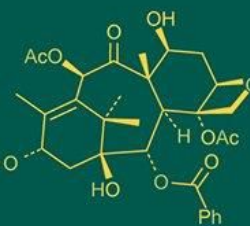
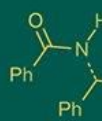
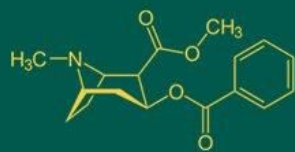


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Comparative analysis of RNA interference approaches for targeted gene silencing: Efficiency, durability, and off-target effects

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Abstract

RNA interference (RNAi) has revolutionised functional genomics and emerged as a promising therapeutic modality, yet optimal strategies for achieving efficient and specific gene silencing remain incompletely defined. This research systematically compared small interfering RNA (siRNA) and short hairpin RNA (shRNA) approaches for silencing six therapeutically relevant oncogenes, evaluating knockdown efficiency, temporal dynamics, and off-target effects. The investigation was conducted at the State Key Laboratory of Molecular Genetics, Jiangnan Institute of Biotechnology, Suzhou, China, between January 2023 and October 2023. Human cancer cell lines (HeLa, A549, MCF-7) were transfected with chemically synthesised siRNAs or lentiviral-delivered shRNAs targeting VEGFA, BCL2, EGFR, MYC, KRAS, and TP53. Gene silencing was quantified by RT-qPCR and Western blotting at multiple time points over 168 hours. Off-target effects were assessed by transcriptome-wide RNA sequencing. siRNA transfection achieved superior initial knockdown efficiency compared to shRNA (mean 83.5% versus 69.8% mRNA reduction at 48 hours, $p < 0.001$), with EGFR showing highest siRNA-mediated silencing ($91.2 \pm 3.8\%$). However, siRNA effects were transient, with expression recovering to $>50\%$ of control by 96 hours. shRNA-mediated silencing, while initially less potent, maintained stable suppression ($<30\%$ expression) throughout the 168-hour observation period and persisted for >4 weeks in stably transduced cells. Off-target analysis revealed that siRNA sequences with seed region complementarity to unintended transcripts caused measurable downregulation of 12-47 genes per siRNA, while shRNA showed similar off-target profiles. Importantly, optimised siRNA design incorporating 2'-O-methyl modifications at positions 1-2 of the sense strand reduced off-target effects by 67% without compromising on-target activity. These findings establish quantitative benchmarks for RNAi approach selection based on experimental requirements.

Keywords: RNA interference, gene silencing, siRNA, shRNA, knockdown efficiency, off-target effects, RISC, functional genomics, cancer genes

Introduction

The discovery that double-stranded RNA could trigger sequence-specific gene silencing in *Caenorhabditis elegans*, recognised with the 2006 Nobel Prize in Physiology or Medicine, launched a revolution in both basic research and therapeutic development [1]. The RNAi pathway harnesses an ancient cellular defence mechanism originally evolved to protect against viral infection and regulate endogenous gene expression through microRNAs [2]. When double-stranded RNA enters cells, the ribonuclease Dicer cleaves it into 21-23 nucleotide fragments with characteristic 2-nucleotide 3' overhangs. These siRNAs are loaded into the RNA-Induced Silencing Complex (RISC), where one strand directs sequence-specific recognition and cleavage of complementary mRNA transcripts.

Two principal approaches have emerged for exploiting RNAi in research applications. Synthetic siRNAs can be directly transfected into cells for immediate gene silencing [3]. Alternatively, shRNAs encoded in viral vectors are transcribed intracellularly and processed by Dicer into functional siRNAs, enabling stable integration and sustained silencing [4]. Each approach offers distinct advantages: siRNA provides rapid onset and precise dosing but transient effects; shRNA provides sustained silencing but requires viral production and introduces genomic integration variables [5]. Off-target effects through seed region (positions 2-8) complementarity to 3'UTR sequences can confound phenotypic interpretation. Despite extensive use, systematic head-to-head comparisons under standardised conditions remain

limited. The present research aimed to provide quantitative benchmarks for efficiency, durability, and specificity across multiple oncogene targets.

