Off-target effects: disturbing the silence of RNA interference (RNAi).

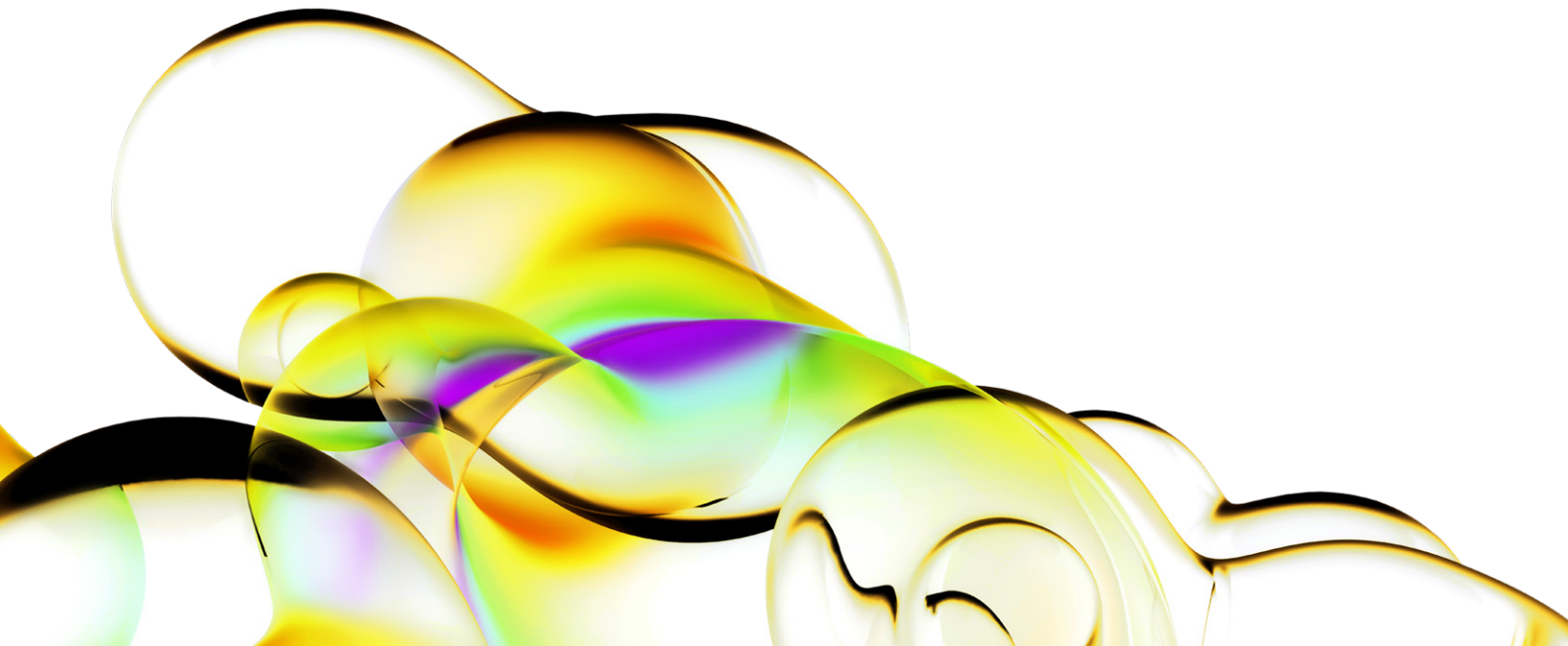
Abstract

Off-target effects occur when an siRNA is processed by the RNA-Induced Silencing Complex (RISC) and down-regulates unintended targets. As these changes in gene expression can lead to measurable phenotypes (such as false positives) it is of great importance to understand the mechanism behind off-targeting so that strategies can be developed to minimize their effects. We have discovered that bioinformatics, novel chemical modifications, and siRNA pooling significantly decrease off-target effects. These three strategies are incorporated into the ON-TARGET*plus*™ and SMARTpool™ siRNA reagents.

