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Exploring miRNA Inhibiting Therapeutic Strategies for Multiple Sclerosis: Insights from Experimental Autoimmune Encephalomyelitis Studies – A Traditional Review

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ABSTRACT
Multiple sclerosis (MS) is a chronic inflammatory disease in which immune cells attack the central nervous system (CNS) and cause degeneration of the myelin sheath. Although disease-modifying therapies are commonly used in the treatment of MS, these therapies cannot fully prevent or reverse the course of neurological deterioration. Furthermore, the response of patients with MS to these drugs may vary from person to person and over time. Therefore, the demand for new and more effective treatments continues. MicroRNAs (miRNAs) have the potential to alter biological processes that lead to various diseases. Hence, dysregulated miRNA levels are thought to be associated with the pathogenesis of MS. Considering the regulatory role of miRNAs in both the CNS and immune response, many studies have proposed them as potential new therapeutic targets for MS in addition to being potential biomarkers. In this traditional review, the primary focus is on miRNA-based therapeutic approaches, followed by a compilation of studies that utilized miRNA therapy to inhibit upregulated miRNAs in the animal model of MS, known as Experimental Autoimmune Encephalomyelitis (EAE).
Keywords: miRNA; multiple sclerosis; therapy; experimental autoimmune encephalomyelitis.

Multipl Skleroz için miRNA İnhibisyonuna Yönelik Terapötik Stratejilerin Keşfi: Deneysel Otoimmün Ensefalomiyelit Çalışmalarından Elde Edilen Bulgular- Geleneksel Bir Derleme

ÖZ
Multipl skleroz (MS), bağışıklık hücrelerinin merkezi sinir sistemine (MSS) saldırarak miyelin kılıfın dejenerasyonuna yol açtığı kronik bir enflamatuvar hastalıktır. MS tedavisinde sıklıkla hastalığı modifiye edici tedaviler kullanılmakla birlikte, bu yaklaşımlar nörolojik bozulmanın seyrini tamamen durdurmakta veya geri çevirmekte yetersiz kalmaktadır. Ayrıca, MS'li bireylerin bu tedavilere verdiği yanıt hem kişiden kişiye hem de zaman içinde değişkenlik gösterebilmektedir. Bu nedenle, daha etkili ve hedefe yönelik yeni tedavi stratejilerine olan ihtiyaç devam etmektedir. MikroRNA'lar (miRNA'lar), çeşitli hastalıklara neden olan biyolojik süreçleri değiştirme potansiyeline sahip küçük düzenleyici RNA molekülleridir. MS patogenezinde düzensiz miRNA ekspresyonunun önemli bir rol oynadığı düşünülmektedir. miRNA'ların hem MSS hem de bağışıklık sistemindeki düzenleyici etkileri göz önünde bulundurulduğunda, birçok çalışma bu molekülleri yalnızca potansiyel biyobelirteçler olarak değil, aynı zamanda terapötik hedefler olarak da önermiştir. Bu geleneksel derlemede, öncelikle miRNA inhibisyonuna yönelik tedavi yaklaşımları ele alınmış; ardından, MS'in deneysel hayvan modeli olan deneysel otoimmün ensefalomiyelit (DOE) kapsamında, aşırı ifade edilen miRNA'ların terapötik inhibisyonunu değerlendiren çalışmalar derlenmiştir.
Anahtar kelimeler: miRNA; multipl skleroz; tedavi; deneysel otoimmün ensefalomiyelit.

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INTRODUCTION

Multiple sclerosis (MS) is a chronic and inflammatory disorder characterized by the dysregulation of the immune system, leading to the targeted attack and demyelination of the central nervous system (CNS) by immune cells (1). MS currently affects 2.9 million people worldwide according to the MS International Federation (2). Neurological dysfunctions, including optic neuritis, motor weakness, numbness, and dizziness manifest subsequent to neuronal demyelination and axonal damage within inflammatory lesions located in the gray and white matter of the brain and spinal cord (1). Although the exact cause of MS is unknown, it is thought that both genetic and environmental factors, including Epstein-Barr virus infection, smoking, and vitamin D deficiency contribute to the risk of the disease (3). There are four defined clinical courses identified in MS. Clinically isolated syndrome (CIS) which is considered a precursor to MS in some cases, refers to a single episode of neurological symptoms caused by inflammation or demyelination in the CNS. These symptoms typically last for at least 24 hours and are not attributable to other known causes. The most common disease course observed is relapsing-remitting MS (RRMS), which affects approximately 85% of the patients with MS. RRMS exhibits periods of symptom aggravations, known as relapses, occurring at irregular intervals, followed by complete or partial neurological recovery. Approximately half of the RRMS patients later develop secondary progressive MS (SPMS) characterized by continuous permanent deterioration with or without relapses. Compared to SPMS, patients with primary progressive MS (PPMS) experience continuous disability progression from the onset without any relapses (1,4).

Treatments currently used for MS can be divided into MS-specific disease-modifying therapies (DMT) and symptomatic treatments that are not specific to MS. DMTs are employed to slow the progression of the disease by reducing inflammation, while symptomatic treatments aim to promptly prevent CNS-related symptoms caused by nerve impairment (3,5). Unfortunately, currently used DMTs cannot completely prevent or reverse the progression of neurological degeneration (5). Moreover, the treatment response of patients to these drugs varies between individuals and all the DMTs have negative side effects with differing degrees of severity. Additionally, the ratio between the benefits and risks of a DMT can change over the course of time, and these drugs are shown to be more effective for RRMS patients while continuing to be insufficient for progressive courses of MS (4). Therefore, there remains a significant need for new and more effective therapeutic drugs.

