2017

The Transdifferentiation of brain derived neurotrophic factor secreting mesenchymal stem cells for neuroprotection

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The transdifferentiation of brain derived neurotrophic factor secreting mesenchymal stem cells for neuroprotection

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biomedical Science (Anatomy)

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Ames, Iowa

2017

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NOMENCLATURE

BDNF	Brain derived neurotrophic factor
ELISA	enzyme linked immunosorbent assay
GDNF	Glial-cell derived neurotorphic factor
GFP	Green fluorescent protein
MSC	Mesenchymal stem cell
NGF	Nerve growth factor
SCs	Schwann cells
TDM	Transdifferentiation media
tMSC	transdifferentiated mesenchymal stem cell
uMSC	undifferentiated mesenchymal stem cell

ACKNOWLEDGMENTS

I would like to thank my mentor of four years, Dr. Donald S. Sakaguchi for his outstanding support and guidance in all aspects of my work for his laboratory. I would also like to commend him for his patience with me during my work as it was never easy to balance my veterinary coursework and master's degree but he was always willing to extend deadlines and offer me assistance whenever needed. I wish to thank Dr. Surya K. Mallapragada for providing feedback to our manuscript. I also want to thank my committee members Dr. Greenlee and Ellinwood for evaluating my progress and providing suggestions for my thesis.

Also, I would like to thank my lab mate Dr. Anup Dutt Sharma without which, completion of my Master's degree would not be possible. The time spent training me on various instrument use, as well as various bench techniques were essential in generating data. Additionally, all of the feedback and editing provided for our submitted manuscript was much appreciated.

Finally, I would like to thank all my other laboratory colleagues, Bhavika, Paul, Dan, Debbie, Svitlana, Elizabeth, and everyone else in the lab for their continued support, friendship, and constructive criticism.

I want to acknowledge the funding support for my research from U.S. Army Medical Department (Medical Research and Material Command-Grand account no. W81XWH-11-1-0700) and stem cell research fund.

CHAPTER 1

RESEARCH OBJECTIVES

1. Introduction

The ultimate purpose of this project was to create a modified stem cell line which could enhance nerve regeneration following peripheral nerve trauma. Specifically, this work was focused upon answering two questions. First, could we chemically transdifferentiate genetically modified mesenchymal stem cells to resemble a Schwann cell-like state? A protocol for the chemical transdifferentiation of MSCs was validated and well tested in the Sakaguchi lab, but no one had ever attempted to chemically transdifferentiate BDNF hyper-secreting MSCs. Second, if we succeeded in creating BDNF hyper-secreting transdifferentiated MSCs (BDNF tMSCs), would levels of BDNF secretion be affected, and, more importantly, would the secreted BDNF still be biologically active? We hypothesized that BDNF tMSCs would still resemble a Schwann cell like phenotype and be able to produce the same or lower amounts of biologically active BDNF when compared to their undifferentiated and GFP control counterparts. Generated data relied largely on the use of immunocytochemistry to quantify the percentage of cells expressing Schwann cell markers. BDNF secretion was quantified by ELISA and bioactivity was tested using the PC12-trkB assay. This study was an important first step in characterizing these BDNF tMSCs by *in vitro* assays and was essentially a proof of concept study to show that genetically modified MSCs could still be chemically transdifferentiated. As a next step, we hope to seed these BDNF tMSCs within a polymeric conduit transplant used in a rat sciatic nerve transection model to test the ability of these cells to aid in nerve regeneration *in vivo*.

2. Summary

The main objectives of this research project are:

1. Chemical transdifferentiation of BDNF MSCs into SC-like phenotypic cells
2. Assessment of SC marker expression levels via immunocytochemistry
3. Measurement of BDNF secretion pre-and post transdifferentiation via ELISA
4. Assessment of BDNF bioactivity via PC12-trkB cell assay

3. Organization of thesis

Chapter 2 is a literature review, which will provide the background context for understanding the importance of peripheral nerve regeneration therapies and the key role that Schwann cells play in this process. The review also discusses the benefits of cell transplants and how, in particular, autologous mesenchymal stem cells offer many advantages such as genetic modification. The chapter continues with a focus on the ways that unmodified and modified MSCs have been used in clinical trials and what nervous system diseases may benefit from their use. Finally, the review ends with a discussion of current and future transdifferentiation methods and trends.

Chapter 3 is adapted from a manuscript we have submitted for publication, detailing the effects of chemical transdifferentiation on BDNF hyper-secreting MSCs. Finally, chapter 4 is a summary of important results and conclusions drawn from this dissertation.

CHAPTER 2

A REVIEW OF MESENCHYMA STEM CELL THERAPIES IN THE CONTEXT OF PERIPHERAL NERVE REGENERATION

1. Introduction

In order to understand the true therapeutic potential for transdifferentiated brain derived neurotrophic factor (BDNF) mesenchymal stem cells (MSCs), it is important to put the research into a clinical perspective. MSCs which have been altered to resemble and act like Schwann cells have key beneficial properties within the context of peripheral nerve trauma such as enhancing neuron survival and improving return to function. The prevalence of peripheral nerve trauma remains surprisingly high and current treatment options have several pitfalls. Newer remedies, such as cell transplants, are in high demand because the traditional gold standard requires the sacrifice of a healthy nerve. In particular, Schwann cells are essential to Wallerian degeneration (1,2,3) and nerve regeneration (4,5,6,7) and are excellent transplant candidates (5,6,7,8). However, Schwann cells are difficult to culture in vitro and are a mature cell line, thus requiring a healthy nerve for harvest (9). Studies within the last twenty years have instead searched for easily harvested cells, capable of transdifferentiating into a Schwann cell phenotype and found that mesenchymal stem cells are capable of expressing Schwann cell markers, promoting neural tissue survival, and improving return to function in peripheral nerve injuries (10, 11, 12, 13, 14). In addition to mimicking Schwann cells, MSCs have their own benefits, such as secreting neurotrophic factors and serving as a vehicle for genetic modification (15, 16, 17, 18, 19). Our project has focused on genetically modified MSCs which can hyper-secrete the growth factor BDNF and have the ability to provide neuroprotection and increased neurite outgrowth. Our lab has used these cells in rat studies of glaucoma models

(20), however, we have never tested them in models of peripheral nerve injury, which is one aim of this study.

Another goal of the study was to determine whether or not these BDNF MSCs could be transdifferentiated to resemble Schwann-like cells and further characterize them with *in vitro* assays in order to add to the growing pool of pre-clinical data necessary for future human trials. Unmodified mesenchymal stem cells have been used in many clinical trials for nervous system disorders and even genetically modified MSCs made to hyper-secrete growth factors have been tested. However, transdifferentiated MSCs have yet to be tested as a treatment for human disorders and require much more extensive *in vitro* studies to ensure their safety and efficacy. A key part of the *in vitro* studies is determining the best method of transdifferentiation. Methods include the use of co-culture or direct transplantation, small molecule/chemical transdifferentiation, or overexpression of a master gene via genetic modification. In particular, our lab has focused on the method of chemical transdifferentiation as it is a relatively rapid process, does not require a viral vector, and has a high success rate of cell conversion. Our overall goal was to test and characterize the ability of BDNF MSCs to undergo transdifferentiation and explore their relevance as a therapeutic treatment option for peripheral nerve injuries. The following chapter will provide the reader with a more in-depth review of current treatment options and their pitfalls; the use of cell transplants, especially Schwann cells and MSCs; and, finally, the use of transdifferentiation to create Schwann-like cells from MSCs and their benefits to peripheral nerve regeneration.

2. Peripheral nerve injuries- Causes & Prevalence

Peripheral nerve injuries (PNIs) may be caused by a variety of etiologies including trauma, metabolic disorders such as Diabetes mellitus, or iatrogenic surgical complications. The most common cause of PNIs are trauma during which nerves may suffer from traction, ischemia, crushing, or penetrating wounds (21). Other less common causes may include thermal, electric shock, radiation, or vibrational injuries (22,23). The majority of incidents are stretch-related injuries, especially in motor vehicle accidents (24), while lacerations by knife, glass, saw, or long bone fractures are only 30% of serious nerve injuries (21). In a study of 1,167 cases of peripheral nerve injury, 5.7% of cases were related to sports (25). In a retrospective study by Kouyoumdjian (2006), 456 cases of PNIs showed upper limb injuries to be the most common, with the ulnar nerve most often injured (26). Again, these injuries were most often due to motor vehicle accidents, particularly motorcycle crashes. In addition to affecting civilians, PNIs can commonly occur in a combative setting, where nerve injuries are commonly caused by shrapnel or blast injury from bombs or improvised explosive devices (27).

After suffering from a peripheral nerve injury, a patient's prognosis depends on the type of functional injury they have experienced. At the anatomic level, nerve injury can be divided into neurapraxia, axonotmesis, and neurotmesis according to Sir Herbert Seddon (28). In neurapraxia, the nerve remains intact but can no longer transmit impulses. Neurapraxia is typically due to segmental demyelination and is the mildest form of nerve injury. Distally, the nerve conducts normally but there is impaired conduction across the lesion due to the focal demyelination. Axons are typically anatomically intact but nonfunctional, which renders a body part paralyzed. There is sensory and motor loss due to demyelination but no Wallerian degeneration occurs. Clinically, muscle atrophy does not develop. Recovery time is typically

rapid and ranges from hours to a few months. Full function is usually expected without any sort of intervention by 12 weeks (21).

In axonotmesis, the axon is damaged but most of the surrounding connective tissue is intact. Wallerian degeneration does occur. Axonotmesis is usually seen in stretch or crush injuries. Recovery and reinnervation depends upon the distance from nerve to muscle and the degree of internal axonal disorganization.

In neurotmesis, the nerve trunk is severed and most of the connective tissue is lost or distorted. Neuroetmesis occurs with massive trauma, nerve avulsions, and sharp, cutting injury. There is loss of nerve trunk continuity and reinnervation typically does not occur. Without surgical intervention, the prognosis is poor. Recovery from this sort of trauma when there is significant axon loss and stromal disruption is usually prolonged and incomplete (29).

When suffering from neurotmesis or axonotmesis, injuries can cause total or partial loss of motor, sensory, or even autonomic function. When left to repair itself, the peripheral nervous system can attempt one of three mechanisms: reinnervation by axonal regeneration, reinnervation by collateral branching of uninjured surrounding axons, or remodeling of the nervous system circuitry; however, left to only these mechanisms, a full functional recovery is often not achieved (29, 30, 31). Failure can be attributed to three problems: First, axons stop elongating and result in neuroma formation. Second, axon sprouts innervate more than one peripheral nerve branch and cause weak or contradicting muscle movements. Third, regeneration into the wrong nerve can occur if, for example, a sensory axon grows into a motor nerve or vice versa (32).

It is important to understand that while the peripheral nervous system retains the ability to reconstruct itself, only 60% of patients suffering from a PNI regain useful function (32). The

occurrence of postparalytic syndromes such as paresis, synkinesis, and dysreflexia are common (33). Additionally, patients can experience chronic neuropathic pain, health care issues, and long periods of sick leave (34, 35).

Due to the high incidence of unsatisfactory return to function, further improvements in peripheral nerve repair and regeneration have become an area of much interest. Today, PNIs have become the focus of new innovations which revolve around many different scientific disciplines. The following section will focus on the two most common areas of clinical treatment: surgery and transplantation. Other disciplines involved such as biomaterial sciences, physical therapy, and pharmacotherapy are outside of the realm of this review.

2.1 Current Treatment Options for PNIs

The most common medical treatments rely largely on reconstructive microsurgery. Although nerve reconstruction has been attempted for centuries, techniques have improved drastically within the past few decades (36). Procedural options include nerve autografts, neurolysis, nerve transfers, and direct suture (end to end neurorrhaphy) (37). The nerve transfer method has seen widespread application in recent years and is used in severe nerve trauma, including brachial plexis avulsions (38, 39).

Although advances in microsurgical techniques have plateaued, a few interesting technological advances have occurred within the past ten years. For example, the use of glue rather than suture has been tried in animal models, and results indicate that glue may be equal or even superior to epi and perineural microsuturing (40, 41). Another area of advancement is robotics assisted surgery. Results from experimental studies are encouraging and robot technologies may be favored by nerve surgeons in the future (42, 43).

Microsurgical treatment alone has relatively low success rates, which is why transplantation is the approach drawing the most interest in regenerative medicine (37). The current "gold standard" includes transplantation of an autologous nerve segment which has been harvested from another healthy, less important nerve. The procedure was first developed by Millesi (1981) (44) and later deemed the standard of care (36). Although autograft is the "gold standard" the harvesting of another healthy nerve represents obvious limitations, which is why veins are sometimes used as an alternative (45). Although vein autografts may lead to satisfactory return of sensation, comparable to nerve grafting, they are only useful for short distances as longer veins tend to collapse (46).

In addition to nerve and vein transplants, skeletal muscle used as guiding fibers has also been tried with relatively good success. Various studies have shown that muscle conduits may potentially bridge peripheral nerve defects (47) and that grafts may even gain some functional recovery (48,49,50).

Apart from tissue transplants, cell transplants are a large area of research. Glial cells, specifically, Schwan cells, are a common cell type studied, as well as mesenchymal stem cells. The purpose and clinical studies of each cell type will be further discussed in sections 4- 6. The following section will explain the process of nerve breakdown and regeneration following a traumatic nerve injury and the essential role that Schwann cells play.

3. Wallerian degeneration

After damage to a peripheral nerve, a complex system of molecular and cellular events take place in order for nerve regeneration to begin. In 1850, August Waller first described Wallerian degeneration which is composed of degeneration in the distal nerve stump, with elongation and regeneration occurring in the proximal nerve stump **(Fig.1)** (1,51).

Soon after a PNI, Schwann cells in the distal nerve rapidly initiate detachment of their myelin sheaths (52). The surrounding myelin and axonal tissue begin to break down. Within hours of injury, histological changes have occurred as neurotubules and neurofilaments become disarrayed (53). Within 24 hours of injury, Schwann cells are stimulated by proteins released from the disintegrating axons (54), and later, by macrophage cytokines, to proliferate. The Schwann cells exhibit an increased mitotic rate as well as nuclear and cytoplasmic enlargement and divide rapidly to form daughter cells (53). These daughter cells produce cytokines and trophic factors which assist in degeneration and repair (55). During this time, local macrophages (Mast cells) interact with the Schwann cells to remove degenerated axonal and myelin debris. Schwann cells and macrophages work together to phagocytose and clear the site of injury. By 36-48 hours, myelin disintegration is quite advanced (53). The elimination of myelin sheaths is important as it clears certain growth inhibitory factors such as myelin-associated glycoproteins (56). At the same time that the distal nerve is degenerating, the nerve cell body is undergoing a process known as chromatolysis. Within 6 hours of injury, the nucleus of the nerve cell body migrates to the periphery of the cells and the rough endoplasmic reticulum (Nissl bodies) break up and disperse (57, 58). In this state, the neuron increases RNA synthesis and cellular protein content, and reduces DNA repression, in order to increase synthesis of growth-associated proteins and membrane structural components (59).

Within two days, Schwann cell daughter cells have undergone rearrangement into a structure known as Bünger bands (60). These bands act as a guidance skeleton for regenerating axon sprouts. Within a week, factors produced by Schwann cells and injured axons leads to recruitment of hematogenous monocytes (61). The new macrophages continue to clear debris and produce factors which facilitate Schwann cell migration (55).

After weeks to months, axon sprouts begin to form, each with a specialized growth cone at the tip which contains multiple filopodia. These filopodia adhere to the basal lamina of the Schwan cells within the Bünger bands, which serve as a guide toward potential new targets of innervation. Both physical and chemotactic guidance from the Schwann cells are important in directing advancement of the growth cone (62, 63). Individual filopodia respond to environmental alterations in calcium (64) and different filopodia can react independently via local changes to actin metabolism (65). Once contacted by regenerative sprouts, Schwann cells re-differentiate, express myelin mRNAs, and begin the process of remyelating and ensheathing fresh axons (21). If axonal sprouts are able to cross the injury site and contact a new peripheral target, then reinnervation may occur. The regeneration and repair phase may last for many months and is not always successful. Regenerating axons may enter surrounding tissue instead of the target organ or may enter into the incorrect endoneurial tube, failing to reinnervate the correct target. After nerve injury and repair, the conduction velocity of regenerated axons, their diameter, and their excitability remain below previous levels for a long time (67,68).

In addition to the complex cellular response, PNIs induce the release of many neurotrophic factors and cytokines in order to create a favorable environment for axon regrowth. These polypeptides assure that the regenerating axons are growing towards the distal nerve stump and stimulate axonal sprouting. The following section will review the role of neuronal growth factors, particularly brain derived neurotrophic factor, during Wallerian degeneration.

4. The importance of neurotrophic factors during peripheral nerve regeneration

In response to a peripheral nerve injury, many neurotrophic factors are upregulated. These molecules may be classified either as neurotrophic factors or neuropoietic cytokines (69). This review will discuss only the neurotrophic factors and will be focused primarily on the role of BDNF.