Introduction

RNA interference (RNAi) is a post-transcriptional gene regulatory mechanism that can be mediated by endogenously encoded small RNA molecules (microRNAs), or synthetic duplexes referred to as small interfering RNAs (siRNAs). In both cases, these molecules partner with the RNA Induced Silencing Complex (RISC) to target messenger RNAs for degradation and/or translation attenuation (Figure 1).

Since the discovery that siRNAs can enter the RNAi pathway and mediate gene knockdown, researchers have invested considerable effort in optimizing the technology for functional genomics studies. As is the case with most new technologies, the transition of RNAi into a research tool has had its obstacles. Initial problems associated with inconsistent siRNA functionality were addressed by detailed studies that identified functional attributes associated with duplex performance[1,2].



More recently, widespread, non-specific effects that complicate the interpretation of data generated from siRNA-mediated knockdown studies have been observed.

Non-specific effects resulting from the introduction of siRNA appear to have three separate origins[3]

1. Lipid-mediated response[4]
2. Interferon response[5]
3. RISC-dependent off-target effects[6-10]

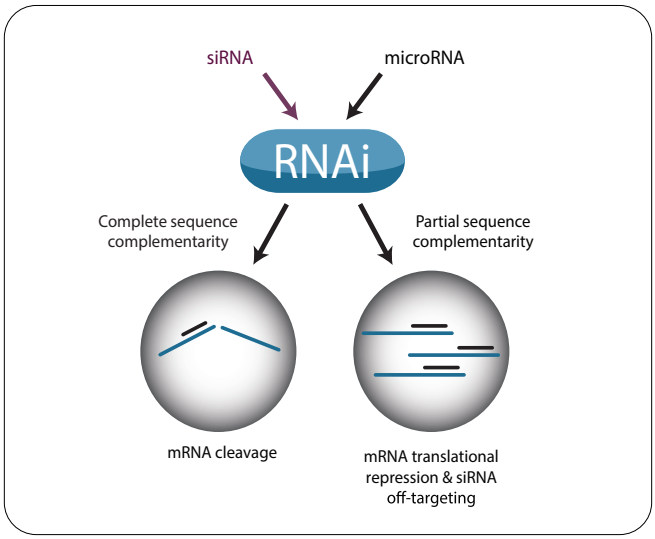


Figure 1: Small RNA molecules, microRNA and siRNA, regulate mRNA translation through RNA interference (RNAi). Depending on the level of complementarity between the small RNA molecule and mRNA, the mRNA will be silenced (complete) or translationally repressed (partial). siRNA off-targeting effects occur through partial complementarity of the siRNA with unintended mRNA targets.

As demonstrated by Fedorov and colleagues, cationic lipids typically used to deliver siRNA can induce broad changes in gene expression profiles. Separately, multiple labs have observed that particular sequence motifs and dsRNA lengths can induce the interferon response pathway in a cell-type specific manner[5,11]. While these unanticipated effects can be eliminated by adopting stringent siRNA design filters and optimizing lipid concentrations and compositions, a more challenging contributor to non-specific siRNA-mediated gene modulation, off-target effects, presents the research community with a surprisingly complex problem[6-10]. Having conducted considerable research on off-target effects, it was found that a combination of bioinformatics, chemical modifications, and siRNA pooling significantly reduce unintended targeting. These three innovations are included in ON-TARGET*plus* SMARTpool siRNA reagents.

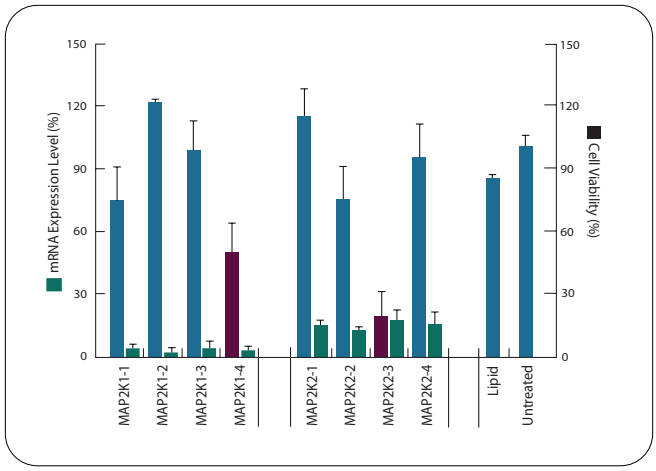


Figure 2: Off-target effects are sequence dependent and target independent. A side-by-side comparison of toxicity and gene knockdown of eight siRNAs targeting two separate genes (MAP2K1 and MAP2K2). While all four duplexes in each cluster provide equivalent levels of gene silencing (> 80%), only a single siRNA in each group is toxic (red bars).

