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Inhibition of aldolase A blocks biogenesis of ATP and attenuates Japanese encephalitis virus production

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ABSTRACT

Viral replication depends on host proteins to supply energy and replication accessories for the sufficient production of viral progeny. In this study, we identified fructose-bisphosphate aldolase A as a binding partner of Japanese encephalitis virus (JEV) untranslated regions (UTRs) on the antigenome via RNA affinity capture and mass spectrometry. Direct interaction of aldolase A with JEV RNAs was confirmed by gel mobility shift assay and colocalization with active replication of double-stranded RNA in JEV-infected cells. Infection of JEV caused an increase in aldolase A expression of up to 33%. Knocking down aldolase A reduced viral translation, genome replication, and viral production significantly. Furthermore, JEV infection consumed 50% of cellular ATP, and the ATP level decreased by 70% in the aldolase A-knockdown cells. Overexpression of aldolase A in aldolase A-knockdown cells increased ATP levels significantly. Taken together, these results indicate that JEV replication requires aldolase A and consumes ATP. This is the first report of direct involvement of a host metabolic enzyme, aldolase A protein, in JEV replication.

1. Introduction

Numerous studies have demonstrated that host factors participate in every step of the viral life cycle [1,2]. These steps include the induction of membrane remodeling [3,4], manipulation of host lipid metabolism [5], recruitment of host factors into the replication complex [1], and targeting of host-cell metabolic machinery during infection [6]. Several cytopathogenetic changes induced by different flaviviruses indicate that viruses affect cellular processes by altering the host's metabolism, yet the underlying mechanisms remain to be elucidated.

Japanese encephalitis virus (JEV) belongs to the *Flaviviridae* family and is transmitted by mosquitoes. In humans, JEV causes acute encephalitis and meningitis, resulting in a high mortality rate and permanent neurologic sequelae in many survivors. JE is therefore a leading form of viral encephalitis worldwide, and it is prevalent mainly in eastern and southern Asia [7]. The JEV contains a single-stranded RNA (~11 kb) genome that encodes a single large, open reading frame (ORF) flanked by a 5′ untranslated region (UTR) of 95 nucleotides (nts) and a 3′ UTR of 585 nts. The ORF is translated into a polyprotein precursor that undergoes proteolytic processing and yields three structural proteins (capsid C, membrane M, and envelop E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [8].

Several studies have shown that host proteins involve the initiation of viral RNA synthesis by interacting with the 5′-UTR and/or 3′UTR of the genome or antigenome [9]. In JEV, cellular Mov 34 and La proteins bind to the 3′-UTR of the viral genome [10–12]. Polypyrimidine tract-binding protein (PTBP) interacts with the 3′ stem-loop region of minus-strand RNA [13]. Heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) binds to the 5′-UTR of the minus-strand JEV RNA [14]. FUSE binding protein 1 binds both UTRs and negatively regulates viral replication [15]. Previously, we showed that the cellular protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binds to the 3′ end of both strands, suggesting a role for promoting asymmetric RNA replication [16]. These interactions have been demonstrated to play roles in regulating viral RNA synthesis and translation.

In this study, we identified aldolase A as a binding partner of antigenome using an RNA affinity capture and mass spectrometry. Aldolase A is a member of multi-enzyme glycolytic complexes and catalyzes the reversible conversion of fructose-1, 6-bisphosphate to glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). Glucose is the primary source of energy for cellular metabolism. Many viruses modulate glucose metabolism to facilitate survival in the hosts [17]. In this study, we found that silencing aldolase A in JEV-infected cells reduces the ATP level, resulting in

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the inhibition of viral replication. Our results indicate that aldolase A is important for JEV reproduction.

2. Materials and methods

2.1. Cells and viruses

BHK-21 cells were grown at 37 °C in RPMI 1640 medium supplemented with 2% fetal bovine serum (HyClone). Human embryonic kidney (HEK293T) cells were grown in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone). JEV RP-9 (GenBank accession No. AF014161) was used in this study.

