The 2009 pandemic A/Wenshan/01/2009 H1N1 induces apoptotic cell death in human airway epithelial cells

Ning Yang1,†, Xiaoxu Hong1,2,†, Penghui Yang3,†, Xiangwu Ju¹, Yugo Wang¹, Jun Tang¹, Chenggang Li², Quanshui Fan³, Fuqiang Zhang³, Zhongwei Chen³, Li Xing³, Zhongpeng Zhao³, Xiao Gao³, Guoyang Liao², Qihan Li², Xiliang Wang³,*, Fangsheng Li⁵, and Chengyu Jiang¹,*

1 State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Peking Union Medical College, Tsinghua University, Chinese Academy of Medical Sciences, Beijing 100005, China
2 Department of Viral Immunology, Institute of Medical Biology, Chinese Academy of Medicine Science, Peking Union Medical College, Tsinghua University, Chinese Academy of Medical Sciences, Beijing 100005, China
3 State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China
4 Centre for Disease Prevention and Control, Cheng Du Military Region, Kunming 650032, China
5 Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
* Correspondence to: Chengyu Jiang, E-mail: chengyujiang@gmail.com; Xiliang Wang, E-mail: xiliangwang@126.com

In 2009, a novel swine-origin H1N1 influenza virus emerged in Mexico and quickly spread to other countries, including China. This 2009 pandemic H1N1 can cause human respiratory disease, but its pathogenesis remains poorly understood. Here, we studied the infection and pathogenesis of a new 2009 pandemic strain, A/Wenshan/01/2009 H1N1, in China in human airway epithelial cell lines compared with seasonal H1N1 influenza virus. Our results showed that viral infection by the A/Wenshan H1N1 induced significant apoptotic cell death in both the human nasopharyngeal carcinoma cell line CNE-2Z and the human lung adenocarcinoma cell line A549. The A/Wenshan H1N1 virus enters both of these cell types more efficiently than the seasonal influenza virus. Viral entry in both cell lines was shown to be mediated by clathrin- and dynamin-dependent endocytosis. Therefore, we discovered that the 2009 pandemic H1N1 strain, A/Wenshan/01/2009, can induce apoptotic cell death in epithelial cells of the human respiratory tract, suggesting a molecular pathogenesis for the 2009 pandemic H1N1.

Keywords: apoptosis, respiratory, S-OIV H1N1, influenza virus

Introduction

In April 2009, a new strain of influenza virus was detected that caused disease and was transmitted in humans by Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team [Centers for Disease Control and Prevention (CDC), 2009]. This new swine-origin H1N1 influenza A virus (S-OIV H1N1) spread efficiently around the world, leading the World Health Organization (WHO) to declare the outbreak of a pandemic on June 11, 2009. Although the infection was mild for most individuals, the young and those with certain underlying conditions (including asthma, diabetes, morbid obesity, and pregnancy) seemed to be at great risk of severe disease progression (Jain et al., 2009). On August 10, 2010, the WHO announced that the H1N1 influenza virus had moved into the post-pandemic period. However, localized outbreaks of various magnitudes are likely to continue. According to the latest WHO statistics, the virus has killed more than 18 000 people, ~4% of the 250 000–500 000 annual influenza deaths. The Health Protection Agency (HPA) reported that 195 of the recent 214 confirmed deaths in the UK had been infected with the 2009 H1N1 strain, suggesting that this S-OIV H1N1 may return.

The 2009 pandemic H1N1 derived from two unrelated swine H1N1 viruses (Garten et al., 2009; Taubenberger et al., 2010), one of them a ‘classic’ swine derivative of the 1918 human virus and the other of European avian-like H1N1 lineage (Dunham et al., 2009). Sequence analysis of its whole genome has failed to identify any previously recognized virulence markers. Animal studies have indicated that 2009 pandemic H1N1 is slightly more pathogenic than contemporary human seasonal H1N1 viruses (Itoh et al., 2009; Maines et al., 2009; Munster et al., 2009). In a mouse model, the 2009 H1N1 virus also replicated more efficiently and caused greater morbidity and mortality than seasonal influenza virus (Itoh et al., 2009; Maines et al., 2009).

