Letter to the Editor

Enhancement of Virus Replication in An Influenza A Virus NS1-Expresssing 293 Cell Line*

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The nonstructural protein 1 (NS1) of influenza A virus, which is absent from the viral particle, but highly expressed in infected cells, strongly antagonizes the interferon (IFN)-mediated antiviral response. We engineered an NS1-expressing 293 (293-NS1) cell line with no response to IFN stimulation. Compared with the parental 293 cells, the IFN-nonresponsive 293-NS1 cells improved the growth capacity of various viruses, but the introduction of NS1 barely enhanced the propagation of Tahyna virus, a negative-strand RNA virus. In particular, fastidious enteric adenovirus that replicates poorly in 293 cells may grow more efficiently in 293-NS1 cells; thus, IFN-nonresponsive 293-NS1 cells might be of great value in diagnostic laboratories for the cultivation and isolation of human enteric adenoviruses.

In general, the innate immune response is launched by host cells within several hours after cellular receptor binding with a virus, which is the first line of defense against viral infection in the infected cell. The complex mechanisms of innate immune response have not yet been fully elucidated, but the induction and expression of interferon (IFN) are known to play important roles in the antiviral responses of host cells. Thus, virus replication in infected cells depends on a race between virus growth and the establishment of an IFN-mediated antiviral state. To proliferate despite the antiviral actions of IFN, several viruses have developed mechanisms to circumvent the IFN response by blocking IFN-induced intracellular signaling or IFN induction[1]. As a consequence, IFN-nonresponsive cells may be beneficial for the replication of viruses, particularly those that are sensitive to IFN.

Interestingly, some negative-strand RNA viruses, such as Sendai virus, Simian virus 5 (SV5)[2-3], Ebola virus[4], and human influenza A virus, possess mechanisms for counteracting the IFN-mediated antiviral reaction. For example, the V protein of SV5 blocks IFN signaling by targeting STAT1, an essential transcription factor for IFN signaling, for proteasome-mediated degradation[2-3]. Similarly, the nonstructural protein 1 (NS1) of influenza A virus, which is absent from the viral particle, but highly expressed in the infected cell, greatly inhibits cellular gene expression and prevents the activation of key components in the IFN system, particularly by antagonizing the IFN-mediated antiviral response[5-6]. A previous study showed that engineering the stable expression of SV5 V protein in laboratory cell cultures blocked IFN signaling and increased the growth of several viruses[7]. In the present study, we extended this method by engineering an NS1-expresssing cell line based on IFN-competent 293 cells, and we also investigated the effects of introducing NS1 on viral kinetics and the possible yield.

We used the NS1 protein from influenza A virus A/PR/8/34 (GenBank No. 956533) because this protein has a defect in the domain responsible for the shutdown of host cell mRNA processing, and thus, it should not be cytotoxic[5]. To produce a cell line expressing NS1 protein, we first constructed a recombinant lentivirus that expressed NS1 protein. A fragment with the PR8 NS1 protein gene was synthesized by Shanghai Biosynthesis Biotechnology (China) and subsequently cloned into the lentivirus vector pCDH cDNA (System Biosciences). The recombinant vector was designated as pCDH-NS1, and it was cotransfected with accessory vectors (pPACKH1-GAG, pPACKH1-REV, and pVSV-G) in HEK293T cells to generate lentivirus particles. Monolayer cultures of 293 cells were infected with the recombinant lentivirus expressing NS1 protein. The cells obtained were then cultured in the

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presence of 3 μg/mL of puromycin to select resistant colonies, and dilution cloning was performed to obtain the NS1-expressing 293 cell line, which was designated as 293-NS1. The expression of NS1 protein by 293-NS1 cells was examined using an indirect immunofluorescence assay. NS1 protein was detected by a mouse polyclonal antibody in conjunction with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody. After staining to detect immunofluorescence, the cell monolayers were examined using a Nikon Microphot-FXA immunofluorescence microscope. A comparison of NS1 protein staining with that of 4, 6-diamidino-2-phenylindole (DAPI), which stains the nucleolus, showed that NS1 protein was expressed at sufficiently high levels to be detected in the nucleolus (Figure 1A). To further confirm the expression of NS1, we verified the chromosomal integration of the NS1 gene and its expression in 293-NS1 cells by PCR and reverse transcription (RT)-PCR, respectively. An amplification fragment of about 660 bp was only detected by PCR in the chromosomal DNA or by RT-PCR using total RNA samples from passaged 293-NS1 cells (Figure1B). These results indicate that the 293-NS1 cell line stably expressed the NS1 protein from influenza A virus A/PR/8/34.

