ROLE OF HUMAN TYROSINE KINASES IN DENGUE VIRUS LIFE-CYCLE

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Certificate

This is to certify that this dissertation entitled “Role of human tyrosine kinases in dengue virus life-cycle” submitted towards the fulfilment of the BS-MS dual degree programme at the Indian Institute of Research and Science Education, Pune represents the original research work carried out by “Yovhan V. Landge” at the Translational Health Science and Technology Institute, Faridabad under the supervision of “Dr. Guruprasad Medigeshi” during the academic year 2014-2015.

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Declaration

I hereby declare that the matter embodied in the report entitled “Role of human tyrosine kinases in dengue virus life-cycle” are the results of the investigations carried out by me at, Translational Health Science and Technology Institute, under the supervision of “Dr. Guruprasad Medigeshi” and the same has not been submitted elsewhere for any other degree.

Yovhan V. Landge

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Abstract

Dengue virus is a pathogen of global concern and infects about 400 million humans worldwide. Host cellular factors play an essential role at every stage of dengue life-cycle, nevertheless, very few of cellular factors that are necessary for dengue virus infection have been identified. We used a human tyrosine kinase siRNA library to identify tyrosine kinases involved in dengue virus life-cycle. C-terminal Src kinase (csk) was one of the positive hits identified in primary siRNA screening. Knock-down of csk by specific siRNA reduced dengue virus titers in the supernatants. Inhibition of csk by a chemical inhibitor reduced dengue virus titers at 24 hrs post infection. Dengue infection led to increase in the phosphorylation of csk suggesting activation of csk during infection. Csk knock-down by siRNAs also led to reduction in replication of Japanese encephalitis virus a related member of flavivirus genus suggesting that csk is a host factor that is involved at later stages of flavivirus life-cycle.

Introduction

Dengue virus (DENV) is a vector transmitted RNA virus belonging to genus flavivirus in *Flaviviridae* family. The genus comprises of around 77 species which includes important pathogens like West Nile virus (WNV), Japanese encephalitis virus (JEV) and Yellow fever virus (YFV). *Aedes aegypti* and *Aedes albopictus* are primary mosquito vectors of DENV. Currently there are four distinct serotypes; DENV-1, DENV-2, DENV-3 and DENV-4. Most of these serotypes co-exist in several tropical and subtropical regions of the world. Humans are infected upon being bitten by dengue carrier mosquito.

Since last few decades, dengue disease is extremely progressive in more than 100 countries with frequent outbreaks. As per recent investigations and the epidemiological model, 2.5 billion people are at risk of primary infection, which may result in approximately 20,000 deaths annually (Bhatt et al., 2013). Currently there are no drugs or vaccines specifically targeting DENV.

Genome and proteins of DENV

Genome of dengue virus is a single stranded 11 kb long RNA molecule which can be directly transcribed to a single polyprotein. It has type I cap at 5' end while there is no 3' polyadenylation. At the 5' end, first few hundred nucleotides termed as 5' untranslated region (UTR) which are non-coding and highly structured along with regulatory elements of viral replication and translation. Similarly, conserved RNA elements in the 3'UTR mediate viral genome replication. DENV genome has a single open reading frame encoding a single polyprotein. Further, this polyprotein is processed into three structural proteins (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

Replication cycle of DENV:

DENV can replicate in both human and mosquito hosts. DENV carrier mosquitoes inject the virus from salivary glands to blood stream of humans during feeding. DENV can infect mammalian cells like B cells, T cells, endothelial cells, neuronal cells, hepatocytes, monocytes, macrophages and dendritic cells. DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) (Lozach et al., 2005), and mannose receptors in macrophages (Miller et al., 2008) are some of receptor proteins to promote dengue infection. Recently TIM (T-cell immunoglobulin domain

and mucin domain) and TAM (Tyro3, Axl and Mer) were reported to mediate entry of DENV (Meertens et al., 2012). Intracellular life cycle of dengue virus can be depicted as shown in Figure 1 and described below.

The receptor-bound dengue virus is internalized by endocytosis (van der Schaar et al., 2008) and low pH in endosome triggers structural rearrangement of E protein resulting in fusion of viral envelope with endosomal membrane leading to the release of nucleocapsid into cytoplasm (Zaitseva, Yang, Melikov, Pourmal, & Chernomordik, 2010). After disassembly of nucleocapsid, dengue genome is translated by ER-associated ribosomes (Selisko, Wang, Harris, & Canard, 2014). The genome has a single open reading frame encoding a single polyprotein (~3400 amino acids) which is further co- and post-translationally processed by cellular as well as viral proteases. This polyprotein is processed into three structural proteins (C, prM/M, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Structural proteins are part of virions. NS5 along with other viral non-structural proteins and presumably various host proteins forms replication complex within an intracellular membrane structure induced by virus. Viral RNA polymerase (NS5) transcribes viral RNA to produce negative sense strand which serves as template for synthesis of subsequent viral RNA copies. This newly synthesized viral RNA can be used for translation or packaged into virus particles.

