Requirement for Siva-1 for replication of influenza A virus through apoptosis induction

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Infection with influenza A virus causes acute respiratory tract infections in humans and may lead to lethal diseases including pneumonia. Identifying host factors that are involved in the severity of infectious diseases caused by influenza A virus is considered important for the prevention and treatment of these viral infections. This report demonstrated that Siva-1 is crucial for the induction of apoptosis caused by infection with influenza A virus and is involved in virus replication. Susceptibility to apoptosis induced by influenza A virus infection was increased in human lung-derived A549 cells, which stably express Siva-1. In addition, induction of apoptosis after influenza A virus infection was strongly inhibited by knockdown of Siva-1 expression. Furthermore, the replication of influenza A virus was significantly suppressed in A549 cells in which Siva-1 expression was inhibited and the effect of Siva-1 knockdown was eliminated by treatment with Z-VAD-FMK. These findings suggest that the caspase-dependent pathway for induction of apoptosis is involved in Siva-1-mediated influenza A virus replication.

INTRODUCTION

Influenza is a respiratory tract infectious disease caused by influenza virus. Influenza virus is an enveloped RNA virus belonging to the family Orthomyxoviridae, and its genome consists of segmented negative-sense RNAs encoding virus-specific proteins. There are three types of influenza virus, A, B and C. Only influenza A virus has caused pandemics in the past, of which there were several in the previous century (Johnson & Mueller, 2002; Kobasa et al., 2004; Trilla et al., 2008). Influenza A virus is also the cause of almost annual epidemics known as ‘seasonal flu’. The estimated death rate from seasonal flu is less than 0.1 % of infected patients, and the virus causing seasonal flu is relatively weaker than the virus that has caused pandemics in the past. However, it is estimated that about 250 000–500 000 patients still succumb to seasonal flu every year worldwide (Suzuki, 2005). Influenza A virus may also be a cause of lethal infectious diseases culminating in severe pneumonia. Certain population groups are known to be at higher risk, including older adults, very young children, pregnant women and individuals with chronic diseases, and infection can lead to death in susceptible individuals. Therefore, identification of the factors contributing to the severity of infectious diseases caused by influenza A virus is necessary to provide effective treatment and prevent lethal infections.

Apoptosis is the process of programmed cell death and is physiologically important for maintaining the homeostasis
of individuals. Dysfunction of the regulation of apoptosis causes cancer (Brown & Attardi, 2005; Hajra & Liu, 2004), autoimmune diseases (O’Riordan & Korsmeyer, 2003; Tanaka & Miyake, 2007), underdevelopment (Suzuki et al., 2009) and neuronal diseases (Abou-Sleiman et al., 2006; Nakamura & Lipton, 2009). In addition, apoptosis is closely related to the pathogenicity of influenza A virus (Hinshaw et al., 1994; Takizawa et al., 1993). One example is the aberrant production of inflammatory cytokines, the so-called ‘cytokine storm’ (Nakamura et al., 2010), occurring in organs infected with influenza A virus, which causes multi-organ disorders through the induction of apoptosis in a wide variety of tissues (Cheung et al., 2002). In particular, inflammatory cytokines belonging to the tumour necrosis factor (TNF) ligand superfamily, such as TNF-α, Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL), are known to be closely implicated in the pathology of the ‘cytokine storm’ (Makhija & Kingsnorth, 2002). These ligands are known as death ligands, and stimulation of the receptor by a death ligand activates the caspase-dependent signalling pathway for the induction of apoptosis (Ashkenazi & Dixit, 1998). Importantly, the stimulation of receptors by death ligands is also involved in the replication of influenza A virus. It has been reported that the expression levels of FasL and TRAIL are increased by infection with influenza A virus (Ishikawa et al., 2005), and the stimulation of the receptors by these ligands in virus-infected cells is required for the effective replication of influenza A virus (Wurzer et al., 2004).

