Repair Schwann Cells: Bridging the Gap for Successful Nerve Repair in the PNS

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Abstract of the Dissertation

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Dissertation Director:

Dr. Haesun Kim

Peripheral nerve injuries remain a very prominent issue in today's society all over the world, and can be sustained while working with machinery, playing sports or simply driving. Our current knowledge of the topic has limited applications for medical professionals in regards to treatments and surgeries they can perform to allow a patient to regain function in the nerve, which clearly leaves room for drastic improvement. Some organisms like mice and other murine species have demonstrated an ability to enact a Schwann cell facilitated nerve repair program that not all other organisms demonstrate when inflicted with a peripheral nerve injury particularly a severed nerve. As it stands our current comprehension of how organisms like these respond to peripheral nerve injuries on a cellular/molecular level is not complete and is still developing, but by studying these organisms over several decades we have made significant progress in the endeavor regardless. This review will discuss the known differences in nerve repair programs in human and mice, the most studied molecules and their molecular pathways that initiate the nerve repair program based on some of the most recently published literature on the topic and what we can take away from nerve injury models to develop future treatments and surgeries.

Preface

I knew upon my acceptance into the Graduate School of Rutgers University-Newark that I would eventually have to decide which faculty member's laboratory I would like to work in/study under so I could get started on my thesis work. While I was reviewing all of the labs available to join, I came across the laboratory of Dr. Haesun Kim on the university website and saw that she did research on the molecular mechanisms of de-myelinating diseases such as Charcot Marie Tooth Disease. Since her area of study had medical application and the thought of studying demyelinating diseases (despite not having experience in that particular field of neuroscience) sounded interesting to me, I decided to schedule an interview with her to discuss the possibility of joining. Upon that moment, I was granted a research project in which I was investigating the molecular mechanisms of nerve injury response and she inundated me with tons of literature that contained all of the background information that I needed to know. It did not initially occur to me the scope of this project, but I was up for the challenge and wanted to commit myself to it. For the next two years I would be training and performing experiments involving characterization of gene expression in mouse sciatic nerve after injury. Before I knew it, I was developing an affinity for this field of study, and I was fully invested. As a result, I tried to produce findings or experimental results each week, which sometimes involved me working in the lab absurdly late into the night as the last person in the lab (but I did not mind. When I was in the lab, I knew I was in my happy place which helped me realize that I wanted to work in research labs in the future as a career).

When it finally became time to work on my thesis, I gathered and read more scientific literature about nerve injury response in mice (and humans) than I have collectively in my entire life time and truly became quite knowledgeable on the topic, which definitely helped write faster and enjoy myself when writing this thesis. This review is the culmination of almost a year's worth of learning through reading dozens of recent articles in scientific literature about the topic, condensed and written in the span of a little over 3 weeks. I learned so much regarding peripheral nerve injury response and its molecular mechanisms during the development of this review and I am truly proud of the product. I hope that this review allows you to quickly gain knowledge and insight into a less recognized, yet equally important and intriguing branch of neuroscience. However, most of all I hope that this review catches your interest, unlocks your creativity and inspires you to search for the answers to life's mysteries like it did for me.

Acknowledgements and Dedication

I would like to thank everyone in Dr. Kim's Lab especially Alexandra Adams, Corey Heffernan, Akash Patel, Henri Antikainen and of course Dr. Kim. Without your patience, hospitality, training and guidance, it would have taken longer to develop my creative, detail oriented, experimental, analytical and critical thinking skills that enable me to be the intellectual I am today.

I would especially like to thank my parents who helped pave a path for success that has led me to where I am today. They have sacrificed so much throughout my life time to enable me to have every possibility to move forward in life. I am forever in their debt and I couldn't ask for better parents.

Dedicated to my late grandfather, Walter Gilmore Sr (1930-2020), the man whom I considered to be my hero, my life instructor, and second father. Now departed, but never forgotten.

With Great Love and Affection,

Your First Grandson,

Evan

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I. Introduction:

History of Peripheral Nerve Injuries and How They Are Relevant Today

Although studies regarding peripheral nerve injuries and or nerve regeneration have been documented as far back as the 4th Century B.C, the microscopic mysteries regarding this type of physical trauma have only truly begun to unveil themselves within the last 170 years dating back to the pivotal publication of research from neurophysiologist Augustus Volney Waller in the year 1850. Titled "Experiments on the section of the Glossopharyngeal and Hypoglossal nerves of the frog, and observations of the alterations produced thereby in the structure of their primitive fibers", this publication which aimed to provide insight to methods of surgical repair on the glossopharyngeal and hypoglossal nerves performed in frogs, was also amongst the first to document the progressive disorganization of the axons downstream of the injury site, and the presence of the myelin sheath during peripheral nerve repair leaving most scientists with the impression that there were going to be many more questions that need to be answered to understand the mechanisms that orchestrate the entire process (Battiston *et al*, 2009).

Even in the present, peripheral nerve injuries remain relevant in the world of medical care since they happen frequently, which is why there is a growing urgency/emphasis on studying the phenomenon at many educational institutions. Peripheral nerve injuries can come in several different forms including over extension, crushed nerve, partially severed nerve, and complete severing of a nerve. Unfortunately, the most common types of nerve injuries experienced in humans have the longest lasting and most detrimental effects on one's day to day life, in the form of a partial or complete

severing of the nerve (Guena, 2015). Each of these forms of peripheral nerve injury have been documented to occur from some of the most common everyday activities such as impacts from falls while walking on flat or elevated surfaces and physical contact in sports, to more extreme scenarios of puncture trauma from sustaining gunshot wounds or injuries from knife stabbings. However, in a study conducted in 2017 it was unveiled that the most common cause that resulted in severed nerves are car collisions (Kouyoumdjian, *et al,* 2017). This study which analyzed 1124 cases of peripheral nerve injury resulting in either complete denervation or partial denervation of nerves saw motor vehicle collisions responsible for 47.1% of these injuries, while falls, sports related injuries, and penetration injuries from gunshot or by other means accounted for 10.5%, 2.3%, and 29.2% respectively (Kouyoumdjian, *et al,* 2017). Assuming that this link between car collisions and the occurrence of severed nerves are consistent and can be replicated in multiple settings where large populations of humans exist and regularly travel via car, the chances of succumbing to a peripheral nerve injury increases. In the same year as the study (2017) the U.S alone saw 6,452,000 police reports regarding car collisions (figure doesn't include incidents where police were not called to the scene of a crash), resulting in around 2.7 million injured people (National Highway Traffic Safety Administration, 2017). It is possible that a relatively large subset of these 2.7 million reported cases of injured people may have been inflicted with injuries resulting in severed nerves. If this is the case, then the U.S alone is responsible for submitting many cases of severe nerve damage to its medical professionals on an annual basis, and when the remainder of the world's population is considered, this only raises the level of importance regarding the

progression of research and understanding for what occurs during and after severed nerve injury on a microscopic scale.

II. Background:

Wallerian Degeneration and Peripheral Nervous System Repair in Humans and Mice

With relatively large sample sizes being affected by severed nerve injuries annually in the US alone, it would be reasonable for an average person to assume that the medical/scientific community has seen this problem millions of times over the years and that they have a firm understanding of the events that take place in an injured peripheral nervous system to a great extent. Although the scientific community has made great strides towards understanding the events that take place in an injured peripheral nervous system in their entirety, many answers still elude them. This is in part due to the observations of differing microscopic reactions and events that follow nerve injury in humans and for instance a mouse which is a commonly used model organism in scientific research. After a nerve is severed and the events of Wallerian Degeneration occur shortly after, which involves the degradation of axons and associated myelin distal to the site of injury and a change in morphology and functioning in Schwann cells distal to the injury site as well (Llobet Rosell and Neukomm, 2019), many differences in the injury response to severed nerves begin to unveil themselves, which results in ineffective nerve repair in the former and highly effective nerve repair in the latter.

Observations of Peripheral Nerve Injury Response in Human:

As convenient and as cost effective as it would be for humans to possess an effective natural ability to repair damaged nerves, the reality is that humans are not afforded such a luxury. Some of these differences include a slower rate of axon regeneration/growth, tissue degeneration, longer distances to travel to bridge the proximal and distal ends of the nerves, poor guidance of regenerating axons resulting in poor re-innervation and the presence of extracellular matrix which inhibits axonal growth. However, the biggest overarching obstacle identified in a human model with severed nerves is a lacking presence of the peripheral nervous system's glial cells called Schwann cells. Schwann cells are highly regarded in the neuroscience community as the facilitators for maintaining intact and efficiently functioning nerves in uninjured organisms to provide the body with quick information relay systems. Upon severing a nerve in the human model, Schwann cell populations at the injury site seem to vanish and the ones that are capable of being detected simply don't promote functions that would be required to sustain a natural and gradual healing/nerve repair process over time (Jessen and Mirsky, 2019). Eventually, this leads to a failure to naturally repair severed nerves in any capacity for humans.

Observations of Peripheral Nerve Injury Response in Mice:

As briefly mentioned before, mice do not appear to have as many limitations in achieving a method of efficient nerve repair like humans. Unlike humans, mice can be regularly observed with many attributes that promote the successful re-innervation and regained function, such as a large populations of repair Schwann cells that are dedicated to promoting/facilitating the repair of severed nerves, little tissue degeneration, faster

regeneration of axons, secretion of trophic support, bridge formation, macrophage recruitment and more. In fact, the severed nerve in the mouse model is often able to achieve near 100% regained function in the nerve. It is due to these differences that mice make for an excellent model organism to perform comparative studies regarding the peripheral nerve injury response in humans.

Repair Schwann Cell Facilitated Nerve Repair in Mice

The differences in nerve regeneration programs in humans and mice as stated above, are largely a result of facilitated repair by repair Schwann cells that takes place after Wallerian Degeneration. One of the first actions these repair Schwann cells undertake is the clearance and degradation of myelin debris that has permeated throughout the surrounding areas of the injury site of the severed nerve. Myelin proposes a problem for regenerating axons, since it has properties that inhibit axon growth and can obstruct the axons from reaching their target (Kang and Lichtman, 2013; Grinsell and Keating, 2014), which will inhibit the overall functionality of the nerve after reinnervation. Although repair Schwann cells demonstrate the ability to effectively "engulf" the myelin debris via phagocytosis, this step in the repair process is coupled with the recruitment and assistance in myelin degradation by macrophages that are recruited from the nearby blood vessels of the circulatory system (Rotshenker, 2011; Martini *et al*, 2008; Jessen and Mirsky, 2019).

Even though the formerly myelinating Schwann cells had de-differentiated just before the initiation of the nerve repair program into repair Schwann cell, they undergo yet another rendition of genetic activation and deactivation after receiving signals from

the microenvironment, which results in another morphologically and functionally distinct sub-population of repair Schwann cells called bridge Schwann cells (Figure 1).

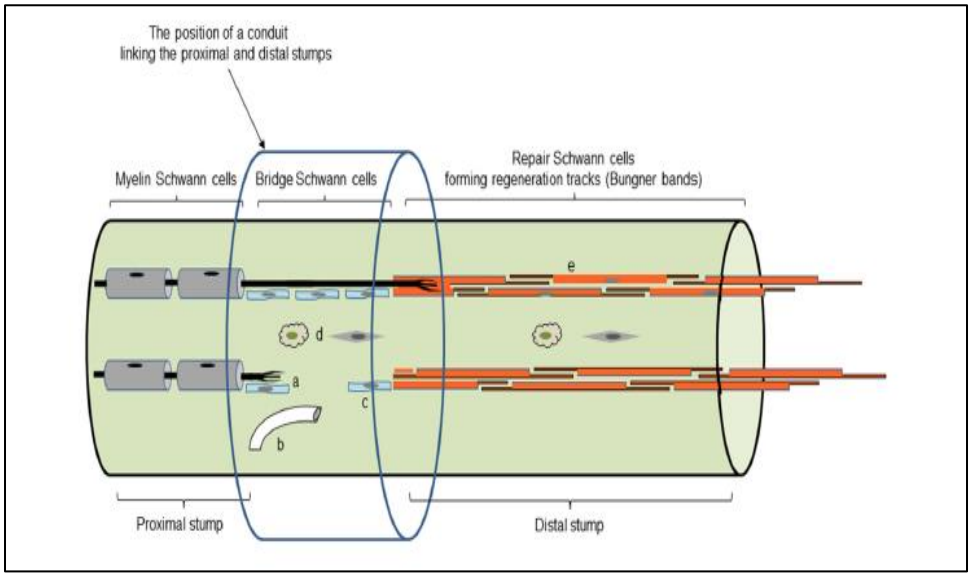


Figure 1: The Peripheral Nerve Injury Response in Mice (a) blue represents bridge Schwann cells of the proximal nerve end. (b) represents blood vessels adjacent to the severed nerve. (c) bridge Schwann cells that stem from the distal nerve end. (d) fibroblasts and macrophages. (e) repair Schwann cells that form the Bands of Büngner (Jessen and Mirsky, 2019).

The bridge Schwann cells migrate to the proximal and distal ends of the partially demyelinated severed nerve and form two parallel chains composed of bridge Schwann cells on each side of the demyelinated proximal nerve end. Meanwhile, other elongated repair Schwann cells that have not become bridge Schwann cells add themselves to the ends of these parallel chains by associating with the bridge Schwann cells and extend distally through the gap where the axons have not regenerated through yet. This section of repair Schwann cells is called the Bands of Büngner or regeneration tracks, which serves to bridge the gap between both ends and safely and directly guide the regenerating axons to the distal nerve stump. Regenerating axons will grow within this bridge and will

be nourished by trophic factors secreted by the bridge Schwann cells in order to promote axon growth and survival. Due to the guidance and trophic support provided by the bridge Schwann cells, the axons are able to reconnect to the distal nerve stump and function once more (Jessen and Mirsky, 2019).

The Purpose of this Research:

Scientists proceed to study the events after a severed nerve injury in mice with two goals in mind. One, to identify the differences between both mouse and human nerve repair programs and two, using that knowledge to develop new treatments that can accurately mimic the processes taking place in mice in order for humans to reap their benefits, and achieve successful re-innervation and functioning of nerves. Although surgeries and other therapies for nerve damage have developed over recent years, even the most advanced methods of nerve treatment and repair are unable to achieve a 52% success rate of motor recovery. The chances of regaining functionality of damaged sensory nerves drop even further to a maximum of 43% success (Grinsell and Keating, 2014). Research in nerve injury response remains relevant, and many of the answers to our questions that would likely lead to the development of new treatments likely lay in the mysteries that shroud our understanding of the molecular mechanisms that facilitate this process. For this reason, the majority of this paper will be reviewing research discoveries in mice regarding several key proteins within Schwann cells that regulate their morphology, functioning and the overall injury response to severing a nerve since it is the most common form of nerve injury in humans. Questions regarding future developments in treatments and surgical repair will also be investigated.

