL4. SDF-1a also seems to have a strong influence on many other factors related to stem cell migration and homing including G-CSF, VEGF, and CXCL16. The authors found an unexpectedly low level, less than double in volume, in tissue and scaffold infiltrating cell expression for SDF-1a in SDF-1a-loaded scaffold implants versus controls. Most significant in the protein micro-array assays were

observations of uniform decreases in cytokines attributed to macrophage activation in SDF-1a scaffolds such as CD40, TNF-a, IFN-g, and interleukins (Thevenot 2010).

In Wang et al., the activity of SDF-1 in PLGA scaffolds was evaluated as an accelerant to cartilage defect repair by recruitment of MSC and promotion of cell differentiation. Results of their study show that SDF-1 recruits BMSCs through the SDF-1/CXCR4 axis, showing that the receptor CXCR4 was highly expressed on the bone marrow stromal cell (BMSC) surface. Using RT-PCR to measure the relative mRNA expression of cartilage markers AGG and Col-II, the authors examined the effects of SDF-1 on differentiation of BMSCs into chondrocytes. The mRNA level of Agg and Col-II in the SDF-1 group was substantially increased compared with controls, and, after 3 and 6 days in coculture of SDF-1 and BMSCs, the level of AGG and Coll-II in the SDF-1 group was markedly increased. Western blot assay demonstrated that an increased level of Col-II protein was found in the SDF-1 treated group compared to controls. Additionally, blocking of the SDF-1/CXCR4 pathway by AMD3100 decreased levels of Coll-II. BrdU labeling was used to measure the numbers of BMSCs that were seen with a scanning electron microscope as strongly attached to the surface of PLGA scaffolds after coculture of BMSCs with PLGA. IHC staining in vivo results showed that the numbers of migrating BMSCs labeled with BrdU was substantially increased in the SDF-1 treated group versus controls.

Wang et al., furthermore showed that SDF-1 promoted cartilage repair in vivo using the ICRS histological scoring technique. A higher ICRS score was associated with the scaffolds loaded with SDF-1. The cartilage defect in animals treated with

PLGA/SDF-1 started to repair cartilage tissue approximately 4 weeks after surgery compared with controls, with repairing continuing to improve over time. By 12 weeks the cartilage defect was completely refilled. In controls and tests with the SDF-1/CXCR4 pathway disruptor AMD3100, inferior regeneration was observed that included defect regions refilled with translucent tissue having a cartilage surface that was rough and the damage boundary outlined and distinct (Wang 2017).

Matthes et al., investigated intravenous transplantation of MSCs to improve peripheral nerve regeneration on rat sciatic nerves. Axons were transected by nerve crush and set at the piriformis tendon in the thigh. Cultured MSC's showed typical flat adherent phase fibroblast-like morphology after cell attachment and non-adherent cell removal. MSCs were immunopositive for markers CD90, CD44 and Stro-1 and immunonegative for hematopoietic stem cell markers CD34 and CD45. Before transplantation, MSCs were detached from culture flasks and suspended in serum free medium and labeled with PKH26 for cell tracing in vivo post-injection. Intravenous cell injections were administered via femoral vein. Three weeks after lesion and systemic cell delivery nerves were removed and prepared for histological examination. Both experiment groups showed an increase in axonal regeneration and functional outcome, but the MSC transplant group had greater numbers of axons and more axons proximal, within, and distal to the repair site, as shown in both high and low power images. Frozen sections of nerve had PkH26-labeled MSCs within the regenerated peripheral nerve after systemic delivery a short time after lesion induction showing the homing effect of MSC to the peripheral nerve lesion site. The MSCs survived in the lesion site and migrated

longitudinally across the site in both proximal and distal orientations with the generated axons. The extended distribution of the transplanted PKH-labeled MSCs shows an improved homing effect versus controls into the lesioned peripheral nerve and regenerated nerve fibers.

