Regulation of oligodendrocyte differentiation and myelination by p38 mitogen-activated protein kinase

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ABSTRACT

Oligodendrocytes (OLG) in the central nervous system (CNS) extend multiple processes to ensheath nerve axons with the spiral wrapped membrane known as myelin. The signal transduction pathways that regulate OLG differentiation and CNS myelination are only beginning to be elucidated. We hypothesized that p38 mitogen-activated protein kinase could regulate OLG differentiation, since this kinase has been found to regulate the growth of peripheral nervous system myelinating Schwann cells. Using cultured oligodendrocyte progenitors, we found that treatment with PD169316, a selective p38 MAPK inhibitor, prevented accumulation of mRNA and protein of cell-stage specific markers characteristic of differentiated OLGs, including myelin basic protein (MBP), myelin-associated glycoprotein (MAG), and the glycosphingolipids, galactosylceramide and sulfatide. In addition, the cell cycle regulator $p27^{KIP1}$ and the transcription factor Sox10 were also significantly reduced. Most significantly, p38 inhibitors completely and irreversibly blocked myelination of dorsal root ganglion neurons by OLGs and prevented the axolemmal organization of the axo-glial adhesion molecule Caspr.

Furthermore, we found that a p38 substrate, mitogen-activated protein kinase activated protein kinase 2 (MK2) is a downstream element of the p38 signaling pathway in OLGs responsible for effecting their differentiation. Thus, inhibition of MK2 activity in OLGs decreased the mRNA and protein levels of myelin-specific proteins MAG and MBP and upregulated transcriptional repressors that normally block OLG differentiation, including transcription factor 4, Notch, and inhibitor of differentiation 2. A genome-wide analysis of OLGs treated with PD169316 confirmed that p38 regulates myelin gene expression and identified a large group of upregulated genes involved in cell cycle regulation and cytokinesis. These data suggest that p38 controls OLG differentiation through the repression of genes involved in cell cycle and cytokinesis and by promoting the expression of pro-myelin gene activators. Overall, our results suggest important roles for p38 signaling in the differentiation of OLGs.

RÉSUMÉ

Dans le système nerveux central (SNC), les oligodendrocytes (OLG) forment de multiples prolongements qui s'enroulent autour des axons formant ainsi la gaine de myéline. Les voies de signalisation qui régulent la différenciation des OLG et la myélinisation du SNC commencent seulement à être élucidées. Nous avons émis l'hypothèse que la MAP-kinase p38 (mitogen-activated protein kinase p38) pouvait réguler la différenciation des OLG puisqu'elle régule la croissance des cellules myélinisantes du système nerveux périphérique, les cellules Schwann. Dans des cultures de progéniteurs d'OLG, nous avons trouvé que le traitement avec un inhibiteur sélectif de MAPK-p38, PD169316, empêchait l'accumulation d'ARNm et de protéines de marqueurs spécifiques du stade cellulaire caractérisants les OLG différenciés, incluant la protéine basique de la myéline (MBP), la glycoprotéine associée à la myéline (MAG) et les glycosphingolipides galactosylceramide et sulfatide. De plus, le régulateur du cycle cellulaire $p27^{KIP1}$ et le facteur de transcription Sox10 étaient également réduits significativement. De façon plus marquante, les inhibiteurs de p38 ont bloqué complètement et irréversiblement la myélinisation de neurones de ganglions de la racine dorsale par les OLG et ont empêché la localisation axolemmale de la molecule d'adhésion axo-gliale Caspr.

En outre, nous avons montré qu'un substrat de p38, la protéine kinase MK2 (MAPK-activated protein kinase 2), est impliqué en aval de la voie de signalisation de p38 dans les OLG et est un effecteur de leur différenciation. Ainsi, l'inhibition de l'activité de MK2 dans les OLG réduit les niveaux d'ARNm et de protéines de MAG et de MBP et sur-régule les répresseurs transcriptionnels qui bloquent normalement la différenciation des OLG, dont le facteur de transcription 4, Notch, et l'inhibiteur de différenciation 2. Une analyse génomique d'OLG traités avec PD169316 a confirmé que p38 régule l'expression de gènes codants pour les protéines de la myéline et a identifié un important groupe de gènes sur-régulés impliqués dans la régulation du cycle cellulaire et la cytokinèse. Ces résultats suggèrent que p38 contrôle la différenciation des OLG à travers la répression de gènes impliqués dans le cycle cellulaire et la cytokinèse et en soutenant l'expression d'activateurs de gènes

pro-myéline. En résumé, nos résultats suggèrent d'importants rôles de la signalisation de p38 dans la différenciation des OLG.

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Preface

This thesis is written in manuscript format as permitted in the guidelines of the McGill Faculty of Graduate Studies, and consists of four manuscripts as follows:

Chapter 2: Fragoso, G.*, Haines, J.D.*, Robertson, J., Pedraza, L., Mushynski, W.E., Almazan, G. p38 mitogen-activated protein kinase is required for central nervous system myelination. *Glia*. 55, 1531-44 (2007). [*denotes equal contribution]

Chapter 3: Haines, J.D., Fang, J., Mushynski, W.E., Almazan, G. Mitogen-activated protein kinase activated protein kinase 2 (MK2) participates in the p38 MAPK regulated control of oligodendrocyte differentiation. *Glia.* 58, 1384-93 (2010).

Chapter 4: Haines, J.D., Richard, S., Almazan G. Transcriptional profiles of p38 mitogen-activated protein kinase regulated genes in oligodendrocytes. *In preparation* (2010).

Chapter 5: Haines, J.D., Fragoso, G., Hossain, S., Mushynski, W.E., Almazan, G. p38 mitogen-activated protein kinase as a regulator of myelination. *J. Mol. Neurosci.*, 35, 23-33 (2007).

Contribution of Authors

Chapter 2: Experiments were performed by J. Haines (Figure 3, 4A, 4B, 7, 8B, Table 2) and G. Fragoso (Figure 1, 2, 4C, 6, 8A, Table 1). G. Almazan and W. Mushynski conceived the experimental design. The manuscript was mainly written by G. Almazan, with contributions from J. Haines, and editing from W. Mushynski. G. Fragoso performed the western blot densitometric quantifications and statistical analysis. L. Pedraza provided reagents, and J. Robertson helped to conceive some of the original experiments.

Chapter 3: Experiments were performed by J. Haines. J. Fang assisted with primary culture preparations and immunoprecipitations (Figure 9). The manuscript was written by J. Haines with editing and revisions by W. Mushynski and G. Almazan.

Chapter 4: All experiments and analysis were performed by J. Haines. S. Richard funded part of the project and provided useful discussions on the experimental approach. The manuscript was written by J. Haines with editing and revisions by G. Almazan

Chapter 5: This paper/article is primarily a review, although it contained some original experiments performed by either J. Haines (Figure 1, 4, 5) and G. Fragoso (Figures 2 and 3). J. Haines wrote much of the review, with assistance from G. Almazan. W. Mushynski and S. Hossain provided useful comments on the manuscript and editing.

I dedicate this thesis

to my parents & family

Original contribution to knowledge

- p38 mitogen activated protein kinase regulates the expression of OLG differentiation-stage markers, including CNP, MAG and MBP.
- p38 inhibitor (PD169316) irreversibly blocks OLG myelination of dorsal root ganglia neurons as determined by immunoblotting and immunocytochemistry for MBP.
- PD169316 prevents the axolemmal organization of the axo-glial adhesion molecule Caspr associated with myelination.
- p38 is phosphorylated (activated) when OLG progenitors make initial contact with dorsal root ganglia neurons.
- p38 inhibitor arrests OLGs at an early stage of differentiation and the effects are reversible.
- p38 inhibitor reversibly blocks the accumulation of myelin-specific proteins and glycosphingolipids (galactosylceramide and sulfatide) in maturing OLGs, since removal of PD169316 restores myelin protein expression.
- p38 inhibitor decreases mRNAs of OLG-specific transcripts including CNP, MAG, MBP, CGT, PLP, MOBP, and the transcription factor Sox10.
- p38 inhibitor decreases levels of the cell cycle inhibitor ($p27^{kip1}$) that are normally upregulated for proper OLG differentiation.
- The abundantly expressed p38α isoform localizes to the cytoplasm and nucleus of OLPs and localizes to the nucleus of maturing OLGs.
- The p38 inhibitors SB203580 and PD169316 are not toxic at the concentrations used in our studies to three different developmental stages of OLGs.

- p38 inhibitors increase mRNA levels of p38 upstream activator kinases (MKK6, PBK, DLK-1).
- PD169316 upregulates genes involved in cytokinesis (cell cycle regulators, cell division proteins, replication factors, kinesins, and cytoskeletal factors).
- p38 inhibitor increases mRNA levels of transcriptional repressors (e.g., Id2, Tcf4) and decreases mRNA encoding transcriptional activators (e.g., HDAC11 and Fyn).
- Small-interfering RNA to selectively knockdown p38α or MK2 reduces MAG and GalC expression.
- Protein levels of phosphorylated (activated) MK2 increases during OLG differentiation.
- Activated MK2 is localized mainly to the cytoplasm in OLG progenitors and becomes successively localized to the nucleus as OLGs mature.
- MK2 is a downstream effector of p38 that regulates OLG differentiation by regulating CNP, MAG, CGT and MBP.
- MK2 inhibitor does not affect survival or proliferation of OLG progenitors.
- MK2 inhibitor decreases mRNA levels of myelin-specific gene transcripts (MBP, MAG, Opalin, Myt1, OLIG2), and increases mRNAs which encode transcriptional repressors (Notch, Id2, Tcf4).
- MK2 forms protein complexes with p38α in oligodendrocyte progenitors. In maturing OLGs, MK2 forms protein complexes with p38α and hsp27. p38α, MSK1 and CREB form a separate co-immunoprecipitatable complex in OLGs.

LIST OF ABBREVIATIONS

ASK1 - apoptosis signal-regulating kinase 1

AP-1 – activator protein 1

ARE – AU-rich element

ATM - ataxia telangiectasia mutated

AU - adenine-uridine

BDNF – brain-derived neurotrophic factor

bFGF- basic fibroblast growth factor

bHLH - basic helix-loop-helix

BMK – big MAPK

BMP – bone morphogenetic protein

BMPR – BMP receptor

BrdU - bromodeoxyuridine

BSA- bovine serum albumin

CA - constitutive active

cAMP - cyclic adenosine monophosphate

Caspr - contactin associated protein

CBP – CREB binding protein

CDKI – Cdk inhibitor

Cdk - cyclin dependent kinase

C/EBP - CAATT enhancer binding protein

CGT - UDP galactose:ceramide galactosyltransferase

CHOP - C/EBP homology protein

CNP – 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS - central nervous system

CNTF - ciliary neurotrophic factor

cPLA2 – cytoplasmic phospholipase A2

CRE - Ca^{2+}/cyclic AMP-response element

CREB - cAMP response element binding protein

CSF-1 – colony-stimulating factor 1

CST - galactosylceramide 3'-sulfotransferase

C-term-pSMAD1 – C-terminal-phosphorylated-SMAD1

DAPI - 4,6-diamidino-2-phenylindole dihydrochloride

dbcAMP - dibutyrl cyclic adenosine monophosphate

DCC – deleted in colorectal cancer

dCKO – double conditional knockout

DMEM - Dulbecco's modified Eagle's medium

DN – dominant negative

DRG – dorsal root ganglion

DRGN - dorsal root ganglion neuron

DTT - dithiothreitol

EAE – experimental autoimmune encephalomyelitis

eIF – eukaryotic initiation factor

eIF4E - eukaryotic initiation factor 4E

E-NCAM – embryonic neural cell adhesion molecule

ER – endoplasmic reticulum

ERK - extracellular signal regulated kinase

FAK – focal adhesion kinase

FCS - fetal calf serum

GalC - galactosylceramide, galactosylcerebroside

GFAP – glial fibrillary acidic protein

GluC - glucosylceramide

Golli – genes of the oligodendrocyte lineage

GPCR – G protein coupled receptor

GRK2 – GPCR kinase 2

GSK3 - glycogen synthase kinase 3

GSL - glycosphingolipid

HBP1 - HMG-box protein 1

HBSS - Hank's balanced salt solution

HDAC - histone deacetylase

hINV – human involucrin

HLH - helix-loop-helix

HMG - high-mobility group

hnRNP - heterogeneous nuclear RNA-binding protein

HRP - horseradish peroxidase

Hsp25 / 27 - heat shock protein 25 / 27

HUVEC – human umbilical vein endothelial cells

IBD – inflammatory bowel disease

IE - immediate early

IGF-1 – insulin growth factor-1

IL - interleukin

JNK – c-Jun amino terminal kinase

K_v^+ - voltage-gated potassium channels

LIF - leukemia inhibitory factor

LIMK1 - LIM domain kinase 1

LPR6 - lipoprotein receptor-related protein 6

LPS - lipopolysaccharide

MAG - myelin-associated glycoprotein

MAPK - mitogen-activated protein kinase

MAPKK / MKK - MAPK kinase

MAPKKK / MKKK - MAPKK kinase

MAPKAPK2 - MAPK activated protein kinase 2

MBP - myelin basic protein

MEF – mouse embryonic fibroblast

MEF2C / 2A – myocyte enhancer factor 2C / 2A

MDM2 - murine double minute 2

miRNA – microRNA

MITF - microphthalmia-associated transcription factor

MK2 / 3/ 5 - MAPK activated protein kinase 2 / 3 / 5

MLK - mixed lineage kinase

MNK - MAPK-interacting kinases / MAPK signal-integrating kinases

MOBP - myelin-associated oligodendrocyte basic protein

MOG – myelin/oligodendrocyte glycoprotein

MRF – myelin gene regulatory factor

MS - multiple sclerosis

MSK – mitogen and stress activated protein kinase

Myt1 – myelin transcription factor 1

Myt1L – Myt1-like

Na_v – voltage-gated sodium channel

NER – nucleotide excision repair

NF - neurofilament

NF-155 - neurofascin-155

NFκB – nuclear factor κB

NFH- neurofilament heavy-chain

NGF - nerve growth factor

NPC – neural precursor cell

Nrg1 – neuregulin-1, type III

NT-3 – neurotrophin-3

OLIG1/2 – oligodendrocyte transcription factor 1/2

OLG - oligodendrocyte

OLP - oligodendrocyte progenitor

OMgp – oligodendrocyte myelin glycoprotein

Opalin - Oligodendrocytic myelin paranodal and inner loop protein

p-p38 – phosphorylated (activated) p38

PcG – polycomb group

PDGFAA - platelet derived growth factor AA

PDGRα - platelet derived growth factor receptor α

PKC – protein kinase C

Plk1 – polo-like kinase 1

PLP - proteolipid protein

PMD – Pelizaeus-Merzbacher disease

PNS - peripheral nervous system

PRAK - p38-regulated and -activated protein kinase

PRC – polycomb repressive complex

PSF - polypyrimidine-tract binding protein-associated splicing factor

PTP - protein tyrosine phosphatase

RA – rheumatoid arthritis

RANKL – receptor activator of NFκB ligand

Rb - retinoblastoma

RISC – RNA-induced silencing complex

RSK - 90 kDa ribosomal S6 kinase

sGalC – sulfated galactosylceramide / sulfatide

SFM - serum-free medium

Sox – sex-determining region on Y box

SRF – serum response factor

TAK1 - TGFβ activated kinase-1

TAO - thousand and one

Tcf4 / Tcf7L2 – transcription factor 4 / 7L2

TCR – T cell receptor

TF - transcription factor

TGFβ – transforming growth factor β

TLR – toll-like receptor

TNF - tumour necrosis factor

TTP - tristetraprolin

TSA - trichostatin A

UTR - untranslated region

VPA – valproic acid

YY1 - yin yang 1

Zfp – zinc finger protein

Rationale and Objectives

Oligodendrocytes (OLGs) are the cells responsible for the production and maintenance of myelin in the central nervous system (CNS). Myelination of axons is required for rapid impulse transmission in the nervous system. Myelin also maintains the integrity of associated nerve fibers through the activation of signals that affect nerve fiber structure and function. Erosion of the myelin sheath therefore causes neurological impairments such as those seen in multiple sclerosis patients. In order to better understand the process myelination, it is essential to characterize the sequence of events that occur during OLG differentiation. OLG differentiation is a complex process requiring the interplay of many transcription factors, cell-cycle regulators, epigenetic modifiers, and transcriptional repressors that control myelin formation. However, the signal transduction pathways that promote an oligodendrocyte progenitor (OLP) to become a mature myelinated OLG are not well defined.

Previous studies in Drs. Almazan and Mushynski's laboratories have shown that p38 mitogen activated protein kinase (p38 MAPK) was important for Schwann cell differentiation and peripheral nervous system myelination. However, the roles for p38 MAPK in OLG differentiation and CNS myelination still remained to be determined. Thus, the objectives of this project were as follows: 1) to determine the role(s) of p38 MAPK in OLG differentiation and CNS myelination; 2) to elucidate possible downstream substrates of p38 MAPK responsible for mediating OLG differentiation and 3) to delineate possible molecular mechanisms by which p38 MAPK regulates OLG differentiation.

CHAPTER 1: General Introduction

1.0. Introduction to Oligodendrocytes and Myelin

During the evolution of higher vertebrates the challenge of accommodating large numbers of axons in a small space has been overcome by myelin producing glial cells. These cells permit space saving economy by allowing larger numbers of smaller diameter axons in the nervous system. Indeed, unmyelinated axons are one-hundred times greater in diameter compared to myelinated axons conducting nerve impulses at the same velocity (Ritchie 1984). Oligodendrocytes (OLGs) of the central nervous system (CNS) and Schwann cells of the peripheral nervous system (PNS) are the glia responsible for myelin sheath production. CNS myelination is an active and plastic process that continues into adulthood. In fact, myelination levels are influenced by many genetic and environmental factors, and diseases such as schizophrenia, depression, bipolar disorder, leukodystrophies, and multiple sclerosis (MS) (Aston et al. 2005; Davis and Haroutunian 2003; Hakak et al. 2001; Tkachev et al. 2003; Uranova et al. 2001). MS is the most common CNS neurodegenerative disease afflicting Canadians with a prevalence rate ranging from 180 to 350 per 100,000 (Beck et al. 2005), and this number continues to increase yearly (Orton et al. 2006). MS is characterized by an autoimmune attack on CNS myelin resulting in focal demyelination and plaque formation. In MS plaque regions, populations of immature OLGs exist which attempt to remyelinate damaged tissue; however these cells fail to differentiate into fully mature OLGs (Chang et al. 2000; Franklin and Ffrench-Constant 2008; Levine and Reynolds 1999). Therefore, identifying mechanisms by which OLGs differentiate and ultimately myelinate axons may provide clues to restoring myelin sheaths in individuals afflicted by demyelinating diseases such as MS. This thesis will review the molecular composition of myelin, mechanisms that regulate OLG differentiation and myelination, and the roles of p38 mitogen-activated protein kinase (p38 MAPK) in regulating cell development, including our work on its roles in OLG differentiation.

1.1. Myelin

In the CNS, OLGs synthesize large quantities of myelin, a lipid-rich, multi-layered spiral-wrapped membrane which insulates nerve axons (Figure 1). Myelinated segments on axons are referred to as internodes and are separated by small gaps of bare axon known as the nodes of Ranvier, which are responsible for propagating neurotransmitter signals along the axon (see below). A single OLG can myelinate up to fifty internodes on multiple axons (reviewed in (Baumann and Pham-Dinh 2001; Pfeiffer et al. 1993)). In contrast, an individual Schwann cell establishes a 1:1 relationship with an axon, producing a single internode (Hildebrand et al. 1994; Jessen and Mirsky 2005). A transverse section of myelin sheath reveals a multi-lamellar structure with alternating layers of electron-dense and light layers. The major dense line (electron dense) is formed from apposed cytoplasmic surfaces of the OLG membrane extension, while the two outer membrane leaflets form the intraperiod line (light density). Compact CNS myelin is traversed by a radial component which retains small pockets of cytoplasm that may play roles in myelin assembly and compaction (Dermietzel and Kroczek 1980; Karthigasan et al. 1996; Kosaras and Kirschner 1990; Peters 1961; Radhakrishna and Almazan 1994). At the inner and outer ends of the spiral membrane myelin is less compacted and forms paranodal loops, which are structures responsible for anchoring myelin to the underlying axon, and segregating ion-channels. The innermost and outermost wraps of myelin are termed the inner- and outer-mesaxon, respectively (reviewed by (Baumann and Pham-Dinh 2001)). The two mesaxonic compartments are connected by Schmidt-Lanterman incisures, which are cytoplasmic channels that balance ionic concentrations between the inner layers of myelin and the extracellular milieu (Arroyo and Scherer 2000; Luxoro 1958; Robertson 1958).

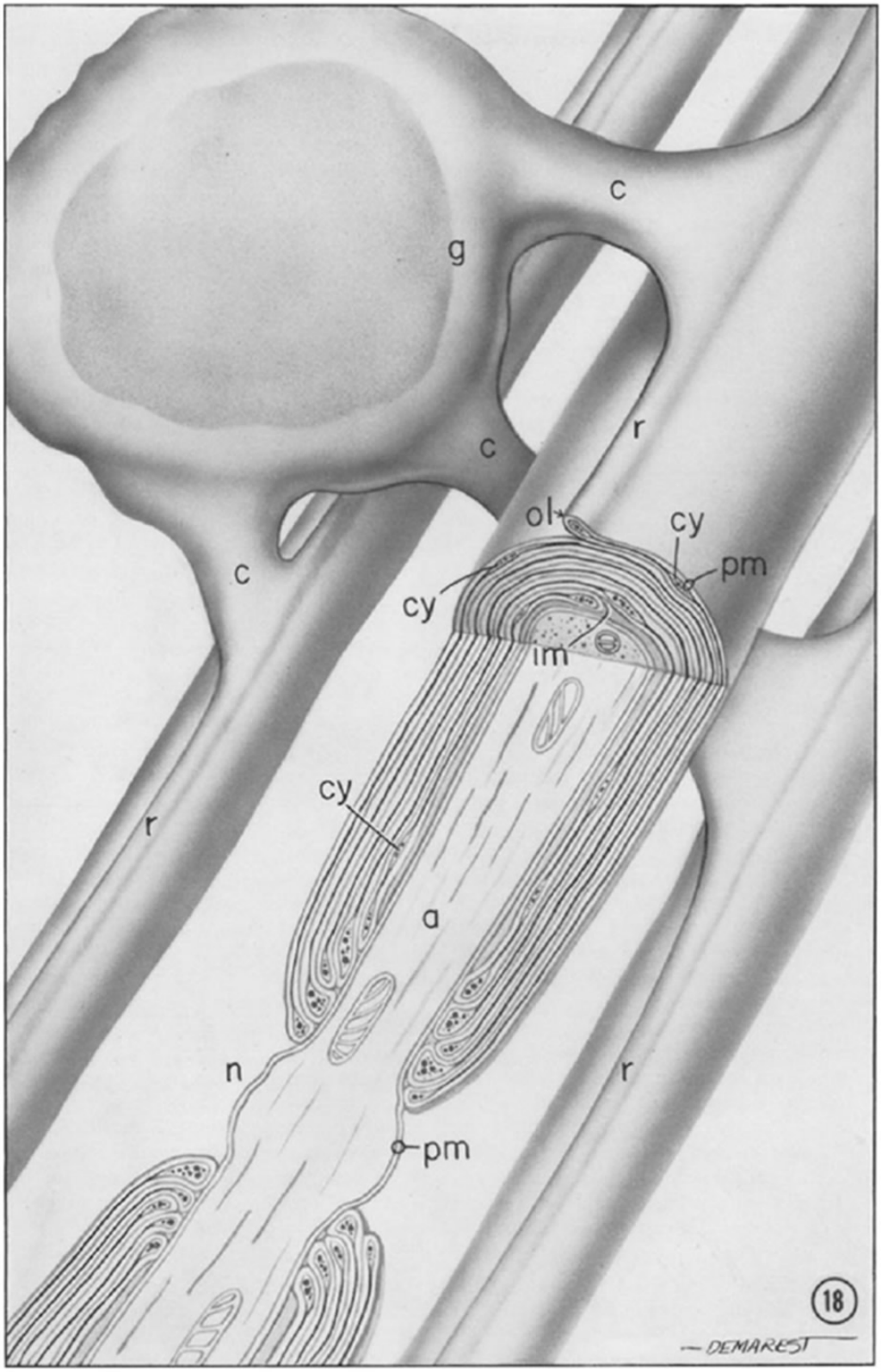

Figure 1

Figure 1. A single oligodendrocyte (g) extends multiple processes (c) from its plasma membrane (pm) to myelinate many axons (a). Myelinated internodes are separated by the nodes of Ranvier (n). A transverse section of myelin depicts alternating layers of dark (cytoplasmic / major dense line) and light (extracellular / intraperiod line) densities. Small pockets of cytoplasm (cy) are retained at the radial component and other non-compact regions of myelin including paranodal loops, inner mesaxon (im), outer mesaxon (ol), and myelin ridges (r). Reproduced from (Bunge et al. 1961) with copyright permission from The Rockefeller University Press.

1.2. Oligodendrocyte/myelin functions

In humans, myelination is an ongoing process that starts *in utero*, peaks during the first postnatal year, and continues into adulthood (Benes 1989). Experience early in life can also affect the number of OLGs and levels of myelination. For example, childhood neglect is associated with region-specific decreases in white matter (Teicher et al. 2004), whereas children who were involved in extensive piano practicing exhibited enhanced white matter tract organization in adulthood (Bengtsson et al. 2005). Furthermore, young rodents raised in enriched environments have higher numbers of OLGs, larger white matter volume, and increased myelination (Sirevaag and Greenough 1987; Szeligo and Leblond 1977). Similarly, rhesus monkeys reared in enriched environments develop greater white matter volume, and have increased cognitive abilities (Sanchez et al. 1998). These observations are a significant advancement to the idea that myelin is simply a passive insulating membrane that facilitates nerve transmission. Rather, myelin participates in an activity-dependent nervous system plasticity (Fields 2005).

Classically, the roles identified for myelin were as a membrane that 1) permits the rapid conduction of nerve impulses along axons, 2) allows the nerve impulse to travel large distances, 3) affords space saving economy by allowing thinner diameter axons, and 4) provides trophic support to axons. Conduction velocities in myelinated axons can reach 150 m/s, which are markedly faster than velocities of 0.5 to 10 m/s measured in unmyelinated axons (Purves et al. 2008). The low capacitance and high resistance of myelin confer these rapid conduction velocities (Hartline 2008). An action potential is generated by membrane depolarization at the axonal hillock (initial segment), which opens voltage-gated sodium (Na_v) channels, causing Na^+ ion influx into the cell. The action potential at one node is sufficient to depolarize the membrane at an adjacent node, triggering further Na^+ channel opening. Thus, the electrical signal in a myelinated axon "jumps" or "hops" from node to node by a method termed saltatory conduction (reviewed in (Baumann and Pham-Dinh 2001)). Juxtaparanodal-localized voltage-gated potassium (K_v^+) channels are responsible for membrane repolarization and are kept physically separated from nodal Na_v channels to

conserve electrical circuitry and maintain fidelity of nerve conduction. Therefore, it is the unique insulating property of myelin, and the physical separation of the Na_v and K_v^+ channels that allow the action potential to rapidly propagate along axons.

Conduction failure resulting from myelin damage can lead to cognitive and motor defects eventually causing paralysis, as seen in debilitating diseases such as MS. MS is a chronic progressive autoimmune disease characterized by extensive myelin damage, OLG cell death, microglial activation, peripheral immune cell infiltration, astrocyte proliferation (astrogliosis), and axonal loss (Pittock and Lucchinetti 2007). A small population (5-8%) of oligodendrocyte progenitors (OLPs) reside in the brain and are capable of generating new OLGs after damage (Polito and Reynolds 2005; Scolding et al. 1999). In early stages of MS periods of remyelination occur; however, for unknown reasons, compact myelin fails to form (reviewed by (Franklin and Ffrench-Constant 2008)). More notably, the efficiency of remyelination decreases with age (Li et al. 2006; Shields et al. 1999). Genetic diseases of CNS myelin (inherited leukodystrophies) include Pelizaeus-Merzbacher disease, Canavan disease, Krabbe disease, Alexander disease and vanishing white matter disease (for reviews see (Di Rocco et al. 2004; Kaye 2001; Kumar et al. 2006; Schiffmann and Boespflug-Tanguy 2001; Schiffmann and van der Knaap 2004; Suzuki 2003)). Common features of these diseases include damage to OLGs/myelin, improper myelin formation, and/or demyelination. There are currently no cures to treat myelinating disorders. Therefore, considering the many diseases affecting OLGs and myelin, understanding the process of OLG development is critical for initiating remyelination in diseases where myelin fails to form correctly, or where it has been lost or destroyed. Animal models of demyelination/remyelination have provided insights into OLG physiology during disease and include experimental autoimmune encephalomyelitis (EAE), viral induced (Theiler's virus, murine hepatitis virus), and toxin induced (cuprizone, ethidium bromide, and lysolecithin) models (Rodriguez 2007; Torkildsen et al. 2008).

1.3. Oligodendrocyte development

Oligodendrocytes, similar to astrocytes and neurons, arise from neuroepithelial precursor cells of the CNS (Wegner 2008). Competing waves of proliferating OLPs migrate from different regions of the subventricular zone to populate white matter tracts destined for myelination (Richardson et al. 2006; Wegner 2008). During migration, OLPs proliferate in excess numbers and eventually compete for axon attachment, resulting in death of ~20% of cells that fail to initiate axonal contact. On the other hand, OLPs that have established axonal contact elaborate numerous membrane processes that spiral around axons which then compacts to form the multi-layered myelin membrane (Barres and Raff 1999; Trapp et al. 1997).

OLG differentiation *in vivo* can be recapitulated *in vitro* using cells purified from rodent brain. McCarthy and de Vellis developed a cell culture technique for the purification of OLPs from mixed glial cultures isolated from neonatal rat brain (McCarthy and de Vellis 1980). Purified OLPs express the platelet-derived growth factor-α receptor (PDGFRα) (Hart et al. 1989) and can be expanded for a limited number of cell divisions using platelet-derived growth factor-AA ($PDGF_{AA}$) (Besnard et al. 1987; Noble et al. 1988), a mitogen mainly secreted by astrocytes (Raff et al. 1988; Richardson et al. 1988). Bögler and colleagues later found that OLPs cultured in the presence of both $PDGF_{AA}$ and basic fibroblast growth factor (bFGF) could undergo continuous self-renewal as progenitors (Bogler et al. 1990) through upregulation of PDGFRα (Fressinaud et al. 1993; Goddard et al. 2001; Grinspan et al. 1993; Hoffman and Duncan 1995; Lachapelle et al. 2002; Mayer et al. 1993; McKinnon et al. 1991; McKinnon et al. 1990; McKinnon et al. 1993; Murtie et al. 2005). Removal of these two growth factors spontaneously initiates OLP differentiation, which is characterized by increased branching and the expression of myelin-specific lipids and proteins (Figure 2). This well-described series of developmental steps of OLG differentiation are extensively reviewed elsewhere (Pfeiffer et al. 1993). Briefly, the pre-OLP, and OLP cell stages are proliferative and migratory, and can be immunostained with antibodies to embryonic neural cell adhesion molecule (E-

NCAM), or GD3/A_2B_5 gangliosides, respectively. In the next stage, late progenitors, which are proliferative but non-migratory, are characterized by sulfatide expression (recognized by the O4 antibody). Late progenitors then differentiate into immature OLGs, which are post-mitotic and characterized by decreased PDGFRα expression and increased synthesis of the lipid galactosylceramide (GalC) (recognized by O1 antibody), and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) expression. Mature OLGs are post-mitotic and characterized by expression of myelin basic protein (MBP), proteolipid protein (PLP) and myelin-oligodendrocyte glycoprotein (MOG) (Pfeiffer et al. 1993). In the absence of axons, mature OLGs produce lacy membrane sheet structures that have a protein and lipid composition similar to that of the myelin sheath. This suggests that some signal(s) inherent to the OLG (or present in culture medium) is sufficient to induce a myelin-specific gene expression program and yield myelin-like membranes (Behar et al. 1988; Raff et al. 1983). This differs from Schwann cells, which require axonal contact and basal lamina synthesis to initiate myelination (Bunge et al. 1982; Jessen and Mirsky 2005). The OLG developmental stages *in vitro* are similar to those observed *in vivo* (Warrington et al. 1992; Warrington and Pfeiffer 1992). Furthermore, isolated rat or human OLPs transplanted into hypomyelinated mouse brain can produce normal myelin (Warrington et al. 1993; Windrem et al. 2008). Purified OLPs seeded on dorsal root ganglia neurons (DRGNs) can also myelinate axons, permitting the study of CNS myelination *in vitro* (Fragoso et al. 2007; Wood and Bunge 1986b).

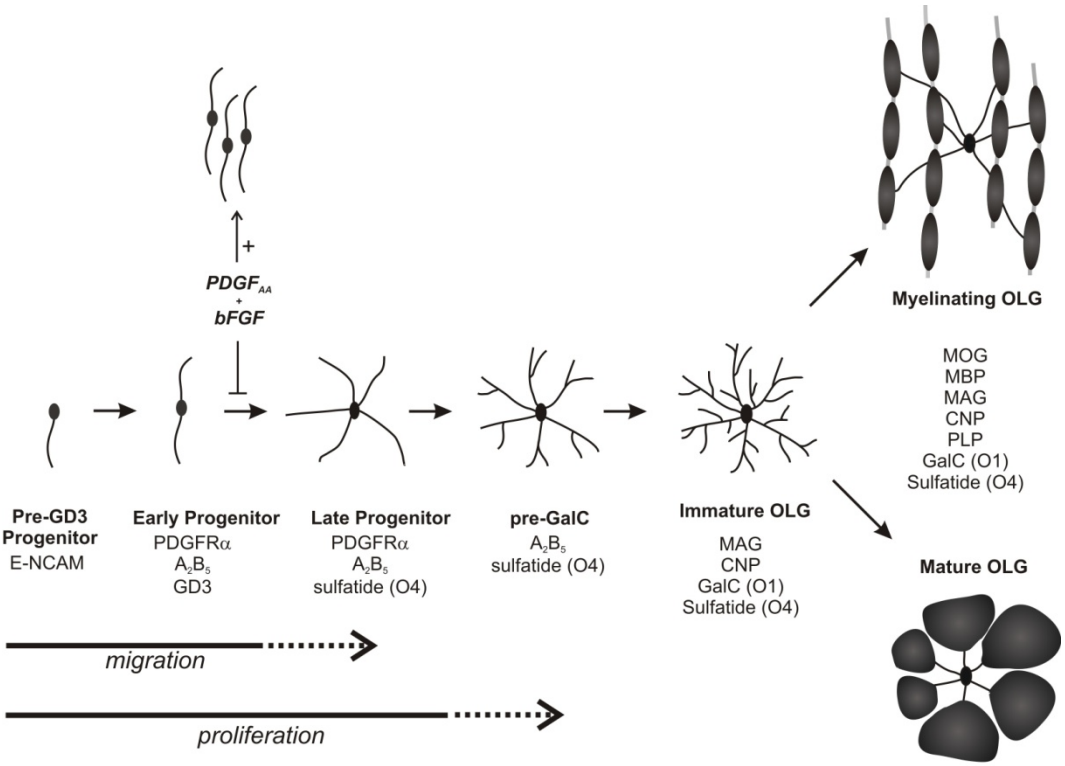

Figure 2. Developmental stages of the OLG lineage. Early OLPs differentiate through stage-specific morphological changes, which are associated with the expression of specific lipids and protein markers. The mitogens $PDGF_{AA}$ and bFGF prevent OLG differentiation and expand OLP numbers by maintaining the cells in a proliferative state. When the two mitogens are removed, OLPs differentiate into OLGs producing either spiral-wrapped myelin membranes (in the presence of axons), or lacy membrane structures (in the absence of axons). Myelinating OLGs and mature OLGs share identical protein and lipid markers. The migratory and proliferative stages are indicated by arrows. Adapted from (Pfeiffer et al. 1993) and (Zhang 2001), with copyright permission from the Elsevier Limited and the Nature Publishing Group, respectively.

1.4. Axo-glial interactions

The unique insulating property of myelin confers rapid impulse transmission along an axon, but also provides trophic support to the underlying fiber, influencing axonal morphology and long-term survival. Moreover, axons provide trophic support to OLPs, influencing their survival and proliferation (Laursen and Ffrench-Constant 2007; Nave 2010a).

Electrical conductivity in axons influences myelination *in vivo* and *in vitro* since myelin levels are increased by α-scorpion toxin or decreased by tetrodotoxin, chemicals that block or stimulate electrical activity in axons, respectively (Barres and Raff 1993; Demerens et al. 1996). In addition, OLPs regulate extracellular neurotransmitter concentrations and fine-tune action potentials during impulse transmission along axons. In fact, OLPs express many of the same neurotransmitter receptors as neurons, including glutamate (Liu and Almazan 1995; Liu et al. 1999; Liu et al. 1997), adenosine (Agresti et al. 2005; Fields 2006), adrenergic (Cohen and Almazan 1993; Khorchid et al. 2002) acetylcholine (Cohen and Almazan 1994), and GABA (Lin and Bergles 2004) (reviewed by (Bakiri et al. 2009; Belachew and Gallo 2004; Fields 2008)).

Neurons also secrete numerous mitogens which influence many aspects of OLP physiology both *in vitro* and *in vivo* (Hardy and Reynolds 1993). For example, DRG and cerebellar neuron conditioned media induce OLP proliferation (Bottenstein et al. 1988; Levine 1989; Wood and Bunge 1986a; Wood and Williams 1984), and purified axo-lemma membranes induce mitogenic effects on cultured OLGs (Chen and DeVries 1989). These findings suggest that many neural-derived factors influence OLP proliferation and survival (Barres et al. 1993). The mitogens released from neurons include insulin growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) (Barres et al. 1994). IGF-1 plays important roles in OLP survival and proliferation (Barres et al. 1992a; Barres et al. 1992b; Masters et al. 1991; McMorris and Dubois-Dalcq 1988; McMorris et al. 1986), by signaling through the phosphatidyl-inositol-3-kinase (PI3K), Akt, Src and extracellular signal regulated kinase (ERK) pathways (Bibollet-Bahena and

Almazan 2009; Cui and Almazan 2007; Cui et al. 2005). At the molecular level, OLG gene expression is affected by the presence of axons in the developing CNS (Kidd et al. 1990), including the expression of developmentally regulated PLP transcripts (Scherer et al. 1992). Furthermore, PLP and MBP mRNAs are elevated when OLGs are cocultured with spinal cord neurons (Macklin et al. 1986). However, in contrast to the pro-survival and proliferation cues received from neurons, OLPs do not appear to require dynamic axonal support for migration or differentiation (Barres and Raff 1999; Ueda et al. 1999). In fact, embryonic and neonatal axons express ligands that are inhibitory to OLP differentiation (see section 3.7 - Notch signaling, below). Furthermore, OLG differentiation does not require dynamic signaling from DRGNs, but rather can be induced by geometric packing constraints between neighbouring OLGs, or polystyrene beads (Rosenberg et al. 2008).

Myelin also dynamically signals to axons, influencing their structure, morphology and survival. The insulating property of myelin keeps long axonal tracts metabolically isolated from trophic factors in the CNS extracellular milieu; thus, myelin must also provide trophic support to axons for their long term survival (Nave 2010a). The roles of myelin in providing metabolic and pro-survival support to axons is clearly demonstrated by the various axonal pathologies seen in many mouse strains with myelin-specific gene mutations/deletions (see below, and (Nave 2010a)). Furthermore, following extensive periods of demyelination in MS, severe axonal damage and eventual transection occurs (Rammohan 2003).

Morphologically, axonal segments underlying myelinated internodes have larger calibres due to increased expression and phosphorylation of neurofilaments (de Waegh et al. 1992; Pigano et al. 2006; Sanchez et al. 1996; Sanchez et al. 2000). In addition, OLGs receive specific instructional cues from certain axons, since they only myelinate fibers ≥ 1 μm in diameter, and not small-calibre c-fibers or dendrites (Lubetzki et al. 1993). Furthermore, a constant relationship (g-ratio) exists between axonal diameter and the number of myelin wraps around that particular fiber (Hildebrand and Hahn 1978; Rushton 1951). In the PNS, type III

neuregulin-1 (Nrg1), interacts with the ErbB2 receptor tyrosine kinases to signal the amount of myelin sheath deposition on axons, and thus regulates the g-ratio. In mice, increased expression of axonal-Nrg1 causes hypermyelination, and wrapping of small-diameter fibers that are not normally ensheathed. In contrast, reduced PNS Nrg1 expression causes hypomyelination (Michailov et al. 2004). In the CNS, a single OLG must determine the appropriate number of myelin wraps for multiple axons of varying diameters. The roles for Nrg1 in this process, however, are less clear. In the corpus callosum of $Nrg1^{+/-}$ mice, reduced myelin sheath thickness has been reported (Taveggia et al. 2008). However, another study showed that CNS myelination was normal in $Nrg1^{-/-}$ mice (Brinkmann et al. 2008). Therefore the signal(s) that regulate the CNS g-ratio remain to be fully elucidated. Possible candidates include the OLG-expressed $\alpha_6\beta_1$-integrin membrane receptor which binds laminin found in axonal tracts at the time of myelination (Colognato et al. 2002). In OLGs expressing dominant negative (DN)-β_1-integrin, larger diameter axons are required to initiate timely myelination (Camara et al. 2009). In addition, transgenic knockout of the α_6 subunit of $\alpha_6\beta_1$-integrin showed this receptor normally promotes survival of the competing waves of OLPs that have established axonal contact. On the other hand, OLPs that fail to make contact with axons undergo apoptosis (Colognato et al. 2002). Considering this, however, the roles for β_1-integrin in CNS myelination have been contradictory. Conditional knockout of the C-terminal domain of β_1-integrin causes hypomyelination in optic nerve and spinal cord, with no myelination defects in corpus callosum (Lee et al. 2006). However, another study showed that conditional inactivation of β_1-integrin in pre-myelinating OLGs caused no defects in axon ensheathment, myelination or remyelination (Benninger et al. 2006).

The intricate relationship between axon and myelin is also observed at the paranodal loops which are regions responsible for myelin membrane attachment to the axon and ion channel compartmentalization (Figure 3). In unmyelinated axons, Na_v and K_v^+ channels remain dispersed along axons. However, when an axon is myelinated, Na_v and K_v^+ channels are segregated into specialized domains

by the paranodal loops which act as a physical barrier to lateral diffusion (Peles and Salzer 2000; Salzer 1997; Scherer and Arroyo 2002). Paranodal loop anchoring to axons is mediated by transverse band structures which are composed of a heterotrimeric protein complex of axonal Contactin, Contactin-associated protein (Caspr) and glial Neurofascin-155 (NF-155) (Tait et al. 2000). In unmyelinated axons, Caspr is diffusely organized throughout the length of the fiber and becomes successively clustered with Contactin and NF-155 into discrete domains during myelination. Genetic ablation of these proteins causes transverse band disorganization, paranodal loop anchoring defects, and lateral redistribution of K_v^+ channels into paranodal regions, resulting in conduction velocity defects. Paranodal and nodal disruption are common defects observed in mouse mutants of spectrin, dystroglycan, deleted in colorectal cancer (DCC), Netrin-1, and some myelin-specific gene mutants including myelin-associated glycoprotein (MAG), and the myelin galactolipid-synthesizing enzymes UDP-galactose: ceramide galactosyltransferase (CGT), and galactosylceramide 3'-sulfotransferase (CST) (see below and reviewed by (Poliak and Peles 2003)). DCC and its ligand Netrin-1 are involved in cell migration, axonal guidance, and cell-cell, and cell-matrix interactions (Baker et al. 2006). Knockout of DCC or Netrin-1 causes paranodal region defects characterized by the disappearance of transverse bands, and disorganization of K_v^+ channels and axonal Caspr (Jarjour et al. 2008). Importantly, these changes are also seen following demyelination in MS. In MS plaque and periplaque regions, damage to myelin results in disorganization of Na_v, K_v^+, Caspr, and NF-155 with many of these proteins inefficiently segregating following remyelination attempts (Coman et al. 2006; Moll et al. 1991; Wolswijk and Balesar 2003).

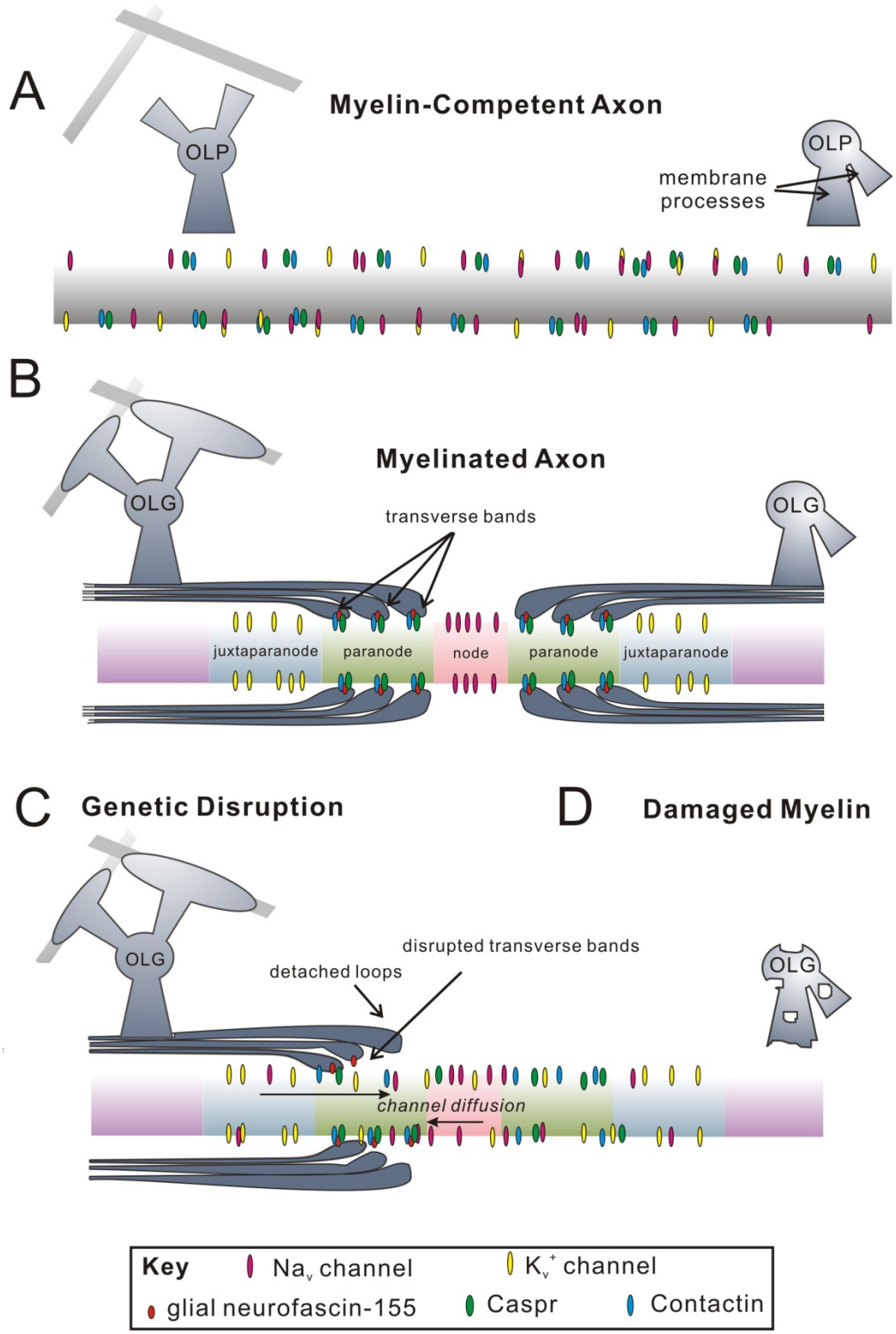
A
Myelin-Competent Axon
OLP
membrane processes
OLP
B
Myelinated Axon
OLG
transverse bands
OLG
juxtaparanode
paranode
node
paranode
juxtaparanode
C
Genetic Disruption
D
Damaged Myelin
OLG
disrupted transverse bands
detached loops
OLG
channel diffusion
Key
Na$_v$ channel
K$_v^+$ channel
glial neurofascin-155
Caspr
Contactin

Figure 3

Figure 3. Oligodendrocytes cluster axonal proteins into specialized domains. A) OLPs extend many processes to ensheath myelin-competent axons. Prior to myelination, Na_v and K_v^+ channels, Caspr and Contactin remain diffusely organized along axons. B) Once an OLG has myelinated an axon, ion channels and structural proteins (Caspr, contactin, NF-155) are clustered into discrete domains including Na_v channels at nodes, and K_v^+ channels at juxtaparanodes. The paranodal loops are anchored to the membrane by a protein complex formed between Contactin, Caspr and glial NF-155 and act as a barrier to keep ion channels physically separated. C) Genetic disruption of structural proteins (e.g., MAG, CGT, Netrin/DCC, Caspr) causes paranodal loops detachment and transverse bands disorganization causing lateral diffusion of ion channels ultimately resulting in conduction defects. D) Demyelination also causes ion channel and structural protein disruption. Modified from (Poliak and Peles 2003) with copyright permission from Nature Publishing Group.

2.0. Molecular composition of myelin

Myelin is a specialized membranous structure composed of 70% lipid and 30% protein (Boggs et al. 2004). Each macromolecular constituent comprises unique structural and signaling molecules necessary for myelin synthesis, maintenance and tight multi-lamellar compaction (Figure 4). Numerous biochemical, molecular, and animal knockout studies have revealed clues to the functions of these lipids and proteins in OLGs and myelin, some of which are reviewed below.

2.1. Myelin galactolipids, synthesizing enzymes and cholesterol

Myelin is cholesterol- and glycosphingolipid- (GSL) enriched, and has the highest lipid content of any biological membrane. Two abundant GSLs, GalC and its sulfated form, sulfatide (sGalC) represent ~20% and 7% of myelin galactolipids, respectively, and play roles in membrane architecture and signaling (Morell and Jurevics 1996) (and reviewed by (Boggs et al. 2004)). Synthesis of these two GSLs peaks at the time of myelination in rats, and during OLG lacy membrane sheet formation *in vitro* (reviewed by (Boggs et al. 2004)). GalC is synthesized by the galactosylation of ceramide by the enzyme UDP-galactose: ceramide galactosyltransferase (CGT). A proportion of GalC is then sulfated by galactosylceramide 3'-sulfotransferase (CST), forming sGalC. The roles of these two GSLs have been ascertained through knockout of the enzymes responsible for their synthesis (Bosio et al. 1996; Coetzee et al. 1996; Honke et al. 2002). $CGT^{-/-}$ mice are viable and myelin forms correctly in the absence of GalC and sGalC due to compensation from glucosylceramide (GluC). Myelin sheaths of $CGT^{-/-}$ mice have ultrastructural defects including vacuolization, and altered paranodal loop interactions with the axon resulting in K_v^+, Caspr, and NF-155 diffusion into axonal nodes and paranodes (Dupree et al. 1999; Ishibashi et al. 2002; Marcus et al. 2002). Knockout of CST from mice results in formation of normal compact myelin formation with paranodal loop defects similar to those observed in $CGT^{-/-}$ mice (Honke et al. 2002; Ishibashi et al. 2002). Proteomic analysis of myelin

isolated from $CGT^{-/-}$ and $CST^{-/-}$ mice also reveals changes in proteins that regulate cytoskeletal dynamics, energy metabolism, vesicular trafficking or adhesion (Fewou et al. 2010). Interestingly, OLGs cultured from $CGT^{-/-}$ and $CST^{-/-}$ mice have enhanced levels of differentiation and morphological maturation, suggesting sGalC is a negative regulator of OLG differentiation (Hirahara et al. 2004).

