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# A pilot study on interaction between donkey tetherin and EIAV stains with different virulent and replication characteristics

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ABSTRACT

Tetherin (BST-2) is an important host restriction factor that can inhibit the release of a diverse array of enveloped viruses from infected cells. Conversely, to facilitate their release and spread, many viruses have evolved various strategies to overcome the antiviral effect of tetherin in a species-specific manner. During the development of an attenuated equine infectious anemia virus (EIAV) vaccine in our laboratory, we found that serial passage of a field-isolated virulent EIAV strains in horse and donkey as well as the cultivated donkey cells, produces several typical EIAV strains, including $EIAV_{DV}$, $EIAV_{DLV}$, and $EIAV_{FDDV}$, which exhibit distinct virulence and replication features *in vivo* and *in vitro*. However, the role of host restriction factors in EIAV evolution during the serial passage is not well understood. This study aimed to evaluate whether these newly generated strains adapt differently to donkey tetherin (do-tetherin) based on their virulence. We found that do-tetherin exerts an inhibition on the release of the viral particles produced by all three strains, albeit with varying intensity: $EIAV_{DV} < EIAV_{DLV} < EIAV_{FDDV}$. Additionally, all three EIAV strains could counteract the restriction mediated by do-tetherin via their envelope proteins (Env) with varying strength: $EIAV_{DV} > EIAV_{DLV} > EIAV_{FDDV}$. These results indicate that donkey tetherin is involved in shaping of EIAV evolution during serial passage.



## 1. Introduction

Recent studies have extensively characterized a number of host restriction factors that restrict viruses at different stage during infection [1–5]. Tetherin was first identified by Neil et al. (2008) [6], who firstly characterized its ability to restrict the release of HIV-1 from the cell surface. Subsequently, more evidence demonstrates that tetherin exerts a broad antiviral effect on various viruses [7–9]. Indeed, many viruses encode viral proteins to antagonize the restriction mediated by tetherin through different strategies [10–15].

Equine infectious anemia virus (EIAV) has the simplest genome structure among all lentiviruses [16], which makes it an ideal model system in which to study the role of specific viral genes in lentiviral replication and persistent infection [17]. Our laboratory has previously developed stably attenuated virulent EIAV strains using a specific passage attenuation system [18,19]. The major strains generated by this process were $EIAV_{DV}$, $EIAV_{DLV}$, and $EIAV_{FDDV}$, each of which exhibit significant differences in virulence and replication both *in vivo* and *in vitro*.

Although equine and donkey tetherin were known to inhibit EIAV release from infected cells, it is unclear whether the arms race between tetherin and EIAV influences either the dynamic evolution of these EIAV strains or the attenuation of their virulence during the adaptive evolution process. To address this question, we evaluated the interplay between do-tetherin and these three different EIAV stains ($EIAV_{DV}$, $EIAV_{DLV}$, and $EIAV_{FDDV}$). Interestingly, we found that do-tetherin has a different antiviral effect on virulent vs. attenuated EIAV strains. Additionally, Env proteins of all three EIAV strains could antagonize the inhibition mediated by do-tetherin in different extent. These results advance our understanding on the interplay between host restriction factors and EIAV evolution.

## 2. Materials and methods

### 2.1. Cell culture

Human embryonic kidney (HEK) 293T cells were maintained at

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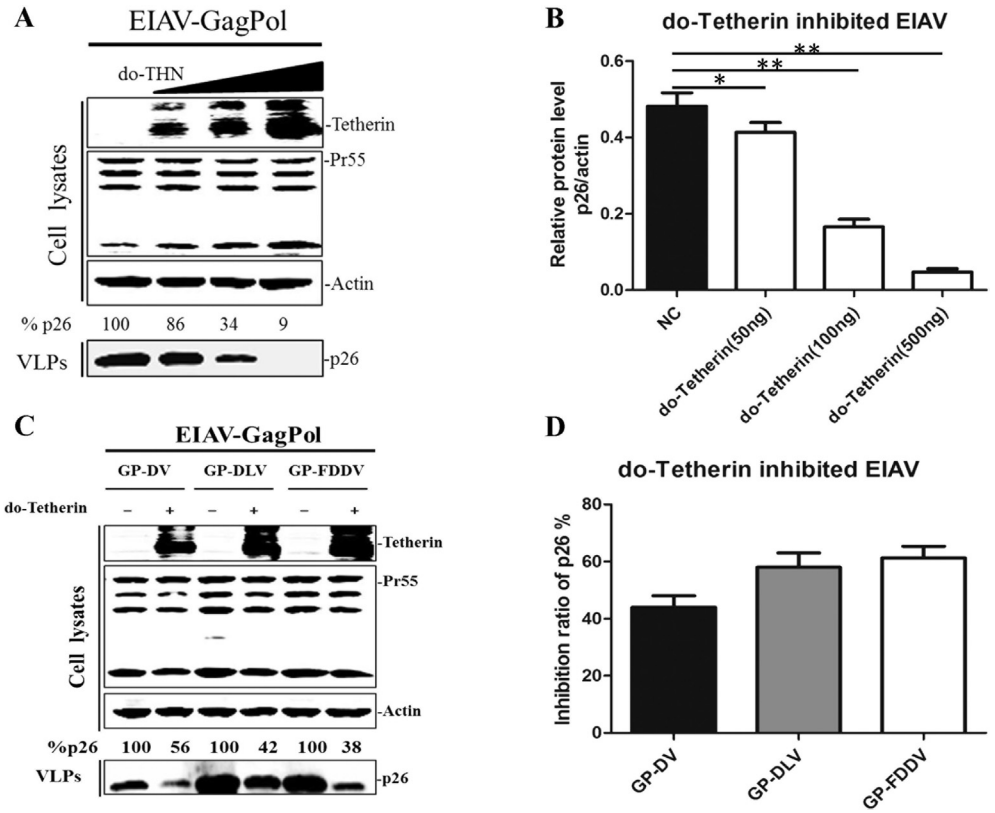


