THE ROLE OF E-CADHERIN IN THE REGULATION OF MYELINATION IN THE PERIPHERAL NERVOUS SYSTEM

By

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ABSTRACT OF THE DISSERTATION

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Dissertation Director:
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In the mammalian nervous system, saltatory conduction is necessary for rapid velocities of action potentials down long axonal trunks. Myelination is the key to this near instantaneous transmission of signals. Oligodendrocytes of the central nervous system and Schwann cells of the peripheral nervous system, are the cells that myelinate axons. While there is some understanding of how these cells concentrically wrap their plasma membranes around the axon to form this insulative layer, the precise induction mechanism is not clearly determined. A potential molecule involved in this process is E-cadherin, which has been previously identified to be localized to regions of compact myelin and various adhesion enriched areas. This study focuses on the role of E-cadherin, a member of the classical cadherin family of calcium dependent adhesion molecules, and its role in the initiation of myelination in the Schwann cells. Protein lysates of rat sciatic nerve demonstrate that expression of E-cadherin was shown to increase with development and that E-cadherin associated with ErbB2, a receptor tyrosine kinase known to function in Schwann cell development, promotes proliferation and myelination. Knockdown of E-cadherin has a negative effect on myelination *in vitro*, while ectopic expression of E-cadherin induces myelination. Additionally, upon

perturbation of beta catenin binding to E-cadherin, enhanced proliferation and myelination was observed. This enhancement was found specifically in Superior Cervical Ganglion neurons (SCG), suggesting that beta-catenin coupling may also have an effect in myelination through an un-determined cadherin-catenin mechanism. These results suggest that cadherins have a critical role in the complex process of myelination.

Acknowledgements

“Education is what survives when what is learned is forgotten…”
-- B.F. Skinner

"When we are motivated by goals that have deep meaning, by dreams that need completion, by pure love that needs expressing, then we truly live life."
-- Greg Anderson

I would like to dedicate this thesis to my unconditionally loving parents, Dr. Vibhuti Desai and Mr. Jitendra Desai. Any success and happiness that I have enjoyed in my life is due to the relentless support and unconditional love they have given me all my life. To say that I am the luckiest son, is an understatement, universal in size. I can never thank them enough for everything and anything. I love you both!

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Introduction

1.1 Function of myelination

Myelination allows for the rapid propagation of action potentials along the axon of a neuron, the primary cell of the nervous system. Most importantly, it is a prerequisite for normal function of the nervous system and an impressive example of differential protein distribution.

Myelination is achieved by the function of highly specialized myelin-forming glial cells. Within their respective systems, these cells are identified as oligodendrocytes and Schwann cells (Zujovic et al.) of the central- and peripheral- nervous systems (CNS & PNS). Early in development, these cells will align onto their respective axons, begin wrapping their plasma membranes in a concentric pattern, which causes an exclusion of the cytoplasm to form a compact region of insulation. This results in the molecular machinery responsible for the rapid propagation of action potentials that are concentrated at regular, discontinuous sites along the axonal trunk (Jessen and Mirsky, 2005b; Lobsiger et al., 2005; Mirsky and Jessen, 1999). Areas where these cells do not cover the axon are called Nodes of Ranvier. Due to the insulatory properties of myelin, membrane depolarization only takes places at these specific nude sites along the axon, thus allowing for rapid or saltatory conduction of the axon potential (Jessen and Mirsky, 1991, 2005a; Koepp et al., 1981; Lobsiger et al., 2005; Mirsky and Jessen, 1996). The genesis of this unique myelin membrane requires complex machinery along with the synthesis of a specific set of lipids and proteins in the correct spatio-temporal orientation (Mirsky and Jessen, 1996).

In addition to their insulative properties, these specialized glial cells also support and modulate neuronal function (Jessen and Mirsky, 2005b; Jessen and Mirsky, 2008; Mirsky and Jessen, 1996). Perturbation of myelination results in several pathologies. For example, multiple sclerosis is a progressive disorder of myelination in the central nervous system. There are 250,000 to 350,000 cases of patients with multiple sclerosis in the United States alone. It is a complex disease with an unknown etiology and no effective cure (Noseworthy et al., 2000). The hallmark of this disease is the deterioration of the myelin sheath by continuous migration of inflammatory immune cells to the CNS (Noseworthy et al., 2000). Having crossed the blood-brain-barrier, these cells attack the myelinated structures, and leave scarring in the white matter (myelin) in the brain and spinal cord, which consequently leads to deficits in motor and sensory functions due to the formed lesions (Compston and Coles, 2008).

Another disorder related to myelin integrity is Charcot-Marie-Tooth disease (CMT). First described in the late 19th century, this inherited incurable pathology of the PNS has two forms characterized by degeneration of the axon leading to loss of motor control. Only one of the two forms relates to destruction of the myelin sheath (Berger et al., 2002). CMT affects 1 in every 2500 people world wide, but has a prevalence of 150,000 in the United States alone; it is one of the most common peripheral neuropathies (Berger et al., 2002). Patients of CMT often have lower limb weakness, followed by loss of function, but no deleterious effect on life span is observed (Berger et al., 2002). These are just a couple of the many neuropathies that result in the disruption or destruction of

the myelination process and thus investigation in to the development of myelin may provide a therapeutic approaches to alleviate suffering in patients with these diseases.

1.2 The Cyto-architecture of myelin

The myelin sheath has a distinct cytoarchitecture made of several morphological domains including the abaxonal/outer and the adaxonal/inner membranes, the node, paranodes and the juxtaparanodes, and the non-compact and compact myelin. (Figure 2A). Compact myelin (CM) is formed as each continuous layer of the Schwann cell plasma membrane wraps around the surface of the axon. This results in a compression of the plasma membrane and exclusion of the cytosol from the region (Fannon et al., 1995; Hasegawa et al., 1996; Hirano et al., 2003; Jessen and Mirsky, 1997). Located on the lateral ends of the myelin sheath are the paranodes, which contain adhesion molecules and channels. The paranodes also help define the border of each myelinating Schwann cell as well as marking the Nodes of Ranvier, the un-myelinated regions of axons found between myelin segments. Additionally, within the non-compact myelin are the Schmidt-Lanterman Incisures, which are funnel-shaped cytoplasmic channels that cross the CM from the abaxonal to the adaxonal membranes. These channels are thought to be required for molecular trafficking between the pericellular and the periaxonal cytoplasm (Arroyo and Scherer, 2000; Fannon et al., 1995; Jessen and Mirsky, 2005a) (Figure 2A).

Another distinct subdomain are the nodes of Ranvier, which are more than un-myelinated regions between myelin segments. They are specified organizations of distinct subdomains that are centralized around the un-myelinated regions, and they are

vital for saltatory conduction in myelinated axon/fibers to occur (Figure 2B) (Salzer, 2003). Additionally, since this area, generally 1μmeter in length, is exposed to the extracellular environment, it is also thought to be the region where communication between the axon and the extracellular environment occurs (Salzer, 2003). Nodes of Ranvier are also enriched with voltage-gated sodium channels, which enables regeneration of the impulse down the length of an axon (Salzer, 2003). One predominate sodium channel member of the node is $Na_v1.6$ (Caldwell et al., 2000), which is thought to be important during high frequency firing that is required here (Zhou and Goldin, 2004). Various voltage-gated potassium channels are also present at nodal sites, including KCNQ2/3 (Pan et al., 2006). These two specific channels play a role in repetitive discharges, which are also necessary for the rapid transmission of action potentials (Pan et al., 2006).

In addition to voltage gated channels, cell adhesion molecules are also localized at the nodes. Two members of the L1 CAM family, NrCAM and neurofacin, specifically the isoform of 186kD (NF186), have been identified (Davis et al., 1996). These two molecules seem to allow for bridging interactions by allowing binding to Schwann cell components to the axon cytoskeleton (Davis et al., 1996), and more specially, gliomedin, a glial matrix protein, known to promote PNS node formation (Eshed et al., 2005). Additionally, these two adhesion molecules also interact with ankyrin G, an other axonal cytoskeletal proteins (Mohler and Bennett, 2005). Ankyrin G is a multivalent cytoskeletal protein, which provides the scaffold that targets and stabilizes various groups of proteins at specialized membrane domains in a great number of cell types (Mohler and

Bennett, 2005). Interestingly, ankyrin G also has an important role in the organization and subsequent stabilization of the nodal/axonal complex by interacting with voltage gated sodium and potassium channels (Dzhashiashvili et al., 2007; Pan et al., 2006).

The nodes of Ranvier are flanked by distinct specialized axo-glial junctions at the paranodes. It is at these junctions where promotion of adhesion between the axon and the glial cell are observed (Figure 2B). Furthermore, this sub-domain is thought to provide a limited barrier to the diffusion of ions between the node and internode during impulses (Rios et al., 2003; Rosenbluth et al., 2003). Similar to the nodal region, the paranodes also have cell adhesion molecules, particularly prominent are a complex of contactin and the contactin-associated proteins (Caspr) (Bhat et al.; Poliak et al.) (Charles et al., 2002). It is thought that this complex interacts directly with NF155, another neurofacin isoform of 155kDa, but the function of this binding has yet to be determined (Charles et al., 2002). Moreover, in 1995 Fannon et al. identified another cell adhesion molecule, E-cadherin, a type I cadherin of the classical cadherin, to be localized at the paranode and other distinct structures of the myelinating Schwann cell (Fannon et al., 1995).

Beyond the paranodes lies the sub-domain called the juxtaparanodes. This region is found under the compact myelin sheath. This area is also rich in cell adhesion molecules such as Caspr2 and TAG-1, a GPI-anchored adhesion molecule (Poliak et al., 1999; Poliak et al., 2003). Also enriched here are Shaker type potassium channels, more specifically Kv1.1 and 1.2, although their exact function has yet to be elucidated (Rasband et al., 2002).

The last domain is the internode, which in terms of size, is the largest of the group. Specifically the internode is the region beneath the compact myelin sheath. On average, the distance an internodal region spans is 1 mm in length, if not more in larger fibers of the adult PNS (Abe et al., 2004). Obviously to maintain SC contact with the axon at such lengths, cell adhesion molecules are clearly found; Neural-cadherin (Ncad) (Letourneau et al., 1991; Letourneau et al., 1990; Pla et al., 2001) as well as the nectin-like cell adhesion molecules (Necl) are present here (Maurel et al., 2007; Perlin and Talbot, 2007). Another molecule enriched within the internodal region is myelin-associated glycoprotein (MAG), which is an Ig-like adhesion molecule (Trapp, 1990). MAG is known to have interactions with numerous axonal components as well (Hannila et al., 2007).

1.3 Schwann cell development

While myelination in the CNS and PNS serve the same function, the cells and the mechanism by which it occurs differ. In the CNS, oligodendrocytes are the glial cells that myelinate the axon (Lu et al., 2002), while in the PNS Schwann cells melinat the axon.

Oligodendrocytes and the onset of myelination are spatially and temporally regulated (Watkins et al., 2008). Oligodendrocytes have only a brief period of time for myelination early during differentiation, and are relatively incapable of myelinating once they are mature (Watkins et al., 2008). Furthermore, the ability to myelinate numerous axons by a single oligodendrocyte is a highly coordinated event: oligodendrocytes do not

ensheath different axons sequentially at different time points, but are done within a brief window of time (Watkins et al., 2008).

In the PNS Schwann cells are derived from neural crest cells. In the developing embryo, neural crest cells emerge from the tip of the neural fold at the dorsal end of an embryo (Garratt et al., 2000a) (Figure 1A). Neural crest cells begin traveling along lateral and ventral migratory paths and further differentiate into various cells types (Garratt et al., 2000a). Schwann cells derived from neural crest cells migrate ventrally toward the notocord and associate with developing motor and sensory neurons (Jessen et al., 1994; Jessen and Mirsky, 1991, 1992, 1999a, 2003, 2005a; Mirsky and Jessen, 1986, 1999; Mirsky et al., 2001; Mirsky et al., 2008) (Figure 1A).

Prior to reaching their final fate of either myelinating or non-myelinating (ensheathing) Schwann cells, two irreversible transitions occur. First, neural crest cells have to transform into Schwann cell precursors followed by becoming immature Schwann cells (ISC). *In vitro* experiments have demonstrated that a number of survival factors, mitogens and differentiation signals initiate this transition from neural crest cells to Schwann cell precursors (Mirsky et al., 2008; Nave and Trapp, 2008; Salzer et al., 1980). In developing rat embryos, neural crest cells develop into Schwann cell precursors between embryonic days 14 and 15(Jessen and Mirsky, 2005a) (Figure 1B). Schwann cell precursors develop in close association with the axons which provide molecular signals to promote Schwann cell precursors to develop into immature Schwann cells (Mirsky et al., 2008; Nave and Trapp, 2008; Salzer et al., 1980). Immature

Schwann cells surround axons and further defasciculate them and will differentiate into either myelinating or non-myelinating cells(Figure 1B).

The myelinating versus non-myelinating phenotypes are determined by the diameter of the axon to which the ISC align. Schwann cells that associate with large diameter axons (>1 micrometer) initially differentiate into pro-myelinating Schwann cells and establish a 1:1 relationship with the associating axon and later myelinate (Sherman and Brophy, 2005). On the other hand, axons smaller than 1 micrometer in diameter induce non-myelinating (ensheathing) phenotypes (Voyvodic, 1989); here, numerous small diameter axons (< 1 micrometer) are sorted together by a single Schwann cell, followed by wrapping its plasma membrane around these thinner axons to form a distinct structure called Remak bundles.

1.4 Molecular mechanism that regulate myelination

Myelination is signaled and regulated by the axon. Originally, it was thought that the size of the axon dictated whether the Schwann cell would achieve either the ensheathing, non-myelinating phenotype responsible for cable conduction, or the myelinating phenotype, responsible for rapid saltatory conduction (Peters et al., 1991). Interestingly, it was identified that surface proteins on the axon, neuregulins, have a role in determining whether a Schwann cell will ensheath or myelinate (Garratt et al., 2000a; Garratt et al., 2000b).

Neuregulins bind to their receptors on the Schwann cell membrane. Neuregulin-1 isoforms promote proliferation and survival of Schwann cell precursors in cultures (Dong et al., 1995). Previous *in vitro* and *in vivo* studies also demonstrated that neuregulin-1

isoforms were shown to promote a similar proliferative and survival effect in Schwann cells aligned with axons (Grinspan et al., 1996; Morrissey et al., 1995). More specifically, neuregulin-1 type III was previously identified as the key signal that not only initiates myelination, but has been shown to regulate the thickness of the myelin sheath formed by the Schwann cell associated with the axon (Michailov et al., 2004; Taveggia et al., 2005). While there are four different neuregulin genes identified, neuregulin-1 has been characterized best (Garratt et al., 2000a). Neuregulin-1 has three isoforms, types I through III. Originally, all neuregulin-1 were thought to be membrane bound molecules, but later it was identified to be a soluble molecule (type II) (Adlkofer and Lai, 2000). Other isoforms were shown also to have a membrane bound version (Type I and III) (Adlkofer and Lai, 2000; Falls, 2003). All neuregulin-1 isoforms have a common EGF-like domain in the amino terminus of the protein, which is sufficient to induce receptor binding and activation (Buonanno and Fischbach, 2001). In type I an Ig-like domain and a site that may also be a potential glycosylation site is present. Type II is characterized by the presence of a hydrophobic region, and type III also has a similar hydrophobic region in combination with a cysteine rich domain. Interestingly, type III was identified to be the key neuregulin-1 isoform involved in Schwann cell development and myelination (Adlkofer and Lai, 2000; Falls, 2003; Michailov et al., 2004; Taveggia et al., 2005).

The receptor for neuregulin-1 was identified to be the ErbB receptors. ErbB receptors belong to the epidermal growth factor family of receptor tyrosine kinases. In the Schwann cell, ErbB3 and ErbB2 are widely present on the plasma membrane.

Neuregulin-1 binding induces receptor dimerization which leads to activation of the tyrosine kinase and subsequent tyrosine phosphorylation of the cytoplasmic domain (Schlessinger, 2002). Following activation and phosphorylation, there is also activation of downstream intracellular signal transduction pathways, notably the PI3-kinase/Akt pathway. Inhibition studies identified that PI3-kinase/Akt was shown to be involved in Schwann cell proliferation in response to a neuregulin-1 isoform binding. Though later on, it was speculated that this effect might have been induced by a survival effect of the mitogen itself (Maurel and Salzer, 2000). This same study demonstrated that PI3-kinase is crucial to the generation and initial differentiation of Schwann cells (Maurel and Salzer, 2000).

Studies using transgenic mice provided the first documentation for a function of neuregulin-1 signaling in myelination. By use of Cre-Flox system, knockdown of ErbB2 was induced upon Krox20 expression, a transcription factor vital to early Schwann cell differentiation; this ablation allowed for normal development of the Schwann cell, followed by myelination. However, g-ratio analysis identified that the myelin formed was thinner compared to controls (Garratt et al., 2000b). This result was similar to another study where neuregulin-1 expression was manipulated and resulted in thinner myelin sheaths (Michailov et al., 2004). Taken together, neuregulin-1/ErbB signaling is vital to Schwann cell development, proliferation and subsequent myelination.

1.5 Stages of myelination

The process of myelination begins early in the development of the Schwann cell. First, immature Schwann cells begin by forming a basal lamina, followed by the Schwann cells encircling axons. They then begin to sort axons based on their diameter and adopt a 1:1 ratio, a process termed radial sorting (Figure 1C&D). Formation of the lamina is a critical step which was identified when deletion of various lamina isoforms impeded radial sorting (Chen and Strickland, 2003; Yang et al., 2005). In addition to helping sort axons, basal lamina formation also functions to initiate cellular asymmetry, as well as establish an axis of polarity which allows the Schwann cell to orient itself to the extracellular environment versus the axonal membrane. Following sorting of the axons by the Schwann cells, myelination begins by assembly of the membrane. After polarized Schwann cell processes have attached to the axon, specific sorting machinery allows for precise delivery of myelin-membrane proteins to the axo-glial binding sites (Fitzner et al., 2006; Taylor et al., 2002).

1.6 Cell-Cell Adhesion Molecules: Cadherins

Epithelial cadherin (E-cadherin) was first characterized by Takeichi in 1977. Originally named uvomorulin, antibodies generated against this cell surface molecule induced a conversion of an organized morula into a disorganized group of cells tethered together similar to a shape of grape bundles, suggesting a role in cell adhesion (Takeichi, 1995; Takeichi et al., 1986). In 1985, François Jacob's group later found a glycoprotein

of 84 kDa (GP84) that seemed to perturb cell-cell interaction and prevent the compaction of pre-implantation embryos (Peyrieras et al., 1985). Later, GP84 was identified as the ectodomain of a stable 120 kDa protein (Takeichi, 1995; Takeichi et al., 1986). In 1984, electron microscopy studies localized this 120kDa protein to adherens junctions or intermediate junctions of interstitial epithelial cells. This observation led Tekeichi and Yoshida-Noro to rename GP84/120kDa protein Epithelial cadherin, commonly identified now as E-cadherin. E-cadherin characterization was followed by the identification of Neural, or N-cadherin, and Placental, or P-Cadherin, which have localizations respective to their names, but share common functional and structural similarities with E-cadherin (Takeichi, 1995).

Being the prototypical cadherin, E-cadherin has three main domains. Beginning with the extracellular domain (EC) found in the N-terminus of the protein, there are 5 extracellular repeat motifs (EC1-EC5), which are recognized by histidine, alanine and valine tripeptides and are necessary for cadherin-based cell-cell interactions (van Roy and Berx, 2008a). These repeats bind calcium and produce the rigid rod-like structure of the EC. Of the five conserved repeats, the fifth, called EC5, is distinct in that it has four cysteines. Deletion of this site affects the formation of strong cell-cell contacts (McCrea and Gumbiner, 1991). Adjacent to the EC is the transmembrane domain, which anchors E-cadherin to the plasma membrane (van Roy and Berx, 2008a). The C-terminus of cadherins contains the cytoplasmic domain (CD), which is partitioned into two sub-domains: the membrane proximal cytoplasmic/conserved domain (MPCD), also known as the juxtamembrane domain (JMD), and the β-catenin-binding domain (Rimm and

Morrow, 1994; van Roy and Berx, 2008b). The cytoplasmic domain plays a vital role in intracellular signaling via its binding partners of various armadillo repeat catenin family members, primarily p120- and β-catenin. Upon binding to the JMD, p120-catenin has been shown to regulate lateral clustering and stabilization of cadherins at the membrane (Kwiatkowski et al., 2007). Meanwhile β-catenin binds to a distal region in the C-terminus of the protein, which then recruits α-catenin, an unrelated catenin that can also bind directly to F-actin and a number of actin binding proteins (Kwiatkowski et al., 2007). This demonstrates that cadherin-catenin protein complexes are involved in the cytoskeletal structure of epithelial cells by cytoskeletal anchoring and signaling (Yagi and Takeichi, 2000) Through these interactions, cadherin family members provide signals to shape and move cells (Geiger and Ayalon, 1992).

