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Selective miRNA Modulation Fails to Activate HIV Replication in In Vitro Latency Models

María Rosa López-Huertas,1 Matías Morín,2 Nadia Madrid-Elena,1 Carolina Gutiérrez,1 Laura Jiménez-Tormo,1 Javier Santoyo,3 Francisco Sanz-Rodríguez,4,5 Miguel Ángel Moreno Pelayo,2 Laura García Bermejo,6 and Santiago Moreno1,7

1Servicio de Enfermedades Infecciosas, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS) and Hospital Universitario Ramón y Cajal, 28034 Madrid, Spain; 2Servicio de Genética, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS) and Hospital Universitario Ramón y Cajal, CIBERER, 28034 Madrid, Spain; 3Edinburgh Genomics, The Roslin Institute, University of Edinburgh, Scotland, UK; 4Fluorescence Imaging Group, Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain; 5Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS) and Hospital Universitario Universitario Ramón y Cajal, 28034 Madrid, Spain; 6Grupo de Biomarcadores y Dianas Terapéuticas, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS) and Hospital Universitario Ramón y Cajal, 28034 Madrid, Spain; 7Facultad de Medicina y Ciencias de la Salud, Universidad de Alcalá de Henares, 28871 Alcalá de Henares, Spain

HIV remains incurable because of viral persistence in latent reservoirs that are inaccessible to antiretroviral therapy. A potential curative strategy is to reactivate viral gene expression in latently infected cells. However, no drug so far has proven to be successful in vivo in reducing the reservoir, and therefore new anti-latency compounds are needed. We explored the role of microRNAs (miRNAs) in latency maintenance and their modulation as a potential anti-latency strategy. Latency models based on treating resting CD4 T cells with chemokine (C-C motif) ligand 19 (CCL19) or interleukin-7 (IL7) before HIV infection and next-generation sequencing were used to identify the miRNAs involved in HIV latency. We detected four upregulated miRNAs (miRNA-98, miRNA-4516, miRNA-4488, and miRNA-7974). Individual or combined inhibition of these miRNAs was performed by transfection into cells latently infected with HIV. Viral replication, assessed 72 h after transfection, did not increase after miRNA modulation, despite miRNA inhibition and lack of toxicity. Furthermore, the combined modulation of five miRNAs previously associated with HIV latency was not effective in these models. Our results do not support the modulation of miRNAs as a useful strategy for the reversal of HIV latency. As shown with other drugs, the potential of miRNA modulation as an HIV reactivation strategy could be dependent on the latency model used.

INTRODUCTION

According to the United Nation’s HIV/AIDS program UNAIDS, in 2015, 35 million people worldwide were affected by HIV.1 Antiretroviral therapy (ART) can successfully suppress HIV viremia and significantly delay disease progression. However, the infection remains incurable, and lifetime treatment is required because of viral persistence in latent reservoirs that are not accessible to ART and are undetectable by the immune system.2,3 Latency is a reversible, nonproductive state of infection of individual cells, and therefore the latently infected cells do not produce infectious virus particles but retain the capacity to do so.4 This life-long latent reservoir is quickly established in vivo after infection and consists mainly of several populations of memory resting (r)CD4 T cells, including central memory, transitional, and effector memory cells.5,6 The reservoir size is quite small in patients on ART, estimated at 10–100 replication-competent latent proviruses per million rCD4 T cells.7 However, it is sufficient to refuel viral replication after an ART interruption.8,9

A plausible strategy for curing HIV is to activate viral gene expression in these latently infected cells, using pharmacological drugs known as latency-reversing agents (LRAs). Viral reactivation should take place along with the removal of HIV-expressing cells via the action of immune cytotoxic effector cells: a strategy commonly known as “shock and kill.” To avoid undesirable side effects, only approaches that trigger HIV reactivation with minimal cellular activation may be considered for therapeutic use.8,10 To date, a major problem following LRA treatment has been that ex vivo assays show great diversity in the HIV reactivation responses that do not always correlate with the results of in vitro assays.11,12 Therefore, new anti-latency compounds that effectively reduce the size of the HIV reservoir in vivo are needed. However, because of the low frequency of latently infected cells in vivo, the use of in vitro models of latency is essential in the search for new anti-latency compounds prior to their testing in ex vivo and in vivo assays. These latency in vitro models must allow efficient viral integration while maintaining low levels of viral expression in rCD4 T cells, and each model should be considered complementary to...
MicroRNAs (miRNAs) are small, endogenous, non-coding RNA transcripts of 20–23 nt involved in the transcriptional regulation of gene expression.\cite{15,16} They have been shown to regulate the persistence and reactivation of HIV reservoirs through the deregulation of specific functional pathways important for HIV replication,\cite{17} but few data are available. The miRNAs involved in HIV infection are classified as viral or host encoded and then are further divided into those that target HIV transcripts directly or that indirectly affect the viral cycle through the regulation of cellular factors.\cite{18} Changes in the host’s miRNA profile in response to HIV latency persistence have been reported,\cite{19,20} and notably, miRNA-29 has already been suggested as a good candidate for incorporation into HIV eradication strategies.\cite{20} Furthermore, the simultaneous inhibition of miRNA-28, miRNA-125b, miRNA-150, miRNA-223, and miRNA-382 has also been shown to reactivate viral replication in ex vivo assays of rCD4 T lymphocytes isolated from ART-treated HIV-infected patients.\cite{19} However, there is no agreement on which specific miRNAs are involved in viral latency and reactivation, and there have been several contradictory results, including which miRNAs have an exclusive tendency toward downregulation.\cite{21,22}

In the current work, different in vitro latency models and next-generation sequencing (NGS) have been used to find new miRNAs involved in latency persistence and to explore the modulation of these miRNAs as a potential novel anti-latency strategy.

