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Andrias davidianus ranavirus *1R* encoding a delayed-early protein promotes cell proliferation by driving cell cycle progression into S phase

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Summary. – *Andrias davidianus* ranavirus *1R* (*ADRV-1R*), a core gene of the family *Iridoviridae*, is predicted to encode a viral transcription factor (vTF) since the protein contains a virus late transcription factor-3 like (VLTF3 like) domain. However, its characteristics and function are still unclear. In this study, the transcription and expression of *ADRV-1R* were investigated in Chinese giant salamander thymus cells (GSTCs). *ADRV-1R* transcription starts 6 hours post-infection (hpi), while the protein expression starts 8 hpi. Drug inhibition assay showed that the transcripts are inhibited by cycloheximide (CHX), a *de novo* protein synthesis inhibitor, indicating that *ADRV-1R* is a viral delayed-early (DE) gene. Subcellular localization showed that ADRV-1R is distributed in the cell nucleus and cytoplasm. The effect of ADRV-1R overexpression on cell proliferation and virus titer was analyzed. ADRV-1R overexpression significantly promoted the cell proliferation starting at day 2. Flow cytometry analysis further indicated that the protein promotes the GSTC cell cycle progression from G1 phase into S phase (G1/S transition). Moreover, ADRV-1R overexperession significantly increased ADRV titer in GSTCs. The virus titer was 6.3–6.9-fold higher at 36 hpi and further after than the control GSTC lines. These data showed that ADRV-1R is a delayed-early protein promoting cell proliferation and virus titers.

Keywords: ranavirus; Andrias davidianus ranavirus; core gene; cell cycle; cell proliferations

Introduction

Transcription factor (TF) binds to the specific DNA site and regulates downstream gene expression, which is an important process in living organisms (Lo *et al*., 2017; Lee *et al*., 2018). Viral transcription factors (vTFs) from higher vertebrate viruses, e.g. cytomegalovirus (CMV), have been widely studied and they are involved in cell proliferation (Sugata *et al*., 2016), viral gene transcription-activating and expression (Pan *et al*., 2018; Castañeda and Glaunsinger, 2019), etc. However, the knowledge about vTFs of lower vertebrate viruses is very limited, as well as of iridovirids. Therefore, the study on the characteristics of vTF genes in iridovirids, the effects of their expression on cell proliferation and virus replication might be helpful to elucidate the function of vTFs in viral life cycle.

Members of the genus *Ranavirus* in the family *Iridoviridae* are large, double-stranded DNA viruses infecting a wide range of cold-blooded vertebrates (Chinchar *et al*., 2017a,b), and are emerging threats to the global aquaculture (Bandín and Dopazo, 2011; Chinchar and Waltzek, 2014; Zhang and Gui, 2015). In previous study, *Andrias davidianus* ranavirus (ADRV) was isolated from diseased Chinese giant salamanders (*Andrias davidianus*, CGS) in China, and was identified as a novel member of *Ranavirus* (Chen *et al*., 2013). Other ranaviruses such as CGSIV were also isolated from CGS (Dong *et al*., 2011; Geng *et al*.,

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Abbreviations: ADRV = *Andrias davidianus* ranavirus; DE = delayed-early; FV3 = frog virus 3; GSTC(s) = Chinese giant salamander thymus cell(s); hpi = hour(s) post-infection; hpt = hour(s) post transfection; RGV = *Rana grylio* virus; SGIV = Singapore grouper iridovirus; VLTF3 = virus late transcription factor-3; vTF(s) = viral transcription factor(s)

2011). The complete genome of ADRV has been sequenced and found to have high homology with *Rana grylio* virus (RGV), another ranavirus isolated from diseased pig frog (*Rana grylio*) (Zhang *et al*., 2001; Lei *et al*., 2012). Up to now, only a few viral genes of ADRV, such as major coat protein gene (*MCP*) and putative AAA ATPase gene (*ATPase*) have been studied (Zhou *et al*., 2015; Zhang and Zhang, 2018). Recently, ADRV *2L* can be used as a DNA vaccine to effectively protect CGS against this viral disease (Chen *et al*., 2018).

The open reading frame (ORF) of *ADRV-1R* is 771 bp long and encodes protein containing a virus late transcription factor-3 like (VLTF3 like) domain and therefore it is predicted to be vTF. The vaccinia virus A2L containing a VLTF3 domain was identified as a trans-activator, which is necessary for the transcription of the viral late genes (Keck *et al*., 1993; Iyer *et al*., 2006). *ADRV-1R*, a core gene of the family *Iridoviridae*, had homologues in most sequenced iridovirids and its deduced protein had high similarity to the homologues in ranavirus, such as 98.8% identity with frog virus 3 1R (FV3-1R) (Tan *et al*., 2004) and 98.0% identity with RGV-1R (Lei *et al*., 2012). However, the effects of ADRV-1R on cell proliferation and virus replication are remaining unclear. In this study, we investigated the transcription and expression of *ADRV-1R* in Chinese giant salamander thymus cells (GSTCs) and analyzed the effects of its overexpression on the cell proliferation and virus titer. The study is aimed to understand the function of vTFs in ranavirus.

Materials and Methods

Virus and cells. Andrias davidianus ranavirus (ADRV) was originally isolated from diseased Chinese giant salamander, *Andrias davidianus* (Chen *et al*., 2013). Chinese giant salamander thymus cell line established by Yuan *et al.* was grown in M199 medium supplemented with 10% fetal bovine serum at 25°C (Yuan *et al*., 2015).

