Cellular protein HSC70 promotes fowl adenovirus serotype 4 replication in LMH cells via interacting with viral 100K protein

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ABSTRACT   Fowl adenovirus serotype 4 (**FAdV-4**), the predominant causative agent of hepatitis-hydropericardium syndrome (**HHS**), has caused severe economic losses to poultry industry since 2015. Although fiber2 and hexon have been confirmed to be the virulence-related factors, the roles of nonstructural viral proteins in pathogenicity of FAdV-4 remain poorly understood. Here, a tandem mass spectrometry (**MS**) was used to identify host factors interacted with 100K protein of hypervirulent FAdV-4 isolate (CH/HNJZ/2015), and 2595 cellular proteins associated with many biological processes and pathways were identified according to Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses. Among the proteins, HSC70 was verified to interact with 100K through co-immunoprecipitation assay. Notably, overexpression of HSC70 promoted the replication of FAdV-4 in LMH cells, whereas blocking HSC70 with inhibitor ver-155008 markedly suppressed viral replication. Collectively, these findings suggested that many cellular proteins involved in FAdV-4 infection through interacting with 100K and HSC70 positively regulated virus replication.

Key words: FAdV-4, HSC70, 100K, interaction, replication

INTRODUCTION

Fowl adenovirus serotype 4 (**FAdV-4**), belonging to FAdV-C species of the *Aviadenovirus* genus in the *Adenoviridae* family, is a non-enveloped and double-stranded DNA virus (Benkő et al., 2022). Its genome encodes four major capsid proteins, hexon, fiber1, fiber2, and penton base, with 2 fibers being noncovalently linked to the penton base (Marek et al., 2012). FAdV-4 is the primary causative agent of hepatitis-hydropericardium syndrome (**HHS**), which is characterized by accumulation of clear, straw-colored fluid in the pericardial sac, and multifocal areas of necrosis in liver (Li et al., 2016; Schachner et al., 2018). HHS primarily affects 3- to 6-wk-old broiler chickens, with mortality rate between 20 and 80% (Balamurugan and Kataria, 2004). HHS was first reported in Pakistan in 1987, and subsequent outbreaks have been recorded in Germany, Canada, Mexico, Russia, Kuwait, Japan, Hungary, Korea, India, Chile, and South and Central Americas (Hess et al., 1999; Kajan et al., 2013). Since May 2015, severe HHS caused by a novel genotype FAdV-4 with hypervirulence has emerged in China (Liu et al., 2016), resulting in significant economic losses to poultry industry.

Currently, fiber2 and hexon genes were reported to be closely associated with FAdV-4 pathogenicity. The recombinant virus with fiber2 or hexon from highly pathogenic strain at the background of non-pathogenic one showed typical clinical and histological signs of HHS in SPF chickens (Zhang et al., 2018; Wang and Zhao, 2019), and 188R in the hexon protein was the key amino acid determining the virulence to animals (Zhang et al., 2021). However, both the virulent FAdV-4 AG234 strain and it's in vitro-attenuated counterpart INT4 bore 188R in hexon, and amino acid mutations were attributed to ORF24, fiber2, ORF19, and ORF16, indicating that besides 188R in hexon, other viral proteins may serve as virulence-modulatory factors (Schachner et al., 2019). Structural protein PX was reported to positively regulate FAdV-4 pathogenicity through inducing apoptosis in leghorn male hepatocellular (**LMH**) cells (Zhao et al., 2020). Apart from viral proteins, some cellular factors have been identified to involve in FAdV-4 pathogenesis. Chicken coxsackie and adenovirus receptor (**CAR**) protein was confirmed as receptor to facilitate viral entry into LMH cells through

interacting with fiber1 protein (Pan et al., 2020). T-complex polypeptide 1 subunit eta (**CCT7**) contributed virus replicating in LMH cells via binding to hexon protein, in order to affect viral capsid assembly (Gao et al., 2019). Karyopherin alpha 3/4 (**KPNA3/4**) efficiently assisted the replication of FAdV-4 in LMH cells through interacting with the N-terminus of fiber2 (Xie et al., 2021). The expression of SUMO-1 activating enzyme subunit (**SAE1**) was greatly upregulated to restrict FAdV-4 infection in LMH cells (Hou et al., 2021). Additionally, FAdV-4 elicited different expressions of long noncoding RNA (**lncRNA**), microRNA, and mRNA (Wu et al., 2020) in infected LMH cells and lncRNA 54128 was identified to greatly suppress the virus induced apoptosis (Wen et al., 2021). All these results suggested that viral and host factors jointly contributed to the pathogenesis of hypervirulent FAdV-4 strains.

