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

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

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Akt	anti-apoptotic serine threonine kinase
Arp 2/3	actin related protein 2/3
ASAP1	Arf GTPase-activation protein GAP 1
CNP	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
E	Early
ECM	Extracellular Matrix
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FERM	FAK N-terminal ezrin/radixin/moesin homology
Fn	Fibronectin
GAP	GTPase-activation protein
GEF	Guanine exchange factor
Graf	GTPase regulator associated with FAK
ILK	integrin linked kinase
L	Late
Ln	Laminin-2
MAPK	mitogen-activated protein kinase
MBP	Myelin basic protein
MS	Multiple Sclerosis
N-WASP	neural-Wiskott Aldrich Syndrome protein

OPC	Oligodendrocyte precursor cell
P	Postnatal
PDGFαR	Platelet-derived growth factor α receptor
PI3K	Phosphotidylinositol 3-kinase
PLP	Proteolipid protein
PRD	Proline rich domain
PRR	Proline rich region
PTK 2	protein tyrosine kinase 2
SFK	Src family kinase
SH2	Src homology 2 domain
SH3	Src homology 3 domain
Shh	Sonic hedgehog

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.

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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 ($Fak^{flox/flox}$: $PLP/CreER^{T}$ 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 oligodendrocytes (Kaplan *et al.* 1997, Kaplan *et al.* 2001, Dupree *et al.* 2005).

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 in 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

some debate as to what constitutes an early glial progenitor (Jakovcevski & Zecevic 2005, Miller 2002, Masahira *et al.* 2006, Nicolay *et al.* 2007, Wegner 2008).

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 mictrotubules 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 mictrotubule 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 fillopodial 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 μm^2 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 is a ubiquitously expressed 125 kDa non-receptor protein tyrosine kinase that was characterized by Steve Hanks, Jun-Lin Gaun, and Michael Schaller in 1992 (Schaller *et al.* 1992, Guan & Shalloway 1992, Hanks *et al.* 1992). FAK plays a key role in integrin-mediated focal adhesion signaling. During association of integrin receptors with ECM proteins FAK activity is enhanced via both autophosphorylation and transphosphorylation by SFKs (Mitra *et al.* 2005, Schlaepfer *et al.* 1999, Guan & Shalloway 1992, Hanks & Polte 1997).

FAK FERM domain

FAK contains an N-terminal FERM (FAK N-terminal ezrin/radixin/moesin homology) domain which binds growth factor receptors such as PDGFαR (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.

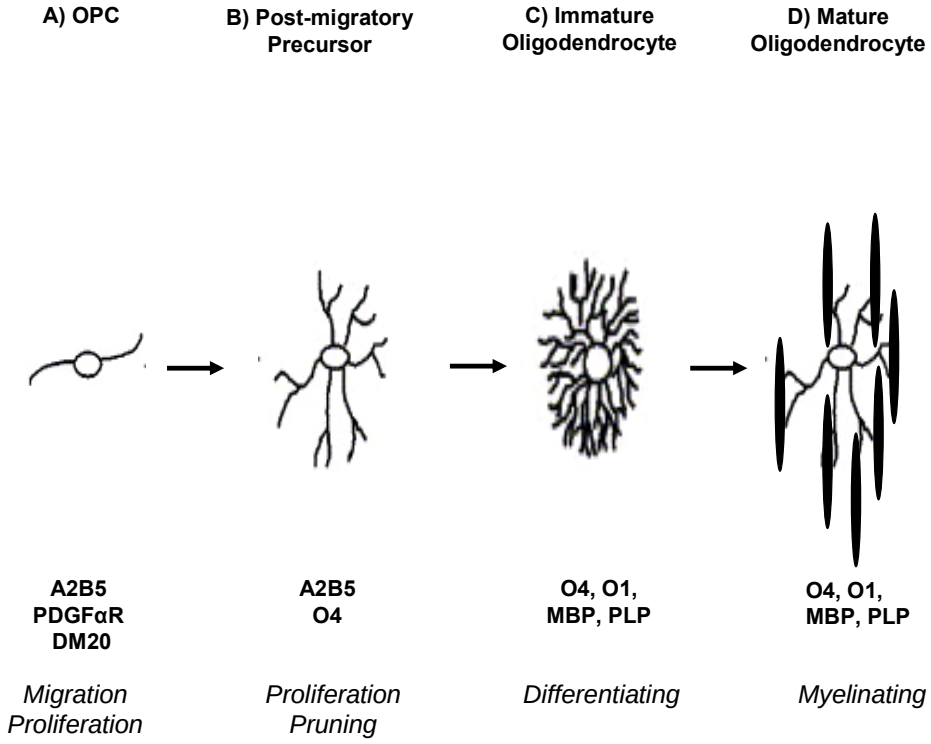


Figure 1.1 Development of an oligodendrocyte from a neuroepithelial cell to a mature oligodendrocyte. A) oligodendrocyte precursor cells (OPC) which migrate to their site of action and further differentiate into B) post-migratory precursor oligodendrocytes which begin sending out processes. C) Immature 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.

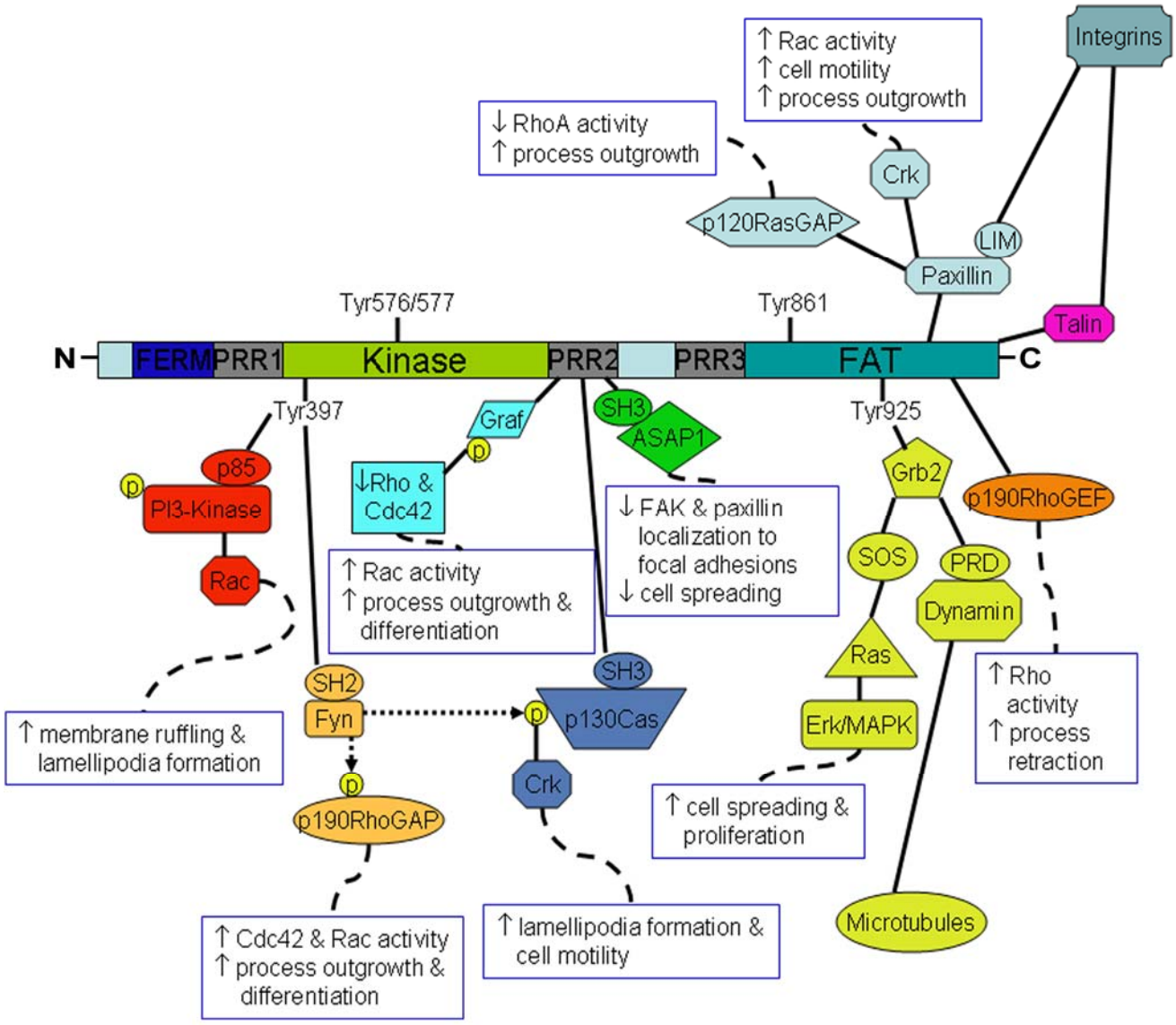


Figure 1.2 Schematic of signaling pathways regulated by FAK via interactions with focal adhesion molecules. FAK promotes cytoskeletal remodeling via association of phosphotidylinositol 3-kinase (PI3K) with the kinase domain of FAK (red pathway). The proline rich regions (PRR) of FAK associate with p130Cas (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

(This chapter was accepted as a paper in the Journal of Neuroscience Research in February of 2009. The work reported for this manuscript was preformed with vital assistance from Dr. Ray Colello and Dr. Jeff Dupree.)

Introduction

During development of the central nervous system (CNS), differentiation of oligodendrocytes, the myelinating cells, and the process of active myelination itself are regulated by complex interactions of the oligodendrocytes' cell surfaces with their extracellular environment (Lubetzki-Korn *et al.* 1983, Fridman *et al.* 1985, Notterpek & Rome 1994, Oh & Yong 1996, Buttery *et al.* 1999, Szuchet *et al.* 2000, Siskova *et al.* 2006, Colognato *et al.* 2007). These interactions are mediated to a large extent by the integrin class of extracellular matrix (ECM) receptors. (Malek-Hedayat & Rome 1994, Milner & ffrench-Constant 1994, Frost *et al.* 1999, Relvas *et al.* 2001, Colognato *et al.* 2002, Colognato *et al.* 2004, Gudz *et al.* 2002, Baron *et al.* 2005, Olsen & ffrench-Constant 2005, Benninger *et al.* 2006, Lee *et al.* 2006). In support of integrin-ECM signaling playing a pivotal role in the regulation of oligodendrocyte differentiation and CNS myelination, signaling molecules that are effectors in integrin-mediated signaling cascades have also been implicated in these processes (Chun *et al.* 2003, Fox *et al.* 2004, Liang *et al.* 2004, Hoshina *et al.* 2007, Sloane & Vartanian 2007).

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*$^{flox/flox}$: *PLP/CreER*^{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*$^{flox/flox}$ 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*^{T} 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*^{T} locus and females homozygous for the floxed *Fak* locus but negative for the *PLP/CreER*^{T} 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/CreER*^{T} mice were additionally bred to *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ reporter mice, which contain an enhanced yellow fluorescent protein (*EYFP*) gene inserted into the *Gt(ROSA)26Sor* 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 *loxP*-flanked STOP sequence and only induced on successful Cre-mediated recombination at the *ROSA26* locus. *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$:*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^{T} reverse (5-ACTATATCCGTAACCTGGAT-3) at 50°C and FAK-flox-P1 (5-GACCTTCAACTTCT CATTTCTCC-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)26Sor$^{tm1(EYFP)Cos}$:PLP/CreER^{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 *PLP/CreER^T* Locus

To determine the *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 *Fak* is flanked by *loxP* sites (*Fak$^{flox/flox}$* 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 (*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 *Fak* locus and either heterozygous for the *PLP/CreER^T* locus (*Fak$^{flox/flox}$:PLP/CreER^T* mice) or lacking the *PLP/CreER^T* locus (*Fak$^{flox/flox}$* littermate controls). It has been previously established that only upon

administration of tamoxifen is $CreER^T$ 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^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$^{flox/flox}$: PLP/CreER^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$^{flox/flox}$:PLP/CreER^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 *Fak*$^{flox/flox}$*:PLP/CreER*^{T} mice compared with their *Fak*$^{flox/flox}$ 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 *Fak*$^{flox/flox}$*:PLP/CreER*^{T} mice compared to *Fak*$^{flox/flox}$ mice (Figure 2.2B,C). No such differences were noted when analyzing vehicle-treated *Fak*$^{flox/flox}$*: PLP/CreER*^{T} and *Fak*$^{flox/flox}$ mice (data not shown).

