RNA*i* Its potential applications

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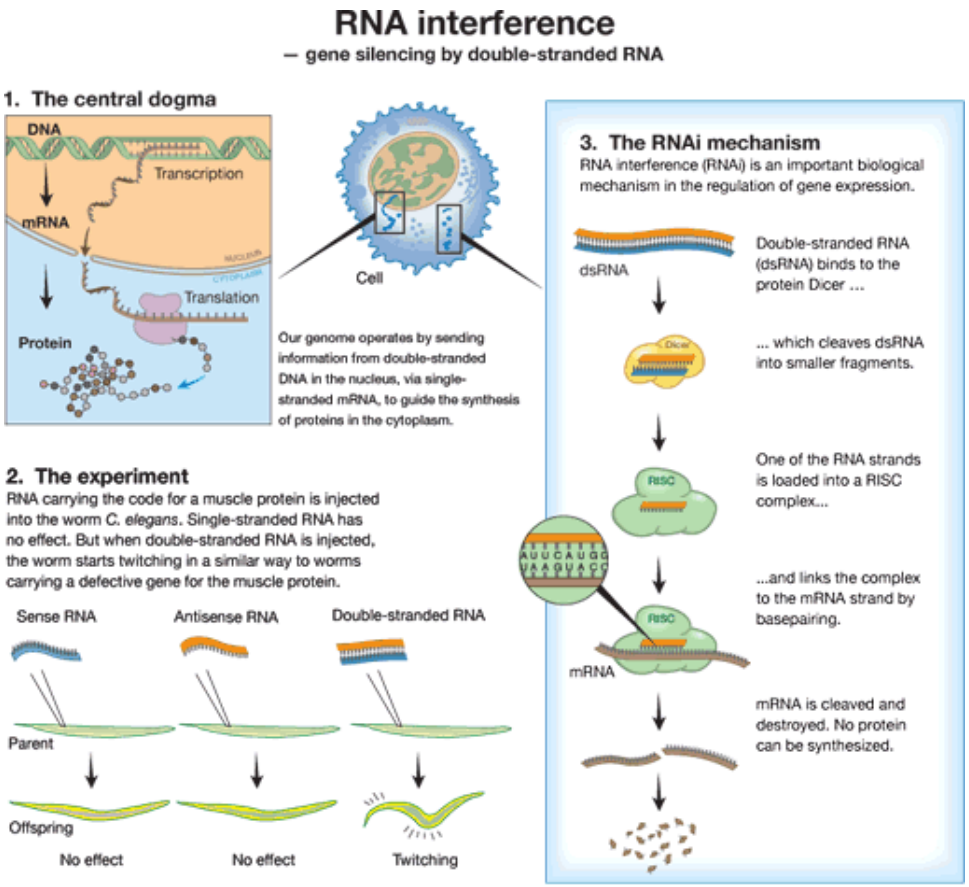
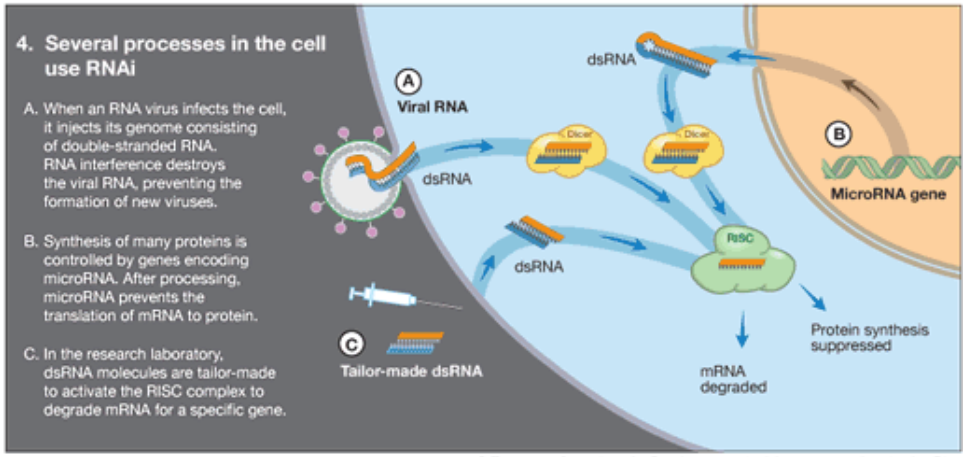
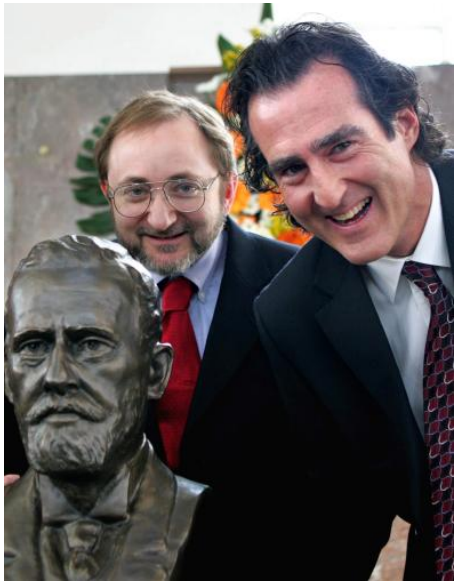
RNAi





