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RNase P-Associated External Guide Sequences Inhibit HIV-1 Infection by Shutting Down Human CCR5 Expression

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Abstract: Background: External guide sequences (EGSs) are small RNA molecules capable of hybridizing to a target mRNA and rendering the target RNA susceptible to degradation by ribonuclease P (RNase P), a tRNA processing enzyme. **Methods:** In this study, natural tRNA-originated and engineered variant EGSs were constructed to target the mRNA encoding human CC-chemokine receptor 5 (CCR5), an HIV co-receptor. **Results:** The EGS variant was about 100-fold more efficient in inducing RNase P-mediated cleavage of the CCR5 mRNA sequence in vitro than a natural tRNA-derived EGS. Furthermore, the expressed variant and natural tRNA-originated EGSs decreased CCR5 expression by 98% and 73–77% and reduced infection by the CCR5-tropic $HIV_{Ba\text{-}L}$ strain in cells by more than 900- and 50-fold, respectively. By contrast, cells expressing these EGSs exhibited no change in the expression of CXCR4, another HIV co-receptor, and showed no reduction in infection by the CXCR4-tropic HIV_{IIIB} strain, which uses CXCR4 instead of CCR5 as the co-receptor. Thus, the EGSs specifically targeted CCR5 but not CXCR4. **Conclusions:** Our results demonstrate that EGSs are effective and specific in diminishing HIV infection and represent a novel class of gene-targeting agents for anti-HIV therapy.

Keywords: RNase P; HIV; CCR5; gene targeting; gene therapy

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1. Introduction

Human immunodeficiency virus (HIV), a zoonotic pathogen, is the causative agent of AIDS. Chemokine receptors serve as co-receptors for HIV-1 entry [1,2]. In particular, human CC-chemokine receptor 5 (CCR5) and CXCR4 serve as the primary co-receptors for macrophage tropic (M-tropic or R5) HIV strains (e.g., $HIV_{Ba\text{-}L}$) and T cell tropic (T-tropic or X4) HIV variants (e.g., HIV_{IIIB}), respectively [3,4]. Studies have been performed to understand the normal physiological functions of CCR5 and suggested potential clinical benefits and pathological consequences due to its absence or reduced expression [5–9]. These results showed that CCR5 represents an excellent anti-HIV target because reduced CCR5 expression correlated with improved resistance to HIV infection and slowed HIV progression [10,11].

Nucleic acid gene interference and editing technologies, including ribozymes, antisense oligonucleotides, small interfering RNAs (siRNAs), and CRISP/Cas9 guide RNAs, have been explored as methods for studying and treating human diseases, such as HIV infection and AIDS [11–18]. Ribonuclease P (RNase P) catalyzes a hydrolysis reaction to generate 5′ mature termini of all tRNAs by cleaving a precursor tRNA leader sequence [16,19,20]. RNase P recognizes its diverse substrates primarily by their structures instead of their sequences (Figure 1A). Thus, this enzyme can cleave a target mRNA in the presence of a

custom-designed RNA molecule called an external guide sequence (EGS) as long as the EGS binds to the mRNA in a complex resembling a pre-tRNA molecule (Figure 1A,B) [21,22]. EGS expression in human cells has been shown to reduce the gene expression and replication of HIV, herpes simplex virus (HSV-1), and human cytomegalovirus (HCMV) by targeting their encoded mRNAs [23–25]. Moreover, EGS variants were generated using evolution in vitro procedures and exhibited better targeting activity in vitro than the EGS constructed from natural tRNA sequences [25]. However, whether these active EGS variants can be used to block HIV infection has not been investigated. Using CCR5 mRNA as the target, in this report, we investigated whether engineered EGS RNAs can effectively block CCR5 expression and shut down HIV infection in cultured cells.

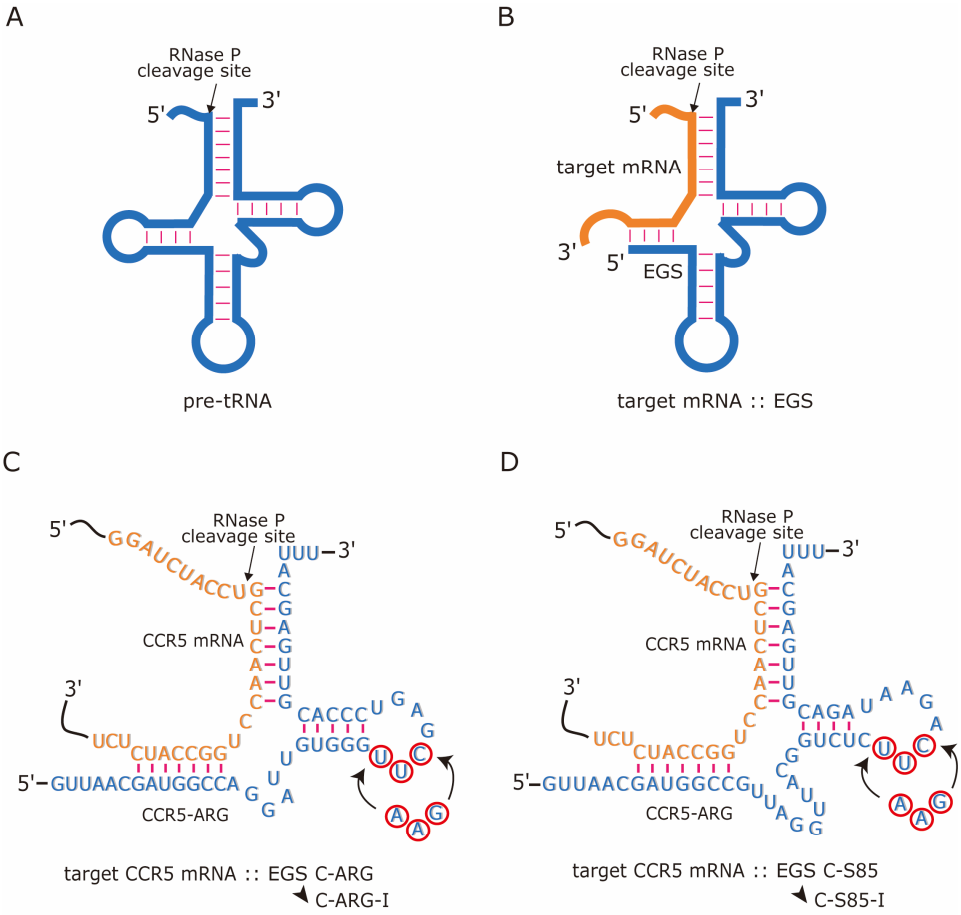


Figure 1. Various RNase P substrates. (**A**) Pre-tRNA. (**B**) Complex of a target mRNA and an EGS resembling a pre-tRNA. (**C**) Complexes between the CCR5 mRNA sequence and active EGS C-ARG and inactive control EGS C-ARG-I, and (**D**) complexes between the CCR5 mRNA sequence and active EGS C-S85 and inactive control EGS C-S85-I. The C-ARG sequences equivalent to the T-stem, T-loop, and variable regions of a tRNA molecule were from $tRNA^{arg}$ [26]. The arrowhead indicates the RNase P cleavage site, which is 204 nucleotides downstream from the translation initiation site of CCR5 [27]. The three nucleotides marked in circles in C-ARG and C-S85 were mutated to generate control EGSs C-ARG-I and C-S85-I, respectively.

