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A new chimeric protein represses HIV-1 LTR-mediated expression by DNA methylase

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ABSTRACT

Once the human immunodeficiency virus (HIV) genome is inserted into the host genome, the virus cannot be removed, which results in latency periods and makes it difficult to eradicate. The majority of strategies to eradicate HIV have been based on preventing virus latency, thereby enabling antiretroviral drugs to act against HIV replication. Another innovative strategy is permanently silencing the integrated virus to prevent the spread of infection. Epigenetic processes are natural mechanisms that can silence viral replication. We describe a new chimeric protein (IN3b) that consists of a HIV-1 integrase domain, which recognises the HIV long terminal repeat (LTR) and the catalytic domain of DNA methyltransferase DNMT3b. Our objective was to silence HIV replication by the specific delivery of the catalytic methyltransferase domain to the LTR promoter to induce its methylation. We found that our IN3b chimeric protein was expressed in the nucleus and decreased LTR-associated HIV genome expression and HIV replication. Therefore, the IN3b chimeric protein may be an effective tool against HIV replication and maybe used in a new line of research to induce or maintain HIV latency.

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1. Introduction

Significant international efforts have been made in the fight against the AIDS epidemic. Highly active antiretroviral therapy (HAART) effectively halts HIV replication and profoundly increases the survival of patients; however, a cure has not yet been achieved due to the persistence of long-lived reservoirs of cells latently infected with HIV (Perelson et al., 1997). Multiple approaches for the reactivation and depletion of the latent reservoir have been evaluated clinically (Chun and Fauci, 2012; Geeraert et al., 2008), and these efforts aim to reactivate latently infected cells to render them susceptible to viral cytopathic effects, antiviral immune responses, antiviral drugs and other means of targeted cell killing. However, the complete depletion of the latent reservoir remains an unachieved goal. Therefore, it is crucial to develop new strategies and improve current approaches to attack the latent reservoir.

DNA modification by CpG nucleotide methylation can control mRNA transcription (Boyes and Bird, 1992). Epigenetic modifications can affect gene transcription by regulating the accessibility of transcription factors to DNA (Bogdanovic and Veenstra, 2009; Robertson, 2002). CpG motifs in DNA that is clustered in regions within promoters can be methylated or demethylated (Hsieh, 2000). The methylation of CpG motifs causes the condensation of chromatin, whereas demethylation leads to the relaxation of chromatin and increased accessibility to target sequences by transcription factors (Huehn et al., 2009; Wilson et al., 2009). Epigenetic control is a well-established mechanism for gene regulation within the immune system, which governs cell fate by controlling lymphocyte development and determining the molecular mechanisms that contribute to the epigenetic imprinting of essential lymphocyte lineage markers, such us cytokine genes (Ansel et al., 2003; Kioussis and Georgopoulos, 2007; Lee et al., 2006).

The different members of the mammalian DNA methyltransferase (DNMT) family have different actions on DNA. DNMT1 exhibits a high affinity for hemi-methylated DNA sequences during DNA replication (Liu et al., 2003). DNMT3a and 3b participate in "de novo" methylation, which is important for normal embryogenic development (Okano et al., 1999; Yokochi and Robertson, 2002). Other members of the DNMT family (DNMT2 and DNMT3L) have not demonstrated methylation activity (Dong et al., 2001; Gowher et al., 2005). De novo methylation is a natural mechanism by which the cell or genome is protected from the expression of foreign DNA, such as viruses, and DNMTs have a role in the control of HIV

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replication. Several authors have reported that the acute infection of CD4 T cells with HIV-1 increases DNMT expression and activity (Mikovits et al., 1998). The increase in DNMTs was due to an overall increase in methylated genomic DNA in HIV-infected cells and the de novo methylation of a single CpG dinucleotide in the gamma interferon (IFN-γ) gene promoter, which subsequently down regulates the expression of IFN-γ in purified HIV-infected CD4 T-cells(-Fang et al., 2001; Mikovits et al., 1998).

Our objective was to design a new chimeric protein that linked the N-terminal domain of the HIV integrase enzyme with the C-terminal domain of DNMT3b to inactivate HIV expression in host cells. In this study, we propose a new molecular mechanism that causes long-term silencing by the transcriptional control of HIVLTRs.

