

# Lentiviral miRNA Expression

Lentiviral Transduction for Specific miRNA Expression

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Catalog number: mir-LV###

April 2010

Size: 1 ml / vial

Storage Temperature: -70°C

## Product Description

Human microRNA (hsa-miRNA) precursors and approximately 100bp upstream and downstream flanking genomic sequences were PCR amplified and cloned into a self-inactivated (SIN) lentiviral vector to generate a lenti-miRNA collection (Fig. 1). The cloning site of pre-miRNA genomic fragments is within the intron of human EF1 $\alpha$  promoter region. The miRNA lentiviral stock was prepared by cotransfecting HEK 293T cells with the lenti-miRNA vector and plasmids expressing Gag-Pol gene products and the vesicular stomatitis virus envelope G (VSV-G). The lentiviral supernatants were collected at 48 hours post transfection and stored at -70°C. The titer of the virus is generally above  $1 \times 10^7$  infection units per ml (IU/ml).

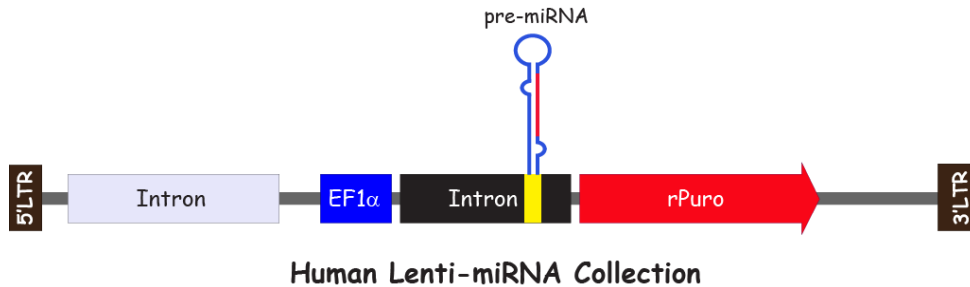


Figure 1. Human miRNA lentiviral expression system.

## Advantages of the Product

1. The lenti-miRNA is a ready-to-use lentiviral stock
2. Lentiviral transduction is one of the most effective delivery system to express miRNA, shRNA, cDNA, etc. Unlike the retroviral system, the lentiviral integration is cell cycle independent. The genetic materials encoded by the lentivirus can be efficiently delivered into both dividing and non-dividing cells.
3. The lenti-miRNA viral genome is integrated into the host chromosome, thus the miRNA is stably expressed in transduced cell lines.
4. The human EF1 $\alpha$  promoter it is unlikely to be silenced in cells. It has been reported that the activities of some viral promoters such as CMV and SV40 are potentially silenced by DNA methylation after a period of time. The human EF1 $\alpha$  promoter, used to express miRNA precursors and the puromycin selection marker, is a house-keeping gene promoter. Therefore, it is unlikely to be silenced by methylation *in vitro* and *in vivo*.
5. The rPuro gene product, expressed from the EF1 $\alpha$  promoter, is the red fluorescent puromycin-N-acetyltransferase. The model cells transduced by lenti-miRNA can not only be selected in the presence of puromycin in the medium, but also display red fluorescence at excitation/emission wavelengths of 587/610 nm (Fig. 2).
6. Each individual miRNA in Biosettia's human lentiviral collection was cloned from its native context, including the stem-loop precursor sequence and approximately 100bp upstream and downstream flanking sequences. This ensured that the miRNA was properly expressed and processed, and would function similarly to its endogenous form.



Figure 2. Microscopy images of 293T cells transduced with hsa-mir-24-1 lentivirus.

## Protocol

### Lentiviral Transduction for Functional Analysis.

This protocol may be used with 96-well, 48-well, 24-well, 12-well and 6-well plates.

Day 0: Seed cells at appropriate density.

Suggestion: Plate cells so that cell density will be ~10-25% confluent at the time of transduction.

Day 1: Transduction. Remove the medium from the tissue culture plate by aspiration and replace it with fresh complete medium containing 5-8  $\mu\text{g/ml}$  polybrene. Gently mix lentivirus with 1ml pipette tip, and add appropriate amount of virus to each well.

Note: Transduction efficiency varies in different cell lines, and polybrene may be toxic to some cell lines.