2. Materials and Methods

2.1. Cells and Viruses

HeLa cells, PA317 cells, human PM1 cells, HIV R5 strain Ba-L ($HIV_{Ba\text{-}L}$), and X4 strain IIIB (HIV_{IIIB}) were obtained from Dr. Shibo Jiang and the NIH AIDS Research and Reference Reagents Program [28].

2.2. Construction of EGS RNAs and RNA Substrate

The DNA sequence that encodes substrate ccr5-s was constructed by PCR using pGEM3zf (+) as a template and oligonucleotides AF25 (5′-GGAATTCTAATACGACT CACTATAG-3′) and CCR-s-3 (5′-GGTTCGGTCAGAGATGGCCAGGTTGAGCAGGTAGAT CCCCTATAGTGAGTCGTATTA-3′) as the 5′ and 3′ primers, respectively. Polymerase chain reaction (PCR) was used to generate the DNA sequences of the EGSs with construct pTK112 DNA [23] as the template. To construct the DNA that encodes EGS C-ARG, the 5′ and 3′ primers were oligoARG5 (5′-GGAATTCTAATACGACTCACTATAG GTTAACGATGGCCAGGATTGTGGG–3′) and oligoARG3 (5′-AAGCTTTAAACGCTCAAC GTGGGACTCGAACCCACAATCCT-3′), respectively. To construct the DNA that encodes EGS C-ARG-I, the 5′ and 3′ primers were oligoARG5 and oligoARG-I3 (5′-AAGCTTTAAACGCTCAACGTGGGACTCCTTCCCACAATCCT-3′), respectively. To construct the DNA that encodes EGS C-S85, the 5′ and 3′ primers were oligoS855 (5′-GGAATTCTAATACGACTCACTATAGGTTAACGATGGCCGUUAGGUUACGG-3′) and the oligoS853 (5′-AAGCTTTAAACGCTCAACGTCTATTCTGAAGAGACCGTAACCTAA-3′), respectively. To construct the DNA that encodes EGS C-S85-I, the 5′ and 3′ primers were oligoS855 and the oligoS85-I3 (5′-AAGCTTTAAACGCTCAACGTCTATTCTCTTGAGACCG TAACCTAA-3′), respectively.

2.3. Binding and Cleavage Assays In Vitro

Human RNase P (purified from HeLa cells) was allowed to react with [^{32}P]-labeled ccr5-s in the presence of different EGSs at 37 °C in buffer A (100 mM NH_4Cl, 50 mM Tris, pH 7.4, and 10 mM $MgCl_2$) [22,25,29]. The cleavage reactions were analyzed by gel electrophoresis followed by use of a STORM 840 Phosphorimager (Molecular Dynamics, Mountain View, CA, USA). The kinetic parameters were determined as described previously [25,29]. The equilibrium dissociation constants (K_d) of the complexes of the EGSs and substrate ccr5-s were determined following the previously described protocol [25,29]. The values were the average of three experiments in duplicate.

2.4. Construction of the EGS-Expressing Cell Lines

The DNAs coding for the EGSs were subcloned into retroviral vector LXSN under the control of the U6 RNA promoter and transfected to PA317 cells [30,31]. At 48 h post transfection, the retrovirus-containing culture supernatants were used to infect human PM1 cells, and those cells containing EGSs were then selected and cloned in the presence of neomycin (800 μg/mL) (Thermo Fisher, Waltham, MA, USA). Northern and western blot analyses were used to determine the mRNA and protein levels of actin, CXCR4, and CCR5, respectively, following the procedures described previously [23,32]. In northern blot analysis, total RNAs were harvested from culture cells, separated in formaldehyde gels, transferred to nitrocellulose membranes, subjected to hybridization of [^{32}P]-labeled DNA probes, and analyzed using a a STORM 840 Phosphorimager. The radiolabeled DNA probes to detect human H1 RNA, EGS RNAs, CXCR4 mRNA, and CCR5 mRNA were cloned from plasmid constructs pH1 RNA, ptRNA^{arg}, pEGS-S85, pCXCR4, and pCCR5, respectively, and prepared as described previously [23,32].

2.5. Studies of HIV Gene Expression and Infection

PM1 cells that either expressed EGSs or did not express EGSs were infected with HIV_{Ba-L} or HIV_{IIIB} (MOI of 0.05). Culture media were harvested at different times and HIV production was assessed by quantifying the p24 level with a p24 ELISA kit (Abcam, Waltham, MA, USA). To determine intracellular HIV RNA levels, RNA samples were isolated from infected cells collected at different times and quantified us-

ing a qRT-PCR assay that contained SYBR Green and amplified the *tat* sequence using actin mRNA as the internal control. The PCR reactions were run in a Bio-Rad iCycler with HIV primers 5-TA-5 (5′-CATCCAGGAAGTCAGCCT-3′) and 3-TA-3 (5′-TTCCTGCCATAGGAGATGC-3′) in PCR mix (1× titanium Taq PCR buffer, 1 mM dNTPs, SYBR Green (1:50,000), 10 nM fluorescein, 1× titanium Taq DNA polymerase (Bio-Rad)) [33]. The actin mRNA level was used as the control and quantified by qRT-PCR with 5′ primer Actin5 (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′) and 3′ primer Actin3 (5′-CTAGAAGCATTGCGGTGGCAGATGGAGGG-3′) [34].

2.6. Statistical Analysis

Experiments were carried out in duplicate and repeated three times. We assessed the data using GraphPad Prism software (version 10) and conducted statistical analyses with (one-way) analysis of variance (ANOVA). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Targeting CCR5 mRNA by RNase P-Associated EGSs In Vitro

We engineered EGSs (Figure 1C,D) to bind to a CCR5 mRNA sequence with no sequence homology with other members of the CC-chemokine receptor family [1,2]. The target CCR5 sequence in our study contained the RNase P cleavage site, which is 204 nucleotides downstream from the CCR5 translation initiation site [27]. The choice of this targeting sequence with no sequence homology with other CC-chemokine receptors would ensure no potential cross-targeting of other chemokine receptors by the anti-CCR5 EGSs. Additionally, the target CCR5 sequence may have lacked a secondary structure or protein binding and was likely accessible to EGS binding because our experiments showed that this CCR5 mRNA region was modified by dimethyl sulphate (DMS) in DMS-treated cells.

