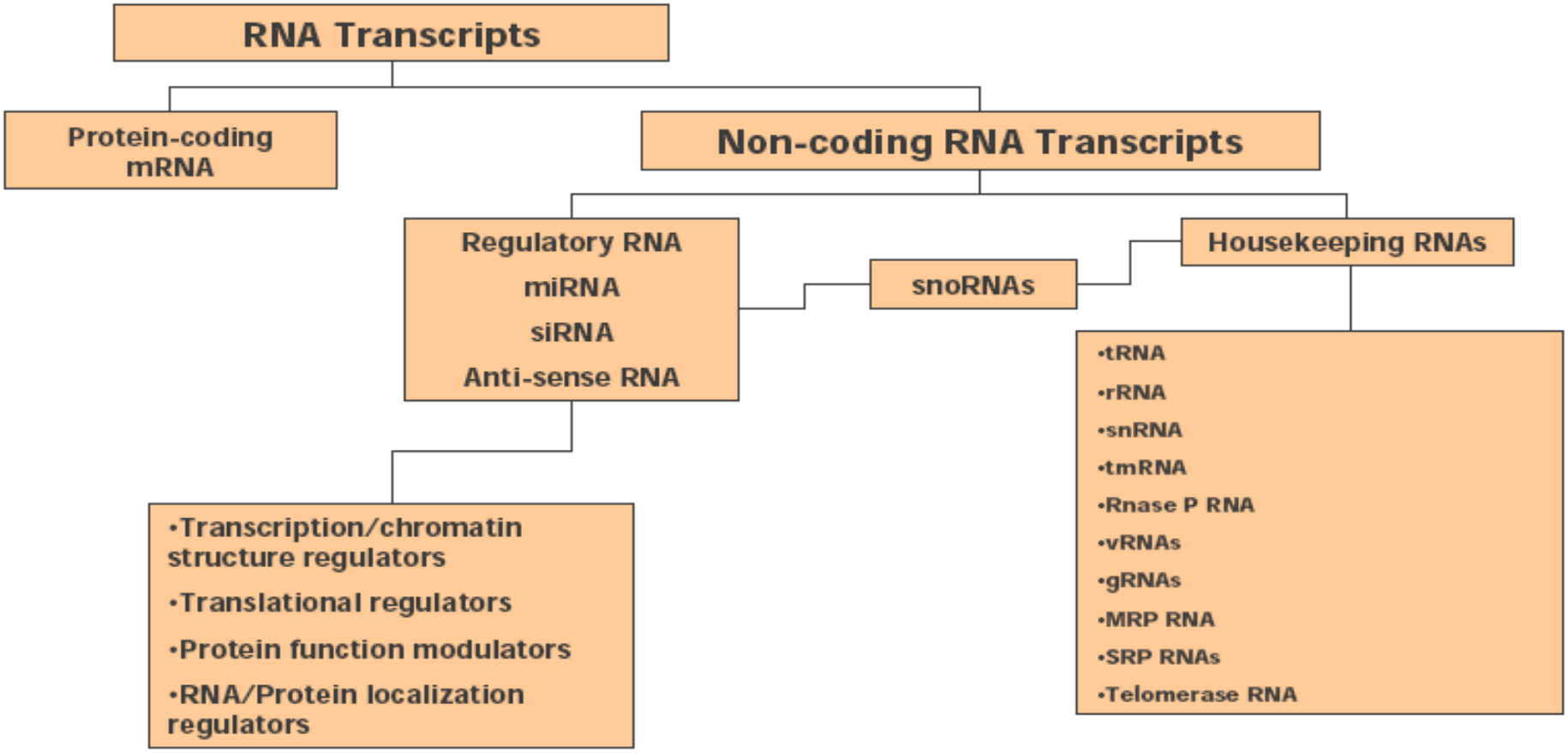
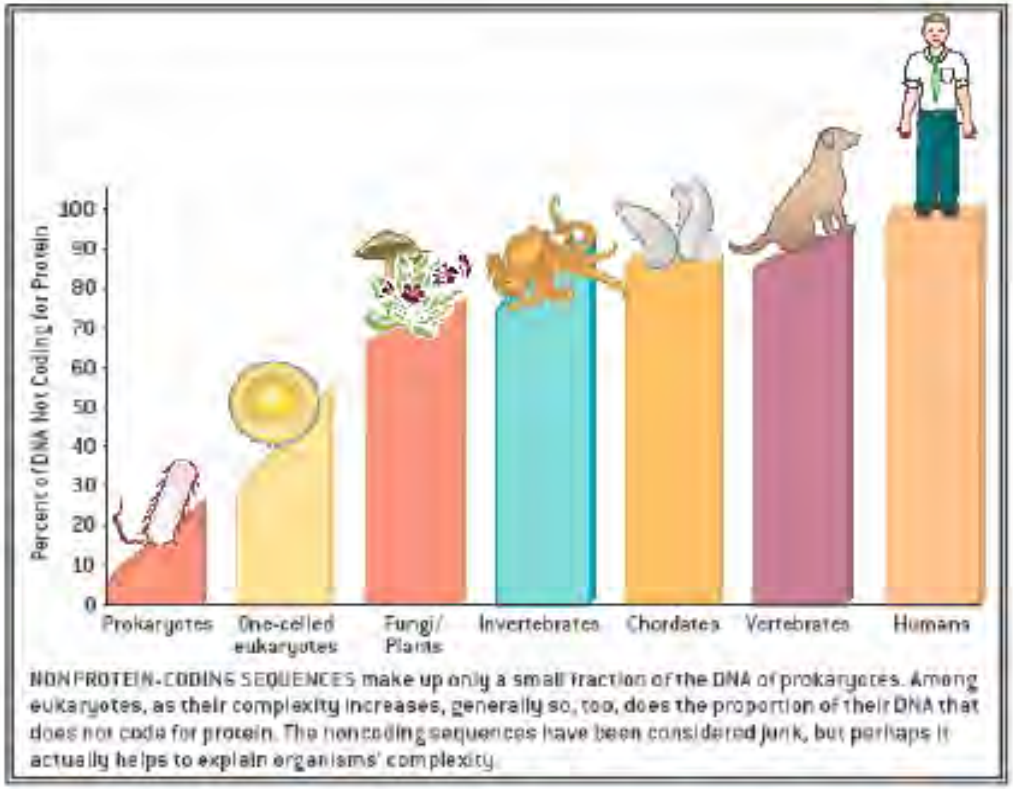

Small regulatory
RNAs

Non-Coding RNA: Formerly known as *"JUNK"*



NC-RNAs compose majority of transcription in complex genomes

Non-Coding RNA: A Key to Eukaryotic Complexity?



NON PROTEIN-CODING SEQUENCES make up only a small fraction of the DNA of prokaryotes. Among eukaryotes, as their complexity increases, generally so, too, does the proportion of their DNA that does not code for protein. The noncoding sequences have been considered junk, but perhaps it actually helps to explain organisms' complexity.

Organism	Percent of Transcriptional Output	
	Protein Coding RNA	Non Coding RNA
E.coli	84	16
S. cerevisiae	71	29
C.elegans	27	73
D. melanogoster	13	87
H. sapiens	2	98

Data suggesting role in diverse mechanisms:

- **RNAi**
- **Gene co-suppression**
- **Imprinting/DNA Methylation**

Possible roles in:

- **Cancer**
- **Neurological Disorders**
- **Host-pathogen interactions**

Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡

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Silence is golden: Craig Mello (left) and Andrew Fire.

Nobel prize 2006 Physiology and Medicine to Craig Mello and Andrew Fire for their report on RNAi.

Key breakthrough: dsRNA is the actual trigger of specific mRNA degradation, with the sequence of dsRNA determining which mRNA is degraded

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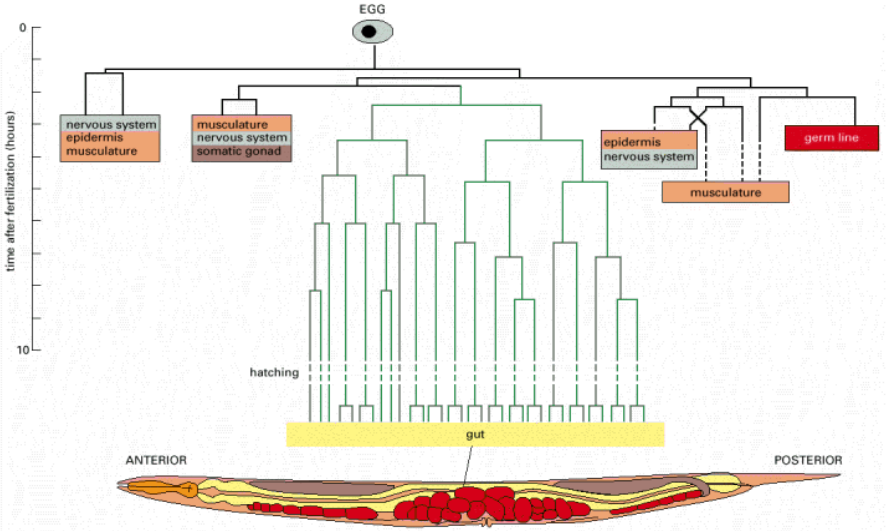
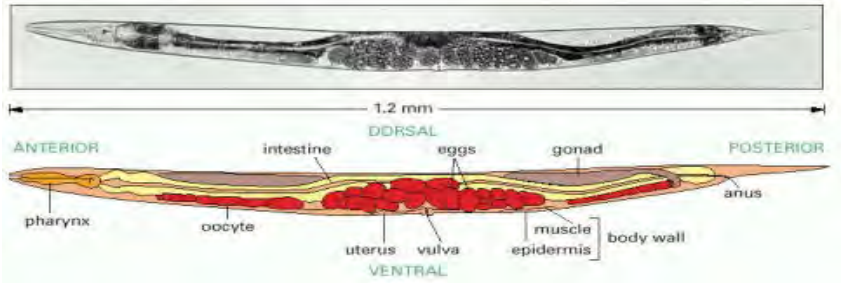
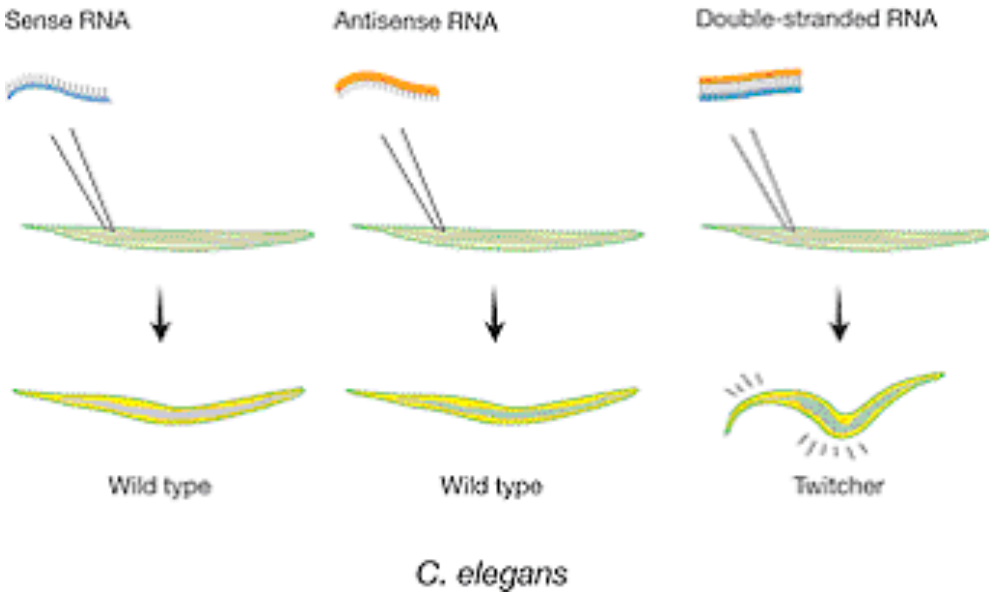
Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene[1,2]. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression[3,4]. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stochiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process.





C. elegans is a precious tool in developmental biology:
- it is tiny and grow rapidly
- females are composed of 956 cells
- males are composed of 1031 cells
- the fate of every cell is characterized

Conclusions of Fire&Mello's study:



*Phenotypic effect after injection of single-stranded or double-stranded unc-22 RNA into the gonad of C. elegans. The **unc-22** gene encodes a myofilament protein. Decrease in unc-22 activity is known to produce severe twitching movements. Injected double-stranded RNA, but not single-stranded RNA, induced the twitching phenotype in the progeny.*

1) silencing was triggered efficiently by injected **dsRNA**, but weakly or not at all by sense or antisense single-stranded RNAs.
2) silencing was **specific** for an mRNA homologous to the dsRNA; other mRNAs were unaffected
3) the dsRNA had to correspond to the mature mRNA sequence; neither intron nor promoter sequences triggered a response. This indicated a **post-transcriptional**, presumably **cytoplasmic** mechanism
4) the targeted mRNA disappeared suggesting that it was **degraded**
5) only a few dsRNA molecules per cell were sufficient to accomplish full silencing. This indicated that the dsRNA was amplified and/or acted **catalytically** rather than stoichiometrically
6) the dsRNA effect could spread between tissues and even to the progeny, suggesting a **transmission** of the effect between cells

Small RNAs and Gene Silencing

Name	Organism	Length (nt)	Proteins	Source of trigger	Function
miRNA	Plants, algae, animals, viruses, protists	20–25	Drosha (animals only) and Dicer	Pol II transcription (pri-miRNAs)	Regulation of mRNA stability, translation
casiRNA	Plants	24	DCL3	Transposons, repeats	Chromatin modification
tasiRNA	Plants	21	DCL4	miRNA-cleaved RNAs from the *TAS* loci	Post-transcriptional regulation
natsiRNA	Plants	22	DCL1	Bidirectional transcripts induced by stress	Regulation of stress-response genes
		24	DCL2		
		21	DCL1 and DCL2		
Exo-siRNA	Animals, fungi, protists	~21	Dicer	Transgenic, viral or other exogenous dsRNA	Post-transcriptional regulation, antiviral defense
	Plants	21 and 24			
Endo-siRNA	Plants, algae, animals, fungi, protists	~21	Dicer (except secondary siRNAs in *C. elegans*, which are products of RdRP transcription, and are therefore not technically siRNAs)	Structured loci, convergent and bidirectional transcription, mRNAs paired to antisense pseudogene transcripts	Post-transcriptional regulation of transcripts and transposons; transcriptional gene silencing
piRNA	Metazoans excluding *Trichoplax adhaerens*	24–30	Dicer-independent	Long, primary transcripts?	Transposon regulation, unknown functions
piRNA-like (soma)	*Drosophila melanogaster*	24–30	Dicer-independent	In *ago2* mutants in *Drosophila*	Unknown
21U-RNA piRNAs	*Caenorhabditis elegans*	21	Dicer-independent	Individual transcription of each piRNA?	Transposon regulation, unknown functions
26G RNA	*Caenorhabditis elegans*	26	RdRP?	Enriched in sperm	Unknown

adapted from Ghildiyal & Zamore. 2009. Nat Rev Genet. 10:94

miRNA: microRNAs

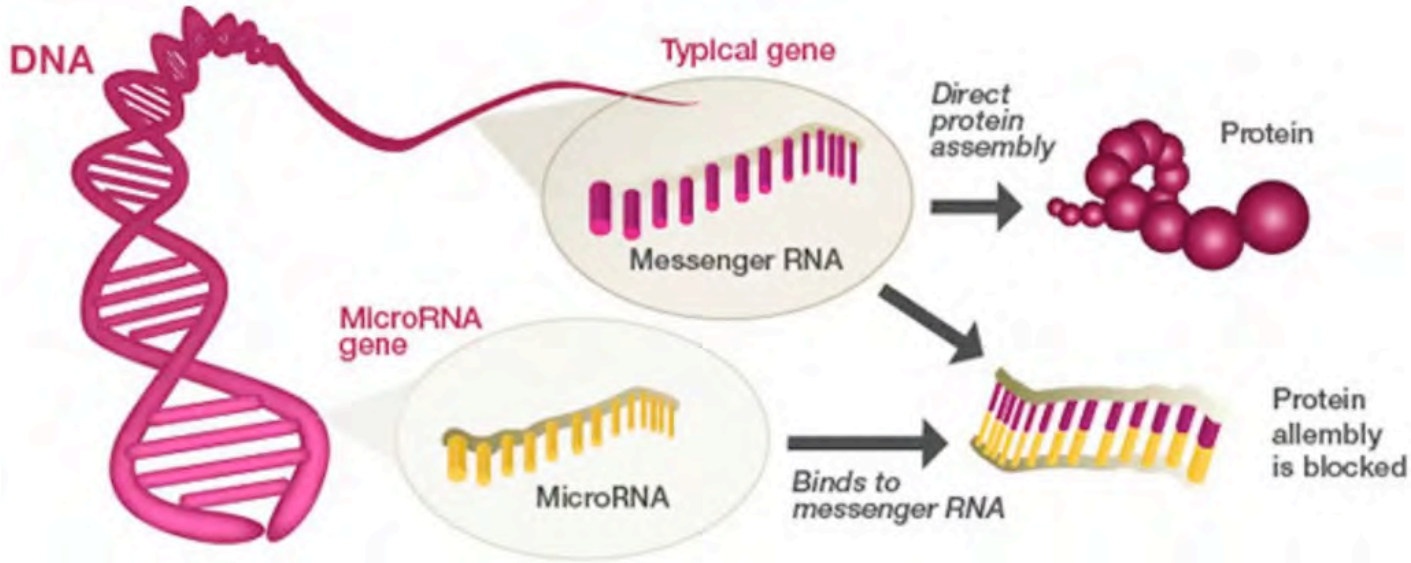
miRBase

http://www.mirbase.org/

miRBase is the central online repository for microRNA (miRNA) nomenclature, sequence data, annotation and target prediction

Griffiths-Jones et al. 2008. NAR. 36:D154

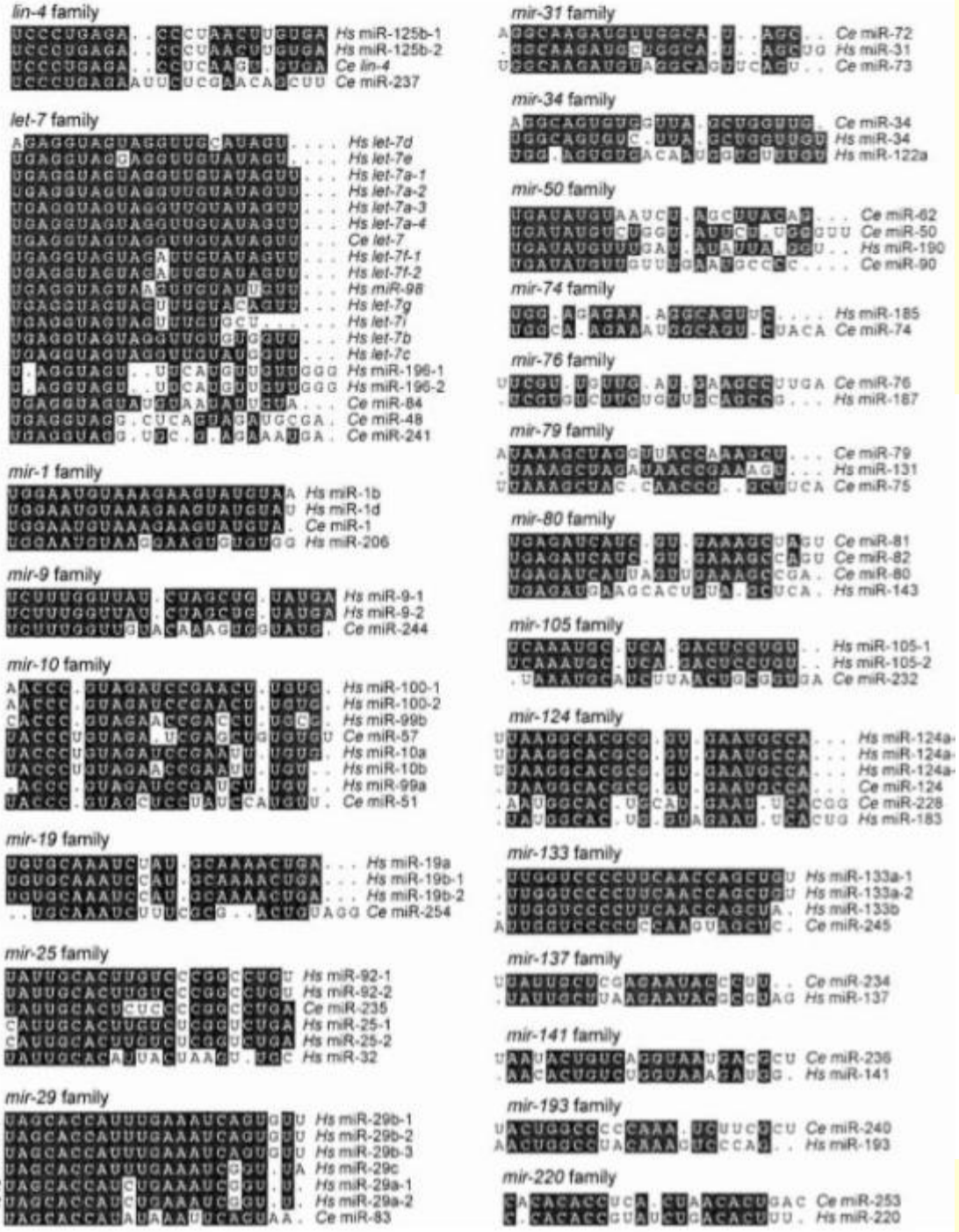
What are miRNAs?



- ✓ miRNA genes are encoded in our genome
- ✓ miRNAs are small dsRNA molecules of 21-22 nt
- ✓ Their derive from precursors of 70-100 nt, transcribed by RNA PolII
- ✓ miRNAs interact with the 3′ untranslated region (3′ UTR) of target mRNAs to induce mRNA degradation and translational repression
- ✓ interaction of miRNAs with other regions, including the 5′ UTR, coding sequence, have also been reported
- ✓ Each miRNA is predicted to regulate hundreds of targets

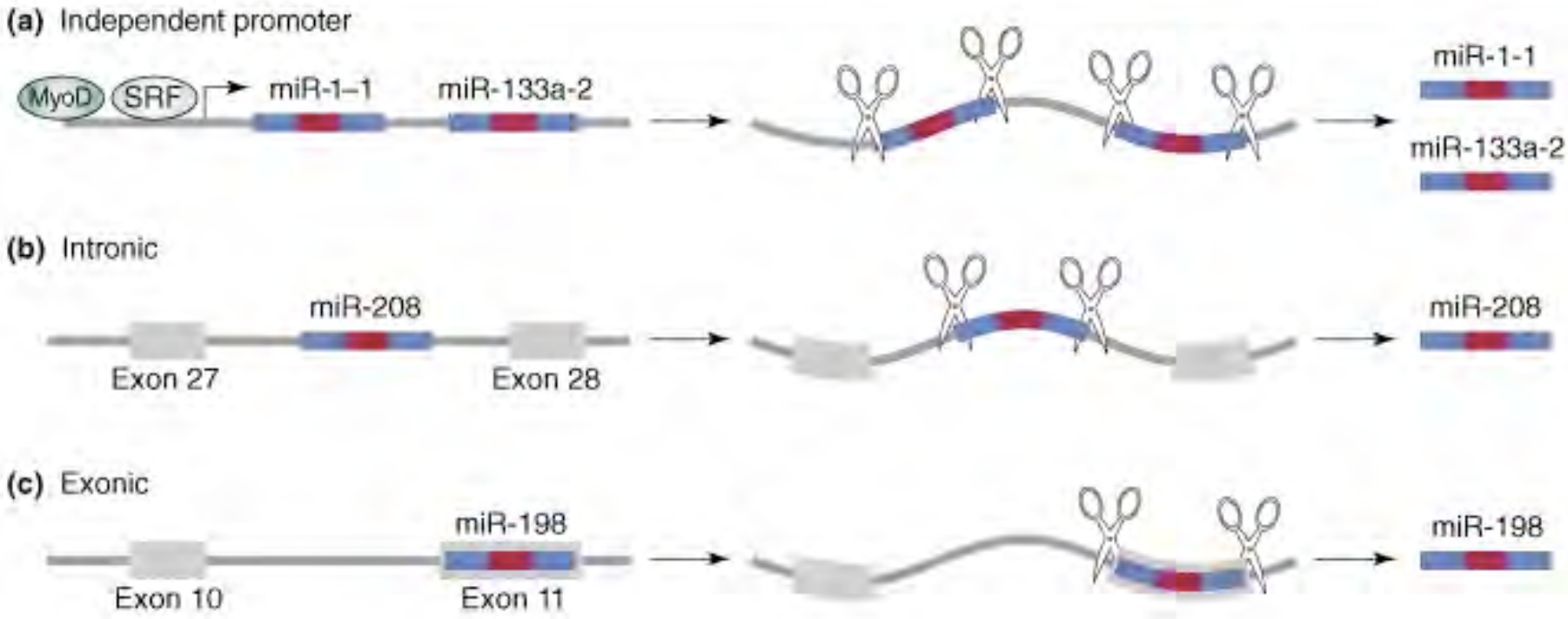
- ✓ miRNAs can be secreted into extracellular fluids and transported to target cells via vesicles, such as exosomes.
- ✓ Extracellular miRNAs function as chemical messengers to mediate cell-cell communication.