Development of The Schwann Cell Lineage and The Expression of Proteins Associated with The Nerve Injury Response

Based on the events described in the prior section, it is evident that any chance of successful nerve repair rests in the presence of Schwann cells and their ability to perform their reparative tasks, which is why it is important to understand them first. In order to establish an understanding of the Schwann cells throughout this extensive nerve repair process we must analyze their cellular development and its genotypic attributes which we would define as its normal myelinating attributes.

Neural Crest Cell to Schwann Cell Precursor:

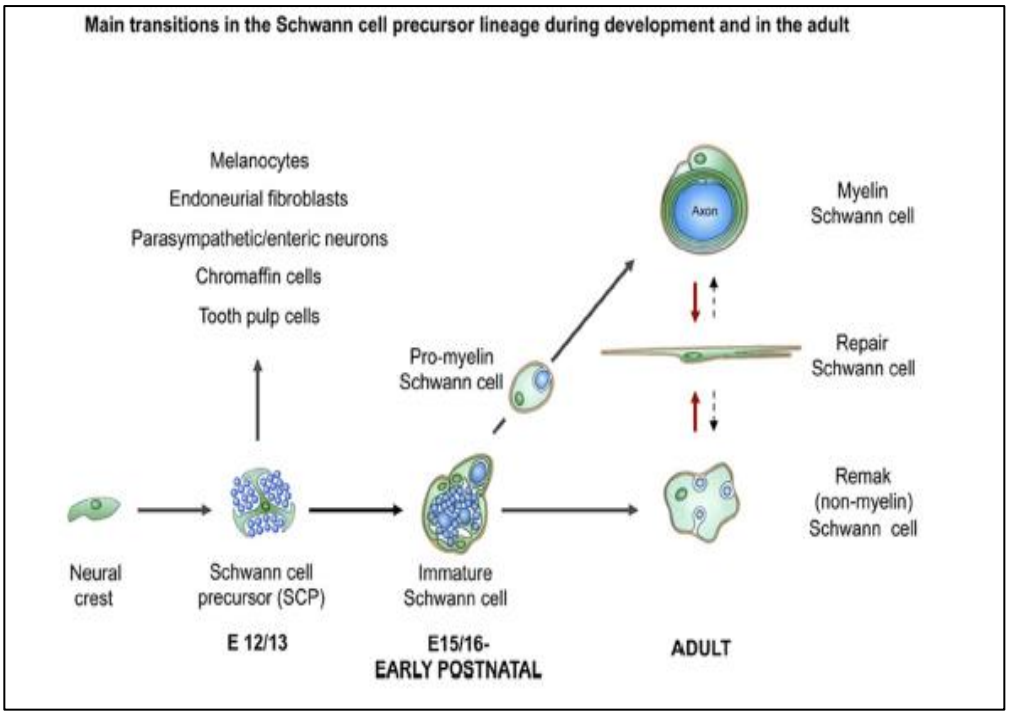
Just like a developing embryo which has to physically develop and mature over time, so do the Schwann cells. In fact, the origins of the Schwann cells can also be traced back as far as the origins of an embryo in its early development. It all starts during the developmental stage of neurulation, in which the embryo develops a crevice on its dorsal surface and the adjacent ectoderm forms folds that rise upward toward one another until both folds connect forming what is called a neural tube. At the site where the folds of the ectoderm fuse, primitive yet pluripotent cells called neural crest cells disassociate from the tips of the folds and migrate ventrally in order to differentiate into neurons and Schwann cells (amongst many other cell types). By embryonic day 12-13 in mice, the neural crest cells express glial differentiation genes that cause it to differentiate into Schwann cell precursors (Figure 2). Schwann cell precursors generally associate with many early embryonic nerves since they are a source of transmembrane type III-

neuregulin 1 (type III-NRG1), which keeps them alive, since they're incapable of producing it on their own and they would die without it. Depending on what combination of genes the precursor cell expresses it will differentiate into four possible cell types including Melanocytes, Endoneurial fibroblasts, Parasympathetic neurons and Schwann cells (Jessen *et al*, 2015).

Schwann Cell Precursors to Immature Schwann Cells:

By embryonic day 13-15, the Schwann cell precursor will begin to express genes responsible for the translation of myelin structural proteins such as P0 and PLP (proteolipid protein) and promyelinating transcription factors Zeb2 (ligand for TGFβ) and Notch (ligand for ErbB3) which promote differentiation deeper into the Schwann cell lineage. Additionally, there is a downregulation of extracellular molecules such as AP2α and endothelin which delay Schwann cell development (Jessen *et al*, 2015). The combination of changes in genetic expression results in the Schwann cell precursor differentiating into an immature Schwann cell (Figure 2). Immature Schwann cells are slightly elongated circular cells that envelope groups of developing axons and they are covered with a basal lamina and extracellular matrix. Eventually over the next few days (E19-E20) the axons from these groupings separate and the immature Schwann cells are sorted in a fashion that usually leaves one immature Schwann cell ensheathed around only one axon in preparation for myelination (Jessen *et al*, 2015). The immature Schwann cells that are now associated with individual axons will eventually receive extracellular signals on their surfaces from the axons in the form of type III-NRG1, Notch, TGF-β, and from the basal lamina in the form of laminin which acts as a ligand for dystroglycan and

β1 integrin on their surface. The combination of these signals promotes the differentiation of the immature Schwann cell to either a mature myelinating Schwann cell



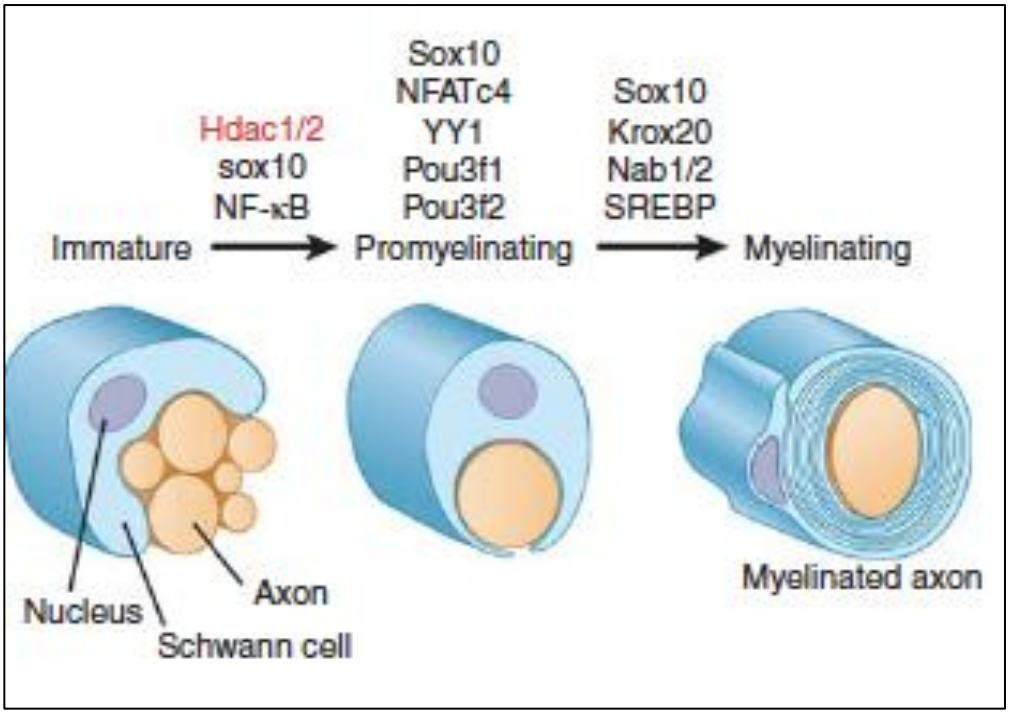
or a non- myelinating Schwann cell (Remak cell) (Figure 2) (Jessen *et al,* 2015).

Myelinating Schwann Cells and Remak Cells:

In response to these axonal signals mentioned earlier, signaling cascades involving the activation of transcription factors Hdac1/2, Sox10, and NF-κB within the immature Schwann cell are initiated (Salzer, 2015). Once initiated, more transcription factors become activated such as Sox10 (continued activation from before), NFATc4, YY1, Oct6 and Brn2, which results in the immature Schwann cells differentiation into an intermediate phenotype of the myelinating Schwann cell called a promyelinating Schwann cell (Figure 3) (Salzer, 2015). Lastly and most importantly, the expression and

Figure 2: Development of the Schwann Cell Lineage (Jessen and Mirsky, 2019)

activation of NFATc4, YY1, Oct6 mentioned earlier, promotes the expression of the transcription factor Krox20 which is largely responsible for promoting the mature



myelinating Schwann cell phenotype and function (Salzer, 2015).

Common proteins that are seen after the expression of Krox20 and are unique to myelinating Schwann cells and not Remak cells are myelin protein zero (MPZ or P0), myelin basic protein (MBP), myelin associated glycoprotein (MAG), PMP22 and periaxin (Jessen and Mirsky, 2019).

The determining factor that decides the fate for immature Schwann cells to Remak cells is far less understood compared to its myelinating brethren. There is a growing collection of evidence that supports the theory that what decides a Remak cells fate is a concentration dependent interaction between the surface receptors on immature

Figure 3: Signal Cascades Involved in Developing a Myelinating Schwann Cell (Salzer, 2015).

Schwann cells (Erb3/2) and an axon type III-NRG1. The most current proposition is that the concentrations of this transmembrane protein differ based on the diameter of each axon, with larger diameter axons expressing it more heavily than smaller diameter ones. This would explain why it is commonly seen that myelinating Schwann cells associate with axons with a diameter larger than 1µm and Remak cells associate with axons with a diameter less than 1µm (Taveggia *et al*, 2005). However, further testing needs to be conducted before this is officially declared the deciding factor in Remak cell fate. Despite not being certain regarding the signaling cascades that destines an immature Schwann cell to have a Remak cell fate, we have been able to identify highly expressed proteins that are found uniquely to Remak cells and not myelinating Schwann cells. These include neural cell adhesion molecule (NCAM), p75 neurotrophin receptor (p75NTR), glial fibrillary acidic protein (GFAP) and L1 NCAM (Jessen and Mirsky, 2019).

As reviewed before, myelinating Schwann cells and Remak cells result from differentiating immature Schwann cells, each of which develop different functions. The myelinating Schwann cells, are tasked with the responsibility of myelinating individual axons by wrapping a membranous extension of the Schwann cell itself that is composed of myelin proteins and lipids around axons in order to enhance the speed of electrochemical signal propagation (Ben Geren, 1954). This method of Schwann cell wrapping occurs after the myelinating Schwann cell associates with an axon. This membranous extension of the Schwann cell begins with myelin proteins and lipids being transported intracellularly from the endoplasmic reticulum to the outgrowing edge of the extending membrane via designated myelin channels that are visible during initial myelination and re-myelination (Velumian *et al*, 2011). As these myelin proteins and

lipids are incorporated with the extending membrane, this membrane slips under each previous layer, continuously wrapping around the axon until the proper myelin thickness is achieved (Bunge *et al,* 1961). Although Remak cells are non-myelinating, they still serve an important function as well in which they provide metabolic support to small axons of the peripheral nervous system making sure that they remain functional and do not degrade (Beiroski *et al,* 2014).

Krox20 Expression and Function During Development:

Krox20 is a transcription factor that is typically seen being constitutively active in the promyelinating stages of Schwann cell development from the initiation of myelination until adulthood and is considered to be transcription factor that controls the myelinating state of a Schwann cell. However, during development, Krox20 seems to play other roles than just regulating promyelinating genes. Through studies analyzing developing Krox20 negative mutants, it became apparent that this transcription factor plays a role in the cell cycle, as the mutant samples of Schwann cells had a great level of difficulty of exiting the S phase of the cycle and the population of cells stuck replicating DNA was 5x greater than that of the controls (Zorick *et al,* 1999). Additionally, Krox20 negative Schwann cells at P12 have also been observed to undergo apoptosis at 16x the rate of controls and 5x the rate of themselves at P3 (Zorick *et al,* 1999). These findings suggest that in developing mice the presence of Krox20 positively affects cell survival and maturation, while its absence it hastens programmed cell death. Additionally, it has been concluded that mutant Schwann cell specimens that are Krox20 negative are still

capable of associating with nerve fibers, but are essentially blocked from performing any myelinating activity often resulting in severe hypomyelination (Topilko *et al,* 1994).

c-Jun Expression and Function During Development:

Although it is commonly known as a transcription factor that upregulates demyelinating genes within hours of administering the nerve injury during the early stages of postnatal life when immature precursor Schwann cells are differentiating into mature myelinating Schwann cells, c-Jun is still expressed within them, albeit in very low concentrations. Since these low concentrations of c-Jun were initially observed in proliferating Schwann cells, leading researchers believed that c-Jun had a secondary role in the Schwann cell which was responsible for cell division. However, this theory was debunked in 2004 when Parkinson *et al* managed to remove proliferating Schwann cells from perinatal nerves and induced quiescence *in vitro*, yet they were still expressing very low levels of c-Jun. This implied that the expression of c-Jun alone is not enough to induce proliferation (Parkinson *et al,* 2004) as previously thought.

Despite expressing Krox20 in a larger proportion to c-Jun during development it appears that even the slightest presence of c-Jun prevents an early death. Ablating c-Jun during embryonic development seems to carry significantly more of an impact on mice mortality in comparison to Krox20 effects on developing mice. In studies that tried to breed c-Jun homozygous knockout mutant mice, not a single one was alive after birth having died *in utero* (Hilberg *et al,* 1993; Roffler-Tarlov *et al,*1996). Analysis of their tissues often displayed many morphological anomalies. It appeared that unlike Krox20, c-Jun didn't affect the cell cycle or differentiation at all. However, cells became apoptotic

or necrotic in important organs like the brain and liver, which likely caused metabolic issues within the mice and led to their deaths (Hilberg. *et al,* 1993). These experiments demonstrate that the presence of c-Jun is essential for embryonic survival/further development.

Transmembrane Type III Neuregulin 1 (type III-NRG1) Expression and Function During Development:

The success of a neuronal crest cell to achieve proper Schwann cell development, migration and myelination are highly dependent on the expression of two proteins. The first of these critical proteins is derived from the membrane of the axons and it is type III-NRG1 (ligand for ErbB3/2 receptor). There are 15 known isoforms of this protein, that are divided into three groups named type I, type II, and type III, but the isoform of NRG1 that plays a large role in neural crest cell migration, promoting Schwann cell development, axon myelination and ensheathment, is transmembrane type III-NRG1 (Falls *et al*, 2003).