Since FAdV-4 sporadically causes HHS outbreaks in many areas in China, accelerating to elucidate the pathogenesis of FAdV-4 will undoubtedly facilitate the development of vaccine and antiviral drugs to control the disease. Apart from structural protein, nonstructural ones play key roles in pathogenicity of FAdV-4 through various ways. 100K, a nonstructural protein, was encoded by L4 gene and synthesized during the late stage of virus infection. It was indispensable in viral life cycle through assisting hexon in trimerization to efficiently generate progeny virus (Hong et al., 2005; Koyuncu et al., 2013). Identifying the cellular factors interacting with 100K and exploring their effects on virus replication will be beneficial for further explore the molecular mechanisms underlying FAdV-4 pathogenesis. In this study, a tandem mass spectrometry (**MS**) was performed to identify the cellular factors interacting with 100K. The peptide segments were matched and analyzed by using Gene Ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) databases. Among those proteins, the interaction between the heat shock cognate 71kDa protein (HSC70) and 100K was verified through co-immunoprecipitation (**Co-IP**) and its effect on viral replication was further evaluated.

MATERIALS AND METHODS

Cell and Virus

Leghorn male hepatocellular (LMH) cells (ATCC, CRL-2117), an immortalized chicken liver cell line, were cultured in DME/F-12 (Hyclone) supplemented with 10% fetal bovine serum and 5% CO_2 at 37°C. The recombinant hypervirulent FAdV-4 strain rCH/HNJZ/2015-△1966/EGFP, with EGFP gene being inserted into 1966-bp deletion on the right end region of viral genome, was constructed in the background of hypervirulent FAdV-4 strain CH/HNJZ/2015 (GenBank accession NO. KU558760) and preserved in our laboratory.

Table 1. Primers for 100K and HSC70 cloning.

Primers	Sequence (5'-3') *
pCAGGS-N-HA-F	AGCGTAATCTGGAACATCGTATG
pCAGGS-N-HA-R	TTAAGGATCTTTTTCCCTCTGCC
FAdV4-100K-F	cagattacgct ATGTCGAATCCGTCGGT
FAdV4-100K-R	aaagatccttaaTTACCTCGGGGTGCTCG
pCMV-3 × flag-F	CGGGTGGCATCCCTGTGACC
pCMV-3 × flag-R	CTTGTCATCGTCATCCTTGTAATCG
HSC70-F	gatgacaagTCAAAGGGACCAGCTGTTG
HSC70-R	TgccacccgTTAATCCACCTCCTCAATGGTTG

*Lower case letters represent the homologous arms.

Plasmids and Antibodies

For plasmids construction, 100K and HSC70 were amplified by using genome DNA of rCH/HNJZ/2015-△1966/EGFP and cDNA of LMH cells as templates with specific primers shown in Table 1, respectively. The amplicons were subsequently cloned into linearized pCAGGS-N-HA and pCMV-3 × flag, respectively, to generate pCAGGS-N-HA-100K and pCMV-3 × flag-HSC70. Mouse anti-HA monoclonal antibody (66006-2-Ig), rabbit anti-flag polyclonal antibody (20543-1-AP), and mouse anti-actin monoclonal antibody (66009-1-Ig) were obtained from Proteintech (Wuhan, China). Mouse anti-chicken HSC70 antibody (LS-C108953) was purchased from LSBio (Seattle, WA). HRP-conjugated goat anti-mouse IgG(H+L) (SA00001-1) and anti-rabbit IgG(H+L) (SA00001-2) antibodies were purchased from Proteintech.

MS Analysis

LMH cells, seeded in 6-well plate, was transfected with 2 μg/well pCAGGS-N-HA-100K or pCAGGS-N-HA plasmids by using Lipofectamine 2000 Transfection Reagent (Invitrogen, United States) according to the manufacturer's instructions. Forty eight h (h) later, the cells were harvested and lysed with IP lysis buffer (Pierce, Rockford, IL) containing a complete protease inhibitor cocktail (Roche, Switzerland) for 30 min on ice. The supernatants, incubated with mouse anti-HA monoclonal antibody, were rocked at 4°C overnight, and then protein G-agarose beads (Roche, Switzerland) were added and rocked for further 6 to 8 h at 4°C. After being washed 5 times with lysis buffer and boiled in 2 × SDS loading buffer, the bound proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**). Subsequently, the gel containing interest proteins was sent to National Center for Protein Science Shanghai and analyzed through MS.