During assembly the nucleocapsid buds into the lumen of the endoplasmic reticulum (ER) acquiring the envelope and formulates the immature dengue virus particles. The E protein in immature virions is prevented from premature-fusion by formation of a heterodimer with prM protein. This immature particle is transported through the classic secretory pathway. During this transportation virion maturation is assisted by furin, an endoprotease residing in *trans*-Golgi network, which processes prM to mature M protein (Boon & Ahlquist, 2010) and virus particles were secreted.

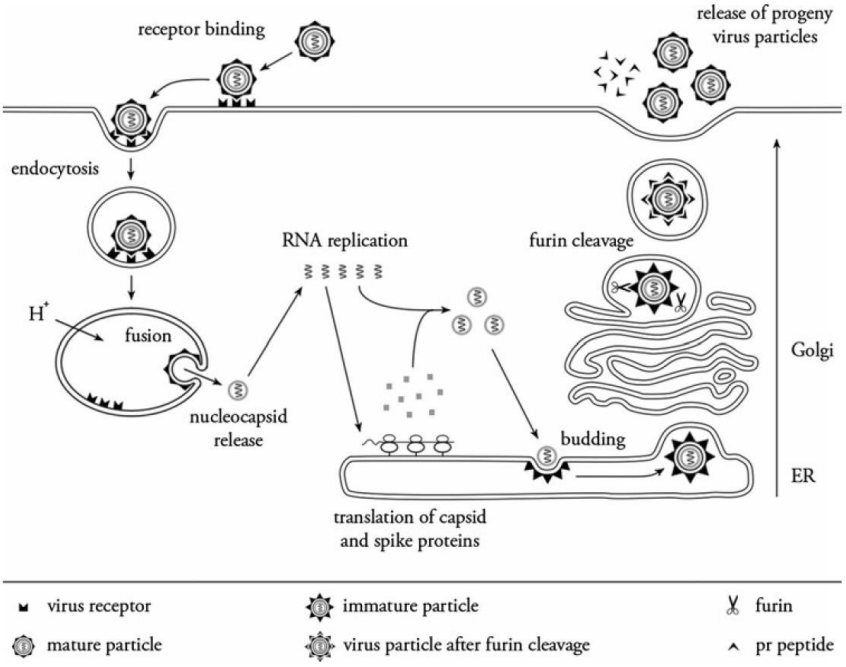

Figure 1. Intracellular life cycle of dengue (Rodenhuis-Zybert, Wilschut, & Smit, 2010)

Host proteins in dengue life-cycle:

Viruses are intracellular parasites. They depend entirely on host for survival. Viruses, during their long association with hosts, have evolved sophisticated methods to exploit host resources for their propagation and subvert the antiviral defence mounted by the host cell. Depending on the nature of virus-host interaction the cellular proteins can assist or suppress viral replication. Some of the host proteins identified in dengue virus life-cycle include human sec3 protein (Bhuvanakantham & Ng, 2013) , adaptor protein complex-1 and 3 (Agrawal et.al. 2013), UBE21, CALR, GOLGA2 and TRIP11 (Mairiang et al., 2013). However, only limited investigations have been carried out to elucidate, in detail the role played by cellular proteins in dengue life-cycle. More information in this field can help to develop a better understanding of virus biology and device novel therapeutic interventions.

Tyrosine kinases

Cellular signalling involving phosphorylation of proteins regulates cellular processes like cell motility, proliferation, differentiation, cell survival, cell adhesion and cell cycle. Protein tyrosine Kinases (PTKs) are one such class of kinases that catalyse the transfer of γ phosphate from ATP to hydroxyl group of specific tyrosine residue on substrate proteins (Hunter, 1998). Depending on cellular localization,

tyrosine kinases can be classified into receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs). RTK, embedded in cell membrane, are characterized by a ligand binding extracellular domain, a single transmembrane α-helix and a cytoplasmic region consisting of a tyrosine kinase domain with C-terminal and juxtamembrane regulators (Sandilands & Frame, 2008). RTKs are activated by binding of a ligand that induces conformational changes in receptor. Autophosphorylation of tyrosine within the cytoplasmic region provides docking sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains of downstream signalling proteins (Hubbard & Miller, 2007). On the other hand, non-receptor tyrosine kinases (nRTKs) are cytoplasmic, that are characterized by SH2, SH3, Pleckstrin Homology (PH) and catalytic domains (Pawson, 1995). SH2 and SH3 domains mediate binding to substrate by determining motifs bearing phosphotyrosine and proline-rich peptides respectively. PH is associated with membranes while catalytic domain has phosphotransferase activity.

