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Technote 1

siRNA Off-Target Effects: Causes, Extent & Impact

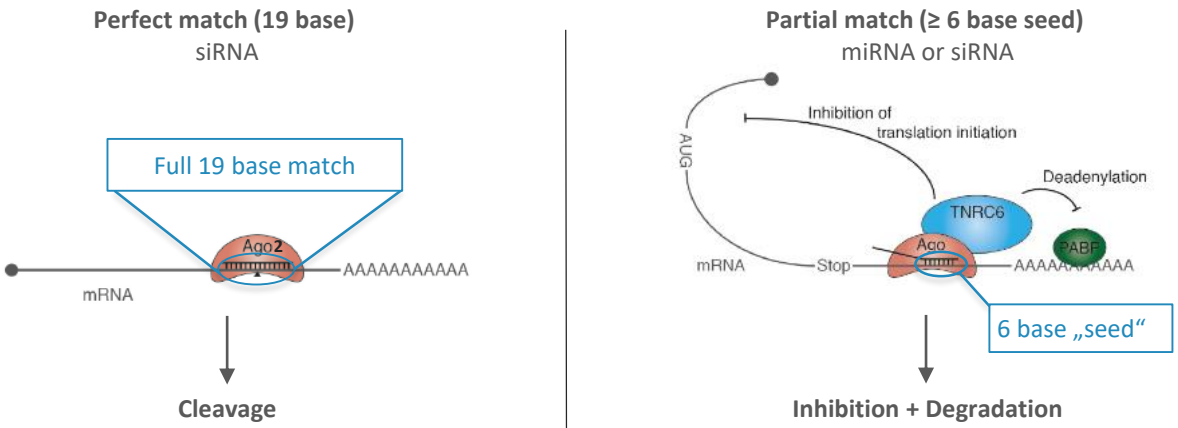
This technote addresses the following questions about siRNA off-target effects:

1. How do they occur?
2. How extensive are they?
3. How do they impact experimental results?

1. How do siRNA off-target effects occur?

a) miRNA-based transcript downregulation

Off-target effects of siRNAs largely arise from **siRNAs behaving like microRNAs (miRNAs)**, downregulating multiple genes with sequence complementarity to the **siRNA seed region** (see Fig. 1).



siRNAs require a **full 19-base match** of the guide strand against the targeted RNA transcript for Agonaute protein 2 (Ago2) to mediate **cleavage** and downregulation of the transcript.

miRNAs require only a **6 base match** between their seed sequence (position 2-7 at 5' end of guide strand) and RNA transcripts. Sites of recognition are usually localized to the 3' untranslated regions (UTR) of transcripts (Birmingham et al., 2006). Transcript downregulation occurs via **translation inhibition** or **deadenylation-induced decay**.

Figure 1. Mechanism of miRNA-based transcript downregulation that contribute to siRNA off-targeting

Large-scale RNAi screens have found that **siRNA seed-based matches played a far greater role** than designed on-target 19-base matches **on influencing observed phenotypes** (Marine et al., 2012, see section 2).

Due to the short sequence complementarity required for miRNA-like siRNA off-targets, designing siRNAs with minimal off-targets is especially challenging. It is also **largely impossible to predict the off-target profile** of siRNAs as few seed-based matches lead to a downregulation of the transcript.

b) Innate immune response

Mammalian systems have evolved a potent antiviral immune response against long double-stranded RNA. This involves the stimulation of interferons and inflammatory cytokines that dramatically alter gene expression and impact various important cellular processes.

siRNAs, notably of lengths > 23 base pairs, trigger potent immune responses that give rise to off-target effects and interfere with functional readouts (Reynolds et al., 2006). Certain siRNA sequence motifs, structures, delivery vehicles and impurities in siRNA preparations may also stimulate immune responses. Immune-mediated effects are sensed by endosomal toll-like receptors (TLRs) or cytoplasmic receptors such as RIG-I and PKR and responses vary between cell types. For a detailed review, refer to Judge and Maclachlan, 2008.

c) Saturation of endogenous RNAi machinery

As siRNA-mediated effects rely on endogenous RNAi machinery, overloading the cell with siRNAs would occupy RNAi effector proteins that miRNAs require for gene expression regulation. A study reported the upregulation of endogenous miRNA targets in a dose-dependent manner corresponding to amount of siRNA used, reinstating the importance of keeping siRNA concentrations low (Khan et al., 2009).

