The broad post-transcriptional gene regulation induced by RNA interference (RNAi) is mediated by microRNAs (miRNAs). These small, highly structured, non-coding RNAs undergo extensive processing in both the nucleus and cytoplasm before partnering with the RNA Induced Silencing Complex (RISC) to target the 3'UTR of messenger RNAs for degradation and/or translation attenuation.

Synthetic 19 base pair (bp) RNA duplexes (small interfering RNAs, siRNAs) can enter RISC directly and induce RNAi-mediated cleavage of targeted transcripts without additional processing. Though this ability has enormous potential, expansion of RNAi technology requires overcoming obstacles associated with siRNA functionality and specificity. Initial problems associated with inconsistent gene knockdown were found to be inherent in the sequence and thermodynamic properties of the siRNA and were addressed by development of algorithms that enabled the selection of highly functional sequences. In contrast, three separate attributes appear to contribute to overall siRNA specificity, thus making precise knockdown a surprisingly onerous problem.

Two of the features that contribute to a lack of siRNA specificity were found to be non-specific in nature. As demonstrated by Fedorov et al (Fedorov et al, 2005), cationic lipids typically used to deliver siRNA can induce broad changes in gene expression profiles. Separately, multiple labs have observed that particular sequence motifs as well as dsRNA lengths can induce broad, non-specific changes in gene expression that have origins in the induction of the interferon response pathway (Hornung et al, 2005; Judge et al, 2005). While these unanticipated effects can be eliminated by adopting stringent siRNA design filters and optimizing lipid concentrations and/or compositions, the most recent challenge, siRNA-mediated off-target effects, has required a more thorough understanding of the mechanism underlying RNAi.

The basic attributes of siRNA off-target effects

Off-target effects were first characterized in detail by Jackson and co-workers in 2003 (Jackson et al, 2003). Using microarray profiling as a method of detection, the authors identified modest, 1.5- to 3-fold changes in the expression of dozens to hundreds 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 profile was unique for each siRNA, suggesting a sequence specific phenomenon.

Initially, the modest changes in expression of off-targeted genes led many to dismiss the event as inconsequential. Unfortunately, this optimism was dispelled by a growing number of reports that demonstrated off-target effects could induce measurable phenotypes. In a broad RNAi-based phenotypic screen designed to identify kinase regulators of HIF-1, Lin and co-workers discovered that the phenotypic effects induced by several of their top candidates were the consequence of HIF-1 off-targeting events (Lin et al, 2005). Similarly, Fedorov demonstrated that a surprising fraction of siRNAs targeting the housekeeping genes cyclophilin B and DBI induced a target-independent, sequence-dependent, cell viability (toxic) phenotype that was contingent upon an intact RNAi pathway (Fedorov et al, 2006). As these findings have noteworthy implications for both research and therapeutic applications of RNAi, investigations have taken place to gain a more complete understanding the mechanism underlying off-target effects.
NEW APPROACHES TO MINIMIZING OFF-TARGET INTERACTIONS

Early studies in RNAi established that single base pair mismatches between the siRNA and the target dramatically alter functionality. From this it was inferred that overall sequence identity played a role in siRNA specificity, thus leading to the adoption of local alignment algorithms (e.g., BLAST and Smith-Waterman) as a method to minimize off-target effects. Birmingham and co-workers recently challenged this premise by comparing a collection of in silico predicted off-targets with a library of validated off-targets identified by microarray gene profiling (Birmingham et al, 2006). Using the Smith-Waterman algorithm, the authors demonstrated that with the exception of cases of near-perfect identity, identity-based algorithms failed to accurately recognize off-targets. In general, the number of in silico predicted off-targets exceeded the true number by 1-2 orders of magnitude with only a small fraction of the experimentally validated off-targets being identified by in silico methods. This article as well as the work of others (Lim et al, 2005; Jackson et al, 2006), has implied that siRNA off-targeting is most likely similar in mechanism to miRNAs and uses different principle for target recognition.

