A novel fusion gene, TRIM5-Cyclophilin A in the pig-tailed macaque determines its susceptibility to HIV-1 infection

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\textbf{Objective}: In Old World monkeys, the tripartite motif 5a (TRIM5a) protein confers resistance to HIV-1 infection following virus entry into host cells. However, the pig-tailed macaque (\textit{Macaca nemestrina}) is an exception and is susceptible to HIV-1 infection. This study dissects the molecular mechanism of the pig-tailed macaque’s susceptibility to HIV-1 infection.

\textbf{Methods}: Genomic sequencing and expression analysis of the \textit{TRIM5a} gene was conducted in the pig-tailed macaque. A novel \textit{TRIM5-Cyclophilin A} fusion gene isoform was identified and subsequently cloned into the pcDNA3.1(+) expression vector. This construct was transfected into HeLa-T4 or HeLa cells which were then infected with the HIV-1\textsubscript{IIIB} or HIV-GFP-VSVG pseudotyped virus, to examine the effects of the TRIM5-Cyclophilin A fusion protein on HIV-1 infection.

\textbf{Results}: A novel \textit{TRIM5-Cyclophilin A} fusion gene (\textit{mnTRIMCyp}) in the pig-tailed macaque was found and its fusion pattern is different from the known fusion gene in the owl monkey (\textit{owlTRIMCyp}). TRIMCyp protein expression in transfected cells was confirmed by western blotting. The tests using HIV-1\textsubscript{IIIB} and HIV-GFP-VSVG pseudotyped virus indicated that \textit{mnTRIMCyp} did not inhibit HIV-1 replication at various multiplicities of infection.

\textbf{Conclusions}: The \textit{mnTRIMCyp} fusion protein does not restrict replication of HIV-1, which provides a potential molecular mechanism that might explain why the pig-tailed macaque is prone to HIV-1 infection, the only known exception in Old World monkeys.

Keywords: TRIM5a, Cyclophilin A, fusion gene, HIV-1 restriction factor, pig-tailed macaque

\textbf{Introduction}

The identification of an appropriate animal model is imperative for investigating the pathogenesis, diagnostic strategies, and treatment options for HIV. The currently used rhesus macaque (\textit{Macaca mulatta}) model, infected by simian immunodeficiency virus (SIV) or simian/human immunodeficiency virus (SHIV), is not ideal for these purposes [1,2]. The Chimpanzee is the closest relative to human and is susceptible to HIV-1 infection [3,4], but this species is not a suitable animal model due to their endangered status and high breeding cost. Among Old World monkeys, the pig-tailed macaque (\textit{Macaca nemestrina}) has been reported to be prone to HIV-1 infection, and developing AIDS-like symptoms [5–7].
There are increasing evidences that host antiretroviral proteins are important innate barriers to retroviral infection, for example, Friend virus susceptibility 1 (Fv1) in mouse, and Apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3G (APOBEC3G) and Zinc-finger antiviral protein (ZAP) in humans [8–10]. These factors act in diverse ways and at different stages of the HIV-1 life cycle. Most of them have broad antiretroviral action, including macaque simian immunodeficiency virus (SIVmac), murine leukemia virus (MLV) and equine infectious anemia virus (EIAV) [8–10].

Recently it was reported that TRIM5α confers resistance to HIV-1 infection in Old World monkeys [11]. The only known exception is the pig-tailed macaque, although the mechanism behind this susceptibility remains unknown [5, 7]. Among New World monkeys, the owl monkey is the only species that is not susceptible to HIV-1 infection [12]. Experiments indicated that a TRIM5-CypA fusion gene in owl monkey (owlTRIMCyp) was responsible for TRIM5α-mediated HIV-1 resistance in the owl monkey [13, 14]. Here we report a novel fusion form of TRIM5-CypA in the pig-tailed macaque (mnTRIMCyp) and its function against HIV replication in vitro. Our data demonstrated that contrary to the owl monkey fusion gene owlTRIMCyp, the fusion gene mnTRIMCyp in the pig-tailed macaque failed to restrict HIV-1 infection.