2. Materials and methods

2.1. IN3b chimeric protein construction, the vectors and the E229A point mutation

We designed the construction of the IN3b chimeric protein, which was produced by BlueSky Bioservices (Worcester, MA, USA). The viral integrase N-terminal domain (nucleotides 1–168) was linked to the SV40 nuclear localisation signal (NLS) (sequence PKKKRKV) and the DNMT3b catalytic domain (nucleotides 1906–2695, GenBank accession). In addition, the chimeric protein was C-terminal-HA tagged. The whole sequence was inserted into a pCI-neo expression plasmid (Promega, Madison, WI, USA) using EcoRI and XbaI sites. The mutation E229A in the DNM3b domain reduces methyltransferase activity (Kioussis and Georgopoulos, 2007). To introduce the mutation, we used the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. We used the oligonucleotides E229A-Fw (GCCCTTCTTCTGGATGTTCGC-GAACGTGGTGGCCATGAA) and E229A-Rv (TTCATGGCCAC-CACGTTCGCGAACATCCAGAAGAAGGGC). The pLTR-luc vector contains the U3-UTR of the LTR from the LAI strain of HIV-1 (nucleotides _644 to _78), and the luciferase gene was provided by Dr. J. L. Virelizier (Institute Pasteur, Paris, France). The pCox-luc vector contains the Cox-2 promoter inserted into the pCOX-Luc reporter plasmid (Nordeen et al., 1998). The pTNF-luc construct was made by inserting 1311 bpupstream from +1 nucleotide of the TNF-α gene promoter (Rhoades et al., 1992).

2.2. Virus production

CXCR4-tropic X4-HIV-1_{NL4-3} was obtained by the transient transfection of pNL4-3 plasmids (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) in the 293T cell line (ATCC-LGC, Teddington, UK). Viral titer was quantified using the-HIVp24^{gag} ELISA kit (INNOTEST® HIV-Antigen mAb, Innogenetics, (INNOTEST® HIV Antigen mAb, Innogenetics, Belgium) (Chonco et al., 2012; Garcia-Merino et al., 2009).

2.3. Cell culture and transfection

The Hek293T cell line (American Type Culture Collection, ATCC) was cultured in Dulbecco's MEM (Biochrom AG, Berlin, Germany), and the CEM lymphoblastic leukaemia cell line (ATCC) was cultured in RPMI 1640 (ATCC) and supplemented with 10% FBS (Lonza, Walkersville, MD), 2 mM L-glutamine, 1% ampicillin and cloxacillin and 0,32% gentamicin (Normon, Madrid, Spain). The Hek293T cells were transfected with calcium phosphate using the "CAPHOS" kit (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. After 48 h, the Hek293T cells were harvested and IN3b expression was analysed using RT-PCR and Western Blot (WB). We used the primers Fw-IN3b-Eco-RI-kozak (CGGAATTCGCCGCCACCATGTTCCTCGACGGTATCGA-CAAG) and Rv-IN3b-HA-XbaI (GCTCTAGACTACTAACCACTCGCG TAGTCGGGGAC) for RT-PCR. We added 5 μM 5-aza-2′-deoxycytidine (5-AzaC) (Sigma–Aldrich) to the transfected Hek293T cells 24 h before the cells were harvested.

2.4. Western blot

The protein extracts from the transfected cells were separated on a 10% polyacrylamide gel and were transferred to nitrocellulose membranes. The blots were probed with an anti-integrase mouse monoclonal antibody (Invitrogen, Merelbeke, Belgium) and an α-tubulin mouse monoclonal antibody (Sigma–Aldrich)(Clemente et al., 2009).

2.5. Flow cytometry

We used the BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences, San Diego, CA) to analyse the intracellular staining using flow cytometry. The Hek293T cells were stained with the anti-integrase antibody or an isotype control. Then, we washed the cells with the Cy3 anti-mouse secondary antibody. IN3b expression was analysed using flow cytometry (Gallios, Beckman Coulter, Fullerton, USA) and Flow Jo software (Tree Star, Inc., Oregon, USA). Each experiment was performed in triplicate.