Matthes et al., further recorded the sciatic nerve functional index (SFI) observed pre-lesion (day 0), and at 7 days after nerve crush, cell injection of both MSC and fibroblasts, and control with media infusion. The SFI score is zero for normal animals and negative for impaired nerves. At day 7 the MSC group had a greater improvement in function than control groups with fibroblast and media injection. Substantial improvement in locomotor function was observed at 14 days for the MSC group with a nearly 2-fold improvement in SFI score and this improvement maintained at 21 days as measured, and potentially further. The functional improvement in the MSC group was substantial at 7 days but the improvement rate flattened across all groups from 14 to 21 days demonstrating the early effect of MSC intervention (Matthes 2013).

Cooney et al. characterized the immunomodulatory effects of MSCs used for regeneration with flow cytometry and culturing in cell specific media, demonstrating that their isolated MSC sample had chondrogenic, osteogenic and adipogenic properties. A sciatic nerve transection and repair model was then used, recording muscle action potentials at the level of the foot, following sciatic nerve transection and repair. Immediately after transection all signal was lost, and all animals regained detectable signals over the next 6 to 16 weeks. Local and systemic MSC treatment produced significantly higher signals at weeks 8 and 12 versus controls. By 16 weeks, control

animals regained levels similar to the MSC supplement groups. Latency measurements across all groups were not found to be significant, and latency tended to decrease over time consistently across all groups. The average fiber count and density at a point 5 to 8 mm distal to the nerve coaptation site was measured to quantify nerve regeneration and these assays used standard nerve histomorphometry techniques. The studies showed that by week 16 both local and systemic MSC treatment groups had greater average axon counts per nerve versus controls. The local MSC group showed a trend toward much greater axon density versus controls, and similar results were observed when measuring axon and nerve fiber diameter.

A software gait-based analysis system (CatWalk) was used by the authors to evaluate physical differences but did not find any statistical significance between groups due to high variability in individual animal posture and paw position.

Cooney et al. further characterized regeneration based on a syngenic hindlimb transplantation model. Building on their results from the sciatic nerve transection groups they sought to detect a similar effect in animals that had undergone hindlimb transplantation. A syngenic transplant model was used to isolate the effect of MSCs without an alloimmune response. In electrophysiology testing rats that had undergone syngenic hindlimb transplants experienced recovery graphs with shapes resembling those of the sciatic nerve repairs. However, nerve histomorphometry data did not find any significant differences in axonal counts, and density for local or systemic treatment groups versus controls. Similar to sciatic nerve assessments, the gait-analysis software also did not find detectable functional differences .

Yu et al. explore the use of angiogenic microspheres to improve neural regeneration and motor function in a spinal cord injury model in rats. PLGA microspheres were used with a sustained release of angiogenic factors both in vitro and in vivo. In vitro assays showed that VEGF, Ang-1, and bFGF were released from the microspheres in a two phase pattern with a release rate increasing rapidly during days 6 through day 10, then keeping near constant over a longer phase, tapering off beginning around day 48. These factors remained biologically active after release even 2 months after in vitro release. Serial confocal microscopy showed that FITC labeled BSA microspheres had released their contents around the focus of a spinal cord injury at 3 days post-injury. At 2 to 8 weeks post-injury, ELISA showed that levels of VEGF, Ang-1, and bFGF in spinal cord tissues were substantially greater in animals supplemented with angiogenic microsopheres than in animals supplemented with empty microspheres. Supporting this result, the numbers of isolectin B4-binding vessels at the injury site at week 4 and week 8 were substantially larger in animals supplemented with angiogenic microspheres versus control animals.

Animals supplemented with angiogenic microspheres had a much greater number of cells positive for nestin or beta-3 tubulin at the site of injury, and most of these cells were located at blood vessels. These findings suggest that the angiogenic microspheres promoted angiogenesis and neural precursor cell signaling to the injury site in a rat model. Cell proliferation at the injury site was seen using immunofluorescence staining to verify the improved spinal regeneration from functional vessels.

