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The heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a host factor required for dengue virus and Junín virus multiplication

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ABSTRACT

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are cellular factors involved in the replication of several viruses. In this study we analyzed the expression and intracellular localization of hnRNP A2 and hnRNP K in cell cultures infected with two viruses that cause human hemorrhagic fevers: dengue virus type 2 (DENV-2) and Junín virus (JUNV). We determined that DENV-2 promoted the cytoplasmic translocation of hnRNP K and to a lesser extent of hnRNP A2, meanwhile, JUNV infection induced an increase in hnRNP K cytoplasmic localization whereas hnRNP A2 remained mainly in the nucleus of infected cells. Both hnRNP K and hnRNP A2 were localized predominantly in the nucleus of JUNV persistently-infected cells even after superinfection with JUNV indicating that persistent infection does not alter nucleo-cytoplasmic transport of these hnRNPs. Total levels of hnRNP K expression were unaffected by DENV-2 or JUNV infection. In addition we determined, using small interfering RNAs, that hnRNP K knock-out inhibits DENV-2 and JUNV multiplication. Our results indicate that DENV-2 and JUNV induce hnRNP K cytoplasmic translocation to favor viral multiplication.

1. Introduction

Dengue (DENV) and Junín (JUNV) viruses are the causative agents of severe hemorrhagic fevers in humans. DENV, member of the *Flaviviridae* family, is transmitted to humans by the mosquitoes *Aedes aegypti* and *Aedes albopictus* and is the most prevalent arthropod-borne viral disease with 50–100 million infections per year. Despite its importance in human health no licensed vaccine or specific antiviral treatment exists for DENV infection. DENV virions contain a positive-polarity RNA genome encoding three structural proteins (capsid protein C, glycoprotein E and pre-membrane pre-M) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Acosta et al., 2014).

On the other hand, JUNV, the etiological agent of Argentine hemorrhagic fever (AHF), belongs to the *Arenaviridae* family. No specific chemotherapy is available to deal with arenavirus infections and even though an effective live attenuated vaccine has been developed to prevent AHF in the endemic area, risks of live vaccines in immunocompromised individuals are not clearly understood. Furthermore, vaccination may not be a definitive solution to JUNV infection because of the emergence of new viral variants and ecological changes of its main reservoir in nature, the cricetid *Calomys musculinus*. JUNV possess a bisegmented RNA genome with an ambisense coding strategy which encodes for N, the nucleoprotein, GPC, the precursor of mature glycoproteins G1 and G2, L, the polymerase and Z, the matrix protein (Olschläger and Flatz, 2013). JUNV is able to cause persistent infections both in its natural reservoir and in cell cultures (Ellenberg et al., 2002, 2004, 2007) however there is still much to understand about viral and host cell factors involved in the development of persistence.

Besides virus-encoded proteins, virus replicative cycle depends on host cell factors and these virus-cell interactions are key determinants of virus replication, host range and pathogenesis. Several viruses are capable to promote cytoplasmic translocation of cellular proteins predominantly located in the nucleus such as the heterogeneous nuclear ribonucleoproteins (hnRNPs) (Castilla and Scolaro, 2012). hnRNPs comprise a family of 21 proteins (from hnRNP A to hnRNP U) involved in mRNA processing, nucleo-cytoplasmic transport, translation and stability (Han et al., 2010). These RNA-binding proteins also exert important and diverse functions in the replicative cycle of different viruses being implied in RNA replication, transcription, splicing and translation (Brunner et al., 2010; Gui et al., 2010; Schmidt et al., 2010; Sola et al., 2011; Levengood et al., 2013; Dinh et al., 2013; Wang et al., 2014; Li et al., 2014). Interestingly, these studies have revealed that hnRNPs may act

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either as favoring factors or as negative regulators of viral multiplication (Castilla and Scolaro, 2012).

Several hnRNPs have been proved to bind to 5′ and 3′ non coding regions (NCRs) of flavivirus genomes. hnRNP E2 and L are required for hepatitis C virus (HCV) RNA replication (Wang et al., 2011; Li et al., 2014) whereas several hnRNPs, such as hnRNP I (also known as polypyrimidine tract-binding protein, PTB), L and D, promote HCV IRES (internal ribosome entry site)-dependent translation (Gosert et al., 2000; Paek et al., 2008; Hwang et al., 2009; Park et al., 2011).

The participation of hnRNP I on DENV multiplication has been investigated by different research groups. Interaction of hnRNP I with DENV RNA and nonstructural proteins NS4A, NS1 and NS3 was proved (Anwar et al., 2009; Jiang et al., 2009). A role of this hnRNP in RNA synthesis was proposed in Huh-7 and Vero cells although hnRNP I accumulation in the cytoplasm of infected cells seems to be cell type dependent (Agis-Juárez et al., 2009; Anwar et al., 2009; Jiang et al., 2009). Like hnRNP I, hnRNPs A1, A2/B1 and Q also bind to the DENV 3′ NCR (Paranjape and Harris, 2007), whereas interaction between hnRNP C1/C2 and NS1 was also described in human 293 T cells infected with DENV-2 (Noisakran et al., 2008). In addition, Chang et al. (2001) demonstrated the interaction between hnRNP K and DENV C protein in 293 T and BHK cells.