Homology Between *C. elegans* and *Homo sapiens* miRNAs



Lim (2003) Genes & Dev. 17: 991-1008

Genomic Organization of miRNA Genes



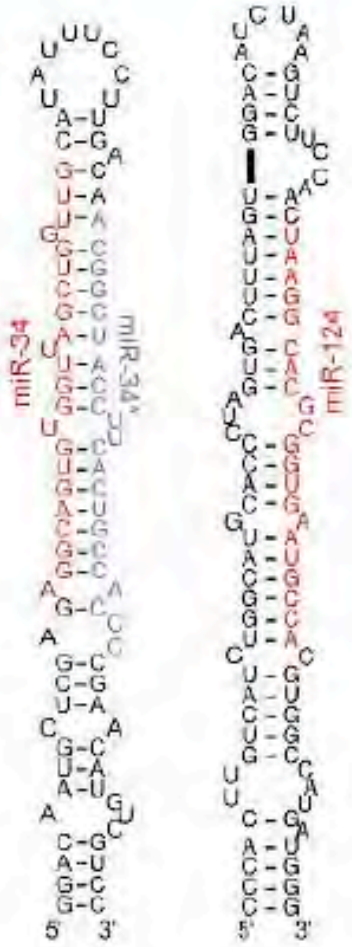
- Intronic miRNAs often in antisense direction, made from own promoter

- Exonic miRNAs - non-coding (or in alternatively spliced exons)

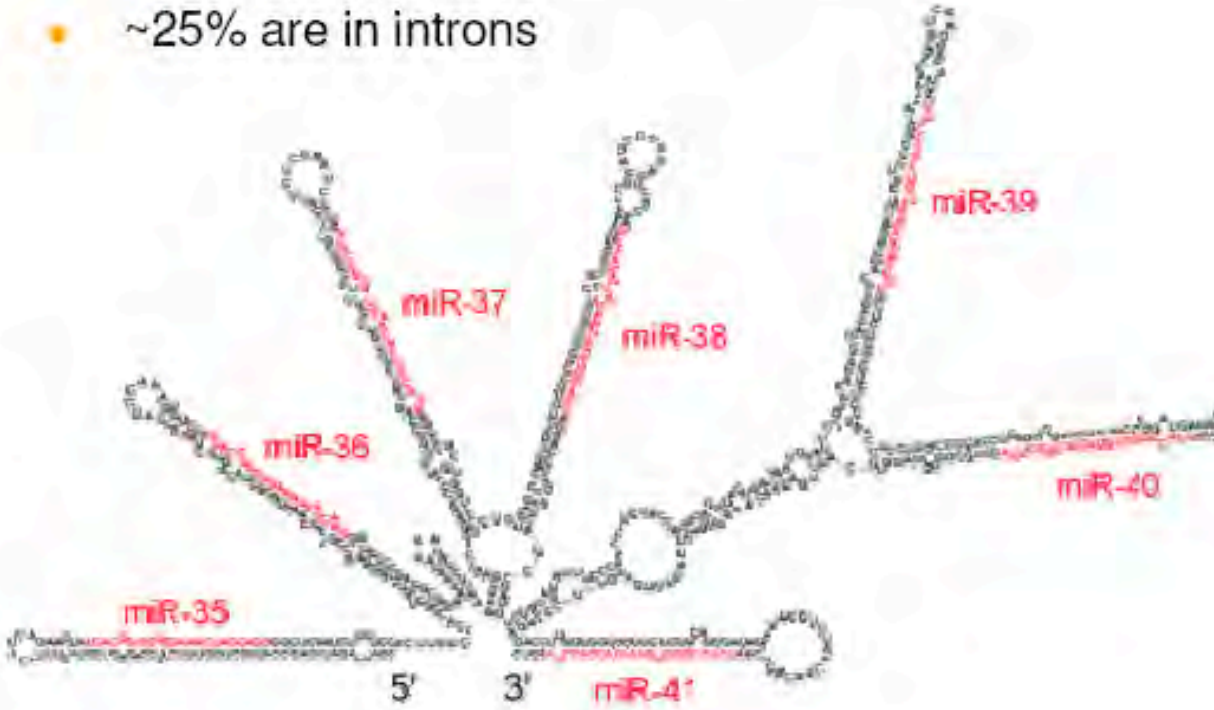
Zhao Y, Srivastava D, TIBS 32:189,2007

Genomic organization of miRNAs

miRNA Genes

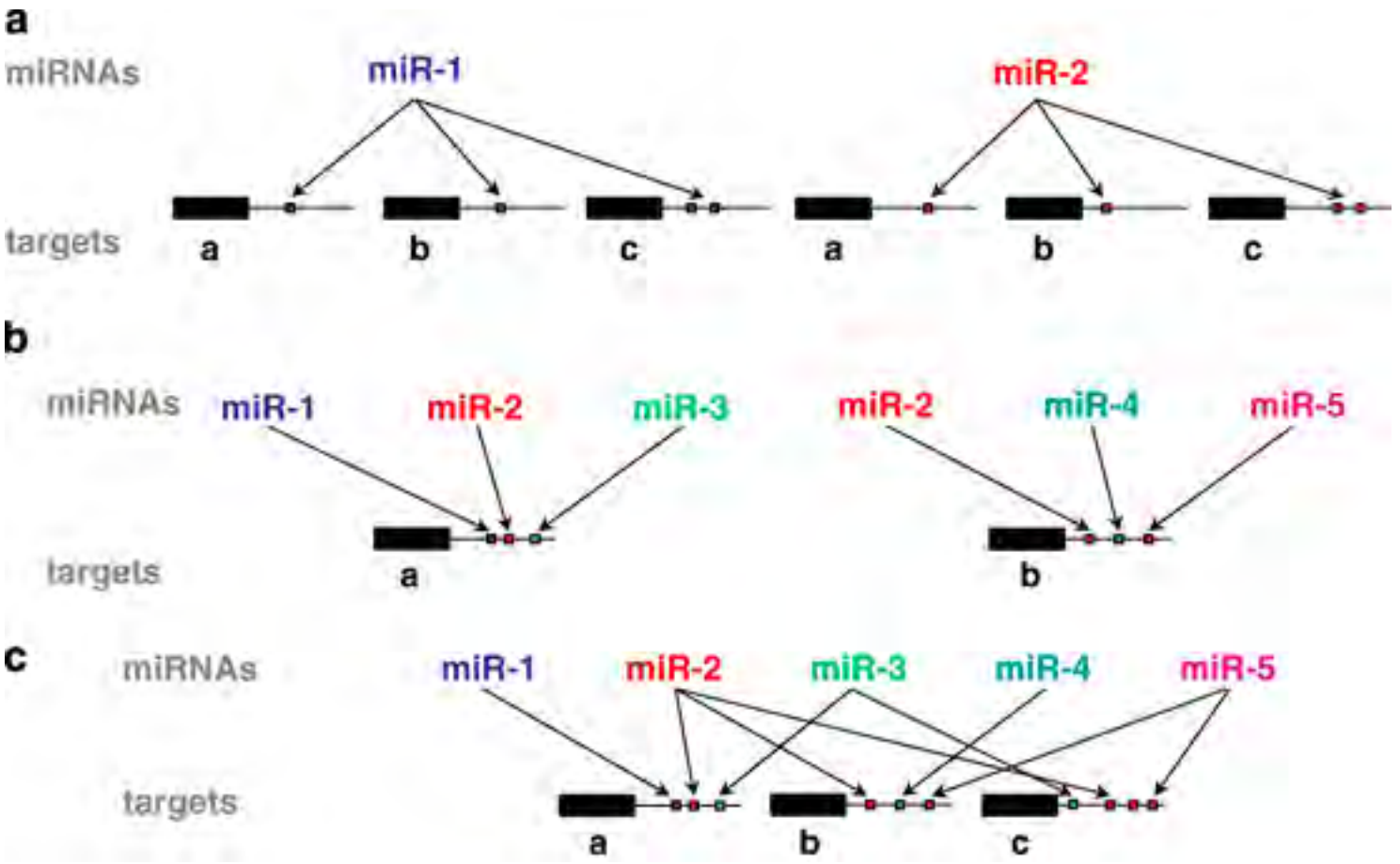


- ~60% of miRNAs are expressed independently
- ~15% of miRNAs are expressed in clusters
- ~25% are in introns



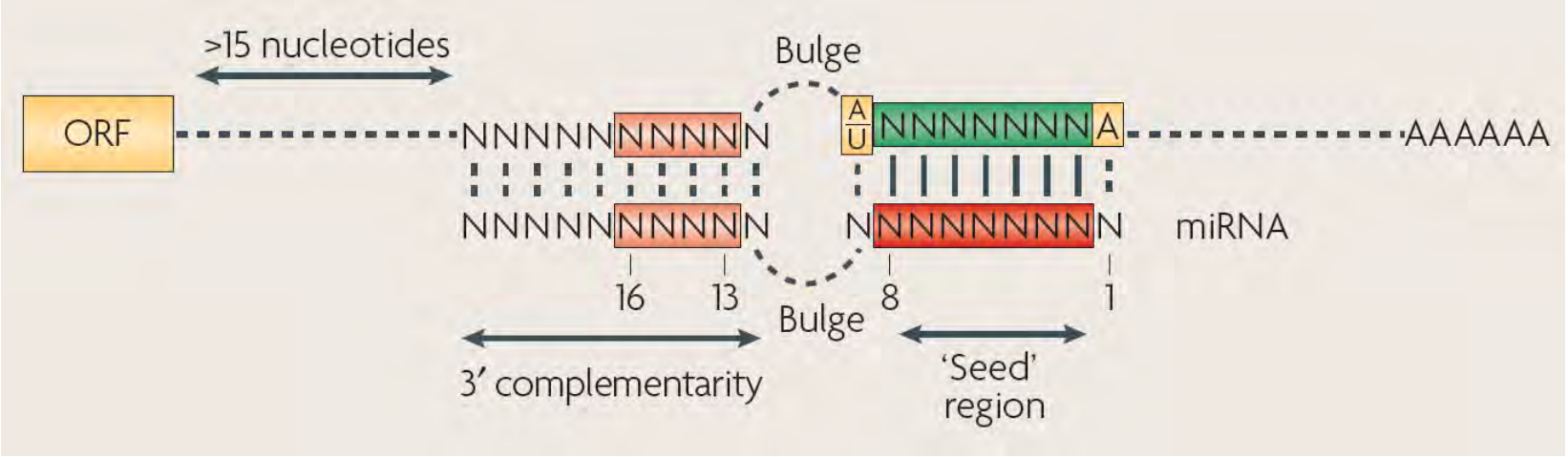
C. elegans

miRNAs target multiple genes and genes are targeted by multiple miRNAs



(**a**) miRNAs have multiple targets. (**b**) Many genes have seed matches for multiple miRNAs in their 3′UTRs. (**c**) A complex network of mutual interactions between miRNAs and mRNAs.

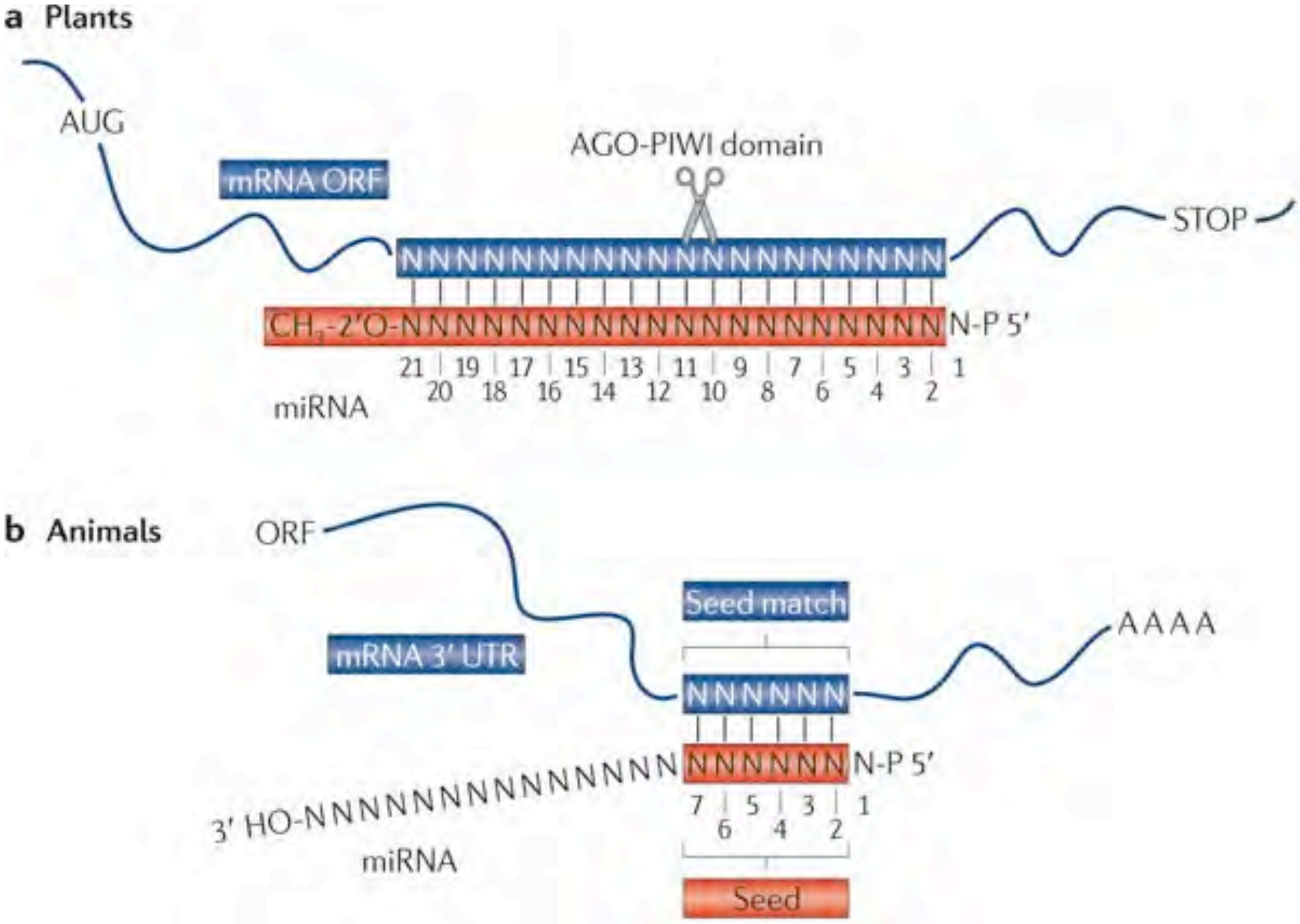
miRNA-mRNA interaction



Filipowicz et al. 2008. Nat Rev Genet. 9:102

miRNA-target base pairing rules:

- perfect and contiguous base pairing of miRNA nucleotides 2 to 8
- bulges or mismatches must be present in the central region of the miRNA-mRNA duplex
- Other factors that can improve site efficacy include AU-rich content, and a position that is not too far away from the poly-A tail or the termination codon

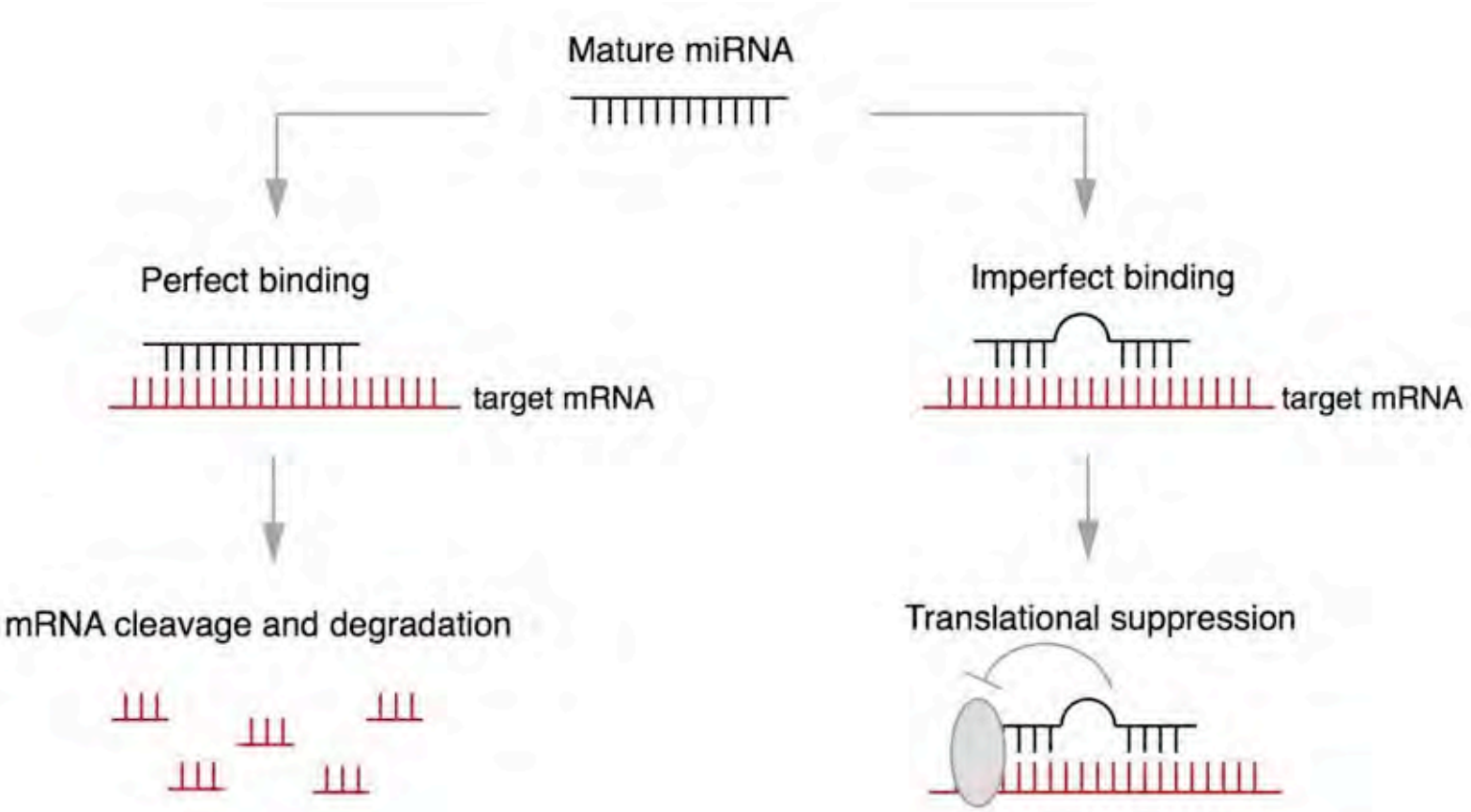

a Plants
AUG
mRNA ORF
AGO-PIWI domain
STOP
NNNNNNNNNNNNNNNNNNNNN
CH3-2'O-NNNNNNNNNNNNNNNNNNNNN N-P 5'
21 19 17 15 13 11 9 7 5 3 1
20 18 16 14 12 10 8 6 4 2
miRNA
b Animals
ORF
Seed match
AAAA
mRNA 3' UTR
NNNNNN
NNNNNN N-P 5'
3' HO-NNNNNNNNNNNNNNN
7 5 3 1
6 4 2
miRNA
Seed

Come funzionano i miRNAs?

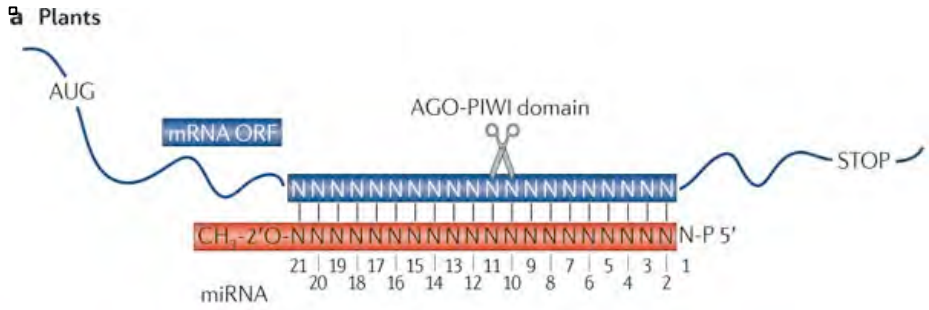
La funzione primaria dei miRNA e' quella di abbassare il livello proteico dei geni target

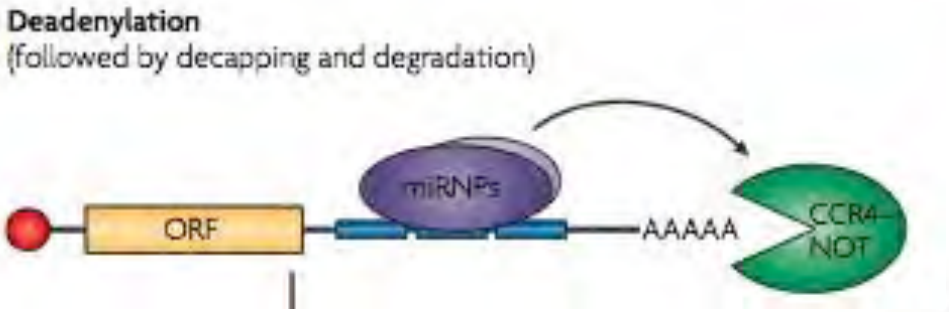
I miRNAs svolgono la propria funzione attraverso due meccanismi:

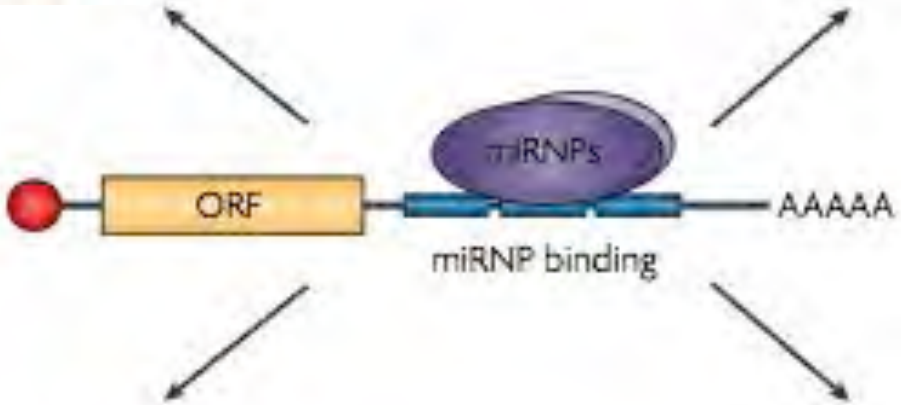
- PERFETTA COMPLEMENTARIETA'→ DEGRADAZIONE DEL mRNA (piante)
- IMPERFETTA COMPLEMENTARIETA'→ REPRESSIONE TRADUZIONALE (animali)



Mechanisms of Regulation by miRNAs



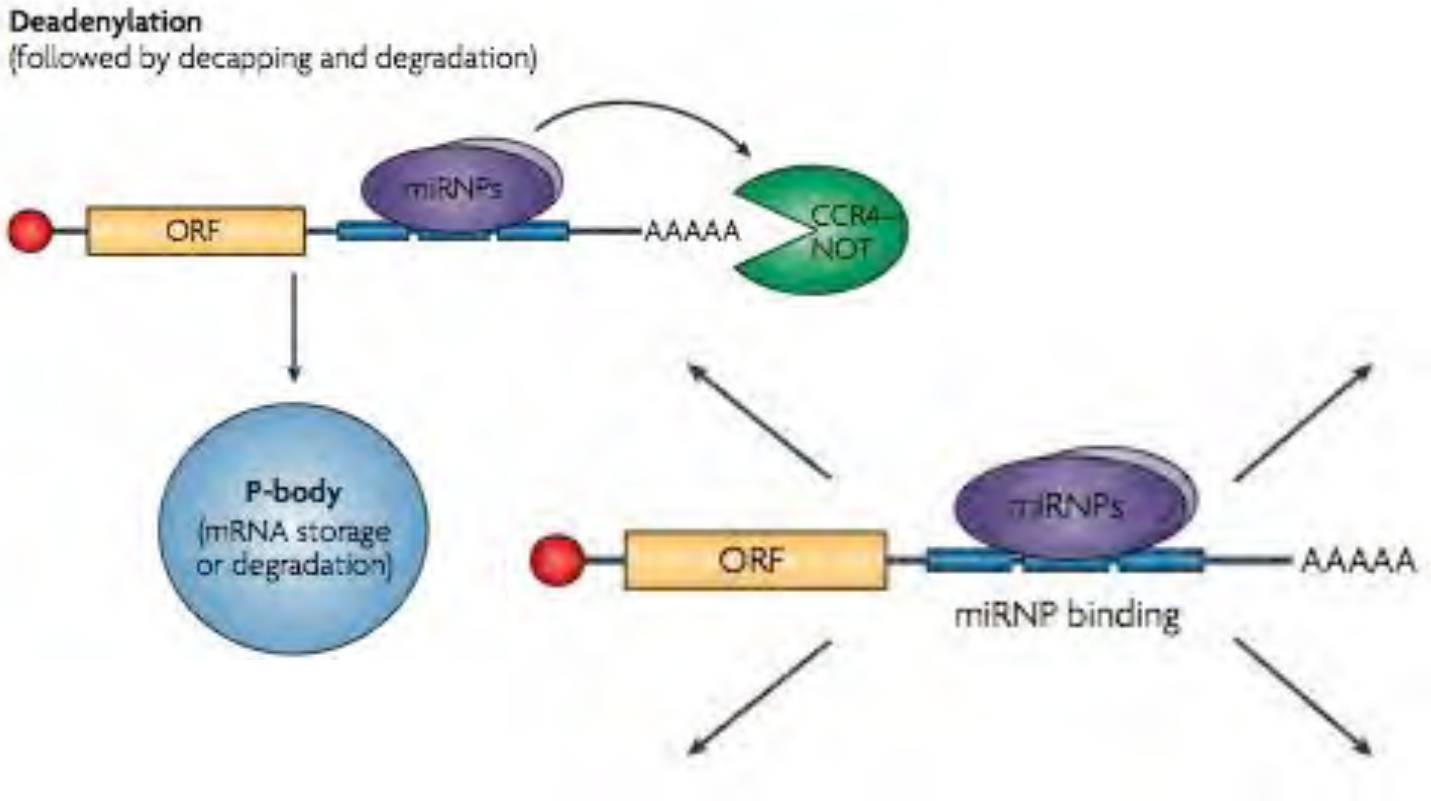




- ✧ Deadenilation
- ✧ Decapping
- ✧ mRNA degradation by the 5'-exonuclease Xrn1.

Filipowicz (2008) Nature Review Genetics 9:102-112.

Mechanisms of Regulation by miRNAs

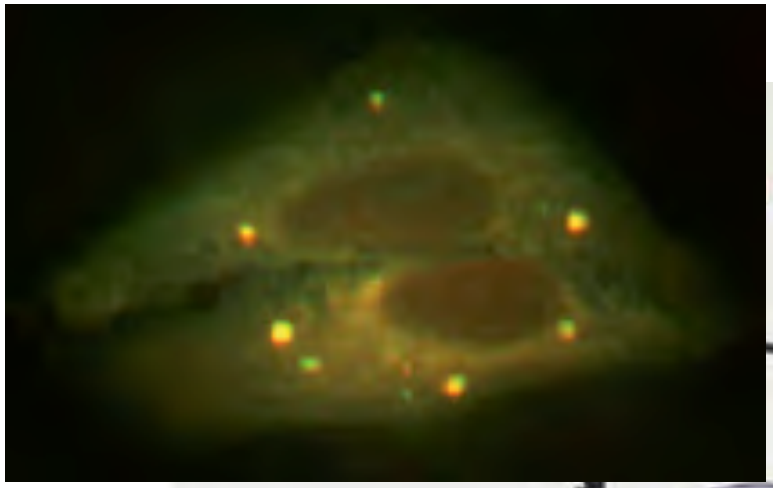


- ✧ Deadenlation
- ✧ Decapping
- ✧ mRNA degradation by the 5'-exonuclease Xrn1.