Type III-NRG1 has been observed to have multiple known functions within a developing embryo in regards to the peripheral nervous systems development. First, type III-NRG1 is required for the migration of the neural crest cells past the dorsal root ganglion to reach ventral regions of the organism for nerve cell development in those distant anatomical regions. Observations made of type III-NRG1 negative mutant mice, lacked ventral nerves or possessed underdeveloped ones (Britsch *et al*, 1998). Secondly, type III-NRG1 is responsible for promoting/committing migrating neural crest cells in the developing peripheral nervous system to differentiate into Schwann cell precursors and

the rest of the cell types in the Schwann cell lineage. In the absence of type III-NRG1, postnatal mice have repeatedly been reported as severely lacking in Schwann cell populations in its peripheral nervous system. Not only do type III-NRG1 promote differentiation into Schwann cell precursors, but they also promote their survival. In situations where embryonic nerves are severed, and Schwann cell precursors are transplanted into culture, what often occurs is that the Schwann cell precursors stop progressing through their differentiation and become apoptotic, eventually dying off. However, with a consistent application of type III-NRG1, it has been documented that you can sustain their survival and continue to promote their differentiation further along the Schwann cell lineage with this treatment alone (Leimeroth *et al*, 2002).

In addition to having significant effects on neural crest cells and Schwann cell precursors during embryonic development, but it appears that type III-NRG1 also serves as a required molecule for myelinating Schwann cells to myelinate and ensheath axons. In studies featuring pre-made separate cultures of neurons from wild type rats and type III-NRG1 negative mutant rats and the addition of many Schwann cells into those neuron cultures, the Schwann cells showed the ability to myelinate the wild type neurons, but the type III-NRG1 neurons were never capable of being myelinated in their own culture, even after they added larger amounts of Schwann cells than the control sample (Taveggia *et al*, 2005). In conjunction with these experiments, it was also determined that type III-NRG1 is the only type of NRG1 isotope available on the axon surface membrane which tells the Schwann cells to myelinate the axon upon contact with it. The amount of the transmembrane protein that it expresses can directly trigger proliferation and differentiation of Schwann cell precursors possibly via the PI3Kinase-Akt-mTorc1 and or

Ras-Raf-Erk signaling pathway and determine how thick it will make the wraps of myelin around the axons (Taveggia *et al*, 2005; Nave and Salzer, 2006; Maurel and Salzer, 2000).

Erythroblastic Oncogene B 3 and 2 (ErbB3/2) Expression and Function During Development:

ErbB3/2 proteins work in conjunction with the former protein/ligand type III-NRG1 as its receptor during a time before axon myelination in developing mice. Early on in development, they are rather heavily expressed on the surfaces of precursor Schwann cells as they search to contact axons throughout the peripheral nervous system. Eventually after the myelination of axons takes place, the expression levels of ErbB3/2 is reduced since myelinating Schwann cells would have located and associated with their

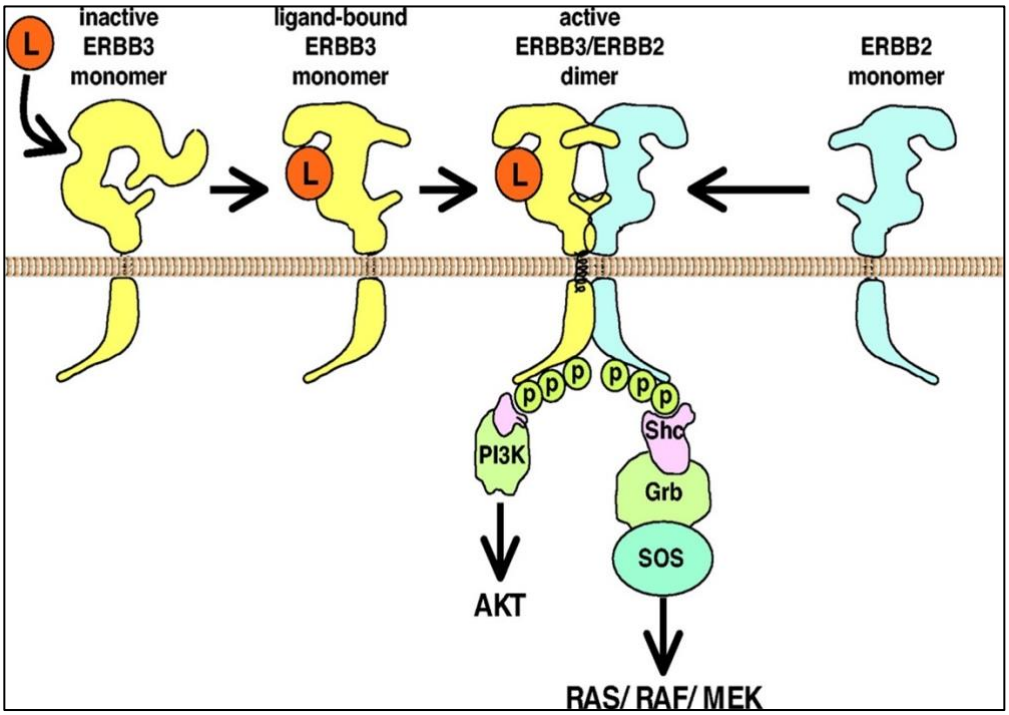


Figure 4: ErbB3/2 Ligand Activation/Dimerization on Schwann Cell Surface. (L) represents any ligand that can bind to ErbB3 and leads to ErbB3/2 activation/dimerization leading to subsequent signaling pathways like PI3Kinase-Akt-mTorc1 and Ras-Raf-Erk (Gilbertson, 2005).

respective axons, thus reaching full maturity. However, during development what happens is that as soon as axon type III-NRG1 enters the active site of ErbB3 on the precursor Schwann cells surface membrane, it subsequently forms a heterodimer with ErbB2 and activates it via phosphorylation thus carrying out the signaling cascade until completion (Figure 4), which results in the determination of the amount of myelin wrapped around axons (the thickness of the sheath) (Nave and Salzer, 2006; Garrat *et al,* 2000; Gilbertson, 2005).

The role of ErbB3/2 was determined in studies using conditional ErbB3/2 gene deletion in mice in which ErbB3/2 was only deleted after neural crest cells had developed into Schwann cell precursors. This was due to the fact that prior attempts had shown that ErbB3/2 knockout mutations in mice caused a reduction in the presence of Schwann cell precursors, which led to few mature Schwann cells after development. The samples from ErbB3/2 ablated mutants can be seen in Figure 5 and clearly showed overall hypomyelination around the nerve fibers as well as a severe myelination decrease in diameter by as much as 2-3x (in the group that were 6 months old). This trend of hypomyelination carried on throughout the specimens lives even for specimens who aged to 14 months. The hypomyelination also extended to other nerves in the lower leg such as the sural and saphenous nerves for all mutant animals in the study (n=28). In addition, the nerve fibers themselves were translucent and thinner than their control sample

counterparts. collectively these effects resulted in physical and behavioral abnormalities as well such as having serpentine or kinked tails, awkward gait, impaired mobility in the hind legs and even weight loss and death. The results from this experiment support the notion that not only does ErbB3/2 function to help the neural crest cells migrate and commit to the Schwann cell lineage during development, but it is also to determine the sufficient myelin thickness for developing nerves and possibly the overall health of the axons themselves. (Garratt *et al*, 2000).

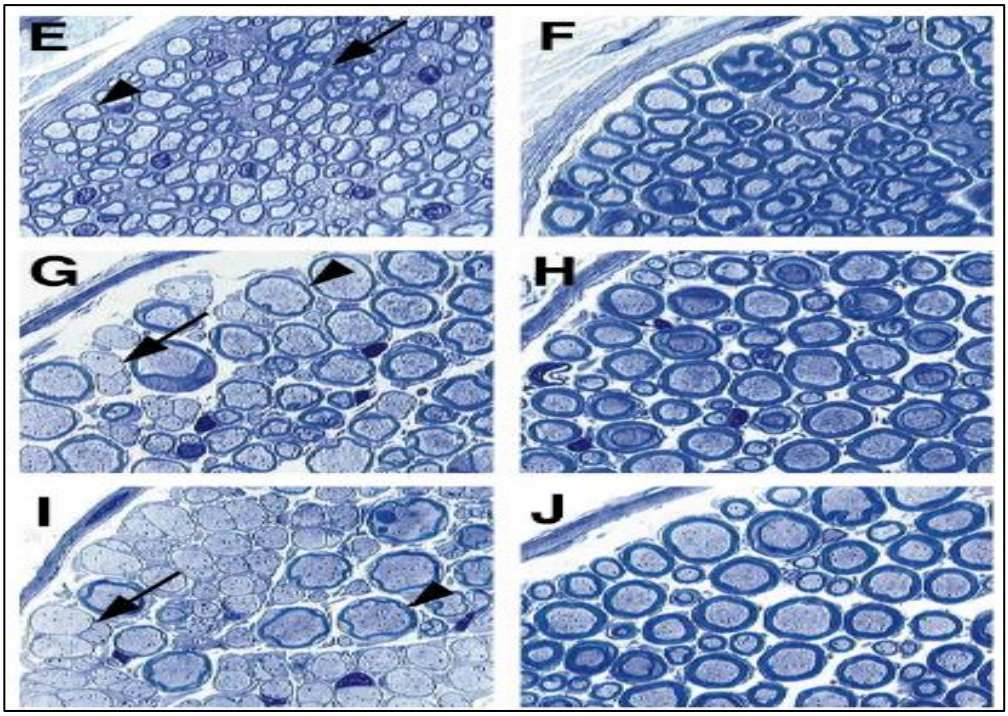


Figure 5: Comparison of a Cross-Section of Axons after ErbB3/2 Ablation During Development. (E, G, I) ErbB2 negative mutant mice axons of the sural nerve with little to no myelination shown by arrows. (F, H, J) wild type control mice with typical axon myelination thickness in sural nerve (Garratt *et al*, 2000).

PI3Kinase/Akt/mTORC1 Pathway Expression and Function During Development:

The protein called PI3Kinase as well as the two other downstream effector proteins called Akt and mTORC1 are highly expressed in precursor Schwann cells and

their activity is thought to inhibit the onset of myelination capabilities (Taveggia, 2016). As briefly mentioned earlier in the type III-NRG1 section, type III-NRG1 has the ability to directly activate PI3Kinase which begins a signal cascade in the precursor Schwann cell that inhibits proliferation and differentiation into a myelinating Schwann cell phenotype during development. Following the activation of PI3Kinase by type III-NRG1, it goes on to activate Akt further downstream. Akt phosphorylates mTORC1's inhibitors TSC Complex and PRAS40, which then indirectly activates the protein mTORC1, which then activates many different target proteins that can affect cell size, proliferation, and differentiation (Lloyd, 2013; Figlia *et al,* 2017). According to a study that analyzed mTORC1's activation throughout Schwann cell development it was found that this activation of mTORC1 gradually decreases as the development of myelinating Schwann cells progresses, meaning that it is at its highest activation levels in Schwann cell precursors and at its lowest activation levels in myelinating Schwann cells (Beirowski *et al,* 2017).

Recently, after analyzing protein expression in mutant myelinating Schwann cells that had PI3Kinase knocked down to varying degrees, and observing an equally proportional inverse pattern of expression and activation between PI3Kinase and Krox20, it has been suggested that PI3Kinase can act as an upstream negative regulator of Krox20 in precursor Schwann cells through mediation by Akt and mTORC1. The activity of these proteins was determined through the analysis of the upregulated and downregulated genes that encode for transcription factors as a result of mTORC1 activation. Among the many genes significantly suppressed by mTORC1, Krox20 was noticed to be among them (Figlia *et al,* 2017). After seeing a possible inverse relationship between mTORC1

activity and Krox20 expression, using pharmacological inhibitors on mTORC1 target proteins unveiled that inhibition of one of its target 4E-BP1 caused a mild rescue of Krox20 expression (Figlia *et al,* 2017). However, pharmacological inhibition of a target protein S6 kinase (S6K) caused a moderate rescue in Krox20 expression (Figlia *et al,* 2017).

This information in combination with the Beirowski, *et al* information agrees with our current understanding of Schwann cell development and suggests that progression through the Schwann cell lineage occurs primarily due to the activation of mTORC1. mTORC1 which is at its highest in Schwann cell precursors will inhibit Krox20 via mediation by 4EBP1 and S6K, thus heavily preventing myelination capabilities. However, as more time passes and the activity of mTORC1 decreases in Schwann cell precursors, which results in the increased expression of Krox20 further resulting in myelinating capabilities the Schwann cell precursors will develop into myelinating Schwann cells (Figure 6).

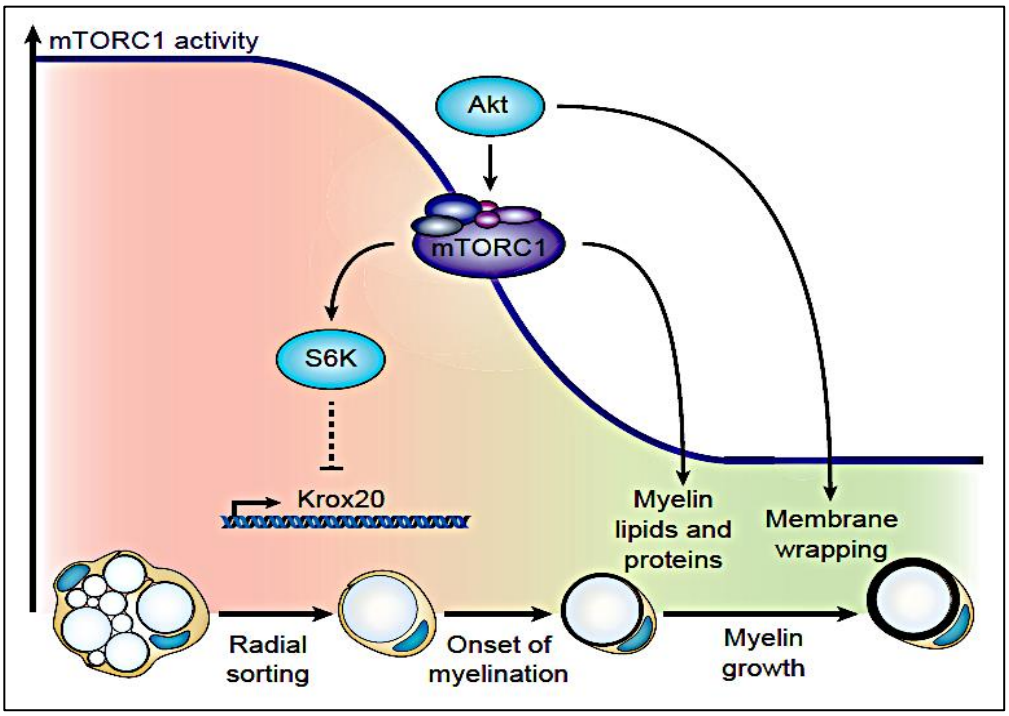


Figure 6: mTORC1 Activity During Myelinating Schwann Cell Development (Figlia *et al*, 2017; Beirowski *et al*, 2017).

