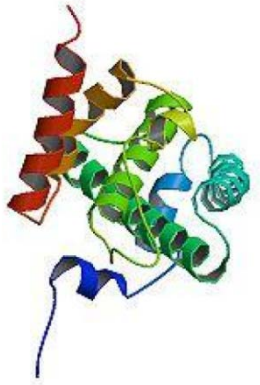
Institute of Biotechnology
Berlin University of Technology

Die RNA – vom kleinen Bruder der DNA zum Multitalent

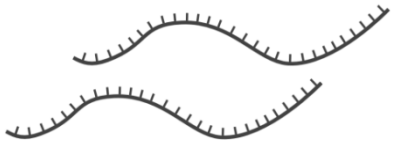
Jens Kurreck

Change of the Paradigm

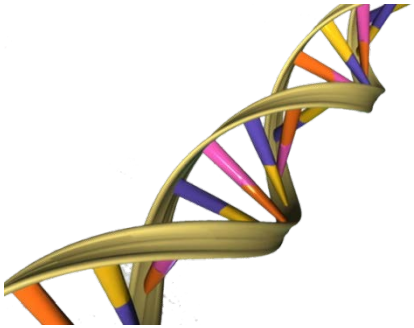
Traditional point of view:



Information

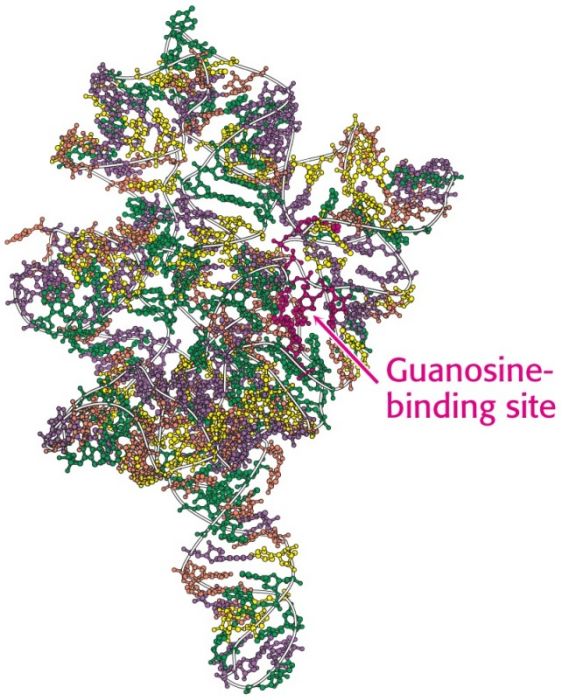


DNA → RNA → **Protein**



Catalysis

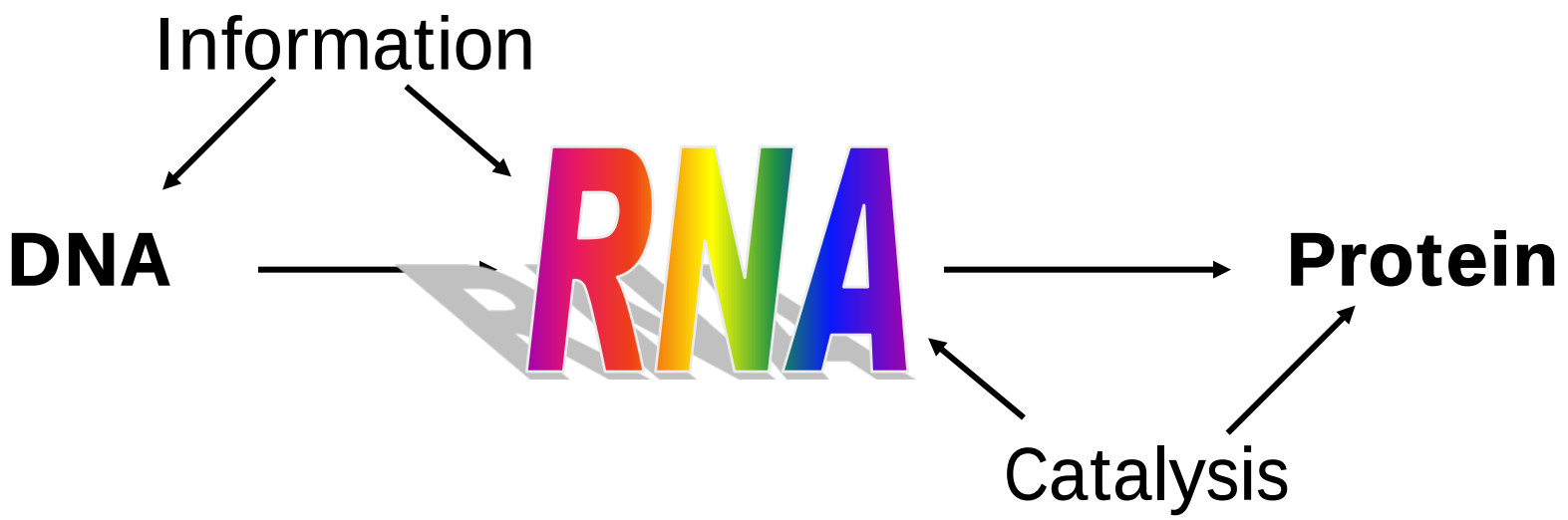
Discovery of Ribozymes



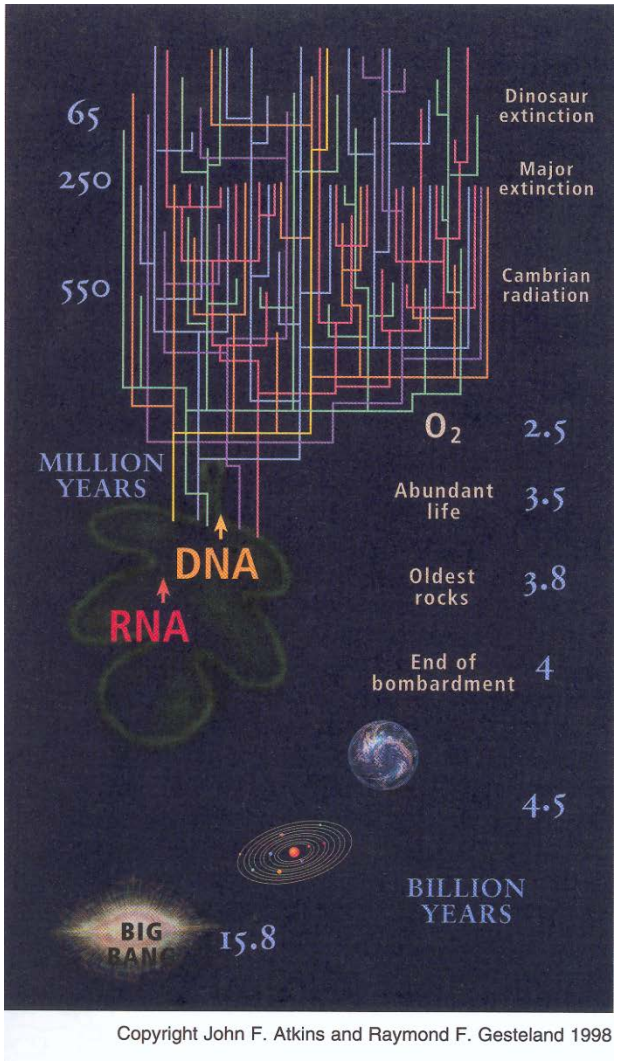
- In the early 1980s, the groups Cech and Altman discovered RNAs with catalytic properties: Ribozymes
- RNA can carry and transmit genetic information and catalyze reactions.

Change of the Paradigm

New point of view:



The RNA World Hypothesis



According to the RNA world hypothesis our current DNA / protein-based world was preceeded by a world dominated by RNA.