Much less is known about arenavirus interactions with hnRNPs. We have previously demonstrated that JUNV infection promotes the cytoplasmic translocation of hnRNP A1 and we also found that this hnRNP acts as a favoring host cell factor for JUNV multiplication (Maeto et al., 2011). The interaction between JUNV N protein and hnRNP A1 was also confirmed and evidences that support a role of N protein in cytoplasmic accumulation of hnRNP A1 were also reported (Maeto et al., 2011). Different results were obtained in Vero cells persistently infected with JUNV (V3 cells). N protein and viral genome can be detected in V3 cells, however these cultures do not produce infectious virus and are highly resistant to a second infection (superinfection) with JUNV or related arenaviruses (Ellenberg et al., 2002, 2004). We demonstrated that hnRNP A1 remains within the nucleus either in V3 or in JUNV-superinfected V3 cells suggesting that persistently infected cells are unable to modify hnRNP A1 nucleo-cytoplasmic trafficking (Maeto et al., 2011).

In this study we analyzed the expression and intracellular localization of hnRNPs A2 and K in DENV-2 and JUNV-infected cell cultures and we examined the effect of hnRNP K silencing on the multiplication of these hemorrhagic viruses.

2. Materials and methods

2.1. Cells and viruses

Vero (African green monkey kidney) and A549 (human lung carcinoma) cell lines were grown at 37 °C in Eagle's minimum essential medium (MEM) (GIBCO) supplemented with 5% calf serum (growth medium). For maintenance medium (MM) serum concentration was reduced to 1.5%.

DENV-2 (New Guinea C strain) stock was obtained in the C6/36 mosquito cell line from *A. albopictus* adapted to grow at 33 °C, JUNV (XJCl3 strain) stock was obtained in BHK-21 cells and vesicular stomatitis virus (VSV, Indiana strain) stock was prepared in Vero cells.

Vero cells persistently infected with JUNV were obtained after infection of Vero cells with JUNV at a multiplicity of infection (MOI) of 0.01 PFU/cell as previously described (Ellenberg et al., 2002) and cells were subcultured in growth medium every two weeks for 1 year. Vero cells were subcultured in parallel to use as control.

2.2. Antibodies

Primary antibodies used for immunofluorescence assays were: mouse mAb anti-glycoprotein E of DENV (ab41349, Abcam), mouse mAb SA02-BG12 anti N protein of JUNV (Sánchez et al., 1989), mouse mAb anti-hnRNP A2 (ab6102, Abcam), rabbit Ab anti-hnRNP K (R332, Cell Signaling), rabbit Ab anti-T7 (ab9115, Abcam) and rabbit immunosera obtained in our laboratory: anti-DENV-2, anti-JUNV and anti-VSV. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) or rhodamine (TRITC), used as secondary antibodies were purchased from Sigma–Aldrich.

Western blot analysis was performed employing mouse mAb anti-hnRNP A2 (ab6102, Abcam), rabbit Ab anti hnRNP K (R332, Cell Signalling), mouse mAb anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab8245, Abcam), rabbit Ab anti-T7 (ab9115, Abcam) or rabbit Ab anti-histone H4 (sc-8660 R, Santa Cruz Biotechnology) as primary antibodies and peroxidase anti-rabbit IgG (W4011, Promega) or peroxidase anti-mouse Ig G (A9044, Sigma–Aldrich) as secondary antibodies.

2.3. Immunofluorescence assays

Monolayers of Vero or A549 cells grown on coverslips were infected with DENV-2 or JUNV at an MOI of 1 PFU/cell or with VSV at an MOI of 0.01 PFU/cell. In the case of V3 cultures they were superinfected with JUNV (MOI = 1). At different times post-infection (p.i.), cells were washed three times with phosphate buffer saline (PBS), fixed with paraformaldehyde 4% in PBS for 10 min at room temperature and permeabilized by incubation in PBS containing 0.2% Triton X-100 during 10 min at room temperature. Then, cells were incubated with primary antibodies during 30 min at 37 °C and after three washes with PBS, incubation with secondary antibodies during 30 min at 37 °C was performed. After three washes with PBS cell nuclei were stained with Hoechst 33258 reagent (1 μg/mL) and coverslips were mounted on a 90% glycerin solution in PBS (pH 7.2) containing 2.5% 1,4-diazabicyclo (2,2,2) octane (DABCO; Sigma–Aldrich) and visualized in a fluorescence microscope. The percentage of infected cells was obtained by counting 20 random selected fields (400× magnification).

2.4. Isolation of nuclear and cytoplasmic fractions

The isolation of nuclear and cytoplasmic fractions from A549 cell cultures infected or not with DENV-2 or JUNV was performed at 4 days after infection using the Nuclei EZ Prep Nuclei Isolation Kit (NUC-101, Sigma–Aldrich) following manufacturer instructions.

2.5. Transfection experiments

Overexpression of hnRNP K was accomplished by transfection of A549 cells with the plasmid pcDNA3.3-T7-hnRNP K (1 μg DNA/culture) using Lipofectamine 2000 (Invitrogen) as transfection reactive. This plasmid allows the expression of hnRNP K fused to T7 epitope allowing a more efficient detection of the cellular protein employing an anti-T7 primary antibody in immunofluorescence assays performed to determine the intracellular localization of hnRNP K. The plasmid pcDNA3.3-GFP, which allows the expression of GFP, was used as control. At 24 h after transfection, cultures were infected with DENV-2 or JUNV (MOI = 1) and at different times p.i. T7-hnRNP K localization was analyzed by immunofluorescence assay and virus yield was quantified by plaque assay on Vero cells.