It has been identified that human tyrosine kinases play a role in the life-cycle of several flaviviruses. Inhibition of c-Src hinders assembly of DENV virion (Chu & Yang, 2007). Csk and Janus kinase 1 were shown to play a significant role in replication of HCV (Supekova et al., 2008). Furthermore, necessity of c-Yes in maturation of WNV has been described (Hirsch et al., 2005). Thus, it appears that tyrosine kinase-mediated signalling pathways could play a significant role at various stages of flavivirus replication. However, understanding the role of tyrosine kinasess in flavivirus infection requires further characterization.

Material and Methods

Materials:

Antibodies:

Table 1. Primary antibodies used in this study.

Antibody for	Raised in	Dilution to be used
Tubulin	Mouse	1:10000
Csk	Rabbit	1:1000
DENV Capsid	Rabbit	1:1000
DENV NS3	Rabbit	1:1000
β-actin	Mouse	1:10000
GAPDH	Rabbit	1:10000
Anti-FLAG epitope	Rabbit	1:1000

Table 2. Secondary antibodies used in this study.

Antibody	Conjugated to	Raised in	Dilution to be used
Goat anti-rabbit	Horseradish peroxide (HRP)	Goat	1:2000
Goat anti-mouse	Horseradish peroxide (HRP)	Goat	1:2000
Goat anti-mouse	Alkaline phosphatase (ALP)	Goat	1:2000

Reagents:

TRIzol reagent, chloroform, isopropanol, , ethanol, mounting antifade reagent, methanol, RNase-free glycogen, Opti-MEM media, RNAimax, Lipofectamine™ 2000 formaldehyde, crystal violet stain, bovine serum albumin (BSA), heat inactivated fetal bovine serum (FBS), trypsin, Penicilin-streptomycin-glutamine (PSG), non-essential amino acids (NEAA), 0.5% carboxy methyl cellulose (CMC), Minimum Essential Media (MEM), Dulbecco's Modified Eagle's Medium (DMEM), protease inhibitor cocktail (PIC), Phenylmethylsulfonyl fluoride (PMSF) and csk inhibitor ASN-2324598 (ASN)

Cells lines

For siRNA screening Huh7, a hepatocyte cell line was used. A porcine kidney (PS) and a baby hamster kidney (BHK21) cell lines were used specifically for plaque assay.

Virus

DENV-2 serotype virus (Accession no. KJ918750.1) and JEV were used for infection experiments.

siRNA

Two siRNAs targeting different regions of csk mRNA were used throughout the study. Non-targeting control (NTC) siRNA was used as negative control.

Methods

Cell cultures:

The mammalian cell lines Huh7 and BHK21 or PS cells were grown in DMEM complete and MEM complete media respectively as monolayer in culture flasks or in required well plate. Cells were maintained at 37°C, 5% CO_2 and 90% relative humidity. Cells were sub-cultured when confluent.

Transfection of Huh7 cells with siRNA:

The siRNA and RNAimax was mixed independently with Opti-MEM and mixed after incubation of 5 minutes at room temperature. This mixture of siRNA and RNAimax was incubated at room temperature for 20 minutes. Meanwhile, Huh7 cells were trypsinized and collected in antibiotic free media and counted. 40,000 cells were seeded in each well containing siRNA. Cells were maintained in 37°C incubator. Next day media was changed to DMEM complete.

Infection of Huh7 cells:

Depending on the experimental requirements a multiplicity of infection (MOI) of 1 or 5 was used. The Huh7 cells were washed once with 1xPBS and virus inoculum prepared in 2% DMEM was added. Volume of inoculum to be used for infection was kept to a minimum for maximizing infection efficiency. The plates were incubated on a rocker for 1 hr at 37°C. The inoculum was removed and cells were washed twice with 1X PBS then replenished with fresh DMEM complete media.

Virus titer estimation by plaque assay:

Plaque assays for DENV and JEV were done using BHK21 and PS cells respectively. Cells were grown in 12 well plates. 60-80% confluent cells were infected with supernatant serially diluted in 2% MEM. Infection was done and incubated on rocker at 37°C for 1h. Subsequently, inoculum was removed and cells were overlaid with CMC then incubated at 37°C for 72 hours. 72-80 hrs post infection, plaque assay was fixed with formaldehyde and then stained with crystal violet.

Isolation of cellular RNA:

Total cellular RNA was isolated using TRIzol reagent by following the manufacture's protocol. Briefly, cells were homogenized using TRIzol reagent by incubating at room temperature for 2 minutes. Then chloroform (2/3rd volume of initially used TRIzol) was added and centrifuged at 12000X g to separate organic and aqueous phase. Aqueous phase was collected and RNA was precipitated using isopropanol for 10 minutes at room temperature. Later, centrifuged at 7500X g for 5 minutes and supernatant was decanted. The pellet was washed with 75% ethanol, centrifuged at 7500X g for 5 minutes and supernatant was decanted. Pellet was kept for drying, and subsequently dissolved in RNase-free water at 60°C.

