The role of microRNAs in epileptogenesis

Modulation of brain inflammation and the extracellular matrix

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CHAPTER 7

General discussion

MicroRNAs in epileptogenesis

Progress in epilepsy research over the past decades has led to the discovery and characterization of the major pathogenic processes associated with epileptogenesis. MiRNAs have emerged as important modulators of signaling pathways associated with these processes in the brain. The involvement of miRNAs in the pathogenesis of epilepsy has been supported by a large number of studies that reported dysregulation of miRNA expression in human epileptogenic brain tissue and in animal models of epileptogenesis [1-3]. Dysregulation of miRNAs may represent both a consequence of pathological changes in the brain as well as a contributing factor to the pathology, leading to the idea that modulation of miRNAs in the brain may be useful in treatment and prevention of epilepsy [4]. However, the identification of these miRNAs and accurate dissection of each miRNA's contribution to epileptogenesis is a challenging task. From more than 2000 mature miRNAs described in mammals [5,6], hundreds of differentially expressed miRNAs have been identified in animal models of epileptogenesis. Each of these miRNAs may be able to influence the expression of multiple mRNA targets, and even entire pathways, in a cell type-specific manner [7]. The deregulated expression of miRNAs can change dynamically during epileptogenesis and also varies depending on the brain region of assessment [8]. Furthermore, the differences between methodologies employed by various laboratories, such as the use of different animal models of epileptogenesis, appear to produce heterogeneous outcomes [9,10]. As a result of these factors, the profiles of miRNA expression have yielded largely discordant results [2,4], representing context-dependent pieces of an intricate, yet incomplete, mosaic.

How can we pinpoint the most relevant miRNAs in epileptogenesis that could be of therapeutic value for the treatment of epilepsy? In chapter 2 we performed a meta-analysis across the published miRNA expression profiles during experimental epileptogenesis and created a database of more than 400 differentially expressed miRNAs [11]. Using a stringent bioinformatical approach we were able to find the most common differentially expressed miRNAs, that formed only a small subset of total analyzed miRNAs. These miRNAs were consistently deregulated across the expression profiles, despite the heterogeneity of factors involved. The identified miRNAs appear to be

associated with the key pathological processes linked to epileptogenesis, such as immune response/inflammation and remodelling of the extracellular matrix (ECM), and these processes represent commonalities in diverse animal models and human epilepsy [12]. Immune response is mediated by the evolutionarily conserved pathways that have been recently targeted in search for novel anti-epileptogenic therapy. Moreover, the activation of the innate immune response and inflammation is responsible for the alterations in the ECM. The miRNAs identified by the meta-analysis, as well as other inflammation-related miRNAs have a potential to modulate epileptogenesis through the regulation of these processes. Therefore, we focused our study on the expression of these miRNAs in various epileptogenic pathologies and the regulation of the pathogenic signaling pathways by these miRNAs in brain cells.

Inflammation-associated miRNAs are expressed by activated glial and immune cells during epileptogenesis

Several miRNAs identified by the meta-analysis and previous observations, including miR132, miR146a, miR155 and miR142 have been associated with the regulation of the immune response and inflammation [11,13-16]. These miRNAs are evolutionarily conserved and their genomic locations are not associated with any protein-coding genes. Knock-out experiments in mice have shown that the lack of these miRNAs may lead to abnormal phenotypes associated with brain development or immune system dysfunctions [7]. In chapters 3, 4 and 5 we validated the increased expression of these miRNAs in human epileptogenic pathologies, including temporal lobe epilepsy (TLE) and traumatic brain injury (TBI), as well as in animal models of TLE and TBI.

