RNA Interference

Diane Carrera
MacMillan Group Meeting
January 21, 2009

Lead References:
Nobel Lectures: Fire, A. Z. Angew. Chem., Int. Ed. 2007, 46, 6966
Mello, C. C. Angew. Chem., Int. Ed. 2007, 46, 6985
Overview

- Introduction to RNAi
  - What is it?
  - Why should we care about RNAi?
  - Biochem Basics

- Discovery of RNAi

- Elucidation of RNAi Mechanism
  - Identifying RISC components
  - Other RNAi triggers

- RNAi and Chromatin Modification

- Possible Therapeutic Applications
**What is RNA Interference?**

- RNA interference (RNAi) is the knockdown of gene expression by small RNA fragments

- 1998 – Mello and Fire publish a seminal *Nature* paper elucidating the trigger for the RNAi process

- 2006 – Mello and Fire awarded Nobel Prize in Physiology and Medicine

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**Andrew Fire**
Stanford

**Craig Mello**
UMass
Why is RNAi Important?

- Provides valuable insight into evolution
  - found in all eukaryotes except some fungi → ancient biochemical mechanism
  - the original form of cellular immunity, signal can also spread horizontally and vertically

- indicates that gene expression is the key to evolving complex organisms

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Nematode</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein encoding genes</td>
<td>$21 \times 10^3$</td>
<td>$19 \times 10^3$</td>
</tr>
<tr>
<td>miRNAs</td>
<td>677</td>
<td>154</td>
</tr>
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</table>
Why is RNAi Important?

- Invaluable tool for functional genomics
  - Knockdowns are easier than knockouts ➡️ Saves time and money
  - Allows for study of function with a variety of controls: positive, negative, rescue

Many public databases (UPenn, Cold Spring Harbor, MIT) now exist for the design of interfering RNAs to target specific genes

- Medicine and Biotech
  - Science "Breakthrough of the Year" 2002
  - Fortune "Billion Dollar Breakthrough"
**Basic Biochem**

DNA and RNA are information containing biopolymers

<table>
<thead>
<tr>
<th>Purines</th>
<th>Pyrimidines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine (A)</td>
<td>Thymine (T)</td>
</tr>
<tr>
<td>Cytosine (C)</td>
<td>Guanine (G)</td>
</tr>
<tr>
<td>Uracil (U)</td>
<td>Adenine (A)</td>
</tr>
</tbody>
</table>

**Chemical Structures:**
- Adenine (A): ![Chemical Structure](image)
- Thymine (T): ![Chemical Structure](image)
- Cytosine (C): ![Chemical Structure](image)
- Guanine (G): ![Chemical Structure](image)
Basic Biochem

DNA and RNA are information containing biopolymers

backbone: ribose
prone to hydrolysis
short term information storage

backbone: deoxyribose
stable to hydrolysis
long term information storage
**Basics of Gene Expression**

- Gene Expression is comprised of Transcription & Translation with distinct roles for DNA & RNA
  - DNA: the architect with the master set of blueprints
  - RNA: the contractor overseeing specific tasks

![Diagram of gene expression process with DNA, RNA, and protein chart]
Transcription

Transcription is the transfer of information from DNA to messenger RNA (mRNA) and has 3 distinct stages

**Initiation:** RNA polymerase enzyme (RNAP) inserts into DNA double helix

**Elongation:** RNAP builds a strand of mRNA from the template DNA strand

**Termination:** mRNA synthesis is complete, RNAP and mRNA are released into solution and double helix reforms

RNAP: 2006 Nobel Prize in Chemistry, Roger Kornberg
Translation

Translation is the synthesis of proteins in 4 distinct stages using the mRNA template.