Materials and methods

Cell lines and viruses

Human HeLa and HeLa-T4 cells were cultured in DMEM–10% heat-inactivated fetal bovine serum (FBS), C8166 cell lines were grown in RPMI 1640–10% heat-inactivated FBS with penicillin and streptomycin. The owl monkey OMK cell line was cultured using the conditions recommended by the American Type Culture Collection. HIV-1IIIB virus infectivity was normalized on C8166 cell line. The 50% tissue culture infectious dose (TCID₅₀) of the HIV-1IIIB supernatants stock per milliliter was determined using the standard method [15].

Vector constructs and transfection

The mnTRIMCyp variant 2 and owlTRIMCyp variant 4 cDNAs were amplified from total mRNA of the pig-tailed macaque whole blood and owl monkey OMK cells, and cloned into pMD 18-T vector (TaKaRa) and confirmed by direct sequencing. All the splicing isoforms were re-amplified using nested primers, TRIM5α-F1 (5’GGCG AATTGCAGATGGCTTCTGGAATCCTGGTTAA TG3’) and CypA-UTR-R (5’ GGCTCTAGACTAAG CAAATGGGGTGGAAGG3’).

Molecular cloning

Total RNA was extracted from pig-tailed macaque whole blood using the TRIzol method (Invitrogen, Carlsbad, California, USA), and reverse-transcribed using oligo-dT and Omniscript Reverse Transcriptase (Qiagen, Valencia, California, USA). The TRIM5α variants were amplified from cDNAs by 3’-RACE with the 3’-Full RACE Core set system (TaKaRa, Dalian, China), including the F-RACE primer (GGTACCGTCCCATCTGCCC TGGAACCTC) and the nested universal Primer A. Polymerase chain reaction (PCR) products were cloned into the pMD 18-T vector (TaKaRa) and confirmed by direct sequencing. The multiple linearly amplified DNA was cloned into the pcDNA3.1 (+) plasmid and digested with EcoRI and XhoI (TaKaRa), and the digested products ligated with a rapid DNA ligation kit (Fermentas, Vilnius, Lithuania). The recombinant pcDNA3.1-mnTRIMCypV2 and owlTRIMCypV4 expression vectors were transfected into human HeLa or HeLa-T4 cell lines by Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions, and the transfected cells were selected in culture medium containing Genetin (Invitrogen).

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Cyclosporin A (CsA, MP Biomedicals, Aurora, Ohio, USA) was dissolved in DMSO at 1 mg/ml and 2.5 μM CsA diluted in tissue culture medium was added to the cells at the time of HIV-1 infection.

Western blot analysis of fusion proteins expression

Fusion proteins of mnTRIMCypV2 and owlTRIMCypV4 stably expressed in HeLa or HeLa-T4 cells were detected by western blotting. Cells were lysed in modified radio immunoprecipitation assay (RIPA) buffer with protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin and pepstatin. Whole cell lysates were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroblotted onto a Polyvinylidene fluoride (PVDF, 0.45 μm) membrane (Millipore, Billerica, Massachusetts, USA). The two proteins were detected using the anti-CypA primary antibody (Upstate, Lake Placid, New York, USA) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, Saint Louis, Missouri, USA) and illuminated with electrochemoluminescent reagent (Cell Signaling, Lake Placid, New York, USA).

Restriction of HIV-1 infection assays

HeLa-T4 cells were seeded in 24-well plates at 2 × 10⁴ cells per well and incubated at 37°C, 5% CO₂ overnight. The transfected cells were infected with the HIV-1IIIB strain at different multiplicity of infection (M.O.I = 0.5, 1, 5, 10) in 1 ml culture medium and
incubated for 2 h. When CsA was used, it was added at the same time as the virus. The HIV-1-infected cells were then washed twice with phosphate-buffered saline (PBS) and incubated at 37°C, 5% CO₂ in 1 ml of fresh culture medium. A total of 200 μl of supernatant from HIV-1-infected cultures was collected at different time-points and lysed by 0.5% Triton X-100. The HIV-1 p24 antigen was measured by enzyme-linked immunosorbent assay (ELISA) assay as previously described [16]. HeLa cells seeded in 24-well plates at a density of 3 × 10⁴ cells/well were incubated in the presence of three-fold serially diluted virus and 8 μg/ml polybrene for 4 h. Cells were washed twice with PBS, fresh culture medium was added and cultured for 48 h, then the percentage of GFP-positive cells well counted by fluorescence activated cell sorter analysis using a FACSCalibur (Becton Dickinson, San Jose, California, USA).

