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Article

A Multifunctional Lentiviral-Based Gene Knockdown with Concurrent Rescue that Controls for Off-Target Effects of RNAi

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Abstract

The efficient, stable delivery of siRNA into cells, and the appropriate controls for non-specific off-target effects of siRNA are major limitations to functional studies using siRNA technology. To overcome these drawbacks, we have developed a single lentiviral vector that can concurrently deplete endogenous gene expression while expressing an epitope-tagged siRNA-resistant target gene in the same cell. To demonstrate the functional utility of this system, we performed RNAi-depleted α-actinin-1 (α-ACTN1) expression in human T cells. α-ACTN1 RNAi resulted in inhibited chemotaxis to SDF-1α, but it can be completely rescued by concurrent expression of RNAi-resistant α-ACTN1 (rr-α-ACTN1) in the same cell. The presence of a GFP tag on rr-α-ACTN1 allowed for detection of appropriate subcellular localization of rr-α-ACTN1. This system provides not only an internal control for RNAi off-target effects, but also the potential tool for rapid structure-function analyses and gene therapy.

Key words: lentivirus, RNAi, shRNA, α-actinin-1, chemotaxis

Introduction

Since the completion of human genome project, an emerging challenge is the development of tools for functional genomics. Small interfering RNA (siRNA) technology has proved to be a rapid and powerful tool in studying gene functions. However, siRNAs introduced into cells via electroporation or liposomes cannot maintain stable under the regulation of target genes due to their progressive degradation or dilution with cell division. Furthermore, some cell lines and many primary cells are very difficult to be transfected or nucleofected with siRNA. To overcome these drawbacks, several viral vectors capable of delivering a small hairpin RNA (shRNA) expression cassette into mammalian cells were developed. Among them, lentiviral vectors hold the most promise, as they are capable of infecting a broad spectrum of cell types, including non-dividing primary cells, and delivered exogenous DNA is integrated into the cell genome, which allows for sustained, stable expression in tissues or cultured cells (*1, 2*). Thus, a lentiviral vector-based shRNA system could provide for a potential therapeutic tool for human diseases (*3-5*).

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Although there are many guidelines and algorithms to predict accurate target sequence for siRNA gene knockdown (*6, 7*), the specificity of siRNA remains a problem. Accumulating evidence indicates that non-specific off-target effects by siRNAs are prevalent. In various studies, dozens to hundreds of genes could be nonspecifically affected, by either the sense or antisense strand of siRNAs (*8-12*). Alternatively, some siRNAs induce an interferon response (*13*). In contrast to siRNAs, shRNAs have a short hairpin loop that forms a closed end with a two-nucleotide overhang at the 3' end. shRNAs may trigger off-target effects by being processed into siRNAs *in vivo*, by Dicer dependent or independent cleavage in the stem or loop, and changing them into an RNAi mechanism (*14*). Furthermore, shRNAs may also function as mimics of miRNAs to cause widespread off-target effects (*15*). These side-effects can lead to misinterpretation of phenotypes as a result of false-positive responses (*9, 12, 16-18*). To control for potential off-target effects of siRNA, various strategies have been employed, such as using multiple siRNAs directed at differing regions in the mRNA, or restoring the expression of target gene with an RNAi-resistant mutant to demonstrate the reversal of phenotype (*19*).

To address three major technical limitations in siRNA and shRNA application, including setting control for non-specific off-target effects of siRNA, enrichment of shRNA-transduced cell population, and stable delivery of shRNA expression cassette into target cells, we developed a single lentiviral vector, which can concurrently knockdown and rescue expression of a target gene in the same cell. We demonstrated the functional application of this system in T cells by shRNA-depleting endogenous α-actinin-1 (α-ACTN1) while rescuing, concurrently, with green fluorescent protein (GFP)-tagged RNAi-resistant α-ACTN1 (rr-α-ACTN1). Using this approach, we found that α-ACTN1 is specifically required for T cell chemotactic migration induced by efficient SDF-1α.

