Scaffold design, function and over-expression of lentiviral-based microRNAs

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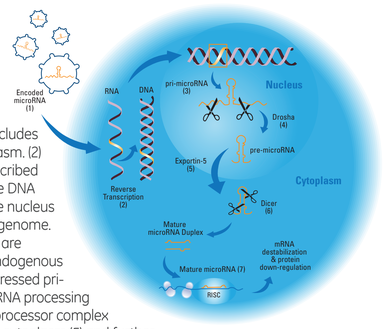
Abstract

MicroRNAs are short non-coding RNAs that regulate gene expression through targeted degradation of one or more genes and have been shown to play an important role in development, differentiation and disease. For example, microRNAs are critical for stem cell function and differentiation. Also, dysregulation of microRNA expression has been associated with the development and progression of many types of cancers. To better understand the functional roles of microRNAs in phenotypes of interest, gain-of-function experiments can be performed by introducing microRNA mimics into cells, which mirror the effects of endogenous microRNAs. Studies with synthetic microRNA mimics are useful in easy-to-transfect cell types as well as instances where phenotypes are readily observed over short time periods. However, additional delivery strategies are required for cells that are refractory to transfection and for phenotypes that develop over longer time points. Here we describe the strategy for scaffold design and highlight the importance of choosing an optimal promoter in cells of interest for the over-expression of lentiviral-based microRNA mimics. We demonstrate down-regulation of gene targets in difficult-to-transfect primary, immune and neuronal cells (HuVEC, K562, SH-SY5Y), and show mesenchymal to epithelial transition markers in MDA-MB-231 breast cancer cells by over-expression of miR-429.

Introduction

Borrowing the endogenous pathway that processes host encoded microRNA transcripts to over-express functional mature microRNA. (1) The lentiviral particle binds to the host cell and delivers its engineered RNA genome, which includes the encoded microRNA, to the cytoplasm. (2) The lentiviral genome is reverse-transcribed in the cytoplasm (*i.e.*, RNA to DNA). The DNA intermediate form is imported into the nucleus and is stably integrated into the host genome. (3) Dharmacon™ shMIMIC™ microRNAs are transcribed in the same manner as endogenous microRNA genes. Native and over-expressed pri-microRNA transcripts enter the microRNA processing pathway, are processed by the Microprocessor complex (4), shuttled out of the nucleus into the cytoplasm (5) and further processed by the Dicer complex into active, mature microRNA sequences (6) which are incorporated into the RNA Induced Silencing Complex (RISC). These microRNA-loaded RISC bind to target mRNAs and cause transcript destabilization, resulting in down-regulation of protein expression (7).



What is the best strategy for microRNA over-expression using lentiviral particles?

microRNA primary transcript strategy

Approximates primary microRNA sequence containing specific microRNA processing requirements

Constructs are microRNA-specific and closer to "endogenous" or "natural regulation"
- Native or non-native promoter
- Native genomic context

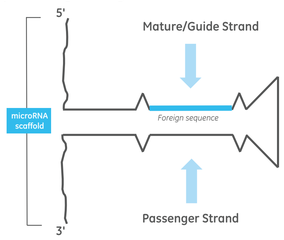
Suitable for examining individual microRNA processing and regulation

Optimized scaffold strategy

Mature human microRNA sequence inserted into a well-characterized primary microRNA sequence (a universal scaffold; the shMIMIC microRNA strategy)

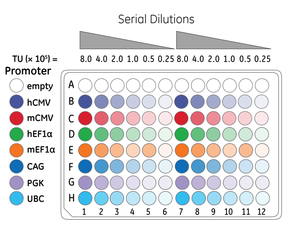
More predictable expression and regulation
- Non-native promoter
- In non-native genomic context

Well-suited for functional analysis of many microRNAs in multiple assays and screening applications



1. Must be processed efficiently and consistently
2. Must accept foreign sequences & maintain accurate processing
3. Must have good functionality from only mature strand
4. Must have minimal passenger strand activity (if passenger strand functional, this could complicate your results)

