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Inhibition of fowl adenovirus serotype 4 replication in Leghorn male hepatoma cells by SP600125 via blocking JNK MAPK pathway

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ABSTRACT

Fowl Adenoviruses (FAdVs) are widely-distributed pathogens across the globe. Fowl adenovirus serotype 4 (FAdV-4), the causative agent for chicken hydropericardium syndrome (HPS) with a high mortality in the infected flocks, has caused severe economic losses to the poultry industry of China in the past few years. Although vaccination against FAdV-4 has been implemented, the prevention and control of FAdV-4 infection is still not very successful. Here, we report that FAdV-4 markedly inhibits Leghorn male hepatoma (LMH) cell growth and this inhibition could be abolished by a small molecule SP600125, a JNK MAPK specific inhibitor. Furthermore, SP600125 considerably suppressed FAdV-4-induced phosphorylation of p38 and JNK MAPK. Importantly, SP600125 promoted type I interferon production associated with inhibition of FAdV-4 replication. Thus, FAdV-4 might employ the JNK MAPK pathway for the benefit of its replication, and SP600125 may have the potential of being used as an anti-virus drug for the control FAdV-4 infection.

1. Introduction

Fowl adenoviruses (FAdVs), members of *Aviadenovirus* belonging to *Adenoviridae*, are non-enveloped double stranded DNA viruses. According to molecular criteria, restriction enzyme digest pattern and serum cross-neutralization test, FAdVs can be divided into 5 species (FAdV-A to FAdV-E) containing 12 serotypes (FAdV-1 to 8a and -8b to 11) (Hess, 2000). The diseased chickens mainly suffer from inclusion body hepatitis (IBH), the gizzard erosions (GE) and the hydropericardium syndrome (HPS) (Nakamura et al., 1999). Among them, IBH can be caused by all those 12 serotypes, and classical IBH is characterized by hepatic necrosis with microscopic eosinophilic or basophilic intranuclear inclusion bodies in hepatocytes and about 10% mortality (Wells and Harrigan, 1974; Domanska-Blicharz et al., 2011).

Hydropericardium syndrome (HPS), also called Angara disease, was first reported in Pakistan in 1987, and subsequently broke out across the world, leading to serious economic losses to poultry industry (Hess et al., 1999). This disease rarely occurs in other species of animals. HPS is mostly caused by FAdV serotype 4 (FAdV-4), characterized by hydropericarditis and hepatitis with a relatively high mortality of 30%–70% in the epidemiological studies (Kim et al., 2008). Many hypervirulent strains have been isolated from diseased chickens with HPS (Pan et al., 2017a, 2017b; Schachner et al., 2014; Ye et al., 2016).

The Mitogen Activated Protein Kinase (MAPK) pathway plays a critical role in cell response to extracellular stimuli, which includes proliferation, differentiation, senescence and so on (Fang and Richardson, 2005; Olsen et al., 2012; Yang et al., 2013). MAPK can be divided into six distinct groups in mammals-ERK1/2, ERK3/4, ERK5, ERK7/8, JNK1/2/3 and the p38 isoforms α/β/γ(ERK6)/δ (Kyriakis and Avruch, 2012; Schaeffer and Weber, 1999). Among them, p38 and JNK MAPKs are involved in the regulation of cell proliferation in mammalian cells in a manner inextricable from other signal transduction system by sharing substrate and cross-cascade interaction (Cheng et al., 2002; Gomez et al., 1996).

p38 MAPK is encoded by four genes: α, β, γ and δ, which have different tissue expression patterns and affinities for upstream activators and downstream effectors (Ono and Han, 2000). Among them, p38α is ubiquitously expressed in most tissues, whereas the others seem to be expressed in a more tissue-specific manner (Cuenda and Rousseau, 2007). p38 MAPK is activated by MKK3 and MKK6 kinases, and the activation of p38 MAPK plays a vital role in the regulation of apoptosis, cell growth inhibition and differentiation (Brancho et al., 2003; Cuadrado and Nebreda, 2010). c-Jun N-terminal kinase (JNK), also known as a stress-activated protein kinase (SAPK), is another important

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member of the MAPK family which contains three genes: JNK1, JNK2, and JNK3 (Yang et al., 1997). Among them, JNK1, JNK2 are expressed ubiquitously and activated by the upstream MKK4 and MKK7 kinase (Fleming et al., 2000), while JNK3 is specifically expressed in a subset of neurons in the nervous system and is activated by threonine and tyrosine phosphorylation (Zhu et al., 2001). The activated JNK can regulate a number of cellular processes, including cell proliferation, DNA repair, autophagy, apoptosis and metabolism (Sabapathy, 2012).