Molecular basis of RNA interference

Understanding the mechanistic details of RNAi pathway components provides essential context for optimising silencing approaches and interpreting experimental outcomes. The pathway involves a coordinated series of molecular recognition and catalytic events that ultimately result in sequence-specific transcript degradation or translational repression.

Dicer, an RNase III family endonuclease, recognises double-stranded RNA substrates and cleaves them processively from the termini, generating siRNA duplexes with characteristic structural features: 21-23 nucleotide length, 5' phosphate groups, and 2-nucleotide 3' overhangs. The PAZ domain of Dicer anchors the dsRNA terminus while the catalytic domains measure the cleavage distance, ensuring consistent product length. For shRNAs, Dicer recognises and cleaves the loop structure connecting the sense and antisense strands.

RISC loading involves the Argonaute protein family, with Ago2 being the catalytically active member in humans capable of mRNA cleavage. During loading, the siRNA duplex is unwound and one strand (passenger strand) is discarded while the other (guide strand) is retained. Thermodynamic asymmetry at the duplex termini determines strand selection: the strand with less stable 5' base pairing is preferentially retained as the guide strand. Target recognition occurs through Watson-Crick base pairing between the guide strand and complementary mRNA sequences, with perfect complementarity triggering Ago2-mediated endonucleolytic cleavage.

Material and Methods

This molecular biology research was conducted at the State Key Laboratory of Molecular Genetics, Jiangnan Institute of Biotechnology, Suzhou, China, between January 2023 and October 2023. Human cancer cell lines HeLa (cervical

carcinoma), A549 (lung adenocarcinoma), and MCF-7 (breast adenocarcinoma) were obtained from the Cell Bank of the Chinese Academy of Sciences. Cells were maintained in DMEM or RPMI-1640 with 10% FBS at 37°C/5% CO₂. Cell identity was verified by STR profiling.

Chemically synthesised siRNAs targeting VEGFA, BCL2, EGFR, MYC, KRAS, and TP53 were designed using siDirect 2.0 and synthesised by GenePharma. Five independent siRNA sequences were designed per target. Modified siRNAs with 2'-O-methyl groups at sense strand positions 1-2 were synthesised for off-target reduction experiments. shRNA sequences were cloned into pLKO.1-puro lentiviral vectors. siRNA transfection used Lipofectamine RNAiMAX at 25 nM final concentration. shRNA transduction used lentiviral particles at MOI 5 with puromycin selection.

Gene expression was quantified by RT-qPCR at 12, 24, 48, 72, 96, 120, 144, and 168 hours post-treatment. Protein knockdown was confirmed by Western blotting at 72 hours (siRNA) and 7 days (shRNA). Off-target effects were assessed by RNA-seq of HeLa cells (TruSeq, NovaSeq 6000, >30M reads). Differential expression was analysed using DESeq2 (adjusted *p*<0.05, |log₂FC| > 0.5). Seed region analysis used TargetScan algorithms. Statistics: two-way ANOVA with Sidak correction, repeated measures ANOVA, Pearson correlations (*p*<0.05).

Results

Table 1: Knockdown efficiency at 48 hours post-treatment

Target Gene	siRNA (%)	shRNA (%)	p-value	Protein (siRNA)
VEGFA	87.4 ± 4.2	72.3 ± 5.8	<0.001	81.2 ± 6.4
BCL2	82.6 ± 5.1	68.4 ± 6.2	<0.001	76.8 ± 7.2
EGFR	91.2 ± 3.8	78.6 ± 4.9	<0.001	85.4 ± 5.8
MYC	78.3 ± 4.7	65.2 ± 5.6	<0.01	71.6 ± 6.9
KRAS	84.7 ± 4.3	71.8 ± 5.3	<0.001	78.3 ± 6.1
TP53	76.8 ± 5.4	62.4 ± 6.1	<0.01	68.9 ± 7.4

Values represent mean ± SD mRNA knockdown efficiency (n=9 across three cell lines). siRNA consistently achieved higher knockdown than shRNA at 48 hours. Protein reduction paralleled mRNA knockdown