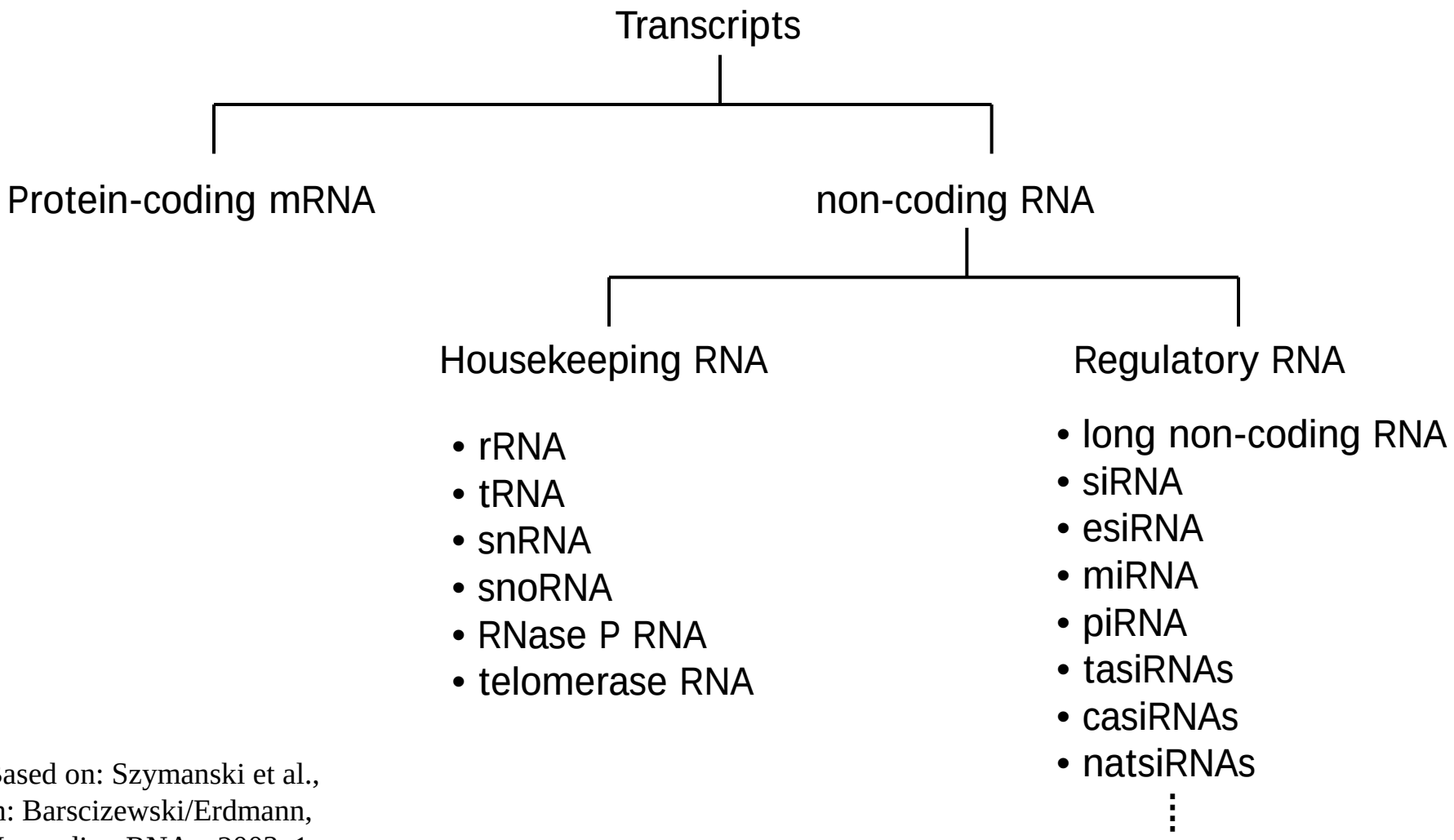
Non-(Protein-) Coding RNA

Organism	Genome Length (kbp)	Protein-Coding Part (%)	Noncoding Part (%)	Number of Genes
Eubacteria				
U. urealyticum	751	88	12	577
E. coli	4639	84	16	4000
M. leprae	3268	73	27	2584
Archaea				
P. horikoshii	1739	87	13	1636
M. jannaschii	1665	83	17	1599
S. solfataricus	2992	77	23	2610
Eukaryota				
E. cuniculi	2900	90	10	2000
S. cerevisiae	12000	71	29	5651
S. pombe	12463	57	43	4824
A. thaliana	115410	29	71	25500
C. elegans	97000	27	73	18424
D. melanogaster	180000	13	87	13600
H. sapiens	3000000	2	98	20.-25.000

Szymanski et al., in: Barscizewski/Erdmann, Noncoding RNAs, 2003, 1.

- While in bacteria almost the complete genome encodes proteins, only 2% of the human genome encodes proteins.
- The ENCODE project demonstrated that more than three quarters of the human genome are transcribed.
- What is the function of the large part of RNA that is transcribed but does not encode proteins?

Diversity of RNAs



Based on: Szymanski et al., in: Barscizewski/Erdmann, Noncoding RNAs, 2003, 1.

Overview

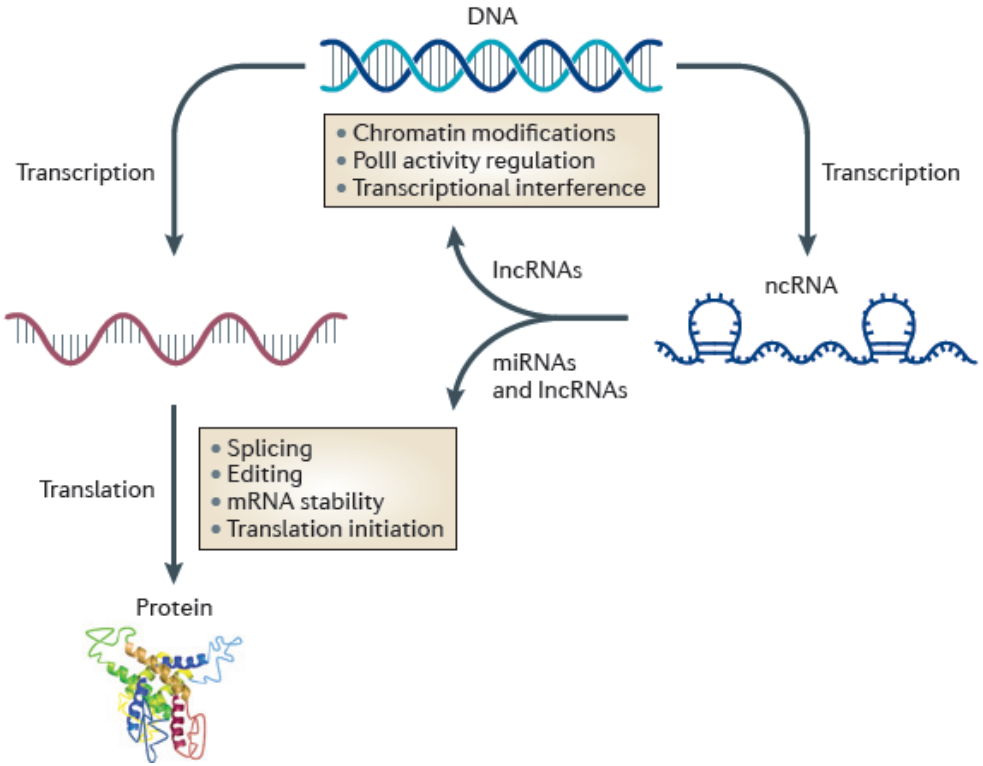
- Long non-coding RNAs
- Small regulatory non-coding RNAs
  - miRNAs
  - piRNAs
  - siRNAs
- Small interfering RNAs as antiviral agents

Long non-coding RNAs

- Long non-coding RNAs (lncRNAs) are 200 nucleotides to several kilobases in length.
- lncRNAs can be subdevided into five classes:
  - Natural antisense transcripts (NATs)
  - Long intergenic non-coding RNAs (lincRNAs)
  - Very long intergenic non-coding RNAs (vlincRNAs or macroRNAs)
  - Sense intronic RNAs
  - Processes transcripts (usually spliced and/or polyadenylated)

Wahlestedt (2013) Nat. Rev. Drug Discov. 12, 433.

Long non-coding RNAs



- Regulatory non-coding RNAs can act at the transcription or translational level.
- They can upregulate or downregulate gene expression.

Wahlestedt (2013) Nat. Rev. Drug Discov. 12, 433.

Long Non-Coding RNAs in Human Diseases

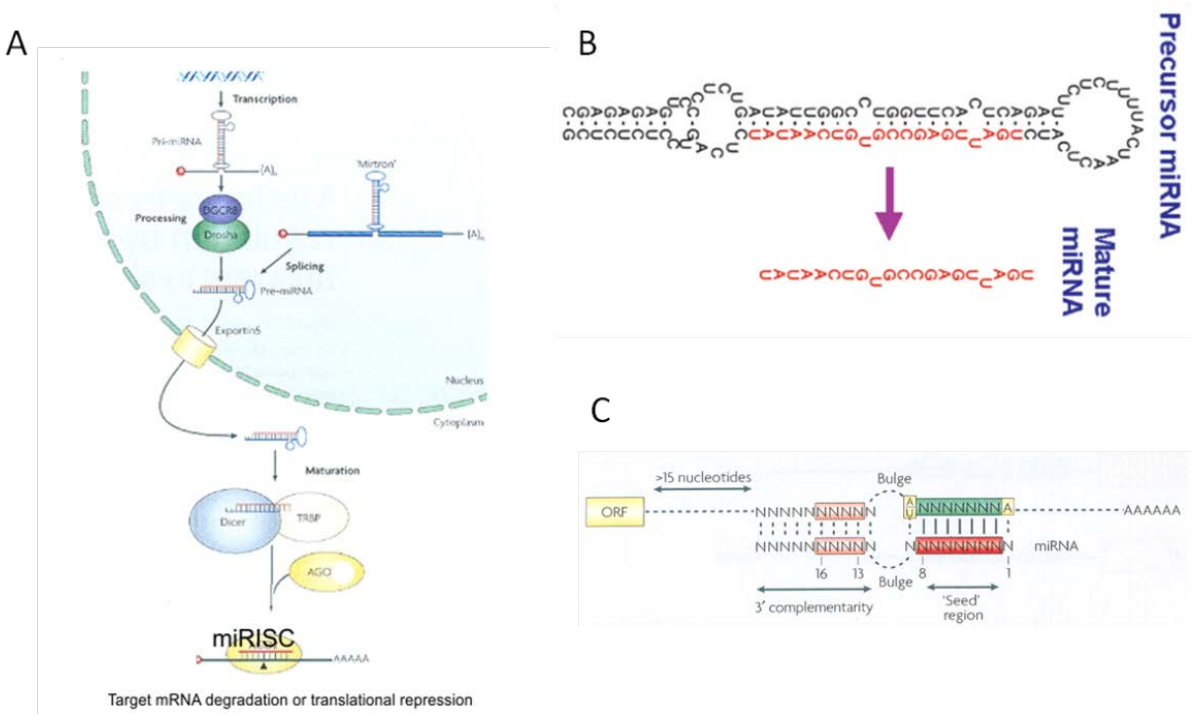
ncRNA	Diseases	Type	mRNA or loci affected	Refs
DBET	Facioscapulohumeral muscular dystrophy	lncRNA	4q35 locus	96
BACE1-AS	Alzheimer's disease	NAT	*BACE1*	88
DISC2	Schizophrenia	NAT	*DISC1*	94
HIF1A	Cancer, myocardial ischaemia	NAT	*HIF1A*	140–142
MALAT1	Cancer	lncRNA	Many	74,75
ATXN8OS	Spinocerebellar ataxia	NAT	*SCA8*	86
FMR4	Fragile X syndrome	lncRNA	*FMR1*	37
FMR1-AS	Fragile X syndrome	NAT	*FMR1*	95
PINK1-AS	Parkinson's disease, diabetes	NAT	*PINK1*	101
CDKN2B-AS1	Cancer, diabetes, cardiovascular disease	lncNRA	*CDKN2A*, *CDKN2B*	143–145
NPPA-AS	Cardiovascular disease	NAT	*NPPA*	146
NAT-*RAD18*	Alzheimer's disease	NAT	*RAD18*	147
BOK-AS	Cancer	NAT	*BOK*	148
HTT-AS	Huntington's disease	NAT	*HTT*	149
HAR1R	Huntington's disease	NAT	*HAR1F*	90
P15-AS	Leukaemia	NAT	*CDKN2B*	150
lincRNA-p21	Cancer	lncRNA	*CDKN1A*	55,151
P21-AS	Cancer	NAT	*CDKN1A*	101
HOTAIR	Cancer	lncRNA	Many	71,72,76,77,151
LSINCT5	Cancer	lncRNA	Many	78
PTCSC3	Cancer	lncRNA	Many	79
TUG1	Cancer	lncRNA	Many	80
lincRNA-EPS	Anaemia	lncRNA	Many	152,153
HELLPAR	HELLP syndrome	lncRNA	Many	92
UCA1	Cancer	lncRNA	Many	81
GAS5	Autoimmune disease, cancer	lncRNA	Many	60,154
DA125942	Brachydactyly type E	lncRNA	Many	93

