Published in final edited form as:

Methods Mol Biol. 2017 ; 1507: 261–268. doi:10.1007/978-1-4939-6518-2_19.

Large-Scale RNA Interference Screening to Identify Transcriptional Regulators of a Tumor Suppressor Gene

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Abstract

RNA interference (RNAi) is a powerful research tool that can be used to silence the expression of a specific gene. In the past several years, RNAi has provided the opportunity to identify factors and pathways involved in complex biological processes by performing unbiased loss-of-function screens on a genome-wide scale. Here we describe a genome-wide RNAi screening strategy to identify factors that regulates epigenetic silencing of a specific tumor suppressor gene, using RASSF1A as an example. The approach we describe is a general RNAi screening strategy that can be applied to identify other factors that drive and/or maintain epigenetic modifications on specific genes, including cancer-related genes.

Keywords

RNA interference; Epigenetic silencing; Positive-selection screen; RNA interference; siRNA; shRNA

1 Introduction

High-throughput RNAi screening provides the opportunities to identify in unbiased manner cellular genes associated with specific biological phenotypes and has the potential to identify new targets for therapeutic interventions. Several factors must be considered when designing a successful RNAi screen. General guidelines for choosing the appropriate RNAi library (e.g., shRNA vs. siRNA, 20 retroviral vs. lentiviral) and screening strategy (single well vs. 21 pooled format, positive vs. negative selection, etc.) have been covered in detail elsewhere, and the reader is referred to several excellent reviews on these topics [1–4].

When planning a genome-scale RNAi screening for epigenetic factors, there are several critical parameters to consider. First, it is important to clone a suitable promoter region. For example, as hypermethylation usually occurs at DNA segments abundant with CpG dinucleotides, it will be of necessary to clone the whole promoter region carrying these CpG islands. Second, it is important to clone the promoter of the gene of interest in a suitable reporter plasmid, which in turn can be used for the selection of cells that eventually expression the tumor suppressor gene of interest. Third, a suitable cell line must be chosen in which the gene of interest is epigenetically silenced, yet can easily be reexpressed by treatment of the cells with epigenetic agents, such as DNA methyltransferase inhibitors and histone deacetylase inhibitors. Fourth, including appropriate negative control will allow one

to evaluate the background of the screen. In most cases, a control non-silencing shRNA or luciferase siRNA is suitable for this purpose. Moreover, in order to discern off-target effects of shRNAs, it is very important to use multiple, unrelated shRNAs/siRNAs targeting the same gene. Finally, the choice of appropriate assays to validate the positive candidates from the RNAi screening is also important to consider because a clear read out will reduce the background and increase the likelihood of success of the RNAi screen.

Here, we describe an RNAi screening that has been published by our group [5] in which we identified factors involved in epigenetic silencing of the tumor suppressor RAS association domain family 1A (RASSF1A). In brief, we generated a reporter construct in which the RASSF1A promoter was used to direct expression of a gene encoding red fluorescent protein (RFP) fused to the blasticidin resistance (Blast^R) gene. This RASSF1A-RFP-Blast^R reporter construct was stably transduced into human MDA-MB-231 breast cancer cells in which the endogenous RASSF1A gene is epigenetically silenced [6]. We then selected cells in which the reporter gene had been silenced as evidenced by loss of RFP expression and acquisition of blasticidin sensitivity. We used a human shRNA library [7] comprising ~62,400 shRNAs directed against 28,000 genes. The shRNAs were divided into ten pools, which were packaged into retrovirus particles and used to stably transduce the MDA-MB-231/RASSF1A-RFP-Blast^R reporter cell line. Blasticidin-resistant colonies, indicative of derepression of the epigenetically silenced reporter gene, were selected, and the shRNAs were identified by sequence analysis. One positive candidate was further confirmed. Indeed, stable transduction of parental MDA-MB-231 cell line with a single shRNA directed against the candidate gene led to derepression of the endogenous, epigenetically silenced RASSF1A gene. Confirmed candidate shRNAs were then tested in a secondary screen for their ability to promote derepression of endogenous epigenetically silenced RASSF1A in three independent NSCLC cell lines: A549, NCI-H23, and NCI-H460 [5].

The approach described here is a general screening strategy that can be used to study other epigenetically silenced genes in different human cancer (or mouse) cell lines.

2 Materials

2.1 Generation of the Reporter Construct, Cell Transfection, and Selection of Stable Clones

1. BAC.
2. pDsRed2-N1 (Clontech).
3. PEF6V5-HisB (Invitrogen).
4. Neomycin.
5. Blasticidin.
6. Transfection reagent, such as Effectene (Qiagen).
7. MDA-MB-231 cell line.

