Reproducible, Long-Term Gene Silencing in a High-Throughput Lentiviral shRNA Screen

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Overview

RNA interference has become a powerful molecular biological tool that is rapidly advancing discoveries in gene function and pathway elucidation. Specific post-transcriptional gene silencing is achieved by the introduction of synthetic small interfering RNA (siRNAs) into cultured cells. Although siRNA can be effective, there are limitations to its usage, such as transient gene silencing effects and the inability to deliver into primary and non-dividing cell types. Alternatively, short hairpin RNA (shRNA) constructs in lentiviral vectors are effective in gene silencing and overcome the limitations of siRNA. We have utilized the Mission™ TRC shRNA library in lentiviral particle format and demonstrated stable, specific, and reproducible gene silencing in a variety of cell types. As part of a large-scale effort to determine the effectiveness of the shRNA constructs in gene knockdown, gene expression was measured for approximately 340 constructs, representing 68 gene targets. To test the long-term stability of the silencing effects, gene expression was measured over time. Knockdown
was assessed at various time points from a continuous culture up to 16 days post infection. The reproducibility of infection with lentiviral particles and subsequent gene silencing was tested over multiple infections for multiple gene targets. Lastly, we have evaluated knockdown of gene expression in human astrocytes, representing a difficult-to-transfect primary cell type, and found that the lentiviral-based delivery system was efficient in delivering shRNA constructs and producing specific gene silencing. Taken as a whole, the data show that the technique of suppressing gene expression through the use of delivering shRNA in a lentiviral format results in effective, reproducible, and stable gene silencing in a wide range of host cells.

Results

As part of an ongoing effort to test the effectiveness of the Mission™ TRC shRNA library, gene expression in shRNA treated cells was measured for approximately 340 constructs, representing 68 gene targets. Minimally, four different shRNA constructs were evaluated for each gene. The set of clones to a target generally span the coding sequence, with at least one shRNA targeting the 3' UTR. Genes were chosen to represent a variety of significant cellular pathways. Five to seven days post infection, and following selection with puromycin, cells were harvested. Gene expression was measured and compared to empty vector control virus infected cells, which serve as a negative control. Varying levels of expression resulted, demonstrating the efficiency of shRNA constructs in knockdown of gene expression. Figure 1, comprising 27 genes associated with the MAP kinase pathway, illustrates typical results.

The reproducibility of infection with lentiviral particles and subsequent gene silencing was evaluated by performing multiple individual infections using the same shRNA lentiviral construct. Additionally, the individual infections were completed using a multiplicity of infection (MOI) of 1 and 3 with the lentiviral particles. Gene expression was measured five days post puromycin selection and assays for gene knockdown were performed in duplicate for each infection. For each shRNA construct tested the results were reproducible and the MOI did not impact the level of knockdown assessed (Figure 2).

Gene expression of ROCK2 in shRNA treated cells was measured over time to determine the stability of the gene silencing effects. Knockdown was evaluated from 5 to 16 days post puromycin selection. A high degree of knockdown, ranging from 82% to 90%, was consistent even after maintaining a continuous cell culture for 16 days (Figure 3).

Lastly, we have evaluated knockdown of gene expression in primary human astrocytes. These cells represent a popular model system; however, the usage of this cell line has previously been limited due to the inherent difficulty to transfect. Four different genes were examined, with a minimum of four shRNA constructs per gene target. The genes chosen represented those identified as relevant in the normal astrocyte cellular pathway: JNK2, p38α, p38δ, and MAP3K4 (Hasselblatt et al, Neuroreport 2006; Yoo et al, Arch Pharm Res 2005). Determined by their resistance to puromycin selection, the shRNA-containing virus preparations successfully transduced the primary astrocyte cultures treated. Varying levels of knockdown resulted (Figure 4).

Materials and Methods

Human Cell Culture

Primary-derived normal human astrocyte cells were obtained cryopreserved from Cambrex (cambrex.com). Cells were seeded in Astrocyte Basal Medium (Cambrex) and cultures established. Human A549 cells were obtained from AATC and were seeded in F12 Ham media. At 70% to 80% confluency, cells were trypsinized and reseeded into 96–well plates at 1.6 × 104 cells/well.

Infection

Cell lines were plated 24 hours prior to infection in 96–well plates and included hexadimethrine bromide (Sigma Cat. No. H9268) at a final concentration of 8μ g/ml. Virus was added as indicated to each appropriate well. Viral constructs were allowed 24 hours incubation with the cell lines prior to replacing media with selective media containing puromycin (Sigma Cat. No. P9620). Media with selective antibiotic was replaced at 48-hour intervals until cell harvest.

