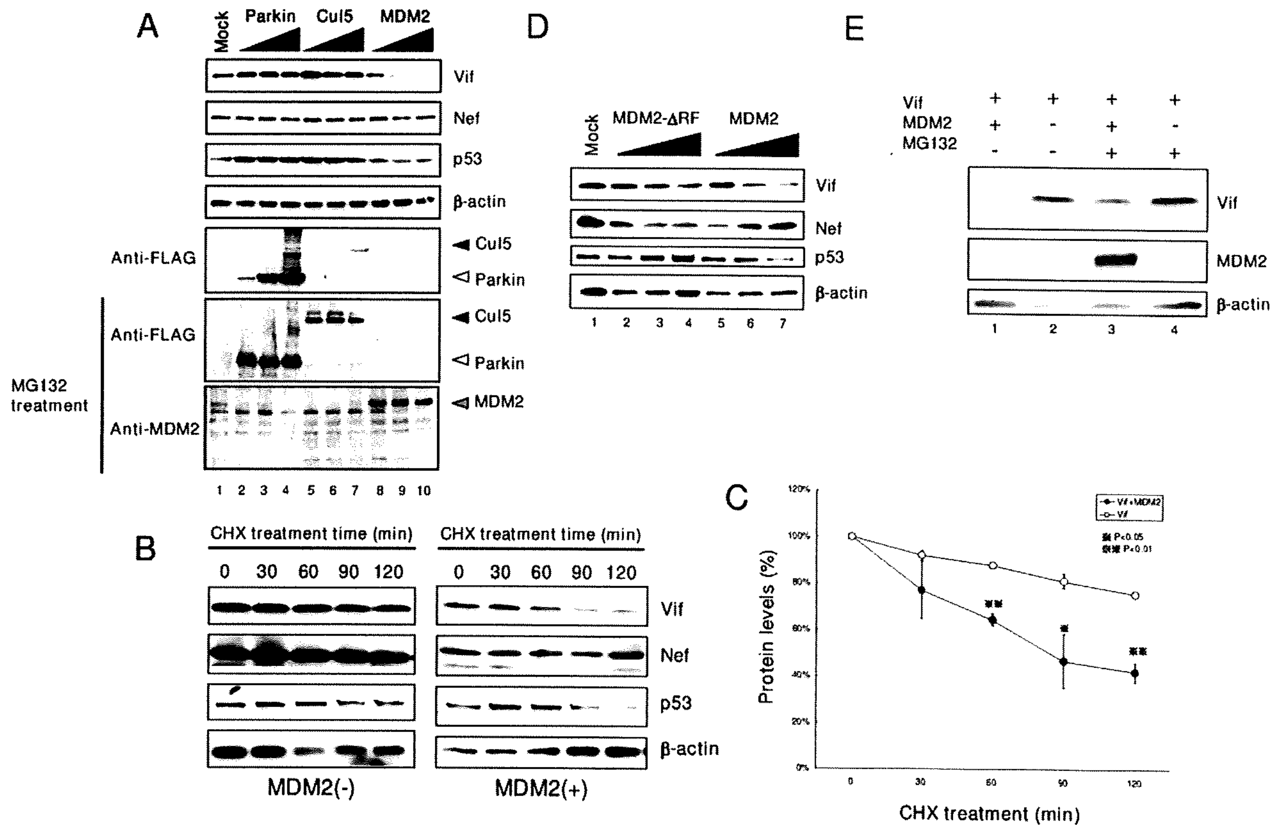


Figure 1
MDM2 downregulated cellular Vif levels in a proteasome dependent manner. (A) MDM2 reduced cellular levels of Vif as well as p53, but not that of Nef. HEK293T cells were cotransfected with expression vectors for the indicated E3 ligases and a subgenomic HIV-1 expression vector pNL-A1. Cell lysates were subjected to immunoblotting with the indicated Abs. We could not detect the expression of FLAG-MDM2 without MG132 treatment, because of a rapid degradation of MDM2. MG132 treatment enabled us to detect expression of MDM2 only with anti-MDM2 Ab, but not with anti-FLAG mAb. (B) Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX)(80 μg/ml) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs. (C) The amounts of Vif and Nef were quantified by densitometry, and Vif protein levels were calculated using Nef protein levels as normalizing loading controls and presented as percentage values relative to that without CHX treatment set as 100%. Values are presented as averages of three independent experiments. (D) MDM2 downregulated Vif, but a ΔRF mutant did not. HEK293T cells were cotransfected with expression vectors for MDM2 and the mutant together with pNL-A1, and cell lysates were subjected to immunoblotting with the indicated Abs. (E) $p53^{-/-}MDM2^{-/-}$ DKO-MEF cells were cotransfected with expression vectors for MDM2 and Vif, and treated with 10 μM MG132 for 6 hrs, and cell lysates were subjected to immunoblotting with the indicated Abs.

regulation of Vif by MDM2 was proteasome-dependent. Treatment with MG132 clearly restored the cellular Vif level that was downregulated by MDM2 (Fig. 1E, top panel, lane 3 as compared with lane 1), supporting that the MDM2-mediated downregulation of Vif was proteasome-dependent. Taken together, we concluded that MDM2 downregulates cellular Vif level by inducing its degradation in a proteasome-dependent manner.

MDM2 specifically binds and downregulates Vif

To further investigate the molecular link between MDM2 and Vif, we next examined the physical interaction of MDM2 with Vif. Immunoprecipitation assays showed that Vif was co-precipitated with MDM2 (Fig. 2A). Glutathione S-transferase (GST) pull-down assays showed that MDM2 was found in GST-Vif-bound, but not GST-bound, material (data not shown). Using a series of MDM2 deletion mutants, we determined that the central region of MDM2 (amino acids 168–320) was necessary for Vif binding (Fig. 2B, left panel &2C). To more precisely

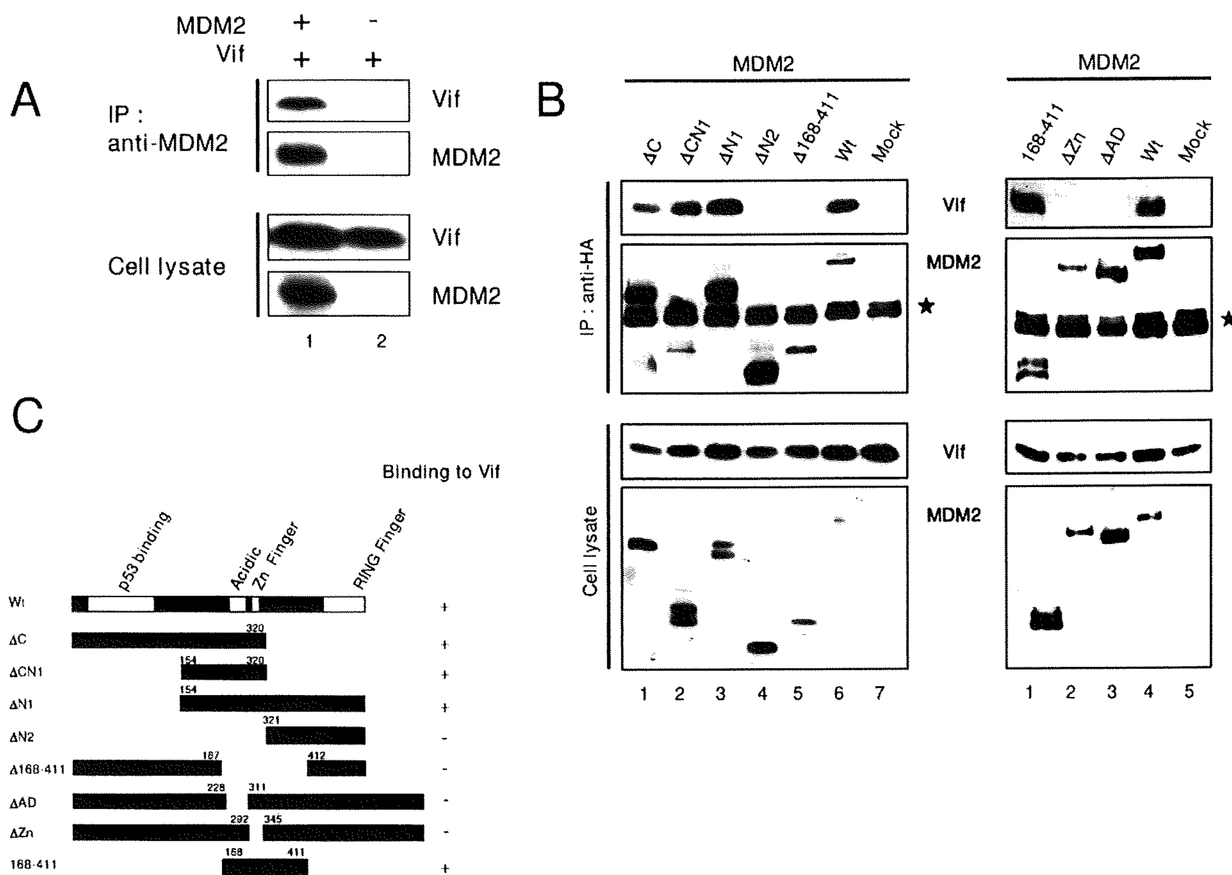


Figure 2
MDM2 bound Vif in its central domain. (A) Immunoprecipitation assays revealed the interaction of MDM2 with Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 and Vif and treated with MG132 for 6 hrs prior to harvest. Cell lysates were immunoprecipitated with anti-MDM2 mAb followed by immunoblotting with the indicated Abs (upper two panels). Cell lysates were also subjected to immunoblotting with the indicated Abs (lower two panels). (B) The interaction domain of MDM2 with Vif. HEK293T cells were cotransfected with expression vectors for HA-tagged MDM2 wild type (Wt) and mutants together with pNL-A1, and cell lysates were immunoprecipitated with anti-HA mAb followed by immunoblotting with the indicated Abs. Asterisk indicates immunoglobulin heavy chains from thenimmunoprecipitation. (C) Schematics of MDM2 mutants binding to Vif are shown.

determine a Vif-binding domain, we further tested mutants deleted in a Zn Finger domain (ΔZn) or in an acidic domain (ΔAD). Neither mutant could bind Vif, whereas the mutant containing amino acids 168–411 was able to bind Vif, suggesting that both domains are necessary and that the central domain is sufficient for Vif binding (Fig. 2B, right panel &2C). Additionally, using a series of Vif deletion mutants, we also found that the N-terminal region of Vif (amino acids 4–22) is needed for MDM2 binding (Fig. 3A &3C). Furthermore, we examined the MDM2-mediated downregulation of Vif mutants. MDM2 was able to efficiently downregulate cellular levels of the MDM2-binding Vif mutants but not that of an MDM2-non binding mutant, Δ4–45 (Fig. 3B). Collectively, these results indicated that the Vif-MDM2 interaction is required for MDM2-mediated downregulation of Vif (Fig. 3C).

MDM2 induces ubiqutination of Vif

Since we found that MDM2 bound Vif and promoted its degradation via a proteasomal pathway, we next examined whether MDM2 is involved in the polyubiquitination of Vif. *In vitro* ubiquitination assays revealed that bacterially expressed GST-MDM2 was able to induce the