History

Off-target effects were first described by Jackson and co-workers in 2003[9]. Using genome-wide microarray profiling as a method of detection, the authors identified modest, 1.5- to 3-fold changes in the expression of dozens of genes following transfection of individual siRNA. The levels of complementarity between the sense or antisense strand of the siRNA and the off-targeted genes varied considerably and the overall off-target expression profile was unique for each siRNA, suggesting a sequence-specific component to the phenomena.

Initially, these "modest" changes in off-target gene expression led many to dismiss the event as inconsequential. Unfortunately, this optimism was recently dispelled by reports that off-target effects could induce measurable phenotypes. In a broad RNAi-based phenotypic screen designed to identify kinase regulators of the Hypoxia-inducible factor 1 (HIF-1), Lin and co-workers discovered that the phenotypic effects induced by several of their top candidates were the consequence of off-targeting of HIF-13 Similarly, Fedorov demonstrated that a significant fraction of siRNAs targeting the housekeeping gene diazepam binding inhibitor (DBI) produced cell viability phenotypes that had characteristic features of off-target effects, such as target independence, sequence dependence, and RISC-reliance (Figure 2)[12]. These findings demonstrated a clear shortcoming of the technology. False positives generated during phenotypic screens can lead to false leads and the

unnecessary use of resources to explore non-productive research tracts. From a therapeutic standpoint, off-target phenotypes represent a drawback that can severely limit applications of siRNAs as future therapeutic agents. For these reasons, understanding the mechanism underlying off-target effects and identifying measures that can enhance siRNA specificity is critical for developing RNAi into a reliable research and therapeutic tool.

Prevention

Early studies in RNAi established that single base pair mismatches between the siRNA and the target transcript dramatically alter siRNA functionality[13]. From this result it was inferred that overall sequence identity played a role in siRNA specificity, thus leading to the adoption of local alignment algorithms, such as BLAST and Smith-Waterman to minimize off-target effects. To achieve this, users identified optimal target sequences using one or more selection criteria, and then filtered the resulting collection to eliminate sequences that shared significant levels of identity (such as > 15/19 bp) with other genes in the target genome. Birmingham and colleagues recently tested this approach by comparing a collection of in silico predicted off-targets with a library of validated off-targets identified by microarray gene expression profiling[14]. Using the Smith-Waterman algorithm, the authors demonstrated that with the exception of cases of near-perfect identity (such as 18/19, 18/20, 19/20) sequence alignment tools failed to accurately recognize off-targets (Figure 3). In general, the number of in silico predicted off-targets exceeded the true number by 1-2 orders of magnitude, regardless of whether one or both strands of the siRNA duplex were included in the analysis. Moreover, only a small fraction of the experimentally validated off-targets were identified by in silico methods, suggesting that overall sequence identity was a poor predictor of the number and identity of off-targeted genes.

While the failings of sequence alignment tools left RNAi users without a viable alternative for lessening off-target effects, recent efforts have identified three approaches for eliminating this source of non-specific gene modulation. These approaches include reducing siRNA concentration, chemically modifying siRNA, and applying more rigorous bioinformatics to siRNA design.

