Focal adhesion kinase, a major regulator of oligodendrocyte morphological maturation and myelination

Audrey Lafrenaye
Virginia Commonwealth University

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Focal adhesion kinase, a major regulator of oligodendrocyte morphological maturation and myelination

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

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<tbody>
<tr>
<td>Akt</td>
<td>anti-apoptotic serine threonine kinase</td>
</tr>
<tr>
<td>Arp 2/3</td>
<td>actin related protein 2/3</td>
</tr>
<tr>
<td>ASAP1</td>
<td>Arf GTPase-activation protein GAP 1</td>
</tr>
<tr>
<td>CNP</td>
<td>2', 3'-cyclic nucleotide 3'-phosphodiesterase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>E</td>
<td>Early</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FAT</td>
<td>Focal adhesion targeting</td>
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<td>FERM</td>
<td>FAK N-terminal ezrin/radixin/moesin homology</td>
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<td>Fn</td>
<td>Fibronectin</td>
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<td>GAP</td>
<td>GTPase-activation protein</td>
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<td>GEF</td>
<td>Guanine exchange factor</td>
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<td>Graf</td>
<td>GTPase regulator associated with FAK</td>
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<tr>
<td>ILK</td>
<td>integrin linked kinase</td>
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<tr>
<td>L</td>
<td>Late</td>
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<tr>
<td>Ln</td>
<td>Laminin-2</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>N-WASP</td>
<td>neural-Wiskott Aldrich Syndrome protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal</td>
</tr>
<tr>
<td>PDGFαR</td>
<td>Platelet-derived growth factor α receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphotidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
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<tr>
<td>PRD</td>
<td>Proline rich domain</td>
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<tr>
<td>PRR</td>
<td>Proline rich region</td>
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<tr>
<td>PTK2</td>
<td>Protein tyrosine kinase 2</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
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<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
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<tr>
<td>SH3</td>
<td>Src homology 3 domain</td>
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<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
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Abstract

FOCAL ADHESION KINASE, A MAJOR REGULATOR OF OLIGODENDROCYTE MORPHOLOGICAL MATURATION AND MYELINATION

By Audrey D. Lafrenaye

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Major Director: Babette Fuss, Ph.D., Associate Professor, Department of Anatomy and Neurobiology

The formation of the myelin sheath is a crucial step during development because it enables fast and efficient propagation of signals within the limited space of the mammalian central nervous system. During the process of myelination, oligodendrocytes actively interact with the extracellular matrix (ECM). These interactions are considered crucial for proper and timely completion of the myelin sheath. However, the exact regulatory circuits involved in the signaling events that occur between the ECM and oligodendrocytes are currently not fully understood. Therefore, in this dissertation we investigated the role of a known integrator of cell-ECM signaling, namely, focal adhesion kinase (FAK), during oligodendrocyte morphological maturation in vivo and in vitro.

Conditional and inducible FAK-knockout mice (Fak\textsuperscript{flox/flox}, PLP/CreERT\textsuperscript{T} mice) were generated to observe the effect of FAK loss on myelination in vivo. The role of FAK during post-migratory premyelinating oligodendrocyte morphological maturation was explored in vitro using primary rat oligodendrocyte
cultures in combination with siRNA or inhibitor treatment.

When inducing FAK knockout just prior to and during active myelination *in vivo*, we observed a significant reduction in the number of myelinated fibers following initial myelination. In addition, our data revealed a decreased number of primary processes extending from oligodendrocyte cell bodies at this stage of development under induction of FAK knockout. In contrast, myelination appeared normal on postnatal day 28.

Our *in vitro* data demonstrated that reduction of FAK expression or activity affected the oligodendrocyte process network maturation in a way that is unique to the ECM substrate present. More specifically, our data suggest that FAK restrains the expansion of the oligodendrocyte process network in the presence of fibronectin and promotes expansion of the oligodendrocyte process network in the presence of laminin-2. We showed that the FAK-mediated restriction of oligodendrocyte morphological maturation is tightly developmentally regulated; being highly active during early stages of post-migratory premyelinating oligodendrocyte morphological maturation but greatly diminished at the later stages of oligodendrocyte development. Thus, our data suggest that FAK controls the efficiency and timing of CNS myelination during its initial stages, at least in part, by regulating oligodendrocyte process network morphological maturation.
Chapter 1

An introduction to oligodendrocyte biology and focal adhesion kinase

The oligodendrocyte is the myelin producing cell of the central nervous system (CNS), responsible for proper conduction of axonal signals. In certain diseases these cells and the myelin that they produce is targeted leading to a wide range of dysfunctions. Although there are oligodendrocyte progenitor cells (OPCs) found within areas of myelin damage there is limited, if any, remyelination (Nait-Oumesmar et al. 1999, Chang et al. 2002). The process of initial myelination during normal CNS development is not well understood; therefore identifying potential key regulators of normal CNS myelination could lead to promising therapeutic targets in disease states. The following dissertation research focuses on elucidating the role of focal adhesion kinase (FAK), a known integrator of cell-ECM signaling and regulator of cytoskeletal remodeling, during oligodendrocyte morphological maturation and myelination.

Why we need myelin

The primary function of an oligodendrocyte in the CNS is to produce a uniquely lipid-rich myelin sheath that enwraps the axon. Myelin is one of two evolutionary mechanisms utilized in the nervous system to expedite axonal signal transduction over great distances. The first of these mechanisms is the enhancement of axonal diameter in proportion to the distance signals travel, however, if this were the only mechanism utilized the human spinal cord would
be the width of a tree trunk to accommodate the diameter of all the axons
traveling to our extremities. The second evolutionary mechanism is the addition
of the myelin sheath to invest the axons at specific intervals, allowing the speed
of conduction to increase exponentially without a concomitant increase in the
diameter of the axon (Sanders & Whitteridge 1946). In saltatory conduction
myelin functions to drastically increase the resistance to outward current flow,
limiting the area of ion exchange to small unmyelinated sections throughout the
length of the axon, drastically reducing the energy required to propagate the
signal (Kettenmann 2005, Lazzarini 2004).

The myelin sheath functions as it does due to both its high-lipid
composition and the domains that it forms in association with the axonal surface
(Uzman & Rumley 1958, Simons & Trajkovic 2006). The domains formed by the
myelin sheath are: the node of Ranvier, the internode, the paranode, and the
juxtaparanode.

The node of Ranvier is the myelin-free zone of the axonal surface in which
ion exchange is allowed, producing electrical current. This area only constitutes
0.1-0.3% of the total axonal surface and is characterized by high sodium channel
concentration, allowing for ion exchange and subsequent membrane
depolarization (Salzer et al. 2008, Rosenbluth 2009). The formation of the node
is well characterized in the peripheral nervous system, however remains largely
unclear in the CNS. Clustering of the sodium channels on the axonal surface in
the CNS does, however, appear to be regulated by contact with mature
The large area in which the myelin membrane is in direct apposition to the axonal surface is the internode (Salzer et al. 2008). The wraps of myelin at the internode compact with myelin maturation; squeezing the cytoplasm from between the cellular membranes forming distinct regions, an electron dense major dense line and a space between the two extracellular-facing myelin membranes called the intraperiod line (De Robertis et al. 1958, Kettenmann 2005). The axon diameter regulates whether it requires myelination (Camara et al. 2009, Matthews & Duncan 1971). The number of myelin wraps of the internode around the axon is also determined by the diameter of the axon being myelinated (Hildebrand & Waxman 1984, Fraher et al. 1988, Schwab & Schnell 1989).

The areas adjacent to the node of Ranvier were once thought to be one structure called the paranode, however, two domains with very different molecular characteristics have now been identified: the paranode and the juxtaparanode (Kettenmann 2005). The paranode is the region in which the lamella of the myelin sheath terminate on the axonal surface in cytoplasmic-filled arms called paranodal loops. The paranodal loops are uniquely associated with the axonal surface through association of axonal contactin with myelin membrane surface neurofasin 155 forming paranode specific adhesions called transverse bands, which are stabilized by spectrins (Salzer et al. 2008, Simons & Trajkovic 2006).

The second domain, the juxtaparanode, is more proximal to the internode than the paranode. The axonal surface of the juxtaparanode expresses
potassium channels as well as unique adhesion molecules that associate with the myelin membrane (Salzer et al. 2008). The function of the potassium channels within this region is still poorly understood, however, it is hypothesized that the placement of the potassium channels underneath the myelin sheath reduces the amount of energy required to repolarize the neuronal membrane following an action potential and prevents after potentials (Rosenbluth 2009).

Active myelination occurs in four distinct phases 1) axonal recognition, 2) synthesis of myelin components, 3) axonal wrapping, and 4) myelin compaction (Simons & Trajkovic 2006). While the domains of myelin in the CNS are well characterized the process of myelin formation by the oligodendrocyte is still largely unknown. Studies have implicated axonal electrical capability and signals sent from the axonal surface in enhancing the initiation of oligodendrocyte myelination, however, these mechanisms are still poorly understood (Ishibashi et al. 2006, Spiegel & Peles 2006, Simons & Trajkovic 2006).

Multiple Sclerosis (the reason for the research)

The requirement of the myelin sheath for normal nervous system function is evident in diseases in which myelin is disrupted. There are two main types of myelin injury, those that disrupt myelin as a result of another insult (secondary myelin diseases) and those injuries that directly affect myelin and oligodendrocytes in the CNS (primary myelin diseases) (Kettenmann 2005, Lazzarini 2004). The primary myelin disorders are further subdivided into those that disrupt the proper formation of the myelin sheath (dysmyelinating diseases),
such as in the case of leukodystrophies, and diseases which destroy properly formed myelin (demyelinating diseases) (Lazzarini 2004).

Multiple sclerosis (MS), the major primary demyelinating disease of the CNS, affects approximately 400,000 people in the United States, with approximately 200 new cases daily (Multiple Sclerosis Society 2010). The cause of MS remains largely unknown; however, various environmental and genetic factors have been linked to increased prevalence. MS is usually diagnosed in young adults between the ages of 20 to 50 years old and is more common in females; being more than twice as prevalent as in males (Multiple Sclerosis Society 2010). The signs and symptoms of MS vary widely based on the stage of the disease and the area of the CNS most affected.

There are four main types of MS: preclinical, relapsing-remitting, secondary progressive and primary progressive (Fox et al. 2006b). The preclinical stage is characterized as the time prior to onset of symptoms. The majority (85%) of patients diagnosed with MS are in the relapsing-remitting stage of the disease, which is characterized by relapsing symptoms that resolve over time. Of people diagnosed with relapsing-remitting MS half will develop the secondary-progressive stage of the disease within ten years; in which remissions become infrequent and neurological symptoms progressively worsen (Multiple Sclerosis Society 2010). Approximately 15% of MS patients initially present with a form of MS in which there are no remissions and the neurological symptoms continuously worsen: primary progressive MS (Fox et al. 2006b).
There are three main issues to address in MS: the auto-immunological aspect, the loss of myelin and oligodendrocytes, and the axonal damage. Current therapeutic strategies to combat MS are focused on modulating the immune system (Fox et al. 2006b), which has been well characterized in MS. Neuronal damage could be precipitated by the immune response or following the loss of myelin and oligodendrocyte support, however this is still a point of debate within the field (McFarland & Martin 2007, Halfpenny et al. 2002, Geurts & Barkhof 2008, Collin et al. 2007). There are also changes in extracellular matrix (ECM) composition within MS lesions which could be associated with the pathological symptoms (Sobel & Mitchell 1989, Comabella & Martin 2007, Satoh et al. 2009, Cossins et al. 1997, Sobel 1998, van Horssen et al. 2007).

While the immune response is well characterized in MS there is still much that is not well understood regarding oligodendrocyte myelination and there are currently no therapies to enhance remyelination. Premyelinating oligodendrocytes are found in the MS lesion, however, they do not properly develop and there is limited remyelination (Chang et al. 2002, Kuhlmann et al. 2008). Due to the inherent limited capacity for remyelination within MS lesions and the lack of knowledge regarding molecular players required for initial myelin formation, the examination of molecules, such as focal adhesion kinase (FAK), known to regulate morphological remodeling in various cell types could bring us one step closer to enhancing remyelination in multiple sclerosis lesions.
Oligodendrocyte development

To elucidate potential factors that would promote remyelination in disease states we first need to better understand the normal developmental progression of oligodendrocyte differentiation and myelination. Oligodendrocytes in dissociated cell culture develop remarkably similar to in vivo conditions where it has been easier to study their biochemical properties during development (Abney et al. 1981, Miller 1996).

The specification of oligodendrocytes throughout the CNS most likely occurs in waves throughout the later aspect of the third trimester in humans or early postnataly in the rodent (Kessaris et al. 2006). Specification of oligodendrocytes has been most well characterized in caudal regions of the CNS, namely the spinal cord, and is understood to occur similarly within the various regions of the CNS. Oligodendrocytes begin as a constituent of the neuroepithelium in the ventral neural tube under the induction of sonic hedgehog (Shh) (de Castro & Bribian 2005, Nery et al. 2001, Wegner 2008). A small percentage of oligodendrocytes also arise from the dorsal aspect of the neural tube, however the signaling involved in their specification is not well understood, accordingly in this section we will focus on the ventrally derived oligodendrocytes (Miller 2002, Richardson et al. 2006). Shh induces the production of the basic helix-loop-helix transcription factors olig1 and olig2 (Nery et al. 2001, Nicolay et al. 2007).

Motor neurons expressing olig2 also arise from roughly the same area of the neural tube prior to glial specification under the control of shh leading to

In OPCs the transcription factor olig2 is thought to induce the expression of the transcription factor sox10 as well as the platelet-derived growth factor α receptor (PDGFαR) (Figure 1.1 A) (Wegner 2008, Zhou et al. 2000). Olig2 also plays a role in the expression of Nkx2.2, which has been surrounded by some controversy due to the differences in timing of Nkx2.2 expression. The expression of olig2 in association with sox10 and Nkx2.2 has been shown to be required for oligodendrocyte fate specification in both the chick and mouse (Liu et al. 2007).

OPCs have a specific membrane constituent including the A2B5 antigen and PDGFαR, the ligand for which is expressed by both astrocytes and neurons and functions to promote OPC proliferation (Miller 1996, Schnitzer & Schachner 1982, Timsit et al. 1995, Sim et al. 2006, Simons & Trajkovic 2006). These progenitors migrate vast distances from their periventricular origin to their site of action. Once OPCs reach their final destinations their migratory capabilities drop drastically and they differentiate into O4 antigen expressing post-migratory oligodendrocytes, which also transiently express the A2B5 antigen early during the transition from an OPC (Figure 1.1 B) (Sommer & Schachner 1981, Miller 1996). Post-migratory O4+ oligodendrocytes are morphologically distinct from OPCs in that O4+ oligodendrocytes have multiple processes, as opposed to the more simple bipolar morphology of OPCs. O4+ post-migratory oligodendrocytes
send out a multitude of processes in search of their axonal targets regulated, in part, by extracellular cues (Fox et al. 2006a).

The O4+ post-migratory oligodendrocytes keep their proliferative abilities until they further differentiate into O1+ immature oligodendrocytes, which lack the ability to proliferate but continue to mature morphologically (Figure 1.1 C) (Miller 1996). During development there is an overproduction of OPCs which are pruned during this developmental stage based on the number and area of axons available to be myelinated (Barres et al. 1992, Miller 2002).

With the expression of the appropriate transcription factors, O1+ oligodendrocytes further differentiate into myelinating oligodendrocytes, which express myelin basic protein (MBP) and proteolipid protein (PLP) (Figure 1.1 D) (Miller 1996, Sim et al. 2006). The myelinating oligodendrocytes will again remodel their process morphology extensively, producing myelin membrane to wrap axonal segments (Bauer et al. 2009, Kettenmann 2005).