Real time - polymerase chain reaction (RT-PCR):

One step RT-PCR was done using Taqman RNA-to-Ct™ 1-Step kit. Primers specific to DENV UTR and human β-actin were used. Reaction was performed on Stratagene Mx3005P (Agilent Technologies).

Protein isolation:

Protein lysates from the mammalian Huh7 cells were prepared using RIPA buffer containing PMSF and PIC. After collecting the cells in RIPA buffer, same were incubated on ice for 10 minutes. Centrifugation is done at 17,000X g, 4°C for 20 minutes and supernatant was collected as protein lysates. Protein quantification was done using Bradford reagent.

Western blotting:

Protein lysates were mixed with Laemlli buffer and incubated at 95°C for 5 minutes. Equal amount of proteins were resolved by using 8%, 10% or 12% (as per requirement) SDS-PAGE which was run initially at 130 V, and subsequently at 150

V. Resolved proteins were immobilized on polyvinylidene fluoride (PVDF) membrane by electrophoretic transfer. Membrane was blocked using 5% non-fat dry milk as blocking buffer for 30 minutes at room temperature. This was followed by incubation with primary antibody for 1 hr at room temperature or overnight at 4°C. Membrane was washed three times with 1X TBST followed by incubation with secondary antibody for 1 hr at room temperature. After 3 washes with 1X TBST and 2 washes with 1X TBS, blot was developed on X-ray film or using gel documentation system. Densitometric analysis was done by Image Lab 4.1 or ImageJ.

Immunoprecipitation

Huh7 cells were plated in a 12 well plate and next day infected with DENV-2 at 5 MOI. Lysates were collected at two time points, 24 and 48 hrs post infection (hpi) in phosphobuffer containing PIC and PMSF. Relative quantitation was done by Bradford method. The lysates were subjected to a preclearing step using rabbit IgG and 30 uL slurry of protein A (prewashed beads in phosphobuffer) and incubated on spin rotor at 4^0C for 1hr. Lysates were centrifuged at 2000 rpm for 5 mins and supernatant was collected. To this pre-cleared lysate, rabbit csk antibody was added and incubated overnight at 4^0C. Next day, to this 30 ul prewashed slurry was added and incubated for 1 hr at 4^0C. Then beads were washed three times with phosphobuffer and then directly boiled in Laemlli buffer at 95^0C for 5 minutes. The supernatant from the boiled beads was resolved on SDS-PAGE gel and immunoblotted for phospho-csk and csk. All the steps for western blotting were same as mentioned in methodology, except that the blocking and antibodies were incubated in 5% BSA in 1X TBST. Total lysates were used to blot for csk and viral capsid. Tubulin was used as loading control.

Results

Previously, C-terminal Src kinase (csk), Twinfilin-2 (TWF2) and Protein tyrosine kinase 6 (PTK6) were identified as positive hits in primary screening for siRNA library against human tyrosine kinases in Huh7 cells (Unpublished data). Therefore, to further gain insights into the role of these kinases in dengue infection, we sought to independently validate the results of primary screen and proceed for further characterization of the mechanism of action.

1. Knockdown of csk, TWF2 and PTK6 in Huh7 cells

siRNA library used for primary screening was a smart-pool of four individual siRNAs targeting each tyrosine kinase (TK). To further validate this observation and to rule out off-target effects, Huh7 cells were transfected with non-targeting siRNA (NTC) or validated single siRNA targeting the indicated kinases. 48 hours post transfection, knockdown efficiency was checked by western for csk or by RT-PCR for TWF2 and PTK6. Knockdown of csk, TWF2 and PTK6 found to be around 79%, 87% and 67% respectively (Figure 2A-C). 48 hrs post siRNA transfection, cells were infected with DENV-2 at an MOI of 1 pfu/cell and 24 hrs post-infection (hpi), cells were collected in TRIzol reagent to isolate RNA. Viral infection was checked by relative quantitation of DENV genomic RNA by RT-PCR. Knockdown of csk significantly reduced the DENV RNA levels by almost 50% while there was no significant change in the case of TWF-2 and PTK6 knockdown (Figure 2D). Since csk knockdown showed significant reduction in virus genomic RNA, subsequent experiments were performed to explore its role in dengue virus life-cycle.