In addition to the roles of GalC and sGalC in paranodal loop maintenance, these GSLs normally interact at apposed extracellular surfaces of compact myelin. The carbohydrate interactions between GSLs promote *trans*-membrane signaling, resulting in clustering of myelin basic protein (MBP), and depolymerization of microtubules and actin microfilaments (Boggs et al. 2008; Boggs et al. 2009). GalC and sGalC are also found in cholesterol-enriched lipid rafts, which are microdomains that cluster signaling molecules involved in myelinogenesis (reviewed by (Debruin and Harauz 2007; Gielen et al. 2006)). Cholesterol also plays a fundamental role in proper myelin formation since mice with an OLG-specific mouse knockout of squalene synthase (a key enzyme in sterol biosynthesis) have reduced myelination and neurological impairments (Saher et al. 2005).

2.2. Proteolipid protein (PLP / DM20)

Proteolipid protein (PLP) is the major component of myelin, constituting 30 to 45% of total protein (Eng et al. 1968; Greer and Lees 2002). PLP is a highly-hydrophobic integral membrane protein that is ~100% conserved at the amino acid level among species (reviewed by (Nave 1994)). Alternative splicing gives rise to PLP (25 kDa), and the developmentally regulated splice variant, DM20 (20 kDa), which appears before full-length PLP (Campagnoni and Skoff 2001). Numerous functions have been proposed for PLP/DM20, including membrane adhesion, myelin compaction, formation of the intraperiod line and OLG maturation (reviewed in (Campagnoni and Skoff 2001). PLP may also play important roles in axo-glial interactions, including myelin wrapping, maintenance and survival of axons (Griffiths et al. 1998). Myelin from PLP^{null} mice is compacted, although ultrastructural analysis shows a condensed intraperiod line, suggesting PLP normally regulates intraperiod line spacing. PLP^{null} mice live for

two years, and develop focal axonal swellings leading to eventual axonal degeneration. OLGs isolated from *jimpy* mice (a strain with a PLP mutation) display enhanced proliferation, differentiation defects and die prematurely by apoptosis (Knapp and Skoff 1987; Knapp et al. 1986; Nave 1994; Vermeesch et al. 1990). In addition, *rumpshaker* mice, which have an amino acid substitution in PLP, exhibit CNS dysmyelination (Griffiths et al. 1990). In humans, genetic point mutations or gene duplications of PLP result in a severe CNS dysmyelinating disease known as Pelizaeus-Merzbacher disease, which is characterized by weak muscle tone, delayed development, lack of motor skills and premature death (Garbern 2005).

2.3. Myelin basic protein (MBP)

Myelin basic protein (MBP) is a family of cytoplasmic, peripheral membrane proteins produced from the genes of the oligodendrocyte lineage (golli) cassette. The *golli* gene cassette gives rise to many different MBP isoforms (21.5, 18.5, 17 and 14 kDa), which are extensively post-translationally modified, producing numerous charge isomers. Many MBP isoforms are developmentally regulated, and differentially localized (for review, see (Campagnoni and Campagnoni 2004; Harauz et al. 2009)). The most abundant MBP isoform (14 kDa in rodent, 18.5 kDa in human) is localized to the major dense line of compact myelin, whereas the 17 and 21.5 kDa MBP isoforms are localized to compact myelin and the radial component (Karthigasan et al. 1996). The highly basic charge of MBP (+19 at neutral pH) promotes its electrostatic interactions with the anionic-charged lipid headgroups of the cytoplasmic membrane leaflet, maintaining the tight compaction of the myelin sheath. In *shiverer* mice, which have a natural gene deletion of MBP, or *shiverer*^{mld} mice which have MBP gene duplication, only a small amount of myelin is formed in the CNS (Fremeau and Popko 1990; Nave 1994; Rosenbluth 1980; Tosic et al. 1990), and is non-compacted at cytoplasmic surfaces (Bird et al. 1978; Privat et al. 1979). However, this hypomyelination defect appears to be CNS region specific (Kirschner and Ganser 1980), and no abnormalities in PNS myelin are observed (Kirschner and Ganser 1980; Privat et

al. 1979) due to compensation from a major PNS structural glycoprotein, P0, which is functionally similar to PLP (Martini et al. 1995).

In addition to the function of MBP in molecular compaction of myelin, it may also participate in signal transduction. MBP contains a SH3-domain ligand which can tether signaling proteins such as Fyn, a tyrosine kinase which regulates MBP promoter activity and is important for myelin synthesis (see below) (Harauz et al. 2009; Homchaudhuri et al. 2009). Moreover, MBP mRNA transcripts are localized to OLG distal processes where they are locally translated at the time of myelination (Ainger et al. 1997; Ainger et al. 1993; Brophy et al. 1993).

2.4. Myelin oligodendrocyte basic protein (MOBP)

Myelin oligodendrocyte basic protein (MOBP) is the third most abundant protein found in CNS myelin and has five splice isoforms (12, 20, 25, 28 and 30 kDa) that are cytoplasmically localized (Montague et al. 2006). The exact functions of MOBPs are unknown; however their similarities to MBP suggest roles in myelin sheath compaction or stabilization (Montague et al. 2006). Similar to MBP, MOBP mRNAs are also localized to OLG processes during development, where they are translated at the time of myelination (Holz and Schwab 1997). Transgenic mouse knockout of MOBP results in no clear overt clinical phenotype and no defect in the process of myelination, suggesting compensatory effects from MBP (Montague et al. 2006). Minor ultrastructural abnormalities are observed in $MOBP^{null}$ mice, including increased axonal diameter (Sadahiro et al. 2000), and greater organization of the radial component (Yoshikawa 2001).

2.5. 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP)

2',3'-cyclic nucleotide 3-phosphodiesterase (CNP) is an early-expressed and low-abundant myelin protein found in differentiating OLGs. CNP, *in vitro*, has enzymatic activity toward 2',3'-cyclic nucleotides; however these molecules are not found in OLGs or the brain, but are temporary intermediates in RNA metabolism (Heaton and Eckstein 1996; Lappe-Siefke et al. 2003). Thus, the exact functions of CNP have remained somewhat elusive. CNP has two isoforms,

CNP1 (46 kDa) and CNP2 (48 kDa), the former which is developmentally regulated (Gravel et al. 2000). CNP is localized to non-compact regions of myelin including the inner mesaxon, paranodal loops, and Schmidt-Lanterman incisures (Braun et al. 1988; Trapp et al. 1988). As OLGs mature, CNP associates with filamentous actin (Dyer and Benjamins 1989; Wilson and Brophy 1989), anchors tubulin to the membrane (Bifulco et al. 2002; Laezza et al. 1997), and interacts with poly(A)$^{+}$ containing mRNAs (Gravel et al. 2009), suggesting roles for CNP in both cytoskeletal arrangement and RNA metabolism (Bifulco et al. 2002; Schwer et al. 2008). Moreover, CNP may regulate OLG process extension since its ectopic expression in fibroblasts induces membrane expansion and increased filopodia outgrowth (Lee et al. 2005). Over-expression of CNP in mice causes premature OLG differentiation, and defects in MBP deposition and myelin compaction (Gravel et al. 1996; Yin et al. 1997). However, transgenic knockout of CNP does not cause initial developmental defects in myelination, although CNP^{null} mice die earlier in life from severe degeneration due to axonal loss (Lappe-Siefke et al. 2003). Minor ultrastructural abnormalities at the OLG inner-mesaxonic membrane may account for the eventual axonal degeneration seen in CNP^{null} mice (Edgar et al. 2009).

2.6. Myelin-associated glycoprotein (MAG)

Myelin-associated glycoprotein (MAG) is a sialic acid binding, type-I transmembrane glycoprotein localized to the inner mesaxonic layer of the myelin sheath (Quarles 2007). The structure and function of MAG have been extensively reviewed elsewhere (Georgiou et al. 2007; Quarles 2007). Quantitatively, MAG is a minor component of myelin, and its expression begins during the early process of myelination. MAG is comprised of two alternatively spliced isoforms producing a large (L-MAG, 72 kDa) and small (S-MAG, 67 kDa) isoform, which are heavily glycosylated resulting in a average MW of ~100 kDa in the CNS. L-MAG is the predominant isoform in early myelinogenesis, whereas S-MAG levels increase during development (Quarles 2007). L-MAG is phosphorylated by Fyn, a tyrosine kinase involved in myelination (see section 3.12, below). MAG

signaling may function in the initial interactions of OLG processes with axons, or be involved in plasma membrane wrapping. In addition, MAG expression levels remain high in adult animals, suggesting it functions in myelin and axon maintenance. The periaxonal localization and sialic acid binding properties of MAG supports its roles in axo-glial interactions. Thus, MAG may serve as a glial receptor for axonal ligands to promote axonal survival, or as a glial ligand for an axonal receptor that promotes OLG survival and differentiation (Quarles 2007). Both roles for MAG are supported by the defects seen in MAG^{null} mice, which have delayed myelin sheath synthesis, and compact myelin with structural abnormalities such as redundant and detached paranodal loops (Bartsch et al. 1995a; Bartsch et al. 1995b; Li et al. 1994). As MAG^{null} mice age, severe neurodegeneration is observed characterized by degeneration of periaxonal OLG processes, focal axonal swellings and spheroid formation resulting in locomotor deficits and premature death (Pan et al. 2005).

Natural ligands for MAG include the neuronally expressed gangliosides GD1a, and GT1b (Collins et al. 1997; Vyas et al. 2002; Vyas and Schnaar 2001; Yang et al. 1996). Interestingly, neural-specific knockout of genes encoding GM2/GD2 synthase (Sheikh et al. 1999), or Galgt1 (Sun et al. 2004), which are enzymes responsible for complex ganglioside synthesis (including GD1a, and GT1b), produce a similar phenotype as MAG^{null} mice. These observations suggest similar functions for both MAG and its ligands (complex gangliosides) in the maintenance of axo-glial interactions (Pan et al. 2005; Sun et al. 2004; Yamashita et al. 2005).

In order to determine roles of other proteins that cooperate with MAG in axo-glial maintenance, MAG^{null} mice have been crossed to mutants lacking either a structural protein (e.g., MBP, PLP, P0), signaling molecule (Fyn), or lipid enzyme (CGT) (reviewed by (Georgiou et al. 2004)). In all double knockout mice, more severe pathologies are observed and paranodal loop disruption occurs more rapidly than in MAG single knockouts. Furthermore, MAG/Fyn compound deficient mice display more severe CNS hypomyelination than Fyn^{null} mice alone,

due to loss of a MAG/Fyn interaction that may be necessary for myelin formation (Biffiger et al. 2000).

In addition to the role of MAG in maintenance of axo-glial interactions, it can exhibit both growth promoting and inhibitory properties depending on the developmental stage of the nervous system. In the developing nervous system, MAG promotes neurite outgrowth; however, in the mature nervous system MAG becomes inhibitory to axon regrowth, most notably after CNS injury (De Bellard and Filbin 1999; Johnson et al. 1989; McKerracher et al. 1994; Mukhopadhyay et al. 1994; Schafer et al. 1996; Turnley and Bartlett 1998; Wong et al. 2003). In this manner, MAG assembles as a coreceptor with oligodendrocyte myelin glycoprotein (OMgp), Lingo-1, Nogo, Nogo receptor (NgR), $p75^{NTR}$, and gangliosides causing collapse of regenerating axonal growth cones in the damaged CNS (Vinson et al. 2003). The role of MAG and various other inhibitors of axonal regeneration following nervous system damage is reviewed elsewhere (Filbin 2006; Yiu and He 2006).

2.7. Myelin/oligodendrocyte glycoprotein (MOG)

Myelin oligodendrocyte glycoprotein (MOG) is one of latest proteins expressed by OLGs during myelinogenesis. MOG is localized to the outer mesaxon, and is quantitatively a minor component of myelin (Johns and Bernard 1999). MOG (27 kDa) was originally identified as a target for auto-antibody mediated demyelination in EAE (reviewed by (Pham-Dinh et al. 2004)). The exact functions of MOG are unknown; however it contains an extracellular IgG-like domain and MOG-MOG interactions may promote intercellular signaling between adjacent myelin sheaths, or serve as an intracellular OLG receptor to transduce signals from extracellular milieu to inner layers of myelin (Pham-Dinh et al. 2004). Endogenous MOG ligands have not been identified, although receptor-ligand interactions have been mimicked using MOG antibodies which causes membrane clustering of MBP, microtubule depolymerization and loss of OLG membrane sheets, suggesting MOG signals are transduced intracellularly (Dyer

1993; Dyer and Matthieu 1994). MOG knockout mice develop normally and have no apparent defects in myelin or axon structure (Delarasse et al. 2003).

2.8. Opalin

Oligodendrocytic myelin paranodal and inner loop protein (Opalin) was originally identified in microarray screens for OLG-transcripts regulated during myelination (Golan et al. 2008; Kippert et al. 2008). The Opalin gene promoter contains conserved domains for myelin transcription factor 1 (Myt1) and cAMP-response element binding protein (CREB), which are crucial for its proper gene expression (Aruga et al. 2007). In OLGs, Opalin is abundantly expressed in differentiating cells, and not in OLPs, astrocytes, microglia, neurons (Kippert et al. 2008), or Schwann cells (Golan et al. 2008; Yoshikawa et al. 2008). Opalin is a single-spanning (type I) transmembrane glycoprotein with a short N-terminal extracellular domain, and a long C-terminal intracellular domain (Yoshikawa et al. 2008). This protein localizes to rims and processes of OLGs, with lower expression in membrane sheets (Kippert et al. 2008). Opalin contains amino acid sites for *N*- and *O*-linked glycans, which when mutated impairs its cell surface localization (Yoshikawa et al. 2008). *In vivo*, Opalin is localized to OLG soma and processes, and along myelinated internodal regions where it is concentrated at the paranodal loops (Golan et al. 2008; Yoshikawa et al. 2008). The exact functions of Opalin remain unknown; however, its subcellular localization supports possible roles in axo-glial interactions and/or myelin maintenance.

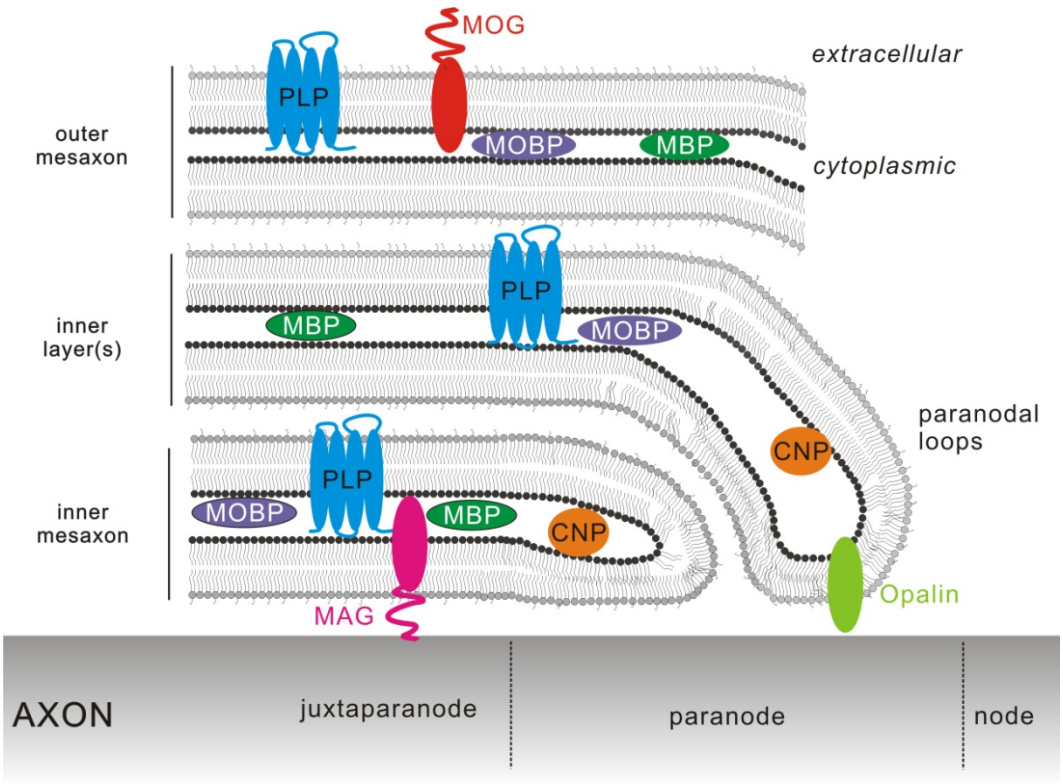

Figure 4. Structure and molecular composition of myelin. Tight multi-lamellar compaction of myelin occurs at the cytoplasmic (major dense line) and extracellular faces (intraperiod line). The compact myelin sheath is comprised of PLP/DM20, MBP and MOBP, providing structural stability to myelin. The inner- and outer-mesaxon contains MAG and MOG, respectively. Paranodal loops and radial component (not shown) are non-compact areas of myelin containing CNP and Opalin. The closely apposed extracellular membrane leaflets contain GalC and sGalC that can signal to alter cytoskeletal dynamics and protein organization in compact myelin. Adapted from Pham-Dinh, Initiatives Santé, 1998 as adapted in (Baumann and Pham-Dinh 2001) with permission from the American Physiological Society.

3.0. Molecular control of OLG development

The progression of an OLP to a mature myelinating OLG requires cell cycle exit, cytoskeletal rearrangement, and synthesis of large quantities of lipids and proteins. The gene regulatory network that controls OLG differentiation involves a complex interplay of factors that normally promote or inhibit OLG development (**Figure 5**). These factors include the activators: OLIG1/2, histone deacetylases (HDACs) Fyn, Sox, Nkx, myelin gene regulatory factor (MRF); and the zinc finger transcription factors (yin yang 1 (YY1), myelin transcription factor 1 (Myt1), and zinc finger proteins (Zfp) 191 and 488). Among the repressors are: bone morphogenetic proteins (BMPs), inhibitors of differentiation (Ids), Notch/Hes5 and Wnt/β-catenin-Tcf4/Tcf7L2. An additional level of regulation of OLG differentiation has been recently discovered which involves microRNA (miRNA) regulation of transcriptional repressors (see (Nave 2010b)). Extensive reviews are available in the literature on transcription factors (Wegner 2000a; Wegner 2000b; Wegner 2001; Wegner 2008) and epigenetic modifiers (Li et al. 2009; Liu and Casaccia 2010) that regulate OLG differentiation. Cytoskeletal modifications during OLG differentiation are reviewed by (Bauer et al. 2009; Maier et al. 2008). Here, the roles of cell cycle regulators and some of the activators and repressors that control OLG differentiation are reviewed. The roles for p38 MAPK in OLG differentiation is covered in Chapters 2-6.

3.1. Cell cycle regulators

A family of cyclin-dependent kinases (Cdks) and Cdk inhibitors (CDKIs) control proliferation and differentiation at the right time and in the correct sequence (Durand and Raff 2000; Lees 1995; Morgan 1995). The Cdk-associated kinase activity depends on the synthesis and association with various cyclins, which promote cell cycle progression. Two major Cdk-cyclin protein complexes are required for the G1-S transition: cyclin D1-Cdk4, and cyclin E-Cdk2 (Ghiani and Gallo 2001). Cyclin-Cdk complexes initiate cell cycle progression by hyperphosphorylating the retinoblastoma (Rb) protein, which promote its release

from E2F transcription factors that can in turn activate replication genes (Lasak et al. 2002; Sellers and Kaelin 1996). Cdk activities are regulated by CDKIs which block the assembly and/or activity of cyclin-cdk complexes (Lees 1995; Morgan 1995; Sherr 1994), thus promoting cell cycle exit and initiating differentiation. CDKIs are divided into two sub-families: Ink4 (p16 ink4a, p15 ink4b, p18 ink4c, p19 ink4d) and Cip/Kip family members ($p21^{Cip1}$, $p27^{kip1}$, $p57^{kip2}$) (Cunningham and Roussel 2001).

In OLPs, an intracellular timer has been postulated to count the number of cell divisions, time cell cycle exit and promote differentiation (Durand and Raff 2000). However, OLP cell cycle exit and differentiation are uncoupled processes (Casaccia-Bonnefil and Liu 2003) since knockdown of cell cycle components is often not sufficient to promote differentiation. In OLPs, cyclin D1-cdk4 is an important regulator of the G1/S transition (Baldin et al. 1993; Bosone et al. 2001), and its induction is an early marker of mitogenic activity of OLPs (Huang et al. 2002; Tang et al. 2001). Furthermore, in the corpus callosum of mice, cyclinE-cdk2 activity is increased as OLPs are dividing before the onset of myelination (Ghiani and Gallo 2001). When myelination commences, or when OLGs undergo cell cycle exit, decreases in protein levels and enzymatic activities of cyclin E-cdk2 and cyclin D1-cdk4 are observed (Ghiani and Gallo 2001; Huang et al. 2002). In most cell types, levels of phosphorylated Rb decrease following cell cycle exit; however, in OLPs Rb protein expression levels also decrease (Huang et al. 2002).

In OLGs cell cycle exit is promoted by $p27^{kip1}$, a CDKI that blocks cdk2 activity, and whose expression levels increase during differentiation (Zezula et al. 2001). However, in $p27^{null}$ mice, OLGs successfully exit the cell cycle by arresting at G1/S through inhibition of cdk2 activity that is mediated by loss of cyclin E (Casaccia-Bonnefil et al. 1999). Furthermore, OLPs isolated from $p27^{null}$ mice divide with a normal cell cycle time; however many cells undergo two or more extra rounds of division before differentiating (Durand et al. 1998). In contrast to $p27^{kip1}$, $p21^{Cip1}$ is not required for OLP cell cycle withdrawal (Zezula et al. 2001), but is necessary for establishing the OLG differentiation program

once growth arrest has occurred (Zezula et al. 2001). During early life, $p21^{null}$ mice are hypomyelinated and exhibit delayed myelination in the cerebellum. OLGs isolated from $p21^{null}$ mice exhibit differentiation defects, which are bypassed by the ERK inhibitor, PD98059, or by ectopic expression of p16 ink4a (Zezula et al. 2001). The Ink4 family member, p18 ink4c, increases as OLPs proliferate, and its over-expression accelerates cell cycle exit causing premature OLG differentiation (Tokumoto et al. 2002). In addition, $p57^{kip2}$ levels continually increase as OLPs proliferate and its expression levels regulate the number of OLP cell divisions before the onset of differentiation (Dugas et al. 2007). Moreover, $p57^{kip2}$ knockdown accelerates morphological maturation of OLGs, and promotes myelin gene expression (Kremer et al. 2009).

3.2. Oligodendrocyte transcription factor 1 and 2 (OLIG1 / OLIG2)

Oligodendrocyte transcription factor 1 and 2 (OLIG1 and 2) are two closely related basic helix-loop-helix (bHLH) proteins important for OLG specification, differentiation and myelination (Ligon et al. 2006). OLIG1 and OLIG2 effect OLG differentiation through homodimeric interactions or heterodimeric complex formation with the ubiquitously expressed bHLH proteins E12 and E47 (Samanta and Kessler 2004). OLIG-E12/E47 complexes are prevented from binding to DNA by Ids, proteins which sequester bHLHs in the cytoplasm, thus preventing gene transcription (Kessaris et al. 2008). Therefore, OLIG subcellular localization is affected by alternating interactions with E2As and Ids (Samanta and Kessler 2004; Sun et al. 2003).

Knockout of the genes encoding OLIG1 and 2 have revealed multiple functions in OLGs. OLIG2 appears to be involved in the initial specification of OLPs, whereas OLIG1 is involved in OLP differentiation and remyelination. Knockout of OLIG2 leads to a complete absence of OLPs in the spinal cord, but a few OLPs remain in the brain due to compensation from OLIG1 (Lu et al. 2002; Zhou and Anderson 2002). However, the roles of OLIG2 appear to be region-specific since ablation from mouse cortices causes OLP myelination arrest (Yue et

al. 2006). OLIG1 regulates MBP, PLP and MAG genes, and OLIG1^{null} mice have defects in OLP differentiation, producing severe neurological defects in these animals (Xin et al. 2005). In OLIG1/2 double knockout mice, OLGs completely fail to develop, suggesting fundamental roles for these bHLHs in OLG specification and differentiation (Lu et al. 2002; Zhou and Anderson 2002).

In contrast to other bHLH transcription factors, OLIGs are considered to be transcriptional repressors, not activators. In consequence, OLIGs activate OLG target genes, such as MBP, PLP and MAG, through repression of transcriptional repressors (Mizuguchi et al. 2001; Novitch et al. 2001; Zhou et al. 2001). One target repressor of OLIG1/2 is G-protein coupled receptor 17 (GPR17), a factor that normally induces Id2/4 expression, resulting in block of OLG differentiation. In fact, knockout of GPR17 promotes precocious OLG differentiation through the early down-regulation of Id2/4. Thus, the OLIG1/2-induced downregulation of GPR17 is necessary to block induction of Id2/4 during differentiation and myelination (Chen et al. 2009).

3.3. Bone morphogenetic proteins (BMPs) and Inhibitors of differentiation 2 and 4 (Id2 / Id4)

The bone morphogenic proteins (BMPs) are members of the transforming growth factor β (TGFβ) family of extracellular ligands (Nohe et al. 2004), which bind to heterotrimeric serine-threonine kinase receptors formed by type I (BMPR1a, BMPR1b, Alk2) and type II (BMPRII) subunits (Samanta et al. 2007; Xiao et al. 2007). Type I BMPRs activate SMADs that cooperate with numerous transcription factors and transcriptional coactivators/corepressors to regulate gene transcription. Genes regulated by BMP signaling include cell cycle regulators, early response genes, Wnt/β-catenin target genes, and inhibitors of differentiation (Ids) (Miyazono and Miyazawa 2002). In addition, BMP signaling is regulated by endogenously secreted extracellular antagonists, including noggin, chordin, gremlin and dan, which bind and inhibit the actions of BMP ligands (Avsian-Kretchmer and Hsueh 2004; Canalis et al. 2003).

In OLPs, multiple lines of evidence have proven that BMP signaling is inhibitory to OLG differentiation. For example, in OLPs, ectopic BMP expression, or treating cells with BMP2/4 decreases OLIG1/2 and increases Id4 expression. Furthermore, high doses of BMP ligands can promote OLPs to become astrocytes (Agius et al. 2004; Cheng et al. 2007; Grinspan et al. 2000; Gross et al. 1996; Hall and Miller 2004; Mabie et al. 1997; Nakashima et al. 2001; Samanta and Kessler 2004; Zhu et al. 1999). In addition, mice with a conditional ablation of BMPR1a gene in OLGs have increased numbers of mature and immature OLGs at the peak of myelination, suggesting BMPR1a normally mediates the suppressive effects of BMP signaling in OLGs (Samanta et al. 2007). Furthermore, injection of noggin-producing Chinese hamster ovary (CHO) cells or BMP neutralizing antibodies into the nervous system of developing chick or *Xenopus* promotes the appearance of OLPs (Cheng et al. 2007; Mekki-Dauriac et al. 2002; Miller et al. 2004).

Mechanistically, the BMP-induced block of OLG differentiation is mediated by nuclear localization of C-terminal-phosphorylated-SMAD1 (C-term-pSMAD1), which induces downregulation of OLIG2. The exclusion of C-term-pSMAD1 from the nucleus is required for the onset of differentiation (Bilican et al. 2008). Furthermore, the BMP-suppressive effect on OLG differentiation is blocked by ectopic OLIG1/2 expression, suggesting OLIG1/2 down-regulation is part of the mechanism by which BMP signaling represses OLG development (Cheng et al. 2007). The BMPR effector, SMAD4 also associates with its cognate binding region on the *Olig2* promoter and normally dissociates from this site during OLG differentiation.

The inhibitors of differentiation (Ids) are DNA helix-loop-helix (HLH) proteins that lack the DNA binding domain of bHLH proteins that function downstream of BMPs (Norton et al. 1998). Ids form heterodimers with bHLH proteins (e.g., OLIG1/2), thus blocking their ability to bind DNA and activate gene transcription (Norton et al. 1998). Therefore, by sequestering bHLHs, Ids inhibit the differentiation program that bHLH proteins would normally promote. In agreement with this, Id over-expression blocks differentiation of many cell

types including B lymphocytes (Sun 1994), myocytes (Jen et al. 1992), mammary epithelial cells (Desprez et al. 1995), myeloid cells (Kreider et al. 1992), erythroid cells (Lister et al. 1995), Schwann cells (Thatikunta et al. 1999) and OLGs (Wang et al. 2001). Accordingly, over-expression of Id2 or 4, but not Id1 or 3, blocks OLG differentiation (Kondo and Raff 2000; Wang et al. 2001), and can promote astrogliogenesis (Samanta and Kessler 2004). During OLG differentiation, Id2 translocates out of the nucleus, and Id2 or Id4 deletion slows proliferation and promotes early OLG differentiation (Wang et al. 2001). In luciferase reporter assays, Id4 decreased the promoter activity of both MBP and CGT, but had no effect on PLP, suggesting Ids differentially regulate myelin gene promoters. In agreement with this, MBP and CGT levels are increased in $Id4^{null}$ mice due to precocious OLG differentiation. Unexpectedly, however, both PLP and MAG are decreased in $Id4^{null}$ mice (Marin-Husstege et al. 2006).

Interestingly, Ids and BMPs are targets of other pathways that repress OLG differentiation, including the Wnt/β-catenin signaling pathway. Namely, the Id2 gene is a target of Tcf4/Tcf7L2, a crucial effector of the Wnt/β-catenin signaling pathway (Memezawa et al. 2007; Rockman et al. 2001). Furthermore, BMPR1a and 1b double knockout mice also have reduced expression of Wnt1, Wnt3a, Id1, Id2 and Id3 (Wine-Lee et al. 2004). In addition, neural precursor cells virally infected with β-catenin have upregulated levels of BMP2, 4 and 7 (Kasai et al. 2005).

The repressive effect of BMPs and Ids on OLG differentiation is eventually relieved by inhibitory complexes formed between YY1 and HDACs which effect myelin gene expression (see below) (He et al. 2007b).

3.4. Wnt/β-catenin

The Wnt/β-catenin signaling pathway controls cell proliferation, cell polarity, and cell fate determination (reviewed by (Barker 2008; Chien et al. 2009; Clevers 2006; Logan and Nusse 2004; MacDonald et al. 2009)). Wnts are secreted glycoproteins that bind to the Frizzled G protein-coupled receptor, and its coreceptor lipoprotein receptor-related protein 6 (LPR6). A key downstream molecule of Wnt/Frizzled/LPR6 is β-catenin, a transcriptional co-activator which

controls developmental gene expression. In the presence of Wnt ligands, an Axin complex constantly degrades β-catenin. This process prevents β-catenin entry into the nucleus, thus repressing the expression of Wnt target genes. However, when Wnt is present, phosphorylated-β-catenin translocates to the nucleus where it forms transcriptional complexes with TCF/LEF which in turn activate Wnt target genes (MacDonald et al. 2009).

Previous studies have shown that Wnt signaling can control distinct cellular processes depending on developmental stage (Kim et al. 2008). For example, at early stages of development, Wnts control cell neural precursor cell proliferation, but can also time cell cycle exit and differentiation (Chenn and Walsh 2002; Megason and McMahon 2002; Zechner et al. 2003). In OLGs, Wnt/β-catenin signaling appears to control multiple processes including specification, proliferation, migration, survival and differentiation. Several lines of evidence support this claim. For example, β-catenin expression is increased at focal adhesion sites in the OLG cell line CG-4 when treated with Dapper, a Wnt antagonist that inhibits OLP migration (Kakinuma et al. 2004); whereas the Frizzled 8a Wnt receptor is important for OLG specification in zebrafish (Kim et al. 2008). In addition, the Wnt antagonist rmFz-8/Fc increases the numbers of immature OLGs in spinal cord explant cultures (Shimizu et al. 2005). Following OLG specification, however, Wnt signaling plays important roles in the timing of differentiation through β-catenin/Tcf7L2 signaling. Consequently, treatment of OLPs with Wnt3a increases expression of two Tcf7L2 target genes Id2 and Id4, but does not affect levels of the Notch signaling effectors Hes1 or Hes5 (Wang et al. 1998; Ye et al. 2009). Moreover, treatment of rat OLPs with Wnt3a decreases GalC and MBP expression without affecting OLP cell fate or proliferation (Feigenson et al. 2009). Mice expressing an OLG-specific constitutively active (CA)-β-catenin have developmentally delayed myelination, recovering to normal levels by adulthood (Feigenson et al. 2009), suggesting β-catenin-Tcf7L2 signaling normally delays OLG differentiation and myelination. This delay is promoted by Tcf7L2-induced activation of Ids (Ye et al. 2009). Interestingly, sustained β-catenin signaling may also be involved in failure of remyelination

following white matter damage. Tcf7L2 was identified in genome-wide screens of transcription factors expressed in rodent remyelinating lesions following cuprizone-, ethidium bromide- and lysolecithin- induced demyelination. Consequently, it was found that Tcf7L2 was specifically expressed in lesioned brain tissue, but not in normal adult white matter, suggesting active β-catenin signaling is responsible for defective remyelination following experimental demyelination (Fancy et al. 2009). Repression of Tcf7L2 activity is achieved through histone deacetylases (HDACs), factors which promote OLG differentiation by competing with β-catenin for Tcf7L2 binding (Ye et al. 2009).

3.5. Histone deacetylases (HDACs)

Histone deacetylases (HDACs) were originally identified as enzymes that remove lysine acetyl groups from the N-terminal tails of histones, resulting in chromatin compaction and repression of gene transcription (de Ruijter et al. 2003; Yang and Gregoire 2005). Multiple lines of evidence suggest that HDACs play important roles in OLG differentiation, myelination, and remyelination. First, OLGs treated with the pan-selective HDAC inhibitors valproic acid (VPA) or trichostatin A (TSA) exited the cell cycle, but arrested at an early stage of differentiation due to reduced myelin gene expression (Marin-Husstege et al. 2002; Shen et al. 2005). Second, in transgenic mice where HDAC1 and 2 are specifically deleted from OLGs, death occurs at the onset of myelination, and these mice display decreased mRNA transcripts encoding PDGFRα, OLIG2, CNP, MBP and PLP (Ye et al. 2009). Mechanistic studies revealed that HDAC1/2 normally promotes OLG differentiation through repression of Tcf7L2, which normally promotes expression of Ids. In agreement with this, the transcriptional repressors Id2 and 4 are increased in HDAC1/2^{null} mice due to lack of repression of Tcf7L2 (Ye et al. 2009). In order to promote OLG differentiation, HDAC1/2 normally form protein complexes with YY1 which leads to repression of Id4 and Tcf7L2 genes, thus relieving the repressive effects of these factors (He et al. 2007a). Notably, the failure of remyelination in aging animals is partly due to

inefficient recruitment of HDACs to the promoters of Id4 and Tcf7L2, leading to their sustained activation, thus inhibiting remyelination (Shen et al. 2008).

Another HDAC family member that promotes OLG differentiation is HDAC11. HDAC11 is primarily expressed in OLG nuclei of mouse brain with expression increasing postnatally until four weeks of age (Liu et al. 2009; Liu et al. 2008). In a rodent OLG cell line, OL-1, reduced histone H3 acetylation levels at promoter regions of *MBP* and *PLP* correlates with increased MBP, PLP and HDAC11 protein expression. In addition, knockdown of HDAC11 with siRNA decreases MBP and PLP gene expression, and blocks OLG process outgrowth (Liu et al. 2009).

3.6. Zinc finger transcription factors (YY1, Myt1, ZFP488, ZFP191)

Four zinc finger transcription factors have been found to play roles in OLG development including yin yang 1 (YY1), myelin transcription factor 1 (Myt1) and zinc finger protein 488 (Zfp488) and 191 (Zfp191).

Myelin transcription factor 1 (Myt1), Myt1-like (Myt1L), and Myt3 are members of the C2HC zinc-finger transcription factors family (Wang et al. 2007a). Myt1 is one of the earliest transcription factors expressed in the developing nervous system and may play roles in regulating proliferation and differentiation of OLGs (Nielsen et al. 2004; Nielsen et al. 2002). Myt1 was originally identified by its binding properties to the *PLP* promoter (Kim and Hudson 1992). Later identification of the Myt1 DNA consensus sequence showed that it is also capable of binding promoters of neuregulin 1 (Law et al. 2006), cannabinoid receptor 1 (McCaw et al. 2004), CGT (Yonemasu et al. 1998), and the RNA binding protein, HuC (Park et al. 2000). Myt1 also interacts with HDACs that may be recruited to gene promoters during neural differentiation (Romm et al. 2005). The exact roles of Myt1 in OLGs is unclear; however, it may also play roles in demyelination and remyelination following white matter damage (Vana et al. 2007). In murine hepatitis virus-induced demyelination the density of $Myt1^{+}$ OLPs is increased during early phases of demyelination and remyelination, suggesting possible roles

for Myt1 in myelin repair (Vana et al. 2007). Myt1 also appears to play roles in development, since Myt1 knockout mice die postnatally, due to confounding developmental defects in many tissues (Wang et al. 2007a). Moreover, a human infant identified with Myt1 gene deletion had severe neurological problems, including learning difficulties, mental retardation and motor defects (Kroepfl et al. 2008). Myt1 also appears to play important roles in neuronal and endocrine islet cell differentiation (Bellefroid et al. 1996; Wang et al. 2008). However, pancreatic-specific knockout of Myt1 shows no overt-phenotype due to compensation from the related paralogs Myt1L and Myt3 (Wang et al. 2007a). The exact functions of Myt1L and Myt3 are unknown; however, Myt1L interacts with the intracellular domain of Lingo-1 and thus may regulate Myt1L transcriptional activity by altering its subcellular localization (Llorens et al. 2008).

YY1 is a multi-functional and ubiquitously expressed transcription factor that can either activate or repress gene expression depending on the cellular context, and its protein binding partners, for example, HDAC1, p300, or Rb (He et al. 2007b). In addition, YY1 regulates the expression of CREB, p53 and c-myc. Early genetic studies in *Xenopus* identified important roles for YY1 in neuronal function (Kwon and Chung 2003; Morgan et al. 2004). In OLGs, YY1 regulates the *PLP* promoter, and its over-expression results in enhanced PLP gene transcription (Berndt et al. 2001). Furthermore, OLG-specific ablation of YY1 impairs myelination due to differentiation arrest following OLP cell cycle exit. Mechanistically, YY1 normally promotes OLP differentiation by repressing transcription of Tcf4/Tcf7L2 and Id4 by recruiting HDAC1 to their gene promoters during OLG development (He et al. 2007a).

Two other zinc finger transcription factors involved in OLG differentiation and myelination are Zfp191 and Zfp488 (Howng et al. 2010; Wang et al. 2006). Zfp191 is a widely expressed transcription factor which plays roles in myelin gene expression. Knockout of Zfp191 from mice causes drastic decreases in MAG, CNP, MBP, MOG and MOBP, leading to defects in myelination and death (Howng et al. 2010). Zfp488 is exclusively expressed in differentiating OLGs and appears to play similar roles to Zfp191 in promoting myelin gene expression.

Knockdown of Zfp488 decreases MBP and CNP expression, and mechanistically promotes OLG differentiation by cooperating with OLIG2 to regulate pro-myelin genes (Wang et al. 2006).

3.7. Notch / Hes5

The Notch signaling pathway is involved in numerous developmental processes, including cell specification, adult homeostasis, and stem cell maintenance (see (Fortini 2009; Tien et al. 2009). Notch is a transmembrane receptor that interacts with numerous ligands on neighbouring cells, including Delta, Serrate/Jagged, and Lag2. Signaling is initiated when Notch interacts with one of these ligands causing an intracellular cleavage of Notch, followed by its subsequent translocation to the nucleus where it inhibits cellular differentiation (Ohtsuka et al. 1999). Cleaved Notch interacts with CSL, a transcription factor required for both the activation and repression of Notch target genes, including Hes1 and Hes5, two bHLHs which also inhibit cell differentiation (Ohtsuka et al. 1999).

Notch and its ligands are expressed in the developing rat optic nerve and their expression is down regulated during myelination (Wang et al. 1998). OLPs normally express Notch, whereas astrocytes and/or neurons express Notch ligands. For instance, Jagged is expressed on non-myelinated axons during early development and is downregulated in adult tissue (Givogri et al. 2002; Wang et al. 1998), and treatment of OLP cultures with purified Jagged or Delta strongly inhibits OLG differentiation (Wang et al. 1998). In MS plaque regions, Jagged expression on astrocytes is upregulated and its levels inversely correlate with remyelination efficiency (John et al. 2002). Conversely, in a cuprizone-induced model of demyelination, no association between Jagged expression level and degree of remyelination was observed. In addition to these findings, an OLG-specific knockout of Notch did not enhance remyelination efficiency (Stidworthy et al. 2004). Another study, however, showed that mice heterozygous for Notch1$^{(-/+)}$ accumulate more myelin-specific gene products during the first few weeks of life (Givogri et al. 2002). Notch heterozygotes also have an early downregulation of Hes5, a Notch effector which normally inhibits OLG

differentiation (Givogri et al. 2002) by inhibiting Mash1 and sequestering Sox10 and Mash1, two factors which normally promote differentiation (Liu et al. 2006). Moreover, $Hes5^{null}$ mice have increased levels of myelin mRNAs and proteins (Liu et al. 2006). Considering the inhibitory nature of Notch signaling on OLG development, this receptor appears to play multiple roles in OLG differentiation and in myelination depending on its ligand. For example, Notch binding to an axonal surface protein, contactin, enhances OLG differentiation, and initiates myelin wrapping (Hu et al. 2003).

3.8. Sex-determining region on Y box (Sox) transcription factors

The sex-determining region on Y box (Sox) are a family of transcription factors involved in tissue development and cell fate specification of many cell types, including neurons and glia (McDonald et al. 2009; Pevny and Placzek 2005). The functions of Sox have been reviewed elsewhere (Kiefer 2007; Lefebvre et al. 2007). The roles of Sox factors involved in OLG development will be reviewed here. Sox9 and 10 are the earliest expressed transcription factors following OLG specification (Stolt et al. 2003; Wegner 2008), with eventual disappearance of Sox9 in late-stage OLPs (Stolt et al. 2003). $Sox9^{null}$ mice have very few OLPs in the spinal cord, which suggests roles in OLP specification (Wegner 2008). However, Sox10 knockout shows no defects in OLP specification, but minor defects in OLG differentiation. In the absence of both Sox9 and 10, OLPs express slightly reduced to normal levels of typical myelin-specific proteins, but have a drastic reduction in PDGFRα, suggesting this gene is a transcriptional target of Sox9/10 during OLG specification. However, other studies have shown that Sox10 regulates *MBP* promoter activity, and Sox10 regulatory elements have been found at *MAG* and *PLP* gene promoters (Wegner 2008).

Another Sox family member, Sox17 is positively regulated during OLG differentiation and its expression correlates with MAG, MOG, PLP/DM20 during post-natal development in mouse OLGs (Sohn et al. 2006). The upregulation of Sox17 is inhibited by conditions that promote OLP proliferation, and its knockdown by siRNA increases proliferation, thereby decreasing OLG lineage progression. In contrast, Sox17 over-expression enhances myelin gene expression,

and stimulates *MBP* promoter activity. Therefore, Sox17 appears to normally regulate both OLP cell cycle exit and differentiation (Sohn et al. 2006).

In contrast to the roles of Sox9, 10 and 17 in OLG specification and differentiation, Sox 5 and 6 block OLG lineage progression, and their deletion promotes precocious OLP specification and differentiation (Stolt et al. 2006). Sox 5 and 6 normally repress OLG differentiation by competing for Sox10 binding sites at myelin gene promoters (Wegner 2008). The repressive actions of Sox5/6 are relieved by their downregulation which is promoted by microRNAs that target Sox6 mRNA transcripts for degradation (see below) (Nave 2010b).

3.9. Nkx

The Nkx homeobox genes are expressed in developing tissues and have been implicated in the control of tissue patterning and cellular differentiation (Cai et al. 2001). One Nkx family member, Nkx2.2, is selectively expressed in migratory OLPs in chick (Fu and Qiu 2001), rodents and mammals (Qi et al. 2001). The Nkx2.2 promoter is also regulated by other transcription factors important for OLP differentiation, including Sox10 and OLIG2 (Liu et al. 2007). In Nkx2.2^{null} mouse mutants, differentiation of MBP^{+} and PLP/DM20^{+} cells is retarded, implicating roles for this factor in OLG differentiation (Qi et al. 2001). However, Nkx2.2^{null} mice have no defects in OLP specification since increased numbers of Olig1/2^{+} and PDGFRα^{+} cells are observed in these mutants. In addition, overexpression of Nkx2.2 in fibroblasts stimulates *PLP* promoter activity (Qi et al. 2001). However, Nkx2.2 expression decreases with OLG development, suggesting this transcription factor plays transient roles in myelin gene induction (Cai et al. 2010). Another Nkx family member, Nkx6.2, is expressed in embryonic neural tube, testis, and differentiating OLGs, but not in immature OLPs or astrocytes, and therefore may play roles in myelinogenesis. Nkx6.2 and a related homolog, Nkx6.1 both regulate the expression of OLIG2. However, Nkx6.2^{null} mice show no developmental defects and no apparent changes in the CNS, suggesting redundant roles with other Nkx family members during OLG differentiation and mouse development (Cai et al. 2001).

3.10. Myelin gene regulatory factor (MRF)

Myelin gene expression regulatory factor (MRF) is a recently identified transcription factor that regulates the expression of numerous myelin genes (Emery et al. 2009). MRF is specifically expressed in the nucleus of post-mitotic OLGs and its expression peaks at the height of myelination. In addition, MRF overexpression or deletion *in vitro*, increases or decreases myelin gene expression levels, respectively. MRF^{null} mice form pre-myelinating OLGs but die at post-natal week three due to lack of myelin formation caused from failure to accumulate myelin-specific gene products (Emery et al. 2009). Based on these observations, it has been proposed that MRF is analogous to Krox20, a master myelin transcription factor essential for Schwann cell myelination (Topilko et al. 1994). In fact, ectopic Krox20 expression in fibroblasts can induce myelin gene expression (Parkinson et al. 2004); however, the ability of MRF to execute a similar role in non-glial cell lines has yet to be determined.

3.11. microRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that play roles in post-transcriptional gene regulation during proliferation, differentiation, apoptosis, stress response, and disease (reviewed in (Bartel 2004; Stefani and Slack 2008). miRNAs are processed by two enzymes Drosha and Dicer, resulting in small ~22-mers which can target numerous mRNAs for degradation by a RNA-induced silencing complex (RISC). The roles of miRNAs in neuronal development have been uncovered by transgenic knockout of Dicer, which causes defective neuron differentiation (Cuellar et al. 2008; Davis et al. 2008). Microarray screens of miRNAs regulated during OLG differentiation revealed dynamic expression of ninety-eight miRNAs. One miRNA identified, miR-9, promotes the downregulation of the mRNA encoding peripheral myelin protein 22 (PMP22), a Schwann cell myelin protein that is not normally expressed in OLGs (Lau et al. 2008). However, the function of the many other miRNAs remained unknown. To address this, *Nestin*, *OLIG*, or *PLP* promoter-driven Cre recombinase mice have

been used to ablate floxed-Dicer alleles from stage-specific OLGs causing a total loss of miRNAs from these cells. Depending on the Cre driver used, Dicer knockout animals either exhibit decreased OLG numbers, hypomyelination, or demyelination and neurodegeneration (Dugas et al. 2010; Kawase-Koga et al. 2009; Shin et al. 2009; Zhao et al. 2010). Further analysis showed that two miRNAs, miR-219 and miR-338, positively regulated OLG differentiation and myelination by degrading mRNAs for PDGFRα, Sox6, Hes5, and other developmental repressors (Dugas et al. 2010; Zhao et al. 2010).

3.12. Fyn

Fyn is a member of the Src family non-receptor tyrosine kinases which include Src, Lyn, Yes, Fgr, Hck, Lck and Blk (Brown and Cooper 1996; Thomas and Brugge 1997). OLGs only express Src, Lyn and Fyn, where the latter plays an important role for CNS myelination and the morphological maturation of cultured OLPs. Fyn activity is highest in brains during the earliest stages of myelination (Kramer et al. 1999) where it is responsible for phosphorylation of L-MAG and activation of hnRNP A2 which promotes translation of MBP RNAs in OLG processes (Lu and Liu 1991; Umemori et al. 1994). Fyn also signals to cytoskeletal proteins involved in process extension and elaboration, such as focal adhesion kinase, Rho, Rac1, Cdc42, and the Rho regulators p190, and p250 RhoGAP (Hoshina et al. 2007; Klein et al. 2002; Liang et al. 2004; Taniguchi et al. 2003; Wolf et al. 2001). Fyn also regulates tau activation (Belkadi and LoPresti 2008; Perez et al. 2009), and the Cdk5-induced phosphorylation of paxillin (Miyamoto et al. 2007). Stimuli that activate or inhibit Fyn can promote or inhibit OLG differentiation, respectively. For example, Fyn is activated by serum withdrawal, IGF-1, laminin stimulation, antibody cross-linking of MAG, or by a netrin-1/DCC receptor interaction (Colognato et al. 2004; Liang et al. 2004; Nakahara et al. 2003; Osterhout et al. 1999; Rajasekharan et al. 2009; Sperber and McMorris 2001; Umemori et al. 1994). In contrast, blocking OLG differentiation with Lingo-1 or myelin debris decreases Fyn activity (Baer et al. 2009; Mi et al. 2005). In addition, Fyn activates the MBP gene promoter (Umemori et al. 1999),

and can tether to MBP via its SH3 domain (Homchaudhuri et al. 2009). In OLGs, inhibition or deletion of Fyn causes decreased process outgrowth and hypomyelination (Osterhout et al. 1999; Wolf et al. 2001). Indeed, Fyn knockout mice have defects in forebrain myelination, but no decreases in cervical spinal cord (Sperber et al. 2001), suggesting region-specific roles for Fyn in myelination. Moreover, Fyn appears to play a unique role in myelinogenesis since no CNS myelination defects are observed in Src, Yes, or Lyn null mice (Jaramillo et al. 1994; Sperber et al. 2001; Umemori et al. 1994). In addition, a Fyn phosphatase, PTPα, which normally activates Fyn, is important for OLG differentiation and *in vivo* myelination (Wang et al. 2009).

