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p57kip2 is dynamically regulated in experimental autoimmune encephalomyelitis and acts as a negative regulator of oligodendroglial maturation

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1. Introduction

1.1 Multiple sclerosis

1.1.1 History and clinical presentation of multiple sclerosis

Multiple sclerosis (MS) which was first described by French neurologist Jean-Martin Charcot in 1868 (Charcot, 1868) is a chronic inflammatory demyelinating disease of the central nervous system of unknown etiology affecting predominantly young adults (Hemmer et al., 2006). MS symptoms include optic neuritis, paresthesia, limb weakness, and spasticity which are all commonly observed at disease onset while cortical signs, such as aphasia, apraxia, seizures, or extrapyramidal signs are observed more rarely (Compston et al., 2005). Following the initial attack, MS patients will experience one of four clinical courses, which have been standardized and classified (Lublin and Reingold, 1996). A relapsing-remitting course is characterized by a sequence of relapses with full or partial recovery between clinical relapse events. About 80 to 90% of patients present this form of the disease at onset. Approximately 40% of patients with relapsing-remitting MS (RRMS) will eventually develop a secondary progressive course. Secondary progressive MS (SPMS) is marked by a gradual progression of symptoms with or without occasional relapses and minor remissions. Eventually, specific signs of CNS dysfunction (e.g. cognitive impairment, progressive motor and sensory loss) accumulate. Alternatively, a primary progressive form (PPMS) may develop from onset, with progressive worsening of symptoms with occasional minor remissions. Patients with this form often have a slowly progressive upper motor neuron syndrome affecting primarily the legs. Finally, a relapsing progressive form of MS, a rare progressive disease from the beginning with clear acute relapses, has also been defined (Lublin and Reingold, 1996).

1.1.2 Epidemiology and Genetics of MS

The prevalence of MS varies greatly worldwide, ranging from 30 cases per 100,000 individuals in northern Europe and North America to fewer than 5 cases per 100,000 (Sadovnick and Ebers, 1993). The prevalence of MS follows a north-south gradient in both hemispheres, with higher prevalences occurring in the north. However, isolated areas of high prevalence are also observed in southern Europe (Sadovnick and Ebers, 1993). Different MS rates have been reported for clusters of genetically disparate populations in the same geographic areas, emphasizing the importance of genetic background for susceptibility to MS. MS, like most

autoimmune disorders, is more common in women than in men, with a ratio of 1.5:1 (Kurtzke, 1993). MS has age-specific incidence rates, with a peak age of onset of 27. Studies of MS in migrants have also been performed to evaluate the combined influence of genetic and environmental factors. Immigrants from high- or medium-risk areas tend to retain the risk of their birthplaces, while immigrants who move from low- to high-risk areas may increase their risk (Kurtzke, 1993). Additional studies have reported that individuals who immigrate after age 15 retain the risk of their birthplace, while those who immigrate before they are 15 acquire the risk of their new country suggesting that an infectious agent acquired before age 15 may influence an individual's likelihood of developing MS. Also, four MS epidemic outbreaks in the Faroe Islands occurred after the occupation by British troops during the Second World War (Kurtzke, 1995), supporting the view of MS as a rare and delayed result of an infection acquired during adolescence. Looking at the families of affected individuals, it seems that inheritance alone is not sufficient to cause the disease, however, genetic factors appear to play a significant role in predisposition to MS. The absolute risk of developing the disease for biological relatives of individuals with MS is 20- to 40-fold higher than for the general population. The risk is greater for siblings, especially sisters, and decreases for second and third relatives (Sadovnick et al., 1988). Additionally, the MS concordance rate is 31% in monozygotic twins and only 5% in dizygotic twins (Sadovnick and Ebers, 1993). This rate of MS disease discordance in twins has been used to support the view that genetics alone cannot explain this disorder and that other factors, such as environmental effects, must also be associated with disease pathogenesis. It is known that an increased risk is related to the presence of the MHC alleles HLA-DR2 and DQw1 on chromosome 6 (Compston, 1994). HLA-DR and DQw1 polymorphisms seem to be associated with MS susceptibility only, but not with the severity and course of the disease. However, the roles of other genes putatively linked to the disease are being evaluated (Chataway et al., 1998). Despite the above mentioned correlations, the pattern of transmission of genetic susceptibility remains unclear. The difficulty in establishing an inheritance pattern for MS may be due to several factors, including, for instance, the difficulty in diagnosing MS and the relatively large age range of high risk (from late teens to late 50s).

1.1.3 Etiology of MS

Concerning the etiology of MS, it is a widely accepted view that multiple sclerosis is an inflammatory disease with autoimmune features influenced by environmental or infectious factors in genetically susceptible individuals (Willer and Ebers, 2000). Although an ideal

system for the classification of different MS stages does not yet exist (Van, V and De Groot, 2000) there is broad consensus that loss of myelin, also known as demyelination, due to oligodendrocyte cell damage or death together with subsequent axonal degeneration leading to reactive glial scar formation are the key hallmarks of this disease (Trapp and Nave, 2008). In this context four different patterns of demyelination have been described (Lucchinetti et al., 2000a). Interestingly, these patterns are quite inhomogeneous in nature with patterns I and II showing close similarities to T cell-mediated or T cell plus antibody-mediated autoimmune encephalomyelitis, respectively, while patterns III and IV are highly suggestive of a primary oligodendrocyte dystrophy, reminiscent of virus- or toxin-induced demyelination rather than autoimmunity. However, it is important to point out that this classification system does not incorporate the common observation of axonal transection seen in MS which is thought to be the pathologic correlate of the irreversible neurological impairment in this disease (Trapp et al., 1998b). In summary, the data available thus far point to the still unclear nature of MS etiology and suggest a polycausal disease process.

1.1.4 Histopathology of MS

Histopathologically, the correlate of demyelination in multiple sclerosis is the so-called MS plaque (Rindfleisch, 1863). These lesions can primarily be found around small veins and venules, which show focal cuffs of perivascular inflammation. When perivenous lesions grow they can fuse with additional adjacent demyelinating areas forming large demyelinating plaques, which may reach several centimeters in diameter (Brownell and Hughes, 1962). MS lesions can also grow radially in which case active demyelination is observed at the periphery of the lesion, resulting in a gradual expansion of the plaque into the surrounding normal white matter. Interestingly, demyelinating lesions are not restricted to the myelin rich white matter but can also occur in gray matter where they are difficult to identify (Brownell and Hughes, 1962). While, in general, lesions can be found in any CNS region, certain neuroanatomical structures are affected more frequently than others such as the periventricular white matter, particularly the lateral angles of the lateral ventricles, the subcortical white matter, the optic nerves, the cerebellar peduncles and the spinal cord. This apprarently irregular distribution of lesions is in part a result of their formation around small to medium-sized veins, an observation which lead Jean Cruveilhier to the statement that „la phlebite domine toute la pathologie“ (“The phlebitis dominates the whole pathology [of multiple sclerosis]”) (Cruveilhier, 1829). As already pointed out above, all MS plaques feature a certain degree of axonal injury and loss, both of which can vary from plaque to plaque in the same brain and even more so between

plaques of different individuals. It has been described that axonal density within plaques ranges from 20 to 80% of that in the periplaque white matter and that within chronically demyelinated plaques the reduction of axonal density is on average 60-70% compared with that in normal tissue of the same area (Lassmann, 2003).

1.1.5 Current and potential treatments for MS

While there is currently no therapy available to directly revert plaque formation in MS affected brains, there are numerous approaches to alter the underlying (auto-)immune mechanisms leading to demyelination. Besides the classical "ABC drugs" for MS, interferon beta-1a, interferon beta-1b and glatiramer acetate new promising MS treatments have emerged during the past 10 years. Among them Natalizumab (O'Connor et al., 2004), a humanized monoclonal antibody against the cellular adhesion molecule α4-integrin and mitoxantron (van de Wyngaert et al., 2001), a type II topoisomerase inhibitor, which disrupts both cellular DNA synthesis and DNA repair, to name only two. Cell based therapies aiming to replace oligodendrocytes lost in the course of inflammatory myelin destruction have thus far yielded unsatisfying results. In animal experiments peripheral Schwann cells (SC) transplanted into demyelinated lesions were oberserved to extensively remyelinate axons while simultaneously supporting axonal regeneration (Blakemore, 1977;Duncan et al., 1981). As SC are easy to obtain by biopsy and can be expanded in culture these cells seem to be ideal candidates for autologous grafting. However, when transplanted into the CNS they do not migrate and are uncapable of integrating into the asctrocyte-rich environment. There have also been studies investigating the potential of oligodendrocyte lineage cells and their precursors as grafts for remyelination (Lachapelle et al., 1983). Similar to SC, extensive remyelination can be seen following grafting of oligodendrocyte precursor cells that integrate well into host CNS tissue. Unlike SC oligodendrocytes can migrate in the host CNS under permissive conditions. The disadvantages of oligodendrocyte transplantation, however, are apparent. Noninvasive isolation of these cells which have to be harvested by stereotactic brain biopsy is currently not possible. Additionally, difficulties committing human stem cells to an oligodendrocyte lineage in culture, be it by epigenetic stimulation or overexpression of certain transcription factors, have not yet been successfully overcome. Another candidate for cell replacement therapy are olfactory ensheathing cells (Franklin et al., 1996) that can be obtained from the olfactory bulb and then autologously engrafted into demyelinated lesions. These cells have better migrational properties than SC but their actual potential for remyelination is still a matter of controverse discussions. Finally, there are numerous studies investigating stem cell based remyelination

therapies (Akiyama et al., 2002;Bonilla et al., 2002;Brustle et al., 1999;Hammang et al., 1997;McKenzie et al., 2006). Although there are several studies confirming that differentiation of stem cells into myelin sheath forming cells under cell culture conditions is generally feasible, the *in vivo* potential of these cells needs to be further examined.

1.2 The oligodendrocyte

1.2.1 Oligodendrogliogenesis during embryonic development

The term oligodendroglia was introduced by Rio Hortega (Rio Hortega DP, 1921) to describe CNS cells that show few processes in material stained by Hortega's metallic impregnations. At this time the oligodendrocyte's function as the myelin producing cell of the CNS was not yet discovered. Today, however, it is known that oligodendrocyte precursors originate from neuroepithelial cells of the ventricular zones, at very early stages during embryonic life. This was first suggested by following the expression of specific markers of oligodendrocytes (Curtis et al., 1988;Hardy and Reynolds, 1993;Levine et al., 1993;Pfeiffer et al., 1993;Hardy and Reynolds, 1991), some of which are transcripts encoding future protein components of myelin such as 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), myelin basic protein (MBP) and proteolipid protein (PLP) (Ikenaka et al., 1992;Peyron et al., 1997;Pringle and Richardson, 1993;Timsit et al., 1995;Timsit et al., 1992;Yu et al., 1994). In the spinal cord, oligodendrocytes initially arise in ventral regions of the neural tube. Looking at specific markers of the oligodendrocyte lineage Warf and colleagues have shown, that oligodendrocyte precursors arise in the ventral spinal cord and then migrate dorsally during ontogenesis (Warf et al., 1991). During subsequent developmental processes, the dorsal regions acquire the capacity for oligodendrogenesis, probably both intrinsically (Cameron-Curry and Le Douarin, 1995;Warf et al., 1991) and through the ventro-dorsal migration of oligodendrocyte precursors. This pattern has been confirmed using cultures of the thoraco-lumbar area of the rat spinal cord, which showed that the ability to give rise to oligodendrocytes is restricted to the ventral region of this area of the spinal cord until E14 (Warf et al., 1991). Signals from the notochord/floor plate, involving the morphogenetic protein sonic hedgehog (Shh), are necessary to induce the development of ventrally derived oligodendroglia (Miller, 1996;Orentas and Miller, 1996;Orentas et al., 1999;Poncet et al., 1996;Pringle et al., 1996). In situ hybridization for mRNAs encoding proteins that are involved in oligodendrocyte maturation has been used to characterize early oligodendrocyte precursors. The data obtained showed that ventral ventricular cells express mRNA for platelet-derived growth factor alpha

receptor (PDGFR-α) (Pringle and Richardson, 1993) while other studies describe a discrete population of cells expressing mRNA for the myelin protein CNPase (Yu et al., 1994) and DM20 (PLP gene) (Timsit et al., 1992) also localized to the same zone of the developing mammalian spinal cord. However, the issue of the identity of these cells still remains unclear as the mentioned markers are not expressed by the same population of oligodendrocyte precursors, indicating cellular diversity already at this stage. DM20 protein is also expressed on embryonic cells that may become oligodendrocyte progenitors as shown by Dickinson and colleagues (Dickinson et al., 1996) in the spinal cord. The initial restricted localization of oligodendrocyte precursors in the ventral plate of the neural tube appears not to be limited to the spinal cord (Yu et al., 1994) but is also observed in mid- and forebrain (Spassky et al., 1998;Timsit et al., 1992). Intestingly, a similar restricted localization of the sites of oligodendrogliogenesis has been described in other vertebrate species, such as human (Hajihosseini et al., 1996), chick (Ono et al., 1997), and *Xenopus* (Yoshida, 1997). The population of oligodendrocytes derived from the restricted loci appears to be regionally distributed in the brain. For instance, in the chick embryo, some of the oligodendrocytes of the optic nerve generate suprachiasmatic foci of precursors located in the medioventral epithelium of the third ventricle (Ono et al., 1997). The developing cerebral cortex of murine embryos, on the other hand, could be populated by oligodendrocytes originating from precursor cells located in the laterobasal plate of the diencephalons and probably also in the telencephalon (Price, 1994). One of the most important cerebral origins of oligodendroglia, however, is the subventricular zone (SVZ) a germinal matrix of the forebrain that first appears during the later third of murine embryonic development (Doetsch et al., 1997). It enlarges during the peak of gliogenesis, between P5 and P20, and then shrinks but persists into adulthood. Lineage tracing studies of perinatal SVZ cells using stereotactically injected retrovirus support the view that the majority of progenitors within this germinal matrix are glial precursors that generate astrocytes on the one hand and oligodendrocytes on the other hand (Luskin et al., 1988;Price and Thurlow, 1988). Although the majority of cells give rise to homogeneous progeny, some SVZ cells differentiate into both oligodendrocytes and astrocytes (Levison and Goldman, 1993).

