RNAi as plant-incorporated protectant: potential for off-target regulation in mammals

Kenneth W. Witwer, PhD

Assistant Professor
The Johns Hopkins University School of Medicine
Department of Molecular and Comparative Pathobiology
Retrovirus Laboratory

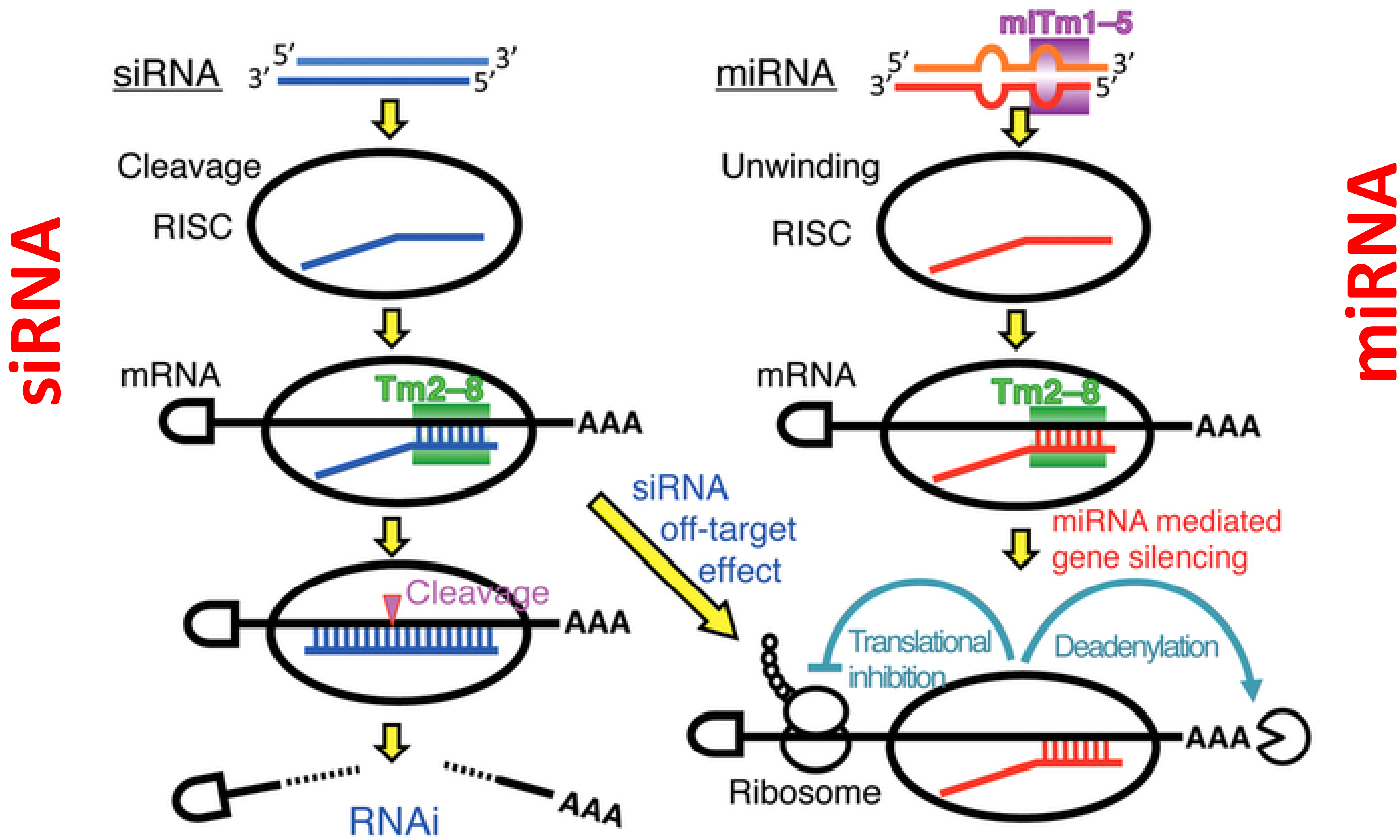
04 June 2014

I have not and do not currently have any financial or contractual relationship with any commercial entity with an interest in RNAi technology. The views expressed are my own and are not necessarily those of my employer or funding organizations.

Off-target effects

- siRNA effects
- Off-target “miRNA-like” effects
- Stimulation of the innate immune system
- Saturation of RNAi machinery

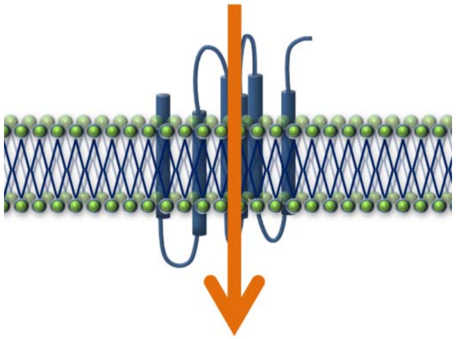
Payload or Passenger: small RNA

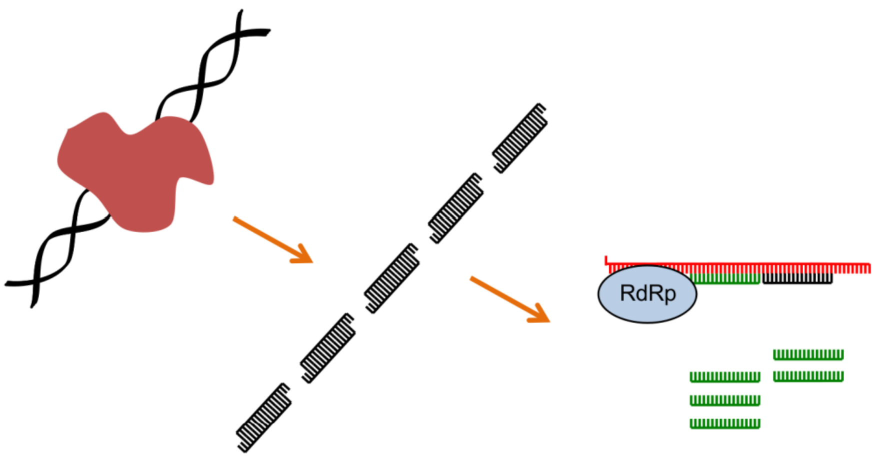


Hibio et al , Sci Reports, 2012

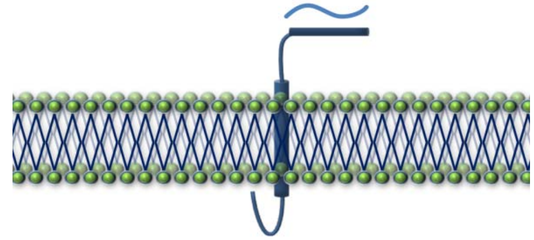
RNAi mechanisms not observed in mammals

Spreading interference: SID-1

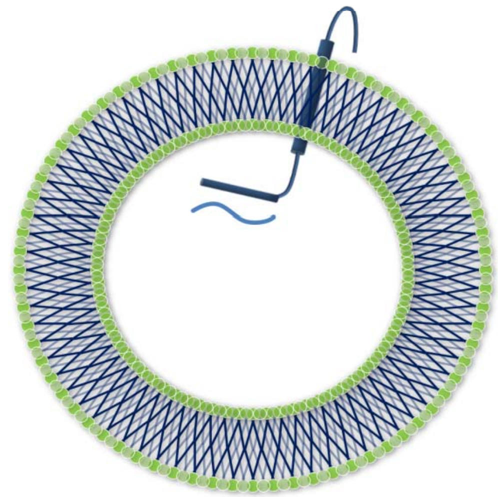




RNAi amplification



Uptake from the environment: SID-2



Witwer and Hirschi, BioEssays 2014

siRNA effects?

- 2009
  - Ivashuta, et al., *Food and Chemical Toxicology*
  - Numerous perfect matches of crop small RNAs to human and other animal genes
  - “History of safe consumption”
- US EPA, 2014 FIFRA SAP on RNAi as a pesticide
  - “RNAi technology as a pesticide: problem formulation for human health and ecological risk assessment”
  - "Bioinformatic analysis can be used to identify specific nucleotide identity in long sequences of dsRNA or processed shorter products that could bind in siRNA-like fashion...“
  - Genes, not genomes