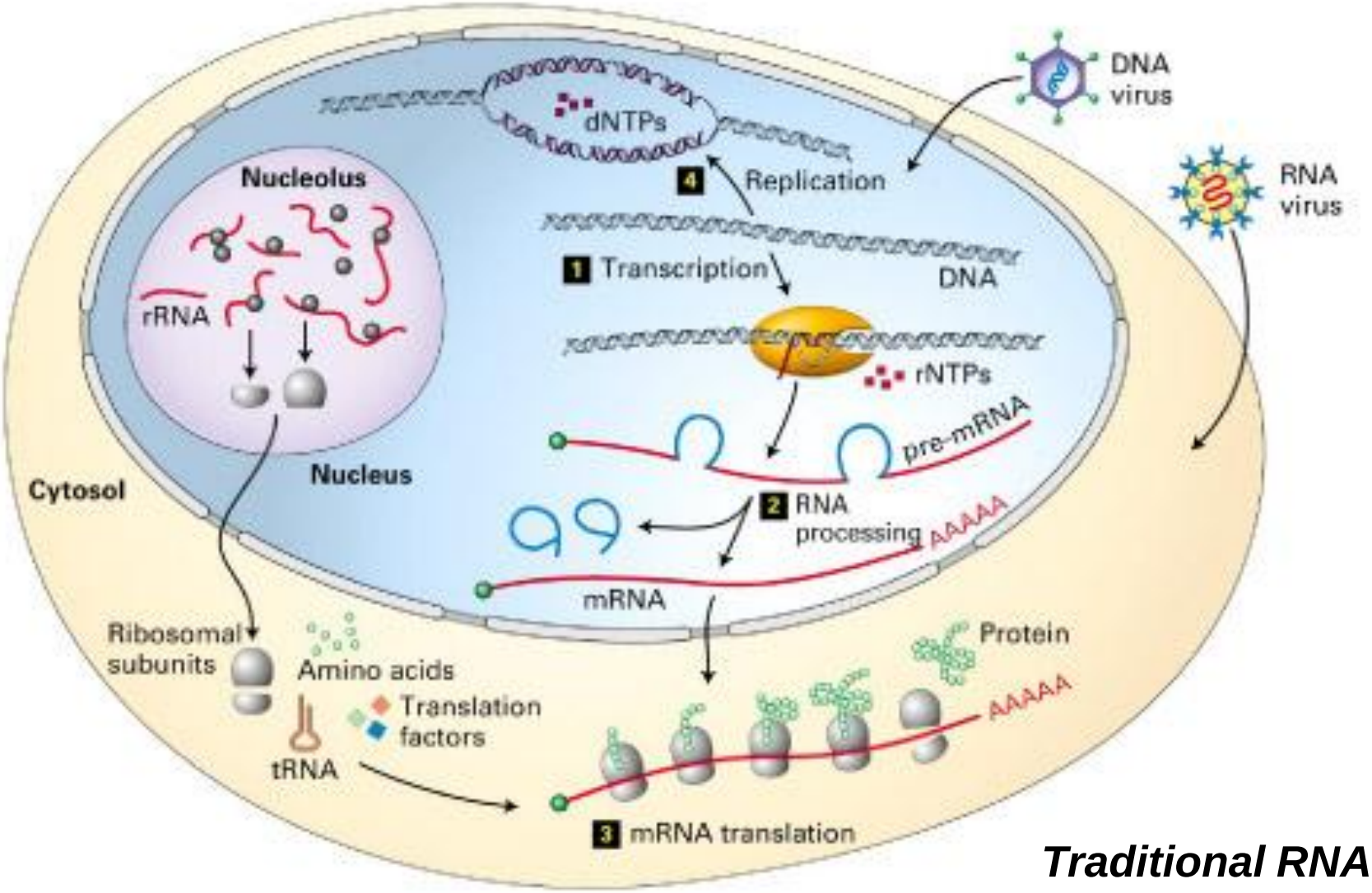


RNA Interference

-gene silencing by double-stranded RNA

Andrew Z Fire(47) & Craig C. Mello(46)

What is RNA?



Traditional RNAs

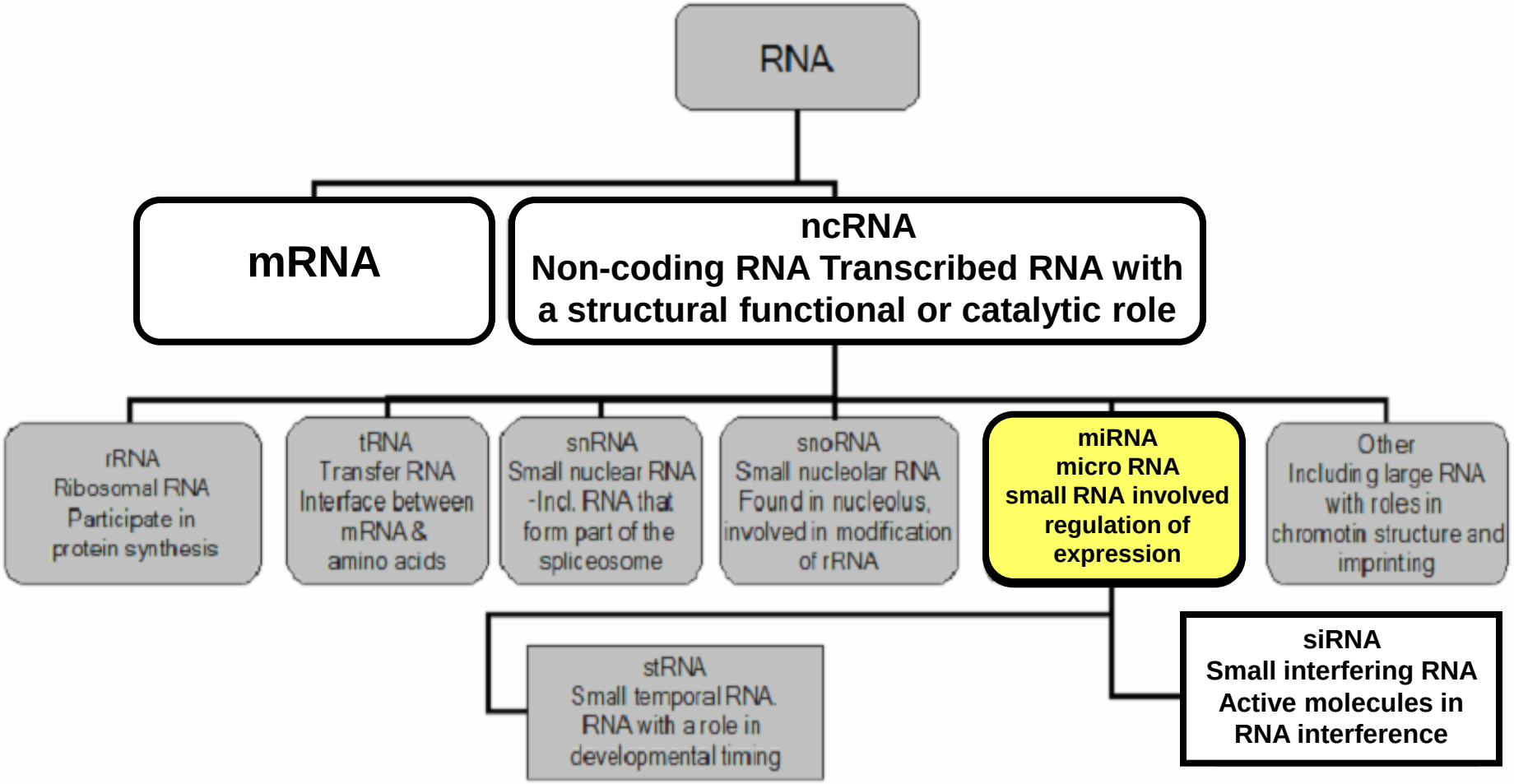
RNA

Coding: messenger RNA (mRNA)

Non-Coding:

- Ribosomal RNA (rRNA)
- Transfer RNA (tRNA)
- Small nuclear RNA (snRNA)
- Small nucleolar RNA (snoRNA)
- **Interference RNA (RNAi)**
- **Short interfering RNA (siRNA)**
- **Micro RNA (miRNA)**