MiRNAs in the brain display cell-type specific expression and functions [17]. One of the most commonly up-regulated miRNAs during epileptogenesis is a brain-specific miR132, which has been studied extensively in neurons [18-22]. We demonstrated that miR132 can be up-regulated in astrocytes and microglia in the epileptogenic hippocampus. This suggests that miR-132 may be involved in the regulation of inflammation in the activated glial cells. The expression of miR146a and miR155 was found in various cell types, including the expression in neurons at basal level, however, their overexpression in the brain of humans

and animals with TLE or TBI is associated with activated astrocytes and microglia, where they function as potent inflammatory regulators. MiR142 is different from others because it is hardly expressed in the brain under normal conditions due to its hematopoietic origin [23]. However, we are the first to demonstrate that it is expressed by activated microglia and macrophages after TBI, as well as by infiltrating T cells, but not by neurons or astrocytes. These findings were also corroborated by the observations in brain tissue of patients with TLE (Fig. 1). Thus, the miRNAs that are commonly up-regulated during epileptogenesis are expressed in activated glial and immune cells, which are the main cells capable of mediating the innate immune response in the brain.

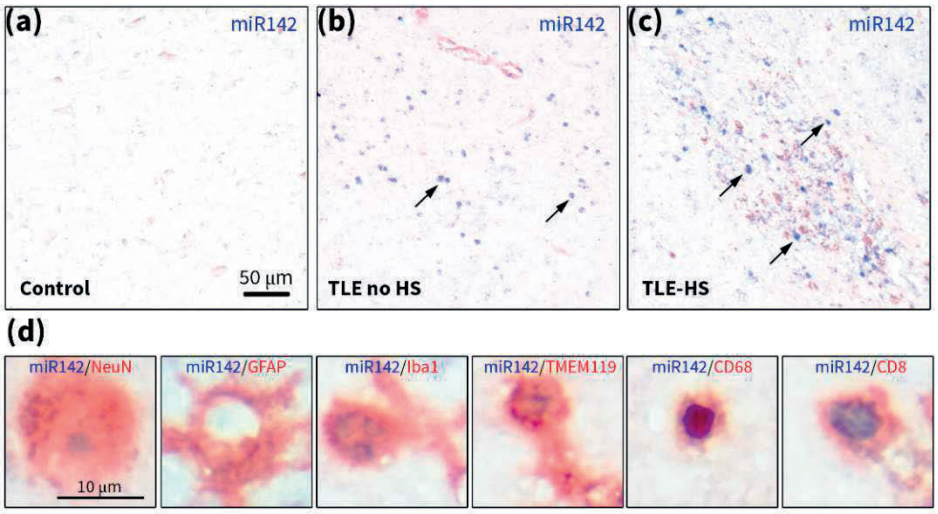

Figure 1. Higher miR142 expression in the human hippocampus. In situ hybridization for miR142 did not reveal the in situ hybridization signal (IHS) in the control hippocampus. In contrast, the stronger IHS was observed in the hippocampus of patients with TLE without hippocampal sclerosis (TLE no HS) (**b**) as well as in patients with TLE and hippocampal sclerosis (TLE-HS) (**c**). Arrows indicate individual cells expressing miR142; (**d**) Double labelling of miR142 with cell type-specific markers showed a weak IHS co-localized with NeuN and GFAP, stronger IHS co-localized with Iba-1 and TMEM119 and the strongest IHS co-localized with CD68 and CD8; Scale bar in **a-c** 50 µm and in **d** 10 µm.

Regulation of inflammation by miRNAs

The overexpression of inflammation-related miRNAs in the epileptogenic brain is also accompanied by increased expression of the markers of inflammation IL-1β, TNF-α and TGF-β1. The activated inflammatory pathways in astrocytes, microglia and macrophages are responsible for the transcriptional activation of

miRNAs, which are further involved in regulatory networks within these pathways. The selected miRNAs may be broadly divided into pro-inflammatory and anti-inflammatory. In human astrocytes the expression of miR146a and miR155 could be increased in response to stimulation with pro-inflammatory cytokines IL-1β and TNF-α, upon which they are involved in the feedback regulation of signal transduction pathways that result in NF-κB and AP-1 activation [24,25]. It has been previously shown that miR146a and miR147b (another miRNA with anti-inflammatory properties) have prominent action in astrocytes, where they suppress the activation of inflammatory signaling [26-28]. However, the overexpression of miR155 in human astrocytes leads to mixed results, with some inflammatory targets decreased and other – increased (Fig. 2). There is also no consensus in the literature on this account, with existing evidence supporting both pro-inflammatory [29] and anti-inflammatory [30] actions of this miRNA. This could be explained by the multitude of target genes in cross-reacting pathways depending on cellular context and time-point of assessment.

