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Suppression of HIV replication using RNA interference against HIV-1 integrase

Tat San Lau[a], Yonggang Li[b], Masanori Kameoka[c], Tzi Bun Ng[a], David Chi Cheong Wan[a,*]

[a] *Department of Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China*
[b] *Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan*
[c] *Department of Biochemistry, Nara Medical University, Japan*

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Abstract RNA interference (RNAi) has become one of the most powerful and popular approach on gene silencing in clinical research study especially in virology due to the gene-specific suppression property of small interfering RNA (siRNA). In this report, we demonstrate that expression of vector-mediated small hairpin RNA (shRNA) against human immunodeficiency virus type 1 (HIV-1) integrase (IN), one of the three important enzymes in HIV infection by controlling the integration of viral RNA to host DNA, could suppress the protein synthesis of EGFP-tagged IN in HeLa cell model efficiently. Furthermore, we show that IN shRNA can successfully reduce the HIV particles production in 293T cells at the level similar to the positive control of HIV-1 tat shRNA. These results provide the therapeutic possibility of HIV replication using RNAi against HIV-1 integrase.

Keywords: Human immunodeficiency virus type 1; RNA interferences; Integrase

1. Introduction

Integration of HIV genome into host DNA is a crucial step in HIV infection. It is absolutely required for viral transcription, translation and replication [1,2]. This final event is catalyzed by HIV-1 integrase (IN) [3] and a functional integrase is necessary for successful virus infection. Besides the function of integration, integrase can act as a co-factor with reverse transcriptase in the reserve transcription process [4,5]. Because of the high selectivity due to the fact that there is no known human homologue, HIV-1 integrase is one of the important targets for development of anti-HIV drugs. Although there are two small molecules that are undergoing clinical trial [6] as IN inhibitors [7,8], the development of therapeutic IN inhibitors suitable for clinical usage is still limited. Recently, the discovery of RNA interference (RNAi) provides a powerful way for functional genomic and disease therapeutic study, especially in the field of oncology and virology [9–12]. RNAi is a gene suppression mechanism through the cleavage of mRNA through the cooperation of Dicer and a 21nt short sequence specific small interfering RNA (siRNA). RNAi has been used in the study of HIV inhibition against different viral proteins such as regulatory proteins: Tat, Rev, co-repectors: CCR5 and CXCR4, etc. [13–16]. Here, we provide evidence to show that the HIV-IN coding region can be one of the potent targets of RNAi for suppression of HIV replication.

2. Materials and methods

2.1. Construction of EGFP-integrase expression vector

Wild-type HIV-1 integrase from a recombinant expression clone [17] was fused with enhanced green fluorescent protein (EGFP) at the N-terminal by cloning into pEGFP-C1 expression vector (Clontech) using PCR with appropriate primers to provide a reporter system for monitoring the RNAi effect.

2.2. shRNA design and plasmid construction

Small hairpin RNA (shRNA) expression vector p*Silencer* 2.0-U6 (Ambion, USA) was used to trigger RNAi. HIV-IN coding sequence (GeneBank accession no. AF078150) was submitted to the Ambion siRNA Target Finder website (http://www.ambion.com/tecchlib/misc/siRNA_finder.html) for siRNA prediction. Four of eighty-four suggested candidates directed against IN were selected. The nucleotide sequences of the siRNA target sites in the HIV-1 IN gene were as follows: IN shRNA1, 5′-GGCCCAAGAAGAACATGAG-3′ (nucleotides [nt] 19–37); IN shRNA2, 5′-CCTACCACCTGTAGTAGCA-3′ (nucleotides [nt] 79–96); IN shRNA3, 5′-GTAGACTGTAGCCCAGGAA-3′ (nucleotides [nt] 158–176); IN shRNA4, 5′-GAGATCAGGCTGAACATCT-3′ (nucleotides [nt] 495–513). All of the position numbers of the IN nucleotide sequence described here correspond to those of the HIV-1 IN cDNA sequence (GenBank accession no. AF078150). A pair of complementary oligonucleotides that contained BamHI site, sense strand of target site, 9 nts hairpin loop sequence (TTCAAGAGA), anti-sense strand of target site and HindIII site were prepared for vector construction according to the manufacturer's instruction. All constructs were verified by sequencing. The p*Silencer* 2.0-U6 Control vector from Ambion was used as a negative control.