Cytoskeletal rearrangement during oligodendrocyte development

The oligodendrocyte cytoskeleton is composed of microtubules and actin microfilaments. The microtubular network is formed by α and β subunits that dimerize and associate to form the characteristic cylindrical shape of a microtubule and these individual microtubules associate to form a complex microtubule cytoskeleton. The formation of microfilaments requires nucleation of globular actin monomers into filamentous actin polymers which then associate to form a comprehensive microfilament cytoskeleton. In general, process formation
and extension occurs first by filopodia formation followed by widening of the narrow filopodial actin bundles to form lamellipodia and finally, the microfilament structure is stabilized by invasion of polymerizing microtubules.

As discussed above, during development oligodendrocytes undergo vast morphological remodeling to transition from an OPC, with a limited process network, to a highly complex immature oligodendrocyte, and finally to a fully developed myelinating oligodendrocyte. These morphological transformations are largely regulated by the cytoskeleton and molecules that mediate cytoskeletal remodeling.

Tubulin destabilizing factors have been implicated in process formation and outgrowth during the extensive morphological changes of the oligodendrocyte process network throughout development (Bauer et al. 2009, Southwood et al. 2007, Richter-Landsberg 2008). While the microtubule cytoskeleton has been shown to be involved in oligodendrocyte process formation, the actin cytoskeleton has been shown to be of great significance for initial cytoskeletal remodeling leading to oligodendrocyte process morphological remodeling (Song et al. 2001).

The most studied molecules involved in directly regulating the actin cytoskeleton in oligodendrocytes are the Rho family GTPases RhoA, Cdc42 and Rac1 (Bacon et al. 2007, Czopka et al. 2009). These molecules are found particularly associated with branch points and the tips of oligodendrocyte processes, areas in which actin is also enriched (Song et al. 2001).
Signals, such as netrin-1, regulate the actin cytoskeleton in opposing ways depending on the stage of oligodendrocyte development. Early during development netrin-1 signaling prevents OPC differentiation and process extension through activation of RhoA, which promotes process retraction (Rajasekharan et al. 2010). Later in oligodendrocyte differentiation, however, the same netrin-1 signal leads to reduced RhoA activation which allows for oligodendrocyte process branching and expansion of the process network (Rajasekharan et al. 2010). The mechanism involved in the switch of GTPase regulation associated with signals such as netrin-1 is currently not known.

The Src family kinases (SFK) member, Fyn, also promotes oligodendrocyte process extension and differentiation during later stages of development via inactivation of RhoA (Wolf et al. 2001). RhoA activity has, however, been linked to enhanced membrane sheet formation in MBP+ mature oligodendrocytes in vitro without affecting the differentiation program of the oligodendrocytes (Czopka et al. 2009). These findings suggest a highly complex role for RhoA-mediated cytoskeletal remodeling during oligodendrocyte development.

Unlike RhoA, the GTPases Rac and Cdc42 have been shown to be involved in promoting oligodendrocyte process extension and enhancing differentiation in vitro (Liang et al. 2004, Hall 1998). While there is no apparent effect on oligodendrocyte development upon Cdc42 or Rac knockout in vivo, there is an increase in the amount of myelin out-foldings and a reduction in
myelin thickness, which points to a role for cytoskeletal modification in appropriate myelin formation (Thurnherr et al. 2006).

Oligodendrocyte process extension in vitro and initiation of myelination in vivo have been shown to involve signaling from neural-Wiskott Aldrich Syndrome protein (N-WASP) which activates the actin related protein 2/3 (Arp2/3) complex (Bacon et al. 2007). The Arp2/3 complex functions to promote actin nucleation and could play a role in lamellipodia formation during oligodendrocyte process remodeling and initial myelination (Bauer et al. 2009).

As described above, molecular interactions which regulate the actin cytoskeleton are crucial for proper and timely myelination. The precise regulatory mechanisms of these complex interactions are still largely unknown; however, focal adhesions, and particularly FAK, have been highly implicated in cell-ECM signaling regulating cytoskeletal rearrangement.

**Focal adhesions: what they are and how they work**

Throughout development changes of the ECM environment induce cellular responses. There are various ways that cells can sense and relate to the environment; however via focal adhesion is the most common. Focal adhesions are intracellular multi-molecular complexes of about 1-10 μm² in size that associate with cell membrane receptors and allow ECM interaction with the cell’s cytoskeleton (Badley et al. 1980).

In any given focal adhesion there are certain required constituents for proper function. Every adhesion site requires a link to the ECM, usually in the
form of an integrin receptor. Integrin receptors also serve as scaffolding proteins that associate with various signaling molecules during focal adhesion formation and turnover (Schwartz & Ingber 1994, Giancotti & Ruoslahti 1999, Playford & Schaller 2004). Other focal adhesion proteins, such as SFKs, are primarily necessary for their catalytic function but also serve to mediate the association of interacting molecules within the focal adhesion (Cobb et al. 1994, Cary et al. 2002, Schwartz & Ingber 1994). A large molecular complex of this magnitude involves so many signaling pathways and therefore requires extensive regulation to mediate proper ECM signal transduction. In this regard FAK has been shown to function as a major regulator and integrator of focal adhesion signaling (Mitra et al. 2005, Parsons 2003).

**Focal adhesion kinase (FAK)**

**FAK FERM domain**

FAK contains an N-terminal FERM (FAK N-terminal ezrin/radixin/moesin homology) domain which binds growth factor receptors such as PDGFRα (Mitra *et al.* 2005, Parsons 2003, Schlaepfer *et al.* 1999). This domain does not, however, appear to play a large role in integrin-stimulated focal adhesion dynamics (Mitra *et al.* 2005). FAK has a central kinase domain that is located just outside of its FERM domain only having ~40% homology to the catalytic domains of other protein tyrosine kinases making it a rather unique non-receptor tyrosine kinase (Schaller *et al.* 1992).

FAK has many phosphorylation sites, both tyrosine and serine, that modulate its activity, however since tyrosine phosphorylation has been more closely linked to ECM-mediated adhesion function the serine residues will not be discussed (Schlaepfer & Hunter 1996). FAK is activated by autophosphorylation on its Tyr397 site, which is within FAK’s kinase domain, leading to a conformational change (Toutant *et al.* 2002, Zhou *et al.* 2006). This allows high-affinity binding for the Src-homology-2 (SH2) domain of SFKs (Hanks & Polte 1997). SFKs are involved in phosphorylating various other tyrosine residues, including Tyr861 and Tyr925, allowing for full activation of FAK (Calalb *et al.* 1996, Schlaepfer & Hunter 1996). The Tyr576/577 sites stabilize FAK in an active conformation and are two of the sites phosphorylated by SFKs (Cary *et al.* 2002, Hanks & Polte 1997). The kinase domain is also a binding site for the p85 subunit of phosphotidylinositol 3-kinase (PI3-Kinase) which promotes membrane
ruffling and lamellipodia formation (Figure 1.2 red pathway) (Hamadi et al. 2005, Clark et al. 1998).

FAK C-terminal domain (FRNK)

The FAK C-terminal domain contains two proline rich regions (PRR) that bind to Src-homology-3 (SH3) domains of signaling proteins involved in focal adhesion formation and turnover, including p130Cas (Figure 1.2 blue pathway), which promotes cell migration by activation of Graf (Ras, GTPase regulator associated with FAK), and ASAP1 (Arf GTPase-activation protein GAP 1) (Mitra et al. 2005). ASAP1 has a role in regulating cell spreading as well as FAK localization to focal adhesions since over expression leads to interruption of both conditions (Figure 1.2 green pathway) (Liu et al. 2002).

Graf is highly expressed in the brain; however, it is inconclusive if Graf is expressed by oligodendrocytes (Taylor et al. 1998). FAK's association with Graf allows Graf activation through phosphorylation by mitogen-activated protein kinase (MAPK) (Taylor et al. 1998). Active Graf inactivates Rho by up regulating the catalysis of Rho bound GTP (active form) to make RhoGDP (inactive form) (Figure 1.2 teal pathway) (Taylor et al. 1998). Graf also inactivates Cdc42, however, Rac is not affected by Graf activation which allows Rac to function without the antagonistic effects of Rho (Taylor et al. 1998). Rac allows for oligodendrocyte process outgrowth, focal adhesion formation, and morphological differentiation and is has been shown to be up regulated during oligodendrocyte differentiation (Clark et al. 1998, Liang et al. 2004).
**FAK FAT domain**

FAK has a focal adhesion targeting (FAT) domain that is necessary for FAK’s localization to focal adhesions (Contestabile et al. 2003, Mitra et al. 2005, Schlaepfer et al. 1999).

The Tyr925 phosphorylation site is located within the FAT domain. The adaptor protein Grb2 binds to phosphorylated Tyr925 linking FAK to the MAPK signaling pathway which is involved in the regulation of various cellular functions (Figure 1.2 yellow pathway) (Mitra et al. 2005, Schlaepfer et al. 1994, Schlaepfer et al. 1999). Grb2 also binds dynamin on its proline rich domain (PRD), which is important for microtubule-induced focal adhesion disassembly, especially in migrating cells. Grb2 probably is the mediator between FAK and dynamin, which allows FAK to relate with microtubule dynamics (Ezratty et al. 2005). In post migratory premyelinating oligodendrocytes phosphorylation of the Tyr925 residue is reduced compared to migrating OPCs (Fox et al. 2004).

The integrin-associated proteins talin and paxillin bind FAK on the FAT domain (Mitra et al. 2005). Phosphorylation of paxillin promotes binding of Crk’s SH2 domain, increasing Rac activity and stimulating cell motility as well as process outgrowth (Figure 1.2 light blue pathway) (Clark et al. 1998). Paxillin also associates with p120RasGAP, which decreases Rho activation through activation of p190RhoGAP, leading to enhanced process outgrowth (Brown et al. 2005). The FAT domain also binds the Rho-family GTPase p190RhoGEF allowing FAK to phosphorylate p190RhoGEF, activating it (Figure 1.2 orange pathway). p190RhoGEF increases the exchange of RhoGDP for RhoGTP.
activating RhoA which leads to process retraction (Zhai et al. 2003). FAK associates with both GEFS (GTPase activators) as well as GAPs (GTPase inactivators) to allow for a cyclical regulation of both Rac, Cdc42 and Rho activity by either activating or inactivating GTPases upstream of these molecules (Tomar & Schlaepfer 2009). Via this cyclical regulation of GTPases FAK enables processes to remain highly dynamic while encountering the ECM environment.

**Life without FAK: FAK null**

Unfortunately the main phenotype found in constitutive FAK null mice is embryonic lethality due to general mesodermal growth defects (Ilic et al. 1995). At this stage of development OPCs have not yet been specified therefore research on FAK’s role in oligodendrocytes has been largely unknown. Fibroblast and endothelial cells collected from FAK null mouse embryos have, however, given some helpful clues as to FAK’s role in focal adhesion dynamics and cytoskeletal rearrangement. FAK null fibroblasts still form focal adhesions showing that FAK is not necessary for focal adhesion formation in fibroblasts; however in neuronal cells FAK plays a role in both adhesion formation and turnover (Mitra et al. 2005, Robles & Gomez 2006). Taken together FAK null fibroblasts were found to display reduced focal adhesion turnover, increased focal adhesion number and size, decreased motility, proliferation defects, actin assembly and location defects, poor cell spreading, and enhanced tyrosine phosphorylation throughout the adhesion (Jiang et al. 2006, Webb et al. 2004, Ilic et al. 1995). The FAK null mice have a similar phenotype to those of ECM
molecule, fibronectin null animals, demonstrating FAK’s necessity in ECM-integrin signaling (Ilic et al. 1995, George et al. 1993). Fibroblasts without FAK also have higher than normal Rho activity, due to an inability to regulate Rho activity, which could explain the decrease in cell motility and actin defects (Ren et al. 2000). Even the reduction of FAK expression, rather than eliminating it altogether, will dramatically reduce the cells ability to function properly (Jiang et al. 2006, Hoshina et al. 2007).

Since Pyk2 is the only other known member of the focal adhesion kinase family it stands to reason that Pyk2 might be compensating for any loss or reduction of FAK. There is an increase in Pyk2 expression in fibroblasts and endothelial cells that lack FAK, however, the functions lost along with FAK are not entirely regained in the Pyk2 over-expressing cells (Schlaepfer et al. 1999, Klingbeil et al. 2001). While there are additional effects of removing Pyk2 from FAKnull cells (Sieg et al. 1998, Lim et al. 2008b) it appears that FAK is the main player in focal adhesion dynamics and cytoskeletal remodeling.

As discussed in the above section FAK is associated with various molecular regulators of cytoskeletal remodeling including, but not limited to, Rho, Rac, Cdc42 and Ras. Phosphorylation by FAK is also involved in the localization and activation of N-WASP, which leads to actin polymerization and oligodendrocyte process extension and initial myelination, as discussed in the previous section (Wu et al. 2004). Due to the high level of involvement for FAK in regulating the cytoskeletal remodeling of the oligodendrocyte process network during development we explored the effects of removing FAK from
oligodendrocytes during a stage of extreme morphological remodeling, i.e. prior to myelination.
Figure 1.1 Development of an oligodendrocyte from a neuroepithelial cell to a mature oligodendrocyte. A) oligodendrocyte precursor cells (OPC) which migrate to their site of action and further differentiate into B) post-migratory precursor oligodendrocytes which begin sending out processes. C) Immature oligodendrocytes undergo drastic morphological maturation ultimately becoming myelin-producing D) mature oligodendrocytes. Markers differentially expressed throughout oligodendrocyte development: A2B5, Platelet derived growth factor α receptor (PDGFαR), DM20, O4, O1, myelin basic protein (MBP), and proteolipid protein (PLP). Adapted from Pfeiffer et al., 1993.
Figure 1.2  Schematic of signaling pathways regulated by FAK via interactions with focal adhesion molecules. FAK promotes cytoskeletal remodeling via association of phosphotidylinositol 3-kinase (PI3K) with the kinase domain of FAK (red pathway). The proline rich regions (PRR) of FAK associate with p130Cas (blue pathway), GTPase regulator associated with FAK (GRAF; teal pathway), and Arf GTPase-activation protein GAP1 (ASAP1; green pathway), affecting various cellular functions primarily involved in cytoskeletal rearrangement. Paxillin (light blue pathway), p190RhoGEF (orange pathway), and Grb2 (yellow pathway) associate with the focal adhesion targeting (FAT) domain of FAK and mediate process dynamics. Adapted from Schlaepfer et al., 1999 and Parsons, 2003.
Chapter 2

Focal Adhesion Kinase (FAK): A Regulator of CNS Myelination

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Introduction

One of the main regulators of integrin-ECM signaling is focal adhesion kinase (FAK). FAK, also known as protein tyrosine kinase 2, is a ubiquitously expressed nonreceptor protein tyrosine kinase that can be activated by a number of extracellular signals (Hanks et al. 1992, Schaller et al. 1992, Schlaepfer et al. 1999, Parsons 2003, Mitra et al. 2005, Mitra & Schlaepfer 2006). FAK has been found to be expressed in cells of the oligodendrocyte lineage and is present in myelin (Kilpatrick et al. 2000, Bacon et al. 2007). Interestingly, phosphorylation of FAK at its Tyr397 site, which represents a critical event for its activation and biological effects, has been described as occurring primarily in postmigratory differentiating oligodendrocytes and not in migratory oligodendrocyte progenitor cells (Liang et al. 2004). Furthermore, additional phosphorylation events of FAK tyrosine residues regulate its overall function (Schlaepfer & Hunter 1996, Hanks & Polte 1997, Cohen & Guan 2005). Phosphorylation of one of these residues, namely, the Tyr925 residue, has been found to be significantly altered during the initial stages of myelination (Fox et al. 2004). Taken together, these data suggest a role for FAK in regulating oligodendrocyte maturation and/or CNS myelination itself. However, this role of FAK has not yet been well characterized.

