RNA interference and DsiRNAs

Yong You
MS, MBA, Associate Product Manager
Integrated DNA Technologies
Starting with a few nomenclatures...

- **dsRNA**
  - Double-stranded RNA (dsRNA) is RNA with two complementary strands
  - dsRNA forms the genetic material of some viruses (double-stranded RNA viruses)
  - Double-stranded RNA such as viral RNA or siRNA can trigger RNA interference in eukaryotes, as well as interferon response in vertebrates

![Struct of Reovirus Core Determined Via X-ray Crystallography](image)
A few nomenclatures

• **NcRNAs - Non coding RNAs**
  - RNA molecules that are not translated into a protein product
  - Includes transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear and small nucleolar RNA, also microRNA and small interfering RNA (siRNA)

• **RNAi - RNA interference**
  - Can refer to the system in living cells that controls gene expression, or the technology that using synthetic RNA to regulate gene expression
  - miRNA and siRNA are central to RNA interference. Major difference between endogenous siRNA and miRNA: precursor of endogenous siRNA is a long dsRNA while the precursor of a miRNA is hairpin-shaped

• **DsiRNA - Dicer-substrate siRNAs**
  - Chemically synthesized 27mer duplex RNA that have increased potency in RNA interference compared to traditional 21mer siRNAs.
  - Developed between IDT and Dr. John Rossi at the Beckman Research Institute at the City of Hope
miRNAs

• MicroRNAs (miRNAs) are naturally occurring short RNA molecules of about 22 nucleotide (nt) long, that regulate gene expression by binding to target mRNA and suppressing its translation or initiating its degradation

• About 1000 human miRNAs discovered, and these have been associated with the regulation of approximately 60 per cent of all human genes.
  • First discovered miRNA: lin-4 small RNA in 1993

• Role in cancer development, cancer characterization and potential cancer therapy

• High-throughput sequencing of small RNAs provides great potential for the identification of novel small RNAs, as well as profiling of known small RNA genes
siRNAs

- Small interfering RNA (siRNA) is a class of double-stranded RNA molecules, 20-25 nucleotides in length, that play a variety of roles in biology

- siRNAs act in the RNA interference (RNAi) pathway, where they interfere with the expression of a specific gene

- In essence, any gene whose sequence is known can be targeted based on sequence complementarity with an appropriately tailored siRNA. This has made siRNAs an important tool for gene function and drug target validation studies in the post-genomic era

- Challenges: avoiding nonspecific effects
<table>
<thead>
<tr>
<th>Similarities</th>
<th>miRNA</th>
<th>siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length and characteristics</td>
<td>~20-25 nt, 5’ phosphate group, 3’ -OH</td>
<td>Both are substrates of Dicer</td>
</tr>
<tr>
<td>Dicer Enzyme</td>
<td>Both are substrates of Dicer</td>
<td>Both need Argonaute protein family</td>
</tr>
<tr>
<td>Argonaute protein family</td>
<td>Both are RISC component, functional boundaries are not clear, might be overlap in the silencing pathway, thus produce on-target and off target effects</td>
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</tr>
<tr>
<td>Functions</td>
<td>Both can block gene expression and mRNA degradation, both work at post-transcription level</td>
<td>Both need Argonaute protein family</td>
</tr>
<tr>
<td>Differences</td>
<td>miRNA</td>
<td>siRNA</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Mechanism</strong></td>
<td>Normal regulatory mechanism, made to fine tune RNA metabolism endogenously</td>
<td>commonly a response to foreign RNA (usually viral), abnormal</td>
</tr>
<tr>
<td><strong>Direct source</strong></td>
<td>Hairpin pre-miRNA</td>
<td>Long strand dsRNA</td>
</tr>
<tr>
<td><strong>Molecular Structure</strong></td>
<td>single strand RNA</td>
<td>dsRNA, have 3’ 2 base overhang</td>
</tr>
<tr>
<td><strong>Action pathway</strong></td>
<td>miRNA pathway</td>
<td>RNAi pathway</td>
</tr>
<tr>
<td><strong>Origination</strong></td>
<td>pri-miRNA-pre-miRNAs-miRNA; In the nucleus and cytoplasm</td>
<td>dsRNA processed by Dicer; Occurring in Cytoplasm</td>
</tr>
<tr>
<td><strong>Complementarity to target</strong></td>
<td>Not 100%, can have mismatches</td>
<td>Almost always 100% complementary</td>
</tr>
<tr>
<td><strong>RISCs</strong></td>
<td>miRISCs/miRNP</td>
<td>siRISCs</td>
</tr>
<tr>
<td><strong>Biological functions</strong></td>
<td>Regulation of development</td>
<td>• Virus defense</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Silence over expressed mRNA</td>
</tr>
<tr>
<td><strong>Location of action</strong></td>
<td>Majorities are on target gene 3’ UTR</td>
<td>Anywhere on mRNA</td>
</tr>
</tbody>
</table>
Both siRNA and miRNA go through RISC (RNA Induced Silencing Complex)
Knock out the RNA