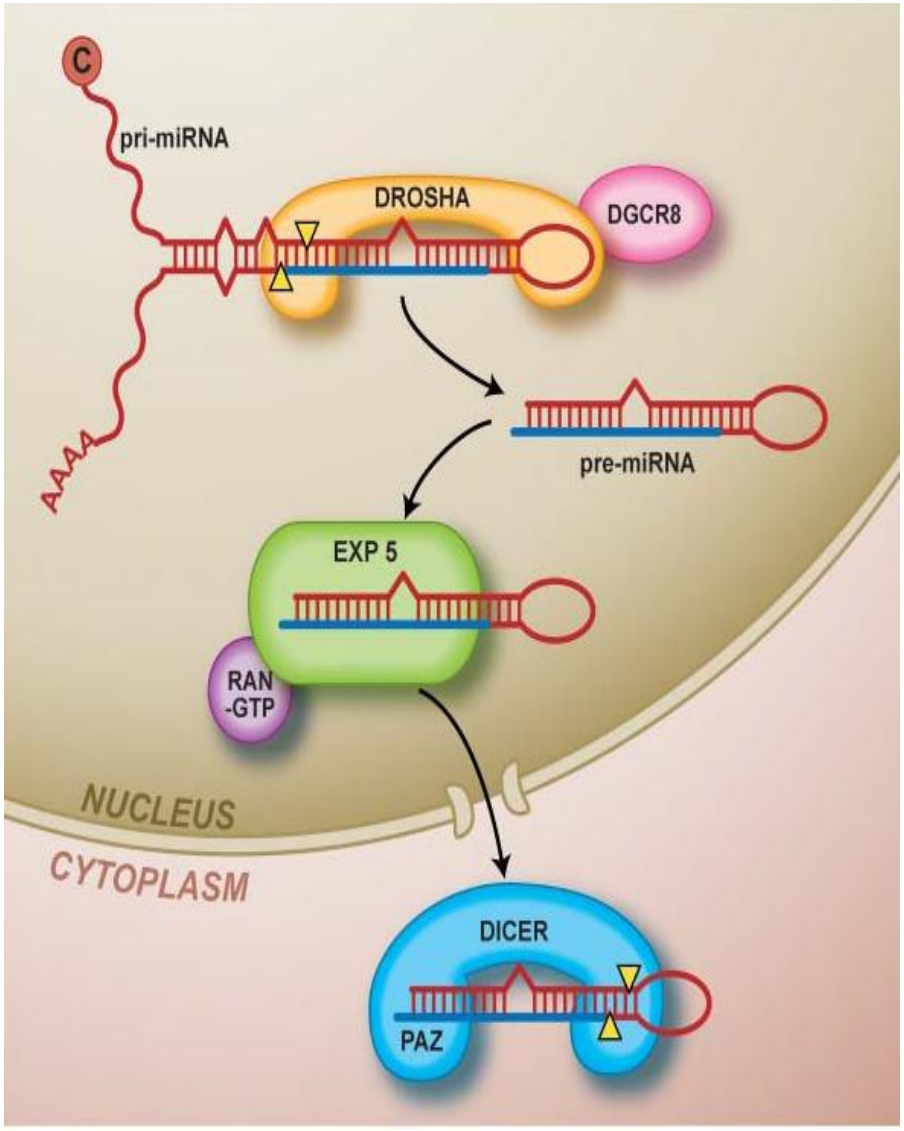
RNA types and its known functions



(Dr. Morten Lindow, Univ. of Copenhagen)

miRNA

- Originate from capped & polyadenylated full length precursors (pri-miRNA)
- Hairpin precursor ~70 nt (pre-miRNA)
- Mature miRNA ~22 nt (miRNA)
- First discovered in 1993 by Victor Ambros at Harvard (*lin-4*)
- *Let-7* discovered in 2000 by Frank Slack as a postdoc at Harvard (Ruvkun lab)



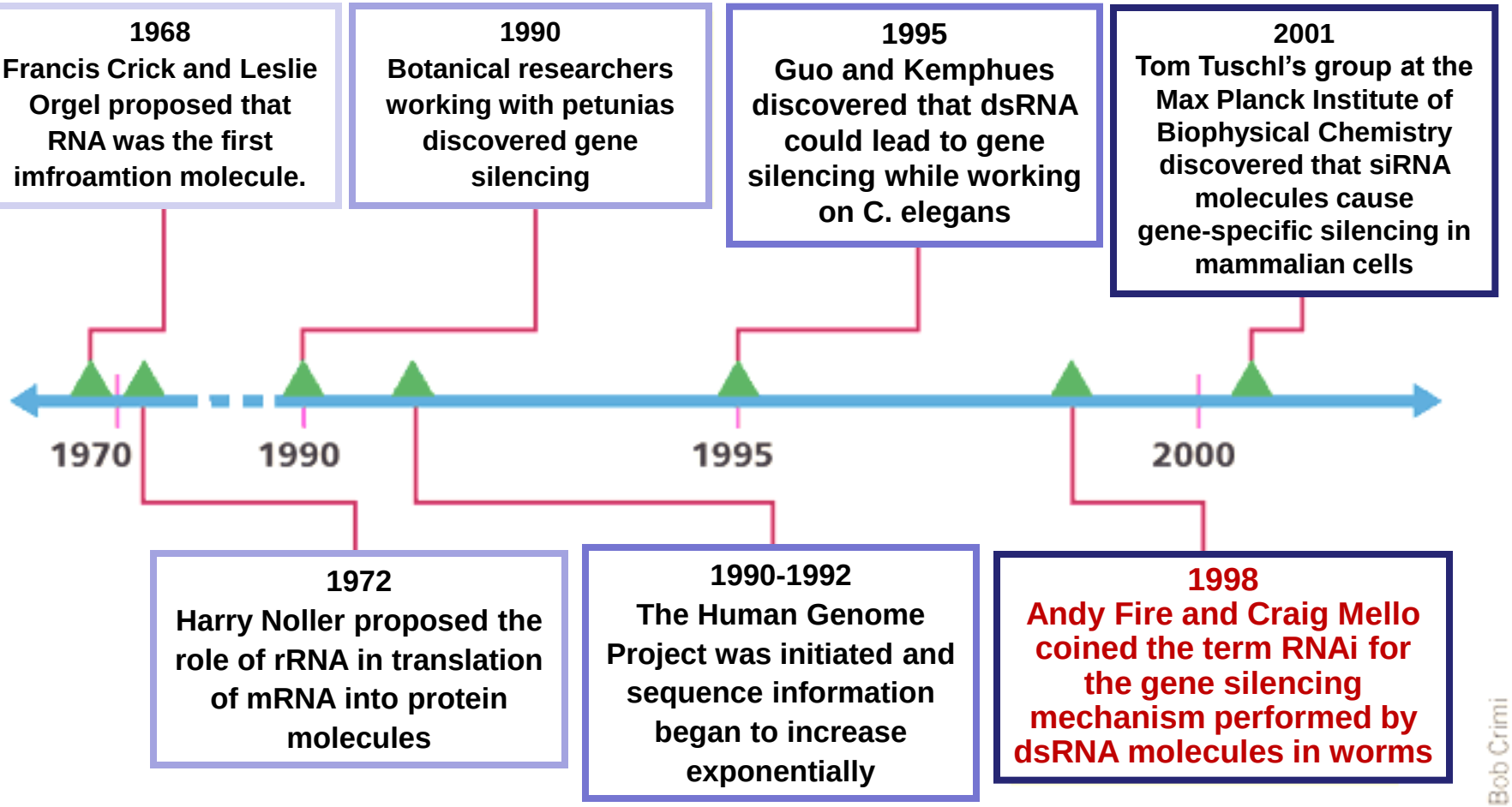
RNAi = RNA interference

A phenomenon in which the introduction of double stranded RNA (dsRNA) into a diverse range of organisms and cell types causes degradation of the complementary mRNA.

The term used to describe the interference of RNA as a natural mechanism, and also as a scientific research tool.

The silencing of gene expression
by double-stranded RNA molecules.