Results and Discussion

As depicted in **Figure 1A**, the lentiviral vector was constructed with two long terminal repeats (LTRs) derived from pFG12 (*2*). The cis-regulatory sequences in the 3' LTR was removed from the U3 region and copied to the 5' LTR after reverse transcription, leading to transcriptional inactivation of both LTRs in target cells. One multiple cloning site (MCS) was created for the insertion of shRNA expression cassette between the HIV flap sequence and the human ubiquitin C (UbiC) promoter, while the other was created between GFP (or YFP) and UbiC promoter for expression of an RNAi-resistant gene or functionally related gene. The presence of GFP (or YFP) or GFP fusion peptide also allows for a determination of transduction efficiency. Since the GFP tag retards migration of the RNAi-resistant gene product on SDS-PAGE, one can readily distinguish it from the endogenous protein by Western blots using antibodies against the endogenous protein directly. It also allows for comparative quantification of exogenous RNAi-resistant product versus endogenous protein, and the extent of endogenous gene product depletion by shRNA. A further benefit of the GFP tag is to ensure that the RNAi-resistant protein is properly localized within the cell, and to make it possible to analyze the functional effects of gene depletion at the single-cell level, if necessary. Finally, the expression of a puromycin-resistance marker allows for removal of non-transduced cells, thereby enhancing the phenotype within a population of cells.

In addition to a C-terminal GFP of the RNAi-resistant rescue proteins, we have also constructed vectors with C-terminal Flag-His6 (FH) (Figure 1A) that facilitate efficient and relatively clean immunoprecipitation (IF) of rescued genes in the presence of endogenous knockdown. With these new lentiviral vectors, we have transduced a variety of human and mouse cell types (*e.g.*, epithelial cells, keratinocytes, fibroblasts, and hematopoietic cells), including tumor cell lines (*e.g.*, HepG2, HaCAT, and Jurkat T cells) and primary cells (*e.g.*, human primary endothelial cells, mouse embryonic fibroblasts, bone marrow derived macrophages, and hippocampal neurons). The efficiency of transduction varies with cell type and virus titer applied, as expected; however, following puromycin selection of transduced Jurkat T cells, more than 98.5% of remaining cells were GFP positive (**Figure 1B**).

To demonstrate the utility of the system, we chose to target α-ACTN1 in human T cells. α-actinins are

