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Review

Involvement of host microRNAs in flavivirus-induced neuropathology: An update

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Flaviviruses are a spectrum of vector-borne RNA viruses that cause potentially severe diseases in humans including encephalitis, acute-flaccid paralysis, cognitive disorders and foetal abnormalities. Japanese encephalitis virus (JEV), Zika virus (ZIKV), West Nile virus (WNV) and Dengue virus (DENV) are globally emerging pathogens that lead to epidemics and outbreaks with continued transmission to newer geographical areas over time. In the past decade, studies have focussed on understanding the pathogenic mechanisms of these viruses in a bid to alleviate their disease burden. MicroRNAs (miRNAs) are short single-stranded RNAs that have emerged as master-regulators of cellular gene expression. The dynamics of miRNAs within a cell have the capacity to modulate hundreds of genes and, consequently, their physiological manifestation. Increasing evidence suggests their role in host response to disease and infection including cell survival, intracellular viral replication and immune activation. In this review, we aim to comprehensively update published evidence on the role of miRNAs in host cells infected with the common neurotropic flaviviruses, with an increased focus on neuropathogenic mechanisms. In addition, we briefly cover therapeutic advancements made in the context of miRNA-based antiviral strategies.

Keywords. Epigenetic regulation; flavivirus; host-response; microRNAs; neurotropism

1. Introduction

Flaviviruses are a member of Flaviviridae family and are single-stranded RNA viruses transmitted by arthropod vectors that subsequently cause severe viral disease. The mosquito-borne flaviviruses include Japanese encephalitis virus (JEV), Zika virus (ZIKV), West Nile virus (WNV), Yellow fever virus (YFV) and the Dengue virus (DENV). The rapidly spreading and continuously emerging class of flaviviruses on a global scale is an ongoing challenge (Pierson and Diamond 2020). No vaccines exist for ZIKV and WNV, while vaccines developed for JEV, YFV and DENV have their unique limitations (Yun and Lee 2014; Barrett 2017; Halstead 2017). Additionally, in the absence of a robust and specific antiviral drug, the clinical management of these viral diseases is still critical and warrants further research. The threat of spread and re-emergence of flaviviruses is the main driving force to conduct further studies and to explore potential therapies and biomarkers. JEV, ZIKV and WNV are the leading viruses that infect the brain and cause neurological disease. These viruses cause encephalitis, paralysis, seizures and cognitive impairment in the infected patient. The neurological debilitation can last long after recovery from acute infection and can lead to life-long functional disability (Pierson and Diamond 2020).

All viruses are obligate parasites and entirely depend on their host cell to survive and propagate. They are known to hijack the cells' housekeeping machinery to their own advantage while the cell attempts to evade the virus by mounting an immune response (Summers 2009). MicroRNAs (miRNAs) are known to be essential components of an antiviral response or even in supporting viral life-cycle through complex gene regulatory networks.

1.1 *MicroRNAs*

First discovered by Lee *et al.* (1993), miRNAs are a class of small, endogenous non-coding RNAs that play

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a role in the post-transcriptional regulation of gene expression in a cell (Lee *et al*. 1993; Bartel 2004; Bhaskaran and Mohan 2014). Each miRNA is 19–25 nucleotides long and predominantly binds to 3′ untranslated regions (UTRs) of target messenger RNAs (mRNAs), leading to repression of translation (partial complementarity) or degradation of the transcripts (complete complementarity) (Mazière and Enright 2007). Interestingly, a single miRNA can target hundreds of mRNAs or a single mRNA can be targeted by multiple miRNAs (Ni and Leng 2015). The most recent miRbase database release has recorded 1917 hairpin precursors and 2654 mature miRNA sequences in the human genome (Kozomara *et al*. 2019). Over the past three decades, there has been a rapid expansion of evidence on the importance of miRNAs in human physiology and disease (Li and Rana 2014; Xue *et al*. 2017; Matsuyama and Suzuki 2019).

The primary function of miRNAs is gene regulation. Mathematical modelling, followed by mounting experimental validation, has discerned nine modes of action for miRNAs, namely, mRNA decay (destabilization), mRNA cleavage, elongation inhibition, transcriptional inhibition through miRNA-mediated chromatin reorganization, followed by gene silencing, 60S ribosomal unit joining inhibition, Cap-40S initiation inhibition, ribosomal drop-off (premature termination), co-translational nascent protein degradation and sequestration of P-bodies (Morozova *et al*. 2012). The biogenesis of miRNAs starts with longer primary transcripts (pri-miRNA) which are usually transcribed by RNA polymerase II. Pri-miRNAs can carry multiple mature miRNA sequences that are processed by Drosha, a type III ribonuclease (RNase), exported into the cytoplasm in a double-stranded form (pre-miRNA) and cleaved by another RNAse III enzyme, Dicer. Either of the strands can act as the mature miRNA post cleavage and can be further loaded onto argonaute (Ago) proteins, to become a part of the multicomponent RNA-induced silencing complexes (RISCs) (Acuña *et al*. 2020). Formerly, miRNAs were annotated as the 'mature strand' (miR) that was highly expressed and the lesser expressed 'passenger strand' (miR*). However, the miR/miR* status could change according to cellular physiology. Eventually, the miR/miR* was replaced by the unbiased 5p/3p annotation according to their position in the pre-miRNA hairpin independent of their functional expression in any tissue (Desvignes *et al*. 2015).

1.1.1 *MicroRNAs and neuronal disease:* A large body of evidence suggests a pivotal role for miRNAs in human diseases involving every tissue of the body including cancer, diabetes, cardiovascular disease and viral infections (Kawaguchi *et al*. 2016; Singh and Sen 2017; Brennan and Henshall 2018; Shaik *et al*. 2018; Zhang *et al*. 2020). The aberrant expression of miRNAs and their life-cycle are being thoroughly researched and exploited to identify their use as biomarkers across various neuronal conditions including Alzheimer's disease, Parkinson's disease, brain injury, stroke, cancer, epilepsy and infections. Features that make miRNAs advantageous biomarkers are as follows: (1) The majority of miRNAs are conserved across species, (2) miRNAs are structurally stable across biofluids and tissues, (3) by targeting miRNAs, multiple pathways and genes in the cell can be altered, and (4) their small sizes makes it easier to intervene with their functions and to develop miRNAs into drug molecules (Sun *et al*. 2018; Xia *et al*. 2019). Specific miRNAs such as miR-155, miR-146a, miR-124, miR-210, miR-206, miR-1 and let-7 have been associated with neuroinflammation and neurodegeneration (Dickey *et al*. 2017; Neal and Richardson 2018; Nuzziello and Liguori 2019). These miRNAs have varied pro- or anti-inflammatory responses depending on the disease state. Microglial activation, apoptotic response, blood–brain barrier (BBB) repair, neuronal death and dysfunction are a few processes regulated by miRNAs (Slota and Booth 2019). Interestingly, these miRNAs play critical roles in flaviviral infections as well.

2. MicroRNAs in flaviviral neuropathology

Current evidence suggests a common theme in the neuropathogenesis of these flaviviruses. JEV, WNV, ZIKA and DENV infect highly specific brain regions that control motor functions such as the thalamus, basal ganglia, brainstem and spinal cord. Their routes of neuroinvasion have been pinned down to hematogenous and axonal transport with their capsid protein (E) playing a key role in neurovirulence. In addition, these flaviviruses primarily cause cellular apoptosis and bystander death upon infection, which ultimately snowballs into a damaging inflammatory response in the brain (Sips *et al*. 2012). It is extremely relevant to explore miRNAs in cells infected with viruses, given the ability of an invading virus to disrupt the host cellular machinery to aid its own replication and survival (Saliminejad *et al*. 2019). In this review, we address the most recent miRNA-related reports of the neurotropic flaviviruses WNV, ZIKV and JEV. In addition, we also explore DENV, which remains vastly understudied in regard to its neurotropism.

