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LETTER

High-Efficiency Rescue of Equine Infectious Anemia Virus from a CMV-Driven Infectious Clone

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Dear Editor,

Equine infectious anemia virus (EIAV) belongs to the macrophage-tropic lentiviruses family and infects mainly equines, including horses, donkeys and mules. EIAV shares many similar characteristics in its viral biology and host-virus immune regulation with other lentiviruses, such as human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), and has been accepted as a model for the study of other lentiviruses, especially HIV-1 (Craigo and Montelaro 2013). Specifically, in the 1970s, Chinese scientists developed an attenuated EIAV vaccine and by using this vaccine, China has successfully controlled EIAV prevalence. This vaccine provides a meaningful reference for lentivirus research (Wang *et al*. 2016).

The full-length genome of EIAV is approximately 8.2 kb, making it one of the simplest lentiviruses. In addition to its structural proteins Env, Gag and Pol, EIAV encodes only three accessory proteins, namely, Tat, Rev and S2 (Cook *et al*. 2013). The Gag proteins are the major structural proteins of lentivirus and are often considered indicators of viral replication. The EIAV Gag-precursor polyprotein (p55) is cleaved into the matrix (MA) p15, the capsid (CA) p26, the nucleocapsid (NC) p11 and p9 by viral protease in the mature virion, among which p26 is the most abundant proteins in the viral particle. After entry into the host cells, EIAV proviral DNA is synthesized from the RNA genome, and a long terminal repeat (LTR) is formed at both ends of the proviral DNA, which is a necessary element for integration of the proviral DNA into the host genome. The 5′ LTR, as the lentiviral promoter, plays an important role in regulating virus replication and gene expression (Frech *et al*. 1996). The EIAV LTR contains three regions, namely, unique 3′ end region (U3), repeated region (R), and unique 5′ end region (U5). The U3 contains regulatory elements and motifs related to viral replication and pathogenicity (Wang *et al*. 2018). The R region contains transcriptional initiation site signals and *cis* activators, termed Tat-activating region (TAR). TAR interacts with Tat, a transactivator viral protein, to promote viral RNA transcription and replication (Carvalho and Derse 1991); the interaction between Tat and the LTR TAR requires the recruitment of cellular factors, including cyclin T1, which is thought to increase the elongation efficiency of the host RNA polymerase II. However, EIAV Tat interacts with only equine or canine cyclin T1 and does not interact with human cyclin T1 (Bieniasz *et al*. 1999).

Reverse genetics systems are powerful genetic tools for studying the life cycles of viruses. The traditional method for the construction of a lentivirus infectious clone is to insert the full-length proviral genome into a clone vector, wherein the 5′ LTR acts as a promoter to trigger viral transcription. The biological functions of EIAV have been studied in detail, owing to several constructs of infectious clones based on the $EIAV_{wyoming}$ strain (Cook *et al*. 1998). Chinese scientists constructed the EIAV infectious clone pLGFD3-8 based on the Chinese vaccine strain FDDV13 (He *et al*. 2003), which greatly promoted the study of the protective and attenuation mechanism of the EIAV attenuated vaccine strain (Shen *et al*. 2006). However, we found that the rescue efficiency of the pLGFD3-8 virus was very low, which became the greatest limitation for its application in the biological study of EIAV. We speculated that the low rescue efficiency of pLGFD3-8 was due to weak promoter activation of the LTR. To test our speculation, we first compared the activities of the LTR promoter and of