Ubiquitous FAK knockout is early embryonically lethal because of general mesodermal defects (Furuta et al. 1995, Ilic et al. 1995). Therefore, to investigate the potential role of FAK in the regulation of oligodendrocyte maturation and/or CNS myelination, we generated oligodendrocyte-specific and inducible FAK-knockout mice using the Cre-loxP system (Fak\textsuperscript{lox/lox}, PLP/Cre\textsubscript{ERT}\textsuperscript{T} mice). Our results revealed that when inducing FAK knockout in these mice just prior to and
during the initial stages of myelination of the optic nerve, myelination was reduced on postnatal day 14 (P14). In addition, our data showed that the induction of FAK knockout resulted in a reduced number of primary oligodendrocyte processes at this developmental age. However, this phenotype appears to be transient because the number of myelinated fibers on postnatal day 28 (P28) was comparable under both control and knockout conditions. Taken together, these data demonstrate that FAK is involved in regulating the efficiency and timing of myelination in its initial stages and suggest that this regulatory role may involve the control of oligodendrocyte process outgrowth and/or remodeling.

Materials and Methods

Animals and Induction of FAK Knockout

Mice in which the second kinase domain exon of Fak is flanked by loxP sites (Fak<sup>flox/flox</sup> mice) were bred to mice that express, under the control of the proteolipid protein (PLP) promoter, Cre recombinase fused to a tamoxifen-inducible mutated ligand-binding domain of the human estrogen receptor (PLP/CreER<sup>T</sup> mice, kindly provided by B. Popko, University of Chicago; (Beggs et al. 2003, Doerflinger et al. 2003)). Both strains are on a C57BL/6 genetic background. Litters used for the present study were derived from males homozygous for the floxed Fak locus and heterozygous for the PLP/CreER<sup>T</sup> locus and females homozygous for the floxed Fak locus but negative for the PLP/CreER<sup>T</sup> locus. Thus, knockout and control mice were derived from the same
breeding pairs. To induce FAK knockout, 300-µL intraperitoneal injections of 3 mg of tamoxifen or vehicle (sunflower oil) were administered daily into lactating mothers from postnatal day 2 (P2) through P12, with P0 referring to the day of birth (Leone et al. 2003). Animals were analyzed at P14 and P28. To confirm successful Cre-mediated recombination under the above-described conditions, $PLP/\text{CreER}^T$ mice were additionally bred to $Gt(\text{ROSA})26\text{So}r^{\text{tm1}(\text{EYFP})\text{Cos}}$ reporter mice, which contain an enhanced yellow fluorescent protein (EYFP) gene inserted into the $Gt(\text{ROSA})26\text{So}r$ locus and are also on a C57BL/6 genetic background (Jackson Laboratory, Bar Harbor, ME; see also (Srinivas et al. 2001). In these reporter mice, expression of EYFP is blocked by an upstream $\text{loxP}$-flanked STOP sequence and only induced on successful Cre-mediated recombination at the $\text{ROSA}26$ locus. $Gt(\text{ROSA})26\text{So}r^{\text{tm1}(\text{EYFP})\text{Cos}}$:PLP/CreER$^T$ mice were treated as described above and analyzed at P14. All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

**PCR Analysis**

For genotype analysis, DNA was extracted from tail clips using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). For analysis of recombination efficiency upon tamoxifen treatment, optic nerves were dissected from P14 animals, and DNA was extracted as described above. Polymerase chain reaction (PCR) was performed using a Taq PCR Core Kit (Qiagen, Valencia, CA) and a PTC-200 DNA Engine Cycler (MJ Research, Waltham, MA). The following primer pairs were used at the indicated annealing temperatures: CreER$^T$ forward (5-
GATGTAGCCAGCAGCATGTC-3) and CreER\textsuperscript{T} reverse (5-
ACTATATCCGTAACCTGGAT-3) at 50°C and FAK-flox-P1 (5-
GACCTTCAACTTCT CATTCTCC-3) and FAK-flox-P2 (5-GAATGCTACAG
GAACCAAATAAC-3) at 55°C. Custom oligonucleotides were obtained from
MWG-Biotech (Huntsville, AL). The PCR cycling conditions were: 2 min at 94°C,
followed by 35 cycles of amplification (45 sec at 94°C; 1 min, 30 sec at the
respective annealing temperature; and 45 sec at 72°C) and 4 min of extension at
72°C. Amplified DNA was analyzed using agarose gel electrophoresis and the
VersaDoc 4000 imaging system (Bio-Rad, Hercules, CA).

**Immunohistochemistry**

Immunohistochemistry was performed on longitudinal sections of optic
nerves dissected from tamoxifen-treated P14
Gt(ROSA)26Sortm1(EYFP)Cos:PLP/CreER\textsuperscript{T} mice. Mice were deeply anesthetized,
transcardially perfused with 4% paraformaldehyde in 0.1M Millonigs buffer (150
mM sodium phosphate monobasic/100mM sodium hydroxide) and postfixed for
24 hr. Optic nerves were removed, cryoprotected in 30% sucrose/PBS,
embedded in Tissue-Tek on dry ice, and 10-µm sections were then cut on a
Shandon SME Cryotome (Thermo Scientific, Philadelphia, PA). Sections were
immunolabeled after a permeabilization step in ice-cold acetone (Dupree et al.
1999) using the following antibodies: mouse monoclonal anti-APC/CC1
(EMD/Calbiochem, Gibbstown, NJ), rabbit polyclonal anti-GFP/YFP (Millipore,
Temecula, CA), and secondary Alexa Fluor 594-conjugated donkey antimouse
IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen/Molecular Probes, Carlsbad, CA). Sections were analyzed by confocal microscopy using a Leica TCS SP2 AOBS System (Leica Microsystems Inc., Bannockburn, IL).

**Light and Electron Microscopic Analysis**

P14 and P28 mice treated with either tamoxifen or sunflower oil were deeply anesthetized and transcardially perfused with 4% paraformaldehyde/2.5% glutaraldehyde in 0.1M Millonigs buffer (Dupree et al. 1998). Mice were postfixed for 1-2 weeks in aldehyde fixative. Optic nerves were dissected out and incubated for 1 hr in 1% osmium tetroxide/0.1M Na-cacodylate buffer (Electron Microscopy Sciences, Ft. Washington, PA). Specimens were dehydrated with ethanol and embedded in Epon resin (Electron Microscopy Sciences, Ft. Washington, PA).

For light microscopic analysis, semithin (1-µm) transverse sections were taken every 1 mm throughout the length of the optic nerve, stained with toluidine blue, and imaged using a Nikon ECLIPSE E800M microscope equipped with a Spot RT CCD camera (Nikon Inc., Melville, NY). The number of myelinated fibers was determined in a blinded fashion for each entire transverse section. The average number for the control sections was set to 100%, and each value was calculated accordingly. Statistical significance was determined using the Student's *t* test.

For electron microscopic analysis, ultrathin transverse sections (90 nm) were taken 1 mm from the lamina cribrosa and at 1-mm intervals along the length
of the optic nerve. Sections were collected on Formvar-coated slotted grids and stained with uranyl acetate and lead citrate. Images were taken using a JEOL JEM1230 transmission electron microscope equipped with an Ultrascan 4000 Gatan CCD camera.

Results

Administration of Tamoxifen to Lactating Females Induces Cre-Mediated Recombination in the Optic Nerve and in Cells of Oligodendrocyte Lineage in Early Postnatal Offspring Containing the {\textit{PLP/CreER}}^T Locus

To determine the \textit{in vivo} role of FAK in differentiating oligodendrocytes during the developmental period of myelination, spatially and temporally controlled transgenic FAK-knockout mice were generated using an inducible Cre-loxP system. More specifically, mice in which the second kinase domain exon of \textit{Fak} is flanked by \textit{loxP} sites (\textit{Fak}^{floxfloxfloxfloxflox} mice; Figure 2.1B) were bred to mice that express, under the control of the PLP promoter, Cre recombinase fused to a tamoxifen-inducible mutated ligand binding domain of the human estrogen receptor (\textit{PLP/CreER}^T mice; Figure 2.1B; (Beggs et al. 2003, Doerflinger et al. 2003)). Because of the breeding strategy used (see Materials and Methods section), all mice analyzed for the potential effects of FAK-knockout induction were homozygous for the floxed \textit{Fak} locus and either heterozygous for the \textit{PLP/CreER}^T locus (\textit{Fak}^{floxfloxflox} ;\textit{PLP/CreER}^T mice) or lacking the \textit{PLP/CreER}^T locus (\textit{Fak}^{floxfloxflox} littermate controls). It has been previously established that only upon
administration of tamoxifen is CreER\textsuperscript{T} translocated from the cytoplasm to the nucleus, where it is able to catalyze recombination at loxP sites (Leone et al. 2003). In addition, it has been shown that the PLP promoter used for the generation of the PLP/CreER\textsuperscript{T} mice is well suited to direct transgene expression to differentiating oligodendrocytes (Wight et al. 1993, Fuss et al. 2000, Fuss et al. 2001, Doerflinger et al. 2003). Thus, the use of the above-described Fak\textsuperscript{flox/flox}: PLP/CreER\textsuperscript{T} mice allowed for design strategies in which to induce Cre-mediated recombination and thus FAK knockout specifically in differentiating oligodendrocytes and during the developmental period of active myelination. Because of the region-specific timing of active myelination in the CNS, however, individual knockout, that is, tamoxifen injection, strategies need to be employed for specific CNS regions (Caley & Maxwell 1968, Foran & Peterson 1992, Leone et al. 2003, Thomson et al. 2005).

For the study presented here, we chose the optic nerve as the anatomical region of interest because of its relatively simple morphology and the well-described chronology of oligodendrocyte differentiation and myelination (Skoff et al. 1976a, Skoff et al. 1976b, Skoff et al. 1980, Tennekoon et al. 1977, Hildebrand & Waxman 1984, Hunter & Bedi 1986, Butt & Ransom 1993, Colello et al. 1995, Thomson et al. 2005). In the optic nerve of developing C57Bl/6 mice, that is, animals of genetic background similar to that of the Fak\textsuperscript{flox/flox}:PLP/CreER\textsuperscript{T} mice, both oligodendrocyte differentiation and myelination proceed from the retinal to the chiasmatic end of the nerve (Thomson et al. 2005). First axonal contact of oligodendrocyte processes can be seen at the retinal end around
postnatal day 6 (P6). At this point, oligodendrocytes also begin to express the PLP isoform of PLP/DM20 and are thus considered to represent a myelinating stage of the lineage (Tennekoon et al. 1977, Trapp et al. 1997, Thomson et al. 2005). At the chiasmatic end, such initially myelinating cells are first observed at P8. Mature, that is, compacted, myelin can be found extended over the whole length of the nerve around P15 (Foran & Peterson 1992). Thus, to induce Cre-mediated recombination in the optic nerve just prior to and during the developmental period of active myelination, tamoxifen or sunflower oil as vehicle control were administered daily to lactating mothers from 2 to 12 days postpartum, and animals were analyzed during the active stages of myelination, that is, at P14, and at a mature stage, that is, at P28 (Figure 2.1A). Successful Cre-mediated recombination at the Fak locus was assessed by PCR analysis (Figure 2.1C). Recombination could be observed as early as P4 (data not shown). At this point, oligodendrocytes in the optic nerve for the most part are at a progenitor stage, at which they express low levels of FAK (Liang et al. 2004). Thus, even in a case of a relatively stable FAK protein, FAK protein levels will be reduced upon FAK-knockout induction because it inhibits the normal developmental up-regulation of FAK expression. At P14, approximately 40% of the cells in the optic nerve are oligodendrocytes (Barres et al. 1992). Thus, the recombination efficiency of approximately 40% observed in our studies (Figure 2.1C) suggests efficient Cre-mediated recombination in cells of the oligodendrocyte lineage, at least at this developmental age. In the other cell types of the optic nerve, which are primarily astrocytes, the PLP promoter is not
operative, and thus recombination cannot be induced upon administration of tamoxifen. To further confirm successful Cre-mediated recombination in cells of the oligodendrocyte lineage under the conditions depicted in Figure 2.1A, \( PLP/CreER^T \) mice were bred to \( Gt(ROSA)26Sor^{tm1(EYFP)Cos} \) reporter mice, in which expression of EYFP is blocked by an upstream \( loxP \)-flanked STOP sequence and only induced upon successful Cre-mediated recombination (Srinivas et al. 2001). As a marker for cells of the oligodendrocyte lineage, an antibody to cytoplasmic APC/CC1 was used (Bhat et al. 1996, Fuss et al. 2000). As shown in Figure 2.1D, EYFP expression could easily be detected in APC/CC1-positive cells of the optic nerve of a tamoxifen-treated P14 \( Gt(ROSA)26Sor^{tm1(EYFP)Cos}:PLP/CreER^T \) mouse. Taken together, the above data confirm that under the conditions depicted in Figure 2.1A, Cre-mediated recombination at \( loxP \) sites occurs efficiently in the optic nerve and in cells of the oligodendrocyte lineage during early postnatal development (P4-P14) of offspring containing the \( PLP/CreER^T \) locus.

At P14, the Number of Myelinated Axons is Decreased in Optic Nerves of Tamoxifen-Treated \( Fak^{flox/flox}:PLP/CreER^T \) Mice

To assess the extent to which FAK may regulate developmental myelination, we determined the number of myelinated fibers in tamoxifen-treated \( Fak^{flox/flox}:PLP/CreER^T \) and control mice at P14 using light microscopy (Figure 2.2). To eliminate the possibility that varying levels of myelination along the length of the optic nerve affect the outcome of our analysis, we examined
sections taken at 1-mm intervals along the whole length of the nerve (Skoff et al. 1980). At all levels, the number of myelinated fibers was found to be reduced in the \( \text{Fak}^{\text{flox/flox}}:\text{PLP/CreER}^T \) mice compared with their \( \text{Fak}^{\text{flox/flox}} \) litter mates (see example in Figure 2.2A). When averaging the number of myelinated fibers for all levels, these were found to be significantly decreased, by more than 30%, in \( \text{Fak}^{\text{flox/flox}}:\text{PLP/CreER}^T \) mice compared to \( \text{Fak}^{\text{flox/flox}} \) mice (Figure 2.2B,C). No such differences were noted when analyzing vehicle-treated \( \text{Fak}^{\text{flox/flox}}:\text{PLP/CreER}^T \) and \( \text{Fak}^{\text{flox/flox}} \) mice (data not shown).

To determine the resolution of our type of analysis, we compared semithin sections (light microscopy) with consecutive ultrathin sections (electron microscopy). As shown in Figure 2.3, such analysis revealed that visualizing transverse sections of the optic nerve using light microscopy allowed myelinated axons enwrapped with as few as four layers of myelin to be distinguished. Thus, our data demonstrate that FAK is involved in the regulation of the initial steps of myelination and/or myelin wrapping.

