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Screening siRNA and Verifying shRNA Knockouts

Key Points

RNA interference (RNAi) is an RNA-dependent gene silencing mechanism that can affect the expression of specific genes by inhibiting translation or suppressing transcription epigenetically. Using Firefly™ assays, RNAi effects such as impact on phosphorylation or silencing can be studied functionally in samples as small as 100 cells. An additional benefit of the small sample size is that a variety of conditions can be studied in a single assay.

Introduction

RNA interference (RNAi) is a useful tool for generating gene knockout model systems and studying pathways and gene function in cell culture and living organisms. Applying RNAi in strategies for drug development offers utility in the determination of biomarkers and development of treatments for disease. However, it can be challenging to design and deliver small interfering RNA (siRNA) constructs that are effective in the cells of interest.

RNA interference can affect expression of specific genes by inhibiting translation or suppressing transcription epigenetically. RNAi is triggered by double-stranded RNA (dsRNA) with homologous sequence to messenger RNA (mRNA) targets in the cell. The dsRNA can be introduced from exogenous sources such as viral or laboratory sources or transcribed endogenously, through micro-RNA (miRNA).

The dsRNA from exogenous sources is cleaved by the RNA-processing enzyme, Dicer, to form siRNA of 20–25 bp with a few unpaired overhanging bases at each end of the molecule.[1,2] The siRNA then undergoes strand separation, and the guide strand integrates into the RNA-induced silencing complex (RISC). When the integrated strand of siRNA binds to its complementary target mRNA, Argonaute nuclease within the RISC degrades the targeted mRNA, preventing its translation into protein.[3]

Another form of dsRNA is short hairpin RNA (shRNA), which is introduced into the cell through transfection of vector constructs or by transduction of viral vector particles.[4] The shRNA is reverse-transcribed into DNA, which is imported into the host nucleus and integrated into the host genome. The shRNA constructs are designed to contain features similar to miRNA.[5] The shRNA is constitutively expressed and processed into short interfering hairpin RNA, which is processed by Dicer to form siRNA. The targeted gene silencing is heritable because the shRNA sequence is integrated into the genomic DNA.

Being able to introduce synthetic dsRNA into cells to suppress specific genes of interest in a systematic way is a useful research tool, but screening and validating siRNA is labor intensive and lengthy. Generally, multiple siRNA candidates must be designed and tested for efficacy against a gene target, including a negative control scrambled sequence siRNA to be tested alongside target-specific candidate siRNA. Unintended off-target transcript silencing has been observed with siRNA,[6] so measuring endogenous transcript levels for hundreds of different targets may be required to validate a candidate siRNA as target-specific. For each of these experiments, entire flasks of cells must be grown—a time-consuming process.

Firefly assays don't require the preliminary cell-growing process because of their minimal sample size. The assays also enable insights into the function of cell signaling proteins that are not possible with Western blots. In this application note, we describe the use of the Firefly System to measure the effectiveness of RNAi in two systems:

- trkA siRNA impact on ERK1 and ERK2 phosphorylation
- shRNA silencing of MEK1 and MEK2 in knockout experiments

Firefly System: ERK and MEK Assays

The Firefly System performs an antibody-probed, capillary isoelectric focusing (IEF) assay, which allows resolution and relative quantitation of protein isoforms using small quantities of sample. Mono- and dual phosphorylated isoforms of ERK and MEK can be distinguished readily on the Firefly System by IEF, but cannot be resolved on size-based Western blots probed with phospho-specific antibodies, because the molecular weight of the phospho-isoforms do not differ significantly. The separation technology and methods are described in an article by O'Neill, et al.[7]

Figure 1: Identification of ERK1 isoform peaks in NGF-treated and untreated rat PC12 cells using different ERK-specific antibodies: ERK1/2 (blues), ERK1 (greens), phospho-ERK1/2 (oranges). Monophospho-ERK1 (pERK1) and diphospho-ERK1 (ppERK1) are induced after NGF treatment.

