Impact of RNAi products on mammalian health

Food stories and food facts

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Retrovirus Laboratory

6 July, 2015

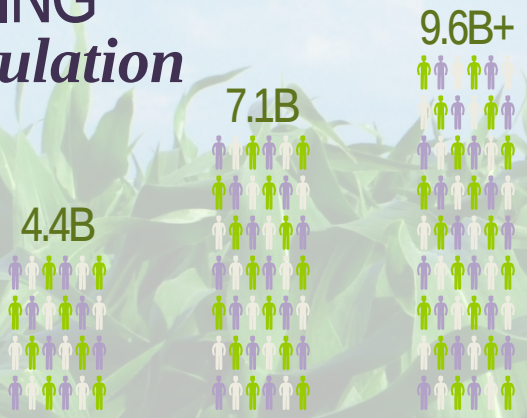
JOHNS HOPKINS MEDICINE

THE RETROVIRUS LABORATORY

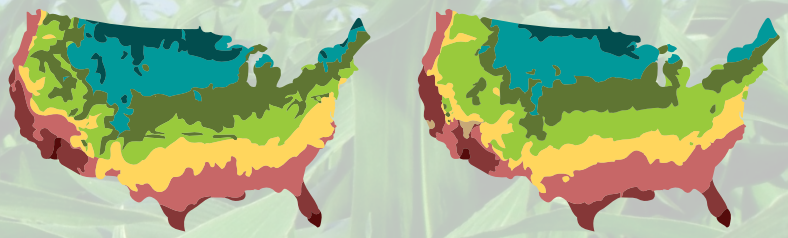
Why GM crops?
Why the strong interest of the US EPA, EFSA, other regulators?

We Will Need to Grow as Much Food in the Next 50 Years as in the Past 10,000 Years Combined

RISING *population*



CHANGING *climate*



DECLINING *arable land*

1 ACRE *per person in* 1961

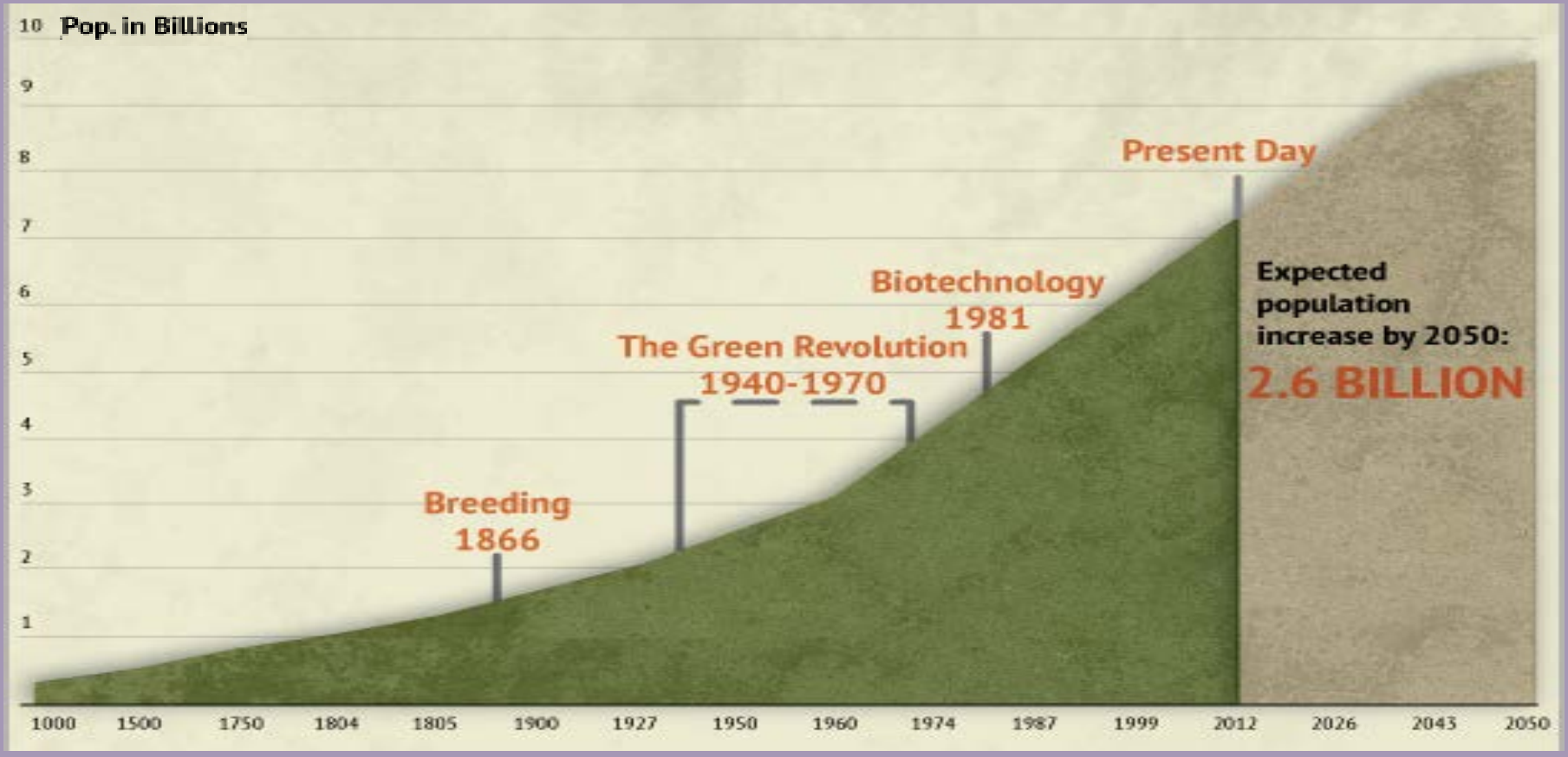
less than 1/3 ACRE *per person in* 2050

CHANGING *economies & diets*

DIETARY PERCENTAGE OF MEAT

Source: The World Bank, Food and Agriculture Organization of the United Nations (FAO-STAT), Monsanto Internal Calculations

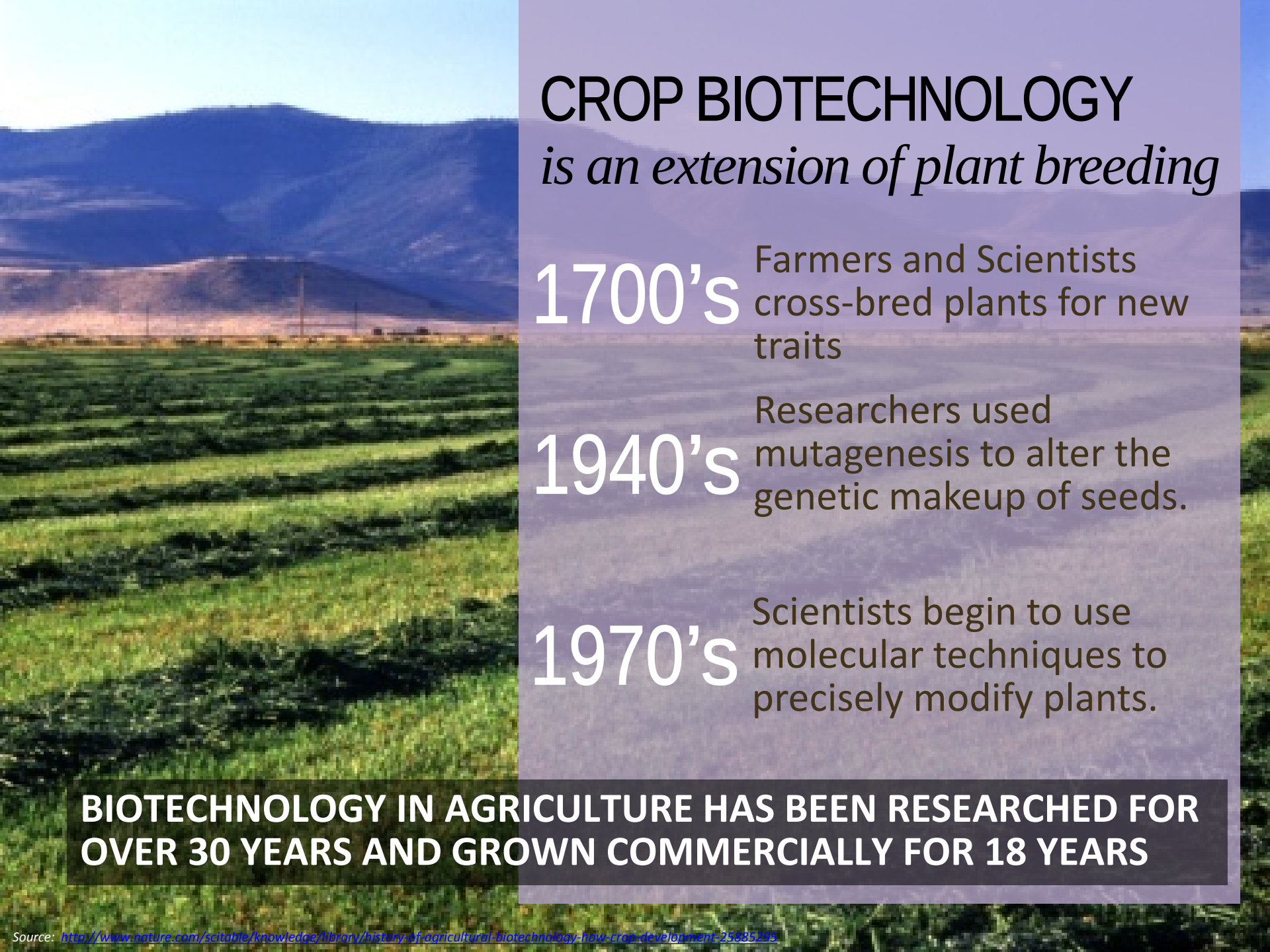
History Shows Us that Advancements in Technology Have a Huge Impact on Agriculture



The rate of population increase exceeds the rate of increase in food production

-Dr. Normal Borlaug

Source: United Nations, The Danforth Center, USDA



CROP BIOTECHNOLOGY

is an extension of plant breeding

1700's Farmers and Scientists cross-bred plants for new traits

1940's Researchers used mutagenesis to alter the genetic makeup of seeds.

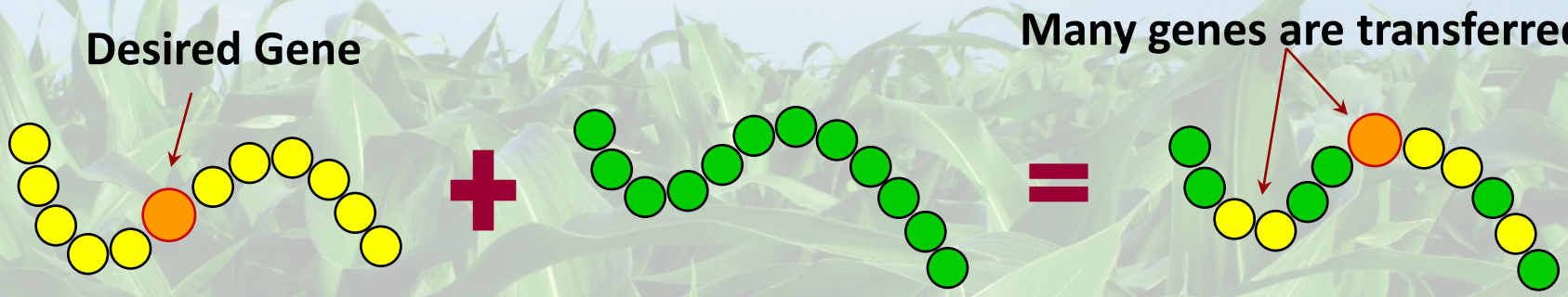
1970's Scientists begin to use molecular techniques to precisely modify plants.

BIOTECHNOLOGY IN AGRICULTURE HAS BEEN RESEARCHED FOR OVER 30 YEARS AND GROWN COMMERCIALLY FOR 18 YEARS

Source: http://www.nature.com/scitable/knowledge/library/history-of-agricultural-biotechnology-how-crop-development-25885295

Crop Biotechnology is an Extension of Traditional Plant Breeding

TRADITIONAL PLANT BREEDING



PLANT BIOTECHNOLOGY

Biotechnology is Used in Many Common Products

CHEESE

Nearly all cheese is produced using rennin produced through biotechnology, instead of naturally occurring rennin, extracted from calf stomachs.

YEAST

Unique flavors are created through biotechnology engineering of yeast varieties, for use in beer brewing and bread making.

MEDICINE

Most insulin used by diabetics is produced using the human DNA sequence of insulin through biotechnology, rather than extracting insulin from the pancreas of pigs or cows.

Source: http://www.nature.com/scitable/knowledge/library/history-of-agricultural-biotechnology-how-crop-development-25885295

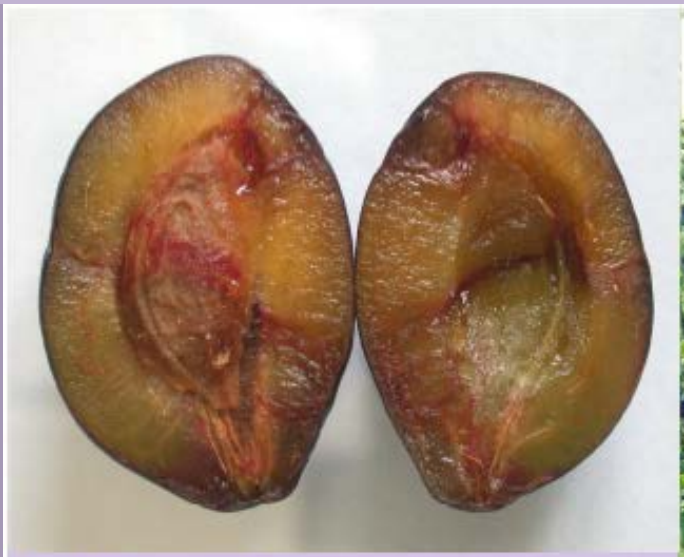
Genetic biotechnology

- **Mutation of an existing gene**
- **Removal of a gene**
- **Introduction of a new gene**
  - **From a different strain or close relative**
  - **From a distant organism**
- **Use of noncoding RNA regulation**

“Rainbow” Papaya: RNAi-based GM

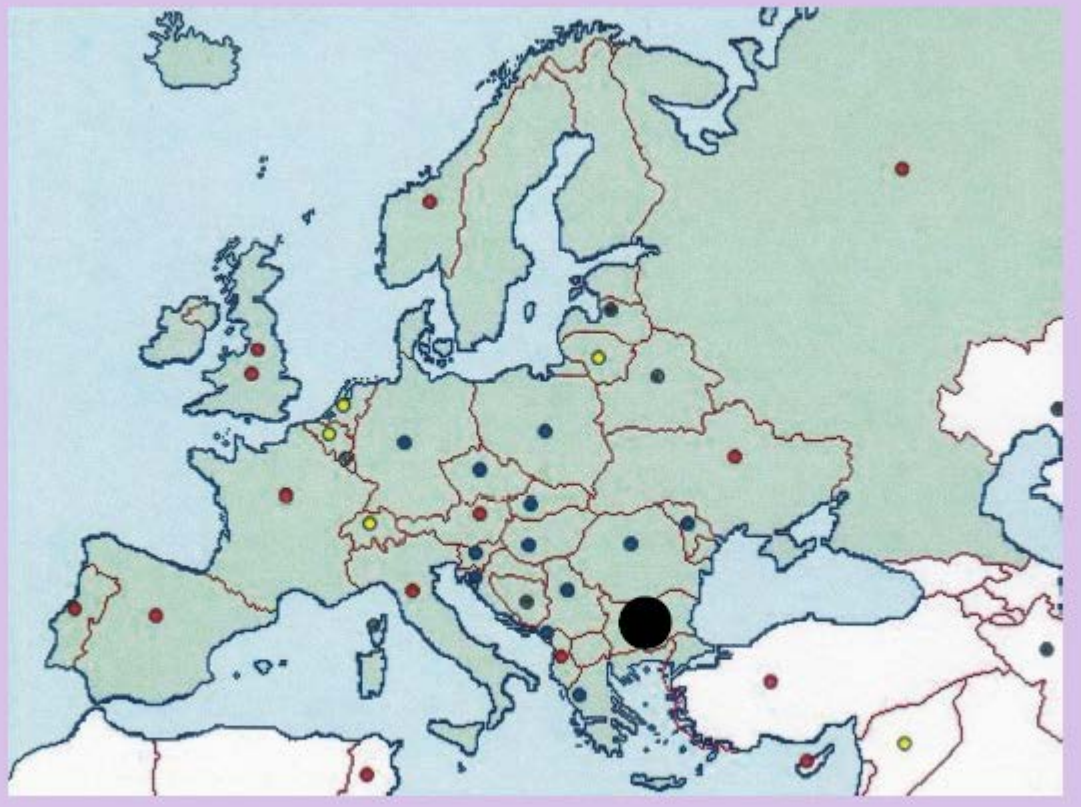
- Papaya ringspot virus a major economic problem
- 1992: field trials started in Hawaii
- 1998: licensure, cultivation
- Pathogen-derived resistance—coat protein gene
  - Actually RNAi

Plum pox virus (PPV)



Sharka (*Plum pox virus*)

PPV SPREAD – EUROPE



widespread
localized
occasional reports

http://www.cabi.org/isc/datasheet/42203

Ralph Scorza

PPV infection in selected European countries:

Bosnia-Herzegovina - **41%** of plum trees infected

Croatia - **51%** plum trees infected

Serbia - **58%** plums infected with PPV

Bulgaria - **62%** infection in plums

Romania – **69%** infection in plums

Moldova - plum yield losses plum are **16-48%**

Greece – apricot production decreased from **35% to 13%** of world production due to PPV

Spain - **2.3 million** PPV-infected trees removed between 1989 and 2006 at a cost of over **63 M Euros**

2006 OEPP/EPPO 36 (2)
Zagrai et al UASMV 67 2010

Ralph Scorza

International 'HoneySweet' Working Group

- 'HoneySweet' co-developed by U.S. and European team
- Field tested in Europe for over 17 years
- Solely the work of publicly-supported scientists, at public research institutions
- For the benefit of growers and consumers

Ralph Scorza

Corn and corn rootworm: RNAi

Control of Coleopteran Pests Through RNA interference

James A Baum, Thierry Bogaert, William Clinton, Gregory R Heck, Pascale Feldmann, Oliver Ilagan, Scott Johnson, Geert Plaetinck, Tichafa Munyikwa, Michael Pleau, Ty Vaughn & James Roberts

November 2007, Volume 25 No 11 pp1187-1328

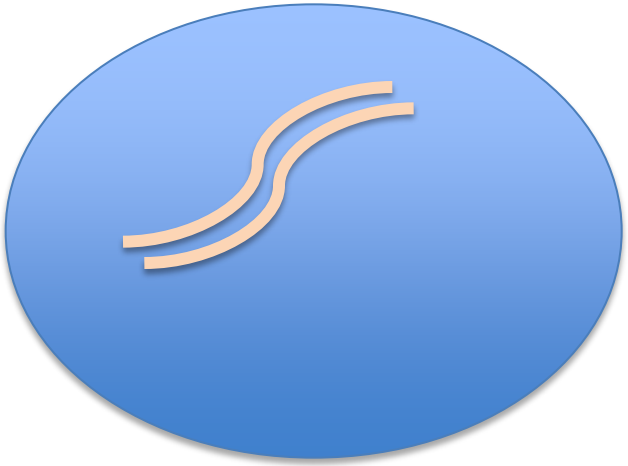
Western Corn Rootworm

Coleoptera: Chrysomelidae:

