Retrovirology: Research and Treatment

ORIGINAL RESEARCH

Inhibition of Feline Leukemia Virus Replication in Chronically Infected Cell Line Utilizing RNA Interference

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Abstract: Feline Leukemia virus (FeLV) is a pathogenic retrovirus endemic among domestic cats, remaining a serious disease since its discovery in 1964. RNA interference (RNAi) is a process in which double-stranded RNA induces the post-transcriptional sequence-specific degradation of homologous messenger RNA. At present, RNAi technology is regarded as a potential strategy for the treatment of various diseases as it can be used to inhibit the expression of desired peptides/proteins. This study aimed to apply RNAi technology to inhibit the replication of FeLV. We examined the effect of vector-mediated transfer and expression of FeLV specific short hairpin RNA (shRNA) against p27 protein expression and replication of FeLV in a feline T-cell line chronically infected with FeLV (3201-EECC). Three shRNA homologous to the FeLV *gag* gene were synthesized, cloned and transfected into a feline fibroblastic cell line (CrFK) expressing FeLV which efficiently reduced FeLV p27 protein expression, consequently decreasing and inhibiting the viral replication in a chronically FeLV infected feline T-cell line (3201-EECC). The expression of shRNA against FeLV *gag* gene showed markedly lower p27 levels and viral replication in both cell lines, 3201-EECC and CrFK. These results provide useful information to pave the road for the development of a gene therapy strategy to control FeLV and related pathogenic retrovirus infections in the future.

Keywords: RNA interference, FeLV, *gag*, p27, cat

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Introduction

The Feline Leukemia Virus (FeLV) is a member of the Retroviridae family, sharing the genus gammaretrovirus with the Xenotropic Murine Leukemia Virus-related gammaretrovirus (MLV) and the Gibbon Ape Leukemia Retrovirus (GALV). FeLV is pathogenic for domestic cats and involved in proliferative, degenerative, and immunosuppressive disorders as well as immunodeficiency, anemia and lymphoma.[1–5] Since its discovery in 1964, FeLV remains a serious pathogen for domestic cats. Most FeLV infections occur after oronasal spread of the virus in the saliva of viremic cats.[6,7] The worldwide prevalence of FeLV infection is not well known. It is remarkably low among cats from single cat households with a prevalence of approximately 1% and higher than 20% in large, multi-cat households that do not practice preventative measures against the introduction of FeLV.[8]

FeLV is a simple retrovirus carrying the necessary genes for replication, lacking cancer-causing oncogenes. Nonetheless the virus is responsible for a large proportion of lymphoid and myeloid malignancies in pet cats.[1,2,6,8–13] The virus contains a diploid RNA genome, each strand encoding the three genes (*gag, pol,* and *env*) required for viral replication. The *gag* gene encodes the structural proteins of the virion core (p10, p15, and p27), the *pol* gene encodes an RNA-dependent DNA polymerase, and the *env* gene encodes the envelope glycoproteins of the virion surface.[3,14]

Cancer development promoted by the virus is hypothesized to occur in two main ways. In the first mechanism the virus acts through the insertional mutagenesis, activating cellular oncogenes driven by proviral sequences involved in virus replication.[15] A second mechanism has been termed transduction, by which the FeLV provirus acquires cellular oncogenes, such as *myc*, by recombination;[12,16] such recombinant viruses can lead to rapid neoplasm development.[10]

In treating cats infected with FeLV, a timely and accurate identification of any diseases affecting the cat is necessary in order to provide an early therapeutic intervention and provide for a successful outcome. Chemotherapeutic drugs, immune modulators, antivirals, and vaccination should be combined to treat FeLV-infected cats.[8,17,18] Therapeutic procedures employing feline omega interferon (ω interferon) and aiming to curb FeLV viremia have been shown to improve the clinical condition significantly and extend the survival time of infected cats, although the procedures did not reverse viremia.[10,19] An antiviral compound routinely used, 3′-azido-2′, 3′-dideoxythymidine (AZT), has been shown to reduce the plasma viral load, improve immunological and clinical status, increase quality of life, and prolong life expectancy in some FeLV-infected cats.[20] However, the manifestation of severe hematological side effects by the use of antiviral drugs and poor clinical improvements in FeLV-infected cats has also been reported.[1,10] Cat vaccination is an essential preventive measure. However, it is unknown whether FeLV vaccines provide 100% protection efficacy, and none prevent infection.[17] Therefore, any effective antiviral procedure for FeLV-infected cats is not yet established.[16]