2. How extensive are siRNA off-target effects?

a) siRNAs downregulate as many genes as miRNAs with significant seed enrichment

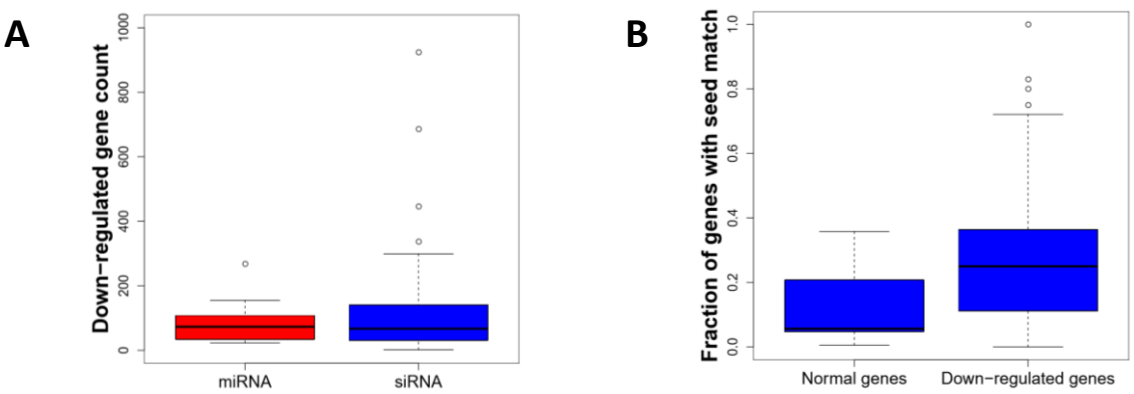


Figure 2. siRNAs downregulate as many genes as miRNAs with significant seed enrichment

To examine the extent of siRNA off-target effects, we looked at overall gene downregulation from 173 published microarray data sets using 162 siRNAs and 11 miRNAs (Garcia et al., 2011). Fig. 2A shows that **siRNAs can downregulate a similar number of genes as miRNAs** (median: ~100 genes). The siRNAs also showed a wider range of activity, with some affecting few genes and others up to nearly a thousand genes.

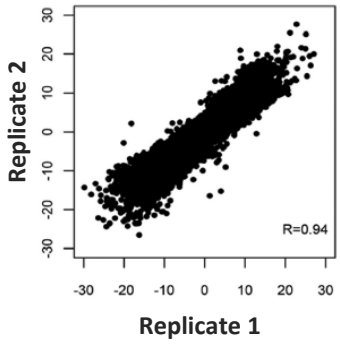
To determine the fraction of downregulated genes that stem from miRNA-based off-target effects, seed enrichment was examined for unchanged (normal) and downregulated genes. In Fig. 2B, **nearly a third of downregulated genes had siRNA seed matches**. For 4 of these siRNAs, around 80% or more genes had seed matches, indicating a high off-target frequency for these siRNAs. Several expression analysis studies reported similar results (Jackson et al., 2006; Caffrey et al., 2011).

b) Seed-based off-target effects dominate RNAi screens

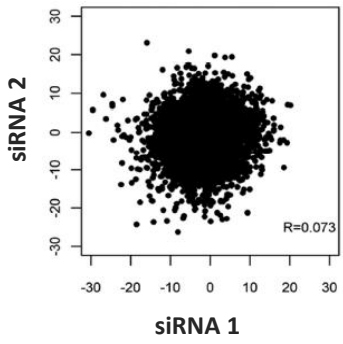
Another method of investigating the penetrance of siRNA off-targets is to compare the correlation between phenotypes obtained by siRNAs targeting the same gene. The assumption being, two siRNAs specific towards a target gene should produce similar phenotypes.

A genome-scale RNAi screen (Marine et al., 2012) performed with siRNAs or low complexity pools of 3-4 siRNAs targeting 6564 and 3086 genes respectively was used to identify genes involved in the Wnt/β-catenin pathway. HT1080 cells were used at siRNA concentrations of 28 nM. The phenotypic readout was luminescence emitted from a luciferase reporter linked to the β-catenin promoter. The plots in fig. 3 depict the **correlation or similarity between two phenotypic read-outs** between **two test conditions**. An R value close to 1 indicates high correlation or similarity in readouts while a value close to 0 indicates little to no correlation.

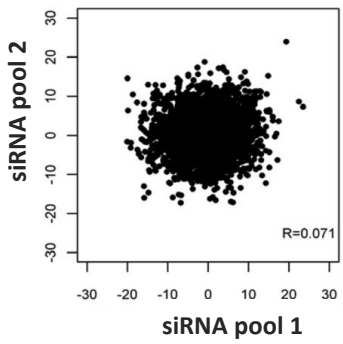
Technical replicates



Single siRNAs



Pools of 3-4 siRNAs



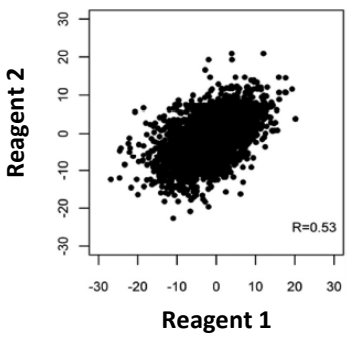
High correlation (R=0.94) between technical replicates indicates the same siRNA reagent performs reproducibly:

