Relieving the epigenetic blockade in progressive MS

RELIEVING THE EPIGENETIC BLOCKADE IN PROGRESSIVE MS

–

MAKING REMYELINATION ACCESSIBLE AGAIN

ASSIA TIANE

Relieving the epigenetic blockade in progressive MS – making remyelination accessible again

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فَإِنَّ مَعَ الْعُسْرِ يُسْرًا

So undoubtedly, along with hardship there is ease.

Quran 94:5

TABLE OF CONTENTS

List of abbreviations

450K	Illumina Infinium HumanMethylation450 BeadChip
850K	Illumina Infinium MethylationEPIC BeadChip
ABCA2	ATP-binding cassette transporter A2
ABCD1	ATP-binding cassette transporter D1
AD	Alzheimer's disease
ADLD	Autosomal dominant leukodystrophy
ALD	Adrenoleukodystrophy
ANOVA	Analysis of variance
APOE4	Apolipoprotein E4
APP	Amyloid precursor protein
ASCL1	Achaete-scute family bHLH transcription factor 1
ATP	Adenosine triphosphate
AZA	Azacitidine
BCAS1	Breast carcinoma amplified sequence 1
BCL2L2	B-cell lymphoma 2-like 2
BMP	Bone morphogenetic protein
BRG1	Brahma-related gene 1
BSA	Bovine serum albumin
CG	Cytosine-guanine (DNA base pair)
CH_3	Methyl group
CNP	2',3'-Cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CNTN2	Contactin-2
CO_2	Carbon dioxide
CPM	Counts per million
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal fluid
DAB	3,3'-Diaminobenzidine
DAKO	A brand of immunohistochemistry and pathology products
DAPI	4’,6-diamidino-2-phenylindole
DE	Differential expression

DEG	Differentially expressed gene
DICER1	Dicer 1, ribonuclease III
DMEM	Dulbecco's Modified Eagle Medium
DMP	Differential methylated probe
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EDSS	Expanded Disability Status Scale
EPIC	Epigenome-Wide Association Study (EWAS) genotyping array
EWAS	Epigenome-Wide Association Study
FACS	Fluorescence-Activated Cell Sorting
FC	Fold Change
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FDR	False Discovery Rate
FGF	Fibroblast Growth Factor
GE	Gene Expression
GO	Gene Ontology
H_2O	Water
H_2O_2	Hydrogen Peroxide
H3	Histone H3
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HES	Hairy and Enhancer of Split
HIS	Histidine
HLA	Human Leukocyte Antigen
HLH	Helix-Loop-Helix
HMG	High Mobility Group
HOG	Heterogeneous nuclear ribonucleoprotein O-like protein
HRP	Horseradish Peroxidase

ID2	Inhibitor of DNA binding 2
ID4	Inhibitor of DNA binding 4
IDAT	Intensity Data files for Illumina microarray
LCM	Laser Capture Microdissection
LINGO	Leucine-Rich Repeat And Ig Domain-Containing Nogo Receptor Interacting Protein
LPAR1	Lysophosphatidic acid receptor 1
LTP	Long-Term Potentiation
MAG	Myelin-associated glycoprotein
MBD	Methyl-CpG binding domain
MBP	Myelin basic protein
MOBP	Myelin oligodendrocyte basic protein
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MWAS	Methylation-wide association study
MYRF	Myelin regulatory factor
NAWM	Normal-appearing white matter
NDRG1	N-myc downstream regulated gene 1
NG2	Neural/glial antigen 2
O4	Oligodendrocyte precursor cell marker
OL	Oligodendrocyte
OLIG	Oligodendrocyte transcription factor
OPC	Oligodendrocyte precursor cell
ORO	Oil Red O
PAD2	Peptidyl arginine deiminase 2
PAM	Protospacer adjacent motif
PARD3	Par-3 family cell polarity regulator
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC1	Principal component 1
PCA	Principal component analysis

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PFA	Paraformaldehyde
PLL	Poly-L
PLO	Probable loss of function
PLP	Proteolipid protein
PMBC	Peripheral blood mononuclear cells
PMI	Post-mortem interval
PMP22	Peripheral myelin protein 22
PPMS	Primary progressive multiple sclerosis
PRMS	Progressive relapsing multiple sclerosis
PRMT	Protein arginine methyltransferase
QC	Quality control
RIN	RNA integrity number
RNA	Ribonucleic acid
RPL13	Ribosomal protein L13
RRMS	Relapsing-remitting multiple sclerosis
RS	Relapse or remission score
SANGER	Sanger sequencing
SD	Standard deviation
SEM	Standard error of the mean
SIRT2	Sirtuin 2
SNP	Single nucleotide polymorphism
SOX	SRY-box transcription factor
SPMS	Secondary progressive multiple sclerosis
SRY	Sex determining region Y
T4	Thyroxine
TALE	Transcription activator-like effector
TBP	TATA-box binding protein
TBS	Tris-buffered saline
TCF	Transcription factor

TET	Ten-eleven translocation enzyme
TMM	Trimmed mean of M-values
TSA	Trichostatin A
UGT8	UDP glycosyltransferase 8
UTR	Untranslated region
VPA	Valproic acid
WB	Western blot
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Chapter 1

General introduction

Progressive multiple sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory autoimmune disease of the central nervous system (CNS) (1). It is one of the major neurological disorders among young adults and affects approximately 2.5 million people worldwide. MS is characterized by inflammation-induced demyelination during the early stages, which eventually results in gradual neurological disability as the disease progresses (1, 2).

The early stage of MS is characterized by acute attacks of infiltrated myelin-reactive lymphocytes and macrophages, resulting in demyelination of the axonal branches. In most cases, these inflammatory relapses are followed by a period of recovery, during which partial or complete remyelination occurs, as demonstrated by thinly (re)myelinated axons (shadow plaques). This clinical form of MS is termed as relapse-remitting MS (RRMS) and affects approximately 80% of the total MS patients (1-3). However, independent of treatment, about 50% of RRMS patients undergo a transition within a period of ten to fifteen years into the progressive form of the disease, labelled as secondary progressive MS (SPMS) (4, 5). Additionally, approximately 10-15% of the MS patients show a gradual increase in disability from disease onset, without experiencing an initial relapsing course. These patients are classified as primary progressive MS patients (PPMS) (6). Together, SPMS and PPMS represent the chronic, progressive stages of MS.

The progressive stages of MS are hallmarked by an increase in neurological deficits, accompanied by a gradual decline in motor and cognitive function (5). This slow progression in disability is shown to develop independently of the acute inflammatory attacks. Progressive MS patients hardly experience new relapses and show little systemic inflammation (7). While available therapies modulate the immune response to temper early disease activity, they have limited efficacy in preventing transition towards the chronic stage and are no longer effective in the progressive stage of MS (8). Thus, there is a high need for novel therapeutic strategies to induce repair mechanisms and combat disease progression during these chronic stages of MS.

Therapies and biomarkers for progressive MS

Over the past years, great advances have been made in the discovery and development of novel treatments, resulting in more than 15 Food and Drug Administration (FDA)-approved disease-modifying treatments for RRMS patients (9). These therapies mainly modulate the immune response to temper early disease activity, yet they have limited efficacy in preventing the transition towards the progressive stage and are no longer effective for progressive MS patients (8). Only two disease-modifying treatments have been approved for progressive MS stages, i.e. ocrelizumab and siponimod. However, as their effect on disability is mediated by their anti-inflammatory properties, these drugs are only effective in a subset of progressive MS patients with an active disease course (10-12). Basic preclinical research has led to numerous clinical trials investigating regenerative compounds to induce myelin repair in the context of MS. Opicinumab, an antibody treatment targeting LINGO-1, has been tested to see whether it could work as an add-on therapy to slow down disability in MS patients. Unfortunately, the study did not meet its goal and further development of the drug has been halted since 2020 (12, 13). Clemastine fumarate has also been identified as a potential remyelinating drug and was successfully validated in a randomized placebo-controlled phase II clinical trial involving 50 RRMS patients (12, 14). Clemastine is currently being investigated in a new clinical trial, involving 74 RRMS patients with chronically demyelinated lesions, with magnetic resonance imaging (MRI)-based evidence for remyelination as the primary outcome (*ClinicalTrials.gov* NCT05359653). However, despite great efforts in the field, there is still a high unmet clinical need for DMTs that target demyelination, axonal loss, and neuronal damage to slow down or halt progression in MS.

One of the major challenges in MS is to accurately monitor and quantify disability over time, as current diagnostics are based on a combination of MRI, neurologic examinations (such as the Expanded Disability Status Scale; EDSS), and the patient's clinical history, concomitant with several limitations (5, 15). The lack of specific and sensitive diagnostic markers for disease progression does not only impact clinical decision making, but also slows down the discovery and validation of new therapeutic agents as current clinical trials mainly depend on traditional clinical imaging outcomes, such as brain atrophy (16). Thus, there is an urgent

need for easily accessible, quantifiable and reliable diagnostic markers for disease progression, associated to remyelination impairment or recovery. Discovery of such biomarkers may furthermore provide new insights into the pathological mechanisms that underlie progressive MS, accelerate and facilitate clinical trials, and could therefore lead to new therapies for progressive MS.

Oligodendrocytes and myelin gene expression

The early stage of MS is characterized by inflammation-induced demyelination, followed by rapid remyelination, as a result of the recruitment and differentiation of oligodendrocyte progenitor cells (OPCs). OPCs can remyelinate affected axons, yielding typical shadow plaques (17). Despite the presence of sufficient numbers of OPCs in the vicinity of the pathological lesions, endogenous repair mechanisms fail in later disease stages, resulting in chronically demyelinated axons and, eventually, neurodegeneration (18). While the processes underlying impaired endogenous repair are poorly understood, there is strong evidence that the reduced ability of OPCs to differentiate into mature myelin-forming oligodendrocytes is an important contributor (19, 20). OPC differentiation can be divided into four distinct stages (Figure 1.1). In the early phases of oligodendrocyte maturation, OPCs proliferate and form bipolar extensions. These motile OPCs differentiate further into pre-oligodendrocytes, characterized by the expression of the O4, an antigen present on the surface of these cells, and the formation of multiple processes. At the end of this stage, the differentiated oligodendrocytes exit the cell cycle and form immature oligodendrocytes. The final maturation stage is defined by the formation of myelin and the expression of the associated myelin protein genes (21).

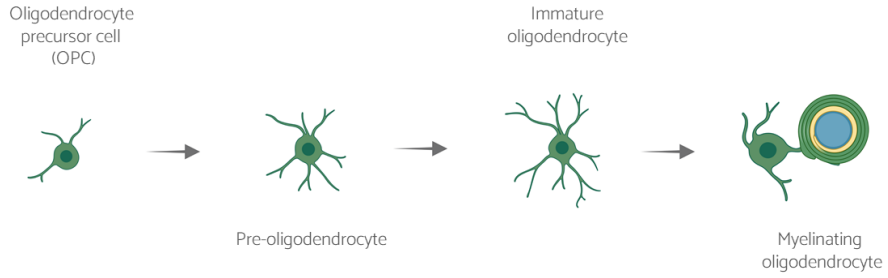

Figure 1.1 - Differentiation stages of oligodendrocyte precursor cells (OPCs) into mature myelinating oligodendrocytes.

OPC differentiation into mature myelinating oligodendrocytes is defined by the expression of myelin genes, such as myelin basic protein (*MBP*), proteolipid protein (*PLP*), and myelin-associated glycoprotein (*MAG*) (21). The expression of these myelin genes is regulated by an upstream transcriptional network, featured by an interplay of positive and negative regulators (Figure 1.2). Positive regulators such as OLIG1/2 activate MYRF, which, like SOX10, binds to the promoter region of genes involved in myelination, hence promoting their expression (22-24). In contrast, increased expression of negative regulators, such as ID2, ID4, and SOX5, inhibits the ability of OLIG2 to induce MYRF expression and, thus, prevents myelination-related gene transcription in an indirect manner. As such, a tight control of the positive and negative upstream regulators is required to orchestrate OPC differentiation during remyelination.

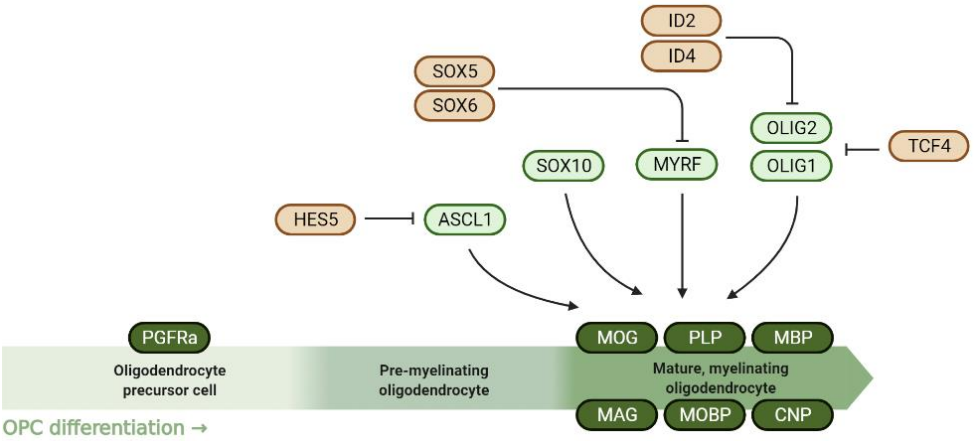

Figure 1.2 - An upstream key regulatory network controls oligodendrocyte maturation and myelin gene expression. Pro-transcriptional factors (green) promote myelin gene expression, while negative regulators (red) antagonize their activity.

Epigenetic mechanisms

Epigenetic fingerprinting allows for a controllable and reversible spatiotemporal regulation of cellular differentiation (25-27). Epigenetic mechanisms are defined as modifications that influence gene expression without altering the DNA sequence itself and are heritable from mother to daughter cell (28, 29). Epigenetic control of gene expression is sustained via DNA methylation, modifications at the histone tails of chromatin and non-coding RNAs. The interplay between these different

modifications changes the physiological form of the DNA, thereby influencing the accessibility of transcription factors to specific genomic regions (29, 30).

DNA methylation is one of the most studied and stable epigenetic modifications. Addition of a methyl-group (-CH_3) to a cytosine base occurs within a 5'cytosine-guanin-3' dinucleotide (CpG) site. So called 'CpG islands' cover regions of more than 300bp with a C/G-content of minimally 50% and are mostly found within promoter regions of protein-encoding genes (31). The regions flanking these CpG islands (<2kb) are called 'CpG shores', whereas the regions flanking the CpG shores (<2kb) are labeled as 'CpG shelves'. Methylation of these CpG-rich regions is generally associated with gene silencing due to the inability of transcription factor binding. DNA methylation is established by DNA methyltransferases (DNMTs) that add a methyl-group to cytosine (forming 5-methylcytosine [5mC]). DNMT1 and DNMT3a/b represent two distinct forms of DNMTs, which either maintain DNA methylation during replication or induce *de novo* methylation, respectively (32, 33). DNA methylation marks can be removed in a passive way through cell division, or more actively, via gradual degradation of 5mC by ten-eleven translocation (TET) enzymes (34, 35). Hydroxylation of 5mC into hydroxy-methylated cytosine (5-hydroxymethylcytosine [5hmC]) is the first step of the demethylation process. Interestingly, 5hmC patterns have shown to be abundantly present in the CNS of mammals (36, 37). While 5hmC was first identified solely as an intermediate epigenetic mark during active DNA demethylation, is has in the meantime also been shown to represent an independent, stable and functionally distinct epigenetic mark in the brain. (38, 39).

Methylation of CpG sites does not only sterically inhibit the binding of transcription factors. DNA methylation is also closely related to other epigenetic mechanisms, such as histone modifications (40). Methylated CpG regions are recognized by methyl-CpG-binding proteins (MBDs) that recruit repressive proteins, resulting in chromatin remodeling. Binding of histone deacetylase (HDACs) enzymes to these MBDs, for instance, removes the acetyl group from histone tails and changes the chromatin structure into a 'closed' format to suppress gene expression (41, 42). Altogether, the epigenome is an intricate system that coordinates the expression of genes in a coordinated spatiotemporal manner.

Methylomic changes in MS

An increasing body of evidence suggests a role of epigenetic mechanisms in the pathophysiology of MS. Numerous studies concerning epigenetics in MS have focused on the early, inflammatory stages of the disease (43-45). Furthermore, links between environmental risk factors and epigenetic changes have been widely studied (46-48). Even though the influence of epigenetics in progressive MS pathology is not clear yet, emerging data suggests a prominent role in oligodendrocyte differentiation and maturation. The presence of DNMTs has been shown to be essential for OPC differentiation and remyelination, following experimental demyelination (49). In line, the levels of demethylation enzymes (TET1-3) decline with oligodendrocyte maturation (50). On the chromatin level, histone deacetylation has been shown to be crucial for the initiation of OPC differentiation (51, 52). This insinuates that negative regulators are epigenetically silenced allowing positive regulators to stimulate OPC differentiation and myelin gene expression. Notably, a recent study reported hypermethylation in the *MBP* gene when comparing DNA derived from normal appearing white matter (NAWM) of MS patients with DNA isolated from white matter of non-neurological controls (53). Generally, hypermethylation of DNA within the promoter region of the *MBP* gene results in lower *MBP* expression and could thus be a major hurdle in oligodendrocyte maturation. Along similar lines, more upstream, DNA methylation changes in positive and negative regulators of myelin-related genes may impact their expression, and, consequently, OPC differentiation during remyelination in MS. Together, these data suggest that a disbalance in the epigenetic coordination of myelin genes and their upstream regulators might be the underlying cause of remyelination impairment in progressive MS.

Overview of the thesis

The research compiled in this thesis is based on three main objectives. The first objective, covered by two chapters, is focused on how DNA methylation influences (physiological) OPC differentiation. The second objective, discussed in Chapters 4 and 5, focuses on how DNA methylation patterns can be altered in the context of MS pathology and whether this could be the underlying mechanism for remyelination impairment. The third and final objective, covered by Chapter 6, is to investigate whether MS-associated epigenetic signatures obtained from studying brain tissue can be applied as a peripheral biomarker for progression in MS.

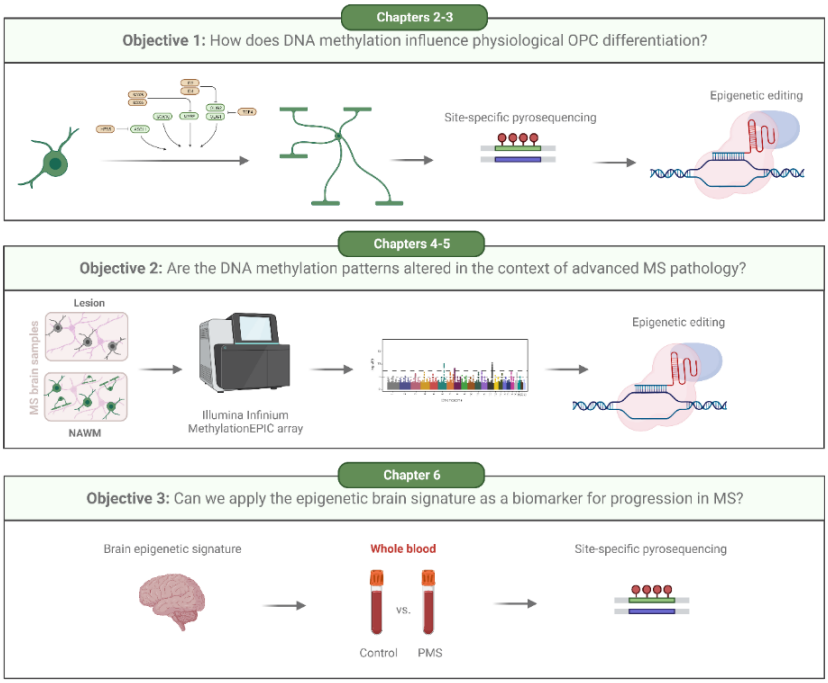

Figure 1.3 – Overview of the thesis objectives and chapters.

More specifically, **Chapter 2** offers more in-depth information on how epigenetic mechanisms influence oligodendrocyte differentiation and myelination. It first of all provides a general overview of the transcriptional network that regulates differentiation. Then, the epigenetic mechanisms, comprising DNA methylation, histone modifications, and micro-RNAs, are each discussed separately based in

view of how they are thought to play a role during physiological OPC differentiation. Finally, the implication of epigenetic dysregulation related to OPC differentiation on demyelinating disorders and ageing is being discussed.

In **Chapter 3**, I describe how I investigated the direct influence of DNA methylation on the transcriptional network that regulates myelin gene expression and OPC differentiation. By applying a pharmacological inhibitor of DNA methylation, as well as CRISPR-Cas9-based epigenetic editing, I assessed which genes are being affected by DNA methylation during physiological OPC differentiation.

Chapter 4 is based on a perspective, in which we discuss the importance of causality assessment in neuroepigenetic research. We propose a workflow, starting from epigenome-wide association studies (EWAS), all the way to applying epigenetic editing as a tool to investigate potentially causal associations between epigenetic modifications of candidate genes and the pathophysiology of neurodegenerative disorders.

In the work described in **Chapter 5**, I applied the proposed workflow from Chapter 4 in the context of progressive MS. Starting from epigenomic and transcriptomic profiles of chronically demyelinated MS lesions, I identified target genes that are differentially expressed and differentially methylated in these lesions, in comparison to the surrounding NAWM. Following cell-specific validation in laser-captured OPCs, I investigated the causal relationship between the methylation of one of the target genes and the differentiation capacity of human iPSC-derived oligodendrocytes by means of epigenetic editing.

In the final study, presented in **Chapter 6**, I aimed to investigate whether the brain methylation pattern of progressive MS patients is mirrored in the blood and thus could be applied as a biomarker for disease progression in MS. Samples from different patient cohorts were used to assess the epigenetic signature of myelin genes, based on the results from Chapter 5.

Finally, **Chapter 7** discusses the results of this thesis, while **Chapters 8** and **9** summarize the main findings. In **Chapter 10**, I further elaborate on the scientific and societal impact of my research.

CHAPTER 2

From OPC to oligodendrocyte: an epigenetic journey

Based on:

From OPC to oligodendrocyte: an epigenetic journey

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Abstract

Oligodendrocytes provide metabolic and functional support to neuronal cells, rendering them key players in the functioning of the central nervous system. Oligodendrocytes need to be newly formed from a pool of oligodendrocyte precursor cells (OPCs). The differentiation of OPCs into mature and myelinating cells is a multistep process, tightly controlled by spatiotemporal activation and repression of specific growth and transcription factors. While oligodendrocyte turnover is rather slow under physiological conditions, a disruption in this balanced differentiation process, for example in case of a differentiation block, could have devastating consequences during ageing and in pathological conditions, such as multiple sclerosis. Over the recent years, increasing evidence has shown that epigenetic mechanisms, such as DNA methylation, histone modifications, and microRNAs, are major contributors to OPC differentiation. In this review, we discuss how these epigenetic mechanisms orchestrate and influence oligodendrocyte maturation. These insights are a crucial starting point for studies that aim to identify the contribution of epigenetics in demyelinating diseases and may thus provide new therapeutic targets to induce myelin repair in the long run.

Introduction

Oligodendrocytes (OLs) are myelinating glial cells within the central nervous system (CNS) that insulate neuronal axons to provide them with trophic, metabolic and functional support. OLs are generated from oligodendrocyte precursor cells (OPCs) via a consecutive process of cell cycle exit, maturation, and differentiation (54). OPCs arise during early development, persist throughout lifetime and occupy around 5-10% of the total number of cells in the brain (55, 56). In response to both intrinsic molecular cues and extracellular signals, OPCs are able to withdraw from their proliferative stage and differentiate into myelin-producing OLs (57). Consequently, alterations in these extrinsic stimuli, such as an increase in inhibitory ECM molecules (LINGO, glycosamineglycans, fibronectin) or secreted factors (BMP, FGF), hamper differentiation, possibly via an upstream effect on transcriptional and epigenetic processes that regulate OL differentiation (58). Indeed, current evidence indicates that epigenetic mechanisms, comprising DNA methylation, histone modifications and microRNAs (miRNAs), play an essential role in the regulation of OL lineage development. As such, epigenetic signatures translate extracellular signals into functional cellular changes and coordinate the transcriptional machinery that is responsible for the differentiation process (27, 59). This review provides an overview of the current understanding of the physiological process of OL lineage development and how the different epigenetic mechanisms are involved in the regulation of this process (Figure 2.1). Furthermore, we discuss how this epigenetic fingerprinting is altered during ageing and in neurological conditions.

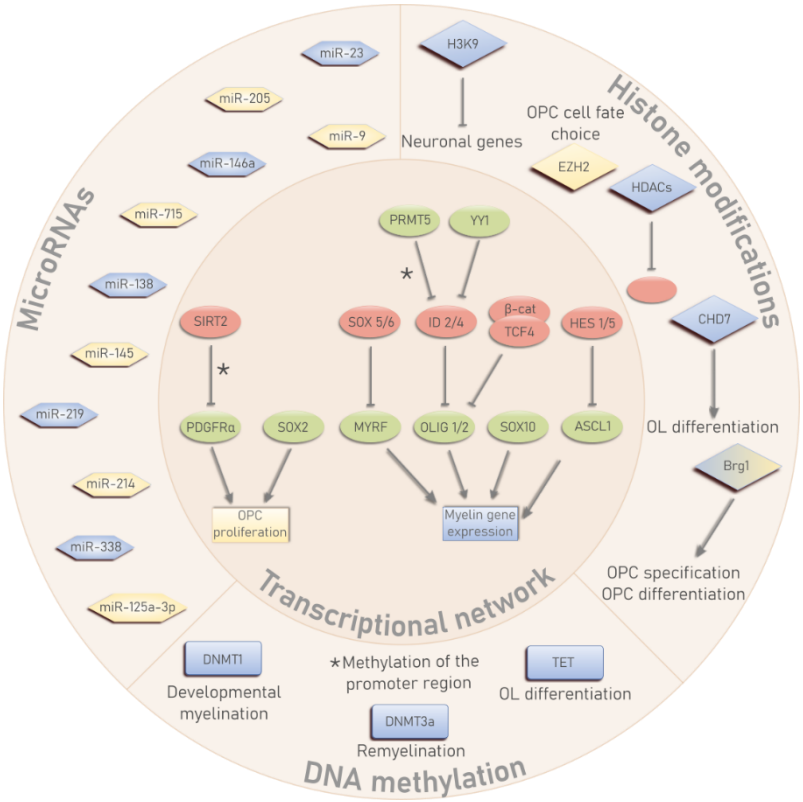

Figure 2.1 – An overview of the transcriptional and epigenetic regulation of OPC proliferation and OL development. Transcription factors that exert a positive or negative effect on these processes are depicted in green and red, respectively. Pro-proliferative factors are visualized in yellow, whereas pro-differentiation factors are blue. *Methylation of the promoter region.

OL differentiation and the transcriptional network

OPCs arise from the ventricular zone during early development, proliferate and migrate their way into the different developing areas of the brain, where they differentiate into myelin-forming OLs (60). Unlike most progenitor cells, OPCs persist throughout life as adult, self-renewing OPCs that can differentiate into newly formed myelinating OLs to maintain myelin plasticity or in response to damaging signals (61). The differentiation of OPC into mature and myelin-producing OLs is a gradual and well-defined process that can be divided into four successive stages: proliferative OPCs, pre-OLs, differentiated OLs and myelinating OLs (21). This process of OL differentiation, both during early development and

in adult stages, is controlled by the combination of OL-specific transcription factors, extracellular signals, epigenetic modifications and signalling pathways. It is necessary to maintain a homeostatic balance between these molecular cues to allow for proper differentiation.

The regulatory network of transcription factors that controls OL lineage development has been extensively studied over the past decades (23, 61, 62). These transcription factors regulate OPC proliferation, migration and differentiation and at the same time serve as stage-specific cell identity markers of the OL lineage (62). In general, a distinction can be made between positive regulators, which boost and stimulate OL differentiation, and negative regulators, which function as inhibitory transcription factors for myelin genes and keep OPCs in a proliferative and non-differentiated state.

The main transcription factors that regulate OL lineage progression belong to the helix-loop-helix (HLH) family, such as the oligodendrocyte transcription factors (OLIG), hairy and enhancer-of-split homologs (HES) and inhibitor or differentiation (ID) proteins. OLIG2 is considered as one of the major and indispensable transcription factors during different stages of OL development. It is an essential factor during OPC specification, enhances OPC migration during early development, but also functions as a promoting factor of OL differentiation and regeneration in the adult life (63-65). In contrast to OLIG2, the closely related OLIG1 is not directly involved during early brain development, but rather promotes OL differentiation and myelination after injury (24, 66). The achaete-scute homolog 1 (ASCL1 or MASH1) is another member of the HLH family that promotes early OPC specification and OL development (67). Although it was considered to be mainly involved in early oligodendrogenesis, ASCL1 is also shown to be important during adult OL regeneration and remyelination (68). In contrast, HES proteins, such as HES1 and HES5, function as differentiation inhibitors either by recruiting other repressor proteins to myelin gene promoters, or by inhibiting ASCL1 (23). Similarly, the ID HLH transcription factors, ID2 and ID4, inhibit OPC differentiation by binding to other members of the HLH family (OLIG1/2, ASCL1) and preventing their translocation from the cytoplasm to the nucleus (69, 70).

Another family of transcriptional regulators are HMG-domain transcription factors, that are classified as the sex determining region Y-box (SOX) family, of which SOX10 is a well-established regulator involved in terminal OL differentiation and

myelination, through its direct binding to the promoter region of myelin genes to enhance their expression (22, 71). Interestingly, SOX10 is expressed in all stages of the OL lineage and can thus serve as a general marker for OPCs/OLs (72). In contrast, SOX5 and SOX6 inhibit OL differentiation by competing with SOX10 binding sites, thereby antagonizing its function (73). SOX2 on the other hand, maintains OPCs in a proliferative and undifferentiated stage, but is indispensable for OPC expansion and OL regeneration during CNS remyelination (74, 75). Transcription factor 4 (TCF4, also known as TCFL2) is another important HMG-domain transcription factor and is a downstream effector of the Wnt signalling pathway. Through its binding to β-catenin, TCF4 acts as an inhibitor of myelin gene expression and impairs (re)myelination (76).

An additional class of OL-related transcription factors are zinc finger proteins (ZFP). Yin Yang 1 (YY1) stimulates OL differentiation by silencing inhibitor proteins, such as ID4 and TCF4 (77). Other ZFPs that enhance OL maturation and differentiation are ZFP191, ZFP488 and the Smad interacting protein 1 (SIP1) (78-81). Myelin regulatory factor (MYRF) was only recently discovered as a crucial regulator of CNS myelination (82). MYRF is exclusively expressed in post-mitotic cells of the OL lineage, which signifies its essential role during terminal differentiation. The synergistic effect of MYRF and SOX10 leads to myelin gene activation and drives CNS myelination (22, 82).

All the transcriptional regulators influence OL differentiation mainly by controlling the expression of genes that encode for the essential myelin-associated proteins, such as the myelin basic protein (MBP), proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) (83, 84). The transcription factors either enhance or inhibit the expression of these myelin genes by directly binding to their promoter region, which eventually results in a spatiotemporal expression of myelin genes during the process of OL lineage development (85).

The epigenetic triumvirate in OL development

OL lineage development and the regulation of the associated transcriptional program is highly influenced by various epigenetic processes. Epigenetic mechanisms are defined as modifications that affect gene expression without altering the DNA sequence itself and are heritable from mother to daughter cell

(28, 29). Epigenetic control of gene expression is sustained via DNA methylation, modifications at histone tails of chromatin, and miRNAs. The interplay between these different modifications changes the physiological form of the DNA, thereby influencing the accessibility of specific transcription factors to their target regions in the genome (29, 30). In the following part of this review, we discuss how the different levels of epigenetic regulation influence OL differentiation and CNS myelination.

DNA methylation

DNA methylation, in particular CG methylation, is one of the most studied and long-lasting epigenetic modification. CG methylation involves the addition of a methyl-group (-CH3) to a cytosine base followed by a guanine nucleotide, referred to as 5'cytosine-guanin-3' dinucleotide (CpG) site. Although various definitions exist, so-called 'CpG islands' cover regions of more than 300bp with a C/G-content of minimum 50% and are mostly found within the promoters of protein coding genes (31). Methylation of these CpG islands is generally associated with gene silencing due to the inability of transcription factors to bind to the methylated promoter region or via an additional recruitment of other repressor proteins (41, 42). DNA methylation is established by DNA methyltransferases (DNMTs) that add a methyl-group to cytosine (5mC). There are two distinct forms of DNMTs, DNMT1 and DNMT3a/b, which either maintain DNA methylation during replication or induce *de novo* methylation, respectively (32, 33). Contrarily, DNA methylation can be removed via gradual degradation of 5mC by the ten-eleven translocation (TET) enzymes (35, 36), although DNMTs may serve the same purpose under certain conditions (86, 87). Hydroxylation of 5mC into hydroxy-methylated cytosine (5hmC) is the first step of the demethylation process. Interestingly, 5hmC patterns have shown to be abundantly present in the CNS of mammals (36, 37). 5hmC was first identified as an intermediate epigenetic mark during active DNA demethylation, but has also been shown to represent a potentially independent and functionally distinct epigenetic marker in the brain. (38, 39).

One of the first studies that linked DNA methylation to OL development showed that neonatal rats treated with the DNMT-inhibitor 5-azacytidine (5-aza), displayed disrupted gliogenesis, concomitant with hypomyelination of the 11-day-old optic nerve. Postnatal inhibition of DNA methylation resulted in a reduced

number of oligodendrocytes, whilst the number of astrocytes was less affected, indicating a higher vulnerability of OPCs to changes in DNA methylation (88). Likewise, ablation of the *Dnmt1* gene in embryonic progenitor cells lead to OPC growth arrest and resulted in severe hypomyelination. Moreover, this loss of *Dnmt1* seemed to alter splicing events, such as exon skipping and intron retention, in genes related to myelination, lipid metabolism and the cell cycle, indicating a crucial role of DNA methylation in relation to alternative splicing during neonatal OL development (49). Although DNMT1 seemed to be an important regulator during developmental myelination, it seems to play a less prominent role during remyelination of the adult CNS (89). After lysolecithin-induced demyelination of adult murine spinal cord white matter, higher levels of DNA methylation in differentiating OLs are accompanied by an increased expression of DNMT3a. Transgenic mice that lack *Dnmt3a* showed impaired OL differentiation and a reduced ability to remyelinate affected axons after injury (89). Together, these studies suggest that maintenance of DNA methylation is important to ensure proper gliogenesis during developmental myelination, whilst *de novo* methylation is needed for the differentiation of adult OPCs into remyelinating OLs. On the opposite side of the methylation spectrum, TET enzymes also strongly influence OL differentiation (50). Even though the three TET enzymes show different subcellular localisation and unique expression patterns, they all seem to be equally important during OL development. Interestingly, knock-down of the *Tet* mRNA levels was associated with increased expression of HLH inhibitory transcription factors, such as ID2 and HES5, leading to suppression of myelin gene expression (50). It however remains unclear whether TET enzymes directly inhibit the expression of these genes or whether the observed transcriptional change is mediated in an indirect manner. In general, epigenome-wide studies of stage-specific cells are still needed to unravel how and which exact CpG sites or islands change in their methylation status during OL lineage progression.