Timeline for RNA*i* Discoveries



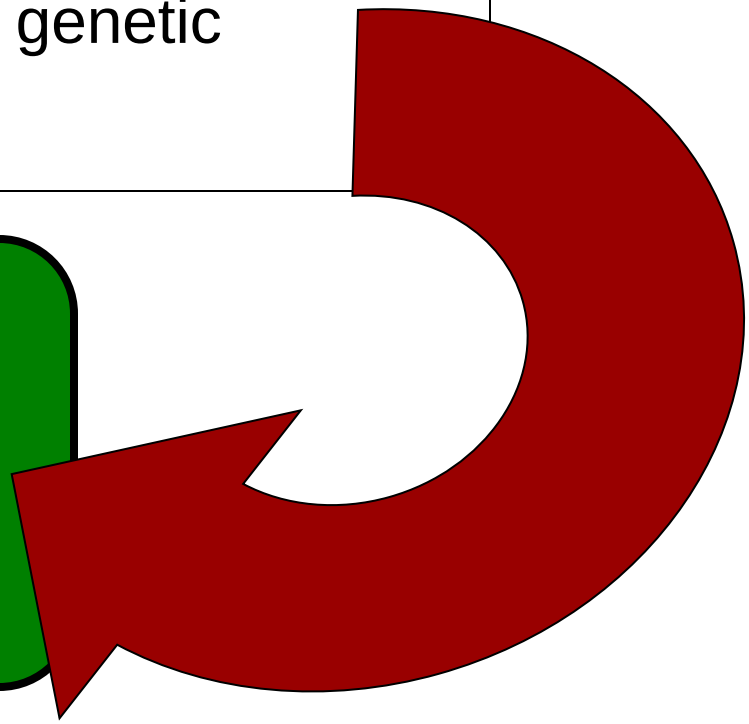
Nature Biotechnology 21, 1441 - 1446 (2003)

RNA*i* = RNA interference

Naturally RNA*i* acts as a gene defense mechanism. It's purpose is to silence types of RNA that are not normally produced by cells such as RNA viruses and rogue genetic elements.

RNA*i* is a useful tool to find out the function of a gene.

- **Determine gene function**
- **Study pathways**
- **Identify and validated targets**
- **Generate knockout models**



Early days of molecular biology:

Modern times:

Forward Genetics

Analysis that starts with phenotype to determine the genotype.

Reverse Genetics

Controlled manipulation of genes to determine phenotype.

Trait Identification

DNA Sequencing

DNA Sequencing

Gene manipulation (siRNA)

Mutation Identification

Gene Function

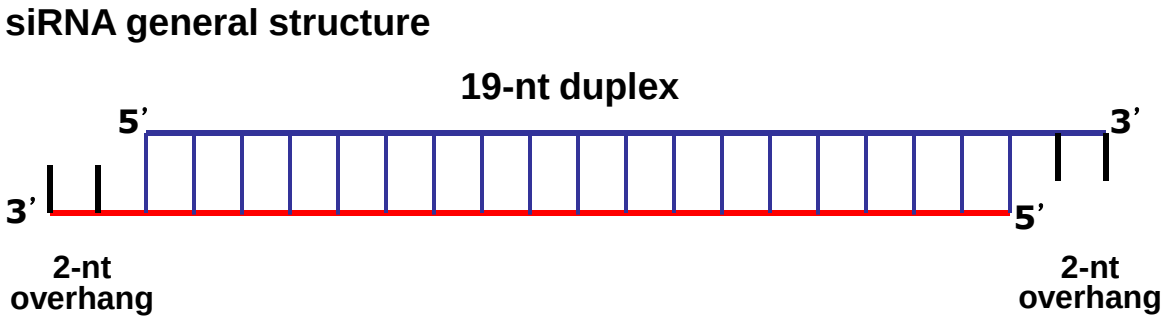
Gene Function

siRNA

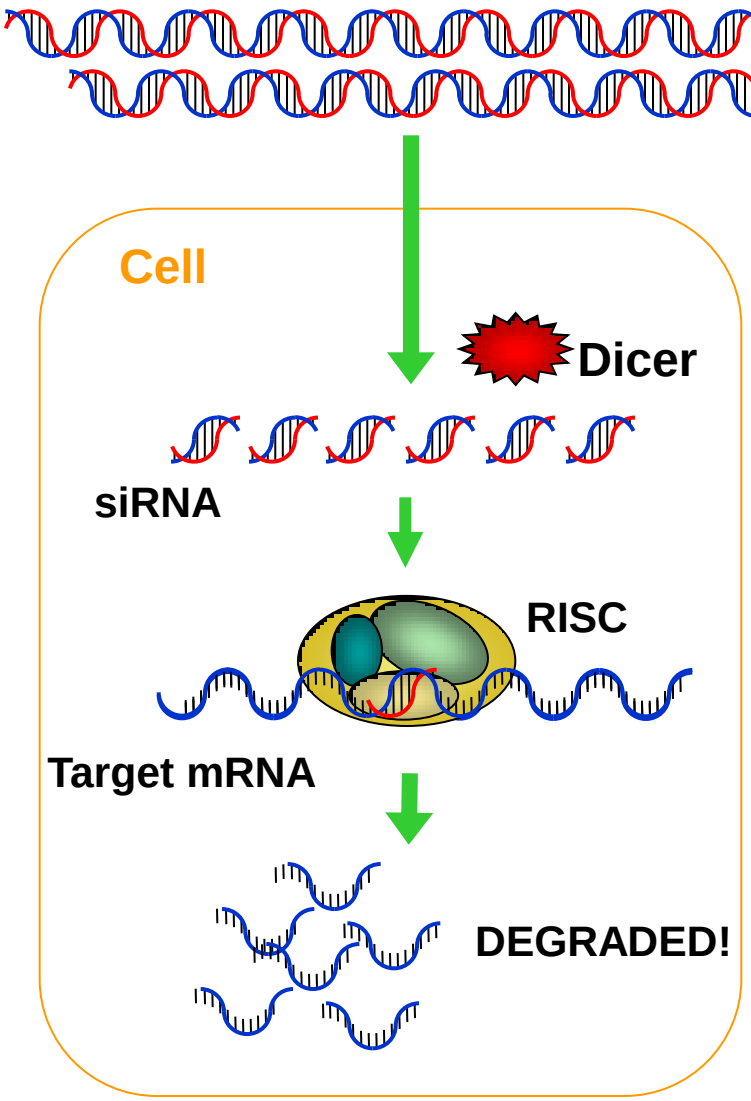
siRNA are short dsRNA molecules

- 21 nucleotide strands of RNA in a staggered duplex

- 19 nucleotides double-stranded, with 2 base overhangs.



Biochemical Mechanism of RNA*i*



dsRNA introduced into the cell.

DICER digests dsRNA into ~21bp dsRNA (short interfering RNAs → siRNA).

The siRNAs are integrated into the RNA Induced Silencing Complex (RISC). The antisense strand then binds to its complementary/target mRNA

Nucleases within RISC degrade the targeted mRNA.

What is the Difference between miRNA and siRNA?