While the failings of identity-based algorithms originally left RNAi users without a viable alternative for reducing off-target effects recent advances in siRNA chemistry and bioinformatics have identified promising approaches for reducing off-targets and unintended gene modulation. The first strategy originates from the observation that off-target effects are concentration dependent. Multiple laboratories have now demonstrated that off-target effects fade as the concentration of the siRNA is reduced. Unfortunately, attempts to employ this approach on a broad scale have failed due to the fact that in most cases, the concentration at which off-target effects decline is comparable to that at which on-target gene knockdown diminishes. Fortunately, this impasse is resolve by adopting the very simple strategy known as “pooling”. Studies have shown that on-target gene knockdown can be achieved with minimal off-target effects if a pool consisting of multiple duplexes is utilized. While individual duplexes can induce sizeable numbers of off-targets, transfection of a pool of siRNAs induces only a fraction of the off-target signature. Most importantly, because all duplexes that make up the pool target the same gene, this approach of eliminating off-targets is achieved without jeopardizing target specific knockdown.

The second approach to minimizing off-target effects involves recent advancements in the field of siRNA chemistry. As reported by Jackson et al (Jackson et al, 2006), a chemical modification pattern has recently been identified that eliminates as much as 80% of the off-target effects. This newly discovered chemistry includes differential addition of 2’-O-methyl moieties to both the sense and antisense strands. On the sense strand, these modifications prevent 5’-phosphorylation, which has been shown to be necessary for RISC entry. On the antisense strand, key nucleotides that are essential for off-targeting, but are less crucial for on-target gene knockdown are modified. Most importantly, the afore-mentioned work by Fedorov and co-workers demonstrated that addition of this modification pattern to toxic siRNA minimizes both the off-target signature (as measured by microarray analysis) and the associated cell viability phenotype.

Finally, the last approach toward eliminating off-target effects is associated with siRNA design. Studies by Birmingham (Birmingham et al, 2006), Lin (Lin et al, 2005) and Jackson (Jackson et al, 2006) revealed that off-targeted genes frequently contained matches between the seed region of the siRNA (positions 2-7) and sequences in the 3’ UTR of the off-targeted gene. As the likelihood of a gene being off-targeted is elevated by the presence of multiple 3’UTR seed matches, these findings intimate a strong mechanistic parallel between siRNA off-targeting and microRNA-mediated gene regulation. Taking these and additional principles into account, Anderson and co-workers have recently developed and validated an in silico method for selecting siRNA that induce low numbers of off-targets (Anderson et al, personal communication), thus promising a new generation of siRNA that provide both potent and specific gene knockdown.

CONCLUSIONS

Unintended gene modulation generated by off-target effects represents a major obstacle to exploitation of RNAi technologies in research, therapeutic, and diagnostic settings. Fortunately, new developments in siRNA bioinformatics, chemistry and experimental design can be used separately and in combination to significantly reduce off-targets without jeopardizing on-target gene knockdown. Implementation of these approaches in future studies offers a means to identify or stratify “hits” derived from screens and increases the inherent reliability of RNAi-based high throughput strategies.

ACKNOWLEDGEMENTS

The authors thank Queta Boese, Jon Karpilow and Anastasia Khvorova for critical reading of this article.

REFERENCES

Birmingham A, Anderson EM, Reynolds A et al. 2006. 3’ UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat Methods, 3, 199-204.
Fedorov Y, Anderson EM, Birmingham A et al. 2006. Off-target effects by siRNA can induce toxic phenotype. RNA, 12, 1-9.
Fedorov Y, King A, Anderson E, Karpilow J, Ilsley D, Marshall W and Khvorova A. 2005. Different delivery methods-different expression profiles. Nat Methods, 2, 241.
Hornung V, Guenthner-Biller M, Bourquin C et al. 2005. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med, 11, 263-270.
Jackson AL, Bartz SR, Schelter J et al. 2003. Expression profiling reveals off-target gene regulation by RNAi. Nature Biotechnol, 21, 635-637.

Jackson AL, Burchard J, Leake D et al. 2006. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA, 12, 1197-1205.

Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol, 23, 457-462.

Lin X, Ruan X, Anderson MG et al. 2005. siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. Nucleic Acids Res, 33, 4527-4535.

SHORT COPYRIGHT STATEMENT

This is an open access article, published under the terms of the Licence for Users available at http://www.libpubmedia.co.uk/RNAiJ/LicenceForUsers.pdf. This licence permits non-commercial use, distribution and reproduction of the article, provided the original work is appropriately acknowledged with correct citation details.