1.7 Cadherins in Schwann Cells

Developmentally, expression of E-cadherin begins to rise with age in mouse sciatic nerves, right after birth, coinciding with the initiation of myelination (Fannon et al., 1995; Menichella et al., 2001). E-cadherin expression is also limited to the Schwann cell and is not observed in neurons (Menichella et al., 2001). In 1995, Fannon established the term "autotypic adherens-type junction" to describe E-cadherin expression patterns observed in the adherens structures between the same contiguous Schwann cell membrane layers found in the myelinating phenotype (Fannon et al., 1995; Young et al., 2002). E-cadherin expression is also localized to SLI, adherens junctions, in the outer and inner loops, and paranodes (Arroyo and Scherer, 2000; Fannon et al., 1995). In myelinated fibers, proper

establishment and maintenance of the abaxonal membrane occurs through E-cadherin (Young et al., 2002). Recently, in Schwann cells expressing a mutated E-cadherin extracellular domain, it has been demonstrated that E-cadherin is required for the maintenance of Schwann cell architecture of the SLI (Tricaud et al., 2005). The function of E-cadherin is mediated through E-cadherin's ability to interact with p120catenin, a protein that binds to the juxtamembrane domain of E-cadherin. p120catenin stabilizes E-cadherin to the cell membrane and prevents entry into the degrading endocytic pathways (Davis et al., 2003; Peifer and Yap, 2003).

We have recently shown that expression of E-cadherin is dependent on direct axonal contact (Crawford et al., 2008). The induction of E-cadherin by axonal contact increases with prolonged exposure to the axon and is mediated by the cAMP pathway (Crawford et al., 2008). Further E-cadherin expression was elucidated in an *in vitro* PNS injury model.. E-cadherin expression was lost upon axonal degradation and resulted in the loss of axonal contacts; however, expression was later restored upon axonal regeneration and re-establishment with axonal surface molecules (Menichella et al., 2001).

N-cadherin appears to be another major cadherin member in Schwann cells as well as DRG neurons (Wanner et al., 2006; Wanner and Wood, 2002). Early in SC-Axon interactions N-cadherin was observed at the SC-Axon interface (Wanner et al., 2006; Wanner and Wood, 2002; Yap et al., 1997). *In vitro*, disruption of N-cadherin, by either blocking antibodies or calcium depletion, limits the ability of the Schwann cell to bind to axons and extend their processes (Wanner et al., 2006; Wanner and Wood, 2002). N-cadherin expression occurs most in initial stages of Schwann cell-Axon interaction; as

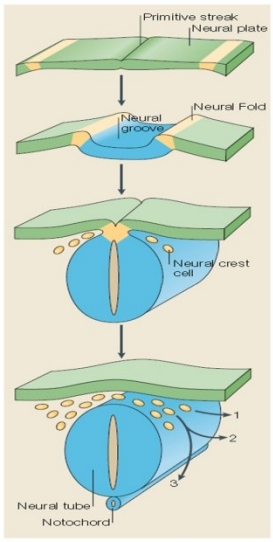
development progresses, N-cadherin levels decrease gradually and are absent in myelinating Schwann cells. (Jessen and Mirsky, 2005a; Mirsky et al., 2008; Wanner et al., 2006; Wanner and Wood, 2002).

The goal of this study is to define the role of E-cadherin in the initiation of myelination. Through its association with RTK in other cell types and its involvement in cell polarization, E-cadherin could have a pivotal role in myelination. While E-cadherin's mechanism of induction has been identified in the Schwann cell (Crawford et al., 2008), the role it may have in the early stages of myelination has yet to be identified.

Figure 1 Schwann Cell development and myelin initiation:
(A) Migration of Neural Crest Cells (NCC) from dorsally located neural groove to the ventrally located notocord (Jessen and Mirsky, 2005); **(B)** Maturation of NCC to Schwann cell precursor, immature Schwann cells and then one of two reversible phenotypes: myelinating Schwann cell and non-myelinating Schwann cells (Jessen and Mirsky, 2005); **(C)** Electron micrograph image of a Schwann cell (SC) beginning to align and ensheath axons and the formation of a basal lamina (BL) (Chan, JR,2007); **(D)** Schwann cell segregating axons (Ax) during radial sorting, then advancing to begin wrapping around the Ax during the initiation of myelination and concluding with the Schwann cell forming concentric circles of myelin (Chan JR, 2007).

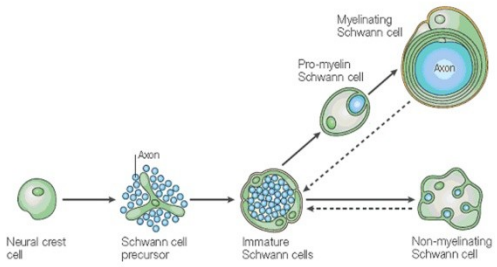
Figure 1 Schwann Cell development and myelin initiation:

1A



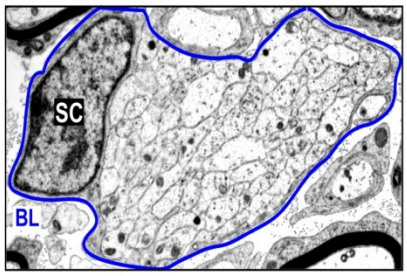
(Jessen & Mirsky, 2005)

1B



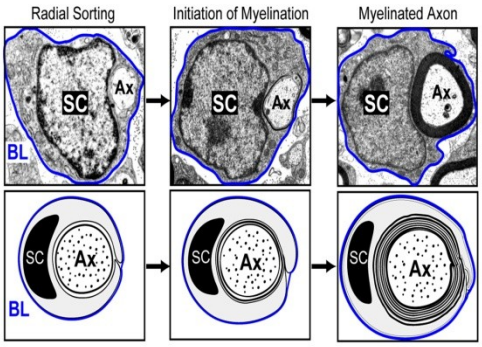
(Jessen & Mirsky, 2005)

1C



(Chan JR, 2007)

1D

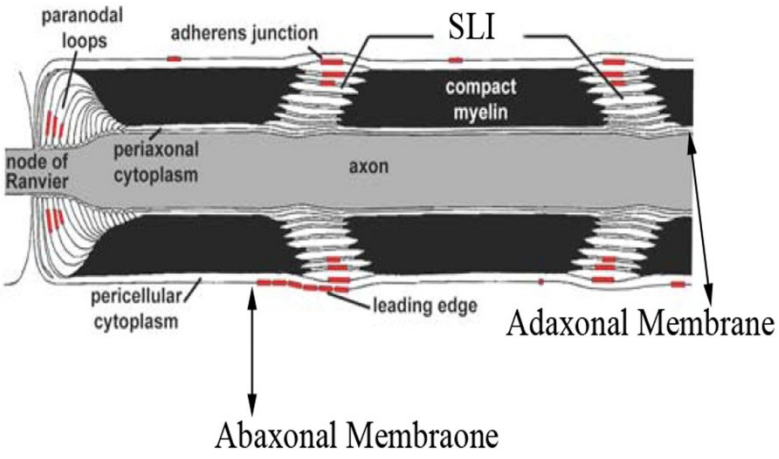


(Chan JR, 2007)

Figure 2. Cytoarchitecture of myelinated axon
(A) Taken from Tricaud et. al. (2005), is a schematic representation of an axon with a myelinating Schwann Cell (SC); this image identifies architectural regions of importance, such as regions of Compact Myelin (CM), Schmidt-Lanterman Incisures (SLI), paranodes, as well as the abaxonal and adaxonal membranes of a myelinating Schwann cell. **(B)** Adapted from Salzer et al. (2008) is a schematic diagram of a myelinating SC, but the nodal region found between adjacent Schwann cells is more emphasized. Regions of interest here are the node of Raniver (purple), the paranodal junctions (green), the juxtaparanodes (orange) and the internodes (red)

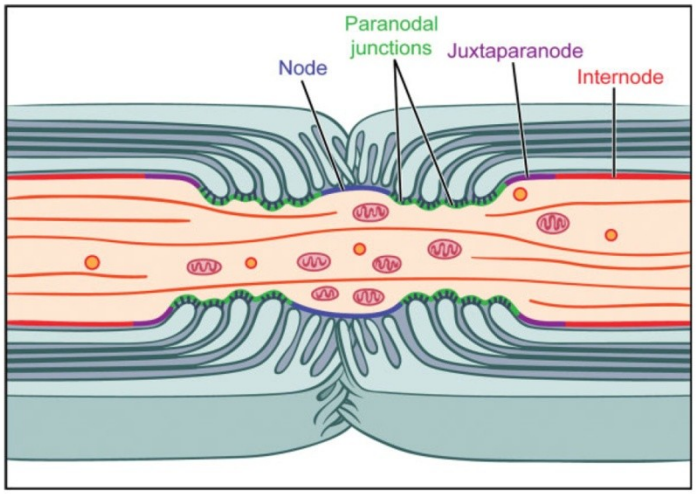
Figure 2. Cytoarchitecture of myelinated axon

2A



(Tricaud et al, 2005)

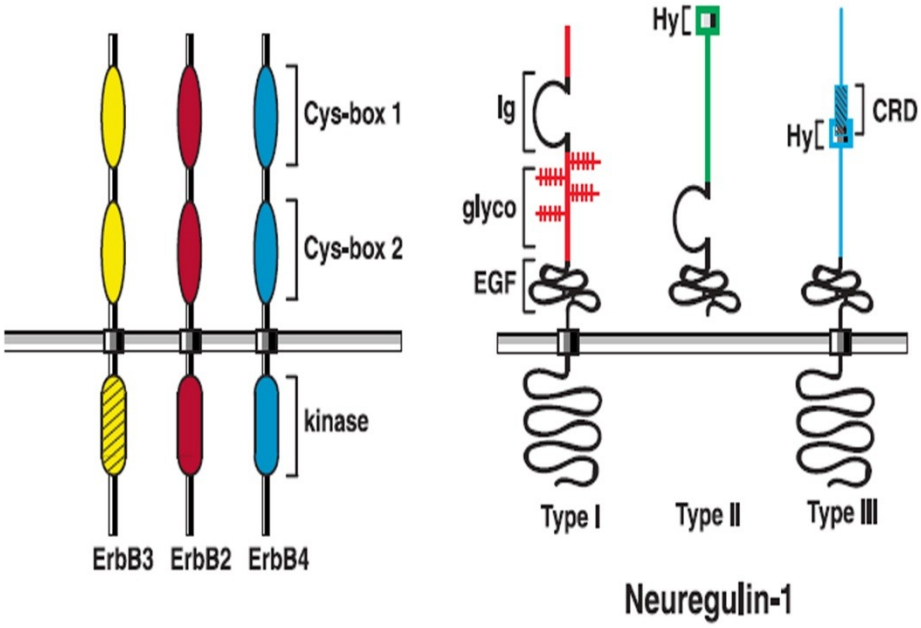
2B



(Salzer et. al., 2008)

Figure 3. Neuregulin and the ErbB family schematic
Taken from Garratt et al. (2000), is a cartoon representation of the three isoforms of Neuregulin I (NRGI), all of which have the EGF-like domain near the extracellular membrane region. This portion of the molecule is sufficient to bind and activate ErbB3/ErbB4 to induce downstream signaling.

Figure 3. Neuregulin 1 isoforms



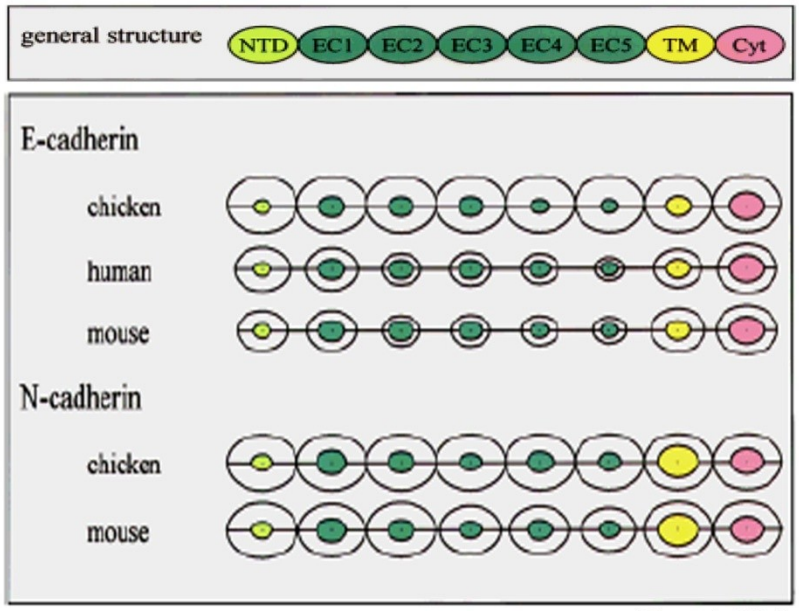
(Garratt et. at., 2000)

Figure 4. Cadherins

(A) Taken from Pla et al. (2001), is a schematic of the domains of E- and N-cadherins. General structure of classical cadherins and protein identities Cadherins are transmembrane proteins that contain five extracellular domains (EC1- EC5) after cleavage of the N-terminal domain (NTD). The protein spans the membrane once through a transmembrane domain (TM). The cytoplasmic domain (Cyt) interacts with catenins (Pla et al., 2001); **(B)** Taken from van Roy and Berx (2008), a schematic identifies various interactions of E-cadherin such as, β-catenin and p120-catenin.

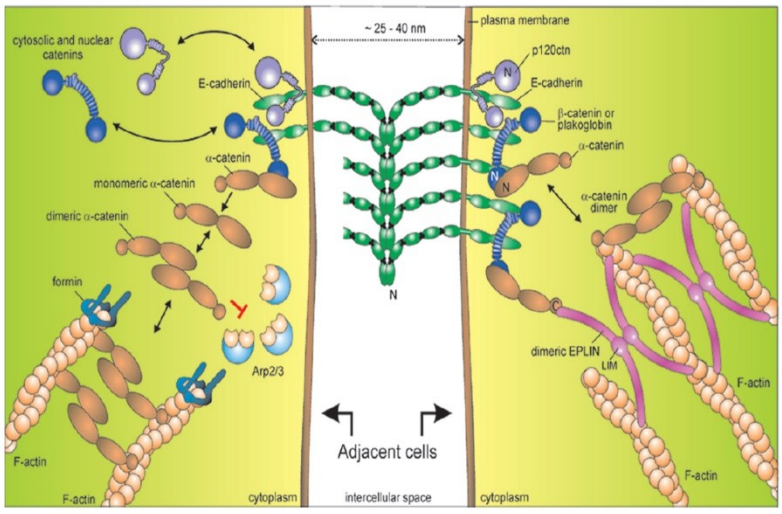
Figure 4. Cadherins

4A



(Pla et. al., 2001)

4B



(van Roy & Berx, 2008)

Research Aims

E-cadherins have been characterized to have a structural and adhesive role in the myelinating Schwann cells of the PNS; they are involved in formation of autotypic junctions among other structural domains within the myelin sheath of the Schwann cell, though recent literature may provide a new regulatory role for cadherins expression in this model.

E-cadherin has been shown to have a signaling function that modulates the activation of receptor tyrosine kinases, including that of ErbB2 (Qian et al., 2004). In Schwann cells, E-cadherin is expressed at the onset of myelination. The signaling function of E-cadherin is mediated through its binding of beta-catenin. Therefore, it is possible that E-cadherin might play a role during the early stage of myelination by modulating the NRG1/ErbB2 signaling that is essential for the initiation of myelination in the PNS.

The goal of this thesis is to elucidate the function of E-cadherin and its relationship with ErbB2 and the subsequent role in the initiation of myelination in the PNS. For the study, I propose four specific aims: 1) compare the temporal expression kinetics of E-cadherin and ErbB2 during Schwann cell myelination and determine whether they form a signaling complex; 2) determine whether E-cadherin expression is regulated during Schwann cell myelination; 3) determine the effect of ectopic E-cadherin expression in Schwann cells; and 4) determine the function of E-cadherin/beta-catenin coupling during Schwann cell myelination.

In the first aim, I determined the expression kinetics of E-cadherin and N-cadherin, and compared them to ErbB2 and its active form (p-ErbB2) as well as the association kinetics of ErbB2 during myelination. Using co-immunoprecipitation and Western blot analysis, the association between E-cadherin and ErbB2 was also determined with rat Schwann cell and purified dorsal root ganglion neuron cocultures.

Within the second aim, a loss of function study, I utilized a lentivirus encoding an shRNA against E-cadherin to knockdown E-cadherin proteins in Schwann cells and determine the effect in Schwann cell myelination. The knockdown of E-cadherin resulted in a defect in myelination. For the gain of function study described in aim three, I generated Schwann cells ectopically expressing human E-cadherin and determined the effect on myelination. Results from this study suggest that E-cadherin elicits a pro-myelinating function in Schwann cells during myelination. More interesting, the effects of the enhancement are shown to be independent of an induction of proliferation.

To investigate the role of E-cadherin associated with beta-catenin, I generated Schwann cells expressing a mutant form of E-cadherin that lacks the binding domain for beta-catenin. Data from this portion of work suggests that E-cadherins association with beta-catenin may have a regulatory effect on Schwann cell myelination. In summary, these results provide a novel regulatory role for E-cadherin in the myelinating Schwann cell of the peripheral nervous system.

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Material and Methods

Antibodies and growth factors

For immunofluorescence staining, monoclonal antibody to E-cadherin (clone 36, BD Transduction Laboratories, Franklin Lakes, NJ) was used at a dilution of 1:200 and chicken polyclonal antibody neurofilament (Covance, Berkley, CA) was used at 1:2000. For Western blot analysis, E-cadherin antibody was used at 1:3000 and monoclonal antibody to N-cadherin (clone 32, BD Transduction Laboratories) was used at 1:2500. Monoclonal antibody to α-actin (AC-15, Sigma, St. Louis, MO) was used at 1:5000. Recombinant glial growth factor (R&D systems, Minneapolis, MN) was used at 10 ng/ml. Polyclonal antibodies against ErbB2 and phospho-ErbB2 (C-18 and p-ErbB2, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1:1000.

Culture Media

Primary rat Schwann cells were grown in Schwann cell growth medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine, and 0.1 mg/ml penicillin-streptomycin, neuregulin-1 EGF-domain (10 ng/ml) and Forskolin (5 μM, Sigma, St. Louis MN). A serum-free defined N2 medium was composed of DMEM and F- 12 at a 1:1 ratio, supplemented with 5 ng/ml Na selenite, 16 μg/ml putrescine, 125 ng/ml progesterone, 0.2 mg/ml apo-transferrin, 0.8 μg/ml insulin, and 1% penicillin-streptomycin. Dorsal root ganglion neurons were maintained in Neurobasal

medium (Invitrogen, Carlsbad, CA), supplemented with 2% B27 supplement (Invitrogen), 1% glutamine, 1% penicillin-streptomycin, 0.4% glucose and 25 ng/ml NGF (Millipore, Billerica, MA). For myelination, co-cultures were maintained in C media (MEM, 10% FBS, 2 mM L- glutamine, 0.4% glucose, and 50 ng/ml 2.5S NGF) supplemented with ascorbic acid (Vitc) at 50µg/mL.

Preparation of Schwann Cells

Schwann cells were prepared as described previously (Raff et al., 1978). Briefly, sciatic nerves were harvested from postnatal day 1 or 2 rats and enzymatically dissociated by incubation with 1% collagenase prepared in DMEM for thirty minutes at 37 °C followed by incubation in 0.25% Trypsin and 1% Collagenase solution for 30 minutes at 37 °C. Cells were spun down and pellets were resuspended in 10% FBS in DMEM, and then plated on two 60 mm culture plates in the same medium. The following day the media was changed to 10% FBS medium containing 10 µM cytosine arabinoside to kill off proliferating fibroblasts. Three days later, in order to remove residual fibroblasts, cells are gently trypsinized, pelleted and incubated for 35 minutes with anti-Thy1 antibody (AbD Serotec, Raleigh, NC) prepared in DMEM at a dilution of 1:1000. This was followed by incubation in rabbit complement (Jackson Immuno, West Grove, PA) for the same length of time. Schwann cells were then plated on poly-L-Lysine-coated 100 mm culture plates and maintained in Schwann cell growth medium.

