Role of autophagy-related proteins and cellular microRNAs in chikungunya and dengue virus infection
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CHAPTER 6

Summarizing discussion and future perspectives
CHAPTER 6

Introduction

The incidence of mosquito-borne human infections has grown dramatically in recent decades. Dengue virus (DENV) infection causes the most widespread arboviral disease to date (1). Annually, an estimated 390 million DENV infections occur and around 96 million people develop a symptomatic febrile illness, with approximately 500,000 individuals developing a severe disease (1). Severe dengue is a leading cause of hospitalization in multiple countries. Moreover, this summer many hospitals in South-East Asia and Latin America were overwhelmed with the number of dengue-infected individuals who required medical care (2). Severe dengue can be fatal if left untreated; currently approximately 20,000 individuals succumb annually from a DENV infection (1).

Another re-emerging mosquito-borne human pathogen of epidemiological importance is chikungunya virus (CHIKV), which emerged in new areas of the world in recent years (3). CHIKV infection leads to symptomatic disease in about 50 - 97% of the cases and to chronic disease in 35% to 52% of the infected individuals (4,5). Zika virus (ZIKV) is yet another mosquito-borne virus that has re-emerged in the last decade, with an estimated 12.3 million cases per year across Latin America and the Caribbean (6). Furthermore, an increasing number of reports report co-infections with these arboviruses as DENV, CHIKV and ZIKV are transmitted by the same mosquito vector and co-circulate in the same regions (7,8). The sudden re-emergence of mosquito-borne human diseases has been linked to variable factors such as global warming, viral adaptation to new mosquito vectors, increased human mobility and other anthropological behavioural patterns (9,10). Moreover, due to massive outbreaks (symptomatic infection rates of 70% are not unusual in new areas), we are witnessing clinical manifestations that have not been reported before and for which no prognostic factors exist to date (11). A recent example is the development of microcephaly in newborns during ZIKV infection in pregnant woman. In case of DENV, the incidence of severe disease continues to increase due to the hyper-endemicity of multiple DENV serotypes in many countries, yet there are no biomarkers available to predict disease outcome (1).

The high burden of disease caused by arboviruses is accompanied with a large socio-economic and public health impact, especially in developing countries that are not prepared for recurrent and usually unexpected epidemics (12). For example, the annual economic cost of dengue in the Americas and Asia was estimated to be at least US$587 million in 2008 (13). These costs come mainly from healthcare expenses, yet the costs are likely to be much higher if the expenses of preventive measures related to environmental management and vector control strategies are kept into account (14). More recently, the estimated cost of chikungunya disease in the Americas in 2016 was reported to be US$184 billion, when accounting for total societal costs for acute illness, chronic inflammatory rheumatism, cognitive delays from neonatal infection, missed
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work or decreased productivity due to disability, and additional sequelae beyond chronic inflammation (15). Furthermore, these diseases are considered to be ‘poverty-promoting’ due to their effect on pregnancy, child health and development, and worker productivity (16).

Due to the staggering number of arboviral infections worldwide, researchers have focussed on the development of preventive and control measures to counteract these diseases. Indeed, novel biological, chemical and mechanical vector control technologies have been developed in recent years. Their use, however, is still limited mainly due to the high cost of implementation, the lack of practicality and the difficulty of community mobilization in real life situations, especially in remote areas (17). Likewise, many innovative vaccine candidates based on numerous platforms like inactivated virus, adenoviral vectors, recombinant subunit and virus-like particles have been reported in recent years (18,19). Some of these strategies have now entered clinical trials yet others are still in the developing stage (19,20). For DENV, a live attenuated chimeric vaccine has been licensed in 2015 (21). However, soon after its licensing, reports were published suggesting that vaccinated naïve individuals are at risk of developing more severe disease during natural infection (22–24). The World Health Organization (WHO) therefore decided against the use of this vaccine for mass immunization (25). According to this, the WHO currently recommends that people are only vaccinated when they show a DENV positive blood test (26). Furthermore, many compounds have been discovered with potent antiviral activity in vitro but unfortunately, most of them are not pursued for clinical development due to various reasons, including toxicity and suboptimal drug properties (27,28). Thus far only few ‘repurposing’ drugs have been tested in clinical trials yet no beneficial effect was measured (27). It is evident from the above that the design of safe and effective vaccines and antivirals for arboviral diseases comes with many challenges. The lack of accurate animal models that resemble the intricate pathogenesis in the human host and the complexity and little understanding of the cellular responses and host factors that control viral replication and pathogenesis, are crucial factors herein. Therefore, a better understanding of the host and cellular factors associated to the pathogenesis of DENV, CHIKV and other important arboviruses is required in order to generate tools with prognostic value and to design antivirals and vaccines that are efficacious and safe.