Good assay reproducibility

Poor correlation (R=0.07) between siRNA reagents (single siRNAs or pools of 3-4 siRNAs) that target the same gene indicates two siRNA reagents, despite targeting the same gene, do not produce similar phenotypes:

Poor siRNA specificity leads to varying phenotypes

Same seed siRNAs



As off-target effects stem from the **seed sequence** of siRNAs *(see Section 1),* correlation between siRNAs having the same seed sequence but designed to target different genes was analysed.

Good correlation (R=0.53) between siRNAs with the same seed sequence, but targeting different genes, indicates seed sequence has a greater influence on siRNA activity than designed on-target effect:

Seed-based off-target effects dominate siRNA-induced phenotypes

Figure 3. miRNA-based siRNA off-target effects exceed designed on-target effect in determining phenotypes produced by single siRNAs/low complexity siRNA pools

3. How do siRNA off-targets impact experimental results?

Off-target effects of siRNAs hinder RNAi data interpretation by producing:

a) False positives – a positive phenotype from an siRNA due to its off-target effect as opposed to on-target gene knockdown.

Example: Lin et al. (2005) performed a kinase-targeted screen for players in the cancer-relevant HIF1α pathway and found that **all three top hits were due to off-target effects**. Two of the top three hits downregulated HIF1α mRNA through miRNA-based seed effects.

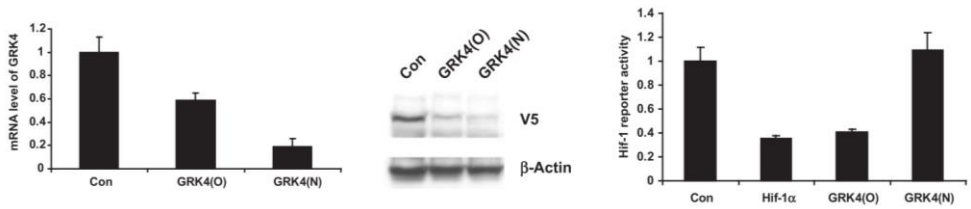


Figure 4. Secondary validation with alternative siRNA sequence for top RNAi screening hit reveals false positive

The figure above shows secondary validation in H1299 cells of a newly designed siRNA, GRK4(N), against a top hit. This siRNA, given at 20 nM, produced more efficient on-target gene knockdown (left panel: mRNA; middle panel: protein immunoblot) but failed to reproduce the same phenotype of lowered HIF1α activity compared to the original siRNA, GRK4(O) (right panel: luciferase reporter assay). The positive results from the original siRNA was therefore due to an off-target effect.

b) False negatives – failure for relevant siRNAs to produce a significant phenotype due to noise caused by off-targeting.

Example: Sigoillit et al., (2012) performed a screen for spindle assembly checkpoint regulators and **failed to identify any new genes**. This was despite identifying known regulators (i.e. positive controls were working).

The was mainly because all 34 active hits identified also strongly decreased expression of known spindle assembly checkpoint gene, MAD2. Half the active siRNAs showed a 7-mer seed sequence match to the 3' UTR of MAD2 (Fig. 5). Seven siRNAs tested confirmed off-targeting due to miRNA-based seed effects. Out of the 324 siRNAs that inactivated the spindle assembly checkpoint, 65% contained a 7-mer seed sequence match the MAD2 3' UTR. This large off-target effect severely impacted the ability of the screen to identify relevant targets.

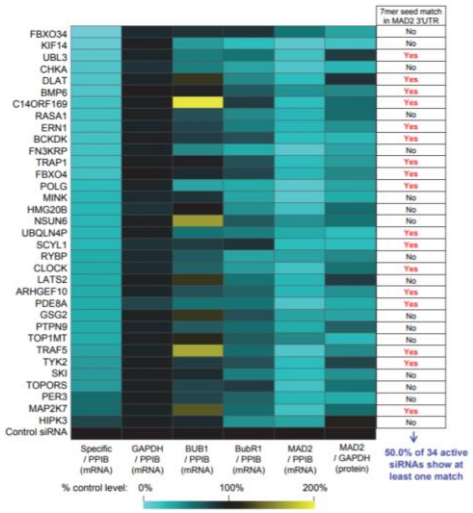


Figure 5. All active siRNAs identified downregulated off-target gene, MAD2

c) Unexpected toxicity: siRNA produces cell toxicity either through general activation of the innate immune response or by affecting off-target genes with a role in cell viability

Example: Federov et al. (2006) reported sequence-dependent cell toxicity induced by siRNAs. Out of 175 siRNAs tested, 51 siRNAs (29%) significantly reduced cell viability. This effect was target-independent as 4 functional siRNAs against the same gene did not produce the same decrease in cell viability for 12 genes tested (Fig. 5).