2.1 *West Nile virus*

2.1.1 *Epidemiology: Culex* (*Cx*) mosquitoes are the principal vectors of WNV, especially *Cx. pipiens* (Sejvar 2016; Habarugira *et al*. 2020). WNV has been reported in west Asia, India, Africa, the Middle East, North America, Europe and Australia (as a WNV subtype) (Hayes *et al*. 2005). Among the infected, the majority of patients are asymptomatic (80%). In the remaining 20% symptomatic patients, a majority present with febrile illness (West Nile fever). However, in 1% of the symptomatic patients, WNV crosses the blood-brain barrier (BBB) to cause neuroinvasive disease in the form of encephalitis, aseptic meningitis and acute flaccid myelitis. Meningitis occurs mostly in paediatric and young populations, whereas encephalitis is observed in older patients (Sejvar 2016).

2.1.2 *Role of host miRNAs during cellular infection:* During the early phase, viral replication takes place in keratinocytes, skin dermal dendritic cells (DCs) and Langerhans cells. Infected DCs migrate to the regional lymph nodes and seed the viral particles; consequently, replication in draining lymph nodes leads to viremia. The viremia causes further infection of the peripheral organs (spleen, kidney, liver) and the infection is subsequently cleared from the system. However, in a subset of human patients, after 6–8 days of infection, WNV can be detected in the brain and spinal cord tissue (Gyure 2009; Sips *et al*. 2012; Suthar *et al*. 2013). Evidence from animal models suggests that the virus infects the central nervous system (CNS) primarily by haematogenous dissemination (Fredericksen 2014). The mechanism underlying WNV invasion of the human host cells, especially in the CNS, is still unclear, but has been well studied in murine models (Suthar *et al*. 2013; Petersen *et al*. 2013; Sejvar 2016). The haematogenous route of neuroinvasion is understudied and could happen by multiple ways: (a) secretion of tumour necrosis factor (TNF) can lead to increased endothelial permeability, (b) breakdown of the endothelial cell junctions by matrix metalloproteinases (MMPs) or (c) by the Trojan horse mechanism, i.e., the virus being carried into the brain tissue by infected immune cells. Currently, there is no evidence of the role of miRNAs in the BBB permeability in the event of WNV infection. A few studies have explored miRNAs in viral neuroinvasion and are interesting leads to pursue (Yang *et al*. 2017; Song *et al*. 2018).

Neurons are the prime target of WNV replication. Virus-associated pathology is characterized by neuronal death, activation of glial cells, BBB disruption, increased production of pro-inflammatory mediators and infiltration of leukocytes in the perivascular space and parenchyma (Kumar and Nerurkar 2014; Sejvar 2016; Peng and Wang 2019; Habarugira *et al*. 2020).

In 2014, Kumar and Nerurkar observed a differential expression of 139 miRNAs in mice infected with WNV in comparison with a non-infected control group. Among the most significant expressions, up to a 1000-fold upregulation of let-7 family (except for let-7c) and a 13000-fold downregulation of miR-196a was observed. This study focussed on miR-155, miR-32, miR-196a, miR-202-3p, miR-449c and miR-125a-3p, and their role in neuroinflammation and neuronal death. Downstream pathway analysis and target validation of these miRNAs revealed the regulation of nuclear factor kappa B (NFκB) and extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway. These pathways are critical for proinflammatory response, antigen presentation and cell survival, regulation of cytokines, chemokines and their receptors. and regulation of cell apoptotic genes such as B-cell lymphoma-2 (BCL-2) family genes. The analysis also identified the disruption of the tight junction protein (TJP) that leads to BBB breakdown as an enriched pathway, although the miRNA–TJP relationship awaits further investigation. This study was conducted on a small cohort (n=4) for each group and certainly warrants a larger cohort validation (Kumar and Nerurkar 2014). In 2019, the same research group focussed on miR-155 in WNV infection. They demonstrated that in miR-155-knockout mice presented with severe neurological disease upon WNV infection when compared with control mice. miR-155 was shown to be regulator of proinflammatory cytokines and chemokines. Further evidence from primary mouse cells and human neuroblastoma cells confirmed that overexpression of miR-155 led to a marked reduction in viral replication and enhanced antiviral immune response, making this miRNA critical for the host to combat WNV infection (Natekar *et al*. 2019). Another study in 2014 showed that the miRNA expression profile of WNV-infected HEK293 cells is independent of toll-like receptor-3 (TLR-3) expression and potentially associated with other pattern recognition receptors (PRRs) such as retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA5) (Chugh *et al*. 2014). It is known that TLRs are responsible for mounting an immune response upon viral invasion and can also alter the expression of miRNAs in host cells (O'Neill *et al*. 2011; Galli *et al*. 2013; Chugh *et al*. 2014). It provides evidence of an early response to infection via PRRs

other than TLR3 that regulate miRNAs and the NFkb pathway upon immediate viral entry. This study recognized and validated a distinct WNV-induced miRNA pattern comprising 70 miRNAs in the cellular model that gives rise to viral pathogenesis and is distinct from the TLR-3-mediated miRNA pattern.

Slonchak *et al.* (2015) performed RNA sequencing of WNV-infected HEK293 cells and identified 24 significant differentially expressed pre-miRNAs and mature miRNAs. The study further validated the antiviral effects of miR-532-5p achieved by regulating and spectrin domain containing 1 (SESTD1), TGF-beta-activated kinase 1 and MAP3K7-binding protein 3 (TAB3), which are essential for efficient WNV replication (Slonchak *et al.* 2015). A high-content immunofluorescence screening performed by Smith *et al.* (2017) identified key miRNAs that show antiflaviviral activity, including miR-34c-5p, miR-1231, miR-517a, miR-876-3p and mir-453 in WNV-infected cells. The miR-34 family was assessed for their regulatory role of Wnt/β-catenin signalling pathway that is postulated to play a role in the antiviral response mechanism of infected cells (Smith *et al.* 2017). The miRNAs discussed in key infection-related mechanisms have been additionally illustrated in figure 1.

2.2 *Zika virus*

2.2.1 *Epidemiology:* ZIKV is a significant and well-established member of the Flaviviridae family and is primarily transmitted by the *Aedes* mosquito (Kazmi *et al.* 2020). Non-vector transmission has also been

