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MICRORNA-125A-3P NEGATIVELY REGULATES OLIGODENDROGLIAL MATURATION AND RE-MYELINATION: MOLECULAR MECHANISMS AND CLINICAL IMPLICATIONS

BIO/14

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ABSTRACT

Multiple sclerosis (MS) is a chronic immune-mediated de-myelinating disease of the central nervous system in which immune system attacks myelin, a substance produced by mature oligodendrocytes that normally surrounds and protects axons, leading to abnormal impulses transmission (Wu et al., 2011).

During their maturation oligodendroglial precursors cells (OPCs) follow a very precise differentiation program, finely orchestrated by transcription factors, epigenetic factors and microRNAs, a class of small non-coding RNAs involved in post-transcriptional regulation (He and Lu, 2013). Any alterations in this program can potentially contribute to dysregulated myelination, impaired re-myelination and neurodegenerative conditions, as it happens in multiple sclerosis.

Based on these considerations, the aim of this study was to investigate the potential role of miR-125a-3p, a brain enriched miRNA, in the regulation of OPC maturation and to assess whether its alteration can contribute to MS pathogenesis or re-myelination failure.

First, our gene ontology based study showed that several of its predicted target mRNAs are involved in glial cell differentiation, myelination, axon ensheathment and oligodendrocyte differentiation, suggesting that miR-125a-3p may have a primary role in the regulation of these processes. Then, we characterized its expression pattern in the CNS, showing that it is expressed in oligodendroglial cells throughout brain development and is progressively up-regulated during OPC *in vitro* differentiation. We also found that the over-expression of this miRNA impairs, whereas its silencing promotes oligodendrocyte maturation *in vitro*, likely acting on different targets at multiple levels of the process.

Interestingly, we observed an up-regulation of miR-125a-3p in two different mouse models of toxic demyelination, induced by cuprizone or lysolecithin administration, suggesting that it may represent a hallmark of de-myelination. Furthermore, the over-expression of miR-125a-3p in the white matter of mice following lysolecithin-induced demyelination maintained oligodendrocytes in the NG2-positive precursor stage, in line with the hypothesis of a delay in both their maturation and the subsequent re-myelination process.

To identify new mechanisms altered by miR-125a-3p during OPC maturation, we performed a transcriptomic analysis after its over-expression in OPCs, showing that miR-125a-3p can modulate different pathways and processes, such as Wnt-signaling and expression of ECM and adhesion molecules.

Globally, our data suggest that miR-125a-3p could represent a new negative regulator of re-myelination and that an antago-miRNA specific for this miRNA may help to foster oligodendrocyte maturation in diseases characterized by impaired myelin repair.

The potential importance of miR-125a-3p in MS was also confirmed by the finding that it is up-regulated in the CSF of MS patients in the active phase of the disease, suggesting that it could represent a potential biomarker for the diagnosis or prognosis of different MS phases.

RIASSUNTO

La sclerosi multipla (SM) è una malattia demielinizzante autoimmune del sistema nervoso centrale (SNC) in cui il sistema immunitario attacca la mielina, una struttura prodotta dagli oligodendrociti maturi che normalmente circonda e protegge gli assoni, portando ad una alterata trasmissione degli impulsi nervosi (Wu et al., 2011).

Durante la loro maturazione, le cellule precursori oligodendrogliali (OPC) seguono un programma di differenziamento molto preciso, finemente regolato da fattori di trascrizione, fattori epigenetici e microRNA, una classe di piccoli RNA non codificanti coinvolti nella regolazione post-trascrizionale (Li and Lu, 2013). Qualsiasi alterazione in questo programma può portare a difetti di mielinizzazione e condizioni neurodegenerative, come avviene ad esempio nella sclerosi multipla.

Sulla base di queste considerazioni, lo scopo di questo studio è stato quello di indagare il potenziale ruolo di miR-125a-3p, un miRNA espresso ad alti livelli nel SNC, nella regolazione della maturazione degli OPC e di valutare se la sua alterazione possa contribuire alla patogenesi della SM o ad ostacolare la rimielinizzazione.

L'analisi *in silico* basata sulle categorie presenti in Gene Ontology ha mostrato che numerosi bersagli predetti di questo miRNA sono coinvolti nel differenziamento degli OPC e nella mielinizzazione, suggerendo che miR-125a-3p possa avere un ruolo primario nella regolazione di questi processi. Abbiamo quindi caratterizzato il suo profilo di espressione nel SNC, dimostrando che esso, durante lo sviluppo, è espresso in cellule oligodendrogliali e che la sua espressione aumenta progressivamente durante la maturazione *in vitro*. Abbiamo anche scoperto che l'eccessiva espressione di questo miRNA ostacola, mentre il suo silenziamento promuove, il processo di maturazione degli OPC, probabilmente agendo a più livelli e su diversi bersagli.

Valutando l'espressione di miR-125a-3p in due diversi modelli murini di demielinizzazione tossica, indotta da somministrazione di cuprizone o lisolecitina, abbiamo riscontrato un aumento della sua espressione nella fase di demielinizzazione, suggerendo che tale alterazione possa rappresentare un segno distintivo di danno demielinizzante. Abbiamo inoltre dimostrato che la sua eccessiva espressione in sostanza bianca di topi, in seguito a demielinizzazione,

mantiene gli oligodendrociti nello stadio precursore, in linea con un ritardo della loro maturazione e del successivo processo di rimielinizzazione.

Per identificare nuovi meccanismi alterati da miR-125a-3p durante la maturazione degli OPC, abbiamo eseguito un'analisi del trascrittoma in seguito alla sua sovraespressione, dimostrando che esso può modulare diversi aspetti dello sviluppo degli OPC, come l'attivazione di specifiche vie di segnalazione e l'espressione di molecole di adesione cellulare.

Globalmente, i nostri dati suggeriscono che l'alterazione di miR-125a-3p potrebbe rappresentare un nuovo meccanismo che regola negativamente la rimielinizzazione e che un inibitore specifico per questo miRNA potrebbe contribuire a promuovere la maturazione degli oligodendrociti in malattie caratterizzate da difetti di rimielinizzazione.

La potenziale rilevanza di miR-125a-3p nella sclerosi multipla è inoltre supportata dall'aumentata espressione riscontrata nel fluido cerebrospinale di pazienti nella fase attiva della malattia, suggerendo che esso potrebbe essere un utile biomarcatore per la diagnosi o prognosi di questa patologia.

1- INTRODUCTION

1.1 MicroRNAs and post-transcriptional regulation

MicroRNAs (miRNAs) are small, highly conserved non-coding RNA molecules involved in the post-transcriptional regulation of gene expression (Bartel *et al.*, 2004), generated from a precursor stem loop (pri-miRNA) through subsequent cleavages and ultimately incorporated into RNA-induced silencing complex (RISC) to function as a guide, directing the silencing of target mRNAs. MiRNA-mediated post-transcriptional regulation results in gene expression repression at both the level of mRNA stability by promoting mRNA degradation and the level of translation (at initiation or after) by inhibiting protein translation or degrading the polypeptides, through binding complementarily to 3'UTR of the target mRNAs. In postgenomic era, the accepted notion is that a single miRNA can regulate hundreds of targets, even if only to a mild degree, however, several miRNAs can bind to their target mRNAs and cooperatively provide fine-tuning of a single mRNA target expression (Valinezhad Orang et al., 2014). In mammals, miRNAs are predicted to control the activity of approximately 30% of all protein-coding genes, and have been shown to participate in the regulation of almost every cellular process investigated so far. As a consequence, changes in their expression has been observed in, and may be responsible for, several human pathologies (Filipowicz et al., 2008). Actually, disease-associated miRNAs represent a major focus of clinical research and a new class of targets for the miRNA-based novel therapeutic or diagnostic/prognostic biomarkers (Rupaimoole et al., 2011).

1.1.1 Discovery of miRNAs

MiRNAs were discovered for the first time in the worm *Caenorhabditis elegans* (Lee *et al.*, 1993; Lau *et al.*, 2001), where, it has been shown that lin-4, a gene known to control the timing of C. elegans larval development, does not code for a protein but instead produces a pair of small RNAs (Lee *et al.*, 1993). The former RNA was 22 nt in length, and the latter approximately 61 nt, which was proposed to be the precursor of the shorter one (Lee *et al.*, 1993). Then, they noticed that these lin-4 RNAs had antisense complementarity to multiple site in the 3'UTR of the lin-14 gene and that this pair led to a reduction in the amount of Lin-14 protein, allowing the transition from cell divisions of the first larval stage to those of the second (Lee *et al.*, 1993).

A second RNA molecule with similar properties was discovered only six years later, known as let-7, another gene in the C. elegans heterochronic pathway (Reinhart *et al.*, 2000). This molecule of RNA is able to bind the 3'UTR of several genes involved in C. elegans larval development, such as lin-14, lin-28, lin-41, lin-42 e daf-12, and promote the transition from late-larval to adult cell fates (Reinhart *et al.*, 2000). Because of their common roles in controlling the timing of developmental transitions, the lin-4 and let-7 RNAs were entitled small temporal RNAs (stRNAs), supposing that other RNAs with similar functions would be discovered (Pasquinelli *et al.*, 2000). Indeed, less than one year later, three labs cloned small RNAs from flies, worms, and human cells reporting more than one hundred additional genes for tiny noncoding RNAs, approximately 20 new genes in Drosophila, 30 in human, and 60 in worms (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). These RNAs were, similar to lin-4 and let-7, expressed endogenously and approximately 22 nt in length, but, since only few of them had a role in the temporal development, the term "microRNA" was used to refer to the stRNAs and all the other tiny RNAs with similar features but unknown functions (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Several studies in the following years allowed to identify additional miRNA genes in mammals, fish, worms and flies, and a specific database was created to facilitate the naming of newly identified genes (Griffiths-Jones 2004). Most of miRNAs, such as lin-4 e let-7, of C. elegans, come from regions of the genome quite distant from previously annotated genes, suggesting that they had independent transcription units (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Nevertheless, about a quarter of human miRNAs are in intronic regions, suggesting that their expression is not under the control of a specific promoter, but it is dependent by the processing of the related mRNA (Aravin *et al.*, 2003). The miRNAs within a genomic cluster are often related to each other, share functional relationship, and related miRNAs are sometimes, but not always, clustered. Nearly all the cloned miRNAs are conserved in closely related animals, such as human and mouse or C. elegans and C. briggsae (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001).

1.1.2 MiRNA biogenesis pathways

Like for coding mRNAs, miRNA transcription is carried out by RNA Pol II and is controlled by RNA Pol II-associated transcription factors and epigenetic regulators (Lee *et al.*,

2004), whereas only some endogenous miRNA-like small RNAs (derived from tRNAs) are transcribed by RNA Pol III (Babiarz *et al.*, 2008). The canonical mammalian miRNA processing pathway (Figure 1.1) is composed by a series of biochemical steps that convert primary miRNA transcripts (pri-miRNAs) into biologically active, mature miRNAs (Kim *et al.*, 2005).

Pri-miRNA is long (typically over 1kb) and contains a local stem–loop structure, in which mature miRNAs sequences are embedded. A typical pri-miRNA consists of a stem of 33-35 bp, a terminal loop and single-stranded RNA segments at both the 5′ and 3′ sides. The nuclear RNase III-type endonuclease Drosha initiates the maturation process by cropping the stem–loop to release a small hairpin-shaped RNA of ~65 nucleotides in length (pre-miRNA). Together with its essential cofactor DGCR8, Drosha forms a complex called Microprocessor (Han *et al.*, 2006). In particular, Drosha liberates the stem-loop pre-miRNAs from pri-miRNAs in cooperation with DGCR8-mediated recognition of the junctional region between the single-stranded and double-stranded portions of pri-miRNAs. The Drosha complex also contains several auxiliary factors such as EWSR1, FUS, numerous heterogeneous nuclear ribonucleoproteins (hnRNPs), p68 and p72 DEAD-box helicases (Gregory *et al.*, 2004).

A recent report showed that additional sequence elements are involved in pri-miRNA processing (Auyeung *et al.*, 2013). These elements reside in the basal region (the UG motif and the CNNC motif) and terminal loop (the UGUG motif) of human pri-miRNAs. At least one of these three motifs is present in 79% of human miRNAs, suggesting the relevance of these elements. Indeed, it has recently been reported that the CNNC motif is also required for DEAD-box RNA helicase p72 binding, which increases processing by Drosha (Mori *et al.*, 2014).

Drosha-mediated processing of intronic miRNAs does not affect splicing of the host pre-mRNA. Accordingly, cleavage of pri-miRNAs is thought to occur co-transcriptionally before splicing catalysis (Kim *et al.*, 2007). Otherwise, when a miRNA hairpin is located in the exonic region, Drosha-mediated cleavage leads to destabilization of the host mRNA (Han *et al.*, 2009). To assure the correct expression level, activity and specificity of Drosha, this important step is under the control of an auto-feedback mechanism; indeed, DGCR8 stabilizes Drosha through protein–protein interactions, whereas Drosha destabilizes DGCR8 mRNA by cleaving it at a hairpin in the second exon (Han *et al.*, 2009). This cross-regulatory loop enables the homeostatic

maintenance of Microprocessor activity and is deeply conserved throughout the animal kingdom.

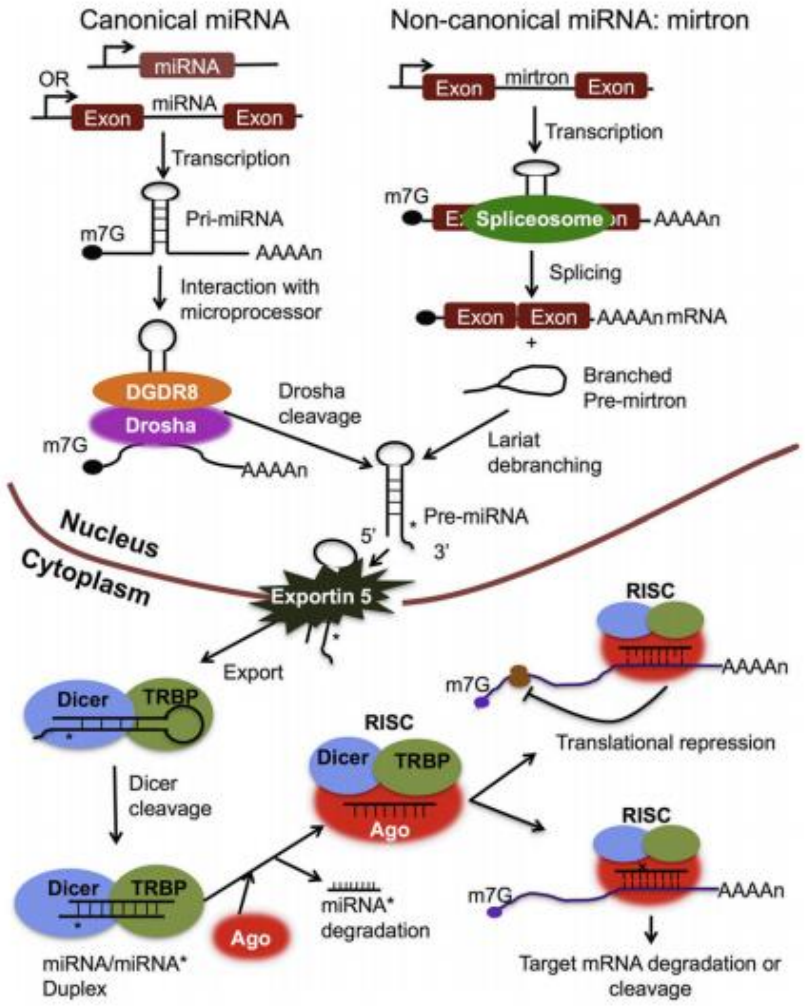

Figure 1.1 miRNA biogenesis by canonical and non-canonical mirtron pathways and mechanism of action (modified by Dai *et al.*, 2011).

Following Drosha processing, pre-miRNA is exported into the cytoplasm to complete the maturation process. The translocation is mediated by the protein exportin 5 (EXP5) that forms a transport complex with GTP-binding nuclear protein RAN-GTP and a pre-miRNA. After translocation through the nuclear pore complex, GTP is hydrolyzed and the pre-miRNA is released into the cytosol (Lund *et al.*, 2004), where the second-round ribonuclease reaction takes place. This step is mediated by another important RNase III-type endonuclease, Dicer, that, in association to other double stranded RNA binding proteins (dsRBP), dices the pre-

miRNAs into a 22 nt long miRNA duplex. Dicer binds to pre-miRNA with a preference for a two-nucleotide-long 3′ overhang that was initially generated by Drosha (Zhang *et al.*, 2004). Dicer cleavage sites are located at a fixed distance from the 3′ end of the terminus of double-strand RNAs (dsRNAs, "the 3′-counting rule"). This distance is typically 21–25 nucleotides in length and depends on the species and the type of Dicer (Macrae *et al.*, 2006). In mammals and flies there is an additional mechanism to determine the cleavage site of pre-miRNA ("the 5′-counting rule"). In this case, Dicer binds to the 5′ phosphorylated end of the pre-miRNA and cleaves it 22 nucleotides away from the 5′ end (Park *et al.*, 2011). Human Dicer1 mRNA contains binding sites for let-7, which results in a negative feedback loop between Dicer and its product let-7. This regulation is thought to contribute to the homeostatic regulation of Dicer activity (Tokumaru *et al.*, 2008).

Small RNA duplex generated by Dicer are subsequently loaded onto an AGO protein to form an effector complex called RNA-induced silencing complex (RISC) (Hammond *et al.*, 2001). Among the four AGO proteins in humans (AGO1–4), only AGO2 can slice perfectly matched target mRNAs. All human AGO proteins can induce translational repression and decay of target mRNAs (Huntzinger *et al.*, 2011). Following miRNA duplex loading, the pre-RISC (in which AGO proteins associate with RNA duplexes) quickly removes the passenger strand to generate the mature RISC. The guide strand is determined during the AGO loading step, mainly based on the relative thermodynamic stability of the two ends of the small RNA duplex (Khvorova *et al.*, 2003) whereas the released passenger strand is quickly degraded. Since the strand selection is not completely stringent, the strand that theoretically is not favored can also be selected, as guide strand, with varying frequency. The less abundant miRNA is also active and can targets different mRNA (Almeida *et al.*, 2012). Alternative strand selection ('arm switching') has been observed in studies comparing miRNA isoforms in multiple tissues (Chiang *et al.*, 2010). These events may be at least partially explained by alternative Drosha processing, which changes the relative thermodynamic stability of the miRNA duplex ends (Wu *et al.*, 2009).

Apart from the canonical miRNA biogenesis pathways, various alternative mechanisms can generate miRNAs. One example is the mirtron pathway, in which pre-miRNAs are generated

by splicing and debranching of short hairpin introns, termed mirtrons, bypassing Drosha cleavage (Ruby *et al.*, 2007; Okamura *et al.*, 2007).

In addition, some small RNAs may originate from other non-coding RNAs, such as tRNAs or tRNA-like precursors, small nucleolar RNAs (snoRNAs), without Drosha processing (Lee *et al.*, 2009). The existence of non-canonical pathways reflects the evolutionary flexibility of miRNA biogenesis. However, the vast majority of functional miRNAs follow the canonical pathway for their biogenesis, and only about 1% of conserved miRNAs are produced independently of Dicer or Drosha in vertebrates.

1.1.3 Nomenclature and target recognition

MiRNA genes constitute one of the most abundant gene families and are widely distributed in all species (Griffiths-Jones, 2008). The latest release of the miRNA database (miRbase 21) has catalogued 434 miRNAs in C. elegans, 466 miRNAs in Drosophila melanogaster and 2,588 miRNAs in humans, although the functional role of many of these miRNAs remains to be determined (Kozomara and Griffiths-Jones, 2014).

In many species there are multiple miRNA loci with very similar sequences that resulted mainly from gene duplication. miRNAs with identical sequences at nucleotides 2–8 of the mature miRNA belong to the same 'miRNA family' (Bartel, 2009) and usually act redundantly on target mRNAs, but distinct roles have also been observed (Ventura *et al.*, 2008). Some miRNAs can also have a common evolutionary origin but some differences in the seed sequence. For example, miR-141 and miR-200 differ by only one nucleotide in their miRNA seeds, but this difference leads to a poor overlap in their target mRNAs, emphasizing the importance of the miRNA seed sequence in miRNA function and evolution (Kim *et al.*, 2013).

The current nomenclature of miRNA genes provides that new miRNAs, identified by cloning or sequencing, receive a sequential numerical name (e.g., the lin-4 homologues in other species are called mir-125). Genes encoding paralog miRNAs, that differ for 1-2 nucleotides, are indicated with lettered suffixes (e.g., mir-125a and mir-125b). In the case that multiple separate loci give rise to the same miRNA, an extra numerical suffix is added at the end of the names of miRNA loci (e.g., mir-125b-1 and mir-125b-2). Each locus produces two mature miRNAs: one from the 5′ strand and one from the 3′ strand of the precursor (e.g., miR-125a-5p and

miR-125a-3p). It is possible that one arm (the guide strand) is more prevalent and active, but it has also been shown that both arms can be biologically active, sharing the same target or not (Huang *et al.*, 2014; Choo *et al.*, 2014). In mammals, most of miRNA-mRNA interactions are characterized by imperfect complementarity (Bartel *et al.*, 2009), whereas perfect matches are very rare (Yekta *et al.*, 2004).

MiRNA targets commonly have at least one region that has Watson-Crick pairing to the 5′ part of miRNA located at positions 2-7 from the 5′ end, known as the 'seed' (Rajewsky *et al.*, 2004). Canonical stringent-seed sites have perfect Watson-Crick pairing and can be divided into four 'seed' types, - 7mer-A1, 7mer-m8, 8mer and 6mer - depending on the combination of the nucleotide of position 1 and pairing at position 8 (see Figure 1.2). 7mer-A1 has an adenine at position 1, while 7mer-m8 has base pairing at position 8. 8mer has both an adenine at position 1 of the target site and base pairing at position 8, whereas 6mer have neither an adenine at position 1 nor base pairing at position 8 and are considered marginal seed sites since usually have reduced efficacy. (Bartel *et al.*, 2009). In addition to this canonical seed matching, moderate-stringent seed matching can be also functional because RISC can tolerate small mismatches or G:U wobble pairing within the seed region (Martin *et al.*, 2014).

Watson-Crick pairing in the 3′ part of miRNA is known to enhance the site recognition efficacy (Grimson *et al.*, 2007). The required number of matches in the 3′ part differs between the site that has stringent seed pairing and the one that has moderate-stringent-seed pairing. Stringent-seeds require 3-4 matches in the positions 13-16, whereas moderate-stringent-seeds require 4-5 matches in the positions 13-19. Sites with this additional 3′ pairing are called 3′-supplementary and 3′-compensatory sites (Bartel *et al.*, 2009).

Several studies have reported that most target sites are in the 3′ UTR segment of target mRNAs, despite miRNA-loaded RISC in theory can bind any segment of the transcript. Target genes tend to have longer 3′ UTR, whereas ubiquitously expressed genes usually have shorter 3′ UTR, potentially to avoid being regulated by miRNAs (Stark *et al.*, 2005). Although functional miRNA sites are preferentially located in 3′ UTR, seed sites in the coding sequence (CDS) and 5′ UTR regions can also mediate miRNA interaction, leading to functional regulation (Lewis *et al.*, 2005; Miranda *et al.*, 2006).

An extremely high number of potential target sites exists for any given miRNA and computational approach to their prediction can facilitate the selection process for the successive experimental validation.

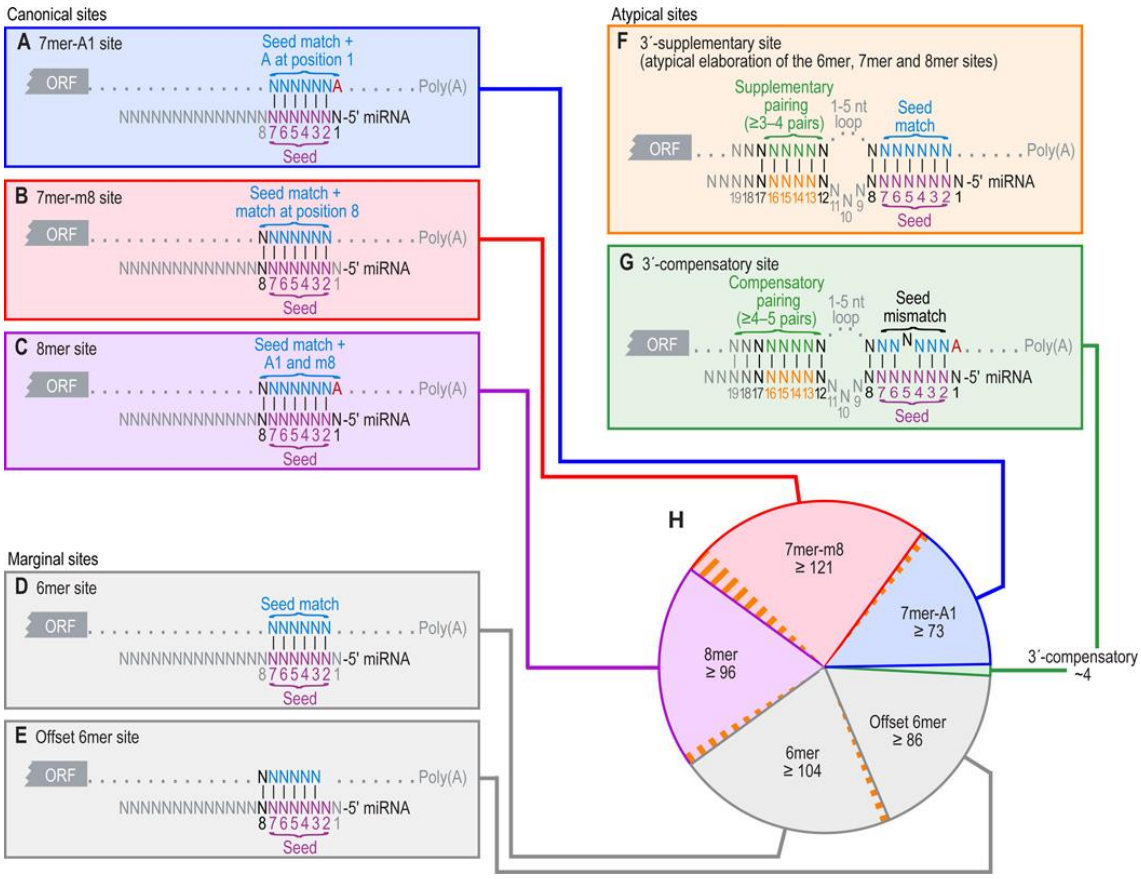

Figure. 1.2 Types of miRNA target sites. A-C canonical sites; D-E marginal sites; F 3′-supplementary site; G 3′-compensatory site. Modified by Bartel *et al.*, 2009.

The available tools for miRNA target prediction encompass a range of different computational approaches, but commonly they take into account key aspects of the miRNA-mRNA interaction, such as site accessibility, seed match, conservation and free energy (Peterson *et al.*, 2014).

1.1.4 Mechanisms of miRNA-mediated gene regulation

The biological outcome of miRNA-mRNA interaction can be altered by several factors that can influence the binding strength and repressive effect of a potential target site (see Figure 1.3) (Carroll *et al.*, 2014). The most crucial factor is a perfect base pairing between the miRNA seed region and target site. Other factors include the number of target sites for the

same miRNA and their relative position, site accessibility, sequences flanking miRNA target site, and their context, and RNA secondary structure can influence the consequences of hybridization (Ohler *et al.*, 2004; Majoros *et al.*, 2007; Brodersen *et al.*, 2009).

Several studies reported that mRNA degradation can be induced either by decapping or deadenylation. Missing poly(A) tail and cap structure can be subjected to the 3′-5′ degradation by cytoplasmic exonucleases (Hausser *et al.*, 2009; Rehwinkel *et al.*, 2005). Alternatively, polysomal ribonuclease 1 (PMR1) can also mediate sequence specific mRNA endonucleolytic cleavage in parallel (Bagga *et al.*, 2005).

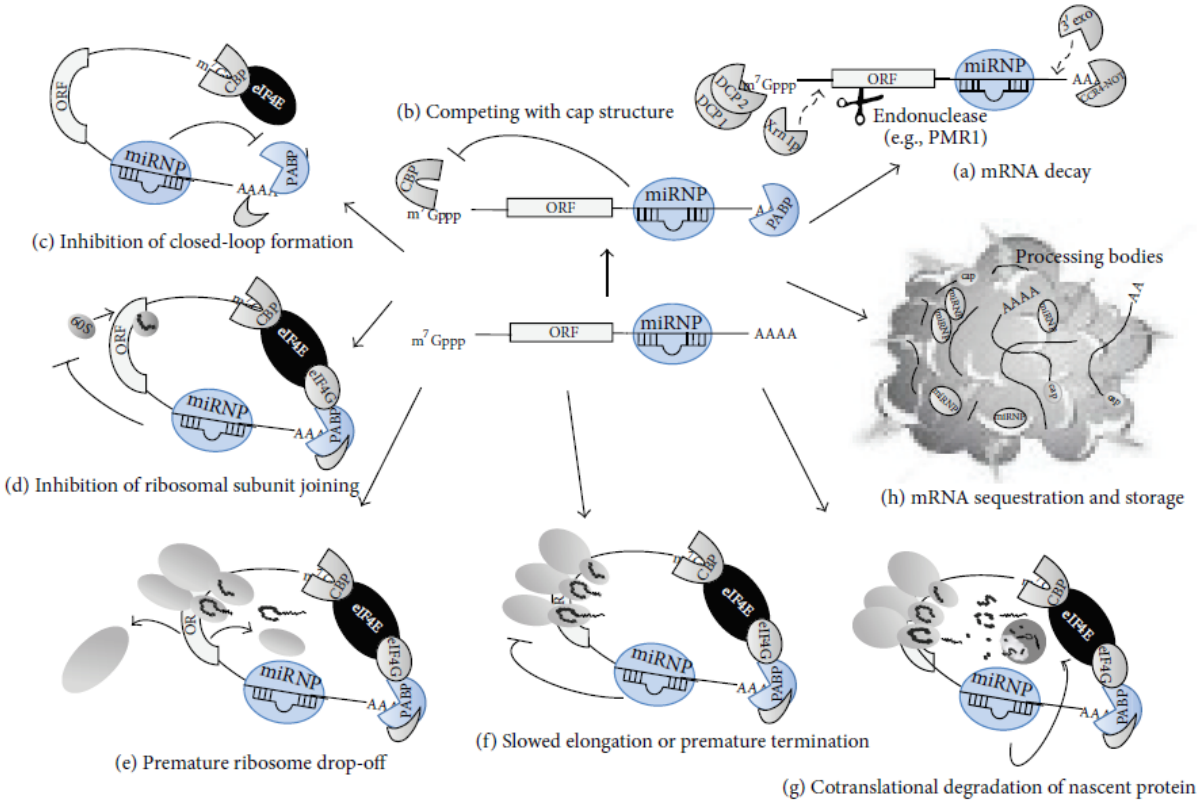

Figure. 1.3 Principal mechanisms of miRNA-mediated downregulation (modified by Orang *et al.*, 2014).

However, several publications have shown that in several cases miRNAs do not promote degradation of their target mRNAs, but inhibit the conversion into protein by preventing the initiation or elongation step of translation (Mathonnet *et al.*, 2007; Pillai *et al.*, 2004). It has been shown that AGO protein can interact with several translation initiation factors, such as eIF4E, eIF4G or eIF4A, and compete for binding to cap structure (Eulalio *et al.*, 2008).

Another possible mechanism of translation initiation block is the Ago interference with the formation of closed-loop mRNAs, normally achieved through interaction between cytoplasmic poly(A) binding protein and cytoplasmic cap binding protein (Eulalio *et al.*, 2008). Several inhibitory mechanisms can also influence the postinitiation or cotranslation steps, leading to ribosome dissociation and/or premature termination (Mathonnet *et al.*, 2007; Chendrimada *et al.*, 2007) and cotranslational protein degradation by proteases recruitment (Pillai *et al.*, 2007). It has been indicated that processing cytoplasmic foci, mostly known as "P bodies," have a central role in mRNA degradation and translation inhibition and it has been proposed that miRNAs can repress translation and promote decay by recruiting P body components to specific target mRNAs (Sen and Blau, 2005).