Off-target “miRNA-like” effects: numbers

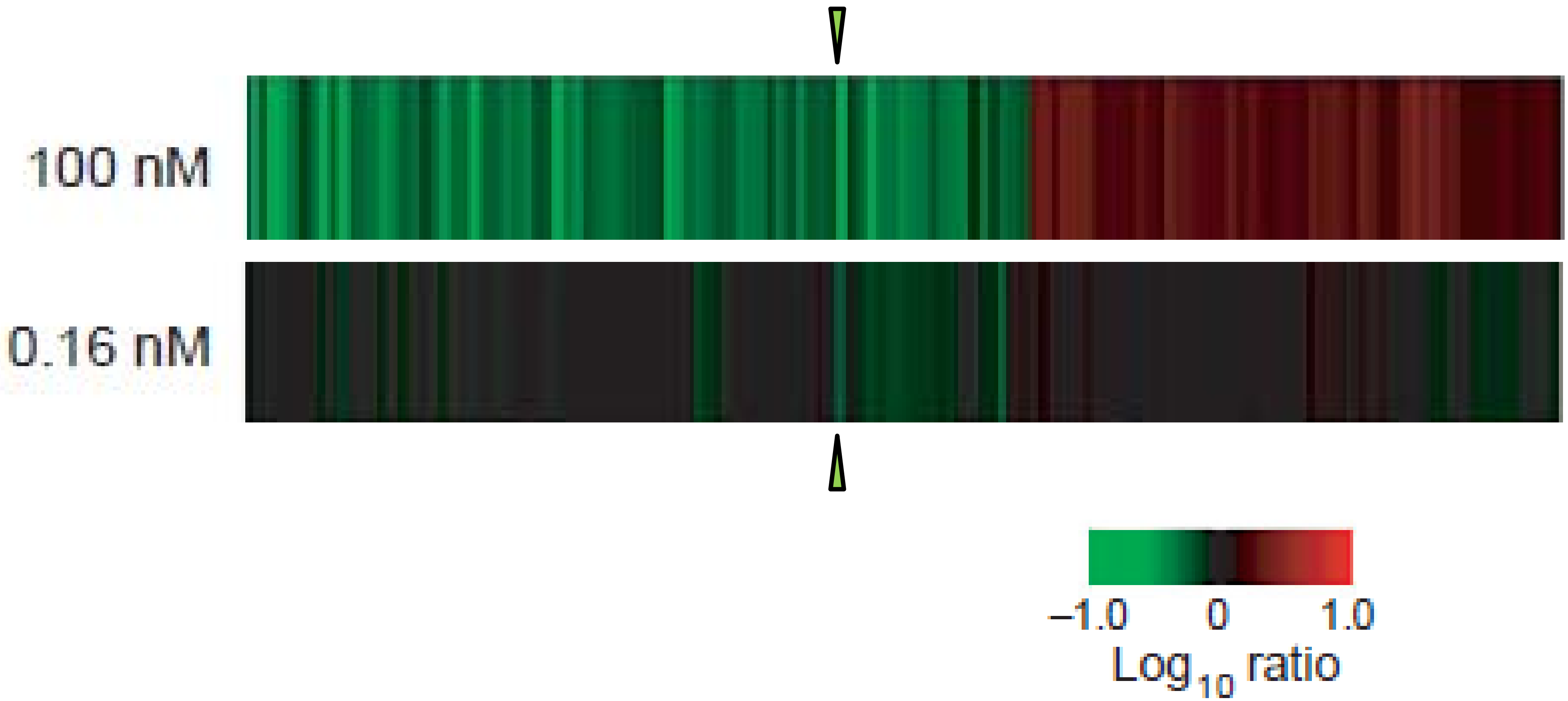
Expression profiling reveals off-target gene regulation by RNAi

Aimee L Jackson[1,2], Steven R Bartz[1,2], Janell Schelter[1], Sumire V Kobayashi[1], Julja Burchard[1], Mao Mao[1], Bin Li[1], Guy Cavet[1] & Peter S Linsley[1]

NATURE BIOTECHNOLOGY VOLUME 21 NUMBER

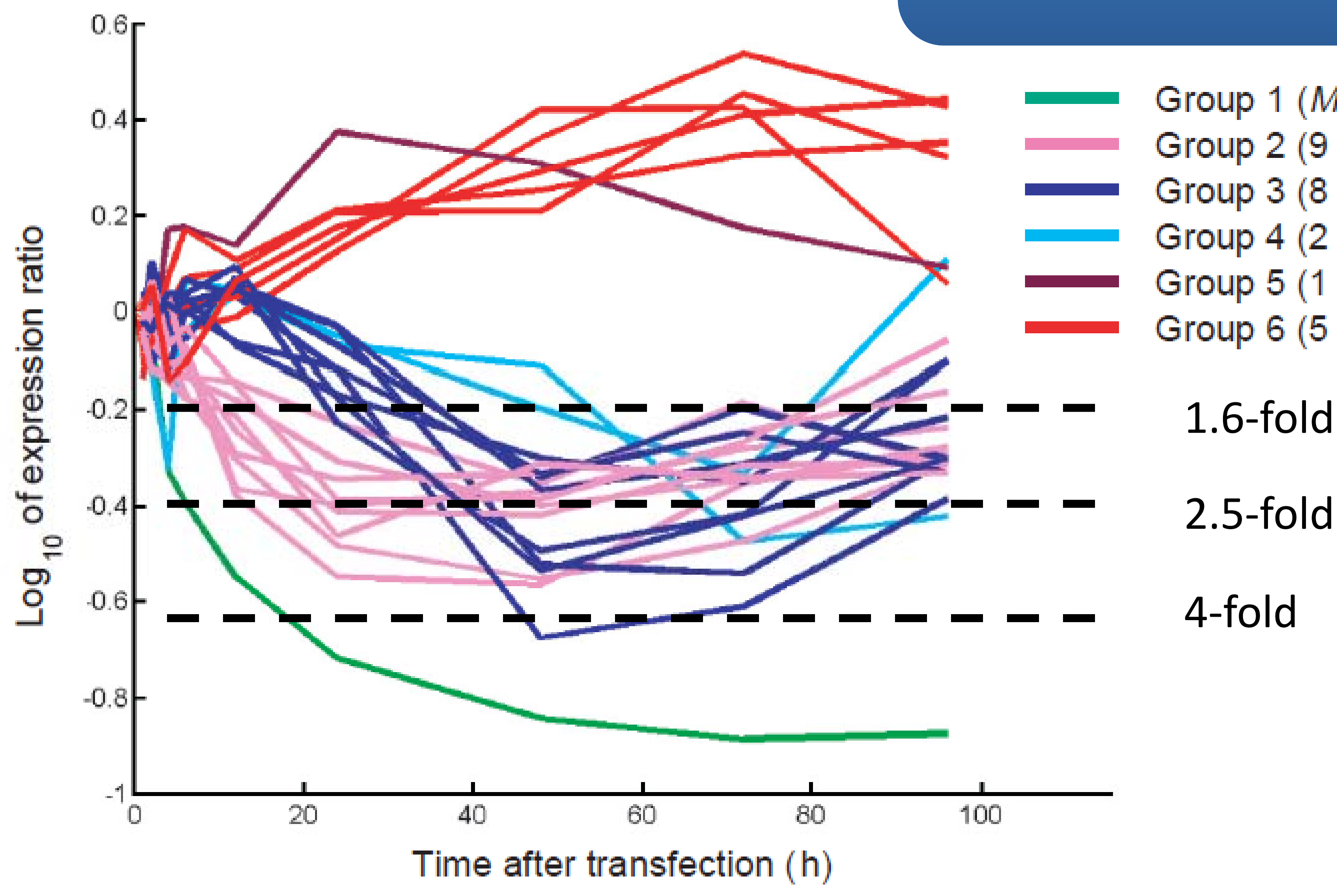
- 6-well plates: approximately one million cells
- RNA added at 100 nM
  - 100 pmol, 750 ng; 1 mL transfection volume
  - Or 60 trillion molecules of RNA
- 60 million molecules of small RNA per cell
- Almost all off-target effects disappeared at 0.16 nM, i.e. at 100,000 copies/cell

Off-target "miRNA-like" effects: dose dependence



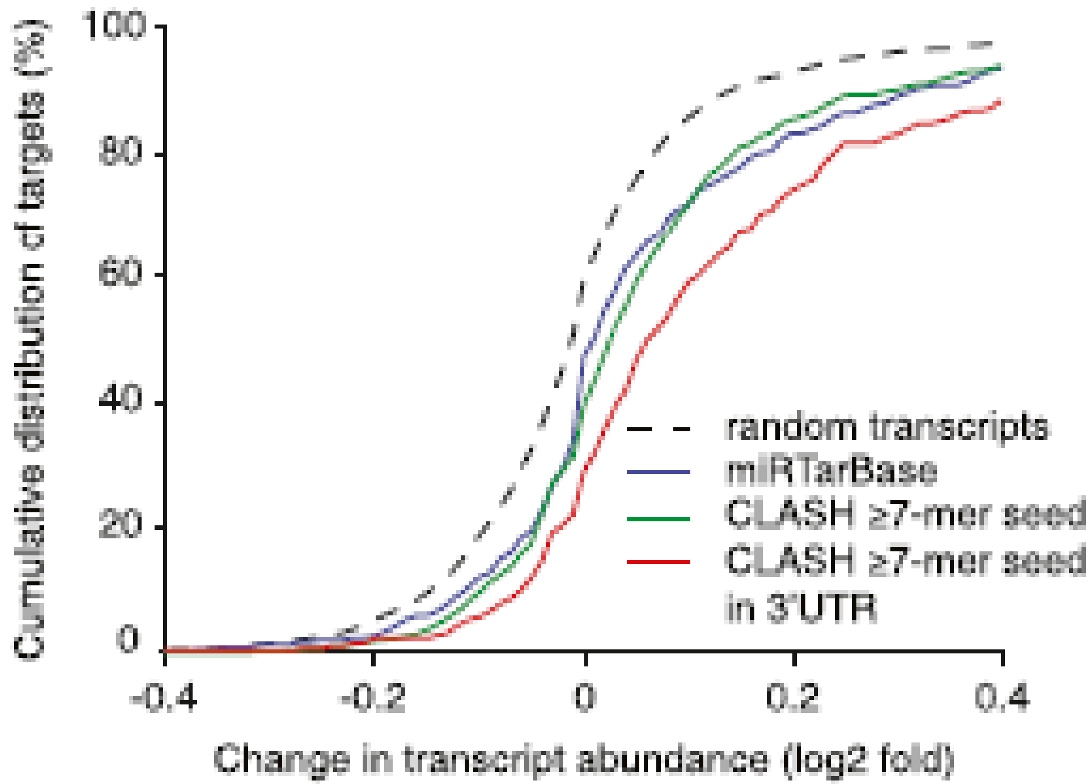
NATURE BIOTECHNOLOGY VOLUME 21 NUMBER 6 JUNE 2003

