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# Synergistic correlation between host angiogenin and dengue virus replication

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**ABSTRACT**

DENV infection poses a major health concern globally and the pathophysiology relies heavily on host-cellular machinery. Although virus replication relies heavily on the host, the mechanistic details of DENV–host interaction is not fully characterized yet. Here, we are focusing on characterizing the mechanistic basis of virus-induced stress on the host cell. Specifically, we aim to characterize the role of the stress modulator ribonuclease Angiogenin during DENV infection. Our results suggested that the levels of Angiogenin are up-regulated in DENV-infected cells and the levels increase proportionately with DENV replication. Our efforts to knockdown Angiogenin using siRNA were unsuccessful in DENV-infected cells but not in mock-infected control. To further investigate the modulation between DENV replication and Angiogenin, we treated Huh7 cells with Ivermectin prior to DENV infection. Our results suggest a significant reduction in DENV replication specifically at the later stages as a consequence of Ivermectin treatment. Interestingly, Angiogenin levels were also found to be decreased proportionately. Our results suggest that Angiogenin modulation during DENV infection is important for DENV replication and pathogenesis.

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## Introduction

The Human RNases superfamily is a large group composed of different types of RNases. They play a significant role in a variety of physiological functions including antitumor activity, angiogenesis, immunity, antibacterial and antiviral activity, etc. The role of RNases against viruses was first reported with respect to Influenza virus (INF) in 1956 by Clerc and co-workers [1]. Studies have also reported RNases like RNASE2 and RNASE3 to inhibit Respiratory Syncytial virus (RSV) life cycle by catalysing the generation of microRNAs (miRNAs) and tRNA-fragments (tRFs). Domachowske and co-worker reported that RNASE 2 interacts with a viral surface molecule and penetrates the capsid to interact with viral RNA [2]. It is also observed that RNASE2 is upregulated in classical monocytes from dengue infected patients [3]. In addition, RNASE 4 and RNASE 5 have also been reported to control HIV infection. Cocchi et al. have shown that CD4(+) and CD8(+) T cells release three beta-chemokines along with the RNases 4 and 5. These soluble factors released from T cells are shown to have suppressive activity against HIV [4]. RNases like onconase possess cytotoxic properties against cancer cells, making them valuable therapeutic agents. These RNases are often referred to as immunoRNases due to their ability to modulate the immune response [5].

Dengue virus (DENV) is a single-stranded RNA virus that belongs to the Flaviviridae family. It has a positive-sense RNA genome which is ~11kb long. It encodes a single polyprotein which is further processed by cellular and viral proteases to form three structural proteins (capsid (C), membrane (M), and envelope (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). It has four serotypes DENV 1, DENV 2, DENV 3, and DENV 4.

Dengue infection is the most common arboviral infection, especially in tropical countries. The environmental conditions of the tropical countries favour the proliferation of *Aedes aegypti* and *Aedes albopictus* which are the primary vectors for DENV infection. According to WHO, dengue cases have increased more than 8-fold over the last two decades. More than 40% of the population is at global risk of DENV infection which is more prevalent in the younger age group. About 390 million cases of dengue have been reported worldwide and the number is increasing annually. As per the ECDC (European Centre for Disease Control) report of 2023 more than 2 million cases 974 deaths have been reported globally.

The global burden of dengue infection poses significant challenges in terms of medical treatment options, as there is a lack of effective therapeutic regimens. This highlights the need to expand our knowledge and deepen our mechanistic understanding of dengue infection in order to develop better strategies for its management. Different studies have reported the roles of gene translation inhibitory molecules like siRNA and miRNA which are known to have potent antiviral

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activities against various viruses including DENV [6–13]. Many drug repurposing studies were also carried out to explore the potential of different drugs having anti-dengue or anti-flaviviral activity [14–17]. In the 1980s, Ivermectin was introduced as the broad spectrum anti-parasitic treatment which interferes with parasitic muscles and the nervous system by activating glutamate-gated chloride channels [18]. Taking into consideration ivermectin's usage as a treatment for parasitic diseases for more than 25 years, its potential was also exploited as anti-flavivirus therapy [19–21]. The study by Mastrangelo et al. suggests that ivermectin interferes with the viral helicase NS3 and mainly exerts its effect during viral RNA replication [19]. Another study suggests ivermectin inhibits the import of NS5 by interfering with IMPα/β1 nuclear importins. NS5 plays a critical role in viral replication within the cytoplasm of the host cell. However, there have been reports suggesting its presence in the cell nucleus during specific stages of viral replication [22,23]. Mutation in the critical residues of IMPα/β1 nuclear importins impairs NS5 nuclear translocation which severely inhibits virus production [17,21]. The specific function of NS5 in a nuclear compartment is not fully understood. However, recently it has been demonstrated that NS5 interacts with U5snRNP components, resulting in the inhibition of host splicing machinery and the inhibition of pre-mRNA splicing in the host cell supports the DENV propagation [24]. It has also been known to play a crucial role in modulating host innate immune response [25]. These studies suggest that targeting DENV with ivermectin can inhibit DENV replication inside the host cell.

RNA and DNA virus encodes for *trans*-acting regulatory RNAs like miRNA, tRNA fragments and sfRNA which regulate host and viral gene expression [26–30]. These regulatory RNAs are produced by the action of various ribonucleases. Angiogenin is known stimulator in blood vessel formation via process of angiogenesis. Angiogenin, also known as RNase 5, belongs to the Ribonuclease A superfamily. It is also known to promote rRNA transcription and translational regulation during cell homoeostasis. It is primarily found in the nucleus with the bounded inhibitor RNH1 [31–34]. Earlier, it was only known to play role in cell growth and survival but now recent studies suggest it play vital role in generation of regulatory RNAs like tRNA fragments. During stress, RNH1 gets unbound from Angiogenin, and it further translocate into the cytoplasm where it cleaves mature/pre-mature tRNA into fragments. Fragmented tRNAs are also known to play various regulatory roles including antiviral activities as reported in the context of the RSV virus [35,36]. Also, in case of viral infection, there are studies that suggest it is crucial for viral replication. It is known to support Kaposi's sarcoma virus replication by promoting protein synthesis [37]. In HIV, angiogenin is known to inhibit viral replication in dose-dependent manner [4]. The study by Design et al. (2010) conducted gene expression profiling on samples from patients with Dengue Shock Syndrome (DSS). Their findings revealed the upregulation of genes involved in tissue repair, remodelling, and anti-inflammatory responses, including Angiogenin [38]. We, therefore, have carried out our study targeting Angiogenin to observe its effect on DENV replication.