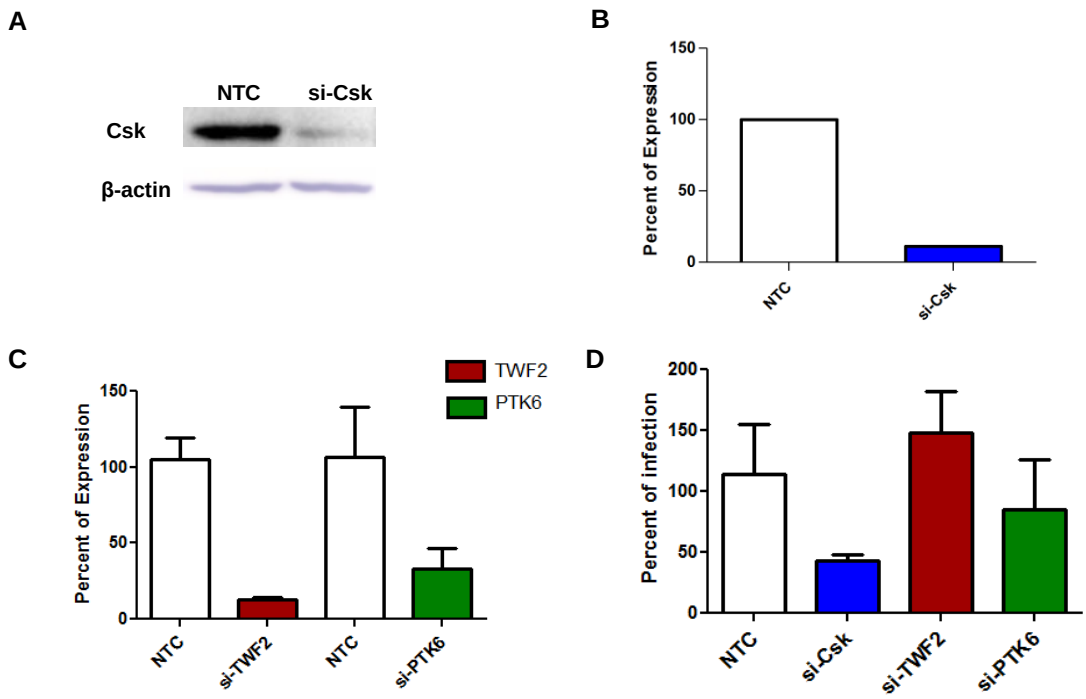

Figure 2. Knockdown of csk, TWF2 and PTK6 and its effect on DENV infection. (A) Western blot to assess csk knockdown and quantitation for the same (B). (C) Knockdown of TWF2 and PTK6 evaluated by RT-PCR. (D) Csk, TWF2 and PTK6 knockdown Huh7 cells were infected with DENV-2 at 1 MOI for 24 hrs. Infection levels were assessed by RT-PCR. NTC: Non-targeting siRNA. Error bars represent standard error of mean (SEM)

2. Effect of csk knockdown on dengue virus propagation in Huh7

We next confirmed knock-down efficiency of csk using two different siRNAs in Huh7 cells, and subsequently its effect on dengue infection was checked. siRNA mediated knockdown of csk was followed by infection with dengue at an MOI of 1 pfu/cell. Supernatant for plaque assay was collected at two time points, 1 hr and 24 hr post-infection. Cells were lysed in RIPA buffer to isolate protein. Viral load was checked by plaque assay. Western blotting for csk and viral capsid protein was done to confirm knockdown and infection respectively. A knockdown of around 80-87% was seen in csk expression (Figure 3A), which led to an almost 50% reduction in viral plaque assay (Figure 3B).

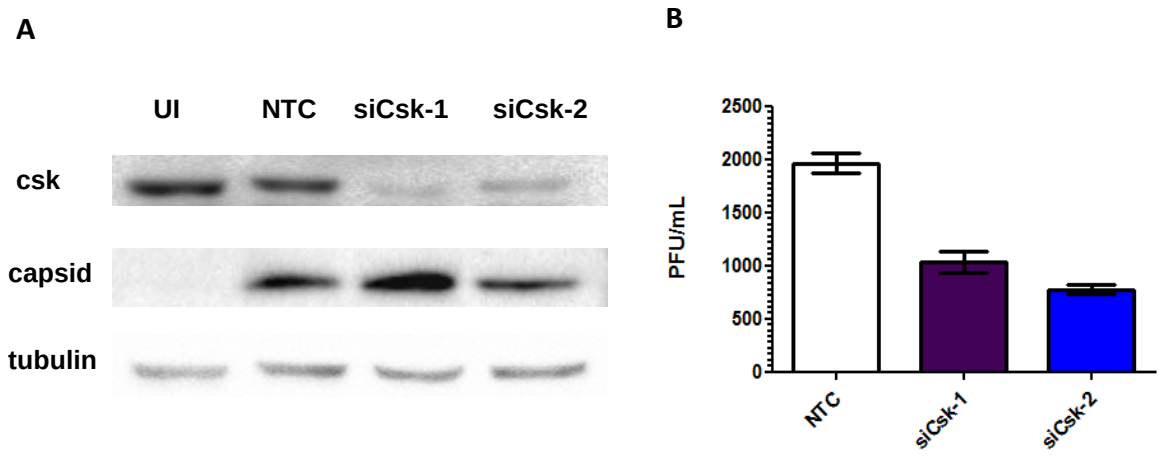

Figure 3. Csk knockdown reduces the DENV-2 infection. Huh7 cells were transfected with siCsk-1 and siCsk-2 followed by infection with DENV-2 at 1 MOI. A) Knockdown of csk and DENV-2 infection was checked by identifying presence of capsid protein by western blotting, tubulin is loading control. B) Plaque assay was done to estimate the plaque forming unit at 24 hrs post infection. siCsk-1 and 2 are targeting two different sites on csk mRNA. NTC is non-targeting control and UI is uninfected. Error bars represent standard error of mean (SEM).

