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The extent of sequence complementarity correlates with the potency of cellular miRNA-mediated restriction of HIV-1

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ABSTRACT

MicroRNAs (miRNAs) are 22-nt non-coding RNAs involved in the regulation of cellular gene expression and potential cellular defense against viral infection. Using *in silico* analyses, we predicted target sites for 22 human miRNAs in the HIV genome. Transfection experiments using synthetic miRNAs showed that five of these miRNAs capably decreased HIV replication. Using one of these five miRNAs, human miR-326 as an example, we demonstrated that the degree of complementarity between the predicted viral sequence and cellular miR-326 correlates, in a Dicer-dependent manner, with the potency of miRNA-mediated restriction of viral replication. Antagomirs to miR-326 that knocked down this cell endogenous miRNA increased HIV-1 replication in cells, suggesting that miR-326 is physiologically functional in moderating HIV-1 replication in human cells.

INTRODUCTION

MicroRNAs (miRNAs) are small 20- to 24-nt non-coding RNAs involved in the post-transcriptional regulation of gene expression through base pairing to complementary sites in messenger RNAs (mRNAs) (1). The miRNA–mRNA interaction, leading to the silencing of mRNA expression, is mediated through an RNA-induced silencing complex (RISC). In plants, miRNA–mRNA interaction is generally perfectly complementary, and this base pairing leads to the cleavage and degradation of the mRNA. In animals, miRNA–mRNA complementarity is largely imperfect and results in the reduction of mRNA levels (2,3) and/or the translational inhibition of the mRNA (4,5).

Initially described in *Caenorhabditis elegans* (6), miRNAs have since been identified in viruses, plants, animals and other eukaryotes (with the exception of yeast) (7). Currently, there are more than 1500 known miRNAs in the human genome (http://www.mirbase.org). In animals, miRNAs are involved in widely divergent cellular processes (8–11), and changes in miRNA expression profiles have been associated with many human pathologies (12–16). It has been suggested by several investigators that cellular miRNAs can serve to defend cells against viral infection [for reviews see refs. (17–20)]. Consistent with this notion, cellular miRNAs that target Primate foamy virus 1 (21), Hepatitis C (22,23), Hepatitis B (24,25), Vesicular Stomatitis Virus (26), influenza virus (27), coxsackie virus (28), Human Papillomavirus (HPV) (29) and herpes viruses (30–32) have been reported. For HIV-1, various reports have identified multiple host miRNAs relevant to virus replication. Two different mechanisms of action are apparently involved. miRNAs can target cellular factors affecting HIV replication, like miR-17-5p and -20a that target PCAF (33) or miR-198 that targets the cyclin T1 protein (34). Alternatively, some miRNAs, like miR-29a, can directly restrict the HIV-1 genome sequence to decrease HIV infectivity (35,36). Indeed, one report has implicated five miRNAs (miR-28, miR-125b, miR-150, miR-223 and miR-382) in targeting the 3′ long terminal repeat (LTR) sequence of the HIV genome leading to viral latency in CD4+ T cells (37); another report has shown that some of these miRNAs

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function similarly in HIV-1 infection of monocytes and macrophages (38).

To date, a systematic evaluation of miRNA susceptible target sequences in the HIV-1 genome has not been performed; nor has it been carefully studied how miRNA selection pressure, if any, shapes HIV-1 replication. Here, we have surveyed the full HIV-1 genome against the known dataset of T-cell-abundant human miRNAs to identify *in silico*, various putative target sites. We then tested 17 discrete miRNAs for their abilities to suppress/augment HIV-1 replication. As an example, we have studied in detail one human miRNA, miR-326, for its endogenous expression in primary T cells and T-cell lines and for its functional interaction with a complementary target sequence in the HIV-1 genome.

MATERIALS AND METHODS

Cell culture

Jurkat and MT4 human T-cell lines were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium with 10% fetal calf serum (FCS) and 2 mM L-glutamine. 293T, TZMbl and the 42CD4 cells (39), derived from HEK-293 cells stably expressing CD4 and CXCR4, were propagated at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 2 mM L-glutamine.

Primary T-cell subsets were isolated from primary blood mononuclear cells after overnight incubation in RPMI 10% FBS with or without staphylococcal enterotoxin B (SEB). Flow cytometry was then used to sort T cells (CD3+) into different populations. Resting cells in the unstimulated culture were identified as CD69− and activated cells from the SEB treatment as CD69+. Cells were then further classed as naïve (CD27+ CD45RO−) or memory (CD45RO+ CD27−). Finally, CD4+ T cells were sorted by staining for cell surface CD4.

DNA constructs

For the luciferase (Luc) reporter plasmids Luc-3X Wt and Luc-3X Tgt, DNA oligonucleotides corresponding to the Wt (forward: 5′-AATGCACTAGTTGGGATGGAGGACCCGGAGGCTGCAGTCTAGAAATGC-3′; reverse: 5′-GCATTTCTAGACTGCAGCCTCCGGGTCCTCCATCCCAACTAGTGCATT-3′) and Tgt (forward: 5′-AATGCACTAGTCTGGAGGAAGGGCCCAGAGGCTGCAGTCTAGAAATGC-3′; reverse: 5′-GCATTTCTAGACTGCAGCCTCTGGGCCCTTCCTCCAGACTAGTGCATT-3′) target sites were hybridized and digested with SpeI+XbaI to be cloned into the SpeI site of pMIR-REPORT- Luc (Ambion) to generate the plasmids containing one target site. The same cloning strategy was used to add two additional target sites to obtain the reporter plasmids Luc-3X Wt and Luc-3X Tgt.

For the LTR-Luciferase reporter constructs, the HIV LTR was amplified from the pILIC-Wt, pILIC-1.3, pILIC-3.1 and pILIC-Tgt plasmids and cloned upstream of the luciferase gene in the pGL3-Basic Vector (Promega).

For pILIC mutant viruses, the BamHI–SphI fragment of pILIC vector (40) containing the HIV LTR was subcloned into the pUC18 plasmid to generate all the mutants by site-directed mutagenesis using the QuikChange Lightning Mutagenesis Kit (Agilent Technologies). Then the SphI–XhoI fragments of the mutated plasmids were cloned into the SphI and XhoI sites of the original pILIC plasmid.

For ΔNef ILIC clones, the DNA fragment corresponding to the *nef*-inactivated locus was amplified from the pNL4.3-let7-scrbl plasmid (41) and cloned between the restriction sites XmaI and XhoI of the pILIC-Wt and pILIC-Tgt plasmids. The pNL4.3-let7-scrbl clone is deficient for Nef due to the replacement of ∼100 bases of sequence encompassing the Nef start codon with ∼100 bases of scrambled sequence, which contains no start codon.

Synthetic RNA

The synthetic miRNAs (Invitrogen) are RNA duplexes without modification. The sense strand is the same as the mature miRNAs. The anti-miR326 hairpin inhibitor and siRNAs against enhanced green fluorescent protein (EGFP) and Dicer are from Dharmacon.

Production of viral stocks and viral infection

Media collected from pNL4-3-transfected 293T cells were filtered using 0.45 μm membrane. Virus was quantified by reverse transcriptase (RT) assay (42). For infection experiments, 0.6×10^6 42CD4 cells were transfected with 40 pmoles of synthetic miRNAs using lipofectamine 2000 (Invitrogen). After 24 h, cells were washed twice with 1 ml phosphate buffered saline and infected with 10^6–10^7 cpm (RT units) of virus prepared from transfected 293T cells. After 15 h of exposure to the virus, cells were washed twice with phosphate buffered saline and new medium was added. Supernatants were harvested 3 days, or 2, 3, 4 and 6 days (time course replication assay) post-infection, and viral replication was monitored by RT quantification.