The influenza A virus NS1 protein is known to be a powerful antagonist of IFN induction. Therefore, 293-NS1 cells should be generally nonresponsive to IFN treatment. To assess the IFN-α response, we compared the expression of an IFN-regulated reporter gene in 293 and 293-NS1 cells. Briefly, 293 and 293-NS1 cells were each transfected with the plasmid pISRE-TA-luc (Clontech), which contains the IFN-stimulated response element (ISRE) upstream of a luciferase reporter gene. Subsequently, the cell cultures were induced with 1000 IU/mL IFN-α(+IFN) or were mock treated (-IFN). The cell lysates were then harvested and firefly luciferase activity levels (relative light units) were measured using the *Renilla* Luciferase Assay System according (Promega) to the manufacturer’s instructions. As shown in Figure 1C, IFN-α induced ISRE-dependent gene transcription by about 12-fold in 293 cells, whereas no significant difference in ISRE-dependent gene expression was observed in 293-NS1 cells. These data indicate that the IFN-nonresponsive mechanism of 293-NS1 cells- was attributable to the expression of influenza A virus NS1 protein.

The efficiency of virus replication is strongly influenced by two opposing processes: the antiviral state caused by IFN production and induction, as well as the capacity for virus replication, and the virus-induced inhibition of the IFN response. Therefore, some viruses should grow better in IFN-nonresponsive cells than the parental cells. To investigate whether the introduction of NS1 protein enhanced virus replication, we analyzed the growth curves for various viruses, including Sindbis virus YN87448 (SV), Japanese encephalitis virus P3 (JEV), Tahyna virus XJ0625 (TAHV), and human adenovirus hAdV-41. As shown in Figure 2A, the virus yield of SV increased by about 1.0 log10 (10-fold) in 293-NS1 cells compared with that in 293 cells. Similarly, the infection kinetics of JEV, another positive single-strand RNA virus, changed greatly in 293-NS1 cells, with a steeper growth difference at 48 h postinfection(hpi) or 64 hpi, i.e., amaximum increase

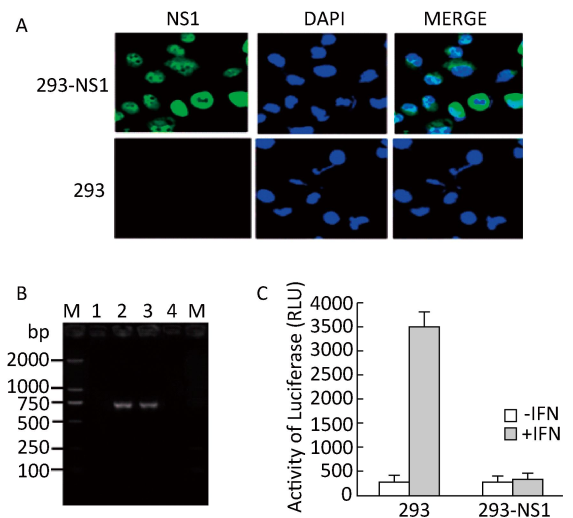

Figure 1. Expression of NS1 protein in 293-NS1 cells. (A) Fluorescence microscopy of 293-NS1 and 293 cells. Monolayers were stained with DAPI, and NS1 protein was stained with FITC-labeled goat anti-mouse secondary antibody, as indicated. The merged images show DAPI staining and the FITC-labeled NS1 protein. (B) Confirmation of NS1 expression in the 293-NS1 cell line. PCR amplification of genomic DNA from 293 (1) and 293-NS1 (2); RT-PCR amplification from the total RNA of 293-NS1 (3) and 293 (4) cells; M: DL-2000 DNA marker. (C) Transient expression assay of IFN responsiveness. The 293 and 293-NS1 cells were transfected with pISRE-TA-luc and then treated with IFN-α (+IFN) or were mock treated (-IFN). Mean±SD luciferase levels are shown.

of approximately 10-fold in 293-NS1 cells (Figure 2B). At all time points, the yield of P3 virus in 293-NS1 cells was approximately 0.8 log10 (6.3-fold) higher than that in the unmodified cell line. In contrast, the yield of TAHV did not differ significantly based on the viral titers in 293-NS1 and 293 cells (Figure 2C). These results indicate that the growth characteristics of Sindbis virus or Japanese encephalitis virus were improved by engineering IFN-nonresponsive 293-NS1 cells, whereas the introduction of NS1 protein barely affected the growth capacity of Tahyna virus, a negative-strand RNA virus.

