Dengue virus non structural protein 1 disrupts the TGF-β/Smad signaling by utilizing E3 ligase Smurf2

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Article

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Abstract

TGF-β signaling is tightly regulated to ensure cellular functions. Role of DENV on the TGF-β/Smad signaling has not been well established. Therefore, we aimed to study the association between DENV replication and TGF-β/Smad signaling. We observed impaired expression of Smad2/3/4 during DENV replication along with significant reduction in the expression of phosphorylated Smad3. Overexpression of Smad6/7 showed inhibitory effect on DENV replication. We observed DENV-NS1 not only physically interacts with Smad2/3/4 but also impaired their expression by utilizing Smurf2 E3 ligase. Co-immunoprecipitation of NS1 and Smurf2 suggests that NS1 may acts as a co-factor to escalate the lysosomal mediated degradation of Smads. Additionally we observed, NS1 is capable of blocking the nuclear translocation of Smad3 and thus further ensuring inhibition of Smad signaling. Therefore, our results confirm that DENV-NS1 interacts with Smads and reduces their expression that may favor in virus replication.

Introduction:

Transforming growth factor-β (TGF-β) is a multifunctional cytokine involved in immense number of biological activities. TGF-β is secreted by many cell types such as lymphocytes, macrophages and platelets. Presence of three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) have been reported in mammalian tissues. Among these, TGF-β1 is the most commonly expressed isoform. TGF-β signaling results in the activation of two different downstream pathways; Smad-dependent and Smad-independent pathways. TGF-β binds to a TGF-β type II receptor, which phosphorylates and heterodimerizes with a type I receptor. Afterward, type I receptor recruits and phosphorylates cytoplasmic receptor called regulated Smads (R-smads; Smad2 and Smad3) which makes complex with Smad4 (co-Smad). This whole complex translocates into nucleus and binds to its promoter sites as transcription factors to regulate the expression of target genes.

TGF-β has been shown to act as a growth activator of several cell types, including mesenchymal stem cells, fibroblasts, smooth muscle cells, endothelial cells and other different cell types. TGF-β works as double edged sword as it can induce cell proliferation (growth) or inhibition (apoptosis) on the same cells according to stimulus.

Certain viruses have been reported to modulate TGF-β pathway by utilizing viral factors to induce or suppress associated receptors and intermediary signaling molecules. Human Papilomavirus, a DNA virus disregulates TGF-β pathway by interrupting TGF-β type II receptor expression and nuclear translocation of p-Smad2/Smad4 complex. However, one of the RNA virus, influenza A virus utilizes a viral protein such as neuraminidase to induce TGF-β signaling and enhanced secondary bacterial infection by up-regulating different host factors.

Flaviviruses such as Hepatitis C virus (HCV) has been also reported to modulate TGF-β expression. NS5A, one of the non-structural protein of HCV has been observed to down regulate TGF-β expression via
blocking transcriptional activator for TGF-β gene expression, AP-1. NS3 of HCV inhibits the binding of Smad3 on its promoter site and suppressed its activation, while NS3-4A increases TGF-β signaling by inhibiting Smurf2, an E3 ligase that controls the activities of Smads.

Dengue virus (DENV) is one of the medically important flaviviruses of the Flaviviridae family responsible for growing global economic and disease burden in humans. DENV is mainly transmitted by mosquitoes of Aedes sp. Clinically apparent DENV infection leads to either mild Dengue Fever or severe Dengue Hemorrhagic Fever and dengue shock syndrome.

The viral particle encapsulates a positive strand RNA of ~11 kb that constitutes the viral genome, with 5’ and 3’ untranslated regions (UTR). Upon infection, the positive strand RNA is translated into a single polypeptide chain and processed into three structural (envelope, precursor-membrane/membrane and capsid) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by viral and host cell proteases. DENV uses all of them to not only replication but also block the host antiviral response and modulate the host-machinery in favor of virus propagation.