Suggestion: Add 1-10  $\mu\text{l}$  lentivirus per 1,000 cells. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at  $1,000 \times g$  for 30-60 min at room temperature helps increase of transduction efficiency.

Day 2: Replace the transduction medium with fresh complete medium to remove lentivirus and polybrene.

Day 3-4: Select transduced cells (>50% confluence is recommended) with medium containing puromycin.

Note: The optimal puromycin concentration varies from cell line to cell line (ranging from 0.5 to 5  $\mu\text{g/ml}$ ).

Suggestion: A pilot experiment should be performed to determine the minimum concentration of puromycin required to kill the untransduced cells before this experiment.

Day 6+: Assay transduced cells.

Suggestion: Expand the culture of cell lines stably expressing miRNA and store the cell line stocks in liquid nitrogen before analyzing the cells.

Note: We have noticed that the level of rPuro gene expression may vary among different cell lines. Therefore, please keep in mind that the red fluorescence may provide a general idea whether the model cells are transduced, but may not quantitatively reflect the percentage of cells transduced by Lenti-miRNA. It is highly recommended to determine the transduction efficiency by puromycin selection, since in some cells, the expression level of red fluorescent puromycin-N-acetyl-transferase is enough to render the cells resistant to puromycin, but not enough for the cells to display detectable red fluorescence.

Please be aware that one miRNA might suppress multiple genes at the same time, some phenotypes may come up or disappear during long-term suppression.

## **Optional: Determining Lentiviral Titer in the Cell Line of Your Choice.**

Follow the protocol below if you would like to determine multiplicity of infection (MOI) to control the copy number of miRNA lentivirus integrated into the chromosomes of the cells of interest. While titrating virus with antibiotic selection, the titrating procedure includes selection of stably transduced cells with puromycin and counting the puromycin-resistant cell colonies. Alternatively, a flow cytometry can be used to determine the viral titer by measuring the number of red fluorescent cells. If you are using both methods to titer your lentiviruses, please keep in mind that the titers may be different due to sensitivities of FACS machine and cell lines resistant to drug selection.

### **A. Determination of lentiviral titers by antibiotics selection.**

Day 0: Seed the cells of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

Day 1: Thaw lentiviral stock, gently mix virus with 1ml pipette tip and then prepare 2 ml 10-fold serial dilutions ranging from  $10^{-3}$  to  $10^{-7}$  in complete medium containing 5-8  $\mu\text{g}/\text{ml}$  polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at  $1,000 \times g$  for 30-60 min at room temperature may be necessary if titrating virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Replace medium with fresh medium containing puromycin to select for stably transduced cells.

Note: At least 48 hours of transduction allows miRNA lentivirus to integrate into the host genome. The optimal puromycin concentration (range 0.5-5  $\mu\text{g}/\text{ml}$ ) varies from cell line to cell line.

Suggestion: A pilot experiment should be performed to determine the minimum concentration of antibiotics required to kill the untransduced cells before this experiment.

Day 5-6: Replace medium with 2 ml fresh medium containing puromycin every 2 days.

Day 7-8: Allow puromycin-resistant colonies to form in dilution wells. No live cells should be growing in the mock control well.

Note: The number of days required for the formation of visible colonies may vary among different cell lines.

Wash wells twice with 2 ml PBS

Stain cells with 1 ml 0.5% crystal violet solution in 20% ethanol and incubate for 30 minutes at room temperature.

Wash wells with distilled water by submerging the plate in a tray full of water, and repeat the wash one more time.

Dry the plate and count the number of blue-stained colonies.

The titer should be the average colony number times the dilution factor.

Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

## B. Determination of lentiviral titers by flow cytometry.

Day 0: Seed the cell of your choice in a 6-well plate so that the cell density that will be ~25-50% confluent at the time of transduction.

Note: The number of cells seeded in the well is required to calculate lentiviral titer later.

Day 1: Thaw lentiviral stock, gently mix virus with 1ml pipette tip and then prepare 2 ml 10-fold serial dilutions ranging from  $10^{-1}$  to  $10^{-4}$  in complete medium containing 5-8  $\mu\text{g/ml}$  polybrene.

Remove the medium from previous day and add 2 ml fresh dilutions into each well.