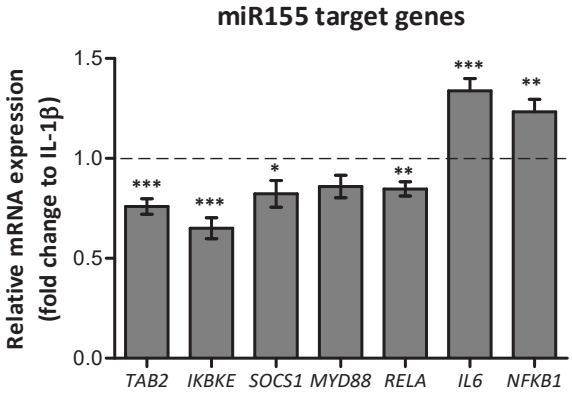

Figure 2. The regulation of miR155 target genes in human astrocytes. RT-qPCR analysis of miR155 target gene expression involved in inflammation in human primary astrocytes (n = 5) showed a lower expression of *TAB2* ($p < 0.001$), *IKBKE* ($p < 0.001$), *SOCS1* ($p < 0.05$) and *RELA* ($p < 0.01$), but higher expression of *IL6* ($p < 0.001$) and *NFKB1* ($p < 0.01$) as compared to control cells following IL-1β stimulation; Mann-Whitney U-test, *$p < 0.05$, **$p < 0.01$,***$p < 0.001$.

MiR132 has also been associated with inflammation via targeting acetylcholinesterase in neurons and promoting cholinergic anti-inflammatory signaling [16,31]. Following our findings that miR132 is expressed in glia in the epileptogenic brain, we demonstrated that miR132 can negatively regulate

IL-1β-mediated inflammatory signaling in astrocytes [32], which is corroborated by a previous report that miR132 can negatively regulate inflammatory signaling in an astrocytic cell line [33]. A potential mechanism for such regulation could be through the down-regulation of its target COX-2. COX-2 expression is increased in both neurons and astrocytes in TLE [34,35] and could be involved in the pathogenesis of epilepsy [36]. Moreover, miR132 may be involved in the regulation of TGF-β pathway since its expression in human astrocytes is increased by the stimulation with TGF-β1 and the predicted target of miR132 TGF-β2 is down-regulated following miR132 overexpression [32]. TGF-β signaling pathway in astrocytes has been shown to be an important contributor to epileptogenesis [37-39]. Thus, in addition to its functions in neurons, miR132 may act to suppress pro-inflammatory signaling in activated astrocytes in TLE.

MiR142 does not appear to be highly expressed in neurons or astrocytes, but it is found in microglia and macrophages, where its overexpression can lead to an increased production of TNF-α. This can both contribute directly to brain inflammation and can activate nearby astrocytes [40]. Indeed, human astrocytes show a higher expression of pro-inflammatory genes, including *IL1B*, *TNF* and *PTGS2*, when treated with the culture medium from miR142-overexpressing macrophage-like cells as compared to astrocytes treated with the medium from control macrophage-like cells. This suggests that miR142-overexpressing cells observed in the brain post-TBI may promote a pro-inflammatory state in surrounding astrocytes. This is in line with previous observations that miR-142 knock-out mice exhibit less inflammation in the cerebellum in the experimental autoimmune encephalomyelitis [41]. Thus, the overexpression of miR142 in the human brain may promote inflammation after a brain insult, which may further contribute to epileptogenesis.

The activation of astrocytes and loss of their homeostatic functions is one of the hallmarks of epileptogenesis [42]. Reactive astrocytes and microglia provide a major source of pro-inflammatory molecules including IL-1β and TNF-α during brain pathology [42,43]. The studied miRNAs act to modulate the pro-inflammatory signaling in astrocytes directly (miR146a, miR155, miR132) or indirectly (miR142). Through such modulation, these miRNAs could act not only to suppress their direct targets, but also influence the expression of the down-stream targets of inflammatory pathways, such as cytokines, prostaglandins, growth factors and ECM molecules.