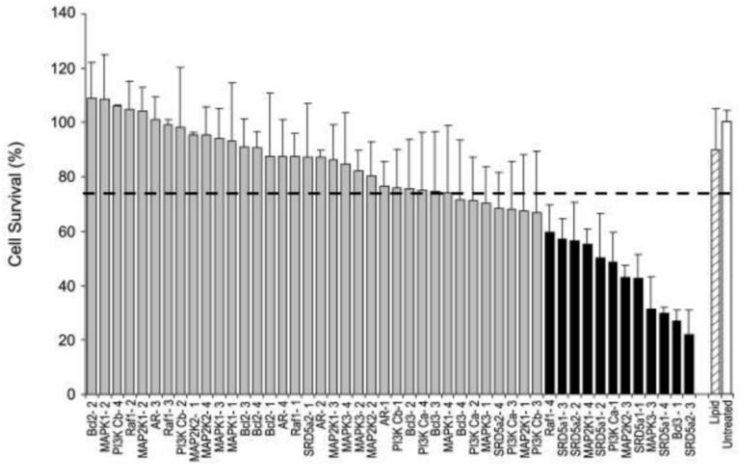


Figure 5. Cell toxicity induced by siRNAs are target-independent
HeLa cells were transfected with 48 different functional siRNAs against 12 genes (4 siRNAs/gene) at 10 nM and cell toxicity measured 72 h after. Data is sorted by cell toxicity which is the mean of 3 independent experiments. Dashed line indicates 75% cell viability, below which siRNAs are considered toxic.

Interestingly, for 4 siRNAs against a gene where 1 siRNA was toxic, pooling and administering all 4 siRNAs reduced toxicity while maintaining on-target knockdown. This was seen for 2 genes – MAP2K1 and MAP2K2 (Fig. 6). Another method that effectively reduced toxicity was the introduction of 2'-O-methyl modifications at the seed sequence region.

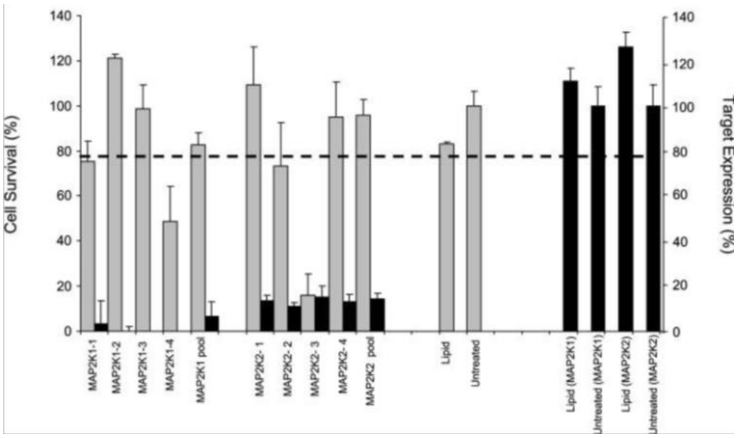


Figure 6. Pooling of siRNAs reduced toxicity while maintaining on-target effect Cell survival shown as grey bars and target expression shown as black bars.

Summary

What causes siRNA off-targeting? The ability of siRNAs to use miRNA-based mechanisms for transcript regulation largely contributes to siRNA off-targeting. Triggering of the innate immune system and saturation of the endogenous RNAi machinery by siRNAs also leads to off-targets.

How extensive are siRNA off-targets? The numerous reports of wide-spread gene deregulation by siRNAs AND the poor performance correlation between different siRNA reagents targeting the same gene demonstrate a high penetrance of siRNA off-targets.

What are the consequences of siRNA off-targets? False positives and false negatives have been reported in numerous RNAi studies. These account for the poor overlap between large RNAi screens designed to identify the same genes (Hasson et al., 2013). Unexpected cell toxicity also compromises phenotypic measurements and produces false positives in screens assaying cell viability.

As a result of the high propensity for siRNA off-targets, scientists often spend **extensive time and resources in siRNA validation efforts.** These include obtaining phenotypes with multiple sequence-independent siRNA reagents, in multiple cell lines, or by carrying out rescue experiments and alternative methods of gene disruption which can be a **frustrating and sometimes fruitless experience**.

siPOOLs™ from siTOOLs Biotech seeks to reduce the workload of the scientist by producing gene knockdown with a cleaner off-target profile that yields more robust and reliable results than current commercial siRNA reagents.

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