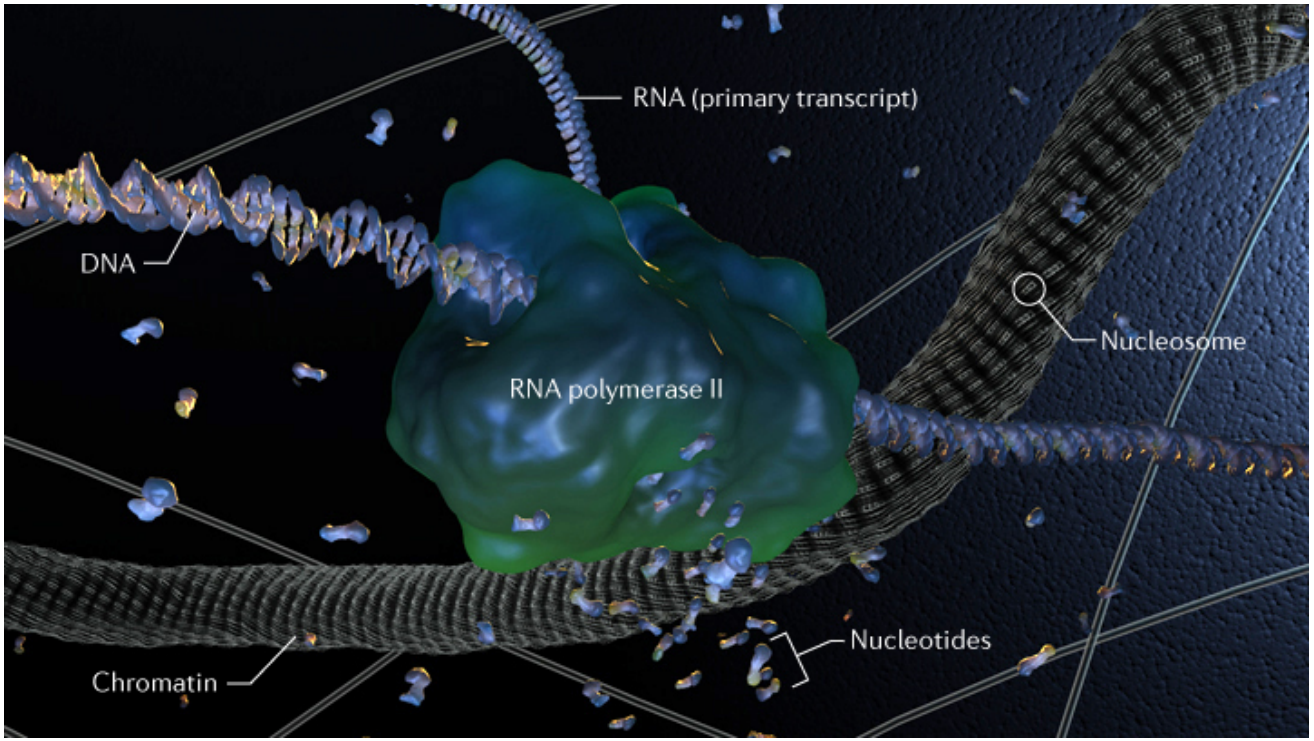
siRNA ve Tedavide Kullanımı

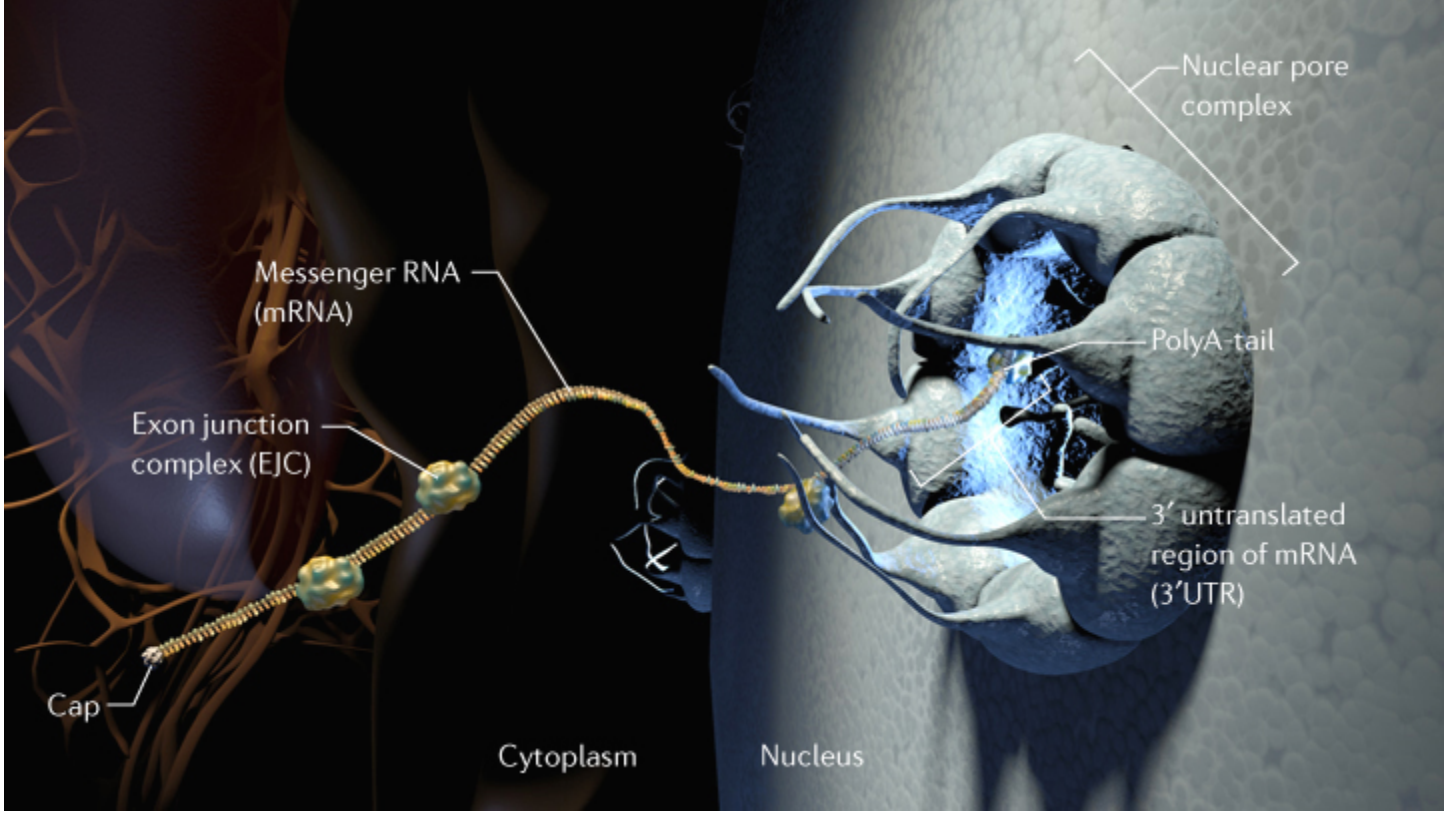
Uzm. Dr. Eyyüp ÜÇTEPE

Dışkapı EAH, Tıbbi Genetik Bölümü