As TGF-β signaling is involved in majority of cellular processes, hence it could be a potential target for DENV. Many researchers have reported increased levels of TGF-β cytokine in DENV infection. So far, the mechanism of TGF-β signaling during DENV infection is not well understood. In this article, we studied the possible mechanism of TGF-β signaling modulation by dengue viral proteins.

Results

DENV infection disrupts TGF-β/Smad signaling by inhibiting phosphorylation and degrading Smad proteins

Smad2 and Smad3 are important members of receptor-activated Smads, upon activation of TGF-β signaling, Smad2/3 get phosphorylated and translocated into nucleus. So, we examined the phosphorylation status of these two Smad proteins in Huh-7 cells. Huh-7 cells were infected with DENV-2 strain NGC and observed the viral replication by specific qPCR at 24 and 48 hr post infection (hpi) (Fig. 1a). To check phosphorylation of Smad2 and Smad3, Huh-7 cells were infected with DENV followed by TGF-β treatment or left un-treated. TGF-β treated control cells were observed with higher expression of phosphorylated Smad2 and Smad3. A significant reduction was noted in the expression of phospho-Smad3 and no effect was observed on phosphorylation of Smad2 in DENV infection (p < 0.05, Fig. 1b and 1c). However, non-treated cells were noted with weak expression of phospho-Smad2/phospho-Smad3 in controls and only difference was observed with phospho-Smad3.

To further investigate, we used constructs of different Smads in HEK-293T cells to confirm their role in DENV infection. HEK-293T cells were infected with DENV-2 strain NGC and replication was noted by qPCR after 24 and 48 hr infection, (Fig. 1d). HEK-293T cells were transfected with constructs of Smad2 and Smad3 and 24 h after transfection, these cells were infected with DENV and maintained till 48 hpi.
Western blot analysis revealed expression of Smad2 and Smad3 was significantly disrupted by DENV infection at 48 hpi when compared with mock infected cells (p < 0.05 and p < 0.01; Fig. 1e and 1f). Smad signaling and regulation requires other assessor proteins such as Smad4 (co-smad), Smad6 and Smad7 (inhibitory smads). We utilized constructs of these Smad proteins similar as Smad2/3 experiments in HEK-293T cells with DENV infection. The western blot analysis of control and infected HEK-293T cells revealed significant reduction in the expression of Smad4, Smad6 and Smad7 at 48 h post DENV infection (p < 0.05; Fig. 1e and 1f). However, the gene expression analysis could not reflect any change in the Smad transcription level with DENV replication (Supplementary Fig. 1). Overall, these results showed that DENV was clearly impairing the TGF-β signaling pathway mostly at 48 hpi.

**Effect Of Smads On DENV Replication**

To establish the effect of different Smads on DENV replication, we checked for viral load in the supernatants of DENV infected Smad overexpressing HEK-293T cells. qPCR data showed significant reduction in viral replication only with Smad6 and Smad7 over expressing cells (p < 0.003), while no significant change was noted in the viral load with Smad2, Smad3 and Smad4 (Fig. 2a). Therefore we believe that inhibitory Smads i.e. Smad6 and Smad7 can potentially reduce the viral replication.

**Dengue NS1 Protein Inhibits TGF-β signalling by reducing the Smad3 and Smad4 expression**

DENV replication derailed the TGF-β/Smad signaling pathway by impairing the expression of Smad proteins. Several viral proteins have been known for their interfering role in the TGF-β/Smad signaling pathway\(^{10,11,17}\). We used two DENV non-structural proteins, NS1 and NS5, as they have been reported to interact with different host proteins\(^{18}\) to find out whether NS1 or NS5 have any role in the TGF-β/Smad pathway. To achieve the same, NS1 or NS5 protein was co-expressed with other Smad proteins i.e. Smad2, Smad3, Smad4, Smad6 or Smad7 in HEK-293T cells. We observed significant reduction in the expression of all Smads when co-expressed with NS1 (p < 0.05, Fig. 2b and 2c) while NS5 had no effect on these proteins (Supplementary Fig. 2).