**RESULTS**

Establishment and Validation of the HIV Latency Models Used in This Study

In this study, we characterized HIV latency models based on exposing rCD4 T cells to chemokine (C-C motif) ligand 19 (CCL19) or interleukin 7 (IL-7) before HIV *in vitro* infection. A schematic of the models is shown in Figure 1A. Briefly, rCD4 T cells were treated with CCL19 or IL-7 for 5 days, infected with an X4-tropic NL4-3 HIV clone, and cultured for an additional 4 days in the presence of CCL19 or IL-7 and with a low concentration of IL-2.

Viral integration and basal replication were assessed at day 4. Proviral integration significantly increased 36- and 659-fold in CCL19- and
A vector expressing non-relevant, scrambled anti-miRNA, together with the reporter carboxyl fluorescein (FAM) (inhibitory [i]-scrambled-miRNA-FAM), was transfected into the HIV-infected CD4 T cells, which was, on average, 29.4% of cells (Figure 1D). The expression of i-scrambled-miRNA-FAM did not affect HIV replication in CCL19- or IL-7-treated cells, since the induced p24/gag levels were similar to those shown in the H2O-transfected cells used as the negative control (Figure 1E).

Identification of Novel miRNAs Potentially Involved in HIV Latency Persistence Using NGS

These HIV latency in vitro models allowed us to identify key miRNAs that play a role in avoiding HIV transcription and/or maintaining viral latency. At day 4 after HIV infection, RNA was extracted in two independent experiments and subjected to NGS. miRNA profiles from non-treated, CCL19-treated, or IL-7-treated HIV-infected rCD4 T cells were compared with those obtained from uninfected cells. Changes in miRNAs expression were considered significant when the fold change was >0.4 or <−0.4 and the adjusted p value (false discovery rate [FDR]) was <0.001. Only deregulated miRNAs found in both latency models and not present in the list of deregulated miRNAs of non-treated HIV cells were selected for further modulation. Based on these criteria, four upregulated miRNAs (miRNA-98-5p [referred to henceforth as miRNA-98], miRNA-4516, miRNA-7974, and miRNA-4488) were selected (Figure 2A; Table 1).

The expression levels of miRNA-98, miRNA-4516, and miRNA-7974 were analyzed by qRT-PCR in CCL19- and IL-7-treated, HIV-infected rCD4 T lymphocytes (Figure 2B). miRNA-4488 could not be measured or modulated, as the synthetic inhibitors and oligonucleotides were neither accurate nor robust enough. The oligonucleotide for miRNA-4488 amplification did not yield a measurable Ct value, even after 55 amplification cycles (data not shown). Therefore, miRNA-4488 was excluded from further study. The relative percentage of miRNA levels was calculated between infected and non-infected cells within the same cytokine treatment (CCL19 or IL-7). Consistent with NGS, qRT-PCR showed that miRNA-98, miRNA-4516, and miRNA-7974 expression was enhanced to 361%, 467%, and 609%, respectively, in the CCL19-treated infected cells (p < 0.05 and p < 0.001; Figure 1E).

The Specific Inhibition of the Upregulated miRNAs in Latently Infected rCD4 T Cells Did Not Enhance HIV Replication

The cells latently infected with HIV were challenged for virus replication by modulating the selected miRNAs for 72 h (Figure 1A). Specific inhibitors of the selected human miRNA-98, miRNA-4516, and miRNA-7974 were transfected alone or combined in CCL19- or IL-7-treated cells 4 days after HIV infection. Vehicle-transfected, latently infected cells were used as the negative control. Phorbol 12-myristate acetate (PMA), a known HIV activator, was added as a positive control.
Table 1. Differentially Expressed miRNAs in Non-, CCL19-, or IL-7-Treated rCD4 HIV-Infected Cells, When Compared with the Equivalent Non-infected Controls

| Name                  | logFC | logCPM | p Value     | FDR    |
|-----------------------|-------|--------|-------------|--------|
| Non-treated rCD4 versus Non-treated HIV rCD4 |       |        |             |        |
| hsa-miR-210-3p        | 3.46  | 7.43   | 5.62E–109   | 2.06E–106 |
| hsa-miR-126-5p        | −1.5  | 6.11   | 1.97E–23    | 3.6E–21 |
| hsa-miR-4532          | 1.41  | 6.51   | 1.04E–18    | 1.26E–16 |
| hsa-miR-143-3p        | −2.87 | 5.41   | 4.68E–09    | 4.28E–07 |
| hsa-miR-210-5p        | 2.31  | 3.14   | 1.74E–08    | 0.00000127 |
| hsa-miR-223-3p        | −1.96 | 6.64   | 6.66E–07    | 0.00000406 |
| hsa-miR-126-3p        | −1.69 | 3.26   | 0.00000249   | 0.0001 |
| hsa-miR-150-3p        | −0.49 | 7.71   | 0.00000522   | 0.0002 |