3. Csk inhibition using known chemical inhibitor

As siRNA knockdown showed that csk has a role to play in dengue virus life-cycle, this was further verified using a known csk inhibitor. Same inhibitor was also used to determine the stage at which csk plays a role in the dengue virus life-cycle. To determine whether csk is interfering at the level of viral entry or post-entry stages, cells were subjected to pre-treatment with the inhibitor before infecting with dengue virus. Huh7 cells were treated with DMSO (vehicle control) or 20 μM csk inhibitor ASN (ASN-2324598) (Kunte et al., 2005) for 6 hrs, and then infected with DENV-2. Samples to be collected after 1 hour of infection (entry experiment) were infected at an MOI of 5 pfu/cell, while samples for the 24 hrs time point were infected with an MOI of 1 pfu/cell. For the entry experiments, cells were collected by trypsinization, then RNA and proteins were isolated using trizol method. The 24 hrs post-infection samples were analysed by preparing protein lysates and checking for NS3 expression.

We found that, amount of DENV RNA was two-fold higher at 1 hr post-infection in ASN-treated samples compared to DMSO treated-samples (Figure 4A). This indicates that inhibition of csk enhances DENV entry. Surprisingly, in 24h post-infection lysates, the level of NS3 was reduced by 50% (Figure 4B). These results suggest that csk is involved at post-viral entry stages in DENV life-cycle.

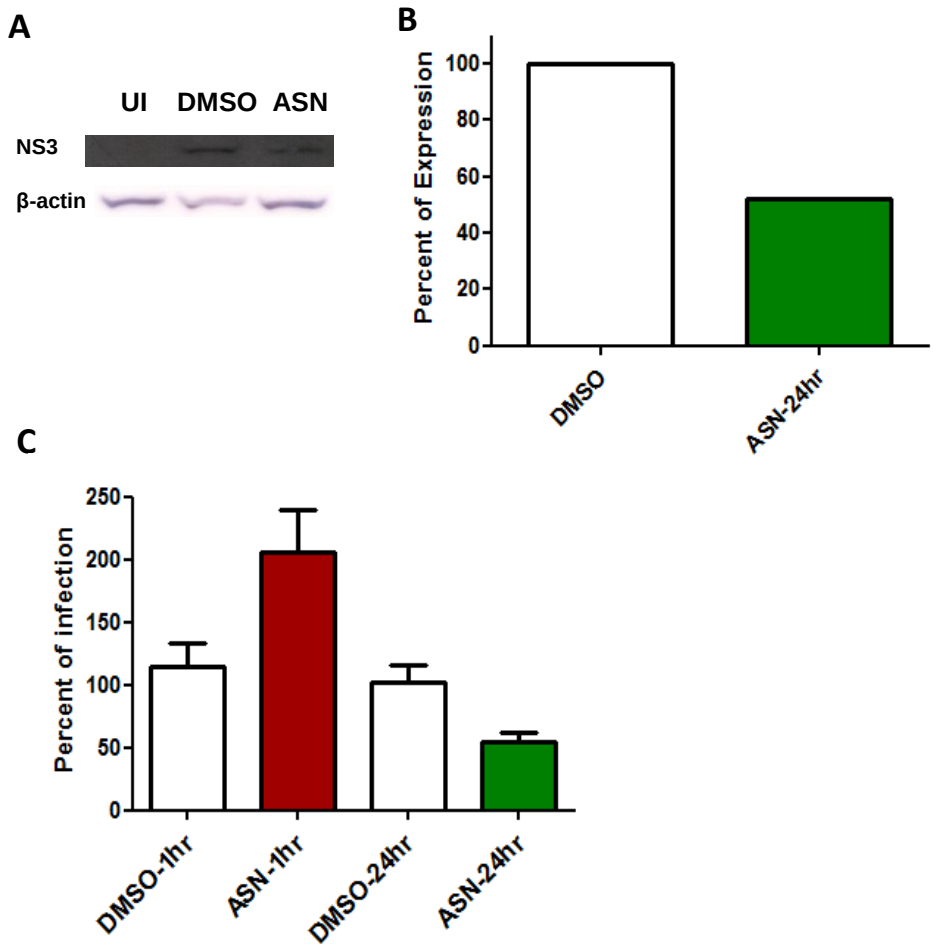

Figure 4. Csk inhibition by csk inhibitor ASN (ASN-2324598) and DENV-2 infection. (A) DENV-2 infection was seen by probing for NS3 in western blotting and β-actin as a loading control. (B) Quantitation of western blot. (C) Effect of inhibiting csk on entry of dengue virus in Huh7 cells. At 1 hr (N=12), and at 24 hrs (N=3) . UI: Uninfected, DMSO: control. Error bars represent standard error of mean (SEM)