RNA interference (RNAi) is a process of sequence specific, post-transcriptional gene silencing (PTGS) triggered by double-stranded RNA (dsRNA) homologous to the target transcripts.[21] The reaction is initiated by the introduction of a double-stranded RNA (dsRNA) into the cell cytoplasm, resulting in the destruction of targeted mRNA and a subsequent reduction in protein production.[22] When successfully manipulated, the RNAi mechanism knocks down single or multiple genes, providing a quick and convenient method of analyzing gene function.[23–29] Recent studies have demonstrated that RNAi can be exploited in order to target genetic sequences of a variety of human and animal viruses including HIV-1, Poliovirus, Human Papillomavirus (HPV), Hepatitis C Virus (HCV), Hepatitis B Virus (HBV), Influenza Virus, Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), and Feline Immunodeficiency Virus (FIV).[9,30–39] These studies confirmed that RNAi technology represents a promising gene therapeutic approach to control viral infection and disease progression. Previous studies applying RNAi directed to structural genes inhibited viral replication of HIV and FIV.[30–33,40]

In this study, in order to investigate the potential use of RNAi technology as a therapeutic strategy for the control of FeLV replication, we first examined the expression of shRNAs against FeLV *gag* gene by the pSUPER vector system in FeLV replication in fibroblastic cells. Sequentially, we generated a retrovirus vector expressing a FeLV-specific shRNA and examined its effect on virus replication in a chronically

FeLV-infected feline T lymphoid cell line, aiming to evaluate the efficient delivery and expression of FeLV-specific shRNA and certify the persistent inhibitory effect of FeLV in these cells.

Material and Methods

Cells

The NIH AIDS reagents and D Looney kindly donated a feline fibroblastic cell line (CrFK) and the Human 293T cells, respectively. Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St Louis, MO) and RPMI 1640 (45%), Leibovitz's L-15 (45%) containing 10% heat-inactivated fetal bovine serum (FBS), and penicillin/streptomycin (GIBCO) maintained established cells including the feline T-cell line, 3201, chronically infected with FeLV-EECC (3201-EECC) NIH strain in the experiments.

Plasmid construction

The general guidelines[22] oriented the design and synthesis (Invitrogen) of target sequences, therefore three selected sequences (F1, F2, and F3) corresponding to the *gag* gene of FeLV Rickard strain, subgroup A (GenBank accession number: AF052723), were based on the conserved sequence of FeLV *gag* gene. Table 1 shows sequences of sense and antisense strands directed to the FeLV *gag* gene. The oligonucleotides strands annealed and cloned into the pSUPER vector essentially as previously described[39] to generate pH1-F1, pH1-F2 and pH1-F3 constructs. The control (pH1-GFP) consisted of a target sequence described before[9,38] against the Green Fluorescent Protein (GFP).[4,22,39]

The DNA sequences containing the H1 promoter and encoding specific shRNA were excised from the pSUPER vector by digestion with *Cla*I and *Xba*I, and subcloned into the same sites in the pNL-SINCMV-BLR vector[40,42,43] (kindly provided by Dr Bryan R Cullen) to generate the pNL-H1-F1 and pNL-H1-GFP constructs as previously described.[40] A similar lentivirus vector termed pNL-H, containing the H1 promoter but lacking any shRNA precursor, was generated in parallel as a negative control.