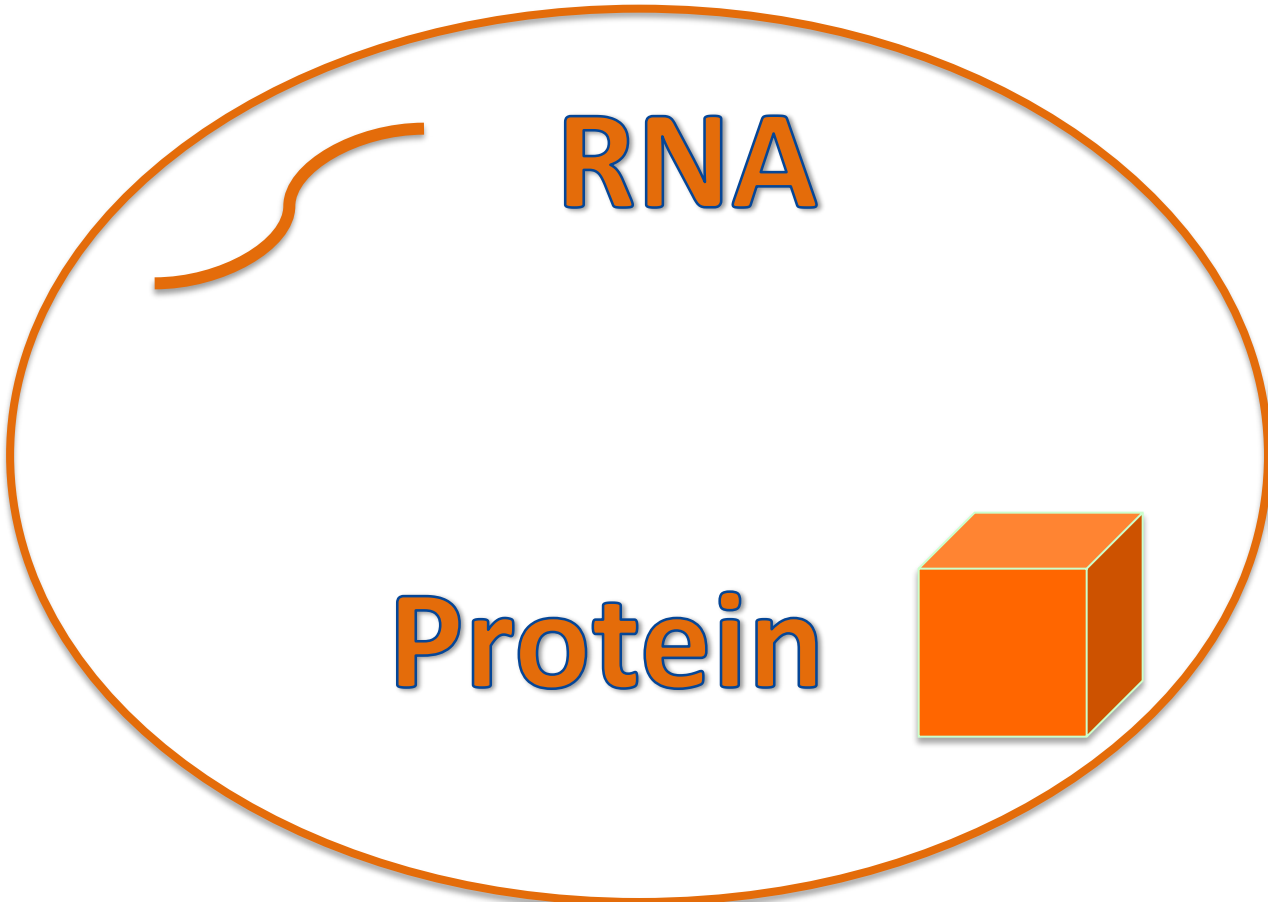
Diabrotica virgifera virgifera

Non-transgenic corn Transgenic corn

What is RNA interference?



DNA

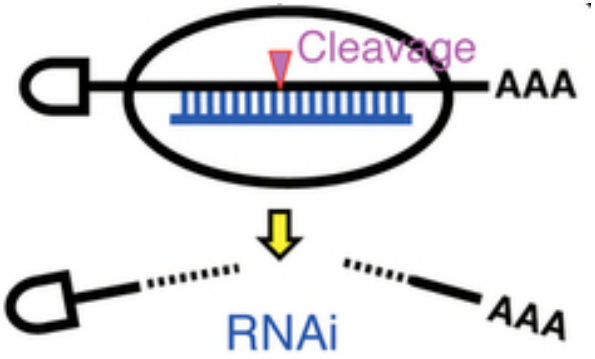


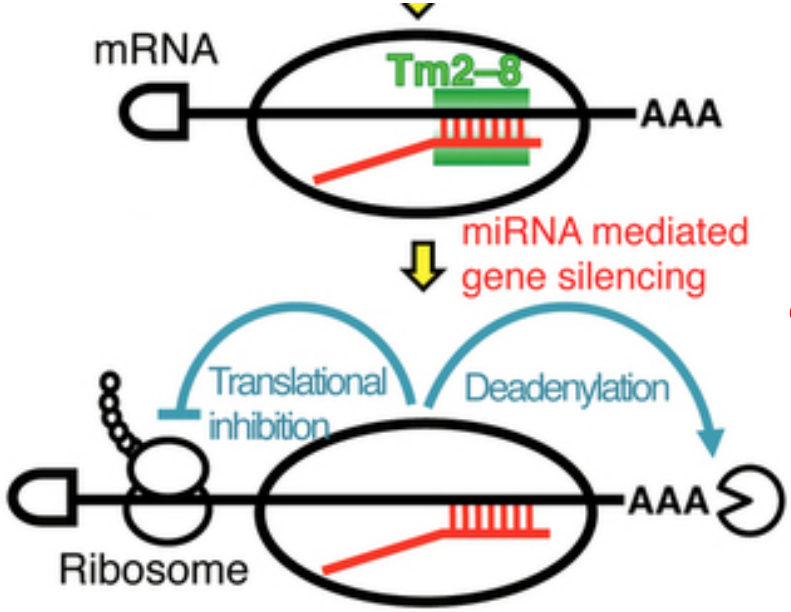
RNA

Protein

RNA interference

siRNA





miRNA

Hibio et al , Sci Reports, 2012

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We always wonder, when we see two people together, particularly when they're actually married, how these two people could have arrived at such a decision, such an act, so we tell ourselves that it's a matter of human nature, that it's very often a case of two people going together, getting together, only in order to kill themselves in time, sooner or later to kill themselves, after mutually tormenting each other for years for for decades, only to end up killing themselves *anyway*, people who get together even though they probably clearly perceive their future of shared torment, who join together, get married, in the teeth of all reason, who against all reason commit the natural crime of bringing children into the world who then proceed to be the unhappiest imaginable people, we have evidence of this situation wherever we look... People who get together and marry even though they can foresee their future together only as a lifelong shared martyrdom, suddenly all these people *qua* human beings, human beings *qua* ordinary people... enter into a union, into a marriage, into their annihilation, step by step down they go into the most horrible situation imaginable, annihilation by marriage, meaning annihilation mental, emotional, and physical, as we can see all around us, the whole world is full of instances confirming this... why, I may well ask myself, this senseless sealing of the bargain, we wonder about it because we have an instance of it before us, [illegible] come to be?

Words ruin one's thoughts, paper makes them ridiculous, and even while one is still glad to get something ruined and something ridiculous down on paper, one's memory manages to lose hold of even this ruined and ridiculous something. Paper can turn an enormity into a triviality, an absurdity. If you look at it this way, then whatever appears in the world, by way of the spiritual world so to speak, is always a ruined thing, a ridiculous thing, which means that everything in this world is ridiculous and ruined. Words were made to demean thought, I would even go so far as to state that words exist in order to abolish thought, and one day they will succeed one hundred percent in so doing. In any case, words (are) bringing everything down. Depression derives from words, nothing else. Words ruin one's thoughts,

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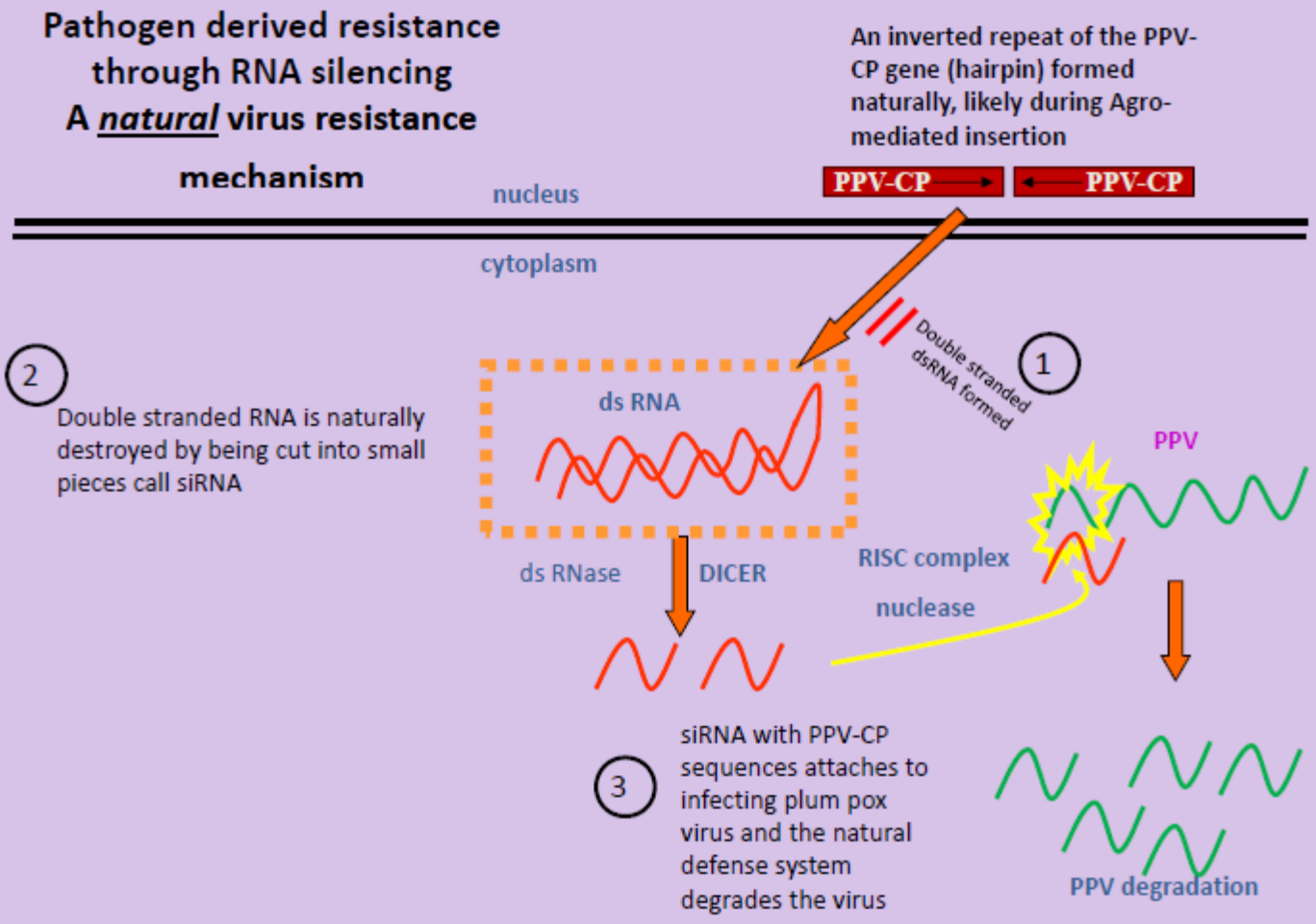
HONEYSWEET PPV RESISTANCE IS RNAi-BASED

Pathogen derived resistance through RNA silencing
A *natural* virus resistance mechanism



Ralph Scorza

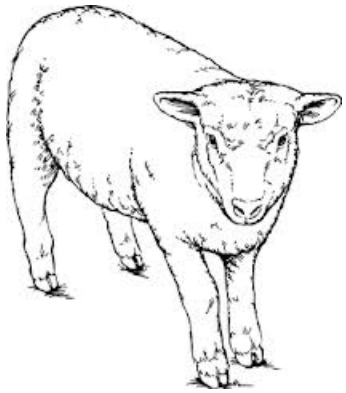
Why would a virologist care about small noncoding RNAs?

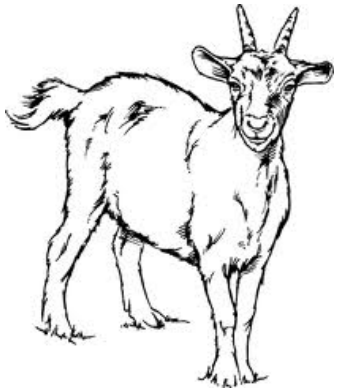
THE RETROVIRUS LABORATORY

The Johns Hopkins University Molecular and Comparative Pathobiology Retrovirus Laboratory

Director: J.E. Clements

Lentiviruses and animal models of HIV disease





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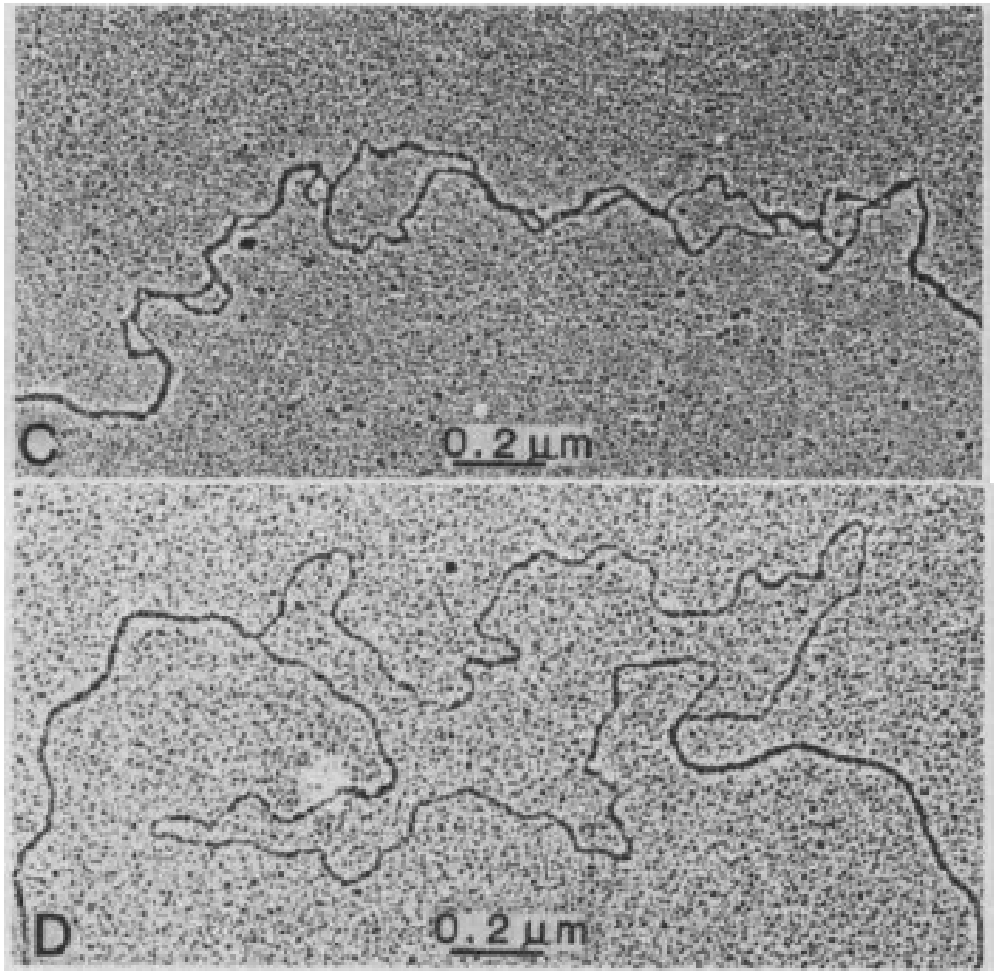
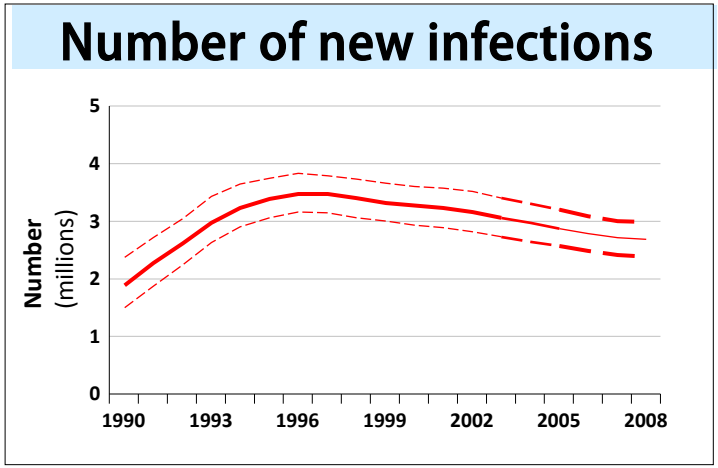
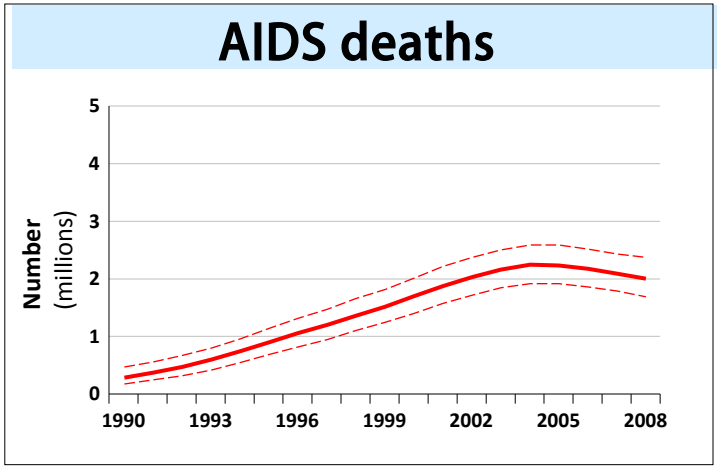


Gonda, et al. Science, 1985

HIV/AIDS: progress…and cure?