Of note, the original assumption that each cluster is a clone, i.e. the complete progeny of a single precursor, was based on the thought that the dispersion of cells in structures like the embryonic cortex was purely radial. However, other retroviral studies have shown that cells disperse considerably and also tangentially during postnatal corticogenesis and embryonic development (Price, 1994). Thus two clones could occupy an overlapping space. Therefore, the issue of OPC dispersion must be considered as still not entirely settled. Nevertheless, based on

studies with tritiated thymidine, immunocytochemistry, and retroviral studies there is good reason to assume that the majority and possibly all oligodendrocytes originate from different cells in the SVZ than those that give rise to astrocytes. In the neonatal rat cerebrum, oligodendrocytes also arise postnatally from the SVZ of the lateral ventricles (Levison and Goldman, 1993;Zerlin et al., 1995). Similarly, immunocytochemical studies have indicated that oligodendrocytes of the cerebellum arise postnatally from the SVZ of the fourth ventricle (Reynolds and Wilkin, 1988b). Oligodendrocyte progenitors have been found to migrate long distances away from these zones and populate the developing brain to form white matter throughout the brain, as shown by developmental (Frost et al., 1996;Levison and Goldman, 1993) and transplantation studies (Espinosa de los et al., 1993;Lachapelle et al., 1983). Since mature oligodendrocytes cannot migrate preventing premature differentiation of progenitors is crucial for ensuring that they successfully make it to their final destination. Oligodendrocytes, first detectable in the optic nerve around birth, continue to increase in number for six postnatal weeks in rodents (Barres and Raff, 1994;Skoff et al., 1976). Indirect evidence suggests that optic nerve oligodendrocytes are derived from precursor cells that migrate into the nerve from the brain rather than from neuroepithelial cells of the original optic stalk. Accordingly, during late embryonic development in the rat (at about E15), cultures from the chiasma but not from the retinal end of the nerve contain significant numbers of oligodendrocyte progenitor cells (Small et al., 1987).

1.2.2 Oligodendrocyte migration: mechanisms and molecules involved

Oligodendrocyte progenitors not only migrate extensively throughout the CNS before their final differentiation into myelin-forming oligodendrocytes (Small et al., 1987) but they also extend processes in a way similar to the extension of neurites from neuronal cell bodies. As for neurons, a number of extracellular matrix (ECM) molecules play an instructive role in the control of migrating oligodendrocytes, among them Tenascin-C and PSA (Bartsch et al., 1994;ffrench-Constant et al., 1988;Wang et al., 1994). After their migration in the mammalian CNS, progenitors settle along fiber tracts of the future white matter and then transform into preoligodendrocytes, multiprocessed cells which keep the property of cell division and acquire the marker O4 (Sommer and Schachner, 1981). At this stage, they are less motile (Orentas and Miller, 1996), or even postmigratory (Pfeiffer et al., 1993), and lose their mitogenic response to PDGF (Gao et al., 1998;Hart et al., 1989;Pringle and Richardson, 1993). The preoligodendrocyte becomes an immature oligodendrocyte, characterized in the rat by the appearance of the marker galactocerebrosidase (GalC), and the loss of expression of GD3 and

A2B5 antigens on the cell surface. CNPase is the earliest known myelin-specific protein to be synthesized by developing oligodendrocytes (Reynolds and Wilkin, 1988a;Reynolds and Wilkin, 1988b;Sprinkle, 1989;Vogel and Thompson, 1988). In rat cerebellum, CNPase is expressed at the same time as GalC (Reynolds and Wilkin, 1988a) while MBP is expressed 2–3 days later along with PLP, immediately before myelin formation. The same sequence also occurs in OPC grown under cell culture conditions where CNPase is expressed at the same time as GalC (Pfeiffer et al., 1993). MBP, myelin-associated glycoprotein (MAG), and PLP appear sequentially both in vivo and in vitro (Dubois-Dalcq et al., 1986;Hardy and Reynolds, 1993;Monge et al., 1986;Pfeiffer et al., 1993) and signify a mature oligodendrocyte. In vitro analyses suggest that maturation of oligodendrocytes from the precursor stage to the mature cell is identical in culture, even without neurons, and in intact tissue. Therefore, the capacity of oligodendrocyte progenitors to differentiate into oligodendrocytes appears to be intrinsic to the lineage (Temple and Raff, 1986). Generally speaking, in the absence of neurons, oligodendrocytes can generate a myelin-like membrane (Sarlieve et al., 1983) though coculture with neurons significantly increases myelin gene expression, such as PLP, MBP, and MAG (Macklin et al., 1986;Matsuda et al., 1997). In culture the presence of myelin oligodendrocyte glycoprotein (MOG) correlates with late stages of maturation of the oligodendrocyte (Solly et al., 1996) which is reminiscent of the oligodendroglial myelin-gene induction by neuronal contact observed in vivo (Kidd et al., 1990). Another indication that myelination is progressing can be seen in the switch to PLP expression in premyelinating oligodendrocytes originally expressing PLP isoform DM20, only. (Trapp et al., 1997). This myelin-gene induction parallels morphological modifications, i.e., in vivo, when axonal contact occurs, there is a dramatic modification of oligodendrocyte morphology with loss of oligodendrocyte processes that have not yet contacted axons (Hardy and Friedrich, Jr., 1996).

1.2.3 Oligodendroglial differentiation and specific markers

During differentiation an oligodendrocyte extends many processes, each of which contacts and wraps around a stretch of axon with subsequent condensation of the multispiral membrane-forming myelin (Bunge et al., 1962;Bunge, 1968). On the same axon adjacent myelin segments belong to different oligodendrocytes whose number of processes forming myelin sheaths can vary from 40 in the optic nerve of the rat (Peters A, 1991) to one in the spinal cord of the cat (Bunge et al., 1961). Before their final maturation involving myelin formation, oligodendrocytes go through many stages of development which are defined by their proliferative capacities, their migratory abilities, changes in their morphology and the

sequential expression of developmental markers (Hardy and Reynolds, 1993;Pfeiffer et al., 1993). Many of these markers were at first identified in tissue cultures and some of them represent characteristic myelin components. Myelination requires a number of sequential steps in the maturation of the oligodendroglial cell lineage (Hardy and Reynolds, 1993;Pfeiffer et al., 1993) accompanied by a coordinated change in the expression of cell surface antigens. Differentiation involves the loss or the acquisition of certain surface or intracellular antigens the most important ones of which should be described below in further detail.

Platelet-derived growth factor α-receptor (PDGFR-α)

Platelet-derived growth factor *α*-receptor (PDGFR-α) transcripts are detected at very early stages of the developmental maturation of myelinating glial cells. The corresponding ligand to the receptor, PDGF, is synthesized during development by both astrocytes and neurons (Mudhar et al., 1993;Yeh et al., 1991). In vitro, PDGF acts as a survival factor for oligodendrocyte precursors (Grinspan and Franceschini, 1995) and as a potent mitogen for oligodendrocyte progenitor cells, although triggering only a limited number of cell divisions (Gao et al., 1998;Raff et al., 1988). This developmental clock (Temple and Raff, 1986) is not related to the loss of PDGFR-α from the surface of oligodendrocyte progenitor cells but is due to the blockade of the intracellular signaling pathways from the PDGF receptor to the nucleus (Hart et al., 1989). PDGF-α receptors disappear at the O4-stage of oligodendrocyte maturation (Ellison and De Vellis, 1994;Nishiyama et al., 1996).

A2B5

The monoclonal antibody A2B5 (Eisenbarth et al., 1979) recognizes several gangliosides (Fredman et al., 1984) that are still uncharacterized. A2B5 is expressed both on neurons and glial cells in vivo and is used essentially in oligodendrocyte cultures to follow the maturation of oligodendrocyte progenitors. In culture, ganglioside GT3 and its *O*-acetylated derivative are among the A2B5-reactive gangliosides (Dubois et al., 1990;Farrer and Quarles, 1999) and undergo downregulation as the cells differentiate into mature oligodendrocytes,

O4

O4, a sulfatide like antigens belonging to the so-called O-family of antigens, is formed postnatally in cell bodies of type I and II (hairy eyeball type) oligodendrocytes (Schachner et al., 1981). During oligodendrocyte differentiation, O4 occurs in pro-oligodendrocytes, but not in O-2A-progenitor cells. O4 is expressed from day 3 onwards in cell cultures of embryonic

mouse brain. Biochemical analyses suggest that anti-O4 antibodies label a proligodendroblast antigen (POA)-like antigen which can, for instance, be found on the surface of chick spinal cord oligodendrocyte precursors.

Proteolipid protein (PLP)

In 1951, it was discovered that a substantial amount of protein from brain white matter could be extracted by organic solvent mixtures (Folch and Lees, 1951). Because these proteins were apparently lipid-protein complexes, they were given the name of proteolipids to distinguish them from watersoluble lipoproteins. Although other myelin proteins can be solubilized in an identical way, the name has been given to a major component of myelin, henceforth known as PLP. This protein constitutes about 50% of myelin proteins and is thus the most common of all existing myelin components. PLP and its isoform DM20 (constituting up to 20% of PLP in the adult) are coded by the same gene (Morello et al., 1986), and formed by alternative splicing (Nave et al., 1986). PLP's structure comprises four hydrophobic α-helices spanning the whole thickness of the lipid bilayer, two extracytoplasmic and three cytoplasmic domains (including the -NH2 and -COOH termini) localized at the intraperiodic and major dense lines of myelin, respectively. Spontaneous mutations involving the PLP gene occur, such as, for instance, in the jimpy (jp) mouse mutant and a number of further animal models, as well as in human dysmyelinating diseases such as Pelizaeus-Merzbacher disease (PMD). Without PLP/DM20 expression, oligodendrocytes are still competent to myelinate axons and to assemble compacted myelin sheaths. However, the ultrastructure of myelin shows condensation of the intraperiodic lines (as also observed in natural PLP mutants), correlating with a reduced physical stability. These observations suggest that PLP forms a stabilizing membrane junction after myelin compaction (Boison et al., 1995), comparable to a molecular "zipper" (Klugmann et al., 1997). On the other hand, the unexpected consequence of the absence of PLP/DM20 is an early occurrence of widespread focal axonal swellings, followed later by axonal degeneration associated with impairment of motor performance in 16-months-old mice (Griffiths et al., 1998).

Myelin basic protein (MBP)

MBP is one of the major proteins of CNS myelin and constitutes as much as 30% of protein (Kornguth and Anderson, 1965). In fact, it is merely a family of proteins, as there are many isoforms of different molecular masses resulting from alternative splicing of the MBP mRNAs (de Ferra et al., 1985). The amino acid composition of the major MBPs was determined by

Eylar and colleagues (Eylar et al., 1971) in the bovine brain, and by Carnegie (Carnegie, 1971) in humans. Direct evidence that MBP molecules play a major role in myelin compaction in the CNS was provided by studies performed on the shiverer mouse mutant which presents a large deletion of the MBP gene (Roach et al., 1985) and in which the major dense line is absent from myelin (Privat et al., 1979). MBP has been associated with cytoplasmatic microtubule association and Ca^{2+}/calmodulin-regulated microtubule stabilization in vitro (Harauz et al., 2004) and is detectable in high density over the whole myelin sheath, but not in regions of loops, somata, or the oligodendrocyte plasma membrane (Brunner et al., 1989a). The different MBP types are a set of membrane proteins that function to adhere the cytoplasmic leaflet of the myelin bilayer. As their biophysical properties may render MBP nonspecifically adhesive to any organelle membranes, oligodendrocytes have developed a mechanism for the transport of MBP mRNAs selectively to intracellular regions where MBP proteins will be necessary for myelin compaction to occur (Colman et al., 1982). Indeed, in situ hybridization has shown that MBP mRNAs are first localized in the cytoplasm of oligodendrocytes, even prior to myelination (Sternberger et al., 1978), and are then dispersed in the oligodendrocyte processes at the beginning of myelination. The myelin sheaths stain for MBP mRNAs because the messages are in part transported within the cytoplasmic channels that surround and infiltrate the sheaths. Spatial segregation of MBP messages begins to function only after oligodendrocytes have differentiated into fully myelinating cells, with cellular extensions being in place first, before the MBP transport machinery is activated (Verity and Campagnoni, 1988). Before that period, MBP is expressed throughout the cytoplasm and quite surprisingly also in the nucleus. MBP is also exceptionally present in the nuclei of apparently mature oligodendrocytes (Hardy et al., 1996) that may be remodeling or remyelinating some of the myelin sheaths that they support (Pedraza et al., 1997). Cytoskeletal elements may be involved in dynamic process of MBP mRNA transport (Ainger et al., 1997).