Sequence alignment and domain analysis. The amino acid sequences of ADRV-1R (AGV20532.1), RGV-1R (AFG73043.1), FV3-1R (AAT09660.1), common midwife toad virus 1R (CMTV-1R, AFA44905.1), tiger frog virus 105R (TFV-105R, ABB92350.1), *Ambystoma tigrinum* virus 91R (ATV-91R, YP_003866.1), epizootic haematopoietic necrosis virus 100R (EHNV-100R, ACO25290.1), european catfish virus 132R (ECV-132R, AFJ52418.1), Bohle iridovirus 1R (BIV-1R, ANK57926.1), Singapore grouper iridovirus 116R (SGIV-116R, AAS18131.1), grouper iridovirus ORF67 (GIV-67, AAV91094.1), lymphocystis disease virus 1 91L (LCDV 1-91L, NP_078747.1) and infectious spleen and kidney necrosis virus 61L (ISKNV-61L, AAL98785.1) were retrieved from the National Center for Biotechnology Information (NCBI) for computer-assisted analysis. Sequence alignment of the thirteen proteins was performed in Clustal X 1.83 (Larkin *et al*., 2007) and edited by the GeneDoc software (Nicholas, 1997). Conserved domains and motifs were predicted by using the NCBI conserved domains search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Genes cloning and plasmids construction. Based on the genome sequences of ADRV (GenBank Acc. No. KC865735), the full open reading frame (ORF) of *ADRV-1R* was amplified by PCR from the total DNA of ADRV infected GSTCs using the primers ADRV-1R-F1/R1. PCR was performed in a volume of 25 μl, containing 1.0 μl of total DNA, 0.2 μM of each primer, 1.0 μl of *TransTaq* HiFi DNA Polymerase, 2.0 μl of 2.5 mM dNTP, 2.5 μl of 10 × *TransTaq* HiFi Buffer II (TransGen Biotech, China), and carried out under the following conditions: 2 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at 52°C, 1 min at 72°C, followed by 72°C for 10 min. The amplified DNA product was then cloned into prokaryotic vector pET-32a (+) (Novagen, USA) to obtain plasmid pET32a-ADRV-1R. The designed primers ADRV-1R-F2/R2 and ADRV-1R-F3/R3 were also used to amplify the *ADRV-1R* ORF to introduce restriction enzyme sites, and the fragments were cloned into eukaryotic vectors pcDNA3.1-HA (Invitrogen, USA) and pEGFP-N3 (Clontech, USA) to obtain the plasmids pcDNA3.1-ADRV-1R and pEGFP-ADRV-1R, respectively.

All primers in the study are listed in Table 1. All constructed plasmids were confirmed by restriction enzyme digestion and DNA sequencing.

Table 1. Primers used in this study (enzyme cleavage site is underlined)

Primers	Primers sequence (5'–3')	Usage
ADRV-1R-F1	CCGGAATTCATGGCATTCTCGACAGAAGATGTGC (*EcoR*I)	pET32a-ADRV-1R
ADRV-1R-R1	CCGCTCGAGTTCACAGGGGGGTAAACTTCCACCCG (*Xho*I)	
ADRV-1R-F2	CCGGAATTCAATGGCATTCTCGACAGAAGATGTGC (*EcoR*I)	RT-PCR, pcDNA3.1-ADRV-1R
ADRV-1R-R2	CCGCTCGAGTCACAGGGGGGTAAACTTCCACCCG (*Xho*I)	
ADRV-1R-F3	CCGGAATTCAATGGCATTCTCGACAGAAGATG (*EcoR*I)	pEGFP-ADRV-1R
ADRV-1R-R3	CGCGTCGACCAGGGGGGTAAACTTCCACCC (*Sal*I)	
β-actin-F	CCACTGCTGCCTCCTCTT	RT-PCR
β-actin-R	GCAATGCCTGGGTACATG	
dUTPase-F	GCACGGAAACTCTCTGCAG	RT-PCR
dUTPase-R	GTGCCTCAGACAAAACTCTCC	

Drug inhibition assay. Cycloheximide (CHX), a *de novo* protein synthesis inhibitor, and cytosine arabinofuranoside (AraC), a viral DNA replication inhibitor, were used as inhibition drugs during virus infection. Briefly, GSTCs monolayer was pretreated with 50 μg/ml CHX or 100 μg/ml AraC for 1 h and inoculated with ADRV at multiplicity of infection (MOI) of 0.1. CHX pretreated GSTCs with virus infected or 1×PBS, and virus infected GSTCs without treatment were harvested 6 hours post-infection (hpi). Simultaneously, AraC pretreated GSTCs with virus infected or 1×PBS, and virus infected GSTCs without treatment were harvested at 48 hpi. Total RNA was isolated using Trizol reagent (Ambion, Life technologies, USA) according to the manufacturer's instructions and *ADRV-1R* transcription was analyzed by RT-PCR with the primers ADRV-1R-F2/R2. The internal control gene, β-actin, and a known delayed-early (DE) gene, *dUTPase* (Zhao *et al*., 2007a), were also analyzed by the RT-PCR. The RNA was pretreated by gDNA Eraser (Takara, China), then reverse transcribed to the first strand cDNA by PrimeScript™ RT reagent kit (Takara, China). The PCR cycle was conducted as described above.