Preparation of Purified rat DRG neuron cultures

Dorsal root ganglia were removed from rat embryos between E14.5-E16.5 and dissociated in 0.25% Trypsin for 30 minutes at 37°C. The dissociated DRG were plated on 12 mm collagen coated glass coverslips in DMEM and 10% FBS supplemented with 25 ng/ml of NGF in a 120 µl droplet. To remove proliferating non-neuronal cells, the following day cultures were flooded in Neurobasal medium with the supplements plus 15 µM Fluorodeoxyuridine (FUdR). Two days later FUdR containing media was removed and cultures were maintained in neurobasal medium with the supplements for two days before starting a new cycle of FUdR treatment. Cycling in FUdR was continued for 10 days until all non-neuronal cells were removed.

Preparation of Purified rat SCG neuron cultures

Superior cervical ganglia (SCG) were harvested from rat postnatal day 1 or 2 pups and dissociated in 0.25% Trypsin for 45 minutes at 37°C. The dissociated SCG were plated on 12 mm collagen coated glass coverslips in DMEM and 10% FBS supplemented with 25 ng/ml of NGF in a 130 µl droplet. To remove proliferating non-neuronal cells, the following day, cultures were flooded in Neurobasal medium with the supplements plus 15 µM Fluorodeoxyuridine (FUdR). Two days later FUdR containing media was removed and cultures were maintained in neurobasal medium with the supplements for two days before starting a new cycle of FUdR treatment. Cycling in FUdR was continued for 10 days until all non-neuronal cells were removed.

SDS PAGE and Western blotting

Cells were lysed in lysis buffer (20 mM Tris pH 7.4, 1% NP-40, 10% glycerol, 2.5 mM EGTA, 2.5 mM EDTA, 1 mM sodium ortho-vanadate, 1 mM sodium fluoride, 1 mM PMSF, 10 μg/ml aprotinin and 20 μM leupeptin) and the cell lysates were centrifuged at 14,000 rpm at 4°C for 15 minutes and supernatants were collected. The protein concentrations were measured using BCA Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer's instructions. Samples (20-100 μg) were fractionated by SDS PAGE and transferred onto PVDF membrane. For Western blot analysis, the membranes were incubated in blocking solution (5% non-fat dried milk in TBS) for one hour followed by incubation with appropriate primary antibodies prepared in TBST (TBS + 0.5% tween20 + 5% BSA) overnight at 4°C. The next day, the membranes were washed 3 times for 10 minutes each in TBST before incubation in secondary antibody prepared in blocking solution with 0.5% Tween20 for 1 hour. The blots were then incubated with ECL Western Blotting substrate for 1 minute and exposed on X-ray films.

Immunoprecipitation

Rat sciatic nerves were harvested at various stages of development, postnatal day 1 to 28. Nerves were snap frozen with liquid nitrogen and stored at -80 C°. Samples were thawed, then using lysis buffer (see above), mechanically homogenized via rotary tool. Cellular debris was cleared by centrifugation and lysate quantified. Samples of 250 to 500 μg were utilized for immunoprecipitation and 20 to 40 μg

were used for Western blots. IP samples were then incubated with E-cadherin (3 µg) antibodies for 60 minutes at 4°C with constant rocking and then incubated with pre blocked Sepharose A beads (Sigma) for an additional 60 minutes at 4°C. Samples were then spun down to pellet the beads and washed 5 times with lysis buffer containing 1 mM sodium ortho-vanadate. The immunoprecipitates were size fractionated on 8% SDS polyacrylamide gels, transferred onto PVDF membranes and Western blot was carried out as above.

Immunofluorescence staining

Cocultures grown on 12mm coverslips were fixed in 4% paraformaldehyde prepared in PBS for 25 minutes at room temperature and permeabilized in methanol for 10 minutes at -20°C. After rehydrating in 1x PBS, the cultures were incubated in blocking solution (10% normal goat serum + 0.3% Triton X-100) for 1 hour at room temperature followed by incubation with primary antibodies overnight at 4°C. The following day, cultures were washed with 1x PBS and incubated with the appropriate secondary antibodies for 45 minutes at room temperature. This was followed by incubation with 4',6' –diamidino-2- phenylindole (DAPI) for 1 minute to visualize nuclei. Coverslips were mounted onto glass slides using Fluoromount G

Lenti Virus production and infection

In collaboration with Dr. Patrice Maurel, constructs for shRNA were designed against E-cadherin (E-cad-shRNA), against Luciferase (Luc-shRNA) as infection controls, as well

as Lentivirus Control construct expressing only GFP (LV-CTL). The 5′-phosphorylated PAGE-purified oligonucleotides were annealed and subcloned into HpaI – XhoI sites of pLL3.7. The lentiviral vector was transfected into 293FT cells together with packaging plasmids Δ8.9 and pCMV-VSVG (a generous gift care of Jeff Milbrant) using Lipofectamine 2000 (Invitrogen). As control, we used the empty pLL3.7 vector or a vector encoding shRNA to Luciferase sequence. Briefly, 293 FT cells (Invitrogen) were grown to 80% confluency in 35mm plates. Cells were then transfected with 4 µg of DNA using Lipofectomine 2000/DMEM (Invitrogen) (Dull et al., 1998) in 293 FT media [(DMEM (Cellgro), .1mM MEM Non-Essential Amino Acids Solution (Invitrogen), 1mM Sodium Pyruvate Solution, 10% Fetal Bovine Serum (Mediatech), 1%Penicillin-Streptomycin, 6 mM L-glutamine)]. Twelve hours post transfection, media was changed to fresh 293 FT and incubated in 7.5% CO_2 at 37 C° for another 48-60 hrs. Cells and the 293 FT medium was collected into 15 ml conical tubes and centrifuged at 5,000g for 15 mins at room temperature to allow for packing of cellular debris. Supernatants were collected without disruption of the pellet and subsequently filter sterilized via a 0.45 micron PVDF syringe filter into a fresh 15 ml conical tube. Virus supernatant supplemented with NRG1 (10ng/mL) was utilized immediately to infect Schwann cell mono-cultures, Schwann cell mono-cultures were at roughly 75% to 80% in density. Virus media/NRG1 was removed 48hrs later and infected cells were visualized for the GFP expression.

Retro Virus production

293 Phoenix cells were plated in 60 mm plates in D-10 [DMEM (Cellgro), 10% Fetal Bovine Serum (Mediatech), 1% Penicillin-Streptomycin and 6 mM L-glutamine] till approximately 60-70% confluency. 4μg of DNA with Lipofectamine 2000/DMEM (Invitrogen) was used to transfect 293 Phoenix cells and were incubated in 7.5% CO_2 at 37 °C. Twelve hours later, transfection medium was replaced with 2mLs of D-10 for virus collection and returned to incubator. Forty to sixty hours after, virus supernatant and cells were collected in a 15 ml conical tube and centrifuged at 5000g for 15 minutes at room temperature. Supernatant was collected without disruption of the pellet and subsequently filter sterilized via a 0.45 micron PVDF syringe filter into a fresh 15 ml conical tube. Infection media was prepared in poly-L-Lysine coated 6-well plates with early passage Schwann cell starting with densities of 80,000 to 100,000 SCs per well. Virus was diluted with 25%, 15%, 10%, and 5% viral component in D-10 with NRGI (EGF domain) (10ng/mL) and Forskolin (5μM final concentration) and Polybrene (1000x) for a final volume of 1ml per well. After 24hrs post infection, infected cells were selected for by the addition of G418 to D-10 media containing growth factors (G418, 500μgram/mL). Growth factor/antibiotic containing media was changed every 48-72 hrs until control, non-infected Schwann cells wells had died, which was approximately 10-14 days post infection. Cells were then re-plated to poly-L-lysine coated 60 mm plates in D-10 plus growth factor and G418 till confluent, then re-plated again in similar media in 100mm plates, maintained in similar media conditions till used.

Myelination quantification

Myelinating cocultures were fixed and immunostained with a primary antibody against Myelin Basic Protein (MBP). After mounting onto slides, 20 random fields per cover-slip were selected and MBP positive segments were counted and averaged per slide.

Proliferation Assay

Schwann cells were plated onto poly-L-lysine coated glass cover-slips in D-10 media at a density of 50,000 cells per cover-slip. Cells were maintained in D-10 without growth factors for 48hours to synchronize cells, then treated with GGF (10 ng/mL)/D-10. Sixteen to eighteen hours post GGF treatment BrdU was added and pulsed for eighteen to twenty-four hours. Cells were fixed in cold methanol for 10 minutes, rehydrated in PBS for 5 minutes, and treated with 2N HCl pre-warmed to 37°C and incubated for 15 minutes at 37°C. Cells were washed three times in 0.1 M borate buffer (pH 8.5, room temperature) over a 10 minute period and then washed three times in PBS. Samples were incubated in blocking solution (5% normal goat serum supplemented with 0.2% Triton X-100, 1x PBS) for 30 minutes and incubated with monoclonal BrdU antibody prepared in blocking solution for 1 hour at room temperature. Alexa 488 goat anti-mouse secondary antibody was added for 1 hour, and before mounting, cells were incubated with 4_,6_-diamidino-2-phenylindole (DAPI) for 1 minute to visualize the nuclei. Using an epifluorescent microscope with a 20x objective, 20 random fields were selected. Within each field, BrdU positive cells (Alexa 488) and DAPI positive nuclei were

counted. The ratio of BrdU positive and DAPI positive cells of the 20 fields was used to calculate the percentage of BrdU positive cells per cover-slip per field.

Results

Aim#1

Axonal contact induces E-cadherin expression in Schwann cells

Schwann cells express E-cadherin (Fannon et al., 1995; Young et al., 2002), but the mechanism by which this protein is induced has not been elucidated. Previously, it has been identified that E-cadherin is expressed at the onset of myelination (Menichella et al., 2001), but exactly when and if axonal contact itself is sufficient to induce E-cadherin expression is not clear, as well as whether the E-cadherin expression is axonal or Schwann cell in origin; therefore, to determine when and which cell expresses E-cadherin, an *in vitro* Schwann cell- purified DRG coculture system was utilized. Schwann cells were prepared from neonatal rat sciatic nerve and maintained in serum-free medium for 3 days, then seeded onto purified rat DRG neurons. Lysates were collected from Schwann cell only monocultures as well as DRG only monocultures. Lysates were also collected from cocultures at day 1, 3, 7 and 9 days. As seen in figure 5A, both Schwann cell only and DRG only lysates are devoid of E-cadherin expression, but as early 1 day after seeding the Schwann cells onto the DRG neurons, there is an observance of E-cadherin expression, followed by a gradual rise in expression levels beginning at day 3 to 9. To further confirm this expression, in figure 5B, immunocytochemistry was preformed on cocultures confirming that E-cadherin (red) membrane expression is confined to Schwann cells seeded on the axons (green neurofilament). Taken together, Schwann cells E-cadherin expression is due to axonal

contact, and expression is limited to the Schwann cells and not the axon, but exactly how the induction is triggered needs to be elucidated.

Axonal contact mediated cAMP-PKA induces E-cadherin expression in Schwann cells

As the previous result showed, axonal contact mediated E-cadherin expression in the Schwann cell, but exactly how that induction was being brought about was not clear. Previous research groups stated that axonal contact induces expression changes in the Schwann cell by the cAMP-PKA pathway. I wanted to identify if the cAMP-PKA is involved in mediating the induction of E-cadherin in rat Schwann cells. Utilizing the *in vitro* coculture model, I again collected lysates from Schwann cell and DRG only monocultures (figure 6A), and upon examination by Western blot with antibodies against rat E-cadherin protein, no E-cadherin protein was detected. To identify if the cAMP-PKA pathway mediated the induction of E-cadherin in Schwann cells, I used an inhibitor to adenyl cyclase, H-89, as well as a vehicle control of DMSO. As seen in figure 6A, Schwann cells DRG cocultures whose lysates were collected at 3 and 6 days, showed clear E-cadherin expression in the DMSO control lanes, but in cocultures treated with the inhibitor there was a clear absence of E-cadherin expression. To provide further support to this data, an additional experiment utilizing a peptide inhibitor to cAMP-PKA, PKI, was similarly performed on another set of cocultures. As seen in figure 6B, treatment with the peptide inhibitor to cAMP induced a similar decrease in Schwann cell E-cadherin expression. These preliminary findings identified that E-cadherin expression in Schwann cells is induced by axonal contact, and subsequently, that induction is mediated

by a cAMP-PKA pathway, but could E-cadherin play a role in myelination in Schwann cells?

Expression kinetics of cadherins and ErbB2 during Schwann cell myelination *in vivo*

In the rat sciatic nerve, myelination is initiated as early as post-natal day 1, but expression kinetics of key adhesion and signaling molecules have not been completely elucidated from this early developmental time point to adulthood; therefore, I wanted to identify the developmental expression kinetics during myelination of E-cadherin, N-cadherin, and both ErbB2 and its activated form, p-ErbB2. To investigate this, lysates were prepared from rat sciatic nerves harvested from various stages of development, ranging from post-natal day 1 to 28 (P1 to P28) and were subjected to Western blot analysis using specific antibodies.

As seen in figure 7, E-cadherin (120 kDa) expression appears to be at the lowest levels early in myelination at P1, but gradually increase afterwards to P28. By contrast, expression of N-cadherin (130 kDa), displays a reciprocal pattern of expression compared to E-cadherin; N-cadherin was clearly present at P1, followed by a significant decrease in protein levels by P7, and was nearly absent at P14 and onward.

Since NRG1/ErbB2 signaling functions early in myelination, the developmental expression kinetics of ErbB2 and its active form, p-ErbB2, were also examined. Western blots showed robust ErbB2 (185 kDa) expression at P1, followed by a clear reduction in expression levels with development, with expression nearly absent at P28. The active form of ErbB2, p-ErbB2 (185 kDa) was initially low at P1 then peaked at P7, followed by

a decrease to P28. Taken together, the result suggest that a functional correlation may exist between members of the cadherin proteins and two forms of ErbB2 due to the converse expression observed between E-cadherin with N-cadherin and ErbB2.

E-cadherin, not N-cadherin associates with ErbB2 *in vivo*

In epithelial cells, E-cadherin has been shown to associate with the EGF-receptor and modulate its signaling function. To determine if a E-cadherin associates with ErbB2 *in vivo* during myelination, I immunoprecipitated ErbB2 from rat sciatic nerve lysates prepared from the developmental stages as described above and determined the association of E-cadherin with ErbB2 by immunoblotting with anti-E-cadherin. The results in figure 8A showed that E-cadherin continually associated with ErbB2 through-out the development, from P1 to P28, although level of association did decrease at P28.

To determine if ErbB2, in its active form, associated with E-cadherin, an immunoprecipitation with p-Erb2 antibody, followed by Western blot with E-cadherin antibodies was performed. As seen in figure 8B, the association between E-cadherin and active ErbB2 was seen mostly between P3 and P14 and was not detected in later stages of myelination. These results suggest that E-cadherin associates with both active and inactive forms of ErbB2, but unlike the association seen with total ErbB2, E-cadherin association with active p-ErbB2 was seen predominately between P3 and P14.

As shown in figure 7, N-cadherin is highly expressed in the PNS from P1 to P7. To determine whether ErbB2 associates with N-cadherin during these stages of development, I performed a similar co-immunoprecipitation with ErbB2 antibodies

followed by immunoblotting with rat N-cadherin antibodies. In figure 8C, Western blots on total lysates of P3 and P5 showed the presence of N-cadherin protein. However, the co-immunoprecipitation experiment showed the absence of N-cadherin association with ErbB2. This suggests that it is E-cadherin that associates with ErbB2 and not N-cadherin.

Association of E-cadherin with ErbB2 is not due to lipid rafts

To address the possibility that the association between E-cadherin and ErbB2 was not due to their containment within a lipid raft, P7 lysates were prepared either with the standard lysis buffer or with a buffer containing β-octylglucoside, a detergent that disrupts lipid rafts. Both lysates were subjected to immunoprecipitation with ErbB2 followed by Western blot with E-cadherin. As seen in figure 8, E-cadherin was observed to be associated with ErbB2, regardless of β-octylglucoside being present or not in the lysis buffer, suggesting that the association between E-cadherin and ErbB2 is independent of lipid raft integrity.

***In vitro* E-cadherin and N-cadherin expression kinetics confers with *in vivo* expression kinetics**

To determine whether the developmental expression kinetics seen *in* vivo would be recapitulated *in vitro*, I used the well established *in vitro* model of Schwann cell myelination. Rat Schwann cell were seeded onto purified DRG neurons and allowed to align and proliferate along axons. After 10 days, the cocultures were treated with ascorbic acid (VitC) to initiate myelination. Cell lysates were collected at days 1to 5, 7, 14, 21 and 28 after the ascorbic acid treatment and the expression of E-cadherin and N-

cadherin was determined as shown. Control cultures were maintained under non-myelinating conditions (- ascorbic acid treatment). Western blot analysis was performed with E-cadherin and N-cadherin antibodies. As shown in figure 9A, under non-myelinating conditions, E-cadherin expression was minimally observed, however, in myelinating conditions, E-cadherin expression was clearly present as early as one day post ascorbic acid treatment, followed by a gradual increase to day 28. On the contrary, N-cadherin was present under both non-myelinating and myelinating conditions, with the level of expression being sustained up to day 14, followed by a gradual decrease to P28.

Upon identifying E-cadherin and N-cadherin expression in the *in vitro* coculture model, I determined whether there was an association between E-cadherin and ErbB2 which was observed *in vivo*. To determine the *in vitro* association between of ErbB2 and E-cadherin, coculture lysates were collected at different stages following initiation of myelination. The lysates were then immunoprecipited with anti-E-cadherin antibody followed by a Western blot for ErbB2. As seen in figure 9B, there was an association observed throughout the developmental stages, from P1 to P28. This is similar to the *in vivo* data shown in figure 5. Taken together, the results show that *in vivo* expression and association kinetics between ErbB2 and E-cadherin is recapitulated in the *in vitro* culture system.

E-cadherin localizes to Schwann cell membrane

Upon Schwann cell alignment to an axon, what is the localization of E-cadherin protein when adherence junctions between the Schwann cell and the axon are formed?

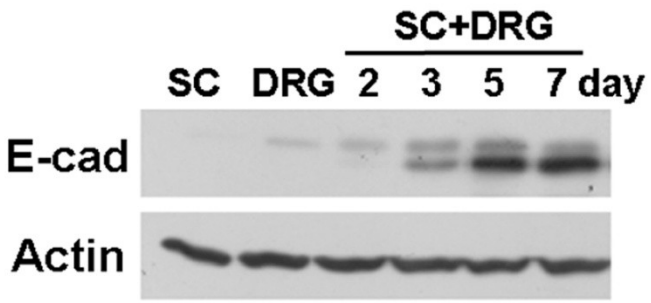
To identify the localization of E-cadherin as a Schwann cell is aligning with an axon, SC/DRG cocultures were established and prepared for immunocyotochemical analysis. Markers for E-cadherin, Glial Fibrillary Acidic Protein (GFAP), and Neurofilament (NF) were used to define membrane protein localization, Schwann cell cytosol and axonal membranes respectively. As shown in figure 10, E-cadherin specific staining (red) was localized to the membranes of a Schwann cell aligned to a DRG axon labeled with NF (blue). E-cadherin staining was also observed to be at the cell periphery, but absent from areas stained with GFAP (green). This result suggests that E-cadherin localizes primarily at the axo-glial contacts and may potentially function to regulate myelination.

Figure 5. Axon-contact induces E-cadherin expression in Schwann cells

(A) Growth factor deprived Schwann cells were plated onto DRG neurons (SC +DRG) and E-cadherin expression was determined at 1, 3, 7 and 9 days following seeding of Schwann cells by immunoblotting. Schwann cell only (SC) and neuron-only (DRG) cultures were used as controls. E-cadherin expression was detected as early as 1day post seeding and levels gradually increased with time. **(B)** Schwann cell specific E-cadherin expression in seen in cocultures (7 day) immunostained with antibodies to E-cadherin (red) and neurofilament (NF, green). Individual Schwann cells are visualized by the nuclear DAPI staining. E-cadherin is detected at the Schwann cell membranes of Schwann cells that are aligned to DRG neural axons.