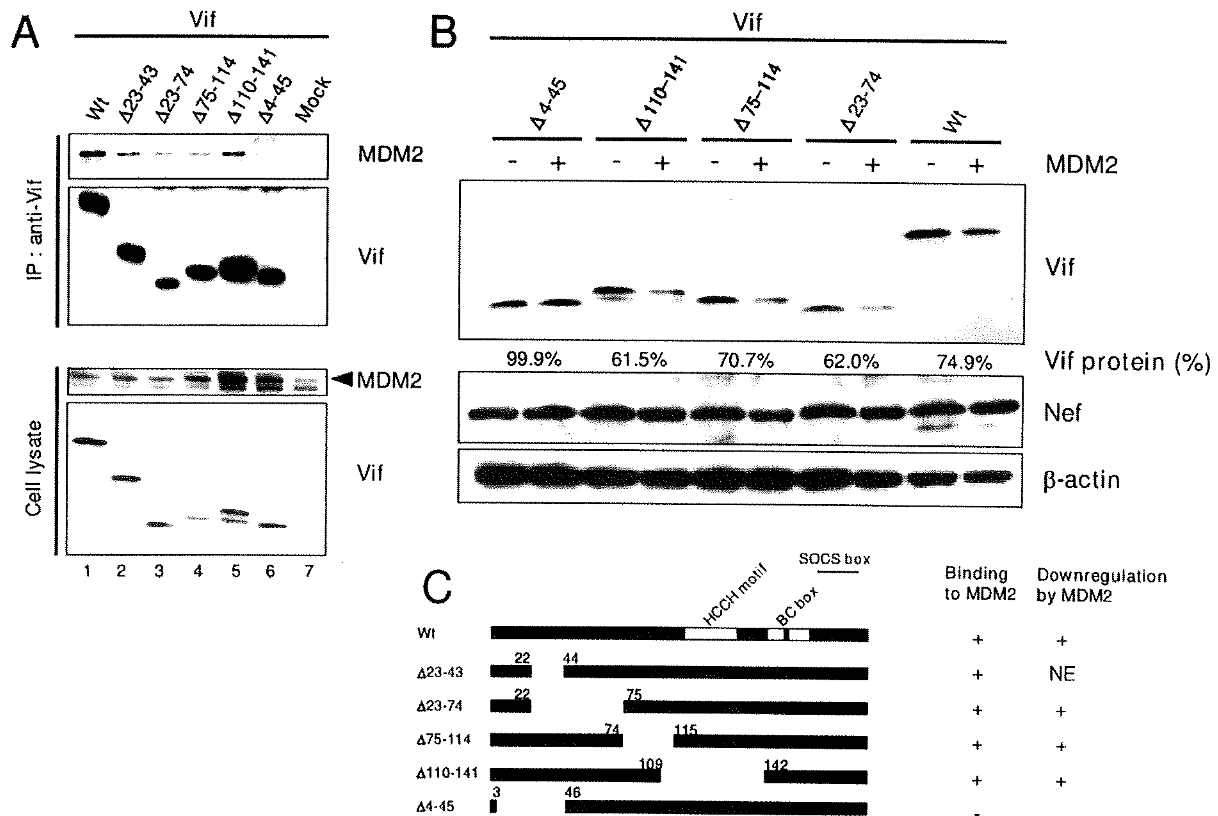


Figure 3
MDM2 specifically bound and downregulated Vif. (A) The interaction domain of Vif with MDM2. HEK293T cells were cotransfected with expression vectors for Vif and mutants together with pCMV/HA-MDM2, and cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with the indicated Abs. Arrowhead indicates MDM2. (B) The downregulation of Vif protein by MDM2. HEK293T cells were cotransfected with expression vectors for Vif and mutants with or without pCMV/HA-MDM2, and cell lysates were subjected to immunoblotting with the indicated Abs. The amounts of Vif were quantified by densitometry and shown as the protein ratio relative to that without expression of MDM2. (C) Schematics of Vif mutants bound by and downregulated by MDM2. NE: not examined.

polyubiquitination of purified GST-Vif protein *in vitro* (Fig. 4A). The ubiquitination of Vif by MDM2 was specific, as the omission of ubiqutin, E1, E2, or MDM2 prevented Vif-ubiquitination as shown in our previous experiments [13]. We also performed *in vitro* ubiquitination assays using immunopurified MDM2 and Cul5. Immunopurified MDM2 was able to induce ubiquitination of Vif *in vitro* to the same extent as Cul5 (Additional file 2, part A), while it could not ubiquitinate the N-terminal Vif deletion mutant Δ22 that was defective for binding MDM2 (Additional file 2, part B). These findings suggest that the interaction with MDM2 is important for Vif ubiquitination. We performed *in vivo* ubiquitination assays to further investigate the importance of MDM2 in Vif ubiquitination. Lysates of cells co-expressing Vif, either with an MDM2 wild type (Wt) or a ΔRF mutant, and His-tagged Ubiquitin (His-Ub) were analyzed for the presence of ubiquitinated Vif conjugates (Fig. 4B). Unfortunately, we detected a Vif band that non-specifically bound to Ni-NTA agarose (arrowhead) due to its nature as a sticky protein. Overexpression of MDM2 induced a ladder detected by anti-Vif Ab, even in the absence of His-Ub (lane 2), suggesting that this ladder represented Vif protein polyubiquitinated with endogenous Ub (arrows with asterisk). Furthermore, in the presence of His-Ub, we detected a doublet of ladder which presumably represented Vif protein polyubiquitinated with endogenous and His-tagged Ub (arrows with asterisk and arrows, respectively). We also obtained similar results using a UbiQapture™-Q Kit (data not shown). We thus concluded that the overexpres-

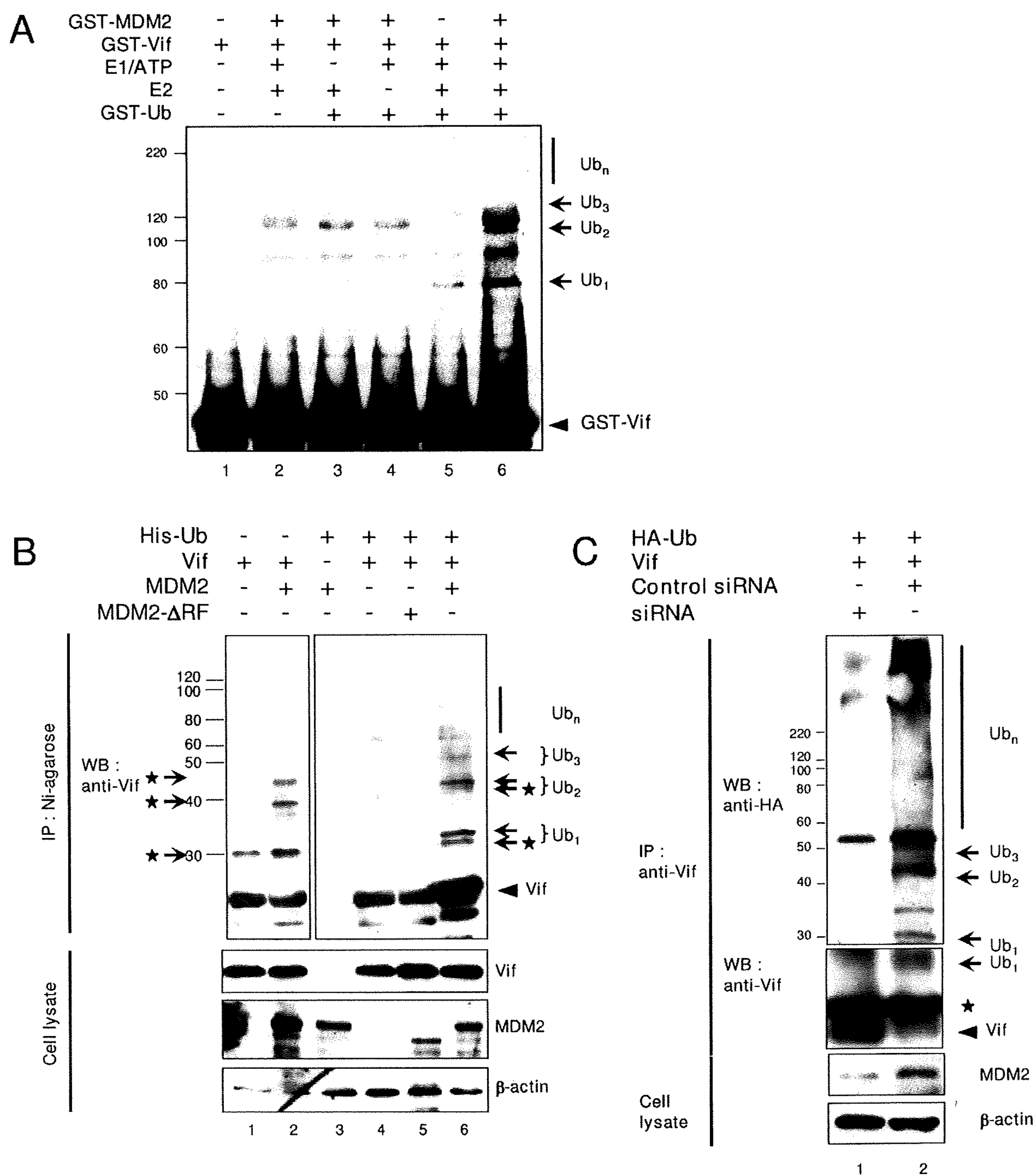


Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
MDM2 induced the polyubiquitination of Vif *in vitro* and *in vivo*. (A) GST-MDM2 induced the polyubiquitination of Vif *in vitro*. Bacterially expressed GST-Vif was subjected to *in vitro* ubiquitination assays. The reaction was performed in the presence or absence of E1, E2, GST-MDM2, and GST-Ubiquitin as indicated. Reactions were subjected to immunoblotting with anti-Vif mAb. Arrows indicate GST-ubiquitin-conjugated Vif. (B) Overexpressed MDM2 induced the polyubiquitination of Vif *in vivo*. HEK293T cells were cotransfected with expression vectors for MDM2 Wt and a ΔRF mutant together with expression vectors for Vif and His-Ubiquitin (His-Ub) as indicated. Cells were treated with MG132 for 6 hrs, and cell lysates were precipitated with Ni-NTA agarose beads followed by immunoblotting with the indicated Abs. Since Vif naturally bound to Ni-NTA agarose, we detected a Vif band itself (arrowhead), whereas no signal was detected in cells lacking Vif (lane 3). Arrows indicate His-Ub-conjugated Vif. Arrows with asterisk indicate Vif conjugated with endogenous ubiquitin. (C) Transduction of siRNA reduced cellular levels of endogenous MDM2 and polyubiquitination of Vif. HEK293T cells were cotransfected with expression vectors for MDM2 siRNA and control siRNA together with expression vectors for Vif and HA-Ubiquitin (HA-Ub). Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblotting with the indicated Abs. Asterisk indicates immunoglobulin light chains from the immunoprecipitation.

sion of exogenous MDM2 efficiently induced polyubiquitination of Vif *in vivo*. Furthermore, the knock-down of endogenous MDM2 expression by introduction of MDM2-specific short interfering RNA (siRNA) resulted in a significant reduction in the amount of polyubiquitinated Vif, commensurate with the extent of reduced MDM2 expression (Fig. 4C). Collectively, these data indicated that MDM2 mediates polyubiquitination of Vif both *in vitro* and *in vivo*.

MDM2 negatively regulates HIV-1 replication in non-permissive cells through ubiqutination and degradation of Vif

Next, we examined the effect of MDM2 on HIV-1 replication. In a single round infection assay (Fig. 5A), in the absence of A3G, viral replication was not affected by expression of MDM2 and/or Vif (lanes 1–6). In contrast, in the presence of A3G in a non-permissive cell setting, without the expression of MDM2, the wild type virus could replicate but the ΔVif virus could not, as previously reported (lanes 7 & 8) [3,8]. Co-expression of MDM2 reduced the cellular level of Vif (Fig. 5B, upper panel, lanes 5 & 11), resulting in the increased virion incorporation of A3G (Fig. 5B, 2nd lower panel, lane 11 as compared with lanes 7) and the greater suppression of viral replication (Fig. 5A, lane 11 as compared with lane 7).

We also tested the effect of MDM2 on HIV-1 replication in the presence of A3F. MDM2 suppressed viral replication in the presence of A3F, similar to results shown for A3G (Additional file 3). These data indicated that the MDM2-mediated Vif downregulation led to upregulated cellular A3G and A3F levels in producer cells, resulting in less infectious HIV-1 virions produced. Since MDM2 was previously reported to upregulate HIV-1 transcription by ubiquitination of Tat, we further examined HIV-1 replication in macrophages knocked down for MDM2 (Fig. 5C). We chose terminally differentiated macrophages as the target, because the knockdown of MDM2 is lethal for proliferating cells. HIV-1 replicated more efficiently in macrophages transfected with MDM2 siRNA than in control siRNA-transfected macrophages. These data indicated that MDM2 negatively regulated HIV-1 replication in non-permissive target cells through the ubiquitination and degradation of Vif.

To obtain further insights into the mechanisms why our MDM2 system did not induce the ubiquitination of A3G which was bound to Vif, we tested the expression levels and the binding affinity of A3G to Vif in transfected cells. Co-expression of MDM2 reduced the cellular levels of Vif and inversely increased the A3G levels in a dose dependent manner (Fig. 5D). Immunoprecipitation assays revealed that the co-expression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner (Fig. 5E). These data suggest that the interaction between MDM2 and Vif precludes A3G from binding to Vif.