Regulation of the ECM by inflammation-related miRNAs

The ECM is the non-cellular component of the brain, which is produced by neurons and glial cells, and the alterations in the ECM composition and structure play an important role in establishing epileptogenic networks [44], contributing to processes such as mossy fiber sprouting, granule cell dispersion and gliosis [45]. Among the deregulated pathways in TLE, the pathways related to the remodeling of the ECM form one of the two largest clusters along with the pathways related to inflammation [46]. Matrix metalloproteinases (MMPs) are the major enzymes responsible for the remodeling of the ECM [47] and their up-regulation in epileptogenic tissue may lead to the degradation of PNNs and BBB, as well as promote neuroinflammation. The increased expression of 4 MMPs (MMP2, MMP3, MMP9 and MMP14) could be observed in human TLE and TSC tissue [48-50] and the increased expression of MMP genes could be observed in the rat hippocampus shortly after SE and during the latent and chronic phases [49,51].

The transcriptional activation of MMP expression is largely dependent on pro-inflammatory stimuli [52-55]. In human astrocytes MMP3 gene expression could be activated by such stimuli as pro-inflammatory IL-1β, TNF-α and TGF-β1, as well as reactive oxygen species-producing hydrogen peroxide stimulation (Fig. 3). In addition, MMP3 transcription was strongly activated in a macrophage/microglia cell model following a classic lipopolysaccharide (LPS) stimulation and the conditioned medium from these cells could induce MMP3 gene expression, but not other MMP genes, in human astrocytes. Moreover, *Mmp3* was the only metalloproteinase gene with increased expression in the cortex of rats 2 weeks post-TBI. MMP3 is normally expressed in astrocytes at low level, but is increased in response to a pro-inflammatory stimulation [56-59]. Thus, MMP3 appears to represent an inflammation-sensitive pathological marker in the brain.

Since miRNAs can modulate pro-inflammatory signaling in astrocytes, they could have a modulatory effect on MMP expression. The overexpression of the anti-inflammatory miR146a and miR132, as well as inhibition of miR155 attenuated the increased expression of MMP3 in human astrocytes. Thus, in addition to modulation of inflammation, miRNAs have a potential to modulate

the alterations in the ECM during epileptogenesis through the control of MMP3 expression.