2.6. Confocal microscopy

Forty-eight hours post-transfection, the Hek293T cells were fixed with paraformaldehyde 4% (Sigma–Aldrich) and permeabilised with Triton X-100 solution (Sigma–Aldrich). The Hek293T cells were stained with either the anti-integrase antibody (Invitrogen) or the isotype control for 30 min at room temperature. After several washes, the cells were incubated with the Cy5 anti-mouse antibody. Coverslips were prepared with specific fluorescent mounting medium (Dako Denmark A/S) and stored at 4 °C. The Hek293T cells were analysed using confocal microscopy (Leica TCS SP2, Germany).

2.7. Luciferase analysis

The luciferase assay was performed using the Luciferase Assay System (Promega) according to the manufacturer's instructions, and luciferase activity was measured usingthe Wallac 1450 Microbeta TriLux Luminescence Counter (GMI, Inc., Ramsey, MN, USA) (Navarro et al., 1998). The levels of luciferase expression in the cells were represented by relative luciferase units (RLUs). Luciferase activity was normalised to β-galactosidase activity. Constant β-gal activity was achieved in each experiment, and luciferase expression was expressed according to β-galactosidase activity.

2.8. IN3b stable transfectants and HIV infection

CEM cells were nucleofected using the Amaxa kit following the manufacturer's recommendations. After nucleofection, the cells were washed with complete RPMI 1640 medium, plated in p6-wellplates, and incubated at 37 °C. After 48 h, 1 mg/ml G418 was added to the complete media. CEM cells that could grow under antibiotic selection were single-cell sorted and cloned. Growing clones were assayed by integrase expression according to flow cytometry, and positive clones were selected. The IN3b chimeric protein, the IN3b-E229A mutant and empty vector CEM clone cells were infected with HIV-$_{NL4.3}$. After 2 h of incubation, the cells were washed twice with complete RPMI 1640 medium. Then, the cells

were plated in p6-wellplates and incubated at different time points. DNA was extracted from the cells, and $P24^{gag}$ antigen was measured using ELISA.

2.9. RNA extraction, reverse transcription and qPCR analysis

Total DNA was extracted from the cell pellets. qPCR was performed in a MicroAmp™ Optical 96-well Reaction Plate (Applied Biosystems, Foster City, CA, USA) in quadruplicate for each DNA sample. PCR reactions were run on an Applied Biosystems StepOne Plus Real-Time PCR System. The LTR-specific primers were as follows: LTR forward, 5′-GCC TCA ATA AAG CTT GCC TTG A-3′; and LTR reverse, 5′-TCC ACA CTG ACT AAA AGG GTC TGA-3′. The data were collected and analysed using StepOne Software, v2.2 (Applied Biosystems) with the comparative delta-delta Ct method (ΔΔCt). Data Assist v1.0 software (Applied Biosystems) was used to derive significance between the independent experiments. The GPDH gene was used for normalisation (Friedrich et al., 2010).

2.10. Statistical analysis

The nonparametric Mann–Whitney *U*-test was used for the variables without a normal distribution. The correlation between the variables was established using Pearson's correlation test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. IN3b chimeric protein expression

First, we analysed the correct expression of the IN3b chimeric vector by transfecting the pCI-IN3b vector and the pCI empty vector in the Hek293T cell line. After 48 h, the Hek293T cells were harvested for mRNA extraction. The IN3b chimeric vector mRNA expression was assessed using-PCR (Fig 1A). As a positive control for the reaction, the pCI-IN3b vector was used. We obtained similar band sizes in the cDNA from the samples that were transfected with the IN3b chimeric vector. Expectedly, the amplification of the cDNA from the samples that were transfected with the pCI empty vector was negative (Fig 1A). We analysed the protein presence in the transfected Hek293T cells using WB with the anti-integrase antibody. We detected the IN3b protein in the samples that were transfected with the pCI-IN3b and pCI-IN3b E229A mutant constructs (Fig 1B). Furthermore, we performed co-transfections with a pE-GFP plasmid, which permitted us to determine the percent of transfection following the expression of GFP, pCI, pCI-IN3b and pCI-IN3b E229A. After 48 h, the Hek293T cells were stained with isotype control and anti-integrase antibodies. We detected the expression of the IN3b chimeric protein in the GFP-expressing Hek293T cells that were transfected with the IN3b and mutant constructs (Fig 1C).The IN3b protein fusion was expressed by the transfected Hek293T cells with both the pCI-In3b and pCI-IN3b E229A constructs. The transfected cells were not affected by the constructs (Supplementary Fig. 1).

