Gene Silencing in Primary Astrocyte Cells using MISSION™ TRC shRNA Lentiviral Particles

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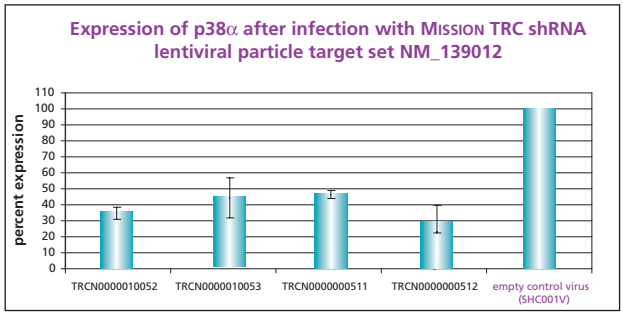
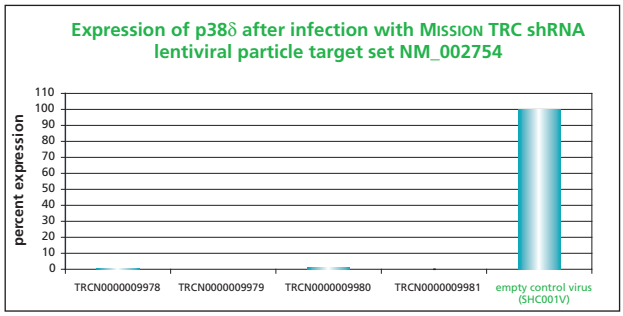
Gene silencing and knockdown utilizing RNAi is quickly becoming a routine laboratory procedure. This silencing technique requires the efficient transfection of the respective short dsRNAs, usually 19-23 bp's in length. While the technique is powerful, it also presents some significant limitations. RNAi has been limited to treating readily transfectable cell lines, such as transformed lines. The efficient delivery of dsRNA to primary cell lines and difficult-to-transfect lines is problematic. Additionally, the silencing effect realized following the transfection of short dsRNA is often transient. The MISSION TRC shRNA lentiviral system is designed to address both of these shortcomings. The advent of retroviral delivery systems, first demonstrated by Barton and Medzhitov (2002, PNAS **99**, 14943-14945), provides an approach to address RNAi-mediated knockdown in primary and hard to transfect lines. In contrast to conventional transfection techniques, this approach is minimally limited by the susceptibility of the particular cell line to lentiviral infection. By using lentiviral-based vectors, easy- and difficult-to-transfect cell lines can be efficiently and readily infected. Transient knockdown issues are overcome by utilizing shRNA constructs. It has been shown that cellular processing of shRNA leads to more stable and long-term gene silencing when compared to transfection of siRNA, allowing the researcher to perform experiments that require a longer timeframe, such as *in vivo* xenograft models.

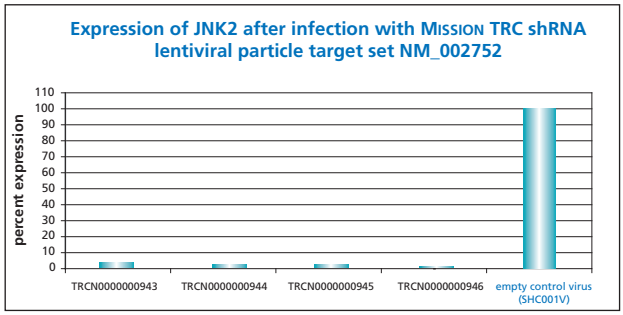
In this communication we demonstrate that the MISSION TRC shRNA lentiviral system can be used to knockdown gene expression in human astrocytes, one of the most difficult to transfect primary cell types. While transfection of siRNA has been demonstrated in a transformed astrocytoma line (Konnikova, L. et. al. 2003. *BMC Cancer* **3**:23), the data here provide the first example of successful transduction and gene silencing in a normal primary astrocyte culture.

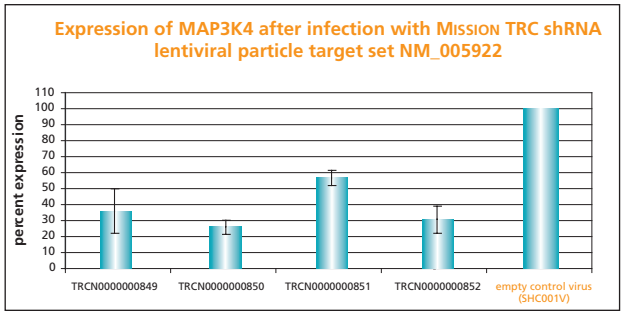
Four different genes were targeted. Minimally, four different shRNA constructs were examined for each gene. The genes chosen represented those identified as relevant in the normal astrocyte cellular pathways: JNK2, p38α, p38δ, and MAP3K4. The shRNA-containing virus preparations successfully transduced 100% of the primary astrocyte cultures treated. Varying levels of knockdown resulted. Five of seven JNK2 constructs knocked down mRNA expression by >90%. The remaining two constructs resulted in no discernable knockdown and 62% knockdown of endogenous mRNA expression.

Seven different constructs designed to target the p38α gene were examined. Levels of knockdown ranged from approximately 70% to 20%. Four different constructs each targeting the p38δ and MAP3K4 genes were tested. Two of the p38δ constructs resulted in knockdown of >95%. The remaining two p38δ constructs, tested in triplicate samples, resulted in no discernable Ct being assigned following 45 cycles of amplification. This shows complete inhibition of the target gene, reducing mRNA levels to below the detection limit of the qRT-PCR technique. The MAP3K4 constructs resulted in silencing ranging from 42% to 74%. All experiments used cells infected with an empty vector control virus (Sigma SHC001V) as a negative control for expression changes.