MicroRNAs (miRNAs), pivotal epigenetic modulators, are non-coding, single-stranded RNAs ranging between 18-25 nucleotides in length. They regulate gene expression post-transcriptionally by either causing degradation of mRNA or inhibit translation (6). Approximately 30% of protein-coding human genes are modulated by miRNAs, thereby playing crucial roles in many biological functions. One such critical role involves the regulation of immune system response, wherein miRNAs control the differentiation and development of immune cells as well as antibody production (6,7). Another essential function occurs within the CNS where over fifty percent of miRNAs are expressed. Here, miRNAs regulate the differentiation of neurons and other CNS cells, as well as the process of myelination (8). Henceforth, dysregulation in miRNA levels may cause alterations in biological processes, contributing to various diseases including neurodegenerative conditions such as MS (6).

Likewise, the regulation of genes through various miRNAs can determine susceptibility to certain diseases and the extent of disease severity (8). Given the need for novel drugs for MS and miRNAs affecting both CNS and immune response, numerous studies propose specific miRNAs as promising therapeutic targets for MS, as well as potential biomarkers (6,9,10).

This narrative review provides a comprehensive overview of the therapeutic approaches utilizing diverse miRNAs and explores research aimed at lowering elevated miRNA levels in a well-established *in vivo* mice model of MS known as experimental autoimmune encephalomyelitis (EAE) (11).

miRNA Biogenesis

miRNAs are first generated with RNA Polymerase II from DNA as double-stranded primary miRNA (pri-miRNA) which is processed into hairpin-shaped precursor miRNA (pre-miRNA, 60-70 nucleotides) by Drosha ribonuclease enzyme and DiGeorge critical region 8 (DGCR8) in the nucleus. The pre-miRNA is then exported out of the nucleus into the cytoplasm with exportin-5 activity, where Dicer transforms it into a double-stranded miRNA duplex (about 22 nucleotides) by cleaving the circular end of the pre-miRNA. One of the strands, called guide miRNA, binds to the Argonaute (AGO) protein within the RNA-induced silencing complex (RISC). This complex uses the seed sequence of the guide miRNA to target the specific mRNA by base pairing with the 3′ UTR of the target mRNA (9,10). Moreover, miRNAs can enter body fluids such as blood upon their release from cells; this group of miRNAs is referred to as circulating miRNAs (Fig. 1) (12).

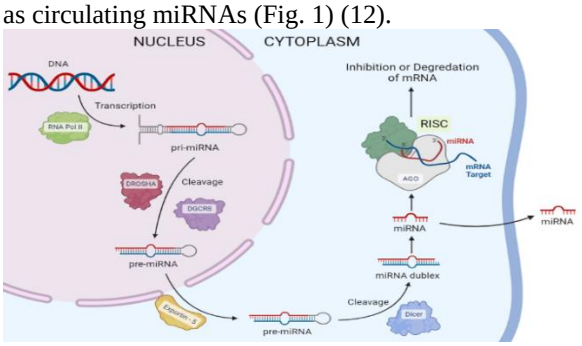

Figure 1. MiRNA biogenesis, pri-RNA: primary miRNA, pre-RNA: precursor miRNA, RNA Pol II: RNA Polymerase II, RISC: RNA-induced silencing complex (Created with BioRender)

The degree of complementarity between the seed sequence and the target mRNA determines which of the two RISC mechanisms will be activated. If the miRNA and mRNA are perfectly paired, mRNAs are degraded upon activation of the RNA-mediated interference pathway. In the second mechanism, a partial match between miRNA and mRNA results in non-perfect binding and suppresses protein translation (13). A single gene could be regulated by multiple miRNAs. Conversely, a single miRNA could exhibit regulatory control over multiple protein-coding genes and could affect various pathways (7).

Therapeutic Approaches for miRNA Treatment

With the understanding of the pivotal function of miRNAs in numerous biological processes and human diseases, the idea of a treatment targeting miRNAs has attracted significant interest across different studies for various diseases (13,14). The pioneering therapy based on miRNAs known as miravirsen was an inhibitor of miR-122 constructed using locked nucleic acid technology to treat hepatitis C viral infections (15). The success of miravirsen in phase I and phase IIa clinical trials have paved the way for miRNAs as treatment targets and development for different approaches (16). These approaches aim to either inhibit or replenish the miRNA activity (Figure 2). There are four different approaches for the downregulation of miRNAs: anti-miR, miRNA sponge, miRNA masking, and small molecule inhibitors. On the other hand, to replenish the downregulated miRNAs of interest another approach called miRNA mimics is employed.