Off-target "miRNA-like" effects: minor



NATURE BIOTECHNOLOGY VOLUME 21 NUMBER 6 JUNE 2003

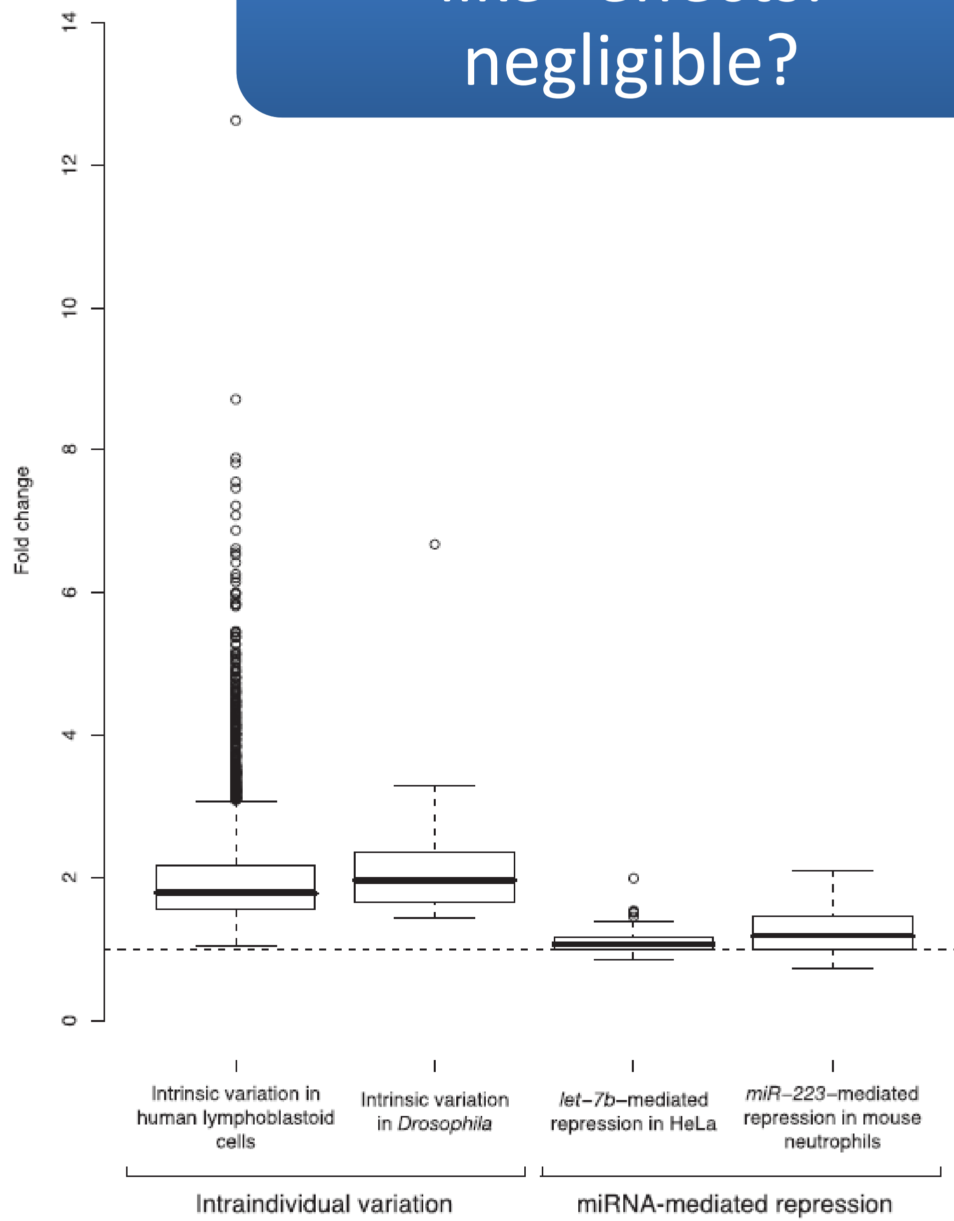
miRNA confirmed targets: << 2-fold change



Helwak, et al., Cell, 2013

Off-target “miRNA-like” effects: negligible?

- 2009: Hervé Seitz, *Current Biology,* “Redefining microRNA Targets”
- Most genes are haplosufficient
- Normal fluctuation in gene expression outweighs most microRNA effects
- Certain miRNA-target pairs have evolved to “work”; others are “decoys”



Saturation of the RNAi machinery? High dose

nature

Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways

Dirk Grimm[1], Konrad L. Streetz[1]†, Catherine L. Jopling[2], Theresa A. Storm[1], Kusum Pandey[1], Corrine R. Davis[3], Patricia Marion[4], Felix Salazar[4] & Mark A. Kay[1]

- One of several studies examining shRNA (i.e., does not bypass Exportin 5)
- shRNA-expressing adeno-associated virus introduced at 100 billion to 1 trillion particles
- Liver toxicity strongest at the highest dose
- Shorter shRNAs (19 nt) were not toxic

Saturation of the RNAi machinery: No

nature

LETTERS

Effective RNAi-mediated gene silencing without interruption of the endogenous microRNA pathway

Matthias John[1], Rainer Constien[1], Akin Akinc[2], Michael Goldberg[3], Young-Ah Moon[5], Martina Spranger[6], Philipp Hadwiger[1], Jürgen Soutschek[1], Hans-Peter Vornlocher[1], Muthiah Manoharan[2], Markus Stoffel[6], Robert Langer[3,4], Daniel G. Anderson[4], Jay D. Horton[5], Victor Koteliansky[2] & David Bumcrot[2]

- Synthetic siRNA in liposomal formulation
- High dose: 5 mg/kg; low dose: 2 mg/kg
- 25 g mouse: ~10 quadrillion siRNA molecules
- Specific targets effectively silenced
- Neither toxicity nor reduction in liver miR-122 were found

Saturation of the RNAi machinery

Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs

Aly A Khan[1,2], Doron Betel[2], Martin L Miller[2,3], Chris Sander[2], Christina S Leslie[2,5] & Debora S Marks[4,5]

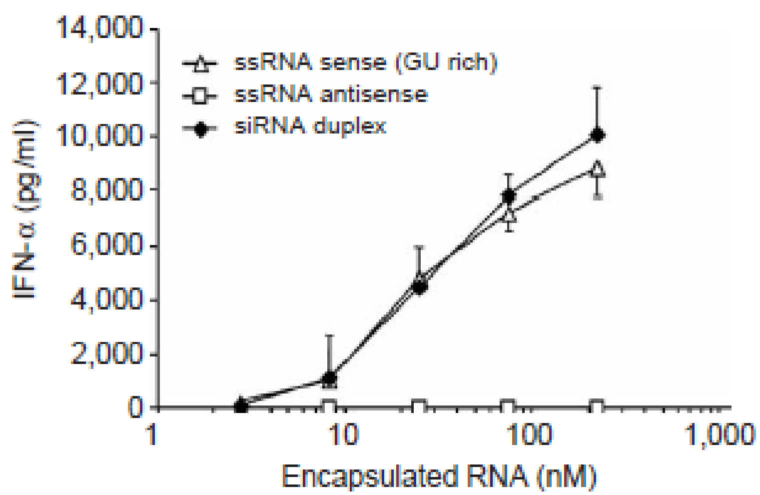
- Examined numerous published datasets
- *In vitro* studies
- Targets of (other) endogenous miRNAs were significantly upregulated at RNA level
- Low fold changes
- Low dose was 100,000 copies per cell!

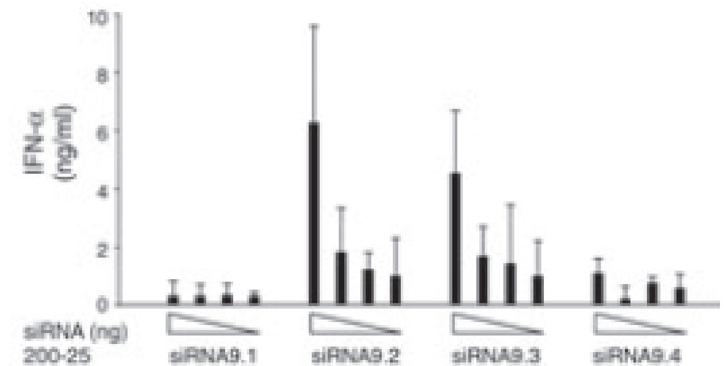
nature biotechnology

Stimulation of the innate immune system

Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA

Adam D Judge, Vandana Sood, Janet R Shaw, Dianne Fang, Kevin McClintock & Ian MacLachlan

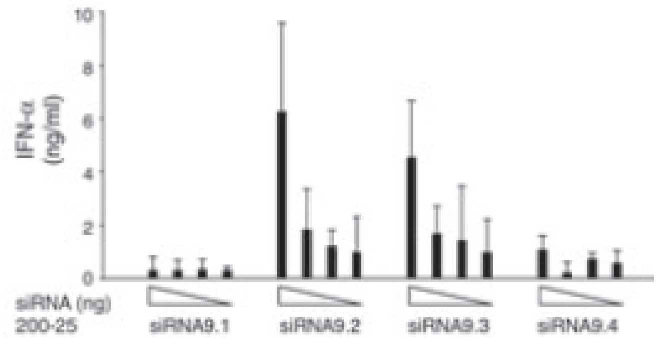




Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7

nature medicine

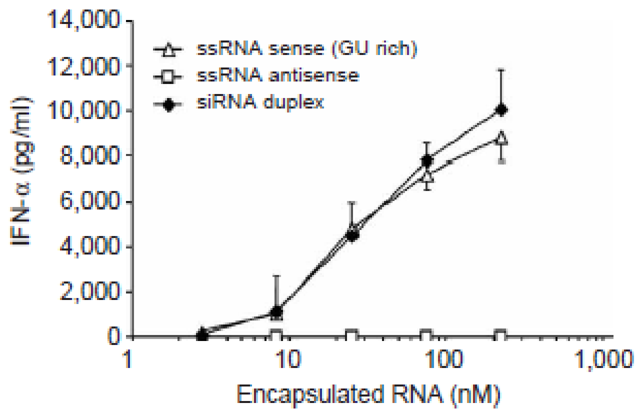
Veit Hornung[1], Margit Guenthner-Biller[1], Carole Bourquin[1], Andrea Ablasser[1], Martin Schlee[2], Satoshi Uematsu[4], Anne Noronha[3], Muthiah Manoharan[3], Shizuo Akira[4], Antonin de Fougerolles[3], Stefan Endres[1] & Gunther Hartmann[1]



Stimulation of the innate immune system

By the numbers: Hornung, et al.