Bioinformatics Analysis

GO and KEGG functions were analyzed using the DAVID Bioinformatics Resources database (ver. 6.8) (https://david.ncifcrf.gov/) (Huang da et al., 2009) to promote better visualization of the proteins.

Co-IP and Western Blot

To verify the interaction between HSC70 and 100K, LMH cells cultured in 6-well plates were transfected with indicated plasmids (2 μg for pCAGGS-N-HA-100K and pCAGGS-N-HA, and 2 or 1 μg for pCMV-3 × flag-HSC70 and pCMV-3 × flag). Cell lysates were prepared at 48 h post-transfection and incubated with mouse anti-HA monoclonal antibody at 4°C overnight, and then protein G-agarose beads were added for further 6 to 8 h at 4°C. After being washed 5 times and boiled in 2 × SDS loading buffer, the bound proteins were separated by 8% SDS-PAGE and transferred onto polyvinylidene difluoride (**PVDF**) membrane (GE Healthcare, United States). After blocking with 5% skim milk for 1 h at room temperature, the membranes were incubated with indicated antibodies and detected by using chemiluminescence reagents.

Cell Treatment

For inhibition of HSC70, ver-155008 (Sigma, United States) was dissolved in dimethyl sulfoxide (**DMSO**) to stock concentration of 50 mM. LMH cells seeded in 24-well plates were pretreated with ver-155008 at final concentrations of 0.1, 0.5, 1, and 5 μM at 37°C for 12 h, and DMSO-treated cells served as control. For overexpression of HSC70, LMH cells were transiently transfected with pCMV-3 × flag-HSC70 or pCMV-3 × flag and incubated at 37°C for 24 h. After removing supernatants by washing 3 times with sterile PBS, the cells were infected with recombinant FAdV-4 strain rCH/HNJZ/2015-△1966/EGFP at an MOI of 0.01 for 2 h at 37°C, then the unbound viruses were removed with PBS. The fluorescence in cells was observed and the supernatants were collected at various time points (hours postinfection) to determine the virus titer on LMH cells.

Virus Titration

To evaluate virus growth kinetics, supernatants, diluted 10^1- to 10^8-fold with DME/F-12 containing 2% FBS, were added onto monolayers of LMH cells. After incubating at 37°C for 2 h, the medium was replaced with fresh 2% DME/F-12 and cultured for further 5 d. The $TCID_{50}$ was determined by fluorescence and calculated by the method of Reed and Muench.

Statistical Analysis

All the experiments were performed at least 3 times, and results were analyzed by using Student's t test. Significant differences were determined as $*P < 0.05$ (significant) or $**P < 0.01$ (highly significant).