To determine the resolution of our type of analysis, we compared semithin sections (light microcopy) 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 *Fak*$^{flox/flox}$*: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 *Fak*$^{flox/flox}$*:PLP/CreER*^{T} mice and their *Fak*$^{flox/flox}$ 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*$^{flox/flox}$*: PLP/CreER*^{T} optic nerves compared to optic nerves from P14 *Fak*$^{flox/flox}$ 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*$^{flox/flox}$ and *Fak*$^{flox/flox}$*:PLP/CreER*^{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*$^{flox/flox}$ and *Fak*$^{flox/flox}$*:PLP/CreER*^{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 $Fak^{flox/flox}$:*PLP/CreER*^{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$^{tm1(EYFP)Cos}$: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$^{tm1(EYFP)Cos}$*: *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

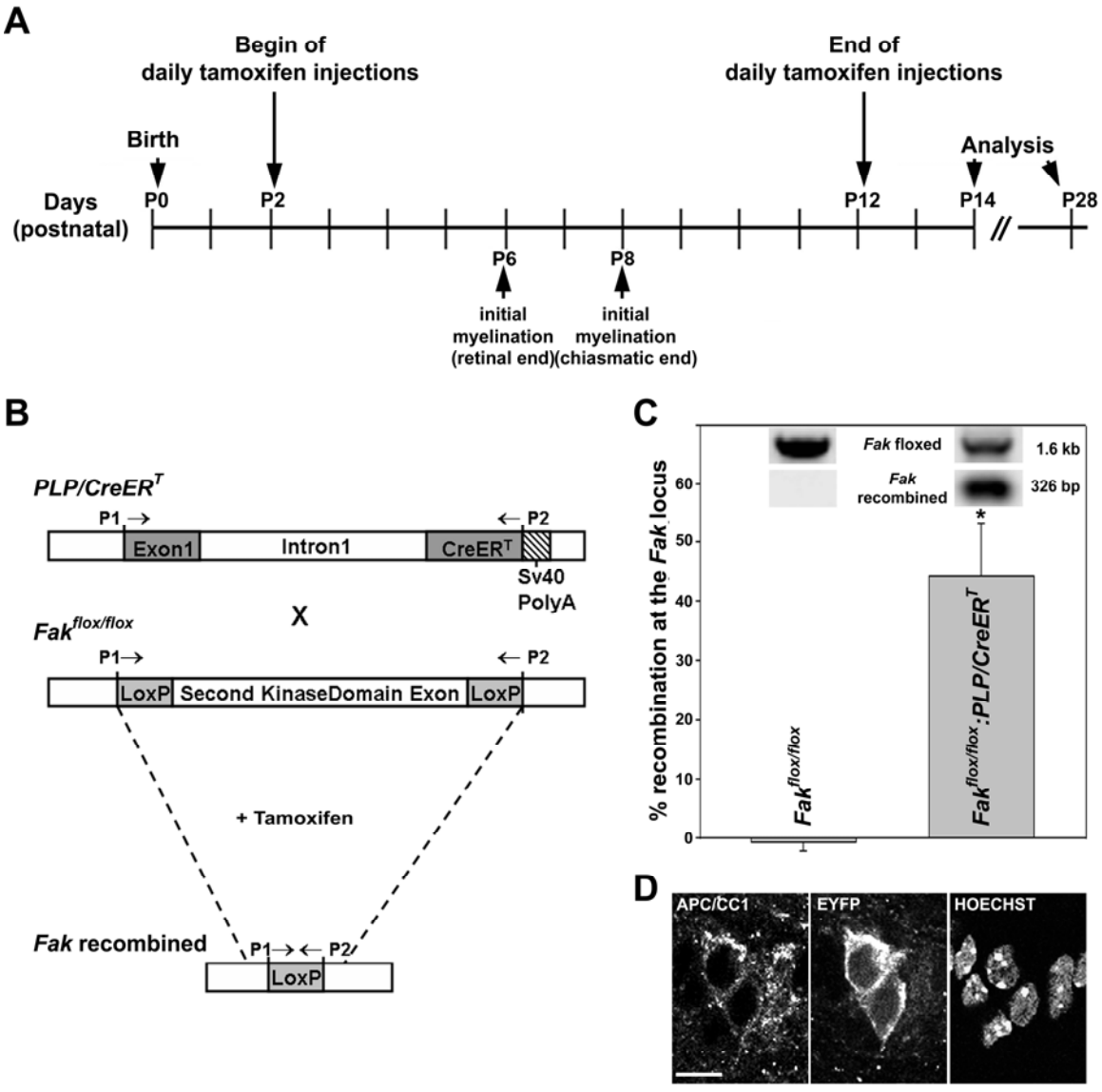
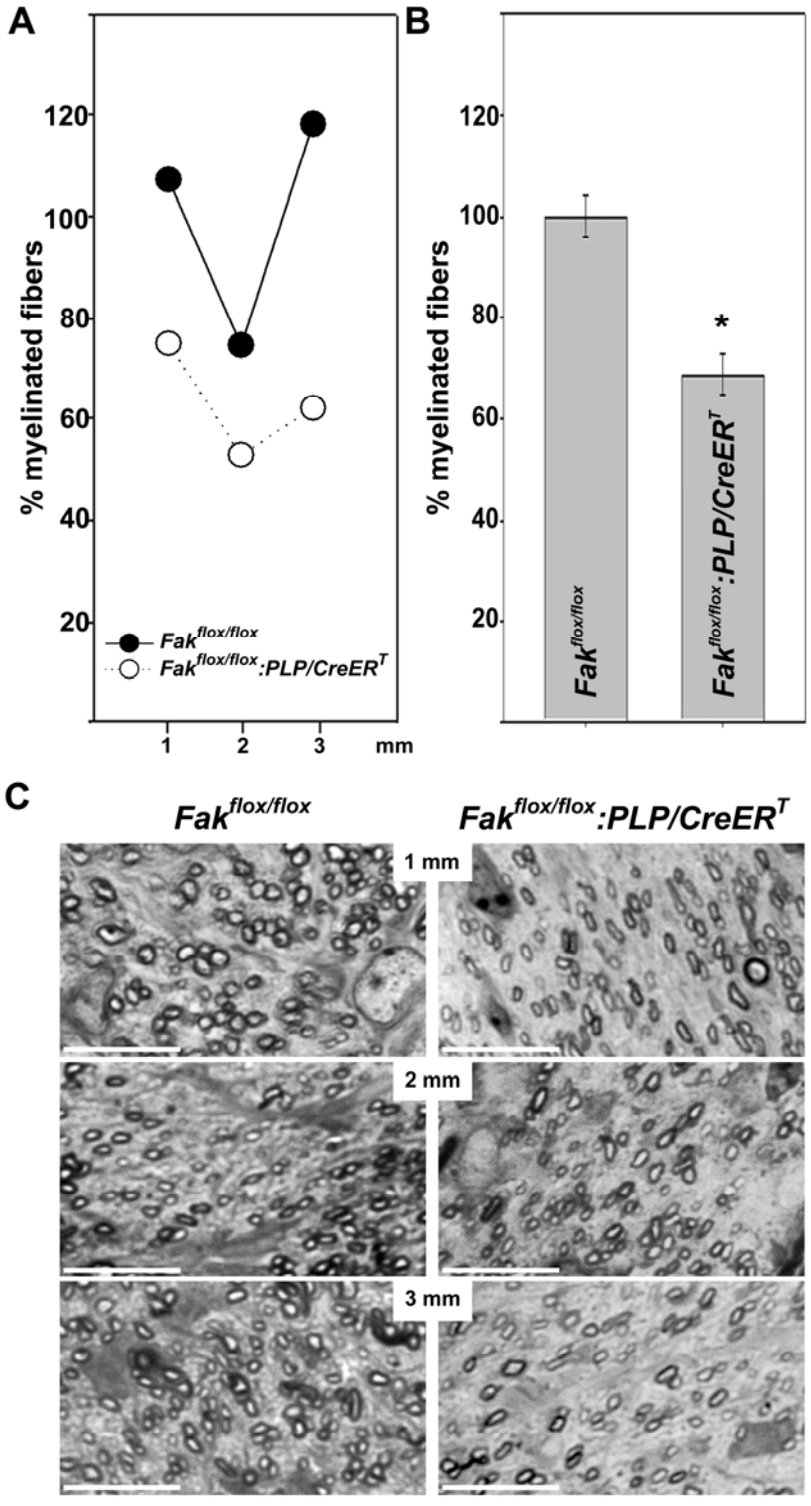

A
Begin of
daily tamoxifen injections
End of
daily tamoxifen injections
Birth
Analysis
Days
(postnatal)
P0
P2
P12
P14
P28
P6
P8
initial
myelination
(retinal end)
initial
myelination
(chiasmatic end)
B
PLP/CreER^T
P1
P2
Exon1
Intron1
CreER^T
Sv40
PolyA
X
Fak^flox/flox
LoxP
Second KinaseDomain Exon
LoxP
+ Tamoxifen
Fak recombined
LoxP
C
Fak floxed
1.6 kb
Fak
recombined
326 bp
% recombination at the Fak locus
Fak^flox/flox
Fak^flox/flox:PLP/CreER^T
D
APC/CC1
EYFP
HOECHST

Figure 2.2 Reduced number of myelinated fibers in optic nerves of tamoxifen-treated *Fak*$^{flox/flox}$*:PLP/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*$^{flox/flox}$ (left) and *Fak*$^{flox/flox}$*:PLPCreER*^{T} (right) optic nerves taken at approximately 1, 2, and 3 mm from the lamina cribosa. Scale bars = 10 μm.

Figure 2.2


A
B
C
% myelinated fibers
120
100
80
60
40
20
1
2
3
mm
Fak flox/flox
Fak flox/flox :PLP/CreER T
*
1 mm
2 mm
3 mm

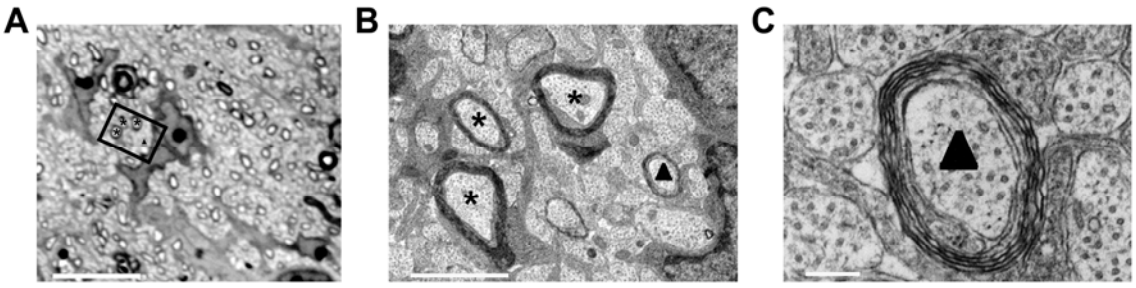


Figure 2.3 Resolution (visual threshold) for the detection of a myelinated fiber in the optic nerve was approximately 4 layers of myelin at the light microscopic level. **A:** Semithin section of the optic nerve imaged at the light microscopic level. Scale bar = 10 µm. **B, C:** Ultrathin sections taken 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).

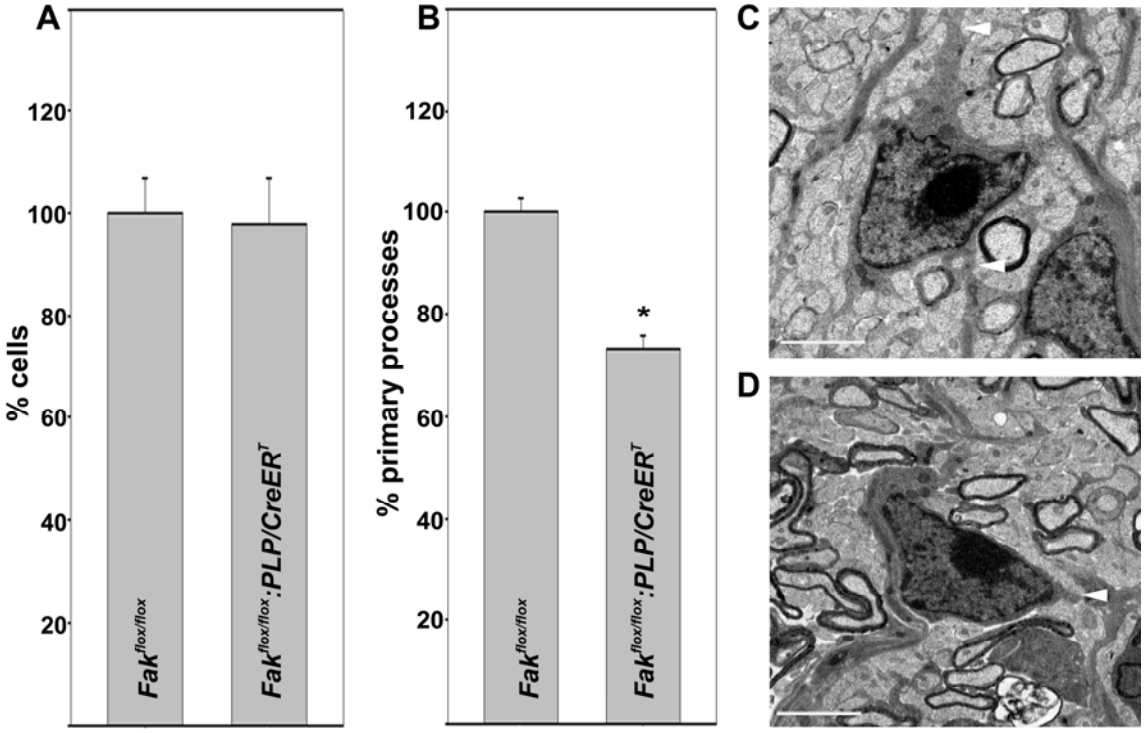


Figure 2.4 Reduced number of primary oligodendrocyte processes in optic nerves of tamoxifen-treated *Fak*$^{flox/flox}$*:PLP/CreER*^{T}. **A:** Total number of cells counted at light microscopic level in semithin P14 optic nerve sections at the 1-mm interval (see Figure 2.2). Mean for all control values 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/CreER*^{T} (**D**) and a *Fak*$^{flox/flox}$ (**C**, control) oligodendrocyte. Scale bars = 2 µm.