To achieve hnRNP K silencing A549 cells were transfected with commercial siRNAs (sc-38282, Santa Cruz Biotechnology) or an RNA used as control (cRNA: 5′-GACCACAATTCTCGATATACAUU-3′). Cultures were transfected with a mix of Lipofectamine and 50 nM RNA and after 5 h cells were subcultured (with a split ratio of 2) and 24 h

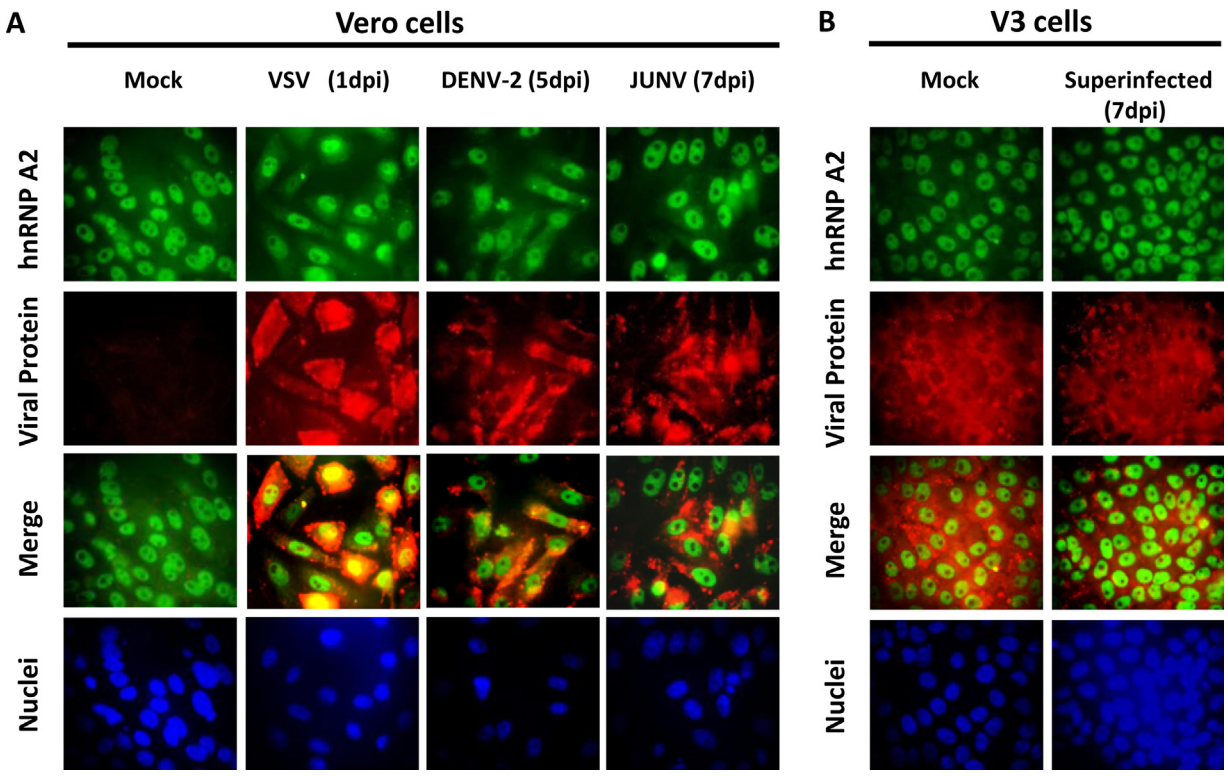

Fig. 1. Intracellular localization of hnRNP A2. A) Vero cells were infected with VSV (MOI = 0.01), DENV-2 (MOI = 1) or JUNV (MOI = 1) and at 1, 5 or 7 dpi, respectively, cells were fixed with paraformaldehyde. B) V3 cells were superinfected or not with JUNV (MOI = 1) and at 7 dpi they were fixed with paraformaldehyde. In A and B hnRNP A2 and viral proteins were revealed by immunofluorescence assays. Cell nuclei were stained with Hoechst (magnification 400×).

later a second round of transfection/subculture was performed. At 24 h post-transfection cultures were infected with DENV-2 or JUNV (MOI = 1) and at different times p.i. virus multiplication was analyzed by quantifying infected cells (by immunofluorescence assay) and virus yield (by plaque assay on Vero cells).

2.6. Western Blot analysis

Vero and A549 mock-infected or infected with DENV-2- or JUNV, A549 cells transfected with the plasmids pcDNA3.3-T7-hnRNP K or pcDNA3.3-GFP and nuclear and cytoplasmic fractions obtained from A549 cultures were lysed in sample buffer (Bio Rad) at different times after infection. SDS-PAGE and protein transference to a PVDF membrane (Hybond P; Amersham, Pharmacia) was performed as previously described (Maeto et al., 2011). After transference, membranes were incubated in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 3% bovine serum albumin (blocking buffer) and further incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. After several washes with TBS containing 0.1% Tween 20, membranes were incubated for 1 h at room temperature with secondary antibodies diluted in blocking buffer. Protein bands were visualized by chemiluminescence detection and quantified by using Image J for Windows.

2.7. Statistical analysis

The 95% confidence intervals (CI) of virus titers were calculated according to Poisson distribution from replicates of one representative experiment. Statistical significance for differences in hnRNP K expression was determined either by 2-tailed paired Student's *t* test or ANOVA analysis using data obtained from three independent experiments. A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Intracellular localization of hnRNP A2 and hnRNP K

In order to establish whether infection of DENV-2 or JUNV induces the translocation of hnRNPs A2 and K to Vero cell cytoplasm we performed double immunofluorescence assays at different days post-infection (dpi). After 7 dpi, DENV-2 induced a poor translocation of hnRNP A2 to the cytoplasm whereas JUNV did not modify the nuclear distribution of hnRNP A2 observed in mock-infected cells (Fig. 1A); when Vero cells were infected with VSV, an RNA virus that alters nucleo-cytoplasmic trafficking of several hnRNPs (Pettit Kneller et al., 2009) partial translocation of hnRNP A2 to the cytoplasm was observed at 1 dpi (Fig. 1A). A nuclear pattern of hnRNP A2 distribution was observed on V3 or JUNV-superinfected V3 cells (Fig. 1B) indicating that JUNV (acute or persistent) infections do not alter hnRNP A2 intracellular localization.