In relation to the transcriptional regulatory network of OL development, it has been shown that DNA methylation can regulate the temporal expression of these transcription factors. In a study of Huang *et al.*, PRMT5 was identified as a pro-differentiation factor that binds to CpG rich islands within the ID2 and ID4 genes. Subsequent DNA methylation of these regions led to silencing of the

transcriptional inhibitors and resulted in OL differentiation (90). In a similar fashion, SIRT2 was shown to translocate to the nucleus, inducing DNA methylation in the platelet-derived growth factor receptor α (PDGFRα) promoter region and initiating glial differentiation (91). Interestingly, both PRMT5 and SIRT2 are classified as histone-modification enzymes, yet they are also known to induce epigenetic changes at the level of DNA methylation, thereby emphasizing the intricate relationship between different epigenetic mechanisms.

Histone modifications

Histone modifications encompass a wide range of post-translational changes on histone tails, such as histone (de)acetylation, methylation, ubiquitination, and phosphorylation. These modifications can act separately or together to orchestrate chromatin dynamics and structure. Depending on the obtained histone code, DNA accessibility for polymerases and transcription factors can be either promoted or hampered (92).

The most prevalent type of histone modifications is (de)acetylation of the lysine (K) residues. Acetylation is established by histone acetyltransferases (HATs), whilst removal of the acetyl groups is maintained by histone deacetylases (HDACs). Histone acetylation neutralises the positive charge of the lysine residues, resulting in a weaker interaction between the histone proteins and the DNA, eventually leading to an 'open' chromatin structure. Consequently, HDACs function to make the chromatin more compact, thereby preventing transcriptional processes to occur (92, 93). Whereas not that many studies have directly assessed the role of HATs in OL development, HDACs have been shown to be heavily involved in different aspects of this process. In general, pharmacological inhibition of HDACs is associated with a decrease in OL maturation and differentiation, suggesting a crucial role of HDACs during OL development (51, 94-96). Treatment of OL *in vitro* cultures with the HDAC inhibitor trichostatin A (TSA), prevented the suppression of inhibitory transcription factors, such as ID2 and SOX11, in rats (95), and ID4, SOX2, and TCF4 in humans (96). These data indicate that HDAC-mediated repression of genes that keep OPCs in a proliferative and undifferentiated state is necessary for the early onset of OL lineage progression. Indeed, it has been shown that HDAC functionality is restricted to a specific temporal window, as HDAC inhibitors seem to only supress myelination

during the early phase of OPC differentiation, but not after onset of myelination (51). These observations are in line with recent findings, which show that HDACs are predominantly expressed in early OPC stages, compared to other stages of OL differentiation (97).

Interestingly, HDACs can also regulate and promote OL development in a (partly) histone-independent manner, as interaction of HDACs with other transcriptional regulators can result in repressive complexes that counteract the expression of OPC differentiation inhibitors. For instance, studies conducted on murine OPCs have shown that the pro-differentiation factor YY1 is recruited via HDAC1 to the promoter region of *Id2*, *Id4* and *Hes5*, where it can block the expression of these genes (98). Protein deacetylation of OLIG1 by HDACs prevents its physical interaction with the inhibitory ID2 protein, stimulates its nuclear transportation and promotes OPC differentiation (99). Furthermore, HDAC1/2 interact with TCF4 and antagonise its binding to β-catenin, thereby preventing its downstream function as an inhibitor of myelin gene expression (76).

Another type of histone modification that has been associated with OL development is histone methylation. Histone methylation can occur either on lysine or arginine side chains, and is associated with both activation and repression of transcription, depending on the site of methylation (93). During OL differentiation, the activity of the Histone H3 Lysine 9 (H3K9) methylation enzyme increases. This is accompanied by an increase of the associated repressive H3K9me3 mark at genes that regulate neuronal lineage development (100). Furthermore, the catalytic subunit (EZH2) of the polycomb repressive complex (PRC) that is responsible for trimethylation of histone 3 (H3K27me3), promotes OPC cell fate choice from progenitor cells and stimulates OPC proliferation (101, 102). A decrease in histone H4R5 methylation via pharmacological inhibition or genetic ablation of PRMT5, results in poor OL differentiation and hypomyelination (103). Likewise, deletion of PRMT1 leads to severe hypomyelination due to impaired OL maturation and disturbed myelin gene expression in OLIG2-positive cells (104).

Next to the abovementioned histone-modifying enzymes, also ATP-dependent chromatin remodeling complexes have been recently shown to influence and orchestrate OPC differentiation. These complexes make use of ATP as an energy source to reposition nucleosomes, thereby altering, histone accessibility and gene

transcription (105). The helicase component of the SWI/SNF-related chromatin remodeling complex brahma-related 1 (Brg1, also known as Smarca4) is highly expressed in OPCs and is an essential factor during OPC specification and at the onset of OL differentiation. BRG1 interacts with the *Olig2* promoter in order to regulate its expression during early development (106). As a positive feedback loop, BRG1 is consequently recruited by OLIG2 to enhance the expression of OL-associated genes (107). One of these targets of BRG1 and OLIG2 is *Cdh7*, an ATP-dependent chromatin remodeler of the chromodomain helicase DNA-binding (CHD) family. CHD7 is highly expressed in differentiating OLs, and functions synergistically with SOX10 to enhance myelin-associated gene expression. Furthermore, CHD7 promotes the expression of other positive transcription factors during OL maturations, such as *Myrf* and *Olig1* (108). Interestingly, deletion of either ATP-dependent remodeler (BRG1 or CHD7) resulted in a dysmyelinating phenotype in mice, suggesting that even though they have different targets and influence OL development at distinct stages, both BRG1 and CHD7 are indispensable factors during OL development and myelination (107, 108).

MicroRNAs

Small non-coding RNAs (ncRNAs) are powerful endogenous regulators of gene expression. Many ncRNAs have been comprehensively described, such as Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs) and miRNAs, with these latter being the most widespread and abundant ncRNAs (109). MiRNAs are small ncRNA molecules with an average length of 21-25 nucleotides and are most often transcribed from non-coding and coding protein introns (110). By means of base-pair complementarity, a mature miRNA binds the seed-sequence at the 3’ untranslated region (3’UTR) of the target mRNA and subsequently negatively regulates its translation by repressing or degrading the mRNA (111-113). Nevertheless, base-pair complementarity between miRNA and target RNA can sometimes be incomplete so that a single miRNA can target multiple 3’ UTR sequencing, leading to a cumulative reduction of gene expression that may orchestrate a common molecular pathway such as cell proliferation, development and differentiation (114).

During OL development, a coordinated interplay between multiple miRNAs determines OPC cell fate by downregulating intrinsic and extrinsic transcription

factor expression (115, 116). The importance of miRNA-mediated gene repression in OPC differentiation is highlighted in animals lacking the DICER1 enzyme which is an essential enzyme responsible for processing pre-microRNA (pre-miRNA) thereby forming mature miRNA. DICER1 mutant mice display a lack of mature miRNAs which is featured by a disrupted CNS myelination pattern due to the lack of differentiated OPCs (117, 118). MicroRNAome studies revealed a 10-100 fold induction of miR-219, miR-338 and miR-138 during OL differentiation (117, 118). Since direct targets of miR-219 include genes essential for maintaining OPC proliferation (e.g. *Sox6*, *Hes5* and *Pdgfra*), its increase stimulates OPCs to exit from the proliferative cycle and enter differentiation (117). By supressing *Hes5* and *Sox6*, miR-219 indirectly elevates the expression of monocarboxylate transporters, leading to increased OL numbers and enhanced protein levels of MBP and CNP, which subsequently attenuates cuprizone-induced demyelination (119). MiR-219 is additionally important for metabolic regulation of lipid formation and maintenance during OL maturation, rendering miR-219 essential in both early and late stages of OL differentiation (118). MiR-219 cooperates synergistically with miRNA-138, which is essential for reaching the immature phase of OL differentiation, to regulate CNS myelination. Boosting the expression of solely these two miRNAs is sufficient to induce OL differentiation *in vitro* (120, 121). Furthermore, differentiation of human endometrial-derived stromal cells towards OLs is stimulated when miR-338 is overexpressed, emphasizing the importance of this miRNA in the regulation of OPC differentiation (122, 123).

In contrast to the induction of several miRNAs, miR-9 is downregulated during OL differentiation (124, 125). In line with this, depleting miR-9 in OPCs stimulates OL differentiation, presumably through an increase in peripheral myelin protein 22 (PMP22) and serum response factor (srf) transcripts (124, 126). During OL differentiation, a comparable expression pattern of the developmentally regulated miR-125a-3p is observed. Oligodendroglial differentiation and maturation is impaired upon miR-125a-3p overexpression, which can be attributed to a decreased expression of genes involved in the differentiation process (e.g. GTPase RhoA, Neuregulin and p38) (127-130). On the contrary, antago-miR treatment that inhibits miR-125-3p expression and subsequently stimulates OL differentiation, indicates the importance of miR-125a-3p suppression during oligodendroglial maturation (127).

Many other miRNAs have been described to be either positively or negatively involved in OL differentiation processes. *In vivo* studies have shown an increased generation of myelin proteins upon miR-146a overexpression in primary OPCs following demyelinating injuries, thereby highlighting the positive relationship between miR-146a and OL differentiation (131, 132). Similarly, miR-23 promotes CNS myelination via the suppression of lamin B1, which is a negatively regulator of OL differentiation (133). On the other hand, many miRNAs inhibit OL differentiation and therefore need to be downregulated during the transition of OPCs to OLs. The translation of essential proteins of the CNS myelin, such as myelin associated oligodendrocyte basic protein (MOBP), claudin11/O4 and MBP is supressed by miR-214 (134, 135), miR-205 (134) and miR-715 (129), respectively. Moreover, miR-145 has been shown to pair to its seeding sequence located in the 3'UTR of the gene coding for Myrf and consequently inhibits OPC differentiation (135, 136). Therefore, downregulating miR-214, miR-205, miR-715 and miR-145 is sufficient for the differentiation of OPCs into mature OLs. In contrast to regulating OL differentiation, at least one miRNA cluster, miR-17-92, has been shown to be involved in OPC expansion by targeting among others PTEN, and therefore regulate OL numbers both *in vitro* and *in vivo* (121, 137). Taken together, miRNAs have been shown to be critically involved in different steps of the process of OL development. Data have demonstrated that miRNA expression is dynamically and precisely regulated to control cellular differentiation, which offers new avenues for further therapeutic target identification for myelin-related pathologies.

Implications in ageing and CNS myelin disorders

Current knowledge about the strong involvement of epigenetic mechanisms in OL development has led to new perspectives on OL- and myelin-related pathologies. Over the past years, a considerable amount of research has been conducted with regard to aberrant epigenetic regulation and its impact on OL regeneration and myelin repair. Hence, in this part of the review, we focus on what is known about epigenetic malfunctioning during OL regeneration and remyelination, both in the context of ageing and myelin-related pathologies.

Ageing

It is generally known that regenerative processes become less efficient with increasing age. A classic example is age-related deficits in remyelination, a process which is entirely dependent on OL regeneration to restore the myelin sheath (138-140). The age-associated decrease in remyelination efficiency is attributed to a reduced level of OPC recruitment. Moreover, recruited OPCs show an impaired ability to differentiate into remyelinating OLs (139). The relationship between ageing and epigenetic alterations has already been proposed before (141-143) and provides an incentive to link age-associated remyelination failure to changes in the epigenome of aged OPCs or OLs.

Up to now, only one study has connected changes in methylation in OPCs/OLs to cellular ageing (144). Rat OPCs from the spinal cord showed an age-dependent decrease in methylation levels. Interestingly, no changes regarding TET activity or expression were observed. The global hypomethylation in aged OPCs rather correlated with a reduced expression and activity of DNMTs, and in particular DNMT1 (144). Regarding histone modifications, mature OLs from the corpus callosum of older animals show increased levels of histone acetylation and a decreased rate of histone methylation, compared to younger mice. These histone changes were correlated with re-expression of inhibitory HLH-transcription factors, such as HES5 and ID4 (145). As mentioned before, HDAC recruitment to these promoter regions is crucial for OPC differentiation and myelin formation. OPCs in demyelinated regions of older mice, however, fail in the recruitment of HDACs, resulting in the accumulation of transcriptional inhibitors and poor remyelination (52).

In a study conducted by Pusic *et al.*, aged rats were exposed to a youthful environment in a Marlau-style enrichment cage to assess the effect on remyelination capacity (146, 147). Environmental enrichment promoted remyelination in aged rats, to a level comparable to younger animals. Interestingly, they found that serum-derived exosomes from both young and environmentally enriched stimulated rats displayed increased levels of miR-219, which is known to inhibit the expression of inhibitory myelin gene regulators and therefore promotes OL differentiation (146). Exosomal delivery of such miRNAs could therefore be regarded as a potential therapeutic strategy to boost remyelination both in young and aged individuals.

Multiple sclerosis

Multiple sclerosis (MS) is a multi-faceted immune-driven demyelinating disease of the CNS. MS is characterized by inflammation-induced demyelination during the early stages, which eventually results in gradual neurological disability as the disease progresses (1, 2). The concordance rate of identical twins to develop MS averages between 6-30%, suggesting that the disease is only partially driven by genetic polymorphisms, but is largely attributed to environmental stimuli (148). Increasing body of evidence suggests a role of epigenetically regulated mechanisms in the pathophysiology of MS. Numerous links have been made between environmental risk factors for MS and epigenetic changes (46-48). Yet, most studies concerning epigenetics in MS are focused on the early, inflammatory stage of the disease (43-45). Another important aspect of the disease is the subsequent endogenous repair process underlying remyelination of axons in order to cope with inflammatory damage. In the chronic stages of MS, however, these repair processes are hampered due to a differentiation block in OPCs (20, 149). New regenerative therapies, such as Opicinimab (anti-LINGO), are currently tested for their potential to boost remyelination in lesions that still contain undifferentiated OPCs (150). Interestingly, even though the influence of epigenetics in progressive MS pathology is not clear yet, emerging data suggest an existing role in OL differentiation and maturation.

Analysis of MS post-mortem samples revealed increased levels of MBP citrullination, a post-translational modification which renders the MBP protein less stable, leads to the degradation of myelin and can eventually result in the development of an auto-immune response against myelin (151, 152). MBP citrullination is carried out by the peptidyl arginine deiminase type-2 (PAD2) enzyme. Interestingly, the promoter region of the *PAD2* gene is hypomethylated in normal appearing white matter (NAWM) of MS patients, compared to control samples (152). This implies that *PAD2* hypomethylation leads to a higher expression of the enzyme, which finally results in the destabilisation and degradation of the myelin sheath in MS white matter. *PAD2* hypomethylation is, surprisingly, not brain-specific but can also be observed in peripheral blood mononuclear cells (PBMCs) of MS patients (153). In a similar fashion, cell-free DNA (cfDNA) in peripheral blood samples of MS patients with an active disease course showed hypomethylated patterns of the *MOG* gene, which is associated

with OL cell death and demyelinating events in the brain (154). The correlation of methylation patterns between the brain and blood has gained interest over the past years for its potential application as a biomarker for neurodegenerative diseases (155-157), and could therefore also be used to monitor disease progression in MS.

An epigenome-wide DNA methylation study (EWAS) was conducted on MS NAWM post-mortem samples. Genes responsible for OL survival (*BCL2L2*, *NDRG1*) and myelination (*MBP, SOX8*) were hypermethylated and decreased in expression in MS affected tissue, compared to controls (53). While representing a valuable study, it is important to note that no distinction has been made between regular cytosine methylation and 5-hydroxymethylation (5hmC). Considering the functional consequences of 5hmC, but also to prevent underrepresentation of methylated cytosine values, 5hmC analysis should be taken along in CNS EWAS studies.

Another study that analysed post-mortem brain tissue of MS patients showed higher levels of histone acetylation in oligodendrocytes within chronic MS lesions, compared to non-neurological controls. These changes are associated with elevated HAT transcript levels and higher expression of inhibitory regulators (*TCF7L2, ID2, SOX2*). In contrast, OLs present in early MS lesions show the presence of deacetylated histones (158). Since histone acetylation impairs OL differentiation and remyelination, these data could partially explain the poor remyelination capacity associated with progressive MS patients.

MiRNA analysis of brain samples of progressive MS patients showed upregulated levels of different miRNAs (miR-155, miR-338, miR-491), which target enzymes that are involved in the production of neurosteroids (159). Opposing results were obtained from another study, in which they show that these miRNAs are downregulated in chronic, inactive MS lesions, compared to control white matter samples (160). The discrepancy between these studies could be attributed to differences in the analysed tissue, their control sample selection or the method of miRNA anal+ysis, which makes it difficult to directly compare them to each other. Interestingly, the most significant downregulated hit from the latter study is miR-219, which, together with miR-338, is essential for OPC cell cycle exit and differentiation into myelin-producing OLs (117, 120, 123). The absence of these miRNAs could thus underlie the differentiation block of OPCs in chronic

demyelinated lesion of progressive MS patients. Moreover, miR-219 expression is also decreased in the cerebrospinal fluid (CSF) of MS patients, rendering it a possible biomarker for MS diagnosis (161).

It is however noteworthy that most of the abovementioned studies have been conducted on bulk tissue, leading to a possible noise introduced by the cellular heterogeneity. Since the observed epigenetic changes could be strongly influenced by cellular variation or cell numbers, cell type specific validation is recommended to circumvent such bias (162, 163).

Other diseases with myelopathy

Even though MS is regarded as the most common myelopathy of the CNS, many other neurological diseases are characterised by oligodendroglial injury and myelin disruption. Here, we briefly discuss how epigenetic changes impact OL regeneration and remyelination in relation to these other demyelinating diseases.

Ischemic stroke, caused by a cerebral artery occlusion, is an important cause of death worldwide and the majority of survivors often struggle from severe neurological disabilities throughout the lifespan. Molecularly, ischemic stroke can be characterized by a disrupted architecture of neuronal synapses, neuronal loss and loss of glial cells, including oligodendrocytes, leading to prominent white matter demyelination (164). During stroke recovery, endogenous repair processes are initiated and include axonal growth, synaptic plasticity, angiogenesis, neurogenesis, and oligodendrogenesis. Interestingly, during early brain recovery following ischemic stroke, HDAC1 and HDAC2 levels were shown to be increased in white matter OPCs at the peri-infarct region (165, 166). Mature OLs showed a retained increase of HDAC2 following stroke, while HDAC1 levels were decreased, indicating that individual HDACs family members play distinct roles during recovery after stroke (165). In line, pan-HDAC inhibitors have repeatedly shown to protect OLs from ischemia-induced cell death and subsequently increase oligodendrogenesis (167-169). However, contradictory results have been observed for the pan HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) as its treatment supressed OPC survival, leading to detrimental effects for the myelinating brain during stroke recovery (170). Interestingly, not only HDAC modifications have shown their importance during oligodendrogenesis following stroke, but also miRNAs have been widely investigated for their therapeutic and

diagnostic properties (171). In ischemic white matter regions, miR-9 and miR-200b levels were decreased, concomitant with an increased differentiation state of OL lineage cells (126, 172). However, the majority of the investigated miRNAs showed an increased expression pattern following stroke. For example, rodent models for ischemic stroke showed a high presence of miR-146a, miR-138, miR-338, miR-423-5p, miR-200b, miR-298, miR-205, miR-107, and miR-145 (131, 173-175), all of which have a negative impact on OPC proliferation, which is actually necessary in the early phase after stroke injury to replenish the pool of lost OPCs. Interestingly, circulating miRNA levels have been measured in stroke patients to provide new therapeutic and minimally invasive diagnostic insights. Measuring miR-146a levels, for example, can segregate the acute phase from the subacute phase during ischemic stroke, thereby highlighting the usefulness of miRNAs for future stroke research (176).

X-linked adrenoleukodystrophy (X-ALD) is a genetic disorder caused by a mutation in the *ABCD1* gene and characterised by progressive demyelination of the CNS (177). An important aspect of this disease is the absence of remyelination capacities, even after successful hematopoietic stem cell transplantation (178). X-ALD patients endure progressive impairment of cognition, vision, hearing and motoric function, eventually leading to total disability (179). An EWAS, conducted on white matter samples of the prefrontal cortex of X-ALD patients, revealed differential DNA methylation in genes involved in OL differentiation. Myelin genes, such as *MBP, PLP1, MOG* and *CNP* were hypermethylated in X-ALD patients compared to age-matched controls. Furthermore, transcriptional inhibitors (*ID4* and *SOX2*) displayed an increased expression in these patients, suggesting a disturbed HDAC activity (178). In line with this, treatment with SAHA prevented OL cell loss both *in vitro* and *in vivo* by counteracting the very long chain fatty acid (VLCFA) derangement associated with X-ALD pathology (180). Another type of leukodystrophy, adult-onset autosomal dominant leukodystrophy (ADLD) is characterised by duplication of the gene that codes for lamin B1 (*LMNB1*), which leads to overexpression of LMNB1 and causes severe myelin loss (181). Interestingly, miR-23 has been identified as a negative regulator of lamin B by targeting its transcript levels, and could therefore be considered as a therapeutic strategy for ADLD (182).

Schizophrenia has also been associated with OL dysfunction. Interestingly, the CpG island within the promoter region of *SOX10* is hypermethylated in brains of patients with schizophrenia, which is directly associated with a decreased expression of *SOX10* and other OL-related genes (183).

Therapeutic perspectives: from pharmaceuticals to (epi)gene therapy to iPSCs

It is clear that epigenetic modifications strongly influence OL development and functional remyelination in a wide variety of diseases. Targeting these epigenetic alterations could therefore be considered as a new therapeutic strategy to overcome remyelination failure. Most attempts to pharmacologically manipulate epigenetic modulations are based on the use of inhibitors of epigenetic enzymes, such as 5-aza, TSA and valproic acid (VPA) (184, 185). However, such pan-epigenetic inhibitors are non-specific due to their pleiotropic impact at a genome-wide level. Furthermore, these compounds are known to have low chemical stability and are cytotoxic at higher doses, which limits their potency to be used in a cellular microenvironment (186, 187). Recent improvements in the field of epigenetic editing has disclosed the use of DNA-binding proteins, such as zinc-finger proteins (ZFPs), transcription activator-like effectors (TALEs) and type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9, as new synthetic epigenomic engineering tools (188-191). These DNA-binding proteins are linked to epigenetic modifiers and serve to guide them to a specific region in the genome, thereby altering the epigenome at specific loci. Even though many advances have been made regarding these new epigenetic editing techniques, their applicability in the clinic may require, next to ethical considerations, additional research as their safety and efficacy remains to be disclosed. In particular, the off-target effects and undesired genomic binding of these DNA-binding proteins are still considered as one of the major hurdles for their therapeutical application (192).

Autologous cell-based therapies have emerged as a promising technique to restore OL dysfunction. Mature and fully differentiated OLs derived from induced pluripotent stem cells (iPSCs) have shown to successfully remyelinate axons in rodents (193). Interestingly, human iPSC-derived OPCs show the same epigenetic

signature during their differentiation process into mature OLs as seen in normal OL development (194). Furthermore, generation of oligodendrocytes from progressive MS patient-derived iPSCs results in functional and myelinating cells, in contrast to the resident non-myelinating OPCs in the CNS (195). Since the epigenetic signature of OPCs/OLs can be disturbed in a pathological context, reprogramming patient-derived iPSCs into OLs and repopulating lesion sites with these cells could be considered as a promising remyelinating strategy.

Concluding remarks

In this review, we have discussed how different epigenetic modifications influence OL development and lineage progression and how this is dysregulated in demyelinating conditions. Epigenetic mechanisms function as a precise gateway control system that governs the transcriptional machinery in a spatiotemporal manner. In CNS demyelinating diseases, these epigenetic mechanisms are found to be altered, concomitant with increased levels of transcriptional inhibitors and resulting in a differentiation block of OPCs. Targeting these epigenetic processes, either by pan-inhibitors or via CRISPR-Cas9 mediated epigenetic editing, could therefore be a potential strategy to boost OL differentiation and (re)myelination. Taken together, epigenetic research has earned its place within the universe of OL development and further studies will contribute to the complete understanding of CNS myelin disorders.

CHAPTER 3

DNA methylation regulates the expression of the negative transcriptional regulators ID2 and ID4 during OPC differentiation

Based on:

DNA methylation regulates the expression of the negative transcriptional regulators ID2 and ID4 during OPC differentiation

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A.T. contributed to the experimental design, data generation, interpretation, analysis, and manuscript writing.

Abstract

The differentiation of oligodendrocyte precursor cells (OPCs) into myelinating oligodendrocytes is the prerequisite for remyelination in demyelinated disorders such as multiple sclerosis (MS). Epigenetic mechanisms, such as DNA methylation, have been suggested to control the intricate network of transcription factors involved in OPC differentiation. Yet, the exact mechanism remains undisclosed. Here, we are the first to identify the DNA-binding protein inhibitors, *Id2* and *Id4,* as targets of DNA methylation during OPC differentiation. By using state-of-the-art epigenetic editing via CRISPR/dCas9-DNMT3a, we confirm that targeted methylation of *Id2/Id4* drives OPC differentiation. Moreover, we show that in the pathological context of MS, methylation and gene expression levels of both *ID2* and *ID4* are altered compared to control human brain samples. We conclude that DNA methylation is crucial to suppress *ID2* and *ID4* during OPC differentiation, a process that appears to be dysregulated during MS. Our data does not only reveal new insights into oligodendrocyte biology, but could also lead to a better understanding of CNS myelin disorders.

Introduction

Oligodendrocytes are derived from a pool of proliferating oligodendrocyte precursor cells (OPCs) that exit the cell cycle and differentiate into mature oligodendrocytes, the myelinating cells of the central nervous system (CNS) (54, 57). This differentiation process is a prerequisite for myelin formation and is strictly coordinated by a complex interplay between extracellular signals, intracellular transcription factors, and epigenetic mechanisms (58, 196). Myelin genes are defined as genes that code for essential proteins of the myelin sheath, such as myelin basic protein (*MBP*), proteolipid protein (*PLP*), myelin oligodendrocyte glycoprotein (*MOG*) and myelin-associated glycoprotein (*MAG*). The expression of these myelin genes during OPC differentiation is regulated by an upstream transcriptional network. This myelin regulatory network is composed of positive regulators, which promote myelin gene expression, and negative regulators, which repress the expression of myelin genes and OPC differentiation, as reviewed more in detail by Tiane *et al.* (23, 85). As such, tight control of both positive and negative upstream regulators is required to orchestrate OPC differentiation during myelin formation.

In many neurodegenerative diseases, such as multiple sclerosis (MS), damaging insults result in demyelination of certain axons, leaving the affected neurons dysfunctional and vulnerable to atrophy (197). Endogenous remyelination is therefore crucial to restore the myelin sheath and prevent further neurodegeneration (20). However, for reasons still not entirely elucidated, these remyelination mechanisms become insufficient as the disease progresses toward the chronic stage of MS, or with age in general (139, 198). Evidence suggests that a significant part of this remyelination failure can be attributed to an impaired OPC differentiation capacity (139). As such, OPCs are present within chronically demyelinated non-fibrotic MS lesions of MS patients, yet they seem to be unable to differentiate into myelinating oligodendrocytes (199, 200). This differentiation and remyelination block is not observed in the early stages of MS, which suggests that changes in the micro-environment, such as accumulated lesion damage due to chronic inflammation, could influence OPC functioning (201-203). Thus, to further comprehend the exact mechanisms causing remyelination failure, we should first gain a better understanding of the biology behind OPC differentiation.

Over recent years, focus has shifted towards the specific involvement of epigenetic mechanisms underlying OPC differentiation and (re)myelination. For example, a large body of evidence has shown that histone modifications are essential to regulate the transcriptional control of myelin genes during OPC differentiation (23, 96, 97, 204). Emerging data also suggest that other epigenetic mechanisms, such as DNA methylation, strongly influence OPC cell fate commitment and (re)myelination. For instance, research has proven the DNA methyltransferase 1 (DNMT1) enzyme to be essential during developmental myelination, while DNMT3a plays a dominant role in adult remyelination after injury (49, 89). Moreover, ten-eleven translocation (TET) enzymes, responsible for DNA hydroxymethylation and DNA demethylation, have shown to be differentially regulated during oligodendrocyte development and remyelination (50, 205). These insights have shed new light on the process of oligodendrocyte development and might unravel new promising strategies to boost OPC differentiation. Nevertheless, which genes are actually targeted by the DNA methylation enzymes during OPC differentiation remains undisclosed.

Accordingly, we hypothesized that the upstream transcriptional regulators of myelin gene expression are themselves regulated by DNA methylation during OPC differentiation. In this study, we show that inhibition of DNA methylation in primary OPCs, by means of incorporation of 5-azacytidine (5-AZA), leads to a decreased OPC differentiation rate, accompanied by an increased expression of *Id2* and *Id4,* two negative transcriptional regulators of myelin genes. Furthermore, we observed that both the *Id2* and *Id4* promotors are hypermethylated during OPC differentiation, which is, in turn, negatively correlated with their gene expression levels. Moreover, CRISPR-dCas9-DNMT3a based targeted methylation of the promoter region of either *Id2* or *Id4* successfully inhibited their expression and boosted OPC differentiation and myelin gene expression. Interestingly, the promoter region of both genes was shown to be hypomethylated in chronically demyelinated inactive lesions of MS patients. To our knowledge, this is the first study that establishes the intricate relationship between DNA methylation of *Id2* and *Id4* and OPC differentiation.

Materials and methods

Primary OPC cultures

All *in vitro* mouse experiments were approved by the Hasselt University Ethics Committee for Animal Experiments. Primary mouse OPCs were obtained from mixed glial cultures, using the standard shake-off method (70). In brief, cortices were isolated from postnatal day 0 mice and cells were enzymatically dissociated by incubation with papain (3U/ml, diluted in Dulbecco's Modified Eagle Medium (DMEM) containing 1 mM L-cystein; Sigma-Aldrich, Bornem, Belgium) for 20 minutes. Mixed glial cells were maintained in DMEM (Sigma-Aldrich), supplemented with 50U/ml penicillin and 50 mg/ml streptomycin (P/S; Invitrogen, Merelbeke, Belgium) and 10% heat-inactivated fetal calf serum (FCS; Hyclone, Erebodegem, Belgium) on poly-L-lysine-coated (5 µg/ml, Sigma-Aldrich) culture flasks. Cells were kept at 37°C in a humidified atmosphere of 8.5% CO2. From the seventh day, cells were maintained in culture medium, supplemented with bovine insulin (5 µg/ml; Sigma-Aldrich) to stimulate OPC formation within the mixed glial cultures. On day 14, the cells were shaken using an orbital shaker at 75 rpm and 37°C for 45 minutes to detach the microglial layer. A second shake-off was performed for 16 hours at 250 rpm, after which the OPC-enriched supernatant was collected, incubated for 20 minutes on a petridish and centrifuged on 300xg for five minutes. All cell cultures had a purity above 95%. OPCs were seeded onto 24-well plates and maintained in DMEM medium (+10 % FCS and 1% P/S) or differentiation medium (DMEM medium, supplemented with 0.5% P/S, 2% horse serum, 0.3 mM transferrin, 0.1 mM putrescin, 0.02 mM progesterone, 0.2 µM sodium selenite, 0.5 µM triiodothyronin, 0.8 mM bovine insulin, 0.5 mM L-thyroxine, 2% B27 supplement; all from Sigma-Aldrich except for P/S, Invitrogen, and B27, in house production as described by Chen *et al*. (206)), depending on the experiment.

5-AZA treatment

Primary OPC cultures were kept in a proliferating state by addition of 5 ng/µl platelet derived growth factor α (PDGFα; Peprotech, Rocky Hill, USA) to the DMEM culture medium, and were treated for three consecutive days with 1 µM 5-AZA (Sigma-Aldrich) or DMSO (Sigma-Aldrich) as a vehicle control. After three or six days of rest in differentiation medium, OPCs were either lysated for RNA isolation

or fixed on coverslips to assess their morphology and protein expression via immunofluorescence.

Transfection

The pdCas9-DNMT3A-PuroR plasmid was a gift from Vlatka Zoldoš (Addgene plasmid #71667). The catalytically inactive pdCas9-DNMT3A-PuroR vector (Addgene plasmid #71684) was taken along as a negative control. Plasmids were transfected into primary OPCs 24 h after seeding, using the OZ Biosciences NeuroMag Transfection Reagent (Bio-connect, Huissen, The Netherlands), following the manufacturer's instructions. In brief, 500 ng of plasmid DNA was diluted in 50 µl DMEM medium, added to 1.75 µl NeuroMag reagent and incubated for 20 minutes on room temperature. DNA/NeuroMag complexes were dropwise added to primary OPC cultures (200 000 cells/well), maintained in P/S free differentiation medium, and placed on a magnetic plate for 30 minutes in an 8.5% CO_2 incubator. Two days after transfection, transfected cells were selected for 72 hours with 5 µg/ml puromycin (Invivogen, Toulouse, France), a dose-optimized concentration of puromycin with 100% mortality in non-transfected cells. OPCs were then kept in standard differentiation medium until further experiments.

CRISPR-dCas9-DNMT3a plasmids

Design guide RNA

The promoter regions of *Id2* and *Id4* were exported from the Ensembl database and were scanned for CpG islands using the default CpG islands track in the UCSC Genome Browser. Specific guide RNAs (sgRNAs) were designed to induce methylation within the promoter region of the *Id2* (chr12:25.097.141-25.097.740) and *Id4* (chr13:48.260.628-48.261.228) genes using Benchling software®. For each gene, the guide with the lowest off-target prediction was used (Supplementary Table S3.1). Guides were synthesized as oligo's with overhangs to fit into the BbsI restriction gap and an additional guanine for increased transcriptional efficiency.

sgRNA cloning and transformation

Plasmid DNA (1µg; Addgene plasmids #71667 and #71684) was incubated overnight on 37°C with 40U BbsI restriction enzyme (Bioké, Leiden, The

Netherlands). Enzyme inactivation was performed by incubation on 65°C for 20 minutes, after which the samples were immediately loaded on an agarose gel (1%). The open vector was extracted from the gel, using the PCR and gel clean-up kit (Macherey-nagel, Düren, Germany), according to the manufacturer's instructions. Annealed sgRNAs were ligated with the T4 DNA Ligase buffer and enzyme system (Bioké) into the linearized vector in a 5:1 insert to vector molar ratio. The ligated product was then transformed into NEB® 5-alpha Competent E. coli cells (Bioké) and plated out on LB-agar plates, supplemented with ampicillin (Amp; 100 mg/ml). Suitable colonies were propagated overnight in LB-Amp medium. Plasmids were extracted using the NucleoBond® Xtra Midi kit, according to the manufacturer's protocol (Macherey-Nagel). SANGER sequencing was carried out on purified plasmid vector to validate the sgRNA incorporation.

