Application Forum

Permanent Knockdown of MicroRNAs using Lentivectors

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Introduction

A new and complicated layer of gene regulation mediated by small RNAs (microRNAs) has begun to revolutionize our understanding of how the cell regulates gene expression on the post-transcriptional level. Many microRNAs are expressed in a tissue-specific and developmental stage-specific manner. These small RNAs bind to target sites within the 3′ untranslated regions (UTRs) of mRNAs and repress translation of that mRNA. What the research field needs currently is a means to stably suppress microRNAs in order to study their signaling functions. In this Application Forum, we describe a new technology to address those experimental needs by providing permanent microRNA inhibition.

Knocking down microRNA function using miRZips™

miRZip anti-sense microRNAs are stably expressed short RNA hairpins that have anti-microRNA activity. These miRZip shRNAs produce short, single-stranded anti-microRNAs that competitively bind their endogenous microRNA target and inhibit its function. The result is the translational de-repression and elevation of the protein levels of the transcripts targeted by the microRNA being "zipped" (Figure 1). The miRZip hairpins are asymmetric and favor the lower strand in generating anti-sense microRNAs that are fully complementary to the specific microRNA being targeted. These fully complementary anti-microRNAs produce a thermodynamically stable duplex that interferes with the endogenous, targeted microRNA much more efficiently and completely than "sponge" half-site approaches. The anti-sense microRNA is continuously expressed at high levels, allowing the investigation of phenotypes which may only emerge over extended time periods.

miRZip lentivector constructs can be used for both GFP sorting and puromycin selection for stable cell lines. This technology provides a means to stably and permanently suppress a given microRNA to investigate multiple roles that a targeted microRNA may play in cellular development, cell patterning, signaling and cancer. SBI's miRZips allow the rapid and efficient delivery of the anti-miR expression constructs, decreasing the time and cost of microRNA inhibition screens and increasing the successful discovery of microRNA functions.

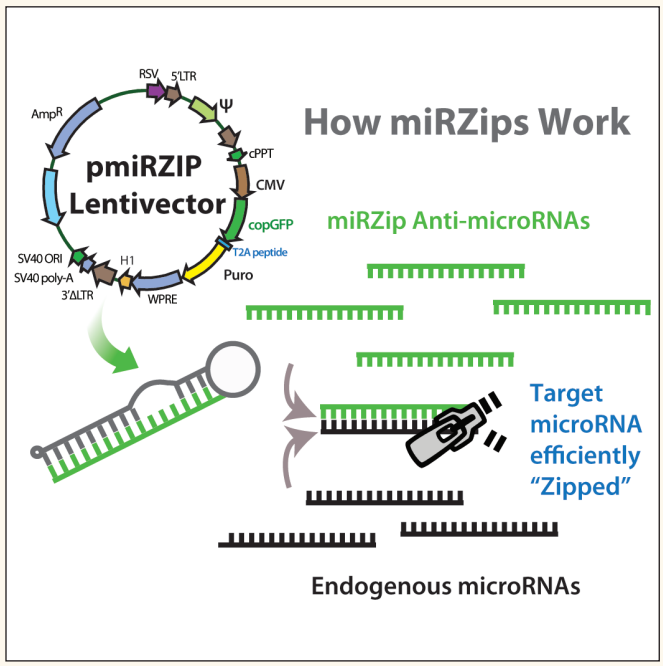

Figure 1. What are miRZips? Anti-sense constructs in SBI's lentivectors feature an H1 promoter driving the miRZip hairpins and CMV expressing the dual-marker cassette for GFP and puromycin. The miRZip hairpins are rationally designed for asymmetry such that the upper strand of the hairpin (gray) does not contain the endogenous microRNA sequence and the lower strand is preferred for producing anti-sense microRNAs (green) that are fully complementary to the specific microRNA being targeted.

Materials and Methods

Enhancing and knocking down miR-29a function

U937 human leukemic monocyte lymphoma cell lines were transduced with control lentivirus (Control), SBI's Lenti-miR-29a (Overexpress) or SBI's miRZip-29a (Knockdown). The cells were FACS-sorted for GFP-positive populations. GFP-positive cells were then propagated in RPMI 1640 + 10% FCS + P/S + beta-ME at 37°C for at least 2 weeks prior to preparation of lysates; no noticeable loss of GFP expression in the established cultures was observed. Whole-cell lysis was performed using standard method with NP-40 based detergent and protease inhibitor cocktail (Pierce). Total