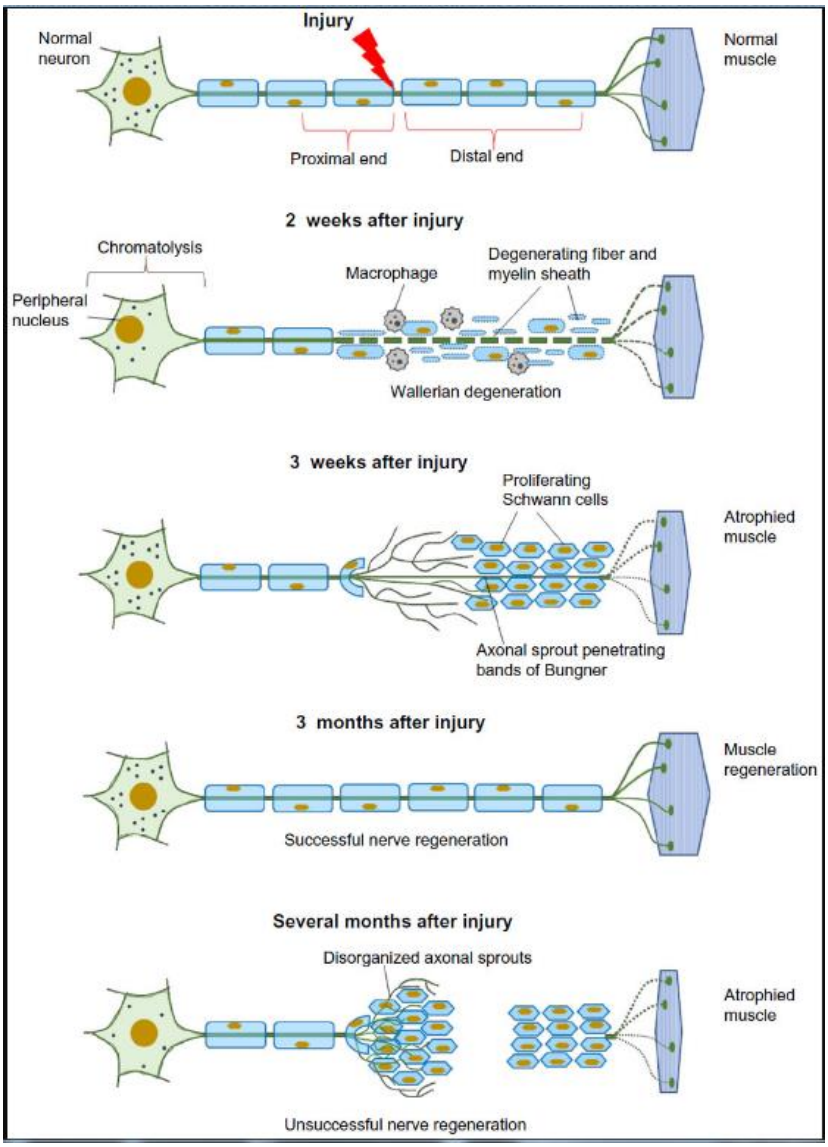

Figure 1. Wallerian degeneration and chromatolysis followed by regeneration. Adapated from (66).

Neurotrophic factors are vital to neurite outgrowth during embryonic development, maintain adult neurons, and aid in regeneration following a PNI (32). The specific neurotrophins involved in regeneration include NGF, BDNF, and neurotrophins 3,4, & 5. Several growth factors are also released, including glial cell-derived neurotrophic factor, fibroblast growth factors, insulin-like growth factors, neuregulins, and neuropeptides (galanin, vasoactive intestinal peptide, etc) (70,71). All neurotrophic factors are believed to be synthesized in target organs and then delivered via retrograde transport to the neuronal soma (72, 73). The neurotrophin members (NGF, BDNF, NT-3/4/5) share a common low-affinity receptor p75 (74) to which they all bind equally. It is thought that p75 interacts with the tropomyosin receptor kinases (Trk) to assist in transport of neurotrophins within the neuronal terminals (75). Each neurotrophin has a specific high affinity receptor: trkA for NGF, trkB is specific for BDNF, and NT-4/5, and NT-3 bind to trkC (76). Every trk receptor is located in a discreet population of primary sensory neurons (77,78) and trkB and C are also present in spinal motoneurons (79). The following section will focus on the trkB receptor and the various roles that BDNF plays in neuronal regeneration.

4.1 Promotion of neuron survival

Activation of each neurotrophin is dependent on the type of neuron damage (motor, sensory, or autonomic). BDNF, in particular, is upregulated in motor neurons, as is its receptor, TrkB, for 48 hours following an axotomy lesion (80). During this time, BDNF acts as a neuroprotectant. It has been shown to rescue motor neurons from natural cell death, as well as prevent their death following axotomy (81, 82, 83). Indeed, external application of BDNF following axotomy or ventral root avulsion reduces motoneuron death (82, 84) and continuous dose-dependent administration of BDNF shows long-term survival effects on adult

motoneurons after sciatic nerve avulsion (85). Additionally, a few studies found that application of NGF, BDNF, and NT-3 can reverse detrimental changes induced by axotomy in adult and neonatal sensory neurons (86,86,88).

The ability of BDNF to rescue motor neurons is carried out through its trkB receptor. Once BDNF binds to TrkB, three different signal transduction cascades are activated. These include insulin receptor substrate-1, Ras, protein kinase C, and many other intermediate proteins. BDNF signaling pathways activate one or more transcription factors (cAMP-response-element-binding protein (CREB), and CREB-binding protein) which regulate the expression of genes encoding proteins that are involved in neural plasticity, stress resistance, and cell survival (89, 90, 91).

4.2 Remyelination

After Wallerian degeneration occurs, the next important step in peripheral nerve recovery is remyelination. Several studies have added exogenous BDNF to a peripheral nerve injury model and examined the effects on myelin protein synthesis and myelin sheath thickness. The first study to examine this phenomenon saw that when applied in combination with CNTF, exogenous BDNF increases myelin thickness of regenerating sciatic nerves (92). This work was continued by a study (93) that used a Schwann cell and dorsal root ganglion (DRG) cell co-culture model, as well as a sciatic nerve *in vivo* model, to test the effects of exogenous BDNF addition following an injury. Immediately following injury, BDNF caused an enhancement in the expression of myelin protein MAG and P0. This effect was seen in both the co-culture and sciatic nerve *in vivo* model. Consequently, when endogenous BDNF levels were reduced in the co-culture via addition of the receptor scavenger TrkB, myelin protein

synthesis was inhibited as was the formation of myelin, demonstrating that BDNF is indeed beneficial during remyelination.

With the use of electron microscopy, Chan et al. demonstrated that the addition of BDNF increased the number of myelinating axons and the thickness of the myelin sheath *in vivo* (93). A similar study (94) created a mouse sciatic nerve injury model and administered exogenous BDNF injections to examine the effects on myelin sheaths in the distal nerve stump. Their results showed that mice receiving BDNF administration had an increased number of myelinated fibers and that myelin sheaths were thicker when compared to control mice. Additionally, mice receiving BDNF antibodies showed significant myelin deterioration in the distal sheath. Furthermore, a study by Zhang et al., 2000, demonstrated that treatment with BDNF antibody reduced the number and density of myelinated axons by 83%, and found that sensory reinnervation was impaired (95). Combined, these results demonstrate that BDNF is critical for preparing nerves for remyelination by increasing myelin proteins such as P0 and MAG, as well as protecting the distal nerve portion from atrophy by promoting remyelination.

4.3 Axonal Sprouting, Regeneration, and Functional Recovery

In addition to examining neuronal survival, regeneration, and re-myelination, several studies have looked at BDNF's role during axonal sprouting. It has been shown that following severe trauma such as ventral root avulsion, exogenous BDNF significantly increases axonal sprouting (71). To support Gordon's findings, another study found that application of BDNF antibodies to a transected facial nerve trunk significantly reduced axon sprouting up to 18% (96). Axonal sprouting may increase in part, due to BDNF's role as a guidance molecule for the growth cone at the end of each axonal sprout. Studies in Xenopus spinal neuron models

show that BDNF and NT-3 can attract or repulse growth cones based on concentration gradients (97, 98).

Although BDNF may increase axonal sprouting, the data is controversial in regards to increased functional return upon application of BDNF. For example, using the sciatic function index (99), gait analysis (100), and force recovery, several studies failed to demonstrate a return to function with exogenous BDNF. One study even showed that local long-term continuous infusion of low dose BDNF had no effect on tibial motoneurons after immediate microsurgical repair (101).

On the other hand, a more recent study found that exogenous BDNF administration accelerates the recovery process in a mouse sciatic nerve injury model while BDNF antibody treatment delayed it (94). After the crush injury, control mice took 12 days to show initial improvement using the toe spreading score of gait analysis, and 24 days for a full recovery. Mice receiving the BDNF treatment required only 7 and 18 days, respectively. Conversely, BDNF antibody treatment delayed the processes to 17 and 30 days.

Another study created control and heterozygote BDNF knockout mice that received a left sciatic nerve crush (102). Nerve function was evaluated using a rotarod test, sciatic function index, and motor nerve conduction velocity simultaneously with histological nerve analysis. Impaired nerve repair was observed in the BDNF heterozygote mice, which was consistent with attenuated function of BDNF. In contrast, the BDNF homozygote mice showed complete functional and histological recovery. These observations support the view that BDNF may play a pivotal role in functional return following a peripheral nerve injury.

Unlike other neurotrophic factors, BDNF is unique in that it regulates and maintains neuronal function, and when given exogenously, it counteracts degenerative changes in both

sensory and motor axons. Unlike NGF, BDNF supports motoneuron survival *in vitro*, rescues from naturally-induced apoptosis, and prevents *in vivo* axotomy-induced cell death (103). While there are benefits of exogenous BDNF application to peripheral nerve lesion sites, its abilities to increase functional return are still controversial, which is why recent research has focused on the adjunct use of BDNF in combination with other therapies such as stem cell therapy, biomaterial conduits, pharmacotherapy, etc. A more in-depth discussion of BDNF therapy combined with stem cell use will be included in sections 6 and 8.

5. Cell Based Therapy for Improving Nerve Regeneration

As discussed above, the gold standard of peripheral nerve repair continues to be the use of nerve grafting combined with direct nerve repair, and occasionally, the use of conduits to bridge larger nerve gaps. Recent research, however, has focused on cell therapy as a promising therapeutic approach for promoting nerve regeneration. Particularly, cell-based therapy has been widely studied as a source of growth-promoting molecule delivery system and graft replacement. This section will focus briefly on the past use of glial cells such as Schwann cells and then discuss the promising potential of bone marrow mesenchymal stem cells.

5.1 Use of Schwann Cells

Schwann cells (SCs) play a key role in axonal regeneration, making them an attractive cell type to use for transplantation. During Wallerian degeneration, Schwann cells remove necrotic tissue and myelin debris together with macrophages (104). In the regeneration phase, glial cells form the Bünger bands which physically guide axons to distal innervation targets. Additionally, SCs increase synthesis of surface cell adhesion molecules and basement membrane proteins such as laminin and fibronectin (105). Schwann cells also produce neurotrophic factors, cytokines, and other compounds which promote neurite growth (106,

107). Experimental evidence shows that transplantation of SCs in vitro supports axonal outgrowth (4), and improves the quality and rate of axon regeneration (5,6,7). Schwann cells combined with a vein conduit have even been used in bridging long nerve gaps (108, 8).

Although Schwann Cells would be an ideal source of cell therapy, there are several technical limitations hampering their use in clinical trials. In the case of acute nerve injuries, use of Schwann cells would be impractical as the time requirement for expanding autologous cells in culture is lengthy (9). Additionally, there is a risk of fibroblast contamination which would lead to unwanted scarring of the nerve (7). In order to obtain a source of autologous SCs, another healthy nerve must be sacrificed for harvesting, making donor site morbidity another concern. All of these limitations have led researchers to seek for a better alternative to SCs for cell transplantation and stem cells have been posed as better candidates.

5.2 Mesenchymal Stem Cells

Stem cells are a distinct population of undifferentiated cells which are characterized by potency, the ability to differentiate into a wide variety of specialized cell types, and the ability to undergo numerous rounds of mitosis while remaining undifferentiated. There are embryonic, fetal, and adult stem cells, of which this review will focus on adult stem cells.

Of all three types, adult stem cells are thought to be the most limited in their potency since their primary role is to repair damaged tissue in which they are found (11). Unlike fetal and embryonic stem cells, adult stem cells raise fewer ethical concerns as they do not require human embryo destruction. Additionally, adult stem cells have a lower risk of tissue rejection as auto-transplantation is a possibility, and the small risk of teratoma formation that embryonic stem cells presents is almost null with adult cells (109). Common sources of adult stem cells include mesenchymal, hematopoietic, or umbilical cord derived. In particular, bone marrow-

derived stem cells are known as mesenchymal stem cells and can differentiate into connective tissue types such as chondrocytes, adipocytes, myocytes, osteocytes, fibroblasts, and tenocytes (110). There is also extensive additional research to suggest that MSCs have the ability to transdifferentiate into ectodermal and endodermal lineages such as glial cells, neurons, hepatocytes, etc **(Fig. 2)** (111, 112, 113). In addition to being a source for many cell types,

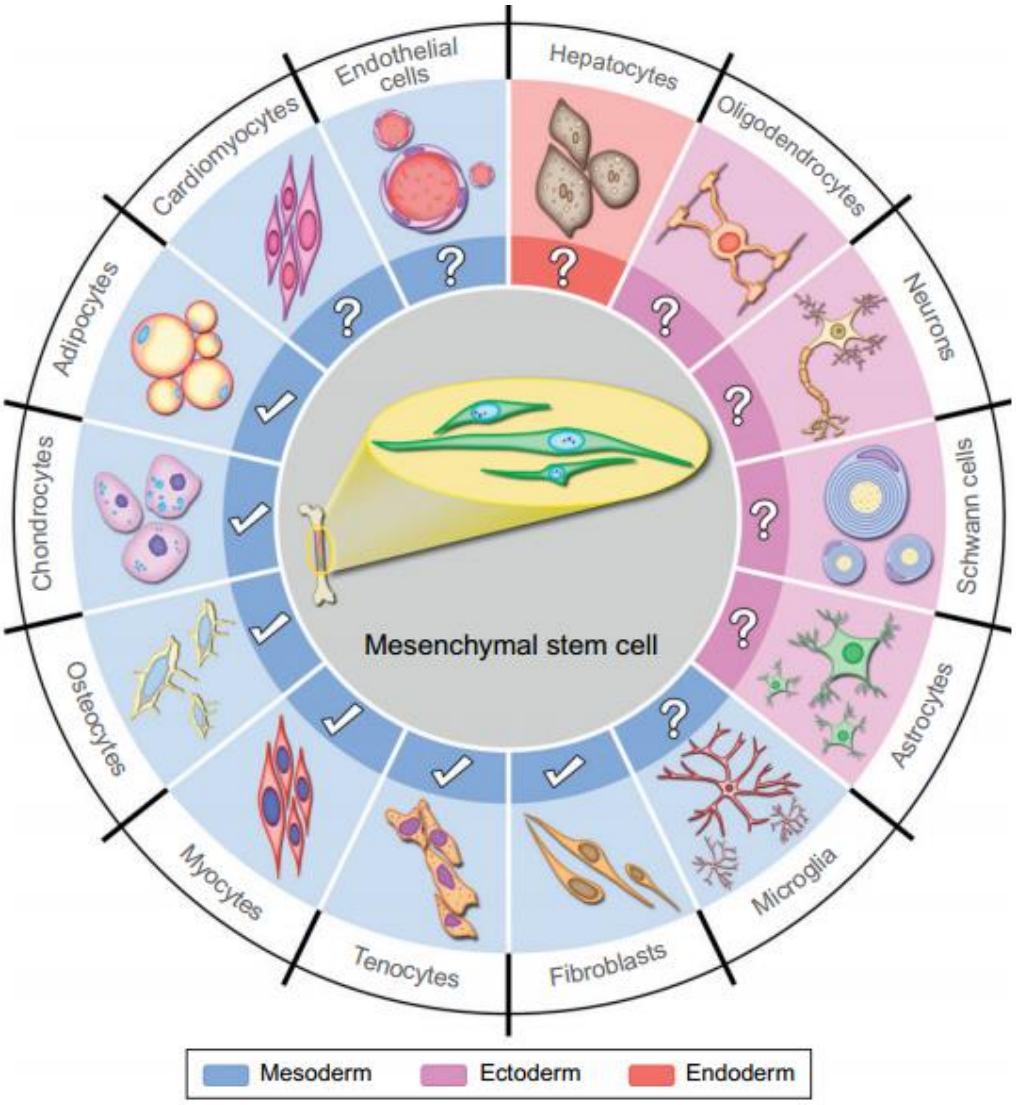

Figure 2. The potential of Mesenchymal stem cells to transdifferentiate into other cell lineages. Adapted from (11).

MSCs are easily accessible and have the ability to rapidly divide under culture, allowing them to meet the requirements of an in vitro cell system. Additionally, MSCs are excellent candidates for allogenic transplantation as they are immune privileged cells and do not require the use of immune suppressive drugs (11). Other advantages include the capacity of MSCs to release paracrine factors, survive and integrate into host tissue, concentrate in injured tissues, and their high safety and efficacy (114).