Immunostaining

Immunocytochemistry

Primary OPCs were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature at day six post 5-AZA treatment or day nine post transfection. Aspecific binding was blocked for 30 minutes with 1% bovine serum albumin (BSA) in 0.1% PBS-T, followed by incubation with primary antibodies (Supplementary Table S3.2) for four hours at room temperature. After three washing steps with PBS, cells were incubated with Alexa 488- or Alexa 555-conjugated secondary antibody (Supplementary Table S3.2) for one hour. Nuclei were counterstained with 4'6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Coverslips were mounted with Dako mounting medium (Dako, Carpinteria, USA) and analyzed using a fluorescence microscope (Leica DM2000 LED). Images were quantified using Fiji, ImageJ software (3 pictures per coverslip). The percentage positive for MBP or O4 was quantified and divided by the percentage positive for DAPI, to correct for cell numbers. Process length was determined by measuring the longest process per cell in pixels.

Immunohistochemistry

Human post-mortem brain tissue was obtained through the Netherlands Brain Bank (www.brainbank.nl) (demographic characteristics described in Table 3.1). MS lesion sections were characterized for demyelination, inflammation, and presence of OPCs by immunohistochemistry. Sections were fixed in ice-cold

aceton for 10 minutes and blocked for 30 minutes with the Dako Protein Block (Dako) at room temperature. Primary antibodies (Supplementary Table S3.2) were added for overnight incubation at 4°C. After repeated washing steps, sections were incubated with horseradish peroxidase (HRP)-conjugated EnVision+ Dual Link System (Dako) for 30 minutes. Unbound antibodies were washed away with PBS and sections were incubated with the DAKO 3,3'-diaminobenzidine (DAB) solution (Dako) for color development. Nuclei were counterstained with haematoxylin for 2 minutes. Following extensive washing in tap water, sections were dehydrated in increasing concentrations of ethanol (70%, 80%, 95%, and 100%) and xylene. Oil Red O (ORO) staining was used to stain for lipid containing phagocytes within MS lesions. Brain sections were stained in 0.3% ORO (Sigma-Aldrich) for 10 minutes, and counterstained with haematoxylin for 1 minute. The stained tissues were mounted with DPX Mountant (Leica Microsystems, Wetzelar, Germany) and visualized with a Leica DM2000 LED Microscope equipped with a Leica MC170 HD Camera (Leica Microsystems).

Quantitative PCR

Total RNA was isolated from cells or brain tissue, using the RNeasy mini kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's instructions. RNA concentration and quality were analyzed with a Nanodrop spectrophotometer (Isogen Life Science, Leiden, The Netherlands). RNA was reverse-transcribed using the qScript cDNA Supermix kit (Quanta, Leuven, Belgium). qPCR was performed to analyze gene expression, using the Applied Biosystems QuantStudio 3 Real-Time PCR System (Life Technologies, Gent, Belgium). The reaction mixture consisted of SYBR Green master mix (Life Technologies), 10 µM forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium), nuclease-free water and cDNA template (12.5 ng), up to a total reaction volume of 10 µl. The primer pairs used for amplification are listed in Supplementary Table S3.3. Results were analyzed by the comparative Ct method and were normalized to the most stable housekeeping genes (*Rpl13a/ Yhwaz* for murine OPCs and *YWHAZ/TBP* for human brain samples), determined by GeNorm.

Genomic DNA isolation and pyrosequencing

Genomic DNA was extracted from transfected OPCs and bisulfite-converted, using the Zymo Research EZ DNA Methylation-Direct Kit (BaseClear Lab Products, Leiden, The Netherlands). For human brain samples, genomic DNA was extracted using a standard chloroform-phenol extraction and ethanol-precipitation method. Human genomic DNA purity was assessed by measuring the A260/A280 ratio using a NanoDrop (Isogen Life Science). A total of 500 ng human genomic DNA was subsequently bisulfite-converted using the EZ DNA Methylation-Direct Kit (Zymo Research). PCR primers were designed using the PyroMark® Assay Design 2.0 software (Qiagen, Supplementary Table S3.4). Product amplification was performed using the following reaction mixture: 1X Buffer with 20 mM MgCl2 (Roche, Bornem, Belgium), 10 mM dNTP mix (Roche), 5 µM forward and reverse primers (Metabion AG, Planegg/Steinkirchen, Germany), 1U FastStart Taq DNA Polymerase (Roche), bisulfite-converted DNA and nuclease-free water to a total volume of 25 µl. PCR cycling was performed as follows: initial denaturation for 5 min at 95°C, 50 cycles of 30 s at 95°C, 30 s at 56°C (mouse *Id2*), 60°C (mouse *Id4*) or 58°C (human *ID2* and *ID4*) and 1 min at 72°C; final extension for 7 min at 72°C. PCR amplicons were sequenced using the Pyromark™ Q48 instrument (Qiagen) with the PyroMark Q48 Advanced CpG Reagents (Qiagen), according to the manufacturer's protocol and quantified with the Pyromark™ Q48 Autoprep software. The human assays for *ID2* and *ID4* were tested for their sensitivity using the EpiTect PCR Control DNA Set (Qiagen). Mouse DNA was manually demethylated by two subsequent whole genome amplification steps using the illustra GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare BioScience, Uppsala, Sweden). After the first (10 µl volume) and the second elution (20 µl volume), the DNA was purified with the DNA Clean & ConcentratorTM-5 Kit (Zymo Research) according to the manufacturer's instructions. A fully methylated "100 % Universal Methylated Mouse DNA Standard" (Zymo Research) was commercially acquired. The mouse assays for Id2 and Id4 were tested for their sensitivity using the aforementioned standards.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0.0 software (GraphPad software Inc., CA, USA). Differences between group means were determined using an unpaired t-test for normally distributed data and a Mann-Whitney test for not normally distributed data. Differences within samples were assessed using one-sample t-test for normally distributed data or Wilcoxon test for not normally distributed data. Correlation analysis was performed by the Spearman's rank correlation test. Differences in methylation at different CpG sites were determined using a two-way repeated measures ANOVA with Šídák's multiple comparisons test. All data are depicted as mean ±SEM, *=$p \leq 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$.

Results

Inhibition of DNA-methylation prevents OPC differentiation and is associated with an increased expression of the negative regulators *Id2* and *Id4*

We pharmacologically treated primary mouse-derived OPCs with 1µM 5-AZA (Supplementary Figure S3.1), a cytidine analogue that prevents DNA methylation transfer during cell divisions, to assess its effect on subsequent cellular differentiation. Immunocytochemical analysis with O4 antibody (a marker for pre-mature oligodendrocytes) and a myelin basic protein (MBP) antibody (a marker for differentiated oligodendrocytes) demonstrated a decreased rate of OPC differentiation six days after 5-AZA treatment, compared to vehicle-treated OPCs (Fig. 3.1A-B). Morphological assessment of the cells showed that 5-AZA-treated OPCs mainly retained a simple morphology, while vehicle-treated OPCs differentiated into oligodendrocytes with longer process extensions (Fig. 3.1C). In line with this, gene expression analysis confirmed a reduced expression of myelin genes at the same time point (Fig. 3.1D). Subsequently, we aimed to address whether the myelin transcriptional regulatory network is already affected at an early stage of inhibition of DNA methylation. To this end, the gene expression of myelin regulatory pathway was measured three days after 5-AZA or vehicle control treatment (Fig. 3.1E-F). Interestingly, while the expression of most positive regulators was unaltered at this stage, the negative regulators *Id2* and *Id4* showed an increased expression upon treatment with the DNA methylation inhibitor (Fig. 3.1F).

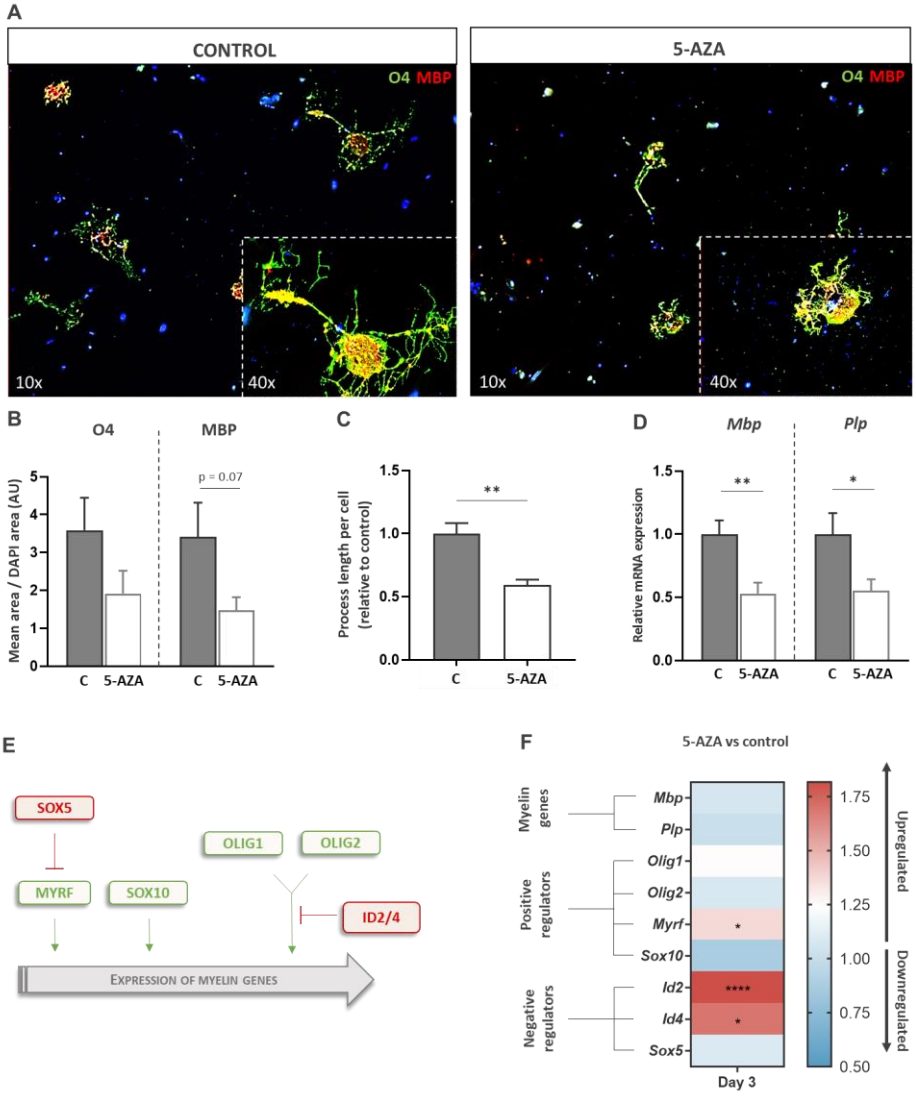


Figure 3.1 – 5-AZA inhibits OPC differentiation and is associated with an increased expression of *Id2* and *Id4*. A-C Representative images and quantification (fluorescence area and process length) of primary OPC cultures treated with 1 µM 5-AZA or DMSO as a vehicle control. **D-F** mRNA expression analysis of myelin genes and the upstream regulatory transcriptional network of 5-AZA-treated primary OPCs, compared to control-treated cells 6 days (D) or 3 days (F) post-treatment. Data are corrected for the most stable housekeeping genes (*Rpl13a* and *Cypa*) and are represented as mean + SEM, n=5, unpaired t-test, *p < 0.05, **p < 0.01, and ****p < 0.0001. 5-AZA = 5-azacytidin, O4 = oligodendrocyte surface marker claudin-11, MBP/*Mbp* = myelin basic protein, *Plp* = myelin proteolipid protein, *Olig1/2* = oligodendrocyte transcription factor 1/2, *Sox10/5* = SRY-related HMG-box protein 10/5, *Id2/4* = inhibitor of DNA-binding protein 2/4.

Id2/*Id4* promoter methylation is negatively correlated with its gene expression levels during OPC differentiation

As we observed an increased expression of the helix-loop-helix (HLH) inhibitory transcription factors *Id2* and *Id4* upon DNA methylation inhibition, we next investigated their expression and methylation profile at the different stages of *in vitro* OPC differentiation. The expression of both *Id2* and *Id4* decreased significantly during the differentiation of murine OPCs into mature oligodendrocytes (Fig. 3.2A). Interestingly, average methylation within the promoter region of *Id2*/*Id4* was increased in mature oligodendrocytes compared to OPCs (Fig. 3.2B). Furthermore, the expression and methylation levels showed a strong negative correlation, suggesting that DNA methylation is necessary for the transcriptional regulation of both genes during OPC differentiation (Fig. 3.2C).

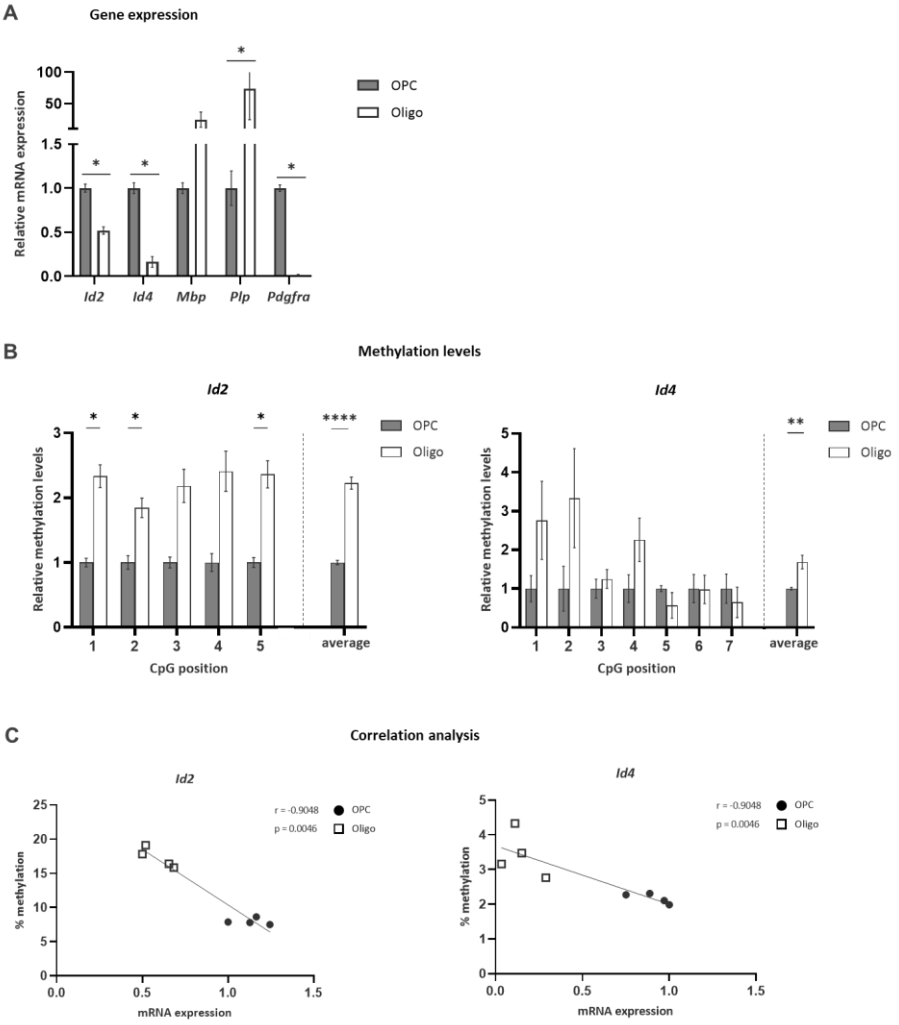


Figure 3.2 – Decrease in *Id2/Id4* expression from OPC to oligodendrocyte stages is associated with an increased methylation profile. A Gene expression analysis in primary murine OPCs (day 0) and differentiated oligodendrocytes (day 12) of *Id2/4* and OPC (*Pdgfra*) and oligo (*Mbp, Plp*) markers. Data are corrected for the most stable housekeeping genes (*Pgk-1* and *Cypa*), n=4, Mann-Whitney test. **B** Methylation analysis of the promoter region of both *Id2* and *Id4*, measured in cells from the same batches (n=4, two-way repeated measures ANOVA with Šídák's multiple comparisons test). **C** Spearman's correlation analysis between expression (A) and methylation (B) levels (n=8). Data are represented as mean + SEM, **=p<0.01, ****=p<0.0001. Oligo = oligodendrocytes

Targeted hypermethylation of *Id2*/*Id4* using CRISPR-dCas9-DNTM3A decreases their expression and stimulates OPC differentiation

To validate the causal relationship between *Id2*/*Id4* methylation and OPC differentiation, we made use of recently developed CRISPR-pdCas9-DNMT3a epigenetic editing plasmids (207). Guide RNAs were designed to target specific CpG-rich regions within the promoter of *Id2* or *Id4* (Fig. 3.3A). In this way, the inactivated Cas9 protein (dCas9), which was attached to the catalytic domain of DNMT3a, was guided to the promoter region of our target genes, inducing methylation at the associated CpG sites. At day six post-transfection, primary OPCs transfected with the CRISPR-pdCas9-DNMT3a constructs showed an overall increase in methylation of the promoter of *Id2* ($p=0.008$) and an increased trend in methylation of the promoter of *Id4 (*$p=0.06$) compared to cells transfected with the catalytically inactive DNMT3a construct targeted to the same sites (Fig. 3.3B). Furthermore, reduced expression levels of the target genes were observed in cells transfected with the active DNMT3a vector compared to inactive constructs (Fig. 3.3C). This pattern was not observed for predicted off-target genes of both guide RNAs (Supplementary Figure S3.2).

We further assessed the impact of our epigenetic editing approach on oligodendrocyte differentiation by evaluating the cellular morphology of transfected cells. Immunostaining for MBP on day nine post-transfection showed an increased immunoreactive area in cells transfected with the active CRISPR-pdCas9-DNMT3a construct targeting either *Id2* or *Id4* (Fig. 3.3D). Quantification of the average process length per cell also revealed longer processes compared to the inactive controls (Fig. 3.3D). Finally, gene expression analysis at the same time point (nine days post-transfection) showed a consistent increase in myelin gene (*Mbp, Mag, Mobp*) expression (Fig. 3.3E).

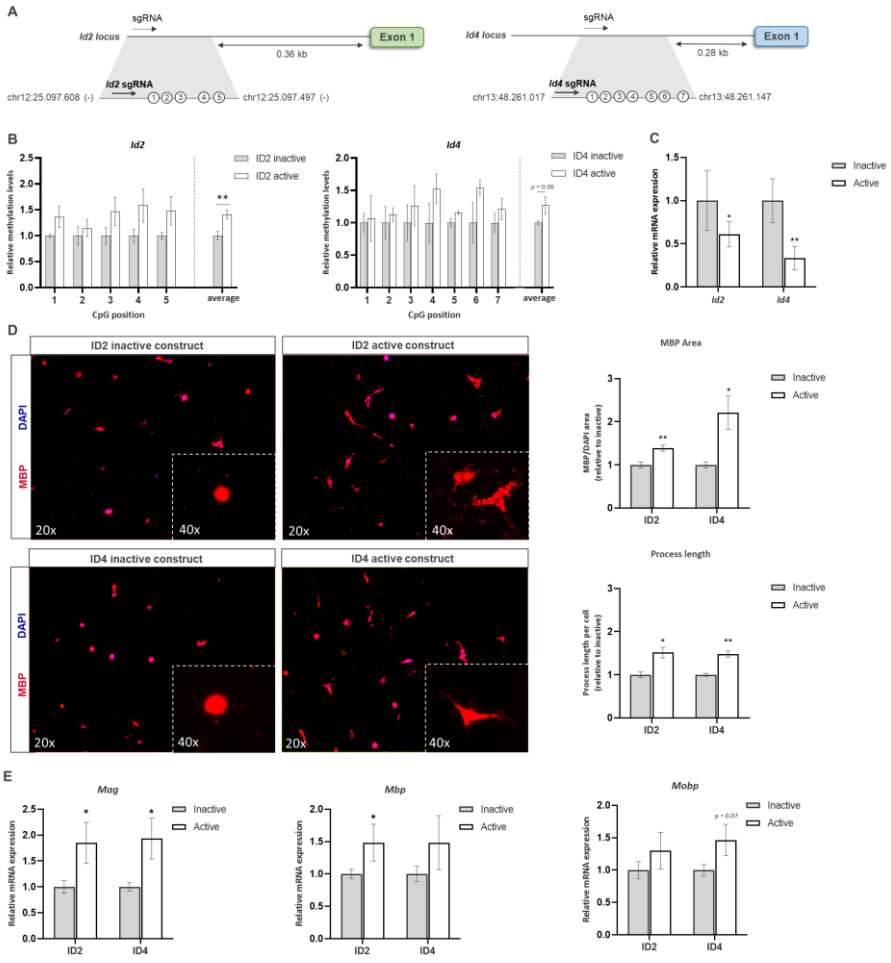


Figure 3.3 – Targeted methylation of *Id2*/*Id4* with CRISPR-dCas9 results in lower gene expression and boosts OPC differentiation. A. Primary OPCs were transfected with a CRISPR-pdCas9-DNMT3a vector targeting the *Id2* or *Id4* promoter region. Numbers reflect the number of CpG sites measured for methylation. **B.** Methylation analysis confirms targeted methylation of the promotor of both genes six days after transfection, compared to control-transfected cells (n=5, two-way repeated measures ANOVA with Šídák's multiple comparisons test). **C.** Gene expression analysis showed lower expression of both genes. Data are corrected for the most stable housekeeping genes (*Rpl13a* and *Ywhaz*), n=5, one sample t-test. **D.** Representative images (20x MBP and DAPI, 40x MBP) and quantification (fluorescence area and average process length) of OPCs, nine days post-transfection (n=5, one sample t-test). **E.** Myelin gene (*Mag, Mbp, Mobp*) expression was increased after targeted methylation of either *Id2* or *Id4* (n=7-8, Wilcoxon test). Data are represented as mean ± SEM, *p < 0.05, **p < 0.01. sgRNA= guide RNA, inactive = catalytic inactive pdCas9-DNMT3A-PuroR vector (control), active = catalytic active pdCas9-DNMT3A-PuroR vector.

Chronically demyelinated MS lesions show altered methylation and expression profiles for both *ID2* and *ID4*

Since *Id2/Id4* appeared to be epigenetically regulated during murine OPC differentiation, we aimed to examine whether the methylation status of both genes was altered in MS lesions. Progressive MS stages are characterized by the abundance of chronically demyelinated lesions due to impaired endogenous remyelination mechanisms. To assess whether such lesions show differential methylation and/or expression levels of *ID2*/*ID4*, we first phenotyped MS brain lesions to include in our analysis (Fig. 3.4A). Chronic non-fibrotic demyelinated lesions were characterized based on absence of PLP staining. Further inclusion criteria were the absence of immune cells (HLA-DR$^-$, ORO$^-$), strictly white matter samples (NeuN$^-$), and the presence of OPCs (NG2$^+$). Lesions were subsequently microdissected from the surrounding normal appearing white matter (NAWM) and gene expression analysis showed, as expected, reduced level of myelin genes in MS lesions, compared to the surrounding NAWM, and to white matter of age- and sex-matched control samples (Fig. 3.4B). Interestingly, significantly higher mRNA expression levels of both *ID2* and *ID4* were observed within MS lesions compared to the surrounding NAWM (Fig. 3.4B). Furthermore, the average DNA methylation levels of both *ID2* and *ID4* were lower in MS lesions compared to control samples (Fig. 3.4C and Supplementary Figure S3.3). Strikingly, particularly ID2 methylation levels within the damage-free NAWM samples followed the pattern observed in the lesions rather than the matched control samples, which suggests that the NAWM might be already affected prior to visible myelin damage (Fig. 3.4C and Supplementary Figure S3.3).

Table 3.1 – Demographic characteristics of the cohort

Characteristic	Non-neurologic controls	MS patients
Gender (male/female)	4/6	4/6
Age, mean (SD)	65.7 (8.90)	64.7 (9.64)
Disease diagnosis (PPMS/PRMS/SPMS/unspecified)	n.a.	3/1/4/2
PMI, mean (SD)	9.12 (1.87)	9.72 (4.09)

Key: MS, multiple sclerosis; PPMS, Primary Progressive MS; PRMS, Primary Relapsing MS; SPMS, Secondary Progressive MS; PMI, post-mortem interval; SD, standard deviation; n.a., not applicable.

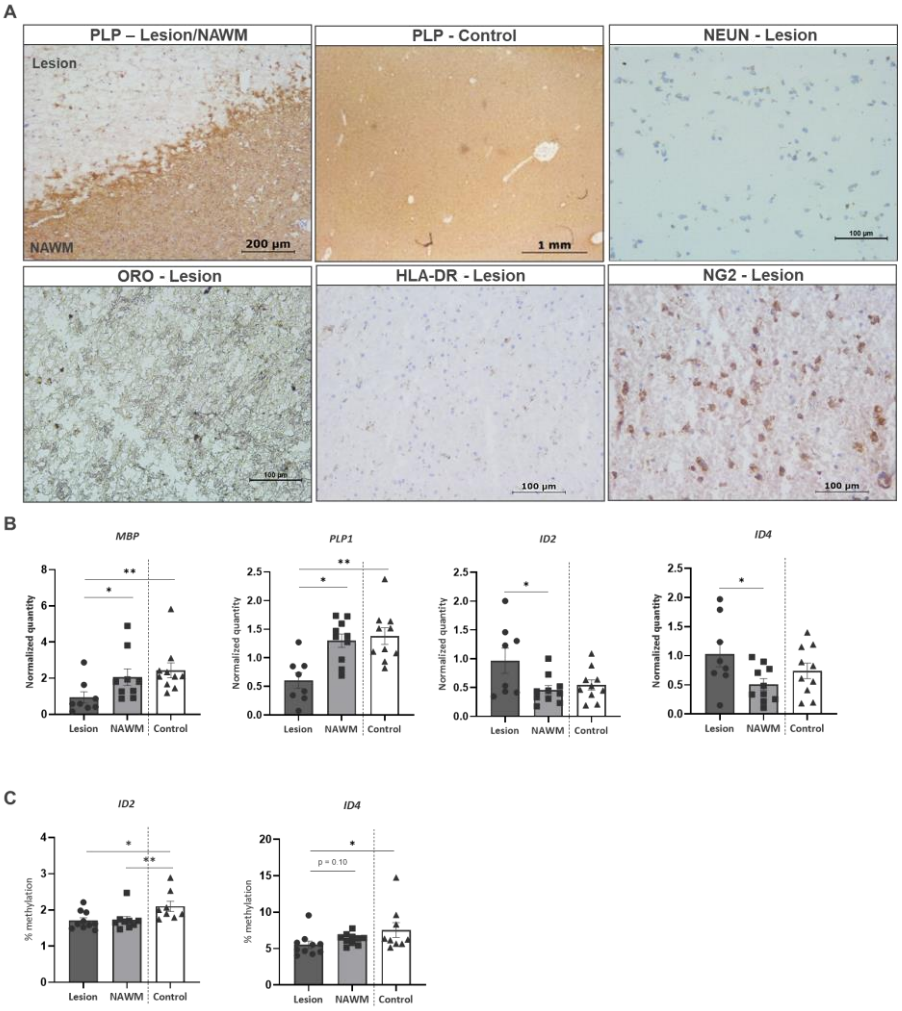


Figure 3.4 – *ID2* and *ID4* are hypomethylated and display increased expression in MS lesions, compared to controls. A Phenotyping of MS lesions by means of immunohistochemistry. Chronically demyelinated lesions were defined as PLP^-, $NeuN^-$, ORO^-, HLA-DR^- and $NG2^+$. Control white matter samples were defined as PLP^+, $NeuN^-$, ORO^-, HLA-DR^- and $NG2^+$. **B** Gene expression analysis of myelin genes (*MBP, PLP*) and the negative regulators (*ID2*, *ID4*) in chronically demyelinated MS lesions, the surrounding NAWM and white matter of matched control samples. Data are corrected for the most stable housekeeping genes (*TBP* and *YWHAZ*), n=8-10, Wilcoxon or Mann-Whitney test. **C** Methylation analysis within the CpG island of the *ID2* and *ID4* promotors in chronically demyelinated MS lesions, the surrounding NAWM and matched control samples (n=10, Wilcoxon or Mann-Whitney test). Data are represented as mean ± SEM, *p<0.05, **p<0.01. NAWM = normal appearing white matter, PLP = myelin proteolipid protein, NeuN = hexaribonucleotide binding protein-3, ORO = Oil red O, HLA-DR = human leukocyte antigen – DR isotype, NG2 = neuron-glial antigen 2, MBP = myelin basic protein.

Discussion

In the present study, we demonstrate that DNA methylation of myelin regulatory genes, in particular the HLH inhibitory transcription factors *Id2* and *Id4*, is crucial for OPC differentiation. Inhibition of DNA methylation in primary OPCs results in significantly increased expression of both genes, eventually inhibiting myelin gene expression and impaired OPC differentiation. Moreover, we show that the decreased expression of *Id2* and *Id4* from OPC to oligodendrocyte stages is negatively correlated with their methylation levels. Our targeted epigenetic editing approach further strengthens evidence for a causal relationship between Id2 and Id4 DNA methylation and OPC differentiation. CRISPR/dCas9-mediated *Id2* and *Id4* hypermethylation resulted in a reduced expression of *Id2* and *Id4*, eventually leading to a boost in OPC differentiation and myelin gene expression. Finally, we show that the promoter regions of *ID2* and *ID4* display decreased methylation in chronically demyelinated MS lesions, resulting in a higher expression of both genes, which could represent a potential key factor in the impaired differentiation capacity of progressive MS patients.

A potential role of DNA methylation enzymes during OPC differentiation and myelination has previously been described (49, 50, 89, 205). Yet, it was unclear which genes are actually epigenetically regulated during oligodendrocyte development. OPC differentiation is featured by the induced expression of myelin genes, which are tightly regulated by an upstream network of activators and repressors (23, 196). Here, we show that pharmacological inhibition of DNA methylation, by means of 5-AZA, mainly affects the repressive transcription factors *Id2* and *Id4* and prevents OPC differentiation. Even though the use of epigenetic drugs has its limitations, such as the lack of specificity and relatively high cytotoxicity, it can provide more insights into DNA methylation biology (186, 187). While we cannot exclude the possibility that other relevant genes may have been affected by the use of 5-AZA, there is evidence from previous studies that show similar effects on OPC differentiation and *ID4* expression when using epigenetic modifiers, such as HDAC inhibitors (96, 145). *Id2* and *Id4* belong to the HLH transcription factors and are highly expressed in OPC stages, a notion confirmed in our current study. Both inhibitory proteins function to keep OPCs in a non-differentiated stage by antagonizing the nuclear translocation of pro-

differentiation HLH transcription factors (OLIG1/2, ASCL1) (70, 208). Indeed, we show that the expression of *Id2*/*Id4* significantly decreased in differentiated oligodendroglial stages. Interestingly, the expression profile of *Id2*/*Id4* was negatively correlated with the methylation profile of the respective promoter regions, thereby suggesting that DNA methylation could represent the mechanism of action behind this stage-dependent regulation. This is in line with previous observations that show that the type II protein arginine methyltranferase PRMT5 associates with the CpG islands of *Id2* and *Id4* and thereby regulating their expression during OPC differentiation (90).

Even though both *Id2* and *Id4* seem to be epigenetically regulated during OPC differentiation, there was still no functional evidence that specific alterations to the methylation profile of the genes will influence oligodendroglial development. To assess this intricate causality, we made use of a recently developed epigenetic engineering system, based on CRISPR-Cas9 technology. Recent advantages have been made to alter the epigenome in a targeted manner, by coupling the nuclease-inactivated dCas9 protein to epigenetic editor domains (such as DNMT3a and TET1). Target-specificity is then achieved by designing a guide RNA towards the desired CpG region (189, 209, 210). For this study, we used the plasmid vector designed by Vojta *et al*. (207). The dCas9 protein was coupled to the catalytic domain of DNMT3a, which allows for targeted methylation of *Id2* or *Id4*, based on the guide RNA that was cloned into the vector. As a control, we took along the DNMT3a-inactive plasmid, which had the same properties as the active vector, but lacked the capacity to induce methylation. Transfection of primary OPCs with the *Id2* or *Id4* plasmids led to higher methylation levels at the targeted region, accompanied by a reduced expression of both genes at day six post-transfection. This timing was specifically chosen, as it has been shown that the peak of methylation is expected to be at the highest point between day six and seven post-transfection (207). However, it must be noted that the methylation levels within the *Id4* promoter region are difficult to measure due to the dense CpG-rich regions, which limits the options for adequate primer design. We only measured the methylation level of seven CpG sites of the *Id4* promotor region due to our limited possibilities in primer design. It might still be possible that other relevant CpG sites show higher changes in % methylation following CRISPR-Cas9. In

general, the overall increase in methylation follows our line of expectancy and does nicely show that our CRISPR-Cas9 vector does induce methylation at the targeted regions. Most interestingly, we also observed a similar increase in the expression of myelin genes and boost in OPC differentiation (MBP area and process length) at day nine post-transfection when targeting either *Id2* or *Id4*, thereby validating the functional importance of DNA methylation of *Id2/Id4* during oligodendrocyte development. However, since we observed a significant increase in *Mag* in both *Id2* and *Id4* targeted samples, and a non-significant increase in *Mbp* and *Mobp*, we assume that the cells were still differentiating at the time of lysation and did not reach the ultimate final stage yet. Interestingly, even though both *Id2* and *Id4* have a similar effector function in the regulation of OPC differentiation, targeted silencing of only one of the genes was already sufficient to boost the expression of myelin genes. This is in line with previous literature, that has shown that both *Id2* and *Id4* function separately as an intracellular timer for oligodendrocyte differentiation. Absence of *Id2* results in premature OPC differentiation and a higher percentage of oligodendrocytes. Similarly, overexpression of *Id4* in OPCs increases their proliferation and inhibits their differentiation into oligodendrocytes (211).