rCD4-CCL19 versus rCD4-CCL19-HIV1

| Name                  | logFC | logCPM | p Value     | FDR    |
|-----------------------|-------|--------|-------------|--------|
| hsa-miR-4488a         | 2.05  | 4.38   | 7.56E–07    | 2.97E–07 |
| hsa-miR-143-3p        | −0.47 | 11.38  | 0.0000186   | 0.0003 |
| hsa-miR-7704          | −0.83 | 5.82   | 0.000013    | 0.0003 |
| hsa-miR-23-2p         | 0.73  | 13.02  | 0.0000193   | 0.0003 |
| hsa-miR-30e-3p        | −0.62 | 8.74   | 0.0000335   | 0.0005 |
| hsa-miR-21-3p         | 0.59  | 12.75  | 0.0000431   | 0.0007 |
| hsa-miR-9794          | 1.33  | 4.16   | 0.0000527   | 0.0007 |
| hsa-miR-151a-3p       | −0.41 | 10.11  | 0.0000479   | 0.0007 |
| hsa-miR-194-5p        | −0.54 | 8.57   | 0.0000523   | 0.0007 |
| hsa-miR-30c-5p        | −0.39 | 10.68  | 0.0000762   | 0.0011 |
| hsa-miR-20b-5p        | 0.84  | 6.21   | 0.000082    | 0.0011 |
| hsa-miR-625-5p        | 0.87  | 6.66   | 0.0000947   | 0.0012 |
| hsa-miR-335-3p        | −1.14 | 6.07   | 0.0001      | 0.0013 |

IL-7-treated rCD4 versus IL-7-treated HIV rCD4

| Name                  | logFC | logCPM | p Value     | FDR    |
|-----------------------|-------|--------|-------------|--------|
| hsa-miR-4532          | 1.23  | 6.48   | 5.49E–17    | 2.04E–14 |
| hsa-miR-4488*         | 1.42  | 4.45   | 2.97E–09    | 5.52E–07 |
| hsa-miR-223-3p        | −2.32 | 6.86   | 6.07E–09    | 7.51E–07 |
| hsa-miR-126-5p        | −1.32 | 6.58   | 9.11E–09    | 8.45E–07 |
| hsa-miR-1307-5p       | 0.6545| 9.2    | 1.48E–08    | 0.0000011 |
| hsa-miR-7974*         | 4.59  | 3.48   | 4.61E–08    | 0.0000285 |
| hsa-miR-143-3p        | −3.11 | 6.08   | 8.22E–08    | 0.0000436 |
| hsa-miR-210-3p        | 2.23  | 8.49   | 0.00000197   | 0.0000914 |
| hsa-miR-98*           | 0.4189| 9.95   | 0.00006699   | 0.0003 |
| hsa-miR-4516*         | 1.25  | 3.66   | 0.000013    | 0.0005 |

Data are fold change of deregulated miRNAs (logFC), the log of counts per million of reads (logCPM), the p value, and the adjusted p value FDR (false discovery rate). *miRNAs shared by both latency models and not presented in non-treated cells.

13-acetate (PMA) was used as a positive control for viral reactivation and was added to mock-transfected cells for the last 18 h. The efficiency of inhibition was evaluated 72 h later for each miRNA by qRT-PCR (Figure 3A; Table 2). Data are the relative percentage of miRNA levels in comparison to mock cells. In the CCL19 model, the expression levels of miRNA-98, miRNA-4516, and miRNA-7974 were reduced to 3.4%, 48.0%, and 32.2%, respectively, after combining all miRNA inhibitors. Similarly, the expression levels of miRNA-98, miRNA-4516, and miRNA-7974 were reduced to 8.8%, 53.9%, and 35.2%, respectively, after the combined modulation. In both latency models, the combination of all the miRNA inhibitors yielded similar inhibition of each miRNA as when used individually (Table 2).

Cell viability and HIV replication were assessed 72 h after miRNA inhibition. Cell viability was not affected by miRNA-98, miRNA-4516, or miRNA-7974 inhibition, alone or combined, in CCL19- or IL-7-treated HIV-infected cells (Figure 3B). PMA was added as a positive control of viral reactivation for the last 18 h. HIV replication was enhanced more than 5-fold after PMA stimulation, in both latency models (Figure 3C). However, HIV latency could not be reversed after the inhibition of miRNA-98, miRNA-4516, and miRNA-7974, alone or combined, in either CCL19- or IL-7-treated cells (Figure 3C), as no significant increase in HIV replication was detected.

The Inhibition of miRNA-98, miRNA-4516, and miRNA-7974 Did Not Enhance the Replication of R5-Tropic HIV, Alone or Combined with LRAs

Most of the viruses that establish the reservoir during early infection are CCR5 tropic. Therefore, rCD4 T cells treated with CCL19 were infected with the JR-CSF HIV strain and then challenged for reactivation by inhibiting altogether the expression of miRNA-98,
miRNA-4516, and miRNA-7974. Vehicle-transfected, latently infected cells were used as mock cells. PMA was used as a positive viral control. The efficiency of inhibition was evaluated 72 h later for each miRNA by qRT-PCR (Figure 4A; Table 2). Data are the relative percentage of miRNA levels in comparison to mock cells. The expression levels of miRNA-98, miRNA-4516, and miRNA-7974 were reduced to 0.5%, 39.3%, and 22.9%, respectively, after combining all miRNA inhibitors. HIV replication was enhanced 9.9-fold after PMA stimulation (p < 0.05), but was not reversed after the inhibition of miRNA-98, miRNA-4516, and miRNA-7974 (Figure 4B).