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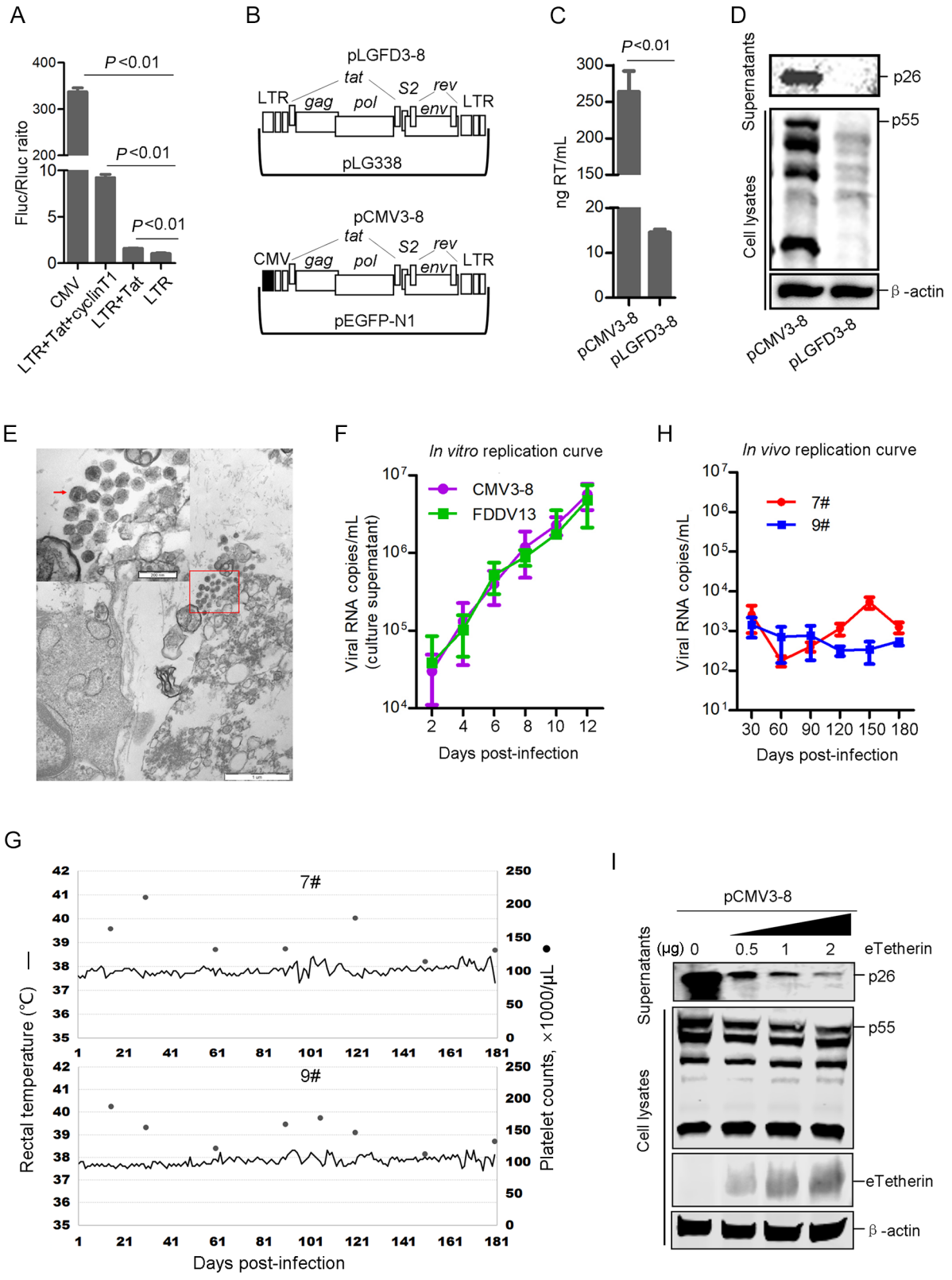
A
Fluc/Rluc raito
P<0.01
P<0.01
P<0.01
CMV
LTR+Tat+cyclinT1
LTR+Tat
LTR
B
pLGFD3-8
LTR
gag
pol
tat
S2
rev
env
LTR
pLG338
pCMV3-8
CMV
pEGFP-N1
C
P<0.01
ng RT/mL
pCMV3-8
pLGFD3-8
D
Supernatants
Cell lysates
p26
p55
β -actin
pCMV3-8
pLGFD3-8
E
F
In vitro replication curve
CMV3-8
FDDV13
Viral RNA copies/mL (culture supernatant)
Days post-infection
H
In vivo replication curve
7#
9#
Viral RNA copies/mL
Days post-infection
G
7#
9#
Rectal temperature (°C)
Platelet counts, ×1000/μL
Days post-infection
I
pCMV3-8
(μg) 0
0.5
1
2
eTetherin
Supernatants
Cell lysates
p26
p55
eTetherin
β -actin