Source: UNAIDS/WHO





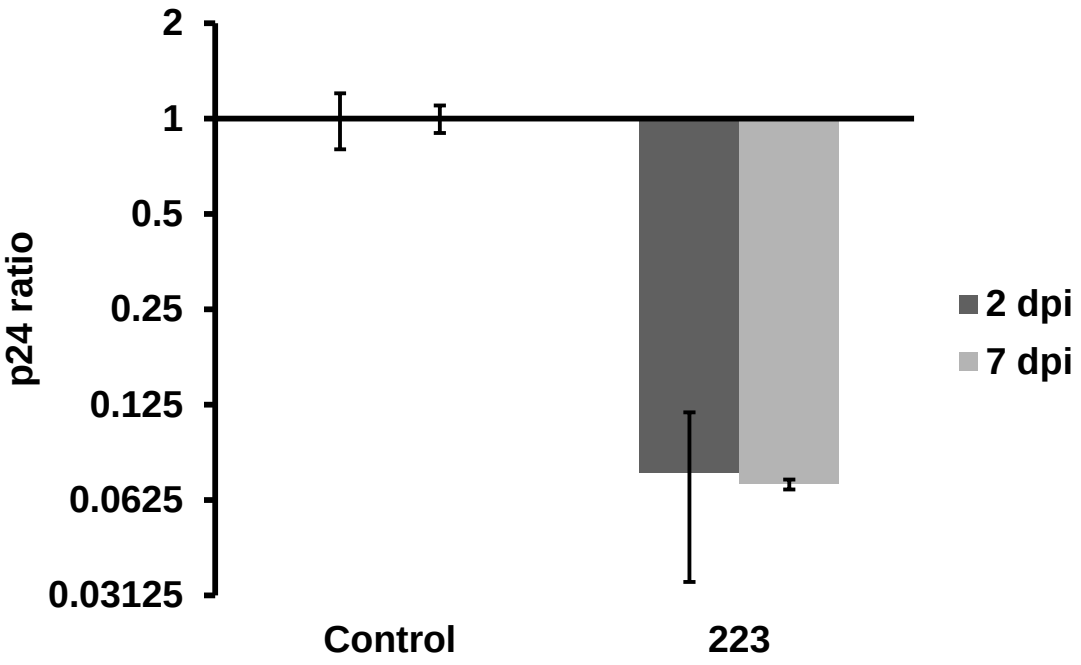
The “Berlin patient”

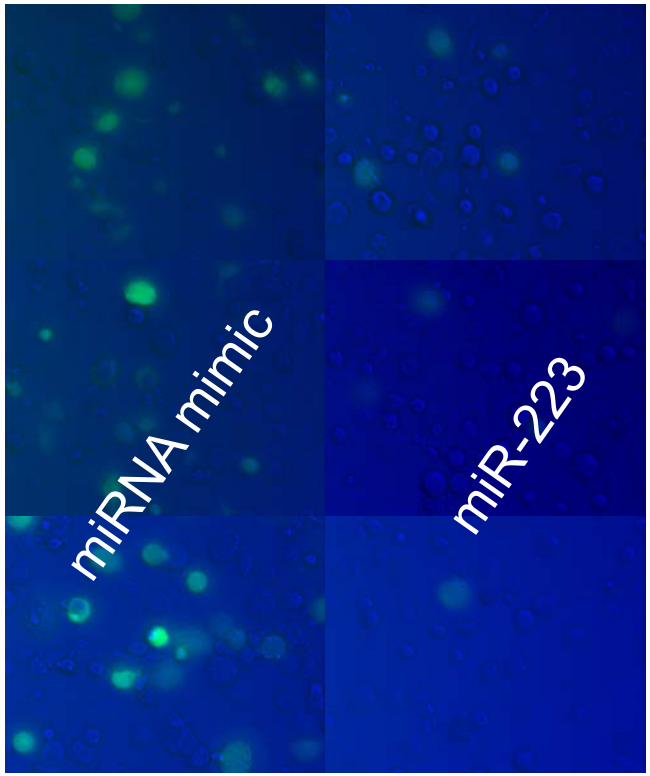
Source: POZ magazine

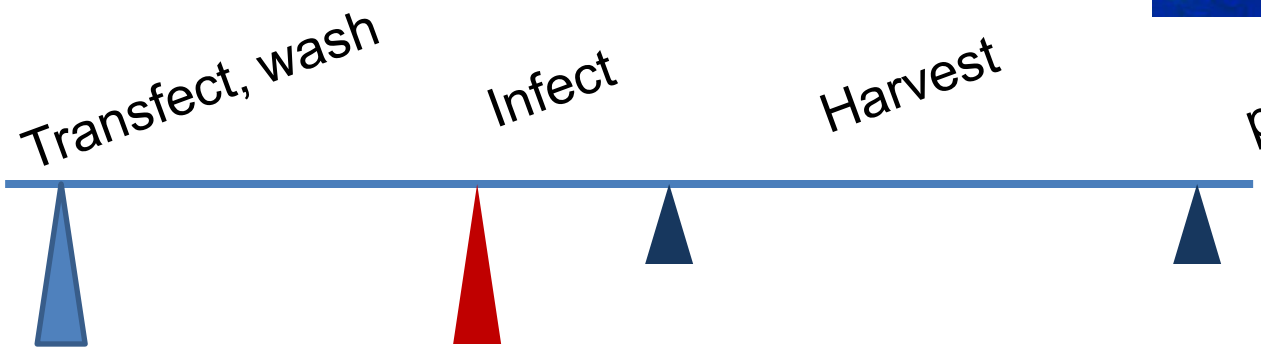
Eradication on the horizon?

- **Stem cell transplant**: Timothy Ray Brown
- **Shock and kill**: activate latent reservoir, immune system does the rest
- **Early treatment** or treatment intensification

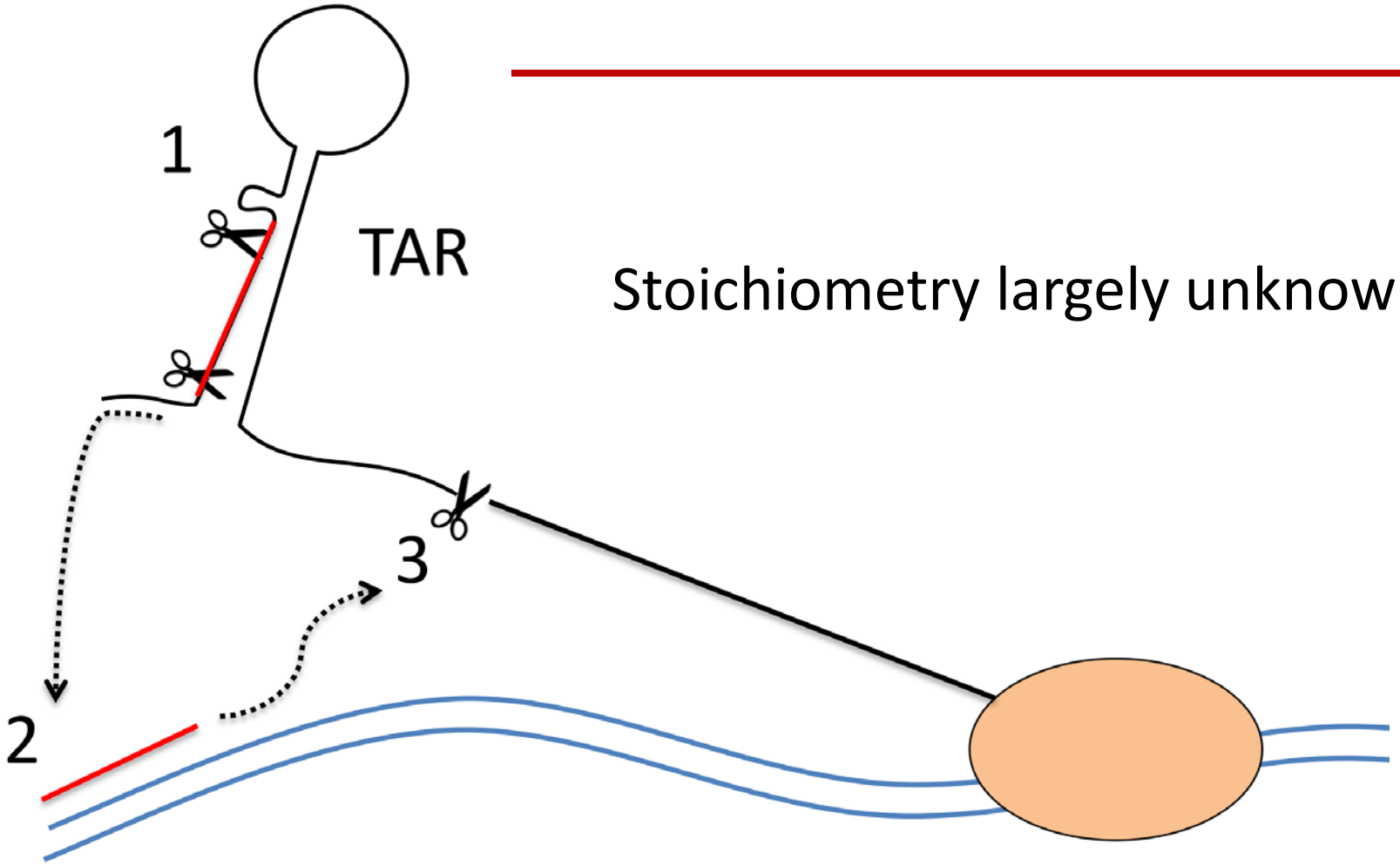
miR-223 vs. control







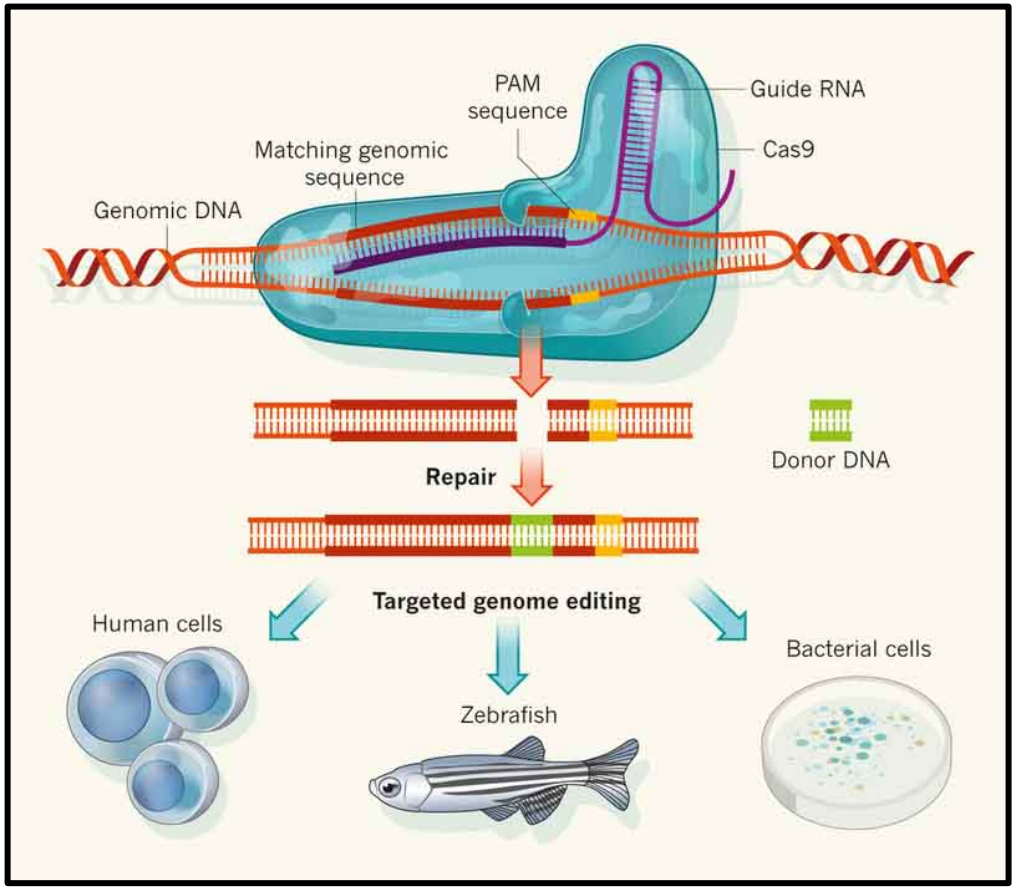
Nuclear HIV sRNA



Stoichiometry largely unknown

- L Wagschal, et al., *Cell*
- Z Klase, et al., *BMC Mol Biol*

Does CRISPR/Cas offer a specific activation option?



Credit: Origene

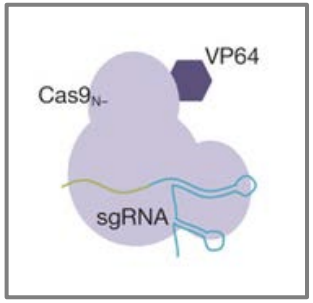
Non-specific activators are:

1) inefficient (activate only a small proportion of the latent reservoir
2) Potentially counterproductive (may promote new infection)

CRISPR/Cas has been reported to excise latent HIV in several models

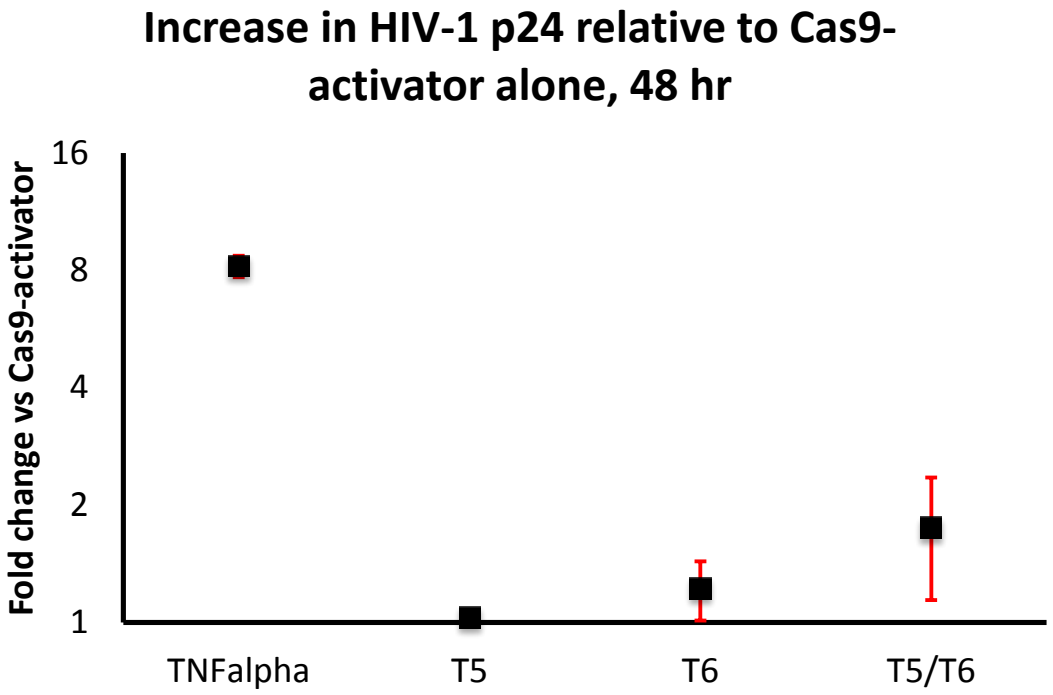
How would this be done in vivo? Even a small percentage of off-target cleavage could result in catastrophic problems.

Another strategy: transcriptional activation



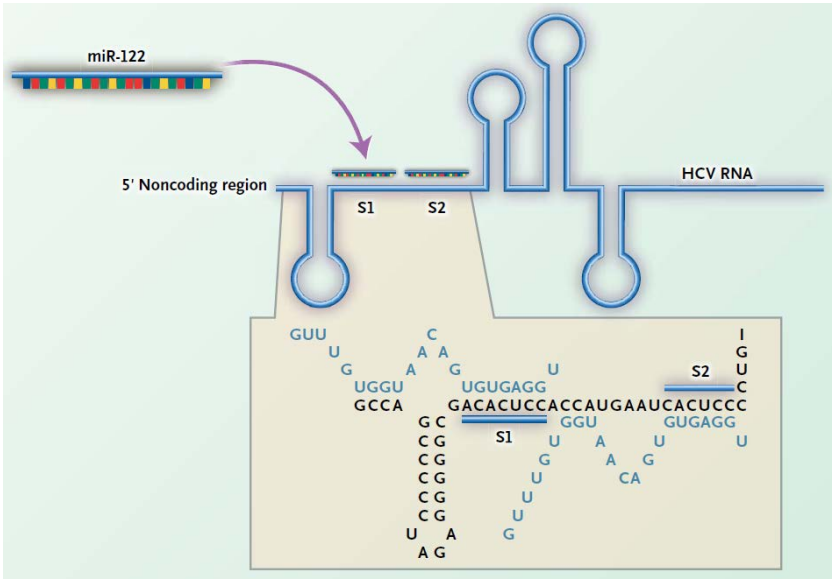
Procedure:

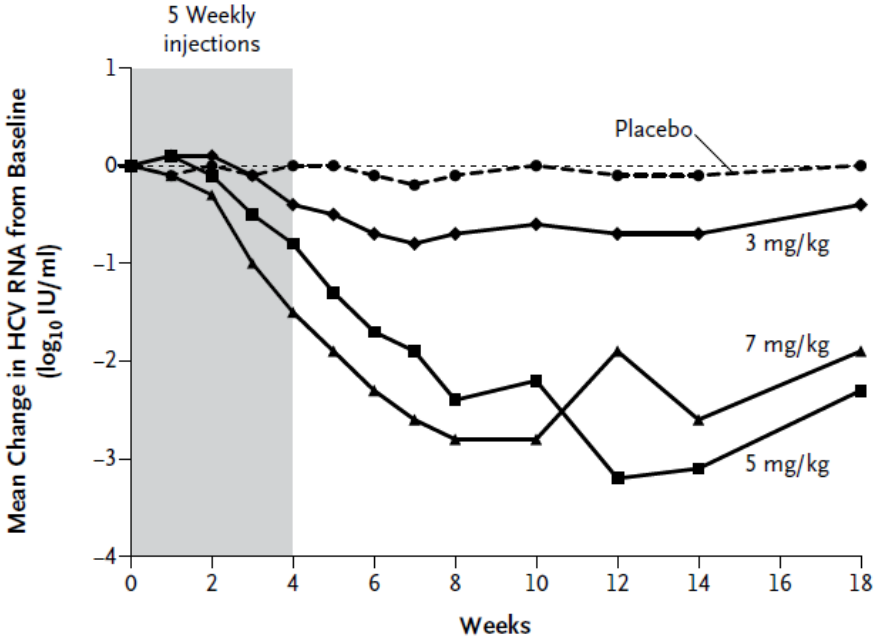
- ACH-2 latency model
- Introduce Cas9-fusion and gRNA sequence(s)
- Measure levels of released HIV-1 p24 protein



Mears, Kim, Witwer, unpublished

Small RNA-based therapy passes Phase 2





Note persistence of effects following injections
Bonus: significant reduction in cholesterol levels

JOHNS HOPKINS MEDICINE

From: Janssen, *et al., NEJM*, 2013

JOHNS HOPKINS MEDICINE

Delivery to liver

- MRX34: liver cancers
- 1st, 2nd, 3rd gen oligonucleotides
- Backbone modifications
  - phosphorothioate
- Other mods and tags

Other exposure routes? Alnylam anti-RSV drug

- Alnylam RSV01 and RSV02
- Delivery of aerosolized naked RNA (siRNA)
  - Inhalation
  - Intranasal
- Promising data, but failed Phase IIb trial, partner backed out
- Claimed evidence of RNAi mechanism was doubted by some (innate immune response?)

Can we exploit oral RNA therapeutically in mammals?

Will mammals be harmed by off-target effects of ingested RNA?