**Accession numbers**
The primate TRIM5-CypA sequences have been deposited in the GenBank database under accession numbers DQ 308404 to DQ 308406.

**Results**

**Retrotransposition of CypA into the pig-tailed macaque TRIM5α locus**

Whilst attempting to sequence the TRIM5α gene of the pig-tailed macaque, we identified a PCR product

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**Fig. 1.** The genomic structure and alternative splicing isoforms of the pig-tailed macaque fusion gene. (a) The genomic structure of the pig-tailed macaque fusion gene. A complete, processed CypA cDNA was inserted into the 3’ UTR region of mnTRIM5 exon 8; The 5’ flank and 3’ flank of the inserted CypA cDNA sequence was aligned with the homologous human CypA sequence. The exons of TRIM/Cyp and the TRIM5 exon 8 is in capital letters, introns in lower cases, and the inserted CypA cDNA sequences are shaded. The ‘natural’ CypA start codon, the CypA/TRIMCyp poly-adenylation signal and poly-A tail are underlined. The insertion
containing a cDNA copy of CypA gene. Sequence analysis indicated that the CypA cDNA copy is retro-transposed into the 3′ regulatory region of TRIM5α (Fig. 1a), resulting in a fusion gene. The novel fusion gene was designated as mnTRIMCyp (Macaca nemestrina TRIM5α-CypA). Three pig-tailed macaque samples were sequenced and all were homozygous for the observed retrotransposition.

Interestingly, the fusion pattern of TRIM5α and CypA in the pig-tailed macaque is different from that of the owl monkey (Fig. 1b; [13,14]). We sequenced four other macaque species and no retrotransposition was observed [17]. Hence, the retrotransposition of CypA into TRIM5α in the pig-tailed macaque is a recent and independent event.

### Analysis of TRIM5-CypA fusion transcripts

We then sought to understand the expression pattern of the putative fusion gene. We designed primers to amplify mnTRIMCyp from mRNA and investigated the expression of this gene in the peripheral blood mononuclear cells (PBMC) of three unrelated adult pig-tailed macaques. Our results indicate that there are three splicing forms encoded by the putative fusion gene (Fig. 1b). Among them, the V1 and V2 forms are the dominant forms (54%, 20/37 and 43%, 16/37, respectively, estimated by the relative abundance of clones). Both V1 and V2 are fusion transcripts of TRIM5α and CypA. The V1 form encodes 260 amino acids (aa) containing the 5′ coding region of TRIM5α (exon 2-4, 250 aa) and the 5′ untranslated region of CypA (10 aa). The V2 form encodes a fusion protein of 468 aa including 291 aa from exon 2-6 of TRIM5α and 177 aa from CypA (12 aa from the 5′ untranslated region and 165 aa of the complete coding region). Exons 7 and 8 of TRIM5α were not transcribed in the V1 and V2 fusion forms. Therefore, these two isoforms do not contain the SPRY domain of TRIM5α, which has been reported as the crucial region for resistance to HIV-1 infection in Old World monkeys [18,19]. Next, we used primers designed to amplify mRNA corresponding to exon 8 of TRIM5α to investigate whether the pig-tailed macaque expresses the
The V2 splicing isoform of mnTRIMCyp that we have detected in the pig-tailed macaque is similar to a fusion gene described in the owl monkey, which showed restrictive effects on HIV-1 infection [13,14]. The putative amino acid sequence of the retrotransposed CypA copy in the V2 form of pig-tailed macaque is highly conserved when compared with the human CypA protein (162/165 = 98.2%). To investigate whether the mnTRIMCyp V2 protein has any effect on the susceptibility of HIV-1 infection, a functional assay was designed to test the ability of mnTRIMCyp V2 to inhibit HIV-1 infection.