Function of both species is regulation of gene expression

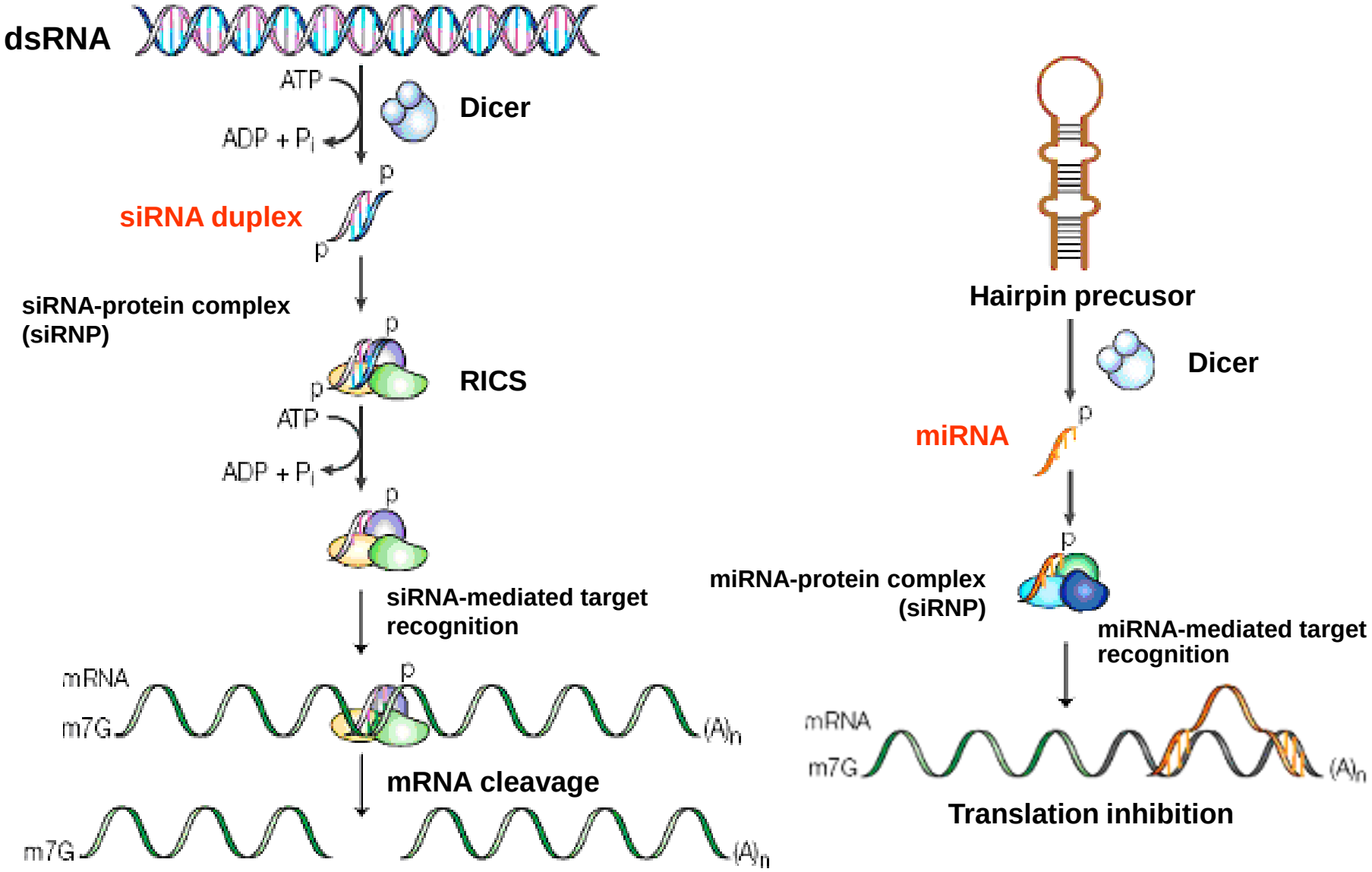
siRNA

- origin: dsRNA
- most commonly a response to foreign RNA (usually viral)
- often 100% commplementary to the target

miRNA

- origin: ssRNA that forms a hairpin secondary structure
- Regulates post-transcriptional gene expression
- often not 100% complementary to the target

What is the Difference between miRNA and siRNA?



RNA*i* : A Technical Overview

Two Main Goals

Excellent Target Knockdown

- Transfection method
- siRNA amount
- Duration time

Low Toxicity

- Prevent Off-Target Effects
- Transfection toxicity itself

siRNA Design

Importance of Good siRNA Design

1. Reduces Off-Target effects
2. Allows for a lower concentration of siRNA
3. Decreases cell toxicity
4. Decreases costs

- For transient expression: duplex RNA can be delivered to the cell
- For a stable expression: a vector containing the DNA to produce a hairpin RNA
- The vector may be plasmid, retrovirus, adenovirus

Basic Requirements: Control

Negative Controls

Scrambled siRNA

- No homology to any known gene
- Shows changes due to presence of foreign body

Non-transfected control cells

- To test for expression changes caused by presence of transfection reagent and buffers.

Positive Controls

- Tests for silencing efficiency
- Test for transfection efficiency

Whither RNAi, *Nature Cell Biology* 5, 489 - 490 (2003)

siRNA design guide and synthesis site

Ambion's siRNA Target Finder (http://www.ambion.com/techlib/misc/siRNA_design.html)

Cold Spring Harbor's RNAi Oligo Retriever (http://katahdin.cshl.org:9331/RNAi/)

Dharmacon's siDesign Center (http://www.dharmacon.com/)

Qiagen's siRNA Target Sequence Design (http://www.qiagen.com/jp/siRNA/sirna_design.asp)

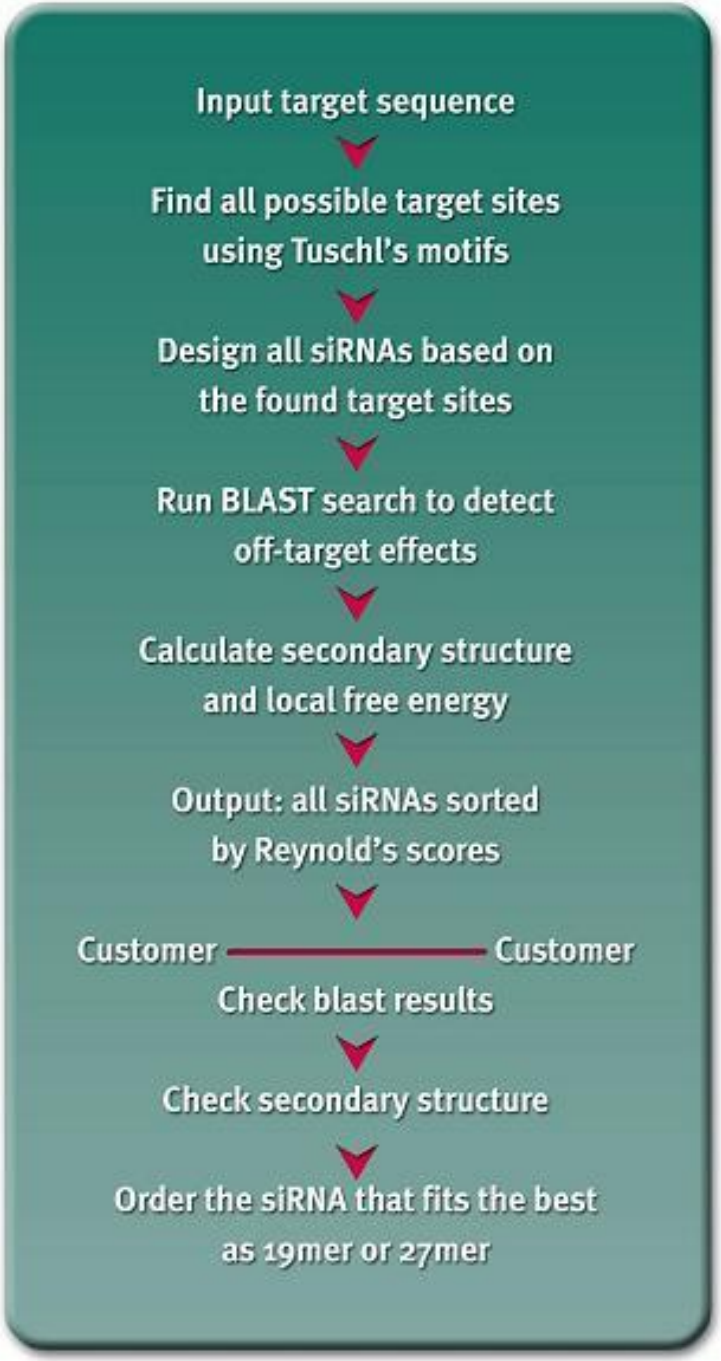
Sirna's Emboss (http://www.biobase.dk/embossdocs/sirna.html)