Wahlestedt (2013) Nat. Rev. Drug Discov. 12, 433.

Overview

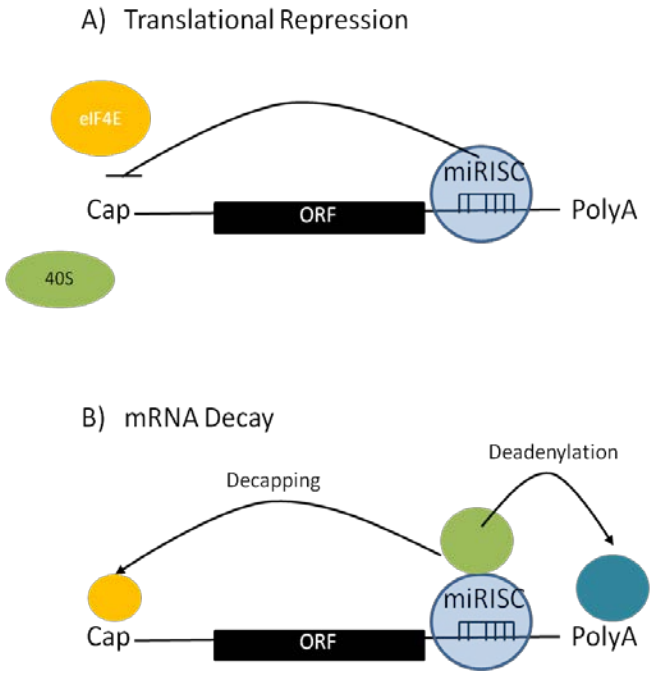
- Long non-coding RNAs
- **Small regulatory non-coding RNAs**
  - miRNAs
  - piRNAs
  - siRNAs
- Small interfering RNAs as antiviral agents

MicroRNA



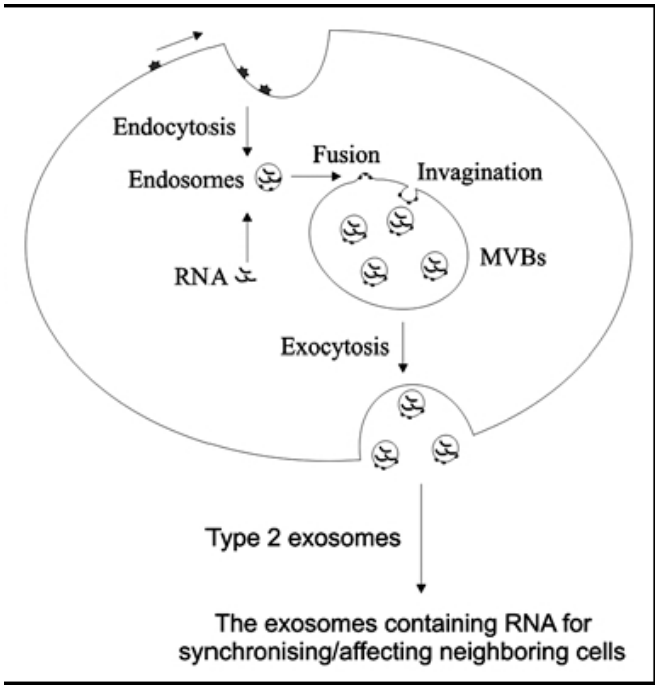
- microRNAs (miRNAs) are small double-stranded RNA molecules that are endogenously expressed and regulate the expression up to 60% of the human genes.

miRNAs



- miRNAs usually bind to the 3‘UTR of mRNAs in an imperfect manner.
- They repress translation and induce mRNA decay.

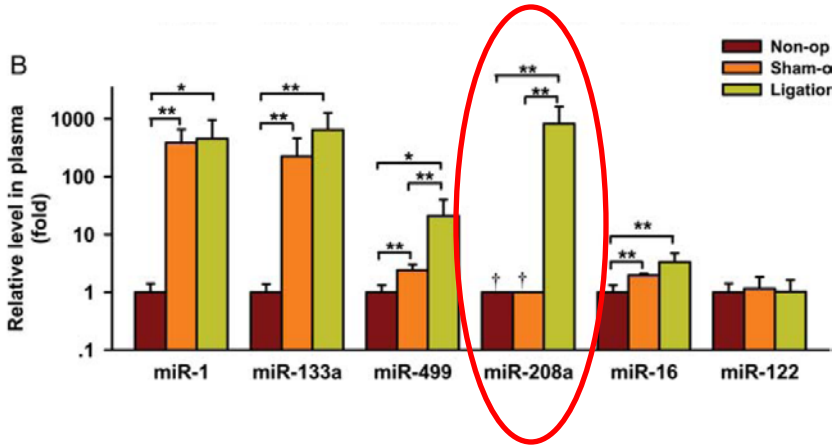
Extracellular miRNAs

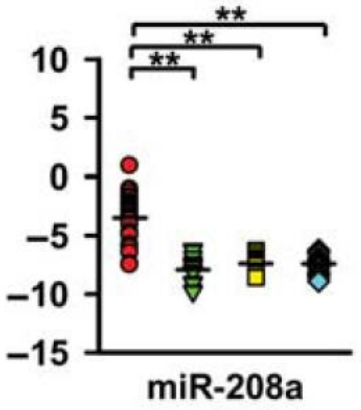


- In recent years, extracellular miRNAs have drawn much attention. miRNAs are secreted from the cells in exosomes.
- Extracelllular miRNAs may have a function in cell-cell communication.
- Furthermore, they can be used as diagnostic biomarkers.

Cell Adh Migr. 2007 Jul–Sep; 1(3): 156–158.; Review: Grimm (2009) Adv. Drug Deliv. Rev. 61, 682.

Extracellular miRNAs and myocardial infarction

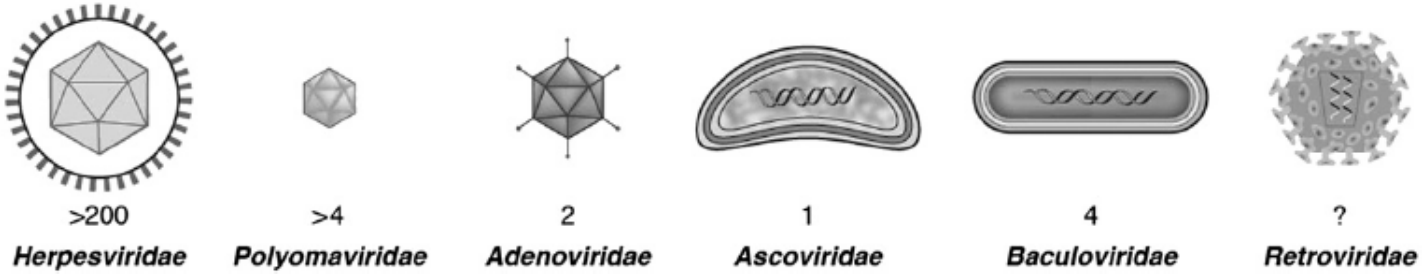




- While miRNA-208a is absent in healthy and sham-operated rats, its level is higher in animals with an induced myocardial infarction.
- The miRNA is also upregulated in patients with a myocardial infarction.
- miRNA-208a was proposed as an early biomarker for myocardial infarction.

Wang et al. Eur. Heart. J. 31, 2010, 659.