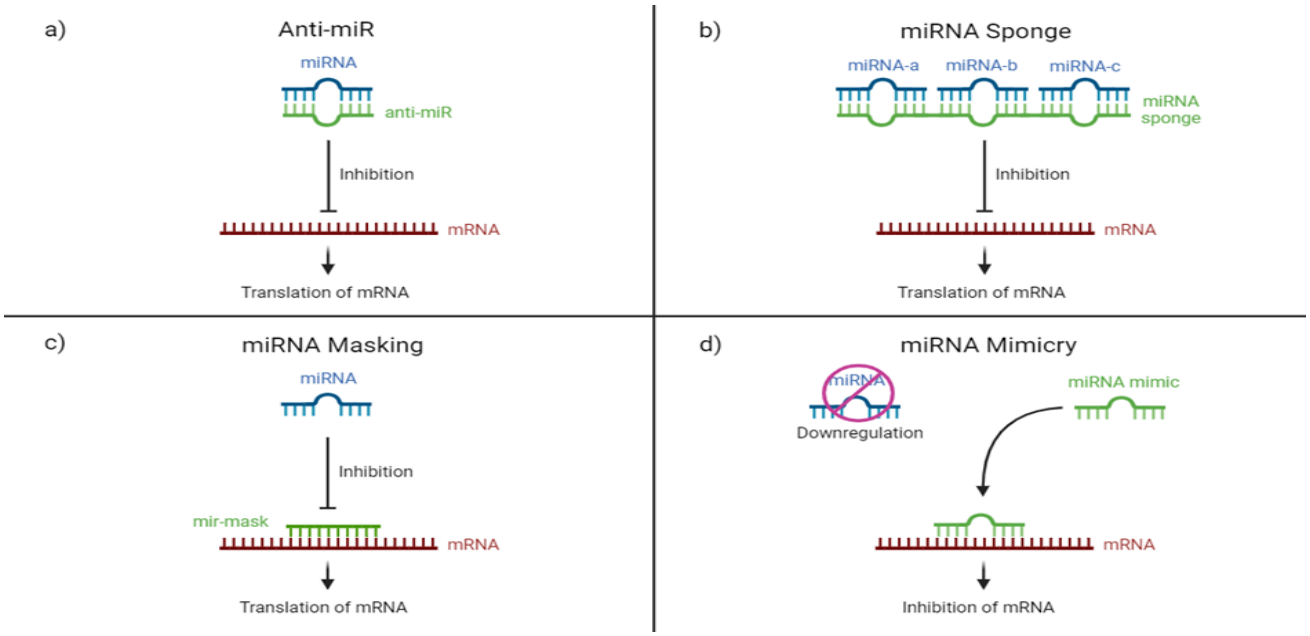

Figure 2. Different therapeutic nucleic acids for miRNAs a) Anti-miR: antagomir b) miRNA sponge c) miRNA masking d) miRNA mimicry (Created with BioRender)

One of the approaches called anti-miR (antagomir) is an antisense oligonucleotide that inhibits the function of miRNA by preventing its binding to its target mRNA; even some could trigger miRNA degradation. It binds to its target miRNA due to being completely complementary to miRNA (13,17). Chemical modifications can be introduced to anti-miRs. Examples of these modified anti-miRs include 2'-O-methyl anti-miR, 2'-O-methoxyethyl anti-miR, and locked nucleic acids (LNAs) (13). LNAs, in particular, provide several benefits encompassing improved thermal stability, enhanced resistance to nucleases, and decreased toxicity (13,18). Notably, LNAs obviate the necessity for the use of delivery vectors and their enhanced binding affinity to the miRNA of interest makes them a more appealing choice (13). Another anti-miR approach is Tough Decoy (TuD) which acts as competitive decoy. It has a hairpin structure containing multiple binding sites. The multiplicity of binding sites allows for more miRNA to bind, increasing binding efficiency and miRNA silencing capacity. The loop in the hairpin structure improves durability by providing resistance to nucleases (19). As a result, TuD can be a potential therapeutic approach in diseases caused by dysregulated miRNA.

miRNA sponges contain multiple binding sites which are complementary to target miRNAs, acting as decoys for miRNAs to bind to. Upon introduction to the cells, multiple binding sites provide simultaneous inhibition of several miRNAs and allow the expression of corresponding mRNA of target miRNA (13,17). Furthermore, it is possible to design miRNA sponges with heptameric seed sequences as binding sites to suppress a miRNA family exhibiting heightened expression (13). This design possibility is an advantage over anti-miRs because the

complementary site of anti-miRs extends beyond the common seed sequence for effective targeting making anti-miRs specific for a single miRNA (20).

Unlike anti-miRs and miRNA sponges that target miRNA, the miRNA masking approach targets the 3' UTR of mRNA with a 2'-O-methyl modified single-strand oligoribonucleotide molecule. This molecule, termed miR-mask, prevents miRNA attachment by perfectly binding to the miRNA binding site within the target mRNA and inhibits the miRNA activity in a competitive manner. A miR-mask exhibits a strong binding affinity to the endogenous target mRNA of the miRNA, thereby establishing a mechanism that effectively avoids off-target outcomes (13,18).

Small molecule inhibitors, unlike other approaches, inhibit miRNAs by disturbing miRNA biogenesis. They can affect at least three steps of miRNA biogenesis. The first two interfere with the transcription or RISC assembly and the third is interrupting the RISC-mRNA interaction (14).

Since miRNAs are downregulated in some diseases, another therapeutic approach is to replenish the miRNA expression with miRNA mimics. These mimics are synthetic double stranded small RNAs that imitate the miRNA duplex, as such, one strand acts as a guide miRNA and triggers mRNA degradation by binding to mRNA while the other strand called the passenger strand is degraded (12-14). In certain instances, the passenger strand could exhibit an undesirable propensity to bind to mRNAs resulting in unintended and potentially adverse effects. Moreover, it is noteworthy that miRNA mimics can be chemically modified to enhance stability and decrease the immune response, just like anti-miRs (12).