Tuschl Laboratory siRNA User Guide (http://www.rockefeller.edu/labheads/tuschl/sirna.html)

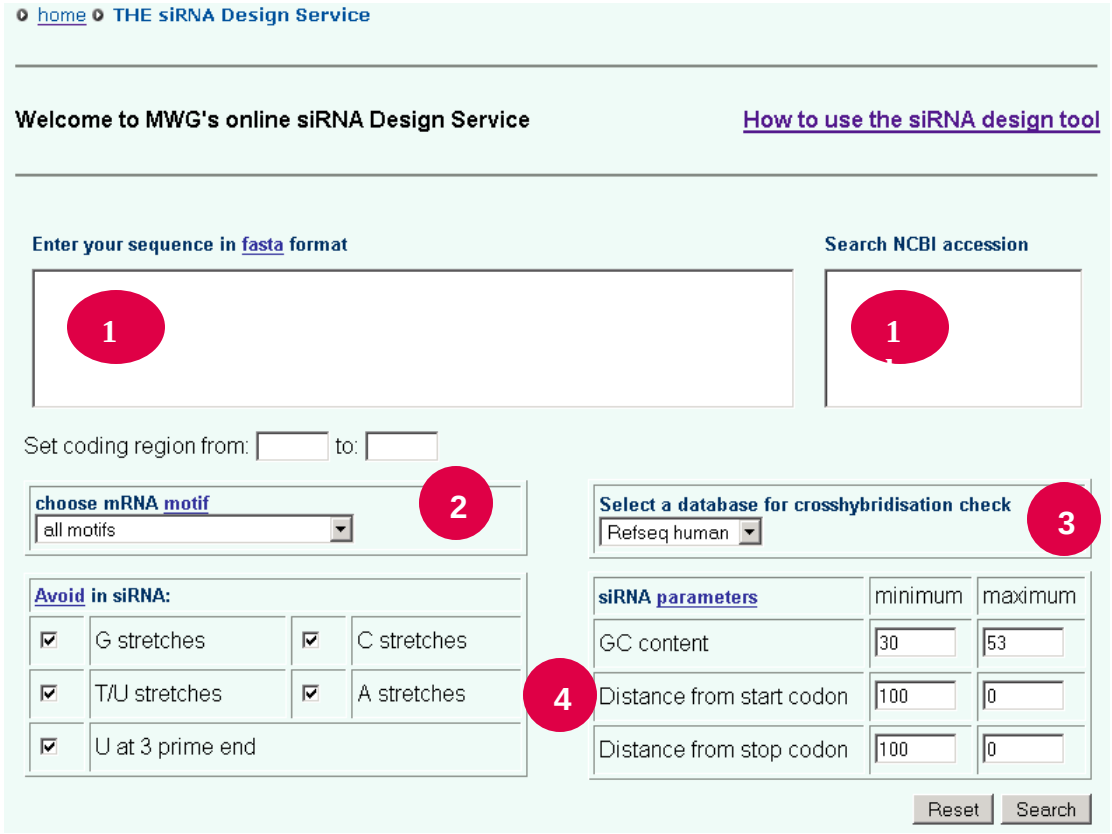
The Whitehead RNAi Selection Program (http://jura.wi.mit.edu/pubint/iona.wi.mit.edu/siRNAext/)

Oligoengine's RNAi software (http://www.oligoengine.com/index.html)

MWG‘sOn-Line Design Tool Flow Chart



MWG‘s On-Line Design Tool



1. **Input either FASTA sequence(s) or accession number(s).**
2. **Define the coding region or mRNA motif**
3. **Select to run Blast Check**
4. **Select parameters such as GC content**

MWG's On-Line Design Tool

Select the siRNA length to order.

Review Blast Results to identify sequences which may have off-target effects.

View the secondary structure to determine mRNA accessibility.

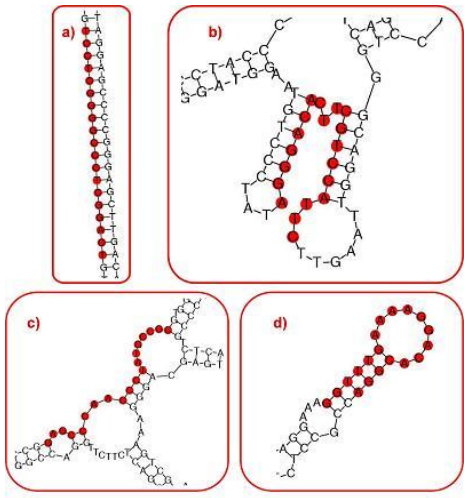
Order 19mer | Order 27mer | Blast Results | Secondary structure | New Search

NM_000075.2 - CDS no. 1 - GI:16936531 - 228..1139

No.	siRNA	mRNA motif	dist. from start	dist. from stop	GC siRNA	Score
1	GCUACCAGAUGGCACUUAC	CA|GCUACCAGAUGGCACUUAC|AC	494	419	52.6	4
2	GAUCUGAAGCCAGAGAACA	GA|GAUCUGAAGCCAGAGAACA|UU	415	498	47.4	4
3	GGACAUAUCUGGACAAGGC	AA|GGACAUAUCUGGACAAGGC|AC	299	614	52.6	2
4	AGUGGUGGAACAGUCAAGC	CA|AGUGGUGGAACAGUCAAGC|UG	445	468	52.6	1
5	CGAUCAAGGAUCUGAUGCG	AA|CGAUCAAGGAUCUGAUGCG|CC	344	569	52.6	1

Secondary Structure

- **Early Design: Algorithms including GC content parameters removed many designs from difficult areas.**
- **Recent articles are revealing that by reviewing the secondary structure of the mRNA target one can increase knockdown success.**