JOHNS HOPKINS MEDICINE

“Holy grail”: oral delivery of small RNA therapeutics?

nature Vol 458|30 April 2009|doi:10.1038/nature07774

LETTERS

Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation

Myriam Aouadi[1]*, Gregory J. Tesz[1]*, Sarah M. Nicoloro[1], Mengxi Wang[1], My Chouinard[1], Ernesto Soto[1], Gary R. Ostroff[1] & Michael P. Czech[1]

TNFα siRNA in a glucan shell
Proposed mechanism: uptake through Peyer’s patches M cells
Phagocytosis by macrophages→acidification→siRNA release
Observed decline in circulating TNFα, macrophage RNA

Pharmaceutical industry has had little or no success with oral delivery of RNA—to the point that the oral route is often used as a negative control

ISEV
INTERNATIONAL SOCIETY FOR EXTRACELLULAR VESICLES

ISEV 2015

Washington, DC

April 23-26

at the Bethesda North Marriott hotel

Nurturing innovative science at US NIH

Francis Collins, April, 2015: International Society for Extracellular Vesicles

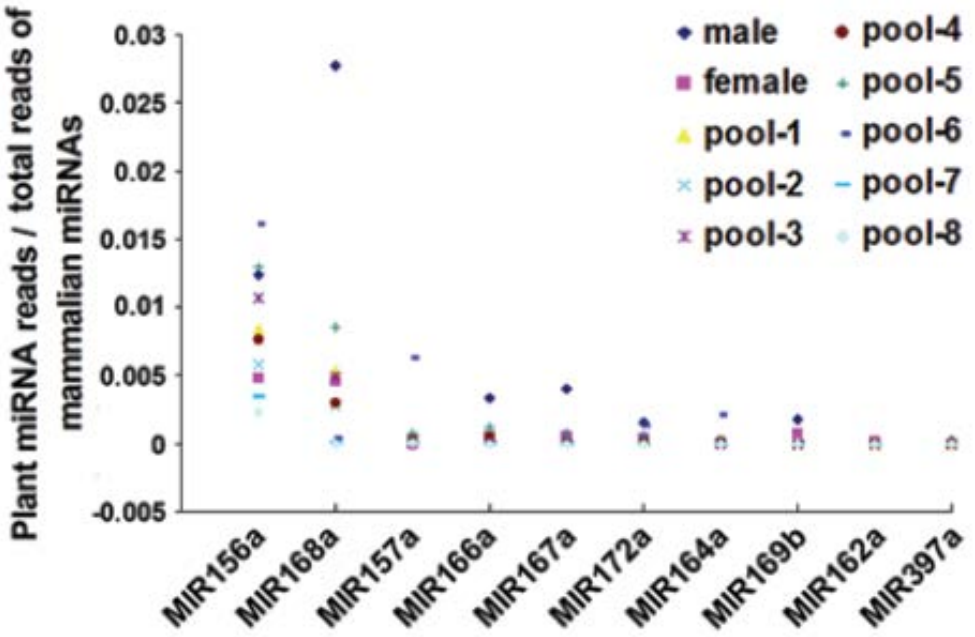
Report: Dietary miRNAs in bloodstream

Cell Research, 2012

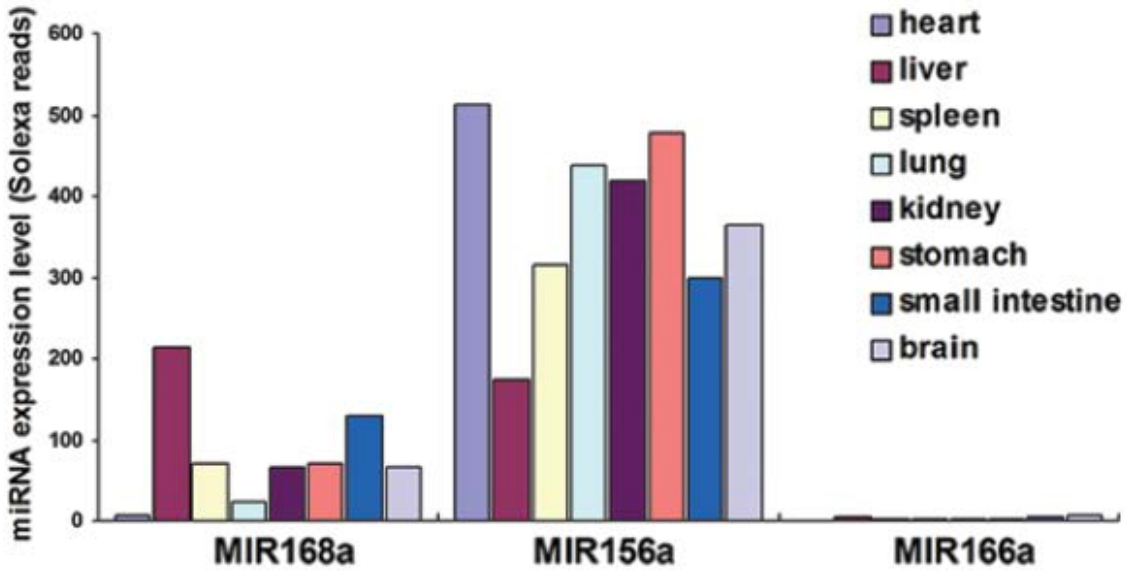
Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA

Lin Zhang[1, *], Dongxia Hou[1, *], Xi Chen[1, *], Donghai Li[1, *], Lingyun Zhu[1, 2], Yujing Zhang[1], Jing Li[1], Zhen Bian[1], Xiangying Liang[1], Xing Cai[1], Yuan Yin[1], Cheng Wang[1], Tianfu Zhang[1], Dihan Zhu[1], Dianmu Zhang[1], Jie Xu[1], Qun Chen[1], Yi Ba[3], Jing Liu[1], Qiang Wang[1], Jianqun Chen[1], Jin Wang[1], Meng Wang[1], Qipeng Zhang[1], Junfeng Zhang[1], Ke Zen[1], Chen-Yu Zhang[1]

Chinese population
Dietary staple: rice
10 pool sera
50 mL each
10-11 samples/pool
RNA-seq



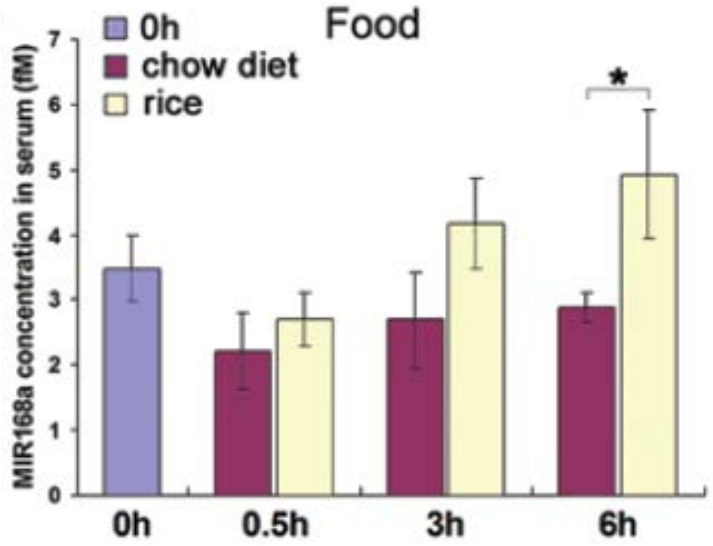
Report: Dietary miRNAs in tissue



Organism: mouse

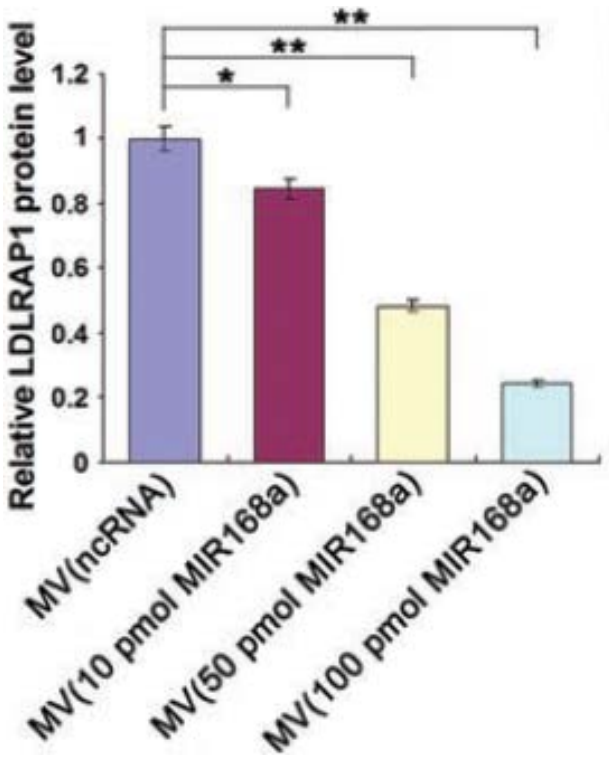
From: Zhang, *et al, Cell Research,* 2012

LDLRAP1: Dietary MIR168 affects an mRNA in liver



Organism: mouse

Dose-dependent effect on a predicted target RNA

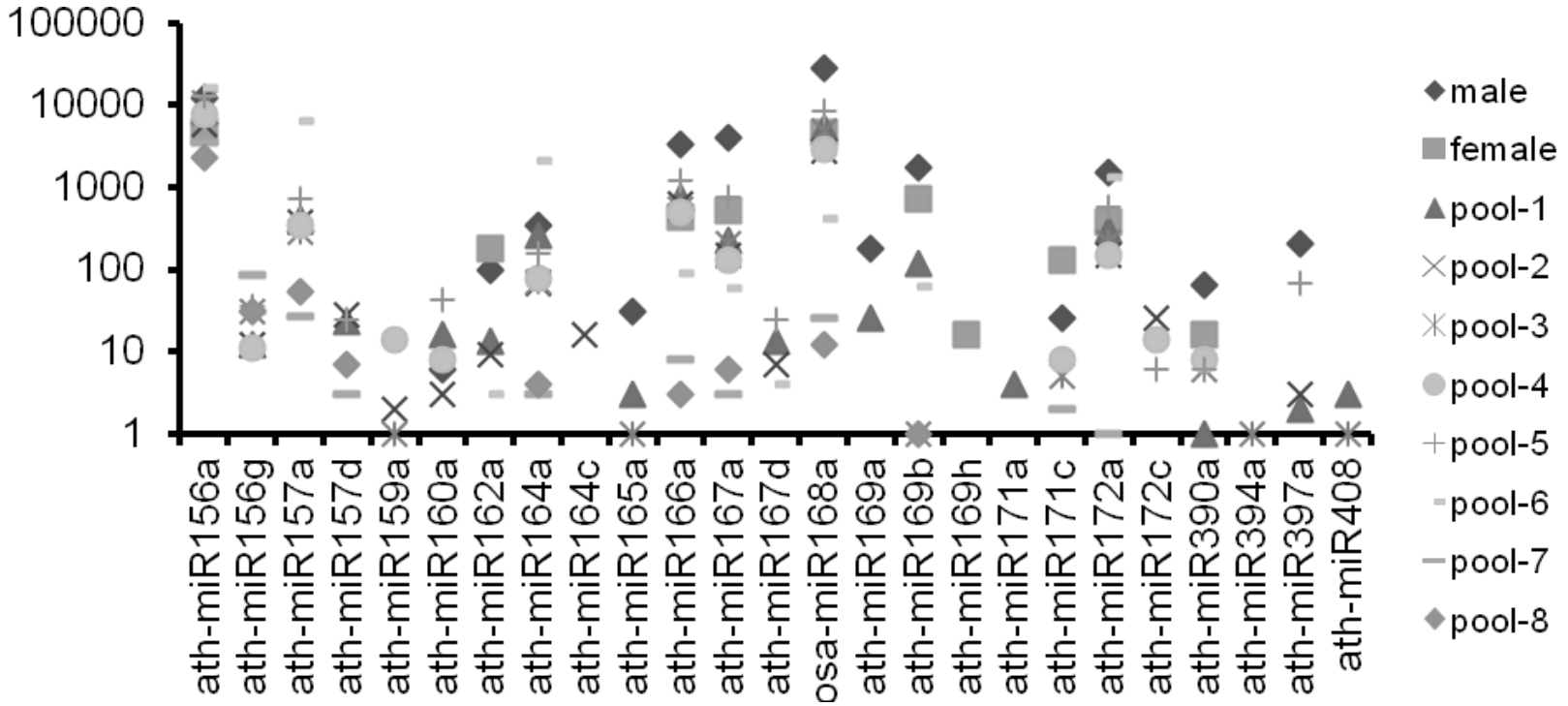


From: Zhang, *et al, Cell Research,* 2012

Why did Francis Collins mention this?

- NIH commitment to extracellular RNA research
- Desire to foster innovation
- Need to balance novelty and sound-bite excitement with solid science
- Need for reproducibility and replication studies
- Cloud of controversy

Dietary plant miRNAs enter the bloodstream of human donors …?



Witwer, analysis of supplementary data
From Zhang, *et al, Cell Research*, 2012

Negative feeding studies

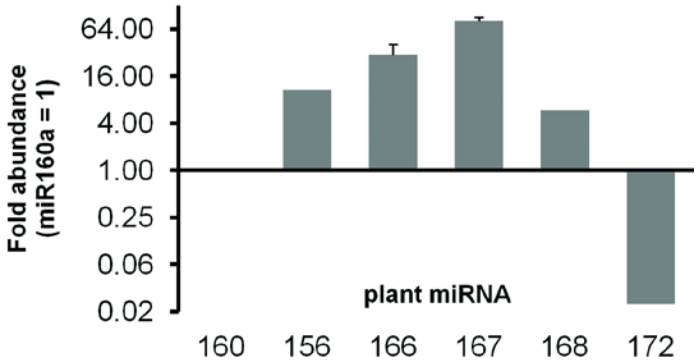
- Snow, et al., *RNA Biology*, 2013
  - Negligible or no detected uptake in bees, mice, humans with diets replete with microRNA
- Witwer, et al. *RNA Biology*, 2013
  - Nonhuman primates: no increase in response to dietary intake; low-level detection was non-specific
- Dickinson, et al., *Nature Biotechnology*, 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

Negative feeding studies

- Petrick, et al., *Regul Toxicol Pharmacol*, 2015
  - Feeding small RNAs or a long dsRNA against an essential gene had no affect on mice
  - 28-day study
  - No evidence of uptake or function of the dietary RNA

Pilot design: mammalian uptake

Pigtailed macaques
Gavage: ~5% of estimated blood volume



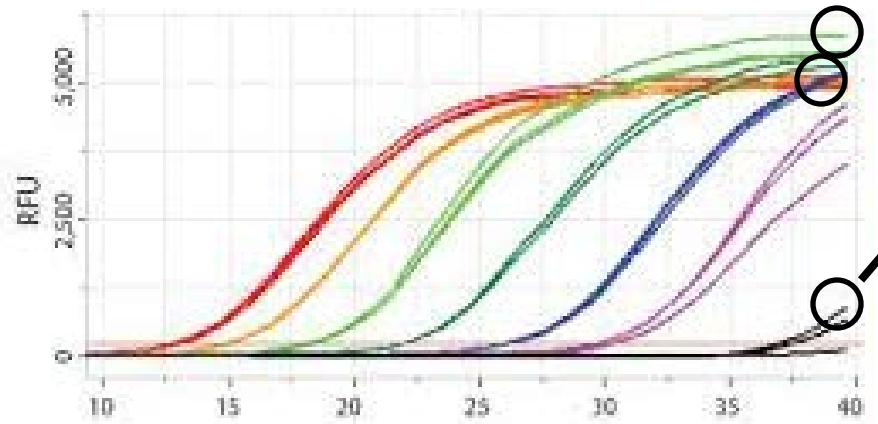
Blood draws: hours post-gavage

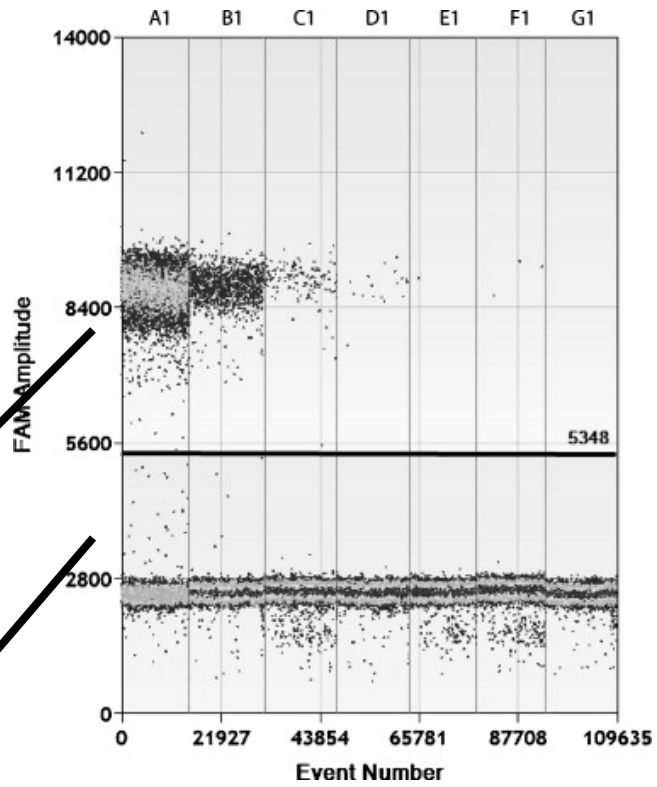
→Immediate processing to platelet-poor plasma
Initial RNA extraction by Ambion mirVana protocol

JOHNS HOPKINS MEDICINE

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

Droplet digital PCR (ddPCR)

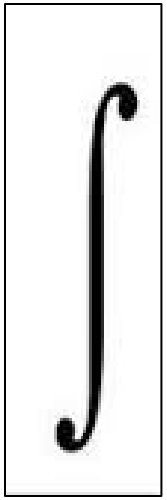


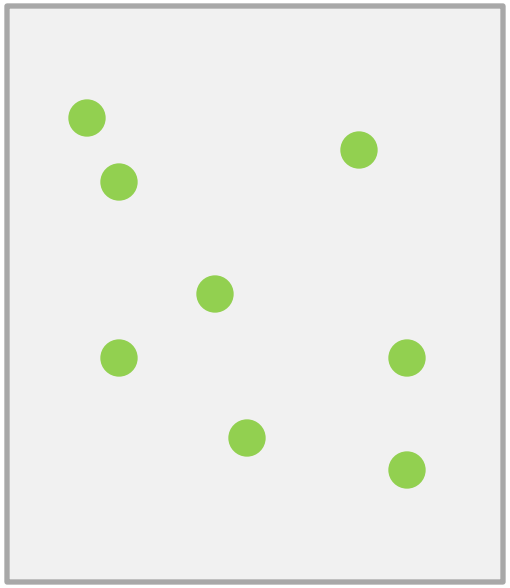


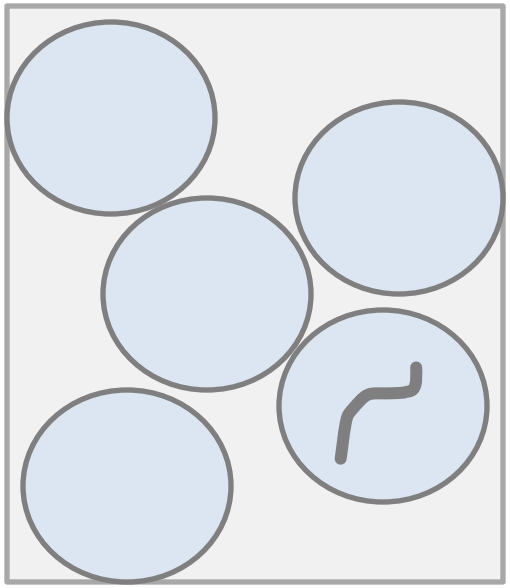
Henrich, *et al., J Virol Methods*, 2012