Using evolution in vitro procedures, we previously generated highly active EGS variants that induced RNase P degradation of the thymidine kinase (TK) mRNA sequence of HSV-1 [25]. In this study, we present the investigation of a newly identified and highly active EGS variant, V85, in directing RNase P cleavage of CCR5 mRNA (see below, Table 1). EGS C-S85 was derived from the EGS domain of V85, resembling the T-loop, T-stem, and variable regions of a tRNA (Figure 1D). EGS C-ARG was constructed from the human $tRNA^{arg}$ sequence [26] (Figure 1C). Control inactive EGSs C-S85-I and C-ARG-I were derived from C-S85 and C-ARG, respectively, and contained three point mutations (5′-UUC-3′ -> AAG) at the T-loop (Figure 1C,D). These three nucleotides are highly conserved [26,35] and critical for RNase P–EGS recognition [25,36,37].

Table 1. Kinetic parameters in the cleavage reaction of the CCR5 mRNA substrate (ccr5-s) by RNase P in the presence of different EGSs. Experimental details are described in Materials and Methods.

Substrate ccr5-s	**$K_{m(apparent)}$ (μM)**	**$V_{max(apparent)}$ (pmol·min^{-1})**	**$V_{max(apparent)}/K_{m(apparent)}$ (pmol·μM^{-1}·min^{-1})**	**K_d (μM)**
+C-ARG	0.85 ± 0.15	0.019 ± 0.006	0.022 ± 0.005	2.7 ± 0.4
+C-ARG-I	ND	ND	ND	2.7 ± 0.4
+C-S85	0.21 ± 0.05	0.53 ± 0.10	2.5 ± 0.5	0.017 ± 0.004
+C-S85-I	ND	ND	ND	0.017 ± 0.004

"ND" not determined.

Both EGS C-S85 and C-ARG efficiently induced human RNase P to cleave the 38 nucleotides long RNA substrate ccr5-s of the CCR5 mRNA sequence, with C-S85 being at least 100 times more active than C-ARG (Table 1). Moreover, C-S85 bound to ccr5-s about 150 times better than C-ARG (Table 1). These findings implied that the engineered EGS C-S85 may have formed additional interactions with ccr5-s and RNase P, leading to increased affinity to ccr5-s and enhanced targeting activity. On the contrary, control EGSs C-S85-I and C-ARG-I exhibited no targeting activity, consistent with previous observations that the T-loop mutations had no effect on EGS binding to mRNA but disrupted RNase P–EGS recognition and abolished the EGS-targeting activity [25,36,37]. Because C-S85-I (K_d = 0.017 ± 0.004 μM) and C-ARG-I (K_d = 2.7 ± 0.4 μM) shared identical antisense sequences (Figure 1C-D) and similar in vitro binding affinities to ccr5-s as C-S85 (K_d = 0.017 ± 0.004 μM) and C-ARG (K_d = 2.7 ± 0.4 μM), respectively (Table 1), we used C-S85-I and C-ARG-I as the controls for the antisense effects.

3.2. EGS Expression in Human Cells

The EGS expression cassettes were inserted into retroviral vector LXSN and driven by the U6 RNA promoter [30,31,38]. Cell clones containing the EGS sequences were generated from PM1 cells, with similar levels of EGSs detected by northern blot analysis with H1 RNA expression as a loading control (Figure 2). The expression of the EGSs appeared to cause little cytotoxicity because the EGS-expressing cells and control cells transfected with the empty LXSN vector DNA alone showed no difference in viability and growth for 2.5 months.

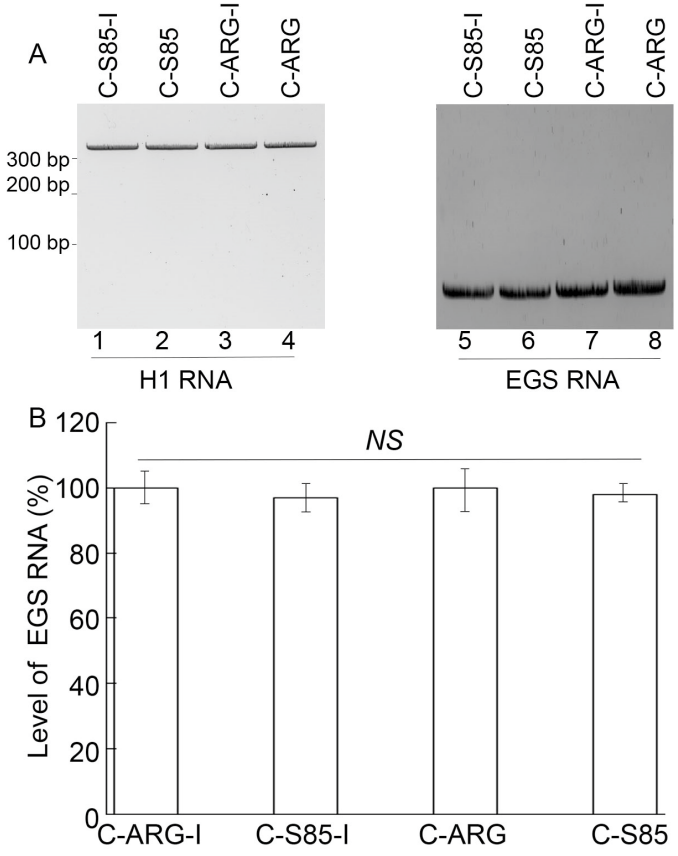


Figure 2. EGS RNA (right panel) and H1 RNA (left panel) expression detected by northern blot analyses in cells with C-S85-I (lanes 1 and 5), C-S85 (lanes 2 and 6), C-ARG-I (lanes 3 and 7), and C-ARG (lanes 4 and 8) (**A**). (**B**) Levels of EGS RNAs shown in % in comparison to those in PM1 cells with C-ARG-I, indicated as the mean ± SD. NS, not significant. Experiments were conducted in duplicate and repeated three times.

3.3. CCR5 Expression Inhibition in EGS-Expressing Cells

Northern blot analyses were used to assay the CCR5 and CXCR4 mRNA levels with actin mRNA as a loading control (Figure 3). Decreases of 98 ± 6% and 77 ± 5% in the CCR5 mRNA level were detected in cells with functional EGSs C-S85 and C-ARG, respectively, while reductions of less than 10% in the CCR5 mRNA level were found in cells with control EGSs C-S85-I and C-ARG-I (Figure 3). Western blot analyses with actin as a loading control showed reductions of 98 ± 6% and 73 ± 5% in the CCR5 protein level in cells with C-S85 and C-ARG, respectively, while reductions of less than 5% in the CCR5 protein level were found in cells with C-S85-I and C-ARG-I (Figure 4). No substantial changes were detected in the CXCR4 mRNA and protein levels among the cells (Figures 3 and 4). Thus, the targeting activities of C-S85 and C-ARG to induce RNase P cleavage appeared to be (a) specific against CCR5 but not CXCR4 and (b) responsible for the substantial CCR5 expression decrease in cells.