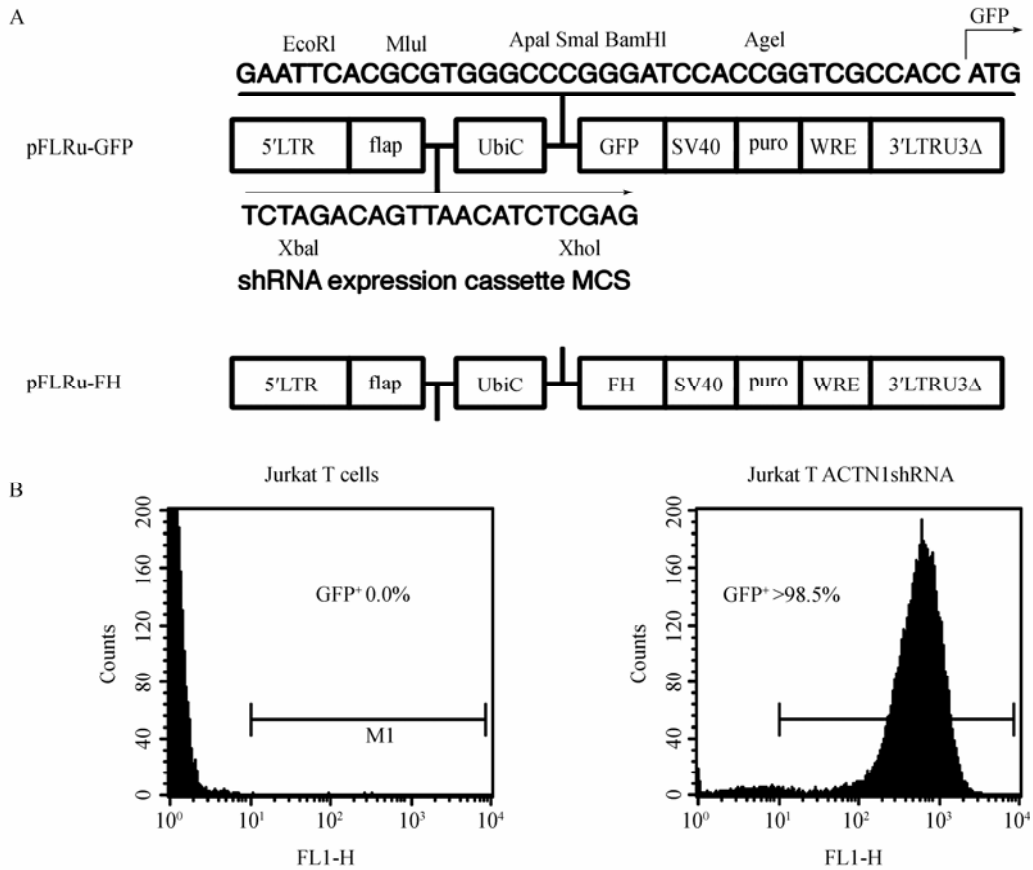

Figure 1 Multifunctional lentiviral vectors. **A**. Map of the lentivrial vectors. pFLRu was generated as described in Materials and Methods. There are two MCSs: 5' of UbiC has the MCS for shRNA expression cassette, and 3' of Ubic has the MCS for RNAi-resistant isoform of shRNA targeted gene containing an in-frame C-terminal GFP tag and FH. It also contains a puromycin (puro) resistance cassette. **B**. Jurkat T cells were infected with freshly produced lentivirus containing pFLRu-α-ACTN1 shRNA, puromycin selected, and FACS analysis performed. The percentages of GFP positive cells are presented. Left panel: parental Jurkat T cells; right panel: lentiviral transduced Jurkat T cells.

actin binding proteins cross-linking F-actin to actin bundles or network, also connecting F-actin to plasma membrane (*20*). There are four α-actinin isoforms identified and characterized. α-ACTN2 and 3 are muscle-specific, which crosslink F-actin in the region of Z-discs of striated muscle cells (*21*), while α-ACTN1 and 4 are ubiquitously expressed. Increased cell motility in α-ACTN4-deficient lymphocytes has been reported (*22*). However, the function of α-ACTN1 on lymphocyte chemotactic migration remains unknown.

To obtain shRNA expression cassette, we performed overlapping PCR to join U6 promoter with α-ACTN1 shRNA target sequence, which produced a putative 27-mer shRNA shunting into a Dicer-dependent interference mechanism *in vivo* (*14*) (**Figure 2A**). Indeed, this predicted 27-mer shRNA exhibited much more potent protein depletion than a corresponding 20-mer counterpart (**Figure 2B and C**).

To rescue α-ACTN1 knockdown, we synthesized an RNAi-resistant isoform of human α-ACTN1 (rr-α-ACTN1). Four nucleotides in the siRNA targeting sequence were changed, and resulted in a new *Ssp*I restriction site that facilitated identification of correct transformants (**Figure 3A**). These nucleotide changes did not alter the encoded amino acids. Endogenous α-ACTN1 level was dramatically reduced in Jurkat T cells infected with the lentivirus expressing the shRNA directed against human α-ACTN1, while control shRNA expressing viruses had no effect (**Figure 3B**). Jurkat T cells were then infected with a lentivirus expressing both α-ACTN1 shRNA and rr-α-ACTN1-GFP. Endogenous α-ACTN1 and rr-α-ACTN1-GFP fusion proteins were detected by Western blots