2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase)

As an early myelin marker, CNPase represents 4% of total myelin proteins and links myelin related proteins to the cytoskeleton (Dyer and Benjamins, 1989). It also interacts with membrane lipids during extension and wrapping of the oligodendroglial process around the axon (Barradas et al., 2000) and can already been detected in mouse spinal cord during embryonic stages (Peyron et al., 1997;Yu et al., 1994). CNPase reactivity is reported to be highest at the myelin/axon interface, and is found in lower concentration over the outer lamellae of myelin sheaths, at the cytoplasmatic face of oligodendrocyte membranes, and

throughout the compact myelin. The enzyme CNPase hydrolyzes artificial substrates, 29,39-cyclic nucleotides into their 29-derivatives. However, the biological role of this enzyme activity is obscure since 29,39-nucleotides have not been detected in the brain yet (Vogel and Thompson, 1988). CNPase is not found in compact myelin but is present in the cytoplasm of noncompacted oligodendroglial ensheathment of axons and in the paranodal loops (Peyron et al., 1997;Trapp et al., 1988;Trapp et al., 1988). The protein is posttranslationally modified, acylated, and phosphorylated (Vogel and Thompson, 1988) and is associated with the cytoplasmic plasma membrane of the oligodendrocyte by isoprenylation (Braun et al., 1990). It also possesses two of the three binding domains for GTP (Braun et al., 1990). Of note, overexpression of CNPase in transgenic mice perturbs myelin formation and creates aberrant oligodendrocyte membrane expansion (Gravel et al., 1996).

Myelin oligodendrocyte glycoprotein (MOG)

Myelin oligodendrocyte glycoprotein (MOG), a CNS-specific integral membrane protein, is an atypical member of the immunoglobulin (Ig) superfamily with two potential transmembrane domains (della et al., 1998;Kroepfl et al., 1996). With only one other exception, all Ig family members possess a single or no membrane spanning region. MOG which has been reported to play a role in cell adhesion, microtubular stabilization and complement activation (Johns and Bernard, 1999) can be preferentially detected on the extracellular surface of myelin sheaths and oligodendrocyte processes (Brunner et al., 1989b) but only in low amounts in the lamellae of compacted myelin and the myelin/axon border zone. MOG was first identified by a polyclonal antibody directed against an antigen called M2 that induces autoimmune encephalomyelitis in the guinea pig and only later it was recognized as a minor glycoprotein specific for CNS myelin (Lebar et al., 1976;Lebar et al., 1986). MOG is a surface marker of oligodendrocyte maturation (Birling et al., 1993;Coffey and McDermott, 1997;Pham-Dinh et al., 1993;Scolding et al., 1989) and its presence correlates with late stages of cell differentiation, possibly restricted to the myelinating oligodendrocytes, as shown using the CG4 oligodendrocyte cell line (Solly et al., 1996). At present, MOG is the only CNS protein that can induce both a T cell-mediated inflammatory immune reaction and a demyelinating antibody-mediated response in an animal model of demyelinating diseases, the experimental autoimmune encephalomyelitis.

1.2.4 Experimental autoimmune encephalomyelitis (EAE)

Probably due to MOG's exposed position on the extracellular surface of myelin sheaths on the one hand and MBP's abundance on the other hand, MOG and MBP are potent immunogenic

agents used to induce EAE an animal model for human multiple sclerosis in a variety of mammals. EAE is induced by the peripheral injection of whole white matter or peptides of myelin proteins such as MBP, PLP, or MOG in complete Freund's adjuvant as well as by passive transfer of autoreactive T cells in susceptible inbred rodent or monkey strains (Wekerle et al., 1994). The initial autoreactivity appears to be $CD4^+$ Th1 dependent, with the exception of MOG-induced EAE, in which a Th2 response inducing demyelinating antibodies seems to play an important role (Linington et al., 1988). Mice deficient in the IL-12 p40 gene, which is expressed in a Th1 response, are not susceptible to EAE (Segal et al., 1998), and anti-IL-12 antibodies are able to prevent EAE (Leonard et al., 1995). However, Th2 cells might also be part of the autoimmune process as Th2 cells from MBP-specific T cell receptor transgenic mice were found to be able to induce EAE in immunodeficient mice (Lafaille et al., 1997). Moreover, marmoset EAE induction using MOG can drive a Th2 response resulting in fatal demyelination (Genain et al., 1995). As for the chronology of EAE it is known that after entering the CNS, $CD4^+$ T cells interact with antigen-presenting cells (APCs) in an MHC class II-restricted manner (Fritz et al., 1985), with a local inflammatory lesion leading to a subsequent antigen-independent recruitment of inflammatory cells into the CNS and BBB alteration. In general, EAE can have an acute or chronically relapsing course, depending on the susceptibility of the species and strains of animals used (Lorentzen et al., 1995). EAE in humanized animal models has also been described. Of particular interest in this context are triple transgenic mice expressing HLA-DR2, a T cell receptor-specific for the encephalitogenic MBP epitope 84–102, and the human CD4 coreceptor (Madsen et al., 1999). These mice were able to spontaneously develop EAE, suggesting a necessary and sufficient role of the described trimolecular complex for the disease in this system. Immune-mediated attack on the myelin sheaths, as observed in EAE and MS, leads to functionally impaired glial cells and may induce oligodendroglial death (Hisahara et al., 2003). Despite a generally limited regeneration capacity of the adult CNS, remyelination does occur, particularly in early disease stages, and can lead to functional improvement (remission) most likely resulting from activation of resident oligodendrocyte precursor cells (OPCs) which can differentiate into functional myelinating cells (Chang et al., 2000). Nevertheless, compared to peripheral nervous system (PNS) lesions in which remyelination of nerve fibers is widely observed, remyelination of CNS lesions is insufficient and rarely leads to a complete clinical remission. This may be due to the limited size of the OPC pool in the CNS as well as incomplete OPC activation and differentiation, respectively. There is evidence suggesting that deficient remyelination may be a result of the influence of factors that specifically block cellular differentiation. As some of these factors

have already been identified and may be of therapeutic relevance it seems necessary to examine them in all possible thoroughness.

1.2.5 Inhibitors of oligodendroglial differentiation

The idea that the observed differentiation block of oligodendroglial progenitor cells in chronic multiple sclerosis might be responsible for remyelination failure (Kuhlmann et al., 2008) and the fact that chronic stage multiple sclerosis lesions were found to contain a relatively quiescent population of oligodendrocyte precursor cells (Wolswijk, 1998) have sparked a search for potential inhibitors of glial differentiation. Transcriptional regulators such as Hes1, Hes5, Id2 and Id4, the Notch signaling pathway and the transmembrane protein LINGO-1 have all been reported to negatively influence oligodendroglial differentiation (Gokhan et al., 2005;Jurynczyk et al., 2005;Kondo and Raff, 2000;Liu et al., 2006;Mi et al., 2005;Wang et al., 2001;Wu et al., 2003). Recently another negative regulator of glial cell differentiation the cyclin dependent kinase inhibitor p57kip2 has been described in Schwann cells (Gokhan et al., 2005;Heinen et al., 2008a) where downregulation of this gene by shRNA mediated knockdown resulted in a massive induction of *in vitro* cell differentiation.

1.2.6 p57kip2

The p57kip2 gene encodes a cyclin dependent kinase inhibitor (CDKI) that belongs to the Cip/Kip family of negative cell cycle regulators. Two subfamilies of CDKIs have been described based on their CDK binding specificity, namely the INK4 family, comprising of p15INK4b, p16INK4a, p18INK4c and p19INK4d (which bind to cyclinD/cdk4 and cyclinD/cdk6 complexes) as well as the Cip/Kip family, comprising p21cip1, p27kip1 and p57kip2 which bind to complexes consisting of cyclinD, -E, -A and cdk2, -4, and -6 (Gokhan et al., 2005;Sherr and Roberts, 1999). CDKIs are known to interfere with the cell cycle either indirectly by binding to cyclin/CDK complexes, leading to hypophosphorylated Rb-family proteins and thus to inactivation of E2F, or directly by binding to MyoD and Neurogenin-2. Cip/Kip proteins share homologous N-terminal CDK binding domains but differ in their additional domains. It has been shown that CDKIs are involved in the differentiation of neural cells and that they are able to modulate cytoskeletal dynamics either by the inhibition of RhoA or ROCK activity [p27kip1, (Besson et al., 2004) p21cip1, (Lee and Helfman, 2004;Tanaka et al., 2002)] or by binding to LIMK-1 [p57kip2, (Yokoo et al., 2003)].

1.3 Goal of the thesis

The goal of this thesis is to describe new inhibitors of oligodendrocyte precursor differentiation that prevent effective remyelination of demyelinated axons in neuroinflammatory diseases such as multiple sclerosis (MS). As the mechanisms of failing neuroarchitectural repair in mammals are so far only poorly understood the discovery of such inhibitors might provide new therapeutic approaches to overcome diseases featuring myelin sheath destruction. In view of the paper by Heinen et al. which describes p57kip2 as an intrinsic inhibitor of Schwann cell differentiation (Heinen et al., 2008a;Heinen et al., 2008b) the study presented here tries to shed light on the question whether this gene has a similar function in oligodendrocyte precursor cell differentiation *in vivo* and *in vitro*.

2. Material and Methods

2.1 Material

Cell culture

Bovine Serum Albumin (BSA)	Gibco, Karlsruhe
Fetal Calf Serum (FCS)	Gibco, Karlsruhe
Cover Slips	Menzel-Glaser, Braunschweig
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Karlsruhe
Dulbecco's Modified Eagle Medium + Hepes (DMEM+Hepes)	Gibco, Karlsruhe
Minimum Essential Medium Eagle (MEM)	Gibco, Karlsruhe
Fugene6	Roche , Mannheim
L-glutamine	Gibco , Karlsruhe
Papain	Sigma-Aldrich, Taufkirchen
L-cysteine	Sigma-Aldrich, Taufkirchen
DNAse I type IV	Sigma-Aldrich, Taufkirchen
Trypsin inhibitor	Sigma-Aldrich,Taufkirchen
Paraformaldehyde (PFA)	Merck, Darmstadt
Penicillin/Streptomycin	Gibco , Karlsruhe
Phosphate buffered saline	PAA, Paschin, Österreich, Linz, Austria
Poly-D-Lysine (PDL)	Sigma-Aldrich, Taufkirchen-Aldrich
Cell culture dishes	Greiner, Frickenhausen
Bovine insulin	Sigma-Aldrich, Taufkirchen
Human transferrin	Sigma-Aldrich, Taufkirchen
Progesterone	Sigma-Aldrich, Taufkirchen
Basic fibroblast growth factor (bFGF)	R&D Systems, Wiesbaden
Platelet-derived growth factor alpha (PDGF-AA)	Peprotech, Hamburg
Putrescine	Sigma-Aldrich, Taufkirchen
Sodium selenite	Sigma-Aldrich, Taufkirchen
Ciliary neurotrophic factor (CNTF)	Chemicon, Temecula, CA

Tri-iodo-thyronine (T3)	Sigma-Aldrich, Taufkirchen
Thyroxine (T4)	Sigma-Aldrich, Taufkirchen

Immunostaining Reagents

rabbit anti-p57kip2 antibody	Sigma-Aldrich, Taufkirchen
mouse anti-APC/CC1 antibody	Calbiochem, San Diego, California
mouse anti-O4 antibody	Chemicon, Temecula, CA
mouse anti-PDGFR-α antibody	Chemicon, Temecula, CA
mouse anti-A2B5 antibody	Chemicon, Temecula, CA
mouse anti-MOG antibody	provided by Prof. B. Hemmer, LMU Munich
mouse anti-MBP antibody	Sternberger Monoclonals, Baltimore, MD
mouse anti-CNPase antibody	Sternberger Monoclonals, Baltimore, MD
rabbit anti-LIMK-1	BD Biosciences, Heidelberg
Alexa Fluor 488 antibody	Molecular Probes
Alexa Fluor 594 antibody	Molecular Probes
Fluoromount-G	Southern Biotech, Birmingham, USA
methyl benzoate	Merck, Darmstadt
Citifluor	Citifluor, Leicester, UK
4',6-Diamidin-2'-phenylindoldihydrochlorid (DAPI)	Roche, Mannheim
Normal goat serum (NGS)	Sigma-Aldrich, Taufkirchen