P bodies:

specific cytoplasmic foci, sites of mRNA decapping and degradation

≥ 35 proteins known, conserved from yeast to man



Where does the action take place ?

P bodies:

specific cytoplasmic foci, sites of mRNA decapping and degradation

≥ 35 proteins known, conserved from yeast to man

Also in p bodies:

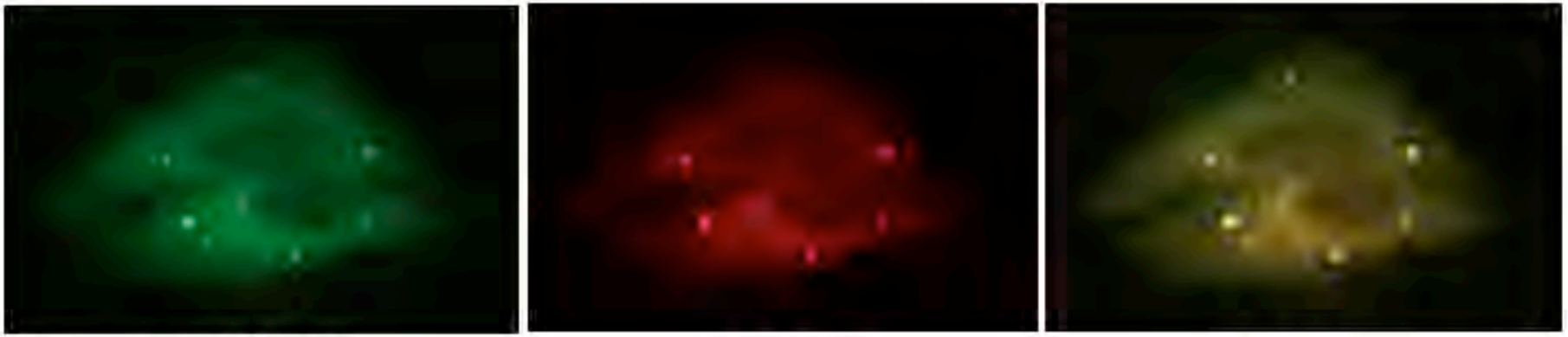
concentration of Argonaute proteins

mRNA targets of miRNAs

Presumable Function:

degradation of mRNAs

storage of mRNAs

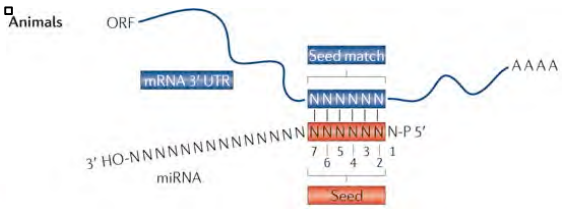


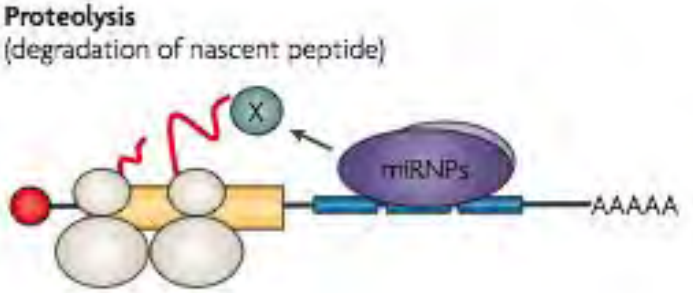
Flag-Dcp1a **Myc-Ago2** **Merge**

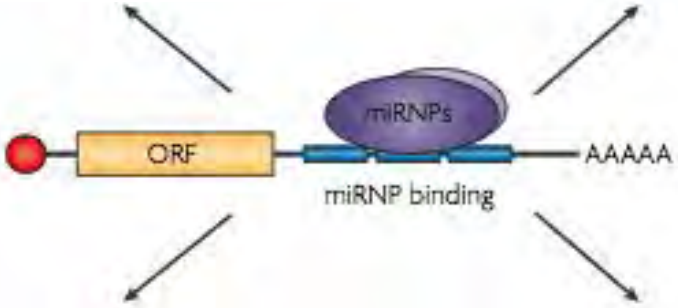
Liu et al., 2005 NatCellBiol 7:719-723

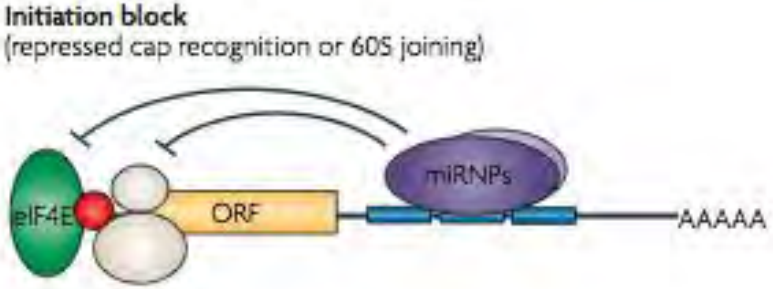
Mechanisms of Regulation by miRNAs

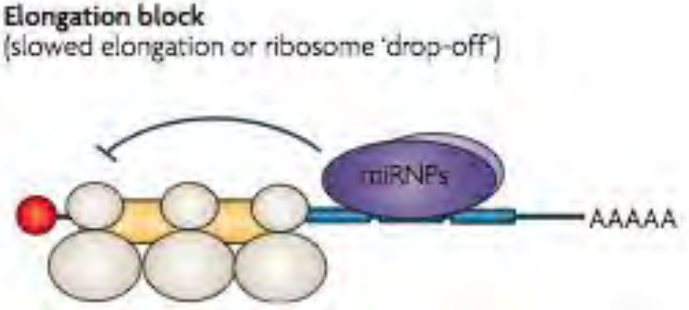
- ✧ Blocco dell'inizio della traduzione
- ✧ Degradazione del peptide nascente
- ✧ Blocco dell'elongazione



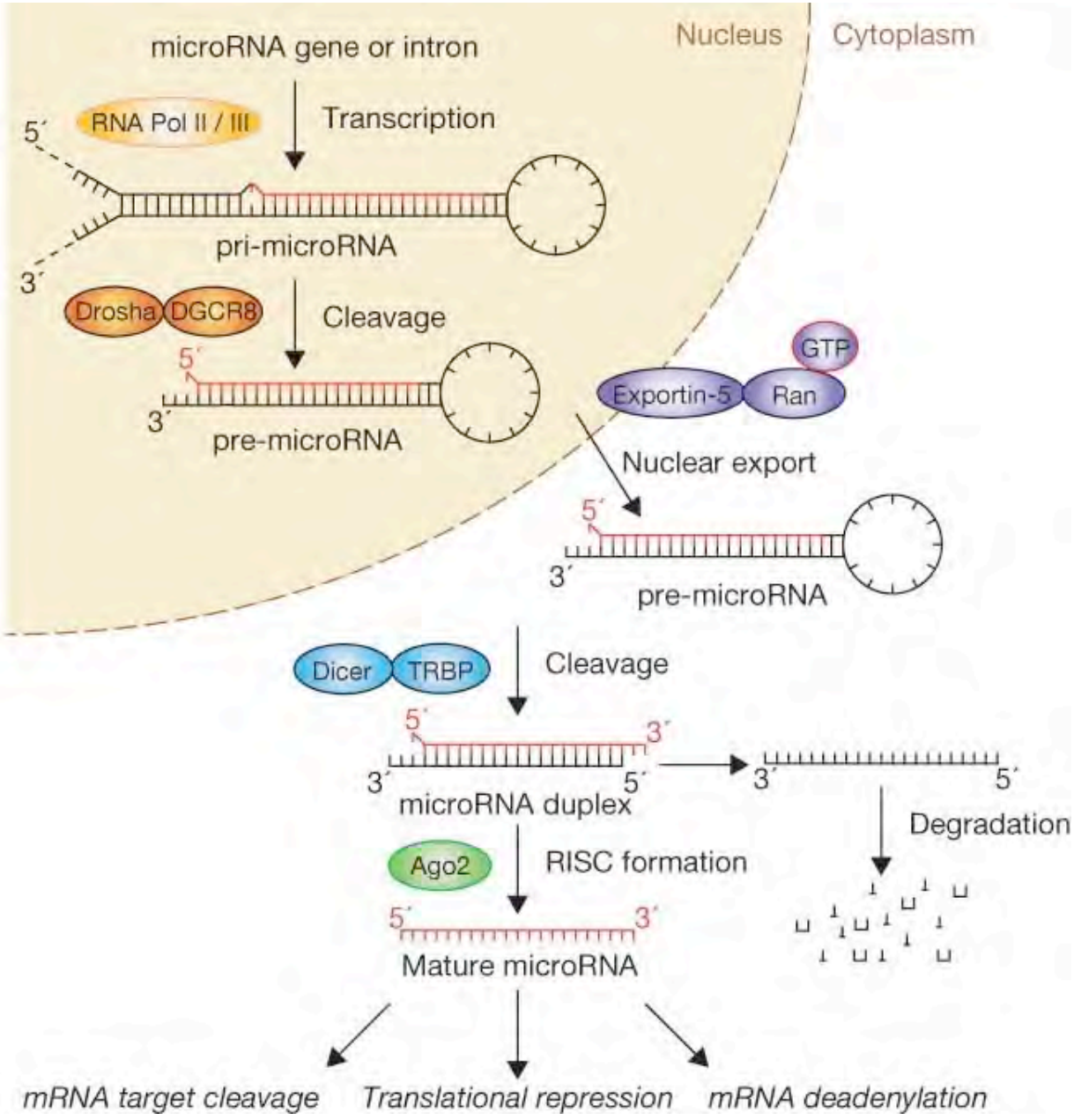






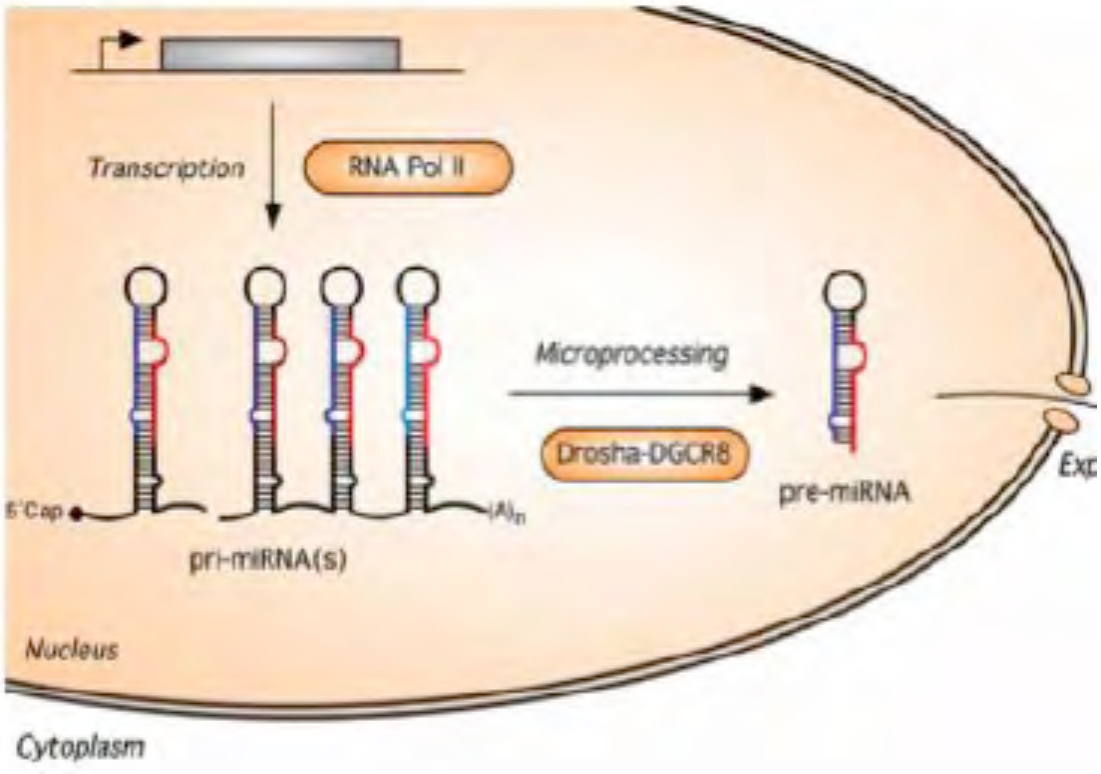


Biogenesis of miRNAs



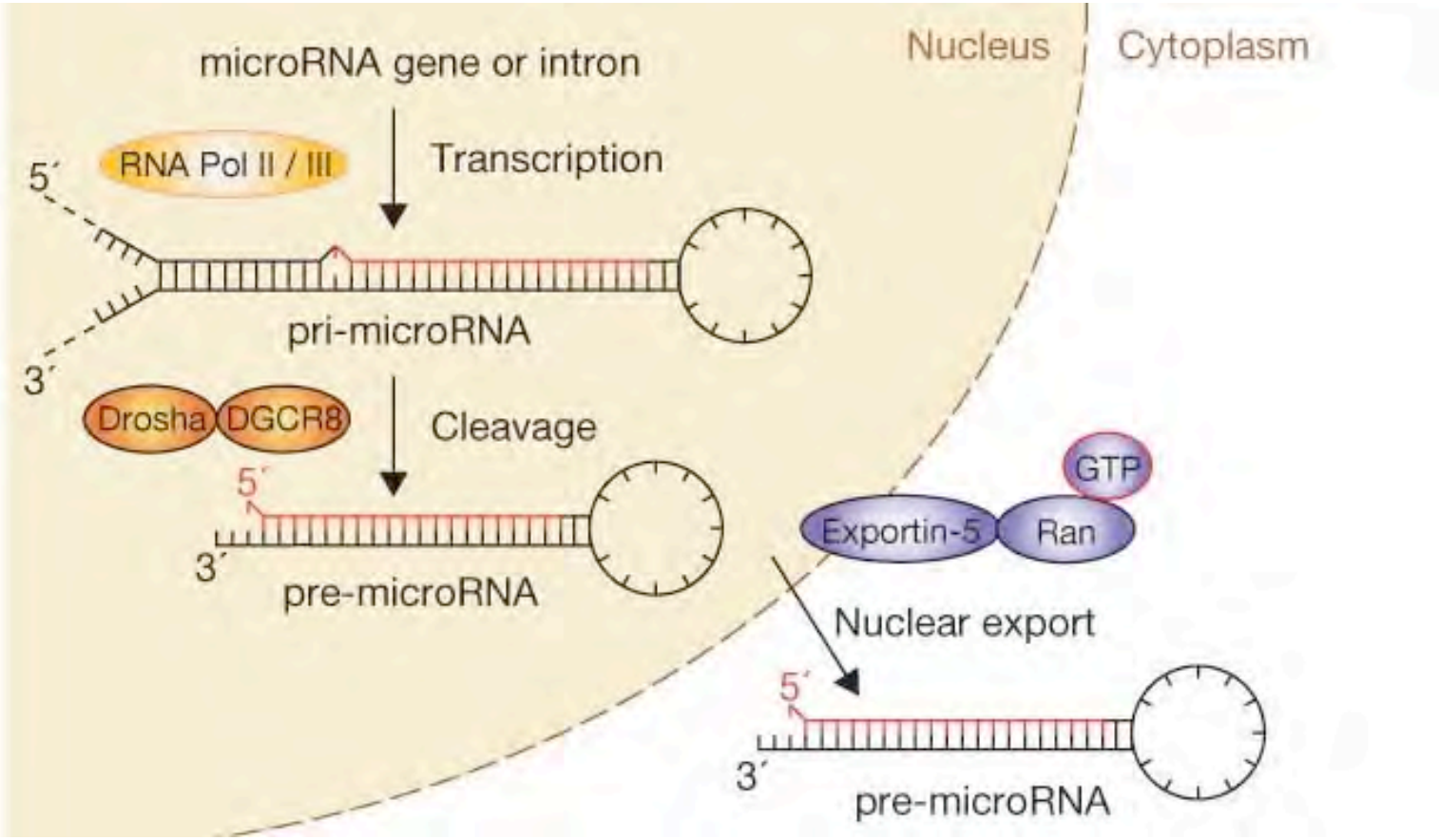
1. **Transcription**
2. **Hairpin release in the nucleus**
3. **Export to cytoplasm**
4. **Dicer processing**
5. **Strand selection by RISC**
6. **Translational repression**

Biogenesis of miRNAs



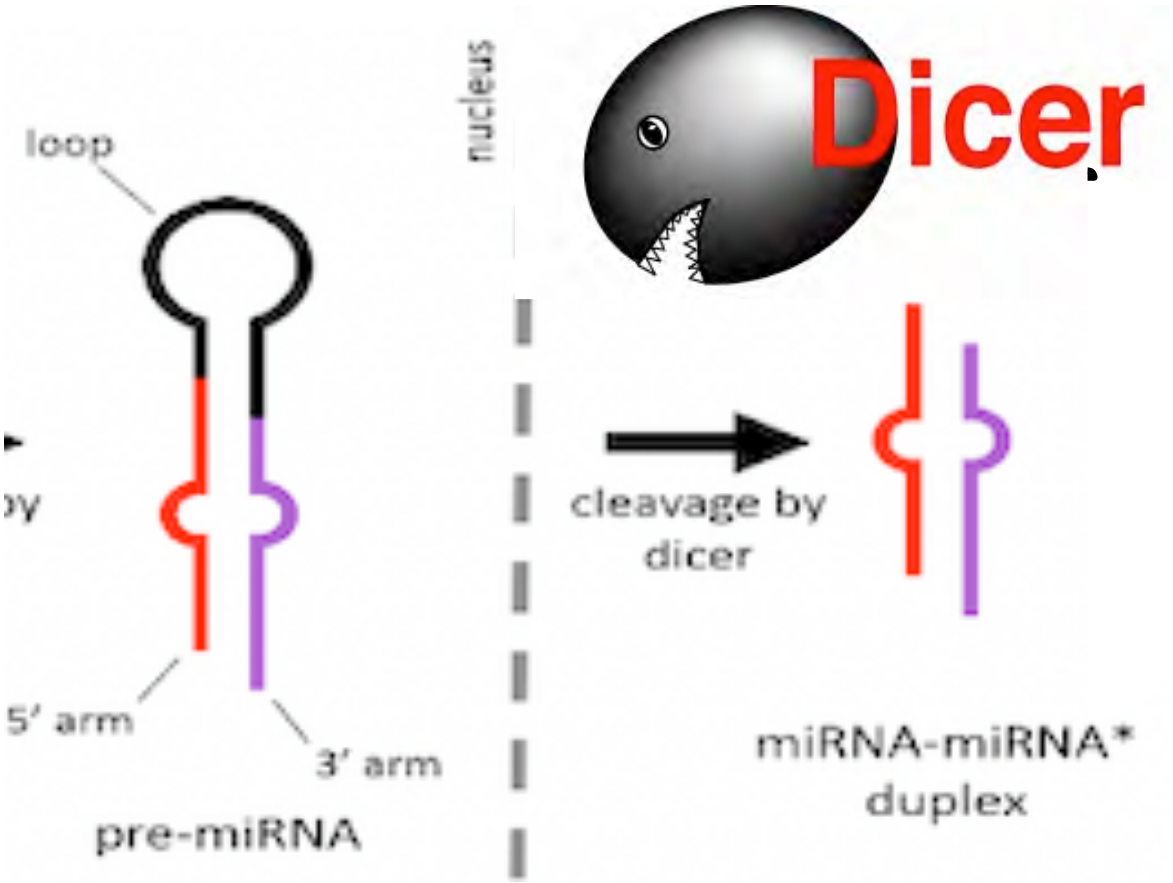
- miRNAs are encoded by the genome.
- RNA polymeraseII transcribes precursors, called **pri-miRNAs (primary miRNAs)**.
- Primary miRNAs are approx 70 nt long, with 3'overhangs 1-4 nt long, stems 25-30 bp long and small hairpins.
- They bear a 5' CAP and a poli A tail.
- The dsRNA-specific ribonuclease **DROSHA** digests the pri-miRNA in the nucleus into single Hairpins, the **pre-miRNAs**.

Biogenesis of miRNAs



NUCLEAR EXPORT- EXPORTIN-5 (Exp5) translocates the mature miRNAs from the nucleus to the cytoplasm.
Exp5 directly binds the pri-miRNA correctly processed.

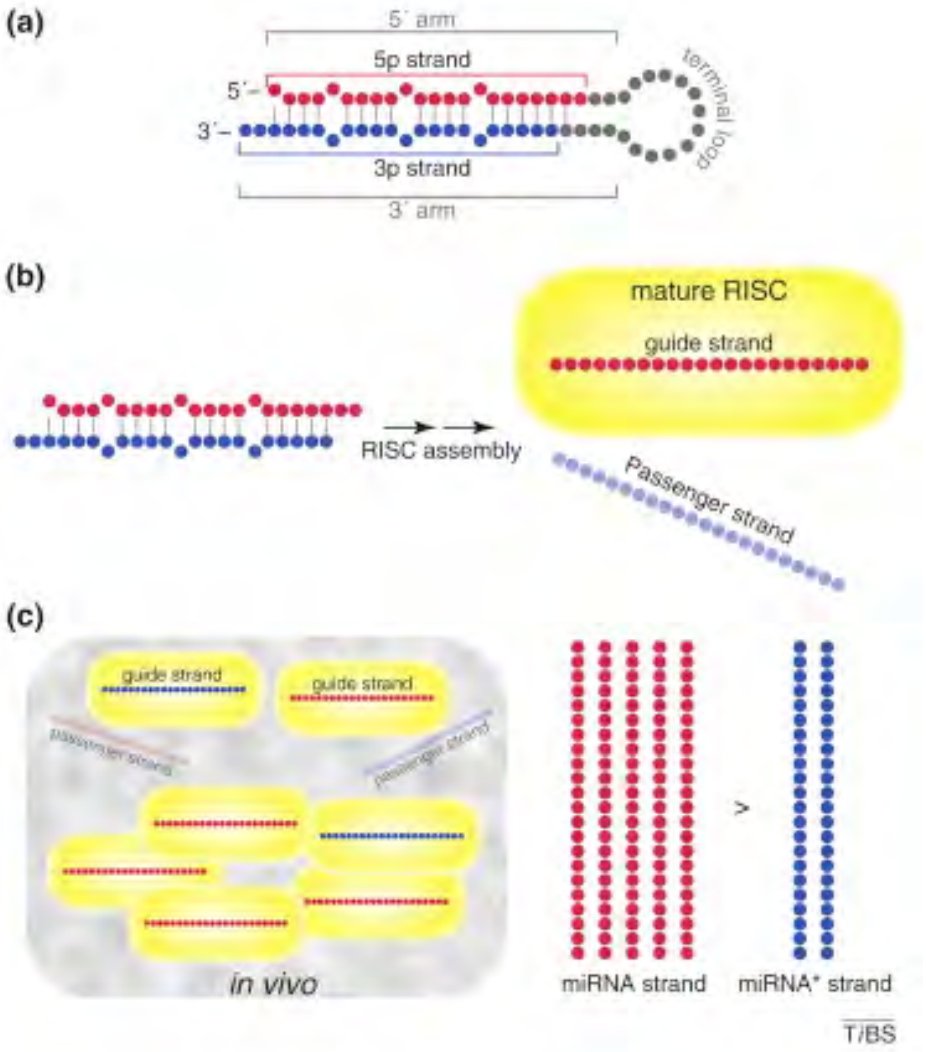
Biogenesis of miRNAs



MATURATION

When the pre-miRNA is in the cytoplasm, the RNAseIII DICER cuts it into:
21-25 nt fragment with 3’ symmentrical overhangs, containing 5’ phosphate groups.

Nomenclature for small RNA strands

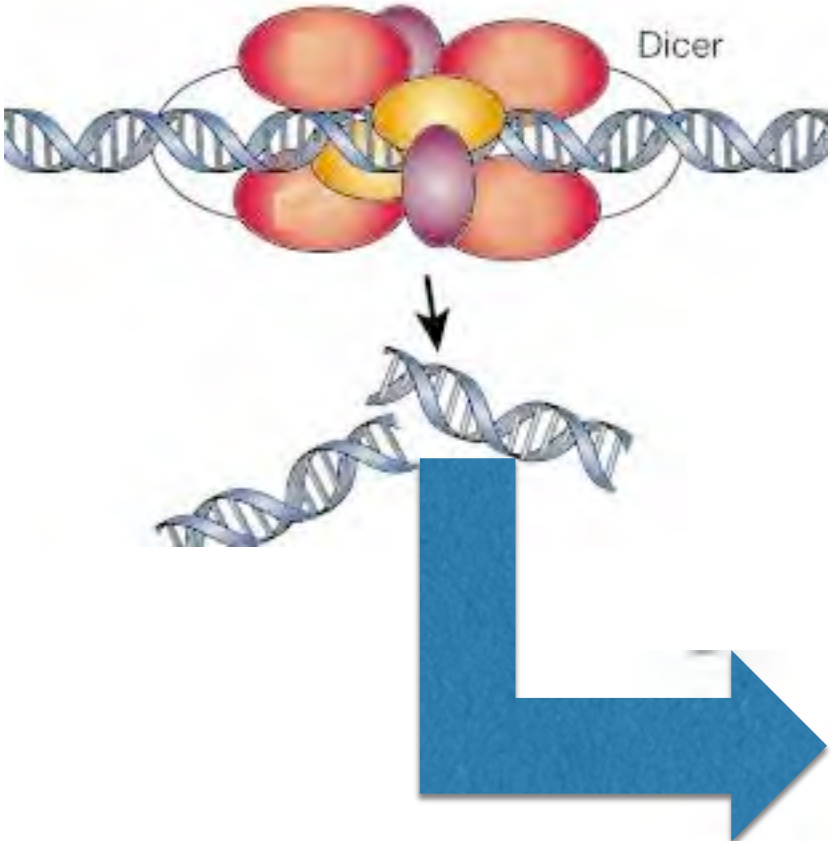


5p and 3p strands: 5p and 3p designate the strands derived from the 5′ arm and 3′ arm of a pre-miRNA, respectively.