- Cultured 50,000 pDCs per well
- Added 25 to 200 ng siRNA per well
- 2 trillion to 16 trillion copies of siRNA per well
- =40 million to 320 million copies per cell



Stimulation of the innate immune system

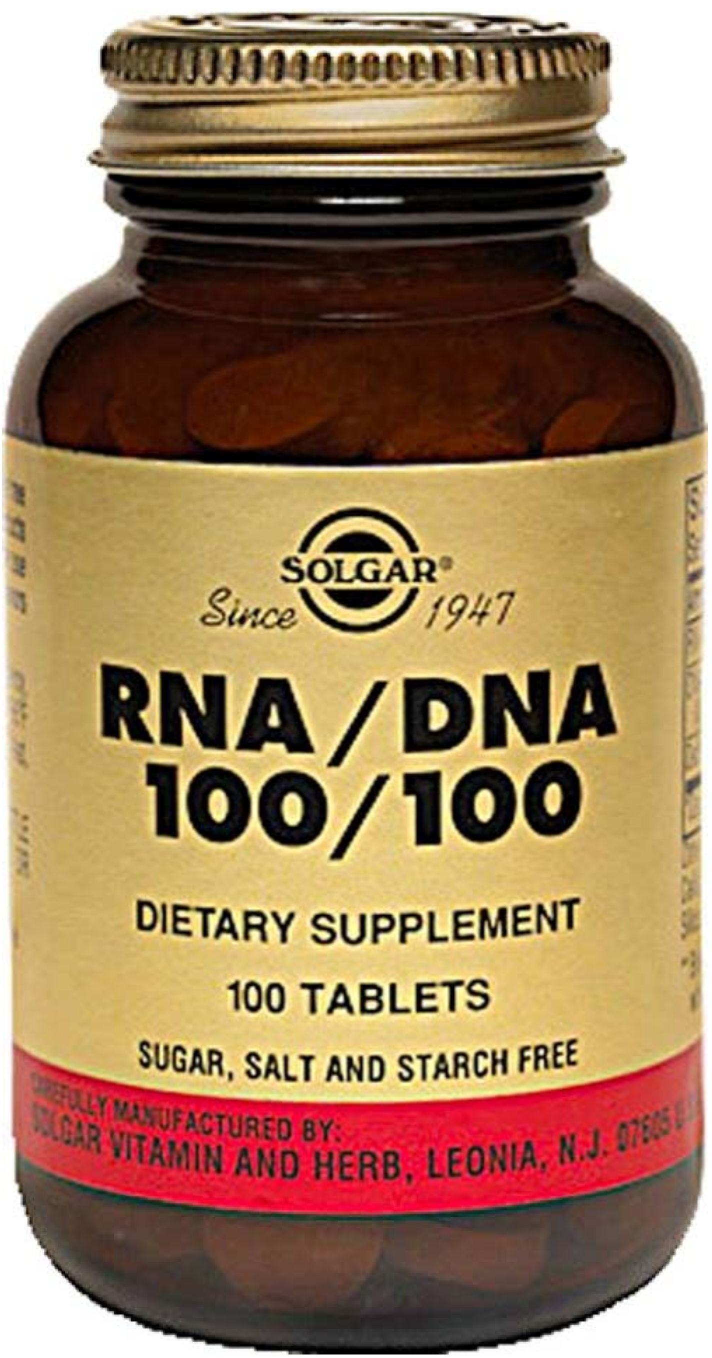
By the numbers: Judge, et al.

- 50 ug injections; 2 mg/kg > 4 quadrillion molecules/mouse
- Low dose for effect *in vitro*: 10 nM w/ transfection
  - No stimulation without transfection!
  - PBMC, 200,000/well
  - 6 million copies/cell

Off-target effect	Observations
Unintended siRNA effects	Not known to occur in mammals despite dietary exposure to exact matches
Off-target miRNA-like effects	*In vitro* studies; off-target miRNA effects vanish below 100,000 copies per cell
Saturation of the endogenous machinery	Conflicting evidence: some RNAs do not saturate; lowest dose examined is 100,000 copies per cell
Stimulation of innate immunity	Stimulation not uniform; appears to require exposure of millions of copies per cell

How much there is there there?

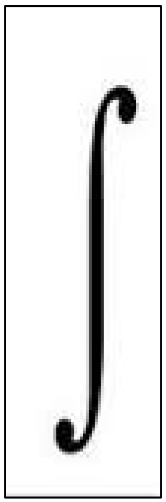
Uptake of dietary RNA by mammals

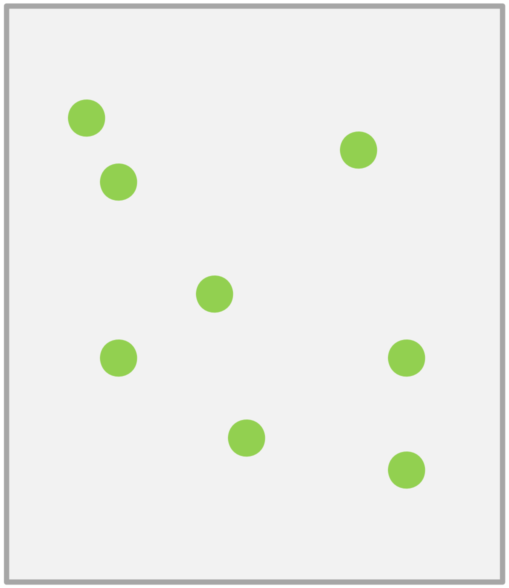


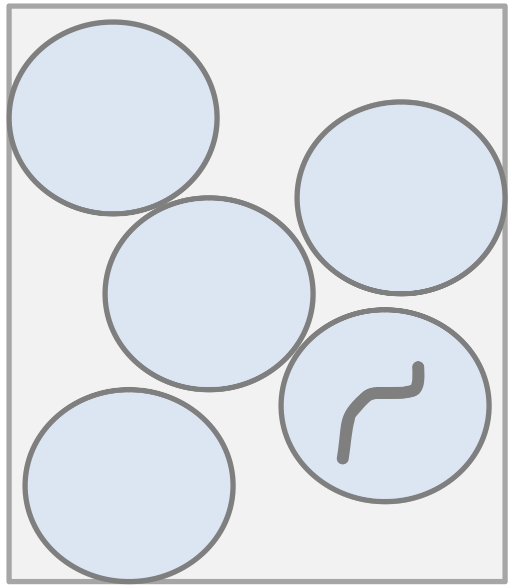
- Nucleic acids in the diet
- Generally regarded as safe (GRAS)—FDA, 1992
- We eat up to gram quantities of RNA/day
- RNA is labile in solution due to its chemical makeup
- Exposure to hostile enzymatic environments