2.3. Cell culture and transfection

HeLa cells (ATCC) were cultured and maintained in RPMI containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of streptomycin–penicillin (Invitrogen) at 37 °C in an 5% CO_2 incubator. Twenty-four hours before transfection, 1×10^6 cells were seeded onto a 6-well plate in RPMI containing 10% FBS. EGFP-C1-IN expression vector and each of p*Silencer* construct or control plasmid were co-transfected into HeLa cells using Lipofectamine+Plus reagent (Invitrogen) in accordance with the manufacturer's instruction. Forty-eight hours after co-transfection, the expression of EGFP tagged IN was screened by means of fluorescence microscopic analysis and Western blot analysis.

*Corresponding author. Fax: +852 2603 7246.
E-mail address: chicheongwan@cuhk.edu.hk (D.C.C. Wan).

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2.4. Fluorescence microscopic analysis

For cellular localization of IN, the glass plate containing transfected cells were rinsed with phosphate-buffered saline, pH 7.5 (PBS). The glass plate was mounted on the stage adaptor (Model: SA-20L/Z) with growth medium. Fluorescence imaging was carried out using a Leica TCS-NT confocal microscope. For RNAi detection, fluorescence imaging was carried out using a Nikon UV microscope directly in a 6-well plate after washing with PBS and staining with DAPI.

2.5. Western blot

The transfected cells were rinsed with PBS and trypsinized. The cell pellet was collected by centrifugation at $5000 \times g$ for 5 min at 4 °C, washed with PBS twice and collected by centrifugation. The cell extract was prepared by lysing in 1% SDS and cleared by centrifugation at $15000 \times g$ for 5 min at 4 °C. The protein concentration of cell lysate was measured using the BCA™ protein assay kit (PIERCE) with bovine serum albumin as a standard in accordance with the manufacturer's instruction. An amount of 30 μg protein was subjected to 12% SDS–PAGE and then transferred to an immobilon-P transfer membrane (Millipore). After blocking with blocking buffer (5% non-fat milk in 1× Tris-Buffered Saline Tween-20 (20 mM Tris–Cl, 150 mM NaCl and 0.05% Tween-20)) for 1 h, membrane were incubated with EGFP antibody (BD Living Colors™ A.v. monoclonal antibody (JL-8)) or β-actin antibody (Sigma) overnight at 4 °C. After incubation with alkaline phosphates (AP) conjugated secondary antibodies and target protein was visualized with BCIP/NBT solution. Band intensity was measured using the AlphaEase™ FC Stand Alone program (Alpha Innotech Corpoation).

2.6. HIV replication analysis

The proviral construct bearing a luciferase reporter gene in place of the viral *nef* gene, pNL-Luc-E$^-$R$^+$ [18,19] was co-transfected with the selected HIV-1 IN shRNA expression vectors (3 μg), pSilencer 2.0-U6 negative control or pBluescript Tat shRNA positive control, in 293T cells using FuGENE 6 transfection reagent (Roche) [20]. After 48 h, HIV virus produced by the proviral construct was measured by ELISA (ZeoptoMetrix Corp., Buffalo, NY) against viral surface antigen p24^{Gag} released into the culture fluid. Gag viral proteins were detected by Western blotting using appropriate antibodies, HIV-1 Gag-p24 (V107) monoclonal antibody [21] and HIV-Gag polyclonal antibody (eENZYME) and was performed as mentioned in Section 2.5.

3. Results

3.1. Expression of active EGFP tagged IN in HeLa cells

To facilitate the screening of the RNAi effect, EGFP-IN expression vector was constructed to provide a reporter system for monitoring the RNAi function. Expression of EGFP-IN was analyzed by both fluorescence localization and immunoblotting (Supplementary 1). The expression of EGFP in HeLa cells was evenly distributed within the cells. In contrast, expression of EGFP-IN was preferentially localized in the nucleus. Globular protein larger than ~50 kDa is limited to import into nucleus through nuclear pore complexes (NPCs) by passive diffusion [22]. The immunoblot analysis for EGFP shows that the molecular weight of EGFP-IN fusion protein is ~60 kDa. The EGFP-IN localization demonstrated that active IN was expressed since nuclear localization signal (NLS) was present within IN at J161-I173 [23] to mediate the nuclear importation. The result indicates that The EGFP-IN localization in live cell imaging was considered a fast screening to demonstrate the shRNA against integrase.