CRISPR-Cas9-based epigenetic editing has gained increasing attention because of its ease-of-use and rapid adaptability. However, one main concern remains the high off-target effects due to the complementarity of the guide RNA with other genomic regions. Even though we did not observe significant predicted off-target effects, we cannot completely rule out misguided dCas9-DNMT3a events. A study has previously shown that the dCas9-DNMT3a tool increases the methylation levels globally, regardless of the use of guide RNAs (212). It is thus suggested that the unspecific activity of epigenetic editing tools is not only a result of off-target guide RNA binding, but also unguided activity of the effector domains, such as DNMT3a, themselves (213, 214).

Since we have shown that *Id2* and *Id4* are epigenetically regulated during normal OPC differentiation, we wondered whether these processes were affected in pathological conditions. MS represents one of the major myelopathies of the CNS and is characterized by early endogenous remyelination, a process that becomes impaired during the progressive stages of the disease (20, 215). It has been suggested that the main reason behind this hampered remyelination is a block in

OPC differentiation within MS lesions (149). We therefore hypothesized that the methylation profile of *ID2* and *ID4* was altered in chronically demyelinated MS lesions, and thus could represent one of the reasons behind the differentiation block. MS lesions are typically very diverse in terms of the degree of demyelination, inflammation and scar formation (216, 217). In the present study, we aimed to include only chronically demyelinated lesions which are inflammatory inactive. These lesions are mostly found in progressive MS patients and represent the main neurodegenerative aspect of the disease. Other important criteria that we applied in our study were the presence of OPCs within the lesions and the exclusion of scar tissue since these have no ability to regenerate and are too advanced in the disease stage. Gene expression analysis showed higher expression of both *ID2* and *ID4* within the lesions, compared to the surrounding NAWM. Even though this observation is in line with our hypothesis this difference could represent the balance between the presence of OPCs within the lesions and oligodendrocytes within the NAWM. However, we also showed that the promoter regions of both *ID2* and *ID4* were hypomethylated within MS lesions, compared to age- and sex-matched controls. Furthermore, the average methylation pattern of ID2 within the NAWM samples resembled the methylation pattern of the lesions, rather than the non-neurological control samples. This suggests that there could already be some OPC dysregulation occurring within MS brains preceding noticeable myelin damage, a notion that has been proposed before by others (53, 218). Interestingly, the average expression levels of both genes was substantially lower in the control cohort compared to the lesions, yet not statistically significant. This could be attributed to multiple aspects, such as the variation between healthy individuals, the RNA integrity of the samples due to the variation in post-mortem interval, or the lack of statistical power due to the low sample size.

Our data demonstrate that chronically demyelinated lesions show dysregulation of *ID2* and *ID4* both on the level of methylation and gene expression, which could be an underlying mechanism behind the OPC differentiation block in progressive MS stages. Our observations are in line with previous research, which has shown that chronic MS lesions show higher histone acetylation levels, associated with an increase in the expression of ID2 and TCF7L2 (158). OPCs have recently also been described as environmental biosensors that can alter their epigenomic signature

in response to chemical and physical stimuli, such as neuronal activity, stiffness of the extracellular matrix and the presence of hormones (219). In line with this rationale, our data could suggest that the accumulation of myelin damage during disease progression induces a change in the epigenetic regulation of *ID2/ID4*, thereby leaving the cells in a blocked differentiation stage. A limitation is that our findings are based on heterogenous bulk tissue, and therefore the presence of other cell types may bias the observed changes in methylation. For example, it has previously shown that Id4 is necessary for astrocyte proliferation after excitotoxic damage, while Id2 has been shown to be upregulated in specific microglia clusters, associated with ageing (220, 221). The cell type heterogeneity within bulk tissue can thus confound analysis and lead to data misinterpretation. Over the recent years, new *in silico* methods have been developed to estimate cell type proportions within bulk tissue for the analysis of epigenome data (222, 223). However, such cell type deconvolution algorithms are not yet applicable for targeted DNA methylation analysis. Especially complex tissues, such as MS brain lesions, of which the cellular composition is very variable and hard to correct for, should therefore be considered with care. Furthermore, the methylation profile does not only differ between different cell types, but can also vary strongly within one cell population, mainly in a pathological context. Indeed, recent studies have revealed distinct OPC and oligodendrocyte populations within MS brain samples, each with different transcriptional, and likely epigenetic, signatures, which could therefore result in misinterpretation of bulk tissue analysis (224, 225). Nevertheless, our observations regarding *ID2/ID4* methylation within MS brain lesions are in line with our previous *in vitro* findings.

Taken together, our study reveals the epigenetic regulation of the inhibitory transcription factors ID2 and ID4 during OPC differentiation. Furthermore, this epigenetic signature appears to be dysregulated in chronically demyelinated MS lesions. Our data provide more insights into OPC biology, while also unraveling new epigenetic targets to boost OPC differentiation that appears relevant in the context of MS.

Supplementary Tables

Table S3.1: Guide RNA targeting *Id2* and *Id4*

Target gene	Guide RNA (5'-3')
Id2	ATC AAG AGG CTC GAA CTG TT
Id4	TCG CCC GCG TCC GGT TCT TG

Table S3.2: Antibody list

Antigen	Company (reference number)	Dilution
O4	R&D systems (MAB1326)	1:1000
MBP	Merck (MAB386)	1:500
PLP	AbD Serotec (MCA839G)	1:100
HLA-DR	eBioscience (14-9956-82)	1:100
NeuN	Millipore (MAB377)	1:200
NG2	Abcam (ab101807)	1:500
Goat anti-mouse IgM	Life Technologies (A21042)	1:600
Goat anti-rat IgG	Life Technologies (A21434)	1:600

Table S3.3: qPCR primer list

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
Id2 (mouse)	GGA CAT CAG CAT CCT GTC CT	CTC CTG GTG AAA TGG CTG AT
Id4 (mouse)	TCC CGC CCA ACA AGA AAG TC	TCA GCA AAG CAG GGT GAG TC
Mbp (mouse)	TCA CAG AAG AGA CCC TCA CAG C	GAG TCA AGG ATG CCC GTG TC
Plp (mouse)	TTG TTT GGG AAA ATG GCT AGG A	GCA GAT GGA CAG AAG GTT GGA
Sox10 (mouse)	GGA GAT CAG CCA CGA GGT AAT G	GTT GGG TGG CAG GTA TTG GT
Sox2 (mouse)	AAC TTT TGT CCG AGA CCG AGA A	CCT CCG GGA AGC GTG TAC T
Myrf (mouse)	GCA TGG GCA CCG CCC CTA AG	GGG GCG AGT CTG GCA GTG TG
Olig1 (mouse)	CGA CGC CAA AGA GGA ACA G	GCC AAG TTC AGG TCC TGC AT
Olig2 (mouse)	CCG AAG CAA TGG GAG CAT	GGA GTG TTC AGC CAA AGA GTC A
Sox5 (mouse)	TGA TTT ACC TCA GGA GTT TGA AAG G	TAC CTC TCC ATC TGT CTC CCC ATA
Mag (mouse)	ACA CCC CCA ACA TTG TGG TT	CGA ACT GCA AGG TGG TGT TG
Pdgfra (mouse)	GGG GAG AGT GAA GTG AGC TG	CAT CCG TCT GAG TGT GGT TG
Dbp (mouse)	ACC GTG GAG GTG CTA ATG	ATG GCC TGG AAT GCT TGA
Pip4k2b (mouse)	CCA GCA AAA CCA AGA CCA AGA A	ACA TCA GGA CGC TGA GAA TCG
Rpl13a (mouse)	GGA TCC CTC CAC CCT ATG ACA	CTG GTA CTT CCA CCC GAC CTC
Cypa (mouse)	GCG TCT CCT TCG AGC TGT T	AAG TCA CCA CCC TGG CA
Yhwaz (mouse)	GCA ACG ATG TAC TGT CTC TTT TGG	GTA CAC AAT TCC TTT CTT GTC ATC
Pgk-1 (mouse)	GAA GGG AAG GGA AAA GAT GC	GCT ATG GGC TCG GTG TGC
MBP (human)	AAG ACA GGC CCT CTG AGT CC	GGA GGG TCT CTT CTG TGAC G
PLP1 (human	GGC CAA CAT CAA GCT CAT TCT T	AGG TGA TGC CCA CAA ACT TGT
ID2 (human)	ATG AAA GCC TTC AGT CCC GT	CGA TCT GCA GGT CCA AGA TG
ID4 (human)	TGA ACA AGC AGG GCG ACA	CGT GCA AAG AAA GAA TGA AAG
YHWAZ (human)	CTT GAC ATT GTG GAC ATC GG	TAT TTG TGG GAC AGC ATG GA
TBP (human)	TAT AAT CCC AAG CGG TTT GC	GCT GGA AAA CCC AAC TTC TG

Table S3.4: Pyrosequencing primer list

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Sequencing primer (5'-3')	Genomic coordinates	Number of CpGs covered	Reference genome
Id2 (mouse)	AGGAAAATTTGAGTAAG GTAAGGTTTGTAA	CTCAAATAATAAATCACTTC CAAACTTAAT	AAGGTAAGGTTTGTAATG	chr12:25.097.141-chr12:25.097.740	5	GRCm38/mm10
Id4 (mouse)	ATTGTTTTTTTGATTGGTT GGTTAT	ATAACTACCCCCCCTCAA	GTTTTATAAATATTGTTGTG	chr13:48.260.426-chr13:48.261.426	7	GRCm38/mm10
ID2 (human)	GGGTTTAGTAGGTATTGA TTAGTTGG	CCCTACAACCTTATCCTC	AGGTATTGATTAGTTGGG	chr2:8.677.850-chr2:8.678.864	11	GRCh38/hg38
ID4 (human)	GTAGTATAGGGAGTGGG GTGAT	ATAACCCACCCCAATATCCT AATC	GGAGTGGGGTGATTT	chr15:19.836.385-chr15:19.837.760	5	GRCh38/hg38

Supplementary Figures

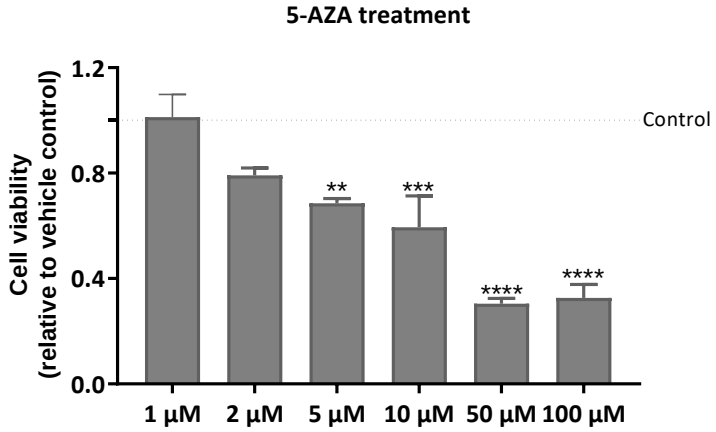


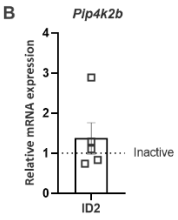
Figure S3.1 – Treatment with 5-AZA does not affect Oli-*neu* cell viability at a concentration of 1 µM. Oli-*neu* cells were treated with different concentrations of 5-AZA for 48 hours. DMSO was used as a vehicle control condition. Cell viability was assessed via an MTT-assay. Data are represented as mean + SEM and are relative to the control condition (n = 6; **p<0.01, ***p<0.001, ****p<0.0001).

A

Off-target hits (ID2 guide)			
Sequence	**PAM**	**Gene**	**Locus**
ATTTAGAGGCTCGAACGGTT	TGG		chr6:-113029600
TAACAGAGGCTCGAACTGTT	CAG	Pip4k2b (ENSMUSG00000018547)	chr11:+97715252
ATCGAGAGGGTGGAACTGTT	AAG		chr15:+25497551

C

Off-target hits (ID4 guide)			
Sequence	**PAM**	**Gene**	**Locus**
TCCCCCGGGCCCGGCTCTTG	CAG	Dbp (ENSMUSG00000059824)	chr7:+45706934
TCTCCCGTGACCGGTTCCTG	CGG		chr6:+114490227
TCGGCGGCTTCCGGTTCTGG	AAG		chr3:+80038745



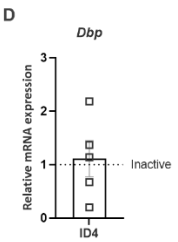


Figure S3.2 – Top 3 off-target hits of the *Id2/Id4* sgRNA. (A,C) The most likely off-targets of the designed sgRNAs are determined by the Benchling software®. The mismatches are depicted in red. (B,D) Off-target effects are analyzed by qPCR of the relevant genes. No difference between the active and inactive constructs is observed. Data are represented as mean ± SEM, n=5 (one sample t-test).

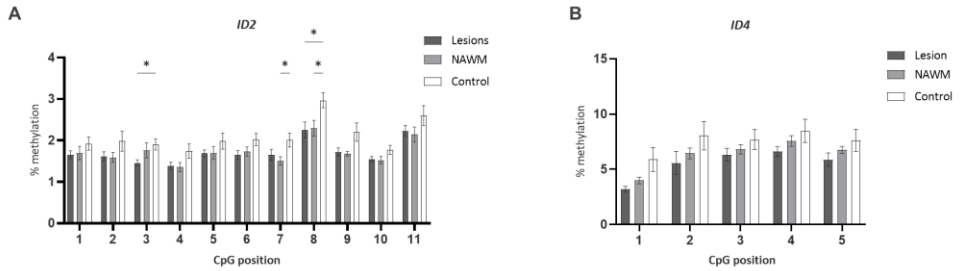


Figure S3.3 – *ID2* and *ID4* methylation levels per measured CpG site. Methylation analysis within the CpG island of the *ID2* and *ID4* genes in chronically demyelinated MS lesions, the surrounding NAWM and matched control samples (n=10, two-way repeated measures ANOVA with Šídák's multiple comparisons test). Data are represented as mean ± SEM, *p<0.05.

Supplementary Methods

Cell viability assay

The effect of 5-AZA on cell viability and cell survival was measured via the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Oli-*neu* cells were seeded in a 96-well plate at a density of 20 x 10^3 cells per well in standard culture medium (DMEM, 10% FCS, 1% P/S). Cells were treated with increasing concentrations of 5-AZA or dimethylsufloxide (DMSO; Sigma-Aldrich) as a control. After 48 hours, medium was removed and cells were incubated with 500 µg/ml MTT (Sigma-Aldrich) in DMEM for four hours at 37°C. After removal of the MTT-solution, a glycine-DMSO mixture was added to induce reduction of MTT to formazan. The absorbance was measured at 540 nm with the iMark Microplate Reader (Bio-rad Laboratories, Temse, Belgium).

CHAPTER 4

A perspective on causality assessment in epigenetic research on neurodegenerative disorders

Based on:

A perspective on causality assessment in epigenetic research on neurodegenerative disorders

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Declaration of own contribution:

A.T. performed literature search, participated in manuscript writing and figure design.

Introduction

Epigenetics refers to heritable and reversible processes regulating gene expression that do not involve a change to the DNA sequence. Epigenetic modifications include DNA modifications (e.g. DNA methylation and hydroxymethylation), histone modifications and non-coding RNAs such as micro RNAs (miRNAs) and long-coding RNAs (lnRNAs) (226). Amongst others, epigenetic mechanisms play a vital role in cell proliferation and development, to ensure the correct genes are being expressed in a differentiating cell type. However, epigenetic mechanisms are also influenced by environmental cues, where they are subject to change during life, and may even mediate transgenerational inheritance (227). In the last decades, research on epigenetics has expanded to study the role of these mechanisms in a plethora of diseases, such as neurodegenerative disorders (228). The most studied epigenetic modifications are DNA modifications, in particular DNA methylation. DNA methylation refers to the process of adding methyl groups to DNA molecules, in particular at the level of CpG dinucleotides, i.e. where a cytosine (C) nucleotide is followed by a guanine (G) nucleotide in the linear sequence of bases. Recent technological advances have led to epigenome-wide-association studies (EWAS), such as methylome-wide association studies (MWAS), allowing for an in-depth analysis of epigenetic changes associated with disease. While EWAS/MWAS represent an important tool to establish a candidate list of genetic loci associated with a specific disorder, they remain purely correlational. Even with robust replicated findings highlighting the same differentially methylated loci and/or regions and showing functional correlations with gene expression, it remains difficult to infer a cause-effect relationship. This notion is especially problematic when studying disorders that are poorly understood. In fact, any epigenetic difference between diseased and healthy subjects could represent a cause or consequence of risk factors, the disease itself, its treatment, or an epiphenomenon, or a combination of one or more of these features. While this limitation is often acknowledged in research across the field, it is rarely addressed properly.

In the last couple of years, epigenetic editing, i.e. altering the epigenome by reversing or restoring e.g. DNA methylation at a specific site, has grown as a powerful tool to further study the involvement of epigenetics in various diseases,

especially in view of addressing causality (229). This perspective proposes a guideline on how to thoroughly investigate potential cause-and-effect relationships for epigenetic alterations in neurodegenerative diseases taking Alzheimer's disease (AD) and multiple sclerosis (MS) as examples.

Major concerns in inferring cause-and-effect relationships in neurodegenerative diseases

Cause-and-effect relationships between observed biological changes and disease-associated phenotypic variation are challenging to infer. Neurodegenerative diseases are particularly suffering from this limitation for a number of reasons. Firstly, these diseases are progressive in nature, posing an enormous challenge to assess the exact disease state. This notion limits the signal-to-noise ratio in EWAS when comparing neurodegenerative patients to healthy controls, while it also makes it difficult to identify those epigenetic changes involved in the early stages of the disease, which often emerge years if not decades before the presentation of its symptoms. Age as such may also interfere in this respect, exerting its own epigenetic imprint (230). Secondly, these diseases are often multi-factorial, with a complex etiology, concomitant with secondary psychological and behavioral changes, or comorbidity, all of which in turn can affect the epigenome. Thirdly, treatment (e.g. pharmacological) interventions can have an impact upon epigenetic changes. Finally, it is of crucial importance to consider the cellular heterogeneity of bulk tissue, on which most of the EWAS studies are being conducted. Such sample heterogeneity does not only limit the reproducibility of the observed data, but can also lead to biased conclusions. Novel techniques, such as single-cell sequencing, could be an ideal strategy to cope with this issue, yet unfortunately such approach is not yet standardized for DNA methylation sequencing.

In this perspective we focus on two neurodegenerative disorders that are both poorly understood, devastating, yet fundamentally different in terms of their etiology, are AD and MS. While the former is characterized by toxic protein aggregates leading to neuronal degeneration and loss, the latter leads to neuronal loss due to demyelination of axons (231). We focus on these disorders to showcase how flexible and versatile this approach to investigate causality is.

Alzheimer's disease

AD is a fatal progressive neurodegenerative disease and the most common form of dementia. It is characterized by intracellular neurofibrillary tangles and extracellular amyloid depositions, leading to memory loss, often accompanied by changes in affective behavior and, eventually, death. AD has devastating implications for patients and care-takers due to rapid cognitive decline. To date, advances in the field have not led to new treatment methods, as the etiology of AD is multi-factorial and remains poorly understood. Approximately 10% of AD cases are considered familial, whereas over 90% are considered sporadic. Sporadic AD cases are most likely caused by a combination of different genetic, environmental and epigenetic factors, such as DNA hypermethylation, deactylation of histones and repressed chromatin states (228). While a recent meta-analysis has highlighted numerous genome-wide significant neuropathology-associated DNA methylation differences in AD, annotated to 121 genes, causality of those genes has not yet been assessed(232).

Multiple Sclerosis

MS is an (auto)immune-driven demyelinating and neurodegenerative disorder of the central nervous system (CNS), caused by autoreactive insults to the myelin sheath. MS is characterized by a sustained toxic pro-inflammatory environment within the CNS parenchyma, both due to resident and infiltrated reactive immune cells, as well as oligodendroglial degeneration and demyelination. The loss of the isolating capsule around the axons does not only affect electrical impulse conduction, but the lack of trophic support also leads to axonal damage, ultimately contributing to the progressive and neurodegenerative aspect of the disease (233). The primary and most studied factor associated with MS pathology is the immune-driven attack in the CNS, accompanied by the breakdown of the myelin sheath. Both innate and adaptive immune cells have been shown to be involved in inflammation observed in MS, yet it remains unclear how these immune cells become autoreactive. The so called 'outside-in hypothesis' suggests that immune cells acquire a pathogenic phenotype in the periphery, possibly due to environmental and epigenetic factors, causing them to invade the CNS where they attack the oligodendrocytes and myelin sheath. In contrast, however, the 'inside-

out hypothesis' states that MS pathology starts with oligodendrocyte dysfunction and cell death, which eventually triggers an autoimmune response (234). This discrepancy, together with the heterogeneity of the disease, are complicating factors when defining causality.

Causality assessment of epigenetic signatures - a proposed workflow

While EWAS studies are highly relevant as they provide new insights into the disease and allow researchers to explore new avenues, they do not give an indication about the cause-and-effect relationship of the studied genes. We therefore propose a workflow to aid in assessing causality of candidate epigenetic signatures in neurodegenerative diseases, such as AD and MS (Figure 4.1).

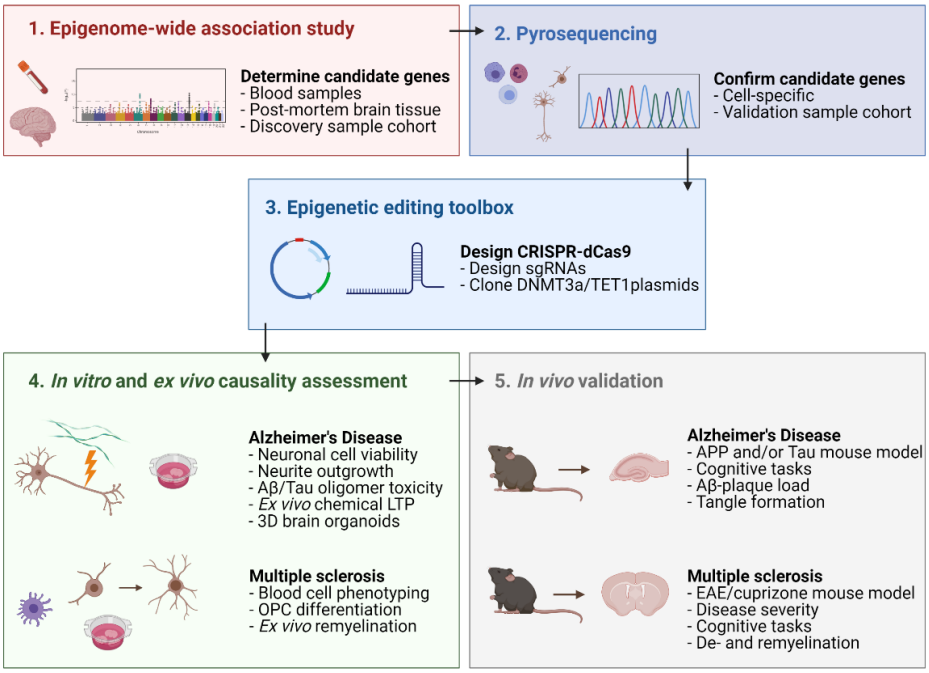

Figure 4.1 - Proposed workflow to aid in assessing causality of candidate epigenetic signatures in neurodegenerative diseases using AD and MS as showcases. Candidate genes, determined in EWAS studies, can be validated in a cell-specific manner using targeted sequencing techniques, such as pyrosequencing. As a functional validation, the epigenetic editing toolbox can be applied to assess the effect of specific epigenetic modifications of the candidate signatures both in vitro and in vivo. CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats, dCas9: deactivated CRISPR associated protein 9, sgRNAs: single guide RNAs, DNMT3A: DNA methyltransferase 3a, TET1: Tet Methylcytosine Dioxygenase 1, Aβ: amyloid-bèta, APP: Amyloid Beta Precursor Protein, LTP: long term potentiation, OPC: oligodendrocyte precursor cell, EAE: experimental autoimmune encephalitis. (Created with BioRender.com)

A general starting point is an EWAS study on a power-based sample size discovery sample cohort to stratify candidate signatures associated with the phenotype of interest. Such candidate signatures can be further validated using targeted sequencing technologies, like pyrosequencing. To control for bulk tissue bias, it would be ideal to consider cell-specific methylation analysis, which can be achieved by technologies such as fluorescent-activated cell sorting (FACS) or laser-captured microdissection (LCM). Once promising candidate signatures are determined and validated, an epigenetic editing toolbox could be applied. The recent introduction of new epigenetic editing tools, such as the CRISPR-(d)Cas9 based system, has opened a new avenue to investigate the potentially causal associations between epigenetic modifications and the pathogenesis of neurodegenerative disorders (235). Based on the nature of the DNA (hydroxy)-methylation signature, one can opt for either a DNMT3a- or TET1-based CRISPR-dCas9 vector and design an optimal sgRNA to the desired genomic region. The epigenetic editing construct can then be transfected into cells of interest, to assess the functional consequence both *in vitro* and *in vivo*. This proposed workflow allows for higher throughput due to a standardized approach, higher chance to identify biologically relevant targets and, therefore, a higher chance to translate findings to patients.

Specific considerations - Alzheimer's disease

One of the many challenges that epigenetic research in AD has to handle is on how to proceed with differentially methylated loci and regions. Firstly, it is unclear if a gene displaying differential methylation when comparing AD and control individuals exerts a causal effect or is differentially methylated as a consequence of its pathophysiology. Secondly, it also has to be assessed if normalizing the degree of methylation of the differentially methylated region has any biological relevance in terms of halting or reversing the disease pathology and functional phenotype. Thirdly, as AD is progressive and involves numerous genes and associated pathways, one has to consider that disease heterogeneity is a highly complicating factor in interpreting the relevance of differential methylation and selecting candidate genes to investigate further.

Currently available epigenetic editing systems offer an ideal toolbox to investigate whether the identified differentially methylated regions represent potential key

players in the development and/or progression of AD (Figure 4.1). As a proposed workflow, one could investigate the effects of inducing hypo- and/or hypermethylation in specific target genes *in vitro* and studying the effects on different parameters, such as cell viability, neuronal growth, plasticity, and metabolic activity in neuronal cells. Here, it is important to consider the potential role of glial cells as effects can be cell type-specific. Focusing on a single cell type increases the signal-to-noise ratio, concomitant with an increase in power, and allows assessing causality in a more reliable manner. A subsequent step could be to culture cells in the presence of Aβ oligomers to investigate if the altered methylation of the candidate genes would exacerbate the toxic effects of Aβ. This approach is not limited to Aβ exposure, but can be extended to pretty much any relevant neuropathological (tau, cytokines, etc.) or environmental (e.g. stress) factor that is relevant to the disease. Furthermore, chemical long-term potentiation (LTP) in *ex vivo* brain slices could be applied as a functional validation as well. However, *in vitro* models suffer from some limitations, such as insufficiently mimicking the neurodegenerative process, which occurs over many years. Alternatively, 3D brain organoids, generated from embryonic stem cells or induced pluripotent stem cells (iPSCs) can be generated to study the pathophysiology of AD. Finally, an *in vivo* approach making use of an AD mouse model could be used to explore the effects of (site and locus-specific) hyper- or hypomethylation, in order to identify the potential functional (e.g. cognitive), and hence putatively even therapeutic, consequences of targeting this locus. Spatiotemporal control of epigenetic modulation in causality assessment can be mimicked using stereotactical injection and cell type-specific promotors.

Specific considerations - Multiple sclerosis

Investigating disease causality in MS would be ideally performed on samples from patients at symptom onset. Since the prodromal MS phase is gaining attention, it would be of great interest to investigate those epigenetic alterations occurring at such an early phase in order to identify individuals at-risk (236). Furthermore, longitudinal blood samples, obtained from MS patients over time, could be of great value to investigate epigenetic alterations acquired as the disease progresses. The identified target genes can then be epigenetically edited to investigate disease

causality. However, even though the epigenetic signature of different subsets of peripheral blood mononuclear cells (PBMCs) of MS patients is already widely investigated by independent research groups, the data does not always reveal reproducible findings (237). This discrepancy could be the result of limited sample size or methods of sample selection, methylation measurements or data-analysis. An overarching meta-analysis of these studies, could potentially correct for methodological dissimilarities and reveal interesting targets that can be further assessed for their potential causative role in MS disease pathology (232).

Investigating epigenetic changes in post-mortem brain tissue and taking into account the differences between lesion types could also potentially reveal new markers or targets for remyelination, neuroprotection, and disease progression in MS. In order to study causation of the observed DNA methylation pattern in MS, the previously mentioned epigenetic editing tools such as CRISPR-dCas9 could be utilized. An interesting approach would be to make use of the CRISPR-dCas9-DNMT3a/TET1 tool to induce DNA (de)methylation at specific loci, which have been associated with oligodendrocyte function. Primary *in vitro* oligodendrocyte cultures could then be transfected with the epigenetic editing plasmid to assess the effects on oligodendrocyte survival and differentiation. Furthermore, *in vivo* epigenetic editing of these genes in for instance cuprizone animal models could reveal whether targeted (de)methylation of these genes does influence remyelination capacity.

Conclusion

In conclusion, causality assessment in epigenetic research remains a challenge. This workflow aims to aid researchers on how to assess candidate epigenetic signatures in neurodegenerative diseases, taking AD and MS as an example. While a single gene is unlikely to be the main contributor to these diseases, it allows for a more thorough understanding of the role a single gene can play in neurodegenerative disorders, allowing to identify whether the epigenetic signature is a cause or merely a bystander or consequential imprint of the pathology. This proposed workflow can be applied to other neurodegenerative disorders as well.

CHAPTER 5

From methylation to myelination: epigenomic and transcriptomic profiling of chronic inactive demyelinated multiple sclerosis lesions

Based on:

From methylation to myelination: epigenomic and transcriptomic profiling of chronic inactive demyelinated multiple sclerosis lesions

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In preparation

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Declaration of own contribution:

A.T. contributed to the experimental design, data generation, interpretation, analysis, and manuscript writing.

Abstract

Introduction: In the progressive phase of multiple sclerosis (MS), the hampered differentiation capacity of oligodendrocyte precursor cells (OPCs) eventually results in remyelination failure. We have previously shown that epigenetic mechanisms, particularly DNA methylation, are highly involved in OPC differentiation and remyelination. In this study, we investigated genome-wide DNA methylation within OPCs derived from chronically demyelinated MS lesions and how certain epigenetic signatures relate to their differentiation capacity.

Methods: We compared genome-wide DNA methylation and transcriptional profiles between chronically demyelinated MS lesions and their matched normal-appearing white matter (NAWM) , making use of post-mortem cortical brain tissue (n=9/group). Cell-type specificity of the DNA methylation differences observed within MS lesions that inversely correlated with mRNA expression of their corresponding genes was confirmed in laser-captured OPCs using pyrosequencing. Based on these results, we epigenetically edited *MBP*, encoding for myelin basic protein, to assess the effect on cellular differentiation using the CRISPR-dCas9-DNMT3a/TET1 system in human-iPSC-derived oligodendrocytes.

Results: Our data show hypermethylation of CpGs within genes that cluster in gene ontologies related to myelination and axon ensheathment. Cell type-specific validation indicates a region-dependent hypermethylation in OPCs obtained from lesions compared to NAWM-derived OPCs. By altering the DNA methylation state of specific CpGs within the promotor region of *MBP* using epigenetic editing, we show that cellular differentiation can be bidirectionally manipulated using the CRISPR-dCas9-DNMT3a/TET1 system *in vitro*.

Conclusion: Our data suggest that OPCs within chronically demyelinated MS lesions acquire an inhibitory phenotype, which translates into hypermethylation of crucial myelin genes. Altering the epigenetic status of *MBP* can restore the differentiation capacity of OPCs and possibly boost (re)myelination.

Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS), characterised by a variety of clinical symptoms, such as visual problems, fatigue, muscle stiffness, and cognitive impairment (1). MS is defined by inflammation-induced demyelination during the early stages, which eventually results in gradual neurological disability as the disease progresses (1, 2).

During the progressive stages of MS, when little inflammation is present, endogenous repair mechanisms (remyelination) become exhausted, resulting in the accumulation of chronically demyelinated lesions. Sustained demyelination within such lesions eventually causes loss of axonal density and neurodegeneration, two major contributors to the progressive nature of MS (238). Even though the exact aetiology of progressive MS remains unclear, it is believed that remyelination is hampered in these stages due to the inability of oligodendrocyte precursor cells (OPCs) to differentiate into mature myelinating oligodendrocytes (20). Indeed, despite the abundant presence of OPCs within chronically demyelinated inactive MS lesions, their differentiation towards myelinating oligodendrocytes is attenuated in these demyelinated areas (239). This suggests that OPCs within chronically demyelinated MS lesions acquire a quiescent phenotype, leading to a differentiation block and, thus, ineffective remyelination.

In support of this idea, it has been shown that OPC differentiation is highly dependent on epigenetic regulation, which can be easily influenced by external stimuli from the surrounding microenvironment, such as sustained inflammation and inhibitory factors of the extracellular matrix (196, 240-243). Epigenetic modifications are highly implicated in oligodendroglial biology (196, 204, 244). DNA methylation, for instance, is a stable yet at the same time dynamic epigenetic mark that translates environmental stimuli to alterations in gene expression and subsequent cellular behaviour. We and others have previously shown that DNA methylation contributes to physiological OPC differentiation (245, 246). On top, DNA methylation is also required for remyelination, as shown in a mouse model for focal demyelination (247). This suggests that in the context of progressive MS, disturbed DNA methylation patterns in the oligodendrocyte lineage might be an acquired underlying feature of remyelination failure. Despite many advances in

the field of neuroepigenetics, the number of epigenome-wide association studies (EWAS) conducted on MS brain tissue is very limited. The majority of EWAS studies in MS have been performed on normal-appearing white matter (NAWM) samples, which revealed important changes in DNA methylation prior to myelin damage, but do not show the epigenetic state in actual demyelinated MS lesions, where OPCs acquire a quiescent phenotype resulting in impaired remyelination (53, 248, 249).

In the present study, we performed transcriptomic and epigenomic profiling of chronically demyelinated inactive MS lesions and their corresponding surrounding NAWM in order to investigate which genes could underlie the differentiation block of OPCs within the lesion environment. Cell-specific validation in laser-captured OPCs showed that OPCs within the lesion exhibit a hypermethylated profile of essential myelin genes, such as *MBP*. By applying the CRISPR/dCas9-mediated epigenetic editing toolbox, we validated the causal relationship between the methylation of these myelin genes and the differentiation capacity of human induced pluripotent stem cell (iPSC)-derived oligodendrocytes.