Guide and passenger strands: the guide strand is retained in the mature RISC whereas the passenger strand is discarded upon unwinding.

miRNA and miRNA* strands: the miRNA strand is the more abundant (and thereby more frequently cloned) strand overall in vivo, whereas the miRNA* strand is the less abundant strand. Note that a passenger strand is quickly degraded as soon as it is discarded from pre-RISC whereas a guide strand is protected from nucleases in the mature RISC. Consequently, the strand that is more likely to serve as the guide strand tends to accumulate and therefore become the 'miRNA strand'.

Biogenesis of miRNAs



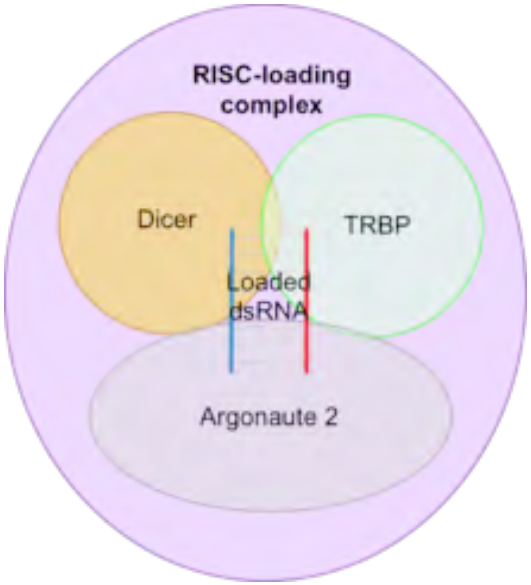
The RISC COMPLEX

RISC EFFECTOR COMPLEX

RISC is a large (~500 kDa) RNA multiprotein complex

Severale enzymes bind to Dicer and to the ds-RNA, to form the Risc-Loading Complex

The main effector at this point is the protein **ARGONAUTE**



AGO proteins are evolutionarily conserved and they are ubiquitously expressed

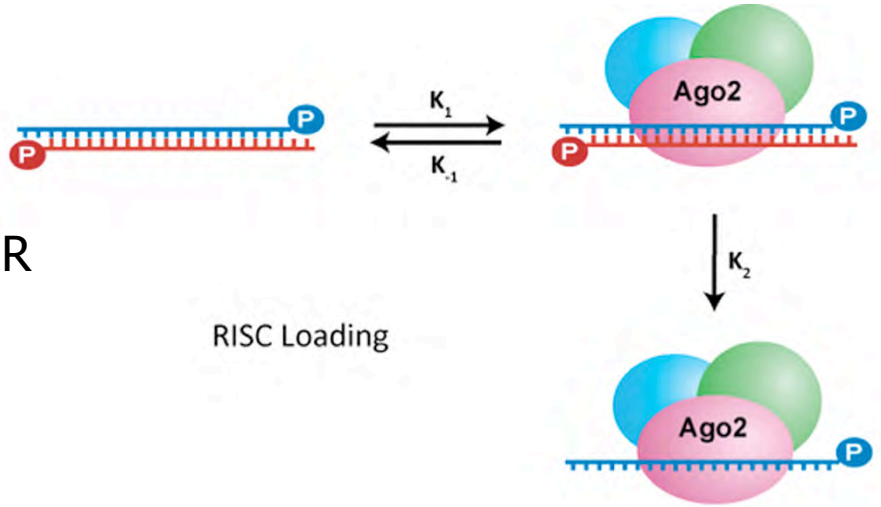
Number of Argonaute family genes in different species

Species	Number of genes
Homo sapiens	8
Rattus norvegicus	8
Mus musculus	8
Drosophila melanogaster	5
Caenorhabditis elegans	27
Arabidopsis thaliana	10
Schizosaccharomyces pombe	1
Neurospora crassa	2

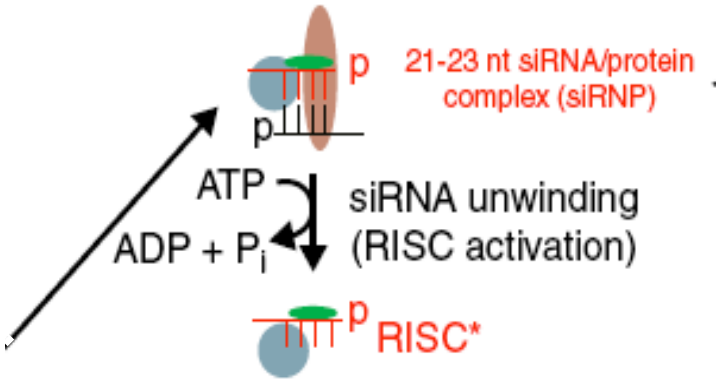
Biogenesis of miRNAs

STRAND SELECTION BY THE RISC COMPLEX

The ds-miRNAs generated by DICER must separate their two strands.



Key component of the RISC is the protein **ARGONAUTE**



ATP-dependent unwinding of the siRNA duplex remodels the complex to generate an active RNA-induced silencing complex (RISC - the asterisk denotes active conformation)

Argonaute

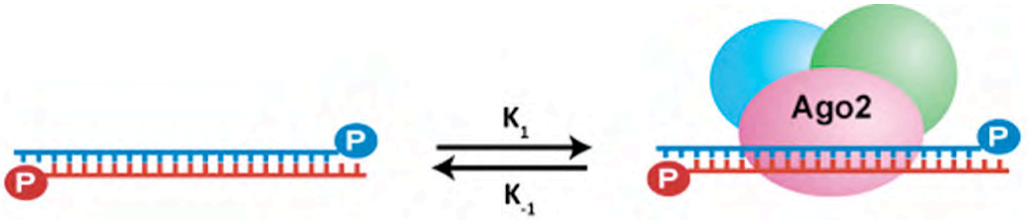
The PAZ Domain has 3' RNA binding activity

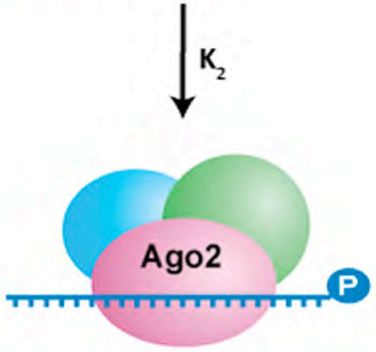
- The PIWI Domain binds the 5'-P RNA end
- It has Rnase H activity

Argonaute

The PAZ Domain has 3' RNA binding activity

- The PIWI Domain binds the 5'-P RNA end
- It has Rnase H activity



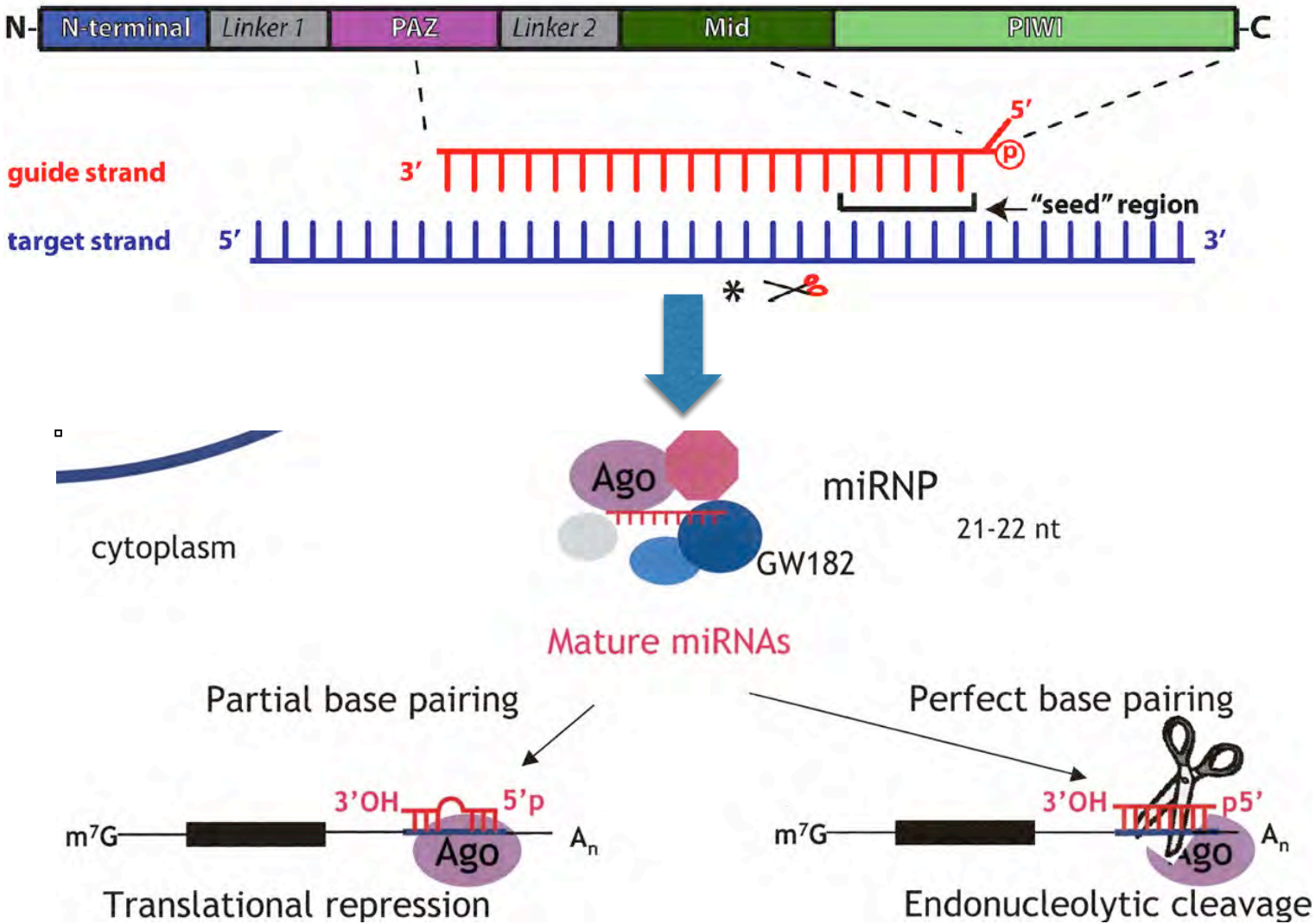


The PIWI domain exerts its RNaseH activity, cleaves the passenger strand leading to its ejection from the complex

Argonaute

- The PAZ Domain has 3' RNA binding activity

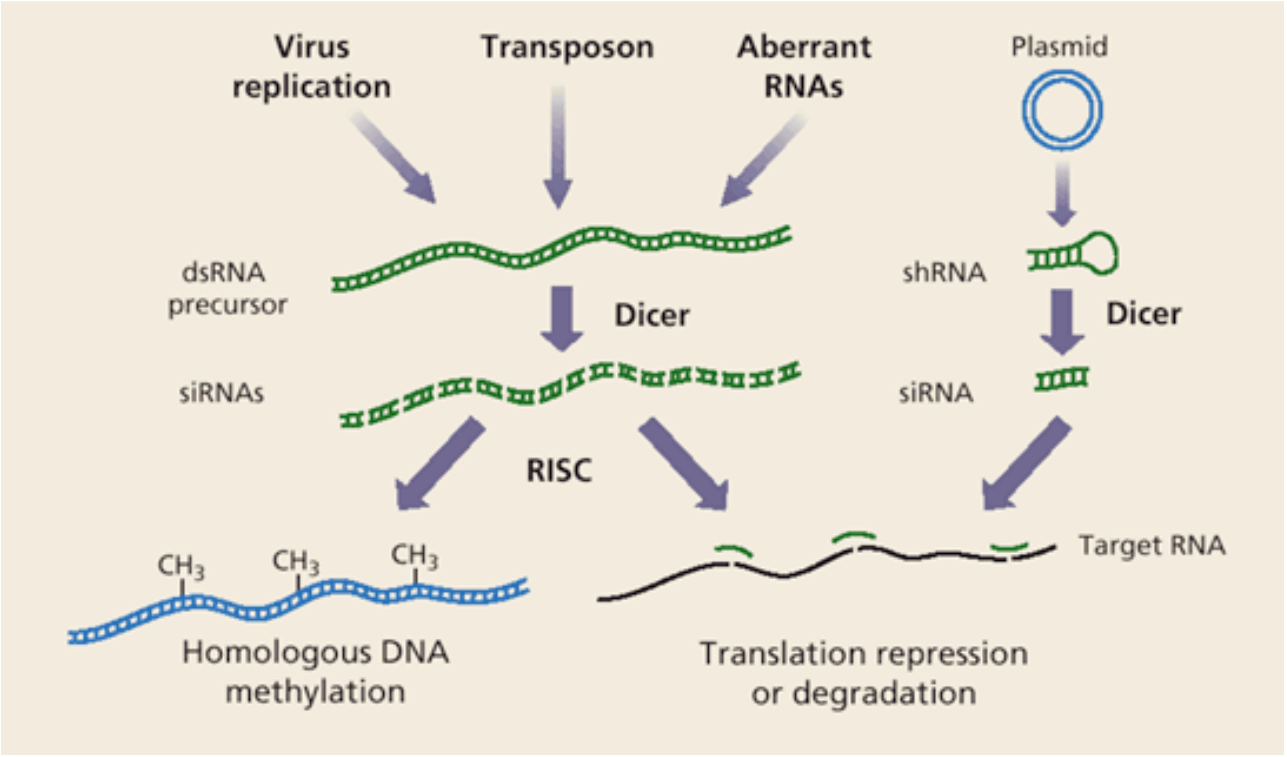
- The PIWI Domain binds the 5'-P RNA end
- It has Rnase H activity



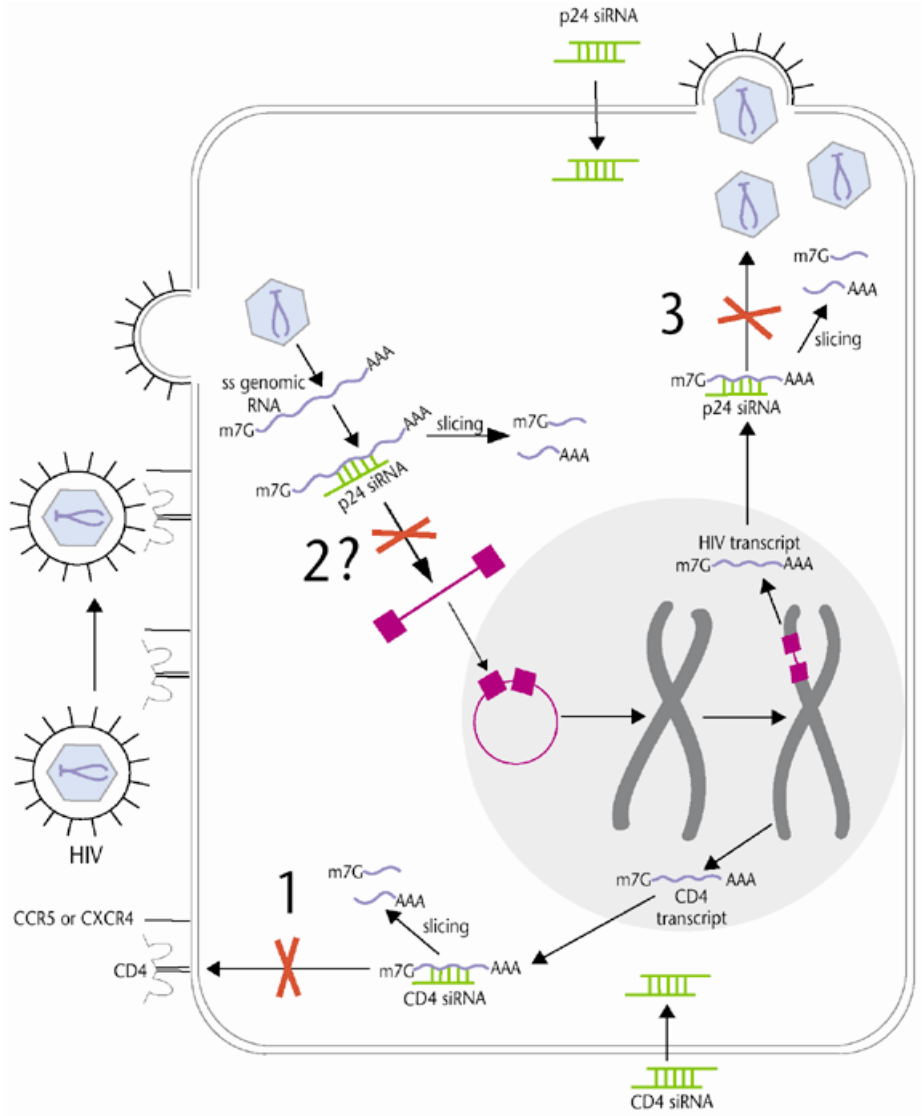
RNA silencing could represent an "immune defense" of the genome

Close to 50% of our genome consists of viral and transposon elements that have invaded the genome in the course of evolution. The RNAi machinery can recognize invading double-stranded viral RNA (or the double-stranded replicative form of the viral RNA) and suppress the infection by degradation of the RNA.

The RNAi system thus shares important features with the vertebrate immune system: it recognizes the invading parasite (dsRNA), raises an initial response and subsequently amplifies the response to eliminate the foreign element.



Silencing viruses by RNAi



siRNA-directed inhibition of HIV-1 infection

Carl D. Novina *et al.*

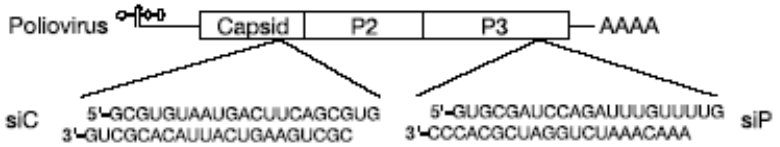
Nature Medicine 8, 681 - 686 (2002)

Modulation of HIV-1 replication by RNA interference

Jean-Marc Jacque, Karine Triques & Mario Stevenson

Short interfering RNA confers intracellular antiviral immunity in human cells

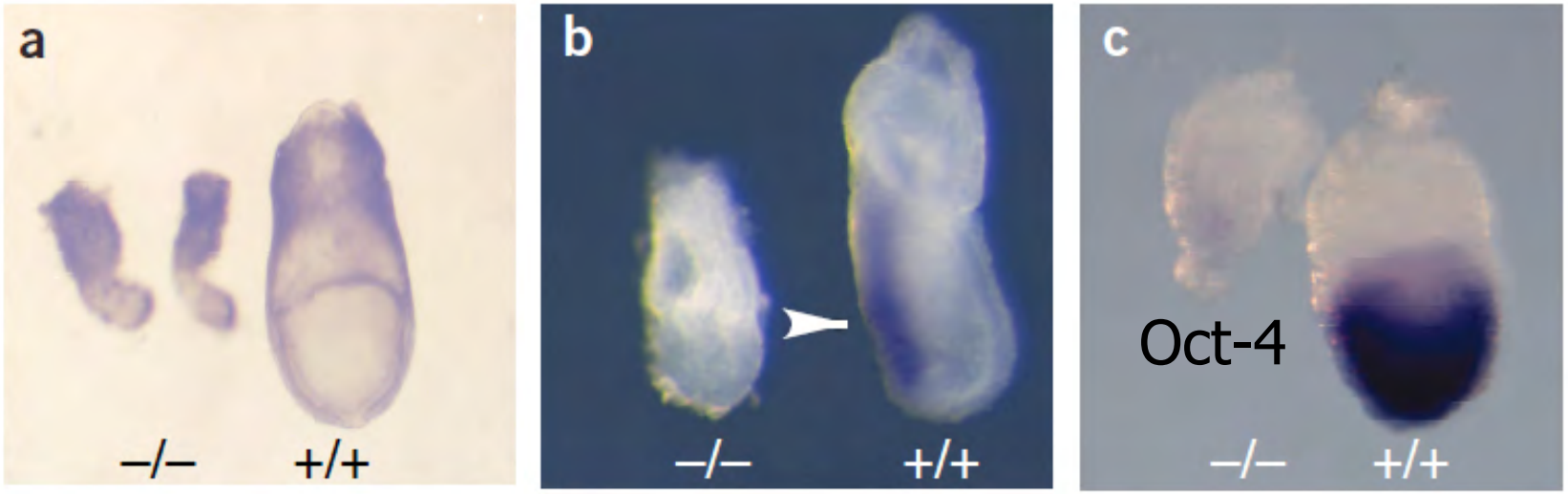
Leonid Gitlin*†, Sveta Karelsky* & Raul Andino*



The endogenous role of RNAi

Dicer is essential for mouse development

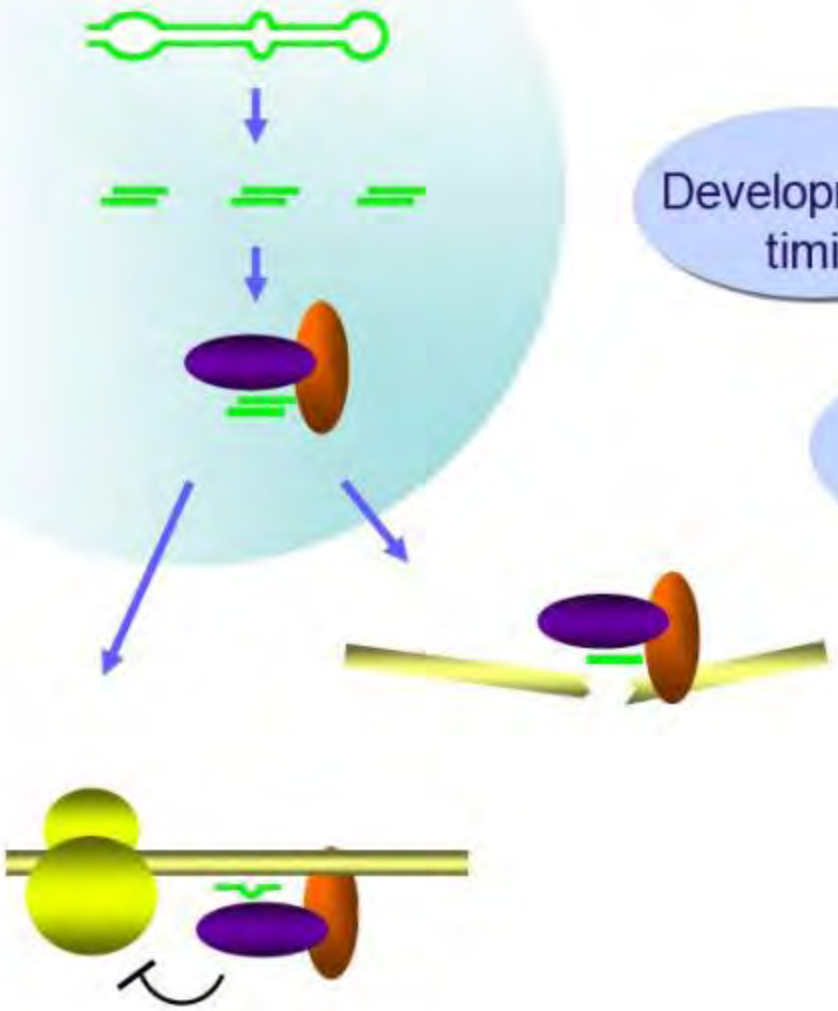
Emily Bernstein[1,2], Sang Yong Kim[1], Michelle A Carmell[1,2], Elizabeth P Murchison[1], Heather Alcorn[3], Mamie Z Li[4], Alea A Mills[1], Stephen J Elledge[4], Kathryn V Anderson[3] & Gregory J Hannon[1]



E7.5 embryos - lack of stem cell development

NATURE GENETICS VOLUME 35 | NUMBER 3 | NOVEMBER 2003

Thousands of microRNAs act in multiple biological events



Developmental timing

Differentiation

Aging

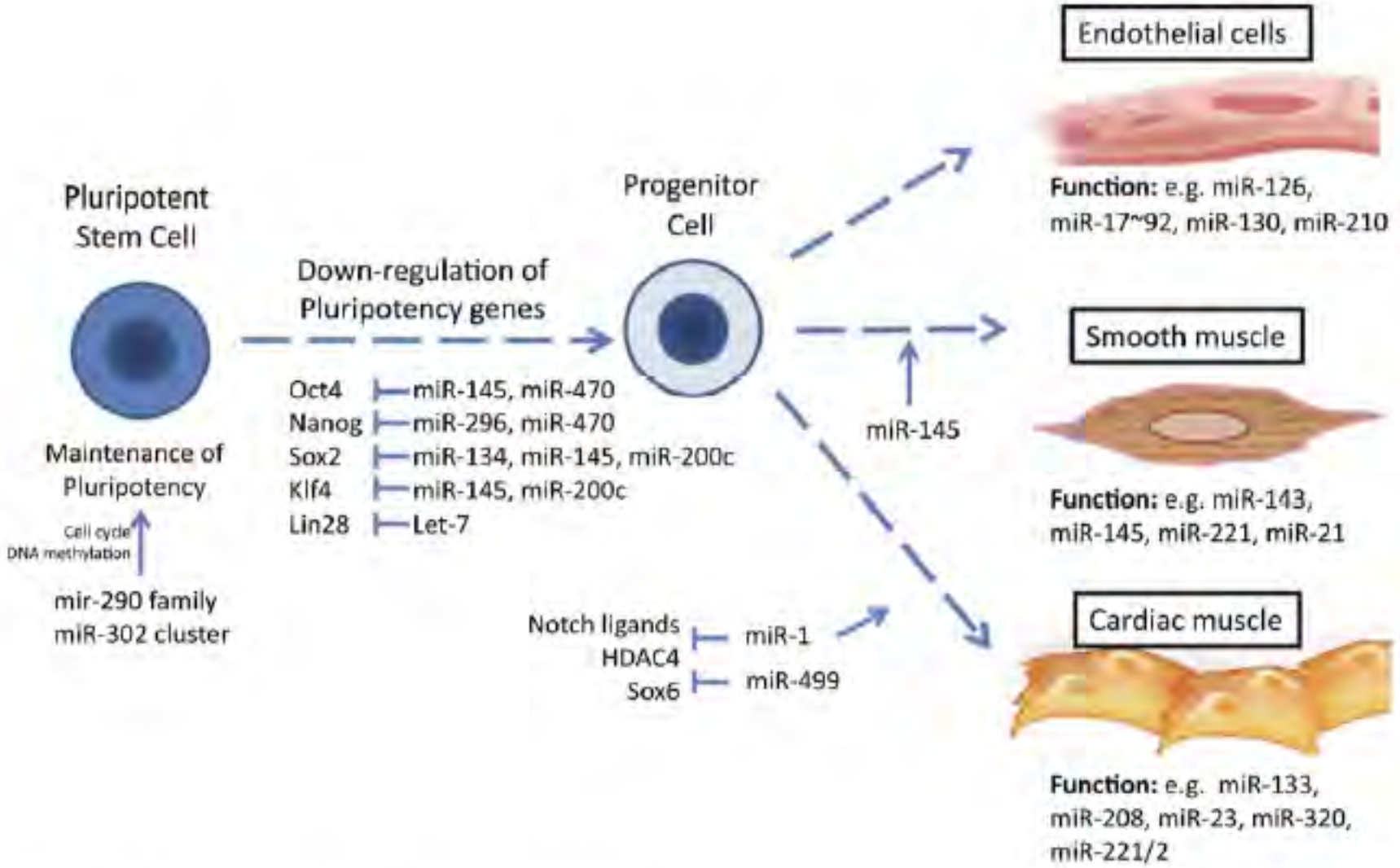
Apoptosis

Metabolism

Cancer

…

Cell fate/differentiation, Cell cycle…



Control of self-renewal and differentiation by microRNAs

miRNA Dysregulation in Human Cancers

Table 2 | **Consequences of microRNA dysregulation in human cancers**

MicroRNA dysregulation	Targets	Consequences
MicroRNA overexpression	Tumour suppressors	Downregulation of tumour suppressors — for example, PTEN, p22, p57, TIMP3 and PDCD4
MicroRNA loss	Oncogenes	Upregulation of oncogenes — for example, *BCL2*, *MCL1*, *RAS*, *HMGA2*, *MYC* and *MET*
MicroRNA loss	DNA methyltransferases	Downregulation of tumour suppressors — for example, p16, FHIT and WWOX
MicroRNA loss	Chromatin silencers	Downregulation of tumour suppressors

BCL2, B cell leukaemia/lymphoma 2; FHIT, fragile histidine triad protein; *HMGA2*, high mobility group AT-hook 2; *MCL1*, myeloid cell leukaemia sequence 1; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homologue; TIMP3, tissue inhibitor of metalloproteinases 3; WWOX, WW domain-containing oxidoreductase.