In this study, we have investigated the effects of RNase Angiogenin on DENV replication. We have performed siRNA-mediated knockdown of Angiogenin to investigate its role in DENV replication. Our results revealed that Angiogenin could not be effectively silenced in the presence of DENV. In fact, we observed an enhancement in Angiogenin levels during DENV infection. Interestingly, as the levels of Angiogenin increased, DENV replication was also increased simultaneously. In order to investigate the DENV-mediated regulation of Angiogenin modulation, Huh7 cells were treated with Ivermectin. Our results suggest that Ivermectin treatment results in a significant decrease in angiogenin. Overall, our results suggest that Angiogenin is modulated during DENV pathogenesis, and this modulation may play a significant role during DENV pathogenesis.

## Materials and methods

### Cell lines and virus propagation

Vero cells (African green monkey kidney cells) and Huh7 cells (hepatocellular carcinoma cells) were procured from NCCS Pune. These cells were cultured in DMEM high glucose media (Hyclone, Cytiva), supplemented with 10% FBS (Gibco) (v/v) and 1% penicillin/streptomycin antibiotic, at 37°C with 5% CO2 under humidified conditions. DENV serotype 2 strain P23085 INDI-60 was propagated in C6/36 cell line in L15 media (Himedia) supplemented with 10% FBS (v/v) and 1% penicillin/streptomycin and incubated at 28°C without $CO_2$. Cultured supernatant was collected by adjusting the FBS concentration to 2% of the total volume. DENV supernatant was then further stored at −80°C until use.

### Immunofluorescence assay (IFA)

IFA was performed to examine the level of infection in the Huh7 cell line. Huh7 cells were seeded onto coverslips in 12 well plate ($4 \times 10$ [5] cells per well), and grown overnight. The next day, the cells were infected with DENV at MOI 1 in DMEM media containing 2% FBS and left for viral adsorption for 2 h. After 2 h adsorption, the culture medium was replaced with complete media after 1X PBS wash. The plate was then incubated for 48 h at 37°C in 5% $CO_2$. Following incubation, cells were fixed with 10% paraformaldehyde, permeabilized with 0.1% Triton X and blocked with 1% BSA. The cells were further incubated with monoclonal anti-dengue virus complex antibody (Sigma: MAB8705) followed by secondary antibody Alexa Fluor 488 goat anti-mouse IgG (Life Technologies: A11034). The nuclei were stained with DAPI stain. Coverslips with grown cells were then mounted onto the slide and observed under a fluorescence microscope (Zeiss, Germany).

### siRNA knockdown and DENV infection

For siRNA transfection, Angiogenin siRNA was the first reverse transfected using Lipofectamine RNAi max (Invitrogen) according to the manufacturer's instructions. Cells were reverse transfected with 10 nM of control

(ACGUGACACGUUCGGAGAAUU) and 10 nM, 30 nM, and 50 nM Angiogenin siRNA (ACGUUGUUGUUGCUU GUGA). Following 24 h of Angiogenin knockdown, DENV infection was given. Cells were harvested in TRIzol for total RNA extraction at different time points (0 h, 4 h, 12 h, 24 h, 48 h). siRNA used were ordered from Eurofin: **siANG** ACGUUGUUGUUGCUUGUGA [39], **Control siRNA** ACGUGACACGUUCGGAGAAUU [40,41].

### RNA extraction and cDNA preparation

RNA was isolated from Trizol extracts. Briefly, 300 µl chloroform was added to the TRIzol extract. Tubes were then inverted to mix well followed by 10 min of incubation at room temperature. Tubes were then centrifuged at 13,000 RPM at 4°C for about 10 min. The aqueous supernatant was collected to which 600 µl of absolute ethanol was added. Mixed it well and transferred it through RNA columns at 8000 RPM for 30 s. The column was then washed with AW1 and AW2 buffers (Qiagen). After 1 min of empty spin, RNA was finally eluted in nuclease-free water.

For cDNA preparation, 1 µg of RNA was DNase (Promega) treated at 37°C for 30 min followed by enzyme inactivation at 65°C for 20 min after adding the stop solution. For the annealing reaction, 1 µl of random primers and 1 µl of 10 nM dNTP mix were added to the DNase-treated RNA and incubated at 65°C for 5 min and immediately chilled on ice. For RT reaction, a mix was prepared with 5X reaction buffer 4 µl, Ribolock (Thermo), 0.5 µl, MLV RT (Promega) 1 µl, and nuclease-free water to make up the volume up to 20 µl with the 1 µg treated RNA. This mix was then incubated at 25°C for 10 min, 42°C for 60 min, and 85°C for 10 min.

### RT-PCR

PCR experiments were carried out using the GoTaq Flexi DNA polymerase kit (Promega). A cocktail mix was prepared on ice containing the following: 4 µl of 5× Green GoTaq Buffer, 5 µM each primer, 0.5 µl of 10 mM dNTP, 0.25 µl GoTaq DNA polymerase (5 U/µl), 2 µl cDNA template. The total reaction mix was adjusted to 20 µl per reaction with nuclease-free water. Cycling conditions were: initial denaturation hold at 98°C for 3 min, amplification (25–30 cycle) denaturation at 98°C for 30 sec, annealing at 52–58°C for 20 s, extension at 72°C for 30 s, final extension at 72°C for 5 min. Soak at 4°C infinite.

qPCR of cDNA was carried out using Sybr Green master mix (Applied Biosystems) according to the manufacturer's protocol. Cycling conditions used were: initial denaturation hold at 95°C for 10 min, denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec, extension at 72°C for 10 sec, with a total of 40 amplification cycles. Beta-actin was used as the internal control. The expressions of genes related to internal controls were detected using the 2−ΔΔCt method. Primers used for RT-PCR and qPCR were common, and the sequence details have been provided in the supplementary table S1.

### Ivermectin treatment

The ivermectin formulation was obtained in a powdered form Sigma-Aldrich (Cat no. I8898). A stock solution was prepared in DMSO at 10 mg/ml concentration, and the aliquots were stored at −20°C to prevent multiple freeze-thawing. Huh7 cells were cultured in DMEM with 10% FBS and were pre-treated with a 50 µM concentration of ivermectin for 4 h [22]. In parallel to ivermectin treatment, DMSO treatment was used as a control. Following 4 h of DMSO/ivermectin treatment, DENV infection was given and the cells were harvested at different time points (0 h, 4 h, 8 h, 12 h, 24 h, 48 h) post-infection.