Discussion

In this study, we report that MDM2 is a novel E3 ligase for HIV-1 Vif. MDM2 physically interacts with Vif and functions as an E3 ligase for Vif to induce its polyubiquitination and proteasomal degradation. Several E3 ligases including Cul5 [17], Nedd4, and AIP4 [18], have been reported to induce Vif ubiquitination, and the roles of Cul5 for Vif ubiquitination and degradation are especially well documented. Dang et al. have recently reported that Cul5 induces A3G degradation not by direct ubiquination of A3G but indirectly through Vif ubiqutination and that polyubiquitinated Vif might serve as a vehicle to transport A3G into proteasomes for degradation [23]. In this manuscript, we show that MDM2 only targets Vif for degradation but not A3G, although MDM2 and Cul5 both induce Vif ubiquitination (Additional file 2, part A). MDM2 reduced cellular Vif levels and inversely increased A3G levels (Fig. 5B &5D), unlike Cul5. One possible explanation is that the binding of MDM2 to Vif precluded A3G from binding Vif (Fig. 5E), whereas a Cul5-Vif complex

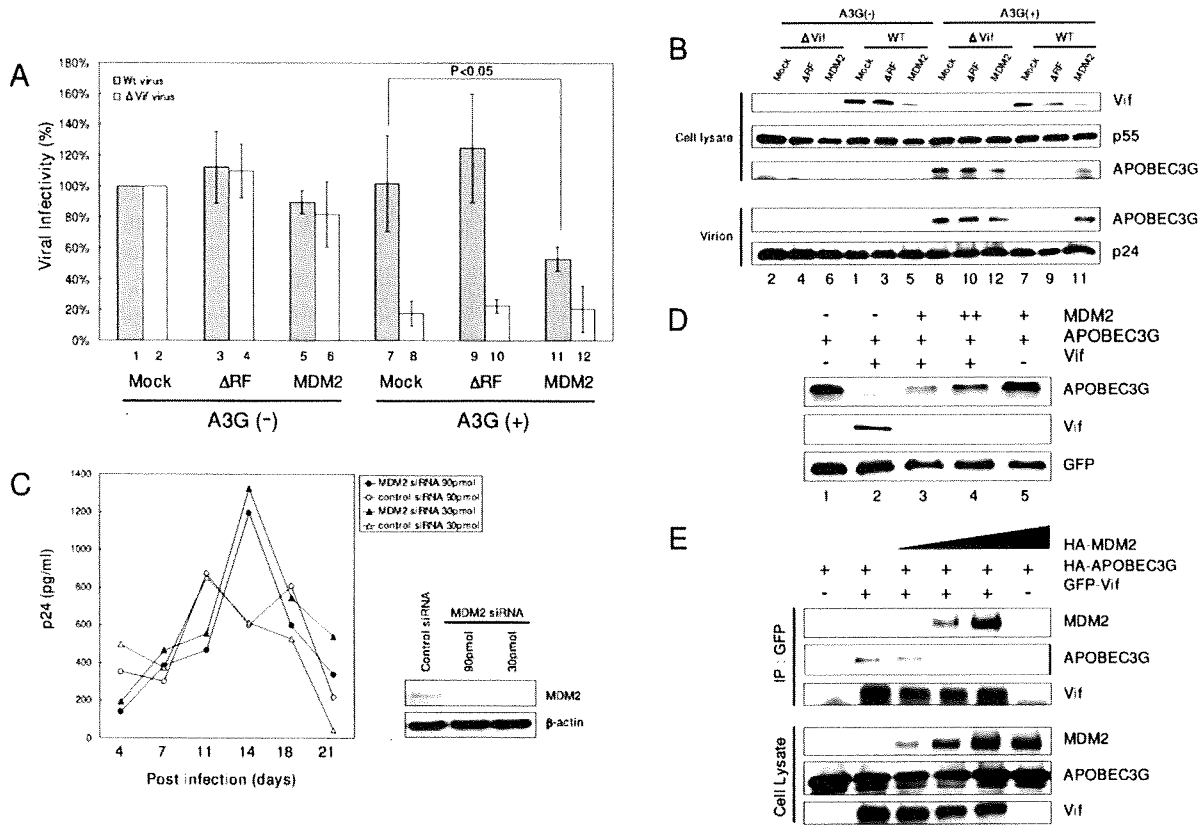


Figure 5
MDM2 negatively regulated HIV-1 replication in non-permissive cells through the degradation of Vif. (A) The overexpression of MDM2 inhibited HIV-1 replication in the presence of A3G. NL-43 Wt and ΔVif viruses were produced from HEK293T cells transfected with expression vectors for MDM2 Wt and a ΔRF mutant in the presence or absence of A3G. The viral infectivity was examined using M8166 cells. Values are presented as averages of more than 3 independent experiments. (B) MDM2 reduced cellular levels of Vif, resulting in more incorporation of A3G into HIV-1 virions. Immunoblotting for cell lysates (upper 3 panels) and precipitated virions (lower 2 panels) was performed with the indicated Abs. Lane numbers correspond to those in Fig. 4A. (C) HIV-1 replication in macrophages transfected with MDM2- and control-siRNA. MDM were transfected with MDM2- and control-siRNA and challenged with R5 HIV-1_{JR-FL} (left panel). Cell lysates were subjected to immunoblotting with the indicated antibodies (right panels). (D) Coexpression of MDM2 reduced cellular levels of Vif and inversely increased A3G levels in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, Vif, GFP, and MDM2 as indicated. Cell lysates were subjected to immunoblotting with the indicated Abs. (E) Immunoprecipitation assays revealed that the coexpression of MDM2 blocked the binding of A3G to Vif in a dose dependent manner. HEK293T cells were cotransfected with expression vectors for A3G, GFP-Vif, and MDM2 as indicated. Cell lysates were immunoprecipitated with anti-GFP mAb followed by immunoblotting with the indicated Abs.

can bind A3G to form a ternary complex. MDM2 binds the N-terminal region of Vif which does not overlap with, but is close to the A3G/A3F binding domain [25]. This binding might affect the interaction of Vif with A3G and/or A3F. Furthermore, the evidence that an MDM2 ΔRF mutant failed to protect A3G indicated that the ubiquitination and degradation of Vif is necessary to protect A3G and A3F from Vif. These findings suggest that different E3 ligases might play different roles in Vif ubiquitination. Further studies on the different roles of Vif ubiquitination by different E3 ligases and their virological significance should be investigated.

We demonstrate that MDM2 negatively regulated HIV-1 replication through Vif degradation. Through the degradation of target proteins (p53, pRB, etc), MDM2 can exert profound physiological effects on the regulation of cell cycle, cell proliferation, DNA repairs and other processes. To our knowledge, this is the first report to show that MDM2 plays an important role in viral replication

through the degradation of viral proteins. Recently, MDM2 was also reported to ubiquitinate HIV-1 Tat protein and activate its transcriptional activity in a non-proteolytic manner [26]. Our experiment using MDM2 knockdown macrophages showed that HIV-1 replication in these macrophages was more efficient than in control siRNA-transfected macrophages. These data are consistent with MDM2 negatively regulateing HIV-1 replication through Vif ubiquitination (Fig. 5C). However, the replication efficiency of HIV-1 in MDM2 knockdown macrophages was only 2-fold higher and was slower than in control siRNA-transfected macrophages. This suggests the possibilities that the ubiquitination of Tat might work as a positive regulatory factor at an earlier phase of infection and that MDM2 might be involved in both positive and negative regulation of HIV-1 replication at different stages. Further studies on the detailed effect of MDM2 on HIV-1 replication are needed.

We also demonstrated that Vif can bind MDM2 directly. We also mapped the interaction domain of MDM2 with Vif to amino acids 168–320 which is located in its central acidic and Zn finger domains. This central domain is different from the primary p53-binding site of MDM2 which is located in its N-terminal region; however, this central deomain was recently reported as a second p53-binding site and was shown to be important for the regulation of p53 stability [27-30] (Fig. 2B &2C). Interestingly, several proteins including p300, p14^{ARF}, and pRB bind to the central domain of MDM2 and regulate the stability and function of p53 via MDM2 [28,31]. Thus, it is possible that Vif might affect the stability and function of p53. Indeed, we confirmed that Vif can stabilize p53 (*Izumi et al., unpublished data*), which could explain why the effect of MDM2 on p53 degradation was weaker than that on Vif as shown in Fig. 1A. A further study is under way to elucidate this new function of Vif (*Izumi et al., HIV-1 Vif induces G2 cell cycle arrest via the p53 pathway, unpublished*).

Finally, expanding evidence suggests that the ubiquitination system plays important roles in many aspects of HIV-1 replication including the degradation of A3G by Vif [9-11], the degradation of CD4 by Vpu [32], HIV-1 viral budding [33], Tat-mediated transactivation [26], and Vpr-induced G2 cell cycle arrest [34,35]. The functional linkage between Vif and MDM2 also suggests that ubiquitin processes such as the A3G/Vif interplay is highly complex. It is obvious that HIV-1 replication in target CD4+ T cells is strongly affected by the interplay of these proteins. From the viral point of view, this interplay might give an advantage to HIV-1 replication. One possibility is that MDM2 regulates cellular Vif levels appropriately, such as not to affect viral replication [36] but just enough to antagonize A3G. Recent studies suggest that the G-to-A mutations induced by A3G may not be the mechanism by which A3G restricts or controls viral replication [37] and that a partially effective Vif inhibitor may actually accelerate the evolution of drug resistance and immune escape [38]. The inhibitory activity of MDM2 toward Vif could be partially effective and therefore could lead to viral evolution of drug resistance and immune escape. More recently, Nathans et al. have reported a small molecule that specifically antagonizes Vif function and inhibits viral replication by targeting the A3G/Vif axis. This compound enhances Vif degradation only in the presence of A3G, but does not induce A3G degradation and rather stabilizes A3G. They suggested the possibility of a new proteolytic enzyme for Vif degradation and that their new compound interferes with Vif interaction with a host protein in a Vif-A3G-host protein complex, thereby making Vif less stable. The precise biological significance of this Vif-A3G-host protein complex requires future elucidation. Nevertheless, modification or intervention of such Vif-A3G-host protein interplay could lead to the development of new therapeutic strategies for HIV-1 infection.

Conclusion

MDM2 is a novel E3 ligase for Vif which induces the polyubiquitination and degradation of Vif to negatively regulate HIV-1 replication.

Methods

Plasmid constructs

Expression vectors for hemagglutinin (HA)- or FLAG-tagged MDM2, pCMV4/HA-MDM2 or pCMV4/FLAG-MDM2, and their mutants were constructed as previously described [19]. An expression vector for HA-tagged human APOBEC3G, pcDNA3/HA-hA3G [39], and HIV-1 reporter plasmids, pNL43/Δenv-Luc (WT) and pNL43/ΔenvΔvif-Luc (ΔVif) [8], were constructed as previously described. Expression vectors for FLAG-tagged Parkin and Cul5 (pcDNA3/FLAG-Parkin and pcDNA3/FLAG-Cul5, respectively) were constructed by the PCR method. Complementary DNA for HIV-1 Vif was also cloned into pDON-AI (TAKARA BIO INC.) and pDON/EGFP for expression of Vif and EGFP-fused Vif (EGFP-Vif). The subgenomic expression vector pNL-A1, which expresses all HIV-1 proteins except for *gag* and *pol* products, and its mutants expressing Vif deletion mutants were kind gifts from Dr. K. Strebel [22].

Co-immunoprecipitation assays

We performed an immunoprecipitation assay for protein-protein interaction *in vivo*, as described previously [8]. HEK293T cells were cotransfected with pCMV4/HA-MDM2 and pNL-A1 by the calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH7.4/150 mM NaCl/1 mM $MgCl_2$/0.5% TritonX-100/10% Glycerol) and complexes were immunoprecipitated with anti-MDM2 monoclonal antibody

(mAb) (SMP-14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Ab-1, Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany) and Protein A-Sepharose beads (Amersham Biosciences Corp.) at 4°C. The beads were washed with RIPA buffer (50 mM Tris-HCl pH8.0/150 mM NaCl/1% Triton-X 100/0.1% SDS/0.1% DOC) and analyzed by immunoblotting with anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) [40] or anti-HA mAb (12CA5). To map the regions of MDM2 necessary for binding to Vif, HEK293T cells were cotransfected with expression vectors for a series of MDM2 deletion mutants together with pNL-A1. Complexes were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb. To map the regions of Vif necessary for binding to MDM2, HEK293T cells were cotransfected with expression vectors for a series of Vif deletion mutants together with pCMV4/HA-MDM2. Complexes were immunoprecipitated with anti-Vif mAb and analyzed by immunoblotting with anti-MDM2 mAb. In all these experiments, transfected cells were treated with MG132 for 6 hrs prior to harvesting in order to stabilize both Vif and MDM2; otherwise we could not detect the expression of MDM2 because of its rapid degradation, as seen in Fig. 1A.

In vitro and in vivo ubiquitination assays

In vitro ubiquitination assays were carried out in ubiquitin reaction buffer (50 mM Tris-HCl/2 mM ATP/5 mM $MgCl_2$/2 μM DTT) with E1(200 ng), E2(Ubc5c)(150 ng), and GST-tagged ubiquitin (GST-Ub) (10 μg) as described previously [13]. MDM2 and Vif were expressed as GST-fusion proteins in Escherichia coli strain DH5α and BL21, respectively. The reactions were incubated at 30°C for 90 min. The samples were subjected to immunoblotting with anti-Vif mAb to detect GST-ubiquitin conjugated Vif.

For *in vivo* ubiquitination assays, HEK 293T cells were cotransfected with plasmids expressing Vif, FLAG-MDM2 or its mutants, and His-tagged ubiquitin (His-Ub) as indicated. Cells were treated with 10 μM MG132 for 6 hrs prior to harvesting. Forty-eight hours post transfection, cell lysates were affinity-purified with Ni-NTA-agarose beads (Invitrogen corporation, Carlsbad, CA) and analyzed by immunoblotting with anti-Vif mAb.

