Evaluating Off-targets of siRNA Drugs: Effects of Sequence, Modifications, Delivery Method, Species, and Cell type

WuXi Biology

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Abstract

SiRNA holds great promise as a therapeutic modality due to its high specificity, developability, and sustained efficacy. However, its application is challenged by sequence-dependent off-target effects, which can compromise both safety and therapeutic effectiveness. A critical step in siRNA drug development is the rigorous evaluation and mitigation of these off-target effects. Off-target assessment can be influenced by multiple variables, including siRNA sequence design, chemical modifications, delivery strategies, as well as species- and cell type-specific responses. To systematically investigate the impact of these factors, we conducted comprehensive transcriptomic profiling to evaluate off-target effects across diverse experimental conditions.

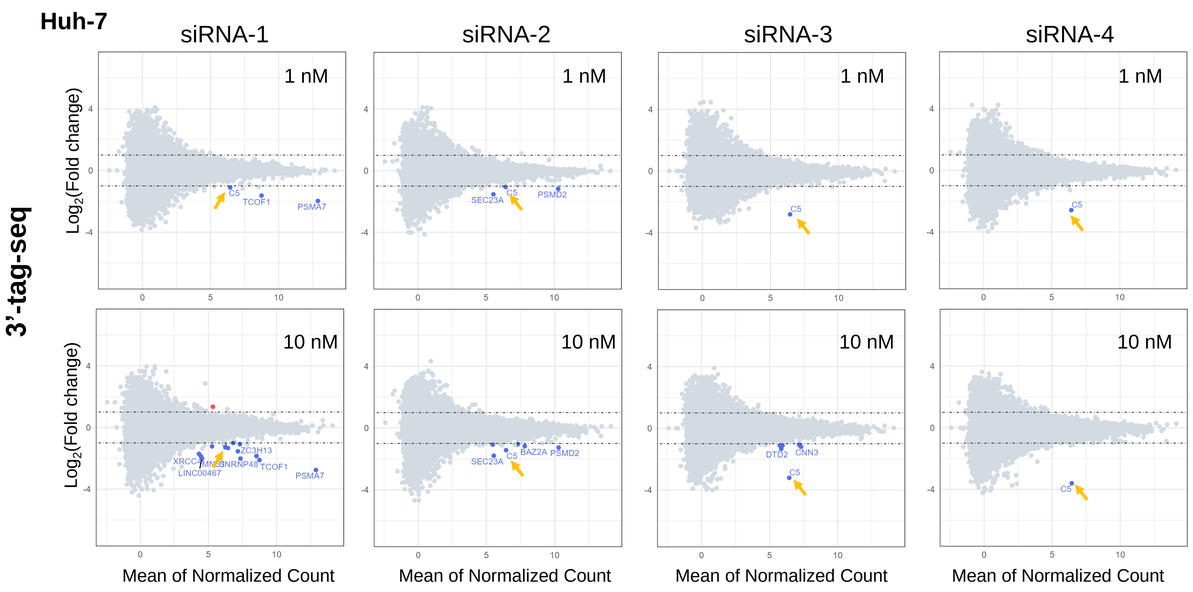
Briefly, we designed four C5-targeting siRNAs with two having higher predicted off-target activity ("dirty") and two with few off-target potential ("clean"). These siRNAs were tested at 1 nM and 10 nM concentrations in Huh-7 cells using our high-throughput 3'-tag-seq platform, which maintains strong correlation with conventional RNA-seq while improving cost-efficiency through multiplexing. The "dirty" siRNAs showed more dose-dependent off-target effects in both Huh-7 and HepG2 cells, with affected genes displaying near-perfect complementarity to siRNA strands. Screening 16 chemical modification patterns on the "dirty" siRNAs revealed that pattern-5 effectively reduced off-target activity without compromising on-target knockdown, identifying a promising strategy for improving siRNA specificity.

We then assessed the impact of different oligo delivery methods on off-targets and found that compared to free uptake/gymnosis, delivery by transfection with lipofectamine led to more differentially expressed genes/potential off-targets, likely due to more efficient siRNA delivery into the cell and perhaps effects of the transfection reagent itself. We also evaluated the off-targets of two siRNAs in PHH (Primary Human Hepatocytes) and PRH (Primary Rat Hepatocytes) and found considerable species differences in potential off-target genes, likely due to transcriptome differences between humans and rats, which is an important limitation for the use of model species to validate siRNA safety and toxicity. Finally, we performed off-target evaluation using human liver primary cells (PHH) and a liver cancer cell line (Huh-7), and detected differences in potential off-target genes, possibly due to differences in isoforms and/or gene expression levels.