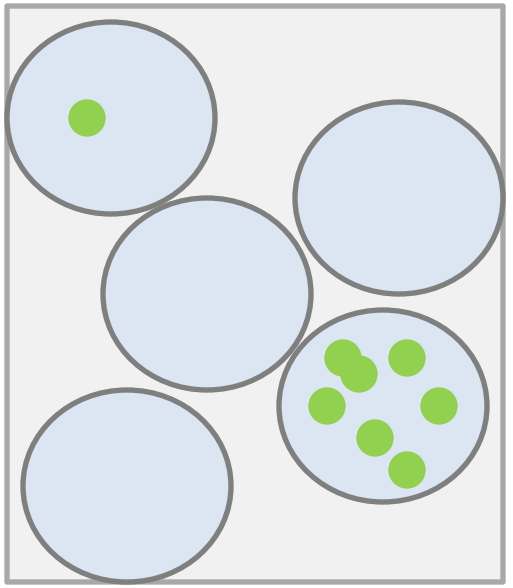
Traditional hydrolysis probe qPCR

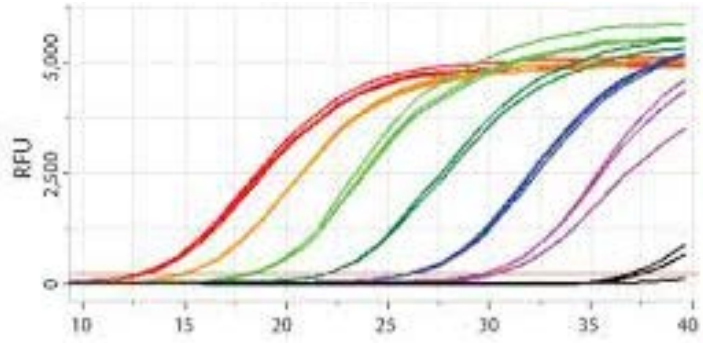
Emulsion “Droplet Digital” PCR





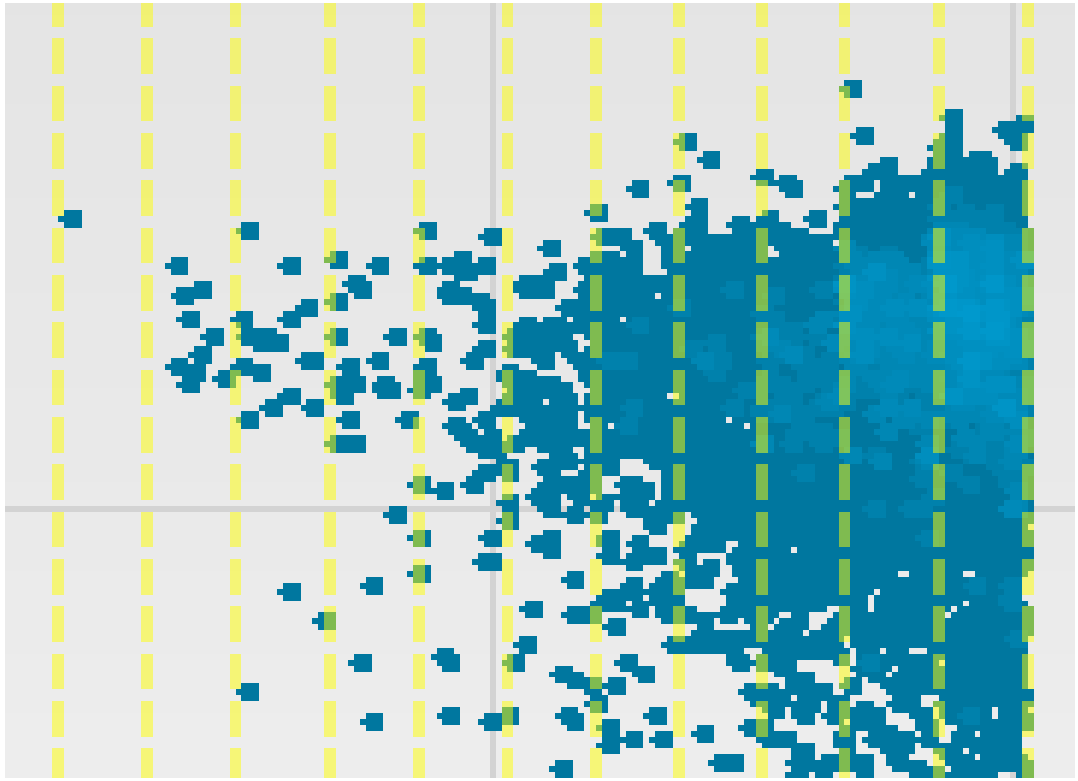






ddPCR Perspective: sensitivity

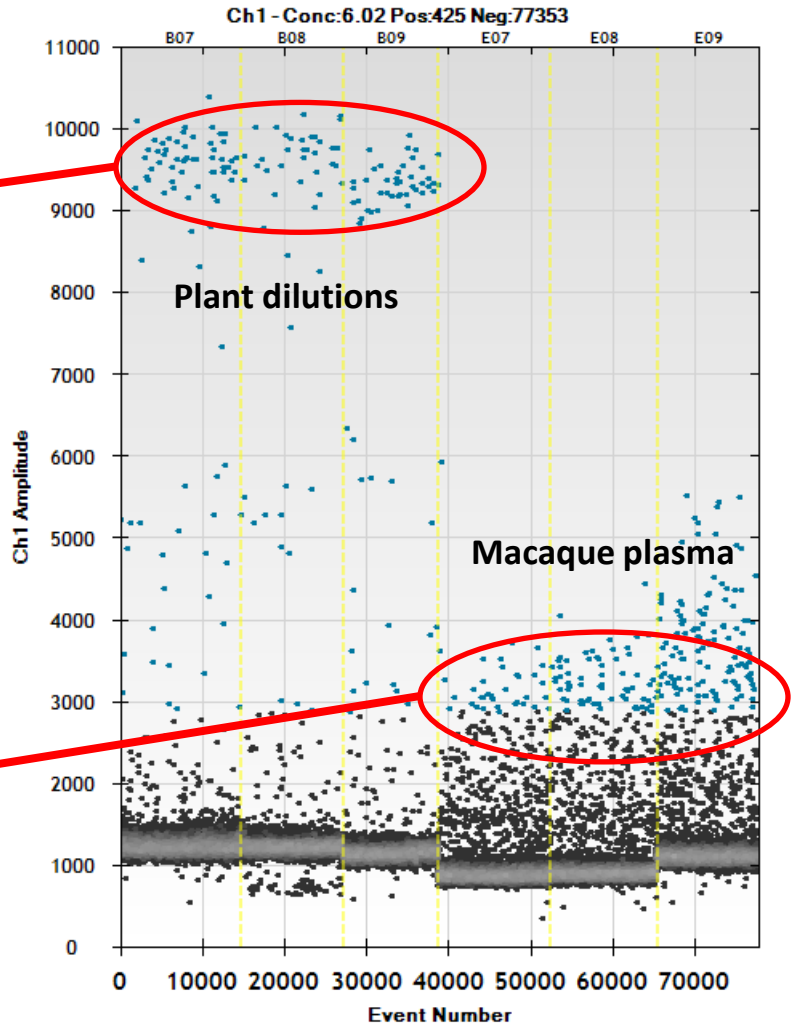
Expected	Observed
256	261
128	134
64	62
32	31
16	17
8	8
4	4
2	2
1	1
0.5	0.8
0	0



Droplet digital PCR results

Single predominant product in highly diluted plant material = specific

Non-specific amplification

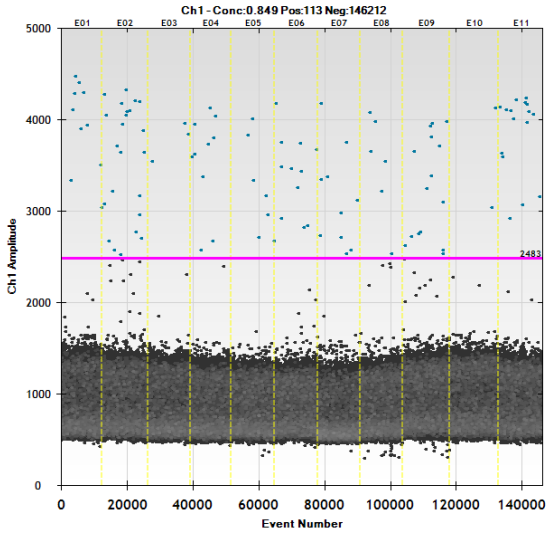


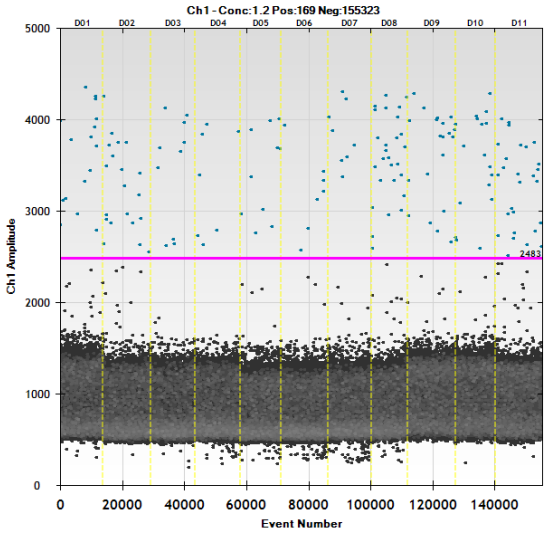
miR168: one of the most abundant miRNAs in the original study

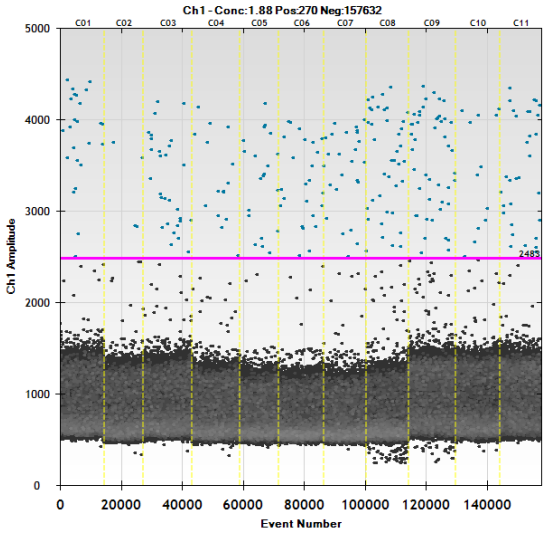
JOHNS HOPKINS MEDICINE

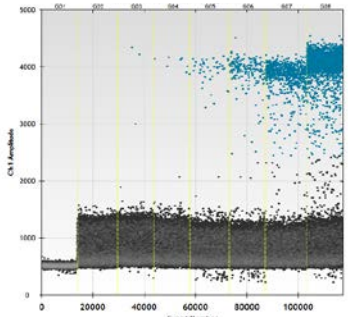
Adapted from: Witwer, *et al*, *RNA Biology*, 2013

Unpublished feeding study: multiple time points pre to post prandial



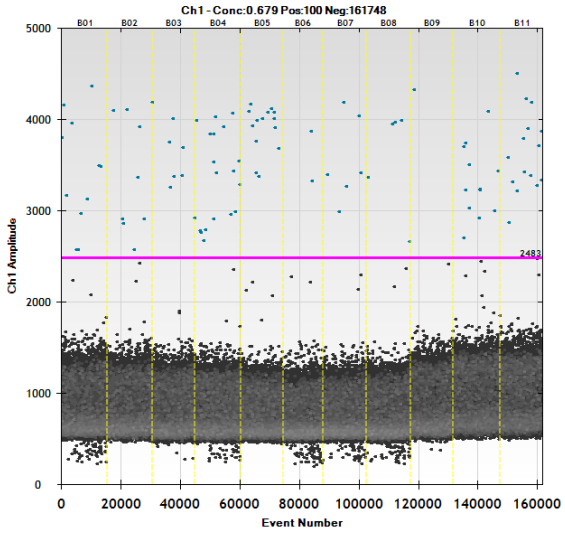


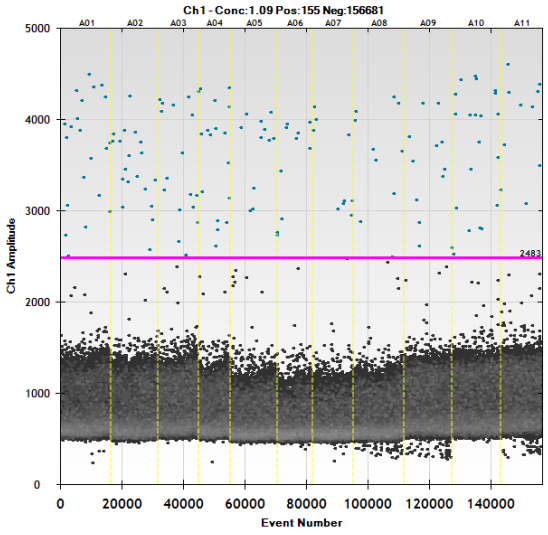


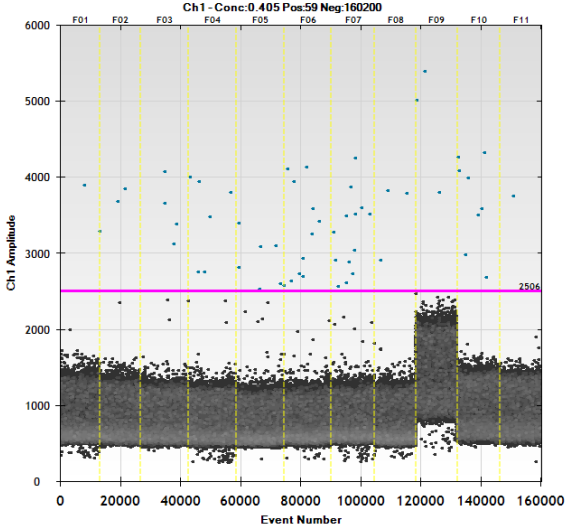


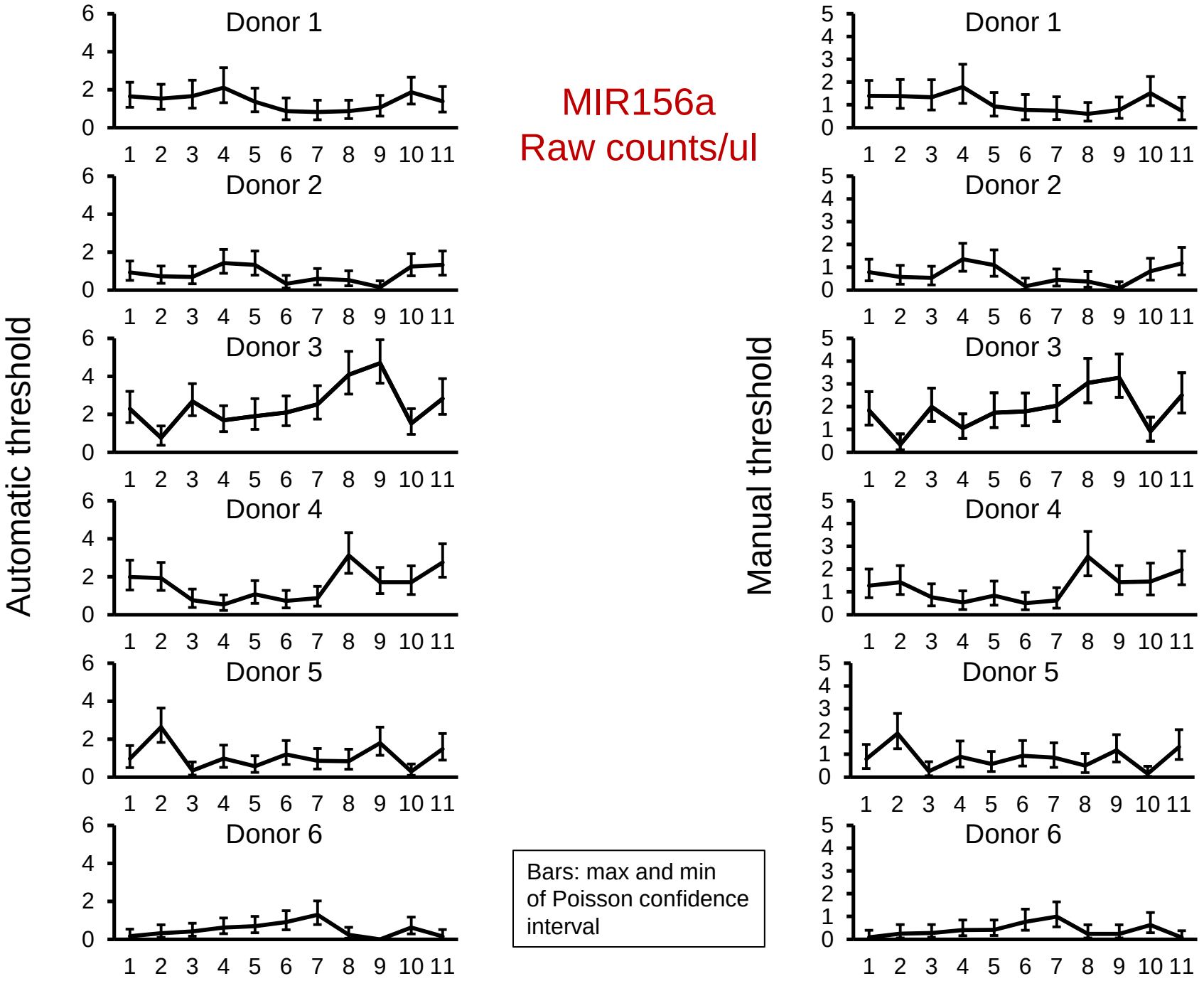
Standards for comparison

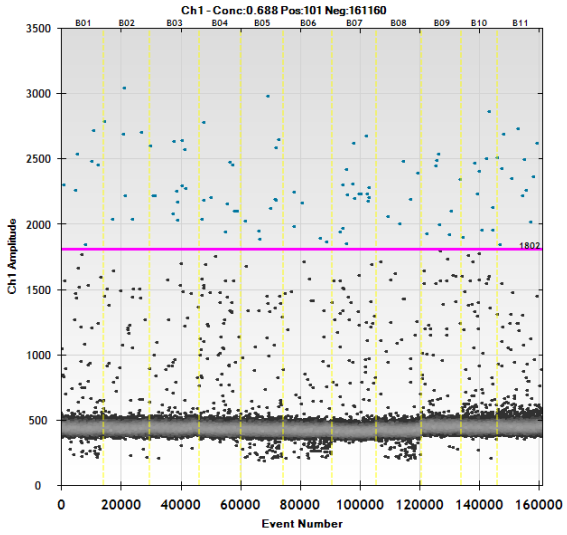
MIR156a
Intensity plots (not necessarily in order by donor #)

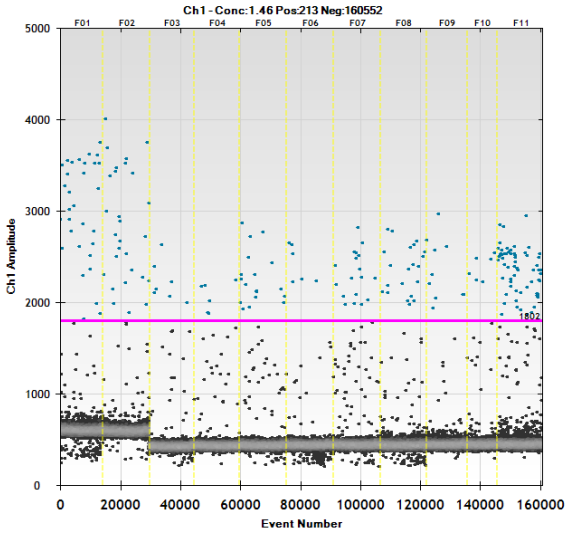


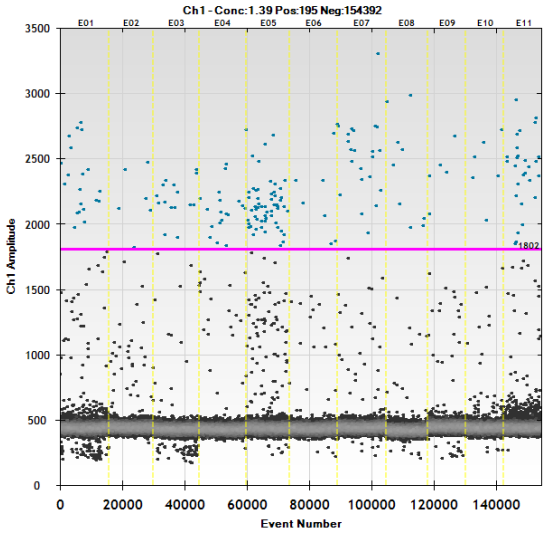


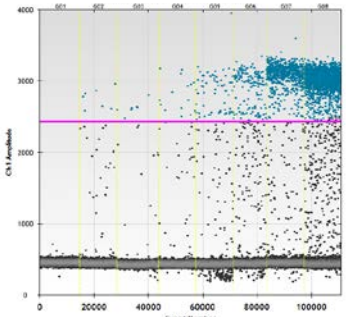



MIR156a
Raw counts/ul
Automatic threshold
Manual threshold
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor 6
Bars: max and min of Poisson confidence interval