Transcription

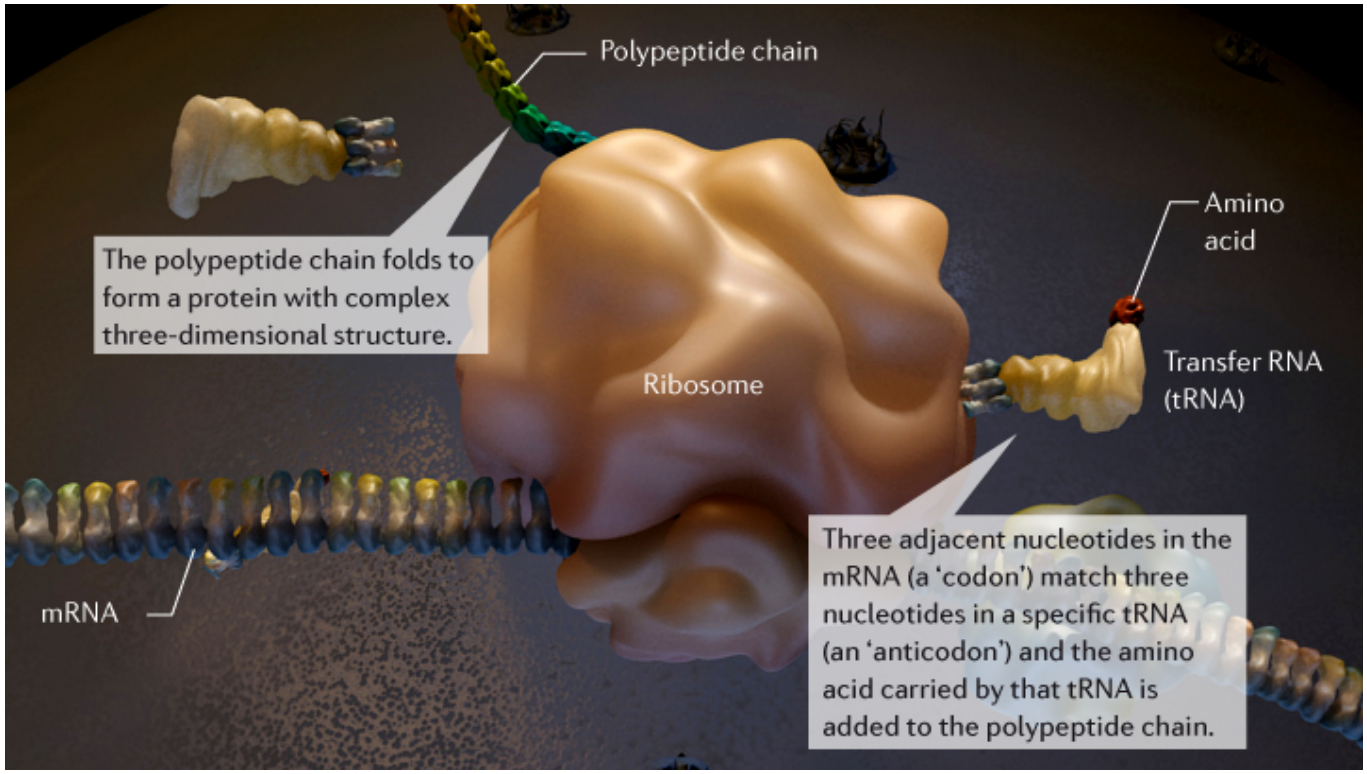


- ✓ DNA sequence as a template
- ✓ RNA polymerase II (RNAPII)
- ✓ the primary transcript
- ✓ regions of sequence that do not code for the protein (introns) are removed by splicing and a 'cap' is added to the 5′ end of the RNA.