In this thesis, we aimed to better understand the replication of DENV and CHIKV, focusing on the identification of cellular host factors relevant in controlling these viral infections. Emphasis was on the role of autophagy and autophagy-related (ATG) proteins in controlling DENV and CHIKV replication, and the importance microRNA (miRNA)-mediated post-transcriptional regulation of cellular gene expression in controlling DENV infection. The results of this thesis and the future perspectives and implications of our results will be discussed in more detail in three distinct sections:
I. Contribution of autophagy and the ATG proteome to DENV infection: unsolved questions and future perspectives

The induction of autophagy during DENV infection was first described in 2008, based on the accumulation of double-membrane vesicles resembling autophagosomes in DENV-infected hepatic cells (29). Subsequently, it has been demonstrated that DENV triggers autophagy in multiple cellular contexts using diverse experimental strategies (30–36). Autophagy induction during DENV infection has been attributed to the expression of the viral non-structural (NS) proteins NS4 and NS1 (31,37). Contrasting data have been published, however, regarding the role of autophagy in controlling virus replication and progeny. Autophagosome formation during DENV infection has been associated with the selective degradation of lipid droplets via autophagy, i.e., lipophagy (38–40). Lipophagy was shown to increase the ATP levels in infected cells, thereby favouring the replication of the virus (38,39). It has also been hypothesized that lipophagy may provide the lipids necessary to facilitate the assembly of nascent viral particles or aid in viral transmission via unconventional secretion of autophagy-associated vesicles containing DENV (41). In contrast, other reports described that autophagy or specific ATG proteins are part of the antiviral cellular response. For example, the induction of autophagy in THP1 cells (42) and reticulophagy (43) in HBMEC cells during DENV infection were described to hamper virus replication. The ATG proteins Beclin-1 (BECN1) and ATG7 in THP1 cells (42); and family with sequence similarity 134-member B (FAM134B) in HBMEC cells (43), were shown to be involved in these antiviral roles. The antiviral role of autophagy in DENV replication is in line with the observation that DENV actively inhibits the selective autophagic flux by inducing proteasomal degradation of p62 (32).

Currently, it is not known which variables could explain the differences across publications. A plausible explanation is that distinct autophagy pathways, i.e., bulk autophagy, lipophagy and reticulophagy, have distinct outcomes on infection. DENV may also subvert parts of the autophagy machinery to promote infection. Alternatively, some ATG proteins may control infection independently of autophagy via unconventional roles. Although it is clear that autophagy is induced during DENV infection, the role of autophagy and specific ATG proteins in controlling DENV infectivity requires further investigation.

II. Autophagy-dependent and -independent functions of ATG proteins and autophagy receptors in CHIKV infection

III. miRNA control of antiviral responses and potential therapeutic approaches
In Chapter 3, we investigated the function of 50 distinct proteins that are part of the autophagy machinery, in order to comprehensively evaluate how the different ATG proteins and autophagy receptors contribute to DENV infection. To this end, we performed an siRNA screen specifically targeting autophagy in the Huh7 hepatic cells infected with DENV. We identified 11 ATG proteins that controlled DENV infection. Among those, depletion of 2 different isoforms of ATG4 (ATG4C and ATG4D) and BECN1 caused the most prominent enhancement of DENV infection. Furthermore, depletion of all the isoforms of ATG4 also significantly favoured viral infection. In addition, knockdown of 2 distinct isoforms of LC3 (LC3A and LC3C), ATG2B, ATG10, ATG13, ZFYVE1 and UVRAG significantly increased DENV infection and/or replication in our model. In contrast, NBR1 depletion was found to decrease the number of infected cells and increased DENV replication in infected cells. Preliminary validation experiments to corroborate the role of ATG4C and NBR1 in DENV infection were performed, yet future research should dissect the function of these, and the other hits identified in our screen, over the course of infection.