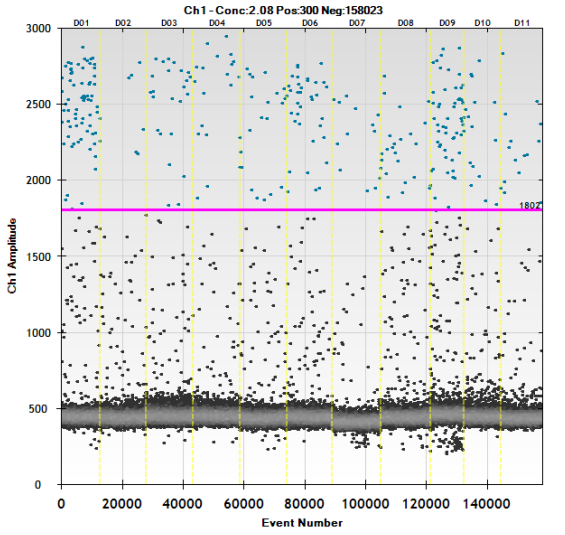


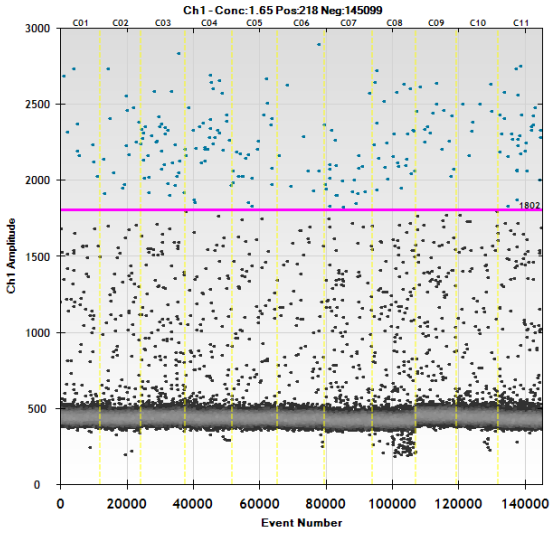


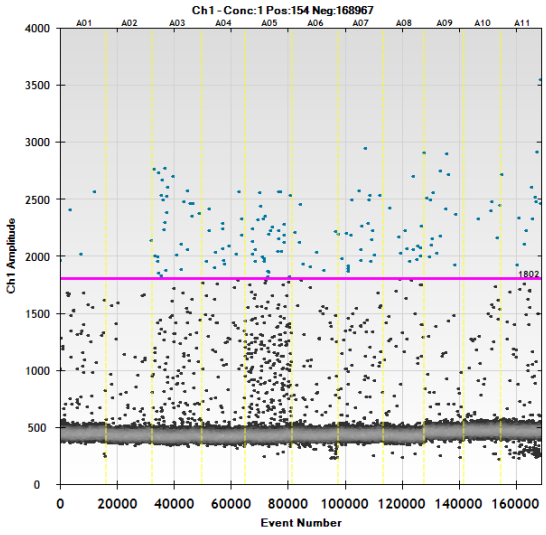


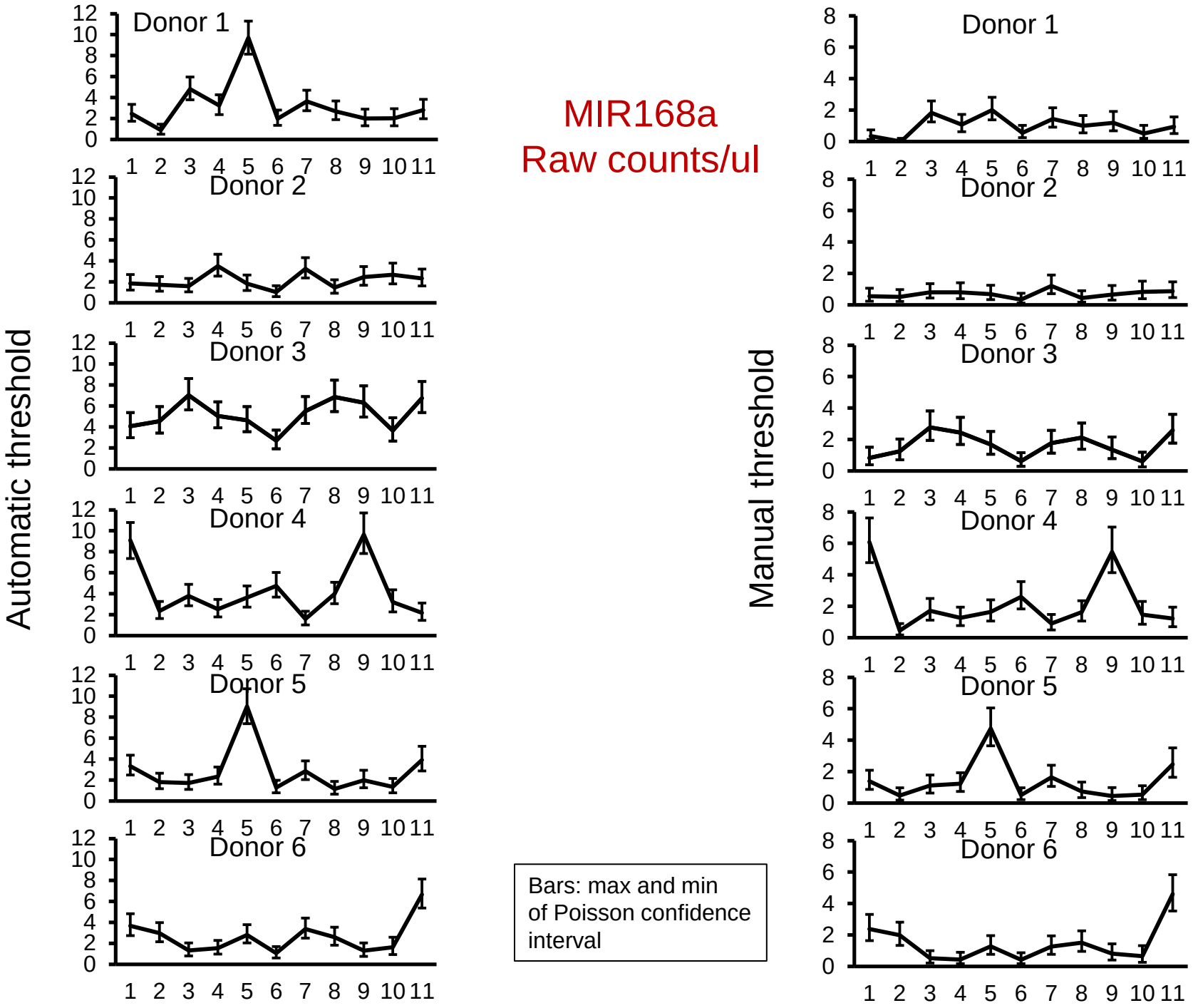
Standards for comparison

MIR168a
Intensity plots (not necessarily in order by donor #)








MIR168a
Raw counts/ul
Automatic threshold
Manual threshold
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor 6
Bars: max and min of Poisson confidence interval

Method optimization needed?

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

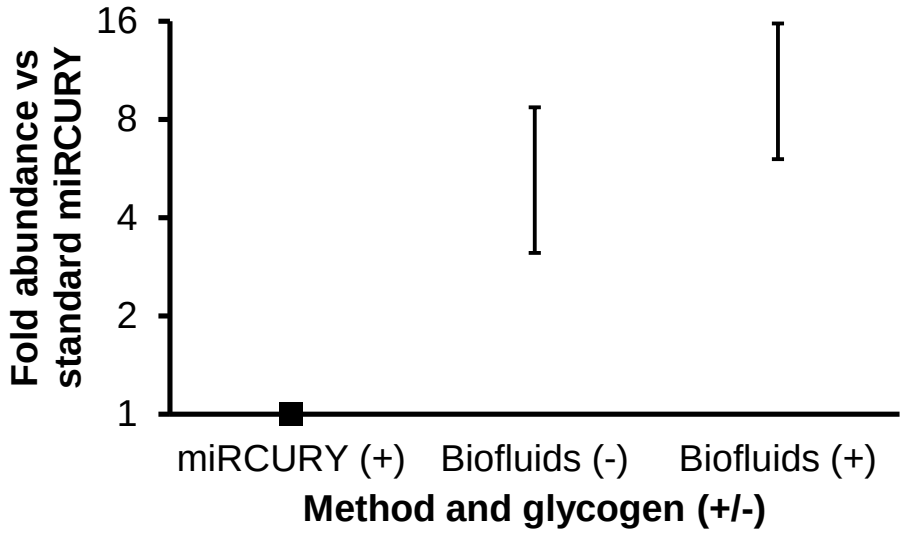
--No; very sensitive detection of plant miRNAs

Low abundance RNAs missing from recovered sample?

Biofluids RNA method from Exiqon (12/2012)

Improved recovery, inhibitor removal

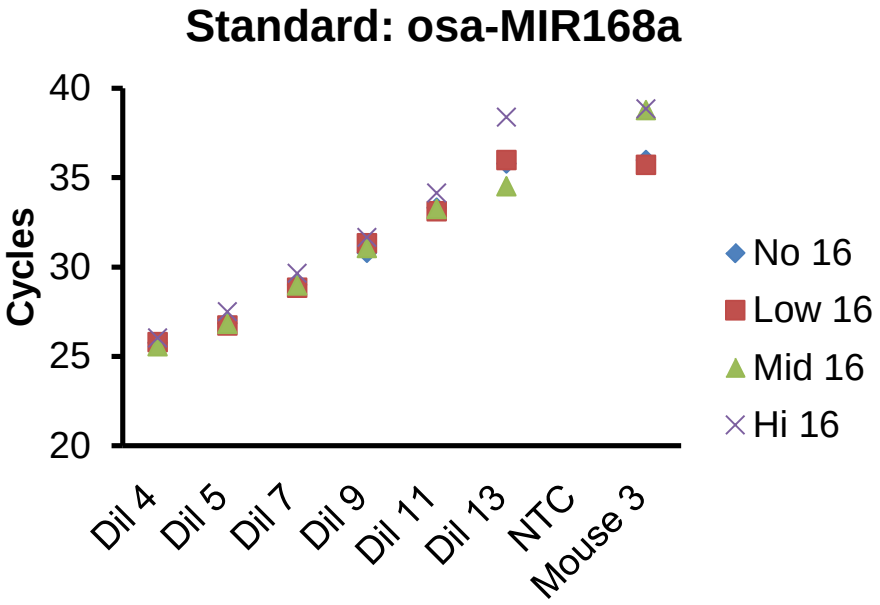
Verified performance

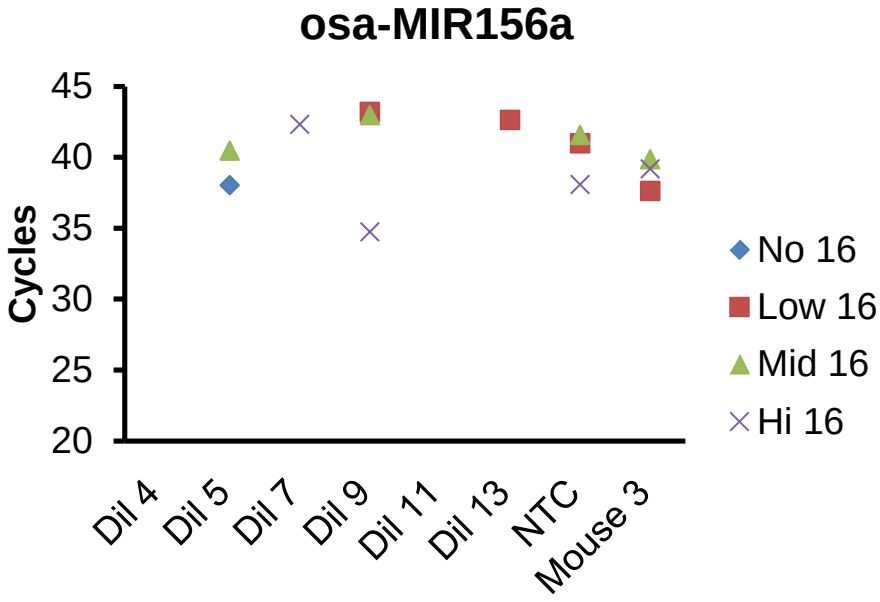


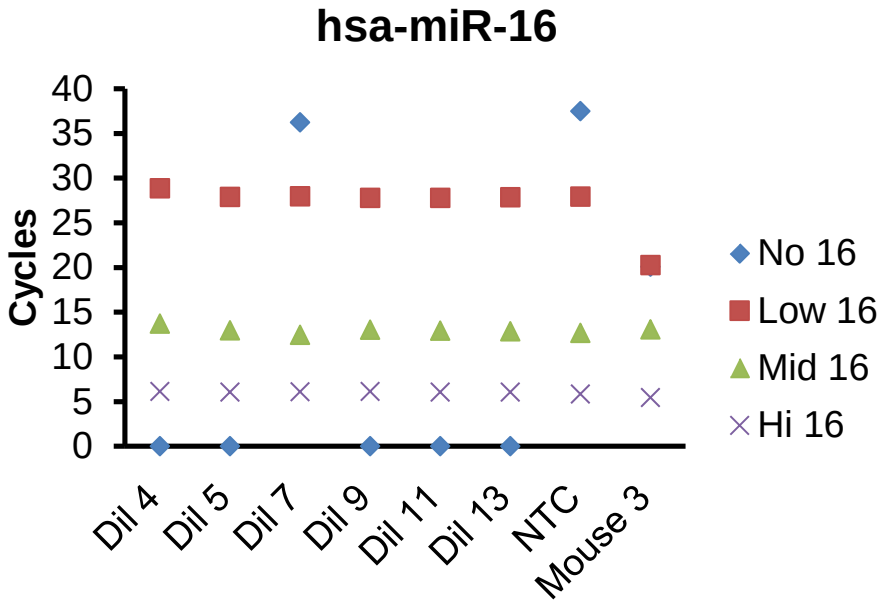
McAlexander, *et al, Trends in Genetics*, 2013

Co-factor needed?

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







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
  - Improved analytic pipeline: no more mapping to MIR168a!
- Wang, et al., *Toxicol Sci*, 2013
  - mouse liver toxicity study; low MIR168a only
- Tosar, et al., *RNA*, 2014
  - Sequencing reads consistent with contamination

Tosar *et al*. explore contamination

- Turtle RNA found in human sperm?
  - Food story vs. food fact: clearly a contaminant
  - Due to work on turtles in lab
  - Eliminated with stringent anti-contam. protocols
- Review of Zhang et al. (rice) and chordate
  - No chordate exposure to plants
  - Almost identical plant miRNA "uptake" with humans
- → Contamination a widespread problem in RNA-seq…especially for endogenous RNA?

When “positive” studies prove the opposite

Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 26 (2015) 505–512

Journal of Nutritional Biochemistry

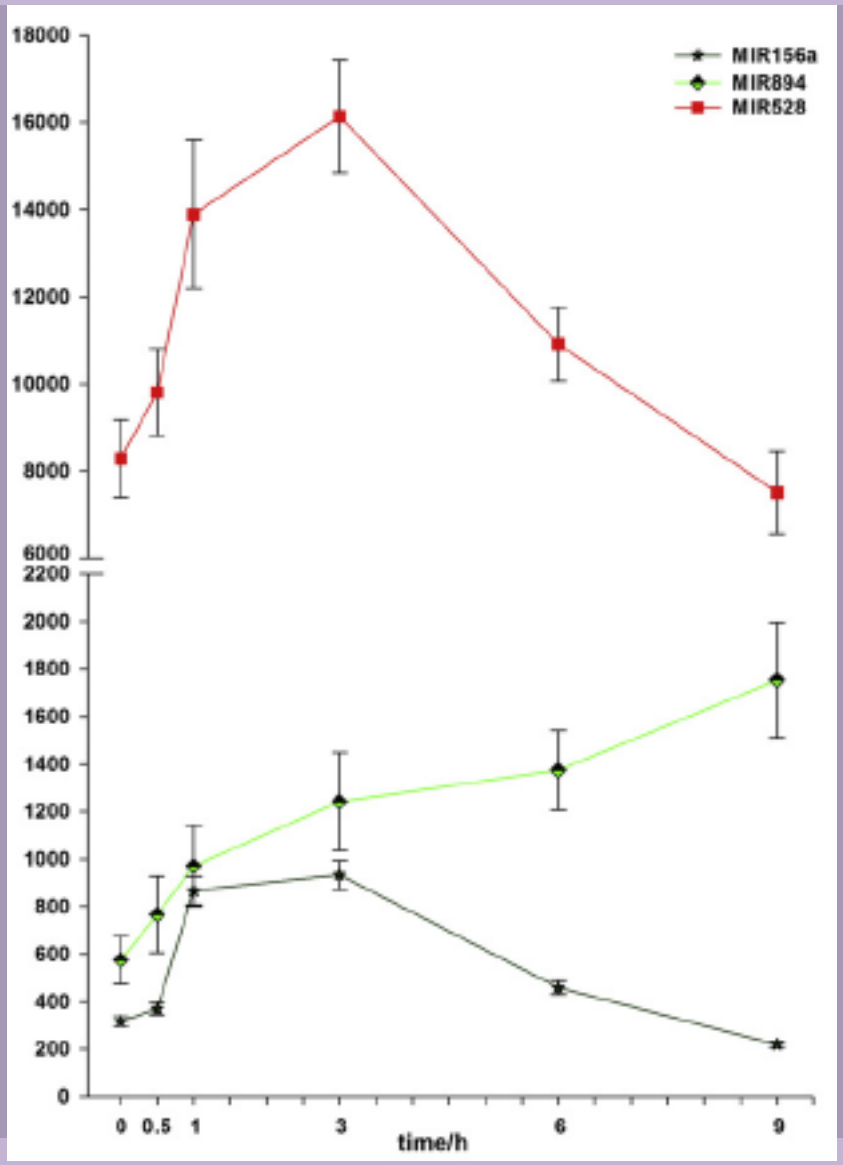
Effective detection and quantification of *dietetically* absorbed plant microRNAs in human plasma

Hongwei Liang[1], Suyang Zhang[1], Zheng Fu[1], Yanbo Wang, Nan Wang, Yanqing Liu, Chihao Zhao, Jinhui Wu, Yiqiao Hu, Junfeng Zhang, Xi Chen*, Ke Zen*, Chen-Yu Zhang*

- Nine humans drank 3 liters of watermelon juice
- Blood draw: before, several time points after ingestion
- >20 miRNAs measured, including 16 plant miRNAs

Watermelon…or not?

- Best response: MIR528
  - Doubling of concentration
  - Rise and fall with time
  - 1.3% uptake: more than all other plant miRNA
- Unfortunately, MIR528 is a miRNA of monocots
- Watermelon is a dicot: no similar seq. in genome
- Contamination?
- “Positive” studies underscore negative findings (see also no template controls!)