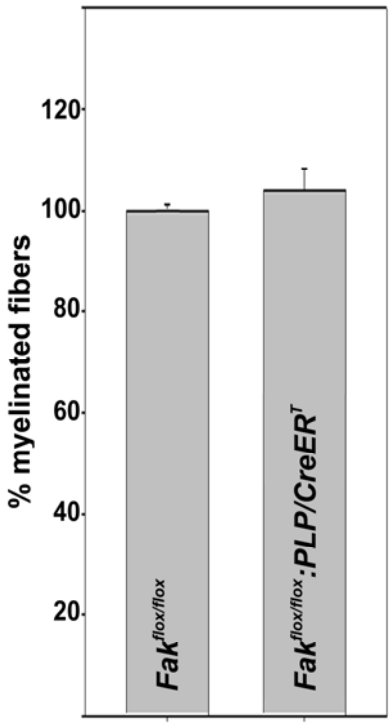


Figure 2.5 Comparable number of myelinated fibers in optic nerves of tamoxifen-treated *Fak*$^{flox/flox}$ and *Fak*$^{flox/flox}$*:PLP/CreER*^{T} mice at P28. Numbers of myelinated fibers were determined within entire transverse sections of optic nerves at approximately 1 mm from the lamina cribosa 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 α chains and one of eight possible β chains. Each subunit (α and β) 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 PDGFα, 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 αvβ1 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 phosphorlyated 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,

Carlsbad, CA); DMEM/T3/N2). 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 Scientifc/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 ($n \geq 3$) 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 hence forth 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 looses 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 premyelinating 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 premyelinating 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.

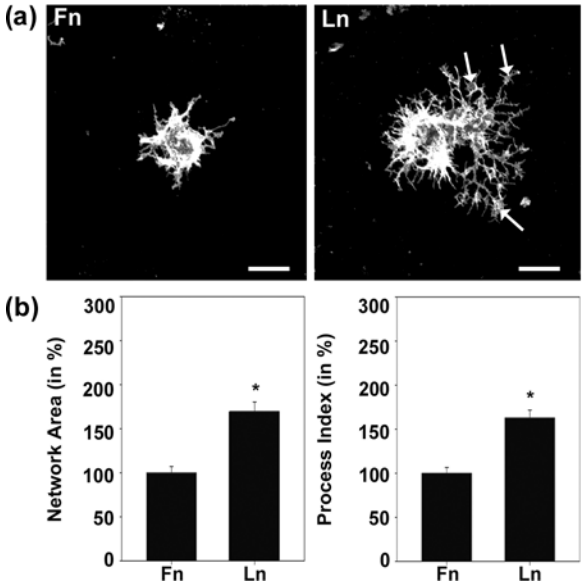


Figure 4.1 Morphological maturation of post-migratory premyelinating oligodendrocytes is uniquely regulated in the presence of fibronectin or laminin-2. Oligodendrocyte progenitor cells were isolated by A2B5 immunopanning from postnatal day 3 rat brains. After 2 days in culture, differentiating oligodendrocytes 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 premyelinating 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 4.2

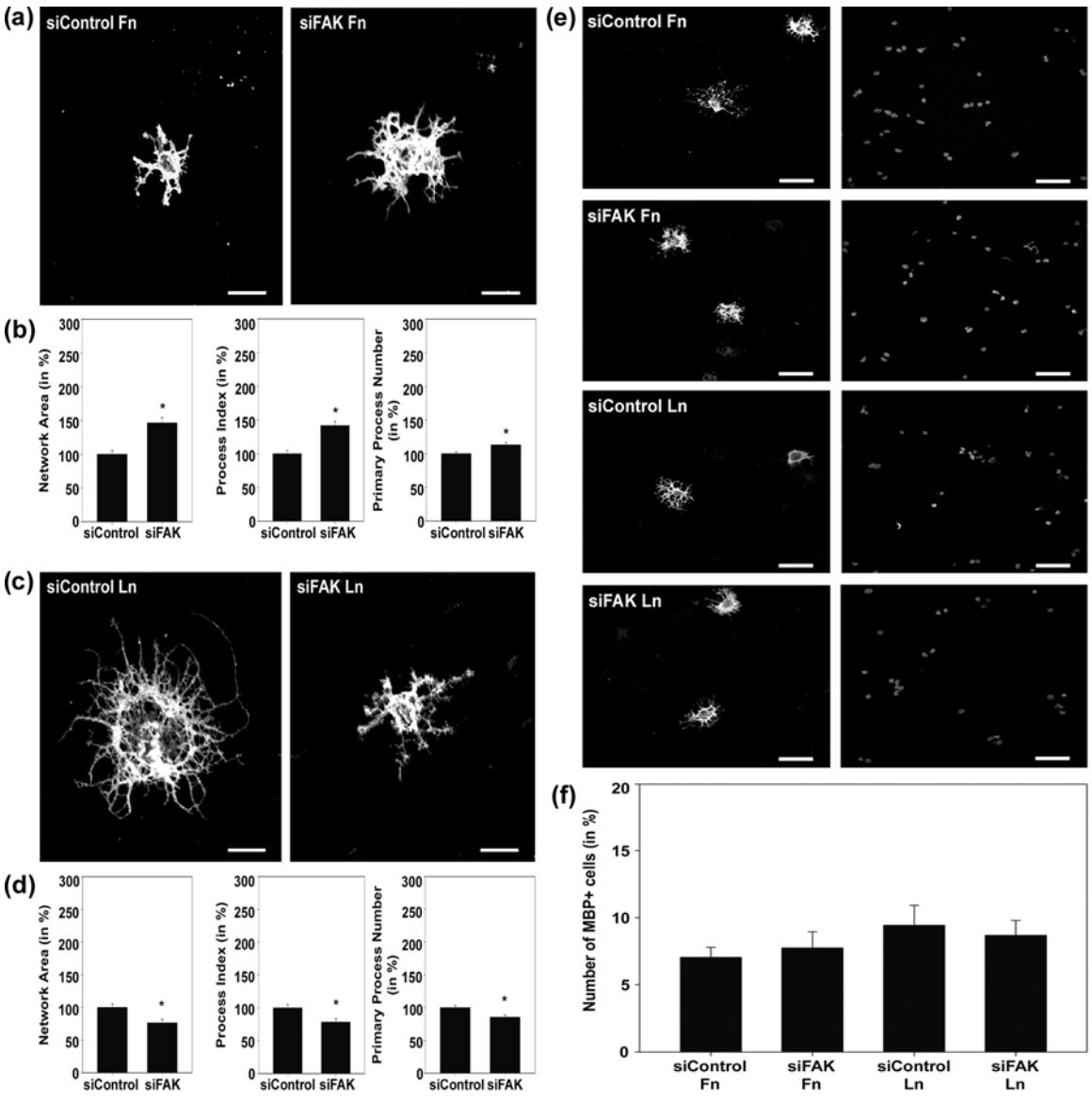
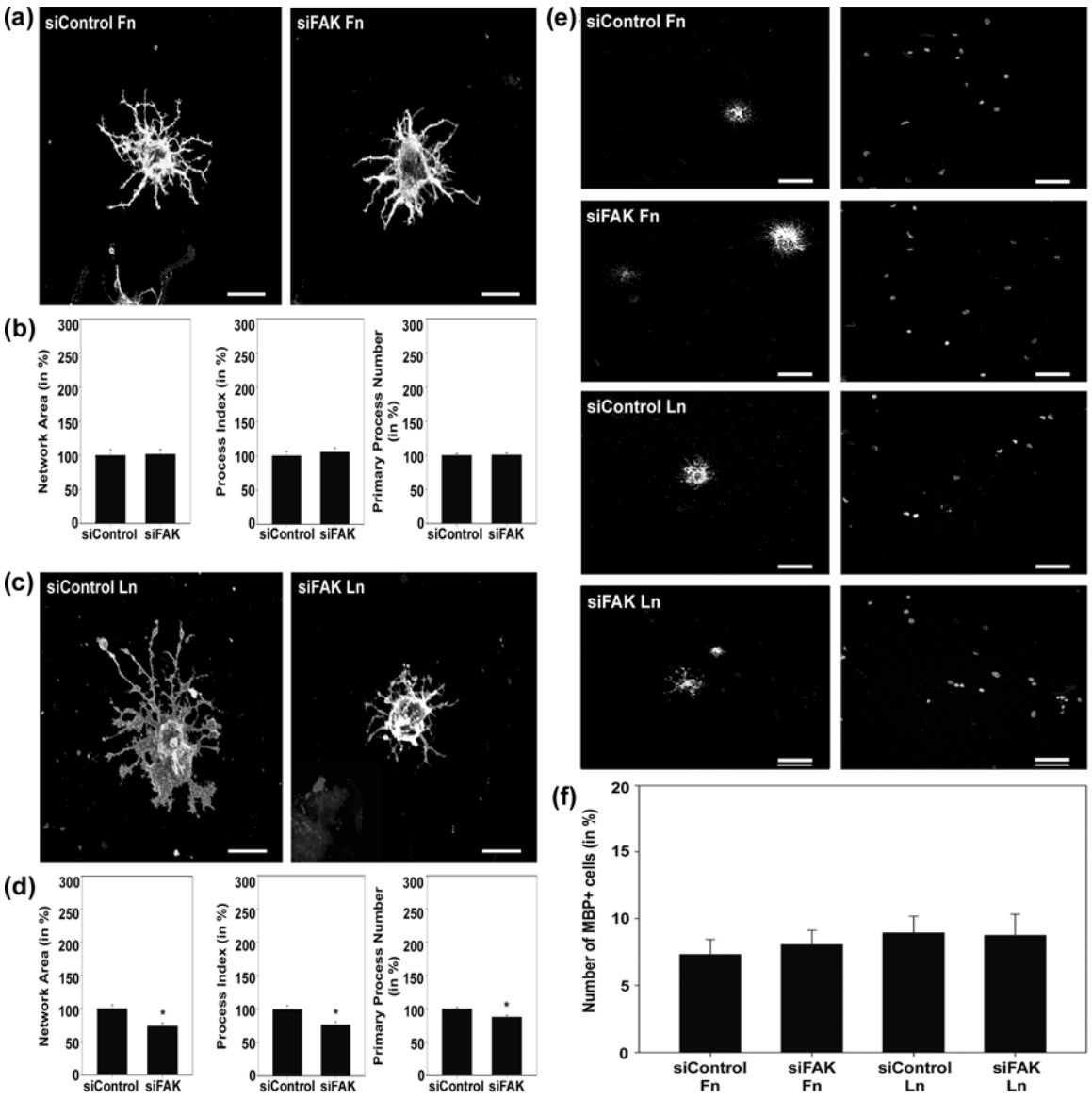

(a)
siControl Fn
siFAK Fn
(b)
Network Area (in %)
Process Index (in %)
Primary Process Number (in %)
siControl siFAK
(c)
siControl Ln
siFAK Ln
(d)
(e)
siControl Fn
siFAK Fn
siControl Ln
siFAK Ln
(f)
Number of MBP+ cells (in %)
siControl Fn
siFAK Fn
siControl Ln
siFAK Ln

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)
Network Area (in %)
Process Index (in %)
Primary Process Number (in %)
300
250
200
150
100
50
0
siControl
siFAK
(c)
siControl Ln
siFAK Ln
(d)
*
(e)
siControl Fn
siFAK Fn
siControl Ln
siFAK Ln
(f)
Number of MBP+ cells (in %)
20
15
10
5
0
siControl Fn
siFAK Fn
siControl Ln
siFAK Ln

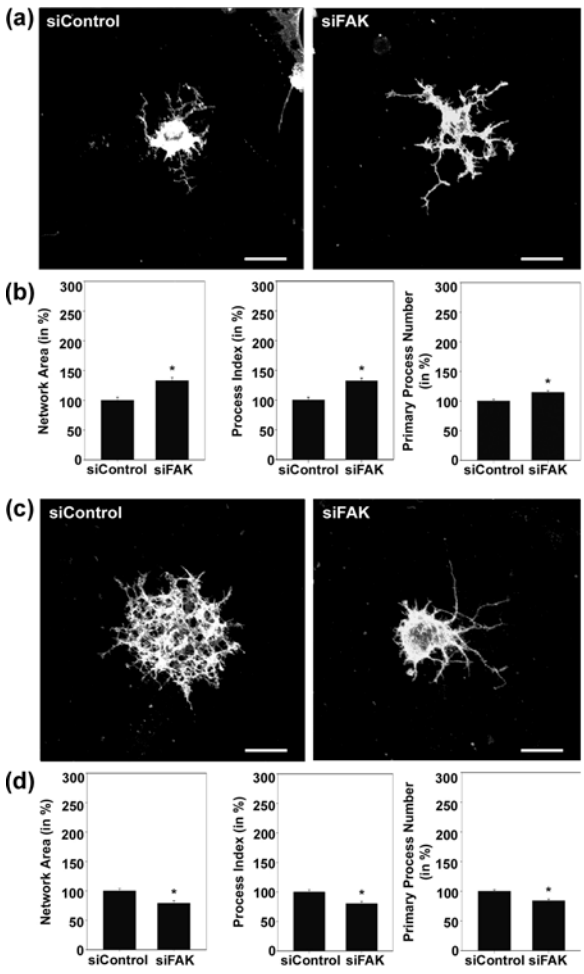


Figure 4.4 siRNA-mediated knock-down of FAK expression in the presence of a mixed fibronectin/laminin-2 substrate affects morphological maturation of early and later stage post-migratory premyelinating oligodendrocytes in a distinctive and opposing fashion. Cells were isolated, differentiated, treated 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.