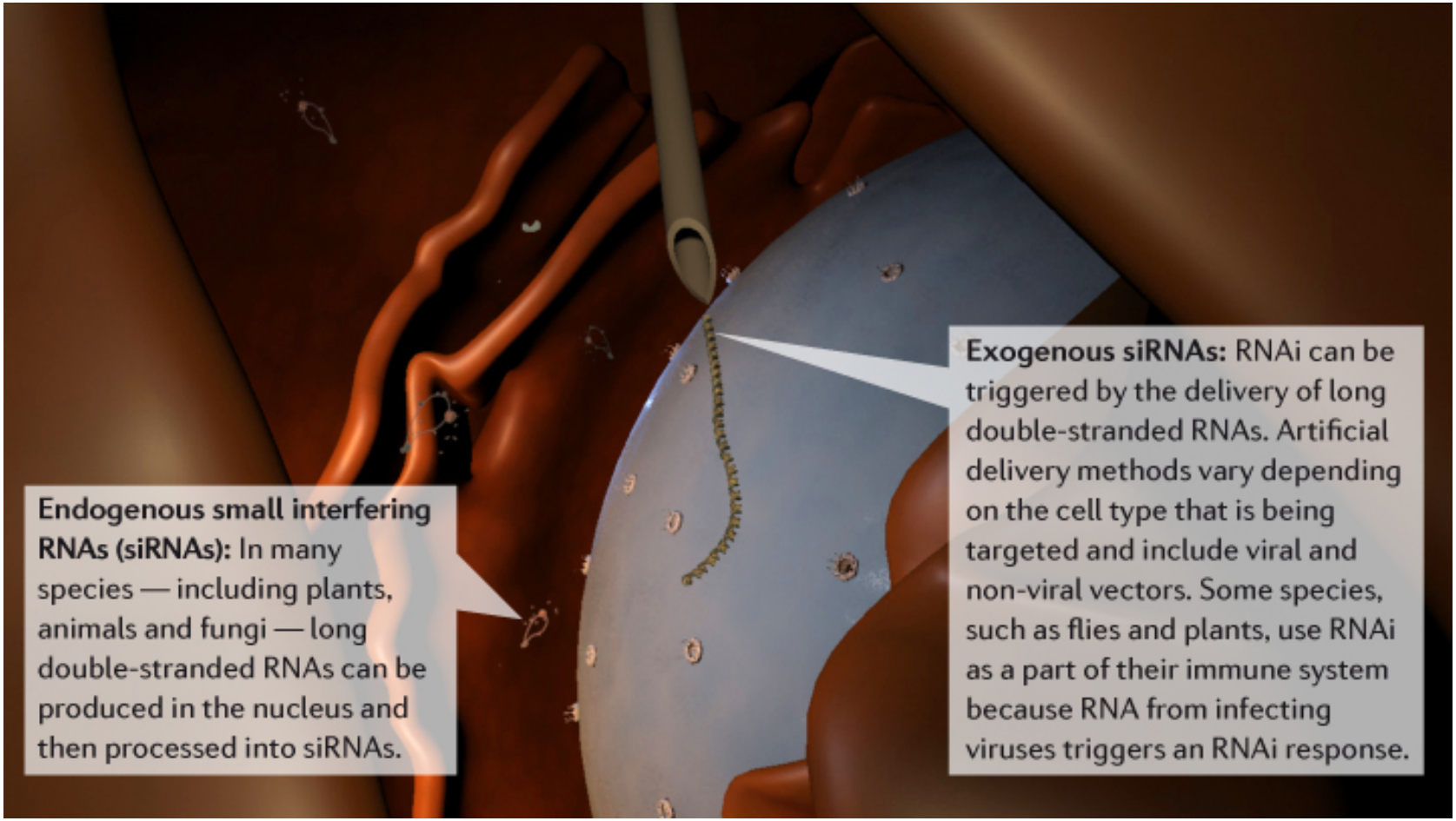
- ✓ Nuclear pore complex
- ✓ Made up of 30 different proteins
- ✓ The central cylinder has an eightfold symmetry.
- ✓ The filaments on the cytoplasmic side help to channel the mRNA towards the protein synthesis machinery.