Additionally, there are studies using small interfering RNA (siRNA) and short hairpin RNA (shRNA) for modulating the expression of miRNAs (21). siRNA is suitable for specific medical conditions due to being easy to manufacture and having a temporary effect per dose. On the other hand, shRNA optimized by the endogenous processing machinery enables enduring effects and high potency with a small quantity of copies while having fewer side effects, especially when embedded in miRNA scaffolds (22).

Direct delivery into the circulation of miRNA mimics or inhibitors is hindered by degradation from RNases in the bloodstream, even when chemically modified. Additionally, these molecules may be taken up by non-target organs, leading to off-target effects. The dense extracellular matrix in certain tissues can also act as a physical barrier, making it more difficult for these molecules to enter cells (12). Therefore, other delivery strategies are being explored.

All these molecules can be introduced into cells through various delivery methods: lentiviral vectors (LV), nanoparticles, and exosomes (23). The disease pathologies and characteristics of the target tissues should be considered for the selection of an appropriate delivery method (17). LVs are highly efficient in the delivery of genes because viruses can be packed with multiple miRNA mimics or anti-miRs, but they carry the risk for triggering the host immune response, limiting its clinical usage (13,14). Progress in materials science has enabled the development of nanoparticles for miRNA therapeutics applications. These particles often contain positively charged domains, allowing efficient binding of negatively charged miRNA mimics or inhibitors to the carrier and they protect the encapsulated molecules from degradation (12,17). An advantage of nanoparticles is the opportunity of surface coatings with molecules for targeting specific tissues or cell types (17). They are increasingly used to target immune cells. Exosomes also have been proposed to be used in miRNA therapeutics delivery. Exosomes are already used by $CD4^+$ regulatory T (Treg) cells to deliver miRNAs to other T cell subsets *in vivo*, during inflammatory conditions (23). Momen-Heravi et al. (24) have suggested that exosomes derived from B cells might be effective in the delivery of miR-155 mimics and inhibitors. In this study, exosomes derived from murine B cells loaded with miR-155 inhibitors successfully delivered their contents to both mouse hepatocytes *in vitro* and mouse liver *in vivo* (24).

Multiple Sclerosis Pathogenesis

The distinctive pathological feature of MS is the demyelination areas called lesions which occur in the CNS (brain and spinal cord) and the optic nerve, in both white and grey matter (4). Ongoing research related to MS suggests that immunopathology plays a major role in the demyelination process, especially the infiltration of immune cells causing inflammation in CNS is demonstrated to be the primary cause (3,7). The pathological process of MS is associated with the breakdown of the blood-brain barrier via cytokines and chemokines, infiltration of immune cells, demyelination followed by oligodendrocyte loss, gliosis, and axonal degeneration (4). The adaptive immune response is initiated with T lymphocytes binding to antigen-presenting cells (APCs) which include B cells, microglia, dendritic cells, and macrophages (5,25). With the cytokines produced by APCs, $CD4^+$ T cells differentiate into T helper 1 (Th1), T helper 2 (Th2), and T helper 17 (Th17) which are capable of releasing certain cytokines (25). Th1 cells induce interferon gamma (IFNγ) and type II interferon and tumor necrosis factor alpha (TNF-α) which are proinflammatory and crucial for both adaptive and innate immunity due to their ability to suppress differentiation of Th2 cells. On the other hand, Th2 cells produce anti-inflammatory cytokines (IL-4 and IL-13). Th17 is another type of $CD4^+$ T cells that facilitate inflammation in MS via its cytokines (IL-17, IL-21, IL-22, and IL-26) (5,25). After the migration of Th1 and Th17 cells into CNS, the observation of demyelination with axonal loss begins (5).

These three subtypes of $CD4^+$ T cells are regulated by Treg cells. Even though Treg cells preserve their number in

patients with MS compared to healthy people, their functionality is diminished (5). In addition to these cells, various studies indicate that $CD8^+$ T cells might have a role in the pathology of MS and can be found in lesions. Although $CD8^+$ T cells secrete cytotoxic proteins to mediate $CD4^+$ T cell suppression, they contribute to the destruction of glial cells, resulting in exposed axons. They also enhance vascular permeability and initiate the oligodendrocyte destruction leading to impaired myelin repair (5,25).

Besides these cells, microglia, circulatory-derived neutrophils, mast cells, and particularly B lymphocytes with their cytokines are also involved in the process of MS pathogenesis. An additional factor in the pathophysiology of MS could be astrocyte activation and proliferation (6).

Recent studies have revealed that the underlying cause of MS is intricately linked to dysregulated levels of miRNA in immune cells (B cells, monocytes, $CD4^+$ and $CD8^+$ T lymphocytes, and neutrophils). For example, while miR-155 was upregulated in $CD4^+$ and $CD8^+$ T cells, monocytes, macrophages and microglia of the patients with MS; miR-20b and miR-320b were downregulated in $CD4^+$ T cells and B cells, respectively (6). Additionally, miRNAs have a role in the regulation of the myelination process and the development of neurons and other CNS cells (8). Because different immune cells exhibit different expression levels of various miRNAs, multiple different miRNAs could be targeted for MS treatment or used as biomarkers (6). miRNAs' stability in serum and plasma could enable them to be biomarkers. In support of this, several miRNAs differentially expressed in blood, serum and plasma of MS patients have been identified. For instance, it has been observed that in MS patients, miR-145 levels are upregulated in plasma, and miR-320a levels are upregulated in serum. Another miRNA that is elevated in peripheral blood miR-326, has been shown to differentiate between the relapsing and remitting stages of MS, whereas miR-181c, which is upregulated in cerebrospinal fluid, has been suggested to distinguish between SPMS and RRMS types (26). The link between several miRNAs and MS is still under investigation.