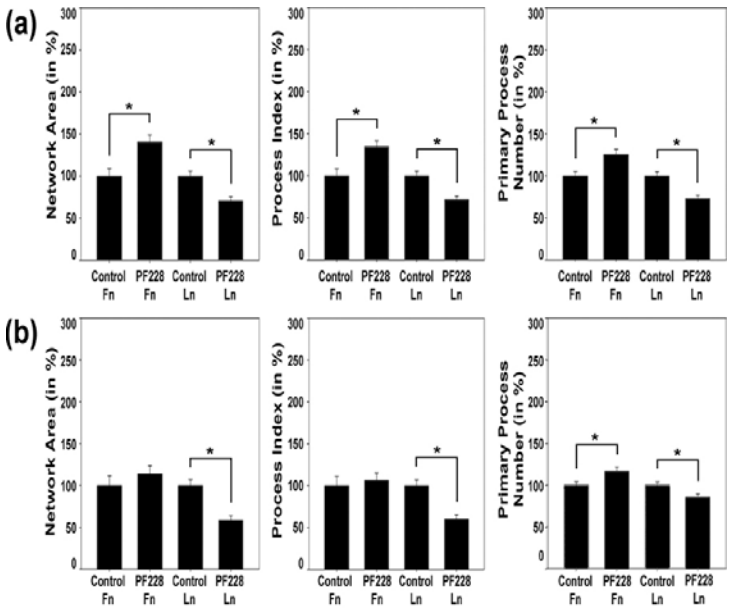


Figure 4.5 Inhibition of FAK's catalytic activity affects morphological maturation of early and later stage post-migratory premyelinating oligodendrocytes in a similar fashion as siRNA-mediated knock-down of FAK expression. Cells were isolated, differentiated and analyzed as described in Figures 4.1. 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.

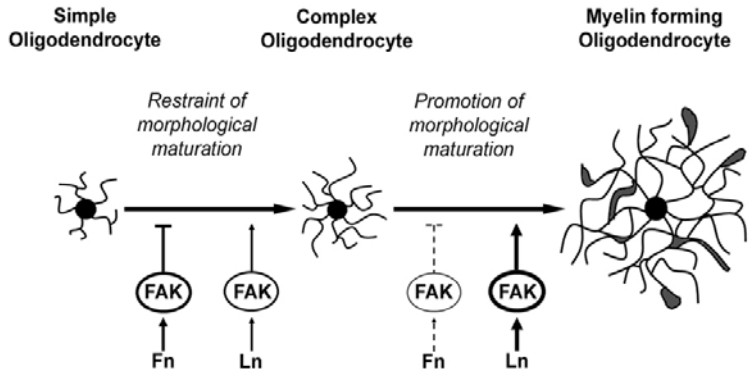


Figure 4.6 Proposed model for the role of FAK in morphological maturation of post-migratory premyelinating oligodendrocytes. The data presented here demonstrate that FAK can have unique and opposing roles depending on the maturation stage of the cell and the nature of the prevalent ECM molecule that 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

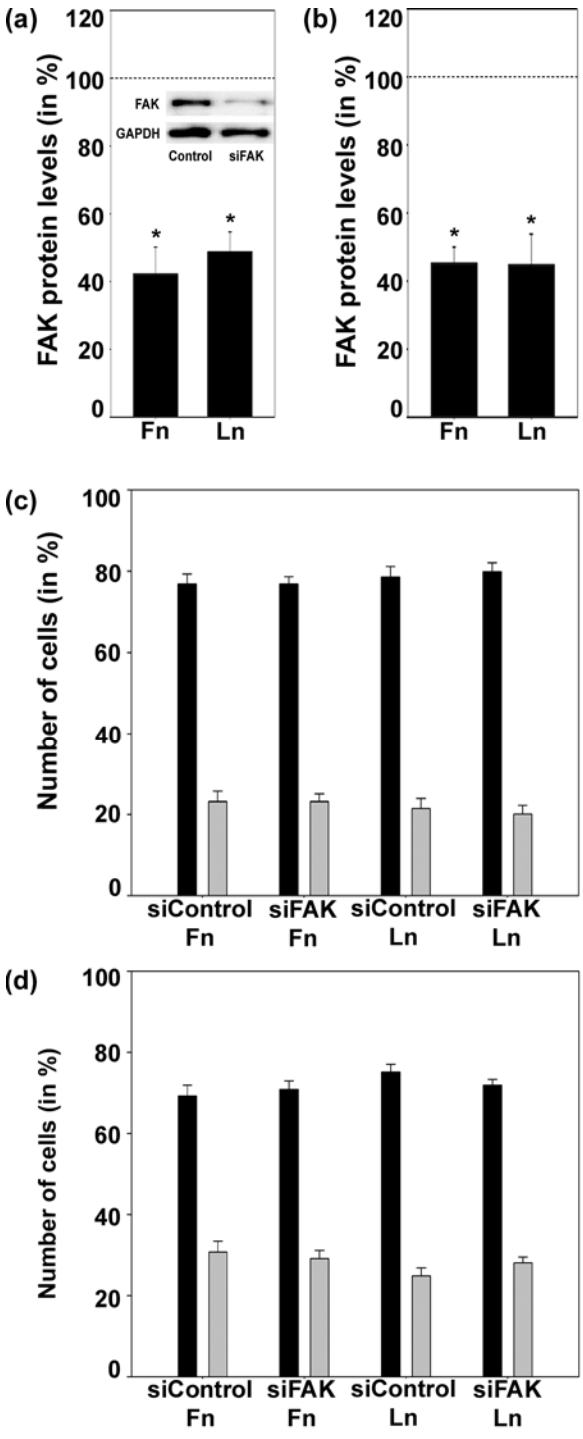
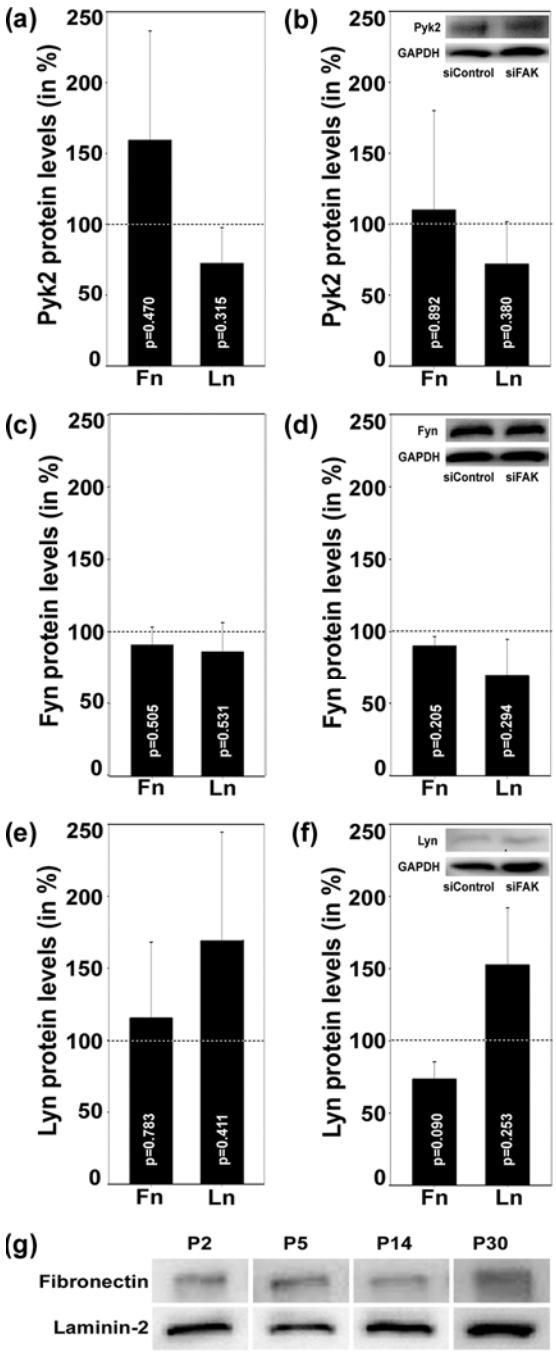

(a)
FAK protein levels (in %)
120
100
80
60
40
20
0
FAK
GAPDH
Control
siFAK
*
*
Fn
Ln
(b)
FAK protein levels (in %)
120
100
80
60
40
20
0
*
*
Fn
Ln
(c)
Number of cells (in %)
100
80
60
40
20
0
siControl Fn
siFAK Fn
siControl Ln
siFAK Ln
(d)
Number of cells (in %)
100
80
60
40
20
0
siControl Fn
siFAK Fn
siControl Ln
siFAK Ln

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


(a)
Pyk2 protein levels (in %)
250
200
150
100
50
0
p=0.470
p=0.315
Fn
Ln
(b)
Pyk2
GAPDH
siControl
siFAK
Pyk2 protein levels (in %)
p=0.892
p=0.380
Fn
Ln
(c)
Fyn protein levels (in %)
p=0.505
p=0.531
Fn
Ln
(d)
Fyn
GAPDH
siControl
siFAK
Fyn protein levels (in %)
p=0.205
p=0.294
Fn
Ln
(e)
Lyn protein levels (in %)
p=0.783
p=0.411
Fn
Ln
(f)
Lyn
GAPDH
siControl
siFAK
Lyn protein levels (in %)
p=0.090
p=0.253
Fn
Ln
(g)
P2
P5
P14
P30
Fibronectin
Laminin-2

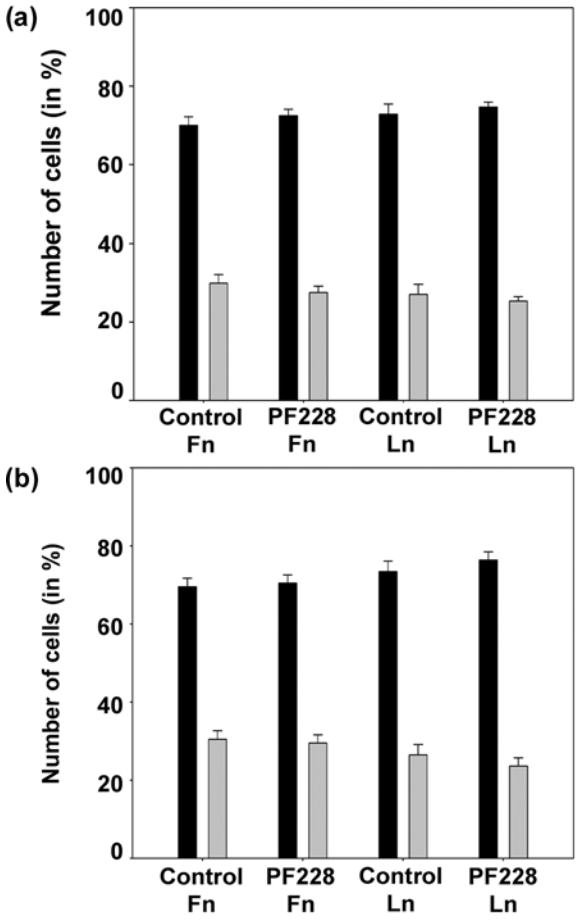


Figure 4.9 Inhibition of FAK's catalytic activity using the inhibitor PF573228 does not significantly affect the viability of early and later stage post-migratory pre-myelinating oligodendrocytes. Bar graphs representing the percentage of enzymatically converted calcein AM-positive (live; black bars) 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 ($FAK^{flox/flox}$; 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 $FAK^{flox/flox}$; 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 asses 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 αvβ3 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 αvβ3 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.