Although the data presented in Chapter 3 requires further validation, the results show that only a limited number of the ATG proteins and autophagy receptors control DENV infectivity in Huh7 cells, suggesting that the identified hits might control DENV via unconventional roles outside autophagy. To the best of our knowledge, except for BECN1 (42), the identified factors have not been shown to control DENV infectivity before. Interestingly, though, non-canonical functions have been described for most identified hits. For example, UVRAG, a membrane trafficking protein, is involved in Influenza A virus and Vesicular Stomatitis virus cell entry (44) and ATG2B silencing was found to cause clustering of lipid droplets independent of autophagy and is upregulated during Japanese Encephalitis virus infection, another flavivirus (45). Alternatively, we might have underestimated the overall importance of ATG genes and autophagy receptors in DENV infection as we had a relatively high percentage of infected cells in our control sample, thereby limiting the measuring range. Future studies should reveal whether the identified hits control DENV infectivity in a canonical or uncanonical fashion.

Another explanation might be that autophagy controls DENV infectivity but that not all components of the machinery are required in this process. For example, a previous study revealed that DENV bypasses the initial stages of autophagy as canonical lipidation of LC3 is not required for infection (46). Indeed, the authors showed that ATG5, BECN1 and ULK1, important factors during the initial phase of autophagy, are superfluous in DENV replication in HeLa cells (46). Similarly, we found that depletion of ATG5 and ULK1 had no effect in the replication of DENV in Huh7 cells. However, the data of our screen supports an antiviral role of BECN1 in DENV infection. This was also observed by others in THP-1 cells but not in A549 cells (42). On top of these discrepancies, another study revealed a proviral role for BECN1 in DENV infectivity, also in Huh7 cells (12), which further underlines the highly controversial role of this protein.
in DENV infection. These discrepancies might be related to the cell type, virus strain and experimental settings used in these studies, and highlights the challenges to conclude whether or not a protein or a pathway is of importance in DENV infection and pathogenesis.

A third explanation is that multiple types of autophagy are induced and the diverse pathways have different effects on DENV infection. Some of the studies published to date have focussed on the role of selective autophagy, e.g., reticulophagy and lipophagy in DENV infection. It is tempting to speculate that both pathways participate in DENV infection in parallel. This is plausible if we consider that lipid droplets originate from the ER, the same organelles that have been proposed as the initiation sites of autophagosome biogenesis (47–49), and the replication site of DENV (50). This is supported by data showing that phosphatidylinositol 3-kinase (VPS34), ATG9 and non-lipidated LC3-I contribute to the ER membrane re-arrangements during the replication of this virus (46). A second hypothesis would be that lipophagy and reticulophagy occur at different stages of the viral replication cycle and therefore at limited time-points. To answer this question, studies in which the dynamics of both processes are evaluated over the course of the replication cycle of DENV could help to decipher if they act independently or in concert.

As described in Chapter 2, the process of selective autophagy is less well understood than bulk autophagy, and future research should focus on revealing the shared and diverging mechanisms among the autophagy pathways (51,52). Essential for lipophagy is the recruitment of LC3-II-enriched membranes that elongate towards the core of lipid droplets (53). Proteins like ATG7, ATG5 and ATG12 are involved in the elongation of these membranes, however, they are not necessarily required for initiation of lipophagy, a step that is poorly understood (51). In that sense, ubiquitination has been suggested as a potential determinant for initiation and cargo selection during lipophagy (54,55). Interestingly, DENV was found to interact with the ancient ubiquitous protein 1 (AUP1) at the surface of lipid droplets to stimulate lipophagy (39). Furthermore, given that AUP1 binds to ubiquitinating enzymes, this may result in ubiquitination of the lipid droplets (56). So far, NS4A and NS4B have been found to be involved in the interaction with AUP1 (39), but the molecular aspects of this interaction and the upstream regulators of these events are not completely understood. In case of reticulophagy, the initiation factors are also not fully characterised. It is known, that DENV, ZIKV and West Nile virus (WNV) are able to subvert reticulophagy by through the NS2B-NS3-mediated cleavage of FAM134B, thereby preventing the oligomerization of FAM134B, which leads to an uncontrolled enlargement of the ER (43). The specificity of this cleavage is not known. Therefore, it would be interesting to investigate whether other ER-located receptors, such as for example preprotein translocation factor (SEC62), cell cycle progression protein 1 (CCPP1), and reticulon 3 (RTN3) (57), could also be cleaved in a similar fashion. Additionally, it has been reported that p62 and BCL2
interacting protein 3 (BNIP3) might also assist ER clearance (58,59), therefore their contribution to reticulophagy during DENV infection should be also assessed. Next to unravelling the initiation factors, it will also be important to identify which ATG proteins are important in distinct autophagy pathways as this may explain some of the controversies observed. Overall, the current literature suggests that both types of autophagy are induced, yet have diverse outcomes in infection. A better understanding of the molecular events in these pathways will lead to better insights into DENV pathogenesis and may unveil novel targets for intervention.