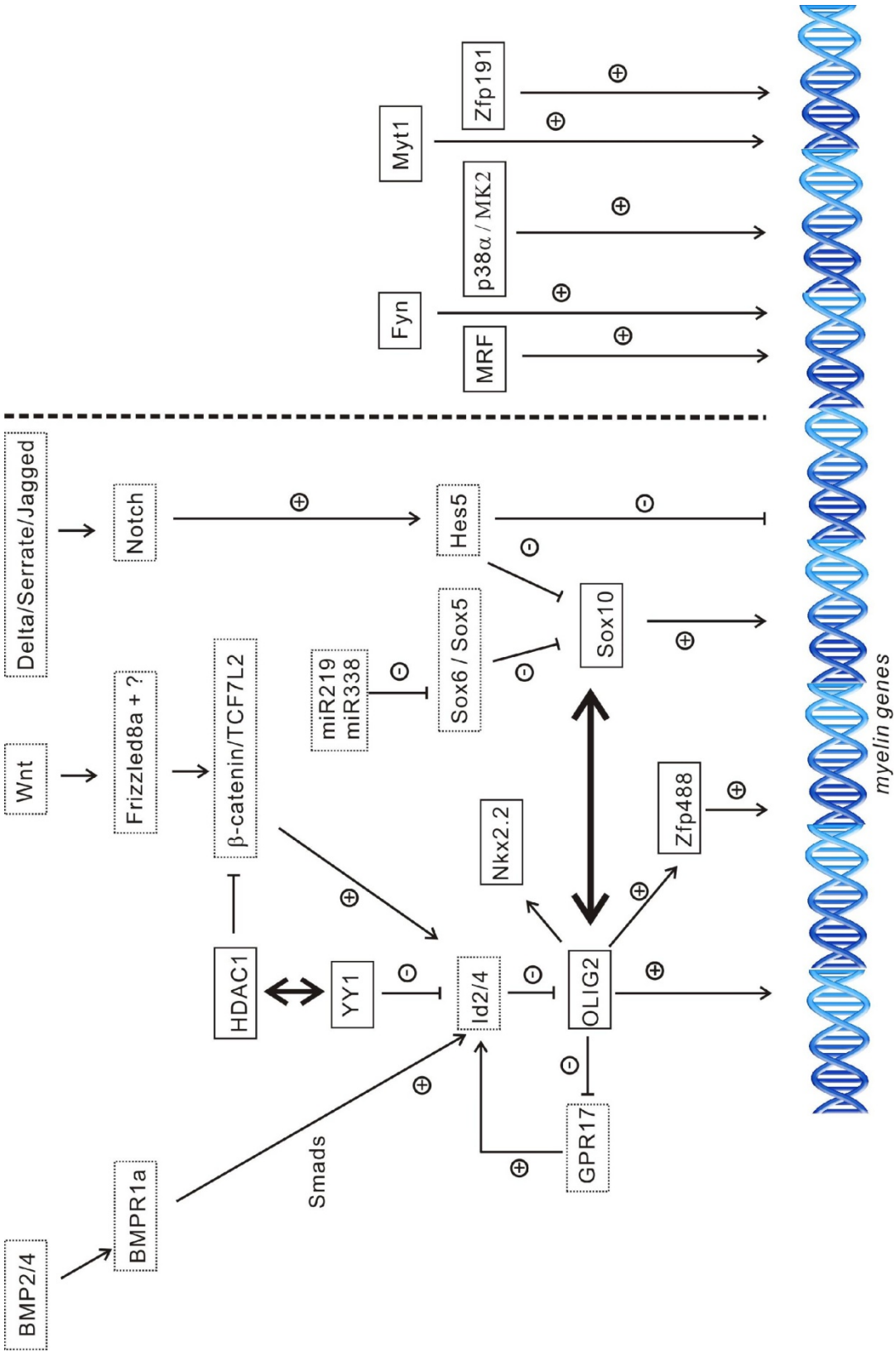
BMP2/4
BMPR1a
Smads
Wnt
Frizzled8a + ?
β-catenin/TCF7L2
HDAC1
YY1
Id2/4
OLIG2
GPR17
Nkx2.2
Zfp488
Delta/Serrate/Jagged
Notch
Hes5
miR219
miR338
Sox6 / Sox5
Sox10
Fyn
MRF
p38α / MK2
Myt1
Zfp191
myelin genes

Figure 5

Figure 5. Molecular control of OLG differentiation. Multiple pathways control the timing of onset of OLG differentiation, including BMP/Ids, Wnt/β-catenin, Notch and microRNAs. These pathways impinge on molecules including HDACs, OLIGs, Sox transcription factors, zinc finger proteins, and MRF. The complex interplay of these factors controls the proper timing of OLG differentiation. Hatched boxes indicate repressors, whereas solid lines represent activators. Solid double-headed arrows represent physical interactions. The dotted line dividing the pathways is to indicate pathways/molecules (e.g., MRF, Zfp191, Fyn, Myt1, p38α/MK2) that are important for OLG differentiation, but the exact mechanism of how they interplay with the other pathways is still under investigation. Adapted from (Wegner 2008) and (Li et al. 2009), with permission from Springer and Elsevier Limited, respectively.

4.0. p38 MAPK

Mitogen-activated protein kinases (MAPKs) are serine-threonine protein kinases which serve as intracellular pathways to regulate numerous cellular functions including cellular stress response, survival, proliferation, migration, and differentiation (Cuenda and Rousseau 2007). The MAPKs are comprised of four sub-families: extracellular-regulated kinases (ERKs), p38, c-Jun N-terminal kinases (JNKs), and big MAPKs (BMKs). All MAPKs contain a Thr-*Xaa*-Tyr motif in the primary amino acid sequence of their activation loop, where *Xaa* differs between the various subfamily members. The functions of the p38 signaling pathway will only be reviewed here since outlining the numerous functions of ERK, JNK, and BMKs are beyond the scope of this thesis.

Early studies showed that macrophages treated with lipopolysaccharide (LPS) induced tyrosine phosphorylation of many proteins which could be blocked by the inhibitor herbimycin A (Han et al. 1993; Lee et al. 1993; Weinstein et al. 1993). One of the phosphorylated proteins identified and cloned was p38 (Han et al. 1994), a kinase which contained a unique Thr-Gly-Tyr (TGY) dual phosphorylation motif (Thornton and Rincon 2009). p38 is activated in response to many inflammatory cytokines including interleukin (IL)-1, -6, -8, and tumour necrosis factor alpha (TNFα) (Dambach 2005; Schieven 2009). In addition, p38 is responsible for cytokine gene activation and their post-transcriptional regulation. Therefore, the dual role for p38 in both the regulation and production of cytokines has generated interest in targeting p38 for the therapeutic treatment of inflammatory diseases, or diseases with an inflammatory component. Such diseases include inflammatory bowel disease (IBD), psoriasis (Pettus and Wurz 2008), rheumatoid arthritis (RA) (Pargellis and Regan 2003), Alzheimer's disease (Culbert et al. 2006), and multiple sclerosis. The role of p38 in inflammation and disease are reviewed by others (e.g., (Cuenda and Rousseau 2007; Ono and Han 2000; Zarubin and Han 2005)) (also see Chapter 6).

In addition to the cytokine-induced activation of the p38 signaling cascade, the pathway can be activated by other stimuli including stress, osmotic shock, reactive oxygen species, UV irradiation, DNA damage, cytokines, chemokines,

GPCR ligands, and growth factors (Thornton and Rincon 2009). The p38 MAPK cascade is initiated by both receptor and non-receptor induced phosphorylation of MAPK kinase kinase (MKKK), which phosphorylates MAPK kinase 3 or 6 (MKK3/6), which in turn activate the four p38 MAPK isoforms, alpha (α), beta (β), gamma (γ), or delta (δ) (Zarubin and Han 2005) (Figure 6). Activated p38 then phosphorylates many substrates, including kinases, phosphatases, cytoskeletal modifiers, and transcription factors which all play roles in cell apoptosis, survival, migration, division, senescence and differentiation. The functions of p38 isoforms, upstream activator kinases, downstream substrates, and mechanisms of regulation of cell growth and development will be reviewed below.

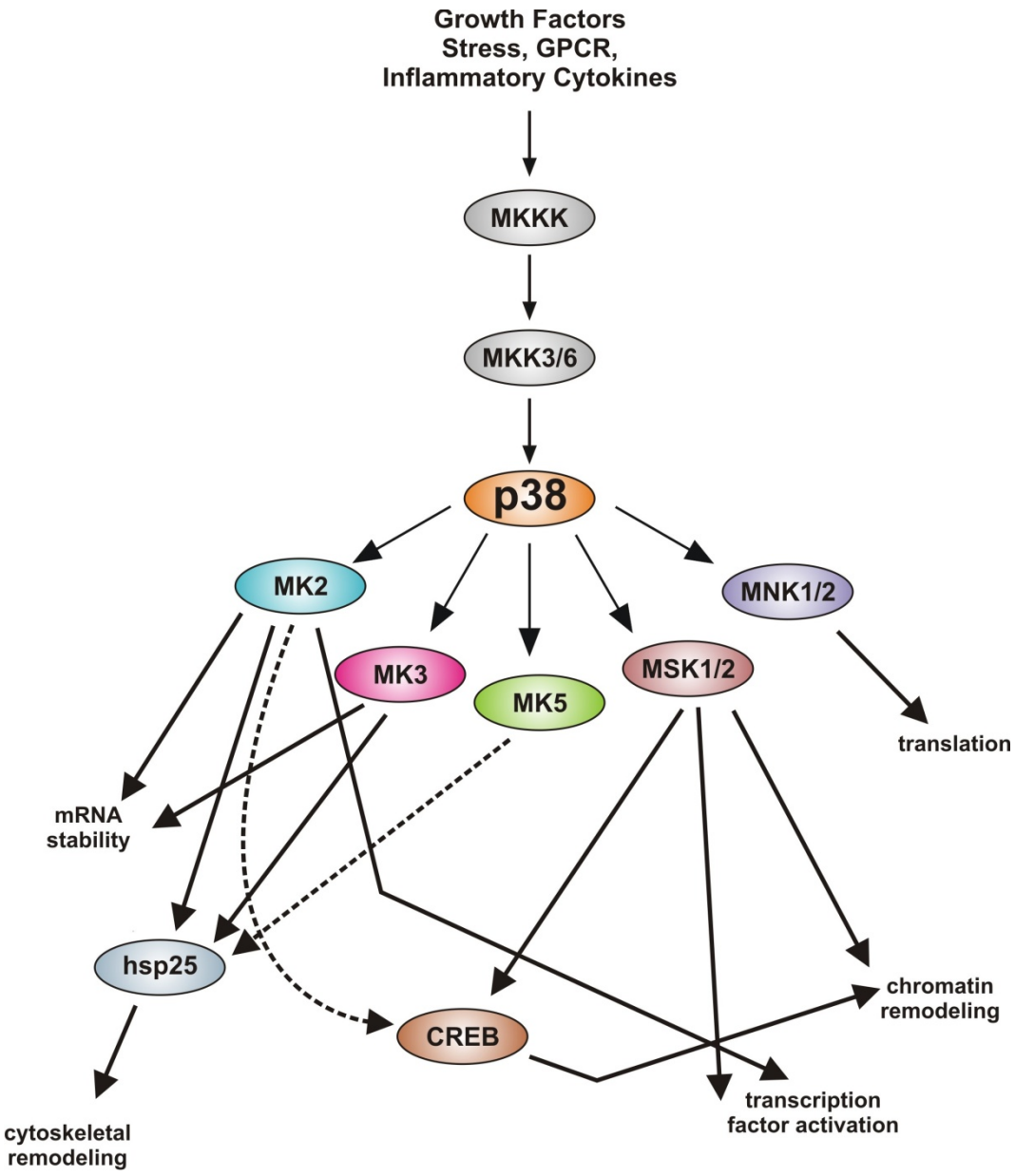

Figure 6. Schematic representation of the p38 signaling pathway. A number of growth factors, stress, GPCRs and inflammatory cytokines stimulate this pathway, leading to activation of the MKKK-MKK3/6-p38 signaling pathway. p38 can, in turn, activate numerous effector kinases, transcription factors, and cytoskeletal remodeling proteins. p38 signaling can affect translation, mRNA stability, cytoskeletal/chromatin remodeling. Dashed arrows represent possible activation pathways. Modified from (Haines et al. 2008).

4.1. p38 isoforms

p38 MAPK has four separate genes encoding four structurally related p38 isoforms α, β, δ and γ which are ~60% identical in their primary sequences (Thornton and Rincon 2009). The p38 isoforms vary in their tissue distribution, subcellular localization, and activation of downstream targets (Gaestel 2006). All p38 isoforms phosphorylate targets containing serine-proline or threonine-proline consensus motifs; however, differential activation of downstream targets occurs, for example, p38α/β, but not p38γ/δ, activate the effector kinases MAPK-activated protein kinase 2/3 (MK2/3) (Cuenda et al. 1997; Goedert et al. 1997). Based on their substrate specificity and inhibition by small molecule inhibitors, p38 isoforms can be classified into two groups: p38α/β and p38γ/δ. The pyridinyl imidazoles-based inhibitors SB203580 and PD169316 selectively inhibit p38α/β isoforms, and have no effect on p38γ/δ activities. These inhibitors compete with ATP for the p38α and p38β ATP-binding pocket, thus preventing their activation of downstream targets. Broad range, pan-selective p38 isoform inhibitors have also been developed (e.g., BIRB796) which block the function of all p38 isoforms *in vitro* and *in vivo* (Kuma et al. 2005) by indirectly competing with the ATP-binding site (Pargellis et al. 2002). Interesting p38 isoform-specific functions have been revealed through pharmacological inhibitors, *in vitro* knockdown, or by p38 isoform-specific gene inactivation in mice.

4.1.1. p38α

p38α is the most abundant expressed isoform, and is ubiquitously expressed in all cell types (Nishida et al. 2004). The many functions ascribed to the p38 signaling cascade using pyridinyl imidazole inhibitors implicate p38α as a major regulator of cell motility, cell cycle regulation, and differentiation, since p38β is much less abundantly expressed than p38α. Total knockout of p38α in mice causes embryonic lethality due to developmental defects in placental organogenesis and stress-induced erythropoiesis (Adams et al. 2000; Aouadi et al. 2006; Tamura et al. 2000). Interestingly, when embryonic $p38\alpha^{null}$ mice are transplanted into wild-

type placentas, the developmental defect is rescued, and embryos develop to full gestational term and are normal in appearance (Adams et al. 2000). However, when p38α is specifically deleted from the mouse embryo (and not the placenta), foetuses develop to term, but die shortly after birth due to lung defects (Hui et al. 2007a). This suggests multiple roles for p38 at different stages of murine development.

Due to the many developmental defects seen in p38α^{null} mice, the exact cellular functions of p38α remained largely unknown. However, accumulating evidence suggests the normal function of p38α is a tumour suppressor (Cuenda and Rousseau 2007; Hui et al. 2007b). For example, fetal hematopoietic cells and mouse embryonic fibroblasts (MEFs) from p38α^{null} mice have increased proliferation and tumour development due to enhanced activation of the JNK/c-Jun pathway (Hui et al. 2007a). In cardiomyocytes, p38α overexpression abrogates cell proliferation, whereas a DN-p38α promotes cell division (Engel et al. 2005). Furthermore, cardiomyocyte-specific p38α knockout mice show a robust increase in cell proliferation (Engel et al. 2005).

More strikingly, lack of p38 activity is associated with increased tumourogenesis *in vivo*. For example, natural lack of p38 activity causes uncontrolled proliferation in a childhood solid muscle tumour known as rhabdomyosarcoma (Puri et al. 2000). Restoring p38 activity in rhabdomyosarcoma-derived cells using CA-MKK6 arrests proliferation and restores muscle cell differentiation (Puri et al. 2000). Furthermore, reduced p38 activity has been observed in other human solid tumours including breast, liver, lung, colorectal, and in malignant lymphoma and Schwannnoma (Liao and Hung 2003).

4.1.2. p38β

p38β is a widely expressed, but low abundant p38 isoform that is 75% homologous to p38α (Cuenda and Rousseau 2007). p38β gene deletion from mice causes no phenotypic differences or health defects (Beardmore et al. 2005). In p38β^{null} MEFs, expression and activation of p38α, ERK1/2 and JNK is not affected in response to anisomycin, a stimulus which specifically activates the p38

pathway (Beardmore et al. 2005). In addition, p38β^{null} MEFs have no alterations in activation of the p38 substrates MK2 or MSK1, and no effects on transcription of p38-dependent immediate early genes. In addition, p38β^{null} mice have normal T-cell development, and LPS-induced cytokine production is unaffected (Beardmore et al. 2005).

In order to study the roles of p38β in inflammatory disease progression in a mouse model of RA and IBD, p38β^{null} mice can be crossed to mice over-expressing TNFα (Kontoyiannis et al. 1999). In these animals, inflammatory response and disease progression is not altered which suggests p38α is the major regulator of the immune response in animal models of inflammation (Beardmore et al. 2005). Considering the functional redundancies between p38α and p38β, some distinct roles for p38β have been determined. For example, during keratinocyte differentiation down-regulation of the E2F1 transcription factor is regulated by p38β-induced activation of specific protein kinase C (PKC) isozymes (Ivanova et al. 2006). In murine C2C12 myoblasts, p38β also specifically phosphorylates glycogen synthase (Kuma et al. 2004).

4.1.3. p38γ

p38γ is most abundantly expressed in skeletal muscle where its functions are best understood (Li et al. 1996; Mertens et al. 1996). Specific stimuli/activation cues appear to regulate p38γ more than other p38 isoforms. For example, p38γ is rapidly and strongly activated in response to hyperosmotic shock, and its expression levels are regulated by muscle contraction (Goedert et al. 1997; Sabio et al. 2004). In response to muscle contraction, p38γ controls glucose uptake by regulating expression of the GLUT4 transporter (Ho et al. 2004). Moreover, p38γ plays roles in the differentiation of myoblasts into mature myotubes (Lechner et al. 1996). This is supported by the observation that p38γ over-expression enhances C2C12 muscle cell differentiation, whereas an inactive p38γ mutant inhibits C2C12 cell differentiation (Lechner et al. 1996). However, when the p38γ gene is deleted from mice, no phenotype or developmental defects in muscle are observed, suggesting compensation from other p38 isoforms (Sabio et

al. 2005). Furthermore, double knockout of both p38γ and p38δ results in no phenotypic alterations, suggesting that p38α and p38β can compensate for p38γ function in muscle development (Sabio et al. 2005).

A unique feature of p38γ is a PDZ-binding motif in its C-terminus, which can serve to dock other substrates containing this domain (Sabio et al. 2004). In this manner, p38γ interacts with a1-syntrophin (Hasegawa et al. 1999), SAP90/PSD95 (Sabio et al. 2004), SAP97/hDlg (Sabio et al. 2005), Lin-7C, Scribble, and outermembrane protein 25 (OMP25) (Court et al. 2005). These p38γ-PDZ-domain protein interactions can result in p38γ localization to the mitochondrial membrane (Court et al. 2005), gap or neuromuscular junctions (Cuenda and Rousseau 2007; Hasegawa et al. 1999), or neuronal synapses (Sabio et al. 2004). Interestingly, not all p38γ-PDZ domain interacting partners contain a p38 consensus phosphorylation sequence, suggesting p38γ can regulate protein scaffolding independent of its kinase function.

4.1.4. p38δ

The functions of p38δ remain much less understood than other p38 isoforms, although it appears to play an important role in keratinocyte survival, differentiation and senescence (Efimova 2010). p38α, β and δ are the only isoforms expressed in keratinocytes (Dashti et al. 2001), and p38δ appears to specifically regulate the aforementioned functions (Efimova et al. 2003; Efimova et al. 2004). Treatment of keratinocytes with okadaic acid, a protein phosphatase inhibitor which promotes differentiation, causes specific activation of p38δ, and decreases ERK1/2 activation. Interestingly, p38δ forms protein complexes with ERK1/2, and suppresses ERK1/2 activation (Efimova et al. 2003). This suggests that crosstalk between MAPK pathways can regulate keratinocyte differentiation. During keratinocyte development, elevated p38δ activity is associated with increased binding of AP1 and CAATT enhancer binding protein (C/EBP) to the promoter of human involucrin (hINV) (Efimova et al. 2003), a marker of differentiated keratinocytes (Murphy et al. 1984). The unique role of p38δ in hINV expression is substantiated by the finding that a DN-p38δ, and not SB203580, abrogates hINV induction (Efimova et al. 2003). In spite of the role

of p38δ in keratinocyte development *in vitro*, p38δ knockout mice show no obvious phenotypic differences and are fertile (Sabio et al. 2005). However, p38δ knockout mice subjected to chemical carcinogens show increased resistance to mutagen-induced skin papillomas (Schindler et al. 2009), suggesting p38δ normally promotes cell proliferation in the epidermis.

p38δ also regulates cytoskeletal remodeling through phosphorylation of stathmin (Parker et al. 1998), and tau (Feijoo et al. 2005), two proteins involved in microtubule assembly. Moreover, in human epithelial KB cells, p38δ phosphorylates eukaryotic elongation factor 2 (eEF2) suggesting this kinase participates in the regulation of translation (Knebel et al. 2002).

4.2. p38 upstream activators

The p38 signaling cascade is initiated by numerous stimuli such as cytokines, growth factors, stress and hyperosmotic shock. Activation of p38 is triggered by a MAPK-kinase kinase (MKKK)-MKK3/6 module, which in turn activates specific p38 isoforms. A diverse number of receptors are coupled to the MKKK-MKK3/6-p38 signaling cascade, including GPCRs, toll-like receptors (TLRs), T-cell receptors (TCRs), interleukin receptors, and growth factor receptors (reviewed by (Ono and Han 2000; Zarubin and Han 2005)). Furthermore, p38 activation is not only dependent on stimulus, but also on cell type, since the same stimuli do not activate p38 to a similar manner in all cells (Zarubin and Han 2005). In addition, a MKKK-MKK3/6-independent mechanism of p38 activation exists through an auto-phosphorylation cascade (Ashwell 2006; Ge et al. 2002; Salvador et al. 2005). Reviewing the many stimuli and receptors coupled to the p38 pathway are beyond the scope of this thesis; however, the direct upstream activators responsible for initiating the p38 cascade are covered below.

4.2.2. Mitogen activated protein kinase kinase kinase (MKKKs / MAP3Ks)

The mitogen-activated protein kinase kinase kinases (MKKKs) are a diverse family of Ser/Thr kinases which are shared between some, or all of the MAPK sub-family members (reviewed by (Craig et al. 2008)). The MKKKs that have

been reported to activate p38 signaling include ASK1, TAK1, TAO kinase, MLKs, and MEKKK3/4. Many of the MKKKs responsible for activating the p38 pathway play roles in stress-response, apoptosis, growth and development. Some of the functions of the MKKKs responsible for activating the p38 cascade are reviewed below.

Apoptosis signal-regulating kinase 1 (ASK1) is responsible for the activation of MKK4/7 and MKK3/6, which activate JNK and p38, respectively (Matsukawa et al. 2004). ASK1 is activated in cells treated with death receptor ligands (e.g., TNFα, or Fas ligand), H_2O_2, chemotherapy drugs, or by growth factor deprivation (Matsukawa et al. 2004). Other means of ASK1 activation include endoplasmic reticulum (ER) stress, calcium signaling and GPCR signaling (McDonald et al. 2000; Nishitoh et al. 2002; Takeda et al. 2004). Furthermore, depending on the stimulus and cell type, ASK1 over-expression or CA-ASK1 can cause apoptosis, survival, or differentiation (Matsukawa et al. 2004). For example, ASK1 can induce PC12 cell survival and neurite outgrowth, promote keratinocyte differentiation, or induce neuronal cell death in response to nitric oxide or ER stress (Han et al. 2001; Nishitoh et al. 2002; Sayama et al. 2001; Takeda et al. 2000). In addition, forced expression of ASK1 in neural stem cells increases Mash1 expression resulting in neural differentiation, while potently and irreversibly inhibiting astrocyte development, even in the presence of astroglial-inducers such as BMP6 and leukemia inhibitory factor (LIF) (Faigle et al. 2004). Considering this, the physiological roles of ASK1 *in vivo* appear to be less important, since $ASK1^{null}$ mice have no overt phenotype; however, MEFs isolated from these animals are resistant to H_2O_2-induced apoptosis (Tobiume et al. 2001).

TGFβ activated kinase-1 (TAK1) is another MKKK upstream of both MKK3/6 and MKK4/7, and therefore can result in p38 and/or JNK activation (Craig et al. 2008). TAK1 was originally identified as a downstream effector of TGFβ, a cytokine family that plays essential roles in cell proliferation and differentiation during embryonic development (Craig et al. 2008; Delaney and Mlodzik 2006). In addition, TAK1 is downstream of TNFα and toll-like receptors (TLRs), where

it promotes either cell survival by caspase modulation, or pro-inflammatory response through modulation of NFκB activity (Sakurai et al. 1998). *In vivo*, TAK1 appears to play important roles in murine embryonic development and tissue homeostasis, since total mouse knockout of TAK1 in mice results in embryonic lethality due to vascular defects, absence of vascular smooth muscle, and overall delayed growth (Jadrich et al. 2006). Moreover, time-specific knockout of TAK1 from adult mice causes bone marrow and liver failure due to increased apoptosis of hematopoietic cells and hepatocytes (Tang et al. 2008b).

Another family of MKKKs are the mixed lineage kinases (MLKs), which are comprised of five family members (MLK1, 2, 3, 4, 7), and are activated in response to cellular stress. MLK1/2 double knockouts and $MLK3^{null}$ mice develop normally with no phenotypic differences, suggesting redundant roles of these kinases in growth and development (Bisson et al. 2008; Brancho et al. 2005). Other MKKKs include MEKK3, MTK1/MEKK4 and thousand and one (TAO) kinase. MEKK3 appears to be a master regulator for developmental epithelial to mesenchymal transition in the heart (Craig et al. 2008). $MEKK3^{null}$ mice die *in utero* from abnormalities in embryo and yolk sac vasculature, and angiogenesis-defects due to increased endothelial cell apoptosis (Deng et al. 2007). The related kinase, MEKK4 regulates activation of p38, JNK, ERK1/2, BMK and NFκB through many intermediary MKKs. p38 and JNK pathways are activated by MEKK4 in response to osmotic shock and other stressful stimuli, but not by inflammatory cytokines, suggesting distinct stimuli can differentially regulate MKKK activation (Takekawa et al. 1997). In mice where MEKK4 is deleted or functionally inactivated, animals die shortly after birth due to neural tube and skeletal malformations (Abell et al. 2005; Chi et al. 2005). The TAO kinase family is comprised of three isoforms, which are implicated in cell survival, cytoskeletal remodeling and GPCR signaling. TAO activation is primarily responsible for activating MKK3 in response to cellular stress and DNA damage. TAO kinase is activated through a pathway involving ATM (ataxia telangiectasia mutated) kinase which signals to p38 in response to DNA damage to establish a G2/M cell cycle checkpoint (see below) (Raman et al. 2007).

4.2.3. Mitogen activated protein kinase kinase (MKK3/MKK6)

Mitogen activated protein kinase kinases (MKKs) are dual-specificity kinases responsible for the phosphorylation of tyrosine and threonine residues in ERK, p38, JNK and BMKs (Johnson and Lapadat 2002). The two upstream kinases of p38 are MKK3 and MKK6, which share 80% sequence homology (Enslen et al. 1998; Moriguchi et al. 1996; Stein et al. 1996). Accumulating evidence suggests that these MKKs are differentially activated by distinct stimuli, and that they can differentially regulate the p38 isoforms. For example, MKK6 more effectively activates p38β than MKK3 (Keesler et al. 1998).

MKK3 and 6 also play important roles in inflammatory responses. In human synovial tissue, higher levels of activated MKK3/6 are seen in RA synovium than osteoarthritic synovial fluid (Chabaud-Riou and Firestein 2004), indicating these kinases are specifically activated during inflammatory disease. Transgenic mouse knockout of either MKK3 or MKK6 does not affect viability, and produces no overt phenotypic defects (Tanaka et al. 2002; Wysk et al. 1999), implicating functional redundancy between these kinases *in vivo*. Mice compound deficient for both MKK3 and MKK6 die *in utero*, due to major placental defects and deficiencies in embryonic vasculature (Brancho et al. 2003). These defects are almost identical to those observed in $p38\alpha^{null}$ mice, further substantiating the roles for MKK3/6 in p38 activation. Furthermore, MKK3/6 double knockout MEFs exhibit increased proliferation, similar to $p38\alpha^{null}$ MEFs (Brancho et al. 2003). In addition, $MKK3^{null}$ MEFs show reduced p38 activation and lower levels of inflammatory cytokine expression (i.e., IL-1α/β, IL-6, and TNFα) in response to TNFα, suggesting defects in the inflammatory mediated response *in vitro* (Wysk et al. 1999). In $MKK3^{null}$ synoviocytes, TNFα-induced activation of p38 and expression of IL-1β and IL-6 are also reduced (Inoue et al. 2006). In addition, the TNFα-, but not LPS-, induced expression of IL-6 is NFκB-dependent and requires MKK3 activity, suggesting distinct stimuli differentially regulate p38 upstream activity (Inoue et al. 2006). Also, $MKK6^{null}$ T cells are more resistant to TCR-induced cell apoptosis (Tanaka et al. 2002).

In an experimental model of arthritis, MKK3^{null} mice show decreased arthritic severity and reduced synovial inflammation due to decreased levels of activated p38 and MK2 and lower levels of IL-1β, CXC ligand 1, IL-6 and MMP3 (Inoue et al. 2006). In addition, in an LPS-induced model of systemic inflammation, IL-6 production was similar in wild-type and MKK3^{null} animals indicating the toll-like receptor 4 (TLR4)-mediated host-defence system is independent of MKK3 function (Inoue et al. 2006). Some distinct functions for MKK3 and MKK6 have been elucidated using T cells cultured from these mice. For example, MKK3^{null}, but not MKK6^{null} T cells show reduced susceptibility to TCR-induced cell death as a result of decreased p38 activity (Tanaka et al. 2002), suggesting differential regulation of p38 by MKK3 and MKK6 in T cells (Kuida and Boucher 2004).

Considering the aforementioned evidence for MKK3/6 regulation of p38 activity, over-expression studies have revealed activation of p38δ by MKK4, an upstream activator of JNK (Jiang et al. 1997). Furthermore, in the absence of MKK3 and 6, MKK4 can induce p38 phosphorylation in response to UV light (Brancho et al. 2003). This is most likely due to compensatory mechanisms, as the MKK4-induced phosphorylation of p38 has not been observed *in vivo.*

Interestingly, p38 can also regulate the expression levels of its upstream activators which provides a further mechanism to control p38 activation. Namely, the upregulation of MKK6 protein and mRNA is observed when p38α is pharmacologically inhibited, or when p38α is deleted from cardiomyocytes. Mechanistically, p38α regulates MKK6 expression through a mRNA stability mechanism that normally decreases the transcript half-life of MKK6 (Ambrosino et al. 2003).

4.3. p38 MAPK downstream effectors

p38 MAPK signals to a large number of downstream effectors including kinases, phosphatases, transcription factors, cell cycle regulators and chromatin modifying enzymes. Some of the cellular targets include MK2, MK3, MK5, MSK1/2, and MNK1/2, which mediate some of the p38-regulated functions including cell migration, proliferation and differentiation. The p38 signaling cascade can activate numerous other targets including transcription factors, cell cycle

regulators and ion pumps, among others (Figure 7) (reviewed by (Cuenda and Rousseau 2007; Shi and Gaestel 2002; Zarubin and Han 2005)). Many of these factors are involved in the regulation of genes encoding other transcription factors, cell cycle regulators, inflammatory cytokines, and cell-surface receptors (Ambrosino et al. 2003). The function of these many targets is beyond the scope of this thesis; however, the roles of the well described downstream kinases (e.g., MK2, MK3, MK5, MNK, MSKs), the small heat shock protein 27 (hsp27) and cAMP response element binding protein (CREB) are reviewed below.

4.3.1. Mitogen activated protein kinase activated protein kinase 2 (MAPKAPK2 / MK2)

Mitogen activated protein kinase activated protein kinase 2 (MK2) was originally isolated from skeletal muscle as an enzyme phosphorylated by ERK *in vitro* (Stokoe et al. 1992); however, p38α/β appear to specifically activate it both *in vitro* and *in vivo*. One of the major cellular targets phosphorylated by MK2 is the small heat shock protein 27 (hsp27). MK2 plays roles in cytoskeletal and chromatin remodeling, activation of cell cycle regulators, and inflammation (Gaestel 2006). For example, MK2 phosphorylates the cell cycle checkpoint protein phosphatases Cdc25-B1, -2, and -C (Manke et al. 2005). MK2 also regulates the p53- dependent checkpoint (Gaestel 2006), and interacts with the cell cycle protein Plk1 (polo-like kinase 1) at spindle poles during prophase and metaphase (Tang et al. 2008a).

In addition, MK2 regulates the differentiation of trophoblast stem cells (Winger et al. 2007) and myofibroblasts *in vitro* and *in vivo* (Hagood and Olman 2007; Sousa et al. 2007). $MK2^{null}$ MEFs contain less actin stress fibers, and have lower levels of α-smooth muscle actin, a marker of differentiated myofibroblasts (Sousa et al. 2007). The reduced differentiation of $MK2^{null}$ myofibroblasts is due to reduced activation of serum response factor (SRF), which normally activates the α-smooth muscle actin gene, a marker of differentiated myofibroblasts (Heidenreich et al. 1999). Targeted disruption of MK2 in mice causes no phenotypic differences; however, animals show increased resistance to stress, and have a decreased rate of death following LPS-induced endotoxic shock

(Kotlyarov et al. 1999). Furthermore, fibroblasts from MK2^{null} mice have significantly reduced hsp27 phosphorylation in response to stress, confirming that MK2 normally activates this cytoskeletal modulating protein. In MK2/3^{null} neutrophils, cell motility is increased, whereas their directionality is lost (Hannigan et al. 2001).

In addition to the roles of MK2 in cell cycle, differentiation and motility it may also play roles in neuronal function. In the PC12 and other neuronal cell lines, MK2 is the primary response gene induced by depolarization with KCl or forskolin (Thomas et al. 2008a; Vician et al. 2004). The roles for MK2 in response to membrane depolarization are unknown, although it may be related to a stress response. Indeed, MK2 mRNA levels are elevated following kainic acid induced seizures in rat (Vician et al. 2004). However, when kainic acid is administered to MK2^{null} mice, reduced cell death and neurodegeneration are seen, suggesting normal roles for MK2 in excitotoxicity (Thomas et al. 2008a). Furthermore, MK2^{null} mice have increased protection to ischemic injury, and enhanced protection to inflammatory cytokine-induced neuronal cell death (Thomas et al. 2008b; Wang et al. 2002).

4.3.2. Mitogen activated protein kinase activated protein kinase 3 (MK3 / MAPKAPK3 / 3pk)

MK3 shares a high degree of sequence homology (75% in humans) to MK2 (Ronkina et al. 2008), and may share many of the functions of MK2; although some distinct roles for MK3 do exist (Aberg et al. 2006). MK3 protein partner interaction studies have shown that it binds human polyhomeotic protein 2 (HPH2), a component of the polycomb repressive complex 1 (PRC1) which is involved in maintenance of chromatin structure and stable gene silencing (Levine et al. 2004). MK3 also regulates chromatin remodeling through via phosphorylation of the polycomb group (PcG) protein Bmi1 (Voncken et al. 2005). In addition, in MK2^{null} MEFs the residual hsp27 phosphorylation, and p38-dependent cytokine synthesis seen are attributed to MK3 activity (Aberg et al. 2006; Kotlyarov et al. 2002).

4.3.3. Heat shock protein 27 (Hsp27)

Small heat shock protein 25 (human) / 27 (rodents) is a non-ATP-dependent chaperone involved in cytoskeletal remodeling, and cell motility, growth and differentiation (Kostenko and Moens 2009). Both MK2 and MK3 are primarily responsible for hsp27 activation, which affects its cytoskeletal rearrangement properties. The non-phosphorylated form of hsp27 inhibits actin polymerization *in vitro*, whereas the phosphorylated form loses this inhibitory activity (Benndorf et al. 1994; Miron et al. 1991). Hsp27 is critical for the migration of smooth muscle and endothelial cells (Hedges et al. 1999; Rousseau et al. 1997). In addition, hsp27 promotes growth-factor induced actin stress fiber formation, whereas a non-phosphorylatable hsp27 mutant inhibits actin stress-fiber formation (Lavoie et al. 1993). Hsp27 ablation in *Xenopus* causes improper heart tube formation (Brown et al. 2007), and mouse knockout of hsp27 causes embryonic lethality due to extensive embryonic stem cell apoptosis during early differentiation (Arrigo et al. 2005; Mehlen et al. 1997).

4.3.4. Mitogen activated protein kinase activated protein kinase 5 (MK5 / PRAK)

MK5, or p38-regulated and -activated protein kinase (PRAK), was originally identified as a kinase activated by both p38 and ERK, but not JNK (New et al. 1998; Ni et al. 1998). MK5 has a wide distribution and is 45% and 46% homologous with MK2 and MK3, respectively (Ni et al. 1998). MK5 differs from MK2/3 due to its preferential activation by p38β *in vitro* (New et al. 1998). However, *in vivo,* MK5 may be activated independently of p38 altogether (Shiryaev and Moens 2010). This is supported by the observation that stimuli that induce p38 activity do not induce MK5 activation, and no binding interaction, or chaperoning properties between p38 and MK5 have been detected (Shi, et al., 2003). Indeed, MK5 activity may be regulated by ERK3, an atypical MAPK related to ERK2. This is supported by the observation that ERK3 and MK5 form protein complexes, and their activities positively correlate during PC12 cell differentiation (Seternes et al. 2004).

The exact functions of MK5 are unknown, however it may play roles in cell senescence by regulating p53 (Sun et al. 2007; Yaswen and Campisi 2007). Furthermore, MK5 regulates murine embryonic development since knockout mice die *in utero*, although this observation appears to be mouse strain-specific (Gaestel 2006). In addition, $MK5^{null}$ mice have increased susceptibility to mutagen-induced skin carcinogenesis, supporting its roles in cell senescence (Sun et al. 2007).

4.3.5. Mitogen activated protein kinase-interacting kinases / Mitogen activated protein kinase signal-integrating kinases (MNKs)

The MAP kinase-interacting kinases (or MAP kinase signal-integrating kinases) (MNKs) are comprised of two forms MNK1 and MNK2. MNK1 was first identified in a yeast-two hybrid screen using ERK as bait; however it was later found that both ERK and p38α/β were responsible for its phosphorylation (Fukunaga and Hunter 1997; Waskiewicz et al. 1997). Activated MNKs phosphorylate a number of cellular targets involved in RNA binding, lipid intermediate formation, and translation, including eukaryotic initiation factor 4E (eIF4E), cytoplasmic phospholipase A2 (cPLA2), and hnRNP A1 (Waskiewicz et al. 1997). The essential role of MNK1/2 in eIF4E phosphorylation is clearly demonstrated in $MNK1/2^{null}$ mice, since no activation of eIF4E is observed after LPS-induction of the p38 cascade (Ueda et al. 2004). MNK1/2 knockout mice are viable and develop normally with no apparent phenotypic defects, or alterations in protein synthesis or cap-dependent translation (Ueda et al. 2004).

4.3.6. Mitogen and Stress Activated Protein Kinase (MSK)

Mitogen and stress activated protein kinase (MSK) 1 and 2 are serine-threonine protein kinases which are downstream of both ERK1/2 and p38 MAPK pathways. The two MSK isoforms are 64% homologous and are related to the 90 kDa ribosomal S6 kinases (RSKs). Both p38α and p38β can phosphorylate MSK1 (Deak et al. 1998). However, MEFs cultured from p38α and p38β knockout mice

showed that only the p38α isoform is responsible for MSK1 activation *in vitro* (Beardmore et al. 2005; Darragh et al. 2005). Substrates of activated MSK1/2 include transcription factors such as NFκB, CREB, and HMGN1, as well as Histone H3 (Arthur 2008). Overexpression of MSK induces its nuclear localization, consistent with its functions in transcription factor activation (Arthur 2008). The lack of selective inhibitors to MSK1 and 2 has made it difficult to elucidate their functions, yet accumulating evidence suggests roles in synaptic plasticity (Arthur 2008). MSKs are responsible for regulating immediate early (IE) genes, while transcription of CREB-dependent IEs is compromised in MSK knockout mice. Stimuli that activate the p38 signaling pathway, such as UV light or anisomycin activate MSKs through p38 and not through ERK1/2. Other stimuli that induce MSK activation include IL-1, lysophosphatidic acid, endothelin-1, oxidative stress, exercise in rats, and light stimulation of the suprachiasmatic nucleus in murine brain (Butcher et al. 2005).

4.3.7. cAMP response element binding protein (CREB)

CREB is a member of the leucine zipper family of transcription factors that binds to the Ca^{2+}/cyclic AMP-response element (CRE) located in the promoter regions of target genes (Montminy et al. 1990; Sheng et al. 1991). CREB is ubiquitously expressed and plays important roles in cell growth, development, and survival (Johannessen et al. 2004; Lonze et al. 2002). In addition, CREB plays a crucial role in learning and memory in *Drosophila* and mice (Silva et al. 1998). CREB is phosphorylated at Ser^{133} by a number of activators including protein kinase A, RSK, MSK and p38 (Delghandi et al. 2005). Activated CREB associates with CREB binding protein (CBP), p300, or CRE modulator (CREM) to effect target gene activation or repression (Groussin and Bertherat 1998). The numerous functions of CREB are beyond the scope of this thesis; however its roles in OLGs are outlined below.

It is estimated that CREB and its coactivators regulate the transcription of ~4000 target genes in the human genome (Zhang et al. 2005). In OLGs, Opalin is

the only myelin-specific gene identified to date, with a CRE consensus sequence in its promoter (Aruga et al. 2007). This suggests that CREB regulates most myelin-specific genes indirectly. In OLGs, the roles for CREB appear to be dependent both on stimulus, and stage of cell development (Saini et al. 2005). The numerous stimuli that can induce CREB phosphorylation in OLGs include forskolin, dbcAMP, apotransferrin, sphingosine-1-phosphate, and neutrophin-3 (NT-3) (Bhat et al. 2007; Garcia et al. 2004; Saini et al. 2005). For example, in immature OLPs, NT-3 induces cell proliferation, whereas apotransferrin promotes cell cycle exit and differentiation, both in a CREB-dependent manner (Garcia et al. 2004; Johnson et al. 2000; Marta et al. 2002; Paez et al. 2004; Paez et al. 2006). In late OLPs, CREB stimulates MBP expression in response to cAMP-mediated pathways (Afshari et al. 2001; Sato-Bigbee and DeVries 1996), and astrocyte-derived endothelin-1 regulates CREB- and ERK- induced OLP migration and differentiation (Gadea et al. 2009). In addition, OLG nuclear thromboxane receptor activation increases OLG survival and stimulates CREB phosphorylation and MBP transcription (Mir and Le Breton 2008). Antisense knockdown of CREB in OLGs results in MBP^+ cells with shorter and less complex processes, and cell soma-restricted MBP immunoreactivity (Sato-Bigbee and DeVries 1996). Moreover, during OLG differentiation, protein kinase C-, ERK- and p38-dependent increases in CREB phosphorylation have been observed (Bhat et al. 2007; McNulty et al. 2001; Shiga et al. 2005).

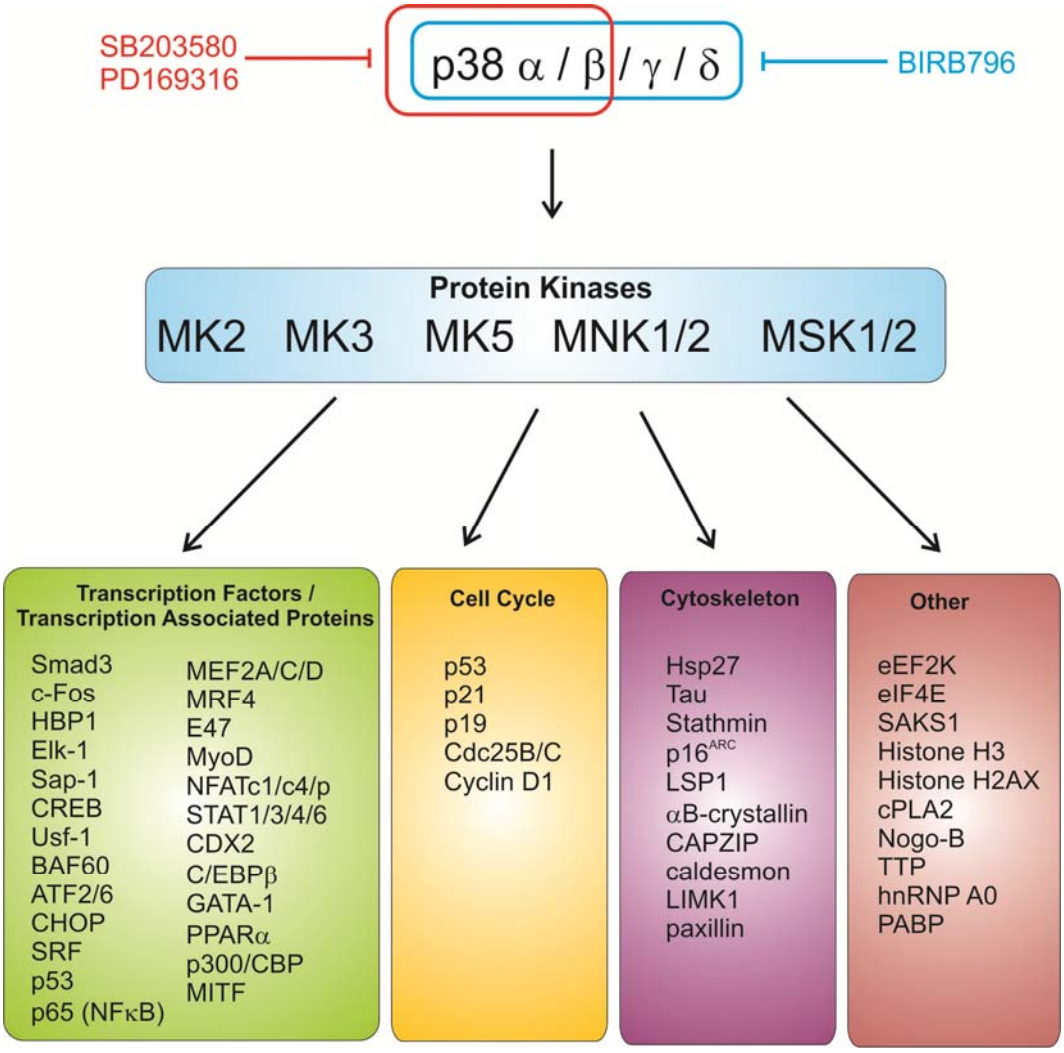

Figure 7. Activated p38 MAPK isoforms signal to many downstream kinases and substrates including transcription factors, transcription associated proteins, cell cycle modifiers and cytoskeletal proteins. Adapted from (Lluis et al. 2006) with copyright permission from Elsevier Limited.

4.4. p38 inactivation - MAPK phosphatases

The four different MAPK signaling cascades are inactivated by phosphatases that dephosphorylate the Thr-*Xaa*-Tyr motif. The dual specificity phosphatase MAPK phosphatase-1 (MKP-1) was originally identified as a stress-responsive enzyme that dephosphorylated ERK (Hu et al. 2007), and was later found to inactivate p38 and JNK. Nine MKPs have been identified, and *in vitro* studies have shown that MKP-1, -4, and -5 can dephosphorylate p38α and p38β. Interestingly, MKPs are unable to inactivate p38γ or p38δ, suggesting differential regulation of p38 isoforms (Ono and Han 2000). Recently, protein tyrosine phosphatase H1 (PTPH1), a phosphatase that inactivates p38γ has been identified through PDZ-domain interaction studies (Hou et al. 2010). In addition, ceramide-activated protein phosphatases (PP1 / PP2A) may inactivate p38δ (Kitatani et al. 2009).

Transient over-expression of MKP-1 in leukocytes prevents LPS-induced TNFα expression (Nimah et al. 2005). p38 and MK2 can also regulate MKP-1 expression level through a post-transcriptional mechanism. Furthermore, MKP-1 protein expression is blocked when mouse macrophages are treated with SB203580, or when MK2 is knocked down (Hu et al. 2007). Furthermore, p38 regulates the expression of other phosphatases, for example, Ser/Thr phosphatase PP2C and protein tyrosine phosphatase (PTP) (Takekawa et al. 2000; Takekawa et al. 1998). Another phosphatase, PP2Cα is able to inactivate MKK6 and MKK4 suggesting different phosphatases regulate p38 signaling at different levels of the cascade. A MAPK phosphatase independent mechanism of p38 inactivation also exists. Namely, GPCR kinase 2 (GRK2) can phosphorylate the p38 docking groove resulting in p38-GRK2 complex formation, which sterically blocks MKK6 access to p38 (Peregrin et al. 2006).

5.0. p38 mechanisms

The p38 MAPK pathway was originally identified as a stress-induced kinase in response to stress and inflammatory cytokines; however, novel functions for this kinase family were soon realized using pharmacological inhibitors and gene

ablation approaches. Some of these other functions include: 1) cytoskeleton remodeling, 2) regulation of mRNA transcript stability, 3) cell cycle control and proliferation 4) differentiation, and 5) regulation of chromatin remodeling. These mechanisms are further discussed below.

5.1. p38 and cytoskeletal remodeling

p38 can affect cytoskeletal remodeling, cell motility, cancer cell metastasis, angiogenesis, and invasion (for reviews see (Cuenda and Rousseau 2007) and (Huang et al. 2004)). For example, p38 mediates endothelial cell, mast cell, neutrophil, and vascular smooth muscle cell migration in response to numerous chemokines, cytokines and chemotactic agents (e.g., VEGF, PDGF, TGFβ, and IL-1β, among others) (Hedges et al. 1999; Heit et al. 2002; Heuertz et al. 1999; Ishizuka et al. 2001; Rousseau et al. 1997). However, p38-mediated regulation of cell migration is cell- and stimulus-type specific, or in other cases, p38 may not be involved at all. For example, the $PDGF_{AA}$ induced migration of OLPs is ERK-, but not p38- dependent (Frost et al. 2009).

In rat Schwann cells treated with SB203580 or PD169316, cells failed to align with axons, a crucial step in the initiation of PNS myelination. Accordingly, SB203580-treated cultures had reduced levels of phosphorylated hsp27 accounting for reduced Schwann cell migration/alignment (Fragoso et al. 2003). In addition to the roles of hsp27 in cytoskeletal rearrangement and migration, p38 and MK2 can activate other cytoskeletal proteins involved in cell shape changes and motility. For example, MK2 can phosphorylate the p16-Arc subunit of actin related protein 2/3 (ARP2/3) (Singh et al. 2003), an actin filament nucleator (Dominguez 2009). Furthermore, MK2 can regulate intermediate filament, microtubule and actin organization through phosphorylation of lymphocyte-specific protein 1 (LSP1) (Huang et al. 1997), vimentin (Cheng and Lai 1998), and αB-crystallin (Kato et al. 1998). MK2 also phosphorylates CapZ-interacting protein (CAPZIP), which interacts with the actin capping protein CapZ (Eyers et al. 2005). In smooth muscle cells the actin- and myosin-binding protein, caldesmon, is phosphorylated in a p38-dependent manner following urokinase plasminogen activator (uPa) stimulation (Goncharova et al. 2002). In nerve

growth factor (NGF)-stimulated PC12 cells, altered focal adhesion linkages between the extracellular matrix and cytoskeleton are mediated through a p38-dependent phosphorylation of paxillin (Huang et al.). In VEGF-stimulated endothelial cells, MK2 phosphorylates LIM domain kinase 1 (LIMK1), a cytoskeletal modulator responsible for cofilin phosphorylation that results in actin depolymerization (Kobayashi et al. 2006). p38 activity can also regulate cell motility and invasion by regulating matrix metalloproteinase (MMP)-1, -9 (Simon et al. 1998; Woo et al. 2004), and -13 (Johansson et al. 2000) expression, resulting in extracellular matrix remodeling.

5.2. p38 and mRNA stability

Regulation of mRNA stability is a mechanism that control the proper expression levels of certain gene transcripts (Wilusz and Wilusz 2004). mRNAs containing an adenine/uridine (AU)-rich repeat in the 3' untranslated region (UTR) are candidates for regulated mRNA turnover (Bakheet et al. 2003; Wilusz and Wilusz 2004). Over 900 genes contain AREs in their 3'UTR (Bakheet et al. 2001), and many of these gene transcripts have been confirmed to be controlled by an mRNA stability mechanism. The many mRNAs regulated by this mechanism include inflammatory cytokines (TNFα and many ILs), c-*fos* and c-*myc* (Chen et al. 1994; Winzen et al. 1999), cyclin B1, cyclin A, and p21 (Wang et al. 2000a; Wang et al. 2000b).

A function of p38 in mRNA stability was first described for cyclooxygenase-2 (COX-2), an enzyme that produces the inflammatory mediator prostaglandin H2 (Ridley et al. 1998). Treatment of HeLa cells with SB203580, or DN-MK2 causes destabilization of COX-2 mRNA resulting in its rapid degradation (Dean et al. 1999; Lasa et al. 2000). Other p38-regulated mRNA targets include TNFα, IL-6 (Neininger et al. 2002), IL-24 (Guo et al. 2008), IL-10 (Tudor et al. 2009), p21 (Lafarga et al. 2009), MMP2, and MMP9 (Kumar et al. 2010). mRNA transcript stability also occurs *in vivo*. Specifically, $MK2^{null}$ mice have reduced levels of TNFα and IL-6 due to reduced transcript stability (Kotlyarov et al. 1999; Neininger et al. 2002). In addition, mice engineered with

a gene deletion of the TNFα ARE become irresponsive to LPS-stimulated induction of TNFα (Kontoyiannis et al. 1999).

Moreover, a p38-dependent mechanism of transcription factor stability has been discovered in chondrocytes. In this manner, p38 positively regulates the stability of mRNA transcripts encoding Sox9, a major regulator of chondrocyte differentiation (Tew and Hardingham 2006; Wang et al. 2004).

