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GENE REGULATORY MECHANISMS IN THE OLIGODENDROCYTE LINEAGE IN DEVELOPMENT AND DISEASE

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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POPULAR SCIENCE SUMMARY

Multiple sclerosis (MS) is a disease that affects the communication of the brain and spinal cord with the rest of the body. This communication is carried out by neurons that transmit information through their axons in the form of electrical signals. Oligodendrocytes are cells that form fatty layers around neuronal axons called myelin sheaths and they protect the axon and promote signaling. When this insulating layer is damaged, the electrical signal leaks and the efficiency of conduction is compromised. This is exactly what happens in MS. Oligodendrocytes are attacked by the immune system resulting in the degeneration of the myelin sheaths. The underlying axons are now exposed and signaling is disrupted. This leads to a wide variety of symptoms in MS patients, depending on the site of the immune attack. An example of a common symptom is the feeling of weakness in one or both legs.

The underlying cause of MS is still unknown but research has shown environmental factors in people with a genetic predisposition for the disease. In this thesis, we investigate the role of oligodendrocytes, the target cells in MS. We first investigated how they are generated during development, as this could help us recognize how we could aid these cells in disease. We studied an enzyme called peptidylarginine deiminase (PAD) that modifies proteins resulting in altered function. We discovered that PAD2 modifies proteins important for the regulation of gene transcription. In the absence of PAD2, there is a failure in the transcription of critical genes required for oligodendrocyte differentiation. PAD2 also modifies proteins important for the myelin sheath, therefore loss of PAD2 results in unstable myelin and motor dysfunction.

As oligodendrocytes are targeted by the immune system in MS, we also studied how these cells are affected by the immune response. The immune system releases cytokines, which are signaling molecules that communicate to other cells that inflammation is ongoing. We discovered that oligodendrocytes exposed to cytokines are captured in an immune state and actively communicate with the immune cells. This directly affects their ability to produce myelin. We therefore investigated the mechanisms of this switch from a myelin to an immune state. Surprisingly, we identified that regions in the DNA containing immune genes are accessible within oligodendrocytes. These regions can contain a variation in the DNA associated with increased risk of MS. These variations were thought to be only relevant in immune cells, but we identified that they could also be relevant in oligodendrocytes.

Lastly, we developed a method to investigate how different DNA regions interact within different brain cell types in 3D, including oligodendrocytes. Interactions between DNA regions influence which genes can be transcribed. We found interactions that are specific for certain cell types in the brain and could therefore identify different mechanisms that regulate gene transcription. This information is important as future research could use this to determine defects within cells that are associated with diseases such as MS.

With our research, we hope to shed light on the mechanisms of how oligodendrocytes develop and how they respond and are affected in disease. We specifically looked at the mechanisms of gene regulation in these processes. This will hopefully lead to the discovery of new future therapies that could improve the symptoms in MS patients.

POPULAIR WETENSCHAPPELIJKE SAMENVATTING

Multiple sclerose (MS) is een ziekte die de communicatie van de hersenen en het ruggenmerg met de rest van het lichaam aantast. Neuronen geven informatie door in de vorm van elektrische signalen via hun axonen. Oligodendrocyten zijn cellen die vetlagen vormen rond neurale axonen, wat ook wel myeline genoemd wordt. Wanneer deze laag beschadigt, lekt het elektrische signaal en wordt de communicatie belemmerd. Dit is precies wat er gebeurt in MS. Oligodendrocyten worden aangevallen door het immuunsysteem, wat resulteert in het afbreken van myeline. De onderliggende axonen worden blootgelegd en de signalering is verstoord. Dit leidt tot een breed scala aan symptomen bij patiënten, afhankelijk van de plaats van ontsteking. Een veel voorkomende klacht is bijvoorbeeld krachtvermindering in de benen.

De onderliggende oorzaak van MS is nog niet bekend, maar onderzoek wijst uit naar omgevingsfactoren in personen met een genetische aanleg voor de ziekte. In dit proefschrift doen we onderzoek naar de oligodendrocyten. We onderzoeken eerst hoe ze tijdens de ontwikkeling worden gevormd, waardoor we hopelijk deze cellen kunnen beschermen. We onderzoeken een enzym, genaamd peptidylarginine deiminase (PAD), dat eiwitten verandert. We ontdekten dat PAD2 eiwitten wijzigt die belangrijk zijn voor de regulatie van gentranscriptie. Wanneer PAD2 ontbreekt, worden de oligodendrocyten niet op de juiste manier gevormd tijdens de ontwikkeling. PAD2 verandert ook eiwitten die belangrijk zijn voor myeline, daarom resulteert verlies van PAD2 in instabiele myeline en motorische afwijkingen.

Omdat oligodendrocyten het doelwit zijn van het immuunsysteem in MS, hebben we ook onderzocht hoe deze cellen worden beïnvloed door de ontstekingsreactie. Het immuunsysteem communiceert door middel van cytokines dat er een ontsteking aan de gang is. We ontdekten dat oligodendrocyten die aan cytokines worden blootgesteld actief communiceren met immuuncellen, wat hun myeline productie beïnvloedt. Daarom onderzochten we de mechanismen die deze verandering teweegbrengen. We vonden stukjes DNA die immuungenen bevatten en bereikbaar zijn in oligodendrocyten. Deze stukjes DNA kunnen een variatie in het DNA bevatten die gepaard gaat met een verhoogd risico op MS. Van deze variaties werd gedacht dat ze alleen relevant waren in immuuncellen, maar we hebben vastgesteld dat ze ook relevant kunnen zijn in oligodendrocyten.

Ten slotte hebben we een methode ontwikkeld om te onderzoeken hoe verschillende stukjes DNA met elkaar in verbinding staan in de verschillende soorten cellen van de hersenen. Deze interacties beïnvloeden welke genen kunnen worden overgeschreven. We vonden interacties die specifiek zijn voor bepaalde soorten cellen in de hersenen en konden daarom verschillende mechanismen vaststellen die gentranscriptie reguleren. Deze informatie is belangrijk omdat dit door toekomstig onderzoek gebruikt zou kunnen worden om defecten in cellen vast te stellen die verband houden met verschillende ziektes zoals MS.

Met ons onderzoek hopen we duidelijkheid te kunnen scheppen hoe oligodendrocyten zich ontwikkelen en hoe ze worden aangetast in ziekte. We hebben specifiek gekeken naar de mechanismen van genregulatie in deze processen. Dit zal hopelijk leiden tot de ontwikkeling van nieuwe therapieën die de symptomen bij MS-patiënten zouden kunnen verbeteren.

ABSTRACT

Oligodendrocytes are the myelinating cells of the central nervous system (CNS). They contribute to the neuronal network through the insulation of neuronal axons, facilitating communication between neurons and providing metabolic support. In multiple sclerosis (MS), oligodendrocytes are attacked by the immune system leading to a wide variety of symptoms. Remyelination is necessary for functional recovery, which can occur through the recruitment and differentiation of oligodendrocyte precursor cells (OPCs) that reside in the adult CNS. During development and in disease, oligodendrocytes and OPCs (oligodendroglia) undergo significant changes at the transcriptional level. However, the genomes remain the same within these cells, so how do these transcriptional changes occur? In this thesis, we investigate gene regulatory mechanisms in the oligodendrocyte lineage in development and disease.

In **Paper I** we investigate the role of citrullination in the differentiation of oligodendrocytes. We identify peptidylarginine deiminase 2 (PAD2) as the major citrullinating enzyme in oligodendrocytes, promoting oligodendrocyte differentiation through the upregulation of myelin genes. Interestingly, the main targets of PAD2 are proteins involved in transcriptional and posttranscriptional regulation. Other PAD2 targets are myelin proteins, which might explain the motor and cognitive deficits and the decrease in myelinated axons we observe upon loss of PAD2.

In **Paper II** we characterize how the oligodendrocyte lineage is affected in disease, using single-cell transcriptomics in the MS mouse model experimental autoimmune encephalomyelitis (EAE). Oligodendroglia in EAE mice show an increase in immune pathway genes including major histocompatibility complex (MHC) class-I and -II genes involved in antigen processing and presentation. Furthermore, OPCs stimulated with interferon-gamma interact with and activate CD4 positive T cells. Thus, oligodendroglia might have a more active role in mediating the inflammatory response in MS than previously thought.

In **Paper III** we investigate how oligodendroglia transition to the immune state, using single-cell ATAC-seq in EAE mice. We find that immune genes are primed and increase their expression in an inflammatory environment through changes in the histone modification landscape, in chromatin interactions, and in transcription factor binding. Overall, we identify gene regulatory mechanisms of the immune program in oligodendroglia that could be possible therapeutic targets for MS.

In **Paper IV** we develop an extension of the method genome architecture mapping (immunoGAM), which we apply to study genome-wide chromatin interactions in intact brain tissue. We find interactions and mechanisms that are specific for different brain cell types. Long neuronal genes that are active, often show decondensation or ‘melting’. Furthermore, topologically associating domains and A/B compartments reorganize extensively upon differentiation, and cell type-specific interactions form mediated by specific transcription factor pairs.

To conclude, this thesis examines different layers of gene regulation including chromatin accessibility, histone modifications, genome interactions, and transcription factor binding. More specifically, we investigate how these different layers are involved in the transitioning of oligodendroglia during differentiation or to disease states. The findings in this thesis will hopefully contribute to the development of improved treatment strategies for MS.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Transcriptional convergence of oligodendrocyte lineage progenitors during development
Sueli Marques*, David van Bruggen*, Darya Pavlovna Vanichkina*, Elisa M Floriddia, Hermany Munguba, Leif Väremo, Stefania Giacomello, Ana Mendanha Falcão, **Mandy Meijer**, Åsa Kristina Björklund, Jens Hjerling-Leffler, Ryan James Taft, and Gonçalo Castelo-Branco
Developmental Cell *2018, Vol. 46, Page 504-517. DOI: 10.1016/j.devcel.2018.07.005*

Developmental landscape of human forebrain at a single-cell level identifies early waves of oligodendrogenesis
David van Bruggen, Fabio Pohl, Christoffer Mattson Langseth, Petra Kukanja, Hower Lee, Alejandro Mossi Albiach, Mukund Kabbe, **Mandy Meijer**, Sten Linnarsson, Markus M Hilscher, Mats Nilsson, Erik Sundström, and Gonçalo Castelo-Branco
Developmental Cell *2022, Vol. 57, Page 1-16. DOI: 10.1016/j.devcel.2022.04.016*

LIST OF SCIENTIFIC PAPERS

I. **PAD2-mediated citrullination contributes to efficient oligodendrocyte differentiation and myelination**
Ana Mendanha Falcão, **Mandy Meijer**, Antonella Scaglione, Puneet Rinwa, Eneritz Agirre, Jialiang Liang, Sara C Larsen, Abeer Heskol, Rebecca Frawley, Michael Klingener, Manuel Varas-Godoy, Alexandre ASF Raposo, Patrik Ernfors, Diogo S Castro, Michael L Nielsen, Patrizia Casaccia, and Gonçalo Castelo-Branco
Cell Reports *2019, Vol. 27, Page 1090-1102. DOI: 10.1016/j.celrep.2019.03.108*

II. **Disease-specific oligodendrocyte lineage cells arise in multiple sclerosis**
Ana Mendanha Falcão*, David van Bruggen*, Sueli Marques, **Mandy Meijer**, Sarah Jäkel, Eneritz Agirre, Elisa M Floriddia, Darya P Vanichkina, Anna Williams, André Ortlieb Guerreiro-Cacais, and Gonçalo Castelo-Branco
Nature Medicine *2018, Vol. 24, Page 1837-1844. DOI: 10.1038/s41591-018-0236-y*

III. **Epigenomic priming of immune genes implicates oligodendroglia in multiple sclerosis susceptibility**
Mandy Meijer*, Eneritz Agirre*, Mukund Kabbe, Cassandra A van Tuijn, Abeer Heskol, Chao Zheng, Ana Mendanha Falcão, Marek Bartosovic, Leslie Kirby, Daniela Calini, Michael R Johnson, M Ryan Corces, Thomas J Montine, Xingqi Chen, Howard Y Chang, Dheeraj Malhotra, and Gonçalo Castelo-Branco
Neuron *2022, Vol. 110, Page 1193-1210. DOI: 10.1016/j.neuron.2021.12.034*

IV. **Cell-type specialization is encoded by specific chromatin topologies**
Warren Winick-Ng*, Alexander Kukalev*, Izabela Harabula*, Luna Zea-Redondo*, Dominik Szabó*, **Mandy Meijer**, Leonid Serebreni, Yingnan Zhang, Simona Bianco, Andrea M Chiariello, Ibai Irastorza-Azcarate, Christoph J Thieme, Thomas M Sparks, Sílvia Carvalho, Luca Fiorillo, Francesco Musella, Ehsan Irani, Elena Torlai Triglia, Aleksandra A Kolodziejczyk, Andreas Abentung, Galina Apostolova, Eleanor J Paul, Vedran Franke, Rieke Kempfer, Altuna Akalin, Sarah A Teichmann, Georg Dechant, Mark A Ungless, Mario Nicodemi, Lonnie Welch, Gonçalo Castelo-Branco, and Ana Pombo
Nature *2021, Vol. 599, Page 684-691. DOI: 10.1038/s41586-021-04081-2*

CONTENTS

LIST OF ABBREVIATIONS

CNS	Central nervous system
MS	Multiple sclerosis
OPC	Oligodendrocyte precursor cell
PAD	Peptidylarginine deiminase
EAE	Experimental autoimmune encephalomyelitis
CFA	Complete Freund's adjuvant
MHC	Major histocompatibility complex
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
GAM	Genome architecture mapping
TAD	Topologically associating domain
P	Postnatal day
E	Embryonic day
ER	Endoplasmic reticulum
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
bHLH	Basic-helix-loop-helix
HDAC	Histone deacetylases
H3K27	Lysine 27 at histone H3
SNP	Single-nucleotide polymorphism
PGN	Pyramidal glutamatergic neurons
DN	Dopaminergic neurons
mESC	Mouse embryonic stem cells

1 INTRODUCTION

1.1 Oligodendrocytes and myelination

Oligodendrocytes are a critical part of a highly complex and heterogeneous network of various cell types that together compose the central nervous system (CNS). Oligodendrocytes enwrap neuronal axons with myelin sheaths, thereby facilitating electrical signal transduction within neurons. The myelin sheaths allow for saltatory conduction, which is the jumping of action potentials between nodes of Ranvier. In this way, the signaling speed increases, being able to cover greater distances in a shorter time (Hartline and Colman, 2007). This is particularly important for neurons in the brain that extend their axons through the spinal cord, covering distances of several meters in large vertebrates (Nave, 2010a; Salzer and Zalc, 2016). Saltatory conduction is also important for the synchronization of neuronal signals, for example with relaying sensory and motor signals between the thalamus and the much larger cortex (Salami *et al.*, 2003). The timing of signals is crucial in this neuronal network and is regulated partially by the differential coverage of myelin sheaths. Electrical signaling through longer axons becomes faster with myelination and therefore can match signaling speed of less myelinated shorter axons (Salami *et al.*, 2003; Seidl, 2014). Thus, oligodendrocytes can contribute to the timing of neuronal signaling over long and short distances forming a functional, synchronized neuronal network.