When distribution of hnRNP K was analyzed we found that partial cytoplasmic relocation of this protein was detected at 5 or 7 dpi for DENV-2 or JUNV, respectively, in Vero cell cultures (Fig. 2A). On the contrary, no change in hnRNP K intracellular localization was observed in V3 cells or JUNV-superinfected V3 cells up to day 7 post-superinfection (Fig. 2B). On the other hand, translocation of hnRNP K to the cytoplasm was observed in DENV-2- and JUNV-infected A549 cells from 2 dpi onward and was more evident at 5 dpi (Fig. 2C). Cytoplasmic and nuclear proteins were fractionated from mock-infected or infected A549 cells and hnRNP K expression was analyzed by Western blot. Histone 4 was employed as nuclear marker whereas GAPDH, which is localized predominantly in the cytoplasm, was used as cytoplasmic marker. A minor but significant increment in the amount of hnRNP K protein in cytoplasmic extracts was observed in DENV-2- and JUNV-infected cells in comparison to uninfected ones in different independent experiments (Fig. 2D).

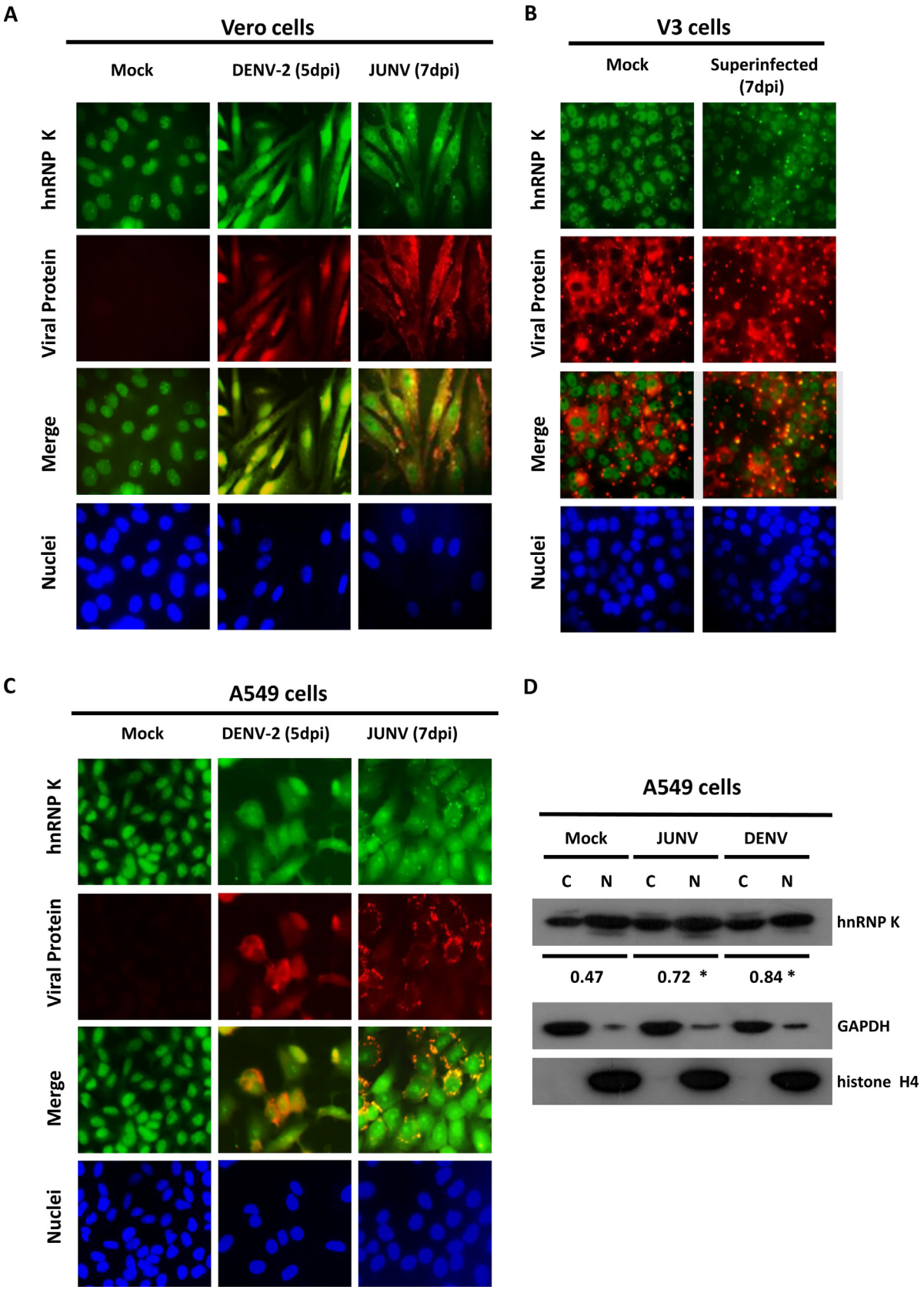

Fig. 2. Intracellular localization of hnRNP K. (A) Vero cells or (C) A549 cells were infected with DENV-2 or JUNV (MOI = 1) and at 5 or 7 dpi, respectively, cells were fixed with paraformaldehyde. (B) V3 cells were superinfected or not with JUNV (MOI = 1) and fixed with paraformaldehyde at 7 dpi. In A, B and C hnRNP K and viral proteins were revealed by immunofluorescence assays. Cell nuclei were stained with Hoechst (magnification 400×). (D) Nuclear and cytoplasmic extracts were obtained from A549 cells mock-infected or infected with DENV-2 or JUNV (MOI = 1) at 4 dpi and hnRNP K, histone H4 and GAPDH expression was analyzed by Western blot. The numbers indicate the mean rate of cytoplasmic/nuclear hnRNP K expression from three independent experiments and asterisks indicate significant differences ($p < 0.05$) between infected and mock-infected cells.