In conclusion, we systematically explored the impact of siRNA sequence, modification, delivery method, species and cell type on off-target evaluation using high-throughput sequencing, and offer recommendations for off-target evaluation to help develop safe and effective oligonucleotide therapies.

Methods and Results

1. Off-target gene detection of C5-targeting siRNAs by 3'-tag-seq in Huh-7 & HepG2 cells



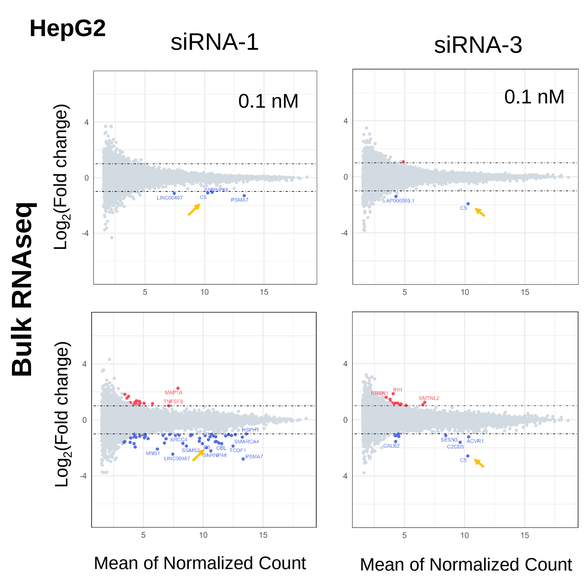


Figure 1. MA plot of gene expressions (treatment *vs.* non-treatment control)

Grey, no significant differential expression; Blue, down-regulated genes; Red, up-regulated genes; Yellow arrow, target gene (C5).

Table 1. Alignment of siRNAs with differentially expressed gene sequences

siRNA ID	Strand	Off-target gene	Alignment (5'->3' from siRNA strand)
siRNA-1	sense	ALCAM	||||||*|||*||*|||||
siRNA-1	sense	ALCAM	*:|||||||||||||:*|*||
siRNA-1	sense	PSMA7	|||:|||**||||||||||:
siRNA-1	antisense	LINC00467	|||||:||:||||*||||||||:
siRNA-1	antisense	SEC23A	|||*||||*|||*||||*||*|
siRNA-1	sense	TCOF1	:|||||||:|||||||||:||
siRNA-1	sense	TCOF1	:||:||:*||||||||||||
siRNA-1	sense	ZC3H13	||||:||*||:|*|:||||||
siRNA-1	sense	ZC3H13	**||||||:|||||||*|||
siRNA-2	antisense	BAZ2A	|*|||||:|||||||*|*|:|
siRNA-2	sense	CNN3	:*|:||**:||:||||*||
siRNA-2	antisense	SEC23A	||||||||||*||||||||||
siRNA-2	antisense	PSMD2	|||||:|:||||||*||*:*||

➢ Alignment status: "|", Watson-Crick pairing; ":", GU pairing; "*", Mismatch;

Results-1

- ✓ 3'-tag-seq is a high-throughput and highly reproducible method for detection of potential off-target genes
- ✓ The majority of differentially expressed genes observed by 3'-tag-seq had nearly perfect match with the sense or antisense strands of the siRNAs
- ✓ Strand exchange and nearly perfect pairing with the off-target genes are likely to be the main causes of siRNA off-target effects
- ✓ siRNAs with higher potency are likely to have fewer off-targets

2. Effects of different modifications on off-targets in Huh-7 cells

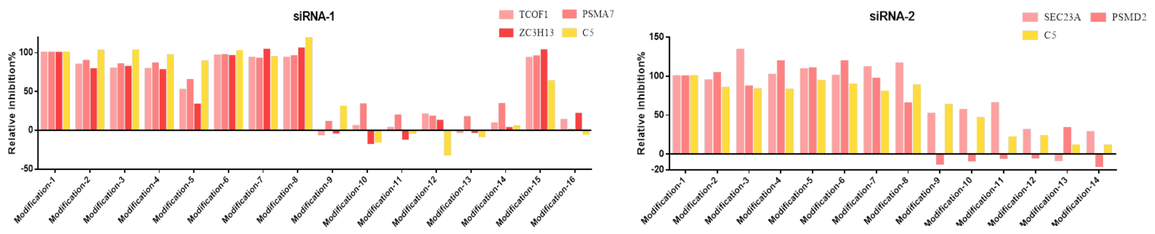


Figure 2. Relative inhibition of on-target gene (C5) and off-target genes by siRNAs with different modification patterns