4. Phosphorylation of csk

Phosphorylation of serine residue at position 364 on csk suggests the regulation of its activity by upstream regulator csk binding protein (CBP). To analyse this, Huh7 cells were infected with dengue virus at an MOI of 5 pfu/cell. At 24 and 48 hr post infection, lysates were prepared in phosphobuffer and csk was immunoprecipitated as described in methods section and phosphorylated csk was detected by phospho-serine antibody. As shown (Figure 5), phosphorylation of csk increased by almost 50% and 100% at 24 and 48 hrs post-infection respectively suggesting that dengue infection leads to activation of csk.

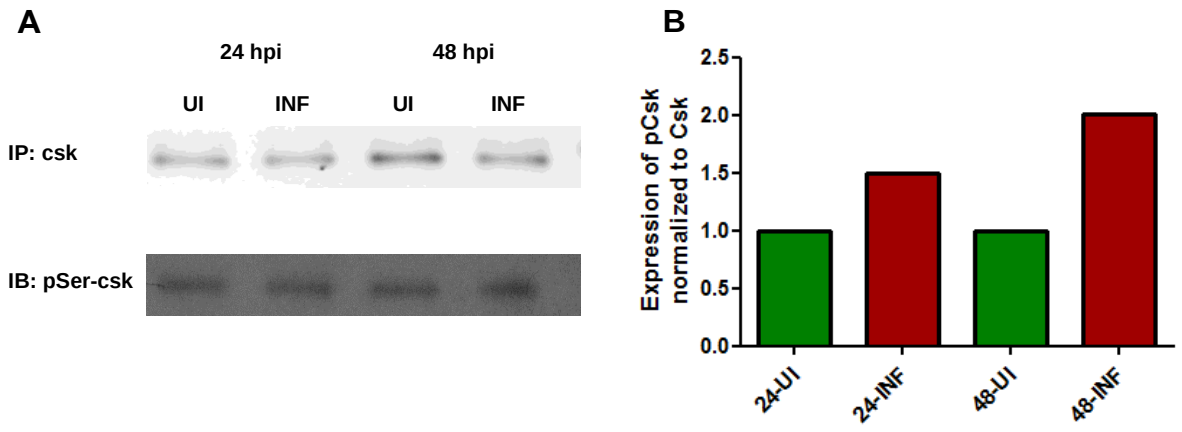

Figure 5. Post-infection phosphorylation of csk. A) 24 and 48 hrs post infection (hpi) time point were immunoprecipitated (IP) for csk and immunoblotted (IB) for pSer-csk. B) Quantitation for the same. UI: Uninfected, INF: Infected, hpi: hrs post-infection.

5. Effect of csk knockdown on Japanese encephalitis virus (JEV)

In order to determine whether csk activity is dengue-specific or conserved across flavivirus, we performed csk knockdown by siRNA followed by infection with JEV at an MOI of 1 pfu/ml. At 24 hrs post infection, viral titers were estimated by performing plaque assay from culture supernatants and JEV genome levels were estimated by RT-PCR using RNA from infected cells. After csk knockdown, JEV viral RNA was reduced by almost 50% (Figure 6A) and there was reduction in JEV titer by 55% as compared to NTC (Figure 6B).

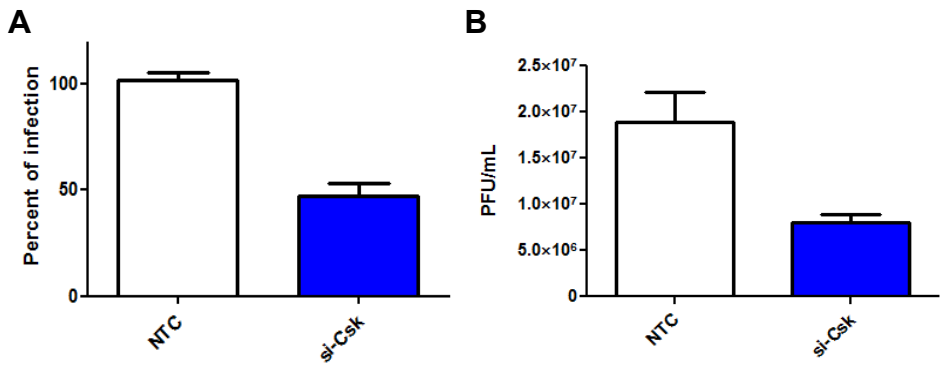

Figure 6. Effect of csk knock-down on JEV infection. A) Percent of infection determined by RT-PCR. B) JEV viral titers by plaque assay. si-Csk: siRNA targeting Csk, NTC: Non targeting siRNA. Error bars represent standard error of mean (SEM)