Reverse Transcription and PCR reagents

High Capacity cDNA Reverse Transcription Kit	Applied Biosystems/Ambion, Darmstadt
mirVana Kit for RNA purification	Applied Biosystems/Ambion, Darmstadt
Power SybrGreen universal master mix	Applied Biosystems, Darmstadt
RNeasy Mini Kit for RNA purification	Qiagen, Hilden
Trizol reagent	Invitrogen, Karlsruhe
PCR amplification primers	MWG Biotech

Technical Equipment

Axioplan 2 fluorescence microscope	Zeiss, Jena

CM 3050 microtome	Leica, Wetzlar
FACS Aria	BD Biosciences, Heidelberg
GeneAmp 7000 Sequence Detection System	Applied Biosystems
TRIO Block Thermocycler	Biometra, Göttingen
Heraeus Hera Safe incubator	Heraeus, Hanau
Excella E24 Incubator Shaker	New Brunswick Scientific, Nürtingen

Vectors

pSuper	OligoEngine
pSuperkip2	see Heinen et al., 2008
pIRES-EGFP	Invitrogen, USA
rp57kip2-IRES2-EGFP	see Heinen et al., 2008
pcDNA3-HygB-citrin	see vector as described in Heinen et al., 2008

2.2 Methods

2.2.1 MOG-induced experimental autoimmune encephalomyelinitis (MOG-EAE)

All rat experiments were performed in accordance with institutional guidelines. MOG-EAE was induced in 10 to 14 weeks old female DA (RT1av1) rats (Harlan) by active immunization with the recombinant MOG protein corresponding to the N-terminal sequence of rat MOG (amino acids 1-125) in complete Freund's adjuvans, as described by Schroeter and colleagues in 2003 (Schroeter et al., 2003). The clinical status of the animals was scored as follows: 0 = no clinical signs; 1 = loss of tail tone; 2 = complete tail paresis; 3 = hind limb weakness; 4 = complete hind limb paraplegia; 5 = tetraparesis; 6 = moribund state; 7 = death. Animals were followed for a maximum of 35 days and developed a multiphasic disease course with two episodes reaching their peak at days 11-12 and 21-22, respectively (see Fig. 1B). For isolation of total RNA, animals were killed at days 9, 11, 18, 22, and 28 after immunization (n=3-4 per time point), corresponding to onset and peak of the first (days 9, 11) and second episode (days 18, 22), and the time point of remission from the second episode (day 28), respectively. As controls, naïve non-immunized DA rats (n=3) were used.

2.2.2 Oligodendroglial cell culture

Purification and culturing of OPCs was performed according to McCarthy and colleagues (McCarthy and De Vellis, 1980). Briefly, the cortices of newly born rats were seperated from the remainder of the brains, and collected in MEM-Hepes medium. After a 30sec centrifugation step the medium was discarded and fresh MEM-Hepes medium containing 30 U/ml papain, 0,24 mg/ml L-cysteine and 40µg/ml DNAse I type IV was added to the cortices. After a 45min incubation step at 37°C, 1ml of ovomucoid trypsin inhibitor solution was added. Ovomucoid trypsin inhibitor solution consisted of 1mg/ml trypsin inhibitor, 50mg/ml BSA V and 40µg/ml DNAse I type IV in 1ml L-15 medium. After 5min of incubation at room temperature the solution was discarded and another ml of the above described solution was added. After thorough trituration with a glass Pasteur pipette 10ml of DMEM-medium containing 10% FCS were added followed by centrifugation for 8min at 1500rpm. Supernatant was then discarded and the dissociated rat cortices were resuspended in 20ml DMEM medium containing 10% FCS, 4mML-glutamine and P/S 5000 and cultured on poly-D-lysine (PDL) coated T-75 cell culture flasks. After 10 days, flasks were shaken at 250 rev/min for two hours in order to remove microglial contamination. Then flasks were shaken for another 20 hours during which OPCs were dislodged from the underlying astrocyte-layer. Subsequently, cells were replated on

PDL coated culture dishes or glass cover slips in high glucose DMEM-Sato-based medium containing bovine 5 µg/ml insulin, 50 µg/ml human transferrin, 100 µg/ml bovine serum albumin, 6,2 µg/ml progesterone, 16 µg/ml putrescine, 5 ng/ml sodium selenite and 4mM L-glutamine. Anti-A2B5 staining revealed that at this point the cultures consisted of 98% oligodendroglial cells. OPCs were either kept in proliferation medium (Sato medium with 10 ng/ml bFGF and 10 ng/ml PDGF-AA) whereas differentiation was initiated by Sato medium which was depleted from growth factors and supplemented with either 0,5% FCS, 10 ng/ml CNTF or 400 ng/ml T3/T4 thyroid hormones.

2.2.3 Transfection of oligodendrocyte precursor cells

Generation of pSUPER based suppression vectors or pIRES2EGFP based expression vectors has previously been described (Heinen et al., 2008a) and was not performed by the author himself. Transfection of vectors was performed using Fugene 6 Transfection Reagent. Briefly, after cells were grown in FGF and PDGF containing proliferation medium for 24h citrin containing plasmids were mixed with pSUPER and pSUPERkip2 plasmids, respectively, in a ratio of 1:5. In parallel, Fugene 6 reagent was added to DMEM + Hepes medium in a ratio of 3:100 and allowed to blend at room temperature for 10 min. Then, Fugene 6/DMEM + Hepes mix was added to the plasmid mix followed by another 10 min incubation at room temperature. Finally, 60 µl of the resulting reaction solution were added to 30.000 OPC grown in 500 µl cell culture medium. Transfection success was evaluated by fluorescence microscopy after 24h. Isolation of citrin positive OPC was performed by fluorescence activated cell sorting in the presence of the author using a BD FACSAria Cell-Sorting System at the Institute for Genomic Research of Microorganisms at the Heinrich-Heine-University Düsseldorf (Head: Prof. Dr. Hegemann). Prior to cell sorting, cells were dislodged from 6 cm diameter cell culture dishes using 250 µl TrypLE Express without Phenol Red. After a brief incubation at 37°C for 2 min, the reaction was stopped with 50 µl FCS. Finally, in order to avoid potential cell clotting, 40 µl EDTA were added and the whole mixture was stored on ice. Cells were then directly sorted into RLT buffer for subsequent preparation of RNA, cDNA and RT-PCR analysis.

2.2.4 Assessment of oligodendrocyte morphology by fluorescence microscopy

Successfully transfected citrin-positive oligodendrocyte precursor cells were fixed with 3,7% PFA, and mounted in Citifluor. Using fluorescence microscopy, cells were then evaluated for their morphological differentiation degree. To this end, a morphology assessment key was

designed based on overall OPC cell diameter, number of branches and degree of secondary branching (see Fig. 3)

2.2.5 Immunostainings

Immunostainings on paraformaldehyde-fixed cultured cells were performed according to the following protocol: Cultured cells were fixed with 3,7 % PFA for 10 min then rinsed three times with PBS at room temperature. Following a 30 min blocking step using 10% normal goat serum (Sigma-Aldrich, Taufkirchen), cells were then incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted as follows: rabbit anti-p57kip2 antibody (1/200), mouse anti-APC/CC1 (1/1000), mouse anti-O4-, mouse anti-PDGFR-α-, mouse anti-A2B5 antibodies (1/100, 1/300, 1/200, respectively), mouse anti-MOG antibody (1/1000), mouse anti-MBP- and mouse anti-CNPase antibodies (1/1000 and 1/500, respectively) and rabbit anti-LIMK-1 (1/500). 24h later cells were incubated with secondary fluorescent antibodies for 2h at room temperature and then rinsed three times with PBS. Secondary antibodies were Alexa Fluor 488-, Alexa Fluor 594-, or horseradish peroxidaseconjugated antibodies (1/500). Nuclei were stained with DAPI. All cell culture immunostainings were mounted in Citifluor and analyzed with an Axioplan 2 fluorescence microscope. Immunostainings on paraffin sections from paraformaldehyde-perfused rat spinal cords were performed acoording to the following protocol: Tissues were fixed with 3,7% PFA at 4°C for 48h. Fixed tissues were then rinsed with PBS for 5 min and dehydrated accoding to a protocol using increasing concentrations of ethanol as follows: 60 min 50% EtOH, 60 min 70% EtOH, 60 min 90% EtOH, 60 min 100% EtOH and 30 min100% EtOH with eosine. Tissue was then incubated overnight in 100% methyl benzoate. Tissues were then incubated in liquid paraffin at 63°C for a total time of 2h after which paraffin was discarded. In the final step, spinal cords were covered in fresh paraffin which was allowed to solidify. After the paraffin blocks had completely hardened 20-40 serial slices of 8 µm thickness were prepared using a microtome. Slices were then put on a 40°C water bath, placed on slides and dried overnight at 37°C. For immunostainings, slices on slides were then incubated in xylole for 3h in order to remove the paraffin. Spinal cord slices were then subjected to incubation in decreasing ethanol concentrations as follows: 10´ 100% EtOH; 10´ 100% EtOH; 5´ 90% EtOH; 5´ 70% EtOH; 5´ 50% EtOH. Finally, slides were rinsed twice with PBS and demasked in a microwave at 780 Watt for 6 min. After two further 5 min washing steps in PBS slices were blocked in 10% normal goat serum, stained overnight at 4°C and incubated with secondary antibodies at room

temperature for 45min (for concentrations, see above). Then slices were washed twice with PBS and mounted in Fluoromount.

2.2.6 RNA preparation, cDNA synthesis and quantitative RT-PCR

For purification of total RNA from spinal cord and cultured cells the mirVana Kit and the Trizol reagent were used, respectively. Purifications according to the mirVana Kit were carried out as follows: Adherent cells were lysed in 300 µl lysis buffer. Then, 30 µl miRNA homogenate additive were added and after vortexing, the mixture was left on ice for 10 min. Subsequently, 300 µl acid-phenol:chloroform were added and the entire liquid was vortexed for 1 min. Note that all following centrifugation steps were carried out at room temperature. After a 5 min centrifugation step at 14.000 rpm the aqueous phase was removed and mixed with 1,25 volumes of 100% ethanol. The mixture was then pipetted onto a filter cartridge and spun down at 10.000 rpm for 2 min. Flow through was discarded and 700 µl of miRNA wash solution 1 were added onto the cartridge which was spun down for 2min at 10.000 rpm. After discarding the flow through, two further centrifugation steps followed using 500 µl of miRNA wash solution 2/3, each of them performed for 2 min at 10.000 rpm. Finally, in order to remove residual fluid from the filter, cartridges were centrifuged for 5 min at 14.000 rpm, collection tubes were discarded and filters were put on fresh tubes. For Elution of RNA, 50 µl pre-heated 95°C Elution Solution was applied to the center of the filter and after 2 min cartridges were spun down at 14.000 rpm for 5 min. Eluate was then stored at -20°C. Total RNA of rat EAE spinal cord tissue was extracted using the Trizol-reagent. Spinal cords were cut into pieces and put into 700 µl Trizol-reagent. Homogenization of nerves was performed for 30 seconds using a polytron power homogenizer and the homogenate was incubated for at least 5 minutes at room temperature. The homogenate was then mixed with 140 µl chloroform, vigorously shaken and centrifuged for 15 minutes at 12.000 rpm and 4°C. Of the three phases which were thus generated, the upper aqueous phase (approximately 500 µl) was carefully pipetted into a new tube. The content of each tube was mixed with 1.1 µl glycogen and 350 µl 2-propanol, vortexed and incubated for 30 minutes at room temperature to allow precipitation of RNA. Samples were centrifuged for 10 minutes at 12.000 rpm and 4°C, supernatants were discarded and RNA pellets were washed with 1 ml ethanol (75% in ddH_2O). Samples were again centrifuged for 10 minutes at 10.000 rpm and 4°C and supernatants were discarded carefully. After drying of the RNA pellets, RNA was dissolved with 30 µl nuclease free ddH_2O and incubated for 10 minutes at 57°C to maximize the yield. Samples were stored at -20°C. Isolated RNA was reverse transcribed using the high capacity cDNA Reverse Transcription Kit. Briefly,

15 µl RNA were added to 15µl of the RT master mix which contained the following components: 3µl 10x RT Buffer, 1.2µl 25x dNTP Mix (100mM), 3µl 10x RT Random Primers, 1.5 µl MultiScribe Reverse Transciptase, 1.5 µl RNase inhibitor and 4.8 µl Nuclease-free H_2O. RT reaction was then carried out according to the following protocol: Step 1, 25°C, 10 min; Step 2, 37°C, 120 min; Step 3, 85°C, 5 sec. Quantitative determination of gene expression levels was performed on an ABI 7000 sequence detection system using Power SybrGreen universal master mix. Primer sequences were determined by means of PrimerExpress 2.0 software by Applied Biosystems and subsequently tested for the generation of specific amplicons. Sequences of primers used at a final concentration of 0.30 pmol/µl were as follows: Q_rp57kip2_fwd: CAG GAC GAG AAT CAG GAG CTG A, Q_rp57kip2_rev: TTG GCG AAG AAG TCG TTC G, Q_rCNP_fwd: ATG CTG AGC TTG GCG AAG AA, Q_rCNP_rev: GTA CCC CGT GAA GAT GGC C, Q_rGAPDH_fwd: GAA CGG GAA GCT CAC TGG C, Q_rGAPDH_rev: GCA TGT CAG ATC CAC AAC GG, Q_rODC_fwd: GGT TCC AGA GGC CAA ACA TC, Q_rODC_rev: GTT GCC ACA TTG ACC GTG. The default two step amplification profile used was 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. GAPDH and ODC were used as reference genes, and relative gene expression levels were determined according to the Applied Biosystems' ΔΔCt method. Each sample was measured in quadruplicate; data are shown as mean values +/- SEM.