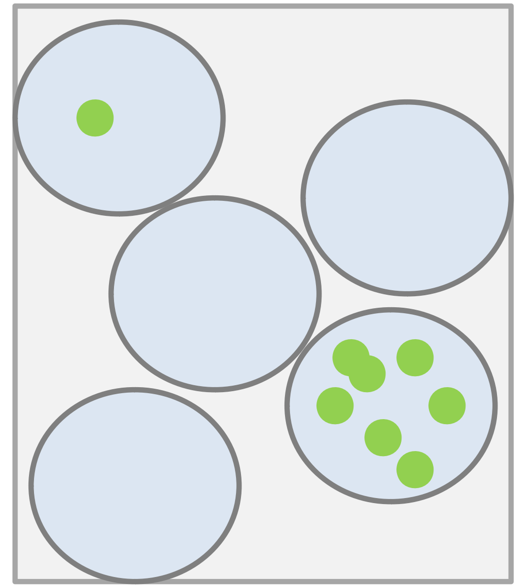
Traditional hydrolysis probe qPCR

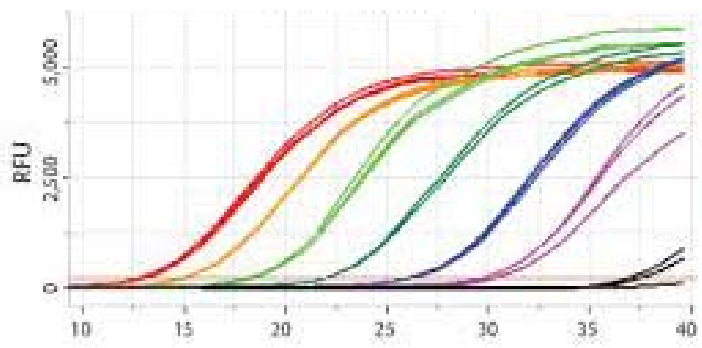
Emulsion “Droplet Digital” PCR



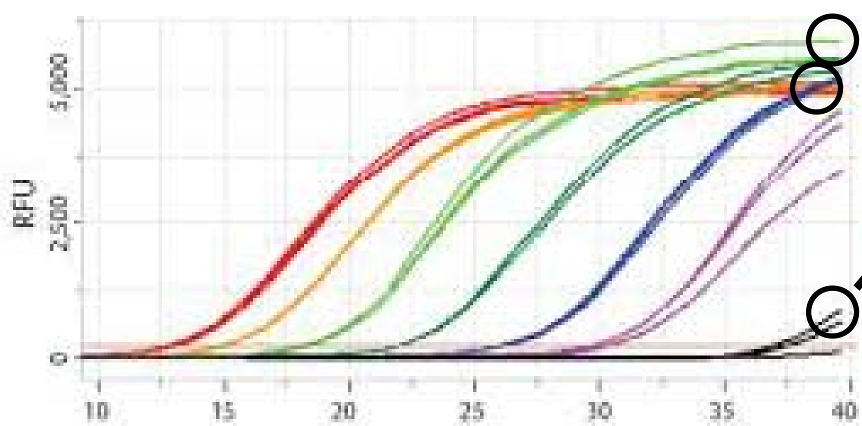


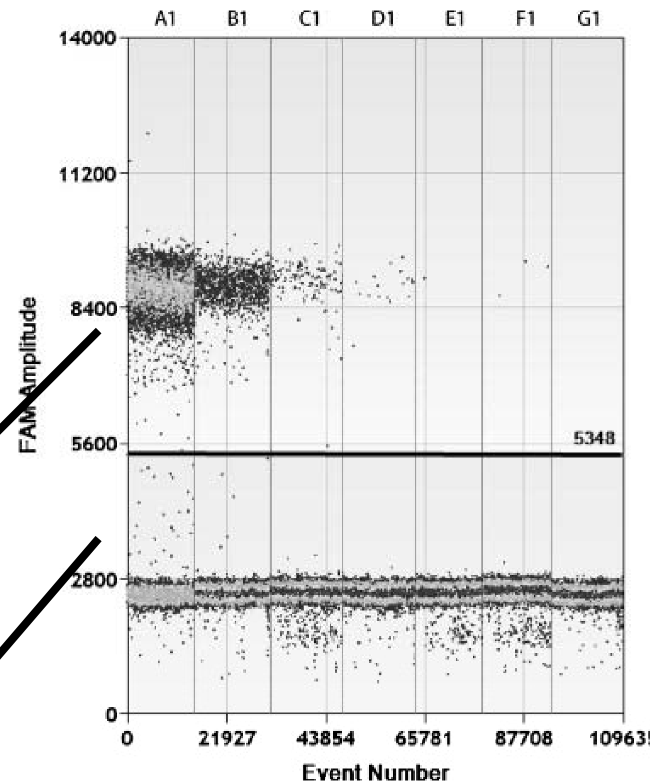






Droplet digital PCR (ddPCR)

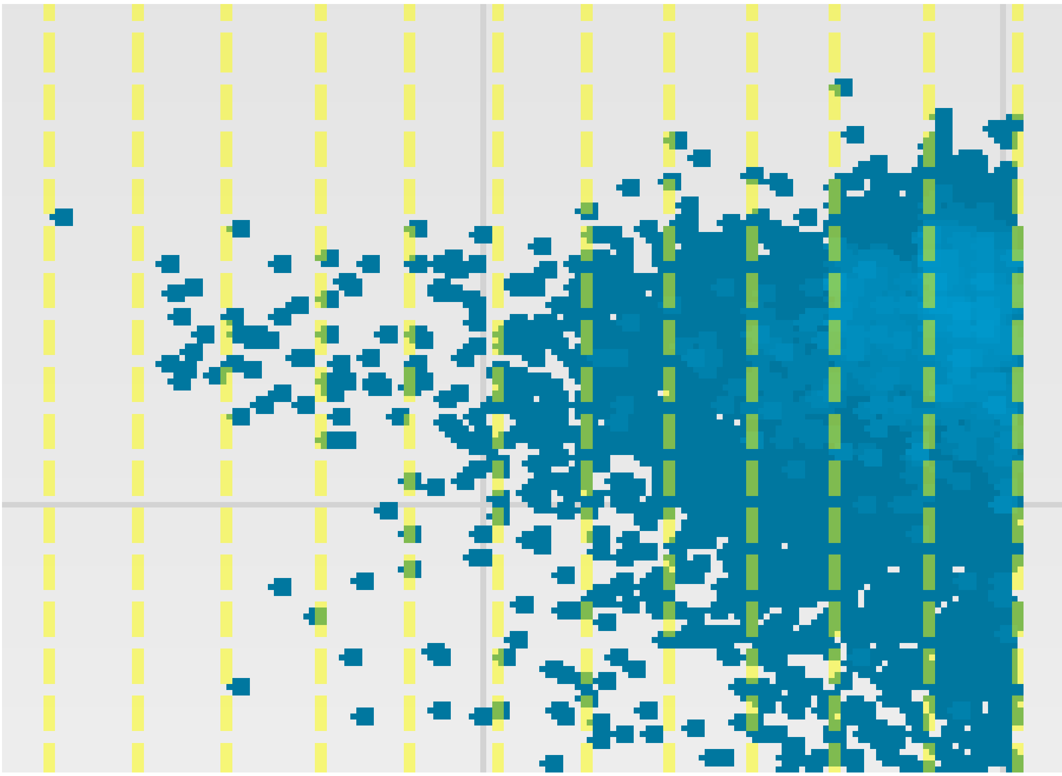




Henrich, *et al., J Virol Methods*, 2012

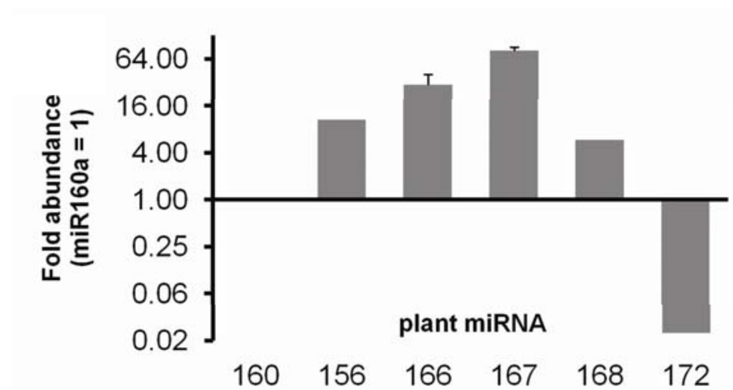
ddPCR Perspective: sensitivity

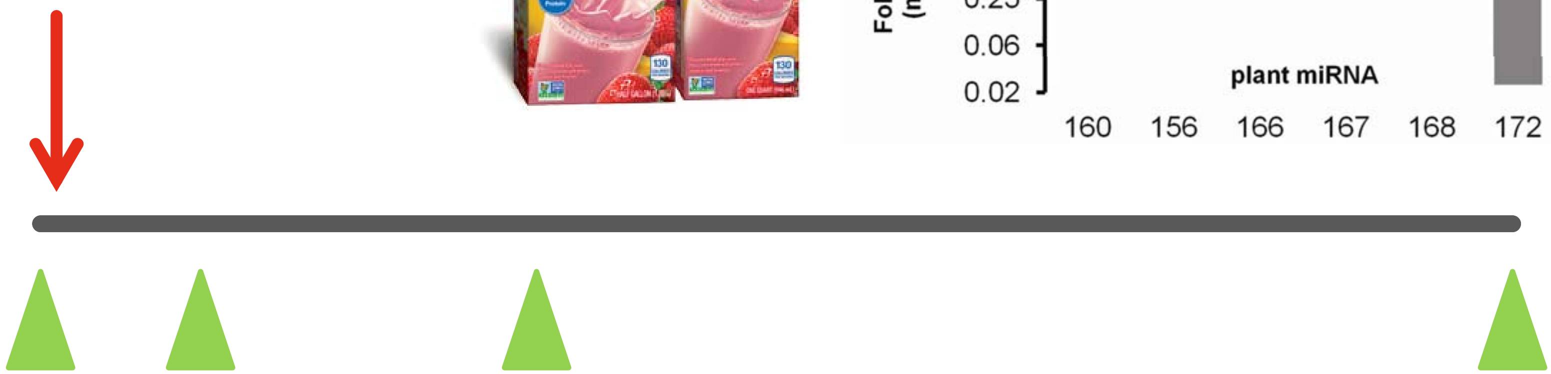
Expected	Observed
256	261
128	134
64	62
32	31
16	17
8	8
4	4
2	2
1	1
0.5	0.8
0	0