2.2 Generation of Retroviral Particles, Determination of the Multiplicity of Infection, and Cell Infection

1. Genome-wide shRNA library and a control shRNA, such as a non-silencing shRNA (Open Biosystems or Sigma-Aldrich).
2. Phoenix-gp helper-free retrovirus producer cell line (Garry Nolan, Stanford University; 1).
3. pCI-VSVG plasmid (Addgene).
4. pGag-pol (Addgene).
5. Transfection reagent, such as Effectene.
6. 0.45 μ m filters.
7. Culture medium: DMEM high glucose (1 $\times$, liquid, with L-glutamine and sodium pyruvate), 10 % FBS, and penicillin–streptomycin.
8. Polybrene.
9. Puromycin.
10. Crystal violet staining solution: 40 % methanol, 10 % acetic acid, 50 % ddH₂O, 0.01 % crystal violet.
11. MDA-MB-231 cell line.

2.3 Isolation of Genomic DNA and Identification of Candidate shRNAs by DNA Sequencing

1. Trypsin-EDTA (0.25 %, Invitrogen).
2. Genomic DNA preparation buffer: 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5 % (v/w) SDS, 50 mL of proteinase K.
3. Phenol–chloroform–isoamyl alcohol (25:24:1).
4. Chloroform.
5. NaCl (5 M).
6. Ethanol (70 and 100 % solutions).
7. TE buffer (1 $\times$): 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
8. Spectrophotometer or NanoDrop.
9. 5 $\times$ Go-Taq PCR buffer.
10. Taq DNA polymerase.
11. Primers for sequencing shRNA inserts in pSM2 library: For-pSM2 (5′ - GCTCGCTTCGGCAGCACATATAC-3′) and Rev-pSM2 (5′ - GAGACGTGCTACTTCCATTGTC-3′).
12. DNase- and RNase-free agarose for gel electrophoresis.
13. Ethidium bromide solution (10 mg/mL).

14. QIAquick gel extraction kit.
15. pGEM-T Vector system I (Promega).
16. Bacterial competent cells that allow for blue/white selection, such as DH5a, and that have a transformation efficiency of $>10^6$ colonies/ μ g.
17. LB-agar plates with 100 μ g/mL ampicillin, 40 μ L X-gal (50 mg/mL), and 10 μ L IPTG (1 M).
18. LB liquid.
19. QIAprep Miniprep kit (Qiagen).
20. SP6 sequencing primer (sequence 5'-ATTTAGGTGACAC TATAG-3').

3 Methods

3.1 Generation of the RASSF1A-RFP-Blast^R Reporter Construct, Cell Transduction, and Selection of Stable Clones

1. Excise the CMV promoter from pDsRed2-N1.
2. PCR amplify the blasticidin resistance gene from a plasmid, such as PEF6V5-HisB.
3. Clone the blasticidin resistance gene into pDsRed2-N1 to generate an in-frame fusion with DsRed2 gene.
4. Amplify 2.5 Kb of the RASSF1A promoter from a BAC.
5. Clone the RASSF1A promoter fragment into the derivative of DsRed2-N1 with the blasticidin resistance gene.
6. Transfect MDA-MB-231 cells with the RASSF1A-RFP-Blast^R reporter construct by using a transfection reagent, such as Effectene (Qiagen).
7. After 24 h enrich for the stable clones by selecting with neomycin.
8. Use in the subsequent RNAi screen the clones that show epigenetic silencing of the RASSF1A promoter, as observed by blasticidin sensitivity and lack of RFP expression.

3.2 Generation of Retroviral Particles

1. Plate 3×10^6 Phoenix-gp cells in ten individual 100 mm tissue culture dishes. Plate one additional dish, to be infected with a retrovirus expressing a control non-silencing shRNA.
2. After 36 h, transfect cells with 10 μ g pooled shRNA plasmid DNA (*see Note 1*), 1 μ g Gag-pol plasmid DNA, and 1 μ g pCI-VSVG plasmid DNA using a transfection reagent.

¹DNA amount along with the packaging plasmids should be optimized to obtain high retroviral titer.

3. After 48 h, collect the culture supernatants, which contain retroviral particles.
4. Filter the culture supernatants using 0.45 µm filters. Aliquot 1 mL supernatant into microfuge tubes and freeze at –80 °C (*see Note* ²).