MISSION TRC shRNA lentiviral particle target sets directed toward p38α, p38δ, JNK2, and MAP3K4 were used to transduce human primary astrocyte cells. 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%).

As controls, no RNA template samples as well as samples including no reverse transcriptase were examined for each primer/probe used. In every instance, no Ct was assigned, demonstrating complete removal of genomic DNA. To examine potential off target affects, samples silenced with constructs specific for the JNK2 gene (TRCN0000010277, TRCN0000010278, and TRCN0000010279) were assayed with the MAP3K4 primer/probe. While JNK2-silenced targets assayed for JNK2 expression exhibited a range of effects: TRCN0000010277 - no knockdown; TRCN0000010278 - 62%; TRCN0000010279 – 95%, those same targets assayed for MAP3K4 expression demonstrated no or little knockdown of the JNK2 gene (data not shown).

Materials & Methods

MISSION TRC shRNA Lentiviral Transduction Particle target sets (Sigma SHVRS (see Table 1)): Twenty-three individual shRNA lentiviral particle constructs, covering four different genes, were utilized for this study. All knockdown was compared to empty vector control virus (Sigma SHC001V)

Astrocyte Culture: Primary-derived normal human astrocyte cells were obtained cryopreserved from Cambrex (cambrex.com). Cells were seeded at a density of 5,000 cells/cm^2 in Astrocyte Basal Medium (Cambrex) and cultures established. At 70% - 80% confluency, cells were trypsinized and reseeded into 96 well plates at 1.6 x 10^4 cells/well.

Infection: In a 96 well plate, 1.6 x 10^4 astrocyte cells containing 110 µl of media (AGM media, Cambrex Bio Science) were seeded in each of the 96 wells 24 hours prior to infection. 2 µl of Hexadimethrine Bromide, final concentration of 8 µg/ml were added to each well followed by 10^5 ~ 10^6 transducing units of lenitiviral particles corresponding to each shRNA construct. Media was changed out 24 hours following infection and replaced with AGM media containing puromycin (1 µg/ml). At 114 hours following puromycin selection, cells were lysed and the RNA isolated for use in qRT-PCR.

Validation of Knockdown: Total RNA was purified and subsequently analyzed using the appropriate TaqMan® Gene Expression Assay (ABI). Master mixes were prepared using Sigma's Quantitative RT-PCR ReadyMix (QR0200) supplemented with additional $MgCl_2$ (M8787) to 4.875 mM and water (W1754). Reference dye was also added to all samples as an internal monitor for fluorescence. Total volume reactions of 25 µl were prepared with primer/probe mixes at 1x concentration for all analysis. Moloney Murine Leukemia Virus Reverse Transcriptase (M1302) was used for the reverse transcription portion of the reaction (15 units per) and diluted in the provided buffer. Each sample was multiplexed in order to simultaneously measure the target as well as housekeeping gene levels. No template controls and no RT controls were run for each sample. PCR conditions were 42 °C for 15 minutes; 94 °C for 3 minutes (initial denaturation and JumpStart Taq activation); 45 cycles of 94 °C 15 seconds and 60 °C 1 minute, with data collection during the 60°C extension portion, endpoint detection. All reactions were run and analyzed using Stratagene Mx3000 qPCR machine and software. Results were normalized for input RNA using GAPDH. The difference in Ct between the infected samples and uninfected control cells along with the efficiency of PCR were used to generate percentage expression levels. Percentage is expressed as a level of the empty vector control, which is set to 100%.

Conclusion

RNA interference technology has been one of the key biological breakthroughs of the last decade and has revolutionized basic biology and gene function studies. It has not only changed the way we perform high content screens, target identification and target validation proof-of-concept studies, but also holds promise to revolutionize the field of therapeutics. The MISSION ™ TRC shRNA libraries overcome the limitation of synthetic siRNAs by providing a system for long term silencing and phenotypic observation, and the ability to generate lentiviral particles for infection and integration of primary cells such as astrocytes. We demonstrate here a high level of stable target knockdown even in a hard-to-transfect primary cell culture.

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Table 1

p38α NM_139012	
TRCN0000010052	GTTCAGTTCCTTATCTACCAA
TRCN0000010053	GACATAATTCACAGGGACCTA
TRCN0000000511	CCATGAGGCAAGAAACTATAT
TRCN0000000512	CGAGGTCTAAAGTATATACAT
p38δ NM_002754	
TRCN0000009978	CCCTTTCAGTCCGAGATCTTC
TRCN0000009979	GCGCAACTTCTATGACTTCTA
TRCN0000009980	CTGTGAATGAGGACTGTGAAC
TRCN0000009981	TCTGTGGGCTGTATCATGGCA
JNK2 NM_002752	
TRCN0000000943	GCTGTCGATGATAGGTTAGAA
TRCN0000000944	GATGTGTATTTGGTTATGGAA
TRCN0000000945	CTGTGAGGAATTATGTCGAAA
TRCN0000000946	AGGGATTGTTTGTGCTGCATT
MAP3K4 NM_005922	
TRCN0000000849	CCGTGACATTAAAGGTGCCAA
TRCN0000000850	GCTTCGCCTTTGTTAGAGATA
TRCN0000000851	GCCCGGAAGTGGATGAATTAT
TRCN0000000852	CAACCAAACATCAGAGGAATA

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