Results-2

- ✓ Modification patterns 1-8 and 9-14 show similar trends for inhibition efficacy of C5 across both siRNA-1 and siRNA-2
- ✓ For siRNA-1, modification pattern 5 reduces off-targets without significantly impacting on-target efficacy
- ✓ For siRNA-2, modification pattern 5 does not reduce off-targets significantly possibly due to different off-target mechanisms (strand-exchange for siRNA-1 and microRNA-like off-target for siRNA-2)

3. Effects of different delivery methods on off-target genes

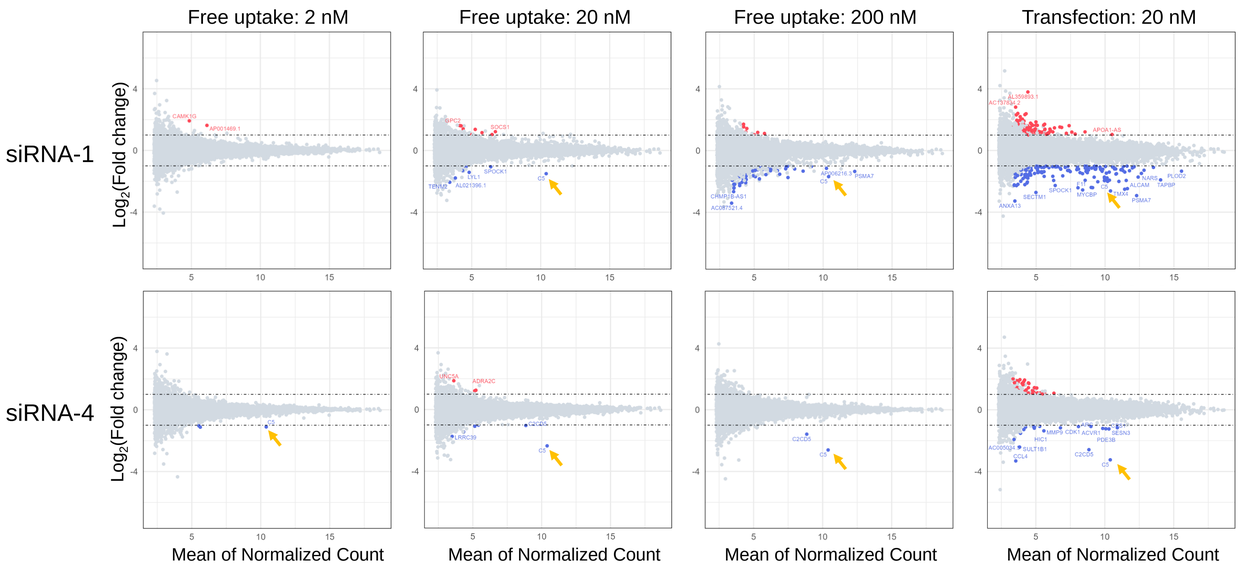


Figure 3-1. MA plot of gene expressions across different delivery methods (treatment *vs.* non-treatment control)

Grey, no significant differential expression; Blue, down-regulated genes; Red, up-regulated genes; Yellow arrow, target gene (C5).

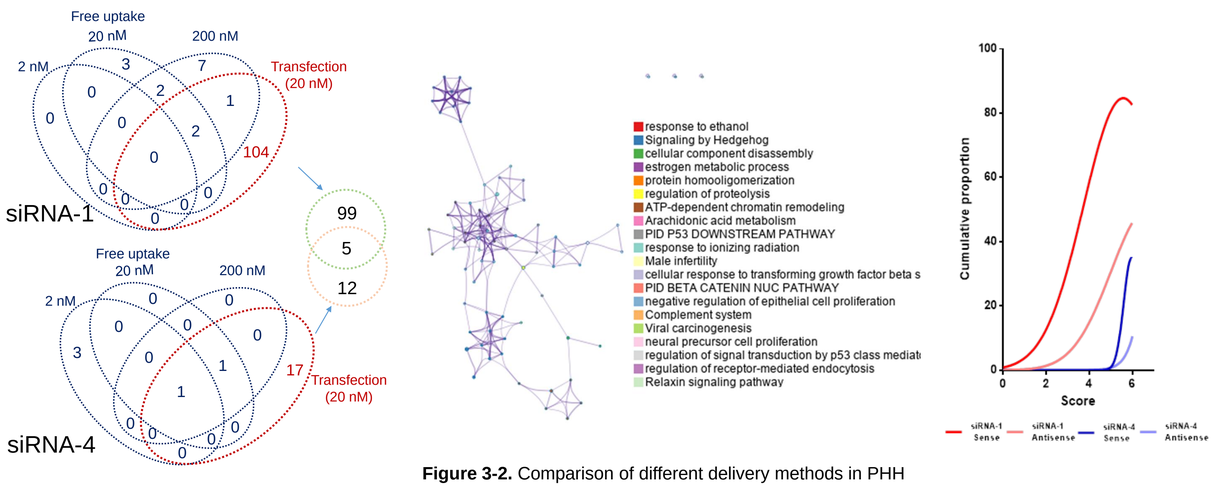


Figure 3-2. Comparison of different delivery methods in PHH

Left, Venn diagram for number of differentially expressed (DE) genes following different treatment conditions; ***Center***, Functional enrichment for DE genes specific to lipofectamine transfection group; ***Right***. Cumulative proportion of transfection-specific DE genes against mismatch score with their siRNAs.