JNK and p38 MAPKs gene-regulated cascades are activated post infection of several members of different viral families, thus inducing apoptosis in host cells and increasing viral replication (Clarke et al., 2004; Hirasawa et al., 2003). In fact, the activation of the JNK MAPK has been observed during infection with various DNA and RNA viruses, revealing a vital role in viral replication (Banerjee et al., 2008; Ceballos-Olvera et al., 2010; Clarke et al., 2001; Holloway and Coulson, 2006). Importantly, JNK MAPK activation is a common feature in the infection states of some viruses, which suggests that it may be an important target for antiviral treatment.

In the present study, we show that infection of Leghorn male hepatoma (LMH) by FAdV-4 inhibits cell growth, and this inhibition could be significantly mitigated by SP600125, an inhibitor for JNK MAPK. Importantly, SP600125 markedly suppressed FAdV-4-induced phosphorylation of JNK MAPK, promoted the production of type I interferon and inhibited FAdV-4 replication, suggesting that SP600125 holds a promise as an anti-virus drug in control of FAdV-4 infection.

2. Materials and methods

2.1. Cell lines and virus

Leghorn male hepatoma (LMH), a chicken hepatic cell line (CRL-2117), was purchased from ATCC, and cells were maintained with Waymouth's MB725/1 medium supplemented with 10% fetal bovine serum (FBS) in 0.1% (w/v) type A gelatin coated 75 T flasks in 5% CO_2 incubator at 37°C. For all exposure studies, cells were cultured for 1 h in opti-MEM medium and the dimethyl sulfoxide (DMSO) in the cell culture medium was used as a control medium 0.1% (v/v). FAdV HB1502, a FAdV-C (FAdV-4) strain isolated from diseased chickens with HPS, was saved in our laboratory (GenBank accession number: KX421401.2).

2.2. Reagents and antibodies

anti-β-actin (sc-1616-R), anti-p38 (sc-535) and anti-p-p38 (sc-166182) antibodies were obtained from Santa Cruz Biotechnology (USA). Anti-JNK (ab208035) and anti-p-JNK (ab76572) antibodies were purchased from Abcam (UK). Anti-GAPDH antibody was obtained from GBC Company (Beijing). Hexon monoclonal antibody was purchased from CAEU Biological Company (Beijing). p38 MAPK inhibitor SB203580 and JNK MAPK inhibitor SP600125 were purchased from Enzo Life Sciences (USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG antibodies were obtained from DingGuo (China). An enhanced chemiluminescence (ECL) kit was purchased from Merck Millipore (Germany). OPTI-MEM I was purchased from Invitrogen.

2.3. Viral infection and measurement of FAdV-4 growth in LMH cells

LMH cells were seeded in 6-well plates (1×10^6 cells/well) or 96-well plates (1×10^4 cells/well) and cultured for 24 h before cells were mock infected or infected with FAdV-4 at an multiplicity of infection (MOI) of 5, which was diluted in Waymouth's MB725/1 medium with 2% FBS. The cells were collected at the indicated time points post virus infection and used for further analysis. LMH cells were pretreated with SB203580 (20 μM), SP600125 (20 μM) or DMSO (0.1%, v/v), washed once before infected with FAdV-4 at an MOI of 5, and cell cultures were collected at the indicated time points (12, 24, 48, 72 h) after infection. The cell culture samples were freeze-thawed three times and centrifuged at 2, 000 × g for 10 min, and the viral contents in the supernatants were titrated using 50% tissue culture infective doses ($TCID_{50}$) in LMH cells. Briefly, the viral solution was diluted by 10-fold in DMEM. 100 μL of diluted samples were added to each well. Cells were cultured for 5 days in 5% CO_2 incubator at 37°C. Tissue culture wells with a cytopathic effect (CPE) were determined to be positive. The titer was calculated on the basis of a previously described method (Nadgir et al., 2013).

2.4. Cell viability assay

The LMH cells (1×10^4 cells/well) were seeded in 96-well plate before mock infected or infected with FAdV-4 at an MOI of 5, and cell viability was evaluated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) colorimetric assay (Carmichael et al., 1987). Briefly, culture medium was carefully removed and changed for a fresh one. 10 μL of MTT solution (5 mg/mL PBS, Sigma-Aldrich, St. Louis, MO, USA) was then added to each well, followed by incubation at 37°C for 4 h. After incubation, the MTT reagent was removed from wells, and 110 μL of DMSO was added to each well and mixed thoroughly. Fifteen minutes after addition with DMSO, the colorimetric absorbance was measured at 490 nm wave length using a plate reader (Sunrise™, TECAN, Switzerland).

2.5. Signaling pathway inhibition assay

LMH cells (1×10^6) were seeded in 6-well plates and cultured for 12 h before incubated with SB203580 (20 μM), SP600125 (20 μM) or DMSO (0.1%, v/v) as control for 30 min., and then the cells were washed once before they were mock infected or infected with FAdV-4 at an MOI of 5. The procedures were done on the basis of a previously described method (Cuenda et al., 1995). Cells were collected at the indicated time points and cell lysates were examined by Western Blot assays using specific antibodies against p38, p-p38, JNK or p-JNK.