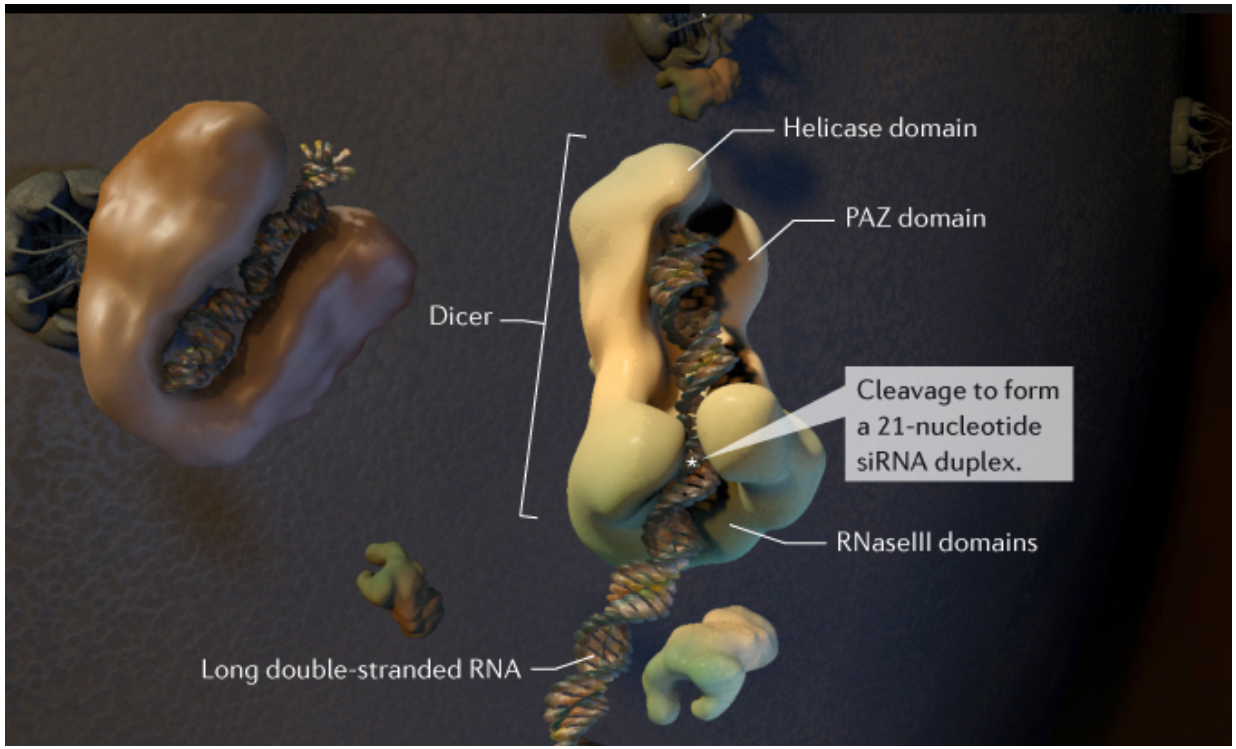
Translation



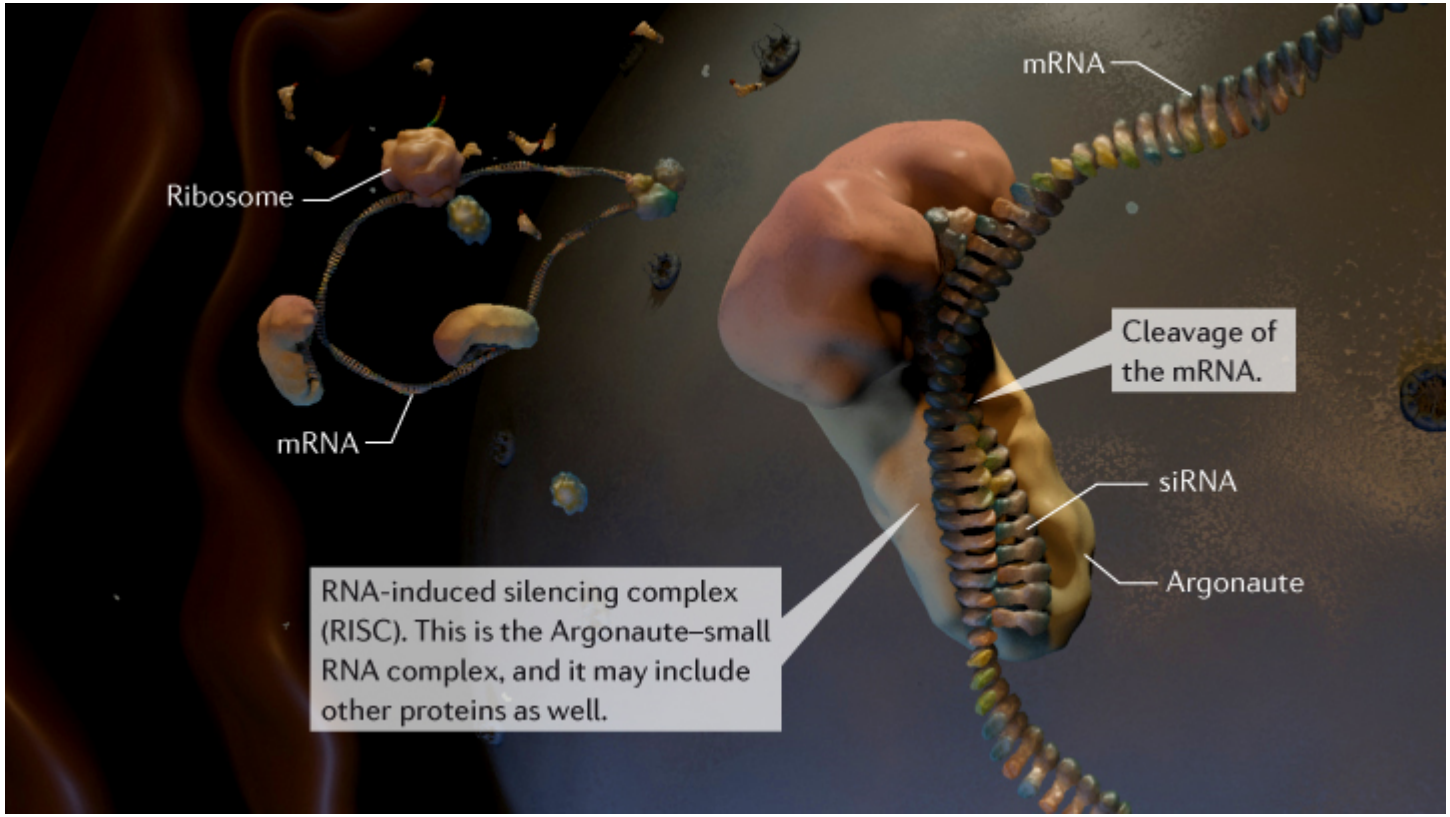
Some protein folding happens during translation, but the endoplasmic reticulum is an important site of protein folding. RNAi needs to target the mRNAs to stop this synthesis of proteins.