CrFK cells transfection

CrFK cells were co-transfected with 0.5 μg of pEECC-FeLV (NIH AIDS reagents) and 3.5 μg of pH1-F1, pH1-F2, pH1-F3 or pH1-GFP, respectively, utilizing the calcium phosphate method as previously described.[44] The cells were plated at 2×10^5 cells per well (9.6 cm^2) with DMEM in six-well plates for 24 hours. The culture medium was replaced 16 hours later, and the supernatant media harvested approximately 48 hours post-transfection. After cell pellet lysis, the shRNA efficiency was analyzed by the immunoblotting detection of p27 protein expression or RT assay.

Immunoblotting analysis

CrFK cells were harvested approximately 48 hours post-transfection and ressuspended in lysis buffer (10 mM Tris-Cl [pH 7.5], 140 mM NaCl, 1 mM PMSF, 1% Triton X-100). Thirty micrograms of total obtained protein were electrophoresed on 10% polyacrylamide gel containing SDS. Electroblotted proteins on PVDF membranes (Hybond P, GE Healthcare, Wien) were blocked overnight with 5% nonfat dry milk in TBS-T (20 mM Tris-Cl [pH 7.6], 280 mM NaCl, 0.1% Tween-20) and incubated for 1 hour at room temperature with goat anti-FeLV p27 (diluted 1:2000; NCI Repository) or anti-GAPDH (diluted 1:500 in TBS-T containing 5% nonfat dry milk; FL-335; Santa Cruz Biotechnology). After washing, the membranes were incubated for 1 hour at room temperature with a secondary anti-goat or anti-mouse antibody conjugated with Alkaline Phosphatase (diluted in TBS-T 1:1000; abcam). Protein immunodetection was performed using NBT/BCIP Stock Solution (ROCHE) according to the manufacturer's instructions. For analysis of the Western blotting data, densitometric analysis was performed using ImageJ software (NIH).

Table 1. Target sequences and corresponding regions in genome of FeLV-A clone.

Identification	Target sequence	FeLV-A genome position (nt)
F1-sense	5′-GGTAAAGCAGGTTGTACAAGG-3′	1911–1931
F2-sense	5′-GCAGCTAGTGTTATACTATCC-3′	2020–2040
F3-sense	5′-GCAAGCAGCTAGTGTTATACT-3′	2016–2036

Preparation of virus stocks and transduced cells

The HIV-1-based lentivirus vectors expressing shRNAs were prepared by co-transfection of 293T cells with 1.8 μg of the lentivirus vector, 0.1 μg of pcRev, 0.1 μg of pcTat, and 50 ng of pHIT/G as previously described.[40] The culture medium was replaced 16 hours later, and supernatant media was harvested approximately 48 hours post-transfection and passed through a filter with a pore size of 0.45 μm. Retroviral RNA was measured by the Reverse Transcriptase Assay.

A feline T-cell line chronically infected with FeLV, 3201-EECC, was seeded into a 12-well plate at 2×10^5 cells/well and inoculated with the lentivirus vectors at a multiplicity of infection (MOI) of 10 in a final volume of 1 mL of RPMI 1640 (45%), Leibovitz's L-15 medium (45%) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (GIBCO). After incubation at 37 °C for 48 hs, the cells were washed twice with PBS, and then cultured in the presence of 8 μg/mL of blasticidin S hydrochloride (Sigma) for 15 days to generate stably transduced cells.

Reverse transcriptase (RT) activity assay

FeLV particles were precipitated and concentrated with polyethylene glycol (PEG 6000). Initially, 3201 EECC cells were centrifuged at 250 × g for 10 minutes to remove cell debris. Obtained supernatant was centrifuged at 2000 × g for 30 min. Sequentially, 1 mL of PEG solution was added to 2 mL of cleared supernatant and incubated overnight at 4 °C. The pellet containing virions was obtained by centrifugation at 800 × g for 45 minutes and stored at −60 °C. All centrifugation procedures were carried out at 4 °C. Virions were lysed using 10% Triton X-100 (1% final concentration) in RPMI medium supplemented with 10% FBS, and RT units were measured using EnzChek Reverse Transcriptase Assay Kit (Invitrogen). The assay was performed in triplicate.