Expression constructs were produced by inserting mnTRIMCyp V2 and owlTRIMCyp V4 into pcDNA3.1 (+). These constructs, as well as pcDNA3.1(+) alone, were transfected into HeLa-T4 cells, and transfected cells were selected using media containing Geneticin. All transfected cell lines were then infected with HIV-1IIIB. Western blotting confirmed the expression of the mnTRIMCyp V2 and owlTRIMCyp V4 proteins in transfected cells. (Fig. 2).

The effects of these constructs on HIV-1 infection were examined by measuring the HIV-1 p24 antigen via ELISA. Our results demonstrate that mnTRIMCyp does not inhibit HIV-1 replication after virus entry into the cells at various M.O.I (Fig. 3a), whereas the cell lines transfected with the owl monkey fusion gene show strong restrictive effects at low M.O.I (0.5 and 1.0), consistent with the previous reports [13,14]. However, when the M.O.I of HIV-1 is elevated to 5 and 10, the restrictive effect of the owl monkey fusion gene is greatly decreased, probably due to a saturation effect. It should be noted that in our experiments, the restrictive effect of the owl monkey fusion gene is not as strong as previously reported [13,14].

To confirm the effect of mnTRIMCyp, we investigated the response of the fusion gene activity to Cyclosporin A (CsA), a drug that disrupts the interaction between the HIV-1 capsid and CypA. CsA treatment of cells expressing mnTRIMCyp inhibited HIV-1 infection (data not shown), consistent with previous reports [20,21]. However, in the owl monkey, the opposite effect was reported, with CsA treatment increasing the infection by HIV-1 [13,14].

Next we investigated the effect of mnTRIMCyp on the infectivity of HIV-GFP-VSVG pseudotyped virus. The mnTRIMCyp and owlTRIMCyp constructs, as well as pcDNA3.1(+) alone, were transfected into human HeLa cells and selected in the media containing Geneticin. Cells stably expressing the fusion proteins were detected by immunoblot (Fig. 2). The ability of these constructs to restrict HIV-GFP-VSVG pseudotyped virus infection was quantified by comparing the percentage of infected HeLa cells. Our results demonstrated that owlTRIMCyp clearly inhibited HIV-1, as shown by a three to four-fold reduction in infection in owlTRIMCyp-transfected HeLa cells compared with empty pcDNA3.1(+) transfected HeLa cells (Fig. 3b). But no difference was observed between the infection of the mnTRIMCyp and empty pcDNA3.1(+) transfected HeLa cells.

The effects of mnTRIMCyp on pseudotyped virus infection were in accordance to p24 core antigen ELISA assay in HIV-1IIIB infection. Again, mnTRIMCyp does not restrict the replication of HIV-GFP-VSVG, but owlTRIMCyp does.

**Discussion**

In Old World monkeys, it has been reported that the restriction of HIV-1 infection was mainly due to the sequence divergence of TRIM5α when compared with humans and apes [17,18]. The RING domain of TRIM5α is not necessarily required for retroviral restriction but can affect the expression level of the gene. The B-Box2 and SPRY domains are essential for efficient retroviral resistance [19,22,23], and a single amino acid site in the SPRY domain was reported to be responsible for the restricting HIV-1 infection in Old World monkeys [19].

CypA interacts with the capsid of HIV-1 and is known to be involved in HIV-1 infection [24–27]. In the human
genome, more than 60 processed CypA pseudogenes have been reported across the genome by retrotransposition [28]. We observed a similar pattern in chimpanzees by genome-wide sequence analysis (data not shown), implying that CypA retrotransposition is a common phenomenon during primate evolution.