Prokaryotic expression, purification and antibody preparation of ADRV-1R. The recombinant plasmid pET32a-ADRV-1R was transformed into *Escherichia coli* BL21 competent cells and fusion protein expressed by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 6 h. The fusion protein was purified according to the protocol supplied with the HisBind purification kit (Novagen). To obtain antibody against ADRV-1R, the purified fusion protein was mixed with equal volume of Freund's adjuvant (Sigma, USA) and mice were immunized by subcutaneous injection at 7-day intervals. The anti-ADRV-1R serum was collected at the fifth day after the fifth immunization. This experiment was carried out in strict accordance with the recommendations in the Regulations for the Administration of Affairs Concerning Experimental Animals of China. The protocol was approved by Animal Center of Disease Control and Prevention of Hubei Province (approval ID: SCXK 2015-0018). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Temporal transcription and expression analyses. To analyze the temporal transcription of the *ADRV-1R*, RT-PCR was performed on 0.1 MOI ADRV infected GSTCs at different infection times. Total RNA was isolated from virus infected cells at 0, 2, 4, 6, 8, 12, 16, 24, 36 and 48 hpi. The first strand cDNA was prepared as described above. PCR was conducted by using gene-specific primers ADRV-1R-F2/R2. Detection of β-actin mRNA was used as an internal control. In addition, to examine the protein expression pattern, Western blot was performed on 12 μl total protein extracts from the ADRV infected GSTC samples at the same time points as described above. Equivalent amounts of cell extracts were separated in 12% SDS-PAGE and subsequently transferred to PVDF membrane (Millipore, USA). The anti-ADRV-1R serum was used as the primary antibody at a dilution of 1:500 and followed by the secondary antibody – alkaline phosphatase anti-mouse IgG (H+L) (Vector Laboratories, Inc., USA) at a dilution of 1:2,500. Simultaneous internal control was performed by detecting β-actin protein with anti-β-actin antibody (Wuhan Boster, China; 1:2,500).

Subcellular localization of ADRV-1R. GSTCs were plated on coverslips in a 6-well plate and transfected with the plasmid pEGFP-ADRV-1R or pEGFP-N3 using Lipofectamine 3000 transfection kit (Invitrogen) according to the manufacturer's instructions. At 48 h post transfection (hpt), the cells were rinsed with 1×PBS (pH 7.4) and fixed with 4% paraformaldehyde for 30 min. Subsequently, the cells were permeabilized with 0.2% Triton X-100 for 15 min and stained with Hoechst 33342 (1 μg/ml) for 5 min. Finally, the cells were rinsed with 1×PBS, mounted with 50% glycerol, and visualized under a fluorescence microscope (Olympus DP80, Japan).

Selection of stable transfectants and cell growth curves. To obtain ADRV-1R or empty vector stably expressed cells, GSTCs were transfected with pcDNA3.1-ADRV-1R or empty vector pcDNA3.1-HA. At 48 hpt, G418 (Geneticin, Gibco, USA) was added to the medium at a final concentration of 400 μg/ml. After 5 to 6 weeks of selection, the stable transfectants were confirmed by PCR and RT-PCR analysis. The pcDNA3.1-ADRV-1R stable GSTCs and pcDNA3.1-HA stable GSTCs were termed as G/ADRV-1R and G/Con, respectively. For cell proliferation assay, G/ADRV-1R and G/Con cells were seeded in 24-well plates at an initial concentration of 4×10^4 cells per well. The cell numbers from triplicate wells were counted using haemacytometer under a light microscope (Leica, Germany) at day 1, 2, 3, 4, 5, 6, 7 and 8. The micrographs of cell morphology were recorded under the fluorescence microscope (Olympus DP80). Student's *t*-test was used to evaluate the statistical significance of G/ADRV-1R and G/Con cell numbers.

Cell cycle analysis by flow cytometry. To test the impact of ADRV-1R overexpression on the cell cycle, GSTCs were transfected with pcDNA3.1-ADRV-1R or empty vector pcDNA3.1-HA as described above. The cells were harvested after culturing for 36 h and fixed in 70% ice-cold ethanol overnight at -20°C. After washing with 1×PBS, the cells were centrifuged at 1,500 rpm for 5 min, and resuspended in 1×PBS containing 20 μg/ml RNase A (Solarbio, USA) and 50 μg/ml of propidium iodide for 30 min. The propidium iodide fluorescence was measured by a CytoFLEX flow cytometer (Beckman, USA) and the percentage of cell numbers in G1, S and G2 phases was analyzed using the Modfit software. Student's *t*-test was used to evaluate the statistical differences of the cell proportions between ADRV-1R transfected cells and empty vector transfected cells.

Viral replication kinetics assay. To investigate the effect of ADRV-1R overexpression on virus replication in GSTCs, viral replication kinetics were evaluated on the ADRV infected G/ADRV-1R and ADRV infected G/Con cells. Briefly, the stably transfected cells were separately seeded in 24-well plates and infected with virus at a MOI of 0.1. The virus infected cell sus-