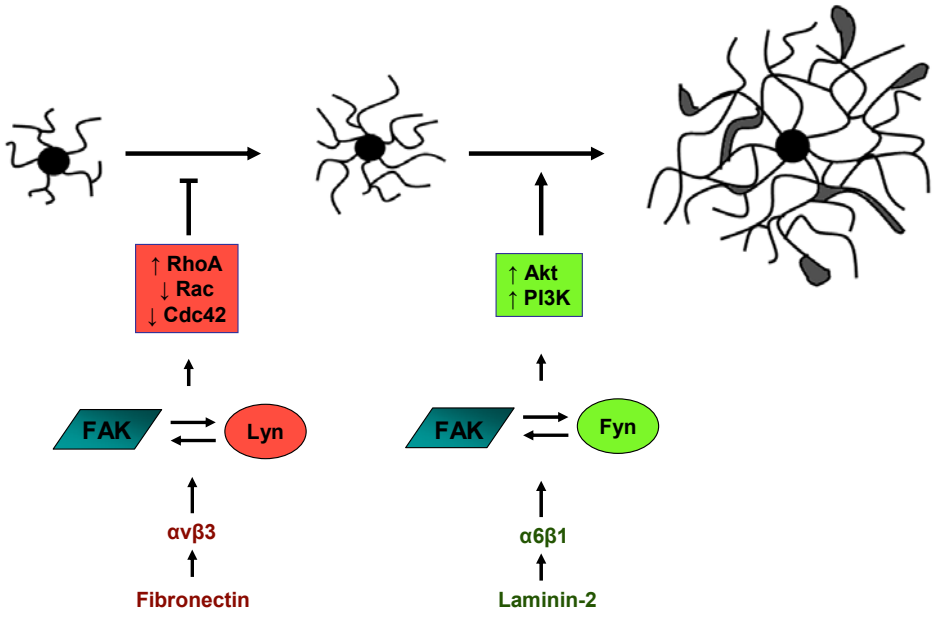


Figure 6.1 Theoretical schematic for FAK-mediated signaling pathways regulating morphological maturation of post-migratory premyelinating oligodendrocytes. Upon association of fibronectin with the integrin receptor αvβ3 a complex of FAK and Lyn is activated. This activation could lead to the regulation 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 α6β1, however, activation of a FAK-Fyn complex initiates activation of the PI3K/Akt pathway leading to enhanced oligodendrocyte morphological maturation and myelination.

List of References

Abney, E. R., Bartlett, P. P. and Raff, M. C. (1981) Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. *Dev Biol,* **83,** 301-310.

Antonyak, M. A. and Cerione, R. A. (2009) Ras and the FAK paradox. *Mol Cell,* **35,** 141-142.

Avraham, H., Park, S. Y., Schinkmann, K. and Avraham, S. (2000) RAFTK/Pyk2-mediated cellular signalling. *Cell Signal,* **12,** 123-133.

Bacon, C., Lakics, V., Machesky, L. and Rumsby, M. (2007) N-WASP regulates extension of filopodia and processes by oligodendrocyte progenitors, oligodendrocytes, and Schwann cells-implications for axon ensheathment at myelination. *Glia,* **55,** 844-858.

Badley, R. A., Woods, A., Carruthers, L. and Rees, D. A. (1980) Cytoskeleton changes in fibroblast adhesion and detachment. *J Cell Sci,* **43,** 379-390.

Bansal, R., Warrington, A. E., Gard, A. L., Ranscht, B. and Pfeiffer, S. E. (1989) Multiple and novel specificities of monoclonal antibodies O1, O4, and R-mAb used in the analysis of oligodendrocyte development. *J Neurosci Res,* **24,** 548-557.

Baron, W., Colognato, H. and ffrench-Constant, C. (2005) Integrin-growth factor interactions as regulators of oligodendroglial development and function. *Glia,* **49,** 467-479.

Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D. and Raff, M. C. (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell,* **70,** 31-46.

Barros, C. S., Nguyen, T., Spencer, K. S., Nishiyama, A., Colognato, H. and Muller, U. (2009) {beta}1 integrins are required for normal CNS myelination and promote AKT-dependent myelin outgrowth. *Development,* **136,** 2717-2724.

Bauer, N. G., Richter-Landsberg, C. and ffrench-Constant, C. (2009) Role of the oligodendroglial cytoskeleton in differentiation and myelination. *Glia,* **57,** 1691-1705.

Baumann, N. and Pham-Dinh, D. (2001) Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev,* **81,** 871-927.

Beggs, H. E., Schahin-Reed, D., Zang, K., Goebbels, S., Nave, K. A., Gorski, J., Jones, K. R., Sretavan, D. and Reichardt, L. F. (2003) FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. *Neuron,* **40,** 501-514.

Benninger, Y., Colognato, H., Thurnherr, T. et al. (2006) Beta1-integrin signaling mediates premyelinating oligodendrocyte survival but is not required for CNS myelination and remyelination. *J Neurosci,* **26,** 7665-7673.

Berrier, A. L. and Yamada, K. M. (2007) Cell-matrix adhesion. *J Cell Physiol,* **213,** 565-573.

Bhat, R. V., Axt, K. J., Fosnaugh, J. S., Smith, K. J., Johnson, K. A., Hill, D. E., Kinzler, K. W. and Baraban, J. M. (1996) Expression of the APC tumor suppressor protein in oligodendroglia. *Glia,* **17,** 169-174.

Bibollet-Bahena, O. and Almazan, G. (2009) IGF-1-stimulated protein synthesis in oligodendrocyte progenitors requires PI3K/mTOR/Akt and MEK/ERK pathways. *J Neurochem,* **109,** 1440-1451.

Biffiger, K., Bartsch, S., Montag, D., Aguzzi, A., Schachner, M. and Bartsch, U. (2000) Severe hypomyelination of the murine CNS in the absence of myelin-associated glycoprotein and fyn tyrosine kinase. *J Neurosci,* **20,** 7430-7437.

Blaschuk, K. L., Frost, E. E. and ffrench-Constant, C. (2000) The regulation of proliferation and differentiation in oligodendrocyte progenitor cells by alphaV integrins. *Development,* **127,** 1961-1969.

Bronner-Fraser, M. (1986) An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. *Dev Biol,* **117,** 528-536.

Brown, M. C., Cary, L. A., Jamieson, J. S., Cooper, J. A. and Turner, C. E. (2005) Src and FAK kinases cooperate to phosphorylate paxillin kinase linker, stimulate its focal adhesion localization, and regulate cell spreading and protrusiveness. *Mol Biol Cell,* **16,** 4316-4328.

Burgaya, F. and Girault, J. A. (1996) Cloning of focal adhesion kinase, pp125FAK, from rat brain reveals multiple transcripts with different patterns of expression. *Brain Res Mol Brain Res,* **37,** 63-73.

Burgaya, F., Menegon, A., Menegoz, M., Valtorta, F. and Girault, J. A. (1995) Focal adhesion kinase in rat central nervous system. *Eur J Neurosci,* **7,** 1810-1821.

Burgaya, F., Toutant, M., Studler, J. M., Costa, A., Le Bert, M., Gelman, M. and Girault, J. A. (1997) Alternatively spliced focal adhesion kinase in rat brain with increased autophosphorylation activity. *J Biol Chem,* **272,** 28720-28725.

Butt, A. M. and Ransom, B. R. (1989) Visualization of oligodendrocytes and astrocytes in the intact rat optic nerve by intracellular injection of lucifer yellow and horseradish peroxidase. *Glia,* **2,** 470-475.

Butt, A. M. and Ransom, B. R. (1993) Morphology of astrocytes and oligodendrocytes during development in the intact rat optic nerve. *J Comp Neurol,* **338,** 141-158.

Buttery, P. C. and ffrench-Constant, C. (1999) Laminin-2/integrin interactions enhance myelin membrane formation by oligodendrocytes. *Mol Cell Neurosci,* **14,** 199-212.

Buttery, P. C. and ffrench-Constant, C. (2001) Process extension and myelin sheet formation in maturing oligodendrocytes. *Prog Brain Res,* **132,** 115-130.

Buttery, P. C., Mallawaarachchi, C. M., Milner, R., Doherty, P. and ffrench-Constant, C. (1999) Mapping regions of the beta1 integrin cytoplasmic domain involved in migration and survival in primary oligodendrocyte precursors using cell-permeable homeopeptides. *Biochem Biophys Res Commun,* **259,** 121-127.

Cahoy, J. D., Emery, B., Kaushal, A. et al. (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci,* **28,** 264-278.

Calalb, M. B., Zhang, X., Polte, T. R. and Hanks, S. K. (1996) Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochem Biophys Res Commun,* **228,** 662-668.

Caley, D. W. and Maxwell, D. S. (1968) An electron microscopic study of neurons during postnatal development of the rat cerebral cortex. *J Comp Neurol,* **133,** 17-44.

Camara, J., Wang, Z., Nunes-Fonseca, C. et al. (2009) Integrin-mediated axoglial interactions initiate myelination in the central nervous system. *J Cell Biol,* **185,** 699-712.

Cance, W. G. and Golubovskaya, V. M. (2008) Focal adhesion kinase versus p53: apoptosis or survival? *Sci Signal,* **1,** pe22.

Cary, L. A., Chang, J. F. and Guan, J. L. (1996) Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J Cell Sci,* **109 (Pt 7),** 1787-1794.

Cary, L. A., Klinghoffer, R. A., Sachsenmaier, C. and Cooper, J. A. (2002) SRC catalytic but not scaffolding function is needed for integrin-regulated tyrosine phosphorylation, cell migration, and cell spreading. *Mol Cell Biol,* **22,** 2427-2440.

Chang, A., Tourtellotte, W. W., Rudick, R. and Trapp, B. D. (2002) Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. *N Engl J Med,* **346,** 165-173.

Chen, H. C. and Guan, J. L. (1994) Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A,* **91,** 10148-10152.

Chen, X., Fu, W., Tung, C. E. and Ward, N. L. (2009) Angiopoietin-1 induces neurite outgrowth of PC12 cells in a Tie2-independent, beta1-integrin-dependent manner. *Neurosci Res,* **64,** 348-354.

Chun, S. J., Rasband, M. N., Sidman, R. L., Habib, A. A. and Vartanian, T. (2003) Integrin-linked kinase is required for laminin-2-induced oligodendrocyte cell spreading and CNS myelination. *J Cell Biol,* **163,** 397-408.

Clark, E. A., King, W. G., Brugge, J. S., Symons, M. and Hynes, R. O. (1998) Integrin-mediated signals regulated by members of the rho family of GTPases. *J Cell Biol,* **142,** 573-586.

Cluzel, C., Saltel, F., Lussi, J., Paulhe, F., Imhof, B. A. and Wehrle-Haller, B. (2005) The mechanisms and dynamics of (alpha)v(beta)3 integrin clustering in living cells. *J Cell Biol,* **171,** 383-392.

Cobb, B. S., Schaller, M. D., Leu, T. H. and Parsons, J. T. (1994) Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. *Mol Cell Biol,* **14,** 147-155.

Cohen, L. A. and Guan, J. L. (2005) Mechanisms of focal adhesion kinase regulation. *Curr Cancer Drug Targets,* **5,** 629-643.

Colello, R. J., Devey, L. R., Imperato, E. and Pott, U. (1995) The chronology of oligodendrocyte differentiation in the rat optic nerve: evidence for a signaling step initiating myelination in the CNS. *J Neurosci,* **15,** 7665-7672.

Colello, R. J., Pott, U. and Schwab, M. E. (1994) The role of oligodendrocytes and myelin on axon maturation in the developing rat retinofugal pathway. *J Neurosci,* **14,** 2594-2605.

Collin, L., Doretto, S., Malerba, M., Ruat, M. and Borrelli, E. (2007) Oligodendrocyte ablation affects the coordinated interaction between granule and Purkinje neurons during cerebellum development. *Exp Cell Res,* **313,** 2946-2957.

Colognato, H., Baron, W., Avellana-Adalid, V., Relvas, J. B., Baron-Van Evercooren, A., Georges-Labouesse, E. and ffrench-Constant, C. (2002) CNS integrins switch growth factor signalling to promote target-dependent survival. *Nat Cell Biol,* **4,** 833-841.

Colognato, H., ffrench-Constant, C. and Feltri, M. L. (2005) Human diseases reveal novel roles for neural laminins. *Trends Neurosci,* **28,** 480-486.

Colognato, H., Galvin, J., Wang, Z., Relucio, J., Nguyen, T., Harrison, D., Yurchenco, P. D. and ffrench-Constant, C. (2007) Identification of dystroglycan as a second laminin receptor in oligodendrocytes, with a role in myelination. *Development,* **134,** 1723-1736.

Colognato, H., Ramachandrappa, S., Olsen, I. M. and ffrench-Constant, C. (2004) Integrins direct Src family kinases to regulate distinct phases of oligodendrocyte development. *J Cell Biol,* **167,** 365-375.

Comabella, M. and Martin, R. (2007) Genomics in multiple sclerosis--current state and future directions. *J Neuroimmunol,* **187,** 1-8.

Contestabile, A., Bonanomi, D., Burgaya, F., Girault, J. A. and Valtorta, F. (2003) Localization of focal adhesion kinase isoforms in cells of the central nervous system. *Int J Dev Neurosci,* **21,** 83-93.

Corley, S. M., Ladiwala, U., Besson, A. and Yong, V. W. (2001) Astrocytes attenuate oligodendrocyte death in vitro through an alpha(6) integrin-laminin-dependent mechanism. *Glia,* **36,** 281-294.

Corsi, J. M., Houbron, C., Billuart, P., Brunet, I., Bouvree, K., Eichmann, A., Girault, J. A. and Enslen, H. (2009) Autophosphorylation-independent and dependent functions of Focal Adhesion Kinase during development. *J Biol Chem*.