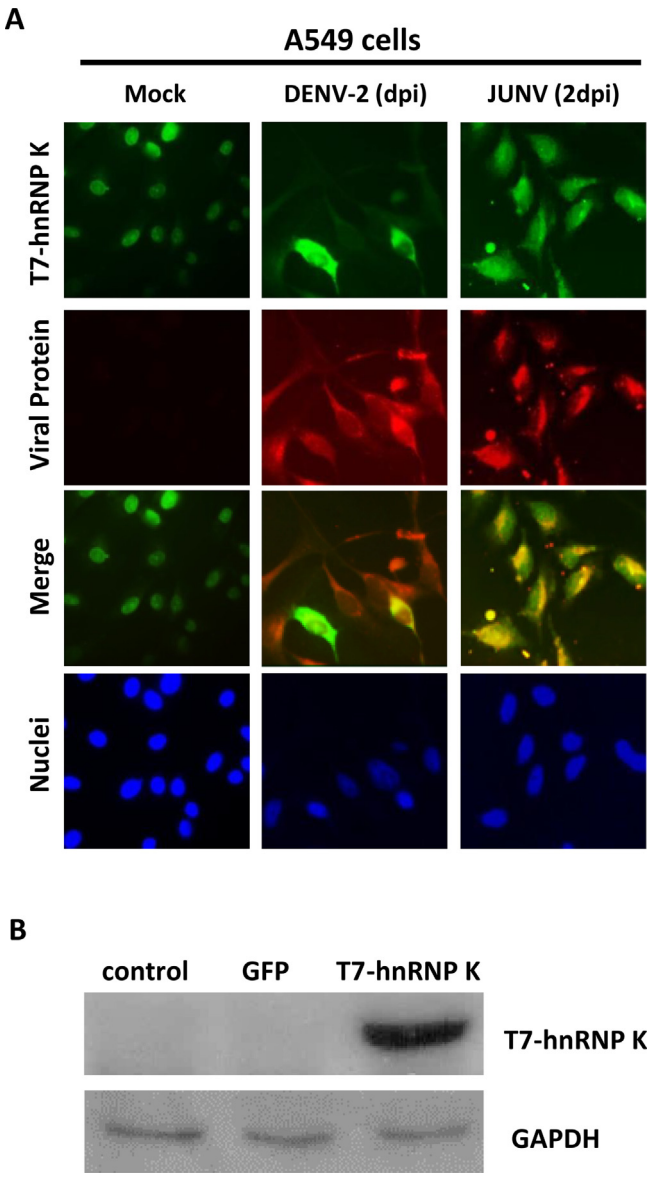

Fig. 3. Intracellular localization of overexpressed hnRNP K. (A) A549 cells were transfected with a plasmid that allows the expression of T7-hnRNP K and at 24 h post-transfection cells were infected with DENV-2 or JUNV (MOI = 1). At 2 dpi cells were fixed with paraformaldehyde and T7-hnRNP K and viral proteins were revealed by immunofluorescence assays. Cell nuclei were stained with Hoechst (magnification 400×). (B) A549 cells (control) or A549 cells transfected with plasmids that allow the expression of GFP or T7-hnRNP K were lysed at 3 days post-transfection and T7-hnRNP K overexpression was analyzed by Western blot using anti-T7 as primary antibody.