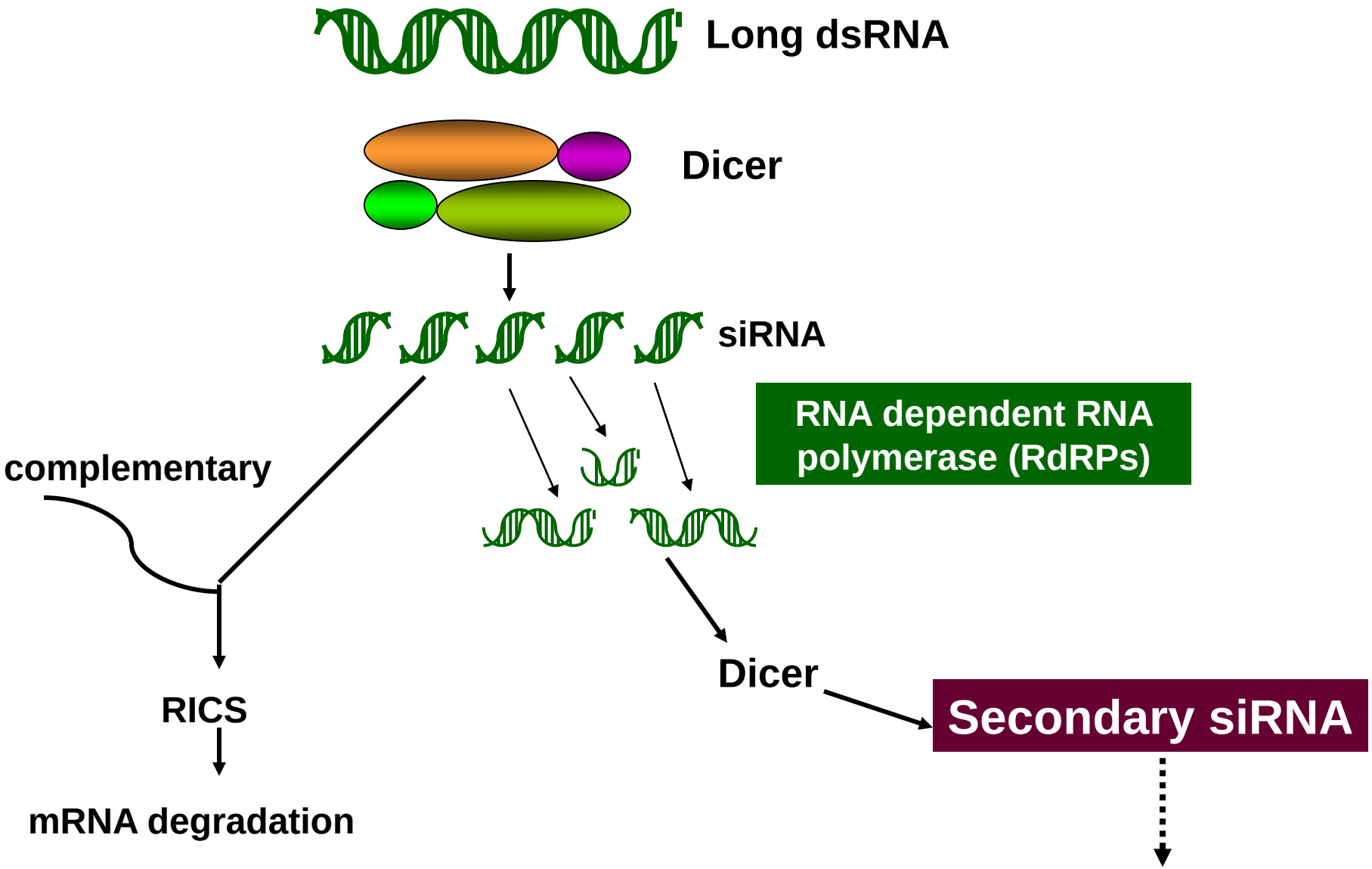


References:

Schubert, S. Grunweller, A.; Erdmann, V. A.; Kurreck, J. (2005). Local RNA target structure influences siRNA efficacy: Systematic analysis of intentionally designed binding regions. *J. Mol. Biol.* 348, 883-893.

Yiu, et al. *Filtering of ineffective siRNAs and improved siRNA design tool.* Bioinformatics. 2005 Jan 15;21(2):144-51.

Heale, et al. *siRNA target site secondary structure predictions using local stable substructures.* Nucleic Acids Res. 2005 Feb 18;33(3):e30.


Long dsRNA
Dicer
siRNA
RNA dependent RNA polymerase (RdRPs)
complementary
RICS
mRNA degradation
Dicer
Secondary siRNA

Basic Requirements: QC

Multiple Designs

- Confirms knockdown due to presence siRNA

Monitor mRNA Changes

Monitor Protein Changes

Monitor Off-Target and Antiviral Effects

- Microarrays : downside cost
- Q-PCR

Nature Cell Biology 5, 489 - 490 (2003)

Methods for Inducing RNA*i*

Direct delivery of siRNAs

- Chemical synthesis of RNA nucleotides
- In vitro transcription of short RNAs
- In vitro transcription of long RNAs followed by RNase cleavage