RESULTS

Cellular Proteins Interacted With 100K in LMH Cells

In order to comprehensively investigate the cellular proteins interacted with 100K, Co-IP was performed and the interest gels were analyzed by MS. A total of 2,595 proteins were identified by mapping the obtained peptides against the chicken reference database, and DAVID 6.8 was used to perform GO and KEGG analysis. In biological processes, these proteins were mostly involved in mRNA splicing. In terms of cellular components, they were mainly distributed in the nucleus. For molecular function, most proteins had protein binding and poly(A) RNA binding activity (Figure 1). KEGG pathway analysis showed that most identified proteins were closely associated with RNA processing, transporting, and biosynthesis (Figure 1), consistent with the results in biological processes of GO analysis. These
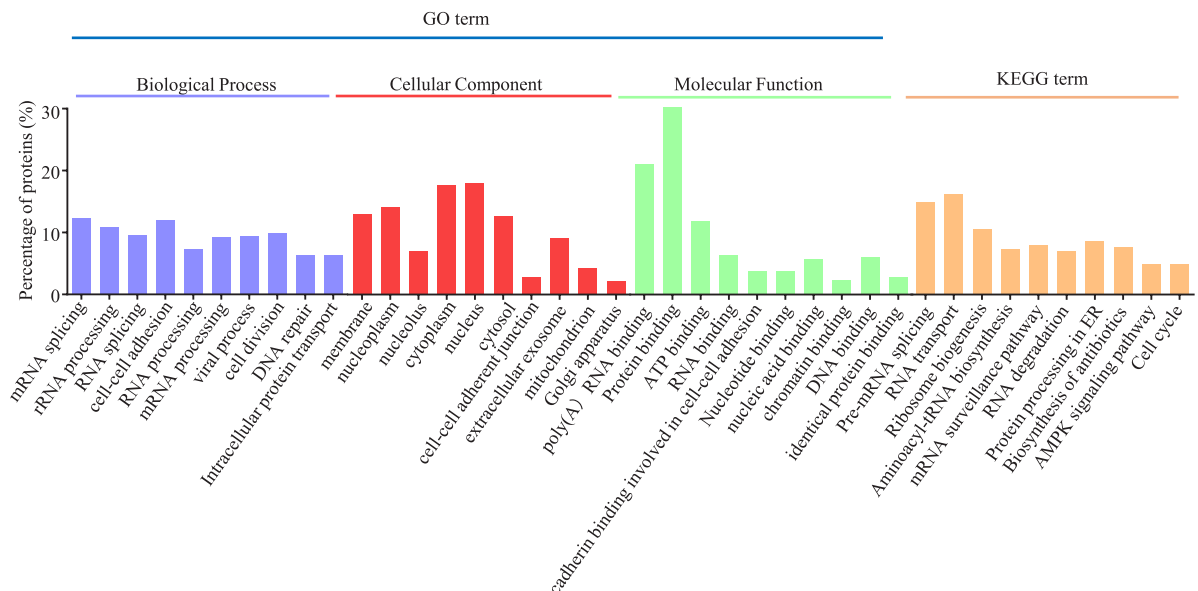
Figure 1. GO and KEGG analysis of identified cellular proteins. Top 10 GO categories in biological process, cellular components, and molecular function with P-adjust value < 0.01. And KEGG pathways enriched in biological process with P-adjust value < 0.01. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

data indicated that targeting these factors might influence FAdV-4 infection through interfering the biological functions of cellular RNAs.

Verification of Interaction Between 100K and HSC70 Through Co-IP

Among the identified cellular proteins, heat shock cognate 71kDa protein (HSC70) was mostly reported to be deeply involved in different viruses' infection through various ways (Wang et al., 2020). However, whether HSC70 affected FAdV-4 infection remains unclear. Therefore, HSC70 was selected to verify its interaction with 100K via Co-IP. LMH cells were transfected with 2 μg pCAGGS-N-HA-HNJZ/100K and 2 or 1 μg pCMV-3 × flag-HSC70, individually or in combination. Cell lysates were immunoprecipitated with anti-HA antibody, followed by western blotting with antibodies against HA or flag tag. For LMH cells transfected with equal amounts of the two plasmids, flag-tagged HSC70 was coimmunoprecipitated with HA-tagged 100K when they were co-expressed, but not in the absence of 100K, suggesting that HSC70 interacted with FAdV-4 100K in LMH cells (Figure 2A). Meanwhile, similar results were shown in cells transfected with plasmids of 2:1 in mass, and a clear band with approximately 70kDa was identified when two proteins were simultaneously expressed (Figure 2B), suggesting a robust interaction between 100K and HSC70. To further investigate the interaction between 100K and endogenous HSC70, LMH cells were transfected with pCAGGS-N-HA-HNJZ/100K, and an immunoprecipitation assay was performed

with anti-HA antibody. The endogenous HSC70 was detectable in cells expressing 100K, but not those transfected with pCAGGS-N-HA empty vector (Figure 2C), further confirming that HSC70 served as a binding partner of the viral nonstructural 100K protein.

## HSC70 Positively Regulated FAdV-4 Replication in LMH Cells

To study the role of HSC70 in FAdV-4 infection, LMH cells transfected with pCMV-3 × flag-HSC70 or pCMV-3 × flag for 24 h were infected with rCH/HNJZ/2015-△1966/EGFP at an MOI of 0.01, and supernatants were collected to evaluate viral growth kinetics in vitro. Results showed that rCH/HNJZ/2015-△1966/EGFP grew more rapidly in HSC70 overexpressed cells, which exhibited greater bright green fluorescence than pCMV-3 × flag transfected cells and control cells (Figure 3A). And virus titers in HSC70 overexpressed cells were approximately 100- to 250-fold higher than those in pCMV-3 × flag transfected cells and control cells at 24, 36, and 48 h p.i. (Figure 3B). These results clearly demonstrated that HSC70 positively modulated FAdV-4 replication in LMH cells.