2.2.7 Microarray analysis

All DNA Microarray experiments were performed by an independent subcontractor (Arrows Biomedical GmbH, Münster). Prior to analysis, pSuper/citrin and pSuperkip2/citrin transfected OPC were FACsorted and lysed in lysis buffer. After RNA purification using the mirVANA RNA purification kit genetic material was analysed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer. Briefly, oligonucleotide probes were generated by reverse in-vitro-transcription (IVT) of purified total RNA and then fluorescently labeled. Subsequently, fluorescent cDNAs were hybridized to rat genome microarrays which were then scanned and analyzed.

3. Results

3.1 Dynamic regulation of p57kip2 expression in the diseased spinal cord.

We performed quantitative RT-PCR analysis of spinal cord RNA in order to determine changes in p57kip2 expression during the course of EAE induced by immunization of DA rats with MOG protein. Most prominent was a strong down-regulation of p57kip2 expression during the first bout (at 11 days) and a moderate down-regulation during the second bout (at 22 days), indicating that lowered p57kip2 expression levels correlate with the onset of remission (Fig. 1 A and B). Beyond 28 days, no further regulation of p57kip2 expression was observed. Immunostaining revealed many p57kip2 expressing cells throughout the healthy spinal cord (Fig. 1C). EAE-related inflammation resulted in decreased transcript levels reflected by a lower number of p57kip2-positive cells (shown for 11 days after MOG immunization in Fig. 1D). Interestingly, we observed that the reduction of the p57kip2 signal was overall and not dependent on direct contact with infiltrating immune cells (dashed line in Fig. 1D'). In the white matter of healthy adult spinal cord (Fig. 1 E and E') and at the end of the second bout (24 days after MOG immunization; Fig. 1 F and F'), p57kip2-positive cells were mostly CC1-positive oligodendrocytes. A lower number of p57kip2-positive cells expressed the oligodendrocyte precursor marker platelet-derived growth factor receptor-α (PDGFR-α; Fig. 1 G and G'). We detected no strong p57kip2 signals in infiltrating immune cells (Fig. 1D') and no overlap with GFAP signals, indicating that astrocytes did not contribute to the p57kip2 signals. Of note, in the healthy spinal cord the majority (>90%) of p57kip2-expressing cells displayed strong nuclear as well as perinuclear p57kip2 signals (Fig. 1E, arrows), whereas a lower number of cells showed only cytoplasmic/ perinuclear signals of weaker intensity (Fig. 1E, arrowhead). This latter subpopulation was substantially increased in the diseased spinal cord (Fig. 1F and F'), indicating that p57kip2 down-regulation might be accompanied by protein relocation. The observation that p57kip2 down-regulation fades over time can therefore be interpreted as this gene acting as oligodendroglial inhibitor in (advanced) pathophysiological situations.

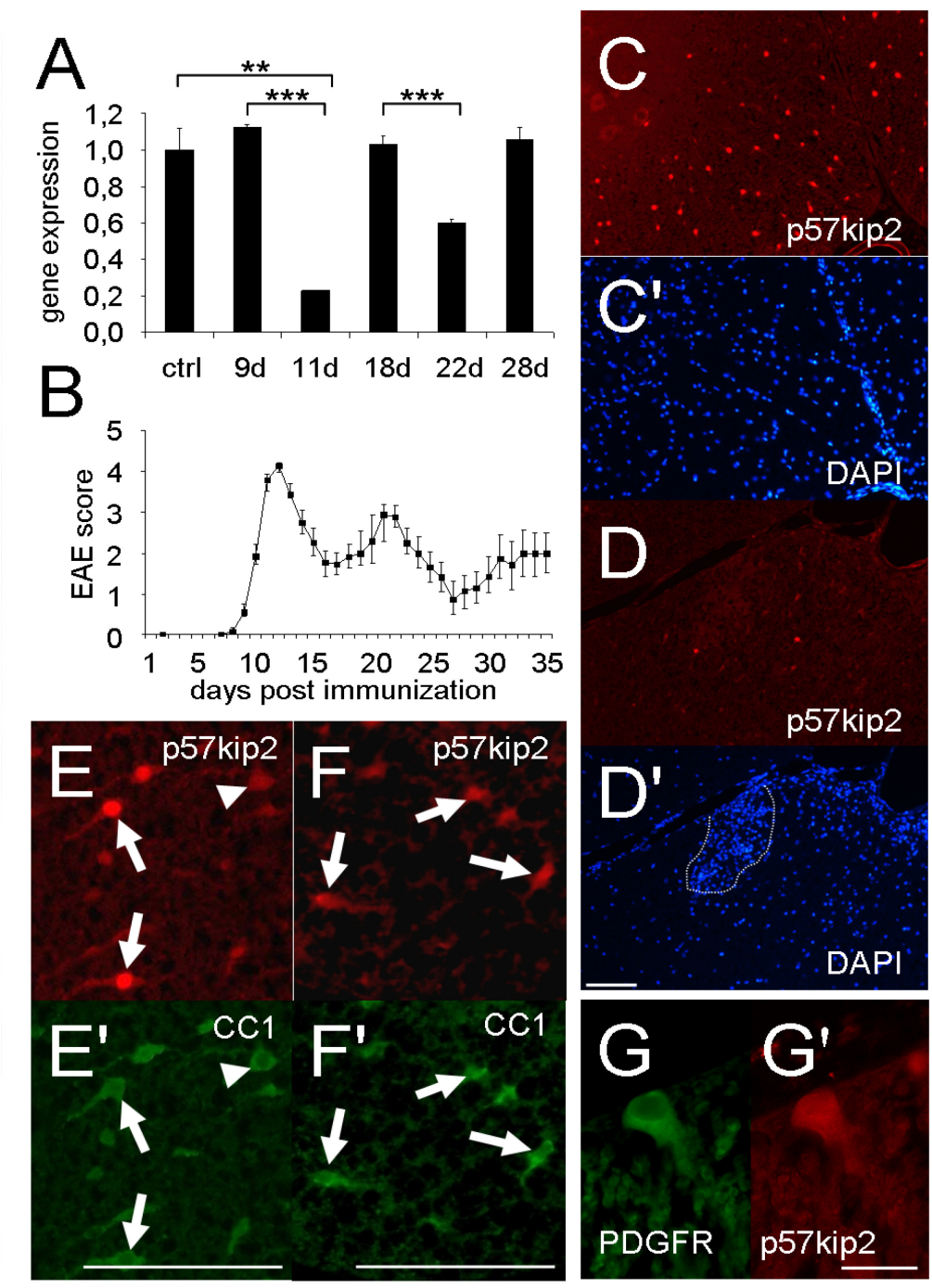

Fig. 1. Regulation of p57kip2 expression in MOG-EAE spinal cords. (A) Quantitative RT-PCR analysis of p57kip2 expression in the diseased spinal cord. Downregulation is observed at the first and, to a lesser degree, at the second bout, as revealed by the peak EAE scores near days 11 and 22 shown in (B) [One out of three independent measurements is shown; GAPDH expression was used as reference and data are mean values +/- SEM (t-test: **P<0.01, ***P<0.001)]. (C-D') Anti-p57kip2 immunostainings and DAPI counter stains of healthy spinal cord- (C,C') and 11 day MOG-EAE spinal cord sections (D,D'); dotted line in (D') marks infiltrating immune cells. Double immunostainings for p57kip2 and CC1 (E,E') and for p57kip2 and PDGFR-α (G,G') indicating that within the healthy spinal cord white matter, p57kip2-expressing cells are oligodendrocytes and oligodendroglial precursor cells. Note that in the healthy tissue the majority of cells feature strong nuclear p57kip2 expression (arrows) whereas few cells show low cytoplasmic/perinuclear expression levels (arrowhead). At the end of the second bout (24 days) CC1-positive cells were found to express p57kip2 again (F,F'). Scale bars: 100µm (D',E',F'), 20ìm (G').

3.2 Regulation of p57kip2 expression in cultured oligodendroglial cells.

As a next step the expression and regulation of p57kip2 in cultured primary oligodendroglial cells derived from newborn rat cortices were investigated. Oligodendrocyte precursor cells (OPCs) were induced to differentiate in culture by means of growth factor withdrawal and then analyzed for gene and protein expression. Determination of gene expression levels by means of real-time quantitative RT-PCR demonstrated an initial increase of p57kip2 expression concomitant with cell cycle exit and differentiation onset followed by decreasing transcript levels during the course of cellular maturation (Fig. 2A). A similar biphasic expression profile has already been described for cultured OPCs derived from P7 optical nerves (Dugas et al., 2007). Following the observation that p57kip2 is downregulated in both the remyelinating spinal cord as well as in differentiation OPCs it was determined to what degree p57kip2 gene suppression affects the cellular differentiation process by applying RNA interference in order to decrease this gene's activity in cultured oligodendroglial cells. However, in these experiments, silencing RNA (siRNA) dependent approaches led only to transient suppression of p57kip2 expression (data not shown). As an alternative a small hairpin RNA (shRNA) cassette (Heinen et al., 2008a) was used which allows long-term p57kip2 suppression and had been previously shown to be specific for p57kip2 and free from off-target effects such as an interferon response induction (Heinen et al., 2008a). Using this approach, oligodendroglial p57kip2 expression could be reduced for up to nine days as shown by quantitative RT-PCR of transfected and sorted OPCs two days following transfection (Fig. 2B) as well as by anti-p57kip2 immunostaining six days following transfection (Fig. 2C,D). Note that also among cultured OPCs, cells with strong nuclear p57kip2 expression (double arrowhead) as well as cells with perinuclear and cytosolic expression only (arrowhead) were detected.

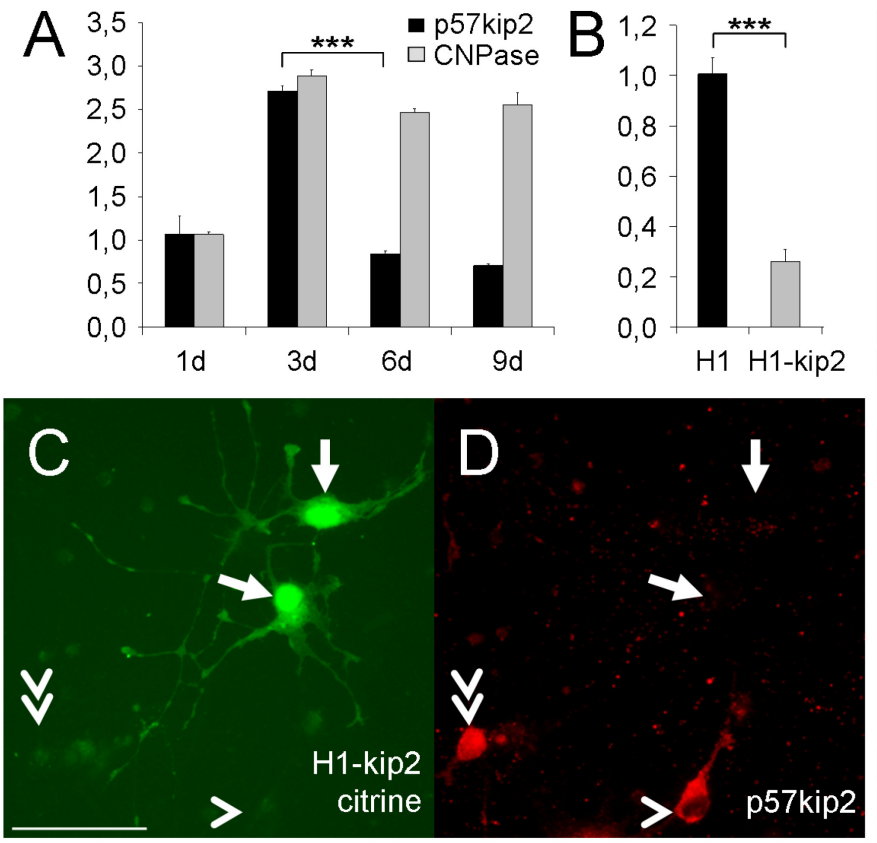

Fig. 2. p57kip2 regulation in cultured oligodendroglial cells. Quantitative RT-PCR analysis revealed an initial gene induction followed by a downregulation of p57kip2 expression during the oligodendrocyte differentiation process which is accompanied by an induction of CNPase expression (A). This analysis also confirmed p57kip2 downregulation in suppressed and sorted oligodendroglial cells (B) [One representative experiment out of five is shown; GAPDH expression was used as reference and data are mean values +/- SEM (t-test: ***P<0.001); H1: control transfected cells, H1-kip2; p57kip2-suppressed cells]. (C,D) Anti-p57kip2 immunostaining (D) demonstrated that six days following transfection p57kip2-suppressed- OPCs (C; marked by expression of citrine) were still devoid of p57kip2 expression. The arrows point to transfected cells, the arrowhead marks a cell with p57kip2 signals outside the nucleus, the double arrowhead points to a cell with strong nuclear p57kip2 expression. Scale bar: 50µm (C).