Validation of Knockdown

Gene expression was measured by using the QuantiGene RNA Quantitation assay from Panomics (panomics.com) as well as in some instances by performing qRT –PCR. The QuantiGene assay was performed according to provided instructions. Briefly, oligonucleotide probes hybridize to complimentary target mRNA and also oligonucleotide-coated capture wells. Separate oligonucleotide probes bind to complimentary target mRNA and branched DNA molecules. Following the addition of chemiluminescent substrate, emitted light is measured on a luminometer. Results were normalized using cyclophilin housekeeping gene. For qRT –PCR, total RNA was purified and subsequently analyzed using the appropriate TaqMan® Gene Expression Assay (ABI). In both quantitation methods, the difference in signal between the infected samples and the empty vector virus treated cells was used to calculate percent expression. Percentage is expressed as a level of the empty vector control (Sigma Cat. No. SHC001V), which is set to 100%.

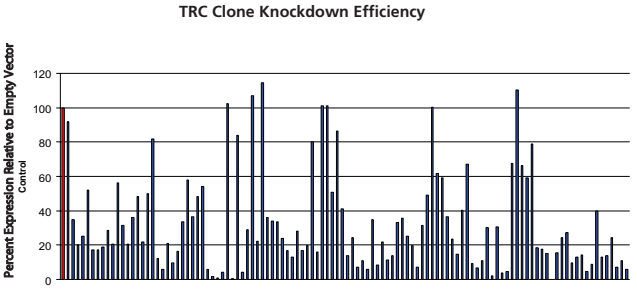


Figure 1. Gene expression was measured and compared to cells treated with empty vector control virus treated cells. The results for a subset of 27 gene targets all belonging to the HeLa cells MAP kinase pathway are shown. Of 113 shRNA clones tested, 61% gave greater than 70% knockdown, with 58% giving greater than 80% knockdown.

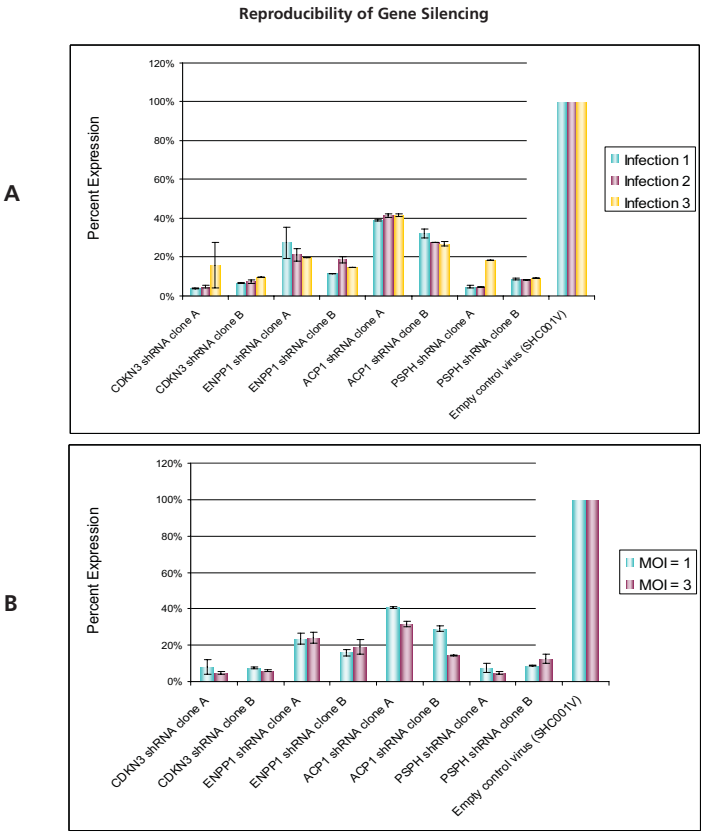


Figure 2. A) Three infections were performed, at a multiplicity of infection (MOI) of 1, in triplicate with eight different lentiviral particles containing shRNA constructs. Gene expression was measured in duplicate for each infection. Although expression varied among the shRNA constructs, in most cases gene expression was consistent among the infections. **B)** The degree of gene silencing achieved when cells were infected with lentiviral particles at an MOI of 1 was compared to silencing achieved when infecting with an MOI of 3. Three infections were performed in triplicate and assayed in duplicate for eight different shRNA constructs at an MOI of 1 and 3. Overall, the level of gene expression was comparable.

