Journal Club

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April 9, 2012

Cell Research (2012) ***22:107–126***

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

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Received 11 August 2011; Revised 23 August 2011; Accepted 26 August 2011; Published online 20 September 2011.

Abstract

Our previous studies have demonstrated that stable microRNAs (miRNAs) in mammalian serum and plasma are actively secreted from tissues and cells and can serve as a novel class of biomarkers for diseases, and act as signaling molecules in intercellular communication. Here, we report the surprising finding that exogenous plant miRNAs are present in the sera and tissues of various animals and that these exogenous plant miRNAs are primarily acquired orally, through food intake. MIR168a is abundant in rice and is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. Functional studies *in vitro and in vivo demonstrated that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in liver, and consequently decrease LDL removal from mouse plasma. These findings demonstrate that exogenous plant miRNAs in food can regulate the expression of target genes in mammals.*

A non-coding RNA (ncRNA) is a functional RNA molecule that is not translated into a protein.

Non-coding RNA genes include highly abundant and functionally important RNAs such as :

- **transfer RNA (tRNA)**
- **ribosomal RNA (rRNA)**
- **microRNA (miRNA)**
- **small nucleolar RNAs (snoRNAs)**
- **small interferrig RNAs (siRNAs)**
- **PIWI-interacting RNAs (piRNAs)**
- **and the long ncRNAs:**
  - **transcribed ultraconserved regions (T-UCRs)**
  - **large intergenic non-coding RNAs (lincRNAs)**

RNAs involved in protein synthesis:

Type	Abbr.	Function	Distribution
Messenger RNA	mRNA	Codes for protein	All organisms
Ribosomal RNA	rRNA	Translation	All organisms
Signal recognition particle RNA	7SL RNA or SRP RNA	Membrane integration	All organisms
Transfer RNA	tRNA	Translation	All organisms
Transfer-messenger RNA	tmRNA	Rescuing stalled ribosomes	Bacteria

RNAs involved in post-transcriptional modification or DNA replication:

Type	Abbr.	Function	Distribution
Small nuclear RNA	snRNA	Splicing and other functions	Eukaryotes and archaea
Small nucleolar RNA	snoRNA	Nucleotide modification of RNAs	Eukaryotes and archaea
Ribonuclease P	RNase P	tRNA maturation	All organisms
Ribonuclease MRP	RNase MRP	rRNA maturation, DNA replication	Eukaryotes
Y RNA		RNA processing, DNA replication	Animals
Telomerase RNA		Telomere synthesis	Most eukaryotes

Regulatory RNAs:

Type	Abbr.	Function	Distribution
Antisense RNA	**aRNA**	**Transcriptional attenuation / mRNA degradation / mRNA stabilisation / Translation block**	**All organisms**
Cis-natural antisense transcript		Gene regulation	
Long noncoding RNA	Long nc RNA	Various	Eukaryotes
MicroRNA	**miRNA**	**Gene regulation**	**Most eukaryotes**
Piwi-interacting RNA	piRNA	Transposon defense, maybe other functions	Most animals
Small interfering RNA	siRNA	Gene regulation	Most eukaryotes
Trans-acting siRNA	tasiRNA	Gene regulation	Land plants
Repeat associated siRNA	rasiRNA	Type of piRNA; transposon defense	Drosophila

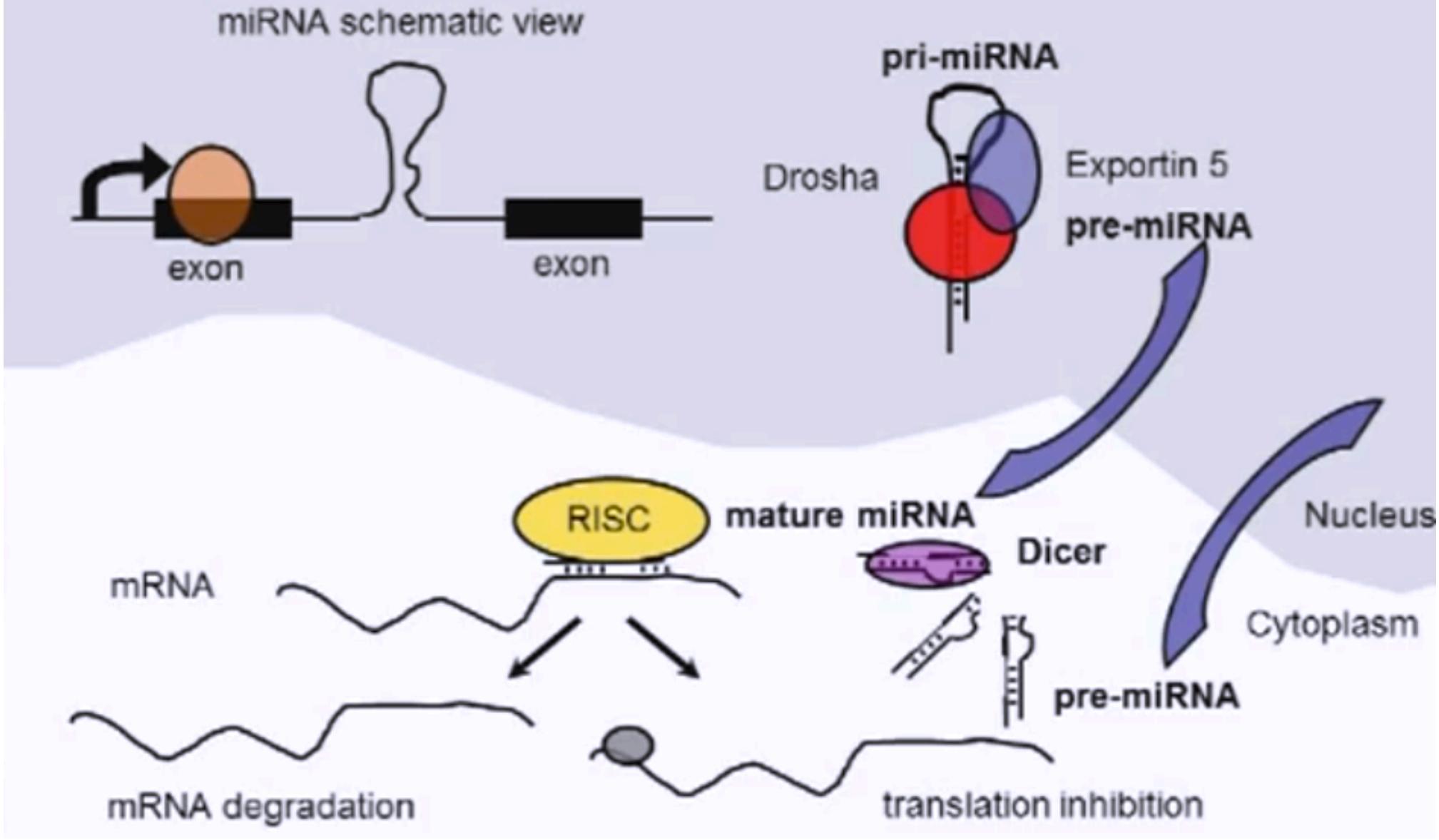
MicroRNAs

miRNAs are a class of post-transcriptional regulators.