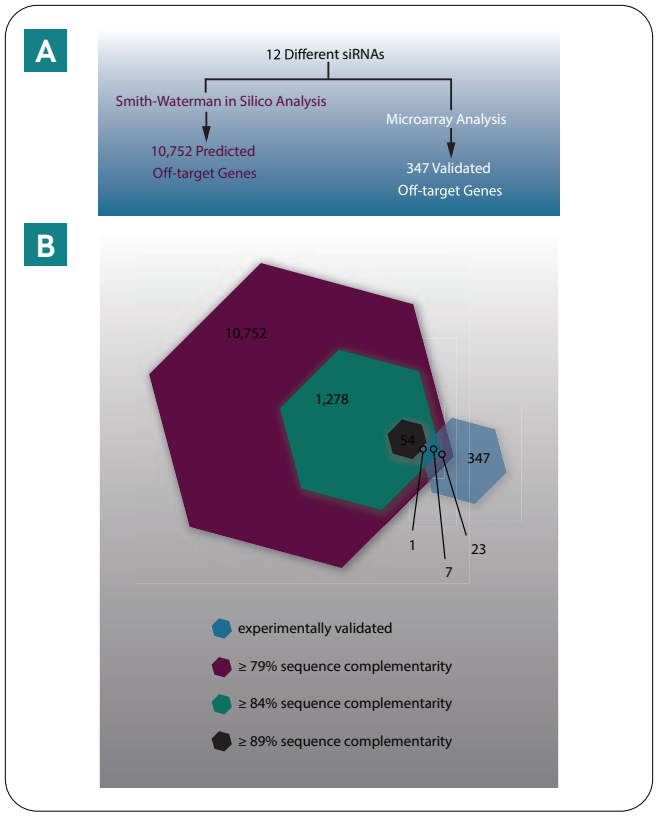


Figure 3: Sequence alignment tools fail to accurately recognize off-target effects. **A.** Investigational approach—12 siRNAs were computationally analyzed with the Smith-Waterman (S-W) alignment algorithm as well as experimentally tested by microarray analysis. **B.** Venn diagram shows overlap between 347 experimentally identified off-targets and in silico off-target genes predicted by the S-W alignment algorithm. Blue hexagon = 347 experimentally validated off-target genes for 12 siRNAs. Red, green and black hexagons represent the number of off-target genes predicted by S-W using 79% (such as 15/19 or better, 10,752 off-targets), 84% (such as 16/19 or better, 1,278 off-targets) and 89% (such as 17/19 or better, 54 off-targets) complementarity filters, respectively. The associated numbers (23, 7, and 1) represent the number of genes that are common between the experimental and predicted groups at each of the complementarity filter levels (79%, 84%, and 89%, respectively).

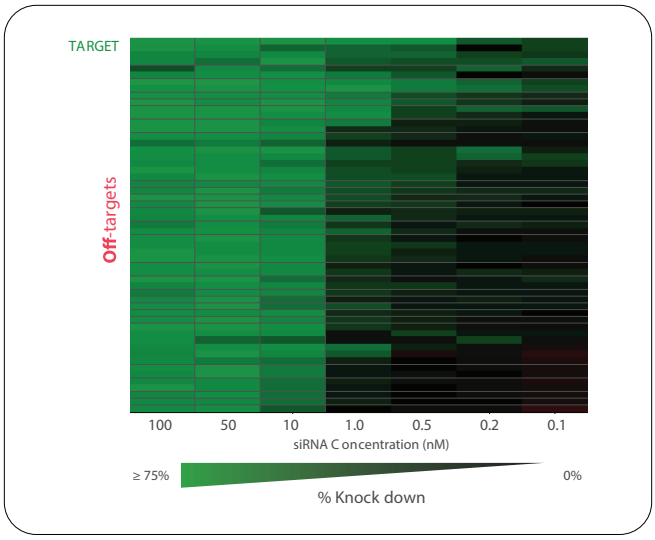


Figure 4: Both off-target and on-target effects greatly diminish with siRNA concentration. Heatmaps from microarray expression studies of HeLa cells transfected with GAPDH (Accession #NM_002046) SMARTselection designed siRNA at various concentrations (0.01-100 nM) at 24 hours. Black represents no change in gene expression, while green represents greater than or equal to 75% reduction in mRNA level.