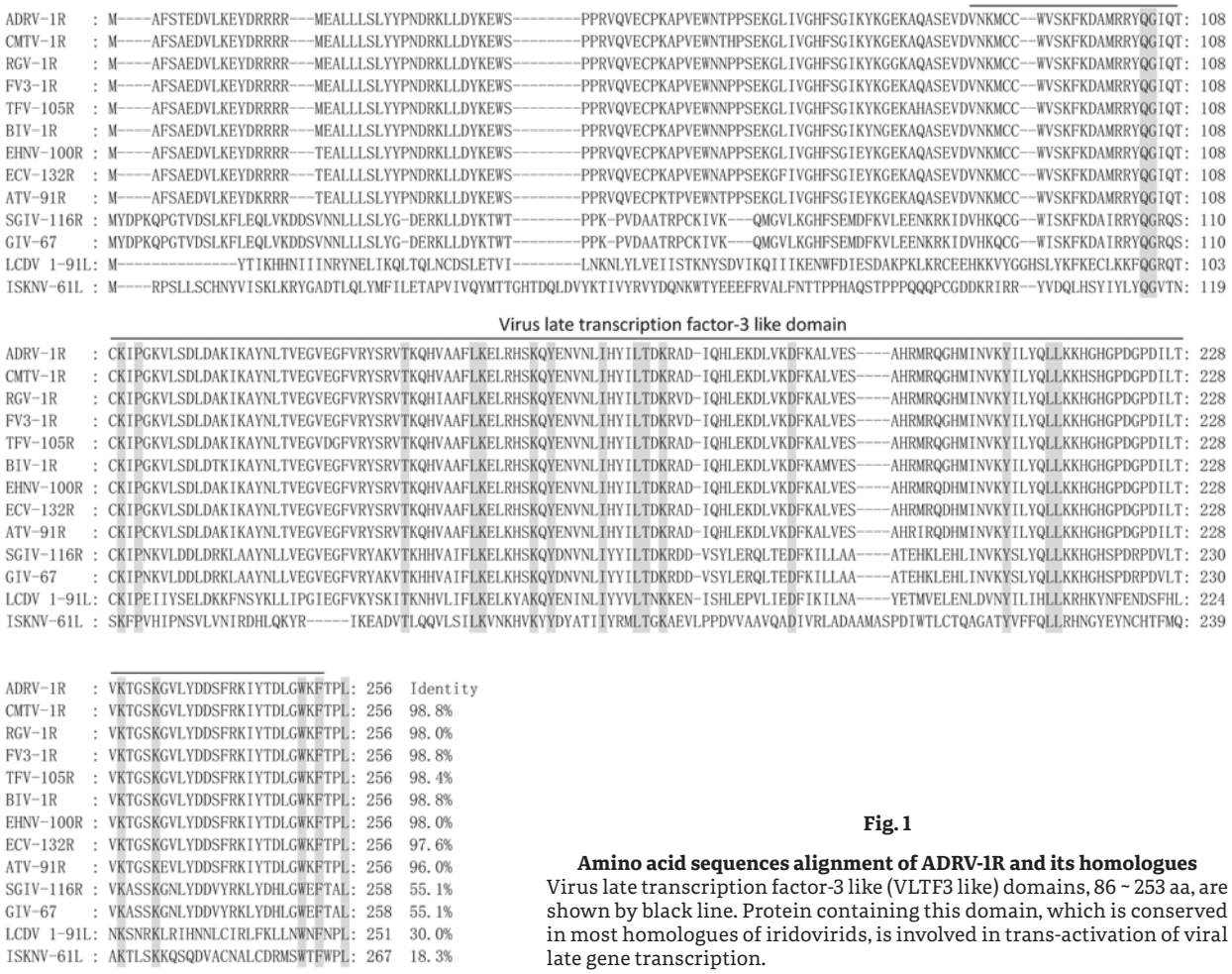

Fig. 1

Amino acid sequences alignment of ADRV-1R and its homologues
Virus late transcription factor-3 like (VLTF3 like) domains, 86 ~ 253 aa, are shown by black line. Protein containing this domain, which is conserved in most homologues of iridovirids, is involved in trans-activation of viral late gene transcription.

pensions were harvested at the time points 0, 4, 8, 16, 24, 36, 48 and 72 hpi. The virus titers were determined by using a 50% tissue culture infectious dose ($TCID_{50}$) assay. Student's *t*-test was used to evaluate the statistical significance.

Results

Amino acid sequence analysis of ADRV-1R

The complete ORF of *ADRV-1R* is 771 bp long and encodes a protein with a predicted molecular mass of about 30 kDa. Except GIV-67 and SGIV-116R, ADRV-1R had at least 96.0% sequence identity to homologues in ranaviruses. Bioinformatic analysis indicated that ADRV-1R contains the virus late transcription factor-3 like (VLTF3 like) domain (Fig. 1). This predicted domain shares ~ 30% sequence identity to the VLTF3 domain (Cdd: pfam04947), which is necessary for the viral late gene transcription of poxvirus (Keck *et al*., 1993). Thus, the ADRV-1R is predicted to have the role of viral transcription factor (vTF).

ADRV-1R is a viral delayed-early (DE) gene

In order to determine whether the *ADRV-1R* was a viral delayed-early (E) gene, CHX and AraC were used in drug inhibition assays. The RT-PCR showed that *ADRV-1R* transcripts were not detected in the CHX pretreated cells with or without virus infection (Fig. 2a, Lane 1 and 2), but they were detected in the virus infected cells with no CHX treatment (Fig. 2a, Lane 3). Meanwhile, *ADRV-1R* transcripts were not detected in the cells without virus infection (Fig. 2a, Lane 4), but were detected in virus infected cells with AraC pretreatment (Fig. 2a, Lane 5), and their amount was less than that in the untreated samples (Fig. 2a, Lane 6). As a control, consistent results were obtained from the known DE gene, *dUTPase*. These results showed that *ADRV-1R* transcription is blocked by *de novo* protein