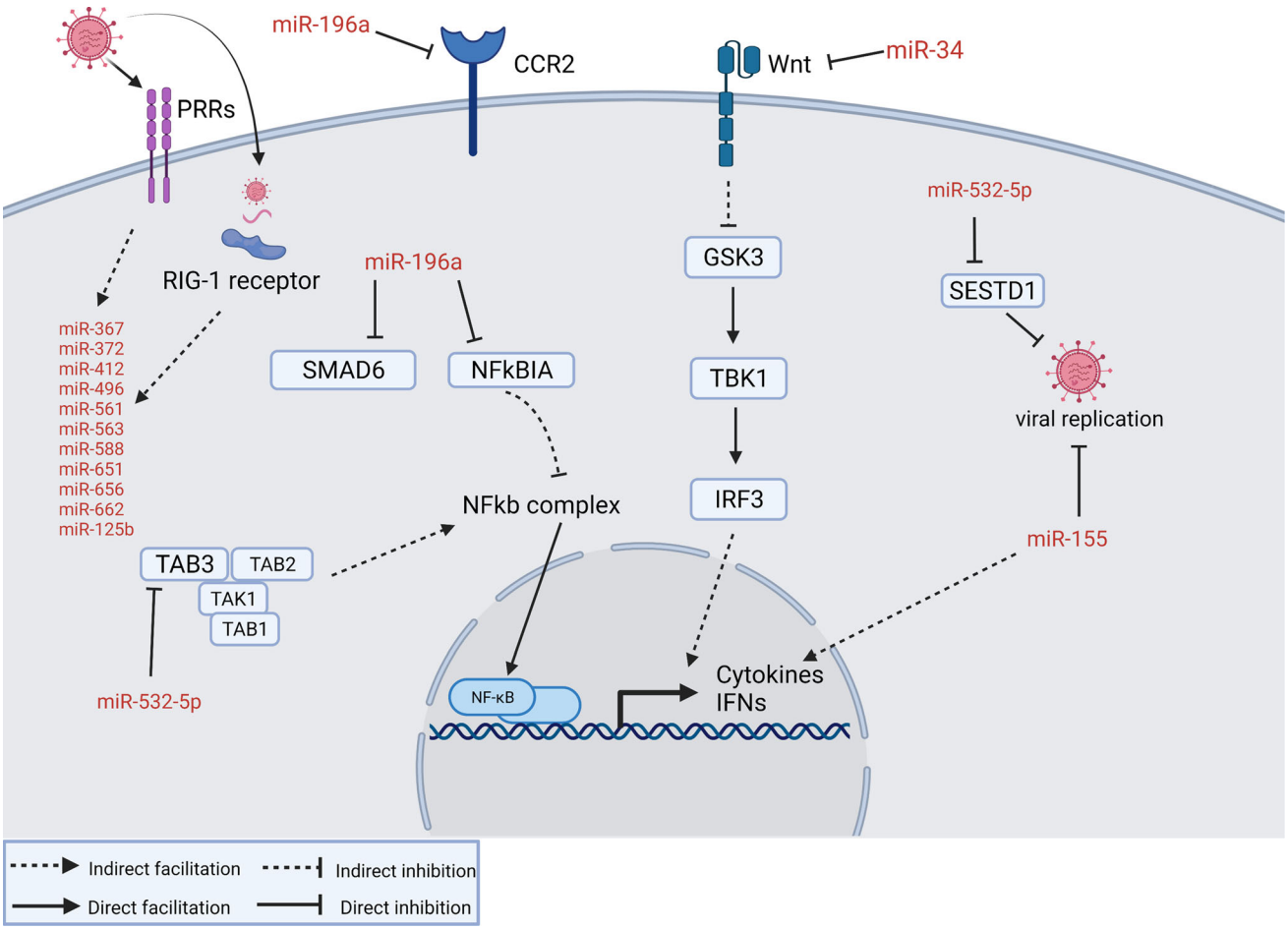

Figure 1. Key miRNAs and their targets involved in host cell response to WNV invasion. miR-196a targeted *SMAD6* and *NFκBIA* genes, and 532-5p-targeted *TAB3* gene regulate NFκB pathway. miR-34a-targeted *Wnt* genes have a regulatory effect on cytokine and IFN expression. miR-155 and mir-532-5p inhibit viral replication. miR-196a also targets the *CCR2* gene, essential for inflammatory response. Viral interaction with pattern recognition receptors such as RIG-1 modulate multiple miRNAs in the host cell (listed).

reported by the mode of blood transfusion, gestational transmission, sexually or by organ transplant (Musso and Gubler 2016; Nugent *et al*. 2017). The virus is known to have originated from Asia and Africa with the first case reported in 1952 in Uganda. Almost after 60 years of latency, the virus emerged and is reported to be spreading across the Asian subcontinent, North Africa, the Pacific ocean islands and South America (Calvet *et al*. 2016; Kazmi *et al*. 2020).

Once introduced into the human host, the virus has an incubation period of roughly 2–14 days. In adults, most infected patients are asymptomatic (80%). Clinical manifestations include mild flu-like symptoms, dizziness, fever, maculopapular rash and conjunctivitis (Kazmi *et al*. 2020; Masmejan *et al*. 2020). The paediatric population shows similar or relatively mild symptoms with ZIKV infection (Masmejan *et al*. 2020). Unfortunately, the ZIKV infection along with its geographical spread has also shown more severe clinical manifestations over the years in the form of severe neurological defects and the life-threatening Guillain-Barré Syndrome (GBS). In pregnant females with prenatal exposure to ZIKV, 6 to 14% of the cases have experienced developmental abnormalities in the foetus, including microcephaly, structural defects and ocular anomalies among other complications (Masmejan *et al*. 2020).

2.2.2 *Role of host miRNAs during cellular infection:* A large variety of cells are receptive to ZIKV including skin keratinocytes, dermal fibroblasts, placental cells, placental macrophages, cells of the CNS, retinal cells, spermatozoa, monocytes and dendritic cells (Sirohi and Kuhn 2017; Lee and Shin 2019). The exact mechanism that makes ZIKV cellular invasion highly aggressive is still largely unexplored.

The ZIKV-collaborative database (CDB) was generated in 2016, which utilized an *in silico* model to predict human miRNAs that interact with the ZIKV genome and viral miRNAs that may recruit human genes to facilitate ZIKV survival (Pylro *et al*. 2016). Virus-encoded miRNAs merit a separate discussion and have not been included in the scope of this review. In addition, a comprehensive list of >5000 human miRNAs with complementary target regions across 20 ZIKV strains was presented in this study, including miR-34a, miR-324-3p, miR-15b-5p,miR-21-5p, miR-335-5p, miR-615-3p and miR-193-3p which are known to target genes involved in microcephaly.

In 2017, Kozak *et al.* performed the first miRNA expression analysis of ZIKV-infected cells. A human foetal cell line, SVG-A, was infected with ZIKV and their global miRNA expression change was analysed using next-generation sequencing. Among the 15 upregulated miRNAs in this study, miR431-5p and miR30e-5p were already known to be involved with other viral pathologies and influence innate immunity and neurological development (Pedersen *et al*. 2007; Zhu *et al*. 2014; Makkoch *et al*. 2016; Kozak *et al*. 2017). Innate immunity genes, *CD59* and pentraxin 3 (*PTX3*), were found to be downregulated and regulated by miR-194-5p and miR-411-3p. Further, miR-17-5p was predicted to regulate genes involved in cell cycle, DNA damage and viral replication, and miR-9 was predicted to regulate the genes involved in mRNA processing, gene expression and viral processes. An overall trend of miRNA downregulation was observed in the infected cells. This seemed to be a consequence of the downregulated expression of DICER1 and in line with increasing viral burden of the cell. Similarly, another study on HEPG2 (liver), A549 (lung) and MA104 (kidney) cell lines infected with ZIKV showed significant dysregulation of Drosha, Dicer, AGO2 and AGO3, belonging to the miRNA biogenesis pathway. This dysregulation coincided with the peak of viral infection and suggests the silencing of miRNA-driven antiviral responses in the cell to promote pathogenesis (Ferreira *et al*. 2018). This phenomenon warrants further investigation due to its potential therapeutic value.

Bhagat *et al.* (2018) demonstrated the alteration of miRNA profile of human fetal neural stem cells (fNSCs) in the presence of ZIKV envelope (E) protein. They observed 25 dysregulated miRNAs including miR-1273g-3p and miR-204-3p, which were shown to target the paired box gene 3 (*PAX3*) and neurogenic locus notch homolog protein 2 (NOTCH2). In further bioinformatics analysis, the most enriched pathway involved with the differentially expressed miRNAs was the Wnt signalling pathway, an observation supported by previous literature (Smith *et al*. 2017; Lin *et al*. 2017; Bhagat *et al*. 2018). Cell cycle and developmental pathways such as NOTCH, epidermal growth factor (EGF), fibroblast growth factor (FGF) and tumour protein 53 (p53) were the most affected in this study and associated with immature neuronal differentiation, apoptosis and microcephaly. In our opinion, this toxic effect on hNPCs may be exacerbated with increased proinflammatory markers released by neighbouring infected microglia (Wang *et al*. 2018c), warranting further evidence. Following this, Azouz *et al* . (2019) performed a high-throughput miRNA and mRNA expression analysis in ZIKV-infected primary mouse neurons. They reported 112 miRNAs that were significantly dysregulated including the upregulated