2.2. Identification of aldolase A protein

The method for capturing RNA-interacting proteins has already been described [18]. Briefly, in vitro-transcribed RNAs (7 μg) corresponding to the 5′-terminal region at nucleotides 10431–10566 on the JEV antigenome was heated at 85 °C for 5 min and annealed to the 5′-biotin-labeled oligonucleotide (5′-TGTAAATGAGAAAATGC ATGCATA-3′) at 45 °C for 30 min. Streptavidin beads were washed three times with binding buffer (20 mM HEPES, pH 7.5, 1 × Protease inhibitor (Roche), 1 mM DTT, 100 mM NaCl) and mixed with the RNA:biotin-labeled duplex for 30 min at 4 °C. The immobilized RNA was washed three times with binding buffer and incubated with 0.6 mg precleared protein extract from BHK-21 cells (or 1.5 μg of NS5 protein as positive control) for 30 min at room temperature. The RNA–protein complexes were washed three times with binding buffer, and the bound proteins were eluted from the streptavidin beads by heating at 95 °C for 5 min in sample buffer and visualized by SDS-10% PAGE. A 40-kDa protein band bound to biotin-RNA duplex but not to the control tRNA was excised from the gel for its identification by mass spectrometry, as described previously [19].

2.3. Mobility shift assay

RNA–protein interactions were analyzed by electrophoretic mobility shift assays (EMSAs), as previously described [16]. Recombinant protein aldolase A (Sigma) was incubated with heat-denatured radioactively labeled riboprobe (10^5 cpm per reaction) in renaturing buffer (40 mM HEPES pH 7.9, 120 mM KCl, 4 mM $MgCl_2$) at room temperature for 30 min. The RNA–protein complexes (RPCs) were resolved by electrophoresis through a 4% native polyacrylamide gel in 0.5 × TBE and analyzed by autoradiography.

2.4. Plasmid

The pEGFP-ALDOA was constructed by cloning the PCR products amplified with the forward (5′-ggatccATGCCCCACCCATACCC-3′) and reverse (5′-ggatccTTAGTAGGCATGGTTAGAGATG-3′) primers into the pEGFP-C1 expression vector (Clontech) and verified by sequencing.

2.5. Immunofluorescence assay

HEK293T cells (1×10^4 cells/well) were seeded onto a 96-well imaging plate (BD Falcon) and incubated overnight. First, cells were transfected with pEGFP-ALDOA (0.2 μg/well) using Lipofectamine 2000 in OptiMEM according to the manufacturer's instructions (Invitrogen). At 12 h post-transfection, cells were mock-infected or infected with JEV at an MOI of 10. Cells were fixed with ice-cold methanol at 24 h postinfection for 20 min and permeabilized using 0.1% Triton X-100 in PBS for 10 min at room temperature. For the detection of dsRNA, the mouse monoclonal antibody J2 (English and Scientific Consulting Bt.) and Alexa Fluor 594 anti-mouse secondary antibody (Invitrogen) were used. The nuclei were stained with Hoechst 33342 (Invitrogen). Images were acquired using a BD pathway 435 Bioimager (BD Biosciences).

2.6. Western blot analysis

For testing endogenous aldolase A expression, 6×10^5 HEK293T cells in 6-well plates were mock infected or infected with JEV at an MOI of 10. Cell lysate was extracted at 12, 24, 36, and 48 h postinfection. Mock-infected cell lysate was extracted simultaneously at each time point. The procedure for transfection pEGFP-ALDOA (or control pEGFP-C1 vector) was performed as described before [20]. Proteins were separated on a SDS-10% PAGE for Western blot analysis using anti-NS5, anti-Aldolase A (Chemicon international), anti-GAPDH (GeneTex), or anti-β-actin (Sigma) antibodies. Proteins were visualized using the ECL detection system (Amersham Biosciences), according to the manufacturer's instructions. Signals were detected with a luminescent image analyzer (LAS-3000, Fujifilm) and analyzed using MultiGauge software (Fujifilm).