3.3 Reduced p57kip2 levels accelerate morphological differentiation of OPC

A number of different pathways are known to promote oligodendroglial differentiation featuring different patterns of gene expression (Billon et al., 2004;Tokumoto et al., 2001). Growth factor withdrawal results in immediate cell cycle exit and differentiation onset, whereas environmental cues such as retinoic acid or thyroid hormone appear to depend on an intracellular molecular clock (Barres et al., 1994;Raff et al., 1988). To determine to what degree decreasing levels of p57kip2 affect differentiation kinetics, mitogen withdrawal was used to study maturation of postmitotic cells and determined the distribution of cellular morphologies of control-transfected- and p57kip2-suppressed oligodendroglial cells (Fig. 3). For visualization, OPCs were cotransfected with a citrine expression vector as described previously (Heinen et al., 2008a). Differentiation of cultured OPCs is not synchronized and

thus is seen as a heterogeneous population of cells with various degrees of maturation, featuring increasing number of processes and secondary branches. In the analysis conducted in this study six different morphologies (see bar at the bottom of Fig. 3E) from “very low” number of processes in progenitor cells to multiple process-bearing cells (“low”, “medium”, “high”) to mature cells with “very high” degree of arborization or flattened appearance (“sheaths”) were distinguished. Twenty four hours after transfection, PDGF-AA and bFGF were withdrawn from the culture and OPCs were exposed to low serum containing medium stimulating differentiation. After 24 hours in this differentiation-promoting medium, p57kip2-suppressed cells appeared to be slightly advanced in their morphological maturation (data not shown). However, at later time points (three, six and nine days post differentiation onset) a strong acceleration of morphological maturation was observed in p57kip2 suppressed cells (grey bars) compared to control transfected cells (black bars; Fig. 3C,D,E). This maturation promoting effect was also observed when additional myelin-enhancing stimuli were provided such as thyroid hormones and/or ciliary neurotrophic factor (CNTF) (Dugas et al., 2007;Mi et al., 2005;Stankoff et al., 2002) in the presence of growth factors (data not shown), indicating that this differentiation promoting effect is a specific consequence of lowered p57kip2 levels.

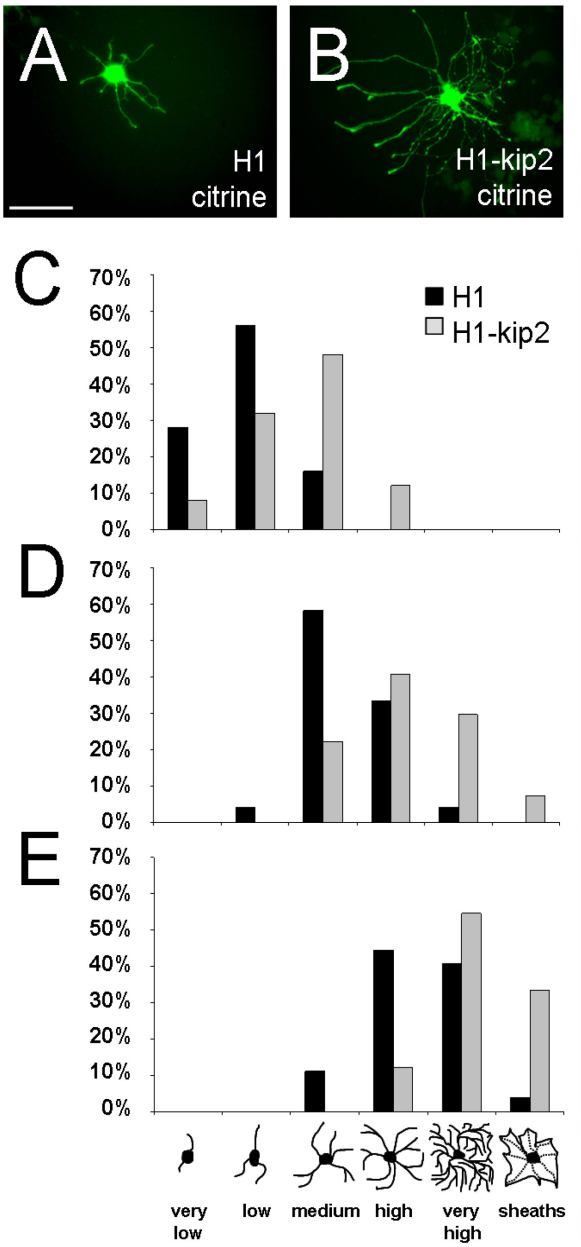

Fig. 3. p57kip2 suppression accelerates morphological maturation of cultured oligodendroglial cells. (A,B) Representative citrine expressing control-transfected- (A) and p57kip2-suppressed OPCs (B) three days following induction of differentiation. The control transfected cell (H1) features a medium- whereas the p57kip2-suppressed cell (H1-kip2) features a high degree of morphological maturation. (C-E) Determination of OPC morphology distribution of control-transfected- (H1; black bars) versus p57kip2-suppressed cells (H1- kip2, grey bars) at three (C), six (D) and nine days (E) post differentiation onset. At all time points p57kip2-suppressed cells were morphologically advanced as compared to control transfected cells. Six different morphologies were distinguished; one representative experiment out of seven is shown. Scale bar: 50µm (A).

3.4 Reduced p57kip2 levels accelerate expression of mature oligodendroglial markers

Oligodendroglial maturation is also reflected by the induction and expression of specific marker proteins. Therefore, it was determined whether the pattern of marker expression was altered upon p57kip2 suppression (Fig. 4). Control- and p57kip2-suppressed cells were fixed at various time points and subjected to immunofluorescent staining with antibodies against the

early markers O4 and CNPase (2',3'-cyclic nucleotide 3'-phospho-diesterase) as well as the late markers MBP (myelin basic protein) and MOG (myelin oligodendrocyte glycoprotein). It could be demonstrated that downregulation of p57kip2 leads to a significant induction of oligodendrocyte markers at all time points investigated and shown for CNPase, O4 and MBP two days and MOG four days following initiation of cell differentiation (Fig. 4). Similar to the morphological analysis presented above this marker induction was observed under a variety of culture conditions stimulating OPC differentiation (data not shown).

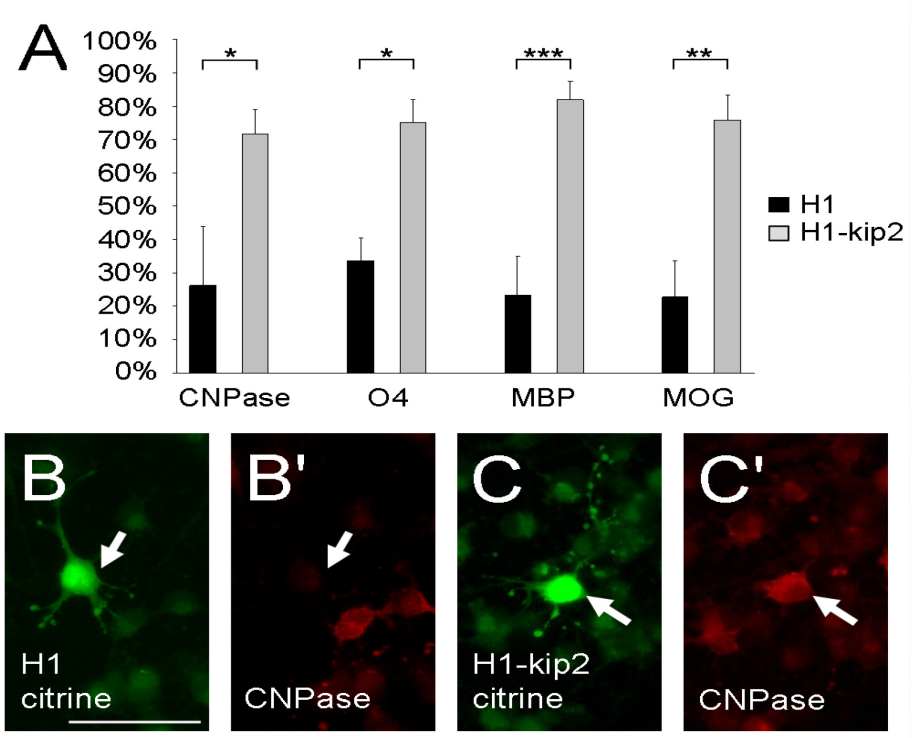

Fig. 4. p57kip2 suppression stimulates oligodendroglial marker expression. (A) Two days following differentiation induction, significantly more p57kip2-suppressed cells (H1-kip2; grey bars) expressed the early oligodendroglial marker O4 and the myelin proteins MBP and CNPase compared to control-transfected (H1; black bars). Similarly, four days postdifferentiation onset, the number of MOG expressing cells was increased [One representative experiment out of nine shown; data are mean values +/- SEM (t-test: *P<0.05, **P<0.01, ***P<0.001)]. (B,B') Representative examples of CNPase-negative controltransfected (H1) and (C,C') CNPase-positive p57kip2-suppressed (H1-kip2) cells. Arrows point to transfected and citrine positive cells. Scale bar: 50µm (B).

3.5 p57kip2 overexpression can induce LIMK-1 nuclear translocation

It has previously been shown that the p57kip2 protein can directly interact with LIM domain containing binding partners such as LIMK-1 (Yokoo et al., 2003), and in a different study our research group gathered evidence that in Schwann cells such specific protein/protein contacts can occur (Heinen et al., 2008b). Given that LIMK-1, through phosphorylation of cofilin, can directly affect actin filament stability, and turnover, hence cell shape and motility, such an interaction might be part of p57kip2's mode of action in inhibiting oligodendroglial

differentiation. Therefore the subcellular localization of LIMK-1 in cultured OPCs was investigated. In addition, the impact of p57kip2 overexpression on an existing subcellular LIMK-1 distribution was evaluated (Fig. 5). OPCs were either cotransfected with a p57kip2-overexpressing- or an empty control vector together with the citrine expression vector (Heinen et al., 2008a). Differentiation promoting medium was added and after 48 hours cells were scored for LIMK-1 subcellular localization by means of immunofluorescence staining. In non-transfected and control-transfected cells two subpopulations of LIMK-1 expressing OPCs were observed. In the majority of cells, signals could be detected in cellular processes and soma (including cell nucleus; Fig. 5C-C''), whereas a minority of cells displayed strong nuclear signals and reduced LIMK-1 in processes (Fig. 5B-B'). Following p57kip2 overexpression, a significant increase in the number of cells with nuclear signals could be observed (Fig. 5A), indicating that in oligodendroglial cells p57kip2 can translocate LIMK-1.