The combination of LRAs is considered a plausible strategy to reduce the size of the HIV reservoir in vivo.11,28 We therefore studied whether combining the inhibition of these miRNAs with one of the most common LRAs would reverse HIV latency. CCL19-treated and JR-CSF-infected rCD4 T cells were transfected with the inhibitors of miRNA-98, miRNA-4516, and miRNA-7974 together (all-i-miRNAs) and then treated with romidepsin, panobinostat, or bryostatin-1 during the last 18 h (Figure 4C). Mock-transfected cells were also treated. PMA was used as a positive control of viral reactivation, which was enhanced around 9.0-fold in both mock and all-i-miRNA-transfected cells (Figure 4C). Viral replication was enhanced 14.4- and 8.5-fold, respectively, in mock and all-i-miRNA-transfected cells treated with romidepsin. This represents a 0.7-fold reduction in all-i-miRNA-transfected cells versus the mock ones (p < 0.01). HIV replication in mock cells treated with panobinostat or bryostatin-1 was enhanced 10.9- and 7.9-fold, respectively, and was similar overall in cells with inhibited miRNAs.

**Inhibition of miRNAs Previously Described to Be Associated with HIV Reactivation Did Not Reverse HIV Latency in CCL19-and IL-7-Based In Vitro Models**

It has been shown that combined inhibition of miRNA-28, miRNA-125b, miRNA-150, miRNA-223, and miRNA-382 resulted in HIV replication in cells in vitro transfected with an NL4.3-expressing vector and also in rCD4 T cells isolated from HIV-infected individuals on ART.19 Therefore, we evaluated whether inhibition of these miRNAs would affect HIV replication in the latency models used in this study.
In the CCL19-based model, the expression levels of miRNA-28, miRNA-125b, miRNA-150, miRNA-223, and miRNA-382 were reduced to 3.1%, 1.7%, 0.1%, 3.8%, and 1.4%, respectively, in the sample transfected with the inhibitors for each miRNA altogether (Figure 5A; Table 2).

A similar inhibition was achieved in IL-7-treated cells, with expression levels reduced to 3.5%, 0.3%, 0.05%, 3.3%, and 8.1% for miRNA-28, miRNA-125b, miRNA-150, miRNA-223, and miRNA-382, respectively, after combined inhibition (Figure 5A; Table 2).

Cell viability was not significantly affected in any experimental condition (Figure 5B). HIV replication was not induced after individual or combined inhibition of these miRNAs in either of the two models tested (Figure 5C). However, PMA stimulation induced HIV replication by around 2.5-fold in both models.

**Prediction of Targets of These miRNAs Potentially Related to HIV Latency**

Putative targets of miRNA-98, miRNA-4516, miRNA-7974, and miR-4488 were predicted by means of the miRDB and miRTarBase databases. Common predicted targets are represented in Figures 6A and 6B, respectively. As expected, prediction of targets was different, depending on the database, due to different algorithms. miRDB was less restrictive and identified polio virus receptor-like (PVRL)1 (CD111 or nectin-1) and MiRTarBase pointed out CDKN1A (p21) as a common target for these four miRNAs. Functional enrichment

| HIV Latency Model miRNA Modulated | miRNA-98 | miRNA-4516 | miRNA-7974 |
|-----------------------------------|---------|-----------|-----------|
| mock                             | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 |
| rCD4 T cells i-miRNA-98          | 3.3 ± 0.7 |          |          |
| CCL19 treated i-miRNA-4516       |          | 75.6 ± 11.6 |          |
| HIV infected i-miRNA-7974        | 3.4 ± 0.9 | 48.0 ± 19.0 | 32.2 ± 15.9 |
| all-i-miRNA together             | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 |
| rCD4 T cells i-miRNA-98          | 3.3 ± 0.7 |          |          |
| IL-7 treated i-miRNA-4516        |          | 75.6 ± 11.6 |          |
| HIV infected i-miRNA-7974        | 3.4 ± 0.9 | 48.0 ± 19.0 | 32.2 ± 15.9 |
| all-i-miRNA together             | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 |

Data are the mean ± SEM, and are expressed as the percentage of change using 5S levels as a reference.
of the common targets was studied using the GOstats and Panther databases (Figures 6C and 6D, respectively). Common targets of the four miRNAs appeared to be involved in immune process, metabolic process, or stimuli response. Using the same methodology, we analyzed the common targets for the five miRNAs identified by Huang et al.19 miRDB did not identify any common target and miRTarBase identified TP53 as a common target for four of the five miRNAs (Figure 6E).

Finally, the mRNA expression of the common miRNAs’ putative targets nectin-1 and p21 was analyzed by qRT-PCR in CCL19- and IL-7-treated cells infected with the NL4.3 strain of HIV, compared with untreated cells (Figure 6F). Consistent with the increased expression of the four selected miRNAs, nectin-1 and p21 mRNA levels were downregulated 0.30- and 0.21-fold in the IL-7 model (p < 0.05 and p < 0.01), respectively. In CCL19-treated cells, nectin-1 and p21 levels were downregulated 0.81- and 0.57-fold, respectively, although this decrease was not statistically significant.