- no protein, what was its function?
Strategies for targeting RNA

ss oligos w/modified bases (Antisense) vs dsRNA (RNAi technology)

- DsiRNA (IDT)
- 21mer (Traditional)
siRNA and Antisense oligos enter 2 different pathways

siRNA/ dsRNA

Antisense (ss oligos/modified bases)

Gene silencing
Antisense and RNAi Comparison

- **Similarity:**
  - Specific
  - Directional

- **Differences:**
  - **Antisense**
    - Single stranded oligo
    - Need modifications
    - Need RNase H functions
  - **RNA interference**
    - Double stranded RNA
    - Need Dicer, RISC functions
Antisense - Mechanisms of Action (using ss oligos)

1) Recycled mRNA degradation (10-100 fold more potent)

2) RNA + AS oligo

RNase H

Steric blockade

mRNA degradation

Recycled
Antisense - Single Strand Oligos

- If unmodified
  - Oligonucleotides can be rapidly degraded by nucleases in tissue culture and in plasma

- If modified
  - Most modifications that block nuclease degradation also interfere with RNase H activity

- SS oligos need to be modified to be
  a) RNase H active
  b) but not degraded by other nucleases
Antisense design: Modifications offered by IDT

RNase H Inactive
- 2’-modified bases
  - 2’-OMethyl RNA
  - 2’-fluoro C, U, A, G

RNase H Active
- Unmodified
- DNA Phosphorothioate

5’-3’ Phosphodiester linkage

5’-3’ Phosphorothioate linkage
Phosphorothioated oligos used in Antisense studies, cytogenetic studies

- Increased resistance to exonuclease, protects from degradation
2’ O-methyl RNA in antisense and *in vivo* siRNA applications

- It is resistant to single-stranded ribonucleases and is typically 5 to 10-fold less susceptible to DNases than a regular DNA.

- It is commonly used in antisense oligos as a means to increase stability and binding affinity to the target mRNA.

- Also used in modified siRNA duplexes as it helps in evading an immune response.
Antisense design: Ideal Design is a mix of mods

- [http://www.idtdna.com/Scitools/Applications/AntiSense/Antisense.aspx](http://www.idtdna.com/Scitools/Applications/AntiSense/Antisense.aspx)

- **Allows use of modification**
  - Increased nuclease resistance (esp. 3’-end)
  - Increased Tm
- **Keeps RNase H activity**

![Diagram of antisense design](image.png)
RNA interference

RNAi (RNA interference) is a form of post-transcriptional gene silencing in which double-stranded RNA induces degradation of the homologous endogenous transcripts, mimicking the effect of the reduction, or loss, of gene activity.

2 types of small non coding RNA molecules are central to RNAi
- microRNA (miRNA)
- Small interfering RNA (siRNA)
RNA interference

Brief History:

1990: Introducing additional copies of a flower pigmentation gene into pink petunia, instead of getting darker flowers, obtained almost white flowers, indicating that the activity of gene had been decreased.

1998: Craig C. Mello and Andrew Fire’s 1998 Nature paper reported a potent gene silencing effect after injecting double stranded RNA into C. elegans. The term RNAi was coined.

2006: Drs. Fire & Mello shared the Nobel Prize in Physiology or Medicine
**RNAi: Mechanism**

**Mechanism of RNAi**
- dsRNA in the nucleus
- Cytoplasm

**Endogenous processing by Dicer**
- Dicer: an endoribonuclease which cleaves dsRNA into siRNA

**Dicer**
- Long dsRNA
- Dicer (RNase III)
- 27mer dsRNA
- siRNAs
- 21mer

**RISC assembles (RNA-induced silencing complex)**
RISC assembly

Sense strand is first degraded in RISC

RISC finds complementary sequence in mRNA

Target mRNA

Cuts up target RNA

3’ degraded mRNA
RNAi animation – Nature Reviews
DsiRNA - Collaborative Project

John Rossi
Dongho Kim
Mohammed Amarzguioui
Beckman Institute, City of Hope
Duarte, CA

Mark Behlke
Scott Rose
Integrated DNA Technologies
Coralville, IA
More recently, studies relating to the in vivo use of DsiRNA have been pursued with the laboratory of Prof. Edouard Cantin, also at the Beckman Research Institute of the City of Hope National Medical Center. Studies into interaction of these RNAs with the innate immune system have been pursued with the laboratory of Prof. Bryan Williams, at the Lerner Research Institute of the Cleveland Clinic Foundation and the Monash Institute of Medical Research.

Dicer-substrate Publications

For additional information and/or examples of use of these methods, see also:

RNAi: IDT approach

What if you use duplexes that mimic the substrate that enters Dicer?

- Mimic the natural siRNAs that enters Dicer

- Traditional method is using 21-mer RNA duplexes
RNAi: IDT

DsiRNA duplexes are as much as 10x more effective than traditional 21mer siRNAs.