Croce Nat Rev Genet. 2009 Oct;10(10):704-14

miRNAs are involved in all aspects of cardiovascular function

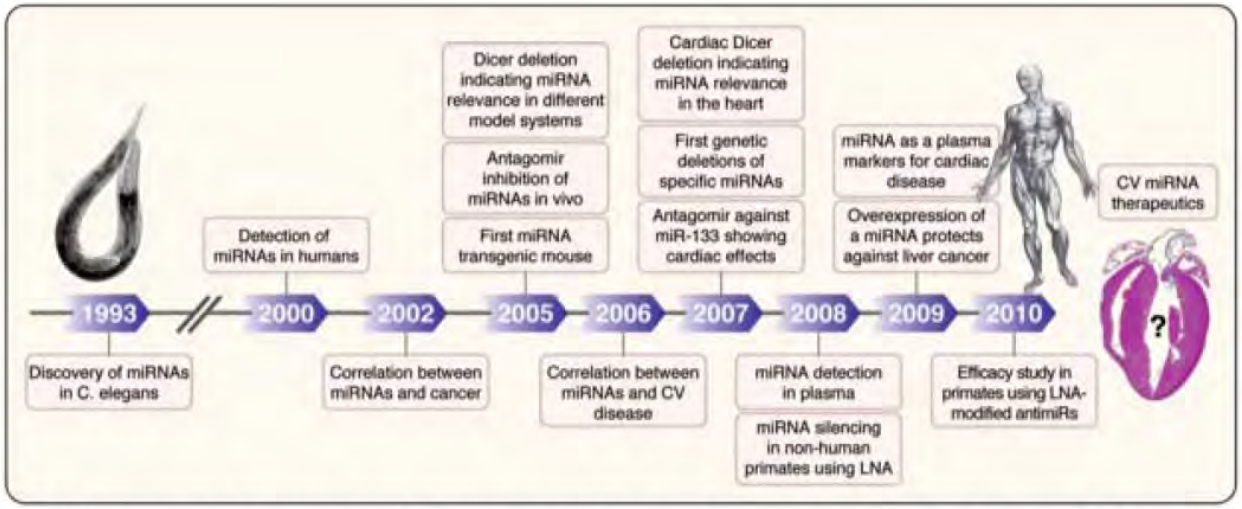


Figure 1. Breakthrough discoveries in miRNA biology. Time line indicating seminal discoveries in miRNA biology with a special focus on the cardiovascular field.

Circ Res. **2011;108:219-234**

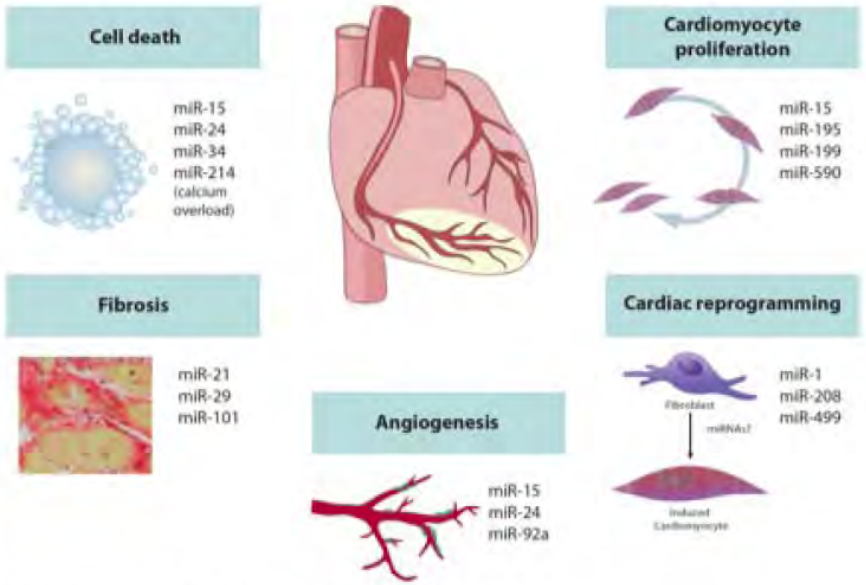


Figure 2. MicroRNAs (MiRNAs) in postinfarction repair.

Arterioscler Thromb Vasc Biol. **2013;33:1739-1746**

MicroRNA therapeutics: towards a new era for the management of cancer and other diseases

Rajesha Rupaimoole and Frank J. Slack

Abstract | In just over two decades since the discovery of the first microRNA (miRNA), the field of miRNA biology has expanded considerably. Insights into the roles of miRNAs in development and disease, particularly in cancer, have made miRNAs attractive tools and targets for novel therapeutic approaches. Functional studies have confirmed that miRNA dysregulation is causal in many cases of cancer, with miRNAs acting as tumour suppressors or oncogenes (oncomiRs), and miRNA mimics and molecules targeted at miRNAs (antimiRs) have shown promise in preclinical development. Several miRNA-targeted therapeutics have reached clinical development, including a mimic of the tumour suppressor miRNA miR-34, which reached phase I clinical trials for treating cancer, and antimiRs targeted at miR-122, which reached phase II trials for treating hepatitis. In this article, we describe recent advances in our understanding of miRNAs in cancer and in other diseases and provide an overview of current miRNA therapeutics in the clinic. We also discuss the challenge of identifying the most efficacious therapeutic candidates and provide a perspective on achieving safe and targeted delivery of miRNA therapeutics.

The Therapeutic Potential of microRNAs

A novel mechanism of action, the ability to function as master regulators of the genome and an apparent lack of adverse events in normal tissue make microRNA a promising technology for current and future therapeutic development.

Table 1: miRNAs in therapeutic development

miRNA	Indication	Status of development
miRNA antagonists		
miR-122	Hepatitis C virus	Phase 2 clinical trials
miR-208/499	Chronic heart failure	Preclinical development
miR-195	Post-myocardial infarction remodelling	Preclinical development
miRNA replacement		
miR-34	Cancer	Preclinical development
let-7	Cancer	Preclinical development

miRna THERAPEUTICS

pioneering **microRNA Replacement Therapy**

MRX34 is a first-in-class cancer therapy and the first microRNA mimic to enter clinical trials.

Mirna has secured an exclusive license from Marina Biotech, Inc. to the patent estate covering the SMARTICLES® liposomal delivery technology for several of our lead microRNA product candidates, including miR-34, let-7 and two other undisclosed targets. The SMARTICLES formulation offers key efficacy and safety benefits, including the ability to deliver high numbers of microRNA mimic molecules to cancers cells in the liver, spleen and other highly vascularized tissues, as well as bone marrow and malignant lymphocytes.

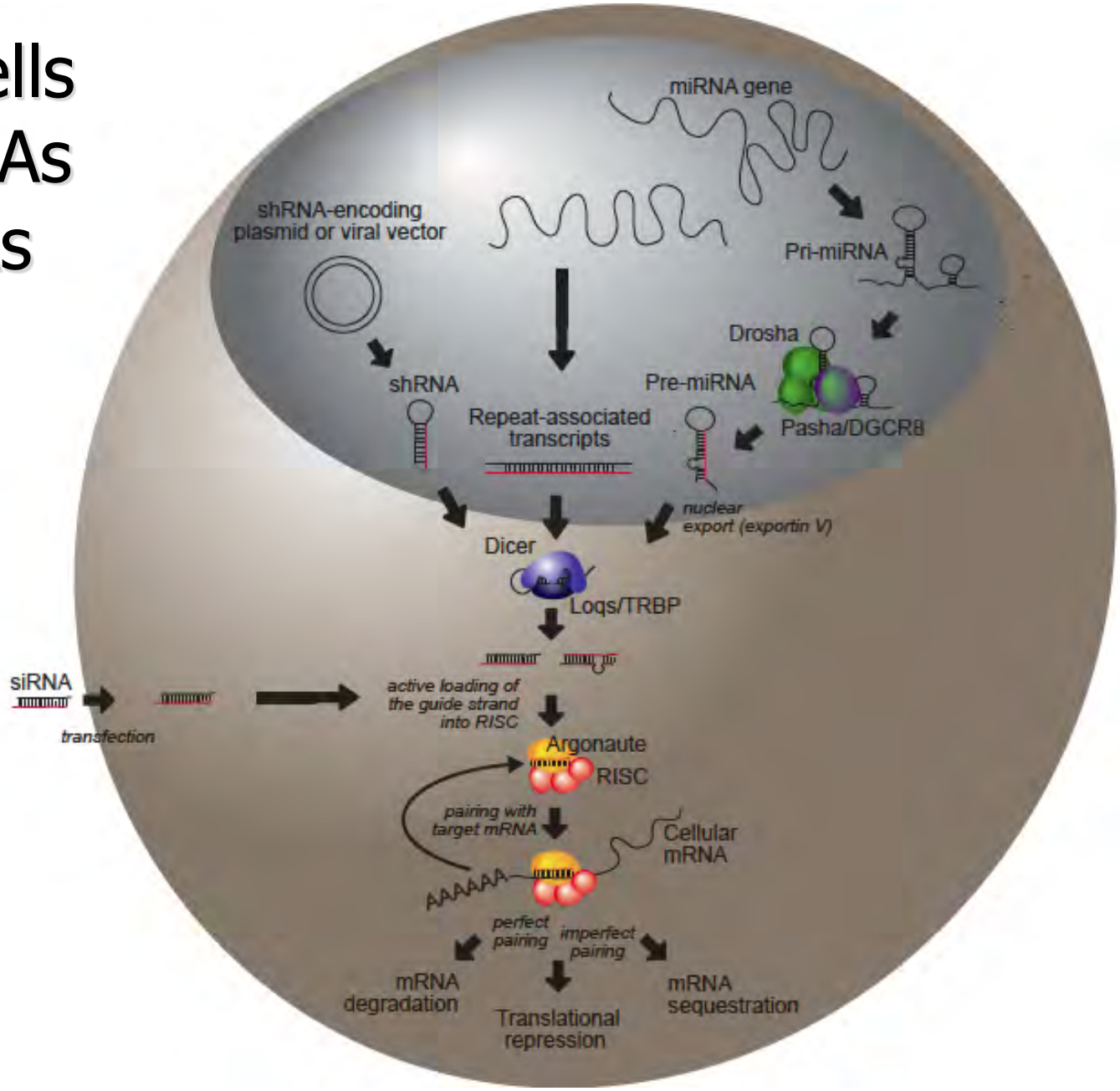
Program	Key Oncogenic Targets	Indication	Discovery	In Vivo Formulation	Preclinical	Phase 1	Phase 2
MRX34 miR-34 mimic	BCL2, E2F3, HDAC1, MET, MEK1, CDK4/6, PDGFR-α, WNT1/3, NOTCH-1	Primary liver cancer & solid cancers with liver metastases				●	
		Hematological malignancies				●	
miR-Rxlet-7 let-7 mimic	RAS, MYC, HMGA2, TGFBR1, MYCN, Cyclin D2, IL6, ITGB3				●		
miR-Rx06	UNDISCLOSED				●		
miR-Rx07	UNDISCLOSED				●		
miR-Rx16 miR-16 mimic	BCL2, VEGF-A, Cyclin-D1, HMGA1, FGFR1, CDK6, BMI1				●		

Table 2 | **Selected list of miRNA therapeutics in clinical trials**

Name (company)	Therapeutic agent	Delivery system	Target diseases	Trial details	ClinicalTrials.gov identifier
miRNA-based therapeutics					
Mirvirasen (Santaris Pharma A/S and Hoffmann-La Roche)	AntimiR-122	LNA-modified antisense inhibitor	Hepatitis C (chronic infections included)	Single-centre phase I, completed	NCT01646489
				Multicentre phase II, completed	NCT01200420
				Multicentre phase II, ongoing	NCT01872936
				Single-centre phase II, ongoing	NCT02031133
				Single-centre phase II, ongoing	NCT02508090
RG-101 (Regulus Therapeutics)	AntimiR-122	GalNAc-conjugated antimiR	Chronic hepatitis C	Phase I, completed	–
				Multiple phase II, ongoing	–
RG-125/AZD4076 (Regulus Therapeutics)	AntimiR-103/107	GalNAc-conjugated antimiR	Patients with type 2 diabetes and non-alcoholic fatty liver diseases	Single-centre phase I, ongoing	NCT02612662
				Single-centre phase I/IIa, ongoing	NCT02826525
MRG-106 (miRagen Therapeutics)	AntimiR-155	LNA-modified antisense inhibitor	Cutaneous T cell lymphoma and mycosis fungoides	Multicentre phase I, ongoing	NCT02580552
MRG-201 (miRagen Therapeutics)	miR-29 mimic	Cholesterol-conjugated miRNA duplex	Scleroderma	Single-centre phase I, ongoing	NCT02603224
MesomiR-1 (EnGeneIC)	miR-16 mimic	EnGeneIC delivery vehicle	Mesothelioma, non-small cell lung cancer	Multi-centre Phase I, ongoing	NCT02369198
MRX34 (Mirna Therapeutics)	miR-34 mimic	LNPs (Smarticles)	Multiple solid tumours	Multicentre phase I, terminated	NCT01829971

DOPC, 1,2 dioleoyl-sn glycero-3 phosphatidylcholine; eIF, eukaryotic initiation factor; GalNAc, N-acetyl-D-galactosamine; HBV, hepatitis B virus; LNA, locked nucleic acid; LNPs, lipid nanoparticles; miRNA, microRNA; PEI, polyethylenimine; RSV, respiratory syncytial virus.

RNAi in mammalian cells works by siRNAs and by miRNAs



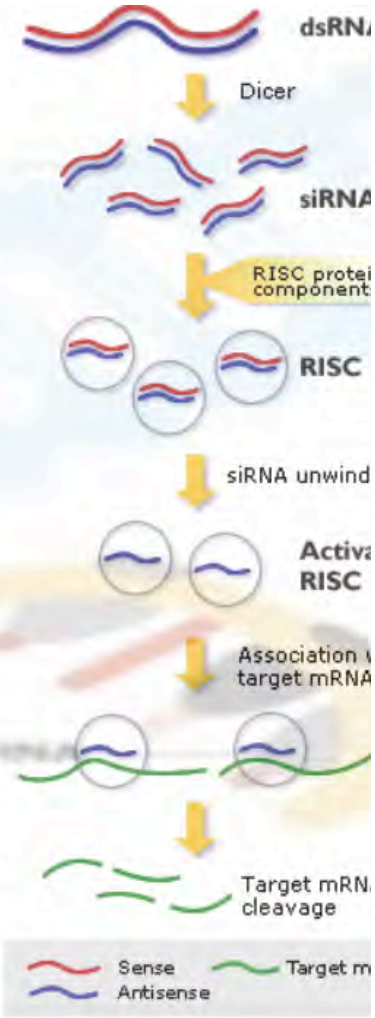
RNAi is controlled by **RISC** and is initiated by short dsRNA molecules in a cell's cytoplasm, where they interact with the catalytic RISC component argonaute.

dsRNAs is cleaved by the **Dicer enzyme** into short fragments of ~20 nucleotides that are called **siRNAs**.

Each siRNA is unwound into two single-stranded (ss) ssRNAs (**passenger** strand and the **guide** strand).

The passenger strand is degraded (red), and **the guide strand (blue) is incorporated into the RNA-induced silencing complex (RISC).**

The most well-studied outcome is post-transcriptional gene silencing, which occurs when the guide strand base pairs with a complementary sequence in a messenger RNA molecule (green) and induces **cleavage by Argonaute**, the catalytic component of the RISC complex.



siRNA

Long double-stranded siRNA precursors (pre-siRNAs)

siRNA duplexes

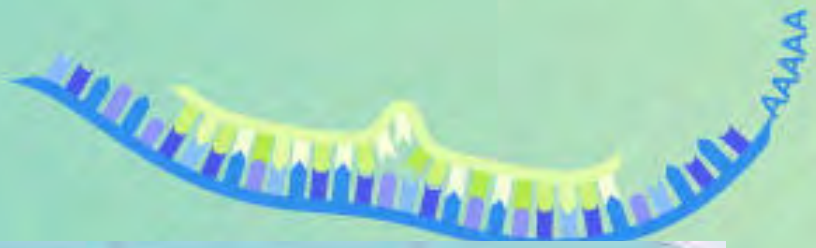
Perfect siRNA match with mRNA targets

miRNA

Short hairpin miRNA precursors (pre-miRNAs)

miRNA duplex

Partial miRNA mismatch with mRNA targets



	Length	Where Found?	Target Recog	Mechanism
miRNA	19-25 nt	Endogenous	Imperfect Match	Translational Repression
siRNA	19-21 nt	Exogenous	Exact Match	mRNA Cleavage

miRNAs typically inhibit the translation of many different mRNAs with similar sequences. In contrast, siRNAs typically inhibit only a single, specific target.

Table 1
Composition and encapsulation efficacy of siRNA delivery systems.

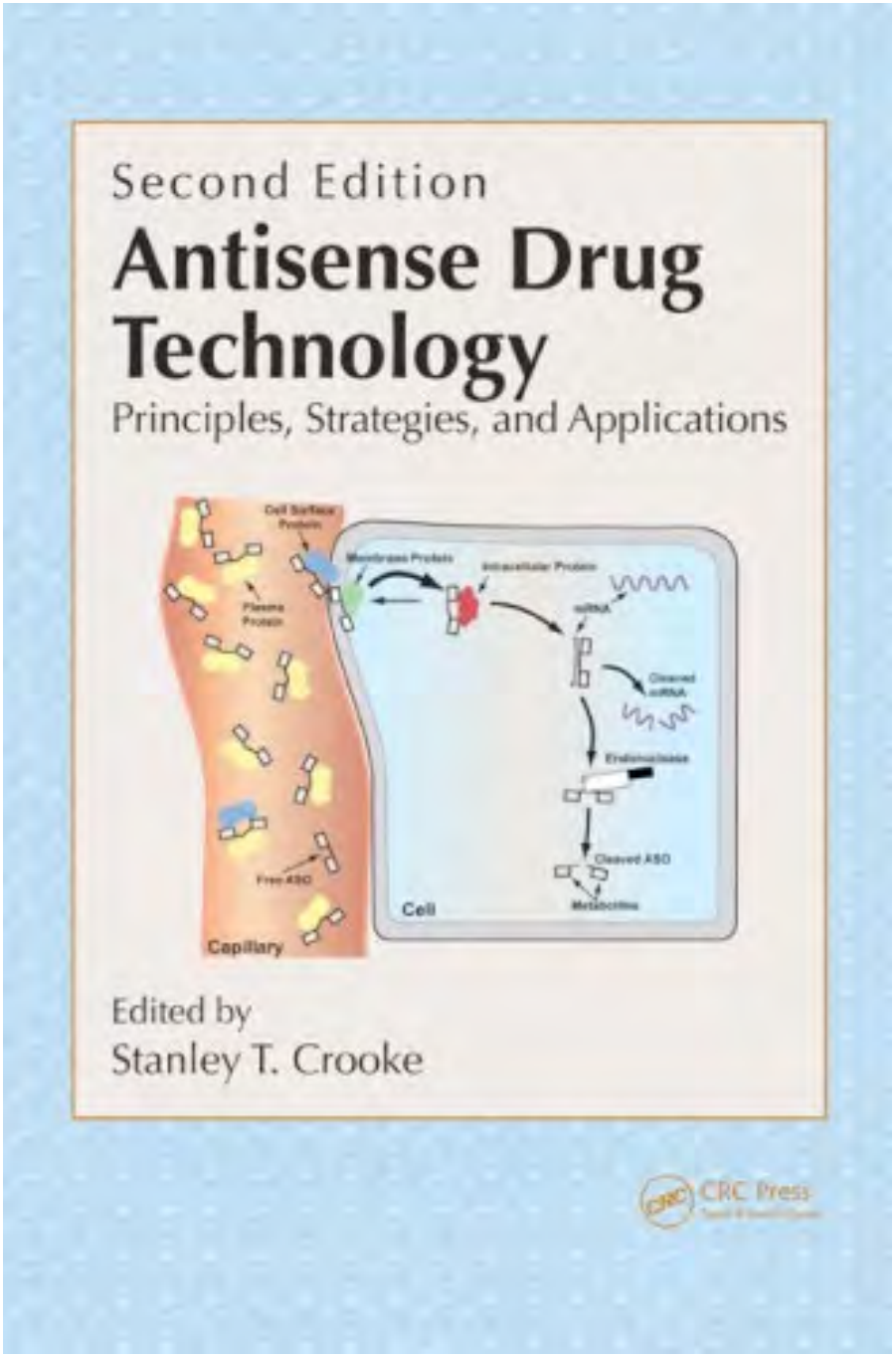
Type	Encapsulation	Composition	Encapsulation yield (%)	Refs.
Liposome	Entrapment	DC–Chol, DOPE, DSPE–PEG, F'ab Her2	80	[10]
	Entrapment	DC-6–14, POPC, Chol, DOPE, DSPE–PEG	90	[9]
	Entrapment	DOTAP, DOPE, Chol, PEG–C16Ceramide	90	[7]
	Entrapment	DNA, protamine, DOTAP, Chol, DSPE–PEG	95	[8]
Nanoparticle	Entrapment	PEG, lecithin, triglycerides, DOTAP, DOPE	65	[13]
	Entrapment/matrix	BHEM–Chol, mPEG–PLA	90	[18]
	Entrapment	Apolipoprotein A-I, Chol, CE, PC, Oligolysine	>90	[15]
	Entrapment	Dextran thiol, Dextran stearylamine, PEG-thiol	95	[16]
	Adsorption	Chitosan, Plurol, Ammonium nitrate, PIBCA	90	[17]
	Matrix	CaP, DOTAP, Chol and DSPE–PEG,	40	[11]
	Matrix	CaP, PEG–Pasp(DET–Aco)	90	[12]
	Matrix	PEI, Tripalmitin, DSPE–PEG–folate, Chol, Lecithin	85	[14]
Complex	Matrix	PEI, Glycol chitosan polymer	100	[19]
	Matrix	PEI, HSA	100	[20]
Micelle	Adsorption	poly(DMAEMA), mAB-SA	100	[21]
	Adsorption	PEG-SS-Pasp (DET)	100	[22]
	Adsorption	Acetal-PEO-b-PCCL	>90	[23]

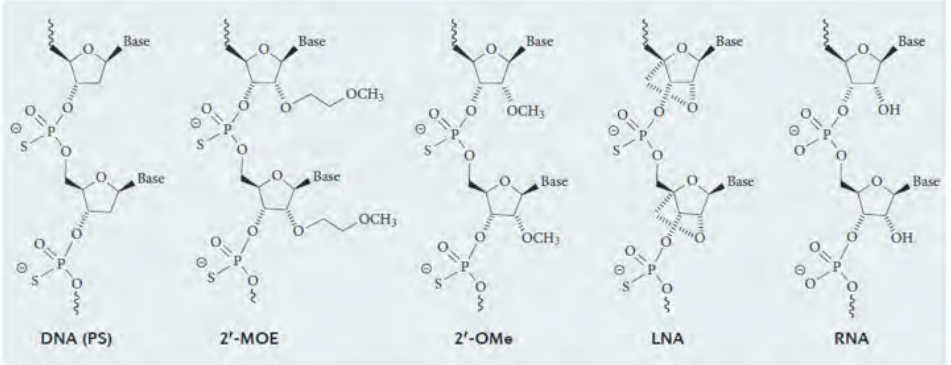
Abbreviations, DC–Chol: 3β-[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol; DOPE: dioleoylphosphatidylethanolamine; DSPE–PEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine methoxy (polyethyleneglycol)-2000; F'ab Her2: antibody fragment of human endothelium growth factor receptor; DC-6–14: O,O'-ditetradecanoyl-N-(α-trimethyl ammonio acetyl) diethanolamine chloride; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Chol: cholesterol; DOTAP: 1,2-DiOleoyl-3-TrimethylAmmonium-Propane; PEG–C16Ceramide: polyethylene glycol–C16–ceramide; PEG: polyethylene glycol; BHEM–Chol: N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide; mPEG–PLA: poly(ethylene-glycol)-b-poly(D,L-lactide); CE: cholesteryl oleate; PC: phosphatidylcholine; PEG-thiol: polyethylene glycol-thiol; PIBCA: poly(iso-butyl-cyanoacrylate); CaP: calcium phosphate; PEG–Pasp(DET): poly(ethylene glycol)-SS-poly[N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide]; PEI: polyethyleneimine; HSA: human serum albumin; poly(DMAEMA): poly(2-(dimethylamino)ethyl methacrylate; mAB-SA: streptavidin-conjugated monoclonal antibody against CD22; acetal-PEO-b-PCCL: acetal-poly(ethylene oxide)-block-poly(ε-caprolactone).