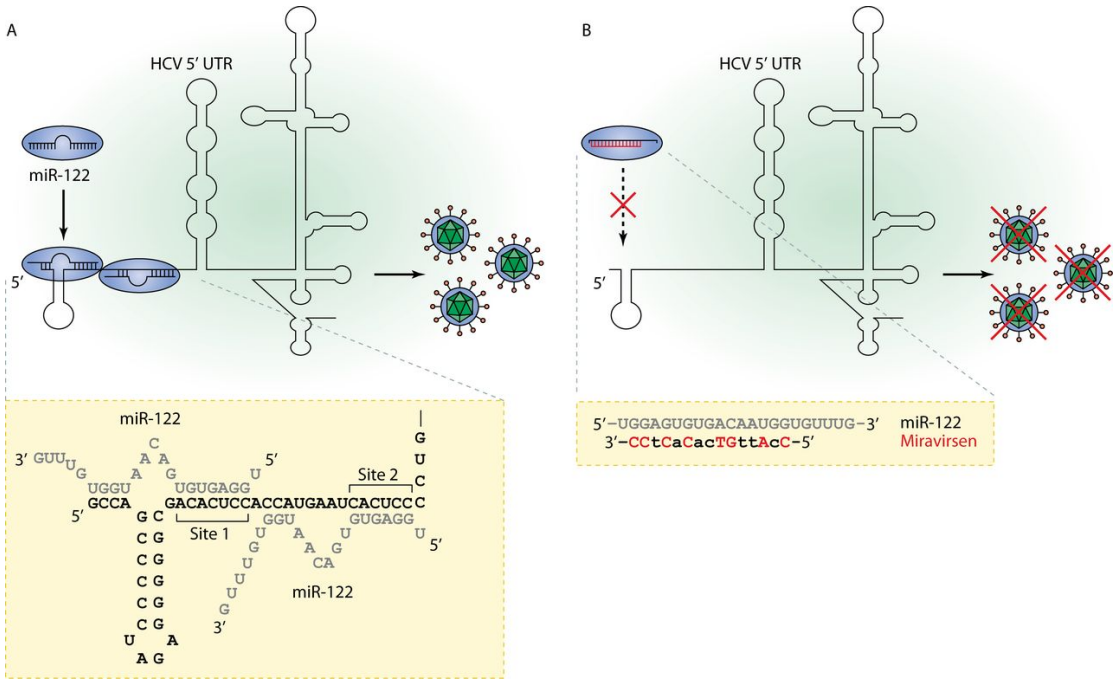
miRNAs in Viral Infections



- Viruses encode more than 200 miRNAs with diverse functions:
  - Autoregulation of viral gene expression
  - Inhibition of host factors to block the immune response
  - Maintanance of latent infections.
- Some human miRNAs inhibit viruses.

Grundhoff A, Sullivan CS. Virology. 2011 Mar 15;411(2):325-43.

miR-122 and HCV

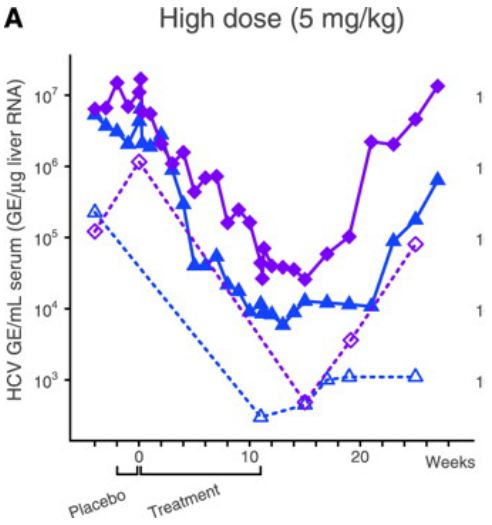


- HCV requires the human miR-122 for efficient replication. The miRNA binds to the 5‘UTR and stabilizes the viral RNA. Inhibition of miR-122 suppresses HCV.

Lindow M , Kauppinen S J Cell Biol 2012;199:407-412

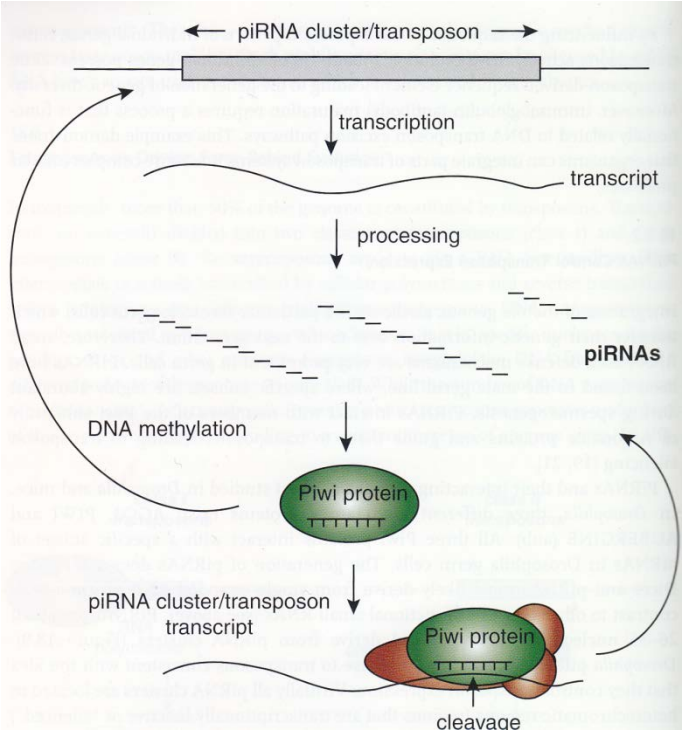
miRNA-Inhibitor for Antiviral Treatment

- An LNA-modified antisense molecule targeting miR-122 is being developed to treat HCV infections.
- Treatment of non-human primates resulted in a significantly reduced HCV level.
- In 2012, results of a phase II study were published demonstrating that the antisense inhibition of miR-122 inhibits HCV in human patients.



Lanford et al. Science 327, 2010, 198.

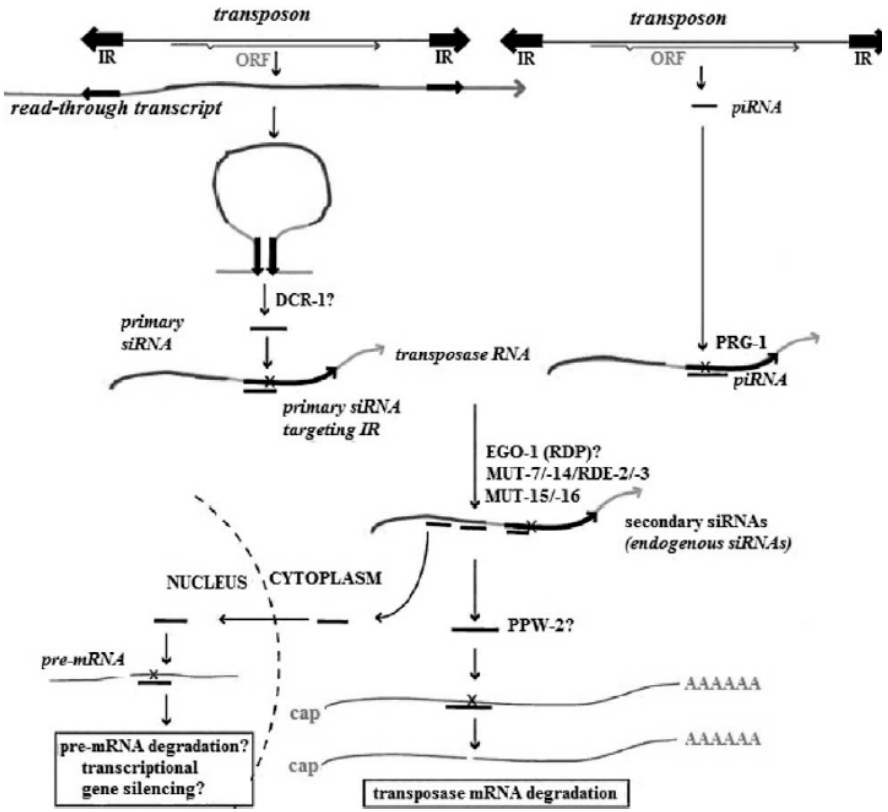
piRNAs



- In 2006, Piwi-interacting RNAs (piRNAs) were discovered in mouse testis.
- They are 24-30 nucleic acids in length.
- In contrast to miRNAs and siRNAs, piRNAs are single-stranded.
- According to their initial discovery in testis, piRNAs seem to play a role in spermiogenesis.

Gunter Meister (2011) RNA Biology. Wiley-VCH, S. 260

piRNAs



- piRNAs arise from repetitive intergenic elements including transposable elements (TEs).
- They target RNAs and degrade them post-transcriptionally.
- piRNAs are involved in maintaining the genetic stability.

Huang et al., Biochemistry (Moscow) 78, 2013, 221.