This study found that Siva-1, which is known to be a host-cell apoptosis-related protein, is crucial for the induction of apoptosis triggered by influenza A virus infection. It was found that A549 cells stably expressing Siva-1 exhibited a phenotype of higher sensitivity to apoptosis induction by influenza A virus infection. In addition, knockdown of Siva-1 expression in A549 cells indicated a phenotype resistant to the apoptosis caused by influenza A virus infection. Furthermore, knockdown of Siva-1 in A549 cells inhibited influenza A virus replication, and this was presumably caused by inhibition of virus particle formation.

RESULTS

Identification of Siva-1 as a polymerase basic protein 2 (PB2)-binding protein

The RNA polymerase complex of influenza A virus consists of PA, PB1 and PB2, and is responsible for the transcription of viral RNA, viral mRNA and viral cRNA (Engelhardt & Fodor, 2006). Previous reports using the highly pathogenic H5N1 subtype avian influenza A virus isolated from patients in Hong Kong in 1997 during the outbreak of the H5N1 avian influenza A virus epidemic demonstrated that mutation of the PB2 protein was correlated with effective replication of avian influenza virus in mammalian cells (Hatta et al., 2001). The PB2 protein is also known to be a viral factor involved in adaptation of influenza A virus to different hosts, and these observations suggest that host-cell molecules that bind to PB2 are important for replication or adaptation of the virus (Neumann & Kawaoka, 2006; Subbarao et al., 1993).

To find host-cell molecules that bind to influenza A virus PB2, a screening was performed using a commercially available antibody array (Signal Transduction Antibody-Array, Hypermatrix) (Wang et al., 2000). This antibody array is a membrane with spots of antibodies against various signalling molecules and is able to identify binding proteins for the molecule of interest. Following incubation with a lysate prepared from cells expressing the molecule of interest, the target protein binds to the membrane by binding to particular signalling molecules trapped by the membrane-spotted specific antibody. Therefore, binding proteins can be identified by detection of the membrane-bound target protein. We used lysate prepared from HEK293T cells expressing Flag-tagged PB2 for the screening and identified Siva-1 as a candidate PB2-binding protein.

To confirm the result of this screening, a co-immunoprecipitation assay was also performed. The expression vectors for Flag-tagged PB2 and haemagglutinin (HA)-tagged Siva-1 were transiently transfected into HEK293T cells. The cells were harvested and whole-cell extracts were subjected to immunoprecipitation using anti-Flag antibody. As shown in Fig. 1(a), the results showed that Siva-1 was co-immunoprecipitated with the PB2 protein, indicating that PB2 binds to Siva-1 in HEK293T cells.

Next, we constructed an expression vector plasmid for Siva-1 fused with EGFP (Siva-1–EGFP) and investigated the subcellular localization of Siva-1. The expression vector for Siva-1–EGFP was transfected into A549 cells. After 24 h, the cells were infected with the PR8 strain of influenza A virus (m.o.i. = 10). The subcellular localization of Siva-1–EGFP was seen to be mainly in the nucleus when it was expressed alone and was not changed by influenza A virus infection (Fig. 1b). In addition, the PB2 subunit was detected mainly in the nucleus of the virus-infected cells, and the subcellular localization of Siva-1–EGFP and PB2 overlapped almost completely. These results suggested that the PB2 protein may affect the function of Siva-1 in the nucleus.

A549 cells stably expressing Siva-1 are susceptible to apoptosis induction by influenza A virus infection