2.7. RNA interference

The lentiviral vectors expressing the short hairpin (sh) RNA (TRCN-314787) targeting the human aldolase A gene (GenBank accession No. NM_001127617.2) and the negative control shRNA (GFP) were obtained from Taiwan National RNAi Core Facility (NRC). Lentivirus stocks were prepared according to the manufacturer's instructions. To knock down aldolase A expression, 1×10^6 HEK293T cells were transduced with the sh-aldolase A or sh-GFP lentiviruses and selected with puromycin (1 μg/ml). At 2 weeks posttransduction, cells were seeded on 60-mm plates (approximately 2×10^6 cells per plate) and infected with JEV at an MOI of 10. The cells were harvested with PBS at 48 h postinfection and divided in half for Western blot and Northern blot analyses. Equivalent amounts of proteins (20 μg/sample) were resolved by SDS-10% PAGE, followed by Western blotting. Total RNA was extracted using REzolTM C&T reagent (Protech) and analyzed by Northern blotting, as described previously [21].

2.8. ATP assays

The ATP Colorimetric/Fluorometric Assay Kit (BioVision) was used for analyzing ATP consumption, according to the manufacturer's instructions. Briefly, HEK293T cells (1×10^6) were mock-infected or infected with JEV at an MOI of 10. At 48 h postinfection, the cells were lysed in 100 μl ATP Assay Buffer and followed by centrifugation. The supernatant (50 μl) was mixed with 50 μl of ATP reaction mix in a 96-well plate. The optical density at 570 nm was measured in an Enspire Multilabel reader (PerkinElmer). Values were compared with the ATP standard curve. Assays were performed in triplicate.

2.9. Statistical analysis

Changes in virus titers and ATP levels were analyzed by Students *T* test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Identification of fluctose-bisphosphate aldolase A associated with JEV RNA

Host proteins have been shown to participate in viral translation and replication by means of binding to the termini of viral genomes and/or antigenomes. To investigate host factors involved

in RNA–protein interactions within JEV-infected cells, a pull-down assay was executed using streptavidin beads to capture biotinylated RNA-associated proteins (described in Section 2). A 40-kDa protein bound to the JEV(−)10431–10566 RNA but not to the negative control (Fig. 1A) was excised from the gel and analyzed by mass spectrometry. The result showed 100% identity with fructose 1, 6-bisphosphate aldolase A (aldolase A). To confirm the interaction between aldolase A and the JEV RNA, we performed a mobility shift assay using recombinant aldolase A protein interacting with JEV(−)10431–10566 as well as JEV(−)1–160 RNAs. RNA–protein complex shift signals were detected in a dose-dependent manner with JEV(−)1–160 and JEV(−)10431–10566 RNAs (Fig. 1B), indicating that both RNAs bind to aldolase A protein but not to BSA (data not shown).

To analyze whether replication of JEV requires the recruitment of aldolase A, we used immunofluorescent staining to examine the localization of aldolase A with double-stranded RNA using confocal microscopy of the mock-infected and JEV-infected cells. To visualize aldolase A, the gene was cloned in-frame with GFP (pEGFP-ALDOA) and transfected into HEK293T cells. The cells were then infected with JEV at an MOI of 10 at 12 h post-transfection and fixed at 24 h postinfection. The expression of aldolase A was detected in both the mock-infected and infected cells (Fig. 1C, panels a and e). Upon JEV infection, active expression of viral double-stranded RNA confirmed by immunofluorescence using the J2 anti-dsRNA antibody was detected (Fig. 1C, panel f), but not in the mock-infected cells (Fig. 1C, panel b). The images of dual-labeled immunofluorescence showed yellow coloration, indicating that aldolase A colocalized with the active replication of the double-stranded RNA (Fig. 1C, panel h).

3.2. Infection with JEV elevated endogenous aldolase A expression

To examine whether infection with JEV caused an increase in aldolase A expression, we compared the expression of aldolase A at 48 h postinfection with uninfected cell extracts and found that JEV infection increased aldolase A expression by 33% (Fig. 2A). We then performed time course experiments and monitored the endogenous aldolase A expression upon JEV infection. As shown in Fig. 2B and C, the expression of aldolase A increased 3.4% at 12 h postinfection and continued to increase to 20% at 48 h postinfection, indicating that JEV infection caused up-regulation of aldolase A (Fig. 2B and C).