### Western blot analysis

DENV infected Huh7 cells were harvested in RIPA buffer (50 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 10 mM MgCl2, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM sodium vanadate, 10 mM sodium fluoride) at different time points (0 h, 4 h, 8 h, 12 h, 24 h and 48 h). The cell lysate was run in 12.5% SDS-PAGE gel and transferred to the PVDF membrane (Amersham). The membrane was then blocked with 5% skimmed milk in 1X TBS for about 1 hr at room temperature followed by antibody incubation at 4°C overnight. The next day, after three times wash with 1X TBST (0.05% tween 20) blot was incubated with a secondary antibody for 1 hr at room temperature. Blot was then finally developed using ECL reagent (Thermo). Primary antibodies used were anti-ANG (Invitrogen: PA534422), Anti-Beta actin (Abbkine: A01010), anti-tubulin (Abbkine: A01030), anti-NS1 (PA5–32207), anti-DV (MAB-8705)

### Cytotoxicity assay

Cell cytotoxicity was determined using MTT assay. Briefly, Huh7 cells were seeded in 96-well plates with a density of 10,000 cells per well. Cells were given the treatment and incubated for the respective time points. After incubation, 15 µl of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) reagent was added and incubated for 3 h at 37°C. After 3-h incubation, DMSO was added as the solubilization/stopping solution to each well and incubated for 30 min at 37°C. The optical density (OD) of each well was then observed at 540 nm.

### Statistical analysis

The experiments were performed in triplicates, and the statistical significance was carried out using analysis of variance (Two-way ANOVA) and the Graph Pad Prism software (Graph Pad Software Inc., San Diego, CA). P-value of less than or equal to 0.05 was considered significant. Error bars are expressed as Standard Error Mean (SEM).

## Results

### Angiogenin level is enhanced during DENV infection

The infectivity of Dengue virus is known to vary between cell lines, and further, a permissive cell line may show serotype-

specific differential infectivity [42,43]. Therefore, to study the kinetics of the laboratory strain used in this study, virus grown in mosquito C6/36 cells were allowed to infect Huh7 cells at a **M**ultiplicity-**o**f-**i**nfection or MOI of 1 and the number of infected cells were determined by Immunofluorescence assay (IF). The results showed that at 48-h post-infection (hpi) >90% of the cells showed the presence of intracellular viral antigen, and thus an MOI of 1 was used for subsequent experiments (supplementary figure S1). Since the role of RNASES like Angiogenin in DENV life cycle is not clear yet, we investigated ANG levels in Huh7 cells following virus infection. Briefly, Huh7 cells infected with an MOI of 1 were lysed at different time points (0 h, 4 h, 8 h, 12 h, 24 h, 48 h) post-infection and the total RNA extracted and purified. The total RNA was reverse-transcribed using random hexamers and the cDNA used for PCR amplification. RT-PCR and quantitative PCR (qPCR) for the Angiogenin transcript normalized to that of Beta-Actin showed an increase in Angiogenin levels with the increase in DENV replication time points. A significant reduction in the Angiogenin transcript level was noticed immediately after infection, specifically at the 0th hour, which corresponds to 2 h after the adsorption of DENV, as compared to the mock infected control group (Figure 1, panel A and B, lane 1 and 2) and at 48 h post-infection Angiogenin levels were found to be significantly higher compared to its level in mock-infected cells (Figure 1, panel A and B, lane 1 and lane 7). In addition, immunoblotting was performed to further confirm the RT-PCR and qPCR results. The results demonstrated a gradual rise in Angiogenin levels over time (Figure 1, panel C and D, lane 1 to lane 7). A qPCR for DENV genomic RNA using primers binding to the coding region of either Env or NS1 protein, and an immunoblotting for DENV NS1 protein, respectively showed a concomitant increase in replication of the viral genome and translation of viral proteins (Figure 1, panel B, C and D). As a proof of intracellular viral replication responsible for inducing ANG expression levels, incubation of Huh7 cells with heat-inactivated DENV at different time points (0 h, 4 h and 48 h) did not show any transcriptional or translational modulation of Angiogenin (Figure 1, panel E and F). The results suggested that early events following DENV infection suppress the expression of ANG while eventually viral replication leads to an induction in its expression.

### *Silencing of ANG in DENV infected cells*

A concomitant rise in Angiogenin levels was observed alongside the enhanced replication of DENV. This suggests that the ribonuclease activity of Angiogenin may still play a role in regulating the virus replication to some extent. We further conducted Angiogenin knockdown in virus-infected cells using siRNA and assessed its impact on virus replication. In order to silence Angiogenin expression in Huh7, different concentrations (10 nM, 30 nM and 50 nM) of Angiogenin-specific siRNA (siANG) were reverse-transfected and harvested the samples at 48-h post transfection. The total RNA from transfected cells was extracted, purified and analysed by RT-PCR for relative level of Angiogenin transcript Level normalized to that of -actin. The results showed increased silencing of ANG mRNA with an increase in siANG concentration, with 50 nM of siANG reducing the steady-state level of the transcript to <85% (Figure S2). However, transfection of 50 nM siANG led to a significant degree of cell death in the transfected cells upon subsequent infection with DENV, which was not observed at the 10 nM and 30 nM concentration of siANG (data not shown). Since transfection