Studies on MS Treatment Targeting miRNA Inhibition

Ongoing studies explore miRNA inhibition as a therapeutic approach for various diseases, including hepatitis C viral infections (15), atherosclerosis, and cardiovascular diseases (14). While numerous investigations primarily focus on different types of cancer, our review will mainly address the studies conducted on the animal model of MS called EAE and transgenic mice (Table 1).

Table 1. Summary of the applied miRNA treatments of the reviewed studies

Organism	Target miRNA	Manipulation effect	Employed therapeutics	Delivery method	Result	Reference
EAE mice	Target miRNA	Manipulation Effect	miRNA Sponge	Lentiviral	Developed milder EAE	27
EAE mice	miR-326	Down	anti-Mir	LNA modification	Reduction in clinical severity	29
EAE mice	miR-155	Down	anti-Mir	Nanoparticle-based	Reduced severity of EAE More enhanced clinical recovery	30
EAE mice	miR-155	Down	miRNA mimic	Nanoparticle-based	Developed severe EAE	30
EAE mice	miR-155	Up	anti-Mir	Lentiviral	Developed milder EAE	32
EAE mice	miRNA let-7e	Down	miRNA mimic	Lentiviral	Developed severe EAE	32
EAE mice	miRNA let-7e	Up	miRNA Sponge	Lentiviral	Delayed onset of EAE Less severe clinical symptoms	33
EAE mice	miR-873	Down	miRNA mimic	Lentiviral	Developed more severe EAE	33
EAE mice	miR-181c	Down	shRNA	Lentiviral	Attenuated EAE clinical symptoms	21
Transgenic Mice	miR-182	Knockdown	-	-	Reduced EAE course Alleviated symptoms	34
Transgenic Mice	miR-182	Knockdown	-	-	Shortened disease progression Slightly alleviated symptoms	35
Transgenic Mice	miR-182	Over expression	-	-	Decreased induction period Severe symptoms	35
EAE mice	miR-125a-3p	Down	TuD	Lentiviral	Promoted remyelination	37
EAE mice	miR-125a-3p	Up	TuD	Lentiviral	Negative effect on OPCs maturation	37
EAE mice	miR-7188-5p	Down	Adoptive Transfer of silenced CD4+ cells	-	Alleviation of EAE disease	38
EAE mice	miR-7235	Down	Adoptive Transfer of silenced CD4+ cells	-	Alleviation of EAE disease	38
EAE mice	miR-7235	Down	anti-Mir	LNA modification	Delayed onset Attenuated EAE disease progression	39
Transgenic Mice	miR-451a	Knock-out	-	-	Did not reduce EAE clinical scores Moderately aggravated the clinical symptoms	40

EAE: Experimental Autoimmune Encephalomyelitis TuD: Tough Decoy

One of the pioneering studies in this area has been conducted by Du et al. (27), who generated three different LVs to manipulate miR-326 levels in EAE. The objective was to elucidate the correlation between miR-326 and EAE development. These LVs contained pre-miR-326 (LV-326), mutated miR-326 control (LV-ctrl), or sponges of miR-326 (LV-sponge), serving as inhibitors before immunization. They reported that mice injected with LV-326 developed a severe form of EAE, while those injected with LV-sponge developed a milder form of EAE compared to LV-ctrl-injected mice. In addition, the frequency and the absolute number of Th17 cells were decreased in LV-sponge-infected mice, suggesting miR-326 correlates with the Th17 differentiation and thus, the disease pathology. The authors argue that miR-326 may be a promising target for the treatment of MS (27).

miR-155 appears to be important in autoimmune disorders as it promotes inflammatory T cell development, making it an attractive therapeutic target. Consistent with this notion, it has previously been shown that miR-155 deficient mice exhibited high resistance to EAE (28). Therefore, it is not surprising that miR-155 is one of the most studied miRNAs.

One of the studies regarding miR-155 was conducted by Murugaiyan et al. (29), who investigated the effects of an antisense oligonucleotide modified by LNA-anti-miR-155 in EAE mice, before and after the onset of clinical symptoms. Both LNA therapy administered before and after the onset led to a reduction in clinical severity. Furthermore, *in vivo*, silencing of miR-155 affected both $CD4^+$ T cells and APCs while preventing the upregulation of IL-17 and IFN-γ in the CNS. Even though it is found that IL-17 and IFN-γ are not requirements for the initiation of MS, numerous studies support the argument that their elevated levels are associated with the disease severity as IL-17 and IFN-γ are crucial pro-inflammatory mediators. These findings imply that the direct impact of anti-miR-155 treatment on the immune response may effectively suppress the development and progression of EAE (29).