Figure 5. Axon-contact induces E-cadherin expression in Schwann cells

5A



5B

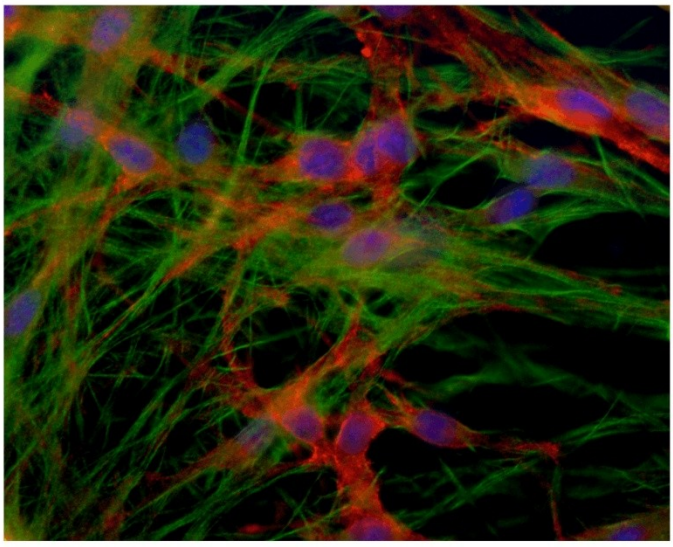
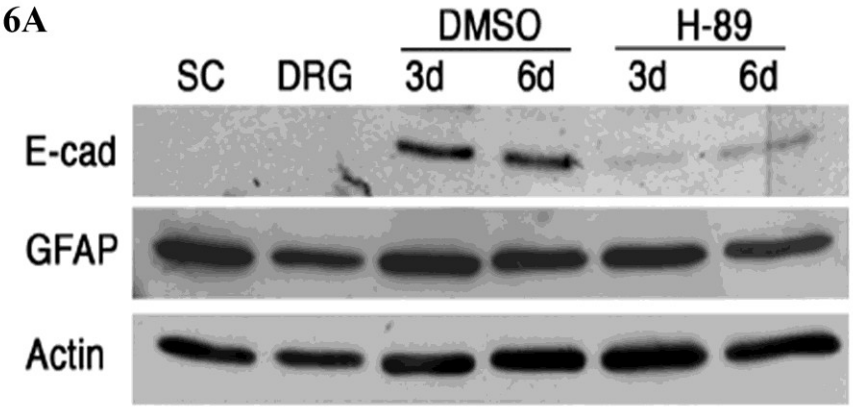


Figure 6. Axonal contact mediates cAMP-PKA induced E-cadherin expression in Schwann cells

(A) Schwann cells were seeded onto DRG neurons and the following day, cultures were treated with DMSO or H-89 (10 μM). Cell lysates were prepared at 3- and 6-days following seeding of the Schwann cells. Schwann cell only (SC) and neuron only (DRG) cultures were used as controls. Levels of E-cadherin (E-cad), GFAP and actin were determined by immunoblotting. Treatment with the adenyl cyclase inhibitor, H-89, resulted in no detection of E-cadherin compared to DMSO control, which had E-cadherin protein detected. **(B)** Schwann cells were preincubated with peptide cAMP-PKA inhibitor, PKI (100μM) for 40 mins, followed by two washes then seeded onto DRG neurons. Three days later, E-cadherin expression was assessed by Western blot analysis. No E-cadherin was detected in Schwann cells treated with PKI, while control Schwann cells did present with E-cadherin protein.

Figure 6. Axonal contact mediates cAMP-PKA induced E-cadherin expression

6A



6B

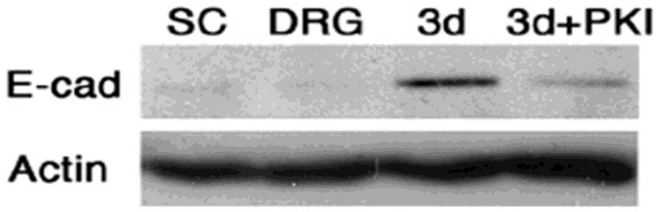


Figure 7 *In vivo* developmental expression profiles study.

Western blot analysis of rat post-natal (P) sciatic nerve lysates starting from P1 to P28 days. Starting with the top panel, E-cadherin (Ecad) shows a gradual increase in expression with development. N-cadherin (Ncad) and ErbB2 both how a gradual decrease in expression with development. The active phosphorylated form of ErbB2 (p-ErbB2) starts with low levels of expression at P1 then rise to peak at P5 to P7 followed by a decrease from P14 all the way to P28. Actin was used as a loading control (40µgrams/lane) (N=3).

Figure 7. *In Vivo* developmental expression profile study

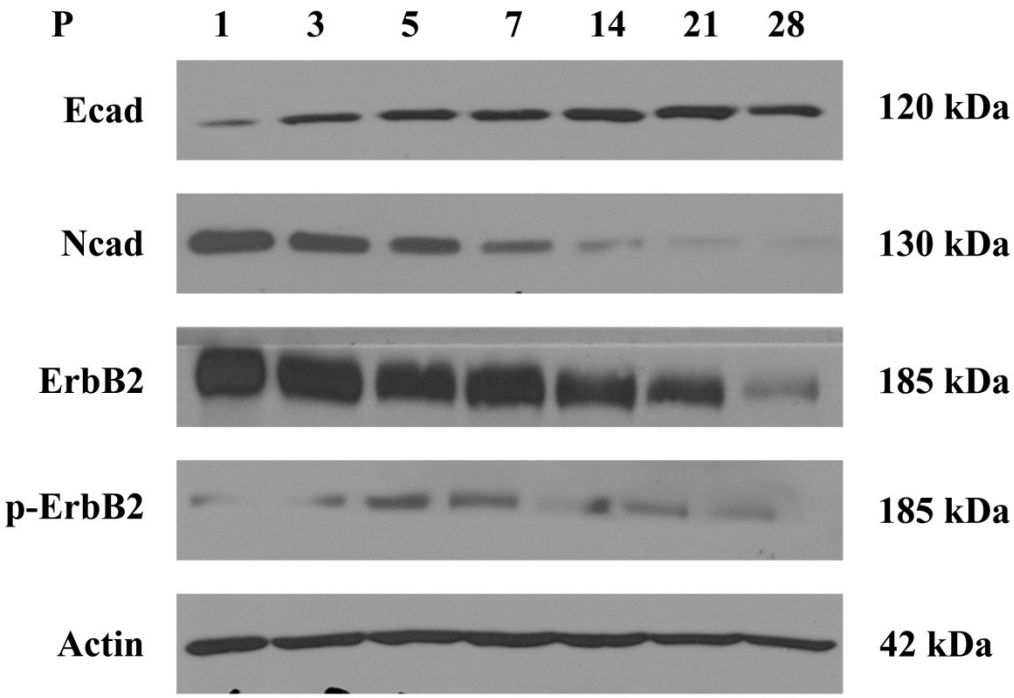


Figure 8. E-cadherin, not N-cadherin associates with ErbB2 and p-ErbB2

(A) Immunoprecipitation using P1 to P28 rat sciatic nerve lysates. Lysates were precipitated with anti ErbB2 followed by Western blot analysis with anti E-cadherin (anti Ecad).**(B)** Utilizing the same developmental time point lysates, except that precipitation was preformed with the phosphorylated form of ErbB2 (p-ErbB2), then followed by Western blot analysis with anti E-cadherin. **(C)** Rat sciatic nerve lysates from P3 and P5 time points were utilized, followed by immunoprecipitation with ErbB2 and subsequent Western blot analysis of both total lysate and IP with antibodies against N-cadherin (anti Ncad) (N=3).

Figure 8. E-cadherin, not N-cadherin, associates with ErbB2 and p-ErbB2

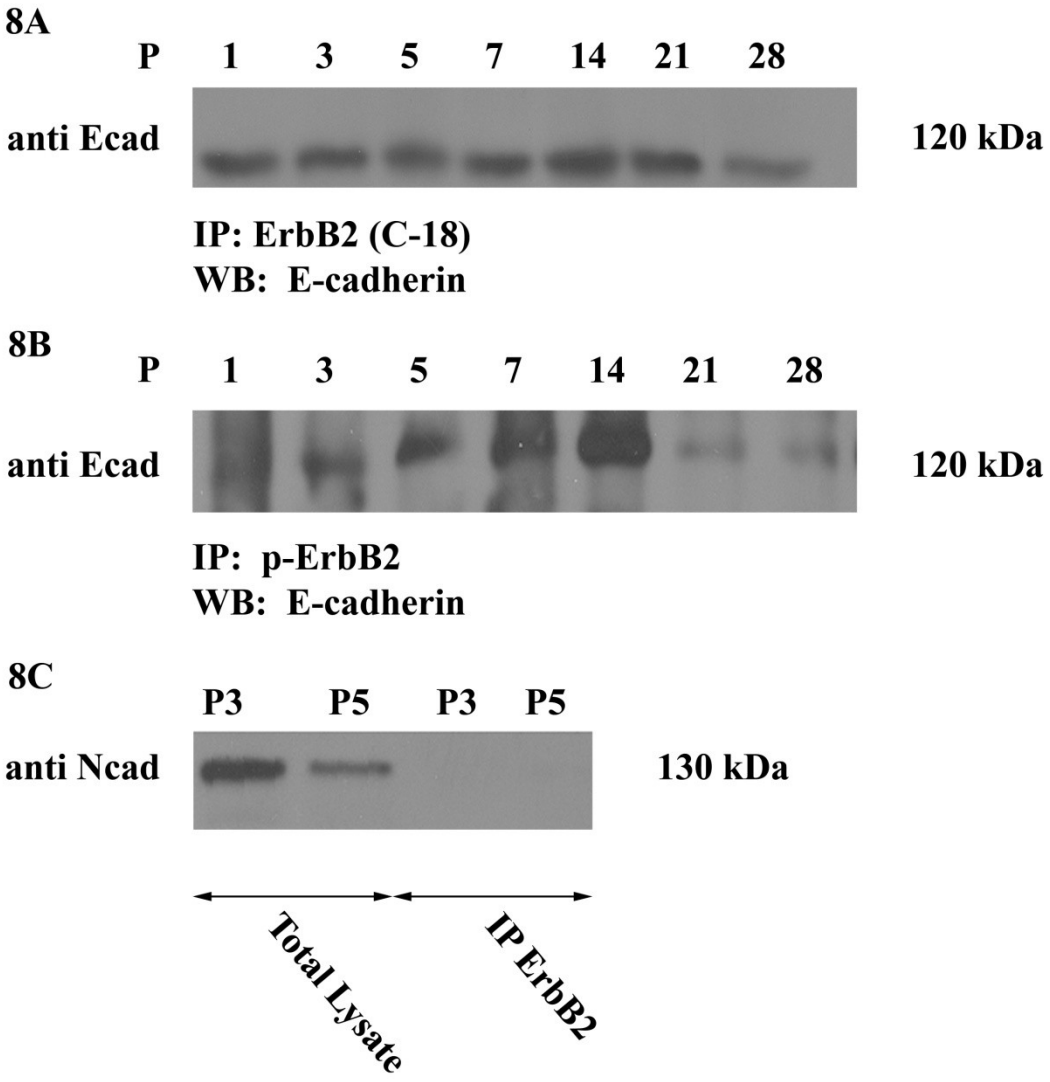


Figure 9. Association of ErbB2 with E-cadherin is not due to their co-existence in a lipid raft

Immunoprecipitation (IP) experiment with rat P7 sciatic nerve lysates that was generated either with standard buffer or with Beta-octylglucoside, to disrupt lipid rafts. Both lysates were immunoprecipitated with anti-ErbB2 antibodies (ErbB2) and followed by Western blot analysis with anti-E-cadherin antibodies (Ecad). Total lysates Western blots were used to identify that E-cadherin or ErbB2 was present.

Figure 9 Association of ErbB2 with E-cadherin is not due to their co-existence in a lipid raft.

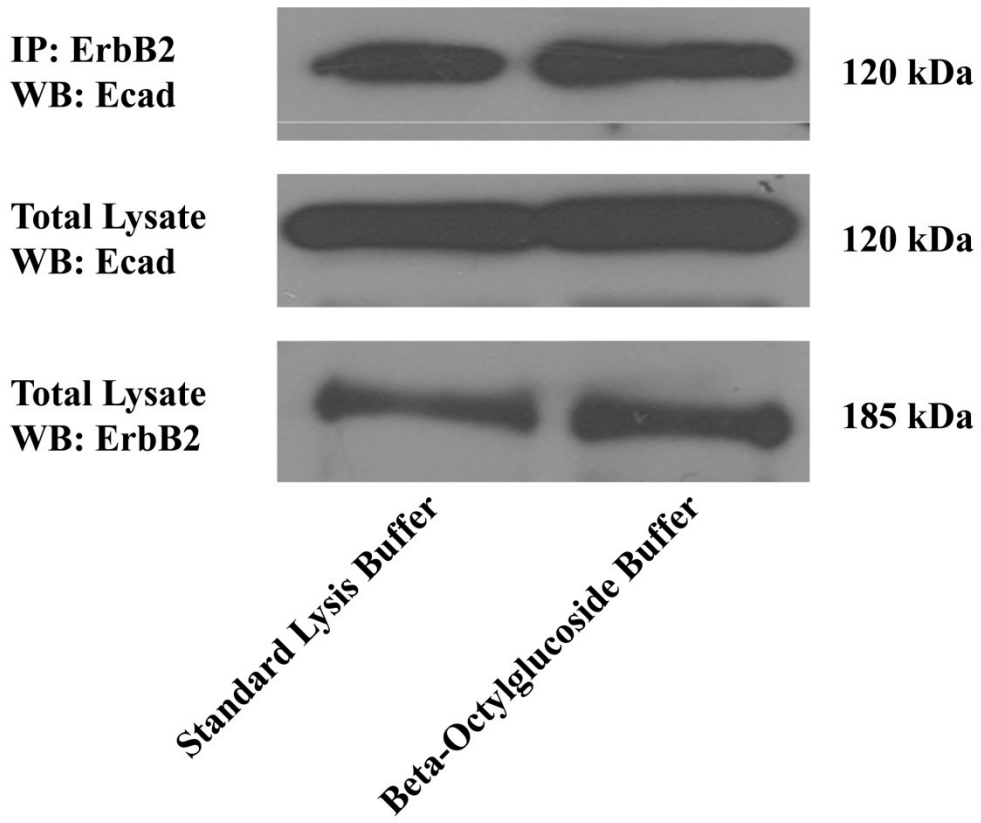
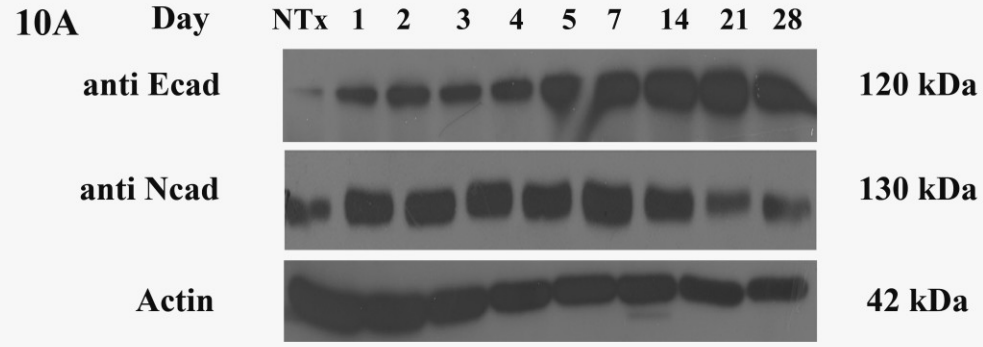
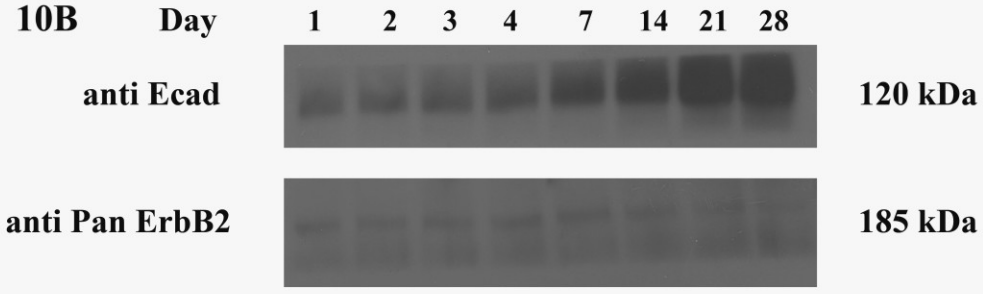


Figure 10 *In vitro* E-cadherin expression supports *in vivo* expression data

(A) Lysates collected from post ascorbic acid treatment (P) SC/DRG co-cultures were analyzed by Western blot to identify E-cadherin (anti Ecad) and N-cadherin (anti Ncad) expression (40 µg/lane). **(B)** Immunoprecipitation was performed on SC/DRG cocultures at time points similar to *in vivo* experiments, once again, a developmental association was observed between E-cadherin and ErbB2 (500µg/IP) (N=3).

Figure 10 *In vitro* E-cadherin and N-cadherin expression supports *In vivo* expression data



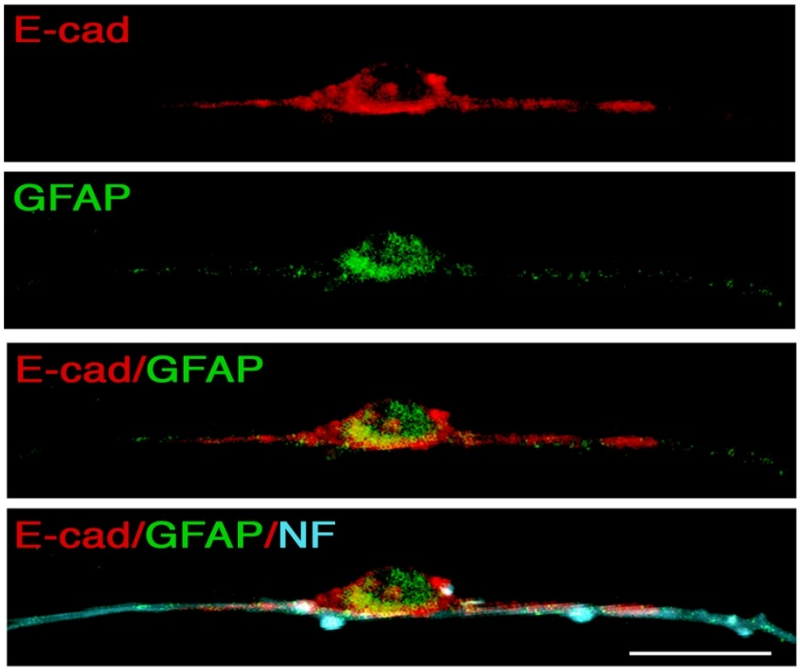


IP: E-cadherin
WB: E-cadherin
WB: Pan ErbB2

Figure 11. E-cadherin localizes to the membrane

E-cadherin is expressed on the surface of axon-associated Schwann cells. Confocal images of an axon-associated Schwann cell immunostained for E-cadherin (red), GFAP (green), and neurofilament (blue). The merged image is shown below. Most of the E-cadherin expression is seen on the Schwann cell surface, which is devoid of cytoplasmic GFAP expression. A small amount of the E-cadherin is seen within the cytoplasm, likely representing the unprocessed population of the protein. The Schwann cell is seen in close contact with the axon visualized by the neurofilament (NF) staining. Scale bar: 2 micrometers

Figure 11. E-cadherin localizes to the membrane upon alignment to the axon



(Crawford et. al., 2008)

Aim#2

Schwann cell E-cadherin is required for myelination

Schwann cell E-cadherin expression is successfully diminished by an shRNA strategy

With the expression kinetics of E-cadherin and N-cadherin, as well as both the active and pan forms of ErbB2 developmental expression kinetics identified, we wanted to examine if E-cadherin has a functional correlation to myelination. Therefore, I utilized an RNA interference strategy to knockdown E-cadherin in Schwann cells. Therefore, short hairpin RNA (shRNA) sequences (2.9 and 3.4) were designed against two separate regions of E-cadherin's coding sequence and then subcloned into the GFP expressing pLL3.7 lentiviral vector (Lois et al., 2002). Next, purified Schwann cells were infected with lentivirus encoding either the 2.9 or 3.4 shRNA constructs against E-cadherin (E-cad-shRNA). As controls, Schwann cells were infected with viruses carrying an empty pLL3.7 vector (GFP-CTL) or a pLL3.7 containing an shRNA against luciferase (Luc-shRNA). A Western blot of Schwann cell lysates showed that both non-infected and GFP-CTL infected Schwann cells had E-cadherin expression. Lanes with a single construct against E-cadherin (either 2.9 or 3.4) showed little knock down of E-cadherin; Schwann cells infected with 2.9 or 3.4 alone did not show a decrease in E-cadherin expression. Simultaneous use of two vectors against E-cadherin, 2.9 and 3.4 achieved successful knockdown of E-cadherin. Based upon this result, all further experiments to knockdown E-cadherin utilized the simultaneous dual infection strategy (Figure 12A).