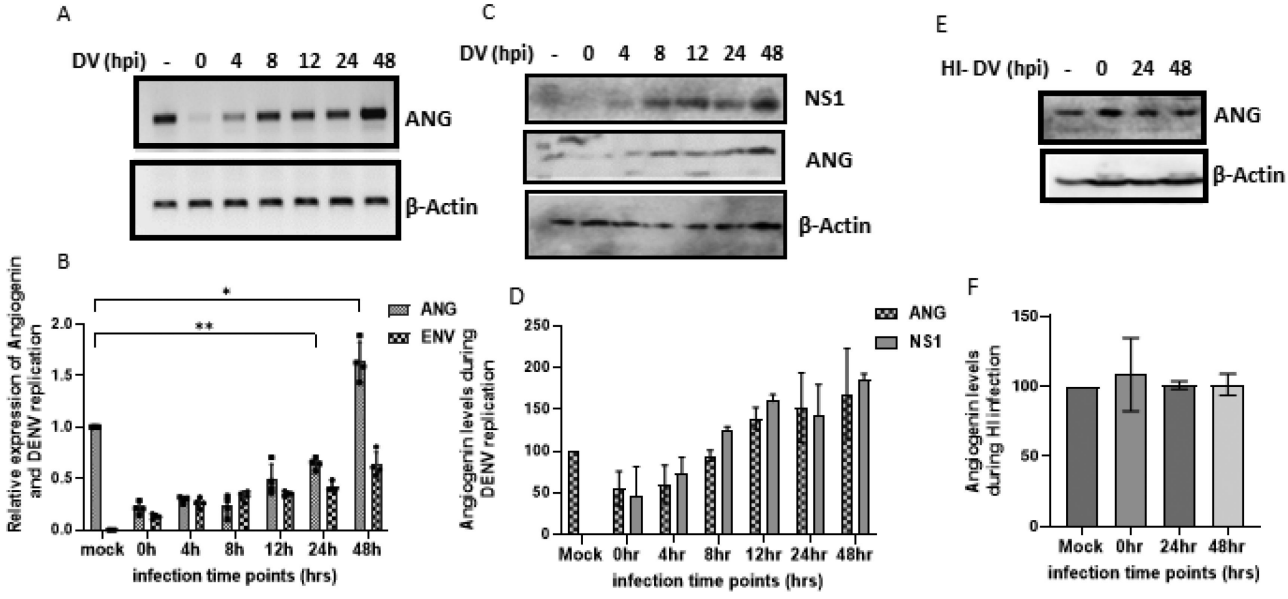


**Figure 1. DENV infected Huh7 cells at different time points.** (A)RT-PCR (B) qPCR analysis and (C) and (D) Western blot analysis showed decrease in the ANG levels at the initial stages of replication while an increase was observed at the later stage of DENV replication (E) and (F) heat inactivated DENV infection was given at different time points (0 h, 24 h and 48 h) and the endogenous levels of angiogenin were compared with the mock levels. Heat inactivated DENV (HI-DV) virus does not show any effect on angiogenin level.

RT-PCR and qPCR analysis is the result of three individual experiments. Error bars show Mean ± SD. Statistical analysis was performed using Two-way ANOVA (Bonferroni method). (*) shows P-value ≤0.05, (**) shows P-value ≤0.005. Western blots were performed twice for validating qPCR results.

of siANG at 10 and 30 nM was respectably able to achieve ~65% and ~80% silencing of the Angiogenin transcript without any significant cytotoxicity upon subsequent DENV infection, these two concentrations were used for all subsequent experiments. Next, a time kinetics for ANG silencing, following transfection of either 10 nM or 30 nM of siANG or scramble siRNA control (siC), was performed at 24 or 48 or 72 h post-transfection through real-time PCR (qPCR) based comparison of ANG transcript level normalized to that of Beta-Actin (Figure 2, panel A). At 24-h post transfection, a substantial and consistent reduction in Angiogenin levels, approximately 65–70%, was observed using 10 nM and 30 nM siANG, respectively. The maximum knockdown efficiency was achieved at 48 h (70–80% reduction) and at 72 h, the knockdown levels were comparable to those observed at the 24-h time point with both 10 nM and 30 nM siANG concentrations (Figure 2, panel A).

To validate the efficacy of Angiogenin knockdown, we assessed the levels of Angiogenin protein through western blot at 24 and 72 h after transfection. For both 10 nM and 30 nM siRNA concentrations, we used 24-h siC transfection control. In the siC transfected cells, Angiogenin levels remained unchanged at both 10 nM and 30 nM siC concentrations. However, in the case of siANG transfection, we observed a knockdown of 55%–65% at 24 and 72 h with 10 nM siANG. For 30 nM siANG, there was an approximate 75% sustained knockdown of Angiogenin at both 24 and 72-h post transfection (Figure 2, panel B and C).

To investigate the impact of Angiogenin silencing on Dengue virus (DENV) replication, Huh7 cells were transfected with two concentrations 10 nM and 30 nM of siANG followed by mock infection or DENV-infection at an MOI of 1. The steady-state levels of ANG transcript normalized to β-actin transcript level, were quantified in DENV infected and mock infected Huh7 cells at 48 h post-infection (72-h post transfection) by qPCR (Figure 2 panel D) using total RNA extracted and purified from these cells. Although Angiogenin silencing was achieved in mock-infected cells. However, Angiogenin knockdown cannot be seen at either of the siANG concentration in DENV infected cells. In fact, Angiogenin level was found to be higher in DENV infected cells compared to mock-infected controls (Figure 2, panel D).