Zhang et al. (30) have provided evidence supporting miR-155 as a promising target for MS treatment. In their research, mice were injected with miR-155 mimics, inhibitors, or controls using a nanoparticle-based delivery system. miR-155 mimic-injected mice developed severe EAE while exhibiting significant inflammatory infiltration and demyelination. In contrast, mice treated with miR-155 inhibitor demonstrated reduced severity of EAE and more enhanced clinical recovery. Furthermore, miR-155 inhibitor treatment resulted in reduced Th17 cell frequency in splenocytes, lymph nodes, and CNS, and decreased IL-17A and IFN- γ cytokine production from Th17 and Th1, respectively. In conclusion, these findings support miR-155 as a potential T cell-associated therapeutic target (30).

Mycko et al. (31) performed a microarray of $CD4^+$ cells after stimulating peripheral lymph node (PLN) cells from immunized mice *in vitro* and found out that miR-21, miR-155, and miR-301a were upregulated. *In vivo* investigation has also demonstrated a significant several-fold increase in the expression levels of these three miRNAs, alongside a substantial rise in T cells throughout the course of EAE. To decrease the expression levels of these miRNAs, $CD4^+$ T cells which isolated from PLN of MOG_{35-55} immunized mice, were transfected in vitro with specific antagomirs. This resulted in the alteration of cytokine secretion profiles, without influencing the T cell proliferation. IFN-γ secretion was downregulated in response to antagomirs against miR-21 and miR-155. Response of all three antagomirs resulted in slightly upregulated IL-4 secretion. Importantly, transfection of miR-301a antagomir led to diminished IL-17 secretion, whereas other antagomirs had barely any effect on the secretion of this cytokine. The researchers additionally discovered that compared to the other T-helper cell subsets, Th17 cells expressed miR-301a at extremely high levels *ex vivo*. These findings indicate that the Th17-like cytokine secretion profile is significantly influenced by miR-301a. Furthermore, it has been revealed that miR-301a contributes to the change of Th17 cells by inhibiting protein inhibitor of activated STAT 3 (PIAS3), an inhibitor of the signal transducer and activator of transcription 3 (STAT3) pathway (31).

Guan et al. (32) have suggested in their study that miRNA let-7e has a role in the regulation of encephalitogenic T cell differentiation and is involved in regulating EAE pathology. Splenocytes derived from EAE-induced wild type (WT) and knock-out mice were transfected with pre-miR to mimic endogenous let-7e, anti-miR against endogenous let-7e, or a negative control (mock). Silencing via anti-miR decreased IFN-γ production and promoted IL-4 production, while overexpression with pre-miR had the opposite effect. Moreover, *in vitro* polarization study of $CD4^+$ cells from not immunized WT mice after transfection, exhibits that anti-miR promotes Th2 differentiation while reducing Th1 differentiation. Two *in vivo* experiments were performed to manipulate miRNA let-7e expressions with LV vectors containing pre-miR let-7e, anti-miR let-7e, and control and these three LVs were injected into WT mice 7 days before immunization or 5 days after immunization. Both experiments similarly resulted in the development of severe EAE in LV-pre-miR injected mice and the development of milder EAE in LV-anti-miR injected mice. Researchers also observed that there was a decreased numbers of encephalitogenic Th1 and Th17 cells, and an increase in Th2 cells with the silencing of miRNA let-7e, whereas overexpression of this miRNA resulted in an opposite observation (32).

Liu et al. (33) examined the expression of miRNAs using miRNA arrays in EAE mice and IL-17 stimulated astrocytes, revealing the upregulation of 11 different miRNAs in both cases. They chose to continue their study with miR-873 as a target due to having stronger inhibition on the A20 (TNF alpha induced protein 3, *TNFAIP3*) gene. First, mouse primary astrocytes were subjected to

transfection with three different agents: LNA-ctrl, miR-873 mimics, and LNA-anti-miR-873, then the cells were subjected to IL-17 stimulation *in vitro*. Compared to the control, 10 times more miR-873 expressions in mimic transfected cells and 2 times less expression in anti-miR-transfected cells were observed. In addition, some of the proteins associated with inflammation were shown to follow a similar trend. To further investigate the effects of miR-873, injections of LV-miR-873 (encoding pre-miR-873), LV-873 sponges, and LV-control were employed on EAE mice *in vivo*. Delayed onset of EAE with noticeably diminished demyelination and minor inflammation in CNS was observed when mice were treated with LV-873 sponge, leading to less severe clinical symptoms. Opposite results were observed when LV-short hairpin RNA of ubiquitin-editing enzyme A20 was applied to inhibit the A20 gene which was thought to be targeted by miR-873. This study further demonstrated that A20 silencing by miR-873 contributes to inflammatory cytokine production by promoting the activation of NF-κB. Knocking down of miR-873 was also shown to alleviate the pathological changes associated with EAE. Therefore, it is suggested that targeting specific miRNAs of astrocytes can be a viable strategy for MS treatment (33).

Zhang et al. (21) employed siRNA and shRNA as the inhibitors of miR-181c *in vitro* and *in vivo* settings, respectively. Mice were injected with LV shRNA (LV-sh) to knockdown miR-181c, leading to the amelioration of EAE symptoms and observable improvements in CNS pathology compared to the control group. Additionally, only miR-181c was downregulated in miR-181 family in various organs. While a decreased percentage of Th17 cells in peripheral lymphoid organs and CNS of LV-sh infected mice was observed, the percentages of Th1 and Treg did not exhibit comparable differences between the control and shRNA infected mice, revealing that miR-181c particularly alters Th17 cells. The study continued with an *in vitro* T cell differentiation assay resulting in higher expression of miR-181c in Th17 cells. Then, to inhibit miR-181c, the isolated $CD4^+CD62L^+$ T cells were transfected with siRNA *in vitro* and cultured under Th17-polarizing conditions. With the knockdown of miR-181c, IL-17-producing cells decreased significantly with expression of Th17-associated transcription factors, surface receptors, and cytokines. Following treatment with miR-181c mimics, a considerable increase in the Th17 percentage with the upregulation of genes unique to Th17 cells was observed. This study revealed that miR-181c promotes Th17 differentiation *in vitro*. Zhang et al. claimed that in this study a new miRNA was identified as a regulator of autoimmunity and Th17 differentiation, making miR-181c a potential treatment target in patients diagnosed with MS (21).