6. Mechanisms behind Nerve Regeneration potential of MSCs

Although MSCs are highly regarded for their plasticity and ability to differentiate into many cell types, there are other mechanisms by which MSCs are thought to promote and

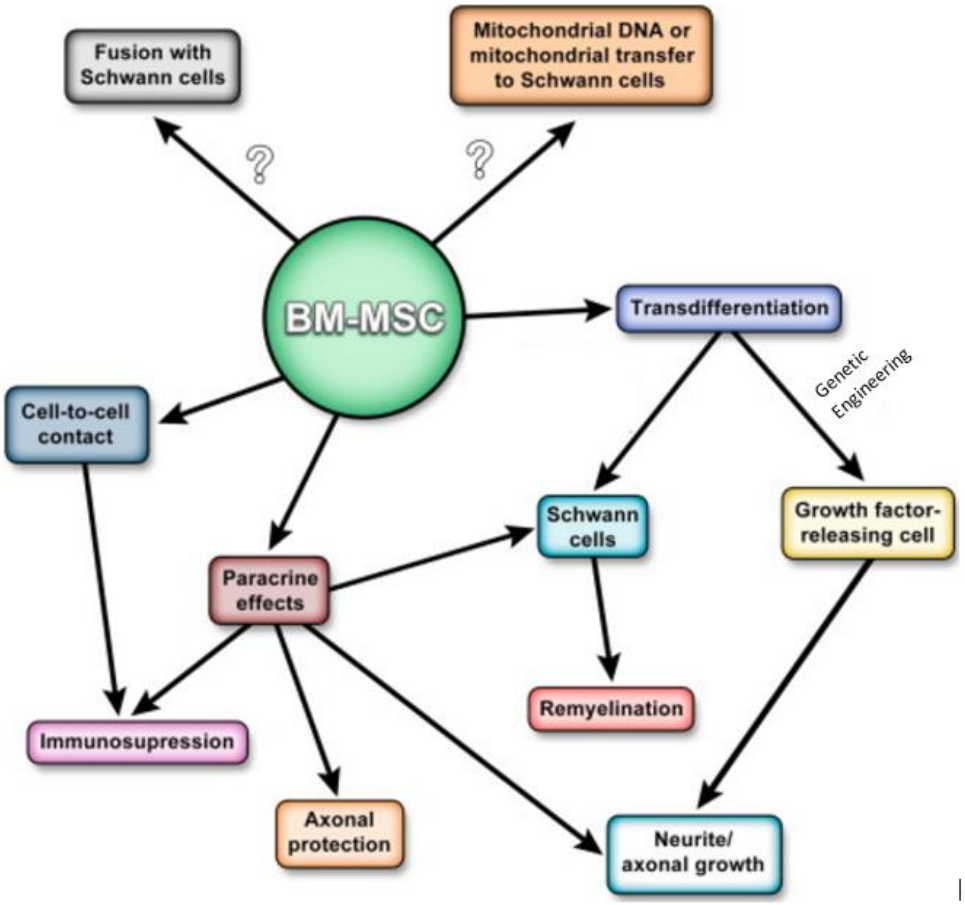

Figure 3. Various proposed mechanisms of neuronal support by MSCs. Adapted from (11).

support nerve regeneration. Such mechanisms include immunomodulation, transdifferentiation into SCs, paracrine secretions, genetic manipulations, or mitochondrial transfer/cellular fusion **(Fig. 3).**

6.1 Secretion of Neurotrophins

As discussed already, neurotrophins promote neuronal survival, help to reverse the negative effects of PNIs, and lead to Schwann cell proliferation and differentiation. One of the key ways that MSCs are thought to help in regeneration is through paracrine production of neurotrophic substances. A recent proteomic study (115) studied DRG explants and neurons co-cultured with MSCs and showed enhanced neurite outgrowth and neuronal cell survival due to the production of NGF, CNTF, BDNF, and basic fibroblast growth factor (bFGF) by MSCs. In the culture system, there was no direct contact between the neurons/explants and MSCs, leading researchers to believe that positive effects were due to the release of soluble growth factors. A similar study found that dorsal root ganglion explants treated with MSC-conditioned media also showed increased neurite outgrowth, presumably due to the presence of growth factors in the media (15). In addition to their direct paracrine effects, MSCs can induce SCs to produce neurotrophic mediators as well. In co-culture studies of rat SCs and MSCs, increased survival and proliferation rates of SCs was noted as well as high expression mRNA and protein levels of NGF, BDNF, and Trk/$p75^{NTR}$ receptors (16). The same group also examined the effect of MSCs on Schwan cells in a rat peripheral nerve repair model and showed that MSCs increased the generation of SCs and promoted SC-mediated neurotrophic functions.

After *in vitro* co-culture studies, the next step was to determine whether or not implanted MSCs continued to produce growth factors *in vivo* and if these factors were biologically active. Several studies were able to document expression of GDNF, CNTF, FGF, and even BDNF by MSCs *in vivo,* allowing for survival and elongation of the growth cone (116, 117, 17, 118). A similar study (15) implanted MSCs at a rat sciatic nerve lesion and the results demonstrated improved regeneration of motor and sensory axons due to the production of growth factors. Other studies incorporated conduits filled with mesenchymal stem cells in order to test models of long sciatic nerve gaps. For example, one group implanted a collagen conduit filled with MSCs at a mouse sciatic nerve transection lesion and saw enhanced axon regeneration and remyelination (17). Additionally, high levels of NGF and BDNF were detected, suggesting that MSCs were expressing these neurotrophins *in vivo*.

Combined, these results demonstrate that MSC-based therapy improves peripheral nerve regeneration through direct secretion of neurotrophic factors which may act locally as well as on glial cells further away.

6.2 Immuno-modulatory effects

One of MSCs most interesting features is their ability to modulate the immune system. When transplanted into tissues, MSCs actually decrease tissue inflammation and can have immunosuppressive effects by suppressing T-cell proliferation and inhibiting natural killer T cell signaling (119). Additionally, MSCs promote anti-inflammatory T helper 2 cells (120). MSCs also suppress monocyte differentiation into dendritic cells, thus decreasing the amount of antigen presentation to T cells (121). In a spinal cord injury model, MSC transplantation favored the development of M2 macrophages and suppressed M1 activation (122). M2 macrophages have anti-inflammatory activity while the classic M1 phenotype has deleterious

effects in damaged tissue (122). The complex mechanisms behind MSCs immunomodulatory properties are still being uncovered but their ability to decrease inflammation has been widely described, supporting the therapeutic merits of stem cells.

6.3 Cellular Fusion

In addition to the various nerve regeneration mechanisms discussed, a few studies have documented the spontaneous transfer of mitochondria from MSCs with a variety of other cell types. MSCs can form tunneling nanotubes through which mitochondria and nuclear DNA can be transferred. Several studies have utilized MSCs in acute pulmonary damage models to demonstrate mitochondrial transfer from MSCs to alveolar cells and airway epithelial cells (123, 124, 125). Mitochondrial transfer has also been demonstrated between mesenchymal stem cells and cardiomyocytes, causing increased proliferation and, in Acquistapace's study, reprogramming towards a progenitor-like state (126, 127,128). The majority of these studies involve use of epithelial or muscle cells; however, one study found that bone marrow derived MSCs were able to fuse with neuronal cell types, including Purkinje cells (129). To date, there is no evidence of mitochondrial transfer or MSC fusion with Schwann cells, but this could represent an alternative mechanism by which MSCs support Schwann cell activity and regeneration.

7. Clinical Trials with MSCs for Neurological Disorders

Extensive *in vitro* and *in vivo* data suggest that mesenchymal stem cells secrete several trophic factors, support neuritogenesis and neurite growth, and promote survival and elongation of damaged peripheral nerves. An even larger body of work exists, demonstrating the benefits of MSCs within the context of central nervous system disorders and spinal cord trauma, which is not covered in this review. Combined, the data has proven the safety and

efficacy of MSCs and allowed the cells to be used in human clinical trials, which is a key stepping stone to them being commonly used as a clinical therapy.

A large number of studies have reported the use of MSCs in treatment of neurological disease and trauma (130). Clinical trials range from treatment of Multiple Sclerosis to Alzheimer's disease to treatment of traumatic injury, with spinal cord injury models having the largest number of trials (Fig 4.) (131).

For clinical treatment of spinal cord injuries, Ra et al. conducted a phase 1 clinical trial in which eight patients who had suffered a spinal cord injury were infused with autologous adipose MSCs (133). After three months, no unwanted side effects were noted. A more recent phase 1 trial involved 14 patients with chronic spinal cord damage who received autologous injections of bone marrow MSCs. Patients displayed improvements in tactile sensitivity and over 50% of patients had increased lower limb motor function (134). Altogether, these clinical trials show promising uses for autologous MSCs in treating spinal cord injuries, as well as for central nervous system disorders including ALS and MS.

Fewer clinical trials have utilized mesenchymal stem cells within a peripheral nerve context. The few trials that have been performed focus on diabetic peripheral neuropathy patients. For diabetic patients, MSCs are an effective therapeutic agent due to secretion of bFGF and VEGF, and their potential to differentiate into neural cells such as astrocytes, oligodendrocytes, and Schwann cells (135). Current clinical trials are in stage II and III and revolve around change of nerve conduction velocities before and after stem cell IV transfusion. The results have yet to be published. There are no current clinical trials examining the use of MSCs in traumatic peripheral nerve damage, which may be another large area for future clinical use.

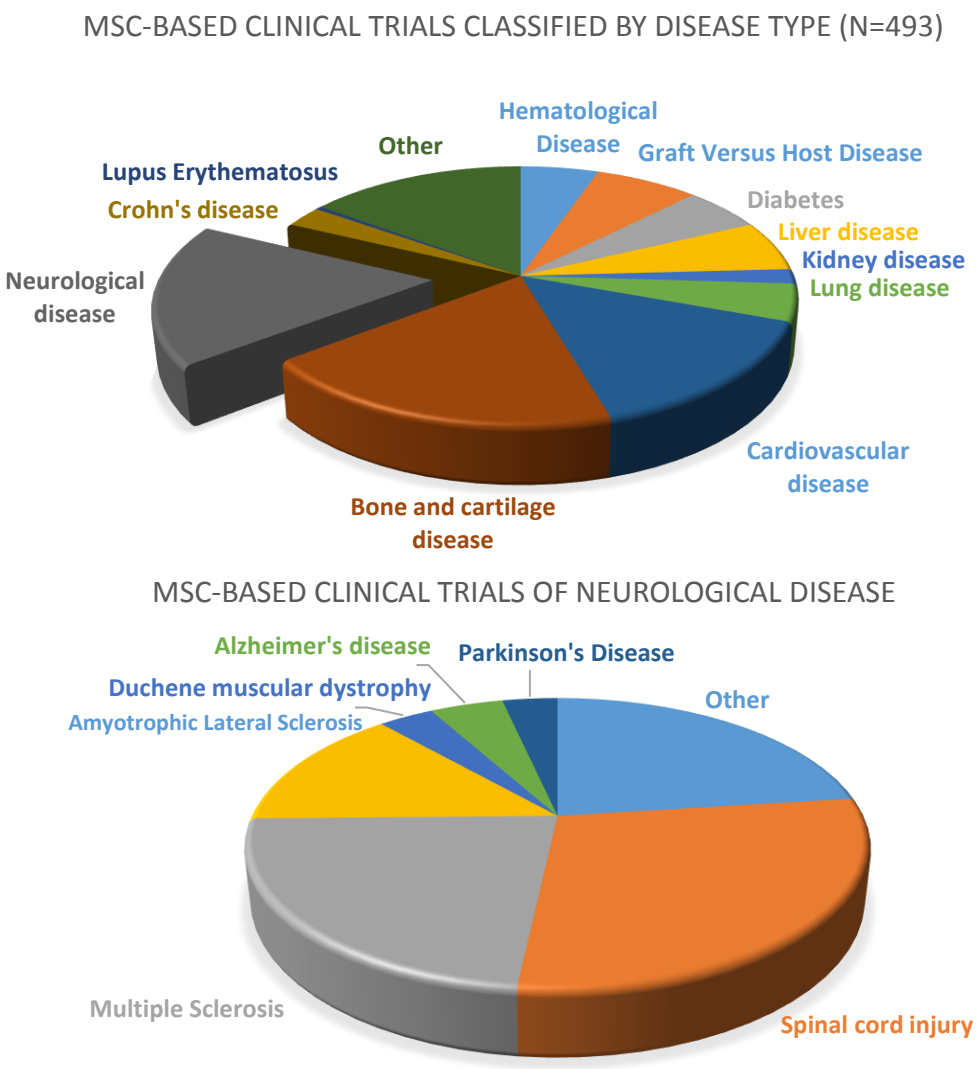

Figure 4**.** The percentage of MSC-based clinical trials classified by disease type followed by a subclassification of MSC-based clinical trials for neurological disease only. Adapted from (131).

The data obtained from clinical trials, as well as *in vitro* and *in vivo* studies shows that unaltered MSCs offer many benefits for nerve regeneration, mainly by secretion of neurotrophic factors, as well as by support of Schwann cells. However, MSCs may hold even greater potential when transdifferentiated into another cell type, such as Schwan cells. The various benefits and methods of transdifferentiated MSCs will be discussed below.

8.Transdifferentiation

Bone mesenchymal stem cells were once thought to be fairly restricted in their differentiation patterns but more studies are demonstrating that they are endowed with versatility and greater plasticity. In response to a variety of culture conditions, specialized *in vivo* microenvironments and genetic manipulations, MSCs can turn into different phenotypes such as glial cells. In particular, turning MSCs into a Schwann cell-like phenotype is of high interest due to the beneficial effects on nerve regeneration. MSCs can be transdifferentiated with a variety of methods, including the use of transplantation, small molecule cues, genetic manipulation, or as most recently described, through electric stimulation. Each method will be discussed in greater detail below.

8.1 Transdifferentiation via transplantation

During Wallerian degeneration and nerve regeneration, a wide variety of cytokines and growth factors are released, creating a specialized microenvironment which has the capacity to greatly influence cell differentiation patterns. Although controversial, these environmental signals have been utilized to transdifferentiate MSCs in response to injury or inflammation. Bone marrow derived MSCs injected at the site of a rat sciatic nerve transection were capable of surviving and migrating, as well as differentiating into an SC-like phenotype, based off of S100 immunoreactivity patterns (10). In this study, it was presumed that MSC transdifferentiation occurred in response to physiological environmental cues, as no MSC medium changes were made. Although transdifferentiation may have occurred, the percentage of cells positive for S100 was so low that this may not be a very efficacious method. Another

2010 study demonstrated similar results, with few numbers of transplanted MSCs at an injury site converting to an SC-like phenotype (136).

8.2 Transdifferentiation via co-culture

A more simplistic approach to changing a cell's microenvironment is to adjust its neighboring interactions using co-culture methods. One study showed that direct contact co-cultures of DRG neurons and MSCs could cause a phenotypic and morphological change in MSCs to resemble Schwan cells (137). Researchers suggested that the release of cytokines and other neuronal molecules on the axonal surface may play a role in the transdifferentiation process. However, this method alone did not allow tMSCs to form compact myelin, suggesting that further molecular cues are necessary for a complete transdifferentiation process. Another study looked at co-culture of MSCs with olfactory ensheathing cells and saw a dramatic increase in the number of MSCs resembling a neural morphology which were immunoreactive to various neural markers such as GFAP, $p75^{NTR}$, and MAP2 (12). These studies demonstrate that a co-culture method may be sufficient to begin the transdifferentiation protocol, but additional small molecules may be needed to affect a functional change in tMSCs.

8.3 Use of small molecules in media

Although transdifferentiation via transplantation and co-culture has shown some success, this method is not as successful or efficient as the addition of small molecules to culture medium. These specific molecules can trigger cell-signaling pathways and rapidly modulate cell phenotype. In 2001, Dezawa et al., discovered a cell medium protocol for transdifferentiation of MSCs into an SC-like morphology(138). After induction, these cells physically resembled SCs and expressed several Schwann cell markers.

Newer studies have utilized compounds such as valproic acid and other histone deacetylase

inhibitors along with neural inducing signaling molecules to create mature neural cells (139). A 2014 study used a two-step method to first create neural precursor cells, and then induced Schwann cells from human foreskin fibroblasts (13). These cells may potentially be used to treat peripheral nerve injuries in the future.

8.4 Genetic modification for transdifferentiation

A newer transdifferentiation method can now convert adult differentiated cells to a specific terminal cell type without going through pluripotency. This newer methodology is based on the idea of 'master control genes' in somatic cells which can be overexpressed to induce a cascade of cell fate changes (140, 141, 142). The earliest evidence of this possibility was provided by Weintraub et al, who confirmed conversion of fibroblasts to myogenic lineage by transfection of a master regulatory gene (*MyoD*) (143). Later, *Pax6* was recognized as a master gene responsible for neuronal differentiation. Vierbuchen et al. identified the combination of *Asc11*, *Brn2* and *Myt11* as able to convert mouse embryonic fibroblasts into mature neurons (144). Unfortunately, this method of generating target cells through transdifferentiation relies on viral expression of exogenous transcription factors which makes demonstration of safety for clinical trials difficult; however, the method holds promise for direct cellular conversion.

8.5 Electrical Transdifferentiation

Finally, a very recent study by Das et al., 2017 described a novel procedure for transdifferentiation of MSCs through the application of electrical stimuli via graphene-based electrode (145). Rat MSCs were immobilized on a graphene interdigital electrode and subjected to either electrical or chemical transdifferentiation, then expression of cell surface markers such as p75, S100, and S100β was analyzed with immunocytochemistry after 15 days. The results for electrical tMSCs were compelling, showing the highest degree of preferential

immunolabeling, with more than 85% of cells demonstrating staining for SC markers vs. 75% for chemically transdifferentiated MSCs. Additionally, electrically stimulated cells secreted significantly higher levels of NGF as compared to their chemically transdifferentiated counterparts. Although not statistically significant, higher levels of BDNF and GDNF were also noted. While other reports have shown that electrical stimulus can increase growth factor level production in Schwann cells (146, 147, 148), this paper is the first to describe such effects in transdifferentiated MSCs. Furthermore, Das et al., demonstrated that electrical stimuli alone can transdifferentiate MSCs to an SC-like phenotype without the need for chemical growth factors, thus saving researchers time, labor, and money, while providing a novel system for an artificial neural network circuit.