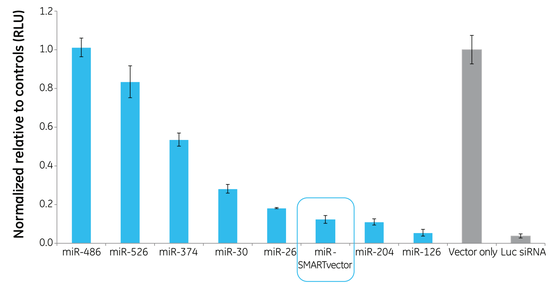
Choose the most active promoter in your cells for best experimental results



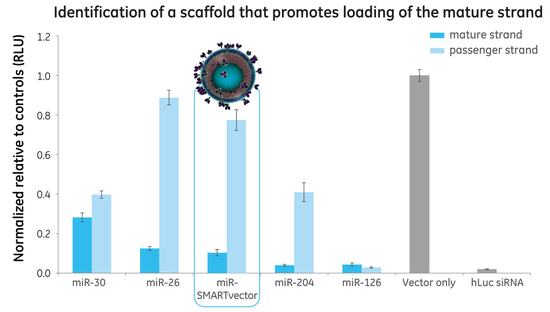
SMARTchoice Promoter Selection Plate
- Arrayed SMARTvector lentiviral particles; one row for each promoter
- Wells contain SMARTvector Non-targeting control expressing TurboGFP™ (Evrogen, Moscow, Russia)
- 2-fold dilutions of particles (TU), in duplicate

Selection of a functional, universal scaffold

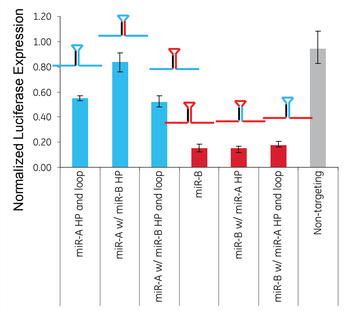
Different microRNA scaffolds produce different levels of functionality



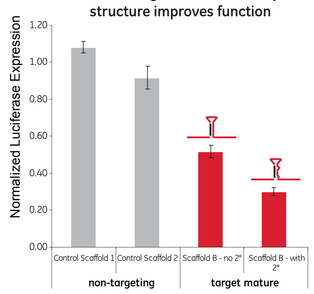
Identification of a scaffold that promotes loading of the mature strand



The flanking region contributes to function



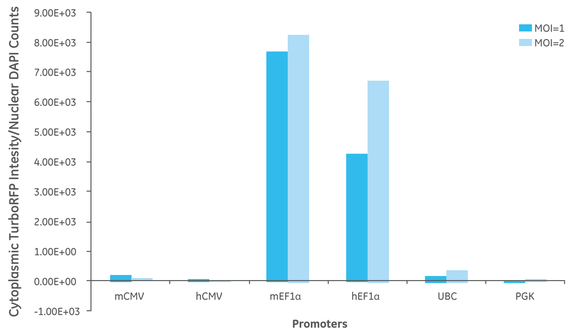
Maintaining natural secondary structure improves function



HeLa cells, co-transfection using 0.3 µL/well Lipofectamine-2000 and 50 ng/well psiCHECK-2 reporter plasmid + 50 ng/well scaffold expressing plasmid. Measured dual-luciferase assay at 48 hours.

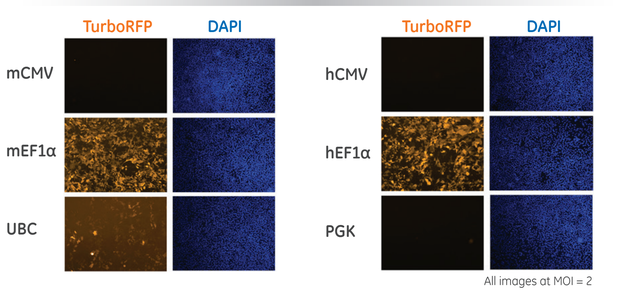
The mouse EF1α promoter is most active in MEL-1 human embryonic stem cells

TurboRFP intensity quantification



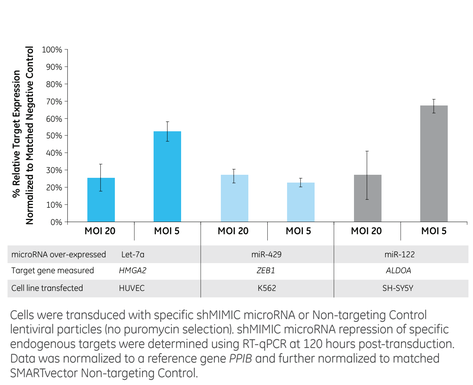
MEL-1 cells were fixed and stained with anti-TurboRFP antibody and DAPI 72 hours post-transduction. Fluorescent images were collected on the GE IN Cell Analyzer 2000 system and quantified using IN Cell Investigator High-content image analysis software.