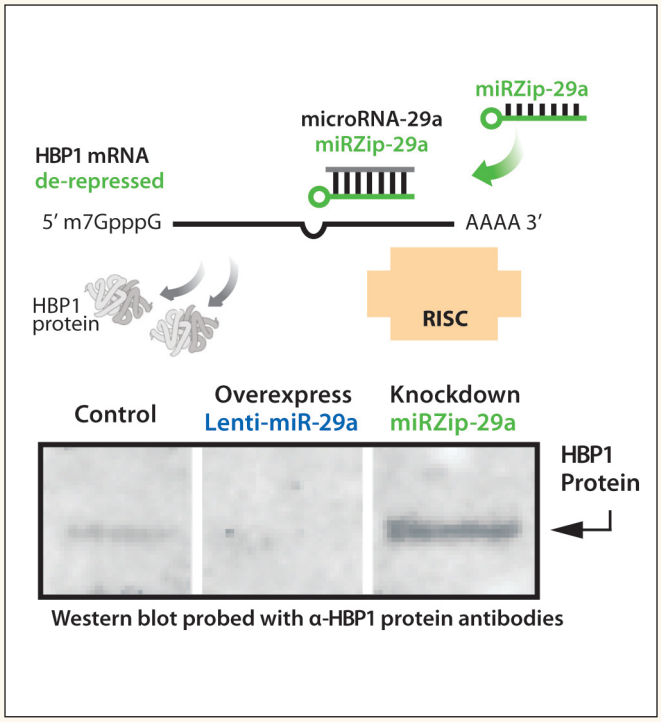

Figure 2. Modulation of HBP1 expression using miR-29a. The schematic at the top of the figure shows how binding microRNA-29a to the messenger RNA for HBP1 influences the protein output from that transcript. If miR-29a binds to the HBP1 mRNA, then less protein is synthesized. If miR-29a is suppressed by miRZip-29a anti-sense RNAs, then this is reversed. Western blot data demonstrating this modulation of HBP1 protein levels using SBI's Lenti-miR-29a (overexpress miR-29a) to reduce HBP1 protein levels—and the converse of inhibiting miR-29a with miRZip-29a—results in increased expression of HBP1 protein (lower panel).

protein concentrations were determined by Nanodrop (A280) and 20 µg equal protein amounts loaded and separated on a 4–12% denaturing protein gel. Standard Western blot protocols were performed using antibodies specific for HBP1 (AbCam).

Knocking down miR-21 and miR-145 functions

To investigate the roles of miR-21 (oncogenic microRNA) and miR-145 (tumor-suppressor microRNA) in breast cancer, cell invasion assays of MDA-MB-231 breast cancer cells were performed after lentiviral transduction with control vector lentivirus (Control) or miRZip-21 or miRZip-145 lentivirus. Matrigel chamber assays (BD Biosciences) were then used to screen for invasive phenotypes. After 20 h, invading tumor cells were fixed followed by staining with 0.05% crystal violet and then counted under a microscope. Representative fields of invasive cells on the membrane are shown for the Control and miRZip-21– or miRZip-145–treated cells. Further analysis of protein target changes for the miRZip-145 experiment was done through standard Western blot analysis with anti–c-Myc and anti–β-actin antibodies (AbCam).