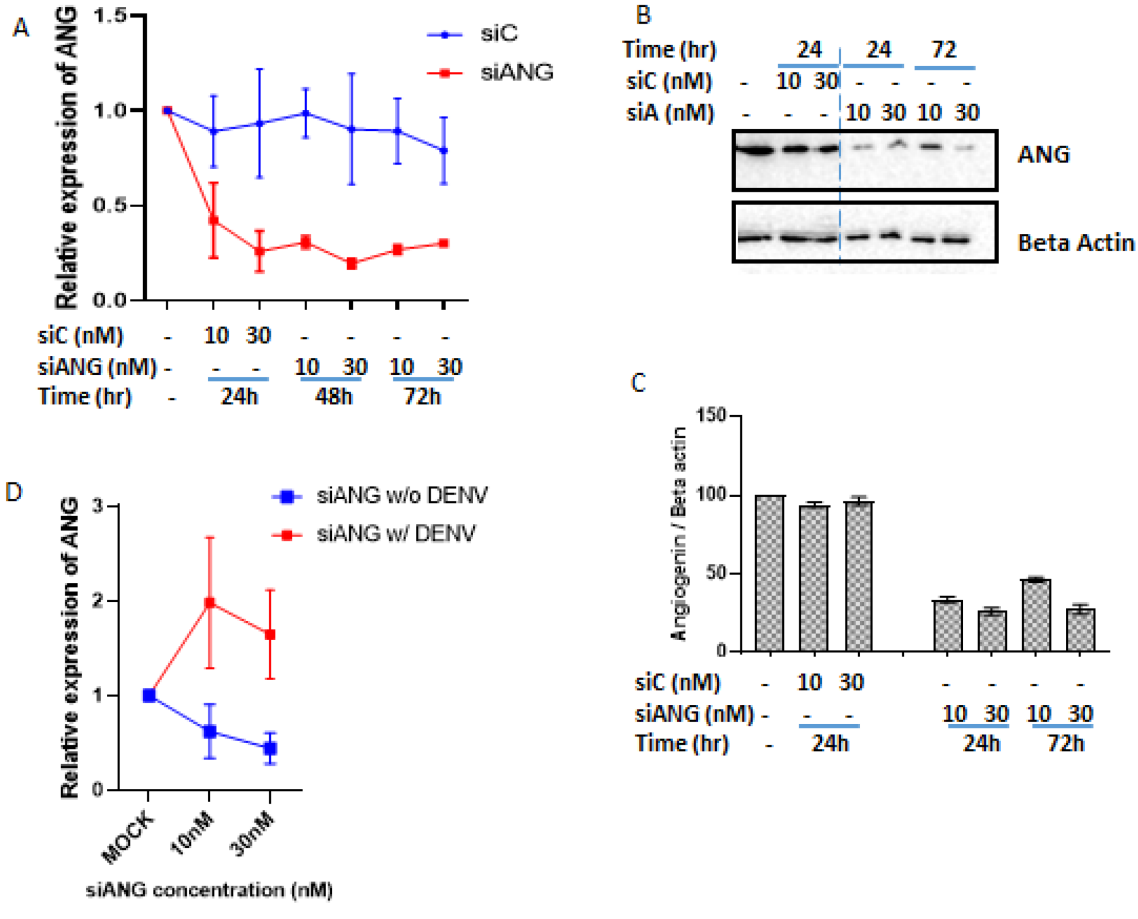


**Figure 2. siRNA mediated knockdown of Angiogenin in DENV-Infected and uninfected cells** A) qPCR analysis shows the knockdown efficiency evaluated by reverse transfecting the cells with 10 nM and 30 nM of control siC or siANG, followed by cell harvested at various time points (24 hours, 48 hours, and 72 hours). It was observed that ANG levels were reduced by approximately 65% up to 72 hours post-transfection with siANG, in the absence of DENV infection. (B) and (C) the efficacy of angiogenin knockdown was confirmed through Western blot analysis conducted at 24 hours and 72 hours following transfection. In the absence of DENV infection, a significant reduction of approximately 80% in angiogenin expression was observed 72 hours post-transfection. (D) qPCR showing angiogenin levels upon 24hrs of siC or siANG transfection, followed by DENV infection. Cells were harvested at 48hpi. Angiogenin levels were found to be increased upon DENV infection. qPCR analysis is the result of three individual experiments. Error bars show Mean±SEM. Western blots were performed twice for validating qPCR results.

### Dengue virus-induced upregulation of angiogenin predominates angiogenin silencing

Despite our efforts to down-regulate Angiogenin expression using siRNA-mediated knockdown techniques, we observed an increase in Angiogenin levels during DENV infection. To investigate further, our study aimed to determine the stage of DENV replication where siRNA-mediated angiogenin knockdown loses effectiveness and determine the time point when maximum Angiogenin upregulation occurs.

To examine the kinetics of this upregulation, we performed reverse transfection of Huh7 cells with 10 or 30 nM of siANG or siC. At 24 h post-transfection, the cells were either mock-infected or infected with DENV at an MOI of 1. Total RNA was extracted from uninfected or DENV-infected cells at various time points post-infection (0, 4, 8, 12, 24, and 48 h). The levels of ANG or DENV genomic RNA were quantified using qPCR, normalized to Beta-actin mRNA levels. The results revealed a significant reduction of ANG mRNA levels 24 h after transfection with both 10 nM and 30 nM siANG concentrations (Figure 3 panel A, compare lane 1, 2 and 3). Following 24 h of transfection, DENV infection showed modulation in Angiogenin levels starting at the 0th hour of DENV infection, which corresponds to 2 h of DENV adsorption. Following DENV absorption, we observed a rapid increase in Angiogenin levels. Between 24 and 48 h after infection, we observed maximum increase in the Angiogenin transcript levels. Interestingly, cells transfected with 10 nM of siRNA showed a higher increase compared to those transfected with 30 nM siRNA (Figure 3 panel A). We have further performed western blot to validate the qPCR analysis (Figure 3 panel B and C). Our result further validated the qPCR findings.

To validate the observed modulation of Angiogenin, a cell cytotoxicity assay was performed using the MTT assay in a 96-well plate. Angiogenin knockdown was carried out for 24 h, followed by DENV infection for 24 h (Figure 3 panel D) and 48 h (Figure 3 panel E). The results showed that the percent cell survival of DENV-infected Angiogenin knockdown cells was comparable to that of uninfected cells subjected to knockdown alone at the 24-h time point. However, at the 48-h time point, approximately 20% and 30% cell death were observed in cells transfected with 10 nM and 30 nM siANG, respectively. These findings indicate a decrease in cell viability over time, with a more pronounced effect observed at the higher siRNA concentration.

Despite the substantial decrease in cell viability, the observed increase in Angiogenin levels suggests that DENV infection can induce significant changes in gene expression within the surviving cell population. These results further support the notion that siRNA-mediated knockdown of Angiogenin during DENV infection does not result in Angiogenin reduction. Instead, an upregulation of Angiogenin levels was observed, indicating that Angiogenin may be transcriptionally regulated during DENV infection.

The observed upregulation of Angiogenin (ANG) during DENV infection is suggestive of its potential role as a proviral factor, contributing to the replication and propagation of the virus.

### Ivermectin treatment suppresses DENV replication and angiogenin expression

To determine the relationship between Angiogenin modulation and DENV replication, we conducted experiments in Huh7 cells treated with either DMSO or Ivermectin (IV). Ivermectin is known to inhibit DENV NS3 protease activity and prevent nuclear localization of NS5, thereby hindering DENV replication and host antiviral activity [17,19]. After a 4-h pre-treatment with DMSO or Ivermectin, DENV infection was initiated at different time points (0 h, 6 h, 12 h, 24 h, 48 h, and 72 h) to assess the dependency of Angiogenin modulation on DENV replication levels.

Following Ivermectin treatment, we observed a gradual decrease in DENV replication starting from 12 h post-infection (hpi). In parallel with the decrease in DENV replication, the levels of Angiogenin showed a similar pattern of reduction as compared to only Ivermectin treated cells in the absence of DENV infection (Figure 4 panel A). Comparing Angiogenin levels in IV/DMSO-treated cells without DENV infection, we found no significant changes with DMSO treatment, indicating that the observed expression levels of Angiogenin during the DENV infection experiment were specific to DENV replication. Moreover, we observed a similar progressive increase in DENV replication and Angiogenin levels over time in the DMSO-treated cells as that of untreated DENV infected Huh7 cells (Figure 4 panel B).

To assess the cytotoxic effects of Ivermectin and its impact on cell viability during DENV infection, a cytotoxicity assay was performed. Huh7 cells were treated with 50 µM Ivermectin for 4 h followed by DENV infection. Cell viability was assessed at 24 hpi (Figure 4 panel C), 48 hpi (Figure 4 panel D), and 72 hpi (Figure 4 panel E). The results revealed no significant difference in cell viability between Ivermectin-treated cells with or without DENV infection. This further emphasizes the importance of Ivermectin treatment in inhibiting DENV replication and subsequently modulating Angiogenin levels.

## Discussion

Dengue is one of the common arboviral diseases which poses a great threat to public health. Currently, there is no licenced antiviral drug against dengue. There is an urgent need for an effective therapeutic agent against dengue. DENV uses host machinery for its replication. Understanding various host-viral interactions is crucial for developing new therapeutic avenues against DENV. Despite the promising nature of several therapeutic drugs, questions regarding their effectiveness and potential toxicity have persisted.