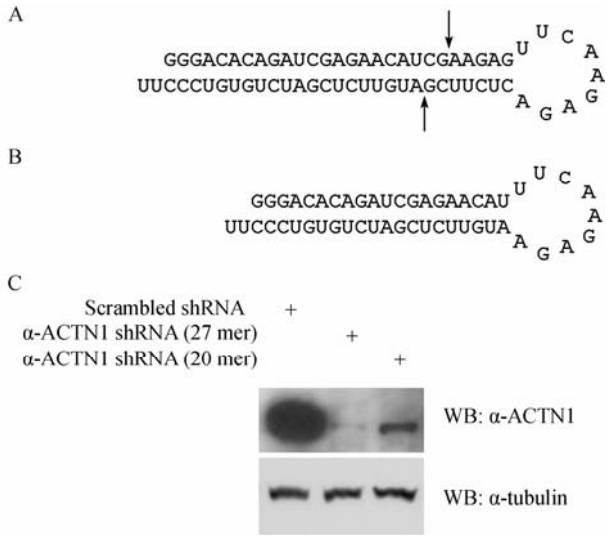

Figure 2 Lentiviral-based Dicer-dependent α-ACTN1 shRNA worked more potent than Dicer-independent counter part. **A**. Putative 27-mer shRNA for α-ACTN1 and predicted shRNA cleavage sites are indicated in arrows. **B**. Putative 20-mer shRNA for α-ACTN1. **C**. Western blot analysis of Jurkat T cells transduced with lentivirus expressing control shRNA (lane 1), α-ACTN1 27-mer shRNA (lane 2), and α-ACTN1 20-mer shRNA (lane 3). Jurkat T cells were transduced by viruses carrying corresponding shRNA expression cassette, and selected by puromycin. The residue cells were collected, and the cell lysate was prepared for Western blots using anti-α-ACTN1 antibody as primary antibody.

using anti-α-ACTN1 antibody (Figure 3B). Immunostaining results showed that rr-α-ACTN1-GFP was found to localize to the same subcellular site as endogenous α-ACTN1, and accumulate around the cell periphery, co-localizing with F-actin (**Figure 3C**). It indicated that the presence of the C-terminal GFP fusion did not affect cellular localization of α-ACTN1.

Finally, we asked whether expression of rr-α-ACTN1-GFP could rescue a phenotype caused by RNAi depletion of endogenous α-ACTN1 protein. RNAi depletion of α-ACTN1 in Jurkat T cells resulted in inhibited chemotaxis in response to an SDF-1α stimulus (**Figure 3D**). This migratory/chemotactic phenotype was rescued by expression of rr-α-ACTN1-GFP in the presence of endogenous α-ACTN1 protein depletion (Figure 3D). Rescued cells expressed 3-fold more rr-α-ACTN1-GFP than endogenous α-ACTN1 (Figure 3B), exhibiting enhanced migration relative to control shRNA infected cells (Figure 3D). These results showed that inhibition of T cell chemotaxis due to RNAi-mediated α-ACTN1 depletion was specific, which is not the result of off-targeting, nonspecific gene silencing.

Our system does not eliminate off-target effects of RNAi, but provides a reliable control for potential off-target effects. The two sets of cells expressing shRNA and shRNA with concurrent rescue have the same shRNA expression and loading in cells, therefore should have similar off-target effects. Our system also provides a tool to determine protein structure-function relationships in cells by rescuing shRNA depletion with RNAi-resistant mutant isoforms or specifically modified posttranslation. In addition, the status of the target protein in a specific signaling pathway can be determined by our system when rescuing with constitutively active isoforms of potential downstream effectors or upstream regulators. Furthermore, our system could also be used for genetic modification of T cells or dendritic cells to target tumors by knocking down immune suppression genes like A2A receptor (*23*) while concurrently expressing a tumor-specific targeting gene like TCR (*24*).

Although we have successfully applied this system in different cell lines for functional studies, under some circumstances, especially in some of primary cells (*e.g.*, bone marrow derived macrophages), we have encountered apoptotic, cytotoxic or osteoclastogenesis phenotypic changes, which cannot be rescued by expression of RNAi-resistant gene isoforms (data not shown). Nonspecific phenotypic changes could be the result of off-target effects of RNAi, or result from oversaturation of the endogenous small RNA pathway by shRNA overexpression (*25, 26*). If this is the case, we would recommend optimization of the shRNA sequence (*25*), or shRNA dosage, by replacing the U6 promoter with an H1 promoter, which is a relative weak promoter (*26, 27*).