DISCUSSION

We identified novel miRNAs potentially involved in latency and explored their modulation as a new LRA approach for switching from viral latency to replication. However, miRNA modulation was an ineffective tool for HIV reactivation in our models of latency.

The profiling of miRNAs in HIV-infected T lymphocytes has been suggested as a valuable tool to identify endogenous miRNAs suitable for modulation to purge latently infected cells,12,13 but no study has been published so far using in vitro models of HIV latency. In this work, cellular miRNAs with a potential role in maintaining HIV latency were identified using NGS in two independent latency models that may be considered complementary, as suggested by another study.14 The NL4.3 HIV strain was used and miRNAs that were shown to be significantly deregulated in both latency models were selected. However, neither the individual nor the combined modulation of these miRNAs yielded viral replication in these HIV latency models. In addition, in CCL19-treated cells, combined inhibition of the selected miRNAs did not affect replication of R5-tropic HIV, which is the phenotype of the viruses that establish the reservoir in vivo.26,27 Furthermore, a combined treatment, including inhibition...
of the selected miRNAs and treatment with one of the best known LRAs, i.e., romidepsin, panobinostat or bryostatin-1, led to the same negative result, and therefore the hypothesis that miRNA modulation may reduce the threshold for viral reactivation was not confirmed.

Both models used in the study fulfilled criteria previously shown to be of importance for robust in vitro HIV latency models: the induction of efficient viral integration, together with restricted viral replication. However, basal replication was around 2.5-fold higher in IL-7- than in CCL19-treated cells probably because viral integration is dramatically higher in IL-7-treated cells, as an action of the cytokine itself, since IL-7 has been shown to reactivate latent HIV in rCD4 T cells from infected individuals. However, this difference does not seem relevant when compared to fully activated cells treated with PHA/IL-2.

In any case, each model resembles different physiological scenarios of HIV infection. CCL19 is in vivo a ligand for CCR7, a molecule constitutively expressed in lymphoid organs that is essential in guiding the migration of naive and memory T cells into lymphoid tissue and in the clustering of T cells with dendritic cells. Interestingly, the latent HIV infection predominantly occurs in rCD4 T cells expressing CCR7, and therefore CCL19 is a critical factor that conditions rCD4 T cells to HIV infection, integration, and latency. For the in vitro CCL19-treated cells, the mechanism associated with the establishment of latency is a rapid de-phosphorylation of cofolin and the subsequent changes in polymerized actin. However, no clinical trial involving the treatment with CCL19 has been implemented so far in ART-treated HIV patients or in other diseases, and the relevance of these in vitro assays has not yet been confirmed. On the other hand, IL-7 contributes to the maintenance of HIV reservoirs via homeostatic proliferation, but it does not disrupt viral latency in latently infected cells, because NF-κB increased slightly and too insufficiently to reverse viral latency. In addition, IL-7 enhances the phosphorylation of the restriction
factor SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase (SAMHD)1, abrogating its antiviral activity, increasing viral RT, and rendering CD4 T cells more susceptible to HIV integration and to the establishment of latent infection.40 Several clinical trials using IL-7 have been performed in HIV ART-treated individuals in order to increase the number and function of lymphocytes and to test IL-7 as a potential anti-latency drug.38,41–44 In some of these assays, IL-7 induced expansion of CD4 T cells42–44 but failed to deplete the viral reservoir and even increased, at least transiently, the HIV DNA copy numbers.43,44 This may be due to the expansion of the pool of T CD4 cells containing provirus but also to the phosphorylation of SAMHD1, leading to HIV integration and reservoir replenishment.40 Nevertheless, IL-7 treatment in clinical trials occurred after HIV infection, whereas IL-7 exposure in our in vitro assays occurred before the infection, and therefore we cannot assume that the same mechanisms were acting.

To our knowledge, this is the first report to identify miRNAs associated with HIV latency in in vitro latency models. The IL7 model involves extensive miRNA alterations, since HIV latency affected more than 30 miRNAs. In contrast, the CCL19 model exhibited a lower number of miRNAs associated with latency. Remarkably, four miRNAs were identified in both models. These results strongly suggest that the miRNA profile associated with latency establishment and maintenance could be model dependent, but a common set of these regulators is always associated with HIV latency. Further studies including other latency models could be done to test this hypothesis. In addition, it is important to verify the information gained from in vitro models within latently infected cells obtained from ART-treated patients.45 In this regard, the quantitative viral outgrowth assay (QVOA) currently remains the gold standard,26 but could not be performed in this study because of the negative nature of our results. However, the reliability of our findings is supported by the fact that the CCL19-based model may represent the best proxy primary in vitro model currently available for analyzing the activation capabilities of LRAs, when compared to QVOA.47 Although the same results have been achieved in both latency models, it should be noted that both models cannot be regarded as equals. The CCL19-based latency model has been widely characterized29,30,37,48,49 and used by others47,50,51 whereas the IL-7-based latency model has only recently been described40,52 and its value remains to be confirmed.
The importance of miRNA modulation in reversing HIV latency was first shown ex vivo more than 10 years ago in rCD4 T cells from ART-treated patients, but particular miRNAs in this context have not been successfully implemented to date. This pioneer study used constructions of 1.9- or 1.2-kb fragments from the 3′ end of HIV, including the reported EGFP gene to identify the miRNAs involved in latency. In this study, miRNA-28, miRNA-125b, miRNA-150, miRNA-223, and miRNA-382 downregulated transcripts containing a 1.2-kb fragment from the HIV 3′ UTR that harbors target sequences for these miRNAs. Combined inhibition of these miRNAs activated HIV replication. However, we were unable to replicate these results despite the successful modulation of the same miRNAs. There were a number of differences between the two studies: the previous study used transfection instead of infection, and incomplete viral genomes that overall do not resemble a natural HIV infection as crucial steps of the viral cycle including viral entry, retro-transcription, and nuclear transport are missing. As explained above, the establishment of latency in the in vitro models used in our study relies on enabling retrotranscription and nuclear transport through the abrogation of coflin or SAMHD1. Despite of the suitability of CCL19- and IL-7-based models, HIV replication was not altered after simultaneous modulation of the five miRNAs previously identified. Indeed, our NGS approach did not indicate the modulation of any of these miRNAs.