1.1.5 Role of miRNAs in the diagnosis and pathogenesis of human diseases

As discovery of human miRNAs increased, the research focus was gradually shifted to the functional characterization of miRNAs, particularly in the context of human diseases. The connection between miRNAs and human disorders was obvious since, in many cases, they define the physiological nature of the cell, influencing several vital biological processes, such as cell division and death (Ng *et al.*, 2012), cellular metabolism (Rayner *et al.*, 2011), intracellular signaling (Zhang *et al.*, 2012), immunity (Taganov *et al.*, 2006) and cell movement (Png *et al.*, 2012). Therefore, aberrant miRNA expression should have a strong impact on these critical processes, thus leading to various pathological and malignant outcomes. In this context miRNAs are emerging either as useful tools for the diagnosis and as a new promising category of druggable targets (see Figure 1.4).

Since the early stages of miRNA research, cancer has been the most prominent human disease with a clear association to miRNA dysregulation. The first evidence came from a study by Calin *et al.* in which they demonstrated a frequent deletion of miRNA genes miR-15a and miR-16-1 in B-cell chronic lymphocytic leukemia (B-CLL) patients (Calin *et al.*, 2002). Since that discovery, many studies were directed into characterizing the expression profile of miRNAs in different tumors. To date, high-throughput miRNA expression profile analysis has been conducted in various human cancers, including glioblastoma, breast cancer, thyroid papillary carcinoma, lung cancer, hepatocellular carcinoma, pancreatic cancer, and colon cancer (Calin *et*

al., 2006; Esquela-Kerscher *et al*., 2006; Spizzo *et al*., 2009). This studies also showed that miRNA profiles of tumor cells are significantly different from normal cells (Calin *et al*., 2005) and that tumors of similar origins have common alterations, providing a tool for cancer diagnosis and prognosis (Yanaihara *et al*., 2006).

In cancer research, miRNAs were divided in two different categories: miRNAs that act as oncogenes and miRNAs that act as tumor suppressor genes. It is worth to note that the same miRNA acting like an oncogene in one type of cells can act like a suppressor in another, due to different targets and mechanisms of action. For example, miR-125b has been shown a tumor-suppressor function in hepatocellular carcinoma targeting the oncogene LIN282B (Liang *et al*., 2010), but an oncogene function in prostate cancer suppressing the pro-apoptotic gene Bak1 (Shi *et al*., 2007).

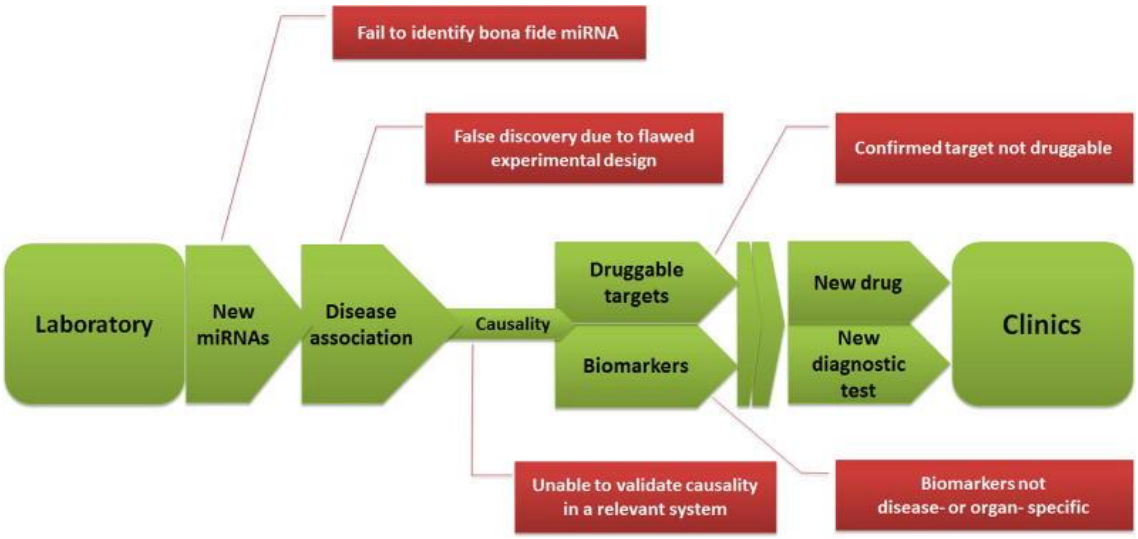

Figure 1.4 The road from laboratory to clinic: the promises and challenges of miRNA research (modified by Li and Kowdley, 2012)

As a consequence, miRNA therapeutics is aimed to downregulate the function of oncogenic miRNAs or to restore the expression of miRNAs that have a tumor-suppressive function. There are two main strategies to target miRNA expression in cancer, which include direct strategies, involving the use of oligonucleotides or virus-based constructs to block the expression of an oncogenic miRNA or to substitute for the loss of expression of a tumor suppressor miRNA and indirect strategies involving the use of drugs to modulate miRNA expression (Giza *et al*., 2014).

Alteration of miRNA homeostasis has been also correlated to neurodegenerative diseases (NDs), a group of late onset progressive disorders of the nervous system, characterized by a complex pathogenesis (Bicchi *et al.*, 2013). Alteration of miRNA levels were associated to numerous pathological processes that can contribute to neurodegeneration in Alzheimer's (AD) and Parkinson's (PD) disease (See Table 1.1), such as response to oxidative stress, cell cycle disorders, neuroinflammation, clearance of pathological proteins and cholesterol trafficking.

For example, several studies reported that miRNAs can modulate the expression of APP (Amyloid Precursor Protein), a key protein involved in AD etiopathogenesis responsible for β-amyloid (Aβ42) accumulation. APP can be proteolyzed directly by α-secretase and then γ-secretase, a process that does not generate Aβ42, or reinternalized into another endosomal compartment containing the β-secretase BACE1 and γ-secretase, leading to the pathological accumulation of Aβ42 (O'Brien *et al.*, 2011). According to Liu *et al.*, production of APP protein is under the control of miR-193b. Furthermore, they measured the concentration of Aβ42 and the expression of exosomal miR-193b in the cerebrospinal fluid, and confirmed a negative correlation between these biomarkers (Liu *et al.*, 2014). Several studies have shown that also the protease BACE1 is regulated by several miRNAs, such as miR-107, miR-195 and miR-339-5p and that their over-expression can reduce the expression of BACE1 mRNA and Aβ42 accumulation (Zhu et al., 2012). Another study from Augustin *et al.*, suggested that also the expression of alfa-secretase ADAM10 is regulated by 2 specific miRNAs, namely miR-107 and miR-103 (Augustin *et al.*, 2012). These findings were confirmed by another study where a RT-qPCR approach was used to show that miR-107 and miR-103 were down-regulated in the peripheral blood of both AD, PD and schizophrenic patients (Leidinger *et al.*, 2013).

Several miRNAs were also linked to PD pathogenesis, a neurodegenerative disease characterized by loss of dopaminergic neurons that is due, at least in part, by the presence of intracellular inclusions, namely Lewy bodies, whose main component is α-synuclein (ASN) (Liu *et al.*, 2012). This process may be promoted by mutations in parkin and α-synuclein genes as well as in leucine-rich repeat kinase 2 (LRRK2) but, it has recently been shown that the metabolism of ASN may also be "fine-tuned" by various miRNAs. For example, it has been

shown that miR-7 cooperates with miR-153 to control the quantity of ASN produced, both in the adult brain and during neuronal development (Doxakis *et al.*, 2010).

miRNA	Source	Change
Alzheimer's disease		
miR-9, -125b, -146a, -155	CSF, BD-ECF	Over-expression
miR-34a, -181b	PBMC	Over-expression
miR-26a, -27b, -30e-5p, -34a, -92, -125, -145, -200c, -381, -422a, -423	Hippocampus, cerebellum, medial frontalgyrus	Over-expression
miR-9, -132, -146b, -212		Down-regulation
let-7f, miR-105, -125a, -135a, -138, -141, -151, -186, -191, -197, -204, -205, -216, -302b, -30a-5p, -30a-3p, -30b, -30c, -30d, -32, -345, -362, -371, -374, -375, -380-3p, -429, -448, -449, -494, -501, -517, -517b, -518b, -518f, -520a*, -526a	CSF	Over-expression
miR-10a, -10b, -125, -126*, -127, 142-5p, -143, -146b, -154, -15b, -181a, -181c, -194, -195, -199a*, -214, -221, -328, -422b, -451, -455, -497, -99a		Down-regulation
miR-29a	Frontal cortex	Down-regulation
miR-9, -125b, -146a, -155	CSF, BD-ECF	Over-expression
miR-26b	SN	Over-expression
let-7d-3p, miR-112, -151a-3p, -161, -5010-3p	Peripheral blood	Over-expression
let-7f, miR-26a, -26b, -103a, -107, -532, -1285		Down-regulation
miR-9	Serum	Over-expression
miR-125b, -181c		Down-regulation
Parkinson's disease		
miR-133b	SNC	Down-regulation
miR-34b, -34c	SNC	Down-regulation
miR-1, -22*, -29a	Peripheral blood	Down-regulation
miR-181c, -331-5p, -193a-p, -196b, -454, -125a-3p, -137	Plasma	Over-expression
miR-21-3p, -224, -373-3p, -26b, -106a, -301b	SN	Over-expression
miR-205	Frontal cortex	Down-regulation
miR-19b, -29a -29c	Serum	Down-regulation

Abbreviations: BD-ECF: Brain Derived Extracellular Fluid; CSF: Cerebrospinal Fluid; PBMC: Peripheral Bl
Substantia Nigra Compacta; P: Patients; C: Controls

Table 1.1 List of miRNAs associated to Alzheimer's and Parkinson's diseases reporting the source and the type of alteration (Prendecki M and Dorszewska J. Austin Alzheimers J Parkinsons Dis. 2014).

Moreover, several studies identified miRNA specifically reduced in post mortem brains of PD patients, such as miR-34b and miR-34c, showing a negative correlation with the expression of Park-7 and Parkin, two proteins associated to familial and sporadic forms of PD (Miñones-Moyano *et al.*, 2011).

As very stable, independent, small genetic entities that are enriched in brain cell cytoplasm, cerebrospinal fluid and in peripheral blood, miRNAs may serve as useful biomarkers for the early diagnosis of human NDs. Leidinger *et al*. investigated the blood samples of 44 AD patients, finding 140 unique mature miRNAs whose expression levels were significantly altered and subsequently, they developed an assay of 12 blood-based biomarkers able to discriminate AD cases from controls with an accuracy of 93%, a specificity of 95% and a sensitivity of 92%. They also showed that miR-107 decreases most significantly in AD and, on the other hand, it was over-expressed in Mild Cognitive Impairment (MCI) (Leidinger *et al*., 2013). Another independent report showed that miR-107 levels were significantly down-regulated in the brains of AD patients at a very early stage of the disease suggesting that testing for miR-107 might provide useful information about a patient's condition in the pre-symptomatic stage of dementia (Wang *et al*., 2008). Sheinerman *et al*. recently identified two miRNA family (miR-132 and miR-134) for early detection of MCI from plasma sample by using RT-qPCR. The miR-132 family biomarkers demonstrated 84%-94% sensitivity and 96%-98% specificity, and the miR-134 family biomarkers showed 74%-88% sensitivity and 80-92% specificity (Sheinerman *et al*., 2013).

Recently, several studies showed that miRNAs play an essential role in the regulation of both innate and adaptive immune responses (Figure 1.5). Thus, it is not surprising that several miRNAs are tightly related to autoimmune inflammatory diseases, a category of disorder that includes many systemic and organ-specific autoimmune diseases (AIDs), such as systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, type 1 diabetes mellitus and Crohn's disease. The cause of these diseases is a negative reaction of the immune system to self-tissues that leads to the formation of autoreactive cells and autoantibodies and to the production of a wide range of pro-inflammatory cytokines and mediators (Baulina *et al*., 2016). It has been shown that specific miRNAs expressed by cells of the immune system and resident cells of tissues can repress the synthesis of these important mediators, thereby contributing to the development of the autoimmune inflammatory response. The sequential activation of specific miRNAs can control the strength level and duration of the inflammatory response induced by activation of toll-like receptors (TLR). For example, induction of miR-155 and repression of miR-

125b and let-7i caused by activation of TLR receptors were shown to promote the production of various pro-inflammatory cytokines and activation of an adaptive immune response.

Whereas miR-21 and miR-147, that are involved in the activation of the inflammatory response, inhibit the synthesis of miR-155 and proinflammatory cytokines (O'Neill *et al.*, 2011). It has also been demonstrated that miRNAs are actively involved in the regulation of the development and differentiation of T and B cells. Indeed, lack of miRNA biogenesis (by Dicer or Drosha mutations) leads to a reduced amount of thymocytes, disruption of B and CD4+ cells differentiation and early development of autoimmune diseases (Koralov *et al.*, 2008; O'Carroll *et al.*, 2007).

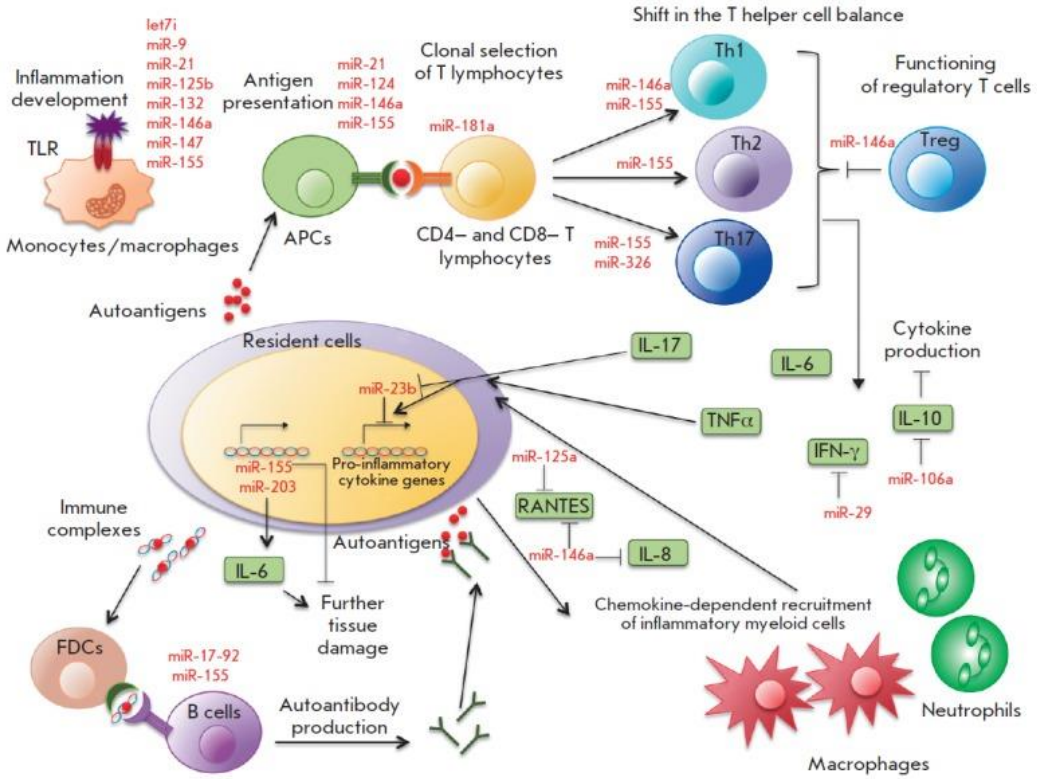

Figure 1.5 Role of miRNAs in autoimmune inflammation (Paulina *et al.*, 2016).

As previously mentioned, the production of several key cytokines is directly regulated by miRNAs. For example, miR-29 in T lymphocytes can bind to IFN-γ mRNA and inhibit its production (Baumjohann *et al.*, 2013), while miR-23b, expressed in resident fibroblast-like synoviocytes, can inhibit NF-κB activation in response to inflammatory cytokines (Zhu *et al.*, 2012). MiRNAs can also be involved in the recruitment of additional inflammatory cells with

participation of chemokines. It was demonstrated that down-regulation of miR-125a causes increase of chemokine RANTES (CCL5) production in activated T cells upon development of systemic lupus erythematosus (Zhao *et al.*, 2010b), and that increased miRNA-146a expression inhibits secretion of chemokines CCL5 and IL-8 in epithelial cells of the human lung (Perry *et al.*, 2008). Moreover, a comparative study of miRNA expression in inflammatory lesions in patients with rheumatoid arthritis, systemic lupus erythematosus, and MS revealed common alterations: expression of miR-23b and miR-30a-5p was enhanced, and expression of miR-214 and miR-146a was reduced (Zhu *et al.*, 2012).

1.1.6 Role of the miR-125 family in neural development and disease

Among the most important miRNA families, miR-125 family (composed by three homologs hsa-miR-125a, hsa-miR-125b-1 and hsa-miR-125-2) has been reported to be implicated in a variety of carcinomas as either repressors or promoters (Sun *et al.*, 2013).

Despite this consolidated role in carcinogenesis, in the last years several studies have also explored the role of miR-125 family members in the context of central nervous system development. MiR-125a and miR-125b were first reported as brain-enriched miRNAs by Sempere and colleagues, which performed an expression profiling of mammalian miRNAs in murine and human brain (Sempere *et al.*, 2004), suggesting a possible role for miR-125 family in neuronal differentiation. Indeed, some years later, it has been shown that over-expression of miR-125b promotes neurite outgrowth and that the physiological up-regulation of miR-125b during neurogenesis is required to repress a subset of genes that normally inhibit multiple pathways involved in neuronal differentiation (Le *et al.*, 2009). Other independent studies reported that miR-125a and miR-125b are able to induce neural commitment and neuronal differentiation by targeting SMAD4 and Nestin (Cui *et al.*, 2012; Boissart *et al.*, 2012).

MiR-125a and miR-125b expression was also linked to astrocyte proliferation and differentiation. In particular, it has been shown that miR-125a and miR-125b expression increase during astrocyte differentiation and that their ectopic over-expression inhibits the proliferation of the relative neural precursors by targeting Musashi1 mRNA (Gioia *et al.*, 2014). Moreover, it has been shown that miR-125a and miR-125b (together with let-7) can

downregulate a common network of targets and cooperate with signaling pathways (such as JAK/STAT) to prime progenitor cells for astroglial differentiation (Shenoy *et al.*, 2015).

MiR-125 family members were also studied in pathological conditions, such as West Nile virus (WNV) neuropathogenesis and glioblastoma multiforme (GBM). In particular, it has been demonstrated that miR-125a-3p may play an important role in regulating anti-viral cytokines in the brain following WNV infection and in the regulation of host immune responses by targeting PTGS2, IL1R1, IL10 and CCL4 (Kumar *et al.*, 2014). miR-125a (and also miR-125b) has been previously demonstrated to constitutively activate NF-κB pathway (Kim *et al.*, 2012), thus, the down-regulation of miR-125a-3p observed after WNV infection could control WNV-induced inflammation by regulating the activation of NF-κB, and represent a sort of protective host response (Kumar t al., 2014). Interestingly, it has been shown that the axis miR-125b/A20/ NF-κB can also have a deleterious effect and be responsible for sustained NF-κB activity in amyotrophic lateral sclerosis (ALS) microglia and toxicity towards motoneurons (MNs).

Several independent reports confirmed the implication of miR-125a also in GBM pathogenesis. The results of these studies showed that the expression of miR-125a-3p is significantly decreased in most malignant glioma and that ectopic over-expression of miR-125a markedly induces the apoptosis and suppresses the proliferation and migration of GBM cell lines by directly targeting Podoplanin (PDPN), Neuregulin1 (NRG1) and TAZ (Cortez *et al.*, 2010; Yuan *et al.*, 2015; Yin *et al.*, 2015).

1.2 Oligodendrogliogenesis and myelination

In the central nervous system, the impulse transmission and the neural network integrity are guaranteed from the activity of specialized glial cells, known as oligodendrocytes. The main function of these cells is to produce a fatty substance called myelin (mainly composed by glycosphingolipids and cholesterol) and to surround axons with myelin layers, ensuring their insulation and the saltatory conduction of nerve impulses. Oligodendrocytes are also implicated in the development and survival of neurons and they are also able to form synapses and actively participate to the CNS activity (Simons *et al.*, 2015).

Oligodendrocytes derived from pluripotent neuroepithelial cells of the neural tube, embryonic precursors that can migrate and proliferate. After reaching their destination, they leave the cell cycle and acquire a post-mitotic mature phenotype and start to produce myelin proteins and lipids (Hu *et al.*, 2009). Oligodendrocyte maturation is under the control of different mechanisms, which include extrinsic factors, such as extracellular molecules and other neural cells, and intrinsic factors, such as transcription factors and epigenetic modulators.

1.2.1 Oligodendrocyte maturation and stage-specific markers

During oligodendrocyte development, differentiation stages can be identified based on the expression of specific markers, the increase in morphologic complexity and the migratory capacity (Barateiro *et al.*, 2014). Oligodendrocyte maturation can be divided into four different stages: oligodendrocyte precursor cells (OPC), pre-oligodendrocytes, immature (or pre-myelinating) oligodendrocytes and mature (or myelinating) oligodendrocytes (Figure 1.6) (Fumagalli *et al.*, 2015).

OPCs are proliferative cells with a high migratory capacity that specifically express platelet-derived growth factor receptor α (PDGFRα), proteoglycan NG2, ganglioside A2B5 and ganglioside GD3. NG2 positive precursors can have different morphologies depending on their location in the brain, but usually they are characterized by a small polygonal soma and a multipolar tree of fine processes. Nevertheless, some studies showed that in white matter they can present a bipolar morphology, characteristic of neural precursor cells, with only a few short processes send out from the opposing poles of the cell body (Chittajallu *et al.*, 2004).

During the differentiation process, OPCs give rise to preoligodendrocytes that extend multipolar short processes, loose the mitogenic response to PDGF and have less migratory capacity (Gao *et al.*, 1998; Pringle *et al.*, 1993). Pre-oligodendrocytes start to express the sulfatide O4 and the receptor GPR17, which persist until the immature oligodendroglial stage. GPR17 has been proposed as a crucial "checkpoint" in the maturation process, indeed, it has been shown that either its silencing in early OPC or its over-expression in pre-oligodendrocytes affects OPC ability to generate mature oligodendrocytes (Fumagalli *et al.*, 2015).

Upon loss of A2B5 and NG2 markers, pre-oligodendrocytes begin to express microtubule-associated protein 1B (MAP1B), galactocerebroside C (GalC), CNPase enzyme and become

immature oligodendrocytes, post-mitotic cells with long ramified branches, that are committed to the oligodendroglial lineage (Pfeiffer *et al.*, 1993; Crociara *et al.*, 2013).

In the final stages of oligodendroglial development, mature oligodendrocytes synthetized myelin proteins, such as myelin basic protein (MBP), proteolipid protein 1 (PLP1) and myelin-associated glycoprotein (MAG). These cells, in response to pro-myelinating stimulus released by neurons or astrocytes, start to enwrap axons with multiple layers of compact myelin, and express high levels of the myelin oligodendrocyte glycoprotein (MOG) (Baumann *et al.*, 2001).

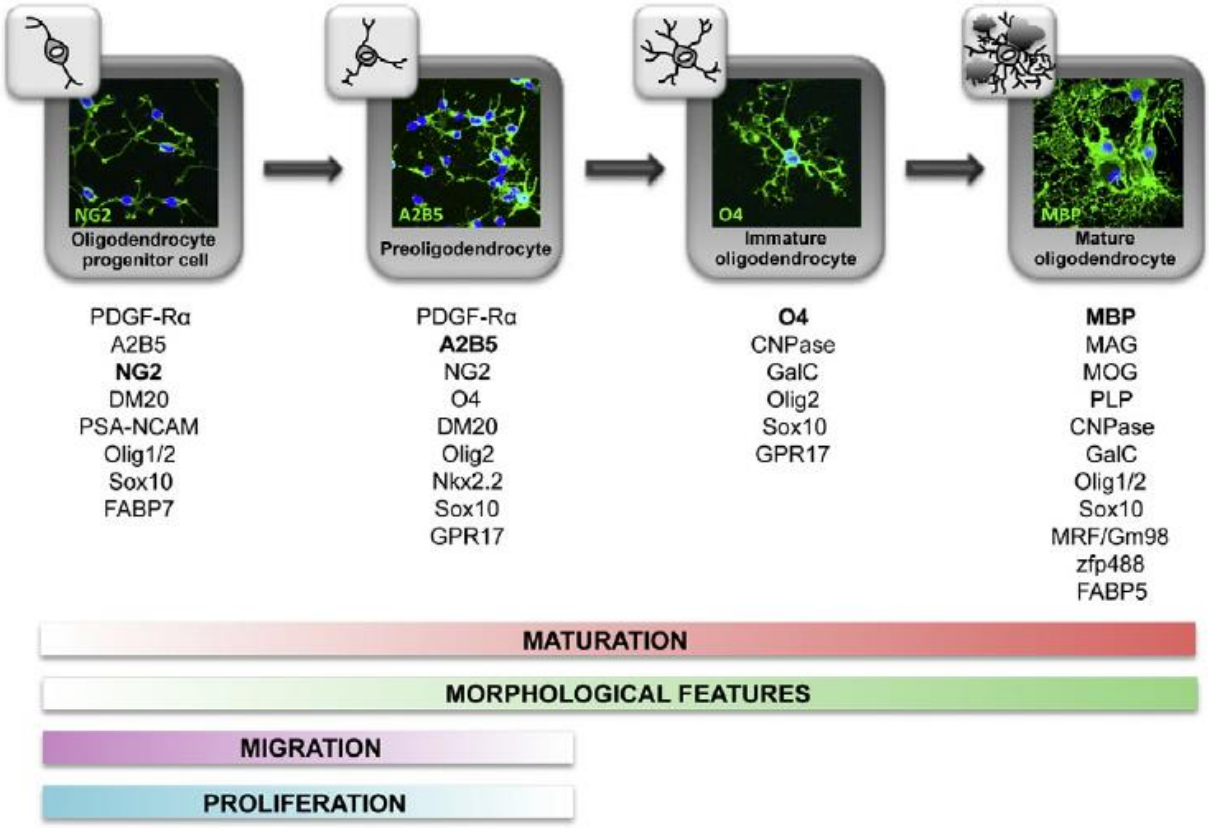

Figure 1.6 Oligodendrocyte differentiation stages and their markers (Barateiro *et al* 2014).

1.2.2 Extrinsic regulation of oligodendrocyte development and myelination

It is widely known that many different factors cooperate in the regulation of oligodendrocyte development and maturation, including growth factors, neuregulins, hormones and neurotransmitters (Figure 1.7).

The most important mitogen for OPCs is PDGF-A (platelet-derived growth factor A), signaling through the receptor PDGFRa expressed by OPC until they start the maturation program

(Pringle *et al.*, 1992). It has been also shown that PDGF alone can induce the commitment of neural multipotent stem cells to the oligodendroglial lineage, through the expression of the transcription factor Olig2 (Hu *et al.*, 2008). PDGF can also be released by neurons and astrocytes and mediate pro-survival signals to oligodendrocytes through association with integrin αvβ3 and the subsequent activation of PI3K/PKC signaling pathways (Baron *et al.*, 2002). Interestingly, axonal signals can also stimulate OPC proliferation and survival via interactions with integrin receptors. In this respect, neuregulin-1 (NRG1) a member of the NRG superfamily of epidermal growth factor-like ligands that are either axonally-bound or secreted, has been shown to bind the oligodendrocyte ErbB tyrosine kinase receptors and promote OPC survival though the PI3K/ Akt pathway (Mitew *et al.*, 2013).

Other important OPC mitogens are FGF2 and IGF1. FGF2 promotes PDGF expression and extends the time in which OPCs and pre-oligodendrocytes are responsive to PDGF. Due to its ability to inhibit OPC differentiation, FGF2, together with PDGF can revert cells from the pre-oligodendrocyte stage to OPC (Baumann *et al.*, 2001). In addition to its potential involvement in OPC specification, IGF1 also helps to promote OPC proliferation and survival by activating the PI3K/Akt pathway through IGF1R (Romanelli *et al.*, 2009). It has been shown that IGF1 over-expression leads to hyper-myelination, whereas, its deletion decreases the number of mature oligodendrocytes and myelin production, suggesting that it may play a role also in oligodendrocyte maturation (Beck *et al.*, 1995; Ye *et al.*, 2002). Moreover, FGF2 and IGF1 can work synergistically, by activating the ERK1/2 pathway, to promote OPC proliferation (Frederick *et al.*, 2007).

Several studies showed that, in addition to mitogen withdrawal, OPCs can also be stimulated to differentiate by triiodothyronine hormone 3 (T3) as part of an intrinsic cell division timer (Durand and Raff, 2000). Although T3 and mitogen withdrawal may work through different signaling pathways, such as p53 or p27/kip1-mediated signaling, respectively, the two mechanisms likely co-operate *in vivo*, regulating a crucial step in myelin development (Durand *et al.*, 1997, 1998; Tokumoto *et al.*, 2001). T3 promotes the morphological and functional maturation of post-mitotic oligodendrocytes binding its intracellular receptors (TRα1, β1, β2), that work as ligand-dependent transcriptional factors (Sarlième *et al.*, 2004). OPC express α1 and

β2 isoforms, whereas, mature oligodendrocytes express α1 e β1. T3 signaling can be modulated by different factors, such as receptor isoforms, co-receptors, presence of co-activators or co-repressors and ligands availability. T3 responsive elements (TRE) were identified in the promoter regions of MBP and PLP genes, suggesting that T3 directly regulates their expression. (Farsetti *et al.*, 1991; Bogazzi *et al.*, 1994).

In specific conditions, OPC should maintain an immature phenotype, in order that myelin is produced in the right place and moment. Thus, oligodendrocyte differentiation onset is also tightly regulated by several extracellular inhibitory factors. Among them, Notch1 receptor, interacting with its axonal ligands Jagged1 and Delta1, prevents OPC maturation and controls when axons should be myelinated (Bozzali & Wrabetz, 2004). Surprisingly, in the presence of the axonally expressed F3/contactin, Notch-1 signaling has been reported to promote OPC differentiation (Hu *et al.*, 2003).

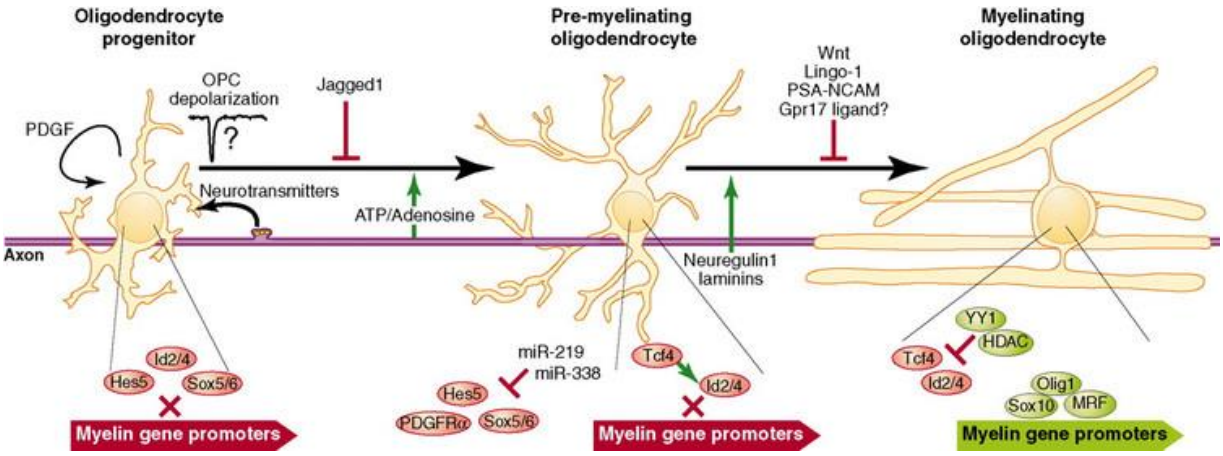

Figure 1.7 Intrinsic (transcription factors and miRNAs) and extrinsic (extracellular ligands and receptors) mechanisms regulating oligodendrocyte maturation (Emery *et al.*, 2010). In red, factors inhibiting, whereas, in green, factors promoting the expression of myelin genes.

Another inhibitory mechanism involves the leucine-rich repeat and immunoglobulin domain-containing-1 (LINGO1), a transmembrane protein expressed on both oligodendrocytes and axons. It has been shown that LINGO1-mediated inhibition of oligodendrocyte maturation works by decreasing the activity of Fyn kinase, and, in turn, increasing RhoA signaling (Mi *et al.*, 2005). Indeed, Fyn kinase is involved in the regulation of process outgrowth and morphological differentiation of oligodendrocytes. Fyn signals through p190RhoGAP that, by inactivation of

RhoA, a member of the Rho family of small GTPases, controls the cytoskeletal structure by mediating actin polymerization. Indeed, it has been demonstrated that the constitutive expression of active RhoA prevents oligodendrocyte process extension (Wolf *et al.*, 2001).