Statistical analysis

Statistical significance was evaluated by one-way analysis of variance (ANOVA), which was used to test for a significant difference in each RT quantification. If a significant difference was found by ANOVA, a student's *t*-test was performed to determine which pair showed the difference. Statistical significance was evaluated at $P < 0.05$.

Results

Inhibition of p27 expression in CrFK transfected cells

We examined the effect of three target sequences homologous to the FeLV *gag* gene (F1, F2, and F3) on the replication of FeLV in CrFK/FeLV cells. A sequence target homologous to GFP was used as a control. Immunoblotting analysis of cell extracts after approximately 48 hours post-transfection demonstrated the reduction of FeLV p27 protein expression in transfected cells with pH1-F1 and pH1-F2 vectors compared to mock-transfected (pH1-GFP) cells (Fig. 1A). Among the three anti-FeLV shRNAs vector expression, pH1-F2 showed the strongest inhibitory effect on FeLV p27 protein expression in CrFK cells. The concentration of FeLV p27 was assessed by measuring the integrated density. The densitometric analysis confirmed p27 reduction after co-transfection of vectors expressing shRNA against *gag* with FeLV plasmid expression, exhibiting the F2 sequence a marked reduction of protein expression, as shown as 1.8 ± 0.38 units for pH1-F2 with pEECC vs. 8.46 ± 1.12 units for pH1-GFP with pEECC expression ($P < 0.05$) (Fig. 1B).

Effect of shRNAs expression on FeLV replication in CrFK transfected cells

The inhibitory effect of anti-FeLV shRNA on FeLV replication in CrFK cells was evaluated by measuring RT activity in the supernatant of co-transfected cells with pEECC and pH1-GFP (Mock) or pH1-F1 or pH1-F2. Co-transfected cells with pH1-F2 showed significantly lower RT units than those of untreated cells, 0.25 ± 0.05 vs. 0.84 ± 0.12 RT units respectively and 0.41 ± 0.07 RT unit reduction in pH1-F1 co-transfected cells ($P < 0.05$) (Fig. 2). These results indicated that shRNA against FeLV *gag* gene could specifically inhibit Feline Leukemia virions release from transfected cells.

Effect of lentivirus vector-mediated expression of FeLV-specific shRNA on the FeLV replication in 3201-EECC cells

The efficiency of lentivirus vector-mediated transference and expression of FeLV-specific shRNA on FeLV replication was investigated in FeLV-infected T cells