They are short ~22 nucleotide RNA sequences that bind to complementary sequences in the 3' UTR of multiple target mRNAs, usually resulting in their silencing. MicroRNAs target ~60% of all genes, are abundantly present in all human cells and are able to repress hundreds of targets each.

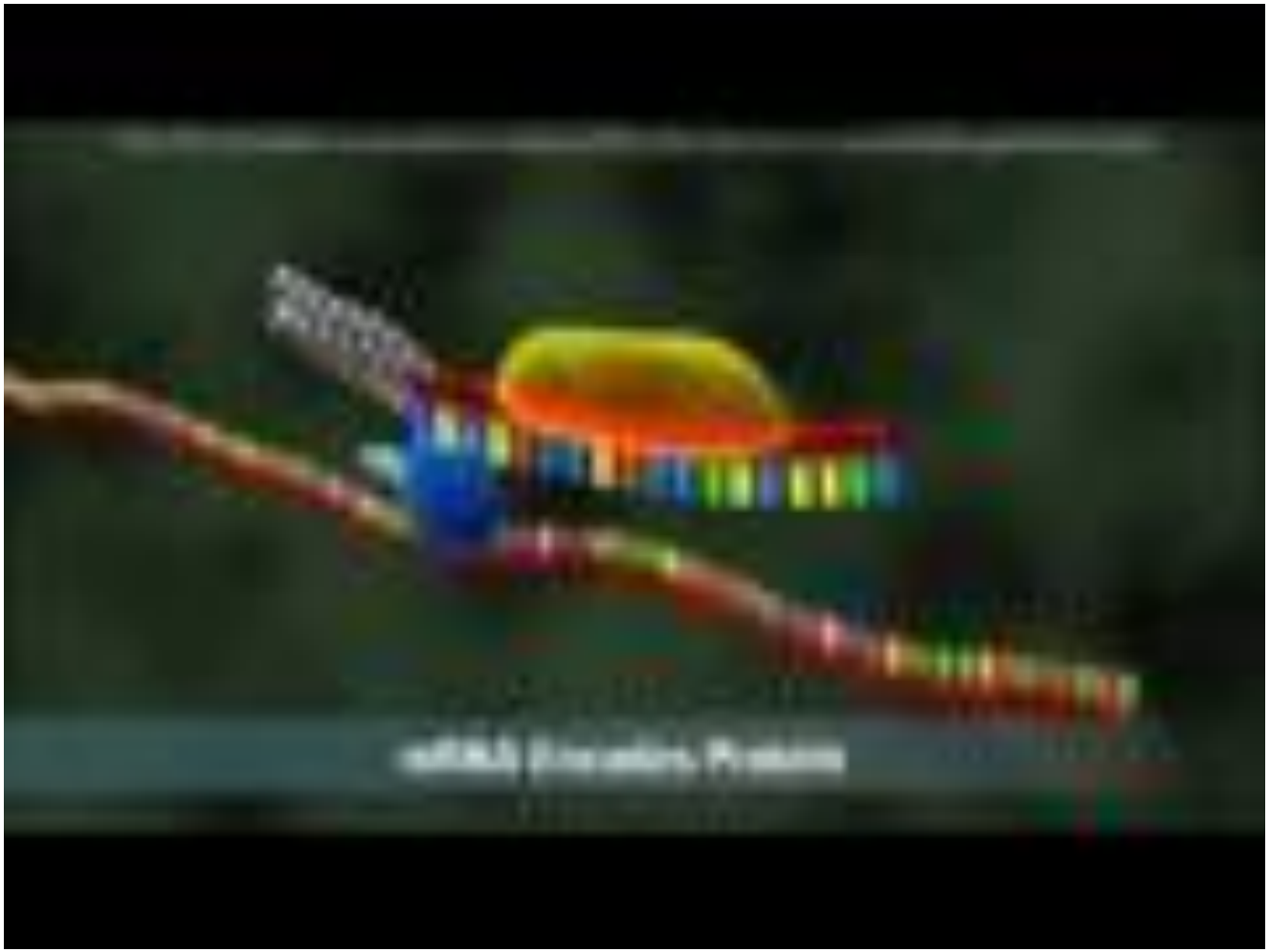
4000 miRNAs have been discovered in all studied eukaryotes including mammals, fungi and plants. More than 700 miRNAs have so far been identified in humans and over 800 more are predicted to exist.

miRNA biogenesis



- MicroRNA short introduction

miRNA by AGTechnologies



What do we know about miRNA functions?

- miRNAs have been widely shown to modulate various critical biological processes, including differentiation, apoptosis, proliferation, the immune response, and the maintenance of cell and tissue identity
- Dysregulation of miRNAs has been linked to cancer and other diseases.
- miRNAs exist stably in the sera and plasma of humans and animals. Specific serum miRNA expression profiles show a great potential to serve as a novel class of biomarkers for the diagnosis of cancer and other diseases
- Numerous reports have shown that unique expression patterns of circulating miRNAs reflect various physiological and pathological conditions.

Plant miRNAs are present in human and animal sera and organs:

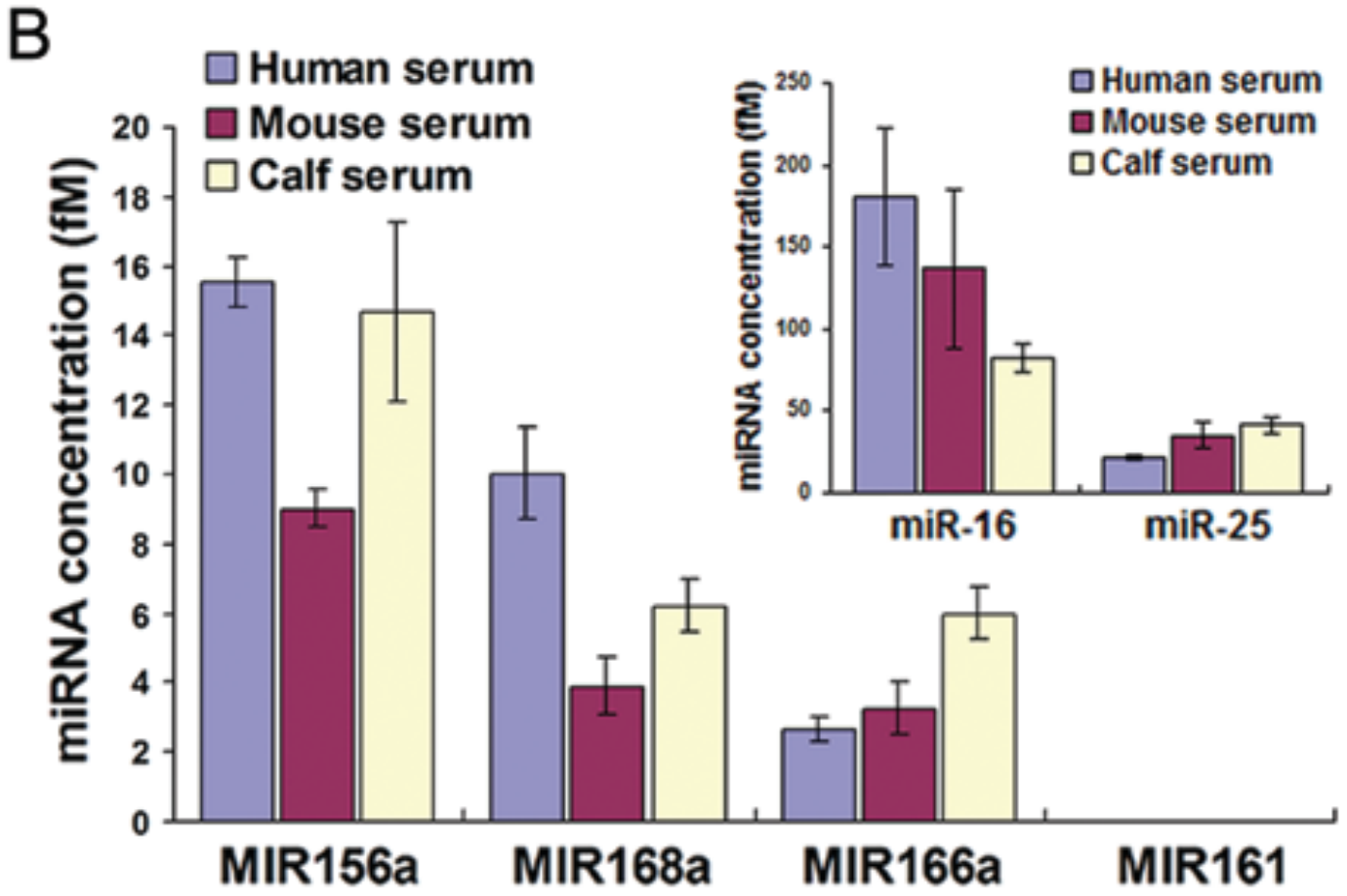
- Exogenous plant miRNAs were consistently present in the serum of healthy (i.e. had no metabolic dysfunction) Chinese men and women, having rice as their main diet.
- Plant miRNAs were also detected in the sera of other animals, such as calves, whose diet was mainly green rich fodder.
- ~30 known plant miRNAs were found in human donors, among which MIR156a and MIR168a showed considerable levels of expression (the mean Solexa reads/total mammalian miRNAs > 0.005).

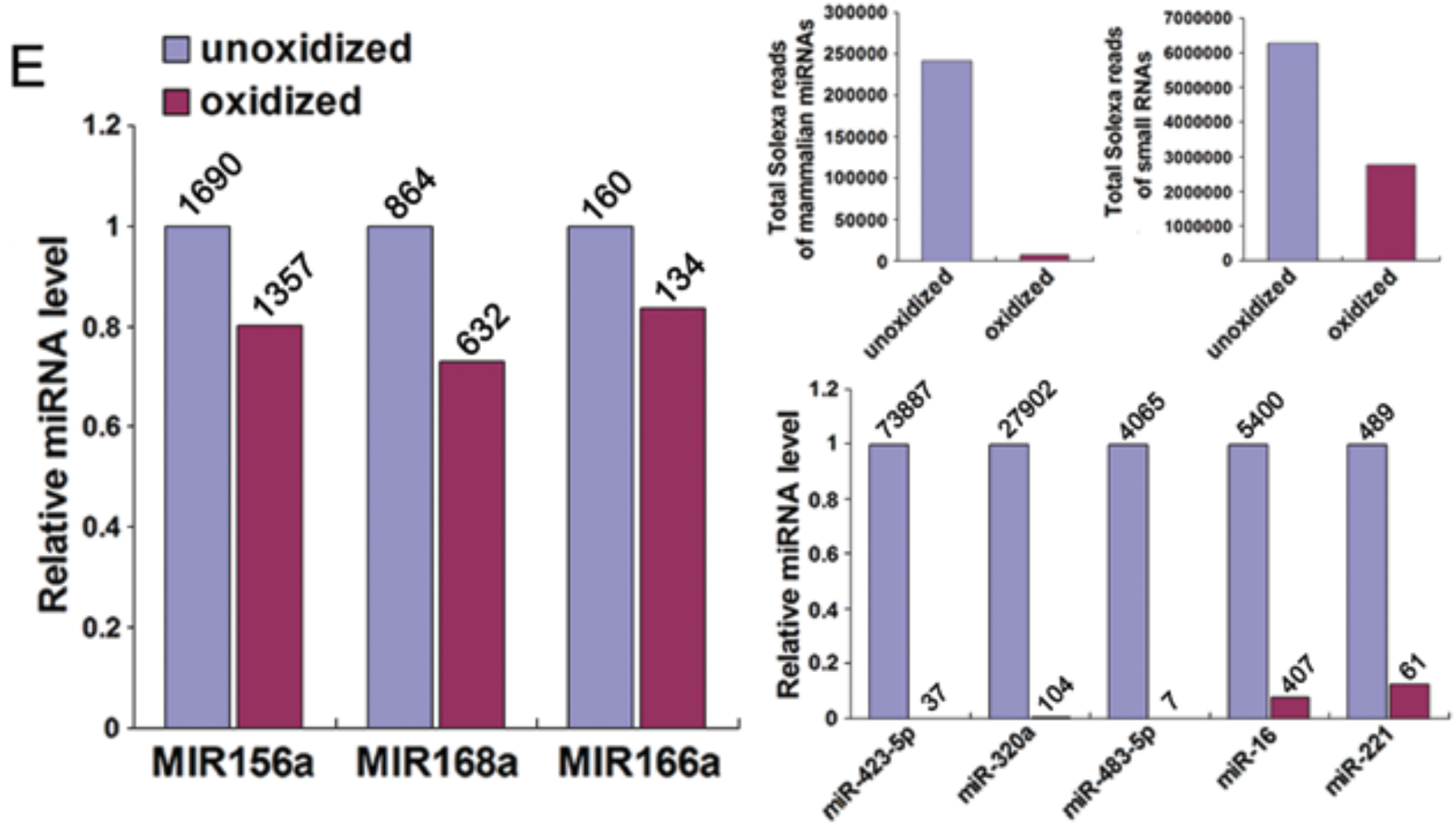
Plant miRNAs are present …

- To confirm the Solexa data, the levels of MIR168a and MIR156a, the two plant miRNAs with the highest levels in the sera of Chinese subjects, and MIR166a, a plant miRNA with modest level, were assessed by a stem-loop quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. MIR161, whose expression level was undetectable, served as a negative control.
- Plant miRNAs at a level as low as 1 fM could be efficiently examined by this assay.

(B) The absolute levels of plant miRNAs in the sera of various mammals detected by qRTPCR

(n = 6). Endogenous animal miRNAs, miR-16, and miR-25 serve as controls (insert).