2.6. RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from LMH cells using a Fastagen RNAfast 200 kit and treated with DnaseI according to the manufactures' instructions. cDNA synthesis was performed via reverse transcription using an RT-PCR kit (TakaRa). The specific primers for chicken AP-1 (5′-CTGTCCGTCTCTAGTGCCAACTT-3′ and 5′-ATCTGTCTCCGCTTGGA GCGTAT-3′), IFN-α1 (5′−CCAGCACCTCGAGCAAT-3′ and 5′-GGCGCT GTAATCGTTGTCT-3′), IFN-β (5′-GCCTCCAGCTCCTTCAGAATACG-3′ and 5′-CTGGATCTGGTTGAGGAGGCTGT-3′), and GAPDH (5′-TGCCAT CACAGCCACACAGAAG-3′ and 5′-ACTTTCCCCACAGCCTTAGCAG -3′) were designed with reference to previous publications (Abdul-Careem et al., 2008; Li et al., 2007; Liu et al., 2010a, 2010b). The real-time PCR was performed and products were detected using the Light Cycler 480 System (Roche, USA). The PCR was performed in a 20 μL containing 1 μl of cDNA, 10 μl of 2 × SYBR green Premix Ex Taq (TaKaRa Bio Inc., Japan), and a 0.4 μM of each gene-specific primer. Thermal cycling parameters were as follows: 94°C for 2 min; 45 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s; and 1 cycle of 95°C for 30 s, 60°C for 30 s, and 95°C for 30 s. The final step was to obtain a melt curve for the PCR product to determine the specificity of the amplification. Each sample was run in triplicates on the 96-well sample plate, and the expression level of each gene was calculated relative to the expression of the GAPDH gene.

2.7. Western blot analysis

LMH cells were mock-infected or infected with FAdV-4 as above described. Cells were collected, centrifuged and lysed in the lysis buffer (150 mM NaCl, 50 mM Tris−HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA,

1% Triton X-100, 10% glycerol and 1% protease inhibitor cocktail C). Cell debris was removed by centrifugation at 12,000 × g for 10 min and was resuspended with 10 × SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electropheresis) loading buffer. The samples were boiled for 5 min. before fractionated by 10%–12% SDS-PAGE gels and transferred onto polvinylidene difluoride membrane. After blocking with 5% skimmed milk, the membranes were incubated with anti-hexon and anti-GAPDH antibodies, followed by an appropriate HRP-conjugated secondary antibody. Blots were visualized using enhanced chemiluminescence kit (ECL kit). For signaling pathways analysis, LMH cells were lysed in lysis buffer as above containing 1% 20 mM phosphatase inhibitor (NaF) and examined with Western Blot assay using anti-p-p38, anti-p38, anti-p-JNK, anti-JNK and anti-GAPDH antibodies.

2.8. Statistical analysis

The significance of the differences between FAdV-4 infection and mock infection groups in gene expression, the differences between SB203580, SP600125 and DMSO-treated cells were determined by the Student's *t*-test using GraphPad Prism software. Densitometry quantification was performed with ImageJ software. Viral growth was determined by the Mann-Whitney test and analysis of variance (ANOVA) accordingly.

3. Results

3.1. FAdV-4 infection inhibits the proliferation of LMH cells

It was reported that chickens infected with FAdV-4 suffer from inclusion body hepatitis (IBH) and the hydropericardium syndrome (HPS) (Domanska-Blicharz et al., 2011; Nakamura et al., 1999; Wells and Harrigan, 1974). The viral load in the liver of FAdV-4 infected chickens was significantly higher than that of any other tissues (Pan et al., 2017a, 2017b). To investigate the effect of FAdV-4 infection on the cellular morphology of host cells, we infected LMH cells with FAdV-4 at an MOI of 5 and examined the cells using a phase-contrast microscope. As shown in Fig. 1A&B, cells had morphological changes or typical cytopathic effect (CPE) 24 h post infection, including round shape and cell shrinkage compared with mock infection group. Then, we further investigated the cell biological features associated with FAdV-4 induced CPE in LMH cells using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays at different time points post infection. Our data show that FAdV-4 significantly inhibited the proliferation of LMH cells in a time-dependent manner as compared to that of mock infection groups (Fig. 1C). These data indicate that cell proliferation of host cell is inhibited by FAdV-4 infection.

3.2. The inhibitory effect of FAdV-4 on cell proliferation could be mitigated by SP600125

It was reported that p38 and JNK MAPK are involved in various cellular activities in mammalian cells, including cell proliferation (Cheng et al., 2002; Gomez et al., 1996). Thus we assumed that FAdV-4-mediated inhibition of cell proliferation might be related to the inhibition of p38 or JNK MAPK pathway. To test this hypothesis, LMH cells were treated with SB203580 (a specific p38 MAPK inhibitor), SP600125 (a specific JNK MAPK inhibitor) or DMSO (medium control) for 1 h before mock infected or infected with FAdV-4. The cell morphology was examined by microscopy. As shown in Fig. 2A-F, The CPE of FAdV-4 infected cells, including decreased cell number, round shape and cell shrinkage was markedly inhibited by SP600125. Consistently, Meanwhile, SP600125 reduced FAdV-4 induced suppression of cell growth as examined by MTT assay to some extent, especially at 24, 48 or 72 h post infection (Fig. 2G). These results suggest that SP600125 can be used as a novel inhibitor in control of FAdV-4 infection.