3.4. HIV Infection Inhibition in the EGS-Expressing Cells

It is reasonable to suggest that a CCR5 expression decrease would inhibit infection by an M-tropic HIV strain (e.g., $HIV_{Ba\text{-}L}$) with CCR5 as the infection co-receptor [39,40]. Experiments were performed to study $HIV_{Ba\text{-}L}$ infection of the EGS-expressing cells. The intracellular HIV RNA level, measured by qRT-PCR of the *tat* sequence using actin mRNA as the control, was reduced by 500- and 10-fold in cells with C-S85 and C-ARG, respectively (Figure 5). $HIV_{Ba\text{-}L}$ replication in these cells was further assayed by measuring the level of viral p24 protein in the supernatant. The p24 level was reduced by more than 900- and 50-fold in cells with C-S85 and C-ARG, respectively, at 12 days post infection. By contrast, no changes in the intracellular HIV RNA and supernatant p24 levels were observed in cells with C-S85-I and C-ARG-I (Figure 6). Thus, HIV infection inhibition in cells with C-S85 and C-ARG appeared to be due to the RNase P cleavage of CCR5 mRNA induced by the EGSs.

3.5. Specific EGS-Mediated Inhibition of M-Tropic but Not T-Tropic HIV Strains

PM1 cells expressed both CCR5 and CXCR4, the co-receptors for the M-tropic (e.g., $HIV_{Ba\text{-}L}$) and T-tropic HIV strains (e.g., HIV_{IIIB}), respectively [28,39]. Our results indicated that those cells expressing functional EGSs C-S85 and C-ARG exhibited reduced CCR5 expression but no change in CXCR4 expression (Figures 3 and 4). Thus, it was reasonable to suggest that these cells should be resistant to infection by M-tropic viral strains but not T-tropic strains. To investigate this, we infected cells with X4 strain HIV_{IIIB} and R5 strain $HIV_{Ba\text{-}L}$. At day 15 post infection, we assayed HIV infection and virion production by quantifying the viral p24 level in the culture supernatant. Cells with C-S85 and C-ARG were substantially resistant to $HIV_{Ba\text{-}L}$ infection. Decreases of ~1000- and 60-fold in the p24 level were detected in strain Ba-L-infected cells with C-S85 and C-ARG, respectively (Figure 7). By contrast, these cells were susceptible to HIV_{IIIB} infection as no change in the p24 level was detected in the strain IIIB-infected cells with no EGS or with C-S85, C1-ARG, C-S85-I, and C-ARG-I (Figure 7). These results implied that the inhibition of viral replication by C-S85 and C-ARG was specific because the same cells protected from CCR5-tropic $HIV_{Ba\text{-}L}$ were still susceptible to CXCR4-tropic HIV_{IIIB}. It was noted that cells with control inactive EGSs C-S85-I and C-ARG-I exhibited no resistance to infection by both $HIV_{Ba\text{-}L}$ and HIV_{IIIB}. These two EGSs exhibited similar affinity to the CCR5 mRNA sequence as C-S85 and C-ARG, respectively, but had little targeting activity. Thus, EGS-targeting activity to induce RNase P cleavage of the target CCR5 mRNA appeared to be responsible for reducing $HIV_{Ba\text{-}L}$ replication.

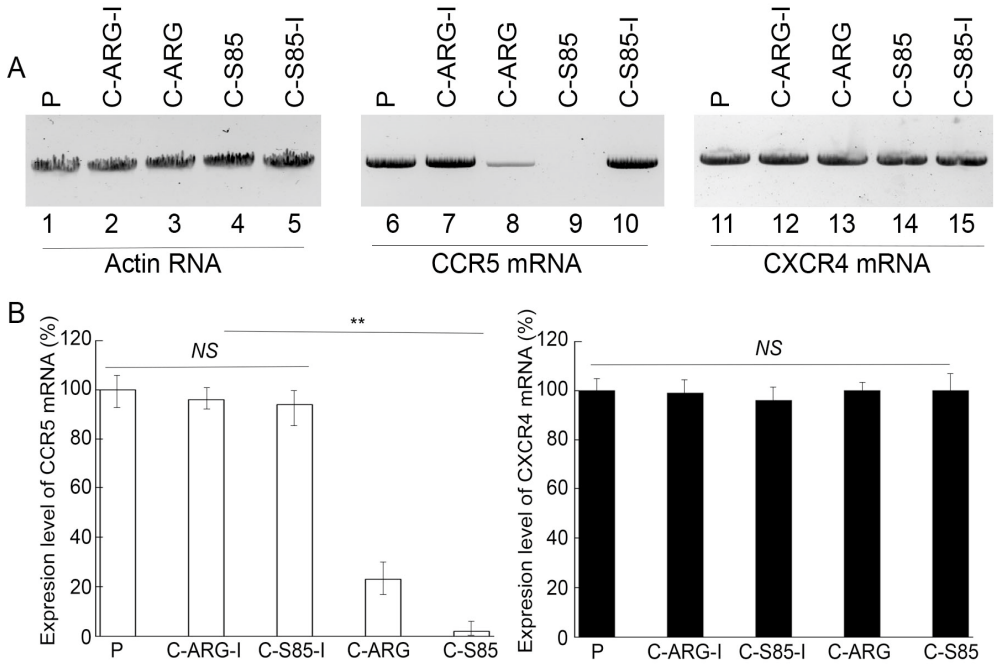


Figure 3. Levels of CCR5 (~4.0 kb) (lanes 6–10) and CXCR4 (~1.7 kb) (lanes 11–15) mRNAs detected by northern blot analyses in PM1 cells with empty LXSN vector (P, lanes 3, 8 and 13) and cell lines with C-ARG (lanes 1, 6, and 11), C-ARG-I (lanes 2, 7, and 12), C-S85-I (lanes 4, 9, and 14), and C-S85 (lanes 5, 10, and 15), with actin mRNA (~1.9 kb) as the loading control (lanes 1–5) (**A**). (**B**) CCR5 and CXCR4 mRNA levels shown in % in comparison to those in PM1 cells with empty LXSN vector, indicated as the mean ± SD. ** $p < 0.05$. NS, not significant.