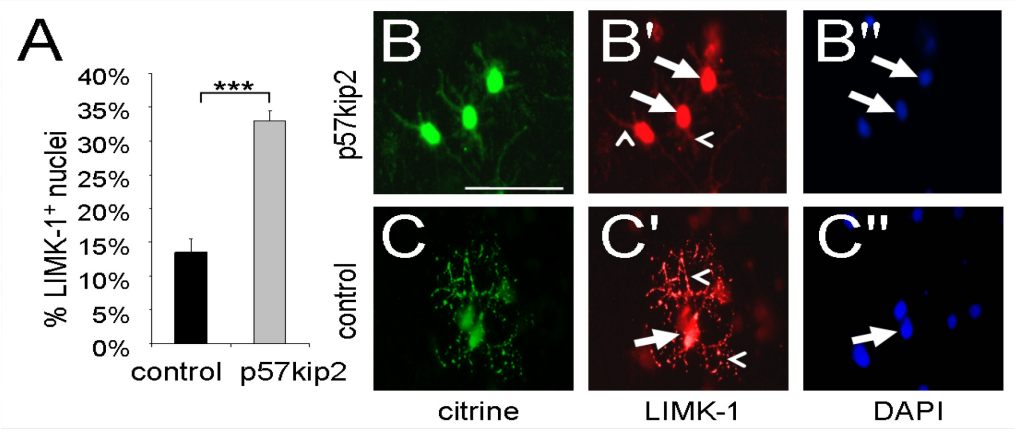

Fig. 5. p57kip2 overexpression leads to nuclear accumulation of LIMK-1. (A) Quantitative determination of the degree of nuclear LIMK-1 expressing oligodendrocytes in control transfected- (black bar) versus p57kip2 overexpressing OPCs (grey bar) [One representative experiment out of two shown, data are mean values +/- SEM (t-test: ***P<0.001)]. (B,C) Anti- LIMK-1 immunostainings of cultured oligodendroglial cells two days following differentiation onset. (C) Most of the control-transfected cells showed a widespread LIMK-1 expression. (B) Upon p57kip2 overexpression more cells showed strong nuclear- and reduced or absent LIMK-1 signals in processes. Arrows mark nuclear- and arrowheads mark localization in processes. Scale bar: 50µm (B).

3.6 Microarray analysis of p57kip2 suppressed OPC

In order to search for possible mechanisms underlying the above described differentiation effects observed in p57kip2 suppressed OPC we conducted a transcriptome analysis aiming at the identification of possible effector genes being affected by altered p57kip2 expression levels. Briefly, OPC were cultured in expansion medium, transfected using Fugene6 after 24h and then enriched using FACsorting. Following RNA purification microarray analysis of the genetic material of the cells was performed using the AB1700 microarray platform (Applied Biosystems). Out of 26857 gene signatures 289 genes were found to be differentially expressed

in p57kip2 suppressed OPC in comparison to control cells. Among them were genes that had previously been identified in the context of cell differentiation. We therefore focused our attention on them: One of them, Catenin1b (Ctnnb1) was found to be downregulated 3-fold on average in p57kip2 suppressed OPC and has been reported to play an important role in neural cell fate commitment and differentiation. (Joksimovic et al., 2009;Otero et al., 2004) Ctnnb1 is part of the canonical Wnt signalling pathway which has been identified as a factor that directly inhibits oligodendrocyte development by preventing differentiation of OPC into a more mature state (Shimizu et al., 2005). Downregulation as found in p57kip2 suppressed OPC could thus result in enhanced oligodendroglial differentiation suggesting a functional connection between this CDKI and the Wnt pathway. Another gene, Lrp2 (Lipoprotein receptor-related protein-2 / megalin) was found to be upregulated approx. 3-fold in p57kip2 suppressed OPC and has been demonstrated to be present in spinal cord oligodendrocytes during myelination (Wicher et al., 2006). Having been detected in both the nuclei of oligodendrocytes and their membranes it was hypothesized that megalin translocates signals from the cell membrane to the nucleus of oligodendrocytes during the formation and maintenance of myein. We also identified Rock1, as a “target gene” of p57kip2. This kinase links Rho GTPases like RhoA to LIMK2, thereby modulating cell morphology (Maekawa et al., 1999;Vardouli et al., 2005;Amano et al., 2001) and was found to be upregulated approximately 2-fold in p57kip2 suppressed OPC. Rock1 has been described as a key player in the coordinate progression of the Schwann cell membrane around the axon during myelination in the PNS (Melendez-Vasquez et al., 2004). It is conceivable that this gene plays a similar role in CNS myelination through maturing oligodendroglial cells. In summary, the microarray data presented here are, as mentioned above, of preliminary nature and require further validation. At this point, it is important to stress that since we were interested in identifying early gene regulatory events all experiments were performed on OPC that had only been grown in differentiation promoting medium for one day prior to fluorescence activated cell sorting. In order to appreciate the complex dynamics of OPC differentiation it will be inevitable to incorporate later time points as well.

4. Discussion

4.1 p57kip2 as a regulator of CNS and PNS glial maturation

In this study it was demonstrated that expression of the cell cycle inhibitor gene p57kip2 is transiently downregulated under inflammatory demyelinating pathophysiological conditions prior to the onset of disease remission. Mimicking downregulation by means of long-term shRNA dependent suppression illustrated that differentiation of oligodendroglial cells is accelerated in response to lowered p57kip2 levels. Previous studies performed by the author's research group revealed a similar function in the related Schwann cell lineage (Heinen et al., 2008a), so that taken together these data strongly suggest that p57kip2 encodes an intrinsic inhibitor of myelinating glial cell differentiation.While cultured Schwann cells are not able to differentiate spontaneously, at least when p57kip2 levels are unchanged, they readily do so in the lesioned or diseased peripheral nervous system, which contributes to successful nerve repair (Son and Thompson, 1995). Cultured OPCs can differentiate but in vivo their capacities to adapt and to de- and redifferentiate are highly limited, resulting in conduction deficits and subsequent axonal degeneration. (Bitsch et al., 2000;Trapp et al., 1998a). This is the reason why CNS diseases such as mulitple sclerosis are progressive with partial remissions only, while PNS diseases such as Guillan-Barré-Syndrome can feature complete remissions when treated adequately (van Doorn et al., 2008). Apart from inhibitory influences from the surrounding tissue, intrinsic blockades such as that provided by p57kip2 may also account for these differences between glial cell types (Kuhlmann et al., 2008;Wolswijk, 1998) and, thus, the ability to control inhibitor expression levels may dictate whether or not remyelination and repair can occur. Considering that substantial regulation by p57kip2 is only seen in early CNS disease stages, this protein could be responsible for the limited regeneration capacity of the inflamed CNS seen later in disease. These data all suggest that disease-stage specific regulation of p57kip2 could constitute a novel promising approach for future remyelination therapies.

4.2 Diverging methodical approaches to clarify p57kip2's regulatory role

In a recent study on cultured P7-P8 optical nerve oligodendrocytes it was shown that the induction of p57kip2 is an element of the intracellular timer mechanisms that control the differentiation onset in the presence of mitogens and upon specific differentiation cues (Dugas et al., 2007). In the study presented here it has now been demonstrated that the secondary decrease in p57kip2 expression is functionally coupled with the oligodendroglial maturation

process and does not only constitute the downside of the peak expression. It therefore appears that p57kip2 controls oligodendrocyte differentiation at various levels and via several pathways. The extent of up- and downregulation is probably dependent on the OPC source and differentiation state (e.g. whether or not additional selection markers such as O4 (Dugas et al., 2007) were used). Such differences might even be more widespread among the different oligodendroglial lineages (Kessaris et al., 2006) and could include contributions from p21cip1 and p27kip1 (Durand et al., 1998;Zezula et al., 2001). However, precursor cell cycle control in the adult CNS appears not to be dependent on p57kip2 induction, since upregulation was not detected during the course of MOG-induced EAE in the experiments performed in this study. It is therefore conceivable that such a (p57kip2-dependent) timer function does not act on adult OPC differentiation and that adult remyelination is primarily regulated by epigenetic mechanisms (Shen et al., 2008). In contrast to the data presented here, Dugas and colleagues (Dugas et al., 2007) reported a negative effect on OPC differentiation of siRNA-dependent suppression in presence of growth factors, an effect which was substantially reduced upon withdrawal of growth factors. Under these conditions, combined suppression of p57kip2 and p27kip1 appeared to have a more sustained effect, supporting the idea that several CDKIs act together. The findings presented in this thesis, on the other hand, show that p57kip2 suppression promotes maturation (Figs. 3 and 4). Although it cannot be excluded that OPCs from different sources respond differently to RNA interference, this difference could be simply of technical nature since stable suppression of p57kip2 using siRNAs proved to be not feasible in primary Schwann cells (Heinen et al., 2008a) nor in OPCs (own observations, unpublished), which is why shRNA encoding constructs were used instead. However, for both procedures, identical interference sequences recognizing all known cDNA sequences of the rat p57kip2 gene were employed. This DNA approach was previously shown to be specific for p57kip2 and free from off-target effects (Heinen et al., 2008a) and it was verified that suppression was not only achieved but also maintained over time (Fig. 2). It thus appears that a sustained downregulation is imperative for an effect on OPC differentiation and that the vector based shRNA approach is highly efficient. Since long-term p57kip2 suppression was found to accelerate differentiation parameters under a number of different culture conditions, including that used for P7-P8 optical nerve cells (Dugas et al., 2007), it is unlikely that functional differences were due to different culture media compositions. It is generally assumed that mature oligodendrocytes are not able de- and redifferentiate and that therefore remyelination must be a consequence of resident precursor cell activation. It was therefore surprising to see that almost all cells within the spinal cord clearly downregulated p57kip2, independent on

whether they were mature or precursor cells or whether they were close to immune infiltrates. In light of this strong overall regulation in early phases of MOG-induced EAE, one could speculate that in such situations, the majority of cells at least attempt to regenerate or to attain a cellular state during which redifferentiation and repair is facilitated. Whether the observed overall downregulation of p57kip2 in early phases allows both cell types (resident precursor cells as well as CC1-positive oligodendrocytes) to adapt at least partially, with precursor cells being more efficient or successful in executing differentiation, is currently unknown and awaits functional experiments in vivo. Downregulation of p57kip2 in vivo appears to be a consequence of inflammation. Nevertheless, the data presented here suggest that either the signaling molecules controlling p57kip2 expression differ at different stages of disease or that the oligodendroglial capacity to respond diminishes over time. Either mechanism could be responsible for the observation that less regulation can be seen in later attacks and during progression to more chronic stages.

4.3 Biomedical relevance of the presented data and their applicability to the human organism

When we try and translate the aforementioned findings and the conclusions drawn from them to the human organism and multiple sclerosis, we are faced with several issues that require further discussion. Despite certain deficits there is broad consensus that MOG-EAE is to be considered the most applicable paradigm for mimicking human neuroinflammatory demyelinating CNS diseases (Furlan et al., 2009;Holmoy, 2008). As a consequence, the next logical step to be taken in order to further examine p57kip2's potential as a target for biomedical therapies are investigations on human CNS tissue.

Firstly, in order to study the expression of p57kip2 in the diseased MS-afflicted human brain it will be necessary to gather high-quality bioptical material from humans (Bitsch et al., 2000;Lucchinetti et al., 2000b). Once obtained, such material could be analyzed using several approaches in parallel. QPCR profiling could provide valuable information regarding the regulation of p57kip2 during relapses and remissions in human multiple sclerosis. Immunohistochemistry of MS tissue might reveal which CNS resident cells express p57kip2 and where in the cell it can be found. Finally, using Western Blot one might be able to evaluate overall protein quantities of p57kip2 in the diseased human CNS. However, while seeming feasible, the described approaches harbor several caveats one needs to be aware of. Firstly, it will be virtually impossible to analyse material from individuals that have not received medication for multiple sclerosis. As the agents that are currently in use for MS therapy (see 1.1.5) are very potent drugs which possibly exert a substantial effect on all CNS cells including

OPC, they constitute a major experimental confounder. In order to minimize the resulting effects it will therefore be imperative to match patients with similar drug regimens and application periods which will, in turn, lead to significant reduction in available patient numbers. Aside from these considerations, the mere isolation of MS material is problematic as CT-based stereotactical biopsies are not routinely performed in MS diagnosis. Yet, this particular technical problem may be the least difficult one to solve since there are several medical facilities that have at their disposal an abundancy of such bioptical material (Barnett and Prineas, 2004). In this context it is of importance to be aware of the fact that it would be nessecary to also analyse CNS material from non-MS affected individuals for gene expression baseline measurements and negative controls respectively. Since the indication for brain biopsy is subject to strict clinical criteria the resulting biopsy pool is likely to be rather small. In addition, most individuals undergoing such biopsies suffer from other neurological conditions that might in a yet unknown way exert a certain influence on the development or the course of multiple sclerosis. On the cell culture level the best approach to study p57kip2's relevance for human glial cell differentiation is to replace the rat oligodendrocyte precursor cell culture used in this study by human precursor cells, a project that has just recently been initiated by the author's research group. As the structural homology between rat and human p57kip2 only reaches about 81% on DNA- and 55 % on protein level (Potikha et al., 2005) it is conceivable that knock-down of human p57kip2 in culture might interfere with effector proteins different from those relevant in animals or that the human p57kip2 is not acting as a glial cell differentiation inhibitor at all while other proteins/genes cover this function. In lhis context it will be also important to investigate to what extent p57kip2 downregulation affects the expression and/or activity of other known myelinating glial cell inhibitors. This will reveal whether oligodendroglial differentiation blockade is due to a simple (master) mechanism or whether multiple negative regulatory pathways exist and act in parallel.