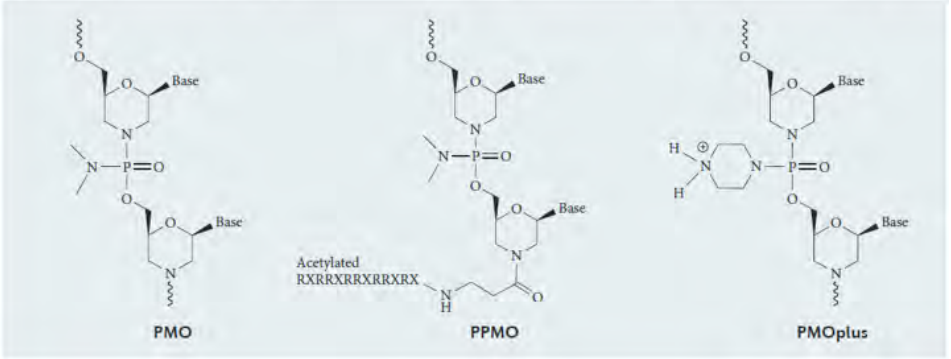
siRNA/shRNA Therapeutics in Clinical Trials

Disease	Stage	RNAi reagent	Delivery	Company/institution
Ocular diseases				
AMD	Preclinical stage	siRNA	Direct intravitreal injection	Quark Biotech
	Clinical trial phase I	siRNA	Direct intravitreal injection	Sirna
	Clinical trial phase II	siRNA	Direct intravitreal injection	Acuity
Viral infections				
Hepatitis B and C	Preclinical stage	shRNA	Liganded nanoparticle	Nucleonics/Intradigm
RSV	Clinical trial phase I	siRNA	Aerosol	Alnylam
HIV	Clinical trial phase I (scheduled for 2007)	shRNA	Lentivirus	Benitec/City of Hope
Cancer				
Hepatic cancer	Preclinical stage	siRNA	Liganded nanoparticle	Calando
Solid tumour cancers	Preclinical stage	siRNA	Liganded nanoparticle	Intradigm
Other disease types				
ALS	Preclinical stage	siRNA	N/A	CytRx
Inflammatory diseases	Preclinical stage	siRNA	Peptide	Nastech

ALS, amyotrophic lateral sclerosis; AMD, age-related macular degeneration; RNAi, RNA interference; RSV, respiratory syncytial virus; shRNA, short hairpin RNA; siRNA, small interfering RNA.







RNA therapeutics: beyond RNA interference and antisense oligonucleotides

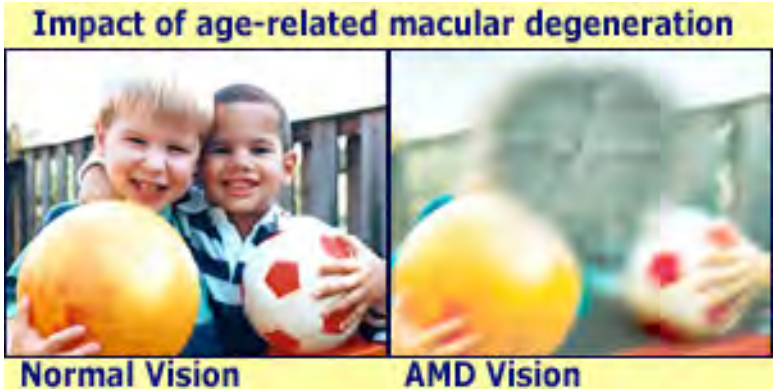
Ryszard Kole[1], Adrian R. Krainer[2] and Sidney Altman[3]

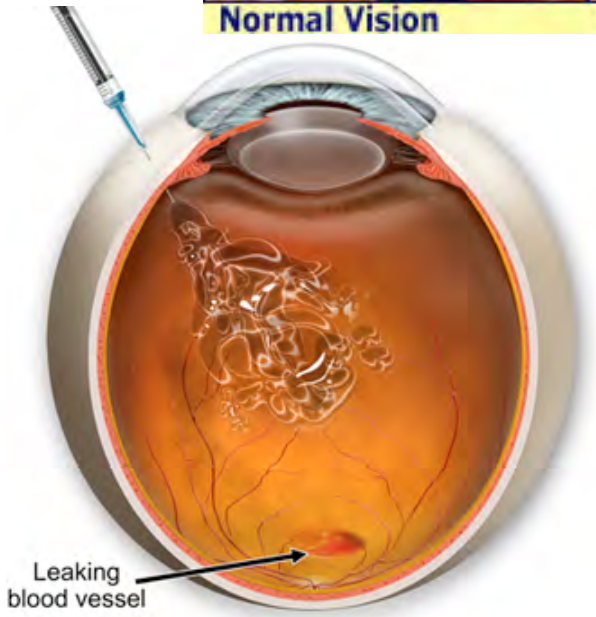
Abstract | Here, we discuss three RNA-based therapeutic technologies exploiting various oligonucleotides that bind to RNA by base pairing in a sequence-specific manner yet have different mechanisms of action and effects. RNA interference and antisense oligonucleotides downregulate gene expression by inducing enzyme-dependent degradation of targeted mRNA. Steric-blocking oligonucleotides block the access of cellular machinery to pre-mRNA and mRNA without degrading the RNA. Through this mechanism, steric-blocking oligonucleotides can redirect alternative splicing, repair defective RNA, restore protein production or downregulate gene expression. Moreover, they can be extensively chemically modified to acquire more drug-like properties. The ability of RNA-blocking oligonucleotides to restore gene function makes them best suited for the treatment of genetic disorders. Positive results from clinical trials for the treatment of Duchenne muscular dystrophy show that this technology is close to achieving its clinical potential.

Anti-VEGF for wet AMD

Macugen (Pegaptanib sodium - pegylated aptamer that binds VEGF165)

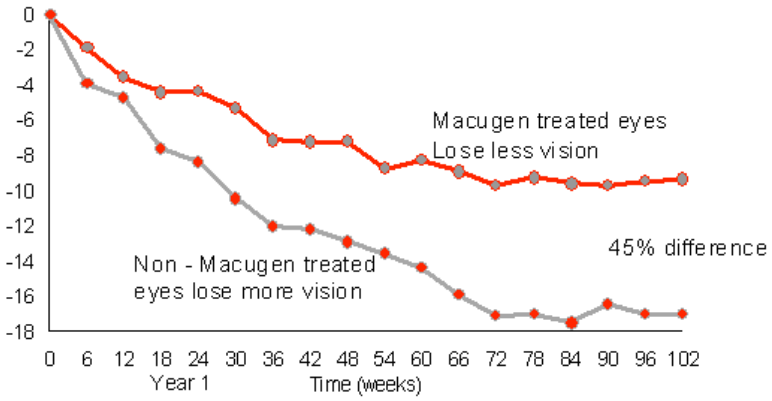
Lucentis (ranibizumab) - recombinant humanized Fab that binds all VEGF isoforms





Intravitreal injection into the back of the eye

Macugen Treatment
Average change in vision over 2 years



MACUGEN®
(pegaptanib sodium injection)

HIGHLIGHTS OF PRESCRIBING INFORMATION
These highlights do not include all of the information needed to use MACUGEN safely and effectively. See full prescribing information for MACUGEN.

MACUGEN® (pegaptanib sodium injection)
Intravitreal Injection
Initial U.S. Approval: 2004

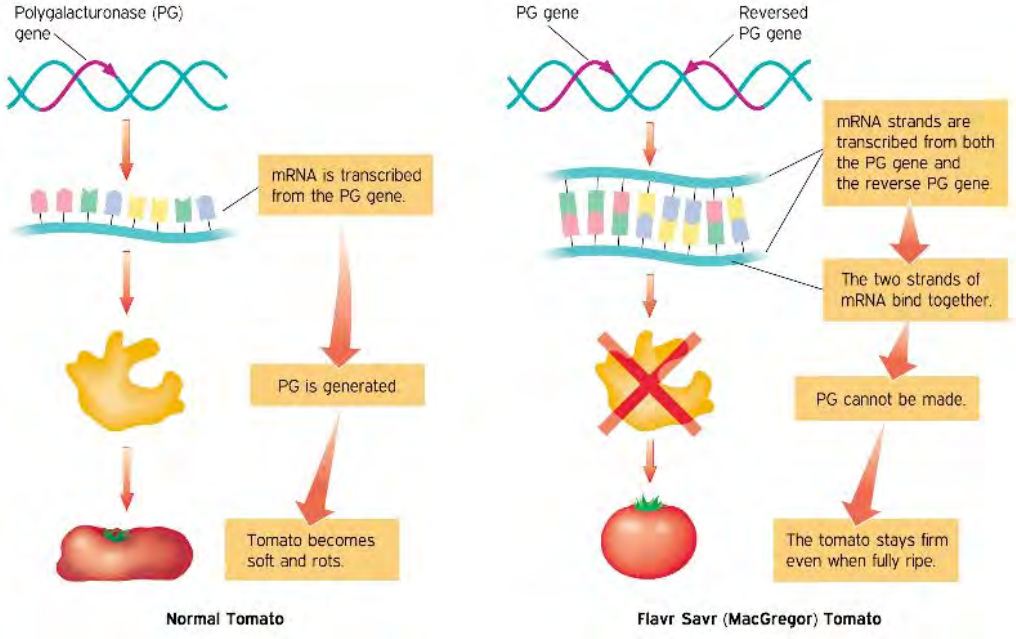
INDICATIONS AND USAGE
MACUGEN is indicated for the treatment of neovascular (wet) age-related macular degeneration. (1)

DOSAGE AND ADMINISTRATION
- FOR OPHTHALMIC INTRAVITREAL INJECTION ONLY. (2.1)
- MACUGEN 0.3 mg should be administered once every six weeks by intravitreous injection into the eye to be treated. (2.2)

DOSAGE FORMS AND STRENGTHS
- 0.3 mg/90 µL solution in a single-use syringe for intravitreal injection. (3)

CONTRAINDICATIONS
- Ocular or periocular infections. (4.1)
- Hypersensitivity. (4.2)

Longer lasting tomatoes by RNA antisense technology



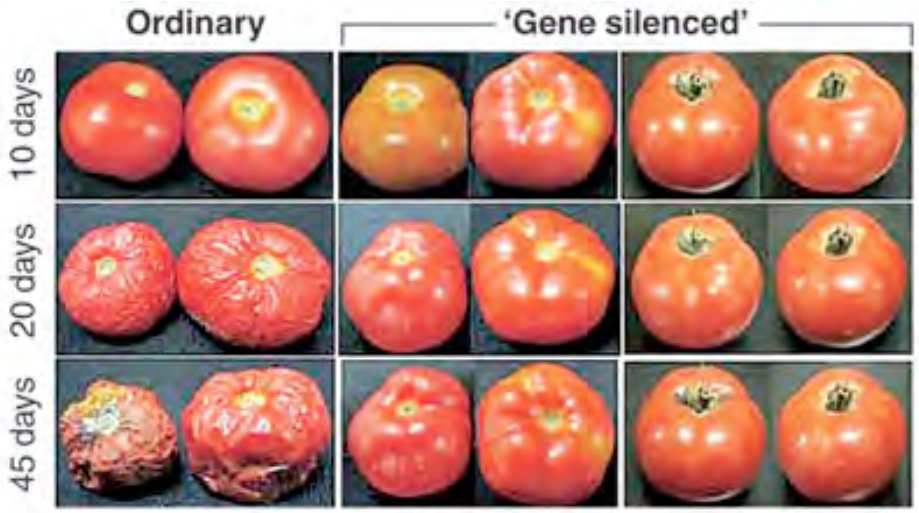
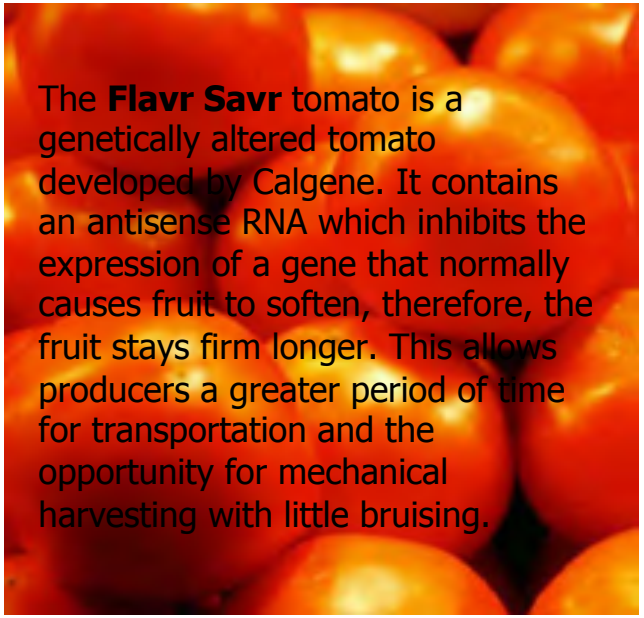


Image shows three sets of tomatoes. The ordinary control tomatoes (extreme left) soften and shrivel up, while texture of gene-silenced tomatoes remains intact for up to 45 days.

Photo credit: Asis Datta, Subhra Chakraborty, National Institute of Plant Genome Research, New Delhi



The **Flavr Savr** tomato is a genetically altered tomato developed by Calgene. It contains an antisense RNA which inhibits the expression of a gene that normally causes fruit to soften, therefore, the fruit stays firm longer. This allows producers a greater period of time for transportation and the opportunity for mechanical harvesting with little bruising.

Come disegnare un siRNA in lab

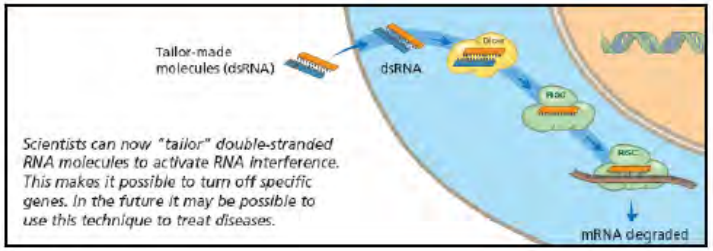
siRNA PROFILING TECHNOLOGIES

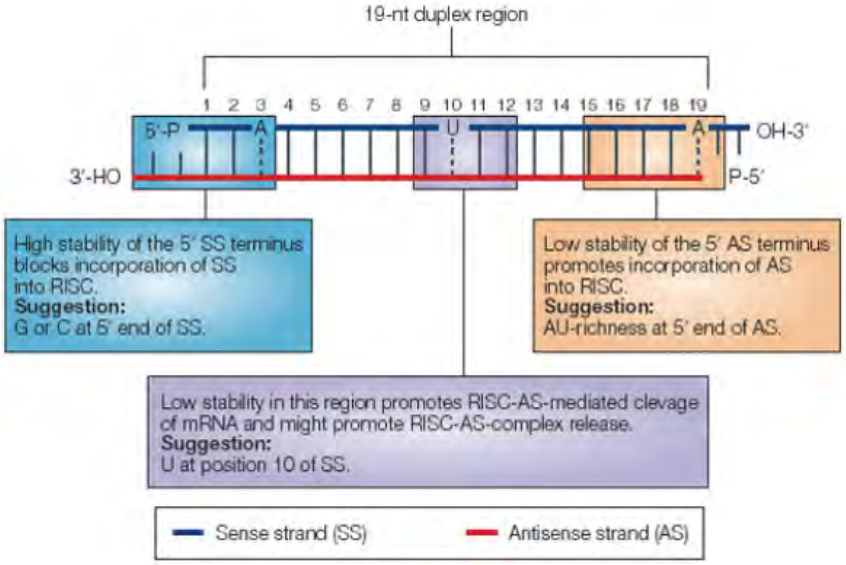
Disegno di siRNA

- **il siRNA possiede un gruppo UU al terminale 3'**
- **19 nucleotidi**
- **un contenuto in G/C < 50% è preferibile.**

Algoritmo di nuova generazione

- **Tm dell'siRNA**
- **Effetti della posizione nucleotidica**
- **Contenuto nucleotidico dei 3' overhangs**
- **Distribuzione nucleotidica**
- **Controllo della specificità**





Preventing Off-Target Effects

Overabundance of the siRNA activates the interferon pathway, as antiviral response

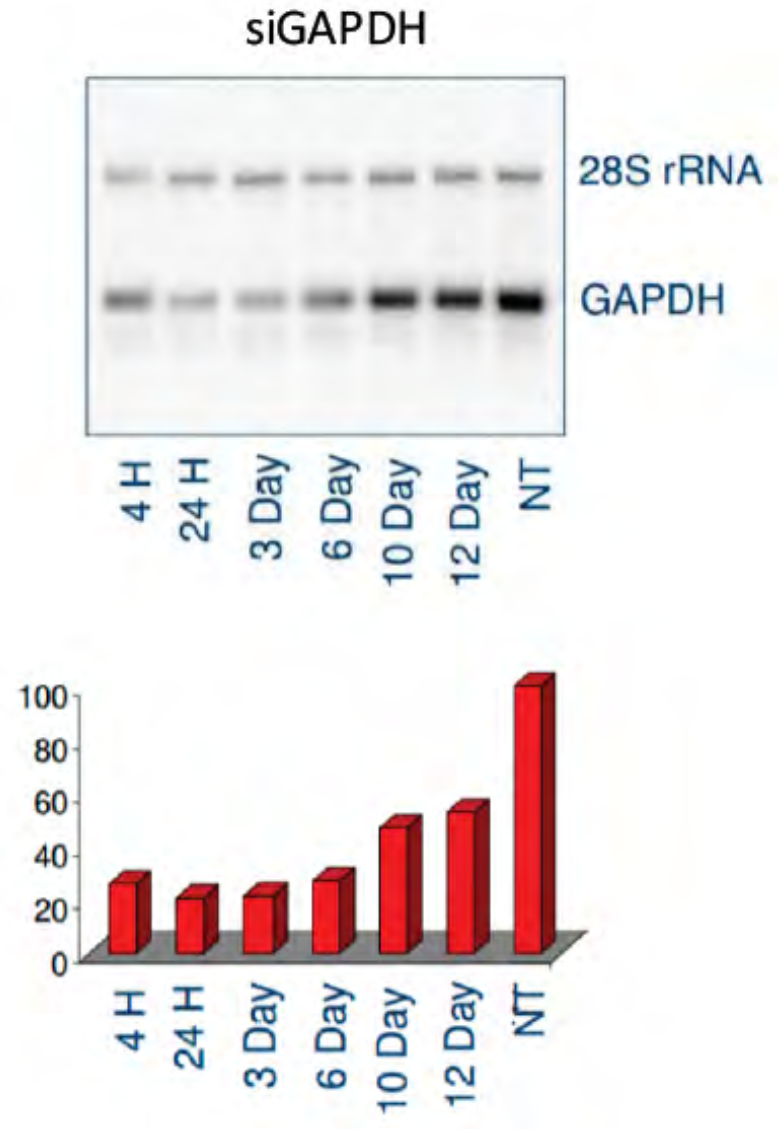
Low concentrations (~5-30nM) of single siRNA minimizes:

- **chances of off-target effect**
- **induction of interferon response**

It is currently preferable to use **ONE** highly potent siRNA than a **MIXTURE** of siRNAs that raise overall siRNA conc.

Verify specificity of RNAi effect by testing independent siRNAs to the same target

Durata del silenziamento transiente



PRO

- La trasfezione con siRNA è davvero molto efficiente in molti tipi di cellule
- Coi siRNA il silenziamento è immediato

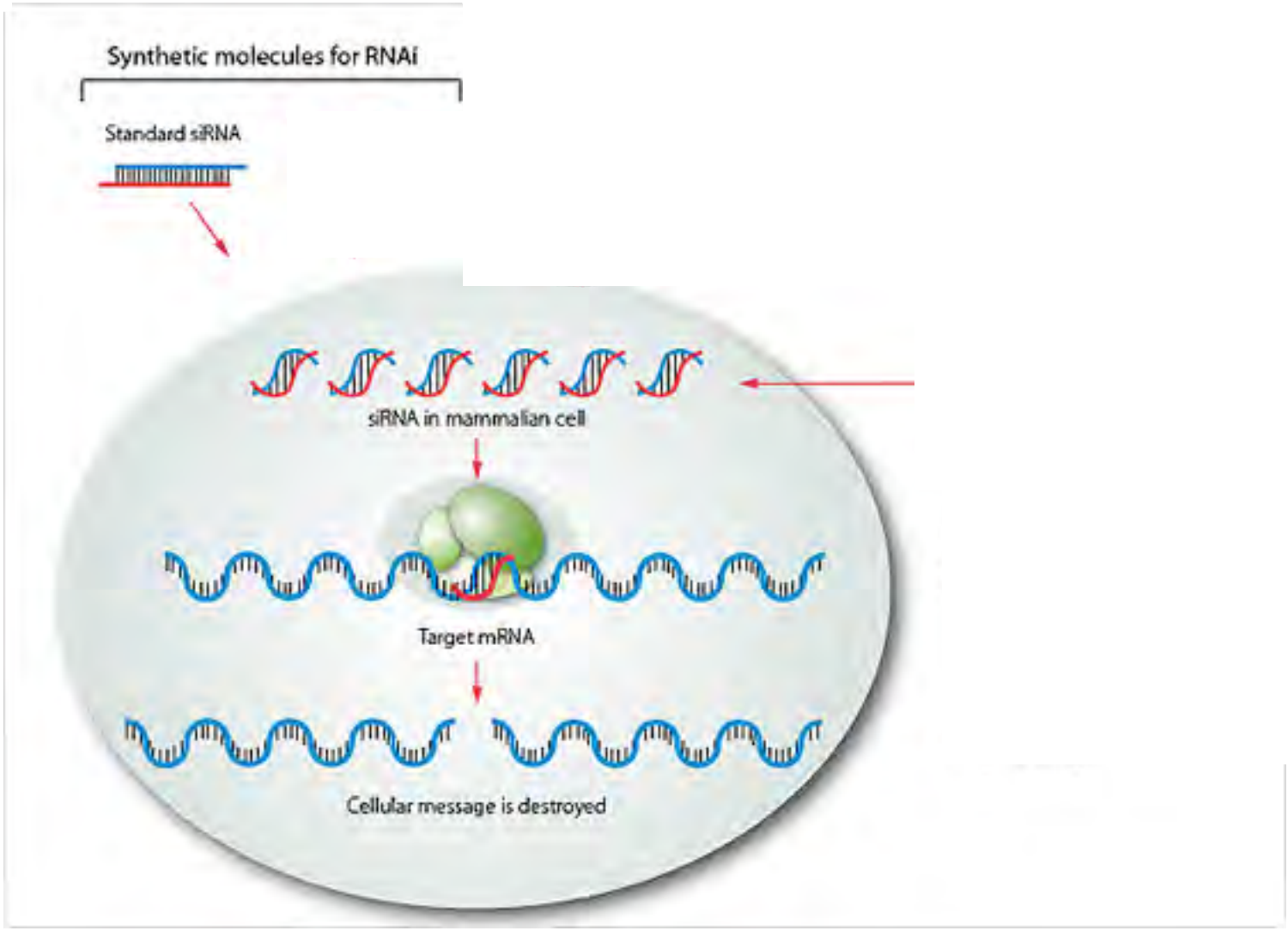
CONTRO

- Alcune cellule sono refrattarie alla trasfezione e la loro elettroporazione spesso causa morte cellulare
- I siRNA sono stabili, ma la trascrizione può risultare transiente se le cellule si duplicano molto in fretta diluendo il silenziamento e la vita media della proteina

Superamento del problema mediante……

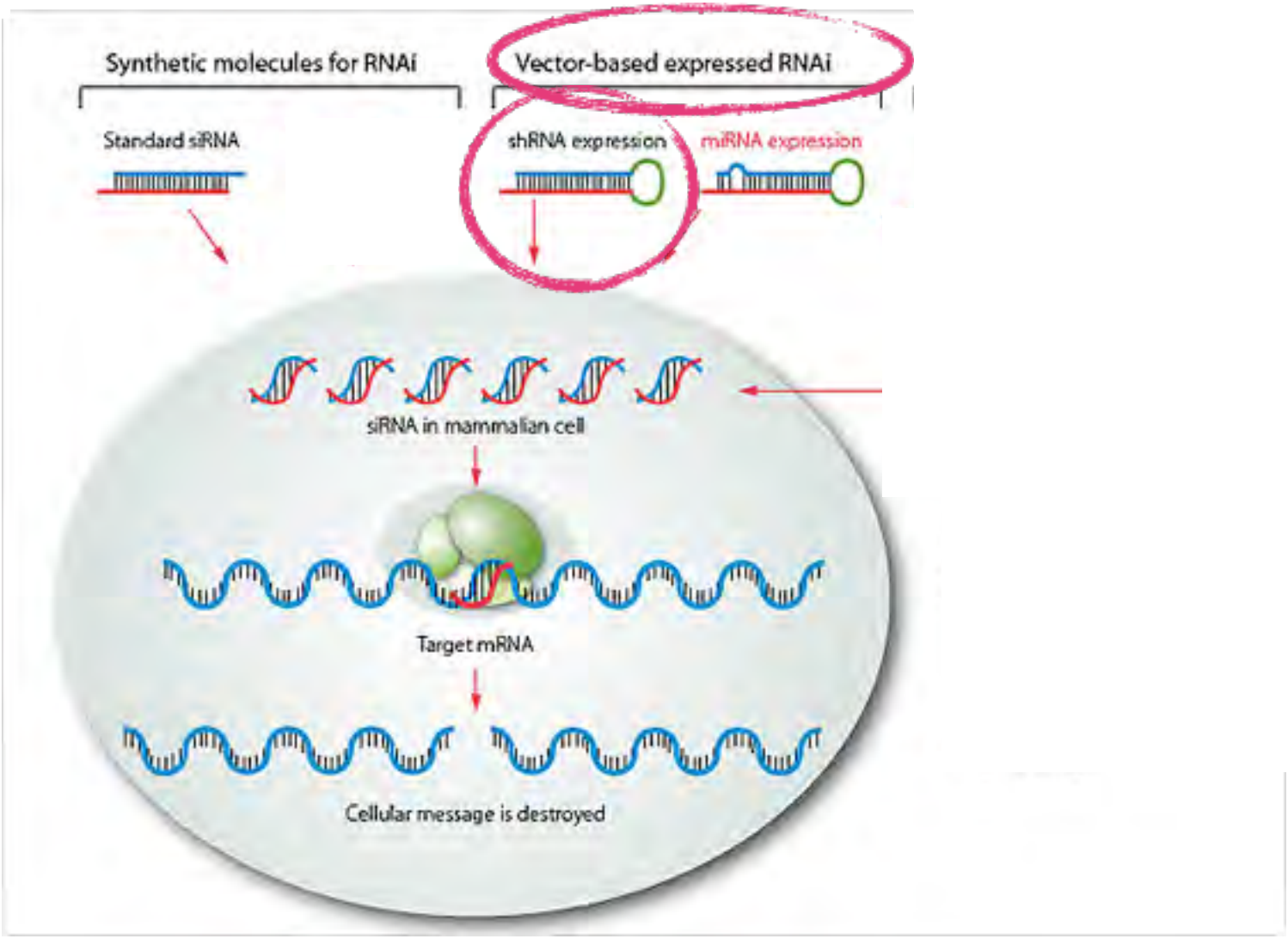
RNAi in mammalian cells - a precious tool for gene silencing

Persistent expression!