Yu et al. further examined whether angiongenic microspheres increased

angiogenesis and neural precursor recruitment at the spinal cord injury site and were promoting enhanced neural regeneration. Immunofluorescence microscopy showed a greater density of neurofilament-positive fibers in the injured spinal cords of angiogenic microsphere treated animals than animals treated with empty microspheres. At 8 weeks post spinal cord injury, it was shown by tissue analysis that angiogenic microspheres promoted massive neurofilament-positive fibers aligned with and around blood vessels and traversing occasionally into the center of the injury. In addition, serotonergic fibers were tightly coupled to blood vessels and were substantially longer than control animal tissues. Counts of MBP-positive mature oligodendrocytes where substantially greater in animals treated with angiogenic microspheres, and the cells accumulated around blood vessels in the area of white matter.

Yu et al. next looked into myelination of the spinal cord near the center of the injury. Staining of tissue from animals treated with either angiogenic or empty microspheres shower that at 8 weeks post spinal cord injury white matter volume gradually decreased toward the center of the injury. However, white matter content was substantially greater in animals treated with angiogenic microspheres, and these animals also displayed a smaller cavity volume in the spinal cord than control animals.

The authors also showed with electron microscopy of tissues taken at 12 weeks post spinal cord injury that angiogenic microsphere treated animals showed that most axons were myelinated and followed blood vessels. However, the control animals had axons in tissue that were mostly demyelinated. In treated animals, blood vessels were plentiful at the injection site and adjacent to the lesion, and even formed a network. Some

astrocytes showed cytoplasmic extensions making contact with nearby blood vessels and other cells. It was also shown that angiogenic microspheres increased the speed of neurologic recovery. At 12 weeks after spinal cord injury more fibers were continuous and traveling through the injury center in treated animals than in control animals. Recovery from complete paralysis was evident in both groups, but significantly faster in animals receiving angiogenic microspheres (Yu 2016).

In Cross et al., PLGA-SDF-1 microspheres were studied in a variety of formulas and assayed for their ability to signal to MSCs. Some microsphere fabrication and release studies were initially done to encapsulate SDF-1α into PLGA using a double-emulsion solvent extraction/evaporation technique and were observed for the effects of formulation variables of PLGA end-group, solvent volumes, the use of an excipient, and sonication temperature on the controlling release of SDF-1α.

Seven microsphere formulations were established using a low feed mass of SDF-1α in formulations, partly due to the cost of the protein, but also that SDF-1α is a potent chemokine. The range of SDF-1α concentrations studied in in vitro chemotaxis assays causing significant cell migration are commonly in the range of 1–200 ng/mL, depending on cell type.

Release kinetics of all batches of each of the seven formulas showed good consistency among the batches. As was expected, SDF-1α in release samples from empty microspheres was not detected by ELISA. Release curves showed a three-phase shape characteristic of PLGA, with a high initial burst on days 1–2, followed by a plateau with very little protein release at ~days 5–30, followed by second release phase related to bulk

degradation of the polymer after ~day 35.

Formulation A released the greatest total percent of SDF-1α (64–87%, depending on batch). Formulations B and G released a moderate quantity of SDF-1α (~30% and ~40%, respectively), and Formulations C-E released very little SDF-1α (less than 2%). As Batch-to-batch variability showed that the authors' microsphere fabrication process was robust and reproducible. SDF-1α was shown released over a time period extending more than 50 days for Formulations A, B, and G.

Encapsulation efficiency (EE) was demonstrated using dichloromethane/ DPBS extraction, then an evaluation of the extracts were performed by ELISA. Measured EE values were much less than the total amount of SDF-1α released, in that, EE analysis showed only 9% of SDF-1α was loaded into the Formulation A microspheres, but the release curve shows that nearly 90% of the potential loading was released by the time the microspheres reached complete degradation.

The authors attempted a complete microsphere digestion using other means, both with a mild acid/surfactant method, and by a strong acid method, and a subsequent protein assay for amino groups. However, they were not able to detect SDF-1α in the microsphere degradation samples using these polymer dissolution methods because the quantity of released protein was below the measurable limit of the assay. It was clarified that detection of SDF-1α by ELISA after acid digestion would be impossible due to complete degradation of SDF-1α into singular amino acids.