The parental lentiviral vector described herein has been successfully used for germline transmission and *in vivo* analysis in mouse tissues (*28*). Indeed, we performed microinjection of concentrated lentivirus into the fetus or neonatal ventricle of the mouse brain and found significant expression of GFP in the neurons, indicating a successful delivery of viral packaged transcripts containing shRNA expression cassette (data not shown). Therefore, this new system could also be applied in an *in vivo* setting or for a gene therapy, possibly, by knocking down both

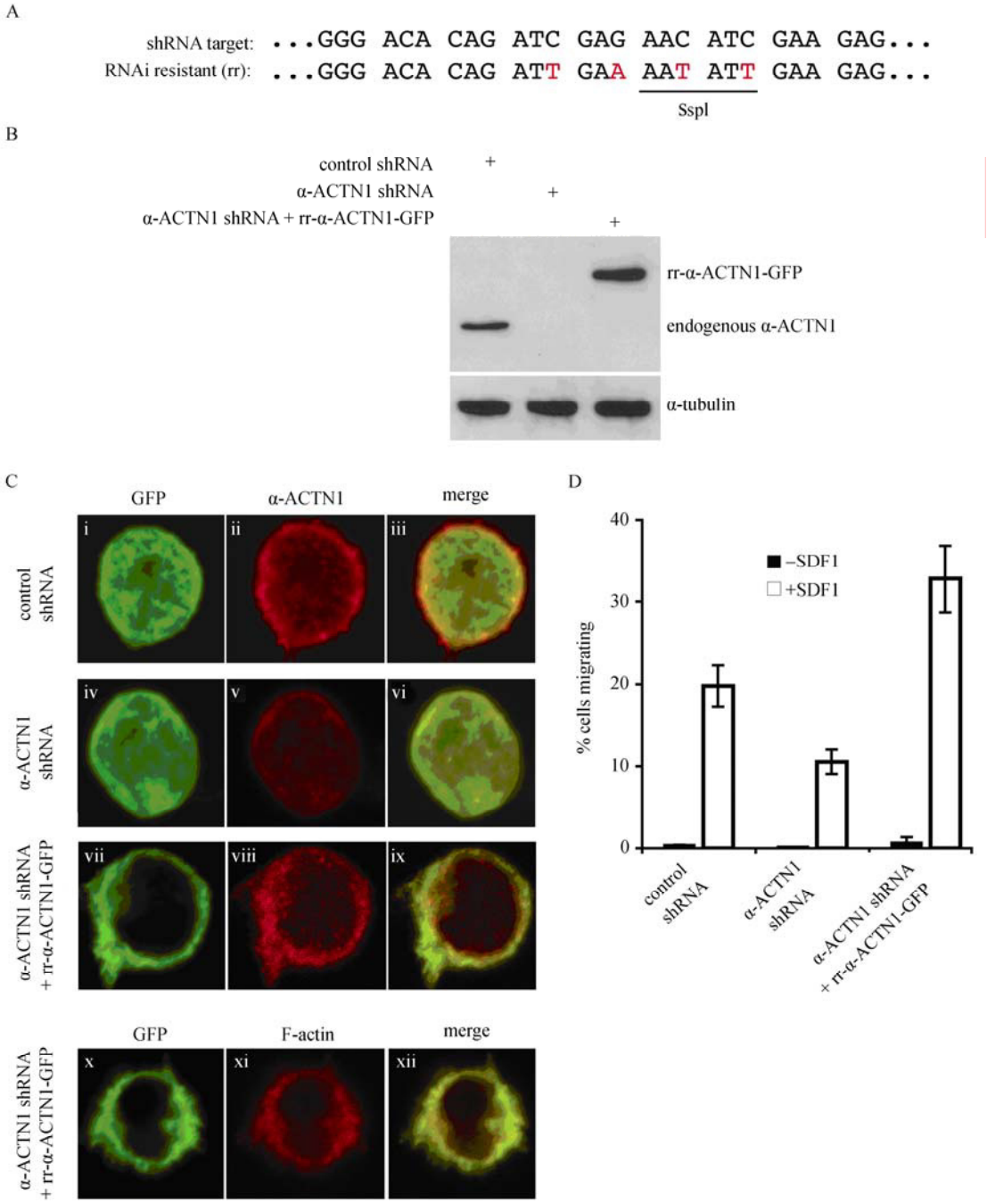

Figure 3 Cellular and functional characterization of multifunctional lentivirus. **A**. Mutations in RNAi-targeted sequence of human α-ACTN1 (red) to generate rr-α-ACTN1. **B**. Western blot analysis. α-ACTN1 levels in Jurkat T cells transduced with control shRNA lentivirus (lane 1), α-ACTN1 shRNA lentivirus (lane 2), and α-ACTN1 shRNA/rr-α-ACTN1-GFP (lane 3) were detected using anti-α-ACTN1 antibody. The α-tubulin levels were detected as a loading control using anti α-tubulin antibody. 30 μg of protein from each cell lysate was applied in each lane. **C**. Subcellular localization of endogenous α-ACTN1 and rr-α-ACTN1-GFP in Jurkat T cells transduced with control shRNA lentivirus (i-iii), α-ACTN1 shRNA lentivirus (iv-vi), and α-ACTN1 shRNA/rr-α-ACTN1-GFP lentivirus (vii-ix). The lower panel (x-xii) demonstrates co-localization of rr-α-ACTN1-GFP and F-actin. Cells were stained with the polyclonal antibody against GFP (i, iv, vii and x), the monoclonal antibody against α-ACTN1 (ii, v and viii), or Rhodamine phalloidin (xi). Confocal images were obtained. **D**. α-ACTN1 shRNA depletion of endogenous α-ACTN1 in Jurkat T cells results in inhibited chemotaxis to SDF-1α that is rescued by co-expression of rr-α-ACTN1-GFP. Values represent average +/− standard deviation of triplicates. The experiments were performed three separate times with similar results.

disease mutation-derived transcripts and wild-type-allele-derived transcripts with an shRNA that targets both, and rescuing with RNAi-resistant wild-type protein or other therapeutic genes. This new system has the potential to advance basic research questions as well as development of gene-based therapeutics.