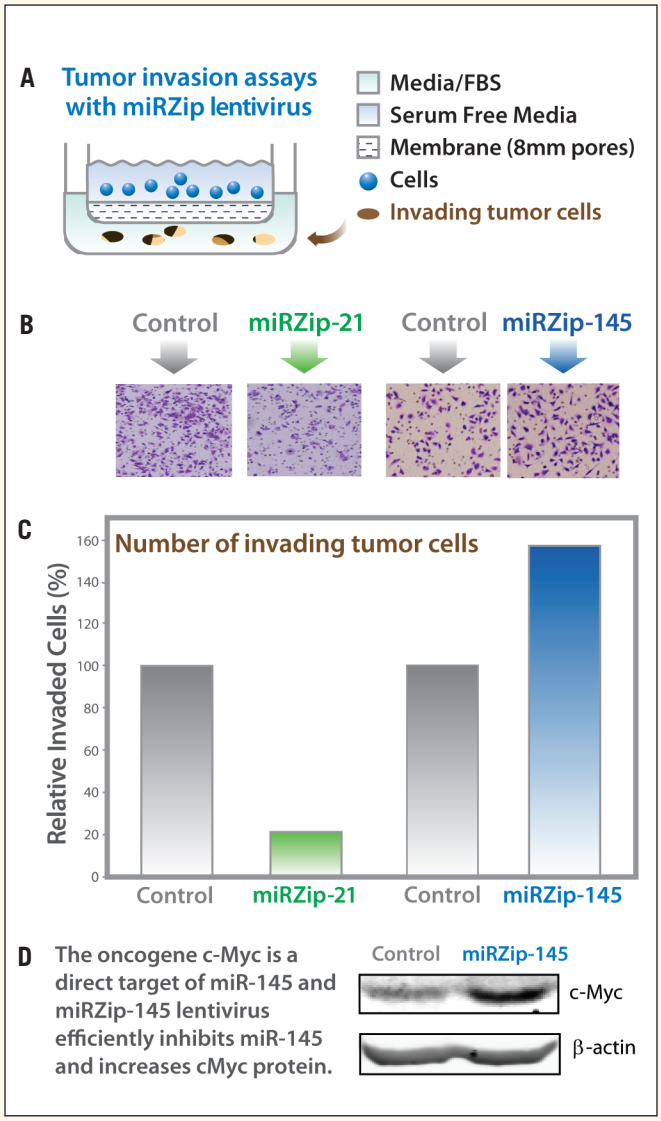

Figure 3. Tumor invasion assays using miRZips. (A) The tumor invasion assay using matrigel chambers and MDA-MB-231 breast cancer cell lines. The cells that have the ability to penetrate the matrigel and move to the bottom of the chamber correspond to the invasive phenotype. (B) After 20 h post-lentiviral transduction, invading tumor cells were fixed then counted under a microscope. Representative fields of invasive cells on the membrane are shown for the Control and miRZip-21– or miRZip-145–treated cells. (C) The number of invading tumor cells from the control, miRZip-21–, and miRZip-145–transduced cells are shown as bar graphs. (D) Western blot data of c-Myc protein levels from the miRZip-145 versus control invasion assays.

Results and Discussion:

The novel miRZip anti-microRNA technology built into lentivectors has been validated in leukemic and breast cancer cell model systems and tested for functional suppression across three distinct microRNAs, miR-29a, miR-21, and miR-145.

Profiling microRNA expression patterns in myeloid cell proliferation disorders revealed that miR-29a was overexpressed (1). To investigate this phenomenon

and discover the mechanisms underlying the roles of miR-29a in leukemic lymphomas, U937 human leukemic monocyte lymphoma stable cell lines that either overexpress miR-29a (Lenti-miR-29a) or permanently suppress miR-29a (miRZip-29a) were generated. Overexpression of miR-29a downregulated histone binding protein 1 (HBP1) protein levels and the suppression of endogenous miR-29a with miRZip-29a significantly de-repressed (upregulated) HBP1 protein levels (Figure 2). These studies demonstrate that the modulation of microRNA-29a directly affects the amounts of HBP1 protein production and that HBP1 mRNA 3′ UTR is a direct binding target of miR-29a (2).

Human breast cancers show a remarkable dysregulation of microRNAs when compared to normal breast tissues (3). In particular, the known oncogenic microRNA miR-21 is overexpressed and the tumor suppressor microRNA miR-145 is reduced in breast cancers. The breast cancer cell line MDA-MB-231 shows this same pattern of microRNA dysregulation and MDA-MB-231 cells are a model system for metastasis using tumor invasion assays (Figure 3A). To study the functions of both miR-21 and miR-145, MDA-MB-231 cells were transduced with miRZip-21 lentivirus expressing anti-mir-21 small RNAs. The result is 80% inhibition of metastasis (Figure 3B). Additionally, we wanted to explore whether the converse would occur if we inhibited miR-145 with the miRZip-145 lentivirus. Suppressing the endogenous miR-145 microRNAs with miRZip-145 showed significant increase of >60% in the number of metastatic breast cancer cells (Figure 3B). When miRZip-145 knocks down miR-145 activity, we also discovered that the oncogene, c-Myc protein levels became elevated (Figure 3C). A recent publication additionally shows the connection between miR-145 and c-*myc* oncogene expression (4). Thus, cancer phenotypic screens can be performed using miRZip lentivirus transductions to identify both oncogenic and tumor-suppressive microRNAs.

Conclusion

MicroRNAs impact nearly all physiological pathways, including apoptosis, proliferation, pluripotency, differentiation, and cancer. In order to begin the process of unraveling the complex regulatory circuits governed by microRNAs, tools for permanent microRNA overexpression and inhibition will play major roles in advancing this research field. SBI has developed a unique and effective technology to express abundant amounts of anti-sense small RNAs that competitively bind and inactivate targeted microRNAs. The anti-miRs are produced from hairpin RNAs expressed by an H1 promoter present in SBI's lentivector system that features a GFP and puromycin dual selection cassette. These anti-miRs, termed miRZips™, can be used to knock down a specific microRNA's function in either transient transfection experiments or permanently using lentivirus transduction.

SBI's miRZip lentivectors are powerful tools to explore microRNAs function and to validate microRNA to 3′ UTR binding activities. The miRZip-205 lentivector was highlighted in the recent Breast cancer studies published by Greene et al (5). In this report, the authors used miRZip-205 to clearly demonstrate the successful derepression of tumor-suppressor gene phosphatase and tensin homolog (PTEN). Other recent publications employing miRZips can be seen in Rajabi et al. where inhibiting the miR-125b tumor suppressor using miRZip-125b unleashes breast cancer cell proliferation mediated by de-repressing Mucin1 (6). A group in Sweden just published a study wherein suppressing miR-18a with miRZip-18a literally kills neuroblastoma cells (7). SBI is building a comprehensive miRZip lentivirus pool to suppress all known microRNAs. This virus pool will enable the "miRNome"-wide screening of microRNA functions all in a single screening experiment. More information on miRZips can be found online at www.systembio.com/mirzips or by calling SBI at 1-650-968-2200.

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