For pILIC Wt and Tgt mutants, the viral stocks were prepared as described previously (40) with minor modifications. The pILIC mutants were digested with BamHI, and the 9.5-kb fragment containing the provirus was eluted from 1% agarose gel and ligated to generate concatemers. The ligated DNA preparations were purified with phenol/chloroform extraction and ethanol precipitation. The 293T cells were transfected with 8 μg of ligated DNA using Lipofectamine 2000 (Invitrogen); and 3 days later, 1 ml of virus containing supernatant was used to infect $4x10^6$ MT4 cells for rapid virus stock amplification. The supernatant of MT4 infected cells was harvested 5 days after infection, and virus was quantified by RT assay.

For TZMbl assay, cells were seeded in a 96-well plate at 15 000 cells/well for 24 h. Medium was then replaced with fresh DMEM containing serial dilutions of viral supernatant. After 24 h post-infection, the cells were washed, fixed and assayed for the presence of β-galactosidase by X-gal enzymatic assay. Blue cells were counted, and the

number of infectious units per volume was computed based on the dilution of infecting supernatant.

Reporter assays

The 293T cells were transfected using Lipofectamine 2000 (Invitrogen) with 1 μg of the pMIR-REPORT-Luc reporter plasmids (Luc-3X Wt and Luc-3X Tgt) alone or co-transfected with 40 pmoles of miR-326 or a negative control siRNA (negative control siRNA #1, Ambion). The pMIR-REPORT-β-gal plasmid (50 ng) was also added to the transfection as a normalization control. After 24 h post-transfection, cells were washed twice with 0.5 ml phosphate-buffered saline and then lysed in Passive Lysis Buffer (Promega). Luciferase assay substrate (Promega) was used according to the manufacturer's protocol, and activity was measured in an Opticom II luminometer (MGM Instruments). Normalization of luciferase activity was based on β-galactosidase activity, measured with Galacto-Star as described by the manufacturer (Applied Biosystem). All luciferase values represent averages ± standard deviation (SD) from at least three independent transfections.

For the LTR-Luciferase reporter constructs, 0.2 μg of reporter plasmid were transfected in 42CD4 and Jurkat cells using Lipofectamine 2000 (Invitrogen) and Nucleofector (Lonza), respectively, with or without pSV-Tat plasmid (0.4 and 2 μg in 42CD4 and Jurkat, respectively). Transfection efficiency of Jurkat cells using the nucleofector was monitored by expression of green fluorescent protein (GFP) from a control plasmid. Efficiency of Jurkat transfection was routinely >60%. The pMIR-REPORT-β-gal plasmid (50 ng) was also added to the transfection as a normalization control. Afetr 24 h post-transfection, luciferase and β-galactosidase activitivities were determined as described above.

Quantitative real-time PCR

To quantify HIV-1 mutant RNA, total RNA from Jurkat cells co-infected with Wt and mutant viruses was harvested by using TRI Reagent (Sigma) following the manufacturer's protocol. Reverse transcription was performed on 0.5 μg of cellular RNA with oligo(dT) as the RT primer, and quantitative PCR for HIV RNAs was performed with 1% of the RT reaction using IQ SYBR Green Supermix (Bio-Rad), and the reaction was carried out with a Bio-Rad CFX96 Real-Time System instrument. The reverse primer was common to all amplification (5′-GTTAGCCAGAGAGCTCCCAG-3′). In co-infection with the Wt virus, the forward primers specific for each mutant virus were the following: Wt (5′-CTGCATGGGATGGAGGA-3′), Tgt (5′-TGCACTGGAGGAAGGGCCCA-3′), 1.1 (5′-CCCTGTGAGCCTGCATT-3′), 1.2 (5′-GTGAGCCTGCATGGGAG-3′), 1.3 (5′-CTGCATGGGATGGAGGG-3′), 2.1 (5′-GTGAGCCTGCATTGGAG-3′), 2.2 (5′-CTGCATGGGAGGGAGGG-3′), 2.3 (5′-CTGCATTGGATGGAGGG-3′), 3.1 (5′-CTGCATTGGAGGGAGGG-3′). In the co-infection with Tgt and 1.3 viruses, the same Tgt-specific primer as below was used, and a different primer was used for 1.3 (5′-GTGAGCCTGCATGGGAT-3′). The glyceraldehyde-3-phosphate dehydrogenase gene and its mRNA quantification were used for normalization (forward, 5′-GCTCACTGGCA TGGCCTTCC GTGT-3′ and reverse, 5′-TGGAGGAGTGGGTGTCG CTGTTGA-3′).

RESULTS

Identification of miRNA target sites in the HIV genome by *in silico* analysis

To identify miRNAs that potentially regulate HIV expression by direct targeting of viral sequences, we performed an *in silico* analysis seeking human miRNA target sites in the HIV-1 genome. Using the target prediction algorithm btarget (43), we assessed the complementarity of the HIV-1 genome with the 400 most T-cell-abundant human miRNAs. From this analysis, we observed 39 potential target sites for 22 different miRNAs (Figure 1A). These miRNA target sites are distributed widely throughout the HIV genome, with a slight tendency to favor the 3′ portion of the viral genome; each of these putative miRNA–mRNA base pairings has a calculated free energy of duplex formation better than −25 kcal/mol. Some examples of miRNA–mRNA complementarities, with seed sequences underlined and with calculated free energies, are shown in Figure 1B. We also show three published examples of characterized miRNA–mRNA target sites, Smo (37), HIV-1 (44) and AURKB (45) as illustrative comparisons (Figure 1C).

Several over-expressed human miRNAs can repress HIV replication

The above *in silico* analyses suggested that many cellular miRNAs could potentially pair with HIV-1 sequences. To assess the biological significance of such interactions, we experimentally tested the effect of 17 cellular miRNAs on HIV-1 replication. Synthetic RNAs corresponding to the mature forms of the respective miRNAs were transfected individually into 42CD4 cells, a cell line derived from 293T, which expresses CD4 and co-receptor. The 42CD4 cells were chosen due to their ease of transfection and the ability to detect viral replication in these cells relatively soon after infection. We checked if the transfected miRNAs would influence HIV-1 replication. After 24 h post-transfection of the synthetic miRNAs, we infected the cells with HIV-1 NL4.3 and virus replication was measured by reverse transcriptase assay 72 h post-infection (Figure 2A). In our assays, we employed as a positive control, miR-29b, which has been reported by others to repress HIV-1 replication (35,36). Indeed, the over-expression of miR-29b decreased HIV replication by ∼60%; and amongst the 17 other miRNAs that we tested, five (miR-133b, miR-138, miR-326, miR-149 and miR-92a) consistently decreased HIV infection by >40%. In contrast, the other miRNAs had either no statistically significant effect on viral replication or slightly augmented (miR-425) the replication of HIV-1 NL4-3 (Figure 2A). When we combined the transfection of two miRNAs and compared those results with the results of the transfection of a single miRNA, we observed that the former had an increased silencing effect over the latter (Supplementary