These authors contributed equally to this work.

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Programmed cell death, or apoptosis, is critical for many physiological processes, including tissue atrophy, development, and tumor biology (Majno and Joris, 1995; Jacobson et al., 1997). Apoptosis also plays an important role in the pathogenesis of many infectious diseases, including those caused by viruses (Young et al., 1997; Ludwig et al., 1999). Many viral infections cause apoptosis in host cells, and the influenza virus induces apoptosis in numerous cell types, both in vivo and in vitro (Mori et al., 1995; Morris et al., 1999). The alveolar epithelial cells or vascular endothelial cells of human patients and chickens infected with H5N1-AVI were reported to undergo apoptosis (Ito et al., 2002; Uiprasertkul et al., 2007). Other reports suggest that apoptosis of these cells is essential in humans for the development of acute respiratory distress syndrome, which is observed in H5N1-AVI-infected patients (Kuwano, 2007). The 1918 H1N1 influenza virus and H9N2 also have been shown to induce apoptosis in infected mice and cell cultures (Baskin et al., 2009; Xing et al., 2009a, b). Until now, however, there has been no report on apoptosis induced by 2009 pandemic H1N1. In this study, we have screened a few 2009 pandemic S-OIV H1N1 strains isolated in China and found that A/Wenshan/01/2009 H1N1 can cause apoptotic cell death in both human airway epithelial cell lines A549 and CNE-2Z. We also found that CNE-2Z cells from the upper respiratory tract are more susceptible to A/Wenshan H1N1 infection than A549 cells, which originate in the lower respiratory tract. When compared with contemporary seasonal H1N1 A/Jinnan virus, A/Wenshan H1N1 displayed higher entry efficiency and virus replication.

Results

A/Wenshan H1N1 virus induces cell death in CNE-2Z cells

We infected cells with A/Wenshan H1N1, a virus strain that emerged in China (A/Wenshan/01/2009 H1N1) and compared its induced cytotoxicity with a seasonal A/Jinnan H1N1 strain (A/Tianjin-Jinnan/15/2009 H1N1). We first used a human

![Graph showing cell viability](image1)

**Figure 1** A/Wenshan H1N1 induced human nasopharyngeal carcinoma CNE-2Z cell death. MTT assay of CNE-2Z cells treated with 2009 pandemic A/Wenshan H1N1 virus and seasonal A/Jinnan H1N1 virus at 1 MOI and 5 MOI at different time points (12, 24, 48, 72 h). *P < 0.01, **P < 0.001.

![Graph showing cell viability](image2)

**Figure 2** A/Wenshan H1N1 induced human lung adenocarcinoma A549 cell death. MTT assay of A549 cells treated with 2009 pandemic A/Wenshan H1N1 virus and seasonal A/Jinnan H1N1 virus at 1 MOI and 5 MOI at different time points (12, 24, 48, 72 h). *P < 0.01, **P < 0.001.

nasopharyngeal carcinoma cell line, CNE-2Z, which is derived from the human upper respiratory tract, to test its sensitivity to virus infection. Cells infected with these two H1N1 strains (multiplicity of infection: 1 MOI and 5 MOI) were incubated at 37°C, and cell viability was measured with the MTT assay at 12, 24, 48, and 72 h post-infection. Virus titer was confirmed by qRT–PCR of the viral M gene in virus stocks (Supplementary Figure S1). Viability decreased among all A/Wenshan H1N1-infected CNE-2Z cells from 24 h post-infection, indicating that the 2009 pandemic A/Wenshan H1N1 virus induced cell cytotoxicity; at 72 h post-infection, cell viability was further decreased (Figure 1).