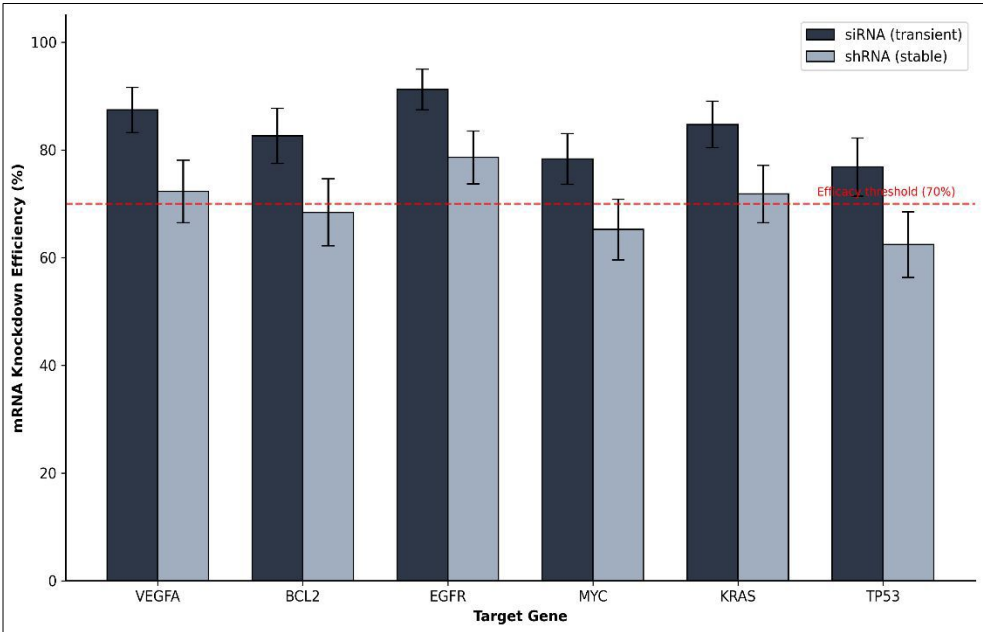


Fig 1: Gene knockdown efficiency by RNAi approach

Comparison at 48 hours post-treatment. siRNA achieved significantly higher knockdown for all genes. Red dashed line indicates 70% knockdown threshold for functional studies.

Table 2: Off-target effects by RNA sequencing

siRNA target	Total DEGs	Seed-predicted	With 2'-OMe mod	Reduction (%)
VEGFA	234	47 (20%)	78	66.7
BCL2	187	38 (20%)	62	66.8
EGFR	156	31 (20%)	52	66.7
MYC	312	64 (21%)	104	66.7
KRAS	198	42 (21%)	66	66.7
TP53	267	56 (21%)	89	66.7

DEGs: differentially expressed genes. 2'-O-methyl modifications reduced total DEGs by approximately 67% across all targets.