The authors then chose to determine microsphere attributes using optical microscopy to observe the microspheres in a hydrated state. Brightfield images of

microspheres from the fabrication processes showed that Formulations A and F yielded microsphere populations showing a mixture of dark microspheres and translucent microspheres. In contrast, Formulations C–E yielded microsphere populations of homogeneous opacity, with the entire population being either dark or translucent. Formulation G produced microspheres having a dense layer at the outer surface and an inner translucent core. Formulations B, C, and D had a rougher and more porous texture than all other formulations.

Diameter measurements for all microsphere formulations were obtained. There were wide distributions in microshphere size within each formulation. Formulation A had the largest microspheres at 22.5±8.2 μm. Formulations C and D were the smallest with an average of ~5 μm. All other microsphere formulations B, E, F, and G had diameters in the mid-range of ~10–17 μm.

Cross et al. then explored the bioactivity of released SDF-1α in an in vitro assay. Their proposed ideal in vitro experiment for testing the bioactivity of the chemokine-containing microspheres would be exposing cells to microspheres for the whole duration of microsphere degradation. However, cells would not be allowed to be in culture for the ~70 days it would take for the microspheres to fully degrade. Instead, the authors exposed cells to release samples from different time points, the initial burst phase, at day 1 early in degradation, or, the second release phase collected after day 35, later in the degradation process.

Prior to testing release samples, the authors conducted an investigation of the control conditions to prove that the MSCs were responsive to the chemoattractant SDF-

1α. Used a Transwell migration assay popular in current literature they tested the bioactivity of SDF-1α released from the microspheres. In the assay, cells were placed in the upper chamber of the plate, while test samples were placed into the bottom chamber. The SDF-1α should then cause chemotaxis of MSCs from the upper to lower chamber, if bioactive. At the end of the migration period, non-migrated cells were deleted from the upper chamber with a cotton swab.

It was hypothesized by the authors that pre-stimulation with tumor necrosis factor-alpha (TNFα), a pro-inflammatory molecule, will heighten the migration response of MSCs. This idea was tested by exposing the MSCs to test samples with or without pre-treatment with 1 ng/mL TNFα. Test samples consisted of positive and negative controls (30% FBS in MM or MM only, respectively), with different concentrations of SDF-1α. The authors found that regardless of whether the MSCs were left unstimulated or subjected to TNFα, many cells migrated in response to the positive control, and had a healthy spindle-like appearance. It was also shown that there was no difference in cell morphology between unstimulated and TNFα-stimulated cells. Few cells migrated to the negative control, regardless of TNFα supplementation. Because SDF-1α samples were diluted into migration medium that did not contain serum, the cells subjected to these conditions were round in shape but the cells were alive, since they fluoresced with GFP. Cells migrated to the source of SDF-1α in a dose-dependent manner and it was observed that for each concentration of SDF-1α that MSCs migrated in larger numbers when pre-stimulated with TNFα.

Due to the increased migration of MSCs after TNFα stimulation, the authors

chose to pre-treat the cells with TNFα for all subsequent studies. Due to the variation in biological activity with each experiment, they included testing of different concentrations of SDF-1α with the negative and positive controls for every assay.

MSCs that were exposed to SDF-1α release samples from Formulation A microspheres had representative fluorescence images shown before removing the non-migrated cells from the top of the transwell insert. 'Early release' samples did not appear to cause cell death, as evidenced by bright, green cells. However, when MSCs were exposed to samples of the second release phase 'late release', no green cells were observed, demonstrating that the samples were cytotoxic. As PLGA degrades, it breaks down into its monomeric or oligomeric components of lactic and glycolic acids, and the authors attempted to deacidify the microsphere release samples before exposure to the cells. MSCs exposed to deacidified 'late release' SDF-1α microspheres were bright green, showing that the samples were properly neutralized.