8.6 Beneficial properties of tMSCs

Once methods of transdifferentiation had been discovered, scientists moved on to *in vivo* studies to determine the effect of tMSCs on models of peripheral nerve injury. Once Dezawa et al. performed their *in vitro* cell characterization, tMSCs were transplanted into the cut end of a rat sciatic nerve. Results showed that the transplanted cells remained in a Schwann-cell like state and were capable of forming myelin sheaths, as well as supporting nerve fiber regrowth (111). Additionally, Dezawa and collaborators also showed that tMSCs colocalized with the myelin-associated glycoprotein antibody signal, suggesting that MSCs may be able to differentiate into myelinating cells. After this initial trial, many labs followed suite by implanting transdifferentiated MSCs into a variety of peripheral nerve and spinal cord injury models. In 2004, Mimura et al. supported Dezawa's work by showing that human and rodent MSC-derived Schwann cells expressed myelin-related markers and contributed to re-myelination when transplanted into a rat sciatic nerve injury (149). Using a similar

transdifferentiation protocol, Keilhoff et al. (2006) also demonstrated that transplanted tMSCs within a muscle conduit promoted remyelination, and electron microscopy showed that single tMSCs were even capable of wrapping more than one axon, similar to an oligodendrocyte (18).

In addition to providing functional support, transdifferentiated MSCs are capable of producing trophic factors at even higher levels than Schwann cells. When transdifferentiated MSCs were placed in a DRG co-culture system without direct contact, tMSCs showed upregulation of BDNF and NGF. Additionally, neurite outgrowth was observed even in the presence of NGF and BDNF blocking antibodies, suggesting that other trophic cytokines or factors may be produced by tMSCs (150). Another interesting study used a combination of two different mediums to transdifferentiate MSCs, causing them to produce large amounts of BDNF and GDNF. Interestingly, cells resembled an astrocyte morphology and expressed certain astrocyte markers. When transplanted, the cells improved muscle reinnervation and restored motor function in a rat sciatic nerve crush model (14). Combined, these results confirm that MSCs display functional characteristics similar to SCs by secreting bioactive neurotrophins.

Soon after the introduction of tMSC transplants, scientists began to question the duration of a Schwann cell-like state once cells were placed in an *in vivo* environment. A study by Shimizu et al., 2007 transplanted MSC Schwan-like cells within a transpermeable tube into a rat sciatice nerve gap (151). After three weeks, tMSCs continued to express SC markers such as p75, GFAP and increased S100 expression. Most importantly, the MSCs expressed myelin-associated markers such as MAG and MBP even after three weeks in vivo, which the authors contend supports the thesis that MSCs may retain SC-like characteristics even after transplantation. It is important to note however, that remeylination was not seen via IHC or

electron microscopy, as in other studies. A different study by Ishikawa et al., 2009 transplanted MSC-derived Schwann cells within chitosan gel sponges and found that cells were able to form myelin sheaths one month after transplant (152). The mean diameter of myelinated fibers increased continuously, even out to four months post-transplant. This study, along with the work by Dezawa et al., 2001, demonstrates that rat tMSCs may contribute to remyelination after transplantation into an injured PNS model. Similar results have been found in spinal cord injury models (153, 154, 155), indicating that MSC-derived Schwann cells are effective for both PNS and CNS regeneration. These studies suggest that MSCs are capable of expressing Schwann cell biomarkers, may express myelin markers, and may even physically form myelin sheaths. Moreover, these effects may last well past the time that MSCs were last exposed to transdifferentiation media, suggesting that the acquired Schwann cell-like state is at least semi-permanent and allows cells to persist well into the acute phase of Wallerian degeneration.

Unfortunately, there have never been clinical trials involving the use of chemically transdifferentiated MSCs for the nervous system. However, a primate study has been completed as an important pre-clinical step. Wakao et al. 2009 used a monkey model and followed subjects out to a year post transplant (156). MSCs were chemically induced to resemble Schwan cells and cell marker expression patterns were confirmed with both immunocytochemistry and reverse transcription-PCR. Cells were transplanted for one year in a median nerve transection model. During this year, no major health abnormalities were observed in the monkeys. Ki-67 immunostaining revealed no signs of massive proliferation and the ^{18}F-FDG-PET scan which detects neoplastic cells, demonstrated no abnormalities. Furthermore, monkeys regained function, and electrophysiology with histology revealed restoration of the severed nerve. This study is particularly important because it demonstrated

not only the efficacy of transdifferentiation, but also the safety of long term implantation of tMSCs.

9. Genetically modified MSCs

The literature has aptly demonstrated that undifferentiated MSCs can produce neurotrophins *in vitro* as well as *in vivo* and that the process of transdifferentiation can even further increase production levels. Only in recent years have researchers begun to investigate the continuous production of these proteins via functional gene insertion. As one of their novel features, MSCs are suitable for transduction and expression of exogenous genes, making them a good candidate system for genetic engineering. The most widely used systems are now either lentivirus or retrovirus based. In regards to nervous system disorders, MSC lines have been created to over express a wide variety of neurotrophins such as GDNF, NGF, and BDNF (19), as well as other growth factors. Pre-clinical studies by Sharma et al., 2015, demonstrated that genetically modified MSCs (BDNF-GFP) had similar viability and proliferation rates when compared to non-genetically modified MSCs (157). One 2009 study by Bauer et al., even went so far as to develop an in depth biosafety model to specifically assess the risk of retro- and lentiviral vectors (158). Human hematopoietic stem cells and MSCs were transduced with Moloney murine leukemia virus and transplanted into 481 immunodeficient mice. There was no detectable evidence of insertional mutagenesis leading to human leukemias or solid tumors during the 18 months animals were studied. Additionally, no vector-associated adverse events were observed and in 117 serum samples analyzed, there was no detectable viral DNA. These findings indicate that virally transfected MSCs are stable and may act biologically similar to the wild type MSC population, making them suitable for *in vivo* study use in a variety of disease and injury models. Genetically modified MSCs have been used in studies ranging from the

treatment of neurodegenerative disorders, to ischemic injury, to spinal cord crush injuries and peripheral nerve transections. Studies in each of these areas will be discussed below.

9.1 Use of genetically modified MSCs in neurodegenerative disorders

Parkinson's Disease

Use of glial derived neurotrophif factor (GDNF) was first described in 1993 as a potential treatment for Parkinson's disease because of its ability to increase dopamine uptake and aid in the survival of embryonic midbrain dopaminergic neurons (159). With the challenge of administering GDNF infusions, cell based strategies to deliver GDNF have received recent attention. In a recent study, MSCs transduced with a GDNF retrovirus vector increased dopaminergic neuron sprouting (160). A similar study found that injections of GDNF MSCs given one week before a lactacystin lesion of the medial forebrain also significantly increased dopamine levels (161). Furthermore, Ren and colleagues (2013) transplanted GDNF MSCs into the brain of non-human primates and saw increased dopamine levels and improved contralateral limb function (162). Preclinical studies provide evidence that GDNF MSCs provide high levels of a functional trophic factor, which, with further safety and efficacy data, could be used in clinical trials as adjunct treatment for Parkinson's disease.

Alzheimer's Disease

Treatment options for Alzheimer's disease are limited and focus on symptoms related to neurotransmitter systems, rather than targeting the underlying pathologies. Given the prevalence of the disease and lack of treatments, new strategies are being developed which focus around the use of nerve growth factor. Autologous fibroblasts engineered to express NGF were transplanted in eight patients with Alzheimer's. Patients saw an improvement of Mini-Mental Status Examination scores and a reduced decline in cognitive scores (163). A phase II

clinical trial is still open for this method (19). MSCs have not directly been used in human clinical trials yet, however, promising work by Li et al. (2008) demonstrated reduced memory deficits in the Morris-water-maze task in a rat model when NGF MSCs were transplanted to the hippocampus (164). The next step in research should include further *in vivo* transplantation trials with NGF MSCs in both rat and human models.

Huntingtin's Disease

Compared to the other neurodegenerative diseases discussed, Huntington's disease is unique in that clinical signs may be directly correlated to reduced levels of a neurotrophic factors, BDNF. Low BDNF levels in the striatum are due to loss of function of the wild-type huntingtin protein. This protein modulates BDNF transcription and plays a role in BDNF transport and secretion (165). The Dunbar laboratory first demonstrated that murine MSCs engineered to overexpress BDNF improved disease progression on a transgenic mouse model of Huntingtin's (166). Important pre-clinical trials by Pollock et al., 2016 utilized a double-blind study to examine the effects of transplanted human BDNF MSCs on disease progression in a mouse Huntingtin's disease model (167). Treatment with MSCs decreased striatal atrophy and significantly reduced anxiety. BDNF MSC treatment also increased the mean lifespan of mice. This study demonstrated the efficacy of BDNF hypersecreting MSCs as a medical therapy for Huntingtin's disease and set the groundwork for future clinical trials.

9.2 MSCs for Ischemic Brain Injury

Ischemic brain injury causes the death of various important cell types such as neurons, glial, and endothelial cells. Regain of function and brain tissue repair necessitates cell replacement and formation of a new network (168). When transplanted into ischemic regions of the rat brain, MSCs reduced functional deficits after 14 days, scar thickness was decreased,

and the number of proliferating cells in the subventricular zone was increased (169, 170, 171). Improvement by MSC treatment has been attributed to decreased apoptosis, MSC differentiation into neuronal cells, and promotion of neurogenesis, angiogenesis, and synaptogenesis (172, 173, 174, 175). Several groups have used genetically modified stem cells that overexpress growth factors known to enhance neuronal survival. One of the first factors studied in MSCs was BDNF and GDNF. When BDNF overexpressing MSCs were delivered to an ischemic brain model via injection, infarct volume was reduced and functional outcome was improved (176, 177, 178). Furthermore, BDNF expressing MSCs can significantly improve behavioral test results and reduce ischemic damage via MRI analysis after 7 and 14 days (177, 179).

9.3 Spinal Cord Injuries

In addition to various therapies within the brain, modified MSCs have been used with variable success in the spinal cord. In a 2005 study by Lu, Jones, and Tuszynski, BDNF MSCs were injected into a crushed spinal cord injury and the extent and diversity of axonal growth was increased (180). Additionally, Schwann cells preferentially migrated to the BDNF secreting grafts. Unfortunately, functional recovery was not achieved for any of the studied rats. A similar study was performed by Sasaki et al., 2009, in which BDNF secreting human MSCs were implanted at a T9 spinal cord lesion (181). After five weeks, locomotor improvement was observed for the BDNF group and there was increased axonal sprouting. Specifically, an increased number of serotonergic fibers were observed in the ventral horn grey matter, an area important for motor controlled movement. Unlike the 2005 Lu study, Sasaki's group demonstrated that BDNF MSCs are associated with improved functional outcome following a spinal cord injury. Due to the conflicting data reports, additional studies are

necessary before the full benefits of BDNF can be determined for the treatment of spinal cord damage.

9.4 Peripheral nerve injury

Of all the disease models discussed so far, peripheral nerve injuries have the fewest published studies involving transplantation of genetically modified MSCs. This may be due to the fact that researchers are now utilizing a multi-disciplinary approach and studies often involve the use of engineered conduits, cell transplants, and now even gene therapy. One of the first studies to use a MSC gene delivery system transplanted MSC spheroids transfected with the BDNF gene (182). Spheroids were combined with a polymeric nerve conduit to bridge a 10 mm rat sciatic nerve transection gap. MRI was used to track the transplanted cells. Animals receiving the BDNF MSC spheroids had the shortest gap bridging time, the largest regenerated nerve, and the thickest myelin sheath at 31 days. Furthermore, BDNF spheroids significantly promoted functional recovery. Another, more recent study (183), combined multi-channel agarose scaffolds with BDNF MSCs to bridge a 15 mm sciatic nerve transection gap. Additionally, the distal sciatic nerve segment was injected with the BDNF lentiviral vector. Twelve weeks after injury, BDNF secreting cells significantly increased axonal regeneration and injection of the lentiviral vector at the distal segment enhanced axonal regeneration beyond the lesion. Finally, a newly published February 2017 study actually looked at the efficacy of BDNF *ex vivo* gene transfer to umbilical cord blood derived MSCs in a rat sciatic nerve crush injury model (184). Four weeks post-surgery, the BDNF expressing MSCs exhibited more peripheral nerve regeneration than the controls. Additionally, sciatic function index, axon counts, and axon density were significantly increased for both the BDNF MSC and regular MSC groups. The results from these works are promising and indicate that

in particular, BDNF hypersecreting MSCs can improve sciatic nerve regeneration. Unlike other areas of research, no pre-clinical characterization studies looking at safety and appropriate dosage ranges have been published and this would be a necessary next step before BDNF MSCs could be tested outside of a rat model.

10. Conclusions and Future Directions

Peripheral nerve injury limits mobility and sensation in up to 2.8% of all trauma patients and often results in unsatisfactory return to function (185). Although the gold standard of microsurgery with autograft has seen advances in the last decade, there are significant drawbacks associated with the procedure. For this reason, scientists have proposed the use of an alternative transplant type, in the form of autologous stem cells. Specifically, research is directed at the conversion of mesenchymal stem cells towards a Schwann cell-like fate in order to aid in Wallerian degeneration, neuronal regeneration, and possibly even remyelination. Additionally, MSCs have their own unique benefits such as immunomodulatory properties, secretion of neurotrophic factors, possible mitochondrial transfer, and the ability to be easily genetically modified. In order to resemble a Schwann cell, MSCs must undergo transdifferentiation which can be achieved through a variety of methods including incorporating specific factors into the growth media, co-culture method, i*n vivo* transdifferentiation, and others. Although these older techniques have their benefits, methods of transdifferentiation have changed drastically within the last ten years and now include master gene modification and the use of specific cell signaling molecules combined with histone deacetylase inhibitors.

As demonstrated by the newer body of literature, scientists are beginning to move away from the use of bone marrow MSCs and are instead using a cell type which is even easier to

harvest such as fibroblasts, adipocytes, and even hair follicle stem cells (186, 187,13).These studies rely largely on immunocytochemical staining, co-culture neurite outgrowth, and gene expression patterns to support transdifferentiation of cells into Schwann cells. Unfortunately, none of these studies have measured growth factor secretion levels from transdifferentiated cells, and only Thoma's study looked at the ability of these cells to create myelin. In order to truly assess whether or not these transdifferentiated cells are Schwann cells, future work should test growth factor secretion, perform patch-clamp recordings, transplant cells into rat sciatic nerve models, and examine myelin formation via electron microscopy (188).

In addition to testing new cell types, researchers are trying new methods of transdifferentiation and emphasizing the use of genetic control and epigenetic cues. Future research may focus on Schwann cell de-differentiation or multi-step transdifferentiation in which a less-differentiated intermediate is first created, and then the fully transdifferentiated cell type is achieved, such as in Thoma et al.'s work with fibroblasts. While these cell fate reprogramming methods are promising, they can often be time consuming, difficult to consistently reproduce, and cost prohibitive. Additionally, studies have yet to be performed which examine the tumorigenic capacity of these cells and their long term genetic stability. While the field of transdifferentiation still has many challenges to overcome, it is a promising focus in the study of regenerative medicine and offers new insight into cell fate plasticity.

Specifically, in regards to the peripheral nervous system, researchers have shown that a variety of regenerative cell types may act like Schwann cells by secreting trophic factors, supporting re-myelination, and decreasing time to functional return of severed nerves. Additionally, when transdifferentiated cells are combined with multiple neuro-regenerative strategies such as *ex vivo* gene delivery, and biomaterial conduits, they may become powerful alternatives to traditional peripheral nerve regeneration therapies.

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CHAPTER 3

TRANSDIFFERENTIATION OF BDNF-SECRETING MESENCHYMAL STEM CELLS SIGNIFICANTLY ENHANCES SCHWANN CELL MARKER PROTEINS AND MAINTAINS BDNF SECRETION LEVELS

Modified from a paper submitted to *Journal of Bioscience and Bioengineering*

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1. Abstract

The use of genetically modified mesenchymal stem cells (MSCs) is a rapidly growing area of research targeting delivery of therapeutic factors for neuro-repair. Cells can be programed to hypersecrete various growth/trophic factors such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) to promote regenerative neurite outgrowth. In addition to genetic modifications, MSCs can be subjected to transdifferentiation protocols to generate neural cell types to physically and biologically support nerve regeneration. In this study, we chose to combine these two different strategies and evaluated the impact of transdifferentiating genetically modified MSCs into a

Schwann cell-like phenotype. After 8 days in transdifferentiation media, approximately 30-50% of transdifferentiated BDNF-secreting cells immunolabeled for Schwann cell markers such as S100, S100β, and p75NTR. Similar results were observed 20 days after inducing transdifferentiation with minimal decreases in expression levels. BDNF production was quantified by ELISA, and its biological activity tested via the PC12-TrkB cell assay. Importantly, the bioactivity of secreted BDNF (45.2 ng/mL/10^6cells/day) was verified by the increased neurite outgrowth of PC12-TrkB cells. These findings demonstrate that not only is BDNF actively secreted by the transdifferentiated BDNF-MSCs, but also that it has the capacity to promote neurite sprouting and regeneration. Given the fact that BDNF production remained stable for over 20 days, we believe that these cells have the capacity to produce sustainable, effective, BDNF concentrations over prolonged time periods and should be tested within an in vivo system for future experiments.