HSC70 Inhibitor Ver-155008 Blocked FAdV-4 Replication in LMH Cells

To further analyze the effect of HSC70 on FAdV-4 replication, LMH cells were pretreated with 0.1, 0.5, 1, and 5 μM ver-155008 for 12 h at 37°C, and then infected with rCH/HNJZ/2015-△1966/EGFP
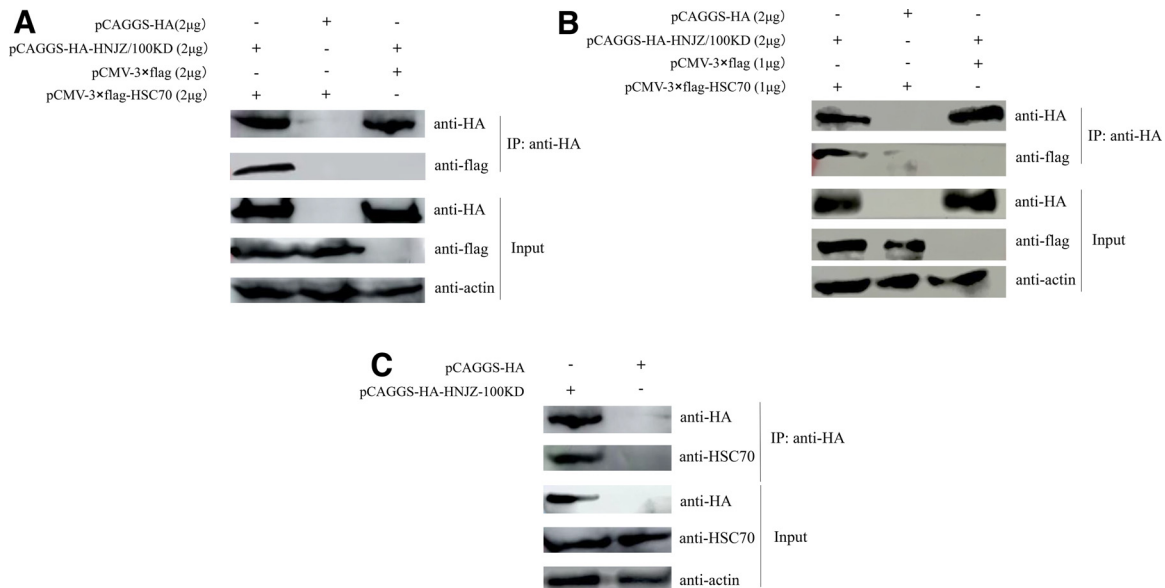
Figure 2. FAdV-4 100K interacted with HSC70 in LMH cells. (A, B) The interaction of 100K with exogenous HSC70. LMH cells were transfected individually or in combination with 2 μg pCAGGS-N-HA-HNJZ/100K and 2 μg (A) or 1 μg (B) pCMV-3 × flag-HSC70. Cell lysates were immunoprecipitated with anti-HA antibody and were subjected to western blotting using anti-HA and anti-flag antibodies to examine the presence of 100K and HSC70, respectively. (C) The interaction of 100K with endogenous HSC70. LMH cells were transfected with pCAGGS-N-HA-HNJZ/100K or empty vector as control. Immunoprecipitation assay was performed with anti-HA antibody and HSC70 in the immune complex was examined with western blotting using mouse anti-chicken HSC70 antibody. Abbreviations: FAdV-4, Fowl adenovirus serotype 4; LMH, leghorn male hepatocellular.
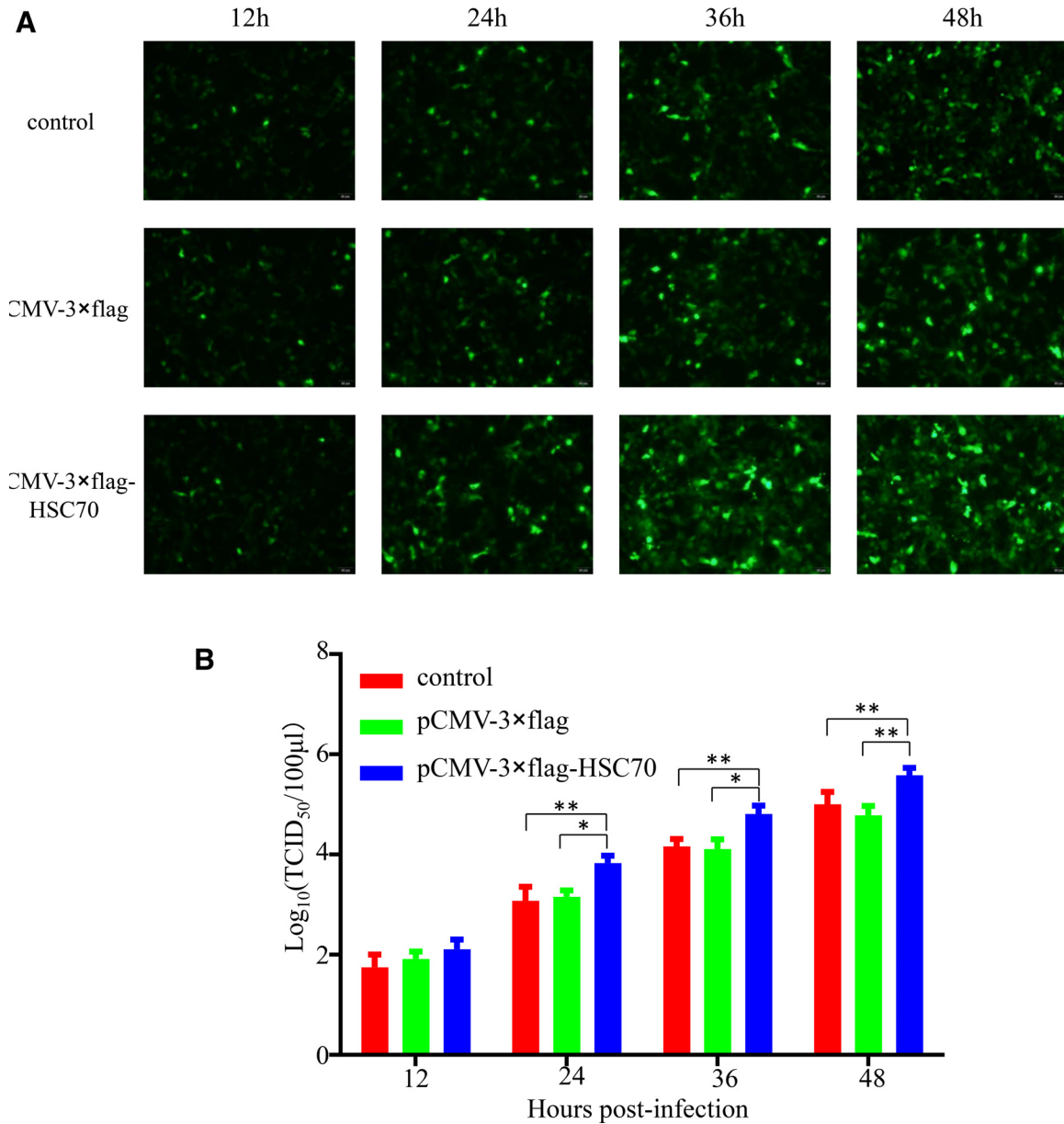