miR-203, miR29a, mir-29b and miR-155 and down-regulated miR-124-3p, miR-883-5p and miR-2137. In particular, miR-155 and miR-203 are known to modulate innate immunity and possess an antiviral role in multiple viral pathologies (Swaminathan *et al*. 2012; Jiang *et al*. 2014; Pareek *et al*. 2014; Zhang *et al*. 2018b; Natekar *et al*. 2019). The brain-enriched miR-124 is a key player in CNS development (Sun *et al*. 2015; Vuokila *et al*. 2018) and would be a valuable target to understand ZIKV-related neurodegeneration. In a contrasting result, Dang *et al*. (2019) showed significant upregulation of miR-124-3p in human neural stem cells (hNSCs) which was shown to target the transferrin receptor (TFRC) that plays a role in maintaining cell stemness and cell cycle. This opposing expression of miR-124 could be due to different cellular models. They also explored the role of upregulated let-7c and its target, high mobility group A2 (HMGA2), in disrupted neurogenesis and microcephaly. Over past few years, multiple high-throughput global miRNA expression studies have been conducted in cellular models of ZIKV (Castro *et al*. 2019; Seong *et al*. 2020; Tabari *et al*. 2020; Bagasra *et al*. 2021).

A study on transgenic mice showed the role of brain enriched miR-9 in promoting ZIKV infection. In this study, *miR-9-TG mice* were bred with cortical-specific *Emx1-cre* mouse line to produce conditional miR-9 overexpression in the resulting progeny. Here, miR-9 is shown to enhance neuronal apoptosis and loss of progenitor cell pool, resulting in reduced brain size and microcephaly. The authors also confirm a relationship between miR-9 and its target, glial cell line-derived neurotrophic factor (GDNF), with the latter known for its neuroprotective role (Sawada *et al*. 2000; Tenenbaum and Humbert-Claude 2017; Zhang *et al*. 2019). The miRNAs in key infection-related mechanisms have been additionally illustrated in figure 2.

2.3 *Japanese encephalitis virus*

2.3.1 *Epidemiology:* JEV is the leading cause of encephalitis in the Asia-Pacific region. Infections have been reported in tropical and subtropical regions including Southeast Asia, parts of Africa and Northern Australia (CDC 2019). *Culex* and *Aedes* mosquitos are vectors of JEV with humans as a dead-end host, i.e., an infected person is less likely to amplify and transmit the virus further via mosquitos (Filgueira and Lannes 2019).

Once a human is infected via a mosquito bite, the incubation period of the virus in host cells is 5–15 days before the onset of clinical symptoms such as fever, headache or vomiting. In addition, JEV has also been shown to invade peripheral organs (Banerjee and Tripathi 2019). The majority of infected cases are asymptomatic with less than 1% developing encephalitis (Turtle and Solomon 2018). JEV infection of the CNS leads to acute encephalitis syndrome, which involves neurological symptoms, cognitive issues, movement disorders, seizures, and is even associated with GBS, similar to ZIKV infection (Turtle and Solomon 2018).

2.3.2 *Role of host miRNAs during cellular infection:* Initially JEV replicates in keratinocytes, fibroblasts, dermal dendritic cells (DCs) and lymph nodes. Infected immune cells such as DCs and macrophages migrate to the CNS, leading to viral neuroinvasion (Banerjee and Tripathi 2019; Filgueira and Lannes 2019). In recent years, much progress has been made in understanding the mechanisms of JEV neuroinvasion, which are similar to the ones proposed for WNV entry (Li *et al*. 2015; Wang *et al*. 2018a; Hsieh *et al*. 2019). In the brain, JEV is known to infect neuronal as well as non-neuronal cell types, with neurons supporting better viral replication (Lannes *et al*. 2017; Mukherjee *et al*. 2017; Banerjee and Tripathi 2019). Considerable evidence on the role of miRNAs in JEV neuropathology has been accumulated over the years.

One of the earliest studies on miRNAs focussed on restricting neurovirulence of JEV using miRNA engineering (Heiss *et al*. 2011;Wu *et al*. 2011; Yen *et al*. 2013). Thounaojam *et al*. (2014) for the first time described the role of host cell miRNA in JEV-induced inflammation. They identified 11 miRNAs to be dysregulated in JEV-infected microglial cells (BV-2 and primary mouse cells), with miR-29b being the most significantly upregulated. Further, miR-29b was shown to target TNF-alpha-induced protein 3 (TNFAIP3), a negative regulator of the NFκB signalling pathway, causing sustained inflammation (Thounaojam *et al*. 2014a, b).

The same group explored the role of miR-155 in JEV-induced microglial activation. They showed the miR-155 targeted SH2-containing inositol 5-phosphatase 1 (SHIP1) to induce NFκB signalling and synthesis of proinflammatory cytokines (Thounaojam *et al*. 2014a, b). In a contrasting study, miR-155 was observed to suppress inflammation via NFκB signalling and limiting JEV replication in microglial cells (Pareek *et al*. 2014). Later on, PEL1 (renamed PGS1) was also validated as a target for miR-155 that led to the suppression of the non-canonical NFκB pathway