Although myelination improves signaling, its insulation limits access of the axon to the extracellular space containing metabolites such as glucose (Nave, 2010b). Oligodendrocytes facilitate myelinated axons in their energy consumption by producing lactate, which they provide to the axons through monocarboxylate transporters (Lee *et al.*, 2012; Fünfschilling *et al.*, 2012). In the absence of glucose, axons take up the released lactate from the periaxonal space between the axon and myelin sheath for their mitochondrial ATP generation. Saltatory conduction also reduces axonal energy consumption, as most sodium channels are concentrated at the nodes of Ranvier and therefore fewer channels are needed. Oligodendrocytes additionally contribute to restoring ionic imbalance after neuronal activity, for example with buffering of potassium from the periaxonal space (Menichella *et al.*, 2006; Larson *et al.*, 2018). Most of the potassium channels are located beneath the myelin sheaths in the juxtaparanodal area, where potassium is released after an action potential and subsequently cleared by oligodendrocytes. In this way, oligodendrocytes do not only contribute to faster signaling, but also to energy saving and maintenance of ion homeostasis. Oligodendrocytes, together with neurons, provide an axon-myelin framework for accurate functional signaling.

1.1.1 Development

Oligodendrocytes are derived from oligodendrocyte precursor cells (OPCs), which are generated from neuroepithelial cells in the developing CNS. In the mouse spinal cord, OPCs are generated in two different waves (Figure 1). The first wave starts at embryonic day (E)12.5 in the ventricular zone of a ventral progenitor domain called pMN, where

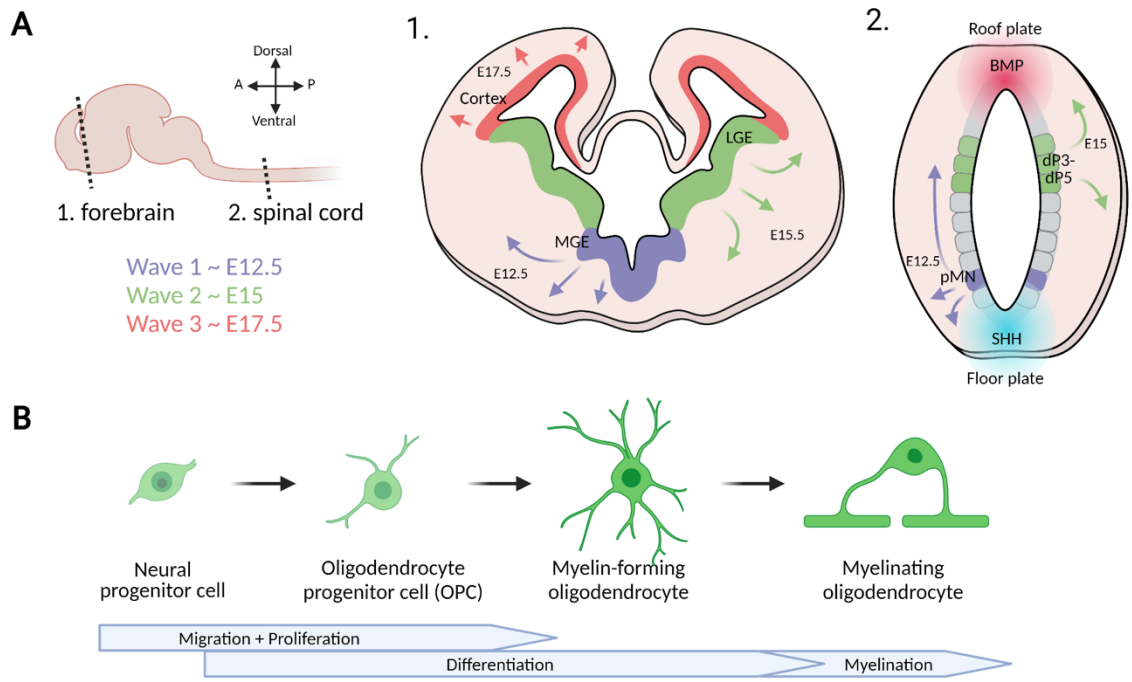

Figure 1. Schematic overview of oligodendrocyte development. A) Depicted are the different developmental waves and their origin in the different regions of the forebrain and spinal cord. B) OPCs are generated from neural progenitor cells and differentiate into myelin-forming oligodendrocytes.

neuroepithelial cells are exposed to the morphogen Sonic hedgehog (SHH) secreted from the notochord and floor plate (Ericson *et al.*, 1996). SHH regulates the expression of patterning transcription factors via a concentration gradient, resulting in the overlap of NKX6.1 and PAX6 at the height of the pMN domain (Ericson *et al.*, 1997; Sun *et al.*, 1998; Liu *et al.*, 2003). This overlap leads to the expression of OLIG2 in this domain, which first induces the generation of motor neurons and then OPCs (Lu *et al.*, 2002; Takebayashi *et al.*, 2002; Zhou and Anderson, 2002; Liu *et al.*, 2003). Although the first wave of OPCs depends highly on SHH (Pringle *et al.*, 1996; Poncet *et al.*, 1996; Orentas *et al.*, 1999), the second wave in the spinal cord is SHH independent and starts around E15 from the dorsal domains dP3-dP5 (Cai *et al.*, 2005). These domains are under the influence of BMP signaling from the roof plate and co-express PAX7 and ASCL1 (MASH1; Liem *et al.*, 1997; Lee *et al.*, 2000; Müller *et al.*, 2002). BMP inhibits OLIG2 expression and the induction of OPCs (Mekki-Dauriac *et al.*, 2002; Miller *et al.*, 2004), however, BMP signaling in the dorsal domains diminishes over time (Vallstedt *et al.*, 2005). Furthermore, FGF signaling inhibits BMP and promotes OLIG2 expression (Bilican *et al.*, 2008). FGF signaling together with reducing levels of BMP allow for the expression of OLIG2 and the generation of OPCs in the dorsal domains (Chandran *et al.*, 2003; Vallstedt *et al.*, 2005). Dorsal OPCs give rise to around 10-15% of the final population of OPCs in the spinal cord while the ventral OPCs dominate with 85% (Richardson *et al.*, 2006).

In the mouse brain, the first wave of OPCs is generated around E12.5 in the ventricular zone of the medial ganglionic eminence and the anterior entopeduncular area in the ventral telencephalon. This domain is defined by NKX2.1 expression and, similar to the ventral spinal cord OPCs, relies on SHH signaling (Nery *et al.*, 2001; Tekki-Kessaris *et al.*, 2001).

After their generation, OPCs migrate laterally and dorsally throughout the entire telencephalon and populate the cerebral cortex from around E16 (Tekki-Kassaris *et al.*, 2001). Another wave of ventral OPCs is generated around E15.5 in the lateral and caudal ganglionic eminences. These OPCs are GSX2 positive (GSH2) and rely also on SHH for their generation. This second wave enters the cerebral cortex around E18. Lastly, a third late embryonic/postnatal wave of OPCs is generated from the cortical subventricular zone (Ivanova *et al.*, 2003; Kessaris *et al.*, 2006; Winkler *et al.*, 2018). This dorsal wave of OPCs comes from an EMX1 positive lineage (Gorski *et al.*, 2002). It was long thought that this wave was SHH independent, however, a population of EMX1 positive cells depends on SHH secreted by migrating interneurons and choroid plexus at late-embryonic stages to generate OPCs (Winkler *et al.*, 2018). EMX1 positive OPCs take over from the earlier generated ventral OPC waves in populating the cortex (Kessaris *et al.*, 2006). In the adult brain, OPCs generated from the third wave are the main residing population in the dorsal areas of the forebrain, whereas the second wave is enriched in the ventral areas (Richardson *et al.*, 2006; Crawford *et al.*, 2016).

As described above, OPCs migrate away from their place of birth toward other areas of the brain and spinal cord. They populate the entire CNS in a homogenous distribution pattern because of self-repulsive mechanisms (Zhang and Miller, 1996). The process of migration is highly dynamic and coincides with ongoing proliferation, differentiation, and apoptosis to maintain the correct number of OPCs and to generate sufficient numbers of myelinating oligodendrocytes (Barres *et al.*, 1992; Hughes *et al.*, 2013). OPC density reaches its peak in the first postnatal week. From then onwards, OPCs begin differentiating into myelinating oligodendrocytes starting in the caudal part of the spinal cord continuing rostrally, with the cortex to be myelinated last (Coffey and McDermott, 1997). Differentiation reaches its peak around postnatal day (P)14 and then slowly declines (Sturrock *et al.*, 1980). A substantial number of OPCs remain in the progenitor state in the adult CNS and take up 2-9% of the total cell population (Dawson *et al.*, 2003). These adult OPCs can be recruited to differentiate and integrate into the cellular network when needed (Fancy *et al.*, 2004). Proliferation, differentiation, and myelination continue in the adult brain, contributing to the plasticity of the neuronal network but also to remyelination in the case of injury (Young *et al.*, 2013; McKenzie *et al.*, 2014; Gensert and Goldman, 1997; Franklin *et al.*, 1997; Levine and Reynolds, 1999).

1.1.2 Heterogeneity of OPCs

The regional and temporal differences of the origins of OPCs and their distribution in the adult CNS could suggest that OPCs are heterogeneous. Interestingly though, when any of the three forebrain OPC waves are ablated, the other two waves can compensate by populating the areas that are lacking OPCs (Kessaris *et al.*, 2006). At later stages, no major differences are observed, which suggests that the different waves have no intrinsic restrictions on populating other areas of the forebrain and contributing to myelination. Similar in the spinal cord, the dorsal wave can also populate ventral regions upon the loss of ventral OPCs

(Vallstedt *et al.*, 2005). Furthermore, postnatal OPCs derived from ventral or dorsal areas of the brain or spinal cord have no, or very little, transcriptional differences and similar electrical properties (Tripathi *et al.*, 2011; Marques *et al.*, 2018; Zeisel *et al.*, 2018). Thus, evidence points to that OPCs derived from different regions in the CNS are homogeneous and functionally redundant. Nevertheless, more and more studies show that differences exist in, for example, a disease context. Dorsal spinal cord OPCs that extend into the ventral domain upon loss of ventral OPCs obtain an altered morphology and distribution and fail to aid injured motor neurons, which ventral derived OPCs would normally do (Starikov *et al.*, 2020). Dorsal OPCs also contribute more to remyelination in both the brain and spinal cord (Crawford *et al.*, 2016). In normal conditions, dorsal OPCs have a preference to myelinating dorsal areas and dorsal axonal tracts, such as the dorsal corticospinal tract (Kessaris *et al.*, 2006; Tripathi *et al.*, 2011; Crawford *et al.*, 2016). Why this preference exists is not known, although it has been suggested that competition between the different waves probably plays a role.

Although OPCs generated in different areas of the CNS do not have major cell-intrinsic differences in homeostatic conditions, their local environment might influence their behavior differently. The cell composition of white and gray matter regions is quite different. White matter regions contain more OPCs, oligodendrocytes, myelinated axon tracts, and fewer neuronal cell bodies (Dawson *et al.*, 2003; Dimou *et al.*, 2008). These characteristics can affect the properties of OPCs, for example, both proliferation and differentiation are more extensive in white matter (Rivers *et al.*, 2008; Kang *et al.*, 2010; Zhu *et al.*, 2011; Young *et al.*, 2013). White matter OPCs are more prone to differentiate even when displaced into the gray matter, and conversely gray matter OPCs are less prone to differentiate in white matter, which suggests some form of long-lasting environmental influence (Viganò *et al.*, 2013). White matter OPCs also respond differently to growth factors and have different ion channel compositions and electrical properties (Chittajallu *et al.*, 2004; Hill *et al.*, 2013). These studies suggest that environmental cues have a critical role in shaping short- and long-term properties of OPCs.

A unique aspect of OPCs is their ability to proliferate and populate the entire CNS throughout life to be able to differentiate when needed. However, the prenatal environment differs from the postnatal and adult CNS. OPCs in the ventral forebrain and spinal cord have the potential to generate astrocytes in the gray matter at embryonic stages (Zhu *et al.*, 2008a; Zhu *et al.*, 2008b). Postnatally they lose this potential and OPCs are restricted to the oligodendrocyte lineage (Rivers *et al.*, 2008; Zhu *et al.*, 2011). In adulthood, their proliferation and differentiation frequency declines (Zhu *et al.*, 2011; Young *et al.*, 2013), likely due to a saturation in myelinated axons. They also undergo transcriptional changes such as an increase in the expression of myelin genes, and the downregulation of *Pdgfra* and *Cspg4* (Moyon *et al.*, 2015). They become more heterogeneous in terms of electrophysiological properties, as they obtain a differential expression of ion channels influenced from their environment (Spitzer *et al.*, 2019). However, upon injury, adult OPCs can become ‘reactivated’ and increase their migration, proliferation, and differentiation potential (Moyon *et al.*, 2015).

To conclude, OPCs derived from different areas of the CNS have functionally different properties to a certain extent. OPCs rely on the interaction with other cell types and signaling factors such as chemokines and growth factors, which can alter their migration, proliferation, and differentiation potential (Calver *et al.*, 1998; van Heyningen *et al.*, 2001; Tsai *et al.*, 2002). Furthermore, these potentials can also be regulated by neuronal activity (Gibson *et al.*, 2014; Gautier *et al.*, 2015). This shows that OPCs are a highly adaptive cell type in order to aid in neuronal signaling whenever needed.

1.1.3 Heterogeneity of oligodendrocytes

Del Río-Hortega was the first to describe the heterogeneity of oligodendrocytes and their morphology in 1928. He could identify four distinct types based on their myelination pattern. Type 1 has many different myelin segments on small diameter axons in diverse orientations and is found in both gray and white matter. Type 2 has many different myelin segments on small diameter axons parallel to each other and is only present in white matter. Type 3 has few myelin segments on axons of large diameter, found only in white matter. Lastly, type 4 has its flat and elongated cell body adjacent to a single very large axon of medium or large diameter, which it myelinates. Similar subtypes were later identified based on morphology and biochemical properties (Butt *et al.*, 1995). However, it is not clear if these differences are intrinsically regulated or determined by the neuronal axons.

Neuronal subtype populations are located in different regions of the CNS and differ in many properties including axon thickness and length. They also have different needs in myelin sheaths, for example in number, length, and thickness. Often larger diameter axons have longer and/or larger diameter myelin sheaths. It has been suggested that oligodendrocytes do not show any preference for axon size upon myelination (Fanarraga *et al.*, 1998). They can myelinate axons of differing sizes at the same time, and they adjust the sheath thickness to the axon diameter (Friedrich and Mugnaini, 1983; Waxman and Sims, 1984). Furthermore, deep layer cortical neurons are less myelinated than neurons in superficial layers, despite the availability of OPCs in the area (Tomassy *et al.*, 2014). Neurons affect myelin through different signals, such as electrical activity, GABA signaling, and synaptic vesicle release (Demerens *et al.*, 1996; Wake *et al.*, 2015; Koudelka *et al.*, 2016; Hamilton *et al.*, 2017). These findings suggest that myelin sheath generation is determined and affected by the axon. However, a group of researchers investigated the intrinsic properties of oligodendrocytes to regulate myelin sheath length, by using microfibers of various diameters to remove any axonal influence. They observed that oligodendrocytes generate longer myelin sheaths on larger diameter microfibers (Bechler *et al.*, 2015). They also noticed that spinal cord and cortical oligodendrocytes myelinate the same number of microfibers, although spinal cord oligodendrocytes generated longer sheaths than cortical oligodendrocytes, indicating that oligodendrocytes in different regions have intrinsic factors contributing to myelin sheath length determination.