As previously mentioned, once oligodendroglial cells become mature oligodendrocytes, they must receive the right signals to myelinate axons. The extracellular signals regulating the myelination process can be divided in 2 groups: those that modulate oligodendrocyte process extension and axonal contact, and factors that dictate subsequent myelin thickening.

Most axonal signals identified are inhibitory and prevent the initial myelination or that over-myelination occurs. Canonical examples include the axonal PSA-NCAM, which is developmentally downregulated simultaneously to the myelination onset (Fewou *et al.*, 2007) and the already mentioned Notch1 and Lingo1 signaling pathways. However, there are also pro-myelinating axonal signals, for example, axonal lamininα2 promotes the initial process extension binding to β1 integrin receptors on oligodendrocytes (Hu *et al.*, 2009). This results in an activating dephosphorylation of the tyrosine kinase Fyn at the inhibitory Tyr531 site and subsequent initiation of actin polymerization and microtubule assembly (Bauer *et al.*, 2009).

In accordance, genetic ablation of Fyn in mice reduced the number of myelinating axons and produced thinner myelin sheats (Goto *et al.*, 2008). Laminin-α2 and β1 integrin mediated signaling is also required for determining the correct myelin thickness and axon selection. Other modulators of myelin thickness include axonal NRG1 and neuronal BDNF. It has recently been shown that BDNF pro-myelinating effects are likely mediated by ERK1/2 and that BDNF receptor deletion in oligodendrocytes reduces myelin thickness during development (Xiao *et al.*, 2012)

1.2.3 Intrinsic regulation of oligodendrocyte development and myelination

In the CNS, oligodendrocyte development is tightly regulated by intrinsic time-dependent mechanisms, which modulate cell proliferation and maturation via signalling cascades, culminating in the activation of several transcription factors and epigenetic modulators, final effectors that can directly influence gene expression (Figure 1.8).

During embryonic phase, OPC formation is driven by the interaction of the morphogen Sonic hedgehog (Shh) with its receptor Patched in neuroepithelial cells, by inducing the expression of the transcriptional factors Pax6 (Paired box 6), Nkx6.1, Nkx6.2 e GLIF, necessary for the initial

ventrally-derived wave of OPCs (Liu *et al.*, 2003). These factors regulate the expression of the bHLH transcription factor Olig2, the best-defined transcription factor in oligodendrocyte specification (Lu *et al.*, 2000).

Several studies demonstrated the fundamental role of Olig2 in oligodendrocyte specification, for example it has been shown that Olig2 knock-out mice fail to produce OPCs within most regions of the CNS and that ectopic expression of Olig2 in nestin-positive progenitor cells leads to an increase number of OPCs (Maire *et al.*, 2010). Nevertheless, Olig2 is not essential for the formation of OPCs in some CNS regions, likely because its loss can be compensated by the very close bHLH transcription factor Olig1 (Lu *et al.*, 2002). A stage-specific conditional-KO approach suggested that Olig2 plays a role also in OPC differentiation, indeed, its ablation in post-mitotic OPCs decreases the number of mature oligodendrocytes. In contrast, its ablation in mature cells does not affect myelination (Mei *et al.*, 2013), suggesting a role during OPC maturation rather than in control of myelination.

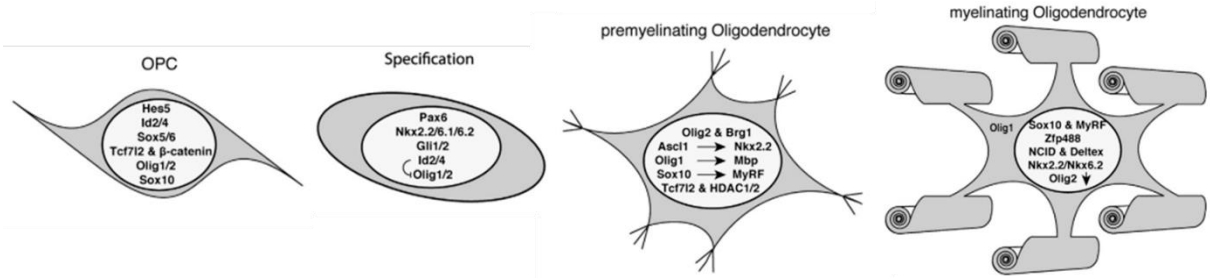

Figure 1.8 Intrinsic regulation of oligodendrocyte differentiation involves the action of stage-specific transcription factors and epigenetic modulators (Mitew *et al.*, 2013).

In the last decades, several transcription factors were identified to be active in maintaining OPCs in their undifferentiated stage (mostly Id2, Id4, Sox6, Hes5), leading to hypothesize a de-repression model of OPC differentiation in which the progressive shutdown of inhibitory signals allows the expression of pro-differentiation factors and the induction of OPC maturation (Emery *et al.*, 2010). For example, Id2 and Id4 (induced by BMP signaling) inhibit OPC differentiation by sequestration of Olig1 and Olig2, thereby preventing their function. Hes5, a bHLH transcription factor, induced by Notch signaling, can recruit HDAC-containing repressive complexes and inhibit the expression of positive regulators of myelin gene expression, such as Sox10 (Liu *et al.*, 2006).

Olig2 expression precedes other important markers of the oligodendroglial lineage, such as Olig1, Nkx2.2, YY1, Sox10, TCF7L2 and MYRF (Wegner *et al.*, 2008), that have a primary role in the induction of OPC differentiation and myelination.

Although Olig1 is expressed from the time of oligodendrocyte specification, some studies suggest that it may be also important for the myelination onset, indeed, it has been shown that Olig1 KO mice fail to produce fully myelinating oligodendrocytes (Xin *et al.*, 2005). Moreover, Olig1, by cooperating with Sox10, can induce the transcription of the MBP gene (Li *et al.*, 2007). When oligodendrocytes become matures, a change in the phosphorylation state of Olig1 causes a change in its localization, moving from the nucleus to the cytoplasm, where it is necessary to promote full membrane extension (Niu *et al.*, 2012). One of the inhibitory factors down-regulated after Olig1 translocation to the cytoplasm is Nkx2.2. Although Nkx2.2 is necessary for post-mitotic OPC formation, it has to be down-regulated concurrent to myelination onset, since it can repress MBP transcription (Wei *et al.*, 2005).

As mentioned before, Sox10 is required for the expression of MBP and the generation of myelinating oligodendrocytes. Recent studies showed that Sox10 can also act as co-factor of other transcription factors essential for OPC differentiation and myelination, such as TCF7L2 (Zhao *et al.*, 2016).

TCF7L2 is transiently expressed in oligodendrocytes and it can have either pro- or anti-differentiation effects, depending its association partners. In OPCs TCF7L2 form a complex with β-catenin (produced in response to Wnt signaling activation) to block OPC differentiation (Fancy *et al.*, 2009). Otherwise, follow OPC differentiation onset, TCF7l2 binds Kaiso and form complexes with HDAC to repress Wnt-β-catenin signaling and promote OPC maturation. Moreover, it has been shown that, in committed oligodendrocytes, TCF7L2 can also bind Sox10 and promote their terminal differentiation (Zhao *et al.*, 2016).

Sox10 can also induce myelination by promoting the expression of the pro-myelinating factor MYRF. Unlike other transcription factors, that are expressed also in OPCs, MYRF is specifically expressed in committed oligodendrocytes (Emery *et al.*, 2009) and is required for the progression of pre-myelinating oligodendrocytes to a mature, myelinating state. Conditional

ablation of MYRF in mature oligodendrocytes results indeed in a complete loss of myelin genes and demyelination, even in adult mice (Koenning *et al.*, 2012).

Another important regulatory mechanism that controls oligodendrocyte development is carried out by HDAC, enzymes that can modify the chromatin acetylation state or physically interact with transcription factors to repress gene expression (Figure 1.9). It has been shown that, unlike other neural lineages, oligodendrocyte differentiation of progenitor cells is initiated by a global histone deacetylation program (Marin-Husstege *et al.*, 2002). During the early phases of oligodendrocyte development, HDACs repress the choice of alternative lineages and contribute to oligodendrocyte identity by blocking critical transcription factors, in particular Sox2 (Lyssiotis *et al.*, 2007; Shen and Casaccia-Bonnefil, 2008). Indeed, it has been demonstrated that the pharmacological inhibition of HDACs during the differentiation of neural stem cells causes a significant reduction in oligodendrocytes in favor of astrocytes and neurons (He *et al.*, 2007; Liu *et al.*, 2007; Siebzehnrubl *et al.*, 2007). Moreover, HDAC inhibition in rodent models delays oligodendrocytes differentiation and myelination (Shen *et al.*, 2005).

The central role of HDACs in oligodendrocyte development was also remarked by an *in vivo* study that showed how the conditional ablation of HDAC1/HDAC2 in Olig1-expressing oligodendrocytes resulted in sever hypo-myelination and post-natal death of mice (Ye *et al.*, 2009).

Another important aspect is the crosstalk between transcription factors, such as REST and YY1, and HDACs, which usually act in combination to repress factors that normally block OPC maturation. REST is a transcription factor involved in OPC specification and in the formation of chromatin remodeling complexes. It has been shown that REST can recruit the co-repressor Sin3a and HDAC1/2 to block the expression of neuronal genes and promote oligodendroglial specification (Dewald *et al.*, 2011). Another example is the crosstalk between HDACs and the multifunctional protein YY1, a transcriptional factor that can act either as activator or repressor. The mechanism involves the recruitment of HDAC1 by YY1 at Id2 and Tcf4 promoter and the inhibition of their expression, allowing OPC differentiation. Accordingly, YY1 KO mice show high expression levels of the inhibitors Id2 and Tcf4, and defective myelination (He *et al.*, 2007).

Sirt2, a cytosolic enzyme highly expressed in oligodendrocytes, that belongs to class III HDACs, can instead explicate its deacetylasic activity on alfa-tubulin, probably modulating morphological differentiation of oligodendrocytes (Tang and Chua, 2008).

Lastly, it has been shown that class IV HDACs, such as HDAC11, play a role in myelination. HDAC11 is indeed highly expressed during oligodendrocyte maturation and its silencing causes a strong decrease in the expression of myelin genes and OPC maturation blockade (Liu *et al.*, 2009).

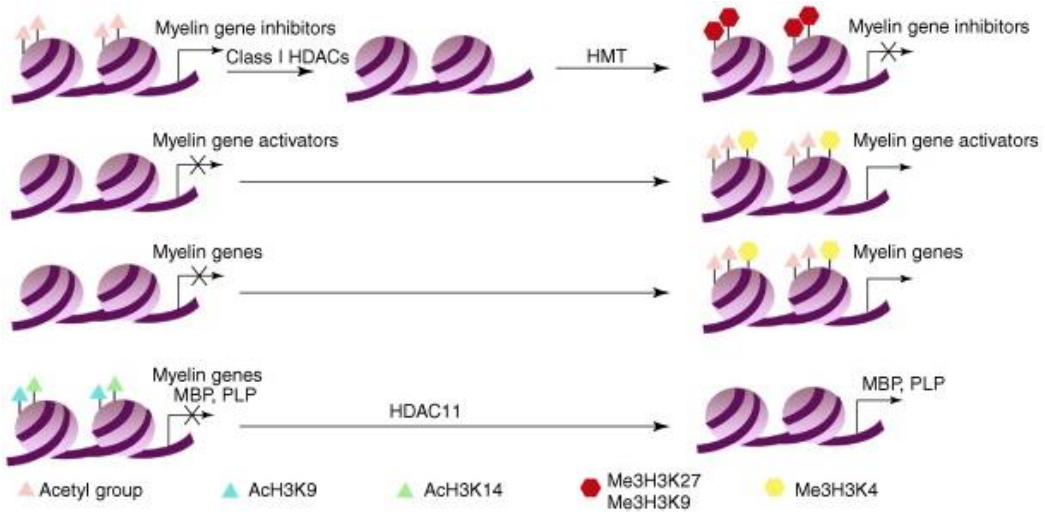

Figure 1.9 Epigenetic mechanisms modulating the transition from OPC (left side) to myelin-forming cells (right side). In OPCs myelin gene inhibitors recruit HDACs on myelin gene promoters, preventing their expression. Then, the progressive decline of inhibitors expression allows the acetylation of activator and myelin gene promoters, inducing their expression (Liu and Casaccia 2010).

1.2.4 Role of miRNAs in the regulation of oligodendrocyte differentiation.

Several recent studies have addressed the important role of miRNAs in the regulation of oligodendrocyte development. First evidences were obtained by Dicer-null experiments, which have shown how mice lacking Dicer1 gene die prenatally (Bernstein *et al.*, 2003).

Gene expression profile studies on oligodendrocytes allowed to identify miRNAs that belong to the miR-17~92 cluster, previously categorized as onco-miRs (Ota *et al.*, 2004; Hayashita *et al.*, 2005). It has been shown that conditional ablation of miR-17~92 cluster in CNP-expressing OPC decreases, whereas, their over-expression increases the number of oligodendrocytes (Budde *et al.*, 2010). Subsequent works revealed that this miRNA cluster control OPC proliferation by targeting PTEN, which inactivation causes an increase in the proliferation rate

through Akt signaling (Harrington *et al.*, 2010). However, as shown by Dicer conditional ablation experiments, miRNAs are required for both cell cycle exit and terminal differentiation of oligodendrocytes. Indeed, conditional ablation of Dicer in OPCs leads to an initial increase in the proliferation, followed by maturation fail, suggesting the existence of miRNA that normally inhibit proliferation and promote terminal maturation (Dugas *et al.*, 2010; Zhao *et al.*, 2010a).

Microarray analysis of mature oligodendrocytes vs. OPCs allowed to identify miRNA that are specifically expressed at high levels by mature cells (Figure 1.10), such as miR-219, miR-338 and miR-138 (Lau *et al.*, 2008; Dugas *et al.*, 2010). Later, it has been shown that miR-219 is a potent activator of oligodendrocytes maturation. Moreover, its exogenous administration rescues myelination defects in Dicer-null mice. The mechanism involves the repression of factors that normally block OPC maturation, such as Pdgfr-α, Zfp238, Hes5, Sox6 and Foxj3. It has been shown that miR-219 is expressed at high levels also in adult mice. Interestingly, conditional ablation of Dicer1 in mature oligodendrocytes leads to hypo-myelination, suggesting that specific miRNAs, such as miR-219, control myelin formation and maintenance. It has been proposed that miR-219 expression in mature cells is necessary to repress its direct target Elovl7 (Shin., 2009), an enzyme involved in the formation of very-long chain fatty acid (VLCFA), essential components of the myelin sheaths. VLCFA over-production was related to different de-myelinating disorders (Dubois-Dalcq *et al.*, 1999), thus, miR-219 may be necessary to repress Elovl7 activity once axonal myelination is completed.

Another miRNA highly expressed during oligodendrocytes maturation is miR-338 (Lau *et al.*, 2008). It has been shown that, similarly to miR-219, miR-338 inhibition increases, whereas its over-expression promotes, the differentiation of OPC into mature oligodendrocytes, directly repressing Hes5 and Sox6 (Zhao *et al.*, 2010a). It is worth to note, that the expression levels of crucial miRNA in oligodendrocytes, such as miR-219 and miR-338, are dependent upon Olig1 for their expression, suggesting the relevance of miRNA/transcription factors crosstalk in oligodendrocyte maturation (Zhao *et al.*, 2010a).

In addition to miR-219 and miR-338, also miR-138 is more expressed in mature oligodendrocytes compared to OPC (Dugas *et al.*, 2010), although it is not as potent as the other in the promotion of OPC maturation, performed by Sox4 inhibition. Moreover, it has been

shown that sustained expression of miR-138 leads to a reduction in MOG protein, suggesting that it can have a transient role in OPC maturation and a Janus role in the terminal phases (Galloway *et al.*, 2016).

miR-23a was initially discovered as a regulator of laminin B1, an inhibitor of myelin genes expression (Lin and Fu, 2009). Indeed, miR-23a over-expression in OPC increases CNPase and MBP levels. Nevertheless, further studies showed that miR-23a over-expression in CNPase-expressing oligodendrocytes results in hind-limb paralysis, loss of extensor tone and kyphosis, due to hyper-myelination (Lin *et al.*, 2013). The mechanism of action underlies these phenotypic changes involves the repression of PTEN, that normally control myelination by limiting Akt and IGF1 signaling (Harrington *et al.*, 2010; De Paula *et al.*, 2014).

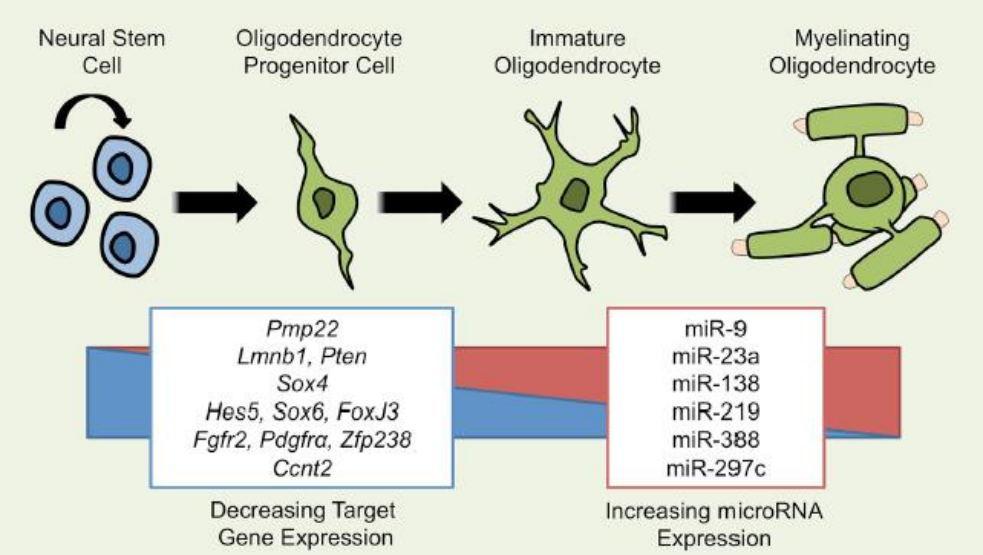

Figure 1.10 MiRNAs implicated in oligodendrocyte maturation and their target mRNAs (Galloway *et al.*, 2016)

MiR-9 play an important role in the regulation of oligodendrocytes maturation by repressing PMP22, a peripheral myelin protein. miR-9 is enriched in OPCs of the CNS, but absent in the peripheral nervous system (PNS), suggesting that it can have a role in the silencing of peripheral genes in the CNS, that are not necessary (Lau *et al.*, 2008; Dugas and Notterpek, 2011).

Another miRNA recently found up-regulated during oligodendrocyte maturation is miR-297c-5p, which represses its direct target Cyclin T2, a regulator of cell cycle progression and a negative regulator of OPC differentiation (Kuypers *et al.*, 2016).

1.3 Multiple sclerosis

Multiple sclerosis (MS) is a multifactorial chronic immune-mediated de-myelinating disorder of the CNS in which immune system attacks myelin, a substance produced by oligodendrocytes that normally surrounds and protects axons, leading to abnormal impulses transmission. The etiology of MS is still unclear, however, it includes a genetic predisposition combined with environmental influences.

It is estimated that 2.5 million people worldwide are affected by MS, with a prevalence range of 100 cases per 100000 people. MS typically affects women more than men. Presentation of MS symptoms usually begin between 20 and 50 years of age, less than 1% can occur in childhood and approximately 2-10% after 50 years of age (Ghasemi *et al.*, 2017). Patients commonly suffer from visual changes, muscle weakness, ataxia leading to falls, paroxysms such as tonic spasms and seizures, cognitive impairments, and fatigue.

MS patients can be divided into 3 subtypes based on the clinical pattern (Figure 1.11). Relapsing remitting MS (RR-MS) is the most common subtype (85% of MS patients) and is characterized by unpredictable acute attacks (relapses) followed by full or partial recovery (remissions), without disease progression between relapses.

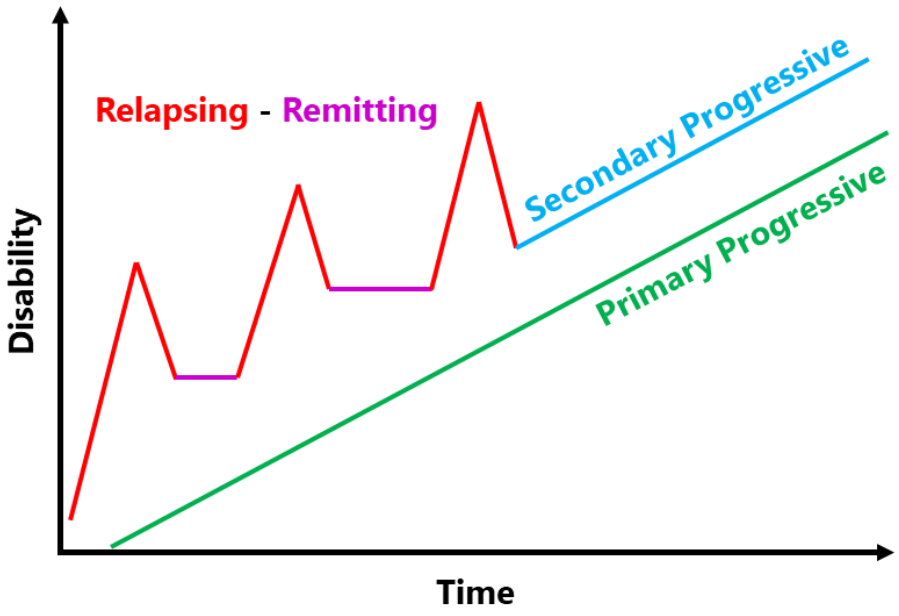

Figure 1.11 Disease progression of different subtypes of MS.

Primary progressive MS (10-15 % of MS patients) is a type of MS (PP-MS) with no acute relapses or remissions, characterized by accumulation of disability from the onset of symptoms. These patients never return to their baseline. Sixty-five percent of RR-MS patients developed into secondary progressive MS (SP-MS) with progressive worsening of neurological disability. In SP-MS, patients can present minor relapses and remissions but they never return to their baseline.

1.3.1 MS etiology and epidemiology

Up to date, the reasons for MS incidence have not been found, however, a combination of external and environmental factors and genetic predisposition were proposed to be involved (Figure 1.12). Since initial exposure to numerous viruses, bacteria and other microbes occurs during childhood, and since viruses are well-recognized as causes of demyelination and inflammation, infectious agents such as Epstein Barr virus (EBV), human herpes virus type 6, and mycoplasma pneumonia were proposed as agents associated to MS onset (Ghasemi *et al.*, 2017).

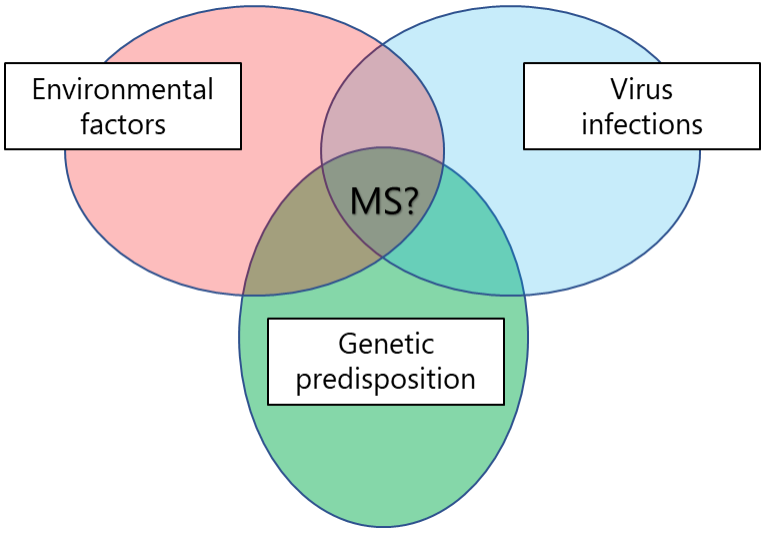

Figure 1.12 Factors potentially responsible for MS development.

In accordance, it is well-documented that in human many viruses were related to demyelination Encephalomyelitis and that viral infection also induced demyelination in animal models (Gilden *et al.*, 2005). Others environmental factors, such as smoking, vitamin deficiency and exposure to UV radiation, can change the risk to develop MS. Smoking leads to the production of nitric oxide (NO) and carbon monoxide (CO), substances that can induce mitochondrial damage and

lipid peroxidation and, in turn, to oligodendrocyte apoptosis, axonal degeneration and demyelination (Mitrovic *et al.*, 1995).

Growing evidence support that also vitamin D and sunlight exposure can play an important role in MS onset. MS prevalence is indeed higher in North America and Europe, due to the decrease of sunlight exposure, that, in turn, determines a lower amount of naturally-produced vitamin D. On the contrary, people who live closer to equator have a lower risk to develop MS (Figure 1.13). Accordingly, MS rate in Europe compared to North Europe moves from 100 to 200 MS cases per 100000 people (Milo *et al.*, 2010).

The risk of developing MS could be also associated to genetic components. Studies have shown that having a first-degree parent with MS does significantly increase the individual risk. It has been estimated that the risk for such those individuals and for dizygotic twins is 2-5% (Willer *et al.*, 2003). The risk of MS in family members of a patient depends on the amount of genetic information they share. Thus, the risk rate in homozygotic twins (that have 100% genetic similarity) is 25%, whereas for second degree relatives is 1-2% (Ebers *et al.*, 1995). Moreover, it has been shown that in the HLA region of chromosome 6 exists a group of genes associated to an increased risk of developing MS (Baranzini *et al.*, 2011).

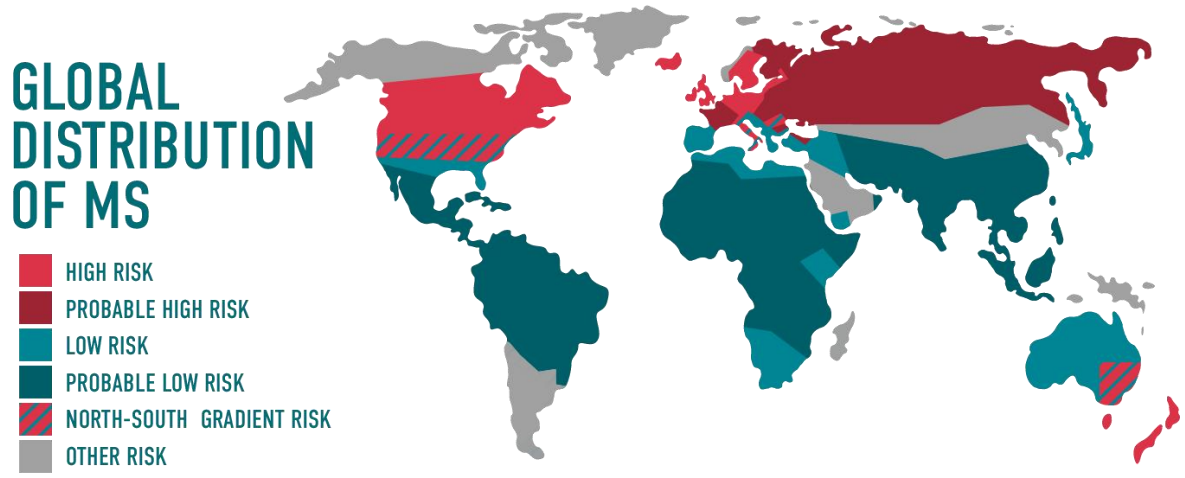

Figure 1.13 Distribution map of MS in the world.

1.3.2 MS pathophysiology

The hallmark of demyelinating disease is the formation of sclerotic plaques, that represent the final stage of a process involving inflammation, demyelination and remyelination,

oligodendrocyte depletion, astrogliosis, neuronal and axon degeneration. However, the order and relation of these separate components remain fully to be resolved (Compston *et al.*, 2008). The lymphocytic presence within plaques and bordering areas suggests that inflammatory destruction in MS is driven by antigen-specific targeting of myelin and other CNS components. In particular, adaptive immune responses by T lymphocytes are thought to mediate injury to myelin and nerves within the CNS during MS. T cells from MS patients can indeed recognize a variety of myelin protein targets, including MBP, PLP, MOG and MOBP, among others.

Pathogen-associated molecules simultaneously bind to toll-like receptors on APCs (dendritic cells, microglia and B cells), that respond by producing specific cytokines, such as IL-12, IL-23 and IL-4, that induce $CD4^+$ T cell differentiation into Th1, Th2, or Th17 phenotypes (Ghasemi *et al.*, 2017). The cytokines produced by Th1 cells are proinflammatory cytokines such as interferon gamma, whereas Th2 cells secrete anti-inflammatory cytokines such as IL-4 and IL-13. Th17 is a newly recognized $CD4^+$ T cell subset that produces IL-17, IL-21, IL-22 and IL-26. Like Th1 cells, Th17 cells promote inflammation in MS (Loma *et al.*, 2011). Interestingly, IL-17 receptors are present in acute and chronic MS plaques. Moreover, studies in IL-17 deficient mice show reduction of clinical severity (Komiyama *et al.*, 2006). *In vitro* mobilization studies suggest that Th17 cells cross the blood brain barrier (BBB) more efficiently than other T cells, and that are capable of eliciting damage to the BBB (Kebir *et al.*, 2007), allowing a greater influx of other inflammatory cells.

Regulatory T cells (T reg) are another $CD4^+$ T cell type involved in the pathogenesis of MS. The role of T-reg cells is to regulate effector Th1, Th2 and Th17 cells. The number of T reg cells is the same between MS patients and controls, however patients with MS have reduced T reg function (Haas *et al.*, 2005).

Besides the involvement of $CD4^+$ T cells in MS pathogenesis, studies have shown that $CD8^+$ T cells are present in MS lesions and may have regulatory function in the progression of disease. $CD8^+$ T cells transect axons, promote vascular permeability and activate oligodendrocyte death (Kasper *et al.*, 2010).

In addition to T cells, also B cells and their released antibodies are involved in the pathogenesis of MS. *In situ* deposition of MOG-specific antibodies has been detected in MS lesions, along

with MOG- and MBP-specific Ig complexed with myelin within macrophages (Genain *et al.*, 1999). Modulation of T cell function may be an equally important function of B cells in the immune dysregulation in MS patients. B cells may promote neuro-inflammation in MS via direct and indirect effects on T cells, such as the secretion of pro-inflammatory cytokines.

Another key element that contributes to the immune-mediated damage during MS is the process by which immune cells are able to gain access to the CNS compartment, generally considered restricted. The BBB normally serves to actively restrict cellular and macromolecular movement between CNS tissue and the blood and appropriate function of the BBB depends from several anatomic and cellular features, such as tight junctions between endothelial cells, specific expression of transporters, and vasculature (Daneman et a., 2009).

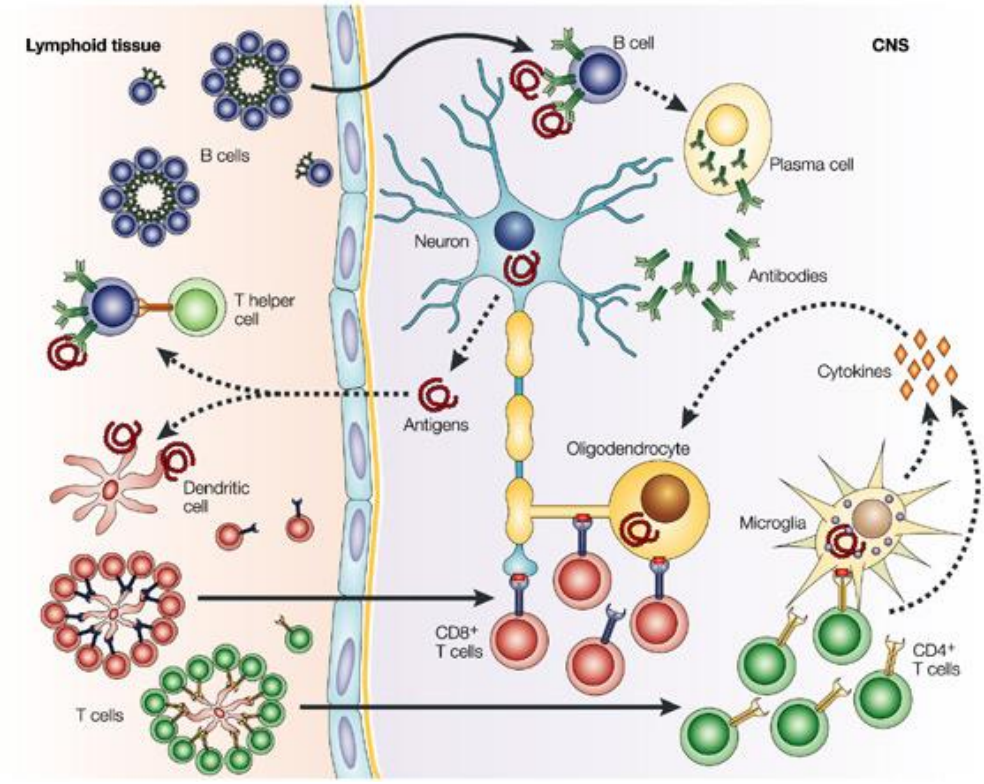

Figure 1.14 Principal mechanisms contributing to MS pathogenesis (Hemmer *et al.*, 2002).