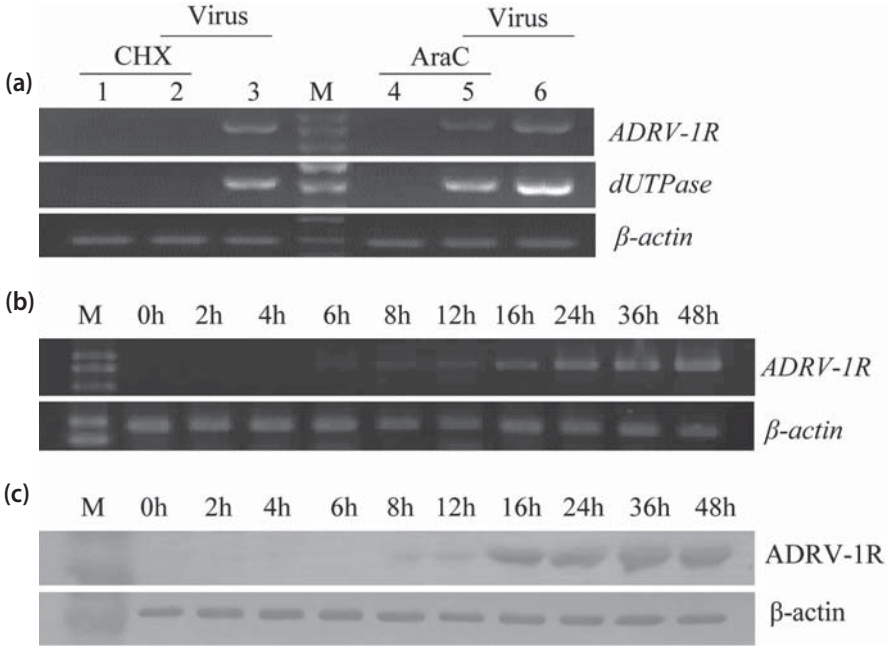

Fig. 2

Drug inhibition assay, temporal transcription and expression patterns of *ADRV-1R*

Drug inhibition assay of *ADRV-1R* by CHX and AraC (**a**). Total RNA was isolated from uninfected CHX-treated cells (Lane 1), virus infected CHX-treated cells (Lane 2), and virus infected untreated cells (Lane 3) at 6 hpi; uninfected AraC-treated cells (Lane 4), virus infected AraC-treated cells (Lane 5), and virus infected untreated cells (Lane 6) were collected at 48 hpi. RNA expression (**b**) and protein expression (**c**) patterns of *ADRV-1R* after virus infection. GSTC cells were infected by ADRV at 0.1 MOI at different times (0, 2, 4, 8, 12, 16, 24, 36 and 48 hpi) and analyzed by RT-PCR or Western blot. β-actin mRNA or protein was detected under the same condition as an internal control.

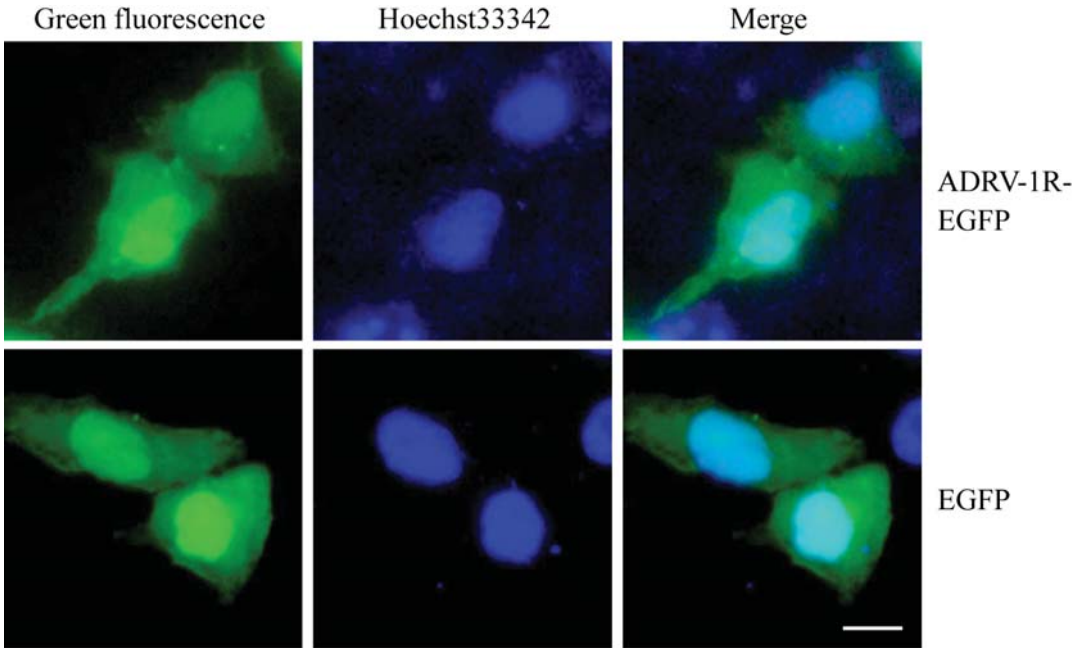

Fig. 3

ADRV-1R localization in cytoplasm and nucleus

ADRV-1R localized in the cytoplasm and nucleus in GSTCs at 48 hpt. Cells were transfected with pEGFP-ADRV-1R or pEGFP-N3. Green fluorescence signal indicates ADRV-1R-EGFP or EGFP protein, and blue fluorescence indicates the nuclei of cells. Scale bar, 5 μm.