RNAi in mammalian cells - a precious tool for gene silencing

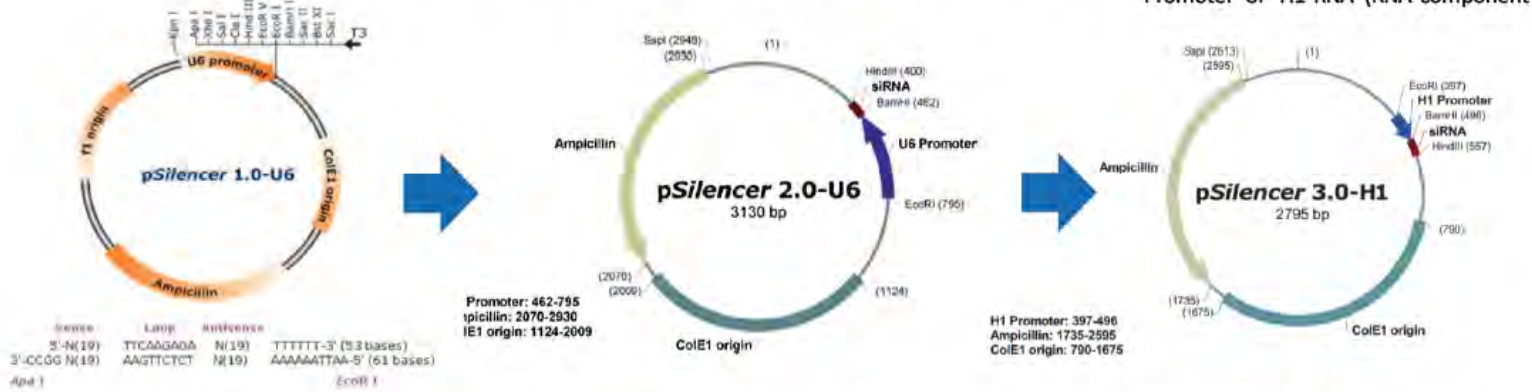
Persistent expression!



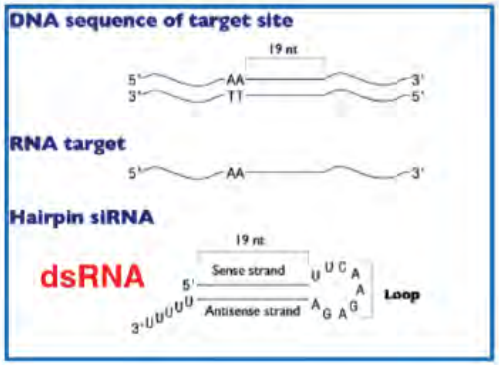
Dal transiente alla trasfezione con vettori

Silenziamento tramite vettori a DNA

Important:
Use RNA Pol III promoters (short RNA)
Promoter or U6 snRNA
Promoter of H1 RNA (RNA component of human RNase P)



Mimicking miRNA production



- Una sequenza stampo per un "hairpin siRNA" viene clonata in un opportuno vettore per trascrivere una molecola di RNA
- Produzione di siRNA *in vivo direttamente* nelle cellule trasfettate
- Trasfezione stabile nella linea cellulare di cui si vuol silenziare il gene target dell'RNAi
- **Silenziamento a lungo termine del gene target**

- **Clonati in vettori plasmidici** con promotori adatti per la produzione di RNA
- **Clonati in vettori virali**
  - **Oncoretrovirus**: MoMuLV o MSCV, le cellule devono duplicanti per poter essere infettate
  - **Lentivirus**: HIV-1, per infettare cellule quiescenti

Trasfezione con siRNA: le APPLICAZIONI

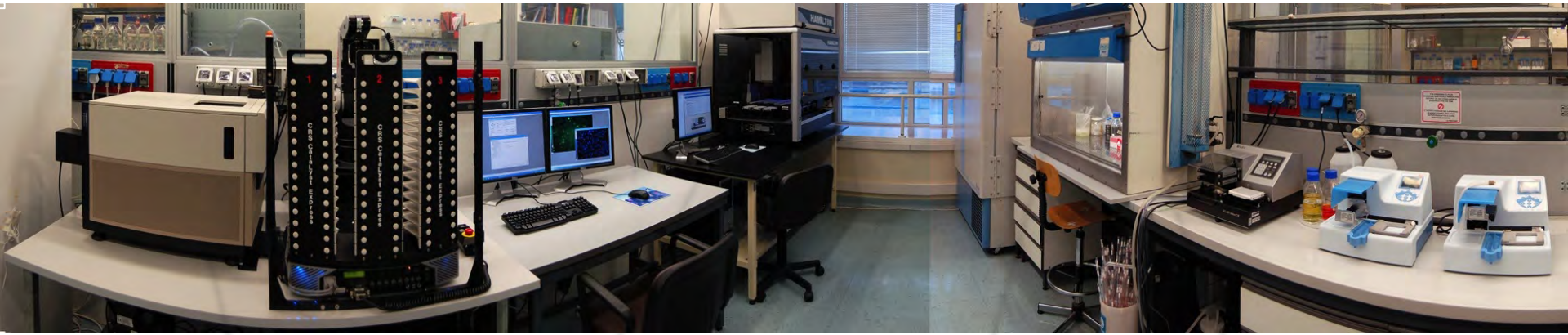
- Silenziamento genico specifico, efficiente e stabile nel tempo (economico e veloce)
- È un approccio di «genetica inversa»
- Screening delle funzioni genomiche (Genome-wide functional screenings)
- Terapia genica (es. antitumorale)
- Creazione di modelli per lo studio di agenti farmacologici (es. murini)
- Rivoluzione nello studio dei meccanismi di regolazione dell'espressione genica

Come possiamo studiare i microRNA?
(sono migliaia!!)

Come possiamo sapere l'effetto del silenziamento genico sulle cellule?

La tecnologia ci aiuta…

High Throughput Screening

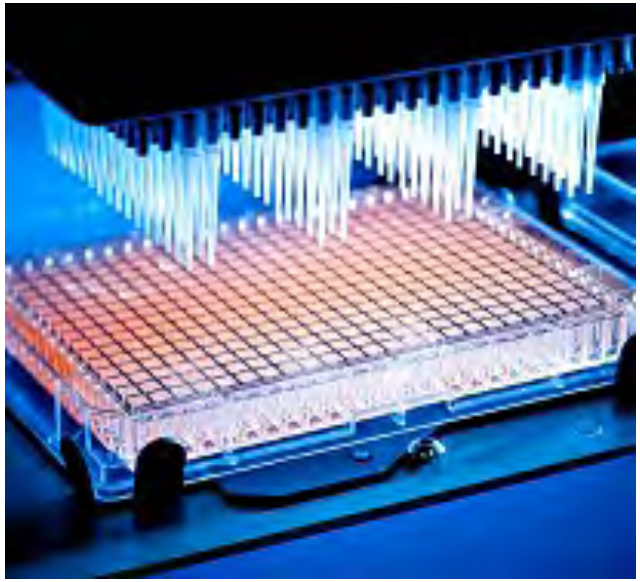


MICROSCOPIO

BRACCIO ROBOTIZZATO PER DISTRUBUZIONE REAGENTI

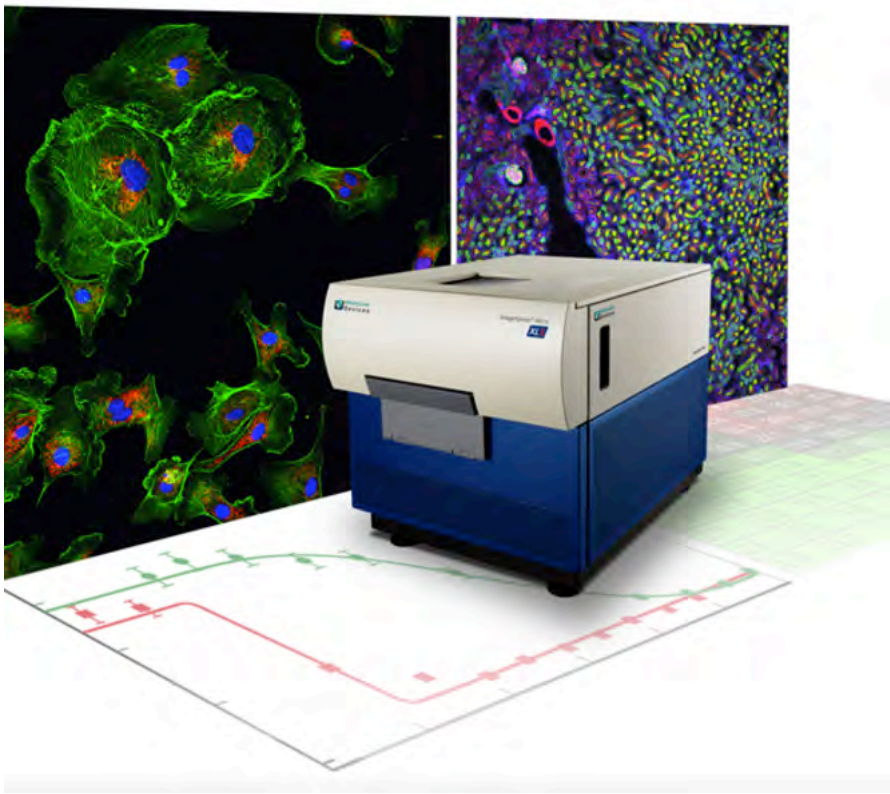
CAPPA CELLULE

DISPENSATORE CELLULE

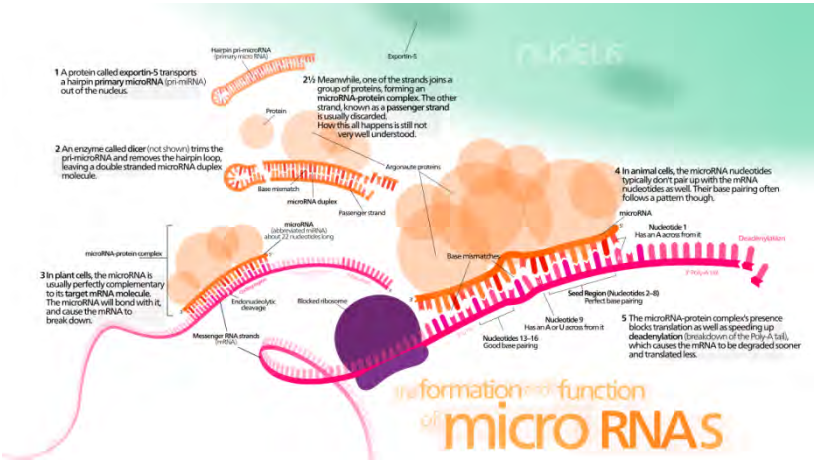


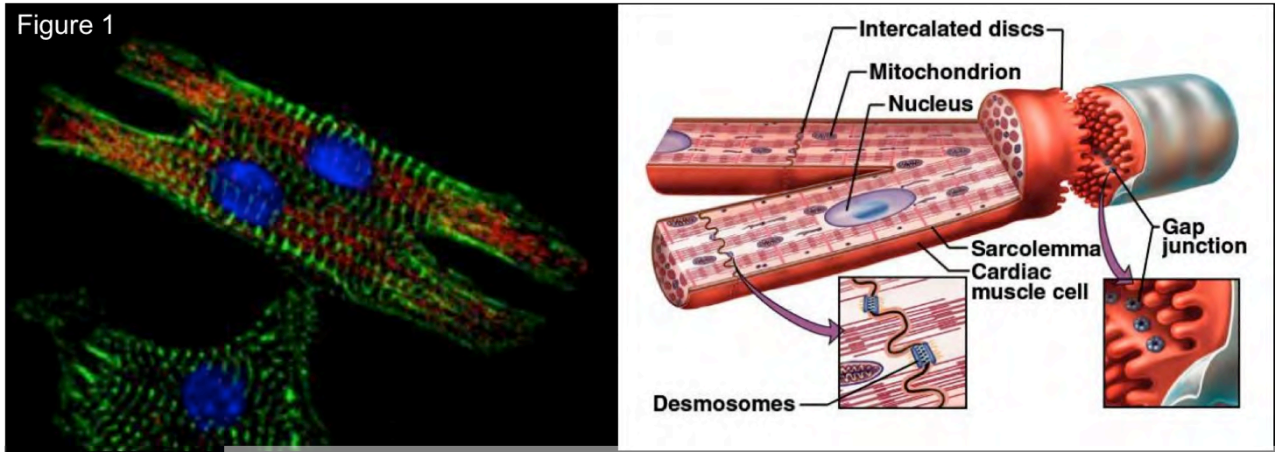
Il microscopio

- Acquisizione numerose immagini per ogni pozzetto
- Diversi tipi di obiettivi e ingrandimenti
- Posizionamento automatizzato
- High-throughput screening: capacità di acquisizione superiore ai 10 milioni di cellule/ora a bassa risoluzione e 1milione/ora ad alta risoluzione



E’ possibile riattivare la divisione cellulare dei cardiomiociti usando i miRNA?





Screening for cardiomyocyte proliferation using a library of microRNA mimics

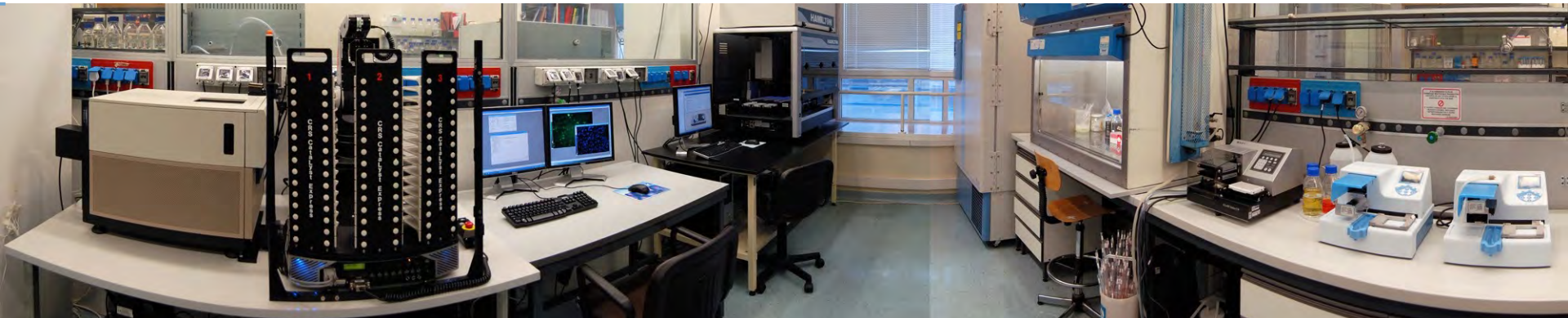
microRNA mimics
arrayed on 96-well plates
(988 mature sequences)

0h — add transfection reagent
add cardiomyocytes isolated from newborn rats

52h — add EdU

72h

cell fixation and fluorescence staining
(Hoechst, alpha-actinin, Ki-67 and EdU)



ARTICLE

doi:10.1038/nature11739

Functional screening identifies miRNAs inducing cardiac regeneration

Ana Eulalio[1]†, Miguel Mano[1], Matteo Dal Ferro[1,2], Lorena Zentilin[1], Gianfranco Sinagra[2], Serena Zacchigna[1] & Mauro Giacca[1]

In mammals, enlargement of the heart during embryonic development is primarily dependent on the increase in cardiomyocyte numbers. Shortly after birth, however, cardiomyocytes stop proliferating and further growth of the myocardium occurs through hypertrophic enlargement of the existing myocytes. As a consequence of the minimal renewal of cardiomyocytes during adult life, repair of cardiac damage through myocardial regeneration is very limited. Here we show that the exogenous administration of selected microRNAs (miRNAs) markedly stimulates cardiomyocyte proliferation and promotes cardiac repair. We performed a high-content microscopy, high-throughput functional screening for human miRNAs that promoted neonatal cardiomyocyte proliferation using a whole-genome miRNA library. Forty miRNAs strongly increased both DNA synthesis and cytokinesis in neonatal mouse and rat cardiomyocytes. Two of these miRNAs (hsa-miR-590 and hsa-miR-199a) were further selected for testing and were shown to promote cell cycle re-entry of adult cardiomyocytes *ex vivo* and to promote cardiomyocyte proliferation in both neonatal and adult animals. After myocardial infarction in mice, these miRNAs stimulated marked cardiac regeneration and almost complete recovery of cardiac functional parameters. The miRNAs identified hold great promise for the treatment of cardiac pathologies consequent to cardiomyocyte loss.

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Screening for cardiomyocyte proliferation by high content microscopy

original microscopy

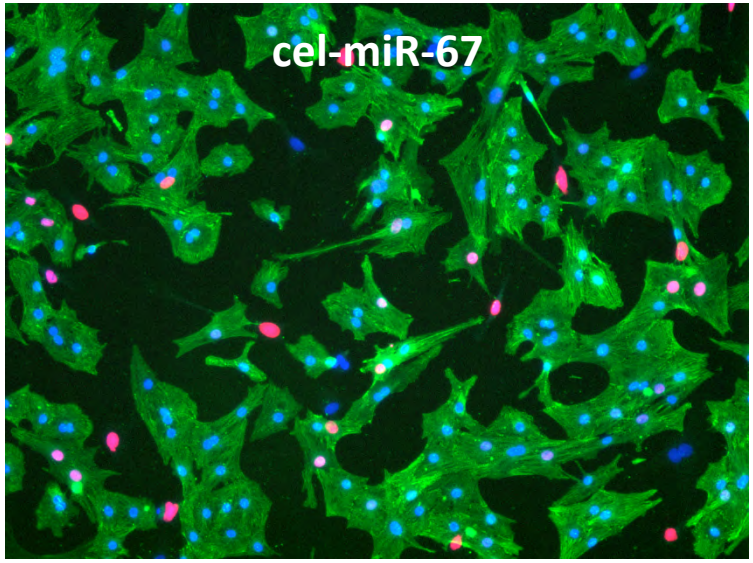
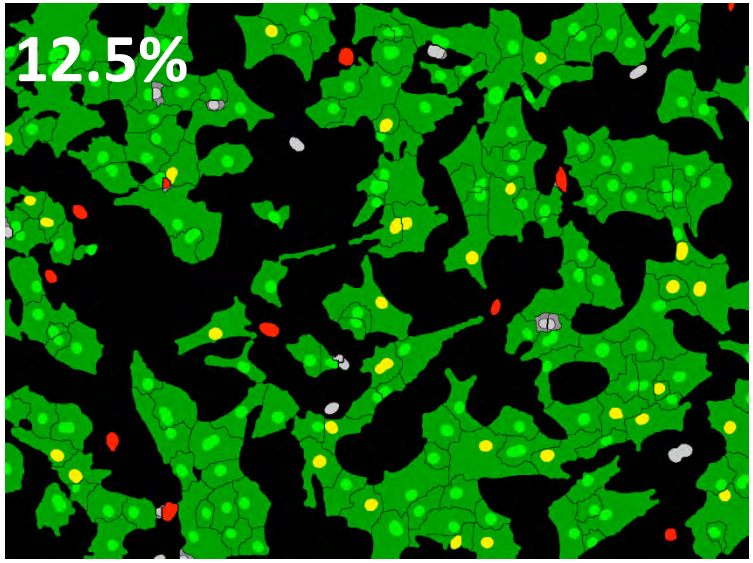


image analysis



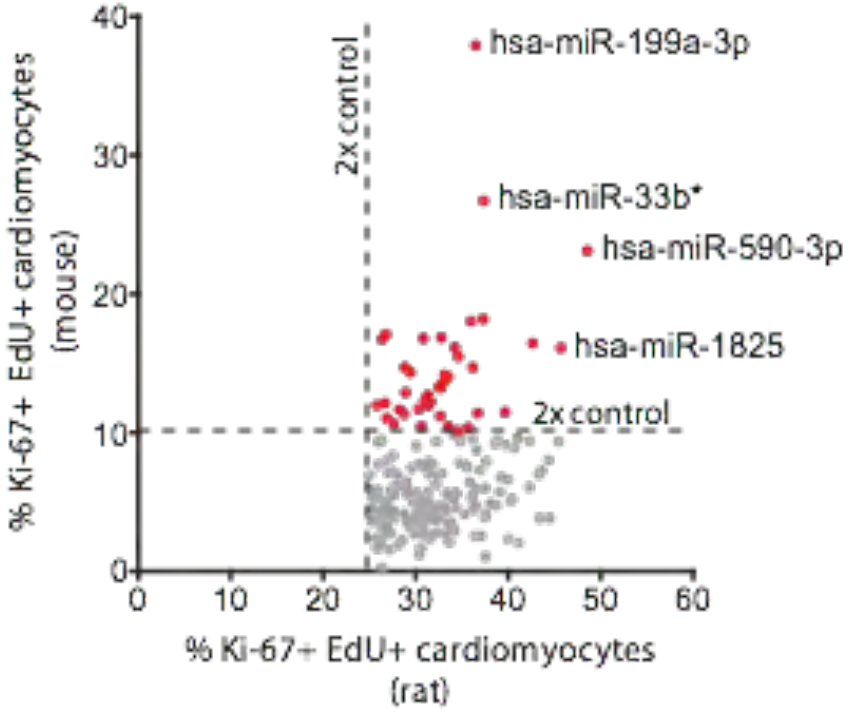
Hoechst – nucleus

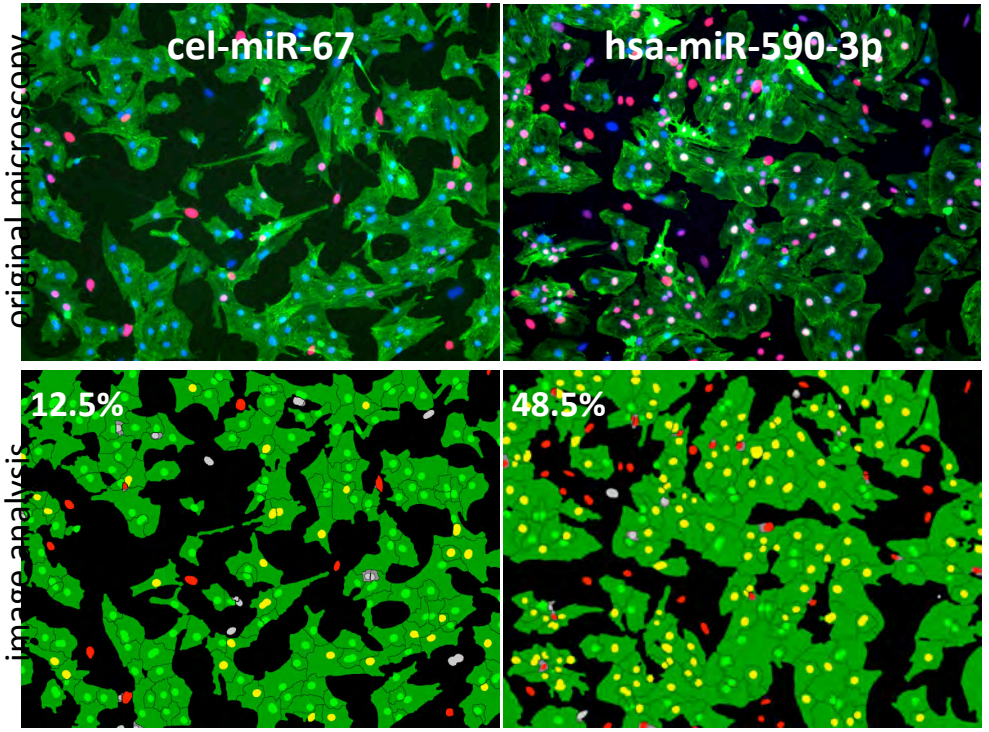
α-actinin – cardiomyocyte marker

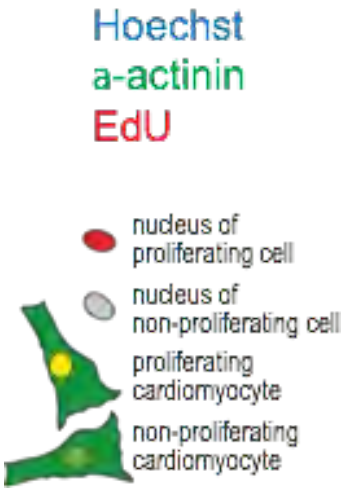
EdU – DNA synthesis

16 images per well - approx. 3000 cells analyzed per miRNA and replicate

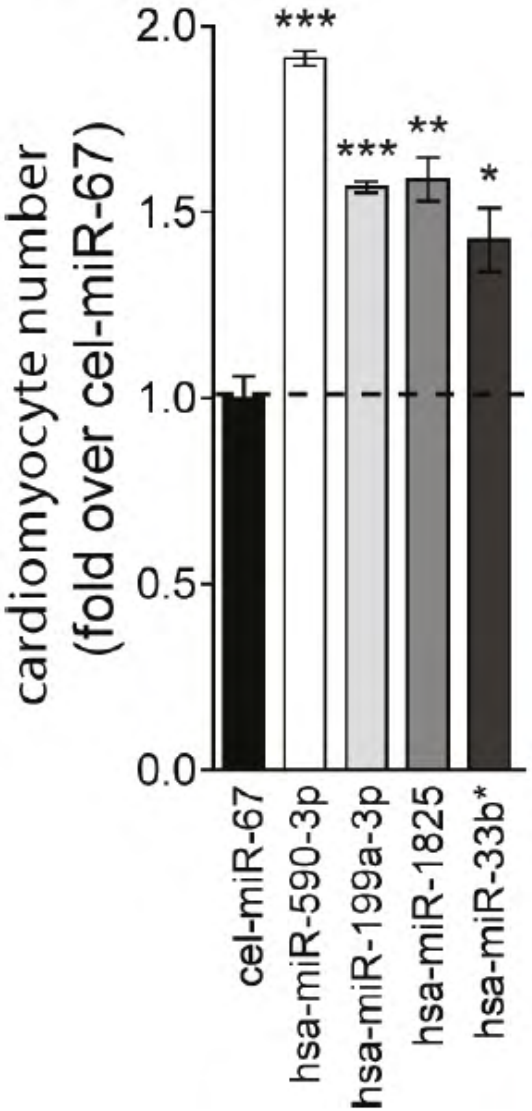
40 human miRNAs increase both rat and mouse cardiomyocyte proliferation

