

L1 retrotransposon-mediated stable gene silencing

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ABSTRACT

RNA interference (RNAi) is widely used for functional studies and has been proposed as a potential therapeutic agent. Current RNAi systems are largely efficient, but have limitations including transient effect, the need for viral handling and potential insertional mutations. Here, we describe a simple L1 retrotransposon-based system for the delivery of small interfering RNA (siRNA) and stable silencing in human cells. This system demonstrated long-term siRNA expression and significant reduction in both exogenous and endogenous gene expression by up to 90%. Further characterization indicated that retrotransposition occurred in a controlled manner such that essentially only one RNAi-cassette was integrated into the host genome and was sufficient for strong interference. Our system provides a novel strategy for stable gene silencing that is easy and efficient, and it may have potential applications for *ex vivo* and *in vivo* molecular therapy.

INTRODUCTION

As a potent and specific gene silencing mechanism, RNA interference (RNAi) has revolutionized reverse genetic approaches for functional studies in a variety of organisms, particularly mammals (1,2). Another intriguing prospect of RNAi is its therapeutic potential, which has been demonstrated by the prevention of fulminant hepatitis (3,4), viral infections (5,6) and malignancies of various tissues (7–11) in both primary mammalian cells and animal models. RNAi involves processing of long double-stranded RNA (dsRNA) precursors to 21–23 nt siRNAs (12), which target the endonucleolytic cleavage of homologous transcripts (13). Several strategies have been developed to produce siRNA *in vitro* or *in vivo* (1,2). For example, pol III promoter transcribed short-hairpin RNA (shRNA) can be processed into siRNA *in vivo* and this has become a commonly used strategy for specific gene silencing in mammalian systems. Various vectors have been used

for shRNA delivery, including plasmid, retrovirus, adenovirus and adeno-associated virus. Although largely efficient, current delivery vehicles have limitations, such as transient effect, demanding viral handling and potentially causing undesirable insertional mutations.

L1s are non-LTR retrotransposons that comprise ~17% of the human genome (14). A consensus full-length human L1 is ~6 kb, containing a 5'-untranslated region (5'-UTR), two open reading frames (ORF1 and ORF2) and a 3'-UTR with poly(A) signal and a poly(A) tail. During retrotransposition, L1 sequence is transcribed from its internal promoter located within the 5'-UTR (15) and the transcript is exported to the cytoplasm where a ribonucleoprotein (RNP) complex is formed with translated ORF1 (16). ORF1 has RNA-binding activity (17) and ORF2 has endonuclease (18) and reverse transcriptase (19) activities. L1 integrates into the genome by target primed reverse transcription (TPRT) using the free 3'-OH at the endonuclease cut site on the genomic DNA as a primer and the L1 RNA as template (20) (Figure 1A).

In this study, we describe a novel system utilizing L1 retrotransposition for stable siRNA expression and gene silencing in human cells. This system was demonstrated to significantly reduce both exogenous and endogenous gene expression and may have potential applications in molecular therapeutics.

MATERIALS AND METHODS

Cell culture

HeLa cells were grown in high glucose (4.5 g/l) DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Invitrogen, Carlsbad, CA) in a humidified 5% CO₂ incubator at 37°C.

Plasmid constructs

Oligonucleotide primer sequences used in this study are available upon request. The *neo*-cassette tagged L1_{RP} was inserted into pBluescript II KS(–) phagemid vector (Stratagene, La Jolla, CA) to create pBSKS-L1_{RP}-*neo*. DNA oligonucleotides encoding control shRNA sequence, 5'-ACTACCG-TTGTATAGGTG-3', which expresses an siRNA with