**Fig. 1. Do-tetherin more strongly inhibited attenuated EIAV strains than virulent strains.** Five micrograms of EIAV-GagPol-FDDV was co-transfected into 293T cells along with varying amounts of do-tetherin (0 ng, 50 ng, 100 ng, and 500 ng). An empty vector was used to equalize the total amount of transfected DNA. After 48 h, the supernatants were collected, and the cells were lysed using cell lysis buffer. (A) The cell lysates and VLPs in the supernatant were analyzed by SDS-PAGE and western blot. (B) The relative expression level of p26 was determined based on the ratio between the p26 expression level in the cell supernatant and the β-actin expression level in the cells. In the experiment shown here, 100 ng of do-tetherin was co-transfected into 293T cells with 5 μg of GagPol-DV, GagPol-DLV, or GagPol-FDDV. As a negative control, 5 μg of GagPol-DV, GagPol-DLV, or GagPol-FDDV alone was transfected. After 48 h, the cell supernatants were collected, and the cells were lysed using lysis buffer. (C) The cell lysates and virus particles in the supernatant were analyzed by SDS-PAGE and western blot. (D) The ability of do-tetherin to inhibit each EIAV strain was determined by analyzing the p26 expression level in the supernatant. (*: $0.01 < P < 0.05$; **: $P < 0.01$).

37 °C in a 5% $CO_2$ incubator in Dulbecco's Modified Eagle's Medium (HyClone, Utah, USA) supplemented with 10% fetal bovine serum (SAFC, Buchs, Switzerland) and 1% penicillin/streptomycin (100 units/ml, HyClone, Utah, USA).

### 2.2. Construction of expression plasmids

$EIAV_{DV}$, $EIAV_{DLV}$, and $EIAV_{FDDV}$ were grown in culture, and total RNA was extracted separately from each culture medium using a QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Germany). cDNA was then synthesized using a Reverse Transcriptase Kit (Life Technologies, California, USA). GagPol-DV, GagPol-DLV, and GagPol-FDDV were amplified and cloned into the $VR_{1012}$ vector using the *Eco*RI and *Not*I restriction sites via PCR with the following primers: GagPol forward, 5'-caccgaattcatggga-gactctttgac-3′ and GagPol reverse, 5'-caccgcggccgctcaatttggtct-tattaataa-3′. The do-tetherin (pEF-do-Tetherin-HA-Flag) and EIAV-Env (pcDNA3.1-Env-DV/pcDNA3.1-Env-DLV/pcDNA3.1-Env-FDDV) plasmids were constructed in our laboratory as previously described [20].

### 2.3. Transfection and western blotting

5 μg of each EIAV-GagPol expression plasmid along with do-tetherin plasmid were transfected into the 293T cells using the classical calcium-phosphate method. Both the cells and the culture supernatant were harvested at 48 h post-transfection, and the cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China). The lysates and the supernatant were then centrifuged separately for 5 min at 10,000 g to remove any cellular debris, followed by centrifugation of the supernatant for 2 h at 20,000 g to precipitate the virus-like particles (VLPs). The lysates and the VLPs were separated by SDS-PAGE, transferred to nitrocellulose (NC) membranes (Millipore, Massachusetts, USA), and then probed with the appropriate antibodies. The membranes were scanned and analyzed using the Near-infrared Fluorescence Scanning Imaging System (Licor Odyssey, USA).

### 2.4. Statistical analysis and software

All of the western blots performed in this study were repeated at least three times. The blots were processed and analyzed using the Near-infrared Fluorescence Scanning Imaging System, which was also used to determine the relative expression levels of each protein. The histograms were created using GraphPad Prism 5 (GraphPad Software, Inc.). For the statistical analysis, the data were analyzed using Student's *t*-test, and differences were considered to be statistically significant when the *P* value was <0.05.

## 3. Results

### 3.1. Do-tetherin inhibited all three EIAV strains with varying degrees of strength

To determine whether do-tetherin could restrict the budding of virulent strains of EIAV, do-tetherin was co-transfected with the