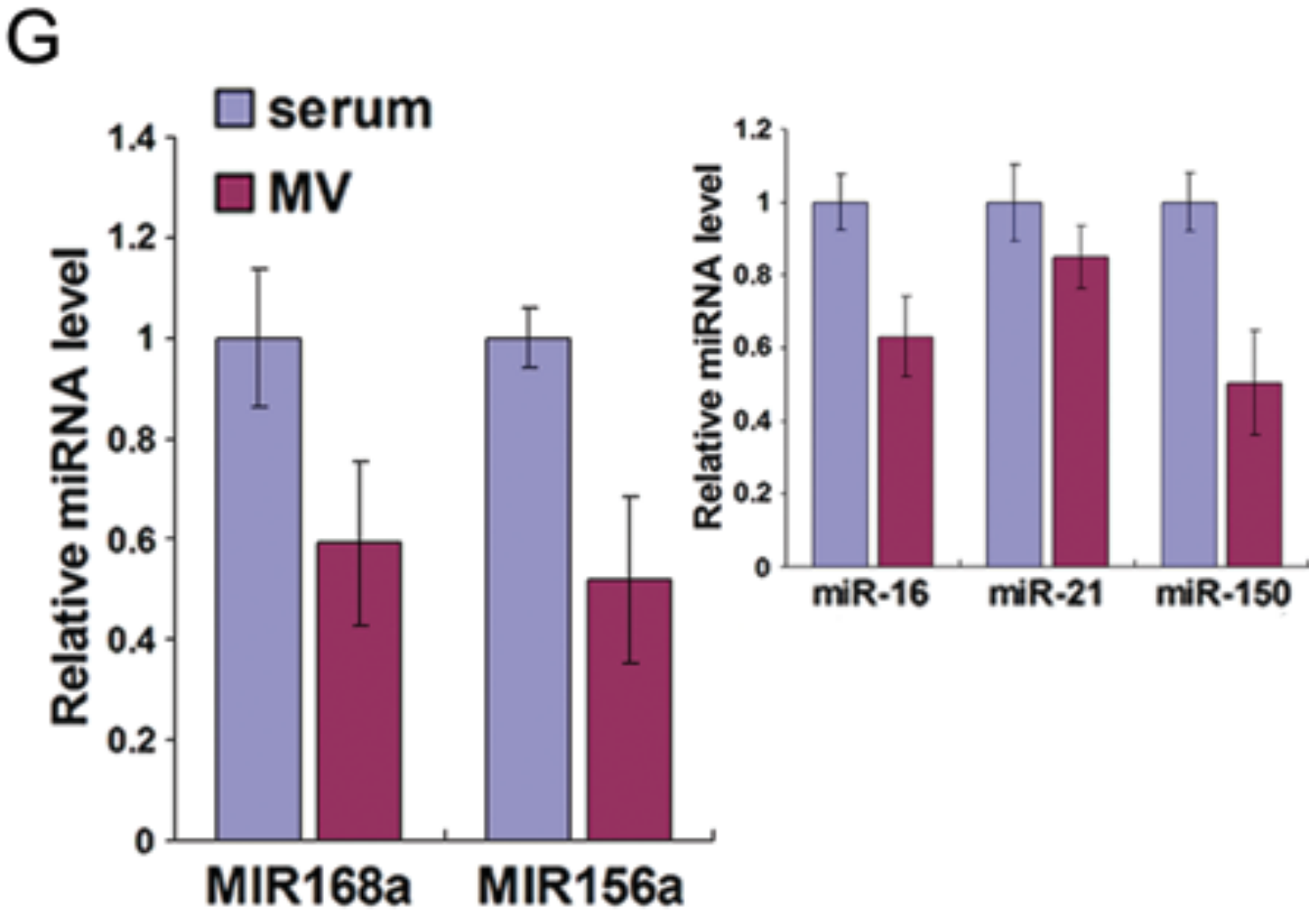


(E) ***Equal amount of total small RNAs (each from 100 ml of human serum) were treated with/without sodium periodate. After the reactions, the RNAs were purified and then subjected to Solexa sequencing. Solexa reads of the plant miRNAs in oxidized and unoxidized groups were compared. Total and individual mammalian miRNAs were compared to serve as controls (insert). The absolute Solexa reads of miRNAs are indicated. Plant miRNAs are 2´ -O-methyl modified on their terminal nucleotide, which renders them resistant to periodate***

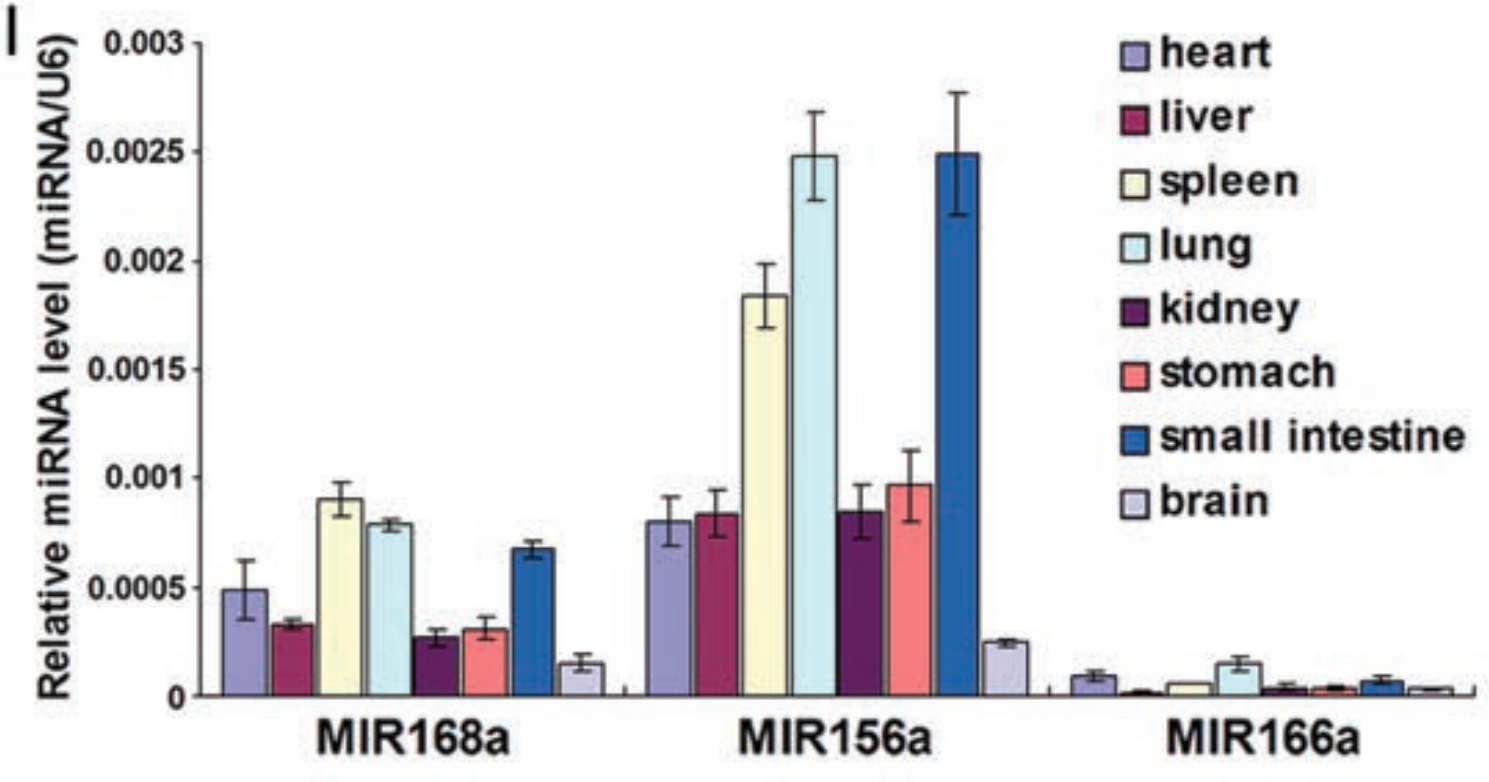
Microvesicles as a possible carrier of circulating miRNAs?