Discussion

Genome-wide RNAi screens have been employed by earlier studies to identify host cellular factors modulating life-cycle of flaviviruses like Hepatitis C virus (HCV) (Li et al., 2009), West Nile Virus (WNV) (Krishnan et al., 2008) and DENV (Sessions et al., 2009). Genome-wide RNAi screening in *Drosophila melanogaster,* revealed 116 genes required for DENV propagation. Among these, 42 out of 82 identifiable human homologues had a significant effect on viral life-cycle in Huh7 cells. A comparative analysis of candidates generated by RNAi screens in both WNV and DENV indicate limited overlaps between them. Number of identified genes in these studies remains to be validated in cell lines that are physiologically relevant for dengue infection.

We performed screening of tyrosine kinase siRNA library in Huh7 cells to identify tyrosine kinases that affect dengue virus titers. C-terminal Src kinase (csk), Twinfilin-2 (TWF2) and Protein tyrosine kinase 6 (PTK6) were identified as positive hits in the primary screening. However, secondary validation with individual siRNAs confirmed only csk as a positive hit. siRNA knockdown of csk led to a reduction in virus titer by 50%. This effect was further confirmed by using an inhibitor of csk which was also used to determine the stage of virus life-cycle affected by csk. It is interesting to note that virus entry was not affected by csk inhibition, but a reduction in viral load by 50% at 24 hrs suggests a role at post-entry level. Immuno-precipitation results showed that DENV infection led to increase in phosphorylation of csk. This confirms that activity of csk is required for dengue virus infection. Inhibition of JEV replication by csk knock-down further suggests that csk plays a role in other flavivirus infection.

It is a widely accepted fact that csk negatively regulates the activity of Src family kinases (SFKs) (Okada, 2012). SFKs consist of c-Src, c-Yes, Fyn, c-Fgr, Lyn, Hck, Lck and Blk. It has been shown that csk, Janus kinase and Vaccinia related-kinase play a significant role in replication of HCV (Supekova et al., 2008). Previously, role of c-Src has been described in dengue virus assembly and maturation (Chu & Yang, 2007). Furthermore, c-Yes was shown to hamper intra-cellular trafficking of West Nile Virus (Hirsch et al., 2005). Therefore, our finding indicates that csk may play a crucial role in flavivirus infection by regulating src kinases or other interacting proteins involved at later stages of infection. We are

further exploring the domains in csk that are essential for its functions with respect to dengue infection to characterize the exact mechanism of action.

Appendix I

Buffer solutions:

1X Phosphate buffered saline (PBS)

NaCl	8 gm
KCl	0.2 gm
Na_2HPO_4	1.44 gm
KH_2PO_4	0.24 gm
dH_2O	1000 ml

5X SDS RUNNING BUFFER (for 1L)

Tris base	15.1gm
Glycine	94gm
10% SDS	50 ml
dH_2O	1 L

Dilute to 1X as working concentration.

TRANSFER BUFFER (For 2L)

Tris base	6.06gm
Glycine	28.8gm
Methanol	400ml
dH_2O	1600ml

10X TBS (For 1L, pH 7.6)

Tris base	24.2gm
NaCl	80gm

Dilute to 1X as working concentration.

1X TBST

1ml of Tween-20 in 1000ml 1X TBS

Blocking milk (5%)(For 20ml)

Non-fat milk powder	1gm
1X TBST	20ml

RIPA buffer (for 10 ml)

1M Tris (pH 7.4)	500 ul
3M NaCl	1.5 ml
Na-deoxycholate	500 ul
10% SDS	100 ul
10% Triton	1 ml

Luminol (For 10ml)

Luminol	0.44gm
DMSO	10ml

p-Coumaric acid (For 10ml)

p-Coumaric acid	0.15gm
DMSO	10ml

4X Laemlli buffer (For 10ml)

1M Tris-Cl (pH=6.8)	2ml
20% SDS	4ml
Glycerol	4ml
β-Mercaptoethanol	500µl
Bromophenol blue	pinch to colour

10% DMEM media (100 ml)

DMEM	88 ml

FBS	10 ml
100X NEAA	1 ml
100X PSG	1 ml

2% DMEM media (100 ml)

DMEM	96 ml
FBS	2 ml
100X NEAA	1 ml
100X PSG	1 ml

10% MEM media (100 ml)

MEM	89 ml
FBS	10 ml
100X PSG	1 ml

2% MEM media (100 ml)

MEM	97 ml
FBS	2 ml
100X PSG	1 ml

Carboxy methyl cellulose (CMC) (300 ml)

Low viscous CMC	0.75 gm
High viscous CMC	0.75 gm
0.5X PBS	50 ml
2% MEM	250 ml

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