Present and future therapeutic strategies should be focused on reduced host toxicity. Upon viral infection, multiple-signalling cascades are activated. This leads to secretion of various molecules to combat the infection. RNases are one of those molecules that are activated upon infection [44,45]. Therefore, they can be ideal candidates for targeting dengue without causing significant toxicity. Several studies have shown modulation of RNases

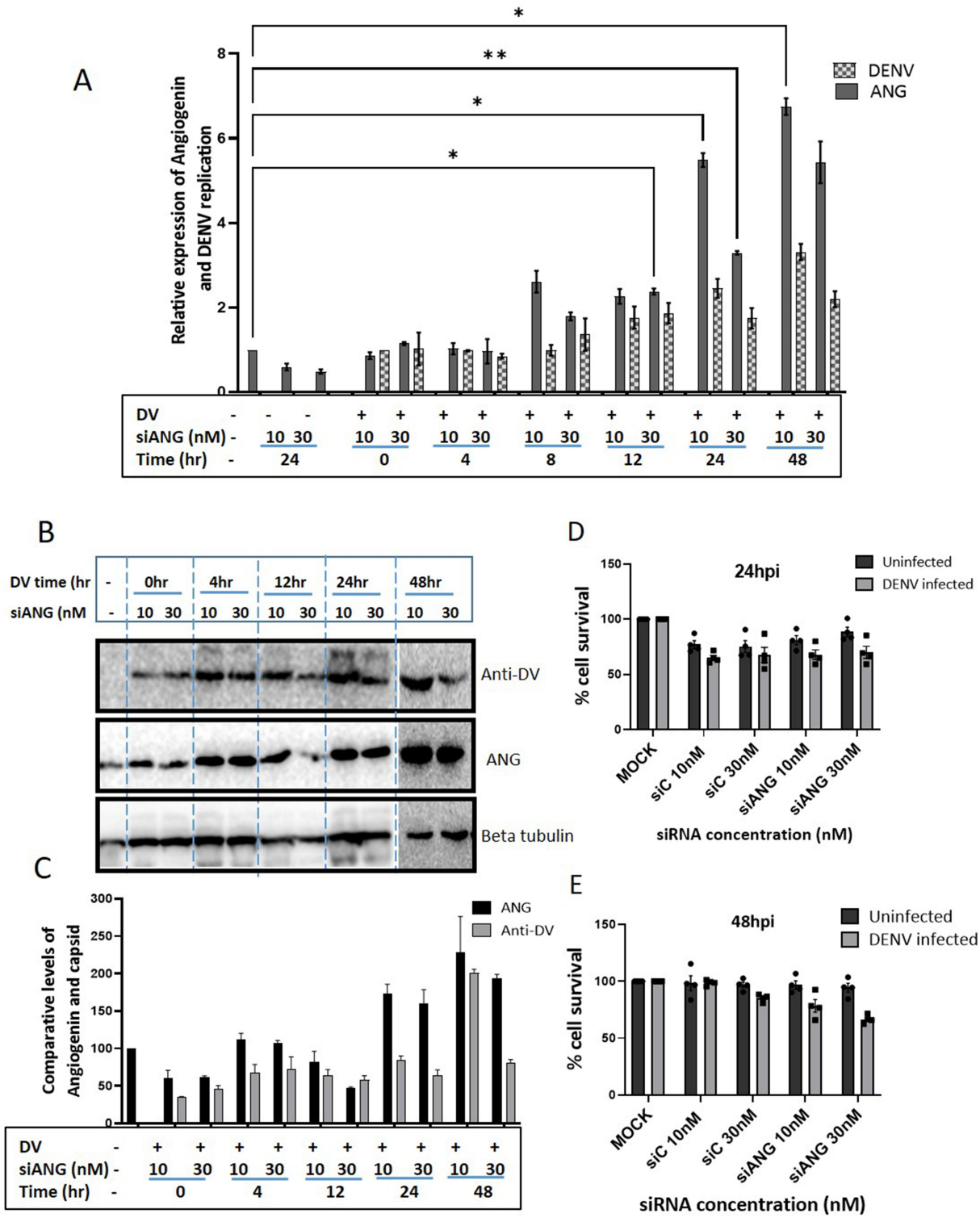


**Figure 3. Angiogenin expression at various time points during siRNA mediated knockdown of angiogenin in presence of DENV infection** (A) qPCR analysis: shows angiogenin levels upon DENV infection at different time points following 24hrs of angiogenin knockdown. The angiogenin levels were assessed at different time post infection (0 hr, 4 h, 8 h 12 h 24 h, 48 h). Angiogenin levels found to be increased immediately after DENV adsorption (0th hpi) and the levels further changes with the increasing time post infection. For further validation, (B and C) Western blot analysis was conducted, yielding similar results as the qPCR analysis. The levels of angiogenin were found to increase with the duration of DENV infection. Cytotoxicity assay of angiogenin knockdown cells and angiogenin knockdown (KD) followed by DENV infected cells. (D) cell cytotoxicity at 24hrs of angiogenin KD followed by 24hrs of DENV infection. (E) cell cytotoxicity at 24hrs of angiogenin KD followed by 48hrs of DENV infection.

qPCR analysis is the result of three individual experiments. Error bars show Mean±SD. Statistical analysis was performed using Two-way ANOVA (Bonferroni method). (*) shows P-value ≤0.05, (**) shows P-value ≤0.005. Western blots were performed twice for validating qPCR results. Cytotoxicity assay is the result of five individual experiments.