- **Microvesicles (MVs) are small vesicles that are shed from almost all cell types under both normal and pathological conditions**. They bear surface receptors/ligands of the original cells and **have the potential to selectively interact with specific target cells and mediate intercellular communication by transporting bioactive lipids, mRNA, or proteins between cells.** The authors recent results demonstrated that MVs from human plasma are a mixture of microparticles, exosomes, and other vesicular structures and that many types of MVs in human plasma contain miRNAs

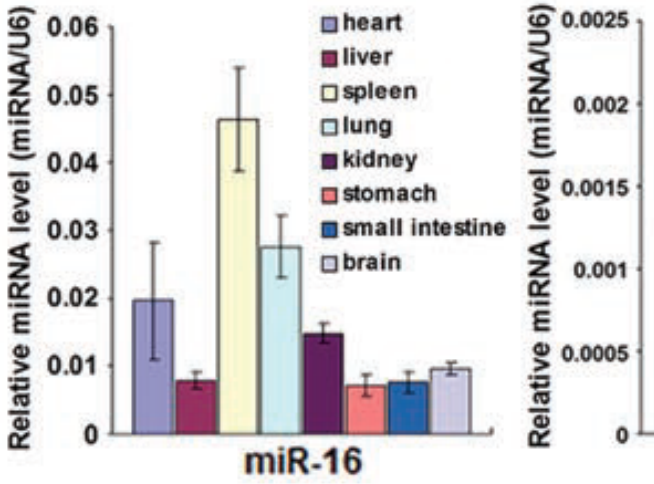
- => *Next they determined the portion of circulating plant miRNAs in MVs compared to MV-free plasma.*



G The levels of plant miRNAs detected by qRT-PCR in MVs isolated from C57BL/6J mouse plasma (n = 4).



Plant MIR168a and MIR156a were detected in various mouse tissues, including liver, small intestine, and lung. Interestingly, MIR166a was nearly undetectable in various mouse tissues, although it was present in the serum. In these studies, the levels of miRNAs were normalized to U6 snRNA and other small RNAs such as snoRNA146 and snoRNA251.



What is the source of these exogenous plant miRNAs in the sera and organs of mammals?

- **Given that plant MIR168a and MIR156a have been reported to be enriched in various plants, including rice (Oryza sativa) and crucifers (Brassicaceae), we hypothesized that plant miRNAs in the sera and tissues of humans and animals are primarily a result of food intake.**

- the expression levels and stability of plant miRNAs in various foods were investigated:

✓ plant MIR168a, MIR156a, and MIR166a were detected in chow diet in the concentrations of 0.43, 0.54 and 0.66 fmol/g (miRNA level/weight of chow diet)

✓ the levels of these three miRNAs were higher, respectively 2.44, 5.72 , 2.25 (fmol/g), in fresh rice than in chow diet

✓ In contrast to chow diet no mammalian miRNA could be detected in rice

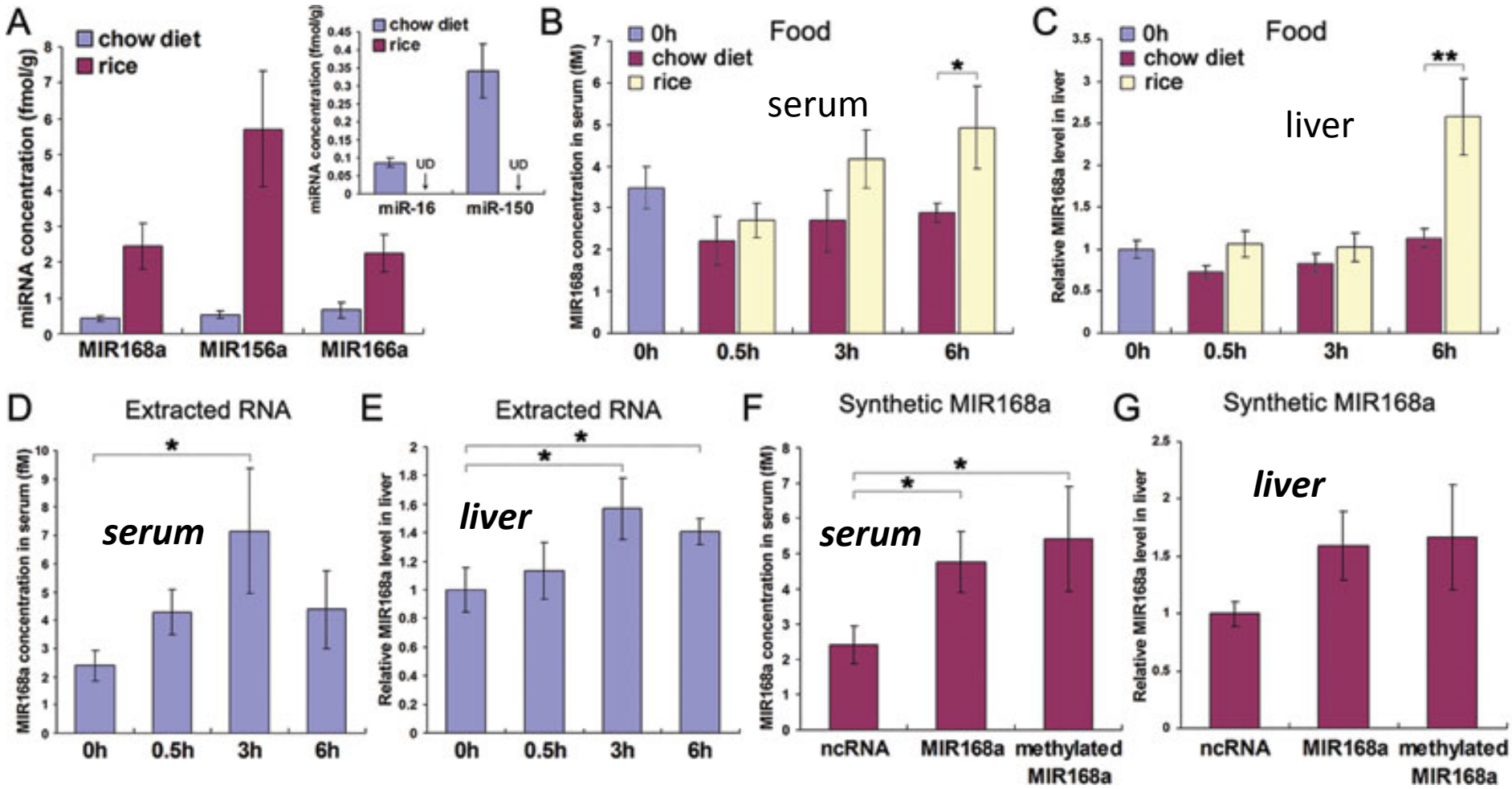
✓ Interestingly, plant miRNAs were stable in cooked foods

✓ Total RNA isolated from rice or mouse liver was adjusted to pH 2.0 and kept at 37 °C for several hours. Acidification did not significantly affect the yield and quality of miRNAs. The majority of plant miRNAs and mammalian miRNAs can survive under acidic condition for at least 6 h. Methylation appears to enhance plant miRNA stability to acidification.