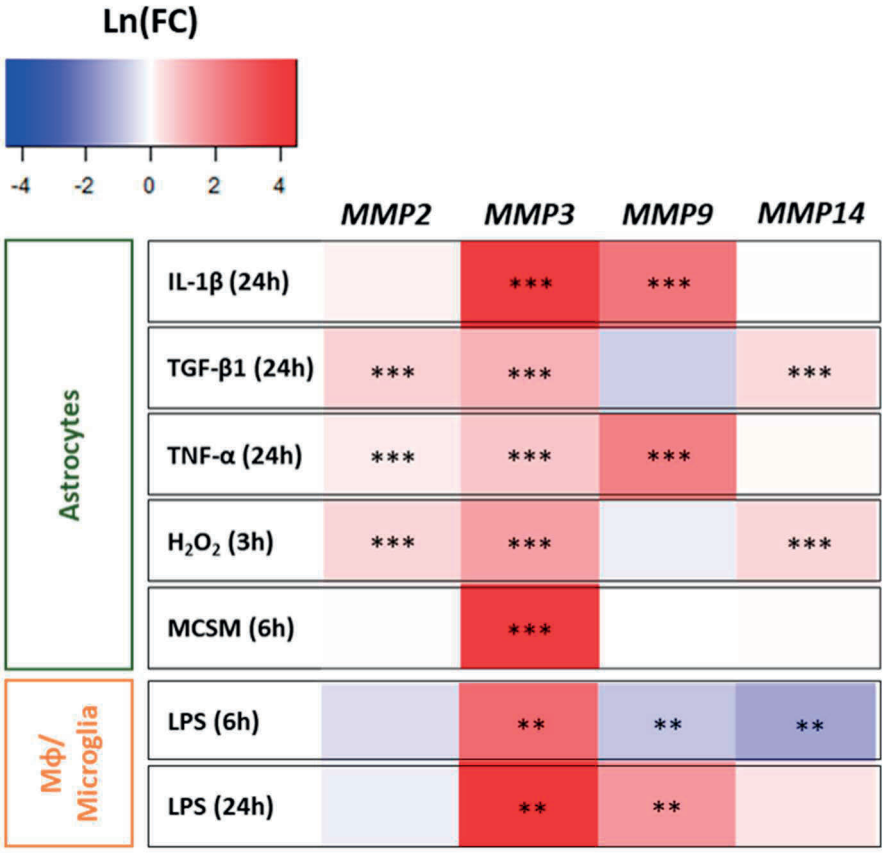

Figure 3. The heatmap of MMP gene expression in vitro. RT-qPCR analysis in human primary astrocytes and THP-1 cell line showed that of *MMP2*, *MMP3*, *MMP9* and *MMP14* could be induced by various pathological stimuli in human cells; H_2O_2 – 100 µm hydrogen peroxide; MCSM – conditioned culture medium from THP-1 cells following stimulation with lipopolysaccharide (LPS); Mann-Whitney U-test, variable n =3–5; **p < 0.01,***p < 0.001.

miR34a overexpression may affect cerebral cortex development in TSC

Apart from the regulation of processes, such as inflammation and ECM remodeling, which contribute to epileptogenesis in the adult brain, miRNAs can also regulate neurodevelopmental processes, the dysregulation of which may lead to malformations of cortical development and early-onset epilepsy [60]. Previously we identified miR34 family (miR34a, miR34b and miR34c) among the most up-regulated miRNAs in TSC cortical tubers compared to autoptic control

brain cortex [61]. A follow-up study performed in chapter 6 showed that overexpression of miR34a in TSC was observed during fetal and early postnatal brain development and may have an impact on corticogenesis and contribute to the pathology in several ways. First of all, the overexpression of miR34a in mice led to a disturbed migration of neural progenitor cells. The genes associated with neuron migration showed a strong enrichment among the predicted targets of miR34a and previous reports have linked miR34a to aberrant migration [62,63]. Further, miR34a is normally highly expressed in the adult brain, but the prematurely high expression during the first years of life may affect neurite outgrowth, as miR34a has been shown to regulate this process [64]. In addition, miR34a can down-regulate the expression of a cell-adhesion molecule contactin-3 (CNTN3) in vitro. This CAM has been shown to possess a neurite outgrowth-promoting activity [65] and its expression is lower in TSC during the first years of life compared to the age-matched controls. Both overexpression of miR34a and the defects in CNTNs, including CNTN3, have been linked to ASD [66,67] and ASD frequently accompanies TSC [68]. Lastly, miR34a has been known as a pro-apoptotic miRNA, since it can be activated by p53 protein and is further involved in the feed-forward regulation of p53 [69]. Apoptosis is a paramount process for both embryonic neurogenesis and postnatal brain development [70]. However, we did not observe an increase in apoptotic cells following miR34a overexpression in mice, suggesting that the increase of miR34a alone may be insufficient for triggering apoptosis.

It is important to note that miR34a overexpression in fetal brain could be observed not only in giant cells, but also in other cells throughout the developing cortex. The increase in miR34a was contingent on the activation of p53 in the developing cortex, whereas pS6 activation was mostly restricted to giant cells. This suggests that miR34a overexpression in TSC cortex may not be directly related to mTOR pathway activation, but rather depend on the pathological microenvironment in the brain areas affected by tuber formation. One of such factors could be oxidative stress, since miR34a could be increased in vitro by stimulation of neuronal cells with hydrogen peroxide, and we have previously shown an increased expression of oxidative stress markers in TSC tubers [71].