Results-3

- ✓ Delivery of the same concentration of siRNA (20 nM) by transfection resulted in a greater number of differentially expressed genes compared to delivery by free uptake/gymnosis
- ✓ This is likely due to transfection being more effective in delivering the siRNA into the cell compared to free uptake, but potentially also due to the effects of the transfection reagent itself

4. Effects of different species and cell type on off-target gene evaluation

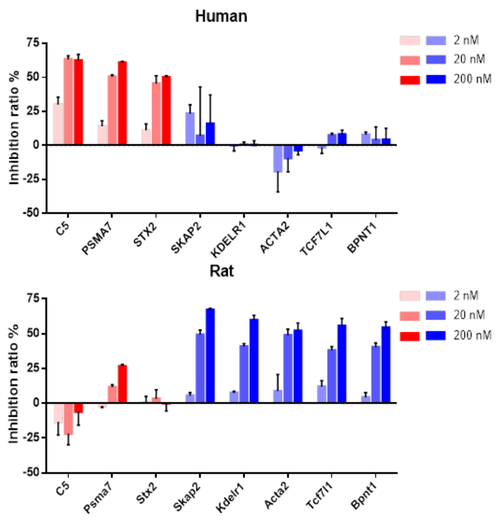


Figure 4. Cross-species comparison between PHH and PRH for detected potential off-target genes

Red represents dose-dependent differentially expressed genes detected in PHH; Blue represents dose-dependent differentially expressed genes detected in PRH

Table 2. Alignment of siRNAs to their on/off-target genes in human and rat

Strand	On/Off-target gene	Alignment	
		Human	Rat
Antisense	C5	||||||||||||||||||||	|*||**|:|||||||**|||
sense	PSMA7	|||:|||**||||||||||:	|||:||:**:|||||||||:
Sense	STX2	:|||||**||||||||**	|:||:||*||*|||:||:|
Sense	SKAP2	||||||*|||*||*|||:|	:||||::|:|||*||:|:**
Sense	KDELR1	||:|*:::|:::|||:||*:::|	|||||**|||||||||:|**
Sense	ACTA2	:|:||||:|:|*||||:*|	:|*|*:|||:||||:||||:*:
Sense	TCF7L1	||:|:|:*|:::|||||||||	|*|||||||||||:||||||
Sense	BPNT1	||||||*|||*||*|||||	|||||**||||||||||*||

➢ Alignment status: "|", Watson-Crick pairing; ":", GU pairing; "*", Mismatch;

Results-4

- ✓ Testing of the same siRNA in primary human hepatocytes (PHH) and primary rat hepatocytes (PRH) showed species-specific patterns of differentially expressed potential off-target genes due to different degrees of siRNA sequence match with the targeted gene sequences
- ✓ Testing of the same siRNA in PHHs and the Huh-7 human cell line showed that TCOF1 was detected as a potential off-target gene in Huh-7 but not PHH. Further analysis showed the siRNA targets a specific isoform of the gene that is expressed in Huh-7 but not PHH (data not shown)

Summary

Off-target evaluation is an integral part of the oligonucleotide drug discovery process. In this study, we used C5-targeting siRNAs as an example to evaluate how siRNA sequence, modifications, delivery method, species and cell type affect off-target evaluation by 3'-tag-seq, a high-throughput method we established for rapid and cost effective identification of potential off-targets of oligonucleotide drugs.

We show that our 3'-tag-seq method could detect the expected off-targets of our "dirty" and "clean" siRNA designs. These off-targets may potentially be reduced by using different modification patterns. Delivery by free uptake enables off-target evaluation without artifacts caused by the transfection reagent. Careful selection of species and cell type is important to enable meaningful off-target evaluation.

Assessment of off-targets of oligonucleotide drugs is one part of WuXi Biology's integrated platform to support oligonucleotide drug discovery. Our platform also offers capabilities for sequence design, synthesis of oligos at different scales, optimization of modifications and delivery tools, and the assessment of in vitro and in vivo safety and efficacy of oligonucleotides.

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