3.3. SP600125 suppressed the activations of p38 and JNK MAPKs signaling pathways

To determine the effect of FAdV-4 infection on the activation of p38 or JNK MAPK signaling pathway and the effect of SB203580 or SP600125 on p38 or JNK MAPK activation, we treated LMH cells with those inhibitors before mock infected or infected cells with FAdV-4 and examined the phosphorylation of p38 and JNK MAPKs using Western Blot assay. As shown in Fig. 3A, p38 or JNK phosphorylation was markedly enhanced in FAdV-4 infected cells as compared to that of controls. Phosphorylation of p38 could be inhibited by both SP600125 and SB203580 (Fig. 3B), while JNK phosphorylation could be only inhibited by SP600125 (Fig. 3C). Besides, the protein levels of viral structural protein hexon were significantly inhibited by SP600125 post FAdV-4 infection (Fig. 3D), indicating the inhibitory role of SP600125 in FAdV-4 replication.

3.4. SP600125 enhances FAdV-4–induced the expression of type I interferon in LMH cells

It was reported that type I interferon plays an important role in the control of adenovirus in vivo (Toth et al., 2015). To determine whether FAdV-4 infection induces the expression of type I interferon in cells, we infected LMH cells with FAdV-4 at an MOI of 5, and examined the mRNA expressions of AP-1, IFN-α or IFN-β at different time points (0, 6, 12 h) post FAdV-4 infection. We found that FAdV-4-infection markedly induced the expression of transcriptional regulator AP-1 and type I

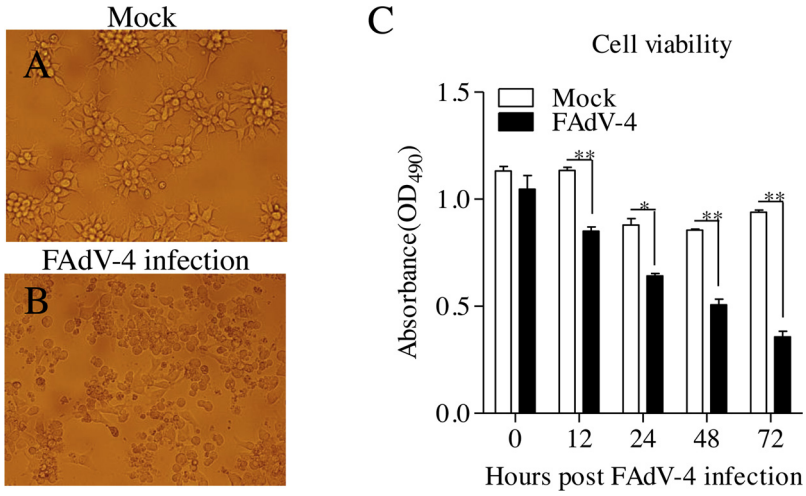

Fig. 1. FAdV-4 infection inhibits the proliferation of LMH cells. (A &B) FAdV-4 infection caused a CPE in LMH cells. LMH cells (4×10^5) were mock infected or infected with FAdV-4 at an MOI of 5 and cellular morphology was observed under a light microscope 24 h post infection (Magnification, ×200). (C) The cell proliferation was measured by MTT assays. LMH cells (1×10^4) were mock infected or infected with FAdV-4 at an MOI of 5 and harvested at different time points (0, 12, 24, 48 or 72 h) post infection. The cell viabilities of LMH were quantitated by absorbance measured at 490 nm plate reader. The cell viability of 0 hpi group was set as 1, and the relative levels of mock infection groups or FAdV-4 infection groups were calculated as follows: absorbance of mock infection or FAdV-4 infection/ absorbance of 0 hpi group. Results are representative of three independent experiments with similar results. Data are shown as means ± SD; n = 3. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