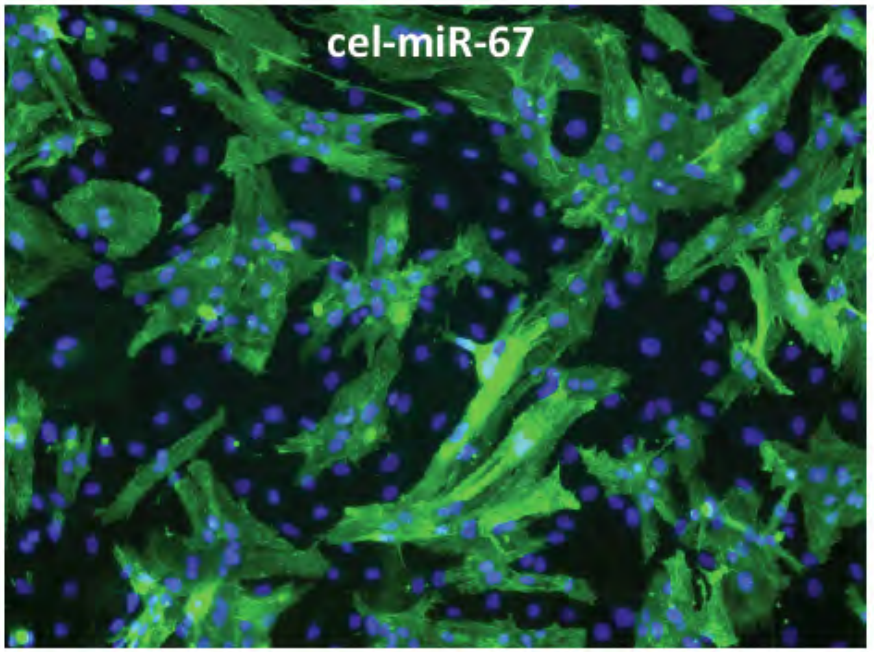


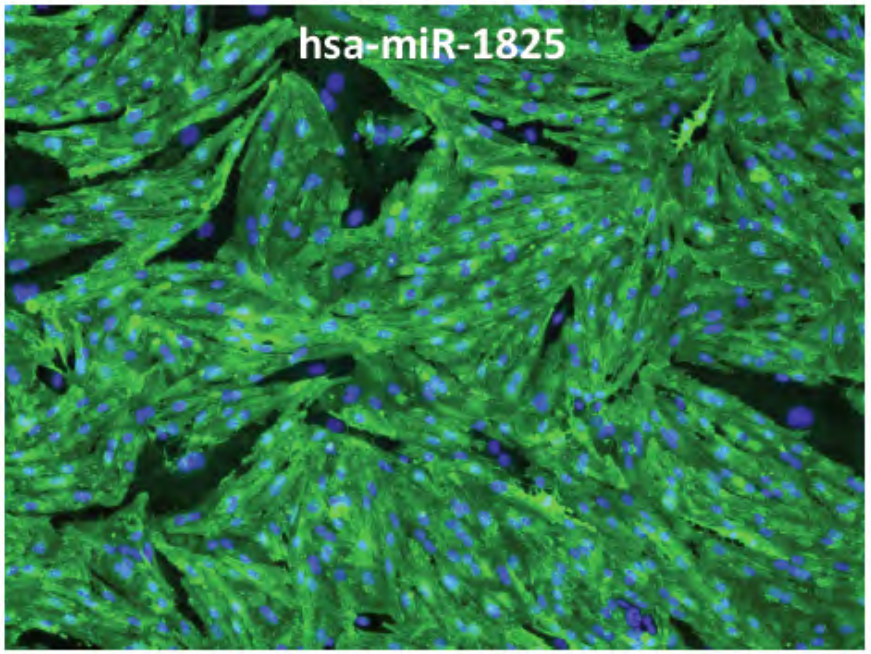




miRNAs inducing cardiomyocyte proliferation



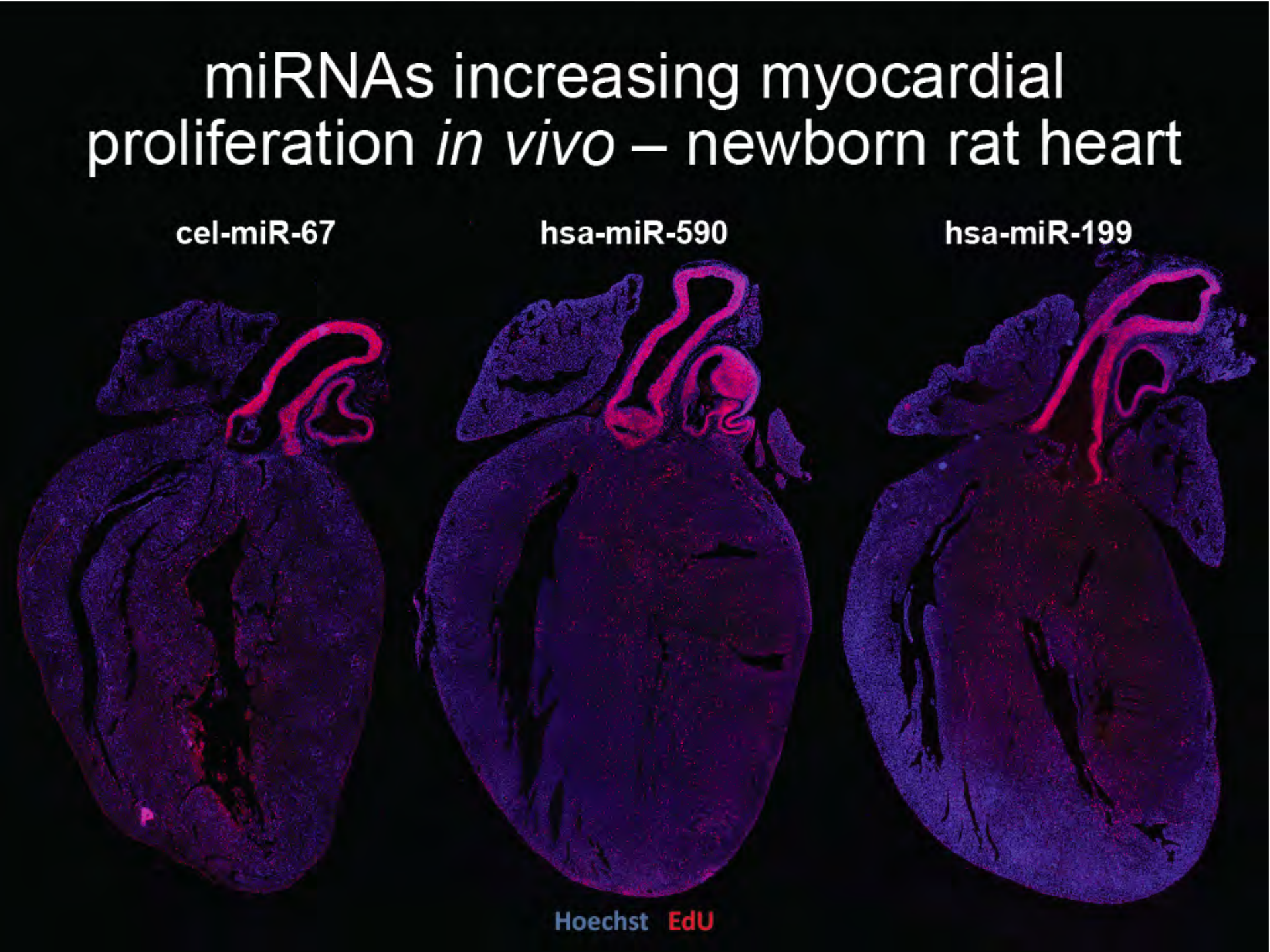




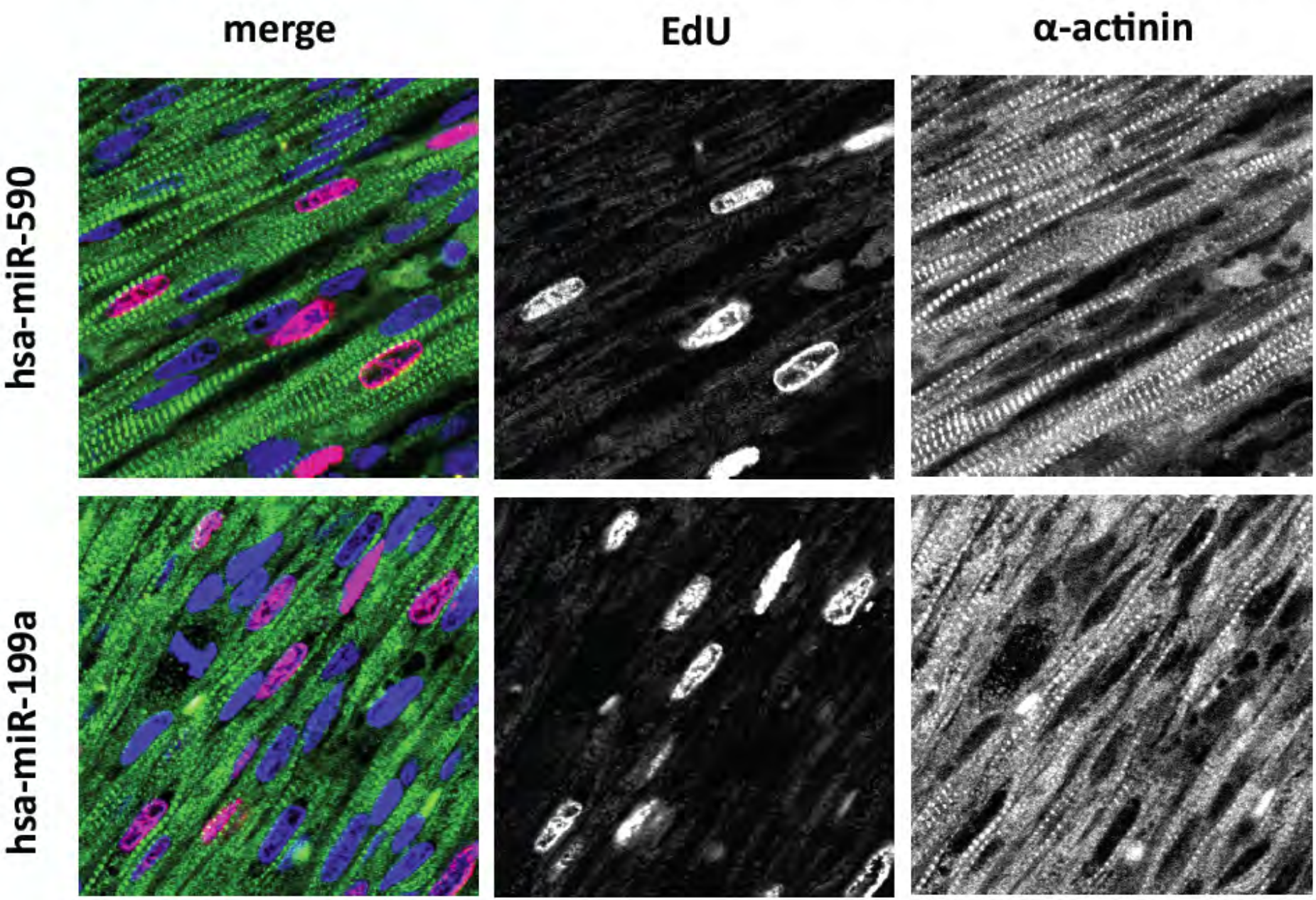
6 days after transfection

Effect of miRNA prolonged expression in vivo?
Regeneration?

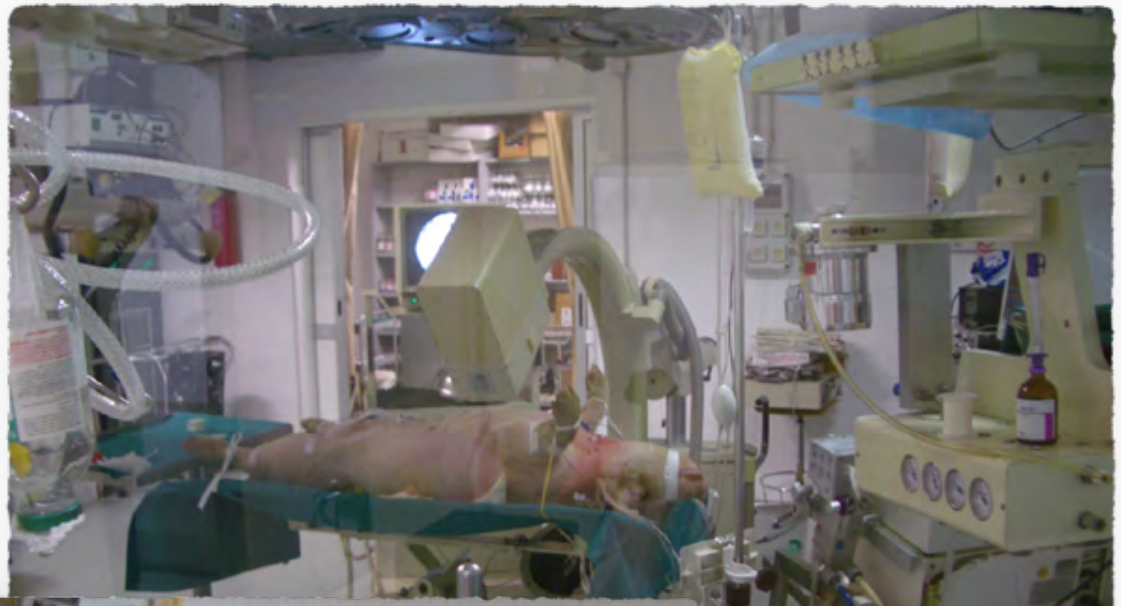
miRNAs increasing myocardial proliferation *in vivo* – newborn rat heart

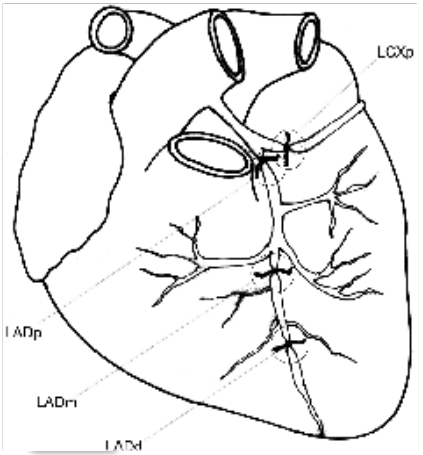


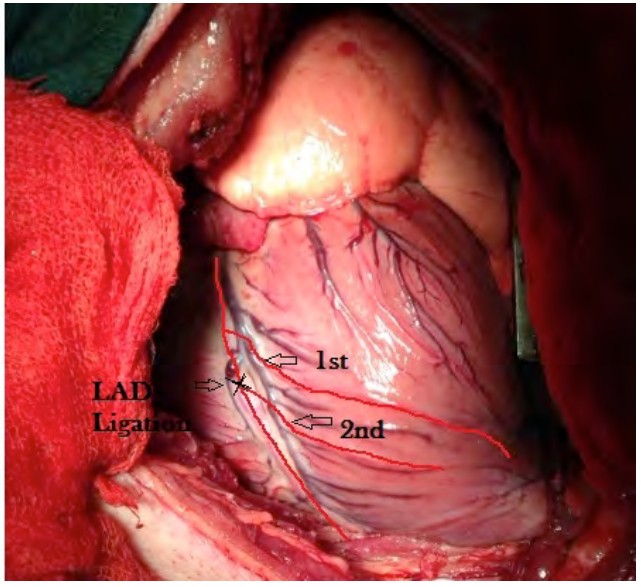
miRNAs increasing CM proliferation *in vivo*



Open chest MI model in farm pig







LETTER

https://doi.org/10.1038/s41586-019-1191-6

MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs

Khatia Gabisonia[1,8], Giulia Prosdocimo[2,8], Giovanni Donato Aquaro[3,8], Lucia Carlucci[1], Lorena Zentilin[2], Ilaria Secco[2,4], Hashim Ali[2,4], Luca Braga[2,4], Nikoloz Gorgodze[1], Fabio Bernini[1], Silvia Burchielli[3], Chiara Collesi[2,5], Lorenzo Zandonà[5], Gianfranco Sinagra[5], Marcello Piacenti[3], Serena Zacchigna[5,6], Rossana Bussani[5], Fabio A. Recchia[1,3,7,9]* & Mauro Giacca[2,4,5,9]*

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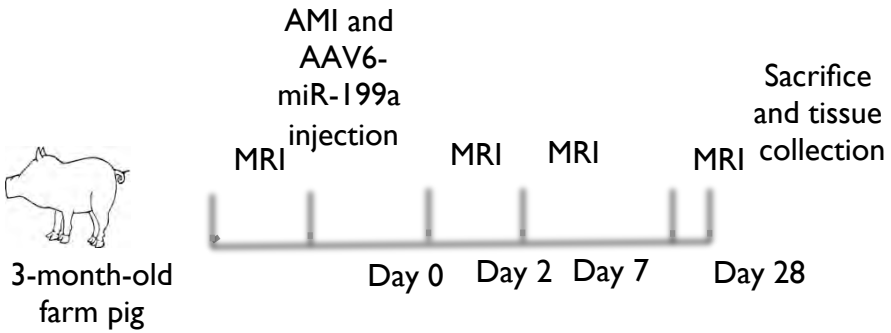
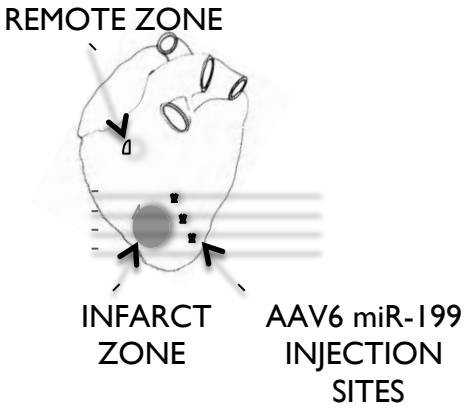
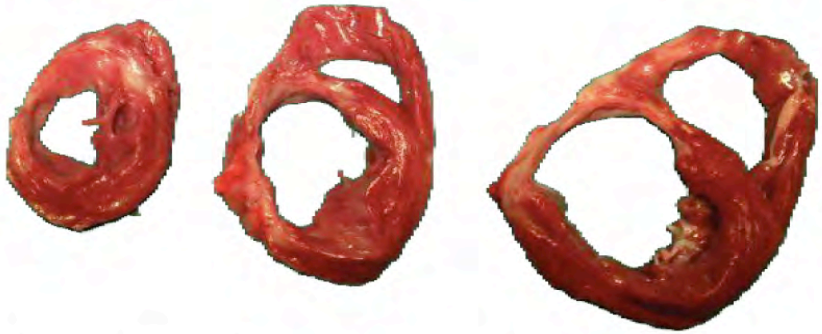
AAV6-miR-199a reduces infarct size after MI

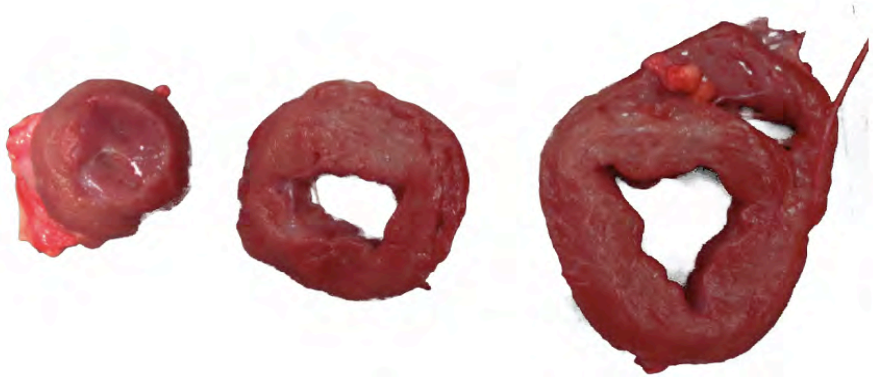


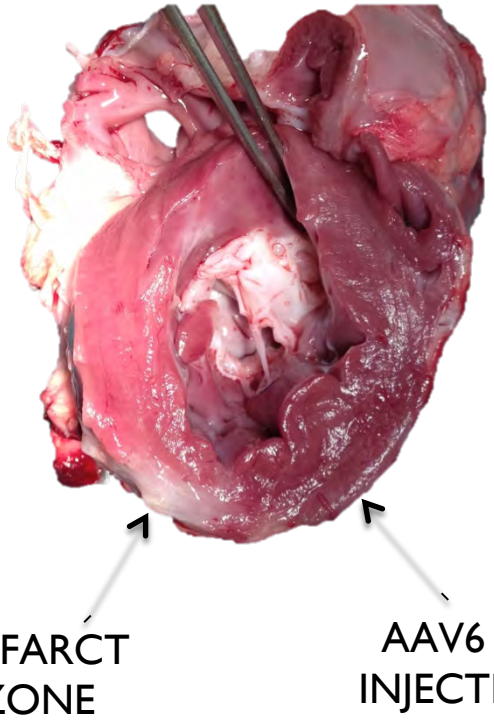


AAV6 empty

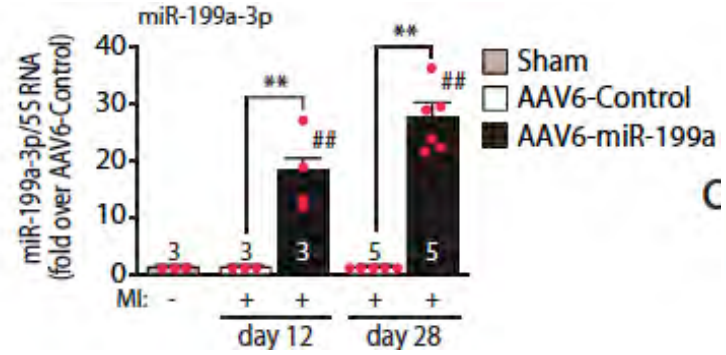


AAV6 miR-199a

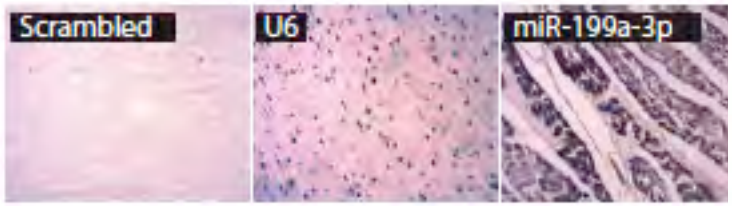


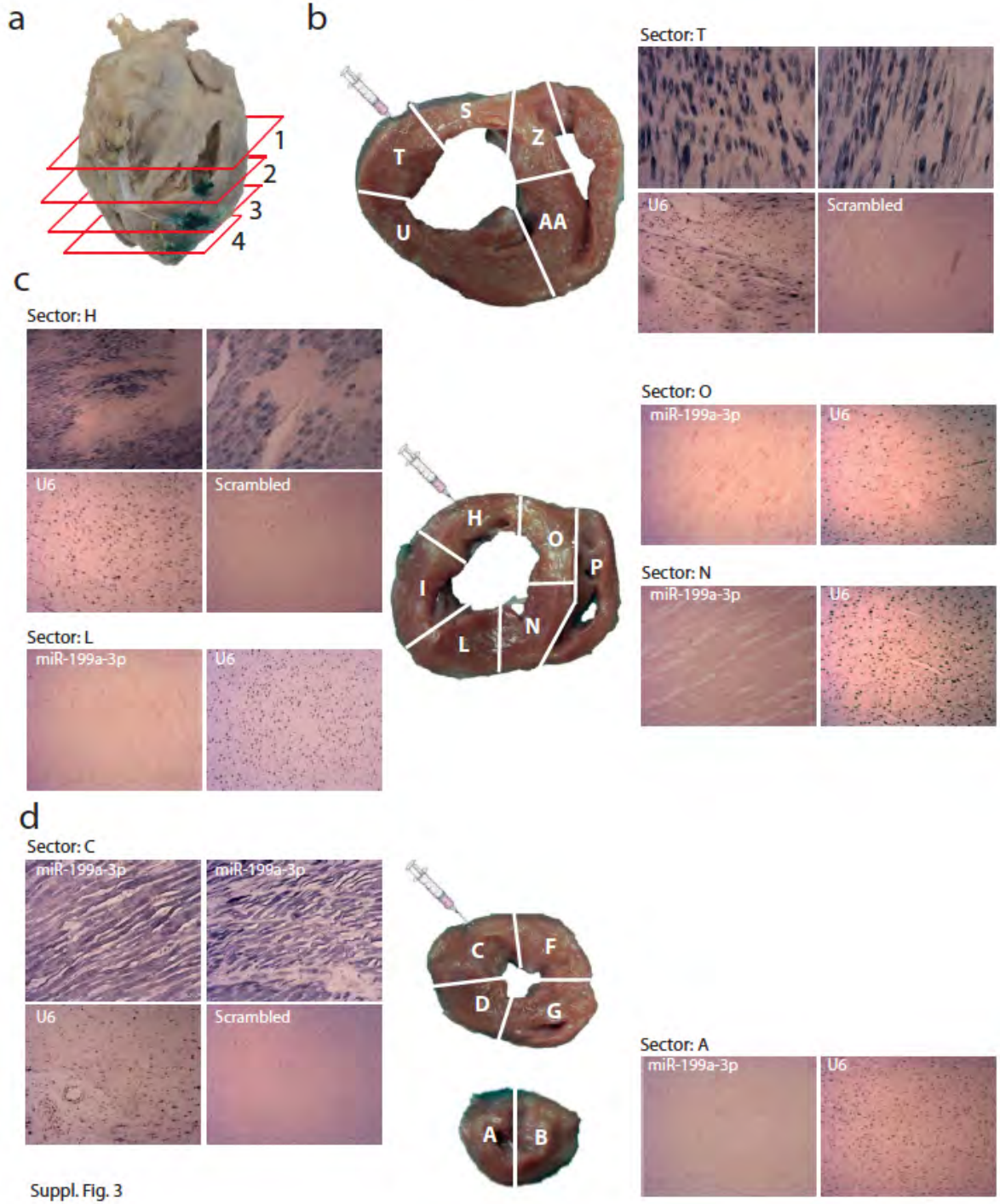


MiR-199a expression is persistent next to the injection sites



In situ hybridization





Suppl. Fig. 3

Goal achieved?