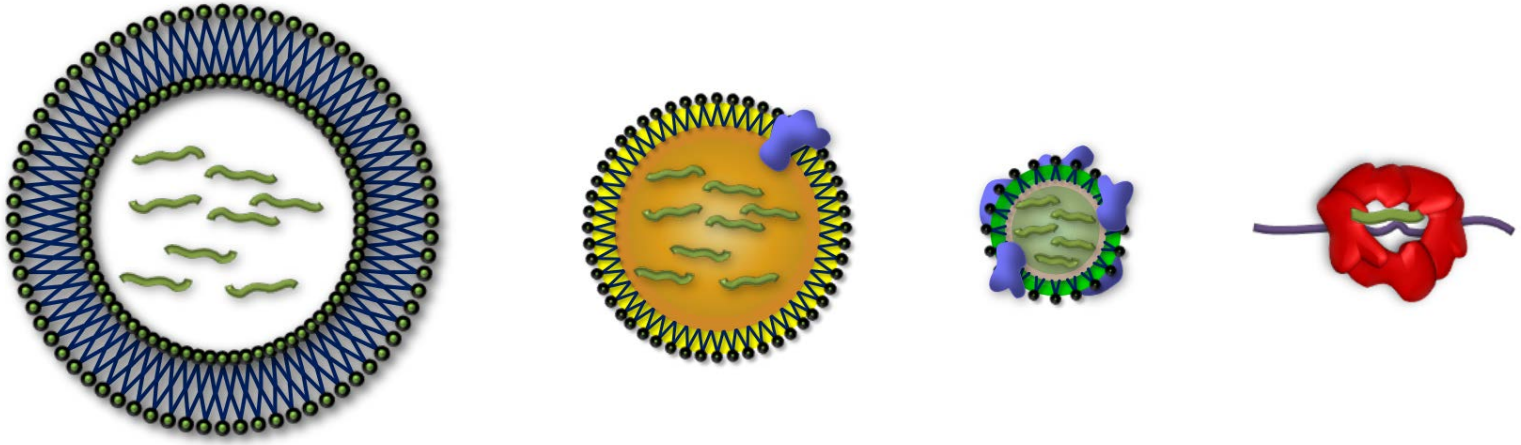


Positive mammalian studies: in common

- Ambiguous or nonsensical results and statistics issues
- Lack of controls
- Mechanistic studies lacking
- Wide-reaching claims not supported by evidence

Principles: uptake and function

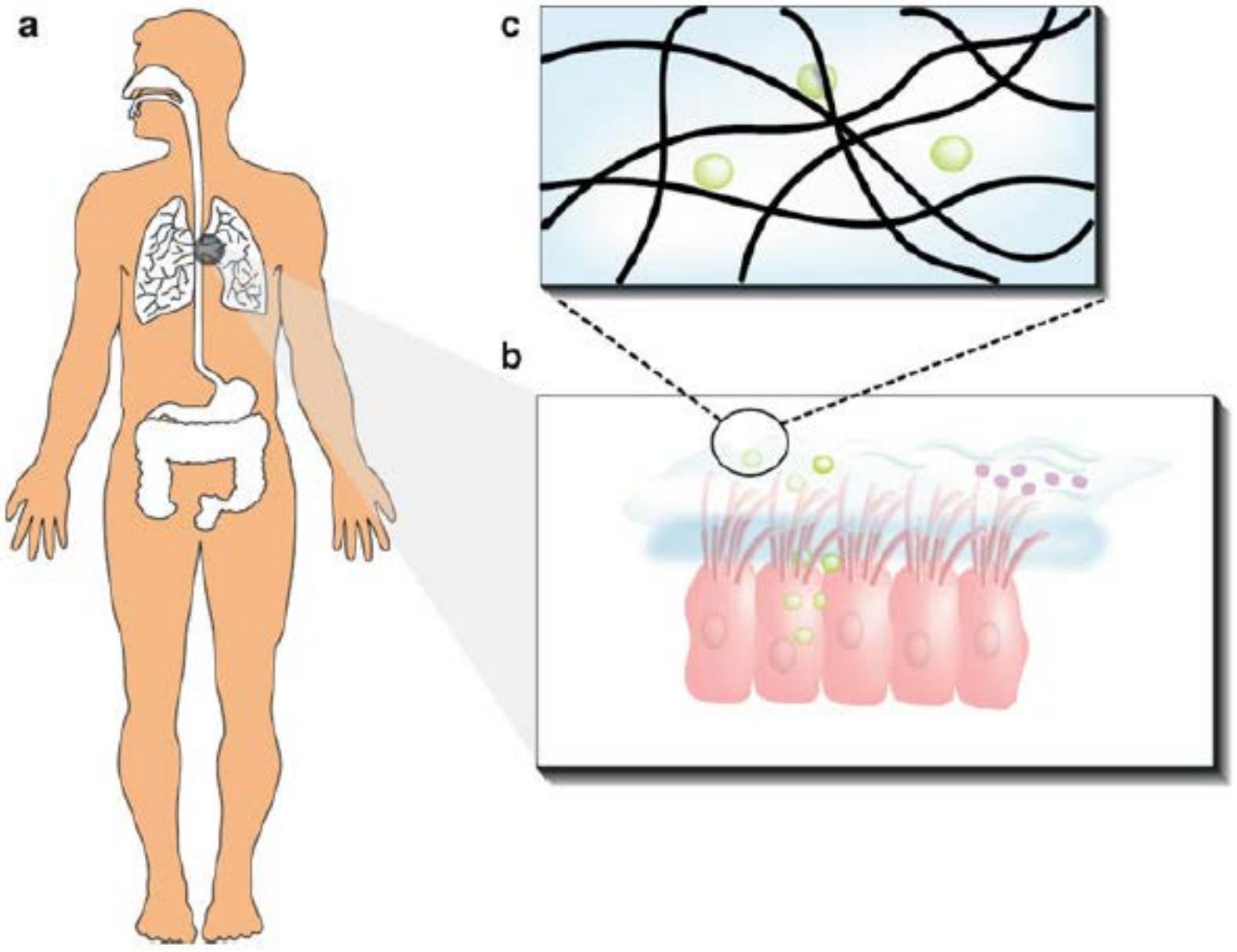
Small RNA “vehicles” carry and protect



EV HDL LDL Protein complex

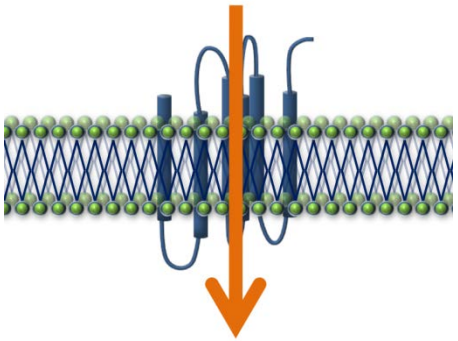
Witwer & Hirschi, BioEssays, 2014

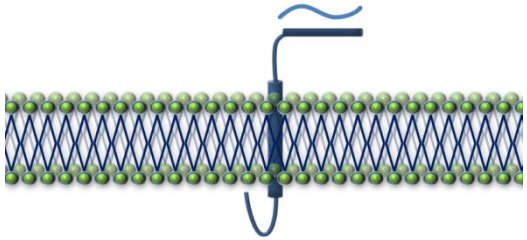
RNases: the piranhas of the body



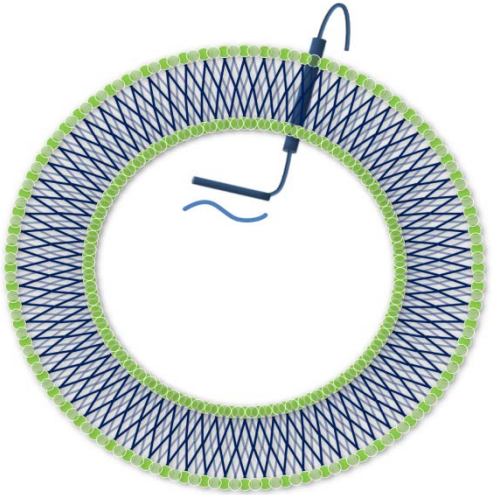
RNAi mechanisms not observed in mammals

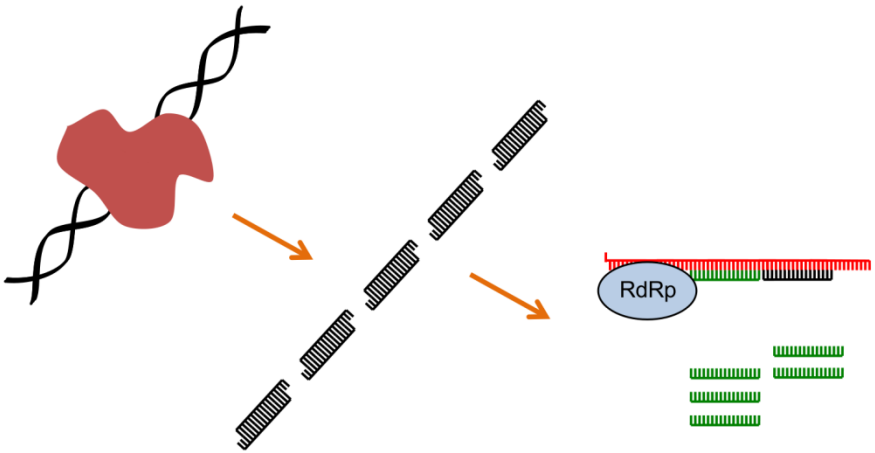
Spreading interference: SID-1





Uptake from the environment: SID-2





RNAi amplification

Witwer and Hirschi, BioEssays 2014

A long road

- RNases: are dietary RNAs protected by Argonaute? In “vesicles”?
- Across the mucus layer
- Across the intestine and through the blood
- The next barrier: cell membrane, endosome
- Could a plant Argonaute-complexed plant small RNA function in a mammalian cell?
- How many copies of a *functional* RNA needed?
- What is the effect of an off-target interaction?

In vivo, most miRNAs are NOT in active complexes

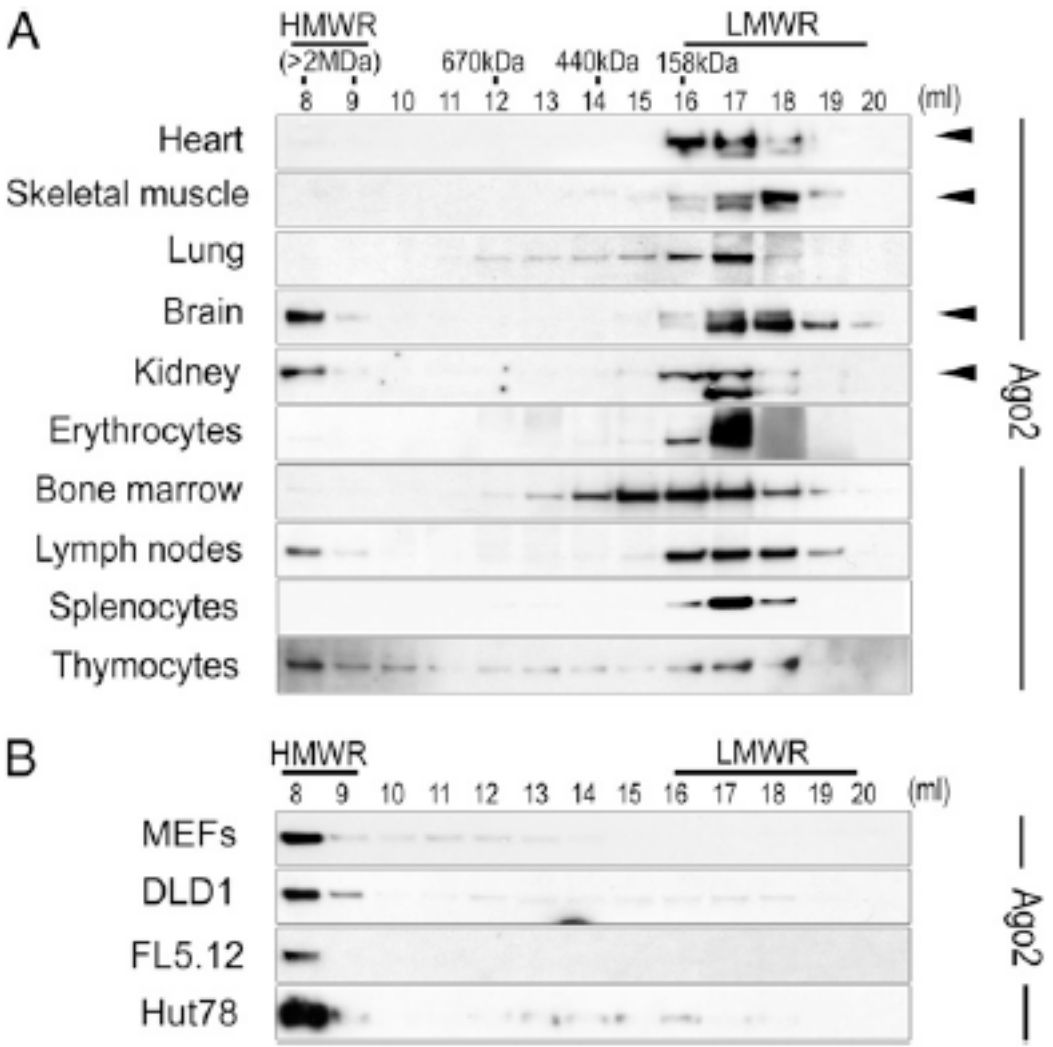
PNAS

In vivo, Argonaute-bound microRNAs exist predominantly in a reservoir of low molecular weight complexes not associated with mRNA

Gaspare La Rocca[a,1], Scott H. Olejniczak[a,1], Alvaro J. González[b], Daniel Briskin[c], Joana A. Vidigal[a], Lee Spraggon[a], Raymond G. DeMatteo[a], Megan R. Radler[a], Tullia Lindsten[d], Andrea Ventura[a], Thomas Tuschl[c], Christina S. Leslie[b], and Craig B. Thompson[a,2]

[a]Cancer Biology and Genetics Program, [b]Computational Biology Program, and [d]Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065; and [c]Howard Hughes Medical Institute, Laboratory of RNA Molecular Biology, The Rockefeller University, New York, NY 10065

In vivo, most miRNAs are not in an active complex



La Rocca et al, 2014

Off-target effects

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

Off-target "miRNA-like" effects

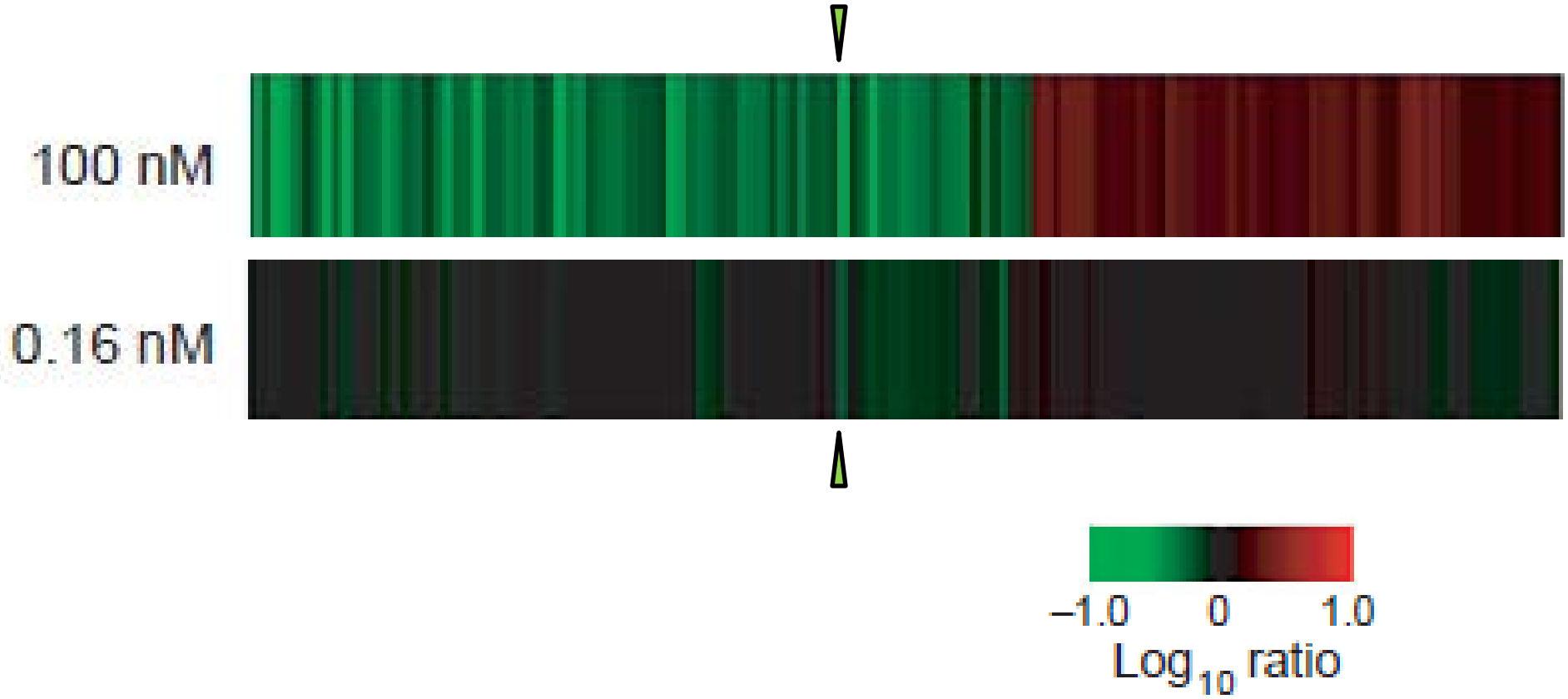
Expression profiling reveals off-target gene regulation by RNAi

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 miRNA per cell
- Almost all off-target effects disappeared at 0.16 nM, i.e. at 100,000 copies/cell

Off-target “miRNA-like” effects



NATURE BIOTECHNOLOGY VOLUME 21 NUMBER 6 JUNE 2003

Cellular 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 dose based on *highest, unconfirmed* reported circulating dietary RNA concentration

Exposure comparisons

- US EPA FIFRA-SAP: public comments
  - PIP dsRNA is at nanogram/gram levels *in planta*

  →Assuming same level in edible parts, a 70 kg human might ingest up to several micrograms RNAi agent

  →Assuming 100% uptake, ~40 nanograms/kilogram

  = 1 ***millionth*** the therapeutic dose for *injected* RNAi
- Biological barriers
- No known mechanism for uptake of dsRNA by mammals…or processing of plant dsRNA into ss effectors…or uptake of small RNA effectors
- Homeopathy?

“Without exposure, there is no risk”

“The dose makes the poison”

“Gene technology has not been shown to introduce any new or altered hazards into the food supply, therefore the potential for long term risks associated with GM foods is considered to be no different to that for conventional foods already in the food supply” -FSANZ

Evidence of harm?

Prevalence and impacts of genetically engineered feedstuffs on livestock populations[1]

A. L. Van Eenennaam[2] and A. E. Young

Department of Animal Science, University of California, Davis 95616

Journal of Animal Science, 2014

Prevalence and impacts of genetically engineered feedstuffs on livestock populations[1]

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Department of Animal Science, University of California, Davis 95616

Table 2. Organic livestock production statistics in the United States (2011)

Industry	Number of organic farms in the United States[1]	Number of animals on organic farms[1]	Total number of livestock animals in the United States[2]	Organic livestock numbers as percent of the U.S. total[3]
Broilers	153	28,644,354	8,607,600,000	0.33%
Layers	413	6,663,278	338,428,000	1.97%
Turkeys	70	504,315	248,500,000	0.20%
Beef cows	488	106,181	30,850,000	0.34%
Dairy cows	1,848	254,711	9,150,000	2.78%
Hogs	97	12,373	110,860,000	0.01%

[1]USDA National Agricultural Statistics Service, 2012.

[2]USDA Economics, Statistics, and Market Information

[3]USDA Economic Research Service, 2013.