2. Introduction

Peripheral nerve injuries occur as the result of sudden trauma and can lead to loss of sensory and motor function to peripheral limbs (1). Many surgical procedures are available to halt the propagation of nerve damage, and the adoption of a particular procedure depends on the extent of injury. Epineural sutures are considered the standard of care in the case of transection injuries (2). Another surgical procedure, autologous nerve grafting, is widely used in cases of gap formation (2, 3, 4). Although these surgical procedures provide many benefits, there are still considerable limitations associated with them such as donor site morbidity, neuroma formation, fascicle mismatch, and scarring (5). To overcome such restrictions, researchers have explored various avenues to improve post-surgical outcomes(6, 7, 8, 9, 10, 11). The most commonly studied methods include: cell transplantation, delivery of growth

factors which stimulate regenerating axons, and implanting nerve regeneration conduits containing replacement cells at the site of injury (7, 12, 13, 14). Schwann cells (SCs), which are peripheral glial cells, play an important role in nerve regeneration by clearing out debris from the site of injury. Additionally, they release growth factors to stimulate myelination and axonal regeneration (15, 16). Various cell types including embryonic stem cells (17), umbilical cord-derived stem cells (18), bone marrow-derived mesenchymal stem cells (MSCs)(19), adipose-derived stem cells (20), olfactory ensheathing cells (21), and dental pulp-derived stem cells (22) have been transplanted to replace native SCs, and each has reported enhanced nerve regeneration. Mesenchymal stem cells in particular are preferred due to benefits like autologous transplantation, routine isolation procedures, and paracrine and immunomodulatory properties [17-19]. Mesenchymal stem cells have been transplanted at the site of injury either directly in their native form (undifferentiated) or in a SC-like form (transdifferentiated). Additionally, some studies have transplanted *ex-vivo* genetically modified MSCs that hypersecrete growth factors normally secreted by Schwann cells during axonal regeneration (23). Here we chose to focus on brain-derived neurotrophic factor (BDNF) delivery because it has been shown to provide neuroprotection and facilitate the rescue and repair of damaged neurons (24, 25, 26, 27) . BDNF is responsible for neurogenesis and helps with survival and growth of various types of neurons such as dorsal root ganglion neurons and cortical neurons. It is also widely explored as a therapeutic agent to target various neurodegenerative conditions (28, 29).In this study, we chose to evaluate the impact of transdifferentiating BDNF hyper-secreting MSCs. Previously, we modified undifferentiated mesenchymal stem cells to hypersecrete neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF)(30).

Additionally, we successfully transdifferentiated these MSCs into an SC-like phenotype on micropatterned substrates (31). Furthermore, MSCs transdifferentiated to SC-like cells have been shown to enhance peripheral nerve regeneration in a number of studies (32, 33, 34, 35). Here, we synergistically combined genetic modification and transdifferentiation to create MSCs that facilitate neurite outgrowth. These transdifferentiated MSCs showed various SC-like characteristics such as bipolar spindle-shaped morphology, expression of SC marker proteins (S100, S100β, and $p75^{NTR}$) and increased release of BDNF. In this study, we successfully transdifferentiated BDNF-hypersecreting MSCs into a SC-like phenotype and quantified their morphological, molecular, and functional changes.

3. Materials and Methods

Mesenchymal Stem Cell Isolation and Culture

Mesenchymal stem cells (MSCs) isolated from adult mice were obtained from the Texas A&M Health Science Center College of Medicine, Institute for Regenerative Medicine. MSCs were maintained as an adherent cell line in Iscove's Modified Dulbecco's Medium (IMDM; 12440-053; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; SH30071.03; Hyclone, South Logan, UT), 10% donor equine serum (SH30074; Hyclone), 2 mM L-glutamine (25030-081; Invitrogen), and antibiotic-antimycotic solution (1%, 15240-096; Invitrogen; 10,000 U/mL penicillin, 10,000 μg/mL streptomycin, 25 ng/mL amphotericin B). Cells were incubated at 37°C with 5% CO_2 humidified atmosphere. When cultures reached 75% confluence, MSCs were harvested from the flask using 0.25% trypsin and 1 mM EDTA solution (**25200-056;** Invitrogen) and were centrifuged at 500 rpm for 5 min. MSCs were subsequently plated into T-25 culture flasks (25 cm^2) at 75-150 cells/cm^2. Fresh media was added every other day to feed the cells. Lentiviral vectors were used to engineer MSCs to

produce and secrete brain-derived neurotrophic factor (BDNF; human cDNA) as well as green fluorescent protein (BDNF-GFP-MSCs), as previously reported (8, 36). A similar method was utilized to generate the GFP expressing mouse MSC line (GFP-MSCs). These cells were obtained from a previous study (30).

In Vitro Transdifferentiation of MSCs

Sub-confluent MSCs were subjected to a three-step chemical transdifferentiation following a previously established induction protocol (31) (modified from Dezawa et al. (35)). First, for 24 hours, cells were placed in Transdifferentiation Media 1 (TDM1) that consisted of IMDM and 1 mM β-mercaptoethanol (BME; M6250; Sigma-Aldrich, St. Louis, MO). Subsequently, for 72 hours, cells were placed in TDM2 that consisted of IMDM, 5% FBS, 5% equine serum, and 35 ng/mL all-trans retinoic acid (ATRA; R2625; Sigma-Aldrich). Finally, cells were placed in TDM3 for 8-20 days. TDM-3 consisted of IMDM, 5% FBS, 5% equine serum, 14 μM forskolin (FSK, 344270; EMD Millipore, Billerica, MA), 5 ng/mL platelet derived growth factor (PDGF; H8291-10UG; Sigma-Aldrich), 10 ng/mL basic fibroblast growth factor (rhFGF, basic; G5071; Promega, Madison, WI), and 200 ng/mL recombinant heregulin β1 (HRG, PF048-50UG; Calbiochem, EMD Millipore). After 8 and 20 days *in vitro* (DIV), cells were counted and plated at 2,000 cells/well in a 96 well plate (655090; Greiner Bio One; Monroe, NC). Immunocytochemistry was used to characterize the control, undifferentiated MSCs (uMSCs), and transdifferentiated MSCs (tMSCs).

Immunocytochemistry

Cells were allowed a total of 48 hours to re-attach and proliferate within the 96-well plate. After this time, all wells were rinsed twice with 0.1 M PO_4 buffer and fixed for 20 minutes with cold 4% paraformaldehyde in 0.1 M PO_4 buffer. Then, cells were rinsed 3 times

with filtered phosphate buffered saline (PBS; BP2944100; Fisher-Scientific, Waltham, MA) every seven minutes. Cells were incubated in blocking solution consisting of PBS with 5% normal donkey serum (NDS; 017-000-001; Jackson ImmunoResearch, West Grove, PA), 0.4% bovine serum albumin (A9647; BSA; Sigma), and 0.2% Triton X-100 (85111; Fisher Scientific).

A panel of antibodies was used for immunocytochemistry (ICC) analysis to compare uMSCs vs. tMSCs (Table 1).

Table 1. Primary antibodies used for immunolabeling of transdifferentiated MSCs

Primary Antibody	Concentration	Marker	Source
Rb-α-S100	1:500	Calcium binding protein – SC marker	Sigma-Aldrich (S2644)
Mo- α-S100β	1:1000	Calcium binding protein – SC marker	Abcam (ab11178)
Rb-α-p75^{NTR}	1:1000	Neurotrophin receptor – glial marker	Promega (G3231)
Mo-α-TUJ1	1:200	βIII-Tubulin – neuronal marker	R & D systems (MAB1195)
Rb-α-GFAP	1:200	Intermediate filament – SC marker	EMD Millipore (MAB360)
Rb- α-Ki67	1:200	Proliferation marker	Abcam (ab16667)

The primary antibodies were diluted with blocking solution and cells were incubated overnight at 4°C. Following incubation, cells were rinsed with PBS 4 times every 8 minutes, and incubated in secondary antibodies diluted in blocking solution. The following secondary antibodies were used: Donkey-α-Mouse Cy3 (715-165-51; Jackson ImmunoResearch Labs, 1:500) and Donkey-α-Rabbit Cy3 (711-165-152; Jackson ImmunoResearch Labs, 1:500). Cell nuclei were stained with DAPI (2-(4-amidinophenyl)-1H -indole-6-carboxamidine) (D3571; Invitrogen, 1:2,000) and incubated at room temperature in the dark for 60-90 minutes. Cells

were then rinsed with PBS 3 times every 7 minutes. Controls included cells incubated without any primary or secondary antibodies, as well as cells with only secondary antibody applied.

Image Analysis

All cell imaging was performed on the ImageXpress Micro high content screening system (Molecular Devices, Sunnyvale, CA). After the ICC, each 96-well plate was loaded into the ImageXpress MICRO and allowed to equilibrate for 20 minutes at 37° C. Plates were imaged using the 20x objective and a total of 64 microscopic fields per well were taken, for a total of 6,144 images per wavelength, per plate. Three wavelengths were selected for our experiments: Cy3 (550 nm), GFP (395 nm), and DAPI (358 nm). Images were analyzed via a multiwavelength cell scoring module on the MetaXpress 4.0 software (Molecular Devices). A threshold of intensity level above local background was set based upon the presumption that uMSCs would express minimum fluorescent levels of the antibody analyzed. Cells with fluorescence levels higher than the threshold were marked as positive. Other parameters such as minimum and maximum cell width, minimum stained area, and cytoplasmic vs. nuclear staining were taken into account during the analysis. For a more detailed procedural description, please refer to Sharma et al. 2015 (31). The percentage of positively stained cells was calculated by dividing the number of cells immunoreactive to each antibody by the total number of DAPI-stained nuclei per image. Every Cy3 and DAPI image was analyzed in order to calculate the average percentage of Cy3 expressing cells per well. Subsequently, the average percentage of Cy3 expression was calculated according to cell type and averaged across a total of four 96-well plate replicates. The Tukey-Kramer Corrections or a student's t-test test was used to compare means for all data analysis using R open software and GraphPad open

software. A p-value ≤ 0.05 was considered significant. Error bars in graphs represent the standard error.

ELISA of BDNF Production

An enzyme-linked immunosorbent assay (ELISA) was used to quantify BDNF release from the genetically modified MSCs (GFP-MSCs and BDNF-MSCs). The E_{max} Immunoassay was used (G7610; Promega, Madison, WI) to measure levels of BDNF in conditioned media from BDNF-MSCs and GFP-MSCs for 48 hours. Cells were plated at 10,000 cells per well in a six-well plate and allowed to grow for 48 hours. Conditioned media was collected and immediately frozen at -20°C. The ELISA was performed as per the kit manufacturer's instructions.

PC12-TrkB cell neurite outgrowth assay

PC12-TrkB cells were used to assess the bioactivity of BDNF released from MSCs. Rat pheochromocytoma derived PC12 cells, which were genetically modified to express the TrkB neurotrophin receptor, were provided by Dr. Moses Chao (Helen L. and Martin S. Kimmel Center for Biology and Medicine at the Skirball Institute for Biomolecular Medicine, New York Univ.). Cells were maintained in RPMI-1650 (ATCC Cell Biology; Manassas, VA) containing 10% heat inactivated equine serum (SH30074; Hyclone, South Logan, UT*)* and 5% fetal bovine serum. Cells were incubated at 37°C with 5% CO_2 . To observe neurite outgrowth, PC12-TrkB cells were plated at 3,000 cells per well of a 96 well plate. Using the condition media collected from MSCs (see above), PC12 cells were grown in a 50:50 mixture of condition media and PC12 maintenance media (MM). Twenty ng of human recombinant BDNF in PC12 MM was used as a control (rhBDNF; 248-BD005; R&D systems, Minneapolis, MN). Cells were allowed to adhere for 48 hours and were subsequently fixed with 4%

paraformaldehyde in 0.1 M PO_4 buffer. Neurite outgrowth was visualized with an Anti-Beta III tubulin Cy3 conjugated antibody (AB15708C3; EMD Millipore, Billerica, MA; 1:100). For image analysis, 25 microscopic fields in each well were taken randomly using a 20x objective on the ImageXpress MICRO high content screening system. The MetaXpress 4.0 software's *neurite outgrowth module* was used to calculate the average neurite length per cell (microns) for each condition.

Morphometric analysis

Changes to cell morphology were analyzed using the parameters of aspect ratio and total cell area. The aspect ratio is the ratio of a cell's length (longest dimension) to its breadth (shortest width), and is expected to be a value of one or greater. A value of one is seen for objects whose length and width are the same, such as circles/regular polygons. Several studies demonstrate that aspect ratio of MSCs may affect their lineage commitment (31, 37). For our purposes, aspect ratio was used as an indicator of cellular elongation when comparing uMSCs and tMSCs.

Thirty cells per condition (BDNF uMSC, BDNF tMSC, GFP uMSC, GFP tMSC) were analyzed. Four experimental replicates were analyzed for a total of 120 cells per condition. This protocol was previously established using the MetaXpress software Morphometric Analysis program (31). A student's t-test test was used to compare means within the same cell type on GraphPad prism v6 software (GraphPad Software, Inc., La Jolla, CA). A p-value $\leq$ 0.05 was considered significant. Error bars in graphs represent the standard error.

4. Results

Characterization of uMSCs

In a previous study by Sharma et al. 2015 (30), the viability and proliferation of BDNF-GFP and GFP mouse MSCs were compared to the original population of non-genetically modified MSCs using a number of *in vitro* assays such as propidium iodide staining, Ki67 immunolabeling, and cellular migration via time-lapse digital imaging. The results demonstrated no significant differences between genetically modified MSCs and unmodified MSCs, confirming the health of BDNF-GFP and GFP expressing MSC lines.

Effect of Chemical Transdifferentiation on Cell Morphology

Cells which were exposed to transdifferentiation media appeared bipolar and assumed a spindle cell appearance, whereas control cells exhibited a typical fibroblast-like morphology (Fig. S1). This observation was analytically supported via MetaXpress morphometric analysis (Fig. 1). The aspect ratio, an indicator of cell elongation, was calculated to compare the phenotype of transdifferentiated cells to control cells. A ratio close to one indicates cells with a more circular shape, whereas a ratio > 1 is produced by cells with a linear, elongated morphology. All four of our cell types had aspect ratios greater than 1, indicating cell length is greater than breadth. The aspect ratio of GFP tMSCs (4.1 ± 0.32) was significantly higher than uMSCs (1.8 ± 0.33), indicating that transdifferentiated cells are more elongated and have a bipolar morphology (Fig. 1A). BDNF tMSCs also had a higher aspect ratio of 3.4 as compared to uMSCs (3.07); however, the difference was not significant (Fig. 1A). Average total cell area was also compared between the cells grown in MM and TDM. Average cellular area was: BDNF uMSCs – 939.5 μm^2, BDNF tMSCs – 1347.1 μm^2, GFP uMSCs – 1106.6

μm^2, and GFP tMSCs – 1192.5 μm^2. While both types of tMSCs had a higher average area, there was no significant difference between any of the cell types (Fig. 1B).

After 8 days growth in TDM3, a significantly larger number of BDNF tMSCs were positively labeled by anti-S100, anti-S100β, and anti-$p75^{NTR}$. Specific staining was not seen for GFAP or TuJ1 for any cell type. S100 immunolabeling was identified in the cytoplasm with only minor staining seen in the nucleus, consistent with expected findings (Fig. S2A and B). Transdifferentiated BDNF cells showed approximately a six-fold higher percentage of S100 immunolabeling compared to uMSCs (Figure 4A): 42% ± 9.9 versus 6.9% ± 4.4, respectively. Anti-S100β binds to the glial specific S100β protein, and is a more exclusive marker than anti-S100, which is expressed by many different cell types (41) . S100β staining was only seen in the cytoplasm (Fig. 2A and B). Again, BDNF tMSCs demonstrated higher levels of staining (29.1% ± 1.9) than uMSCs (4.7% ± 1.3), with a six-fold difference observed (Fig. 4A). Up to 52 ±10% of BDNF tMSCs expressed $p75^{NTR}$ neurotrophin receptor (Fig. 3A and B, and 4). Ki67 was similar for both BDNF cell types, with approximately 25% of cells showing active proliferation, demonstrating results similar to those found by Sharma et al. 2015 (30) (Fig. S3A, B).

Transdifferentiated GFP cells showed markedly different results from the BDNF cells. GFP MSCs showed only a significant difference in antibody immunolabeling for S100β (Fig. 2A). Approximately 23.9% ± 5.2 of GFP tMSCs were immunolabeled by S100β antibody, while only 6.1% ± 0.5 of uMSCs showed labeling. GFP tMSCs had a higher average percentage of cells immunolabeled for S100 and $p75^{NTR}$, but the variability between replicates was too great to be significant. Again, Ki67 was similar for both cell types, with 30% of cells

showing immunolabeling (Fig. S3C, D). In general, both GFP cell types appeared to proliferate more than their BDNF counterparts.

At 20 days growth in TDM3, cells were additionally tested to assess cell proliferation and cell marker profiles. After 20 days growth in TDM3, BDNF cells appeared largely unchanged when compared to day 8 cells. BDNF tMSCs continued to express significantly higher levels of S100, S100β, and $p75^{NTR}$ (Fig. 4B). Again, immunolabeling for GFAP and TuJ1 was not noted for any cell type. When compared to Day 8 there was a decrease (42 vs. 27%) in S100 immunolabeled BDNF-tMSCs on Day 20; however, levels were still significantly higher than BDNF uMSCs. A similar drop was noted for BDNF tMSC levels of $p75^{NTR}$ (52% on Day 8; 34% on Day 20). S100β remained consistent from Day 8 to Day 20 for BDNF tMSCs (29% vs. 32%). The BDNF uMSCs continued to express minimum levels of Schwann cell markers (5-8%). Ki67 immunolabeling demonstrated no significant difference between BDNF cell types and remained close to ~25% as on Day 8.

Post 20 days TDM3, GFP cells appeared further transdifferentiated to resemble a Schwann cell-like phenotype. A significant difference was noted between GFP tMSCs and uMSCs for S100 and p75, however, no difference was seen for S100β (Fig. 4B). GFP tMSC expression of S100 actually increased by approximately 10% (20% on Day 8; 30% on Day 20). The opposite effect was noted for p75 expression, with a drop in expression of 10%. Again, Ki67 levels remained at roughly 25-30%.