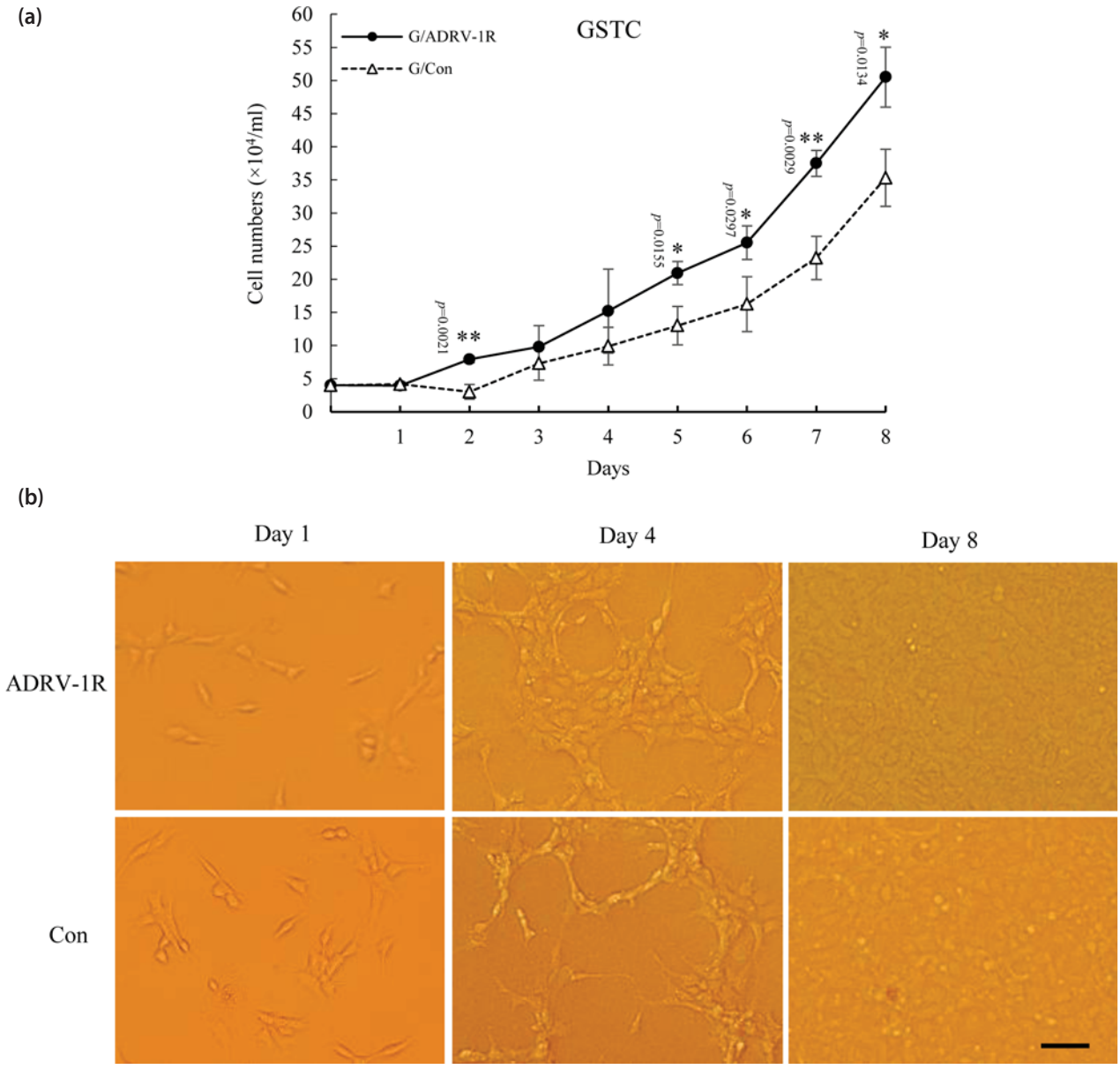

Fig. 4

ADRV-1R promoted GSTC cell proliferation

Cell growth curves of G/ADRV-1R and G/Con cells until day 8 (**a**) and micrographs of cells at day 1, 4, 8 (**b**). G/ADRV-1R and G/Con cells were seeded in 24-well plates at an initial concentration of 4×10^4 cells per well. ADRV-1R promoted cell proliferations on GSTCs compared to empty vector transfected cells. The number of increased cells gradually raised until day 8. Statistical analysis was performed using Student's *t*-test. *, P <0.05; **, P <0.01. Scale bar, 50 μm.

synthesis inhibitor CHX, but not by viral DNA replication inhibitor AraC, indicating that *ADRV-1R* is a viral DE gene.

Temporal transcription and expression analyses of ADRV-1R

To detect the temporal transcription pattern of *ADRV-1R*, its transcriptional kinetics were examined in GSTCs at different time points after ADRV infection. The RT-PCR analysis showed that *ADRV-1R* transcripts were detected as early as 6 hpi, and a higher level of transcripts expression continued until 48 hpi (Fig. 2b). The results were consistent with the drug inhibition analysis and confirmed the *ADRV-1R* to be a viral DE gene.

Meanwhile, the expression pattern of ADRV-1R was analyzed on the samples described above. Western blot