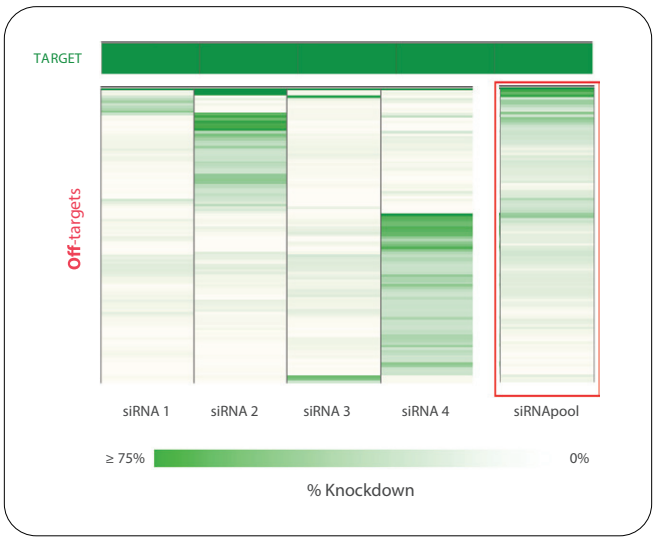


Figure 5: Pooling of four siRNA duplexes greatly reduces off-target effects of individual siRNAs, while maintaining high levels of target silencing. Heatmaps from microarray expression studies of HeLa cells transfected four Cyclophilin B (Accession #NM_000942) SMARTselection designed siRNAs and the corresponding SMARTpool siRNA reagent (Cat. #M-004606-00) at 24 hours. White represents no change in gene expression, while green represents greater than or equal to 75% reduction in mRNA level.

siRNA concentration

The first strategy originated from observations that off-target effects were concentration-dependent[6,9]. As shown in Figure 4, as the concentration of the siRNA is reduced, off-target effects faded. Unfortunately, attempts to employ this approach with all but the most potent siRNAs on a broad scale have failed due to the simultaneous depletion of target gene knockdown along with off-target effects. This impasse is resolved by adopting the very simple strategy known as "pooling." SMARTpool siRNA reagents pool four highly functional SMARTselection designed siRNAs targeting the same gene. Studies show that strong on-target gene knockdown can be achieved with minimal off-target effects if a pool consisting of highly functional multiple siRNA is substituted for individual duplexes. This finding is in contrast to speculation that mixtures of siRNAs can compound off-target effects. As shown in Figure 5, while individual duplexes delivered at 100 nM can induce varying numbers of off-targeted genes, transfection of the corresponding SMARTpool siRNA (100 nM total concentration) induces only a fraction of the total off-target profile. Importantly, the reduction in off-target gene modulation is achieved without jeopardizing target-specific knockdown.

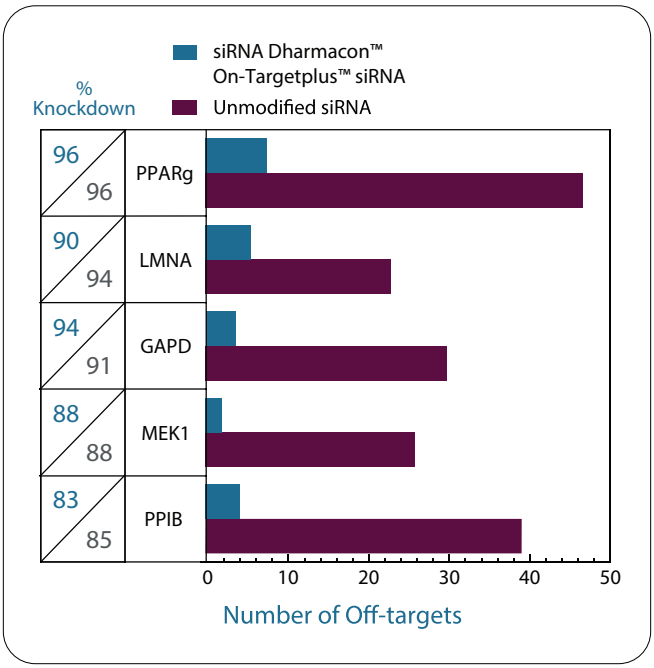


Figure 6: ON-TARGET*plus* siRNA significantly reduce off-target effects while maintaining high levels of target gene knockdown. Five SMARTpool siRNA reagents (PPARg, LMNA, GAPD, MEK1, and PPIB), both unmodified, and modified with ON-TARGET*plus* siRNA (100 nM) were transfected into Dharmacon HEK293 cells using Dharmacon™ DharmaFECT™ 1 Transfection Reagent.