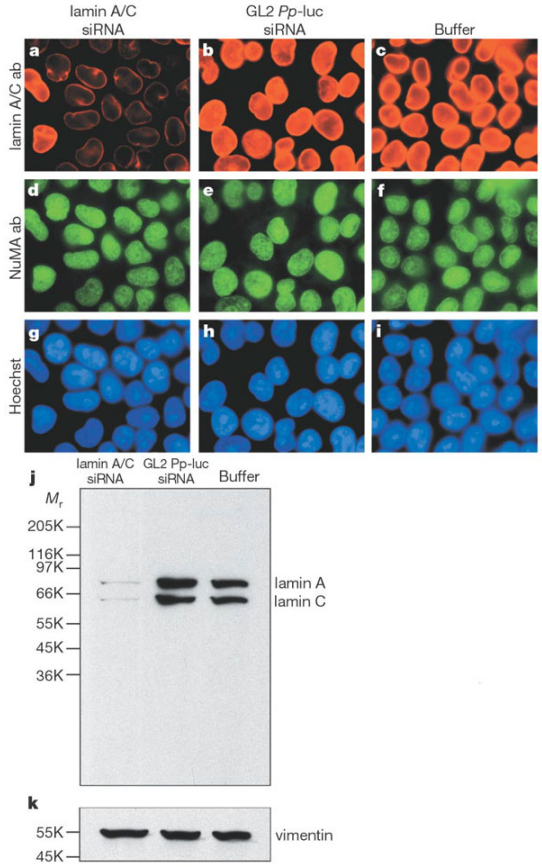
Small noncoding RNAs

Class	Origin	Size, nt	Function	Reference
Small nucleolar RNAs (snoRNAs)	sense, intergenic, or intronic	60-300	RNA modification, including 2′-O-methylation and pseudouridylation	[14]
Promoter-associated RNAs (PASRs)	sense, intergenic (promoter region)	20-200	transcription	[15]
Permini-associated small RNA (TASRs)	antisense, intergenic (3′-UTR end of genes)	20-200	transcription	[15]
Small vault RNA (svRNA)	within vault RNA genes	23-40	drug resistance	[16, 17]
Vault RNA (vRNA)	conserved genomic locus linked to protocadherin gene cluster	88-98	transport and nuclear extrusion of xenobiotics	[18]
Transcription initiation RNA (tiRNA)	downstream to TSS in highly expressed genes	18	transcription	[19]
Transcription start site associated RNA (TSSa-RNA)	found within −250 to +50 nt from TSSs of highly expressed genes	20-90	transcription	[20]
Promoter upstream transcripts (PROMPTs)	−2500 to −50 nt to TSS of actively transcribed protein coding genes	18	transcription	[21]
Small activating RNA (saRNA)	exogenous or endogenous	21	gene activation	[22, 23]
QDE-2-interacting small RNA (qiRNA)	ribosomal DNA locus	20-21	DNA damage response	[24]
MicroRNA-offset RNAs (moRNAs)	regions adjacent to pre-miRNAs	~20	post-transcriptional gene silencing	[25, 26]
MSY2-associated RNAs (MSY-RNAs)	derma cell-specific DNA/RNA binding protein MSY2	~26-30	unknown	[27]
Telomere small RNAs (tel-sRNAs)	G-rich strand of telomeric repeats	~24	telomere maintenance	[28]
Centrosome-associated RNAs (crasiRNAs)	centrosomes	~34-42	guiding local chromatin modifications	[29]
X-inactivation RNAs (xiRNAs)	duplexes of two lncRNAs, Xist and Tsix	~50	X-chromosome inactivation	[29-31]
Sno-derived RNAs (sdRNAs)	small nucleolar RNAs	20-24	RNA silencing	[32-34]
Splice junction-associated RNAs (spliRNAs)	sense, exonic (splice donor site)	17-18	epigenetic regulation	[35]
Mirtron	introns	21-25	post-transcriptional gene silencing	[36-38]

Huang et al., Biochemistry (Moscow) 78, 2013, 221.

- Summary of small noncoding RNAs in eukaryotic cells in addition to siRNAs, miRNAs and piRNAs.

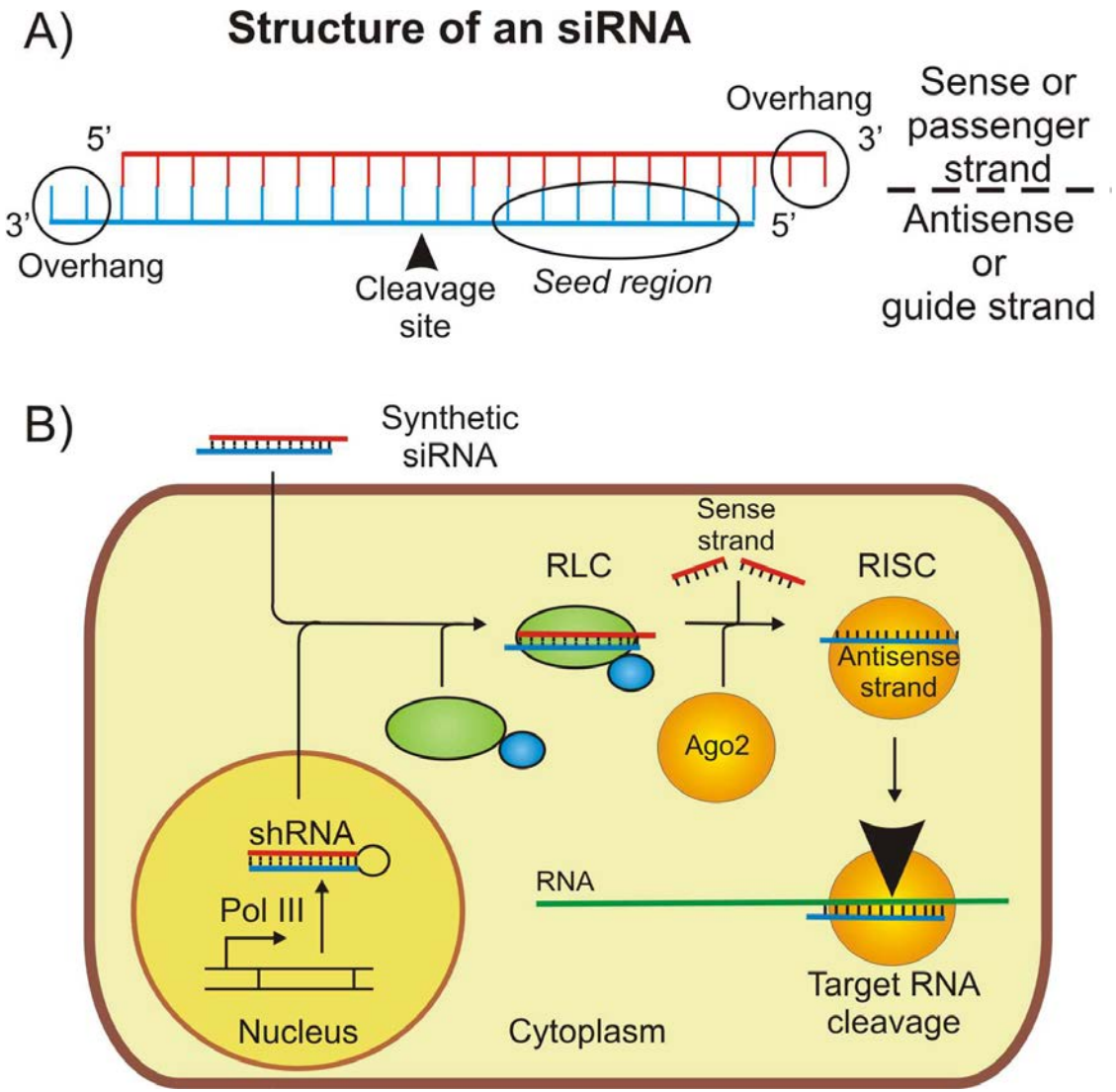
siRNA-Mediated Silencing of Lamin A/C in Mammalian Cells



- In 2001, Tom Tuschl and co-workers demonstrated for the first time that endogenously expressed genes can be silenced siRNAs in mammalian cells.

Elbashir et al., Nature 411, 2001, 494-498

RNA Interference



Kurreck, J. (2009) Angewandte Chemie Int. Ed. **48**, 1378.