**E3 Ubiquitin ligase Smurf2 is required for regulation of R/Co-Smads and I-Smads during DENV infection**

Smad proteins have been regulated by E3 ligases. In our case we hypothesized that either smurf2 or CHIP has a significant role in DENV infection. NS1 and Smad protein constructs were co-transfected with Smurf2 or CHIP in HEK-293T cells. Western blot analysis revealed that Smurf2 has significant role in degradation of Smad3, Smad4, Smad6 and Smad7 and lesser effect on Smad2 (Fig. 3a). However, no difference was observed with CHIP co-transfection (Fig. 3b).

**CHIP Knockdown Increases The Stability Of Smad Proteins**
We utilized CHIP knockdown HEK-293T cells to see the effect on the expression of Smads with or without NS1. CHIP knockdown cells were prepared by using CRISPER-Cas9 method\textsuperscript{19}. Smad2, Smad3, Smad4 and NS1 were co-expressed in the CHIP\textsuperscript{-/-} HEK-293T cells. We observed that higher expression of Smad3 and Smad4 expression in CHIP\textsuperscript{-/-} cells and NS1 had less effect on their expression with these cells (Fig. 3c).

To determine the effect of NS1 on the degradation kinetics of the Smad proteins, we carried out the CHX chase assay in Smad3/4 and NS1-transfected cells. In the presence of NS1, Smad3 and Smad4 exhibited not so significant degradation by 10 h post CHX treatment, while protein levels of these Smads were maintained when transfected alone (Supplementary Fig. 3).

**Lysosome mediated degradation of Smad3 and Smad4 by dengue virus NS1**

Molecular basis of degradation of Smad3 and Smad4 by NS1 was either associated with proteasomal or lysosomal mechanism. To understand the same we utilized inhibitors of these pathways, i.e., MG132 and chloroquine. Smad and NS1 co-transfected cells were treated with MG132 and chloroquine for 8 hrs prior harvesting and investigated levels of Smads by immunoblotting.

Treatment of transiently transfected HEK-293T cells with increasing concentrations of the proteasome inhibitor MG132 could not restore the expression of Smad3 and Smad4 (Fig. 3d). On the other hand, we observed some restoration in the expression of Smad3 and Smad4 protein even in the presence of NS1 with lysosomal inhibitor chloroquine, suggesting NS1 exploits the lysosomal pathway to lower the expression of these Smad proteins (Fig. 3e).

**NS1 Interacts With Smad2, Smad3 And Smad4 Complex**

Smad2 and Smad3 form complex with Smad4 and translocated in to nucleus. To find out the physical interaction of Smad proteins and DENV NS1, we performed co-immunoprecipitation (co-IP) assay by using Smad proteins or NS1 as bait. Our co-IP data suggested that NS1 physically interacts with Smad proteins. We observed increase in degradation of these Smad proteins with NS1 when co-expressed with Smurf2. Therefore, we wanted to know whether smurf2 is also interacting with NS1. Co-IP of NS1 and Smurf2 verified their interactions. Interaction of NS1 with Smad proteins and Smurf2 complex may inhibit the TGF-β/Smad signaling pathway (Fig. 4a).

**Dengue Virus NS1 Blocks The Nuclear Translocation Of Smad3**

Nuclear translocation of Smad proteins is essential step of TGF-β/Smad signaling. We confirmed the interaction of NS1 with Smad2 and Smad3. So we examined whether NS1 altered nuclear translocation of these Smad proteins. Cells expressing Smad2 and Smad3 in the absence or presence of NS1 were
fractionated into distinct cytoplasmic and nuclear fractions and analyzed by western blot. We have observed reduction in Smad3 protein level in nucleus, when co-expressed with NS1 suggesting the possible inhibition of Smad3 nuclear translocation by NS1 (Fig. 4b).

**Discussion:**

TGF-β/Smad signaling in viral infection has been widely explored earlier. This is a crucial host-defense mechanism against viruses. Different viruses and their proteins were found to abrupt the signaling by interacting with downstream signaling molecules such as TGF-β receptors and Smad proteins. In DENV infection, the role of Smad proteins and their interaction with dengue viral proteins has not been studied. Therefore, we have observed the effect of DENV infection on TGF-β signaling molecules.