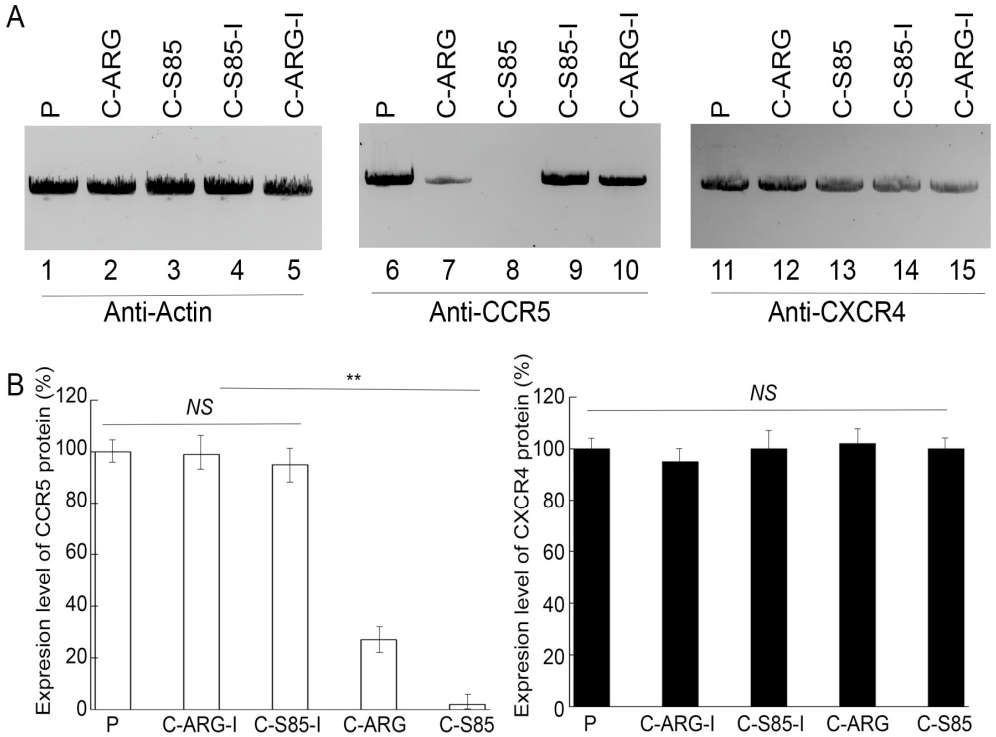


Figure 4. Protein levels of CCR5 (~55 kD) (lanes 6–10) and CXCR4 (~45 kD) (lanes 11–15) detected by western blot analyses in PM1 cells with empty LXSN vector (P, lanes 3, 8 and 13) and cell lines with C-ARG (lanes 1, 6, and 11), C-ARG-I (lanes 2, 7, and 12), C-S85-I (lanes 4, 9, and 14), and C-S85 (lanes 5, 10, and 15), with actin protein (~40 kD) as the loading control (lanes 1–5) (**A**). (**B**) CCR5 and CXCR4 protein levels shown in % in comparison to those in PM1 cells with empty LXSN vector, indicated as the mean ± SD. ** $p < 0.05$. NS, not significant.

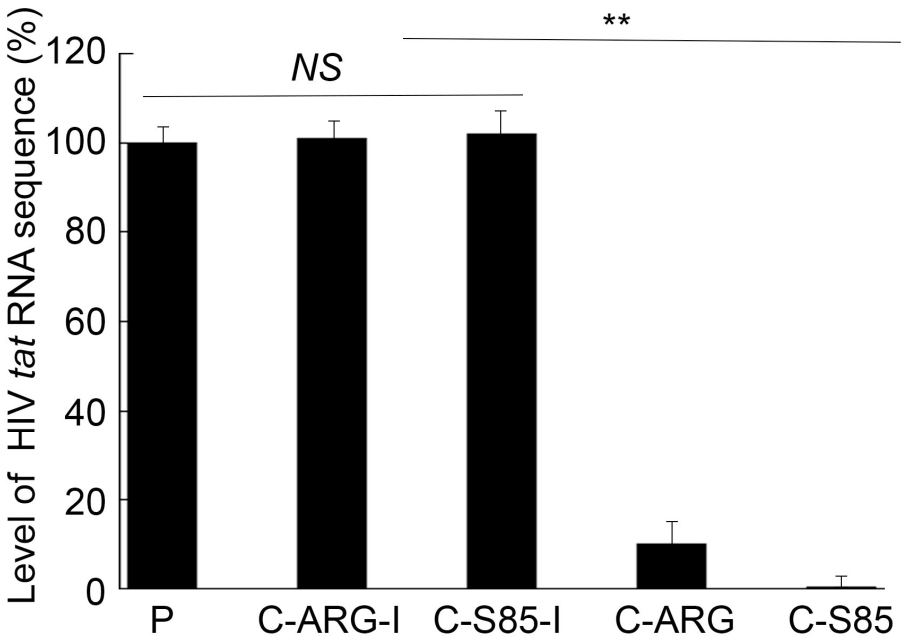


Figure 5. Levels of intracellular HIV RNA quantified with qRT-PCR amplifying the *tat* sequence from PM1 cells with empty LXSN vector or cells with different EGSs. Results are shown in % in comparison to those in PM1 cells with empty LXSN vector, indicated as the mean ± SD. ** $p < 0.05$. NS, not significant.

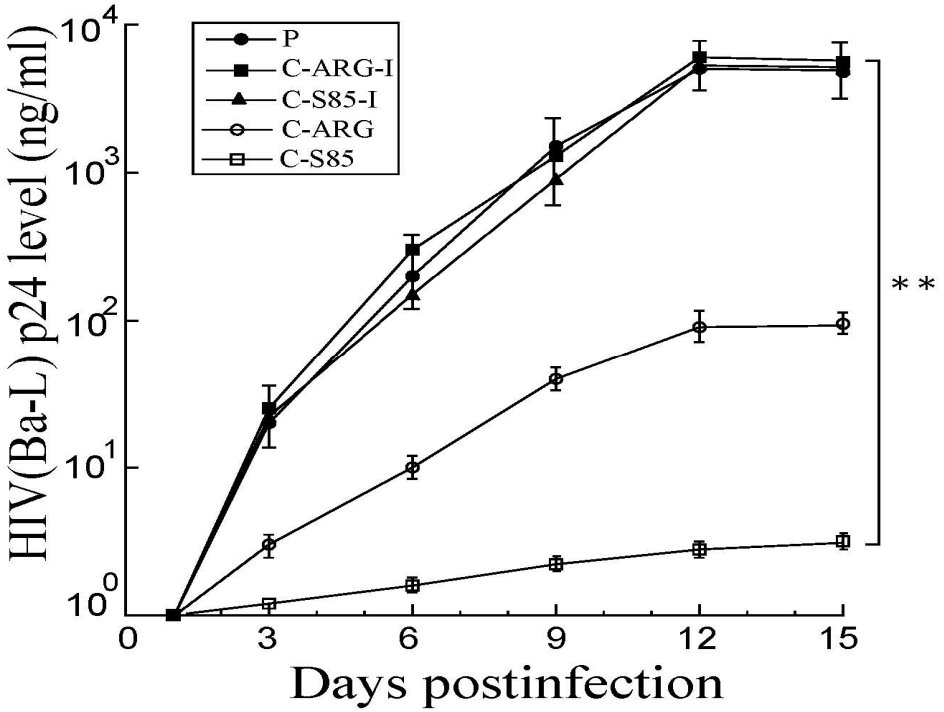


Figure 6. Growth of $HIV_{Ba\text{-}L}$ in PM1 cells with empty LXSN vector and cells with different EGSs. Cells (5×10^5) were infected with $HIV_{Ba\text{-}L}$ at a MOI of 0.05–0.1. Samples were collected at different times and viral production was monitored with the p24 antigen assay. Results are shown as the mean ± SD. ** $p < 0.05$. Experiments were conducted in duplicate and repeated three times.