3.2. Inhibition of HIV-1 integrase protein expression by shRNA expression constructs

Four different target regions were selected for the shRNA vectors construction and the locations of the four shRNA target sites in the HIV-1 IN are shown in Fig. 1A. Monitoring the amounts of fluorescence expressed in transfected HeLa cells were used as fast screening of the RNAi effect against IN. As shown in Fig. 1B, observable reduction of fluorescence was detected in HeLa cells transfected with p*Silencer* IN shRNA 1 and p*Silencer* IN shRNA 4 constructs compared with the p*Silencer* 2.0-U6 Control. In contrast, p*Silencer* IN shRNA 2 and p*Silencer* IN shRNA 3 had similar amounts of fluorescence. The RNAi effect was further confirmed by IN expression analysis using immunoblotting and the inhibition of each shRNA was calculated after measurement of the band intensity. The results are shown in Fig. 1C. Virtually no IN was detected and over 90% of inhibition was obtained in the cell lysate that expressed shRNA 2 and 4 (lanes 3 and 6). We further tested the effectiveness of both potent shRNA, shRNA 1 and 4, on EGFP-IN suppression by using various amounts of shRNA expression vector in the co-transfection. To compensate for the effect on transfection efficiency due to different total amounts of plasmid, p*Silencer* 2.0-U6 Control plasmid was used to keep the total amounts of plasmid at the same level. Through Western blot analysis, IN suppression by shRNA was obtained in a concentration-dependent manner and is shown in Fig. 2A. HIV-IN suppression was only detected by using 0.625–1.25 μg of p*Silencer* IN shRNA 1 (pEGFP-C1-HIV-IN: p*Silencer* ratio in 1:2.5–1.5), suppression was greatly reduced below this range of concentration of p*Silencer* used. Total suppression of HIV-IN was achieved at a concentration of p*Silencer* IN shRNA 4 as low as ~0.1 μg (pEGFP-C1_HIV-IN: p*Silencer* ratio in 1:0.6). Furthermore, shRNA 4 showed a higher effectiveness on IN suppression than that of shRNA 1 in the logarithmic plot in Fig. 2B. Since, a lower IC_{50} value was observed by using shRNA 4 (~400 ng) than that of shRNA 1 (~9.3 ng).

3.3. shRNA directed against IN suppresses single round cycle of HIV-1 replication

The potent IN shRNAs, shRNA 1 and 4, were chosen for further analysis to demonstrate their ability to suppress HIV replication. Two of the potent shRNA constructs with luciferase-expressing HIV-1 construct, pNL-Luc-E$^-$R$^+$ into 293T cells were co-transfected and the amount of p24^{Gag} antigen released into the culture fluid 48 h after transfection was measured. As shown in Fig. 3A, both of the control, pBluescript and pSilencer (lanes 2 and 4) only slightly affected virus production compared with that in proviral vector only (lane 1) and the effect of both are similar. High suppression of HIV synthesis was observed in the positive control of pBluescript Tat shRNA (lane 4) and showed ~80% reduction relative to the pBluescript control (lane 3). In the case of our two p*Silencer* constructs, it was found that both p*Silencer* IN shRNA 1 and p*Silencer* IN shRNA 4 constructs could inhibit new HIV-1 production in the range about 70–80% (lanes 5 and 6) compared to the untreated sample and IN shRNA 4 provided a better suppression of HIV replication than IN shRNA 1. The suppression of HIV synthesis using shRNA targeting IN region is only slightly weaker than that targeting tat. These results suggest that shRNA targeted against IN region significantly suppressed a single replication cycle of HIV-1.

In addition, we examined that whether the infection ability of newly formed virus is being reduced due to the suppression of HIV-IN after RNAi by infecting an equal amount of