We found reduced expression of phosphorylated Smad3 during DENV infection. Virus was also significantly disrupting the normal Smad3 expression. Downregulation of co-Smads was used as a strategy by some viruses to improve their survival. Respiratory syncytial virus inhibits the Smad2/3 signaling in macrophages to block interferon secretion\(^\text{20}\). Hepatitis C virus (HCV) protein NS3, NS5 or core interact with Smad2/3 and block the phosphorylation and function of these Smad proteins\(^\text{11,21}\). Herpes Simplex virus, human cytomegalovirus and HIV inhibits the Smad3 activity to maintain their survival in different cells\(^\text{22–24}\). Not all viruses require suppression of Smad2/3 activities, Rift Valley fever virus requires the phosphorylation of these proteins for its own replication\(^\text{25}\). In other report influenza virus activates the TGF-β signaling to promote secondary bacterial infection\(^\text{9}\).

So, we were curious to know what will be the fate of the co-Smad Smad4 involved in the Smad signaling during DENV replication. Over expression of Smad4 in HEK-293T cells followed by DENV infection resulted in high level of its degradation. Similar results had been observed with Human T-cell leukemia virus type 1 that blocked Smad3 and Smad4 binding with respective promoters to activate the transcription of associate genes\(^\text{26}\). Likewise, BARF1 protein of Epstein-Barr virus downregulated Smad4 to promote cell proliferation and virus induced stomach cancer progression\(^\text{17}\).

In TGF-β signaling the activity of Smad2/3/4 is regulated by inhibitory Smads, i.e. Smad6 and Smad7. Differential expression of Smad6 and Smad7 were observed with viral infection. These inhibitory Smads had impeding effect on virus replication. This could be the key for developing antiviral therapy against DENV infection. Unlike the DENV infection, Smad6/7 induces expression of different receptors i.e. SDC1 and LDLR, that promote the HCV replication\(^\text{27}\).

Not only in virus replication, the viral non-structural proteins evade the host response by interacting with host proteins. Different RNA and DNA viruses use their viral proteins to evade TGF-β signaling by blocking the Smad complex\(^\text{11,21,17}\). DENV NS1 and NS5 have been known to its multifunctional nature\(^\text{18}\). Over expression of these Smads with NS1 results their degradation at different levels but not with NS5. Since NS1 does not possess ubiquitin function, it must do so by taking help from ubiquitinins involved in TGF-β signaling. A handful of ubiquitinins such as Smurf2 and CHIP/Stub1 are known to interact with
Smad family proteins\textsuperscript{12,28−29}. Our results confirmed that these E3 ligases have different preferences during DENV replication and NS1 expression. CHIP and Smurf2 has been reported for their specific regulation of Smad proteins in different environments\textsuperscript{28}. However, CHIP\textsuperscript{−/−} cells showed higher expression of Smad3 and Smad4 suggesting constant requirement of CHIP in maintaining their basal level in normal condition. Next we found that these E3 ligases utilize the lysosomal pathway. Utilization of both proteosomal and lysosomal mediated pathway by E3 ligases in enveloped/RNA virus infection is well reported\textsuperscript{19,30}. Utilization of such selective pathways during infection may be the one of the biological signatures of DENV pathogenesis.

Reduction in expression of Smad proteins in the presence of NS1 allowed us to look for the physical interaction between NS1, Smads and E3 ligases. Our co-IP experimentation established the physical interaction between E3 ligases and Smads which was facilitated by NS1 as a connecting bridge. Further experimentation showed lower level of translocation of Smads into nucleus due to NS1 suggesting abrogation of Smad signalling. Likewise, NS5 of HCV have been earlier reported to block the translocation of Smad proteins into nucleus\textsuperscript{21}.

DENV uses its non-structural proteins to evade the host defense. This study has demonstrated the novel regulatory role of NS1 in TGF-\(\beta\)/Smad signaling and confirmed that DENV NS1 utilizes E3 ligase Smurf2/CHIP to regulate expression of regulatory, co and inhibitory Smads during DENV infection. We have observed another evidence of multifunctional viral protein NS1 playing as an antagonist of host-defense mechanism as depicted in Fig. 5. Thus NS1 would be a perfect target for developing novel therapeutics against DENV infection.