MAPKinases (Ras-Raf-Erk) Expression and Function During Development:

It is widely believed that mitogen activated protein kinases (MAPK's) also play a large role in modulating Schwann cell plasticity like many of the proteins that take part in the PI3Kinase pathway. However, during Schwann cell development it is likely that MAPK's Ras, Raf and Erk don't play as critical of a role as some of the other proteins mentioned prior. These three proteins which actually form a signal cascade in the mature Schwann cells that begins with the activation of Ras followed by Raf and Erk respectively likely fluctuate from an active state and inactive state which is determined by the stages in its development. For instance, activation of the pathway is likely occurring in the time where the Schwann cell precursors and immature Schwann cells are developing but are not ready to differentiate to the next respective stage in the lineage.

When it is time for the precursor Schwann cell or immature Schwann cell to differentiate, Ras-Raf-Erk likely deactivate in order to prepare for differentiation. The reasoning behind this theory is based on findings from other studies that have documented that the activation of Ras followed by Raf and Erk respectively prevented immature Schwann cells from differentiating into their mature myelinating morphology, even with application of cAMP which can trigger differentiation (Harrisingh *et al*, 2004).

Not only does the activation of the Ras-Raf-Erk pathway prevent differentiation through the Schwann cell lineage, but it even seems to reverse it, which implies that it plays a bigger role in the nerve injury response than development (Harrisingh *et al*, 2004). Inversely to the research done by Harrisingh *et al*, other studies were conducted involving the activation of this pathway, and what those researchers found was that *in vivo* Erk activation promotes myelination, and if it is inhibited it prevents de-differentiation (Newbern *et al*, 2011). These contradictory findings have many researches puzzled until this day, but many have tried reconciling these developments by explaining that perhaps low levels of Erk activation are needed for a Schwann cell precursor cell to differentiate into a mature myelinating Schwann cell, and high levels of activation cause the Schwann cell to de-differentiate and proliferate (Napoli et al, 2012).

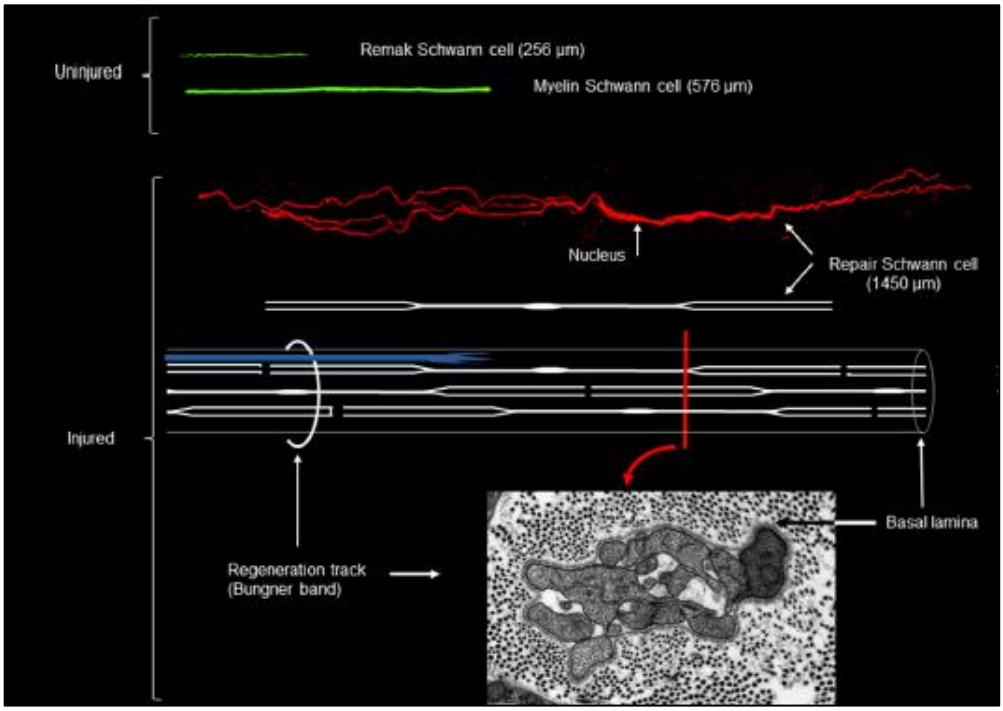
IV Main:

Generation of the Repair Schwann Cell After Severing a Nerve

The Identity of Repair Schwann Cells:

It has been shown that when Schwann cells are prevented from de-differentiating into the repair Schwann cell, the nerve repair program completely fails because the

Schwann cells are incapable of exiting their myelinating state, therefor their repair behavior/activity is dependent on its ability to generate a repair Schwann cell (Arthur-Farraj *et al*, 2012). These findings bring up some important questions. What are repair Schwann cells/how are they unique from the previous two phenotypes of Schwann cells that we have already seen (myelinating Schwann cells and Remak cells) and what triggers their genesis? Based on the most obvious external features in Figure 7, it is clear to see that by size alone, the repair Schwann cell is significantly elongated in comparison to the myelinating Schwann cells and Remak cells, by as much as 2x-3x respectively



(Jessen and Mirsky, 2019). Additionally, it also appears that some repair Schwann cells have 2 or 3 processes that run along parallel with its cellular body axis unlike the myelinating Schwann cell or Remak cell (Jessen and Mirsky, 2019).

Despite the differences in size, it is not fair to say that they are different cellular forms. Examination on a genetic level will have to be made to aid in this distinction. After all, lineage tracing experiments have confirmed, that repair Schwann cells do in fact originate from both myelinating Schwann cells and Remak cells (Arthur-Farraj *et al,* 2017). As mentioned earlier, both myelinating Schwann cells and Remak cells actively express specific genes to synthesize proteins at high levels that the other one does not.

An analysis of genes responsible for producing certain proteins in the repair Schwann cell can be used as identifying markers that can either set itself apart from the other two Schwann cell phenotypes or demonstrate an almost identical relationship with them. Genetic analysis has shown that repair Schwann cells differ in the active expression of the genes Olig1, sonic hedgehog (Shh) and glial cell derived neurotrophic factor (GDNF. This is the case with one exception) (Jessen and Mirsky, 2019). Myelinating Schwann cells, Remak cells and even immature Schwann cells, which are destined to have the myelinating or Remak fate, but are often compared to repair Schwann cells due to their slightly elongated appearance, have not shown active expression of either of these

Figure 7: Size Comparisons of Remak, Myelinating and Repair Schwann Cells Green lines represent the length of Schwann cells in non-injured nerves. Red lines represent the length of the repair Schwann cell (Arthur-Farraj *et al,* 2017). Below that is a diagram that shows how they interlink to form the Bands of Büngner (Gomez-Sanchez *et al,* 2017).

two genes. However, the expression of GDNF can be seen in immature Schwann cells during cellular development, although it is downregulated (Lu et al, 2000; Zhou et al, 2000; Piirsoo et al, 2010; Arthur-Farraj et al, 2012; Fontana et al, 2012; Lin et al, 2015). This might be because these genes are known to be heavily promoted by the transcription

factor c-Jun, which is highly expressed in repair Schwann cells, but not the other three phenotypes just mentioned. From a cellular function standpoint, it has been shown *in vivo* that repair Schwann cells greatly outperform immature Schwann cell types when they are placed in the same denervated environments and are analyzed for their ability to perform myelin clearance, construct a bridge. Immature Schwann cells show no ability to guide axons, breakdown myelin or recruit macrophages (Arthur Farraj *et al*, 2012).

Change in c-Jun/Krox20 Axis Expression After Nerve Injury:

These genetic and physically diverging traits between the myelinating Schwann cells and the repair Schwan cells that make them unique seems to be triggered by an intracellular change in the expression of Krox20 and c-Jun. c-Jun appears to have a unique inverse relationship with the promyelinating transcription factor Krox20 in regards to their expression levels during periods of nerve repair and myelination respectively (this inverse relationship has been referred to as the Krox20/c-Jun axis). Before nerve damage occurs, myelinating Schwann cells in the mouse are constitutively expressing high levels of Krox20, which reinforce its myelinating phenotype and function. However, upon severing a nerve the expression levels of Krox20 drop significantly to negligible levels due to a currently undetermined extracellular signal. Subsequently, transcription factor c-Jun has been documented to massively increase its expression at the same time, which also appears to directly trigger the Schwann cell nerve injury response in the form of de-differentiation into its repair phenotype and function. These observations imply that Krox20 and c-Jun expression are key regulators of the

myelinating and repair Schwann cell phenotype and function respectively (Figure 8) (Arthur-Farraj *et al*, 2012).

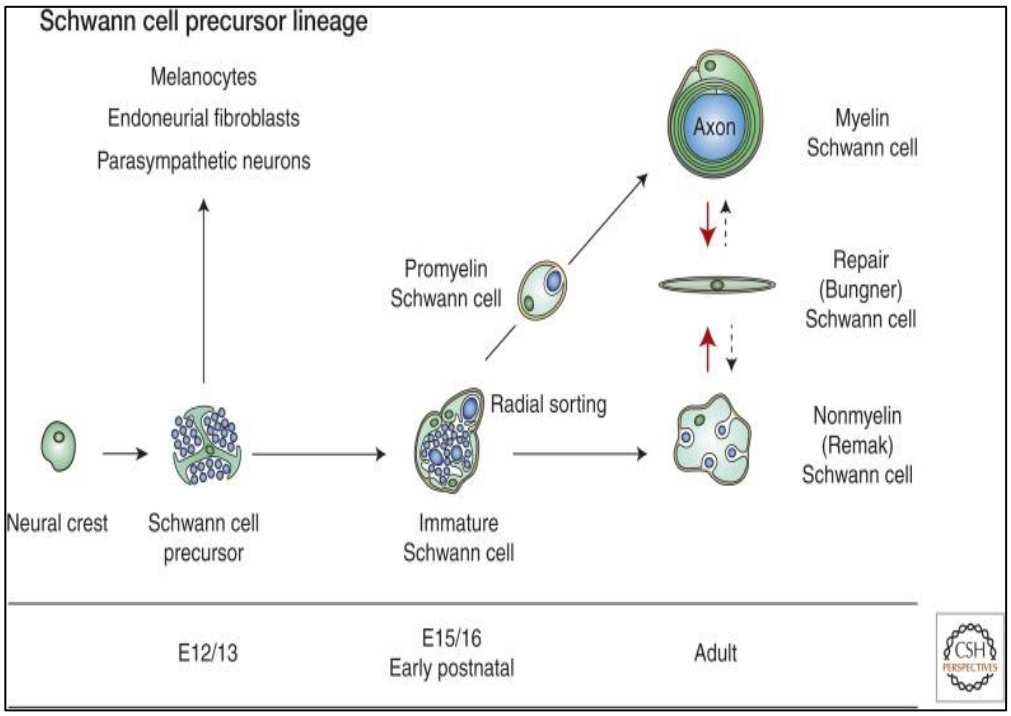


Figure 8: Illustration of Krox20/c-Jun Expression Axis in Each Developmental Stage in The Schwann Cell Lineage (Jess c-Jun Low/Krox20 High).

In studies involving mutant mice that were conditional c-Jun knock outs and had a nerve severed, the absence of c-Jun resulted in a complete failure of the nerve repair c-Jun High/Krox20 Low program usually facilitated by repair Schwann cells at every stage of the process. First of all, c-Jun ablated Schwann cel c-Jun Low/Krox20 High d to be incapable of successfully de-differentiating into their repair phenotype and upregulating the expression/secretion of trophic factors that would normally be increased in order to promote the survival and growth of axons. This is due to the fact that heavy expression of c-Jun is required not only to promote demyelinating genes, but also to suppress promyelinating genes *in vivo*, which would normally initiate the Schwann cell response to nerve injury starting with de-

differentiation into its repair phenotype. Consequently, Schwann cells (and macrophages) that are c-Jun negative have also shown that they are incapable of successfully performing myelin clearance, as c-Jun negative mutant mice regularly displayed a larger concentration of myelin debris at the site of the severed nerve weeks after the cut was inflicted in comparison to controls. Not only that, but it has been shown using electron microscopy that the c-Jun negative Schwann cells who manage to phagocytize some of the myelin debris had bloated waste transfer compartments called autophagosomes filled with myelin debris inside. It appeared as though c-Jun had an effect on the c-Jun negative Schwann cells ability to transfer or degrade the debris in lysosomes unlike the controls (Arthur-Farraj *et al,* 2012).

Lastly, c-Jun negative Schwann cells have demonstrated that they're incapable of forming the Bands of Büngner, also called regeneration tracks which are used to help guide the regenerating axons to the distal end of the nerve and support their growth. Due to lack of an elongated cell morphology that is required to form a sufficient bridge and the insufficient quality of cell to cell associations and disorganized chain construct formations. It is suggested from this observation that c-Jun affects not only whether Schwann cells de-differentiate into repair Schwann cells, but it also has an effect on the resulting cell morphology, cell sorting and cell to cell associations (Parkinson *et al*, 2008; Arthur-Farraj *et al*, 2012; Fontana *et al*, 2012; Gomez-Sanchez *et al,* 2017). Therefore, it has been widely accepted by the scientific community that the role of c-Jun during periods of time after severing a nerve, is to activate an over-arching nerve repair program in the mouse peripheral nervous system.

NRG1 Expression and Function After Nerve Injury:

Two of the biggest questions that would help establish our understanding of what initiates the expression change in the Krox20/c-Jun axis, yet still eludes us is, what extracellular signal (or signals) are the myelinating Schwann cells and Remak cells receiving in order to initiate the process of de-differentiation and from where do they originate? Although there has not been a lot of headway in regards to answering these questions, the research community is not starting from scratch, as they have been able to make theories based on the data at our disposal. So far, research has established that before axonal degeneration occurs, there is upregulation and downregulation of many genes. It just so happens that some of the genes that are downregulated promote myelination or glial differentiation genes that go on to synthesize proteins like Krox20, MBP, and MAG (El Soury *et al,* 2018). It has now been proposed that soluble isoforms of NRG1 are responsible for this de-differentiation process.