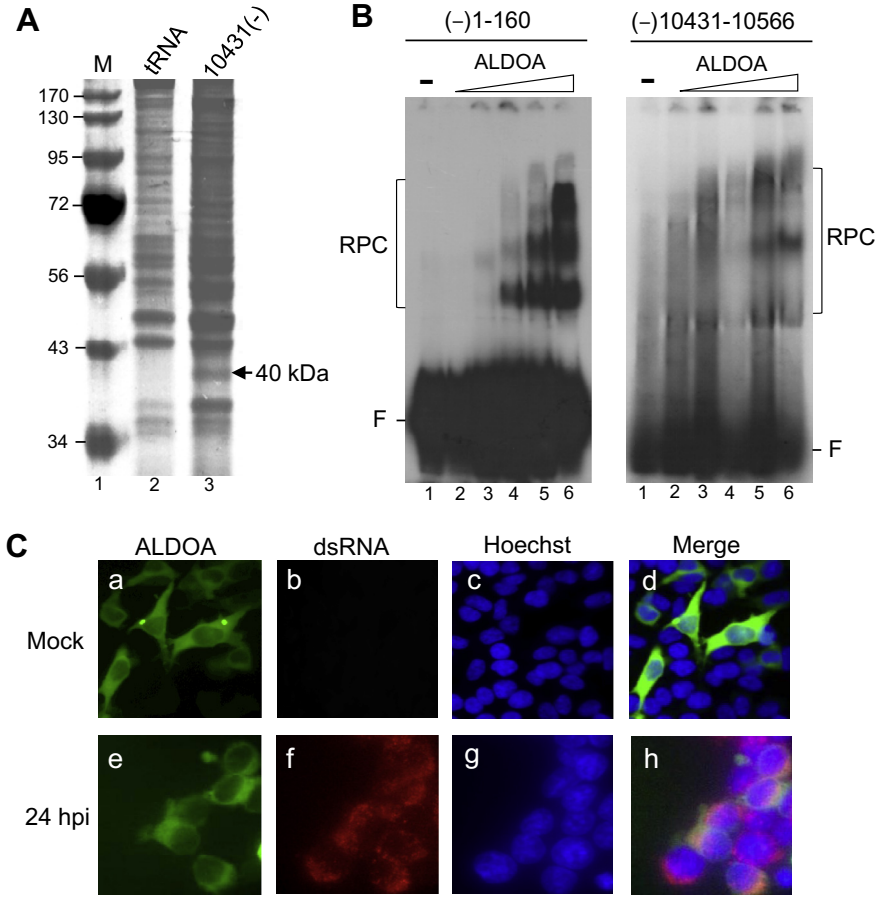

Fig. 1. Interaction of Aldolase A with JEV RNAs. (A) Identification of cellular proteins interacting with JEV(−)10431–10566 RNA by affinity capture pulldown assay, as described in Section 2. The bound proteins were eluted and resolved by SDS-10% PAGE and stained with Coomasie blue. The 40-kDa band, which bound to JEV(−)10431–10566 (lane 3) but not to tRNA (lane 2), was excised for in-gel trypsin digestion and analyzed by MALDI-TOF. The protein was identified as aldolase A. (B) Interaction between the recombinant aldolase A with JEV(−)1–160 and JEV(−)10431–10566 RNAs was confirmed by EMSA. Radiolabeled RNAs were incubated with increasing amounts of aldolase A (0.05, 0.1, 0.5, 1.0, and 2.0 μg) (lanes 2–6, respectively) or without added aldolase A (lane 1). Reaction products were electrophoresed on a 4% native polyacrylamide gel. The RNA–protein complex (RPC) signals are indicated. F, free riboprobe. (C) Expression of aldolase A protein colocalized with JEV double-stranded RNA (dsRNA) in infected cells. HEK293T cells were transfected with pEGFP-ALDOA and mock-infected or infected with JEV at an MOI of 10 at 12 h posttransfection and fixed at 24 h postinfection (hpi). Expression of transfected pEGFP-ALDOA (panels a and e), immunostaining for dsRNA (panels b and f), and DAPI-stained nuclei (panels c and g) are shown. Panels d and h are merged images. Cells were viewed at 400× magnification.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