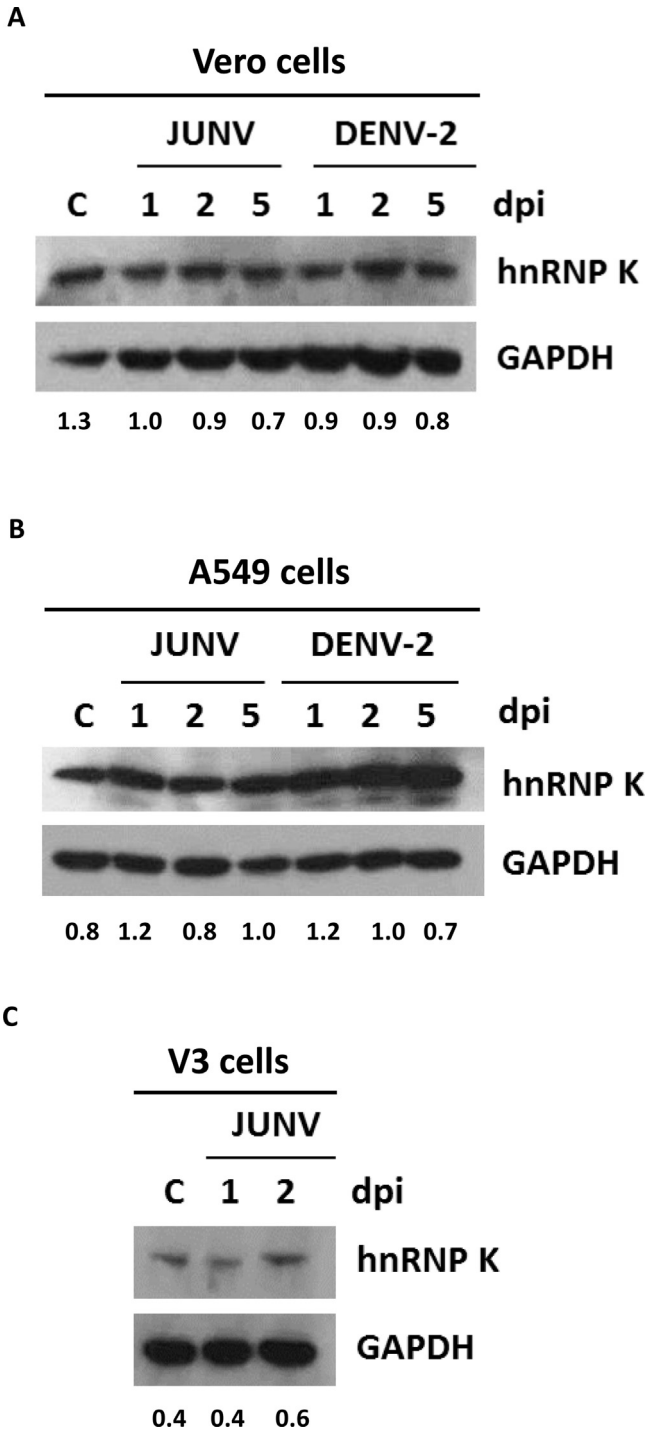

Fig. 4. Expression of hnRNP K in DENV-2 or JUNV-infected cells. (A) Vero cells or (B) A549 cells were infected with JUNV or DENV-2 (MOI = 1) and at 1, 2 and 5 dpi hnRNP K levels were assessed by Western blot. (C) V3 cells were superinfected with JUNV (MOI = 1) and at 1 and 2 dpi the expression levels of hnRNP K were analyzed by Western blot. C: mock-infected cells. The numbers indicate the relative intensity of hnRNP K bands respect to GAPDH bands and data are mean from triplicate experiments. Non-significant differences ($p \square 0.05$) in hnRNP K expression were determined in infected cultures with respect to mock-infected ones.

We decided to corroborate our data by analyzing the intracellular localization of overexpressed hnRNP K in A549 cells. For this purpose, A549 cells transfected with a plasmid that allows the transient expression of T7-tagged hnRNP K were infected with DENV-2 or JUNV and detection of tagged hnRNP K and viral proteins was performed by immunofluorescence technique. As can be seen in Fig. 3 A, at 3 dpi T7-hnRNP K was found mainly in the cytoplasm of DENV-2-infected cells and an evident cytoplasmic translocation was also detected in JUNV-infected cells, confirming that both viral infections affect nucleo-cytoplasmic distribution of hnRNP K.

3.2. *hnRNP K levels of expression in DENV-2 and JUNV infected cells*

We next analyzed whether infection with DENV-2 or JUNV affects hnRNP K total level of expression. To this end Vero (Fig. 4A) or A549 (Fig. 4B) cells were infected with DENV-2 or JUNV (MOI = 1)

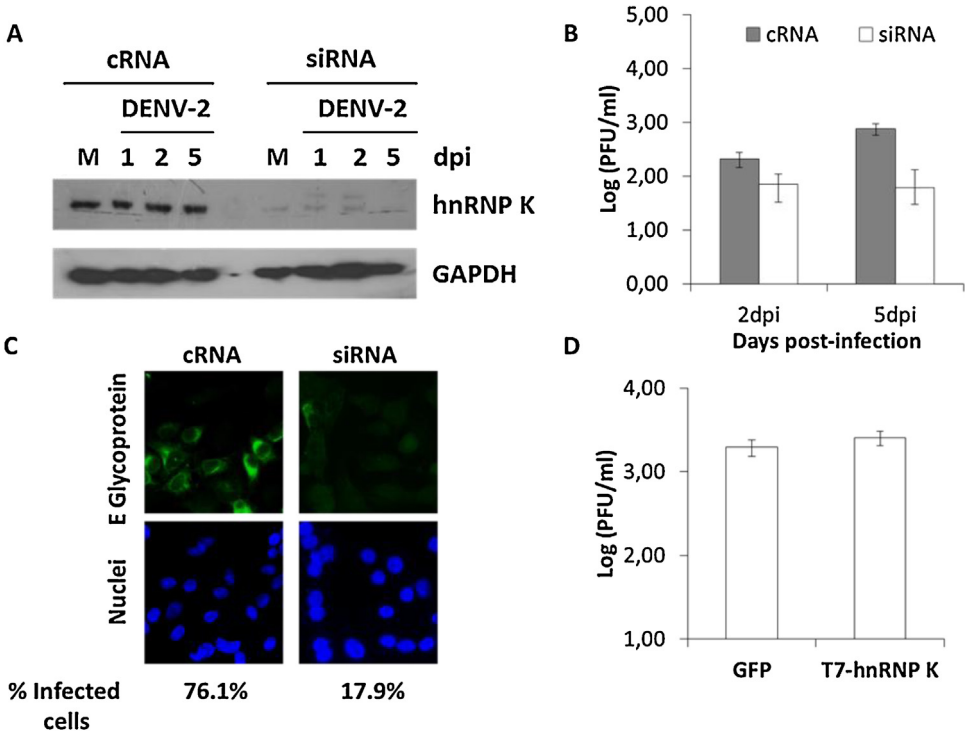

Fig. 5. Effect of silencing or overexpression of hnRNP K on DENV-2 multiplication. A549 cells were transfected with hnRNP K siRNA or cRNA, and 24 h later cells were infected with DENV-2 (MOI = 1). (A) The expression levels of hnRNP K were assessed by Western blot at different dpi (M: mock-infected cells). (B) Viral yields were measured by plaque assay at 2 and 5 dpi. (C) E-protein expression was analyzed by immunofluorescence assay at 2 dpi. The percentage of infected cells of one representative experiment is shown. (D) A549 cells were transfected with plasmids that allow the expression of GFP or T7-hnRNP K and at 24 h post-transfection cells were infected with DENV-2 (MOI = 1), and at 2 dpi viral yields were quantified by plaque assay. Viral titers are mean values from three replicates of one representative experiment ±95% CI.

and at 1, 2 or 5 dpi cells were lysed and hnRNP K expression was examined by Western blot assay. This analysis was also performed in V3 cells and JUNV-superinfected V3 cells at 2 days after superinfection (Fig. 4C). The statistical analysis, using data obtained from three independent experiments, showed that hnRNP K levels of expression in virus-infected Vero or A549 cells were not significantly different from those in mock-infected cells (Fig. 4A and B). In a similar way, JUNV-superinfection of V3 cells did not alter the levels of hnRNP K expression observed in V3 cultures (Fig. 4C).