For production of RNAi within the cells, we used the pSuper vector as described previously [19]. pSuper-MDM2-1 contained the 19 nt derived from the *mdm2* cDNA (nt 404–422) as the target sequence. Double-stranded RNA containing scrambled 19 nt was used as a control. HEK293T cells were transfected with pSuper plasmids together with plasmids expressing Vif and HA-Ub. Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblottimg with anti-HA mAb.

Single round infection assays with HIV-1 luciferase reporter virus

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/Δenv-Luc (Wt) or pNL43/ΔenvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or an expression vector for MDM2 or a mutant in the presence or absence of pcDNA3/hA3G by calcium phosphate as previously described [8]. The reporter viruses were adjusted according to p24 values and used to infect M8166 target cells. Productive infection was measured by luciferase activity and values were presented as percent infectivity relative to the value of each virus without the expression of hA3G.

Knockdown of MDM2 in macrophages and replication assays

Monocyte-derived macrophages (MDM) were cultured for 7 days from CD14+ monocytes isolated from the peripheral blood of an HIV-1-negative healthy individual. Electroporation with Stealth Select RNAi for MDM2 or Control (Invitrogen Corporation) was performed using the Nucleofector machine (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's instructions. Twenty four hours after transfection, MDM were challenged with R5 HIV-1_{JR-FL} at multiplicity of infection of 0.1 at 37°C for 3 hrs. The cells were cultured from day 4 to 21 after infection, and the concentration of p24 antigen in the supernatant was measured with an HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA] kit (ZeptMetrix, Buffalo, NY).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TI. designed research, performed research, contributed vital new reagents, analyzed data, and wrote the paper. ATK designed research, analyzed data, wrote the paper, and organized the research. KS, KIo, and MM prepared the materials and performed a part of the research. KIwai, HK, TS, MT, SI., and HA contributed vital new reagents. YK contributed vital new reagents, performed a part of the research, and analyzed the data. HH, KItoh, and JF designed the research, contributed vital new reagents, and analyzed the data. TU analyzed the data, drafted the paper, and organized the research.

Additional material

Additional file 1

Supplementary figure 1 – the stability of Vif protein in p53-/- MEF and p53-/-MDM2-/- MEF cells. MEF cells were transfected with pDON/Vif or pcDNA3/HA-A3G. Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-6-1-S1.pdf]

Additional file 2

Supplementary figure 2 – immunopurified MDM2 induced the polyubiquitination of Vif in vitro. *(A) MDM2 as well as Cul5 induced the polyubiquitination of Vif. HEK293T cells were transfected with expression vectors for His-MDM2 and His-Cul5. His-tagged proteins were purified using Ni-NTA agarose and subjected to* in vitro *ubiquitination assays as described in a legend to Fig. 4A. Reactions were subjected to immunoblotting with anti-Vif Ab. Arrows indicate GST-Ub-conjugated Vif. Asterisks indicate non-specific bands associated with GST-Vif protein recognized by anti-Vif Ab, as they are seen in lanes 1 and 3. (B) MDM2 induced the polyubiquitination of Vif Wt but not that of Δ22 that was defective for binding MDM2. Filled asterisks indicate non-specific bands associated with GST-Vif protein, while white asterisks indicate those associated with GST-Vif Δ22.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-6-1-S2.pdf]

Additional file 3

Supplementary figure 3 – the overexpression of MDM2 inhibited HIV-1 replication in the presence of A3F. Single round infection assays were performed in the presence or absence of A3F as described in a legend to Fig. 5A. Values are presented as averages of more than 3 independent experiments.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-6-1-S3.pdf]

Acknowledgements

We thank Drs. K. Strebel for the pNL-A1 plasmid and its derivative mutants, D. P. Lane for p53$^{-/-}$MDM2$^{-/-}$ DKO-MEF, and M. Malim for the anti-Vif mAb (#319) through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This study was partly supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, from the Ministry of Health, Labour and Welfare, Japan, from the Naito Foundation, and from Mitsubishi Pharma Research Foundation.

References

1. Goff SP: **Retrovirus restriction factors.** *Mol Cell* 2004, **16**:849-859.
2. Towers GJ: **The control of viral infection by tripartite motif proteins and cyclophilin A.** *Retrovirology* 2007, **4**:40.
3. Sheehy AM, Gaddis NC, Choi JD, Malim MH: **Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein.** *Nature* 2002, **418**:646-650.
4. Goila-Gaur R, Strebel K: **HIV-1 Vif, APOBEC, and intrinsic immunity.** *Retrovirology* 2008, **5**:51.
5. Mangeat B, Turelli P, Caron G, Friedli M, Perrin L, Trono D: **Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts.** *Nature* 2003, **424**:99-103.
6. Harris RS, Bishop KN, Sheehy AM, Craig HM, Petersen-Mahrt SK, Watt IN, Neuberger MS, Malim MH: **DNA deamination mediates innate immunity to retroviral infection.** *Cell* 2003, **113**:803-809.
7. Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L: **The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA.** *Nature* 2003, **424**:94-98.
8. Shindo K, Takaori-Kondo A, Kobayashi M, Abudu A, Fukunaga K, Uchiyama T: **The enzymatic activity of CEM15/Apobec-3G is essential for the regulation of the infectivity of HIV-1 virion but not a sole determinant of its antiviral activity.** *J Biol Chem* 2003, **278**:44412-44416.
9. Marin M, Rose KM, Kozak SL, Kabat D: **HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation.** *Nat Med* 2003, **9**:1398-1403.
10. Sheehy AM, Gaddis NC, Malim MH: **The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif.** *Nat Med* 2003, **9**:1404-1407.
11. Stopak K, de Noronha C, Yonemoto W, Greene WC: **HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability.** *Mol Cell* 2003, **12**:591-601.
12. Yu X, Yu Y, Liu B, Luo K, Kong W, Mao P, Yu XF: **Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex.** *Science* 2003, **302**:1056-1060.
13. Kobayashi M, Takaori-Kondo A, Miyauchi Y, Iwai K, Uchiyama T: **Ubiquitination of APOBEC3G by an HIV-1 Vif-Cullin5-Elongin B-Elongin C Complex Is Essential for Vif Function.** *J Biol Chem* 2005, **280**:18573-18578.
14. Zheng Y-H, Irwin D, Kurosu T, Tokunaga K, Sata T, Peterlin BM: **Human APOBEC3F Is Another Host Factor That Blocks Human Immunodeficiency Virus Type 1 Replication.** *J Virol* 2004, **78**:6073-6076.
15. Shirakawa K, Takaori-Kondo A, Kobayashi M, Tomonaga M, Izumi T, Fukunaga K, Sasada A, Abudu A, Miyauchi Y, Akari H: **Ubiquitination of APOBEC3 proteins by the Vif-Cullin5-ElonginB-ElonginC complex.** *Virology* 2006, **344**:263-266.
16. Fujita M, Akari H, Sakurai A, Yoshida A, Chiba T, Tanaka K, Strebel K, Adachi A: **Expression of HIV-1 accessory protein Vif is controlled uniquely to be low and optimal by proteasome degradation.** *Microbes Infect* 2004, **6**:791-798.
17. Mehle A, Goncalves J, Santa-Marta M, McPike M, Gabuzda D: **Phosphorylation of a novel SOCS-box regulates assembly of the HIV-1 Vif-Cul5 complex that promotes APOBEC3G degradation.** *Genes Dev* 2004, **18**:2861-2866.
18. Dussart S, Courcoul M, Bessou G, Douaisi M, Duverger Y, Vigne R, Decroly E: **The Vif protein of human immunodeficiency virus type 1 is posttranslationally modified by ubiquitin.** *Biochem Biophys Res Commun* 2004, **315**:66-72.
19. Higashitsuji H, Itoh K, Sakurai T, Nagao T, Sumitomo Y, Masuda T, Dawson S, Shimada Y, Mayer RJ, Fujita J: **The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53.** *Cancer Cell* 2005, **8**:75-87.
20. Honda R, Tanaka H, Yasuda H: **Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53.** *FEBS Lett* 1997, **420**:25-27.
21. Yu Y, Xiao Z, Ehrlich ES, Yu X, Yu X-F: **Selective assembly of HIV-1 Vif-Cul5-ElonginB-ElonginC E3 ubiquitin ligase complex through a novel SOCS box and upstream cysteines.** *Genes Dev* 2004, **18**:2867-2872.
22. Strebel K, Daugherty D, Clouse K, Cohen D, Folks T, Martin MA: **The HIV 'A' (sor) gene product is essential for virus infectivity.** *Nature* 1987, **328**:728-730.
23. Dang Y, Siew LM, Zheng YH: **APOBEC3G is degraded by the proteasomal pathway in a Vif-dependent manner without being polyubiquitylated.** *J Biol Chem* 2008, **283**:13124-13131.
24. Honda R, Yasuda H: **Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase.** *Oncogene* 2000, **19**:1473-1476.
25. He Z, Zhang W, Chen G, Xu R, Yu XF: **Characterization of conserved motifs in HIV-1 Vif required for APOBEC3G and APOBEC3F interaction.** *J Mol Biol* 2008, **381**:1000-1011.
26. Brès V, Kiernan RE, Linares LK, Chable-Bessia C, Plechakova O, Tréand C, Emiliani S, Peloponese JM, Jeang KT, Coux O, Scheffner M, Benkirane M: **A non-proteolytic role for ubiquitin in Tat-medi-**

ated transactivation of the HIV-1 promoter. *Nat Cell Biol* 2003, **5**:754-761.
27. Argentini M, Barboule N, Wasylyk B: **The contribution of the acidic domain of MDM2 to p53 and MDM2 stability.** *Oncogene* 2001, **20**:1267-1275.
28. Iwakuma T, Lozano G: **MDM2, an introduction.** *Mol Cancer Res* 2003, **1**:993-1000.
29. Kawai H, Wiederschain D, Yuan ZM: **Critical contribution of the MDM2 acidic domain to p53 ubiquitination.** *Mol Cell Biol* 2003, **23**:4939-4947.
30. Meulmeester E, Frenk R, Stad R, de Graaf P, Marine JC, Vousden KH, Jochemsen AG: **Critical role for a central part of Mdm2 in the ubiquitylation of p53.** *Mol Cell Biol* 2003, **23**:4929-4938.
31. Ganguli G, Wasylyk B: **p53-independent functions of MDM2.** *Mol Cancer Res* 2003, **1**:1027-1035.
32. Margottin F, Bour SP, Durand H, Selig L, Benichou S, Richard V, Thomas D, Strebel K, Benarous R: **A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif.** *Mol Cell* 1998, **1**:565-574.
33. Freed EO: **Viral late domains.** *J Virol* 2002, **76**:4679-4687.
34. Wen X, Duus KM, Friedrich TD, de Noronha CM: **The HIV1 protein Vpr acts to promote G2 cell cycle arrest by engaging a DDB1 and Cullin4A-containing ubiquitin ligase complex using VprBP/DCAF1 as an adaptor.** *J Biol Chem* 2007, **282**:27046-27057.
35. Schrofelbauer B, Hakata Y, Landau NR: **HIV-1 Vpr function is mediated by interaction with the damage-specific DNA-binding protein DDB1.** *Proc Natl Acad Sci USA* 2007, **104**:4130-4135.
36. Akari H, Fujita M, Kao S, Khan MA, Shehu-Xhilaga M, Adachi A, Strebel K: **High level expression of human immunodeficiency virus type-1 Vif inhibits viral infectivity by modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site.** *J Biol Chem* 2004, **279**:12355-12362.
37. Ulenga NK, Sarr AD, Hamel D, Sankale JL, Mboup S, Kanki PJ: **The level of APOBEC3G (hA3G)-related G-to-A mutations does not correlate with viral load in HIV type 1-infected individuals.** *AIDS Res Hum Retroviruses* 2008, **24**:1285-1290.
38. Pillai SK, Wong JK, Barbour JD: **Turning up the volume on mutational pressure: is more of a good thing always better? (A case study of HIV-1 Vif and APOBEC3).** *Retrovirology* 2008, **5**:26.
39. Kobayashi M, Takaori-Kondo A, Shindo K, Abudu A, Fukunaga K, Uchiyama T: **APOBEC3G Targets Specific Virus Species.** *J Virol* 2004, **78**:8238-8244.
40. Simon JH, Southerling TE, Peterson JC, Meyer BE, Malim MH: **Complementation of vif-defective human immunodeficiency virus type 1 by primate, but not nonprimate, lentivirus vif genes.** *J Virol* 1995, **69**:4166-4172.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

BioMedcentral

Virus Genes (2009) 39:165–175
DOI 10.1007/s11262-009-0382-x

Efficient transduction of cytotoxic and anti-HIV-1 genes by a gene-regulatable lentiviral vector

Yasuhiko Shinoda · Kuniko Hieda · Yoshio Koyanagi · Youichi Suzuki

Received: 18 April 2009 / Accepted: 12 June 2009 / Published online: 25 June 2009
© Springer Science+Business Media, LLC 2009

Abstract Lentiviral vectors modified from human immunodeficiency virus type 1 (HIV-1) offer a promising approach for gene therapy, facilitating transduction of genes into non-dividing cells both in vitro and in vivo. When transducing cytotoxic or anti-HIV genes, however, the vector must avoid self-inhibition by the transgene that can lead to a disruption in production of infectious virions. In this study, we constructed two HIV-1-based lentiviral vectors harboring the mifepristone-inducible gene expression unit in either the forward or the reverse orientation with respect to the direction of viral genomic RNA. The ability of these vectors to transduce cytotoxic and anti-HIV genes was evaluated. When human CD14 was used as a transgene, infectious lentiviral vectors were produced by both forward and reverse vector systems. CD14 expression was efficiently induced in cells transduced by both lentiviral vectors following treatment with mifepristone. However, a higher level of basal transgene expression was observed in the forward vector system in the absence of mifepristone. In contrast, high titers of infectious lentiviral vector containing the cytotoxic *vesicular stomatitis virus M* gene were successfully generated using the reverse vector, but not the forward vector. In addition, when a VPS4B-dominant negative mutant against HIV-1 budding was cloned into the reverse vector, significant amounts of lentiviral vector were obtained. Subsequent transduction of cells with the VPS4B mutant resulted in approximately 50% inhibition of HIV-1 production only in the presence of mifepristone. Our study thus demonstrates that incorporation of a mifepristone-regulatable gene expression unit in the reverse orientation makes significant advances toward development of a lentiviral vector that allows transduction of harmful genes.