**Methods:**

**Cell culture and virus propagation.** Dengue virus serotype 2 (DENV-2) strain New Guinea C (NGC) was used in our experiments, prepared by propagating in C6/36 cells. These cells were maintained in L-15 medium (Gibco Life Technologies), supplemented with fetal bovine serum and antibiotic antimycotic solution (Himedia Laboratories). Virus containing cell supernatants were harvested upon observation of cytopathic effect or physical changes in the cells, filtered and stored at -70\(\degree\)C till further use. Virus stocks were titered by plaque assays using BHK21 cells.

The STUB1/CHIP gene in HEK293T cells was knocked out with CRISPR-Cas9 plasmid containing CHIP sgRNA as described previously\textsuperscript{19,31}. DNA oligonucleotides were synthesized by using the sgRNA designer tool available in the web interface of Broad Institute, USA from cDNA sequence of CHIP. Source of control sgRNA was firefly (\textit{Photinus pyralis}) luciferase gene (**Supplementary Table S1**). Oligonucleotides were mixed with phosphonucleotide kinase enzyme reagent mix and kept for 40 min at 37\(\degree\)C. Reaction was stopped by adding 0.1M NaCl and incubated at 65\(\degree\)C for 20 min. Oligonucleotides were annealed by boiling the mix for 5 min at 100 \(\degree\)C and allowed the mix to bring to room temperature. BbsI digested pSpCas9 (BB)-2A-GFP (pX458) plasmid was mixed with reaction mix for ligation with annealed oligos and transformed in to \textit{E. coli} to propagate the plasmid. Clone was confirmed by Sanger sequencing and
used to transfect HEK-293T cells. The said plasmid has GFP protein to sort the positive cells by sorter (BD FACS Aria-II). Sorted cells were maintained in DMEM containing 10% FBS and antibiotics and gene knockdown was checked by western blot.

**Transfections and plasmids.** Transfections were performed using linear Polyethyleneimine (MW 25,000; Polysciences Inc., USA) reagents in accordance with the manufacturer’s instructions. Addgene plasmids pCMV5B-HA-Smad2 (# 11734), pCMV5B-Flag-Smad3 (# 11742), pCMV5-Smad7-HA (# 11733), pCMV5B-Flag-Smurf2 wt (# 11746), pCMV5B-Flag-Smurf2 C716A (# 11747) were gifts from Dr. Jeffrey Wrana. Two addgene plasmids; pcDNA Flag-Smad4M (# 14959) and CS2 HA-Smad6 (# 14962) were gifts from Joan Massague. DENV NS1 encoding plasmid (pCDNA 3.1-His-NS1) was kindly provided by Dr. Ronaldo Mohana-Borges, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil. Dr. Chia-Yi Yu, Department of Microbiology, College of Medicine, National Cheng Kung University, Taiwan kindly provided DENV NS5 encoding Plasmid (pCAG-HA-NS5).

**Western blotting and antibodies.** Supernatant of lysed HEK-293T cells were resuspended in SDS sample loading buffer, resolved on 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Cat # SCNJ8101XXXX101, MDI, Advanced Microdevices Ltd., India). Membrane was blocked with nonfat milk or BSA and probed with different specific primary antibodies, followed by probing with respective HRP-conjugated secondary Abs (Jackson ImmunoResearch, USA). Membrane was washed and developed using chemiluminescence solutions.

Anti-Smad2 (cat#5339), phospho-SMAD2 (Ser465/Ser467; cat#18338), anti phospho-Smad3 antibody (Ser423/425; cat#9520), anti-Lamin A/C antibody (cat#2032) antibodies were obtained from Cell Signaling Technology; anti-GAPDH antibody (cat#Sc-32233) from Santa Cruz Biotechnology; anti-HA tag antibody (cat #M1001010), anti-His tag antibody (cat#M1001020) from Immunotag/G-Biosciences and anti-FLAG® M2 antibody (cat #F1804), anti-DENV NS1 antibody (cat# SAB2702308) from Sigma-Aldrich.