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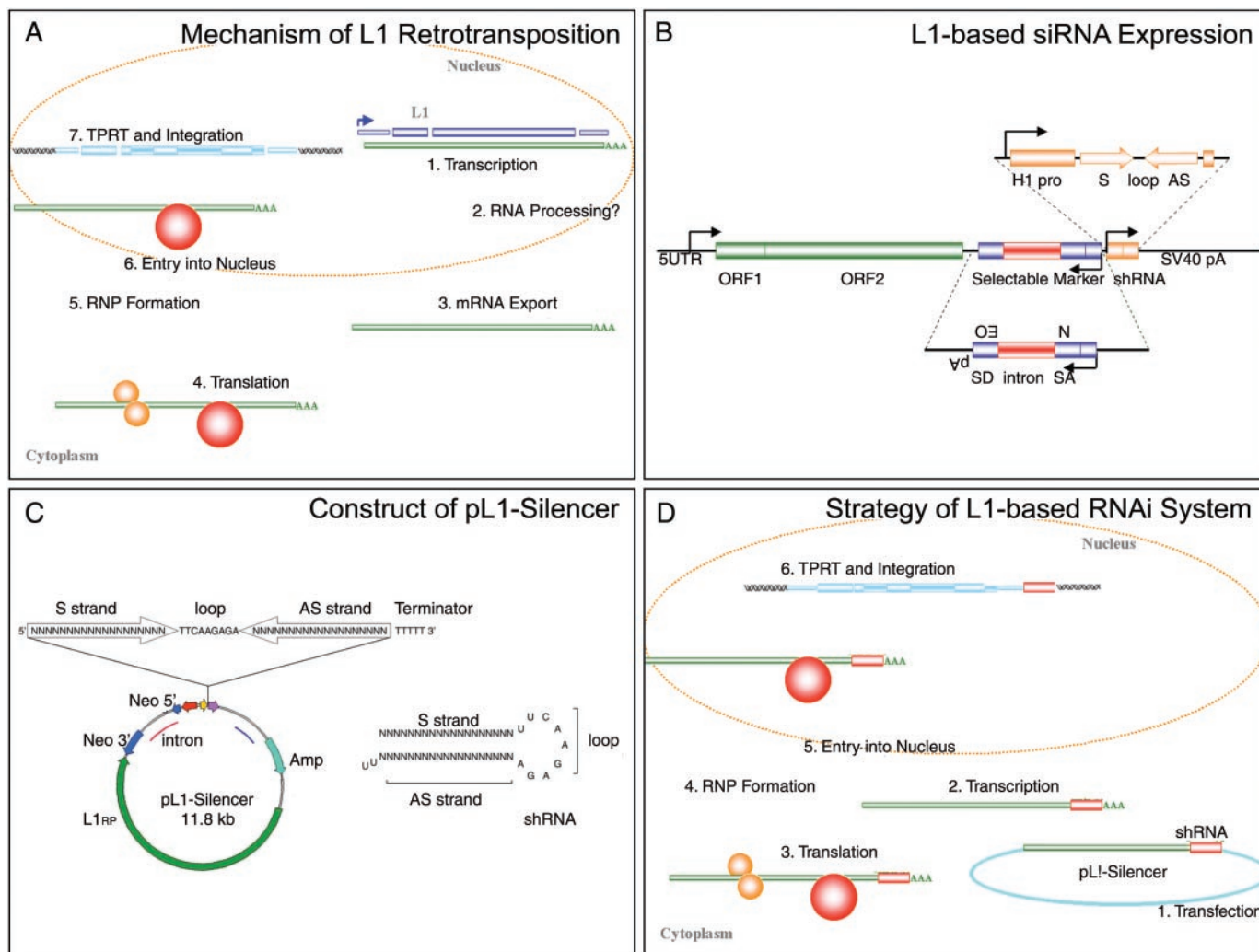


Figure 1. L1 retrotransposon-based RNAi system. (A) Mechanism of L1 retrotransposition. (B) Strategy for L1-based RNAi. A selectable *neo*-cassette for retrotransposition is inserted in the 3'-UTR of L1_{RP}, followed by an RNAi-cassette composed of the H1 promoter, the 19 bp sense and antisense sequences of a specific gene separated by a 9 bp loop sequence and a pol III terminator of six thymidines. Abbreviations: pA, polyadenylation signal; NEO, *neo* gene; SD, splicing donor site; and SA, splicing acceptor site. Arrows indicate transcription start sites. (C) Structure of L1-based RNAi vector (pL1-Silencer) and expressed shRNA. (D) L1-based RNAi system. Transfection of pL1-Silencer into cultured cells is followed by the transcription of L1-RNAi sequence, its integration into the host genome and stable siRNA expression.

limited homology to any known sequences in the human, mouse and rat genomes (Ambion, Austin, TX), green fluorescence protein (GFP) target sequence, 5'-GGCTACGTC-CAGGAGCGCA-3' (21), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) target sequence, 5'-GTGGATAT-TGTTGCCATCA-3' (Ambion), were synthesized by Integrated DNA Technologies (Coralville, IA). The complementary oligonucleotides were annealed and inserted downstream of the H1 promoter into *Apal*-*EcoRI* linearized pSilencer3.0-H1 vector (Ambion) according to the manufacturer's instructions. The RNAi-cassettes composed of H1 promoter and shRNA sequence were amplified by PCR using Expand High Fidelity PCR System (Roche, Mannheim, Germany) and inserted just downstream of the *neo*-cassette in pBSKS-L1_{RP}-*neo* to create pL1-Silencer constructs.

GFP expression plasmid pCEP-puro-GFP was constructed by insertion of the *EGFP* gene from pEGFP-N1 vector (Clontech, Palo Alto, CA) downstream of the cytomegalovirus

(CMV) promoter in pCEP-puro, which is derived from pCEP4 vector (Invitrogen) except that the hygromycin resistance gene in the original vector was replaced with a puromycin resistance gene. Inserted sequences in all constructs were verified by DNA sequencing.

Cell culture and transfection

To establish L1-mediated stable RNAi cell lines, HeLa cells were seeded in six-well dishes at 3×10^5 cells/well and grown to 60% confluence before transfection. Each transfection received 1 μ g plasmid DNA, 3 μ l FuGENE 6 transfection reagent (Roche) and 2 ml complete medium according to the manufacturer's protocol. Antibiotic selection with G418 (400 μ g/ml; Invitrogen) was started 48 h post-transfection. Untransfected cells were used to determine when antibiotic selection had been completed. After G418 application for 24 h, 10% of cells from each well were removed to a 100 mm

culture dish and grown in G418 containing medium until single colonies appeared. In ~1 week, several individual colonies were picked from each 100 mm dish and grown separately in one-well of a six-well dish. When nearly confluent, cells were transferred onto 75 mm² flasks for future analysis.