The exact mechanisms of p38-mediated regulation of transcripts stability is still under investigation (Wilusz and Wilusz 2004). AREs are binding sites for many factors including tristetraprolin (TTP), hnRNP A0 (Rousseau et al. 2002), poly (A) binding protein PABP1 (Bollig et al. 2003), KSRP (Briata et al. 2005), and hnRNP A1 (Buxade et al. 2005). p38 and/or MK2 can directly phosphorylate some of these factors (e.g., TTP, hnRNP A0, PABP) which promotes their ability to stabilize ARE containing transcripts (Wilusz and Wilusz 2004).

5.3. p38 and cell cycle control, proliferation, senescence

The control of proper cell division is maintained by cell cycle checkpoints to ensure high quality undamaged DNA is replicated and passed onto daughter cells. p38 plays roles in the G2/M and G1/S checkpoints in response to DNA damage by regulating the activity of cyclins, CDKIs, phosphatases, and the major cell cycle regulator p53 (reviewed by (Thornton and Rincon 2009)).

5.3.1. G2/M checkpoint

Exposure of cells to γ-irradiation, UV light or chemotherapeutic drugs can induce DNA strand breaks which causes a G2/M delay in order to repair damaged DNA (Bulavin et al. 2002; Mikhailov et al. 2004). Following DNA damage p38 is activated and accumulates in the nucleus. The mechanism of p38 activation in response to DNA damage is not entirely clear; however, one pathway may be through an ATM kinase-induced activation of TAO kinases (MKKKs), which activate MKK3/6 and subsequently p38 (Raman et al. 2007). Activated p38 then initiates the G2/M checkpoint by two mechanisms. First, p38 directly activates the tumour suppressor p53 (Wang et al. 2000c) a major regulator of the G2/M

checkpoint (Bulavin et al. 1999; Huang et al. 1999; She et al. 2001; She et al. 2000). The p38-induced activation of p53 results in p53-induced stabilization and activation of the G2/M target genes Gadd45α (growth arrest and DNA damage-inducible 45α), $p21^{Cip1}$, and 14-3-3 (el-Deiry et al. 1993; Hermeking et al. 1997; Zhan et al. 1999). These proteins promote the G2/M checkpoint by inactivating a cdc2/cyclin B complex which normally drives the G2/M transition. In addition, p38 directly phosphorylates Gadd45α, which promotes GADD45α-p53 association to induce activation of G2/M target genes (Bulavin et al. 2003; Jin et al. 2003). Second, p38 and/or MK2 can phosphorylate and inhibit Cdc25B phosphatase by promoting its association with 14-3-3 and subsequently inducing a G2 delay (Lemaire et al. 2006; Manke et al. 2005). The non-phosphorylated Cdc25B would normally promote G2/M cell cycle progression by activating cyclin B/cdc2 (Bulavin et al. 2001; Lopez-Girona et al. 1999; Morris et al. 2000).

Another study showed that p38 is activated when cells are arrested at the M phase by disrupting the mitotic spindle apparatus with nocodazole. Furthermore, injection of activated p38 into *Xenopus* induces an M-phase checkpoint, which is abrogated by SB203580 (Takenaka et al. 1998).

5.3.2. G1/S checkpoint

The G1/S transition controls passage from the 'gap' phase (G1) into the DNA synthesis (S) phase. A checkpoint is initiated between the G1 to S phase to ensure undamaged and intact DNA is replicated. Several mechanisms exist by which p38 can regulate G1/S checkpoint including p53, p21 and cyclin D1 (Polager and Ginsberg 2009). p38-induced activation of p53 results in accumulation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$, which induces cell cycle arrest at G1/S by inactivating cdk2. Furthermore p38 can phosphorylate $p21^{Cip1}$ leading to its stabilization and further inactivation of cdk2 (Kim et al. 2002; Kishi et al. 2001). In addition, p38 signaling normally decreases cyclin D1 expression at the level of transcription (Lavoie et al. 1996), by promoting induction of HBP1, a factor which inhibits cyclin D1 gene expression and other gene targets involved in replication (Yao et al. 2005; Yee et al. 2004). Furthermore, p38 can directly phosphorylate cyclin D1, resulting in its ubiquitination and subsequent

degradation by the proteasome (Casanovas et al. 2000). Other mechanisms of p38-mediated G1/S checkpoint regulation include degradation of Cdc25A phosphatase (Goloudina et al. 2003), and upregulation of the CDKIs p16 ink4a, and p19ARF (Bulavin et al. 2004; Faust et al. 2005; Ito et al. 2006).

5.3.3. G1/G0 and cell cycle exit

In addition to the role of p38 in cell cycle checkpoints, it can also promote G1/G0 arrest by antagonizing the JNK/c-Jun pathway. The increased proliferation of $p38\alpha^{null}$ erythroblasts is correlated with increased JNK/c-Jun activity leading to sustained activation of cyclin D1 and cdc2. Moreover, the upregulation of cyclin D1 and cdc2 is abolished when $p38\alpha^{null}$ MEFs are treated with JNK inhibitors (Perdiguero et al. 2007). In contrast, mice lacking both c-Jun and p38α in hepatocytes have reduced liver tumour growth (Hui et al. 2007b). Furthermore, p38 activity is increased in regenerating livers from $c\text{-}Jun^{null}$ hepatocytes (Stepniak et al. 2006). Interestingly, the p38α regulation of proliferation appears to occur at different levels of the JNK signaling cascade. For example, the JNK kinases HKP1 and MKK7 are increased in $p38\alpha^{null}$ hematopoietic cells and MEFs, respectively (Hui et al. 2007a; Perdiguero et al. 2007). As a consequence of p38 abrogation of JNK/c-Jun activity, cells exit G1/G0 and can either remain quiescent or progress through a differentiation program.

5.4. p38 and differentiation

p38 regulates the differentiation of numerous cell types, including adipocytes (Bost et al. 2005), myocytes (Zetser et al. 1999), keratinocytes (Efimova et al. 2003), chondrocytes (Zhen et al. 2001), cardiomyocytes (Davidson and Morange 2000), erythroblasts (Di Giacomo et al. 2009), neurons (Iwasaki et al. 1999; Morooka and Nishida 1998), osteoclasts (Li et al. 2002), osteoblasts (Wang et al. 2007b), monocytes (Ayala et al. 2000), OLGs (Baron et al. 2000; Bhat et al. 2007; Fragoso et al. 2007; Haines et al. 2008) and Schwann cells (Fragoso et al. 2003). Notably, in many of these cell types, p38 inhibitors block the accumulation of differentiation stage markers. For example, keratinocytes fail to accumulate

hINV, whereas myocytes have reduced α-smooth muscle actin levels. Likewise, Schwann cell myelination is blocked by the p38 MAPK inhibitors SB203580 or PD169316, with reductions in P0, MBP and MAG (Fragoso et al. 2003). Similarly, treatment of OLPs with high concentrations of SB203580 reduces sulfatide levels and maintains cells in a bipolar state (Baron et al. 2000). We have also shown that p38 plays a crucial role in OLG differentiation and myelination of DRGNs (see chapters 2, 3, 4, 5 of this thesis).

Furthermore, CA- and DN- constructs of p38 (or MKK3/6) alone, or in combination with pyridinyl imidazole inhibitors have confirmed the important role for this kinase in differentiation. For instance, the insulin-induced differentiation of 3T3 L1 fibroblasts into adipocytes is blocked by SB203580 (Engelman et al. 1998), whereas CA-MKK6 induces adipocytic differentiation (Engelman et al. 1999). In PC12 cells, NGF-induced neuronal differentiation is blocked by SB203580, DN-MKK6, or DN-p38. In contrast, CA-MKK6 can induce PC12-neurite outgrowth in the absence of NGF, which is blocked by SB203580 (Morooka and Nishida 1998).

In these many cell types, it appears that p38 regulates no single mechanism to effect differentiation. For example, in adipocytes transcriptional targets of p38 include peroxisome proliferator-activated receptor γ (PPARγ), and C/EBPβ (Engelman et al. 1999; Engelman et al. 1998); whereas in osteoblasts, p38 regulates expression of osterix, a master regulator of bone cell differentiation (Wang et al. 2007b). The cellular system in which p38-regulated differentiation is best understood is myocytes. In myocytes, p38 regulates the activity of key muscle transcription factors, including MyoD, an essential transcription activator for myogenesis (for reviews see (Keren et al. 2006; Lluis et al. 2006)). Furthermore, differentiation of C2C12 myoblasts into mature myotubes is correlated with high p38 activity, and SB203580 reduces myoblast alignment and elongation (Cuenda and Cohen 1999). p38 also regulates expression of caveolin-3, a membrane protein necessary for myoblast fusion, a crucial step in myotube formation (Galbiati et al. 1999). In addition, differentiation of L8 myoblasts to multinucleated myotubes is prevented by SB203580, and correlates with reduced

expression of important regulators of myoblast differentiation including MEF2C, MyoD and myosin light chain 2 (Zetser et al. 1999). Mechanistically, p38 can directly phosphorylate MEF2C, a co-activator of the MyoD bHLH transcription factor (Zetser et al. 1999). Moreover, p38 phosphorylates E47, another bHLH transcription factor that heterodimerizes with MyoD to promote muscle-specific gene transcription (Lluis et al. 2005).

5.5. p38 and histone modifications/chromatin remodeling

Chromatin is comprised of DNA wound around core histone proteins H2A, H2B, H3, and H4, forming tightly packed DNA. Higher order chromatin structures are formed by the linker histone H1 and other chromatin proteins (Thoma et al. 1979). Core histones undergo extensive post-translational modifications including phosphorylation, methylation and acetylation that alter their DNA packing ability. In this manner, p38-induced activation of MSK can lead to histone H3 phosphorylation, resulting in chromatin relaxation, thus providing access to transcription factors, replication factors, or DNA repair enzymes (Zhao et al. 2008). In addition, p38 can phosphorylate the histone acetyltransferase coactivator CBP/p300, resulting in acetylation of protein targets including histone H3, or the p65 subunit of NFκB (Saha et al. 2007; Simone et al. 2004).

Multiple lines of evidence support the aforementioned conclusions. For example, shear stress induces a p38-dependent phosphorylation and acetylation of histone H3 in human umbilical vein endothelial cells (HUVECs) (Illi et al. 2003), and UV-induced phosphorylation of histone H3 is both ERK- and p38-, but not JNK-, dependent (Zhong et al. 2000). Other studies have shown that treatment of mouse JB6 cells with TSA increases histone H3 acetylation and phosphorylation through activation of ERK and p38 (Zhong et al. 2003). A report has also suggested that p38 can phosphorylate H2AX, a variant of the histone H2A family, in response to ionizing radiation, to repair DNA damage (Lu et al. 2008). Furthermore, in fibroblasts, forced activation of p38 (by CA-MKK6) reduces histone H1 protein levels, a necessary step in induction of cell senescence (Funayama et al. 2006).

In addition to stress, inflammatory cytokines can induce a p38-dependent phosphorylation and acetylation of histone H3 (Saccani et al. 2002). The p38-dependent phosphorylation of histone H3 at cytokine gene promoters relaxes chromatin, and allows increased access to NFκB to promote cytokine gene transcription (Saccani et al. 2002). Interestingly, the p38-dependent phosphoacetylation of histone H3 appears to be specific to cytokine genes, since this regulation is not seen at muscle-specific gene promoters (Simone et al. 2004).

p38 also regulates the activity of the multiprotein SWI-SNF chromatin remodeling complexes that regulate both the activation and repression of target genes. During muscle cell differentiation, p38 phosphorylates the BAF60 subunit of SWI-SNF, promoting recruitment of complexes to myogenic loci (Simone et al. 2004). In this context, blocking p38α/β activity represses the transcription of muscle specific genes by preventing SWI-SNF recruitment to myogenic loci (Simone et al. 2004). In contrast, forced activation of p38α/β in myoblasts using CA-MKK6 promotes early recruitment of SWI-SNF to the myogenin promoter. Furthermore, inactivation of SWI-SNF enzymatic subunits blocks the MKK6-dependent induction of myocyte gene expression, suggesting this effect is p38 pathway specific (Simone et al. 2004). In osteoclasts, NFATc1, PU.1, and microphthalmia-associated transcription factor (MITF) are three transcription factors that cooperate with the SWI-SNF chromatin-remodeling complex to regulate osteoclast gene promoters. p38 phosphorylates and activates MITF, a bHLH leucine zipper which regulates target genes of osteoclast differentiation (Luchin et al. 2000; Mansky et al. 2002; Motyckova et al. 2001; So et al. 2003; Weilbaecher et al. 2001). Treatment of bone marrow derived osteoclast precursors with factors that stimulate differentiation causes increased levels of phospho-MITF, phospho-p38 and SWI/SNF complexes to osteoclast-specific gene promoters where they execute chromatin remodeling and gene transcription (Mansky et al. 2002; Sharma et al. 2007; Weilbaecher et al. 2001).

Finally, MK2 and MK3 can regulate chromatin remodeling through polycomb group (PcG) proteins. PcGs form transcriptional complexes with chromatin to control gene activation/deactivation. PcGs comprise two subclasses,

polycomb-group repressive complex 1 and 2 (PRC1 and PRC2). PRCs are composed of numerous proteins which can be regulated by phosphorylation to effect PRC association with chromatin (Kerppola 2009). In this manner, MK3 associates with and phosphorylates Bmi1, a component of PRC1, resulting in PcG dissociation from chromatin and de-repression of target genes, including the cell cycle regulator $p14^{ARF}$ (Voncken et al. 2005). Furthermore, both MK2 and MK3 interact with HPH2 a component of PcGs (Voncken et al. 2005; Yannoni et al. 2004).

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Chapter 2: p38 mitogen-activated protein kinase is required for central nervous system myelination

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Abbreviations

BSA, bovine serum albumin

bFGF, basic fibroblast growth factor

BrdU, 2-bromodeoxyuridine

Caspr, Contactin associated protein

CGT, UDP galactose:ceramide galactosyltransferase

CNP, 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS, central nervous system

DAPI, 4,6-diamidino-2-phenylindole dihydrochloride

DMEM, Dulbecco's modified Eagle's medium

DTT, dithiothreitol

DRG, dorsal root ganglion

DRGN, DRG neuron

ERK, extracellular signal regulated kinase

GalC, galactosylceramide, galactosylcerebroside

FCS, fetal calf serum

HBSS, Hank's balanced salt solution

HSP27, heat shock protein 27

HRP, horseradish peroxidase

MAG, myelin-associated glycoprotein

MAPK, mitogen-activated protein kinase

MBP, myelin basic protein

MOBP, myelin-associated oligodendrocyte basic protein

NGF, nerve growth factor

NFH, neurofilament heavy-chain

OLG, oligodendrocyte

PBS, phosphate buffered saline

$PDGF_{AA}$, platelet derived growth factor AA

PFA, paraformaldehyde

PNS, peripheral myelination

SFM, serum-free medium

SDS-PAGE, SDS polyacrylamide gel electrophoresis

Abstract

The p38 MAPKs are a family of kinases that regulate a number of cellular functions including cell migration, proliferation, and differentiation. Here, we report that p38 regulates differentiation oligodendrocytes. Inhibition of p38 with PD169316 and SB203580 prevented accumulation of protein and mRNA of cell-stage specific markers characteristic of differentiated oligodendrocytes, including myelin basic protein, myelin-associated glycoprotein, and the glycosphingolipids, galactosylceramide and sulfatide. In addition, the cell cycle regulator $p27^{KIP}$ and the transcription factor Sox10 were also significantly reduced. Most significantly, p38 inhibitors completely and irreversibly blocked myelination of dorsal root ganglion neurons by oligodendrocytes and prevented the axolemmal organization of the axo-glial adhesion molecule Caspr. Our results suggest a role(s) for this kinase in key regulatory steps in the maturation of OLGs and initiation of myelination.

Introduction

The p38 mitogen-activated protein kinases (MAPKs) play critical functions in cellular differentiation. Classically, p38 MAPK was identified as a stress-activated protein kinase that regulates cellular response to various stimuli including inflammatory cytokines and UV irradiation (reviewed by (Ono and Han 2000; Zarubin and Han 2005). More recently, novel functions have been ascribed to p38, including the regulation of cell-cycle control, proliferation, survival, and differentiation (Nebreda and Porras 2000). Four p38 isoforms have been identified (α, β, γ, δ, (Jiang et al. 1996; Jiang et al. 1997; Lechner et al. 1996; Li et al. 1996) (Kumar et al. 1997), all of which are activated through phosphorylation on threonine and tyrosine residues in a TGY motif by upstream dual-specificity kinases (Hanks and Hunter 1995), MAPK kinase 6 (MKK6) and MKK3 (p38α, γ and δ) (Han et al. 1996; Moriguchi et al. 1996; Raingeaud et al. 1996). MKK6/3 are in turn activated by a diverse range of MKK kinases, including TAK1, ASK1, MEKK4 and MLK3 (reviewed by (Ono and Han 2000; Zarubin and Han 2005).

All p38 isoforms phosphorylate substrates containing the minimal consensus sequence Ser (Thr)/Pro (Ono and Han 2000). However, substrate selectivity, as well as isoform-specific tissue distribution and degree of activation by the upstream activators MKK6/3, contribute to their diverse biological functions. For example, a major substrate of p38α and β is MAPK-activated protein kinase 2 (MAPKAP2), which regulates HSP27 and is involved in actin cytoskeleton remodeling (Rouse et al. 1994). p38δ can promote microtubule assembly (Feijoo et al. 2005); while p38γ can phosphorylate the scaffolding protein SAP90 (Sabio et al. 2004). p38 isoforms regulate gene transcription of a large number of transcription factors such as ATF2, CREB, ELK-1, STAT-1, C/EBPβ and MEF2 (reviewed by (Ono and Han 2000; Zarubin and Han 2005). A more recent function ascribed to p38 is the stabilization of mRNAs and promotion of translation through AU-rich elements in the 3' untranslated regions (reviewed by (Saklatvala 2004; Zarubin and Han 2005).

p38 can regulate differentiation of the peripheral neuronal cell line PC12 (Iwasaki et al. 1999), chondrocytes, adipocytes, erythrocytes and skeletal myocytes

(Efimova et al. 2003; Engelman et al. 1999). Furthermore, four independent studies reported that p38α deficient mice are embryonic lethal since this kinase appears to be important for erythropoiesis and placental angiogenesis (Adams et al. 2000; Allen et al. 2000; Mudgett et al. 2000; Tamura et al. 2000).

We reported that p38 MAPK activity plays a fundamental role in peripheral nervous system (PNS) myelination (Fragoso et al. 2003). p38 MAPK-selective inhibitors reduced steady-state levels of mRNA for the myelin-specific proteins myelin-associated glycoprotein (MAG), myelin basic protein (MBP) and Po and blocked myelination by Schwann cells (SCs). p38 inhibitors were also found to reduce laminin content as well as extracellular matrix-induced changes in SC shape and axonal alignment in PNS cultures while neither viability nor proliferation was altered (Fragoso et al. 2003). A previous study in oligodendrocyte progenitors (OLPs), found that the SB203580 compound decreased cell proliferation, and reduced sulfatide levels (Baron et al. 2000). In addition, developmental and forskolin-induced MBP gene expression was also blocked by SB203580 (Bhat et al. 2007).

Considering the increasing amount of information on roles of p38 MAPK in cellular differentiation, and our previous findings for regulation of PNS myelination by p38, we set out to determine the roles of this kinase family in oligodendrocyte (OLG) differentiation. We have used OLG cultures alone or in coculture with dorsal root ganglion (DRG) neurons (DRGN) to study the role of p38 in myelination. Inhibitors of p38 were found to decrease steady state levels of mRNA and protein for myelin-specific proteins (2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP), myelin-associated oligodendrocyte basic protein (MOBP), proteolipid protein (PLP), MBP, MAG). We also observed a decrease in mRNA levels for UDP galactose:ceramide galactosyltransferase (CGT), which accounted for the substantial decrease in galactosylceramide (GalC) and its sulfated form sulfatide. Our results suggest a role for p38 MAPK activity in regulating the process of differentiation and myelination in OLGs when cultured alone, or in the presence of DRGN. Portions of this work have been published by our group in abstract form (Fragoso 2006; 2004).

Materials and Methods

Materials

Ham's F12 medium, PBS, HBSS, 7.5% BSA fraction V, and penicillin/streptomycin were purchased from Invitrogen (Burlington, ON, Canada). FBS and DMEM were from Wisent Inc (St-Bruno, QC); NGF (2.5S) from Alomone Labs (Jerusalem, Israel); $PDGF_{AA}$ and bFGF from PeproTech (Rocky Hill, NJ). PD169319, SB203580 and U0126 were from EMD Chemicals (San Diego, CA). Kodak XRP-5 film and nitrocellulose membranes were from Mandel Scientific (Guelph, ON, Canada); DAPI was from Molecular Probes Inc. (Eugene, OR); A_2B_5 mouse monoclonal antibody (mAb) from American Type Culture Collection (CRL 1520). Dr. Ranscht (La Jolla Cancer Research Center) provided mmAb for GalC. ECL Western Blotting Detection reagents were from Perkin Elmer (Boston, MA). Poly-D-lysine, poly-L-ornithine, human and bovine transferrin, insulin, HEPES, Triton-X-100, DTT, BrdU and rabbit polyclonal anti-actin, anti-tubulin and mmAb anti-BrdU were from Sigma-Aldrich (Oakville, ON, Canada). Other primary antibodies were from the following suppliers: mmAb against Western (NFH), MBP and CNP from Sternberger Monoclonals (Lutherville, MD); rabbit polyclonal phospho-specific anti-p38 (Thr^{180}, Tyr^{182}), anti-p38α and anti-p38δ from Cell Signaling Technology (Danvers, MA); anti-p38β from Zymed/Invitrogen; mmAb anti-complement type-3 from Serotec Canada (Toronto, ON); rabbit polyclonal anti-HSP27 from StressGen Bioreagents (Ann Arbour, MI); goat polyclonal anti-β-actin antibody and rabbit polyclonal p38γ from Upstate Biotechnology (Lake Placid, NY). HRP-, FITC- or Texas Red-conjugated secondary antibodies were from Southern Biotechnology, or Jackson Immunoresearch Laboratories (Cedarlane, Hornby, ON) or BIO-RAD Canada (Mississauga, ON). High-performance thin layer chromatography (HPTLC) plates were from Whatman (silica gel 60 Å, 10 x 10 cm). All other reagents were from Fisher Scientific (Whitby, ON), or VWR (Mont-Royal, QC).

Dorsal root ganglion neuron cultures

Purified DRGN cultures were prepared using methods described previously (Giasson and Mushynski 1996). DRGs were obtained from Sprague-Dawley rat embryos at 15-16 days gestation, dissociated with trypsin and plated onto rat-tail collagen-coated dishes. The cultures were maintained with 12.5 ng/mL NGF in serum-free N1 medium and treated with anti-mitotic agents to rid them of proliferating Schwann cells and fibroblasts. Myelination was initiated in the third week of culture by the addition of OLPs, plated at a density of 0.7×10^5 cells/cm^2. At this stage, DRGN are morphologically mature, displaying a profuse axonal network. The medium was replenished every 2 days.

Oligodendrocyte progenitor cultures

Primary cultures of OLPs were prepared from the brains of newborn Sprague-Dawley rats as described previously (Almazan et al. 1993; McCarthy and de Vellis 1980). OLPs were plated on poly-D-lysine-coated culture dishes and grown for 6d in SFM consisting of a DMEM-F12 mixture (1:1), 10 mM HEPES, 0.1% bovine serum albumin, 25 µg/mL human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 µg/mL insulin, 16 µg/mL putrescine, 30 nM selenium and 2.5 ng/mL each of $PDGF_{AA}$ and bFGF. Cultures were characterized immunocytochemically with cell type-specific antibodies. More than 95% of the cells were positive for monoclonal antibody A_2B_5, a marker for OLPs in culture while less than 5% were GalC-positive OLGs, GFAP-positive astrocytes or complement type-3-positive microglia (Cohen and Almazan 1994; Radhakrishna and Almazan 1994).

All animal use was approved by the McGill Animal Care Committee (protocol number 4373-020331) in accordance with Canadian Council on Animal Care guidelines.

Western Blot Analysis

After treatment, cells were lysed in buffer (2% SDS, 62.5 mM Tris-HCl (pH 6.8). Protein content was determined using the bicinchonic acid assay, and 20 μg of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween20 and incubated with appropriate primary and secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence and quantified by densitometry.

RNA extraction and real-time PCR

Total RNA was extracted from cultures using the TRIzol reagent according to manufacturer's instruction (Invitrogen). Two μg of DNaseI-treated RNA was reverse-transcribed using the AMV reverse transcriptase (Roche Diagnostics, Laval, Quebec). Primers were designed to span exon-exon junctions (sequences are available upon request). A 1/25 of the total cDNA sample was analyzed per reaction, using the Roche LightCycler 2000, and the SuperArray SYBR Green master mix (SuperArray, Frederick, MD, USA). PCR amplifications were performed as follows: heat inactivation (10 min, 95ºC); followed by 35-45 cycles of 94ºC, 15 sec; 59ºC, 30 sec; 72ºC, 20 sec. Fluorescence detection of product was detected at the end of the PCR extension step, and melting curves were analyzed by monitoring the continuous decrease in fluorescence of the SYBR Green signal. PCR products were verified for a single amplification product using melting curve analysis, and the molecular weight of each product was confirmed using PAGE. Standard curves were generated for each primer set to determine primer efficiencies, and quantification relative to β-actin controls was performed using the Pfaffl method (Pfaffl 2001). This equation calculates the difference in the critical threshold (C_T) values for the target and reference genes in the control vs. treated samples while accounting for the differences in amplification efficiencies between primer sets.

siRNA knock-down for p38

Small interfering RNAs (siRNAs) designed to target p38α, and fluorescently-tagged controls (siGLO-Alexa^{594}) were purchased from Dharmacon (Chicago, IL). Primary OLGs were transfected using the siIMPORTER siRNA reagent (Upstate Biotechnology, Lake Placid, NY). Briefly, siRNAs were mixed with transfection reagent to a final concentration of 80 nM, and lipid complexes were allowed to form for 5 min at RT. The transfection mix was added to cells, and cultures were analyzed by immunoblotting 48 h later. p38α siRNA-treated cells were co-transfected with siGLO to verify transfection using immunocytochemistry.

Immunofluorescence staining

Immunofluorescence was performed as described (Cohen and Almazan 1994); (Radhakrishna and Almazan 1994). Briefly, cultures on glass coverslips were exposed to A_2B_5 or anti-GalC monoclonals at 37°C, fixed with 4% PFA in PBS, and incubated with TexasRed or FITC-conjugated secondary antibodies. For MBP and NF immunostaining, cells were postfixed with methanol for 5 min at -20°C prior to incubation with appropriate antibodies.

BrdU incorporation, as an index of cell proliferation, was performed as described (Cohen et al. 1996). Cells were incubated in BrdU (10 μM for 24 h), fixed and immunostained. Coverslips were mounted on DAPI-containing mounting medium and examined using a Leitz Diaplan epifluorescence or Zeiss confocal microscope. Percentage of $BrdU^+/DAPI^+$ was determined by counting ~500 cells per coverslip. Data represent the results of three independent experiments.

Visualization of apoptotic nuclei (TUNEL labeling)

Fragmented DNA was detected in PFA-fixed cells by incorporating fluorescein-12-dUTP at 3'-OH ends using Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) assay as described (Cui et al. 2005).

Electron Microscopy

Myelinating cell cultures were fixed with 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer, pH 7.3, followed by postfixation in 1% osmium tetroxide. Samples were stained with 1% uranyl acetate, dehydrated in ethanol gradients and embedded in Epon. Semithin sections (0.5 μm) were cut and stained with methylene blue to select myelinated areas. Thin sections were cut and stained with uranyl acetate and lead citrate and examined with a JEOL 2000FX electron microscope.

Lipid extraction and high-performance thin-layer chromatography

Lipids were double-extracted from OLG cultures using chloroform/methanol (2:1, v/v), according to published protocols (Bligh and Dyer 1959; Simons et al. 2000). The lipid layer was dried under a stream of nitrogen, resuspended in 50 μL of chloroform and spotted in a 1 cm band onto HPTLC plates. The plates were resolved in chloroform:methanol:water (14:6:1, v/v/v), and visualized by charring with 50% sulfuric acid, followed by incubation at 120°C for 10 min. Lipid bands were identified by eluting purified rat brain myelin lipids in parallel to lipid extracts from treated and non-treated OP cultures. Plates were scanned and quantified using Alpha Innotech quantification software.

Drug treatments

The p38 inhibitors PD169319 and SB203580 and the MEK1 inhibitor, U0126 (10 μM), were applied to OLG at the initiation of differentiation (removal of mitogens $PDGF_{AA}$ and bFGF) for 1 to several days. For DRGN/OLG cocultures, OLGs were allowed to attach to DRGN for 3 h, then medium was replaced in the presence (or absence) of inhibitors, and changed every other day.

Statistical analysis

Statistical analysis was performed by one-way Analysis of Variance (ANOVA), followed by Tukey-Kramer post-hoc test (Graph Pad Prism). Differences were considered statistically significant when p-values were < 0.05.

Results

Characterization of myelinating DRGN/OLG cocultures

OLG cocultured with rat embryonic DRGNs made contact with axons, and initiated myelination spontaneously. Immunofluorescence analysis for MBP shows an OLG beginning to myelinate a DRGN three days post seeding (Fig 1A). After 2 weeks, the cultures exhibit extensive myelination (depicted in large microscopic fields) where multiple internode formation by many OLGs is observed (Fig 1B-D). Ultrastructural analysis by EM of 2-week myelinated cultures showed sheath formation and tight compaction of myelin lamellae (Fig 1E).

Activation of p38 in DRGN / OLG cultures

Relative levels of p38 expression in isolated DRGN and OLG cultures was first examined by Western blotting using specific antibodies to p38α, β, δ or γ. The main isoforms expressed by both OLG and DRGN are p38α and p38γ. Low levels of p38β are detected in DRGN (Fig 2A). Changes in p38 activation in the cocultures were assessed with phospho-specific p38 antibodies, following the addition of OLP to DRGN. A rapid and transient increase in p38 activation was observed at 15 min following the seeding of OLPs on DRGN, declining to basal levels by 24 h. HSP27, which is phosphorylated by MAPKAPK2, a downstream substrate of p38, was activated in a similar time course (Fig 2B).

Inhibition of p38α/β prevents differentiation of OLG alone or in coculture with DRGN

OLP cultured in the presence of the mitogens PDGF-AA and bFGF are mostly bipolar cells and express gangliosides recognized by the A_2B_5 antibody (Dubois et al. 1990). Removal of mitogens initiates morphological differentiation and sequential expression of lipid antigens, sulfatide and GalC, and myelin specific proteins, including CNP, MBP, PLP, MAG and MOBP. To determine whether p38 plays a role in OLG differentiation, OLP alone or cocultured with DRGN were treated with the pyridinyl imidazole compounds SB203580 and PD169316, selective inhibitors of

p38α/β, and lipid, mRNA and protein levels were examined using biochemical and immunocytochemical approaches.

Inhibition of p38 with 5 μM PD169316 caused a dramatic reduction in GalC levels as determined by immunofluorescence in pure OLG cultures (Fig 3A). To assess whether treatment with the drug was preventing the morphological differentiation of OLP, in addition to inhibiting lipid synthesis, cultures were stained with the A_2B_5 antibody, and an anti-tubulin antibody. Inhibition of p38 did not have an apparent effect on the branching of differentiating OLGs as observed with A_2B_5 surface staining and tubulin staining, which labeled the primary, secondary and tertiary cellular processes (Fig 4C). Furthermore, PD169316 (0.5 μM and 5 μM) did not produce a significant cytotoxic effect as shown by TUNEL staining, which detects DNA condensation/fragmentation in apoptotic cells (Table 1). A reduction in the total number of cells could be attributed to a decrease in cellular proliferation. Thus, as previously reported (Baron et al. 2000) for SB203580, PD169316 decreased the mitogenic activity of $PDGF_{AA}$ by ~50% in a 24h period, although under differentiating conditions the number of proliferating progenitors is very low (Table 1).

Lipid levels in both culture systems were quantified using HPTLC (Fig 3B,C). Cultures were grown for 7d in the absence or presence of 5 μM PD169316, and lipids were extracted with chloroform:methanol. The drug caused significant reductions (~50%) in both GalC and sulfatide for both the α-hydroxylated and non-hydroxylated fatty acid forms, while non-significant reductions were observed in phospholipids, sphingomyelin, and cholesterol.

Steady-state levels of mRNAs encoding myelin proteins was determined in OLG differentiated for 2d in the absence or presence of 5 μM PD169316. Real-time PCR analysis showed that p38 inhibition decreased CNP1 and MAG (~100%), PLP (~70%), MBP and MOBP (~50%) mRNA levels (Table 2). A significant decrease (~50%) was also observed in the mRNA levels for CGT, the transferase essential for galactolipid formation, consistent with the substantial decrease in GalC and sulfatide levels. In addition, the levels of Sox10, a transcription factor required for terminal differentiation of OLG (Stolt et al. 2002), were reduced by 25%.

To test the effect of p38 inhibition on myelin protein expression, DRGN/OLG cocultures were treated with PD169316 (5 μM) or SB203580 (2.5 and 5 μM) for 7d. PD169316 was more effective in blocking MBP accumulation than SB203580. In contrast, U0126 (10 μM), an inhibitor of the upstream kinase of extracellular-regulated kinase (ERK), MEK1, did not affect MBP or CNP expression (Fig 5A). Furthermore, a dose-dependent decrease in MBP and CNP expression was seen with increasing concentrations (0.01 to 5 μM) of PD169316 (Fig 5B). The block in OLG differentiation with PD169316 was corroborated using an siRNA to knock-down p38α. Following siRNA transfection, p38α decreased by 60% with a parallel reduction in MAG, an early marker of myelination (Fig 4A). A dramatic decrease in GalC expression was also observed in cells co-transfected with p38α and negative control siRNAs (Fig 4B).

The biochemical results were confirmed by immunofluorescence with antibodies to MBP. DRGN/OLG cultures treated for 7 days with PD169316 failed to form myelinated internodes, showing a reduction of more than 90%, as compared to non-treated controls. Neuronal integrity was not affected by PD169316 treatment since NFH immunostaining shows a normal pattern and intensity (Fig 6). In contrast, PD169316 prevented axolemmal organization of Caspr, which is expressed diffusely on unmyelinated axons but becomes localized to paranodal junctions after the onset of myelination (Fig 6) (Einheber et al. 1997).

To address whether p38 activity is required for the initiation of myelination, DRGN/OLG cultures were treated with PD169316 starting on d0 to d4 and continued to grow until d7 for all conditions. Examination of MBP, CNP and MAG levels showed that myelination is most significantly blocked when treatment is started in the first 2 days of coculture. Less significant reductions were observed when treatment was initiated on d4 (Fig 7A). Most notably, treatment of the cocultures from d0-d1 and d0-d2, followed by removal of the drug was sufficient to completely block MBP and significantly decrease CNP expression, illustrating an irreversible effect (Fig 8A). Identical experimental conditions in cultures of OLG alone showed that PD169316 significantly reduces MBP, CNP and MAG when present throughout the culture period. However, the drug was not effective when

applied to cultures differentiated for more than 3 days (Fig 7B). In contrast to the irreversible block seen in DRGN/OLG cocultures, removal of the drug reversed the inhibitory effect on OLG differentiation (Fig 8B). These results suggest that p38 is required for OLG differentiation and for the initial establishment of axonal contact and/or ensheathment both of which are required for myelination.

We also examined levels of $p27^{KIP1}$, a cyclin-dependent kinase inhibitor which increases with OLG differentiation (Casaccia-Bonnefil et al. 1997). Inhibition of p38 activity decreased accumulation of $p27^{KIP1}$ in both DRGN/OLG and OLG cultures (Fig 7A/B).

Discussion

The p38 MAPK family consists of α, β, δ and γ isoforms, and their differential activation may control downstream cellular processes, depending on cell type and context (reviewed by (Saklatvala 2004; Zarubin and Han 2005). In addition to its role as a stress-activated kinase, p38 may be involved in neural function and development. Furthermore, p38α and β were found activated in adult brain tissue, mostly in neuronal nuclei (Lee et al. 2000) and in myelin-like structures (Maruyama et al. 2000). p38 can also modulate synaptic function by acting on neurotransmitter receptors and ion channels (Brust et al. 2006; Poolos et al. 2006). In addition, associative learning in rabbits increased p38 activation and both events were blocked with the selective p38 inhibitor, SB203580 (Zhen et al. 2001), while cAMP-stimulated neurite outgrowth of PC12 cells also required p38 activation (Hansen et al. 2000). Based on these and other observations, we hypothesized that p38 could play an important role in myelination (Fragoso et al. 2003).

Relatively little is known about the signals that initiate OLG differentiation and myelination. In contrast to the PNS, where initiation of the developmental program requires contact of Schwann cells with axons and with the extracellular matrix protein laminin, removal of the mitogens $PDGF_{AA}$ and bFGF is sufficient to trigger morphological differentiation of cultured OLP and expression of myelin-specific lipids and proteins. However, formation of myelinated internodes requires the presence of myelination-competent neurons. We have established a DRGN culture system as previously described (Wood and Bunge 1986), where extensive myelination of axons by Schwann cells or OLGs can be produced under serum-free conditions. Using this system, we demonstrated that p38 MAPK is involved in peripheral myelination (Fragoso et al. 2003). In this study, we investigated the requirement of p38 MAPK activity during OLG differentiation and myelination using pharmacological inhibition of p38 with SB203580 and PD169316. We observed that p38 activity is essential for the initial stages of OLG differentiation including synthesis of myelin-specific lipids (sulfatide and GalC) and proteins (CNP, MAG, and MBP). The effects were specific to the p38 pathways since treatment of

the cultures with U0126, an inhibitor of MEK1, the kinase upstream of ERK1/2, had no significant effect on MBP, or CNP expression.

Analysis of the specific p38 proteins expressed by the cultures showed that p38α and γ were the main isoforms found in OLG and DRGN, while p38β was only detected in the latter. Since the p38 inhibitors used are selective for the α and β isoforms, the results suggest that p38α regulates OLG differentiation. This was further corroborated with a specific siRNA to reduce p38α expression. Transfection of OLP at the initiation of differentiation with p38α siRNA caused a reduction in p38α protein and significant decreases in GalC, and MAG, both early markers of differentiation.

In differentiating OLG, p38 inhibition prevented the accumulation of the cell cycle inhibitor $p27^{KIP1}$, which normally increases during OLG differentiation (Casaccia-Bonnefil et al. 1997). This is in line with the observations that p38 is implicated in the G1 and G2/M phases of the cell cycle (Molnar et al. 1997; Yee et al. 2004), while inhibitors prevented the expression of the cell cycle regulator $p21^{waf1}$ and the differentiation of muscle cells (Wu et al. 2000). In addition, increased expression of $p27^{KIP1}$ in OLGs enhanced MBP promoter activity (Miskimins et al. 2002) through stabilization of the transcription factor Sp1 (Wei et al. 2004). Furthermore, coexpression of $p27^{KIP1}$ with Sox10, a member of the high-mobility group factors that activates MBP expression (Stolt et al. 2002), resulted in further increases in promoter activity (Wei et al. 2004). Our observations that p38 inhibitors can decrease both Sox10 mRNA expression and $p27^{KIP1}$ protein accumulation can partly explain their effects on MBP expression in differentiating OLGs. However, the effect of p38 on transcription of other myelin-specific genes, including CNP, MAG, MOBP and PLP suggests that this kinase exerts a transcriptional regulatory control on myelination, and further studies are required to address the level at which this regulation occurs. Other transcription factors reported to play a role in OLG differentiation, including Olig2, Nkx2.2 and Sox17 (Gokhan et al. 2005; Qi et al. 2001; Sohn et al. 2006; Zhou et al. 2001), might also be regulated by p38. In muscle, p38 can directly phosphorylate or induce synthesis of MEF2, Myf5 and MRF4

transcription factors, which regulate early and late stages of myogenesis (Keren et al. 2006).

Treatment of DRGN/OLG cocultures with p38 inhibitors blocked not only accumulation of myelin lipids and proteins but also formation of myelinated internodes. These effects were concentration and time-dependent and required the presence of the inhibitor for the first one to two days after initiation of the coculture. Interestingly, the addition of OLP to DRGN initiated a rapid and transient activation (phosphorylation) of p38 with a maximum increase at 15 minutes and return to basal levels 24 h later. In the cocultures the first 24-48 h appear to be most critical for the requirement of p38 in the initiation of the differentiation program since addition of PD169316 on the third to fourth day has much less effect on the accumulation of CNP, MAG, and MBP. Furthermore, the differentiation block was irreversible since removal of the p38 inhibitor from DRGN/OLG cocultures on day 1-7 failed to restore myelination. In contrast, treatment of OLP cultures with the same drug concentration for the first two days following mitogen removal reduced MBP and CNP levels by only 50%, and removal of PD169316 from days 2-8 allowed for the recovery of myelin protein accumulation as compared to non-treated controls. These results suggest that in addition to its effect on OLP differentiation, p38 is also required at an early stage of myelin formation. Consistent with this notion, inhibition of p38 disrupted the axolemmal organization of Caspr in the DRGN/OLG cocultures. Caspr is an adhesion molecule, concentrated with contactin at the paranodal junctions that are formed between axons and the terminal loops of OLGs (Einheber et al. 1997; Menegoz et al. 1997). One possibility is that PD169316 blocks the ensheathment of axons by OLP cellular processes, an early critical step for the formation of myelinated internodes. Our previous study showed that p38 inhibitors prevented laminin-induced Schwann cell elongation and alignment with axons without altering SC proliferation or survival (Fragoso et al. 2003). These morphological changes require actin cytoskeleton remodeling (Rouse et al. 1994), which in other systems is regulated through the action of p38 on MAPKAP2 followed by HSP27 phosphorylation (Lavoie et al. 1993).

Another interesting observation is that the p38 inhibitors did not appear to alter the early morphological changes associated with OLP process formation as observed by tubulin immunostaining, which delineated the branching of primary, secondary and tertiary cellular processes. These results suggest that OLP treated with PD169316 differentiated morphologically although GalC was reduced by more than 50% in both OLG alone or in coculture with DRGN.

Future studies should be aimed to identify the specific extracellular cues which induce the p38 pathway(s) involved in myelination, the transcriptional specificity of p38 in the activation of myelin-specific genes, and potential roles of other p38 isoforms expressed in OLG and DRGN, including the abundantly expressed p38γ. p38 MAPK α/β isoform-specific inhibitors, and dominant negative constructs have already revealed novel functions regulated by this kinase in various cells (Lee et al. 1999). A non-selective p38 inhibitor, BIRB796, has been shown to block the stress-induced phosphorylation of the scaffold protein SAP97, a physiological substrate of p38γ (Kuma et al. 2005).

In summary, our results show that p38 MAPK is a key regulatory component of OLG differentiation and initiation of myelination. We are currently investigating the mechanisms through which p38 plays a regulatory role in OLG differentiation, as well as the mechanism responsible for the lack of myelination and loss of recovery in our coculture system. Clues from this work could possibly result in the development of strategies to re-initiate myelination of neurons whose myelin sheath has been lost or destroyed.

Acknowledgements

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Tables and Figures

Table 1. Effect of PD169316 on proliferation and survival of OLG

	BrdU/DAPI (%)	Tunel positive cells (%)
Control	70.5 ± 2.5	5.2 ± 0.5
0.5 μM PD169	60.6 ± 3.3	6.9 ± 0.9
1 μM PD169	50.8 ± 1.9**	5.2 ± 0.7
5 μM PD169	40.4 ± 1.6***	7.2 ± 0.9

OLPs were treated with PD169316 for 30 min prior to the addition of $PDGF_{AA}$ (5 ng/mL) and BrdU (10 μM) BrdU incorporation was assessed by immunofluorescence as a percentage of total DAPI-labeled nuclei. Apoptotic cell numbers were determined by TUNEL in cultures growing for 48 h in differentiation medium (SFM without mitogens) and PD169316. Values are mean±SEM for three experiments in triplicate. Comparison is made to control values: $^{**}p<0.01$, $^{***}p<0.001$.

Table 2. Inhibition of p38 reduces steady-state levels of myelin-specific mRNA transcripts

Gene	Control (mean) (%)	PD169316 treatment (% of control)
MBP	100	59.5 ± 2.50**
CNP1	100	0.1 ± 0.38***
MAG	100	0.0 ± 1.70***
PLP	100	31.3 ± 3.41**
MOBP169	100	48.5 ± 2.39**
CGT	100	56.0 ± 1.94**
SOX10	100	76.6 ± 1.21**

RNA was extracted and reverse-transcribed from 2d-differentiated OLG grown in the absence or presence of PD169316 (5 μM). Quantifications were performed using real-time PCR relative to β-actin. Values represent mean±SEM for 3 culture preparations in triplicate. Comparison is made to control values: $^{**}p<0.01$, $^{***}p<0.001$.

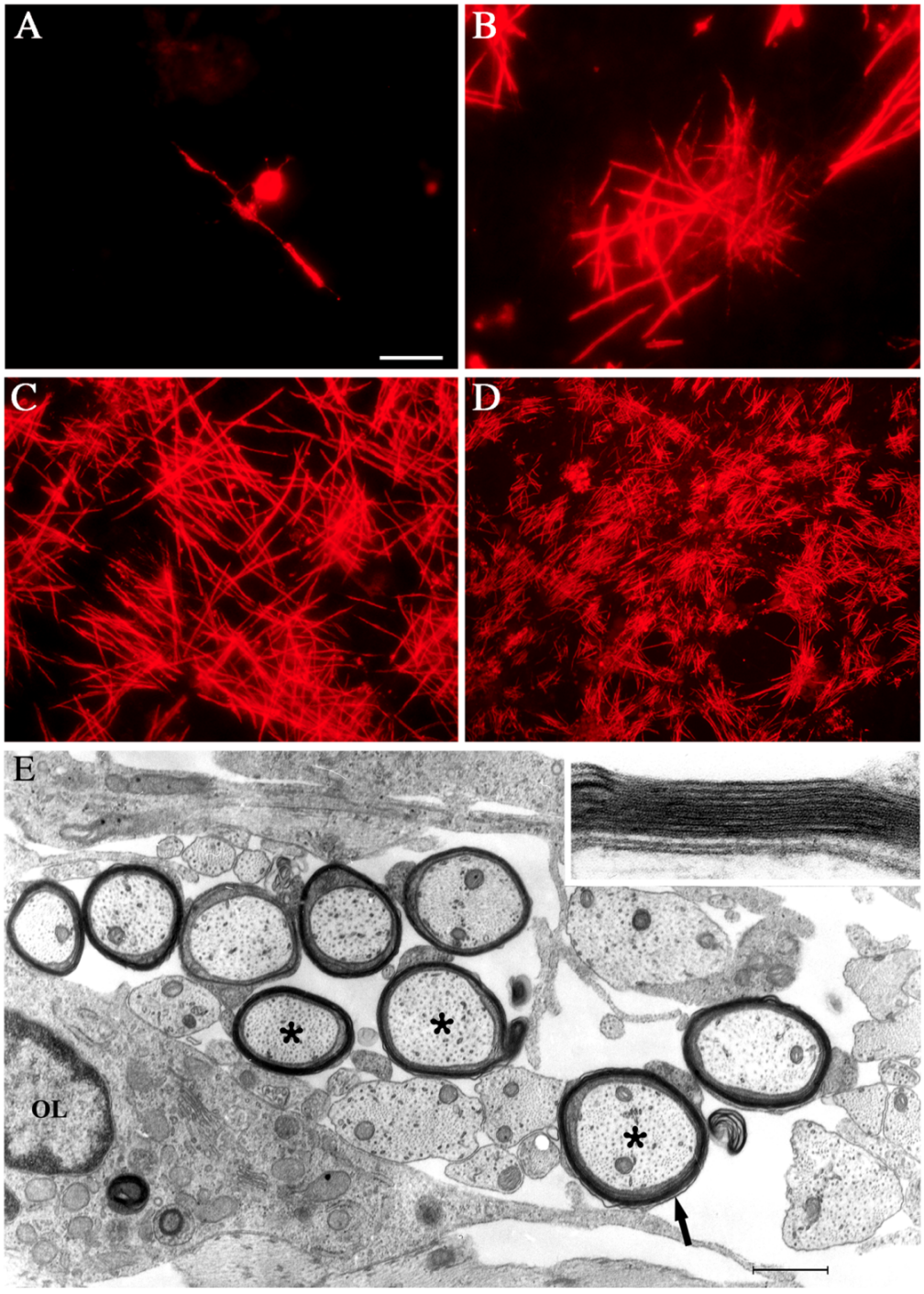


Figure 1

Figure 1. Myelination of DRGN by OLGs. Cultures were stained with anti-MBP and TxR-conjugated antibodies at 3 d (A) and 15 d (B, C, D) after OLP seeding. In A, and B, a single OLG is myelinating one or multiple internodes, respectively. In C and D, a panoramic field of mature OLG myelinating many neurites. In E an EM micrograph shows a transverse section of a 2 week myelinating DRGN/OLG coculture. Higher magnification insert depicts compact myelin with multiple lamellae. Arrow indicates compact myelin sheaths surrounding axons (asterisk). Scale bars represent ~20 μm (A, B), ~50 μm (C), ~80 μm (D) and ~1 μm (E).