Siva-1 was originally identified as a CD27-binding protein responsible for apoptosis induction by CD27 (Prasad et al., 1997; Yoon et al., 1999). Siva-1 is also involved in various types of apoptosis (Barkinge et al., 2009; Lin et al., 2007; Shuyu et al., 2009), such as activation-induced cell death (Gudi et al., 2006; Py et al., 2004), UV irradiation (Chu et al., 2004; Xue et al., 2002) and oxidative stress-induced cell death (Cao et al., 2001). Therefore, it was thought that
Siva-1 expression might be related to the apoptosis induced by influenza A virus infection. To investigate whether Siva-1 expression affected the apoptosis induced by infection with influenza A virus, we established a cell line that stably expressed Siva-1 using the Flp-In system (Invitrogen) with the human lung adenocarcinoma epithelial cell line A549. This system is based on site-directed recombination technology by Flp recombinase (Zhu & Sadowski 1995). By using the Flp-In system, an expression cassette can be integrated into a specific locus on the genome. Therefore, the genetic background of the established cell lines differs only by the inserted gene in terms of expression. As shown in Fig. 2(b, c), A549 cells stably expressing Siva-1 exhibited a higher expression level of Siva-1 mRNA and protein than control cells carrying an empty expression cassette. The cells constitutively expressing Siva-1 and the control cells were infected with influenza A virus PR8 at an m.o.i. of 2 and analysed using a phase-contrast microscope (Fig. 2a). The results showed that the cells stably expressing Siva-1 clearly showed a higher susceptibility to cell death following influenza A virus infection than the control cells. To confirm this observation, A549 cells expressing Siva-1 were infected with PR8 (m.o.i. = 2) and cell viability was measured using a trypan blue exclusion assay (Fig. 2d). The results showed that the percentage of trypan blue-positive (dead) cells was significantly increased in the influenza A virus-infected cells that stably expressed Siva-1 compared with the control cells. These data suggested that Siva-1 is involved in the induction of apoptosis caused by influenza A virus. However, the replication of influenza A virus in
Fig. 2. A549 cells stably expressing Siva-1 are susceptible to apoptosis induced by influenza A virus infection. (a) A549 cells stably expressing Siva-1 were infected with the PR8 strain of influenza A virus (m.o.i. = 2). After 48 h, the cells were analysed by phase-contrast microscopy. (b) The expression level of Siva-1 mRNA was analysed by semi-quantitative RT-PCR. Total RNA isolated from HA-tagged Siva-1-transfected cells was used as a positive control (+) and a PCR without template cDNA was used as the negative control (−). Amplification of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was used as a loading control. (c) The expression level of the Siva-1 protein was analysed by Western blotting (WB). Siva-1 was detected by anti-Siva antibody (FL-175; Santa Cruz Biotechnology). Actin was used as an internal control and detected by anti-actin mAb (clone C4; Millipore). (d) A549 cells stably expressing Siva-1 were infected with PR8 (m.o.i. = 2). After 48 h, the cell viability rate was determined using a trypan blue exclusion assay. Error bars indicating SD were calculated from three independent experiments. (e) A549 cells stably expressing Siva-1 were infected with PR8 (m.o.i. = 2). After 24, 36 and 48 h, virus titres were quantified using a plaque assay. Error bars indicating SD were calculated from three independent experiments. (f) A549 cells stably expressing Siva-1 were infected with PR8 (m.o.i. = 2). After 48 h, the cells were harvested and subjected to a DNA fragmentation assay.
A549 cells was not affected by the stable expression of Siva-1. As shown in Fig. 2(e), the viral titres were not significantly different in the culture medium of the Siva-1-expressing and control cells at each time point.

Apoptosis is the process of programmed cell death, and one of the morphological features of apoptosis is known to be cell shrinkage (Majno & Joris, 1995). As shown in Fig. 2(a), many shrunken cells were observed by phase-contrast microscopy, suggesting that the death of cells expressing Siva-1 caused by influenza A virus infection was a result of apoptosis induction. To confirm that Siva-1-mediated cell death was caused by the progression of apoptosis, DNA fragmentation assays were performed. The fragmentation of DNA into multiples of approximately 180 bp by cleavage of nucleosome units is known to be a biological change specifically observed in the process of apoptosis induction (Samejima & Earnshaw, 2005). A549 cells expressing Siva-1 were infected with PR8 (m.o.i. = 2). At 48 h post-infection (p.i.), the fragmented DNA isolated from the cells was analysed by agarose gel electrophoresis. The results showed that Siva-1 significantly enhanced the DNA fragmentation caused by apoptosis induction through infection with influenza A virus (Fig. 2f).