Genomic DNA extraction and PCR

Genomic DNA was extracted using QIAamp Blood kit (Qiagen, Valencia, CA) and 100 ng of genomic DNA was used for PCR by using PCR Core kit (Roche). A forward primer located within the *neo* sequence upstream of the γ -globin intron and a reverse primer located within the H1 promoter were designed so that a 775 bp PCR product would be produced if the intron had been correctly spliced out in contrast to a 1679 bp product with an intact intron. An aliquot of 100 ng of genomic DNA was used in each 50 μ l PCR performed by using PCR Core kit with an initial denaturation step at 94°C for 2 min, followed by 42 cycles of 94°C for 15 s, 61°C for 30 s, 72°C for 2 min and a final extension at 72°C for 10 min. The 2 min extension time also allowed for the amplification of unspliced sequence. A positive control for unspliced product using 10 ng pBSKS-L1_{RP}-*neo* plasmid as template was performed in parallel. An aliquot of 5 μ l of PCR product was electrophoresed and purified with QIAquick PCR Purification kit (Qiagen) for sequencing.

Real-time PCR and RT-PCR

An aliquot of 1 μ g of total RNA isolated with RNeasy RNA Isolation kit (Qiagen) and treated with DNase I (Invitrogen) was used for cDNA synthesis with random hexamer and SuperScript First-Strand Synthesis System (Invitrogen). Real-time amplification was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using Sybergreen PCR Core Reagent kit (Applied Biosystems) with reverse transcribed cDNA or Taqman PCR Core Reagents (Applied Biosystems) with genomic DNA. Real-time RT-PCR of β -actin and GAPDH was performed as control. Standard calibration curves were constructed and the relative expression of each sample was calculated as described previously (22). Each sample was analyzed twice and each real-time RT-PCR experiment contained two non-template control wells. PCR products were confirmed as single bands on gel electrophoresis.

Northern-blot analysis

Probe for siRNA detection was composed of 19 nt siRNA sequence in either sense or antisense direction, synthesized and 5'-digoxigenin labeled by Integrated DNA Technologies. RNA highly enriched for small RNA species (no larger than 200 nt) was extracted from wild-type and knockdown HeLa cell lines using *mirVana* miRNA Isolation kit (Ambion) following the manufacturer's protocol. Only 1.5 μ g of each sample and 0.1 ng of probe was loaded on a 15% TBE-Urea gel (Invitrogen) and electrophoresed at 180 V for 1.5 h. The gel was stained in 1 \times TBE plus 1 μ g/ml ethidium bromide for 40 min and photographed before electroblotting onto positively charged nylon membrane (Roche) in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) at 20 V for 1.5 h. The membrane was hybridized with 100 ng/ml probe at 37°C overnight. On the following

day, the membrane was washed twice in 2 \times SSC at room temperature (21°C) for 5 min each and twice in 0.1 \times SSC at 37°C for 15 min each. Digoxigenin signals were detected with DIG Northern Starter kit (Roche) following the manufacturer's instructions.

Western-blot analysis

Total protein from stable HeLa RNAi cell lines was extracted with M-PER mammalian protein extraction reagent (Pierce, Rorkford, IL). Of the total protein, 7.5 μ g was electrophoresed in a 10% SDS-PAGE gel, transferred onto a PVDF membrane (Millipore, Billerica, MA) in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 15 V for 30 min, blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TTBS) and incubated overnight with primary polyclonal anti-GFP antibody (Clontech) diluted 1:400, monoclonal anti-GAPDH antibody (Chemicon, Temecula, CA) diluted 1:200 or monoclonal anti- β -actin antibody (Sigma-Aldrich, St Louis, MO) diluted 1:20 000. The membrane was then probed with secondary peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:5000 dilution in TTBS; Amersham Biosciences, Piscataway, NJ) for 1 h and developed with an ECL[®] Detection kit (Amersham Biosciences) according to the manufacturer's instructions.

Fluorescence-activated cell scanning

Established RNAi cell lines targeting GFP were transfected with 1 μ g of pCEP-puro-GFP for each well of six-well dish. Puromycin selection was started 48 h post-transfection. On day 5, cells were analyzed by using fluorescence-activated cell scanning (FACS) as described previously (23).

Immunofluorescence

Cells were fixed with ice-cold acetone for 10 min, then blocked in 10% normal goat serum, incubated in primary mouse anti-human GAPDH antibody (1:100; Chemicon) and rabbit anti-human cytokerin antibody (1:500; DAKO, Carpinteria, CA), and visualized using species appropriate secondary IgG conjugated with fluorescein isothiocyanate or Texas Red (Vector, Burlingame, CA) diluted 1:40 in phosphate-buffered saline. DAPI (Vector) counterstaining for nuclear DNA was performed.

Retrotransposition assay

Retrotransposition assay was performed on days 3, 4, 5, 6, 7, 8, 9 and 10 post-transfection as described previously (23,24).

Statistical analysis

Data analysis was performed using the SPSS statistics software package. All the results were expressed as means \pm SE, and $P < 0.05$ was used for significance.