Future outlook and therapeutic potential of miRNAs

Epilepsy is a complex pathology produced by multiple intricately interwoven pathogenic processes. MiRNAs are estimated to target more than 45,000 sites in the 3' untranslated regions of about 60% of all existing messenger RNAs [72], and thus the evolutionarily conserved miRNAs deserve to be considered as major components of the gene regulatory units in epileptogenic networks. The dysregulation of many miRNAs during epileptogenesis represents an additional level of complexity to the gene expression analysis, but also provides an opportunity for therapy [3,73] or diagnostics as biomarkers [74-76]. MiRNAs are network regulators of gene expression, and the development of new bioinformatics methods and statistical analysis may help to predict the most potent "master regulators" among them.

The current knowledge of the differentially-expressed miRNAs in epileptogenesis is mostly based on microarray- and qPCR array-based studies. This knowledge is waiting to be confirmed and refined by the studies using the more advanced and unbiased high-throughput RNA-Seq techniques. The benefits of RNA-Seq include a transcriptome-wide scale and the analysis of isomiRs – variants of mature miRNA sequences with minute differences in nucleotide composition at the 3'and 5'ends [77]. Further, the advent of the singe-cell expression analysis will further deepen our understanding of miRNA expression in a cell type- and context-specific manner. This is essential, since even miRNAs established to be expressed in certain cell types may be expressed in different cells depending on pathological states. One example is a neuronal miR132, the inhibition of which has been proposed as a potential neuroprotective treatment based on a study in a post-SE model [78]. Our findings suggest that miR132 is expressed and may have anti-inflammatory functions in astrocytes during epileptogenesis [32], whereas its inhibition may promote inflammation and potentially aggravate the pathology.

The field of non-coding RNA biology is a relatively young branch of science, but it has already produced results that are translatable to the clinics [79-81]. This includes treatment of epilepsy, since the use of an RNA-based inhibitor may soon enter the phase II of clinical trials for the treatment of the drug-resistant epilepsy in Dravet syndrome (https://dravetsyndromenews.com/cur-1916/). Studies in preclinical models of

epilepsy have shown an efficacy of modulation of neuronal miRNA expression in modification of epileptogenesis [82,83]. Our results described in chapter 6 allow us to hypothesize that normalization of neuronal miR34a levels in the developing TSC brain may be of therapeutic value. Furthermore, the potential to combine inhibition of miR34a with the established drugs like rapamycin or everolimus, as well as anti-oxidant treatments, could be further explored in order to achieve a synergistic therapeutic effect in treatment of TSC and associated neurological impairments (Fig. 4). In a similar manner, the overexpression of miR146a, aimed at suppression of brain inflammation in order to alleviate epileptogenic pathology, has shown some promising results in vivo [84,85]. The administration of miR132 or inhibition of miR155 may also have an anti-epileptogenic action through the modulation of pro-inflammatory signaling and down-stream targets, such as IL-1β, COX-2 and MMP3. Indeed, silencing of miR155 in vivo has been shown to reduce seizure frequency in rats and mice post-SE [86,87] (Fig. 4).