◀**Fig. 1** **A** Comparison of the transcriptional activities of the CMV promoter and the EIAV LTR without or with Tat and equine cyclin T1 in HEK293T cells. The results are the means of three independent experiments. **B** Schematic diagram of the EIAV infectious clones pCMV3-8 (top) and pLGFD3-8 (bottom). **C** Comparison of released virions from pCMV3-8- and pLGFD3-8-transfected HEK293T cells by detecting RT activity at 48 hpt. The results are the means of three independent experiments. **D** Comparison of EIAV expression in cell lysates and supernatants from pCMV3-8- and pLGFD3-8-transfected HEK293T cells via Western blotting at 48 hpt. The experiment was performed three times, and a representative result was shown. **E** TEM image of EIAV from HEK293T cells transfected with pCMV3-8 (Scale bar = 1 μm). An enlarged image of the red boxed area is shown in the top left corner (Scale bar = 200 nm), and red arrows indicate cone-shaped core virions. **F** Growth kinetics of the cloned virus vCMV3-8 and the parental virus FDDV13. Viral RNA was prepared from the supernatants of FDD cells infected with either vCMV3-8 or FDDV13 and was quantitatively measured by real-time RT-PCR. The results are the means of three independent experiments. (**G** and **H**) Changes in body temperature, platelet count and viral load in two horses inoculated with vCMV3-8. **G** The rectal temperature (—) was measured daily. An automatic blood analyzer was used to measure the platelet counts (●). **H** The plasma viral loads were measured by real-time RT-PCR. The results are the means of three independent experiments. **I** Equine tetherin-inhibited release of EIAV particles by cotransfected pCMV3-8 and eTetherin expression plasmids in HEK293T cells was validated via Western blotting at 48 hpt. The experiment was performed three times, and a representative result is shown.

CMV promoter (the human cytomegalovirus immediate early promoter, a widely used eukaryotic promoter with high promoter efficiency). The sequences of the LTR derived from pLGFD3-8 (GenBank accession no. GU385361.1, nucleotide positions 1–328) and that of the CMV promoter derived from the pEGFP-N1 vector (GenBank accession no. GU385361.1, nucleotide positions 1–589) (Clontech, USA) were cloned into the *Kpn*I and *Xho*I restriction sites of pLG3-Basic vector (Promega, USA), and the constructs were named as pGL-LTR and pGL-CMV, respectively. We then compared the promoter activities of CMV and the LTR in the absence or presence of Tat and cyclin T1 (Du *et al*. 2015). Equivalent amounts (0.5 μg) of pGL-CMV and pGL-LTR without or with a Tat-expressing plasmid (0.5 μg) and an equine cyclin T1-expressing plasmid (0.5 μg) were cotransfected into HEK293T cells in 24-well culture dishes by using the polyJet transfection reagent (SignaGen, USA). The pRL-CMV vector (Promega) (0.02 μg per well) was used as an internal control. The cells were lysed at 48 h posttransfection (hpt), and the luciferase activity in the cell lysates were analyzed by the Dual-Luciferase Reporter Assay System (Promega, USA). The results showed that the promoter activity of CMV was much higher than that of the LTR, regardless of whether tat or cyclin T1 were present (Fig. 1A).