Recently, a fusion protein (TRIMCyp) was reported in owl monkey, a New World monkey species [13,14]. The fusion was also caused by retrotransposition of CypA into TRIM5α, and the fusion gene conferred strong post-entry restriction of HIV-1 infection in owl monkey. It has been shown that in Old World monkey cells, CypA is required

Fig. 3. Restriction analysis of the fusion gene to HIV-1 infection. (a) Expression of p24 antigen of HIV-1 in the HeLa-T4 cell lines transfected with TRIMCyp fusion genes. The cells transfected with the owl monkey fusion gene (owlTRIMCyp) and the empty pcDNA3.1(+) vector were used as positive and negative controls respectively. The HIV-1 p24 antigen was used as the marker to measure HIV-1 replication using an enzyme-linked immunosorbent (ELISA) assay. A virus gradient was used in the infection tests as determined by the multiplicity of infection (M.O.I), including M.O.I = 0.5, 1.0, 5.0 and 10.0. The columns indicate the HIV-1 p24 ELISA optical density (OD) values measured in a time period (day 1, 3, 5, 7, 9, 11). Each column is the mean of triple wells with standard error indicated. Results are representative of three independent experiments. (b) Restricting effects of mnTRIMCyp on HIV-GFP-VSVG pseudotyped virus infection. HeLa cells stably expressing mnTRIMCyp, owlTRIMCyp or empty vector pcDNA3.1(+) were infected with two-fold serially diluted HIV-GFP-VSVG pseudotyped virus in the present of 8 μg/ml polybrene. Infected cells (the percentage of GFP-positive cells) were enumerated 48 h later by FACS. Results are representative of three independent experiments.
The different effects of the two fusion genes on HIV-1 infection in pig-tailed macaque and owl monkey are intriguing. In the case of owl monkey, it was suggested that although the fusion gene TRIM5a has lost the SPRY domain, the retrotransposed CypA supplemented the function of the SPRY domain of TRIM5a, enabling the fusion protein to interact with the capsid proteins of HIV-1, resulting in resistance to infection [13,14]. However, our data on the pig-tailed macaque suggests that the nmTRIMCyp fusion protein has no restrictive activity on HIV-1 infection. It is likely that the cells of pig-tailed macaque differ from those of other Old World monkeys in allowing HIV-1 infection to reach the stage of reverse transcription. The comparison of putative amino acid sequences of the owl monkey V4 and the pig-tailed macaque of Guest TRIMCyp fusion protein are shown in Fig. 1c.

The fusion gene in pig-tailed macaque is defective in defending against HIV-1 infection, which raises a question of what its evolutionary function may be. There are several possible explanations. First, the fusion gene might have other potential benefits for pig-tailed macaques; for example it might be resistant to infection of other viruses. Second, the fixation of the nmTRIMCyp fusion gene may be due to genetic drift.

The narrow host range of HIV-1 has impeded the development of a suitable animal model for the study of AIDS pathogenesis and evaluation of therapeutic drugs and vaccine strategies. Although other New World monkeys are also susceptible to HIV-1 infection, they do not manifest the symptoms. Furthermore, in the phylogeny of primate evolution, the relationship between New World monkey and human is further than Old World monkey with human. Hence, the pig-tailed macaque, as the only known Old World monkey species prone to HIV-1 infection, may serve as a potential animal model for HIV-1 studies. Recently, the pig-tailed macaque AIDS models have been explored by Pullium and Otten et al. using HIV-1 virus [32,33]. Their studies demonstrated that pig-tailed macaque is a very promising AIDS non-human primate in the pathogenesis and prophylactic methodology.

In conclusion, a novel TRIM5-Cyclophilin A fusion gene (nmTRIMCyp) in the pig-tailed macaque was found and its fusion pattern is different from the owlTRIMCyp [13]. It provides a potential molecular mechanism that could explain why the pig-tailed macaque is susceptible to HIV-1 infection. The fusion of TRIM5a and CypA disrupts the open reading frame of TRIM5a, resulting in a chimeric protein that has lost the ability to restrict HIV-1 restriction in pig-tailed macaque. Our study confirms that the pig-tailed macaque might be an optimal AIDS animal model which allows investigators dissecting the mechanism of HIV infection and disease development during the early stage of HIV-1 infection.

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