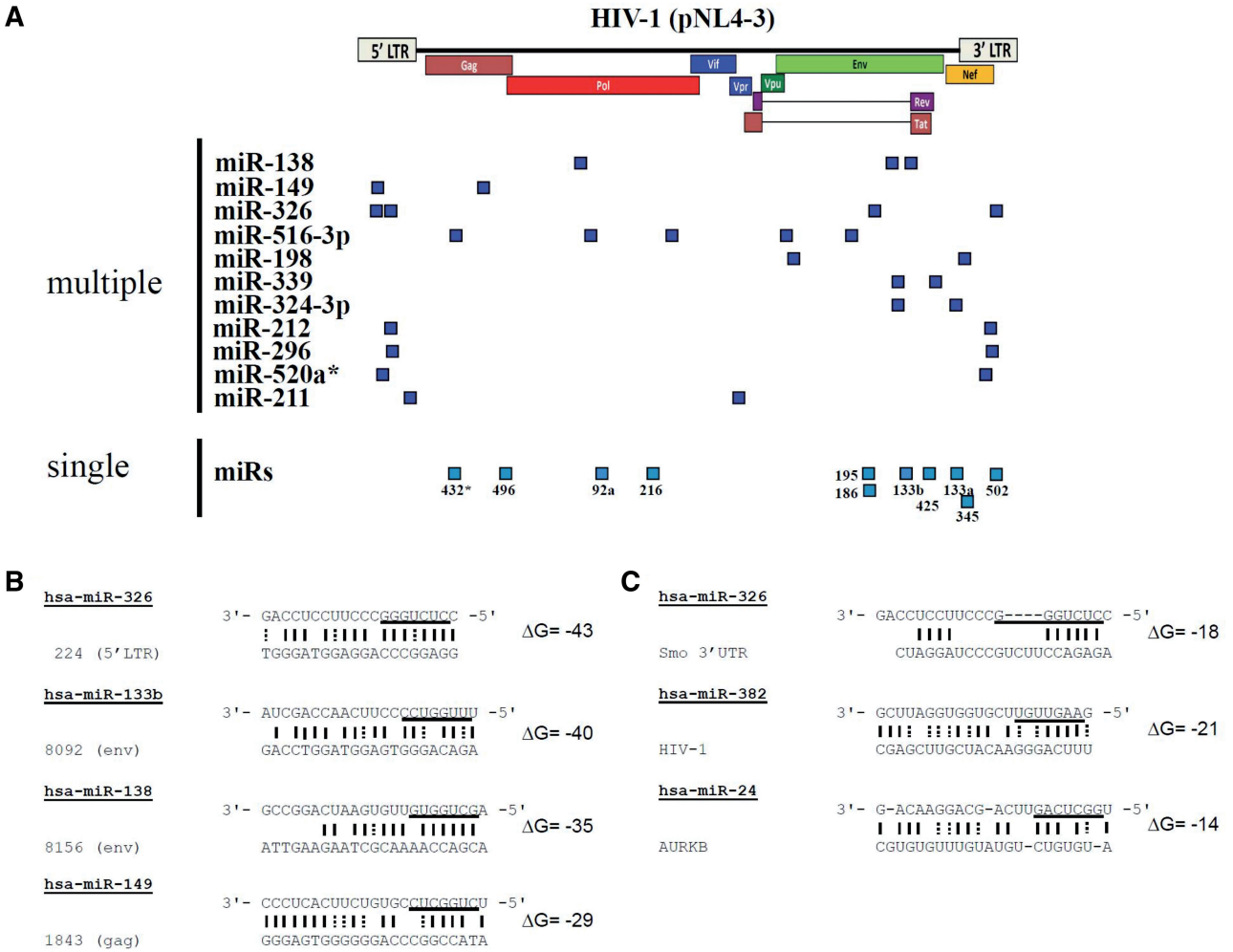

Figure 1. *In silico* screening of human miRNA target sites in the HIV genome. (**A**) Positions of 39 predicted target sites in the HIV NL4-3 genome for the indicated 22 human miRNAs are shown. The miRNAs with multiple putative target sites are shown at the top, and those miRNAs with single putative target sites are shown at the bottom. (**B**) Examples of predicted target duplexes for four miRNAs, miR-326, miR-133b, miR-138 and miR-149, are shown. The miRNA sequence (top) is shown with its seed sequence underlined, and its complementary HIV sequence indicated below. The numbering is based on the pNL4.3 sequence (left, nucleotide position numbers). (**C**) Three examples of published miRNA–mRNA target site duplex with mismatches or G:U pairings in the seed sequence are shown.

Figure S1). Overall, the extent of miRNA-(miR-133b-, miR-138-, miR-326-, miR-149-, miR-92a-) mediated repression of HIV-1 replication in these short term infection assays is consistent with findings elsewhere that such repression is generally modest in magnitude (5).

To move beyond single round assays, we next tested four miRNAs (miR-138, miR-326, miR-149 and miR-195) to assess their effects over 6 days of spreading HIV-1 infection (Figure 2B). Compared with control cells transfected with an irrelevant miRNA mimic (Oligo Fluo), we found that miR-138, miR-149 and miR-326 reproduced their single round effects (Figure 2A) in significantly reducing a spreading HIV-1 infection at levels similar to that seen with the positive control miR-29b (Figure 2B). On the other hand, miR-195, which had no significant single-round effect (Figure 2A), behaved like the negative control (Oligo Fluo) in producing no effect on a spreading HIV-1 infection (Figure 2B).

Increasing miRNA–mRNA complementarity enhanced the potency of repressing HIV-1 replication

The various cellular miRNAs that target HIV-1 anneal with imperfect complementarity to viral sequences (Figure 1B). Potentially, some of these miRNA–mRNA mismatches could originally have been perfectly matched, and the current sequence may have arisen from changes that arose to reduce the RNAi-mediated restriction of viral replication (Figure 2B); accordingly, changes in viral sequences away from perfect base pairing (strong restriction) to imperfect base pairing (weaker restriction) might be favored. In this line of thinking, one would expect the virus to mutate its sequence just enough to skirt endogenous RNAi restriction, whereas not so dramatically as to create fitness deficits. To test the notion that miRNA-restriction of HIV-1 correlates with the extent of sequence complementarity, we next asked if 'correcting' miRNA–mRNA