Our study, however, has not been the only controversial publication in the context of miRNAs in HIV replication. As an example, contradictory findings have been published in regard to the modulation of the miRNA-17/92 cluster during HIV replication, probably because of the different time points and half-lives of the cultures, which resulted in a differential regulation of cellular pathways involved in T cell activation. Indeed, only miRNA-143-3p and miRNA-10a-5p exhibited similar trends of suppressed expression in different approaches. The effect of host miRNAs on HIV replication appears to be highly dependent on the experimental parameters, the quantification method, the type of cell, and the HIV variants. Our results suggest that this principle might affect HIV latency as well.

Finally, HIV acute infections achieved by transfection of the NL4.3 clone have been shown to downregulate 312 host miRNAs, with no upregulations observed. Our results, based on latent HIV infection using a similar clone, gave downregulated and also a set of upregulated miRNAs, suggesting that different miRNA profiles are exploited during latent persistence. These differences may account for active versus dormant infection but may also reflect the poor concordance already described among studies describing the HIV-mediated modulation of host miRNAs. It is also worth pointing out that highly expressed miRNAs may be important for normal cellular homeostasis. Thus, using pooled anti-miRNAs to activate HIV in latently infected cells could result in toxicities. However, we did not report them.

The accuracy of the reagents used to modulate and amplify these miRNAs may partially account for these unexpected results. In fact, miRNA-4488 could not be neither quantified nor modulated because validated probes are not available yet. Specific inhibition of miRNA-4516 and amplification of miRNA-7974 were also difficult to attain with the provided commercial tools. We observed great variations among the biological replicates. Technical replicates within each biological sample reproduced the results and confirmed the disparity. Replicate measurements are not independent tests and cannot provide evidence of the reproducibility of the results. Therefore, only one value—the mean of all the technical replicates for each biological sample was considered as a result to be represented and used in the statistical analyses. Nevertheless, reasonable biological arguments for the observed results are provided above.

In any case, none of the miRNAs identified in the present manuscript directly controls genes involved in HIV latency. Altogether, our results indicated that the four miRNAs do not act cooperatively in HIV latency establishment or reactivation and, in fact, do not share many targets. Depending on the database, p21 and nectin-1 were identified. Remarkably, nectin-1 and p21 mRNA levels were downregulated in the IL-7-based model, and to a lower extent, also in CCL19 latently infected cells, strongly suggesting both proteins to be real miRNAs targets. The cyclin-dependent kinase inhibitor p21 has been linked to the inhibition of HIV replication. Indeed, p21 blocks HIV replication at the RT step through the increase of the antiviral activity of SAMHD1 and acts downstream of p53 in non-cycling human macrophages. Accordingly, the four miRNAs here identified, through p21 modulation, could affect HIV replication more than the establishment of viral latency. Particularly, the inhibition of the four miRNAs would lead to p21 upregulation, and therefore inhibition of HIV replication could be expected. Changes in HIV replication after miRNA inhibition was not observed in our models in correlation with this supposition. In any case, further experiments should be done to test this hypothesis. Nectin-1 is a key protein in cell-cell adhesion and acts also as a viral receptor of herpes simplex virus (HSV) and Pseudorabies virus. Moreover, Nectin-1 is sequenced to adhere junctions and facilitates HSV infection in epithelial cells exposed to HIV through the regulation of matrix metalloproteinases. In any event, the predicted cooperative regulation of nectin-1, mediated by the miRNAs deregulated in our latency models, would not affect HIV latency or replication, according to our findings. Remarkably, functional analysis of the five miRNAs identified by Huang et al. did not yield any cooperative common targets. Consequently, because of this lack of functional cooperation, the modulation of the five miRNAs in our models did not lead to HIV reactivation. In summary, although we have identified differentially expressed miRNAs associated with latency in two in vitro models, the inhibition of these selected miRNAs, individually or combined, was ineffective for HIV latency reversal in vitro. It could be that miRNA modulation alone is not sufficient to reactivate HIV and may be due to HIV’s counteracting mechanisms keeping a selective pressure that stimulates latency preservation.

Overall, the number of miRNAs identified as potential tools in HIV re-activation is growing, but the selection of particular miRNAs in
this context appears to be cell model dependent and still lacks efficacy for most candidates.