At P14, the Number of Primary Oligodendrocyte Processes is Reduced in Optic Nerves of Tamoxifen-Treated \( \text{Fak}^{\text{flox/flox}}:\text{PLP/CreER}^T \) Mice

To further investigate the above role of FAK during developmental myelination of the optic nerve, we determined the total number of cells in the semithin 1-µm transverse sections taken at 1-mm intervals (measured from the lamina cribrosa). This analysis revealed no differences between P14 \( \text{Fak}^{\text{flox/flox}}:\text{PLP/CreER}^T \) mice and their \( \text{Fak}^{\text{flox/flox}} \) littermates (Figure 2.4A),
suggesting that the effect of FAK-knockout induction on myelination may not be a result of changes in the number of oligodendrocytes. However, when analyzing the number of primary processes, that is, processes that directly extend from oligodendrocyte cell bodies, a significant decrease, of approximately 30%, was observed in P14 $Fak^{\text{floxflox}}: PLP/\text{CreERT}^T$ optic nerves compared to optic nerves from P14 $Fak^{\text{floxflox}}$ littermates (Figure 2.4B-D). Oligodendrocyte cell bodies can be easily distinguished from cell bodies of the major other cell type found in the optic nerve, namely, astrocytes, as astrocytes exhibit abundant glycogen granules and contain intermediate filaments. Thus, we are confident that to a large extent, our analysis was restricted to oligodendrocytes. Taken together, the above data therefore suggest that the decrease in the number of myelinated fibers seen upon induction of FAK knockout is a result, at least in part, of impaired oligodendrocyte process outgrowth and/or remodeling.

At P28, the Number of Myelinated Axons within Optic Nerves is Comparable Between Tamoxifen-Treated $Fak^{\text{floxflox}}$ and $Fak^{\text{floxflox}}: PLP/\text{CreERT}^T$ Mice

To assess the extent to which the above-observed effects of FAK-knockout induction during periods of active myelination are persistent into adulthood, we determined the number of myelinated axons at P28. At this age, the number of myelinated axons in the optic nerve was found to be comparable between $Fak^{\text{floxflox}}$ and $Fak^{\text{floxflox}}: PLP/\text{CreERT}^T$ mice (Figure 2.5). These data suggest that the role of FAK may be of particular importance for the efficiency and timing of the initial stages of myelination.
Discussion

In the present study, we have demonstrated that induction of FAK knockout just prior to and during active stages of myelination results in hypomyelination in the optic nerve at a time-point when normally myelinated fibers can be found extended throughout the whole length of the nerve, that is, at P14. Furthermore, our data suggest that this effect of FAK-knockout induction is a result at least in part of reduced outgrowth and/or impaired remodeling of primary oligodendrocyte processes. However, myelination appears to have reached normal levels by P28. Thus, our data suggest that in vivo in the optic nerve, FAK promotes efficient and properly timed myelination during the active phases of myelin sheath formation.

In light of the known role of FAK in integrating ECM-integrin signaling events, our data are in support of the previously suggested but somewhat controversial regulatory role of integrin signaling during CNS myelination (Relvas et al. 2001, Benninger et al. 2006, Lee et al. 2006, Colognato et al. 2007). Furthermore, they are in good agreement with findings that demonstrated developmental myelination to be controlled by signals up- and downstream of the integrin-FAK axis, such as laminin2/merosin and Fyn, respectively (Biffiger et al. 2000, Sperber et al. 2001, Chun et al. 2003). Interestingly, expression of dominant-negative β1 integrin, laminin2/merosin deficiency, and Fyn knockout all result in hypomyelination in a region-specific manner, with only the optic nerve affected to a similar extent in these mutant mice. Thus, the roles of ECM proteins, integrins, and FAK may differ in different CNS areas. Those areas...
potentially not controlled by FAK may depend on the FAK-related kinase proline-rich tyrosine kinase 2 (Pyk2). Although Pyk2 expression in oligodendrocytes has not been well defined, it represents a good candidate for functionally substituting FAK (Avraham et al. 2000, Klingbeil et al. 2001, Orr & Murphy-Ullrich 2004, Nakamura et al. 2007). However, future studies will be necessary to dissect the exact roles of FAK and Pyk2 in the regulation of CNS myelination.

FAK has been characterized as a regulator of morphological remodeling and, in particular, has been implicated in the regulation of process outgrowth from both neurons and oligodendrocytes (Beggs et al. 2003, Falk et al. 2005, Robles & Gomez 2006, Hoshina et al. 2007). Our data demonstrating a reduction in the number of primary processes on induction of FAK knockout are consistent with these previous findings. A pivotal role of FAK in the regulation of morphological oligodendrocyte differentiation is further supported by the fact that Fyn, a known FAK effector, has been found to be important for the development of the extensive oligodendrocyte process network (Osterhout et al. 1999, Buttery & ffrench-Constant, Klein et al. 2002, Liang et al. 2004). In the case of Fyn, this morphological maturation can be regulated independent of changes in gene expression typically associated with oligodendrocyte differentiation (Osterhout et al. 1999). The extent to which FAK is involved in the control of gene expression in differentiating oligodendrocytes, however, has not yet been characterized. Nevertheless, the above data support the idea that impaired process outgrowth and/or remodeling, at least in part, may be responsible for the hypomyelination seen in the optic nerves of tamoxifen-treated $Fak^{\text{flox/flox}}$:PLP/CreERT$^T$ mice.
In our studies, the effect of FAK-knockout induction on myelination was found to be transient, with normal levels of myelination detectable at P28. Thus, FAK’s role appears to mainly affect the efficiency and timing of myelination. However, as discussed above, Pyk2 may be able to substitute FAK’s function not only in a region-specific manner but also in the case of loss of FAK. Future studies will be necessary to assess such a potentially important role of Pyk2 in myelination.

Taken together, our data suggest that FAK promotes efficient and properly timed myelination in the optic nerve, where it likely acts as an effector of integrin signaling activated by oligodendrocyte-ECM interactions. Our data further suggest that this signaling event promotes process outgrowth and potentially remodeling during the initial stages of myelination. Impairment of these steps of oligodendrocyte maturation appears to be responsible for, at least in part, the limited repair of the myelin sheath seen in lesions of patients suffering from the major demyelinating disease in humans: multiple sclerosis (Chang et al. 2002, Franklin & ffrench-Constant 2008, Kuhlmann et al. 2008). Thus, increasing the understanding of the role of FAK in CNS myelination not only furthers our understanding of normal CNS development but may also reveal novel targets suitable to stimulate remyelination under pathological demyelinating conditions.
Figure 2.1 Induction of Cre-mediated recombination in $Fak^{flox/flox}$:PLP-CreER$^T$ and $Gt$(ROSA)26Sor$^{1m1(EYFP)Cos_5}$:PLP-CreER$^T$ mice. A: Injection paradigm used to induce Cre-mediated recombination at the floxed fak or ROSA26 locus just prior to and during myelination of the optic nerve. Times of initial myelination defined as described by Thomson et al. 2005 (Thomson et al. 2005). B: Schematic at the top depicts the transgene construct used to generate the PLP/CreER$^T$ mice (Doerflinger et al. 2003). The PLP cassette contains 2.4 kb of the 5'-flanking DNA, exon 1 and intron 1 of the Plp gene. The cDNA sequence coding for CreER$^T$ was inserted 3' of intron 1. For transcription termination, a simian virus (SV) 40 poly(A) signal sequence was added. Middle schematic depicts the floxed Fak locus, in which the second kinase domain exon of Fak is flanked by loxP sites (Beggs et al. 2003). Upon crossbreeding and tamoxifen application, recombination at the Fak locus was induced as depicted in the bottom schematic. P1 and P2 indicate the location of PCR primers used for genotyping. C: PCR analysis of recombination at the Fak locus in optic nerves of tamoxifen-treated $Fak^{flox/flox}$:PLP-CreER$^T$ and $Fak^{flox/flox}$ mice at P14. Primers P1 and P2 depicted in A were used, and the amount of each amplification product was determined using the VersaDoc 4000 imaging system (Bio-Rad, Hercules, CA). Bar graph depicts means ± SEM ($n = 3$ per genotype); *, statistically significant as determined by Student's $t$ test. D: Representative confocal images of P14 optic nerve sections taken from tamoxifen-treated $Gt$(ROSA)26Sor$^{1m1(EYFP)Cos_5}$: PLP-CreER$^T$ mice after double-labeling for APC/CC1 and YFP. Nuclei were stained using Hoechst. Images depict single channel representations of a single optical section (approximately 0.2-µm x-z resolution). Scale bar = 10 µm
Figure 2.1

A

B

C

D
Figure 2.2 Reduced number of myelinated fibers in optic nerves of tamoxifen-treated $Fak^{\text{flox/flox}}:PLP/\text{CreER}^T$ mice at P14. A: Numbers of myelinated fibers within entire transverse sections of P14 optic nerves at approximately 1, 2, and 3 mm from the lamina cribosa. Mean for all control values was set to 100%, and percentages for all data points were calculated accordingly. Results for a representative optic nerve littermate pair are shown. B: Numbers of myelinated fibers averaged over all three intervals of the optic nerve. Percentages were calculated as described in A. Bar graph depicts means ± SEM ($n = 3$ per genotype); *, statistically significant as determined by the Student's $t$ test. C: Representative pictures of semithin sections from $Fak^{\text{flox/flox}}$ (left) and $Fak^{\text{flox/flox}}:PLP\text{CreER}^T$ (right) optic nerves taken at approximately 1, 2, and 3 mm from the lamina cribosa. Scale bars = 10 µm.
Figure 2.2

A

% myelinated fibers

\( Fak^{\text{flox/flox}} \)

\( Fak^{\text{flox/flox}}:\text{PLP/CreER}^T \)

1 2 3 mm

B

% myelinated fibers

\( Fak^{\text{flox/flox}} \)

\( Fak^{\text{flox/flox}}:\text{PLP/CreER}^T \)

C

\( Fak^{\text{flox/flox}} \)

\( Fak^{\text{flox/flox}}:\text{PLP/CreER}^T \)

1 mm

2 mm

3 mm
Figure 2.3 Resolution (visual threshold) for the detection of a myelinated fiber in the optic nerve was approximately 4 layers of myelin at the light microscopic level. **A**: Semithin section of the optic nerve imaged at the light microscopic level. Scale bar = 10 µm. **B, C**: Ultrathin sections taken immediately adjacent to the semithin section shown in **A** and imaged at the electron microscopic level. Scale bar = 2 µm (**B**) and 200 nm (**C**). Rectangle in **A** marks the area shown in **B** ( *, myelinated fibers that can be identified as such at both the light and the electron microscopic levels; Δ, myelinated fiber that can only be identified as such at the electron microscopic level).
Figure 2.4 Reduced number of primary oligodendrocyte processes in optic nerves of tamoxifen-treated $Fak^{flox/flox}:PLP/CrERT$. A: Total number of cells counted at light microscopic level in semithin P14 optic nerve sections at the 1-mm interval (see Figure 2.2). Mean for all control values was set to 100%, and percentages for all data points were calculated accordingly. Bar graph depicts means ± SEMs ($n = 3$ per genotype). B: Number of primary processes per cell counted at electron microscopic level and over an entire transverse section for each nerve analyzed. Mean for all control values was set to 100%, and percentages for all data points were calculated accordingly. Bar graph depicts means ± SEMs ($n = 3$ per genotype, 258 cells for each condition); *, statistically significant as determined by the Student's $t$ test. C, D: Representative transmission electron micrographs for a $Fak^{flox/flox}:PLP/CrERT$ (D) and a $Fak^{flox/flox}$ (C, control) oligodendrocyte. Scale bars = 2 µm.
Figure 2.5 Comparable number of myelinated fibers in optic nerves of tamoxifen-treated $Fak^{\text{flox/flox}}$ and $Fak^{\text{flox/flox}}$:PLP/CreER$^T$ mice at P28. Numbers of myelinated fibers were determined within entire transverse sections of optic nerves at approximately 1 mm from the lamina cribosa and as described in Figure 2.2. Mean for all control values was set to 100%, and percentages for all data points were calculated accordingly. Bar graph depicts means ± SEMs ($n = 3$ per genotype).
Chapter 3

FAK associated mediators of oligodendrocyte cytoskeletal remodeling

Integrins

Integrins are ubiquitously expressed transmembrane heterodimer receptors formed from the combination of one of eighteen possible $\alpha$ chains and one of eight possible $\beta$ chains. Each subunit ($\alpha$ and $\beta$) is composed of a single transmembrane domain, to tether the integrin to the membrane, a large extracellular domain, which interacts with ECM ligands, and a cytoplasmic domain, that interacts with signaling molecules, such as FAK, within the cell (Zamir & Geiger 2001, Romer et al. 2006).

Activation of integrins involves a conformational change which induces receptor clustering allowing for further recruitment and activation of focal adhesion molecules (Ye et al. 2010, Schwartz & Ingber 1994). Signaling through integrins is a bidirectional process with information traveling in an outside-in (signals sent from the extracellular environment are transduced into reactions by the cell) or inside-out (signals originating within the cell affect a change in the integrin signaling to various other molecules which then leads to other cellular reactions) direction (Romer et al. 2006). One of the signaling molecules most commonly activated by integrin signaling is FAK (Buttery et al. 1999).
Integrins in developing oligodendrocytes

To date five heterodimer pairs of integrins have been shown to be expressed on the membranes of oligodendrocytes: \( \alpha_6\beta_1, \alpha_v\beta_1, \alpha_v\beta_3, \alpha_v\beta_5, \) and \( \alpha_v\beta_8 \) (Milner & ffrench-Constant 1994, Buttery et al. 1999, Baron et al. 2005). The fibronectin/vitronectin receptor \( \alpha_v\beta_1 \) integrin is highly expressed in OPCs then declines drastically with differentiation while \( \alpha_v\beta_3 \) integrin is most highly expressed following OPC differentiation and prior to myelin sheet formation in vitro (Milner & ffrench-Constant 1994, Milner et al. 1997, Blaschuk et al. 2000, Baron et al. 2005). Fibronectin and vitronectin along with growth factor signaling, such as PDGF\( \alpha \), acting through the \( \alpha_v\beta_1 \) integrin receptor enhance OPC migration while signaling through \( \alpha_v\beta_3 \) integrin is more important for OPC proliferation (Lee et al. 2006, Baron et al. 2005). The other fibronectin/vitronectin-binding integrin, \( \alpha_v\beta_5 \), replaces the declining \( \alpha_v\beta_1 \) integrin during early oligodendrocyte differentiation. The expression of \( \alpha_v\beta_5 \) increases with differentiation and is found associated with PLP (Milner & ffrench-Constant 1994, Blaschuk et al. 2000, Gudz et al. 2002).

Early oligodendrocytes have been shown to contain integrin aggregates composed mainly of \( \alpha_v\beta_3 \) integrin (Cluzel et al. 2005). The Lyn SFK associates with \( \alpha_v\beta_3 \) on OPCs, however there has been little investigation into Lyn’s role during oligodendrocyte differentiation and myelination (Klein et al. 2002, Colognato et al. 2004).

Various in vivo studies have been preformed to elucidate the role of the different integrin subunits during oligodendrocyte development. The most
commonly studied subunit is the β1 integrin subunit due to its constitutive presence during oligodendrocyte development. Studies have been contradictory until recently. Region specific myelin deficits and decreases in oligodendrocyte differentiation have been reported in mice expressing inactive forms of the β1 integrin, (Barros et al. 2009, Lee et al. 2006) while in mice with an early knock out of β1 expression no myelin defects were reported (Benninger et al. 2006). These contradictory findings have been theorized by ffrench-Constant to be due to differences in experimental design. In the inactive β1 expresser studies there could be an exaggeration of the phenotype since the inactive integrin might out-compete other receptors. While the knockout study could have eliminated β1 from the system at an early point during development thus allowing compensatory mechanisms to take affect. Due to these possibilities ffrench-Constant preformed a study in which a dominant negative β1 integrin (a fusion protein containing the intracellular β1 domain bound to the nonsignaling human interleukin-2 extracellular domain) under control of a later stage oligodendrocyte promoter (MBP) was expressed in mice (Camara et al. 2009). In these mice a transient myelin deficit was observed specifically affecting small-diameter fibers and a decrease in FAK signaling was seen (Camara et al. 2009). As mentioned in chapter 2 these results were in line with what we had previously observed in our FAK-null mice. These results insinuate a role for integrin signaling, possibly through FAK, in initial myelin formation during development.