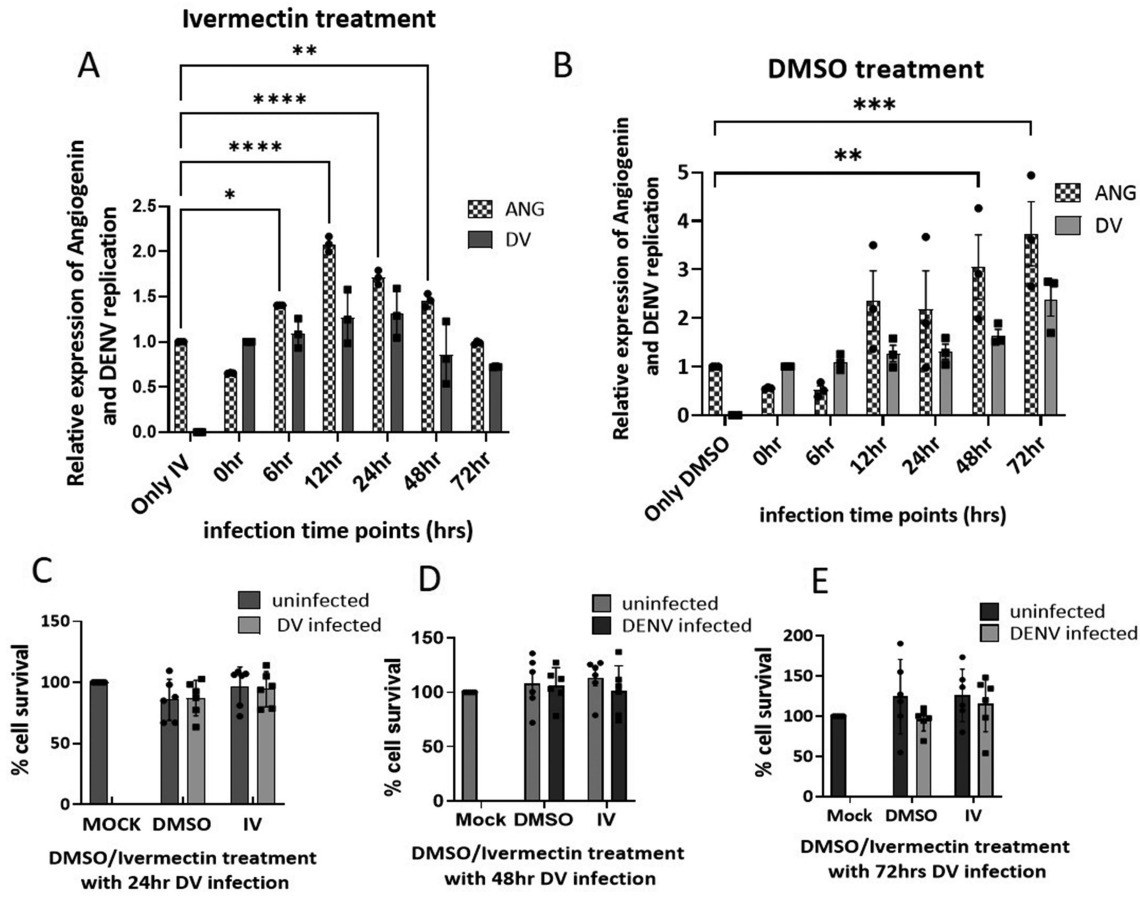


**Figure 4. Ivermectin treatment leads to a reduction in the DENV replication and the expression Angiogenin:** Huh7 cells were pre-treated with (A) 50 µM ivermectin (IV) and (B) DMSO for 4 h followed by DENV infection. Cells were harvested at different time points and the results were analysed with qRT-PCR. A decrease in the levels of angiogenin was observed with the decrease in DENV replication in case of ivermectin treated cells, while no effect was seen in DMSO treated cells. Cytotoxicity assay during IV/DMSO treatment with and without DENV infection was observed at (C) 24 hr (D) 48 hr and (E) 72 hr. Percent cell survival was found to be similar in IV/DMSO in presence of DENV infection as compared to only IV/DMSO treated condition.

qPCR shows the result of three individual experiments and Cytotoxicity assay is the result of five individual experiments. Error bars show Mean±SD. Statistical analysis was performed using Two-way ANOVA (Bonferroni method). (*) shows P-value ≤0.05, (**) shows P-value ≤0.005 (***) shows P-value ≤0.0005 (****) shows P-value ≤0.0001.

upon DENV infection. Following DENV infection, the expression of Wolbachia RNase HI was observed to be induced in mosquito cells [46]. RNaseHI degrades DENV ssRNA genome and thus reduces its replication. Reduced levels of RNaseHI under knockdown conditions were found to promote viral replication [46]. Modulation in RNases levels or activity might impact the viral RNA stability or affect the fidelity of viral RNA replication, which might also lead to mutations in the 3' UTR region [47–49]. Mutations occurring in the 3' untranslated region (3' UTR) have the potential to affect the production of sub-genomic flaviviral RNA (sfRNA) and its interaction with host proteins, which play a crucial role in facilitating effective viral replication [27].

The results of our study indicate an enhancement in Angiogenin levels during DENV infection. We investigated the kinetics of Angiogenin levels during DENV infection and observed that the levels of Angiogenin gradually increased at later time points of DENV infection. This indicates a potential role of Angiogenin during translation to replication switch and virus assembly. We observed that upon DENV infection, Angiogenin levels were reduced at the early stages of DENV replication, but after 4 h, its levels started to increase gradually (Figure 1 panel A-D). The increase in Angiogenin levels was observed only in the presence of live DENV infection and not with heat-inactivated virus (Figure 1 panel E and F). These results suggest that the changes in Angiogenin levels are solely due to DENV replication. We also performed angiogenin knockdown experiment at different siRNA concentrations and the knockdown efficiency of Angiogenin was evaluated at different time points. Our results demonstrate a consistent reduction of approximately 80% in Angiogenin levels after siANG transfection compared to siC transfection (Figure 2 panel A, B and C). Further experiments were performed to examine the impact of Angiogenin silencing on DENV replication in Huh7 cells. The results showed that siRNA-mediated knockdown of Angiogenin during DENV infection did not result in Angiogenin reduction but instead led to an upregulation of Angiogenin levels (Figure 3A-C). This suggests that Angiogenin may be transcriptionally regulated during DENV infection to an extent that predominates the silencing effect of the siRNA. Interestingly, the upregulation of Angiogenin was

observed in the presence of DENV infection but not in mock-infected cells (Figure 1). The increase in Angiogenin correlated with enhanced DENV replication. As discussed by Kato et al., DENV viral proteins are translated within 1–5 h post-viral infection, Viral RNA synthesis takes place after 5 h, and assembly and release occur after 12 h [50]. Therefore, the upregulation of Angiogenin at later stages of infection suggests that it might be playing role in the transition from viral translation to viral RNA synthesis and replication complex formation. Previous studies suggest that the host protein plays an important role during immune response, viral translation and replication switch. A study on DENV infected dendritic cells shows the upregulation of mitochondrial protein CMPK2 that regulates different immune responses [51]. Ray et al. (2011) on Hepatitis C Virus demonstrated that host factors La autoantigen proteins bind to HCV IRES which is responsible for HCV IRES-mediated translation. The protease domain of NS3 HCV protein competes with La protein to bind at the HCV IRES site. This competition regulates the switch from translational to replication HCV [52]. Another protein RBM24 has also been shown to interact with 3' and 5' UTR of HCV and inhibits IRES-mediated translation by preventing 60S ribosomal recruitment at IRES site whereas, it promotes HCV replication by linking 3' and 5' UTR [53]. These studies support the notion that the molecular switch of viral translation to replication can be regulated by host proteins. Based on our results, we suggest that modulation of Angiogenin may play a role in translation to the replication switch during DENV infection.