Figure 3. HSC70 overexpression contributed to the replication of FAdV-4 in LMH cells. LMH cells were transfected with pCMV-3 × flag-HSC70 or empty vector as control, followed by infection with rCH/HNJZ/2015-$\triangle$1966/EGFP at an MOI of 0.01. At different time points postinfection, the green fluorescence was observed (A) and virus titers in cultural supernatants were determined by $TCID_{50}$ in LMH cells (B). *Stands for $P < 0.05$, ** stands for $P < 0.01$. Abbreviations: FAdV-4, Fowl adenovirus serotype 4; LMH, leghorn male hepatocellular.

at an MOI of 0.01. The expression of green fluorescence was observed and culture supernatants were collected at different timepoints to evaluate viral titers. Results demonstrated that the intensity of green fluorescence negatively correlated with the inhibitor concentrations, indicating the repressive effect of ver-155008 was dose-dependent. LMH cells treated with 5 μM ver-155008 only expressed a little amount of fluorescence at every timepoint p.i.. However, the number of fluorescence increased gradually without ver-155008 treatment (Figure 4A). Meanwhile, inhibiting HSC70 led to an about 100- to 300-fold decrease in progeny virus titers compared with those of control cells (Figure 4B), indicating that ver-155008 blocked FAdV-4 replication through inhibiting HSC70.