In MS, a sequence of critical events allows to auto-reactive lymphocytes to enter in the CNS compartment. Initially, leukocytes take contact with endothelium of the BBB, a process facilitated by up-regulation of adhesion molecules, such as ICAM1 and VCAM, by the vasculature (Piccio *et al.*, 2002). It has been proposed that changes in the vascular endothelium

could be mediated from the action of circulating pro-inflammatory mediators, including tumor necrosis factor (TNF) and lipopolysaccharide (LPS). Subsequently, migration of cells through and between endothelial cells takes place (Holman *et al.*, 2011). Extravasation of immune cells in perivascular cuffs within the CNS parenchyma can also occur and lead to a breach in the BBB, an essential component in the process of inflammatory destruction of the white matter in MS.

In MS, leukocyte influx to the CNS is also influenced by the presence of specific chemokines. For example, SDF1 is typically localized to the basolateral microvasculature of the CNS and functions to retain leukocytes within the perivascular space. Interestingly, redistribution of SDF1 to the luminal microvasculature was observed in autopsy specimens from MS patients, which would induce dissemination of lymphocytes into the CNS parenchyma (Mc Candless *et al.*, 2008).

As mentioned before, MS is the most common de-myelinating disease. In the early phase of the disease, myelin repair mechanisms are activated in response to the formation of de-myelinating sclerotic plaques, to produce new myelin. This re-myelination process is carried out by OPC, which migrate toward lesions, proliferate and differentiate to oligodendrocytes (Franklin, 2002). Nevertheless, after repeated cycles of damage, the re-myelination efficiency decreases and oligodendrocytes fail to restore myelin and axonal function. As a consequence, axons remain unprotected and degenerate. The reasons for re-myelination failure are not well understood, however, patient age, the hostile environment in lesions (myelin debris and inflammation) and disease progression itself may negatively influence OPC ability to re-myelinate (Keough *et al.*, 2013).

1.3.3 MS Diagnosis and Prognosis

The diagnosis of MS is based on medical history, neurological examination by imaging techniques such as magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) and blood sample analysis, and evoked potentials (Røsjø *et al.*, 2014; Figure 1.15). The first neurological event that lasts at least 24 hours is termed a "clinically isolated syndrome" (CIS), that is named "monofocal", when a single lesion leads to a single neurological symptom or "multifocal", when more than one lesion causes multiple symptoms (Sturm *et al.*, 2014).

The presence of elevated immunoglobulins or oligoclonal bands in the CFS of a patient who presents a CIS is consistent with a diagnosis of MS (Polman *et al.*, 2010).

MRI shows focal or confluent abnormalities in white matter in more than 95% of patients, however, their presence alone does not allow the diagnosis of multiple sclerosis, since lesions can also appear in people without clinical signs of disease and many individuals older than 50 years can present non-specific white matter lesions (Compston *et al.*, 2008).

Given the nature of MS, MRI findings will change over the course of time. For this reason, specific criteria that take in account these changes were defined to simplify the diagnosis, maintaining sensitivity and specificity (Montalban *et al.*, 2010).

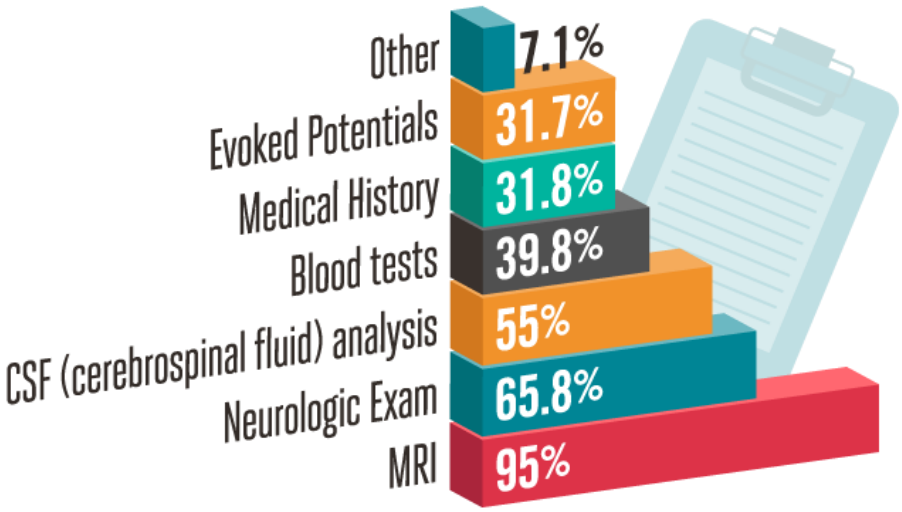

Figure 1.15. Diagnostic tests used to make initial diagnosis

According to McDonald criteria, a patient with CIS should also presents specific MRI changes to support a MS diagnosis and must include the documentation of lesions disseminated in space (DIS) and in time (DIT). DIS requires one or more T2 lesions[1] in two or more MS locations of the CNS, typically periventricular, juxtacortical, infratentorial, and spinal cord. DIT can be established by the presence of a new lesion compared with a previous scan or the simultaneous presence of an asymptomatic enhanced lesion plus a T2 non-enhancing lesion on the same scan (Polman *et al.*, 2010).

[1] hyperintense lesions, or "bright spots", in T2-weighted MRI scans, often referred to as plaques.

When CIS are also accompanied by white matter abnormalities, detected by MRI at clinically unaffected sites, the chance of a second attack of demyelination (fulfilling the diagnostic criteria for RR-MS) increases from 50% at 2 years to 82% at 20 years. As mentioned before, 65% of RR-MS patients develop in SP-MS, whereas in 20% of patients the illness is progressive from the onset. In both situations, the progression typically starts around 40 years and patients often manifest a spinal disease. Children with MS are usually women, they reach the secondary progressive in more time with respect to adults, but do it at a younger age (Renoux *et al.*, 2007). A minority of MS patients present a first attack after 50 years of age and were defined late-onset MS (LOMS) (Polliack *et al.*, 2001). Since LOMS patients often have atypical presentations, misdiagnosis and delayed diagnosis are more common compared to young-onset MS (YOMS) patients (Martinelli *et al.*, 2004).

In RR-MS patients, the risk of a relapse is doubled after viral exposure especially for upper respiratory and gastrointestinal infections (Andersen *et al.*, 1993). Conversely, persistent parasitic infection seems to protect from disease activity, likely by promotion of T-regulatory-cell activity (Correale *et al.*, 2007).

The median time to death is around 30 years from disease onset, representing a reduction in life expectancy of 5–10 years (Bronnum-Hansen *et al.*, 2004).

1.3.4 Biomarkers for MS

The intrinsic heterogeneity of MS causes that the detection and the prediction of disease progression is very difficult, also for the lack of sensitivity in current clinical assessments. Treatment decisions are based more on risk rate and trial and error than on objective assessments that predict who will respond to any given disease-modifying therapy (DMT, for details on DMTs see section 1.3.6). In this respect, the identification of specific biomarkers that reflect the disease activity, would allow an individualized clinical management of MS patients and to predict treatment response (Harris *et al.*, 2016). Conventional biomarkers are molecular markers detectable in body fluids that can sensitively and specifically reflect either a normal or pathological process. In MS, the CSF is the most direct source of biomarkers, whereas blood can indirectly reflect alteration in CNS mechanisms (Kuhle *et al.*, 2016).

Biomarkers could have a high relevance in CIS patients, in order to predict their conversion to clinically defined MS. For example, biomarkers correlated to oligodendrocytes loss or axonal damage would help to choose the right treatment to prevent or delay the disease progression. Since CSF collection is a routine analysis in CIS patients, several biomarker discovery studies were performed (Figure 1.16) and many are still ongoing, however the current challenge is to understand the reliability of these predictive biomarker and their value in the identification of subtype of patients with a poor prognosis. In this respect, Chitinase 3-like-1 (CH3L1) has been proposed as a predictive biomarker for the conversion of CIS in clinically defined MS. It has been shown that elevated CH3L1 in the CSF correlate with higher probability, and a shorter time, to convert from CIS to MS, in addition to a more rapid development of long term disability (Hinsinger *et al.*, 2015; Canto *et al.*, 2015). Other markers used to predict the conversion of CIS in MS are IgG and IgM oligoclonal bands (OCB) in the CSF (Petzold *et al.*, 2013). Moreover, IgM OCBs are also predictive of a more aggressive disease progress, correlate with brain atrophy and to CXCL13 levels, chemokine that controls B cell migration (Ferraro *et al.*, 2015). Some biomarkers can also be used to monitor the therapeutic effect of DMTs. For example, interferon-β (IFN-β) and Natalizumab efficacy usually decreases following the formation of neutralizing antibodies, thus, serum antibodies are commonly assessed as biomarker of treatment response (Harris *et al.*, 2009). Another biomarker used to monitor IFN-β response is myxovirus resistance protein (MxA), a gene rapidly induced in peripheral blood mononuclear cells (PBMCs) by IFN-β. It has been shown that Natalizumab also reduced levels of CH3L1 (Stoop *et al.*, 2013).

Biomarkers can be also useful to prevent the onset of specific pathological conditions resulting from the use of some DMTs. For example, Natalizumab, an immunosuppressive drug that reduces relapses in RR-MS patients, has been associated to multifocal leukoencephalopathy (PML), a potentially dangerous infection caused by CNS reactivation of the John Cunningham virus (JCV). The risk of develop PML in patients under treatment with Natalizumab is reduced by monitoring the serum levels of JCV antibody (Lee *et al.*, 2013). It has been shown that the levels of some biomarkers in CSF, such as Fetuin-A, osteopontin, and CXCL-13, can correlate with disease activity. These factors are present at higher levels in the CSF of CIS, RRMS, SPMS,

and PPMS patients compared to symptomatic controls and they decrease in response to treatments (Kuhle *et al.*, 2016).

In the last years, light (NF-L) and heavy (NF-H) neurofilaments are emerging as new biomarkers of MS in the monitoring of axonal damage and neurodegeneration, based on their release in the extracellular space following axonal injury. It has been shown that levels of NF are high in the CSF of MS patient, during relapses. NF-L levels correlate more with the inflammatory components of the disease, whereas, NF-H reflect more the progression disability and ongoing neurodegeneration (Teunissen *et al.*, 2012).

A recent study has shown the prognostic value of NF-H, demonstrating that elevated levels of NF-H in the CSF of RR-MS patients is predictive of atrophy measured 15 years later (Petzold *et al.*, 2016). NF-L levels in the CSF can also correlate with other biomarkers. For example, it has been shown that CFS NF-L inversely correlate to serum levels of vitamin D, suggesting that the last can also protect from axonal injury (Sandberg *et al.*, 2016).

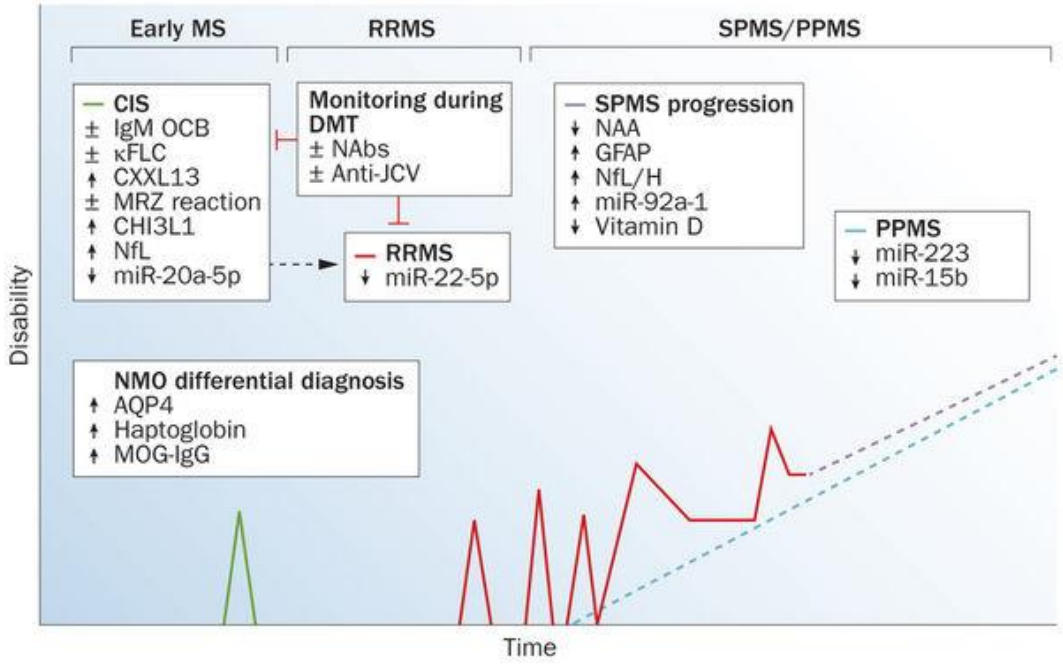

Figure 1.16. Biomarkers associated with different clinical phases in MS. (Teunissen *et al.*, 2015)

Currently, several studies are investigating whether NF blood levels sufficiently correlate with disease activity, in order to allow less-invasive analysis.

Several studies have explored the use of circulating miRNA as biomarker of MS, revealing miRNAs that are differentially expressed in MS vs controls, associated to the conversion of CIS in MS or that change in response to DMTs. In these studies, miRNA expression was evaluated in the CFS, whole blood, plasma, PBMCs, or MS lesions (Table 1.2).

Junker and colleagues analyzed the expression of 365 miRNAs in 16 active and 5 inactive white matter MS brain lesions compared to 9 control white matter specimens. They identified some miRNAs expressed by astrocytes, such as miR-155, miR-326 and miR-24a, that are specifically up-regulated in MS active lesions, proposing that they can have a role in the regulation of macrophages activity (Junker *et al.*, 2010).

Another study analyzed the expression profile of miRNAs in the CSF of 53 MS patients compared to 39 patients with other neurological diseases (OND). Subsequent validation experiments by qPCR on the selected candidates revealed that miR-922, miR-181c, and miR-633 are differentially expressed in patients with MS compared with OND and that miR-181c and miR-633 differentiated RRMS from SPMS with 82% specificity and 69% sensitivity (Haghikia *et al.*, 2012). Given its greater accessibility and less invasive procedures, several studies in the last years evaluated miRNA expression profile in blood samples, such as PBMC, plasma or serum, to identify possible biomarkers for MS. For example, miR-20a-5p was reported to be down-regulated, whereas miR-22-5p was up-regulated, in the blood samples of MS patients compared to healthy controls (HC) (Cox *et al.*, 2010; Siegel *et al.*, 2012). Interestingly, it has also been shown that miR-20a-5p is involved in the regulation of T cell activity. In another study miRNA expression profile was performed using plasma samples from 10 RRMS, 9 SPMS, and 9 HC. This study revealed that specific miRNAs can distinguish between MS patients and HC and between different MS subtypes. The expression of selected miRNAs was then assessed in a larger cohort of MS patients (100) compared to amyotrophic lateral sclerosis (ALS) patients and HC. miR-92a resulted useful to distinguish RRMS from SPMS and HCs, and showed association with EDSS (Expanded Disability Status Scale) and disease duration (Gandhi *et al.*, 2013). Let-7a, instead, differentiated SPMS from RRMS and HC. Interestingly, these 2 miRNAs are also useful to distinguish RR-MS from ALS and they don't change between SP-MS and ALS, suggesting they can have common pathological features.

A recent study identified other 2 differentially expressed miRNAs, miR-233 and miR-15b, useful to discriminate between RR-MS and PP-MS (Fenoglio *et al.*, 2013).

miRNA	Findings	Proposed pathways/targets*
let-7a	Serum increase SPMS[87] and plasma increase RRMS[88]	IL-12, TGFβ, and TLR[87]
let-7c	Serum increase RRMS[87] and whole blood decrease after natalizumab[86]	LIN28B, SMAD2, ZCCHC11, DICER1, and EIF2C2[86]
let-7d	Serum increase RRMS[87] and PBMC increase MS[89]	IL-1β,[89] SMARCAD1, FAM178A, LIN28B, LRIG3, GATM, and IGDCC3
let-7g	PBMC decrease MS[90]	HTATIP2, LRRK1, and TLR4[90]
miR-16	PBMC increase MS and normalized after AHSCT[84]	FOXP3 and PDCD1[84]
miR-16-2-3p	Whole blood increase CIS/RRMS[81]	CUL2, RAB6A, PLCXD3, INTU, SGIP1, FAM126B, and CLTC
miR-18a	Whole blood increase after natalizumab[85]	MAPK, NF-κB,[85] NEDD9, BBX, ZBTB47, PHF19, RORA, and INADL
miR-18b	PBMC increase MS[80] and increase RRMS[91]	PERQ1, GAB1, SIM2, GLRB, REXO2, BTG3, HSF2, MDGA1, UBTD2, TSHZ3, C7orf42, HMBOX1, CLIP3, and UBE2Z[91] CDKN1A[92]
miR-20a-5p	Whole blood decrease CIS/RRMS[81]	
miR-20b	Whole blood increase after natalizumab[85] and PBMC decrease MS[93]	MAPK, NF-κB,[85] ZNFX1, PTPN4, PDCD1LG2, ADARB1, PKD2, and ZNF800
miR-22	Plasma increase MS[94] and increase RRMS[87]	BTG-1 and ESR-α[94]
miR-26a	PBMC increase RRMS relapse[95]	TGFβ (SMAD1, SMAD4, p300, and c-Myc)[95]
miR-26a-5p	Serum increase PPMS and SPMS[96] and PBMC increase MS and in IFNβ responders[80]	HOMER1, GRIN3A, SLC1A1, SLC38A1, and DLG4[80]
miR-142-3p	PBMC increase MS, normalized after AHSCT,[84] increase MS,[93] increase RRMS, and reduced after glatiramer acetate[97]	FOXO1,[84] FAM208B, WASL, HECTD1, CLDN12, RLF, and MTUS1
mir-145	Serum increase RRMS,[87] increase MS,[89] plasma increase MS,[89] PBMC increase MS,[89] increase MS,[93] and CSF increase MS[83]	IFNβ,[98] ABCE1, MPZL2, DAB2, KCNA4, and ABHD17C
miR-146a	PBMC increase RRMS,[99] reduced after glatiramer acetate[97]	NOVA1, SRSF6, BCORL1, SEC23IP, ZBTB2, and EIF4G2
miR-146b	PBMC increase MS[100] and increase RRMS[99]	SRSF6, NOVA1, SEC23IP, BCORL1, EIF4G2, and ZBTB2
mir-150	Serum increase MS after natalizumab, decrease after fingolimod,[83] PBMC decrease MS,[90] CSF increase RRMS, increase CIS-RRMS converters, and reduced after natalizumab[83]	SOCS1, SPI1, and EPHB2[90]
miR-155	PBMC increase MS, normalized after AHSCT,[84] increase MS,[80] and increase RRMS[97]	FOXP3 and IRF2BP2[84]
miR-181c	Serum increase vs. CSF,[82] PBMC decrease MS,[90] CSF increase MS,[101] and increase CIS-RRMS[82]	MeCP2, XIAP, HMGA1, GDNF, and VEGF[102]
miR-210	Serum increase RRMS[87] and PBMC increase MS[100]	FGFRL1, ISCU, RRP1B, DENND6A, and IGF2
miR-223	Serum decrease PPMS[103] and PBMC increase MS[93]	FBXW7, RRAS2, CRIM1, HSP90B1, and INPP4A
miR-326	Whole blood decrease after natalizumab,[85] PBMC increase MS,[80] increase RRMS relapse,[95] and increase RRMS[97]	MAPK, NF-κB,[85] ETS1,[104] CEP85, GGT7, PALM, PRR14L, and SAMD4B
miR-422a	Plasma increase MS[94] and PBMC increase MS[93]	CYP7A1,[94] NR2C2, KIAA1522, TMEM245, and SLC7A6
miR-572	Serum decrease PPMS, increase SPMS and RRMS relapse,[105] and plasma increase MS[94]	NCAM1,[94] SEPT8, TAOK2, and QRICH2
miR-599	PBMC increase MS[80] and increase RRMS relapse[91]	LRRC4C, ZSWIM6, NFIA, ROCK1, TGFβ2, and ATMIN
miR-648a	Plasma increase MS[94] and decrease RRMS remission[88]	ONECUT2, HBP1, LRRC16A, IMPDH1, MLLT4, KIF13A, and MBD5
miR-922	Serum increase CIS-RRMS conversion,[82] CSF decrease MS CSF,[101] and increase CIS-RRMS[82]	UCHL1,[106] APH1A, UCHL1, CLIC5, STX17, RNF2, and HIF1AN

Table 1.2 List of miRNAs potentially useful as biomarker of MS with the relative source and proposed targets. Upper numbers refer to the original reference (Harris et al., 2016 doi.org/10.2147/DNND.S98936).

It has been demonstrated that the efficacy of some DMTs can be partially related to change in the expression level of specific miRNAs that regulate key aspects of MS pathogenesis; thus, circulating miRNAs can also be used as treatment response biomarker in MS. For example, it

has been shown that Natalizumab treatment caused down-regulation of miR-326b and up-regulation of miR-20b, which in turn can regulate Th17 immune response and BBB breakdown (Ingwersen *et al.*, 2015).

Another independent study reported that 20 miRNAs take part in the mechanism of action of IFN-B and that some of them, such as members of the mir-29 family, are associated with apoptosis or have direct relationship with IFN-responsive genes (Hecker *et al.*, 2013).

In another treatment response study, the expression of 1059 miRNAs was tested in B cells isolated from 10 untreated and 10 Natalizumab treated MS patients using microarrays analysis. This study showed that 10 miRNAs were differentially expressed in B cells of Natalizumab-treated patients compared to untreated patients. They also found that the expression of the miR-106b-25 and miR-17-92 clusters was dysregulated both in untreated and Natalizumab-treated MS patients compared to HC, suggesting a role for these miRNAs in MS pathogenesis (Sievers *et al.*, 2012).

1.3.5 Models to study de-myelination and re-myelination

In order to better understand de-myelination and re-myelination dynamics, several experimental models of MS have been developed in the last decades, however, not a single model can mimic all the pathophysiological processes that occur in the human disease. For this reason, different *in vitro*, ex vivo and *in vivo* models are commonly used in combination to achieve a complete picture of a mechanism.

OPCs are the most common *in vitro* model, widely used to understand myelin development and to assess the effects of new agents, in relation to their migration, survival, proliferation and differentiation, however, they cannot be used for myelination studies (Osorio-Querejeta *et al.*, 2017). Given the high difficult to obtain human OPCs, most of the studies make use of OPCs obtained from brain cortex of young and adult rodents. Nevertheless, in the last years, human iPSCs are emerging as a useful source of human OPCs and can also represent a potential therapeutic approach, since they can be obtained by MS patients themselves (Ogawa *et al.*, 2011). *In vitro* models based on OPCs are simple and low cost, and can represent an initial point to test a compound or to manipulate a specific pathway, to improve their re-myelinating capabilities. Other *in vitro* models make use of co-cultures, that involve the presence of axons

(either natural, e.g. dorsal root ganglion, or artificial, e.g. nanofibers) and myelinating cells (i.e. OPCs). Co-cultures can be used to assess the myelination capabilities of the cells under study or to investigate the effects of drugs on these cells. Analysis on co-cultures include quantification of myelin and g-ratio[2] analysis (Osorio-Querejeta *et al.*, 2017).

Among ex-vivo models, organotypic cultures represent the most common used 3D cultures and can be considered an intermediate step between *in vitro* and *in vivo* studies. Organotypic cultures consist of approximately 300 um slices that mimic the structure and the cell types of the origin organ. They can be generated by different CNS structures, such as brain, spinal cord and cerebellum (Kipp *et al.*, 2012).

Usually, for de-myelination/re-myelination studies, organotypic cultures are treated with the lysophosphatidylcholine (LPC), a detergent that destroys myelin. Following LPC removal, OPCs activate and proliferate in response to de-myelination, and differentiate to mature oligodendrocytes, that start the re-myelination process. This model is widely used to test the regenerative capacity of endogenous cells and how pharmacological or biotechnological interventions can modulate the re-myelination process. Despite they cannot replace *in vivo* models, organotypic cultures can represent a useful tool for screening treatments before *in vivo* testing (Zhang *et al.*, 2011).

In vivo models for MS have been developed to study pathological processes related to demyelination and re-myelination and can be classified in 4 categories: autoimmune, toxic, viral and genetic. Animal models can be monitored during disease by magnetic resonance imaging and several data can be collected with post mortem analysis (e.g. myelin quality, g-ration, gene expression analysis).

Experimental autoimmune encephalomyelitis (EAE) is the best characterized autoimmune model of MS and exhibits several histopathological features of the human disease. In this model, animals are immunized against a myelin peptide to induce an T-cell mediated autoimmune response against myelin. Depending on the peptide, animal host and viral strain different models can be generated, which resemble human SP-MS or RR-MS. Like human MS, EAE presents features such as myelin destruction, development of lesions into the CNS, and

[2] ratio of the inner to the outer diameter of the myelin sheath of a myelinated axon.

presence of immunoglobulins in the cerebrospinal fluid and CNS, however de-myelination and re-myelination processes are less extensive (Kipp *et al.*, 2012). Chronic EAE is the most used for de-myelination studies and this can be achieved by immunizing C57BL7/6 mice or Dark Agouti rats against myelin oligodendrocyte glycoprotein (MOG 35–55) peptide, emulsified in Freund's adjuvant that is supplemented with *Mycobacterium tuberculosis* extract. Chronic EAE is the gold standard for pre-clinical proof of concept studies to test new drugs (Constantinescu *et al.*, 2011). Nevertheless, it has proven poorly predictive of treatment efficacy in human MS, in particular regarding the use of inflammatory cytokines. During EAE development, disease progression can be monitored by clinical scores. The main limitations of the model are the high complexity and the low reproducibility, which can make interpretation of results very complicated. Moreover, EAE cannot provide new insight on progressive-MS and it is not the more suitable model to study remyelination, since lesions occur stochastically in time and location (Ransohoff *et al.*, 2012).

In toxic models of MS oligodendrocytes are depleted following the injection or administration of specific toxic agents. The most widely used toxins are lysophosphatidylcholine (LPC) and cuprizone. LPC can damage myelin altering specifically the membrane composition of oligodendrocytes. In this model, the demyelinating lesions, characterized by astrogliosis and microgliosis, are obtained by stereotaxic injection of LPS in the white matter of mice. The damage induces OPC proliferation and migration towards lesions, where they start to differentiate in oligodendrocytes and begin the regenerative process (Osorio-Querejeta *et al.*, 2017).

Cuprizone is a copper chelating agent that induced selective death of oligodendrocytes and, as a consequence, demyelination. The exact mechanism is still unknown, however, it has been proposed that cuprizone causes errors in the mitochondrial respiratory chain (Ransohoff *et al.*, 2012). In this model, cuprizone (0.2% in chow) is usually fed to mice for 4-6 weeks and after 3 weeks of administration an extensive death of oligodendrocytes in the corpus callosum and hippocampus can be observed. Depending on the length of treatment (6 or 12 weeks), cuprizone can induce acute or chronic de-myelination. Demyelination and re-myelination simultaneously occur as the damage evolves, in a similar way to the human disease. After

cuprizone withdrawal, robust re-myelination occurs, but in the chronic models it is slower and limited (Kipp *et al.*, 2009).

In contrast to EAE, T cells do not play a role in the generation of de-myelinating lesions in toxic models. These models are simpler and highly reproducible, and can be useful to investigate demyelination and re-myelination processes. They can also be used to evaluate the effects of drugs or cell therapy (in the absence of high levels of inflammation) on re-myelination, a crucial point for neuroprotection in MS, thus representing the most significant tools for translational research (Ransohoff *et al.*, 2012).

Genetic models of MS (also known as myelin mutants) are animals with specific mutations that lead to a global failure in myelin production, thus characterized by extensive demyelination. These models are widely used for transplantation studies, to test the myelinating capabilities of exogenous cells (Duncan *et al.*, 2011). The most commonly used myelin mutant in transplant experiments is the *shi* mouse, which has a mutation in the myelin basic protein (MBP) gene, but it is not a model of human disease. Shi mice are characterized by absence of myelin and they die at approximately 120 days (Duncan *et al.*, 2011).

Development of viral models of MS was based on the hypothesis that some virus may cause demyelinating disorders such as MS. Given that viral infections are one of the potential causes of MS onset in human, these models allow to investigate the potential effects of remyelinating and immunomodulatory agents in cells infected by neurotrophic virus (Osorio-Querejeta *et al.*, 2017).

1.3.6 Drugs available in clinics and new therapeutic perspectives in MS

Currently there is not a definitive cure for MS, however, treatments available can efficiently decrease symptoms and prevent the progression, reducing the number of relapses. There are 3 general approach to MS treatment: (1) corticosteroids (CS) for acute relapses; (2) DMT to reduce relapses and prevent disability; (3) minimize the impact of quality life treating MS-associated symptoms (Sturm *et al.*, 2014).

Corticosteroids are potent anti-inflammatory and immune-suppressive agents, which are commonly used to treat MS related exacerbations (relapses). Benefits of corticosteroids are related to their capability to restore the integrity of the BBB and to reduce the migration of

inflammatory cells into the CNS, and subsequently increase the speed of recovery after MS relapses (Ontaneda *et al.*, 2009).

In 2016, 11 disease-modifying agents have been approved by the U.S. Food and Drug Administration (FDA) for the relapsing-remitting MS subtype: daclizumab, glatiramer acetate, interferon beta-1a, interferon beta-1b, dimethyl fumarate, fingolimod, teriflunomide, alemtuzumab, mitoxantrone, natalizumab and ocrelizumab. Currently, interferons and glatiramer acetate are the best treatments, indeed both are effective in reduce MS relapses by 30% and are safe for longer therapy (Johnson *et al.*, 2009). Although the exact mechanism of action of beta-interferons is unknown, it may be related to suppression of T-helper cell response. Glatiramer acetate, instead, mimics myelin basic protein and induces Th2 cells activation.

Natalizumab can reduce relapses more efficiently, but it has several side effects and, for this reason, it is used in non-responsive or MS advance patients (Tsang *et al.*, 2011), similarly to Mitoxantrone. In the last years, several oral drugs, such as teriflunomide, fingolimod, and dimethyl fumarate, have shown efficacy in RR-MS patients. Phase 3 clinical trials (FREEDOMS, TEMSO and DEFINE) showed reduction of the absolute relapse rate (ARR), reduction in the number of new T2 lesions and in gadolinium enhancing-lesions, but their uses are limited due to lack of long term data efficacy and safety (Killestein *et al.*, 2011).

There are also new treatments making use of monoclonal antibodies such as ocrelizumab, rituximab and ofatumumab. These category of drugs targets the antigen CD20, a transmembrane protein expressed by B cells with possible role in activation and differentiation. Different phase 2 and 3 clinical trials showed that anti-CD20 mAbs can significantly reduce new brain MRI lesions and relapses in RRMS and be effective in a subset of PPMS patients (Barun at al., 2011).

Despite all the mentioned immunosuppressive agents have shown a beneficial effect in the treatment of RR-MS, they are almost useless in the progressive forms. Only recently, a phase 3 clinical trial completed in December 2016 showed that PP-MS patients treated with Ocrelizumab had a slower disease progression compared to placebo treated patients

(Montalban *et al.*, 2017). Thus, Ocrelizumab is the only agent approved by FDA for the treatment of PP-MS.