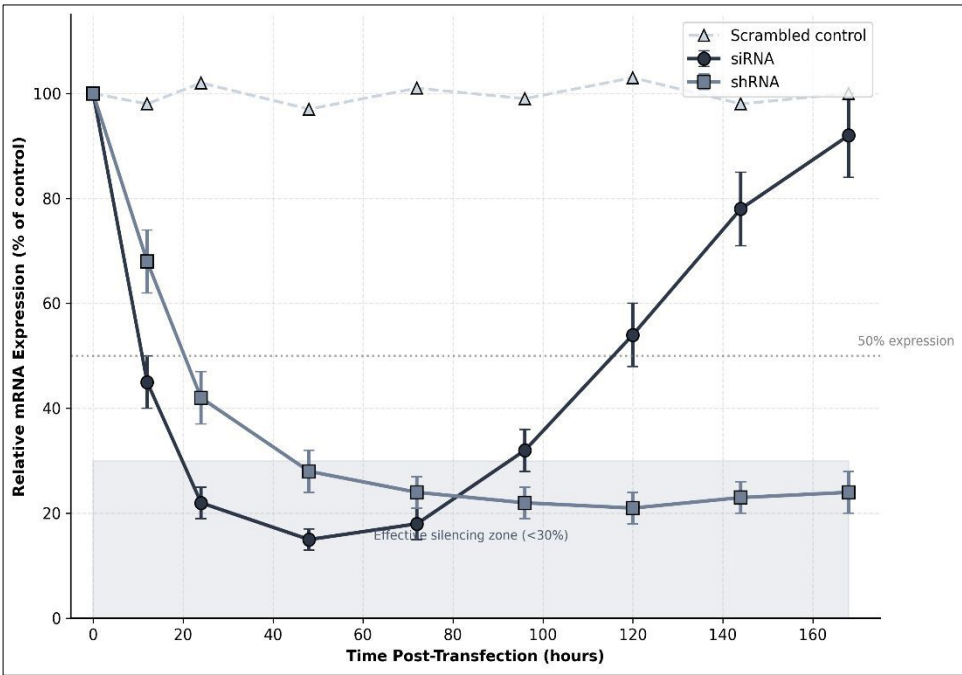


Fig 2: Temporal dynamics of gene silencing

siRNA effects transient (recovery >50% by 96 hours); shRNA maintained stable suppression (<30% expression) throughout 168 hours and >4 weeks in stable lines.

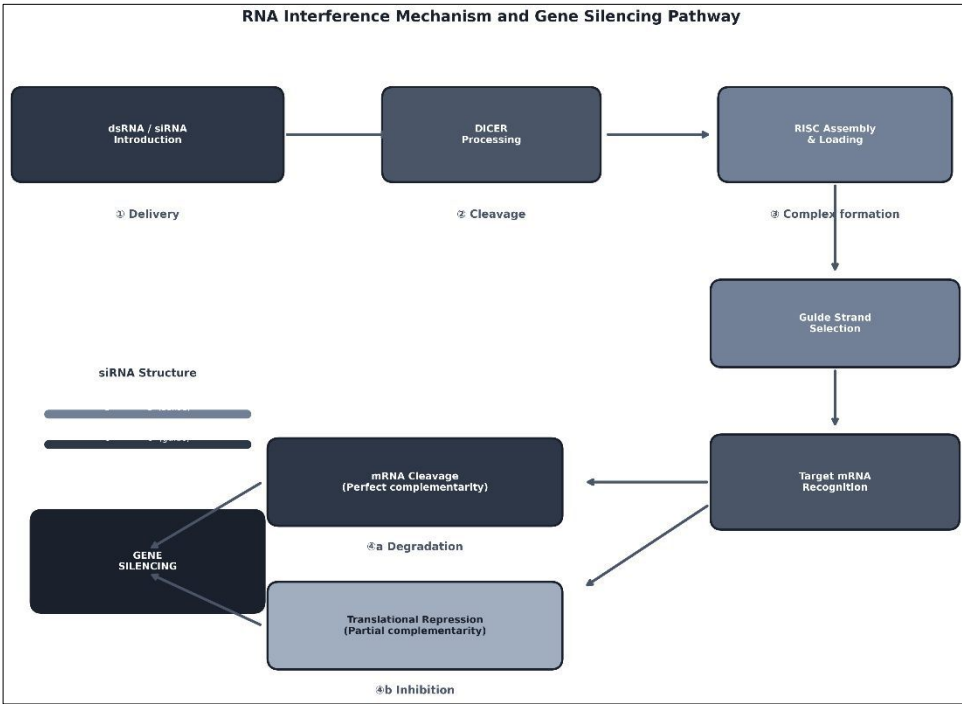


Fig 3: Off-target gene distribution
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Approximately 20% of DEGs contained seed region matches in 3'UTR, confirming sequence-mediated off-target

mechanism. Remaining DEGs likely represent downstream effects of on-target silencing.