Throughout development oligodendrocytes express the α6β1 integrin, which binds laminin-2 (Baron et al. 2005). This integrin receptor is involved in
oligodendrocyte survival throughout development, possibly by associating with an antigen on the membrane surface of adjacent astrocytes (Gudz et al. 2002, Frost et al. 1999, Corley et al. 2001). The β1 integrin of this heterodimer seems to play a large role during myelination, since reducing the expression of β1 inhibits the activation of Rac and Cdc42 thus inhibiting oligodendrocyte morphological maturation and decreasing the number of oligodendrocytes exhibiting mature membranes in vitro (Buttery & ffrench-Constant 1999, Liang et al. 2004). Oligodendrocytes grown only on laminin-2 (the substrate for integrin α6β1) also have greater process extension and an increased membrane formation (Buttery & ffrench-Constant 1999).

The binding of laminin-2 to α6β1 integrin activates the SFK, Fyn, which is expressed by oligodendrocytes throughout development (Grant et al. 1995, Klein et al. 2002, Colognato et al. 2004). Fyn associates with FAK and activates p190RhoGAP by tyrosine phosphorylation allowing the upregulation of Cdc42 and Rac activity, which increases process extension in vitro (Figure 1.2, peach pathway) (Wolf et al. 2001, Liang et al. 2004, Cobb et al. 1994, Cary et al. 1996).

Fyn is highly expressed in oligodendrocytes during differentiation and myelination and is found associated with myelin membrane microdomains (Klein et al. 2002, Osterhout et al. 1999, Kramer et al. 1999). There is increased Fyn expression in more differentiated, premyelinating oligodendrocytes than in OPCs leading to the conclusion that Fyn may be more functional in process outgrowth and myelination than migration and proliferation of oligodendrocytes (Colognato et al. 2004).
While Fyn may not be needed for general growth and differentiation, without Fyn mice have impaired myelination and a reduced number of oligodendrocytes throughout development with less complexity in the oligodendrocytes that do survive (Ponniah et al. 1999, Sperber & McMorris 2001, Colognato et al. 2004, Sperber et al. 2001, Biffiger et al. 2000). Fyn null mice also have reduced levels of phosphorylated FAK (Grant et al. 1995).

It has been shown that β1-integrin inhibition decreases the activity of Fyn and disrupts oligodendrocyte differentiation (Liang et al. 2004). It appears that Fyn, activated by association with α6β1 integrin in the focal adhesion, instigates FAK’s association with many proteins via tyrosine phosphorylation. These FAK-mediated molecular associations activate various proteins including Rac and Cdc42 which then lead to increased oligodendrocyte process outgrowth and morphologic differentiation (Colognato et al. 2004, Wolf et al. 2001, Liang et al. 2004).

**Extracellular matrix in the CNS**

The ECM is the conglomeration of various molecules, which allow cells to respond appropriately to environmental cues primarily via rearrangement of the cytoskeleton. Major constituents of the CNS ECM are hyaluronic acid and proteoglycans, as well as tenascins and adhesion-associated ECM molecules, such as fibronectin and laminin-2 (Sobel 1998, Bronner-Fraser 1986). It has been well characterized that a change in the ECM environment affects the morphology of developing oligodendrocytes (Notterpek & Rome 1994, Fridman et
al. 1985, Oh & Yong 1996). The most well characterized ECM molecules involved in oligodendrocyte morphological remodeling are fibronectin and laminin-2; therefore we will focus on these two molecules henceforth.

*In vitro* studies have linked fibronectin signaling to enhanced OPC proliferation and migration (Hu *et al.* 2009). This fibronectin-mediated promotion of OPC proliferation requires Lyn activity (Colognato *et al.* 2004). Fibronectin is also involved in keeping OPCs from differentiating too early during development (Hu *et al.* 2009). More differentiated oligodendrocytes in association with fibronectin have a smaller, less mature process morphology (Olsen & ffrench-Constant 2005, Liang *et al.* 2004). This restriction of the oligodendrocyte process network has been shown to involve RhoA activation (Liang *et al.* 2004). The effect of fibronectin on oligodendrocyte process morphology could be due to issues with membrane-directed transport since oligodendrocytes plated on fibronectin have transport issues that are alleviated when integrin signaling, and hence the signaling of fibronectin is blocked (Siskova *et al.* 2006).

Laminin-2 is the ECM ligand for two receptors expressed by oligodendrocytes, α6β1 integrin and dystroglycan (Colognato *et al.* 2007, Buttery & ffrench-Constant 1999) and is found in direct opposition to unmyelinated axons during development (Colognato *et al.* 2002). Laminin-2 is involved in growth-factor induced oligodendrocyte survival and enhanced oligodendrocyte differentiation (Baron *et al.* 2005, Colognato *et al.* 2002). The laminin-mediated promotion of oligodendrocyte survival and differentiation requires Fyn activity and α6β1 integrin function (Colognato *et al.* 2004, Camara *et al.* 2009).
While fibronectin plays a role in holding oligodendrocytes in a less mature morphology, laminin-2 is involved in promoting a mature oligodendrocyte process morphology and myelin sheath formation (Buttery & ffrench-Constant 1999, Olsen & ffrench-Constant 2005, Sypecka et al. 2009). *In vivo* animals with mutations of laminin-2 have a reduction in oligodendrocyte differentiation and a reduced number of myelinated small diameter axon fibers in various regions of the CNS (Chun et al. 2003, Relucio et al. 2009). The laminin-mediated effects on oligodendrocyte morphology and myelin formation require integrin-mediated PI3K and integrin linked kinase (ILK) activity (Chun et al. 2003). The laminin-induced effect on myelin formation appears to be, at least in part, mediated by the dystroglycan receptor, however little is known about the exact effects of laminin-2 association with dystroglycan receptors (Colognato et al. 2007). Fyn and FAK activity have been shown to be enhanced and appears to be required for laminin-2’s affect on oligodendrocyte morphology and myelination (Relucio et al. 2009, Hoshina et al. 2007).

Although FAK is a well known integrator of cell-ECM interactions, the involvement of FAK in the ECM-mediated effects on oligodendrocyte morphology during development, as discussed above, has remained a mystery. To unravel this mystery we utilized an *in vitro* system which allows for stringent control of the oligodendrocyte developmental stage as well as the ECM environment.
Chapter 4

Focal adhesion kinase (FAK) can play unique and opposing roles in regulating the morphological maturation of differentiating oligodendrocyte

(This chapter will be submitted as a paper to the Journal of Neurochemistry.)

Introduction

Focal adhesion kinase (FAK), also known as protein tyrosine kinase 2 (PTK2), is a ubiquitously expressed non-receptor tyrosine kinase that functions as an important regulator of cell shape and adhesion in response to environmental signals (Mitra et al. 2005, Parsons 2003, Schaller 2010, Hanks & Polte 1997, Schlaepfer et al. 1999). In particular, components of the extracellular matrix (ECM) are known to interact with transmembrane receptors of the integrin family and to subsequently recruit FAK to intracellular multi-molecular complexes, termed focal adhesions (Berrier & Yamada 2007, Giancotti & Ruoslahti 1999, Geiger et al. 2009, Schaller et al. 1992). FAK-containing focal adhesions function as key sensory machineries that integrate extracellular signals, interconnect them with the cell’s actin cytoskeleton and thus ultimately mediate complex cellular responses.

In the central nervous system (CNS) FAK expression has long been recognized to occur in neurons (Burgaya et al. 1995, Burgaya & Girault 1996, Burgaya et al. 1997, Grant et al. 1995, Stevens et al. 1996, Contestabile et al. 2003). Only more recently, however, its expression was characterized in cells of
the oligodendrocyte lineage (Bacon et al. 2007, Kilpatrick et al. 2000).

Oligodendrocytes, the myelin forming cells of the CNS, undergo extensive morphological remodeling when they differentiate from migratory bipolar oligodendrocyte precursor cells to post-migratory premyelinating oligodendrocytes that extend a complex process network and finally to mature oligodendrocytes that generate the myelin sheath (Pfeiffer et al. 1993, Baumann & Pham-Dinh 2001, Jackman et al. 2009). Completing these distinct steps of morphological maturation requires extensive remodeling of the cytoskeleton (Wang et al. 2008, Bacon et al. 2007, Kim et al. 2006, Miyamoto et al. 2007, Sloane & Vartanian 2007, Song et al. 2001, Bauer et al. 2009, Richter-Landsberg 2008, Southwood et al. 2007, Liang et al. 2004). Thus, FAK, as a key player in regulating cytoskeletal organization, is likely involved in the regulation of oligodendrocyte differentiation and myelination. Indeed, FAK has been found to mediate process outgrowth from cells of the rat-derived oligodendrocyte cell line CG4 (Hoshina et al. 2007). In addition, phosphorylation of FAK at its autophosphorylation site, which represents a critical event for FAK’s activation and catalytic function, has been described to occur primarily in post-migratory differentiating oligodendrocytes and to a lesser extent in migratory oligodendrocyte progenitor cells, suggesting a role for FAK primarily in maturing cells of the oligodendrocyte lineage (Liang et al. 2004). Furthermore, FAK has been implicated in a number of signaling pathways regulating oligodendrocyte differentiation (Wang et al. 2009, Rajasekharan et al. 2009, Miyamoto et al. 2007, Fox et al. 2004). Most importantly, both our lab and ffrench-constant’s lab
recently documented that conditional knock-out of FAK in maturing oligodendrocytes results in an inhibition and/or delay of normal developmental myelination (Camara et al. 2009, Forrest et al. 2009). Taken together, these studies highlight the importance of FAK in regulating myelination. However, the molecular mechanisms mediated by FAK in maturing oligodendrocytes underlying the observed phenotype in the conditional FAK knock-out mice are not fully understood.

Morphological maturation of cells of the oligodendrocyte lineage occurs in the context of a diverse extracellular environment. In particular two ECM proteins have been characterized with regard to their importance in oligodendrocyte differentiation and myelination, namely fibronectin and laminin-2. Both are present in the CNS during the time of normal developmental myelination (see Figure 4.7b). Fibronectin has been detected in the developing CNS in a likely diffuse manner, while non-basal lamina laminin-2 was found to be present on the axonal surface (Tom et al. 2004, Colognato et al. 2002, Zhao et al. 2009). Functionally, fibronectin was found to attenuate process outgrowth in oligodendrocytes, while laminin-2 has been implicated in stimulating myelin sheath formation (Buttery & ffrench-Constant 1999, Chun et al. 2003, Buttery & ffrench-Constant 2001, Olsen & ffrench-Constant 2005, Laursen & ffrench-Constant 2007, Colognato et al. 2005, Siskova et al. 2009, Maier et al. 2005). The opposing effects seen in the presence of fibronectin versus laminin-2 raise the question of whether either one or both ECM protein’s effects require FAK and how these effects may relate to the in vivo phenotype seen in the conditional
FAK knock-out mice.

In an attempt to better understand the role of FAK, as an integrator of ECM signaling, the current study investigated the role of FAK on the morphological maturation of post-migratory premyelinating oligodendrocytes in the presence of fibronectin and laminin-2 in a well defined *in vitro* system. The data presented here demonstrate the unique and opposing roles of FAK that are dependent on the ECM substrate present and on the developmental stage of the maturing oligodendrocytes. Thus, these data provide novel insight into the role of FAK and they highlight the multi-functionality of FAK in the context of developmental myelination.

**Materials and methods**

**Antibodies**

Hybridoma clone A2B5 (ATCC, Manassas, VA) was used for immunopanning of oligodendrocyte progenitor cells. Hybridoma clone O4 (gift from S. Pfeiffer) was used to identify post-migratory, premyelinating oligodendrocytes (Sommer & Schachner 1981, Bansal *et al.* 1989). Anti-MBP (SMI99; Covance, Princeton, NJ) was used for immunostaining. Anti-laminin alpha2, anti-fibronectin, anti-GAPDH, anti-FAK (Millipore, Billerica, MA) anti-Fyn and anti-Lyn (Cell Signaling, Beverly MA) antibodies were used for Western blots. For all immunostainings, Alexa 594 or 488-conjugated antibodies (Invitrogen/Molecular Probes, Carlsbad, CA) were used as secondary antibodies.
Horseradish peroxidase (HRP)-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) were used for Western blots.

**Animals**

Sprague–Dawley female rats with early postnatal litters were obtained from Harlan Laboratories (Indianapolis, IN). All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

**Primary Oligodendrocyte cultures**

Primary rat oligodendrocytes were isolated from postnatal (day 3 or 5) rat brains as described previously (Barres *et al.* 1992, Fox *et al.* 2003). Briefly, cerebral hemispheres were minced and incubated in Hanks balanced salt solution supplemented with 0.25% trypsin (Invitrogen, Carlsbad, CA), and 1 μg/ml DNase (Sigma, St. Louis, MO). After titration, single cells were collected by centrifugation, resuspended in Dulbecco's Modified Eagle Medium (DMEM)/10% FCS (Invitrogen, Carlsbad, CA) and subjected to A2B5 immunopanning. Immunopanned cells were plated onto fibronectin (10 μg/ml)-coated 6-well tissue culture dishes and cultured in serum-free proliferation medium (DMEM containing 10ng/ml PDGF (R&D Systems; Minneapolis, MN) and 5ng/ml bFGF (Sigma, St. Louis, MO); DMEM/PDGF/bFGF) for 15-20 hrs followed by 20-24 hrs in differentiation medium (DMEM containing 40 ng/ml tri-iodo-thyronine (T3; Sigma, St. Louis, MO) and 1× N2 supplement (Invitrogen,
Cells were then trypsinized and re-plated onto the various ECM substrates in the presence of differentiation medium and cultured for an additional 20-24 hrs, at which time cells were analyzed.

**siRNA-mediated knock-down of FAK expression**

Oligodendrocytes were isolated by A2B5-immunopanning from postnatal day 3 or 5 rat brains and plated onto fibronectin (10 μg/ml)-coated 6-well tissue culture dishes. Cells were cultured in serum-free proliferation medium (DMEM/PDGF/bFGF) for 15-20 hrs. Subsequently, cells were switched into differentiation medium (DMEM/T3/N2) and siRNA transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA). For siRNA transfection a siGLO green transfection indicator along with either a SMARTpool siRNA directed against rat FAK or a control non-targeting SMARTpool siRNA, all obtained from Thermo Fisher Scientific/Dharmacon, Inc. (Lafayette, CO), were used. Transfection medium containing siRNA-lipofectamine complexes was replaced with serum-free differentiation medium (DMEM/T3/N2) after 3 hrs and cells were cultured for an additional 15-20 hrs. Subsequently, cells were re-plated onto fibronectin (10 μg/ml), laminin-2 (10 μg/ml), or mixed substrate (5 μg/ml fibronectin and 5 μg/ml laminin-2)-coated glass coverslips and cultured for an additional 15-20 hrs. No significant reduction in total FAK protein levels was detected at the time of replating (data not shown).
**PF573228-mediated FAK inhibition**

Oligodendrocytes were isolated by A2B5-immunopanning from postnatal day 3 or 5 rat brains and plated onto fibronectin (10 μg/ml)-coated 6-well tissue culture dishes. Cells were cultured in serum-free proliferation medium (DMEM/PDGF/bFGF) for 15-20 hrs followed by an additional 20-24 hrs in differentiation medium (DMEM/T3/N2). Oligodendrocytes were then re-plated onto fibronectin (10 μg/ml), laminin-2 (10 μg/ml), or mixed substrate (5 μg/ml fibronectin and 5 μg/ml laminin-2)-coated glass coverslips, allowed to settle for 2-4 hrs and cultured in the presence of a specific inhibitor of FAK’s catalytic activity (PF573228 (Tocris Bioscience, Bristol, England) at 100nM or 0.1% DMSO as control). Cells were analyzed after an additional 15-20 hrs in culture.