Combined, the ICC results demonstrate that both GFP and BDNF MSCs assumed a Schwann cell-like phenotype, based off the increased immunolabeling for specific markers. In general, BDNF cells showed a significant change in their immunolabeling profile faster than

GFP cells, and at higher levels. Both cell types retained their SC-like phenotype for up to 20 days in TDM3.

Quantification of BDNF Production from MSCs

ELISAs were performed using conditioned media samples, to quantitatively determine BDNF levels. After 8 days growth in TDM3, BDNF cells were secreting significantly higher levels of the neurotrophic factor than the GFP control cells. Secretion of BDNF from control GFP uMSCs was 2.74 ± 2.7 ng/mL/ million cells/day. Levels of BDNF were undetectable from the conditioned media collected from the GFP tMSCs (Fig. 6A). BDNF uMSCs and tMSCs secreted significantly higher amounts of BDNF than both GFP cell types (45.16 ± 14.0 and 39.8 ± 6.3 ng/mL/ million cells/day, respectively). A Tukey-Kramer test revealed significant differences between both BDNF cell types and GFP cells. There was no significant difference between BDNF uMSCs and tMSCs.

ELISAs conducted after 20 days growth in TDM3 revealed continued BDNF production and secretion by the BDNF uMSCs (71.32 ± 17.78 ng/mL/million cells/day) and BDNF tMSCs (102.26 ± 30.37 ng/mL/million cells/day) (Fig. 6A). Again, there was no significant difference between BDNF cell types. There was no significant difference in BDNF levels between 8 vs. 20 days in TDM3. GFP uMSCs and tMSCs appeared to be producing similar levels of BDNF, approximately 16.9 ng/mL/million cells/day. A Tukey-Kramer test revealed significant differences between BDNF tMSCs and both GFP cell types. A student's t-test calculated a significant p-value of 0.04 for the difference between BDNF uMSCs and GFP tMSCs. The difference between BDNF uMSCs and GFP uMSCs was very close to statistical significance, with a p-value of 0.054. These results demonstrated that the

transdifferentiation process did not alter the ability of the BDNF MSCs to produce and secrete significant quantities of BDNF.

BDNF Bioactivity: PC12-TrkB neurite outgrowth assay

The PC12-TrkB cell assay was utilized in order to assess the bioactivity of secreted BDNF. The original PC12 cells are a clonal cell line derived from a rat pheochromocytoma, which project long neurites when exposed to NGF (42) . The PC12-TrkB cells were genetically programmed to over-express the BDNF neurotrophin receptor, TrkB (43). By comparing neurite length between conditions, PC12-TrkB cells were used to assess the bioactivity of MSC secreted BDNF. Immunolabeling for TuJ1 showed the extent of neurite outgrowth for PC12-TrkB cells cultured in the following conditioned medias: GFP uMSC (Fig. 5), GFP tMSC (B), PC12 growth media (C) BDNF uMSC (D), BDNF tMSC (E), and 20 ng rhBDNF control. Eight-day conditioned media from both BDNF uMSCs and tMSCs visibly enhanced PC12-TrkB neurite outgrowth when compared to GFP conditioned media (Fig. 6B), with an average neurite outgrowth of 50.0 ± 1.8 μm and 41.6 ± 6.0 respectively, vs. 4.4 ± 0.4 and 5.5 ± 1.4 μm for GFP MSCs. No statistical difference was found between both BDNF cell conditioned medias and the rhBDNF positive control (data not shown). Similarly, there was no significant difference between both GFP cell conditioned medias and the negative control (data not shown). Very similar results were confirmed for the conditioned media collected after 20 days in TDM3 (Fig. 6B). The neurite outgrowth for BDNF uMSCs vs. tMSCs was 43.7 ± 4.2 μm and 54.3 ± 7.3 respectively, vs. 5.0 ± 1.1 and 8.5 ± 1.9 μm for GFP MSCs. Together these results demonstrate that BDNF MSCs (both uMSCs and tMSCs) are capable of producing and secreting bioactive BDNF with potent neurite outgrowth promoting activity.

5. Discussion

Peripheral nerve injury limits mobility and sensation in up to 2.8% of trauma patients and often results in unsatisfactory return to function (9, 44). The gold standard for severe transected peripheral nerve damage involves microsurgery replacement with an autologous nerve graft. However, due to donor site morbidity, many studies have shifted focus to glial cell transplants. Schwann cells are the primary glial cells of the peripheral nervous system and are necessary for nerve damage repair and regeneration. Specifically, SCs remove myelin debris and guide the directed growth of regenerating axons by undergoing dedifferentiation, proliferation, and migration (45, 46). Additionally, SCs produce neurotrophic factors such as BDNF, NGF, NT-3, and NT-4/5, which are necessary for neuronal growth and survival (47). Unfortunately, SCs can only be obtained by sacrificing a healthy nerve, and the process of cell culture is often arduous (48). In search of an alternative to Schwann cells, many studies have looked at mesenchymal stem cells, especially the process of transdifferentiation into a Schwann cell-like phenotype (38, 39, 40). For our purposes, we chose to study MSCs not only for their plasticity, but also because of our past success in genetically modifying these cells as delivery vehicles to hypersecrete neurotrophic factors (8, 30, 36). Since neurotrophic factors such as BDNF promote nerve regeneration, they hold great therapeutic potential. Current clinical use of BDNF is limited, however, due to absence of safe and reliable delivery systems that can provide sustainable effective concentrations over time (49). As an alternative to traditional nerve regeneration therapies, we combined dual strategies to investigate the transdifferentiation of BDNF hypersecreting MSCs into an SC-like phenotype.

In the current study, we subjected both BDNF and GFP expressing mouse MSCs to a transdifferentiation protocol and subsequently analyzed their morphology and SC

immunolabeling profile. Upon exposure to transdifferentiation media, cells became elongated and spindle shaped, with tMSCs demonstrating a larger on average aspect ratio. Several other studies have found similar morphological changes (31, 33, 35). Furthermore, transdifferentiated cells had higher average cellular areas than uMSCs, though no significant differences were observed.

Cells were further characterized by immunostaining using Schwann cell markers such as S100, S100β, $p75^{NTR}$, GFAP, and TuJ1. 30-50% of BDNF tMSCs were preferentially immunolabeled for Schwann cell markers such as S100, S100β, and $p75^{NTR}$ after 8 days in TDM3, and even out to 20 days in TDM3, with minimal decreases in expression seen. Zaminy et al. (50) and Ladak et al. (51) reported ~ 50% S100β expression levels and 75% $p75^{NTR}$ levels after 6 days in TDM. However, their studies were performed on rat MSCs. Additionally, our cells have the added metabolic stress of expressing GFP and/or producing BDNF, and that might lead to lower transdifferentiation levels. No specific staining for GFAP and TuJ1 was noted, which is consistent with our previous results (31).

After 8 days in TDM3, GFP tMSCs only showed significant immunolabeling for S100β, but after 20 days *in vitro,* cells showed significantly higher levels for both S100 and $p75^{NTR}$. These findings suggest that BDNF itself may facilitate conversion of cells to an SC-like phenotype faster. Several studies, in fact, reported mouse and human MSC transdifferentiation protocols, which rely on BDNF, among several agents (41, 52). Furthermore, a recent publication by (53) found that secreted BDNF can influence phenotype modulations of SCs and neurons, forming a positive feedback for nerve development and regeneration , leading us to believe that exposure to BDNF may convert cells to an SC-like phenotype faster.

After exposure to TDM conditions, BDNF production was quantified by ELISA, and its biological activity tested via the PC12-TrkB bioassay. The ELISA results showed that BDNF production was well above the GFP baseline both after 8 and 20 days in TDM3. In a previous study on a different BDNF MSC cell line, Harper et al. (2011) (8) found that unaltered BDNF MSCs secrete approximately 41 ng BDNF/million cells/day. This is very similar to our findings of 45.2 and 39.8 ng/mL/ million cells/day after 8 days in TDM3. These results are very promising, given that Harper's results were obtained from unaltered cells, signifying that the process of transdifferentiation does not repress BDNF secretion. The bioactivity of secreted BDNF was verified by the increased neurite outgrowth of PC12-TrkB cells. These findings suggest that not only is BDNF actively secreted by our cells, but also that it has the capacity to promote neurite sprouting and regeneration. Were these cells to be used for *in vivo* studies, we believe they could increase axonal outgrowth and survival. Given the fact that BDNF production remained stable for over 20 days, we believe these cells have the capacity to produce sustainable, effective BDNF concentrations over prolonged time periods.

Our research group has previously shown that lentiviral induced BDNF MSCs have the capacity to survive and protect neuronal function within the retina (8) . For future studies, we hope to transplant BDNF tMSCs within a biodegradable conduit into a rat sciatic nerve gap model in order to assess the *in vivo* effects on peripheral nerve regeneration. In addition to peripheral nerve transections, BDNF hyper-secreting MSCs could be used in the treatment of spinal cord trauma (54), ischemic stroke (55), Parkinson's (56), and many other neurodegenerative disorders. We hope our results from this study encourage the future use of transdifferentiated genetically modified MSCs as a reliable and effective system for delivery of neurotrophic or other therapeutic factors.

6. Figures

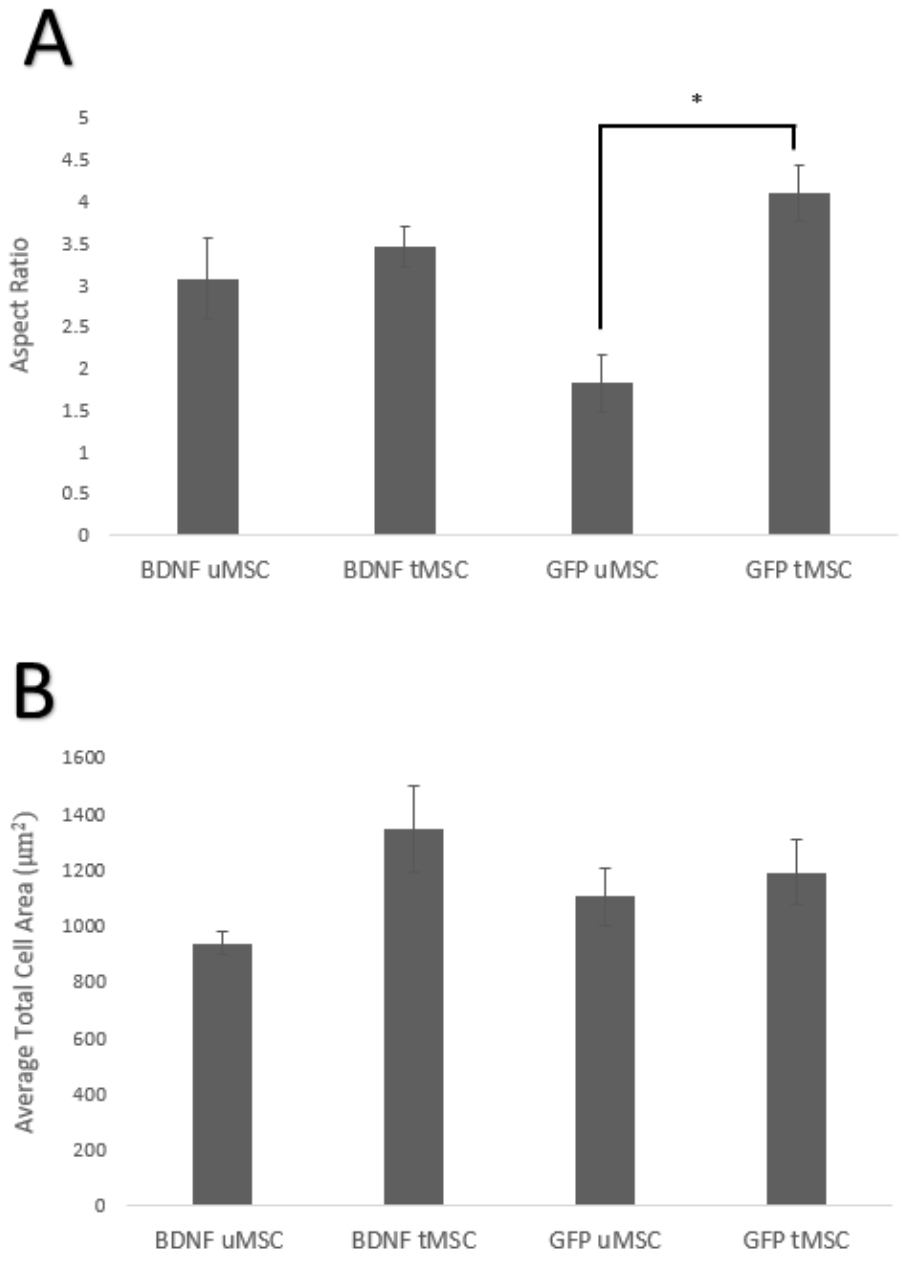

Figure 1. The aspect ratios and cellular areas of undifferentiated versus transdifferentiated MSCs. **(A)** Aspect ratio of GFP and BDNF cells. Aspect ratio is the ratio of length vs. breadth of the cell. A ratio greater than one implies cellular elongation. GFP tMSCs had an aspect ratio significantly greater than GFP uMSCs, implying a greater average cell length. **(B)** Average total cellular area of GFP and BDNF MSCs. There were no significant differences between cell types. Error bars represent standard error of the mean. N = 30 cells per condition, four independent transdifferentiation experiments carried out. Total of 120 cells per condition. *Significantly different at $p \leq 0.05$.

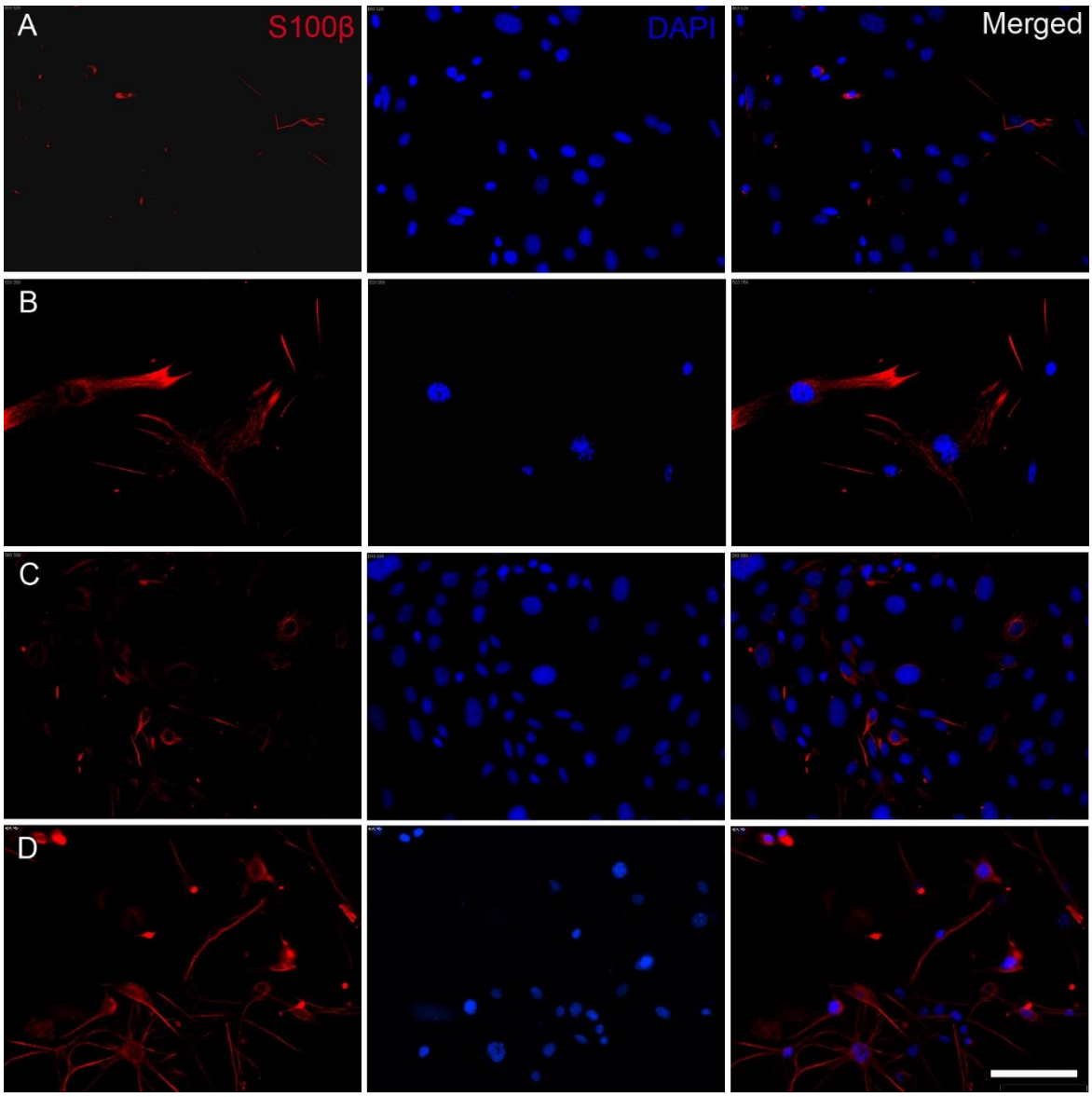

Figure 2. Anti-S100β immunolabeled BDNF and GFP uMSCs vs. tMSCs at 8 days in TDM3. (A) BDNF uMSCs (B) BDNF tMSCs (C) GFP uMSCs (D) GFP tMSCs. BDNF tMSCs expressed a significantly higher percentage of Cy3 immunostaining for S100β when compared to BDNF uMSCs. A greater percentage of GFP tMSCs immunolabeled for S100β after 8 days in TDM3 but not after 20 days TDM3. All cells immunolabeled for S100β in the cytoplasm. Calibration bar represents 100 μm.