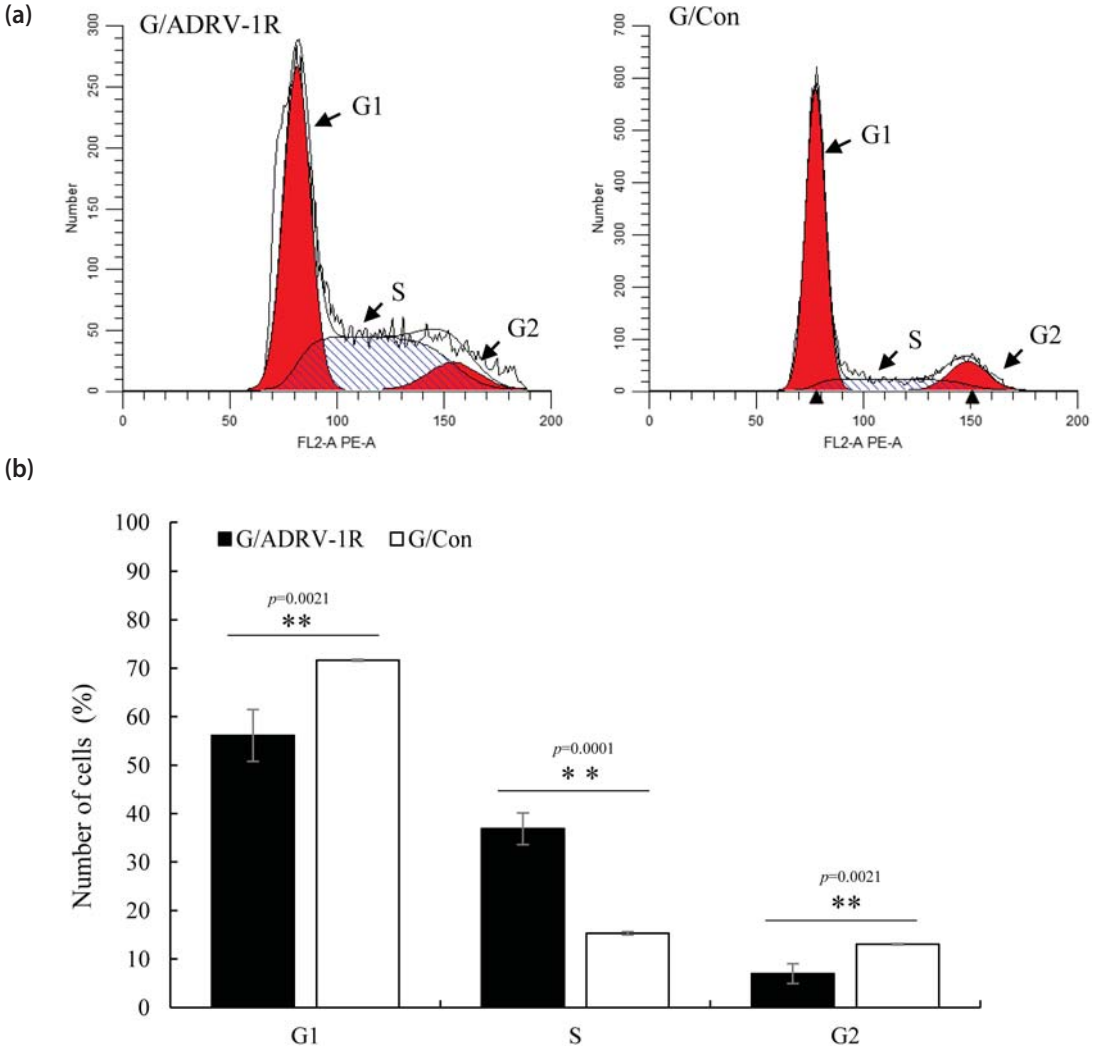

Fig. 5

Flow cytometry analysis of cell cycle profiles

Cell cycle profiles of pcDNA3.1-ADRV-1R and pcDNA3.1-HA transfected GSTCs were analyzed by flow cytometry **(a)**. Cell proportions of G1, S and G2 phases at 36 hpt are shown **(b)**. The percentage of cells in different phases are shown in the graph. Statistical analysis was performed using Student's *t*-test. *, P <0.05; **, P <0.01.

analysis showed that a specific immunoreactive band of approximate 30 kDa was firstly detected at 8 hpi, and the amount gradually increased until 48 hpi (Fig. 2c). The ADRV-1R expressed as early as 8 hpi after virus infection, implies that it has ability to perform the functions of viral gene transcription.

ADRV-1R is localized in the cytoplasm and the nucleus

The subcellular localization of ADRV-1R was analyzed by detecting the fluorescence distribution of ADRV-1R-EGFP fusion protein. In pEGFP-ADRV-1R transfected cells, the green fluorescence signals were observed in the cytoplasm and the nucleus, indicating that ADRV-1R was localized in the cytoplasm and the nucleus in GSTCs (Fig. 3). As a control, the vector expressing EGFP was also distributed in the cell cytoplasm and the nucleus.

ADRV-1R overexpression promoted cell proliferation and affected cell cycle progression

The effect of ADRV-1R overexpression on cell proliferation was investigated. As shown in Fig. 4a, the growth of G/ADRV-1R cells was faster than G/Con cells at day 2, with over about 4.9×10^4 cells/ml. The number of increased

cells continued to rise until day 8. The increased cells were highest at day 8, with about 1.52×10^5 cells/ml more than in G/Con cells. Statistically, there were significant differences of cell numbers at day 2 and from day 5 to day 8 between the G/ADRV-1R and G/Con cells (Fig. 4a). The cell numbers on micrographs were consistent with the cell growth curves (Fig. 4b). These results indicate that ADRV-1R overexpression promotes the cell proliferation in GSTCs.

We further assessed the effect of ADRV-1R overexpression on cell cycle progression. As shown in Fig. 5a, comparing with the vector expressed GSTCs, ADRV-1R overexpression was associated with a decreased proportion of cells in G1 and G2 phases and was accompanied by a significantly increased cell proportion in the S phase. In detail, the populations of G1 phase, S phase and G2 phase were 56.15%, 36.88% and 5.5% in the ADRV-1R expressed GSTCs, respectively, whereas they were 71.65%, 15.31% and 13.03% in the control group (Fig. 5b). Comparing with the empty vector expressed cells, an approximatively 2.4-fold higher proportion of S phase cells were detected in ADRV-1R expressed cells ($P < 0.01$). These data indicate that ADRV-1R can promote cell cycle progression from G1 phase into S phase (G1/S transition) and consequently enhance cell proliferation.