Pilot design: mammalian uptake

Two pigtailed macaques
Gavage: ~5% of estimated blood volume





0 1 4 12

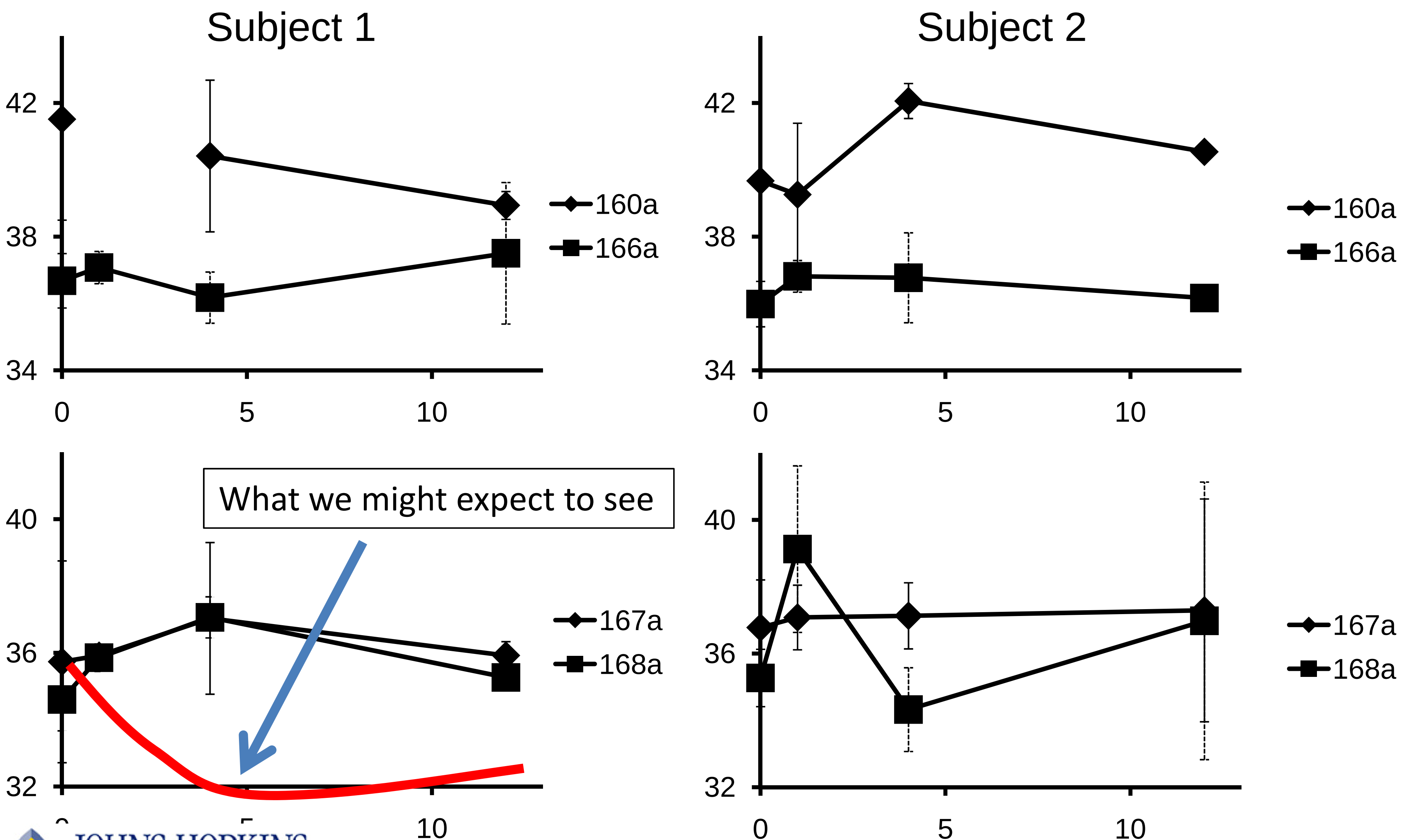
Blood draws: hours post-gavage

→Immediate processing to platelet-poor plasma
Initial RNA extraction by Ambion mirVana protocol

JOHNS HOPKINS MEDICINE

Witwer, *et al*, *RNA Biology*, 2013

Results with optimized Exiqon Biofluids RNA



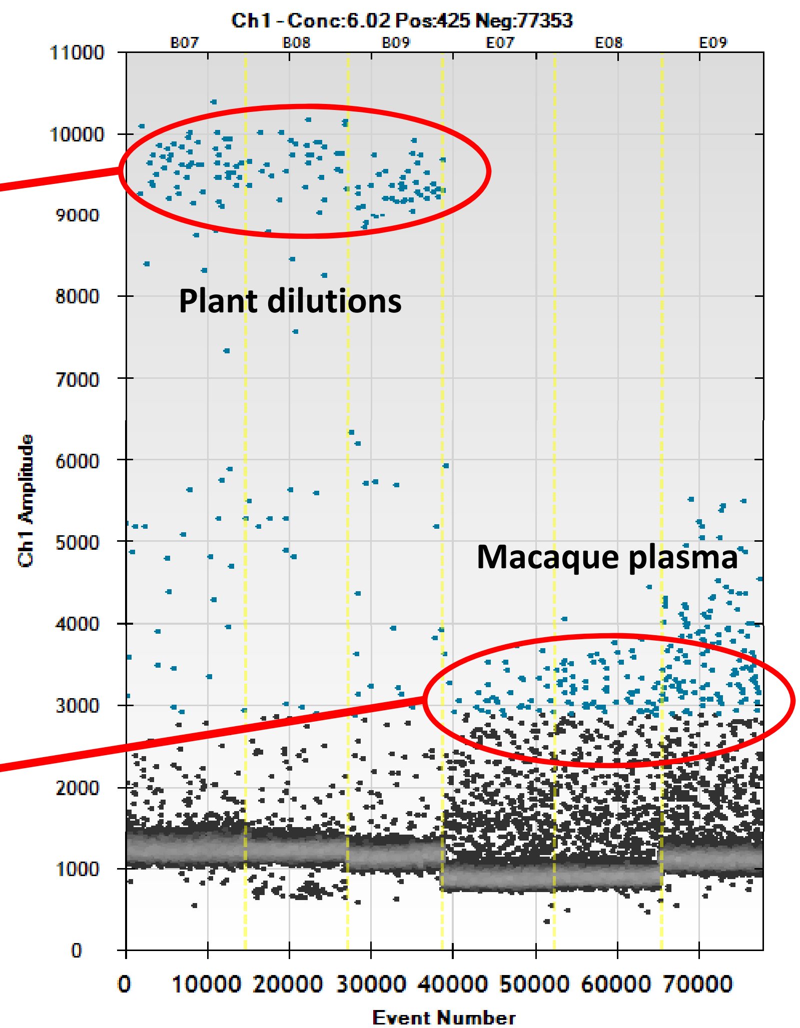
JOHNS HOPKINS MEDICINE

Witwer, *et al*, *RNA Biology*, 2013

Droplet digital PCR results

Single predominant product in highly diluted plant material = specific

Non-specific amplification



miR168: one of the most abundant miRNAs in the original study

Adapted from: Witwer, *et al*, *RNA Biology*, 2013

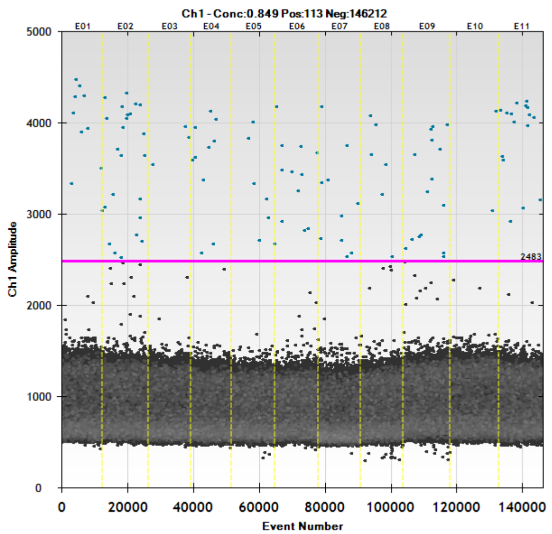
Negative feeding studies

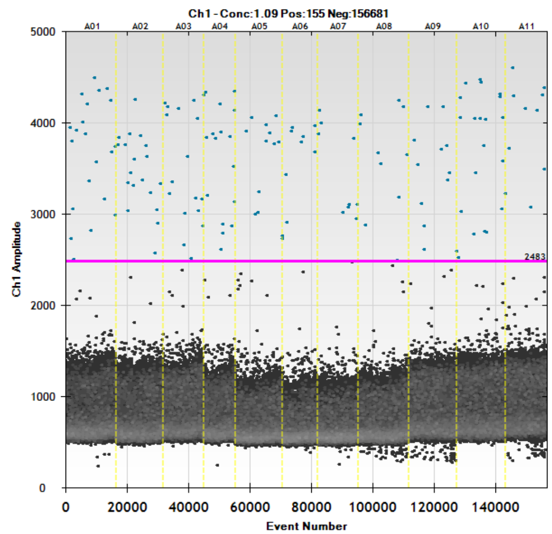
- Snow, et al., *RNA Biology*, 2013
  - Negligible or no detected uptake in bees, mice, humans with diets replete with microRNA
- Witwer, et al. *RNA Biology*, 2013
  - Nonhuman primates: no increase in response to dietary intake; low-level detection was non-specific
- Dickinson, et al., *Nature Biotechnology*, 2013
  - Negligible uptake in mice with rice diets (more MIR168a than in Zhang, et al.)
  - No LDLRAP1 response to feeding
  - Mouse LDL increase was due to nutritional insufficiency

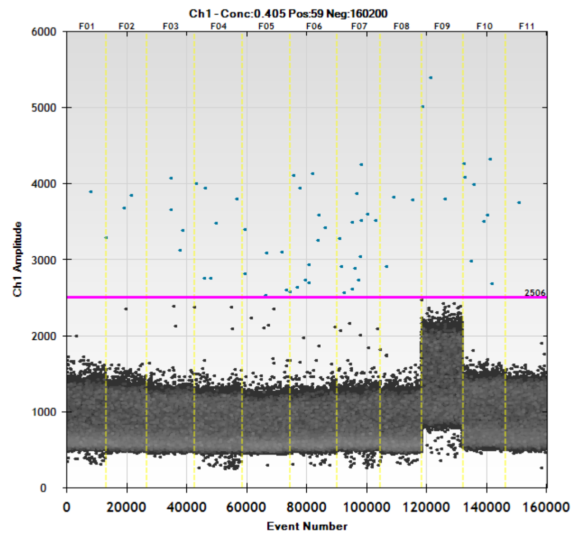
Additional negative findings

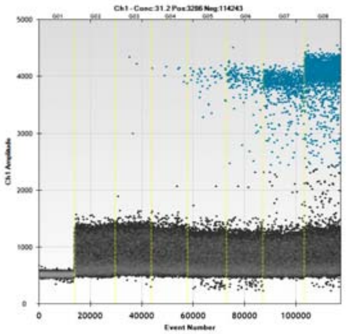
- Zhang, et al., *BMC Genomics,* 2012
  - Public dataset analysis
  - Few plant miRNAs detected, at low copy numbers
  - MIR168a consistent with artifact
- Wang, et al., *PLOS One*, 2012
  - human study; low read numbers of MIR168a only
  - No increased uptake with colitis, colon cancer
- Wang, et al., *Toxicol Sci*, 2013
  - mouse liver toxicity study; low MIR168a only
- Tosar, et al., *RNA*, 2014
  - Sequencing reads likely due to contamination

Specificity: Where to draw the line?