In experiments that involved severing nerves that were associated with myelinating Schwann cell populations *in vivo* and *in vitro,* and were treated with the soluble isoform of NRG1 called NRG1β1, it was observed that many of the same myelination and differentiation genes that would normally become downregulated due to injury were replicated by its application (El Soury *et al,* 2018). In further experimentation which analyzed myelinating Schwann cell gene regulation after the severing of the nerve, it was discovered that the severed nerve stimulates the distal stump to heavily express soluble NRG1 isoforms (the identities of which isoforms are expressed has not been heavily investigated and requires further study). When analyzing the gene expression of the myelinating Schwann cells that were associated with the axons of the distal stump,

they too were seen downregulating promyelinating, cell differentiation inducing and apoptotic genes (El Soury and Gambarotta, 2019). Additionally, experiments similar to this were able to document large quantities of phosphorylated (active) ErbB2 and mTORC1 after a severed nerve injury in repair Schwann cells, indicating that soluble NRG1 isoforms are a potential candidate that is capable of activating the nerve injury response in myelinating Schwann cells (Parkinson *et al*, 2008; Yang *et al*, 2012; Guertin *et al* 2015; Arthur-Farraj *et al* 2017). The expression of soluble NRG1 in the distal nerve stump in theory is able to initiate the necessary de-differentiation in myelinating Schwann cells and Remak cells located distal to the site of the cut in the nerve to promote an efficient nerve repair program (El Soury and Gambarotta, 2019).

ErbB3/2 Expression and Function After Nerve Injury:

Just as type III-NRG1 has shown it is capable of binding to ErbB receptors, the same can be said about soluble NRG1 isoforms. Experimental evidence in recent years has demonstrated that the binding that takes place between soluble NRG1 isoforms and ErbB3/2 is involved in initiating de-differentiation in myelinating Schwann cells and Remak cells that are distal to the injury site of the severed nerve. Soluble NRG1isoforms, which have been demonstrated to increase expression in axons distal to nerve injuries, have shown a great affinity for ErbB3/2 receptors that are found on the surfaces of myelinating Schwann cells and Remak cells. Within 10 minutes of severing a nerve, the

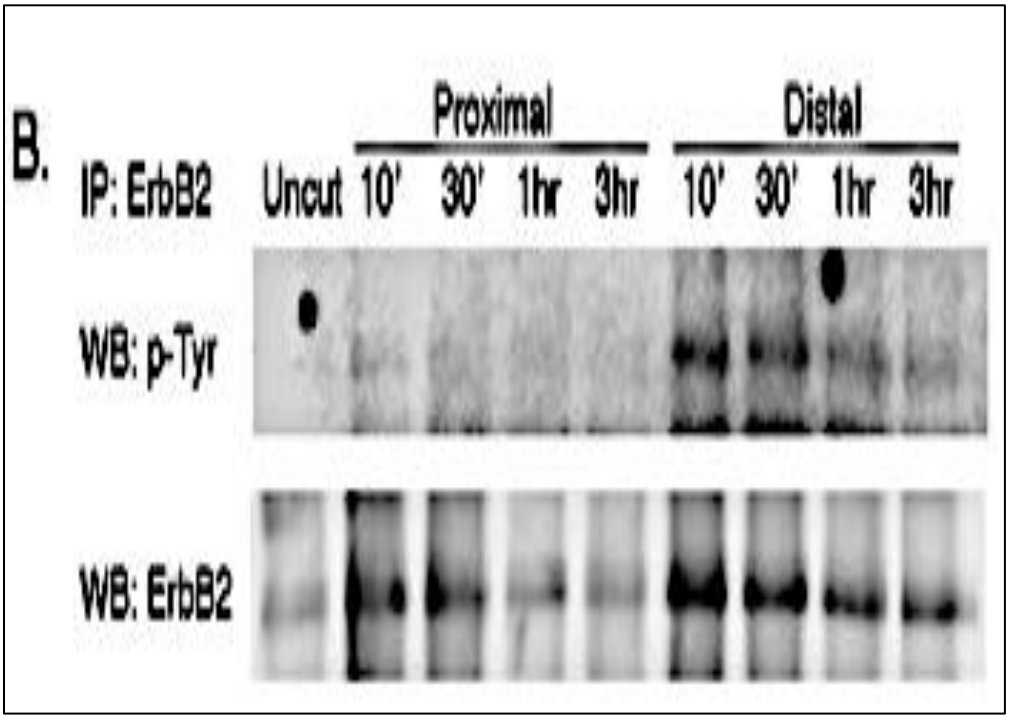


Figure 9: Time Lapse Western Blot of ErbB2 Expression and Activation After Severing a Nerve in Both Proximal and Distal Nerve Ends. P-Tyr represents active ErbB2, while the other row represents the expression of ErbB2 (Guertin *et al*, 2005).

expression and activation of these ErbB receptors of both myelinating Schwann cells and Remak cells membranes of the distal nerve stump increase dramatically in comparison to

their levels in the proximal stump or during myelination as seen in Figure 9 (period without having a severed nerve) (Guertin *et al,* 2005). At 30-60 min post nerve cut, ErbB3 activation peaks and eventually decreases in the hours afterwards (Guertin *et al,* 2005). After its activation, ErbB3 dimerizes with ErbB2, activating ErbB2. In this brief time frame lasting about 1-hour post injury where expression of these receptors is at their highest, the expression/activation of them is enough to induce de-differentiation of myelinating Schwann cells and Remak cells *in vivo* followed by demyelination of axons and myelin clearance (Guertin *et al,* 2005).

Pharmacological intervention has also aided in the quest to determine the role that ErbB3/2 serves in Schwann cell de-differentiation. PKI166 is a specific antagonist to ErbB2 which inhibits the receptors activation and yields no side effects on the Schwann cells as a result. After treatment with the ErbB2 specific inhibitor PKI166, followed by the severing of a nerve, the myelinating Schwann cells were blocked from executing de-differentiation, performing demyelination/myelin clearance activity (Figure 10), and in separate cultures already containing de-differentiated Schwann cells and axons which they were actively demyelinating, it halted their demyelinating functioning, thus attributing some level of control on the repair Schwann cell functioning after their generation from myelinating Schwann cells (Guertin *et al,* 2005).

How ErbB3/2 goes about accomplishing this feat is another story that requires further analysis. From what is currently understood, the number of downstream proteins that are targeted within the distally located myelinating Schwann cells and Remak cells by active ErbB2 are quite numerous providing a multitude of possible suspects that are downstream of these receptors and lead to Schwann cell de-differentiation. However, the

two downstream signaling pathways that have been mentioned earlier in this review (PI3Kinase-Akt-mTORC1 and Ras-Raf-Erk) have been the most heavily studied due to the suspected relationship it shares between the activation of ErbB2 in myelinating Schwann cells and Remak cells and the generation of the repair Schwann cell.

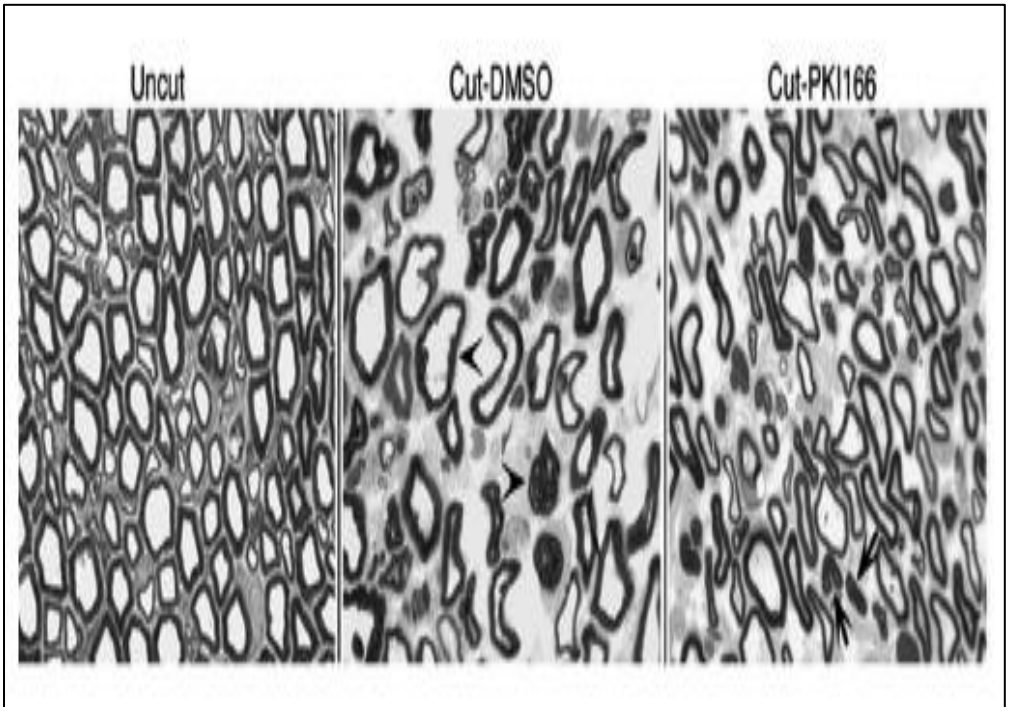


Figure 10: Cross-Section Images of Myelinated Axons After Nerve Cut and Treatment with ErbB2 Inhibitor PKI166 (Guertin *et al*, 2005) The concentration of myelin debris in ErbB2 inhibited mutants compared to the wild type control specimens that had their nerves severed.

PI3Kinase/Akt/mTORC1 Pathway Activation After Nerve Injury:

One of the suspected pathways that are activated by ErbB2 to some capacity is the PI3Kinase-Akt-mTORC1 pathway. In studies investigating the source of the extracellular signal that causes the myelinating Schwann cell's severed nerve injury response to activate, it had been confirmed that it did not come from macrophages which are usually

the only other outside cell type that can be in the same vicinity of the Schwann cells (Norrmen *et al,* 2018). With that possibility eliminated, the most likely source of the extracellular molecule are the distal axons themselves which do have physical contact with the Schwann cells prior to Wallerian Degeneration (Norrmen *et al,* 2018). The reasoning behind this theory comes from studies that documented severed axons on the distal side of the nerve heavily expressing soluble NRG1 isoforms after being cut (Figlia *et al,* 2017), which is capable of activating PI3Kinase. In addition to the resulting mTORC1 activation after soluble NRG1 treatment, the severing of a nerve in mice results in the upstream activator of mTORC1 named Akt to be phosphorylated and active at this time, as well as mTORC1's downstream effector protein S6K (Norrmen *et al,* 2018). As mentioned earlier S6K has been previously documented to inhibit Krox20 expression, which allows for the expression of c-Jun to increase and further repress expression of Krox20. To confirm the phosphorylated S6K in fact does repress expression of Krox20 and promote c-Jun expression, studies have utilized mutant mice that had defective mutant mTORC1 which couldn't phosphorylate downstream proteins. Observations were made to see if there were any changes to Krox20 and c-Jun expression. Both myelinating Schwann cells and Remak cells that were associated with the axons of the distal nerve stump and also possessed the defective mTORC1 were unable to affect the expression levels of either transcription factor, nor could they successfully perform de-differentiation leading to poor myelin clearance/demyelination of the injury site after the nerve was severed (Norrmen *et al,* 2018). However, after severing the nerves in the mice with control Schwann cells, these formerly myelinating Schwann cells displayed the presence of phosphorylated S6K, followed by significantly decreased expression of Krox20 and a

massive increase in c-Jun expression, which even went on to generate repair Schwann cells (Norrmen *et al*, 2018)

In summation, these experiments provide significant evidence that Schwann cell de-differentiation can be activated through the use of the PI3Kinase-Akt-mTORC1 pathway. This theoretical response to a severed nerve injury suggests that when the axons are severed, the distal nerve stump begins to heavily express soluble NRG1 isoforms which quickly binds to the surface receptors ErbB3/2 which activate the proteins participating in the PI3Kinase-Akt-mTORC1 pathway (and many other target proteins) of myelinating Schwann cells and Remak cells. In accordance with the research and findings made in the Beirowski *et al*, study it appears that mTORC1 activity increases dramatically from this point onward, which goes on to activate its target proteins S6K and 4E-BP1. These two proteins repress the expression of Krox20 and allow for the expression of c-Jun to occur which further represses the expression of Krox20 and initiates de-differentiation (Figure 11) (Figlia et al, 2017; Beirowski et al, 2017; Norrmen et al, 2018). When soluble NRG1 binds with ErbB3/2 on the Schwann cells surface PI3Kinase can be activated and the following downstream target activations result in de-differentiation and thus begins the peripheral nerve injury response (Jessen and Arthur-Farraj, 2019). Now, although this signaling pathway has demonstrated that it is a likely candidate that initiates de-differentiation in myelinating Schwann cells and Remak cells, it doesn't mean that it is the only one capable of doing this nor is it the only signaling pathway that becomes active following events that result in a severed nerve.

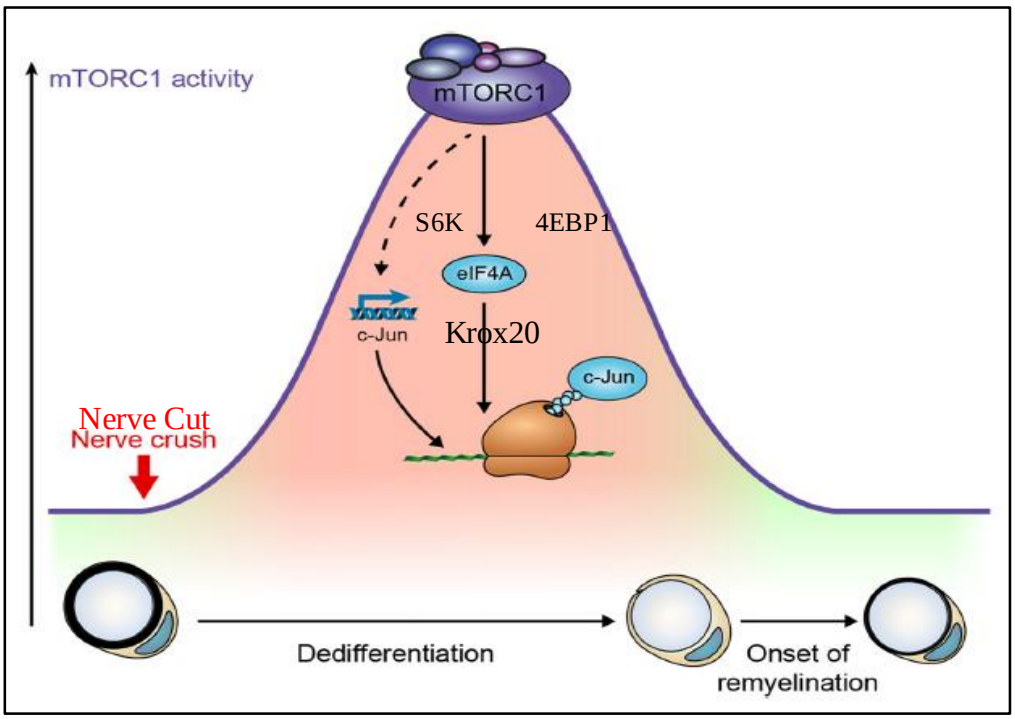


Figure 11: mTORC1 and Effector Protein Activity After Severing a Nerve (Figlia *et al,* 2017; Newbernn *et al,* 2018). Shortly after nerve cut, mTORC1 activity increases dramatically and through the following signaling pathway, it most likely initiates de-differentiation in myelinating Schwann cells. After nerve repair is completed, mTORC1 activity decreases back to basal levels.