1. Activation: amino acids bind to transfer RNA (tRNA)

2. Initiation: small subunit of ribosome binds 5' end of mRNA

3. Elongation: peptide chain grows as amino acids are brought in by tRNAs with anticodons corresponding to mRNA codons

4. Termination: peptide released upon reaching stop codon (UAA, UAG, UGA)
**The Biochemist's Best Friend**

- *Caenorhabditis elegans (C. elegans)* is an ideal system for studying gene expression

  - nematode roundworm
  - 1mm in length
  - lives in soil

  the developmental timeline of every cell has been mapped

  the developmental timeline of every cell has been mapped

  phenotypic response to gene expression is well defined

  - unc-22 repression: twitching
  - par-1 repression: symmetrical cell division

- Microinjection technique allows for the direct introduction of macromolecules into the animal

  - ability to affect large populations
  - RNA, DNA and proteins can all be injected
Unexplained Results with Antisense

In the 1990's, antisense looks like the next big thing

antisense mediated silencing

Some experiments are giving unexpected results

Guo & Kemphues discover that both sense and antisense strands mediate silencing

antisense experiments with unc-22 give same result

supression effects persist and are passed on through germline

Cell 1995, 81, 611

Fire Development 1991, 113, 503
Kuwabara Genetics 1996, 144, 597
**Finding the Trigger**

- Electrophoresis shows RNA preparations are contaminated with dsRNA

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A Very Potent Trigger

- Dilution studies show suppression is obtained with as little as a few molecules per cell.
Vital Control Experiments

Control experiments reveal dsRNA interference is gene specific and spreads throughout the worm cells.

complex RNA

microinjection

A  B

on  off

dsRNA mediated interference is specific

GFP - mitochondrial gfp
LACZ - nuclear gfp

non-striated muscles are only unaffected cells

Control RNA (ds-unc-22)  ds-gfpG RNA  ds-lacZL RNA

Young larva

Adult

Adult body wall

Control RNA (ds-unc-22)  ds-gfpG RNA  ds-lacZL RNA

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RNAi: So Easy a First Year Can Do It

- Injection into gonads is not required for RNAi to be effective
  improperly placed needles still lead to interference

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>Injection Site</th>
<th>Phenotype</th>
<th>Progeny Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>gonad or cavity</td>
<td>no twitching</td>
<td>no twitching</td>
</tr>
<tr>
<td></td>
<td></td>
<td>strong GFP</td>
<td>strong GFP</td>
</tr>
<tr>
<td>unc22</td>
<td>gonad</td>
<td>weak twitchers</td>
<td>strong twitchers</td>
</tr>
<tr>
<td></td>
<td>body-cavity</td>
<td>weak twitchers</td>
<td>strong twitchers</td>
</tr>
<tr>
<td>gfpG</td>
<td>gonad</td>
<td>weak GFP</td>
<td>absent GFP</td>
</tr>
<tr>
<td></td>
<td>body-cavity</td>
<td>weak GFP</td>
<td>absent GFP</td>
</tr>
<tr>
<td>lacZL</td>
<td>gonad</td>
<td>weak nuclear GFP</td>
<td>absent nuclear GFP</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

- Ingestion of dsRNA expressing _E. coli_ and soaking also work

"Do experiments that your advisor would never condone or suggest" – Fire, Nobel lecture

Mello *Science*, 1998, 282, 430
What's in the Black Box?

- Early experiments reveal target mRNA degradation is initiated by dsRNA

*mex-3* mRNA can be detected by staining

**negative control**
no staining in wild type without hybridization

**positive control**
staining in wild type with hybridization

*mex-3* antisense RNA

*mex-3* dsRNA

dsRNA

RNAi

degraded mRNA
What's in the Black Box?