Materials and methods

Sample collection

Human post-mortem brain tissue was obtained through the Netherlands Brain Bank (www.brainbank.nl). Chronic, inactive demyelinated white matter lesions from progressive MS patients (n=10) were selected and characterised for demyelination (Proteolipid protein [PLP^-]), inflammation (Human leukocyte antigen [$HLA\text{-}DR^-$], Oil Red O [ORO^-]), and presence of OPCs (Neural/glial antigen 2 [$NG2^+$]) by immunohistochemistry (245). Lesions were manually dissected from the surrounding NAWM, using the proteolipid protein (PLP) staining as a reference. Slices of 30 µM were made using a cryostat (Leica) and were alternately collected for either RNA or DNA isolation (Figure 5.1a). For laser-capture microdissection and immunohistochemistry, slices of 10 µM were cut, using a CM3050 S cryostat (Leica), and collected on glass microscopy slides.

Transcriptomic profiling

Total RNA was extracted from lesions and their surrounding NAWM, using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA concentrations were analysed with a Nanodrop spectrophotometer (Isogen Life Science). RNA integrity was checked using the Agilent RNA 6000 Pico Bioanalyzer (Agilent Technologies). RNA integrity number (RIN) values ranged between 2,40 and 6,70. Samples were processed and sequenced by the Genomics Core Leuven (Leuven, Belgium). Library preparation was performed using the Lexogen 3'mRNA-Seq Library Prep Kit (Isogen Life Science). Libraries were sequenced on the Illumina HiSeq4000 sequencing system. Quality control (QC) of raw reads was performed with FastQC v0.11.7 (250). Adapters were filtered with ea-utils fastq-mcf v1.05 (251). Using the default parameters, splice-aware alignment was performed with HISat2 against the human reference genome hg38 (252). Reads mapping to multiple loci in the reference genome were discarded. The resulting BAM alignment files were handled with Samtools v1.5 (253). Read counts for each gene were compiled using Rsubread (version 2.8.2) by reading in and processing each bam file. A minimum threshold of 15 counts per million reads for at least 40% of all samples was used to determine whether a gene was expressed, leaving 8399 genes for analysis. The package EdgeR (version 3.36.0) was used to

normalise and transform counts to log counts-per-million, using the Trimmed Mean of M-values (TMM) normalisation method.

Methylomic profiling

Genomic DNA was extracted using a standard chloroform-phenol extraction and ethanol-precipitation method. DNA concentration was assessed with the Qubit dsDNA HS Assay Kit (Invitrogen). A minimum of 500 ng per sample was used for the Illumina Infinium MethylationEPIC array BeadChip (850K), which was carried out by the Epigenomic Services from Diagenode (Liège, Belgium; Cat nr. G02090000). The DNA was deaminated with the EZ-96 DNA Methylation Kit (Zymo Research) according to Illumina's recommended deamination protocol. Methylation data processing and statistical analyses were performed using the programming language R (version 4.1.2.) and RStudio (version 2021.09.1). Raw IDAT files were loaded into R using the minfi package (254). To confirm that matched lesion and NAWM samples were from the same individual, we made use of 59 single nucleotide polymorphism (SNP) probes on the Illumina EPIC array to cluster genetically identical samples. Cell proportion estimates were generated using the Houseman method (255). Samples with a $NeuN^+$ estimation of more than 5% were excluded from the analysis. Cross-hybridizing probes and probes containing SNPs were removed (256). Probe filtering was performed using the *pfilter* function from the wateRmelon package (version 2.0.0) to exclude probes with >1% of samples with a detection p-value >0.05. The remaining data were normalised using the *dasen* function from the wateRmelon package, and probes on the X and Y chromosomes were excluded from the dataset. As principle component analysis (PCA) trait analysis showed a significant correlation with the EPIC chip IDs, we corrected for this batch effect using the *ComBat* function from the *sva* package (version 3.20.0), which applies a Bayesian method to adjust for known batch covariates (257). After data processing, eight lesion and nine NAWM samples remained, as well as 769,804 probes.

Laser captured microdissection

Sections (10 µM) of the human post-mortem MS tissue blocks, covering both lesions and the surrounding NAWM, were mounted on glass cover slides. OPCs were stained using an accelerated protocol to maintain DNA integrity. Briefly,

sections were fixed in ice-cold acetone for 10 minutes and dip-washed in TBS/TBS-T/TBS. Endogenous peroxidase activity was neutralised with 1.5% H_2O_2 in TBS for 10 seconds, followed by a rinse with TBS and a 30-minute blocking step with the Dako Protein Block (Dako) at room temperature. Sliced were incubated with a primary antibody against NG2 (1:200, Abcam Ab101807) for 30 minutes, followed by a quick wash step in TBS. Sections were incubated with horseradish peroxidase (HRP)-conjugated EnVision + Dual Link System (Dako) for 15 minutes, washed with TBS and incubated with an avidin-biotinylated horseradish peroxidase complex for 10 minutes, after which visualisation of the staining was accomplished using 0.3% ammonium nickel sulphate and 0.025% diaminobenzidine (pH 7.8) in TBS. After sequential dehydration steps (30 seconds in 75%-95%-100% ethanol and five minutes in xylene), the samples were ready for immediate laser-captured microdissection using a PALM MicroBeam (Zeiss). 50 cells were isolated per region and collected into 0.1 ml tube caps containing 10 µl PBS.

CRISPR-dCas9 plasmids

Guide design: A specific single guide RNA (sgRNA) was designed to induce (de)methylation within the promoter region of the MBP (chr18:74,690,791-74,691,721) gene using Benchling software (Supplementary Table 1). Guides were synthesised as oligos with overhangs to fit into the BbsI restriction gap and an additional guanine for increased transcriptional efficiency**.**

sgRNA cloning: Guides were cloned into the DNMT3a plasmids (Addgene #71667 and #71684) using a one-step digestion and ligation protocol. Briefly, 100 ng of plasmid was added to a mixture of 1 µM of the annealed guide oligos, 20 U BbsI restriction enzyme (Bioké), 1x cutsmart buffer (Bioké), 400 U T4 ligase (Bioké), 1x T4 ligase buffer (Bioké) and H_2O to an end volume of 20 µl and incubated for 30 cycles of 5 minutes on 37°C and 5 minutes on 23°C. The product was then transformed into NEB 5-alpha Competent E. coli cells (Bioké) and plated on LB-agar plates, supplemented with ampicillin (Amp; 100 mg/ml). Suitable colonies were propagated overnight in LB-Amp medium. Plasmids were extracted using the NucleoBond Xtra Midi kit, according to the manufacturer's protocol (Macherey-Nagel). SANGER sequencing was carried out on purified plasmid vector to validate the sgRNA incorporation. For the TET1 vectors (Addgene #129025 and #129026), we performed subcloning from the DNMT3a vectors using the PvuI and XbaI

restriction enzymes (Thermofisher). One µg of each vector was incubated overnight at 37°C with 10 U of both restriction enzymes, 1x Tango buffer, and H_2O up to a total volume of 50 µl. The samples were loaded on an agarose gel (1%) and both insert (from the DNMT3a vectors), as well as vectors (from the TET1 vectors) were extracted from the gel, using the PCR and gel clean-up kit (Macherey-Nagel), according to the manufacturer's instructions. Inserts and vectors were ligated with the T4 DNA Ligase buffer and enzyme system (Bioké) into the linearized vector in a 2:1 insert to vector molar ratio. Plasmid transformation and purification was performed as described above.

Cell culture and transfection

Human-derived iPSC-oligodendrocytes: Inducible SOX10-overexpressing iPSCs were used to generate O4+ and MBP+ oligodendrocyte cultures as described previously and kindly provided under a mutual transfer agreement (MTA) by Catherine Verfaillie (KuLeuven, Leuven, Belgium) (258, 259). Differentiated iPSC-oligos were frozen in liquid nitrogen and thawed for transfection experiments. Cells were seeded at a density of 250,000 cells/well in a PLO/laminin-coated 24-well plate and maintained in differentiation medium with doxycycline (4 µg/ml). The DNMT3a plasmids were a gift from Vlatka Zoldoš (Addgene #71667 and #71684), and the TET1 plasmids were a gift from Julia K Polansky (Addgene #129025 and #129026). Plasmids were transfected into human iPSC-derived OPCs 48 hours after seeding, using the OZ Biosciences NeuroMag Transfection Reagent (Bio-connect), following the manufacturer's instructions. In brief, 1 µg of plasmid DNA was diluted in 50 µl DMEM/F12 medium, added to 1.75 µl NeuroMag reagent and incubated for 20 minutes at room temperature. DNA/NeuroMag complexes were dropwise added to iPSC-oligo cultures (250,000 cells/well), maintained in differentiation medium, and placed on a magnetic plate for 4 hours in a 5% CO_2 incubator. Medium change with fresh differentiation medium, containing doxycycline (4 µg/ml), was performed 72 hours after transfection. Cells were lysed or fixated on day five post-transfection for further experiments.

Human oligodendroglioma (HOG) cell line: The human oligodendroglioma cell line HOG was maintained in culture medium (DMEM, 10% FCS, 1% P/S) at 37°C and 5% CO_2. For transfection experiments, cells were seeded in poly-L-lysine (PLL, Sigma-Aldrich) coated 24-well plates at a density of 37,500 cells per well. After

attaching to the plate, cells were transfected on the same day as the seeding, using the protocol described above, with a minor adjustment (3 µl NeuroMag reagent and 500 ng DNA per well for 30 minutes on the magnetic plate). Cells were maintained in differentiation medium (DMEM, 1% P/S, 0.05% FCS, 5 µg/ml bovine insulin, 5 µg/ml transferrin, 0.03 nM sodium selenite, 30 nM L-thyroxine; all from Sigma-Aldrich), with one medium change 48 hours after transfection. On day four post-transfection, cells were fixated for further experiments.

Pyrosequencing

Genomic DNA was extracted from laser-captured OPCs, as well as transfected iPSC-OPCs, and bisulfite-converted, using the Zymo Research EZ DNA Methylation-Direct Kit (BaseClear Lab Products). PCR primers were designed using the PyroMark Assay Design 2.0 software (Qiagen, Supplementary Table 2). The assay for *MBP* was tested for its sensitivity using the EpiTect PCR Control DNA Set (Qiagen). Product amplification was performed using the following reaction mixture: 1X Buffer with 20 mM MgCl2 (Roche), 10 mM dNTP mix (Roche), 5 µM forward and reverse primers (Metabion AG), 1U FastStart Taq DNA Polymerase (Roche), bisulfite-converted DNA and nuclease-free water to a total volume of 25 µl. PCR cycling was performed as follows: initial denaturation for 5 minutes at 95 °C, 50 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C and 1 minute at 72 °C; final extension for 7 minute at 72 °C. PCR amplicons were sequenced using the Pyromark Q48 instrument (Qiagen) with the PyroMark Q48 Advanced CpG Reagents (Qiagen), according to the manufacturer's protocol and quantified with the Pyromark Q48 Autoprep software.

Quantitative PCR

Transfected iPSC-OPCs and post-mortem human MS samples were lysed in Qiazol (Qiagen), and RNA was isolated using a standard chloroform extraction and ethanol precipitation method. RNA concentration and quality were analysed with a Nanodrop spectrophotometer (Isogen Life Science). RNA was reverse-transcribed using the qScript cDNA Supermix kit (Quanta). qPCR was performed to analyse gene expression using the Applied Biosystems QuantStudio 3 Real-Time PCR System (Life Technologies). The reaction mixture consisted of SYBR

Green master mix (Life Technologies), 10 µM forward and reverse primers (Integrated DNA Technologies), nuclease-free water and cDNA template (12.5 ng), up to a total reaction volume of 10 µl. The primers used for amplification are listed in Supplementary Table 3. Start fluorescence values were calculated for the human MS sample validation of the RNAseq data. Transfection results were analysed by the comparative Ct method and were normalised to the most stable housekeeping genes (RPL13a and TBP).

Immunocytochemistry

Transfected cells were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Aspecific binding was blocked for 30 minutes with 1% bovine serum albumin (BSA) in 0.1% PBS-T, followed by incubation with primary antibodies (Supplementary Table 4) for four hours at room temperature. After three washing steps with PBS, cells were incubated with Alexa 488- or Alexa 555-conjugated secondary antibody (Supplementary Table 4) for one hour. Nuclei were counterstained with 4'6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Coverslips were mounted with Dako mounting medium (Dako) and analysed using a fluorescence microscope (Leica DM2000 LED). Per coverslip, three images were quantified using Fiji ImageJ software.

Statistical analysis

Differential expression analysis was performed using the *limma* package (version 3.50.0). Age, sex, and post-mortem interval (PMI) were included as covariates and individual was treated as a random-effect variable, using the *dupplicateCorrelation* function from the *limma* package. P-values were FDR-corrected for multiple testing to determine differentially expressed genes (DEG, FDR p-value < 0.05 and absolute fold change >1.5) between lesion and NAWM samples.

We extracted all the CpG probes from the Illumina methylationEPIC array that were annotated (Illumina UCSC annotation) to DEGs from the RNAseq analysis. Out of the 769,804 probes, 29,446 probes were used as input for the differential methylation analysis using the same approach as for the DEG analysis. The *dupplicateCorrelation* function from the *limma* package (version 3.50.0) was

applied to block individual as a random effect. Age, sex, and PMI were included as covariates in the regression model and FDR correction for multiple testing was applied to the nominal p-values to identify differentially methylated probes (DMPs, FDR p-value < 0.05).

All DE genes that contained a DMP were subjected to a Pearson's correlation analysis between expression (LogCPM) and methylation (bèta values) levels. A final list of genes that displayed differential expression and methylation, and a significant correlation between both expression and methylation, was used as input for a gene ontology (GO) analysis using the *enrichGO* function from the *clusterProfiler* package (version 4.2.2), focusing on the 'Biological Process' ontologies. An overview of the data analysis workflow is provided in Figure 5.1b.

Statistical analysis of the transfection and pyrosequencing experiments was performed using GraphPad Prism 9.0.0 software (GraphPad software Inc., CA, USA). Differences between group means were determined using an unpaired t-test for normally distributed data and a Mann-Whitney test for not normally distributed data. Differences in methylation at different CpG sites were determined using a two-way repeated measures ANOVA with Šídák's multiple comparisons test. All data are depicted as mean ±SEM, *=p≤0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

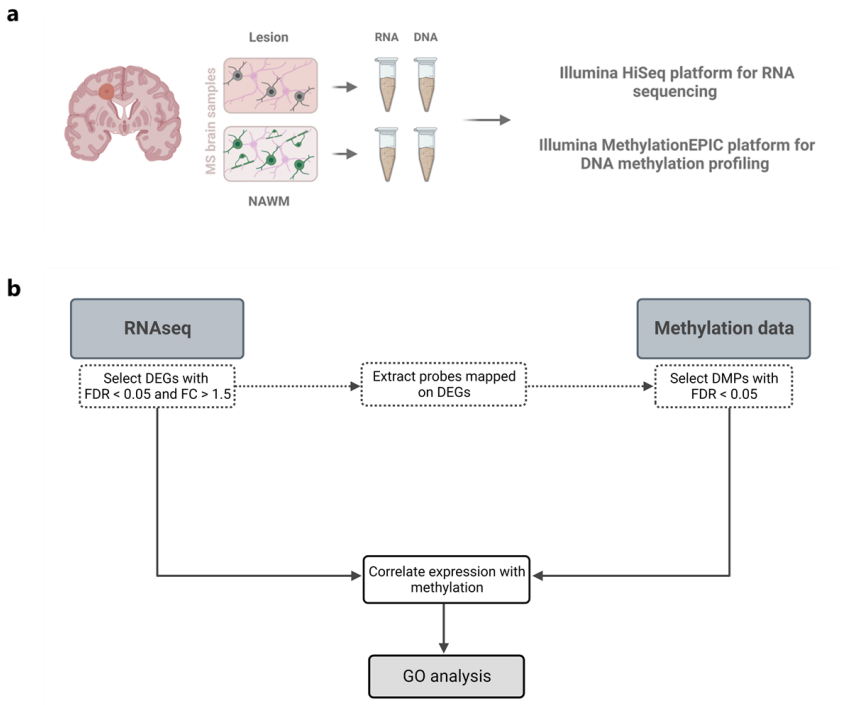


Fig. 5.1 | Overview of the sample preparation and data analysis workflow. a Multiple sclerosis (MS) lesions were dissected from the surrounding normal appearing white matter (NAWM), and both were collected for RNA and DNA isolation. Transcriptomic and methylomic profiling was carried out using the HISeq sequencing and Illumina MethylationEPIC array platform, respectively. **b** Illustration of the data analysis workflow integrating the transcriptomic and methylomic datasets. NAWM: normal-appearing white matter, DEGs: differential expressed genes, FDR: false discovery rate adjusted p-value, FC: fold change, DMPs: differential methylated probes, GO: gene ontology.

Results

Transcriptomic profiling of chronic inactive demyelinated MS lesions and the surrounding NAWM

Bulk RNA sequencing was performed on chronically demyelinated MS lesions and the corresponding surrounding NAWM. After stringent data pre-processing and QC filtering, 17 samples (9 lesions and 8 NAWM) were included in the RNA data analysis. Gene clustering based on absolute expression levels indicated clustering of the lesions separately from the NAWM (Fig. 5.2a). PCA based on the logCPM values showed that 63% of the variance could be explained by PC1, which highly correlated ($p = 0.00059$) with the sample group (Fig. 5.2b). Out of the total of 8,399 genes that were subjected to a differential gene expression analysis, 641 genes were found to be significantly differentially expressed between lesion and NAWM, with an absolute fold change above 1.5 (Fig. 5.2c, Supplementary Data S5.1). Interestingly, the distribution was roughly balanced between upregulated (242) and downregulated (399) genes.

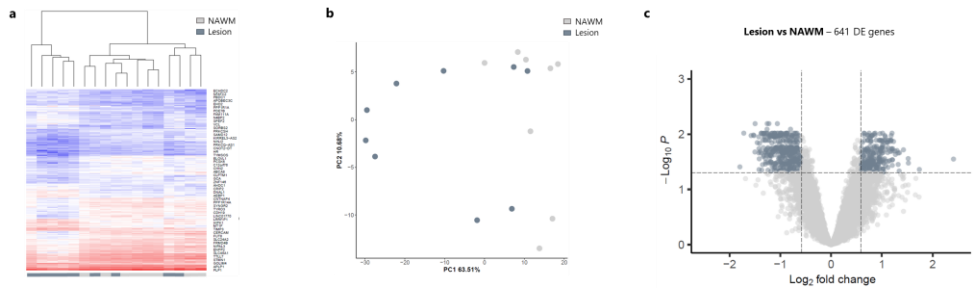
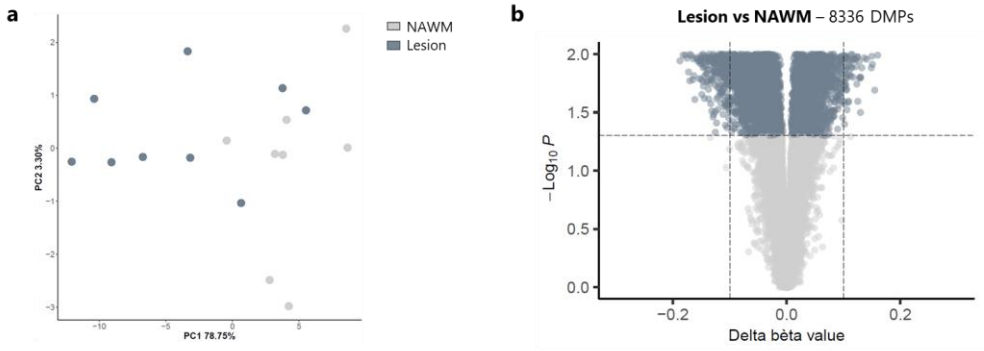


Fig. 5.2 | Chronically demyelinated lesions are transcriptionally distinct from the surrounding normal appearing white matter (NAWM). Based on the transcriptomic profile, chronic multiple sclerosis (MS) lesions can be distinguished from the surrounding NAWM, as determined by (**a)** gene clustering based on absolute expression levels and (**b)** a principle component analysis (PCA). **c** Differential expressed genes (DEGs) analysis revealed 641 genes that are significantly differentially expressed between lesion and NAWM (FDR p-value < 0.05), with an absolute fold change above 1.5.

Genes involved in glial cell development and myelination are differentially methylated in chronic MS lesions

An EWAS was conducted using the Illumina methylationEPIC array to analyse the DNA methylation state of the chronically demyelinated lesions and NAWM samples. PCA revealed clustering of the samples based on the methylation bèta values, similar to those observed in the RNA sequencing data (Fig. 5.3a). Out of the 769,804 CpGs that passed the initial quality control, 29,446 CpG sites were annotated to the DEGs from the transcriptome analysis. Differentially methylation analysis of these genes showed that 8,336 CpG positions were significantly (FDR p-value < 0.05) differentially methylated between lesions and NAWM (Fig. 5.3b, Supplementary Data S5.2). These differentially methylated positions (DMPs) were then subjected to a correlation analysis with the matching expression data. Interestingly, 508 genes showed a significant (FDR-adjusted p-value < 0.05) and strong correlation between their expression and methylation profile (Supplementary Data S5.3). Fig. 5.3c shows the top ten correlating CpGs, nine of which showing a strong negative correlation between DNA methylation and RNA expression. The final set of 512 genes, which were differentially expressed, differentially methylated and correlated between both expression and methylation, was used for the GO analysis, with a focus on Biological Process (Fig. 5.3d). Clustering of the significantly enriched GO terms showed two main clusters, related to glial cell development/myelination and cytoskeleton organisation (Fig. 5.3d).



c

Probe ID	Gene	Pearson's *r*	P-value	FDR adjusted p-value
cg12139813	BTBD3	-0.959261755	1.23E-09	5.11E-06
cg23017573	PIP4K2A	-0.960319767	1.01E-09	5.11E-06
cg14541848	SEMA4D	-0.954591255	2.73E-09	7.58E-06
cg23874669	DDR2	0.948755478	6.64E-09	1.29E-05
cg12126243	DAAM1	-0.945613192	1.03E-08	1.29E-05
cg19094243	TMEFF2	-0.945180383	1.09E-08	1.29E-05
cg23644066	RNF220	-0.947357677	8.09E-08	1.29E-05
cg02249322	PCSK6	-0.942158496	1.62E-08	1.64E-05
cg16100530	SH3GL3	-0.942155344	1.62E-08	1.64E-05
cg26360766	LPAR1	-0.938317066	2.59E-08	2.34E-05

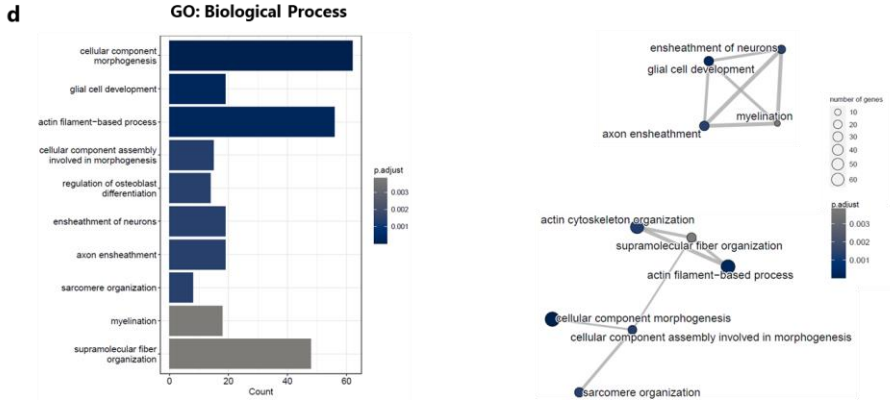
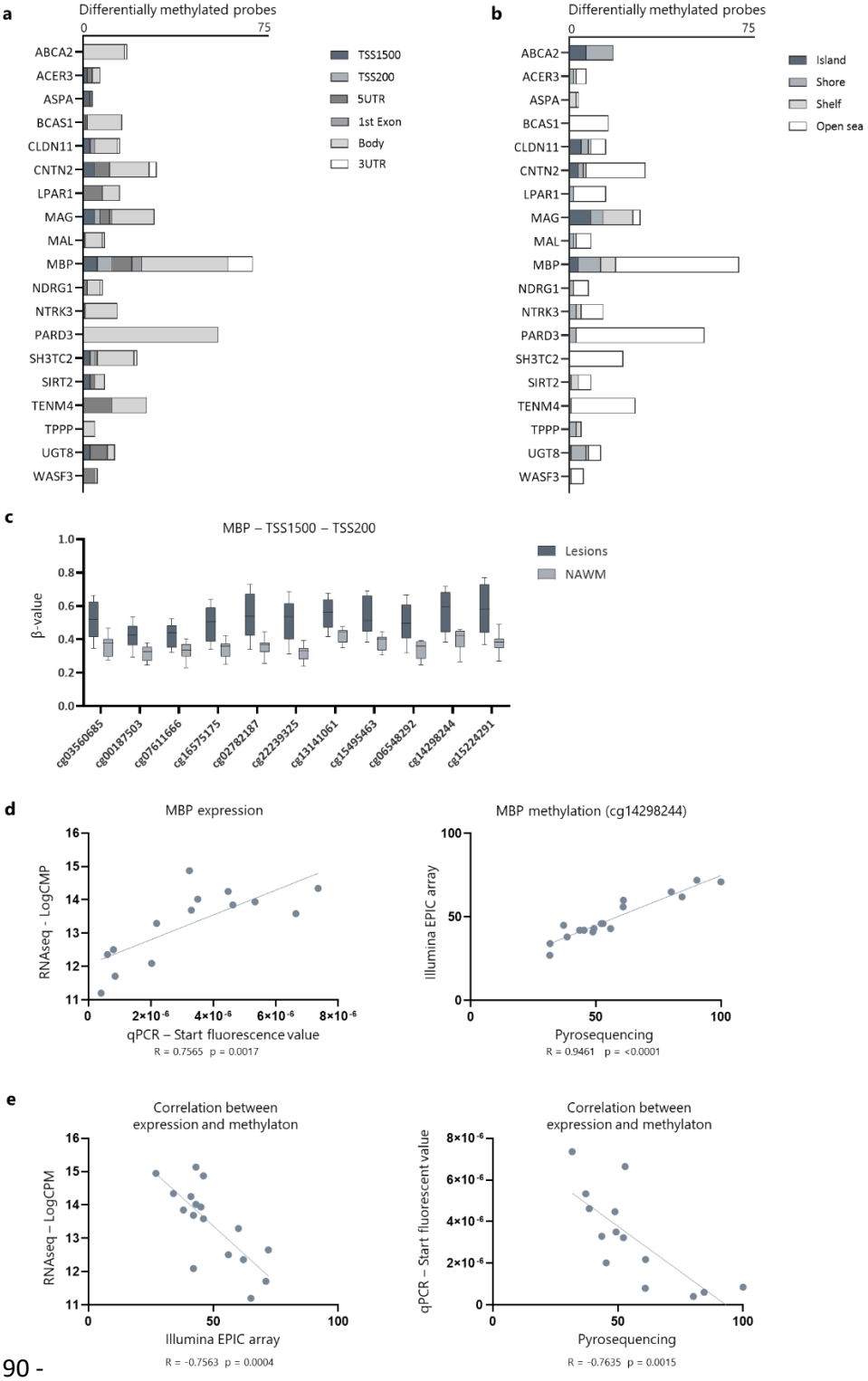


Fig. 5.3 | Differentially methylation analysis between lesions and NAWM reveals enriched gene ontologies (GOs) related to glial cell development and myelination. a Principal component analysis (PCA) shows the clustering of the samples based on the methylation bèta values. **b** Out of the 29,446 analysed CpG sites, 8,336 CpGs are differentially methylated between lesions and normal appearing white matter (NAWM; FDR<0.05). **c** Pearson's correlation analysis between methylation and expression levels of the significantly differentially methylated CpGs. **d** Gene ontology analysis of the 512 genes that were differentially expressed and correlated significantly with their differential methylated probes (DMPs) revealed two main significantly enriched clusters related to the cytoskeleton and glial cell development/myelination.

As we are particularly interested in the contribution of DNA methylation to (re)myelination in the MS lesions, we focused on the genes that were part of the enriched GO clusters related to glial cell development/myelination (GO:0021782, GO:0007272, GO:0008366, GO:0042552). We explored the distribution of those DMPs across gene features (Fig. 5.4a) and CpG-related island features (Fig. 5.4b). Interestingly, the gene *MBP*, coding for myelin basic protein, the second most abundant protein in central nervous system myelin, did contain the highest number of DMPs in general as well as the highest number of DMPs that were located in the promotor region (TSS1500, TSS200) (Fig. 5.4a). An essential portion of these DMPs was furthermore situated in CpG islands or shores (Fig. 5.4b). Interestingly, all the CpGs within the promotor region of the gene were consistently hypermethylated in lesions compared to the surrounding NAWM (Fig. 5.4c). To technically validate our findings from the RNAseq and EWAS data, we performed targeted analysis of the expression and methylation profile of *MBP* using qPCR and pyrosequencing, respectively. The correlation analyses for both expression and methylation showed a strong and significant correlation between the two techniques, serving as a robust validation of the RNA sequencing and EWAS discovery data (Fig. 5.4d). Furthermore, we observed a significant negative correlation between *MBP* expression and methylation levels (Fig. 5.4e). Altogether, these data suggest an important role of DNA methylation for the regulation of *MBP* expression.


a
Differentially methylated probes
0
75
ABCA2
ACER3
ASPA
BCAS1
CLDN11
CNTN2
LPAR1
MAG
MAL
MBP
NDRG1
NTRK3
PARD3
SH3TC2
SIRT2
TENM4
TPPP
UGT8
WASF3
TSS1500
TSS200
5UTR
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Differentially methylated probes
0
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Island
Shore
Shelf
Open sea
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MBP – TSS1500 – TSS200
β-value
Lesions
NAWM
cg03560685
cg00187503
cg07611666
cg16575175
cg02782187
cg22239325
cg13141061
cg15495463
cg06548292
cg14298244
cg15224291
d
MBP expression
RNAseq - LogCMP
qPCR – Start fluorescence value
R = 0.7565 p = 0.0017
MBP methylation (cg14298244)
Illumina EPIC array
Pyrosequencing
R = 0.9461 p = <0.0001
e
Correlation between expression and methylaton
RNAseq – LogCPM
Illumina EPIC array
R = -0.7563 p = 0.0004
Correlation between expression and methylaton
qPCR – Start fluorescent value
Pyrosequencing
R = -0.7635 p = 0.0015

Fig. 5.4 | In-depth overview of the genes that are part of the enriched GO clusters related to glial cell development/myelination (GO:0021782, GO:0007272, GO:0008366, GO:0042552) Distribution of DMPs within the GO clusters related to myelination across gene features **(a)** and CpG-related island features **(b)**. The height of the bars represents the number of DMPs annotated to the gene. **c** The beta values of the DMPs located in the promoter region (TSS1500, TSS200) of *MBP* indicate consistent hypermethylation within lesions compared to the surrounding NAWM. **d** Technical validation of the expression and methylation levels of MBP, as determined by qPCR and pyrosequencing, respectively. Pearson's correlation analysis shows a significant correlation between both techniques on the expression level, as well as DNA methylation level (n=14). **e** Expression and methylation levels of *MBP* are significantly negatively correlated (Pearson's correlation analysis), both for array-based techniques (RNAseq, Illumina EPIC array), as well as targeted techniques (qPCR, pyrosequencing) (n=14).

Cell-type-specific validation indicates hypermethylation of *MBP* in OPCs obtained from lesions, compared to NAWM-derived OPCs

The methylation signature within MS lesions suggests a potential differentiation and (re)myelination block, directly acting on essential myelin genes, such as *MBP*. However, as the Illumina EPIC array was performed on bulk tissue, the observed degree of methylation of *MBP* could be explained by cellular heterogeneity of the samples. As we were particularly interested in whether there is a contribution of OPCs to the observed epigenetic signature of *MBP*, we stained OPCs within the samples, laser capture micro-dissected, and collected them for targeted methylation analysis of the *MBP* promotor region by means of pyrosequencing (Fig. 5.5a). Strikingly, we again observed a hypermethylated profile in OPCs obtained from lesions compared to OPCs that were located in the NAWM (Fig. 5.5b,c).

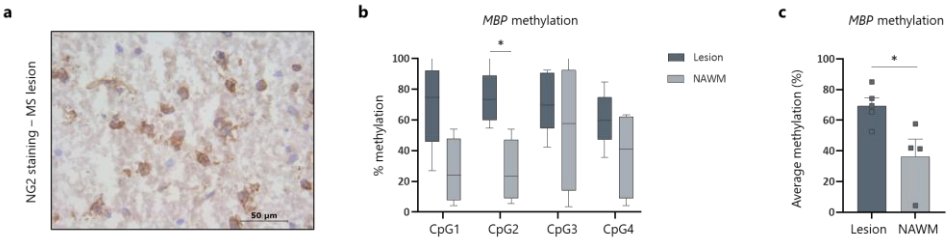

Fig. 5.5 | Cell-specific validation of the hypermethylated profile of *MBP* within OPCs derived from lesions, compared to OPCs isolated from the surrounding NAWM. a OPCs were stained for the NG2 marker and laser capture microdissected from either lesions of NAWM. Batches of 50 cells per sample were subjected to bisulfite pyrosequencing to determine the methylation profile of the *MBP* promoter region. **b, c** OPCs within the promoter region of *MBP* show a hypermethylated profile compared to OPCs isolated from the NAWM (n=4-5, two-way ANOVA with Šídák's multiple comparisons test for b and Wilcoxon test for c). Data are represented as mean ±SEM, *p<0.05.