3.2. IN3b chimeric protein sub-cellular location

The IN3b chimeric protein had the nuclear localisation signal (NLS) to localise the protein into the nucleus. To study the functionality of the NLS sequence, Hek293T cells were transfected with the pCI, pCI-IN3b and pCI-IN3b-E229A constructs and were fixed, permeabilised and stained with the anti-integrase antibody and DAPI, which was used to label the nuclei. The samples were analysed using confocal microscopy. Both the IN3b and IN3b mutant proteins were expressed in the nuclei of the Hek293T cells (Fig 2). The IN3b chimeric protein expressed nuclear localisation.

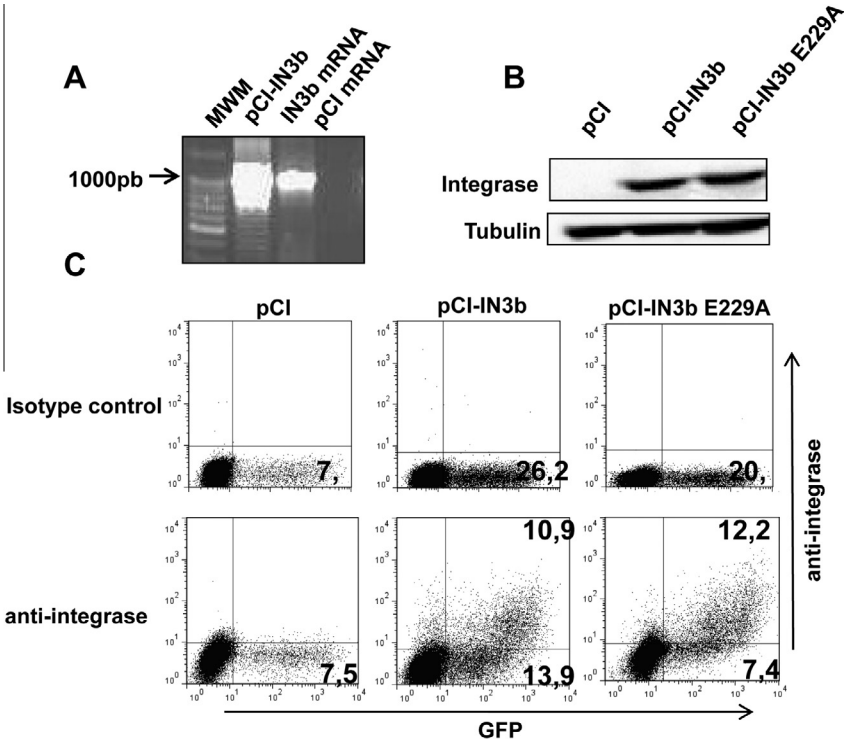

Fig. 1. IN3b chimeric protein expression. Hek293T cells were transfected with pCI-neo (empty vector), pCI-IN3b and pCI-IN3b E229A mutant constructs. The Hek293T cells were harvested 48 h post-transfection, and the IN3b chimeric protein and mutant expression were studied. (A) mRNA expression was assayed using RT-PCR. A representative experiment of two independent assays is shown. (B) The Western blot of the transfected Hek293T cells. A representative experiment of three independent assays is shown. (C) Flow cytometry of Hek293T cells co-transfected with GFP and pCI-neo, pCI-IN3b and pCI-IN3b-E229A mutant constructs. A representative experiment of five independent assays is shown.