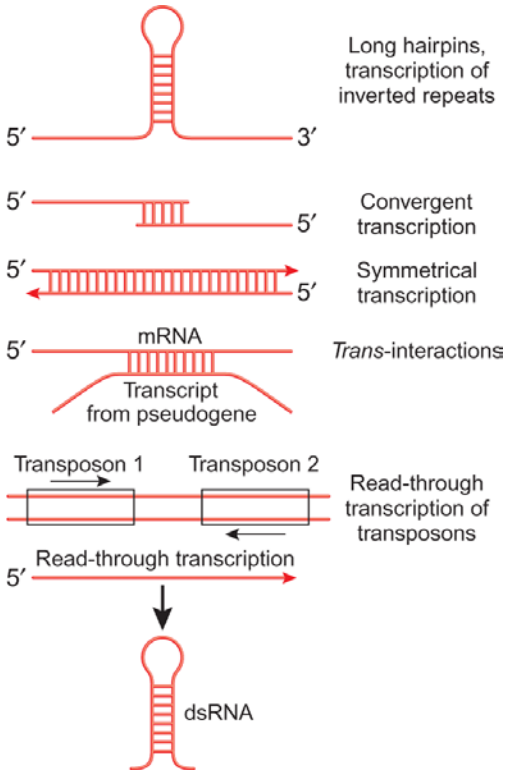
RNAi Therapeutics in Clinical Pipeline

Table 1 RNAi therapeutics clinical pipeline

Year of IND/CTA	*Candidate*	*Indication*	*Target*	*Delivery*
2004	Cand5	Wet AMD, diabetic macular edema	VEGF	Intravitreal needle injection (retina; local)
2004	Sirna-027/AGN-745	Wet AMD	VEGF-R1	Intravitreal needle injection (retina; local)
2005	ALN-RSV01	RSV infection	Viral RNA	Inhalation of unformulated siRNAs (lung epithelium; local)
2007	DGFI	Acute kidney injury, delayed graft function	p53	Intravenous naked siRNA (proximal tubule cells; systemic)
2007	PF-4523655	Wet AMD, diabetic macular edema	RTP801/REDD1	Intravitreal needle injection (retina; local)
2007	rHIV-shI-TAR-CCR5RZ	HIV infection	Viral RNA and host factors	Lentiviral (hematopoietic stem cells; *ex vivo*)
2007	NucB1000	Hepatitis B viral infection	HBV RNAs	Liposomal plasmid (hepatocytes; systemic)
2008	TD101	Pachyonychia congenita	Mutant keratin	Intradermal needle injection (skin; local)
2008	Therapeutic vaccine	Metastatic melanoma	Immunoproteasome	Electroporation (autologous monocytes; *ex vivo*)
2008	Excellair	Asthma	Syk kinase	Inhalation of unformulated siRNAs (lung epithelium; local)
2008	CALAA-01	Nonresectable or metastatic solid tumors	M2 subunit of ribonucleotide reductase	RONDEL (solid tumor cells; systemic)
2008	ALN-VSP02	Liver cancer, cancer with liver involvement	VEGF, KSP	SNALP liposome (hepatocytes; systemic)
2009	Atu027	Advanced solid tumors	PKN3	AtuPLEX lipoplex (vascular endothelial cells; systemic)
2009	QPI-1007	Chronic nerve atrophy, nonarteritic ischemic optic neuropathy	Caspase 2	Intravitreal needle injection
2009	SYL040012	Intraocular pressure and glaucoma	β-Adrenergic receptor 2	Eye drop (ciliary epithelial cells; local)
2009	TKM-ApoB	Hypercholesterolemia	Apolipoprotein B	SNALP liposome (hepatocytes; systemic)
2009	bi-shRNAfurin/GMCSF	Ovarian cancer, advanced melanoma	Furin	Electroporation plasmid (autologous tumor samples; *ex vivo*)
2009	ALN-TTR01	Transthyretin amyloidosis	Transthyretin	SNALP liposome (hepatocytes; systemic)
2010	siG12D LODER	Operable pancreatic ductal adenocarcinoma	Mutated KRAS	LODER local drug elution
2010	TKM-PLK1	Solid cancers and lymphoma	Polo-like kinase 1	SNALP liposomal (solid tumor cells; systemic)
2011	CEQ508	Familial adenomatous polyposis/ colon cancer prevention	-Catenin	Bacterial (mucosal layer of small and large intestine; oral)
2011	ALN-PCS02	Hypercholesterolemia	PCSK9	SNALP liposome (hepatocytes; systemic)
2011	TKM-EBOLA	Ebola infection (biodefense)	Viral RNA	SNALP liposome (hepatocytes and phagocytes; systemic)
Select preclinical candidates				
2012 (est.)	RXI-109	Dermal scarring	CTGF	Intradermal needle injection (skin; local)
2012 (est.)	To be named	HIV infection	CCR5	Lentiviral transduction transduction (hematopoietic stem cells; *ex vivo*)

Haussecker (2012) Mol. Ther. Nucl. Acids 2, e8

Endo-siRNAs (esiRNAs)



- Initially, only organisms encoding an RdRP were considered to generate endogenous siRNAs.
- Interestingly esiRNAs were detected even in mouse oocytes and drosophila, both of which do not produce an RdRP.
- esiRNAs are generated from hairpin structures or complementary RNAs.
- esiRNAs originate from retrotranposons and control mobile genetic elements. In addition esiRNAs were found in pseudogenes, which regulate protein-coding mRNAs.

Kommentar von: Nilsen, TW
Nat. Struc. Mol. Biol. 15, 2008, 546

RNAi as an Antiviral Mechanism in Mammalian Cells

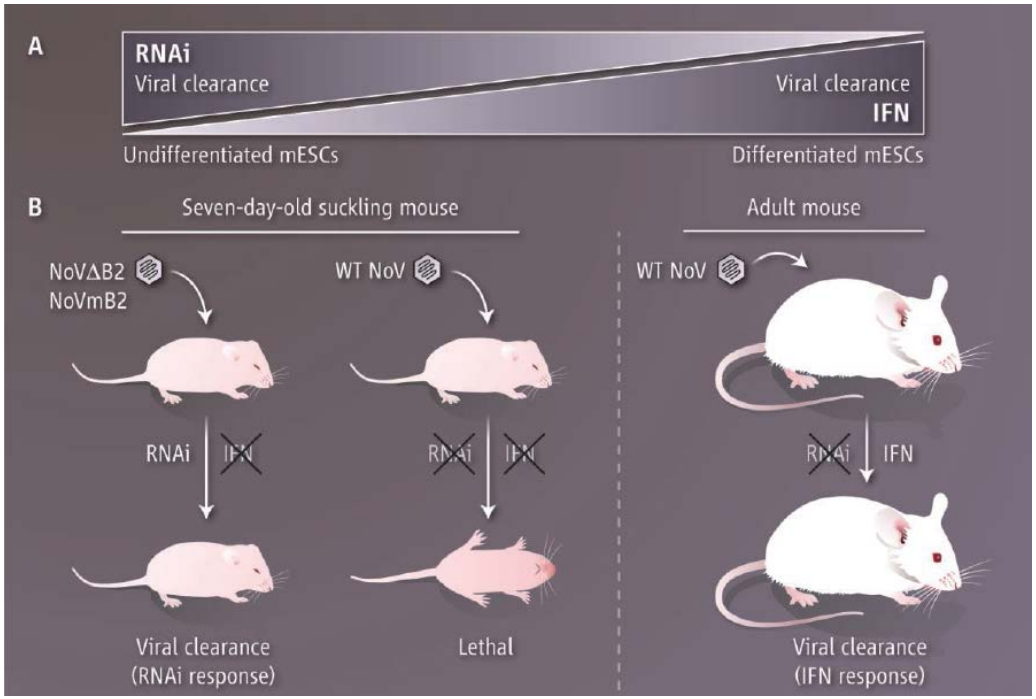
- The antiviral activity of RNAi in plants and invertebrates has been well established.
- However, it remained elusive, whether RNAi also has antiviral activity in mammalian cells, or if the innate IFN immune response supplanted the RNAi defense.
- Evidence for virus-derived small RNAs (vsRNAs) was provided, but it was still questioned, whether the vsRNAs were functional.

RNAi as an Antiviral Mechanism in Mammalian Cells

- In October 2013, two groups independently demonstrated antiviral RNAi in mammalian cells:
  - Murine embryonic stem cells lack the IFN response. vsRNAs associate with Ago2.
  - The Nodamura virus produces an RNAi suppressor. Deletion mutants lacking the suppressor are suppressed by RNAi.
  - The same is still true in 7-day old suckling mice. Mutated NoV lacking the RNAi suppressor are inhibited by a potent antiviral RNAi response, while the wt virus lacking the suppressor escapes inhibition by RNAi. In adult mice the virus is cleared by the IFN response.

Maillard et al. Science 342, 2013, 235; Li et al. Science 342, 2013, 231.

RNAi as an Antiviral Mechanism in Mammalian Cells



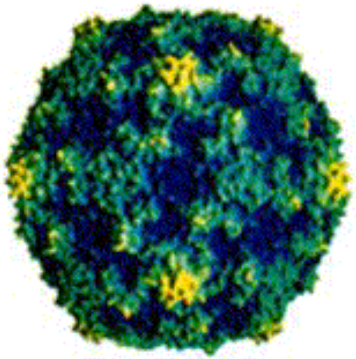
- In young mice the RNAi response can clear NoV lacking the RNAi suppressor B2. NoV with the RNAi suppressor is lethal.
- In adult mice, the IFN response clears the virus.

Maillard et al. Science 342, 2013, 235; Li et al. Science 342, 2013, 231.

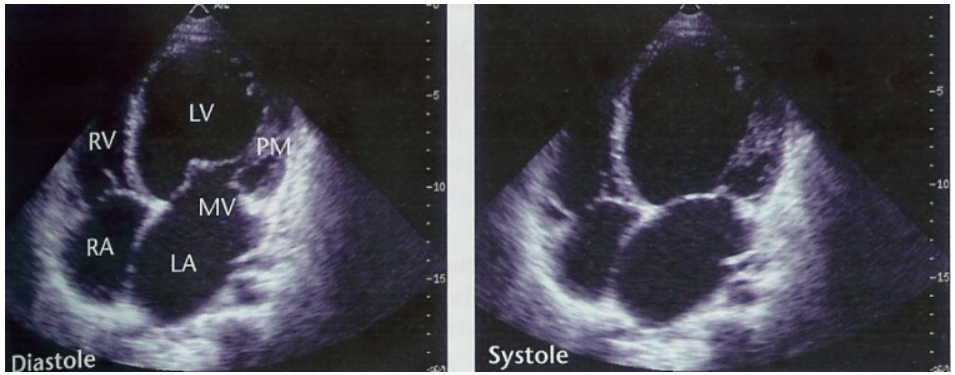
Overview

- Long non-coding RNAs
- Small regulatory non-coding RNAs
  - miRNAs
  - piRNAs
  - siRNAs
- **Small interfering RNAs as antiviral agents**

Coxsackievirus B3 (CVB-3)