RESULTS

Development of an L1-based RNAi system

To obtain stable siRNA expression *in vivo*, we combined pol III promoter driven shRNA production with L1-mediated integration. An active human L1, L1_{RP} (25), was tagged in its 3'-UTR with an antisense *neo*-cassette driven by a

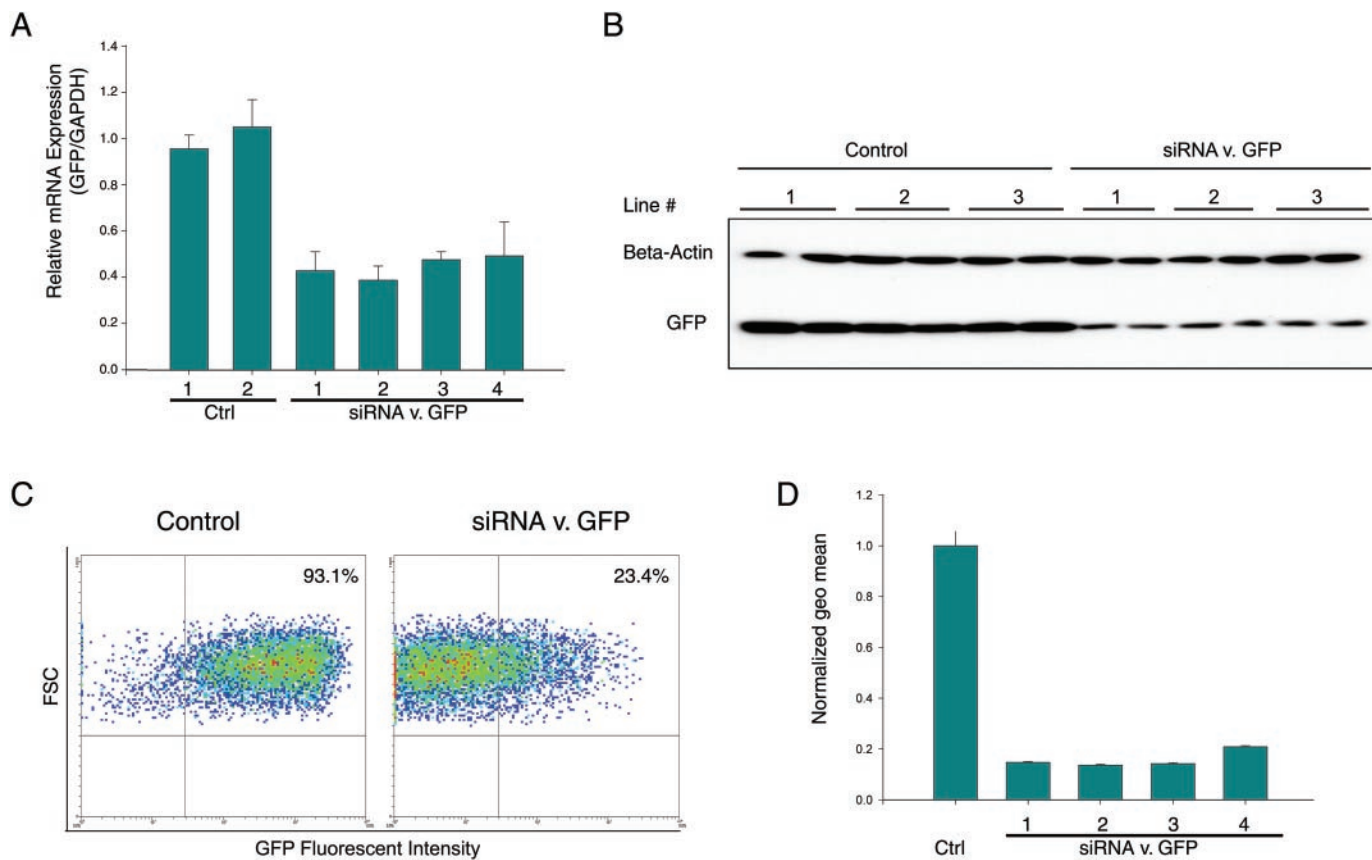


Figure 2. Inhibition of exogenous GFP expression using L1-based RNAi system. (A) Real-time RT-PCR results of GFP mRNA expression in stable HeLa lines expressing control or GFP-targeted siRNA ($n = 6$). (B) Western-blot analysis of GFP expression in control or knockdown clones. (C) Typical FACS dot plots of GFP expression in control and knockdown clones. (D) Normalized GFP fluorescence in control and knockdown clones by FACS ($n = 4$).

heterologous promoter and followed by an SV40 poly(A) signal (Figure 1B). The *neo* gene was disrupted by a γ -globin intron in the same direction as L1 such that *neo* could only be expressed following L1 retrotransposition (26). An RNAi-cassette containing H1 promoter and specific shRNA targeting sequence was then inserted downstream of the *neo*-cassette tagged L1 to create the L1-RNAi vector, pL1-Silencer (Figure 1B and C). After transfection of such a vector into wild-type HeLa cells, individual clones in which tagged L1 had retrotransposed were selected by G418. Most importantly, since the RNAi-cassette is located downstream of the *neo*-cassette, the TPRT mechanism during retrotransposition essentially guarantees that G418 resistant clones will also integrate the RNAi-cassette into the host genome (Figure 1D). This strategy provides an easy and efficient method for stable siRNA expression *in vivo*.