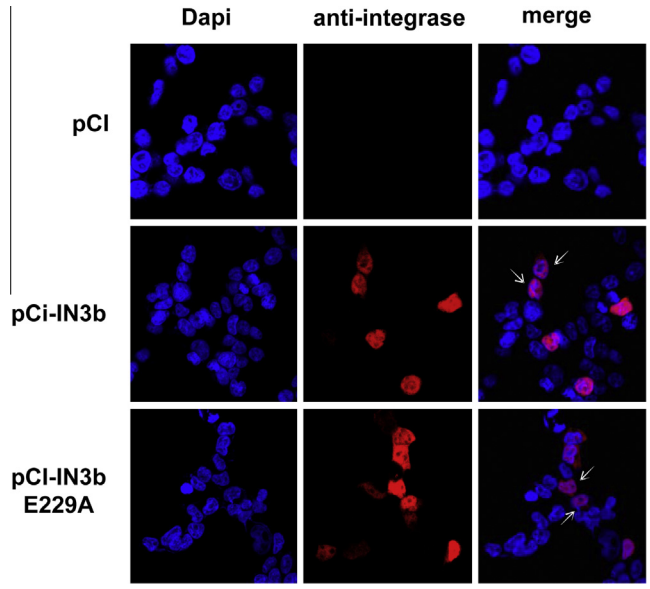

Fig. 2. IN3b chimeric protein cellular localisation. Hek293T cells were transfected with pCI-neo, pCI-IN3b and pCI-IN3b E229A mutant constructs. At 48 h post-transfection, the Hek293T cells were stained with an anti-integrase antibody and DAPI. The samples were analysed using confocal microscopy. A representative experiment of four independent assays is shown.

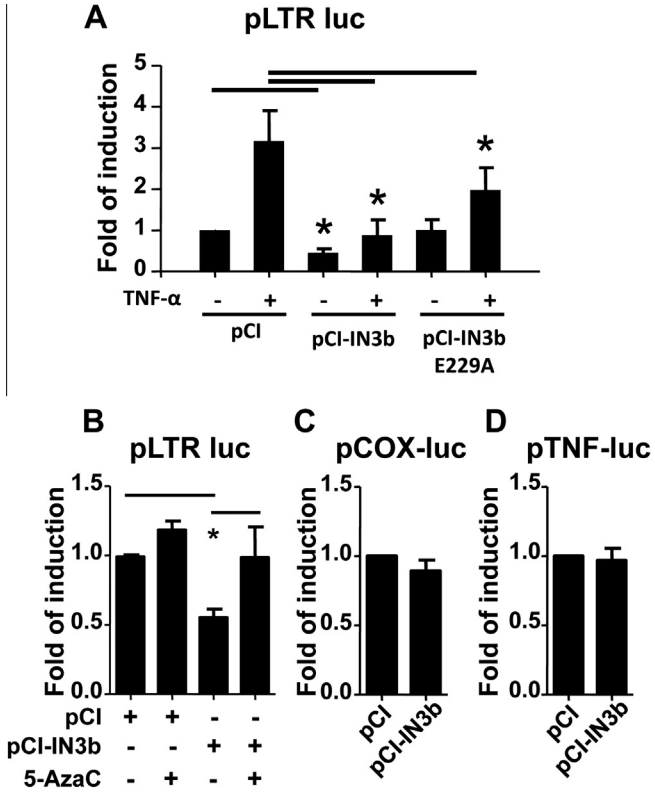

Fig. 3. IN3b chimeric protein has LTR repressing activity. (A) Hek293T cells were co-transfected with a pLTR-luc plasmid and pCI-neo, pCI-IN3b and pCI-IN3b E229A mutant constructs. The Hek293T cells were harvested 48 h post-transfection, and luciferase expression was quantified. (B) The transfected cells were treated with 5 μM 5-Aza-C. (C) The Hek293T cells were co-transfected with COX-luc or (D) TNF-Luc plasmids and pCI-neo or pCI-IN3b, and luciferase expression was quantified. The data are represented as the fold induction normalised to β-galactosidase expression. An average of four to six experiments is shown. The error bars show the standard deviation. $^{*}p < 0.05$, Mann–Whitney *U*-test.

Therefore, IN3b could interact with the HIV genome that had integrated into the nuclei.