Wan al. (34) conducted two studies on miR-182 in EAE mice. In their first study, they confirmed that Treg cell differentiation was inhibited by miR-182 via its putative target Foxo1-dependent pathway. They generated transgenic mice by LV infection of the embryonated zygotes to decrease miR-182 levels, which led to a reduced EAE course as researchers anticipated, along with alleviation of the symptoms. $CD4^+$ T helper cells from the spleen and axillary lymph nodes exhibited greater levels of miR-182 during the acute phase of EAE in WT mice compared to transgenic mice, and both Foxo1 mRNA and protein levels were found out to have an inverse correlation with miR-182 levels. Researchers then delivered miR-182 inhibitors (shRNA) with LV to isolated $CD4^+$ T cells derived from the draining lymph nodes of MOG_{35-55} immunized WT mice *in vitro*, to eliminate any potential systematic effect of transgenic mice. Consistent with *in vivo* observations, miR-182 silencing *in vitro* resulted in increased expression of Foxo1, Foxp3 transcription factor, and higher Treg cell proportion (34).

In their second study, Wan et al. (35) first documented an upregulation of miR-182 levels in Chinese cohort patients with RRMS. They introduced miR-182 mimics or antagomirs to the peripheral blood mononuclear $CD4^+$ cells from healthy donors *in vitro*. Consequently, following the treatment with mimics, both IFN-γ production by Th1 cells and IL-17 production by Th17 cells increased. However, after the treatment with inhibitors, only IFN-γ levels decreased, with no significant change observed in IL-17 levels. Thus, they concluded that miR-182 primarily stimulates Th1 differentiation in peripheral blood mononuclear cells. In the continuation of the study, transgenic mice were generated with the same method from the previous study for the knockdown or overexpression of miR-182. In the lymphoid tissue of knockdown mice, a 50% decrease in miR-182 expression was observed while overexpressed mice exhibited twofold expression. Then these mice were employed for EAE induction and overexpressed mice demonstrated a decreased induction period with more severe symptoms. In knockdown mice, shortened disease progression and slightly alleviated symptoms were demonstrated without an extended induction period. Moreover, cytometric analyses of lymph nodes and spleen indicated alterations in the frequency of Th1 and Th17 cells. Overexpressed mice exhibited an increase, whereas knockdown mice showed a significant decrease. The researchers concluded that miR-182 levels may be a good indicator of the degree of autoimmune inflammation in general as well as the activation state of T cells (35).

Previously, Lecca et al. (36) initially showed that the -3p strand of miR-125 regulates the development of oligodendrocyte precursor cells (OPC) *in vitro* and its over-expression hinders cell maturation. Later, Marangon et al. (37) first assessed that miR-125a-3p exhibits higher expression levels both in active lesions of patients with MS and OPC from the spinal cord of chronic EAE mice but not in OPCs from spontaneously remyelinating corpus callosum. These results imply that terminal maturation of

OPC is negatively affected by the upregulation of miR-125a-3p, consequently resulting in impaired remyelination. To further investigate this, mice were first treated with lysophosphatidylcholine (LPC) to induce demyelination in mice. Some of the mice were infected with a LV which simultaneously expressed the target miRNA miR-125a and green fluorescent protein (GFP) reporter (LV-miR-125a-GFP) or control (LV-ctrl-GFP). They observed that 10% of the total oligodendroglial population was infected, and approximately 85% of the infected population still demonstrated immature features which indicates immature oligodendrocyte precursor maturation while the noninfected oligodendroglial population exhibited no such defect. To evaluate whether the inhibition of miR-125a-3p promotes the remyelination, the remaining mice were infected with LV-decoy-miR-125a which is a TuD and designed to inhibit miR-125a-3p after 5 days of LPC induction. As a result, researchers observed considerably increased $GSTpi^+$ oligodendrocytes and myelin production, however they couldn't determine if this effect was a direct or indirect result of silencing. Furthermore, the results from inhibiting the miRNA with LV in an *ex vivo* model of demyelination confirmed the findings. The conclusions from this study suggest that silencing of miR-125a-3p might be in use to promote remyelination in demyelinating diseases like MS (37).

In a study by Ibrahim et al. (38) in which several previously undiscovered miRNAs were associated with MS via bioinformatics methods, the possibility of these miRNAs as biomarkers and therapeutic targets was examined through *in vitro* and *in vivo* experiments. Using the *in vivo* adoptive transfer model of $CD4^+$ cells with silenced miR-7188-5p and miR-7235 from control mice to EAE mice, researchers confirmed altered CNS pathophysiology and pattern of astrocytes following the silencing of miR-7188-5p and miR-7235. The suppression of these two miRNAs revealed a noticeable amelioration of the EAE disease, affirming their regulatory roles. This study thereby indicates that miR-7188-5p and miR-7235 exhibit encouraging therapeutic potential for managing MS (38).