Currently, all the treatments available for MS are immunomodulating and anti-inflammatory agents, which can delay the progression and decrease the pathological symptoms, however, they do not halt the ongoing de-myelination and the subsequent neurodegeneration. Therefore, other strategies such as use of stem cell-based therapy or re-myelinating agents are under investigation as potential novel paradigms for the treatment of MS.

Several studies on animal models of MS showed efficacy of MSC (mesenchymal stem cells), NSC (neural stem cells), OPC or ADSC (adipose-derived stem cells) transplantation. It has been proposed that the beneficial effect of MSC transplantation could be due to their immunomodulatory properties and paracrine effects (Mohyeddin Bonab *et al.*, 2013). Functional recovery observed after NSC transplantation is instead associated to re-myelination improvements (Pluchino *et al.*, 2003). Other studies have shown that also ADSC transplantation can decreased pathological signs and de-myelination in rodent model of MS (Ghasemi *et al.*, 2016; Shalaby *et al.*, 2016). ADPC can differentiate in other cell lineages and secrete neurotrophic factor, suggesting that their transplantation could promote tissue regeneration (Ghasemi *et al.*, 2017).

As previously discussed, re-myelination is essential to restore axonal conduction and plays a primary role in clinical recovery and remissions. Extensive re-myelination can occur during early disease and in the remitting phases, however, it declines dramatically in the progressive phase, contributing to neurodegeneration. Thus, foster endogenous re-myelination could have a strong impact in these progressive forms. Moreover, they can also be used in a combination therapy with the already available DMTs.

High-throughput screening of previously FDA-approved drugs allowed to identify several classes of pro-myelinating drugs. For example, anticholinergics, such as benzotropine and clemastine, have shown pro-myelinating effects, likely via antagonism of M1/M3 muscarinic acetylcholine receptors directly expressed by oligodendrocytes (De Angelis *et al.*, 2012). Another small molecule screening identified miconazole, an antifungal agent, and clobetasol, a corticosteroid used to treat eczema. It has been shown that these molecules can exert they re-

myelinating properties by influencing eIF2, thyroid hormone and cholesterol signaling directly on oligodendrocytes (Najm *et al.*, 2015). The safety profile of these agents was already established, thus, a phase 2 clinical trial could be done in the next future.

Olesoxime, a mitochondrial pore modulator originally developed to treat ALS, accelerates oligodendrocyte differentiation and myelination either *in vitro* and *in vivo* without affecting oligodendrocyte survival or proliferation (Bordet *et al.*, 2007). A phase 1b study to evaluate the safety of this agent in MS patients is currently ongoing.

Another possible strategy to foster re-myelination is to modify the local environment, in order to make it more permissive for endogenous OPC. In this respect, one promising target is LINGO1, an already discussed negative regulator of myelination (see 1.2.2). A phase 2 clinical trial with IgG1 anti-LINGO1 was already done and produced good results in the treatment of optic neuritis, leading to faster nerve impulse conduction, indication of myelin repair (Harlow *et al.*, 2015). Another promising target is represented by semaphorin 4D (SEMA4D), normally expressed by mature oligodendrocytes. It has been demonstrated that SEMA4D is up-regulated after injury and increases oligodendrocytes apoptosis. Moreover, its over-expression in DRG co-cultures inhibits myelination, whereas, its silencing in oligodendrocytes after spinal cord injury promotes functional recovery (Zhang *et al.*, 2014). A recent study has shown that antibodies against SEMA4D improved BBB integrity, OPC maturation and myelination (Smith *et al.*, 2015) in the EAE model of MS. A phase 1 trial for the evaluation of humanized anti-SEMA4D safety was recently completed.

As discussed above, several strategies to foster endogenous re-myelination are under investigation, however, up to date, advancements in the detection of myelin specific changes are necessary to fully evaluating the efficacy of re-myelinating agents in clinical trials. However, several studies suggest that the next frontier in MS therapy will be supplement immunomodulators to pro-remyelinating agents, in order to block disease progression and recover neurological functions.

1.3.7 MiRNA as therapeutic targets in MS

As previously discussed, several studies have shown the important role of miRNAs in the regulation of development, homeostasis and maturation of either OPCs and immune cells. Thus,

it is not surprising that some miRNAs were directly linked to MS pathogenesis and recently proposed as new potential therapeutic targets for MS (Junker *et al.*, 2011; Dugas *et al.*, 2010; Thamilarasan *et al.*, 2012; Wang *et al.*, 2017). Depending on the target, modulators of miRNA expression can act as pro-remyelinating agents or as disease-modifying therapy.

Recent studies have demonstrated that miR-155 silencing could repress the activity of Th1 and Th17 cells and subsequently reduce disability in EAE mice. Accordingly, administration of anti-miR-155 in mice significantly decreased EAE development. miR-155 was also found up-regulated in plasma and active lesions in MS patients, thus, representing a good therapeutic target in MS (Ma *et al.*, 2014). Similarly, miR-326 is up-regulated in MS active lesions and its expression highly correlates with MS patient's disability. Moreover, miR-326 *in vivo* silencing reduced, whereas its over-expression increased, the number of Th17 cell and EAE severity (Du *et al.*, 2009).

Another promising therapeutic target is miR-23b, that can repress IL-17 production and NFkB activation in EAE mice (Zhu *et al.*, 2012). Down-regulation of miR-125a-5p in MS patients was recently associated to BBB dysregulation and thus it could be used to rescue BBB integrity in MS treatment (Reijerkerk *et al.*, 2013). It has been reported that miR-27b, miR-128 and miR-340 can induce the shift from a Th2 to a Th1 cytokines production and that they are up-regulated in CD4+ T cells isolated from MS patients. Interestingly, *in vitro* silencing of these miRNAs restores Th2 responses in T cells from MS patients, suggesting that they could represent new targets for MS treatment (Guerau de Arellano *et al.*, 2011).

As previously discussed, re-myelination failure in MS is frequently due to an impairment in the maturation of oligodendrocytes, thus, the study of miRNAs regulating OPC differentiation could help to identify novel targets to foster endogenous re-myelination in MS patients. In this respect, miR-219 and miR-338, miRNAs highly expressed by mature oligodendrocytes, were found to be strongly down-regulated in MS brain lesions, suggesting that restoring of their physiological levels could promote re-myelination in MS (Junker *et al.*, 2009)

The property of miRNAs to simultaneously regulate the expression of multiple genes is the main feature that attracted the attention of researchers and clinicians to consider miRNAs as a new promising category of drugs, but is also the major critical point. Indeed, the main issue about

the systemic use of miRNAs in therapy would be to overcome potential side effects arising from the ability of miRNAs to regulate multiple genes in different cell types, leading to unwanted silencing effects (De Faria *et al.*, 2012). Moreover, miRNAs share common problems with other drugs, such as stability, cytotoxicity and delivery to target cells. In this respect, chemical modifications have proven efficacy to increase miRNA stability to endo- and eso-nuclease (Van Rooij *et al.*, 2014), whereas, packaging of miRNAs into lipid vesicles conjugated to appropriate receptors could help to delivery miRNAs to the correct cell type and reduce off-target effects (Baumann and Winkler, 2014). Interestingly, it has been shown that exogenous miRNAs can also successfully delivered by HDL particles purified from the blood (Vickers *et al.*, 2011).

Given the high heterogeneity of cell types involved in MS pathogenesis and the pleiotropic effect of miRNAs, understand how a candidate miRNA can affect the phenotype of individual cell types in the CNS is a critical point for the identification of new miRNA-based drugs for the treatment of MS.

2- AIM

The discovery of microRNAs (miRNAs) unveiled a new level in the regulation of gene expression and opened new directions in all fields of science. Up to date, thousands of miRNAs were identified and, given that a single miRNA can modulate hundreds of targets, they represent a powerful system in the fine tuning of gene expression. It has been shown that specific miRNAs are expressed at high levels in the central nervous system (CNS) and that they participate in the regulation of crucial processes related to neural cell development (Petri *et al.*, 2014). As a consequence, dysregulation in miRNA levels can contribute to the onset and to the pathogenesis of several neurological diseases, such as multiple sclerosis (MS). MS is a chronic autoimmune demyelinating disease in which immune system attacks and disrupts myelin, a fatty substance produced by oligodendroglial cells that surrounds and protects axons, leading to an incorrect transmission of nerve impulses and severe disability (Wu *et al.*, 2011). In MS, remyelination can occur as a spontaneous regenerative process following demyelination, but this process is often incomplete, likely due to the fail in the maturation of oligodendrocyte precursors, the cells responsible for myelin production (Kuhlmann *et al.*, 2008).

Interestingly, several studies on animal models of MS and human specimens suggested that miRNAs can contribute to MS pathogenesis and may play an important role in the re-myelination failure (Junker *et al.*, 2011; Dugas *et al.*, 2010; Thamilarasan *et al.*, 2012; Wang *et al.*, 2017).

Unfortunately, the number of miRNAs fully characterized in MS context is still poor, therefore, the main aim of this study was to evaluate the potential role of miR-125a-3p, a brain enriched miRNA, in OPC maturation and myelination.

In particular, we aimed to:

i. Assess the relevance of miR-125a-3p to oligodendrocyte maturation;
ii. Characterize its expression in the CNS and oligodendroglial cells;
iii. Evaluate the effects of miR-125a-3p manipulation in oligodendroglial cells;
iv. Analyze its expression in animal models of de/re-myelination;
v. Evaluate the effects of miR-125a-3p manipulation in demyelinating conditions;
vi. Analyze its expression in the cerebrospinal fluid of human MS patients;
vii. Identify the mechanisms altered by miR-125a-3p in oligodendroglial cells;

The identification of new miRNAs regulating oligodendrocyte maturation may help to find new therapeutic targets to foster endogenous re-myelination in diseases characterized by myelin defects, such as MS. Moreover, miRNA levels in body fluids can reflect disease activity and their restoration could be a novel means to evaluate the efficacy of available treatments, thus representing useful biomarkers of disease progression and in personalized pharmacology.

3- METHODS

3.1 *In silico* and statistical analysis

3.1.1 *In silico* analysis. MyMir (http://www.itb.cnr.it/micro), a system based on integration, filtering and re-ranking of outputs produced by different miRNA databases (i.e. TargetScan, RNAHybrid, miRanda, PITA) was used for the prediction of miRNA target transcripts. STRING database was used to obtain the list of the Gene Ontology biological processes (GO BPs) significantly enriched (p value < 0.05) for miR-125a-3p targets. For the analysis, the false discovery rate (FDR) adjusted p-value was used, to reduce the chance of false-positive results. The fold enrichment in miR-125a-3p was calculated using the following formula as previously described:

(1) Fold enrichment = $\frac{m \div n}{M \div N}$

m = genes target in a BP; n = all genes target in the list; M = all genes in a BP; N = all genes in the genome. A fold enrichment greater than 1 (expected value) indicates that the category is overrepresented in the input list.

QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used to build the network model for the synergic regulation of MBP and to perform the upstream regulator analysis on differentially expressed genes after miR-125a-3p over-expression. Z score > 2 indicates that a transcriptional regulator is likely activated, whereas Z score < 2 indicates that it is likely inhibited in the experimental condition. The software Metacore™ was used to perform an ontology-based clusterization on differentially expressed genes after miR-125a-3p over-expression in OPCs, to identify common processes.

3.1.2 Statistical analysis. Data are presented as mean ± standard error (SEM) of replicates. GraphPad Prism 6.0 was used for statistical analysis. For all comparisons between two groups with a normal distribution, two-tailed unpaired t-test was performed. For multiple comparison testing, one-way analysis of variance (ANOVA) accompanied by Dunnett's post-hoc test was used. For CSF samples, data with a Median Absolute Deviation (MAD) > 3.5 were considered outliers and discarded from the analysis. P values <0.05 were considered statistically significant.

3.2. Cell cultures and miRNA transfection

3.2.1 Primary cultures and OPC isolation. Primary mixed glial cultures were obtained from postnatal day 2 (P2) Sprague-Dawley rat cerebral cortices. After the removal of meninges, cortical tissues were diced with a sterilized razor blade and transferred into a 50 ml sterile centrifuge tube (Euroclone) containing ice-cold Hanks balanced salt solution with calcium and magnesium (HBSS, Euroclone). Afterwards, HBSS was aspired and tissues were washed with HBSS without calcium and magnesium and incubated with 10 ml trypsin-EDTA solution containing 1% DNAse I (final concentration 0.01 mg/ml) (Sigma-Aldrich) for 30 minutes in a water bath at 37°C for tissue disaggregation. After the incubation, trypsin was inactivated with HBSS containing 10% of fetal bovine serum (FBS, Euroclone) and tissues were further triturated mechanically with a Pasteur pipet. The cellular suspension was passed through a 100 μm cell strainer (BD) in order to eliminate undissociated tissue residues, collected into a 50 ml sterile centrifuge tube and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in the medium for cortical astrocytes containing: DMEM high glucose (Euroclone), 2 mM L-glutamine (Sigma-Aldrich), 1 mM Sodium pyruvate (Sigma-Aldrich), Penicillin 100 U/ml-Streptomycin 100 μg/ml (Euroclone), 2,5 μg/ml Fungizone (Euroclone) and 20% FBS. The cellular suspension was spread over the entire T75 poly-D-lysine (final concentration 10 μg/ml, Sigma-Aldrich) -coated flask surface. Then, flasks were put in a tissue culture incubator (Steri Cult 200) at 37°C, 5% CO_2 and 95% relative humidity. The medium was changed every 2-3 days until cell confluence (about 8 days after the plating).

Flasks were then removed from the incubator, well fixed with the plug-seal cap tightly screwed on a horizontal orbital shaker and shaken for 20 minutes at 200 rpm at room temperature to remove microglial cells. The medium containing microglial cells was discarded from the flasks by aspiration and 12 ml of fresh DMEM containing 10% of FBS were added to each flask. The flasks were put again on the shaker for further 3-4 hours to isolate OPCs. The cell suspension from each flask was then collected by a pipette, transferred to an untreated Petri-dish and incubated for 20 minutes in tissue culture incubator at 37 °C for differential adhesion of contaminating microglia and astrocytes. The cell suspension was transferred into a 50 ml tube and centrifuged at 1200 rpm for 15 minutes. The supernatant was carefully discarded and the

pellet was resuspended and dissociated in a small amount of Neurobasal (Life Technologies) containing 2 mM L-Glutamine, 1% of Penicillin 100 U/ml-Streptomycin 100 µg/ml and 2% of B27 (Life Technologies). Living cells were counted using the Trypan blue which allows the exclusion of death cells. OPCs were plated onto poly-D,L-ornithine-coated (final concentration 50 µg/ml; Sigma-Aldrich) 13-mm glass coverslips for immunocytochemistry (2-3 x 10^4 cells/coverslip) and poly-D,L-ornithine-coated 6-wells plates (10^5 cells/coverslip) or 6-cm dishes (2.5 x 10^5 cells/coverslip) for qRT-PCR assays. Cells were plated in Neurobasal medium supplemented with 2% B27, 2 mM L-glutamine, 10 ng/ml human platelet-derived growth factor BB, and 10 ng/ml human basic fibroblast growth factor to promote proliferation. When OPCs reached a 60% confluency, cultures were switched to a Neurobasal medium lacking growth factors and containing triiodothyronine 15 nM (T3, Sigma-Aldrich) to allow differentiation. In selected experiments, T3 was not added to induce a slower maturation.

The primary cultures used for the experiments were derived from rats. All the procedures were performed in accordance with the approved national law, with the implementation of European Union Directive nr. 86/609/CEE regarding the Protection of animals used for experimental research. The experiments were approved by the Council of the Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Italy, which is legally entitled for the use of animals for scientific proposes (D.M. of Italian Ministry of Health, Authorization #295/2012-A 12/20/2012, according to the D.lgs 116/92).

3.2.2 Transfection of primary OPCs with miR-125a-3p mimics and inhibitors. OPCs were transfected immediately after switching from proliferating to differentiating medium (in the absence or in the presence of T3). MiR-125a-3p mimics or hairpin inhibitors (Dharmacon) were transfected at the final concentration of 50 nM. A scrambled miRNA transfection was included as negative control. MiRNAs were transfected with Lipofectamine RNAiMAX reagent (Life Technologies) following the manufacturer's protocol.

3.3 Immunofluorescence and in situ hybridization

3.3.1 Immunocytochemistry and cell counting. Cells were fixed in a 4% paraformaldehyde phosphate-buffered solution containing 4% sucrose. The following primary antibodies were

used: rabbit anti-Olig2 (1:100; Millipore), rabbit anti-GPR17 (1:100; Cayman Chemical), rat anti-MBP (1:200; Merck Millipore), goat anti-MAP1B (1:300; Santa Cruz), mouse anti-O4 (1:100; R&D), mouse anti-GFAP (1:100; Cell signalling). Incubation of primary antibodies were performed 2.5 hours at room temperature or over-night at 4°C. Cells were then incubated for 1 h at room temperature with secondary antibodies conjugated to either AlexaFluor 488 or AlexaFluor 555 (1:600; Life Technologies). All the antibodies were diluted in a phosphate-buffered blocking solution (pH 7.4) containing 0.3% Triton X-100, with the exception of O4, which did not require detergents. Nuclei were labeled with the UV fluorescent dye Hoechst 33258 (1:10,000; Life Technologies). Coverslips ere then mounted in a fluorescent mounting medium (Dako). Positive cells for the selected markers were counted from 20 random fields for each coverslip (0.07 mm^2/field). The result was expressed as a percentage over the number of nuclei, and then normalized versus controls (transfected with negative miRNA) set to 100%.

3.3.2 MicroRNA in situ hybridization in combination with immunohistochemistry. After anaesthesia, post-natal P7, P14 and adult rats (or mouse) were perfused with 4% paraformaldehyde (PFA; Sigma) in phosphate buffered saline (PBS). Brains were explanted and post-fixed in 4% PFA for 1 hour. Embryos and P2 rat brains were fixed by immersion in 4% PFA for 4 hours. Tissues were washed in PBS, dehydrated overnight in 30% sucrose, and then embedded in OCT (Bio-optica). Twelve micrometer sections were cut on a cryostat in RNase-free conditions, transferred to Superfrost/plus microscope slides (Thermo Scientific), and stored at 20°C. Buffers and reagents were prepared using DEPC-treated (Sigma) water and autoclaved. Detection of miRNA was performed with miRCURY LNA miRNA detection probes (Exiqon), using a previously described protocol63 with minor modifications. Briefly, tissues were further fixed in 10% formalin (Sigma) and then subjected to acetylation. Pre-hybridization was carried out for 2 hours at room temperature, followed by hybridization with scramble/miR-125a-3p DIG-LNA probes (20nM; Exiqon) overnight at 55°C. The following day, endogenous peroxidases were blocked with 1% H2O2 (Sigma). For probe detection, the sections were incubated with a sheep anti-DIG-POD primary antibody (1:600; Roche), diluted in blocking buffer (0.05% Tween-20, 1% BSA and 1% sheep serum, all from Sigma) at room temperature for 2 hours, then with

biotynilated-tyramide for signal amplification (Perkin Elmer) and finally streptavidin Alexa Fluor 555 conjugated (1:1000; Life Technologies) for 50 minutes.

For detecting the epitopes of interest, immunohistochemistry (IHC) was combined with in situ hybridization (ISH) by using the following primary antibodies: rabbit anti-Olig2 (1:100; Millipore), rabbit anti-Iba1 (1:500; Wako), mouse anti-GFAP (1:100; Cell Signaling), mouse anti-NeuN (1:100; Millipore), mouse anti-Nestin (1:100; Millipore), rabbit anti-NG2 (1:100; Millipore), mouse anti-CC1 (1:50; Calbiochem). Alexa Fluor 488 or 633 (1:600 and 1:300, respectively; Life Technologies), were used as secondary antibodies in double or triple staining. Stainings with Alexa Fluor 633 were always shown in either white or blue pseudocolor. Nuclei were labelled with the UV fluorescent dye Hoechst 33258 (1:20,000; Life Technologies). Finally, slides were mounted in a fluorescent mounting medium (Dako).

3.4 Gene expression analysis

3.4.1 Total RNA extraction, retrotranscription and gene expression analysis. Total RNA was extracted from rodent cells or tissues using Trizol reagent (Life Technologies). Human RNAs (total brain, brainstem, frontal cortex, kidney and liver) were bought from Ambion. For qRT-PCR of miRNA, 10 ng of total RNA was reverse-transcribed with TaqMan® MicroRNA Reverse Transcription Kit and then subjected to Taqman microRNA assay (Life Technologies). Expression level of miR-125a-3p was normalized to the U87 snRNA in rat samples and to the U6 snRNA in mouse and human samples by the ΔCt method. For gene expression analysis, cDNA synthesis was performed starting from 800 ng of total RNA using SuperScript® II Reverse TranscriptiScript cDNA synthesis kit (Biorad). The expression of all genes was analyzed with Universal SYBR Green Supermix (Biorad) and normalized to GAPDH expression using CFX96 real time PCR system (Bio-rad) following the manufacturer's protocol.

3.4.2 Isolation of miRNAs from cerebrospinal fluid (CSF) and real-time PCR. Human samples of CSF have been obtained from the Institute of Experimental Neurology (INSpe) Biobank at the San Raffaele Hospital after thorough evaluation of neuropathology and consisted of MS, Alzheimer, and neurologically normal control subjects. The distinction between patients who had radiologic or clinical evidence of MS activity was done through the

assessment of gadolinium-enhancing lesions on cranial MRI and presence of recent clinical relapses (Fox *et al.*, 2014). According to the recently updated McDonald criteria (Polman *et al.*, 2011), patients have been considered to be in a clinical relapsing phase of the disease if they experienced a relapse in the 30 days prior to lumbar puncture. Patients with one or more gadolinium-enhancing lesions at baseline MRI (with or without corresponding clinical symptoms) were classified the same as in a relapsing (active) phase of the disease.

The specimens were collected from adult subjects under informed consent in accordance with the laws and with the internal guidelines and regulations of the San Raffaele Hospital. The protocols were approved by the Ethical commitee of the San Raffaele Hospital. The analysis on human specimens were also approved by the Ethical Committee of the University of Milan (prot. 71/14). CSF samples were collected by diagnostic lumbar puncture or puncture of Ommaya reservoirs[3] from individual MS patients, centrifuged at 13,000g for 5 minutes and stored at -80°C. RNA was isolated from 200 µl of CSF using miRNeasy Serum/Plasma Kit (Qiagen, Milan) following the manufacturer's protocol and reverse-transcribed with TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies). Expression level of miR-125a-3p was analyzed with TaqMan® MicroRNA Assays and normalized to spiked-in cel-miR-39 by the ΔCt method.

3.4.3 Microarray. RNA quality was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies). RNA with RNA integrity number (RIN) > 7 was used for microarray analyses. Labeled cRNA was synthesized from 100 ng of total RNA using the Low Input Quick-Amp Labeling Kit, one color (Agilent Technologies) in the presence of cyanine 3-CTP. Total RNA was hybridized on SurePrint G3 Rat Gene Expression Microarrays (#G4858A-074036, Agilent Technologies). This microarray consists of 60-mer DNA probes synthesized in situ, which represent 30,584 rat transcripts. Hybridization was performed at 65°C for 17 hours in a rotating oven. One-color gene expression analysis was performed according to manufacturer's procedure. Feature Extraction 10.7.3 software (Agilent Technologies) was used to obtain microarray raw-data. A fold change of ±1.5 and a FDR-adjusted p-value < 0.05 were considered to obtain the list of genes differentially expressed between the two experimental conditions.

[3] surgically implanted device that provides direct access to the cerebrospinal fluid.

3.4.4 Pathway-focused PCR arrays. The PCR arrays "Wnt signaling targets" (PARN-243ZD) and "Extracellular Matrix & Adhesion Molecules" (PARN-013ZD) were used to identify genes differentially expressed in OPCs after miR-125a-3p over-expression compared to negative control (See Appendix B and C for the list of genes analyzed). For each PCR array, cDNA synthesis was performed starting from 500 ng of DNase pre-treated total RNA using RT^2 First Strand Kit (Qiagen), following manufacturer's protocol. RT^2 Profiler PCR Array (SABiosciences) and RT^2 SYBRgreen Mastermix (Qiagen) were used to measure gene expression levels. Each array includes five housekeeping genes, that enable normalization of data, a genomic DNA control, that specifically detects genomic DNA contamination, a reverse transcription control, that tests the efficacy of the reverse-transcription reaction and a positive PCR control, that tests the efficacy of the polymerase chain reaction itself. Data were analysed by RT^2 Profiler PCR Array data analysis center v. 3.5 (Qiagen).

3.4.5 Cloning and luciferase reporter assay. The web tool RNA22 was used to identify 7 hypothetical binding sites for miR-125a-3p on GPR17 3'UTR. To construct the reporter plasmid pmirGLO-UGPR17, a 3'UTR region of 736 bp, containing the 3 more conserved binding sites between rat and mouse, was amplified from rat cDNA using the following primer: Fw-CTTCACAAAGCCCAGGTCAG; Rv-CTGGGGAAGGAAGGGTCAAA. The PCR product was digested with SacI and XbaI and was cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). 10 ng of plasmid were transfected alone or together with miR-125a-3p mimics (or scramble miRNA, 50 nM) in COS7 cells. Cells were lysed 24 hours after transfection. Dual luciferase reporter assay was performed to evaluate the luminescence signal produced in the different conditions.

3.5 Models of de/re-myelination

3.5.1 Cuprizone-induced demyelinating model. 40 male C57BL/6 wild-type mice were housed on-site under normal light/dark cycle conditions. Mice (8-week-old age) were fed with 0.2% (w/w) oxalic bis-cyclohexylidenehydrazide (cuprizone; Sigma-Aldrich) supplemented diet ad libitum for 5 weeks and were then switched to normal diet for further 3 weeks to allow spontaneous re-myelination. Real time PCR analysis was performed on the corpus callosum

only. Groups of 5 animals were sacrificed at 0, 1, 2, 3, 5, 6, 7 and 8 weeks from the beginning of the experiment. Whole brain was explanted and allocated in a brain matrix to produce 5 coronal sections of 1 mm interval. Section were fresh frozen in dry ice and lastly processed one by one to excide the corpus callosum. Corpus callosum sections were lysed in 500 µl of Trizol reagent (Life Technologies).

3.5.2 Organotypic cerebellar slices. 250 µm-thick parasagittal slices of postnatal day 10 (P10) mouse cerebellum were cut using a Tissue Chopper. The slices were placed on Millicell-CM culture plate inserts (Millipore) in a pro-survival medium composed by 50% basal medium with Earle's salts, 25% Hanks' buffered salt solution, 25% horse serum, and 5 mg/ml glucose at 37°C. For the induction of demyelination, at 4 days *in vitro* (DIV) slices were incubated with fresh medium including lysolecithin (Lysophosphatidylcholine, LPC, Sigma-Aldrich) overnight (15–17 hr) at 37°C. After incubation, the LPC-containing medium was replaced with fresh medium.
Slices from 3 millicells (12 slices) were lysed together 3-days post-demyelination with 500 µl of Trizol (Qiagen) for the subsequent RNA extraction.

3.5.3 Lysolecithin-induced de-myelinating model and lentiviral infection. Focal demyelination was obtained in 3-4 month old C57BL/6 mice by a bilateral stereotaxic injection of 1ul of a 1% LPC in 0.9% NaCl solution into the subcortical white matter at coordinates: 1 mm medio-lateral, 0.1 mm rostral to bregma and at 1.2 from the cortical surface. Lentiviral infection (stereotaxic injection of 1ul of 10^9-concentrated replication-incompetent viral particles) was performed in the same sites 9 days later. Animals were sacrificed by intracardial perfusion at 21 days post lesion. Brains were explanted and post-fixed in 4% PFA for 1 hour. Immunohistological procedures were performed according to standard protocols (Boda *et al.*, 2011). Brain slices were stained to detect the expression of different antigens: GFP (1:700, Invitrogen); NG2 (1:200, Millipore). Quantitative evaluations (marker coexpression in GFP-tagged infected cells) were performed on confocal images by employing the Neurolucida (MicroBrightfield, Colchester, VT) or ImageJ (Research Service Branch, National Institutes of Health, Bethesda, MD) softwares. Measurements derived from at least 3 sections per animal. At least three animals were analyzed for each experimental condition.

4- RESULTS

4.1 MiR-125a-3p potentially targets genes involved in oligodendrocyte differentiation

To evaluate the potential relevance of miR-125a-3p in oligodendrocyte differentiation and myelination, we first performed an *in silico* analysis on mouse databases. We used MyMir, a software that performs meta-predictions based on integration, filtering and re-ranking of outputs produced by different miRNA databases and we obtained a list of 1,673 unbiased putative target transcripts of this miRNA. Then, by using STRING, a Gene Ontology (GO) based tool, we found that these targets were significantly clustered in 485 GO biological processes (GO BPs), suggesting that miR-125a-3p can take part in their regulation. According to previous findings showing that miR-125a-3p is a brain-enriched miRNA, we highlighted that many GO BPs include nervous system development, neurogenesis, glial cell differentiation, myelination, and oligodendrocyte differentiation (Fig. 4.1). For all of them, we calculated and reported the fold enrichment in miR-125a-3p targets (see equation (1) in Methods) and we found that the BPs related to oligodendrocyte differentiation have a higher enrichment among all the CNS-related BPs, strongly supporting the hypothesis that miR-125a-3p takes part into oligodendroglial cell maturation.

4.2 Time-regulated expression of MiR-125a-3p in cultured OPCs

To assess the expression of miR-125a-3p in the CNS, we measured its levels by means of real-time PCR in several rodent and human tissues and cells. The analysis showed that indeed, miR-125a-3p it is more abundantly present in the CNS of both rat and human. In adult rat, the highest expression was found in both total brain and spinal cord, whereas very low levels were found in kidney, heart, lung, stomach and liver (Fig. 4.2a). We performed the same analysis on human samples, and, among the analysed tissues, miR-125a-3p was found mainly expressed in brain tissues, with a significant enrichment in frontal cortex compared to whole brain and also in brain stem and corpus striatum (Fig. 4.2b). In line with the rodent data, lower expression levels were found in kidney and liver, confirming the brain enrichment of this miRNA. Then, we asked whether miR-125a-3p is regulated in rat developing brain. To this aim, we collected RNA from total rat brain at different ages starting from embryonic day 14 (E14) to adulthood (6 months of age).