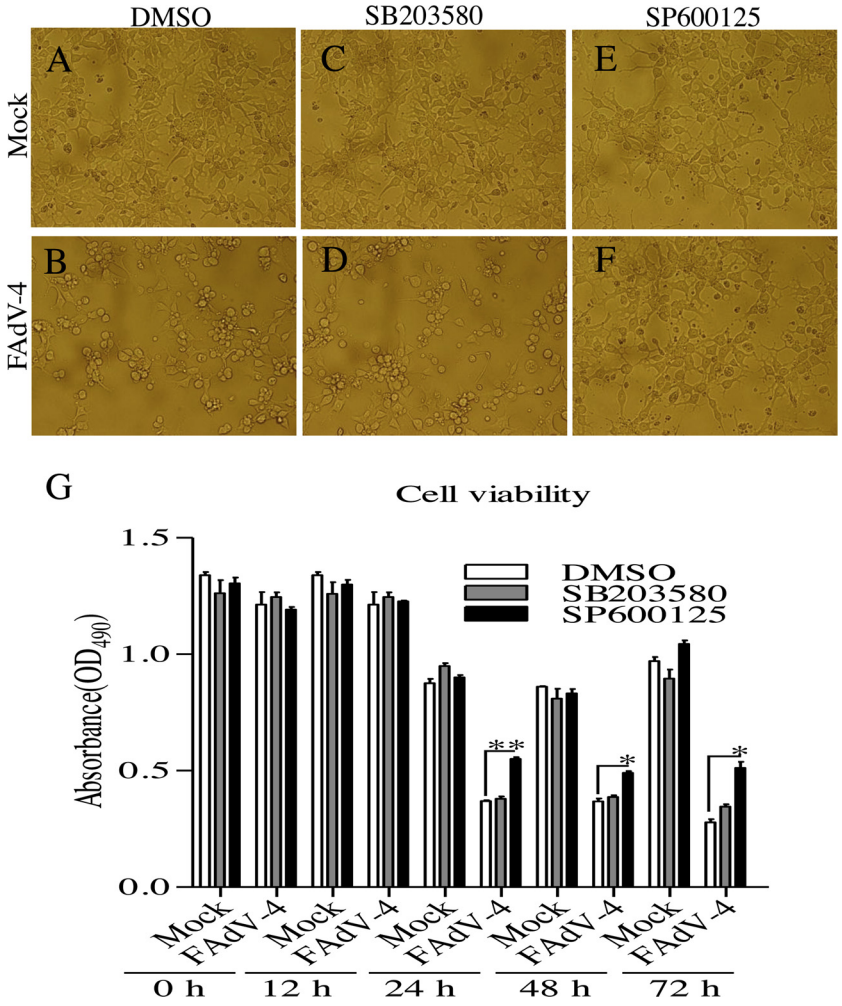

Fig. 2. FAdV-4 infection inhibits the proliferation of LMH cells via JNK MAPK signaling pathway. (A–F) Effects of MAPK inhibitors on cell proliferation. LMH cells (4×10^5) were mock infected or infected with FAdV-4 at an MOI of 5 and cellular morphology was observed under a light microscope 24 h post infection (Magnification, ×200). (G) The cell proliferation was measured by MTT assay. LMH cells (1×10^4) were treated with SB203580, SP600125 or DMSO before infected with FAdV-4 at an MOI of 5 and harvested at different time points (0, 12, 24, 48 or 72 h) post infection. The cell viabilities of LMH were quantitated by MTT assays as described in Fig. 1. Results are representative of three independent experiments with similar results. Datas are shown as means ± SD; n = 3. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