Suggestion: Leave one well uninfected for mock control. Spin transduction in a desktop centrifuge (e.g. Sorvall RT6000) at  $1,000 \times g$  for 30-60 min at room temperature may be necessary if titering virus in cell lines other than 293T.

Day 2: Replace the medium containing virus and polybrene with 2 ml of complete medium.

Day 3-4: Follow your lab protocol to collect and resuspend cells for flow cytometry to determine the percentage of green or red fluorescent cells.

Note: At least 48 hours of transduction allows lenti-miRNA to integrate into the host genome.

The titer should be the average number of live fluorescent cells times the dilution factor.

Note: The transduction efficiency varies from cell line to cell line. The lentiviral titer in the cell line of your interest may be lower (sometimes more than 10-fold) or higher than the virus titer in commonly used cell lines such as 293T cells.

Example:  $1 \times 10^5$  cells were seeded on Day 0 and the cell doubling time is around 24 hours. The FACS data showed 5% and 40% of green fluorescence cells in the  $10^{-3}$  and  $10^{-2}$  dilution wells, respectively. The lentiviral titer is calculated by multiplying the fraction of transduced cells by  $2 \times 10^5$  (the expected number of cells in the well on Day 1, the time of transduction), and by the dilution factor.

$$0.05 \times (2 \times 10^5) \times 10^3 = 1 \times 10^7$$

$$0.4 \times (2 \times 10^5) \times 10^2 = 8 \times 10^6$$

The average lentiviral titer is  $9 \times 10^6$  IU/ml

### **Safety Guidelines for Working with Lenti-miRNA.**

The recombinant lentiviruses have been designated as Level 2 organisms by NIH and CDC. A Biosafety Level 2 (BSL-2) facility is required in order to work with lentiviruses. The information of Biosafety in Microbiological and Biomedical Laboratories (BMBL) can be downloaded from the following link:

[http://www.cdc.gov/od/ohs/biosfty/bmbl5/BMBL\\_5th\\_Edition.pdf](http://www.cdc.gov/od/ohs/biosfty/bmbl5/BMBL_5th_Edition.pdf)

**Please be aware that you are working with media containing lentiviral particles which could transduce human cells.**

## Examples of miRNA Expression

To validate the expression of miRNAs from the precursor and flanking sequences inserted in the EF1 $\alpha$  intron in the Lenti-miRNA expression system, a variety of analyses such as RT-PCR, Northern Blot, functional analysis and reporter assays have been carried out. The results are shown below:

### RT-PCR

HEK 293 cells were transduced with Lenti-miRNA viruses expressing let-7a-1, let-7a-2, and let-7a-3 (catalog# mir-LV001-003; Fig. 3A), or LV-[hsa-mir-ctrl] (catalog# mir-LV000; Fig. 3B) in which no DNA sequence was inserted into the EF1 $\alpha$  intron. The total RNAs were extracted 72 hours post-transduction, followed by RT-PCR to detect the transcripts of let-7a precursors. The forward primers used in PCR are let-7a-specific (let-7a-1, gccttcctgtgggtctcaactgtg; let-7a-2, taacttgtaatttcctgcttaag; let-7a-3, gtccccaggagggtcctctggaag), while the reverse primers are specific for the EF1 $\alpha$  intron (cgctactccaaaagctcgagctagc and ctcagtgtggggaaactccatcgc for primer sets 1 and 2, respectively) (Fig. 3).

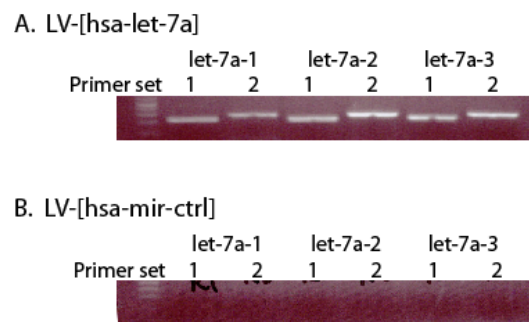


Figure 3. Detection of the expression of let-7a transcripts from the Biosettia LV-[hsa-let-7a] lentiviral transductions by RT-PCR.