**Cycloheximide chase assay.** Degradation kinetics of Smad proteins was studied by cycloheximide chase assay. Briefly, HEK-293T cells were transfected with respective plasmids for 24 h and then treated with Cycloheximide (cat #239764, Sigma-Aldrich). Cell lysates were prepared at different time points (0,2,4,6,8 and 10 h) and analyzed by immunoblotting.

**Activators and inhibitors.** Human Transforming Growth Factor-beta 1 (#TC298; Himedia) was used to stimulate Smad signaling. Lysosomal function inhibitor chloroquine (#C6628, Sigma-Aldrich) and MG132 (#C2211, Sigma-Aldrich), a membrane permeable proteasome inhibitor were used to treat HEK-293T cells for 8 hours prior harvesting for immunoblotting.

**Preparation of nuclear and cytoplasmic extracts.** Nuclear and cytoplasmic fractions were prepared from cells transfected with different plasmids of interest using Nuclear and Cytoplasmic Extraction Kit (G-Biosciences, USA) as per manufacturer's instructions and subjected to immunoblotting. In our experiments, Lamin A/C and GAPDH were used as a nuclear and cytosolic loading control respectively.
Gene expression analysis. Total cellular RNA was extracted from DENV-2 infected or control HEK293T cells using RNeasy Mini Kit (Qiagen) as per the manufacturer’s instructions. The quantity and quality of RNA were determined on a Nanodrop instrument (ThermoFisher Scientific). 500 ng of RNA was subjected to cDNA synthesis by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative Real time PCR was performed on cDNA samples using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) on a ABI 7500 Fast Real Time PCR system (Applied Biosystems) as described in manufacturer’s protocol. Set of primers used in this study were described in Supplementary Table 2. Gene expression analysis was performed by ddCt method and fold changes were calculated.

Quantitative real-time PCR for viral replication. Viral RNA was extracted from cell supernatants harvested from DENV infected HEK-293T cells using QIAamp Viral RNA Mini Kit as per manufacturer instructions (Qiagen). Eluted RNA samples were subjected to qRT-PCR with very specific primers and probe of DENV-2 capsid region and TaqMan fast virus one step master mix (Applied Biosystems) as described previously\(^3^3\). Estimation of viral RNA (copies/mL) was carried out by using the standard curve generated from DENV-2 transcripts.

Statistical analysis. Western blot images were analyzed by using imageJ software. All results represented here as mean ± standard error obtained from three independent experiments. For the calculation of fold changes GAPDH was used as endogenous control. Statistical significance was calculated by using student t test and data was considered significant if p < 0.05.

Declarations

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Author contributions

A.L., R.P.A and A.C.B. conceived and designed the experiments, analyzed data and wrote the manuscript. A.L., R.P.A. and V.K. performed the experiments.

Competing interests

The authors declare no competing interests.

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**Figures**
Figure 1

DENV interacts with Smad proteins and impaired their expression. (a) Huh-7 cells were infected with DENV (1 MOI) and virus replication was assessed at 24 and 48 hr post infection by quantitative RT-PCR. (b) DENV infected Huh-7 cells were treated with TGF-β or left untreated. Expression of phosphorylated Smad2/3 in DENV infected cells was observed by western blot. (c) Expression of p-Smad2/3 showed as column graphs (mean±SE). (d) HEK-293T cells were infected with DENV (1 MOI) and virus proliferation was monitored by quantitative RT-PCR at 24 and 48 hr post infection. (d) Reduced expression of Smad2, Smad3, Smad4, Smad6 and Smad7 was noted following transfection with individual Smad constructs and DENV infection (1 MOI) in HEK-293T cells. (e) Presented as column graphs (mean±SE).
Figure 2