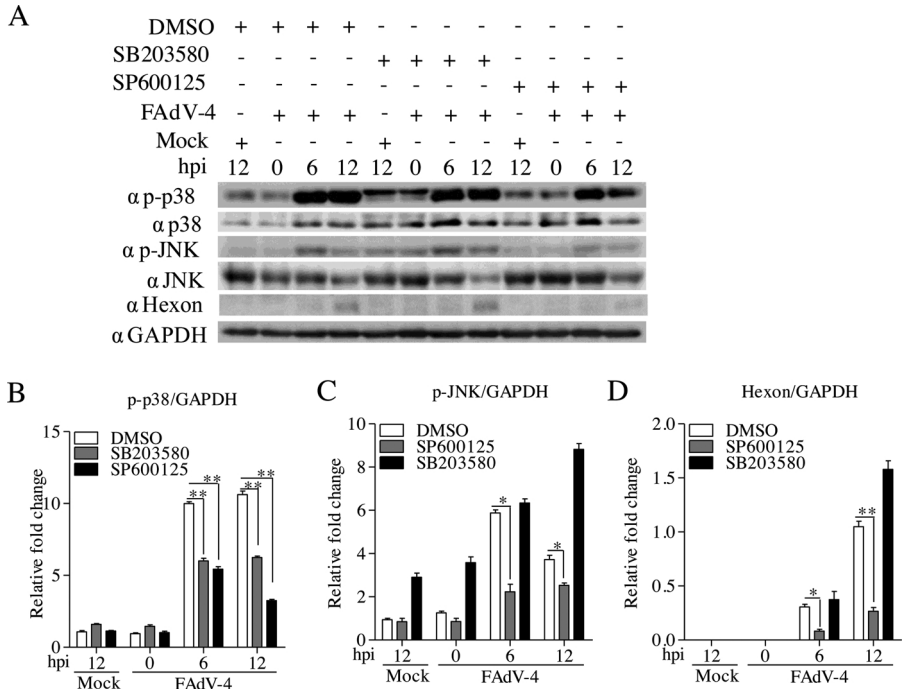

Fig. 3. The suppressive effect of MAPK inhibitors on p38 or JNK activation post FAdV-4 infection. (A) The LMH cells were seeded in 12-well plates at the density of 2×10^5 cells per well and treated with SB203580, SP600125 or DMSO before mock infected or infected with FAdV-4 at an MOI of 5. Cells were harvested at indicated time points post infection and examined with Western Blot using antibodies against p-p38, p38, p-JNK, JNK or GAPDH. (B&C) The histograms represented the changes of relative protein levels of p-p38 (B) or p-JNK (C). The band density of p-p38 or p-JNK in (A) was quantitated by densitometry, and the relative levels of p-p38 or p-JNK were calculated as follows: band density of p-p38 or p-JNK/band density of GAPDH. The relative levels of p-p38 or p-JNK in mock infection cells treated with DMSO was set as 1. (D) The histogram represented the changes of relative protein level of hexon in (A). The relative levels of hexon were calculated as follows: band density of hexon/band density of GAPDH. The relative levels of hexon in 12 hpi of FAdV-4 infection cells were set as 1. Results are representative of three independent experiments with similar results. Data are shown as means ± SD; n = 3. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

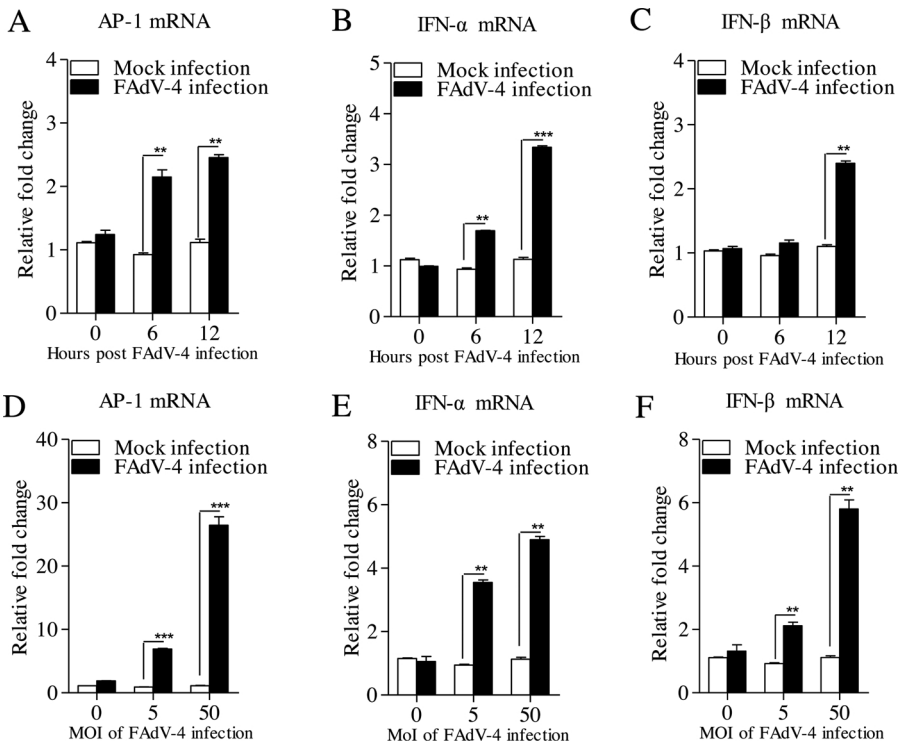

Fig. 4. FAdV-4 infection induces the expression of type I interferon in LMH cells. (A to C) LMH cells (1.0×10^6) were infected with FAdV-4 at an MOI of 5, and cells were harvested at different time points post infection. (D to F) LMH cells were infected with FAdV-4 at an MOI of 0, 5 or 50, and cells were harvested at 12 h post infection. The mRNA expression levels of AP-1, IFN-α or IFN-β were measured by qRT-PCR using specific primers. The expression levels of mRNA were calculated in relation to the expression level of GAPDH. Results are representative of three independent experiments with similar results. Data are presented as means ± SD. Data are shown as means ± SD ; n = 3. ***, P < 0.001; **, P < 0.01; *, P < 0.05.