Less than 1% of livestock in the US are on "organic" farms

→Trillions of meals of GM plant material during a period of improving health for livestock

Journal of Animal Science, 2014

Table 3. Estimated cumulative number of livestock raised in the United States during the period from 2000 to 2011

Industry[1]	United States
Broilers	94,683,600,000
Layer Hens	3,722,708,000
Turkeys	2,733,500,000
Beef cattle	339,350,000
Dairy Cows	33,550,000
Hogs	1,219,460,000
Total	102,732,168,000

Genetically Modified Crops

Produce Food as Safe and Nutritious as Conventional

3.95 Billion ACRES OF FARMLAND
USED FOR GMO CROPS SINCE 1996

63 COUNTRIES
WHERE GM CROPS HAVE BEEN APPROVED FOR CULTIVATION OR IMPORT

1000+ ACADEMIC STUDIES
SUPPORT THAT GMO CROPS ARE JUST AS SAFE AS THOSE DEVELOPED THROUGH TRADITIONAL BREEDING

13 YEARS
ON AVERAGE TO DEVELOP AND TEST GM SEEDS BEFORE THEY'RE GROWN COMMERCIALLY IN THE U.S.

30 YEARS
THAT GMO CROPS HAVE BEEN RESEARCHED AND DEVELOPED

Sources: ISAAA.org, biofortified.org, croplife.org/PhillipsMcDougalstudy

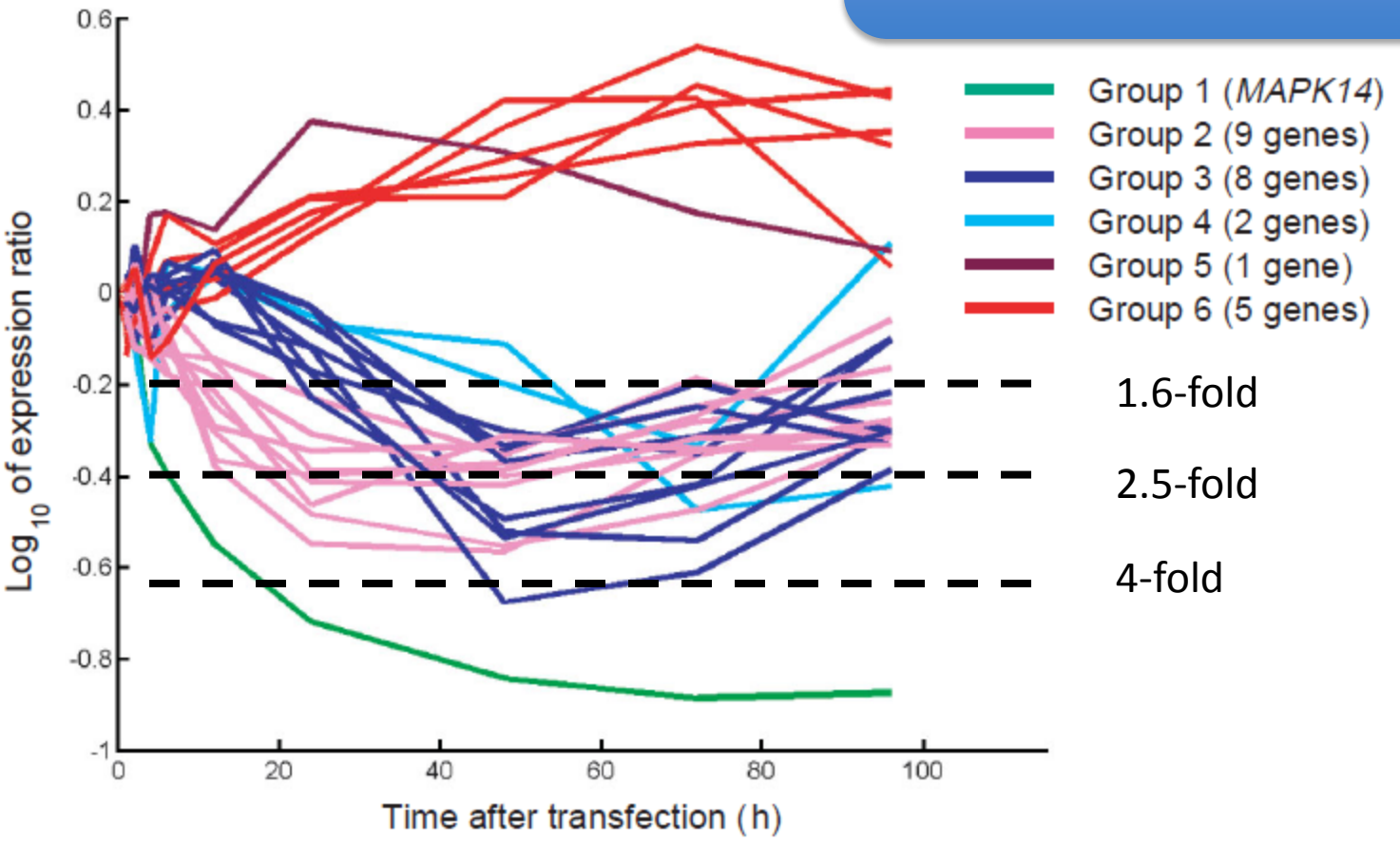
Conclusions

- RNA-based therapeutic strategies can be exploited in mammals—especially injectables, liver-targeted
- Oral delivery is difficult and unlikely to succeed
- Weight of the evidence: little uptake of dietary RNA in any form
- Studies claiming uptake and function are overshadowed by serious doubts, up to the level of the director of NIH
- Off-target effects of dietary, environmental RNA exposure are highly unlikely

Thank you!

Sasha Vlassov, Life Technologies

Off-target "miRNA-like" effects



NATURE BIOTECHNOLOGY VOLUME 21 NUMBER 6 JUNE 2003

Saturation of the RNAi machinery

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

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]

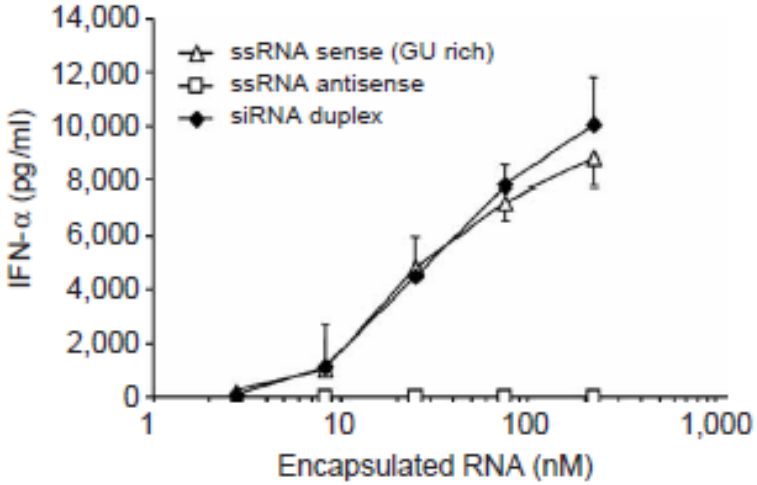
- Examined numerous published datasets
- *In vitro* studies
- Targets of (other) endogenous miRNAs were significantly upregulated at RNA level
- Low fold changes
- Low dose was 100,000 copies per cell

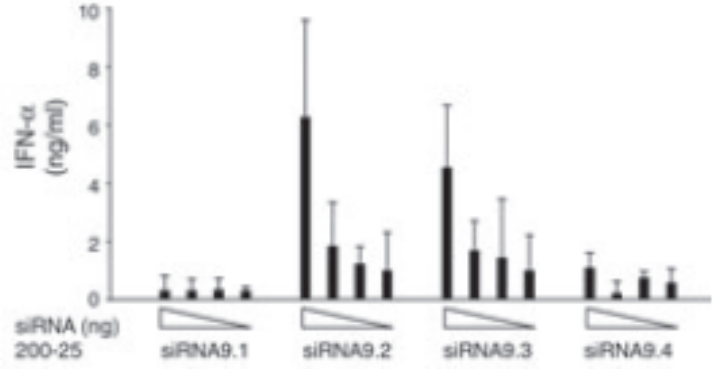
nature biotechnology

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

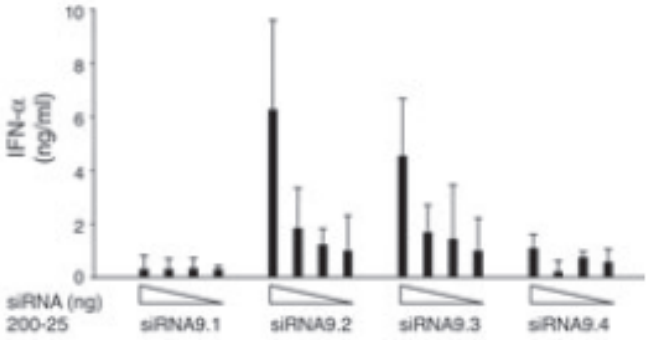




Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7

nature medicine

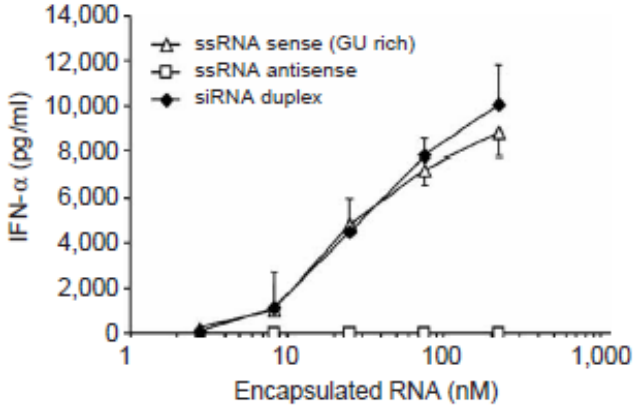
Veit Hornung[1], Margit Guenthner-Biller[1], Carole Bourquin[1], Andrea Ablasser[1], Martin Schlee[2], Satoshi Uematsu[4], Anne Noronha[3], Muthiah Manoharan[3], Shizuo Akira[4], Antonin de Fougerolles[3], Stefan Endres[1] & Gunther Hartmann[1]



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

- Federal Insecticide Act of 1910
- Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA): 1947

Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA): 1947

- Prompted by widespread use of potentially dangerous synthetic organic pesticides
- Included herbicides
- All new products to be registered with USDA

- Labeling: contact info, ingredients, warnings, directions
- Little enforcement mechanism

FIFRA amendments: 1959, 1964

- 1959
  - Nematicides
  - Additional plant controls: desiccants, defoliants

- 1964
  - Federal ID number intro'd
  - Mandate of toxicity-related keywords
  - Authority to stop sale of hazardous pesticides: Secretary of Agriculture

1970s

- EPA formed (1970)—for FIFRA
- 1972 Environmental Pesticide Control Act
  - General use
  - Restricted use (certification required)
  - EPA could deny registration if adverse effects

- 1975 Amendment
  - Scientific Advisory Panel mandated for review of regulations
  - Secretary of Agriculture and economic impact

Today: EPA

- "…regulate the use and sale of pesticides to protect human health and preserve the environment"

1. Put the burden of proof on the manufacturer for suitability, lack of adverse effects

2. Enforce decisions re: banned products
3. Establish regulatory framework

FIFRA SAP per 1972 Federal Advisory Committee Act and following…

- Public deliberation
- Public participation
- Minutes within 90 days
- 5-7 meetings per year
- Process for each
  is shepherded by a designated federal official
- 7 NIH, NSF permanent members + ad hoc

Image: Sharlene Matten, EPA

Purposes of the FIFRA-SAP

- Improve effectiveness and quality of:
  - scientific analyses, reports and operating guidelines
  - scientific testing and of data submitted to EPA
- Peer review major scientific studies
- Scientific basis underlying pesticide regulatory activities
- Enhance EPA's scientific credibility and integrity

Source: EPA

Most common topics for FIFRA meetings

Product(s) or Program	Science Issue(s)	Number of Meetings
Plant-Incorporated Protectants	Human health and/or ecological risk assessment, and/or IRM	11
Bt corn and Bt cotton PIPs	IRM (primary focus)	6
Atrazine	Human Health: cancer and non-Cancer risk, Epidemiology studies, Incident data Environmental Fate: surface water monitoring, drinking water Ecological effects: aquatic ecosystem -- plants, amphibians, invertebrates	8
Endocrine Disruptor Screening Program	Human health and ecological effects: Tier 1 battery, Tier 2 studies, Weight of Evidence, Prioritization using computational toxicology methods	7

Source: EPA

2012-2014

2012	SAP Topics
Jan 31 - Feb 2, 2012	**Common Aquatic Effects Assessment Methodology** Developed in the Office of Pesticide Programs and Office of Water
March 6-7, 2012	Methods for Efficacy Testing of **Bed Bug** Pesticide Products
April 10-12, 2012	Health Effects of **Chlorpyrifos**
June 12-15, 2012	Problem Formulation for the Reassessment of Ecological Risks from the Use of **Atrazine**
Sept 11-14, 2012	**Pollinator** Risk Assessment Framework
2013	
Jan 29 - 31, 2013	Prioritizing the Universe of Endocrine Disruptor Screening Program (**EDSP**) Chemicals Using Computational Toxicology Tools
Mar 19-21, 2013	Draft **Product Performance** Data Needs Assessment for Products Claiming **Efficacy Against Invertebrate Pests**
May 21-23, 2013	Endocrine Disruptor Screening Program (**EDSP**) Tier 1 Screening Assays and Battery Performance
June 25-28, 2013	Endocrine Disruptor Screening Program (**EDSP**) Tier 2 Ecotoxicity Tests
Jul 30-Aug 2, 2013	Weight-of-Evidence: Evaluating Results of **EDSP** Tier 1 Screening
Dec 4-5, 2013	**Corn Rootworm Resistance Monitoring** for Bt corn Plant Incorporated Protectants (PIPs)
2014	
Jan 28, 2014	**RNAi Technology** as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment

Source: EPA

Human Health Considerations

Question 1. Please discuss the nature and extent of uncertainty in the specificity of long sequences of dsRNA targeted at pest species, if bioinformatic analysis shows no significant similarity to mammalian genes?

Question 2. Based on data indicating degradation of the majority of dsRNA in the digestive system, please discuss the strengths and limitations in concluding there will not be significant absorption of dsRNA with possible mammalian effects on oral exposure?

Question 3. To what extent does the specific structure of dsRNA, if it is super coiled or in a hairpin structure, make it more likely to survive degradation in the gut and lead to possible mammalian effects with oral exposure?

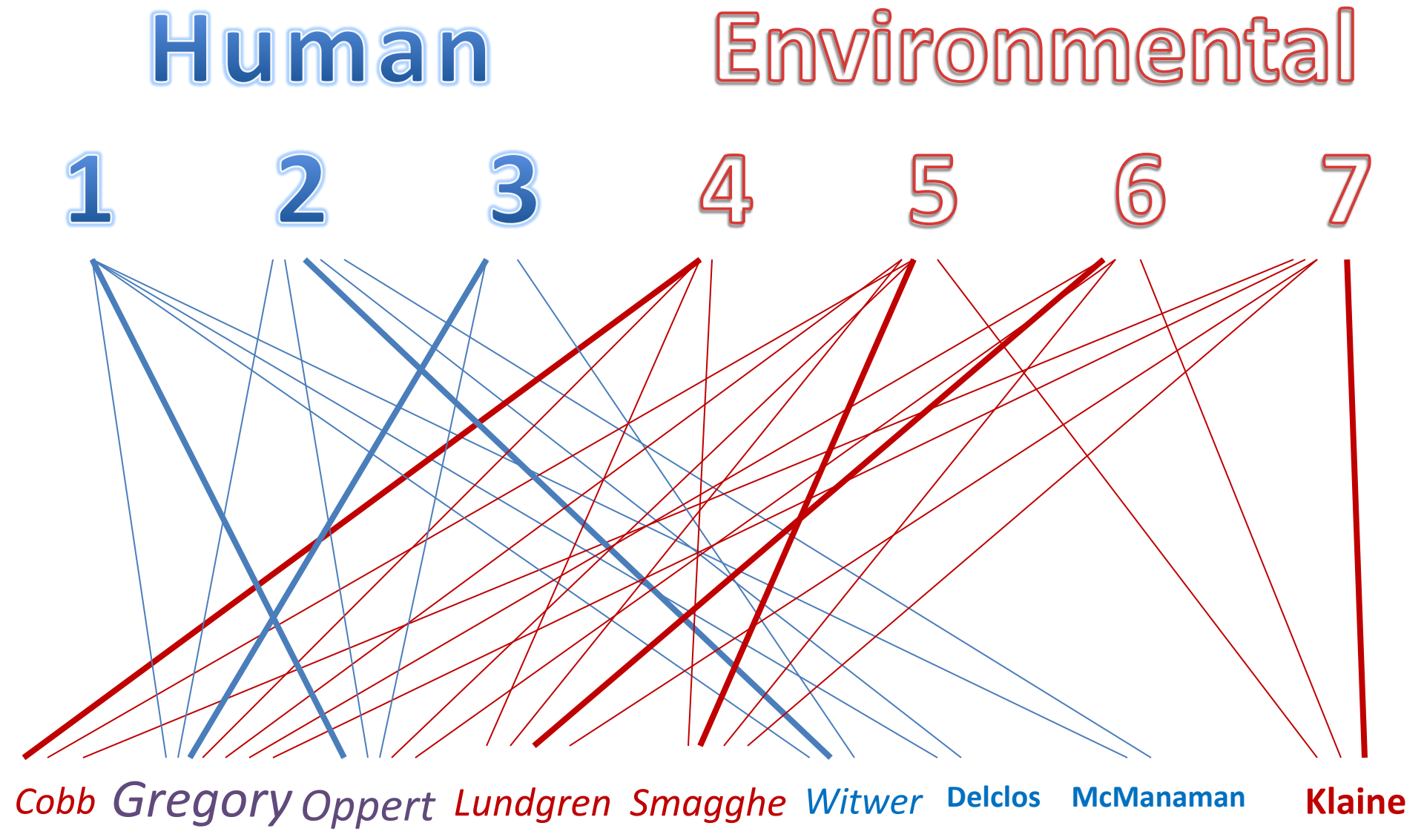
Environmental Considerations

Question 4. Environmental fate of dsRNA and tests needed

Question 5. Routes of exposure and non-target taxa

Question 6. Off-target effects in non-target organisms; information needed to reduce uncertainty

Question 7. Current Framework
Non-target organism and off-target effects testing;
role of bioinformatics;
other information needed


Human
Environmental
1
2
3
4
5
6
7
Cobb
Gregory
Oppert
Lundgren
Smagghe
Witwer
Delclos
McManaman
Klaine
Ad hoc members
Permanent members

To keep in mind…

- Guidance, not policy
- Unanimity not required
  - "a panel member suggested that…"
- Separation between the Charge Questions required and enforced before and after the meeting
- The FIFRA SAP meetings usually last two days;
  - RNAi meeting: one day

EFSA workshop

Risk assessment considerations for RNAi-based GM plants

June 4-5, 2014

Brussels, Belgium

- Molecular biology of RNAi
  - Fire, Vaucheret, Meister, Bellés
- RNAi-based GM plant applications
- Risk assessment considerations

EFSA Day 2: Three Workshops

- Molecular characterization
- Food/feed risk assessment
- Environmental risk assessment
  - Different emphases, but each break-out session included deliberation on off-target effects
- Participants
  - Regulatory officials; citizens' groups; academic scientists; industry scientists and representatives; consultants

EPA and EFSA: an outcome?

Science and other considerations…

EPA and EFSA

- **Significant uptake**? **No**.
- **Functional consequences unlikely…**but PiP-specific studies needed (?)
- **Degradation in the mammalian gut**…but in the sick? Spray applications? And are dermal/inhalation tests needed?
- Other questions: Microbiome?

- **Significant uptake**? Unknown.
- **Function**: no consensus on off-target effects
- Saturation of the endogenous machinery **unlikely**
- Immune stimulation (?)
- RNA in the air, breathed by workers or public?
- Other questions: Microbiome?