Oligodendrocytes are heterogeneous in their morphology and their generation of myelin sheaths. However, axonal input largely contributes to that heterogeneity. To investigate the

cell-intrinsic contribution to this heterogeneity further, single-cell transcriptomics has been used (Marques *et al.*, 2016). Here, twelve distinct populations along the oligodendrocyte lineage were identified, from OPCs to multiple myelin-forming and mature oligodendrocyte subclusters. They found two different myelin-forming oligodendrocyte states (MFOL1-2) and six different mature oligodendrocyte states (MOL1-6). The two myelin-forming states and the first four mature states were enriched in the juvenile CNS at P21, while MOL5 and 6 are enriched in the adult. This suggests that a higher heterogeneity exists during development, probably representing intermediate transitional stages, and that mature oligodendrocytes in the adult are more homogeneous. Nevertheless, different mature oligodendrocyte states locate to specific areas in the spinal cord in the adult (Floriddia *et al.*, 2020). MOL2 is preferentially located in the spinal cord compared to the cortex and corpus callosum. Even in the spinal cord, MOL2 is enriched in white matter, while its occurrence in the gray matter is lower and decreases over time. MOL5 and 6 are located more in the gray matter of the spinal cord and their density increases over time. In the dorsal column, MOL2 has specific localization to the fasciculi gracilis and cuneatus containing the ascending sensory tracts, while MOL5 and 6 localize specifically to the dorsal corticospinal tract in the adult. The different mature populations do not have a preference for dorsal or ventral areas and OPCs from different waves contribute to different mature states (Floriddia *et al.*, 2020). Overall, the transcriptome of OPCs converges during development, whereafter they go through a single differentiation pathway and diverge when they mature. This diverging most likely is linked to where they are located and which axonal tracts they are myelinating. While MOL5 and 6 are the main mature oligodendrocytes in the adult, other smaller populations might contribute to specific areas and axonal tracts. The observed heterogeneity is probably for the most part regulated by axonal and environmental input, rather than cell-intrinsic factors.

1.2 Disease

One of the major diseases affecting oligodendrocytes is multiple sclerosis (MS). MS is characterized by inflammatory demyelinating lesions, leading to a variety of symptoms in the patients depending on the site of inflammation. In most cases, remyelination occurs early in the course of the disease and patients recover. However, in more severe cases, a chronic demyelination of axons can lead to axonal degeneration and long-term loss of function (Franklin, 2017).

Two different hypotheses that try to explain the cause of MS have been around in the field (Trapp and Nave, 2008; Stys *et al.*, 2012; Stys, 2013). The first and most accepted hypothesis is the ‘outside-in’ hypothesis, which proposes dysregulation of the immune system as a primary event leading to an autoimmune response against myelin peptides. The immune cells infiltrate the CNS, possibly because of a leaky blood-brain barrier, and subsequently attack myelin. This would then result in the demyelination observed in MS. The second hypothesis is the ‘inside-out’ hypothesis, which proposes that myelin degeneration or injury is the primary event, which triggers an autoimmune reaction.

1.2.1 Demyelination and remyelination

Demyelination has severe consequences for the transmission of electrical signals in neurons and can lead to a cascade of detrimental events. Neurons can partially compensate for the loss of saltatory conduction by the redistribution and upregulation of ion channels along its axon where demyelination has occured (Smith *et al.*, 1982; England *et al.*, 1990; Craner *et al.*, 2004). However, this leads to a local increase in sodium signaling along the demyelinated area which increases energy demand. Persistent sodium currents and an increase in sodium/calcium exchangers lead to rising levels of sodium and calcium within the axon (Kapoor *et al.*, 2003; Craner *et al.*, 2004) and in turn, increased calcium levels can be toxic as it activates calcium-dependent enzymes and degradation pathways (Lehning *et al.*, 1996; Bechtold and Smith, 2005). Furthermore, the exposed axons are subject to reactive oxygen species from ongoing inflammation. Nitric oxide released by microglia diffuses into demyelinated axons and disrupts mitochondrial ATP generation (Su *et al.*, 2009; Mancini *et al.*, 2018). Reduced levels of ATP lead to the failure of ATP-mediated sodium export that is required for uninterrupted neuronal signaling. Demyelination also results in loss of trophic and metabolic support from the oligodendrocyte, which leads to a further reduction of ATP generation. Overall, demyelination leads to an increase in energy demand of neurons that are energy depleted, have an ion imbalance, and are exposed to a toxic environment, all cumulating in axonal degeneration.

To prevent axonal degeneration and loss of long-term function, remyelination needs to occur. Remyelination often results in the recovery from symptoms in MS, which is why it is important to understand what leads to successful remyelination (Bodini *et al.*, 2016). In rodents, adult OPCs respond to demyelination by migrating to the lesion site where they proliferate and differentiate (Figure 2; Gensert and Goldman, 1997; Levine and Reynolds, 1999; Watanabe *et al.*, 2002; Kang *et al.*, 2010; Moyon *et al.*, 2015). The ability to migrate and differentiate, however, also declines with age (Sim *et al.*, 2002; Crawford *et al.*, 2016; Neumann *et al.*, 2019), and the progenitor pool becomes depleted over time (Mason *et al.*, 2004). Differentiation itself is also inhibited by the accumulation of myelin debris at the site of lesions (Robinson and Miller, 1999; Kotter *et al.*, 2006; Plemel *et al.*, 2013). Macrophages can aid in the clearing of myelin debris, especially in newly demyelinated lesions (Brück *et al.*, 1995; Bogie *et al.*, 2011). Macrophages can also promote the recruitment and differentiation of OPCs, independent of myelin debris clearing (Kotter *et al.*, 2005). Astrocytes and microglia within the lesion can promote the recruitment of OPCs through the secretion of chemotactic molecules, such as SEMA3F (Boyd *et al.*, 2013). However, OPC differentiation is inhibited by cytokines secreted by inflammatory cells, such as interferon-gamma (Turnley *et al.*, 1991; Agresti *et al.*, 1996; Chew *et al.*, 2005; Kirby *et al.*, 2019). This illustrates that remyelination in the context of inflammatory disease is very complex and the interplay between oligodendroglia and other cell types within the lesion, such as neurons, astrocytes, microglia, and immune cells all affect the remyelination potential and disease outcome.

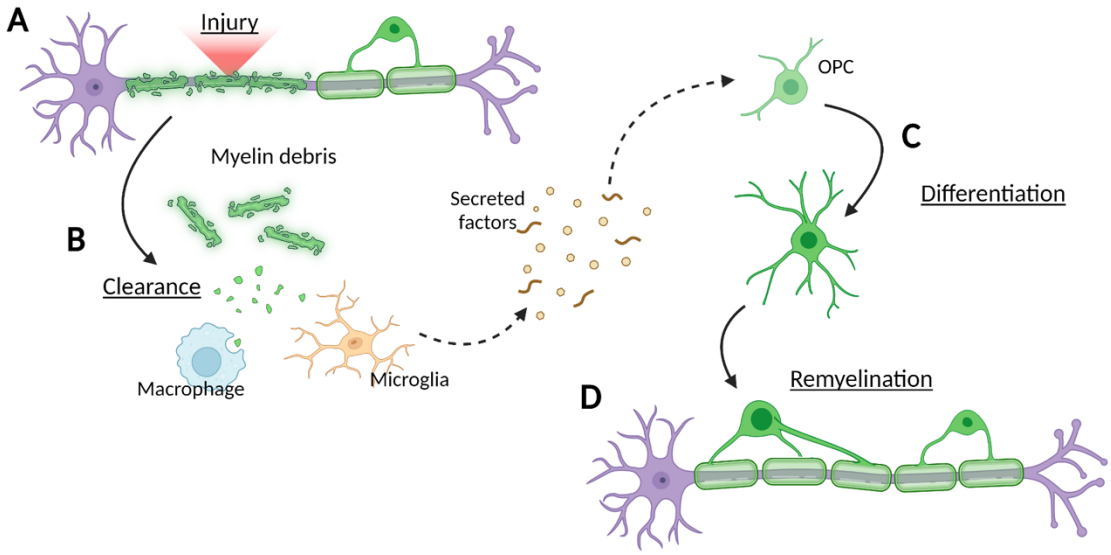

Figure 2. Schematic overview of remyelination after myelin injury. A) Injury can result in myelin degeneration. B) Myelin debris clearance by macrophages/microglia. C) Secreted factors from cell types at the injury site can recruit OPCs. D) Differentiated OPCs than contribute to remyelination.

In MS, different lesion characteristics within the same patient or among the population complicates the situation even more (Lucchinetti *et al.*, 2000; Patrikios *et al.*, 2006). For example, active lesions contain oligodendrocytes and show signs of remyelination, but mixed and inactive lesions show areas of oligodendrocyte loss, especially in the center of the lesions where they can be almost completely depleted (Lucchinetti *et al.*, 1999; Heß *et al.*, 2020). Remyelination might occur in some lesions early in the disease, in the presence of macrophages that clear debris, yet repeated demyelination of the same area could deplete the progenitor pool and affect the remaining oligodendrocytes (Prineas *et al.*, 1993a, 1993b). Furthermore, in the case of chronic lesions, oligodendrocytes fail to remyelinate due to axonal degeneration (Chang *et al.*, 2002). Similar to the mouse models, an increase in the number of proliferating OPCs is found in some MS lesions, but the number of OPCs decreases with age or duration of the disease (Maeda *et al.*, 2001; Wolswijk *et al.*, 2002; Kuhlmann *et al.*, 2008). Also, heterogeneity of chemokines is observed in different lesions (Williams *et al.*, 2007). SEMA3F, which attracts OPCs, has higher expression in active lesions with signs of remyelination, while SEMA3A, which inhibits OPC recruitment, has higher expression in chronic active lesions where less remyelination occurs.

While the focus has been mostly on OPC recruitment and promoting differentiation, some studies suggest that remyelination rather occurs by remaining oligodendrocytes that survive the immune attack and generate new myelin sheaths (Yeung *et al.*, 2019; Jäkel *et al.*, 2019). A technique using carbon dating can assess if cells have undergone cell division or if they have been around for longer, which has been termed ‘old’ (Yeung *et al.*, 2019). A large amount of newly generated oligodendrocytes were only found in patients with very aggressive MS and short disease duration leading to death. In other patients, remyelinated lesions had mainly old oligodendrocytes, suggesting that not proliferating OPCs, but already existing mature oligodendrocytes contribute to remyelination instead (Yeung *et al.*, 2019). Single-nuclei RNA-seq also showed that remyelinated lesions mainly consisted of a specific

stable mature oligodendrocyte state and that OPCs and intermediate oligodendrocytes were depleted in MS, especially in remyelinated lesions (Jäkel *et al.*, 2019). Mature oligodendrocytes in MS had in general upregulated myelin gene expression, suggesting that their myelination program is activated. Moreover, active lesions had enrichment of an actively myelinating oligodendrocyte state, which corresponds to earlier studies where active lesions have ongoing remyelination (Williams *et al.*, 2007; Heß *et al.*, 2020). A possible explanation for the lack of OPCs could be that they have already differentiated or have been depleted because of disease duration (Prineas *et al.*, 1993a, 1993b; Maeda *et al.*, 2001; Wolswijk *et al.*, 2002; Kuhlmann *et al.*, 2008). We can also not exclude that remyelinated lesions could represent demyelinating lesions instead. It is difficult to assess these aspects in the human CNS, as we can only analyze oligodendroglia composition post-mortem. It could be that early in the disease, OPCs contribute to myelination, but these newly formed oligodendrocytes might be more susceptible to subsequent inflammatory attacks. One specific mature oligodendrocyte state was strongly depleted in MS, while others were enriched (Jäkel *et al.*, 2019). This could suggest that some oligodendrocytes are more vulnerable to disease and that others are very stable and remain intact, even after their myelin sheaths have been attacked. Remyelinated lesions might therefore not require OPCs while other more affected areas, such as active lesions, depend on recruited OPCs.

More research has to be done to understand the heterogeneity of oligodendroglia and if certain oligodendrocytes or areas of the CNS are more susceptible to degeneration than others, which could also explain the heterogeneity of lesions. Depletion and enrichment of certain subtypes could also represent transcriptional subtype switching in response to disease, instead of the loss of a certain subtype. Lastly, an oligodendroglia cell state with immune gene expression was also found in MS patients (Jäkel *et al.*, 2019; Kirby *et al.*, 2019), which suggests that some oligodendroglia are captured in an immune state.

These studies indicate that MS is a very complex condition and remyelination depends on many factors. Either OPCs or remaining oligodendrocytes within the lesion could contribute to remyelination if they are not severely affected by the ongoing inflammatory insult. Within the cellular environment, many other cell types such as microglia, astrocytes, neurons, and immune cells affect the migration, proliferation, differentiation, and myelination potential of OPCs and oligodendrocytes.

1.2.2 Animal models

To be able to study such diseases as MS, the use of model organisms is needed due to the difficulty of studying progressive diseases at the cellular level in humans. The most common MS mouse model is experimental autoimmune encephalomyelitis (EAE), a model used to study the immune response targeting oligodendrocytes and supports the ‘outside-in’ sequence of events. EAE is induced in rodents through immunization with a myelin antigen, such as a myelin oligodendrocyte glycoprotein (MOG) peptide, together with an immune booster (CFA, see below). Additional boosting of the immune system occurs with the addition of pertussis toxin, which opens the blood-brain barrier to facilitate the infiltration of T cells into

the CNS. The pathology in EAE is mostly observed in the spinal cord. The model can also be induced with the adoptive transfer of myelin antigen-specific T cells instead of the immunization with myelin peptides (Paterson, 1960; Pettinelli and McFarlin, 1981).

The development of the model is based on an observation during the development of the rabies vaccine in 1888 (Baxter, 2007). Sporadic cases of paralysis were reported when people were injected with spinal cord tissue from rabbits. Other researchers inoculated animals with CNS tissue, which resulted in similar paralysis. Later, the observed paralysis was associated with demyelination in the CNS (Rivers *et al.*, 1933; Rivers and Schwentker, 1935). As the paralysis specifically occurred when CNS-specific antibodies were used, they reasoned that the demyelination must be caused by an immune response. Around the same time, Freund developed complete Freund's adjuvant (CFA), which is a strategy to boost an immune response against an antigen by injecting an emulsion of that antigen with killed *Mycobacterium tuberculosis* (Freund and McDermott, 1942). Using this adjuvant in combination with CNS tissue resulted in a strong immune reaction against myelin, which was the beginning of the EAE model. The first induction of EAE in mice occurred in 1949 (Olitsky and Yager, 1949). After that, the immunization was improved with the use of CNS white matter, then with only myelin, and lastly, with myelin peptides.

EAE can be used to study the interactions of immune cells with oligodendroglia and other cell types of the CNS. A disadvantage of the EAE model is the difficulty to study demyelination and remyelination events. Therefore, toxin-induced demyelination models have been developed (Blakemore and Franklin, 2008). Cuprizone and lysolecithin are the most commonly used toxins, they induce widespread, or focal demyelination, respectively. To study both aspects of the disease, a combination of the EAE and cuprizone models has been developed (Baxi *et al.*, 2015). The model works by first inducing cuprizone-mediated demyelination and then transferring myelin-reactive CD4 positive T cells. This allows for the possibility to study the effect of inflammation on remyelination.