3.3. The effect of the IN3b chimeric protein on LTR expression

As we have demonstrated, the IN3b chimeric protein was correctly expressed in the nuclei of the transfected Hek293T cells. Therefore, we studied the functional effect of this IN3b chimeric protein on LTR expression. The Hek293T cells were co-transfected with a p-LTR-luc plasmid with the pCI, pCI-IN3b or pCI-IN3b E229A constructs. The Hek293T cells were treated with TNF-α to induce luciferase gene expression, which is controlled by the HIVLTR. The Hek293T cells that were transfected with the IN3b chimeric protein reduced the expression of LTR-luciferase by TNF-α by more than 65% compared with the Hek293T cells that were transfected with the empty vector. In contrast, the IN3b-E229A mutant did not affect LTR luciferase expression in the non-stimulated Hek293T cells, and the reduction of LTR luciferase expression in TNF-α-stimulated cells was approximately 30%. Therefore, the mutation in the catalytic domain of DNA methyltransferase could partially revert the outcome that occurred with the unmutated IN3b. The IN3b chimeric protein has repressive activity on HIV LTR expression (Fig 3A). To associate LTR repression with IN3b methyltransferase activity, we treated the transfected Hek293T cells with the CpG methylation inhibitor 5-azacytidine and we observed that the treatment reverted HIV LTR repression (Fig 3B). Therefore, this result suggests that the repression of LTR expression by IN3b, which was reverted by 5-AzaC, could be caused by DNA methylation. Furthermore, we co-transfected the pCI-IN3b construct with pCOX-Luc and pTNF-α-luc constructs. These constructs contain the COX and TNF-α promoters that drive luciferase expression. We selected these promoters because these proteins are implicated in the inflammatory pathway and are activated during HIV-1 infection. We detected no effect on luciferase expression by the COX and pTNF-α promoters when the cells were co-transfected with the pCI-IN3b construct compared with the cells that were transfected with the pCI empty vector (Figs 3C and D). Therefore, IN3b acts through CpG DNA methylation and is specific to the LTR promoter.

3.4. The stable transfection of the IN3b chimeric protein decreases HIV replication

To study the effect of the IN3b chimeric protein on HIV infection, we generated stable transfectants that were derived from CEM cells. After multiple rounds of cloning and cell sorting, we selected cells that stably expressed the IN3b protein and IN3b-E229A mutant (Fig 4A). We compared the expression of the CD4 HIV receptor and the CXCR4 HIV co-receptor in the selected clones, and we found no significant differences compared with the parental lines. Our data indicate that the clones were unlikely to affect HIV entry by the CD4 receptor or CXCR4 co-receptor (Fig. 4B). Moreover, we analysed the expression of other T-cell markers to select clones with the closest phenotype to the parental control lines (Fig. 4B). The selected cells were infected with HIV-$1_{NL-4.3}$. We quantified the $P24^{gag}$ amount in the supernatant of the CEM-IN3b cell culture after 1, 3 and 7 days post-infection (Fig 5A). The CEM-IN3b cells had a smaller amount of $p24^{gag}$ than the clones that were transfected with the IN3b E229A mutant or empty vector. This reduction in HIV-$1_{NL-4.3}$ replication was detected as early as 3 days after infection (Fig. 5A). To confirm this result, DNA from the CEM stable clones was harvested at day 7 and quantitative PCR was performed to detect the HIV LTR. The average amount of HIV LTR detected in the CEM cells transfected with IN3b was 50% less than that in the CEM cells transfected with the IN3b E229A mutant or empty vector (Fig. 5B). In summary, the IN3b chimeric protein reduced HIV replication.