**Process morphology analysis**

Process morphology was analyzed as previously described (Dennis et al. 2008). siRNA or FAK inhibitor-treated cells along with their respective control cells were immunostained with O4 antibodies. For siRNA-treated cells only those containing the siGLO green transfection indicator were analyzed. Images of at least 25 cells were taken randomly for each treatment group in each experiment (n ≥ 3) using an Olympus BX51 inverted fluorescent microscope (Olympus America Inc., Center Valley, PA). IP Lab imaging software (BD Biosciences Bioimaging, Rockville, MD) was used to determine process index (total area found to be O4-positive minus the cell body) and network area (total area within the radius of the process network surrounding the cell body minus the cell body).
In addition, the number of primary processes (any process directly extending from the cell body) was counted for each cell used for the above process morphology analysis. For the bar graphs representing network areas, process indices and primary process number the mean value for cells cultured under control conditions was calculated. This mean value was set to 100% and adjusted, i.e. normalized, values for all cells were averaged for each experimental condition.

A confocal laser scanning microscopy (TCS SP2 AOBS, Leica Microsystems, Exton, PA) was used for the generation of representative images. Images represent 2D maximum projections of stacks of 0.4µm optical sections.

**Live/Dead Viability Assay**

siRNA or FAK inhibitor-treated cells were assayed for cell viability using 2µM calcein AM/4µM ethidium homodimer-1 as described by the manufacturer (Live/Dead Viability Assay kit, Invitrogen Corp., Carlsbad, CA). Images of 8 fields at 10X magnification were taken randomly for each treatment group in each experiment \( (n = 3) \) using an Olympus BX51 inverted fluorescent microscope (Olympus America Inc., Center Valley, PA). The percentage of live cells (labeled with calcein) or dead cells (labeled with ethidium homodimer-1) was analyzed. Bar graphs represent the mean value of live or dead cells under each experimental condition.
Results

For early stage post-migratory premyelinating oligodendrocytes, FAK plays unique and opposing roles in regulating morphological maturation in the presence of fibronectin versus laminin-2

The ECM substrates laminin-2 and fibronectin have been previously described to differentially affect the maturation of post-migratory premyelinating oligodendrocytes (Buttery & ffrench-Constant 1999, Buttery & ffrench-Constant 2001, Olsen & ffrench-Constant 2005, Siskova et al. 2006, Siskova et al. 2009). To assess the effect of these ECM molecules in our tissue culture paradigm, we compared network areas and process indices as described previously (Dennis et al. 2008). In addition, we analyzed the effect of laminin-2 and fibronectin at two developmental stages, namely early and later stage post-migratory premyelinating oligodendrocytes. To obtain the early stage oligodendrocytes, oligodendrocyte progenitor cells were isolated from postnatal day 3 rat brains by A2B5 immunopanning and allowed to differentiate for 20-24 hours before re-plating onto the different ECM substrates. At the time of immunopanning, oligodendrocyte progenitor cells express only the progenitor cell marker recognized by the A2B5 antibody (Fox et al. 2004). In contrast, the cells henceforth referred to as later stage post-migratory premyelinating oligodendrocytes were isolated from postnatal day 5 rat brains by A2B5 immunopanning, at which stage the majority of cells express both the early marker recognized by the A2B5 antibody as well as a later stage surface antigen recognized by the O4 antibody.
(Fox et al. 2004). As described above, cells were allowed to differentiate for 20-24 hours before re-plating onto the different ECM substrates. In all cases, cells were analyzed after an additional 20-24 hours of culture in differentiation medium.

As shown in Figure 4.1, early stage oligodendrocytes plated on laminin-2 developed much larger network areas and process indices compared to oligodendrocytes plated on fibronectin. Early stage oligodendrocytes on laminin-2 revealed an increased occurrence of membrane sheet-like structures as denoted by the arrows in Figure 4.1a (right panel). The later stage post-migratory premyelinating oligodendrocytes showed a similar effect (network area fibronectin: 100%± 7, network area laminin-2: 155%± 10, p<0.05; process index fibronectin: 100%± 7, process index laminin-2: 153%± 10, p<0.05) with an even more obvious occurrence of membrane sheet-like structures (compare Figure 4.2c (left panel) with 3c (left panel)). Taken together, these studies demonstrate that the effect of fibronectin versus laminin-2 on oligodendrocyte maturation is in agreement with previous findings and that the effect of fibronectin versus laminin-2 on oligodendrocyte maturation occurs for both early and later stage oligodendrocytes and.

One of the major intracellular signaling molecules known to regulate cellular morphology is the nonreceptor tyrosine kinase FAK. To determine the extent to which FAK may play a role in mediating the differences in oligodendrocyte maturation seen in cells plated in the presence of fibronectin or laminin-2, early stage post-migratory premyelinating oligodendrocytes were
treated with a siRNA pool against FAK or a non-targeting siRNA pool as a control. Cells were cultured and analyzed as described above. As shown in Figure 4.2a and b, siRNA-mediated FAK knock-down resulted in a significant increase in both network area and process index for cells plated in the presence of fibronectin. In addition, the number of primary processes was found to be increased under these conditions. In contrast, when cells were plated in the presence of laminin-2, all three parameters were found to be decreased upon siRNA-mediated FAK knock-down (Figure 4.2c and d).

The effects on cell morphology described above were unlikely due to an interference with initial cell spreading, since at the time of re-plating no significant differences in FAK protein levels were noted (data not shown). At the time of analysis, however, FAK protein levels were found decreased by approximately 50-60% in cells treated with the FAK-specific siRNA pool as compared to cells treated with the control siRNA pool (see Figure 4.7a). It has been previously demonstrated that FAK not only acts as a regulator of actin dynamics and cell shape but can also be involved in signaling events controlling cell survival (Lim et al. 2008a, Westhoff et al. 2004, Mitra et al. 2005, Cox et al. 2006). In addition, elevated expression of Pyk2, a cytoplasmic tyrosine kinase closely related to FAK, was found to functionally compensate for a loss of FAK and to cause alterations beyond effects due to a loss of FAK in certain cell types under some circumstances (Weis et al. 2008, Lim et al. 2008b, Sieg et al. 1998). Thus, we assessed both cellular viability and Pyk2 protein levels. No significant differences were observed at the time of analysis (see Figures 4.7a and 4.8a). These
findings suggest that FAK regulates morphological maturation of post-migratory premyelinating oligodendrocytes without a significant change in cell viability or a considerable compensatory increase in Pyk2 expression.

During normal development morphological maturation of the oligodendrocyte’s process network occurs concurrently with the establishment of a protein expression profile characteristic for mature oligodendrocytes (Hardy et al. 1996, Emery et al. 2009, Dugas et al. 2006). Under experimental conditions, however, an independent regulation of these two processes has been observed indicating that morphological and gene expression profile maturation may be regulated by distinct molecular mechanisms (Buttery & ffrench-Constant 1999, Osterhout et al. 1999, Sloane & Vartanian 2007, Younes-Rapozo et al. 2009). To assess the extent to which the effect of siRNA-mediated FAK knock-down on cell morphology was associated with a change in the oligodendrocyte’s protein expression profile, the number of cells expressing one of the most extensively studied proteins associated with oligodendrocyte maturation, namely myelin basic protein (MBP), was determined. As shown in Figure 4.2e and f, no significant changes in the number of the MBP-expressing cells were noted under either of the conditions analyzed.

Thus, the above data demonstrate that FAK can regulate the morphology of the early stage post-migratory premyelinating oligodendrocyte’s process network in a unique and opposing manner depending on the ECM substrate present. In particular, FAK limits the maturation of the oligodendrocyte’s process network in the presence of fibronectin, while it promotes process maturation in
the presence of laminin-2. In both cases, this regulation appears to be
independent of survival and/or the expression of myelin proteins.

For later stage post-migratory premyelinating oligodendrocytes,
morphological maturation is significantly less dependent on FAK in the
presence of fibronectin but still remains significantly controlled by FAK in
the presence of laminin-2

Our previous in vivo data demonstrated a significant decrease in the
number of primary processes upon induction of FAK knock-out; an effect
resembling the one seen in the presence of laminin-2 but not fibronectin for the
early stage post-migratory Premyelinating oligodendrocytes (Figure 4.2c and d).
The in vivo data were obtained from P14 optic nerves and thus at more
advanced developmental stages than those analyzed in Figure 4.2. Based on
these observations, we hypothesized that the role of FAK in regulating the
oligodendrocyte's process network may change during developmental
maturation. To investigate this hypothesis, we treated and analyzed later stage
differentiating oligodendrocytes (isolated from postnatal day 5 rat brain) as
described above. Compared to the early stage post-migratory premyelinating
oligodendrocytes, the later stage cells were found to express similar levels of
FAK mRNA and protein (our unpublished data and (Dugas et al. 2006)). In
addition, there was no significant difference in the efficiency of siRNA-mediated
FAK knock-down between the two stages of oligodendrocytes (Figure 4.7a).
Furthermore, as observed for the early stage post-migratory premyelinating
oligodendrocytes, viability and Pyk2 expression remained unchanged in the later stage cells upon siRNA-mediated FAK knock-down (Figures 4.7b and 4.8b).

Unlike the early cells, however, there was no discernable change in the later stage post-migratory Premyelinating oligodendrocyte’s process network morphology in the presence of fibronectin upon siRNA-mediated FAK knock-down (Figure 4.3a and b). In contrast, in the presence of laminin-2 the later stage differentiating oligodendrocytes displayed a similar change in the process network morphology as seen for the early stage post-migratory premyelinating oligodendrocytes (compare Figure 4.2c and d with Figure 4.3c and d). As was observed for the early stage oligodendrocytes there was no notable change in the number of MBP expressing oligodendrocytes under either of the conditions (Figure 4.2e and f and 4.3e and f).

The above data demonstrate that with developmental maturation, FAK loses its capacity to and/or effectiveness in regulating the process network morphology of post-migratory pre-myelinating oligodendrocytes in the presence of fibronectin. FAK’s role in enhancing process network morphology in the presence of laminin-2, however, appears to remain unchanged.

For early stage post-migratory premyelinating oligodendrocytes, FAK’s constraining role for morphological maturation appears to predominate while for later stage oligodendrocytes FAK’s stimulatory role seems to prevail.

Both, fibronectin and laminin-2 have been found present in the developing
CNS during oligodendrocyte maturation (Figure 4.8g and (Fox et al. 2004, Colognato et al. 2002, Tom et al. 2004). Thus, we wished to determine the role of FAK for the morphological maturation of post-migratory premyelinating oligodendrocytes under conditions in which both fibronectin and laminin-2 are present in the environment. Early and later stage post-migratory premyelinating oligodendrocytes were cultured, treated and analyzed as described above, with the exception that cells were re-plated on a mixed substrate of equal concentrations of fibronectin and laminin-2 (instead of the individual substrates). Interestingly, even though FAK was found to regulate morphological maturation of early stage post-migratory premyelinating oligodendrocytes in an opposing manner on fibronectin versus laminin-2, its role on the mixed substrate appeared to be a primarily constraining one, i.e. similar to the one observed on fibronectin alone (Figure 4.4a and b). For the later stage post-migratory premyelinating oligodendrocytes, in which FAK appeared to have lost its capacity and/or effectiveness in regulating the process network morphology in the presence of fibronectin, the role of FAK was expectedly a stimulatory one, i.e. similar to the one seen on laminin-2 alone (Figure 4.4c and d).

These data suggest that during the developmental maturation of post-migratory premyelinating oligodendrocytes FAK’s role switches from a predominantly morphology restraining to a mostly morphology enhancing one.
FAK’s unique and opposing roles in regulating morphological maturation of post-migratory premyelinating oligodendrocytes are mediated by its catalytic activity

FAK’s role in regulating morphological remodeling in various cell types has at least *in vitro* been well documented to depend on its catalytic activity, i.e. autophosphorylation (Parsons 2003, Hanks *et al.* 2003, Playford & Schaller 2004, Schlaepfer *et al.* 1999, Cary *et al.* 1996). However, recent *in vitro* and *in vivo* studies highlight the importance of both catalytic-dependent and catalytic-independent mechanisms in mediating FAK’s physiological functions (Corsi *et al.* 2009, Lim *et al.* 2008a, Cance & Golubovskaya 2008). To assess the extent to which the above described effects of FAK in oligodendrocyte maturation were dependent on its catalytic activity, we used the FAK kinase inhibitor PF573228. This inhibitor has been shown in a variety of cell types to effectively block FAK autophosphorylation and downstream signaling without significantly affecting cell survival (Slack-Davis *et al.* 2007, Chen *et al.* 2009). Thus, to prevent potential effects of initial cell spreading differentiating oligodendrocytes were treated with PF573228 following re-plating. As described previously, treatment with PF573228 did not significantly affect the viability of differentiating oligodendrocytes (see Figure 4.9).

As shown in Figure 4.5, for both early and later stage post-migratory premyelinating oligodendrocytes treatment with PF573228 resulted in changes in cell morphology similar to the ones seen upon FAK knock-down. A concentration of 100 nM was reported to yield approximately half-maximal inhibition of FAK
autophosphorylation in various cell types (Slack-Davis et al. 2007). Thus, in both paradigms, siRNA-mediated FAK knockdown and PF573228 pharmacological inhibition, residual FAK activity likely remains. These data, therefore, suggest that a significant downregulation of FAK expression and/or FAK catalytic activity is sufficient to block the majority of FAK’s roles in regulating morphological maturation of differentiating oligodendrocytes. Interestingly, however, for later stage post-migratory pre-myelinating oligodendrocytes treatment with PF573228 in the presence of fibronectin resulted in a slight increase in the number of primary processes, while network area and process index remained unchanged. FAK’s role in limiting initial process outgrowth in later stage post-migratory pre-myelinating oligodendrocytes in the presence of fibronectin seems to be particularly sensitive to the inhibition of FAK’s catalytic activity.

Discussion

The current study demonstrates that FAK can regulate morphological maturation of post-migratory premyelinating oligodendrocytes in a unique and opposing fashion that is dependent on the nature of the ECM substrate present (i.e. fibronectin or laminin-2) and that is, for the most part, mediated by FAK’s catalytic activity. In addition, the regulatory role of FAK in the morphological maturation of post-migratory premyelinating oligodendrocytes was found to be tightly regulated with the cell’s stage of maturation. More specifically, for the early stage post-migratory premyelinating oligodendrocytes FAK’s constraining role on morphological maturation predominated, while its stimulatory role prevailed for
the later stage oligodendrocytes (see Figure 4.6). Taken together, the data presented here provide novel insight into the complex role that FAK plays in regulating the morphological maturation of differentiating oligodendrocytes in the context of developmental changes and spatial differences in the molecular composition of the extracellular environment.

FAK’s role in limiting the establishment of an expanded process network in the presence of fibronectin was found to be significantly diminished for later stage post-migratory premyelinating oligodendrocytes (see Figure 4.6). Fibronectin receptors αvβ1, αvβ3, αvβ5 and αvβ8 integrins are expressed and present on the surface of oligodendrocytes at all developmental stages (Milner & ffrench-Constant 1994, Milner et al. 1997, Cahoy et al. 2008, Dugas et al. 2006). Thus, while the functional role of FAK in the presence of fibronectin likely involves the activation of integrin type receptors, it seems unlikely that a reduced expression of fibronectin receptors is the primary cause of the observed reduction in FAK-mediated function for the later stage cells. It, therefore, appears to be the signaling pathway downstream of FAK that is altered in the more mature cells. Due to the complex nature of FAK-containing signaling complexes, multifaceted studies will be necessary to better understand the molecular mechanism responsible for the developmental difference observed here.