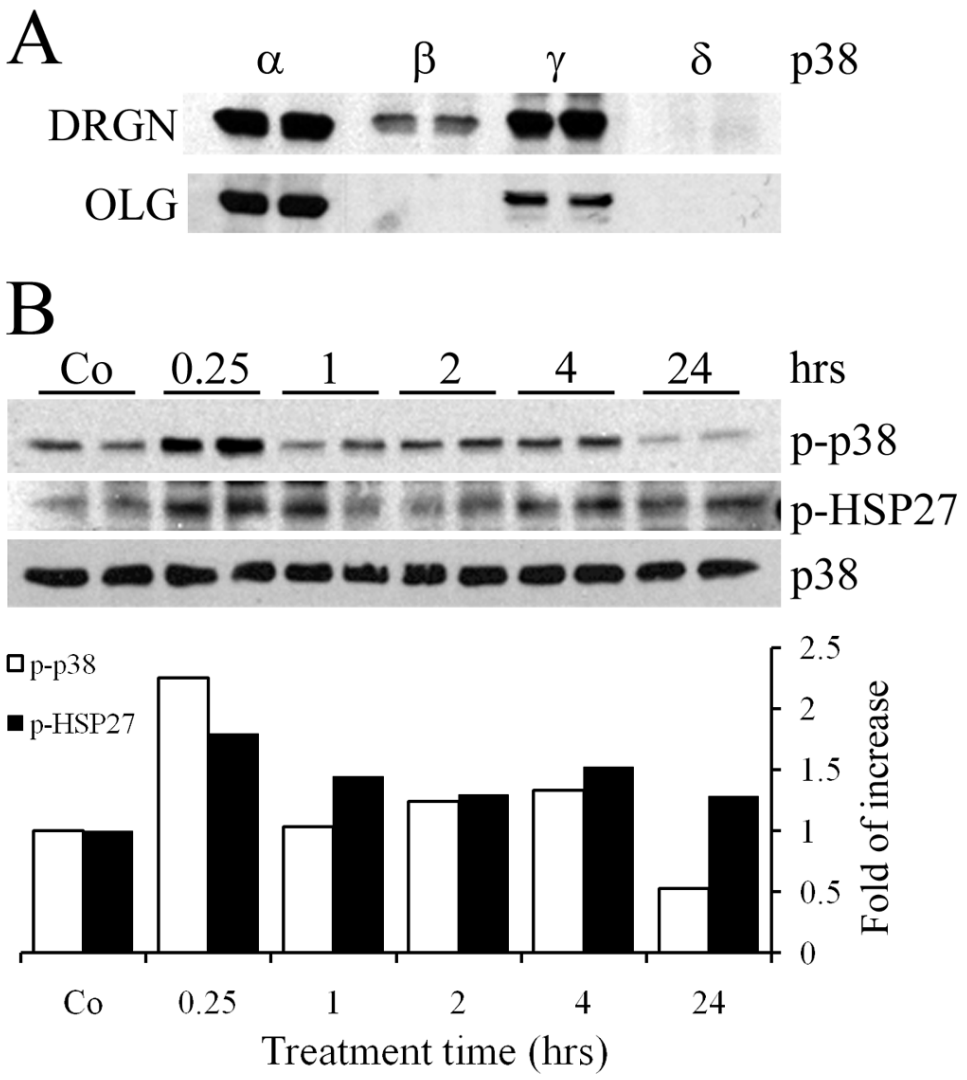
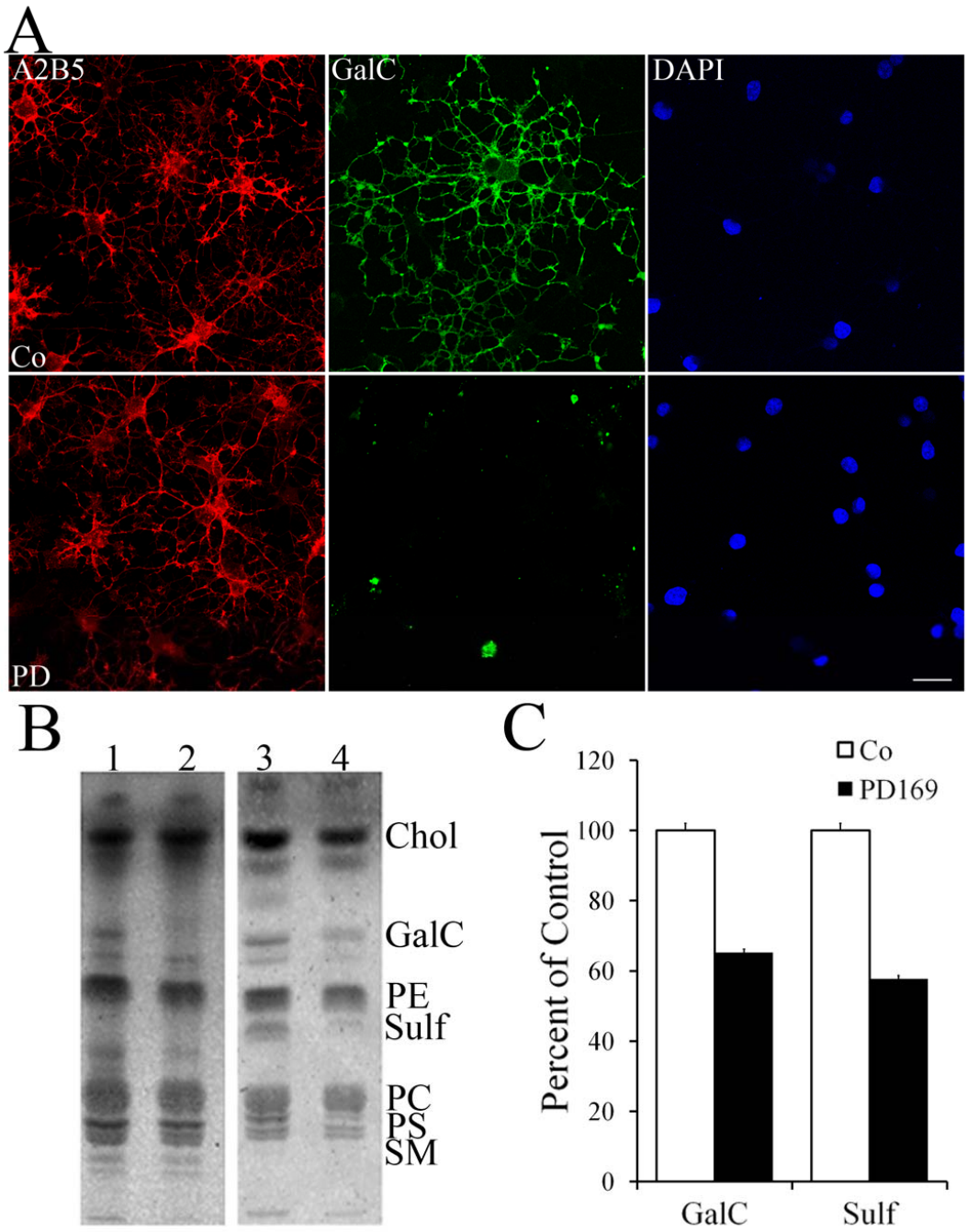


Figure 2. Expression and activation of p38 in DRGN and OLG cultures. (A) Relative levels of p38 isoforms were examined in both OLG and DRGN cultures. OLGs and DRGN most abundantly expressed p38α, followed by lower levels of p38γ. DRGN also expressed low levels of p38β. (B) Activation of p38 and a downstream target, HSP27, was assessed at different time points following the seeding of progenitors on DRGNs. Controls represent a mixture of individually lysed and pooled DRGN and OLP. Levels of phospho-p38 and -HSP27 were determined by Western blots and quantified by densitometry. Maximal phosphorylation levels occurred at 15 minutes, decreasing thereafter. Values were calculated as fold increase above controls, corrected for p38 as internal control.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A2B5
GalC
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4
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PD169
Percent of Control
120
100
80
60
40
20
0
GalC
Sulf

Figure 3

Figure 3. Decreases in GalC and sulfatide expression in OLG or DRGN/OLG cultures treated with a p38 inhibitor. (A) In a 2d-differentiated OLG culture, 5 μM PD169316 produced a dramatic decrease in GalC staining in OLG arborized processes, while A_2B_5 immunostaining was retained. Nuclei are stained with DAPI (blue). Scale bar represents 20 μm. (B) HPTLC of lipids in DRGN/OLG and OLG cultures. Each lane contains lipids extracted from 3 wells of a 6-w plate for OLG, or 1X 6-w plate for DRGN/OLG cultures. Lane 1: DRGN/OLG coculture untreated control; Lane 2: DRGN/OLG coculture treated for 2d with 5 μM PD169316, followed by a 6d drug removal; Lane 3: OLG culture control, Lane 4: OLG culture treated with 5 μM PD169316 for a total of 7 d. (C). Densitometric analysis of GalC and sulfatide from three separate OLG cultures (in triplicate) treated for 7d with 5 μM PD169316 showed a significant reduction compared to controls ($p<0.001$, paired t-test). Chol, cholesterol; GalC, galactosylceramide; PE, phosphatidylethanolamine; Sulf, sulfatide; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin.

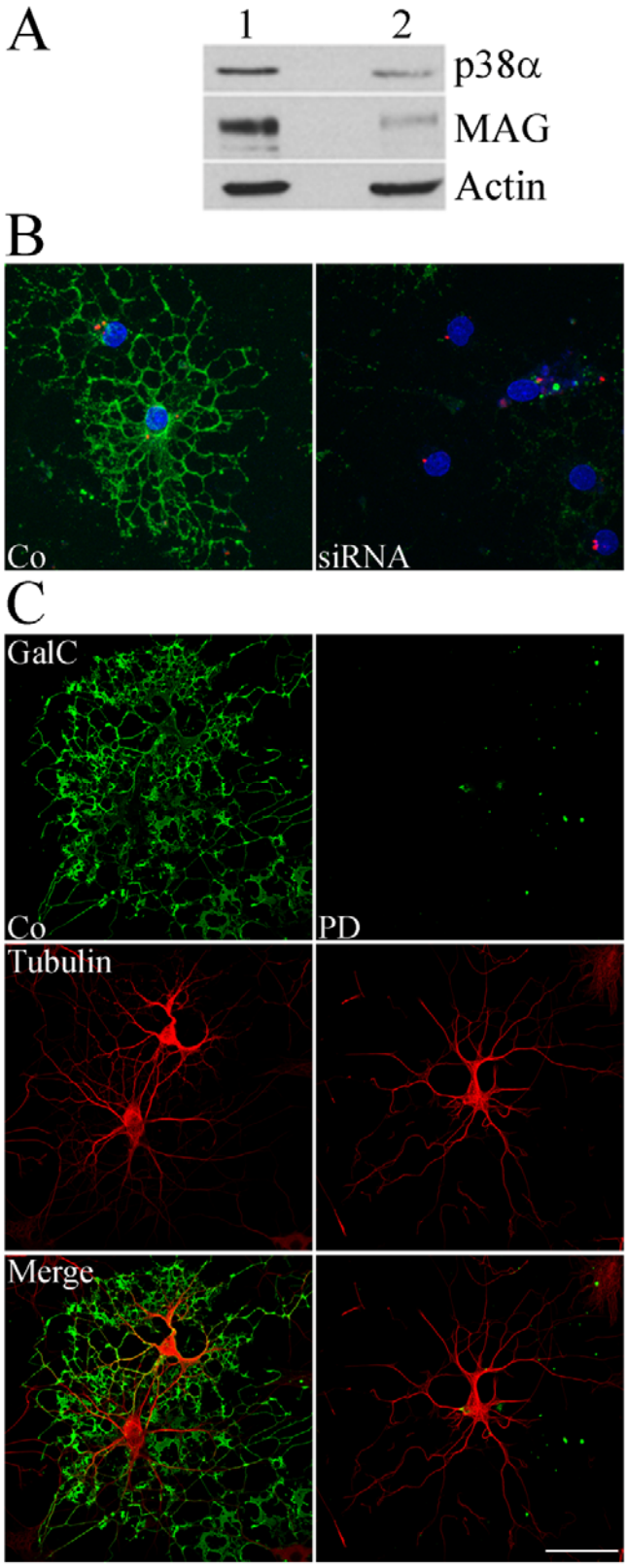


Figure 4

Figure 4. Decreases in GalC and MAG expression in OLG cultures treated with a p38α siRNA or p38 inhibitor (A) OLG were transfected with a fluorescently-tagged negative control siRNA (lane 1), or a p38α-specific siRNA (lane 2). Cell extracts were analyzed by Western blotting for p38α to confirm its knock-down, followed by MAG and β-actin (B) GalC immunocytochemical analysis of control transfected (siGLO-Alexa^{594}), or p38α siRNA/siGLO co-transfected OLG cultures. A dramatic reduction in GalC staining was observed in p38α knock-down cells. (C) In a 2d-differentiated OLG culture, 5 μM PD169316 reduced GalC, while branching of the cellular processes was normal as determined by tubulin immunostaining. Scale bar in B and C represents ~30 μm.

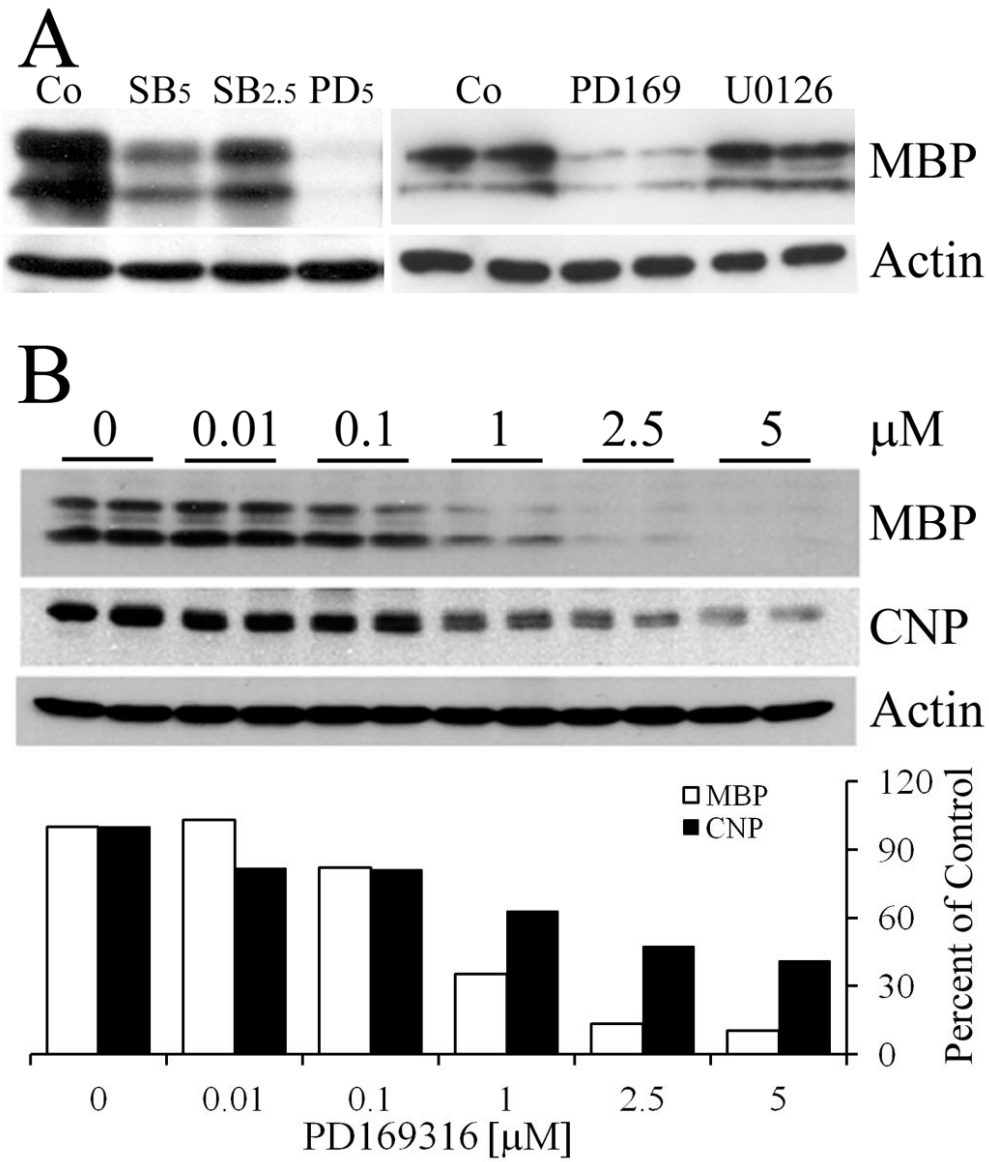


Figure 5. Decreases in MBP and CNP expression in DRGN/OLG cocultures treated with p38 inhibitors. (A) Treatment of cocultures for 7d with SB203580 (SB) (2.5 and 5 μM) or PD169316 (PD) (5 μM) caused a significant reduction in the accumulation of MBP. In contrast, the MEK1 inhibitor, U0126 (10 μM) had no effect. (B) The effects of PD169316 were dose-dependent. Blots for MBP, and CNP were quantified by densitometry and expression levels, relative to β-actin, were calculated as a percentage of non-treated controls. Values were obtained from duplicate experiments.

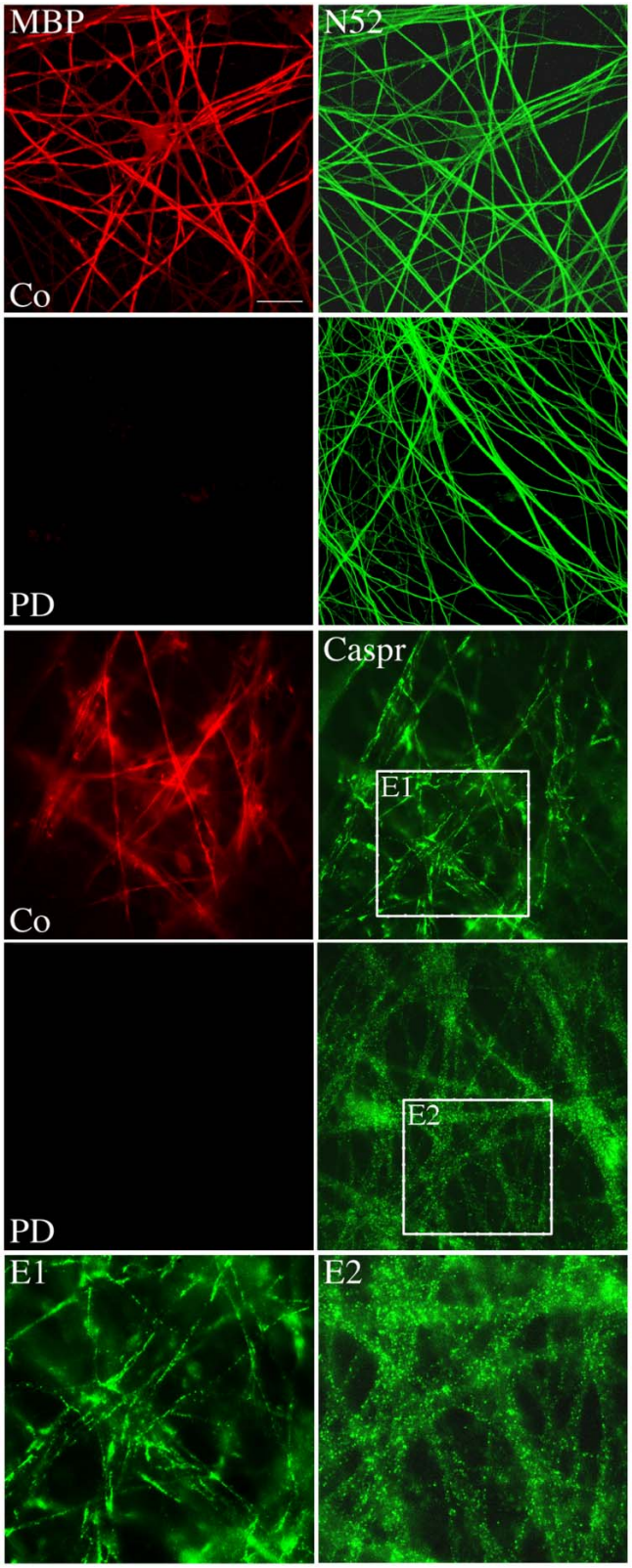
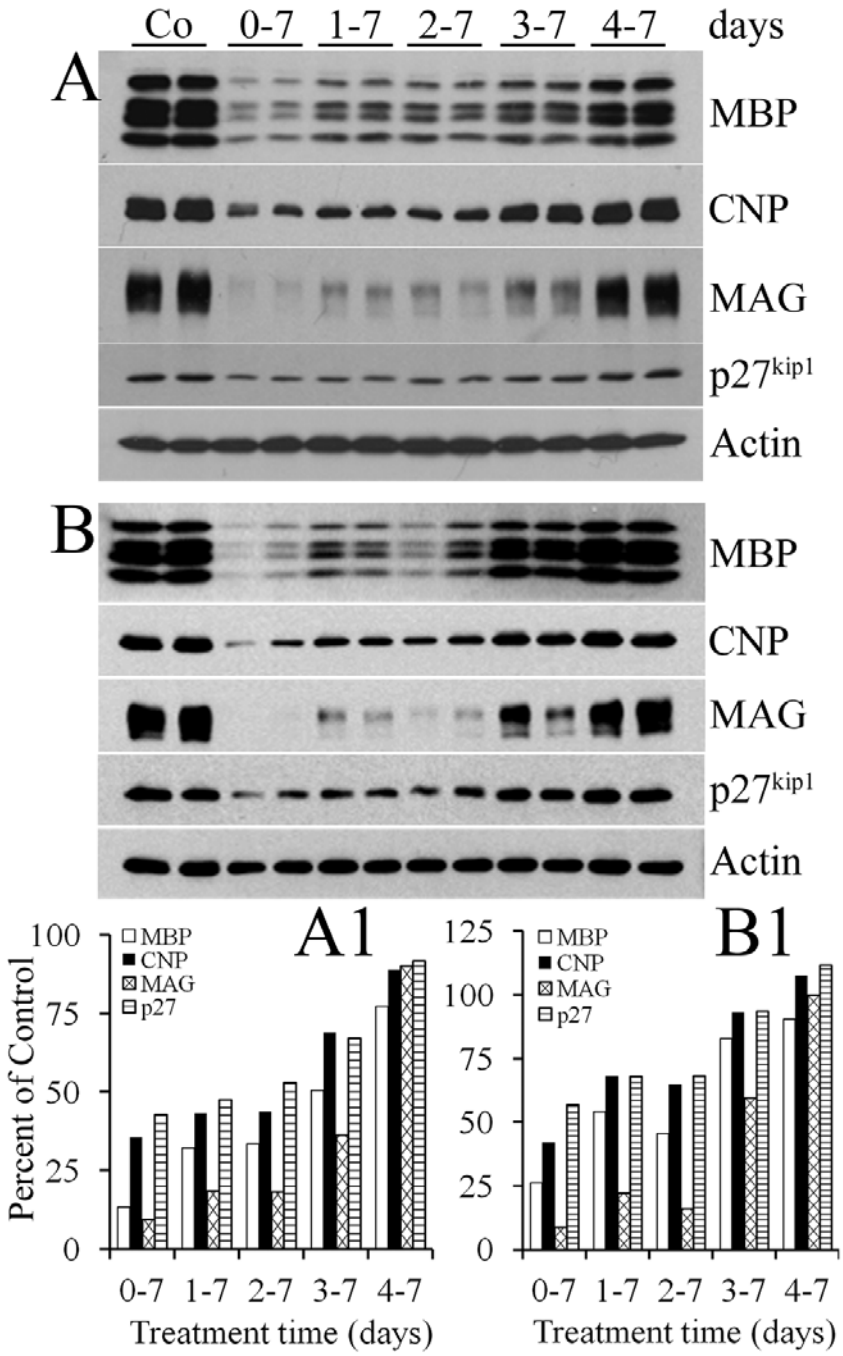


Figure 6

Figure 6. Expression of neuronal markers and MBP in PD169316-treated cocultures. DRGN/OLG were cultured in the absence or presence of PD169316 (5 μM) for 7d. Immunostaining with an anti-NFH antibody showed normal neurofilament organization in PD169316-treated cultures as compared to non-treated controls. The neuronal protein Caspr was found to cluster into discrete domains underlying myelinated (MBP^+) internodes in control cultures. However, Caspr, remained diffusely dispersed in PD169316-treated cultures in the absence of myelinating OLG. Scale bar = 20 μm. The inserts show higher magnification of Caspr staining for Control (E1) and PD169316-treated cultures (E2).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p27
Percent of Control
100
75
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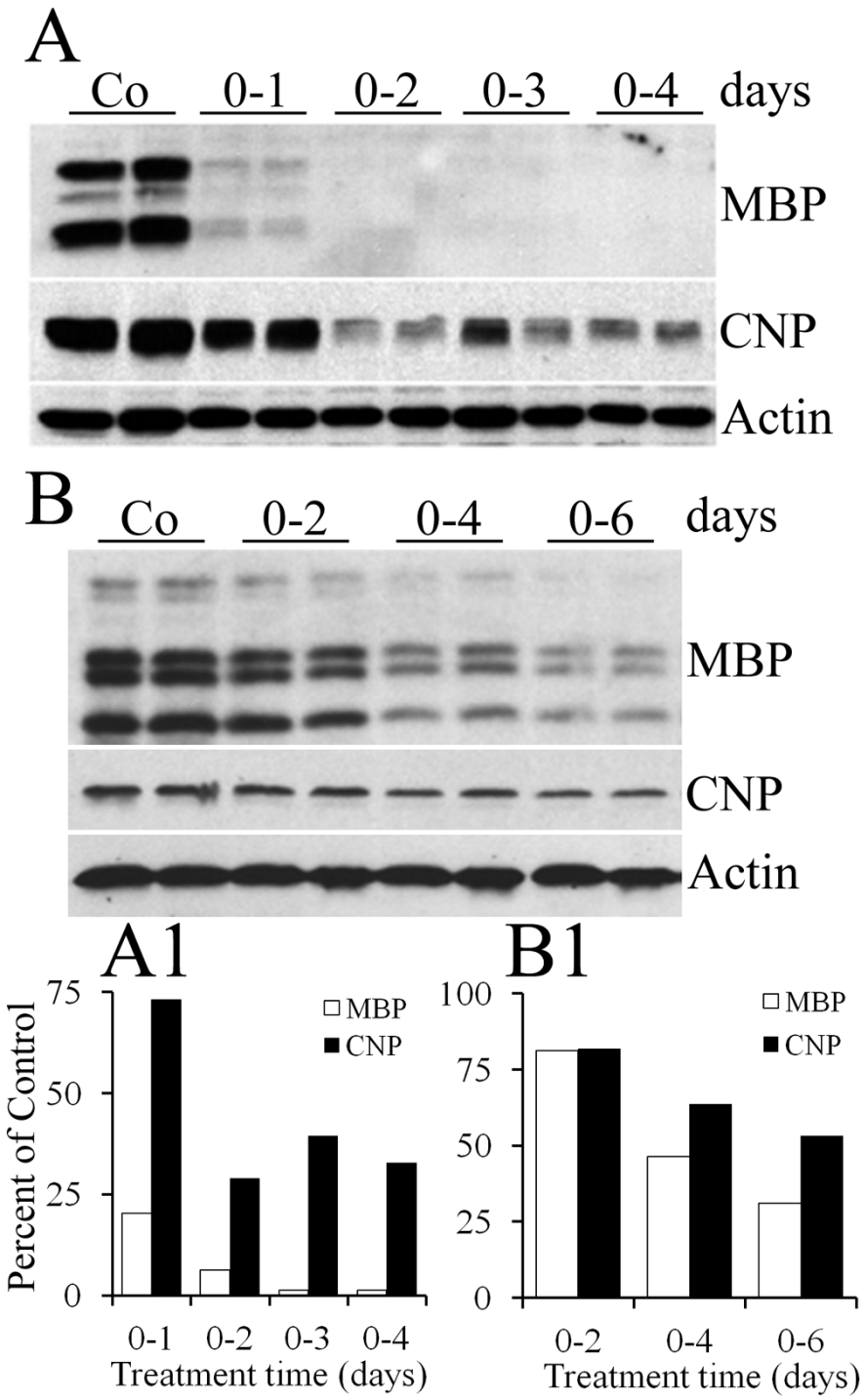
125
0-7 1-7 2-7 3-7 4-7
Treatment time (days)

Figure 7

Figure 7. Time-dependent decreases in myelin specific proteins and the cell cycle-inhibitor $p27^{KIP1}$. Cells were exposed to 5 μM PD169316 starting on d0-d4 and continued to grow until d7 for all conditions. MBP, CNP, MAG, $p27^{KIP1}$ and actin levels were determined by Western blots. Most significant effects were obtained with continuous PD169316 treatment. Blots for MBP, CNP, MAG and $p27^{KIP1}$ were quantified by densitometry and expression levels, relative to β-actin, were calculated as a percentage of non-treated controls. Values were obtained from duplicate experiments ((A), DRGN/OLG coculture (B), OLG alone).


A
Co
0-1
0-2
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days
MBP
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Actin
B
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0-2
0-4
0-6
days
MBP
CNP
Actin
A1
Percent of Control
75
50
25
0
MBP
CNP
0-1
0-2
0-3
0-4
Treatment time (days)
B1
100
75
50
25
0
MBP
CNP
0-2
0-4
0-6
Treatment time (days)

Figure 8

Figure 8. PD169316 irreversibly blocks myelination of DRGN/OLG. DRGN/OLG (A) or OLG (B) cultures were treated with 5 μM PD169316 starting on d0, and drug was removed on d1, d2, d3, d4 or d6. All groups were harvested on d7 and subjected to Western blotting for MBP, CNP and actin. Blots were quantified by densitometry and expression levels, relative to β-actin, were calculated as a percentage of non-treated controls. Values were obtained from duplicate experiments. Presence of the p38 inhibitor for 24-48 h after initiation of coculture was sufficient to fully block MBP accumulation in DRGN/OLG cocultures. In OLG cultures alone, the continuous presence of PD169316 is required to produce maximal effect.

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INTERVENING SECTION 1

After characterizing the role of p38 MAPK in OLG differentiation and CNS myelination, we sought to identify the downstream targets of p38 signaling responsible for regulating this process. p38 signals to many downstream effectors including MK2, MK3, MK5, MSKs, MNK, and CREB. In this section, experiments were designed to determine the role of MK2 in OLG differentiation using a pharmacological inhibitor and a siRNA. Co-immunoprecipitation experiments were used to verify that MK2 interacts with p38α during OLG differentiation.

Chapter 3: Mitogen-activated protein kinase activated protein kinase 2 (MK2) participates in p38 MAPK regulated control of oligodendrocyte differentiation

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Abbreviations

CGT – UDP: galactose ceramide galactosyltransferase

CMPD1 - 4-(2'-Fluorobiphenyl-4-yl)-N-(4-hydroxyphenyl)-butyramide

CNP – 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS - central nervous system

CREB – cAMP response element binding protein

GalC - galactosylcerebroside / galactosylceramide

HPH2 – human polyhomeotic 2

Hsp25 - heat shock protein 25

Id2 – inhibitor of differentiation 2

MAG – myelin-associated glycoprotein

MBP - myelin basic protein

MAPK - mitogen-activated protein kinase

MK2 - MAPK activated protein kinase 2

MSK - mitogen- and stress-activated kinase

Myt1 – myelin transcription factor 1

OLIG2 – oligodendrocyte transcription factor 2

OLG - oligodendrocyte

OLP - oligodendrocyte progenitor

PcG – polycomb group

pMK2 – phosphorylated MK2

SFM - serum free media

siRNA – small interfering RNA

SRF – serum response factor

Tcf4 – transcription factor 4

Abstract

The p38 mitogen-activated protein kinases (p38 MAPKs) are a family of kinases that regulate a number of cellular functions including cell migration, proliferation, and differentiation. We have previously reported a role for p38 MAPK in the regulation of oligodendrocyte (OLG) differentiation and Schwann cell myelination. Here, we extend our previous findings by showing that a p38 substrate, mitogen-activated protein kinase activated protein kinase 2 (MK2) is a downstream element of the p38 signaling pathway responsible for effecting oligodendrocyte differentiation. Inhibition of MK2 activity in oligodendrocyte progenitors (OLPs) using CMPD1 [4-(2'-Fluorobiphenyl-4-yl)-N-(4-hydroxyphenyl)-butyramide] blocked the activation of MK2 and resulted in decreased accumulation of myelin-differentiation markers, including myelin-associated glycoprotein (MAG) and myelin basic protein (MBP). We corroborated these findings using a small-interfering RNA to MK2, which decreased the myelin-specific lipid galactosylceramide and MAG. Treatment of cultures with CMPD1 decreased the steady state levels of mRNA encoding myelin transcription factor 1 (Myt1), MAG, MBP and Opalin, a transmembrane sialylglycoprotein expressed in OLGs. In contrast, increases were observed in the mRNA levels of OLG transcriptional repressors, including transcription factor 4 (Tcf4), Notch1, and inhibitor of differentiation 2 (Id2). Furthermore, we found that the predominantly expressed isoform of p38 in OLGs, p38α, and MK2 can form co-immunoprecipitatable complexes in OLPs and OLGs. Our results demonstrate that the p38-MK2 pathway is a component of the signaling cascade regulating OLG differentiation.

Introduction

We previously reported that p38 mitogen-activated protein kinase (MAPK) effects OLG differentiation and myelination (Fragoso et al. 2007; Haines et al. 2008). p38, composed of four isoforms α, β, γ, and δ, are serine/threonine kinases involved in stress responses, cell migration, proliferation and differentiation (Nebreda and Porras 2000). Treatment of oligodendrocyte progenitors (OLPs) with p38 inhibitors, prevented their differentiation and myelination of dorsal root ganglion neurons. These effects were corroborated with a small-interfering RNA (siRNA) to p38α which decreased levels of myelin associated glycoprotein (MAG), and galactosylceramide (GalC) staining in OLG membrane sheets (Fragoso et al. 2007). From these results, we concluded that p38α was essential for OLG differentiation; however, the downstream targets of p38 and molecular mechanisms that regulate this process still remained to be determined.

p38 MAPK signals through a number of downstream kinases including the mitogen-and stress-activated protein kinases (MSKs), mitogen signal interacting kinases (MNKs), and the MAPK-activated protein kinases (MKs). The MK family members are serine/threonine kinases comprised of MK2, MK3, and MK5 that vary in their function and activation of downstream targets. The most studied kinase, MK2, is involved in cell cycle regulation, migration, actin cytoskeleton organization, and chromatin remodeling (reviewed by (Gaestel 2006; Ronkina et al. 2008). p38 MAPK phosphorylates MK2 on multiple amino acid residues, including Thr-25, -222, -334 and Ser-272, which result in its activation (Ben-Levy et al. 1995)**.** More recently, MK2 has been implicated in cellular differentiation, for example, the conversion of fibroblasts to myofibroblasts (Hagood and Olman 2007; Liu et al. 2007; Sousa et al. 2007).

Here, we extend our previous findings to report that MK2 is a key player in the regulation of OLG differentiation. We used a p38α/MK2 docking inhibitor, CMPD1 [4-(2'-Fluorobiphenyl-4-yl)-N-(4-hydroxyphenyl)-butyramide] (Davidson et al. 2004), and MK2 siRNA to study its role(s) in OLG differentiation. Treatment of OLPs with CMPD1 decreased myelin proteins and

GalC staining in OLG membrane sheets. MK2 siRNA decreased MAG expression and reduced GalC staining, confirming the effects with CMPD1. Levels of phosphorylated MK2 (pMK2) were highest in early stages of OLG development, decreasing slightly with differentiation. CMPD1 also reduced mRNA levels of myelin-specific markers while increasing those of factors inhibitory to OLG differentiation, thereby implicating MK2 in the regulation of OLG differentiation at the transcriptional level. Furthermore, we showed that p38α co-immunoprecipitates with MK2, suggesting these kinases form functional complexes in developing OLPs. As OLP differentiation progressed, small heat shock protein 25 (hsp25) became associated with MK2 and p38α. In addition, p38, MSK1 and cAMP-response element binding protein (CREB) associated in a separate complex that did not contain MK2. Together our data show that the MK2 is an effector of the signaling cascade regulating OLG differentiation.

Materials and Methods

Reagents and Supplies

Ham's F12 medium, 7.5% BSA fraction V, penicillin/streptomycin, and DAPI were purchased from Invitrogen (Burlington, ON, Canada); fetal calf serum and DMEM from Wisent Inc (St-Bruno, QC); $PDGF_{AA}$ and bFGF from PeproTech (Rocky Hill, NJ); CMPD1 from EMD Chemicals (San Diego, CA); Kodak XRP-5 film and nitrocellulose membranes from Mandel Scientific (Guelph, ON, Canada); A_2B_5 mouse monoclonal antibody (mmAb) from American Type Culture Collection (CRL 1520). ECL Western Blotting Detection reagents were from Perkin Elmer (Boston, MA); poly-D-lysine, poly-L-ornithine, human transferrin, insulin, HEPES, Triton-X-100, and DTT from Sigma-Aldrich (Oakville, ON). Other primary antibodies were from the following suppliers: mmAb against MBP and CNP from Sternberger Monoclonals (Lutherville, MD); anti-OLIG2 (Santa Cruz Biotechnology, CA); rabbit polyclonal antibodies to phospho-MK2 (Thr334), MK2, CREB, MSK1 and p38α were from Cell Signaling Tech. (Danvers, MA); rabbit polyclonal anti-hsp25 was from StressGen Bioreagents (Ann Arbour, MI). HRP-, FITC- or Texas Red-conjugated secondary antibodies were from Southern Biotech., or Jackson Immunoresearch (Cedarlane, Hornby, ON) or BIO-RAD (Mississauga, ON). All other reagents were from Fisher Scientific (Whitby, ON), or VWR (Mont-Royal, QC).

Cell cultures

OLP primary cultures were prepared from newborn Sprague-Dawley rat brains as described (Almazan et al. 1993; McCarthy and de Vellis 1980). Experiments were approved by the McGill Animal Care Committee. OLPs were plated on poly-D-lysine-coated dishes and grown in serum free medium (SFM) consisting of a DMEM-F12 mixture (1:1), 10 mM HEPES, 0.1% bovine serum albumin, 25 µg/mL human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 µg/mL insulin, 16 µg/mL putrescine, 30 nM

selenium and 2.5 ng/mL each of $PDGF_{AA}$ and bFGF. Under these conditions, progenitors keep dividing, while removal of the mitogens initiates their differentiation. Culture medium is replaced every 2 days under all experimental conditions. Cultures were immunocytochemically characterized with cell-type-specific antibodies to determine their purity. More than 95% of the cells were A_2B_5 positive, a gangliosides marker for OLPs, while less than 5% were GalC-positive OLGs, glial fibrillary acidic protein-positive astrocytes or complement type-3-positive microglia. The progressive expression of stage-specific markers was studied previously in detail. Cells acquire surface sulfatides ($O4^+$) on day 1-2 following growth factor removal, galactocerebroside ($O1^+$) and MAG on day 2-3 and myelin basic protein on day 2-4 (Cohen and Almazan 1994; Radhakrishna and Almazan 1994).

Western blotting

After treatment, cells were lysed in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and protein quantified with the bicinchoninic acid assay. Proteins were resolved by SDS-PAGE, and Western blotted as described (Fragoso et al. 2007).

Proliferation Assay

Cells were grown to a density of ~1.5×10^5 cells/cm^2 in 24-well dishes. Following the removal of $PDGF_{AA}$ and bFGF, OLPs were incubated with 1 μCi/mL [^{3}H]-thymidine in SFM +/- CMPD1 (1-10 μM). After 24 h, the medium was aspirated and cultures were rinsed three times with ice-cold 5% trichloroacetic acid and solubilized in 0.2 N NaOH and 0.1% Triton-X-100. Radioactivity was quantified liquid scintillation counting.

MTT Assay

Cell viability was assessed using the MTT assay which measures mitochondrial dehydrogenase activity as described (Mosmann 1983). Following the removal of $PDGF_{AA}$ and bFGF to initiate differentiation, cells were incubated with CMPD1 (1-10 μM). After 24h, cultures were incubated with 0.5 mg/ml MTT at 37°C for

3 h after which the formazan crystals were solubilized in acidified isopropanol. Absorbance was measured at 595 nm by spectrophotometry.

siRNA transfections and knockdown of MK2

Small interfering RNAs (siRNAs) designed to target MK2, and fluorescently-tagged controls (siGLO-DY^{547}) were purchased from Dharmacon (Chicago, IL). Primary OLGs were transfected using the siIMPORTER reagent (Upstate Biotechnology, Lake Placid, NY) and 80 nM siRNAs for 48 h as described (Fragoso et al. 2007).

RNA Extraction and Real-Time PCR

Total RNA was extracted from OLGs using the TRIzol reagent according to manufacturer's instruction (Invitrogen). One microgram of DNaseI-treated RNA was reverse-transcribed using the AMV reverse transcriptase (Roche Diagnostics, Laval, Quebec). Primers were designed to span exon-exon junctions (sequences are available upon request). One µL of the total cDNA sample was analyzed, using the Roche LightCycler 2000, and SYBR Green master mix (SABiosciences, Frederick, MD, USA). PCR amplifications were performed as described (Fragoso et al. 2007). Changes in mRNA levels were determined using the $\Delta\Delta C_T$ method. The C_T (crossing point value) of the treated samples are compared to that of controls, which are normalized to the reference gene GAPDH using the equation $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T(sample)} - \Delta C_{T(reference)}$ (Livak and Schmittgen 2001).

Immunoprecipitation

OLPs were harvested in RIPA buffer supplemented with protease and phosphatase inhibitors. Lysates were centrifuged and the post-nuclear supernatant was pre-cleared with 20 µL of protein A agarose for 1 hr at 4°C. Pre-cleared supernatant (250 µg protein) was incubated with 1-2 µg of primary antibody and rocked gently overnight at 4°C. Immune complexes were captured using 20 µL of protein A resin, centrifuged at a 100 X g and washed three times with RIPA buffer. A small volume of 1X sample buffer was added, boiled for 5

min, electrophoresed and analyzed by Western blotting using anti-light chain- or anti-heavy chain-specific secondary antibodies. Thus, detection of MK2 and CREB were not obscured by denatured IgG heavy chains (~ 50 kDa) from primary antibodies used for immunoprecipitation. For hsp25, the anti-heavy chain antibody was used so denatured light chains (~ 25 kDa) did not obscure its detection.

Immunocytochemistry

Immunofluorescence was performed as described (Fragoso et al. 2007). Briefly, cultures on poly-D-lysine-coated glass coverslips were exposed to A_2B_5, anti-O1 (GalC), or anti-O4 (sulfatide) monoclonal antibody (Sommer and Schachner 1981) at 37°C, fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), and incubated with the second primary antibody (pMK2 (1:200), or tubulin (1:200)) overnight. Coverslips were washed three times with PBS, and incubated with TexasRed or FITC-conjugated secondary antibodies for 1 h at room temperature, followed by DAPI staining, and mounting.

Quantification and statistical analysis

Densitometric quantifications of Western blots were performed using AlphaEase (AlphaInnotech, San Leandro, CA) and statistically analyzed by GraphPad 5.0 software. Unpaired t-tests were used when comparing two groups and Dunnett's for multiple comparisons. Results were expressed as mean ± SEM from 3 separate experiments. $P < 0.05$ was considered statistically significant.

Results

MK2 activation and subcellular localization in developing OLGs

OLGs were harvested at four time points during differentiation, and pMK2 levels were determined by Western blot analysis. Day 0 (d0) represents OLPs expressing gangliosides ($A_2B_5^+$), d2 are sulfatide-positive pre-OLGs ($O4^+$), d3 are GalC-positive ($O1^+$) and d4 are MBP-positive maturing OLGs. We found that pMK2 levels increased as OLPs differentiated and accumulated MAG and MBP, two markers of OLG differentiation (Figure 1A, B). Analysis of pMK2 by immunocytochemistry during OLG development showed that the protein is diffusely localized to the cytoplasm and nucleus in $A_2B_5^+$ OLPs. In contrast, pMK2 is mostly detected in the nucleus in maturing OLGs (Figure 2).

MK2 inhibitor decreases expression of myelin-specific markers

We have reported that p38α MAPK is implicated in OLG differentiation (Fragoso et al. 2007). To further characterize the molecular mechanisms involved in this process, CMPD1, a p38α/MK2 docking inhibitor was used. CMPD1 selectively blocks the activation of MK2, and not ATF-2, MSK1, or MSK2 (Supplemental Figure 1A) (Davidson et al. 2004; Lukas et al. 2004). OLPs cultured in the presence of the mitogens $PDGF_{AA}$ and bFGF are mostly bipolar and express gangliosides recognized by the A_2B_5 antibody. Removal of mitogens initiates morphological differentiation and sequential expression of lipid antigens, sulfatide and GalC, and myelin specific proteins, including 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), MBP, proteolipid protein (PLP), and MAG. To determine whether MK2 plays a role in OLG differentiation, OLP were treated with CMPD1 and the levels of lipid, mRNA and protein were examined using biochemical and immunocytochemical approaches. CMPD1 did not affect cell proliferation or survival up to 5 μM (Supplemental Figure 1B, C).

Steady-state levels of mRNAs encoding myelin proteins were determined in OLG differentiated for 2 days in the absence or presence of 5 μM CMPD1 (Figure 3). Real-time PCR analysis showed that CMPD1 decreased mRNA levels

for MAG by 80% ($p < 0.001$), MBP by 50% ($p< 0.001$) and reduced mRNA of Opalin, a recently identified myelin transcript, by 50% ($p < 0.01$). Interestingly, we observed a 95% decrease ($p < 0.001$) in mRNA levels of myelin transcription factor 1 (Myt1), a zinc-finger transcription factor which binds the promoter of proteolipid protein, and has been shown to regulate proliferation and differentiation in OLGs (Nielsen et al. 2004; Romm et al. 2005). We also observed a decrease in OLIG2 mRNA levels (25%, $p < 0.05$), an OLG-specific transcription factor that regulates differentiation (Wang et al. 2006). However, mRNA encoding Sox10, a transcription factor shown to regulate the expression of MBP in OLGs (Stolt et al. 2002), was not significantly increased by CMPD1 treatment ($p > 0.05$).

To further understand the mechanisms by which MK2 regulates OLG differentiation, we assessed mRNA levels of factors that are inhibitory to OLG lineage progression. Activation of the Wnt/β-catenin pathway in OLGs prevents the accumulation of myelin differentiation markers (Fancy et al. 2009; Feigenson et al. 2009; Ye et al. 2009) and is mediated through the β-catenin transcriptional target, Tcf4. Treatment with 5 μM CMPD1 increased Tcf4 by 170% ($p < 0.01$) and the inhibitor of differentiation 2 (Id2) by 90% ($p < 0.01$). We also determined mRNA levels of Notch, a transmembrane receptor which normally represses OLG differentiation (Popko 2003). The levels of Notch mRNA were increased by 270% ($p < 0.01$) in CMPD1-treated OLGs, however, no increase in Hes5, a downstream effector of Notch signaling was observed (Figure 3).

CMPD1 treatment reduces myelin protein expression

To assess the effect of CMPD1 on OLG differentiation, cells were treated for 8d with the MK2 inhibitor, and myelin protein levels were determined by Western blotting (Figure 4). CMPD1 dose-dependently decreased MAG and MBP expression, reaching ~70% at 5 μM ($p < 0.001$). However, it had no effect on OLIG2, and caused only a small decrease in CNP and CGT levels (~25% at 10 μM CMPD1, $p < 0.01$). Furthermore, the cell cycle regulator, $p27^{kip1}$, was only slightly reduced compared to the results we previously reported with the p38

inhibitor, PD169316 (Fragoso et al. 2007). To determine whether the activity of MK2 was required for OLP differentiation throughout the culture period, CMPD1 was added at different time points from d0 to d8 and all groups were harvested on day 8 (Figure 5A, B). The results showed that continuous presence of CMPD1 maximally reduced MAG protein expression (~50%, $p < 0.001$); while its addition on d2 or d4 decreased the effect ($p < 0.01$) and on d6 the drug had no effect ($p > 0.05$). Furthermore, the effect of the inhibitor was fully reversible as 2d treatment with CMPD1 followed by a 6d recovery period restored MAG protein levels to non-treated controls (Figure 5C, D). In cultures treated for the first 4 or 6 days and recovered for 4 or 2 days, respectively, MAG levels were only partially restored ($p < 0.05$ and $p < 0.01$). Immunocytochemical analysis revealed that GalC levels were significantly reduced in CMPD1 treated cultures, although the cells remained morphologically branched as demonstrated by tubulin staining (Figure 6A).

Knock-down of MK2 with siRNA corroborated effects of CMPD1

To further substantiate the results with CMPD1, a small interfering RNA was used to knock-down MK2. OLPs were transfected with 80 nM siRNA and analyzed 48 hrs later for the expression of MK2 and OLG markers. Western blot analysis with an antibody for total MK2 revealed a ~90% knock-down of MK2 following siRNA treatment. Samples were also analyzed for MAG that decreased by ~50% following treatment with MK2 siRNA (Figure 6B). Furthermore, immunostaining of MK2-siRNA treated cultures showed a reduction in GalC staining (Figure 6C), similar to the results we observed with CMPD1.

MK2 can form co-immunoprecipitation complexes with p38α and hsp25

If MK2 is indeed a target of p38 signaling in OLPs responsible for mediating the downstream effects of p38α, we hypothesized they would form a protein complex which could be co-immunoprecipitated from cells. Three other potential binding partners are the downstream mitogen- and stress-activated protein kinases (MSKs), the transcription factor cAMP-response element binding protein (CREB) and small heat shock protein 25 (hsp25). OLPs and 2d differentiated OLGs were harvested, and co-immunoprecipitated with antibodies to p38α MK2, CREB, MSK1 or hsp25. Prior to OLP differentiation, we found that p38α and MK2 were co-immunoprecipitated in a protein complex (Figure 7). As the OLPs differentiated, hsp25 was detected with the p38α-MK2 signaling complex, suggesting possible roles for this association in cytoskeletal remodeling. We next determined if CREB associated with this complex, and found that it could be immunoprecipitated with p38α and MSK1, but not with MK2. Normal rabbit serum IgG did not immunoprecipitate any of the protein complexes (results not shown).

Discussion

OLG differentiation and myelination (Fragoso et al. 2007; Haines et al. 2008) requires p38 MAPK activity but the downstream effectors remained unknown. Here, we show that MK2 is involved in OLG lineage progression since a pharmacological inhibitor and siRNA to MK2 decreased myelin-specific lipids and proteins while increasing several factors that prevent differentiation.

Our observation that pMK2 levels were increased in early-stage OLGs, suggested a role for activated MK2 in OLG differentiation which could affect cell cycle control, migration and cytoskeletal remodeling as reported in other systems (Gaestel 2008). The MK2 inhibitor CMPD1 decreased several myelin-specific proteins, including MAG, and MBP, and reduced GalC staining in OLG membrane sheets. These findings were corroborated in experiments with an siRNA to MK2, where decreases in MAG and GalC were also observed. These results are similar to those reported with the p38 inhibitors (PD16936) or siRNA; however, the magnitude of the effects was different. For example, PD169316 treatment caused larger decreases in CNP, and the cell cycle inhibitor $p27^{kip1}$ protein levels as well as a more significant reduction in GalC staining in OLG membrane sheets. Furthermore, we observed a 25% decrease in Sox10 in PD169316-treated OLPs (Fragoso et al. 2007), however this was not observed with CMPD1. MK2, therefore, appears to mediate certain downstream functions of p38 in OLG differentiation. Other downstream targets of p38, for example, MK3, MK5 or MSK1/2, may regulate other aspects of OLG differentiation. In addition, MK2 activity is important for the early stages of differentiation, since the effect of the inhibitor was more pronounced when applied at the OLP stage. Furthermore, the effects were reversible, suggesting that the MK2 inhibitor delays OLG lineage progression.

To investigate possible mechanisms through which MK2 regulates OLG differentiation, we examined gene transcripts levels for myelin-specific proteins, transcription factors, and factors that inhibit OLG maturation. When MK2 activity was blocked in OLPs, we observed reduced levels of myelin-specific mRNAs, including those encoding MAG, MBP and Opalin. Opalin is an OLG-

specific protein expressed in differentiated OLGs (Aruga et al. 2007), where it is localized to the paranodal loop regions (Yoshikawa et al. 2008), and may play a role in myelin formation and maintenance of its cytoarchitecture. The Opalin gene promoter contains consensus sequences for binding of Myt1 and CREB (Aruga et al. 2007). One of the most dramatic effects of CMPD1 was on the levels of the transcription factor Myt1, which is involved in OLG lineage progression (Nielsen et al. 2004). Myt1 levels were reduced by ~90%, suggesting that MK2 is involved in an early stage in progression of OLPs to a differentiated phenotype. Furthermore, we found that MK2 signaling regulated mRNA levels of various transcriptional repressors in OLGs. For example, Tcf4, a transcription factor downstream of the Wnt/β-catenin signaling pathway which is inhibitory to OLG differentiation and myelination, (Ye et al. 2009), was increased in OLGs treated with the MK2 inhibitor. In addition, the inhibitor of differentiation, Id2, which normally decreases during OLG differentiation (Gokhan et al. 2005; Samanta and Kessler 2004), was increased with CMPD1 treatment. Id2 is a direct gene target of transactivated Tcf4 (Memezawa et al. 2007; Rockman et al. 2001), and mutation of the Tcf4 binding site in the Id2 promoter abolishes the β-catenin induced promoter activity (Rockman et al. 2001). We found that MK2 also increased the expression of Notch1 mRNA, a transmembrane receptor involved in cell fate decisions and neural development (Liu et al. 2006). Notch signaling is inhibitory to OLG differentiation, as heterozygous gene knock-out mice show an accumulation of myelin-gene products in post-natal brain (Givogri et al. 2002). Notch 1 signals to downstream effectors, including Hes5, a member of the basic helix-loop-helix family of transcription factors (Wegner 2008), which also decreases during OLG differentiation (Kondo and Raff 2000; Wang et al. 1998). In contrast to the increase in Notch, we observed no change in Hes5 mRNA levels in OLPs treated with MK2 inhibitor. This suggests that MK2 regulates Notch signaling at the level of the receptor, but Hes5 is not involved. OLIG2 mRNA, an essential regulator of OLG differentiation (Takebayashi et al. 2002), was also decreased by the MK2 inhibitor. Taken together, these data suggest that MK2 normally suppresses the expression of some factors involved in OLG maturation,

although MK2 is not the only p38 downstream effector that could regulate this process. The exact mechanism by which MK2 regulates the expression of these inhibitory factors remains to be determined. One possibility is through regulation of chromatin-associated polycomb group (PcG) proteins. MK2 interacts with the human polyhomeotic 2 (HPH2) transcriptional regulator, and it targets components of the polycomb maintenance complex, PRC1 (Yannoni et al. 2004). Furthermore, MK2 physically associates with Edr1/2 and Ring1B, which are components of PRC1 (Schwermann et al. 2009). The roles of PcG proteins in OLG specification and differentiation are only starting to be unraveled. For example, Yin Yang 1 belongs to the PcG family of proteins and regulates OLG differentiation (He et al. 2007), while Ring1B-deficient neural stem cell progenitors display decreased OLG generation (Roman-Trufero et al. 2009). Other mechanisms could include the regulation of serum response factor (SRF), a direct target of MK2 that activates the α-smooth muscle actin gene, a marker of differentiated muscle cells (Heidenreich et al. 1999; Sousa et al. 2007). SRF is expressed in OLGs, and OLPs derived from neuronal SRF-deficient animals failed to mature into CNP^+ OLGs (Stritt et al. 2009).