Targeted epigenetic editing of the *MBP* gene influences the differentiation capacity of human iPSC-derived oligodendrocytes and human oligodendroglioma cells

As we discussed elaborately in our recently published perspective (260), most EWAS observations remain correlational, making it difficult to infer a cause-effect relationship. In the last couple of years, epigenetic editing, i.e., altering the epigenome by directing e.g. DNA methylation at a specific site, has grown as a powerful tool to further study the role of epigenetics in health and disease, especially in view of addressing causality. Hence, we applied CRISPR-dCas9-based epigenetic editing to investigate potential cause-and-effect relationships for epigenetic alterations of *MBP* regarding oligodendrocyte differentiation. A sgRNA was designed to target the promotor region of *MBP* and cloned into CRISPR-dCas9-DNMT3a or CRISPR-dCas9-TET1 vectors, to respectively methylate or demethylate the CpG sites within the *MBP* promotor region. We explored the impact of epigenetic editing on human iPSC-derived oligodendrocyte differentiation. As MBP is a solid marker for mature oligodendrocytes, we performed immunostaining for MBP to observe the effects on the protein level, as well as to visualise and assess the cellular morphology (Fig. 5.6a,b). Cells transfected with an active TET1 construct targeting *MBP* showed increased MBP protein expression (as determined by the MBP-positive area per transfected cell). Human iPSC-derived oligodendrocytes that were transfected with the DNMT3a construct to methylate the *MBP* promotor showed a tendency towards decreased MBP expression. To evaluate the differentiation capacity of the transfected cells, we furthermore performed a Sholl analysis (Fig. 5.6c-f). Analysis of the ending radius (Fig. 5.6d), the sum of intersections (Fig. 5.6e) and the average number of intersections per Sholl ring (Fig. 5.6f) all showed that modulation of the *MBP* promotor methylation status influences cellular differentiation. Interestingly, we observe an overall more pronounced effect in the TET1-mediated demethylation experiments compared to DNMT3a-driven targeted methylation. In line, we observed a trend towards lower methylation levels and higher expression levels of *MBP* after targeted demethylation (Fig. 5.6g-h). However, next to the low statistical power, our heterogeneous bulk cultures consisted of both transfected as well as untransfected cells, leaving our expression and methylation results confounded by the background noise of unmodified cells. Off note, the functional

readouts, which were based on transfected cells only, showed strong and significant results after epigenetic editing of *MBP*. To validate our findings, we also transfected a human oligodendroglioma cell line with the epigenetic editing vectors (Fig. 5.6i). Similar effects were observed as to iPSC-derived oligodendrocytes, both in terms of cellular complexity (Fig. 5.6j) and MBP fluorescence area (Fig. 5.6k) of the transfected cells. Altogether, these results show that by altering the methylation profile of the *MBP* gene, it is possible to influence the differentiation capacity of human oligodendrocytes.

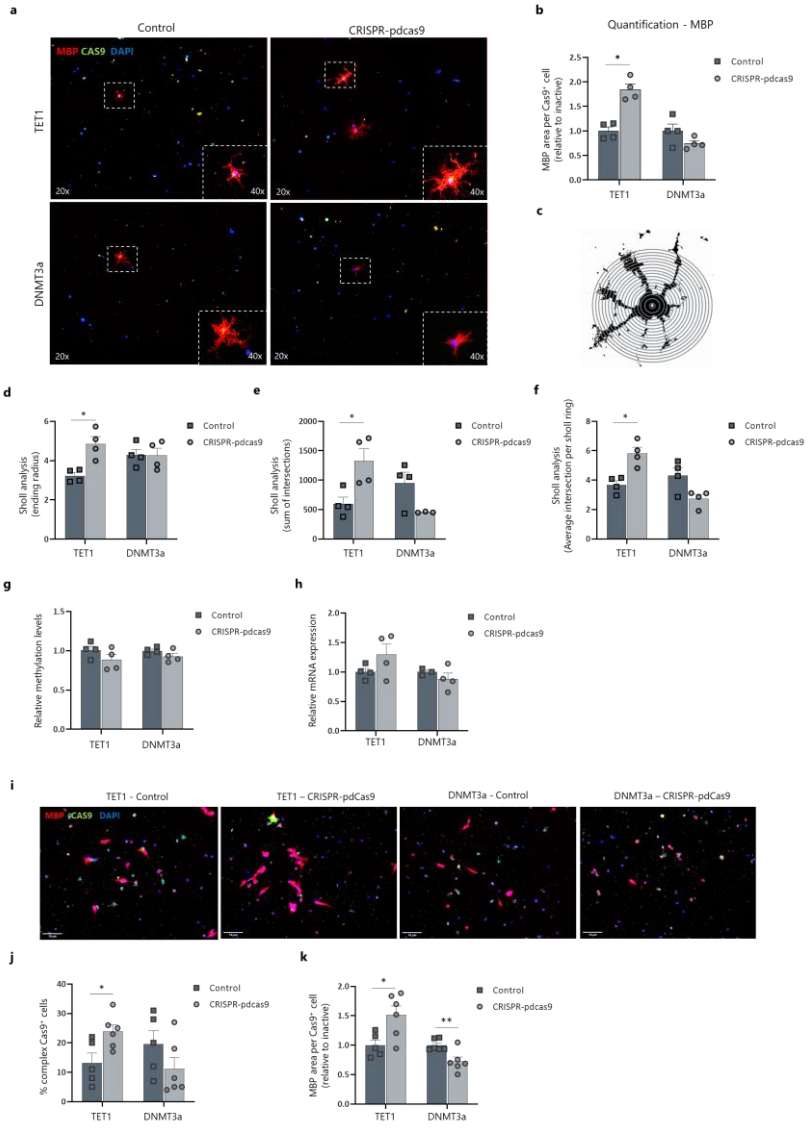

Fig. 5.6 | Epigenetic editing of the *MBP* promotor region in human iPSC-derived oligodendrocytes and a human oligodendroglioma (HOG) cell line influences the differentiation capacity. Human iPSC-derived oligodendrocytes and human oligodendroglioma cells were transfected with either a CRISPR-pdCas9-DNMT3a or CRISPR-pdCas9-TET1 vector to methylate or demethylate the promotor region of the *MBP* gene. Inactive constructs harbouring a catalytical inactive DNMT3a or TET1 were used as control. **a** Representative image of transfected human iPSC-derived oligodendrocytes. **b** Quantification (MBP fluorescence area) of transfected human iPSC-derived oligodendrocytes show an effect on MBP protein expression after epigenetic editing (n=4, Wilcoxon test). **c-f** Representation and quantification of the sholl analysis of transfected iPSC-derived oligodendrocytes (n=4, Wilcoxon test). **g** Methylation analysis of the *MBP* promotor region in transfected cells (n=4). **h** Gene expression analysis showed a tendency towards an altered expression profile of *MBP* after targeted (de)methylation. Data are corrected for the most stable housekeeping genes (RPL13a and TBP), n=4. **i-k** Representative images and quantification (complexity and MBP fluorescence area) of transfected human oligodendroglioma cells also show an impact of epigenetic editing on cellular behaviour (n=6, unpaired t-test). Data are represented as mean ±SEM, *p<0.05, **p<0.01.

Discussion

In the current study, we investigated the transcriptomic and epigenomic profile of chronically demyelinated lesions and the surrounding NAWM from 10 donors, with the final goal of understanding the molecular mechanisms underlying the hampered differentiation capacity of OPCs within the MS lesion microenvironment. We found 641 genes to be differentially expressed between lesions and NAWM. Subsequent methylation analysis on this geneset revealed a total of 8,336 CpGs located on 512 different genes displaying differential methylation between lesions and NAWM. Gene ontology analysis revealed enriched clusters of genes related to glial cell development and myelination. We then further explored *MBP*, the gene with the highest number of DMPs within the promotor region among these clusters. This gene displayed decreased expression as well as hypermethylation in lesions. Cell-specific validation of *MBP* methylation in lesion-derived OPCs revealed a similar hypermethylated profile compared to NAWM-derived OPCs. Finally, we functionally validated the influence of *MBP* methylation on oligodendrocyte differentiation by means of epigenetic editing.

The involvement of DNA methylation in oligodendrocyte differentiation has been investigated previously by us and other colleagues (196, 245, 246, 261). Using rodent-derived OPCs or mouse models for MS, it has been shown that the presence of DNA methylation enzymes, such as DNMT1 and DNMT3a, is crucial for oligodendrocyte differentiation during development and remyelination (246, 247). Furthermore, we have recently established that the myelin regulatory pathway is under epigenetic control during physiological OPC differentiation (245). Yet, the direct impact of DNA methylation in relation to remyelination failure in MS remained to be investigated. One of the first studies to use an epigenome-wide approach investigated methylomic alterations within NAWM brain samples of MS patients and compared them to matched non-neurological white matter control samples (53). Pathology-free MS samples show differentially methylated regions within genes related to oligodendrocyte development and survival. In line with this notion, recent studies by Kular *et al.* showed specific DNA methylation profiles of neuronal and glial cells isolated from the NAWM of post-mortem MS brains (248, 249). As for lesions, one study has investigated the difference in the methylation patterns between demyelinated and intact hippocampi of progressive MS patients

using the Illumina Methylation 450K array. Chomyk et al. elegantly highlighted several DMPs related to neuronal survival and memory function yet did not reveal any methylation changes related to oligodendrocyte biology (262). Altogether, it is evident that the DNA methylation is affected in MS post-mortem brain tissue, but how this relates to the block on OPC differentiation in chronically demyelinated lesions has remained unclear up to now.

In the present study, we aimed to investigate the methylomic signature of chronically demyelinated MS lesions in order to understand the direct contribution of DNA methylation to the hampered differentiation state of OPCs within these lesions. One of the main strengths of this study is the unique within-comparison between lesions and their surrounding NAWM isolated from each patient. This setup increased our statistical power and allowed us to investigate DNA methylation changes specifically related to the lesion microenvironment, where OPC differentiation is hampered. Furthermore, we examined both transcription and DNA methylation in these samples, allowing us to directly correlate our transcriptional data to the methylation profile of these genes.

Our GO analysis based on genes that displayed both differential expression and methylation, as well as a significant correlation between these two features, revealed two main clusters, i.e., 'cytoskeleton organisation' and 'glial cell development and myelination'. Genes within these clusters ranged from important myelin genes (*MBP, MAG*) and genes that regulate myelin formation (*CNTN2*, *LPAR1*) or OPC differentiation (*PARD3*, *BCAS1*), to genes important for lipid metabolism (*UGT8*, *ABCA2*) (263-268). Intriguingly, we found *MBP* to contain both the highest number of DMPs overall and the highest number of DMPs located within the promotor region and on CpG islands or shores. Moreover, all the probes within the promotor region of *MBP* were consistently hypermethylated in lesions compared to the surrounding NAWM. One could advocate that the *MBP* gene would be an obvious suspect to be altered within a demyelinated lesion. Yet again, interestingly, *MBP* has also been shown to be hypermethylated in NAWM samples of MS patients compared to non-neurologic controls (53). These findings suggest a possible step-wise methylation change in the *MBP* gene, already initiated in pathology-free regions and becoming more pronounced in the actual lesion site, where myelin damage has already occurred. Interestingly, *MBP* has also been shown to be differentially methylated in other neurodegenerative diseases with

white matter pathology, such as Alzheimer's disease (AD). A recent meta-analysis, which combined data from six independent brain AD methylation studies (n=1,453 individuals), investigated the methylation profile of 485,000 CpG sites, of which one of the differentially methylated CpG sites in the prefrontal cortex reaching genome-wide significance was located within the *MBP* gene (269). Altogether, this emphasises the importance of DNA methylation in the regulation of *MBP* expression and its susceptibility to changes during disease.

The observed hypermethylated profiles of *MBP* within NAWM and MS lesions represent interesting independent observations, yet they could potentially be explained by differences in cellular composition between patients and controls and between lesions and NAWM, respectively (53). We however hypothesized that the epigenetic block on *MBP* was present in OPCs within lesions, thereby inhibiting their differentiation into myelin-forming oligodendrocytes. As such, we laser-captured OPCs both from lesions as NAWM samples and performed bisulfite pyrosequencing of the *MBP* promotor region. Altogether, these results indicate that the *MBP* promotor becomes hypermethylated in OPCs located within the lesion microenvironment, possibly preventing them from differentiating into mature oligodendrocytes.

These observations regarding *MBP* methylation in MS lesions and lesion-derived OPCs are novel and unexpected, yet remain correlational. As we have suggested previously, it is important to investigate the potential cause-and-effect relationship between epigenetic signatures and functional read-outs, such as oligodendrocyte differentiation (260). Over the past years, epigenetic editing, using CRISPR-dCas9 engineered systems, has proven to be a powerful tool to provide evidence of functional consequences of epigenetic changes at specific loci (270). In the present study, we made use of both a CRISPR-dCas9-TET1 and a CRISPR-dCas9-DNMT3a vector to target and demethylate or methylate, respectively, the *MBP* promotor region with the final aim of assessing the influence on oligodendrocyte differentiation capacity (271, 272). We used two cell culture models, i.e. human iPSC-derived oligodendrocytes and HOG cells, which we transfected with the epigenetic editing vector to target the *MBP* gene, and assessed the effects on MBP protein expression and cellular morphology. As a control for transfection and steric hindrance, we transfected cells with a catalytic inactive version of the vectors that are unable to (de)methylate. Interestingly, we

observed significant functional effects after targeted demethylation of the *MBP* gene, resulting in higher MBP expression and a more differentiated cellular morphology, both in iPSC-derived oligodendrocytes and in the HOG cell line. Targeted methylation showed less pronounced effects, yet did reveal a consistent trend towards reduced MBP protein expression and lower cellular complexity. A possible explanation for this could be that the baseline default methylation status of both cell culture types already levelled around 80%, leaving little room for effects of additional (hyper)methylation by the CRISPR-dCas9-DNMT3a vector.

Collectively, our data demonstrate strong differences in DNA methylation between chronically demyelinated MS lesions and the NAWM, which furthermore correlate with the expression profile of the corresponding DEGs. We identified an epigenetic block on *MBP* within OPCs located in the lesions and showed that this could have a major impact on the differentiation capacity of these cells. Notably, more than 8,000 CpG sites displayed differential methylation within MS lesions, with numerous of them potentially impacting upon cellular behaviour within the lesion site. It is therefore important to further characterise MS-associated epigenetic signatures, preferably in a cell-type-specific manner, in order to fully understand the contribution of DNA methylation to remyelination failure in progressive MS stages. Which specific molecules and factors within the microenvironment of demyelinated lesions drive the observed epigenetic changes remains to be elucidated. Our study represents a starting point for important research regarding DNA methylation signatures in chronic MS lesions with the final aim to discover new targets to restore the remyelination capacity in the progressive MS stages.

Supplementary information

Supplementary Table S5.1 - Guide RNA targeting MBP

	Forward oligo (5'-3')	Reverse oligo (5'-3')	Off-target score
MBP sgRNA	ACTGACTCCAAGCGCACAG	CTGTGCGCTTGGAGTCAGTC	63

Supplementary Table S5.2 - Pyrosequencing primer list

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Sequencing primer (5'-3')	Genomic coordinates	Number of CpGs covered	Reference genome
MBP	GTTTGGTAGGATGTTTATTTAGTTGA	TCTATAACCCCATCACATCCAAACTCTC	GGATGTTTATTTAGTTGATTTAGG	chr18: 77016996-77017182	4	GRCh37 (hg19)

Supplementary Table S5.3 - qPCR primer list

	Forward oligo (5'-3')	Reverse oligo (5'-3')
MBP human	AAGACAGGCCCTCTGAGTCC	GGAGGGTCTCTTCTGTGACG
TBP human	TATAATCCCAAGCGGTTTGC	GCTGGAAAACCCAACTTCTG
RPL13a human	AAGTTGAAGTACCTGGCTTTCC	GCCGTCAAACACCTTGAGAC

Supplementary Table S5.4 - Antibody list

Antigen	Company (reference number)	Dilution
MBP (iPSC-oligo's)	Merck (MAB386)	1:500
MBP (HOG cells)	MilliporeSigma (AB980)	1:500
CAS9	Merck (MAC133)	1:1000
Goat anti-mouse IgG	Life Technologies (AB_2534069)	1:600
Goat anti-rat IgG	Life Technologies (A21042)	1:600
Goat anti-rabbit IgG	Life Technologies (A27039)	1:600

Supplementary Table S5.1 – Top 100 differential expressed genes between MS lesions and NAWM

Gene	logFC	AveExpr	t	P.Value	adj.P.Val
USP32P2	1.25823099	3.92743783	6.25889685	1.6528E-06	0.00646743
KLHL32	1.05859356	6.43731719	6.14710947	2.1779E-06	0.00646743
HS3ST5	1.49686001	3.60983135	6.02911843	2.9183E-06	0.00646743
HAGLR	1.22394726	3.8907478	6.00742916	3.0801E-06	0.00646743
CLASP2	0.79909723	7.58694172	5.79290782	5.2642E-06	0.00815848
CNOT2-DT	1.26169314	3.93003791	5.7523627	5.8282E-06	0.00815848
CPEB2	0.8740346	5.32321546	5.60150615	8.5216E-06	0.00961256
ELMO1	1.04036328	5.84409089	5.53015178	1.0206E-05	0.00961256
PLD1	1.31558753	5.92102907	5.45242817	1.2426E-05	0.00961256
UNC5C	0.9955468	5.04190957	5.4141829	1.3692E-05	0.00961256
CYP2J2	1.06662353	4.17891905	5.3499863	1.6117E-05	0.00961256
MOG	1.71979541	5.84094543	5.32656571	1.7107E-05	0.00961256
CTNNA3	1.16260446	6.63582068	5.28575994	1.8979E-05	0.00961256
MBNL2	0.67181466	7.86317329	5.28532032	1.9E-05	0.00961256
PTPRK	0.70968524	5.15443344	5.20128367	2.3539E-05	0.00961256
COLGALT2	0.83947417	6.89352203	5.18045883	2.4823E-05	0.00961256
USP32P3	0.97960906	5.43527453	5.16693804	2.5695E-05	0.00961256
REEP3	0.86996254	7.58086617	5.16262926	2.5979E-05	0.00961256
DNM3	1.11633938	8.11041551	5.13611977	2.7799E-05	0.00961256
SLITRK2	0.87100588	5.15566313	5.1233503	2.8721E-05	0.00961256
RASGRP3	1.37074839	5.94556519	5.08827272	3.1415E-05	0.00961256
LOC101930421	1.37751306	4.14798796	5.07346326	3.2627E-05	0.00961256
CNTNAP4	1.28338634	6.71518726	5.06625744	3.3234E-05	0.00961256
SPTLC2	0.99797075	7.57847196	5.06073028	3.3707E-05	0.00961256
USP54	0.73848535	6.86599195	5.05210214	3.446E-05	0.00961256
SILC1	1.26474502	7.16112078	5.04847542	3.4781E-05	0.00961256
ABCA8	1.11007816	6.46831903	5.0404321	3.5504E-05	0.00961256
SLC44A1	0.90555429	9.89334578	5.01078274	3.8303E-05	0.00969665
PDE1C	1.32234302	7.66980189	4.99683331	3.9696E-05	0.00969665
FOLH1	1.24805274	5.79417991	4.97237909	4.2262E-05	0.00969665
PLCL1	1.14680514	6.83299805	4.96733278	4.2811E-05	0.00969665
CD47	0.69229537	6.7338353	4.95778856	4.3871E-05	0.00969665
MVK	1.33100521	4.05021907	4.94593027	4.5224E-05	0.00973949

RASGRF2	0.90022663	5.30959689	4.92150045	4.8146E-05	0.0098049
PROX1	0.95566233	6.75119502	4.90532749	5.0184E-05	0.0098049
MOBP	1.35018343	12.0536142	4.88124161	5.3381E-05	0.0098049
DOCK10	1.10487443	7.31324403	4.8795357	5.3615E-05	0.0098049
SPOCK3	1.18373392	6.63463837	4.87892146	5.37E-05	0.0098049
CNDP1	1.53018055	7.8510607	4.83195095	6.0576E-05	0.01040087
PPP2R3A	0.79578271	5.83093932	4.82602462	6.1504E-05	0.01040087
DACT3	0.98489839	5.23903309	4.82518229	6.1637E-05	0.01040087
EDIL3	1.14645267	8.61721281	4.79437489	6.6707E-05	0.01063261
LRRC8D	0.87543132	5.93249777	4.77037607	7.0945E-05	0.01063261
CLDND1	1.17844952	7.94298668	4.76489355	7.1951E-05	0.01063261
ATP8A1	1.00568333	7.68905518	4.75904114	7.304E-05	0.01063261
LRP2	1.51818712	7.32829521	4.75460688	7.3876E-05	0.01063261
SECISBP2L	0.75823139	9.32882733	4.73700183	7.7292E-05	0.01063261
PRRG1	1.00166268	6.73307252	4.72380253	7.9956E-05	0.01063261
TMEM165	1.01116793	7.42777484	4.72325106	8.0069E-05	0.01063261
PEX5L	0.95818724	8.25193288	4.711616	8.2497E-05	0.01063261
ARHGEF37	1.0819822	5.65422884	4.70399561	8.4128E-05	0.01063261
FAM107B	1.00237321	9.25400049	4.70000887	8.4993E-05	0.01063261
TMC7	1.19201676	4.66793017	4.69052848	8.7088E-05	0.01063261
TRAFD1	1.02809165	4.22618994	4.67687316	9.0196E-05	0.01063261
ANLN	1.16836362	9.12740124	4.67680571	9.0211E-05	0.01063261
GCA	0.94907131	5.49168922	4.66740824	9.2415E-05	0.01063261
APBB2	1.02071265	5.99897652	4.65944318	9.4325E-05	0.01063261
ALCAM	0.90878923	8.97722256	4.65724504	9.4859E-05	0.01063261
NT5DC1	0.6489285	6.3677861	4.6487015	9.6963E-05	0.01063261
TF	1.22806135	10.5635754	4.64455542	9.8001E-05	0.01063261
ENPP2	1.20543847	8.16372181	4.63342974	0.00010084	0.01063261
CHN2	0.89464961	6.22904991	4.63100135	0.00010147	0.01063261
BTBD3	1.0141059	5.42436793	4.62618259	0.00010274	0.01063261
PCSK6	1.57561943	5.76919251	4.62123171	0.00010405	0.01063261
SLC22A23	0.62676871	6.48751804	4.61764627	0.00010501	0.01063261
ACER3	0.76822136	7.025997	4.61742844	0.00010507	0.01063261
PIP4K2A	1.13127713	8.9625397	4.59138722	0.00011234	0.01103709
FRMD4B	1.14139632	7.62261331	4.59128321	0.00011237	0.01103709
PPP1R14A	1.32054327	6.98197901	4.58906738	0.00011301	0.01103709
PLP1	1.12897565	13.4595407	4.56928197	0.0001189	0.0111779

AIF1L	1.20185011	6.84031181	4.56470925	0.00012031	0.0111779
FAR1	0.77651576	7.35439991	4.55877234	0.00012216	0.0111779
SLC24A2	1.0040096	7.9252435	4.55124257	0.00012454	0.0111779
POPDC3	1.12856936	3.65260666	4.55015391	0.00012489	0.0111779
SLCO1A2	1.11066626	9.66894231	4.54618252	0.00012617	0.0111779
OSBPL1A	0.71374979	9.21809042	4.5423758	0.00012741	0.0111779
ERMN	1.21683647	9.91282618	4.54012957	0.00012815	0.0111779
SLAIN1	1.10149411	7.32889834	4.53070848	0.00013129	0.0111779
CFL2	0.83344471	7.1523973	4.52819268	0.00013214	0.0111779
LPAR1	1.29965748	7.51028715	4.52458333	0.00013337	0.0111779
ASPA	1.07551079	7.52338556	4.52060742	0.00013474	0.0111779
MTRR	0.74437233	4.92369751	4.52045132	0.00013479	0.0111779
SGCD	0.66615198	5.93988068	4.51391051	0.00013707	0.0111779
RASSF2	1.03875188	8.10250949	4.51090898	0.00013813	0.0111779
BCAS1	1.42455823	11.3423906	4.50300104	0.00014097	0.01120123
CPOX	1.08351356	4.88061132	4.50190485	0.00014137	0.01120123
ACSL1	0.85410654	5.26663485	4.4942613	0.00014417	0.0113165
MRPL48	0.6819198	6.15925636	4.44208126	0.00016484	0.01258597
SYT9	0.94771048	4.00212834	4.40559203	0.00018102	0.01290394
SYNJ2	1.21125412	9.14775207	4.39410011	0.00018644	0.01290394
LOC101929341	1.28643486	4.83054903	4.37898243	0.00019381	0.01290394
LINC00320	1.34594019	5.04694236	4.3768827	0.00019486	0.01290394
ARFGEF3	0.89159673	8.0261369	4.37011058	0.00019828	0.01290394
TULP4	0.69275125	6.81067224	4.36835413	0.00019917	0.01290394
TMTC2	0.74467752	6.71198104	4.36188169	0.00020251	0.01290394
ADCY5	0.82617331	5.23718986	4.36175819	0.00020257	0.01290394
TARS3	0.8574697	6.5603623	4.36131444	0.0002028	0.01290394
ZEB2-AS1	1.30759096	7.58201027	4.35110417	0.00020818	0.012973
SNX6	0.60571073	6.95005288	4.3470484	0.00021036	0.012973
NKX6-2	1.24143333	6.78842702	4.34675549	0.00021052	0.012973

Supplementary Table S5.2 – Top 100 DMPs between MS lesions and NAWM

CpG probe	P.Value	adj.P.Val	CHR	MAPINFO	UCSC_RefGene_Name	UCSC_RefGene_Group	Relation_to_UCSC_CpG_Island
cg04168675	1.99E-06	0.006671016	2	225809655	DOCK10	Body	
cg16374517	2.16E-06	0.006671016	4	119779202	SYNPO2	Body	
cg13361275	4.16E-06	0.006671016	2	159331163	PKP4;PKP4;PKP4;PKP4;PKP4	5'UTR;5'UTR;5'UTR;5'UTR;5'UTR	
cg01433296	4.28E-06	0.006671016	8	41164606	SFRP1	Body	N_Shore
cg15913157	4.89E-06	0.006671016	2	206223286	PARD3B;PARD3B;PARD3B	Body;Body;Body	
cg00854315	4.99E-06	0.006671016	12	100305873	ANKS1B	Body	
cg09111971	5.38E-06	0.006671016	3	134573293	EPHB1	Body	
cg17411918	5.67E-06	0.006671016	14	50942455	MAP4K5;MAP4K5	Body;Body	
cg22559881	5.86E-06	0.006671016	12	65700713	MSRB3;MSRB3;MSRB3;MSRB3	5'UTR;5'UTR;5'UTR;Body	
cg26055210	8.56E-06	0.006671016	11	62310752	AHNAK;AHNAK	5'UTR;5'UTR	N_Shelf
cg09317128	8.84E-06	0.006671016	4	56265301	TMEM165	Body	S_Shelf
cg13599415	9.59E-06	0.006671016	11	83285825	DLG2;DLG2;DLG2;DLG2;DLG2;DLG2	Body;Body;Body;Body;Body;Body	
cg17117049	1.08E-05	0.006671016	8	17579735	MTUS1;MTUS1;MTUS1;MTUS1	TSS200;Body;5'UTR;Body	

cg03649589	1.11E-05	0.006671016	8	19460935	CSGALNACT1;CSGALNACT1;CSGALNACT1	TSS1500;Body;5'UTR	S_Shore
cg01829163	1.16E-05	0.006671016	16	87871160	SLC7A5	Body	
cg15681096	1.20E-05	0.006671016	12	50922317	DIP2B	Body	
cg04423025	1.20E-05	0.006671016	11	82542561	PRCP;PRCP	Body;Body	
cg19223211	1.25E-05	0.006671016	8	17579771	MTUS1;MTUS1;MTUS1;MTUS1	TSS200;5'UTR;Body;Body	
cg05902884	1.30E-05	0.006671016	3	183543862	MAP6D1	TSS1500	S_Shore
cg04628938	1.34E-05	0.006671016	17	58824885	BCAS3;BCAS3	Body;Body	
cg00858400	1.38E-05	0.006671016	16	87904580	SLC7A5	TSS1500	S_Shore
cg13625631	1.39E-05	0.006671016	15	70340707	TLE3;TLE3;TLE3	3'UTR;3'UTR;3'UTR	
cg22052672	1.59E-05	0.006671016	2	74663416	RTKN;RTKN;RTKN	Body;5'UTR;Body	Island
cg16180353	1.64E-05	0.006671016	11	31819219	PAX6;PAX6;PAX6	Body;Body;Body	N_Shore
cg06665333	1.76E-05	0.006671016	16	87873837	SLC7A5	Body	
cg07006935	1.77E-05	0.006671016	7	55620641	VOPP1	Body	
cg01869632	1.77E-05	0.006671016	8	33457822	DUSP26	TSS1500	S_Shore

cg09547820	1.84E-05	0.006671016	2	236568802	AGAP1;AGAP1;AGAP1	Body;Body;Body	
cg16095148	1.91E-05	0.006671016	2	73197100	SFXN5	Body	
cg24940096	1.92E-05	0.006671016	10	65374705	REEP3	Body	
cg06284898	1.98E-05	0.006671016	14	64669350	SYNE2;SYNE2;MIR548AZ	Body;Body;Body	
cg08885854	2.05E-05	0.006671016	1	171909155	DNM3;DNM3	Body;Body	
cg27408049	2.13E-05	0.006671016	1	205044990	CNTN2	3'UTR	
cg11369927	2.18E-05	0.006671016	12	51008361	DIP2B	Body	
cg16988986	2.19E-05	0.006671016	1	109941118	SORT1	TSS1500	S_Shore
cg01168185	2.36E-05	0.006671016	11	118498071	PHLDB1;PHLDB1;PHLDB1	Body;Body;Body	
cg07081339	2.38E-05	0.006671016	9	96862110	PTPDC1;PTPDC1;PTPDC1;PTPDC1	Body;Body;Body;Body	
cg13727237	2.39E-05	0.006671016	3	58567776	FAM107A;FAM107A	Body;Body	
cg12555907	2.43E-05	0.006671016	18	74845422	MBP;MBP	TSS1500;TSS1500	Island
cg17255375	2.49E-05	0.006671016	16	19025910	TMC7;TMC7;TMC7	Body;Body;5'UTR	
cg07645671	2.52E-05	0.006671016	8	53062531	ST18;ST18	ExonBnd;Body	

cg00606190	2.69E-05	0.006671016	12	16761702	LMO3;LMO3;LMO3;LMO3;LMO3	TSS1500;TSS1500;TSS1500;TSS1500;TSS1500	
cg08190615	2.70E-05	0.006671016	11	116760808	SIK3	Body	
cg04568895	2.71E-05	0.006671016	4	41145263	APBB2;APBB2;APBB2	5'UTR;5'UTR;5'UTR	
cg06962436	2.76E-05	0.006671016	5	83675737	EDIL3;EDIL3	Body;Body	N_Shelf
cg27437585	2.78E-05	0.006671016	19	41729988	AXL;AXL	Body;Body	N_Shelf
cg11454957	2.80E-05	0.006671016	2	193004694	TMEFF2;TMEFF2	Body;Body	
cg12251910	2.81E-05	0.006671016	4	185728262	ACSL1;ACSL1;ACSL1;ACSL1;ACSL1	TSS1500;5'UTR;5'UTR;5'UTR;5'UTR	S_Shelf
cg06970090	2.85E-05	0.006671016	10	73848823	SPOCK2;SPOCK2	TSS200;TSS200	S_Shore
cg16648368	2.85E-05	0.006671016	13	99676432	DOCK9;DOCK9	Body;Body	
cg01647560	2.87E-05	0.006671016	11	83404491	DLG2;DLG2;DLG2	Body;Body;Body	
cg12592297	2.92E-05	0.006671016	11	12455524	PARVA	Body	
cg00558702	2.93E-05	0.006671016	12	16726818	LMO3;LMO3	Body;Body	
cg16197568	3.03E-05	0.006671016	13	78270580	SLAIN1	TSS1500	N_Shore
cg14008998	3.06E-05	0.006671016	14	64542631	SYNE2;SYNE2	Body;Body	

cg14486782	3.08E-05	0.006671016	10	34594444	PARD3;PARD3;PARD3;PARD3;PARD3;PARD3;PARD3	Body;Body;Body;Body;Body;Body;Body	
cg24229206	3.12E-05	0.006671016	10	11277563	CELF2;CELF2;CELF2;CELF2	Body;Body;Body;Body	
cg12435792	3.15E-05	0.006671016	4	619375	PDE6B;PDE6B;PDE6B;PDE6B	1stExon;1stExon;5'UTR;5'UTR	N_Shore
cg19252328	3.18E-05	0.006671016	13	99624413	DOCK9;DOCK9;DOCK9;DOCK9	Body;Body;Body;Body	
cg05101437	3.22E-05	0.006671016	7	92262970	CDK6;CDK6	Body;Body	
cg20823481	3.22E-05	0.006671016	4	185736247	ACSL1	5'UTR	
cg20049923	3.25E-05	0.006671016	7	92395820	CDK6;CDK6	Body;Body	
cg17537683	3.26E-05	0.006671016	2	55239067	RTN4;RTN4;RTN4;RTN4	Body;Body;Body;Body	
cg14373167	3.27E-05	0.006671016	11	117187742	BACE1;BACE1;BACE1;BACE1	TSS1500;TSS1500;TSS1500;TSS1500	S_Shore
cg18731860	3.40E-05	0.006671016	10	65280618	REEP3	TSS1500	N_Shore
cg02645407	3.43E-05	0.006671016	3	105179112	ALCAM;ALCAM;ALCAM;ALCAM	Body;Body;Body;Body	
cg26521139	3.46E-05	0.006671016	2	236658091	AGAP1;AGAP1	Body;Body	
cg12228627	3.59E-05	0.006671016	1	90374649	LRRC8D;LRRC8D	5'UTR;5'UTR	
cg03087372	3.59E-05	0.006671016	20	48030335	RASSF2	5'UTR	Island

cg21327469	3.63E-05	0.006671016	15	88633822	NTRK3;NTRK3;NTRK3;NTRK3	Body;Body;Body;Body	
cg09934219	3.65E-05	0.006671016	15	79254338	RASGRF1;RASGRF1;RASGRF1	3'UTR;3'UTR;3'UTR	
cg17371404	3.66E-05	0.006671016	19	39029649	RYR1;RYR1	Body;Body	
cg00944067	3.69E-05	0.006671016	7	37129597	ELMO1;ELMO1;ELMO1	Body;Body;Body	
cg25406657	3.69E-05	0.006671016	15	63342033	TPM1;TPM1;TPM1;TPM1;TPM1;TPM1;TPM1	Body;Body;Body;Body;Body;Body;Body	S_Shore
cg14921326	3.80E-05	0.006671016	8	119358052	SAMD12	Body	
cg08882528	3.84E-05	0.006671016	3	135689675	PPP2R3A;PPP2R3A	5'UTR;5'UTR	
cg05931860	3.86E-05	0.006671016	8	133664099	LRRC6;LRRC6	Body;Body	
cg01345338	3.95E-05	0.006671016	22	45949437	FBLN1;FBLN1;FBLN1;FBLN1	Body;Body;Body;Body	
cg08369777	3.96E-05	0.006671016	1	168054972	GPR161;GPR161;GPR161;GPR161;GPR161;GPR161;GPR161	Body;Body;Body;Body;Body;Body;Body	
cg26341831	3.97E-05	0.006671016	1	226036279	TMEM63A	Body	
cg16608348	3.97E-05	0.006671016	1	214724481	PTPN14	5'UTR	Island
cg15824100	4.02E-05	0.006671016	1	77980005	AK5;AK5	Body;Body	
cg08598383	4.06E-05	0.006671016	12	99175188	ANKS1B;ANKS1B;ANKS1B;ANKS1B;ANKS1B;ANKS1B;ANKS1B	Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body	