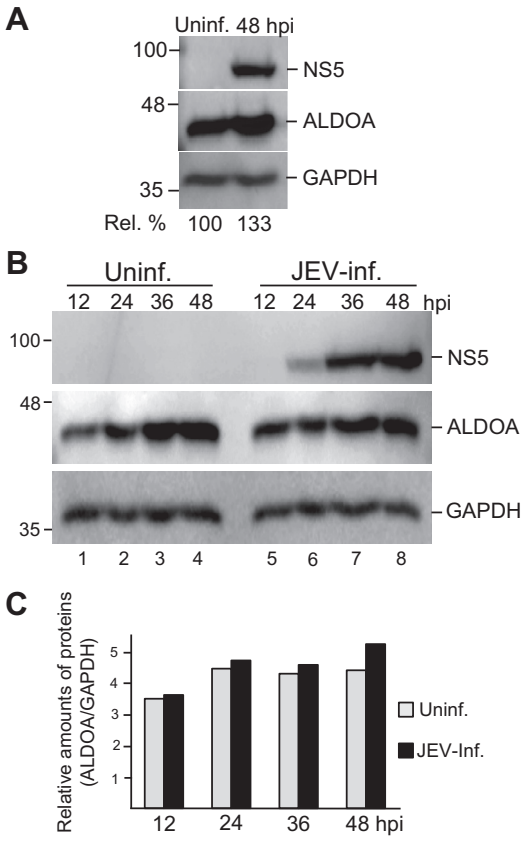

Fig. 2. Infection with JEV caused up regulation of endogenous aldolase A. (A) HEK293T cells were uninfected (uninf.) or infected with JEV at an MOI of 10. The cell lysates were extracted at 48 h postinfection and subjected to Western blot analysis using anti-NS5, anti-aldolase A, and anti-GAPDH antibodies. The relative amounts of proteins (aldolase A/GAPDH) were quantified with image densities. (B) Infection was performed as in (A), and the proteins were extracted at the time indicated at the top. (C) Band densities on (B) were quantified and plotted versus time.

3.3. Aldolase A is important for JEV replication

To determine if JEV-mediated aldolase A activation is relevant to viral replication, we knocked down aldolase A (Aldoa-KD) by gene silencing prior to JEV infection. The expression of aldolase A decreased significantly to 27%, compared to that of the sh-GFP control WT cells (Fig. 3A, lane 3). The cells were then infected with JEV at an MOI of 10, followed by the isolation of total RNA and protein and collection of supernatant from both the control and silenced cells at 48 h postinfection. The NS5 protein expression as well as the viral genome decreased to approximately 41% in Aldoa-KD cells, compared to those of WT cells (Fig. 3A and B). Virus titers in culture fluid samples were determined by plaque assay on BHK-21 cells. Fig. 3C shows that the suppression of aldolase A significantly reduced virus titers. These results indicate that aldolase A is important for JEV replication.

The reduction in virus production might be due to the inhibition of host metabolism, which decreases the efficiency of cell propagation. To examine this possibility, we seeded equal numbers of wild-type and Aldoa-KD cells and measured the cell numbers at 6- to 12-h intervals by counting the cell numbers and performing cell viability assay. The proliferation of Aldoa-KD cells showed no significant difference from the wild-type cells determined by both methods (data not shown).

To test whether aldolase A overexpression facilitates JEV replication, pEGFP-ALDOA was transfected into HEK293T cells. There is no significant difference in viral translation, replication, or virus titer, compared to the mock-transfected, JEV-infected cells (data not shown), which suggests that abundant endogenous aldolase A is sufficient to support JEV replication, whereas the overexpression of aldolase A does not contribute substantially to facilitate viral replication.