Representative images showing optimal promoter (mEF1α)

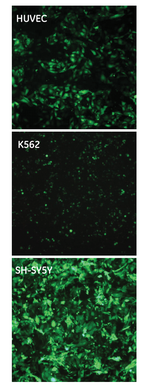


All images at MOI = 2

Efficient down-regulation of endogenous microRNA targets on mRNA level and transduction of cells (GFP expression) in primary, immune and neuronal cell lines

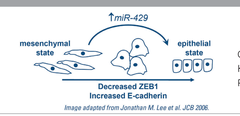


microRNA over-expressed	Let-7a	miR-429	miR-122
Target gene measured	*HMGA2*	*ZEB1*	*ALDOA*
Cell line transfected	HUVEC	K562	SH-SY5Y

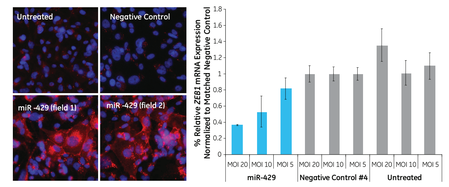


Cells were transduced with specific shMIMIC microRNA or Non-targeting Control lentiviral particles (no puromycin selection). shMIMIC microRNA repression of specific endogenous targets were determined using RT-qPCR at 120 hours post-transduction. Data was normalized to a reference gene *PPIB* and further normalized to matched SMARTvector Non-targeting Control.

MET phenotype induced by transduction of hsa-miR-429 shMIMIC microRNA



Gregory *et al. Nat. Cell Biology* 2008
Korpal *et al. Biol. Chem* 2008
Park *et al. Genes & Development* 2008



- Over-expression of miR-429 results in over-expression of E-cadherin and initiation of the morphological change from mesenchymal to epithelial cells
- Down-regulation of *ZEB1* (a putative miR-429 target) expression is also observed

MDA-MB-231 cells were transduced in triplicate with either miR-429 shMIMIC microRNA or negative control (no puromycin selection). Cells were fixed in 2% paraformaldehyde 96 hours post-transfection. Immunofluorescence was used to visualize E-cadherin expression and cellular localization. E-cadherin protein localization is in red, while nuclei stained with Hoechst 33342 are in blue. Silencing of *ZEB1* expression (120 hours) was measured using RT-qPCR.

Conclusions

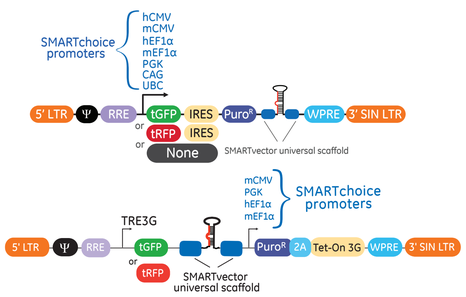
Preferred design strategy for expressing microRNAs

Optimized scaffold design derived from native human microRNAs
- Secondary structure retained to result in efficient processing and loading of the mature microRNA
- Scaffold selected to minimize star (*) strand or passenger strand processing and activity

Over-expression in neuronal, immune and primary cell lines possible

Research tool for long-term studies of microRNA function and creation of stable cell lines

shMIMIC microRNA features
- Lentiviral delivery results in stable integration of microRNA into the host cell genome
- Provided as cost- and time-saving lentiviral particles for direct transduction
- shMIMIC designs created for human, mouse and rat microRNAs in miRBase
- TurboGFP or TurboRFP (Evrogen) expression allows visual tracking of shMIMIC microRNA expression
- Puromycin-selectable for creation and maintenance of stable cell lines
- Pseudotyped with broadly trophic VSVg envelope
- Multiple promoter options for optimal expression in your cells of interest



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