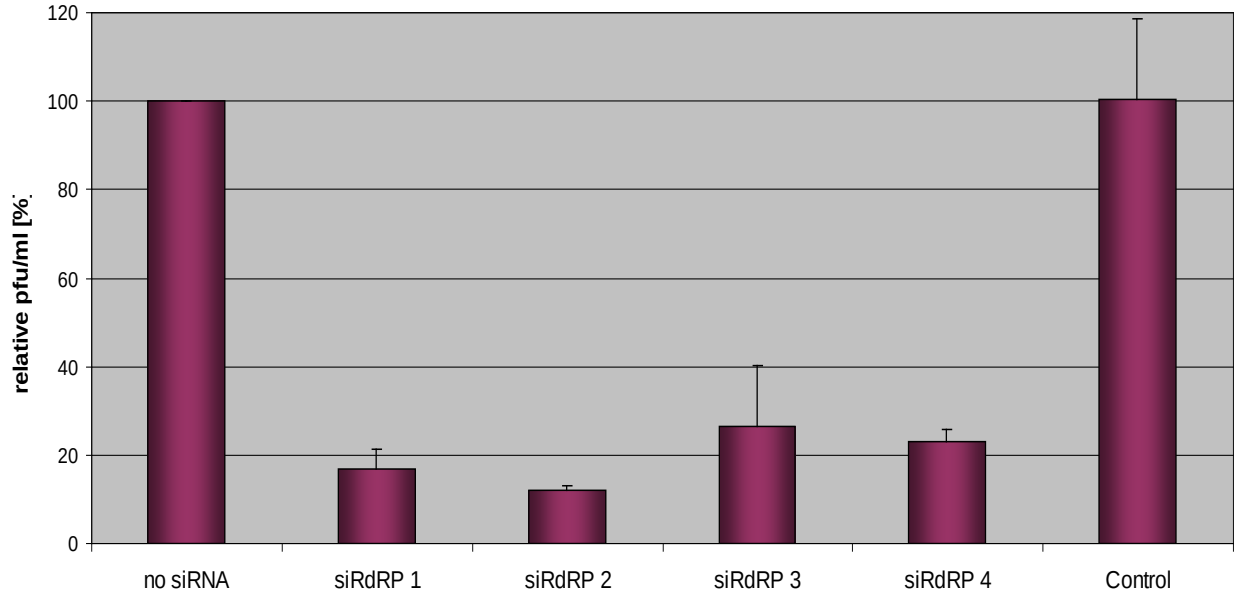
Muckelbauer et al. (1995)
Structure 3, 653.



From: Renz-Polster et al., Basislehrbuch Innere Medizin

- Member of the picornavirus family
  - Plus-strand RNA viruses
  - Cytoplasmic replication-cycle
- High clinical relevance:
  - Meningoencephalitis, pancreatitis
  - CVB-3 is one of the major causes of acute myocarditis that can persist chronically and develop into a dilated cardiomyopathy.

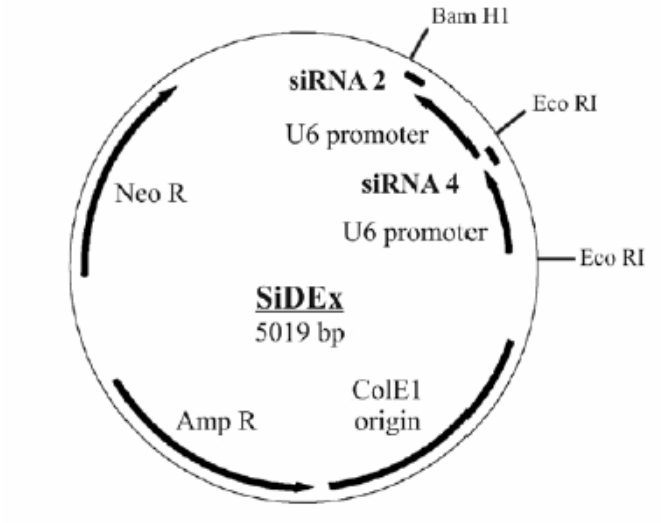
Inhibition of Coxsackievirus B3



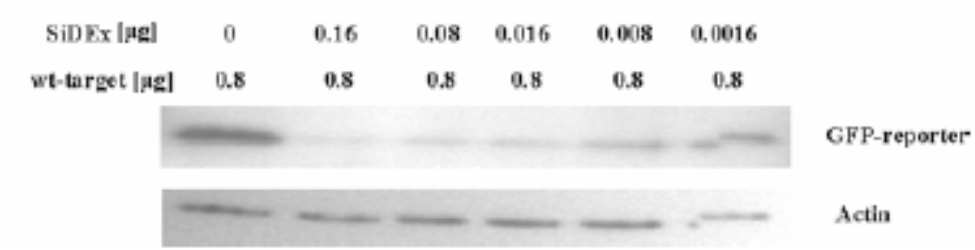
- Plaque reduction assay:
  Up to 90% reduction of virus propagation.

- Schubert, Grunert, Zeichhardt, Werk, Erdmann, Kurreck (2005) J. Mol Biol. 346, 457.
- Werk, Schubert, Lindig, Grunert, Zeichhardt, Erdmann, Kurreck (2005) Biol. Chem. 382, 857.

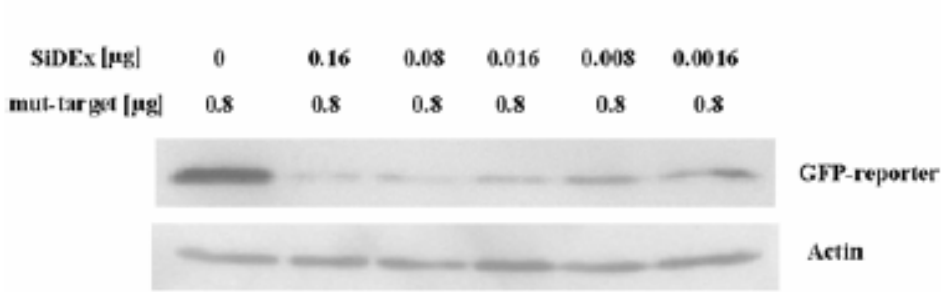
SiRNA Double Expression Vector (SiDEx)



wt RdRP



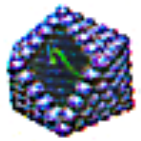
Mutated RdRP



- SiDEx silences
  - wt RdRP
  - and mutated RdRP

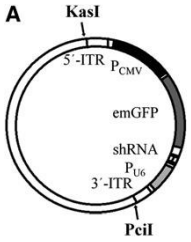
Schubert, Grunert, Zeichhardt, Werk, Erdmann, Kurreck (2005) J. Mol. Biol. 346, 457.

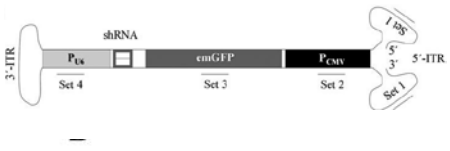
AAV Vectors for Knockdown

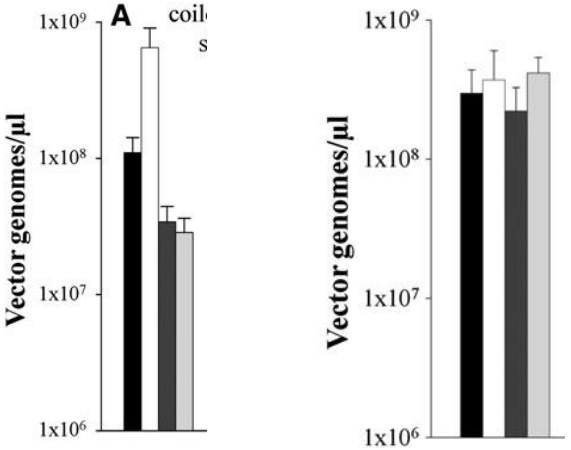


- Advantages: Low pathogenicity, transduction of quiescent cells, serotypes with specific tissue tropism
- Disadvantage: Low packaging capacity

- Challenge: Determination of vector concentration





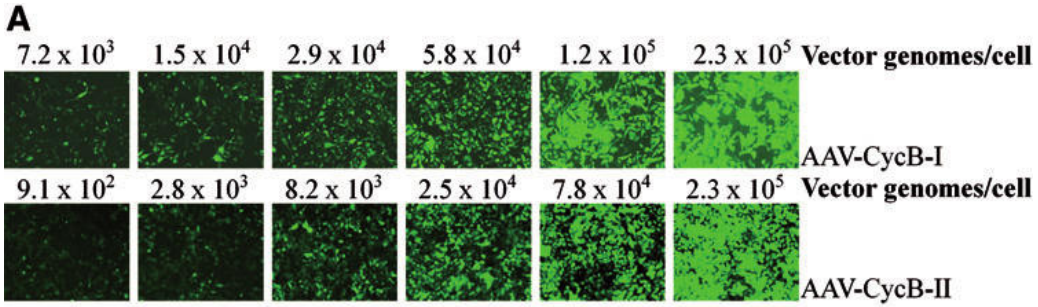


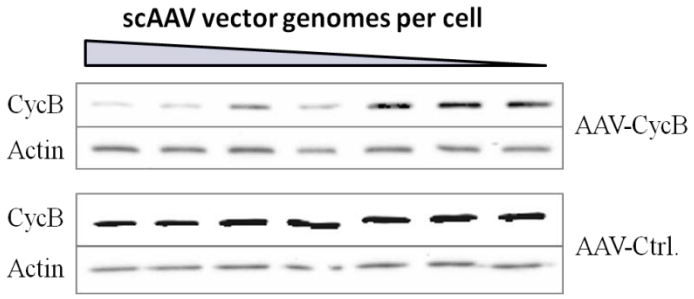
- Use of plasmid standard in qPCR gives variable concentrations depending on the primer set.
- Only the use of isolated genomic AAV DNA as a standard gives reliable results.