Supplementary information, Table S3 The level of plant miRNAs in chow diet, rice, Chinese cabbage, wheat, and potato.

	MIR156a (fmol/g)	MIR166a (fmol/g)	MIR168a (fmol/g)
chow diet	0.54	0.66	0.43
rice (*Oryza sativa*)	5.72	2.25	2.44
cooked rice	0.33	0.01	0.95
Chinese cabbage (*Brassica rapa pekinensis*)	2.62	2.29	1.68
cooked Chinese cabbage	0.58	1.8	0.28
wheat (*Triticum aestivum*)	0.35	0.04	undetectable
cooked wheat	0.47	0.17	undetectable
potato (*Solanum tuberosum*)	2.01	0.02	0.23
cooked potato	0.49	0.01	undetectable

=>The exogenous mature plant miRNAs in food can pass through mouse GI tract and enter the sera and organs.

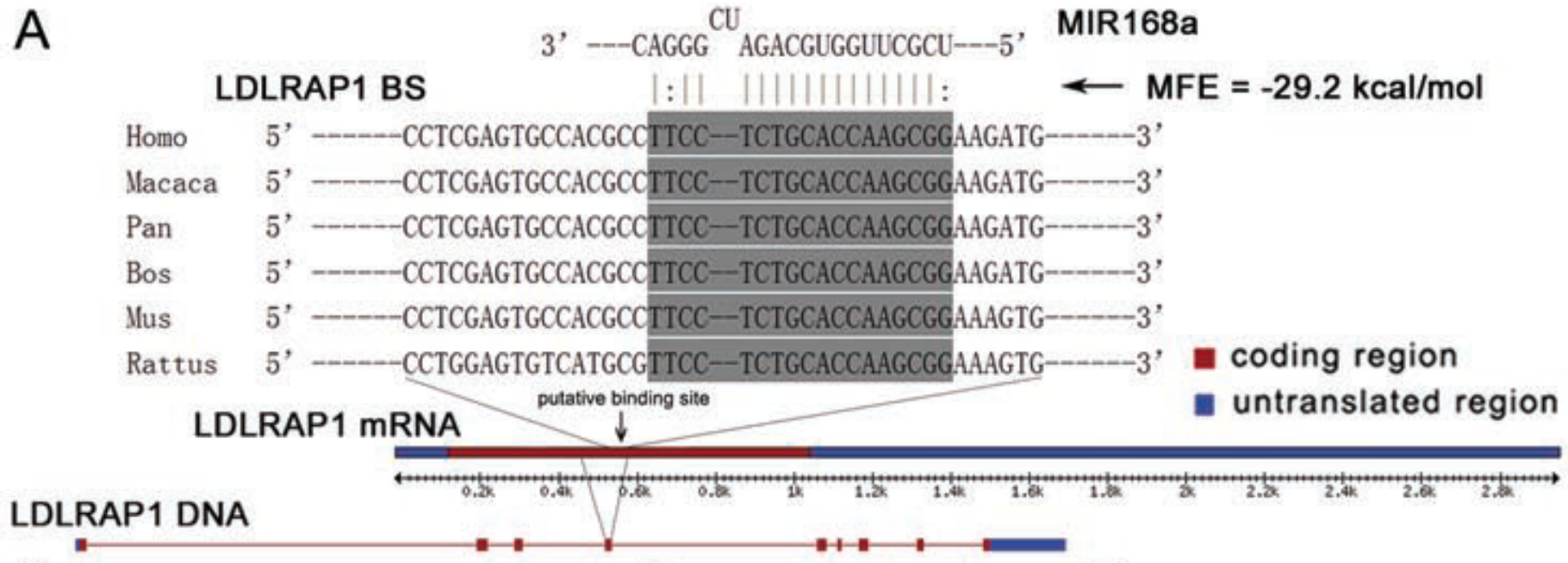


D, E, F, G : following gavage feeding with synthetic MIR168a and synthetic methylated MIR168a (n = 8)

What are the potential plant miRNA targets?

- Because most plant miRNAs can act like RNA interference (RNAi), which requires a high degree of complementarity between miRNAs and target RNAs, **bioinformatic analysis** to identify any sequences in the human, mouse, or rat genome with perfect or near-perfect match to MIR168a was performed. **Approximately 50 putative target genes were identified as the target genes of MIR168a.**

- **The most highly conserved sequence of a putative binding site among various species is located in exon 4 of the low-density lipoprotein receptor adapter protein 1 (LDLRAP1)**



(A) Schematic description of the hypothesized duplexes formed by interactions between the exon 4 of LDLRAP1 and MIR168a. Paired bases are indicated by a black oval and G:U pairs are indicated by two dots. The predicted free energy of the hybrid is indicated. Note that the potential binding site of MIR168a to LDLRAP1 mRNA is highly conserved across species.

LDLRAP1 (the low-density lipoprotein receptor adapter protein 1):

- is a liver-enriched gene that plays a critical role in facilitating the removal of LDL from the circulatory system.
- LDL molecules, are the major carriers of cholesterol in the blood, and each one contains approximately 1,500 molecules of cholesterol ester. The shell of the LDL molecule contains just one molecule of apolipoprotein B100, which is recognized by the LDL receptor in peripheral tissues.
- When the cell has abundant cholesterol, LDL receptor synthesis is blocked so new cholesterol in the form of LDL molecules cannot be taken up.
- As a result many LDL molecules appear in the blood without receptors on the peripheral tissues. These LDL molecules are oxidized and taken up by macrophages, which become engorged and form foam cells. These cells often become trapped in the walls of blood vessels and contribute to artherosclerotic plaque formation.

Plant MIR168a binds to exon 4 of mammalian LDLRAP1 and decreases LDLRAP1 protein level *in vitro*

- Transfection of HepG2 cells (a hepatocyte carcinoma cell line) with MIR168a precursor (pre-MIR168a) resulted in a 1 000-fold elevation in MIR168a, suggesting that plant pre-MIR168a can be properly processed in mammalian HepG2 cells. Additionally, the LDLRAP1 protein level in these cells was significantly reduced, whereas the LDLRAP1 mRNA level was not affected.