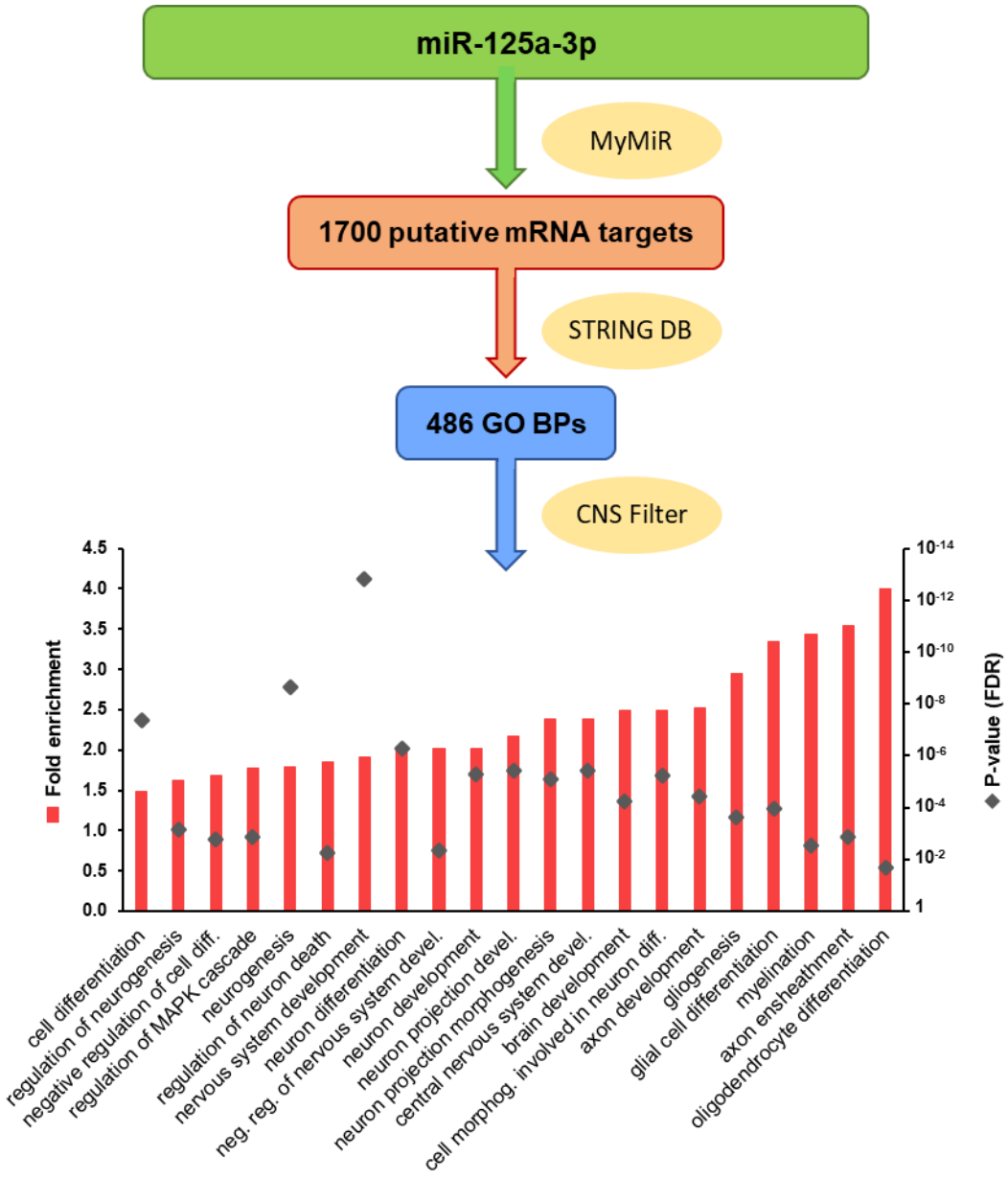

Figure 4.1 Gene Ontology biological processes (GO BPs) related to oligodendrocyte development are enriched in miR-125a-3p targets. The genome-wide miRNA target identification tool MyMir was used to obtain the list of 1700 miR-125a-3p putative targets. These potential target genes were then significantly clustered in 486 GO BPs by means of STRING database. For each category related to nervous system development the fold enrichment in miR-125a-3p targets was calculated (see methods). Histograms in red show the fold enrichment in miR-125a-3p targets for each selected GO BP (fold enrichment compared to expected value = 1; see left axis). Gray diamonds represent p-value (with FDR correction) of the prediction for each GO BP (see right axis; $\log_{10}$ scale).

Levels of miRNA were already high at E14, gradually declined up to postnatal day 0 (P0), remained low after birth up to P5, increased immediately before myelination onset and then remained mostly stable until adulthood (Fig. 4.2c).

To evaluate the distribution of this miRNA in the different neural cell types during brain development, we took advantage of in situ hybridization (ISH) in combination with immunohistochemistry for cell markers, namely Nestin for multipotent neural stem cells, NeuN for neurons, GFAP for astrocytes, Olig2 for oligodendrocytes and Iba1 for microglia. In embryonic brain, miR-125a-3p was found widespread in the whole parenchyma with a higher signal in the ventricular zone (Fig. 4.2d), where it mostly co-localized with Olig2, which, at this stage, labels a pool of precursor cells that will give rise to both OPCs and neurons (Takebayashi *et al.*, 2000), and to a lower extent with Nestin. In postnatal brain, the expression of miR-125a-3p is restricted to neurons, both in cortex and striatum, and oligodendrocytes (Fig. 4.2e), whereas only a few cells also expressed GFAP and no apparent co-localization was found with microglia.

Then, we focused on oligodendrocytes, and evaluated whether miR-125a-3p is preferentially expressed in early OPCs expressing the proteoglycan NG2, or in more mature $CC1^+$ oligodendrocytes. To this aim, we performed a triple immunofluorescence staining using the same ISH technique described above for miR-125a-3p in parallel with anti-NG2 and anti-CC1 antibodies in both postnatal (P7) and adult brains, in particular, corpus callosum, where a large number of oligodendrocytes could be analysed. In postnatal brain, some cells already expressed CC1 and virtually all of them were also decorated with the probe for miR-125a-3p (Fig. 4.2e). As expected, the number of $CC1^+$ cells in the adult brain was much higher, but also in this case, virtually all cells expressed miR-125a-3p. Both in P7 and adult brains, co-localization between miR-125a-3p and NG2 was also found, but only in a subpopulation of cells, with a further trend toward a decrease in the adult, suggesting that miR-125a-3p serves a more critical function in mature oligodendrocytes. Then, to assess if the expression timing of miR-125a-3p indeed changes during oligodendrocyte maturation, we used cultured OPCs to measure its expression during their *in vitro* differentiation.

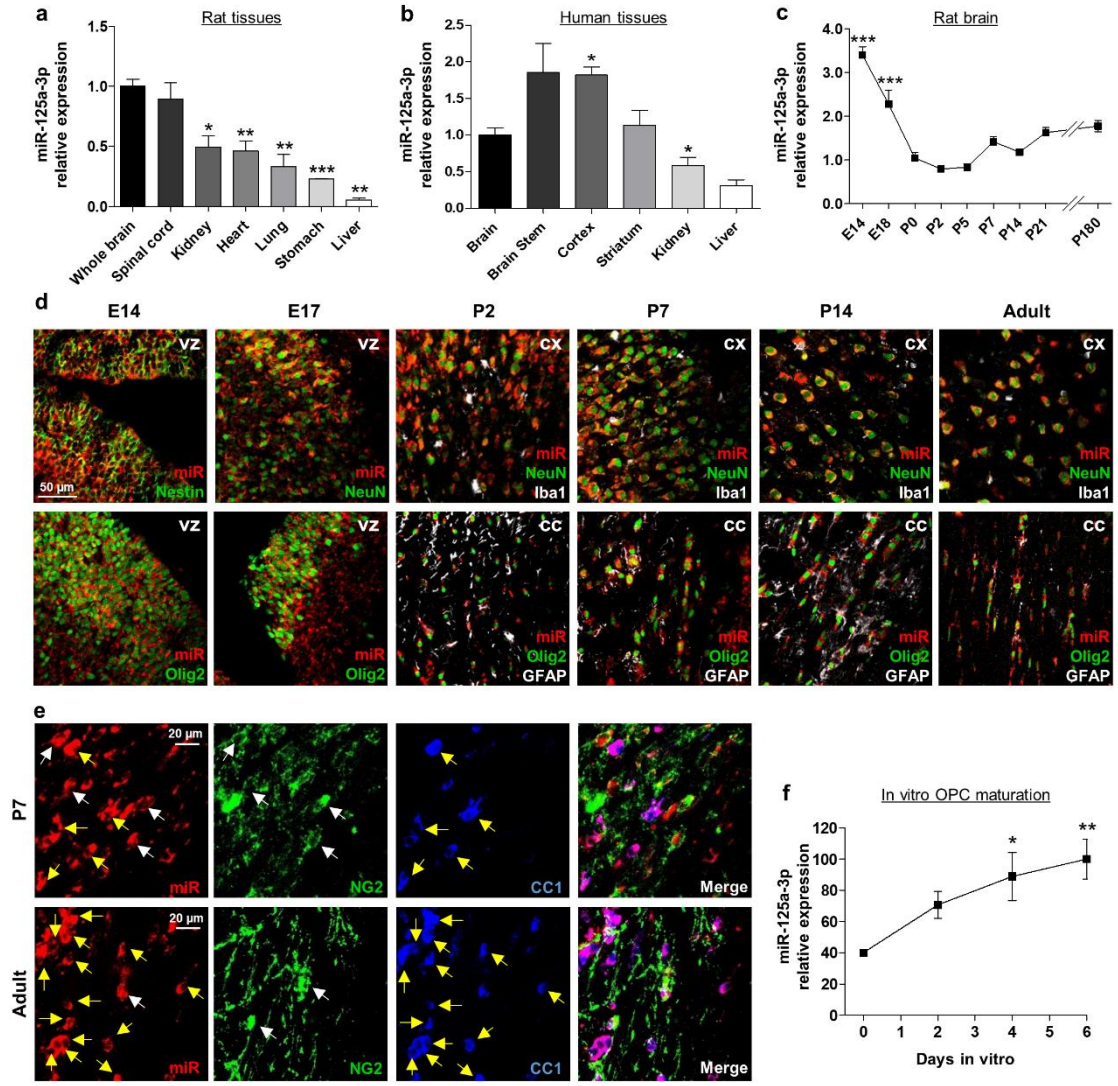

Figure 4.2 Expression of miR-125a-3p in physiological conditions. (**a, b**) miR-125a-3p relative expression was analyzed using Taqman assay in different rat and human tissues and compared to level measured in brain. Neural tissues showed higher expression with respect to other tissues. Data were expressed as mean ± SEM, n = 3; two-tailed unpaired t test *p < 0.05, **p < 0.01, ***p < 0.001 vs. total brain. (**c**) The levels of miR-125a-3p were analyzed during rat brain development and normalized to the levels at P0. Data were expressed as mean ± SEM, n = 4 animals for each time-point; one-way ANOVA with Dunnett's post-test ***p < 0.001 vs. P0. (**d**) In situ hybridization of miR-125a-3p (red fluorescence) in combination with different lineage markers during rat brain development from embryonic day 14 (E14) to adulthood. In embryonic brain, the ventricular zone (vz) was chosen. In postnatal brain neurons stained with NeuN were shown in cerebral cortex (cx), whereas Olig2^{+} oligodendrocytes were shown in corpus callosum (CC). Iba1 and GFAP were shown in triple stainings (white pseudocolor) to identify microglia and astrocytes, respectively. (**e**) In situ hybridization of miR-125a-3p (in red) in double staining with both NG2 (early OPC marker; in green), and CC1 (marker of mature oligodendrocytes; in blue). Micrographs show representative pictures of corpus callosum of P7 rats (upper panels) and adult rats (lower panels). White arrows highlight co-localization of miR with NG2, while yellow arrows refer to co-localization with

CC1. (**f**) Time-course of mir-125a-3p expression in OPCs was analyzed in differentiating conditions and normalized to the levels in undifferentiated OPCs (day 0). MiR-125a-3p levels progressively raised, reaching a 2.5-fold increase after 6 days in culture. Data were expressed as mean ± SEM, n = 6; one-way ANOVA with Dunnett's post-test; *p < 0.05, **p < 0.01 vs. day 0.

Interestingly, the levels of miR-125a-3p progressively increased in OPCs kept in cultures under differentiating conditions with the triiodothyronine hormone (T3), reaching a 2.5-fold increase after 6 days (Fig. 4.2f) compared to the initial proliferating conditions (day 0, in the presence of growth factors and in the absence of T3).

Globally, the *in vivo* and *in vitro* data suggest multiple developmental roles for miR-125a-3p, that, during embryonic life, is expressed at high levels in neural undifferentiated precursors likely to prevent their untimely commitment, and that, within cells of the oligodendroglial lineage, it could crucially regulate maturation by repressing early transcripts important for the maintenance of OPCs at an undifferentiated state.

4.3 Over-expression of miR-125a-3p delays oligodendrocyte precursor cell maturation and re-myelination.

Since we were interested in myelination, we investigated more in detail the possible role of miR-125a-3p during physiological differentiation of OPCs to mature oligodendrocytes, performing gain- and loss-of-function experiments.

When OPCs are cultured in the absence of growth factors and in the presence of the T3 hormone, they undergo rapid *in vitro* differentiation, whose phases can be easily monitored by staining cells for progressively more mature oligodendrocyte markers (drawing in Fig. 4.3).

To evaluate the possible role of miR-125a-3p in OPC differentiation, we overexpressed it by transfecting cells with a specific miR-125a-3p mimic the same day of growth factors withdrawal when the endogenous levels of miR-125a-3p were low. Then, we performed immunofluorescence staining for the myelin basic protein (MBP), a typical marker of mature oligodendrocytes and counted cells in mimic-transfected cultures and in control conditions (cells transfected with scramble double-stranded RNA). Two days after miR-125a-3p mimic transfection, the number of MBP^+ cells was strongly reduced compared to controls, suggesting an effect on terminal maturation (Fig. 4.3a-c).

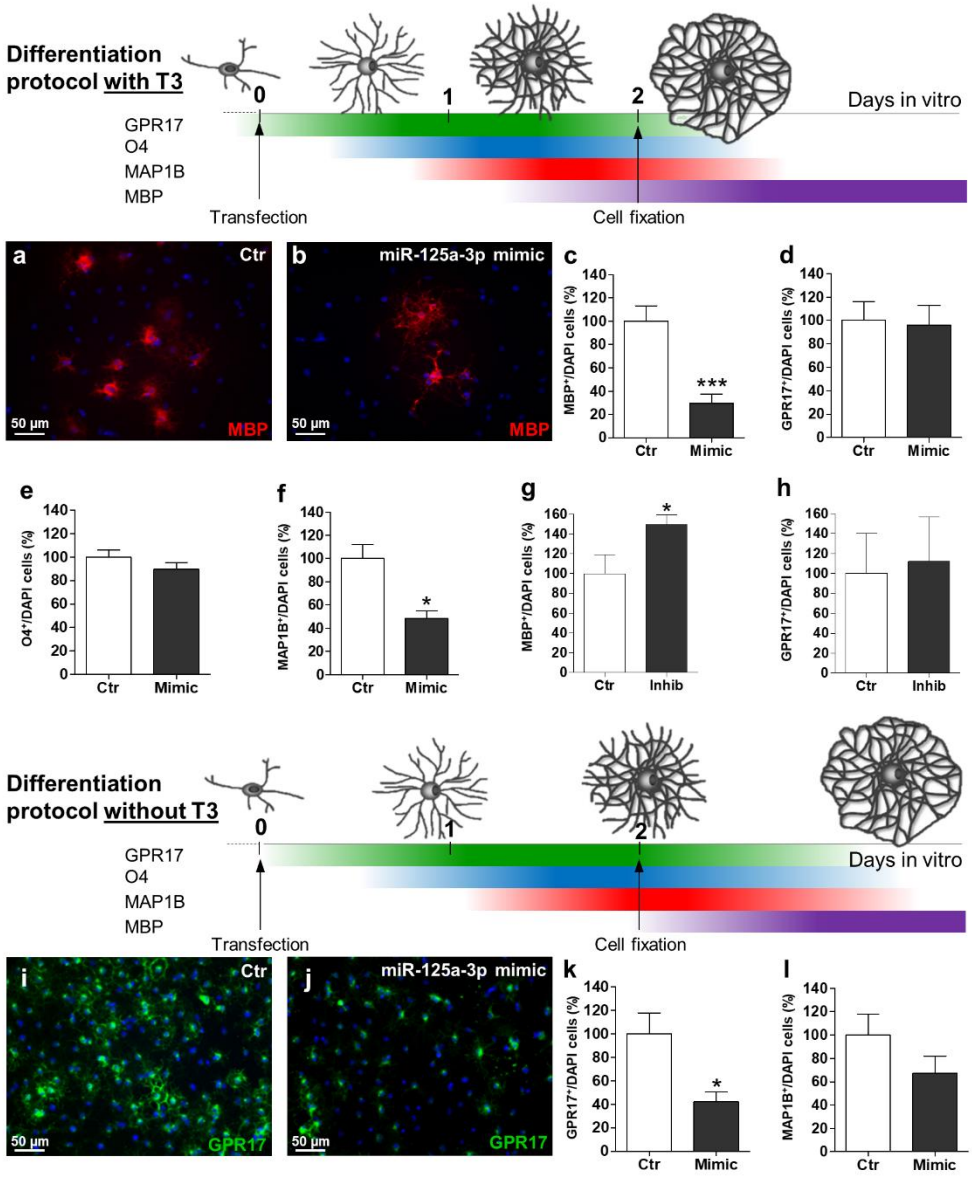

Figure 4.3 Effect of over-expression or inhibition of miR-125a-3p during oligodendroglial differentiation. To evaluate the time-dependent effects of either the overexpression or the silencing of miR-125a-3p on OPC maturation 4 different markers of progressive differentiation stages were analysed by immunocytochemistry, GPR17 and O4 (which label pre-oligodendrocytes), MAP1B (which labels a slight more advanced stage) and MBP (which labels terminally differentiated cells). Cells were stained for the selected markers 48 h after transfection of miR-125a-3p mimic or inhibitor, in the presence or in the absence of the T3 hormone. (**a-h**) The drawing represents a schematic timeline of the fast differentiation protocol in the presence of T3, showing the presented markers. (**a, b**) Representative micrographs of MBP-positive oligodendrocytes after transfection of miR-125a-3p mimic in presence of T3 were shown. (**c, d, e, f**) Histograms show the changes in the number of cells positive for MBP (n=10), GPR17 (n=8), O4 (n=2) and MAP1B (n=3) with respect to negative controls. Data were expressed as mean ± SEM, two-tailed unpaired t test *p < 0.05, ***p < 0.001 vs. negative control. (**g, h**) Transfection of OPCs with the

inhibitor of miR-125a-3p (in presence of T3) led to a significant increase in the number of MBP-positive (n=4; p-value=0.0376), but not GPR17-positive (n=7) cells. Data were expressed as mean ± SEM, two-tailed unpaired t test *p < 0.05 vs. negative control. (**i-l**) A second differentiation protocol in the absence of T3 (slower protocol) was used. Also in this case, the drawing represents a schematic timeline showing the presented markers. (**i, j**) Representative micrographs of GPR17-positive oligodendrocytes after transfection of miR-125a-3p mimic in absence of T3 were shown. (**k, l**) Histograms show a considerable decrease in the number of GPR17- (n=7) and MAP1B-positive (n=3) cells after transfection with the mimic in the absence of T3 with respect to the negative control. Data were expressed as mean ± SEM, two-tailed unpaired t test *p < 0.05 vs. negative control.

Since MBP is not present in the list of miR-125a-3p putative targets obtained from MyMir, we reasoned that miR-125a-3p inhibitory effect is likely exerted on upstream players in the pathway culminating with OPC maturation and MBP expression. Thus, we also counted cells positive for earlier oligodendroglial players, such as, sequentially, GPR17 (a marker of early precursors and intermediate immature oligodendrocytes), O4 (a marker of immature oligodendrocytes), and the microtubule associated protein MAP1B (that labels a slightly more advanced differentiation stage compared to O4, see drawing in Fig. 4.3). While the number of cells positive for GPR17 and O4 were unaltered (Fig. 4.3d, e), the number of $MAP1B^+$ cells was reduced by approximately the 50% after mimic miR-125a-3p treatment (Fig. 4.3f), suggesting inactivation of transcripts specifically involved in the progression between the O4 and the MAP1B stage. To confirm the importance of miR-125a-3p in OPC maturation, in a parallel experiment, OPCs maintained under the same culture conditions were transfected with a hairpin inhibitor RNA that specifically inactivates miR-125a-3p. A marked increase in the number of MBP^+ cells was found, yet with no significant effect on the number of $GPR17^+$ cells (Fig. 4.3g, h). Globally, these data suggest that miR-125a exerts a silencing effect on transcripts typical of OPC differentiation stages that are upstream to MAP1B but downstream to GPR17 and O4.

Then, we wondered whether mimic overexpression in cultured OPCs maintained under slower differentiating conditions could also affect the expression of these earlier transcripts.

To test this hypothesis, we cultured OPCs in the absence of both growth factors and T3. Under these settings, OPCs did undergo spontaneous differentiation in culture, but did not reach terminal maturation within the experimental observation times, thus representing a useful paradigm for our purpose (see drawing in Fig. 4.3). As expected, under this culturing protocol, almost no MBP^+ cells were detected; however, upon mimic overexpression, the number of both

GPR17$^+$ cells and of MAP1B$^+$ cells were strongly reduced (of -60% and -35%, respectively, Fig. 4.3i-l) compared to controls.

These data demonstrate that if differentiation proceeds at a lower speed, miR-125a-3p can also act on early targets, whereas, if differentiation is faster, miRNA can only act on later transcripts that are closer to terminal maturation. These data also suggest that the post-transcriptional regulation of genes involved in oligodendrocyte maturation vary depending on basal or stimulated conditions such as those occurring upon CNS damage, when recruitment and differentiation of OPCs is rapidly and markedly improved to sustain myelin repair (Reynolds *et al.*, 2002).

To evaluate whether miR-125a-3p over-expression could delay myelination or exacerbate a pathological condition, we took advantage of the lysolecithin model, in which the detergent lysolecithin (LPC) is used to induce a focal demyelination injury in the white matter.

In this model OPCs start to activate and proliferate in response to the toxic damage 3 days post lesion (dpl), they differentiate from 7 dpl and start to re-myelinate from 10 dpl, completing the process at 21 dpl. Stereotaxic injection of 1% lysolecithin was performed in the subcortical white matter of adult mice. The over-expression of miR-125a-3p was performed by lentiviral infection in the same site at 9 dpl, then animals were sacrificed at 21 dpl (Fig. 4.4a). The lentivirus is not selective for any cell types, but its infection can be followed by the GFP reporter protein (Fig. 4.4b), whose gene is carried by the vector. To evaluate the degree of maturation of infected cells we performed a double staining for NG2, marker of oligodendroglial precursor cells, and the reporter GFP. The analysis was performed comparing the number of NG2/GFP double-positive cells on the total amount of infected oligodendroglial cells, between mice receiving the lentiviral vector expressing miR-125a-3p and mice infected with lentiviral control. Interestingly, we found that miR-125a-3p over-expression increased the number of GFP positive infected cells that still express the early marker NG2 at 21 dpl compared to control (Fig. 4.4c), suggesting that miR-125a-3p over-expression delayed the maturation of these cells and hindered the re-myelination process.

Globally these data showed that high levels of miR-125a-3p can negatively modulate OPC maturation either in physiological and pathological context.

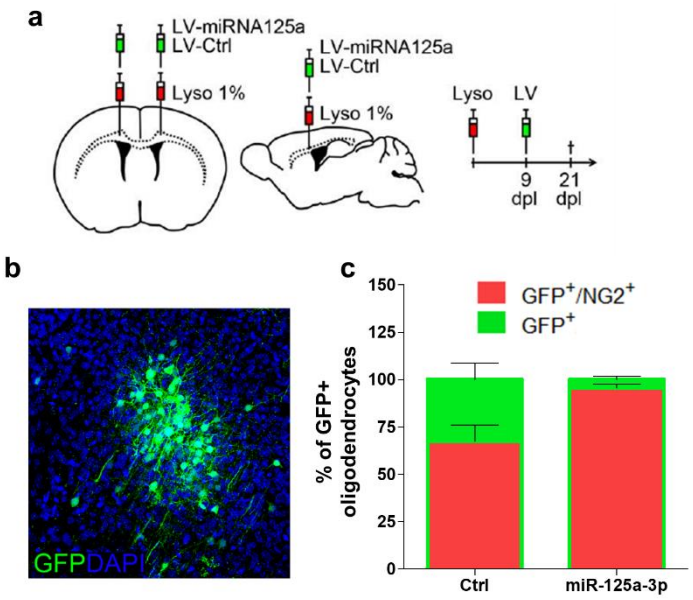

Figure 4.4 MiR-125a-3p over-expression inhibits re-myelination following lysolecithin (LPC) induced de-myelination. (a) Stereotaxic injection of the detergent lysolecithin (1%, 2 µl) was performed in the subcortical white matter of C57BL/6 adult mice to produces a focal demyelinating injury. Over-expression of miR-125a-3p was performed by lentiviral infection in the same site 9 days later, then animals were sacrificed at 21 days post-lesion. **(b)** Infected cells start to express the reporter green fluorescent protein (GFP). **(c)** The histogram shows the proportion of $GFP^+/NG2^+$ and $GFP^+/NG2^-$ oligodendroglial cells after miR-125a-3p (n=5) over-expression compared to control condition (mice infected with lentivirus-CTR, n=3). Over-expression of miR-125a-3p caused an increase in the amount of oligodendrocyte precursors expressing NG2 over the total infected oligodendroglial cells (GFP positive oligodendrocytes), suggesting that miR-125a-3p over-expression produced an impairment in the maturation process.

4.4 MiR-125a-3p levels are up-regulated in experimental models of toxic de-myelination

To better elucidate whether miR-125a-3p could have a pathogenetic role in de-myelinating diseases, such as MS, we analysed its expression in two different models of toxic demyelination induced by either cuprizone or lysolecithin injection.

For the cuprizone induced demyelination study we used C57BL6 8 weeks old male mice. Animals received a 0.2% cuprizone supplemented diet for up to five weeks to induce de-myelination of the corpus callosum. Then, they were switched to standard diet to allow spontaneous re-myelination. 5 animals were sacrificed every week and gene expression analysis was performed on the explanted corpus callosum of each animal (Fig. 4.5a).

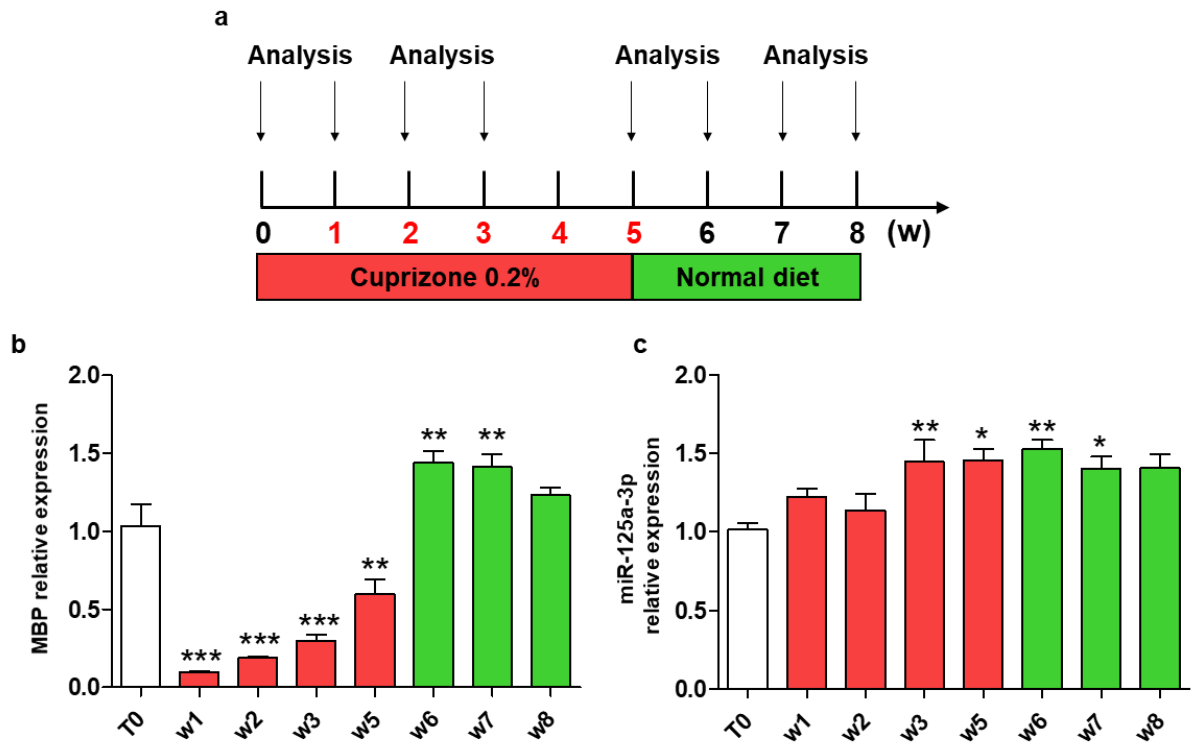

Figure 4.5 miR-125a-3p is up-regulated in the corpus callosum of mice in response to cuprizone-induced de-myelination. (a) C57BL6 adult mice were fed with a 0.2 % cuprizone supplemented diet up to 5 weeks to induce de-myelination of the corpus callosum and then they were switched to normal diet up to 3 weeks to allows spontaneous re-myelination. 5 animals were sacrificed for each time-point and the corpus callosum was explanted from each animal for the independent evaluation of MBP and miR-125a-3p expression levels. **(b)** MBP expression levels strongly decreased after just a week of cuprizone administration (W1), remained low until the switch to normal diet (W2-3-5) and then they returned to basal levels (W6-7-8), confirming a strong de-myelination of the corpus callosum followed by a successful re-myelination. Data were expressed as mean ± SEM; * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ One-way ANOVA with Tukey's multiple comparison test. **(c)** MiR-125a-3p levels were up-regulated in the corpus callosum after 3 weeks of cuprizone administration (W3), suggesting that the detrimental behavior can induce its expression. Data were expressed as mean ± SEM; * $p \leq 0.05$, ** $p < 0.01$ One-way ANOVA with Tukey's multiple comparison test vs T0.

As expected, MBP expression levels dramatically decreased after just 1 week of cuprizone treatment, confirming the correct de-myelination of the corpus callosum, and, albeit progressively rising, remained lower than control until the switch to normal diet. Then, MPB levels returned to basal levels, demonstrating the successful re-myelination (Fig. 4.5b). Interestingly, we found that miR-125a-3p expression was up-regulated after 3 weeks of cuprizone administration and remained high also after the switch to the normal diet (Fig. 4.5c).

To univocally correlate miR-125a-3p alteration to demyelinating conditions we took advantage of organotypic cerebellar cultures, another well-established model to investigate demyelination and re-myelination processes. Cerebellar slices were obtained from p10 mice and then cultured with high concentration of growth factors to promote their survival (see Methods).

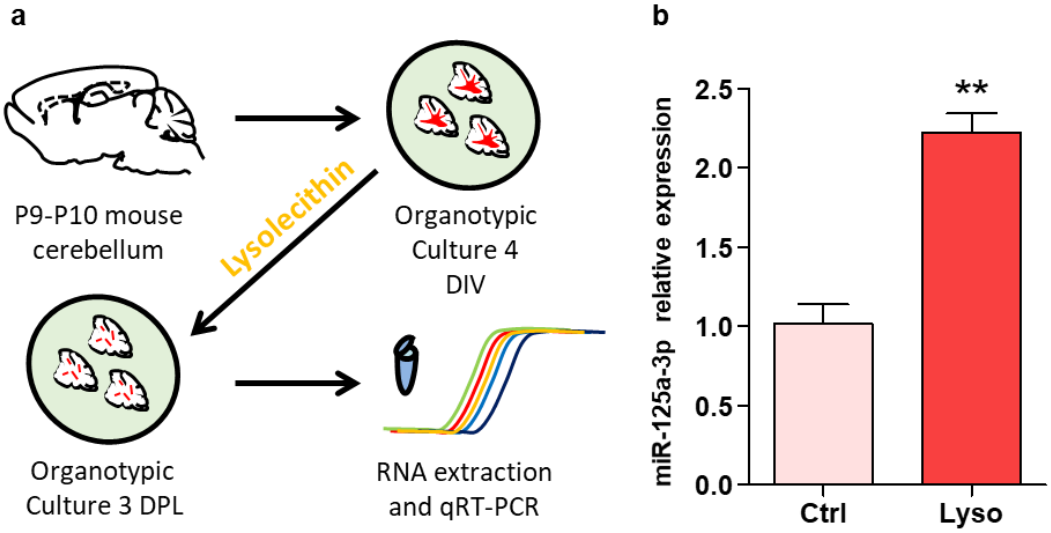

Figure 4.6 MiR-125a-3p is up-regulated in organotypic cerebellar cultures following lysolecithin induced de-myelination. (a) Cerebellar slices were obtained from p10 mice and cultured in presence of growth factors for 4 days. Then they were treated with the detergent lysolecithin to induce local demyelination and lysed at 3-days post-lesion to analyze miR-125a-3p expression levels. DIV = days in vitro **(b)** A significant up-regulation of miR-125a-3p levels was found 3 DPL (days post lesion), suggesting that this alteration could be a hallmark of white matter demyelination. Data were expressed as mean ± SEM; ** $p<0.01$ Unpaired t test.

Slices were treated over-night with the detergent lysolecithin to induce local de-myelination and they were lysed 3 days post lesion for gene expression analysis (Fig. 4.6a). Interestingly, also in this model miR-125a-3p was significantly up-regulated in the de-myelination phase (Fig. 4.6b), suggesting that its up-regulation could indeed represent a hallmark of white matter de-myelination.

4.5 MiR-125a-3p is up-regulated in the cerebrospinal fluid of multiple sclerosis patients

MiRNAs are stable in body fluids and their alterations in plasma and cerebrospinal-fluid (CSF) may represent novel biomarkers for the diagnosis and prognosis of human diseases. It

has been proposed that circulating miRNAs are originated by passive leakage from broken cells, thus reflecting the disease state of injured tissues.