A/Wenshan H1N1 virus induces cell death in A549 cells

We also infected the human lung adenocarcinoma cell line A549, which originates from the lower respiratory tract. A/Wenshan H1N1 infection (1 MOI and 5 MOI) can also induce significant cell death starting at 48 h post-infection (Figure 2). Although we used a high dose (10 MOI) of seasonal A/Jinnan H1N1, cell death was not observed in either CNE-2Z or A549 cells (Supplementary Figure S2). This finding suggests that infection with 2009 pandemic A/Wenshan H1N1 virus can induce progressive, irreversible cytotoxicity in A549 cells, whereas infection with seasonal A/Jinnan H1N1 virus does not induce significant cytotoxicity. Taken together, we found that A/Wenshan virus is more virulent than A/Jinnan virus, and the CNE-2Z cell line was more susceptible to A/Wenshan H1N1 infection than the A549 cell line, indicating epithelial cells derived from the upper respiratory tract may be more easily damaged by A/Wenshan H1N1 infection.

The growth kinetics of A/Jinnan and A/Wenshan viruses in both CNE-2Z and A549 cells

To assess the relationship between cytopathicity and virus replication in the tested cell lines, we studied the growth kinetics of A/Wenshan H1N1 and A/Jinnan H1N1 viruses. The total RNA from infected cells at different time points was harvested and the M, NS1, and NP mRNA copy numbers in infected cells were...
normalized to GAPDH. In CNE-2Z cells, A/Wenshan H1N1 produced high copies of viral mRNA from 24 h post-infection; in contrast, A/Jinnan H1N1 did not produce significant levels of viral mRNA (Figure 3A). Similarly, in A549 cells, A/Wenshan H1N1 produced high copies of viral mRNA from 24 h post-infection; in contrast, A/Jinnan H1N1 did not produce significant levels of viral mRNA (Figure 3B). These results suggest that the cell death in the A/Wenshan H1N1-infected cell lines may be due to its unusually high replication rate.

**Caspase 3-dependent apoptosis is involved in A/Wenshan H1N1 virus-induced CNE-2Z cell death**

To elucidate which type of cell death is involved during A/Wenshan H1N1 infection, we tested if A/Wenshan H1N1 virus infection induces apoptotic cell death. The hallmark assay of apoptosis, the TUNEL assay, was performed using terminal deoxynucleotidyl transferase (TdT), which catalyzes the addition of FITC-labeled dUTPs onto nicks in DNA (Gavrieli et al., 1992). As can be seen in Figure 4A, the apoptotic cells were clearly observed in A/Wenshan H1N1-infected cells (the black dots) but not in mock-infected cells and A/Jinnan H1N1-infected cells at 24 h post-infection. Statistical analysis showed that the percentage of apoptotic cells was much higher in A/Wenshan H1N1-infected cells (Figure 4B).

To further confirm our observation, we analyzed other hallmarks of apoptosis, PARP and caspase 3 activation, by immunoblot analysis (Martin et al., 1994). The activation of caspase 3 requires proteolytic processing of its inactive proenzyme into active 17 and 12 kDa subunit proteins (Nicholson et al., 1995). The anti-caspase 3 antibody used in this analysis recognizes 35 kDa procaspase 3 and the 17 kDa subunit protein. At 24 h post-infection, activated caspase 3 was detected in A/Wenshan H1N1-infected CNE-2Z cells, but not in the mock-infected control and seasonal A/Jinnan H1N1-infected cells (Figure 4C). Following the increase in virus MOI, the caspase 3 cleaved bands increased gradually in groups of A/Wenshan H1N1 infection (Figure 4D). The death substrate PARP, which is a key substrate for active caspase 3 (Tewari et al., 1995), was also proteolytically cleaved to generate an 89 kDa fragment in A/Wenshan H1N1-infected cells, but not in the mock-infected control and A/Jinnan H1N1-infected cells. The degree of PARP activation corresponds to the caspase 3 activation in A/Wenshan H1N1 groups (Figure 4E). Last, we wanted to distinguish if the apoptosis was induced in antigen-positive cells. We found that abundant TUNEL-positive cells were detected in A/Wenshan H1N1-infected CNE-2Z cells (Figure 4D), but few TUNEL-positive cells were detected in control and A/Jinnan H1N1-infected cells. These results indicate that caspase 3-dependent apoptosis was involved in A/Wenshan H1N1-induced CNE-2Z cell death.