### Real-Time PCR

The human foreskin (BJ) cells were transduced with LV-[hsa-mir-ctrl] (catalog# mir-LV000) or LV-[hsa-mir-17\_18a\_19a\_20a\_19b-1\_92a-1] (catalog# mir-LVc02) viruses. The total RNA were extracted 72 hours post-transduction, followed by TaqMan miRNA assays (Applied Biosystems). The relative level of individual miRNA expression was determined by comparing with the negative control, LV-[hsa-mir-ctrl] (Fig. 4).

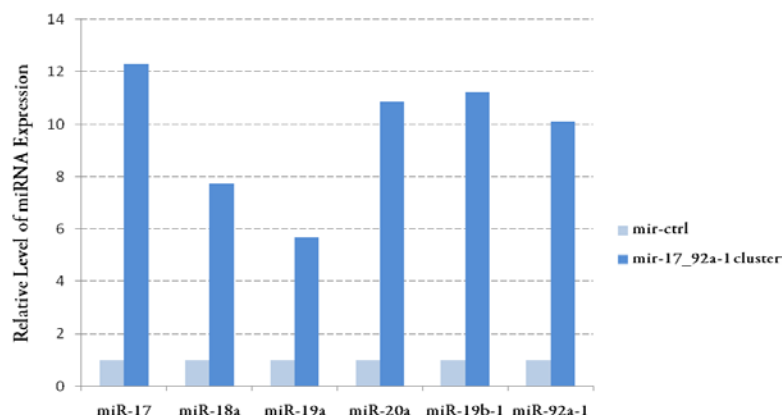


Figure 4. Detection of Lenti-miRNA by real-time PCR.

## Northern Blot

HEK 293 cells were transduced with LV-[hsa-mir-ctrl] (catalog# mir-LV000) or LV-[hsa-let-7g] (catalog# mir-LV010) viruses. The total RNA were extracted 72 hours post-transduction and subjected to Northern Blotting analysis as described [1]. The total RNA from HeLa cells with high expression levels of let-7g was used as positive control (Fig. 5, lane 1). Co-transduction of Lin28 with LV-[hsa-let-7g] reduced the expression of let-7g (Fig. 5, lane 5), confirming a reported finding that the let-7g biogenesis is suppressed by Lin28 [2, 3]

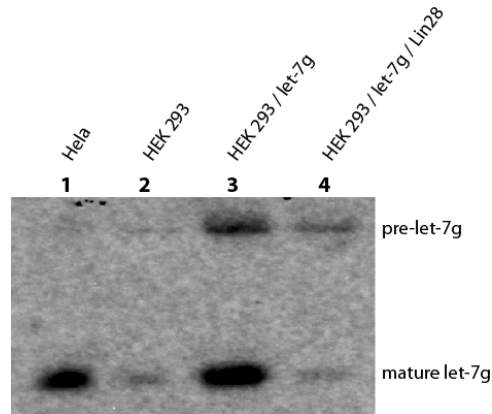
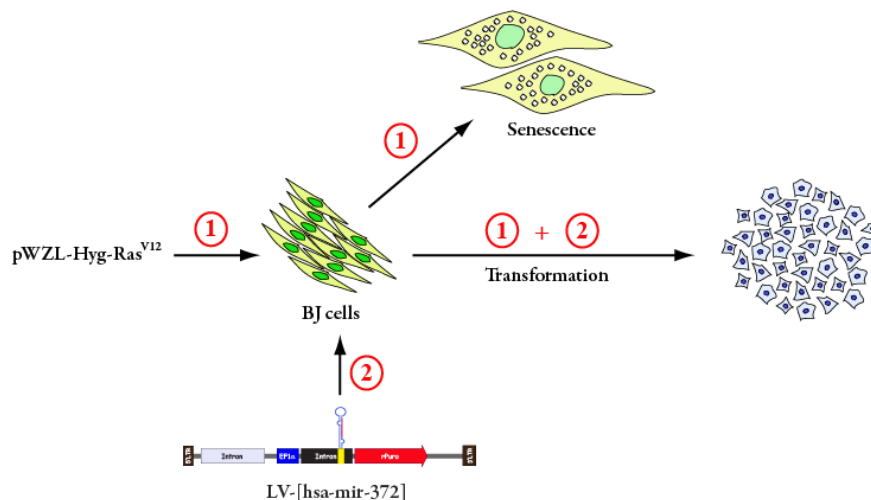


Figure 5. Detection of pre-let-7g and mature let-7g expressed from the Biosettia LV-[hsa-let-7g] lentiviral transductions by Northern Blotting.