1.2.3 Immune response in oligodendroglia

Oligodendrocytes are exposed to cytokines secreted by T lymphocytes in MS (Link, 1998). One of the main cytokines secreted is interferon-gamma, which can be harmful to oligodendroglia and can even lead to apoptosis *in vitro* (Vartanian *et al.*, 1995). However, some studies show that susceptibility to apoptosis is dependent on its developmental stage. For example, OPCs appear to be more sensitive to interferon-gamma-induced apoptosis than mature oligodendrocytes (Baerwald and Popko, 1998) and a possible explanation could be that interferon-gamma affects the cell cycle, which would leave OPCs more vulnerable (Chew et al., 2005; Horiuchi *et al.*, 2006). Furthermore, OPCs are more sensitive to endoplasmic reticulum (ER) stress than mature oligodendrocytes and interferon-gamma can induce ER stress because of overloading with major histocompatibility complex (MHC) class I proteins (Baerwald *et al.*, 2000; Lin *et al.*, 2005). Newly formed myelinating oligodendrocytes could also be more vulnerable to ER stress as they process an enormous amount of myelin proteins to generate myelin sheaths, which reduces the ER capacity to

process additional proteins (Lin *et al.*, 2005). Accordingly, interferon-gamma overexpression during development is associated with ER stress and leads to loss of oligodendrocytes, hypomyelination, infiltration of lymphocytes, and the upregulation of MHC molecules (Corbin *et al.*, 1996; Baerwald *et al.*, 2000; Lin *et al.*, 2005). On the other hand, overexpression of interferon-gamma after development leads to similar responses, including demyelination (Horwitz *et al.*, 1997). Another way by which interferon-gamma could be detrimental in oligodendroglia is through the upregulation of chemokines. For example, the chemokine CXCL10 is induced in oligodendroglia by interferon-gamma, and through the locally produced concentration gradient, it chemoattracts immune cells (Balabanov *et al.*, 2007). Additionally, it has been shown that inhibition of CXCL10 activity reduces inflammation in EAE (Liu *et al.*, 2001).

Nevertheless, the negative effect of interferon-gamma on oligodendroglia is controversial and interferon-gamma can even be beneficial (Billiau *et al.*, 1988; Lublin *et al.*, 1993; Ferber *et al.*, 1996; Krakowski *et al.*, 1996; Willenborg *et al.*, 1996). Some studies show that interferon-gamma promotes signaling pathways that protect oligodendroglia from apoptosis. For example, the expression of interferon-gamma in the pre-symptomatic phase of EAE activates the integrated stress response in oligodendrocytes which is protective and prevents demyelination, axonal damage, and oligodendrocyte loss (Lin *et al.*, 2007). Interferon-gamma also induces protective mechanisms against oxidative stress in EAE (Espejo *et al.*, 2002), possibly through the induction of genes with antioxidant function and the promotion of proteasome activity (Balabanov *et al.*, 2007). The proteasome is involved in the removal of oxidized proteins that accumulate, which may otherwise lead to cell death (Poppek and Grune, 2006). Microglia release nitric oxide in response to interferon-gamma, which is toxic to oligodendrocytes (Merrill *et al.*, 1993). Inflammation in general releases reactive oxygen species, which oligodendrocytes are more sensitive to than other cell types (Juurlink *et al.*, 1998; Goldbaum *et al.*, 2006; Lassmann and van Horssen, 2016). Inhibiting interferon-gamma signaling in oligodendrocytes leaves them susceptible to early apoptosis and increased severity of EAE (Balabanov *et al.*, 2007). A possible explanation could be that oligodendrocytes undergo early apoptosis because of oxidative stress, which promotes a stronger inflammatory response resulting in a worse outcome of disease symptoms (Hisahara *et al.*, 2000; Balabanov *et al.*, 2007; Traka *et al.*, 2016). While interferon-gamma signaling seems mainly protective in EAE, it is the timing of the signaling that is important for this protective mechanism to occur. Increased interferon-gamma signaling during the recovery phase of EAE delayed and impaired recovery of symptoms and reduced remyelination (Lin *et al.*, 2006).

From the previous studies, we can conclude that probably the timing and the concentration of interferon-gamma are the most critical factors. Low doses before the onset of symptoms could protect oligodendroglia from inflammation, while prolonged higher doses could lead to ER stress, apoptosis, and inflammation. In the *in vitro* studies, the purity of the culture is important, as contaminating microglia could release nitric oxide in response to interferon-gamma, resulting in apoptosis of oligodendroglia. Other studies have shown that a lower dose

of interferon-gamma in culture does not induce apoptosis, but instead affects the potential of OPCs to differentiate into mature oligodendrocytes (Turnley *et al.*, 1991; Agresti *et al.*, 1996; Chew *et al.*, 2005; Kirby *et al.*, 2019). This could be problematic in MS when OPCs are recruited to the site of injury to differentiate into myelinating oligodendrocytes. Instead, they are exposed to interferon-gamma from T lymphocytes, which induces the expression of MHC-I in OPCs (Suzumura *et al.*, 1986; Turnley *et al.*, 1991; Kirby *et al.*, 2019). Induction of MHC-II expression in OPCs and in some cases in oligodendrocytes has also been observed, but to a much lesser extent (Bergsteindottir *et al.*, 1992; Itoh *et al.*, 2009). OPCs expressing MHC-I are vulnerable to T cell-mediated cytotoxicity. They can present exogenous proteins, such as myelin peptides, and activate CD8 positive T cells, which can lead to OPC death (Kirby *et al.*, 2019). The presentation of antigens by OPCs requires LRP1 (Low-density lipo-protein receptor-related protein 1; Fernández-Castañeda *et al.*, 2020). LRP1 is a phagocytic receptor involved in the clearing of myelin debris (Gaultier *et al.*, 2009), and is highly expressed in OPCs (Fernández-Castañeda *et al.*, 2020). LRP1 prevents OPC differentiation after cuprizone-mediated demyelination and loss of LRP1 reduces EAE symptoms and inflammation. These studies suggest that OPCs mediate inflammation in the CNS through LRP1 and antigen presentation. To conclude, OPCs can contribute to the spreading of the immune response by presenting myelin peptides to T lymphocytes, thereby stalling their own differentiation, limiting their contribution to remyelination, and even inducing their own cell death.

1.3 Peptidylarginine deiminases

Peptidylarginine deiminases (PADs) are enzymes that convert the positively charged peptidylarginine into a neutral peptidylcitrulline, a reaction that is calcium dependent (Figure 3). This conversion is called citrullination or deimination and affects the structure and function of the targeted protein by reducing its positive charge. Five family members (PAD1-4 and PAD6) exist in mammals, which are highly conserved and show many similarities at the mRNA and protein level (Zhang *et al.*, 2004; Chavanas *et al.*, 2004). Nevertheless, PADs show restricted expression patterns to different tissues and show high specificity in their citrullination targets (Darrah *et al.*, 2012).

1.3.1 Citrullination in oligodendroglia and multiple sclerosis

Hypercitrullination of MBP (myelin basic protein) has been implicated in MS as it could affect myelin stability. MBP has an abnormally high positive charge and is by itself extremely disordered. MBP becomes neutral and forms a stabilized highly compacted network through binding with negatively charged myelin membranes (Aggarwal *et al.*, 2013). This results in the stacking of myelin layers ensuring a tightly compacted myelin sheath that covers the neuronal axon. However, regions of decompacted or loose compacted myelin are necessary to form cytoplasmic channels connecting the oligodendrocyte with the underlying axon. These channels are important for metabolic support and ion homeostasis. CNP, another

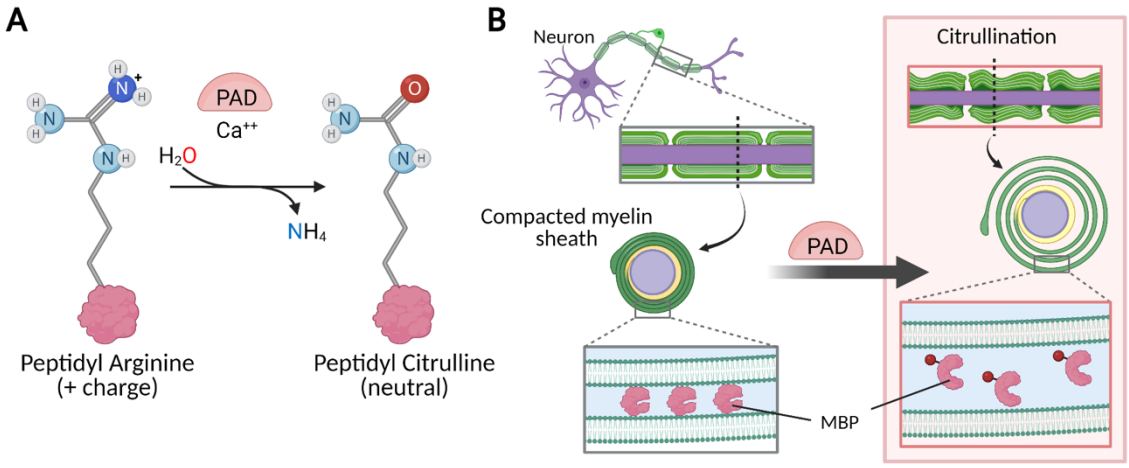

Figure 3. Schematic overview of PAD2 mediated citrullination of MBP. A) PADs convert peptidylarginine into peptidylcitrulline. B) Binding of MBP to the myelin membrane leads to the formation of compacted sheaths. Citrullination of MBP results in a change in the conformation of the protein and the affinity to bind to the membrane, which results in decompacted myelin sheaths.

myelin component, contributes to the formation of these channels, as it antagonizes the function of MBP through association with the actin cytoskeleton to keep regions decompacted (Snaidero *et al.*, 2017).

Citrullination of MBP results in the reduction of the positive charge of the protein and therefore also reduced myelin compaction (Beniac *et al.*, 2000). MBP contains 19 arginine residues of which 6 are citrullinated in its citrullinated charge isomer (Brady *et al.*, 1985; Wood and Moscarello, 1989). In the healthy adult brain, 20% of total MBP is found in its citrullinated form compared to 45% in chronic MS (Moscarello *et al.*, 1994). The increase of citrullinated MBP in MS could affect the myelin compaction and stability. In comparison, the majority of MBP is citrullinated in human infants up to 2 years of age. This amount of citrullinated MBP decreases as the CNS matures. The percentage of citrullinated MBP found in MS is comparable with infants of 3-4 years old, which suggests that the myelin found in MS is developmentally immature. While during development high levels of citrullinated MBP are functional, in MS it could have severe consequences. The amount of arginine residues citrullinated further affects myelin compaction. In an acute, fulminating type of MS, 90% of MBP is found citrullinated and the amount of arginine residues citrullinated goes up to 18 (Wood *et al.*, 1996). This increase in the number of arginines found citrullinated on MBP correlates with the severity of the disease.

In the mouse CNS, PAD enzyme and activity are mainly found in myelin (Pritzker *et al.*, 1999). The mRNA and protein were detected at P5 in the mouse brain with peak expression levels at 1 month and peak protein level and enzyme activity at 2 months after birth. In compact myelin, PAD activity reached its peak at P15 and reduced significantly at 1 and 2 months of age, while in loose myelin, PAD activity remained at high levels up to 8 months of age. Thus, PAD activity in compact myelin correlates highly with the timing of myelination and decreases when the myelin matures. The family member responsible for MBP citrullination is PAD2, which is highly expressed in oligodendrocytes and in myelin

(Akiyama *et al.*, 1999; Gould *et al.*, 2000). Also, the promoter of *Padi2* is hypomethylated and the PAD2 protein levels are upregulated in the CNS of MS patients (Mastronardi *et al.*, 2007). While it has been suggested that PAD4 is also localized in myelin, more evidence points out to the role of PAD2 (Wood *et al.*, 2008). In a transgenic demyelination animal model, where demyelination occurs around 3 months of age, increased levels of PAD2 protein are observed at 1 month of age. This increase was followed by MBP citrullination and preceded the spontaneous demyelination, which suggests that citrullinated MBP is an early change in demyelination (Moscarello *et al.*, 2002). Accordingly, overexpression of *Padi2* in the CNS of transgenic mice leads to demyelination and thinner myelin sheaths (Musse *et al.*, 2008). In EAE, an inflammatory mouse model mimicking some aspects of MS, an increase in citrullinated proteins is observed within inflammatory lesions in the spinal cord (Nicholas *et al.*, 2005; Raijmakers *et al.*, 2005). Inhibiting PAD activity in different models of demyelination and/or inflammation, resulted in decreased PAD activity, a reduction of disease symptoms and an increase in remyelination (Moscarello *et al.*, 2013; Caprariello *et al.*, 2018). Also, a decrease in CD3 positive T cells and interferon-gamma was observed. Nevertheless, induction of EAE in *Padi2* knockout mice did not show any difference in disease onset or development despite the decrease of citrullinated proteins in the CNS (Raijmakers *et al.*, 2006), possibly because of compensatory mechanisms by other PADs. To conclude, increased levels of citrullinated proteins can lead to myelin defects, which could evoke an immune response.

Other implications for citrullinated MBP are in degradation and stability of the protein itself, which could cause immune reactivity. As citrullination alters the structure and conformation of the protein, it can become more susceptible for degradation by cleavage enzymes. Citrullination of MBP results in a more neutral and open conformation, which allows for digestion by both cathepsin D and stromelysin-1, releasing an immunodominant epitope of MBP (Pritzker *et al*., 2000; D'Souza & Moscarello, 2006). The citrullinated form of MBP additionally enhances the response of T cells isolated from MS patients compared to controls (Tranquill *et al.*, 2000). However, one study shows that citrullination of MBP prevents degradation by the proteasome (Kuzina *et al.*, 2016). The high positive charge of MBP results in a structure susceptible for degradation. Citrullination leads to reducing this charge, which prevents it from presentation as antigenic peptide and recognition by T cells. Therefore, if hypercitrullination of MBP evokes an immune response resulting in MS or if it protects MBP from being recognized by T cells is still to be investigated.

Overall, these studies imply that elevated levels of PAD2 and subsequent MBP hypercitrullination can lead to demyelination and MBP degradation, thereby possibly inducing an immune response. However, if hypercitrullination by PAD2 is the cause or mediator of disease is currently unknown. Immune infiltration and demyelination could possibly progress without MBP hypercitrullination in different types of MS.

1.3.2 Histone citrullination as an epigenetic modifier

Besides MBP citrullination, increased histone H3 citrullination is observed in MS and in demyelinating animal models (Mastronardi *et al.*, 2006). First it was thought that only PAD4 could citrullinate histones as it is the only PAD with a nuclear localization signal (Arita *et al.*, 2004). However, other PADs have been characterized to be localized in the nucleus as well, including PAD2 (Jang *et al.*, 2011). Since then, numerous of studies have found histones as possible substrates of the different PADs. Especially histones H3, H4 and the linker histone H1 have been described to be citrullinated in various systems (Wang *et al.*, 2004; Zhang *et al.*, 2012; Cherrington *et al.*, 2012).

Like MBP, histones also contain a highly disordered protein structure. Their intrinsic disordered properties are important for their heterodimerization and formation of octamers, crucial for the nucleosome complex (Peng *et al.*, 2012). These properties are also important for the interactions with DNA and other proteins. Intrinsically disordered proteins are highly dynamic and extremely sensitive to changes in the environment, and posttranslational modifications can alter their function and stability (Darling and Uversky, 2018). Posttranslational modifications on histone tails, therefore, regulate critical aspects of their function. Citrullination on histone tails usually results in reduced binding to the DNA and thus chromatin decompaction, as the positive charge of the arginine is reduced. Chromatin decompaction is associated with gene activation as the DNA would be more accessible. Histone citrullination can also serve as a histone arginine demethylase, by replacing the arginine containing methyl groups with citrulline (Wang *et al.*, 2004; Cuthbert *et al.*, 2004). Histone arginine methylation is associated with gene activation and by demethylation, citrullination could act as a repressor. These contradicting outcomes show that citrullination of histones has variable effects on the underlying DNA depending on the context.