**Caspase 3-dependent apoptosis is involved in A/Wenshan H1N1-induced A549 cell death**

A TUNEL assay was performed in A549 cells, and apoptotic cells were clearly observed in A/Wenshan H1N1-infected A549 cells (the black dots), but not in mock-infected and A/Jinnan H1N1-infected cells at 48 h post-infection (Figure 5A). Statistical analysis revealed that the percentage of apoptotic cells in A/Wenshan H1N1-infected cells is much higher than in mock- and A/Jinnan H1N1-infected A549 cells (Figure 5B).

We further analyzed caspase 3 and PARP activation in A549 cells by immunoblot analysis. At 48 h post-infection, activated caspase 3 was detected in A/Wenshan H1N1-infected A549 cells but not in mock-infected and A/Jinnan H1N1-infected cells (Figure 5C). PARP is also cleaved to generate an 89 kDa fragment in A/Wenshan H1N1-infected A549 cells (Figure 5C). Finally, we detected TUNEL-positive cells in A/Wenshan H1N1-infected A549 cells (Figure 5D), but few TUNEL-positive cells were detected in control or A/Jinnan H1N1-infected cells. These results suggest that A/Wenshan H1N1-induced A549 cell death is caspase 3-dependent apoptosis.
Figure 4  A/Wenshan H1N1 induces apoptosis in the CNE-2Z cell line. (A and B) After 24 h infection (1 MOI), CNE-2Z cells were examined by TUNEL assay. (A) Original magnification. The arrows indicate apoptotic cells (black). Scale bar, 400 μm. (B) Statistical analysis of relative proportion of TUNEL-positive cells. For quantification, >1000 cells were scored in three independent experiments. **p < 0.001. (C) After 24 h infection, western blot analysis of mock-infected and H1N1-infected CNE-2Z cell lysate with anti-caspase 3, anti-PARP, and anti-β-actin antibodies. (D) TUNEL assay for detection of apoptosis in CNE-2Z at 24 h post-infection (1 MOI). Parallel samples were cultured with the caspase inducer actinomycin D (4 μg/ml) as a positive control. The arrows indicate apoptotic cells (green). Viral antigen was detected simultaneously with a polyclonal antibody against the NP proteins (red). Cellular nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 100 μm.

Figure 5  A/Wenshan H1N1 induces apoptosis in the A549 cell line. (A and B) After 48 h infection (1 MOI), A549 cells were examined by TUNEL assay. (A) Original magnification. The arrows indicate apoptotic cells (black). Scale bar, 400 μm. (B) Statistical analysis of relative proportion of TUNEL-positive cells. For quantification, >1000 cells were scored in three independent experiments. **p < 0.001. (C) After 48 h infection, western blot analysis of mock-infected and H1N1-infected A549 cell lysate with anti-caspase 3, anti-PARP, and anti-β-actin antibodies. (D) TUNEL assay for detection of apoptosis in A549 cells at 24 h post-infection (1 MOI). Parallel samples were cultured with the caspase inducer actinomycin D (4 μg/ml) as a positive control. The arrows indicate apoptotic cells (green). Viral antigen was detected simultaneously with a polyclonal antibody against the NP proteins (red). Cellular nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 100 μm.
A/Wenshan H1N1 displays higher entry efficiency than A/Jinnan H1N1 in CNE-2Z cells

We infected CNE-2Z cells with H1N1 and carried out a single-hit infection assay (5 MOI) at an early time point of infection (6 h post-infection) based on immunofluorescence microscopy using an anti-NP antibody. We chose this time point because the NP gene does not replicate efficiently before 6 h, and therefore, at such an early time point, the viral RNA seen is solely from the stocks used to infect the cells (Supplementary Figure S3). NP is a fragment component of H1N1 virus. A/Wenshan H1N1 showed higher levels of infection compared with A/Jinnan H1N1 infection (Figure 6A and B). Control cells without viral infection showed no green fluorescence from NP (data not shown). Therefore, we tested whether the difference of entry efficiency between the two virus strains was related to the cell entry pathways. We studied the entry pathways exploited by H1N1 by infecting CNE-2Z cells.