DISCUSSION

As a multifunctional nonstructural protein, 100K played critical roles in Adenovirus infection. It assisted chimeric hexon in trimerization to generate recombinant human adenovirus serotype 5 (**HAdV-5**) vectors and improved proliferation efficiency (Yan et al., 2016). Additionally, hexon was insoluble when expressed alone in vitro, however, soluble trimeric hexons were detected when co-expressed with 100K, further confirming the chaperon role of 100K in hexon folding (Hong et al., 2005). HAdV-5 100K selectively bound to 5' noncoding region of viral late mRNA and formed a complex with initiation factor eIF4G and poly(A)-binding protein to promote ribosome shunting in order to enhance viral protein
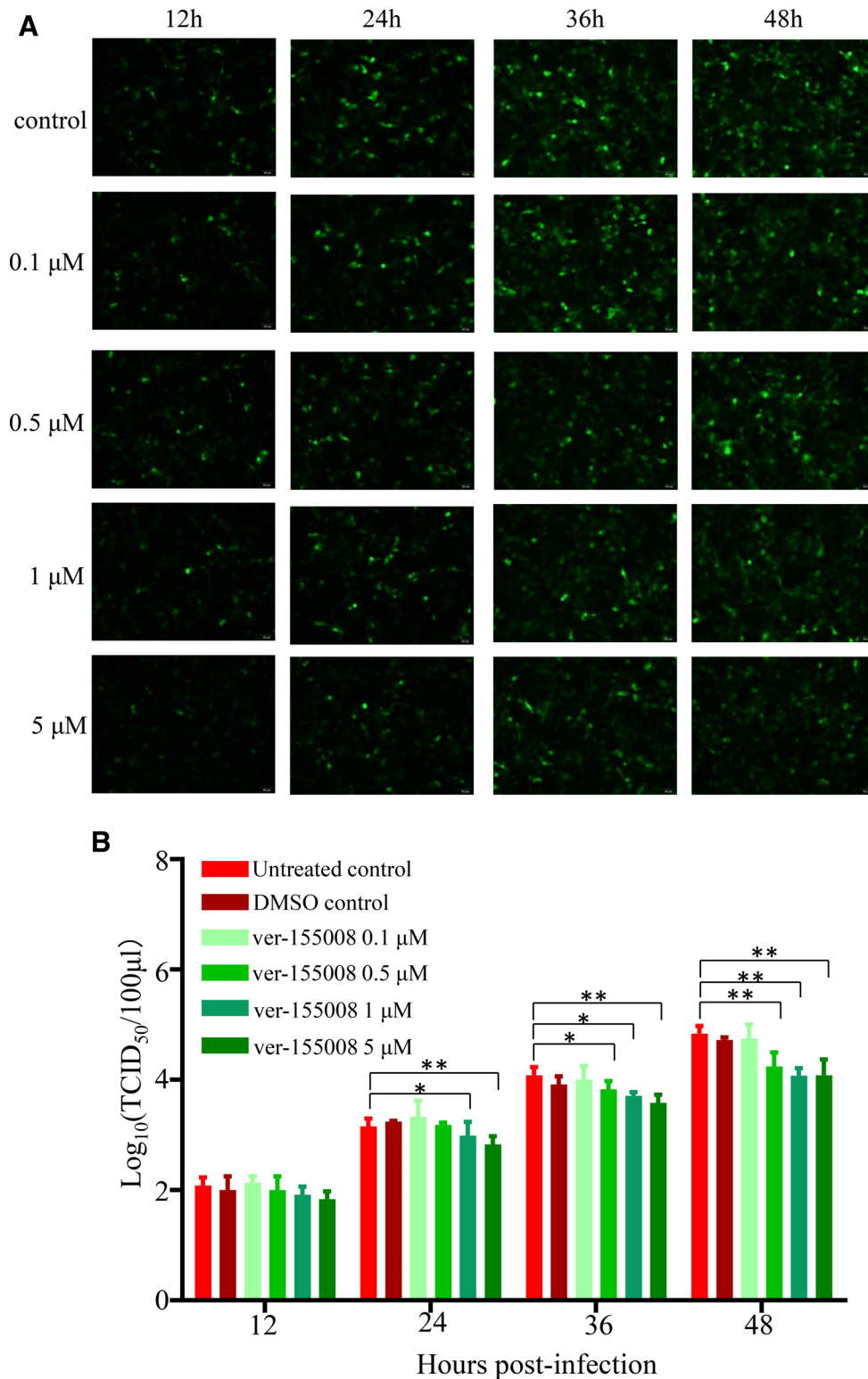
Figure 4. HSC70 inhibitor negatively regulated FAdV-4 replication in LMH cells. LMH cells were pretreated with different concentrations of HSC70 inhibitor ver-155008, followed by infection with rCH/HNJZ/2015-△1966/EGFP at an MOI of 0.01. Then the expression of green fluorescence (A) and virus loads in cultural media (B) were evaluated at different time points post infection. * Stands for $P < 0.05$, ** stands for $P < 0.01$. Abbreviations: FAdV-4, Fowl adenovirus serotype 4; LMH, leghorn male hepatocellular.