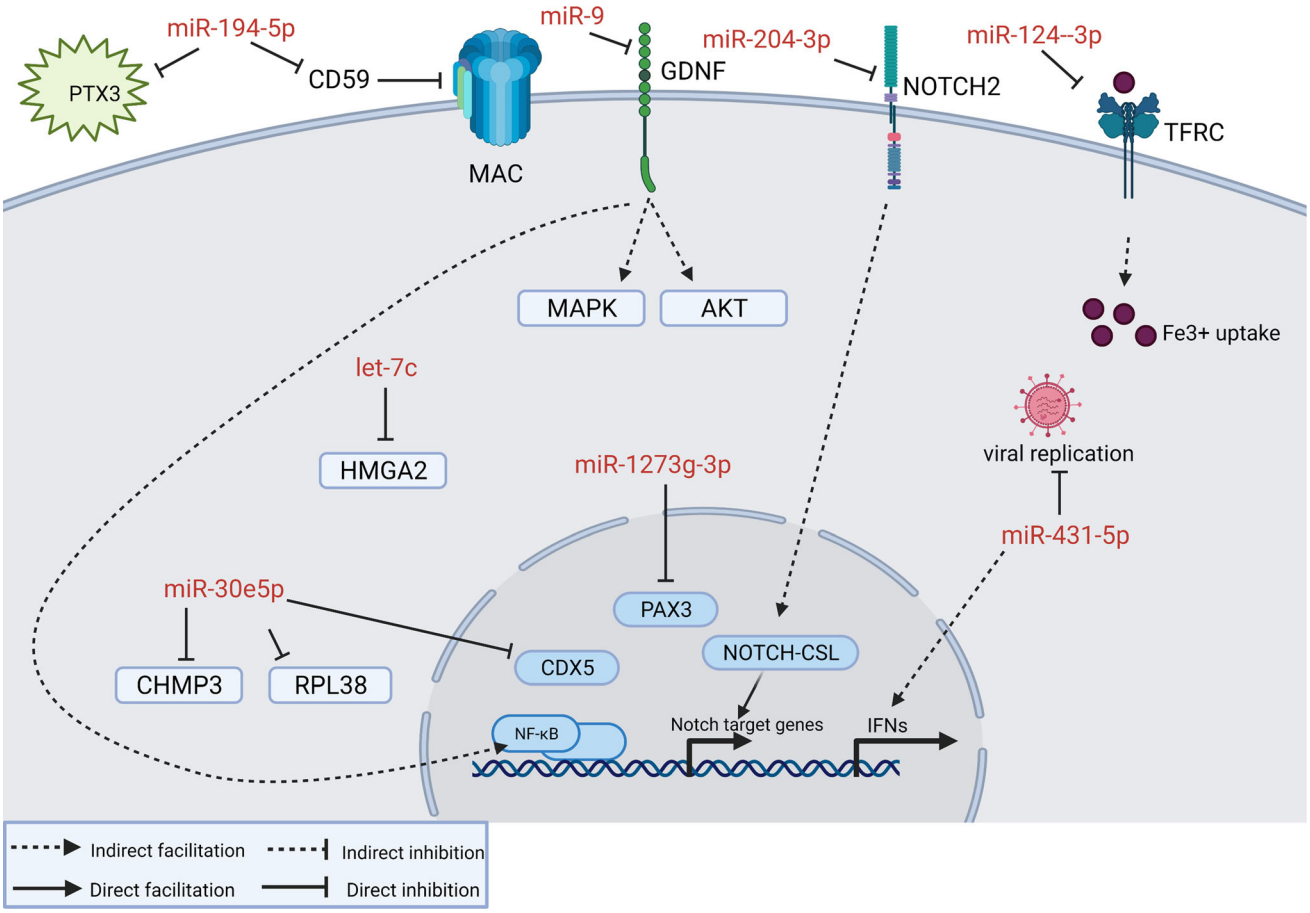

Figure 2. Key miRNAs and their targets involved in the host cell response to ZIKV invasion. miRNA and gene targets that still await elucidation of functional pathways, such as miR-30e-5p-targeted charged multivesicular body protein 3 (CHMP3), ribosomal protein L8 (RPL38) and transcription factor CDX5, let-7c-targeted HMGA2 and miR-1273g-3p-targeted PAX3. Cell surface receptors GDNF, TFRC and NOTCH are targeted by miR-9, miR-124-3p and miR-204-3p, respectively. miR-9 regulation is shown to impact the MAPK, AKT3 and NFκB pathways simultaneously. miR-431-5p, which regulates IFN production, also directly restricts viral replication. miR-194 targets *CD59*, which encodes an inhibitor of the membrane attack complex (MAC) and the *PTX3* gene, which encodes an extracellular inflammatory marker.

and a dampened immune response to JEV (Rastogi and Singh 2020).

Further, upregulated miR-146a was observed to suppress NFκB signalling and disrupt Janus kinases (JAK)/signal transducer and activator of transcription proteins (STAT) pathway in JEV-infected microglial cells (CHME3). However, this observation was specific to the strain JaOArS982 and was not identified in P20778 strain infection (Sharma *et al.* 2015). One more study observed an upregulated miR-146a and its role in modulating proinflammatory cytokines in JEV-infected C8-b4 microglial cells and also in a mouse model. Mechanistically, miR-146 was shown to negatively regulate proinflammatory cytokines and seemed to act as a feedback regulator of virus-induced inflammation of the cell (Deng *et al.* 2017).

The role of miR-15b was studied by Zhu *et al.* (2015) in JEV-induced inflammatory response. Deep sequencing of JEV-infected astrocyte U251 cells was performed and miR15b was found to be significantly upregulated. Ring finger protein (RNF) 125, a negative regulator of RIG1, was identified as a target of miR-15b. Hence, elevated miR15b was shown to activate RIG-1 signalling and lead to increased expression of proinflammatory cytokines and type-1 interferons. *In vivo* knockdown of miR-15b reduced viral burden, decreased cellular damage and had pro-survival effects. In a subsequent study, it was shown that MAPK/ERK and NFκB signalling is activated in a RIG-1-dependent manner that eventually leads to the NFκB subunit c-Rel and cAMP-response element binding protein (CREB), as transcriptional factors, to

bind to the miR-15b promoter and regulate its transcription during JEV infection (Zhu *et al*. 2016).

In a separate study, JEV NS1 protein-induced CREB and c-rel were also shown to bind to the promoter of miR-22 and increase its transcription to block interferon expression via mitochondrial antiviral signalling protein (MAVS) (Zhou *et al*. 2020). Following a microarray screening, the role of a dysregulated miR-370 in U251 cell lines was observed to be associated with viral replication and toxic inflammatory response (Li *et al*. 2016).

Human microglial cells infected with JEV and WNV were profiled by Kumari *et al.* (2016) to observe global miRNA and mRNA expression patterns. This study identified miR-34c-5p-targeted NOTCH signalling to be specifically linked to JEV pathogenesis. Data generated from a previous study was further explored by the authors to describe the role of let-7a/b. The study showed the activity of let-7a/b via the TLR7 and NOTCH signalling pathway in microglial cells. In addition, exosomal secretion of the same miRNA led to bystander neuronal death by caspase activation (Mukherjee *et al*. 2019b). In a sequencing analysis involving human kidney HEK293T cells, miR-33a-5p was observed to be significantly downregulated upon JEV infection. miR-33a-5p was identified as a negative regulator of JEV replication by targeting eukaryotic translation elongation factor 1A1 (EEF1A1), which prevents the latter from interacting with viral replicase complex (Chen *et al*. 2016a). A report by Sharma *et al.* (2016) described the downregulation of an miR-432 JEV-infected cell model that directly targeted suppressor of cytokine signaling 5 (SOCS5) and JAK/STAT signalling. miR-432 knockdown was shown to activate STAT1 phosphorylation and enhance the antiviral response of the cell. Further, Ashraf *et al.* (2016). explored the significant upregulation of miR-19b-3p in the JEV-infected U251 astrocytic cell line and BV2 microglial cell line. RNF11was identified as the direct target of miR-19b-3p which serves as a negative regulator of NFκB signalling. *In vivo* silencing of miR-19b-3p led to reduced inflammatory response, abrogated cellular damage and increased survival (Ashraf *et al*. 2016). The role of miR-124 in suppressing JEV replication was found in PK15 porcine kidney cells by targeting dynamin 2 (DNM2) (Yang *et al*. 2016). Interestingly, miR-124 was found to be significantly downregulated in human neuronal precursor (hNP) and human neuronal stem (hNS1) cells upon JEV infection (Mukherjee *et al*. 2019a, b).

As reported by Hazra *et al.* (2017a, b), JEV induces the expression of miR-301a in an NFκB-dependent manner. miR-301a, in turn, aids the virus in evading innate immune response by suppressing SOCS5 and interferon regulatory factor 1 (IRF1) and reducing interferon production. They demonstrated that neutralizing miR-301a rescues JEV-infected mice from a weakened immune surveillance. In addition, the role of miR-301a in augmenting inflammation by inhibiting NFκB repressor factor (NKRF) was studied in *in vitro* and *in vivo* models (Hazra *et al*. 2019). Further, another study on the innate immune response during JEV infection showed that miR-374b-5p targeted phosphatase and tensin homolog (PTEN) to modulate the phosphoinositide 3-kinases (PI3K)/AKT/interferon regulatory factor 3 (IRF3) pathway and interferon response in infected human HMC3 microglial cells (Rastogi and Singh 2019). Recently, it was determined that JEV non-structural (NS3) protein specifically binds to pre-miRNA 466-3p and leads to the degradation of the mature miRNA that promoted viral replication and inflammation. This miRNA degradation was alleviated by three single amino acid substitutions in the helicase region of NS3 and shows potential in vaccine development strategy (Jiang *et al*. 2020). In the most recent miRNA profiling of JEV-infected BHK1 kidney cell lines, a high expression of miR-125b-5p was detected in acute infection that evidently reduced viral replication. *STAT3*, *MAP2k7* and *TRIAP1* were verified as target genes for miR-125b-5p (Huang *et al*. 2021).

2.3.3 *Role of circular RNAs:* In a novel strategy, a genome-wide RNA sequencing study identified 180 circular RNAs along with 58 miRNAs in JEV-infected mice brain tissue. circ_0000220 and miR-326-3p were validated *in vitro* to be essential in inflammatory cytokine response (Li *et al*. 2020). circRNAs, a new class of non-coding RNAs, are generated by back-splicing a single pre-mRNA, in which the 5′ and 3′ ends are covalently linked (Greene *et al*. 2017). circRNAs have been described to act as miRNA sponges (Yu and Kuo 2019). This paves the way for future studies to understand the potential of the former as an miRNA regulatory machinery, and to a way for future studies to understand the potential of circRNAs *in vivo*.