Because endocytosis requires a low pH environment, we evaluated bafilomycin A1 (BFA), a highly specific inhibitor of vacuolar type H^+^-ATPase (V-ATPase), and chloroquine, a lysosomotropic agent that accumulates preferentially in the lysosomes of cells (Wiesmann et al., 1975; Yoshimori et al., 1991). Treatment of CNE-2Z cells with BFA and chloroquine before infection at 37°C significantly reduced the expression of NP in the nuclei (Figure 6C and D). Thus, it can be concluded that H1N1 requires an acidic intracellular pH to penetrate into CNE-2Z cells, indicating that A/Wenshan H1N1 and A/Jinnan H1N1 entry occurs through a low pH-dependent endocytic route.

We then examined whether clathrin-mediated endocytosis was involved in the infection (Doherty and McMahon, 2009). Immunofluorescence analysis demonstrated that viral NP expression was significantly decreased by the clathrin inhibitor chlorpromazine in a dose-dependent manner (Figure 6E). Next, we pretreated CNE-2Z cells with dynasore, a small molecular inhibitor of dynamin GTPase activity that prevents the scission of clathrin-coated pits from the plasma membrane (Macia et al., 2006). Dynasore diminished virus antigen expression of NP in a dose-dependent manner (Figure 6F). Finally, we defined the role of caveolin-mediated endocytosis in H1N1 infection of CNE-2Z cells. Nystatin was applied to specially block this endocytic pathway (Anderson et al., 1996). Nystatin did not inhibit virus entry at 6 h post-infection (Figure 6G). To assess the effect of
inhibitors on cell viability, high concentrations of drugs were added to the cells. We found that these inhibitors had no obvious effect on cell viability, indicating the virus entry inhibition was not related with any cytotoxic effect of the drugs (Supplementary Figure S4). Therefore, we conclude that H1N1 infection of CNE-2Z cells is mediated by clathrin- and dynamin-dependent endocytosis.

**Discussion**

Our study focused on a new strain of 2009 pandemic H1N1 isolated in China and tested its pathogenicity in two human epithelial cell lines. We found that the A/Wenshan H1N1 we used can produce pronounced cytopathic effects in the tested cell lines, suggesting that those two cell types may also be targeted during 2009 pandemic virus infection, and that this highly pathogenic virus—Wenshan H1N1—can cause cellular damage in human nasopharynx and lung epithelial cells. Our study suggests
that the upper respiratory tract is more sensitive to A/Wenshan H1N1 infection than the lower respiratory tract. The cytopathic effect in cells was related to caspase 3-dependent apoptosis induced by the virus. Due to these results, we speculate that the apoptotic cell death induced by A/Wenshan H1N1 or some homologous strains in airway epithelial cells may account for the critical illness of some patients.

Previous reports have confirmed that several strains of influenza A virus can induce apoptosis in cell lines and animal models (Daidoji et al., 2008; Lam et al., 2008; Xing et al., 2009b). H5N1 induces apoptotic cell death in human lung epithelial cells, and 1918 H1N1 influenza virus also activates the apoptotic signaling pathway in infected animals (Baskin et al., 2009; Ueda et al., 2010). Previous studies have shown that the PB1-F2 protein plays an important role in determining the degree of virulence seen in influenza virus infection (Chen et al., 2001; Zamarin et al., 2005; Gocnikova and Russ, 2007). PB1-F2 is a short viral protein of ~90 amino acids expressed from a +1 reading frame in the PB1 gene segment (Chen et al., 2001). To date, PB1-F2 is the only protein of the 11 proteins coded by influenza virus that has been proven to induce apoptosis directly. However, the 2009 pandemic H1N1 does not encode the full length of PB1-F2 protein (Hai et al., 2010; Herfst et al., 2010; McAuley et al., 2010). Therefore, the apoptosis induced by A/Wenshan H1N1 may be caused by other mechanisms. Previous studies have shown that several cell signaling pathways are involved in the cell death process, including the p38 signaling pathway, the PI3K-Akt signaling pathway, and the extrinsic apoptotic pathway (Ludwig et al., 2006; Ehrhardt et al., 2007; McLean et al., 2009; Nencioni et al., 2009). Recently, Ueda et al. (2010) found that the extracellular Ca\(^{2+}\) influx induced by the highly pathogenic H5N1 influenza virus can lead to apoptosis in avian cells.