- A luciferase reporter constructs WT and exon 4 mutated LDLRAP1 were cloned and transfected into the HepG2 cells. The reporter activity was significantly reduced in WT upon pre-MIR168a transfection, whereas the mutant version remained unaffected. Inhibition was miRNA concentration dependent.
- Same results confirmed with site directed mutagenesis of the binding site and GFP tagged ORF of the receptor adaptor gene.

Hypothesis for *in vivo* plant miRNA action mechanism :

- Epithelial cells, particularly in the small intestine, might take up plant miRNAs in food, then package them into MVs and release them into the circulatory system.
- The secreted MVs from small intestinal epithelial cells could then deliver exogenous plant miRNA to target cells of other organs and regulate recipient cell function.

Mature MIR168a in Caco-2 cell-derived MVs sufficiently reduces mammalian LDLRAP1 protein level in recipient HepG2 cells:

- Human intestinal epithelial Caco-2 cells were transfected with single-stranded mature MIR168a.

=> The MVs released by the Caco-2 cells were collected and used to treat HepG2 cells.

===> The LDLRAP1 protein level in HepG2 cells was then assessed.

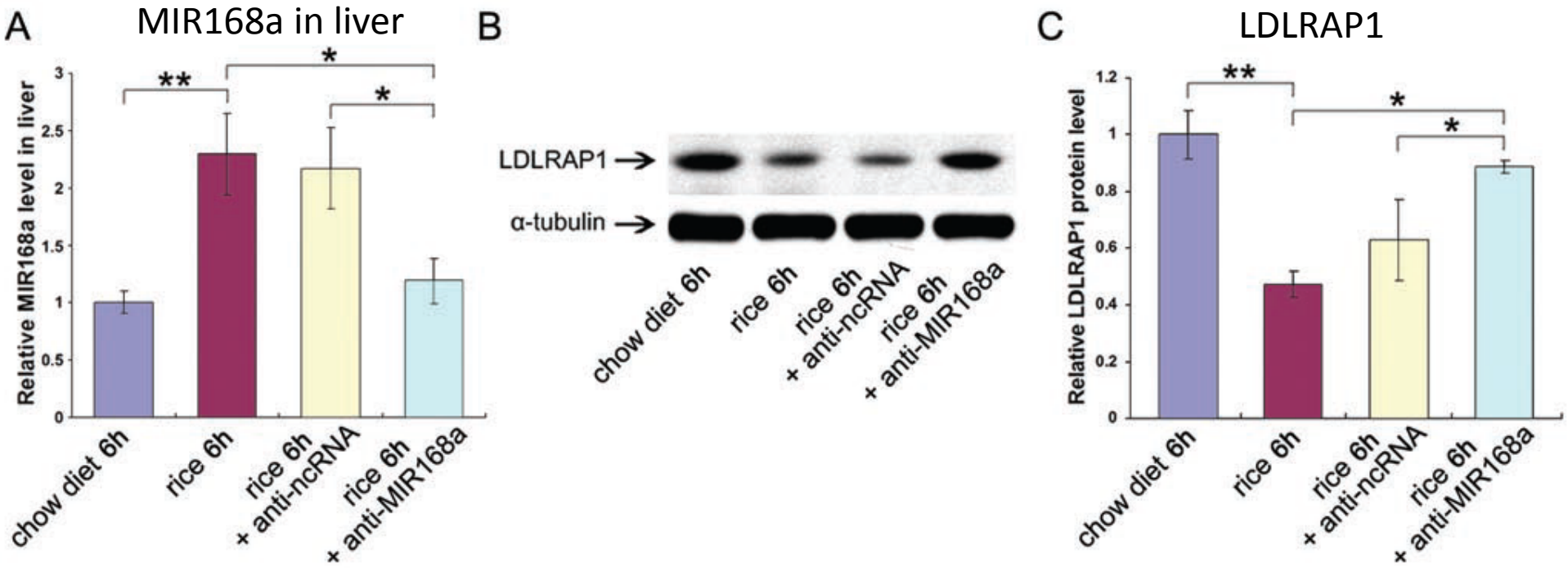
➔ The levels of mature MIR168a were elevated by 200-fold in MVs released by Caco-2 cells transfected with MIR168a and by 100-fold in Caco-2 MV-treated HepG2 cells.

➔ Plant MIR168a delivered into HepG2 cells via Caco-2 MVs significantly decreased the LDLRAP1 protein level in the recipient HepG2 cells, while it had no effect on LDLRAP1 mRNA levels. The repression was dose dependent.

AGO2-association important?

- Because **Argonaute2 (AGO2)** is present in MVs and facilitates miRNA binding to its target gene via the **RNA-induced silencing complex (RISC)** an immunoprecipitation experiment was performed to assess whether plant MIR168a was associated with mammalian AGO2 and LDLRAP1.
- => both MIR168a and LDLRAP1 mRNA were detected in the product precipitated by an anti-AGO2 antibody from the HepG2 cells treated with Caco-2 MVs, while only MIR168a but not LDLRAP1 mRNA was detected in the anti-AGO2 antibody-precipitated product from the Caco-2 MVs.

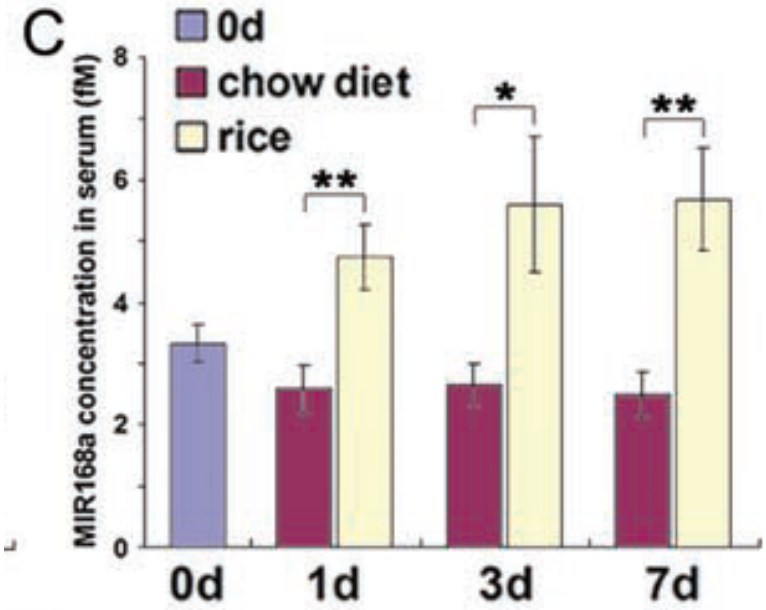
Anti-MIR168a ASO reverses rice feeding-induced reduction of mouse liver LDLRAP1 protein at 6 h feeding