Specific inhibition of exogenous and endogenous gene expression

To test the efficacy of our system, stable knockdown cell lines were first established by transfecting wild-type HeLa with the pL1-Silencer containing either control or GFP-targeted shRNA sequence. The established cell lines were then transfected with plasmid pCEP-puro-GFP such that cells expressing GFP would be selected by puromycin. By real-time RT-PCR, we found that the mRNA level of exogenous GFP

was reduced by 50–60% ($P < 0.001$), in all the four lines that were analyzed, compared with controls (Figure 2A). Consistently, GFP expression was also markedly inhibited in knockdown lines as revealed by western-blot analysis (Figure 2B) and its fluorescence was reduced by up to 87% after FACS analysis ($P < 0.001$, Figure 2C and D). Using our system, the expression of an endogenous gene, *GAPDH*, was also significantly reduced at both mRNA and protein levels ($P < 0.001$, Figure 3). It is noteworthy that all the stable knockdown cell lines had been cultured for over 3 months before analysis was performed to ensure that the episomal plasmid had been lost. In addition, the silencing of targeted genes was observed over multiple generations. Finally, northern-blot analysis was performed to demonstrate successful siRNA expression in stable knockdown cells. We detected specific siRNA expression in all *GAPDH* and GFP knockdown cell lines but not in wild-type HeLa (Figure 4 for *GAPDH* and data not shown for GFP). Furthermore, the siRNA expression level correlated inversely with the *GAPDH* expression observed in each line (Figure 4), providing a possible explanation for the different levels of silencing observed among them.

Knockdown clones are derived from L1 retrotransposition

To confirm that the stable silencing was derived from the retrotransposed RNAi-cassette but not integrated

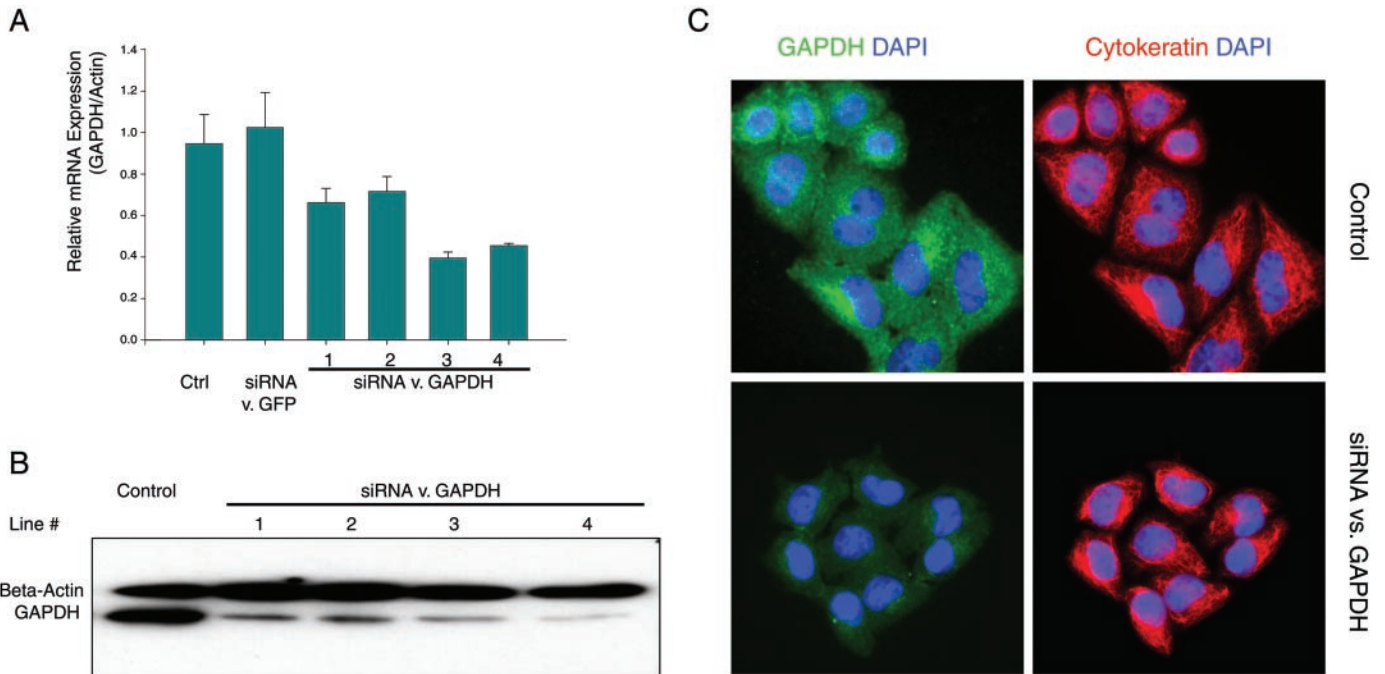


Figure 3. Inhibition of endogenous GAPDH expression using L1-based RNAi system. (A) Real-time RT-PCR results of GAPDH mRNA expression in stable HeLa lines expressing control, GFP-targeted or GAPDH-targeted siRNA ($n=6$). (B) Western-blot analysis of GAPDH protein expression in control or GAPDH knockdown clones. (C) Immunofluorescent staining of GAPDH (green) and cytokeratin (red) in control and knockdown cells. Nucleus is counterstained with DAPI (blue).