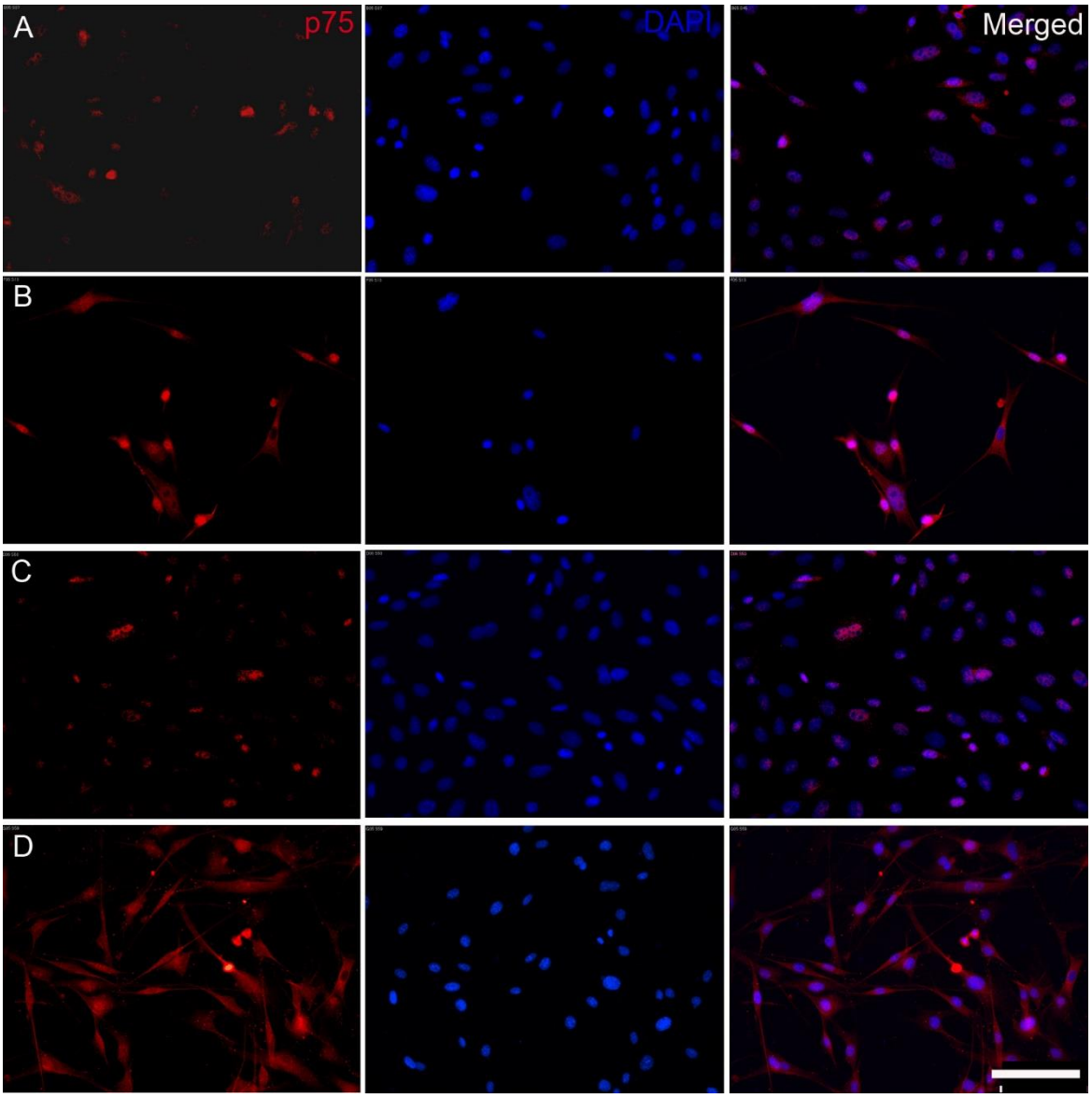

Figure 3. Anti- $p75^{NTR}$ immunolabeled BDNF and GFP uMSCs vs. tMSCs. Minimal immunolabeling for $p75^{NTR}$ was observed in the BDNF uMSCs (A) or the GFP uMSCs (C). (B) In comparison, 50% of BDNF tMSCs immunolabeled specifically for $p75^{NTR}$. (D) Similarly, a significantly higher percentage of GFP tMSCs were immunolabeled for $p75^{NTR}$ after 20 days in TDM3 but did not after only 8 days in TDM3. Cells showed immunostaining for $p75^{NTR}$ in the nucleus and cytoplasm. Calibration bar represents 100 μm.

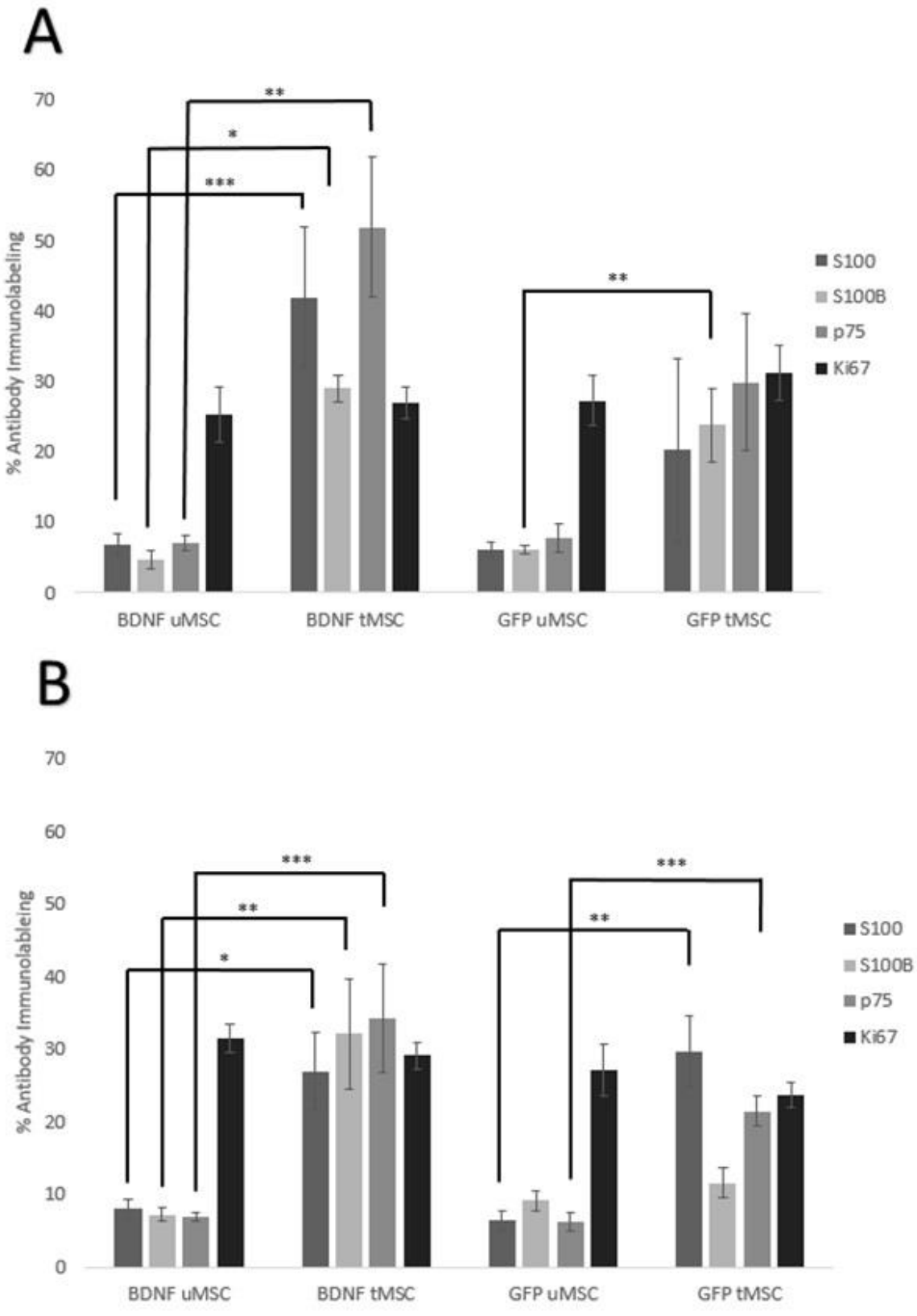
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Figure 4. Immunolabeling characterization of BDNF and GFP uMSCs vs. tMSCs: S100, S100β, $p75^{NTR}$, and Ki67 labeled cells. Specific staining for GFAP and TuJ1 were never noted and are thus not included in this data. **(A)** Immunolabeling for GFP and BDNF MSCs after 8 days growth in TDM3. S100, S100β, and $p75^{NTR}$ staining was significantly higher in BDNF tMSCs than the uMSCs ($p \leq 0.05$). In GFP cells, S100β immunostaining was significantly higher in tMSCs than uMSCs ($p \leq 0.05$). Ki67 was not significantly different between any cell types. **(B)** Immunostaining results for GFP and BDNF MSCs after 20 days growth in TDM3. Again, S100, S100β, and $p75^{NTR}$ staining was significantly higher in BDNF tMSCs ($p \leq 0.05$) than the uMSCs. GFP cells demonstrated a large shift in their immunolabeling profile, with a significantly larger percentage of GFP tMSCs staining positive for S100 and $p75^{NTR}$. Again, no significant differences were noted between cell types for the Ki67 marker. Error bars represent standard error of the mean. N = 4 independent transdifferentiation experiments carried out. *Significantly different at $p \leq 0.05$. ** Significantly different at $p \leq 0.01$. *** Significantly different at $p \leq 0.001$

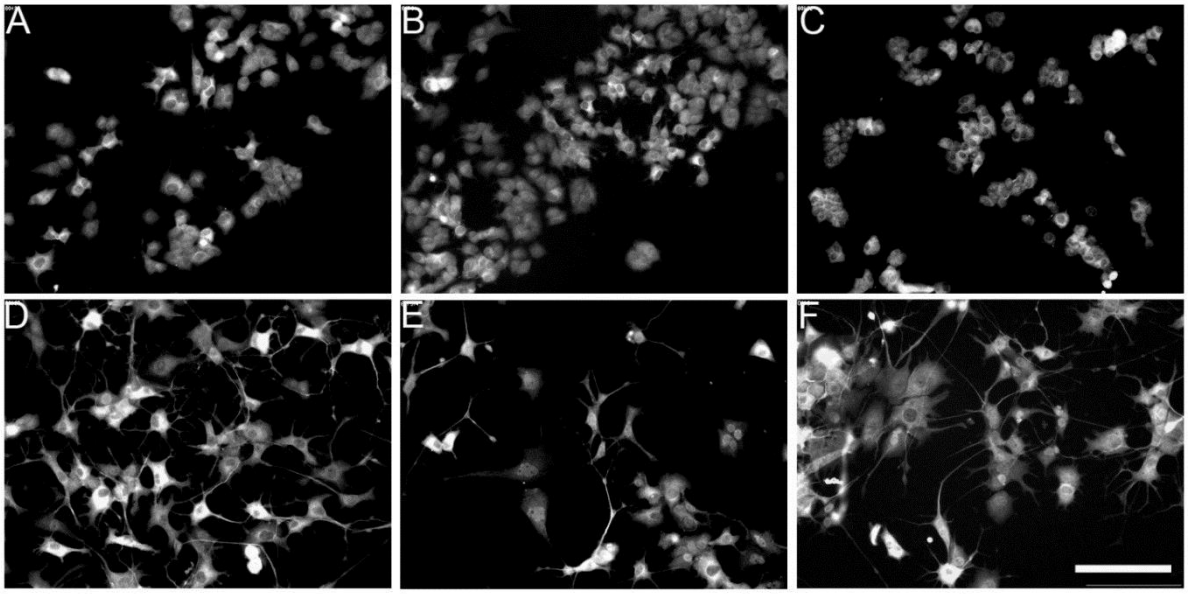

Figure 5. PC12-TrkB neurite outgrowth under several media conditions. Fluorescence images of TuJ1 immunolabeling to detect neurite outgrowth. (A) PC12-TrkB cells grown in GFP uMSC condition media. Cells remained spherical with few detectable neurites. (B) Cells grown in GFP tMSC condition media. No neurite outgrowth was observed. (C) Negative control. Few neurites were observed when PC12-TrkB cells were grown in their own maintenance media - RPMI-1640. (D) PC12-TrkB cells in BDNF uMSC condition media. Cells flattened and often produced several long neurites. (E) Cells in BDNF tMSC condition media. Similar results to (D). (F) Positive control. Cells were grown in the presence of 20 ng rhBDNF and again long neurites were seen, similar to (D) and (E). Scale bar represents 100 μm.