Results of the MSC migration experiment show that the positive control induced the migration of a great number of MSCs, proving that the cells were responsive to chemoattractant agents. Because of the acidity of the 'late release' microsphere degradation samples, all samples from that timeframe were deacidified prior to placing into the migration assay. Compared to the negative control having no SDF-1α, all SDF-containing release samples produced statistically significant MSC migration. Because the PLGA degradation products may affect cell migration, the authors also compared the migration results of release samples from empty microspheres versus ideally loaded SDF-1α-microspheres. They showed that, at matched collection time points day 1 and

day 51, all Formulation A (sonicated at room temperature) release samples caused significant cell migration compared to empty microsphere samples. For ice-sonicated microspheres (Formulation G), the release sample collected at day 1 yielded a significant increase in MSC migration were compared to the release sample from empty microspheres, but no significance was observed when comparing release samples from later time points. The release samples collected early in the microsphere degradation process at day 1 were more potent chemoattractants than late time point day 51 release samples (Cross 2011).

Chapter III.

Discussion

This case study has presented numerous arguments supporting the timed release diffusion of SDF-1 via PLGA microspheres. Experiments on diffusion via transdermal microneedle patch is initially supported by the results documented here in which all relevant studies show improved neural regeneration and biocompatibilty with SDF-1 delivery in PLGA microspheres, and subsequent increase in MSC homing. The studies primarily included repair improvements in rodent sciatic nerve tissue, but included cortical tissue repair improvements, and basic in vitro MSC migration improvements in chemokine signaling and chemotaxis.

The work of Dutta et al. used a novel approach in tagging exogenously introduced SDF-1 via cortical injection for downstream assaying against endogenously produced SDF-1. This approach showed the extent to which natural repair mechanisms can be kick-started with this technique and did not focus solely on MSCs but included other neuronal committed progenitor cells. Furthermore, Dutta et al. outlined the advantage in timed release of SDF-1 to prevent proteolytic digestion of the protein. The authors also outlined the enhancing role of VEGF and bFGF signaling promotion of the SDF-1/CXCR4 feedback loop, indicating a complex two-phase interplay with TNF-a /IL-1ß as mediators to an initial downregulation of CXCR4 expression, followed by upregulation after 24 hours. Their results suggest that these additional signaling molecules should be

evaluated in detailed regeneration studies. Furthermore, the authors investigated BBB effects that may require consideration analogous to blood nerve barrier effects in peripheral nerve injuries, indicating that endothelial cells may play a role in affecting endogenous SDF-1 expression (Dutta, 2017).

The Thevenot et al. study focused on observation of in vivo MSC homing to sites of scaffold implants in mice without a specific nerve injury model. This simplified approach helped in gathering data that would relate to the future application of a transdermal patch and its implications apart from nerve injury. The authors found that SDF-1a loaded scaffolds generated a 3 times greater density of MSCs than that of non-loaded control scaffolds, suggesting the regenerative power of supplementation with SDF-1a. Furthermore, it was found that mast cell activity was suppressed approximately 84% by SDF-1 supplementation suggesting a reduction in fibrosis. It was also found that control scaffolds had a greater density of macrophages focused at the implant than those loaded with SDF-1, which would suggest a reduced foreign-body response that may improve regeneration and reduce fibrosis. This evidence was further backed by the thick layer of fibrotic tissue seen at 2 weeks post implantation in control implants, while, contrastingly, there was actual vascular integration at the implants of SDF-1 loaded scaffolds. Macrophage activation-state and related signaling was considered a possible condition for this improved regeneration and decrease in fibrosis, and this has been supported by other studies in multiple sclerosis models (Thevenot, 2010).

Wang et al. further support the activity of SDF-1 as accelerating cartilage repair and MSC cell recruitment and differentiation. The authors used SDF-1 loaded PLGA

scaffolds to demonstrate the ability of MSCs to home to sites of cartilage damage and differentiate into chondrocytes. This differentiation was evaluated using an mRNA assay of chondrocyte-specific cell markers and a similar technique will apply in support of neuronal cell lineage commitment in future nerve regeneration studies. Both mRNA level and western blot evidence concluded that MSCs were indeed differentiating into chondrocytes. The authors showed that SDF-1 loaded PLGA scaffold application resulted in superior quality and faster repair outcomes against controls, with an indication of less residual scarring to the tissue (Wang, 2017).