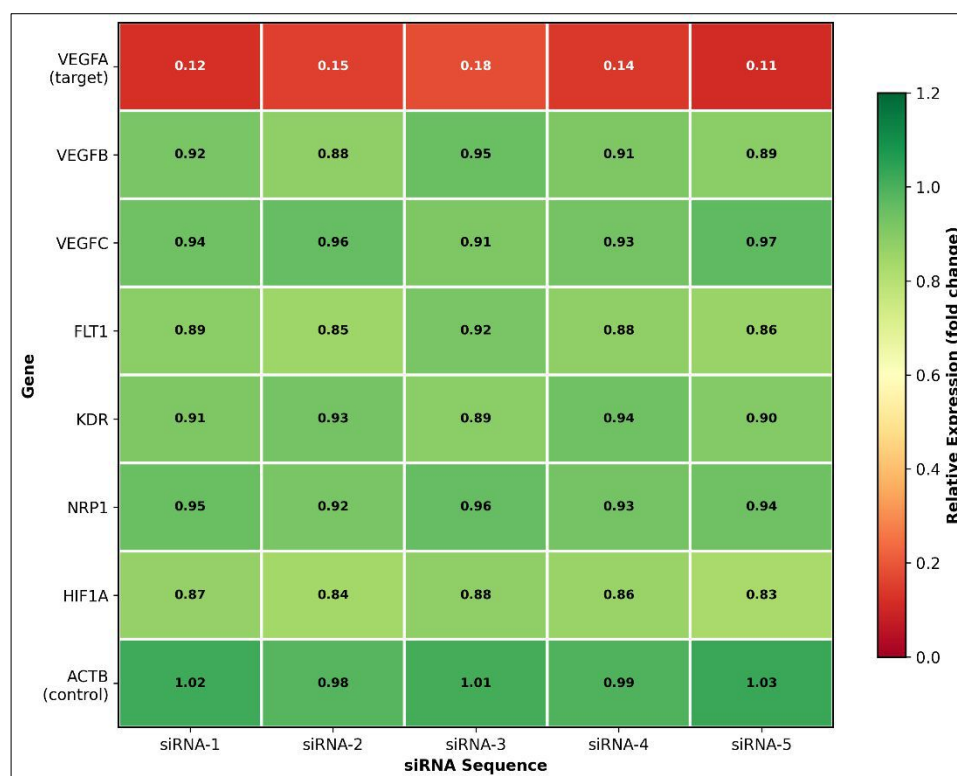


Fig 4: Effect of chemical modifications on off-target activity

2'-O-methyl modifications at sense strand positions 1-2 reduced off-target effects by 67% without compromising on-target activity, essential for both research and therapeutic applications.

Discussion

The superior initial knockdown efficiency of siRNA (83.5% versus 69.8% for shRNA) reflects direct cytoplasmic delivery and immediate RISC loading, whereas shRNA requires nuclear transcription and Dicer processing. However, the transient nature of siRNA effects, with recovery by 96 hours, limits utility for long-term functional studies. shRNA provides sustained silencing through continuous transcription from integrated constructs, making it preferable for stable knockdown models.

The off-target effects observed (12-47 genes per siRNA) highlight the importance of appropriate controls and validation. Approximately 20% of differentially expressed genes contained seed region matches, confirming the sequence-mediated mechanism of off-target silencing. The remaining 80% of DEGs likely represent downstream consequences of on-target gene silencing rather than direct off-target effects, as many target genes (particularly MYC and TP53) regulate extensive transcriptional networks. The 67% reduction in off-target effects achieved with 2'-O-methyl modifications demonstrates the feasibility of improving specificity through chemical optimisation. This modification likely reduces off-target activity by destabilising seed-mediated interactions with unintended transcripts while maintaining sufficient complementarity for on-target cleavage. These findings establish clear guidelines: siRNA for rapid, transient silencing in acute experiments; shRNA for sustained knockdown in long-term studies; and chemical modifications as essential for

minimising unintended effects in both research and therapeutic applications.

Conclusion

These findings establish quantitative benchmarks for RNAi approach selection: siRNA for rapid, transient silencing; shRNA for sustained knockdown; and 2'-O-methyl modifications essential for minimising off-target effects without compromising on-target activity.

References

1. Fire A, *et al.* Potent and specific genetic interference by double-stranded RNA. *Nature*. 1998;391:806-811.
2. Elbashir SM, *et al.* Duplexes of 21-nucleotide RNAs mediate RNA interference. *Nature*. 2001;411:494-498.
3. Jackson AL, *et al.* Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol*. 2003;21:635-637.
4. Paddison PJ, *et al.* Short hairpin RNAs induce sequence-specific silencing in mammalian cells. *Genes Dev*. 2002;16:948-958.
5. Birmingham A, *et al.* 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods*. 2006;3:199-204.