Cossins, J. A., Clements, J. M., Ford, J., Miller, K. M., Pigott, R., Vos, W., Van der Valk, P. and De Groot, C. J. (1997) Enhanced expression of MMP-7 and MMP-9 in demyelinating multiple sclerosis lesions. *Acta Neuropathol,* **94,** 590-598.

Cox, B. D., Natarajan, M., Stettner, M. R. and Gladson, C. L. (2006) New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. *J Cell Biochem,* **99,** 35-52.

Cui, Q. L., Zheng, W. H., Quirion, R. and Almazan, G. (2005) Inhibition of Src-like kinases reveals Akt-dependent and -independent pathways in insulin-like

growth factor I-mediated oligodendrocyte progenitor survival. *J Biol Chem,* **280,** 8918-8928.

Czopka, T., Von Holst, A., Schmidt, G., ffrench-Constant, C. and Faissner, A. (2009) Tenascin C and tenascin R similarly prevent the formation of myelin membranes in a RhoA-dependent manner, but antagonistically regulate the expression of myelin basic protein via a separate pathway. *Glia,* **57,** 1790-1801.

de Castro, F. and Bribian, A. (2005) The molecular orchestra of the migration of oligodendrocyte precursors during development. *Brain Res Brain Res Rev,* **49,** 227-241.

De Robertis, E., Gerschenfeld, H. M. and Wald, F. (1958) Cellular mechanism of myelination in the central nervous system. *J Biophys Biochem Cytol,* **4,** 651-656.

Del Re, D. P., Miyamoto, S. and Brown, J. H. (2008) Focal adhesion kinase as a RhoA-activable signaling scaffold mediating Akt activation and cardiomyocyte protection. *J Biol Chem,* **283,** 35622-35629.

Dennis, J., White, M. A., Forrest, A. D., Yuelling, L. M., Nogaroli, L., Afshari, F. S., Fox, M. A. and Fuss, B. (2008) Phosphodiesterase-Ialpha/autotaxin's MORFO domain regulates oligodendroglial process network formation and focal adhesion organization. *Mol Cell Neurosci,* **37,** 412-424.

Doerflinger, N. H., Macklin, W. B. and Popko, B. (2003) Inducible site-specific recombination in myelinating cells. *Genesis,* **35,** 63-72.

Dugas, J. C., Tai, Y. C., Speed, T. P., Ngai, J. and Barres, B. A. (2006) Functional genomic analysis of oligodendrocyte differentiation. *J Neurosci,* **26,** 10967-10983.

Dupree, J. L., Coetzee, T., Blight, A., Suzuki, K. and Popko, B. (1998) Myelin galactolipids are essential for proper node of Ranvier formation in the CNS. *J Neurosci,* **18,** 1642-1649.

Dupree, J. L., Girault, J. A. and Popko, B. (1999) Axo-glial interactions regulate the localization of axonal paranodal proteins. *J Cell Biol,* **147,** 1145-1152.

Dupree, J. L., Mason, J. L., Marcus, J. R., Stull, M., Levinson, R., Matsushima, G. K. and Popko, B. (2005) Oligodendrocytes assist in the maintenance of sodium channel clusters independent of the myelin sheath. *Neuron Glia Biol,* **1,** 1-14.

Emery, B., Agalliu, D., Cahoy, J. D. et al. (2009) Myelin gene regulatory factor is a critical transcriptional regulator required for CNS myelination. *Cell,* **138,** 172-185.

Endo, M. and Yamashita, T. (2009) Inactivation of Ras by p120GAP via focal adhesion kinase dephosphorylation mediates RGMa-induced growth cone collapse. *J Neurosci,* **29,** 6649-6662.

Ezratty, E. J., Partridge, M. A. and Gundersen, G. G. (2005) Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol,* **7,** 581-590.

Falk, J., Bechara, A., Fiore, R. et al. (2005) Dual functional activity of semaphorin 3B is required for positioning the anterior commissure. *Neuron,* **48,** 63-75.

Flores, A. I., Narayanan, S. P., Morse, E. N., Shick, H. E., Yin, X., Kidd, G., Avila, R. L., Kirschner, D. A. and Macklin, W. B. (2008) Constitutively active Akt induces enhanced myelination in the CNS. *J Neurosci,* **28,** 7174-7183.

Foran, D. R. and Peterson, A. C. (1992) Myelin acquisition in the central nervous system of the mouse revealed by an MBP-Lac Z transgene. *J Neurosci,* **12,** 4890-4897.

Forrest, A. D., Beggs, H. E., Reichardt, L. F., Dupree, J. L., Colello, R. J. and Fuss, B. (2009) Focal adhesion kinase (FAK): A regulator of CNS myelination. *J Neurosci Res,* **87,** 3456-3464.

Fox, M. A., Afshari, F. S., Alexander, J. K., Colello, R. J. and Fuss, B. (2006a) Growth conelike sensorimotor structures are characteristic features of postmigratory, premyelinating oligodendrocytes. *Glia,* **53,** 563-566.

Fox, M. A., Alexander, J. K., Afshari, F. S., Colello, R. J. and Fuss, B. (2004) Phosphodiesterase-I alpha/autotaxin controls cytoskeletal organization and FAK phosphorylation during myelination. *Mol Cell Neurosci,* **27,** 140-150.

Fox, M. A., Colello, R. J., Macklin, W. B. and Fuss, B. (2003) Phosphodiesterase-Ialpha/autotaxin: a counteradhesive protein expressed by oligodendrocytes during onset of myelination. *Mol Cell Neurosci,* **23,** 507-519.

Fox, R. J., Bethoux, F., Goldman, M. D. and Cohen, J. A. (2006b) Multiple sclerosis: advances in understanding, diagnosing, and treating the underlying disease. *Cleve Clin J Med,* **73,** 91-102.

Fraher, J. P., Kaar, G. F., Bristol, D. C. and Rossiter, J. P. (1988) Development of ventral spinal motoneurone fibres: a correlative study of the growth and maturation of central and peripheral segments of large and small fibre classes. *Prog Neurobiol,* **31,** 199-239.

Franklin, R. J. and ffrench-Constant, C. (2008) Remyelination in the CNS: from biology to therapy. *Nat Rev Neurosci,* **9,** 839-855.

Fraser, M. M., Bayazitov, I. T., Zakharenko, S. S. and Baker, S. J. (2008) Phosphatase and tensin homolog, deleted on chromosome 10 deficiency in brain causes defects in synaptic structure, transmission and plasticity, and myelination abnormalities. *Neuroscience,* **151,** 476-488.

Fridman, R., Alon, Y., Doljanski, F., Fuks, Z. and Vlodavsky, I. (1985) Cell interaction with the extracellular matrices produced by endothelial cells and fibroblasts. *Exp Cell Res,* **158,** 461-476.

Frost, E. E., Buttery, P. C., Milner, R. and ffrench-Constant, C. (1999) Integrins mediate a neuronal survival signal for oligodendrocytes. *Curr Biol,* **9,** 1251-1254.

Furuta, Y., Ilic, D., Kanazawa, S., Takeda, N., Yamamoto, T. and Aizawa, S. (1995) Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. *Oncogene,* **11,** 1989-1995.

Fuss, B., Afshari, F. S., Colello, R. J. and Macklin, W. B. (2001) Normal CNS myelination in transgenic mice overexpressing MHC class I H-2L(d) in oligodendrocytes. *Mol Cell Neurosci,* **18,** 221-234.

Fuss, B., Mallon, B., Phan, T., Ohlemeyer, C., Kirchhoff, F., Nishiyama, A. and Macklin, W. B. (2000) Purification and analysis of in vivo-differentiated oligodendrocytes expressing the green fluorescent protein. *Dev Biol,* **218,** 259-274.

Geiger, B., Spatz, J. P. and Bershadsky, A. D. (2009) Environmental sensing through focal adhesions. *Nat Rev Mol Cell Biol,* **10,** 21-33.

George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. and Hynes, R. O. (1993) Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development,* **119,** 1079-1091.

Geurts, J. J. and Barkhof, F. (2008) Grey matter pathology in multiple sclerosis. *Lancet Neurol,* **7,** 841-851.

Giancotti, F. G. and Ruoslahti, E. (1999) Integrin signaling. *Science,* **285,** 1028-1032.

Grant, S. G., Karl, K. A., Kiebler, M. A. and Kandel, E. R. (1995) Focal adhesion kinase in the brain: novel subcellular localization and specific regulation by Fyn tyrosine kinase in mutant mice. *Genes Dev,* **9,** 1909-1921.

Guan, J. L. and Shalloway, D. (1992) Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature,* **358,** 690-692.

Gudz, T. I., Schneider, T. E., Haas, T. A. and Macklin, W. B. (2002) Myelin proteolipid protein forms a complex with integrins and may participate in integrin receptor signaling in oligodendrocytes. *J Neurosci,* **22,** 7398-7407.

Halfpenny, C., Benn, T. and Scolding, N. (2002) Cell transplantation, myelin repair, and multiple sclerosis. *Lancet Neurol,* **1,** 31-40.

Hall, A. (1998) Rho GTPases and the actin cytoskeleton. *Science,* **279,** 509-514.

Hamadi, A., Bouali, M., Dontenwill, M., Stoeckel, H., Takeda, K. and Ronde, P. (2005) Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. *J Cell Sci,* **118,** 4415-4425.

Hanks, S. K., Calalb, M. B., Harper, M. C. and Patel, S. K. (1992) Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc Natl Acad Sci U S A,* **89,** 8487-8491.

Hanks, S. K. and Polte, T. R. (1997) Signaling through focal adhesion kinase. *Bioessays,* **19,** 137-145.

Hanks, S. K., Ryzhova, L., Shin, N. Y. and Brabek, J. (2003) Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Front Biosci,* **8,** d982-996.

Hardy, R. J., Lazzarini, R. A., Colman, D. R. and Friedrich, V. L., Jr. (1996) Cytoplasmic and nuclear localization of myelin basic proteins reveals heterogeneity among oligodendrocytes. *J Neurosci Res,* **46,** 246-257.

Hildebrand, C. and Waxman, S. G. (1984) Postnatal differentiation of rat optic nerve fibers: electron microscopic observations on the development of nodes of Ranvier and axoglial relations. *J Comp Neurol,* **224,** 25-37.

Hoshina, N., Tezuka, T., Yokoyama, K., Kozuka-Hata, H., Oyama, M. and Yamamoto, T. (2007) Focal adhesion kinase regulates laminin-induced oligodendroglial process outgrowth. *Genes Cells,* **12,** 1245-1254.

Hu, J., Deng, L., Wang, X. and Xu, X. M. (2009) Effects of extracellular matrix molecules on the growth properties of oligodendrocyte progenitor cells in vitro. *J Neurosci Res,* **87,** 2854-2862.

Hunter, A. and Bedi, K. S. (1986) A quantitative morphological study of interstrain variation in the developing rat optic nerve. *J Comp Neurol,* **245,** 160-166.

Ilic, D., Furuta, Y., Kanazawa, S. et al. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature,* **377,** 539-544.

Ishibashi, T., Dakin, K. A., Stevens, B., Lee, P. R., Kozlov, S. V., Stewart, C. L. and Fields, R. D. (2006) Astrocytes promote myelination in response to electrical impulses. *Neuron,* **49,** 823-832.

Jackman, N., Ishii, A. and Bansal, R. (2009) Oligodendrocyte development and myelin biogenesis: parsing out the roles of glycosphingolipids. *Physiology (Bethesda),* **24,** 290-297.

Jakovcevski, I. and Zecevic, N. (2005) Olig transcription factors are expressed in oligodendrocyte and neuronal cells in human fetal CNS. *J Neurosci,* **25,** 10064-10073.

Jiang, X., Jacamo, R., Zhukova, E., Sinnett-Smith, J. and Rozengurt, E. (2006) RNA interference reveals a differential role of FAK and Pyk2 in cell migration, leading edge formation and increase in focal adhesions induced by LPA in intestinal epithelial cells. *J Cell Physiol,* **207,** 816-828.

Kaplan, M. R., Cho, M. H., Ullian, E. M., Isom, L. L., Levinson, S. R. and Barres, B. A. (2001) Differential control of clustering of the sodium channels Na(v)1.2 and Na(v)1.6 at developing CNS nodes of Ranvier. *Neuron,* **30,** 105-119.

Kaplan, M. R., Meyer-Franke, A., Lambert, S., Bennett, V., Duncan, I. D., Levinson, S. R. and Barres, B. A. (1997) Induction of sodium channel clustering by oligodendrocytes. *Nature,* **386,** 724-728.

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M. and Richardson, W. D. (2006) Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat Neurosci,* **9,** 173-179.

Kettenmann, H. a. R., B. (2005) *Neuroglia Second Edition*: Neuroglia. Oxford University Press Inc.

Kilpatrick, T. J., Ortuno, D., Bucci, T., Lai, C. and Lemke, G. (2000) Rat oligodendroglia express c-met and focal adhesion kinase, protein tyrosine kinases implicated in regulating epithelial cell motility. *Neurosci Lett,* **279,** 5-8.