Histone citrullination has most extensively been studied as a defense mechanism by neutrophils. Neutrophils are a type of immune cell that can release DNA to bind and kill extracellular pathogens. The release of DNA by neutrophils is called neutrophil extracellular trap formation. This process occurs through PAD4-mediated citrullination of linker histone H1, which results in decondensation of the chromatin and release of DNA in the extracellular space (Li *et al.*, 2010; Dwivedi *et al.*, 2014). PAD4 citrullinates linker histone H1 also in embryonic stem cells, which likewise leads to decondensation of the chromatin (Christophorou *et al.*, 2014). Histone H1 citrullination serves as a mechanism involved in pluripotency through which PAD4 activates genes.

In bone marrow progenitor cells, PAD4 binds to the promoter region of the gene *c-myc*, where citrullinated histone H3 is also found (Nakashima *et al.*, 2013). In cells depleted of PAD4, increased histone H3 arginine methylation at the *c-myc* promoter region was found accompanied by increased histone H3 acetylation levels and the upregulation of *c-myc*. These findings suggest that within bone marrow progenitor cells, histone H3 citrullination prevents histone H3 arginine methylation and acetylation and therefore activation of *c-myc*. Citrullination of histone H3 arginine 8 can also lead to exclusion of HP1a from the chromatin

(Sharma *et al.*, 2012). HP1a normally binds to methylated lysine 9 on histone H3, which is associated with gene repression and the formation of heterochromatin. The release of HP1a from the chromatin because of citrullination of the adjacent residue can lead to gene activation. These are some of the mechanisms by which histone citrullination could affect gene transcription. The role of histone citrullination depends on the cellular context and may vary in different cell types and systems. How histone H3 citrullination affects gene transcription within MS is still to be investigated.

1.4 Gene regulatory mechanisms

Oligodendroglia experience many different changes throughout their life, from cell fate specification to differentiation into myelinating oligodendrocytes. They are influenced by numerous signals from their environment, but they also have cell-intrinsic mechanisms to regulate how they respond to those signals. When oligodendroglia transition to different states, they undergo significant changes at the transcriptional level. Gene regulatory mechanisms mediate those transitions, and they also ensure the maintenance of transcriptional programs in homeostasis. Among those mechanisms are regulatory elements, transcription factors, chromatin-modifying enzymes, posttranslational modifications of histones, chromatin remodelers, chromatin architecture proteins, and genome interactions.

1.4.1 Transcription factors

Transcription factors are important regulators of gene transcription. They can be repressors or activators of transcription. They bind to regulatory elements on the DNA, which are often located in promoters or enhancers. The binding of the transcription factor to the DNA is sequence-specific and depends on the DNA-binding domain. One of the most important transcription factors in OPCs is the basic-helix-loop-helix (bHLH) transcription factor OLIG2 (Lu *et al.*, 2000; Zhou *et al.*, 2000; Takebayashi *et al.*, 2002). OLIG2 is important for the specification of motor neurons and subsequent specification of OPCs, but its expression is downregulated in motor neurons and maintained in the oligodendrocyte lineage. OLIG2 is important for the maintenance of OPC fate in development, as the loss of OLIG2 results in the conversion to astrocytic fate (Zhu *et al.*, 2012; Zuo *et al.*, 2018). OLIG2 is also important for the differentiation of OPCs into oligodendrocytes (Mei *et al.*, 2013). Another bHLH transcription factor important in the oligodendrocyte lineage is OLIG1 (Lu *et al.*, 2000). OLIG1 and OLIG2 functions are partly redundant, however, OLIG1 is not involved in the specification of motor neurons or OPCs, but it is important in differentiation, myelin gene expression, and myelin formation, especially in the brain (Lu *et al.*, 2002; Xin *et al.*, 2005). The high-mobility-group transcription factor SOX9 is important for the specification of astrocytes and OPCs and mediates the switch from neurogenesis to gliogenesis (Stolt *et al.*, 2003). SOX9 is downregulated upon differentiation of OPCs but remains high in astrocytes. After specification, OLIG2 induces the expression of SOX10 by binding to its enhancer (Zhou *et al.*, 2000; Küspert *et al.*, 2011). SOX10 is one of the major regulators of

oligodendroglia-specific gene transcription and is required for terminal differentiation and myelination (Inoue *et al.*, 1999; Stolt *et al.*, 2002). SOX9 and SOX10 are co-expressed in OPCs and regulate the expression of *Pdgfra* (Finzsch *et al.*, 2008). PDGFRA regulates the expansion, survival, and migration of OPCs through PDGF signaling and is often used as an OPC marker (Calver *et al.*, 1998). Another marker of OPCs is NG2, encoded by *Cspg4*. PDGFRA and NG2 are both downregulated upon differentiation and in quiescent OPC stages in the adult CNS (Moyon *et al.*, 2015). *Cspg4* expression is regulated by an enhancer, which contains binding sites for both SoxE and bHLH transcription factors in close proximity (Gotoh *et al.*, 2018). SOX10 and OLIG2 bind to these binding sites to induce *Cspg4* expression.

The transcription factor NKX2.2 is transiently expressed before differentiation and downregulated immediately after differentiation. NKX2.2 promotes OPC differentiation through repression of *Pdgfra* by directly binding to its promoter (Qi *et al.*, 2001; Fu *et al.*, 2002; Zhu *et al.*, 2014). NKX2.2 is also transiently upregulated in adult OPCs in response to demyelination (Fancy *et al.*, 2004; Watanabe *et al.*, 2004). Upon differentiation, SOX10 is responsible for the induction of *Myrf* by binding to its enhancer (Hornig *et al.*, 2013). MYRF is a transcription factor important for myelination as it cooperates with SOX10 to induce myelin gene expression (Emery *et al.*, 2009; Hornig *et al.*, 2013). MYRF is also important for the maintenance of mature oligodendrocyte identity and myelin in the adult CNS (Koenning *et al.*, 2012). Interestingly, MYRF is anchored to the membrane of the ER with its C-terminal region (Bujalka *et al.,* 2013). The N-terminal of MYRF, when cleaved, travels into the nucleus where it binds to and activates the regulatory regions of myelin genes (Bujalka *et al.,* 2013). Several transcription factors have been identified as negative regulators of OPC differentiation, such as HES5, ID2, and ID4, and their expression decreases upon maturation (Kondo & Raff, 2000a, b; Wang *et al.*, 2001). HES5 inhibits *Ascl1* (*Mash1*) expression, which is a transcription factor involved in early OPC specification and differentiation (Sugimori *et al.*, 2008). HES5 also inhibits myelin gene expression through promoter binding (Liu *et al.*, 2006). Increased levels of SOX10 during differentiation inhibit HES5 through sequestration and myelin gene promoter displacement. Another transcription factor, TCF7L2, is temporarily upregulated upon differentiation and involved in remyelination (Fancy *et al.*, 2009). It cooperates with KAISO to antagonize WNT signaling at the onset of differentiation and cooperates with SOX10 to induce myelin gene expression and genes important for cholesterol biosynthesis (Zhao *et al.*, 2016). These are some of the transcription factors that regulate different processes in the oligodendrocyte lineage. Transcription factors often cooperate with other transcription factors to regulate varying sets of genes, thereby being able to have distinct functions dependent on cell type and stage. They often also work together with chromatin-modifying enzymes.

1.4.2 Chromatin-modifying enzymes

Chromatin-modifying enzymes regulate the epigenomic landscape by altering the tails of histones through deposition of modifications, such as methylation and acetylation among

others. These histone modifications can affect the compaction of the chromatin and the recruitment of non-histone proteins, which in turn regulate gene silencing or activation (Kouzarides, 2007). Histone deacetylases (HDACs) remove acetylation marks from histones, while histone acetyltransferases are the enzymes that add acetylation. Acetylation at lysines, for example at lysine 27 of histone H3 (H3K27), results in the loss of a positive charge and a more open chromatin conformation. H3K27ac is an active mark often associated with active enhancers and promoters (Roth *et al.*, 2001). Methylation of lysines is often associated with repression, but at some sites also with activation. For instance, H3K4me3 is associated with active promoters, while H3K27me3 and H3K9me3 are associated with repression. The association can also be dependent on the number of methyl groups added to the lysine, for example, H3K4me1 is associated with active and primed enhancers (Rada-Iglesias, 2018). Methylation is deposited by histone methyltransferases and removed by histone demethylases. Multiple histone modifications often occur in the same loci, even active and repressive marks. When H3K27me3 and H3K4me3 occur in the same locus it is called bivalency. Loci that have bivalent marks are called poised or primed, which suggests that the gene can be activated by removal of the repressive mark or further repressed by removal of the active mark (Voigt *et al.*, 2013). Bivalent marks often resolve into either an active or repressed state when the cell differentiates.

Chromatin-modifying enzymes play a big role in lineage commitment and the maintenance of phenotypes. During development, ventral forebrain OPCs arise from the ganglionic eminences, in which first interneurons are generated. When OPCs commit to oligodendrocyte fate they lose the ability to generate interneurons. However, OPCs have fewer repressive or bivalent marks and more active marks at interneuron genes than other cell types (Boshans *et al.*, 2019). This suggests that these OPCs have an epigenetic memory or potential to express interneuron genes, which they might lose upon differentiation or maturation. Another example is the regulation of oligodendrocyte lineage genes versus astrocytic genes by HDAC3. Loss of HDAC3 in OPCs results in the conversion to astrocytic fate, mediated directly through OLIG2 regulation and is identical to the *Olig2* knock-out phenotype (Zhu *et al.*, 2012; Zhang *et al.*, 2016). HDAC3 cooperates with the histone acetyltransferase p300 and OLIG2 to maintain oligodendrocyte lineage fate and repress astrocytic fate. H3K27me3 is also often associated with the repression of alternative lineages (Sher *et al.*, 2012; Bartosovic *et al.*, 2021). EZH2, the enzyme responsible for H3K27me3, is highly expressed in neural stem cells, reduces expression upon specification into neurons and astrocytes, but remains high upon specification into oligodendrocyte lineage fate (Sher *et al.*, 2008). In premyelinating oligodendrocytes, EZH2 suppresses genes associated with astrocytic and neuronal fate while promoting oligodendrocyte fate (Sher *et al.*, 2012). H3K27me3 is also important in the differentiation into myelinating oligodendrocytes as it promotes the expression of transcription factors important for differentiation, such as MYRF, SOX10, NKX2.2, TCF7L2, through the suppression of NOTCH, WNT and BMP signaling (Wang *et al.*, 2020a, b). The NOTCH pathway promotes astrocytic fate and is suppressed in the oligodendrocyte lineage by H3K27me3 (Wang *et al.*, 2020a). While H3K27me3 is not

necessary for myelin maintenance, it is important in remyelination (Wang *et al.*, 2020b). Another repressive mark is H3K9me3, which suppresses genes associated with the neuronal lineage and promotes OPC differentiation (Liu *et al.*, 2015). Single-cell CUT&Tag profiling showed that astrocyte genes had higher levels of H3K4me3 in OPCs compared to oligodendrocytes but had H3K27me3 in both cell types (Bartosovic *et al.*, 2021). Furthermore, H3K27me3 was observed at OPC genes in astrocytes but not in mature oligodendrocytes. This suggests that astrocyte genes are still poised in OPCs and become more repressed upon maturation. OPC genes are not repressed through H3K27me3 in oligodendrocytes, but other genes from other cell fates, such as astrocytic fate, are.

HDACs are also important during the specification and differentiation of the oligodendrocyte lineage. HDAC2 is a negative regulator of oligodendrocyte specification as it represses *Sox10* and *Sox8* expression (Castelo-Branco *et al.*, 2014). However, HDAC inhibition leads to impaired differentiation into mature oligodendrocytes (Marin-Husstege *et al.*, 2002; Shen *et al.*, 2005). HDACs are required during remyelination in the adult CNS as they directly suppress genes that encode proteins that inhibit the differentiation process, such as *Hes5* and *Sox2* (Shen *et al.*, 2008). These studies indicate that HDACs are important for differentiation and myelin gene expression, but specific HDACs, such as HDAC2 could have a negative effect dependent on context and timing. OLIG2 is involved in the recruitment of the histone methyltransferase SETDB1, which is responsible for H3K9me3, to the *Sox11* locus resulting in reduced expression (Zhang *et al.*, 2022). SOX11 is an inhibitor of differentiation and SETDB1 promotes differentiation and myelination through its repression. OLIG2 also recruits SMARCA4 (BRG1), a chromatin remodeler that acts as a transcriptional activator, to induce myelin gene expression and promote differentiation (Yu *et al.*, 2013). Other important chromatin remodelers are CHD7 and CHD8 as they promote the survival and differentiation of OPCs (He *et al.*, 2016; Marie *et al.*, 2018; Zhao *et al.*, 2018). They simultaneously bind to genes important for these processes, often overlapping with SOX10 and OLIG2 binding. They induce the expression of important regulators in OPC differentiation, such as *Sox10* and *Nkx2.2*. Thus, important transcription factors regulate oligodendrocyte gene expression through the recruitment and interactions with chromatin-modifying enzymes and chromatin remodelers. Chromatin-modifying enzymes can regulate oligodendrocyte fate by repressing other cell fates or activating oligodendrocyte-specific gene programs.

1.4.3 Assay for Transposase-Accessible Chromatin using sequencing

Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) is a method to identify functional regulatory elements to understand gene regulatory mechanisms (Buenrostro *et al.*, 2013). The method uses a hyperactive Tn5 transposase to introduce adapters into open regions on the genome, which is called tagmentation (Figure 4). Tagmentation is followed by amplification of the accessible chromatin regions with sample-specific primers that bind to the inserted adapters. After amplification, the libraries are ready for sequencing, unlike other techniques that require fragmentation and subsequent ligation of

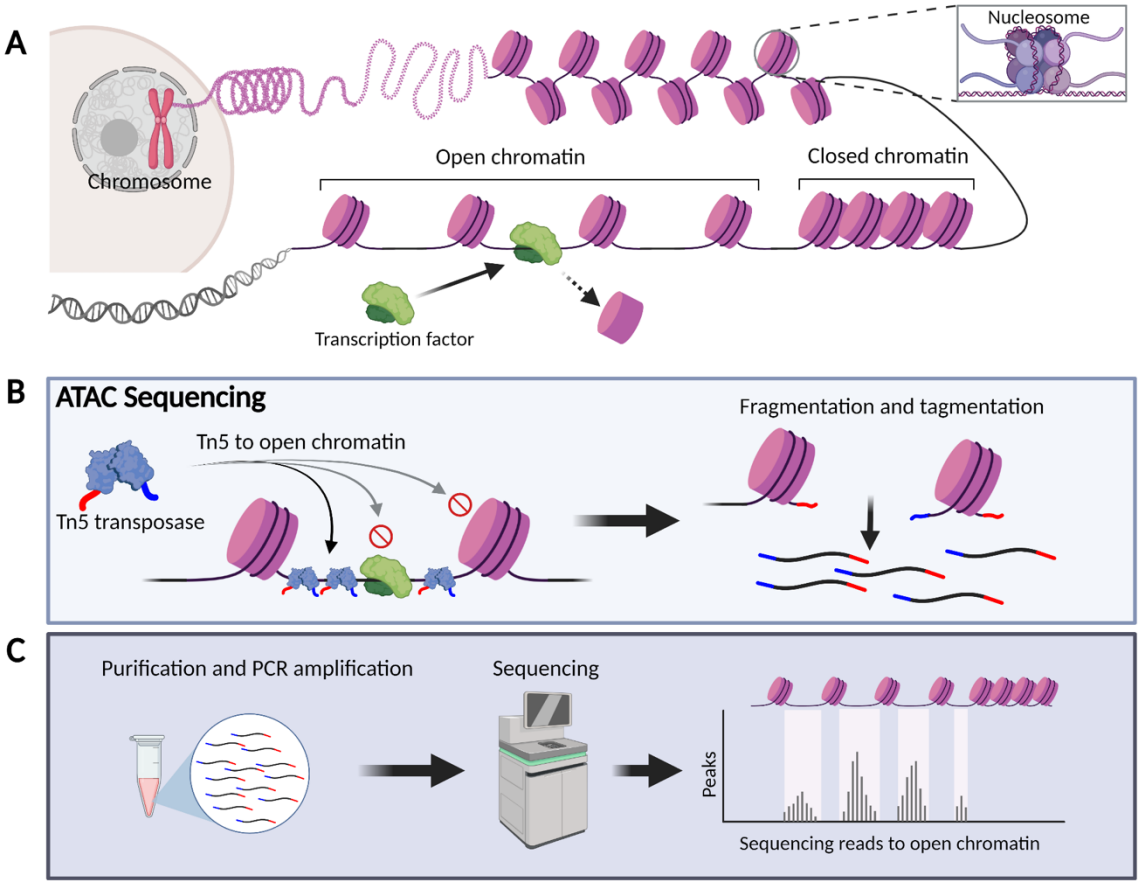

Figure 4. Schematic overview of tagmentation in open chromatin. A) The displacement of nucleosomes leaves chromatin in an open conformation; transcription factors can bind to these open regions. B) The Tn5 transposase tagments open chromatin regions with sample specific adapters. C) The fragments are purified, amplified, sequenced, and analyzed.

the fragments with sequencing adapters. Another advantage of the technique is that it requires only a small number of cells (50.000 cells), which makes it possible to analyze the chromatin of rare cell types.