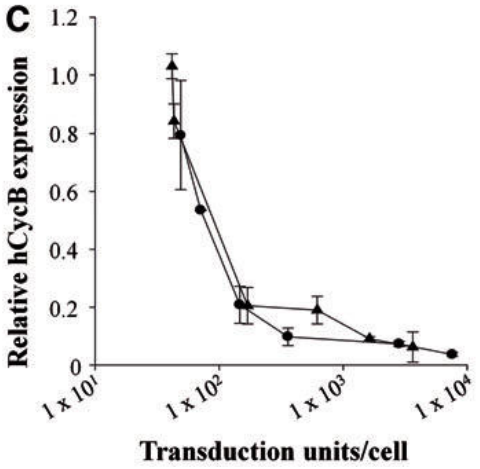
Wagner, Röhrs, Kedzierski, Fechner, Kurreck (2013) Hum. Gene Ther. Meth., in press.

AAV Vectors for Knockdown

- Knockdown of cyclophilin B:
  - Increasing transduction rates at higher concentrations
  - Approximately 150 transduction units required for 80% knockdown.

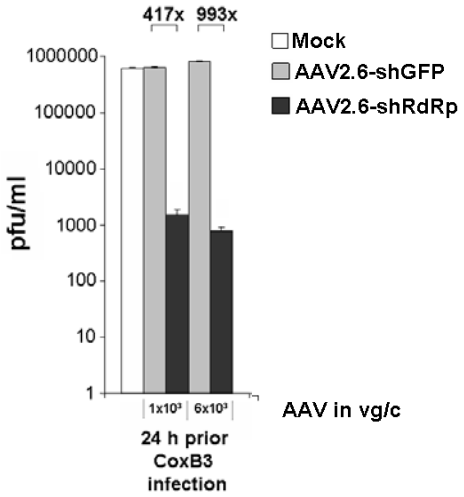


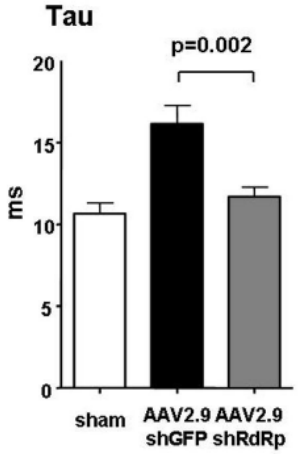




Wagner, Röhrs, Kedzierski, Fechner, Kurreck (2013) Hum. Gene Ther. Meth., in press.

Virus inhibition in rat primary neonatal cardiomyocytes



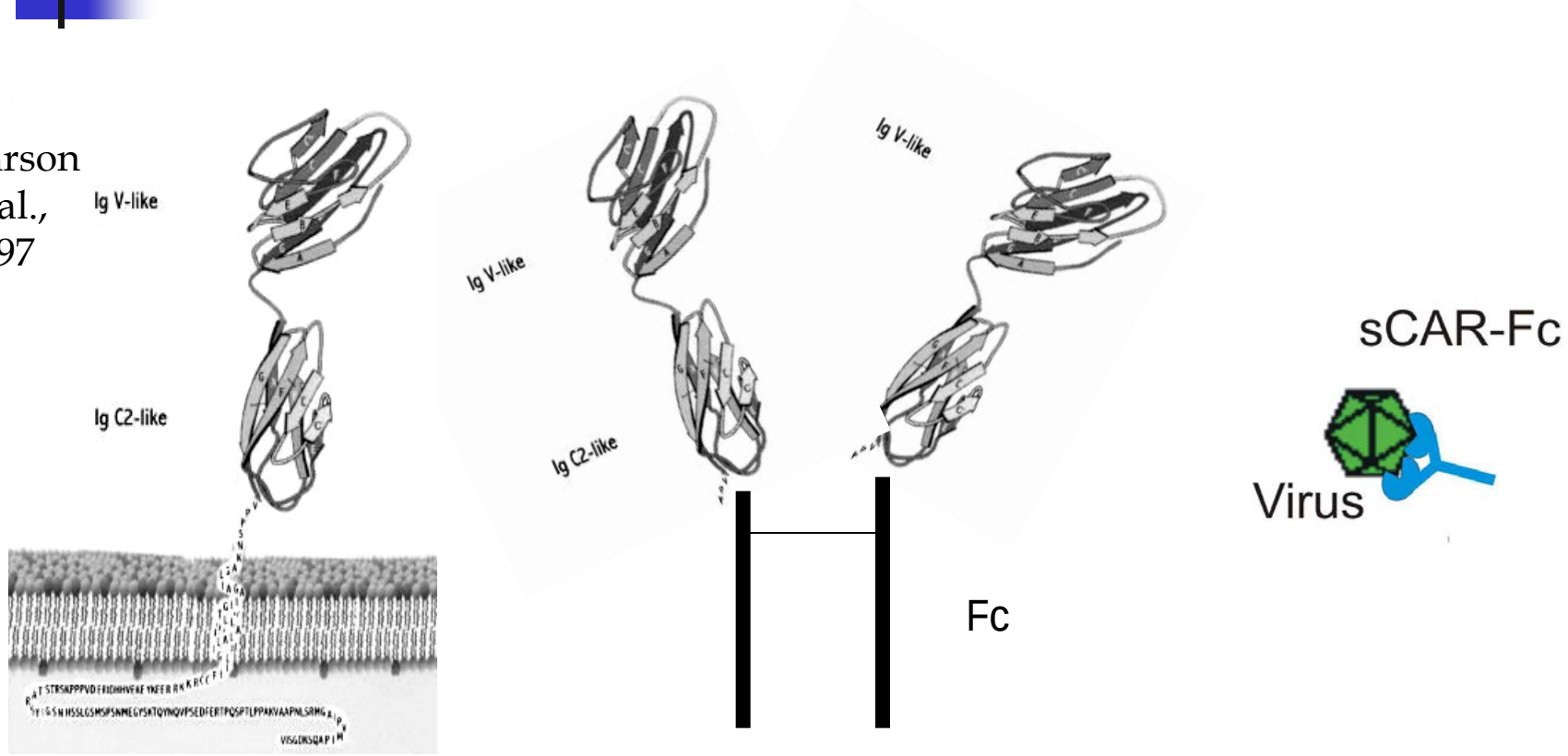


- SiDEx reduces the virus titer in primary neonatal cardiomyocytes by 3 $\log_{10}$ steps.
- Treatment improves cardiac function in mouse myocarditis model.
- However: The therapeutic effect was limited. Reduction of the virus titer in the heart did not reach significance.

Fechner, H.; Sipo, I.; Westermann, D.; Pinkert, S.; Wang, X.; Suckau, L.; Kurreck, J.; Zeichardt, H.; Müller, O.; Vetter, R.; Tschope, C.; Poller, W. (2008) J. Mol. Med. 86, 987.

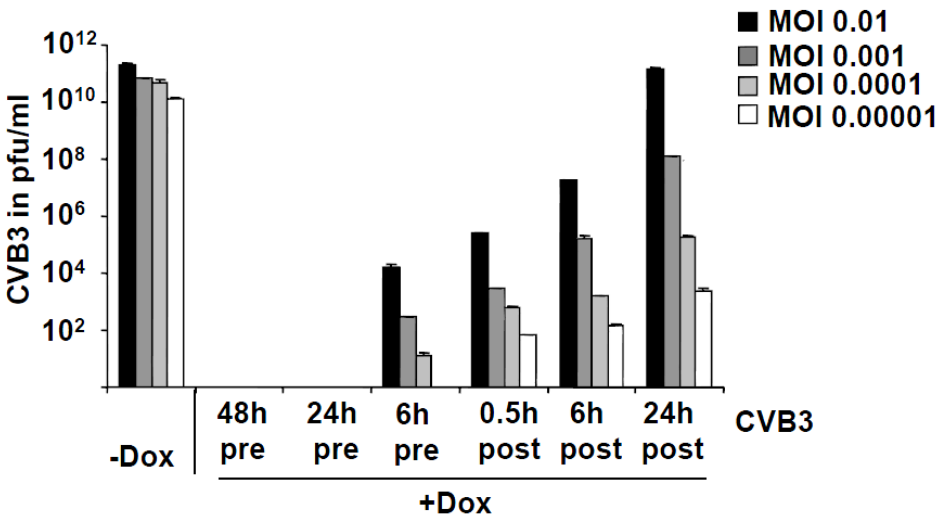
Soluble CAR (sCAR) as virus trap

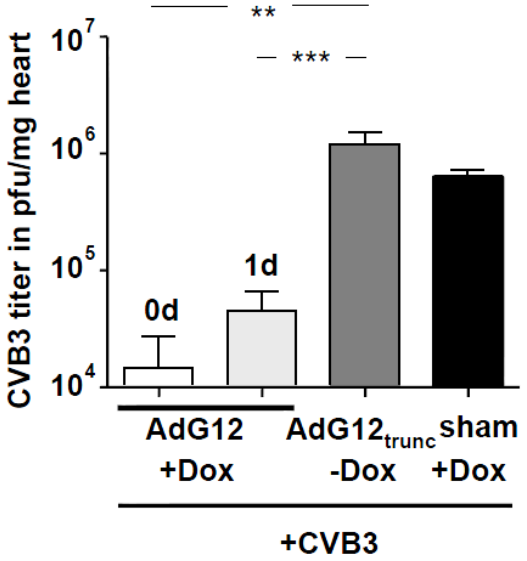
Carson et al., 1997



- Extracellular domains of CAR fused to the Fc domain of an IgG have been shown to trap the virus and prevent its spread.

Antiviral activity of sCAR-Fc





- Pre-treatment of HeLa cells with sCAR-Fc prevents virus infection. Even treatment 24 h after the infection reduces the virus titer by 6 $\log_{10}$ steps.
- sCAR-Fc reduces virus titer *in vivo* and prevents cardiac dysfunction in CVB-3 myocarditis.

Pinkert, ..., Fechner (2009) Circulation 120, 2358.

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