In a recent study, Fujiwara et al. (39) initially assessed miR-92a levels in EAE mice, revealing higher levels of miR-92a compared to controls. Subsequently, they induced EAE in miR-92a deficient mice (Mir92a-/-), which noticeably delayed onset and reduced severity of the disease. When $CD4^+$ cells from both WT and Mir92a-/- mice were incubated, miR-92a loss did not alter Th1 differentiation but diminished both nonpathogenic and pathogenic Th17 cells while significantly increasing Treg polarization. Next, they employed LNA-modified miR-92a anti-miR, which they refer to as power inhibitors to investigate the therapeutic potential of miR-92a inhibition in EAE, both *in vitro* and *in vivo*. In the *in vitro* study, WT $CD4^+$ T cells exhibited higher frequency of Tregs and reduced nonpathogenic and pathogenic Th17 differentiation. When WT mice were treated with inhibitors *in vivo*, delayed onset and attenuated EAE disease progression were observed. This alleviation of EAE was linked to considerably decreased frequencies of $CD4^+$ T cells expressing IFN-γ, IL-17A, and IL-17A+GM-CSF. Lastly, Fujiwara et al. examined the effect of miR-92a silencing on $CD4^+$ T cells of RRMS patients and healthy controls. Consistent with the mice experiments, in both groups of cells, the induction of Treg cells was facilitated, and Th17 differentiation was limited, while Th1 differentiation was not affected. Collectively, the findings of this study indicate that miR-92a affects the MS disease course by maintaining the imbalance between Treg and Th17 cells and suggest silencing of miR-92a as a promising therapeutic target for MS (39).

Nakashima et al. (40) observed a potential correlation between miR-451a contained within extracellular vesicles (EV) and EAE exacerbation, as higher levels of EV miR-451a one day prior to EAE induction in mice were associated with increased EAE scores and worsening clinical symptoms. They conducted miR-451a knock-out analysis to evaluate the involvement of miR-451a in EAE exacerbation in mice and discovered that knock-out of miR-451a did not alleviate EAE scores. Instead, it slightly worsened the clinical symptoms, contrary to their expectation. In vitro experiments confirmed that miR-451a supports Th17 cell development. They noted that since Th17 are involved in the pathogenesis of EAE in mice, increased EAE severity in miR-451a knock-out mice can be a result of this increased Th17 differentiation. With another experiment, they observed that EVs boost IL-6 expression in macrophages when exposed to LPS. Therefore, to understand the impact of miR-451a on EV-mediated cytokine expression, THP-1 macrophage cell line was transfected with control, miR-451a mimic RNA or antagomir-451a for 2 days and then stimulated with LPS. Unexpectedly, IL-6 expression induces by LPS and in the presence of EV, was unaffected by both miR-451a mimic and antagomir-451a (40).

CONCLUSION

In summary, considering the significant impact of certain miRNAs on the immune system and inflammatory responses, their potential application as therapeutic targets in MS treatment appears promising. This review article offers a summary of studies that therapeutically impact the progression of EAE by inhibiting upregulated miRNAs. It is essential not to overlook the replenishing of the downregulated miRNAs as an alternative therapeutic strategy for MS. In support of this strategy, Zhu et al. (41) demonstrated that miR-20b overexpression, achieved through *in vivo* delivery of pre-miR-20b using LVs, reduced the numbers of Th17 cells and alleviated EAE severity (41). In the study from Osorio-Querejeta et al. (42), three delivery systems -Liposomes, Polymeric nanoparticles, EVs- were evaluated for encapsulating and releasing of miR-219a-5p mimic or human miR-219a-5p,

and their potential for remyelination promotion. The authors suggest EVs loaded with miR-219a-5p are capable of enhancing the clinical progression of EAE and potential approach for promoting remyelination in MS patients (42). Moreover, a single miRNA can regulate multiple mRNAs of several genes, thereby influencing diverse pathways. The potential of unwanted side effects occurring due to the alteration of the expressions of numerous off-target genes is an ongoing problem in the therapeutic usage of miRNAs (10). Thus, aggravation of other inflammatory disorders is a possible risk that should be handled carefully. For instance, while miR-20b is downregulated in MS and considered as a possible treatment target in EAE, it exhibits a reverse shift by being upregulated in ulcerative colitis (41). Generally, many anti-miRs are designed to be perfectly complementary to the specific seed region of target miRNA. However, previous studies reported anti-miRs not being able to differentiate between miRNAs within the same family under physiological conditions, especially those sharing identical seed regions. This may lead to anti-miR inhibiting different miRNAs in the same family and creating unexpected effects, which is another challenge in the development of miRNA therapeutics (17). Other limitations are delivery efficiency and dosage, since over-inhibition or overexpression of the miRNAs could impair normal cell functions or even lead to toxicity. To achieve effective delivery of anti-miRs, several delivery strategies must carefully be considered based on the target cell and site (23). Combined, these challenges reflect the challenges of bringing miRNA-based therapies into effective and safe treatments of MS. Even though no clinical trials have yet been conducted on the use of miRNAs in MS treatment (43), promising results obtained *in vitro* and in the EAE model raise hopes that a clinical study on MS therapeutics will soon commence. Hence, further exploration in this field and clinical trials are imperative.

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