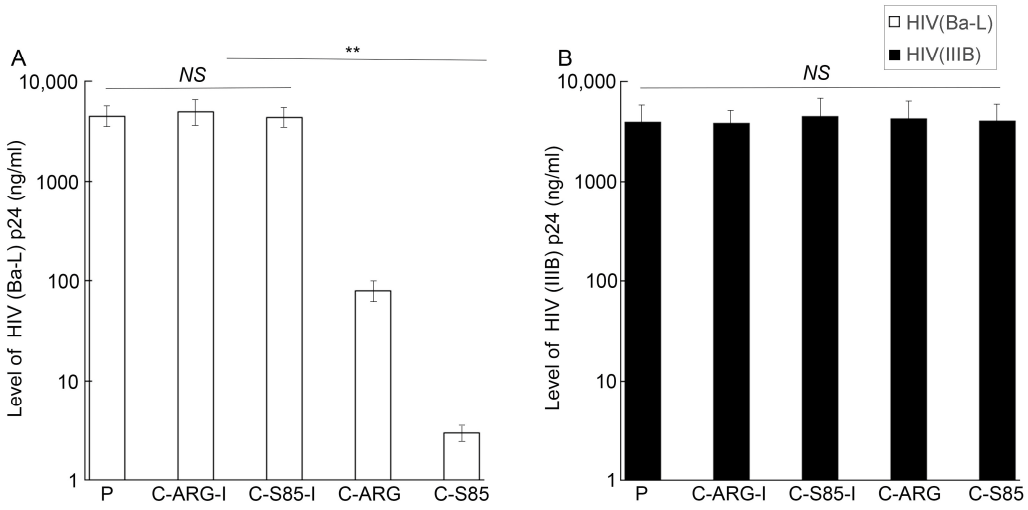


Figure 7. Levels of supernatant HIV-1 p24 protein in cells infected with $HIV_{Ba\text{-}L}$ (**A**) or HIV_{IIIB} (**B**). Culture media were collected at 15 days post infection and the p24 level was quantified with a p24 ELISA kit. Results are shown as the mean ± SD. ** $p < 0.05$. Experiments were conducted in duplicate and repeated three times.

4. Discussion

Being responsible for processing all tRNAs in cells, RNase P is one of the most ubiquitous and active enzymes in nature [16,19,20]. Additionally, the EGS-induced RNase P cleavage of a target mRNA is specific. No irrelevant cleavage associated with RNase H-mediated cleavage directed by antisense phosphothioate molecules was detected during the EGS-induced RNase P cleavage [16,20,41]. Thus, EGSs represent excellent gene-targeting reagents for both basic research and clinical applications.

Several lines of evidence in our results imply that the anti-CCR5 EGS-targeting activity is specific. First, EGS expression demonstrated little cytotoxicity, as we detected no substantial differences in viability and growth between the parental cells and EGS-expressing cells for 2.5 months. Second, the antiviral effects of C-S85 and C-ARG (inhibiting HIV infection) appeared to result from the decrease in CCR5 expression. This is because CCR5 expression was reduced in cells with C-S85 and C-ARG but not with C-S85-I and C-ARG-I (Figures 3 and 4). Third, C-S85 and C-ARG only decreased CCR5 expression but not the expression of other cellular genes, such as CXCR4 and actin (Figures 3 and 4). Cells with C-S85 and C-ARG were resistant to M-tropic $HIV_{Ba\text{-}L}$ but not T-tropic HIV_{IIIB}, demonstrating that C-S85 and C-ARG specifically targeted CCR5 but not CXCR4.

EGSs with enhanced targeting activities can be generated by evolution in vitro procedures [42–44]. In this report, C-S85, which was derived from V85, a variant EGS selected in vitro [25], demonstrated improved targeting activity in vitro and in culture cells. Hence, our results reveal a feasible approach for developing active gene-targeting EGSs with in vitro selection procedures.

It is currently unknown how the C-S85 sequence accounts for its improved activity. C-S85 binds to ccr5-s about 150-fold better and is about 100-fold more active in guiding RNase P-mediated hydrolysis than C-ARG, the EGS constructed from natural $tRNA^{arg}$. Perhaps additional tertiary interactions are present between C-S85 and ccr5-s, enhancing their binding and the formation of the ccr5-s–C-S85 complex. For example, the EGS sequences equivalent to the tRNA T-loop and variant domains (Figure 1) could possibly affect the folding of the mRNA–EGS complex and its binding to RNase P by increasing their interactions with the sequence resembling the D-loop [16,19,20,45]. In the ccr5-s–C-S85 complex, a part of C-S85 equivalent to the T-loop and variant domains may form additional interactions with RNase P and the 3′ region of ccr5-s, which resembles part of the

D-loop (Figure 1D), leading to enhanced C-S85/ccr5-s binding and improved EGS targeting efficacy. Additional studies should elucidate the mechanism of how C-S85 increases the EGS targeting activity.

One of the potential approaches for gene therapy against HIV infection is to engineer anti-CCR5 and anti-CXCR4 EGS molecules and express them in hematopoietic stem/progenitor cells prior to their differentiation into multi-lineage cells such as T cells and macrophages [10,11,15,17,46,47]. One limitation of our current study is that we only tested the effect of EGSs in human PM1 cells with laboratory-adapted HIV strains Ba-L and IIIB. It is important to test additional HIV strains, especially clinical isolates, in human primary cells, including the resting and activated CD4+ T cells that are known to be infected by HIV in vivo. We need to confirm whether EGSs are active in inducing RNase P cleavage of its target mRNA (i.e., CCR5 mRNA) and in shutting down CCR5 expression in these cells. These studies will reveal the potential of the RNase P-EGS approach against HIV infection in clinical settings.

We note that extensive studies have been conducted to understand the normal physiological functions of CCR5 and, more importantly, the potential clinical benefits and pathological consequences due to its absence or reduced expression [5–9]. These results indicated that CCR5 represents an excellent anti-HIV target because reduced CCR5 expression correlated with improved resistance to HIV infection and slowed HIV progression [10,11]. However, these studies also suggested that the absence of CCR5 or reduced CCR5 expression may have potential pathological consequences in vivo, such as increased risk for severe West Nile virus infection and its associated clinical complications [6,7]. Additional studies are needed to understand the CCR5 function in human physiology and the potential unwanted effects of the absence of CCR5 and reduced CCR5 expression. These studies will reduce the potential toxicity and side effects of CCR5 targeting and facilitate the development of new drugs targeting CCR5 for treating human diseases, including HIV [5,9].