Next, PR8 was used to infect A549 cells expressing Siva-1 at a low m.o.i. of 0.1, and growth of the virus-infected cells was monitored using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Only a few of the cells were infected by influenza A virus at the initial time point, and viruses spread from cell to cell in accordance with the release of virus particles. As shown in Fig. 3(a), the difference in viability of cells expressing Siva-1 and the control cells increased with the incubation time p.i. In contrast to Fig. 2(d), these data represented the relative cell viability, which was calculated as a percentage of cell viability at the initial time point. Therefore, the decrease in cell viability in Siva-1-expressing A549 cells at 24 h suggested that most of the cells were already infected by influenza A virus at this time, whilst the viability of the control cells continued to increase by proliferation. Thus, the results suggested that Siva-1 may also be related to the efficiency with which the virus spreads.

To investigate the effects of Siva-1 expression on the induction of apoptosis caused by infection by another influenza A virus strain, the highly pathogenic avian influenza virus strain A/HongKong/483/97 (HK483; H5N1) was used. A549 cells expressing Siva-1 were infected with HK483 or PR8. After 48 h, the viability of the cells was monitored using a Cell Counting kit 8. It has been reported previously that a specific strain of H5N1 that is a highly pathogenic avian influenza virus causes extensive lung injury to mice and ferrets in experimental infections (Martin & Wurfel, 2008; Shinya et al., 2004; Tumpey et al., 2000). Despite the severity of experimental infection in animals, this highly pathogenic H5N1 subtype avian influenza A virus showed a lower level of efficiency of apoptosis induction in human macrophages by in vitro infection (Mok et al., 2007; Zhou et al., 2006). Consistent with these reports, a relatively lower level of apoptosis induction was observed in the A549-derived cells expressing Siva-1 infected by HK483 than in those infected by PR8 (Fig. 3b, c). However, an increase in apoptosis induction by the Siva-1 expression was also observed in the HK483-infected cells. Previous reports have demonstrated that host-cell molecules involved in activation of cell metabolism (e.g. phosphoinositide 3-kinase, AKT and...
nuclear factor-κB) are activated by infection with influenza A virus (Ehrhardt et al., 2006; Hale & Randall, 2007; Ludwig & Planz, 2008; Shin et al., 2007). As these data represent relative cell viabilities, which were calculated by comparison with uninfected cells, the increase in cell viabilities found in these experiments is thought to arise from this. Overall, the data here suggested that Siva-1 is crucial for induction of apoptosis caused by infection with various strains of influenza A virus.

**Knockdown of Siva-1 expression effectively suppresses apoptosis induced by infection with influenza A virus**

To investigate the function of endogenously expressed Siva-1 on cells infected with influenza A virus, we designed short hairpin RNAs (shRNAs) to effectively silence the Siva-1 mRNA, and constructed retrovirus vectors to express these shRNAs (Fig. 4a). The retrovirus vectors were packaged and used to infect A549 cells, and the expression level of Siva-1 mRNA was measured by both semi-quantitative and quantitative RT-PCR. As shown in Fig. 4(b, c), the expression level of endogenously expressed Siva-1 mRNA was strongly suppressed by infection with a retroviral vector expressing shSiva-1A in A549 cells. A549 cells infected with a retroviral vector expressing shSiva-1B or shSiva-1C exhibited moderate inhibition of Siva-1 gene expression. Similar results were obtained by Western blotting using a Siva-1-specific antibody. As shown in Fig. 4(d), expression of the endogenous Siva-1 protein was strongly suppressed by infection with the retroviral expression vector for shSiva-1A.

Next, the sensitivity of the Siva-1-knockdown cells to apoptosis induced by influenza A virus infection was investigated. The PR8 strain was used to infect the Siva-1-knockdown A549 cells, and cell viability was measured by a trypan blue exclusion assay. The results showed that these cells were not subject to apoptosis induced by influenza A virus infection by the knockdown of Siva-1 gene expression, and the gene silencing efficiency of each shRNA correlated with the increase in cell viability (Fig. 4e). For further confirmation of the results, a DNA fragmentation assay was performed. In agreement with the results of the cell viability assay (Fig. 4e), the results showed that shSiva-1A, which efficiently silenced Siva-1 expression, significantly inhibited the DNA fragmentation caused by apoptosis induction by infection with influenza A virus (Fig. 4f).