Our results also showed that pMK2 was diffusely distributed in cytoplasm and nucleus of OLPs, but became more intensely localized to the nucleus as the cells matured, similar to what we observed with p38α staining (Haines et al. 2008). Activated MK2 can influence p38 compartmentalization through regulation of a MK2 nuclear export signal (Engel et al. 1998; Kotlyarov et al. 2002). Therefore, MK2 and p38 may play different roles in OLGs, depending on their subcellular localization during differentiation. In line with this hypothesis, we found that p38α and MK2 form co-immunoprecipitable complexes in OLPs. As OLPs mature, they begin to express the small heat shock protein, hsp25 (Hemdan and Almazan 2008), which plays roles in cytoskeletal organization (Mounier and Arrigo 2002). Hsp25 associated with a p38α/MK2 signaling complex as reported in fibroblasts (Zheng et al. 2006), suggesting roles in cytoskeletal remodeling in developing OLGs. Signaling downstream of p38 also involves CREB activation, a transcription factor phosphorylated by MSK (Arthur 2008; Wiggin et al. 2002).

In maturing OLGs, MSK and CREB associated with p38 but not with MK2, a complex that may result in myelin gene activation. One potential target is Opalin, an OLG specific transmembrane protein, which contains a cAMP-responsive element (Aruga et al. 2007). In addition, activation of p38 and CREB has been reported during OLG differentiation (Bhat et al. 2007); thus our results suggest that MSK is responsible for the phosphorylation of CREB, downstream of p38. Targeted knockdown of MSK1 and CREB in OLGs will provide further insight into the role of these p38α binding partners during differentiation. Furthermore, determining the interactions of p38 and MK2 with other binding partners, including chromatin remodeling factors may reveal mechanistic clues as to how these kinases regulate OLG development.

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Figures

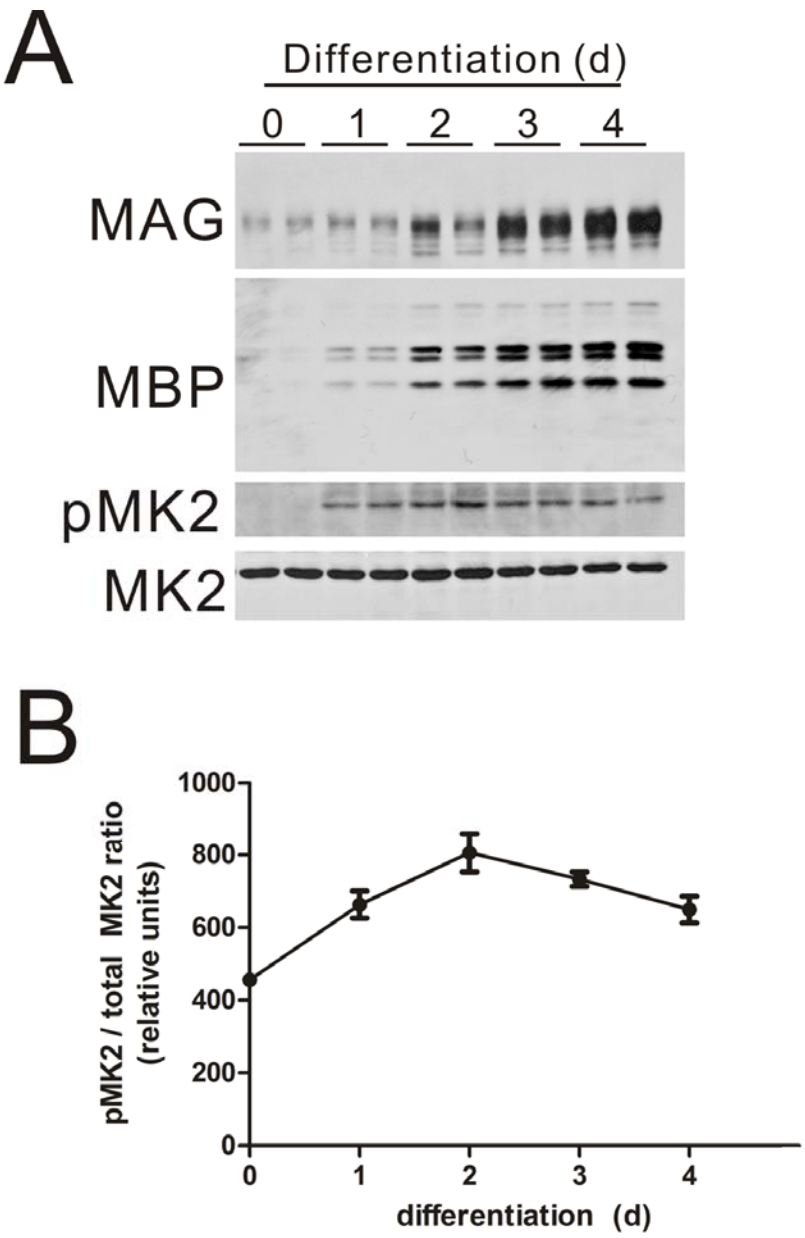


Figure 1. pMK2 levels increase during OLG differentiation. OLPs were allowed to spontaneously differentiate by removing $PDGF_{AA}$ and bFGF from the medium. A) Cells were harvested at d0-d4 of differentiation and lysed for Western blot analysis with antibodies for MAG, MBP, pMK2 and total MK2. B) Relative ratio of pMK2 to total MK2 levels of blots in A).

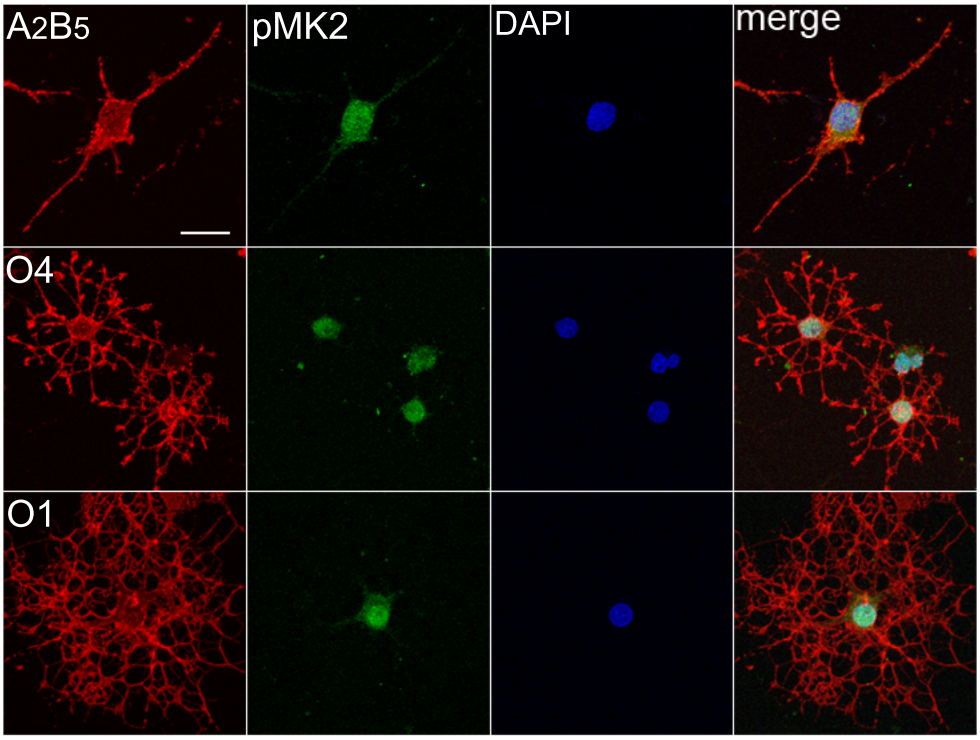


Figure 2. Subcellular localization of pMK2 during OLG development. OLGs at three developmental stages were immunostained with antibodies for either A_2B_5 at day 1, sulfatide (O4) at day 2 or GalC (O1) at day 4 (TexasRed, red) and with anti-pMK2 (FITC, green). DAPI (blue) was used to stain the nuclei. Scale bar represents 10 μm for A_2B_5 image series, and 7.5 μm for O4 and O1 image series.

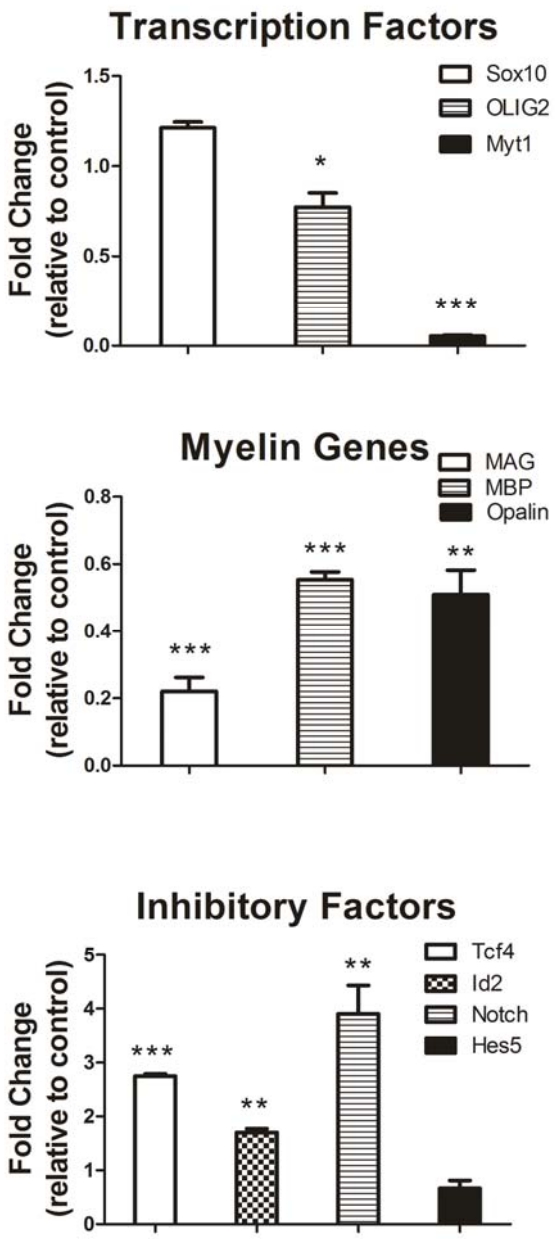


Figure 3. Relative levels of myelin-specific, transcription factors, and inhibitory factor mRNAs determined by real-time PCR. OLPs were treated for 2d with 5 μM CMPD1 and harvested using TRIzol. Complementary DNA was reverse-transcribed from 1 μg of total RNA. All transcript levels were corrected using GAPDH as a house-keeping gene using the $\Delta\Delta C_T$ method. Statistical differences determined using independent t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

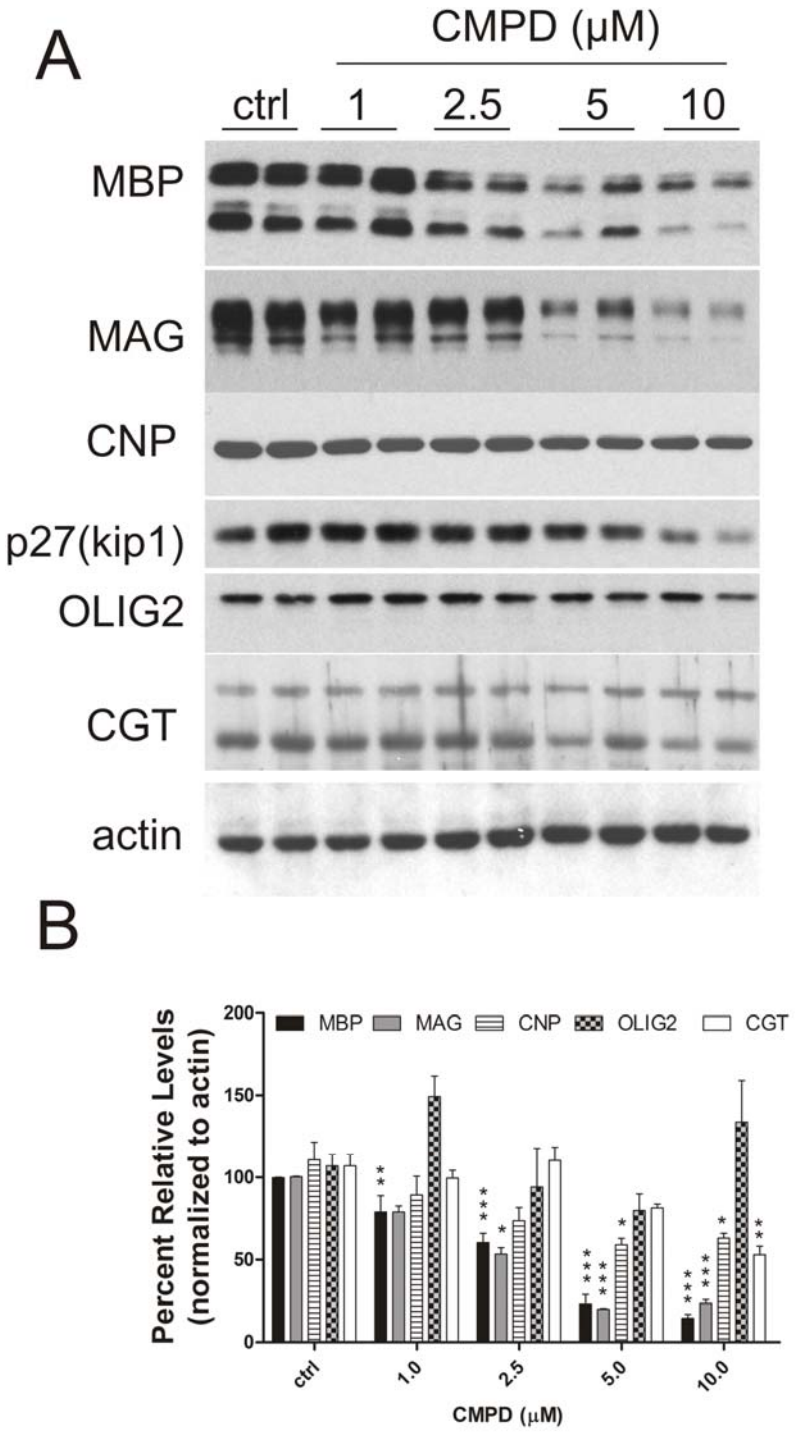

A
CMPD (μM)
ctrl
1
2.5
5
10
MBP
MAG
CNP
p27(kip1)
OLIG2
CGT
actin
B
MBP
MAG
CNP
OLIG2
CGT
Percent Relative Levels (normalized to actin)
200
150
100
50
0
ctrl
1.0
2.5
5.0
10.0
CMPD (μM)

Figure 4

Figure 4. Myelin differentiation markers decrease in CMPD1 treated cultures. A) Treatment of OLPs with increasing concentrations of CMPD1 (1-10 μM) dose-dependently decreased levels of MAG and MBP, while having only small effects on CNP and OLIG2. B) Quantification of blots in A). Statistical differences were determined by Dunnett's multi-comparison test, *p < 0.05, **0.01, ***0.001 as compared to controls, respectively.

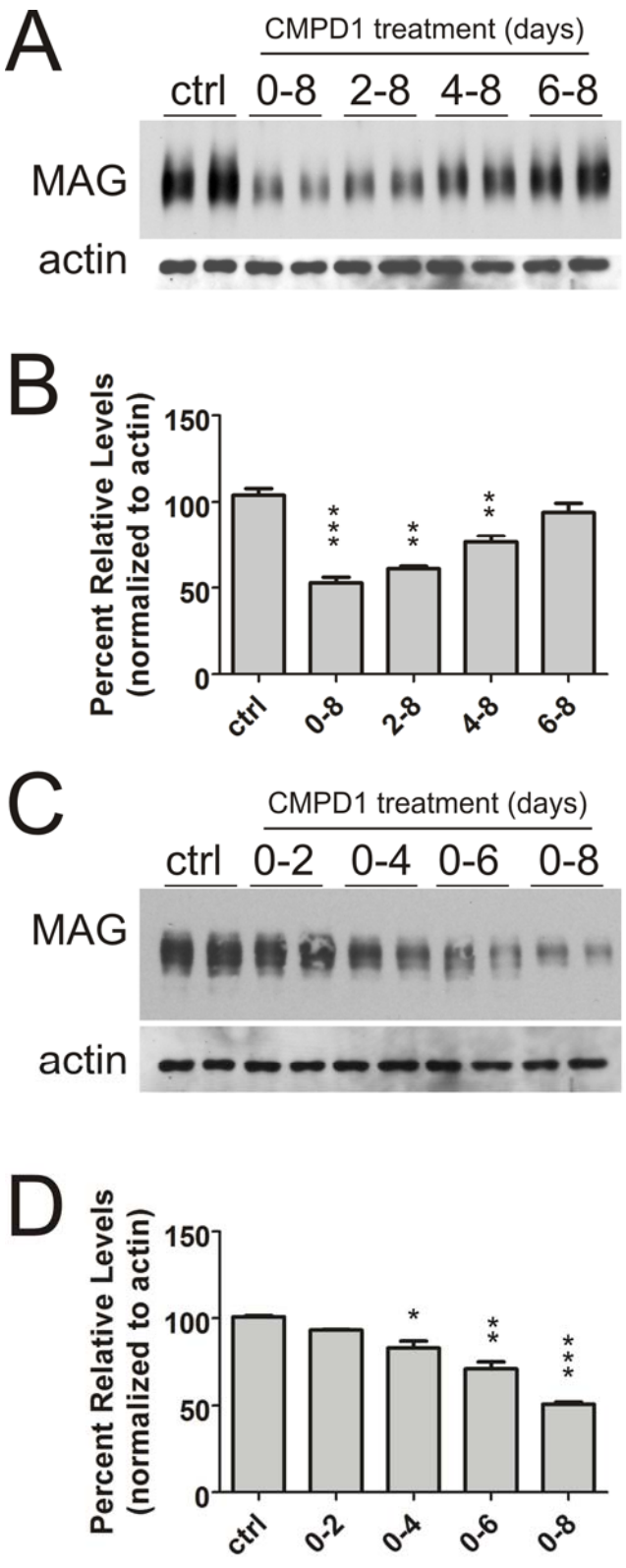

A
CMPD1 treatment (days)
ctrl
0-8
2-8
4-8
6-8
MAG
actin
B
Percent Relative Levels
(normalized to actin)
150
100
50
0
***
**
**
ctrl
0-8
2-8
4-8
6-8
C
CMPD1 treatment (days)
ctrl
0-2
0-4
0-6
0-8
MAG
actin
D
Percent Relative Levels
(normalized to actin)
150
100
50
0
*
**
***
ctrl
0-2
0-4
0-6
0-8

Figure 5

Figure 5. CMPD1 inhibitor blocks OLG differentiation at early stages of development, and the effect is reversible. A) OLPs were treated with 5 μM CMPD1 at different time points following the initiation of differentiation: ctrl (non-treated), d0-8, d2-8, d4-8, d6-8. C) OLPs were treated with CMPD1 for different times (ctrl, d0-2, d0-4, d0-6, d0-8), followed by recovery up to d8. All groups were harvested at d8, followed by Western blotting for MAG and actin. B, D) Densitometric quantifications of blots in A and C. Statistical differences were determined by Dunnett's multi-comparison test, *$p < 0.05$, **0.01, ***0.001 as compared to controls.

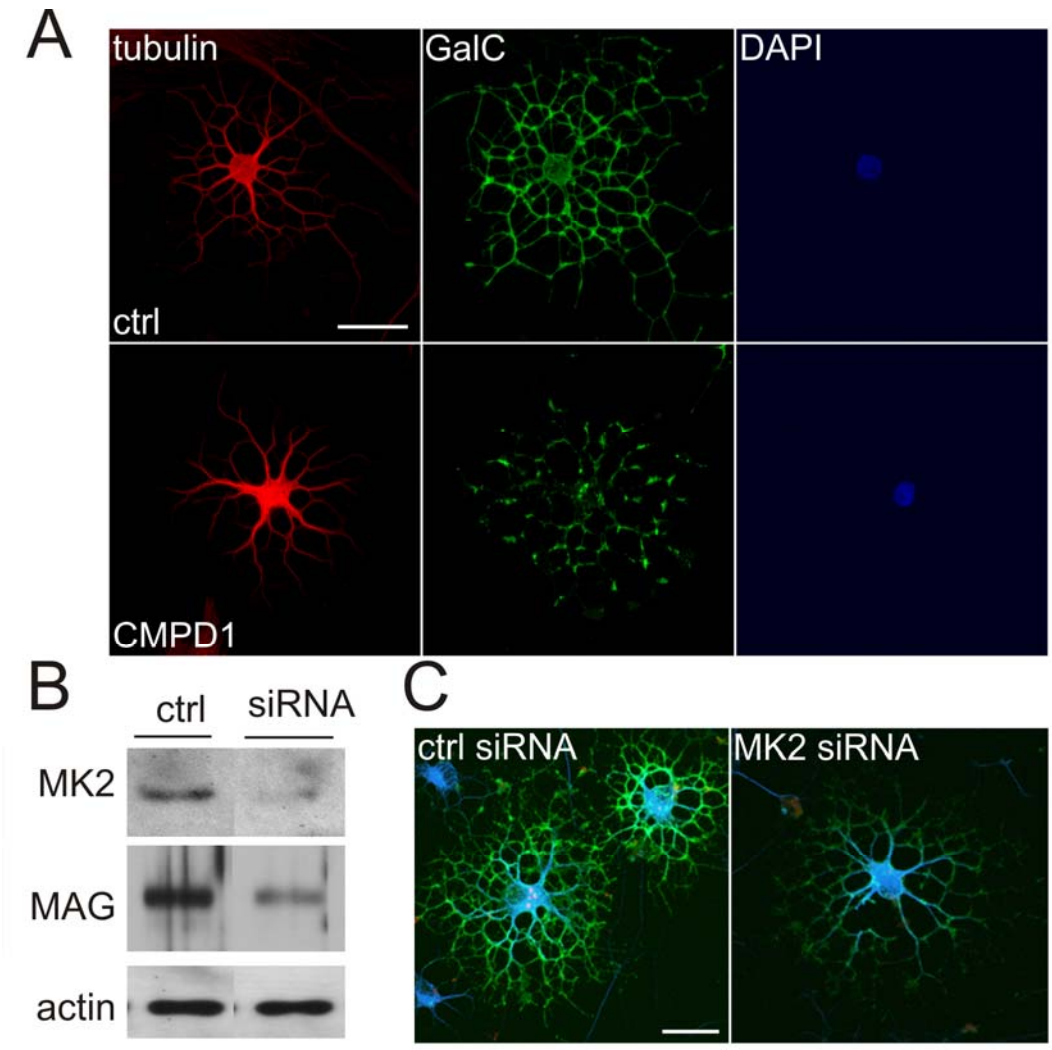

A
tubulin
GalC
DAPI
ctrl
CMPD1
B
ctrl
siRNA
MK2
MAG
actin
C
ctrl siRNA
MK2 siRNA

Figure 6

Figure 6. A) Immunocytochemistry of OLPs treated with 5 μM CMPD1 reveals decreased GalC staining in OLG membrane sheets. Cells were grown for 6d in the presence or absence of CMPD1 and immunostained with antibody for tubulin (TexasRed, red) GalC (FITC, green), and DAPI (blue). Cells were imaged using z-stack layering with a Zeiss dual-laser scanning confocal microscope. Scale bar represents 20 μm. B, C) OLPs were treated with 80 nM negative control or MK2 specific siRNAs for the first 48 hrs of differentiation. Western blots (B) of cell lysates show that MK2 specific siRNA reduced MK2 and MAG protein levels by ~90%, and ~50%, respectively. C) Immunocytochemistry of MK2 siRNA-transfected OLPs reveals a reduction of GalC (FITC, green) staining while branching appears normal as delineated by tubulin staining ($Alexa^{350}$, blue). siRNA delivery into cells was confirmed by co-transfection siGLO (DY^{547}, red). Scale bar represents 15 μm.

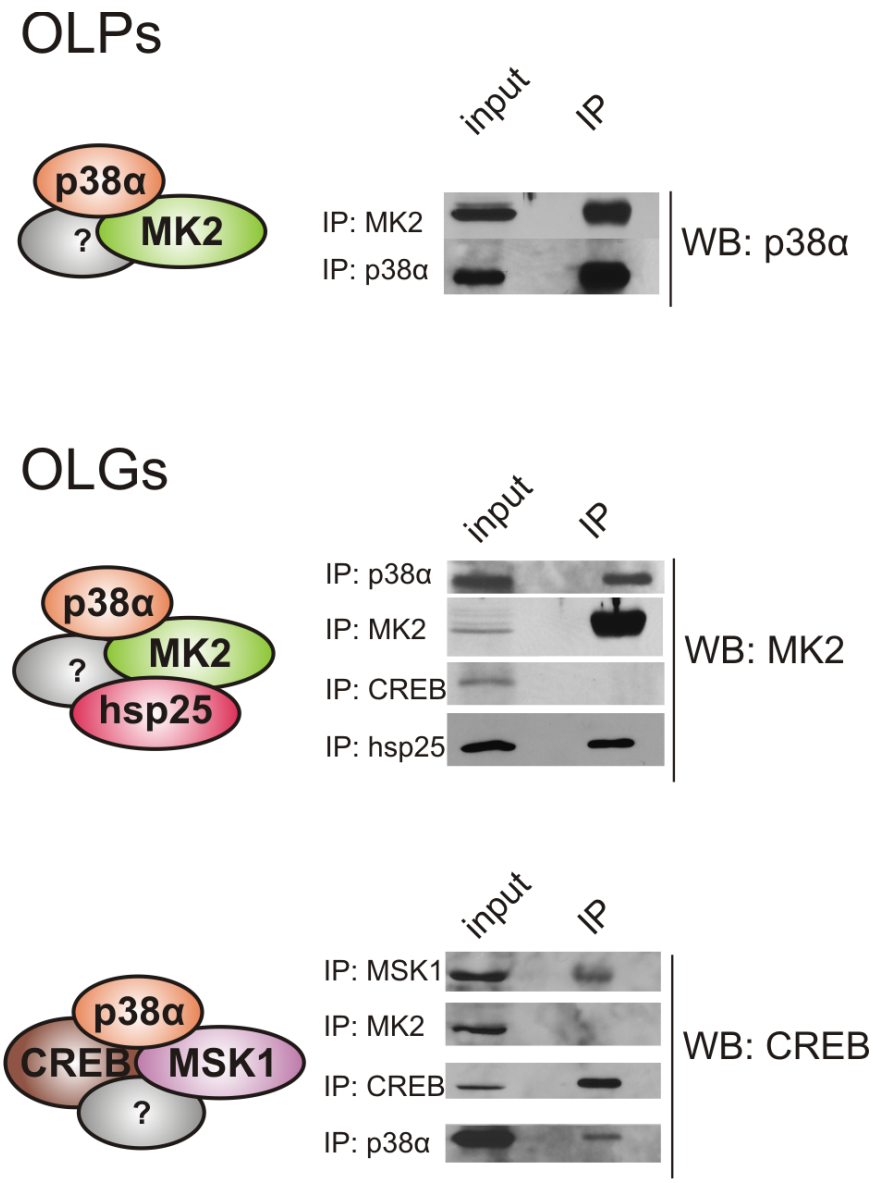
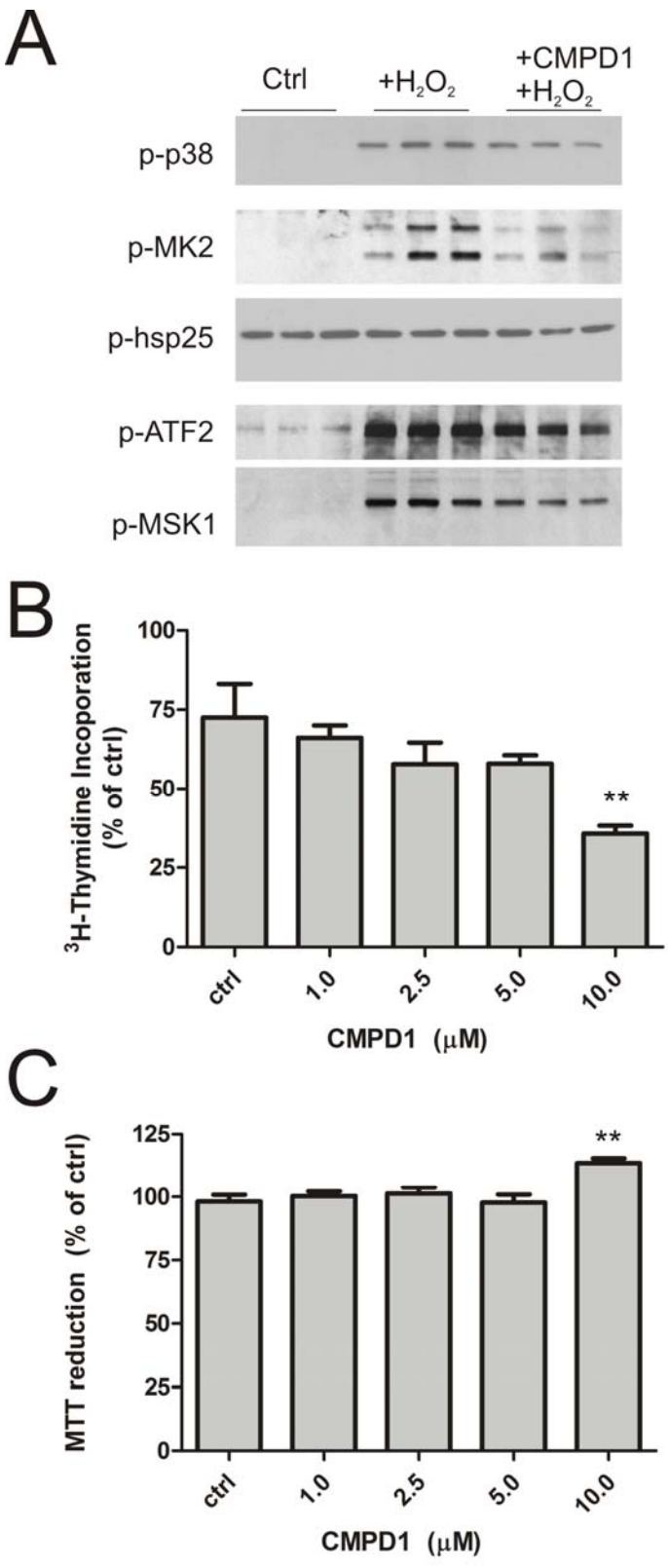


Figure 7. p38α co-immunoprecipitation complexes formed in OLPs and OLGs. Total cell lysates (input) from either OLPs or 2d differentiated OLGs were immunoprecipitated (IP) using p38α, MK2, hsp25, CREB and MSK1 antibodies. Immunoprecipitates were electrophoresed and subjected to Western blotting (WB) with the indicated antibodies.



Supplemental Figure 1

Supplemental Figure 1. A) The p38/MK2 docking inhibitor, CMPD1, potently blocks the hydrogen-peroxide induced p38 activation of MK2 but not MSK1, ATF2, or hsp25. OLGs were pre-treated for 30 min with 5μM CMPD1 prior to stimulation with 100 μM hydrogen peroxide for 10 min. Cells were harvested and subjected to Western blotting with phospho-specific antibodies for p38, MK2, hsp25, ATF2, and MSK1. B) Proliferation and C) survival data for OLPs and OLGs treated with CMPD1. OLPs were treated with CMPD1 (1-10 μM) in serum-free medium for 24 hrs. For proliferation assays, ^{3}H-thymidine was added at the same time as CMPD1, and scintillation counting was performed 24 hours later. For cell viability assays, OLPs were treated with CMPD1 for 24h, followed by the addition of MTT for 4h. Formazan crystals were solubilized and measured spectrophotometrically. Statistical differences determined using Dunnett's multi-comparison test, **$p < 0.01$ as compared to control.

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INTERVENING SECTION 2

In the previous two chapters, we have established that p38 MAPK and its downstream effector MK2 are involved in OLG differentiation. To determine the molecular mechanisms of p38 regulated OLG differentiation, we used a rat whole genome microarray screen and quantitative real-time PCR analysis.

Chapter 4: Transcriptional profiles of p38 mitogen-activated protein kinase regulated genes in oligodendrocytes

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In preparation

Abbreviations

CDKI – cyclin dependent kinase inhibitor

CGT – UDP: galactose ceramide galactosyltransferase

CNP - 2′,3′-cyclic nucleotide 3′-phosphodiesterase

CNS - central nervous system

ERK - extracellular signal regulated kinase

GalC - galactosylcerebroside / galactosylceramide

GPR17 – G protein coupled receptor 17

HDAC – histone deacetylase

Id2 – inhibitor of differentiation 2

JNK - c-Jun amino terminal kinase

MAG – myelin-associated glycoprotein

MBP - myelin basic protein

MAPK - mitogen-activated protein kinase

MK2 - MAPK activated protein kinase 2

MKK – MAPK kinase

MKKK – MKK kinase

MOG – myelin oligodendrocyte glycoprotein

MRF - myelin gene regulatory factor

OLIG2 – oligodendrocyte transcription factor 2

OLG - oligodendrocyte

OLP - oligodendrocyte progenitor

PBK – PDZ domain binding kinase

qRT-PCR – quantitative real-time PCR

SFM - serum free media

siRNA – small interfering RNA

SRF – serum response factor

Tcf4 – transcription factor 4

THRA – thyroid hormone receptor alpha

Abstract

We have previously shown that p38 mitogen-activated protein kinase (p38 MAPK) is important for oligodendrocyte (OLG) differentiation and myelination. However, the mechanisms by which p38 regulates OLG differentiation remain largely unknown. To this end, we performed a microarray analysis on OLGs treated with the p38 inhibitor PD169316 to determine how this kinase may be effecting OLG differentiation. Significant changes in gene expression were grouped according to their functional role. An upregulation of transcriptional repressors including the Wnt/β-catenin target Tcf4, and inhibitor of differentiation 2 (Id2) was observed. In addition, we detected down-regulation of transcriptional activators, including histone deacetylase 11 (HDAC11) and Fyn tyrosine kinase, which are important for OLG differentiation and CNS myelination. We also found a downregulation of myelin-specific transcripts, and an upregulation of cell cycle regulators, suggesting that OLGs treated with PD169316 remain in an immature state. In fact, the largest group of genes upregulated consisted of transcripts encoding proteins involved in cytokinesis, centromere and spindle formation and replication, suggesting that OLPs remain in a proliferative state. However, tritiated thymidine-incorporation assays showed that PD169316 did not induce OLP proliferation. Rather, OLPs remained in a proliferative competent state since application of platelet-derived growth factor-AA (PDGF-AA) and basic fibroblast growth factor (bFGF), two important mitogens for OLPs, to PD169316-treated OLPs was required to induce proliferation. This suggests that PD169316-treated cells exit the cell cycle but fail to differentiate when p38 activity is attenuated. Therefore, our results suggest that p38 controls multiple steps of OLG differentiation through the regulation of genes encoding cell cycle and cytokinesis proteins, and by modulating genes which are normally repressed or activated during normal OLG lineage progression.

Introduction

The regulation of OLG differentiation involves a complex interplay of factors, which properly time the onset of maturation. These include cell cycle regulators, transcriptional activators and repressors, which control OLG differentiation (extensively reviewed by (Li et al. 2009; Wegner 2008; Yu et al. 2010)). We have previously shown that p38 mitogen-activated protein kinase (MAPK) is also responsible for regulating OLG differentiation and central nervous system (CNS) myelination (Fragoso et al. 2007; Haines et al. 2008). Treatment of oligodendrocyte progenitors (OLPs) with p38 inhibitors resulted in a decrease in levels of mRNA and protein for myelin-specific proteins, including myelin basic protein (MBP) and myelin associated glycoprotein (MAG) (Fragoso et al. 2007). In addition, the cell cycle regulator $p27^{kip1}$ and the transcription factor Sox10 were also significantly reduced in OLPs treated with p38 inhibitors. Furthermore, a small-interfering RNA to p38α resulted in decreased levels of MAG, and galactosylceramide (GalC) staining in OLG membrane sheets. From these results, we concluded that p38α was necessary for OLG differentiation. More recently, we have shown that MK2, a downstream effector of p38 MAPK, is an element of the signaling pathway responsible for regulating OLG differentiation (Haines et al. 2010). However, the exact mechanisms by which p38 MAPK signaling regulate OLG differentiation still remained elusive.

Gene expression profiling has had great utility in identifying factors involved in cell proliferation and differentiation. For example, purified rat immature OLPs, pre-myelinating and post-mitotic OLGs have been gene profiled, revealing dynamic regulation of transcripts during OLG differentiation (Dugas et al. 2006; Nielsen et al. 2006). More recently, gene expression profiling has been performed on highly purified, acutely isolated neurons, astrocytes and OLGs from mouse forebrain to further understand neural and glial development (Cahoy et al. 2008). These analyses have lead to the identification of novel factors that are responsible for promoting OLG differentiation including myelin gene regulatory factor (MRF)

(Emery et al. 2009), and G protein coupled receptor 17 (GPR17) (Chen et al. 2009). Furthermore, in cardiomyocytes, gene targets regulated by p38 MAPK have been identified, including genes involved in cell division, cell signaling, inflammation and adhesion (Tenhunen et al. 2006). Therefore, in order to better understand how p38 MAPK signaling regulates OLG differentiation, we performed an Illumina rat whole genome microarray analysis on oligodendrocyte progenitors (OLPs) treated with the p38α/β isoform selective pyridinyl imidazole based inhibitor, PD169316. Microarray data were analyzed using FlexArray software using EB Wright & Simon analysis to determine molecules up- or down-regulated by PD169316 treatment. In agreement with our previous findings, a number of myelin-specific genes were decreased following PD169316 treatment. In addition, we observed changes in transcripts encoding kinesins, actin and microtubule cytoskeletal proteins, a vesicle transport protein, cell cycle regulators, transcription factors, chromatin modifiers, replication, centromere and spindle formation. Furthermore, we found decreased mRNA levels of Fyn tyrosine kinase and histone deacetylase 11 (HDAC11), two factors that normally promote OLG differentiation. From these data, it appears that p38 controls OLG differentiation through the regulation of factors that normally promote or inhibit OLG maturation, including the transcriptional repression of many genes involved in cell division.

Materials and Methods

Reagents and Supplies

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 medium, PBS, 7.5% BSA fraction V, and penicillin/streptomycin were purchased from Invitrogen (Burlington, ON, Canada). Fetal calf serum and DMEM were from Wisent Inc (St-Bruno, QC); PDGF-AA and bFGF from PeproTech (Rocky Hill, NJ). PD169316 was from EMD Chemicals (San Diego, CA). Poly-D-lysine, poly-L-ornithine, human transferrin, insulin, HEPES, Triton-X-100, DTT were from Sigma-Aldrich. All other reagents were from Fisher Scientific (Whitby, ON), or VWR (Mont-Royal, QC).

Cell cultures

Primary cultures of oligodendrocyte progenitors (OLPs) were prepared from the brains of newborn Sprague-Dawley rats as described previously (McCarthy and de Vellis 1980; Almazan, Afar et al. 1993). All experiments were approved by the McGill Faculty of Medicine Animal Care Committee in accordance with Canadian Council on Animal Care guidelines. OLPs were plated on poly-D-lysine-coated culture dishes and grown in serum free media (SFM) consisting of a DMEM-F12 mixture (1:1), 10 mM HEPES, 0.1% bovine serum albumin, 25 μg/mL human transferrin, 30 nM triiodothyronine, 20 nM hydrocortisone, 20 nM progesterone, 10 nM biotin, 5 μg/mL insulin, 16 μg/mL putrescine, 30 nM selenium and 2.5 ng/mL each of PDGFAA and bFGF. The OLPs are changed with media containing mitogens every 2d to maintain the cells in a proliferative state. OLPs spontaneously differentiate upon removal of mitogens. Cultures were characterized immunocytochemically with cell-type-specific antibodies. More than 95% of the cells were positive for gangliosides detected with monoclonal antibody A_2B_5, a marker for OLPs in culture while less than 5% were GalC-positive OLGs, GFAP-positive astrocytes or complement type-3-positive microglia (Cohen and Almazan 1994; Radhakrishna and Almazan 1994). The

culture media was changed every 2d, and PD169316 was used at 5.0 μM for all experiments, unless otherwise indicated.

RNA extraction

Total RNA was extracted from OLGs (~500,000 cells) differentiated in the absence or presence of 5 μM PD169316 for 1d, 2d or 3d using the Qiagen RNeasy kit (Qiagen, Mississauga, ON, Canada). Genomic DNA was eliminated using an on-column DNase digest (DNase set, Qiagen). The RNA was divided into separate aliquots for microarray analysis and quantitative PCR validations. RNA quality was assessed by Genome Quebec using an Agilent RNA BioAnalyzer (Agilent Technologies, Santa Clara, CA), followed by a Qiagen RNA clean-up column, and quantification using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Illumina Microarray

Gene expression was determined in control and PD169316-treated OLGs using Illumina Rat Whole Genome microarrays in collaboration with Genome Quebec. Complementary RNA (cRNA) was prepared from 250 ng of total RNA using the Ambion TotalPrep RNA Amplification kit according to the manufacturer's protocol (found at http://www.ambion.com/techlib/prot/fm_IL1791.pdf). The cRNA (750 ng) generated from this kit was hybridized to a Rat Illumina Whole Genome Microarray according to Illumina's protocol available on their website (http://www.illumina.com/applications.ilmn#gene_expression_analysis).

FlexArray Analysis

The EB (Wright and Simon) statistical correction was used for fold-change values and p-values analysis. The EB (Wright & Simon) analysis provides ratio data for treatment value compared to control samples for each gene, the t-statistic and the associated p value. The ratio data re-calculated as fold-change data using -1/x transformation for ratio values below 1.0. Values above 1.0 were not transformed. Samples were n = 3 for each treatment group. Genes with a fold change value +/-

1.25, with a p value less than 0.05 were considered to be statistically different from control. All of the tabulated microarray data are available in the Supplemental Data online.

Functional Determination using UniProt and literature searches

The "Core Analysis" function included in Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA) was used to interpret the rat whole genome microarray data in the context of biological processes, pathways and networks that were regulated during PD169316-treatment. Genes were identified through their NCBI Accession number. Function of the most upregulated and downregulated genes was determined using UniProt (http://www.uniprot.org/), or by PubMed literature searches (http://www.ncbi.nlm.nih.gov/pubmed/).

Quantitative PCR validations

One microgram of DNaseI-treated RNA was reverse-transcribed using the AMV reverse transcriptase (Roche Diagnostics, Laval, Quebec). Primers were designed to span exon-exon junctions (sequences are available upon request). One µL of the total cDNA sample was analyzed per reaction, using the 96 well-block Roche LightCycler 480, and SYBR Green master mix (SABiosciences, Frederick, MD, USA). PCR amplifications were performed as follows: heat inactivation (10 min, 95°C); followed by 35-45 cycles of 94°C, 15 s; 59°C, 30 s; 72°C, 20 s. PCR products were detected by fluorescence at the end of the extension step, and melting curves were analyzed by monitoring the continuous decrease in fluorescence of the SYBR Green signal. PCR products were verified for a single amplification product using melting curve analysis, and the molecular weight of each product was confirmed using PAGE. The fold change in mRNA levels was determined using advanced relative quantification (Pfaffl) method available on the Roche LightCycler 480 software with normalization correction to 28S rRNA.

Proliferation Assays

Cells were grown to an approximate density of 1.5×10^5 cells/cm^2 in 24-well dishes. Cells were treated with an increasing dose (1 – 10 μM) of PD169316 for 24, 36, or 48 hr in SFM without mitogens, and then incubated with 1 μCi/mL ^{3}H-thymidine for an additional 24 hr. In experiments where mitogens were applied after PD169316 treatment, 2.5 ng/mL PDGF-AA and bFGF were added at the same time as the ^{3}H-thymidine and incubated for 24 hr, as above. Following the ^{3}H-thymidine incorporation period, the medium was aspirated and cultures were rinsed three times with 5% ice-cold trichloroacetic acid and solubilized in 0.2 N NaOH and 0.1% Triton-X-100. Aliquots were mixed with Ecolite liquid scintillation counting fluid and emissions were recorded using a β-counter.

Results

In order to better understand how p38 regulates OLG differentiation, we performed an Illumina microarray analysis on rat primary OLPs that were differentiated in the presence or absence of PD169316 for 24h and 48h. The microarray data were analyzed using FlexArray software to determine ratio differences from control. These ratio data were converted to fold change values by taking the negative inverse (-1/x) of any ratio data values below 1.0. Genes that were up- or down-regulated 1.25 fold, with a p-value less than 0.05 were considered to be significantly different from control. We classified the 50 most upregulated and downregulated genes that were affected by 24 hr PD169316 treatment according to their functional classes by UniProt database querying or by literature searches (Figure 1 and Tables 1-5).

Myelin gene transcripts upregulated with OLG differentiation

Oligodendrocyte progenitors are maintained in a proliferative state when maintained in the presence of PDGF-AA and bFGF. Following removal of these two mitogens, OLPs spontaneously differentiate through a maturation program characterized by the increased expression of myelin-specific genes, including MAG, MBP, myelin oligodendrocyte glycoprotein (MOG), and myelin and lymphocyte protein (MAL) which are all markers of differentiated OLGs. In addition, levels of immature cell-stage markers such as the PDGF receptor α (PDGFRα) and Nestin decreased with differentiation. In addition, the Nkx homeobox gene, Nkx6.2 increased with OLG differentiation, which is in agreement with its roles in OLG differentiation (Liu et al. 2003). Another pro-myelin gene activator HDAC11, which promotes the expression of MBP and PLP during OLP maturation, increased with OLG differentiation (Liu et al. 2009). In contrast, the immature OLP markers, PDGFRα and the early specification marker of the Nkx homeobox gene class family, Nkx2.2, decreased with OLG differentiation (Table 1).

p38 inhibitor decreases levels of transcripts encoding myelin-specific, sterol synthesis enzyme, and transcriptional activators

We had previously found that PD169316 treatment decreased levels of myelin-specific gene transcripts, including MBP, MAG, myelin oligodendrocyte basic protein (MOBP), and UDP-galactose ceramide galactosyltransferase (CGT) (Fragoso et al. 2007). In agreement with our previous findings, we found decreases in the levels of MBP, CGT and MAG by Illumina microarray analysis. In addition, we observed decreases in oligodendrocyte myelin glycoprotein (OMgp). Corroborating our previous findings, we observed a large downregulation of MAG by qRT-PCR analysis at three time points of differentiation in the presence of PD169316 (Figure 2). We also found decreases in non-OLG specific genes including squalene epoxidase, an enzyme involved in a key step in the synthesis of cholesterol, a necessary component of myelin membrane formation (Saher et al. 2005). Importantly, we found a downregulation of transcripts encoding Fyn (Table 2 and Figure 2), a Src-like tyrosine kinase family member which is important for OLG differentiation and CNS myelination (Umemori et al. 1994). PD169316 treatment decreased the transcription of genes encoding Nkx6.2, HDAC11, Sox8 and zinc-finger protein 488 (Zfp488), all of which are implicated in activating myelin gene transcription. We used qRT-PCR analysis to confirm the decreases in HDAC11 and found decreases at three different time points of differentiation, thus confirming the effects observed by microarray analysis (Figure 2). In addition, microarray analysis revealed decreased mRNA levels of thyroid hormone receptor alpha (THRA) which binds thyroid hormone T3 to influence the activation of an intracellular timer that regulates OLP differentiation (Billon et al. 2002).

p38 inhibitor increases mRNA levels of OLG transcriptional repressors, and markers associated with early OLPs

Numerous transcriptional repressors have been identified that inhibit the progression of OLP maturation, and function to properly time the onset of OLG

differentiation. These include inhibitors of differentiation (Ids), the Wnt/β-catenin target Tcf4, Sox5/6, Notch and one of its downstream effectors Hes5. The levels of these repressors must normally decrease with OLG maturation in order for differentiation to properly progress. Following PD169316 treatment, we observed an upregulation of Id1 and Id2 (Table 3). We also found an upregulation in the levels of nestin and Nkx2.2 which are markers of early OLPs. A modest increase was also detected in the levels of Sox6, a Sox family member that acts as a transcriptional repressor to block OLG differentiation. Interestingly, however, the levels of PDGFRα were not affected by PD169316 treatment (Supplemental data, Table S1). Furthermore, there were no increases in Notch, or Hes5 by microarray analysis (Supplemental data, Table S1), although we did observe a modest upregulation of Hes5, Tcf4, Id2 when mRNA from PD169316-treated cells was analyzed by qRT-PCR (Figure 2). In addition, qRT-PCR analysis revealed an upregulation of the early specification marker Nkx2.2 following PD169316 treatment (Figure 3).

p38 inhibitor upregulates levels of p38 MAPK upstream activators

The p38 signaling cascade is initiated by numerous growth stimuli to activate a kinase cassette, which includes a number of MAPK kinase kinases (MKKKs). These, in turn, activate MKK3 or 6 to subsequently activate p38. The MKKs are dual-specificity kinases that directly phosphorylate the Thr-Gly-Tyr activation loop on p38. Interestingly, we observed an upregulation of MKK6, following PD169316 treatment, suggesting p38 itself normally negatively regulates levels of its upstream activators. In addition, an atypical upstream MKK activator of p38, known as PDZ-binding kinase (PBK) was also increased following treatment of OLPs with PD169316. PBK specifically activates p38 during cell cycle progression and proliferation (Ayllon and O'Connor 2007). Moreover, a p38 upstream MKKK known as Dlk-1, which is related to the mixed lineage kinases (MLKs), was also upregulated with PD169316 (Holzman et al. 1994; Nakata et al. 2005) (Table 4).

p38 inhibitor affects cell cycle regulators, early growth response proteins and cytokinesis regulators (kinesins, spindle and centromere formation proteins and actin/tubulin reassembly proteins)

The most abundant group (~45%) of upregulated genes were for mRNAs which encode proteins involved in cell cycle progression, spindle and centromere formation, and cytokinesis (Figure 1). Numerous cyclins (A2, B2, D1), cyclin associated proteins, and cell division proteins were also upregulated following treatment of OLPs with PD169316 by both microarray and qRT-PCR analysis (Table 5 and Figure 3). In contrast, the cyclin-dependent kinase inhibitor (CDKI), $p57^{kip2}$ which normally increases with differentiation was decreased in both the microarray and qRT-PCR (Table 5 and Figure 3). p38 also regulates numerous cell cycle checkpoints through the phosphorylation of p53 (Thornton and Rincon 2009). Although we found no changes in mRNA levels of p53 (Supplemental Data, Table S1), we did observe decreased levels of p53 target genes including $p21^{cip1}$ (Figure 3) and GADD45α (Table 5). $p21^{cip1}$ is another CDKI family member that is required for proper cell cycle exit. We also found an upregulation of two mitotic checkpoint serine/threonine-protein kinases BUB1 (budding uninhibited by benzimidazoles 1 homolog) and a related isoform, BUB1B. Moreover, we found that PD169316 upregulated a large group of kinesins involved in microtubule spindle formation during mitosis (Table 5). In agreement with the upregulation in the kinesins, an upregulation of cytoskeletal genes (e.g., tubulin -β2, -β3, -β5, -β6, -γ and anillin) which are involved in mitotic spindle assembly and chromosome separation were also observed. In addition, the levels of Rab33a, a protein involved in vesicular transport was downregulated by PD169316 (Table 5 and Figure 3). Furthermore, the levels of p21-activated kinase 7 (PAK7) were decreased with PD169316 treatment. PAK are serine/threonine protein kinases that regulate Rac/Cdc42 GTPases and are involved in cytoskeletal dynamics. There were also increased mRNAs levels for genes encoding enzymes involved in replication and DNA synthesis, including minichromosome maintenance deficient (MCM), DNA primase and replication factor C. Moreover, a large group of genes involved in spindle body formation

and centromere formation were upregulated by PD169316 treatment, including aurora kinase B (AURKB), centromere protein T (CENPT), nucleolar and spindle associated protein 1 (NUSAP1) and polo-like kinase 1 (PLK-1), kinetochore associated proteins and protein regulator of cytokinesis (PRC1) (Table 5).

p38 inhibitor alone does not induce cell proliferation, however cells remain proliferation competent following p38 inhibition

Considering the large increase in genes involved in cell cycle and mitosis, and the decrease in mRNAs encoding CDKIs and the thyroid hormone receptor, we next examined whether OLPs were proliferating in response to PD169316 treatment. ^{3}H-thymidine incorporation assays were performed on cells treated with PD169316 for 24, 36 and 48 hr (Figure 4). OLPs did not proliferate in response to PD169316 treatment alone, and thymidine incorporation actually decreased at the 36 and 48 hr time points (Figure 4C, E). We next determined if re-applying mitogens to PD169316-treated cells could promote OLPs to re-enter the cell cycle. At the 36 and 48 hr time point we observed an slight increase in thymidine-incorporation following PDGF-AA and bFGF stimulation of PD169316-treated OLPs (Figure 4D, F), suggesting p38 blocks OLG differentiation while maintaining cells in a proliferation-competent state.