					B;ANKS1B;ANKS1B;ANKS1B;ANKS1B		
cg05254221	4.09E-05	0.006671016	10	61050062	FAM13C;FAM13C;FAM13C;FAM13C	Body;Body;Body;Body	
cg18787420	4.12E-05	0.006671016	20	30309466	BCL2L1;BCL2L1	Body;Body	N_Shore
cg23742209	4.15E-05	0.006671016	4	185118189	ENPP6	Body	
cg22246215	4.17E-05	0.006671016	1	203118486	ADORA1;ADORA1	Body;Body	
cg19523892	4.19E-05	0.006671016	5	148378223	SH3TC2	3'UTR	
cg01326932	4.19E-05	0.006671016	10	22959757	PIP4K2A	Body	
cg13381110	4.20E-05	0.006671016	18	60646614	PHLPP1	Body	
cg20956174	4.29E-05	0.006671016	1	65729786	DNAJC6;DNAJC6	TSS1500;TSS1500	N_Shore
cg04142864	4.42E-05	0.006671016	11	118480576	PHLDB1;PHLDB1;PHLDB1	5'UTR;5'UTR;5'UTR	Island
cg22412989	4.43E-05	0.006671016	7	37389957	ELMO1;ELMO1;ELMO1	5'UTR;5'UTR;5'UTR	
cg10800369	4.46E-05	0.006671016	12	16761930	LMO3	TSS1500	
cg12417955	4.52E-05	0.006671016	10	134587811	INPP5A	Body	S_Shore
cg18758976	4.53E-05	0.006671016	6	158430311	SYNJ2	Body	

cg20954129	4.54E-05	0.006671016	6	33259866	RGL2;RGL2	Body;3'UTR	S_Shelf
cg02107844	4.57E-05	0.006671016	15	92612836	SLCO3A1;SLCO3A1	Body;Body	
cg26234644	4.60E-05	0.006671016	17	10634427	TMEM220	TSS1500	S_Shore
cg12448312	4.61E-05	0.006671016	10	135032219	KNDC1	Body	N_Shore

Supplementary Table S5.3 – Top 100 correlating CpGs and genes

Gene	CpG probe	P-value	Correlation coeff	FDR p-value
BTBD3	cg12139813	1.23E-09	-0.959261755	5.11E-06
PIP4K2A	cg23017573	1.01E-09	-0.960319767	5.11E-06
SEMA4D	cg14541848	2.73E-09	-0.954591255	7.58E-06
DDR2	cg23874669	6.64E-09	0.948755478	1.30E-05
DAAM1	cg12126243	1.03E-08	-0.945613192	1.30E-05
TMEFF2	cg19094243	1.09E-08	-0.945180383	1.30E-05
RNF220	cg23644066	8.09E-09	-0.947357677	1.30E-05
SH3GL3	cg16100530	1.62E-08	-0.942155344	1.50E-05
PCSK6	cg02249322	1.62E-08	-0.942158496	1.50E-05
LPAR1	cg26360766	2.59E-08	-0.938317066	2.16E-05
MAP6D1	cg14785438	5.09E-08	-0.932330154	3.86E-05
SH3GL3	cg03905236	9.21E-08	-0.926598272	4.87E-05
BTBD3	cg07098747	1.14E-07	-0.924376171	4.87E-05
ERMN	cg24833225	1.22E-07	-0.923692067	4.87E-05
HHIP	cg22218015	9.07E-08	-0.926755569	4.87E-05
DAAM1	cg18411994	1.12E-07	-0.924570852	4.87E-05
DAAM1	cg23040687	1.23E-07	-0.923665327	4.87E-05
GRID1	cg10527005	1.24E-07	-0.92354697	4.87E-05
LPAR1	cg11893004	9.41E-08	-0.92638198	4.87E-05
SLC12A2	cg01250678	1.28E-07	-0.923170862	4.87E-05
BTBD3	cg00346716	7.72E-08	-0.928358792	4.87E-05
PALLD	cg20685334	8.72E-08	-0.92715128	4.87E-05
TPD52	cg21582824	1.60E-07	-0.920809721	5.40E-05
TTYH2	cg23460124	1.54E-07	-0.921254294	5.40E-05
EDIL3	cg14430679	1.62E-07	-0.920680949	5.40E-05
DAAM1	cg17326597	1.70E-07	-0.92013684	5.46E-05
GOLIM4	cg01893100	1.77E-07	-0.919708926	5.46E-05
PARD3	cg02538783	1.86E-07	-0.919155814	5.54E-05
FAM107B	cg18833808	2.13E-07	-0.917618447	6.07E-05
GOLIM4	cg07417772	2.18E-07	-0.917342791	6.07E-05
PTPN14	cg00163510	2.80E-07	-0.914454445	6.30E-05
PLCL1	cg12600692	2.40E-07	-0.916258635	6.30E-05
FRMD4B	cg24949040	2.76E-07	-0.914628598	6.30E-05
OSBPL1A	cg02442222	2.83E-07	-0.914336744	6.30E-05
ERMN	cg10812717	2.95E-07	-0.913863346	6.30E-05

SH3GL3	cg03047383	2.82E-07	-0.914371691	6.30E-05
DIAPH1	cg12695286	2.62E-07	-0.915244733	6.30E-05
ERMN	cg03252823	2.53E-07	-0.915646128	6.30E-05
RNF220	cg09139806	2.90E-07	-0.914049774	6.30E-05
DNM3	cg08885854	5.66E-07	-0.905726474	6.41E-05
DLG2	cg03902417	5.43E-07	-0.906263334	6.41E-05
FRMD4B	cg22640764	4.92E-07	-0.907539411	6.41E-05
MBNL2	cg05676204	3.51E-07	-0.911744447	6.41E-05
SH3GL3	cg23454826	4.55E-07	-0.908537938	6.41E-05
FRMD4B	cg00468670	3.29E-07	-0.912530589	6.41E-05
ENPP2	cg23155467	4.96E-07	-0.907439836	6.41E-05
LPAR1	cg14231369	5.13E-07	-0.906999304	6.41E-05
RNF220	cg19401733	4.83E-07	-0.907779568	6.41E-05
TPD52	cg14075772	4.90E-07	-0.907584012	6.41E-05
FRMD4B	cg21961548	4.37E-07	-0.909045491	6.41E-05
PALLD	cg06044751	3.91E-07	0.910422631	6.41E-05
AIF1L	cg14293473	6.15E-07	-0.904625488	6.41E-05
PLCL1	cg09025663	5.60E-07	-0.90586383	6.41E-05
BTBD3	cg20981848	4.95E-07	-0.907457158	6.41E-05
LRRC8D	cg06983052	5.00E-07	-0.907321002	6.41E-05
HIP1	cg03524389	4.73E-07	-0.908038672	6.41E-05
SH3GL3	cg21036778	5.66E-07	-0.905712749	6.41E-05
ERMN	cg01691358	6.15E-07	-0.904626107	6.41E-05
UTRN	cg02832051	5.96E-07	-0.905041283	6.41E-05
HDAC11	cg05446471	4.23E-07	-0.909442469	6.41E-05
C12orf76	cg16047828	4.59E-07	-0.908411387	6.41E-05
PIP4K2A	cg12491257	5.76E-07	-0.905482808	6.41E-05
DDR2	cg21539842	5.78E-07	-0.905443404	6.41E-05
FRMD4B	cg25835936	4.26E-07	-0.90936856	6.41E-05
BTBD3	cg12494488	3.15E-07	-0.913072004	6.41E-05
UTRN	cg22311289	3.88E-07	-0.910519733	6.41E-05
WWTR1	cg12716319	5.48E-07	-0.90613562	6.41E-05
FRMD4B	cg11975222	4.84E-07	-0.907730658	6.41E-05
PARD3B	cg08138586	3.73E-07	-0.911022376	6.41E-05
ANKS1B	cg05967710	5.07E-07	-0.907152438	6.41E-05
LACC1	cg16077991	4.65E-07	-0.908240949	6.41E-05
ABCA8	cg19850503	5.67E-07	-0.905700125	6.41E-05

LPAR1	cg17163538	3.41E-07	-0.912101124	6.41E-05
PXK	cg09627520	4.22E-07	-0.909461452	6.41E-05
FAM107B	cg03641375	3.92E-07	-0.910397092	6.41E-05
HDAC11	cg09019865	5.92E-07	-0.905123078	6.41E-05
GLDN	cg07340020	6.05E-07	-0.904856069	6.41E-05
FAM107B	cg12076357	4.53E-07	-0.908592818	6.41E-05
LPAR1	cg03095453	5.75E-07	-0.905505239	6.41E-05
NIPAL3	cg18588191	4.65E-07	-0.90824137	6.41E-05
FRMD4B	cg27402434	6.40E-07	-0.904092554	6.59E-05
DOCK10	cg17614903	6.66E-07	-0.903565947	6.77E-05
GLDN	cg13351721	6.80E-07	-0.903298217	6.83E-05
HDAC11	cg02762546	7.04E-07	-0.90283011	6.90E-05
BTBD3	cg01091831	6.99E-07	-0.902914655	6.90E-05
UTRN	cg18688142	7.21E-07	0.902493811	6.93E-05
APBB1	cg09042386	7.24E-07	-0.902454892	6.93E-05
BTBD3	cg03643760	7.48E-07	-0.902006803	7.01E-05
GFAP	cg12670990	7.49E-07	-0.901991686	7.01E-05
PIP4K2A	cg21845726	7.96E-07	-0.901152941	7.09E-05
HDAC11	cg21810733	8.12E-07	-0.900877309	7.09E-05
PARD3B	cg03787092	8.23E-07	-0.90070139	7.09E-05
DAAM1	cg04272613	7.79E-07	-0.901450983	7.09E-05
MOBP	cg14133257	7.85E-07	-0.901340709	7.09E-05
LPAR1	cg21161126	8.25E-07	-0.90066593	7.09E-05
GLDN	cg10329200	7.67E-07	-0.901670704	7.09E-05
PPFIBP2	cg19646484	8.18E-07	-0.900784882	7.09E-05
SLCO1A2	cg19659215	8.44E-07	0.900347767	7.16E-05
CNDP1	cg12031346	8.71E-07	-0.899912599	7.16E-05
DDR2	cg11501313	9.08E-07	-0.899332194	7.16E-05

CHAPTER 6

The epigenetic signature of myelin genes as a biomarker for progressive multiple sclerosis - sample storage time matters

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Abstract

One of the major challenges in multiple sclerosis (MS) is to accurately monitor and quantify disability over time. Thus, there is a high need to discover new biomarkers for disease progression. Peripheral blood DNA methylation has been shown to be an easily accessible and quantifiable marker in many neurodegenerative diseases. In this study, we aimed to investigate whether the brain methylation pattern of progressive MS patients is mirrored in the blood and whether it could be applied as a biomarker for disease progression in MS. While our initial analysis showed differences in blood methylation state of important myelin-related genes between progressive MS patients and controls, these findings were not reproducible in other sample cohorts. Our data suggest that sample storage time influences DNA methylation patterns, which might obstruct accurate epigenetic interrogation and should therefore be considered during initial sample selection stage in biomarker studies.

Introduction

Multiple sclerosis (MS) affects around 2.5 million people globally, causing a high healthcare burden (273). Around 85% of all MS patients are initially diagnosed with a relapsing-remitting disease course (RRMS), of which more than 50% will end up developing progressive MS within a period of 10-15 years, independent of treatment (5). Progressive MS is mainly characterized by the accumulation of chronically demyelinated lesions, as a consequence of failed endogenous remyelination. Sustained axonal damage within these lesions eventually leads to neurodegeneration, as reflected by progressive clinical disability of these MS patients (18, 274-276).

One of the major challenges in MS is to accurately monitor and quantify disability over time, as current diagnostics are based on a combination of magnetic resonance imaging (MRI), neurologic examinations (such as the Expanded Disability Status Scale; EDSS), and the patient's clinical history, concomitant with several limitations (5, 15). The lack of specific and sensitive diagnostic markers for disease progression does not only impact clinical decision making, but also slows down the discovery and validation of new therapeutic agents as current clinical trials mainly depend on traditional clinical imaging outcomes, such as brain atrophy (16). Thus, there is an urgent need for easily accessible, quantifiable and reliable diagnostic markers for disease progression, associated to remyelination impairment or recovery. Discovery of such biomarkers may furthermore provide new insights into the pathological mechanisms that underlie progressive MS, accelerate and facilitate clinical trials, and could therefore lead to new therapies for progressive MS.

Epigenetic control, and in particular DNA methylation, is highly involved in oligodendrocyte precursor cell (OPC) differentiation and remyelination (245-247). DNA-methylation has gained great interest over the past years in its application as a biofluid biomarker for neurodegenerative diseases (277-280). Accordingly, blood methylation patterns could reflect the brain epigenome, either by the presence of cell-free DNA derived from the brain due to blood-brain-barrier leakage, or could be a systemic epigenetic imprint also effecting the methylation state of peripheral blood mononuclear cells (PBMCs) (154, 281). Taken together, this provides a great incentive to investigate blood-borne methylation profiles as

accessible biomarkers to monitor the development and course of demyelinating diseases. We therefore investigated whether the blood methylomic profile of myelin related-genes is systemically altered in progressive MS stages and can be used as blood-borne biomarker for remyelination. Newly identified biomarkers could be used to closely monitor ongoing brain damage during the course of the disease and may serve as target for the development of successful treatment regimens for progressive MS.

Material and methods

Sample cohorts and ethical approval

DNA isolated from whole blood from two cohorts of MS and control patients was provided from the Netherlands Brain Bank (NBB; https://www.brainbank.nl/). Demographic characteristics of both cohorts are described in Table 1 and Table 2. Plasma samples of controls, relapsing-remitting patients (RRMS), and secondary progressive patients (SPMS), as well as blood fractions (whole blood, plasma, PBMCs) were provided from the UbiLim biobank (https://www.ubilim.be). All experiments were conducted after approval of the ethical committee of Hasselt University and patient anonymity was assured by handling the tissue samples in a coded fashion.

Pyrosequencing

Genomic DNA was extracted from PBMCs, plasma, or whole blood and bisulfite-converted, using the Zymo Research EZ DNA Methylation-Direct Kit (BaseClear Lab Products). PCR primers were designed using the PyroMark Assay Design 2.0 software (Qiagen, Supplementary Table S6.1). The assays were tested for their sensitivity using the EpiTect PCR Control DNA Set (Qiagen). Product amplification was performed using the following reaction mixture: 1X Buffer with 20 mM MgCl2 (Roche), 10 mM dNTP mix (Roche), 5 µM forward and reverse primers (Metabion AG), 1U FastStart Taq DNA Polymerase (Roche), bisulfite-converted DNA and nuclease-free water to a total volume of 25 µl. PCR cycling was performed as follows: initial denaturation for 5 min at 95 °C, 50 cycles of 30 s at 95 °C, 30 s at the appropriate annealing temperature and 1 min at 72 °C; final extension for 7 min at 72 °C. PCR amplicons were sequenced using the Pyromark Q48 instrument (Qiagen) with the PyroMark Q48 Advanced CpG Reagents (Qiagen), according to the manufacturer's protocol and quantified with the Pyromark Q48 Autoprep software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0.0 software (GraphPad software Inc., CA, USA). Differences between group means were determined using an unpaired t-test for normally distributed data and a Mann-Whitney test for not normally distributed data. Correlation analyses were performed using Spearman's correlation tests. All data are depicted as mean ±SEM, *=p≤0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Results

We have previously shown that genes related to myelination and oligodendrocyte differentiation are differentially methylated in chronically demyelinated lesions of progressive MS patients (see chapter 5). As DNA methylation has gained great interest in its application as a biofluid biomarker for neurodegenerative diseases, we postulated that the methylation state of (part of) these genes was also detectable in peripheral blood samples of progressive MS patients as a reflection of ongoing brain pathology. We therefore obtained DNA from whole blood samples of the same patients as the discovery brain cohort, as well as age- and sex matched non-neurologic control samples (Table 6.1). DNA methylation of five of the top differentially methylated myelin-associated genes (*MBP, MAG, CNTN2, BCAS1, PARD3*) was assessed using bisulfite pyrosequencing. Three (*MBP, MAG, CNTN2*) out of the five genes showed a significant difference in methylation between control and MS samples (Figure 6.1). Interestingly, the methylation profile of these genes followed the pattern as observed in the CNS, implying systemic hypermethylation of these genes in progressive MS patients. This suggests that the DNA methylation profile of these genes could potentially be applied as a peripheral marker of remyelination failure in the disease course of MS.

Table 6.1 – Demographic details of the first cohort of peripheral blood DNA samples obtained from the Netherlands Brain Bank (NBB).

Group	Sex (F/M)	Age (mean ± SD)	PMI (mean minutes ± SD)	Storage time (mean years ± SD)
MS	2/4	66,50 ± 9,69	572,5 ± 81,16	9,33 ± 1,63
Controls	3/3	64,33 ± 6,09	605,0 ± 284,7	24,67 ± 2,25

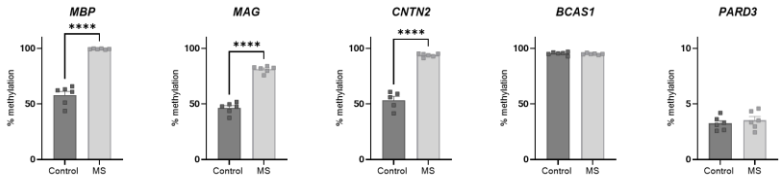

Figure 6.1 – Myelin-related genes show a differentially methylated profile when comparing control and multiple sclerosis (MS) peripheral blood samples. DNA isolated from peripheral blood samples from the same MS patients as the brain discovery (NBB) cohort was used for bisulfite pyrosequencing. Three out of five genes displayed significant differential methylation. Data are represented as mean + SEM. Unpaired t-test, ****=p<0.0001.

An ideal biomarker for progression in MS would differentiate between the RRMS and SPMS stages. Therefore, in the next experiment, we made use of DNA isolated from plasma from healthy controls, RRMS, and SPMS patients and evaluated the methylation profile of one of the myelin-related gene *MBP*. To our surprise, we found no difference between the three groups, with, in contrast to our previous findings, healthy controls now also showing a hypermethylated profile (Figure 6.2a). Since in our discovery cohort we observed significant differences in *MBP* methylation in DNA isolated from whole blood samples, the observed discrepancy could have been explained by the absence of PBMCs in plasma. Therefore, in the next step, we isolated DNA from different fractions (whole blood, plasma, and PBMCs) from new healthy control donors. Interestingly, the *MBP* gene displayed hypermethylation in all of the three blood fractions (Figure 6.2b).

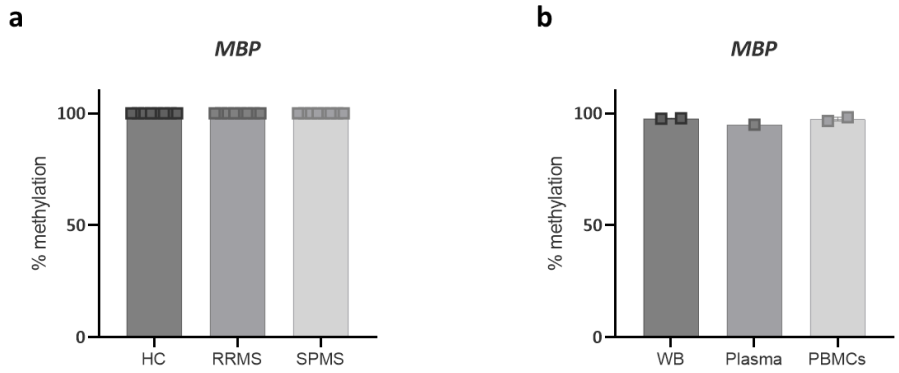

Figure 6.2 – Lack of differential methylation of *MBP* in UbiLim blood cohorts. a. DNA from plasma samples of healthy controls, relapsing-remitting (RRMS) and secondary progressive (SPMS) patients does not show any difference in *MBP* methylation. **b.** DNA isolated from different blood fractions of healthy control samples shows an overall hypermethylated profile of *MBP* in all the blood fractions. Data are represented as mean ± SEM. HC = healthy control, WB = whole blood, PMBCs = peripheral blood mononuclear cells.

The discrepancy in our data suggest that the original findings from the discovery cohort could have been biased by certain covariates. We therefore performed a correlation analysis between the methylation values of *MBP* and different covariates, such as age, sex, post-mortem interval (PMI), and storage time (Table 6.2). As expected, age, sex, and PMI did not show any correlation to *MBP*

methylation. Interestingly, storage time, the only covariate that appeared not to be matched between the groups during the selection of the samples, did show a strong and significant negative correlation to the *MBP* methylation state (Table 6.2). These results suggest that long storage time of the samples might result in a loss of the DNA methylation signature. To investigate this, we ordered new samples from the NBB, yet took into account the storage time of the samples during the sample selection, with an inclusion criteria of a storage time of less than 10 years (Table 6.3). We performed bisulfite pyrosequencing on these samples for the five myelin-related genes. Interestingly, and in contrast to our previous findings, we observed no differences between the two groups for any of the measured genes (Figure 6.3). The hypomethylated profile of the control samples was not reproducible in the new sample cohort with a shorter storage time. Collectively, these data show that the DNA methylation profile can be strongly influenced by certain covariates, such as sample storage time.

Table 6.2 – Storage time correlates significantly with the methylation state of *MBP*. Pearson's correlation analysis was performed between *MBP* methylation and different covariates, such as age, sex, PMI, and storage time. PMI = post-mortem interval.

Covariate	Pearson r	P value
Age	0.1559	0.6286
Sex	-0.2880	0.3641
PMI	-0.0065	0.9839
Storage time	-0.9129	<0.0001

Table 6.3 – Demographic details of the second cohort of peripheral blood DNA samples obtained from the Netherlands Brain Bank.

Group	Sex (F/M)	Age (mean ± SD)	PMI (mean minutes ± SD)	Storage time (mean years ± SD)
MS	3/2	68,00 ± 18,61	524,0 ± 92,29	9,20 ± 1,30
Controls	3/2	69,20 ± 17,30	440,0 ± 37,58	9,00 ± 1,41

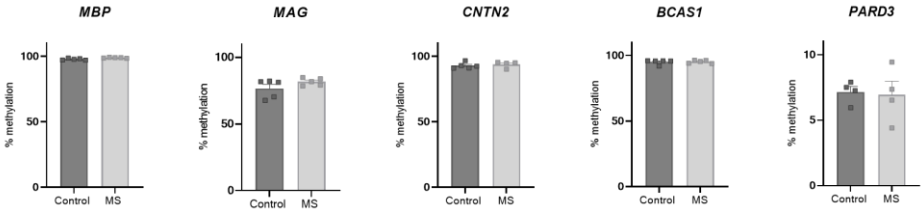

Figure 6.3 – Samples with a shorter storage time do not show differences in methylation between control and multiple sclerosis (MS). DNA isolated from peripheral blood samples from a new cohort of MS patients and non-neurologic controls, with a storage period of maximal ten years, was used for bisulfite pyrosequencing. Data are represented as mean ± SEM.

Discussion

The initial aim of the present study was to investigate whether the DNA methylation state of myelin-related genes could be applied as a blood-borne biomarker for disease progression in MS. While we initially observed significant differences in peripheral DNA methylation between MS patients and non-neurologic controls, we were not able to reproduce these findings in samples obtained from other cohorts. We furthermore observed a strong correlation between the degree of DNA methylation of these genes and storage time of the samples. Our data suggests that the DNA methylation signature can be affected by long-term storage, an important factor that should be taken along in future studies.

The diagnosis of progressive MS is still regarded as a significant challenge since there are no accessible quantifiable markers available yet (15, 282). The current diagnosis of transition from RRMS to SPMS is mainly based on retrospective analysis of clinical parameters, which means that the transition to progressive MS can remain unnoticed with a delay of up to three years (282, 283). Upon recent approval of disease-modifying drugs for SPMS, such as siponimod, an increasing need for timed and accurate diagnosis from the RRMS towards SPMS stages developed (284). As for the development of new drugs that modulate the disease progression, biomarkers for remyelination impairment can be applied in drug screening phases, as well as in human clinical trials. Such markers could give an accurate and valid indication of the effect of a treatment on patients, thereby enabling and accelerating smaller clinical trials (16). There is a great effort within

the MS research domain to discover new accessible and quantifiable markers for disease progression. For example, integration of MRI data with proteomic data from the cerebrospinal fluid (CSF) has shown to be able to distinguish between RRMS and SPMS stages (285). Similarly, the combination of MRI data with cognitive performance accurately discriminated RRMS patients from SPMS patients (286). A new PET tracer ([18F]3F4AP) has been shown to effectively detect myelin loss in primates and is currently being tested in a clinical study with healthy volunteers and MS patients, with the final aim to apply this tracer as a new *in vivo* imaging tool for demyelination (287, 288). However, to date, there are no easily accessible and reliable markers that can define the progressive phase of MS or anticipate the conversion towards SPMS.

DNA methylation has gained great interest in its application as a biomarker for many neurodegenerative or neuropsychiatric diseases. In Parkinson's disease, Alzheimer's disease, and epilepsy, for instance, numerous DNA methylation signatures in peripheral blood samples have been shown to mirror methylation differences within the brain (279, 289, 290). We therefore wondered whether the epigenetic differences we previously observed in MS brain samples could be mirrored in peripheral blood samples and be applied as a marker for progression in MS. We have previously identified important genes regarding myelination and oligodendrocyte differentiation, which were hypermethylated in chronically demyelinated lesions of progressive MS patients (Chapter 5). In the present study, we investigated the methylation state of these genes in whole blood samples, isolated from the same patients as the brain discovery cohort. Interestingly, three genes (*MBP*, *MAG*, *CNTN2*) initially seemed to be significantly hypermethylated in MS blood samples, compared to non-neurologic control samples. This suggested that the hypermethylated profile of these genes within the CNS was also reflected in the blood, potentially rendering them an interesting accessible marker for the ongoing CNS pathology. Similarly, the myelin oligodendrocyte glycoprotein (MOG) gene, another important myelin gene, has previously been described to be demethylated in serum from MS patients with an active and symptomatic disease course, probably reflecting oligodendrocyte cell loss during these stages of the disease (154).

Our main goal was to define a specific marker for disease progression in MS, which would therefore distinguish SPMS patients from RRMS patients. We isolated DNA

from plasma of a new cohort of healthy control subjects, and age- and sex-matched RRMS and SPMS patients. Unexpectedly, we observed no differences in methylation between the three groups, as all samples showed a hypermethylated profile of *MBP* in this cohort. A possible explanation for this discrepancy in our data is that in the first cohort we made use of DNA isolated from whole blood samples, including both cell-free DNA and DNA from PBMCs, whereas in the cohort with the different disease stages, we only looked at cell-free DNA isolated from plasma samples. The absence of PBMCs could thus be a potential factor influencing the reproducibility of our data. To confirm this, we isolated DNA from different blood fractions (whole blood, plasma, PBMCs) from two healthy control donors. Interestingly, all the fractions showed the same hypermethylated state of *MBP* in the control samples, confirming that the observations from the first cohort were not reproducible.

During the selection procedure of the samples of the first cohort, we matched the samples based on age, sex, and PMI. Sample storage time was initially not included as a sample selection criterion. However, correlation analysis between the *MBP* methylation state and all of the abovementioned covariates did show a strong and significant correlation with the sample storage time. Indeed, when we included storage time as an inclusion criterion (less than 10 years on 4°C) during the sample selection of a new set of DNA samples from whole blood, we observed no differences anymore when comparing controls and progressive MS patients. These results suggest that long storage time of the samples might result in a loss of the DNA methylation signature. Previous studies have already investigated the stability of DNA methylation marks after long-term storage (291, 292). Interestingly, no global changes in DNA methylation were observed after 20 years of storage of DNA samples at 4°C (292).We did observe a loss of methylation in three out of the five measured genes in control DNA samples stored for more than 20 years on 4°C. As this loss of methylation was not observed for all genes, this selective loss of methylation could be missed during the screening of global methylation changes of archived samples, as previously conducted by other colleagues (292). Moreover, different storage conditions between different institutions and agencies may also play an important role in this respect. Evidently, the longer samples are being stored, the higher the likelihood for incidents, e.g. related to temporary changes in temperature, to occur.

Interestingly, matching brain samples, which were stored at -80°C for the same time period, did not show loss of methylation of the measured genes. Altogether, our data suggests that the DNA methylation signature in blood can be affected by long term storage, an important factor that had been neglected before yet should be taken into account in future studies.

Unfortunately, in the present study, we were not able to discover new biomarkers for progression in MS based on the DNA methylation of a subset of myelin-related genes. Our targeted approach, based on genes that displayed differential methylation in chronically demyelinated lesions compared to the surrounding non-affected white matter, was proven unsuccessful. As such, it would be of great interest to subject DNA from peripheral blood samples, albeit whole blood or plasma, from both progressive MS patients and control individuals to genome-wide methylation analysis. Data analysis comparing cases versus controls in both brain tissue and peripheral blood could then reveal potential differentially methylated genes that overlap between the brain and periphery. These genes could be further investigated for their role as a biomarker for disease progression, reflecting the ongoing CNS pathology.

Supplementary information

Supplementary Table S6.1: Pyrosequencing primer list

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Sequencing primer (5'-3')
MBP	GTTTGGTAGGATGTTTATTTAGTTGA	TCTATAACCCCATCACATCCAAACTCTC	GGATGTTTATTTAGTTGATTTAGG
MAG	AGGGTGTATAGGGATGGAAGAT	AAAAAAAACACAAAAAAACCCTTATCAC	GGAAAGAGTTAGGAGAATTTA
CNTN2	GAGGGGGGTGAGATAATAGT	CCTACCAACTCTAAAATTCTAAATACTCA	TGAGATAATAGTGATAGTTTGA
BCAS1	GGAGTATATAGTTGAGGGGGTTGATAG	CTCAAAAACTTAAACTCTAACCCTAAATT	GGAAGTATAGTAGTTTGTTTATAAT
PARD3	AGGGAGAGGGTAGGGTAGAAA	CCCCTTCCCCTTTCTTTATC	AGAAATTTAGTAGAGTAAGTTGTAG

Chapter 7

General discussion

The general aim of this thesis was to investigate the role of DNA methylation on oligodendrocyte biology, both in physiological conditions, as well as in the context of progressive multiple sclerosis (MS). The key findings, strengths, limitation, and future perspectives of this thesis are discussed in this chapter.

Key findings of this thesis

Over the recent years, increasing evidence has shown that epigenetic mechanisms, such as DNA methylation, are major contributors to oligodendrocyte differentiation and (re)myelination. Epigenetic signatures translate extracellular signals into functional cellular changes and coordinate the transcriptional machinery that is responsible for the differentiation process. In **Chapter 2**, we provided an overview of the current understanding of the physiological process of oligodendrocyte lineage development and how various epigenetic mechanisms are involved in the regulation of this process. In central nervous system (CNS) demyelinating diseases, these epigenetic mechanisms are found to be altered, concomitant with increased levels of transcriptional inhibitors and resulting in a differentiation block of oligodendrocyte precursor cells (OPCs). Targeting these epigenetic processes, either by pan-inhibitors or via CRISPR/Cas9-mediated epigenetic editing, could therefore be a potential strategy to boost oligodendrocyte lineage differentiation and (re)myelination.

The first objective of this thesis was to investigate how DNA methylation influences OPC differentiation in physiological conditions. Emerging data suggest that DNA methylation enzymes strongly influence OPC cell fate commitment and (re)myelination (246, 247). Nevertheless, which genes are actually targeted by the DNA methylation enzymes during OPC differentiation remained undisclosed. We hypothesized that transcriptional regulators upstream of myelin genes are regulated by DNA methylation during OPC differentiation. Indeed, in **Chapter 3**, we demonstrated that DNA methylation of myelin regulatory genes, in particular the HLH inhibitory transcription factors *Id2* and *Id4*, is crucial for OPC differentiation. The identification of *Id2* and *Id4* as important targets of DNA methylation during OPC differentiation was based on the application of 5-azacytidine (5-AZA), a pharmacological inhibitor of DNA methylation. While we cannot exclude the possibility that other relevant genes may have been affected

by the use of 5-AZA, there is evidence from previous studies that show similar effects on OPC differentiation and *ID4* expression when using epigenetic modifiers, such as HDAC inhibitors (96, 145). To confirm our findings and to assess the intricate causality between DNA methylation of *Id2* and *Id4* and OPC differentiation, we made use of a recently developed epigenetic engineering system, based on CRISPR-Cas9 technology. Site-specific CRISPR/dCas9-mediated *Id2* and *Id4* hypermethylation resulted in a reduced expression of *Id2* and *Id4*, eventually leading to a boost in OPC differentiation and myelin gene expression. Our targeted epigenetic editing approach further strengthens evidence for a causal relationship between *Id2* and *Id4* DNA methylation and OPC differentiation.

In **Chapter 4**, we furthermore suggest the application of epigenetic editing as a tool for causality assessment in neurodegenerative diseases. Recent technological advances have led to epigenome-wide-association studies (EWAS), such as methylome-wide association studies (MWAS), allowing for an in-depth analysis of epigenetic changes associated with disease. While EWAS and MWAS represent important approaches to establish a candidate list of genetic loci associated with a specific disorder, they remain purely correlational. In fact, any epigenetic difference between diseased and healthy subjects could represent a cause or consequence of risk factors, the disease itself, its treatment, or an epiphenomenon, or a combination of one or more of these features. While this limitation is often acknowledged in research across the field, it is rarely addressed properly. For example, the multiple risk factors associated with MS, as well as the heterogeneity in lesion composition, and clinical manifestations, are all complicating factors when defining causality. We therefore propose a workflow, starting from EWAS studies, all the way to applying epigenetic editing as a tool to investigate potentially causal associations between epigenetic modifications of major candidate genes and the pathophysiology of neurodegenerative disorders. The proposed workflow allows for higher throughput owing to a standardized approach, a higher chance to identify biologically relevant targets and, therefore, a higher likelihood to translate findings to patients.