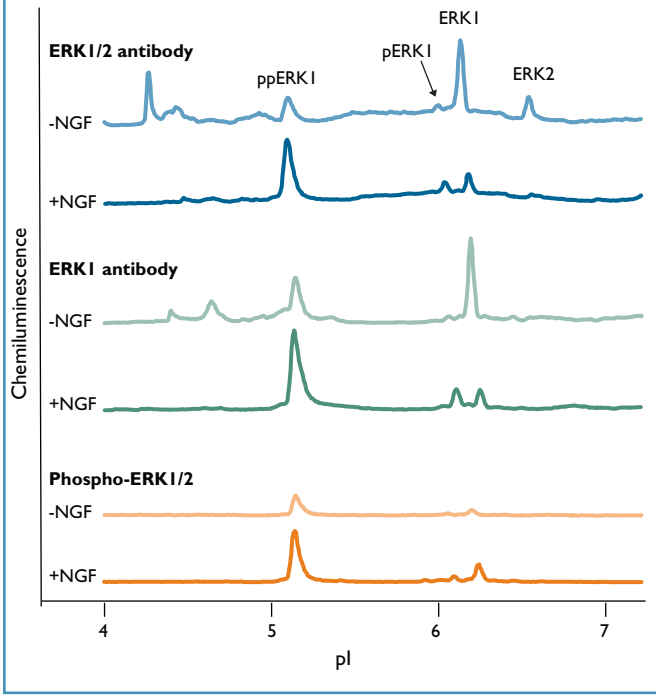
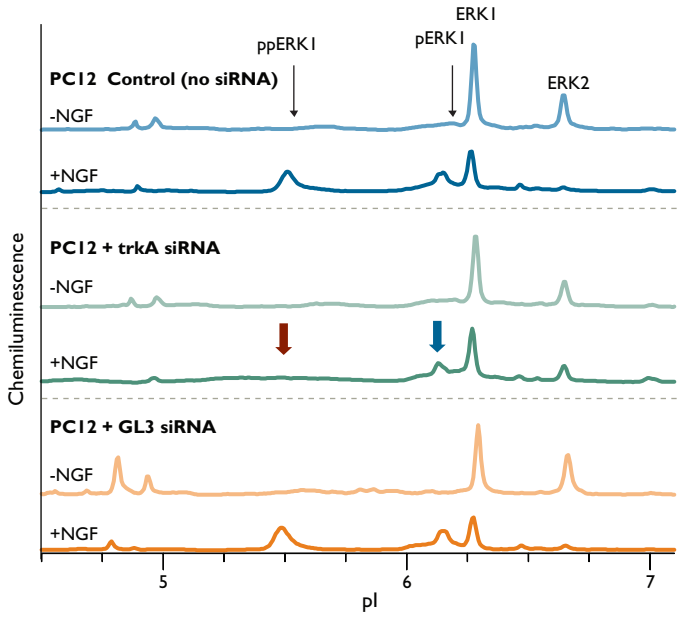


Figure 2: The siRNA targeting trkA suppresses downstream dual phosphorylation of ERK1 (brown arrow) but not mono-phosphorylation (blue arrow) in PC12 cells induced by NGF. Treatment with a sham sequence siRNA (GL3 siRNA) or no siRNA shows the expected induction of phospho-isoforms of ERK1 in NGF treated cells.



Characterization of siRNA Activity

Detection of ERK Isoforms in Rat Neuronal Cells

The ability to distinguish between different ERK isoforms is useful when assessing the impact of siRNA. The Firefly ERK1/ERK2 assay peak profiles reflect the binding of antibodies specific for ERK1 and ERK2 isoforms. By probing the capillary IEF-separated samples with antibodies against total ERK 1/2, ERK1, ERK2 and phospho-ERK, and correlating the presence or absence of peaks with the antibody specificity, one can identify the isoform associated with the pI peak.[7] Although the ERK proteins are highly conserved among species, differences in amino acid sequences and post-translational modifications will affect the pI of the protein isoforms. Therefore, preliminary Firefly ERK1/ERK2 assays were performed on cells from the PC12 cultured rat neuronal cell line in order to characterize isoform peak profiles.

PC12 cells were treated with nerve growth factor (NGF) to induce ERK phosphorylation or left untreated. Cell lysates were then prepared, and the samples were assayed using the Firefly System with antibodies specific for different ERK isoforms. Figure 1 shows the peak profiles and derived peak identifications for ERK1 isoforms in rat PC12 cells. The antibodies used were: anti-ERK1/2 (Millipore-Upstate Cat. No. 06-182), anti-ERK1 (Millipore-Upstate Cat. No. 05-957) and anti-phospho-ERK1/2 antibody (Cell Signaling Technologies Cat. No. 4377).

trkA siRNA Inhibits Downstream Dual Phosphorylation of ERK1

Drugs or treatments designed to act on ERK activation directly or indirectly through interaction with upstream signaling proteins can be efficiently screened using the detailed, isoform-specific data from Firefly assays. We have studied the effect of siRNA molecules targeted at the neuronal growth factor catalytic receptor, trkA, on downstream ERK signaling in NGF-stimulated PC12 cells. Rat PC12 cells were cultured in 96-well plates and treated with trkA siRNA, a negative control siRNA (GL3 siRNA) or no siRNA. After a 48-hour incubation period, samples were stimulated with NGF or left untreated, and then cell lysates were prepared and assayed on the Firefly System. Representative results are shown in Figure 2.

The ability to distinguish between the different ERK isoforms reveals trends that aren't visible in Western blots. The trkA siRNA led to suppression of the dual phosphorylation of ERK1 at the Thr-Glu-Tyr site, but did not suppress mono-phosphorylation. The monophospho-ERK1 detected may be arising from autophosphorylation,[8,9] which would not be subject to trkA siRNA cascade suppression.

Figure 3: Firefly MEK1 assay peak profiles from MCF7 cell lines containing shRNA constructs against GFP (negative control), MEK1, MEK2, and MEK1 and MEK2.