In vivo expression of shRNAs

- Plasmids
- Viral vectors
- PCR products

Chemicall y Synthesized siRNAs

Commercial synthesis of siRNAs

Design effective siRNA

Advantages

- No hands –on time
- Puryity of siRNA
- Synthesis can be scaled up & labeled

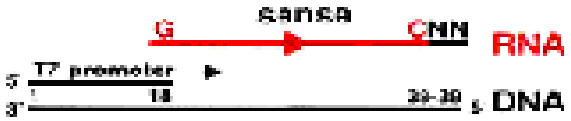
Disadvantages

- Turn around time
- Price

In Vitro Transcribed siRNAs

Equally effective as chemically synthesized siRNAs

Design effective siRNA

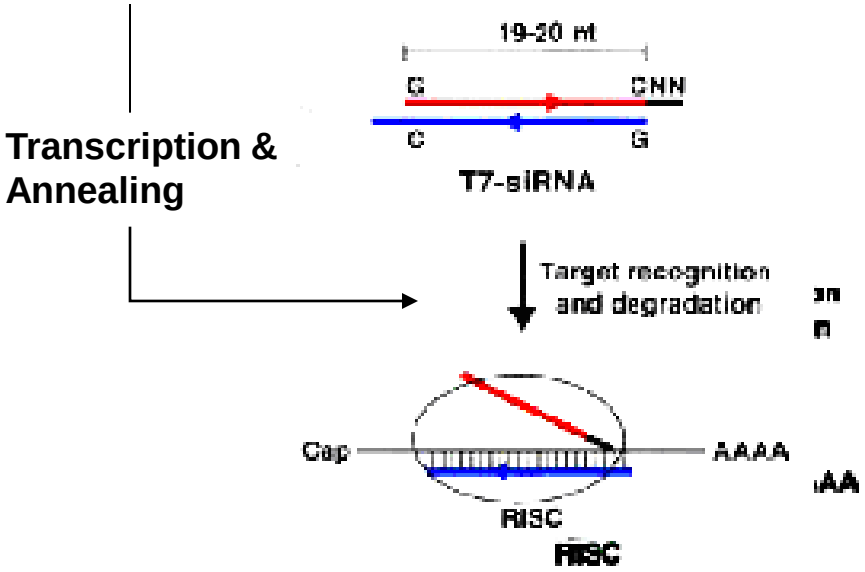


<u>Advantages</u>

- Price
- Short tern-around time

<u>Disadvantafes</u>

- More hands-on
- Scalability



Dicer/RNaseIII Digestion

Population of siRNAs generated by enzymatic digestion of dsRNA

Effective downregulation

Leaves same overhangs as characterized siRNAs

Advantages

Save time and money

Disadvantages

Does not identiry single effective siRNA sequence

Questions about no-specific effects

siRNA Plasmid Expression Vectors

Expression of a short hairpin siRNA by a plasmid

Advantages

No need to work with RNA

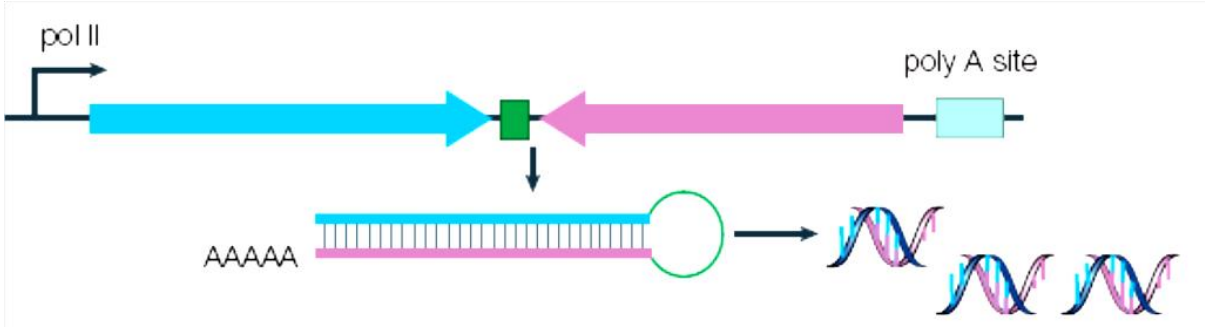
Stable expression

Disadvantages

Not a for siRNA screening method

Need for cloning and verification of insert

Unable to directly label expressed siRNAs



Viral Vectors

Adenovirus

Infects dividing and non-dividing cells

Does not integrate (transient expression)

Lentivirus (retrovirus)

Infects dividing and non-dividing cells

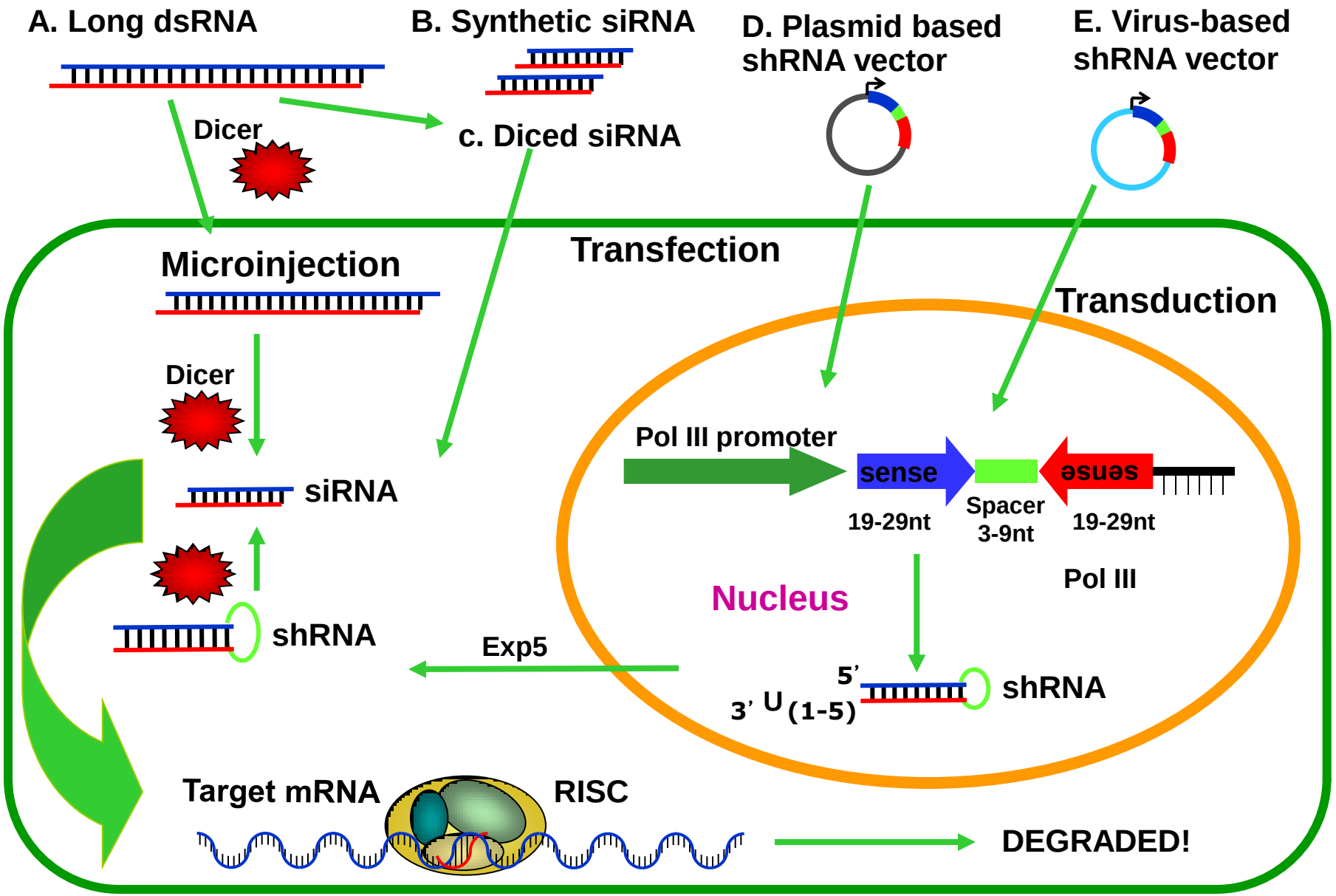
Can integrate (stable expression)

Mouse stem cell virus (retrovirus)

Infects dividing cels only

Can integrate (stable expression)

RNAi Delivery & Processing



Experiment Optimization

- Choice of Transfection Reagent
  - Lipid vs. Amine: Depends on cell type used
- Amount of Transfection Reagent
  - Too much is toxic, too little decreases efficiency
- Plating Density
  - High Density: Low accessibility of siRNA to the cells
  - Low Density: Not enough material to work with
  - Goal: Good Knock-Down levels with a decent amount of cell material for further experimentation.
- Amount of siRNA
  - Goal is to minimize amount needed for each experiment to save costs. Optimal Goal ~10-30nM
  - Reduces off target effects!

Preventing Off-Target Effects

- **Genome-wide array studies give mixed results**
- **Low concentrations(~10-30nM) of single siRNA minimizeds chances of off-target effects**
- **Verify specificity of RNAi effect by testing independent siRNAs to same target**

Chi et al (2003) PNAS 100:6343-6346
Semizarow et al. (woo3) PNAS 100:6347-6352
Jackson et al. (2003) Nature Biotechnol. 21(6): 635-637

siRNA Therapeutics

Therapeutic siRNAs

siRNA target gene	Disease
p53 mutant K-Ras BCR-ABL MDR1 C-RAF Bcl-2 VEGF PKC-α B-Catenin	Cancer
Fas receptor Caspase-8	Acute Liver Failure
TNF-α	Sepsis

Therapeutic siRNAs

siRNA target gene	Disease
HIV-Tat	
HIV-Rev	
HIV-Vif, -Hef	
HPV-E6 and –E7	Viral Infection
HBV-S1, -S2, -S, -X	
CCR5, CXCR4	
CD4	

Problems

- Silencing efficiency
  - Vector-based large dsRNA delivery
- Systemic silencing
- dsRNA-dependent protein kinase response

Further Improvements

- Increase RNAi potency and stability
- Better methods of delivery and expression

Conclusions

The method chosen for inducing RNAi in mammalian cells depends on the needs of the researcher and the goals of the project.

A highly effective siRNA algorithm saves time and reduces cost of screening experiments.

Highly potent siRNAs permit use at low concentrations which provides specificity and limits off-target effects


RNAi
Thank You