- A clever experiment reveals which genes are involved in RNAi

*pos-1* expression is required for *C. elegans* survival

- Feed ds*pos-1* RNA expressing *E. coli*

- Standard genetic mapping

- RNAi mutant

- Viable progeny

*Rde-1* identified as a member of the little known argonaute family of proteins

Fire, Mello Cell 1999, 99, 123
**What's in the Black Box?**

- Two groups take the lead in working out the RNAi biochemical pathway

### 1998

**Nature 1998, 391, 806**

Mello & Fire establish dsRNA as trigger for RNAi

### 2000

**Nature 2000, 404, 293**

Hannon defines RISC complex as cause of mRNA degradation

**Cell 2000, 101, 25**

Tuschl & Sharp find mRNA is cleaved into 22 nt fragments

### 1999

**Genes Dev. 1999, 13, 3191**

Sharp & Tuschl replicate RNAi in vitro

### 2001

**Nature 2001, 409, 363**

Hannon defines roles of Dicer & Slicer, identifies Argonaute in RISC

**Science 2001, 293, 1146**

**Gene Dev. 2001, 15, 188**

**Nature 2001, 411, 494**

Tuschl determines structures of Dicer dsRNA products, siRNA

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**Phil Sharp**

MIT

**Thomas Tuschl**

MIT, Gottingen, Rockefeller

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**Greg Hannon**

Cold Spring Harbor
Overall Scheme of RNAi process

dsRNA trigger

RDE-4 binds dsRNA, recruits RDE-1

RDE-4 & RDE-1 recruit DCR-1 (Dicer)

Dicer degrades dsRNA to short-interfering RNA (siRNA)

Slicer degrades mRNA

RISC binds target mRNA via base pairing

RISC binds target mRNA via base pairing

SiRNA guide strand incorporated into RNA Induced Silencing Complex (RISC)

siRNAs, 22 nt

cut between nucleotides 10 & 11 from 5' end
Crystal Structure Reveals Dicer Mechanism

- Doudna et. al. is able to obtain a crystal structure of *G. intestinalis* Dicer enzyme

Dicing requires both enzyme and Mg$^{2+}$ to produce siRNAs 25-27 nt in length

Glu and Asp perform cleavage in active site

A distance of 17.5 Å between the RNase III domains fits the width of the major groove of dsRNA

*Science* 2006, 311, 195
Crystal Structure Reveals Dicer Mechanism

Analysis reveals Dicer is in effect a molecular ruler

A distance of 65 Å between the PAZ and RNase III domains corresponds to the length of an siRNA fragment containing 25 bp

3' two nucleotide overhang recognition by PAZ OB fold is conserved across many species
Crystal Structure of Argonaute Reveals Slicer Mechanism

Argonaute has been closely linked to RNAi across all species studied.

As seen with Dicer, Slicer also requires Mg$^{2+}$ for activity.

positively charged groove in crescent formed between base (N-terminal, middle and PIWI domains) and overhanging PAZ domain.

phosphate binding

homology to sugar binding lac-repressor

RNA binding

RNase H-like catalytic domain with Asp-Asp-His

Joshua-Tor *Science* 2004, 305, 1434
Crystal Structure of Argonaute Reveals Slicer Mechanism

- Modelling of siRNA guide strand and mRNA places scissile bond in active site

PAZ binds phosphate between 2 overhanging nucleotides of guide strand via H-bonding with His & Tyr residues

human Ago1

Pf Ago

evidence for binding 5' end with PIWI and/or middle domain

base pairing with the guide strand positions mRNA target for cleavage in PIWI domain

Leemor Joshua-Tor
Cold Spring Harbor
RNAi is also Triggered by microRNAs

Ambros discovers the first endogenous short RNA (microRNA) via forward genetics in 1993

*C. elegans* mutant with abnormal embryonic development

*Cell, 1993, 75, 843*

miRNAs use the RNAi pathway to regulate gene expression, controlling cell development throughout animal life cycles

- c-myc overexpression → lymphoma
- miRNA knockout
- miRNA
  - cancer
  - controlled cell growth

short hairpin RNA 70 nt long
miRNAs Are Correlated with Complexity

- miRNAs are found in all animals and attempts to find all miRNA encoding genes are ongoing

Bartel proposes miRNA regulation could explain why complex organisms have the same number of genes as simple ones

*Nature, 2008, 455, 1193*
RNAi Also Directs Heterochromatin Formation

- Discovered in 1928, heterochromatin is darkly staining, covalently modified chromatin that does not unwind at any time in the cell cycle.