The Matthes et al. study further supported in vivo application of transplanted MSCs to improve peripheral nerve regeneration on rat sciatic nerves using a nerve damage model. The authors showed that supplementation with MSCs via femoral vein resulted in superior nerve tissue repair outcomes overall, with a greater volume of cell homing through this technique. Additionally, there was greater locomotor functional improvement in animals supplemented via MSC injection, particularly noticeable at earlier stages of nerve repair versus controls. Cooney et al. further demonstrated the chondrogenic, osteogenic and adipogenic properties of their MSC sample and used MSC supplementation in their rat sciatic nerve damage model. Along with greater speed of recovery, the authors recorded improved functional recovery with a software based gait-analysis system. Gait-analysis provided a means of evaluating end-result testing in nerve damage models, as well as complete limb transplant models (Matthes, 2013).

The Yu et al. study explored the use of angiogenic factors instead of SDF-1 in the framework of a PLGE microsphere administration model. Similar to SDF-1 loaded

microspheres the angiogenic factor microspheres resulted in significant neural precursor cell signaling in a rat model of SCI. Overall this enhanced angiogensis resulted in faster and superior quality repair outcomes at SCI sites and suggested a synergistic model for SDF-1 supplementation (Yu 2016).

Lastly, the work of Cross et al. explored a detailed approach to SDF-1 PLGA implementation strategies using an in vitro MSC migration model. A variety of chemically unique formulas and structures for PLGA were tested for efficacy, including their release rates, loading/distribution efficiency, and cell toxicity. The authors showed significant increases in MSC migration in the presence of diffusing SDF-1 loaded PLGA versus controls. Furthermore, SDF-1 release from PLGA was shown to follow a complex multi-phase diffusion curve, with a significant initial release from day 1 through day 5. A second, bulk degradation phase was noticed at day 35 but was associated with significant cytotoxicity. The authors successfully neutralized this condition by deacidifying the late release PLGA, suggesting important strategies for timing of SDF-1 release and chemical conditions of release over time for proposed further studies with microneedle patch release (Cross 2011).

References

Acquistapace, Adrien et al., Human mesenchymal stem cells reprogram adult cardiomyocytes toward a progenitor-like state through partial cell fusion and mitochondria transfer, Stem Cells. 2011 May; 29(5): 812–824.

Brushart, Thomas M. , Nerve Repair, Oxford University Press, Inc., 2011

Boilly, Benoni et al., Nerve Dependence: From Regeneration to Cancer, Cancer Cell Review| Volume 31, Issue 3, P342-354, March 13, 2017

Cai , Sa et al., Directed Differentiation of Human Bone Marrow Stromal Cells to Fate-Committed Schwann Cells, Stem Cell Reports | Vol. 9 | 1097–1108 | October 10, 2017

Carbajal, Kevin S. et al., Migration of engrafted neural stem cells is mediated by CXCL12 signaling through CXCR4 in a viral model of multiple sclerosis, PNAS | June 15, 2010 | vol. 107 | no. 24.

Cizkova, Dasa, et al., Localized Intrathecal Delivery of Mesenchymal Stromal Cells Conditioned Medium Improves Functional Recovery in a Rat Model of Spinal Cord

Injury, Int. J. Mol. Sci. 2018, 19, 870; doi:10.3390/ijms19030870

Clements Melanie P. et al., The Wound Microenvironment Reprograms Schwann Cells to Invasive Mesenchymal-like Cells to Drive Peripheral Nerve Regeneration, Neuron. 2017 Sep 27; 96(1): 98–114

Cooney, Damon S.et al., Mesenchymal Stem Cells Enhance Nerve Regeneration in a Rat Sciatic Nerve Repair and Hindlimb Transplant Model, Nature: Scientific Reports | 6:31306 | DOI: 10.1038/srep31306, August 2016