Standards for comparison

MIR156a
Intensity plots for selected donors
from unpublished feeding experiment

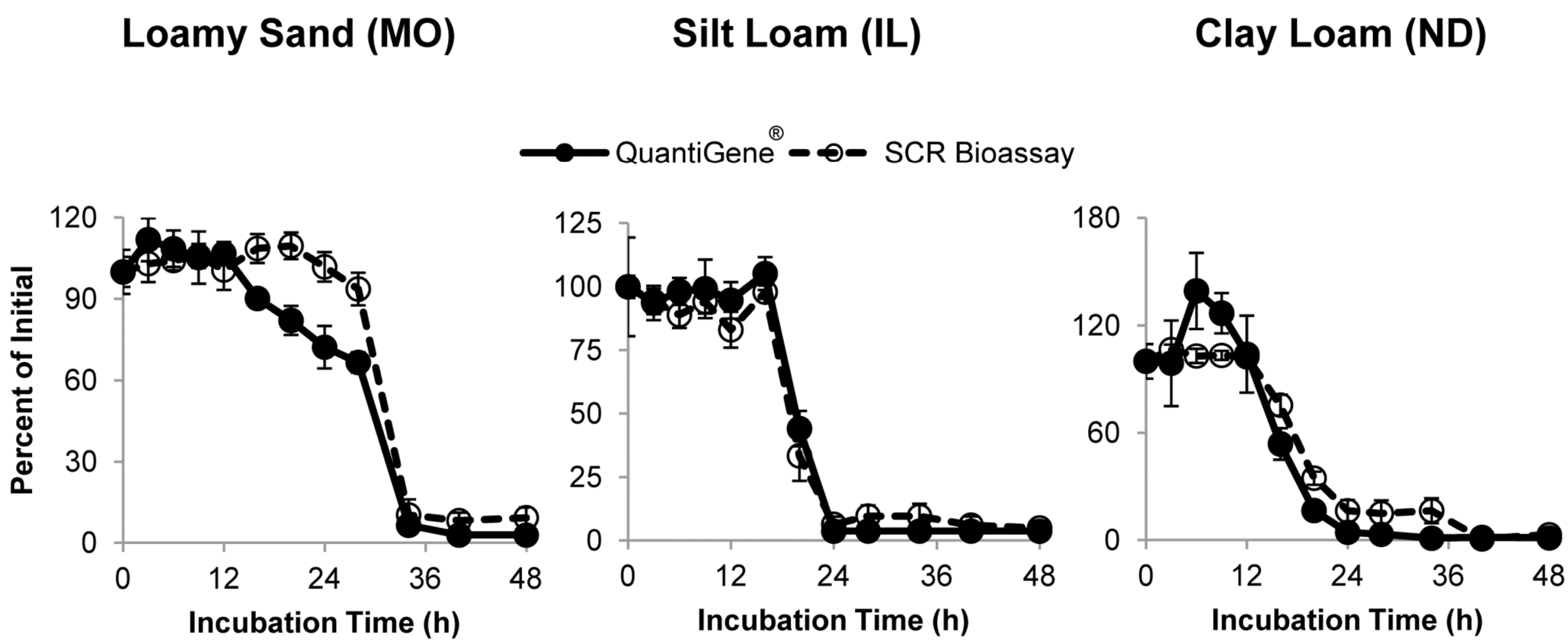
Positive study

- Zhang, et al., *Cell Research*, 2012
- Increase from ~2.5 to 7 fM in mouse blood
  - Maximal concentration: 3 hours post-feeding
- 7 fM, 53 fg/mL, 7 attomoles = 4.3 million molecules of miRNA per mL
- Take a 20 g mouse with 1.1 mL blood and a 1 g liver
  - 4.7 million copies of miRNA/135 million liver cells = 1 copy per 28 liver cells
  - Compare: 60 million copies/cell in luciferase reporter experiments (1.7 billion-fold difference)

Per cell exposure comparison

>1E5	• Off-target miRNA effects • Saturation of machinery
6E6 +	• Immune stimulation (some RNA lengths/sequences)
1E7 +	• Typical treatment for 3’ UTR luciferase assays
< 1	• In vivo max exposure based on *highest, unconfirmed* reported circulating dietary RNA concentration

What *is* exposure level? (Diet, environment)
Lability and low abundance of PIP dsRNA in soil



Dubelman, et al., PLOS ONE, 2014

Conclusions

- To date, no validated finding of dietary RNA transfer→function
- The various off-target reports are:
  - mostly *in vitro* studies
  - sometimes contradictory
  - involve orders of magnitude more *transfected or transduced* RNA than the highest reported concentrations of transferred dietary RNA *in vivo*
- Contamination and specificity concerns may imply even lower dietary exposure than some reported
- Realistic environmental and dietary exposure scenarios are incompatible with mammalian off-target effects of PIP RNAi

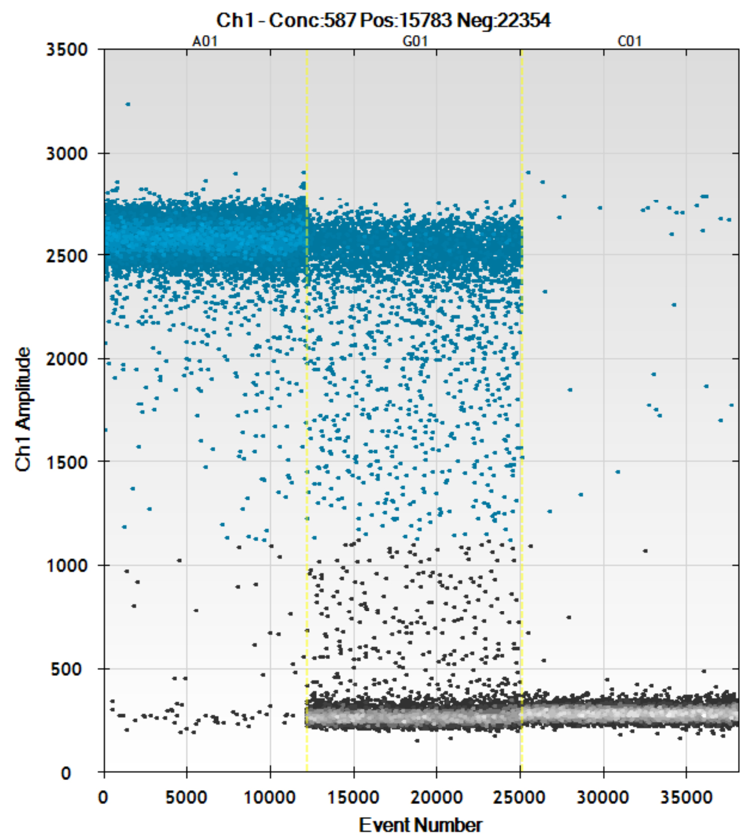
Questions that remain: uptake and function

- Are “absorbed” RNAs protected by Argonaute? How are they transferred into the blood?
- Into the cell
  - How, and could a plant Argonaute-complexed plant small RNA function in a mammalian cell? (No.)
- How many copies of a *functional* RNA needed?
- Are there off-target effects we haven’t yet explored? (Exploratory science, little or no data)

Gut injury model to query low-level uptake?

Herbal treatment→
Gut damage (Kendal Hirschi)

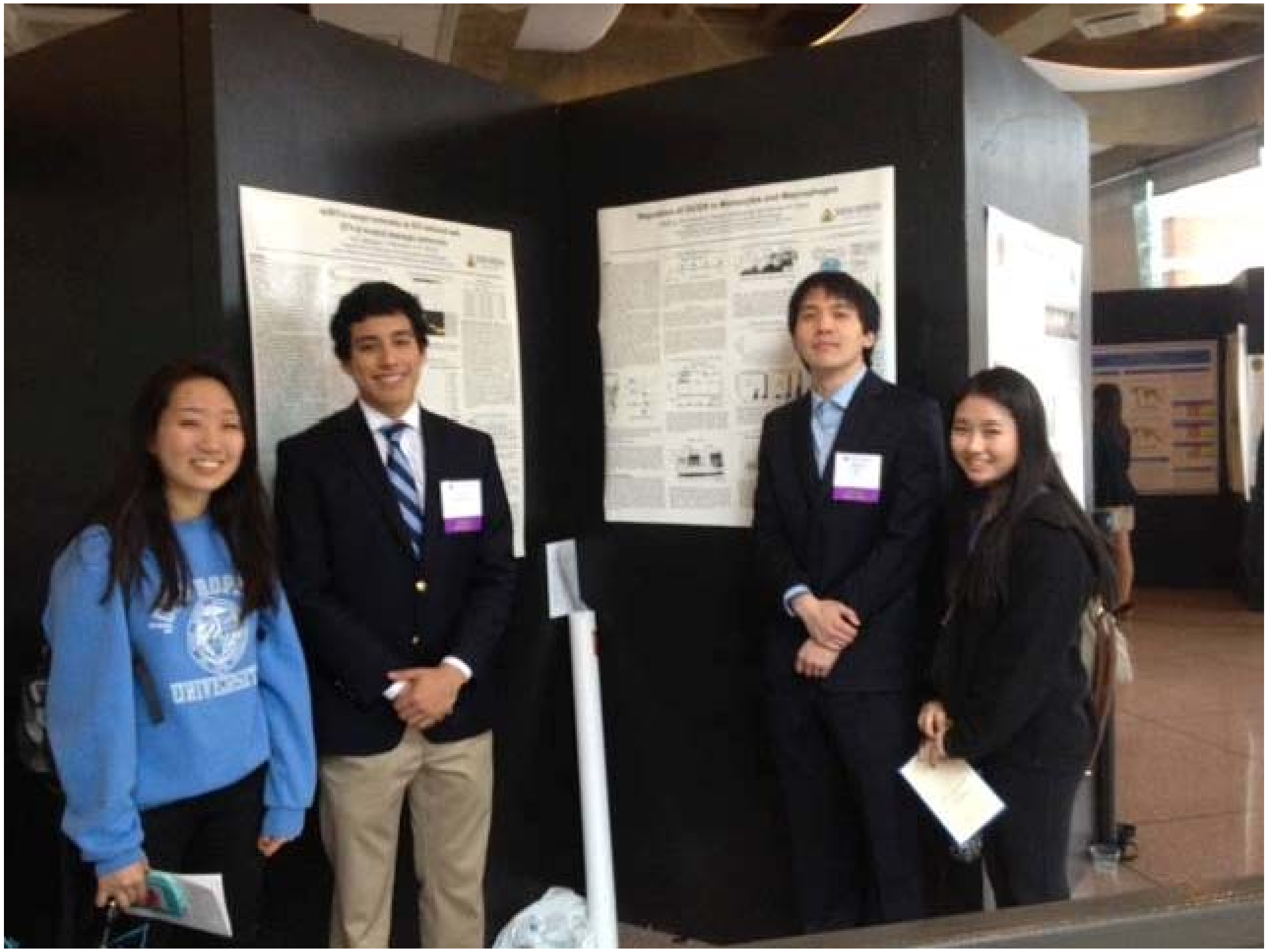
Measured a highly abundant plant small RNA (plasma), MIR2911, derivative of rRNA