2.3.4 *Clinical evidence:* Circulating exosomal miRNAs have already been touted as important clinical markers in multiple diseases (van Harten *et al*. 2015; Mori *et al*. 2019; Xia *et al*. 2019). So far in literature, we found two such clinical analyses in the context of JEV infection. One study by Goswami *et al.* (2017) analysed 87 miRNAs in cerebrospinal fluid (CSF) of JEV patients with acute encephalitis syndrome (AES)

in comparison with patients with AES without JEV and found that miR-21-5p and miR-150-5p regulated cellular pathways such as TGFβ, MAPK and axon guidance. However, no healthy control was included and the overall cohort size of the study was small (n=24). Hence, a follow-up study by scaling up could be beneficial. In the second study, the sera of JEV-infected patients were analysed for the expression of miR-29b and miR-146a. Significant downregulation of miR-146a was found in JEV patients in comparison with healthy controls, while a significant elevation of miR-29b was associated with neurological sequelae after JEV recovery, posing both miRNAs as potential biomarkers to track disease severity (Baluni *et al*. 2018).

Key miRNAs in infection-related mechanisms have been illustrated in figure 3.

2.4 *Dengue virus*

2.4.1 *Epidemiology:* DENV is one of the leading viruses infecting humans. *Aedes aegytpi* and *Aedes albopictus* mosquitoes that thrive in urban environments are the two vectors responsible for human DENV transmission. DENV is most common in tropical and subtropical regions of the world. The viral infection is still on the rise and considered as endemic in more than 128 countries (Schaefer *et al*. 2021). According to WHO, the annual incidence has increased 30 times in the past 5 decades. In a yearly estimate, dengue fever affects approximately 100 million humans, who present with clinical symptoms (Harapan *et al*. 2020; Schaefer *et al*. 2021). However, the true estimate of infection ranges from 300 to 500 million annually (Elduma *et al*. 2020; Harapan *et al*. 2020). In the past decade, the mean age of patients has been 34 years. In one study, the mortality burden of DENV in the year 2013 was calculated to be 13,586 deaths globally with 5838 deaths in children (Shepard *et al*. 2016).

This mosquito-borne virus with a single-stranded RNA genome has four identified serotypes (DENV1–DENV4). DENV infection can lead to clinical manifestations ranging from mild flu-like symptoms known as dengue fever to the life-threatening dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Harapan *et al*. 2020). In cases with severe dengue infection, damaging symptoms can arise across multiple organs of the body, including hepatic, musculoskeletal, renal, gastrointestinal, respiratory and neurological systems (Calderón-Peláez *et al*. 2019). Despite the presence of the virus over decades, there is a surprising lacuna of knowledge regarding the mechanisms of DENV infection, especially regarding its neuroinvasion. Some might argue that DENV cannot be classified as a neurotropic virus, but recent clinical evidence shows DENV-3 and -4 as the most frequently involved serotypes in neurological alterations. Clinical and experimental evidence has allowed comparisons with WNV- and JEV-induced neuroinflammation. The entry of DENV into the CNS is suggested to be via a haematogenous route, subsequently damaging the BBB (Calderón-Peláez *et al*. 2019)

2.4.2 *Role of host miRNAs during cellular infection:* Following a mosquito bite, DENV initially replicates in skin cells and gets transferred to the lymph nodes. In the lymphatic nodes, the virus infects monocytes, dendritic cells and macrophages, and spreads to the blood and peripheral organs (Laureti *et al*. 2018). In one of the earliest studies to explore host cell miRNAs during DENV infection, Qi *et al*. (2013) performed microarray analysis of cultured human peripheral blood mononuclear cells (PBMCs). They observed 19 differentially expressed miRNAs that were associated with a virus-induced cytokine storm and epigenetic regulation of cytokine expression. Jiang and Sung (2018) conducted a similar high-throughput analysis using sequencing technology to identify another set of differentially expressed miRNAs involved in DENV-3-associated pathology and immune response in human PBMCs. Further, DENV-infected THP-1 cells and primary monocytes were observed to have an elevated miR-146 expression, which aids in viral replication in the host cell and attenuates interferon-β (IFN-β) production in parallel via its target gene, TNF-receptor associated factor 6 (*TRAF6*). *TRAF6* has been touted as essential in NFκB activation and type I IFN response, and its function was rescued upon miR-146 inhibition (Wu *et al*. 2013). Here, the proviral role of miR-146 in innate immunity draws a parallel with its previously discussed role in JEV infection. The miR-146-TRAF6 relationship is revisited and confirmed to be involved in DENV-induced autophagy via IFN production (Pu *et al*. 2017). Interestingly, in a separate study on DENV-infected U937 monocytes and HeLa cells, overexpressed miR-30e* showed a similar regulatory effect on NFκB activation-induced IFN-β response. However, unlike miR-146, miR-30e* promoted IFN expression and dampened DENV replication by targeting the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα). The authors deciphered an antiviral role of the miRNA by