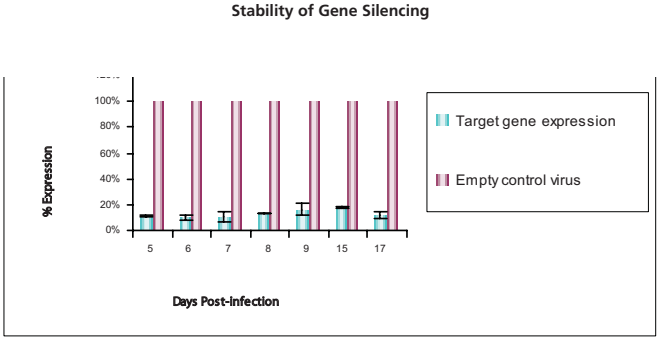
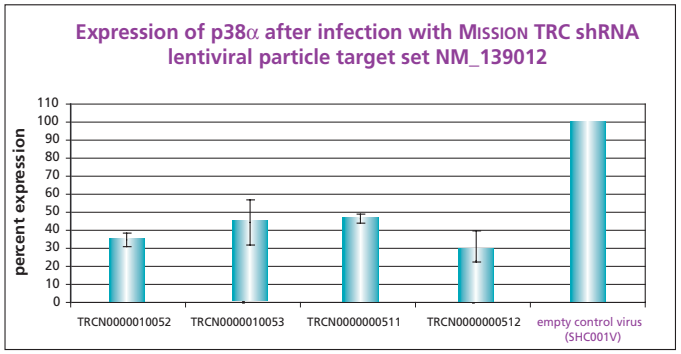
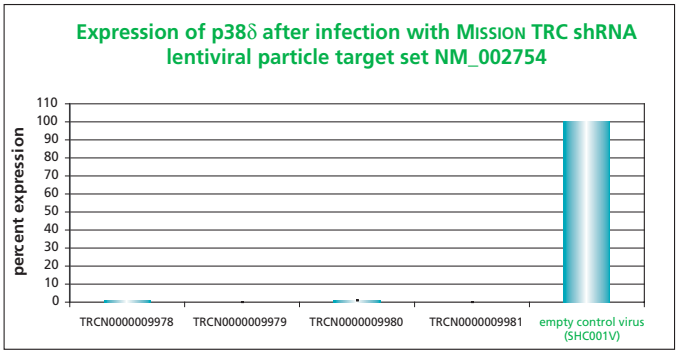
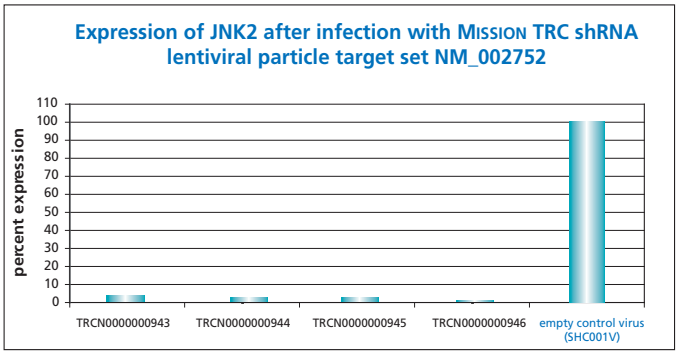


Figure 3. Gene expression of ROCK2 was evaluated at regular time intervals following infection with lentiviral particles. The level of gene silencing achieved when cells were harvested 5 days post puromycin selection was maintained, even when tested 17 days post selection.







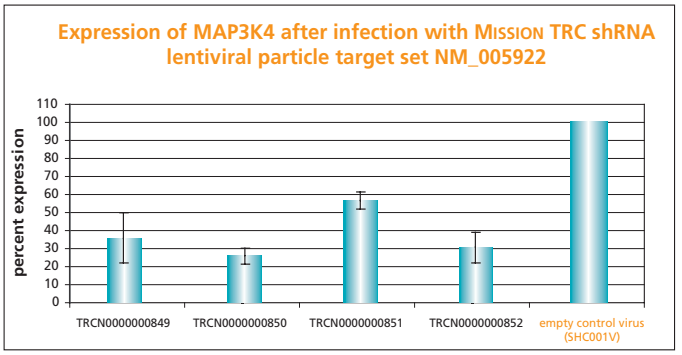


Figure 4. Mission™ TRC shRNA lentiviral particle target sets directed toward p38a, p38d, JNK2, and MAP3K4 were used to transduce human primary astrocyte cells. At 114 hours post selection, total RNA was purified and analyzed with the appropriate TaqMan®, Gene Expression assay. Results were normalized to GAPDH. Percentage is expressed as a level of the empty vector control (100%).

Conclusion

RNA interference is an exciting new tool that has revolutionized gene function studies and the field of therapeutics. The use of lentiviral particles containing shRNA constructs may provide distinct advantages and remediate some to limitations to siRNA. The Mission™ TRC shRNA library demonstrates stable and specific gene silencing in a variety of cell types, even in primary cell lines such as astrocytes. The gene silencing effects achieved by the use of the library are stable and highly reproducible. This library provides an unprecedented tool for discovery.

References

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