3.3. *Effect of silencing or overexpression of hnRNP K on DENV-2 and JUNV infections*

Taking into account that DENV-2 and JUNV infections induce changes in the nucleo-cytoplasmic trafficking of hnRNP K we postulated that this protein could play a role in viral replication. To investigate this possibility we transfected A549 cells twice with specific siRNAs to knockdown hnRNP K expression. At 24 h post-transfection cells were mock-infected or virus-infected (MOI = 1) and at different dpi culture supernatants were harvested and virus titers were determined. In addition, the number of cells expressing E glycoprotein (in DENV-2-infected cells) or N protein (in JUNV-infected cells), determined by immunofluorescence assay, was counted. The efficiency of siRNA-mediated hnRNP K knockdown in mock-infected or virus-infected cultures was assessed by Western blot (Figs. 5A and 6A). Silencing of hnRNP K produced a significant decrease in DENV-2 and JUNV yields at different times p.i. A 92.0 and 92.6% inhibition of virus titers were achieved at 5 dpi for DENV-2 and JUNV, respectively (Figs. 5B and 6B). In addition, the number of virus infected cells, detected by immunofluorescence assay, at 2 dpi for DENV-2 or at 5dpi for JUNV were also reduced (Figs. 5C and 6C). These results indicate that inhibition of hnRNP K expression negatively affects DENV-2 and JUNV multiplication.

We also examined whether overexpression of hnRNP K exerts a promoting effect on viral production. For this purpose, cultures transfected with plasmid pcDNA3.3-T7-hnRNP K, that allows the expression of T7-hnRNP K, or with the plasmid pcDNA3.3-GFP, used as control, were infected with DENV-2 or JUNV (MOI = 1) and at 2 dpi extracellular virus titers were quantified. Similar levels of transfection were achieved with both plasmid constructions (varying between 46 and 60% of transfected cells in different experiments). Virus yields obtained from pcDNA3.3-T7-hnRNP K-transfected cells did not exhibit significant differences with virus yields from pcDNA3.3-GFP-transfected cells (Figs. 5D and 6D) indicating that endogenous levels of cytoplasmic hnRNP K are sufficient to promote viral multiplication.

4. Discussion

Knowledge of virus-cell interactions is critical to the understanding of viral replication and pathogenesis. The identification of host cell factors involved in virus multiplication is a major goal in the virology field and diverse RNA-binding cell proteins are implied in the replication of cytoplasmic RNA viruses. hnRNP K is a poly(C) binding protein that contains a K-interaction (KI) domain and its interactions with different molecular partners result in the involvement of this protein in multiple cellular processes (Bomsztyk et al., 2004). Phosphorylation of hnRNP K by mitogen-activated protein kinase (MAPK) ERK induces its re-localization to the cytosol and it was proved that pharmacological inhibitors of ERK pathway prevent cytoplasmic translocation of hnRNP K (Choi et al., 2009). It has been reported that DENV-infection modulates MAPK signaling pathways in different cell lines and activation of ERK pathway has been proved in endothelial vascular cells and human macrophages (Huerta-Zepeda et al., 2008; Ceballos-Olvera et al., 2010). In addition, we have recently demonstrated that JUNV