3.3 Determining the Multiplicity of Infection for Retroviral shRNA Pools

1. Plate 1×10^5 293 cells in each well of a 6-well plate, using one plate for each pool.
2. Perform serial dilutions of each retroviral shRNA pool. First, label six microfuge tubes as “10⁻¹,” “10⁻²,” “10⁻³,” “10⁻⁴,” “10⁻⁵,” and “10⁻⁶.” Add 1 mL of the DMEM media to the first tube and add 1000 and 900 µL in the tubes from 2 to 6. Add 100 µL of retroviral supernatant in the first tube, resulting in a 1/10 dilution (10⁻¹). Remove 100 µL of the 10⁻¹ dilution and add it to the second tube to create a 10⁻² dilution. Repeat to generate subsequent serial dilutions.
3. Aspirate the media from all the wells and add 1 mL of serially diluted retroviral supernatant with polybrene (10 µL/mL) to the appropriate well.
4. After 24 h, remove the media and add 2 mL of fresh DMEM media.
5. After 24 h, add puromycin (1.0 µg/mL) to select for cells carrying the retroviral shRNA.
6. Change the media with puromycin every 3 days.
7. Between day 10 and 14, depending upon the size of the colonies, stain the colonies that survive the puromycin selection using crystal violet staining solution.
8. Calculate the multiplicity of infection (MOI) of the retroviral supernatants as follows:

$$\text{MOI (particle forming units (pfu) / mL)} = \text{Number of colonies} \times \text{dilution factor} \times 10.$$

For example, if you observe five colonies in the 10⁻⁴ dilution plate, the calculation will be: $5 \times 10^4 \times 10 = 5 \times 10^5$ pfu/mL (*see Note* ³).

3.4 Infection and Selection of Cells After Transduction with Retroviral shRNA Pools

1. Plate 1.2×10^6 MDA-MB-231 cells in ten individual 100 mm tissue culture dishes.
2. After 24 h, transduce the MDA-MB-231 cells with retroviral shRNA pools in a total volume of 5 mL of DMEM media with 10 % FBS/penicillin–streptomycin and polybrene (10 µg/mL) to achieve infection at an MOI of 0.2 (*see Note* ⁴).

²It is important to note that freeze-thawing will lead to drop in virus titer. Therefore, we recommend avoiding multiple freeze-thaw cycles. Storing viral supernatant in the form of aliquots at –80 °C freezer prevents the drop of viral titer and improves the infection.

³While performing the titration, it is important to determine the titer in the cell line that will be eventually used for the screen, rather than in an unrelated cell line, because titer can differ depending upon the infectivity of different cancer cell lines.

3. After 24 h, change the media and add 10 mL of DMEM media with 10 % FBS/penicillin–streptomycin.
4. After 24 h, add puromycin (1.0 µg/mL) to enrich for cells that carry integrated shRNAs. Change the media every 3 days with fresh puromycin.
5. After 5–7 days, when the puromycin selection is over, add blasticidin (2.0 µg/mL) to select for blasticidin-resistant colonies, indicative of derepression of the epigenetically silenced reporter gene.

3.5 Isolation of Genomic DNA from Blasticidin-Resistant and RFP-Positive Cells and Identification of Integrated shRNAs by DNA Sequencing

1. For all ten pools, trypsinize and isolate the cells that acquired blasticidin resistance.
2. Extract genomic DNA using Qiagen genomic DNA isolation kit as per the manufacturers' instructions.
3. To amplify the retroviral shRNA integrated into the genomic DNA, set up the following PCR:

Components	Volume
5× Go-Taq PCR buffer	10 µL
Taq polymerase (5 units/µL)	Taq polymerase (5 units/µL)
Genomic DNA	2 µL [100 ng (50 ng/µL)]
For-pSM2 (10 pmoles/µL)	1 µL
Rev-pSM2 (10 pmoles/µL)	1 µL
dd H ₂ O	35 µL

4. Run the PCR products on a 1 % agarose gel with 10 µL ethidium bromide (10 mg/mL stock) and elute from the gel using a Qiagen gel elution kit.
5. Ligate 100 ng of the eluted PCR product with the TA vector using the TA cloning kit (Promega) as per the manufacturer's instructions.
6. Perform an overnight ligation at 16 °C.
7. Next day, transform ligation mixture into bacterial competent cells, and plate the reaction onto LB-agar plates containing ampicillin, 40 µL of X-gal (50 mg/mL), and 10 µL of IPTG (1 M).
8. Inoculate white colonies into tubes containing 3 mL of LB liquid with 100 µg/mL ampicillin. Grow overnight at 37 °C.