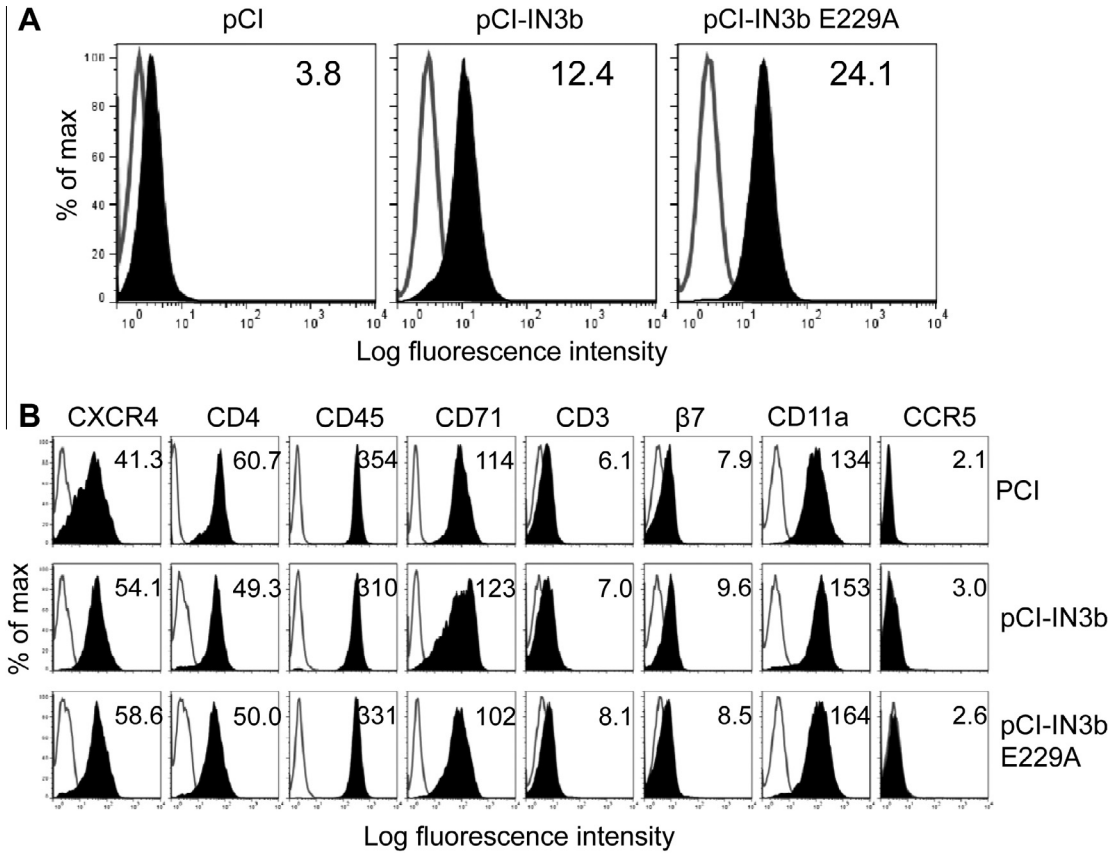

Fig. 4. Generation of IN3b-expressing transfectants. CEM cells were transfected with pCI-neo, pCI-IN3b and pCI-IN3b-E229A mutant constructs**.** The CEM cells were grown under neomycin selection and were selected by multiple rounds of cloning and FACS selection. (A) The CEM cells were stained with isotype control mAbs (the thin line) or an anti-integrase mAb (the black filled). The values represent the MFI. A representative experiment taken from six experiments is shown. (B) A phenotypic analysis of the selected clones. The isotype control mAbs (the thin line) and the indicated mAbs (black filled). The values represent the MFI. A representative experiment of three independent assays is shown.

4. Discussion

Viral genome integration plays a key role in the natural course of HIV infection. HIV genome integration occurs in transcribed and activated sequences in the host genome. However, integration may occur in non-transcriptional sequences in several cases. Therefore, several cells can survive during infection and become dormant, thereby keeping the virus in a latent state in which there is no production of new viral particles (Jordan et al., 2001; Lassen et al., 2004). Under certain circumstances, these cells can be reactivated, which leads to the production of new viral particles. In contrast, epigenetic processes, such as DNA methylation, are mechanisms that can control HIV latency induction (Chavez et al., 2011; Mok and Lever, 2007; Tripathy et al., 2011). Understanding the molecular mechanisms that underlieHIV-1 transcriptional silencing and activation is a major challenge in the fight against HIV infection and AIDS and could lead to the development of new therapeutic tools (Chavez et al., 2011; Mok and Lever, 2007; Tripathy et al., 2011).

In this study, we presented a chimeric protein that was constructed by joining the DNMT3b C-terminal domain and the HIV integrase N-terminal domain to target HIV LTRs for DNA methylation. We observed lower HIV replication in the cells that expressed the IN3b chimeric protein compared with those that were transfected with an empty vector or the IN3b construct mutated in the catalytic domain of DNM3b. This effect was determined to be specific to the HIV LTR because other cellular promoters were not affected by this chimeric protein. Finally, the effect of IN3b was reverted using a methyltransferase inhibitor, which suggests that the mechanism of LTR methylation was the mechanism implicated in the inhibition of HIV replication. These data are in agreement with other studies that demonstrated a chimeric protein that linked the C-terminal domains of DNMT3 to the DNA binding sequences of different transcription factors and produced methylation activity in the transcription factor target sequence, which decreased its transcriptional activity (Li et al., 2007).