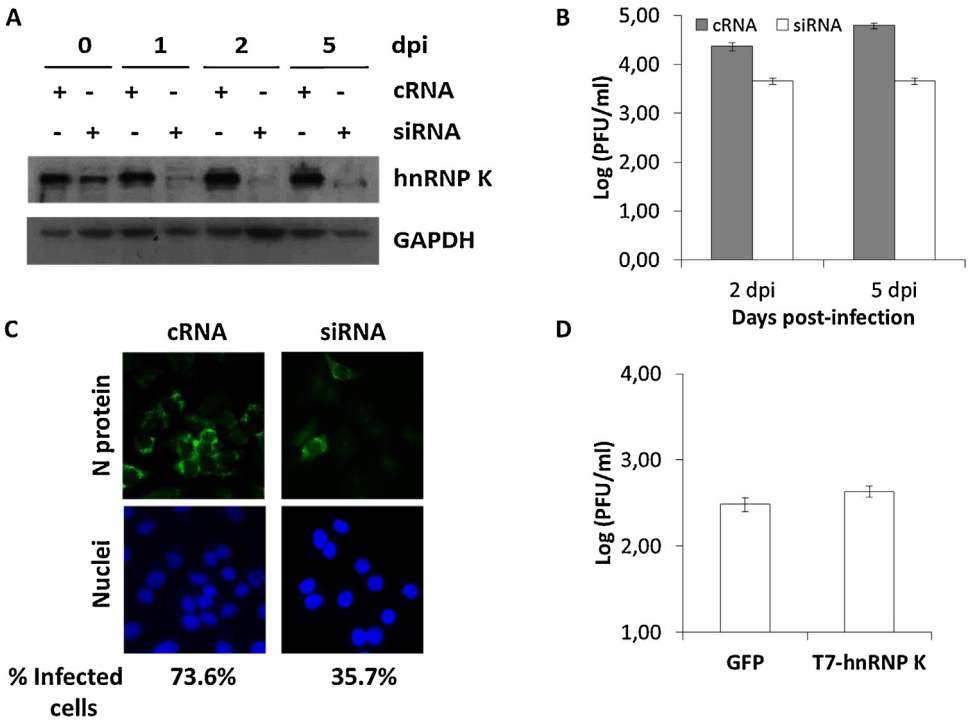

Fig. 6. Effect of silencing or overexpression of hnRNP K on JUNV multiplication. A549 cells were transfected with hnRNP K siRNA or cRNA, and 24 h later cells were infected with JUNV (MOI = 1). (A) The expression levels of hnRNP K were assessed by Western blot at different dpi. (B) Viral yields were measured by plaque assay at 2 and 5 dpi. (C) N-protein expression was analyzed by immunofluorescence assay at 5 dpi. The percentage of infected cells of one representative experiment is shown. (D) A549 cells were transfected with plasmids that allow the expression of GFP or T7-hnRNP K and at 24 h post-transfection cells were infected with JUNV (MOI = 1), and at 2 dpi viral yields were quantified by plaque assay. Viral titers are mean values from three replicates of one representative experiment ±95% CI.

infection induces ERK activation in Vero, A549 and pre-monocytic U937 cells (Rodríguez et al., 2014). Therefore, activation of ERK signaling cascade may account for hnRNP K cytoplasmic accumulation during DENV-2 and JUNV infections. Changes in the total level of expression of hnRNPs have been described for some viruses including DENV (Mishra et al., 2012), however, we could not detect significant alterations in hnRNP K levels in DENV-2 or JUNV-infected cells in comparison with mock-infected cells.hnRNP A2 is a trans-acting factor involved in the intracellular trafficking of RNAs containing the A2 response element (Brumwell et al., 2002). In this work hnRNP A2 was found mainly in the nucleus in mock-infected and JUNV-infected cells indicating that this arenavirus does not produce a general alteration of nucleo-cytoplasmic trafficking, and only a very poor cytoplasmic translocation of hnRNP A2 was detected in DENV-2 infected cultures. Despite the lack of hnRNP A2 cytoplasmic accumulation we have previously shown that hnRNP A2 is a factor that favors JUNV multiplication (Maeto et al., 2011), therefore, we cannot rule out that reduced levels of hnRNP A2 in the cytoplasm, not detected in the assays performed in this work, are sufficient to somehow participate in virus replication. Further studies are needed to ascertain whether hnRNP A2 has a role in DENV-2 multiplication.

On the other hand, in accordance with previous results obtained for hnRNP A1 (Maeto et al., 2011), hnRNP A2 and hnRNP K remained within the nucleus in V3 cells even after superinfection with JUNV. V3 cells are refractory to JUNV multiplication and after superinfection, viral particles are able to penetrate persistently infected cells but protein production of superinfecting virus was totally blocked (Ellenberg et al., 2004). This could be one of the reasons for the lack of hnRNP K cytoplasmic translocation. In addition, the analysis of ERK activation in JUNV persistent cultures is actually been conducted to unravel the implication of cell signaling pathways in V3 nucleo-cytoplasmic trafficking.

Our results show that silencing of hnRNP K reduces DENV-2 replication in line with data reported for HCV (Fan et al., 2014). However, a more recent report points out that knockdown of hnRNP K expression causes a strong enhancement in the release of HCV infectious particles (Poenisch et al., 2015). In the same study no changes in the amount of DENV infectious particles quantified at 2 dpi were observed after hnRNP K silencing. A possible reason for the discrepancy of these results with ours may be ascribed to differences in the cell line, the siRNAs and the knockdown protocol employed. In fact, at 2 dpi we observed a moderate siRNA-mediated reduction in DENV-2 yield whereas a stronger inhibition of viral production was detected at 5 dpi.hnRNP K interacts with the 5′ NCR of enterovius 71 genome and is implied in genome replication (Lin et al., 2008). In addition, a role of hnRNP K on herpes simplex virus type 1 and VSV egress from the cell has been proposed (Schmidt et al., 2010; Dinh et al., 2013). Interestingly, reciprocal immunoprecipitation studies and double immunofluorescence assays performed in EA.hy926 endothelial cells infected with DENV-2 revealed the interaction between hnRNP K and vimentin, component of the intermediate filaments and a strong association of vimentin with NS1 viral protein was also demonstrated. In addition, disruption of vimentin intermediate filaments not only dissociates hnRNPs–vimentin complexes but also reduces extracellular virus yields (Kanlaya et al., 2010). These observations led to speculate about a role of hnRNP K in the release of progeny virus from infected cells. Our data reveal the importance of hnRNP K for DENV multiplication since silencing of this cell factor inhibits viral multiplication and similar results were obtained with the unrelated virus JUNV. It is interesting to note that a previous study showed that the integrity of vimentin intermediate filaments would be necessary for JUNV multiplication, however up to now it is unknown whether there is a direct interaction between viral components and the intermediate filament network (Cordo and Candurra, 2003). The relevance of

the interaction of other viruses such as influenza A and HCV with vimentin filaments for viral protein synthesis has been described (Arcangeletti et al., 1997; Nitahara-Kasahara et al., 2009). In addition, it has been recently proposed that vimentin interaction with DENV NS4A viral protein would be necessary for proper distribution of replicative complex (Teo and Chu, 2014). Hence, we can hypothesize that hnRNP K may play a role in DENV-2 and JUNV protein synthesis and/or RNA replication by facilitating the interaction between viral macromolecules and the cytoskeleton. Further studies are needed to explore this possibility.

In conclusion, our study demonstrates that DENV-2 and JUNV induce the cytoplasmic re-localization of hnRNP K and provides the first experimental evidence that this protein is a host cell factor that promotes DENV-2 and JUNV productive infections.

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