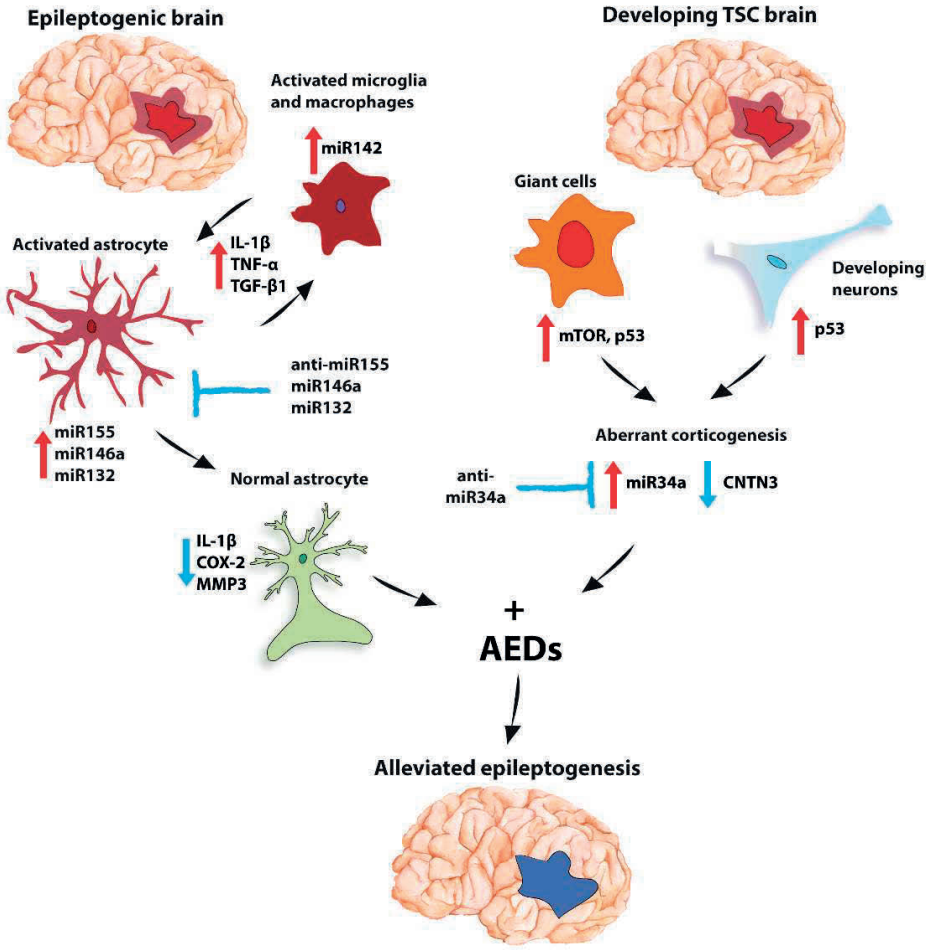

Figure 4. MiRNA regulation of processes associated with epileptogenesis. The activation of glial cells in the adult epileptogenic brain leads to the production of inflammatory mediators, such as IL-1β, TNF-α, TGF-β1. The overexpression of miR142 in the microglia and macrophages may lead to an increased production of at least one of them – TNF-α, which results in the overexpression of pro-inflammatory genes in astrocytes. This leads to the loss of astrocytic homeostatic functions and further perpetuation of neuroinflammation. The pro-inflammatory cytokines also increase the expression of miR146a, miR155 and miR132 in astrocytes. In the developing brain of patients with TSC, the overexpression of miR34a is dependent not only on mTOR activation, but also on p53 activation. The overexpression of miR34a during fetal and early post-natal brain development may lead to a decreased expression of neuronal molecules, such as CNTN3 and overall aberrant corticogenesis. The inhibition or overexpression of these miRNAs may help to normalize dysregulated signaling pathways and rescue pathological consequences of epileptogenic structural lesions. The combination treatment with the established AEDs may have a synergistic effect to alleviate epileptogenesis.

Conclusions

In this thesis we aimed to investigate the involvement of miRNAs in epileptogenesis through the regulation of pro-epileptogenic processes in the brain, including immune response and inflammation, alterations in the ECM and corticogenesis during neural development. Furthermore, we aimed at the identification of novel therapeutic targets and miRNA-based approaches for prevention or modification of acquired epilepsy. The miRNAs miR132, miR146a, miR155 and miR142 are primarily involved in the regulation of innate immune response and inflammation in activated glial cells and can modulate the expression of pro-epileptogenic factors, such as pro-inflammatory cytokines and MMPs. The increased expression of miRNAs associated with neurodevelopmental processes, such as miR34a and miR34b, can lead to aberrant corticogenesis in the immature brain, which may contribute to epileptogenesis and cognitive impairment in TSC. The ability to modulate key pathological processes associated with epileptogenesis suggests that overexpression or inhibition of these miRNAs may be considered for use as a novel therapeutic approach in treatment of epilepsy and associated co-morbidities.

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