ATAC-seq data gives similar data as earlier developed chromatin accessibility techniques, such as DNase-seq (Song & Crawford, 2010), FAIRE-seq (Formaldehyde Assisted Isolation of Regulatory Elements; Giresi *et al.*, 2007), and MNase-seq (Schones *et al.*, 2008). Both DNase-seq and MNase-seq rely on the digestion of open chromatin by nucleases, while FAIRE-seq uses sonication to shear the open chromatin. Using nucleases, all accessible chromatin is digested except for chromatin occupied by nucleosomes or other DNA binding proteins, such as transcription factors and chromatin-modifying enzymes. With FAIRE-seq the open chromatin fractions are not digested, but isolated and sequenced, which results in direct identification of all open chromatin. The use of nuclease digestion leads to the identification of nucleosome positions and DNA binding motifs. The identification of the latter is called footprinting (Boyle *et al.*, 2011; Hesselberth *et al.*, 2009) and when combined with protein-DNA binding methods such as ChIP-seq, Cut&Run, or CUT&Tag, specific transcription factor motifs can be characterized. This is not possible with FAIRE-seq as the resolution of the shearing is not high enough. ATAC-seq allows for direct identification of all open chromatin, nucleosome positioning, and transcription factor footprinting at the same time, because of the high resolution and the capture of both short fragments that are

nucleosome-free and 200-600 bp fragments that contain nucleosomes. As some of these methods are enzyme-based they are biased in their activity towards specific sequences, for example, MNase-seq has a bias towards A/T-rich sequences and the Tn5 used in ATAC-seq has a bias towards G/C-rich sequences (Meyer and Liu, 2014).

To study the variability in accessible chromatin regions between cells, single-cell versions of the method have been developed. The first versions used microfluidics (C1, Fluidigm) with a throughput of 96 cells at a time (Buenrostro *et al.*, 2015) or combinatorial barcoding with a throughput of +- 2.400 cells at a time (Cusanovich *et al.*, 2015). The first approach led to more sequencing reads per cell, while the second approach could process more cells simultaneously. The combinatorial barcoding approach has been optimized and used to identify the chromatin states of 15.000 single nuclei from flash-frozen mouse forebrain tissue at seven developmental stages (Preissl *et al.*, 2018). They find 20 distinct cell populations corresponding to different cell types in the brain during development and in the adult, including one oligodendrocyte cluster. This combinatorial barcoding approach was then used to profile 100.000 cells from 13 different tissues (Cusanovich *et al.*, 2018). They found 85 different chromatin patterns and around 400.000 sites differentially accessible. They used label transfer from a single-cell RNA-seq data set to assign clusters in the single-cell ATAC-seq data set. They used Cicero, which is a computational method that identifies correlations in accessibility between regulatory regions (Pliner *et al.*, 2018). A high correlation means those regions are 'co-accessible', and they could be linked to each other. Based on co-accessibility patterns, Cicero maps enhancer regions to the genes they might regulate (Pliner *et al.*, 2018). Other single-cell ATAC-seq methods have been developed since then, with some of them being commercialized for easier access. First, a nano dispensing technique, in which they use the iCELL8 from Takara bio (Mezger *et al.*, 2018). One chip has space for 5184 cells, but only one-third of the chip is generally used per experiment. This technique allows for fluorescent imaging within the chip prior to processing, which could be used for the identification and exclusion of, for example, dead cells. In this paper, ChromVar is used to identify the variation of transcription factor motif accessibility between cells (Schep *et al.*, 2017). Then, a plate-based approach was developed, protein-indexed ATAC-seq (PI-ATAC), in which you could fix the cells and sort based on transcription factors or other intracellular markers (Chen *et al.*, 2018). This method is non-commercial and easy to implement within any lab space. Lastly, two droplet-based single-cell techniques became available. The first one is through 10x Genomics, in which 6.000 – 10.000 cells per sample can be processed (Satpathy *et al.*, 2019). The second one is through Biorad, in which 10.000 cells per sample can be processed (Lareau *et al.*, 2019). Combining the last approach with combinatorial indexing, the authors could process 100.000 cells per run. Thus, high throughput methods to profile chromatin accessibility at the single-cell level are now available. These assays allow for the identification of novel regulatory regions and the mapping of those regions to the genes they regulate. They also allow for the identification of transcription factor motif variability to point to transcription factors that could be important for different transcriptional programs.

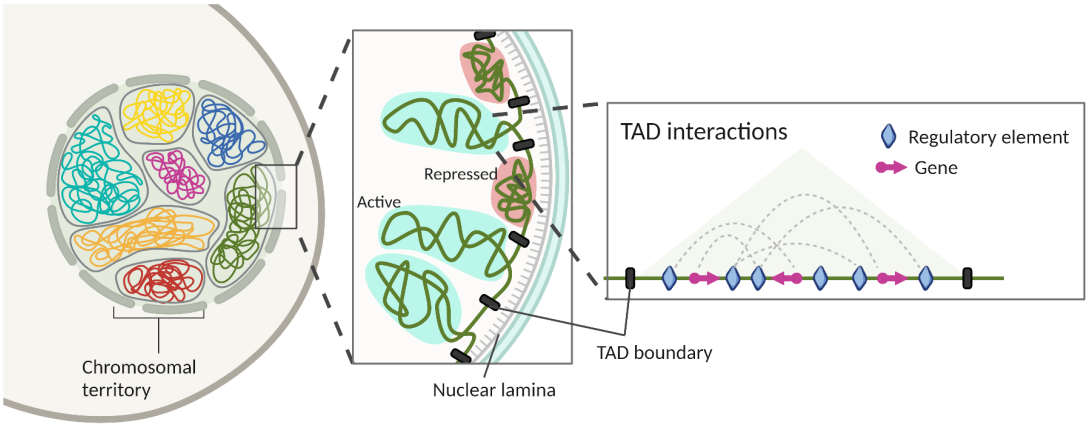

Figure 5. Schematic overview of higher-order chromosome organization. Chromosomes are divided in structural domains in which frequent interaction between regulatory regions take place. Active (A compartments) and repressive (B compartments) domains are shown.

1.4.4 Chromatin organization and looping

To activate gene expression, enhancers are looped towards the promoter they regulate. These are specific and highly dynamic interactions between different loci that are regulated by mechanisms at the higher-order chromatin structure level. Chromosomes are divided in dynamic large-scale domains in which interactions between regions occur more frequently than between different domains (Figure 5). These are called topologically associating domains (TADs; Dixon *et al.*, 2012). TAD boundaries prevent unspecific interactions between neighboring domains. They can differ between cell types, depending on the genes needed to be activated and the location of their regulatory elements (van Bortle *et al.*, 2014). Architectural proteins, such as CTCF, facilitate the formation of chromatin loops and bring promoters and enhancers in contact (Kurukuti *et al.*, 2006).

Chromosomes can also be divided at a larger scale into different domains in which chromatin is either open and more susceptible to activation or chromatin that is in a repressed state. The domains associated with open chromatin are called A compartments and often associate with active gene transcription (Lieberman-Aiden *et al.*, 2009). These domains usually reside in the interior of the nucleus. Multiple TADs can locate to one overlapping A compartment. B compartments are associated with closed chromatin and silenced gene transcription, they reside at the nuclear periphery and frequently associate with the laminin and nucleolus. Compartment A and B regions can alternate on the linear sequence, but in the 3D nuclear space are frequently found close to the regions from their own compartment (Lieberman-Aiden *et al.*, 2009). During differentiation, parts of compartments can switch from A to B and vice versa (Dixon *et al.*, 2015).

1.4.5 Genome Architecture Mapping

Genome architecture mapping (GAM) is a technique developed to map DNA interactions genome-wide (Beagrie *et al.*, 2017). This technique identifies interactions between regulatory elements on the genome and relies on the concept that DNA regions that interact are found more often in close proximity within the nucleus than regions that do not interact. To obtain

such data, first ultra-thin cryosections are made from tissue or cells. These cryosections result in a section through the nuclei in a random orientation. Then, nuclear slices are laser microdissected and the DNA is amplified and sequenced. The frequency that two DNA regions are found together in one slice is determined with computational methods. The technique is similar to Hi-C, a chromatin conformation capture method (3C) that also maps DNA interactions genome-wide (Lieberman-Aiden *et al.*, 2009). However, Hi-C requires millions of cells and ligation of the DNA fragments. GAM only requires 400 cells and is ligation free. Another advantage of GAM over Hi-C is that contacts between multiple regions can be identified simultaneously, while with Hi-C, interactions between only two regions are captured at the same time. GAM identifies gene regulatory mechanisms at the chromatin architecture level, such as A/B compartments, TADs, and long- and short-range interactions. Genome-wide chromatin interaction data was not available for the oligodendrocyte lineage until paper IV in this thesis.

2 RESEARCH AIMS

The overall aim of the thesis is to investigate the gene regulatory mechanisms of the oligodendrocyte lineage in development and disease.

We specifically investigated the following aims:

Paper I
- To identify the role of PAD2 in oligodendrocyte differentiation
- To analyze the citrullination targets of PAD2 in the oligodendrocyte lineage
- To characterize the role of histone citrullination on gene transcription during oligodendrocyte differentiation

Paper II
- To identify the effect of the inflammatory environment of EAE on the oligodendrocyte lineage transcriptional state

Paper III
- To understand the gene regulatory mechanisms involved in the immune response in the oligodendrocyte lineage
- To characterize which transcription factors could be involved in the immune response in the oligodendrocyte lineage
- To map MS-associated single-nucleotide polymorphisms (SNPs) that overlap with accessible regulatory regions in the oligodendrocyte lineage

Paper IV
- To map genome-wide chromatin interactions in intact brain tissue
- To characterize the chromatin conformation of different neural cell types including oligodendrocytes
- To understand the mechanisms of regulation at the chromatin level that could relate to differences in gene transcription between cell types

3 RESULTS AND DISCUSSION

3.1 Paper I: PAD2-mediated citrullination contributes to efficient oligodendrocyte differentiation and myelination

3.1.1 Results

MBP hypercitrullination is a hallmark of MS and *Padi2* overexpression leads to demyelination (Moscarello *et al.*, 1994; Musse *et al.*, 2008). Inhibiting PAD activity leads to the amelioration of disease symptoms (Moscarello *et al.*, 2013; Caprariello *et al.*, 2018). It is clear that too much citrullination and PAD activity are disadvantageous, yet MBP seems to be heavily citrullinated at specific stages during development. While PAD2 is the main PAD enzyme expressed in oligodendrocytes, its function besides MBP citrullination is not completely known. Increased levels of histone H3 citrullination have been observed in MS and could affect gene transcription. In **paper I**, we asked whether PAD2 plays a role in normal oligodendrocyte development and if PAD2 citrullinates histone H3 in this process. We additionally asked if we could identify other potential citrullination targets of PAD2 in oligodendrocytes.

We confirmed that *Padi2* is the main *Padi* expressed in oligodendrocytes and its expression increases with maturation, with the highest expression in adult oligodendrocytes. PAD2 protein levels plateaued around P21 in the spinal cord, at the peak of myelination. Inhibition of PAD activity and knockdown with siRNAs targeting *Padi2* specifically, resulted in decreased upregulation of myelin genes upon differentiation of oli-neu (embryonic mouse OPC cell line; Jung *et al.*, 1995) or mouse and rat primary OPCs. Thus, *Padi2* increases upon differentiation and promotes myelin gene expression.

We next investigated if PAD2 has a similar effect on the differentiation of oligodendrocytes *in vivo*. Loss of *Padi2* under the *Pdgfra* promoter, which results in loss of *Padi2* in the entire oligodendrocyte lineage (*Padi2* cKO), led to a reduced number of CC1 positive oligodendrocytes in the dorsal funiculus of the spinal cord at P21, which recovered in the adult (at 4 months old). We did not observe a change in CC1 positive cells in the corpus callosum and anterior commissure. These results show that while PAD2 is not involved in the generation of oligodendrocytes in the brain, it is involved in the spinal cord and its loss leads to a transient decrease in oligodendrocytes.

We then examined if the loss of *Padi2* leads to motor and/or cognitive symptoms and if its loss affects myelin formation. Both *Padi2* cKO and full *Padi2* KO mice showed impaired motor coordination, spending less time on the rotarod. The *Padi2* cKO also showed an increase in the number of slips on the beam test and they performed worse on the novel object recognition test. The full *Padi2* KO had a decrease in myelinated axons in the corpus callosum and differences in the g-ratio of axons with a smaller diameter. Hence, confirming

the role of *Padi2* in the formation of healthy myelin and showing that loss of *Padi2* results in impaired motor and cognitive functions.

We observed that PAD2 is located both in the nucleus and cytoplasm in OPCs and oligodendrocytes. We therefore further explored the possible role of *Padi2* in the nucleus. Using a SILAC (stable isotope labeling of amino acids in cell culture) proteomics approach in *Padi2* overexpressing oli-neu, we found that citrullination targets were enriched in differentiation. Among those targets, we found different histones (H1, H2b, and H3) and gene regulatory enzymes such as the histone demethylase KDM3b and the chromatin remodeler CHD6. We also found myelin, ribosomal and RNA-binding proteins among the citrullination targets. RNA-binding proteins contain many arginines and are involved in important mechanisms such as RNA splicing, processing, stabilization, transport, and translation. These targets point to the possible additional roles of PAD2 in the oligodendrocyte lineage besides MBP citrullination. We also investigated the potential protein interactors with the same approach, this time by pulling down biotin-tagged PAD2. Among the targets were again proteins involved in translation and posttranscriptional regulation of gene expression and different components of the myelin sheath.

To continue exploring the role of PAD2 in the nucleus we looked at specific histone H3 targets, often citrullinated in other cell types. Citrullination at arginine 26 of histone H3 (H3R26cit) and at arginines 2, 8, and 17 of histone H3 (H3R2+8+17cit) was present in oli-neu and reduced upon PAD inhibition. *Padi2* overexpression increased both H3R26cit and H3R2+8+17cit, with a stronger increase in differentiation. Thus, showing that PAD2 citrullinates histone H3, especially during the differentiation of OPCs. Therefore, we analyzed the chromatin landscape, specifically if PAD2 could affect chromatin accessibility. Indeed, we observed decreased chromatin accessibility at the regulatory regions of *Mag* and *Mbp*. These results show that PAD2 regulates the expression of myelin genes possibly through histone H3 citrullination.