To corroborate the immunoblot data, immunocytochemistry was also used to verify E-cadherin knockdown by shRNA. In figure 12B, control non-infected Schwann cells or Schwann cells infected with empty shRNA vector (GFP-CTL), as well as E-cadherin-shRNA were fixed and immunostained with antibodies against GFP, E-cadherin and DAPI. GFP (green) was observed in both the GFP CTL and E-cadherin-shRNA cells, indicating successful infection with the virus. On the other hand, E-cadherin (red) was only observed in control cells, and was absent in the E-cad-shRNA cells, in agreement with the immunoblot data that also showed knockdown of E-cadherin. These results verify successful knockdown of E-cadherin with the shRNA strategy.

Knock down of E-cadherin inhibits Schwann cell myelination *in vitro*

To determine the effect of E-cadherin knockdown on Schwann cell myelination, E-cadherin shRNA Schwann cells were plated onto DRG neurons and myelination was observed ten days after initiating myelination. The cocultures were fixed and immunostained for GFP and Myelin Basic Protein (MBP). Knockdown of E-cadherin in Schwann cells results in a clear inhibition of myelin segment formation compared to control non-infected Schwann cells, Schwann cells infected with vector alone, or the shRNA to Luciferase. Epifluroescencent microscopy was utilized and MBP positive segments were quantitated. As seen in figure 13A, cocultures with E-cad-shRNA had a significantly lower number of MBP segments per coverslips compared to the controls ($N=2$, $P<=.001$), therefore demonstrating that knockdown in E-cadherin in Schwann cells inhibits myelination.

Knockdown of Schwann cell E-cadherin also down regulated down stream signaling pathways.

With the previously results showing that a knockdown in Schwann cell E-cadherin by shRNA causes a defect in myelination in the coculture system, our lab next wanted to exam what are the effects of the down stream pathways related to myelination. As mentioned in the introduction, myelination in Schwann cells is regulated by two downstream pathways, the ErbB signaling and its down stream AKT and ERK signaling pathways. Could knockdown of Schwann cell E-cadherin be regulating these pathways crucial to myelination? To identify if such an effect was present, our lab utilized E-cadherin shRNA Schwann cells which had E-cadherin knocked down (figure 13B), followed by a treatment with with NRG1. After treatment, lysates were collected and immunoblot analysis revealed that Schwann cell E-cadherin levels were absent in E-cadherin shRNA Schwann cells when compared to control, even with treatment with Forskolin, previously identified by our lab to induce Schwann cell E-cadherin in culture (Crawford et al., 2008). Analysis with antibodies against ErbB2 showed that ErbB2 levels remained consistent, even in the absence of E-cadherin, but upon examination of the active form, there was a clear decrease in activation in lysates collected from the E-cadherin shRNA knockdown Schwann cells (figure 13B). Furthermore, examination of the phosphorylation of AKT and ERK 1/2 by immunoblot of the same lysates identified that knockdown of Schwann cell E-cadherin by shRNA also affects their phosphorylation state compared to control Schwann cell lysates. Interestingly, the non-phosphorylated state of AKT was stable, but there was a decrease in pan ERK 1/2 levels. Taken together, the defect in myelination that was observed by shRNA mediated knockdown of E-

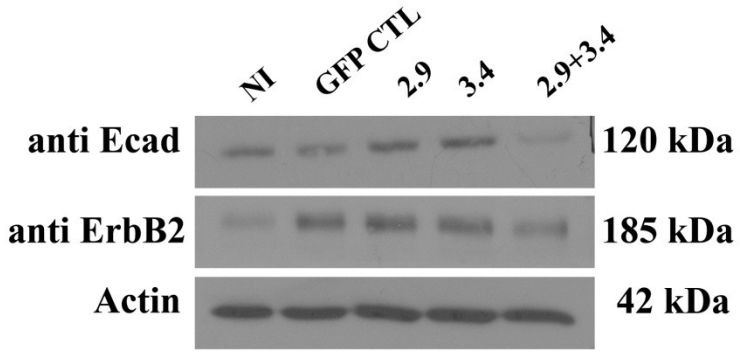
cadherin in Schwann cells could be attributed to knockdown of signaling pathways related to myelination.

Figure 12. shRNA mediated E-cadherin expression knockdown results in inhibition of myelination

(A) Western blot of Schwann Cell monocultures infected with an shRNA to knockdown E-cadherin, two constructs, 2.9 & 3.4 used together demonstrated better knockdown of E-cadherin compared to their use individually (40 µgrams/lane) **(B)** Immunocytochemistry corroborates with Western blot data of figure 10A.

Figure 12. shRNA mediated E-cadherin expression knock down results in inhibition of myelination

12A



12B

E-cadherin staining in SCM (+ Forskolin & NRG)-24hr-Lenti Ecad shRNA SC

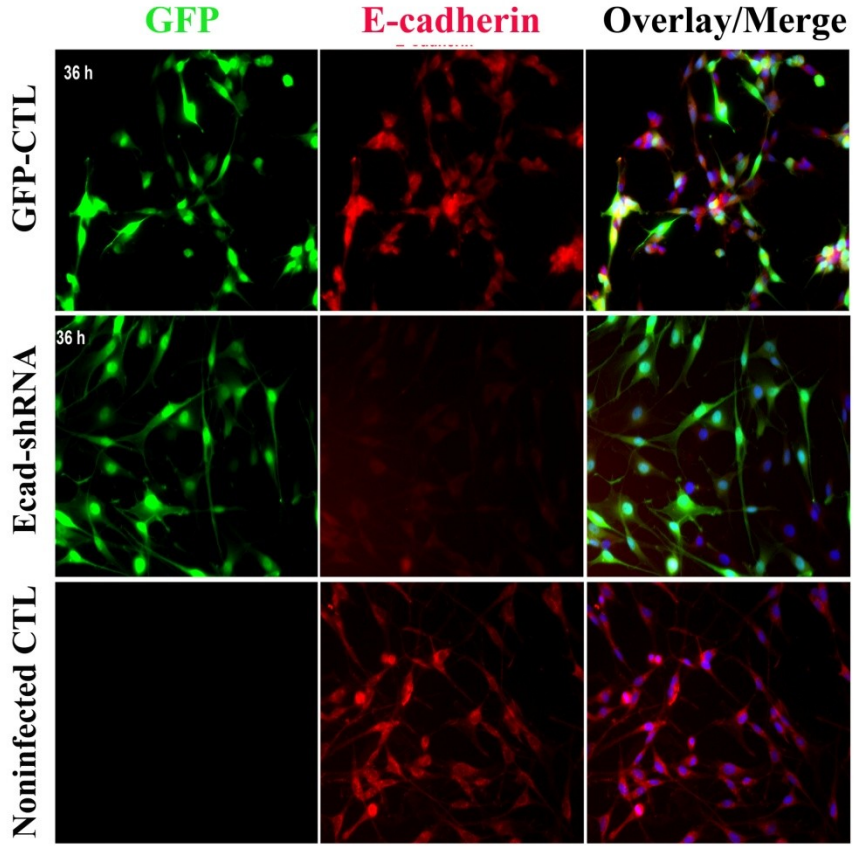


Figure 13A. shRNA mediated knockdown of E-cadherin causes a statistically significant defect in myelination

Quantification of myelin segments using Schwann cells with shRNA mediated E-cadherin knockdown seeded onto DRG neurons shows a defect in myelination, as compared to controls, is statistically significant by one-way ANOVA (**,N=2,P = <0.001). Immunocytochemical analysis of myelinating cocultures were immunostained with antibodies against myelin basic protein.

Figure 13. E-cadherin shRNA inhibits myelination in SC/DRG cocultures

13A

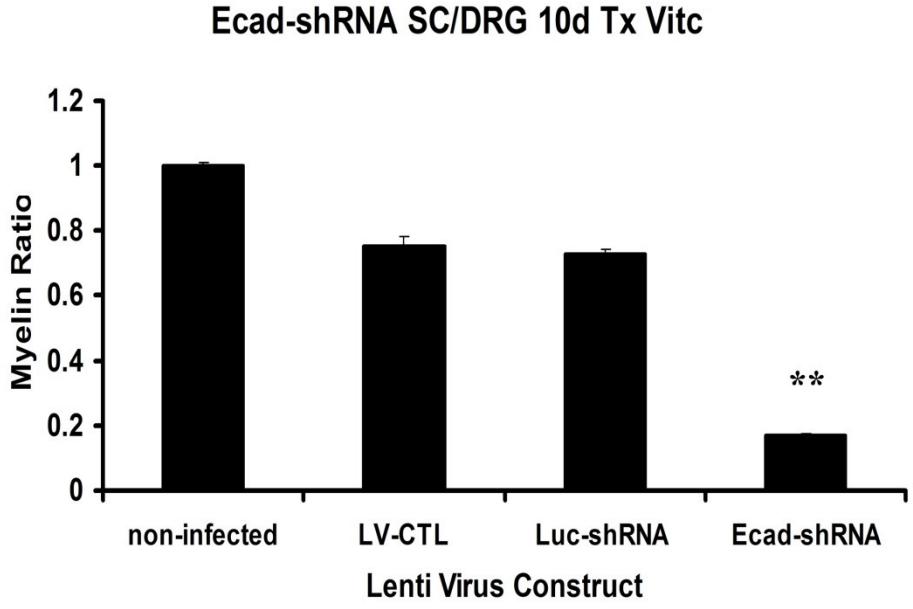
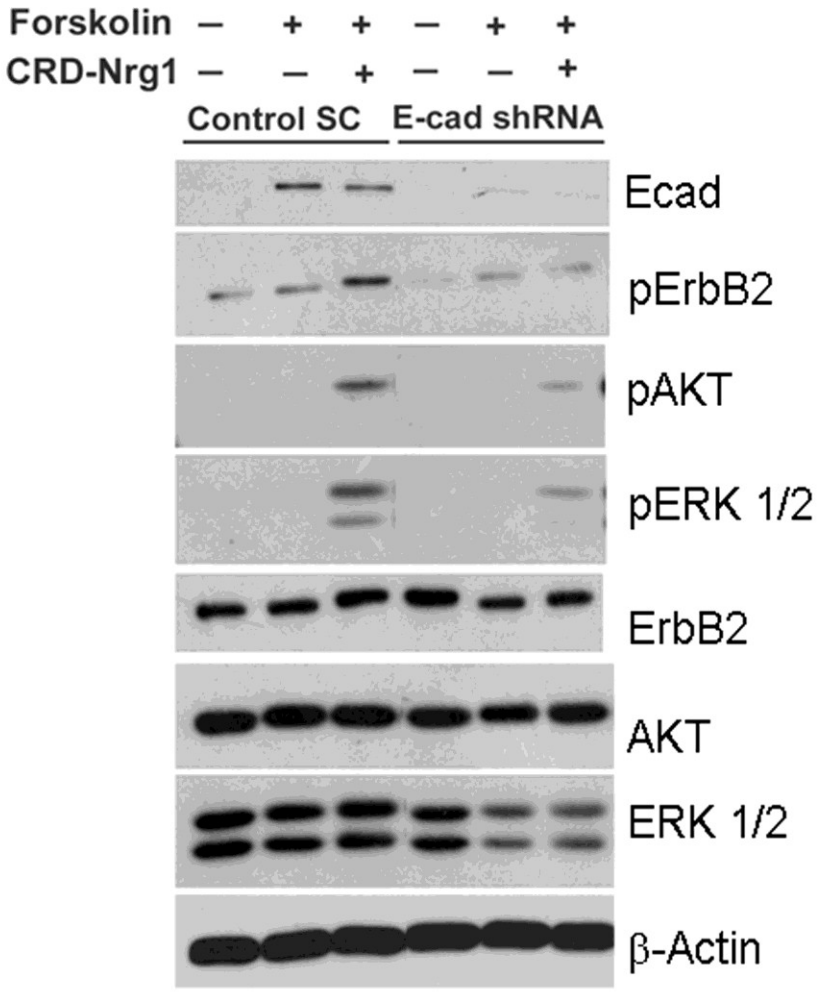


Figure 13B Down stream signaling pathways related to myelination also down regulated with shRNA mediated knockdown of rat E-cadherin

(B) Western blot analysis of rat Schwann cells infected with lentivirus carrying an shRNA to knockdown E-cadherin. Infected (E-cad shRNA) and control Schwann cells (control SC) monocultures were treated with Forskolin, Neuregulin 1 (CRD-Nrg1), or both and lysates were obtained following specified treatment. Immunoblot analysis with antibodies against rat E-cadherin (Ecad) detected E-cadherin in control Schwann cells, but absent in shRNA infected cells. ErbB2 levels were present in both control and infected Schwann cells, but the active form (pErbB2) was only clearly present in control cells and diminished in shRNA infected cells. Lysates were also probed for AKT and ERK 1/2 proteins; AKT was present in both conditions, but ERK 1/2 was detected at a lower level in shRNA Schwann cells in comparison to control cells. The active forms of AKT and ERK 1/2 was observed to noticeably lower in detection in the shRNA infected Schwann cells compared to control cells.

Fig ure 13B Down stream signaling pathways related to myelination also down regulated



Aim #3

Ectopic expression of E-cadherin in Schwann cells promotes myelination

Generation of rat Schwann cells overexpressing Human E-cadherin and N-cadherin

Knockdown of E-cadherin caused a defect in myelination. Next, to determine whether overexpressing E-cadherin is sufficient to induce myelination, I first generated Schwann cells ectopically expressing human E-cadherin (HE-cadherin). Briefly, Schwann cells were infected with a retrovirus carrying a construct to overexpress a human E-cadherin; in control cultures, Schwann cells were also infected with retrovirus which carried a construct to overexpress an empty expression vector (Perego et al.). Schwann cells were also infected with a retrovirus carrying a construct to overexpress human N-cadherin, to identify if N-cadherin may also affect myelination. Ectopic expression was confirmed by preparing lysates of infected Schwann cells, and then analyzing by Western blot with an antibody specific to the human isoform of E-cadherin or N-cadherin. As seen in figure 14A, Schwann cells infected clearly express HE-cadherin, which showed a band present at 120 kDa. Similarly, HN-cadherin infected Schwann cell lysates identified over expression of the 130 kDa HN-cadherin protein.

To compliment the immunoblot data, concurrently infected Schwann cells were fixed and stained for immunocytochemical analysis. These Schwann cells showed an expression of both HE-cadherin and HN-cadherin, respectively. Upon inspection at higher magnification of 60x, HE-cadherin staining is seen at the cell membranes and enriched in regions with cell to cell contacts (figure 14B).

Ectopic expression of Human E-cadherin, not N-cadherin enhances myelination in Schwann cell /DRG neuron cocultures

Upon successful generation of a line of Schwann cells overexpressing cadherins, and based upon our previous loss of function study, we wanted to determine the effect on myelination when there is an overexpression of cadherins in the DRG neuron model. Briefly, HE-cadherin and HN-cadherin ectopically expressing Schwann cells were seeded onto purified DRG neurons at the densities of 1.0×10^5 Schwann cells per coverslip. After seeding, Schwann cells were allowed to align onto axons and proliferate, followed by treatment with ascorbic acid for ten days to allow for myelination. Cocultures were fixed and immunostained with HE-cadherin or HN-cadherin and myelin basic protein (MBP) antibodies. Quantification of MBP positive segments identified that HE-cadherin cocultures had a significantly higher number of MBP positively stained segments (red) compared to the NEO control cocultures ($N=4$, $P<=.001$). By contrast, expression of HN-cadherin had no effect on myelination. These data suggest that HE-cadherin, not HN-cadherin positively effects myelination in the normally myelinating DRG neuron model (figure 15).

These mutant HE-cadherin and control Schwann cell/DRG cocultures were subjected to examination of the internodal length of myelinated axons to identify a change in length due to ectopic expression. Upon evaluation, distances from node to node did not demonstrate a significant change in length (data not shown). Taken

together, ectopic expression did have a positive affect on myelination on DRG axons, but did so without an enhancement in internodal length.

Ectopic expression of E-cadherin increases ErbB signaling during myelination

With the previous result demonstrating the ectopic expression enhances myelination observed in the DRG neurons, our lab next examined if there is also an enhancement in signaling related to myelination, specifically the ErbB signaling pathway and its downstream pathways of AKT and ERK1/2. Utilizing cocultures with ectopically expressing HE-cadherin Schwann cells on purified DRG neurons, lysates were collected at 0 days, 2, 3, 5 and 7 days following the treatment of Ascorbic acid to induce myelination. As you can see by figure 16, throughout the time course of myelination, 0 day (no treatment with Ascorbic acid) to completion, 7 days in Ascorbic acid treatment, there is a steady level of HE-cadherin present, compared to control Schwann cells. Immunoblot analysis with antibodies against ErbB2 demonstrated that levels of ErbB2 receptor were stable in both ectopically expressing HE-cadherin Schwann cells and NEO control cells. However, when the active form of ErbB2 was probed, there was an increase in the active state of ErbB2 present at day 3 in the ectopically expressing HE-cadherin Schwann cells. Upon examination of the downstream signaling pathways of AKT and ERK 1/2 by immunoblot analysis of the same lysates, an expression of both proteins, though ERK 1/2 levels seemed to be lower in the experimental Schwann cells compared to the NEO controls was identified. Expression of AKT was observed to be similar in both control and ectopically expressing Schwann cells. Next these lysates were probed

for the active phosphorylated forms of AKT and ERK1/2. Examination by antibodies specific to these two forms demonstrated that there was a rise in phosphorylated AKT expression in the HE-cadherin overexpressing Schwann cells at days 2 to 5 post initiation of myelination by treatment with Ascorbic acid compared to their NEO control. There was a absence of the phosphorylated form of ERK1/2 expression from day 3 to 7 in the same Schwann cell lysates when compared to NEO controls, which had clear expression of the active form of ERK1/2 throughout the treatment (figure 16).

The myelination experiments were replicated three times and the subsequent Western blots were then analyzed for quantification of the bands for these specific proteins by pixel analysis software (Imagequant). This data was utilized to produce graphical interpretation to further support the Western blot analysis. Again, this quantification verified that ErbB and AKT level were higher in the ectopically expressing Schwann cells compared to NEO controls, as well as ERK 1/2 levels were lower (figure 17) . Taken together, overexpression of E-cadherin not only enhances myelination in DRG neurons, but does so by a mechanism dependent on an increase in ErbB and AKT signaling pathways.

Ectopic expression of E-cadherin overexpression does not enhance Schwann cell proliferation

Results above showed that overexpression of HE-cadherin resulted in the enhancement of myelination. Therefore to rule out the possibility that this pro-myelinating effect by the HE-cadherin was a result of an increase in Schwann cell number, prior to myelination, I determined the effect of ectopic expression of HE-

cadherin on Schwann cell proliferation. NEO, HE-cadherin, and HN-cadherin Schwann cell monocultures were plated onto poly-L-lysine coated glass coverslips and subsequently treated with glial growth factor (GGF)(50ng/mL) a known Schwann cell mitogen (Trachtenberg and Thompson, 1996). Control cultures were left untreated. Sixteen to eighteen hours later, BrdU was added to the culture media. Eighteen to twenty-four hours later, the numbers of BrdU positive cells were determined by immunocytochemistry. In all growth factor control Schwann cells, few if any cell nuclei were positive for BrdU. In cultures treated with GGF, nearly 60% of Neo Schwann cells were observed to have BrdU positive nuclei. Similarly, HE-cadherin Schwann cells treated with GGF also had a greater percent of BrdU positive nuclei compared to that observed in NEO Schwann cells. HN-cadherin Schwann cells treated with GGF did have an increase in the number of BrdU positive nuclei, but upon comparison with NEO and HE-cadherin, it was less ($N=2, P<.0.437$) (figure 18). This result suggests that the enhancement in myelination in the previous study was independent of an increase in proliferation due to ectopic expression of HE-cadherin or HN-cadherin, but rather by an undetermined mechanism.

To determine the effect of HE-cadherin or HN-cadherin on Schwann cell proliferation by axonal contact, Neo, HE-cadherin and HN-cadherin Schwann cells were plated onto purified DRG neuron at a density of 1.0×10^5. The Schwann cells attached and aligned along the axons, BrdU was added to cocultures and eighteen to twenty-four hours later the number of BrdU positive Schwann cell nuclei was determined as shown in figure 19. Upon examination by immunocytochemistry using antibodies against BrdU,

Neo Schwann cell/DRG cocultures were observed to have approximately 60% of the Schwann cells nuclei that were positive for BrdU. In comparison, HE-cadherin Schwann cell/DRG cocultures were observed to have close to 70% BrdU positive nuclei. Upon examination of the HN-cadherin Schwann cell/DRG cocultures, had fewer BrdU positive nuclei compared to Neo or HE-cadherin DRG cocultures, approximately 50% (N=2, P<.112) (figure 19). Taken together, these results of the Schwann cell monoculture and Schwann cell/DRG coculture proliferation studies suggests that ectopic expression of HE-cadherin or HN-cadherin does not affect Schwann proliferation.