**Siva-1 regulates the replication of influenza A virus without inhibiting RNA transcription by the viral polymerase**

In the course of viral infections, apoptosis is important not only for exclusion of virus-infected cells, but also for effective replication of the virus. For instance, inflammatory cytokines, which belong to the TNF superfamily and are responsible for inducing apoptosis, are known to be an important factor both in activating the immune system to exclude the virus and in the activation of virus replication (Ishikawa et al., 2005; Wurzer et al., 2004). To investigate whether Siva-1 functioned to effect the exclusion of influenza A virus or the acceleration of the virus replication, the virus replication in Siva-1-knockdown A549 cells was analysed by monitoring virus titre. As shown in Fig. 5(a), virus replication was significantly inhibited by knockdown of Siva-1 gene expression, indicating that Siva-1 is also involved in the replication of influenza A virus.

Next, we investigated whether transcriptional activity of the influenza A virus RNA polymerase was affected by Siva-1 gene knockdown. A549 cells expressing Siva-1-specific shRNAs were infected with PR8 (m.o.i. = 2). After 24 h, total RNA was isolated from these cells and subjected to real-time reverse transcriptase RNA analysis using a nucleoprotein (NP)-specific primer set. For cDNA synthesis, random hexamers were used for the quantification of total NP RNAs (mRNA, vRNA and cRNA) (Fig. 5b), and oligo(dT), NP-specific sense and NP-specific antisense primers were used for quantification of NP mRNA (Fig. 5c), NP vRNA (Fig. 5d) and NP mRNA and cRNA (Fig. 5e), respectively. These results showed that the transcription of influenza A virus RNAs was not significantly affected by Siva-1 gene knockdown, suggesting that Siva-1 may regulate influenza A virus replication at a later stage in the synthesis of viral genomic RNA.

The results of the RNA quantification analysis above suggested that Siva-1 may regulate replication of the influenza A virus at a late stage in the synthesis of viral genomic RNA. It has been reported that activation of caspase-3 is involved in the effective export of the viral ribonucleoprotein (RNP) complex from the nucleus to the cytoplasm (Wurzer et al., 2003). Caspase-3 is known to be an effector caspase. It is located downstream of initiator caspases such as caspase-8 and caspase-9, and is responsible for activation of a number of factors involved in the execution of apoptosis. Therefore, it was thought that Siva-1 might regulate the replication of influenza A virus in a caspase-dependent manner. To investigate whether caspase activation is involved in the inhibition of influenza A virus replication, we investigated the effect of caspase inhibition on Siva-1 function during virus replication using a pan-caspase inhibitor, Z-VAD-FMK. A549 cells in which expression of Siva-1 was inhibited by the retroviral vector shSiva-1 were infected with PR8 (m.o.i. = 2) and the cells were treated with Z-VAD-FMK. The results show that treatment with Z-VAD-FMK resulted in reduced replication of the virus, whilst there was no reduction in cells where Siva-1 expression was inhibited (Fig. 6a). In addition, the difference in virus replication between the control and Siva-1-knockdown cells disappeared following treatment with Z-VAD-FMK. These results suggested that Siva-1 may be located upstream of caspase activation in the replication of influenza A virus. Influenza A virus-induced apoptosis is also involved in the replication of influenza A virus.
apoptosis was reduced in both control and Siva-1-knockdown cells by the treatment with Z-VAD-FMK (Fig. 6b), and a decrease in cell death was still observed in the Siva-1-knockdown cells by Z-VAD-FMK treatment. These data suggested that Siva-1 may also be involved in regulation of the caspase-independent pathway for cell death. In addition, cell death was inhibited by Siva-1 knockdown in the presence of Z-VAD-FMK (Fig. 6b). These results indicated that cell death is induced by a Siva-1-mediated pathway and another caspase-dependent pathway. Although Siva-1-independent pathways are also involved in the cell death induced by influenza A virus infection, these pathways are thought not to be important for virus replication. Therefore, from the data presented here, we concluded that Siva-1-mediated activation of caspase is crucial for influenza A virus replication.

**DISCUSSION**

This study demonstrated that Siva-1, identified as a PB2-binding protein, is crucial for the induction of apoptosis caused by infection with influenza A virus and is also important for virus replication through the activation of caspase.