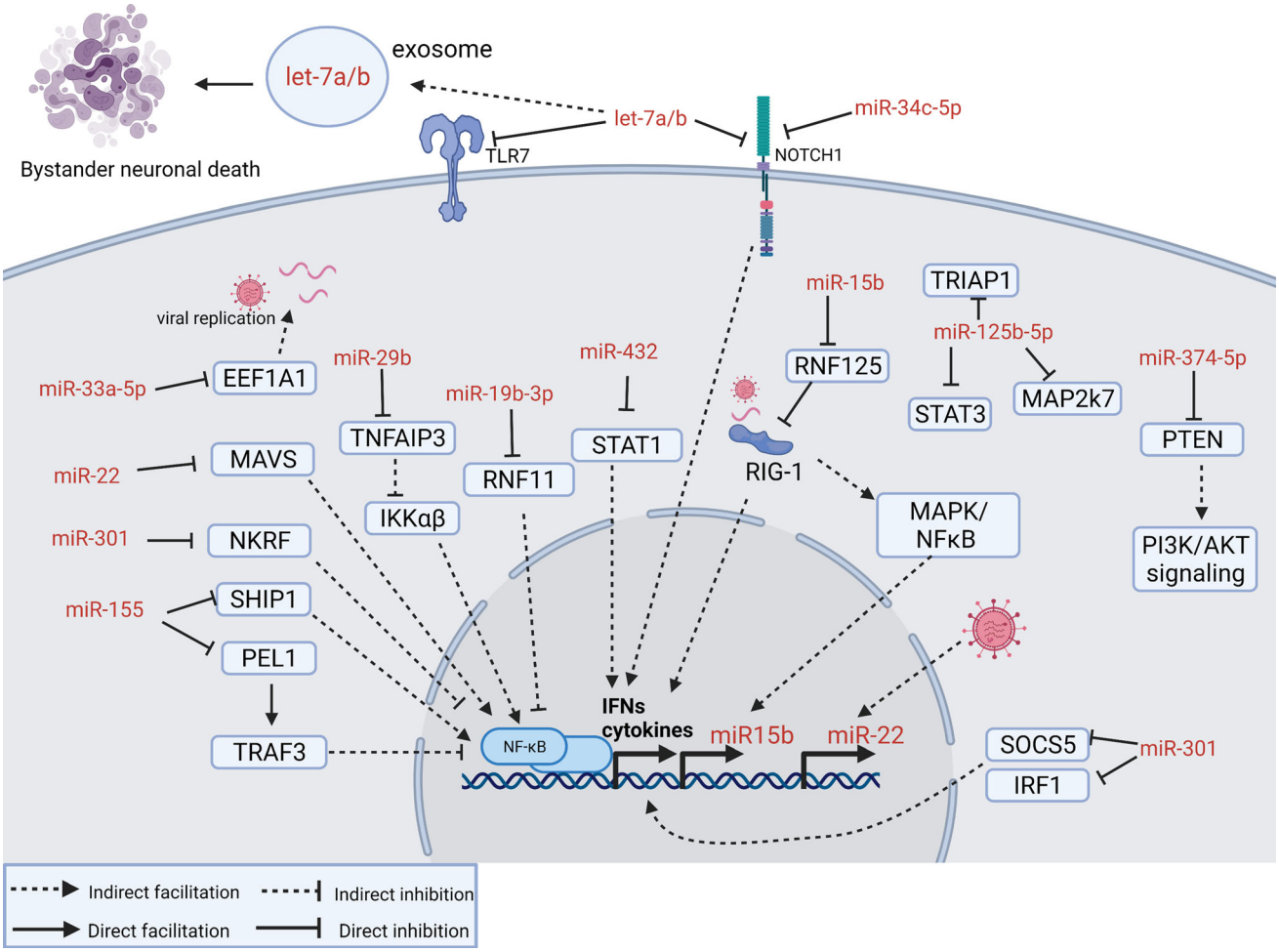

Figure 3. Key miRNAs and their targets involved in the host cell response to JEV invasion. A majority of miRNAs regulate the NFκB pathway, including miR-155-targeted SHIP1 and PEL1, miR-301-targeted NKRF, miR-22-targeted MAVS, miR29-b-targeted TNFAIP3 and miR-19b-3p-targeted RNF11. IFN and cytokine response is regulated by miR-301-targeted SOCS5 and IRF1, miR-432-targeted STAT1 and miR-34c-5p-regulated NOTCH1. RIG1 regulates miR-15 promoter via the MAPK/NFκB pathways. miR-15 regulates RIG-1 via its target RNF125. miR-22 promoter is indirectly regulated by JEV. miR-33a5p targets the translation factor EEF1A1 to restrict viral replication. TRIAP1, STAT3 and MAP2K7 are targeted by miR-125b-5p. miR-374-5p targets PTEN to regulate the PI3K/AKT pathway. let-7a/b targets cell surface receptors, NOTCH1 and TLR7, and is exported in exosomes to cause neuronal apoptosis by caspase activation.

further demonstrating an enhanced viral replication achieved by miR-30e* silencing (Zhu *et al.* 2014).

Another antiviral miRNA, namely, miR-223, was identified in EAhy926 human endothelial-like cells infected with DENV (Wu *et al.* 2014). Viral infection was shown to reduce the expression of miR-223, leading to the upregulation of its target, stathmin1 (STMN1), that ultimately aided in DENV replication (Wu *et al.* 2014). A study by Wen *et al.* (2015) reported the role of miR-548g-3p in inhibiting DENV viral protein translation in cell culture by directly targeting the 5′ UTR of the DENV genome, thus providing evidence of a potential therapeutic strategy. In an miRNA expression profiling study of DENV-infected Huh-7 liver cells and U937-DC-SIGN macrophage-monocytic cells, let-7c was found to be significantly upregulated, supposedly as an antiviral mechanism. It was shown that let-7c targets transcription factor the BTB domain and CNC homolog 1 (BACH1) and significantly reduces DENV replication (Escalera-Cueto *et al.* 2015). In 2015, miRNA expression profiling of PBMCs obtained from DENV-infected patients was conducted and a significant downregulation of miR-30e, miR-27a* and miR-378 was observed. Evidently, miR-378 enhanced expression of toxic molecule granzyme B (GrzB) in natural killer (NK) cells, which plays a protective role against viral replication. *In vivo* analysis showed the

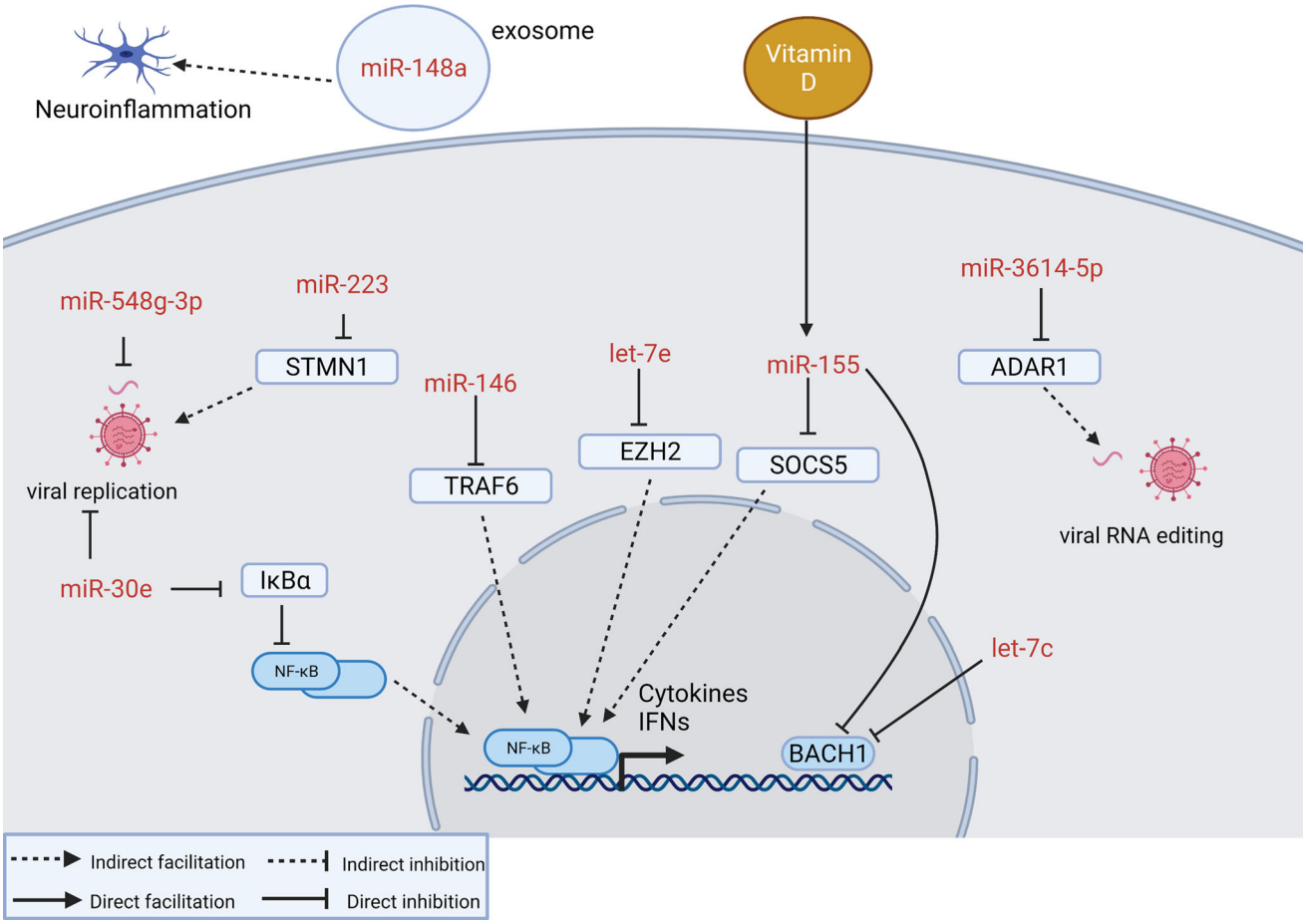

Figure 4. Key miRNAs and their targets involved in the host cell response to DENV invasion. NFκB pathway regulation is most evident by miR-30e-targeted IκBα, miR-146-targeted TRAF6, let-7e-targeted EZH2 and vitamin D/miR-155-targeted SOCS5. miR-30e and miR-548g-3p directly restrict viral replication, while miR-223 has the same effect by targeting STMN1. miR-3614-5p targets ADAR1 to indirectly effect viral genome editing. miR-155 and let-7c directly regulate the transcription factor BACH1. Exosomal miR-148 taken up by microglia activates their inflammatory pathways by targeting the USP3-ATF3 axis.

suppression of GrzB via miR-378 overexpression leads to viral invasion into multiple organs of mice, including the brain (Liu *et al*. 2016). In another report that was aimed at investigating the host miRNAs that were manipulated by DENV to facilitate its own replication, bioinformaticians identified 13 miRNAs with potential target sites on DENV 3′UTR. miR-133 was found to have an antiviral role against all four DENV serotypes by targeting the polypyrimidine tract binding protein (PTB) (Castillo *et al*. 2016).