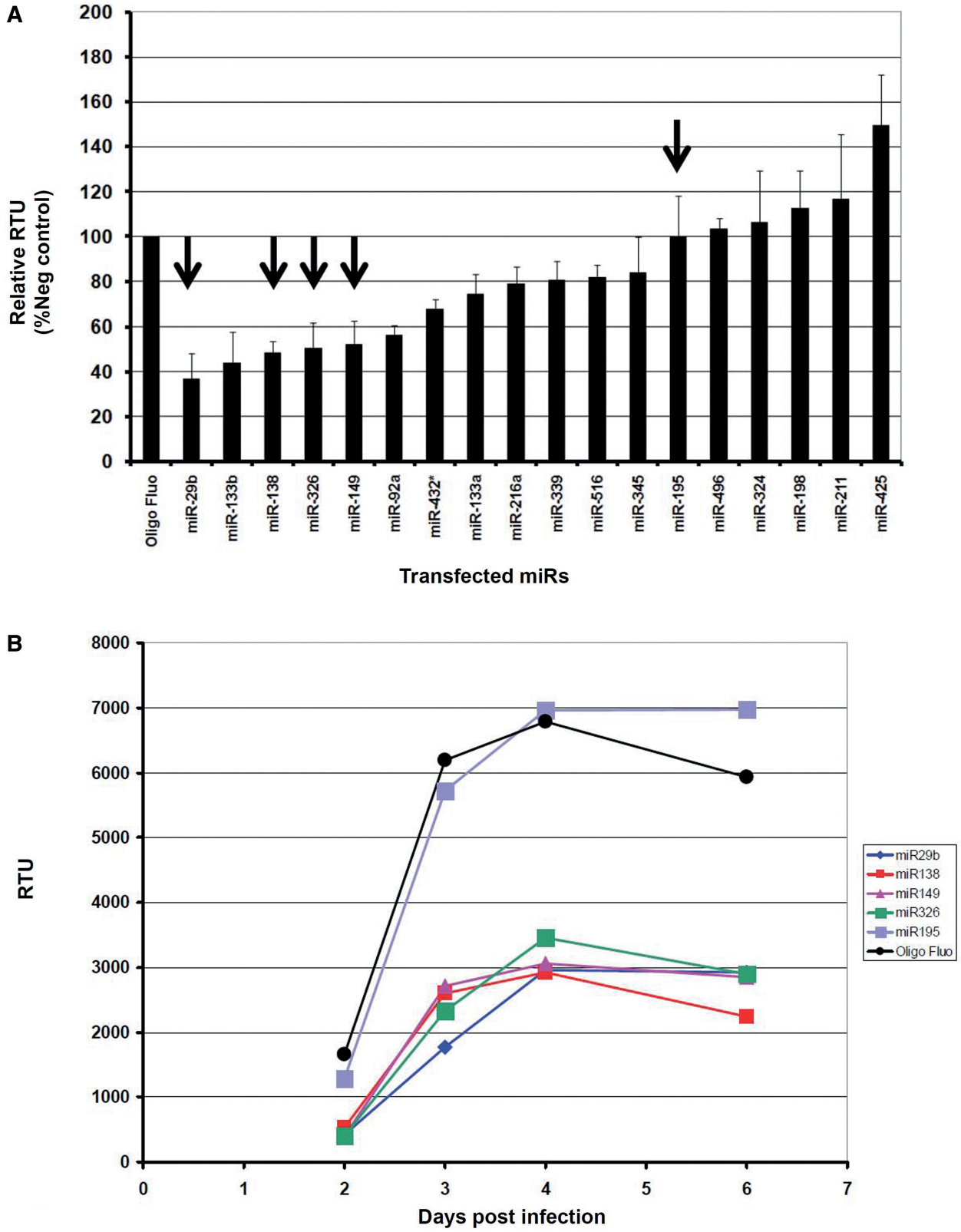

Figure 2. Expression of human miRNAs suppress HIV-1 replication in cell culture. (**A**) Effects of transfection of the indicated individual miRNAs on HIV-1 replication in cultured cells. The 42CD4 cells were transfected with the indicated miRNAs, and cells were infected with HIV-1 NL4-3 24 h later. Viral replication was monitored by measuring RT production in culture supernatants, 3 days after infection. Values are expressed as percent of a control irrelevant RNA oligonucleotide (oligo fluo). Arrows indicate miRNA chosen for follow-up experiments. (**B**) Time course replication curves spanning 2, 3, 4 and 6 days post-infection in cells transfected with miR-138, miR-149, miR-326, miR-195 and miR-29b (indicated by an arrow in panel A) and irrelevant control RNA oligonucleotide (oligo fluo); miR-29b is a positive control; oligo fluo is a negative control. Results are representative of three independent experiments. Values are the mean of three separate experiments with standard deviations.

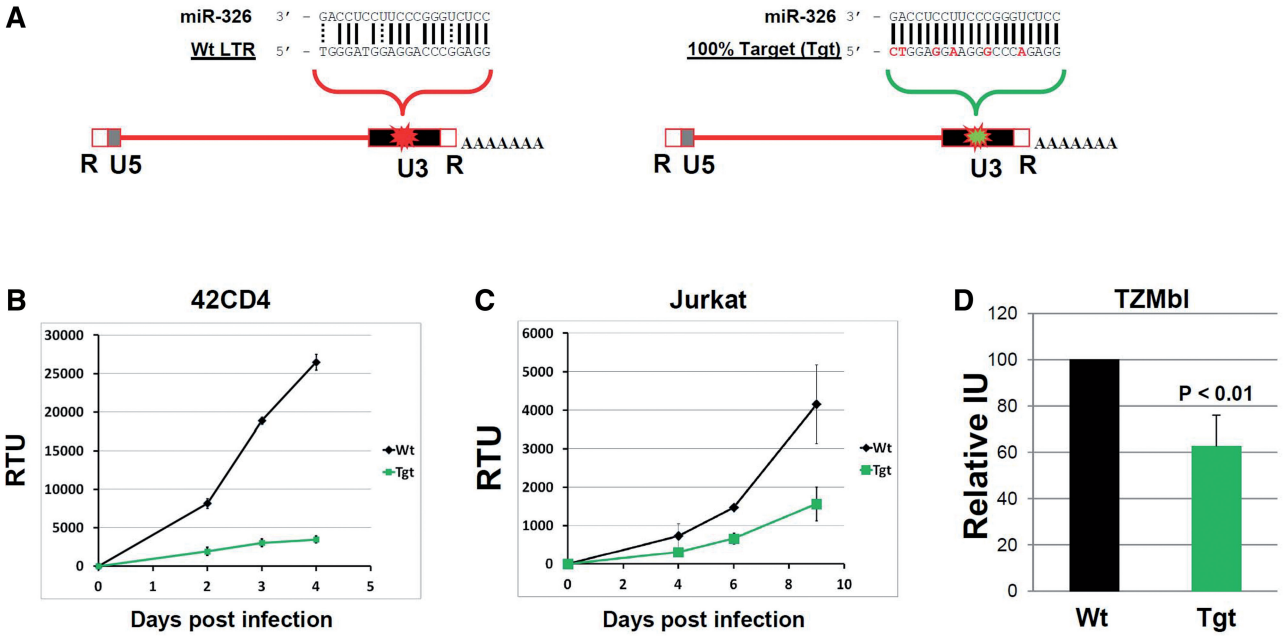

Figure 3. Restriction of an HIV-1 genome containing a 3' LTR site engineered to have perfect complementarity with miR-326. (**A**) A schematic representation of engineered HIV genomes containing a pNL4-3 Wt miR-326 target site or a target site created by changing the indicated nucleotides to create a sequence fully complementary to human miR-326 (Tgt). (**B**) and (**C**) Comparisons of the replication of Tgt and Wt viruses in 42CD4 (B) and Jurkat (C) cells. Cells were infected with the same amount of Wt or Tgt virus (normalized using reverse transcriptase measurements), and the infections were monitored by measuring supernatant RT 2, 3 and 4 days (42CD4) or 4, 6 and 9 days (Jurkat) post-infection. (**D**) TZMbl indicator cells were infected with RT equivalents of Wt or Tgt viruses. On completing 24 h post-infection, cells were fixed and infectivity was measured by β-galactosidase assay. *P*-value was determined by Student's *t*-test comparing Wt with Tgt. Values are the mean of three separate experiments with standard deviations.

mismatches would increase the repression of viral expression/replication.