MAPKinases (Ras-Raf-Erk) Activation After Nerve Injury:

The other signaling pathway that has raised suspicions in researchers as a possible initiator of de-differentiation is the Ras-Raf-Erk pathway. Despite not having a large involvement in developing Schwann cells, the signaling cascade highlighted by proteins Ras-Raf-Erk has shown significant evidence as another pathway that can lead to Schwann cell de-differentiation. In a series of studies, research teams noticed this reverse differentiation effect through Ras-Raf-Erk pathway activation, when they induced a promyelinating Schwann cell culture to differentiate into mature myelinating Schwann cells using cAMP (researchers are not sure why cAMP yields this result or its implications in the PNS development, but it was used knowing that it had this effect on

immature Schwann cells) for 3 days and then activated Raf constitutively, the then myelinating Schwann cells, lost their myelinating function and phenotype and seemingly reverted back to a phenotype from earlier stages in the Schwann cell lineage (Harrisingh *et al,* 2004; Napoli *et al,* 2012). This experiment which demonstrated that activating Raf alone was enough to induce de-differentiation, cause repair Schwann cell proliferation, myelin degradation and the recruitment of macrophages in nerve cross sections that never sustained any type of injuries (Figure 12) was very convincing (Napoli *et al*, 2012*).*

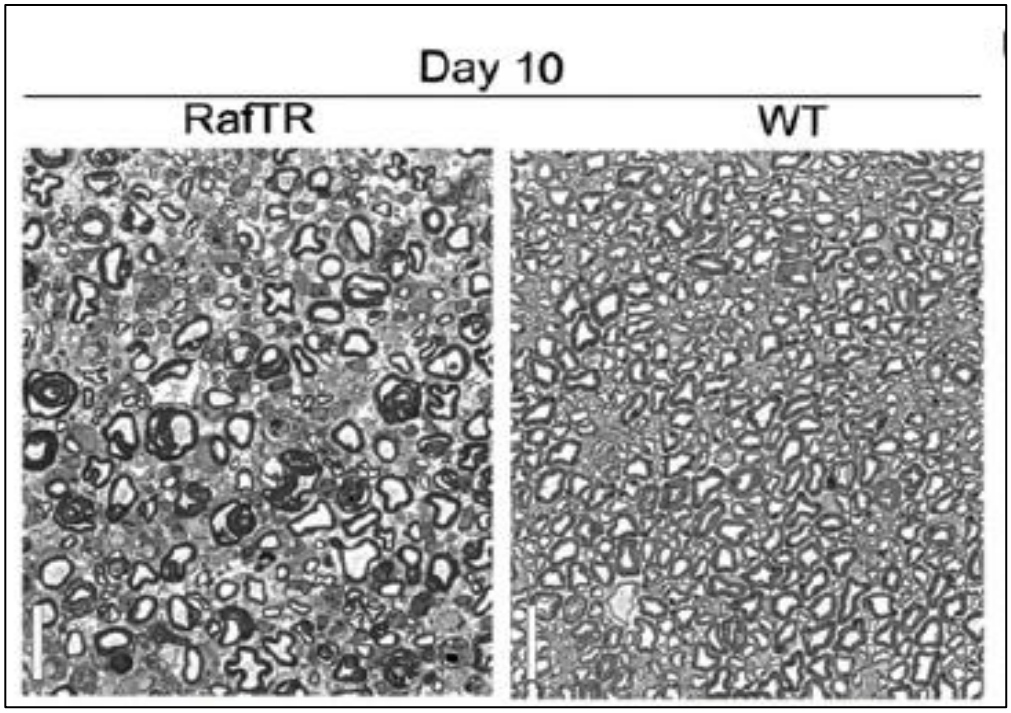


Figure 12: Nerve Cross Section Comparisons Between Constitutively Activated Raf and Wild Type in the Absence of Any Nerve Injury (Napoli *et al*, 2012).

During the analysis of protein expression from these de-differentiated Schwann cells, it was found that there were high levels of expression and activation of the downstream protein of Raf called Erk. This raised suspicions that Raf had accomplished this effect on myelinating Schwann cells through the activation of Erk (Harrisingh *et al,*

2004; Agthong *et al*, 2006). To determine this, the same experiments were repeated, but in the presence or absence of a pharmacological inhibitor of Erk's activator MEK. What was observed was with the constitutive activation of Raf there was a total block of activated Erk (P-Erk) in the presence of the MEK inhibitor, resulting in no downregulation of P0 or Oct-6, while the absence of the MEK inhibitor saw the presence of P-Erk and downregulation of P0 and Oct-6, indicating that Raf induced de-differentiation is mediated by Erk (Figure 12) (Harrisingh *et al*, 2004).

Eventually, by making previously myelinating Schwann cell populations express the upstream activator of Raf (named Ras) which was tagged with green fluorescent protein and injecting it into a myelinating Schwann cell population, it was found that the myelinating Schwann cell population had de-differentiated (Harrisingh *et al*, 2004).

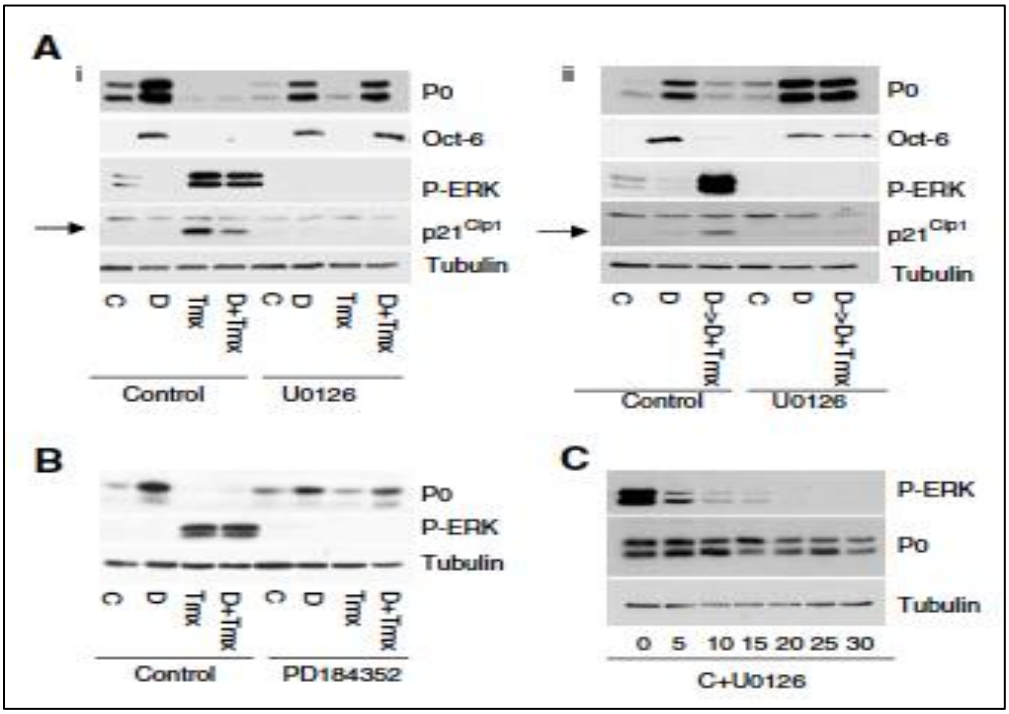


Figure 13 Western Blots of P0, Oct-6 and p-Erk With U0126 and PD184352 MEK Inhibitors. Tmx is a group of mutant mice who have Ras-Raf-Erk constitutively active and D+Tmx is a subpopulation of the same mutant mice, that are also treated with db cAMP (Harrisingh *et al*, 2004).

Not only had that population de-differentiated, but after testing for the presence of proteins associated with myelinating Schwann cells like Krox20, MBP and Oct-6, it was found that they were heavily downregulated (Harrisingh *et al,* 2004). This experiment was also performed with the same MEK inhibitors used prior to make sure that it was performing these functions through P-Erk mediation, and just like the previous experiment with the inhibitor, there was no P-Erk detected and no de-differentiation, suggesting that de-differentiation can be initiated through the Ras-Raf-Erk pathway simultaneously alongside PI3Kinase-Akt-mTORC1 pathway activation (Figure 13).

Myelin Clearance and Macrophage Recruitment

Unlike their glial cell counterparts in the central nervous system (oligodendrocytes), the repair Schwann cells that are generated through likely many signaling pathways including the ones just discussed appear to be morphologically and behaviorally distinct. The activity of the repair Schwann cells revolves primarily around myelin following the severing of a nerve. This myelin destructive behavior that takes place shortly after the generation of repair Schwann cells and which oligodendrocytes are incapable of replicating, is referred to as myelin clearance. Since myelin displays growth inhibitory features, it can be conceived that if enough myelin debris gathers in between both the proximal and distal regions of the nerve it can prevent regenerating axons from growing past them to reinnervate with the distal end (Kang and Lichtman, 2013). Thus, myelin clearance is an important event in the repair program that aims to remove as much of the myelin debris as possible.

Repair Schwann cells achieve this goal through the use of two mechanisms called autophagy (also called myelinophagy and it is categorized as phase 1 of myelin clearance) and macrophage mediated phagocytosis (known as phase 2 of myelin clearance). Starting as soon as two days after a nerve is severed, autophagy related genes quickly activate and become heavily upregulated until 7 days post injury. The particular genes activated are ULK complex, ATG9 cycling system, ATG7, ATG16L, Wipi2 and Beclin1 which are all essential for autophagosome formation (Gomez-Sanchez *et al,* 2015). At this same time of autophagy related gene activation, the presence of key myelin components MBP and P0 in the extracellular environment begin to decrease as well. In fact, by using electron-microscopy one can easily see collections of myelin debris in double membraned autophagosomes. In the event that one of the autophagy related genes are not expressed, it produces a profound effect on the repair Schwann cells and their ability to complete the process of myelin clearance. In experiments that used ATG7 knockout mutant mice and severed one of their nerves, a significant increase in the presence of myelin debris at the injury site could be seen, as well as the presence of repair Schwann cells with a bloated morphology and swollen autophagosomes filled with myelin debris that they could never seem to degrade (the results from this experiment were replicated in another version of this experiment where c-Jun was ablated, which suggests that c-Jun has an effect on the expression of autophagy related genes like ATG7 as well) (Gomez-Sanchez *et al,* 2015; Arthur-Farraj *et al,* 2012).

The events of phase 1 in myelin clearance is highlighted by strict repair Schwann cell myelin autophagy, in which myelin debris and some undamaged myelin sheaths on the proximal nerve end are “engulfed” by the repair Schwann cell and transferred into

internal transport compartments called autophagosomes, which eventually are transferred into lysosomes where chemical degradation occurs allowing the repair Schwann cell to recycle the degradation products for later use (Jessen and Mirsky, 2019). The second phase is highlighted by the recruitment of macrophages by repair Schwann cells. Macrophages invade the injury site from the circulatory system and finish the myelin clearance via phagocytosis and myelin degradation in their own lysosomes (Jessen and Mirsky, 2019).

From 4-7 days post injury, the presence of macrophages at the injury site can be seen in large quantities which indicates the second phase of myelin clearance has begun. However, as mentioned before, the macrophages that are normally circulating through an organism's blood vessels need to receive signals in order for them to know where they are being recruited to and what they need to do. This recruiting process conducted between the repair Schwann cells and macrophages is accomplished through the union of repair Schwann cell secreted monocyte chemoattractant protein-1 (MCP-1 also called CCL2) and its receptor protein on macrophage surfaces called CCR2. Without the binding between these two proteins (as demonstrated by MCP-1 or CCR2 gene deletion mutants), macrophage recruitment to peripheral nerve injury is severely attenuated (Lindborg *et al,* 2017). Intriguingly, in a compensatory reaction to situations like this, (which hinder the recruitment of macrophages), phagocytic repair Schwann cells proliferate and by using a currently unknown method of extracellular signaling, they are able to recruit neutrophils to build a large local population of cells capable of clearing myelin debris, and to quite a great effect (Lindborg *et al,* 2017). It appears though that this is their one and only contingency plan, because as soon as neutrophils are eliminated

through genetic engineering, myelin clearance is not completed (Lindborg *et al,* 2017). Additionally, there is no known compensatory reaction in situations where there are no repair Schwann cells to secrete MCP-1 or other signaling proteins to recruit other phagocytic cells (Lindborg *et al,* 2017).

As researchers would find out through further experimentation, this macrophage recruiting mechanism is regulated by the presence of a protein found in Schwann cells called miR-327 (Zhao *et al,* 2017). As it turns out MCP-1 is a target of miR-327, and when it interacts with MCP-1, MCP-1 is heavily suppressed in the Schwann cells preventing any outgoing recruitment signals for macrophages. Beings that normal myelinating Schwann cells don't ordinarily secrete MCP-1, it is highly likely that miR-327 is at its highest expression levels during this period of time, but as soon as a nerve injury like a severed nerve occurs and the myelinating Schwann cells de-differentiate, the axis shifts so that the expression of miR-327 is suppressed by a currently unknown nerve injury related protein (miR-327 seems to be suppressed by many molecules, but one that is related to nerve injury hasn't been uncovered yet) and that allows for the increased expression and secretion of MCP-1 in order to recruit the M1 macrophages needed to promote more efficient nerve repair (Zhao *et al*, 2017).

Well into phase 2 of myelin clearance, the presence of two types of macrophages will likely be seen. The first type is classified as M1 type macrophages and they are described as pro-inflammatory macrophages. Their job upon invading the injury site is to secrete pro-inflammatory factors, remove myelin debris, phagocytize unwanted foreign microorganisms and elimination of any apoptotic cells (Gensel and Zhang, 2015). M1 macrophages show an enhanced phagocytic ability and increased scavenging activity for

debris and bacteria, in comparison to its M2 macrophage counterparts, therefore contributing the most in regards to the phagocytosis of myelin debris inside and outside the axons (Sindrilaru and Scharffetter-Kochanek, 2013).