Discussion

We previously reported that p38 MAPK played an important role in OLG differentiation and myelination of cultured dorsal root ganglia neurons. In order to better understand how p38 regulates OLG differentiation, we used a rat whole genome microarray analysis to globally survey gene expression changes in primary OLGs treated with the p38 inhibitor PD169316. After grouping the genes into functional classes using either UniProt or literature searches, we found PD169316-treatment upregulated a group of genes involved in cytokinesis, centromere and spindle formation, replication and cell cycle progression. Smaller subsets of genes upregulated by PD169316 treatment included amino acid transporters, cytoskeletal proteins and vesicle transporters, extracellular matrix molecules, and genes involved in oxidative stress. In contrast, the most prominent group of down-regulated genes was generally unclassified or had no known function. Among other downregulated genes were bone morphogenetic proteins, chondroitin sulfate proteoglycans, and cytoskeletal/vesicle trafficking proteins.

Several studies have examined the gene expression profile changes that occur during OLG differentiation (Cahoy et al. 2008). In agreement with these findings, we found a large upregulation of myelin-specific gene products associated with OLG differentiation. In our previous study using PD169316, we observed differential decreases in transcripts encoding myelin genes including MAG, MBP, CNP, PLP and MOBP (Fragoso et al. 2007). This suggested that p38 differentially regulates myelin-specific genes, possibly through the differential regulation of myelin-promoter activities. Although the large majority of genes which were downregulated by PD169316 had no known function, we did observe decreases in myelin-specific genes, including MAG and CGT. We also found decreases in squalene epoxidase, a rate-limiting enzyme that catalyzes the first oxygenation step in cholesterol biosynthesis (Abe et al. 2007). Interestingly, OLG-specific knockout of squalene synthase, an enzyme upstream of squalene epoxidase, reduces the cholesterol content of myelin and causes hypomyelination in mice (Saher et al. 2005). In addition to these decreases in myelin-specific

genes we observed decreases in the levels of pro-myelin gene activators including HDAC11, Zfp488, Fyn, and Nkx6.2. HDAC11 is responsible for activating the MBP and PLP promoter, and siRNA knockdown of HDAC11 causes decreases in MBP and PLP levels (Liu et al. 2009). Zfp488 is exclusively expressed in differentiating OLGs and plays roles in MBP and CNP expression through cooperation with the basic helix-loop-helix factor OLIG2 (Wang et al. 2006), which is also important for OLG differentiation (Zhou et al. 2001). Fyn activity is important for the transport of MBP mRNAs to distal processes of OLGs, and is also responsible for the phosphorylation of MAG, and activating the MBP promoter (Umemori et al. 1999; White et al. 2008). The mechanism of p38-mediated regulation of Fyn expression is unknown, however an interplay between p38 and Fyn has previously been reported in the production of the interleukin-4 (Frossi et al. 2007). In addition, Fyn has been reported to act upstream of p38 in murine mast cells, suggesting potential cross-talk between these two kinase pathways (Samayawardhena et al. 2006; Samayawardhena et al. 2007).

Interestingly, increased expression of p38 upstream activators was observed following treatment of OLPs with PD169316. Thus, p38 may negatively regulate the mRNA levels of its upstream activators, which would ultimately control its activation state. This upregulation of MKK6 has been previously reported in cardiomyocytes when p38 was pharmacologically inhibited or when p38α was deleted (Ambrosino et al. 2003). Mechanistically, MKK6 upregulation is mediated through a p38-induced stability of the MKK6 mRNA transcript which contains an adenine/uridine-rich sequence in its 3' untranslated region that is known to confer stability to numerous genes, including inflammatory cytokines (Frevel et al. 2003). Moreover, the upregulation of PBK, which is an atypical MKK that specifically phosphorylates p38 during the cell cycle, suggests that p38 can regulate levels of upstream activators other than MKK6 (Ayllon and O'Connor 2007; Dougherty et al. 2005). Furthermore, the PD169316-induced upregulation of the MLK-related MKKK DLK-1 suggests that p38 can also regulate levels of kinases upstream of the MKKs (Abe et al. 2000).

We previously observed an upregulation of transcriptional repressors when OLPs were treated with an MK2 inhibitor, suggesting MK2 normally represses levels of transcriptional repressors in order to promote OLG differentiation (Haines et al. 2010). The levels of some transcriptional repressors were also upregulated by PD169316. For example, the Tcf4 is a target of β-catenin which encodes genes involved in repressing OLG differentiation, including Id2 (Ye et al. 2009). We also noted increases in levels of Sox6, a transcriptional repressor that blocks OLG differentiation (Stolt et al. 2006). In addition, the inhibitors of differentiation, Id1 and Id2 were increased, substantiating the findings that PD169316-treated OLPs are arrested at an immature stage of differentiation. Further supporting this hypothesis, the immature OLP marker, nestin was increased following PD169316 treatment, whereas this marker should normally decrease with OLG development (Almazan et al. 2001). In addition, Nkx2.2, which normally decreases with OLG differentiation was increased by PD169316 treatment (Cai et al. 2010). However, p38 appears to differentially regulate immature cell-stage gene targets that are normally downregulated during OLG lineage progression, since no upregulation of PDGFRα was found with p38 inhibitors. Interestingly, short non-coding microRNAs have recently been found to regulate the expression of PDGFRα and other transcriptional repressors (e.g., Sox6) to properly time the onset of OLG differentiation (Dugas et al. 2010; Zhao et al. 2010).

The largest subset of upregulated genes induced by PD169316 treatment was associated with cell cycle, chromosome spindle formation and cytokinesis. We also detected increases in various cell cycle regulators including cyclin A2, B1, D1, and the aurora kinases. Furthermore, reduced levels of thyroid hormone receptor alpha transcripts suggest that p38 inhibition may disrupt the intracellular timer that regulates OLG differentiation. Substantiating this hypothesis were the observed decreases in the cyclin dependent kinase inhibitors, $p57^{kip2}$ and $p21^{cip1}$. $p57^{kip2}$ properly times the exit of OLPs from the cell cycle, and therefore normally increases with differentiation.

We also observed many genes upregulated with PD169316 treatment that are involved in centromere formation, kinesin molecular motors, cell division, microtubule/microfilament organization and spindle complex formation. The function of these many factors in OLGs are unknown; however, they play roles in mitosis, and replication spindle formation in other cells (reviewed by (Miki et al. 2001)). Importantly, these factors normally decrease with OLG differentiation (supplemental data of (Cahoy et al. 2008)). Interestingly, the large upregulation of genes involved in cell cycle regulation are strikingly similar to what was found in cardiomyocytes following treatment with another p38 inhibitor, SB203580 (Engel et al. 2005). Increased cyclin A2 levels were also observed in cardiomyocytes following treatment with p38 inhibitors. Furthermore, when the same SB203580-treated cardiomyocyte microarray data was further analyzed, there was an upregulation of many genes associated with cytokinesis (Engel et al. 2006). Furthermore, application of fibroblast growth factor-1 (FGF1) to SB203580-treated cardiomyocytes resulted in an induction of cell proliferation that was not observed with SB203580-treatment alone (Engel et al. 2006). This is similar to our observations with OLPs, where PD169316-treated cells only re-entered the cell cycle after application of PDGF-AA and bFGF. This suggests OLPs are arrested at a non-proliferating and non-differentiating state. Previous experimental evidence in purified OLPs has found that cell cycle exit and differentiation are uncoupled processes (reviewed by (Casaccia-Bonnefil and Liu 2003)).

A role for p38 in mediating proliferation of cardiomyocytes has also been found, since p38α overexpression or a dominant negative p38α, blocked or increased cell proliferation, respectively (Engel et al. 2005). Furthermore, p38 plays an important role in the regulation of DNA checkpoints including the G2/M, G1/S and G1/G0 checkpoints (Thornton and Rincon 2009), suggesting important roles for this kinase in regulating both the cell cycle and differentiation. Interestingly, a dual function for p38 has been observed in myocytes, where in early satellite cells, p38 regulates unknown targets in the cell cycle; however, its function switches to promote myocyte differentiation once MyoD is expressed

(Jones et al. 2005). Moreover, p38 regulates the differentiation of a large number of cell types, including keratinocytes, neurons, adipocytes (Nebreda and Porras 2000), and Schwann cells (Fragoso et al. 2003). The mechanism of p38-mediated differentiation are starting to be unraveled, and includes cross-talk between the p38 and JNK/c-Jun signaling pathways, the latter which can normally promote cell cycle entry (Hui et al. 2007b). p38 normally represses JNK activity to promote differentiation of hematopoietic cells and mouse embryonic fibroblasts (Hui et al. 2007a). Further studies are underway to address the interplay between p38 and JNK signaling in OLGs to determine the cross-talk between these pathways in regulating OLG differentiation.

In conclusion, our observations suggest that p38 inhibitors modify mRNAs encoding OLG transcriptional activators, repressors, and genes associated with cytokinesis, chromosome spindle formation and replication. Therefore, p38 appears to positively regulate differentiation in OLGs by repressing genes involved in cell cycle and promoting the expression of factors that promote OLGs differentiation.

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Tables and Figures

Table 1: Myelin-specific and other pro-myelin gene transcripts upregulated and/or downregulated during OLG differentiation. OLGs were differentiated by removal of PDGF-AA and bFGF for 2d.

Gene ID	Gene Name	Accession Number	Fold Change	p value
MAL	Myelin and lymphocyte protein	NM_012798.1	12.59	1.065E-05
MOG	myelin associated glycoprotein	NM_022668.1	10.33	9.30E-07
MBP	myelin basic protein, transcript variant 3	NM_001025293.1	6.58	3.75E-06
Nkx6.2	NK6 transcription factor related, locus 2	XM_219447.4	4.15	1.18E-04
HDAC11	histone deacetylase 11	XM_001073226.1	4.09	3.81E-06
MAG	myelin-associated glycoprotein	NM_017190.4	3.24	4.71E-07
CGT/UGT8	UDP galactosyltransferase 8	NM_019276.2	1.79	3.62E-05
PDGFRA	platelet derived growth factor receptor, alpha polypeptide, transcript variant 2	XM_001067631.1	-2.34	6.65E-06
Nkx2.2	NK2 transcription factor related, locus 2 (Drosophila)	XM_001056116.1	-4.68	4.84E-06

Table 2: Myelin genes and transcriptional activators are decreased by a 24h treatment of OLPs with 5 μM PD169316.

Gene ID	Gene Name	Accession Number	Fold Change	p value
Fyn	fyn proto-oncogene	XM_001062721.1	-1.92	8.44E-09
MBP	myelin basic protein, transcript variant 3	NM_001025293.1	-1.51	6.98E-04
Sox8	SRY-box containing gene 8 (predicted)	XM_001060343.1	-1.50	7.26E-07
CGT/UGT8	UDP galactosyltransferase 8	NM_019276.2	-1.46	7.48E-07
SQLE	squalene epoxidase (Sqle)	NM_017136.1	-1.45	1.53E-04
HDAC11	histone deacetylase 11	XM_001073226.1	-1.43	1.27E-06
THRA	thyroid hormone receptor alpha (Thra), transcript variant TRalpha2	NM_031134.2	-1.43	6.98E-07
OMgp	oligodendrocyte-myelin glycoprotein	NM_001005898.2	-1.40	4.55E-06
Nkx6.2	NK6 transcription factor related, locus 2	XM_219447.4	-1.29	0.40 (NS)
Zfp488	zinc finger protein 488	XM_224697.4	-1.29	1.12E-03
MAG	myelin-associated glycoprotein	NM_017190.4	-1.22	1.96E-03

NS, non-significant.

Table 3: Transcriptional repressors and early OLP markers are upregulated following 24h treatment with 5 μM PD169316.*

Gene ID	Gene Name	Accession Number	Fold Change	p value
Id1	inhibitor of DNA binding 1	NM_012797.2	1.73	1.33E-06
NES	nestin (Nes)	NM_012987.1	1.46	1.56E-06
JunD	Jun D proto-oncogene (Jund)	XM_001070425.1	1.37	3.80E-04
Id2	inhibitor of DNA binding 2 (Id2)	NM_013060.2	1.35	2.01E-05
Nkx2.2	NK2 transcription factor related, locus 2 (Drosophila) (predicted)	XM_001056116.1	1.32	0.32 (NS)
Sox6	SRY-box containing gene 6	XM_215016.3	1.22	0.23 (NS)
PDGFRA	*platelet derived growth factor receptor, alpha polypeptide, transcript variant 2*	*XM_001067631.1*	*-1.09*	*0.22(NS)*

*Italics represents gene(s) that did not meet the cut-off criteria (+/- 1.25) with PD169316 treatment; NS, non-significant.

Table 4: PD169316 treatment elevates mRNA levels of p38 upstream activators.

Gene ID	Gene Name	Accession Number	Fold Change	p value
PBK	PDZ binding kinase	XM_224300.4	1.93	3.40E-08
DLK1	delta-like 1 homolog (Drosophila) (Dlk1), mRNA.	NM_053744.1	1.88	1.48E-06
MAP2K6 / MKK6	mitogen-activated protein kinase kinase 6 (Map2k6)	NM_053703.2	1.53	1.67E-05

Table 5: Transcripts encoding cell cycle regulators, kinesins, centromere, spindle and kinetochore associated proteins are upregulated by 5 μM PD169316 treatment.*

Gene ID	Gene Name	Accession Number	Fold Change	p value
cyclins, cyclin associated proteins, cyclin dependent kinase inhibitors				
CCNB2	cyclin B2 (Ccnb2)	NM_001009470.1	1.97	7.82E-08
CCNA2	cyclin A2 (Ccna2)	NM_053702.1	1.80	7.02E-08
CCND1	cyclin D1 (Ccnd1)	NM_171992.2	1.68	9.88E-07
CDKN1C	cyclin-dependent kinase inhibitor 1C (P57) (Cdkn1c), transcript variant 1	NM_001033757.1	-1.85	1.71E-06
CDC2	cell division cycle 2, G1 to S and G2 to M	NM_019296	2.40	1.78E-08
CDCA1	cell division cycle associated 1	XM_573495.1	2.15	4.74E-08
CDC20	cell division cycle 20 homolog	NM_171993.1	1.51	3.25E-07
kinesins				
KIFC1	kinesin family member C1 (Kifc1)	NM_001005878.1	2.16	1.36E-07
KIF22	kinesin family member 22 (Kif22)	NM_001009645.1	1.81	3.54E-07
KIF4	kinesin family member 4 (Kif4)	XM_343797.3	1.53	8.37E-08
KIF20A	kinesin family member 20A (predicted)	XM_341592.3	1.52	6.71E-06
KIF11	kinesin family member 11 (Kif11)	XM_001060913.1	1.48	1.30E-06
KIF23	kinesin family member 23 (predicted)	XM_001073723.1	1.36	8.10E-06
KIF15	kinesin family member 15 (Kif15)	NM_181635.2	1.24	1.03E-03

microtubules, microtubule networks

TUBB5	tubulin, beta 5 (Tubb5)	NM_173102.1	1.37	2.77E-05
TUBB6	tubulin, beta 6 (Tubb6)	NM_001025675.1	1.36	3.26E-06
LOC498736	similar to tubulin, beta 2 (LOC498736),	XM_574013.2	1.35	3.43E-06
TUBG1	tubulin, gamma 1 (Tubg1)	NM_145778.2	1.23	8.53E-05
TUBB3	tubulin, beta 3 (Tubb3)	NM_139254.1	1.21	3.11E-04
PAK7	p21 (CDKN1A)-activated kinase 7	XM_001080088.1	-1.25	7.95E-04

actin assembly, vesicle transport

ANLN	anillin, actin binding protein (scraps homolog, Drosophila)	XM_219687.3	1.34	8.14E-08
Rab33a	RAB33A, member of RAS oncogene family	XM_229145.3	-2.12	2.23E-07

replication fork, DNA synthesis initiation

MCM6	minichromosome maintenance deficient 6	XM_001055953.1	1.99	7.89E-08
PRIM1	DNA primase, p49 subunit (Prim1)	NM_001008768.1	1.89	2.24E-07
RFC3	replication factor C (activator 1) 3	NM_001009629.1	1.73	3.57E-07
MCM7	minichromosome maintenance deficient 7	NM_001004203.1	1.43	9.16E-06
MCM2	minichromosome maintenance deficient 2 mitotin	XM_001072364.1	1.36	3.18E-06
MCM3	minichromosome maintenance deficient 3	XM_236988.4	1.35	6.18E-06
MCM10	minichromosome maintenance deficient 10	XM_001071383.1	1.29	4.39E-07

spindle formation, separation				
AURKB	aurora kinase B	NM_053749.1	2.09	6.10E-09
PRC1	protein regulator of cytokinesis 1	XM_001061201.1	2.06	9.14E-07
SPC25	NDC80 kinetochore complex component, homolog	NM_001009654	1.93	2.55E-07
SPBC24	spindle pole body component 24 homolog	XM_001077474.1	1.86	4.37E-06
ASPM	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	XM_213891.4	1.82	2.65E-07
NUF2	Kinetochore protein Nuf2	NM_001012028	1.73	4.72E-10
NUSAP1	nucleolar and spindle associated protein 1	XM_001075591.1	1.51	1.19E-05
KNTC1	kinetochore associated 1	XM_001074897.1	1.43	2.48E-07
KNTC2	kinetochore associated 2 (predicted)	XM_001055564.1	1.37	3.13E-07
PLK1	polo-like kinase 1 (Drosophila) (Plk1)	NM_017100.1	1.34	6.68E-06
CENPT	centromere protein T	NM_001024257	1.33	1.11E-04
checkpoints				
TTK	Ttk protein kinase (predicted)	XM_001062174.1	1.58	2.91E-07
BUB1	budding uninhibited by benzimidazoles 1 homolog	XM_215849.4	1.50	1.85E-05
BUB1B	budding uninhibited by benzimidazoles 1 homolog, beta	XM_342494.3	1.33	1.29E-06
GADD45A	growth arrest and DNA-damage-inducible 45 alpha	NM_024127.2	-1.37	2.88E-05

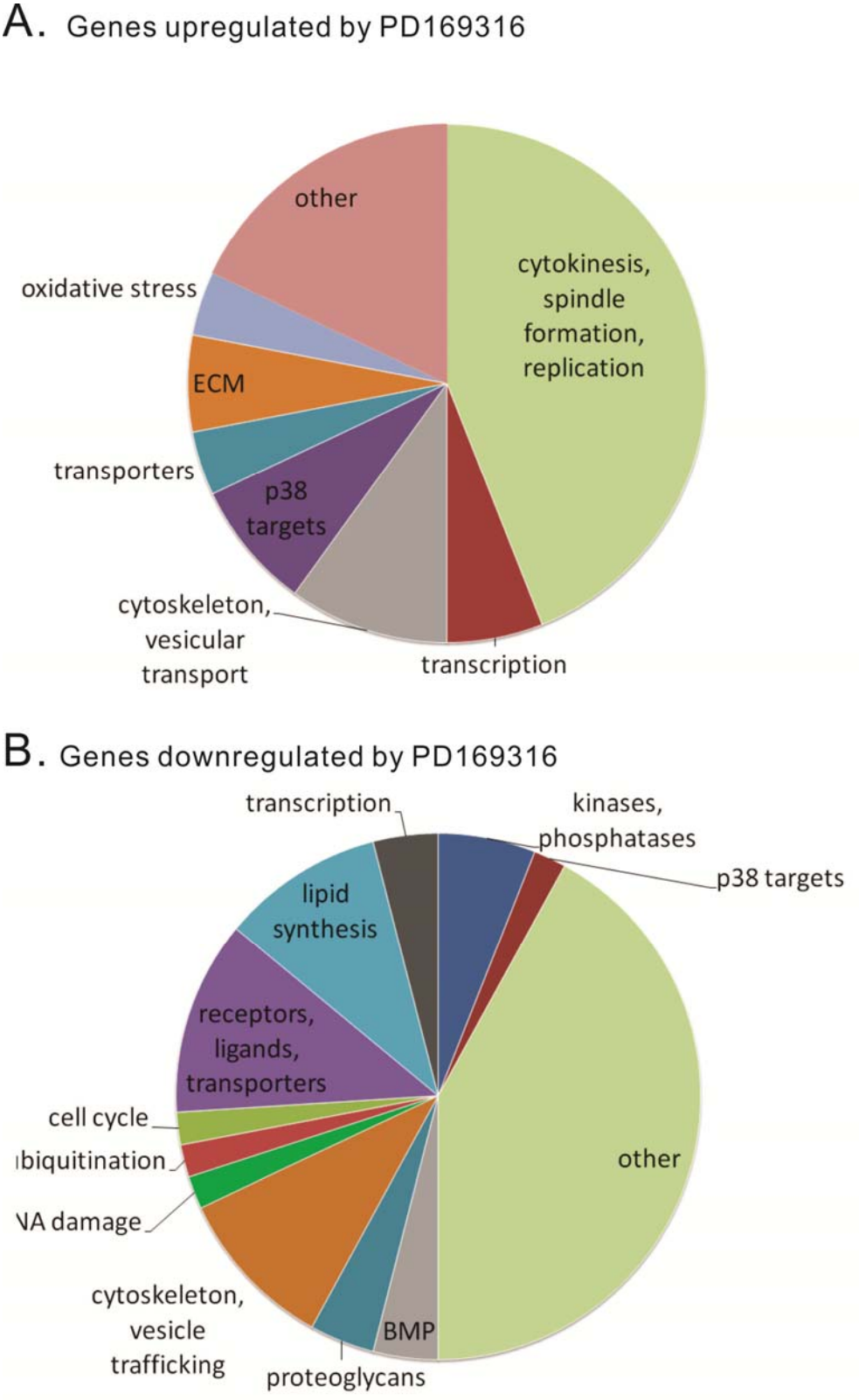
A. Genes upregulated by PD169316
other
cytokinesis, spindle formation, replication
oxidative stress
ECM
transporters
p38 targets
cytoskeleton, vesicular transport
transcription
B. Genes downregulated by PD169316
transcription
kinases, phosphatases
p38 targets
lipid synthesis
receptors, ligands, transporters
cell cycle
biquitination
NA damage
other
cytoskeleton, vesicle trafficking
BMP
proteoglycans

Figure 1

Figure 1. Functional classes of the top upregulated (A) and downregulated (B) gene classes affected by 24 hr treatment of OLPs with 5 μM PD169316. Groups of genes were classified into function by literature search, or by UniProt functional analysis.

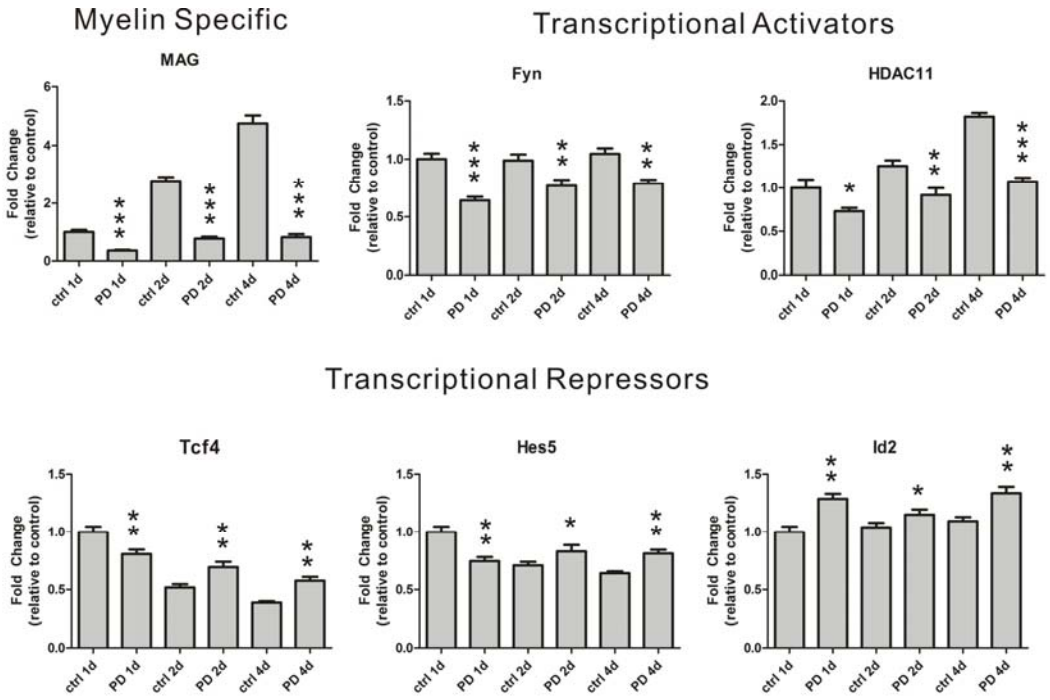

Figure 2. Gene transcript expression levels of myelin specific (MAG), transcriptional activators (Fyn, HDAC11) and transcriptional repressors (Tcf4, Hes5, Id2) changes with PD169316 treatment as determined by qRT-PCR. OLPs were treated with 5 μM PD169316 for either 1d, 2d or 3d and RNA was harvested, reverse transcribed and analyzed by qRT-PCR. Relative values were determined using the Advanced Relative Quantification method in the Roche LightCycler 480 software, relative to 28S rRNA. Statistical differences were determined using independent t-tests, *, **, *** = $p < 0.05, 0.01, 0.001$.

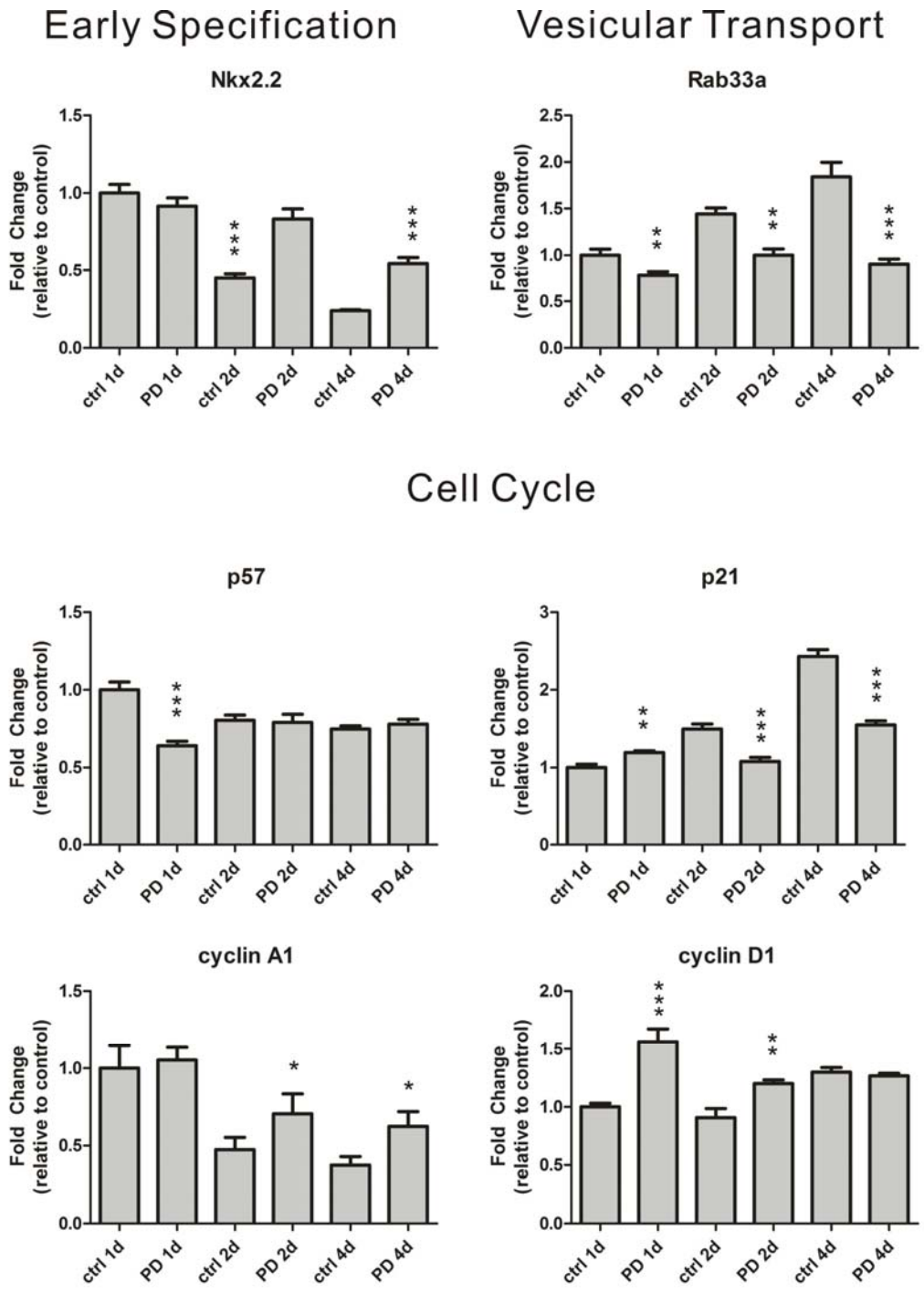
Early Specification
Vesicular Transport
Nkx2.2
Rab33a
Fold Change (relative to control)
ctrl 1d
PD 1d
ctrl 2d
PD 2d
ctrl 4d
PD 4d
Cell Cycle
p57
p21
cyclin A1
cyclin D1

Figure 3

Figure 3. Gene transcript expression levels of early specification markers (Nkx2.2), vesicular transport (Rab33a), and cell cycle (p57, p21, cyclin A1, cyclin D1) changes with PD169316 treatment as determined by qRT-PCR. OLPs were treated with 5 μM PD169316 for either 1d, 2d or 3d and RNA was harvested, reverse transcribed and analyzed by qRT-PCR. Relative values were determined using the Advanced Relative Quantification method in the Roche LightCycler 480 software, relative to 28S rRNA. Statistical differences were determined using independent t-tests, *, **, *** = $p < 0.05, 0.01, 0.001$.

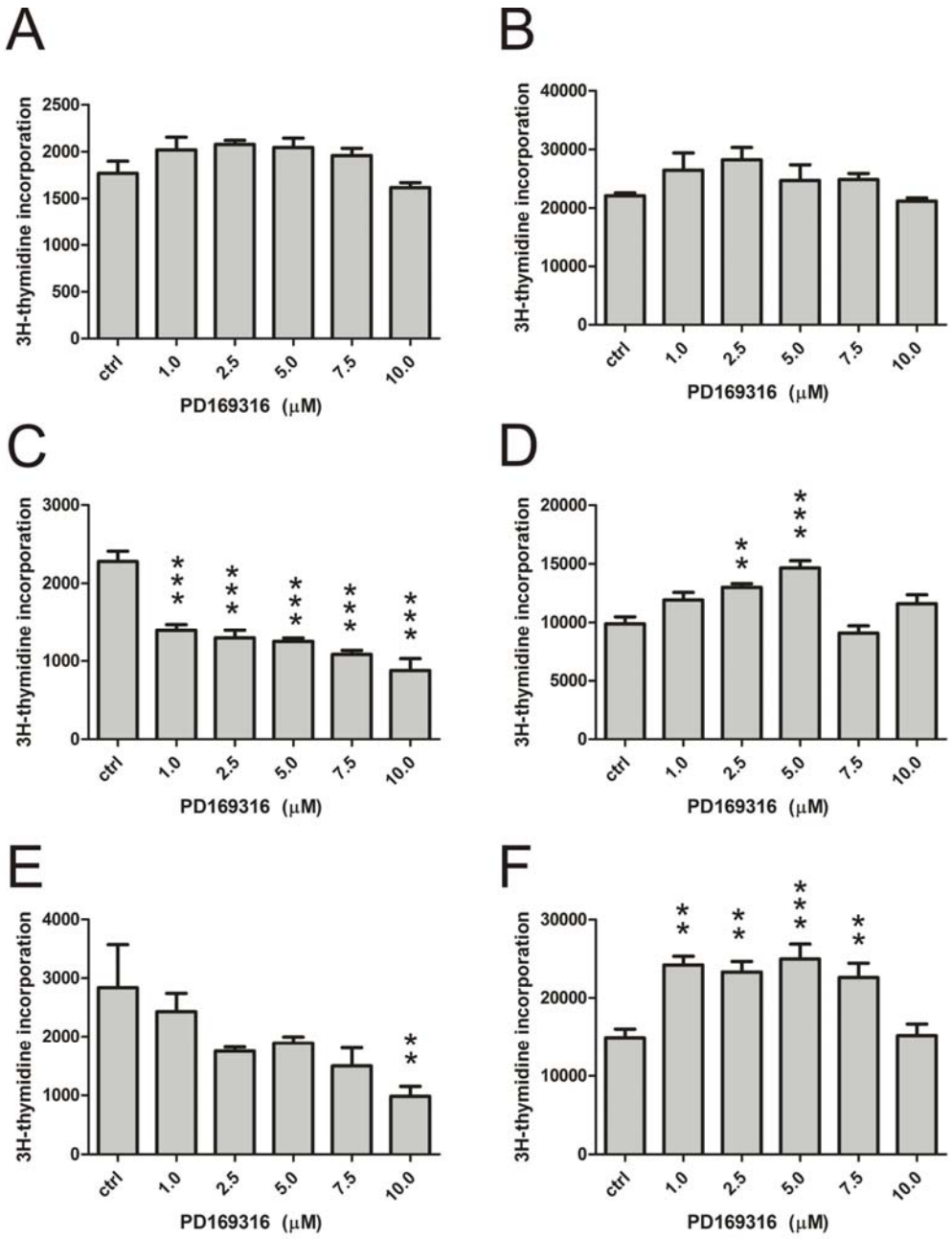
A
B
C
D
E
F
3H-thymidine incorporation
PD169316 (μM)
ctrl
1.0
2.5
5.0
7.5
10.0
***
**

Figure 4

Figure 4. OLPs treated for 24, 36, or 48hr (A, C, E, respectively) with PD169316 have decreased ^{3}H-thymidine incorporation. However, when cultures are maintained for 24, 36, or 48 hr with PD169316 and then stimulated with PDGF-AA and bFGF for an additional 24 hrs, OLPs incorporate thymidine (B, D, F). Statistical differences were determined by Dunnett's multi-comparison test, *p < 0.05, **0.01, ***0.001 as compared to controls, respectively.

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INTERVENING SECTION 3

In the final chapter, we reviewed the roles of p38 in both OLG and Schwann cell myelination. Furthermore, the subcellular localization of the abundantly expressed p38α isoform using immunocytochemistry was determined. In addition, experiments were performed to assess the effects of two p38 inhibitors (PD169316 and SB203580) on cell viability in OLGs at three different developmental stages.

Chapter 5: p38 mitogen-activated protein kinase regulates myelination

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Abbreviations

Caspr - Contactin associated protein

CGT - UDP galactose:ceramide galactosyltransferase

CNP - 2',3'-cyclic nucleotide 3'-phosphodiesterase

CNS - central nervous system

DAPI - 4,6-diamidino-2-phenylindole dihydrochloride

DRG - dorsal root ganglion

DRGN - DRG neuron

ERK - extracellular signal regulated kinase

FGF - fibroblast growth factor

GalC - galactosylceramide, galactocerebroside

HSP27 - heat shock protein 27

JNK - c-*jun* N-terminal kinase

MAG - myelin-associated glycoprotein

MAPK - mitogen-activated protein kinase

MAPKAPK2/3 - MAPK activated protein kinase 2/3

MBP - myelin basic protein

MNK - MAPK-interacting kinase

MOBP - myelin-associated oligodendrocyte basic protein

MSK - mitogen- and stress-activated protein kinase

NGF - nerve growth factor

NFH - neurofilament heavy-chain

OLG - oligodendrocyte

OLP - oligodendrocyte progenitor

PDGF - platelet derived growth factor

PNS - peripheral nervous system

SC - Schwann cell

Abstract

The p38 mitogen-activated protein kinase family is emerging as a crucial signaling molecule for a vast number of cellular functions including cell migration, proliferation, and differentiation. The function of p38 in myelination has only been recently addressed. Using pyridinyl imidazole based p38 α/β selective inhibitors, we have recently reported a critical role for this kinase in the regulation of myelination, specifically, in controlling the differentiation of Schwann cells, and oligodendrocytes, the myelinating glia of the peripheral and central nervous systems, respectively. These compounds inhibited the accumulation of myelin cell-specific markers, including myelin-specific glycosphingolipids, myelin-associated glycoprotein and myelin basic protein. More significantly, myelination of dorsal root ganglia neurons by oligodendrocytes was irreversibly blocked by p38 inhibitors. Our current studies are focusing on the molecular mechanisms by which p38 regulates oligodendrocyte and Schwann cell differentiation, and its role in models of myelination and remyelination.

Introduction

The mitogen activated protein kinases (MAPKs), are a family of serine/threonine protein kinases which are activated in response to extracellular cues including mitogens, growth factors, and environmental stress. The MAPKs are comprised of four groups, the extracellular regulated kinases (ERK), c-*jun* N-terminal kinase (JNK), p38, and big MAPKs (BMKs). The MAPKs share a common Tyr-Xaa-Thr amino acid phosphorylation sequence, and a three kinase module consisting of a MAPK kinase kinase (MKKK), a MAPK kinase (MKK), which activates the specific MAPK (Figure 1). The p38 MAPK family is comprised of four isoforms, α, β, γ, δ, identified by a common Thr-Gly-Tyr dual phosphorylation motif. The α/β isoforms are selectively inhibited by the pyridinyl imidazoles compounds SB203580/SB202190 and PD169316, which have helped to elucidate the

physiopathological roles of p38 as well as its signaling pathways. The four p38 isoforms vary widely in their tissue distribution, and activation by the upstream activators MAPK kinase 3 (MKK3) and 6 (MKK6). Further upstream activation is mediated by a large number of MAPKKKs/MAP3Ks, including ASK1, MLK3, TAB1 and TAK1 (Ono and Han 2000; Zarubin and Han 2005). The p38 protein was originally identified as a stress-activated kinase phosphorylated in response to inflammatory cytokines. It is now clear, however, that p38 signals to modulate numerous cellular processes including proliferation, survival, motility, and differentiation (Nebreda and Porras 2000; Ono and Han 2000). There are a large number of stimuli which can induce phosphorylation of p38 MAPK, including UV irradiation, osmotic stress, and heat shock. Numerous pro-inflammatory cytokines (interleukins, tumour necrosis factor α (TNFα), growth factors (platelet derived growth factor (PDGF), insulin growth factor, nerve growth factor (NGF)), and G protein-coupled receptors have also been found to trigger p38 activation (Ono and Han 2000). Once activated, p38 mediates its downstream effects through serine/threonine phosphorylation of various downstream protein kinases (MAPKAPK2/3, MNK1, MSK1/2) and transcription factors (ATF-2, CHOP, Elk-1, STAT1, MEF2, Myc and CREB). The activation of downstream kinases, or transcription factors culminates in the regulation of gene expression (Ono and Han 2000), and its diverse set of biological functions. Since little is known about the roles of p38 in neural function, this review will also summarize findings in other systems, which might be relevant to myelination.

p38 Functions

Cell cycle control, growth and differentiation

p38 is widely implicated in the control of cellular growth, in particular, its role in the differentiation of various cell types has been well illustrated as recently reviewed (Nebreda and Porras 2000; Ono and Han 2000; Roux and Blenis 2004). These include myocytes (Keren et al. 2006), chondrocytes (Stanton et al. 2003), adipocytes (Bost et al. 2005) and keratinocytes (Efimova et al. 2003).

Genetic knock-out of p38α resulted in embryonic lethality since it appears to be important for erythropoietin and placental angiogenesis (Adams et al. 2000; Allen et al. 2000; Mudgett et al. 2000; Tamura et al. 2000). In contrast, genetic knockout of p38β had no effect, and transcription of p38-dependent genes, and LPS-induced cytokine production were not affected by p38β ablation, suggesting that these effects are mediated through p38α (Beardmore et al. 2005). Similarly, single or double genetic knockouts of p38δ and γ isoforms has no effect on animal viability (Sabio et al. 2005). The embryonic lethality of p38α null mice has resulted in the establishment of conditional knockouts using the Cre-lox system. In adult cardiomyocytes, p38α ablation enabled mitogenesis (Engel et al. 2005), and decreased cell survival in response to cardiac pressure overload (Nishida et al. 2004). When p38α was knocked-out in adult mice, defective differentiation, and increases in lung stem cell and progenitor cell proliferation were also observed (Ventura et al. 2007). Perdiguero et al. used Cre-lox mice to delete each p38 isoform in myoblasts, and showed that p38α is critical for abrogating proliferation and allowing differentiation to occur. Thus, differentiation of myoblasts was disrupted due to a delayed cell-cycle exit and continuous proliferation under culture conditions that normally promote differentiation (Perdiguero et al. 2007). These results are consistent with the previous report showing that p38 is involved in a DNA checkpoint promoting G2 to M transition (Pearce and Humphrey 2001). Furthermore, p38 inhibitors prevented the expression of the cell cycle regulator $p21^{waf1}$ and the differentiation of muscle cells (Wu et al., 2000). The roles of the p38δ isoform in growth-promoting properties remains much less understood, however, it is known to regulate keratinocyte differentiation (Eckert et al. 2003).

p38 may be involved in neural function and development. The NGF-induced neurite outgrowth of PC12 cells was blocked in the presence of SB203580, and these results were confirmed by transfecting kinase-inactive forms of MKK6 or p38 (Morooka and Nishida 1998). More recently, Poolos, et al., showed that p38 can modulate hyperpolarization-activated cyclic nucleotide-gated channels and neuronal excitability (Poolos et al. 2006; Wynne 2006). Furthermore, in the rat CA1 region of the hippocampus, p38 associates with the

adenosine A1 receptor, and contributes to the inhibition of synaptic transmission (Brust et al. 2006). The p38α and β isoforms were found to be phosphorylated in adult brain tissue, mostly in neuronal nuclei (Lee et al. 2000) and in myelin-like structures (Maruyama et al. 2000). Also, p38 has been found to phosphorylate the inhibitor of neurite outgrowth, Nogo-B, via MAPKAPK2 (Rousseau et al. 2005). In addition, associative learning in rabbits increased p38 activation and both events were blocked with the selective p38 inhibitor, SB203580 (Zhen et al. 2001), while cAMP-stimulated neurite outgrowth of PC12 cells also required p38 activation (Hansen et al. 2000). Based on these observations for roles of p38 in neural function, we hypothesized that MAPKs and p38 in particular play an important role in myelination.

Oligodendrocyte and Schwann cell development

Oligodendrocytes (OLGs), and Schwann cells (SCs) are the myelin-producing cells of the central nervous system (CNS) and peripheral nervous system (PNS), respectively. The production of myelin in both cells occurs in a multi-stage process, and is associated with the accumulation of various protein and lipid markers. Although there are many similarities with respect to the molecular composition and cytoarchitecture of myelin, differences do exist, including the ability for OLGs to myelinate multiple internodes, as opposed to SCs that form a 1:1 relationship with myelinating fibers. Another important difference is the requirement for an extracellular matrix for peripheral myelination to take place. We are using DRGN in coculture with SCs or OLGs where myelination is induced spontaneously for OLGs, or through the addition of vitamin C, laminin or extracellular matrix for SCs (Figure 2). Vitamin C permits synthesis of collagen IV, serving as a scaffold for the deposition of other constituents of the basal lamina (Eldridge et al. 1987; Woodley et al. 1983). Our early studies demonstrated a fundamental role for p38 in PNS myelination (Almazan et al. 2003; Fragoso et al. 2003). Using DRGN/SC cultures, we showed that the p38 inhibitors PD169316 and SB203580 blocked the laminin- and ascorbate-induced

myelination (Figures 2, 4A). The fundamental roles for p38 in SC differentiation were further illustrated by the ability of PD169316 to inhibit laminin-induced elongation and alignment of SCs along axons, an early step required for myelination (Fragoso et al. 2003). The involvement of p38 in laminin-stimulated signaling was confirmed by the ability of exogenously applied laminin or ECM to induce phosphorylation of p38. Concomitantly, the small heat shock protein, hsp27, which is activated by the p38 downstream kinase MAPKAPK2, was phosphorylated. The transcription and translation of myelin-specific genes was also affected; steady state levels of mRNA encoding the structural proteins myelin-associated glycoprotein (MAG), myelin basic protein (MBP), and protein zero (P0) were reduced. In contrast to the results obtained with p38 inhibitors, the MEK1 inhibitor U0126 had no effect on myelination, but decreased both viability and proliferation of SCs. This finding is consistent with the report of Harrisingh, et al., showing that the Ras/Raf/ERK pathway is involved in SC proliferation induced by peripheral nerve injury (Harrisingh et al. 2004). Another MAPK family member, JNK, serves to control both proliferation and death of SCs (Parkinson et al. 2004).

A role of p38 in CNS myelination was suggested by the study of Baron, et al., which showed that SB203580 reduced sulfatide levels and the mitogenic effects of PDGF/FGF in OLG cultures (Baron et al. 2000). Maruyama and colleagues detected p38 MAPK in myelin-sheath like structures, which co-localized with 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) in mouse brain (Maruyama et al. 2000). In our recent studies, we have examined the role of p38 in differentiation of OLGs, and myelination of DRGNs (2007; Fragoso et al. 2004b; 2006). We have found that p38α is localized to the cytoplasmic processes and the nucleus of OLPs and in the nucleus of galactosylceramide -positive OLGs as detected by immunocytochemistry (Figure 3). Since the SB203580 and PD19316 compounds inhibit the p38α/β isoforms, we examined the differential expression of p38 in OLG cultures using isoform specific antibodies from different commercial sources, and real-time quantitative RT-PCR. The main isoform detected by both western blots and RT-PCR was p38α. All other p38

isoforms were detected by PCR, but the relative levels of p38γ mRNA was not consistent at the protein levels. The p38β and δ isoforms are barely detectable by western blotting. Inhibition of p38 in OLP cultures with PD169316 reduced both protein (Figure 4B) and steady-state mRNA levels of various myelin constituents including the MAG, MBP, and the early marker CNP. The block in OLG differentiation with PD169316 was corroborated using an siRNA to knock-down p38α, which decreased MAG expression, an early marker of myelination. A dramatic decrease in galactosylceramide (GalC) expression was also observed in cells transfected with p38α siRNA or treated with PD169316. Using RT-PCR, we also showed that mRNA levels of UDP galactose:ceramide galactosyltransferase were significantly reduced, accounting for the substantial decrease in the myelin glycosphingolipids, galactosylceramide and sulfatide. Treatment of DRGN/OLG cocultures with p38 inhibitors blocked not only accumulation of myelin lipids and proteins but also formation of myelinated internodes (Figure 2) and disrupted the axolemmal organization of Caspr. This protein is an adhesion molecule, concentrated at the paranodal junctions formed between axons and terminal loops of OLGs (Einheber et al. 1997; Menegoz et al. 1997).

In OLG cultures, p38 inhibition also prevented the accumulation of the cell cycle inhibitor $p27^{kip1}$, which normally increases during OLG differentiation (Casaccia-Bonnefil et al. 1997). This is in agreement with the finding that p38 is implicated in the G1 and G2/M phases of the cell cycle (Molnar et al. 1997; Yee et al. 2004). In addition, increased expression of $p27^{kip1}$ in OLGs enhanced MBP promoter activity (Miskimins et al. 2002) through stabilization of the transcription factor Sp1 (Wei et al. 2004). Furthermore, coexpression of $p27^{kip1}$ with Sox10, a member of the high-mobility group factors that activates MBP expression (Stolt et al. 2002), resulted in further increases in promoter activity (Wei et al. 2004). Our observations that p38 inhibitors can decrease both Sox10 mRNA expression and $p27^{kip1}$ protein accumulation can partly explain their effects on MBP expression in differentiating OLGs. However, the effect of p38 on transcription of other myelin-specific genes, including CNP, MAG, myelin-associated oligodendrocyte basic protein (MOBP) and proteolipid protein (PLP) suggests that this kinase exerts a

transcriptional regulatory control on myelination. This postulate is supported by the finding that the effects of p38 inhibitors on myelination were only observed when the drugs were present from the beginning of differentiation. The roles of transcription factors that regulate OLG differentiation are reviewed elsewhere (Ligon et al. 2006; Wegner 2000).

Our studies have also shown that the block of OLG differentiation was reversible, where removal of PD169316 was able to restore levels of MBP, MAG and CNP (Figure 4C). In contrast, removal of the p38 inhibitor in the OLG/DRGN coculture system resulted in a lack of recovery of myelination (Fragoso et al. 2007). We are currently investigating the reason for these recovery differences. Another group recently published a study where p38 inhibition was found to interfere with OLG differentiation, showing decreased levels of MAG and MBP in the presence of SB203580, with CREB suggested as a downstream target of the p38 pathway (Bhat et al. 2007). However, the molecular mechanisms regulating cell growth and differentiation of OLGs by p38 remain to be elucidated.

Cytoskeletal dynamics, motility and adhesion

In addition to its roles as a regulator of gene transcription, p38 can also regulate the assembly of the cytoskeleton. The assembly of microtubules is regulated through tau, which shows decreased microtubule assembly properties when phosphorylated by p38 isoforms (in order of preference: p38δ, γ, β, α) (Buee-Scherrer and Goedert 2002; Goedert et al. 1997). The assembly of actin is also regulated via p38, through the action of the small heat shock protein, hsp27 (Dalle-Donne et al. 2001). Hsp27 is phosphorylated by MAPKAPK2/3, resulting in F-actin modulation. In our study with primary rat SC cultures, we showed that the laminin-induced SC alignment with axons required both p38 and hsp27 activation (Fragoso et al. 2003). Laminin is a critical component of the basal lamina, which is required for myelination of the PNS. Mutations of the human and mouse α-chain of the laminin-2 subtype are associated with peripheral nerve

dysmyelination (Russell et al. 2000; Uziyel et al. 2000; Xu et al. 1994). Several studies have shown that the main laminin receptors in myelinating SCs are dystroglycan and the integrins, α6β1 and α6β4 (Einheber et al. 1993; Feltri et al. 1994; Previtali et al. 2001; Yamada et al. 1996). Conditional disruption of β1 integrin causes a severe neuropathy with impaired radial sorting of axons by SCs, and severe dysmyelination in knock-out mice (Feltri et al. 2002). This phenotype is similar to conditional inactivation of Rac1 in SCs, and it has been demonstrated that Rac1 is downstream of β1 integrin activation (Benninger et al. 2007; Nodari et al. 2007). Rac1 is a small GTP-ase which regulates cell spreading and adhesion and is activated upon clustering into lipid microdomains (del Pozo et al. 2004), resulting in p38 activation. The fundamental role of Rac1 in both CNS (Thurnherr et al. 2006) and PNS (Benninger et al. 2007; Nodari et al. 2007) myelination has been demonstrated by gene inactivation in transgenic mice. An important question that remains to be answered is whether p38 is involved in the integrin-Rac1 signaling to regulate myelination.