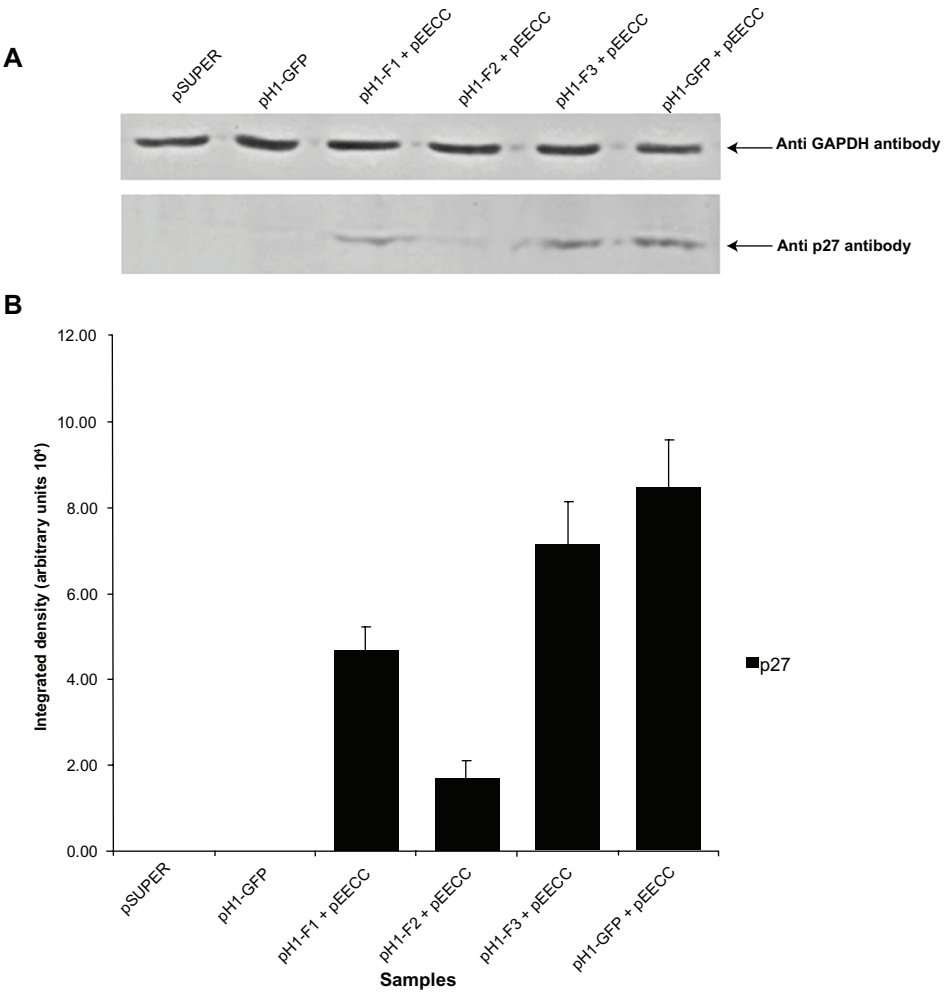

Figure 1. (**A**) Effect of shRNAs on the p27 protein expression in CrFK/FeLV cells. Regulation of p27 protein expression in CrFK cells transfected with pEECC plasmid and shRNA expression vector against *gag* gene. The shRNA expression vector against GFP was used as a control. The immunoblotting assay was performed with polyclonal antibody against p27 and monoclonal antibody against GAPDH (as a control). The first line corresponded to the GAPDH levels in the cells and the second line corresponded to p27 levels in the cells. The p27 levels reduced in the presence of the pH1-F1 and pH1-F2 vectors. (**B**) Densitometric analysis of p27 expression. The p27 protein content was determined by the Immunoblotting analysis and data are expressed as integrated density in arbitrary units $\times 10^4$.
Note: The columns and bars show means and standard deviations, respectively, obtained from the data in triplicate samples.

(3201-EECC cells). The cells stably expressing shRNA after VSV-G pseudotyped lentivirus vector inoculation, were selected utilizing blasticidin. FeLV replication in these cells was examined during 15 days after inoculation with the lentivirus vectors. The RT activity measured in the supernatant of transduced cells with the lentivirus vector carrying shRNA specific to FeLV (shF2) was significantly lower (0.86 ± 0.11 units) than in untreated cells (3.04 ± 0.16 units) ($P < 0.05$), and in the infected cells with the lentivirus vector control with only the H1 promoter (shH1) (2.41 ± 0.28 units) (Fig. 3).

Discussion

Since the RNAi technology has been developed, a plethora of genes from eucaryotes, prokaryotes, and viruses have been studied, and as a therapeutic tool, a variety of viral infections and diseases in humans and animals have been treated. The inhibition of HIV-1 and FIV[29–33,40,45,46] replication among other viruses could be accomplished by the shRNA expression in established and primary cell lines. Here, we investigated the feasibility of using RNAi technology, based in shRNA expression, aiming at controlling FeLV infection in feline cell lines. The present study was carried out to demonstrate the potentiality of RNAi technology approach as a strategy for the control of FeLV infection, and to demonstrate that it is possible to reduce FeLV replication in vitro by RNAi technology. A lentiviral vector expressing shRNA specifically targeting FeLV gene sequences were