Over time as the myelin debris progressively is removed from the injury site, these M1 macrophages will gradually become type M2 anti-inflammatory macrophages of which there are 4 subtypes, each with specific roles after being stimulated by certain extracellular signals. M2a macrophages promote anti-inflammatory effects like Schwann cell proliferation and migration, production of growth factors and assisting in the removal of apoptotic cells. M2b macrophages promote cell maturation, tissue stabilization, angiogenesis, and extracellular matrix synthesis. M2c macrophages accelerate the resolution of inflammation, tissue repair and extracellular matrix synthesis and produce growth factors. M2d macrophages have roles in angiogenesis and wound healing (Ferrante and Leibovich, 2012; Chen et al, 2015; Gensel and Zhang, 2015).

Bridge Schwann Cell Formation/Migration and Facilitated Regeneration and Trophic Support of Axons

For a long time, scientists made the rash error of assuming that the repair Schwann cells that participated in myelin clearance and Schwann cells that migrated and participated in the formation of the bridge were one in the same cell type since both processes tend to occur at the same time. Recent studies have provided evidence that repair Schwann cells undergo yet another change in gene expression in order to develop another specialized form of Schwann cell that is required in order to proceed with axon regeneration.

Through the use of transcriptome analysis seen between the two de-differentiated Schwann cell populations, it is made clear that bridge Schwann cells downregulate genes that are commonly expressed in the distally located repair Schwann cells and are needed to proceed with nerve repair (Figure 13). These include genes that are responsible for inflammation, immune signaling, and the production of extracellular matrix. Inversely, genes that involve cell division, growth and active metabolism were highly upregulated allowing them to be more proliferative than their distal repair Schwann cell counterparts. Additionally, it was discovered that what genetically identifies the bridge Schwann cells in comparison to the repair Schwann cells were their greater expression of mesenchymal genes like Lrrc15 and tenascin-c (Clements *et al*, 2017) which seems to be triggered during migration into the bridge region. Collectively this data supports the notion that repair Schwann cells and bridge Schwann cells are in fact two different sub-populations with their own unique set of active genes responsible for their respective functioning (Clements *et al,* 2017).

This genetic evidence not only suggests that there are two different sub-populations of derived from Schwann cells after the severing of a nerve, but this also meant that at that specific time frame, something is either being expressed within or coming in to contact with repair Schwann cells located distally that triggers this transition to bridge Schwann cells. The molecule TGFβ which is considered to be a master regulator of mesenchymal genes that plays a role in wound healing instantly became a suspect. In experiments that monitored the presence of TGFβ following nerve injury, TGFβ concentration increased in the microenvironment of the bridge region, which differentiated itself from the rest of the nerve microenvironment. Additionally, it was also

determined that the majority of the mesenchymal genes that were specifically activated in bridge Schwann cells were also gene targets of TGFβ, which suggests that TGFβ was the main factor that caused the genetic transition. When TGFβ downregulated mutant rats had their nerve injury sites analyzed, it was observed that many of the bridge Schwann cells were incapable of completing the full length of the bridge which was highly disorganized due to uneven bridge Schwann cell chain extensions and their loose connections with one another. It was later established that TGFβ potentiates the effect of a receptor protein that induces cell sorting named EphB2. The mechanism that facilitates this cellular action involves EphB2 binding with its ligand, which results in it being phosphorylated. The now active EphB2 protein post translationally modifies and stabilizes the pluripotency factor Sox2, which goes on to increase Schwann cell to cell adhesion by means of re-localizing N-cadherin which is a protein found in many cells that is responsible for promoting cell mobility and cell to cell contact/interactions (Figure

14). In short this means that TGFβ enhances the ability of the bridge Schwann cells to associate with other bridge Schwann cells allowing for longer, tighter and more organized chain constructs that compose the bridge (Clements *et al*, 2017; Parrinello *et al*, 2010).

After a couple of years following this study, it had been discovered that Sox2 expression also has an effect on bridge Schwann cell migration guidance into the bridge region and as an indirect result on the directionality of axon regeneration (Dun *et al*, 2019). It is clearly visible in Figure 15 that Sox2 ablated bridge Schwann cells seem hindered in their ability to direct their migration through the bridge region and successfully to the distal end of the nerve. Additionally, following protein analysis after administering a nerve cut in these Sox2 negative mutants, it was observed that repair Schwann cells especially the bridge Schwann cells resulted in a significant decrease in expression of a particular Sox2 target that plays a role in axon guidance called Robo1.

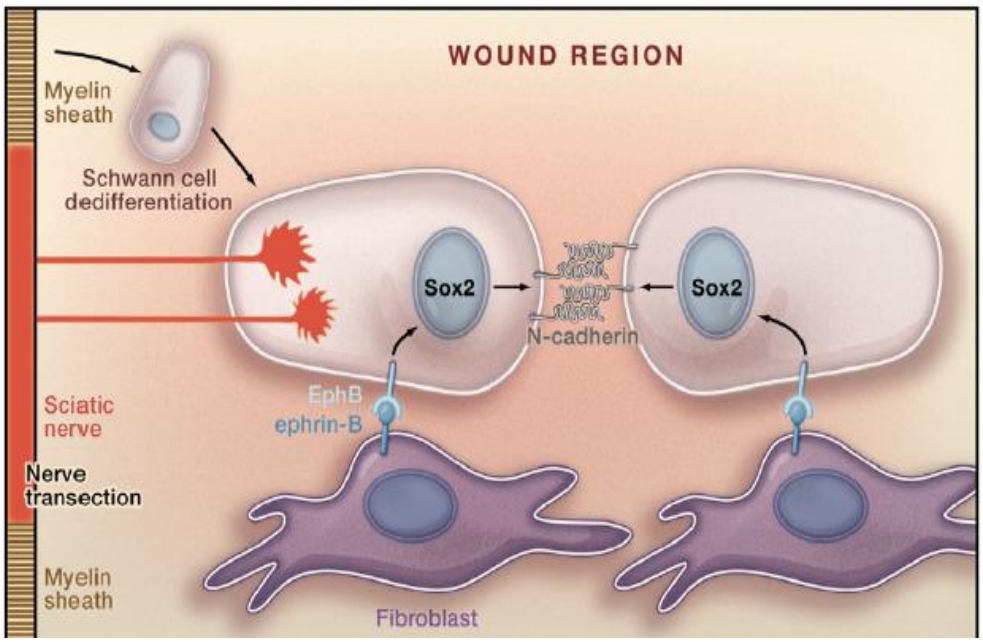


Figure 14: Illustration of EphB Signaling Between Repair Schwann Cells and Fibroblasts (Klein, 2010).

Likewise, the over expression of Sox2 once again coincided with the expression of Robo1, implying that Sox2 is a regulator of the receptor (Dun *et al,* 2019).

Previous studies in rats had shown that the ligand for Robo receptors were Slit proteins (Slit 1, 2 and 3) and their interaction had an effect on Schwann cell migration (Wang *et al,* 2013). Following further experimentation, it was eventually uncovered that not only does Robo1 have a high affinity for Slit3, but the macrophages that are located at the perimeter of the bridge (as well as within the nerve bridge) also highly express Slit3 (Dun *et al,* 2019). The reactions between the bridge Schwann cells and macrophages on the perimeter of the bridge suggested that Slit3 has a repulsive effect on the bridge Schwann cells and an inhibitory effect on the speed of regenerating axons due to experiments which observed Slit3 and Robo1 heterozygote mutants regenerating axons growing at a faster rate and in a multidirectional fashion in comparison to homozygous controls (Dun *et al,* 2019). This relationship that Slit3 has with Robo1 receptors appears to keep the bridge Schwann cells within the confines of the bridge's parallel borders that macrophages help to establish. Additionally, it slows down axon regeneration long enough for bridge Schwann cells to reach the distal end preventing axon regeneration failure caused by a growth rate of axons that exceeds the rate of growth for the bridge resulting in diverging growth of axons (Dun *et al,* 2019). Despite having the important

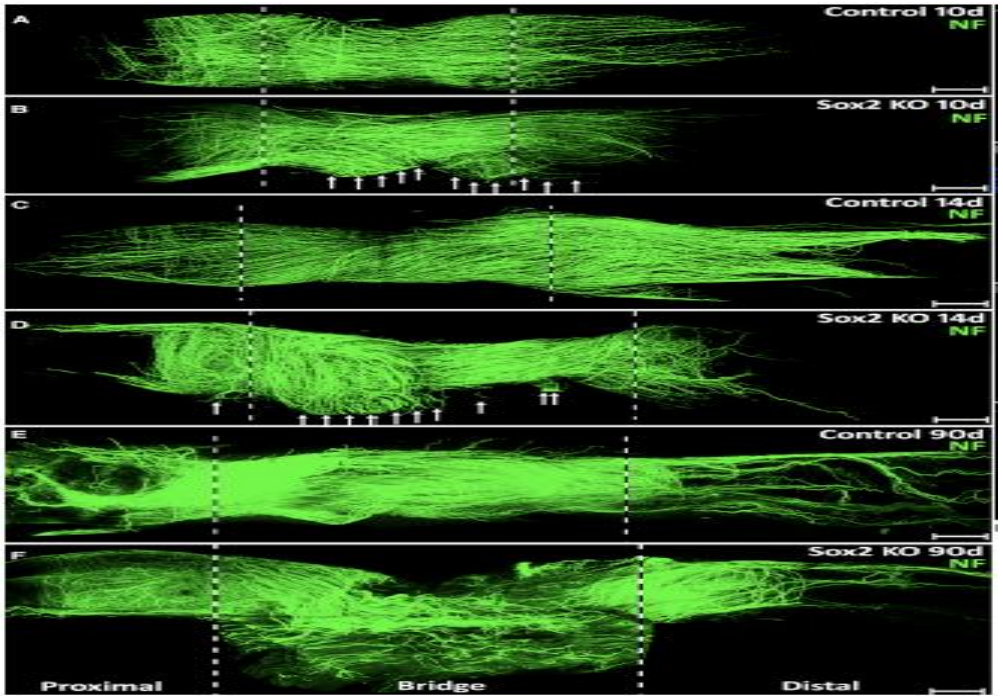


Figure 15: Neurofilament Immuno-Staining Comparing Nerve Regeneration in Sox2 KO mutants and Control Mice During Nerve Regeneration. Sox2 negative mutant axon growth can be seen diverging from the intended path to the distal nerve end (Dun *et al*, 2019).

responsibility of clearing myelin debris and constructing the bridge between both the proximal and distal nerve ends, it is not the only responsibility that both repair and bridge Schwann cells have. After de-differentiation Schwann cells are essentially tasked with the responsibility of being a microscopic caretaker for the damaged axons and other Schwann cells. This is accomplished by secreting a plethora of neurotrophins like glial cell line-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and erythropoietin (cytokine that encourages the production of red blood cells) which keeps the neuron and axons that composed the nerve alive throughout the entire duration of the repair process (Grothe *et al*, 2006; Fontana *et al*, 2012; Brushart *et al*,

2013). In addition to neurotrophins, repair Schwann cells also secrete cytokines like tumor necrosis factor a (TNFa), interleukin-1a (Il-1a), Il-1b, leukemia inhibitory factor (LIF), and as mentioned earlier monocyte chemotactic protein-1 (MCP-1) (Martini et al, 2008; Rotshenker, 2011), which aids in the recruitment of macrophages from the circulatory system in an attempt to accelerate myelin clearance to prevent the blockage of regenerating axons which in turn increases the chances of all axons reattaching to the distal end of the nerve and regaining function. Each one of these molecules play a critical part in the success of the nerve repair program in murine species and they will likely play a big role in enhancing current nerve repair treatments for humans.

V: Discussion

Current Surgical Interventions: Nerve Grafts

Just as discoveries involving the mechanisms of peripheral nerve repair are made, often times, developments in treatment methodology soon follow. Over the last 170 years, the people involved in scientific and or medical professions have developed several treatment methods for people who are diagnosed with peripheral nerve injuries. The surgical methodology that shows the greatest potential for regaining function in severely damaged nerves are nerve grafts. Nerve grafts can be generally described as a surgical method that manages to bridge a gap between both nerve ends utilizing donor tissues or conduits allowing for the patient to maintain a level of functionality in the nerve. Although this is the current pinnacle of surgical treatment/repair for nerves it still has yet to achieve beyond a 50% success rate of regained function. In fact, the simple

mishandling of donor nerves or tissues during the surgery can cause enough microscopic damage that it can begin to undergo fibrosis which would negate the purpose of the surgery. There are several different iterations of nerve grafts that have developed and have tried to improve upon one another over the years. Each nerve graft has their own specialized application dependent upon the severity of the damaged nerve. Some peripheral nerve injuries are graded as more severe than others, and these types of injuries are generally more difficult to treat and require creative solutions like the nerve graft.

A popular type of nerve graft is called a human autograft, which utilizes pieces of tissue from either the same patient or someone else whose tissue will not be rejected by the body, which is used to bridge the gap between proximal and distal ends. Categorized as single grafts, these nerve segments usually come from a donor and are similar in diameter to the one being repaired. In situations where nerve damage effects a large length of the nerve, cable grafts are used. Cable grafts are composed of multiple donor nerves (usually smaller in length) sutured together until it is the approximate length of the original intact nerve (Grinsell and Keating, 2014).

For nerve damage on a large proximal end of a nerve, surgeons will generally use a trunk graft, which comes in the form of a large sized nerve segment harvested from a donor. However, functionality success rates are often poor using this method, because the segments are so large and poorly vascularized, that scar tissue forms inside of the graft, thus blocking re-growing axons from reaching their destination. Vascularized versions of trunk grafts (where donor nerve is transposed along with its blood vessels that nourish it) have been performed since its inception in 1976 by Taylor and Ham, but there is a debate

as to whether it actually stops the formation of scar tissue and increases the functionality success rate by allowing axons to re-grow until they reach their destination. Occasionally, an autologous (nerve sample from the same patient) nerve graft is used, in which a fully functioning sensory nerve is removed and used to bridge the gap between proximal and distal ends of the injury site (Grinsell and Keating, 2014).

It is important to note that all of the various nerve grafts discussed are transposed in reverse orientation, because nerves tend to branch out into smaller segments that can lead in multiple directions the further you look downstream of the distal end. To mitigate the chances of regenerating axons extending away from its destination through branches, its orientation is inverted, thus allowing regenerating axons to grow downstream through one main nerve conduit (Grinsell and Keating, 2014).