Materials and Methods

Antibodies and cell lines

Monoclonal anti-α-ACTN1 antibody was purchased from Sigma (A5044, clone BM-75.2) and used for Western blot and IF. Polyclonal antibody against GFP was purchased from Molecular Probes, and h-SDF-1α was from PerproTech. Jurkat T cells were maintained in RPMI-10 containing 10% FBS, pen/strep and glutamine. Human keratinocyte cell line HaCat and large T antigen immortalized human bronchial epithelial cell line Beas-2B, HEK-293-T cells were cultured in DMEM-10 containing 10% FBS, pen/strep and glutamine.

Plasmid construction

5' LTR and 3' LTRU3Δ from FG12 (*2*) were subcloned into pBluescript to obtain the intermediate vector (p1). PCR fragments of UbiC promoter, GFP (pEGFP-N1 as template), YFP (pCS2-YFP as template), FH, and SV40-puro (pMX-puro as template) were spliced together in various combinations to obtain another intermediate vector (p2). Digestion of p2 with *Xho*I/*Not*I yielded a fragment containing UbiC-MCS-GFP (or YFP, FH)-SV40-puro that was then inserted between the LTRs in p1 to obtain the lentiviral vector shown in Figure 1. The complete vector was sequenced and named pFLRu (Feng's Lenti-Rescue using UbiC promoter). An shRNA expression cassette was constructed by joint PCR. The predicted human α-ACTN1 sequence targeted was GGGACACAGATCGAGAACATCGAAGAG. We obtained the hU6 promoter (f1) by amplifying pBS-hU6-1 template (*2*) with primers 1 and 2, and the shRNA fragment (f2) with primers 3 and 4 (**Table 1**). The last 5-6 base pairs are complementary to the corresponding base pairs before the hairpin in the same sequence. F1 was purified from 1% TAE-agrose gel with gel purification kit (Invitrogen) while f2 was purified from 12% TAE-polyacrylamide gel. Joint PCRs were carried out by using hU6 forward primer (primer 1), shRNA reverse primer (primer 4) and mixed template (2 μL of purified f1 and f2). The PCR products were purified, cut with *Xba*I/*Xho*I and subcloned into the pFLRu. For control shRNA, we used a fire fly luciferase target sequence (5'-gCTTACGCTGAGTACTTCGA) under U6 promoter.