**MATERIALS AND METHODS**

**Cells**

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden). Human rCD4 T lymphocytes were isolated with a CD4 T Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany) and the subsequent depletion of CD25+ and HLA-DR+ cells was performed using CD25 MicroBeads II and anti-HLA-DR MicroBeads (Miltenyi Biotec), respectively.

**Models for In Vitro HIV Latent Infections**

Figure 1A shows a schematic of HIV latency models. The HIV infection of purified primary rCD4 T lymphocytes was performed as previously described, with minor modifications. Briefly, rCD4 T cells were initially cultured for 5 days in the presence of CCL19 (29 nM) or IL-7 (5 nM) (both from R&D Systems, Minneapolis, MN) or left inactivated. HIV infection has been performed as previously described. Briefly, infectious supernatants of NL4.3 or JR-CSF strains were obtained from a calcium phosphate transfection of 293T cells with HIV plasmids and treated with DNase (QIAGEN, Hilden, Germany) before infection. Then, the rCD4 T lymphocytes were infected with 10 pg of p24 per million cells for 2 h with gentle rotation at room temperature. The cells were centrifuged at 600 g for 30 min at 25°C and then extensively washed with 1× PBS. HIV-infected cells were cultured in RPMI supplemented with IL-2 (10 IU/mL) (Sigma-Aldrich, St. Louis, MO, USA), together with CCL19 or IL-7 at the concentrations indicated above for an additional 4 days. At this time, provirus integration and basal viral replication was assessed. Integrated HIV was quantified as previously described. Briefly, strong stop DNA was quantified using specific primers for the R and U5 regions of the HIV long terminal repeat (LTR). Episomal forms of 2-LTR and integrated HIV proviral DNA were quantified by PCR on a LightCycler 480 Instrument II (Roche Diagnostics, Mannheim, Germany). A standard curve of integrated DNA from 8E5 cell line was prepared, and CCR5 was used as the normalization gene.

Basal HIV replication was calculated by measuring the p24/gag antigen in the culture supernatants with a commercial ELISA (Innotest HIV Ag mAb; Fujirebio Europe, Ghent, Belgium) and a microplate in the culture supernatants with a commercial ELISA (Innotest HIV Ag mAb, Fujirebio Europe, Ghent, Belgium). The HIV Ag antigen was measured using the Innotest HIV Ag mAb and influenza expression (Innotest HIV Ag mAb) and miRNA expression levels (qRT-PCR).

**Construction of miRNA Libraries and NGS**

CCL19- or IL-7-treated rCD4 T cells latently infected with the HIV NL4-3 strain from two independent donors were used. Non-treated cells were also included as the negative control. At day 4 after HIV infection, total RNA, preserving the small RNA fraction, was extracted, using the mirVana miRNA Isolation Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. The quantity and quality of the RNAs were evaluated with the Agilent 2200 TapeStation. High-throughput sequencing was carried out on a HiScanSQ platform (Illumina Inc., San Diego, CA). Libraries for sequencing were prepared using TruSeq Small RNA Sample Prep Kit V1 (Illumina), according to the TruSeq Small RNA Sample Preparation Guide. Briefly, ligation of the 3′ adaptor was conducted incubating 1 μg of total RNA from each sample with the adaptor for 2 min at 70°C. Then the 5′ adaptor was added alongside by a truncated T4-RNA ligase 2 (New England Biolabs, Ipswich, MA) for 1 h at 28°C. Half of the ligation product was used for RT with SuperScript II RT (Life Technologies) in a thermocycler for 1 h at 50°C. Next, enrichment of the cDNA was performed using PCR cycling: 30 s at 98°C; 11 cycles of 10 s at 98°C, 30 s at 60°C, and 15 s at 72°C; a final elongation of 10 min at 72°C; and a pause at 4°C. PCR products were resolved on 6% Novex Tris-Borate-EDTA (TBE) PAGE gels (Life Technologies). miRNA fragments between 145 and 160 bp were cut from the polyacrylamide gel and extracted with a MinElute gel extraction kit (QIAGEN), according to the manufacturer’s instructions. The libraries were visualized on a 2100 Bioanalyzer with the High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA) and quantified using qPCR with the Library Quantification Kit (Master Mix and DNA Standards; Kapa Biosystems, Wilmington, MA). The libraries were pooled, and 9 pM pool libraries were sequenced to generate single-ended 50-bp reads. Low-quality reads (Q < 30) and reads that contained only Illumina adapters were filtered out from the raw data. Filtered reads were mapped against the human hg19 genome using the Novoalign program, which strips adapters from the reads before mapping and detects hairpin structures in the mapped region. Unique mapping reads, with a minimum length of 17 nt and a maximum of a single mismatch, were obtained in a sequence alignment map (SAM) file and then sorted, indexed, and converted to a binary alignment map (BAM) file, using Samtools. The total count for each of the miRNAs was obtained with HTSeq-count, using a gff (gene-finding format) file with all human miRNA features from mirBase (release 21). Normalization of miRNA counts and differential expression between treated and non-treated cells was performed using the edgeR package from Bioconductor. Then, dataset comparisons to obtain the differentially expressed microRNA profiles were performed.