4.4 Potential links between p57kip2 and other inhibitors of oligodendroglial differentiation

Among the group of proteins inhibiting oligodendroglial differentiation one can find a remarkably high number of helix-loop-helix transcriptions factors (HLH) such as Hes1, Hes5 or non DNA binding transciption modulators such as Id2 and Id4. HLH proteins can not only bind to and antagonize the action of other HLH proteins (Akazawa et al., 1992;Sasai et al., 1992;Tietze et al., 1992) but can also bind to N-box elements in the DNA and recruit corepressor proteins to inhibit gene transcription (Fisher et al., 1996;Grbavec and Stifani, 1996). Importantly, Id4, for instance, has been shown to stimulate proliferation and to block the differentiation

programs of OPC (Kondo and Raff, 2000). The two transcription factors Hes1 and Hes5 are activated by Notch signalling (Jarriault et al., 1998;Ohtsuka et al., 1999;Wang et al., 1998), which, in turn, has been shown to exert an inhibitory influence on oligodendroglial differentiation (Wang et al., 1998). Interestingly, Jagged1, the ligand of Notch, is re-expressed in multiple sclerosis lesions where it interferes with efficient differentiation of oligodendrocyte precursor cells and remyelination (John et al., 2002). Its expression profile is reminiscent of the observations made in this study regarding p57kip2 whose downregulation appears to be a prerequisite for successful remyelination as well. Two maybe even more intriguing candidates for negative regulators which could, in turn, be regulated via p57kip2 are protein kinase C (PKC) and Fyn-RhoA whose downregulation in OPC has been shown to enhance cell differentiation in presence of inhibitory CNS myelin (Baer et al., 2009). Interestingly, via ROCK, RhoA acts as an upstream regulator of LIMK1 which, in turn, was demonstrated in this work as being translocated into the cell nucleus by p57kip2 thereby altering cell morphology. Another protein inhibiting oligodendroglial differentiation by activation of RhoA is LINGO-1 (Mi et al., 2005). The attenuation of this protein, on the other hand, results in downregulation of RhoA activity, which propels OPC differentiation. Conversely, overexpression of LINGO-1 leads to activation of RhoA and inhibition of oligodendrocyte differentiation and myelination. Whether or not p57kip2 expression modulation affects those factors directly or indirectly remains to be demonstrated. A further potential protein candidate for the role of an inhibitory factor that might be regulated in response to p57kip2 downregulation could be the protein tyrosine phosphatase MKP5. In cell culture experiments, this gene turned out to be an inhibitor of oligodendroglial maturation. This is in line with the observation that knock-out mice for this enzyme were found to be partially protected from experimental autoallergic encephalitis (Zhang et al., 2004). It is therefore conceivable that enhanced oligodendrocyte-mediated remyelination due to downregulation of MKP5 might account for clinically less severe disease courses – a theory that is in line with our conclusions regarding the downregulation of p57kip2 during MOG-EAE remissions.

4.5 Future p57kip2 based MS therapies

So far, MS therapy, as it is clinically applied today, has always been directed at the modulation of immune cell behaviour or localization. Most approaches make use of corticosteroid-based immunosuppression (Wiendl et al., 2008) or of the antiinflammatory properites of the interferon family of proteins interferon beta-1a and interferon beta-1b. These approaches are rather non-specific and target the organism's immune system as a whole (Kieseier et al.,

2008;Tumani et al., 2008;Tumani, 2008). Even the recently introduced therapies using humanized monoclonal antibodies such as natalizumab (Rice et al., 2005) or sphingosine receptor modulators such as fingolimod (Matloubian et al., 2004) are mainly supposed to prevent immune cell penetration through the blood brain barrier (BBB) into the CNS. In search of novel cell-targeted and thus more specific treatments for demyelinating diseases the development of a p57kip2-based remyelination therapy could be a promising approach. However, the implementation of such a therapy largely depends on their technical feasibility which is why the recent discovery that transvascularly applied siRNA can successfully penetrate the blood brain barrier into the brain where it can then modulate the gene expression of neurons is of high scientific importance (Kumar et al., 2007). In theory, siRNA mediated knockdown of p57kip2 during an MS bout could be followed by promoted differentiation of oligodendrocyte precursor cells which then, in turn, would lead to more successful remyelination. This tempting therapeutic scenario, however, requires more detailed knowledge about the intricate interplay of resident CNS cells and the immune system in order to choose the most favorable period for siRNA application and delivery (see above).

Another possible approach to diminish the activity of p57kip2 and to thereby enhance glial differentiation could be the design of small molecules interfering with the normal functions of p57kip2. However, it has to be pointed out that although successful therapies involving small molecules might have been found for immune disorders in animal studies, few have passed the much harder test of treating human diseases (Feldmann and Steinman, 2005). Moreover, peripheral application of siRNA might affect p57kip2 expression within the whole body leading to yet unforseeable consequences in tissues other than the CNS. As several other functions besides the modulation of glial cell differentiation have been attributed to p57kip2 serious caveats about therapeutic specificity must be considered. According to the current scientific literature p57kip2 has been described to be involved in the development of the skeleton (Kim et al., 2008;Takahashi et al., 2000), muscle differentiation (Nakano, 2001;Tintignac et al., 2000), kidney development and growth (Felekkis et al., 2008;Hiromura et al., 2001;Petermann et al., 2002) and probably most importantly in the formation of neoplasias (Jin et al., 2008;Larson et al., 2008;Li et al., 2003;Sato et al., 2005).

Another aspect this study discusses is the interaction of p57kip2 and LIMK-1. Subtle changes in p57kip2 levels in vivo could modulate the equilibrium of enzymes and regulators that control cytoskeletal dynamics (Arber et al., 1998) leading to the observed oligodendroglial morphological changes. However, prior to direct pharmaceutical targeting of this protein it is important to understand how downregulation of a single gene can affect multiple cellular

parameters, to which end further studies addressing p57kip2's mode of action will be necessary. It remains to be seen whether these studies will reveal the extent of induced alterations in gene expression and whether or not they are secondary to morphological events. It is tempting to speculate that interactions with related LIM proteins could affect additional aspects of glial differentiation e.g. gene regulation.

Finally, for comprehensive evaluation of p57kip2's role beyond cell cycle regulation, it will be of importance to find out more about p57kip2's *in vivo* functions. As p57kip2 knock-out mice die shortly after birth (Takahashi et al., 2000), experiments in the context of the pathological adult CNS are impossible to perform on these animals. MOG-EAE for instance, as an inflammatory disease paradigm of the mature CNS, features prolonged disease courses of up to two weeks and is thus not viable in p57kip2 knock-out mice. Therefore, sublethal *in vivo* reduction of p57kip2 levels seems to be the only viable means to address more complicated questions in regard to this regulatory protein. To this end, two molecular approaches can be persued: a) downregulation of p57kip2 using vector-mediated RNA interference, either as a transgene or delivered using retroviral particels or b) the construction of conditional knock-out animals, where tissue specific gene deletion allows to analyse its function in the CNS or PNS only.

5. Summary

This study has shown that a sustained reduction of p57kip2 expression levels in oligodendroglial cells facilitates and promotes their differentiation, indicating that p57kip2 is a novel negative regulator of (re)myelination at the interface between morphogenesis and gene expression. This outcome is of particular interest regarding our still-limited understanding of (re)myelination mechanisms and will be important in defining novel strategies in order to promote endogenous remyelination and CNS repair. In this regard, it will be imperative to determine in future studies to what degree p57kip2 expression directly affects (re)myelination *in vivo*.

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7. Abbreviations

BSA	bovine serum albumin
BBB	blood brain barrier
DNA	deoxyribonucleic acid
CDKI	cyclin dependent kinase inhibitor
CNPase	2',3'-cyclic nucleotide 3'-phosphohydrolase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
EtOH	ethanol
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
ECM	extracellular matrix
Ex	embryonic day x
FACS	fluorescence activated cell sorting
FGF	fibroblast growth factor
FCS	fetal calf serum
GalC	galactosylceramidase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
HLA	human leucocyte antigen
LIMK-1	LIM domain kinase 1
MAG	myelin associated glycoprotein
MBP	myelin basic protein
mM	millimolar
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
O-2A	oligodendrocyte-type-2 astrocyte
ODC	ornithine decarboxylase
OPC	oligodendocyte precursor cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-AA	platelet derived growth factor AA

PDGFR-α	platelet derived growth factor receptor alpha
PDL	Poly-D-Lysine
PFA	paraformaldehyde
PLAOCD	Petrophaga lorioti associated oligodendrocyte calcium deprivation
PLP	proteolipid protein
PNS	peripheral nervous system
POA	proligodendroblast antigen
PPMS	primary progressive multiple sclerosis
PSA-NCAM	polysialylated form of cell surface glycoprotein neural cell adhesion molecule
Px	x days from birth
RNA	ribonucleic acid
RRMS	relapsing remitting multiple sclerosis
RT	reverse transcription
SC	Schwann Cell
SEM	standard error of the mean
shRNA	small/short hairpin RNA
siRNA	small interfering RNA
SPMS	secondary progressive multiple sclerosis
SVZ	subventricular zone
T3	triiodothyronine
T4	thyroxine
YFP	yellow fluorescent protein

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9. Curriculum vitae

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Publications

06/08	Heinen A, Kremer D, Göttle P, Kruse F, Hasse B, Lehmann H, Hartung HP, Küry P: The cyclin-dependent kinase inhibitor p57kip2 is a negative regulator of Schwann cell differentiation and in vitro myelination. Proc Natl Acad Sci U S A. 2008 Jun 24;105(25):8748-53.
09/08	Heinen A, Kremer D, Hartung HP, Küry P: p57 kip2's role beyond Schwann cell cycle control. Cell Cycle. 2008 Sep 15;7(18):2781-6.
04/09	Kremer D, Heinen A, Jadasz J, Göttle P, Zimmermann K, Zickler P, Jander S, Hartung HP and Küry P: p57kip2 is dynamically regulated in experimental autoimmune encephalomyelinitis and acts as a negative regulator of oligodendroglial maturation. accepted by Proc Natl Acad Sci U S A (2009) in press
05/09	Kremer D, Aktas O, Hartung HP and Küry P (2009) Immunobiology of the oligodendrocyte. In "The Biology of Oligodendrocytes", Publisher: Prof. Patricia Armati, Cambridge University Press (2009) in press

Abstract

Die vorliegende Dissertationsarbeit befasst sich mit den Mechanismen glialer Differenzierung und der Frage, inwieweit diese im Rahmen inflammatorischer Erkrankungen des Zentralen Nerversystems (ZNS) wie der Multiplen Sklerose (MS) von Bedeutung sind.

Multiple Sklerose ist eine autoimmune inflammatorische ZNS Erkrankung, die eine Zerstörung der axon-isolierenden Myelinscheiden zur Folge hat. Hierdurch kommt es zunächst zu einer Herabsetzung der elektrischen Überleitungsgeschwindigkeit in den neuronalen Axonen und schließlich zum direkten axonalen Schaden. Die myelinbildenden Zellen des ZNS, die Oligodendrozyten, können die zerstörten Myelinscheiden in der Folge nur unvollständig erneuern, wobei dies nicht zuletzt mit der eingeschränkten Fähigkeit von residenten Oligodendrozytenvorläuferzellen zu differenzieren in Verbindung gebracht wird.

In der vorgelegten Arbeit konnte nun gezeigt werden, dass eine anhaltende Reduzierung der Genexpression von p57kip2, eines Zyklin abhängigen Kinaseinhibitors, in oligodendroglialen Vorläuferzellen zu einer erleichterten und verstärkten Differenzierung dieser myelinisierenden Zellen führt. Zu diesem Zweck wurden primäre Rattenoligodendrozyten aus dem neugeborenen Rattenhirn mit einem Gensuppressionsvektor für p57kip2 transfiziert und anschließend immunzytochemisch und morphologisch analysiert. Neben diesen *in vitro* Studien wurde gezeigt, dass p57kip2 im Rahmen der MOG-EAE (Myelin-Oligodendrozyten- Glykoprotein induzierte experimentelle autoimmune Enzephalomyelitis), einer Modellerkrankung der MS in der Ratte, fast ausschließlich von Oligodendrozyten und ihren Vorläuferzellen exprimiert und schubabhängig reguliert wird. Hierfür wurde Material aus dem gesunden und dem EAE-Rattenhirn mittels qRT-PCR und Immunhistochemie aufgearbeitet und miteinander verglichen. Gemeinsam legen die in dieser Arbeit vorgestellten Ergebnisse nahe, dass es sich bei p57kip2 um einen neuartigen negativen Regulator der (Re-) Myelinisierung handelt, der sich an der Schnittstelle zwischen Genexpression und Zellmorphogenese befindet.

Insbesondere in Hinblick auf das immer noch begrenzte Verständnis der zerebralen (Re-) Myelinisierungsmechanismen kann die vorgelegte Arbeit neue Wege in Richtung auf alternative Therapiestrategien, wie der Stimulierung hirneigener Regenerationsprozesse weisen. Die im Rahmen dieser Doktorarbeit generierten Daten konnten in einem Fachjournal publiziert werden (Kremer et al., PNAS 2009 106:9087-9092).