Construction of rr-α-ACTN1 cDNA

Primers with three or four point mutations inside the siRNA targeted DNA sequence were designed, based upon wobble base pairing rules. For human α-ACTN1, we designed four primers (primers 5-8) (Table 1). N-terminal fragment (N-ter) and C-terminal fragment (C-ter) of rr-α-ACTN1 were obtained by PCR using primer pairs 5 and 6, or 7 and 8, respectively. Full length of rr-α-ACTN1 was obtained by joint PCR using purified N-ter and C-ter mixed template and primers 5 and 8, followed by subcloning the fragment into *Eco*RI/*Age*I sites of pFLRu.

Table 1 PCR primer pairs used for hU6 promoter, shRNA fragment and human α-ACTN1 cloning

Primer	Sequence	Orientation
1	5'-ACAGAATTCTAGAACCCCAGTGGAAAGACGCGCAG	forward
2	5'-GGTGTTTCGTCCTTTCCACAAG	reverse
3	5'-GTGGAAAGGACGAAACACC**GGGACACAGATCGAGAACATCGAAGAG***TTCAAGAGA***CTCTTC**	forward
4	5'-TCCAGCTCGAGAAAAA**GGGACACAGATCGAGAACATCGAAGAG***TCTCTTGAA***CTCTTC**	reverse
5	5'-AGAGAATTCCATGGACCATTATGATTCTCAGCAAAC	forward
6	5'-CCTCTTCAATATTTTCAATCTGTGTCCCCGCCTTCCGGAG	reverse
7	5'-CACAGATTGAAAATATTGAAGAGGACTTCCGGGATGGCCTG	forward
8	5'-CTCGGTACCGGTAGGTCACTCTCGCCGTACAGGCGCGTG	reverse

Note: Nucleotides in italics were hairpin sequence while in bold face were target RNAi sequence.

Lentiviral generation and transduction

For lentivirus production, 8×10^5 of 293-T cells were played in each well of a 6-well plate overnight before transfection. For one well, the cells were transfected with packaging plasmids pHR'8.2ΔR/pCMV-VSV-G at a ratio of 8:1 (1.5 μg total DNA) (kindly provided by Dr. Sheila Stewart, Washington University, St. Louis) and pFLRu plasmid (1.5 μg). 24 h after transfection, the cell culture medium was refreshed and the following day medium containing virus was harvested from transfected 293-T cells and filtered through a 0.45 μm filter to remove any residual 293-T cells. To infect target cells, virus was mixed with fresh medium and protamine sulfate (final 10 μg/mL, Sigma), applied to target cells and incubated from 6 h to overnight. Cells were then fed with fresh, virus-free medium. 24 h later, puromycin was added (final 0.5 μg/mL) until all remaining cells were green under the fluorescent microscope. Dead Jurkat T cells were removed by sedimentation through a Ficoll Paque (Sigma) layer. To avoid multi-entry of viral transcripts, we used the virus from one well of cell culture to infect 1×10^6 Jurkat T cells, which gave about 50% infected cells before selection.

T cell migration assay

Jurkat T cells (3×10^5/mL) were serum-starved in migration medium (RPMI containing 0.25% BSA and 10 mM HEPES, pH 7.0) for 1.5 h. 4×10^5 cells in 100 μL of migration medium were loaded in the upper chamber of uncoated transwell insert (5 μm, Costar) in 24-well plates, and 600 μL of migration medium in the lower chamber, with or without h-SDF-1α (5 ng/mL). All samples were performed in triplicate. After 1.5 h at 37°C and 5% CO_2 in incubator, cells in the lower chamber were collected, pelleted, resuspended in 100 μL of migration medium, and counted.

Authors' contributions

YF and GDL conceived the project;YF performed the experiments; LN, MDT, QS, ZC, YZ constructed part of the intermediate plasmids and performed part of the experiments; YF, LN and MDT plotted the figures; GDL supervised the project; YF and GDL co-wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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