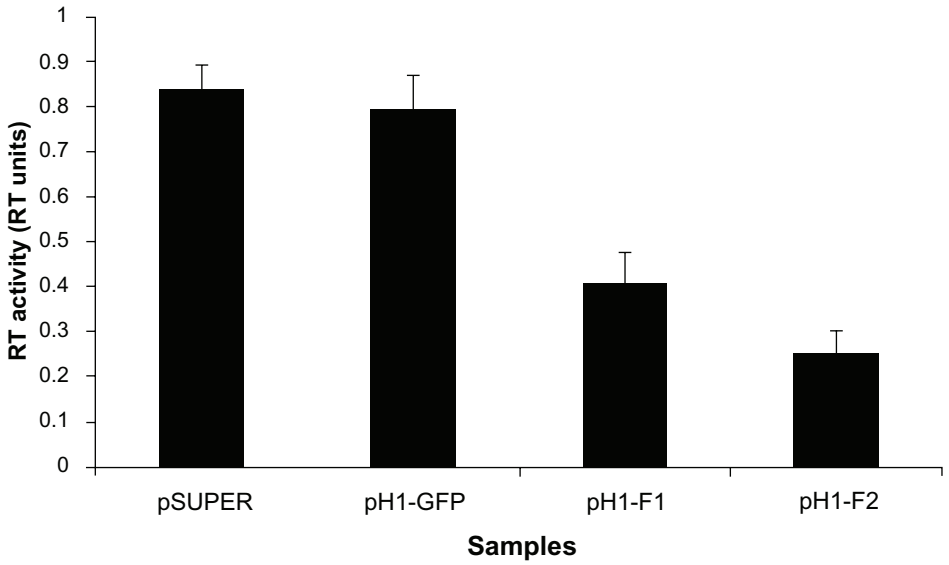

Figure 2. Effect of shRNA expression on the releasing of FeLV in CrFK cells.
Notes: The RT activity in the culture supernatants was measured approximately 48 hours after co-transfection with shRNA vector expression and pEECC vector. The columns and bars show means and standard deviations, respectively, obtained from the data in triplicate samples. A target sequence GFP was used as a control (pH1-GFP).

developed and its effect examined upon the transference into feline cell lines.

In order to identify the target sequence which can efficiently inhibit FeLV replication, the effects of homologous sequences to FeLV *gag* gene on FeLV co-transfected CrFK cells were investigated. Three homologous target sequences to *gag* gene were used to construct three clones against EECC-FeLV clone (pH1-F1, pH1-F2, and pH1-F3) and co-transfected with pEECC plasmid on CrFK transfected cells. We demonstrated that transfection with vectors expressing anti-FeLV shRNAs (F1 and F2) resulted in reduction of p27 protein expression (Fig. 1) and RT activity in the culture supernatant (Fig. 2), indicating that a region of FeLV *gag* gene can be targeted by shRNA, as can other types of retrovirus