Targeting the HIVLTR sequence that contains the essential promoters for viral transcription could be a pivotal strategy for future HIV silencing approaches. The blockade of the HIV LTR using a short-hairpin RNA against a NF-kappaB site of the LTR sequence reduced HIV replication in T lymphocytes (Yamagishi et al., 2009). Moreover, CpG DNA methylation of the HIV LTR sequence could be implicated in viral silencing in vitro and in infected patients (Blazkova et al., 2009; Palacios et al., 2012).

HIV-1 eradication from HIV-infected individuals cannot be achieved with current therapies (Richman, 2001). Viral reservoirs remain unaffected by antiretroviral therapies, and upon interruption of antiretroviral treatment, the reservoirs can replenish systemic HIV infection (Steingrover et al., 2008). Most of the strategies that have been designed to eradicate the virus have been aimed at enhancing viral reactivation while treating HIV patients with HAART. However, this strategy is potentially problematic if reactivation is not complete because the viral reservoir in dormant cells will not be eradicated.

Therefore, alternative strategies are needed to eradicate HIV in infected patients. Our study proposes a new molecular mechanism

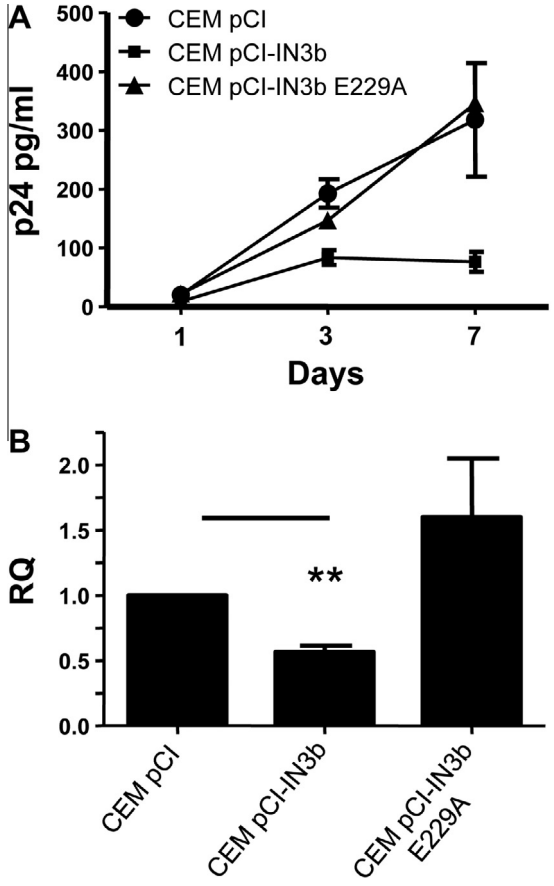

Fig. 5. IN3b chimeric protein suppresses HIV infection. The transfected CEM cells shown in Fig. 4 were infected with HIV-$1_{NL4.3}$. (A) The $p24^{gag}$ antigen was quantified in the cell culture supernatant after 1, 3 and 7 days post-infection. An average of 3 experiments is shown. (B) At day 7 post-infection, the cells were harvested and washed and genomic DNA was extracted. qPCR was performed to detect the HIV LTR. An average of three experiments is shown. $^{*}p < 0.005$, paired *t*-test.

that causes long-term silencing by the transcriptional control of HIV LTRs. Further experiments are necessary to evaluate the feasibility of this approach in patients. However, silencing the HIV genome may be possible because other retrotransposons and human endogenous retroviruses (HERVs) that represent 8% of the human genome (Griffiths, 2001) can be silenced in most cases. Finally, retroposon and HERV expression are in part regulated by DNA methylation, such as HERV-K (HML-2) (Florl et al., 1999; Laska et al., 2013; Lavie et al., 2005). Epigenetic mechanisms can provide new insights into future molecular therapies to control viral silencing in HIV-infected individuals. Our study aimed to develop a method that would deeply silence HIV expression as an alternative to the current strategies that have been designed to silence the HIV genome.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.04.007.

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