Schwann cell expression of E-cadherin is lower on SCG neurons compared to DRG neurons

Data presented so far indicate that E-cadherin elicits a pro-myelinating function in Schwann cells. We have shown previously that E-cadherin expression in Schwann cells is induced by axonal association. Axons of the superior cervical ganglion (SCG) neurons are small in diameter, thus do not become myelinated, whereas the larger diameter axons of the DRG are normally myelinated by the association of Schwann cells. To determine whether there is a correlation between myelination phenotype of the axon and the Schwann cells E-cadherin expression, rat Schwann cells were seeded onto purified DRG or SCG neurons and levels of E-cadherin expression were determined by Western blot using antibodies against rat E-cadherin. As seen in figure 20, little if any E-cadherin was present in the Schwann cell only lysate, and it was completely absent from the DRG neuron lysate, but upon contact with DRG neurons there is an induction of E-cadherin expression. In comparison with Schwann cells allowed to associate with SCG neurons,

there is clearly a lower amount of E-cadherin present. Actin lanes corresponding to each lysates demonstrated uniform loading of lysates (figure 20). This result suggests that SCG axons have a reduced ability to induce E-cadherin expression.

Ectopic expression of cadherins induces myelination in the Schwann cell/SCG coculutre

To determine whether overexpression of E-cadherin in Schwann cells may alter the fate of SCG neurons to a myelinated one, Neo and HE-cadherin Schwann cells were plated onto SCG neurons and myelination was assessed 51 days later by immunostaining for MBP (red). Quantification of MBP positive segments in Neo infected Schwann cells identified very few, if any MBP positive segments present. Upon examination of the HE-cadherin Schwann cell cocultures, many more MBP positive segments were observed. Later quantification found that there was a twenty-five fold higher number of myelin segments compared to NEO (Figure 21A) ($N=5$, $P <= .001$). To verify that Schwann cells were continuing to ectopically express HE-cadherin, Schwann cell/SCG cocultures were also immunostained with antibodies against HE-cadherin (green) (figure 21B). Upon examination of HN-cadherin Schwann cells/SCG cocultures also presented MBP positive segments. When quantified, segments were greatly higher compared to NEO Schwann cells, between 15-20% more segments were observed. Once again immunostaining against HN-cadherin (green) verified that ectopic expression was still present (figure 21B). In a previous study, ectopic expression of HE-cadherin in Schwann

cells induced an enhancement of myelination with DRG neurons, in comparison, the HN-cadherin Schwann cells with DRG neurons data suggests that there was an inhibition of myelination observed, opposite to what was identified when these Schwann cells were seeded onto SCG neurons. Taken together, this suggests that ectopic expression of HE-cadherin has a generally pro-myelinating effect, independent of axon size, while overexpression of HN-cadherin only positively affects myelination in axons of the small diameter SCG neurons.

Figure 14. Verification of cadherin(s) overexpression

(A) Immunoblot of Schwann cells overexpressing Human E-cadherin(HEcad), Hela cell lysates was used as a control for the anti Human E-cadherin antibody (120kDa). Immunoblot of Schwann Cells over-expressing Human N-cadherin (HNcad) (130kDa). **(B)** Immunocytochemistry of Schwann cells, control cells (Non-Infected SC and NEO) only show DAPI nuclear staining (blue), but HE-cadherin and HN-cadherin show distinct membrane localization of the respective proteins (green) (20x). **(C)** Higher magnification (60x) of HE-cadherin cells shows enrichment of E-cadherin at cell to cell contacts.

Figure 14 Verification of Human cadherin(s) overexpression

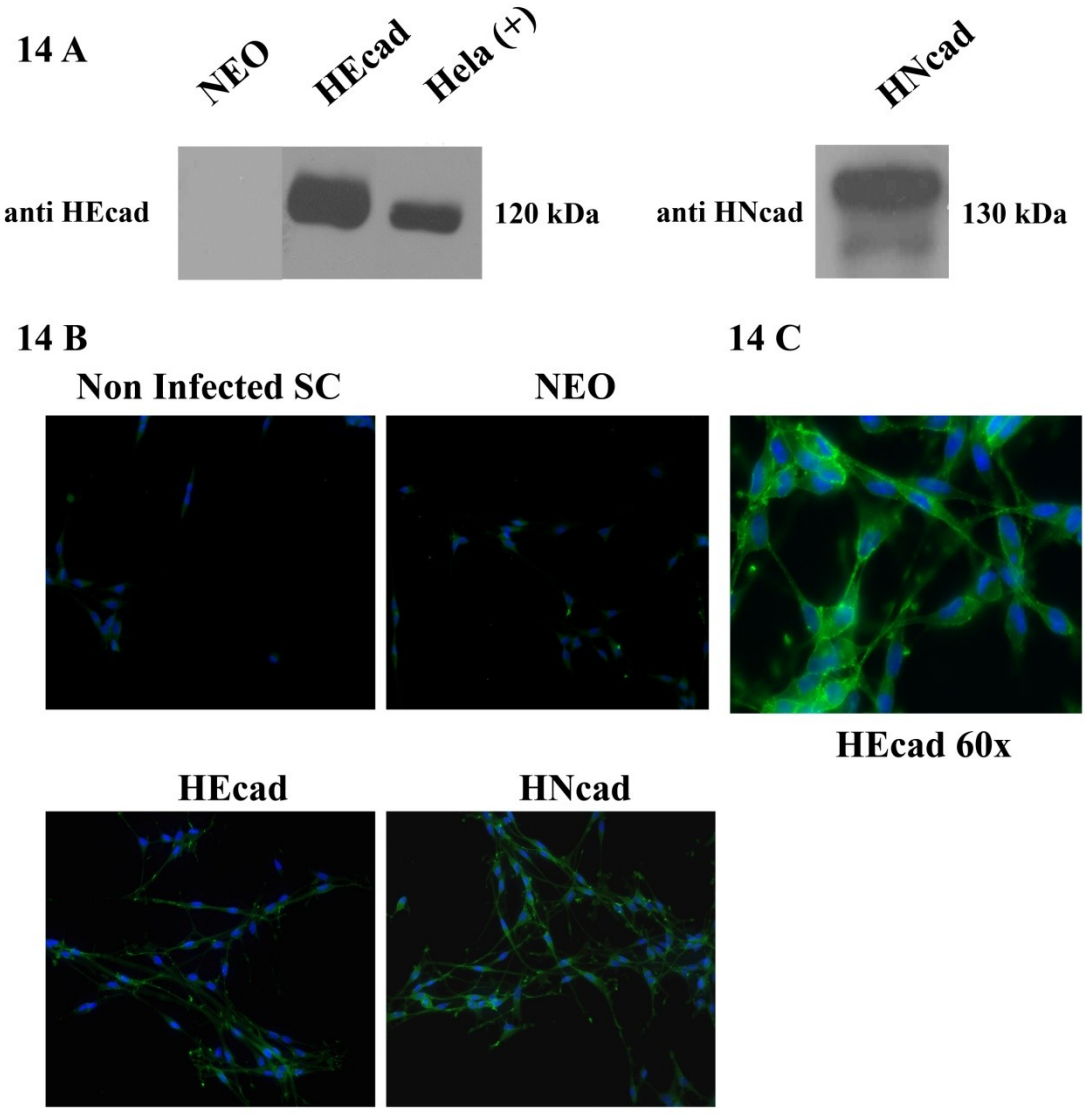


Figure 15. Expression of Human E-cadherin, but not N-cadherin enhances myelination on DRG neurons

Human E-cadherin(HEcad) and N-cadherin (HNcad) overexpressing confirmed retro virus infected Schwann cells and control Schwann cells were plated onto purified Dorsal Root Ganglion neurons (DRG) and placed under myelinating conditions for 10 days by treatment with ascorbic acid. Immunocytochemistry with antibodies against MBP was performed, following which the myelin ratio was calculated and compared to NEO cocultures. HE-cadherin has nearly a 2 fold increase in the number of myelin segments (N=4) (**,P<.001).

Figure 15 Overexpression of Human E-cadherin, but not Human N-cadherin, enhances myelination in SC/DRG cocultures

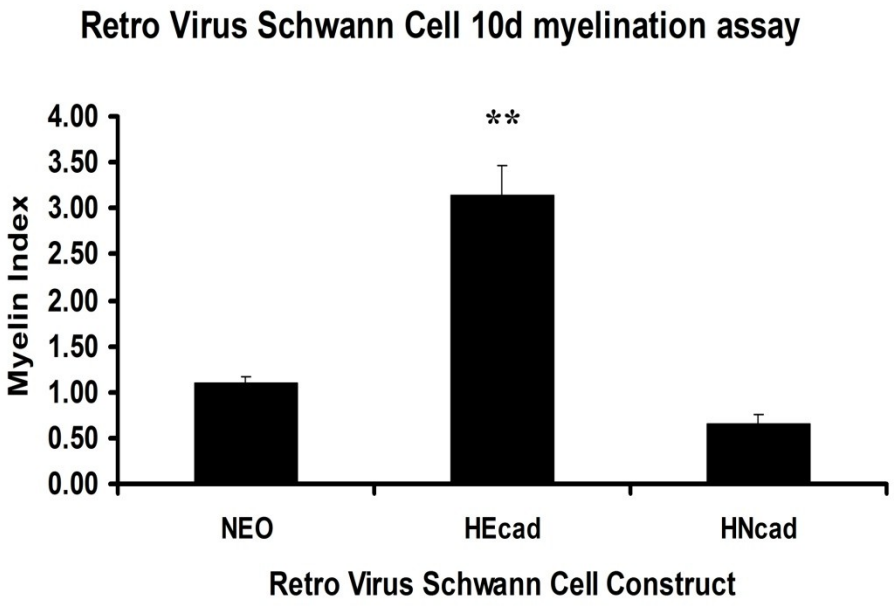


Figure 16. Ectopic expression of E-cadherin increases ErbB signaling during myelination

Control Schwann cells (Perego et al.) or Schwann cells overexpressing human E-cadherin were cocultured with DRG neurons. Following initiation of myelination on Day 0, levels of active ErbB2, AKT and ERK1/2 were determined by Western blot. Human E-cadherin (hE-cad) is only detected in lysates prepared from Schwann cells induced to ectopically express human E-cadherin. Upon probing these lysates with antibodies against ErbB2, AKT and ERK 1/2, all three were detected in both control and ectopically expressing Schwann cells. Examination with antibodies against the active forms of these three proteins identified an enhancement of the active forms of ErbB2 and AKT (p-ErbB2 and p-AKT, respectively), and down regulation of the active form of ERK 1/2 (p-ERK).

Figure 16 Ectopic expression of E-cadherin increases ErbB signaling during myelination

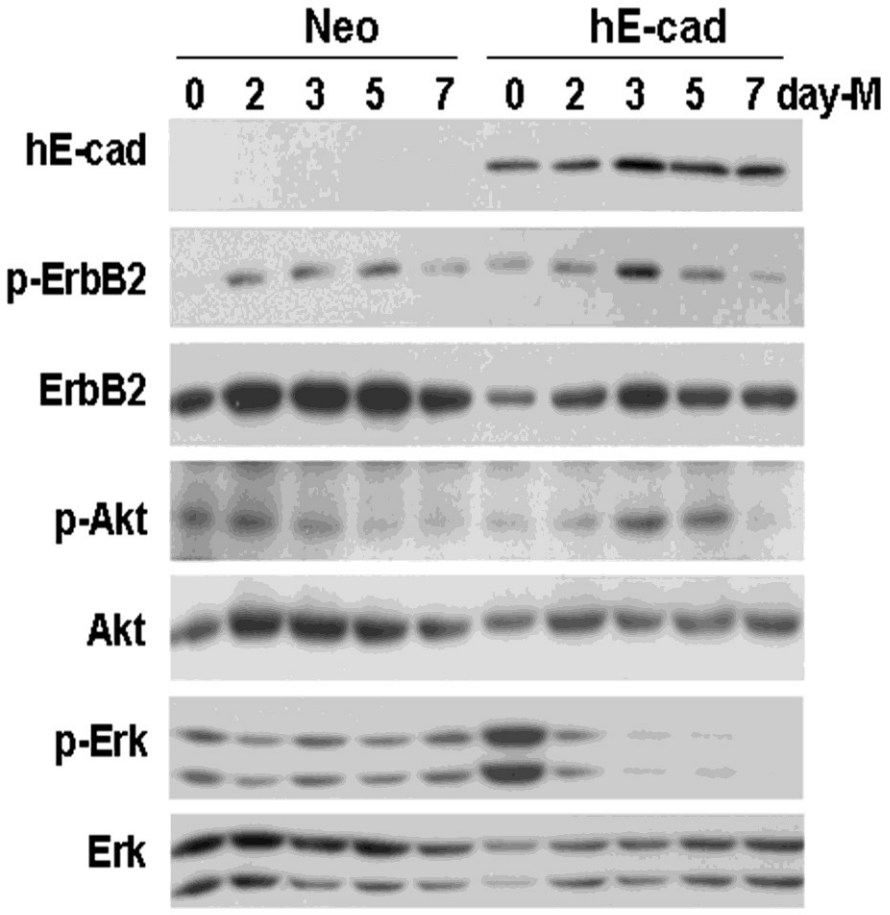


Figure 17. Quantification of Ectopic expression of E-cadherin increases ErbB2 signaling during myelination

Quantification of Western blot data of three experimental trials using the ectopically expressing human E-cadherin and control Schwann cells maintained in myelination cocultures.

Figure 17 Quantification of Ectopic expression of E-cadherin increases ErbB signaling during myelination

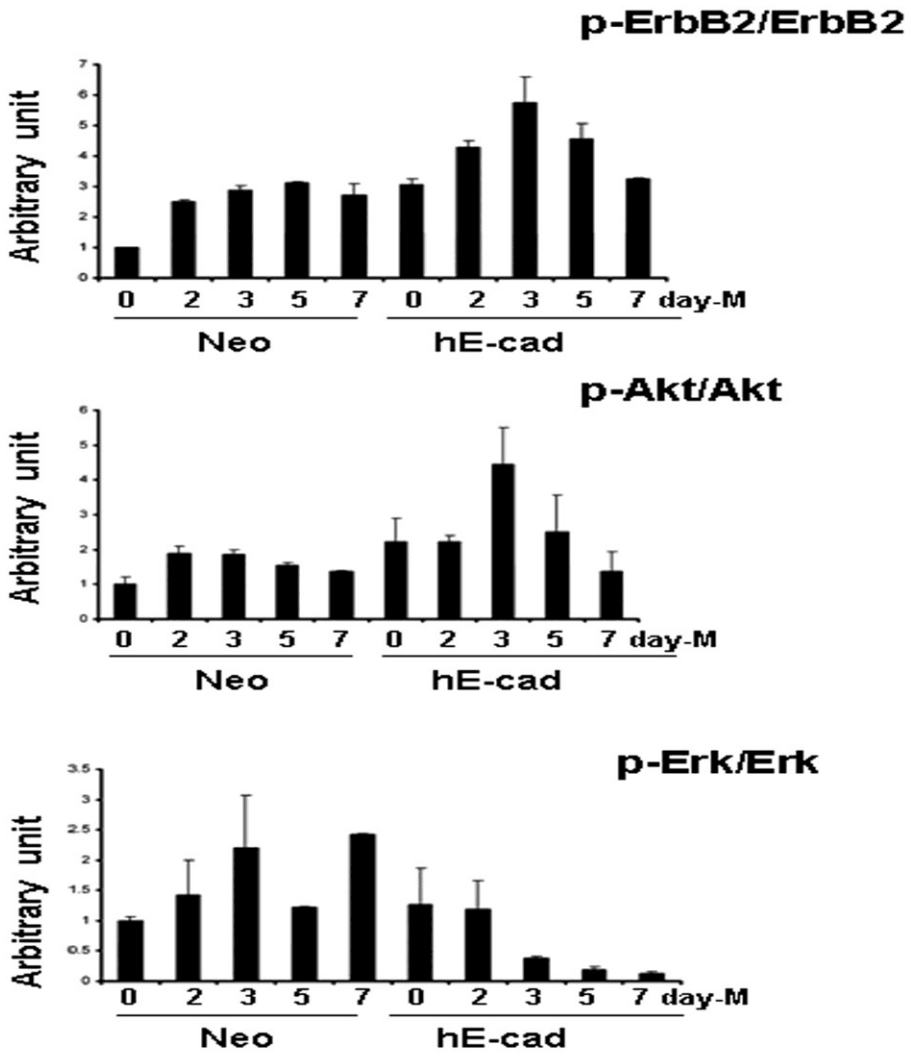


Figure 18. Over expression of HE-cadherin does not alter Schwann cell proliferation in response to growth factor stimulus

HE-cadherin and HN-cadherin Schwann cell monocultures were plated onto poly-L-lysine coated glass coverslips to assess response to glial growth factor (GGF) stimulation. Cells were fixed and stained and BrdU incorporation was assessed and a comparison of BrdU positive cells to DAPI nuclear staining was obtained. GGF treatment has no effect on proliferation on HE-cadherin or HN-cadherin overexpressing Schwann cells ($N=20, P<.0.437$).

Figure 18 Overexpression of Human E-cadherin does not alter proliferation in response to growth factor stimulus

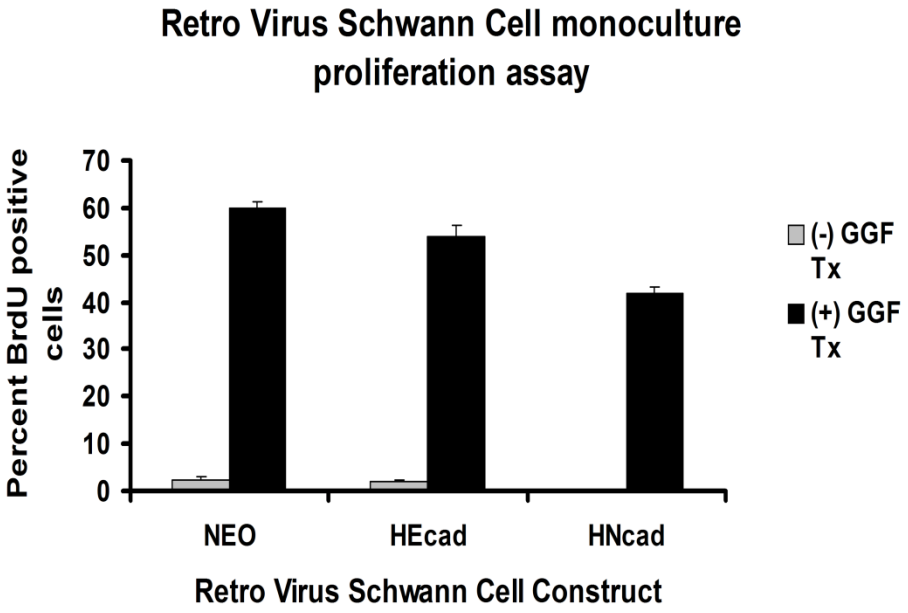


Figure 19. Overexpression of HE-cadherin does not alter Schwann cell proliferation in response to seeding on DRG Neurons.

Expression verified retro virus Schwann cell for HE-cadherin, HN-cadherin, and NEO were seeded onto purified DRG neurons. 4 days post seeding, Schwann cell/DRG cocultures were pulsed with BrdU for 18-24hrs. Immunocytochemistry was used with anti-BrdU and DAPI to identify cells actively proliferating. When compared to NEO controls, neither HE-cadherin nor HN-cadherin was observed to have a statistically significant change in proliferation. Therefore the enhancement in myelination observed in the 10 day treatment with Ascorbic Acid was by another mechanism ($N=20$, $P<.115$)

Figure 19 Overexpresssion of HEcad does not alter Schwann Cell proliferation in response to DRG neuron association.