Since progressive loss of the ability of OPCs to generate mature oligodendrocytes is a well-known feature of both human MS and other demyelinating diseases, we wondered whether miR-125a-3p was also altered in the CSF of MS patients. We thus measured miR-125a-3p levels in CSF samples from 30 MS patients (28 patients with the relapsing-remitting and 2 with the secondary-progressive form), 11 of which had clinically or neuroradiologically active disease (active lesions) at the time of CSF withdrawal. We compared MS patients to a control group mainly encompassing non-Alzheimer dementias and normal pressure hydrocephalus patients, and to a pure Alzheimer's patients group (AD) (demographic data of the patients are summarized in Table 4.1).

Diagnostic group	n	Sex F:M	Age y, mean	miR-125a-3p log2 fold increase	p-value vs CTRL
HC	13	1.6	75.9	0.000 ± 0.141	-
AD	17	2.4	72.6	0.030 ± 0.125	0.645
Active-MS	11	1.75	32.1	0.700 ± 0.185	0.003
Inactive-MS	19	3.75	34.8	0.152 ± 0.178	0.469

Table 4.1 Demographic data and miR-125a-3p log2 fold increase by diagnostic group.

Quantitative real-time PCR analysis revealed a significant upregulation (up to 4-fold) of miR-125a-3p in the active MS group compared to both HC, AD and inactive MS groups (Fig. 4.7). Applying One-way ANOVA to consider multiple comparisons, active MS was still significantly different from the control group ($p=0.02$). Although these findings are not sufficient for proposing miR-125a-3p as a biomarker for MS, our results indeed suggest its potential clinical relevance in this disease.

4.6 MiR-125a-3p can modulate OPC maturation acting on multiple targets

It has been previously shown that the receptor GPR17 play an essential role in the regulation of oligodendrocyte maturation and that its early silencing leads to the block of OPC

differentiation. Interestingly, GPR17 was predicted as a direct target of miR-125a-3p. Thus, we decided to explore the possibility that GPR17 down-regulation observed after miR-125a-3p over-expression (Fig. 4.3k) could be the result of a direct interaction between this miRNA and the GPR17 transcript.

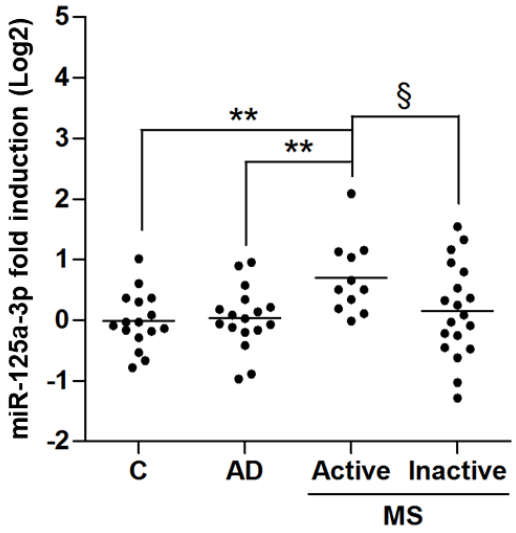

Figure 4.7 MiR-125a-3p is upregulated in the cerebrospinal fluid of MS patients in active phase. MiR-125a-3p levels were analyzed in the cerebrospinal fluid of AD and MS patients and compared to a control group mainly encompassing non-Alzheimer dementias patients. A significant increase in the relative expression of miR-125a-3p was found by qRT-PCR in the cerebrospinal fluid of active (relapsing) MS patients (n=11) compared to control (C; n= 13; p value = 0.004), Alzheimer (AD; n=17; p value = 0.004) and inactive (remitting) MS (n=19; § p=0.0545) groups. Data were expressed as mean ± SEM; * p≤0.05, ** p<0.01 Unpaired t test.

First, we used the web tool RNA22 to identify the number and the location of all miR-125a-3p hypothetical binding sites and their corresponding heteroduplexes in the GPR17 3'UTR. Thanks to this bioinformatic approach we identified 7 hypothetical binding sites in the 4 Kb GPR17 3'UTR and we selected a short region of 736 bp, containing the 3 more-conserved sites in mouse and rat, to be used for a specific reporter assay (Fig. 4.8a).

This region of 736 bp was cloned into a pmiRGLO construct (which owns constitutive luciferase activity) downstream a gene encoding for a luciferase enzyme under the control of a moderate-strength promoter. To assess the ability of miR-125a-3p to bind GPR17 3'UTR, we co-transfected this construct together with the miR-125a-3p mimic in COS7 cells, an easy to transfect cell line usually used in this type of experiments, and we performed a luciferase

reporter assay (Fig. 4.8b), comparing the bioluminescence produced by the luciferase in this condition with respect to control (scramble double-stranded RNA and mock transfection). The interaction of the miRNA mimic with the predicted target sites downstream the luciferase gene should lead to a reduced production of luciferase enzyme and a subsequent decrease in the bioluminescence signal. However, in our experiments miR-125a-3p was not able to decrease bioluminescence (Fig. 4.8c), suggesting that the effective binding site responsible for GPR17 down-regulation could be in another region of the 3'UTR or that the effect on GPR17 expression may be indirect.

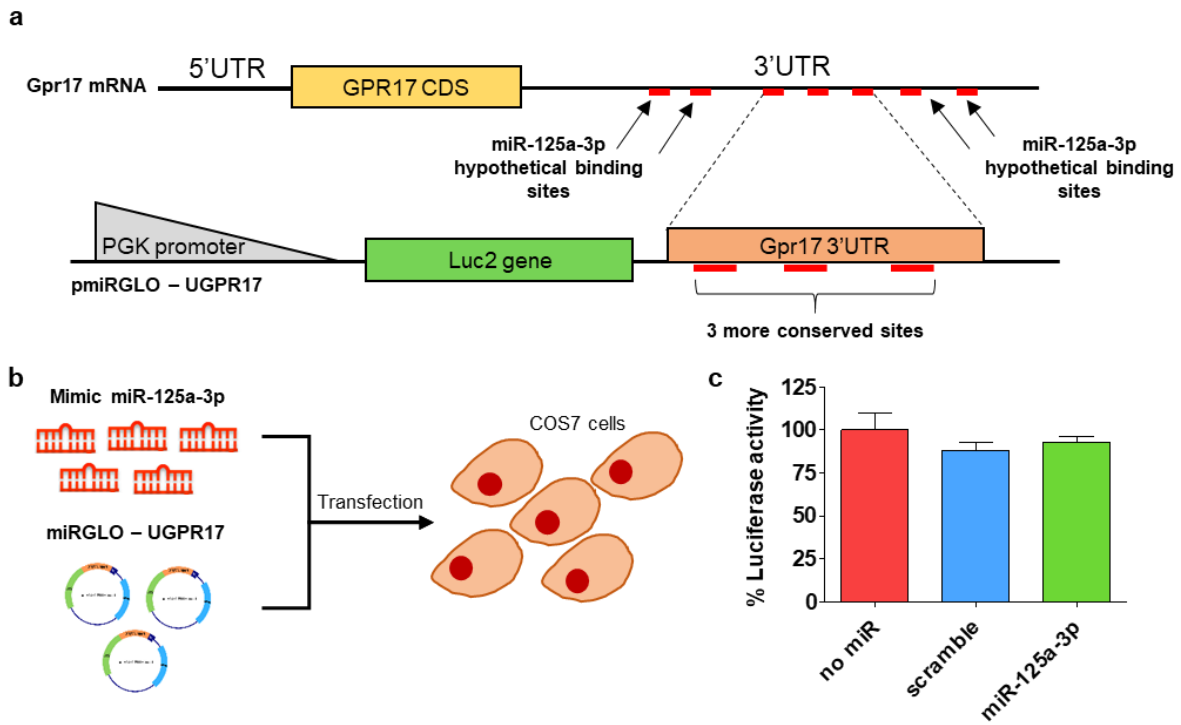

Figure 4.8 Evaluation of GPR17 as a direct target of miR-125a-3p (a) The presence and the location of miR-125a-3p binding sites in GPR17 3'UTR was assessed through the miRNA prediction tool RNA22. 7 hypothetical binding sites were identified. A portion of GPR17 3'UTR of 736 bp length, containing the 3 more conserved sites between rat and mouse, was cloned into a pmiRGLO construct downstream a gene encoding for a firefly luciferase (luc2) under the control of the moderate-strength PGK promoter. **(b)** MiR-125a-3p mimics and the reporter plasmid pmiRGLO-UGPR17 were co-transfected in COS7 cells and the ability of miR-125a-3p to interact with the inserted binding sites and affect the luciferase expression was assessed evaluating the luminescence signal produced compared to control (scramble miRNA or mock transfection) by a dual luciferase reporter assay. **(c)** The histogram shows that in this condition miR-125a-3p was not able to reduce the luminescence signal.

Based on the current knowledge, a single miRNA can interact with hundreds of target transcripts with "imperfect matches"; this unique feature enables a fine titration of several players of the same pathway at the same time. Since, as mentioned, Mbp is not a predicted target for miR-125a-3p, we used QIAGEN's Ingenuity Pathway Analysis tool (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) to evaluate how alterations of already validated targets of the myelination pathway such as SMAD4, Fyn, RhoA, p38 and NRG1 could potentially influence MBP expression, thus being responsible for the observed delay in oligodendroglial maturation. This analysis showed that not only NRG1 and Fyn, but also RhoA and p38 (that are downstream targets of Fyn signaling) can directly or indirectly promote MBP expression, suggesting that miR-125a-3p may limit oligodendroglial maturation through inhibition of some of these targets, if not all (Fig. 4.9a). To confirm this hypothesis, we performed quantitative real time PCR experiments after miR-125a-3p over-expression in OPCs, and found that the expected reduction of MBP mRNA was also accompanied by significant downregulation of both Fyn, NRG1 and MAP1B (Fig. 4.9b).

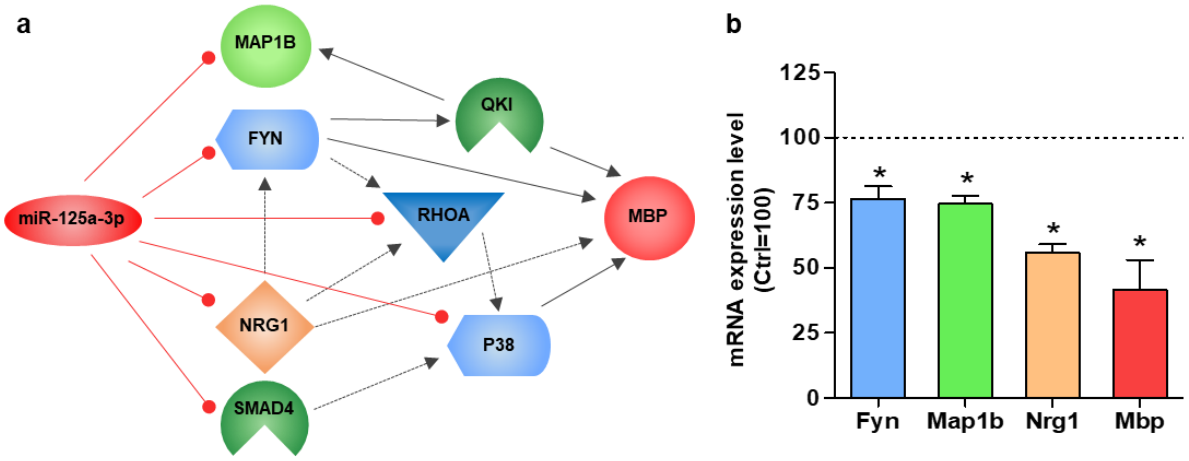

Figure 4.9 Model representing the connections between miR-125a-3p and MBP. (**a**) The model shows how miR-125a-3p can influence MBP expression, acting on some predicted (MAP1B) and validated targets (SMAD4, p38, RhoA, NRG1 and Fyn). All these direct targets converge in a pathway leading to the maturation of oligodendrocytes. (**b**) To confirm this mechanism qPCR experiments were performed after miR-125a-3p over-expression in OPCs, founding that both Fyn (n=3), NRG1 (n=3), MAP1B (n=3) and in turn MBP (n=5) mRNA were significantly down-regulated in these conditions. Data were expressed as mean ± SEM, two-tailed unpaired t test *p < 0.05 vs. negative control =100.

4.7 Transcriptomic analysis on OPC after miR-125a-3p over-expression

After showing the final effect of miR-125a-3p modulation on OPC differentiation, we decided to investigate the pathways influenced by its alteration, which could be responsible of the maturation impairment. To this purpose, we performed a transcriptomic analysis on OPCs after miR-125a-3p over-expression, applying the "fast" differentiation protocol (see Fig. 4.10a). Total RNA was hybridized on SurePrint G3 Rat Gene Expression Microarrays (#G4858A-074036, Agilent Technologies), which represent 30,584 rat transcripts.

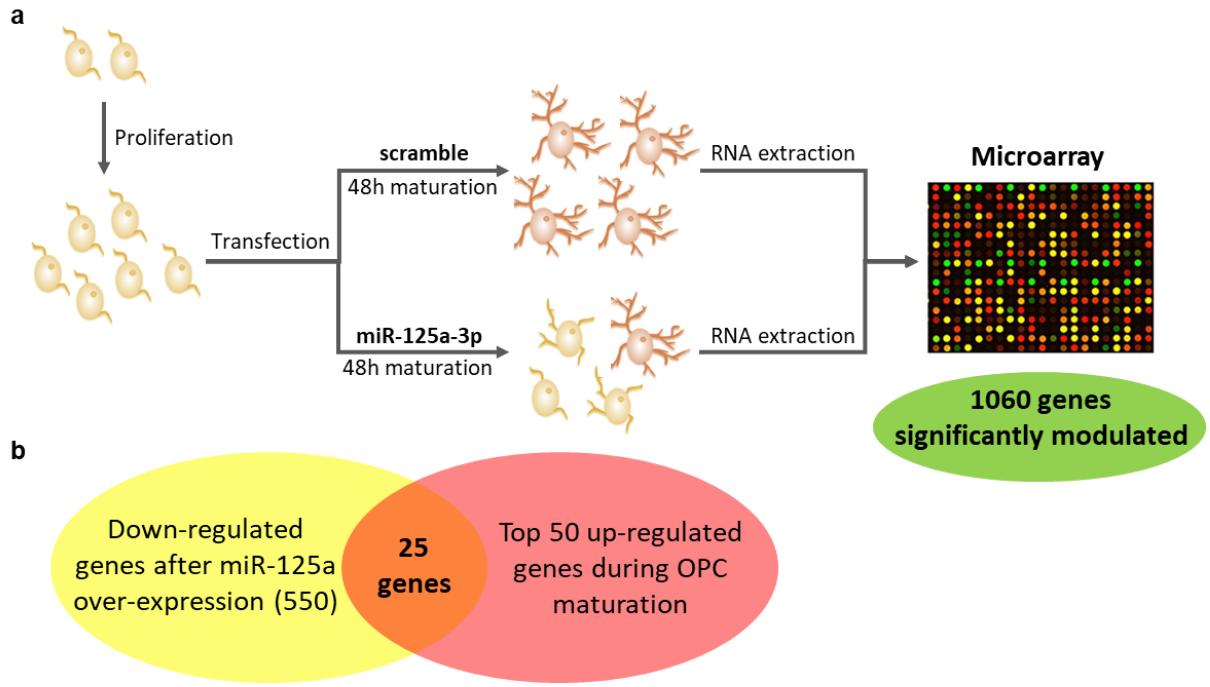

Figure 4.10 Gene expression profiling of OPCs after miR-125a-3p over-expression. (a) Primary cultured OPCs were grown for three days in presence of growth factors, then they were transfected with the negative control or miR-125a-3p mimic in presence of hormone T3 to promote cell maturation and finally lysed 48 hours after transfection, for the subsequent RNA extraction and microarray analysis. The microarray revealed 1060 genes significantly modulated after miR-125a-3p over-expression. **(b)** Among the top 50 up-regulated genes during OPC maturation (Dugas *et al.*, 2006) 25 genes were down-regulated in OPCs after miR-125a-3p over-expression, confirming the strong impairment in the maturation process.

A fold change of ±1.5 and a FDR-adjusted p-value < 0.05 were considered to obtain the list of genes differentially expressed between the two experimental conditions (miR-125a-3p transfected OPC vs scramble RNA transfected OPC). Thanks to this analysis we identified 1060 genes significantly modulated after miR-125a-3p over-expression.

To better understand the effect of miR-125a-3p over-expression on OPC maturation, as a first analysis, we crossed the list of down-regulated genes in our dataset with the top 50 up-regulated genes during OPC maturation (Dugas *et al.*, 2006). Interestingly, among the top 50 up-regulated genes, 25 were down-regulated (Fig. 4.10b), in line with our data demonstrating that miR-125a-3p over-expression exerts a strong negative effect on OPC differentiation.

Then, the list of differentially expressed genes was analysed by two different approaches: pathway oriented (IPA) and Gene Ontology-based analysis. The software IPA was used to perform an upstream regulator analysis, that allows to identify transcriptional regulators that may be responsible for the observed expression changes in the experimental dataset and to understand whether they are likely activated or inhibited.

Master regulator	Molecule type	Prediction	Z Score	p-value	Number of targets
TCF7L2	**transcription factor**	**Inhibited**	**-5.186**	**5.21E-08**	**27**
BDNF	growth factor	Inhibited	-2.381	4.23E-06	26
CXCL8	cytokine		-1.219	8.54E-06	8
1L1B	cytokine		1.349	3.26E-05	30
IFNG	cytokine	Activated	2.541	1.26E-04	36
TGFB1	growth factor		1.451	1.69E-04	55
LIF	cytokine		1.362	3.29E-04	14
STAT1	transcription factor	Activated	2.592	3.95E-04	14
IL6R	receptor	Activated	2.236	6.06E-04	7
MKNK1	kinase	Activated	2.714	6.47E-04	11

Table 4.2 Upstream regulator analysis. Differentially expressed genes were analyzed with the Ingenuity Pathway Analysis tool (IPA) performing an upstream regulator analysis, in order to identify possible alterations in the activity of master regulators of gene expression, that may be responsible for the changes observed in the experimental dataset. The table shows the most promising master regulators resulting from the analysis, with the relative p-value and number of targets modulated. Cut-off: Z= ±1. In green, regulators that are potentially strongly inhibited (Z score < -2); in red, regulators that are potentially strongly activated (Z score > 2). In bold, master regulator selected for further studies.

Among the potential candidates, we focused on TCF7L2 (Table 4.2), an essential effector of the Wnt pathway that can also work in a Wnt-independent manner and promote oligodendrocyte

maturation (Hammond *et al.*, 2015). Indeed, the analysis showed that the down-regulation of 27 genes in our experimental dataset could be related to an inhibition of the TCF7L2 signaling (Fig. 4.11), as suggested by the low Z score (-5.186).

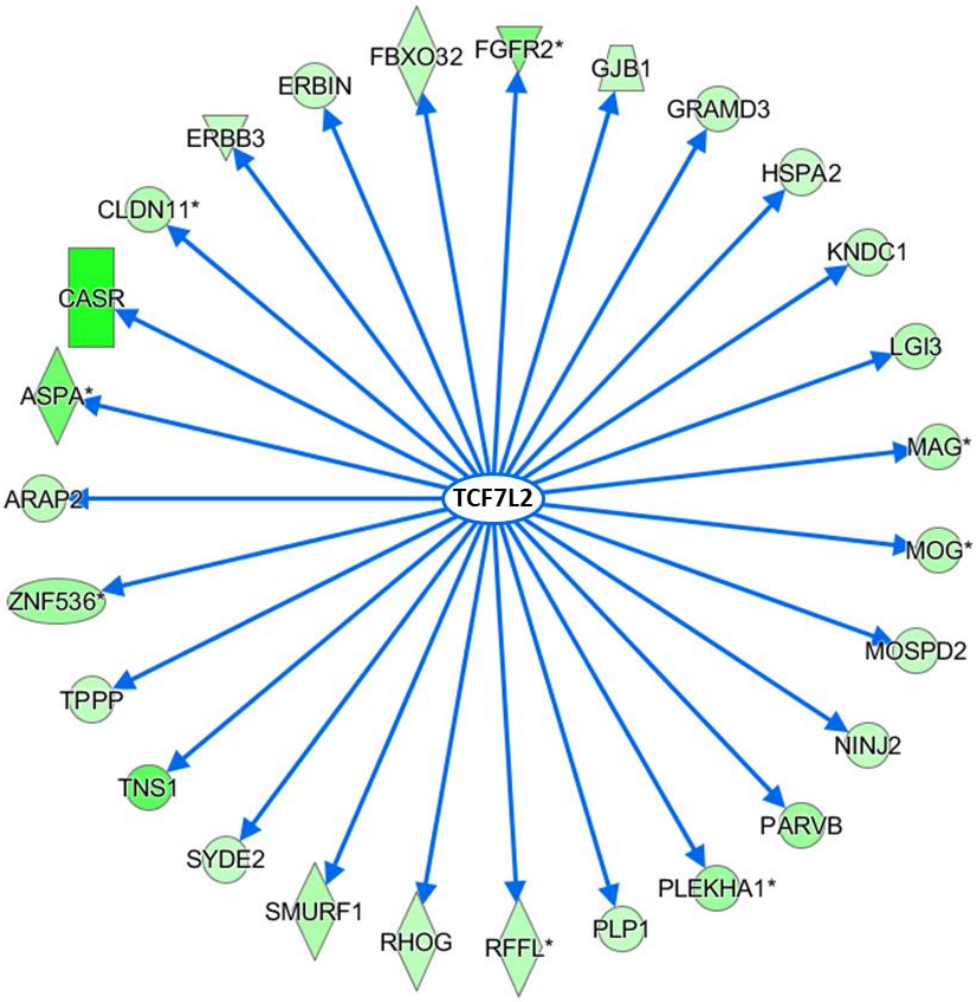

Figure 4.11 MiR-125a-3p over-expression represses TCF7L2 signaling in OPCs. Genes related to TCF7L2 signaling in the experimental dataset. The scheme shows that the alteration of multiple genes after miR-125a-3p over-expression could be related to TCF7L2 signaling inhibition. Blue arrows represent negative interaction (leads to inhibition). Green shapes represent transcripts down-regulated in the experimental dataset. Green intensity directly correlates to the entity of the down-regulation.

Based on this result and the opposite role that TCF7L2 can exert on oligodendrocyte maturation depending on its interaction partners, we hypothesized that the over-expression of miR-125a-3p lead to Wnt signaling over-activation and to an increased association of β-catenin to TCF7L2, preventing its pro-myelinating Wnt-independent effect. To assess this hypothesis, we utilized a

pathway focused "Wnt signaling targets" array, which simultaneously profiles the expression of 84 key genes responsive to WNT signal transduction (see Appendix C). After the over-expression of miR-125a-3p, expression changes were detected in 26 of 84 genes (Fig. 4.12a). The 14 down-regulated genes were: Bmp4, Enpp2, Fosl1, Fst, Jag1, Lrp1, Met, Mmp2, Nrp1, Pdgfra, Sox2, Tcf7l1, Tcf7l2 and Tle1. The 12 up-regulated genes were: Abcb1a, Birc5, Cdh1, Ctgf, Dlk1, Egr1, Fn1, Gja1, Klf5, Smo, Tcf7 and Wnt3a. Changes in the expression levels of Wnt3a, a Wnt ligand, and of Tcf7, Tcf7l1, Tcf7l2, downstream nuclear factors of the Wnt signaling, suggest an over-activation of the Wnt pathway (Fig. 4.12b).

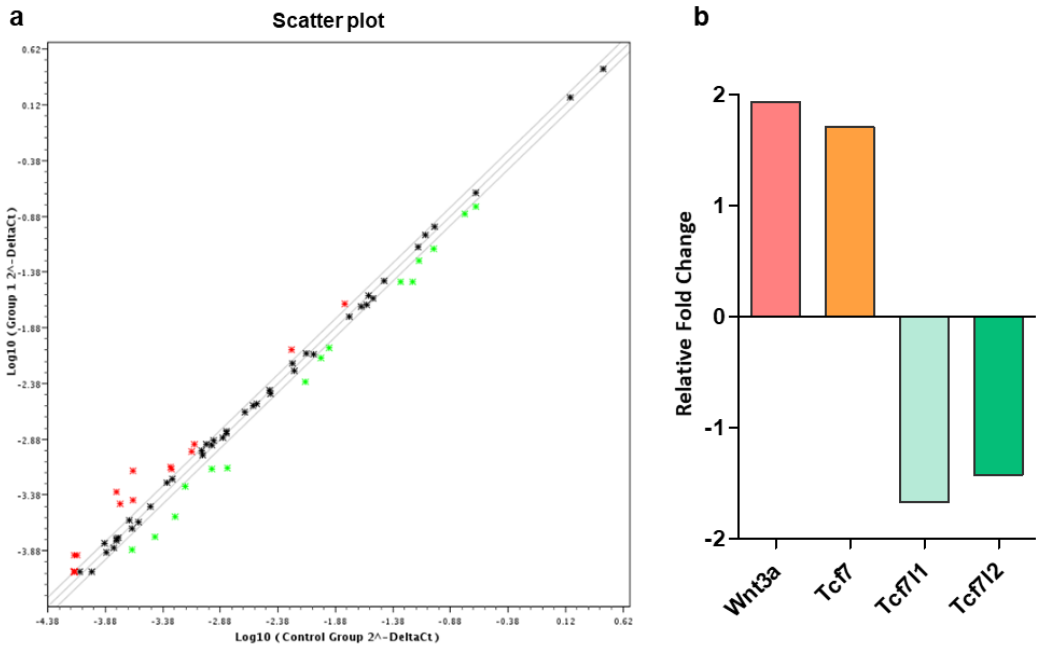

Figure 4.12 Evaluation of Wnt targets expression after miR-125a-3p over-expression in OPCs. (a) Scatter plot comparing the normalized expression of each genes of the "Wnt targets" PCR array between the two groups (miR-125a-3p over-expression vs negative control). Red asterisks represent genes up-regulated, green asterisks represent genes down-regulated. Black asterisks in the central line indicate unchanged gene expression. Cut-off = ±1.3 **(b)** Histogram shows changes in the expression of Tcf7, Tcf7l1 and Tcf7l2 after mir-125a-3p over-expression in OPCs (control set to ±1).

The Gene Ontology-based analysis allowed us to identify common biological processes and functions for differentially expressed genes, suggesting that miR-125a-3p can take part in their regulation. Biological processes resulting from the gene-ontology-based analysis are reported in Table 4.3. The most significant biological process, "Role of cell-cell and ECM-cell interactions in oligodendrocyte differentiation and myelination", was studied more in detail by means of a

specific PCR array, "Extracellular Matrix & Adhesion Molecules", that simultaneously profiles the expression of 84 genes important for cell-cell and cell-matrix interactions (see Appendix D). In the "Extracellular Matrix & Adhesion Molecules" array, after miR-125a-3p over-expression, expression changes were detected in 41 of 84 genes (Fig. 4.13a). The 12 down-regulated genes were: Col3a1, Fn1, Thbs2, Entpd1, Fbln1, Sparc, Icam1, Timp1, Thbs1, Col1a1, Spp1 and Lamb2. The 29 up-regulated genes were: Adamts1, Adamts5, Catna1, Cdh1, Cdh2, Cdh4, Cntn1, Col2a1, Col4a1, Col4a3, Itga3, Itga4, Itga5, Itga9, Itgav, Itgb1, Itgb3, Lama2, Lamc1, Mmp10, Mmp11, Mmp15, Mmp2, Mmp9, Spock1, Syt1, Tgfbi, Timp3 and Vcan.

Biological Process	Total	FDR p-value	N°of targets	Targets in the dataset
Role of cell-cell and ECM-cell interactions in oligodendrocyte differentiation and myelination	34	9.1E-05	9	Claudin-11, PLP1, Connexin 43, Connexin 26, Reticulon 4, MAG, GJC3, Myelin basic protein, Connexin 32
Cell adhesion_Gap junctions	30	1.6E-02	6	PKC, Connexin 43, Connexin 31, Connexin 26, Connexin 32, Tubulin beta
Role of Thyroid hormone in regulation of oligodendrocyte differentiation	48	2.6E-02	7	TR-beta, PLP1, p73, MOG, MAG, Myelin basic protein, OATP-A
ATM / ATR regulation of G2 / M checkpoint	26	3.1E-02	5	Chk1, Chk2, Cyclin B, Claspin, GADD45 beta
Substance P-stimulated expression of proinflammatory cytokines via MAPKs	43	3.9E-02	6	PKC-delta, PLC-beta, Substance P extracellular region, CCL13, c-Jun, GRO-2
Oxidative stress_Activation of NADPH oxidase	59	3.9E-02	7	PKC, PKC-delta, p47-phox, PLC-beta, p22-phox, TRIO, Rac2
HDL-mediated reverse cholesterol transport	44	3.9E-02	6	Pre beta-1 HDL, Nascent HDL, Large apoE-rich HDL, APOA1, PLTP, APOE
Cytoskeleton remodeling	102	5.3E-02	9	Fibronectin, MyHC, MYLK1, MLCK, Collagen I, TGF-beta receptor type I, TRIO, c-Jun, LIMK2

Table 4.3 Ontology-based clusterization with Metacore. The software Metacore was used to perform an ontology-based analysis of the 1067 differentially expressed genes, in order to identify common biological processes and functions. In table are reported the resulting more significant biological processes and the relative associated genes from the experimental dataset. In bold, biological processes selected for further studies.

Differentially expressed genes of interest were divided in two groups: adhesion molecules and ECM proteins. Among adhesion molecules, miR-125a-3p over-expression globally reduced the expression of connectin 1 (Cntn1), integrins (Itg) and laminins (Lam) (Fig. 4.13b). Among ECM

proteins, we observed alterations in the expression of disintegrin and metalloproteinases (ADAMTS) remodeling enzymes (Fig. 4.13c) and their inhibitors (TIMP).

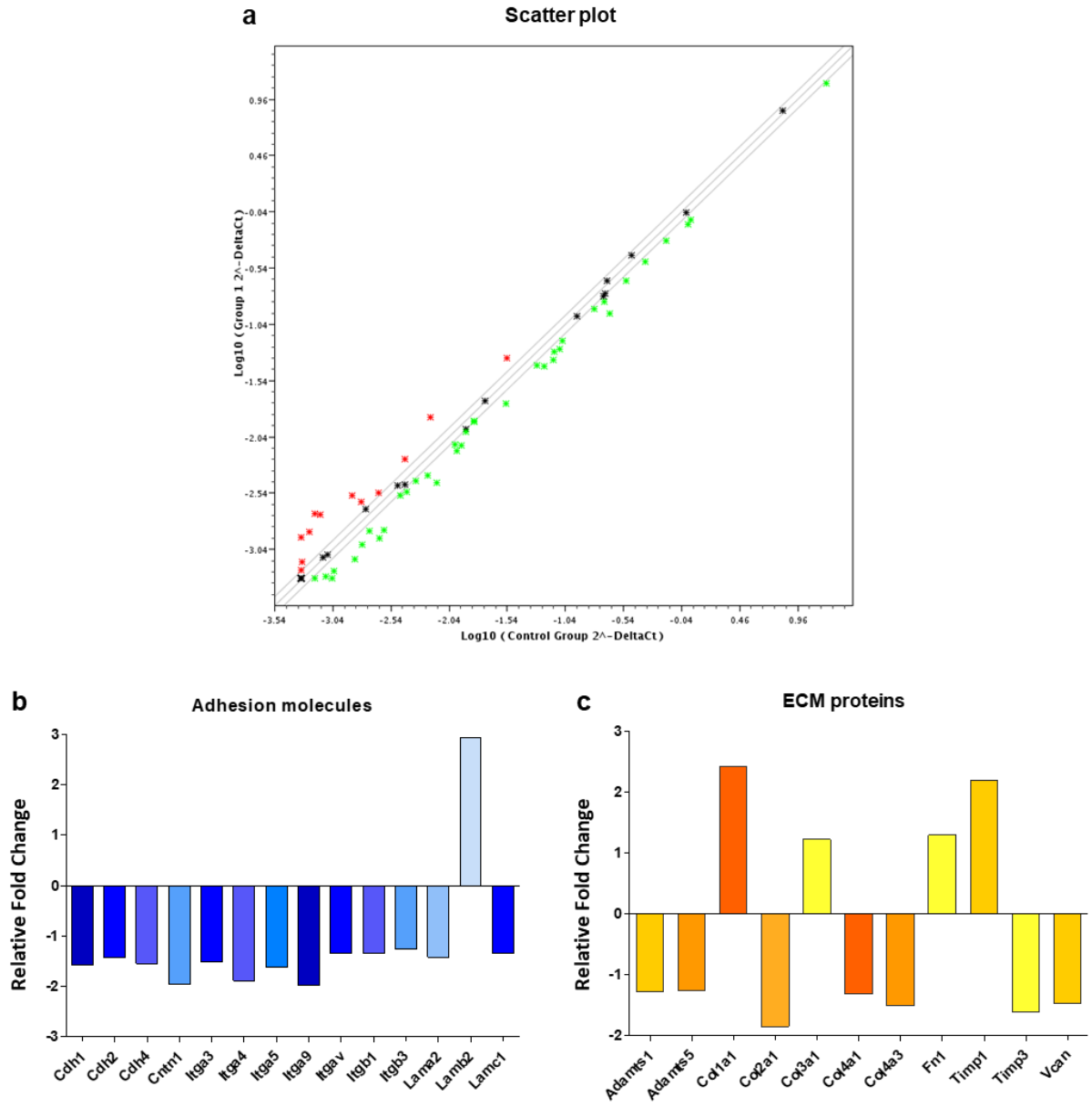

Figure 4.13 Evaluation of Extracellular Matrix & Adhesion Molecules after miR-125a-3p over-expression in OPCs. (a) Scatter plot comparing the normalized expression of each genes of the "Extracellular Matrix & Adhesion Molecules" PCR array between the two groups (miR-125a-3p over-expression vs negative control). Red asterisks represent genes up-regulated, green asterisks represent genes down-regulated. Black asterisks in the central line indicate unchanged gene expression. Cut-off = ±1.3 **(b)** Histogram shows changes in the expression of selected adhesion molecules (control set to ±1). **(c)** Histogram shows changes in the expression of selected ECM proteins (control set to ±1).