Kim, H. J., DiBernardo, A. B., Sloane, J. A., Rasband, M. N., Solomon, D., Kosaras, B., Kwak, S. P. and Vartanian, T. K. (2006) WAVE1 is required for oligodendrocyte morphogenesis and normal CNS myelination. *J Neurosci,* **26,** 5849-5859.

Klein, C., Kramer, E. M., Cardine, A. M., Schraven, B., Brandt, R. and Trotter, J. (2002) Process outgrowth of oligodendrocytes is promoted by interaction of fyn kinase with the cytoskeletal protein tau. *J Neurosci,* **22,** 698-707.

Klingbeil, C. K., Hauck, C. R., Hsia, D. A., Jones, K. C., Reider, S. R. and Schlaepfer, D. D. (2001) Targeting Pyk2 to beta 1-integrin-containing focal

contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells. *J Cell Biol,* **152,** 97-110.
Kramer, E. M., Klein, C., Koch, T., Boytinck, M. and Trotter, J. (1999) Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. *J Biol Chem,* **274,** 29042-29049.
Kuhlmann, T., Miron, V., Cui, Q., Wegner, C., Antel, J. and Bruck, W. (2008) Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain,* **131,** 1749-1758.
Laursen, L. S. and ffrench-Constant, C. (2007) Adhesion molecules in the regulation of CNS myelination. *Neuron Glia Biol,* **3,** 367-375.
Lazzarini, R. A. (2004) *Myellin Biology and Disorders*. Elsevier Academic Press.
Lee, K. K., de Repentigny, Y., Saulnier, R., Rippstein, P., Macklin, W. B. and Kothary, R. (2006) Dominant-negative beta1 integrin mice have region-specific myelin defects accompanied by alterations in MAPK activity. *Glia,* **53,** 836-844.
Leone, D. P., Genoud, S., Atanasoski, S., Grausenburger, R., Berger, P., Metzger, D., Macklin, W. B., Chambon, P. and Suter, U. (2003) Tamoxifen-inducible glia-specific Cre mice for somatic mutagenesis in oligodendrocytes and Schwann cells. *Mol Cell Neurosci,* **22,** 430-440.
Liang, X., Draghi, N. A. and Resh, M. D. (2004) Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. *J Neurosci,* **24,** 7140-7149.
Lim, S. T., Mikolon, D., Stupack, D. G. and Schlaepfer, D. D. (2008a) FERM control of FAK function: implications for cancer therapy. *Cell Cycle,* **7,** 2306-2314.
Lim, Y., Lim, S. T., Tomar, A. et al. (2008b) PyK2 and FAK connections to p190Rho guanine nucleotide exchange factor regulate RhoA activity, focal adhesion formation, and cell motility. *J Cell Biol,* **180,** 187-203.
Liu, Y., Loijens, J. C., Martin, K. H., Karginov, A. V. and Parsons, J. T. (2002) The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly. *Mol Biol Cell,* **13,** 2147-2156.
Liu, Z., Hu, X., Cai, J., Liu, B., Peng, X., Wegner, M. and Qiu, M. (2007) Induction of oligodendrocyte differentiation by Olig2 and Sox10: evidence for reciprocal interactions and dosage-dependent mechanisms. *Dev Biol,* **302,** 683-693.
Lubetzki-Korn, I., Ovadia, H., Vlodavsky, I., Fuks, Z. and Abramsky, O. (1983) Enhanced growth and morphological differentiation of isolated adult rat oligodendrocytes in vitro: use of a naturally produced extracellular matrix. *Brain Res,* **267,** 151-155.
Maier, O., van der Heide, T., van Dam, A. M., Baron, W., de Vries, H. and Hoekstra, D. (2005) Alteration of the extracellular matrix interferes with raft association of neurofascin in oligodendrocytes. Potential significance for multiple sclerosis? *Mol Cell Neurosci,* **28,** 390-401.

Malek-Hedayat, S. and Rome, L. H. (1994) Expression of a beta 1-related integrin by oligodendroglia in primary culture: evidence for a functional role in myelination. *J Cell Biol,* **124,** 1039-1046.

Masahira, N., Takebayashi, H., Ono, K. et al. (2006) Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells. *Dev Biol,* **293,** 358-369.

Matthews, M. A. and Duncan, D. (1971) A quantitative study of morphological changes accompanying the initiation and progress of myelin production in the dorsal funiculus of the rat spinal cord. *J Comp Neurol,* **142,** 1-22.

McFarland, H. F. and Martin, R. (2007) Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol,* **8,** 913-919.

Menegon, A., Burgaya, F., Baudot, P., Dunlap, D. D., Girault, J. A. and Valtorta, F. (1999) FAK+ and PYK2/CAKbeta, two related tyrosine kinases highly expressed in the central nervous system: similarities and differences in the expression pattern. *Eur J Neurosci,* **11,** 3777-3788.

Miller, R. H. (1996) Oligodendrocyte origins. *Trends Neurosci,* **19,** 92-96.

Miller, R. H. (2002) Regulation of oligodendrocyte development in the vertebrate CNS. *Prog Neurobiol,* **67,** 451-467.

Milner, R. and ffrench-Constant, C. (1994) A developmental analysis of oligodendroglial integrins in primary cells: changes in alpha v-associated beta subunits during differentiation. *Development,* **120,** 3497-3506.

Milner, R., Frost, E., Nishimura, S., Delcommenne, M., Streuli, C., Pytela, R. and ffrench-Constant, C. (1997) Expression of alpha vbeta3 and alpha vbeta8 integrins during oligodendrocyte precursor differentiation in the presence and absence of axons. *Glia,* **21,** 350-360.

Mitra, S. K., Hanson, D. A. and Schlaepfer, D. D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol,* **6,** 56-68.

Mitra, S. K. and Schlaepfer, D. D. (2006) Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol,* **18,** 516-523.

Miyamoto, Y., Yamauchi, J., Chan, J. R., Okada, A., Tomooka, Y., Hisanaga, S. and Tanoue, A. (2007) Cdk5 regulates differentiation of oligodendrocyte precursor cells through the direct phosphorylation of paxillin. *J Cell Sci,* **120,** 4355-4366.

Moeller, M. L., Shi, Y., Reichardt, L. F. and Ethell, I. M. (2006) EphB receptors regulate dendritic spine morphogenesis through the recruitment/phosphorylation of focal adhesion kinase and RhoA activation. *J Biol Chem,* **281,** 1587-1598.

Nait-Oumesmar, B., Decker, L., Lachapelle, F., Avellana-Adalid, V., Bachelin, C. and Van Evercooren, A. B. (1999) Progenitor cells of the adult mouse subventricular zone proliferate, migrate and differentiate into oligodendrocytes after demyelination. *Eur J Neurosci,* **11,** 4357-4366.

Nakamura, I., Duong le, T., Rodan, S. B. and Rodan, G. A. (2007) Involvement of alpha(v)beta3 integrins in osteoclast function. *J Bone Miner Metab,* **25,** 337-344.

Nery, S., Wichterle, H. and Fishell, G. (2001) Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain. *Development,* **128,** 527-540.
Nicolay, D. J., Doucette, J. R. and Nazarali, A. J. (2007) Transcriptional control of oligodendrogenesis. *Glia,* **55,** 1287-1299.
Notterpek, L. M. and Rome, L. H. (1994) A protein involved in central nervous system myelination: localization in the extracellular matrix and induction in neuroblastoma cells. *Dev Neurosci,* **16,** 267-278.
Oh, L. Y. and Yong, V. W. (1996) Astrocytes promote process outgrowth by adult human oligodendrocytes in vitro through interaction between bFGF and astrocyte extracellular matrix. *Glia,* **17,** 237-253.
Olsen, I. M. and ffrench-Constant, C. (2005) Dynamic regulation of integrin activation by intracellular and extracellular signals controls oligodendrocyte morphology. *BMC Biology,* **3**.
Orr, A. W. and Murphy-Ullrich, J. E. (2004) Regulation of endothelial cell function BY FAK and PYK2. *Front Biosci,* **9,** 1254-1266.
Osterhout, D. J., Wolven, A., Wolf, R. M., Resh, M. D. and Chao, M. V. (1999) Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. *J Cell Biol,* **145,** 1209-1218.
Parsons, J. T. (2003) Focal adhesion kinase: the first ten years. *J Cell Sci,* **116,** 1409-1416.
Pfeiffer, S. E., Warrington, A. E. and Bansal, R. (1993) The oligodendrocyte and its many cellular processes. *Trends Cell Biol,* **3,** 191-197.
Playford, M. P. and Schaller, M. D. (2004) The interplay between Src and integrins in normal and tumor biology. *Oncogene,* **23,** 7928-7946.
Ponniah, S., Wang, D. Z., Lim, K. L. and Pallen, C. J. (1999) Targeted disruption of the tyrosine phosphatase PTPalpha leads to constitutive downregulation of the kinases Src and Fyn. *Curr Biol,* **9,** 535-538.
Rajasekharan, S., Baker, K. A., Horn, K. E., Jarjour, A. A., Antel, J. P. and Kennedy, T. E. (2009) Netrin 1 and Dcc regulate oligodendrocyte process branching and membrane extension via Fyn and RhoA. *Development,* **136,** 415-426.
Rajasekharan, S., Bin, J. M., Antel, J. P. and Kennedy, T. E. (2010) A Central Role for RhoA during Oligodendroglial Maturation in the Switch from Netrin-1 Mediated Chemorepulsion to Process Elaboration. *J Neurochem*.
Relucio, J., Tzvetanova, I. D., Ao, W., Lindquist, S. and Colognato, H. (2009) Laminin alters fyn regulatory mechanisms and promotes oligodendrocyte development. *J Neurosci,* **29,** 11794-11806.
Relvas, J. B., Setzu, A., Baron, W., Buttery, P. C., LaFlamme, S. E., Franklin, R. J. and ffrench-Constant, C. (2001) Expression of dominant-negative and chimeric subunits reveals an essential role for beta1 integrin during myelination. *Curr Biol,* **11,** 1039-1043.
Remahl, S. and Hilderbrand, C. (1990) Relation between axons and oligodendroglial cells during initial myelination. I. The glial unit. *J Neurocytol,* **19,** 313-328.

Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D. and Schwartz, M. A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci,* **113 (Pt 20),** 3673-3678.
Richardson, W. D., Kessaris, N. and Pringle, N. (2006) Oligodendrocyte wars. *Nat Rev Neurosci,* **7,** 11-18.
Richter-Landsberg, C. (2008) The cytoskeleton in oligodendrocytes. Microtubule dynamics in health and disease. *J Mol Neurosci,* **35,** 55-63.
Rico, B., Beggs, H. E., Schahin-Reed, D., Kimes, N., Schmidt, A. and Reichardt, L. F. (2004) Control of axonal branching and synapse formation by focal adhesion kinase. *Nat Neurosci,* **7,** 1059-1069.
Robles, E. and Gomez, T. M. (2006) Focal adhesion kinase signaling at sites of integrin-mediated adhesion controls axon pathfinding. *Nat Neurosci*.
Romer, L. H., Birukov, K. G. and Garcia, J. G. (2006) Focal adhesions: paradigm for a signaling nexus. *Circ Res,* **98,** 606-616.
Rosenbluth, J. (2009) Multiple functions of the paranodal junction of myelinated nerve fibers. *J Neurosci Res,* **87,** 3250-3258.
Salzer, J. L., Brophy, P. J. and Peles, E. (2008) Molecular domains of myelinated axons in the peripheral nervous system. *Glia,* **56,** 1532-1540.
Sanders, F. K. and Whitteridge, D. (1946) Conduction velocity and myelin thickness in regenerating nerve fibres. *J Physiol,* **105,** 152-174.
Satoh, J. I., Tabunoki, H. and Yamamura, T. (2009) Molecular network of the comprehensive multiple sclerosis brain-lesion proteome. *Mult Scler,* **15,** 531-541.
Schaller, M. D. (2010) Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. *J Cell Sci,* **123,** 1007-1013.
Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992) pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A,* **89,** 5192-5196.
Schlaepfer, D. D., Hanks, S. K., Hunter, T. and van der Geer, P. (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature,* **372,** 786-791.
Schlaepfer, D. D., Hauck, C. R. and Sieg, D. J. (1999) Signaling through focal adhesion kinase. *Prog Biophys Mol Biol,* **71,** 435-478.
Schlaepfer, D. D. and Hunter, T. (1996) Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol,* **16,** 5623-5633.
Schlomann, U., Schwamborn, J. C., Muller, M., Fassler, R. and Puschel, A. W. (2009) The stimulation of dendrite growth by Sema3A requires integrin engagement and focal adhesion kinase. *J Cell Sci,* **122,** 2034-2042.
Schnitzer, J. and Schachner, M. (1982) Cell type specificity of a neural cell surface antigen recognized by the monoclonal antibody A2B5. *Cell Tissue Res,* **224,** 625-636.
Schwab, M. E. and Schnell, L. (1989) Region-specific appearance of myelin constituents in the developing rat spinal cord. *J Neurocytol,* **18,** 161-169.