DNA methylation results in silencing

- Has a regulatory effect on nearby genes, ie fruitfly & maize variegation

histone modification occurs on tails

- Comprised of short repeating sequences, heterochromatin produces repeat associated siRNAs (rasiRNAs) that induce covalent modification of DNA & histone
**RNAi Also Directs Heterochromatin Formation**

Though its exact mechanism is still unclear, the RNA-induced transcriptional silencing (RITS) complex plays the role of RISC and contains an argonaute protein.

Joshua-Tor *Nature Chemical Biology* 2006, 3, 36

1. RNA polymerase II transcribes DNA
2. Pol II transcripts targeted by RITS complex
3. Slicing results in free 3' OH, creates RdRC substrate
4. RdRC creates dsRNA
5. dsRNA enters RNAi pathway
6. Rik1 complex (methyltransferase Clr4) performs histone H3 Lys9 dimethylation, spreading the silencing

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**chromatin modification** → **inherited gene regulation** → **evolution**
**Therapeutic Possibilities for RNAi**

- Using RNAi as an experimental and therapeutic tool requires several considerations

**Innate immune response (interferon) provides viral immunity**

- dsRNA
  - RNAi: < 30 nt
  - Protein kinase catalyzed degradation: > 30 nt

**Design of siRNA**

- Passenger strand (sense)
  - Strand with lower 5' thermodynamic stability (A–T) is incorporated into RISC
  - Seed region has greatest effect on mRNA recognition
  - Cleavage site between nt 10 & 11 from 5' end

- Guide strand (antisense)

**4 Rules for effective siRNAs**

1. Get right strand into RISC (design algorithms)
2. Target several alleles (phenotypic correlation)
3. Use low concentrations (limit off target effects)
4. Use rescue experiments (important control)
**Therapeutic Possibilities for RNAi**

- Endogenous miRNAs have inspired the design of short hairpin RNAs (shRNAs)

  - **HIV**
    - RNAi has been demonstrated to stop spread of HIV in mice
    - High mutation rate poses problems, cellular cofactors are other possible targets (NF-κB, CD4, CXCR4, CCR5)

  - **Hepatitis C**
    - Blocking has been demonstrated but it is temporary due to virus mutation

  - **Cancer**
    - Alnylam compound in clinical trials for treatment of liver cancer
    - Contains 2 siRNAs in lipid nanoparticle (Tekmira) targeting KSP & VEGF

  - **Genetic Disease**
    - Amyotrophic lateral sclerosis treatment targeting single nucleotide mutant of *sod1*
    - Huntingtons disease
Therapeutic Possibilities for RNAi

The path to commercial therapies that utilize RNAi is not necessarily clear

Can the RNAi pathway be saturated?

- therapies hijack the native RNAi machinery which we know is used in gene expression
- RISC saturation has been shown in vitro

How can we delivery these therapies?

- this issue has plagued antisense for over 20 years
- many options exist (backbone modification, virus vectors, transfection) but none work perfectly
- for now targets are set low (liver)

What about off target effects?

- suppression of cofactors may result in disruption of normal cellular processes
- chromatin modification has not yet been identified in humans but endogenous RNAs point to the possibility of its existence

Conclusions

- RNAi is an important part of the cellular machinery that provides viral immunity and a mechanism for the control of gene expression

- A variety of RNA triggers function in the RNAi mechanism result in gene suppression that can be both temporary and permanent

  dsRNA, siRNA, miRNA, shRNA

- Recent studies have implied that RNAi could be an important factor in evolution due to its key role in epigenetics

  miRNA, chromatin modification

- Potential therapeutic applications of RNAi include treatments for viruses, cancer and heritable disease

- The field is relatively young and much remains to be discovered
RNAi is Actually Quite Complicated

- RNAi is still an emerging field with many unanswered questions and potential applications

C. elegans contains 27 argonaute proteins, all of which are involved in RNAi

Mello Cell, 2006, 127, 747