Short synthetic RNA duplexes in the 25-30 bp range are substrates for Dicer processing in mammalian cells.
RNAi: Features of DsiRNA design for maximum potency

What is the optimal length
27 mer

Blunt end or overhang?
2 base 3’-overhang on the AS strand and blunt on the other end;
The blunt end is modified with 2 DNA bases on the 3’ end for sense strand
Since more antisense strand is loaded, get more degradation of sense mRNA

Increased potency than 21mer
Specific effects at lower concentration (IDT recommends DsiRNA at 10nM)
Non specific effects at higher conc. (Dharmacon recommends their siRNAs at 100nM)

Ability to use DsiRNA at lower concentrations will minimize non specific effects
Chemical modifications in siRNA

- Not required for siRNA function
- For 21-mer siRNAs, if a fluorescent RNA is desired, IDT recommends use of 5’ TYE™563 or Cy3™.
- For DsiRNAs, IDT recommends the 3'-end of the sense strand for routine modification needs.
- Incorporation of 2'-O-methyl RNA residues can prevent activation of IFN responses and should be considered for all *in vivo* applications. IDT has developed modified DsiRNAs that evade immune detection and have improved nuclease stability in serum.
RNAi: IDT DsiRNA “TriFECTa” kit

- Predesigned DsiRNAs against RefSeq Database
- Designed to target *only* common exons
- Designed to avoid known SNPs

What does the kit contain?
- 3 Target-specific Dicer-substrate duplexes
  - Not pooled
  - With Sequence information
- TYE 563™-labeled transfection control duplex
- HPRT positive control duplex
- Negative control duplex
- Guarantee
RNAi: Predesigned DsiRNAs Design Criteria

- Human, mouse, rat, cow, dog, chicken, and chimp RefSeq transcripts
- Site selection using a proprietary algorithm with novel Dicer-substrate specific design rules.
- Sequences are screened to minimize the potential for cross-hybridization and off-target effects
- Sites are also eliminated that include known SNPs and alternatively spliced exons (if any are present for that gene in RefSeq).
- Areas of high predicted secondary structure are avoided.
Design Tool
RNAi: Screening DsiRNA at IDT

- Ideal for small scale *in vitro* applications
- Sequences must be between 24 and 30 bases and be 100% complementary with up to a 3-base overhang
- Supplied as 2n mole, 10n mole, 40 n mole scales
- Large-Scale DsiRNAs
  - RNA duplexes are available up to 10 grams.
  - All duplexes are purified using RNase-free HPLC methods
  - Come with purity and ESI-MS QC documents
FAQs for DsiRNA
How many DsiRNAs do I need to try?

- A **minimum** of two **effective** DsiRNAs per gene target should be used (preferably more).

- They should be able to show similar results in effects with two different DsiRNAs for the same gene (so they may have to try more than 2 to find 2 that work well)
How do I assess knockdown?

- qPCR is the best way to do it (PrimeTime qPCR assays)
- At least two spatially distinct qPCR assays should be used.
- Some customers may still use northern blot (not the best option for quantification)
Transfection reagent

- What transfection reagent do I use?
  - Recommend trying a panel of transfection reagent
  - but we can’t guarantee transfection efficiency, it’s dependent on cell line

- Have you used this reagent successfully with this cell line and with RNA before?
  - Optimization with different transfection reagents has to be done for a particular cell line and RNA
  - (doesn’t matter if they have used that reagent with another cell line or RNA, it may not be optimum)
Positive control HPRT and TYE563 labeled control

- **HPRT control:**
  - Very potent DsiRNA, knockdown the mRNA and the protein is long-lived, so the cell is not affected
  - Data has to show 90% knockdown with HPRT + control
  - If you are not getting 90-95% knockdown with the positive control it means that you are probably not getting enough siRNA into cells
  - HAVE TO FIRST OPTIMIZE THE TRANSFECTION REAGENT AND CONC TO USE TO SEE AT LEAST 90% KNOCKDOWN WITH HPRT

- **TYE 563™ labeled oligo control**
  - Visual aid to see if DsiRNA is getting into cells, qualitative measure
  - qPCR is the quantitative measure
What is the best time/conc to assess DsiRNA knockdown?

- 24 - 48 hrs after transfection is the best, 72 hrs maybe, but not as good (we recommend collecting data at different time points)

- Concentration range – 10nM is the guarantee but some DsiRNAs/cell line maybe difficult

- Could try at least 50nM, try a combination of DsiRNAs to see if that makes a difference

- One reagent/concentration does not fit all, they have to try different combinations for a given cell type and given gene
RNAi: Advantages of IDT DsiRNA

- Increased potency
- Minimize nonspecific effects
- Predesigned for 8 species
- TriFecta kit - guarantee
- IDT provide mirCat miRNA cloning kit to detect unknown miRNA
- IDT does not provide miRNA design tool
- IDT accept customer designs for novel pre-miRNAs