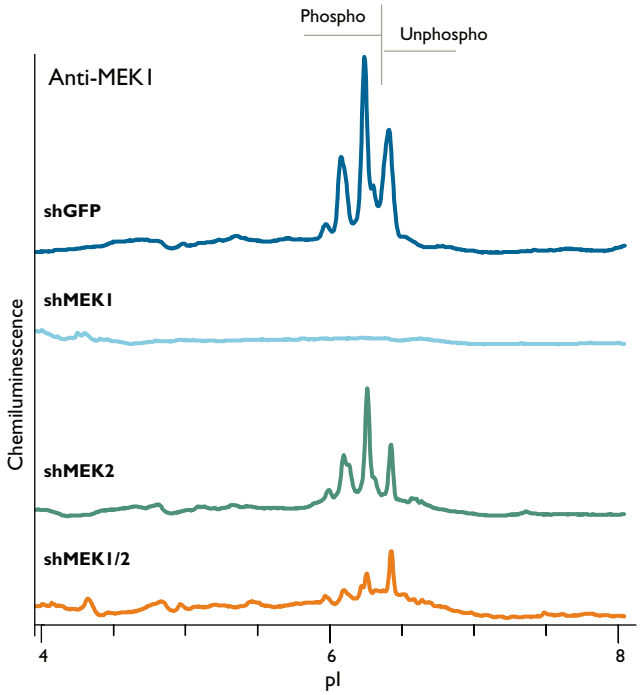
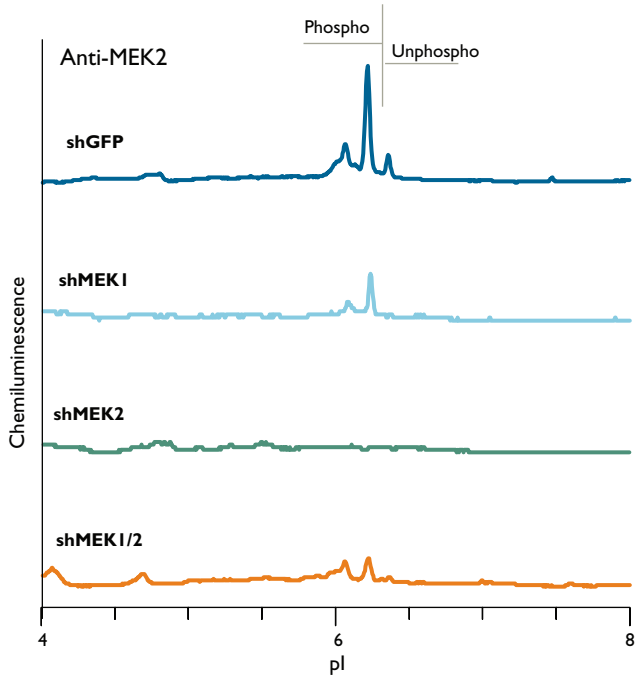


Figure 4: Firefly MEK2 assay peak profiles from MCF7 cell lines containing shRNA constructs against GFP (negative control), MEK1, MEK2, and MEK1 and MEK2.



Verifying shRNA Knockouts

Characterizing shRNA MEK Suppression

Next, the Firefly System's ability to detect protein isoforms was used to examine MEK-targeted gene silencing in shRNA transduced cell lines. Cell lysates were made from four cell lines containing different shRNA constructs targeting MEK1, MEK2, both MEK1 and MEK2, and green fluorescent protein (GFP) as a control. Samples were assayed using anti-MEK1 antibody (Millipore-Upstate, Cat. No. 07-641) and anti-MEK2 antibody (Cell Signaling Technologies, Cat. No. 9125).

Figure 3 shows representative peak profiles from the Firefly MEK1 assay. MEK1 isoform peaks were readily detected in the cell lines containing the shGFP and shMEK2 constructs. The shMEK1/2 cell line had detectable MEK1 protein, as well, although at a lower level. No MEK1 peaks were detected in the cell line containing the shMEK1 construct, verifying knockout of the MEK1 gene. Figure 4 shows representative peak profiles from the MEK2 assay on the same cell lines. As expected, MEK2 protein isoform peaks were detectable in the cell lines containing shGFP, shMEK1 and shMEK1/2. However, no peaks were detected in the shMEK2 cell line, verifying MEK2 gene knockout by the shMEK2 construct.

The Firefly assays showed that the shRNA for MEK1 and MEK2 were well constructed and specifically silenced their targeted genes. The cell line containing the shRNA targeting both MEK1 and MEK2 showed low but detectable levels of MEK1 and MEK2 protein isoforms. Complete silencing of MEK1 and MEK2 would prevent cell proliferation, so low levels of MEK1 and/or MEK2 protein isoforms might be expected in the shMEK1/2 cell line.

A Tool for Characterizing RNAi Constructs

We have presented two examples of functionally characterizing RNAi constructs with the Firefly System. The automated, highly sensitive assays show the effectiveness of siRNA targeting upstream signaling proteins by measuring the phosphorylation levels of proteins downstream in the cascade. By enabling the detection and measurement of different protein isoforms in samples as small as 100 cells, the Firefly System yields information unattainable with Western blots. The functionality of an shRNA gene knockout can be verified and studied, probing with multiple primary antibodies, and the researcher can gain insights into the functions of the RNAi target and its interactions with other cell signaling proteins.

Acknowledgements

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