3.4. Replication of JEV consumes ATP

Aldolase A is a major metabolic enzyme during glycolysis for the biogenesis of ATP. To analyze whether the recruitment of aldolase A during JEV replication changes cellular ATP amounts, we measured the ATP levels in HEK293T and the Aldoa-KD cells with or without JEV infection. As shown in Fig. 4, knocking down aldolase A reduced approximately 70% of total ATP, which indicates that aldolase A is essential for producing ATP. JEV infection consumed approximately 55% of the cellular ATP, and the ATP amounts were further reduced in the Aldoa-KD cells (Fig. 4). To test whether the overexpression of aldolase A can rescue ATP synthesis, we transfected pEGFP-ALDOA into the Aldoa-KD cells. The result showed that the ATP level increased significantly in JEV-infected Aldoa-KD cells,

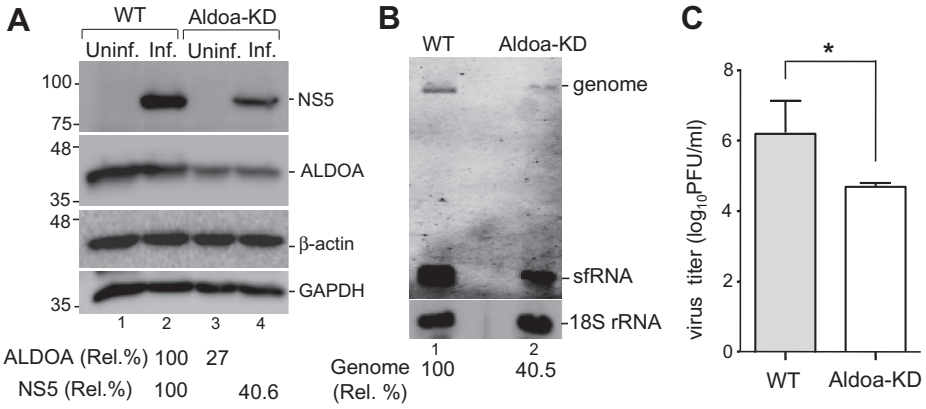

Fig. 3. Silencing of aldolase A inhibits viral translation, replication, and titers. (A) HEK293T (WT) and aldolase A-knockdown cells (Aldoa-KD) were mock-infected or infected (inf.) with JEV at an MOI of 10 and analyzed at 48 h postinfection by Western blotting using anti-NS5, anti-aldolase A, and anti-β antibodies. Rel. % represents percentage of aldolase A (top) and NS5 (bottom) expression normalized to β-actin in Aldoa-KD cells compared to that in WT cells. (B) Cells were treated as described in panel (A). Total RNAs (4 μg) were analyzed by Northern blotting using an Irdye 700-labeled oligonucleotide to detect nts 10433–10566 in the 3′ UTR. Rel. % represents JEV genome normalized to 18S rRNA, compared to that in WT cells. (C) Virus titers (PFU/ml) in the culture supernatants determined by plaque assays executed in triplicate on BHK-21 cells. *P* values were calculated by two-tailed Student's *t*-test. Asterisks indicate statistically significant differences, compared with the WT cells.