ADRV-1R overexpression promoted virus titers

Given our finding that ADRV-1R overexpression was able to promote cell proliferation, we next determined if it is relevant to the contribution of the ADRV replication. As shown in Fig. 6, ADRV titers in G/ADRV-1R were higher than that in G/Con cells, with significant differences at 36 hpi and thereafter. At 36 hpi, the ADRV titer was 1.48×10^7 $TCID_{50}$/ml in G/ADRV-1R, however it was 2.14×10^6 $TCID_{50}$/ml in G/Con cells. Both ADRV titers further increased at 48 hpi in G/ADRV-1R cells to 4.90×10^7 $TCID_{50}$/ml and G/Con cells to 7.76×10^6 $TCID_{50}$/ml, and the highest ADRV titers were at 72 hpi in G/ADRV-1R cells with 5.25×10^8 $TCID_{50}$/ml and G/Con cells with 7.76×10^7 $TCID_{50}$/ml. In summary, during the periods from 36 to 72 hpi, the ADRV titers yielded from G/ADRV-1R were 6.3 ~ 6.9-fold higher than that from the G/Con cells. These results indicate that ADRV-1R overexpression significantly increased ADRV titer in GSTCs.

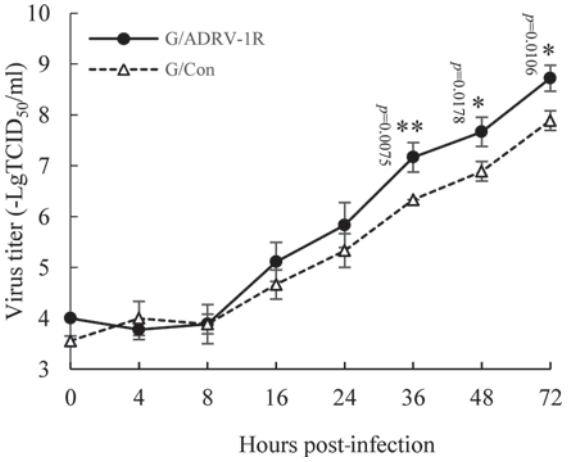

Fig. 6

ADRV replication kinetics in G/ADRV-1R and G/Con cells
ADRV replication kinetics was measured in G/ADRV-1R and G/Con cells. G/ADRV-1R and G/Con cells were infected by 0.1 MOI ADRV and cell suspensions were harvested at 0, 4, 8, 16, 24, 36, 48 and 72 hpi for $TCID_{50}$ assay. ADRV-1R overexpression significantly increased ADRV titer in GSTCs. Statistical analysis was performed using Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$.

Discussion

In this study, ADRV-1R is predicted to be a vTF. Some transcription factors from large DNA viruses have been reported. For example, vaccinia virus A2L containing VLTF3 is a trans-activator, which is necessary for the transcription of the viral late genes. In cytomegalovirus, the transcription factors complex formed from six viral transcription factors are required for progeny virus production (Pan *et al*., 2018; Gong *et al*., 2014). These implied that ADRV-1R might play roles in regulating the viral late gene transcription and virus production.

ADRV-1R was classified as viral DE gene. Similar to other large dsDNA viruses, the *Ranavirus* genes are expressed sequentially in three temporal cascades of immediate-early (IE), delayed-early (DE) and late (L) mRNAs during viral life cycle (Ince *et al*., 2015; Jancovich *et al*., 2015; Majji *et al*., 2009), which are involved in virus infection, replication and assembly at specific period (He *et al*., 2014; Banjara *et al*., 2018; Gui *et al*., 2018; Zhao *et al*., 2017b). Viral IE and DE gene transcripts likely encode catalytic and virulence proteins or key regulatory proteins, such as FV3 transcriptional elongation factor IIS (Majji *et al*., 2009; Sobhy, 2017). Moreover, the ADRV-1R expressed as early as 8 hpi after ADRV infection. The early expressed protein further implied that ADRV-1R might have the role to regulate viral gene transcription.

Subcellular localization indicated that ADRV-1R was distributed in the cytoplasm and nucleus. During the life cycle of ranavirus, the viral genomic DNA synthesis firstly took place in nucleus. Subsequently, the genomic DNA was transported into cytoplasm (Jancovich *et al*., 2015). Furthermore, vTF usually binds to the specific DNA sequences in viral genome. Therefore, the ADRV-1R

localization conforms to the viral replication events and might function in cytoplasm and nucleus.

We observed that ADRV-1R overexpression promoted cell proliferation by regulating the cell cycle progression from G1 phase into S phase (G1/S transition). Cell cycle from G1 phase to S phase transition was regulated by cell cycle regulatory proteins, such as CDK2, p53 and cyclin D2 (Benedict *et al*., 2018; Liu *et al*., 2013; Pei *et al*., 2018). Whether the ADRV-1R affecting the cell cycle was regulated by these proteins needs more evidence. Moreover, it has been reported that the viral proteins encoded by other DNA viruses promote cell proliferation by similar regulation process. For example, two tumor necrosis factor receptor-like proteins encoded by SGIV have roles in promoting cell proliferation by regulating G1/S transition (Yu *et al*., 2015; Huang *et al*., 2013). The nuclear antigen 3C from Epstein-Barr virus facilitates cell proliferation by regulating cyclin D2 thereby promoting G1/S transition (Pei *et al*., 2018). In addition, at this phase viral DNA replication is activated, which might be benefit for the synthesis of viral genomic DNA.

Lastly, ADRV-1R overexpression significantly increased ADRV titer in GSTCs. Comparing with the vector expressed cells, it is possible that ADRV-1R overexpression induced higher cell number which provides more energy and raw materials for virus replication or proliferation. Similar phenomena were observed in several ranavirus genes, such as SGIV *ICP18* and *ICP46* (Xia *et al*., 2009, 2010).

In conclusion, the *ADRV-1R* was identified as a delayed-early gene and its encoded protein is distributed in cytoplasm and nucleus. ADRV-1R overexpression promoted cell proliferation by regulating cell cycle progression from G1 phase into S phase, and thereby increased the ADRV titer in GSTCs. The study extends the knowledge about viral transcription factor in ranavirus.

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