Manipulation of autophagy has been used previously for the development of pharmaceutical compounds aiming at treating cancer, neurodegenerative diseases and ischemia-reperfusion injury, among others (60,61). Drugs like rapamycin have been demonstrated to be effective in the treatment of different types of cancer (62), but this molecule has multiple autophagy-independent effects on cells and organisms (63,64). There are several drugs in clinical use that modulate autophagy (65). In recent years, however, more specific methods to manipulate autophagy have also emerged. In Chapter 2, we described the development of a synthetic peptide which stimulates bulk autophagy and efficiently reduces the mortality of neonatal mice infected with CHIKV and WNV (66). Similar strategies could be developed in order to target p62 or FAM134B during infection with DENV, or to stimulate specific types of selective autophagy. For example, by stimulating the antiviral properties of these proteins or by inhibiting the ability of DENV to interfere with their functions. Conversely, pharmacological inhibition of lipophagy could be used as a therapeutic approach for DENV infection and might be a promising strategy for the treatment of the severe disease.

II. Autophagy-dependent and -independent functions of ATG proteins and autophagy receptors in CHIKV infection

Autophagy induction upon CHIKV infection is cell-type dependent. The formation and degradation of autophagosomes has been observed in HeLa cells and Hek293 cells (67–69), whereas HepG2 cells do not display signs of autophagy induction upon CHIKV infection (70). Furthermore, modulation of autophagy and ATG protein function has been described to alter the replication of CHIKV in diverse ways. For example, autophagy was shown to target CHIKV components for degradation in order to counteract infection (69). Furthermore, super-induction of autophagy in HeLa cells was shown to reduce CHIKV output (66). Moreover, inhibition of autophagy has been correlated with increased cell death and viral propagation in murine models (68). In contrast, depletion of specific ATG proteins like BECN1 or ATG7, restricts CHIKV replication in HeLa and Hek293 cells, suggesting a beneficial role of autophagy in infection (67,69). The proviral functions of ATG7 and BECN1 in CHIKV-infected human cells has been explained in the context of autophagy, as their positive effects correlated
with that of drug-mediated autophagy inhibition or induction. The autophagy receptor receptor calcium binding and coiled-coil domain 2 (NDP52) has been demonstrated to favour CHIKV replication in a process independent of LC3 and therefore was suggested to stimulate CHIKV infection independently of autophagy (69). This particular study underscores the importance of investigating the role of the ATG proteome both in- and outside the autophagy context as individual ATG proteins can also influence viral infections via unconventional functions.