While our current study showed that the constructed EGSs effectively inhibited HIV replication, the exact mechanism of how these EGSs block HIV infection and growth is currently not known. Detailed studies to investigate the effects of EGSs on each step of the HIV replication and infection cycle, such as viral entry, gene expression, and assembly, should reveal which step(s) of HIV infection and replication are blocked in EGS-expressing cells. For example, CCR5 is important for the entry of M-tropic HIV strains (e.g., $HIV_{Ba\text{-}L}$) [3,4]. It is likely that the cellular surface expression of CCR5 is also reduced due to the EGS-mediated inhibition of the overall expression of CCR5 in these cells. To investigate whether EGSs affect viral entry by downregulating CCR5 expression, it is important to determine whether the viral entry of these strains is inhibited and whether the level of inhibition correlates with the level of EGS-mediated reduction in the overall expression of CCR5 in these cells and the level of reduction in CCR5 expression on the surface of these cells. These studies will reveal the exact mechanism of how EGSs block HIV infection and replication and facilitate the development of these RNA molecules for anti-HIV application.

The EGS strategy against CCR5 targets a cellular gene at the HIV entry step and should minimize the emergence of HIV strains with drug-resistant mutations. Improved gene therapy against HIV can be further developed by combining other therapeutic strategies with EGS technology, including those based on ribozyme, RNAi, and CRISPR/gRNA methods [11–18]. Further studies on anti-CCR5 EGS antiviral activity, coupled with research on engineering EGSs with increased activity, will help to develop the RNase P-EGS technology for anti-HIV applications.

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References

1. Deng, H.K.; Unutmaz, D.; KewalRamani, V.N.; Littman, D.R. Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature* **1997**, *388*, 296–300. [CrossRef] [PubMed]
2. Liao, F.; Lee, H.H.; Farber, J.M. Cloning of STRL22, a new human gene encoding a G-protein-coupled receptor related to chemokine receptors and located on chromosome 6q27. *Genomics* **1997**, *40*, 175–180. [CrossRef] [PubMed]
3. Doranz, B.J.; Berson, J.F.; Rucker, J.; Doms, R.W. Chemokine receptors as fusion cofactors for human immunodeficiency virus type 1 (HIV-1). *Immunol. Res.* **1997**, *16*, 15–28. [CrossRef] [PubMed]
4. Berger, E.A. HIV entry and tropism: The chemokine receptor connection. *Aids* **1997**, *11* (Suppl. A), S3–S16.
5. Brelot, A.; Chakrabarti, L.A. CCR5 Revisited: How Mechanisms of HIV Entry Govern AIDS Pathogenesis. *J. Mol. Biol.* **2018**, *430*, 2557–2589. [CrossRef] [PubMed]
6. Ellwanger, J.H.; Kaminski, V.L.; Chies, J.A.B. CCR5 gene editing—Revisiting pros and cons of CCR5 absence. *Infect. Genet. Evol.* **2019**, *68*, 218–220. [CrossRef] [PubMed]
7. Glass, W.G.; McDermott, D.H.; Lim, J.K.; Lekhong, S.; Yu, S.F.; Frank, W.A.; Pape, J.; Cheshier, R.C.; Murphy, P.M. CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J. Exp. Med.* **2006**, *203*, 35–40. [CrossRef] [PubMed]
8. Hutter, G.; Nowak, D.; Mossner, M.; Ganepola, S.; Mussig, A.; Allers, K.; Schneider, T.; Hofmann, J.; Kucherer, C.; Blau, O.; et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N. Engl. J. Med.* **2009**, *360*, 692–698. [CrossRef]
9. Vangelista, L.; Vento, S. The Expanding Therapeutic Perspective of CCR5 Blockade. *Front. Immunol.* **2017**, *8*, 1981. [CrossRef] [PubMed]
10. Chung, J.; Rossi, J.J.; Jung, U. Current progress and challenges in HIV gene therapy. *Future Virol.* **2011**, *6*, 1319–1328. [CrossRef]
11. Zhou, J.; Rossi, J.J. Current progress in the development of RNAi-based therapeutics for HIV-1. *Gene Ther.* **2011**, *18*, 1134–1138. [CrossRef] [PubMed]
12. Scherer, L.J.; Rossi, J.J. Approaches for the sequence-specific knockdown of mRNA. *Nat. Biotechnol.* **2003**, *21*, 1457–1465. [CrossRef]
13. Santoro, S.W.; Joyce, G.F. A general purpose RNA-cleaving DNA enzyme. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4262–4266. [CrossRef] [PubMed]
14. Stein, C.A.; Cheng, Y.C. Antisense oligonucleotides as therapeutic agents--is the bullet really magical? *Science* **1993**, *261*, 1004–1012. [CrossRef] [PubMed]
15. Wong-Staal, F.; Poeschla, E.M.; Looney, D.J. A controlled, Phase 1 clinical trial to evaluate the safety and effects in HIV-1 infected humans of autologous lymphocytes transduced with a ribozyme that cleaves HIV-1 RNA. *Hum. Gene Ther.* **1998**, *9*, 2407–2425. [CrossRef] [PubMed]
16. Jarrous, N.; Liu, F. Human RNase P: Overview of a ribonuclease of interrelated molecular networks and gene-targeting systems. *RNA* **2023**, *29*, 300–307. [CrossRef]
17. Sparmann, A.; Vogel, J. RNA-based medicine: From molecular mechanisms to therapy. *EMBO J.* **2023**, *42*, e114760. [CrossRef] [PubMed]