Although all of these surgical innovations represent the height of our ability to medically intervene to help patients who suffer from severed nerves heal and go back to normal everyday life, it is simply not enough. There are several limitations associated with all of these surgical treatments, which prevents a human patient from reaching 100% regained function. Some of the most limiting problems is the mismatch that occurs in both the size of the repaired nerve and the fascicles. Despite using surgical microscopes and fine utensils to more efficiently view and manipulate tissues, it is still very difficult to perform a perfect repair, since the utensils are often times not fine enough and end up grabbing more fibers then the surgeon intended to suture, which can result in fascicular mismatch, the hand is often unsteady and regularly shaking when viewing through a microscope which also affects the precision of the repair (Grinsell and Keating, 2014). At

the end of the day, surgical repair methods usually see axon regeneration success rates of 25-50% depending on the type of injury. (Grinsell and Keating, 2014).

Future of Nerve Repair Treatments

At the end of the day, current surgical repair methods like nerve grafts are insufficient in fully repairing a nerve included with regained functioning, but with the information we currently know it may be possible to develop a next generation blueprint for a new method of treatment that provides improved nerve repair and functioning for patients. Due to the fact that nerve tissue can undergo fibrosis easily depending upon the physical contact that is used upon them, an ideal method for nerve repair will involve as little as little contact as possible with the nerves in order for them to remain viable. This immediately would remove any type of suturing type repair as a candidate since there must be a significant amount of handling of the nerve to accomplish this. However, there is another avenue of nerve repair treatment that still remains in its infancy, but has potential to provide highly effective nerve repair if perfected. This developing treatment method is known as a nerve conduit.

Nerve conduits in their simplest terms are essentially a protective artificial tube that would be surgically implanted in the gap between the proximal and distal ends of a severed nerve that would allow for the axons of the proximal end to be guided directly to the distal nerve end as they regenerate naturally. However, the implementation of nerve conduits is considered highly controversial in the realm of neuroscience, due to the level of complexity in their construction and function that is needed for them to work cooperatively with the natural biological response from an *in vivo* microenvironment.

There have been attempts at designing ideal nerve conduits in the past several decades but the growing collection of doubts regarding their level of effectiveness that they have provided in comparison to the far simpler nerve grafting method. Although this is certainly a legitimate cause for concern, there is still a possibility that in time it can be improved and possibly perfected using better technology and data that develops alongside it over time.

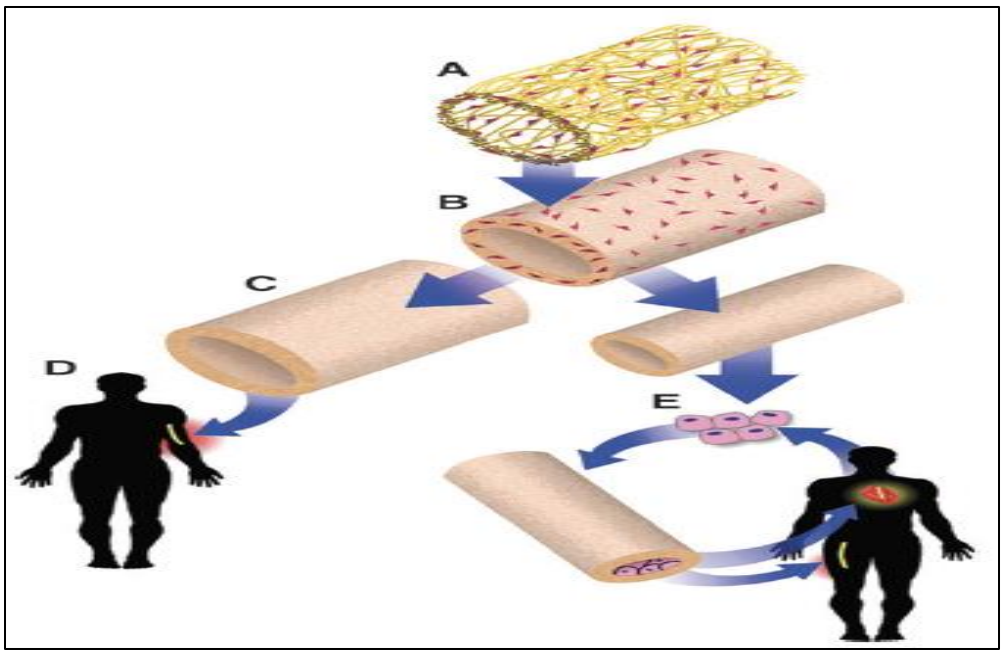
As mentioned previously, the ideal nerve conduit will need to cooperate with a patient's natural biological response to a severed nerve. This is where the first problem is encountered, since it was mentioned at the beginning of this review that the human model of peripheral nerve injury response is completely ineffective on its own. Essentially this means that the nerve conduit will likely not be receiving much assistance to promote growth naturally from the cells and tissues of the severed nerves microenvironment, which also means that additional medical intervention will be needed in order to successfully promote the repair of a severed nerve. This will need to be taken into consideration when developing this prototype nerve conduit.

The nerve conduit itself must possess many unique properties that will overcome the deficiencies in the human severed nerve model and promote nerve regeneration. The most important properties include having an internal framework that is home to a network of repair Schwann cells and their supporting cells in macrophages and fibroblasts, being porous for trophic support and vasculature and having internal microchannel structural guidance (Colazo *et al,* 2019). Other properties such as being biodegradable and conductive would be an added luxury (Collazo *et al,* 2019), but are not necessarily required.

Luckily, it appears that the FDA is currently prepping for phase 3 clinical trials of a new nerve conduit material, provides the critical properties needed to facilitate proper nerve healing. This new material is being referred to as a Tissue Engineered Vascular Graft (TEGF) and has been showing great potential to function as a structural material for the conduit which provides an internal cellular network that can host a multitude of cell types within its walls to facilitate axon regeneration and prevent unwanted immune system reactions that could harm the patient since it is decellularized (Dahl *et al,* 2011). In order to better understand the steps that follow the generation of this material, it is important to understand how it's made (Figure 16). The first steps in its creation is to take either human allogeneic or canine smooth muscle cells and culture them for 7-10 weeks on tubular shaped scaffold made of polyglycolic acid (Dahl *et al,* 2011). During the culturing process the smooth muscle cells will secrete collagen to synthesize vascular tissue, while the polyglycolic acid degrades (Dahl *et al,* 2011). Lastly, this tissue is

decellularized using detergents leaving behind the porous, tubular collagenous matrix (Dahl *et al,* 2011).

Figure 16: Illustration of the Steps Used to Construct a Decellularized Nerve Conduit. (A) Human cells can be cultured on a polymer scaffold that degrades leaving behind a



(B) tissue composed of ECM proteins. That conduit made of tissue will be decellularized (C), and may be seeded with other cells (E) or may not have other cells seeded in them (D) (Colazo *et al,* 2019).

Following the successful harvesting of human myelinating Schwann cells most likely from human cadavers and growing them in culture containing human Schwann cell growth medium, the generation of repair Schwann cells needs to take place. To do this either a preexisting culture of myelinating Schwann cells or a culture of immature Schwann cells treated with cAMP (this induces promyelinating differentiation into the myelinating Schwann cell (Harrissingh *et al,* 2004)) will have to be treated with a soluble isoform of NRG1 such as NRG1β1 (it's the same isoform used in the El Soury *et al,* 2018 study, which seemed to have shown a great deal of success in this venture), since

that is the suspected extracellular signal that triggers the generation of repair Schwann cells from myelinating Schwann cells (or Remak cells). After the application of NRG1β1 to the culture followed by the binding of it with ErbB3/2 signal cascades from PI3Kinase-Akt- mTORC1 and Ras-Raf-Erk pathways (and likely others that are currently unknown) would result in the transition from myelinating Schwann cells to a population of repair Schwann cells.

Separate M1/M2 macrophage and fibroblast cultures will also need to be made after harvesting preferably from elsewhere in the patient or from a matching donor. In theory, the repair Schwann cells that will be transplanted along with the macrophages and fibroblasts, will actively secrete cytokines to recruit/activate them, however this is not a guarantee and active M1 and M2 macrophages and fibroblasts will be needed to assist in myelin clearance and more importantly convert the repair Schwann cells into bridge Schwann cells respectively. To artificially generate M1 and M2 macrophages their separate cultures should be treated with a cytokine called interferon-γ in order to generate M1 macrophages which perform phase 2 of myelin clearance and interleukin-4 to generate M2 macrophages, which secrete anti-inflammatory molecules (Mosser and Edwards, 2008; Truyens and Carlier, 2017). Additionally, the fibroblasts will also need to be activated in order to encourage bridge Schwann cell transitioning and tissue repair. This can be done by adding the enzyme fibroblast activation protein (seprase) to its respective culture before transplantation (Wei *et al,* 2020).

These four cell types will also need to be tested for purity of the populations before transplantation. To do this for repair Schwann cells a small sample from the culture will have to immuno-stained for genetic markers Olig1, Shh and S100 in which

Olig1 and Shh are unique to repair Schwann cells and S100 serves as a positive control for the Schwann cell lineage since it is a universal Schwann cell marker (Jessen and Mirsky, 2019). M1 macrophages will require immuno-staining for genetic markers like CD80, CD86, CD64 and CD32 in combination with the universal macrophage lineage marker CD68 (Tedesco *et al,* 2015). M2 macrophages on the other hand will need to be stained for any one of CD206 or CD163 also in combination with CD68 (Rebelo *et al,* 2018)

At this point in the preparation process, the scaffold would be made and all of the cell cultures would be fully differentiated. Before implantation the cells must be placed on/in the nerve conduit. To do this individual cell suspensions can be made from the main cultures of repair Schwann cells, M1 and M2 macrophages and fibroblasts into separate tubes. Meanwhile, the nerve conduit will be placed in a flask or graduated cylinder with the intention of having the nerve conduit submerged in a mix of all of the cell suspensions after they are poured in so that every surface is coated in all of the cells. After an undetermined period of incubation that provides enough time for the cells to adhere to the surfaces of the nerve conduit, it can be removed from the mixed cellular suspension and either directly sutured to both ends of the patient's severed nerve aseptically so that it bridges the gap in between or frozen for storage. Afterwards, the patient can have their surgical incision sutured and hopefully over time the cells in the nerve conduit will facilitate a type of nerve repair that resembles the efficiency of the mouse and are able to regain function.

As I had mentioned earlier in this prototype blueprint, this procedure will likely involve either manual injections or injections by an automated delivery system to deliver

neurotrophins and growth factors at the site of the severed nerve. These neurotrophins would supplement the active nerve repair taking place by increasing the rate at which both axons and cells alike regenerate and survive. Neurotrophins that would need to be administered would be BDNF, GDNF, NGF, and NT3 in order to deliver several types of neurotrophins that have been shown to enhance nerve repair (Grothe *et al*, 2006; Fontana *et al*, 2012; Brushart *et al*, 2013). However, there is still a large problem that still puzzles researchers until this day and that is the issue of vascularizing the regenerating axons. The damaged axons as well as the damaged tissues around them will need to receive nutrients and oxygen from the endogenous blood vessels to survive. As an attempt to solve this problem and to coax the body to cooperate with the treatment, it would be of interest to see if by injecting certain growth factors that promote angiogenesis and other extra cellular matrix proteins such as IGF-1, VEGF, erythropoietin, platelet derived growth factor (PDGF), collagen I and IV, fibronectin, elastin, fibrin (and others) (Colazo *et al*, 2019; Friedrich *et al*, 2018; Shafiq *et al*, 2015) in order to grow and attract the endogenous blood vessels to the site of the implant where they can nourish the axons with nutrients and oxygen that they will need to survive the long process of nerve healing that lay ahead.

Limitations in Translation of Research in Mice to Humans and How It Is Achieved

Even if a new and innovative treatment for severed nerve repair is developed, it will take many trials in mice in order to perfect all of the variables that are a part of the overall result, such as concentrations of cells and neurotrophins to use, guaranteeing cell culture purity, time to culture and incubate etc. On top of that, there is still the possibility

that after all of that is perfected it still may not function 100% successfully. In fact, history has shown us on multiple occasions that treatments developed in pre-clinical trials based on the observations made in animal models often fail when they're applied to humans for the same purpose. The generalized belief usually held by laypeople and even new/inexperienced researchers, that molecular occurrences that happen in animals will immediately translate into effective treatment in other animals and humans that may be evolutionarily more advanced. Although many features between such organism's genomes would be conserved over time, they will not be identical and this can lead to discrepancies in observed results for differing species.

Since we want to avoid blindly and directly testing experimental treatments on humans without having knowledge of whether it will be safe for them, many research labs use screening technology on potential model species before a study actually begins in order to find the most suitable model candidate that has the most similarities with humans, therefore in theory providing better translation of results (Brubaker and Lauffenburger, 2020). With modern technological advances in computing software, scientists today have the ability to generate simulations that consider the observations from treatments witnessed in an animal species while taking into consideration the differences between the experimental species and humans to predict an outcome for the human. Now, there are many variations of computer-generated simulations used for this general purpose, but in order to acquire some reassurance that a nerve repair treatment like a specially designed nerve conduit which utilizes mimicked molecular interactions to yield a visible result in humans, we will need software that is capable of performing "cross species molecular to phenotypic translation" (Brubaker and Lauffenburger, 2020).

To do this the computational software will need to have the knowledge (in the form of a database) of well documented “cross species molecular to phenotypic” data in order to clearly lay out all similarities or differences that may be experienced in a treatment (Brubaker and Lauffenburger, 2020).

Lastly, it is important to note that just because a computational program predicts a particular result in a human, it too does not mean that it is guaranteed to occur in that manner, possibly due to limitations in the amount or specificity (or lack thereof) of data parameters that you can input into the program in. Therefore, extra measures will have to be taken to assure the accuracy of the predicted human model results. To obtain some gauge of accuracy a comparison between the predicted results of the human model to data that has been analyzed from humans alone must be performed (Brubaker and Lauffenburger, 2020). Luckily, at least one program which has been in development since 2011 called sbv IMPROVER is striving to become the gold standard used for purposes like this (Brubaker and Lauffenburger, 2020; Rhrissorrakrai *et al*, 2015). Developments in technology like this computational program sounds encouraging, but we must temper our excitement and expectations and be patient. There is far more research that needs to be done in order to even begin to develop a prototype method of treatment that aims to provide the solution to this conundrum faced by humans since evolutionary development. As the field in neuroscience continues to grow, and more scientists develop new and innovative studies to shed light on the things still unknown in the repair process for severed nerves, I have confidence that the gap that lies in between where we are now and having full understanding of this phenomenon will be bridged and a solution for this ailment suffered by people around the globe will be found.

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