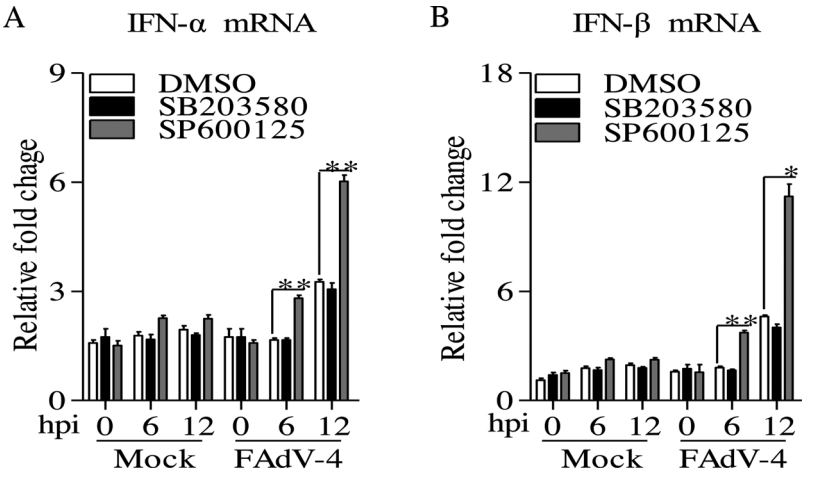

Fig. 5. The effect of SP600125 on type I IFN expression in LMH cells. (A&B) LMH cells (1.0×10^6) were treated with SB203580, SP600125 or DMSO for 1 h before infected with FAdV-4 at an MOI of 5, and cells were harvested at different time points post infection. The mRNA expression levels of IFN-α or IFN-β were measured by qRT-PCR using specific primers. The expression levels of mRNA were calculated in relation to that of GAPDH. Results are representative of three independent experiments with similar results. Data are presented as means ± SD ; n = 3. ***, P < 0.001; **, P < 0.01; *, P < 0.05.

interferon in a time-dependent and dose-dependent manners (Fig. 4A–F). Interestingly, FAdV-4-indued expression of type I interferon was significantly enhanced in cells treated with SP600125 (Fig. 5A&B). These results suggest that SP600125 strengthen the antiviral responses of host cells.

3.5. SP600125 markedly restricts virus growth of FAdV-4

The facts that type I interferon plays a vital role in anti-virus response of host cells and SP600125 can markedly promote type I interferon expression and suppress viral protein hexon expression suggest that inhibition of JNK MAPK by SP600125 inhibit FAdV-4 growth in cells. To test this hypothesis, we treated LMH cells with SB203580, SP600125 or DMSO and then infected the cells with FAdV-4 at an MOI of 5. The viral loads in the supernatants or cell cultures were examined at different time points post infection. As shown in Fig. 6A&B, the viral loads in the supernatants or cell cultures markedly decreased after SP600125 treatment as compared to that of SB203580 or DMSO controls ($p < 0.001$), indicating that SP600125 restricts FAdV-4 growth in cells.

Taken together, our results suggest that SP600125 serves as an antivirus inhibitor in host cells against FAdV-4 infection via inhibiting JNK MAPK signaling pathway and enhancing type I interferon expression.

4. Discussion

In recent years, the clinical cases of IBH, HPS or GE are increasing (Choi et al., 2012; Kajan et al., 2013; Mittal et al., 2014). Among them, there is an increasing trend of IBH or HPS in China, and the outbreaks mostly happened in broilers of 3–5 weeks of age. Besides, FAdVs are highly contagious to chickens and cause considerable economic losses (Dahiya et al., 2002; Mazaheri et al., 1998; Schonewille et al., 2008; Steer et al., 2015). Thus, FAdVs remain a threat to the poultry industry worldwide.

Nowadays, several vaccines, which are efficient to protect chickens against FAdVs, have been developed, including inactivated oil-emulsion vaccine and the new generation vaccine (Alvarado et al., 2007; Schachner et al., 2014). Besides, considering the fact that competent

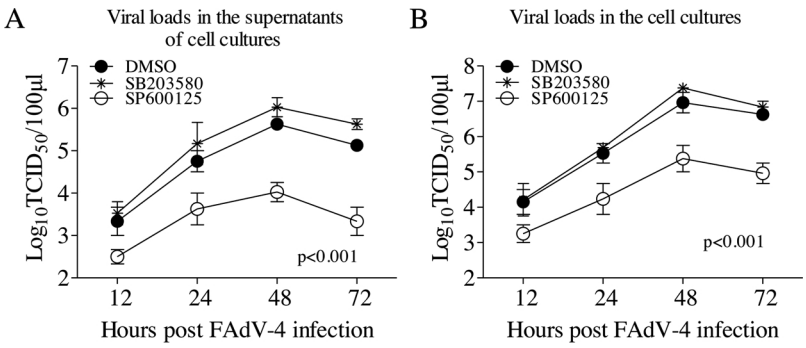

Fig. 6. Restriction of FAdV-4 growth by SP600125. (A&B) Effects of SP600125 or SB20350 on FAdV-4 growth. LMH cells were treated with SB203580, SP600125 or DMSO before infected with FAdV-4 at an MOI of 5. The viral loads in the supernatants (A) and the cell cultures (B) were determined by TCID50 at different time points (12, 24, 48, and 72 h) after FAdV-4 infection. The significance of the differences between SB20350, SP600125 and DMSO treated cells was determined by ANOVA ($P < 0.001$). Results are representative of three independent experiments with similar results. Data are represented as means ± SD; n = 3. ***, $p < 0.001$.

maternally-derived antibodies to FAdVs are capable of providing protection against FAdVs infection, vaccination of breeders is another important approach to protect young chickens against FAdVs (Toro et al., 2002). However, owing to a lack of understandings of the pathogenesis of FAdVs, an effective measure for the prevention and control of FAdVs has not been well implemented in China.