Plant treated control

JOHNS HOPKINS MEDICINE

Acknowledgements



Johns Hopkins
Amanda Brown
Joel Blankson
GRCF DAF
MCP Retrovirus Lab
Bob Adams
Janice E. Clements
Chris Zink
Joe Mankowski
David Graham
Lucio Gama
Kelly Metcalf Pate

Baylor COM
Kendal Hirschi and lab

ISEV
Yong Song Gho, Jan Lötvall

Melissa McAlexander
Erin Buchanan
Suzanne Queen

Diego Espinoza
Grace Hancock
Veronica Kim

Funding
JHU CFAR 1P30AI094189
NIAID R21 AI102659
R01 NS076357, NIMH Pilot Grant

Method optimization needed?

Problem with plant RNA modification(s), e.g. 2'-O-methyl?

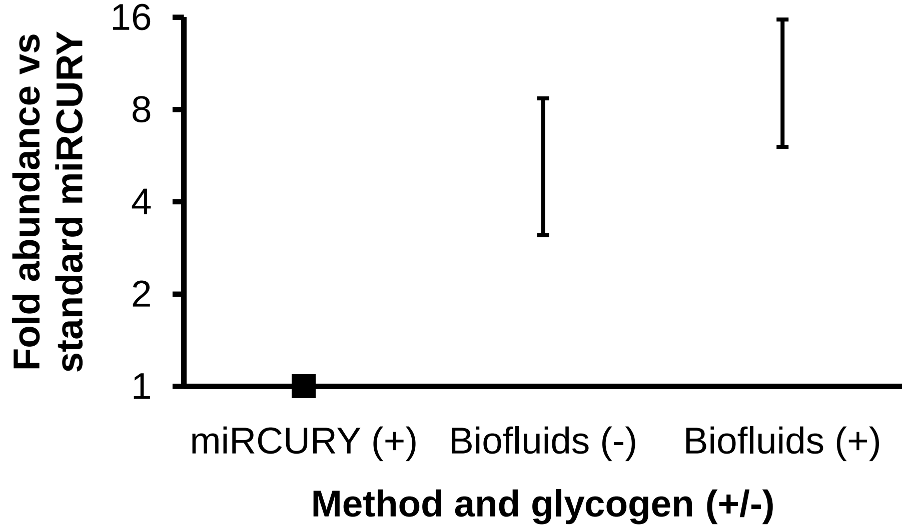
--No; very sensitive detection of plant miRNAs

Low abundance RNAs missing from recovered sample?

New biofluids RNA method from Exiqon (12/2012)

Improved recovery, inhibitor removal

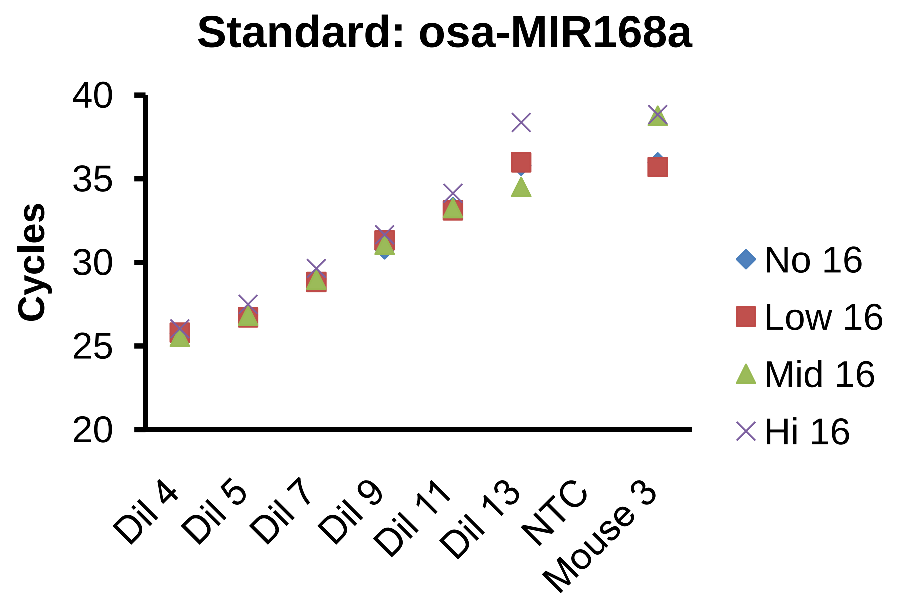
Verified performance

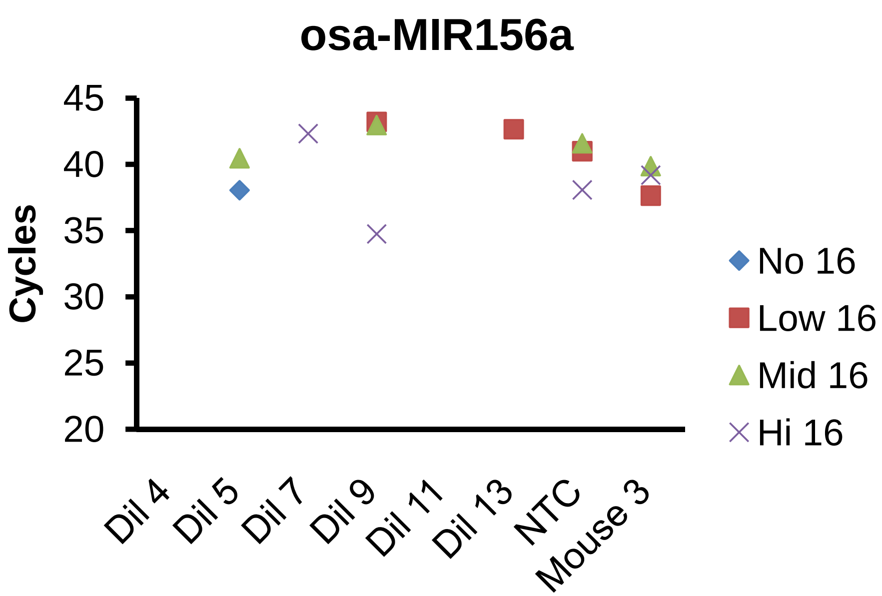


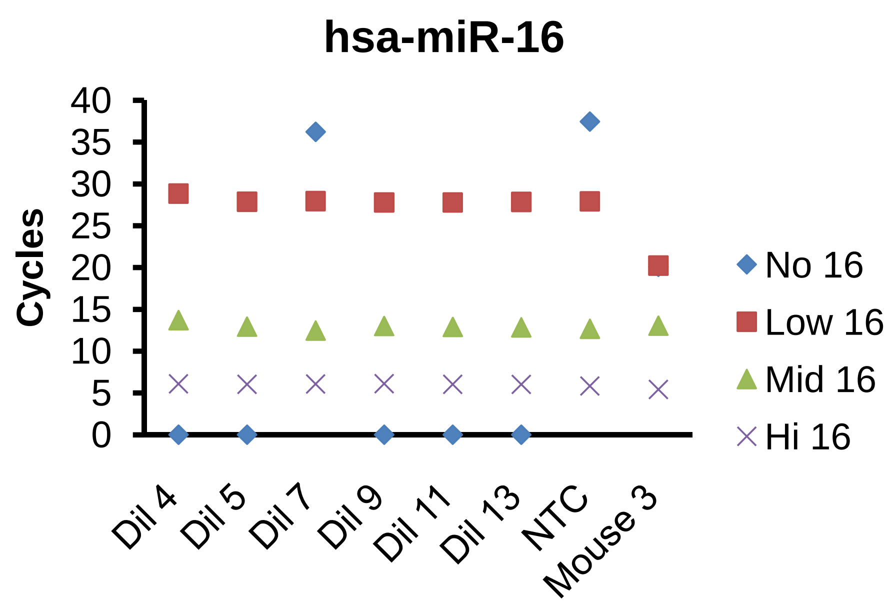
McAlexander, *et al, Trends in Genetics*, 2013

Recent suggestion

Low sensitivity because specific reverse transcriptases work best in presence of excess miR-16.
We pre-incubated synthetic miR-16 at several concentrations before RT of osa-MIR168a standard curve.
→No effect of miR-16 addition







Conclusions from Liang, et al, *Food Science and Nutrition*, 2014

- MIR172 is absent in mice with normal diets
- Uptake seen with 50 ug purified RNA, but not 30 ug or 10 ug
- Perspective
  - 50 ug RNA in a mouse is akin to 175 mg in a human

New mouse blood calculations

- Methods are somewhat unclear; "copies" provided, but no input information
- Assuming RNA extraction from 500 ul blood, resuspension in 20 ul, and qPCR input of 1.25 ul cDNA (based on methods)
- About 300,000 copies of miRNA per mL blood
- > 1 order of magnitude lower than reported for MIR168 by Zhang, et al.
  - At maximal concentration in blood, 1 copy per 500 liver cells
  - If "copies" means copies/mL, much lower!
  - Plus, authors mention: kidney and liver "devoid" of exogenous RNA "even long after a single feeding."