18. Wang, J.Y.; Doudna, J.A. CRISPR technology: A decade of genome editing is only the beginning. *Science* **2023**, *379*, eadd8643. [CrossRef] [PubMed]
19. Evans, D.; Marquez, S.M.; Pace, N.R. RNase P: Interface of the RNA and protein worlds. *Trends Biochem. Sci.* **2006**, *31*, 333–341. [CrossRef]
20. Gopalan, V.; Altman, S. RNase P:structure and catalysis. In *The RNA World*; Gesteland, R., Cech, T., Atkins, J., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2006; Volume 277, Chapter 6.1. Available online: http://rna.cshl.edu (accessed on 15 January 2025).
21. Forster, A.C.; Altman, S. External guide sequences for an RNA enzyme. *Science* **1990**, *249*, 783–786. [CrossRef] [PubMed]
22. Yuan, Y.; Hwang, E.S.; Altman, S. Targeted cleavage of mRNA by human RNase P. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 8006–8010. [CrossRef]
23. Deng, Q.; Liu, Y.; Li, X.; Yan, B.; Sun, X.; Tang, W.; Trang, P.; Yang, Z.; Gong, H.; Wang, Y.; et al. Inhibition of human cytomegalovirus major capsid protein expression and replication by ribonuclease P-associated external guide sequences. *RNA* **2019**, *25*, 645–655. [CrossRef] [PubMed]
24. Kraus, G.; Geffin, R.; Spruill, G.; Young, A.K.; Seivright, R.; Cardona, D.; Burzawa, J.; Hnatyszyn, H.J. Cross-clade inhibition of HIV-1 replication and cytopathology by using RNase P-associated external guide sequences. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 3406–3411. [CrossRef] [PubMed]
25. Zhou, T.; Kim, J.; Kilani, A.F.; Kim, K.; Dunn, W.; Jo, S.; Nepomuceno, E.; Liu, F. In vitro selection of external guide sequences for directing RNase P-mediated inhibition of viral gene expression. *J. Biol. Chem.* **2002**, *277*, 30112–30120. [CrossRef] [PubMed]
26. Gogakos, T.; Brown, M.; Garzia, A.; Meyer, C.; Hafner, M.; Tuschl, T. Characterizing Expression and Processing of Precursor and Mature Human tRNAs by Hydro-tRNAseq and PAR-CLIP. *Cell Rep.* **2017**, *20*, 1463–1475. [CrossRef] [PubMed]
27. Mummidi, S.; Ahuja, S.S.; McDaniel, B.L.; Ahuja, S.K. The human CC chemokine receptor 5 (CCR5) gene. Multiple transcripts with 5′-end heterogeneity, dual promoter usage, and evidence for polymorphisms within the regulatory regions and noncoding exons. *J. Biol. Chem.* **1997**, *272*, 30662–30671. [CrossRef] [PubMed]
28. Lusso, P.; Cocchi, F.; Balotta, C.; Markham, P.D.; Lioue, A.; Farci, P.; Pal, R.; Gallo, R.C.; Reitz, M.S. Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): Failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1. *J. Virol.* **1995**, *69*, 3712–3720. [CrossRef]
29. Kawa, D.; Wang, J.; Yuan, Y.; Liu, F. Inhibition of viral gene expression by human ribonuclease P. *RNA* **1998**, *4*, 1397–1406. [CrossRef]
30. Miller, A.D.; Rosman, G.J. Improved retroviral vectors for gene transfer and expression. *Biotechniques* **1989**, *7*, 980–982, 984–986, 989–990.
31. Liu, F.; Altman, S. Inhibition of viral gene expression by the catalytic RNA subunit of RNase P from *Escherichia coli*. *Genes. Dev.* **1995**, *9*, 471–480. [CrossRef] [PubMed]
32. Jiang, X.; Gong, H.; Chen, Y.C.; Vu, G.P.; Trang, P.; Zhang, C.Y.; Lu, S.; Liu, F. Effective inhibition of cytomegalovirus infection by external guide sequences in mice. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 13070–13075. [CrossRef] [PubMed]
33. Zeng, W.; Vu, G.P.; Bai, Y.; Chen, Y.C.; Trang, P.; Lu, S.; Xiao, G.; Liu, F. RNase P-associated external guide sequence effectively reduces the expression of human CC-chemokine receptor 5 and inhibits the infection of human immunodeficiency virus 1. *Biomed. Res. Int.* **2013**, *2013*, 509714. [CrossRef] [PubMed]
34. Daftarian, P.M.; Kumar, A.; Kryworuchko, M.; Diaz-Mitoma, F. IL-10 production is enhanced in human T cells by IL-12 and IL-6 and in monocytes by tumor necrosis factor-alpha. *J. Immunol.* **1996**, *157*, 12–20. [CrossRef] [PubMed]
35. Jühling, F.; Mörl, M.; Hartmann, R.K.; Sprinzl, M.; Stadler, P.F.; Pütz, J. tRNAdb 2009: Compilation of tRNA sequences and tRNA genes. *Nucleic Acids Res.* **2009**, *37*, D159–D162. [CrossRef] [PubMed]
36. Yuan, Y.; Altman, S. Selection of guide sequences that direct efficient cleavage of mRNA by human ribonuclease P. *Science* **1994**, *263*, 1269–1273. [CrossRef] [PubMed]
37. Zhu, J.; Trang, P.; Kim, K.; Zhou, T.; Deng, H.; Liu, F. Effective inhibition of Rta expression and lytic replication of Kaposi's sarcoma-associated herpesvirus by human RNase P. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9073–9078. [CrossRef]
38. Bertrand, E.; Castanotto, D.; Zhou, C.; Carbonnelle, C.; Lee, N.S.; Good, P.; Chatterjee, S.; Grange, T.; Pictet, R.; Kohn, D.; et al. The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *RNA* **1997**, *3*, 75–88. [PubMed]
39. Berger, E.A.; Murphy, P.M.; Farber, J.M. Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annu. Rev. Immunol.* **1999**, *17*, 657–700. [CrossRef]
40. Bieniasz, P.D.; Cullen, B.R. Chemokine receptors and human immunodeficiency virus infection. *Front. Biosci.* **1998**, *3*, 44–58.
41. Ma, M.; Benimetskaya, L.; Lebedeva, I.; Dignam, J.; Takle, G.; Stein, C.A. Intracellular mRNA cleavage induced through activation of RNase P by nuclease-resistant external guide sequences. *Nat. Biotechnol.* **2000**, *18*, 58–61. [CrossRef] [PubMed]
42. Gold, L.; Polisky, B.; Uhlenbeck, O.; Yarus, M. Diversity of oligonucleotide functions. *Annu. Rev. Biochem.* **1995**, *64*, 763–797. [CrossRef]

43. Szostak, J.W. In vitro genetics. *Trends Biochem. Sci.* **1992**, *17*, 89–93. [CrossRef]
44. Joyce, G.F. Directed molecular evolution. *Sci. Am.* **1992**, *267*, 90–97. [CrossRef] [PubMed]
45. Marvin, M.C.; Engelke, D.R. Broadening the mission of an RNA enzyme. *J. Cell Biochem.* **2009**, *108*, 1244–1251. [CrossRef]
46. Akkina, R.; Banerjea, A.; Bai, J.; Anderson, J.; Li, M.J.; Rossi, J. siRNAs, ribozymes and RNA decoys in modeling stem cell-based gene therapy for HIV/AIDS. *Anticancer. Res.* **2003**, *23*, 1997–2005. [PubMed]
47. Strayer, D.S.; Akkina, R.; Bunnell, B.A.; Dropulic, B.; Planelles, V.; Pomerantz, R.J.; Rossi, J.J.; Zaia, J.A. Current status of gene therapy strategies to treat HIV/AIDS. *Mol. Ther.* **2005**, *11*, 823–842. [CrossRef]

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