To demonstrate that the enhanced virus growth was mediated by the IFN antagonist capacity of NS1, we investigated the IFN sensitivity of the tested viruses. As shown in Figure 3, treatment of 293 cells with IFN-α reduced plaque formation by YN87448 or P3 in a dose-dependent manner, i.e., ca 40% inhibition at 800-1200 IU/mL, whereas infection by XJ0625 was inhibited only marginally (ca 6%). These results indicate that both YN87448 and P3 are sensitive to IFN-α treatment, whereas IFN-α signaling plays a minor role in the replication of XJ0625 virus. Therefore, the influence on virus growth in 293-NS1 cells was mediated at least partly by IFN suppression *via* NS1.

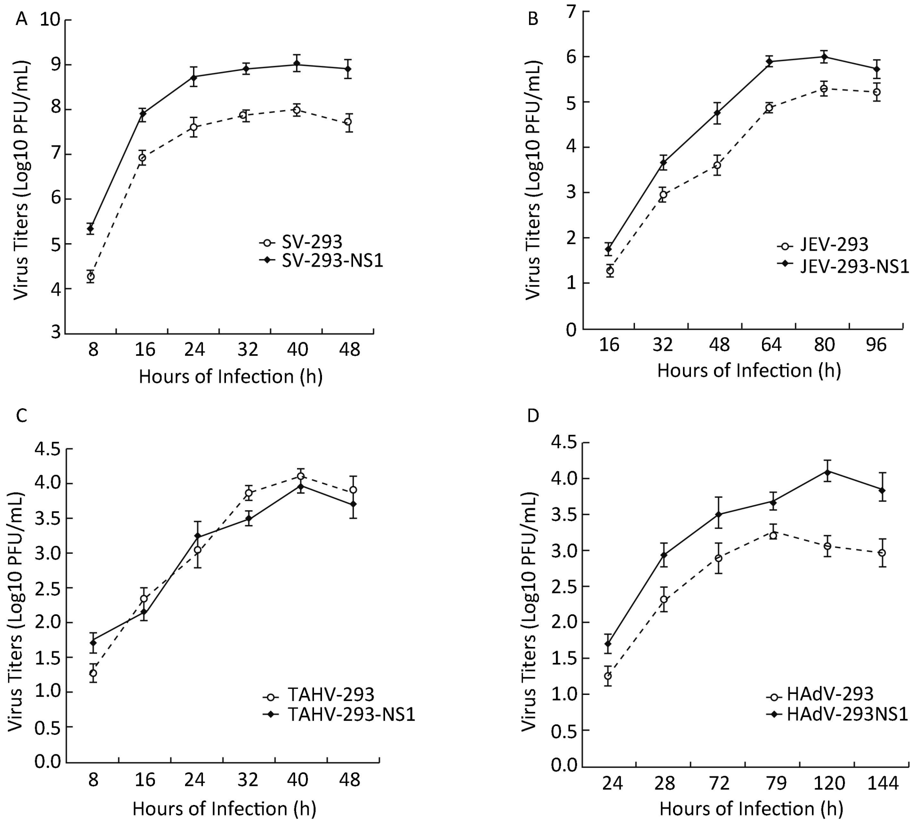

Figure 2. Virus growth curves in 293 cells and 293-NS1 cells. (A), (B), (C), and (D) show the results for Sindbis virus YN87448 (YN-293 and YN-293-NS1), Japanese encephalitis virus P3 (P3-293 and P3-293-NS1), human enteric adenovirus hAdV41 (hAdV-293 and hAdV-293-NS1), and Tahyna virus XJ0625 (XJ-293 and XJ-293-NS1), respectively. Monolayers of 293 cells or 293-NS1 cells were infected at a multiplicity of infection of 0.01 PFU per cell for YN87448, P3, and XJ0625 and 50 infectious units for hAdV-41 virus. After infection for 1 h at 37 °C, the medium was removed and culture medium was added. At each time point, the medium was removed and the virus yield was determined in cell culture medium by plaque titration in BHK-21 cells for YN87448, P3, and XJ0625 (Zhu et al., 2009) and by limiting dilution assay in 293 cells for hAdV-41 (Lu et al., 2009). Values represent the mean±SD based on three independent samples for each time point.