Schwartz, M. A. and Ingber, D. E. (1994) Integrating with integrins. *Mol Biol Cell,* **5,** 389-393.
Shi, Y., Pontrello, C. G., DeFea, K. A., Reichardt, L. F. and Ethell, I. M. (2009) Focal adhesion kinase acts downstream of EphB receptors to maintain mature dendritic spines by regulating cofilin activity. *J Neurosci,* **29,** 8129-8142.
Sieg, D. J., Ilic, D., Jones, K. C., Damsky, C. H., Hunter, T. and Schlaepfer, D. D. (1998) Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration. *EMBO J,* **17,** 5933-5947.
Sim, F. J., Lang, J. K., Waldau, B. et al. (2006) Complementary patterns of gene expression by human oligodendrocyte progenitors and their environment predict determinants of progenitor maintenance and differentiation. *Ann Neurol,* **59,** 763-779.
Simons, M. and Trajkovic, K. (2006) Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. *J Cell Sci,* **119,** 4381-4389.
Siskova, Z., Baron, W., de Vries, H. and Hoekstra, D. (2006) Fibronectin impedes "myelin" sheet-directed flow in oligodendrocytes: a role for a beta 1 integrin-mediated PKC signaling pathway in vesicular trafficking. *Mol Cell Neurosci,* **33,** 150-159.
Siskova, Z., Yong, V. W., Nomden, A., van Strien, M., Hoekstra, D. and Baron, W. (2009) Fibronectin attenuates process outgrowth in oligodendrocytes by mislocalizing MMP-9 activity. *Mol Cell Neurosci,* **42,** 234-242.
Skoff, R. P., Price, D. L. and Stocks, A. (1976a) Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. I. Cell proliferation. *J Comp Neurol,* **169,** 291-312.
Skoff, R. P., Price, D. L. and Stocks, A. (1976b) Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. *J Comp Neurol,* **169,** 313-334.
Skoff, R. P., Toland, D. and Nast, E. (1980) Pattern of myelination and distribution of neuroglial cells along the developing optic system of the rat and rabbit. *J Comp Neurol,* **191,** 237-253.
Slack-Davis, J. K., Martin, K. H., Tilghman, R. W. et al. (2007) Cellular characterization of a novel focal adhesion kinase inhibitor. *J Biol Chem*.
Sloane, J. A. and Vartanian, T. K. (2007) WAVE1 and regulation of actin nucleation in myelination. *Neuroscientist,* **13,** 486-491.
Sobel, R. A. (1998) The extracellular matrix in multiple sclerosis lesions. *J Neuropathol Exp Neurol,* **57,** 205-217.
Sobel, R. A. and Mitchell, M. E. (1989) Fibronectin in multiple sclerosis lesions. *Am J Pathol,* **135,** 161-168.
Society, N. M. S. (2010). http://www.nationalmssociety.org/index.aspx
Sommer, I. and Schachner, M. (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev Biol,* **83,** 311-327.

Song, J., Goetz, B. D., Baas, P. W. and Duncan, I. D. (2001) Cytoskeletal reorganization during the formation of oligodendrocyte processes and branches. *Mol Cell Neurosci,* **17,** 624-636.
Southwood, C. M., Peppi, M., Dryden, S., Tainsky, M. A. and Gow, A. (2007) Microtubule deacetylases, SirT2 and HDAC6, in the nervous system. *Neurochem Res,* **32,** 187-195.
Sperber, B. R., Boyle-Walsh, E. A., Engleka, M. J., Gadue, P., Peterson, A. C., Stein, P. L., Scherer, S. S. and McMorris, F. A. (2001) A unique role for Fyn in CNS myelination. *J Neurosci,* **21,** 2039-2047.
Sperber, B. R. and McMorris, F. A. (2001) Fyn tyrosine kinase regulates oligodendroglial cell development but is not required for morphological differentiation of oligodendrocytes. *J Neurosci Res,* **63,** 303-312.
Spiegel, I. and Peles, E. (2006) A new player in CNS myelination. *Neuron,* **49,** 777-778.
Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol,* **1,** 4.
Stevens, G. R., Zhang, C., Berg, M. M., Lambert, M. P., Barber, K., Cantallops, I., Routtenberg, A. and Klein, W. L. (1996) CNS neuronal focal adhesion kinase forms clusters that co-localize with vinculin. *J Neurosci Res,* **46,** 445-455.
Sypecka, J., Dragun-Szymczak, P., Zalewska, T. and Domanska-Janik, K. (2009) Laminin promotes oligogliogenesis and increases MMPs activity in human neural stem cells of HUCB-NSC line. *Acta Neurobiol Exp (Wars),* **69,** 37-45.
Szuchet, S., Watanabe, K. and Yamaguchi, Y. (2000) Differentiation/regeneration of oligodendrocytes entails the assembly of a cell-associated matrix. *Int J Dev Neurosci,* **18,** 705-720.
Taylor, J. M., Hildebrand, J. D., Mack, C. P., Cox, M. E. and Parsons, J. T. (1998) Characterization of graf, the GTPase-activating protein for rho associated with focal adhesion kinase. Phosphorylation and possible regulation by mitogen-activated protein kinase. *J Biol Chem,* **273,** 8063-8070.
Tennekoon, G. I., Cohen, S. R., Price, D. L. and McKhann, G. M. (1977) Myelinogenesis in optic nerve. A morphological, autoradiographic, and biochemical analysis. *J Cell Biol,* **72,** 604-616.
Thomson, C. E., Vouyiouklis, D. A., Barrie, J. A., Wease, K. N. and Montague, P. (2005) Plp gene regulation in the developing murine optic nerve: correlation with oligodendroglial process alignment along the axons. *Dev Neurosci,* **27,** 27-36.
Thurnherr, T., Benninger, Y., Wu, X. et al. (2006) Cdc42 and Rac1 signaling are both required for and act synergistically in the correct formation of myelin sheaths in the CNS. *J Neurosci,* **26,** 10110-10119.
Timsit, S., Martinez, S., Allinquant, B., Peyron, F., Puelles, L. and Zalc, B. (1995) Oligodendrocytes originate in a restricted zone of the embryonic ventral

neural tube defined by DM-20 mRNA expression. *J Neurosci,* **15,** 1012-1024.
Tohda, C., Nakanishi, R. and Kadowaki, M. (2006) Learning deficits and agenesis of synapses and myelinated axons in phosphoinositide-3 kinase-deficient mice. *Neurosignals,* **15,** 293-306.
Tom, V. J., Doller, C. M., Malouf, A. T. and Silver, J. (2004) Astrocyte-associated fibronectin is critical for axonal regeneration in adult white matter. *J Neurosci,* **24,** 9282-9290.
Tomar, A. and Schlaepfer, D. D. (2009) Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. *Curr Opin Cell Biol,* **21,** 676-683.
Toutant, M., Costa, A., Studler, J. M., Kadare, G., Carnaud, M. and Girault, J. A. (2002) Alternative splicing controls the mechanisms of FAK autophosphorylation. *Mol Cell Biol,* **22,** 7731-7743.
Trapp, B. D., Nishiyama, A., Cheng, D. and Macklin, W. (1997) Differentiation and death of premyelinating oligodendrocytes in developing rodent brain. *J Cell Biol,* **137,** 459-468.
Tyler, W. A., Gangoli, N., Gokina, P., Kim, H. A., Covey, M., Levison, S. W. and Wood, T. L. (2009) Activation of the mammalian target of rapamycin (mTOR) is essential for oligodendrocyte differentiation. *J Neurosci,* **29,** 6367-6378.
Uzman, L. L. and Rumley, M. K. (1958) Changes in the composition of the developing mouse brain during early myelination. *J Neurochem,* **3,** 170-184.
van Horssen, J., Dijkstra, C. D. and de Vries, H. E. (2007) The extracellular matrix in multiple sclerosis pathology. *J Neurochem,* **103,** 1293-1301.
Wang, H., Tewari, A., Einheber, S., Salzer, J. L. and Melendez-Vasquez, C. V. (2008) Myosin II has distinct functions in PNS and CNS myelin sheath formation. *J Cell Biol,* **182,** 1171-1184.
Wang, P. S., Wang, J., Xiao, Z. C. and Pallen, C. J. (2009) Protein-tyrosine phosphatase alpha acts as an upstream regulator of Fyn signaling to promote oligodendrocyte differentiation and myelination. *J Biol Chem,* **284,** 33692-33702.
Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T. and Horwitz, A. F. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol,* **6,** 154-161.
Wegner, M. (2008) A matter of identity: transcriptional control in oligodendrocytes. *J Mol Neurosci,* **35,** 3-12.
Weis, S. M., Lim, S. T., Lutu-Fuga, K. M. et al. (2008) Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *J Cell Biol,* **181,** 43-50.
Westhoff, M. A., Serrels, B., Fincham, V. J., Frame, M. C. and Carragher, N. O. (2004) SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol Cell Biol,* **24,** 8113-8133.

Wight, P. A., Duchala, C. S., Readhead, C. and Macklin, W. B. (1993) A myelin proteolipid protein-LacZ fusion protein is developmentally regulated and targeted to the myelin membrane in transgenic mice. *J Cell Biol,* **123,** 443-454.

Wolf, R. M., Wilkes, J. J., Chao, M. V. and Resh, M. D. (2001) Tyrosine phosphorylation of p190 RhoGAP by Fyn regulates oligodendrocyte differentiation. *J Neurobiol,* **49,** 62-78.

Wu, C. and Dedhar, S. (2001) Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. *J Cell Biol,* **155,** 505-510.

Wu, L., Bernard-Trifilo, J. A., Lim, Y. et al. (2008) Distinct FAK-Src activation events promote alpha5beta1 and alpha4beta1 integrin-stimulated neuroblastoma cell motility. *Oncogene,* **27,** 1439-1448.

Wu, X., Suetsugu, S., Cooper, L. A., Takenawa, T. and Guan, J. L. (2004) Focal adhesion kinase regulation of N-WASP subcellular localization and function. *J Biol Chem,* **279,** 9565-9576.

Ye, F., Hu, G., Taylor, D., Ratnikov, B., Bobkov, A. A., McLean, M. A., Sligar, S. G., Taylor, K. A. and Ginsberg, M. H. (2010) Recreation of the terminal events in physiological integrin activation. *J Cell Biol,* **188,** 157-173.

Younes-Rapozo, V., Felgueiras, L. O., Viana, N. L., Fierro, I. M., Barja-Fidalgo, C., Manhaes, A. C. and Barradas, P. C. (2009) A role for the MAPK/ERK pathway in oligodendroglial differentiation in vitro: stage specific effects on cell branching. *Int J Dev Neurosci,* **27,** 757-768.

Zamir, E. and Geiger, B. (2001) Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci,* **114,** 3583-3590.

Zhai, J., Lin, H., Nie, Z., Wu, J., Canete-Soler, R., Schlaepfer, W. W. and Schlaepfer, D. D. (2003) Direct interaction of focal adhesion kinase with p190RhoGEF. *J Biol Chem,* **278,** 24865-24873.

Zhao, C., Fancy, S. P., Franklin, R. J. and ffrench-Constant, C. (2009) Up-regulation of oligodendrocyte precursor cell alphaV integrin and its extracellular ligands during central nervous system remyelination. *J Neurosci Res,* **87,** 3447-3455.

Zhou, Q., Wang, S. and Anderson, D. J. (2000) Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. *Neuron,* **25,** 331-343.

Zhou, Z., Feng, H. and Bai, Y. (2006) Detection of a hidden folding intermediate in the focal adhesion target domain: Implications for its function and folding. *Proteins,* **65,** 259-265.

Vita

Audrey Diane Lafrenaye was born in Syracuse New York on December, 31, 1981 and is an American citizen. She graduated from Lake Braddock Secondary School in Burke, Virginia in 2000. Her education continued at Christopher Newport University in Newport News, Virginia where she earned her Bachelor of Arts in Biology. She graduated *Cum Laude* from Christopher Newport University in 2004 and immediately began her graduate education; earning a Post-Baccalaureate Premedical Certificate in anatomy from the Department of Anatomy and Neurobiology at Virginia Commonwealth University in 2005. The following fall Audrey transitioned into the Ph.D. program in the Department of Anatomy and Neurobiology and joined the laboratory of Babette Fuss in the spring of 2006. As a graduate student, Audrey earned various honors and awards including the Hgalmer L. Osterud Award for service in the Department of Anatomy and Neurobiology and membership in the Phi Kappa Phi honor society. In the spring of 2008 Audrey received another certificate from the Preparing Future Faculty program. In 2009 Audrey also secured a Ruth L. Kirschstein National Research Service Award for Individual Predoctoral Fellows. Throughout her graduate career she was given the opportunity to present her research at the annual Society for Neuroscience meeting as well as various institutional presentations.

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)

AD Forrest, HE Beggs, LF Reichhardt, JL Dupree, RJ Colello and B Fuss **Focal Adhesion Kinase (FAK) is a regulator of CNS myelination.** Journal of Neuroscience Research **87**, (2009) p.p.3456-3464

J Dennis, MA White, **AD Forrest**, LM Yuelling, L Nogaroli, FS Afshari, MA Fox, and B Fuss. **Phosphodiesterase-I alpha/Autotaxin's MORFO domain regulates oligodendroglial process network formation and focal adhesion organization.** Molecular and Cellular Neuroscience. **37**, (2008), pp. 412-424.