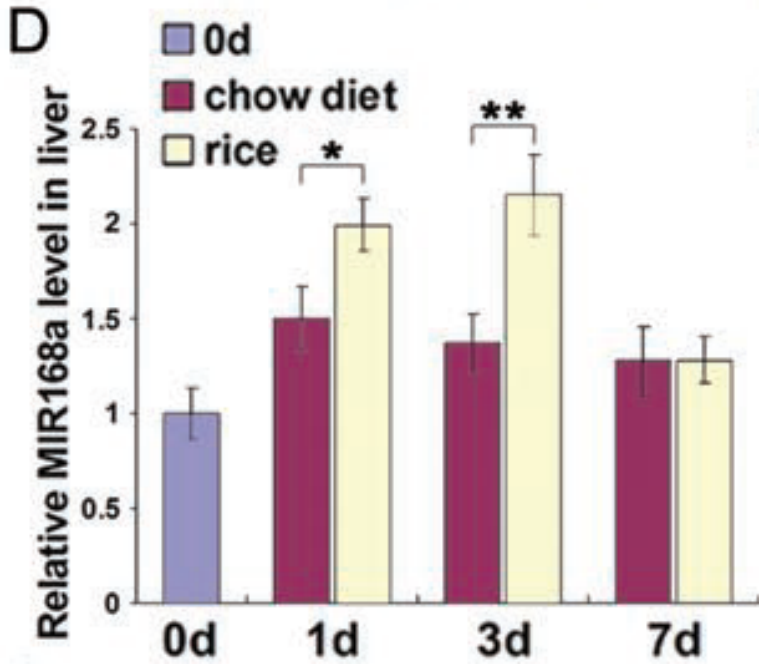


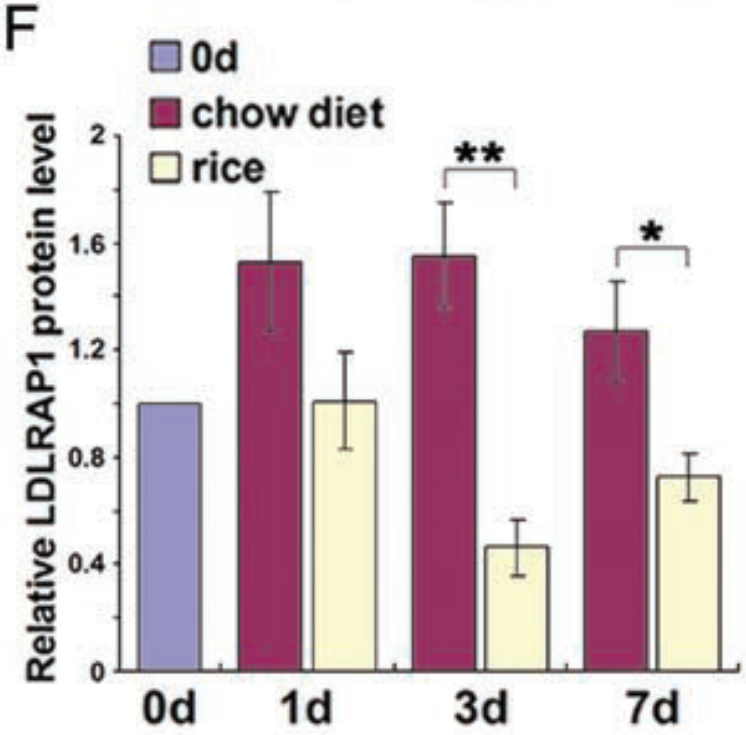
Exogenous MIR168a inhibits mouse liver LDLRAP1 expression and elevates plasma LDL-cholesterol level at 3 days after food intake

! LDL is the major cholesterol-carrying lipoprotein of human plasma and plays an essential role in the pathogenesis of atherosclerosis. Downregulation of LDLRAP1 in the liver causes decreased endocytosis of LDL by liver cells and impairs the removal of LDL from plasma.

- To assess the physiological function of food-enriched MIR168a in mammals, mice were fed with fresh rice and chow diets for 7 days after a 12-h of fasting:


C
0d
chow diet
rice
MIR168a concentration in serum (fM)
0
2
4
6
8
**
*
**
0d
1d
3d
7d


D
0d
chow diet
rice
Relative MIR168a level in liver
0
0.5
1
1.5
2
2.5
*
**
0d
1d
3d
7d


F
0d
chow diet
rice
Relative LDLRAP1 protein level
0
0.4
0.8
1.2
1.6
2
**
*
0d
1d
3d
7d

In vitro & in vivo results are consistent:

- Consistently, the rice-induced elevation of LDL levels in mouse plasma was blocked by the anti-MIR168a ASO
- Furthermore, the association of MIR168a with LDLRAP1 mRNA through AGO2 in mouse liver was tested: For this experiment, AGO2 protein in mouse liver following different treatment was immunoprecipitated by anti-AGO2 antibody and the levels of MIR168a and LDLRAP1 mRNA in anti-AGO2 antibody IP product were assessed.
- ==> In rice-fed mice, AGO2-associated MIR168a and LDLRAP1 mRNA in mouse livers were significantly increased compared to those in chow diet-fed mice and this rice feeding-induced elevation of liver AGO2-associated MIR168a and LDLRAP1 mRNA could be blocked by administration of anti-MIR168a ASO.
- Decreased liver LDLRAP1 levels led to an elevation in the mouse plasma LDL level.

MIR168a is a specific inhibitor of LDLRAP1 expression in mouse liver, but does not affect cholesterol biosynthesis.

- In separate experiments, we tested the link between the downregulation of mouse liver LDLRAP1 and the elevation of mouse plasma LDL level by directly decreasing liver LDLRAP1 by administration of LDLRAP1 siRNA.
- Downregulation of liver LDLRAP1 via LDLRAP1 siRNA resulted in the elevation of plasma LDL level suggesting that LDLRAP1 is responsible for plasma LDL clearance.
- However, the level of liver LDLRAP1 was not related to the levels of plasma cholesterol or triglycerides. Decreased levels of plasma cholesterol, but unchanged plasma ApoA and triglyceride levels further supported the hypothesis that the elevation of fresh rice-derived MIR168a in the mouse liver specifically decreased liver LDLRAP1 expression and thus caused an elevated LDL level in mouse plasma.

Summary:

- Taken together, these results indicate that exogenous plant miRNAs are able to enter the serum and organs of mammals via food intake, and that plant MIR168a can bind to the nucleotide sequence located in exon 4 of mammalian LDLRAP1, leading to the inhibition of LDLRAP1 expression *in vivo.*

Conclusions:

- Mature single-stranded plant miRNAs are stable.
- Neither plant pri-miRNA nor plant pre-miRNA has been detected in the sera and tissues of human and animals. Single-stranded mature MIR168a is likely to be the form taken up by animals via the GI tract.
- Plant miRNAs execute their function in mammalian cells in a fashion of mammalian miRNA.
- AGO2-associated mature MIR168a in MVs is the functional form *in vivo.*
- Food-derived miRNAs may serve as a novel essential nutrient.

You are what you eat

- Anthelme Brillat-Savarin wrote, in *Physiologie du Gout, ou Meditations de Gastronomie Transcendante, 1826:"Dis-moi ce que tu manges, je te dirai ce que tu es."*

Tell me what you eat and I will tell you what you are.

- In an essay entitled *Concerning Spiritualism and Materialism, 1863/4, Ludwig Andreas Feuerbach wrote:"Der Mensch ist, was er ißt."*

That translates into English as 'man is what he eats'.