To optimize the expression of EIAV infectious clone, most of the genome of the EIAV provirus DNA except the 5′ LTR U3 region from pLGFD3-8 was cloned into the pEGFP-N1 vector by PCR within the *Xho*I/*Not*I restriction sites and the plasmid was named as pCMV3-8 (Fig. 1B). Then it has been verified by DNA sequencing. Equal amounts (1 μg) of pLGFD3-8 and pCMV3-8 were transfected into HEK293T cells. After 48 h, culture supernatants were collected and analyzed by a reverse transcriptase (RT) assay (Jiang *et al*. 2011), which is used to detect and quantify retrovirus progeny in cell cultures. The result demonstrated that the EIAVs were successfully rescued and the number of virions in the supernatant of cells transfected with pCMV3-8 was remarkably higher than those in the supernatant of cells transfected with pLGFD3-8 (Fig. 1C). Simultaneously, the expression levels of Gag protein (p55) and viral capsid (p26) cleaved from p55 in cell lysates and culture supernatants of pCMV3-8- and pLGFD3-8-transfected cells were also measured by Western blotting using a mouse anti-p26 monoclonal antibody (9H8). The results showed that the expression levels of p55 and p26 of CMV3-8 were apparently higher than those of pLGFD3-8 in both cell lysates and supernatants (Fig. 1D). In fact, the protein expression of pLGFD3-8 was hardly detected in either cell lysates or culture supernatants under this transfection conditions. Hence, it can be concluded that pCMV3-8 possesses a high rescue efficiency. Furthermore, to further confirm virion rescue from the pCMV3-8, transmission electron microscopy (TEM) was used to observe virion morphology. The results showed that round or oval virions approximately 100 nm in diameter with a conical core of high electron density from pCMV3-8-transfected HEK293T cells could be found via TEM (Fig. 1E), which is the typical morphology of EIAV particles by TEM observation. Furthermore, to verify that no mutation was introduced into the progeny virus during the rescue process of EIAV, the rescued virus was extracted from pCMV3-8, and the sequencing result showed that it was consistent with that from parental virus.

Next, to determine the *in vitro* replication kinetics of the rescued viruses CMV3-8 (vCMV3-8), a dynamic replication curve of the vCMV3-8 in fetal donkey dermal (FDD) cells was measured and compared with that of the parental virus FDDV13. FDD cells were cultured in a 6-well plate and then inoculated with equal amounts (10^4 $TCID_{50}$) of vCMV3-8 or FDDV13. Supernatants from infected cells were collected every 2 days until day 12 postinfection, and virus titers were determined by quantitative real-time RT-PCR of the *gag* gene fragment (Ma *et al*. 2013). The results showed that there was no difference in replication properties between vCMV3-8 and FDDV13 in FDD cells (Fig. 1F). Subsequently, to further detect the replication characteristics of vCMV3-8 *in vivo*, we inoculated two horses intravenously with 2×10^5 $TCID_{50}$ of vCMV3-8,

and the horses were monitored daily for clinical symptoms of equine infectious anemia over 6 months, with two horses inoculated with α-MEM as a mock control (data not shown). Samples of blood, plasma, and serum were collected from each horse every month to detect platelet counts and plasma viral RNA copy numbers. The vCMV3-8-inoculated horses (7# and 9#) did not show abnormalities in body temperature and platelets after inoculation (Fig. 1G). We found similar plasma viral loads between two inoculated horses during the 6-month infection period, which were maintained at 10^2–10^4 copies/mL (Fig. 1H), which is similar to the replication characteristics of FDDV13 *in vivo* (Ma *et al*. 2013).

Equine tetherin (eTetherin) is a host restriction factor that inhibits retrovirus release and can be neutralized by EIAV envelope protein (Yin *et al*. 2014). To confirm the potential application of pCMV3-8 in basic research of EIAV, eTetherin was used to evaluate the the interaction between the virus and host proteins. HEK293T cells were cotransfected with 1 μg of pCMV3-8 and increasing doses of an HA-labeled eTetherin expression plasmid (0, 0.5, 1 and 2 μg). The amounts of vCMV3-8 in the cell lysate and supernatant were measured as the levels of p55 and p26 by Western blotting at 48 hpt. The levels of p55 in the cell lysate were not affected by eTetherin. However, the level of p26 in viral particles released into the culture supernatant was decreased in a dose-dependent manner under the expression of eTetherin (Fig. 1I). The result further confirms that eTetherin has a strong antiviral activity, and indicates that pCMV3-8 can be more widely used than previous viral constructs to elucidate host-EIAV interaction mechanisms.

In this study, the strong promoter of CMV was introduced in place of the 5′ LTR U3 of EIAV to improve the expression level of the molecular clone and to promote the rescue efficiency of the virus. We demonstrated that the EIAV infectious clone pCMV3-8 have a strong capacity to rescue EIAV by viral RT activity assay, Western blotting and TEM. Taken together, these results indicate that pCMV3-8 could be used for genetic manipulation of EIAV and can be applied to the researches on the mechanism of viral replication and attenuated EIAV vaccine.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement Animal experiments in this study were approved by the Ethics Committees of Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences (CAAS).

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