Interestingly, it has been previously reported that FAK can also limit the size of the axonal arbor for a variety of neuronal cell types (Rico et al. 2004). In addition, FAK has been found to limit the length and number of dendritic protrusions (Moeller et al. 2006, Shi et al. 2009). Both functions of FAK were
found to be, at least in part, dependent on FAK’s catalytic activity. Most importantly, FAK was implicated in controlling the establishment of the final pattern of connections between neurons and their targets by enabling efficient pruning of overproduced and/or ‘weak’ connections. With regard to the findings described here, it is, therefore, tempting to speculate that the process network limiting function of FAK observed in the presence of fibronectin, an ECM molecule that is expressed broadly during development, allows for efficient pruning of ‘non-functional’ oligodendroglial processes and represents part of the regulatory mechanism that determines the final number of myelinated segments that are generated by an individual mature oligodendrocyte.

In contrast to FAK’s process network limiting role discussed above, its role in stimulating the establishment of a more mature morphology in the presence of laminin-2 remained unchanged during the maturation of post-migratory premyelinating oligodendrocytes and was thus found to predominate for the later stage cells. Due to the more restricted presence of laminin-2 on the axonal surface (Colognato et al. 2002), this function of FAK may play a more spatially confined role than the one seen in the presence of fibronectin. Notably, the interaction between laminin-2 and the integrin receptor α6β1 has been well demonstrated to be involved in regulating the morphological maturation of post-migratory premyelinating oligodendrocytes in vitro and in vivo (Camara et al. 2009, Lee et al. 2006, Buttery et al. 1999, Barros et al. 2009, Relvas et al. 2001, Olsen & ffrench-Constant 2005, Chun et al. 2003, Colognato et al. 2002, Laursen & ffrench-Constant 2007). Most importantly, hypomyelinating phenotypes
observed upon reducing α6β1 integrin signaling in maturing oligodendrocytes resemble those seen upon inducing FAK knock-out in maturing oligodendrocytes (Camara et al. 2009, Forrest et al. 2009). Thus, in the presence of laminin-2 FAK likely functions as a downstream target of laminin-2-α6β1 integrin interactions. In addition to regulating morphological maturation of differentiating oligodendrocytes laminin-2-α6β1 integrin interactions have been described to promote the transition from oligodendrocyte progenitor cells to mature MBP-expressing oligodendrocytes and to enhance survival of oligodendrocytes in response to limiting concentrations of growth factors (Colognato et al. 2007, Frost et al. 1999, Baron et al. 2005, Colognato et al. 2002). However, no significant changes in the number of MBP-expressing or surviving cells were noted in the studies presented here. These finding are in agreement with the data obtained from conditional FAK knock-out mice, in which no obvious changes in the number of cells were noted (Forrest et al. 2009). Thus, FAK is unlikely to play a significant role in regulating myelin protein gene expression and/or survival for post-migratory premmyelinating oligodendrocytes as a downstream target of laminin-2-α6β1 integrin interactions. This interpretation may be supported by the discovery that the src family kinase (SFK) regulatory proteins Csk and Cbp are critically involved in mediating the transition from oligodendrocyte progenitor cells to MBP-expressing oligodendrocytes downstream of laminin-2-α6β1 integrin interactions (Colognato et al. 2004, Relucio et al. 2009). Taken together, these findings, therefore, strongly suggest a crucial role of FAK in regulating the morphological maturation of post-migratory premmyelinating oligodendrocytes by
functioning as a downstream target of spatially restricted laminin-2-α6β1 integrin interactions that affect the organization of the cell’s cytoskeleton. Interestingly, dystroglycan has been recently identified as a second laminin receptor functionally involved in promoting oligodendrocyte maturation and myelination (Colognato et al. 2007). However, knock-down of dystroglycan was found to significantly affect both myelin gene expression as well as morphological maturation of oligodendrocytes. Thus, it is unclear to what extent laminin-2-dystroglycan interactions may contribute to the FAK-mediated mechanism described here.

The role of FAK in regulating oligodendrocyte morphology in the presence of both fibronectin and laminin-2 was seen to be largely dependent on FAK’s catalytic activity. Phosphorylation of FAK at its autophosphorylation site creates a high affinity binding site for Src-homology 2 (SH2) domain containing proteins, in particular SFKs (Mitra et al. 2005, Mitra & Schlaepfer 2006, Parsons 2003, Cobb et al. 1994). The binding of SFKs to FAK can lead to the formation of a FAK-activated SFK complex, which has the capability to act as a regulator of cell shape. In addition, it has been demonstrated that FAK can directly phosphorylate SFKs at their Y418 activation site (Wu et al. 2008). Two SFKs have been described to be expressed at significant levels in maturing oligodendrocytes, namely Fyn and Lyn (Colognato et al. 2004, Osterhout et al. 1999, Kramer et al. 1999). In agreement with these studies, both Fyn and Lyn were found expressed in post-migratory premyelinating oligodendrocytes at the maturation stages analyzed here (see Figure 4.8c-f). The SFK Fyn has been well documented to be
involved in regulating morphological maturation of differentiating
oligodendrocytes via the laminin-2-α6β1 integrin pathway (Colognato et al. 2004,
Relucio et al. 2009). Taken together with the data presented here, morphological
maturation of post-migratory premyelinating oligodendrocytes appears to be
mediated by the concerted action of FAK and Fyn as downstream targets of
laminin-2-α6β1 integrin interactions (see also (Hoshina et al. 2007). In contrast
to the laminin receptor α6β1 integrin, which has been shown to associate with
Fyn, the fibronectin receptor αvβ3 integrin was found to associate with the SFK
Lyn and not Fyn (Colognato et al. 2004). This association has been functionally
implicated in the regulation of PDGF-mediated oligodendrocyte progenitor cell
proliferation. The data presented here, could indicate an additional role, i.e. a role
in negatively regulating the morphological maturation of post-migratory
premyelinating cells via a fibronectin-αvβ3 integrin-FAK-Lyn pathway.

The current study provides novel insight into the complex and diverse
roles that FAK can play in regulating the morphological maturation of post-
migratory premyelinating oligodendrocytes. In vivo FAK knock-out was found to
lead to a delay in myelination, a phenotype that is associated with a reduction in
the number of primary oligodendroglial processes (Camara et al. 2009, Forrest et
al. 2009). Based on the data presented here, this phenotype appears, at least in
part, to be due to a lack of FAK’s maturation promoting role in the presence of
laminin-2. In the context of demyelinating diseases, such as Multiple Sclerosis,
FAK, therefore, presents itself as a good therapeutic target for promoting
remyelination. However, demyelinated lesions are characterized by an
extracellular environment that is different from the one found in the developing
CNS. In particular, high levels of fibronectin were noted in lesions of Multiple
Sclerosis patients (Satoh et al. 2009, van Horssen et al. 2007, Sobel & Mitchell
1989). Thus, FAK signaling may, at least in part, contribute to the limited repair
seen in such lesions. Taken together, the findings presented here highlight the
complexity of FAK’s role in regulating the maturation of post-migratory
premyelinating oligodendrocytes, and they emphasize the importance of a better
understanding of the signaling pathways involving FAK in order to be able to
design effective therapeutic strategies for promoting remyelination under
pathological demyelinating conditions.
Figure 4.1 Morphological maturation of post-migratory premyelinating oligodendrocytes is uniquely regulated in the presence of fibronectin or laminin-2. Oligodendrocyte progenitor cells were isolated by A2B5 immunopanning from postnatal day 3 rat brains. After 2 days in culture, differentiating oligodendrocytes were re-plated onto fibronectin (Fn) or laminin-2 (Ln)-coated glass coverslips and allowed to differentiate for 20-24 hours. (a) Representative images of cells stained with the O4 antibody. (b) Bar graphs depicting quantitative analyses of the total area occupied by the cells’ process network (network area; left graph) and the total area of O4-positive process surfaces per cell (process index; right graph). Mean control network areas and process indices were set to 100% and all values were adjusted accordingly. Means and standard errors are shown. Four independent experiments were performed and 25 cells per experiment were analyzed. For both parameters, Student’s t-test analysis revealed an overall two-tailed significance level of p<0.05 (indicated by the star). Scale Bar: 20 μm.
Figure 4.2 siRNA-mediated knock-down of FAK expression affects morphological maturation of early stage post-migratory premelinating oligodendrocytes distinctively and in an opposing fashion in the presence of fibronectin or laminin-2. Cells were isolated, differentiated and analyzed as described in Figure 4.1. Cells were, however, treated with a control siRNA pool (siControl) or a siRNA pool against FAK (siFAK) 15-20 hours after initial plating. (a) Representative images of cells stained with the O4 antibody and plated in the presence of fibronectin (Fn). (b) Bar graphs representing quantitative analyses of network areas, process indices and primary process numbers of cells plated in the presence of fibronectin (Fn). (c) Representative images of cells stained with the O4 antibody and plated in the presence of laminin-2 (Ln). (d) Bar graphs representing quantitative analyses of network areas, process indices and primary process numbers of cells plated in the presence of laminin-2 (Ln). (e) Representative images of cells stained with an antibody specific for myelin basic protein (MBP) (left panels) and with Hoechst to visualize nuclei (left panels). (e) Bar graph representing the percentage of Hoechst-positive cells that are also MBP-positive. In all bar graphs, means and standard errors of at least three independent experiments (25 cells each for all conditions) are shown. Stars indicate an overall two-tailed significance level of p<0.05 as determined by Student’s t-test analysis. Scale Bars: 20 μm (in a and b), 50 μm (in e).
Figure 4.3 siRNA-mediated knock-down of FAK expression fails to affect morphological maturation of later stage post-migratory premyelinating oligodendrocytes in the presence of fibronectin but still affects maturation in the presence of laminin-2. Later stage oligodendrocytes were isolated, differentiated, treated and analyzed as described in Figure 4.2. (a) Representative images of cells stained with the O4 antibody and plated in the presence of fibronectin (Fn). (b) Bar graphs representing quantitative analyses of network areas, process indices and primary process numbers of cells plated in the presence of fibronectin (Fn). (c) Representative images of cells stained with the O4 antibody and plated in the presence of laminin-2 (Ln). (d) Bar graphs representing quantitative analyses of network areas, process indices and primary process numbers of cells plated onto laminin-2 (Ln) as substrate. (e) Representative images of cells stained with an antibody specific for myelin basic protein (MBP) (left panels) and with Hoechst to visualize nuclei (left panels). (e) Bar graph representing the percentage of Hoechst-positive cells that are also MBP-positive. In all bar graphs, means and standard errors of at least three independent experiments (25 cells each for all conditions) are shown. Stars indicate an overall two-tailed significance level of p<0.05 as determined by Student’s t-test analysis. Scale Bars: 20 μm (in a and b), 50 μm (in e).
Figure 4.3

(a) siControl Fn  siFAK Fn

(b) 300 300 300
    Network Area (in %) Process efficacy (in %) Primary Process Number (in %)

(c) siControl Ln  siFAK Ln

(d) 300 300 300
    Network Area (in %) Process efficacy (in %) Primary Process Number (in %)

(e) siControl Fn  siFAK Fn

(f) 20
    Number of MECP+ cells (in %)

(siControl Fn  siFAK Fn  siControl Ln  siFAK Ln)
Figure 4.4 siRNA-mediated knock-down of FAK expression in the presence of a mixed fibronectin/laminin-2 substrate affects morphological maturation of early and later stage post-migratory premyelinating oligodendrocytes in a distinctive and opposing fashion. Cells were isolated, differentiated, treated and analyzed as described in Figure 4.2. (a,c) Representative images of cells stained with the O4 antibody. Early stage post-migratory premyelinating oligodendrocytes are shown in (a), while (c) depicts later stage cells. Scale Bars: 20 μm. (b,d). Bar graphs representing quantitative analyses of network areas, process indices and primary process numbers. Means and standard errors of at least three independent experiments (25 cells each for all conditions) are shown. Stars indicate an overall two-tailed significance level of p<0.05 as determined by Student’s t-test analysis.
Figure 4.5 Inhibition of FAK’s catalytic activity affects morphological maturation of early and later stage post-migratory premyelinating oligodendrocytes in a similar fashion as siRNA-mediated knock-down of FAK expression. Cells were isolated, differentiated and analyzed as described in Figures 4.1. Cells were, however, treated with the FAK inhibitor PF573228 (PF228; 100 nM)) or vehicle (control; 0.1% DMSO) (a,b). Bar graphs representing quantitative analyses of network areas, process indices and primary process number of early (in a) and later (in b) stage post-migratory premyelinating oligodendrocytes in the presence of fibronectin (Fn) or laminin-2 (Ln). Means and standard errors of at least three independent experiments (25 cells each for all conditions) are shown. Stars indicate an overall two-tailed significance level of p<0.05 as determined by Student’s t-test analysis.
Figure 4.6 Proposed model for the role of FAK in morphological maturation of post-migratory premyelinating oligodendrocytes. The data presented here demonstrate that FAK can have unique and opposing roles depending on the maturation stage of the cell and the nature of the prevalent ECM molecule that the cell encounters. In early stage post-migratory pre-myelinating oligodendrocytes FAK is involved in restraining morphological maturation in the presence of fibronectin, while it promotes maturation in the presence of laminin-2. Based on the mixed substrate data, it is the restraining role of FAK that predominates in these early stage cells. In the later stage post-migratory pre-myelinating oligodendrocytes FAK’s role in restraining morphological maturation in the presence of fibronectin is significantly diminished or even lost. FAK’s maturation promoting role, however, still remains active. Taken together, these data uncover the complexity of FAK’s role in the regulation of morphological maturation of post-migratory premyelinating oligodendrocytes and they suggest that proper oligodendrocyte maturation requires a well coordinated balance between maturation restraining and promoting mechanisms.
Figure 4.7 siRNA treatment of differentiating oligodendrocytes reduces FAK protein levels without significantly affecting cell viability. (a-b) Bar graphs representing FAK protein levels as determined by Western blot analysis for early (a) or later stage (b) post-migratory premyelinating oligodendrocytes treated with a siRNA pool against FAK (siFAK) or a non-targeting siRNA pool as control (siControl) in the presence of fibronectin (Fn) or laminin-2 (Ln). Protein levels were quantified using enhanced chemiluminescence (ECL) detection in combination with VersaDoc imaging and the use of the QuantityOne software package (BioRad Laboratories, Hercules CA). GAPDH protein levels were used for normalization. A representative Western blot is shown in the inset in (a). Dashed lines represent control levels of FAK protein, which were set to 100% for each condition. The efficiency of the siRNA transfection for all conditions was approximately 70% (data not shown). (c-d) Bar graphs representing the percentage of enzymatically converted calcein AM-positive (live; black bars) or ethidium homodimer-positive (dead; grey bars) cells following treatment with a siRNA pool against FAK (siFAK) or control siRNA pool (siControl) in the presence of fibronectin (Fn) or laminin-2 (Ln). In (c) the results for early stage post-migratory premyelinating oligodendrocytes are shown, whereas in (d) the results for the later stage cells are depicted. In all bar graphs, means and standard errors of at least three independent experiments (25 cells each per condition) are shown. Stars indicate an overall two-tailed significance level of p<0.05 as determined by Student’s t-test analysis.
Figure 4.7
Figure 4.8 The protein levels of the non-receptor tyrosine kinase family member Pyk2 (a and b) and the src kinases Fyn (c and d) and Lyn (e and f) are not significantly affected upon siRNA-mediated FAK knock-down. Total cell homogenates were prepared from early (a, c and e) and late stage (b, d and f) post-migratory premyelinating oligodendrocytes, treated with a siRNA pool against FAK (siFAK) or control siRNA pool (siControl) and plated on fibronectin (Fn) or laminin-2 (Ln). Cell lysates were analyzed by Western blot analysis as described in Figure 4.7 and normalized to GAPDH. Representative Western blots of the later stage oligodendrocytes plated on laminin-2 are shown in the insets in (b, d, and f). Dashed lines represent control levels of protein, which were set to 100% for each condition. Densitometrical measurements were analyzed for at least three independent experiments (p values are indicated in white on each bar).

g) Both fibronectin and laminin-2 are present in vivo in the mouse optic nerve at developmental time points when oligodendrocyte maturation and myelination occur. Whole tissue homogenates were prepared from postnatal day (P) 2, 5, 14 and 30 optic nerves. Homogenates were analyzed using Western blots as described in Figure 4.7
Figure 4.8
Figure 4.9 Inhibition of FAK’s catalytic activity using the inhibitor PF573228 does not significantly affect the viability of early and later stage post-migratory pre-myelinating oligodendrocytes. Bar graphs representing the percentage of enzymatically converted calcein AM-positive (live; black bars) or ethidium homodimer-positive (dead; grey bars) cells following treatment with PF573228 (PF288) or vehicle (control) in the presence of fibronectin (Fn) or laminin-2 (Ln). In all bar graphs, means and standard errors of at least three independent experiments (25 cells each per condition) are shown. Stars indicate an overall two-tailed significance level of p<0.05 as determined by Student’s t-test analysis.
Chapter 5

Final conclusions

Various pathological conditions affect the oligodendrocyte’s myelin sheath and hence disrupt the ability of axonal signals to be communicated effectively leading to a multitude of devastating symptoms. As discussed in chapter one the most prevalent primary demyelinating pathology of the CNS in humans is MS. While there are premyelinating oligodendrocytes found in at least some MS lesion, their final maturation appears blocked and there is little to no successful remyelination (Chang et al. 2002, Kuhlmann et al. 2008). Due to the lack of remyelination in pathologies involving oligodendrocyte and myelin sheath destruction, identifying potential targets that will enhance the derelict endogenous remyelination could lead to potential cures for these pathologies. In order to fully understand the optimal therapeutic targets that will promote remyelination in pathologies, such as MS, it is first essential to understand the factors involved in developmental oligodendrocyte maturation and myelination.