p38 and JNK MAPKs signaling pathways have been reported to be involved in many pathological conditions (Cuenda et al., 1995; Jiang et al., 1996; Kumar et al., 2001), and they are mostly indicated to be the central signaling pathways of growth stimulation (Kim et al., 2002). The MAPKs family members, especially the p38 and JNK MAPKs are known as promising drug targets for the treatment of oxidative stress-induced diseases, indicating their critical roles in anti-oxidation behavior (Ozaki et al., 1999; Saeki et al., 2002; Soh et al., 2003). In this study, we found JNK MAPK specific inhibitor SP600125 could markedly restrict virus replication in LMH cells, suggesting that JNK MAPK could serve as a novel target for antiviral therapy.

Our data show that FAdV-4 infection could inhibit the growth of LMH cells, and this inhibition could be markedly mitigated by treatment with SP600125, but not with SB203580 (Fig. 1 and 2). Furthermore, FAdV-4 infection induced phosphorylation of p38 or JNK MAPK and type I interferon expression (Fig. 3 and 4). Moreover, consistent with these observations, SP600125 significantly promoted the FAdV-4-induced type I interferon expression (Fig. 5) and inhibited viral growth in cells (Fig. 6). These data indicate that SP600125 may serve as a potential anti- FAdV-4 drug for clinical use.

Our results showed that FAdV-4 induced 2.5–4 fold higher levels of AP-1, IFN-α and IFN-β transcripts than mock-infection groups post infection. Besides, we got similar results when LMH cells were infected with FAdV-4 at different MOIs. The results are similar to the data on FAdV-9 molecular virology, which upregulated IFN-α and IFN-β by 2.7 and 3.3 fold, respectively (Deng et al., 2016). These results indicate that type I interferons play an important role in the host response against FAdVs infection.

JNKs have attracted intense interest owing to the increasing evidence of the involvement of JNK-dependent signaling events in the development of several pathological conditions since their discovery in the early 1990s. JNK-specific inhibitors have been applied in the treatment of different human diseases, from cancer, diabetes, and ischemia to viral diseases (Kaneto et al., 2005; Liu et al., 2010a, 2010b; Mizutani et al., 2005). Besides SP600125, there still exists some other small molecule compounds inhibiting JNK MAPK activity with higher efficacy and selectivity (Cargnello and Roux, 2011), and the combination of these inhibitors may be beneficial for HPS treatment.

It has been reported that viral infection can result in JNK and p38 MAPK activation, which is required for viral replication and release (Huerta-Zepeda et al., 2008; Mizutani et al., 2005; Si et al., 2005; Zapata et al., 2007). In this study, we observed a strong activation of p38 and JNK post infection of LMH cells with FAdV-4. SP600125 could suppress the phosphorylation of both JNK and p38 MAPKs, indicating that JNK and p38 MAPKs may share substrate and cross-cascade interaction. Furthermore, SP600125, as a JNK MAPK inhibitor, could significantly promote the FAdV-4-induced type I interferon expression, indicating that it might induce the activation of some DNA sensor-mediated signaling pathway(eg, cGAS-STING pathway), which still need to be dissected in our future study. Moreover, SP600125 dramatically reduced viral titers in both cell cultures and the supernatants, which is consistent with the previous findings from the studies of dengue virus, rotavirus and circovirus (Ceballos-Olvera et al., 2010; Holloway and Coulson, 2006, Wei et al., 2009). In contrast, the p38 MAPK inhibitor SB203580 did not reduce the viral yields in LMH cells, suggesting that p38 is not a critical component in cell response to FAdV-4 infection. Several questions need to be addressed, for example: does SP600125 affect cell cycles since it suppresses LMH cell growth? If yes, what is the underlying molecular mechanism? In addition to type I interferon, does SP600125 affect other anti-virus responses of host cells, such as cell apoptosis, autophagy, and inflammatory responses? More efforts will be required to elucidate these questions.

In summary, our data reveal that FAdV-4 suppresses the proliferation of LMH cells, induces the activation of p38 or JNK MAPK, and enhances type I interferon expression. We observed that inhibition of JNK MAPK signaling pathway by small molecule inhibitor SP600125 could inhibit FAdV-4-mediated suppression of cell growth. Furthermore, we found that inhibition of JNK MAPK activation promoted type I interferon expression and restricted viral replication in FAdV-4 infected LMH cells, indicating that JNK MAPK signaling pathway might be taken advantage of by FAdV-4 for its own benefit. Thus, SP600125 shows a promising antiviral agent for the control FAdV-4 infection.

Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

SZ, YW and ZH conceived and designed the experiments; ZH performed the experiments; SZ, YW and ZH analyzed the data; SZ, YW, XC, MF, JT, WF, XL and HC contributed reagents/materials/analysis tools; SZ, YW and ZH wrote the paper.

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