To further investigate the link between DENV replication and Angiogenin modulation, we performed ivermectin treatment followed by DENV infection using DMSO as a control. It has been reported that ivermectin inhibits DENV NS3 activity which functions as a helicase important for genome unwinding and further replication [19]. It has also been reported that ivermectin prevents nuclear localization of NS5 by targeting important host nuclear importins IMPa/β1 which are required for NS5 nuclear localization [21]. Role of NS5 in the host nucleus is not yet fully understood. However, a study has been shown that NS5 modulates cellular splicing events within the nucleus. NS5 interacts with the spliceosome complex, which leads to a dysregulation of pre-mRNA processing in the nucleus. In this way, DENV makes the host environment less hostile for its propagation and replication [24]. The localization of NS5 May play a critical role in DENV pathogenesis as all the four DENV serotypes have been shown a decrease in infection as a result of ivermectin treatment [17]. In a study by Yap et al. in 2007, it has been demonstrated that the antiviral activity of ivermectin is due to the action on importins, rather than inhibition of NS5 RdRp activity [54]. Following Ivermectin treatment, a decrease in DENV replication levels observed, which correlated with a decrease in Angiogenin levels. In contrast, no significant changes were observed in DENV replication and Angiogenin levels in cells treated with the control DMSO (Figure 4 panel A and B). These results suggest that Angiogenin levels are modulated in parallel with DENV replication.

The observed upregulation of Angiogenin during DENV infection raises the question about its potential role as a proviral factor, contributing to the replication and propagation of the virus. Angiogenin upregulation may facilitate DENV replication by promoting conditions favourable for viral propagation. It is possible that elevated levels of Angiogenin may create a microenvironment that supports viral replication, such as increased angiogenesis, or enhanced vascular permeability, which in turn could aid in viral dissemination [31,55]. Angiogenin is known to have implications in immune modulation, including effects on inflammatory responses and leukocyte migration. Its upregulation during DENV infection could influence the host immune response, potentially facilitating viral immune evasion or dampening antiviral defences, thus promoting viral persistence [56,57]. Angiogenin has been shown to interact with and recruit RNA-binding proteins to stress granules [34]. These RNA-binding proteins play a crucial role in RNA sequestration and translational repression within stress granules. Additionally, Angiogenin exhibits endoribonuclease activity and can cleave tRNA molecules during stress conditions. This cleavage results in the generation of small tiRNAs, which have been implicated in stress granule assembly and regulation of stress response pathways. Angiogenin has also been demonstrated to contribute to the translational repression of mRNAs during viral stress [34]. In Figure S3, we have provided an investigative overview of the potential roles of Angiogenin in the context of DENV replication.

Earlier studies have also reported the modulation of Angiogenin during viral infection. Design and co-workers performed gene expression profiling of Dengue Shock Syndrome (DSS) patient samples. They observed upregulation of repair/tissue remodelling genes and anti-inflammatory genes including Angiogenin [38]. Hanley and co-workers performed immune-transcriptome profiling in the DENV serotype 2 infection model and identified upregulation of certain genes after 28 days of DENV infection, referred as post-viraemia genes. Pathway analysis revealed that angiogenesis was one of the majorly affected pathways associated with the upregulation of post-viraemia genes [58]. Furthermore, studies have reported the role of Angiogenin during other viral infections. Cocchi and co-workers demonstrated the suppression of HIV replication by Angiogenin in a dose-dependent manner. They found that CD4+ and CD8+ T-cells produce anti-HIV factors like chemokines and RNases including Angiogenin and RNase4, which are effective HIV suppressors [57]. Wang et al. 2013 observed the upregulation of a tRNA half (tRF5-Glu-CTC), upon RSV infection in A549 cells. Knockdown analysis of Angiogenin, Dicer, Drosha, and ELAC2. revealed that downregulation of Angiogenin decreases the levels of tRF5-Glu-CTC significantly. Further study by Deng and co-workers showed that tRF5-Glu-CTC has a *trans*-silencing activity that targets APOER2 mRNA and thus leads to the suppression of the host immune system thereby promoting RSV replication [35,36,59]. In addition to Angiogenin, other small non coding RNAs such as miRNAs have also been identified to be differentially expressed during DENV infection [8,60–63]. For example, miR-146a, miR-21, miR-188, miR-152 and miR-590 have been reported to be upregulated in response to DENV infection. These miRNAs play roles in modulating the inflammatory response and

regulating the expression of immune-related genes. They can target key signalling molecules, such as IRAK1, TRAF6, and SOCS1, thereby influencing the host immune response to DENV infection [64]. Additionally, miRNAs have been found to directly target the DENV genome. For instance, miR-146a has been shown to target conserved regions within the DENV genome, leading to the inhibition of viral replication. This miRNA interferes with viral RNA translation and the assembly of the viral replication complex [65,66]. Differential expression of miRNAs, such as miR-146-5p, miR-126-5p and miR-122a-5p, have also been reported during DHF (Dengue haemorrhagic fever) [10]. These miRNAs are associated with liver pathogenesis in DHF and play roles in endothelial repair, regulation of vascular permeability, control of homoeostasis, and modulation of inflammatory cytokine expression [10]. Angiogenin has been reported to regulate the expression of miR-141 in endothelial cells, thereby influencing vascular permeability [67]. Another miRNA, miR-142, is known to target TIM-1, a type I cell-surface glycoprotein expressed on endothelial cells that plays a role in the internalization of the virus. miR-142 reduces TIM-1 expression and thus prevents the viral replication [68]. The dysregulation of angiogenin and miRNAs during DENV infection can have significant implications for disease progression. Angiogenin's involvement in promoting vascular leakage may contribute to the development of severe dengue manifestations, such as dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) [69]. Dysregulated miRNA expression can impact the antiviral response, immune regulation, and vascular integrity, influencing disease severity and outcome.

## Conclusion

In conclusion, our study reveals that Angiogenin levels are enhanced during DENV infection and that Angiogenin knockdown does not effectively reduce its expression during DENV infection. The results suggest a complex interplay between Angiogenin expression, DENV infection, and cellular responses. The findings also indicate that Angiogenin may play a role in DENV replication, highlighting the need for further investigation to elucidate the underlying molecular mechanisms involved.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Author contributions

D.M., S.M., and B.V. conceptualization; D.M., S.M., S.B., S.P., and B. V. methodology; D.M., S.M., S.B., and B.V. formal analysis; D.M., S.M., and B.V. investigation; D.M., S.M., R.K.S., and B.V. writing – original draft; B.V. supervision; B.V. funding acquisition.

## Data availability statement

All data described within the article are included in this article.

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