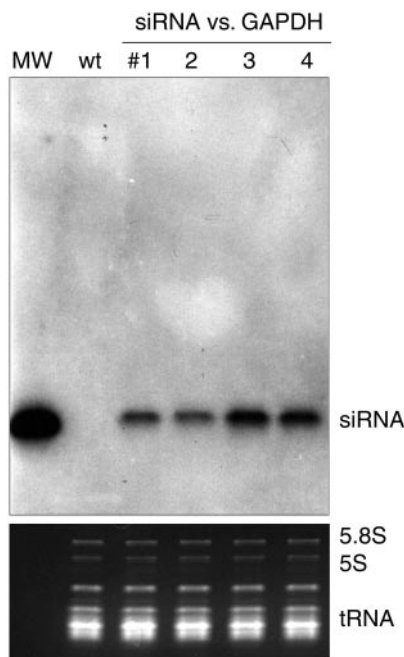


Figure 4. Northern-blot analysis of siRNA expression in GAPDH knockdown cells. Upper panel (from left to right): lane 1, 19 nt probe containing the sense strand sequence of GAPDH siRNA as a molecular weight marker (MW), which was also used as the probe to detect antisense siRNA in the test samples; lane 2: wild-type HeLa (wt); lanes 3–6: four GAPDH knockdown lines (#1, 2, 3 and 4). Position of antisense siRNA is indicated. Note the inverse relationship of siRNA expression seen here with GAPDH mRNA and protein expression (Figure 3A and B). Lower panel: gel post-electrophoresis. Positions of 5.8S, 5S and tRNAs are indicated.

pL1-Silencer plasmid in the host genome, PCR was performed using genomic DNA extracted from G418 resistant clones and a pair of primers flanking the γ -globin intron that disrupts the *neo* gene (Figure 5A). As expected, the intron was correctly spliced out in all stable knockdown clones (Figure 5B), i.e. a retrotransposition event had occurred. Although permitted by the PCR condition, no unspliced product was detected, indicating the absence of the integrated pL1-Silencer plasmid in the host genome. We also performed PCR with a reverse primer further downstream and confirmed by sequencing that the intact RNAi-cassette had been integrated into the genomes of the knockdown clones (data not shown).

Characterization of L1 retrotransposition in human cells

Since our RNAi system is based on L1 retrotransposition, we further characterized its properties in human cells, using a GFP-cassette to replace the *neo*-cassette for retrotransposition detection (24). Consistent with a previous report (24), we found that retrotransposition was not detectable until 2–3 days post-transfection and the percentage of cells hosting a retrotransposition event increased in a linear manner up to day 7 (Figure 6A). We also found that retrotransposition indeed reached a plateau around day 7 and few new events occurred after that (Figure 6B).

We then used a real-time PCR strategy to estimate the number of retrotransposition events per cell (Figure 6C). A pair of primers was designed within the GFP gene flanking the γ -globin intron and a bridging Taqman probe was designed such that no fluorescence would be emitted unless the intron

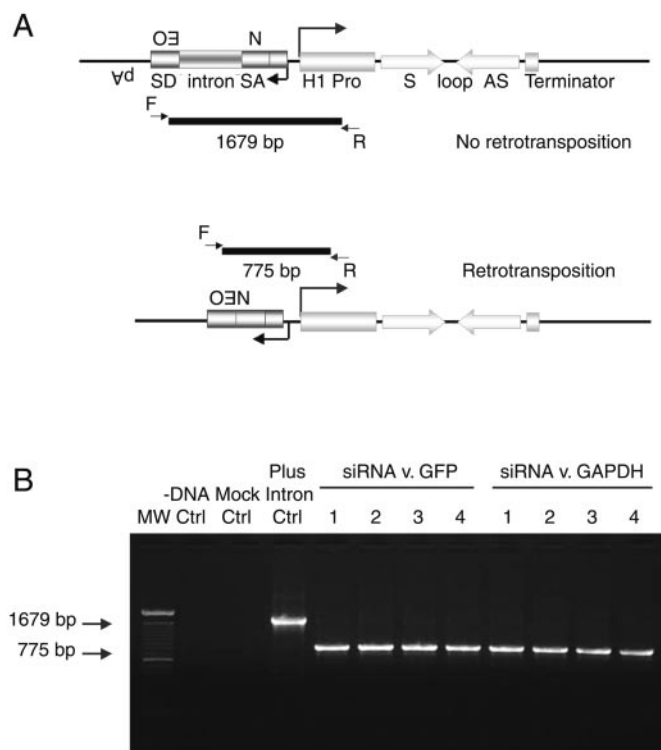


Figure 5. G418 resistant clones are derived from retrotransposition. (A) PCR strategy to characterize retrotransposition of tagged L1. The primer pair (F and R) produces a PCR product of 1679 bp before or of 775 bp post retrotransposition of tagged L1. (B) Electrophoresis of the PCR product of genomic DNA from knockdown clones, plus controls with no DNA (-DNA), wild-type HeLa DNA (Mock) or pL1-Silencer plasmid (Plus Intron). MW, molecular weight marker. Arrows indicate the positions of expectant bands.