Human enteric adenoviruses propagate poorly in conventional human cell lines that are used to grow other adenovirus serotypes. In a previous study, the growth capacity of the human enteric adenovirus type 40 (hAdV-40) was improved in a modified IFN-nonresponsive cell line *via* the constitutive expression of the V protein from SV5 (Sherwood et al., 2007), thereby suggesting that IFN sensitivity may be at least partly responsible for the fastidious growth of human enteric adenoviruses. To investigate whether NS1 expression could enhance the propagation capacity of human enteric adenovirus, we performed a quantitative comparison of the yield of another fastidious human adenovirus, hAdV-41, in 293 cells and 293-NS1 cells. Comparison of hAdV-41-infected 293-NS1 cells with parental 293 cells showed that the recombinant cell line generated more viral progeny with 0.7 log10 (5.0-fold) higher titers (Figure 2D), which suggests that the defect in hAdV-41 in counteracting the IFN response can be overcome at least partly by using the 293-NS1 cell line. The results were particularly striking with hAdV-41 virus and other fastidious adenoviruses. Fastidious adenoviruses that replicate poorly in 293 cells may grow more efficiently in 293-NS1 cells; therefore, IFN-nonresponsive cells might be of great value in diagnostic laboratories for rapid isolation of known and/or unknown adenoviruses.

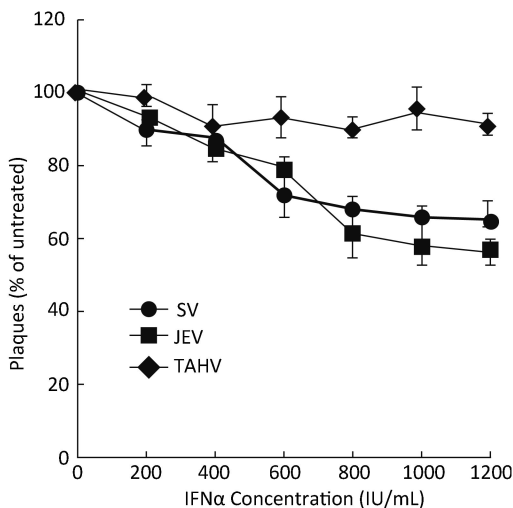

Figure 3. Effect of IFN-α treatment on plaque formation. Confluent 293 cell monolayers were treated with IFN-α at the concentrations indicated. After washing three times with phosphate-buffered saline, the cells were infected with viruses at a multiplicity of infection of 0.01 PFU per cell (YN87448, P3, and XJ0625). Plaque formation was analyzed, and the values represent the mean±SD based on three independent samples for each time point.

Previous studies have shown that expression of viral IFN antagonist proteins is a simple and efficient method for reducing IFN signaling and increasing the virus yield[7-9]. In our study, we found that NS1-expressing 293 cells exhibited significantly reduced IFN signaling, there by facilitating an increase in virus replication and the final virus yield for some positive-strand RNA viruses and fastidious adenoviruses, such as SV, JEV, and hAdV, whereas there was no significant effect on the yield of TAHV. These findings agree well with those obtained in other studies, which showed that the growth of NS1-deleted influenza viruses with the capacity for IFN induction was promoted when IFN signaling was absent or inhibited[4,10-11]. However, other wild-type influenza virus strains with the capacity for IFN suppression obtained only slightly increased yields in NS1-transfected cells. In particular, IFN-nonresponsive 293-NS1 cells failed to improve the yield of TAHV, possibly because of its insensitivity to IFN. In addition, the 293-NS1 cells constructed in this study retained their genetic stability, and their morphological and proliferation characteristics were similar to those of the parental 293 cells, where NS1 expression was detected continuously in 293-NS1 cells after ≥10 repeated passages. In conclusion, IFN-nonresponsive NS1-293 cells could play an important role in the cultivation and isolation of various viruses, especially human enteric adenoviruses.

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