Stress Response, inflammation and apoptosis

Originally, p38 was identified as a protein rapidly tyrosine phosphorylated in response to lipopolysaccharide in macrophages (1994; Han et al. 1993). Many other studies followed, and implicated p38 in cell death and apoptosis of a large number of cells, however, few are related to myelinating glia. In Schwann cells (SCs), methylglyoxal-induced apoptosis was p38-dependent (Fukunaga et al. 2004). In contrast, the SC morphological and proliferative changes effected by endothelin were blocked by SB202190 (Berti-Mattera et al. 2001). In oligodendrocytes (OLGs), hydrogen peroxide-induced toxicity of progenitors and mature cells was only partly mediated through p38 (Fragoso et al. 2004a). Similarly, in a central glia cell line, CG4, hydrogen peroxide stimulated p38 activation, but SB203580 failed to have a protective effect (Bhat and Zhang 1999). In contrast, p38 mediated the production of inducible nitric oxide synthase (iNOS) in response to inflammatory cytokines in CG4 cells (Bhat et al. 1999). Similarly,

SB203580 blocked ceramide-induced apoptosis in OLG cultures (Hida et al. 1999). In another OLG cell line, MO3.13, the galactocerebroside-break down product, psychosine induced phosphorylation of p38 along with ERK and JNK, the first are known to phosphorylate cytoplasmic phospholipase A2 (Giri et al. 2006). Furthermore, differential activation of p38 and other MAPK families has been observed in OLGs in response to inflammatory cytokines. For example, TNFα-induced death of adult human OLGs was mediated through JNK, but not through p38 (Jurewicz et al. 2003), which is in contrast to findings in other systems where TNFα strongly induces p38 phosphorylation (Grethe et al. 2004). Treatment of primary SCs with TNFα activated p38, resulting in decreased cell viability, which was blocked by SB203580 (Myers et al. 2003). Furthermore, the TNFα-induced expression of monocyte chemoattractant protein-1 mRNA in SCs was reduced by inhibitors of nuclear factor-κB and p38 MAPK (Subang and Richardson 2001). Another cytokine, interleukin-1, increased the phosphorylation of p38 in primary rat OLG progenitors in parallel with mitotic arrest and an increase in differentiation (Vela et al. 2002). All together, these results suggest a limited role for p38 in regulation of stress response and apoptosis in myelinating glia, and suggest a more important role for promoting differentiation. A recent study concluded that p38 is essential for mature OLG survival when SB203580 was used to inhibit p38 activity (Hamanoue et al. 2007). However, our results in OLG cultures alone, or in combination with DRGNs show that under the experimental conditions used for differentiation and myelination (which includes high levels of insulin in serum-free media and NGF) both SB203580 and PD169316 do not reduce cell viability. This point is illustrated in figure 5 and (2007; Fragoso et al. 2003), where we measured mitochondrial dehydrogenase activity as an indicator of cell survival.

p38 MAPK Mechanisms

The molecular mechanisms by which p38 regulates cellular growth is an active area of research. It has been postulated to function by four general mechanisms: 1) direct phosphorylation of transcription factors, 2) regulation of mRNA stability 3) translational control, and 4) regulation of chromatin remodeling and histone H3 phosphorylation (Schieven 2005) (Figure 1). Although generally thought to be mechanisms for response and production of pro-inflammatory cytokines, it is becoming clear that these mechanisms may be shared for regulation of cell differentiation by p38. A p38-induced mRNA stability has also been reported for various cytokine mRNAs (Dean et al. 2004) (e.g., TNFα), and for the transcription factor, Sox9, which serves to regulate chondrocyte differentiation (Tew and Hardingham 2006). However, the exact mechanism by which p38 signaling stabilizes mRNA transcripts remains unknown. It appears mRNA stability is mediated through the downstream kinase MAPKAPK2, and the presence of adenine/uridine-rich elements in the 3'-untranslated region of an mRNA can be a destabilizing determinant, although it is not an absolute requirement (Dean et al. 2004). The roles of p38 in translational regulation through MNK1, and chromatin remodeling through MSK1/2 remain much less understood. The p38-MNK pathway was found to regulate TNFα expression in T-cells (Buxade et al. 2005), however its roles in growth and development do not appear crucial, as knock-out animals were normal (Ueda et al. 2004). Early embryonic stem cell differentiation involved chromatin remodeling mediated through the p38-MSK pathway which stimulated histone H3 phosphoacetylation (Lee et al. 2006). A mouse knockout of MSK1/2 showed reduced mitogen- and stress-induced phosphorylation of CREB and ATF1 in cultured fibroblasts (Wiggin et al. 2002). Along with MSK, CREB transcriptional activity can be regulated by a number of kinases including Ca^{2+}/calmodulin-dependent kinases, protein kinase C, RSK1-3, and MAPKAPK2/3 (Gonzalez et al. 1989; Herdegen and Leah 1998; Shaywitz and Greenberg 1999; Sheng and Greenberg 1990).

Potentially, p38 could regulate peripheral myelination at the transcriptional level through the subsequent activation of CREB followed by Krox20, a master regulator of SC differentiation (Topilko et al. 1994; Zorick et al. 1999). The Krox20 promoter contains cAMP response element (CRE) which is required for gene activation (Watanabe et al. 2005).

The role of p38 in transcriptional regulation is best understood in myogenic differentiation, where several lines of investigation have illustrated that p38 is master regulator (Keren et al. 2006; Lluis et al. 2006). During myoblast differentiation, the phosphorylation of both p38α and β gradually increases (Cuenda and Cohen 1999; Wu et al. 2000; Zetser et al. 1999), and SB203580 implicated these isoforms in myogenesis. More recent studies demonstrated that p38 regulates the activities of transcription factors from the MyoD and MEF2 families, resulting in chromatin remodeling at muscle-regulatory sites (Keren et al. 2006). A number of these transcription factors are phosphorylated by through p38 direct phosphorylation or by MAPKAPK2 (Lluis et al. 2006).

In adipocytes, the phosphorylation of the CCAAT/enhancer-binding protein β (C/EBPβ) protein was blocked in the presence of SB203580 (Engelman et al. 1998), and a constitutive active MKK6 increased its transcriptional activity in 3T3 L1 cells (Engelman et al. 1999). The C/EBPs are a large family of regulatory factors involved in gene transcription, and knock-down of C/EBPδ was found to inhibit P0 mRNA induced by an interleukin-6 receptor/ligand (Kamaraju et al. 2004), implicating these factors in myelination. In adipocytes, a member of the peroxisome proliferator-activated receptor family, PPARγ, was reduced in the presence of SB203580 (Engelman et al. 1998). This family of nuclear transcription factors is required for terminal differentiation of adipocytes (Ailhaud 1990). Interestingly, in OLG cultures, a PPARδ agonist stimulated cell differentiation (Saluja et al. 2001), however, targeted disruption of this gene in a mouse model did not affect expression of MBP and PLP (Peters et al. 2000). These results do not exclude the possibility that another PPAR isoform has a compensatory effect.

Future Directions and Conclusions

Classically, p38 was identified as a signaling molecule activated in response to environmental stress, and apoptosis. In the last decade, novel roles for p38 in many cellular programs emerged, including survival, cytoskeletal remodeling, proliferation and differentiation. Presumably, the huge diversity in the number of activating upstream and downstream kinases contributes to the diverse set of functions. It is clear that identification of both natural upstream activators of p38, and downstream phosphorylation targets will be the key to understanding how this kinase regulates myelination. Our current studies are identifying both p38 upstream activators and downstream targets in both SCs and OLGs. Understanding the p38-mediated regulation of myelination in these cell systems may reveal clues to remyelination attempts in diseases where myelin sheaths are lost or destroyed, for example, multiple sclerosis, or Charcot-Marie Tooth disease, in the CNS and PNS, respectively.

Acknowledgements

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Figures

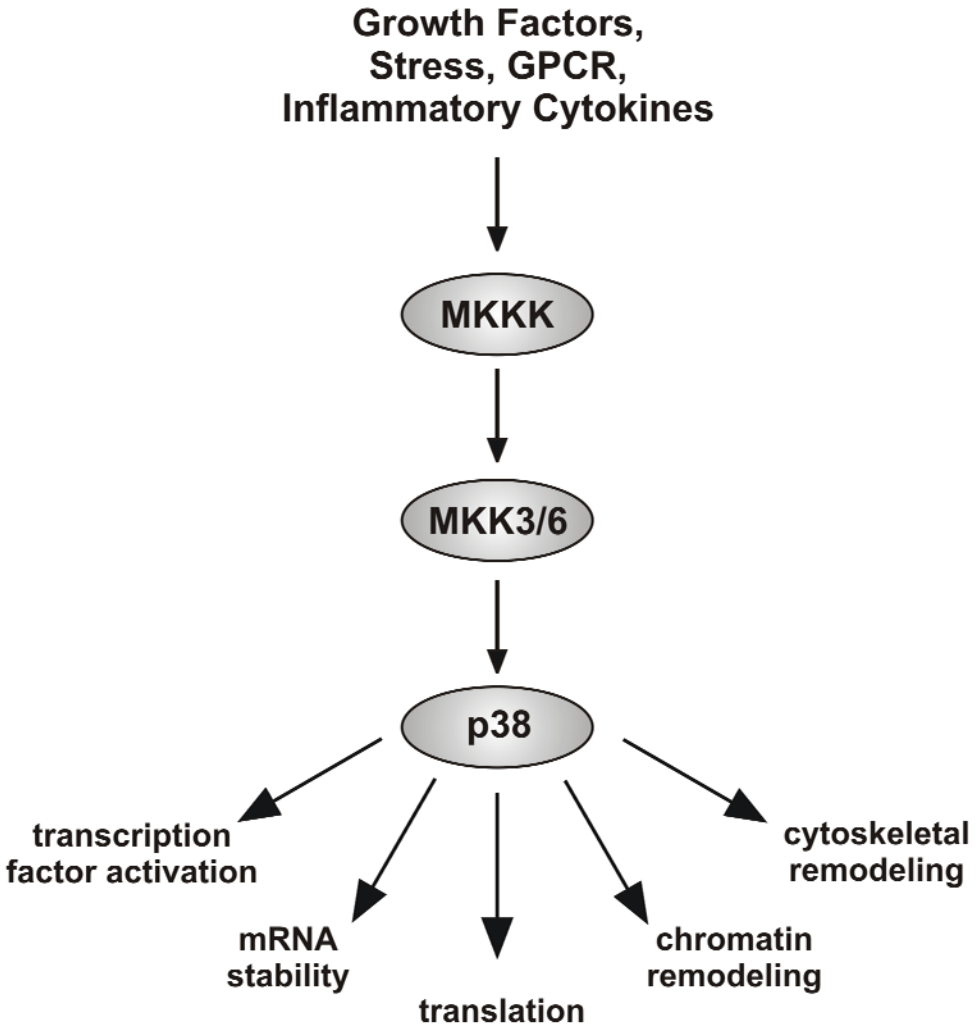

Figure 1. Schematic representation of the three kinase module (MKKK, MKK3/6, p38 MAPK) which constitutes the p38 MAPK signaling pathway. A number of growth factors, stress, GPCRs and inflammatory cytokines activate this pathway, leading to downstream effects (transcription factor activation, translation, mRNA stability, chromatin remodeling).

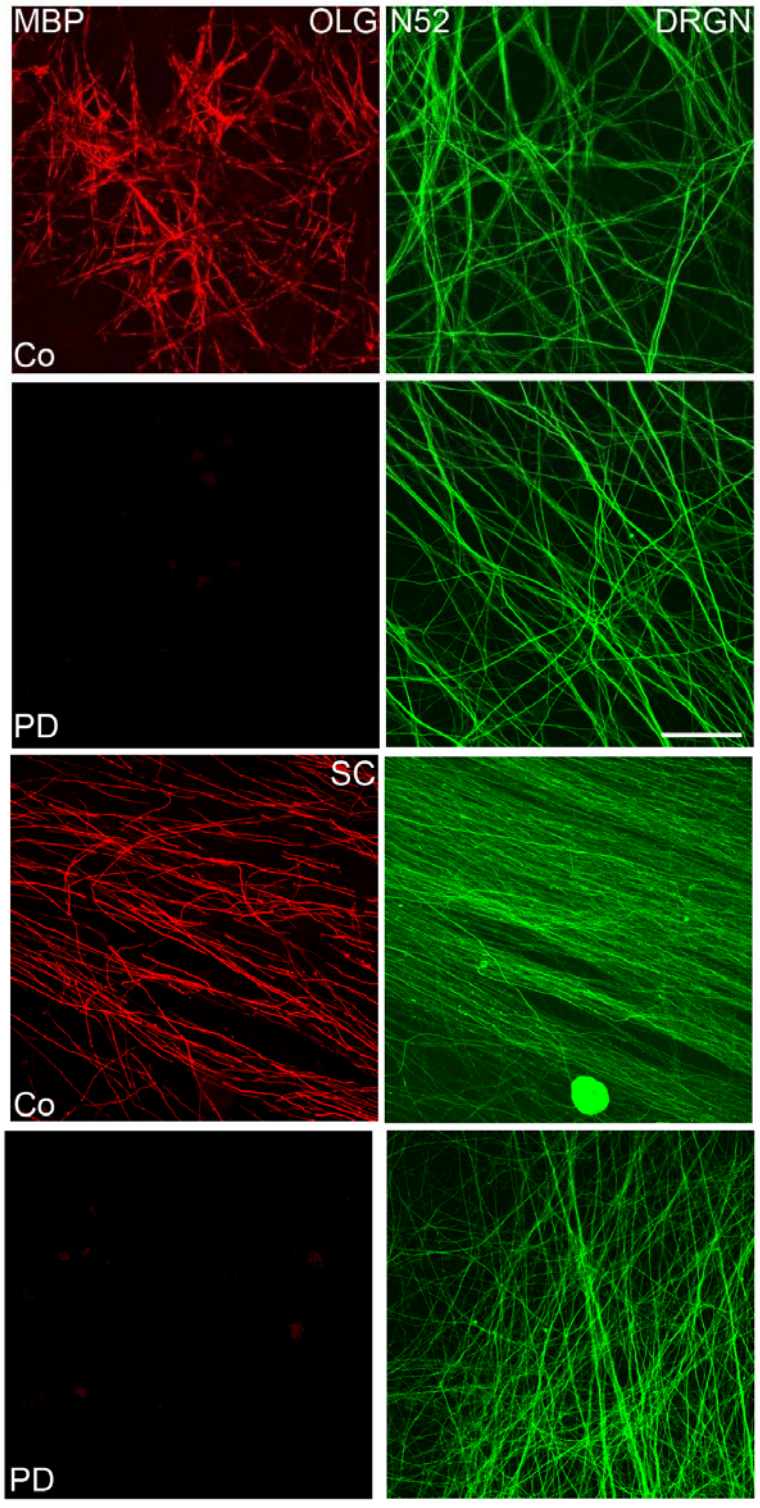

Figure 2

Figure 2. Inhibition of p38 blocks myelination of dorsal root ganglion neurons (DRGNs) by oligodendrocytes (OLGs) or Schwann cells (SCs) without altering neurofilament expression. DRGs were obtained from Sprague–Dawley rat embryos at 15–16 days gestation, dissociated with trypsin, and plated onto rat tail collagen-coated wells. The SC/DRGN cultures were maintained in serum-free N1 media supplemented with nerve growth factor, and myelination was initiated in the third week through the addition of vitamin C. For OLG/DRGN cocultures, neurons are pre-treated with anti-mitotic agent (to rid cultures of proliferating SCs and fibroblasts) prior to the seeding of OLGs at the third week of culture. Following the initiation of myelination, cocultures were maintained in N1 media in the presence or absence of PD169316 (5 μM) for 8d (OLGs), and 10d (SCs). Cells were stained with anti-MBP (TexasRed) and neurofilament heavy chain (N52 antibody) (FITC). Scale bar represents ~50 μm (for OLG) and ~100 μm (for SCs).

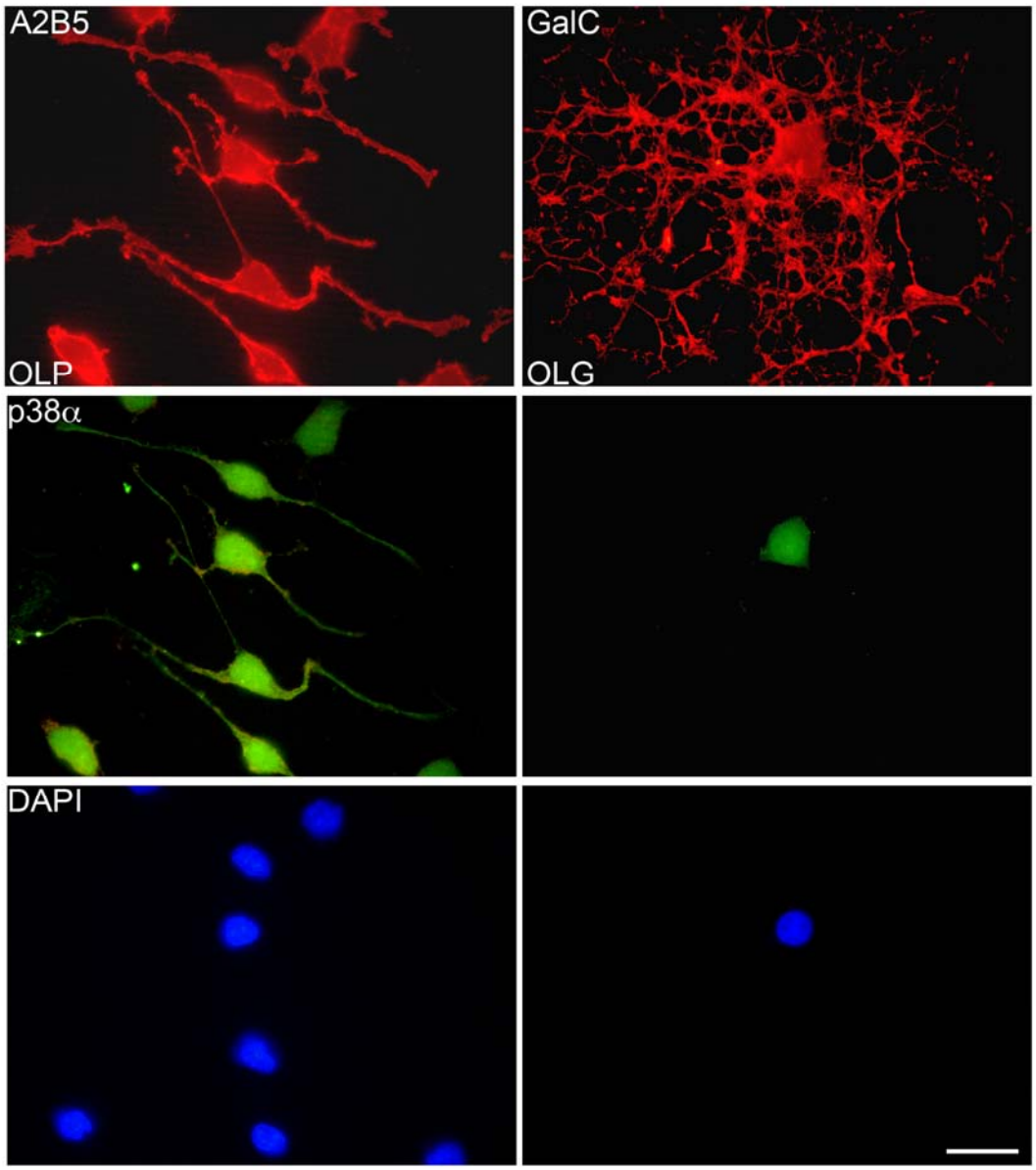

Figure 3. Subcellular localization of p38α in OLPs and OLGs. OLPs were stained with A_2B_5 (TexasRed), and OLGs with GalC (TexasRed) followed by immunostaining for p38α (FITC) and nuclear staining (DAPI). Scale bar represents ~12 μm.

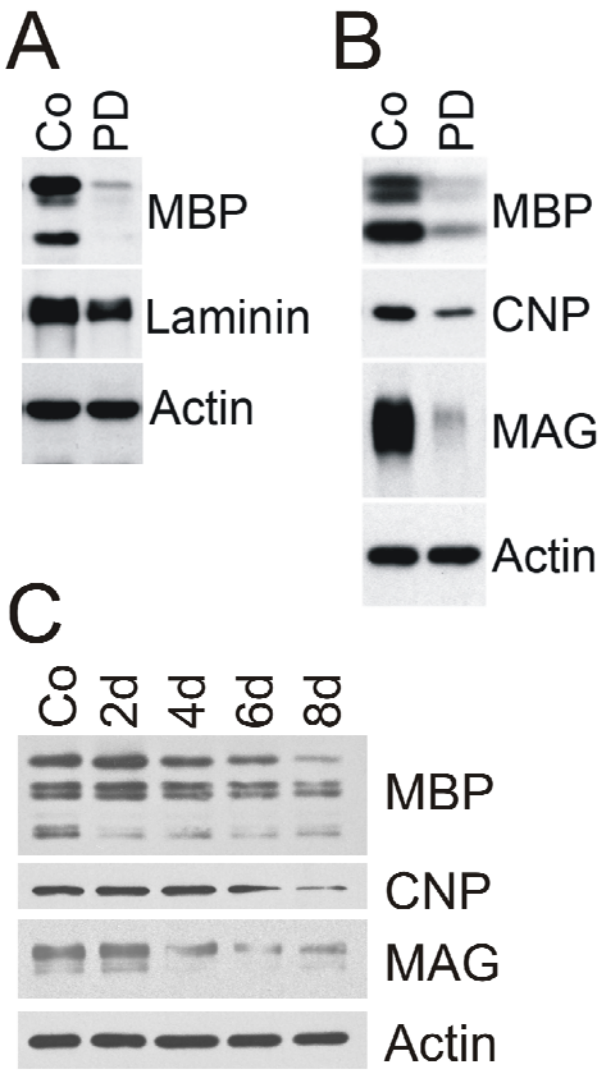

Figure 4. PD169316 (5 μM) decreases myelin protein expression in SC/DRGN, OLG/DRGN, and OLG cultures. (A) SC/DRGN maintained for 10d in the absence or presence of PD169316 also showed block of myelination assessed by reductions in myelin basic protein (MBP) and laminin. (B) OLG/DRGN maintained for 8d in the absence or presence of PD169316 showed almost a complete block of myelin markers (MBP, CNP, MAG). (C) OLG cultures were treated with 5 μM PD169316 for different time periods (2, 4, 6, 8 days) beginning at day zero, when OLP begin to differentiate. All groups were harvested on day 8 and subjected to western blots as in (A) and (B).

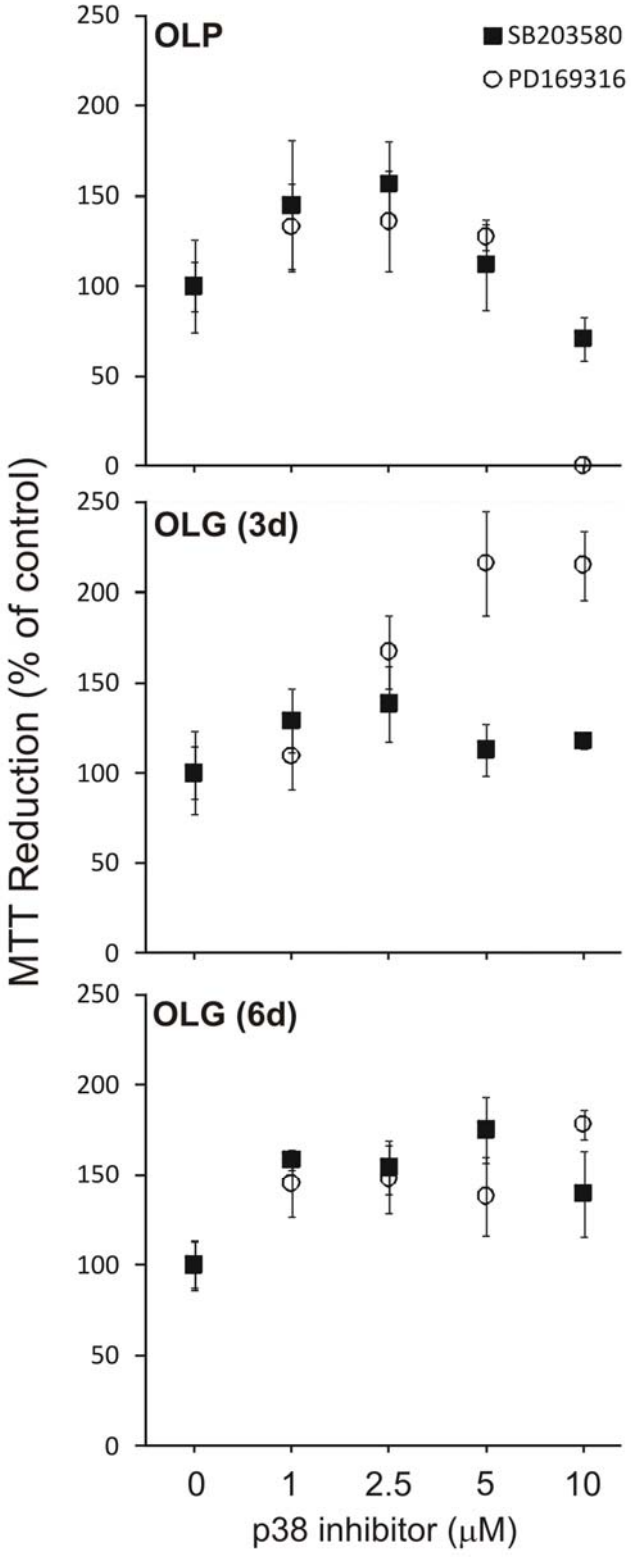
OLP
SB203580
PD169316
OLG (3d)
OLG (6d)
MTT Reduction (% of control)
p38 inhibitor (μM)
0
50
100
150
200
250
0
1
2.5
5
10

Figure 5

Figure 5. p38 inhibitors do not affect OLG viability. OLG at different developmental stages (OLP, 3 and 6 day differentiated) were treated with increasing concentrations of PD169316 and SB203580 (1, 2.5, 5, 10 μM) for 2d. Cell viability was determined by mitochondrial dehydrogenase reduction of MTT, followed by spectrophotometry. The values represent mean +/- standard error of the mean for five determinations. The results show that the 10 μM PD169316 and SB203580 were toxic to OLPs.

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CHAPTER 6: GENERAL DISCUSSION

Summary of Findings

The differentiation of an OLP to a mature OLG involves a complex interplay of repressors and activators that ultimately times the onset of myelination. However, the signal transduction pathways that regulate the transition from an immature OLP to a mature myelinating OLG are only beginning to be elucidated. The main objective of this thesis was to assess the potential involvement of the p38 MAPK signaling pathway in CNS myelination using OLG primary cultures alone or in coculture with dorsal root ganglion neurons (DRGN).

In chapter 2, we investigated the role of p38 MAPK in OLG differentiation. OLG cultures treated with the p38 inhibitor PD169316 reduced the lipid and protein levels of myelin differentiation markers. In addition, the myelination of DRGN was blocked by p38 inhibitors as evidenced by the lack of MBP internodal staining and absence of Caspr clustering at paranodal regions. p38 inhibitors also blocked the expression of myelin-specific gene transcripts. Furthermore, a siRNA to p38α confirmed the effects of the p38 inhibitor by preventing MAG accumulation and GalC staining in OLG membrane sheets. In contrast to the results with the p38 inhibitors, the MEK/ERK inhibitor U0126 had no significant effect on MBP accumulation in OLG/DRGN cocultures.

In chapter 3, we explored potential downstream substrates of p38α that were responsible for effecting OLG differentiation. A number of observations suggest that mitogen-activated protein kinase activated protein kinase 2 (MK2) is an important component of the signaling pathway, including: 1) phosphorylated (activated) MK2 levels increased during OLG differentiation, and by using an MK2 inhibitor we showed that myelin differentiation markers were decreased at the protein and mRNA level; 2) a siRNA to MK2 reduced MAG and GalC levels in OLG membrane sheets; 3) a number of factors that are inhibitory to OLG differentiation were upregulated following treatment of the cultures with the MK2 inhibitor, among them Notch, Tcf4 and Id2; and 4) p38α and MK2 form complexes in OLPs and OLGs, and as cells differentiated, hsp25 became

associated with p38/MK2 complexes, and p38/MSK1/CREB formed separate complexes that did not contain MK2.

In chapter 4, we performed an Illumina rat whole genome microarray screen to determine the molecular mechanisms by which p38 signaling regulated OLG differentiation. We found that p38 regulates the expression levels of many factors that regulate OLG differentiation including transcriptional repressors (Id2, Tcf4, Notch), vesicular transport proteins (Rab33a), kinases (Fyn), and a histone modifier (HDAC11). Furthermore, we observed a large upregulation of genes encoding proteins involved in cell cycle regulation and cytokinesis (e.g., kinesins, centromere/spindle formation, cell cycle regulators).

In chapter 5, we found differential p38α localization depending on the maturity stage of the cells. We also showed that p38 inhibitors (SB203580 and PD169316) reduce markers of differentiated OLGs and Schwann cells, and have no effect on cell viability of OLGs at three stages of their development.

General Discussion and Future Directions

p38 MAPK regulates the differentiation of numerous cell types. Our finding that p38 inhibitors reduce the accumulation of OLG differentiation markers led us to conclude that p38 plays important roles in OLG lineage progression. We found that the MEK/ERK inhibitor had no effect on the accumulation of MBP in OLG/DRGN cocultures. In OLPs, the MEK/ERK pathway is important for survival and proliferation (Bibollet-Bahena and Almazan 2009; Cui and Almazan 2007). Others have shown that neuronal precursor cell ablation of B-Raf, the MKKK upstream of ERK, causes CNS dysmyelination and defective OLG differentiation (Galabova-Kovacs et al. 2008). ERK may also regulate OLP process extension (Stariha et al. 1997; Younes-Rapozo et al. 2009), suggesting that ERK is involved in early developmental events. Our findings that p38 regulates OLP maturation are in agreement with conclusions drawn from other systems where p38 appears to be a major regulator of cellular differentiation. In fact, p38 plays important roles in promoting differentiation of both Schwann cells and OLGs. In Schwann cells, p38 plays multiple roles in their progression to a myelinated phenotype, including the regulation of cell alignment with neuronal

axons, basal lamina synthesis, and differentiation (Fragoso et al. 2003; Hossain et al. 2010). Interestingly, reduced p38 activation (Liao and Hung 2003), and increased JNK signaling is seen in human Schwannomas (Kaempchen et al. 2003). Therefore, p38 may antagonize JNK signaling in Schwann cells to initiate growth arrest and promote differentiation, since c-Jun is a negative regulator of Schwann cell myelination (Parkinson et al. 2008). Moreover, the aggressive human oligodendroglioma cell line Hs683 exhibits uncontrolled proliferation when levels of activated p38 are low. However, following long-term culturing with an alkylating chemotherapy agent, activated p38 levels are elevated and correlate with oligodendroglioma proliferative growth arrest (Lamoral-Theys et al. 2010). In addition, our microarray data suggests that a large number of genes involved in cell cycle regulation and cell division are upregulated following treatment with p38 inhibitors. Taken together, these findings suggest that p38 activity is required for normal growth arrest and the subsequent differentiation of myelinating glial cells.

At the protein level, we found that p38α and p38γ were the most abundant isoforms expressed in OLGs. This is in contrast to previous studies where p38γ transcripts were found to be exclusively expressed in skeletal muscle as determined by Northern blotting (Li et al. 1996). Interestingly, p38α negatively regulates p38γ expression during stress response (Qi et al. 2007). Furthermore, a recent report has suggested that p38γ actually inhibits differentiation of primary muscle satellite cells (Gillespie et al. 2009), which is in contrast to what was shown previously for p38γ in immortalized myocyte cell lines. Therefore, in muscle satellite cells, p38γ negatively regulates differentiation, whereas p38α promotes it. Mechanistically, p38γ phosphorylates MyoD on sites which reduce its activity when bound to the myogenin promoter. However, at later stages of development, p38α phosphorylates alternate MyoD amino acid residues to promote transcription of muscle specific genes. Thus, the opposing actions of p38α and p38γ appear to properly time the onset of muscle differentiation. Whether this type of opposing regulation of p38α and p38γ operates in other cell systems has yet to be explored.

The important roles for p38 in the differentiation of numerous cell types has been well documented; however, a single mechanism of p38-mediated regulation of differentiation has not been identified. For example, p38 can phosphorylate/activate master transcription factors involved in differentiation, including MyoD, a critical bHLH regulator of myocyte differentiation. In other cell types, p38 regulates expression of mRNA and protein levels of master gene regulators, for example, osterix a transcription factor necessary and sufficient for osteoblast differentiation (Wang et al. 2007). In chondrocytes, p38 regulates the expression levels of Sox9, a transcription factor considered to be a "master switch" for chondrocytic differentiation (Hoffman et al. 2003). In addition, p38 appears to regulate Schwann cell differentiation through control of Krox20, the master transcription factor of PNS myelination (Hossain et al. 2010). However, the lack of evidence for an OLG master transcription factor makes it unlikely that p38 regulates a single molecule in these cells to promote differentiation. Supporting this notion is our observation that both p38 and MK2 inhibitors increase levels of multiple inhibitory factors that normally repress OLG differentiation. In addition, p38 inhibitors upregulate a large number of genes involved in cell division. Therefore, p38 most likely regulates multiple steps of the OLG differentiation pathway. Indeed, in muscle, p38α/β act as a molecular switch that can regulate multiple stages of myocyte growth. Namely, in myocyte satellite cells that do not express MyoD, p38α/β regulate cell cycle entry; however, following specification and MyoD expression, p38α/β switch its function to regulate differentiation (Jones et al. 2005).

A genome-wide screen of p38 regulated mRNAs identified a number of transcripts involved in OLG differentiation, including an upregulation of gene targets that are normally inhibitory to OLG differentiation, including Tcf4, and Id2. Furthermore, we observed increases in Hes5 when we treated OLPs with p38 inhibitors. Increases were also seen with these many repressors following treatment with MK2 inhibitors. Therefore, both p38 and MK2 downregulate transcripts that normally repress OLG differentiation through an unknown mechanism. We observed increased levels of activated MK2 during OLG

differentiation. Increases in activated MK2 have also been observed in trophoblast stem cells (Winger et al. 2007).

The Wnt/β-catenin target, Tcf4 is also upregulated following treatment of OLPs with p38 or MK2 inhibitors. One possible link between β-catenin signaling and p38 signaling is through the glycogen synthase kinase 3β (GSK3β) signaling pathway. Indeed, the Wnt/β-catenin pathway activator, Wnt3a was able to strongly activate p38 MAPK (Bikkavilli et al. 2008). In addition, the p38 inhibitors SB203580 and SB230963, or DN-p38 block the Wnt3a-induced accumulation of β-catenin and subsequent Tcf gene activation (Bikkavilli et al. 2008). In another study, sustained activation of β-catenin led to reduced p38 activity, possibly through a β-catenin mediated downregulation of Rac1 and Cdc42, both of which can activate p38 (Kim et al. 2010). Furthermore, in chondrocytes, DN-p38 results in sustained Wnt7a activation and inhibition of differentiation (Jin et al. 2006). These data suggest that p38 signaling can normally impinge on Wnt/β-catenin signaling to promote differentiation, possibly through regulation of GSK3β. Therefore, p38 may be involved in the correct timing of OLG differentiation by regulating Wnt/β-catenin signaling through GSK3β.

Another important question to be addressed is the upstream stimuli and/or receptors that stimulate p38 signaling to promote differentiation. In myocytes, Cdo cell surface receptors are coupled to p38 to regulate differentiation. The Cdo receptor is an IgG superfamily member that promotes myocyte differentiation both *in vitro* and *in vivo* (Cole et al. 2004; Kang et al. 2003; Kang et al. 1998). Cdo^{null} myocytes fail to differentiate, and this phenotype can be rescued by CA-MKK6 (Takaesu et al. 2006). Mechanistically, the Cdo receptor scaffolds Bnip2 and JLP to serve as docking proteins for Cdc42 and p38α/β, respectively. Cdc42, in turn, activates p38 by an unknown mechanism (Kang et al. 2008). Purified mouse OLGs express Cdo transcripts (supplemental data of (Cahoy et al. 2008)), although the functions of this receptor in OLGs remains completely unknown. In fact, the ligand/signal that initiates OLG differentiation has not been conclusively identified. Several lines of evidence point towards surface immunoglobulin or

integrin receptors to regulate OLG differentiation. These IgG members include MAG, the immuglobulin Fc receptor γ (FcRγ) (Nakahara et al. 2003), L1-cell adhesion molecule (L1-CAM), integrin receptors, or F3/contactin (Laursen et al. 2009). FcRγ signals through Fyn to regulate OLP differentiation, and $FcR\gamma^{null}$ mice are hypomyelinated and have drastic reductions in MBP staining.

The mechanism of p38 activation in OLGs may also be through laminin-integrin receptor interactions. We observed upregulation of activated p38 following the seeding of OLPs onto DRGNs, suggesting that OLG-neuron contact is sufficient to induce activation of this kinase. Furthermore, in Schwann cell cultures, stimulation with laminin induced a p38 dependent phosphorylation of hsp27, a downstream effector of MK2, which could be inhibited by SB203580 (Fragoso et al. 2003). Integrin receptors couple to Fyn tyrosine kinase through p190RhoGAP, an activator of the Rho family of proteins including Rho, Rac1 or Cdc42. OLGs express various integrin receptors (e.g., $\alpha_6\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_4$, $\alpha_v\beta_5$), which play multiple roles during OLG development. For example, the integrin $\alpha_v\beta_1$ is downregulated during OLG differentiation, whereas $\alpha_v\beta_5$ is upregulated (Milner and Ffrench-Constant 1994). In addition, Fyn may also signal through Cdc42 to promote OLG differentiation. Both Cdc42 protein levels and enzymatic activity are increased as OLPs differentiate, and DN- and CA- Cdc42 constructs have shown that Cdc42 (along with other GTPases like Rac1) play important roles in morphological maturation of OLGs (Liang et al. 2004). Therefore, an integrin-Fyn-p190RhoGAP-Cdc42 pathway may be coupled to the p38 pathway to promote OLG differentiation. In Schwann cells, laminin-integrin signaling is coupled to p38 activation and myelination (Fragoso et al. 2003), which may also be coupled through Cdc42. In fact, specific ablation of Cdc42 from Schwann cells causes severe myelination defects (Benninger et al. 2007). In OLGs, however, transgenic ablation of Cdc42 does not affect OLP proliferation, migration, or their *in vitro* differentiation. Only a minor myelination defect is observed by the enlargement of the OLG inner mesaxon (Thurnherr et al. 2006). Therefore, in OLGs the interplay between p38, Fyn and Cdc42 requires further investigation.

The ability of cultured OLPs to spontaneously differentiate in the absence of laminin or axons raises the possibility that multiple pathways actually promote OLG differentiation. Considering the observation that OLPs differentiate simply by mitogen withdrawal suggests that an intrinsic stimulus exists to promote differentiation. Indeed, OLGs can secrete growth factors, for example, IGF-1, in either an autocrine or paracrine fashion to regulate OLG differentiation and myelination ((Ye et al. 2002; Zeger et al. 2007) and reviewed by (Pfeiffer et al. 1993)). We have found that IGF-1 and other growth factors induce p38 activation in cultured OLPs (Haines, J.D., unpublished observations). Interestingly, packing constraints between OLGs induce differentiation, suggesting that intercellular contact or volume receptors may be able to regulate OLG growth. For example, contact between membranes of neighbouring OLGs can trigger signaling through myelin glycosphingolipid carbohydrate interactions, resulting in cytoskeletal rearrangement; however, this signaling is not p38-dependent (Boggs et al. 2008).

The downstream effectors of p38 signaling that induce cellular differentiation has also generated intense interest. In myocytes and neurons, the Cdo receptor regulates cellular differentiation by promoting p38 induced phosphorylation of E47 and subsequent dimerization with bHLHs. E47 dimerizes with MyoD in myocytes, and neurogenin-1 in neurons to promote cell-specific gene transcription (Lluis et al. 2005; Oh et al. 2009). E47 is also a cofactor for the OLG-specific bHLH, OLIG2 that promotes OLG differentiation (Wegner 2008). Therefore, determining the p38-induced activation of E47 and subsequent dimerization with OLIG2 will establish if a similar mechanism exists in OLGs. In fact, determining protein complex formation between p38 and various upstream activators and downstream substrates will provide further mechanistic clues to their functions in OLGs. We found protein complexes formed between p38 and MK2. It has been previously reported that complexes form between p38, MK2, hsp27 and Akt to regulate apoptosis (Zheng et al. 2006). However, the function(s) of these complexes during normal cell growth and differentiation has yet to be fully determined.

Interestingly, the MKK6/p38 and IGF/PI3K/Akt pathways have been found to be interdependent. Akt1/2 can promote the association of MyoD with the p300 and PCAF histone acetyltransferases (HATs) via a direct phosphorylation of p300. Furthermore, mutating amino acid residues on chromatin targets that are normally phosphorylated by Akt prevents the induction of the myogenic program, even after forced induction of p38 (Serra et al. 2007). p38 also regulates Akt at the transcriptional level and can promote its activation in C2C12 myocytes (Cabane et al. 2004). In OLGs, Akt plays an important role in differentiation since transgenic mice overexpressing CA-Akt have enhanced myelination (Flores et al. 2008). The targets of Akt-induced OLG differentiation and myelination are still under investigation, but appear to involve the mammalian target of rapamycin (mTOR) (Narayanan et al. 2009), a protein that regulates OLG differentiation (Tyler et al. 2009) via protein translation (Bibollet-Bahena and Almazan 2009). Therefore, deciphering the interplay between p38 and Akt during OLGs differentiation may provide information as to how these two factors regulate myelination.

Determining protein complexes formed between p38, MSK and CREB, and other binding partners, e.g., histone H3, may provide clues to chromatin changes occurring during OLG differentiation and myelination. Similar to Akt, p38 can phosphorylate CREB binding protein (CBP) and p300 which alters their capacity to activate chromatin structure through acetylation of histones (Bratton et al. 2009; Chan and La Thangue 2001). Equilibrium between the acetylation and deacetylation status of histones affects chromatin structure and subsequent gene transcription (Lehrmann et al. 2002). For example, during the onset of rodent myelination, the protein levels of the CBP and p300 acetyltransferases decrease (Shen et al. 2005); therefore allowing HDACs to deacetylate histones associated with transcriptional repressor genes that normally block OLG differentiation (Li et al. 2009). Interestingly, p38 can phosphorylate p300 at the two sites that target it for proteosomal degradation (Poizat et al. 2005). Therefore, p38 signaling can indirectly affect chromatin remodeling through regulation of acetyltransferase activity. In addition, p38 downstream effectors can alter chromatin structure

through the regulation of polycomb group proteins (PcGs), which are transcriptional regulatory multiprotein complexes that control gene expression (Kerppola 2009). For example, MK3 can regulate the phosphorylation of Bmi1, a component of the polycomb repressive complex 1 (PRC1) subfamily of PcGs (Voncken et al. 2005). In addition, MK2 and MK3 can associate with HPH2, another component of PRC1. The protein composition of PcG complexes in OLGs, and their roles differentiation and myelination remain unknown (Copray et al. 2009).

Finally, it is essential to determine the roles that p38 plays in the process of myelination and remyelination *in vivo*. The importance of p38α for OLG differentiation and *ex vivo* myelination of DRGN may not hold true for *in vivo* myelination, most likely due to compensation from other p38 isoforms. Selective knock-out of p38α from OLGs using floxed p38α mice crossed to CNP promoter-driven Cre recombinase mice would address the *in vivo* roles of p38α in myelination. The roles of p38 in remyelination versus myelination also should be established. Some evidence suggests that the process of developmental myelination and remyelination are different processes. For example, OLIG1 plays less important roles in OLG development than remyelination (Arnett et al. 2004). Furthermore, the inflammatory environment present during multiple sclerosis (MS) is not seen during normal development. Therefore, application of p38 inhibitors in an experimental autoimmune encephalomyelitis (EAE)-model of MS may shed light on the roles of this kinase during an inflammatory demyelination process. The use of p38 inhibitors in other inflammatory diseases of the CNS has not been widely explored. One study showed that a blood brain barrier-permeable p38 inhibitor attenuated cytokine expression and reduced behavioural deficits in a mouse model of Alzheimer's disease (Munoz et al. 2007)

Therapeutic Implications in the CNS

p38 is considered a major target of the inflammatory response for production and regulation of inflammatory cytokines. Therefore, the p38 MAPK signaling cascade has been considered one of the most desirable targets for the treatment of inflammation and autoimmune diseases. After identification of its role in the

regulation of IL-1 and TNFα, huge interest was generated in therapeutically targeting p38 to treat inflammatory diseases. The upregulation of these cytokines is a hallmark associated with many diseases including rheumatoid arthritis, endotoxic shock, psoriasis and MS (Lin et al. 2006; Salituro et al. 1999). Moreover, aberrant p38 signaling has been suggested for diseases with an inflammatory component, such as Alzheimer's disease (Johnson and Bailey 2003), where increased phospho-p38 levels have been detected in plaques (Hensley et al. 1999). However, it appears that the phosphorylation status of proteins in MS plaque tissue has never been examined, although, microarray analysis of MS patient blood revealed an upregulation of p38α mRNA during relapsing phases of the disease. The upregulation of p38α transcripts correlated with increased expression of cytokines and other genes involved in the immune response (Arthur et al. 2008).

The etiology of MS is unknown; however the pathological hallmarks of the disease include peripheral T-cell infiltration, microglial activation, demyelination and OLG cell death, astrogliosis, and eventual axonal transection. Following disruption of the blood brain barrier, peripheral T cells invade the CNS which results in microglial activation. During disease progression T cells secrete matrix metalloproteinases (MMPs) to degrade extracellular proteins, further promoting their transport through the blood brain barrier and eventually leading to the tissue destruction seen in MS (Chandler et al. 1997; Lee et al. 1999; Ozenci et al. 2000). p38 MAPK among other kinases, positively regulates MMP expression (Reuben and Cheung 2006). Therefore, pharmacological inhibition of p38 may be efficacious in abrogating T-cells infiltration into the CNS, since inhibitors can reduce MMP expression. Once activated by T cells, microglia release both pro- and anti-inflammatory cytokines. These cytokines have both neurotoxic and neuroprotective roles to OLGs and neurons. Importantly, the imbalance of these cytokines is a milestone of MS, namely an upregulation of pro-inflammatory (IFNγ, TNFα, IL-6, IL-12, IL-17, IL-23), and downregulation of anti-inflammatory (IL-4, TGFβ1, IL-10) cytokines are observed (Mihailova et al. 2005; Navikas and Link 1996; Olsson et al. 1990). p38 regulates the expression of

many of these cytokines, and p38 inhibition may prove to be beneficial in attenuating the pro-inflammatory cytokine production. However, the relative contribution of p38 in regulating the balance between pro- and anti-inflammatory cytokines in MS has yet to be determined, but may hold promise in restoring cytokine homeostasis. In addition, T lymphocytes produce IFNγ, a cytokine that becomes detectable during the symptomatic phase of MS and EAE, and may play roles in OLG-induced cell death, or have anti-inflammatory properties (Muhl and Pfeilschifter 2003; Wheeler and Owens 2005). IFNγ induces a strong activation of p38 in human microglia (D'Aversa et al. 2002). Furthermore, activated microglia produce IL-23, whose levels can be decreased by blocking the p38 MAPK pathway or by application of NFκB inhibitors (Li et al. 2008). Interestingly, the tetracycline antibiotic minocycline has shown moderate clinical efficacy for the treatment of MS by reducing pro-NGF production from activated microglia by a mechanism dependent on p38 (Yune et al. 2007). In addition, microglia serve as scavengers to remove axonal and myelin debris following injury, which has consequences on axon regrowth (Tanaka et al. 2009). The axon damage and eventual axonal transection that occurs in MS presents a major therapeutic obstacle for treating disease. The axonal debris engulfment properties of microglia requires p38 activity (Tanaka et al. 2009). Furthermore, several studies have shown that p38 (or p38 homologs in *C. elegans*) is required for synapse formation, growth cone initiation, and axon regeneration following axotomy (Hammarlund et al. 2009; O'Brien and Sagasti 2009; Verma et al. 2005). In fact, p38 inhibitors cause collapse of regenerating growth cones following injury (Verma et al. 2005). Interestingly, however, p38 appears to play opposite roles in the PNS, where p38 inhibitors actually promote axon regrowth (Myers et al. 2003).

In response to demyelination and other CNS injuries, astrogliosis occurs. This is a process where astrocytes hyperproliferate, become hypertrophic, extend long processes and secrete large amounts of chondroitin sulfate proteoglycan (CSPG). These processes may occur in order to protect damaged areas from further destruction (Rolls et al. 2009). However, CSPGs along with myelin

inhibitors (MAG, OMgp, Nogo-A, NgR, Lingo-1, p75, gangliosides) create a plaque environment inhibitory to *de novo* axonal regrowth (Domeniconi and Filbin 2005; Xie and Zheng 2008). Interestingly, MK2 phosphorylates Nogo-B, a splice-isoform related to Nogo-A (Rousseau et al. 2005). In addition, in models of injury, reactive astrocytes have upregulated activation of p38β in response to kainic acid treatment. Furthermore, treatment of mouse astrocytes with p38 inhibitors induces process outgrowth (Heffron and Mandell 2005). Our laboratory also has evidence to suggest that p38 inhibitors induce astrocyte proliferation (unpublished observations). In addition, in MS and EAE, astrocytes express increased levels of ICAM-1, an IgG superfamily member that is important for T cell invasion through brain capillary walls, and subsequent binding to astrocytes. ICAM-1 engagement in rat primary astrocytes causes production of IL-6 which is dependent on both p38 and ERK (Lee et al. 2000).

Finally, the small population of OLPs that reside in the adult brain migrate to plaque areas in an attempt to remyelinate axons. However, these OLPs fail to differentiate into mature OLGs, suggesting a differentiation process is blocked leading to the failure of remyelination. We have shown important roles for p38 in regulating OLG differentiation and myelination. Therefore, p38 inhibitors might have detrimental effects on OLG remyelination if these drugs were to be administered for therapeutic purposes in MS. Considering the aforementioned evidence for the multiple roles of p38 in OLG differentiation, axon regrowth, astrogliosis, attenuating microglial activation and infiltrating T cells, the consequence of p38 inhibitors is unknown. As such, it is important to determine the relative contribution of p38 function in glial, neuronal and inflammatory cells during the pathological progression of MS.

p38 inhibitors for the treatment of other inflammatory diseases

Studies in the past ten years have brought to question the efficacy of p38 inhibitors in inflammatory diseases, and clinical efficacy is becoming doubtful. Pre-clinical problems associated with p38 inhibitors include hepatotoxicity, and

CNS inflammatory syndrome in dogs following chronic p38 inhibitor exposure (Dambach 2005). A number of p38 inhibitor compounds have showed modest to no clinical efficacy in the treatment of RA (Hammaker and Firestein 2010). For example, the p38 pan-isoform inhibitor BIRB796 showed no efficacy in the treatment of Crohn's, RA, or psoriasis (Genovese 2009). Furthermore, a p38α isoform selective inhibitor, pamapimod (RO4402257), showed no clinical efficacy in the treatment of RA, compared to methotrexate, a commonly used treatment for RA that does not target the p38 pathway (Cohen et al. 2009). Strikingly, at least twenty-two p38 inhibitors have been investigated in phase I/II clinical trials, without a single compound advancing into the phase III stage (Firestein 2009). Many reasons have been speculated for the block of advancement of these inhibitors, including the failure of animal models to accurately reflect human disease (Firestein 2009). Thus, the role, if any, that p38 inhibitors will serve in the treatment of inflammatory diseases is highly debatable. Alternative approaches for targeting the p38 signaling cascade to treat inflammation are still under investigation. For example, inhibiting either p38 upstream activators (e.g., TAK1) or downstream effectors (e.g., MK2) may ameliorate the inflammatory response and show clinical efficacy for the treatment of inflammation and autoimmune diseases (Hammaker and Firestein 2010).

Conclusions

We have found that p38 MAPK signaling cascade is a crucial effector of both OLG differentiation and CNS myelination. This contribution of knowledge will help us to further understand the complex process of OLG differentiation, and may provide clues to stimulating remyelination in diseases such as MS.

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