To address the above, we focused on a putative miR-326 target in pNL4-3. This miR-326 target sequence in NL4.3 virus was designated as 'Wt'. In its current form, it contains three nucleotide mismatches with the cellular miR-326, although it retains a fully matched seed region [nucleotide positions 2–7 of the miRNA critical for miRNA-targeting and function (46,47)] and has an overall duplex free energy of −43 kcal/mol (Figure 1). In addition, the miR-326 target site is located in the Nef ORF located in the 3′ U3 of all the viral transcripts. Moreover, we found that miR-326 is expressed at several hundred copies per cell in cell lines and primary T cells (Supplementary Figure S2A), amounts that have been shown to be relevant for miRNA-mediated functions; and this miR-326 target site is well conserved among various HIV-1 isolates (Supplementary Figure S2B).

Starting from Wt, we created a version of the virus that is fully complementary to miR-326 by correcting the three mismatched nucleotides and converting the three GU wobble base pairing to the prototypic Watson–Crick GC and UA base pairing. We designated this latter fully matched sequence to miR-326 as 'Tgt' (Figure 3A). These base changes in Tgt did not alter major LTR transcription factor binding sites such as SP1 and NF-κB. Next, we compared the effect of miR-326 on an HIV that contains either a Tgt or a Wt site in its 3′ LTR. We first monitored the replication of the Tgt and Wt viruses in 42CD4 cells that were used for our initial screening, and next we replicated the virus in Jurkat cells (Figure 3B and C). The results showed that the Tgt-virus replicated reproducibly 2- to 5-fold less well than the Wt-virus in Jurkat and 42CD4 cells, respectively (Figure 3B and C). On the other hand, when we made a virus with a miR-326 target site that is even less complementary to miR-326 than that in Wt virus, this altered virus, replicated better than Wt virus in Jurkat cells (Supplementary Figure S3). We also evaluated the differences between Wt and Tgt viruses in a single cycle infection using the TZMbl cell line (Figure 3D). Infection of TZMbl cells with equal RT counts of Wt and Tgt virus showed that the Tgt infection yielded ∼40% less virus positive cells than Wt. We note that the experiments in Figure 3B–D, were all conducted in cell settings without exogenous over-expression of miR-326. Collectively, these results are consistent with an interpretation that cell endogenous miR-326 acts in a complementarity-driven manner to modulate HIV-1 replication in cells.

Tgt has 6 nt changes from Wt in its miR-326 site. The difference in replication seen in Figure 3 between these two viral genomes could be due to miR-326-mediated restriction or alternatively, it could be due to *cis*-sequence effects elicited by the 6 nt changes. To verify between an RNAi-dependent effect versus a *cis*-sequence difference between Wt and Tgt, we used siRNA against Dicer to knockdown Dicer expression in TZMbl cells (Figure 4A). SiRNA against Dicer (siDicer) decreased Dicer mRNA by >70%, whereas control

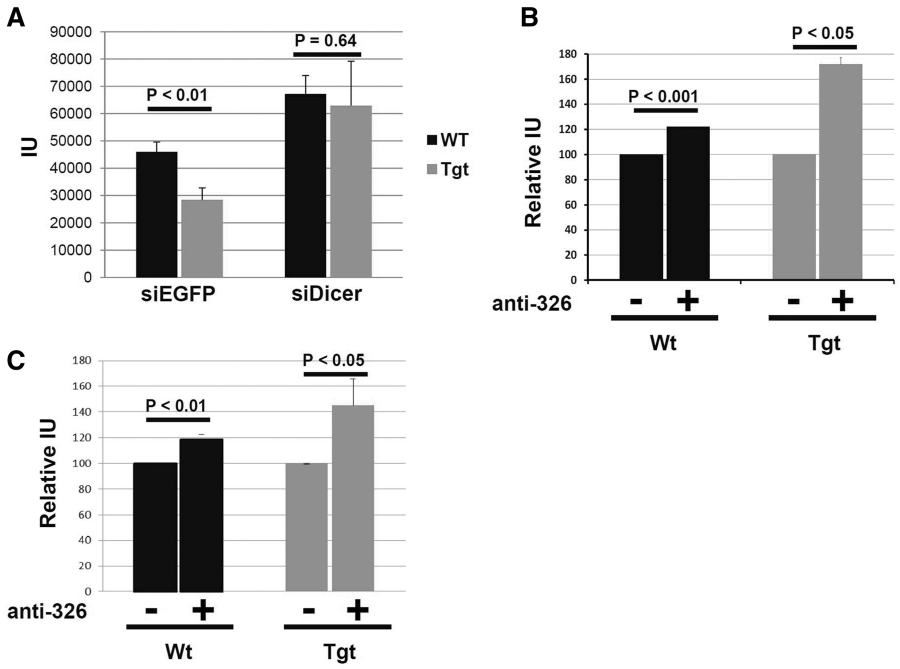

Figure 4. Deficit in Tgt replication is miR-326-specific. (**A**) SiRNA against Dicer (siDicer) equalizes the replication of Wt and Tgt viruses in TZMbl cells. HeLa-derived TZMbl indicator cells were transfected with siRNA against EGFP (control) or Dicer. After 24 h post-transfection, the cells were infected with equal amounts of Wt or Tgt virus normalized by RT. The cells were fixed and the amount of infectious virus was determined by β-galactosidase staining at 24 h post-infection. Values of Wt and Tgt viruses are plotted relative to each other. (**B**, **C**) Anti-miR326 antagomir increases relative Tgt-virus replication to a greater extent than Wt-virus replication. Anti-miR326 antagomir or control 'scrambled' antagomir was transfected into 42CD4 (B) or Jurkat (C) cells. After 24 h post-transfection, the cells were infected with equal amounts of Wt or Tgt virus. Infections were monitored by measuring supernatant RT at 2 days (42CD4) or at 6 days (Jurkat). *P*-values were determined using Student's *t*-test to compare Wt with Tgt. Values of Wt and Tgt viruses are plotted independently of each other. Note that in each case (Wt or Tgt) anti-326 (+) virus sample was graph-normalized to the respective control scrambled antagomir (−) sample, which was arbitrary set as '100'. In actual value, the Tgt scrambled antagomir (−) sample arbitrarily set at '100' is ∼60% (Figure 3D) of the value relative to the Wt scrambled antagomir (−) sample, which is also graphed as arbitrarily set at '100'. Because the values of Wt and Tgt viruses are plotted independently of each other, the graphs in (B) and (C) as drawn are intended to compare the Tgt (−) anti-326 relative to Tgt (+) anti-326 and the Wt (−) anti-326 relative to Wt (+) anti-326, but not intended to cross-compare the Wt samples with Tgt samples (in which case, the latter values should be corrected by a factor of ∼0.6).

siRNA directed against EGFP did not change the level of Dicer mRNA (Supplementary Figure S4). In the presence of siEGFP, Wt virus, as expected (similar to the results in Figure 3D), replicated better than Tgt virus (Figure 4A, left, siEGFP). On the other hand, when we knocked down Dicer, we observed that both Wt and Tgt viruses replicated equivalently (Figure 4A, right, siDicer). If the difference between Wt and Tgt viruses is due to a *cis*-sequence effect, then the knock down of Dicer should not normalize the difference; on the other hand, if the difference between Wt and Tgt viruses is due to miR-326-mediated RNAi-restriction, then the knock down of Dicer is indeed expected to normalize the difference between Tgt and Wt virus (Figure 4A). Thus, the findings in Figure 4A favor a miR-326 RNAi effect over a *cis*-sequence effect in explaining the difference between Tgt and Wt virus. In Supplementary Figures S5 and S6, we also show that the 6 nt change in Tgt versus Wt does not create a *cis*-Nef-function difference or a *cis*-LTR-promoter difference between Wt and Tgt virus (Supplementary Figures S5 and S6).