Keywords Lentiviral vector · Mifepristone-regulatable system · VSV M · VPS4B

Y. Shinoda · K. Hieda · Y. Koyanagi
Laboratory of Viral Pathogenesis, Research Center for AIDS, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan

Y. Suzuki (✉)
Laboratory for Host Factors, Center for Emerging Virus Research, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan
e-mail: ysuzuki@virus.kyoto-u.ac.jp

Present Address:
K. Hieda
Microbiological Research Institute, Otsuka Pharmaceutical Co., Ltd, Kawauchi-cho, Tokushima 771-0192, Japan

Introduction

A variety of gene-transfer vectors based on RNA and DNA viruses have been developed to deliver foreign genes to target cells in vitro and in vivo [1]. Retroviral vectors derived from gammaretroviruses and lentiviruses have the potential advantage of sustained expression of transgenes in transduced cells, because of their ability to stably integrate viral DNA into the host genome. While gammaretroviruses require cell division to establish infections, lentiviruses including human immunodeficiency virus type 1 (HIV-1) are capable of infecting both dividing and non-dividing cells [1]. Lentivirus-based vector systems thus potentiate long-term gene expression in non-dividing cells such as neurons and hematopoietic stem cells [2, 3].

Lentiviral vectors hold great promise for a gene therapy approach to inherited and acquired diseases such as cancer and acquired immunodeficiency syndrome (AIDS).

A potential application of lentiviral vectors would be the transduction of a gene cytotoxic to tumor cells or virus-infected cells resulting in the eradication of these unwanted cells from the body. For the treatment of AIDS, an alternative approach would be to deliver an anti-HIV gene to a population of cells rendering them resistant to HIV infection. However, insertion of toxic or anti-HIV genes into an HIV-based lentiviral vector can create problems for production of the vector itself. Expression of anti-HIV transgenes in vector packaging cells can interfere with production of lentivirus particles, blocking the ability to make lentiviral vector [4]. One strategy to solve this problem is the use of a regulatable system in which the target transgene is kept silent during vector production and expression is subsequently switched on following transduction in the context of a lentiviral vector.

The first generation of regulatable gene expression systems was based on naturally occurring inducible promoters [5]. However, this type of system had limitations due to high levels of "leaky" or basal expression driven by such promoters, modest induction of transgene expression, and pleiotropic activity of the inducer. For these reasons, the last two decades have seen development of chimeric regulatable systems engineered from a number of prokaryotic, eukaryotic, and viral elements designed to enhance specificity and activity of transgene expression [6]. Amongst the reported chimeric regulators is one based on a mutated human progesterone receptor which is unable to bind endogenous hormone but is activated by binding the progesterone antagonist, mifepristone (RU-486) [7, 8]. The chimeric transactivator (regulator) protein of this so-called GeneSwitch system comprises the GAL4 DNA-binding domain from *Saccharomyces cerevisiae* fused to the ligand-binding domain of a mutant progesterone receptor and the activation domain of the p65 subunit of human NF-κB [8]. In the presence of mifepristone, this transactivator binds to GAL4 activation sequences upstream of the inducible transgene, stimulating transcription of the target gene by more than 200-fold in cultured cells [9]. An advantage of the GeneSwitch system is that the majority of its components are modified human proteins having no impact on cell viability. In addition, although mifepristone has anti-progesterone and -glucocorticoid activities, the concentration needed for ligand-inducible transactivation of the target gene (10^{-8} to 10^{-11} M) is much lower than the concentration producing an anti-progesterone effect in humans [10]. Furthermore, use of a mifepristone-inducible (autoinducible) promoter to regulate expression of the chimeric transactivator dramatically reduced basal expression of the transgene in the absence of the inducer, thereby improving the dynamic range of in vivo transgene regulation [9].

Many types of regulatable gene expression systems have been incorporated into lentiviral vectors [11–21]. The most commonly used inducible system is based on the bacterial tetracycline-responsible gene expression system (Tet system) [22]. While representing an important tool for controlling target gene expression, Tet-regulatable systems in the context of lentiviral vectors have shown high basal levels of transgene expression without induction [22]. Such leakiness would be undesirable especially in the production of lentiviral vectors aimed at transducing toxic proteins into target cells.

To generate a viral vector in which transgene expression was tightly controlled, we combined an HIV-1-based lentiviral vector with the GeneSwitch system described above. The mifepristone-inducible lentiviral vector reported here minimized the interference of transgene expression during virus production and permitted efficient transduction of cytotoxic and anti-HIV genes into target cells.

Materials and methods

Cells

GeneSwitch-293 cells (a HEK293-derived human cell line expressing the GeneSwitch protein) were purchased from Invitrogen. GeneSwitch-293 and human 293T cells were maintained in Dulbecco's modified Eagle medium (D-MEM) containing 10% fetal calf serum (FCS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (D-MEM/10% FCS).

Construction of lentiviral vector plasmids

The mifepristone-inducible promoter sequence, GAL4/TATA, was amplified by polymerase chain reaction (PCR) from the vector pGene/V5-His5C (Invitrogen) and subcloned into LITMUS28 (New England Biolabs) to create pLITMUS28-GAL4/TATA. A GAL4/TATA fragment was then inserted into a Gateway-compatible lentiviral vector plasmid pYK005C [23] producing the plasmid for the forward vector (fragment positioned in the forward orientation).

To generate a plasmid for the reverse vector, a fragment containing the Gateway cloning cassette, internal ribosome entry site (IRES), and humanized *Renilla* green fluorescent protein (hrGFP) sequences together with a fragment containing bovine growth factor hormone poly(A) (BGH pA) sequence were isolated from pYK005C and pSwitch (Invitrogen), respectively. These were subcloned into LITMUS28. Then, the GAL4/TATA sequence derived from pLITMUS28-GAL4/TATA was inserted into the subcloning plasmid. To prepare a vector backbone, the internal human

elongation factor 1 α subunit promoter of a self-inactivating (SIN) lentiviral vector plasmid, CSII-EF-MCS [24], was replaced with a fragment containing GAL4/TATA, Gateway-cassette, IRES, hrGFP, and BGH pA sequences, thus generating the final plasmid.

Preparation of all Gateway plasmids containing the *ccdB* gene was carried out using *Escherichia Coli* (*E. Coli*) strain DB3.1.

Cloning of transgenes

All transgenes were cloned into the lentiviral vector plasmid via the Gateway cloning system [23]. Entry plasmid clones encoding transgenes were constructed as follows. Human CD14 was amplified by PCR using primers containing the *att*B1 tail (5′-GGGGACAAGTTTGTACAAAAAAGCA GGCT-3′) at the 5′-end of the forward primer and the *att*B2 tail (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′) at the 5′-end of the reverse primer [23]. PCR products were subcloned into the entry plasmid, pDONR201 (Invitrogen), using a Gateway BP reaction [23]. Vesicular stomatitis virus matrix protein (VSV M) cDNA fused with a FLAG epitope tag sequence (FLAG-VSV-M) was generated from a VSV M-expressing plasmid, pEGFPN3-M [25]. This involved use of a forward primer containing the *att*B1 tail and subsequent FLAG tag sequence (5′-ATGGATTACAAGGATGACGA CGATAAG-3′) and a reverse primer containing the *att*B2 tail. The PCR fragment encoding FLAG-VSV-M was subcloned into entry plasmid, pDONR221 (Invitrogen), by Gateway BP reaction. Fragments encoding the VPS4B K180Q mutant (VPS4B-KQ) or firefly luciferase (Luc) were amplified by PCR from VPS4B K180Q-expressing plasmid [26] and pGL3-Basic (Promega), respectively. These fragments were inserted into *Eco*RI-*Mlu*I sites downstream of three FLAG epitope tags in pCMV-SPORT6-3xFLAG, a Gateway-compatible pCMV-SPORT6 (Invitrogen)-derived entry plasmid. After sequence confirmation, individual transgenes in entry plasmids were transferred to lentiviral vector plasmids by Gateway LR reaction.

Lentiviral vector production

293T cells were seeded to appropriate densities 20 h prior to transfection. Infectious lentiviral vectors pseudotyped with VSV G protein were produced by lentiviral vector plasmid, VSV G- and HIV-1 Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev), and HIV-1 Gag-Pol-expressing plasmid (pCAG-HIVgp) via calcium phosphate-mediated transfection, as described previously [27]. Conditioned medium was harvested 48-h post-transfection and concentrated 40-fold by ultracentrifugation at 4°C at 100,000×*g* for 90 min.

For titration of the lentiviral vectors, GeneSwitch-293 cells were infected with serial dilutions of vector stocks supplemented with 10 nM mifepristone (Invitrogen) 24-h post-infection. Vector titers (transduction units: TU) were determined 48 h after induction by quantitative flow cytometric analysis for hrGFP positive cells.

In the following text, the use of "p" and "v" to prefix nomenclature denotes vector plasmid and infectious lentiviral vector, respectively.

Transduction and induction of transgenes

GeneSwitch-293 cells were seeded in 6-well plates at a density of 1×10^5 cells/well with D-MEM/10% FCS 20 h prior to infection. Cells were then exposed to lentiviral vectors for 24 h at multiplicities of infection (MOI) of 0.1 (for vF-CD14 and vR-CD14) or 5 (for vR-VSV-M). To induce transgene expression, culture medium was replaced with D-MEM/10% FCS containing 10 nM mifepristone and cells were analyzed 48 h (vF-CD14 and vR-CD14 transductions) or 24 h (for vR-VSV-M transduction) later.

Flow cytometric analysis

Transduced cells were incubated with anti-human CD14 mouse monoclonal antibody (61D3, eBioscience) for 20 min at 4°C and then stained with Cy5-conjugated anti-mouse IgG donkey polyclonal antibody (Chemicon International Inc.) for a further 20 min at 4°C. Data were collected using the FACScalibur system (BD Bioscience) and analyzed with WinMDI software.

Western blotting analysis

Cells were lysed in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, 0.9% β-mercaptoethanol). Boiled samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilion Transfer Membranes (Millipore). Primary antibodies used were (i) anti-FLAG mouse monoclonal IgG (M2, Sigma) to detect FLAG-VSV-M; (ii) biotinylated anti-FLAG mouse monoclonal IgG (BioM2, Sigma) to detect FLAG-VPS4B-KQ and FLAG-Luc; and (iii) anti-α-tubulin mouse monoclonal IgG (DM1A, Sigma) to detect α-tubulin. Biotinylated anti-mouse IgG (BA-2000, Vector Laboratories) was then used for the detection of FLAG-VSV-M. Proteins were detected using horseradish peroxidase (HRP)-conjugated streptavidin (ZYMED Laboratories, for FLAG-tagged proteins) and HRP-conjugated anti-mouse IgG (Cell Signaling, for α-tubulin) using a Western Lightning Chemiluminescence Reagent Plus

(PerkinElmer). Signals were analyzed using a luminescence image analyzer, LAS-3000mini (Fujifilm).

Microscopy analysis

293T cells transfected with pF-VSV-M and pR-VSV-M were examined by light and fluorescent microscopy using an Eclipse TS100 microscope (Nikon) at the point of harvesting lentiviral vector. Images were obtained with a DFC480 digital camera and IM500 image manager software (Leica Microsystems).

To detect FLAG-VSV-M, transduced cells were fixed with 4% paraformaldehyde and incubated with PBS containing 5% normal goat serum and 0.05% Triton X-100 at room temperature for 1 h. This was followed by incubation with anti-FLAG-mouse monoclonal IgG (M2) at 4°C overnight. Samples were then incubated with Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen) at room temperature for 1 h and nuclei were stained using Hoechst 33342 (Invitrogen). Images were obtained using a CTR 6500 fluorescent microscope and FW4000 software (Leica Microsystems).