3.1.2 Discussion

The loss of PAD2 in development has not been investigated before. *Padi2* overexpression had previously been shown to lead to demyelination (Musse *et al.*, 2008). Here we show that *Padi2* loss can also result in myelin sheath dysregulation. MBP is heavily citrullinated during development around the time that myelin sheaths are formed. A decompacted state of the myelin sheath is necessary during myelin sheath elongation. This allows for the formation of cytoplasmic channels that facilitate the transport of membrane material, such as MBP mRNA, to the active growth zone (Snaidero *et al.*, 2014). Citrullination of MBP, and possibly also other myelin sheath components, is one way to maintain decompaction. Loss of *Padi2* could affect the decompaction and therefore the transport of membrane material. However, if loss of *Padi2* results in a decrease in cytoplasmic channels remains to be investigated. Also, a reversal of arginine citrullination has thus far not been found, so how would MBP citrullination levels decrease after development? Possibly through replacement of citrullinated MBP by newly synthesized MBP. PAD2 activity reduces in compact myelin

upon maturation so newly synthesized MBP will not be exposed to PAD2 activity to the same extent (Pritzker *et al.*, 1999). For this, a high turnover of MBP protein is required.

As mentioned in the previous paragraph, the reversal of arginine citrullination has not been found thus far. The turnover of nucleosomes at promoters and enhancers is important for transcription factor binding and gene activation (Klemm *et al.*, 2019). A high nucleosome turnover rate is associated with active promoters and enhancers. As histone H3 citrullination is considered an active mark, high turnover rate could be a mechanism to decitrullinate histone H3. The maintenance of histone H3 citrullination in that case, has to occur through continues active citrullination.

Many RNA-binding proteins were found among the citrullination targets, especially in differentiation. RNA-binding proteins are involved in mRNA processing and transportation. *Mbp* mRNA is transported to the myelin sheath in granules and locally translated (Müller *et al.*, 2013). Some of the proteins that are involved in this process, such as HnRNPa2b1 and HnRNPk, are among PAD2 citrullination targets in the differentiation condition. These proteins bind to *Mbp* mRNA directly and regulate its transport to the processes (White *et al.*, 2008; Laursen *et al.*, 2011). RNA-binding domains contain many arginines as they are positively charged, citrullination could therefore result in reduced binding affinity of these proteins to mRNA. This is one way in which PAD2 could affect *Mbp* mRNA trafficking and translation. Thus, PAD2 could regulate MBP at the transcriptional, posttranscriptional, and posttranslational level. Furthermore, increased levels of calcium stimulate MBP synthesis (Wake *et al.*, 2011; Friess *et al.*, 2016), which is one way in which neuronal activity regulates myelin formation (Krasnow *et al.*, 2018; Baraban *et al.*, 2018). As citrullination is calcium-dependent, calcium influx could affect PAD2 activity and citrullination of RNA-binding proteins and MBP during myelination. PAD2 could have a dual role in the active growth zone, maintaining an open decompacted myelin structure through MBP citrullination, and release of *Mbp* mRNA through citrullination of RNA-binding proteins. Further research would be necessary to examine in what way PAD2 contributes to healthy myelin sheath formation and what the role of citrullination of MBP and other proteins is during oligodendrocyte development.

We observed a transient decrease of mature oligodendrocytes in the dorsal column of the spinal cord of *Padi2* cKO mice, which was not observed in the brain regions that we analyzed. The dorsal spinal cord population seems to be more susceptible to *Padi2* loss, despite the uniform expression of *Padi2* in all oligodendrocyte populations. If the spinal cord origin contributes to this susceptibility or because they are from this specific dorsal region, is not known. It could be that the brain regions also had a delay but recovered faster. Future research could consider more time points and areas to understand why the brain populations are unaffected at P21 and in the adult. Compensatory mechanisms from other PADs could also have affected this difference, although other PADs are very low or not expressed in the oligodendrocyte lineage.

Although we observed an increase in histone H3 citrullination upon *Padi2* overexpression and a decrease upon PAD2 inhibition, we could not link the histone H3 citrullination directly to the loss of chromatin accessibility and the increased myelin gene expression upon *Padi2* knockdown. We would need to assess in which genomic loci histone H3 citrullination is found on the genome, for example by ChIP-seq or CUT&Tag. We have tried multiple experimental set-ups, but the antibody, unfortunately, did not work in our hands in such applications. Thus, the changes at the chromatin level could be due to citrullination of histone H3, other histones, chromatin-modifying enzymes, or other mechanisms.

Previous studies have shown that hypercitrullination can be detrimental. In our study, we showed that loss of *Padi2* is equally harmful. This is an important aspect to consider when modulating PAD2 activity in future applications.

3.2 Paper II: Disease-specific oligodendrocyte lineage cells arise in multiple sclerosis

3.2.1 Results

Oligodendrocytes are attacked by the immune system in MS, which leads to oligodendrocyte degeneration and demyelination. Activated lymphocytes within the lesion secrete cytokines, which can affect the cells residing there. These lymphocytes and cytokines also affect OPCs that are recruited to contribute to remyelination. In **Paper II**, we performed single-cell RNA-seq on oligodendroglia from the spinal cords of mice at the peak of EAE, to investigate how oligodendroglia are affected by the inflammatory environment.

We found disease-specific populations of oligodendroglia in EAE, which had enriched expression of MHC-I genes and genes involved in antigen processing. Interestingly, some smaller disease populations also had increased expression of MHC-II genes, normally only expressed in professional antigen-presenting cells. MHC-II protein was also found in OLIG1/2 positive cells in postmortem brain tissue of MS patients. Another interesting group of genes enriched in EAE are the *Serpins*, which are serine protease inhibitors. Granzyme-B is a serine protease secreted in EAE by cytotoxic T cells that causes neuronal damage. One of the *Serpins* upregulated in EAE populations is *Serpina3n*, which has been shown to inhibit Granzyme-B resulting in neuroprotection against T cells and reduced EAE severity (Haile *et al.*, 2015). Thus, the upregulation of *Serpins* could have a protective effect in oligodendroglia. Genes identified to be involved in MS susceptibility have been associated with microglia, however many of the non-MHC locus associated genes were enriched in OPCs and in a specific population of mature oligodendrocytes enriched in EAE (MOL1/2-EAE).

We identified that CD45 positive immune cells isolated from the spinal cord of EAE mice specifically induce MHC-II expression in OPCs in culture. We found that interferon-gamma was responsible for MHC-II induction in oligodendroglia and the upregulation of many of the gene modules we found in EAE. Furthermore, OPCs were able to phagocytose myelin,

independent of interferon-gamma treatment. MOG peptide-specific CD4 positive memory T cells were activated in co-cultures with OPCs pre-treated with interferon-gamma and MOG peptide. These CD4 positive T cells had increased survival, proliferation, and cytokine production. This shows that OPCs can present antigen to CD4 positive memory T cells.

To conclude, oligodendroglia undergo a major switch in the transcriptional program in inflammation. Some of the genes are protective against disease, while others could promote interactions with the immune system. OPCs could mediate and spread the immune response by phagocytosing myelin and presenting antigens to CD4 positive T cells.

3.2.2 Discussion

Previous research on the effect of interferon-gamma on oligodendroglia is controversial. Some studies suggest that interferon-gamma is harmful and could even lead to apoptosis (Vartanian *et al.*, 1995; Baerwald and Popko 1998; Chew et al., 2005; Horiuchi *et al.*, 2006), while others showed that interferon-gamma could be protective (Billiau *et al.*, 1988; Lublin *et al.*, 1993; Ferber *et al.*, 1996; Krakowski *et al.*, 1996; Willenborg *et al.*, 1996). In our study, we did not observe any signs of apoptosis in primary cells treated with interferon-gamma. We did observe differential expression of genes encoding ER proteins, suggesting ER stress might occur in oligodendroglia in EAE. ER stress in oligodendroglia has been observed previously, in response to overexpression of interferon-gamma during development (Baerwald *et al.*, 2000; Lin *et al.*, 2005). On the other hand, protective mechanisms were also induced in oligodendroglia in EAE, such as the upregulation of *Serpins*, which suggests oligodendroglia might regulate their own survival upon inflammatory insult.

MHC-II upregulation in oligodendroglia has only been reported in a few studies (Bergsteindottir *et al.*, 1992; Itoh *et al.*, 2009). Although MHC-I expression is much more widespread, MHC-II expression was observed in about 3.4% of *Sox10* positive cells in the lesion area in EAE and in a large percentage of primary oligodendroglia treated with interferon-gamma. This result was surprising, as normally MHC-II is only expressed by professional antigen-presenting cells. We showed that OPCs could phagocytose myelin debris and present antigen to CD4 positive T cells through MHC-II, which is in agreement with a study showing OPCs phagocytosing myelin debris and presenting antigen to CD8 positive T cells through MHC-I (Kirby *et al.*, 2019). OPCs could therefore be involved in the initiation or amplification of an immune response. What this implies for MS still needs to be investigated.

3.3 Paper III: Epigenomic priming of immune genes implicates oligodendroglia in multiple sclerosis susceptibility

3.3.1 Results

Oligodendroglia in an inflammatory environment are captured in an immune transcriptional state, which inhibits their capacity to contribute to remyelination and could leave them vulnerable to cytotoxicity. In **Paper III**, we wanted to understand how oligodendroglia

transition to the immune state by identifying their gene regulatory mechanisms. We used single-cell ATAC-seq in EAE at peak to map the chromatin accessibility landscape and to identify transcription factors that could be important in the transition.

Single-cell ATAC-seq revealed chromatin signatures in EAE which were in accordance with the transcriptional state, as enriched chromatin accessibility was mainly found at gene loci involved in immune processes. However, a subset of immune genes had already accessibility in control, while they were not or low expressed. The transcription of these immune genes was induced in EAE, but we did not observe such changes at the chromatin accessibility level. This suggests that other gene regulatory mechanisms are involved in the activation of these genes. We additionally performed multi-ome profiling of oligodendroglia in EAE, where we measured chromatin accessibility and RNA from the same nuclei. This confirmed our earlier observation, that many immune genes were primed in control.

To investigate how immune genes are induced, we treated OPCs with interferon-gamma in culture and performed bulk ATAC- and RNA-seq. There was a considerable induction of genes at the RNA level, including interferon response genes, and MHC-I and -II genes. However, at the chromatin level, the changes that were observed were limited. Single-cell ATAC-seq revealed a set of transcription factors that had enriched motif accessibility in oligodendroglia in EAE. Among those transcription factors were IRF1, STAT1, STAT3, and BACH1. Knockdown of *Bach1* in primary OPCs treated with interferon-gamma resulted in induced expression of MHC-I genes, *H2-Q4* and *H2-Q7*, and MHC-II pathway gene *Cd74*. BACH1 could therefore be involved in the negative regulation of MHC-I and -II expression in OPCs in EAE. In contrast, knockdown of *Stat1* in primary OPCs treated with interferon-gamma resulted in decreased upregulation of interferon response genes, and MHC-I and -II genes. CUT&Tag using an antibody against STAT1 showed increased binding of STAT1 at those genes upon interferon-gamma treatment. This shows that STAT1 activates the immune profile in OPCs upon interferon-gamma signaling.

To understand how primed genes are induced, other than STAT1 activation, we profiled the histone modification landscape with Cut&Run and H3K27ac HiChIP in interferon-gamma-treated OPCs. We observed an increase in H3K27ac and CTCF binding at immune genes and increased enhancer-promoter contacts. This suggests that chromatin remodeling rather than chromatin accessibility is involved in the induction of immune genes. Profiling H3K4me3 and H3K27me3 showed that many MHC-I and -II genes were bivalent, which means they had both marks. Upon interferon-gamma treatment, the repressive mark H3K27me3 was removed, which then induced expression. Primed genes switch from a bivalent state (H3K4me3 and H3K27me3) to an active state (H3K4me3, CTCF, and H3K27ac) upon interferon-gamma treatment. Inhibiting EZH2, the enzyme responsible for H3K27me3, led to an increased upregulation of MHC-I and -II genes in interferon-gamma-treated OPCs. Thus, STAT1 binding, together with H3K27ac, chromatin remodeling, and H3K27me3 removal induces expression of immune genes in OPCs.

Next, we were interested if the priming of immune genes also occurred in the healthy human brain. We performed single-cell multi-omics to assess the chromatin and RNA profiles of different CNS cell types. We observed that especially MHC-I genes were primed in all cell types of the CNS, while MHC-II accessibility was more restricted. Some MS-associated SNPs overlapped with chromatin accessibility sites in all cell types, for example at the HLA-B locus. Some SNPs also overlapped with regions in mice that had no accessibility in control, but upon interferon-gamma treatment would gain accessibility. For example, at the *Socs1* locus, where an enhancer region gained accessibility, H3K27ac, CTCF binding, and predicted interactions with the *Socs1* promoter. Thus, MS-associated SNPs could also be relevant in other cell types besides microglia.

3.3.2 Discussion

Within the general immune response, only a small subset of genes was found enriched in oligodendroglia, even in EAE. The hallmarks 'interferon-gamma' and 'interferon-alpha response' were enriched in EAE oligodendroglia, but other signaling pathways, such as 'IL6 JAK STAT3 signaling' and 'IL2 STAT5 signaling' were only enriched in microglia. While the inflammatory environment in EAE changes the transcriptional profile of oligodendroglia, they remain committed to their own lineage, showing many differences compared to microglia. OPCs might be able to perform certain microglial functions, such as phagocytosis of myelin debris and antigen presentation *in vitro* (Kirby *et al.*, 2019; Fernández-Castañeda *et al.*, 2020), however, they might be missing other pathways important for other microglial functions. These data imply that microglia have specialized functions compared to immune OPCs and probably are still the dominating cell type for immune regulatory functions in the CNS.

Although many genes enriched in EAE were also enriched in interferon-gamma treated OPCs, we found only low overlap in different gene categories (primed vs. induced) between EAE and interferon-gamma OPCs. In EAE many other cytokines and signals are around that could affect the chromatin and transcriptional state of OPCs, which might contribute to those differences. For example, interleukin-17 signaling has been shown to inhibit the differentiation potential of OPCs but does not alter MHC-I and -II gene expression (Kirby *et al.*, 2019).

H3K27me3 has previously been shown to suppress astrocytic and neuronal fate in oligodendroglia (Sher *et al.*, 2012). We show here that besides the suppression of those cell fates, we also observed suppression of MHC-I and -II genes and some chemokine genes. However, those genes remained repressed upon EZH2 inhibition and were only induced when OPCs were spiked with interferon-gamma, then their induction was much greater than treatment with interferon-gamma without EZH2 inhibition. Some of these genes even showed bivalency. These results indicate that an additional cue, besides H3K27me3 removal, is necessary for MHC-I and -II genes to be induced and their induction is potentiated by H3K27me3 removal.

We observed one SNP within a regulatory region with predicted interactions with the *Bcas1* promoter, a gene important for early myelination. The SNP region overlaps with high levels of H3K27me3. H3K27me3-rich regions have been shown to repress genes through chromatin interactions (Cai *et al.*, 2021). *Bcas1* levels are reduced upon interferon-gamma treatment, possibly through increased interactions with this region. These results show an example of how MS-associated SNPs could affect neighboring genes in other cell types than microglia in the context of disease.