Here, we show that the 2009 pandemic H1N1 new strain—A/Wenshan H1N1—can induce apoptosis in human cells, and as far as we know, this is the first demonstration that a 2009 pandemic H1N1 strain can induce apoptosis in human cells, a pathway that is likely to contribute to virus pathogenesis. A/Wenshan H1N1 displays a higher replication rate than A/Jinlan H1N1, which may partially account for the higher virulence of A/Wenshan H1N1. However, the key factors determining the higher replication rate of A/Wenshan H1N1 are yet to be studied.

Our study also showed that A/Wenshan H1N1 entry into cells is more efficient than the entry of contemporary seasonal H1N1–A/Jinlan H1N1. Other studies have shown that influenza virus infection of target cell is a multi-step process. After binding to sialic acid on the cell surface, influenza virus is internalized into the endosome by receptor-mediated endocytosis. The fusion between the virus and the endosomal membrane leads to the release of vRNPs into the cytoplasm. vRNPs are then imported into the nucleus and replicated (Sieczenkarski et al., 2002a; Lakadamyali et al., 2004). Influenza virus has been reported to exploit clathrin-mediated endocytosis, caveolin-mediated, or clathrin- and caveolin-independent pathways, depending on the type of target cells (Sieczenkarski et al., 2002b; Nunes et al., 2004; Rust et al., 2004). Here, we determined the dominant entry pathway for the two virus strains. Although they differ in cell entry efficiency, they exploit the same entry pathway to enter the cells, implying that different influenza A virus strains tend to utilize the same entry pathway when infecting the same cell line.

Thus, in this report, we have elucidated a molecular pathogenesis for 2009 pandemic H1N1, which might be useful to prepare for the possible return of such a pandemic.

**Materials and methods**

**Cell lines and antibodies**

The human lung adenocarcinoma A549 cell line was purchased from ATCC and cultured in DMEM (Hyclone) supplemented with 10\% FBS (Hyclone) and 100 U/ml penicillin/streptomycin at 37°C with 5\% CO\(_2\). The human nasopharyngeal carcinoma CNE-2Z cell line was purchased from the cell culture center of Peking Union Medical College and cultured in DMEM supplemented with 10\% FBS, 100 U/ml penicillin/streptomycin at 37°C, and 5\% CO\(_2\).

The primary antibodies used in the analysis, anti-PARP and anti-caspase 3, were purchased from Cell Signaling Technology. The anti-NP antibody was purchased from Millipore. The Alexa Fluor 488 goat anti-mouse antibody was purchased from Invitrogen. The anti-β-actin antibody was purchased from Sigma-Aldrich. Horse radish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

**Influenza virus preparation and infection of cells**

The wild-type pandemic 2009 H1N1 virus A/Wenshan/01/2009 H1N1 was kindly provided by the Centre for Disease Prevention and Control, Cheng Du Military Region. The wild-type seasonal 2009 H1N1 virus A/Tianjin-Jinan/15/2009 H1N1 was kindly provided by the Institute of Medical Biology, Chinese Academy of Medical Science. Influenza virus was grown in 10-day-old embryonated eggs, and working stocks were stored at −80°C. Live virus experiments were performed in biosafety facilities under governmental and institutional guidelines.

Before infection, cells were washed three times in DMEM to remove FBS and then incubated with influenza virus diluted in DMEM for 1 h at 37°C. After 1 h, cells were washed and maintained in growth medium (DMEM containing 10\% FBS) for the indicated amount of time.

For the inhibition experiments of virus entry, cells were pre-treated with BFA, chloroquine, chlorpromazine, dynasore, and nystatin (Sigma-Aldrich) at the indicated concentration for 30 min and then infected with influenza virus. Every reagent was present during H1N1 infection. After 1 h incubation, the reagents and virus were washed and maintained in growth medium.