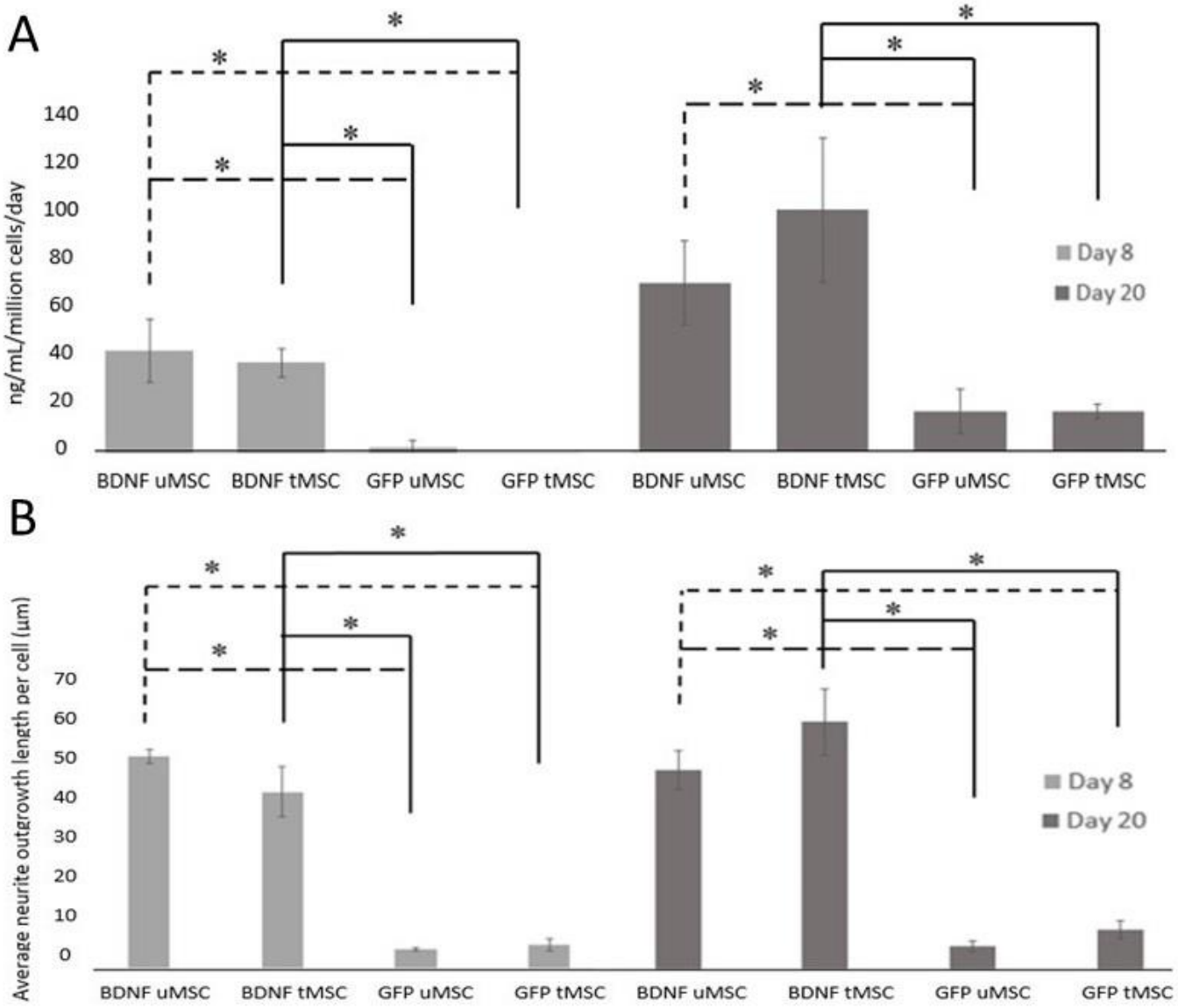
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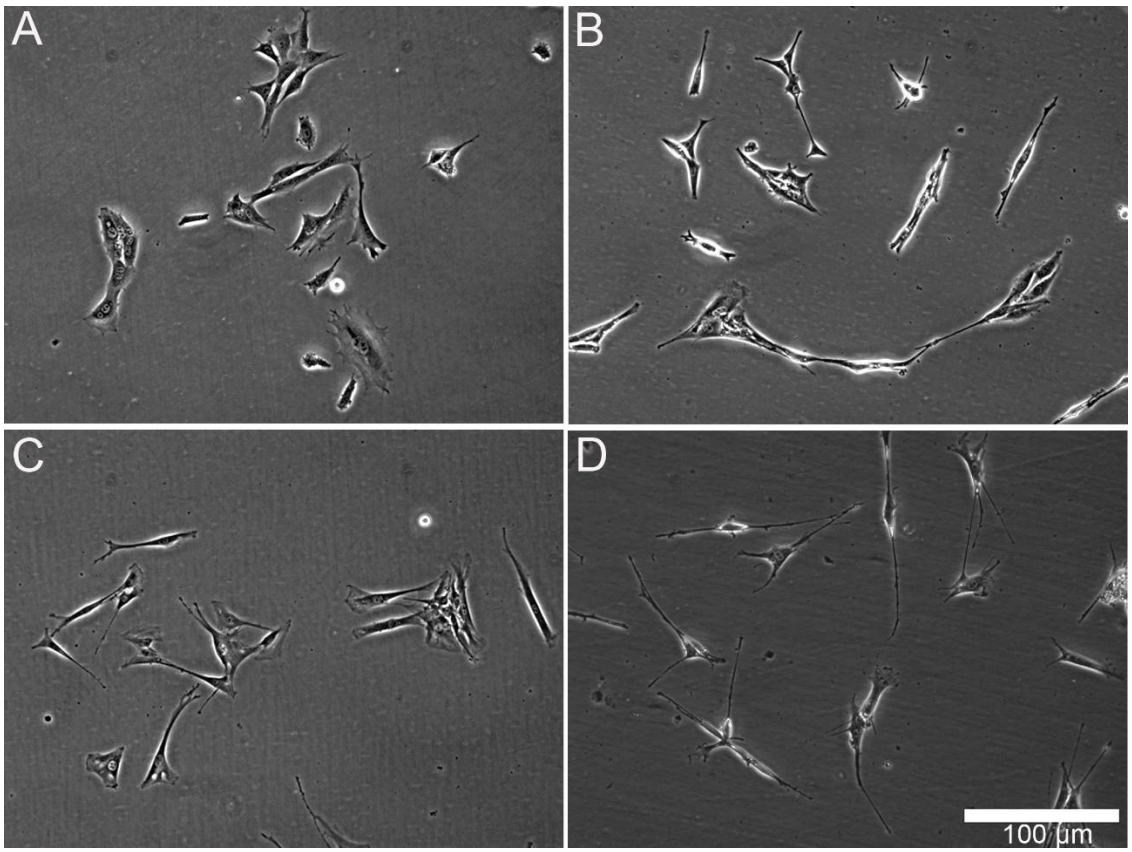
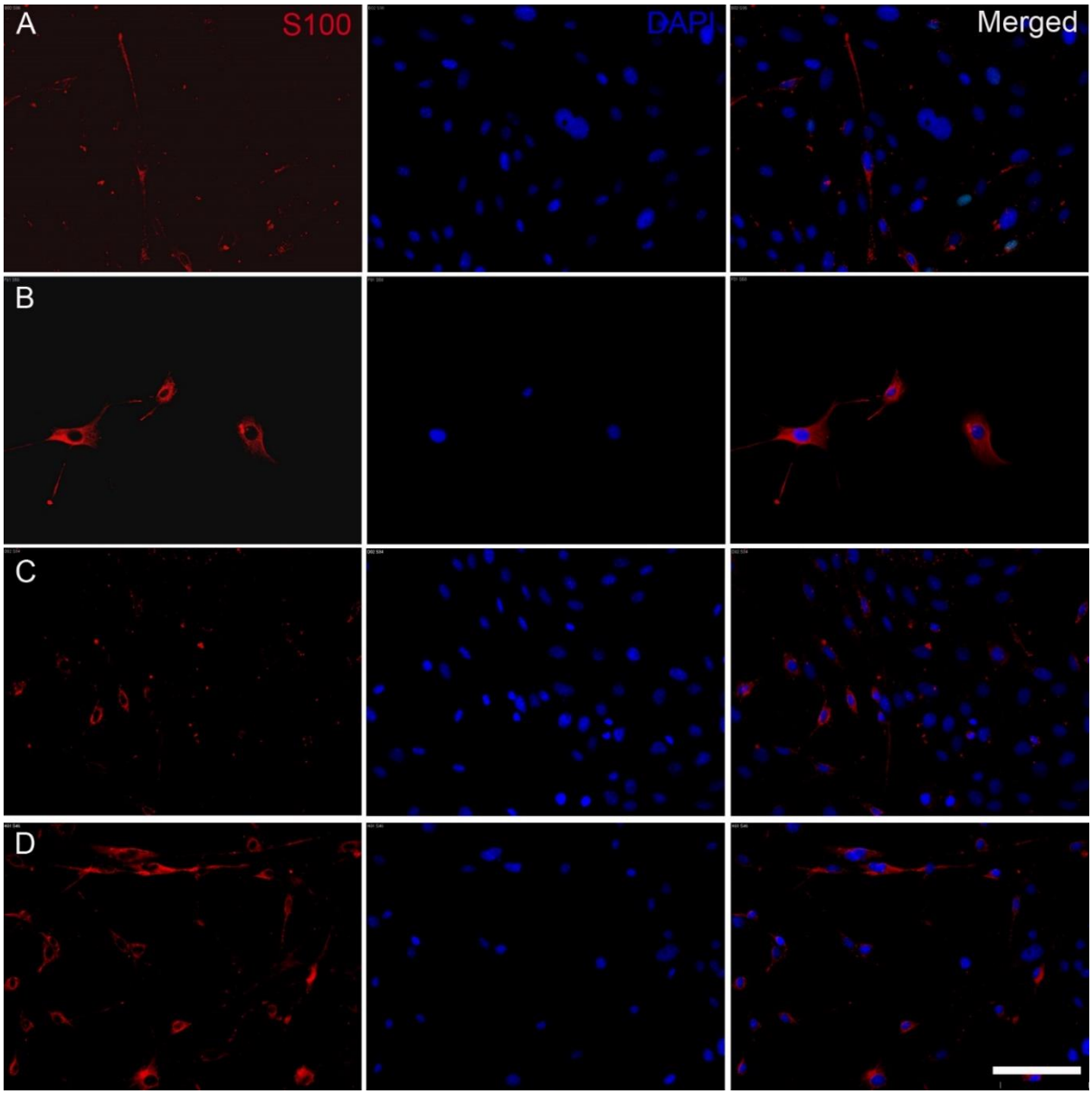
Figure 6. Estimation of BDNF secreted and assessment of its bioactivity. (A) BDNF secretion from BDNF and GFP, uMSCs and tMSCs was measured using ELISA. Lighter bars shows day 8 data and darker bars show day 20 data of BDNF secretion. Day 8-ELISA analysis revealed BDNF uMSCs secrete 45.16 ± 14.0 ng/mL/million cells/day and tMSCs secrete similar levels: 39.8 ± 6.3 ng/mL/ million cells/day. After 20 days, both BDNF cell types continued to secrete similar levels of the factor: 71.33 ± 17.8 ng/mL/ million cells/day for uMSCs and 102.3 ± 30.37 for tMSCs. From day 8 to day 20, a relatively higher increase in BDNF secretion was observed from BDNF tMSCs as compared to BDNF uMSCs indicating cells secrete higher amount of BDNF as number of transdifferentiation day's increase. However, no significant differences were observed at $p \leq 0.05$ among BDNF cell types. GFP cell types secreted a significantly lower amount of BDNF at both day 8 and day 20. Error bars represent standard error of the mean. N = 3 independent transdifferentiation experiments. * represents significant differences at $p \leq 0.05$. **(B)** Quantification of neurite outgrowth using high throughput imaging system and automated analysis. Average neurite outgrowth (μm) for PC12-TrkB cells cultured in a variety of conditioned media. PC12-TrkB cells subjected to both BDNF cell type conditioned media grew significantly longer neurites compared to both GFP uMSC and tMSC conditioned media ($p \leq 0.0001$) at day 8 (Lighter bars) and 20 (Darker bars). Day 20 BDNF tMSCs showed an increase in neurite outgrowth as compared to BDNF uMSCs but Neurite outgrowth was not significantly different between cells grown in BDNF tMSC vs. uMSC-conditioned media for either Day 8 or 20no significant differences were observed at $p \leq 0.05$. Extensive neurite outgrowth across all four BDNF cell type condition implies that BDNF threshold required for differentiation of PC12-TrkB cells is very low and using conditioned media from smaller number of BDNF cells might help in detecting the differences in neurite outgrowth. Error bars represent standard error of the mean. N = 4 independent PC12 conditioned media experiments carried out. * represents significant differences at $p \leq 0.05$

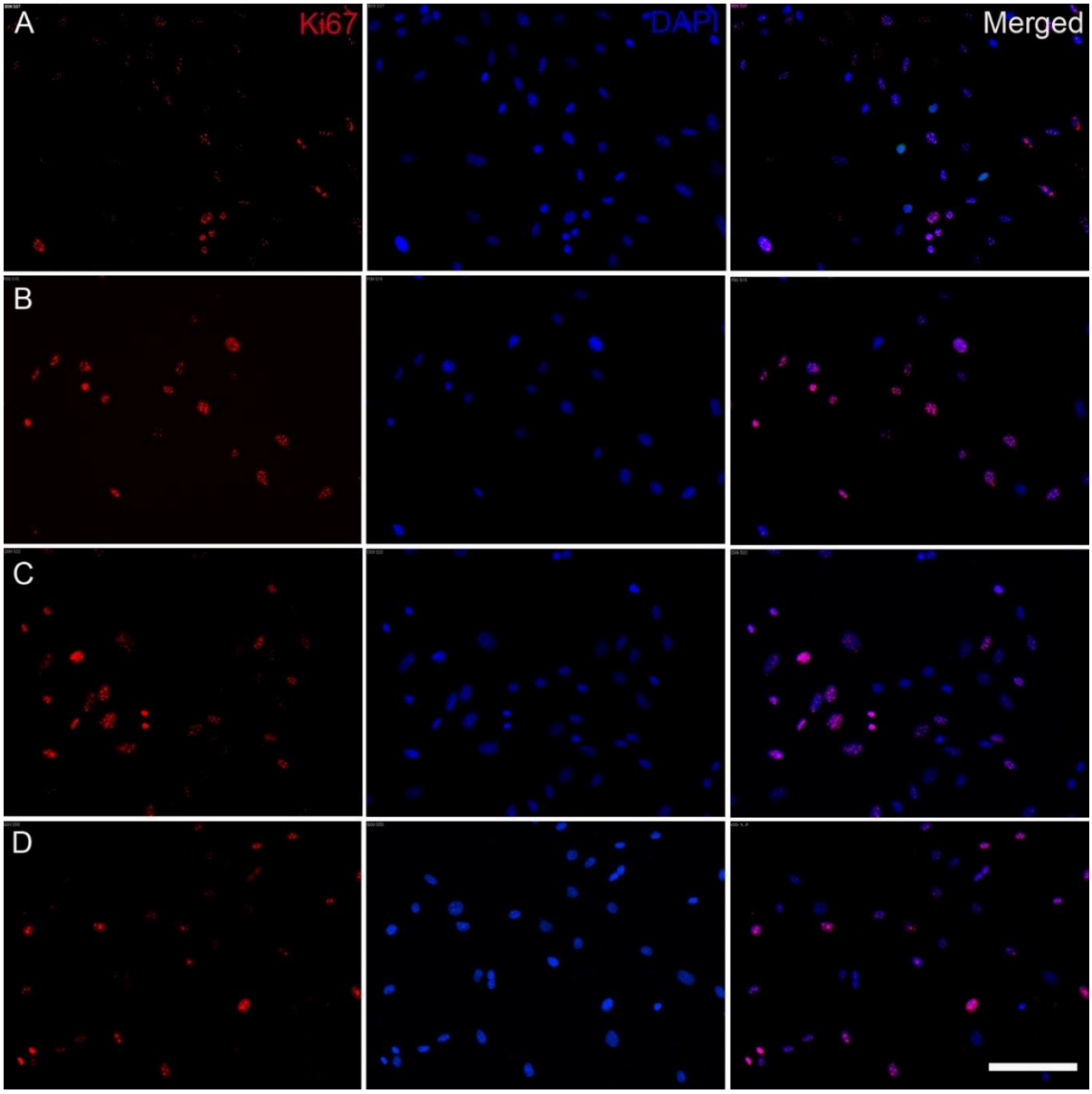
7. Supplemental material



Supplemental Figure S1. Morphology of mouse mesenchymal stem cells (A) BDNF uMSCs in maintenance media, B) BDNF tMSCs, 6 days growth in TDM3, C) GFP uMSCs in MM, D) GFP tMSCs, 5 days growth in TDM3. Cells subjected to transdifferentiation media became more elongated and spindle-shaped compared to their control counterparts.



Supplemental Figure S2. Anti-S100 immunolabeled BDNF and GFP uMSCs vs. tMSCs. (A) BDNF uMSCs demonstrate minimal S100 staining. (B) In comparison, 40% of BDNF tMSCs immunolabeled specifically for S100. (C) Similar to BDNF cells, GFP uMSCs showed minimum labeling for S100. (D). GFP tMSCs expressed significantly higher levels of S100 after 20 days in TDM3 but did not after only 8 days in TDM3. All cells immunolabeled for S100 in the cytoplasm. Calibration bar represents 100 μm.



Supplemental Figure S3. Anti-Ki67 immunolabeled BDNF and GFP uMSCs vs. tMSCs. (A) BDNF uMSCs, (B) BDNF tMSCs, (C) GFP uMSCs, and (D) GFP tMSCs. No significant differences in Ki67 immunolabeling was found between any cell types, suggesting that transdifferentiation did not affect cell proliferation in any significant fashion. All cells demonstrated nuclear staining. Calibration bar represents 100 μm.

8. Acknowledgements

The authors thank B. Patel and Dr. E. Sandquist for comments on the manuscript. This work was supported by a US Army Medical Research and Materiel Command grant [W81XWH-11–1–0700] and the Stem Cell Biology Support Fund. MBDR was in part funded by an ISU College of Veterinary Medicine Summer Scholars internship.

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CHAPTER 4

CONCLUSION

The bulk of our study contributes toward the *in vitro* characterization of a new cell line – chemically transdifferentiated, genetically modified MSCs, made to resemble Schwann cells. The first step of the project was to grow cells in a series of transdifferentiation medias and then test for morphological as well as cell surface marker differences via immunocytochemistry. This part of the project was successful as BDNF tMSCs preferentially immunolabeled for Schwann cell markers such as S100, S100β, and $p75^{NTR}$. Initially, cells were grown in transdifferentiation media for a total of 12 days. This brief timeline gives our particular transdifferentiation protocol an advantage over some of the newer, more labor-intensive protocols, which may take double this amount of time (1,2). Studies indicate that ideal time for neural stem cell transplant is approximately one week after nerve injury (3), which would make our protocol a more clinically feasible option.

In addition to being grown for 12 days, cells were also kept in transdifferentiation media for a total of 32 days. Several studies have demonstrated that long-term culture can alter genetic composition of MSCs (4,5), and cause changes in proliferation and expression patterns in surface markers (6). We kept cells in media for 32 days in order to observe the long-term effects of transdifferentiation media on MSC replication rates, and ability to express Schwann cell markers. Immunocytochemistry for Ki67 revealed no drastic changes in replication rates, and Schwann cell marker expression were very similar for 12 vs. 32 days in media. These results indicate that the transdifferentiation media itself does not cause alterations in proliferation and cell surface markers. However, to assess genomic stability and mutation rates,

a more complete genetic panel using methods such as SNP genotyping would be necessary, and would be highly recommended for moving forward with transplantation studies.

The second part of our project was to quantify BDNF production via ELISA and demonstrate its bioefficacy via PC-12 cell assay. BDNF MSCs, both transdifferentiated and non, produced significantly higher levels of BDNF than the GFP MSCs. Not only was BDNF secreted, but it was biologically active, as the PC-12 cells showed significant neurite outgrowth when grown in the presence of BDNF conditioned media vs. GFP MSC media. These results demonstrate that genetically modified cells continued to over produce BDNF, even after being in transdifferentiation media for both 12 and 32 days.

While these results are very promising, our BNDF tMSCs have yet to be tested for ability to myelinate cells. As a next step, tMSCs could be co-cultured with dorsal root ganglion cells to see if tMSCs would be capable of wrapping around these cells and forming a myelin-like structure. Additionally, patch clamp voltage testing could be used to determine whether these cells can act like voltage gated Schwann cells. To further characterize changes caused by transdifferentiation, genomic testing should be performed on uMSCs vs. tMSCs, to determine what genes may be changing in response to transdifferentiation media, and what roles these genes may play in cell physiology and morphology.

Since we have effectively shown that our cells can transdifferentiate and still produce significant quantities of BDNF, future experiments should consider seeding this cell line into a bioengineered conduit, and transplanting these cells into a transected sciatic nerve model. Important questions to consider include: Can these cells survive and remain within the conduit? Will the cells continue to secrete BDNF *in vivo*? Once no longer exposed to transdifferentiation media, will these cells revert back to their original MSC phenotype or will they continue to

express Schwann cell markers? After transplant, will the tMSCs continue to support neurite outgrowth and will rats functionally improve more rapidly with the addition of BDNF tMSCs? All of these questions are essential in determining whether or not our BDNF tMSCs could have any future relevant clinical applications.

The goal of this Master's project was to describe a novel protocol, demonstrating that genetically modified MSCs could still be transdifferentiated and assume a dual role in nerve regeneration by hyper-secreting BDNF and assuming a Schwann cell-like morphology. We have successfully supported the conclusion that BDNF tMSCs preferentially express Schwann cell markers and promote neurite outgrowth. While our research has established the foundations of a promising new cell line, many more questions have yet to be answered and further data will dictate the role of BDNF tMSCs in peripheral nerve regeneration.

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