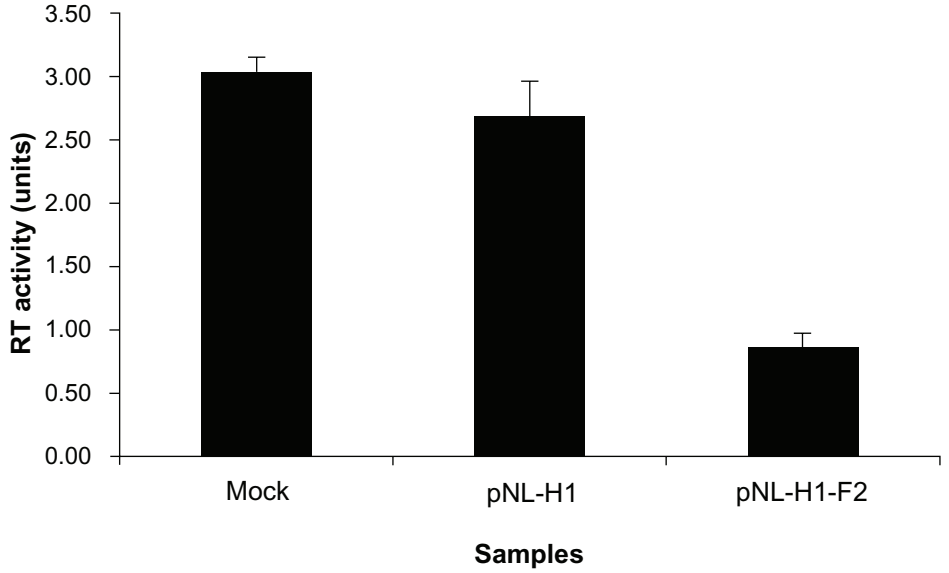

Figure 3. Effect of lentivirus vector-mediated transfer and expression of FeLV-specific shRNA on the replication of FeLV in 3201 EECC cells.
Notes: The cells were transduced with the lentivirus vector, which express shRNA (F2 target sequence) or only H1 promoter and then selected under blasticidin treatment. The RT activity in the culture supernatants were measured 15 days after lentivirus vector-mediated transfection. The columns and bars show means and standard deviations, respectively, obtained from the data in triplicate samples.

like HIV and FIV, for example.[30–33,40,41] The F3 vector expressing anti-FeLV shRNA failed to reduce p27 protein expression, despite its complementarity to the *gag* sequence. Distinct factors could influence the inhibition mechanisms exerted by the anti-FeLV shRNA, including technical errors.

Lentiviral vectors can infect both dividing and nondividing cells, changing the expression of their target cell genes and being capable of providing highly effective gene therapy devices.[47] Therefore, we examined the effect of the lentivirus vector mediated transfer and expression of the shRNA in feline T-cell line. The amount of RT activity in the culture supernatant of the 3201 EECC cell line were markedly decreased even 15 days after transduction with a lentiviral vector which expresses shRNA specific to FeLV (pNL H1-F2) (Fig. 3). This reduction in RT activity observed in chronically FeLV-infected cell line, 3201, supports a future use of this tool in FeLV gene therapy. However, while the interference RNA virus escape was observed in other virus systems,[48,49] in the FeLV system the results could be distinct as significant changes in FeLV render the virus less viable than FIV and HIV.[3] In this case, the shRNA could be a new treatment strategy in the future for FeLV infected cats.

Further investigation is necessary to improve the transduction efficiency of the lentivirus vector in primary T cells of cats. Additionally, hematopoietic stem cells are promising target candidates for RNAi gene therapy to control FeLV infection. The development of antiviral gene therapy strategies targeting these cells has been proposed as a long-term treatment for AIDS. Nevertheless, the isolation and purification system of feline hematopoietic stem cells has not been established, and the viral vector-mediated transfer of anti-FeLV shRNA and other genes into these cells will probably be a useful gene therapy strategy for the control of FeLV infection and treatment of infected cats in the future.

The goal of the gene therapy strategy for the control of FeLV infection is to achieve FeLV-resistant hematopoietic cells and/or inhibit the disease progression. It is highly plausible that the F2 target sequence inhibits the replication of various FeLV field strains as well as the EECC strain and may be used to protect the cells. Gene silencing by RNAi mechanism requires almost perfect complementarity between the shRNA and target sequence besides clinical trials would result differently from in vitro studies. The efficacy of shRNA generated in this research study needs to be investigated in other FeLV strains. In any case, this work demonstrates the reduction of FeLV infection by the RNAi technology and opens an opportunity to continue experiments aimed at developing experimental gene therapy models.

Conclusions

In summary, we demonstrated that expression of shRNAs homologous to the FeLV *gag* gene efficiently inhibited p27 expression and FeLV replication in the CrFK cell line. In addition, we showed that lentivirus vector-mediated transfer and expression of FeLV-specific shRNA could inhibit virus replication in chronically infected FeLV lymphoid cells. The present study indicated that the RNAi technology could be useful as a gene therapy strategy for the control of FeLV infection.

Authors' Contributions

SSO performed the experiments and prepared the manuscript; GB contributed with the RT assay; LIBK supervised the work. The final manuscript was read and accepted by all authors.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

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