5- DISCUSSION

5.1 MiR-125a-3p over-expression in OPCs represents a new pathogenetic mechanism that can contribute to re-myelination failure following a demyelinating injury

Several papers have described the miR-125 family (composed by miR-125a and 125b) as one of the most important for its implication in cancer (Sun *et al.*, 2013). Only recently, the role of miR-125a has been investigated also under physiological settings, when a fine balance between proliferation and differentiation is responsible for cell lineage commitment. Despite previous studies demonstrating its presence in oligodendrocytes, with higher abundance of the -3p compared to the -5p strand (Jovicic *et al.*, 2013), no functional roles for this miRNA in these cells was demonstrated yet. Here, we describe miR-125a-3p as a new regulator of oligodendroglial differentiation and myelination and we propose that its pathological up-regulation could represent a new pathogenetic mechanism involved in re-myelination failure.

Our gene ontology based study showed that several of the predicted target mRNAs of this miRNA are involved in nervous system development, with higher enrichment in glial cell differentiation, myelination, axon ensheathment and oligodendrocyte differentiation (see Fig. 1), suggesting that miR-125a-3p may have a primary role in the regulation of these processes. We also showed that, both in rodents and humans, miR-125a-3p is more abundantly expressed in CNS with respect to peripheral tissues, with a higher expression in neurons and oligodendrocytes. Our expression studies during brain development showed very high levels of miR-125a-3p at embryonic day E14, mainly in neural precursors (see Fig 2). This is in line with previous literature data demonstrating that miR-125a directly binds SMAD4, a key regulator of pluripotent stem cell lineage commitment, thus potentiating early neural specification (Boissart *et al.*, 2012). Moreover, during postnatal life, SMAD4 and its cascade inhibit oligodendrogenesis by inducing the Id and Hes genes and by repressing Olig1 and Olig2 (Cheng *et al.*, 2007). Interestingly, by means of in situ hybridization, we found that miR-125a-3p is expressed throughout post-natal age in $Olig2^{+}$ oligodendrocytes, suggesting that, in these cells, may be necessary for silencing SMAD4, to promote the expression of these genes and the subsequent oligodendroglial specification.

Several miRNAs have been demonstrated to regulate the transition from neural stem cells to mature oligodendrocytes. Indeed, as OPCs differentiate, the levels of several miRNAs, including miR-138, -219 and -338, increase and promote maturation through the inhibition of repressors of oligodendrocytes differentiation, such as Hes5, Sox6, Foxj3, and of genes that promote OPC proliferation, such as Fgfr2 and Pdgfrα (Dugas *et al.*, 2010; Zhao *et al.*, 2010a). In line with the hypothesis of the modulation of early targets, during *in vitro* OPC differentiation miR-125a-3p is progressively up-regulated (Fig. 2f). In situ hybridization studies showed that almost all mature oligodendrocytes, positive for CC1, express miR-125a-3p, whereas only a subset of $NG2^+$ cells express it (Fig. 2e), suggesting a role in the repression of early genes when oligodendrocytes become mature and/or myelinating.

Recent literature has demonstrated that a single miRNA can regulate hundreds of transcripts, thus having a very broad array of functional consequences (Wu *et al.*, 2007). Thus, integration of the information on multiple targets of a given miRNA can shed light on very complex regulatory networks like those of transcription factors. In this respect, our data on the role of miR-125a-3p on OPCs maturation under "strong" or "mild" differentiation paradigms unveil that myelination may be regulated in different ways even by the same miRNA depending upon distinct pathophysiological conditions. Specifically, to understand the effect of miR-125a-3p on OPC differentiation *in vitro*, we took advantage of 4 different markers that identify 4 distinct sequential stages of OPC differentiation, namely: GPR17, O4, MAP1B, and MBP (Fig. 3). Among them, GPR17 is an oligodendroglial marker expressed in early OPCs up to the stage of immature pre-oligodendrocytes (Fumagalli *et al.*, 2011) and widely adopted to specifically label OPCs at an intermediate differentiation stage partially overlapping with O4 (Fig. 3; Boda *et al.*, 2011; Crociara *et al.*, 2013; Fumagalli *et al.*, 2015). O4 is instead a well-established marker of immature oligodendrocytes (Franke *et al.*, 2013). MAP1B is the major microtubule associated protein found in microtubules early during development (Wu *et al.*, 2001). In oligodendrocytes, MAP1B expression is slightly delayed compared to O4 and immediately precedes morphological differentiation, after which MAP1B is strongly down-regulated (Vouyiouklis and Brophy, 1993). Finally, MBP is known to label mature oligodendrocytes, being a protein associated to myelin (Baumann *et al.*, 2001). When miR-125a-3p was overexpressed by mimic

transfection in the presence of T3 ("strong" differentiation protocol), the block of terminal maturation was accompanied by a strong reduction of MAP1B and MBP expression, whereas earlier markers were not affected. However, when miR-125a-3p overexpression was performed using the "mild" differentiation protocol (i.e., in the absence of T3), OPCs proceeded slower along their lineage and late antigens were not visible, but the number of earlier $GPR17^+$ and $MAP1B^+$ precursors was diminished, to suggest that, under these conditions, miR-125a-3p can regulate OPC maturation not only downstream but also upstream to GPR17.

Globally, our *in vitro* data suggest that when miR-125a-3p levels are high, oligodendrocyte maturation is delayed, and this could explain the high expression levels found during embryogenesis, to suggest that miR-125a-3p may, at the same time, promote neural specification and prevent the untimely expression of genes necessary for oligodendrocyte commitment/maturation and the subsequent myelination process that, indeed, does not take place at this stage. Moreover, these findings show that the biological role of miR-125a-3p changes depending on the developmental stage, and that, from embryonic life to adulthood, its expression moves from brain ventricles to parenchyma up to neurons of the cerebral cortex, where it is likely to repress the expression of non-neuronal genes.

Recently, several studies have correlated alterations of miRNA homeostasis to the development of pathological conditions, typical of neurodegenerative diseases, showing that miRNAs can influence disease pathogenesis (Bicchi *et al.*, 2013). Our *in silico* and *in vitro* data suggested that alterations in miR-125a-3p levels may have a high relevance in demyelinating diseases such as multiple sclerosis, in which extensive re-myelination failure is responsible of the lesion formation and the subsequent neurological outcomes. To investigate the role played by miR-125a-3p both during demyelination process and re-myelination attempts, we evaluated its expression in two different mouse models of toxic de-myelination. In the first model, the toxicant cuprizone was supplemented to the diet and administered to animals for five weeks to induce de-myelination of the corpus callosum. During the de-myelination phase, we observed a significant up-regulation of miR-125a-3p after 3 weeks of cuprizone administration (see Fig. 4.5). At this time point, mature oligodendrocytes are almost completely depleted by the toxicant, whereas, NG2-positive precursors start to proliferate in response to damage and

migrate towards the corpus callosum (Gudi *et al.*, 2014), suggesting that miR-125a-3p up-regulation take place in these cells. In the cuprizone model, re-myelination is very robust and starts directly after demyelination is complete, regardless if cuprizone is further administered to animals (Gudi *et al.*, 2014). Thus, the persistent up-regulation of miR-125a-3p observed after cuprizone withdrawal could be partially explained by the increase in the number of mature oligodendrocytes, that, as we showed (see Fig. 4.2), normally express higher levels of miR-125a-3p compared to OPCs.

To further demonstrate the link between miR-125a-3p up-regulation and de-myelinating conditions we took advantage of organotypic cerebellar slices, a widely used model for study de-myelination and re-myelination processes (Zhang *et al.*, 2011). In this toxic model, the detergent lysolecithin is used to produce local de-myelination. As in the cuprizone model, OPCs respond to damage and start the re-myelination process, but in a faster and more reproducible way (Tan *et al.*, 2017). Interestingly, at 3-days post-lesion, we found a significant up-regulation of miR-125a-3p expression in cerebellar slices treated with lysolecithin (see Fig. 4.6), supporting our hypothesis.

Globally, our data in different models of de-myelination, show that OPCs up-regulate miR-125a-3p in response to a de-myelinating injury, suggesting that this alteration could represent a hallmark of de-myelination damage, however, this alteration is not sufficient to counteract the spontaneous re-myelination typical of these models.

To overcome such spontaneous re-myelination and assess if miR-125a-3p up-regulation may have a role in a pathological context, we over-expressed it by means of lentiviral infection in the white matter of mice following lysolecithin induced de-myelination. Interestingly, we found that miR-125a-3p infection maintained oligodendrocytes in the NG2-positive precursor stage (see Fig. 4.4), in line with a delay of their maturation and of the subsequent re-myelination process, suggesting that miR-125a-3p could represent a new pathogenetic mechanism that negatively regulate re-myelination. Other studies have recently shown how the modulation of a single miRNA can influence re-myelination efficacy. For example, it has been shown that infusion of miR-219 mimic promotes, whereas its inhibition represses, *in vivo* re-myelination

after lysolecithin-induced demyelinating injury (Wang *et al.*, 2017), likely modulating the expression of multiple inhibitors of myelination.

The identification of miR-125a-3p as a modulator of oligodendrocyte differentiation and myelination provide new findings in the complex regulation of de/re-myelination processes. Moreover, results from this study suggest that an antago-miRNA specific for miR-125a-3p may help in promoting oligodendrocyte maturation in diseases characterized by impaired myelin repair.

5.2 MiR-125a-3p controls oligodendrocyte maturation and myelination acting on multiple pathways

In the literature, several transcripts involved in pathways eventually linked to myelination have been so far identified as direct targets of miR-125a-3p. By using the Ingenuity Pathway Analysis tool, we connected several of these players to each other, building a model for the synergic regulation of MBP and myelination (see Fig. 4.9). Among these players, Fyn-kinase plays important roles in neuronal functions, myelination, oligodendrocytes formation and cytoskeletal rearrangements (Klein *et al.*, 2002). NRG1 is a potent chemoattractant that selectively regulates OPC migration during early CNS development stages of CNS through interaction with the ErbB4 receptor (Ortega *et al.*, 2012). In line with the prediction of our IPA analysis, overexpression of miR-125a-3p resulted in a reduction of Fyn, NRG1 and MAP1B (see Fig. 4.9). It is worth to note that despite a striking effect on MBP expression, the reduction of these genes was quantitatively smaller, supporting the hypothesis that the mechanism of action consists in a fine modulation of a network of genes. Moreover, miRNAs do not necessarily degrade their target mRNAs, but they can also impair their translation into protein (Pasquinelli *et al.*, 2012), thus, the downregulation observed by qRT-PCR may not fully reflect the observed functional effect. These data are also consistent with the previous demonstration that the mRNAs of both MAP1B and MBP are stabilized by the RNA-binding protein QKI, which is, in turn, regulated by Fyn-Kinase. Lack of Fyn-kinase, as detected after mimic transfection, leads to failure of MBP to incorporate into myelin (White *et al.*, 2008). Moreover, Fyn activation is mediated by phosphorylation of a tyrosine residue (Y420), which, in turn, depends on the interaction between NRG1 (which is downregulated after mimic transfection) and its receptor

ERBB4 (Bjarnadottir *et al.*, 2007). All these data support the hypothesis that miR-125a-3p synergistically inhibits different mechanisms that normally promote the expression of myelin genes. Interestingly, the miRNA prediction tool myMir reported that also GPR17 is a putative target of miR-125a-3p, suggesting that it could be regulated by miR-125a-3p during OPC maturation. Nevertheless, in our studies we were not able to demonstrate a direct interaction between the miRNA and GPR17 transcript, suggesting that the effect on GPR17 expression could be indirect.

To identify new mechanisms altered by miR-125a-3p during OPC maturation, we performed a transcriptomic analysis after its over-expression using the "strong" differentiation protocol (see Fig. 4.10). Interestingly, by matching the down-regulated genes in the dataset with the top 50 up-regulated genes during OPC maturation (Dugas *et al.*, 2006), we found 25 genes down-regulated, confirming our data showing that miR-125a-3p exerts a strong negative effect on OPC maturation.

Differentially expressed genes were analyzed with two complementary approaches to identify common pathways and biological processes.

A first pathway-based analysis, performed with IPA tool, suggested that the expression changes of several genes in the dataset may be related to TCF7L2 signaling inactivation (see Fig. 4.11). TCF7L2 is an important effector of the Wnt/β-catenin pathway, but, as recently shown, it can also act in a Wnt-independent manner by interacting with other co-factors (Kaiso and Sox10), to promote oligodendrocyte maturation (Zhao *et al.*, 2016). Since it is widely known that constitutive activation of Wnt/b-catenin inhibits oligodendrocyte maturation (Hammond *et al.*, 2015), we hypothesized that miR-125a-3p over-expression could induce an over-activation of the Wnt pathway, which, in turn, leads to an increased association of β-catenin to TCF7L2, preventing its pro-myelinating effects. Our PCR array analysis on Wnt signaling after miR-125a-3p over-expression in OPCs revealed an up-regulation of the Wnt ligand Wnt3a coupled to up-regulation of Tcf7 and down-regulation of Tcf7l1 and Tcf7l2 downstream effectors, expression changes that have been previously associated to Wnt-signaling stimulation (Kuwahara *et al.*, 2014), supporting our hypothesis.

A second ontology-based approach allowed us to identify common biological process for differentially expressed genes. The analysis revealed that miR-125a-3p can modulate several processes related to oligodendrocyte maturation and myelination, such as "adhesion molecules and ECM proteins", "gap junctions" and "thyroid hormone signaling". PCR array specific for "Extracellular Matrix & Adhesion Molecules" was used to simultaneously profile the expression of 84 genes involved in cell-cell and cell-matrix interactions. Our data showed that miR-125a-3p over-expression in OPCs altered the expression of several class of ECM and adhesion molecules, such as contactin (cntn), collagens (Col), integrins (Itg), laminins (Lam) and disintegrin and metalloproteinases (Adamts). Despite some of these class of molecules may have a dispensable role during OPC *in vitro* maturation, several reports in literature showed that such alterations are usually associated to dysmyelinating conditions, suggesting that our data could have a high relevance *in vivo*. For example, it has been reported that disruption of integrin-ECM connection leads to aberrant process and myelin sheath formation (Olsen *et al.*, 2005). Moreover, antibodies blocking β1-integrin reduce the ability of OPCs to extend their processes *in vitro* (Buttery and Ffrench-Constant, 1999). Interestingly, one of the downstream mechanisms that mediate integrin effects on OPC morphological differentiation is the activation of the Fyn kinase, which, in turn, regulates several downstream signaling, such as Rac1 and RhoA (O'Meara *et al.*, 2011), suggesting that, also in this case, miR-125a-3p can influence several actors of a common pathway. Other reports have shown that β1-integrin can also form a functional signaling unit by associating to contactin 1 (cntn1) and regulate Fyn activation by controlling its phosphorylation state (Laursen *et al.*, 2009). These data suggest that miR-125a-3p over-expression can not only directly influence Fyn expression, but also indirectly control its activity by modulation of upstream signaling molecules.

Down-regulation of Adamts1 and Adamts5, and their inhibitor Timp3, as well as up-regulation of Col1a1 and Col3a1, were instead found in human MS active lesions (Haddock *et al.*, 2006; Mohan *et al.*, 2010), suggesting that miR-125a-3p over-expression can recapitulate in OPCs some typical pathological features that they acquire in a demyelinating milieu.

Considering the mechanism of action of miRNAs, that not necessarily lead to the degradation of target mRNAs, a future proteomic analysis after miR-125a-3p over-expression will help to interpret and strengthen these transcriptomic data.

Globally, these data suggest that miR-125a-3p can modulate different aspects of OPC development, playing an essential role in cell differentiation, myelination and dysmyelination. The identification of new miR-125a-3p direct targets will allow not only to clarify the networks that orchestrate these mechanisms, but also to identify new pharmacological targets to foster re-myelination processes in diseases characterized by myelination defects.

5.3 MiR-125a-3p alteration in the CSF from human MS patients correlates with disease activity

Reports from oncology, cardiovascular research and infectivology have demonstrated the potential diagnostic and prognostic significance of miRNAs passively leaked or actively released from cells into biological fluids including circulating blood and CSF (Corsten *et al.*, 2010), either contributing to disease pathogenesis or reflecting response to treatment. Living neurons, but also oligodendrocytes and other CNS cells secrete miRNAs into the extracellular space packaged in exosomes or microvesicles (Saman *et al.*, 2012; Verderio *et al.*, 2012). Recent evidence support that miRNAs are altered in bodily fluids in Parkinson's and Alzheimer's Disease (Gui *et al.*, 2015), suggesting that their release does reflect not only activation of immune cells, but also the undergoing neurodegenerative process.

In a previous study, the -5p arm of miR-125a was found to act as a key regulator of brain endothelial integrity and its levels to be increased in MS brain lesions as compared to surrounding normal appearing white matter (Reijerkerk *et al.*, 2013). Coherently, treatment of MS patients with the anti-VLA4 monoclonal antibody (Tysabri) that inhibits inflammatory cell migration to the brain parenchyma and thus favours endothelial integrity, results in decreased blood levels of miR-125a-5p (Munoz-Culla *et al.*, 2014). However, up to the present study, changes of the -3p arm of this miRNA had not been investigated. Here we show that miR-125a-3p is up-regulated in the CSF of relapsing MS patients compared to control subjects. This alteration was specific for MS, since it was not found in Alzheimer's disease patients.

This increase could be explained by passive release of miR-125a-3p from neural cells such as neurons or oligodendrocytes undergoing destruction in active MS patients. We cannot obviously exclude that the aberrant presence of this miRNA in CSF could also reflect the inflammation state of the CNS during the relapsing-remitting phase of the disease (Romme Christensen *et al.*, 2014), since previous reports have demonstrated the role of miR-125a in immune cells activation (Kumar *et al.*, 2014).

The transposition of the *in vitro* and *in vivo* results presented in this thesis to human MS subjects should of course take in account the high heterogeneity of OPCs, that do not necessarily behave in the same exact way. However, our data showing increased levels of miR-125a-3p in the CSF of MS patients in the relapsing phase could serve as a basis for further studies on larger cohort of patients to validate miR-125a-3p as a biomarker for different MS stages, providing a previously unrecognized venue for medical interventions.

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APPENDIX A

AD	Alzheimer's Disease
AIDs	Auto-Immune Diseases
BPs	Biological Processes
CC	Corpus Callosum
CX	Cerebral Cortex
CSF	CerebroSpinal Fluid
DIG	Digoxigenin
DIV	Days *In vitro*
DPL	Days Post Lesion
ECM	ExtraCellular Matrix
FDR	False Discovery Rate
GBM	Glioblastoma Multiforme
GO	Gene Ontology
HDACs	Histone Deacetylase
IPA	Ingenuity Pathway Analysis
LNA	Locked Nucleic Acid
LPC	Lysolecithin
LV	Lentivirus
MCI	Mild Cognitive Impairment
MNs	Motoneurons
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NDs	Neurodegenerative Diseases
OPCs	Oligodendrocyte Precursor Cells
PD	Parkinson's Disease
PP-MS	Primary Progressive Multiple Sclerosis
RISC	RNA-Induced Silencing Complex
RR-MS	Relapsing Remitting Multiple Sclerosis
SP-MS	Secondary Progressive Multiple Sclerosis
TLR	Toll-Like Receptor
UTR	UnTranslated Region
VZ	Ventricular Zone

APPENDIX B

Bioinformatic tools:

NCBI	http://www.ncbi.nlm.nih.gov/
BLAST	http://www.ncbi.nlm.nih.gov/blast
STRING	https://string-db.org/
Gene Ontology	http://www.geneontology.org/
miRanda	http://www.microrna.org
miRwalk 2.0	http://zmf.umm.uni-heidelberg.de /apps/zmf/mirwalk2/
MyMir	http://www.itb.cnr.it/micro/
RNA22 v2	https://cm.jefferson.edu/rna22/Interactive/

Software:

IPA® (Qiagen)	www.qiagen.com/ingenuity
Metacore™	https://portal.genego.com/
ClustalX 2.1	http://www.clustal.org/clustal2/
ImageJ	https://imagej.nih.gov/ij/

APPENDIX C

RT² Profiler™ PCR Array Rat WNT Signaling Targets (PARN-243Z)

Symbol	Description	GeneBank
Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	NM_133401
Ahr	Aryl hydrocarbon receptor	NM_013149
Angptl4	Angiopoietin-like 4	NM_199115
Antxr1	Anthrax toxin receptor 1	NM_001044249
Axin2	Axin 2	NM_024355
Bglap	Bone gamma-carboxyglutamate (gla) protein	NM_013414
Birc5	Baculoviral IAP repeat-containing 5	NM_022274
Bmp4	Bone morphogenetic protein 4	NM_012827
Btrc	Beta-transducin repeat containing	NM_001007148
Cacna2d3	Calcium channel, voltage-dependent, alpha2/delta subunit 3	NM_175595
Ccnd1	Cyclin D1	NM_171992
Ccnd2	Cyclin D2	NM_022267
Cd44	Cd44 molecule	NM_012924
Cdh1	Cadherin 1	NM_031334
Cdkn2a	Cyclin-dependent kinase inhibitor 2A	NM_031550
Cdon	Cdon homolog (mouse)	NM_017358
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	NM_013154
Ctgf	Connective tissue growth factor	NM_022266
Cubn	Cubilin (intrinsic factor-cobalamin receptor)	NM_053332
Dab2	Disabled homolog 2 (Drosophila)	NM_024159
Dkk1	Dickkopf homolog 1 (Xenopus laevis)	NM_001106350
Dlk1	Delta-like 1 homolog (Drosophila)	NM_053744
Dpp10	Dipeptidylpeptidase 10	NM_001012205
Efnb1	Ephrin B1	NM_017089
Egfr	Epidermal growth factor receptor	NM_031507
Egr1	Early growth response 1	NM_012551
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	NM_057104
Ets2	V-ets erythroblastosis virus E26 oncogene homolog 2	NM_001107107
Fgf20	Fibroblast growth factor 20	NM_023961
Fgf4	Fibroblast growth factor 4	NM_053809
Fgf7	Fibroblast growth factor 7	NM_022182
Fgf9	Fibroblast growth factor 9	NM_012952
Fn1	Fibronectin 1	NM_019143
Fosl1	Fos-like antigen 1	NM_012953
Fst	Follistatin	NM_012561
Gdf5	Growth differentiation factor 5	XM_001066344

RT² Profiler™ PCR Array Rat WNT Signaling Targets (PARN-243Z)

Gdnf	Glial cell derived neurotrophic factor	NM_019139
Gja1	Gap junction protein, alpha 1	NM_012567
Id2	Inhibitor of DNA binding 2	NM_013060
Igf1	Insulin-like growth factor 1	NM_178866
Igf2	Insulin-like growth factor 2	NM_031511
Il6	Interleukin 6	NM_012589
Irs1	Insulin receptor substrate 1	NM_012969
Jag1	Jagged 1	NM_019147
Klf5	Kruppel-like factor 5	NM_053394
Lef1	Lymphoid enhancer binding factor 1	NM_130429
Lrp1	Low density lipoprotein-related protein 1	NM_001130490
Met	Met proto-oncogene	NM_031517
Mmp2	Matrix metallopeptidase 2	NM_031054
Mmp7	Matrix metallopeptidase 7	NM_012864
Mmp9	Matrix metallopeptidase 9	NM_031055
Myc	Myelocytomatosis oncogene	NM_012603
Nanog	Nanog homeobox	NM_001100781
Nrcam	Neuronal cell adhesion molecule	NM_013150
Nrp1	Neuropilin 1	NM_145098
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	NM_012731
Pdgfra	Platelet derived growth factor receptor, alpha	NM_012802
Pitx2	Paired-like homeodomain 2	NM_019334
Plaur	Plasminogen activator, urokinase receptor	NM_017350
Pou5f1	POU class 5 homeobox 1	NM_001009178
Ppap2b	Phosphatidic acid phosphatase type 2B	NM_138905
Ppard	Peroxisome proliferator-activated receptor delta	NM_013141
Ptch1	Patched homolog 1 (Drosophila)	NM_053566
Ptgs2	Prostaglandin-endoperoxide synthase 2	NM_017232
Runx2	Runt-related transcription factor 2	NM_053470
Sfrp2	Secreted frizzled-related protein 2	NM_001100700
Six1	SIX homeobox 1	NM_053759
Smo	Smoothened homolog (Drosophila)	NM_012807
Sox2	SRY (sex determining region Y)-box 2	NM_001109181
Sox9	SRY-box containing gene 9	XM_001081628
T	T, brachyury homolog (mouse)	NM_001106209
Tcf7l1	Transcription factor 3	NM_001107865
Tcf4	Transcription factor 4	NM_053369
Tcf7	Transcription factor 7 (T-cell specific, HMG-box)	XM_006220666
Tcf7l2	Transcription factor 7-like 2 (T-cell specific, HMG-box)	NM_001191052
Tgfb3	Transforming growth factor, beta 3	NM_013174

RT² Profiler™ PCR Array Rat WNT Signaling Targets (PARN-243Z)

Tle1	Transducin-like enhancer of split 1	NM_001173433
Twist1	Twist homolog 1 (Drosophila)	NM_053530
Vegfa	Vascular endothelial growth factor A	NM_031836
Wisp1	WNT1 inducible signaling pathway protein 1	NM_031716
Wisp2	WNT1 inducible signaling pathway protein 2	NM_031590
Wnt3a	Wingless-type MMTV integration site family, member 3A	NM_001107005
Wnt5a	Wingless-type MMTV integration site family, member 5A	NM_022631
Wnt9a	Wingless-type MMTV integration site family, member 9A	NM_001105783

APPENDIX D

RT² Profiler™ PCR Array Rat Extracellular Matrix & Adhesion Molecules (PARN-013Z)

Symbol	Description	GeneBank
Adamts1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	NM_024400
Adamts2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	NM_001137622
Adamts5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	NM_198761
Adamts8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	NM_001106811
Catna1	Catenin (cadherin associated protein), alpha 1	NM_001007145
Cd44	Cd44 molecule	NM_012924
Cdh1	Cadherin 1	NM_031334
Cdh2	Cadherin 2	NM_031333
Cdh3	Cadherin 3	NM_053938
Cdh4	Cadherin 4	XM_001061943
Cntn1	Contactin 1	NM_057118
Col1a1	Collagen, type I, alpha 1	NM_053304
Col2a1	Collagen, type II, alpha 1	NM_012929
Col3a1	Collagen, type III, alpha 1	NM_032085
Col4a1	Collagen, type IV, alpha 1	NM_001135009
Col4a2	Collagen, type IV, alpha 2	XM_001076134
Col4a3	Collagen, type IV, alpha 3	NM_001135759
Col5a1	Collagen, type V, alpha 1	NM_134452
Col6a1	Collagen, type VI, alpha 1	XM_215375
Col8a1	Collagen, type VIII, alpha 1	NM_001107100
Ctgf	Connective tissue growth factor	NM_022266
Ctnna2	Catenin (cadherin associated protein), alpha 2	NM_001106598
Ctnnb1	Catenin (cadherin associated protein), beta 1	NM_053357
Ecm1	Extracellular matrix protein 1	NM_053882
Emilin1	Elastin microfibril interfacer 1	NM_001106710
Entpd1	Ectonucleoside triphosphate diphosphohydrolase 1	NM_022587
Fbln1	Fibulin 1	NM_001127547
Fn1	Fibronectin 1	NM_019143
Hapln1	Hyaluronan and proteoglycan link protein 1	NM_019189
Icam1	Intercellular adhesion molecule 1	NM_012967
Itga2	Integrin, alpha 2	XM_345156
Itga3	Integrin, alpha 3	NM_001108292
Itga4	Integrin, alpha 4	NM_001107737
Itga5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	NM_001108118
Itgad	Integrin, alpha D	NM_031691
Itgae	Integrin, alpha E	NM_031768

RT² Profiler™ PCR Array Rat Extracellular Matrix & Adhesion Molecules (PARN-013Z)

Itgal	Integrin, alpha L	NM_001033998
Itgam	Integrin, alpha M	NM_012711
Itgav	Integrin, alpha V	NM_001106549
Itgb1	Integrin, beta 1	NM_017022
Itgb2	Integrin, beta 2	NM_001037780
Itgb3	Integrin, beta 3	NM_153720
Itgb4	Integrin, beta 4	NM_013180
Lama1	Laminin, alpha 1	NM_001108237
Lama2	Laminin, alpha 2	XM_008758643
Lama3	Laminin, alpha 3	XM_003753026
Lamb2	Laminin, beta 2	NM_012974
Lamb3	Laminin, beta 3	NM_001100841
Lamc1	Laminin, gamma 1	NM_053966
Mmp10	Matrix metallopeptidase 10	NM_133514
Mmp11	Matrix metallopeptidase 11	NM_012980
Mmp12	Matrix metallopeptidase 12	NM_053963
Mmp13	Matrix metallopeptidase 13	NM_133530
Mmp14	Matrix metallopeptidase 14 (membrane-inserted)	NM_031056
Mmp15	Matrix metallopeptidase 15	NM_001106168
Mmp16	Matrix metallopeptidase 16	NM_080776
Mmp1	Matrix metallopeptidase 1a (interstitial collagenase)	NM_001134530
Mmp2	Matrix metallopeptidase 2	NM_031054
Mmp3	Matrix metallopeptidase 3	NM_133523
Mmp7	Matrix metallopeptidase 7	NM_012864
Mmp8	Matrix metallopeptidase 8	NM_022221
Mmp9	Matrix metallopeptidase 9	NM_031055
Ncam1	Neural cell adhesion molecule 1	NM_031521
Ncam2	Neural cell adhesion molecule 2	NM_203409
Pecam1	Platelet/endothelial cell adhesion molecule 1	NM_031591
Postn	Periostin, osteoblast specific factor	NM_001108550
Sele	Selectin E	NM_138879
Sell	Selectin L	NM_019177
Selp	Selectin P	NM_013114
Sgce	Sarcoglycan, epsilon	NM_001002023
Sparc	Secreted protein, acidic, cysteine-rich (osteonectin)	NM_012656
Spock1	Sparc/osteonectin, (testican) 1	NM_001271297
Spp1	Secreted phosphoprotein 1	NM_012881
Syt1	Synaptotagmin I	NM_001033680
Tgfbi	Transforming growth factor, beta induced	NM_053802
Thbs1	Thrombospondin 1	NM_001013062

RT² Profiler™ PCR Array Rat Extracellular Matrix & Adhesion Molecules (PARN-013Z)

Thbs2	Thrombospondin 2	NM_001169138
Timp1	TIMP metallopeptidase inhibitor 1	NM_053819
Timp2	TIMP metallopeptidase inhibitor 2	NM_021989
Timp3	TIMP metallopeptidase inhibitor 3	NM_012886
Tnc	Tenascin C	NM_053861
Vcam1	Vascular cell adhesion molecule 1	NM_012889
Vcan	Versican	NM_001170558
Vtn	Vitronectin	NM_019156