Further, Casseb *et al*. (2016) infected A549 lung cells with DENV-4 and observed a significant reduction in miRNA biogenesis-related proteins such as Dicer, Drosha and DGCR8. In a separate sequencing analysis of human primary macrophages, it was observed that differentially expressed miRNAs in DENV-infected cells did not play a significant role in viral amplification. However, it was shown that miR-3614-5p, upregulated in uninfected cells, played an antiviral role by targeting adenosine deaminase RNA-specific (ADAR) that promotes DENV infectivity in cells (Diosa-Toro *et al*. 2017). As part of the emerging evidence, the proviral role of miR-21 and the antiviral role of miR-484 and miR-744 were identified in HEPG2 and Vero cells, respectively, in separate cell culture screening studies (Castrillón-Betancur and Urcuqui-Inchima 2017; Kanokudom *et al*. 2017). To understand the mechanisms of DHF/DSS better, Zhang *et al*. (2018a) in their study of DENV-2-infected PBMCs explored a novel let-7e/enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2)/H3K27me3/NFκB axis in regulating TNFα production. In another study focussing on DHF symptoms, six miRNAs, including miR-320e, miR-485-3p and mir-

Table 1. Common host cell microRNAs and their functions in multiple viral pathologies

Host cell miRNA	Virus	Pathways targeted	Functional	References
miR-34a	WNV, ZIKV, JEV, DENV	Wnt/β-catenin signalling, IFN response	Host cell surface receptor signalling, immune response	Fineberg *et al.* 2012; Kumari *et al.* 2016; Pylro *et al.* 2016; Smith *et al.* 2017; Dang *et al.* 2019
miR-34c-5p	WNV, DENV JEV	Wnt signaling, NOTCH signalling	Viral replication and life cycle in host cell, cell fate decisions	Kumari *et al.* 2016; Smith *et al.* 2017
miR-1231, miR-517a, miR-876-3p, mir-453	WNV, JEV, DENV	?*	Viral replication and life cycle in host cell	Smith *et al.* 2017
miR-155	WNV, JEV, ZIKV, DENV	TLR signalling, Cytokine/ chemokine response, NFκB (canonical/ noncanonical) signalling	Inflammatory response	Thounaojam *et al.*2014a, b; Sharma *et al.* 2015; Azouz *et al.* 2019; Natekar *et al.* 2019; Rastogi and Singh 2020
miR-146	JEV, DENV	NFκB signaling, JAK/ STAT signaling, type I IFN response		
miR-124	JEV, ZIKV	Iron homeostasis, cell stemness and differentiation pathways, endocytic pathway	Host cell-cycle and cell development, intracellular membrane trafficking	Dang *et al.* 2019; Mukherjee *et al.* 2019a, b

* Unknown pathways.

4498 were identified in microvascular endothelial cells (MECs) to be significantly associated with vascular development processes and the acute migratory phenotype of the cells (Álvarez-Díaz *et al.* 2019). In a separate study, vitamin D was shown to modulate the NFκB-induced cytokine response of DENV-infected macrophage cells via the miR-155–SOCS1 axis, reflecting its therapeutic potential (Arboleda *et al.* 2019). In addition, overexpression of miR-155 in mouse model provided protection against severe DENV infection and reduced viral titre by directly targeting the BTB domain and CNC homolog 1 (BACH1) and antiviral interferon response (Su *et al.* 2020). Even though there is a large body of evidence on the neuroinvasion and neuropathogenesis of DENV especially in the form of clinical case reports, we found only one report on miRNA-induced neuroinflammation. This *in vitro* study demonstrated the mechanism of extracellular vesicle export of miR-148a by DENV-infected monocytes and the subsequent uptake by microglial cells and an enhanced inflammatory response via the miR-148a-ubiquitin-specific peptidase 33 (USP33)-activating transcription factor 3 (ATF3) axis (Mishra *et al.* 2020). Given the evident neurological manifestations caused by DENV and the significant role of miRNAs observed in other neurotrophic viruses, studies focusing on this aspect are the need of the hour.

2.4.3 *Clinical evidence:* Over the past few years, there have been attempts to identify circulatory miRNAs from serum or peripheral blood cells in DENV patients as clinical biomarkers of dengue infection and disease progression, with the most recent analysis suggesting miR-122-5p as a potential biomarker for DENV-1 (Ouyang *et al.* 2016; Shahen *et al.* 2018; Hapugaswatta *et al.* 2020; Saini *et al.* 2020; Teng *et al.* 2021).

miRNAs discussed in key infection-related mechanisms have been additionally illustrated in figure 4.

3. RNA interference (RNAi) therapeutic strategies

In 2018, as an antiviral therapeutic strategy, three artificial miRNAs (amiRNA) were delivered into neuronal cells using a plasmid vector to check their activity against JEV. Two of these amiRNAs were successful in reducing JEV replication and also diminished mature virion release by 95% with minimal toxicity to the host cells (Sharma *et al.* 2018). A similar strategy was

adopted for WNV, where two-vector-cloned amiRNAs targeting the NS5 and NS2A proteins of the virus reduced replication, and NS5 amiRNA was successful in reducing viral titer upto 97.11% (Karothia *et al*. 2020). The inhibition of DENV replication and expression was most successfully achieved by amiRNA DENV-128 among 21 amiRNAs tested by Xie *et al.* (2013). No such advances have been seen in Zika virus research yet.

In recent years, extracellular vesicles (EVs) have garnered much attention for their essential role in intercellular communication. EVs, also known as exosomes or microvesicles, are secreted from cells to transport DNA, mRNAs, proteins, miRNAs and viral genomes to bring about physiological changes in recipient cells. EVs also play key roles in viral pathogenesis with miRNAs as one of their cargos and their ability to both facilitate and suppress viral propagation in a multicellular environment (Koppers-Lalic *et al*. 2013; Yoshikawa *et al*. 2019). Unravelling of the EV structure and their cargos are still in early stages of research with a promising potential to manipulate them as RNA interference (RNAi) vehicles (Dogrammatzis *et al*. 2020; O'Brien *et al*. 2020). EVs have low immunogenicity, are stable in biofluids and, unlike other drug molecules, are able to cross the BBB. All these factors make them a potential therapeutic tool against neurotropic viruses (Chen *et al*. 2016b; Kumar *et al*. 2020; Yang *et al*. 2021). miR-133b-, miR-30d-and miR-124-3p-enriched EVs have been shown to alleviate brain injury in neurodegenerative models (Munir *et al*. 2020). miR-401-enriched EVs have been designed to block herpes simplex virus-1 (HSV-1) replication *in vitro* (Wang *et al*. 2018b). These limited but important findings pave the way towards resolving flavivirus-induced brain injury using similar EV-based strategies.

4. Conclusion

In summary, we comprehensively reviewed a significant number of miRNAs employed by the host in response to flaviviral infections. Across all four viruses, the majority of evidence has been generated from *in vitro* and *in vivo* model systems. Tissue-specific or circulatory miRNA profiles from WNV- and ZIKV-infected patients would further enrich our knowledge. Each virus induces unique miRNA profiles that aid in the individualized understanding of their pathogenesis. However, certain miRNAs, as shown in table 1, commonly involved across multiple viral pathologies, may have a higher chance of success in regard to clinical interventions and are worth pursuing.

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