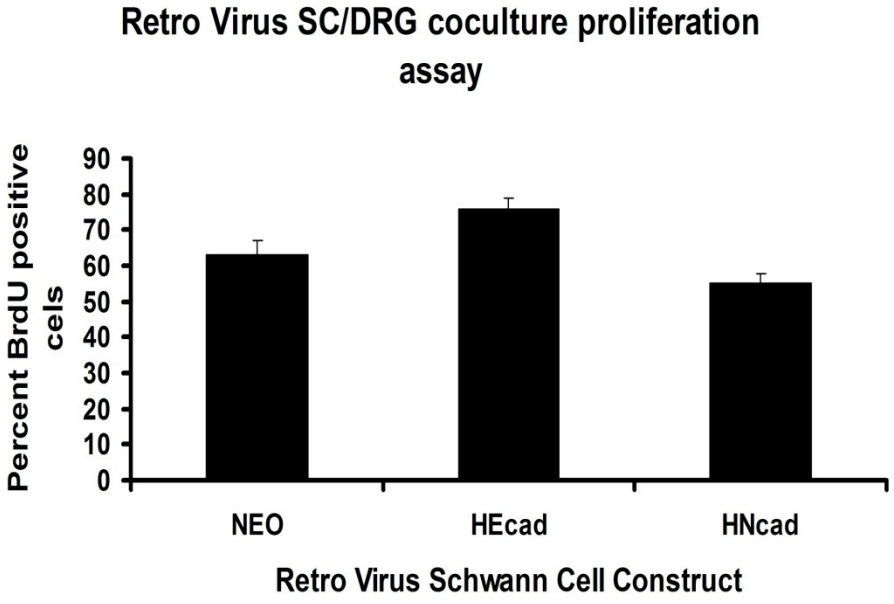


Figure 20. E-cadherin levels are lower in the Schwann cell/SCG cocultures compared to Schwann cell/DRG cocultures.

Immunoblot of lysates (50µgram/lane) collected from wild type rat Schwann cells only, DRG neuron only, Schwann cell/DRG cocultures, SCG neuron only and Schwann cell/SCG cocultures. In the Schwann cell only lane, some E-cadherin expression is observed even after 48hrs without treatment with Forskolin (5µM), and no E-cadherin expression is observed in either neuron only lanes. But upon comparison of the Schwann cell/DRG to Schwann cell/SCG cocultures, there is more E-cadherin present in the Schwann cell/DRG lane. Bottom panel shows even loading by use of the same lysates, but probing with Actin.

Figure 20 E-cadherin levels are lower in SC/SCG cocultures compared to SC/DRG

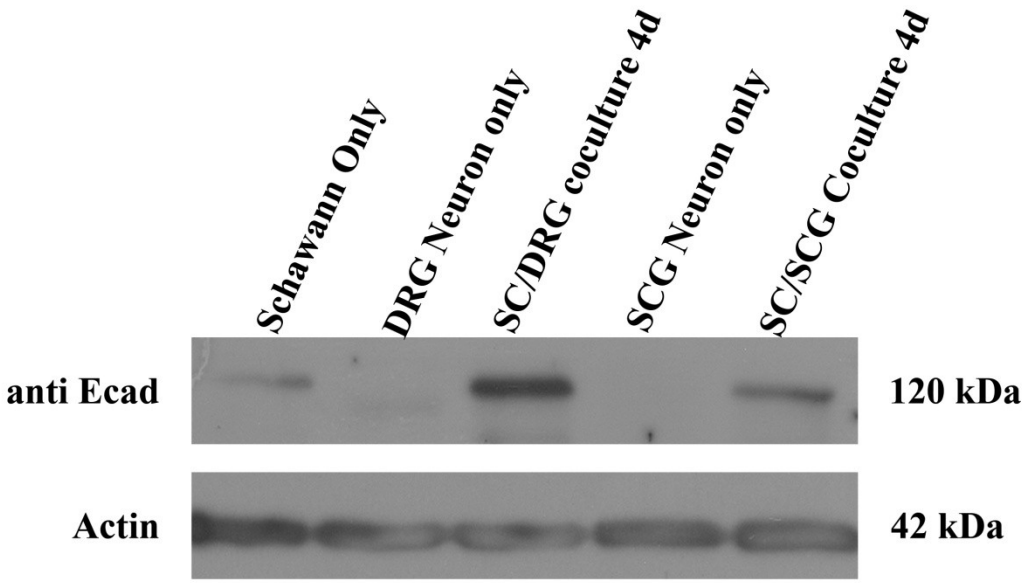
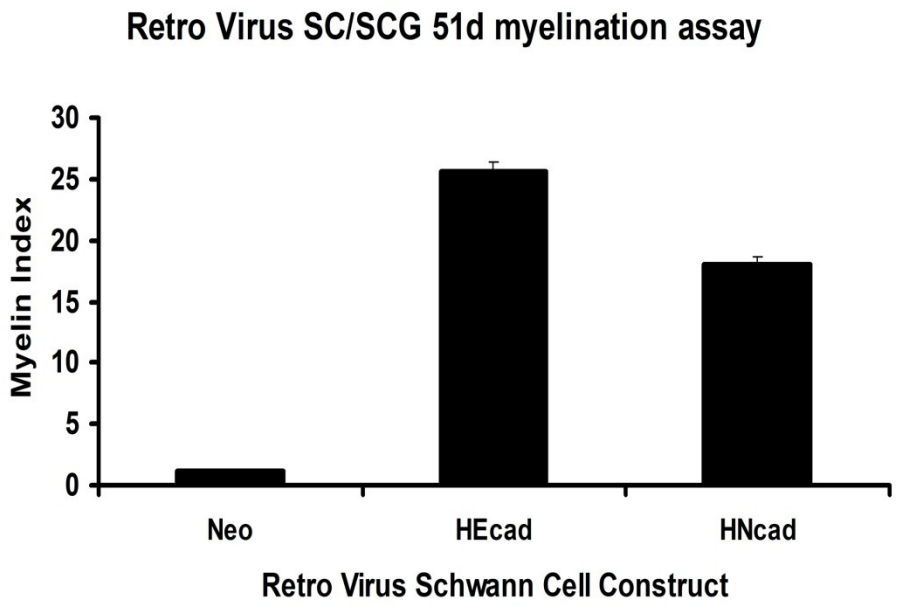


Figure 21. Overexpression of cadherins in Schwann cells induces myelination on SCG Neurons.

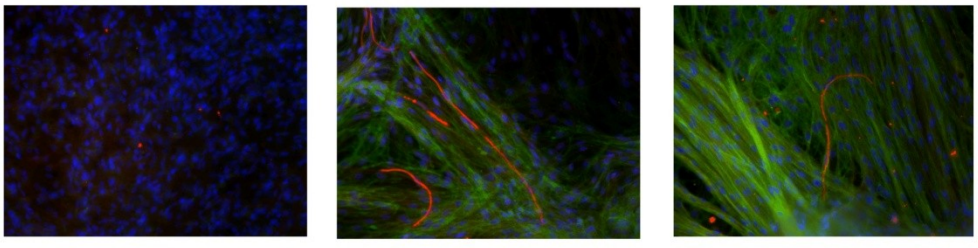
(A) Human E-cadherin (HEcad) and N-cadherin (HNcad) overexpressing confirmed retro virus infected Schwann cells and control were plated onto purified superior cervical ganglion neurons (SCG) and placed under myelinating conditions for 51 days by treatment with ascorbic acid. Myelin ratio was calculated compared to NEO. HE-cadherin and HN-cadherin induces myelination even in the lower levels of NRG1 type III present on the SCG segments. Upon analysis by one-way ANOVA, it was found to be statistically significant (N=5) ($P<.001$). **(B)** NEO, HE-cadherin, HN-cadherin cocultures were fixed and stained for DAPI to visualize nuclei, MBP (red) for myelin segments and either HE-cadherin or HN-cadherin (green) to identify Schwann cell expressing respective proteins. Only DAPI staining is observed in NEO, since neither HE-cadherin or HN-cadherin is present in these cocultures. HE-cadherin and HN-cadherin cocultures have DAPI, MBP and cadherin specific staining, with HE-cadherin having more segments present than HN-cadherin

Figure 21 Overexpression of cadherin(s) in Schwann Cells induces myelination in SCG/SC cocultures

21A



21B



NEO **HEcad** **HNcad**

Aim#4

Uncoupling of β-catenin affects myelination and proliferation

Generation and verification of Human E-cadherin with mutant β-catenin binding site

In the previous aims of this research, cadherins were shown to have an effect on myelination. Briefly, knockdown of E-cadherin caused a defect in myelination with DRG neurons, and ectopic expression of E-cadherin showed a proliferation independent pro-myelinating effect. Taken together, these findings lead us to question what mechanism might be involved in these observations. Is it possible that upon formation of adherence junctions, cytoplasmic binding of an accessory protein, such as β-catenin may induce these effects?

In a previous study, it was suggested that E-cadherin binding to β-catenin is a regulatory function utilized by cells to prevent transactivation by nuclear translocation of β-catenin and binding to TCF/Lef to initiate transcriptional activity (Orsulic et al., 1999). Interestingly, Gess et al. (2008) showed that knock-down of β-catenin, a cytoplasmic binding partner of both E-cadherin and N-cadherin, causes a defect in Schwann cell proliferation both in Schwann cell monocultures and Schwann cells associated with DRG axons (Gess et al., 2008). These findings lead to the question of whether β-catenin binding to E-cadherin may be a plausible mechanism through which E-cadherin might relay its effects.

To characterize the function of β-catenin decoupling to E-cadherin, a retrovirus carrying a construct for human E-cadherin with a mutant β-catenin (HEcadΔCB) binding site were generated. Schwann cells were infected and the expression was verified by

Western blot, as seen in figure 22A. An immunoblot using antibodies against HE-cadherin verified that lysates prepared from infected and selected Schwann cells express the human E-cadherin isoform (120 kDa).

With the successfully infected Schwann cells expressing the human E-cadherin isoform, next we confirmed that β-catenin was not binding to the mutant E-cadherin. In order to verify this mutation, a co-immunoprecipitation with antibodies against human E-cadherin and β-catenin demonstrated that E-cadherin does not associate with β-catenin in the HEcadΔCB Schwann cell lysates. A similar co-immunoprecipitation was performed using lysates prepared from Neo Schwann cells, which did verify association of E-cadherin to β-catenin (figure 22B). Following the successful generation of Schwann cells expressing HEcadΔCB, the next goal was to characterize this mutation with regards to proliferation and myelination.

Uncoupling of β-catenin from E-cadherin increases proliferation

The next question was to identify if the deletion of the β-catenin binding site on E-cadherin affects Schwann cell proliferation. To identify if β-catenin uncoupled to E-cadherin affects proliferation in Schwann cells, HECADΔCB Schwann cells construct were subjected to proliferation assays. HECADΔCB Schwann cells, along with HE-cadherin, HN-cadherin and NEO control Schwann cells were plated onto poly-L-lysine coverslips. Cells were treated with GGF (50ng/mL) and control cultures were left untreated. Sixteen to eighteen hours following treatment, both control and GGF treated cultures were pulsed with BrdU for eighteen to twenty-four hours, and then analysized

by immunocytochemistry using antibodies against BrdU. As expected, all growth factor control cultures had few Schwann cell nuclei that were BrdU positive. In comparison, growth factor treated Schwann cells cultures had an increase in BrdU positive nuclei. Upon examination, NEO Schwann cells nuclei were nearly 60% positive for BrdU staining; in comparison, HE-cadherin Schwann cell cultures had nearly 50% of their nuclei positive for BrdU staining. Lastly, HN-cadherin Schwann cell cultures had approximately 40% of their nuclei positive for BrdU staining, but interestingly, HECADΔCB had more BrdU positive Schwann cells nuclei compared to the control (Perego et al.) (figure 23A) (N=2, P<=.001).

Previously, a proliferation assay was performed using Schwann cells with HE-cadherin, HN-cadherin, and NEO control cells, associated with DRG neurons; this identified that HE-cadherin and HN-cadherin did not have a significantly higher rate of proliferation as compared to NEO Schwann cells (figure 15); therefore the next question was would a mutation in the β-catenin binding site alter the rate of proliferation of Schwann cells in association with DRG axons? To assess the effect on proliferation due to DRG neurons association with HECADΔCB Schwann cells, in addition to HE-cadherin and HN-cadherin Schwann cells, as well as NEO controls were seeded onto purified DRG neuron axons and allowed to align on to the axons followed by a pulse of BrdU for eighteen to twenty-four hours. Cocultures were again examined by immunocytochemical analysis with antibodies against BrdU. Upon examination, 60% of NEO Schwann cells/DRG cocultures Schwann cells were positive for BrdU immunostaining; close to 70% of HE-cadherin/DRG cocultures Schwann cells were

positive for BrdU immunostaining; nearly 50% of HN-cadherin/DRG coculture Schwann cells were positive for BrdU immunostaining, but interestingly, cocultures with HECADΔCB Schwann cells had nearly 80% of their Schwann cells positive for BrdU immunostaining (figure 23B) (N=2, P<=.001). Taken together, these two results suggest that uncoupling of β-catenin with E-cadherin has a positive effect on proliferation, and that it is irrespective of soluble GGF treatment or contact with DRG axons.

Uncoupling of β-catenin from E-cadherin inhibits myelination

In the previous myelination assays on purified DRG neurons, HE-cadherin cocultures had a statistically significant increases in myelination compared to Neo controls (figure 15). To determine the effect on myelination with HECADΔCB Schwann cells associated with DRG neurons, HECADΔCB and NEO Schwann cells were plated onto purified DRG neurons. Ten days following initiation of myelination, immunocyotochemical analysis with anti MBP antibodies was performed. Quantification of MBP positive segments identified that HECADΔCB had a significantly lower number of myelin segments compared to NEO and HE-cadherin, graphically shown in figure 24A (N=4, P = <0.001). This result suggests that decoupling of β-catenin with E-cadherin has a negative effect on Schwann cell myelination on DRG neurons.

Lastly, HECADΔCB Schwann cells were seeded onto purified SCG neurons to determine the effect of this phenotype on the small diameter, normally non-myelinating SCG neurons. Following 51days under myelinating conditions by treatment with ascorbic acid, cocultures were processed for immunocytochemical analysis to identify

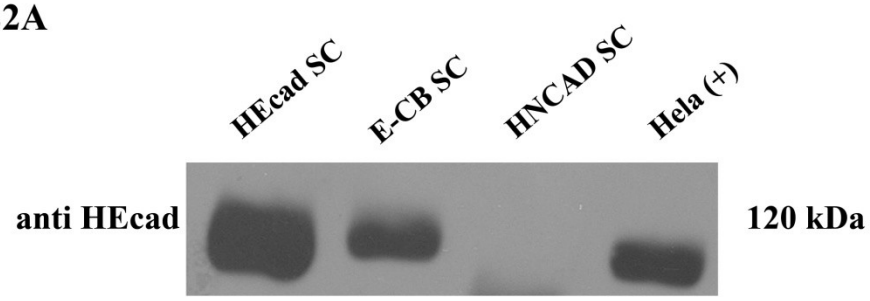
MBP segments with anti-MBP. Once again, myelin segments were observed with HECADΔCB cocultures, but upon quantification of the MBP positive segments in the cocultures, it was identified that there was a statistically significant increase in the number of segments present compared to NEO and HE-cadherin (N=5, P<=.001) (figure 24B). In summary, mutation of β-catenin binding site of E-cadherin has a negative effect on DRG neurons, but interestingly, there is a pro-myelinating effect on SCG neurons.

Figure 22. Generation of Schwann cells expressing human E-cadherin with mutant β-catenin binding site.

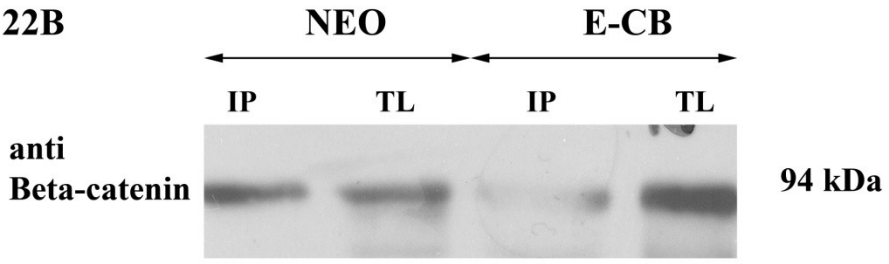
(A) Schwann cells infected with retro viruses to induce overexpression of HE-cadherin with mutant β-catenin binding site (E-CB) were probed by immunoblot analysis using antibodies against the extracellular region of the HE-cadherin protein. Lysates were collected from HE-cadherin overexpressing Schwann cells, HN-cadherin overexpressing Schwann cells (used as a negative control for the HE-cadherin antibody), HECADΔCB expressing cell and Hela cells were used as a positive control for the antibody. As expected, all cell lysates from Schwann cells expressing the HE-cadherin protein were identified, while the negative control of HN-cadherin was empty, verifying that the Schwann cells were accurately expressing the HE-cadherin protein (50µg/Lane). **(B)** Immunoprecipitation (IP) further identified that HECADΔCBSC were also not associating with the HE-cadherin protein expressed, though still present in the total lysate (TL) lane, but conversely, NEO showed association and β-catenin as shown by the 92 kDa band(500 µg/IP).

Figure 22 Generation of Schwann Cells expressing human E-cadherin with mutant beta-catenin binding site.

22A



22B



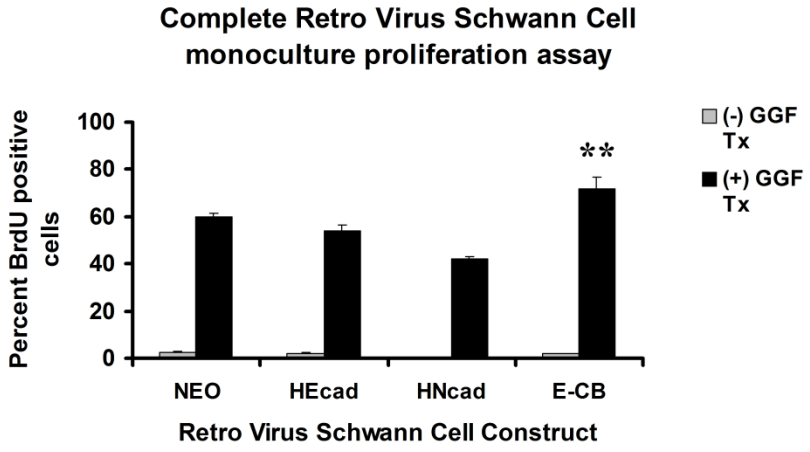
IP: Human E-cadherin
WB: Beta-catenin

Figure 23. Human E-cadherin with β-catenin binding mutation has increased proliferation in response to growth factor and axon alignment.

Similar to the HE-cadherin and HN-cadherin proliferation experiments, HECADΔCB verified Schwann cells were treated with growth factor or seeded onto purified DRG neurons; cells or cocultures were then pulsed with BrdU for 18-24 hrs and immunocytochemistry was utilized to observe the number of cells in S-phase of the cell cycle. Upon quantification, it was observed that HECADΔCBSC proliferated in both conditions at statistically significant levels compared to NEO control Schwann cells, but not compared to HE-cadherin or HN-cadherin Schwann cells (N=3) (**,P = <0.001).