was correctly spliced out, i.e. upon retrotransposition. In our experiment, we examined retrotransposition of the GFP-cassette tagged L1 in HeLa cells by both FACS and real-time PCR. FACS analysis revealed that on day 7 post-transfection 3.4% of transfected cells hosted retrotransposition event(s) from a tagged L1_{RP}, whereas ~21.3 copies of GFP sequence resulting from retrotransposition were detected per 1000 GAPDH gene copies (i.e. 500 HeLa cells) or 4.3 GFP copies per 100 cells (Figure 6D). Thus, on average 1.25 (4.3/3.4) retrotransposition events of tagged L1_{RP} occurred per cell. A similar result was obtained with tagged L1.3. Therefore, most transfected cells host no more than one retrotransposition event from a tagged L1, meaning that most knockdown clones contain only one RNAi-cassette in their genome.

DISCUSSION

Current siRNA production methods include both *in vitro* and *in vivo* strategies. *In vitro* chemically or enzymatically generated siRNA is limited, costly and produces only a transient effect; in addition, special cautions have to be taken when handling and working with RNA. All these reasons limit the application in whole animal models or clinical therapy (2,27). A more practical strategy is to produce siRNA *in vivo* from an expression cassette. The most popular delivery

vectors used are plasmid- and retrovirus-based, because they are efficient and capable of conferring stable RNAi. However, inclusion of plasmid sequence into the host genome and insertional mutation caused by retroviral integration (28) are always a concern, especially when clinical therapy is contemplated (2).

L1 retrotransposon has long been proposed as a novel molecular delivery tool. However, because reverse transcription during retrotransposition infrequently produces products >3 kb in length (29,30), its delivery capacity is limited. In this study, we avoided this problem by delivering a short siRNA expression cassette of ~150 bp. Our L1-based RNAi system allowed successful siRNA expression (Figure 4) and stable reduction of both exogenous and endogenous gene expression (Figures 2 and 3) and thus provides a simple and efficient method for stable gene silencing *in vivo*. Actually, the GFP silencing induced by our system (up to 87%, Figure 2) was greater than that induced by chemically synthesized siRNA of the same sequence (~64%) (21). Although GAPDH reduction was not as dramatic as that of GFP, we probably approached its limit since GAPDH is a critical gene involved in cellular metabolism and greater reduction may lead to cell death. Indeed, we observed that GAPDH knockdown clones were smaller and grew slower (data not shown). Most importantly, using the same H1 promoter and shRNA sequence against GAPDH our system delivered silencing similar to that of the pSilencer3.0-H1 plasmid (Ambion) and much greater silencing than that of the pSIREN-derived retroviral delivery system (Clontech) (data not shown).

In summary, our L1-mediated RNAi system has unique advantages over current delivery vehicles: (i) L1 proteins have no immunogenicity even upon inadvertent expression from heterologous promoters in the genome. (ii) Unlike retroviral integration that always includes an LTR with strong promoter activity, L1 usually loses its internal promoter during integration and is thus unable to activate host genes. (iii) A single copy of the integrated RNAi-cassette is sufficient to elicit strong silencing, which is consistent with the previous reports (31). Therefore, in comparison with the multiple integrations of retrovirus and their preference for active genes (32) and the tandem repeat insertions of plasmid, the random and single integration of L1 retrotransposon is especially beneficial because it reduces the likelihood of insertional mutations and non-specific interferon response owing to the overexpression of siRNA from multiple transgenes (2,27). (iv) All the G418 resistant clones tested were positive for silencing, suggesting that the integrated RNAi-cassette probably resided in an active chromatin structure owing to its proximity to the expressed *neo* gene. Interestingly, we also observed an inverse correlation between siRNA expression and gene silencing (Figure 4) in GAPDH knockdown clones. Variation in GAPDH siRNA expression among different clones was probably owing to position effect at the different genomic integration sites. Also, all the knockdown clones contained a retrotransposed RNAi-cassette but not an integrated pL1-Silencer plasmid (Figure 5B). Taken together, our system is more efficient at producing stable knockdown clones compared with plasmid vector delivery. (v) No expertise in viral handling is required; and compared with a recently reported transposon-based RNAi delivery method (33), our system avoids co-transfection of two plasmids. In conclusion,