In **Chapter 5**, I applied the workflow proposed in Chapter 4 in order to investigate how the methylome is altered in progressive MS lesions, which is the second main objective of this thesis. MS lesions are typically very diverse in terms of the degree of demyelination, inflammation and scar formation (216, 217). In our study, we

aimed to include only chronically demyelinated lesions, which are inactive from an inflammatory point-of-view. These lesions are mostly found in progressive MS patients and represent the main neurodegenerative aspect of the disease. Other important criteria that we applied in our study were the presence of OPCs within the lesions and the exclusion of scar tissue since these have no ability to regenerate, representing an advanced disease stage. We then investigated the transcriptomic and epigenomic profile of chronically demyelinated lesions and their surrounding NAWM, with the final goal of understanding the molecular mechanisms underlying the hampered differentiation capacity of OPCs within the MS lesion microenvironment. Cell-specific validation making use of laser-captured OPCs showed that OPCs within the lesion exhibit a hypermethylated profile of the essential myelin gene, *MBP*. By applying the CRISPR/dCas9-mediated epigenetic editing toolbox, we validated the causal relationship between the methylation of *MBP* and the differentiation capacity of human induced pluripotent stem cell (iPSC)-derived oligodendrocytes. Interestingly, *MBP* has previously been shown to be hypermethylated in NAWM samples of MS patients compared to white matter samples derived from non-neurologic controls (53). These findings suggest a possible gradual methylation change in the *MBP* gene, already initiated in regions devoid of lesions and becoming more pronounced at the actual lesion site, where myelin damage has already occurred. Moreover, *MBP* has also been shown to be differentially methylated in other neurodegenerative diseases with white matter pathology, such as Alzheimer's disease (AD) (269). Altogether, this emphasises the importance of DNA methylation in the regulation of *MBP* expression and its susceptibility to changes during disease. Notably, many more CpG sites displayed differential methylation within MS lesions, with numerous of them potentially impacting upon cellular behaviour within the lesion site. Altogether, it is important to further characterise MS-associated epigenetic signatures, preferably in a cell-type-specific manner, in order to fully understand the contribution of DNA methylation to remyelination failure in progressive MS stages.

DNA-methylation has also gained great interest over the past years in its application as a biofluid biomarker for neurodegenerative diseases (277, 278). The blood methylation pattern could reflect the CNS epigenome, either by the presence of cell-free DNA derived from the CNS due to blood-brain-barrier leakage, or could be a systemic epigenetic imprint also affecting the methylation

state of peripheral blood mononuclear cells (PBMCs) (154, 281). Taken together, this provides a great incentive to investigate blood-borne methylation profiles as accessible biomarkers to monitor the development and course of demyelinating diseases. We therefore, in **Chapter 6**, investigated whether the methylomic profile of myelin related-genes is systemically altered in progressive MS stages and can be used as blood-borne biomarker for disease progression. While we initially observed significant differences in peripheral DNA methylation between MS patients and non-neurologic controls, we were not able to reproduce these findings in samples derived from other cohorts. We did, however, observe a strong inverse correlation between the degree of DNA methylation and sample storage time. Previous studies have already investigated the stability of DNA methylation marks after long-term storage (291, 292). Interestingly, no global changes in DNA methylation were observed after 20 years of storage of DNA samples at 4°C (292).We did observe a loss of methylation in three out of the five measured genes in control DNA samples stored for more than 20 years on 4°C. As this loss of methylation was not observed for all genes, this selective loss of methylation could be missed during the screening of global methylation changes of archived samples, as previously conducted by other colleagues (292). Moreover, different storage conditions between different institutions and agencies may also play an important role in this respect. Evidently, the longer samples are being stored, the higher the likelihood for incidents, e.g. related to temporary changes in temperature, to occur. Altogether, our data suggests that the DNA methylation signature can be affected by long-term storage, an important factor that had been neglected before yet should be taken into account in future studies.

Strengths and limitations

Aside from gaining a better insight into the involvement of DNA methylation in oligodendroglial biology, the strength of this thesis is reflected by the application of state-of-the art technologies. It is noteworthy that the results of this thesis are based on a unique combination of different biological materials (i.e. murine primary OPCs, human iPSC-derived oligodendrocytes, human post-mortem brain samples, peripheral blood samples) and a wide range of advanced experimental techniques (i.e. sequencing technologies, bioinformatic analysis, CRISPR/dCas9-

mediated epigenetic editing, laser-capture microdissection, and functional cellular assays). This unprecedented unique experimental design allowed us to unravel the epigenetic signature of OPCs and chronically demyelinated MS lesions and evaluate causality of the identified genes in view of OPC differentiation.

In this thesis, we applied different techniques to investigate DNA methylation signatures, either in a targeted fashion, e.g. via pyrosequencing, or on an epigenome-wide level, using the Illumina MethylationEPIC array. These techniques however, are all based on bisulfite conversion of genomic DNA. A downside to the use of classical bisulfite conversion is that this method does not allow for discrimination between DNA methylation (i.e. 5-methylcytosine [5mC]) and hydroxymethylation (i.e. 5-hydroxymethylcytosine [5hmC]). Hydroxylation of 5mC into 5hmC is the first step of active DNA demethylation. Interestingly, 5hmC patterns have shown to be abundantly present in the CNS of mammals (36, 37). 5hmC was first identified as merely an intermediate epigenetic mark during active DNA demethylation, but has in the meantime also been shown to represent an independent functionally distinct epigenetic mark in the brain (38, 39). It is therefore important to be able to distinguish between these two methylation states. Recent technological advances have led to the application of an oxidation step prior to bisulfite conversion, which only leaves 5mC signatures to be sequenced. By performing classical bisulfite parallel to oxidative bisulfite conversion on the same DNA sample, followed by sequencing both templates, one can distinguish between unmethylated, methylated and hydroxymethylated CpG sites within the genome. It would be of great added value for future studies to distinguish between 5mC and 5hmC in order to obtain the full picture of the methylation changes in MS brain lesions.

As discussed in chapter 4 of this thesis, it is important to investigate potential cause-and-effect relationships for epigenetic alterations. One of the major strengths of this thesis is that we successfully applied CRISPR-Cas9 mediated epigenetic editing to validate the effect of DNA methylation of our target genes on OPC differentiation. The introduction of epigenetic editing tools has opened a new avenue to investigate the causal relationships between epigenetic modifications and disease pathology. Epigenetic editing tools generally consist of a DNA-binding domain that is used as a vehicle to target epigenetic modifiers to exact genomic loci (188). For instance, CRISPR-Cas9 mediated epigenetic editing makes use of

DNA-RNA based complementarity to recruit a nuclease-deactivated Cas9 endonuclease (dCa9) (209, 293). The inactive dCas9 protein is fused to epigenetic modifying domains, such as DNA methyltransferase (DNMT) 3a or TET1, to allow for straightforward and easy modulation of the epigenome (271, 294-297). As a control, a plasmid with an inactivated epigenetic modifying domain that has the same properties as the active vector, but lacks the capacity to alter the epigenome, can be used. The different properties of epigenetic editing tools, such as their size, efficiency and specificity, can greatly impact the success rate of epigenetic editing and should therefore be considered when selecting an epigenetic engineering system. The simplicity and adaptability of the CRISPR-Cas9 based system is considered as a major advantage and has greatly facilitated programmable epigenetic targeting. The major concern of the use of CRISPR-Cas9 mediated epigenetic editing is the relatively high chance of off-target activity due to binding to similar sequences in the genome. The CRISPR-Cas9 system relies on target-specific sgRNAs, adjacent to a protospacer adjacent motif (PAM) sequence for Cas9 protein binding. However, mismatches up to five nucleotides with unwanted genomic target sites can be tolerated by the sgRNA, resulting in off-target activities (298). On top of this, a recent study has also shown that the dCas9-DNMT3a tool can lead to an unspecific increase in methylation, regardless of the use of sgRNA to guide the complex (299). These data suggest that not only the sgRNAs are responsible for off-target effects, but also the effector domains, such as DNMT3a, themselves can exert unguided off-target activities. Fortunately, the constant development of algorithmic tools has led to a better prediction of optimal sgRNAs with minimized off-target effects and recent adaptations to the dCas9-DNMT3a tool, such as modulation of dCas9 expression by different promotors or the use of different dCas9 orthologs, have been shown to effectively reduce off-target effects, while maintaining on-target specificity (214). Finally, other features such as the size of the epigenetic modifying domain, the efficiency of plasmid delivery in both *in vitro* and *in vivo* systems and the immunogenic response should be weighted out thoroughly when considering the optimal epigenetic editing tool.

Another strength of the work in this thesis is the unique within-comparison between lesions and the surrounding NAWM isolated from each patient. This setup increased our statistical power, despite the low sample size, and allowed us to

investigate DNA methylation changes specifically related to the lesion microenvironment, where OPC differentiation is hampered. However, as it is not sure yet whether the NAWM is completely unaffected, it would be of interest to also compare the methylation profile of the NAWM to non-neurologic control brain samples. Unfortunately, as our sample size was limited to 10 MS donors, the statistical power was not sufficient to detect epigenome-wide differences between NAWM and non-neurological control samples. Interestingly, in chapter 3, *Id2* and *Id4* have been shown to be regulated via DNA methylation during OPC differentiation. However, we did not observe significant differences of these genes between lesions and NAWM in our targeted analysis. In contrast, the average methylation pattern of *ID2* and *ID4* within NAWM samples resembled the methylation pattern of the lesions, rather than the non-neurological control samples. In chapter 5, we observed significant differences in *MBP* methylation in lesions compared to NAWM samples. In the literature, *MBP* methylation has also been shown to be differentially methylated between NAWM and control samples (53). Together, this suggests that there could indeed already be some OPC dysregulation occurring within MS brains preceding noticeable myelin damage, a notion that has been proposed before by others (53, 218).

Our findings regarding chronically demyelinated MS lesions are mainly based on heterogenous bulk tissue, and therefore the presence of other cell types may bias the observed changes in methylation. Cellular heterogeneity within bulk tissue can thus confound analyses and lead to data misinterpretation. Especially complex tissues, such as MS brain lesions, of which the cellular composition is very variable and hard to correct for, should therefore be considered with care. In chapter 5, we applied laser-capture microdissection to validate our top hit in a cell-specific manner in OPCs, allowing us to eliminate cellular heterogeneity-induced bias. While the methylation profile does not only differ between different cell types, it can also vary strongly within one cell population, mainly in a pathological context. Indeed, recent studies have revealed distinct OPC and oligodendrocyte populations within MS brain samples, each with different transcriptional, and likely epigenetic, signatures, a notion that should be considered carefully (224, 225, 300).

The main focus of this thesis is on how DNA methylation influences OPC differentiation. As the title of this thesis states, we aimed to investigate the

'epigenetic signature' underlying remyelination failure in progressive MS. It is important to note, however, that epigenetics comprises more than DNA methylation only. Posttranslational modifications at the level of histone tails and non-coding RNAs are two other important epigenetic modifications that have been shown to influence OPC differentiation and remyelination (196, 301). DNA methylation is furthermore closely related to these other epigenetic mechanisms and only by investigating the interplay between all the three epigenetic modifications, we can obtain the complete epigenetic signature of progressive MS.

Future perspectives

It is clear that epigenetic modifications strongly influence OL development and functional remyelination in a wide variety of diseases. Targeting these epigenetic alterations could therefore be considered as a new therapeutic strategy to overcome remyelination failure. Most attempts to pharmacologically manipulate epigenetic modulations are based on the use of inhibitors of epigenetic enzymes, such as 5-AZA and valproic acid (VPA) (184, 302). However, such pan-epigenetic inhibitors are non-specific due to their pleiotropic impact at a genome-wide level. Furthermore, these compounds are known to have low chemical stability and are cytotoxic at higher doses, which limits their potential to be used in a cellular microenvironment (186, 187). Recent improvements in the field of epigenetic editing have disclosed the use of DNA-binding proteins, such as zinc-finger proteins (ZFPs), transcription activator-like effectors (TALEs) and CRISPR/Cas9, as new synthetic epigenomic engineering tools (188, 190, 191, 298). Even though many advances have been made regarding these new epigenetic editing techniques, their applicability in the clinic may require, next to ethical considerations, additional research as their safety and efficacy remain to be disclosed. In particular, the off-target effects and undesired genomic binding of these DNA-binding proteins are still considered as one of the major hurdles for their therapeutic application (303).

Unfortunately, in the study presented in chapter 6, we were not able to discover new peripheral biomarkers for disease progression in MS based on the DNA methylation of myelin-related genes. Our targeted approach, based on genes that displayed differential methylation in chronically demyelinated lesions compared to

the surrounding non-affected white matter, was proven unsuccessful. As such, it would be of great interest to subject DNA from peripheral blood samples, albeit whole blood or plasma, from both progressive MS patients and control individuals to genome-wide methylation analysis. Data analysis comparing cases versus controls in both brain tissue and peripheral blood could then reveal potential differentially methylated genes that overlap between the brain and periphery. These genes could be further investigated for their role as a biomarker for disease progression, reflecting the ongoing CNS pathology.

Our findings provide novel and important insights into oligodendrocyte biology. Even though this work is mainly centered around progressive MS, the results of this thesis can also be extrapolated to other neurodegenerative diseases that are marked with oligodendroglial dysfunction. For instance, over recent years, neuroimaging studies have identified white matter degeneration as a long-overlooked yet vital process in the pathophysiology of AD (304-307). Disruption of myelin sheaths and oligodendroglial cell death is accompanied by axonal damage and neurodegeneration, eventually resulting in cognitive decline. Indeed, evidence is emerging that OL cells are altered along the course of the disease, both in terms of numbers and morphology, in post-mortem human AD brain tissue and mouse models of AD (304, 308-310). A recent study also nicely revealed a previously unknown role for oligodendrocytes (OLs) in AD, involving the *APOE4* risk variant (311). In parallel, recent evidence suggests that epigenetic dysregulation plays an important role in the development and course of AD (269, 312). Bridging these findings, it would be interesting to investigate and modulate the epigenetic signature of myelin genes in oligodendroglial cells of AD patients to understand its role in myelin formation during disease pathology.

Altogether, this dissertation provides more insights into the influence of DNA methylation on OPC differentiation and MS pathology. The studies presented in this thesis contribute to a better understanding of the molecular mechanisms underlying remyelination impairment and set the stage for future research on epigenetic changes in relation to progressive MS stages. The data generated in this research is a valuable addition to the current epigenetic data collection on MS brain samples and contributes to the efforts of the scientific community to identify novel markers for disease progression as well as targets for therapeutic drug development.

Chapter 8

Summary

The work presented in this thesis investigates the influence of DNA methylation on oligodendrocyte biology, both in physiological conditions, as well as in the context of progressive multiple sclerosis (MS).

Chapter 2 offers in-depth information on how epigenetic mechanisms influence oligodendrocyte differentiation and myelination. It provides first of all a general overview of the transcriptional network that regulates the differentiation process. Then, the epigenetic mechanisms, comprising DNA methylation, histone modifications, and miRNAs, are each discussed separately based on how they are known to play a role during physiological oligodendrocyte precursor cell (OPC) differentiation. Finally, the implication of epigenetic dysregulation related to OPC differentiation on demyelinating disorders and ageing is discussed.

Even though the literature suggests that DNA methylation enzymes strongly influence OPC cell fate commitment and (re)myelination, it remained undisclosed which genes are actually targeted by the DNA methylation enzymes during OPC differentiation. In **Chapter 3**, I investigated the direct influence of DNA methylation on the transcriptional network that regulates myelin gene expression and OPC differentiation. I did not only confirm that DNA methylation is crucial for the differentiation process, but also showed that the negative transcriptional regulators, *Id2* and *Id4*, are mainly affected by DNA methylation going from OPC to oligodendrocyte stages. Moreover, I showed that in the pathological context of MS, methylation and gene expression levels of both *ID2* and *ID4* are altered compared to control human brain samples. Based on these data, we can conclude that DNA methylation is crucial to suppress *ID2* and *ID4* during OPC differentiation, a process that appears to be dysregulated during MS. These results do not only reveal new insights into oligodendrocyte biology, but could also lead to a better understanding of myelin disorders, such as MS.

Chapter 4 is based on a perspective, in which we discuss the importance of causality assessment in neuroepigenetic research. We propose a workflow, starting from epigenome-wide association studies (EWAS), all the way to applying CRISPR-Cas9 based epigenetic editing as a tool to investigate the potentially causal associations between epigenetic modifications of top hit genes and the pathophysiology of neurodegenerative disorders.

In the work described in **Chapter 5**, I applied the proposed workflow from chapter 4 in the context of progressive MS. Starting from epigenomic and transcriptomic

profiles of chronically demyelinated MS lesions, I identified target genes that are differentially expressed and differentially methylated in these lesions, in comparison to the surrounding normal-appearing white matter (NAWM). Cell-specific validation of one of the strongest differentially methylated genes in relation to myelination, *MBP*, in laser-captured OPCs showed that OPCs within the lesion exhibit a hypermethylated profile of this essential myelin gene. By applying the epigenetic editing toolbox, I validated the causal relationship between the methylation of *MBP* and the differentiation capacity of human induced pluripotent stem cell (iPSC)-derived oligodendrocytes.

In the final study, presented in **Chapter 6**, I investigated whether the brain methylation pattern of progressive MS patients is mirrored in the blood and could thus be applied as a biomarker for disease progression in MS. The dysregulated epigenetic signature of the myelin genes, observed in the EWAS study from chapter 5, was not reflected in the blood samples of progressive MS patients. However, we did observe a strong correlation between DNA methylation of these genes and the storage time of the samples. Our data from this study suggests that the blood DNA methylation signature can be affected by long-term storage, an important factor that should be taken along in future studies.

To conclude, this dissertation provides more insights into the influence of DNA methylation on OPC differentiation and MS pathology. The studies presented in this thesis contribute to a better understanding of the molecular mechanisms underlying remyelination impairment and set the stage for future research on epigenetic changes in relation to progressive MS stages.

CHAPTER 9

Samenvatting

Het werk in dit proefschrift onderzoekt de invloed van DNA-methylatie op het gedrag van oligodendrocyten, zowel in fysiologische omstandigheden als in de context van progressieve multiple sclerose (MS).

Hoofdstuk 2 biedt diepgaande informatie over hoe epigenetische mechanismen de differentiatie van oligodendrocyten en myelinisatie beïnvloeden. Het geeft allereerst een algemeen overzicht van het transcriptionele netwerk dat het differentiatieproces reguleert. Vervolgens worden de epigenetische mechanismen, bestaande uit DNA-methylatie, histon-modificaties en miRNA's, elk afzonderlijk besproken op basis van hun gekende rol tijdens fysiologische oligodendrocyteprecursorcel (OPC) differentiatie. Ten slotte wordt het gevolg van epigenetische ontregeling gerelateerd aan OPC-differentiatie in myeline-gerelateerde aandoeningen en veroudering besproken.

Hoewel de literatuur suggereert dat DNA-methylatie-enzymen een sterke invloed hebben op de differentiatie van OPCs en (re)myelinisatie, bleef het ongeweten welke genen het doelwit zijn van deze enzymen tijdens OPC-differentiatie. In **Hoofdstuk 3** onderzocht ik de directe invloed van DNA-methylatie op het transcriptionele netwerk dat myeline-genexpressie en OPC-differentiatie reguleert. Ik heb niet alleen bevestigd dat DNA-methylatie cruciaal is voor het differentiatieproces, maar ik heb ook aangetoond dat de negatieve transcriptionele regulatoren, *Id2* en *Id4*, voornamelijk worden beïnvloed door DNA-methylatie tijdens OPC-differentiatie. Bovendien heb ik aangetoond dat in de pathologische context van MS de methylatie- en genexpressieniveaus van zowel *ID2* als *ID4* veranderd zijn in vergelijking met hersenstalen van gezonde controles. Op basis van deze resultaten kunnen we concluderen dat DNA-methylatie cruciaal is om *ID2* en *ID4* te onderdrukken tijdens OPC-differentiatie, een proces dat tijdens MS ontregeld lijkt te zijn. Deze resultaten onthullen niet alleen nieuwe inzichten in de biologie van oligodendrocyten, maar kunnen ook leiden tot een beter begrip van myelineaandoeningen, zoals MS.

Hoofdstuk 4 is gebaseerd op een perspectief, waarin we het belang van causaliteitsbepaling in neuro-epigenetisch onderzoek bespreken. We stellen een workflow voor, beginnend bij epigenoom-wijde associatie studies (EWAS), tot het toepassen van op CRISPR-Cas9 gebaseerde epigenetische bewerking als een hulpmiddel om de mogelijk causale associaties tussen epigenetische modificaties

van tophit-genen en de pathofysiologie van neurodegeneratieve aandoeningen te onderzoeken.

In het werk beschreven in **Hoofdstuk 5** heb ik de voorgestelde workflow uit hoofdstuk 4 toegepast in de context van progressieve MS. Uitgaande van epigenomische en transcriptomische profielen van chronisch gedemyeliniseerde MS-laesies, identificeerde ik doelgenen die differentieel tot expressie komen en differentieel gemethyleerd zijn in deze laesies, in vergelijking met de omringende normaal uitziende witte stof (NAWM). Cel-specifieke validatie van een van de sterkste differentieel gemethyleerde genen in relatie tot myelinisatie, *MBP*, toonde aan dat OPCs in de laesie een hypermethyleerd profiel vertonen van dit essentiële myelinegen. Door de CRISPR-Cas9 gebaseerde epigenetische bewerkingstoolbox toe te passen, heb ik het verband tussen de methylering van *MBP* en het differentiatievermogen van menselijke iPSC-afgeleide oligodendrocyten gevalideerd.

In de laatste studie, gepresenteerd in **Hoofdstuk 6**, heb ik onderzocht of het methyleringspatroon van de hersenen van progressieve MS-patiënten wordt weerspiegeld in het bloed en dus kan worden toegepast als een biomerker voor progressie bij MS. De ontregelde epigenetische signatuur van de myelinegenen, uit de EWAS-studie uit hoofdstuk 5, werd niet weerspiegeld in de bloedstalen van progressieve MS-patiënten. We hebben echter een sterke correlatie waargenomen tussen DNA-methylering van deze genen en de bewaartijd van de stalen. De resultaten uit deze studie suggereren dat het DNA-methylatieprofiel in het bloed kan worden beïnvloed door langdurige opslag, een belangrijke factor waar in toekomstige studies rekening mee moet genomen worden.

In conclusie, biedt dit proefschrift meer inzicht in de invloed van DNA-methylatie op OPC-differentiatie en MS-pathologie. De resultaten van dit proefschrift dragen bij aan een beter begrip van de moleculaire mechanismen die ten grondslag liggen aan remyelinisatiestoornissen en vormen de basis voor toekomstig onderzoek naar epigenetische veranderingen in relatie tot progressieve MS-stadia.

Chapter 10

Impact paragraph

The research described in this thesis identified epigenetic signatures underlying impaired oligodendrocyte precursor cell (OPC) differentiation and remyelination within lesions of progressive multiple sclerosis (MS) patients as a first step towards the identification of new targets for the development of novel treatment strategies. Furthermore, it linked brain and peripheral epigenetic marks in view of the potential application of blood methylation profiles as new biomarkers for disease progression in MS.

Societal impact

MS is one of the most common neurological conditions among young adults in the Western world, affecting approximately 2.5 million people worldwide. Around 1 million people are diagnosed as progressive MS patients, including primary progressive (PPMS) and secondary progressive MS patients (SPMS). The relatively high prevalence of MS (1:1000) is accompanied by high costs for patients and their family, as well as for society. In Europe, the annual costs for an MS patient with moderate disease severity is estimated at €37,100. Importantly, these costs increase with approximately 50% as the disease progresses (313, 314). In the early stages of the disease, overall costs are mainly driven by disease-modifying drug treatments. As the disease progresses, the overall cost increase is mainly affected by indirect costs, such as the loss of productivity for patients and their caretakers (315).

Available Food and Drug Administration (FDA)-approved therapies mainly modulate the immune system and temper early disease activity, but have limited efficacy in preventing transition towards the chronic phase and are no longer effective in progressive MS stages (8, 316, 317). Thus, there is a high medical need for novel therapeutic strategies to induce repair mechanisms and prevent or attenuate disease progression during the chronic stages of MS. Notably, the emphasis within MS research has strongly shifted towards understanding the molecular mechanisms underlying progression in MS, as supported by the Progressive MS Alliance (318), which represents a global collaboration of MS organisations, researchers, health professionals, the pharmaceutical industry, companies, trusts, foundations, donors and people affected by progressive MS, aimed at accelerating the development of effective treatments for people with

progressive MS in order to improve quality of life. Accordingly, in the present project, we aimed to uncover new mechanisms and pathways that underly remyelination impairment in order to identify novel therapeutic targets for progressive MS. We identified multiple epigenetic target genes that play an important role in oligodendrocyte precursor cell (OPC) differentiation. Targeting these epigenetic alterations, e.g. by CRISPR-Cas9-based epigenetic editing, could therefore be considered as a potential therapeutic strategy to overcome remyelination failure.

The second aim of this thesis was to investigate whether brain methylation profiles are mirrored in the blood and could serve as a biomarker for disease progression in MS. Unfortunately, in our study, we were not able to discover new biomarkers for progression in MS when it comes to DNA methylation signatures of myelin-related genes. Yet, this does not exclude the possibility of blood-born DNA methylation biomarkers to be of added value in this respect. Such a biomarker would benefit progressive MS patients and the healthcare system on multiple levels. First of all, a new bloodborne surrogate marker to define disease progression is easily accessible and reduces the need of magnetic resonance imaging (MRI), the current golden standard. Moreover, this can lead to an early adaptation of the treatment regimen so that patients will not be unnecessarily treated with ineffective drugs, eventually leading to a cost reduction for both the patients and society. As for the development of new drugs that modulate the disease progression, biomarkers for remyelination impairment can be applied in drug screening phases, as well as in human clinical trials. Such theranostic markers give an accurate and valid indication of the effect of a treatment on patients, thereby enabling and accelerating clinical trials.

Scientific impact

The research described in this thesis is one of its kind, since it is the first to reveal the epigenetic signature within chronically demyelinated lesions of progressive MS patients. Similar research has been conducted in the context of other neurological diseases, such as Alzheimer's disease or schizophrenia, and has revealed innovative targets related to disease development (319, 320). Yet, within the scope of progressive MS, data on the epigenetic imprint of remyelination

impairment was still lacking at this stage. We aimed to explore these new avenues to unravel the molecular links between environmental changes and disease progression in MS. To achieve this, we applied a set of innovative experimental techniques, such as laser-capture microdissection (LCM) and CRISPR/Cas9-based epigenetic editing system, to assess the specificity of our targets and the functional effect on OPC differentiation, respectively. Our work represents a starting point for important research regarding DNA methylation signatures in chronically demyelinated MS lesions with the final aim to discover new targets to restore the remyelination capacity in progressive MS stages.

Even though in our current study, we did not identify new biomarkers for disease progression in MS, blood-based methylation marks may still be assessed and proven useful in view of disease prognosis by e.g. performing an epigenome-wide association study in the blood, as our group has previously shown in other disease domain, including Alzheimer's disease (321). Moreover, our data on myelin-related gene methylation in MS suggest that the degree of DNA methylation in the blood can be affected by long-term sample storage, depending on the gene assessed. This is an important factor that had been neglected before yet might lead to false epigenetic discoveries. Sample storage time should therefore be considered during the initial sample selection stage in future studies.

Altogether, this dissertation provides more insights into the influence of DNA methylation on OPC differentiation and MS pathology. The work in this thesis is a first step in the field of myelin-related epigenetics and lays the foundation for future research on epigenetic changes in relation to progressive MS stages. The data generated in this research is a valuable addition to the current epigenetic data collection on MS brain samples and contributes to the efforts of the scientific community to identify novel markers for disease progression as well as targets for therapeutic drug development.

Chapter 11

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Chapter 12

Curriculum Vitae

Curriculum Vitae

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Education

2019 – present	PhD student, FWO-SB Fellow Hasselt University (BE) / Maastricht University (NL)
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Publications

Published:

1. **A perspective on causality assessment in epigenetic research on neurodegenerative disorders.** Tiane A, Koulousakis P, Hellings N, Prickaerts J, van den Hove D, Vanmierlo T. Neural Regen Res. 2023 Feb;18(2):331-332. doi: 10.4103/1673-5374.343898. PMID: 35900421; PMCID: PMC9396512. (IF 6.058)
2. **DNA methylation regulates the expression of the negative transcriptional regulators ID2 and ID4 during OPC differentiation.** Tiane A, Schepers M, Riemens R, Rombaut B, Vandormael P, Somers V, Prickaerts J, Hellings N, van den Hove D, Vanmierlo T. Cell Mol Life Sci. 2021 Oct;78(19-20):6631-6644. doi: 10.1007/s00018-021-03927-2. Epub 2021 Sep 5. PMID: 34482420; PMCID: PMC8558293. (IF 9.207)
3. **From OPC to Oligodendrocyte: An Epigenetic Journey.** Tiane A., Schepers M., Rombaut B., Hupperts R., Prickaerts J., Hellings N., van den

Hove D., Vanmierlo T. Cells., 2019 Oct 11;8(10). pii: E1236. Review. (IF 4.366)

Publications in preparation:

1. **From methylation to myelination: epigenomic and transcriptomic profiling of chronic inactive demyelinated multiple sclerosis lesions** Assia Tiane, Ehsan Pishva, Melissa Schepers, Lieve van Veggel, Rick Reijnders, Sarah Chenine, Ben Rombaut, Emma Dempster, Catherine Verfaillie, Kobi Wasner, Anne Grünewald, Jos Prickaerts, Niels Hellings, Daniel van den Hove, Tim Vanmierlo

Co-authored publications:

1. **24 (S)-Saringosterol Prevents Cognitive Decline in a Mouse Model for Alzheimer's Disease.** Martens N., Schepers M., Zhan N., Leijten F., Voortman G., Tiane A., Rombaut B., Poisquet J., van de Sande N., Kerksiek A., Kuipers F., W Jonker J., Liu H., Lütjohann D., Vanmierlo T., T Mulder M. Mar Drugs 2021 Mar 27;19(4):190. (IF 6.083)
2. **Oxidative stress and impaired oligodendrocyte precursor cell differentiation in neurological disorders.** Spaas J., van Veggel L., Schepers M., Tiane A., van Horssen J., M Wilson D., R Moya P., Piccart E., Hellings N., O Eijnde B., Derave W., Schreiber R., Vanmierlo T. Cell Mol Life Sci. 2021 May;78(10):4615-4637. (IF 9.207)
3. **PDE inhibition in distinct cell types to reclaim the balance of synaptic plasticity**. Rombaut B., Kessels S., Schepers M., Tiane A., Paes D., Solomina Y., Piccart E., van den Hove D., Brône B., Prickaerts J. and Vanmierlo T. Theranostics 2021; 11(5): 2080-2097. (IF 11.556)
4. **Sphingosine-1-phospate receptor modulators and oligodendroglial cells: beyond immunomodulation** Roggeri A., Schepers M., Tiane A., van Veggel L., Hellings N, Prickaerts J, Pittaluga A, Vanmierlo T. Int J Mol Sci. 2020 Oct 13;21(20):E7537. (IF 5.92)
5. **Edible seaweed-derived constituents: an undisclosed source of neuroprotective compounds.** Schepers M., Martens N., Tiane A., Vanbrabant K., Liu H-B., Lütjohann D., Mulder M, Vanmierlo T. Neural Regen Res. 2020 May;15(5):790-795. Review. (IF: 5.14)

Scientific grants and awards

Fellowship:

- FWO-SB PhD fellowship

Grants:

- Belgian Charcot foundation research grant - €40k (2019-2021)
- EURON mobility grant - €1500 (2019)
- UHasselt Doctoral School mobility grant - €500 (2019)
- Rotary, Hope in Head research grant - €7k (2019-2020)

Awards and prizes:

- First prize oral presentation Flemish MS Research days 2023, Antwerpen (BE, May 2023)
- Laureate of "Diversiteitsprijs 2018" UHasselt/HBVL, Hasselt (BE, Oct 2018)
- First price poster presentation Animal behaviour symposium 2018, Hasselt (BE, Sept 2018)
- First price poster presentation MOSA Conference 2018, Maastricht (NL, June 2018)
- First price best intern higher education, VOKA Limburg Qstage, Hasselt (BE, May 2018)

International stays

The UK - Visiting researcher at the Complex Epigenetics Research Group in the lab of dr. Emma Dempster at the university of Exeter, The UK as a training for bioinformatic analysis of epigenomic data in R programming language (Nov – Dec 2019)

Luxembourg – Visiting researcher at the lab of prof. dr. Anne Grünewald at the university of Luxembourg, Grand Duchy of Luxembourg to perform laser-captured microdissection (Jan 2020)

Oral and poster presentations

Oral presentations:

Title: Relieving the epigenetic blockade in progressive multiple sclerosis - making remyelination accessible again
Conferences:
- EURON PhD days 2018 (Brussels, Belgium): selected talk
- Dutch Neuroscience meeting 2019 (Lunteren, The Netherlands): invited talk

Title: Epigenomic and transcriptomic profiling of chronically demyelinated MS lesions.
Conferences:
- GliaNed 2022 (Utrecht, The Netherlands): selected talk
- MS research days 2022 (Rotterdam, The Netherlands): selected talk

Poster presentations:
Title: Relieving the epigenetic blockade in progressive multiple sclerosis - making remyelination accessible again
Conferences:
- Glia 2019 (Porto, Portugal)
- MHeNS research days 2019 (Maastricht, The Netherlands)
- WOG MS 2019 (Antwerpen, Belgium)

Title: DNA methylation regulates the expression of the negative transcriptional regulators ID2 and ID4 during OPC differentiation.
Conferences:
- EURON PhD days 2021 (Hasselt, Belgium)
- Glia 2021 (Marseille/Online)
- Myelin Gordon Research Seminar and Conference (Lucca, Italy)

Academic activities

Membership of scientific organisations:
- Member of the junior board of the Belgian Society for Neuroscience (BSN) (2021 – 2022)
- EURON PhD representative, Maastricht University (2019 – 2022)

Institutional responsibilities

- Elected member of the department council neuroscience of Hasselt University (2020 – present)
- Member of the PhD council of Hasselt University (2019 – present)
- Invited member of the jury 'Diversiteitsprijs' of Hasselt University (Sept 2019)
- Invited guest for the GLW faculty strategy day at Hasselt University (March 2019)
- Academic coaching of students with a foreign background, UHasselt (2014 - 2017)

Chapter 13

Acknowledgements

Do a PhD, they said. It will be fun, they said.

And fun it was. Next to all the stress, pressure, and workload of course. The past four years have been a wild ride. But looking back, I would do it all over again. I had the most amazing opportunities and have collected so many great memories during my PhD. Yet, I would never have been able to do this all by myself. I consider myself lucky to be surrounded by amazing, empowering, and motivational people who all had their fair share in helping me arrive at the finish line. I would therefore like to take the opportunity to express my gratitude to a number of people.

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