synthesis but inhibit cellular mRNA translation (Xi et al., 2004). HAdV-5 100K specifically formed a stable complex with granzyme B to inhibit apoptosis of infected cell and promote virus replication (Andrade et al., 2001). Therefore, it's essential to explore the roles of 100K in pathogenesis of hypervirulent FAdV-4. In this study, we firstly used MS to examine critical cellular proteins interacted with 100K and 2,595 potential host factors were identified. By comprehensive GO and KEGG analysis, most identified host proteins were found to be related with cellular RNA processing, transporting, and biosynthesis, indicating the possible mechanism of how they might be involved in FAdV-4 pathogenesis.

The heat shock proteins 70 (**HSP70s**) family consists of molecular chaperons of approximately 70 kDa in size (Radons, 2016) and all the members share the similar structural domains, including an N-terminal ATP-binding domain (**NBD**), a substrate-binding domain (**SBD**), and a C-terminal lid domain (Rauch et al., 2016). Thereinto, HSC70 is constitutively expressed and responsible for maintaining host protein homeostasis and protecting cells from physical and chemical damage (Stricher et al., 2013). Additionally, HSC70 has been reported to be involved in regulating various viruses' infections. As an intracellular and membrane-anchored protein, HSC70 facilitated the entry step of dengue virus into C6/36 cells through interacting with glycoprotein E (Vega-Almeida et al., 2013). Many enveloped viruses were internalized through clathrin-mediated endocytosis and HSC70 assisted in the release and recycle of clathrin to contribute virus invasion (Newmyer and Schmid, 2001). Therefore, in HSC70-knockdown C6/36 cells, Japanese encephalitis virus cannot be taken up to form acidified endosomes, let alone release viral RNA for further translation on endoplasmic reticulum (**ER**) membrane (Ren et al., 2007). Furthermore, HSC70 can be hijacked by murine latency-associated nuclear antigen, a conserved protein of murine γ herpesvirus 68, to form viral replication complexes in nucleus and further promoting viral DNA replication (Salinas et al., 2015). Hepatitis C virus NS5A protein directly interacted with HSC70 to promote the degradation of hepatocyte nuclear factor 1 alpha via chaperone-mediated autophagy pathway and thereby facilitated virus pathogenesis (Matsui et al., 2018). Here, we demonstrated that HSC70 positively involved in FAdV-4 infection through interacting with 100K protein, further enriching the biological functions of HSC70 regulating virus pathogenesis.

Although the data indicated the requirement of HSC70 for the replication of FAdV-4 in LMH cells via interaction with 100K, further investigation is needed to reveal the exact mechanism underlying the process. Meanwhile, further studies will be necessary to elucidate the roles of other identified proteins in FAdV-4 infection.

In summary, our data firstly identified the cellular proteins interacting with FAdV-4 100K protein through MS approach, and demonstrated HSC70 served as a binding partner of 100K and positively regulated FAdV-4 replication in vitro. Further functional analysis to explore the underlying mechanism will be beneficial for not only elucidation the pathogenesis of hypervirulent FAdV-4 but the development of antiviral strategies.

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DISCLOSURES

The authors declare no conflict of interest.

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