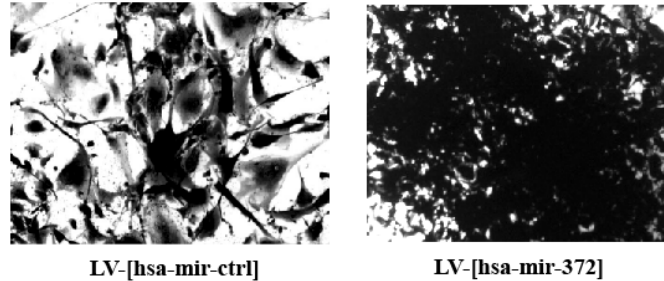
## Functional Analysis

It has been shown that the presence of microRNAs mir-372 and mir-373 in human primary fibroblast cells allows the cells to bypass H-Ras<sup>V12</sup> mediated growth arrest [4]. We have transduced BJ cells with retrovirus pWZL-Hyg-Ras<sup>V12</sup>. After selection with hygromycin, these cells were transduced with LV-[hsa-mir-ctrl], LV-[hsa-mir-372], or LV-[hsa-mir-373] (catalog# mir-LV000, 219, and 220) 4 days after pWZL-Hyg-Ras<sup>V12</sup> transduction. The BJ cells were grown for another 14 days before microscopic analysis and crystal violet staining (Fig. 6B). Our data showed that mir-372 and mir-373 abolished H-Ras<sup>V12</sup>-dependent senescence, consistent with the results reported by Voorhoeve *et al.* [4].

A.



B.



C.

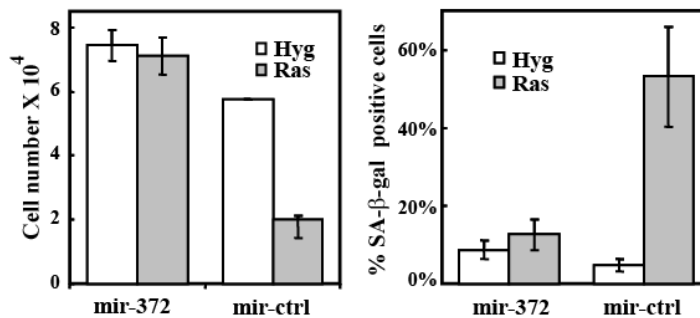


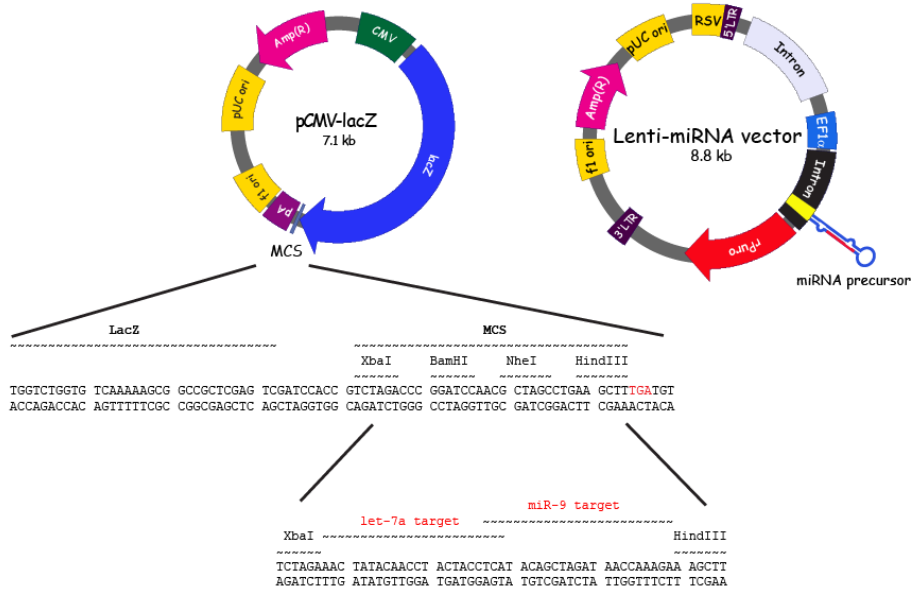
Figure 6. Functional analysis of mir-372 expressed from the Biosettia LV-[hsa-mir-372] lentiviral transductions. (A) Collaboration between oncogenic RAS and mir-372 in the transformation of primary cells. (B) Expression of mir-372 suppresses RAS-mediated senescence and transforms BJ cells. Early passage BJ cells were transduced with pWZL-Hyg-RasV12 (Ras), and then with LV-[hsa-miR-372] or control LV-[hsa-mir-ctrl] from the Biosettia Lenti-miRNA collection. Cells were stained with crystal violet for microscopic analysis at day 14 after RAS transduction. (C) Cell proliferation analysis and senescence SA-beta-gal assay. Early passage BJ cells were transduced with pWZL-Hyg-RasV12 (Ras) or pWZL-Hyg control (Hyg), and then with LV-[hsa-miR-372] or control LV-[hsa-mir-ctrl] from the Biosettia Lenti-miRNA collection. Cells were counted (left panel) and stained for SA-beta-gal (right panel) 8 days after RAS transduction.