Assay for HIV-1 release from VPS4B mutant-transduced cells

GeneSwitch-293 cells were seeded at a density of 1×10^3 cells/well in a 96-well plate 20 h prior to infection and exposed to vR-VPS4B-KQ and vR-Luc at a MOI of 1 for 24 h. Transduced cells were then expanded for 4–5 weeks and reseeded at a density of 4×10^6 cells in a 15-cm diameter dish 20 h prior to induction. A portion of transduced cells was subjected to flow cytometric analysis to monitor hrGFP expression after induction with 10 nM mifepristone. To enrich transduced cells, single hrGFP positive cells were positively selected using the FACSAria cell sorting system (BD Bioscience). Sorted cells were cultured for a further 4–5 weeks and cell clones with the strongest hrGFP signals in respective transductions were used for an assay of HIV-1 release.

For the assay of HIV-1 release, 4 μg of pNL4-3 [28] was transfected to transduced cells with Lipofectamine 2000 (Invitrogen). Four hours post-transfection, culture medium was replaced with D-MEM/10% FCS containing 10 nM mifepristone. Culture supernatant was harvested 24 h later and virus production was monitored by HIV-1 $p24^{CA}$ enzyme-linked immunosorbent assay (ELISA, ZeptoMetrix).

Statistical analysis

Student's *t* test was used to determine statistical significance. A *P* value of <0.05 was considered significant.

Results

Generation of mifepristone-inducible lentiviral vectors

To establish a regulatable viral vector system, we cloned a mifepristone-inducible promoter sequence (as an internal promoter) into a self-inactivating (SIN) lentiviral vector plasmid in which the U3 region of the 5′-long terminal repeat (LTR) was replaced with the cytomegalovirus (CMV) promoter and the enhancer/promoter unit was deleted from the U3 region of the 3′-LTR [23, 27]. The inducible promoter was a hybrid consisting of the yeast GAL4 upstream activating sequences linked to the adenovirus major late E1b TATA box (GAL4/TATA) [7]. Gene expression from the GAL4/TATA promoter was controlled by a chimeric regulatory protein termed GeneSwitch. Binding of mifepristone to GeneSwitch induced a conformational change in the regulator to an active state, resulting in transcription of the gene of interest [7]. Besides an inducible promoter, our lentiviral vectors contained a Gateway cloning system reading frame cassette, facilitating cloning of genes of interest by site-specific recombination-based Gateway technology [23]. In addition to a conventional lentiviral vector containing the GAL4/TATA-Gateway component in the forward orientation (forward vector, Fig. 1a), we also constructed a version of the lentiviral vector plasmid in which the inducible gene expression unit was located in the reverse orientation, with the intention of reducing interference from the CMV promoter (reverse vector, Fig. 1a).

To evaluate our gene delivery system, the human *CD14* gene was cloned into both forward and reverse vector plasmids (designated pF-CD14 and pR-CD14, respectively). Infectious lentiviral vectors (vF-CD14 and vR-CD14) were produced following co-transfection of VSV G/Rev-expressing plasmid and Gag-Pol-expressing plasmid into 293T cells. Flow cytometric analysis of transfected cells showed that even in the absence of the GeneSwitch protein and mifepristone, expression of *CD14* and cistronic *hrGFP* genes occurred in pF-CD14-transfected producer cells (40.7 ± 3.1%), while such expression was repressed in pR-CD14-transfected cells (1.4 ± 0.4%) (Fig. 1b). This leaky expression of transgenes from the forward vector plasmid in virus producer cells might be due to transcriptional interference arising from the presence of a heterologous promoter in the same orientation (i.e., the CMV promoter), which was required for Tat-independent transcription of the viral RNA genome [27]. Culture supernatant containing the lentiviral vector was harvested and viral titer determined by quantification of the number of hrGFP-positive cells in viral vector-transduced GeneSwitch-293 cells, expressing the GeneSwitch regulatory protein, in the presence of mifepristone [8]. Lentiviral vectors produced by pF-CD14 showed 34.4-fold higher

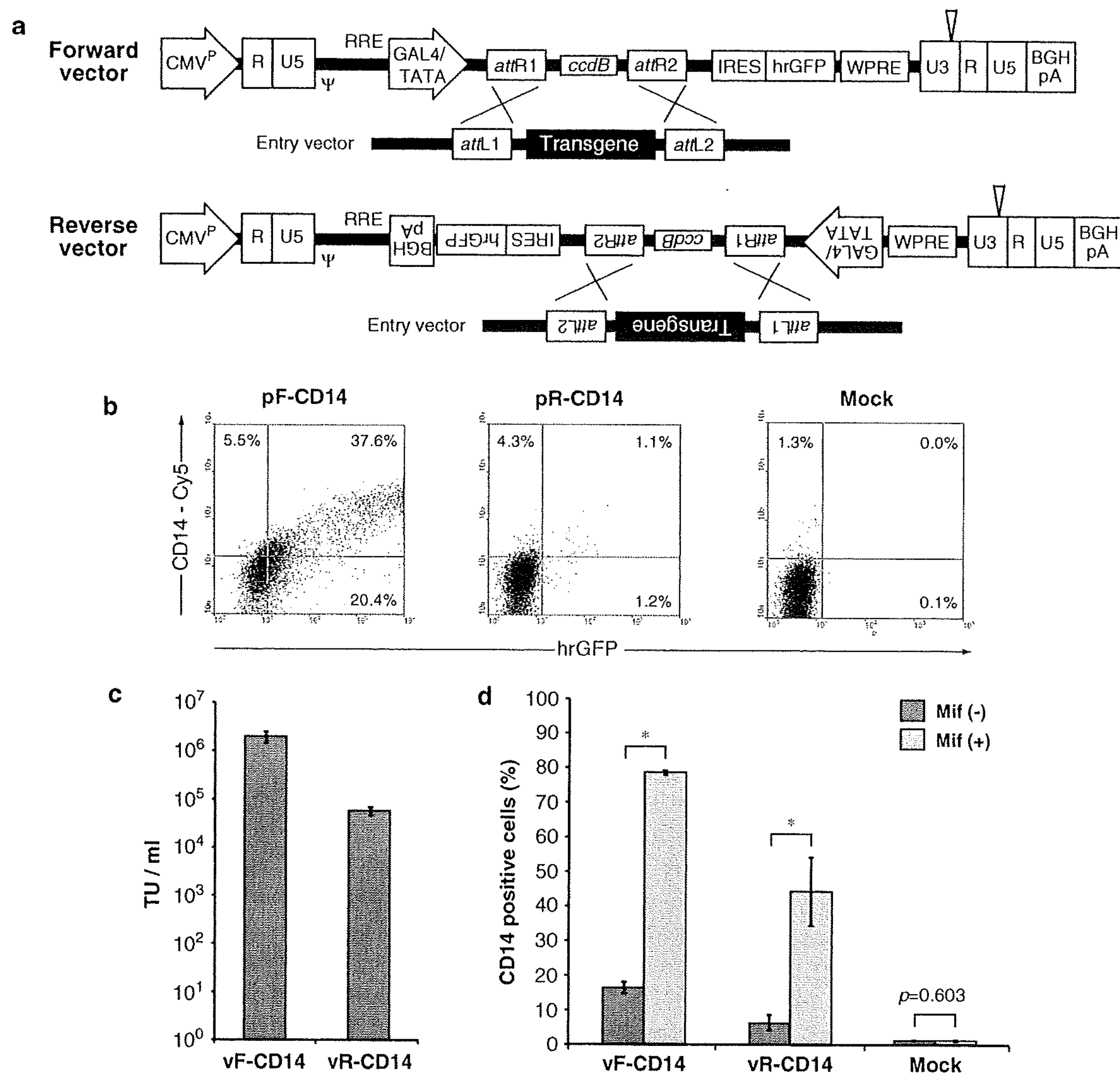


Fig. 1 Transduction of the *CD14* gene by mifepristone-inducible lentiviral vectors. **a** Schematic of forward and reverse vectors. In these SIN lentiviral vector plasmids, the U3 region of the 5′-LTR has been replaced with the CMV promoter (CMV^P) resulting in Tat-independent transcription. A portion of the U3 region containing the enhancer/promoter unit has been deleted from the 3′-LTR (represented as *triangles*). The gene of interest (transgene), flanked by *att*B sites, was subcloned into an entry plasmid and then transferred to the vector plasmid by a Gateway reaction. Note that site-specific recombination between *att*R sites on the vector plasmid and *att*L sites on the entry vector gave rise to new *att*B sites on the final lentiviral vector plasmid. The GAL4/TATA promoter regulated expression of the transgene and IRES-controlled hrGFP gene in the presence of the transactivator protein (GeneSwitch) and mifepristone. Ψ, packaging signal; *RRE* Rev responsive element; *WPRE* woodchuck post-regulatory element, *BGH pA* bovine growth factor hormone poly(A). **b** Expression of CD14 in vector producer cells. 293T cells were co-transfected with a VSV G/Rev-expressing plasmid, a Gag-Pol-expressing plasmid, and a forward or reverse vector plasmid coding *CD14* and *hrGFP* genes (pF-CD14 and pR-CD14, respectively). Expression of CD14 (*y*-axis) and hrGFP (*x*-axis) at the time of vector harvest (48 h after transfection) was analyzed by flow cytometric analysis. Untransfected cells (mock) served as a negative control. The representative results of three independent experiments are shown. **c** Infectious titers of lentiviral vectors. Vector titer (transducing units [TU]/ml) was determined by quantitative flow cytometric analysis for hrGFP positive cells on transduced GeneSwitch-293 cells in the presence of mifepristone. Values represent the mean ± standard deviation (SD) for triplicate determinations. **d** Transduction and induction activities of lentiviral vectors. GeneSwitch-293 cells were infected with vF-CD14 or R-CD14 at a MOI of 0.1 (or uninfected, mock) and cultivated in the presence or absence of 10 nM mifepristone (Mif (+) and Mif (−), respectively). Expression of CD14 protein 48 h after induction was determined by flow cytometric analysis. Values represent the mean ± SD of three independent experiments. The *P* value versus no mifepristone treatment is <0.05 by Student's *t* test (*)

viral titers (vF-CD14, $2.0 \pm 0.5 \times 10^6$ TU/ml) than viral vectors obtained by pR-CD14 (vR-CD14, $5.8 \pm 1.1 \times 10^4$ TU/ml) (Fig. 1c). The transduction and induction efficiencies of lentiviral vectors were then assessed following infection of GeneSwitch-293 cells at a MOI of 0.1. As shown in Fig. 1d, a low level of CD14 expression in

transduced cells was observed in both vF-CD14- and vR-CD14-infected cells in the absence of mifepristone. However, basal gene expression in vF-CD14-infected cells (16.5 ± 1.7%) was higher than that recorded in vR-CD14-infected cells (6.3 ± 2.2%), indicating that transgene expression from the reverse orientation vector was tightly controlled in both transduced cells and virus producer cells (Fig. 1b, d). In the presence of mifepristone, expression of CD14 increased significantly, with the percentage of CD14-positive cells rising to 78.6 ± 0.6% (vF-CD14) and 44.3 ± 9.9% (vR-CD14) (Fig. 1d). The regulation factor (ratio of maximal induced expression to basal expression levels in the absence of the inducer [29]) of vR-CD14 was higher than that of vF-CD14 (7.0-fold vs. 4.7-fold). These results demonstrated that our mifepristone-inducible lentiviral vectors functioned as an efficient gene-delivery system with a tight on–off switch for regulating transgene expression.