Effect of DENV infection on Smads: a) Identification of Smads that inhibit DENV replication. HEK-293T cells (1×10^5) were seeded on 6-well plates and the next day transfected with Smad constructs (Smads 2, 3, 4, 6 and 7) or left untransfected. At 24h post-transfection, cells were infected with 1 MOI of DENV. Viral RNA copy number was determined from isolated supernatants collected at 48hr post-infection by qRT-PCR. Smad6 and Smad7 were found in correlation with reduction in DENV RNA copy number. (b) DENV-NS1 is responsible for degradation of Smad proteins. HEK-293T cells were transfected with DENV NS1 and any one of the Smad constructs (Smad2/3/4/6/7). Western blot analysis reveals that the degradation is NS1 mediated. All the blots are representative of three independent experiments. (c) Representative image of DENV-NS1 expression following co-transfection with individual Smads in HEK-293T cells.
Figure 3

The ubiquitin ligases Smurf2 and CHIP assists in DENV-NS1 mediated degradation of Smads (a) HEK-293T cells were co-transfected with 3 constructs, i.e., DENV-NS1, Smurf2 and Smad2/3/4/6/7 and harvested at 48h followed by western blot analysis. Densitometry measurements indicate that Smurf2 has connection in degradation of Smad3, Smad4, Smad6 and Smad7, while leaving smad2 less affected. (b) Effect of CHIP in the expression of Smad2, Smad3 and Smad4. Smad2/3 or Smad4 was transfected with CHIP construct in HEK-293T cells. Co-expression of CHIP did not change the normal level of expression of Smad2, Smad3 and Smad4. Smad2/3 or Smad4 was transfected with CHIP construct in HEK-293T cells. Co-expression of CHIP did not change the normal level of expression of Smad2, Smad3 and Smad4. Smad2/3 or Smad4 was transfected with CHIP construct in HEK-293T cells. Co-expression of CHIP did not change the normal level of expression of Smad2, Smad3 and Smad4. (c) Expression of Smad2, Smad3 and Smad4 in CHIP knockdown HEK-293T cells. CHIP knockdown cells showed higher expression of Smad3 and Smad4 and had no effect of NS1. (d) HEK-293T cells were transfected with Smad2/3/4 alone or with DENV NS1; at 36h post-transfection, cells were treated with 20 and 40 uM MG132 and (e) lysosomal inhibitor Chloroquine (100uM) for 8h followed by western blotting. Only chloroquine treatment reduced the effect of NS1.
Figure 4

Co-immunoprecipitation of DENV-NS1 with Smad2/3/4 and Smurf2. (a) His-NS1 and Smad2/3/4 or Smurf2 co-expressed in HEK-293T cells for 48 hr. Cells were lysed and supernatant was subjected with Ni-NTA resin. His-tagged NS1 bounded proteins were released from the cell lysate during elution. The eluted samples were subjected for western blot and probed for Smad or smurf2 proteins. Western blot analysis showed binding of Smad2/3/4 or smurf2 with NS1. (b) Western blotting of nuclear and cytoplasmic extracts of HEK-293T cells transfected with DENV NS1 and respective Smads. Lamin A/C and GAPDH were used as markers for nuclear and cytoplasmic extract respectively. DENV NS1 blocks the nuclear translocation of Smad3.
Figure 5

The graphic represents the events during dengue virus infection in TGF-β signalling. (1) TGF-β binds to its receptors and the receptors activate the downstream signalling cascades. TGF-βRI and TGF-βRII phosphorylate Smad2/3. (2) p-Smad2/p-Smad3 binds with Smad4. (3) This complex goes into nucleus and binds with specific site of DNA for transcription. (4) DENV enters into the cells and releases its RNA genome for translation of viral structural and non-structural proteins and their replication. (5) NS1 blocks the phosphorylated Smad2/3 and (6) binds with Smad4, (7) binds with Smad2/3/4/Smurf2 complex for (8) lysosomal degradation. Smurf2 an E3 ligase, interacts with Smad2/3 and Smad4. NS1 interacts with Smad6 and Smad7. (10) The Smad6/7 over-expression reduced the replication of dengue virus.

Supplementary Files

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