### Reporter Assay

It has been reported that miRNA is able to cleave mRNA if the mature miRNA sequence is fully complementary to the mRNA target [5]. A 42-bp DNA oligonucleotide (aactatacaacactactacatcacagctagataaccaaga) containing sequences complementary to both mature hsa-let-7a (tgaggtagtaggttgtagtt) and hsa-mir-9 (tccttgggttatctagctgtatga) were inserted into the 3' end of the  $\beta$ -galactosidase gene to serve as miRNA targets (Fig. 7A). The  $\beta$ -galactosidase activity is suppressed when either mature let-7a or mir-9 miRNAs is present and targeted to the  $\beta$ -galactosidase fusion mRNA. The  $\beta$ -galactosidase activity is not suppressed by the other let-7 miRNAs that do not perfectly match the inserted target sequence (Fig. 7B). The mir-ctrl, a lentiviral vector without miRNA insertion, is used as negative control.



A.



B.

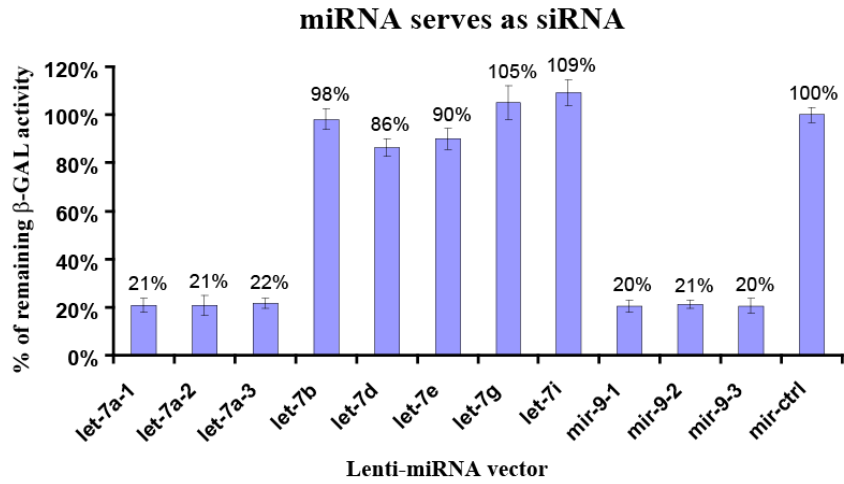


Figure 7. The lacZ reporter assay. The pCMV-lacZ and Lenti-miRNA vectors in a molar ratio of 1:10 were co-transfected into 293T cell. The  $\beta$ -galactosidase activity was determined 24 hours post-transfection.

## References

1. Bagga, S., et al., *Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation*. Cell, 2005. **122**(4): p. 553-63.
2. Heo, I., et al., *Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA*. Mol Cell, 2008. **32**(2): p. 276-84.
3. Piskounova, E., et al., *Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28*. J Biol Chem, 2008. **283**(31): p. 21310-4.
4. Voorhoeve, P.M., et al., *A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors*. Cell, 2006. **124**(6): p. 1169-81.
5. Zeng, Y., R. Yi, and B.R. Cullen, *MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms*. Proc Natl Acad Sci U S A, 2003. **100**(17): p. 9779-84.

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