The results showed that knockdown of Siva-1 expression significantly inhibited the replication of influenza A virus (Fig. 5a). However, overexpression of Siva-1 did not
significantly affect virus replication (Fig. 2e). These observations suggested that a specific expression level of Siva-1 is critical for influenza A virus replication and that induction of apoptosis by Siva-1 is involved in this process. Activation of caspase-3 is known to be crucial for effective exportation of the viral RNP complex to the cytoplasm (Wurzer et al., 2003). However, activation of caspase-3 does not accelerate other replication steps of influenza A virus, such as viral transcription and protein synthesis. Therefore, strong acceleration of apoptosis induction before synthesis of the viral RNAs in sufficient amounts may cause a decrease in viral particle production.

The functional relationship between the influenza A virus PB2 protein and Siva-1 remains unclear, but the data here demonstrated that Siva-1 is crucial for induction of apoptosis after influenza A virus infection. As described in previous reports, Siva-1 is expressed in a wide variety of tissues and overexpression of Siva-1 induces apoptosis in a number of cell lines (Prasad et al., 1997; Yoon et al., 1999). Taken together, this suggests that transcriptional regulation of the expression of Siva-1 mRNA is critical for apoptosis induction by influenza A virus infection. To investigate this possibility, a semi-quantitative RT-RNA analysis was performed. However, it was not possible to detect changes in the expression levels of Siva-1 mRNA in A549 cells following infection with influenza A virus (data not shown). The absence of changes suggested that Siva-1 function in apoptosis induction caused by infection with influenza A virus is regulated by post-translational modifications. Although the activation mechanism for Siva-1 function in viral infection has not been established, it can be postulated that oxidative stress in particular may be involved in the activation of Siva-1 by influenza A virus infection. It has been reported that Siva-1 is involved in oxidative stress-induced apoptosis and that oxidative stress is increased by infection with influenza A virus (Hennet et al., 1992). Therefore, the oxidative stress induced by influenza A virus infection may be a trigger for Siva-1 activation.

Previous studies have described the molecular mechanism for Siva-1-mediated apoptosis, where it was demonstrated that the cellular molecules X-linked inhibitor of apoptosis protein (XIAP) (Resch et al., 2009), B-cell lymphoma 2 (Bcl-2)/Bcl-XL (Chu et al., 2004, 2005; Xue et al., 2002) and

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**Fig. 5.** Knockdown of Siva-1 expression inhibits replication of influenza A virus, but does not affect the transcriptional activity of the viral RNA polymerase. (a) A549 cells in which Siva-1 expression was suppressed were infected with the PR8 strain of influenza A virus (m.o.i. = 2). After 24, 36 and 48 h, virus titres were quantified using a plaque assay. (b–e) A549 cells in which Siva-1 expression was suppressed were infected with PR8 (m.o.i. = 2). After 24 h, total RNA was isolated from the cells and cDNA was synthesized by reverse transcription using random hexamers (b), oligo(dT) primers (c), nucleoprotein (NP)-specific sense primers (d) or NP-specific antisense primers (e). The RNA transcription level was quantified using a real-time RT-PCR assay with a gene-specific primer set for NP. The data represent relative amounts, normalized to the expression level of G3PDH. Error bars indicating SD were calculated from at least three independent experiments.
Abl-related gene (ARG) tyrosine kinase (Cao et al., 2001) bind to Siva-1 and are involved in Siva-1-mediated apoptosis induction. These molecules are also thought to be important in Siva-1 function for influenza A virus-induced apoptosis; in particular, the inhibitory activity of Siva-1 for XIAP through binding could be important for Siva-1 to regulate replication of influenza A virus, as XIAP belongs to the family of inhibitor of apoptosis proteins, and is known to be a caspase inhibitor by binding directly to the activated form of caspases including caspase-3 (Dubrez-Daloz et al., 2008). This function of Siva-1 has been suggested to be a more direct effect in the replication of influenza A virus and in apoptosis caused by viral infection. However, it remains unclear whether these molecules are involved in Siva-1-mediated apoptosis induced by influenza A virus infection. Further investigations are required to understand the molecular mechanism of the induction of apoptosis through activation of Siva-1 by influenza A virus infection, and to understand the relationship between the function of Siva-1 and the pathogenicity of influenza A virus.