In Chapter 4, we studied the involvement of 50 ATG proteins and autophagy receptors in CHIKV infection by performing an imaged- and siRNA-based screen in human U2OS cells. Analysis of the screen data revealed that 10 different proteins potentially regulate CHIKV infection. Depletion of 9 ATG proteins (i.e., ULK1, ULK4, ATG4C, ATG7, LC3B, GABARAPL2, ATG9B, NBR1 and BNIP3) significantly increased CHIKV infection, suggesting a role of these proteins or their associated pathways, in controlling virus infectivity. Depletion of UVRAG, on the other hand, was shown to significantly downregulate viral infection, suggesting that this protein stimulates CHIKV propagation. The most prominent antiviral effect was observed for the mitochondrial protein and mitophagy receptor BNIP3. Subsequent experiments revealed that BNIP3 depletion increased CHIKV infection in a unique fashion that is independent of the ATG machinery and cellular death pathways. We showed that BNIP3 depletion has no role in LC3 lipidation in CHIKV-infected cells. Moreover, our data shows that ATG7 depletion increased CHIKV infectivity and simultaneous depletion of BNIP3 and ATG7 further enhanced the number of infected cells. Similar results were obtained in ATG7 knockout U2OS cells. Furthermore, we showed that BNIP3 depletion has no effect on the total mitochondrial mass and the mass of polarized mitochondria in CHIKV-infected cells. Finally, on the basis of the lack of LC3 lipidation and formation of WIPI-positive puncta in CHIKV-infected U2OS cells, we conclude that autophagy is not induced during CHIKV infection in U2OS cells. Collectively, our results demonstrate that basal autophagy limits CHIKV infectivity and that BNIP3 controls CHIKV infectivity in an unconventional way. With regard to cell death, we observed a multiplicity of infection (MOI)- and time-dependent increase in the number of dead cells during CHIKV infection, yet BNIP3-depletion did not inhibit/delay cell death during infection. A detailed analysis of the viral replication cycle of CHIKV revealed that BNIP3 controls CHIKV infectivity early in infection, but this occurs after virus cell uptake and viral membrane hemifusion. BNIP3 depletion was shown to increase the number of intracellular viral RNA copies early in infection. The increased number of RNA copies translated in a higher number of infected cells, an overall higher expression level of viral proteins and enhanced release of viral progeny. Furthermore, BNIP3 depletion was shown to increase virus progeny release of two distinct circulating CHIKV genotypes and that of the closely related Semliki Forest virus (SFV).
Overall, the results presented in Chapter 4 indicate that the ATG proteome largely controls CHIKV replication in U2OS cells, which is in line with previous reports suggesting an antiviral function of autophagy in HeLa cells and in murine models (68,69). The antiviral function of autophagy in CHIKV infection has thus far been almost exclusively attributed to p62, which co-localizes with ubiquitinated CHIKV capsid protein in autolysosomes (69). The specific factors that were identified in our screen have not yet been associated with an antiviral function during CHIKV infection. Also, our study revealed that ATG7 has antiviral properties towards CHIKV whereas another study showed that ATG7 promoted infection (69). A possible explanation for this discrepancy might be the cell type used also because the proviral effect of ATG7 was observed in cells that induce autophagy during the course of infection. Within our hits, ULK1 has thus far only been linked to the initiation of autophagy (71). The proteins ATG4C, LC3B and GABARAPL2, have been reported to be predominantly involved in the elongation and closure of autophagosomal membranes (72,73). LC3-I was also reported to function independently of autophagy, in a process that does not appear to involve most of the other ATG proteins (74). Lastly, NBR1 is an autophagy receptor that has been associated with distinct forms of selective autophagy (75), however little is known about its function in viral infection. Future research should focus on validating the role of these proteins in CHIKV replication and demonstrate whether or not they function in the context of autophagy.

We observed that BNIP3-depletion increases the viral RNA copy number early in infection yet no major effect was seen on membrane hemifusion. Our first hypothesis is that BNIP3 interferes with nucleocapsid delivery into the cytosol. To study this in more detail, cellular fractionation can be performed to separate endosomes from the cytosol and determine whether the nucleocapsid, detectable by RT-qPCR, is released in the cell cytosol in the presence or the absence of BNIP3. A second hypothesis is that BNIP3 interferes with the nucleocapsid uncoating required to release the genomic RNA into the cytoplasm. The process of nucleocapsid uncoating is ill-understood and it is currently unknown whether host cell factors are involved in this process (76,77). Imaging techniques at the super-resolution scale using viruses with fluorescently-labelled RNA may shed light on the process of nucleocapsid uncoating and the potential role of BNIP3 herein. Furthermore, techniques like fluorescence-based in situ hybridization and immunoprecipitation could be implemented to determine viral RNA- or viral protein-BNIP3 interactions, respectively. A third hypothesis is that BNIP3 affects the early formation of the alphavirus replication complexes inside spherules, by targeting the viral proteins or the RNA genome in the first rounds of replication. Essential to the formation of proper replicase complexes is the translation of the non-structural proteins in the form of P1234 or as a combination of P1234 and P123 polyproteins (78,79). Partial processing of the replicase into P123 + nsP4 leads to the synthesis of negative strand RNA, whereas complete replicase processing is associated
with a switch to positive sense sub-genomic RNA (sgRNA) synthesis (80). The assembly of CHIKV replication complexes, including replicase formation and processing, are tightly balanced processes. Thus, BNIP3 interference at any of these steps may determine the fate of viral replication. To determine whether BNIP3 interferes with the formation of the replication complexes, evaluation of the potential interactions between this cellular protein and the viral non-structural proteins could help to describe this potential mechanism in more detail. Furthermore, determining the levels of sgRNA versus negative strand RNA at a given time point might provide more insight into BNIP3-mediated alphavirus regulation. However, selective amplification of negative strand RNA has been proven difficult to assess, as often false priming of the positive RNA strand interferes with the specific quantification of the negative strand (81).