Throughout development oligodendrocytes undergo many drastic morphological metamorphoses; developing from bipolar OPCs into highly complex premyelinating oligodendrocytes and finally into fully myelinating mature oligodendrocytes. Such extreme morphological remodeling requires drastic cytoskeletal rearrangement. FAK is a known leading regulator of cytoskeletal rearrangement and hence an ideal candidate for further exploration into its role during developmental oligodendrocyte morphological maturation.
In vivo analysis of FAK during initial CNS myelination

In order to ascertain if FAK is involved in later stage oligodendrocyte morphological maturation and initiation of myelination, temporally-regulated oligodendrocyte-specific FAK knockout mice were analyzed (Figure 2.1). These studies demonstrate that during development FAK expression is required for appropriate initiation of myelination. Mice lacking FAK in oligodendrocytes during later stages of morphological remodeling and initiation of myelination have fewer myelinated fibers than control littermates (Figure 2.2). The hypomyelinating phenotype without FAK expression in oligodendrocytes was also observed by ffrench-Constant’s group, finding that this phenomenon is primarily restricted to the small diameter axonal fibers during initiation of myelination in conditional knockout mice (FAK^{floxflox}, CNP/Cre) (Camara et al. 2009). This additional information is of interest since the small diameter axonal fibers are the last to be myelinated during normal development (Hildebrand & Waxman 1984, Fraher et al. 1988, Schwab & Schnell 1989).

We further explored the possible explanations for this hypomyelination and discovered that the reduction in myelinated axonal fibers could be due to inappropriate morphological remodeling of the oligodendrocyte process network prior to and during initial myelination. FAK null oligodendrocytes have a reduced number of primary processes (Figure 2.4). These data could indicate that normal myelination can progress to a point at which time the resources of the FAK null oligodendrocyte are depleted and the last fibers to be myelinated, the small diameter fibers, are left unmyelinated. Alternatively this could involve the relation
of axonal diameter size to the number of internodal segments myelinated by a single oligodendrocyte, i.e. that oligodendrocytes that myelinate large diameter axons myelinate fewer internodal segments than those that myelinate small diameter fibers (Butt & Ransom 1989, Remahl & Hilderbrand 1990). Due to the reduction in the number of primary processes the FAK-null oligodendrocytes could be ideal for myelinating the larger diameter fibers, however, they might not have the required number of processes to efficiently myelinate the small diameter axon’s internodal segments.

In both oligodendrocyte-specific FAK-null mouse systems myelin appears normal in the adults suggesting a transient effect in which the timing of myelination is negatively affected (Figure 2.5 (Camara et al. 2009)). The normal myelination seen in the adult FAK^{floxflox}; CNP/Cre mice could be due to early knock-out of FAK under the CNP promoter. The early elimination of FAK from oligodendrocytes could allow for the upregulation of a compensatory mechanism that could repair the initial hypomyelination observed leading to the transient phenotype. One of the main candidates for such a compensatory mechanism is Pyk2 since it is highly expressed in the brain as well as being expressed in oligodendrocytes (Figure 4.8) and is the only other family member of the non-receptor tyrosine kinase family (Menegon et al. 1999). Pyk2, however, has been shown incapable of replacing all of the functions FAK fulfills, including those involved in cytoskeletal dynamic regulation (Schlaepfer et al. 1999, Klingbeil et al. 2001). The levels of Pyk2 are also shown to be upregulated in MS lesions weakening the idea that Pyk2 could effectively compensate for FAK and promote
normal myelination since it is highly expressed during a pathological condition in which remyelination is limited (Comabella & Martin 2007).

A more likely candidate involved in a possible compensatory mechanism would be ILK, which is associated with focal adhesion molecules and is expressed in the CNS (Chun et al. 2003, Wu & Dedhar 2001). ILK has also been shown to be involved in myelin sheet formation in vitro; dominant negative ILK reduces the formation of myelin sheets on the permissive laminin-2 ECM substrate (Chun et al. 2003). Therefore ILK could signal to many of the molecules also regulated by FAK and could compensate for FAK function in myelin formation providing that it is activated at an early enough time point.

Our system also displays proper myelination in the adult, an effect that could possibly be due to the timing of induced FAK knock-out rather than reflecting a transient effect. To remain consistent with the developmental studies, tamoxifen injections, and thus induction of FAK knock-out, were carried out from P2 through P14 allowing tamoxifen to be flushed from the system around P16-17, a time point at which myelination is still highly active in the optic nerve. Without tamoxifen in the system Cre recombinase is unable to translocate to the nucleus effectively inhibiting FAK knockout in oligodendrocytes derived following tamoxifen elimination. A second wave of oligodendrocytes, expressing normal levels of FAK, could have migrated to the areas of hypomyelination and myelinated any of the small diameter fibers that the FAK null oligodendrocytes were unable to myelinate, effectively repairing the hypomyelinating phenotype (Colello et al. 1994, Kessaris et al. 2006).
Currently the exact nature of possible compensatory mechanisms is unknown. The most elegant way to assess the actual role of FAK throughout development, from oligodendrocyte morphological maturation through myelin maintenance, would be to continue tamoxifen injections in our inducible FAK null mice throughout adulthood, thus eliminating the possibility of any unaffected oligodendrocytes repairing the hypomyelinated region. Additional studies in which the timing of tamoxifen injection is altered to target the development of oligodendrocytes in other CNS regions could also determine whether the effect of FAK on oligodendrocyte initial myelination is ubiquitous throughout the CNS or is region specific.

**In vitro analysis of FAK in post-migratory premyelinating oligodendrocyte morphological maturation**

Since we discovered that inappropriate oligodendrocyte morphological remodeling could be responsible for the hypomyelination observed during initial myelination *in vivo* we began exploring the possibility that FAK could be involved in oligodendrocyte morphological maturation leading up to myelination. To further elucidate FAK’s role during oligodendrocyte morphological remodeling prior to myelination we utilized an *in vitro* system which allowed for strict regulation of both the environment and developmental stage of the oligodendrocytes analyzed.

These studies revealed that FAK can regulate morphological maturation of post-migratory premyelinating oligodendrocytes in a unique and opposing fashion
that is dependent on the nature of the ECM substrate present and that is, for the most part, mediated by FAK’s autophosphorylation and catalytic activity. Early post-migratory premyelinating oligodendrocytes with a reduction in appropriate FAK signaling (either via a siRNA knockdown of FAK protein levels or an inhibition of FAK catalytic function) in the presence of fibronectin have unbridled process networks, whereas when in the presence of laminin-2 oligodendrocytes with reduced FAK signaling are unable to form or maintain the appropriately substantial morphology required at that stage of development (Figure 4.2 and Figure 4.5). Both signaling pathways in the presence of fibronectin and in the presence of laminin-2 converge on FAK and, based on the ECM signal that FAK receives, it is able to uniquely modulate the oligodendrocyte process network.

In the proposed mechanism fibronectin most likely signaling through the $\alpha_v\beta_3$ integrin receptor, would signal for morphological remodeling at early developmental time points (Olsen & ffrench-Constant 2005, Lee et al. 2006). This association is thought to lead to recruitment of FAK and the SFK member, Lyn, to the focal adhesion. Lyn is the most likely candidate for this association since it is upregulated at an early time point during oligodendrocyte development and has been shown to be associated with the $\alpha_v\beta_3$ integrin receptor (Colognato et al. 2004, Kramer et al. 1999).

The restriction of the oligodendrocyte process network in the presence of fibronectin could be associated with FAK signaling to cytoskeletal regulators. This signaling pathway could involve signaling to Rho family GTPases such as RhoA, Rac, and Cdc24, which have been well characterized to modulate the
cytoskeleton in various cell types and specifically during oligodendrocyte development, as discussed in chapter one (Sypecka et al. 2009, Mitra et al. 2005, Romer et al. 2006, Hoshina et al. 2007, Rico et al. 2004, Moeller et al. 2006, Wang et al. 2009). FAK associates with both GEFs, which promote activation of GTPases, and GAPs, which decrease the activity of GTPases, allowing for tightly regulated mediation of process morphology (Tomar & Schlaepfer 2009). A limited activation of p190RhoGAP and/or GRAF by FAK would enhance RhoA activity, previously demonstrated to mediate cytoskeleton contraction and process retraction, which could lead to reduced process network formation in the early oligodendrocytes (Liang et al. 2004, Taylor et al. 1998). The decrease in FAK association with p130Cas could also lead to restricted Rac activation which would reduce process outgrowth limiting the ability of an early oligodendrocyte to efficiently differentiate morphologically (Mitra et al. 2005, Liang et al. 2004, Schlomann et al. 2009).

Inappropriate membrane transport, which is observed in oligodendrocytes in the presence of fibronectin, could keep proteins from being over produced and transported, which would reduce process network expansion, thus reducing the risk of over utilizing and depleting resources required for later stages of morphological maturation and myelination (Siskova et al. 2006).

The FAK-mediated effect on oligodendrocyte morphology in the presence of fibronectin was also found to be, at least in part, developmentally regulated. Later stage post-migratory premyelinating oligodendrocytes no longer respond to
the FAK-mediated restriction of the process network in the presence of fibronectin (Figure 4.3 and Figure 4.5).

Interestingly fibronectin levels are upregulated and incongruous fibronectin deposits are found throughout MS lesions (Comabella & Martin 2007, Sobel 1998). The remodeling of fibronectin as well as other ECM molecules is also skewed in MS lesions (Cossins et al. 1997, Sobel 1998). In concert with the above proposed mechanism of early oligodendrocyte morphological remodeling, the fibronectin deposits within the MS lesion could prevent efficient maturation of differentiating oligodendrocytes and thus restrict remyelination.

The association of oligodendrocytes with laminin-2 is most likely mediated through the α6β1 integrin. As discussed in chapter three, laminin-2 is a well characterized regulator of oligodendrocyte morphological remodeling and the α6β1 integrin receptor has been implicated in regulating oligodendrocyte maturation and myelination (Baron et al. 2005, Colognato et al. 2007, Olsen & ffrench-Constant 2005, Chun et al. 2003, Colognato et al. 2002). This association recruits FAK and the SFK member, Fyn, which in turn signals FAK to perform various cellular functions. Fyn has been greatly implicated in the morphological differentiation regulated by laminin-2 signaling and is highly associated with α6β1 integrin (Colognato et al. 2004, Osterhout et al. 1999, Antonyak & Cerione 2009).

Due to the observation that neither Fyn nor Lyn levels change in our system (Figure 4.8c-f) it is unlikely that there is a feedback loop affecting the expression of SFKs, or if there is a feedback loop the small percentage of SFK
found in association with FAK (only 3-5%) is not substantial enough to show an overall change (Cobb et al. 1994).

A strong candidate for a downstream signaling target of FAK involved in the morphological maturation of oligodendrocytes in the presence of laminin-2 is anti-apoptotic serine threonine kinase (Akt) via activation of PI3K. FAK has been shown to activate PI3K which could then activate Akt signaling for various cellular functions (Chen & Guan 1994, Del Re et al. 2008).

The requirement of Akt activity for oligodendrocyte survival has been well characterized in vitro (Cui et al. 2005, Bibollet-Bahena & Almazan 2009). More importantly, the activation of the PI3K/Akt signaling pathway has also been associated with cytoskeletal rearrangement and morphological remodeling (Del Re et al. 2008, Endo & Yamashita 2009). PI3K-induced Akt activation is enhanced with the progression of oligodendrocyte differentiation (Tyler et al. 2009). Activation of the PI3K/Akt signaling pathway enhances CNS myelination while inhibition of this pathway has been shown to reduce the efficiency of myelination (Flores et al. 2008, Barros et al. 2009, Fraser et al. 2008, Tohda et al. 2006). Therefore it is possible that upon association of laminin-2 with α6β1 integrin Fyn enhances FAK-mediated activation of PI3K which could lead to increased activation of Akt and oligodendrocyte differentiation and the expanded process network observed.

The data presented in this dissertation reveal the importance of FAK signaling during oligodendrocyte morphological maturation and myelination in CNS development. Due to the opposing roles of FAK in the presence of the
different ECM molecules, fibronectin or laminin-2, meaningful direct regulation of FAK in pathological conditions may be difficult to achieve. A potential approach to indirectly modulate FAK-mediated oligodendrocyte morphological maturation during pathology would be to manipulate the ECM environment or activation of FAK-associated molecules, primarily Fyn and Lyn or integrin receptors. Thus, the findings presented here highlight the importance of better understanding the molecular mechanisms regulating developmental myelination in order to develop novel therapies which would promote remyelination in MS and other oligodendrocyte pathologies.
Figure 6.1 Theoretical schematic for FAK-mediated signaling pathways regulating morphological maturation of post-migratory premyelinating oligodendrocytes. Upon association of fibronectin with the integrin receptor $\alpha_v\beta_3$ a complex of FAK and Lyn is activated. This activation could lead to the regulation of the Rho family of GTPases, primarily RhoA, Rac, and Cdc42, enhancing oligodendrocyte process retraction and restricting oligodendrocyte process extension thus holding the early stage post-migratory premyelinating oligodendrocyte in a less mature morphology. Upon association of laminin-2 with the integrin receptor $\alpha_6\beta_1$, however, activation of a FAK-Fyn complex initiates activation of the PI3K/Akt pathway leading to enhanced oligodendrocyte morphological maturation and myelination.
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Vita

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Manuscripts resulting from Audrey’s work at Virginia Commonwealth University:

**AD Lafrenaye** and B Fuss Focal adhesion kinase (FAK) can play unique and opposing roles in regulating the morphological maturation of the differentiation oligodendrocyte (in preparation)