**qRT-PCR Assays**

The isolation of total RNA containing miRNAs was carried out using the mirVana miRNA Isolation Kit (Life Technologies), and cDNA was synthesized from 100 ng of total RNA using the miRCURY LNA Universal RT microRNA PCR System (Exiqon), according to the manufacturer’s instructions. cDNA synthesis was carried out under the following conditions: 42°C for 60 min and 95°C for 5 min. The expression levels of miRNA-98, miRNA-4516, miRNA-7974, miRNA-28, miRNA-125b, miRNA-150, miRNA-223, and miRNA-382 were quantified with a specific mirRNA-LNA PCR primer set.
for PCR amplification for each miRNA (Exiqon). Primers used for amplification of the putative targets nectin-1 and p21 have been described. Small 5S rRNA was used as a housekeeping gene for data normalization; a 5S rRNA PCR primer set (Exiqon) was used. ExiLENT SYBR Green PCR Master Mix (Exiqon) was used according to manufacturer’s instructions. PCR cycle conditions were 95°C, 10 min; 55 cycles: 95°C, 10 s; and 60°C, 1 min, with a subsequent melting-curve analysis. All reactions were performed in a LightCycler 480 Real-Time PCR System (Roche Diagnostics). The Cts observed for all the miRNAs were lower than 35, although miRNAs inhibition rendered higher Cts, as expected. Data analysis was performed using the second-derivative method. Once the Ct was obtained, data were normalized with the Ct values of 5S rRNA and analyzed with the formula 2−ΔΔCt, with mock-transfected cells as the reference control.

Electroporation and HIV Re-activation with LRAs
CCL19- or IL-7-treated rCD4 T cells were transfected 4 days after HIV infection with synthetic inhibitors for specific human miRNAs, alone or using the combinations described above, with the use of a Gene Pulser Electroporator (Bio-Rad, Hercules, CA). In brief, 3 million cells were collected in 350 μL RPMI 1640 medium without supplements and mixed with 100 nM each miRNA inhibitor. The cells were transfected in a cuvette with a 4-mm electrode gap (Bio-Rad) at 250 V, 950 microfarads, and maximum resistance. After transfection, the cells were incubated for 72 h at 37°C in supplemental RPMI including IL-2 (10 IU/mL) together with CCL19 (29 nM) or IL-7 (5 nM). A scrambled miRNA inhibitor control expressing 5-carboxyfluorescein (FAM) as a reporter gene (i-scrambled-miRNA-FAM) (Power inhibitor negative control; Exiqon) (100 nM) was used as the control of transfection efficiency and measured by flow cytometry on a FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA), using CellQuest software. PMA was used as a positive control for HIV replication and was added to mock-transfected cells for the last 18 h. In some experiments, PMA or an LRA was added to mock-transfected cells or to cells transfected with inhibitors for all the selected miRNAs during the last 18 h, before harvesting. The LRAs and concentrations used were romidepsin (40 nM), panobinostat (30 nM), and bryostatin-1 (10 nM).  

Cell Viability
Cell viability was measured in latently infected rCD4 T lymphocytes after inhibition of selected miRNAs using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Briefly, 2 x 10^5 cells were incubated for 10 min at room temperature in CellTiter-Glo Reagent, which contained lysis buffer and thermostable luciferase. The luminescent signal correlated with cell viability was analyzed on an Orion Microplate Luminometer with Simplicity software (Berthold Detection Systems, Pforzheim, Germany).

Ethics Statement
The fresh human whole-blood samples used in this study were obtained from healthy anonymous volunteers at the Transfusion Center of Madrid (Spain). Appropriated informed consent was obtained from each subject in accordance with the Spanish legislation on blood donor regulations, in compliance with the confidentiality and privacy rules. The procedures carried out in this study were approved by the Institute Ramón y Cajal for Health Research (IRYCIS) ethics committee, in compliance with the Declaration of Helsinki.

Bioinformatics Studies
Predicted target genes of selected miRNAs were recovered from miRTarbase 8 (http://mirtarbase.mbc.nctu.edu.tw/php/index.php/) and miRDB 67,70 databases (http://www.mirdb.org/). miRTarBase has accumulated more than 360 thousand miRNA-target interactions (MTIs), collected from the literature after filtering research articles related to functional studies of miRNAs. The recently updated MirDB includes 2.1 million predicted gene targets regulated by 6,709 miRNAs. Targets genes were selected using as the criterion that a gene had to have at least two positive computational predictions with different algorithms or reported biological evidence. Functional enrichment analysis was performed using GOSstats (http://gostats.com/) and Panther (http://www.pantherdb.org/) databases. A threshold of p < 0.001 was selected to filter significant GO terms.  

Statistical Analysis
The Kruskal-Wallis test with Dunn’s post-hoc analysis or two-way ANOVA with the Bonferroni post-test analysis was performed, using Prism 5.0 (Graph Pad, San Diego, CA).

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.06.006.

AUTHOR CONTRIBUTIONS
S.M. and L.G.B. conceived the study. M.R.L.-H. and L.G.B. led the design of the study. All authors discussed results and participated in the design. M.R.L.-H. implemented the HIV latency models. M.R.L.-H and L.J.-T. performed the experiments. M.M., M.A.M.P., and J.S. analyzed the NGS data and suggested the deregulated miRNA. M.R.L.-H., L.G.B., and S.M. wrote the manuscript. All authors read and approved the submission of the manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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