The increased CHIKV RNA levels that we observed early in infection in BNIP3-depleted cells likely increases the chance for the virus to productively infect a cell. In turn, the higher number of infected cells correlates with the overall higher protein expression late in infection. Moreover, the higher number of infected cells also correlates with increased output of progeny particles. Collectively, we have pinpointed the mode of action of BNIP3 in controlling CHIKV infection to the early steps in infection and further research is required to delineate the exact mode of action. Although technically challenging, a more detailed understanding of the early events in CHIKV infection will uncover the function of BNIP3 and possibly lead to the identification of other novel host factors controlling the outcome of infection.

BNIP3 might regulate CHIKV infection together with still unknown host factors, reason why elucidating the interacting partners of BNIP3 might shed light on the mechanism involved. Given that BNIP3 is localized on the surface of mitochondria, other mitochondria-associated proteins might be involved in its antiviral function, especially since other mitochondrial-associated signalling events have been linked with the regulation of viral infection (82–84). Intriguingly, BNIP3 and other mitochondrial proteins, have also been detected in RAB5-positive endosomes (85), organelles from which CHIKV particles fuse (86). The localization of BNIP3 in RAB5-positive endosomes has been suggested to mediate the clearance of mitochondria under normal cellular conditions and when cells are treated with the mitochondria uncoupler FCCP (85). Due to the localization of BNIP3 in early endosomes, it will be interesting to identify and study potential viral and cellular interacting partners of BNIP3 in RAB5-positive vesicles. At the structural level, the function of BNIP3 might be elucidated by investigating its functional domains. BNIP3 comprises of a PEST domain, an LC3 interacting region (LIR), a BH3 domain and a transmembrane domain (TM). The TM and the BH3 domains are both associated with the pro-apoptotic and autophagy-related function of BNIP3 (87). The LIR motif, is responsible for the interaction of BNIP3 with lipidated LC3-II during the formation of autophagosomes (88). In Chapter 4 we show that the function of BNIP3 in controlling alphavirus infection is likely not mediated by its role as an
autophagy receptor, reason why we hypothesise the conventional functions of the LIR and the BH3 domains are not relevant for BNIP3 antiviral function. This is supported by the fact that NIX depletion has no effect on CHIKV replication, despite sharing 55% sequence similarity with BNIP3, especially in the domains related to autophagy and apoptosis (89). Finally, the function of BNIP3 in alphavirus infection might also be determined by studying the post-translational modifications of this protein, like phosphorylation and ubiquitination, or by changes in its expression levels, which are currently unknown under viral infection conditions.

To conclude, our data suggests that BNIP3 participates in other processes unrelated to cell death and autophagy to control CHIKV and SFV infections, and future research is therefore required to pinpoint more specifically which other host factors participate in this antiviral mechanism.