**TCID\(_{50}\) assay**

The virus stocks were 10-fold serially diluted with PBS and applied in quadruplicate to 2.5 × 10\(^5\) MDCK cells/well on a 96-well plate. The cells were supplied with DMEM containing TPCk-trypsin (5 μg/ml). On the fourth day after infection, the virus titer was determined by observing cytopathogenic effects and confirmed by hemagglutination. TCID\(_{50}\) was determined on the basis of the Reed–McUench method as described previously.
tion. dUTPs were visualized using HRP-conjugated antivariated with 3% hydrogen peroxide in methanol. After the TdT reaction, dUTPs were fixed with paraformaldehyde in PBS and permeabilized for another 2 h. Absorbance was then recorded at 490 nm.

Western blotting

Cells were seeded in 96-well plates at 1 x 10^5 cells/ml. Influenza virus or allantoic fluid was added to the wells the next day. Each group had triplicate wells. After incubating for the indicated times, 20 μl CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was added to each well and incubated for another 2 h. Absorbance was then recorded at 490 nm.

Real-time quantitative PCR

Total RNA was isolated using Trizol reagent (Invitrogen). The first-strand cDNA was synthesized with 1.5 μg of each total RNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR amplification assays were performed with the LightCycler® 480 SYBR Green Master (Roche Diagnostics) on The LightCycler® 480 Real-Time PCR System (Roche). Each value was calculated automatically by the LightCycler® 480 software, using human GAPDH as the reference. The specific primers that could detect both the virus and the reference gene used were as follows: NS1 forward, 5′-ATGAGGATGTCAAAAATGCA-3′, reverse, 5′-TTTCCAAAGCCGATCTCTGTAGA-3′; NP forward, 5′-TGTCCTTCCAGGGGGC-3′, reverse, 5′-TACCTCTGACATTGTCTC-3′; M forward, 5′-AAGACCAATCTGACATTGTCTC-3′, reverse, 5′-CAAAGGCTCTAGCAGGATT-3′; human GAPDH forward, 5′-GGTGGTCTCCCTCTGATCTCAACA-3′; reverse, 5′-GTTGCTGTAGGCAATTTGTTG-3′.

TUNEL assay

Cells were seeded on coverslips in 24-well plates. One day later, virus infection was performed at an MOI of 5. Then, A549 cells were cultured for 48 h, and CNE-2Z cells were cultured for 24 h. Parallel samples were cultured with the caspase inducer actinomycin D (4 μg/ml) as a positive control. Apoptotic cells were characterized by positive terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining according to the manufacturer’s instructions (In Situ Cell Death Detection Kit, POD; Roche). Briefly, cells were fixed with paraformaldehyde in PBS and permeabilized using 0.1% Triton X-100. Endogenous peroxidases were inactivated with 3% hydrogen peroxide in methanol. After the TdT reaction, dUTPs were visualized using HRP-conjugated anti-fluorescein antibody and 3,3′-diaminobenzidine.

Indirect immunofluorescence microscopy

Virus- or mock-infected cells were fixed with 4% paraformaldehyde in PBS at room temperature (rt) for 20 min, after which cells were washed with PBS and permeabilized in blocking buffer (PBS containing 10% FBS, 3% BSA, and 0.5% Triton X-100) at rt for 15 min. Cells were then incubated with primary antibodies against influenza NP in blocking buffer either at rt or at 4°C overnight. Cells were washed with wash buffer (PBS containing 0.2% BSA and 0.1% Triton X-100) and incubated with secondary antibodies diluted in blocking buffer at rt for 1 h. Cell nuclei were stained with Hoechst 33342 (Sigma-Aldrich) diluted in PBS for 10 min at rt. Images were captured by a Nikon Eclipse TE2000-U inverted fluorescence microscope. Analysis was performed with Image Pro Plus software.

Statistical analysis

All data are shown as mean ± SEM, and statistical analyses are conducted using the Student t-test.

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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Conflict of interest: none declared.

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