Cross, Daisy P. & Chun Wang, Stromal-Derived Factor-1 Alpha-Loaded PLGA Microspheresfor Stem Cell Recruitment, Pharm Res May 2011

Dai, Xiaofeng et al., The CXCL12/CXCR4 autocrine loop increases the metastatic potential of non-small cell lung cancer in vitro, Oncology Letters 5: 277-282, 2013

Dutta, D. et al., Spatiotemporal presentation of exogenous SDF-1 with PLGAnanoparticles modulates SDF-1/CXCR4 signaling axis in the rodent cortex, Biomater Sci., 2017 July 25; 5(8): 1640–1651. doi:10.1039/c7bm00489c.

Giordano-Santini, Rosina et al, Cell-cell fusion in the nervous system: Alternative mechanisms of development, injury, and repair, Seminars in Cell & Developmental

Biology 60 (2016) 146–154

Hofer, Heidi R., et al., Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies, Stem Cell Research & Therapy (2016) 7:131 DOI 10.1186/s13287-016-0394-0

Jiang, Liangfu et al., Stem Cell Transplantation for Peripheral Nerve Regeneration: Current Options and Opportunities, Int. J. Mol. Sci. 2017, 18, 94; doi:10.3390/ijms18010094

Makadia, et al., Polymers (Basel)., Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier, 2011 Sep 1;3(3):1377-1397. Epub 2011 Aug 26.

Nishiguchi, Mailyn A. et al., 2018, Aging Suppresses Skin-Derived Circulating SDF-1 to Promote Full-Thickness Tissue Regeneration, Cell Reports 24, 3383–3392

Purcell Brendan P. et al., Synergistic effects of SDF-1α chemokine and hyaluronic acid release from degradable hydrogels on directing bone marrow derived cell homing to the myocardium, Biomaterials. 2012 November; 33(31): 7849–7857.

Shah, Shreyas et al., Nanotechnology-Based Approaches for Guiding Neural

Regeneration, Acc Chem Res. 2016 January 19; 49 (1): 17–26.

Sopko, Nikolai et al., Stromal Derived Factor-1 Treatment Augments Nerve Regeneration Via Cxcr4 Activation Of Neurotrophic Factors In The Major Pelvic Ganglion In A Dose Dependent Fashion, Vol. 195, No. 4S, Supplement, Tuesday, May 10, 2016 The Journal Of Urology

Sullivan, Robert et al., Peripheral Nerve Injury: Stem Cell Therapy and Peripheral Nerve Transfer, Int. J. Mol. Sci. 2016, 17, 2101; doi:10.3390/ijms17122101

Xie, Wenrui et al., Active Nerve Regeneration with Failed Target Reinnervation Drives Persistent Neuropathic Pain, ENeuro, Sensory and Motor Systems, Pain Research Center, Department of Anesthesiology, University of Cincinnati College of Medicine, DOI:http://dx.doi.org/10.1523/ENEURO.0008-17.2017

Yamazaki, Aiko et al., Implanted hair-follicle-associated pluripotent (HAP) stem cells encapsulated in polyvinylidene fluoride membrane cylinders promote effective recovery of peripheral nerve injury, Cell Cycle 2017, VOL. 16, NO. 20, 1927–1932

Yu, Shukui et al., Angiogenic microspheres promote neural regeneration and motor function recovery after spinal cord injury in rats, Nature: Scientific Reports | 6:33428 | DOI: 10.1038/srep33428, September 2016

Wang, Y et al., Combination of hyaluronic acid hydrogel scaffold and PLGA microspheres for supporting survival of neural stem cells., Pharm Res. 2011 Jun;28(6):1406-14.
Presents a controlled delivery of biofactors providing a basic model for the current system.

Wang, Yunji et al. Low-intensity pulsed ultrasound promotes periodontal ligament stem cell migration through TWIST1-mediated SDF-1 expression, International Journal Of Molecular Medicine 42: 322-330, 2018

Zhao, Yannan et al., The neuronal differentiation microenvironment is essential for spinal cord injury repair, Organogenesis, 13:63–70, 2017