III. miRNA control of cellular responses during DENV infection and potential therapeutic approaches

miRNAs are known for their ability to target and control the expression of almost the entire human transcriptome and thus are involved in the regulation of multiple cellular processes, including the ones that take place during viral infection. In the case of DENV, it has been reported that infection of cells changes the miRNA expression landscape (miRNAome) and both upregulation and downregulation of individual miRNAs has been observed (90–94). Furthermore, some of the studies have shown that differentially expressed miRNAs have a beneficial or detrimental effect in DENV replication, depending on the involved miRNA. For example, increased expression of the interferon (IFN)-inducible miR-146a favours DENV replication by targeting TNF receptor associated factor 6 (TRAF6), thereby decreasing the IFN-β response in both the monocytic cell line THP-1 and human primary monocytes (95). In addition, it has been proposed that miRNA regulation of gene expression in DENV-infected peripheral blood mononuclear cells (PBMCs) has an effect on cytokine expression, creating a favourable cellular environment for viral replication (90). On the other hand, expression of miRNAs that regulate the innate immune response have also been reported to have antiviral functions. For example, miR-30e* has been shown to increase the antiviral IFN-β response in DENV-infected PBMCs, U937 and HeLa cells (96). Moreover, miRNAs have been described to reduce DENV infectivity via both regulation of the oxidative stress response by Let-7 (91) and destabilization of microtubule dynamics by miR-223 (92). Additionally, direct targeting of miR-548g-3p to the 5’ UTR in the DENV genome has also been reported as an antiviral mechanism (97). Altogether, it is evident that individual miRNAs can have both detrimental as well as beneficial effects on DENV replication via both direct as well as indirect functions.
In **Chapter 5**, we examined the involvement of miRNA-mediated post-transcriptional regulation of cellular gene expression in primary monocyte-derived macrophages (MDMs) exposed to DENV. Macrophages are considered one of the most important target cells during natural infection (98), and the role of miRNAs in controlling DENV infectivity in this cell type is poorly understood. MDMs cultures were challenged with DENV for 24 h after which the cells were sorted in DENV-infected MDMs and non-infected MDMs that were exposed to DENV. The miRNA expression profile was determined in both cell populations and in mock-infected MDMs. Furthermore, UV-inactivated DENV was used as a non-replicating virus control to also determine the importance of viral replication on the miRNAome. Overall, we observed limited changes in the miRNAome of MDMs upon DENV infection. No differences were seen between the cells exposed to UV-inactivated DENV and the mock-infected cells. The deep-sequencing data revealed that DENV infection upregulates the expression of miR-4508, miR-3960, and miR-4301 in DENV-infected cells and miR-4508, miR-3960, miR-3614-5p and miR-181a-3p in non-infected MDMs that have been exposed to DENV when compared to mock-infected cells. Interestingly, miR-3614-5p and miR-181a-3p were solely upregulated in non-infected cells that were exposed to DENV, which suggests that these miRNAs may have antiviral properties. Indeed, overexpression of miR-3614-5p was found to reduce the production of DENV progeny. No effect was observed for miR-181a. Subsequent proteomic analysis of cells overexpressing miR-3614-5p revealed that this miRNA appears to regulate multiple proteins. We validated the effect of miR-3614-5p on the expression level of protein adenosine deaminase acting on RNA 1 (ADAR1). We found that miR-3614-5p indeed controls the expression of ADAR1, and downregulation of ADAR1 was found to impair DENV replication.

Mounting evidence suggests that DENV infection only has a limited effect on the miRNA expression profile of human cells (90,92). We identified only 3 miRNAs that were differentially expressed in DENV-infected MDMs. This is in line with a study describing the miRNAome profile during DENV infection in EA.hy926 cells, a vascular endothelial cell line, which identified 8 miRNAs that are upregulated and 4 miRNAs that were downregulated during infection (92). Similarly, a study in PBMCs infected with DENV-2, identified 11 upregulated and 4 downregulated miRNAs as determined by microarray analysis (90). Furthermore, the miRNAs identified vary between distinct studies. In fact, little overlap is seen between the studies published to date. These discrepancies can be explained by the cell type and the infection conditions, as the miRNA response is largely dependent on the cellular context (99). Likewise, the technique used to identify the differentially expressed miRNAs, e.g., miRNA microarray versus deep sequencing, can also have a significant impact in the results obtained (100). Collectively, the current literature suggests that miRNA expression is not drastically changed by DENV infection.

From the miRNAs that we identified in our study, miR-3614-5p overexpression was shown to have an antiviral effect on DENV replication in Huh7 cells. This is line with
previous evidence suggesting that cellular miRNAs might indeed have antiviral properties (91,92,96,101,102). The mechanism by which miRNAs regulate viral replication depends on the cellular target mRNAs. Our proteomic analysis revealed that overexpression of miR-3614-5p downregulated the expression of 9 cellular proteins and upregulated 20 cellular proteins. Interestingly, some of the proteins regulated by miR-3614-5p, e.g., protein S100-A9, dynein light chain 1 and heterogeneous nuclear ribonucleoproteins A2/B1 have been previously associated with the replication of several viruses, including DENV (103–108). We confirmed the function of miR-3614-5p in controlling the expression of one of these proteins, i.e., ADAR1, which has been previously identified as a host factor that facilitates the replication of yellow fever virus, WNV, Venezuelan equine encephalitis virus and CHIKV (109). In line with this observation, siRNA-mediated depletion of ADAR1 has been shown to inhibit DENV (110). Our data is in agreement with these findings, as ADAR1 knockout MEFs were less susceptible to DENV in comparison to WT MEFs, specifically during the first round of replication within the first 24 hpi. Furthermore, overexpression of miR-3614-5p also reduced the replication of WNV, which suggest that this miRNA has a similar antiviral effect on viruses of the same virus family. The contribution of other proteins regulated by miR-3614-5p in DENV infection and the specific mechanism by which ADAR1 promotes DENV infectivity, however, remains to be elucidated.