To further confirm the virus modulating role of cell endogenous miR326, we next employed anti-miR326 antagomir. Control 'scrambled' or anti-miR326 antagomirs were transfected into 42CD4 (Figure 4B) and Jurkat (Figure 4C) cells, and the cells were infected with equal amounts of either Wt or Tgt virus. We documented that the anti-miR326 antagomir effectively reduced the amount of miR-326 (Supplementary Figure S4), and in both cells, anti-326 compared with control scrambled antagomir increased Tgt replication to a greater degree ($P < 0.05$, 65% increase in Figure 4B; 42% increase in Figure 4C) than anti-326 compared with control scrambled antagomir increasd Wt replication ($P < 0.001$, 21% increase in Figure 4B; $P < 0.01$, 19% increase in Figure 4C). Taken together, the results in Figure 4 are consistent with the interpretation of a Dicer-dependent RNAi effect arising from cell endogenous miR-326, which exerts a more prominent restriction on Tgt than Wt virus.

To additionally validate that miR-326 more strongly represses the Tgt versus the Wt target site sequence, we created two Cytomegalovirus (CMV) promoter-driven constructs that contain three copies of either the Wt or the Tgt target positioned downstream of a luciferase reporter (Figure 5A). The two reporters were transfected into 293T cells, and relative luciferase expression was measured. In agreement with the virus replication results in Figures 3 and 4, the Tgt-reporter was consistently expressed at a lower magnitude than the Wt-reporter

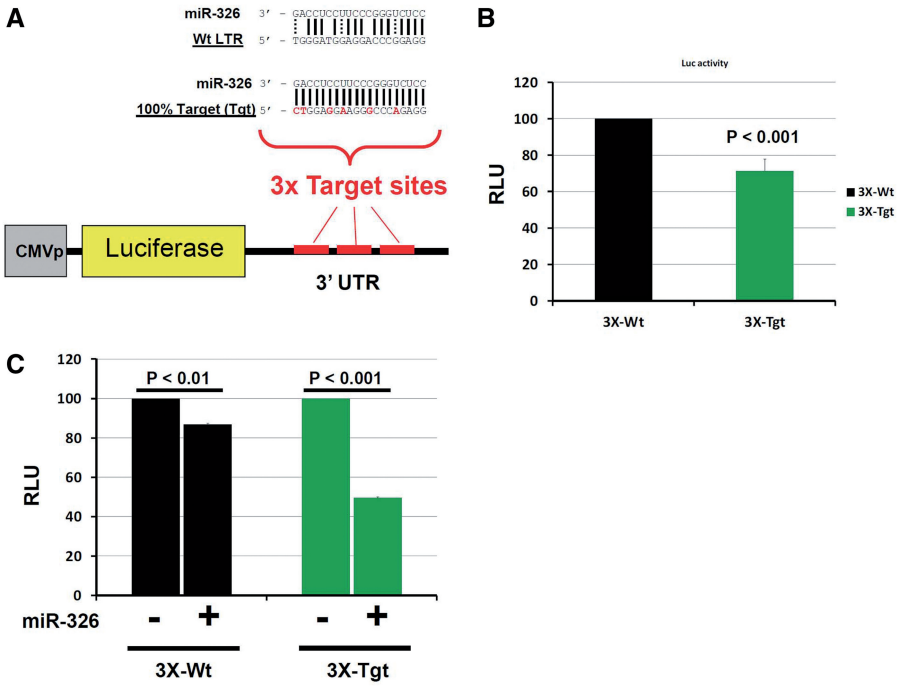

Figure 5. Increased miRNA–mRNA complementarity correlates with greater reduction of expression in reporter assays. (**A**) Schematic representation of CMV promoter-driven reporter constructs engineered to contain three tandem copies of either the Wt or Tgt target sites positioned downstream of the luciferase gene. (**B**) Effect of miR-326 on the Wt and Tgt target sites. The 293T cells were transfected with Luc-3X Wt or Luc-3X Tgt together with an internal control pMIR-βgal plasmid. After 24 h post -transfection, cells were lysed and luciferase and βgal activities were measured. The luciferase activity from Luc-3X Tgt, after normalization to βgal, was expressed relative to that from the Luc-3X Wt construct. (**C**) Effect of transfected miR-326 on Luc-3X Wt and Luc-3X Tgt. miR-326 (+) or negative control (−) siRNA (negative control siRNA #1, Ambion) was transfected into 293 T cells with either the Luc-3X Wt or Luc-3X Tgt constructs and the internal control pMIR-βGal. Luciferase and βgal activities were monitored 24 h post-transfection. Values (+) are given as percent of the value from the negative control siRNA (−) transfected cells which was set to 100%. Values are the mean of three separate experiments presented with standard deviations.

($P < 0.001$; Figure 5B) in cell settings where no miR-326 over-expression was performed. To check that this repressive effect can also be dose-dependent on miR-326 expression, we repeated the experiment in the context of miR-326 over-expression. Wt- and Tgt- luciferase reporters were co-transfected with a synthetic miR-326 or a negative control random RNA oligonucleotide. In the setting of miR-326 over-expression, Tgt-linked luciferase expression was decreased by 2-fold more than Wt-linked expression (Figure 5C).

To compare the replication of the two viruses in a rigorous manner, we performed 'head-to-head' competition, co-infecting cells with both viruses together in a single culture. This type of assay measures the relative replication potency of the two viruses more stringently than infections conducted in parallel culture flasks (48–50). 'Head-to-head' competitive co-infections with Wt and Tgt viruses in Jurkat cells were conducted at 1:1 or 1:4 (Wt:Tgt) ratios (Figure 6). On completing 9 days after infection, the cells were harvested, and total cellular RNA was extracted. Wt and Tgt viral RNAs were quantified using sequence-specific qPCR primers (Figure 6A). In the 1:1 co-infection, the levels of Wt and Tgt viral RNAs produced were, 4×10^5 (Wt) and 1×10^5 (Tgt) RNA copies at Day 9. As expected, by increasing the starting amount of Tgt virus relative to the Wt virus in the 1:4 infection, we observed an increase in the amount of Tgt viral RNA (Figure 6A) measuring 3.5×10^5 and 6×10^5 copies for Wt and Tgt viral RNAs, respectively at Day 9. Over a longer time course of passaging, the competition results illustrated clearly that the Tgt virus was more strongly repressed than the Wt virus. For instance, we passaged the culture supernatant obtained after 9 days of the first co-infection in a second round of co-infection, which was then harvested 9 additional days later and passaged in a third round of co-infection (Figure 6B). For each round, total RNA was extracted, and Wt and Tgt viral RNAs were quantified using sequence-specific qPCR. It was evident from comparing the amounts of viral RNAs at each round that the percentage of Tgt-RNA decreased more rapidly in proportion to Wt-RNA. Indeed, whether starting with a 1:1 (Wt:Tgt) ratio or a 1:4 (Wt:Tgt) ratio, by the third 9-day passage of co-infection with Wt and Tgt virus, the Tgt virus became <2–18% of the total virus population (Figure 6B). These results conducted in cells without miR-326 over-expression are consistent with a stronger restriction of Tgt-virus versus Wt-virus.