siRNA modification

A second approach to minimizing off-target effects involves recent discoveries in the field of siRNA chemical modification. In the process of performing a structure-function analysis of chemically modified siRNA, Our scientists have identified a unique combination of modifications that eliminate as much as 80% of off-target effects[15]. The modification pattern includes both sense and antisense strand enhancements. On the sense strand, modifications have been added to impede strand entry into RISC, thus eliminating off-target effects by this strand. To address off-targets generated by the antisense strand, key nucleotides that are essential for off-targeting were identified. Modification of these positions eliminates the majority of antisense strand off-target effects while preserving on-target knockdown (Figure 6). The combination of both sense and antisense strand modifications has recently been validated[15].

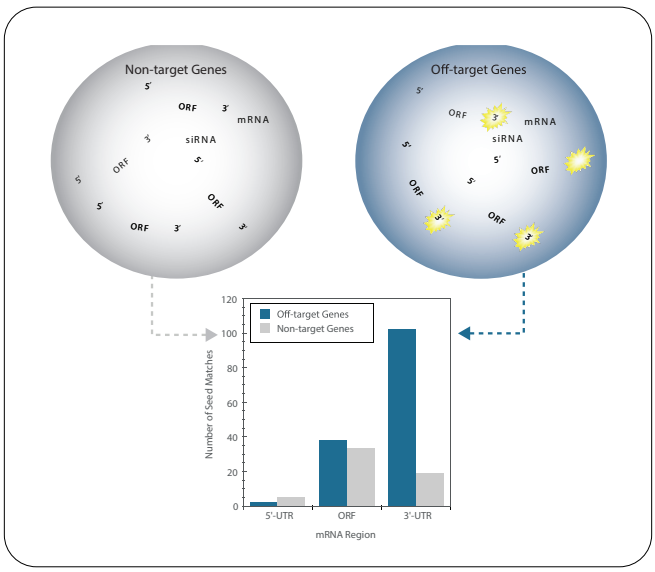


Figure 7. Exact complementarity between the siRNA seed region and the 3′-UTR (but not 5′-UTR or ORF) distinguishes off-targeted from non-targeted genes. A search for complementarity between the siRNA antisense seed region (positions 2-7) and 5′-UTRs, ORFs, and 3′-UTRs of off-targeted (green) and non-silenced (blue) genes was performed. The number of seed matches in the 3′-UTR region is almost five-fold greater in the off-targeted genes than the non-silenced genes.

Bioinformatics

The last approach toward eliminating off-target effects is associated with siRNA design. In the studies by both Birmingham[14] and Lin[3], the investigators observed that off-targeted genes often contained matches between the seed region of the siRNA (positions 2-7) and sequences in the 3′-UTR of the off-targeted gene. Furthermore, Birmingham found evidence that the likelihood of a gene being off-targeted is elevated by the presence of multiple 3′-UTR seed matches (Figure 7). microRNAs also utilize the seed region to mediate gene knockdown, thus these findings intimate a strong mechanistic parallel between siRNA off-targeting and microRNA-mediated gene regulation. The role of the 3′-UTR seed match can be taken into consideration during siRNA design, allowing users to minimize off-target effects.

These three principles have been incorporated into ON-TARGET*plus* siRNA reagents, part of the pre-designed genome-wide collection of siRNA reagents. The ON-TARGET*plus* product line is comprised of four individual four siRNAs, and SMARTpool reagents which are chemically modified and rationally designed to minimize off-target effects. ON-TARGET*plus* siRNA reagents represent the most advanced RNAi silencing products available and promise to provide users with potent, highly specific gene knockdown.

Conclusions

For RNAi to become a more rigorous technology, issues pertaining to functionality, specificity, and delivery must be addressed. Unintended gene modulation generated by off-target effects represents a prominent roadblock to RNAi technologies in research, therapeutic, and diagnostic settings. As described above, pooling strategies, chemical modifications, and novel rational design filters can significantly reduce off-targets without jeopardizing on-target gene knockdown. Implementation of these approaches, available with ON-TARGET*plus* siRNA reagents, will greatly reduce the presence of off-target phenotypes and enhance researcher confidence in the specificity of RNAi.

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