Although virus-induced changes in the miRNAome have been reported, little is known about the underlying mechanisms by which viral replication induces such variations. The regulation of miRNAs during viral infection can be mediated by indirect mechanisms e.g., caused by cellular stresses and antiviral responses, or via direct mechanisms due to the viral genome and/or proteins. For example, DNA viruses from the *Herpesviridae*, *Polyomaviridae*, and *Papylomaviridae* families can directly affect miRNA expression as these viruses encode viral miRNA genes (111). Furthermore, the expression of viral proteins can also directly induce the expression of specific miRNAs (112,113). For DENV, it has been suggested that the viral protein NS4B participates in the general suppression of miRNA response (94), although more accurate studies are required to validate this result. An example of an indirect mechanism is the induction of miRNAs as part of the viral innate immune response. In this scenario, active viral replication is required, as viral RNA intermediaries are recognized by immune receptors such as retinoic acid-inducible gene I or the endosomal Toll-like receptors, rapidly modulating the expression of miRNAs that control the strength and duration of the antiviral response (114). Our sequencing data showed that UV-inactivated DENV did not induce changes in miRNA expression, corroborating that active replication is required to alter the cellular miRNAome during DENV infection. Similarly, viral-induced ER expansion and viral egress through the secretory pathway might contribute to induction and/or repression of determined miRNAs, a possibility that should be the subject of future research. The underlying mechanisms by which the identified miRNAs
in our study regulate infection warrants further investigation as this will further increase our understanding of the role of miRNAs in DENV infection.

An advantage of our study was that we used primary MDMs, which are important natural targets of DENV during infection (115). Primary cells have the advantage of maintaining many of the important markers and functions that are observed in vivo (116). The use of primary cell lines, however, comes also with some limitations. In this context, our main challenge was to validate the role of miR-3614-5p in MDMs. We attempted to overexpress this miRNA in either primary cells or cell lines resembling monocytes or macrophages, but this overexpression was difficult to achieve. It is generally known that monocytes and macrophages are difficult to transfect, mainly due to their high differentiation stage and specialization. Even more, these cells activate the type-I IFN response upon transfection, especially when lipophilic transfection reagents are used (117,118). In line with these observations, transfection of MDMs with miRNA-encoding DNA vectors resulted in a low transfection efficiency and cell death (data not shown). On the other hand, when overexpression of the miRNA was successful, cells were found to be refractory to infection because of the activation of the innate immune response. Therefore, with the techniques and tools currently available, it is difficult to specifically validate the role of miR-3614-5p in the context of macrophage-like cells. So far, the only report in which a macrophage-like cell line has been efficiently transfected used nucleofection technology, which represents an optimized electroporation approach for DNA transfection (119). To the best of our knowledge, this technique has not been used in MDMs, and future studies would therefore determine its efficiency in primary MDMs. Overall, although studies in primary cell lines provide many advantages for virology studies, there is an urgent need to improve and make more readily available methodologies to induce RNA and DNA overexpression in these type of cells.

Due to their capacity to modulate gene expression, miRNAs are currently being considered for therapeutic strategies against viral infections. In the case of hepatitis C virus (HCV), for example, the 5'UTR of the viral genome is targeted by the liver-specific miR-122, which promotes HCV protein translation by stabilizing the viral genomic RNA (120,121). This function led to the design of a synthetic antagonizing locked nucleic acid against miR-122 for HCV treatment (Miravirsen®). Miravirsen has demonstrated antiviral activity in vitro against all HCV genotypes and has produced long-lasting suppression of HCV, with no evidence of viral resistance, in both an HCV-infected chimpanzee model and a human phase 2 clinical trial (122,123). Similar mechanisms could be used to control transcription, stability or translation of viral genes over the course of infections caused by other viruses. Furthermore, long-non-coding RNAs are another type of RNAs that are induced during viral infection and have the ability to regulate innate immune responses (124,125). Finally, study of miRNAs and other types of non-coding RNAs in the context of DENV infection should be investigated further with the aim to develop new therapeutic strategies or tools with prognostic value.
References


Discussion and future perspectives

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