3.4 Paper IV: Cell-type specialization is encoded by specific chromatin topologies

3.4.1 Results

GAM is a method to delineate genome-wide interactions from low cell numbers in a ligation-free manner (Beagrie *et al.*, 2017). In **Paper IV**, we adapted GAM, now called ImmunoGAM, to enable the use of antibodies to detect cell type-specific proteins or reporters. This addition allows for the selection of specific cell types from intact heterogeneous tissues. We applied ImmunoGAM to characterize the chromatin conformation of different cell types in the brain. Ultra-thin (220 nm) cryosections are antibody labeled and nuclear slices are laser microdissected from selected cells, therefore avoiding tissue dissociation. The DNA content from each nuclear slice is then amplified and sequenced. As only few cells are needed (~1000 cells), ImmunoGAM profiles can be generated from single animals. We characterized the chromatin conformation of three cell types: oligodendroglia from the somatosensory cortex, pyramidal glutamatergic neurons (PGNs) from cornu ammonis 1 of the dorsal hippocampus, and dopaminergic neurons (DNs) from the ventral tegmental area of the midbrain. We compared the profiles of these cells to the profiles of mouse embryonic stem cells (mESC) that were publicly available (Beagrie *et al.*, 2021).

We were able to show that ImmunoGAM detects differences in both short- and long-range interactions between cell types. An example of this is at the *Pcdh* locus, a cluster of genes encoding protocadherins. Contacts between the three genes, *Pcdha*, *Pcdhb,* and *Pcdhg*, correlated with gene expression. The contacts were stronger in neurons and oligodendroglia, where all three genes are expressed, compared to mESC, where only *Pcdhg* is expressed. Short-range interactions were often mediated by specific transcription factor pairs. NEUROD1/2 were most often associated with interactions in PGNs, while CTCF was the most enriched in DNs, followed by FOXA1. ImmunoGAM also detected cell type-specific TAD boundaries. Many TAD boundaries were unique for each cell type and often contained genes that were expressed and related to cell type-specific functions. For example, oligodendroglia had genes involved in mechanical stimulus and fatty acid biosynthesis.

Extensive reorganization also occurred at the compartment level. Compartment B to A switching, from mESC to brain cell type, often contained genes more strongly expressed in the brain cell types related to specific functions such as behavior and regulation of synaptic transmission. A to B transitions often contained genes that were silent in all cell types. 50 of

those genes were highly expressed in mESC and were involved in transcriptional regulation. A-B switching also often contained sensory genes, such as the *Vmn* (vomeronasal) and *Olfr* (olfactory) receptor gene clusters. These genes were in B compartments in brain cells and had strong contacts with other B compartments, possibly mediating their sustained repression. To conclude, we found cell type-specific chromatin interactions that were functional. The interactions mainly linked genes that should remain repressed, or active genes with special functions for that cell type. Also, short-range interactions between active genes were often mediated by specific transcription factor pairs.

Lastly, we identified a distinct feature for long, active genes in neurons. Long neuronal genes (>300 kb) lose contact density when active in that cell type. *Grik2* is highly expressed in PGNs and loses many of its interactions compared to mESC. The same was observed for *Dscam* in DNs, although *Grik2* mostly lost contacts at the transcription start site and end site while *Dscam* lost contacts throughout the entire gene body. We called this decondensing of the gene locus 'melting' and it was often observed in highly expressed genes in PGNs and DNs but not in oligodendroglia or mESC. Melting genes also often had higher levels of chromatin accessibility and often belonged to compartment A. Two other examples are *Nrxn3* (Neurexin 3) and *Rbfox1* (RNA binding Fox1 homolog 1). We showed with polymer modeling and cryo-FISH that their loci decondensed in DNs (*Nrxn3*) and PGNs (*Rbfox1*).

3.4.2 Discussion

Pluripotent mESC have a relatively open chromatin landscape as all genes need to be able to be transcribed depending on the differentiation path they will initiate (Mattout and Meshorer, 2010; Gaspar-Maia *et al.*, 2011). Other local regulatory mechanisms are in place to keep lineage-specific genes silent in mESC. Upon differentiation, more defined chromatin structures are formed, reflected in our study at the TAD boundary and compartment level. TAD boundaries that were shared between mESC and brain cell types had stronger insulation in the brain cell types, suggesting that brain cell types have more specific and more frequent interactions between the same loci. In mESC, these similar interactions are probably more sparse because of the open chromatin landscape. Furthermore, A to B compartment switching, from mESC to brain cell type, often contained genes that were silent in both cell types, suggesting other mechanisms are in place to maintain silencing of genes in A compartments in mESC.

We showed that chromatin interactions occur to maintain genes in either a repressed or active state. Short-range interactions between active genes often involved cell-specific transcription factor and/or CTCF motifs, suggesting that CTCF and transcription factors mediate such interactions. Cell type-unique TAD boundaries were often associated with active genes for that cell type. The data from this paper show that, upon differentiation, cell type-specific interactions are formed that facilitate gene transcription for that specific cell type. Some of these mechanisms were already known to correlate with gene expression. However, here we show that we can identify these mechanisms in specific cell types in heterogeneous tissues.

Only around 1000 nuclei are necessary, which makes it possible to uncover cell-specific chromatin interactions in rare cell populations.

4 CONCLUSIONS

This thesis has presented some of the gene regulatory mechanisms that regulate the transcriptional states of oligodendroglia during development and in disease.

In **Paper I** we have shown that PAD2 is involved in oligodendrocyte development by promoting differentiation through the chromatin accessibility and upregulation of myelin genes and through histone H3 citrullination. PAD2 also contributes to healthy myelin sheaths, important for motor skills and cognitive function. We also showed that PAD2 citrullinates myelin proteins and proteins involved in transcriptional and posttranscriptional regulation. Among the citrullination targets were histones, chromatin-modifying enzymes, and RNA-binding proteins. This suggests that PAD2 could have additional roles in regulating gene expression and RNA processing.

In **Paper II** we have shown that the oligodendrocyte lineage transitions to an immune state in the inflammatory environment of EAE. Oligodendroglia in EAE upregulate immune pathway genes including MHC-I and -II genes involved in antigen processing and presentation. We have shown that OPCs are able to phagocytose myelin debris and present antigen to CD4 positive memory T cells. In this way, OPCs could mediate and amplify the inflammatory response in MS.

In **Paper III** we have shown some of the mechanisms behind the oligodendroglia immune state transitioning. A subset of immune genes exists in a primed conformation in oligodendroglia, showing open chromatin or bivalency in a healthy state. Immune gene transcription in OPCs is induced through the removal of H3K27me3 and the increase in CTCF-mediated interactions of active enhancers with promoters. Transcription factors BACH1 and STAT1 are also involved in this process. In the human healthy brain, MHC-I genes are also in a primed state in oligodendroglia, while MHC-II genes are more restricted. We have shown that MS-associated SNPs can be accessible in other cell types than microglia and that chromatin accessibility at SNPs can be induced in specific conditions, such as inflammation.

In **Paper IV** we have shown that immunoGAM detects differences in both short- and long-range interactions between cell types. Sort-range interactions were often mediated by specific transcription factor pairs for that cell type. We were also able to detect cell type-specific TADs, with unique boundaries containing genes expressed and related to cell type-specific functions. We have shown extensive reorganization at the compartment level, with A to B and B to A compartment switching upon differentiation. We also showed that long active genes in neuronal populations undergo decondensation or melting.

The findings of these four papers involve different mechanisms that regulate gene expression in oligodendroglia in development and in disease contexts. We have investigated citrullination of proteins, transcriptional changes in disease, chromatin accessibility, CTCF binding, histone modifications, transcription factor binding, chromatin-modifying enzymes,

and genome interactions. There are many layers of gene regulation and together they regulate different functional aspects of the cell. The transcriptional state of oligodendroglia is affected by the inflammatory environment in MS. To protect oligodendroglia and promote remyelination, we need to understand the changes they go through in disease and how we can regulate those changes. Understanding the regulatory mechanisms of gene transcription in oligodendroglia in development and disease provides us with new targets for therapeutical approaches relevant to MS.

5 POINTS OF PERSPECTIVE

Based on the concepts discussed in this thesis and the conclusions from the presented papers, we can speculate how oligodendroglia are affected in disease, how an inflammatory attack could be initiated, and in what way gene regulatory mechanisms play a role.

It has been proposed that MS is caused by a dysregulation of either the immune system (outside-in) or a component within the CNS, such as myelin (inside-out; Trapp and Nave, 2008; Stys *et al.*, 2012; Stys, 2013). Many theories which involve PAD2-mediated MBP citrullination support the inside-out hypothesis (Yang *et al.*, 2016; Luchicchi *et al.*, 2021). Neuronal activity during development can lead to rising levels of calcium within oligodendrocytes, which mediates myelin sheath formation (Krasnow *et al.*, 2018; Baraban *et al.*, 2018). However, elevated calcium levels in later stages, because of axonal damage or infection among others, could lead to destabilized myelin due to MBP displacement from the myelin membrane (Weil *et al.*, 2016), possibly through PAD2-mediated citrullination. Citrullinated MBP might be more susceptible to degradation, releasing an immunodominant epitope (Pritzker *et al*., 2000; D'Souza & Moscarello, 2006). The immunodominant epitope of MBP could be phagocytosed and presented to CD4 positive T cells, which would evoke a myelin-specific inflammatory response.

On the contrary, citrullination could be important for the stabilization of MBP, reducing its charge and preventing it from proteasomal degradation (Kuzina *et al.*, 2016). Upon myelin degeneration, because of injury or inflammation, MBP could be released from the membrane in its highly disordered and unstable form. PAD2-mediated citrullination could protect MBP from degradation and recognition by the immune system. This might lead to the accumulation of citrullinated MBP observed in MS (Moscarello *et al.*, 1994). Another possibility could be that increased levels of citrullinated MBP are found because of remyelination. It has been proposed, based on citrullinated MBP levels, that myelin in MS is immature (Moscarello *et al.*, 1994). PAD2-mediated citrullination is important for myelination in development (**Paper I**) and might be equally as important in remyelination.

As both injury and inflammation can lead to the accumulation of calcium within the myelin sheath, both the outside-in and inside-out hypotheses could explain the increased levels of MBP citrullination. Citrullinated MBP is probably not the actual trigger in all MS patients, but it could be the case in some. The higher levels of citrullinated MBP in MS might facilitate remyelination at the start. However, sustained levels, due to ongoing inflammation and injury, might lead to unstable myelin which is vulnerable to degradation and leads to the release of myelin epitopes with the potential of stimulating and amplifying the immune response.

We and others have shown that OPCs can phagocytose myelin debris and present antigen to T cells (**Paper II**, Kirby *et al.*, 2019; Fernández-Castañeda *et al.*, 2020). This capability can induce their own cell death (Kirby *et al.*, 2019) and could possibly lead to the OPC depletion observed in MS (Chang *et al.*, 2000; Jäkel *et al.*, 2019). However, if OPCs with immune

properties are functional in EAE or MS, is not yet understood. We have shown that only a small subset of genes expressed by oligodendroglia in EAE overlaps with genes expressed by microglia (**Paper III**). The immune gene upregulation observed in oligodendroglia could be merely a signal to the cell that it is under attack and could in some cases even be beneficial. For example, interferon-gamma can promote different protective mechanisms (**Paper II**, Espejo *et al.*, 2002; Lin *et al.*, 2007; Balabanov *et al.*, 2007). Newly differentiated OPCs in an inflammatory environment could be extremely vulnerable due to the high upregulation of myelin proteins and associated ER stress (Lin *et al.*, 2005). Interferon-gamma could, by inhibiting OPCs from differentiation (Turnley *et al.*, 1991; Agresti *et al.*, 1996; Chew *et al.*, 2005; Kirby *et al.*, 2019), therefore be protective.

While interferon-gamma might have some protective effects, it is not recommended for use as a therapeutic agent. The effect of interferon-gamma on the cell is highly dependent on the timing and the dose, and administration, while inflammation is ongoing, can increase the release of reactive oxygen species and ER stress, leading to increased apoptosis of oligodendroglia and axonal degeneration. Some of the protective mechanisms could, on the other hand, be considered as potential therapeutic targets, for example, research into the role of *Serpins* (**Paper II**) in oligodendroglia in the context of inflammation would be worth pursuing.

The transcriptional changes in response to inflammation or interferon-gamma signaling in oligodendroglia are not observed to the same extent at the chromatin accessibility level (**Paper III**). A subset of immune genes resides in a poised or primed state, presenting chromatin accessibility or bivalency in a non-disease context. Chromatin accessibility depends on nucleosome turnover rates and binding of transcription factors and other molecules to the DNA (Klemm *et al*., 2019). A high nucleosome turnover at the promoter allows for potential transcription factors to bind and activate genes. Open chromatin at primed immune genes, therefore, allows for activation. However, activation still relies on environmental cues, such as interferon-gamma signaling, to induce activation pathways including transcription factor binding, H3K27ac, CTCF binding, genome interactions, and/or removal of H3K27me3.

If primed or poised genes are activated faster than non-primed genes is still up for debate, however, it has been shown in mESC that bivalent genes are not activated faster or with higher levels than non-bivalent genes (Kumar *et al.*, 2021). It could be that priming or poising of genes allows for greater plasticity, the possibility for a gene to be expressed in the future. For example, alternative lineages become more repressed upon differentiation in mESC. This has also been shown in the oligodendrocyte lineage in which astrocytic and neuronal fates become more repressed through combined mechanisms such as H3K27me3, HDAC3, and H3K9me3 (Sher *et al.*, 2012; Liu *et al.*, 2015; Zhang *et al.*, 2016; Bartosovic *et al.*, 2021).

Ultimately, the activation of a gene depends on many regulatory levels that cooperate. If a region is not accessible, the gene can often not be activated by transcription factors, except for some transcription factors with pioneering functions. Pioneer transcription factors can

bind to the nucleosome containing DNA and can displace nucleosomes (Cirillo *et al.*, 1998, 2002; Zaret and Carroll, 2011), while other transcription factors are dependent on chromatin remodelers to actively displace nucleosomes before they can bind to the DNA (Klemm *et al.*, 2019). It would therefore be interesting to investigate which chromatin remodelers are involved in immune gene priming and upregulation in oligodendroglia. Also, immunoGAM in oligodendroglia in an inflammatory context would be very valuable to identify which higher-order chromatin processes are involved in immune gene regulation. We already observed differences in CTCF binding and predicted genome interactions (**Paper III**), but immunoGAM could identify specific short- and long-range interactions and changes at the TAD and A/B compartment level (**Paper IV**). This type of information could give us insights into which regulatory regions map to which genes and what the factors are that regulate those interactions.

To conclude, in this thesis we unveil several new facets of oligodendroglia in development and disease, finding that PAD2 is important in differentiation, myelination, and gene regulation at the transcriptional and posttranscriptional level. Further research on the role of PAD2 in MS would be necessary to unveil if higher citrullinated MBP levels could contribute to remyelination or if they contribute to inflammation instead. We could identify that oligodendroglia upregulate immune genes in inflammation and we characterized the gene regulatory mechanisms responsible for that upregulation. The function of immune OPCs in MS is still unclear and future research will unravel if they are involved in the initiation and/or mediation of inflammation in MS. Lastly, we identified that interactions at the higher-order chromatin level are cell type-specific and associated with gene expression. Thus, our results highlight the considerable impact of epigenomics in development and disease. The advancement of new technologies to study gene regulatory mechanisms is very important as it strengthens our knowledge of how genes are regulated and the potential to influence those processes for future therapies in diseases such as MS.

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