The extent of sequence complementarity with miR-326 correlates with HIV-1 replication in cells

In principle, a strong restriction of viral replication generally selects rapidly for 'escape' mutations, whereas weak or inconsequential restriction does not (50,51). To ask if

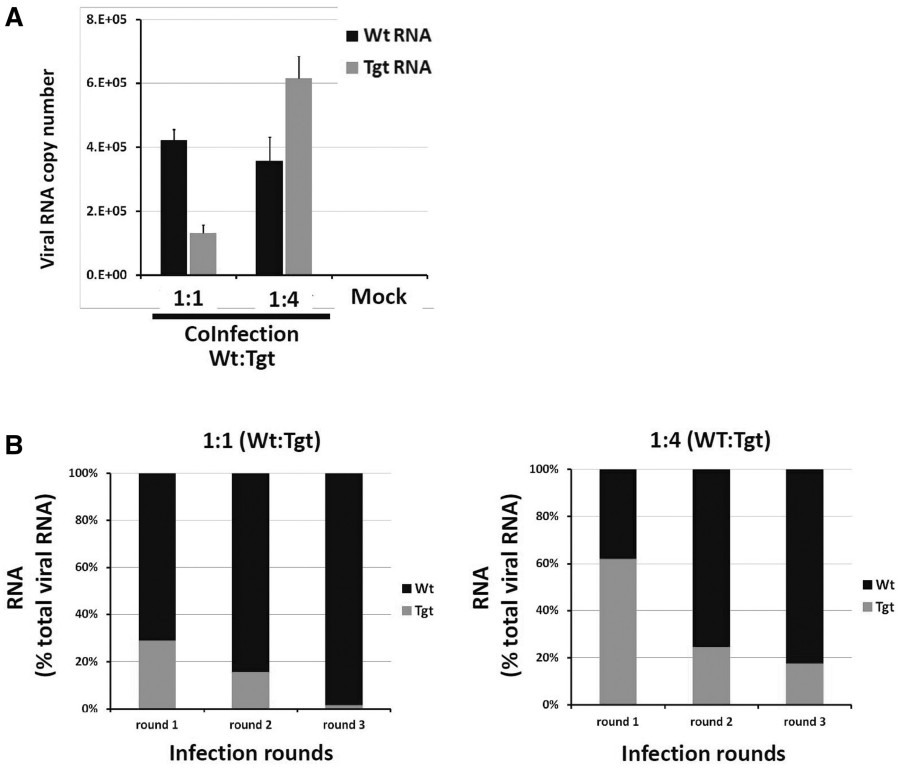

Figure 6. Virus replication competition assays in cells co-infected with Wt and Tgt viruses. (**A**) Quantification of Wt and Tgt viral RNAs in co-infected cells. Jurkat cells were co-infected with 1:1 or 1:4 (Wt:Tgt) ratios (normalized by RT) of input viruses. On completing 9 days after infection, cells were harvested and total cellular RNA was extracted. Viral RNAs for Wt and Tgt virus were then quantified using real time RTQPCR with primers specific for Wt and Tgt viruses. The corresponding RNA copy numbers are graphed. (**B**) Assessment of the Tgt RNA:Wt RNA ratios at the different rounds of infection. Supernatants from 9 days Wt+Tgt (1:1 and 1:4) co-infected cells (infectious round 1) were used to infect new Jurkat cells (round 2). After 9 days of infection, supernatants from round 2 of infection were used to initiate a third round of infection (round 3). The Wt and Tgt viral RNAs were quantified by QRT–PCR in infected cells at the end of each infection to calculate their relative amount at the end of each infection cycle (in percent of total virus genomic RNA). Data points represent geometric mean values from duplicate experiments, with error bars indicating standard deviation.

miR-326 restriction correlates with better viral RNA target complementarity to miR-326, we created seven new HIV viruses (1.1, 1.2, 1.3, 2.1, 2.2, 2.3 and 3.1), all of them fully isogenic, except for 1, 2 or 3 base changes in their miR-326-targeted Wt sequence to increase progressively (+1, +2 or +3 matches) their base pairing complementarity with miR-326 (Figure 7). These mutant viruses were individually tested in three rounds of co-infection paired with the Wt virus, and viral RNAs were quantified at the conclusion of each round of infection (Figure 8A). We noted that all mutant viruses were created to be better base pair-matched to miR-326 than Wt virus; and indeed all the mutant viruses were more strongly repressed than Wt virus in head-to-head replication competition (Figure 8), consistent with the extent of sequence complementarity with miR-326 correlating with the robustness of HIV-1 replication in cells.

DISCUSSION

Here, we show that target sites for more than 22 miRNAs are putatively represented in the HIV genome. Based on the current understanding of RNA biology, it is perhaps not surprising that among the many miRNA target sites in HIV-1, no site has perfect complementarity to its cognate miRNA. Nevertheless, when 17 human miRNAs were individually tested in transfections, we found 5 that significantly reduced HIV-1 replication by $\geq$40% (Figure 2A). We note that a 40% reduction per round of viral replication is a significant biological effect because natural virus infection in a host population likely transpires through thousands, if not millions, of 'parallel' rounds of replication during the diseases course and during this course, small single round effects quickly manifest as large overall changes.

An important question asked here is whether ambient cellular miRNAs (without ectopic miRNA-over-expression) could exert biologically relevant modulation of HIV-1 replication. Three findings support the likelihood that endogenous miRNA can restrict viral replication in an RNAi-dependent manner. First, the differential miR-326 effect on Tgt versus Wt virus occurs in a Dicer-dependent fashion (Figure 4A) consistent with an RNAi-effect. Second, antagomir-specific knockdown of cell endogenous miR-326 increases HIV-1 replication in Jurkat T-cells (Figure 4C) demonstrating that the observed effect is not dependent on miR-326 over-expression. Third, in eight different viruses with point nucleotide changes in their miR-326-targeted sequence, we found a correlation between increased miR-326–mRNA