Transduction of a cytotoxic gene by the mifepristone-inducible lentiviral vector

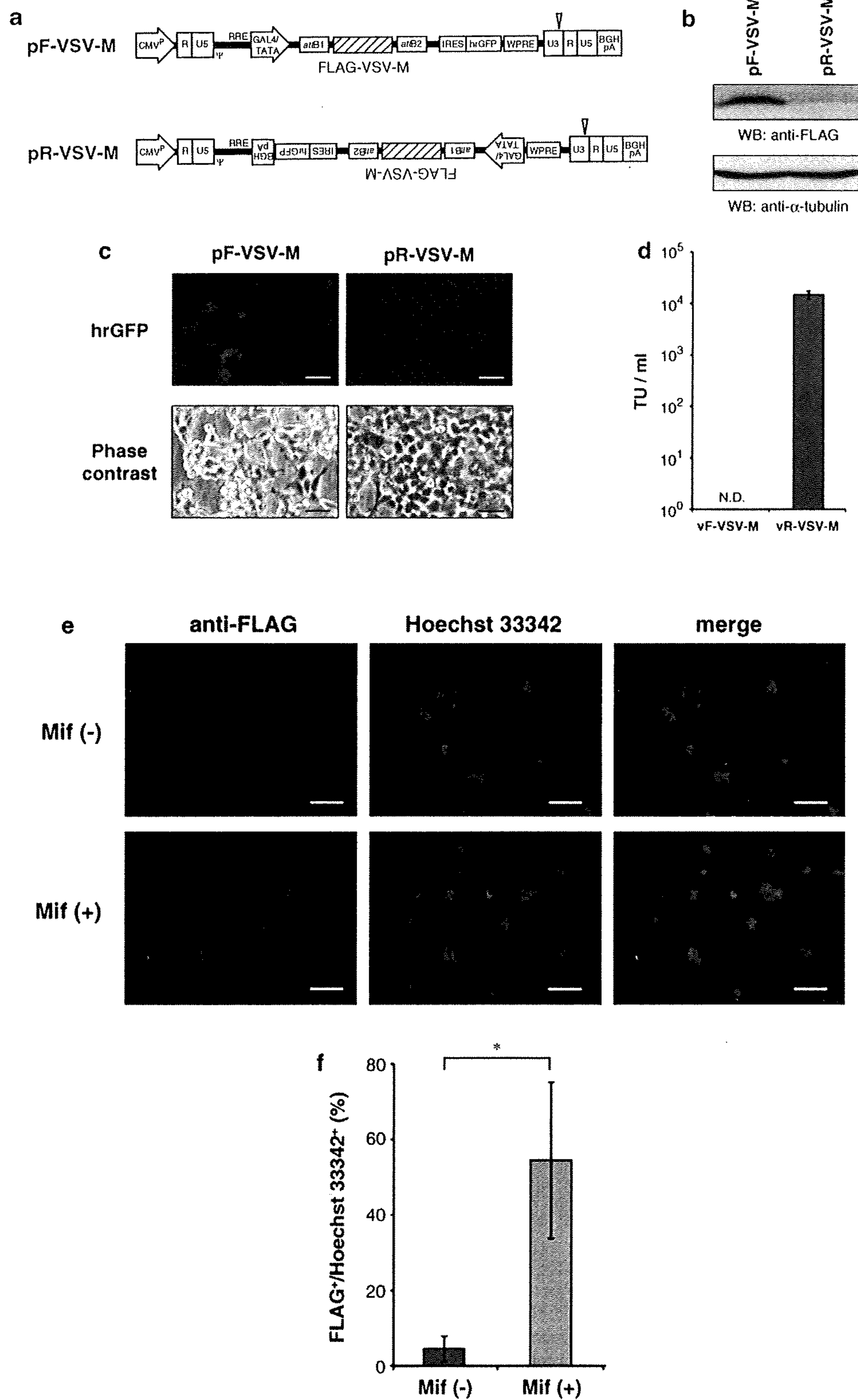
In gene therapies for cancer patients, delivery of a cytotoxic gene by lentiviral vector is one strategy for destroying tumor cells. However, expression of a toxic gene in a lentiviral vector genome would hamper vector production resulting in a reduction in titer. Expression of such a cytotoxic gene thus needs to be blocked during the process of vector production. The lentiviral vector described here, containing a mifepristone-inducible gene expression unit in the reverse orientation, shows great promise as a carrier for a cytotoxic transgene with CD14 expression tightly repressed in pR-CD14-transfected producer cells (Fig. 1b). We examined this system further to ascertain if it could produce infectious lentiviral vector containing a cytotoxic gene and transduce that gene into target cells. Forward and reverse vector plasmids containing FLAG tag-fused VSV matrix protein (FLAG-VSV-M) were constructed (pF-VSV-M and pR-VSV-M, Fig. 2a) and transfected into 293T cells to produce VSV G-pseudotyped infectious vector. VSV M was chosen as it inhibits nuclear export of cellular RNA by interacting with nucleoporin. This protein is responsible for most of the cytopathic effects observed in VSV-infected cells [30]. Analysis of the level of FLAG-VSV-M in virus producer cells showed that, even in the absence of inducers, transgene expression was activated in pF-VSV-M-transfected cells, while expression was undetectable in pR-VSV-M-transfected cells (Fig. 2b). As predicted by Western blotting analysis, activated expression of the downstream *hrGFP* cistron, together with the rounded phenotype of infected cells (a hallmark of VSV infection in cell culture [30]), was observed in pF-VSV-M-transfected cells (Fig. 2c). In contrast, pR-VSV-M-transfected cells

Fig. 2 Effective transduction of VSV M by the reverse vector. ▶ **a** Forward and reverse vectors containing FLAG-tagged VSV M (FLAG-VSV-M) as a transgene (pF-VSV-M and pR-VSV-M, respectively). **b** Expression of FLAG-VSV-M in vector producer cells. 293T cells were co-transfected with a VSV G/Rev-expressing plasmid, a packaging plasmid, and pF-VSV-M or pR-VSV-M to produce lentiviral vectors. Cell extracts were prepared 48 h after transfection and subjected to Western blotting (WB) analysis using anti-FLAG (*upper panel*) and anti-α-tubulin (*lower panel*) antibodies. **c** Leaky expression of VSV M caused the death of producer cells. Fluorescent (*upper panels*) and light (*lower panels*) microscopic analysis of producer cells was undertaken at the time of vector harvest. Scale bar, 40 μm. **d** Titers of lentiviral vectors bearing the *VSV M* gene. TU was determined by hrGFP positive cells in GeneSwitch-293 cells in the presence of mifepristone. The expression of hrGFP in vF-VSV-M-infected cells was below the detection limit (not detected, N.D.). Value of vR-VSV-M represents the mean ± SD of three independent experiments. **e** Inducible expression of FLAG-VSV-M in vR-VSV-M-transduced cells. GeneSwitch-293 cells were infected with vR-VSV-M at a MOI of 5 and fixed with paraformaldehyde 24 h after induction. Expression of FLAG-VSV-M was examined as described in materials and methods. Hoechst 33342 indicates nucleus. Mif (−), without mifepristone treatment; Mif (+), with 10 nM mifepristone treatment; scale bar, 100 μm. **f** Quantification of inducibility. FLAG-VSV-M positive cells observed by immunofluorescence analysis were counted in a randomly selected visual field. Data are expressed as the percentage of FLAG$^+$ cells in Hoechst 33342$^+$ cells (mean ± SD of four independent transductions). The *P* value versus no mifepristone treatment is <0.05 by Student's *t* test (*)

exhibited neither hrGFP expression nor cytopathic effect, demonstrating the ability of the reverse vector to suppress the cytotoxic gene in virus producer cells (Fig. 2c). Importantly, the ability of pR-VSV-M to suppress VSV M expression was reflected in the production of infectious lentiviral vector from transfected cells. Transfection with pR-VSV-M yielded lentiviral vector (vR-VSV-M) with titers of $1.5 \pm 0.5 \times 10^4$ TU/ml, while no infectious vector could be obtained by transfection with pF-VSV-M (Fig. 2d). The ability of vR-VSV-M to transduce the transgene was then analyzed by infection of GeneSwitch-293 cells. Immunostaining analysis indicated that FLAG-VSV-M expression was induced in response to mifepristone in transduced cells (Fig. 2e) and the regulation factor (i.e., inducibility) was 11.8-fold (FLAG positive cells per Hoechst 33342 positive cells, 4.6 ± 3.2% without mifepristone treatment versus 54.5 ± 20.6% with mifepristone treatment (Fig. 2f). These data demonstrated that our reverse vector system is suitable for the transduction of a harmful gene.

Inhibition of HIV-1 release by mifepristone-inducible lentiviral vector

To evaluate whether the reverse vector could overcome self-inhibition caused by an anti-HIV transgene in producer cells, a dominant negative mutant of human VPS4B was



selected as a transgene. VPS4B is one of two known isoforms of AAA-ATPase VPS4 and is thought to catalytically remove endosomal sorting complex required for transport (ESCRT) complex III proteins from the plasma membrane, resulting in budding of HIV-1 from infected cells [26]. The ATPase activity of VPS4B is essential for

HIV-1 budding with dominant negative mutants of VPS4 (such as K180Q, where the residues required for ATP binding have been mutated), previously reported to block HIV-1 release and infectivity [26]. We inserted a FLAG tag-fused VPS4B K180Q mutant (FLAG-VPS4B-KQ) into both the forward and the reverse vector plasmids and constructed vector plasmids encoding FLAG tag-fused luciferase (FLAG-Luc) as control vectors (Fig. 3a). When these vector plasmids were used to produce lentiviral vector, expression of the transgenes (i.e., FLAG-VPS4B-KQ or FLAG-Luc) was observed in producer cells transfected with forward vector plasmids, but not in cells with reverse vector plasmids (Fig. 3b). While transfection with pR-VPS4B-KQ yielded infectious lentiviral vector, vR-VPS4B-KQ, transfection with pF-VPS4B-KQ did not generate infectious vector, indicating that leaky expression of the VPS4 dominant negative mutant from the forward vector plasmid self-inhibited lentiviral vector production (Fig. 3c). Titers of vR-VPS4B-KQ ($8.7 \pm 2.4 \times 10^4$ TU/ml) were comparable to those of lentiviral vector obtained by transfection with pR-Luc (vR-Luc, $5.9 \pm 4.1 \times 10^4$ TU/ml), indicating that titers of lentiviral vectors containing the gene

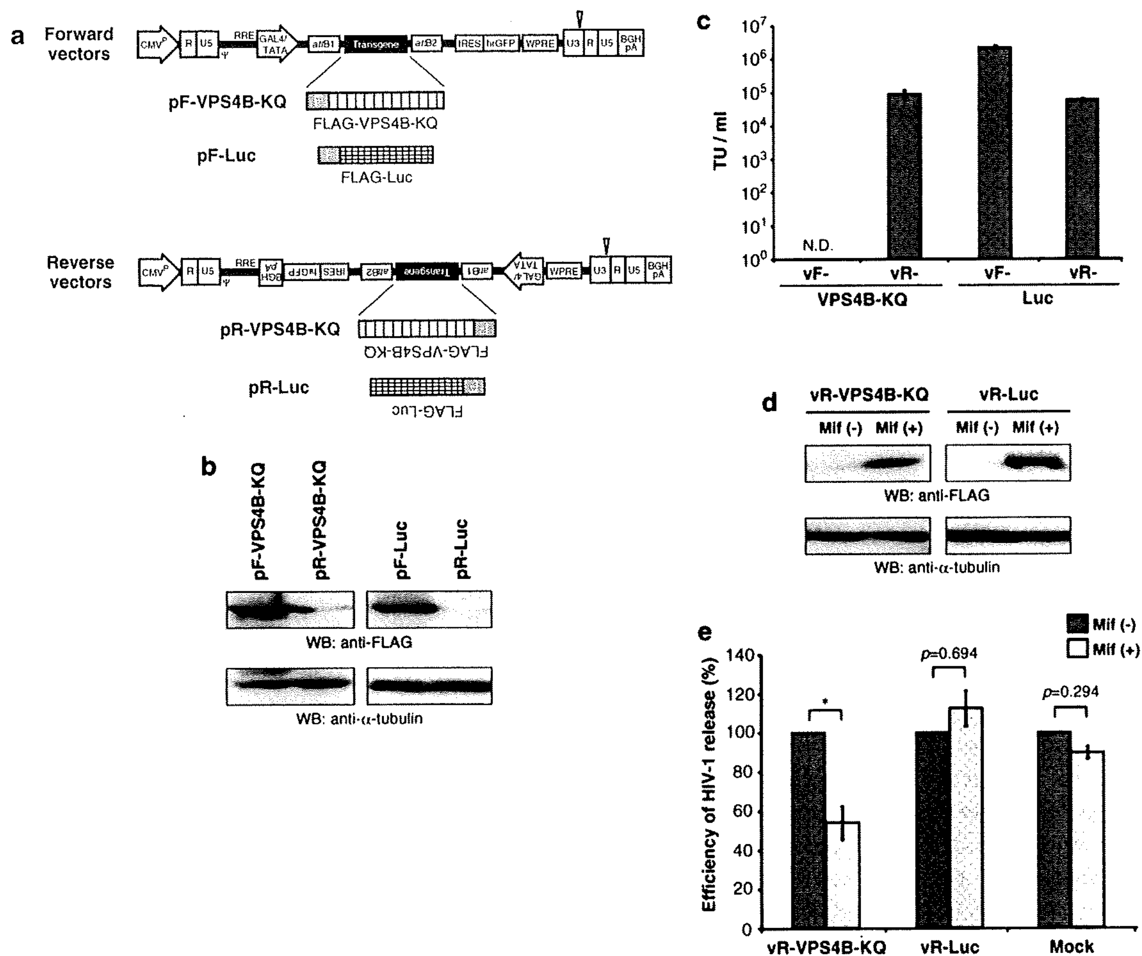


Fig. 3 Inhibition of HIV-1 release by a reverse vector encoding VPS4B dominant negative mutant. **a** Schematic representation of forward and reverse vectors bearing FLAG-tagged VPS4B K180Q (FLAG-VPS4B-KQ) or luciferase (FLAG-Luc). **b** Expression of transgenes in vector producer cells. The lentiviral vectors were produced as described in "Materials and methods". Producer cells were collected at the time of virus harvest and subjected to Western blotting (WB) analysis using anti-FLAG (*upper panels*) and anti-α-tubulin (*lower panels*) antibodies. **c** Titers of lentiviral vectors. N.D. means below the detection limit of flow cytometric analysis for hrGFP expression. **d** Induction of FLAG-VPS4B-KQ and FLAG-Luc expressions by reverse vectors. GeneSwitch-293 cells were infected with vR-VPS4B-KQ or vR-Luc at a MOI of 1 and cultivated in the absence of mifepristone. After sorting of cell clones that were capable of expressing hrGFP, the inducibility of FLAG-VPS4B-KQ and FLAG-luciferase expression was analyzed by western blotting. Mif (−) and Mif (+) indicate before and after induction with mifepristone, respectively. **e** Analysis of HIV-1 production in transduced cells. GeneSwitch-293 cell lines transduced with either vR-VPS4B-KQ or vR-Luc (and untransduced cells, mock) were transiently transfected with pNL4-3. The amount of HIV-1 virions present in the culture supernatant was measured by $p24^{CA}$ ELISA. The efficiency of HIV-1 release in the presence of 10 nM mifepristone is shown as a percentage of the value in the absence of mifepristone (mean ± SD of three independent transfections). The *P* value versus no mifepristone treatment is <0.05 by Student's *t* test (*)

expression unit in a reverse orientation were not affected by the anti-HIV transgene (Fig. 3c).