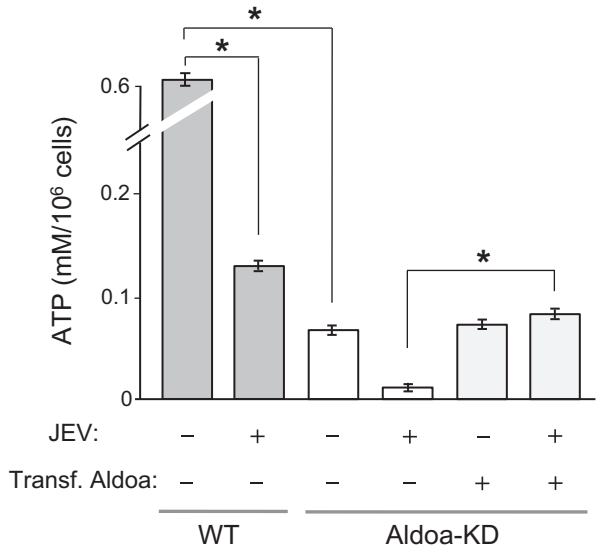

Fig. 4. Infection with JEV consumes ATP, and aldolase A is responsible for ATP synthesis. The symbol "–" represents untreated and "+" treated with JEV infection or pEGFP-ALDOA transfection (Transf. Aldoa.). Cells were harvested at 48 h post-infection to measure the ATP level normalized to ATP standard curve. Error bars indicate the standard deviations of results from three independent experiments. $^{*}P < 0.05$, compared to the control group.

however the amounts of ATP were not as high as those in the WT cells (Fig. 4, lane 6), indicating that the overexpression of aldolase A partially rescued ATP synthesis.

4. Discussion

A variety of intracellular pathogen infections are known to change the host metabolism through interaction with host metabolic enzymes to benefit the invaders [22]. In this study, we found that JEV infection elevated aldolase A expression throughout the 48-h infection period, compared to the mock infection (Fig. 2). Similar findings have been reported, wherein infection with Dengue viruses up-regulated several glycolytic enzymes, including fructose-bisphosphate aldolase, which suggests that stimulating the glycolysis pathway may benefit the infection process [23,24]. Glycolysis and glucose metabolism have been shown to be important for viral replication [25,26]. Various viral infections decrease the ATP level, which indicates that ATP is required to support active viral replication [27,28]. We showed that besides silencing aldolase A expression, JEV infection also reduced cellular ATP levels significantly (Fig. 4). Ectopic expression of aldolase-GFP in Aldoa-KD cells increased ATP levels significantly, which indicates that aldolase-GFP fusion protein contributed to ATP synthesis (Fig. 4). However, there is no effect on increasing virus titer, which suggests that sufficient amounts of ATP are required to support effective viral reproduction. Ectopic expression of aldolase-GFP in WT cells did not facilitate viral replication, indicating that either an abundance of endogenous aldolase A in WT cells is sufficient for supporting JEV replication, or the in-frame expression with GFP may alter protein conformation such that it did not contribute to proper functioning.

Several studies have shown that aldolase A binds to UTRs of viral genome or cellular mRNA. In simian hemorrhagic fever virus, aldolase A binds to a stemloop of the 3′-UTR, yet the function of this binding has not been further investigated [29]. The authors suggested that aldolase A may play a role in regulating the function of the genomic 3′-UTR or involvement of the organizing cytoskeleton. Three aldolase isozymes (A, B, and C) are conserved through evolution in vertebrates. Aldolase A and C expressed in neural cells bind to the 3′-UTR of light neurofilament mRNA, a prominent neuron-specific transcript, and modulate mRNA stability by competing interaction with poly A binding protein [30]. Aldolase A in synergy with GAPDH bound to 3′-UTR of myosin heavy chain (MyHC) implicates the post-transcriptional regulation of the MyHC [31]. Myosin has been shown to be essential for JEV entry in neuronal cells [32]. We have shown that GAPDH binds to 3′-ends of both plus and minus strands [16]. We demonstrated that aldolase A interacted with JEV RNA and is part of the replication complex (Fig. 1). Whether aldolase A in synergy with GAPDH promotes JEV RNA replication or facilitates viral entry will be investigated in future studies to clarify the molecular mechanisms in which aldolase A is involved.

The successful establishment of viral infection relies on host factors and physiological processes for the completion of the virus's life cycle. Identifying host factors and cellular pathways not only elucidates the disease pathogenesis but also enhances the discovery of potential antiviral cell targets for the therapeutic development of medically significant flaviviruses. Silencing aldolase A expression did not affect cell propagation but significantly reduced virus titers, which suggests that aldolase A may be an optimum candidate as an antiviral target. This is the first report demonstrating that aldolase A participates in JEV replication and the biogenesis of ATP.

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