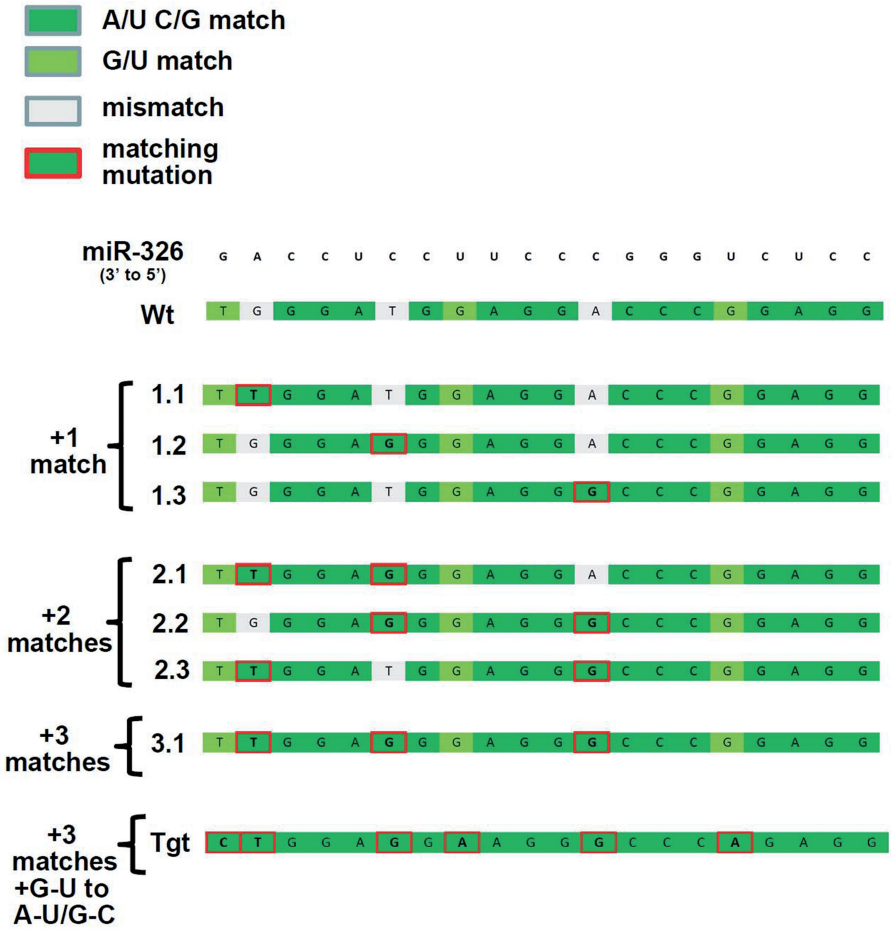

Figure 7. Creation of multiple miR326 target site mutants. Sequences are shown of various target sites with the indicated changed nucleotides designed to increase complementarity with miR-326. Top two rows show the miR-326 sequence and the Wt target sequence. Three mismatched positions between miR-326 and Wt (G, T, A) are shown in gray. The next three rows (+1 match) show single nucleotide changes to the Wt sequence that increase complementarity with miR-326. In 1.1, G is changed to T; in 1.2, T is changed to G; in 1.3, A is changed to G. The following three rows (+2 matches) show two nucleotide changes to the Wt sequence that increase complementarity with miR-326. In 2.1, the G, T are changed to T, G; in 2.2, the T, A are changed to G, G; in 2.3, the G, A are changed to T, G. In the last two rows (+3 matches), the G, T, A bases in the Wt sequence are changed to T, G, G in 3.1; and in Tgt, three other bases T, G, G are changed to C, A, A. Light and bold green represents either G-U or classical A-U/G-C base pairing, respectively. Red box indicates changed nucleotide.

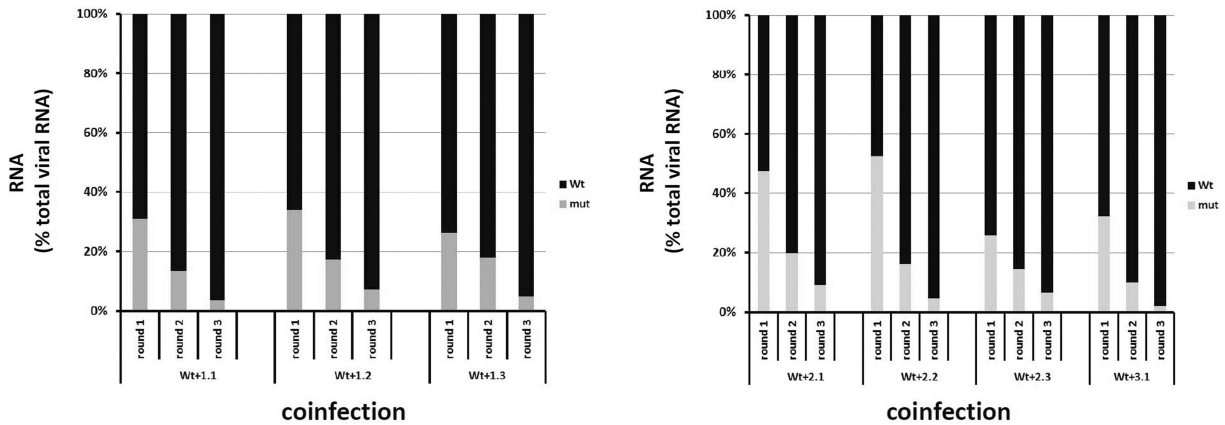

Figure 8. Increasing the complementarity of HIV sequence to miR-326 leads to greater restriction of the virus. Co-infection experiments in Jurkat cells with mutant and Wt viruses. Each of the seven changed viruses (1.1, 1.2, 1.3, 2.1, 2.2, 2.3 or 3.1) in A was used in head-to-head co-infection experiments with the Wt virus for three successive rounds of infection. The relative amounts of Wt and mutant RNA (in percent of total genomic RNA) at rounds 1, 2 and 3 are given for co-infection of Wt with 1.1, 1.2 or 1.3 virus (left graph) or Wt with 2.1, 2.2, 2.3 or 3.1 virus (right graph).

complementarity and increased miR-326-mediated repression of viral replication. Indeed, in head-to-head competitive co-infections between Wt and mutant viruses (where the latter viruses were constructed with target sites having better complementarity to miR-326), all mutant-viruses were more strongly restricted than the Wt-virus (Figure 8). While other explanations may be possible, the most parsimonious interpretation is that greater miRNA–mRNA complementarity correlates with better miRNA-mediated restriction of the target RNA.

Here, we have studied the complementarity between a miRNA and its target viral sequence as a determinant of restriction. Many other factors may influence the strength of a miRNA effect. We are aware that simple linear complementarity is likely an over-simplified determinant for several reasons. These reasons include that, the secondary RNA structure surrounding the target site may block access by miRNA-RISC [indeed, studies have shown that the U3 region is rich in secondary structures (52)], and cell endogenous miRNAs with good target complementarity may be functionally impotent unless their copy numbers are appropriately abundant to be loaded into an appreciable percentage of RISC complexes. A commonly demonstrated threshold needed for physiological functionality is 100 miRNA copies per cell (53). Indeed, the expression of in primary T cells and T-cell lines exceeds this level (Supplementary Figure S2); indicating that miR-326 meets this criterion for functional sufficiency. Other factors that affect miRNA potency include the expression of RNA-binding proteins that may shield mRNAs from being targeted by miRNAs, or the expression of strong or weak viral suppressors of RNAi that may modulate cellular miRNA-mediated restriction (20,54). In this context, the HIV-1 Tat protein has been reported by several investigators to have suppressor of RNAi activity (55,56); however, the demonstration of this activity in some assays has been difficult because of Tat's non-specific activation of promoters (57). How to integrate all of these observed effects is complex and will require further study.

Our current investigation of miRNA–HIV-1 interaction is a proof-of-principle study on the engagement of a host cell miRNA, miR-326, with a virus target sequence. Our findings and the accumulating literature converge in demonstrating roles for the RNAi machinery (19,33,58–60) and human miRNAs in cellular defense against various viral infections (21–25,27,61,62). The function of RNAi/miRNAs in plants and invertebrates as antiviral defenses is well established (63). Although the RNAi machinery found in lower eukaryotes is conserved in higher animals, it remains debated whether RNAi functions as an antiviral mechanism in mammals. Nevertheless, in the case of virus–miRNA interaction, in addition to the findings noted here and elsewhere, there is also increasingly persuasive evidence that the tissue tropism of animal viruses is guided by host tissue-specific miRNA expression (64,65).

Although our work is consistent with the view that ambient host miRNAs may provide a first encounter defense in cells against infecting viruses, the effectiveness of this defense remains to be carefully assessed in the light of adaptive immune mechanisms such as cytotoxic T cells and humoral immunity. Whether miRNA-mediated restriction mounts a sufficiently robust selection pressure to affect HIV-1 sequence changes also remains to be carefully evaluated.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

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