Figure 23. Human E-cadherin with beta-catenin binding mutation has increased proliferation in response to growth factor treatment and axonal alignment

23A



23B

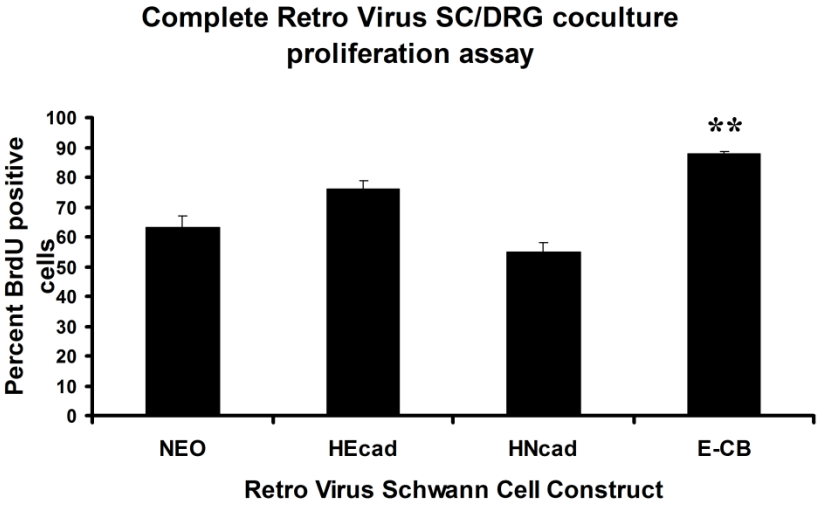
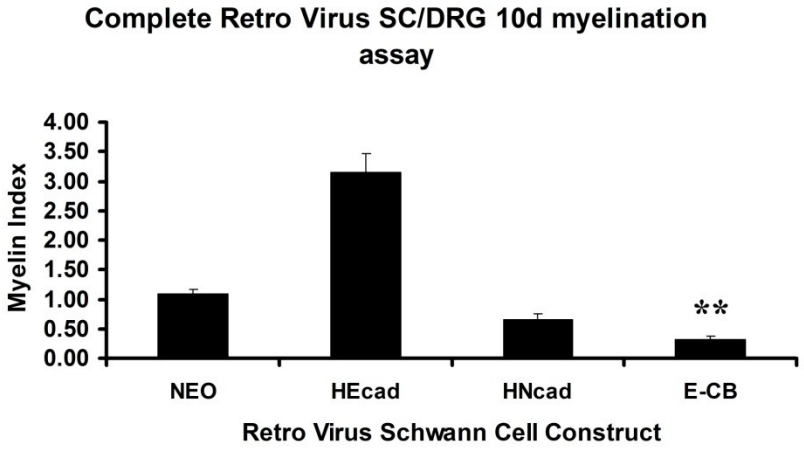


Figure 24 . **Human E-cadherin with β-catenin binding mutation induces myelination in SCG/**Schwann cell **cocultures**

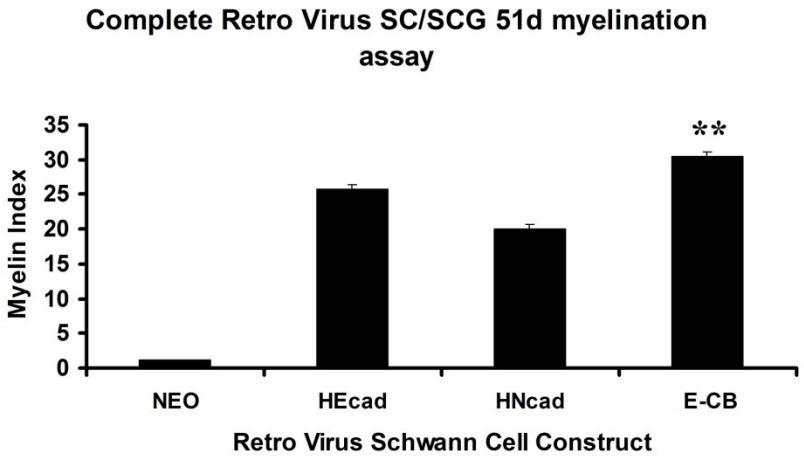
Similar to previous experiments, HECADΔCB (E-CB) verified Schwann cells were seeded onto purified SCG or DRG neurons and treated with ascorbic acid to induce myelination. As seen in graph **A**, HECADΔCB seems to cause a defect in myelination in comparison to the other conditions. While in graph **B**, an adverse observation is seen, HECADΔCB , similarly to HE-cadherin and HN-cadherin expressing cells, induces statistically significant myelination, in the SCG model, when compared to all the other conditions (NEO, HE-cadherin, and HN-cadherin) (N=5) (P = <0.001) (N=4) (**,P = <0.001).

Figure 24 Human E-cadherin with beta catenin binding mutation induces myelination in SCG/SC cocultures

24A



24B



Discussion

E-cadherin is expressed at the onset of myelination: a possible role of cadherin switch in Schwann cell myelination

The experiments described in this thesis identify another potential pathway involved in PNS myelination, specifically the role of E-cadherin in the initiation of myelination. This process began by first characterizing the developmental expression kinetics of the E-cadherin, N-cadherin, ErbB2, and the activation kinetics of ErbB2 during the course of myelination *in vivo*. As shown in this thesis, developmentally from post-natal day 1 to day 28 E-cadherin levels increase, while, in contrast, N-cadherin and ErbB2 levels have a reciprocal expression. The phosphorylated active form, p-ErbB2 was identified to have activation that was low early in development, peaking at P7, followed by a subsequent drop to P28. This data set has provided a clear kinetic pattern of expression for key adhesion and signaling molecules during PNS myelination.

One possible explanation of the developmental increase in E-cadherin could be due to myelination itself. As the Schwann cell plasma membrane concentrically wrap around the axon in myelination, autotypic adhesion junctions appear and these adherence junctions are predominantly formed by E-cadherin (Fannon et al., 1995). With the increase in myelination, there is an increase in the formation of these structures, resulting in an increase in E-cadherin expression.

In support of Fannon et al. data, Menichella (2001) previously described the *in vivo* expression kinetics of E-cadherin in rat sciatic nerves where they reported E-cadherin expression to begin at postnatal day 2; with a rise in expression to day 15, followed by a slight decrease to day 30, suggesting that E-cadherin functions at early

stages of myelination. Interestingly, our data parallel the Menichella findings of the E-cadherin expression kinetics, though in comparison, ours begins at postnatal day 1 and provide a more precise detail of the incremental increase in expression observed from day 3, 5, 7, 14, 21, and 28.

Another plausible rationale for the developmental expression of E-cadherin may be related to a finding that E-cadherin down regulates RTK activation (Lampugnani et al., 2006; Qian et al., 2004). Previously, Qian et al. (2004) identified that E-cadherin mediated adhesion inhibits RTK activity via an interaction between its extracellular domains interaction and RTK, which causes a decrease in receptor mobility as well as ligand-affinity. This interaction may subsequently lead to preventing proliferation and possibly promoting differentiation. In the Schwann cell, the RTK ErbB2 functions early in myelination to initiate the Schwann cell to proliferate followed by a cue to myelinate. Our data showed that as E-cadherin expression increased with myelination, while the activity state of ErbB2 ranges from low, early in myelination (P1-3), followed by a peak (P5-7) and then a decrease. It may be possible that E-cadherin may potentially be acting to regulate this state. E-cadherin, through its association with ErbB2 maybe inhibiting the activation of ErbB2, as shown by our lab data in figure 13B, and subsequently, also down regulating key down stream signaling pathways, such as PI3-K and ERK.

We have previously shown that E-cadherin expression is mediated by axonal components via up-regulation of the cAMP-PKA pathway (Crawford et al., 2008). Therefore, as the Schwann cell wraps around the axon, this contact also induces E-cadherin expression. Interestingly, this mechanism, which induces E-cadherin

expression, down-regulates N-cadherin (Crawford et al., 2008). Previous reports identified that N-cadherin is expressed with migration in Schwann cells, meanwhile E-cadherin is associated with differentiated myelinating Schwann cells (Crawford et al., 2008; Fannon et al., 1995; Wanner et al., 2006). This switch in expression of the cadherins might facilitate the differentiation of the Schwann cell. Initially, Schwann cells utilize N-cadherin to strengthen glia-glial interactions, and it may be necessary to weaken these contacts, via down-regulation of N-cadherin for the correct Schwann cell-axon contacts. Following this decrease in N-cadherin, the increase in E-cadherin may be necessary to aid Schwann cell differentiation, first by organizing and then polarizing the Schwann cell, similar to that observed in other cell types.

The reciprocal expression kinetics of E-cadherin and N-cadherin may have similarites to that seen during epithelial-mesenchymal transition, which typically occurs at critical phases in development, as in the case of neural crest cells. Previous reports identified that neural crest cells develop from a small portion of the dorsal neural tube (Huang and Saint-Jeannet, 2004; McKeown et al., 2005). Soon after epithelial-mesenchymal transition by the neural crest cells, there is a migration away from the neural tube followed by differentiation into various cell types, such as melanocytes, smooth muscle, bones and peripheral nerves and glia (Huang and Saint-Jeannet, 2004). A hallmark of this period is the loss of E-cadherin expression and an increase in N-cadherin expression, (Lee et al., 2006). As observed in epithelial cells, early contacts are mediated by E-cadherin, which cluster into small junctional complexes that expand to establish stable adherens junctions, requiring, E-cadherin (Imhof et al., 1983).

Previous studies and our data suggest that N-cadherin functions primarily during the early development of the Schwann cell lineage, prior to myelination, specifically for formation of Schwann cell-Schwann cell junctions and Schwann cell process growth in terms of alignment with axons (Wanner and Wood, 2002). N-cadherin expression was shown to be restricted to axon growth and regulated by NRG1 expression, which also decreases its expression during development (Wanner et al., 2006; Wanner and Wood, 2002). Additionally, N-cadherin has also been reported by Corell et al. (2010) to be primarily in Schwann cell precursors, early in development (Corell et al., 2010). This study also observed, that with myelination, N-cadherin expression decreases as Schwann cell precursors differentiate to Schwann cells (Corell et al., 2010). Furthermore, this report states that N-cadherin found exclusively in non-myelinating Schwann cells and suggests that N-cadherin functions mostly primarily in the adhesion between glial cells during post-natal development (Corell et al., 2010). In my study, I show a similar decrease in expression with myelination which corroborates the previous findings. The lack of association of N-cadherin with ErbB2 observed in our data may be related to the expression levels of N-cadherin present in the neuron and not in the Schwann cell itself, where ErbB2 is solely observed. Another possible explanation for these two molecules not demonstrating an association may be that E-cadherin is in such an abundant level, it may out compete N-cadherin for the association with ErbB2.

The decrease in N-cadherin observed developmentally may imply that its functions are utilized early in myelination versus later. N-cadherin has been identified to act as a positive regulator of other RTK, more specifically FGF receptors (FGFr)

(Suyama et al., 2002). N-cadherin in the presence of the ligand, FGF-2, stabilizes FGFr at the surface, causing sustained activation of the MAPK-ERK pathway; MAPK-ERK has been identified in Schwann cell development where it functions in differentiation (Harrisingh and Lloyd, 2004; Harrisingh et al., 2004). N-cadherin dependant activation of FGFr was also found to generate an anti-apoptotic signal (Erez et al., 2004). By using an endothelial cell line, plated at high density and blocking N-cadherin function by treatment with an N-cadherin specific antagonist, large numbers of cells underwent apoptosis (Erez et al., 2004). This increase apoptotic effect could also be rescued by administration of exogenous FGF ligand (Erez et al., 2004). With N-cadherin levels high in early stages of myelination, it is plausible that N-cadherin could be promoting a similar anit-apoptotic function in the Schwann cell via the NRG1/ErbB3/ErbB2 pathway.

With the developmental association of E-cadherin to both the pan and active forms of ErbB2 observed, the question of whether this association may function in myelination lingered. I conducted a loss-of-function and gain-of-function study for E-cadherin during myelination. In the loss of function study, we observed a clear knockdown of E-cadherin expression in the Schwann cells, even with treatment with Forskolin which induced E-cadherin expression similar to axonal contact. When these E-cadherin knock-down Schwann cells were utilized in the myelination experiments, fewer myelin segments were observed, compared to control Schwann cells. This suggested that E-cadherin does function in myelin programming. It would be of interest to investigate which downstream signaling pathways related to myelination are affected to further characterize the effect of E-cadherin knockdown in relation to myelination.

Furthermore, when Schwann cells with deficient E-cadherin expression were treated with a soluble neuregulin 1 and these cells showed reduced ErbB2, AKT and ERK activation upon treatment (data not shown, unpublished Kim lab data). This *in vitro* loss of function data suggest that knockdown of E-cadherin not only affects NRG1/ErbB2 reception activation, but also pathways downstream involved in myelination.

To additionally support the *in vitro* data, we utilized E-cadherin ablated transgenic mice to show a delay in myelination. Examination of mice sciatic nerves at P2 to P3 showed a significant number of axons remained in a pro-myelinating stage compared to controls, verified by sustained expression of Krox20 and P0 (Kim lab, data unpublished). As expected, a significant percentage of axons were not myelinated (Kim lab, data unpublished). Electron-microscopy was used to further characterize the myelination defect in the E-cadherin transgenic mouse dorsal roots at the P3 stage and revealed that in mutant nerves, Schwann cells had formed a 1:1 relationship with an axon, but these remained unmyelinated (Kim lab, unpublished data). Taken together, the loss of function of E-cadherin does negatively affect myelination *in vitro* and it appears to delay it *in vivo.*

The defect in myelination observed with the loss of E-cadherin expression is different from previous findings by Young et al. (2002). Young's study demonstrated that E-cadherin did not have a significant function in myelination through use of a Cre-lox deletion of E-cadherin, under the control of a Krox20 promoter. They observed that E-cadherin ablation did not disrupt the onset of myelination; Schwann cells aligned onto axons in a 1:1 ratio and did subsequently myelinate. This study did observe that E-

cadherin is necessary for the proper formation of the outer mesaxons (Young et al., 2002). They further stated the E-cadherin deficiency did not cause significant demyelination up to the age of 15 months (Young et al., 2002). By contrast, our *in vitro* coculture results demonstrated that knockdown of E-cadherin had a defect in myelination. The contradiction in these two results may be explained by the method of knockdown. While clearly, Young's group did knock-down E-cadherin protein by use of the Cre/LoxP system, but the time of ablation may have been developmentally too late. In our study, E-cadherin knockdown was confirmed, following which, these Schwann cells were utilized in myelination experiments. Also, due to the stability of the lentivirus infection, any axonal induced E-cadherin expression would also be dampened. Therefore, no *de novo* E-cadherin protein would be present in the cocultures. A comparable *in vivo* experiment to the Young work might be to use a promoter whose expression is at a earlier developmental time point, such as Desert Hedge Hog, whose expression is observed in Schwann cell precursor cells, as well as in Schwann cells to at least postnatal day 10 (MIRSKY et al., 1999).

The defect in myelination observed with the loss of E-cadherin may also be due to a loss of polarization early in the Schwann cell myelination program. Polarity of the Schwann cell is integral to initiate Schwann cell myelination (Chan et al., 2006) and interestingly, E-cadherin has been previously been identified to mediate cell-cell adhesion polarization (Desai et al., 2009). Chan demonstrated that the role of Par protein Par3 in glial cells polarity is vital to initiate myelination; Par3 accumulates at the axon-glial contact sites and recruit and associates with $p75^{NTR}$ and thus promotes myelination.

Recent unpublished data from our lab identified that Par3 also associates with E-cadherin *in vivo* shown by co-immunoprecipitation experiments using development rat sciatic nerve lysates. If E-cadherin is in complex with Par3, this suggests that it may play a role in Par3's function in the crucial Schwann cell polarization step. It would be of interest to identify if Par3 expression or localization is altered with the knockdown of E-cadherin.

Another role of E-cadherin may be regulating cytoskeletal activity through Rac1 and cdc42 activity. Using dominant negative mutants of these members of Rho family of small GTPases, it has been shown that Rac1 and cdc42 activation are rapidly induced in E-cadherin containing cells, following E-cadherin engagement (Betson et al., 2002). Rac1 has been shown to be required for the extension of the Schwann cell radial process in culture and for axonal sorting (Benninger et al., 2007). Rac1 activation has also been mediated by cadherin adhesion, through a PI-3 kinase activation mechanism (Kovacs et al., 2002b). Kovacs group also identified that cadherin-mediated adhesion can activate Rac1 in PI-3 kinase independent mechanism, through a complicated association with receptor tyrosine kinases (Kovacs et al., 2002a). Cdc42 is also a key player in the control of cell orientation, where it is involved in controlling baso-lateral sorting (Folsch, 2008). Taken together, down regulation of E-cadherin could hamper crucial developmental processes involved in myelination.

E-cadherin functions as pro-myelinating signal

In support of the loss of E-caherin function work, the next question was what would the effect of ectopically expressing E-cadherin be on myelination? In this portion

of the thesis, HE-cadherin was ectopically expressed in Schwann cells and these cells were utilized for myelination studies with DRG neurons. As the data showed, it caused an enhancement of myelination in the DRG cocultures. Interestingly, this enhancement in myelination was not in conjunction with an increase in Schwann cell numbers, as evidenced by the proliferation data. Therefore this positive effect functions via an undetermined mechanism. One plausible pathway may be via an alternative pathway to activate ErbB2 and downstream signaling related to myelination. This theory has been tested by Kim lab members by the use of E-cadherin overexpression Schwann cells and has shown an increase in ErbB2 activation in response to neuregulin 1 treatment (data not published). Additionally, there was also an increase in Rac, and AKT activity observed; these signaling pathways related to myelination versus proliferation (data not publish).

Ectopic expression of HE-cadherin also had a positive effect on myelination with SCG neurons. Again, these neurons are smaller in diameter, with lower levels of neuregulin 1 type III present on their membranes. Therefore, they do not myelinate. As seen in our data, the lack of neuregulin 1 type III needed to myelinate the axon was successfully overcome with overexpression of E-cadherin. A mechanism that may explain this result could be through the Rac1 cdc42 activation that often accompanies E-cadherin adherence junction formation. With the overexpression of HE-cadherin, there is an increase in Rac1 activity, which allows for the cells to initiate the myelination program. Furthermore, E-cadherin has been shown to activate RTKs in a ligand independent manner previously described by Pece and Gutkind (2000), where cadherin

clustering can recruit RTK family members and lead to an increase in MAPK activity (Pece and Gutkind, 2000).

Interestingly, Taveggia (2005) was also able to overcome this non-myelinating phenotype in the SCG neurons via transfection of a construct that overexpressed WHAT?? (Taveggia et al., 2005). While we do see a similar induction of myelination, a more precise comparison needs to be performed. It may be of interest to repeat Taveggia's study and identify if there also is an induction of E-cadherin. Upon examination of E-cadherin expression in Schwann cells associated with DRG versus SCG neurons, it was identified that there was a lower level of E-cadherin expression observed in the Schwann cell/SCG cocultures; this, in conjunction with lower levels of neuregulin 1 type III might be a mechanism that does not promote a myelinating phenotype in the SCG neurons. Interestingly, transgenic mice studies with knockdown of axonal neuregulin 1 type III showed a similar decrease in E-cadherin expression. Although treatment with soluable CRD-Nrg1 partially rescued E-cadherin expression, the Schwann cells aligned to neurons of a heterozygous mouse, which has expression of neuregulin 1 type that is lower and not completly ablated (data not published).

While overexpressing HE-cadherin had induced a similar effect in myelination as the Taveggia study, this led us to focus on E-cadherin's downstream signaling. Gess (2008) demonstrated that loss of β-catenin, the intracellular binding partner of E-cadherin, expression by siRNA decreased proliferation in both mono-cultures and co-cultures. An interesting question proposed dealt with loss of β-catenin binding to overexpression of E-cadherin. β-catenin by itself has proliferative functions (Chenn and

Walsh, 2002, 2003; Hirabayashi et al., 2004; Machon et al., 2003; Otero et al., 2004), but for myelination, proliferation has to cease and differentiation to a myelination state needs to initiate. Therefore, it is interesting to note that Schwann cells expressing the mutant β-catenin binding site had a statistically significant increase in both proliferation and myelination. More specifically, HECADΔCBSC had more myelin segments compared to HE-cadherin or HN-cadherin in the SCG/Schwann cell model and HECADΔCB demonstrated more mitotic activity compared to the control NEO cell and HE-cadherin and HN-cadherin cells. Intriguingly, the increase in myelination and proliferation was not recapitulated when the same HECADΔCBSC were seeded on the DRG neurons, where there was a statistically significantly lower level of myelination observed. One potential theory is that because these cells maybe in a state of continual and active proliferative cycle, they are not able to initiate the myelination programming, but that needs further investigation.

Future Directions

With β-catenin having a dramatic effect on this system, it would be interesting to further investigate the cross-talk between β-catenin and ErbB2 and its signaling pathways. In other cell types, a functional association was observed where β-catenin was the link between cadherins and RTK signaling. Since we have shown an increase in both myelination and proliferation with the β-catenin binding mutants, it may be of interest to identify what specific effect the free β-catenin has on the two major signaling pathways downstream of NRG1/ErbB2/ErbB3 activation.

Additionally, it would be interesting to observe what is occurring to the β-catenin that is now not able to bind to E-cadherin or N-cadherin. Previous reports have identified that β-catenin has one of three major fates: it remains bound between cadherins and α-catenins at adherence junctions; it is utilized in the canonical WNT signaling pathway leading to binding with TCF/Lef to act as an transcription factor and lastly, it is degraded.

If β-catenin in the Schwann cell model is working through the WNT signaling pathway to activate genes related to myelination and proliferation of the Schwann cell, it would be interesting to identify which genes are being regulated. One would surmise that genes involved with differentiation would be the first targets of β-catenin. Upon their identification, one could perform characterization of these genes and functional studies related to the Schwann cell model would be intersting.

While Gess et al. did perform a loss of function study with β-catenin in Schwann cells, a gain of function study was not completed. Therefore, an appealing study would identify the effects of overexpression of β-catenin in the Schwann cell model and further

characterize the effects on proliferation and myelination in the SCG and DRG neuron models.

With the HE-cadherin overexpressing cells, it may be of significance to observe downstream signaling. Curiously, since overexpression has shown a positive functional myelination phenotype, it would be of value to characterize the precise pathway of this phenomenon.

In accordance with the *in vitro* E-cadherin knock-down, use of a more efficient knock-down of both E-cadherin and N-cadherin may allow better elucidation of a functional role for these competing, yet important molecules in myelination. Use of a Cre/Loxp strategy to have tissue and time specific knockdown of these proteins will further help clarify their roles.

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