The ability of our lentiviral vectors to transduce the VPS4B dominant negative mutant for inhibition of HIV-1 release was then tested. GeneSwitch-293 cells were infected with vR-VPS4B-KQ and vR-Luc and sorted for hrGFP positive cells. When transduced cells were cultured with mifepristone, induction of transgene expression was seen (Fig. 3d). The inhibitory effect of transgenes on HIV-1 release from transduced cells was examined by transiently transfecting a plasmid DNA producing infectious HIV-1 virions (pNL4-3). In the presence of mifepristone, levels of HIV-1 production from vR-VPS4B-KQ-transduced cells dropped to 53.9 ± 8.5%, approximately half that seen in the absence of mifepristone treatment. However, no significant inhibitory effects on HIV-1 release were observed in vR-Luc or mock-transduced cells (112.3 ± 9.0% and 89.7 ± 3.1%, respectively). This indicated that induced expression of dominant negative mutant VPS4B proteins in transduced cells accounted for the observed inhibition of HIV-1 release. These results demonstrate the utility of our reverse vector to transduce an anti-HIV gene that functionally suppresses HIV-1 release in target cells.

Discussion

The basic principle of current gene therapy is to deliver genetic material to a population of cells in the body, thereby preventing a disease or improving the clinical status of a patient. Although, a key factor in successfully implementing gene therapy is the development of effective vector systems, a number of issues need to be addressed to apply them in a clinical setting. In terms of viral vector systems, one of the major problems is that insertion of cytotoxic or antiviral transgenes adversely affects viral titers during vector production. In this study, we incorporated a mifepristone-inducible gene expression unit into HIV-1-based lentiviral vectors to solve the problem of vector self-inhibition.

Previous studies have reported the delivery of various anti-HIV genes by HIV-1-based vectors in vitro and in vivo [31–36]. Some of the transgenes used in these studies target HIV-1 RNA sequences either directly or indirectly, aiming to inhibit transcription, nuclear translocation, or translation of viral RNA [32–36]. In these types of approach, the problem of self-inhibition can be solved by modifying the nucleotide sequence of a lentiviral vector such that the function of the vector RNA does not interfere with the anti-HIV transgene in producer cells. However, if the transgene targets a fundamental process of the HIV-1 life cycle, such as virion formation, another strategy to avoid self-inhibition is to express the transgene in a regulated manner such that its expression is blocked in producer cells and induced in target cells. This kind of approach would be of value in the transduction of a harmful gene into target cells. The data presented here demonstrate that a lentiviral vector bearing a regulatable gene expression unit is indeed capable of transducing cytotoxic (VSV M) and anti-HIV (VPS4B K180Q) genes into target cells without significant decrease in vector titer (Figs. 2, 3). In addition, induction of anti-HIV genes in transduced cells resulted in approximately 50% inhibition of HIV-1 release (Fig. 3e).

Expression of VPS4B-KQ mutant by transfection has been reported to inhibit HIV-1 release >100-fold [26]; although, the VPS4B-KQ expression induced by our mifepristone-regulatable system produced about 2-fold reduction in HIV-1 production (Fig. 3e). When we looked at the IRES-controlled hrGFP expression in mifepristone-induced cells that had been transduced by vR-VPS4B-KQ or vR-Luc and sorted, hrGFP expressions were only observed in 10.5% (vR-VPS4B-KQ) or 12.2% (vR-Luc) of the cells (data not shown). We speculate that uninducible population of cells was still permissive to HIV-1 production and thus lead to the observed 50% inhibition in the vR-VPS4B-KQ-transduced cells. During expansion of these transduced cells after cell sorting it is possible that some (e.g., gene shut-off) lost their ability to be induced by mifopristone. Besides, the cell sorting step might lead to this issue of lost inducibility. Therefore improving the way to enrich transduced cells should help to alleviate this problem.

To achieve tight regulation of transgene expression, enabling production of infectious vectors, it was necessary to place the mifepristone-inducible gene expression unit in the reverse orientation in the context of the lentiviral vector. Sirin and Park [12] tested the forward and the reverse orientations of a mifepristone-inducible gene expression unit in HIV-1-based lentiviral vectors and reported basal levels of transgene expression that were higher in lentiviral vectors bearing the expression cassette in the reverse orientation than those containing it in the forward orientation. This was in contrast to the findings presented here, where basal expression of the CD14 transgene in reverse vector-infected cells appeared to be lower than that in forward vector-infected cells (Fig. 1d). Similarly to the Sirin and Park [12] study, we used an HIV-1-based SIN vector in which the woodchuck post-regulatory element (WPRE) was inserted into the 3′-untranslated region of the viral genome (Fig. 1a). WPRE has been reported to increase the stability of RNA transcripts, thereby enhancing transgene expression from retroviral and lentiviral vectors [37]. Interestingly, WPRE functions only when placed in the sense orientation of a transgene and antisense WPRE actually shows an inhibitory effect on transgene expression [37]. In our reverse vector, WPRE

was positioned in the opposite orientation to the inducible gene expression unit (Fig. 1a), while the vector designed by Sirin and Park [12] contained WPRE in same orientation as the expression unit. Orientation-dependent elements such as WPRE can thus enhance basal expression of a transgene in both producer and transduced cells. In addition to WPRE, the SIN vector used in our study contained a hybrid 5′-LTR in which the U3 region was replaced with the CMV promoter [27]. We speculate that, in the context of our forward vector, these *cis*-acting sequences should increase background activity of the mifepristone-regulatable gene expression unit without induction, leading to leaky expression of cytotoxic/anti-HIV genes in producer cells and significant loss of vector titers (Figs. 2d, 3c).

One general drawback of regulatable gene expression systems, including the Tet and mifepristone systems, is that they necessitate delivery of two expression units into a target cell; one to express the transactivator and the other to express the transgene in response to the activator. To exclude differences in experimental conditions due to differing levels of transactivator expression, a cell line stably expressing the GeneSwitch transactivator was used as a target cell in this study. While Sirin and Park [12] also described a two-lentiviral vector system in which GeneSwitch and inducible gene expression units were cloned into separate vectors, this type of binary approach would produce populations of singly transduced cells with either transactivator or transgene, resulting in low inducibility. Single-lentiviral vectors bearing the entire regulatable unit have been developed in Tet systems [11, 38, 39]. This single-vector approach would be an attractive option for the mifepristone-regulatable system, bypassing the need for co-transduction of target cells with high amounts of virus. However, RNA virus-based vectors, such as lentiviral vectors, are limited in their cloning capacity for larger genes. Theoretically, lentiviral vectors can accommodate 7–7.5 kb of foreign DNA [1], yet this packaging capacity will be decreased by the insertion of additional regulatory sequences. Improvements to the mifepristone system that would allow incorporation of both transactivator and inducible units into a single-lentiviral vector would be necessary to design a more versatile vector.

The mifepristone-regulatable gene expression system reported here has a number of potential advantages that suit it to gene therapy applications in humans. First, the majority of the system consists of modified human proteins with no impact on cell viability. Second, the induction response is specific and rapid. Third, mifepristone is orally effective and the dose required for induction is within the range acceptable for clinical use [10]. Importantly, mifepristone has been approved by the Food and Drug Administration (FDA) for use in humans. Although, no gene regulatory system has yet been approved by the FDA for clinical use, lentiviral vectors in conjunction with a mifepristone-regulatable gene expression system are a promising step toward achieving successful gene therapy.

Acknowledgments We thank Hiroyuki Miyoshi (RIKEN BioResource Center) for providing pCMV-VSV-G-RSV-Rev and pCAG-HIVgp, Elisa Izaurralde (European Molecular Biology Laboratory) for EGFP-fused VSV M-expressing plasmid, and Wesley Sundquist (Department of Biochemistry, University of Utah) for dominant-negative mutant VPS4B-expressing plasmid. We are also grateful to Joanne Martin for proofreading of the manuscript and members of the Laboratory of Viral Pathogenesis and the Laboratory for Host Factors for support of experimental techniques and helpful discussions. This work was supported by grants from the Ministry of Health, Labour and Welfare and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. I.M. Verma, N. Somia, Nature **389**, 239–242 (1997)
2. L. Naldini, U. Blomer, P. Gallay, D. Ory, R. Mulligan, F.H. Gage, I.M. Verma, D. Trono, Science **272**, 263–267 (1996)
3. H. Miyoshi, K.A. Smith, D.E. Mosier, I.M. Verma, B.E. Torbett, Science **283**, 682–686 (1999)
4. J.A. Taylor, L. Vojtech, I. Bahner, D.B. Kohn, D.V. Laer, D.W. Russell, R.E. Richard, Mol. Ther. **16**, 46–51 (2008)
5. M. Fussenegger, Biotechnol. Prog. **17**, 1–51 (2001)
6. S. Agha-Mohammadi, M.T. Lotze, J. Clin. Invest. **105**, 1177–1183 (2000)
7. Y. Wang, B.W. O'Malley Jr., S.Y. Tsai, B.W. O'Malley, Proc. Natl Acad. Sci. USA **91**, 8180–8184 (1994)
8. M.M. Burcin, G. Schiedner, S. Kochanek, S.Y. Tsai, B.W. O'Malley, Proc. Natl Acad. Sci. USA **96**, 355–360 (1999)
9. R.V. Abruzzese, D. Godin, V. Mehta, J.L. Perrard, M. French, W. Nelson, G. Howell, M. Coleman, B.W. O'Malley, J.L. Nordstrom, Mol. Ther. **2**, 276–287 (2000)
10. J.L. Nordstrom, Steroids **68**, 1085–1094 (2003)
11. T. Kafri, H. van Praag, F.H. Gage, I.M. Verma, Mol. Ther. **1**, 516–521 (2000)
12. O. Sirin, F. Park, Gene **323**, 67–77 (2003)
13. B. Mitta, C.C. Weber, M. Rimann, M. Fussenegger, Nucleic Acids Res. **32**, e106 (2004)
14. S.C. Beutelspacher, N. Ardjomand, P.H. Tan, G.S. Patton, D.F. Larkin, A.J. George, M.O. McClure, Exp. Eye Res. **80**, 787–794 (2005)
15. F. Galimi, E. Saez, J. Gall, N. Hoong, G. Cho, R.M. Evans, I.M. Verma, Mol. Ther. **11**, 142–148 (2005)
16. S. Hartenbach, M. Fussenegger, J. Biotechnol. **120**, 83–98 (2005)
17. H.L. Heine, H.S. Leong, F.M. Rossi, B.M. McManus, T.J. Podor, Methods Mol. Med. **112**, 109–154 (2005)
18. B. Mitta, C.C. Weber, M. Fussenegger, J. Gene Med. **7**, 1400–1408 (2005)
19. K. Okamoto, J. Fujisawa, M. Reth, S. Yonehara, Genes Cells **11**, 177–191 (2006)
20. W. Weber, W. Bacchus, F. Gruber, M. Hamberger, M. Fussenegger, J. Biotechnol. **131**, 150–158 (2007)
21. H. Hurttila, J.K. Koponen, E. Kansanen, H.K. Jyrkkanen, A. Kivela, R. Kylatie, S. Yla-Herttuala, A.L. Levonen, Gene Ther. **15**, 1271–1279 (2008)
22. S. Goverdhana, M. Puntel, W. Xiong, J.M. Zirger, C. Barcia, J.F. Curtin, E.B. Soffer, S. Mondkar, G.D. King, J. Hu, S.A. Sciascia, M. Candolfi, D.S. Greengold, P.R. Lowenstein, M.G. Castro, Mol. Ther. **12**, 189–211 (2005)