⁴The purpose of infecting the cells at the MOI of 0.2 is to prevent superinfection and to ensure that each cell receives no more than one shRNA, and thus the observed phenotype is driven due to a single-gene knockdown.

9. Isolate plasmid DNA from white colonies using Qiagen's miniprep kit as per the manufacturer's instructions.
10. Sequence the plasmid DNA using the SP6 primer.
11. To identify the genes targeted by the shRNAs, perform a nucleotide BLAST search using the shRNA sequence as a query.

3.6 Secondary Assays to Confirm the Candidates Identified from the Initial Screening

1. Select the individual shRNA corresponding to the candidate gene from the RNAi library and prepare the retrovirus as described above in Subheading 3.1.
2. Plate 1×10^5 MDA-MB-231 cells in a well of a 6-well for each candidate shRNA to be tested, and infect with the retrovirus particle(s).
3. After 24 h, change the media and add 2 mL of DMEM media.
4. After 24 h, add puromycin (1.0 $\mu\text{g/mL}$) to enrich the cells that carry integrated shRNAs. Change the media every 3 days with fresh puromycin.
5. Validate the candidates by using several assays to assess if the knockdown promotes the derepression of the endogenous epigenetically silenced target gene. These assays include qRT-PCR as well as immunoblot.
6. Validate the positive candidate by using other shRNAs, unrelated in sequence to the first shRNA, to rule out "off-target" effects (*see Note*⁵).
7. To further confirm the candidates, repeat the validation in several cell lines in which the gene of interest is epigenetically silenced.

Acknowledgments

We gratefully acknowledge grants from the National Institutes of Health: R21CA197758-01 (N.W.), R21CA191364-01 (N.W.), R21CA195077-01A1 (N.W.), and R01CA200919-01 (N.W.). N.W. is also supported by Research Scholar Grant from American Cancer Society (128347-RSG-15-212-01-TBG) and grants from the Melanoma Research Alliance and the Melanoma Research Foundation.

References

1. Ashworth A, Bernards R. Using functional genetics to understand breast cancer biology. *Cold Spring Harb Perspect Biol.* 2010; 2(7):a003327. doi: 10.1101/cshperspect.a003327, cshperspect.a003327 [pii]. [PubMed: 20519343]
2. Lizardi PM, Forloni M, Wajapeyee N. Genome-wide approaches for cancer gene discovery. *Trends Biotechnol.* 2011; 29(11):558–568. doi: 10.1016/j.tibtech.2011.06.003, S0167-7799(11)00105-3 [pii]. [PubMed: 21757246]
3. Mullenders J, Bernards R. Loss-of-function genetic screens as a tool to improve the diagnosis and treatment of cancer. *Oncogene.* 2009; 28(50):4409–4420. doi: 10.1038/onc.2009.295, onc2009295 [pii]. [PubMed: 19767776]

⁵It is recommended that at least two to three additional shRNAs are used against the validated candidates to ensure the gene-specific effect and to rule out off-target effects. Additionally, when possible an shRNA-resistant cDNA can be used to perform the rescue experiments.

4. Westbrook TF, Stegmeier F, Elledge SJ. Dissecting cancer pathways and vulnerabilities with RNAi. *Cold Spring Harb Symp Quant Biol.* 2005; 70:435–444. DOI: 10.1101/sqb.2005.70.031 [PubMed: 16869781]
5. Palakurthy RK, Wajapeyee N, Santra MK, Gazin C, Lin L, Gobeil S, Green MR. Epigenetic silencing of the RASSF1A tumor suppressor gene through HOXB3-mediated induction of DNMT3B expression. *Mol Cell.* 2009; 36(2):219–230. doi: 10.1016/j.molcel.2009.10.009, S1097-2765(09)00744-8 [pii]. [PubMed: 19854132]
6. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet.* 2000; 25(3):315–319. DOI: 10.1038/77083 [PubMed: 10888881]
7. Silva JM, Li MZ, Chang K, Ge W, Golding MC, Rickles RJ, Siolas D, Hu G, Paddison PJ, Schlabach MR, Sheth N, Bradshaw J, Burchard J, Kulkarni A, Cavet G, Sachidanandam R, McCombie WR, Cleary MA, Elledge SJ, Hannon GJ. Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet.* 2005; 37(11):1281–1288. doi: 10.1038/ng1650, ng1650 [pii]. [PubMed: 16200065]