**METHODS**

**Cell culture and transfection.** HEK293T cells and MDCK cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) with 10% FBS (Invitrogen), 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (both from Sigma-Aldrich) at 37 °C with 5% CO₂; A549 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Ageing and Cancer, Tohoku University, Japan, and were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ at 37 °C with 5% CO₂. Transfection assays were performed using Lipofectamine 2000 transfection reagent (Invitrogen) or FuGENE HD (Roche Diagnostics) according to the manufacturers’ protocols.

**Vector construction.** To construct the expression vector for Siva-1, we amplified the entire coding region of Siva-1 by PCR from human placental cDNA and inserted the amplified fragment into pcDNA3.1(+) -HA(C), pcDNA5/FRT (both from Invitrogen) or pEGFP-c2 (Clontech). The expression vector for Flag-tagged PB2 was constructed by insertion of a fragment encoding PB2, amplified from an infectious clone of the A/Puerto Rico/8/34 (PR8 strain; H1N1) strain, into pcDNA3-Flag (Iwai et al., 2003).

**Subcellular localization analysis.** A549 cells expressing EGFP-fused Siva-1 were infected with influenza A virus (PR8 strain). After 24 h, the cells were washed with PBS and fixed with 4% paraformaldehyde. After permeabilization with 0.2% Triton X-100 for 5 min, the cells were immunostained using a combination of anti-PB2 antibody (Hatta et al., 2000), anti-GFP (Medical & Biological Laboratories) and Alexa Fluor 55-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit antibodies (Invitrogen). The slides were mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories) and subcellular localization was observed using a confocal laser-scanning microscope (FluoView FV1000; Olympus).

**Establishment of an A549 cell line stably expressing Siva-1.** Establishment of an A549 cell line stably expressing Siva-1 using cells was carried out using a Flp-In Expression System (Invitrogen) according to the manufacturer’s protocols.

**Retroviral vectors for shRNAs.** Retrovirus vectors for constitutive expression of shRNA in the cells were constructed using piShlencer5-1-U6 (Applied Biosystems) in accordance with the manufacturer’s protocols. The following Siva-1 mRNA-specific target sequences were used for construction of the vectors: siSiva-1A: 5'-GTGGCCCTGTA-CCCTGTGCTGGGCTCT-3'; siSiva-1B: 5'-ACTGTGGGGGTGCGACGGGAGG-3'; siSiva-1C: 5'-GTGCCGTCGTACCTGCCAGGAGTC-3'. Packaging and infection of the retrovirus vector were carried out using commercially available systems (Platinum Retroviral Expression System, Ecotropic; Cell Biolabs) according to the manufacturer’s protocols.

**RT-PCR.** Gene-specific primers sets for Siva-1 (sense: 5'-CGTGGG-CCCTGACCCCTGGTGTG-3'; primer: 5'-CGACGACCCAGATTCTCAGGAC-AC-3'), NP (sense: 5'-CCAGGGGCGGGGAATCTCTGGAGTCCT-GGA-3'; antisense: 5'-TACTCTCCTGTGATCGGCTGGAAGAATA-3') and G3PDH (sense: 5'-CTACTGGGGGTGCGACGGGAGG-3'; antisense: 5'-GTGTTTGTGCTGTTGAAATGCT-3') were used. Real-time RT-PCR was performed using an Mx3000P Real-time PCR System (Stratagene) in accordance with the manufacturer’s instructions.

**Analysis of cell death and virus titres.** For the trypan blue exclusion assay, cells were washed briefly with PBS, trypsinized and collected by centrifugation. The cells were resuspended in PBS.
containing 0.4% trypan blue and the proportion of dead cells was determined using a Countess Automated Cell Counter (Invitrogen). A modified MTT assay was carried out using a Cell Counting kit 8 (Dojindo Molecular Technologies) according to the manufacturer’s protocols. Plaque assays and DNA fragmentation assays were performed as described previously (Ikeda et al., 1998; Tsuda et al., 2009).

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