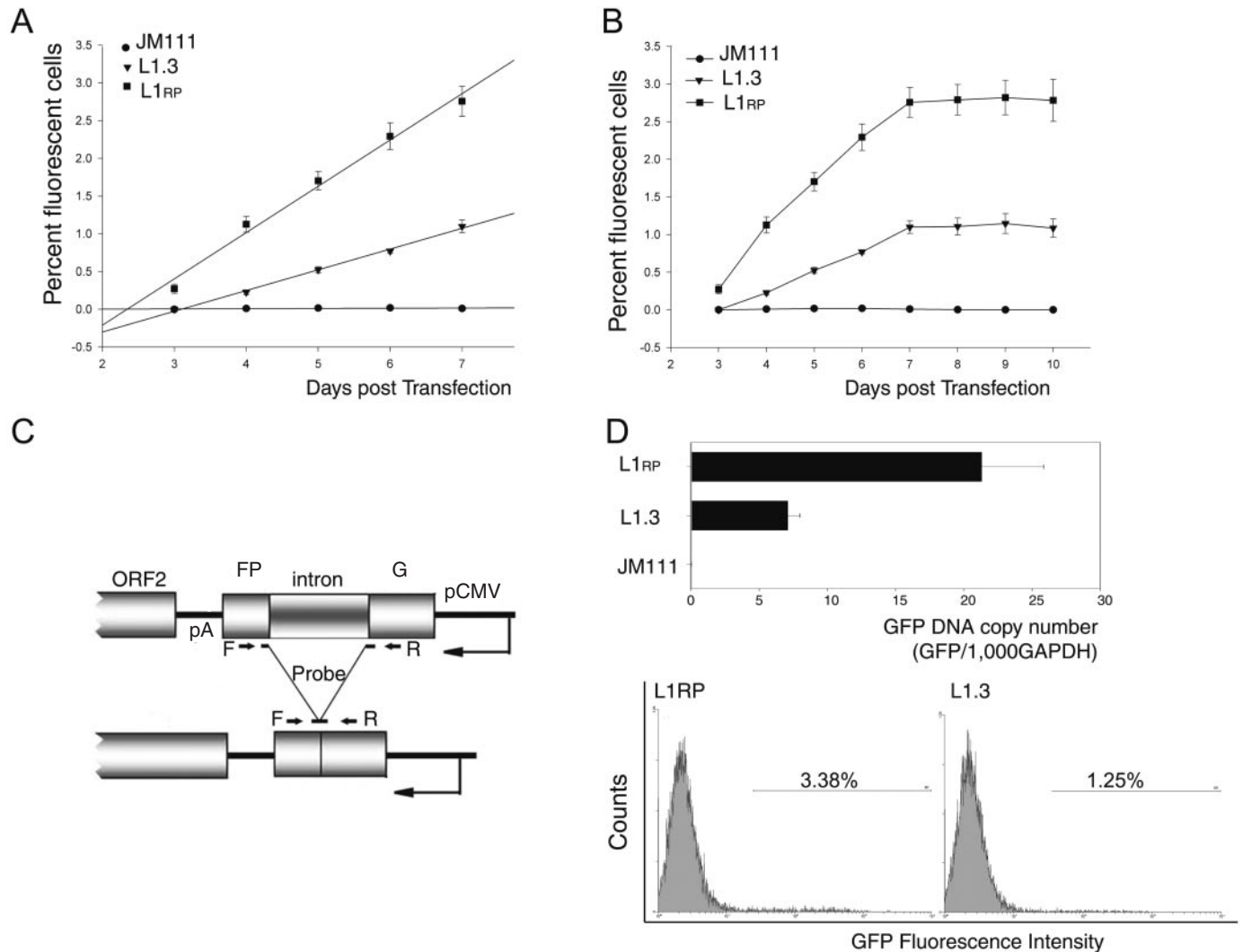


Figure 6. L1 retrotransposition occurs in a controlled manner. (A) Retrotransposition frequency is linear in cultured cells up to day 7 post-transfection as revealed by FACS ($n = 4$). JM111, a negative control of L1 with eliminated retrotransposition activity. (B) Retrotransposition frequency reaches a plateau around day 7 post-transfection ($n = 4$). (C) Real-time PCR strategy to characterize retrotransposition of GFP-cassette tagged L1. Arrows, primers; bar, Taqman probe. (D) Retrotransposition frequency of exogenously introduced L1_{RP}, L1.3 and JM111 in HeLa on day 7 post-transfection as revealed in parallel by real-time PCR (upper panel) in which data are represented as copy numbers of intact GFP per 1000 GAPDH and by FACS (lower panel) in which data are represented as percentage of GFP positive cells among transfected cells.

all these advantages make our system a promising option for stable gene silencing in mammalian cells; and more importantly, it may represent a highly attractive tool for molecular therapy. For example, L1 retrotransposition probably occurs in human lymphoid cells (23,34); therefore, *ex vivo* therapy targeting host factors involved in HIV life cycle, e.g. TSG101 and AIP1 (35), or pathogenic oncoproteins associated with lymphoid malignancies, e.g. TEL-AML1 and E2A-HLF (36), can be contemplated.

This is among the first studies using L1 retrotransposon for transgene delivery. The system certainly has limitations at this infant stage. For example, delivery of the plasmid-based construct into certain cells can be difficult. However, this problem may be alleviated with the improvement of present methods (e.g. electroporation and liposome carrier). Also, L1 retrotransposition may have cell type restrictions (37). This may be overcome by the modification of L1 5'-UTR,

e.g. inclusion of a heterologous promoter to get L1 transcripts in other cell types (38), since transcription is proposed as the rate-limiting step of retrotransposition (15,23). One could also create synthetic L1 with novel properties (39). In conclusion, our L1-based RNAi system can provide efficient stable gene silencing in human cells and has promising practical applications in molecular therapeutics.

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