Endojen & Eksojen siRNA

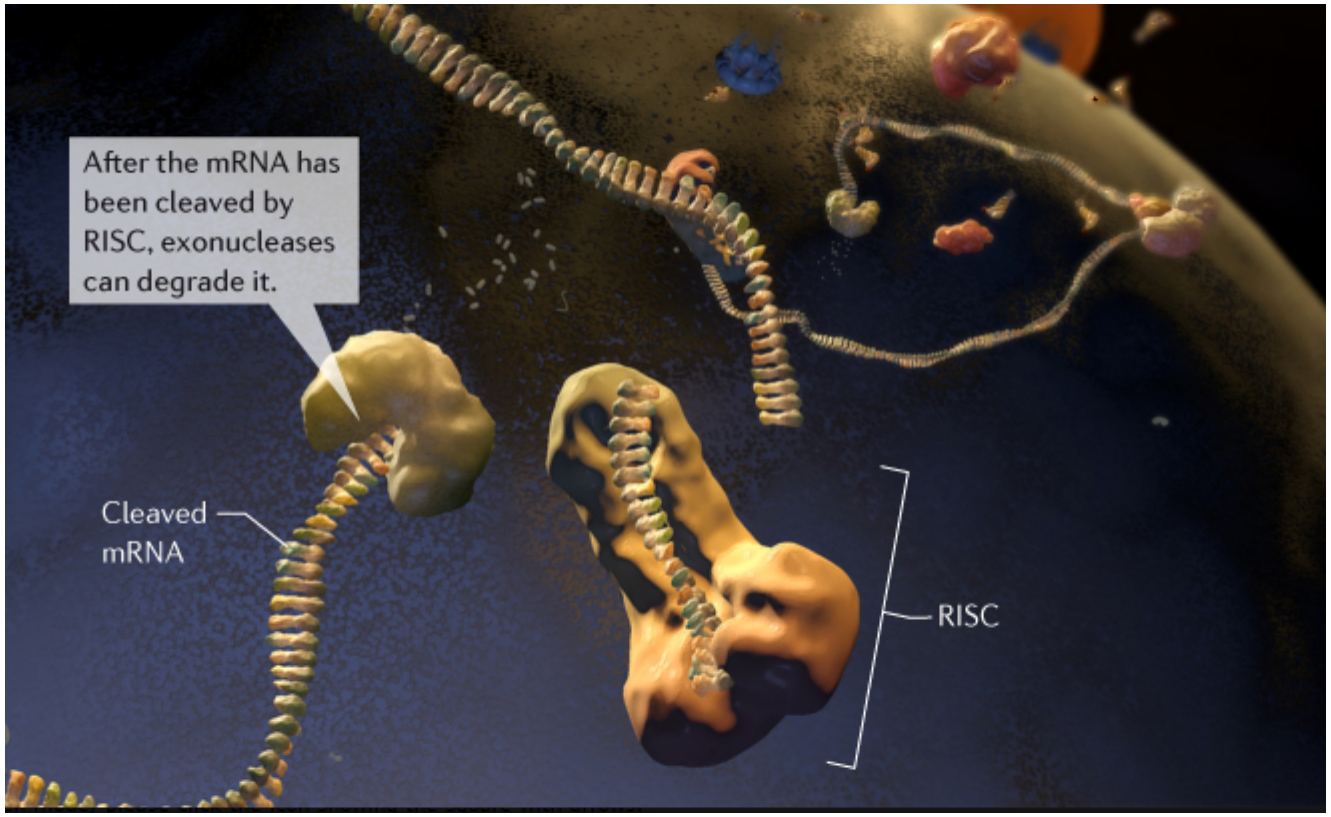




- Dicer is a double-stranded-RNA-specific ribonuclease from the RNase III protein family.
- Dicer produces double-stranded siRNAs that are ~21 nucleotides long.
- Two-nucleotide overhang at their 3′ end, as well as a 5′ phosphate and a 3′ hydroxyl group.

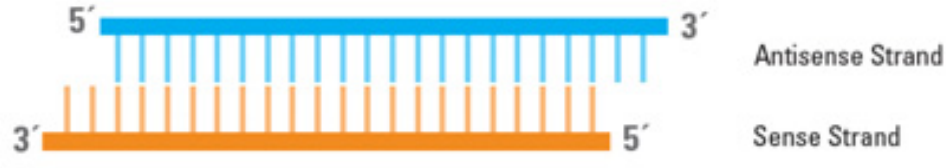


- Argonaute catalyses cleavage near the centre of the region of the mRNA
- There are more than 25 Argonautes in the nematode worm *C.elegans* compared with five in the fly *Drosophila melanogaster*.



- siRNAs can trigger degradation of specific mRNAs
- 'Knock down' the products of genes that are being studied.
- In the clinic to reduce the production proteins that are not functioning correctly.

What is siRNA?



- The most commonly used RNA interference (RNAi) tool for inducing short-term silencing of protein coding genes.
- A synthetic RNA duplex designed to specifically target a particular mRNA for degradation
- Their utility is limited to cells that are amenable to transfection
- Experiments are limited to relatively short time frames on the order of 2-4 days

How is siRNA delivered to a cell?

Technique	Delivery Mode	Advantages	Disadvantages
Transfection	Cationic liposomes or polymer based	Delivery of siRNA, microRNAs, and shRNA into most cell types	Not all cell types amenable to transfection reagents
Electroporation	Electrical pulse	Effective for difficult-to-transfect cells	Cell death often increased
Viral-mediated Delivery	Lentivirus Retrovirus Adeno-associated virus	Effective for difficult-to-transfect cells For use in stable selection In vivo application	Requires BSL2 facilities May trigger antiviral response in some cell types
Modified siRNA	Modified siRNA (Accell) To enable passive uptake by many cell types	Effective for difficult-to-transfect cells Repeated dosing possible for longer-term silencing In vivo application	Delivery efficiency inhibited by presence of >3% serum during application

Transfection Reagent

- Dharmafect
- Lipofectamin
- **Polyethylenimine** (**PEI**)
- HEK 293T hücresi için

(12'lik well: 1 ml medyum)

- -2mg DNA, 4ug PEI, 100 ul OptiMEM

Specificity
off-target & on target

- The sequence complementarity-based mechanism
- Chemical modifications to the siRNA for preferential loading of the intended antisense (guide) strand into the RISC complex
- Chemical modifications or thermodynamic-based design for siRNA seed region to discourage undesired interactions
- The strategy of pooling several independent siRNAs

Applications

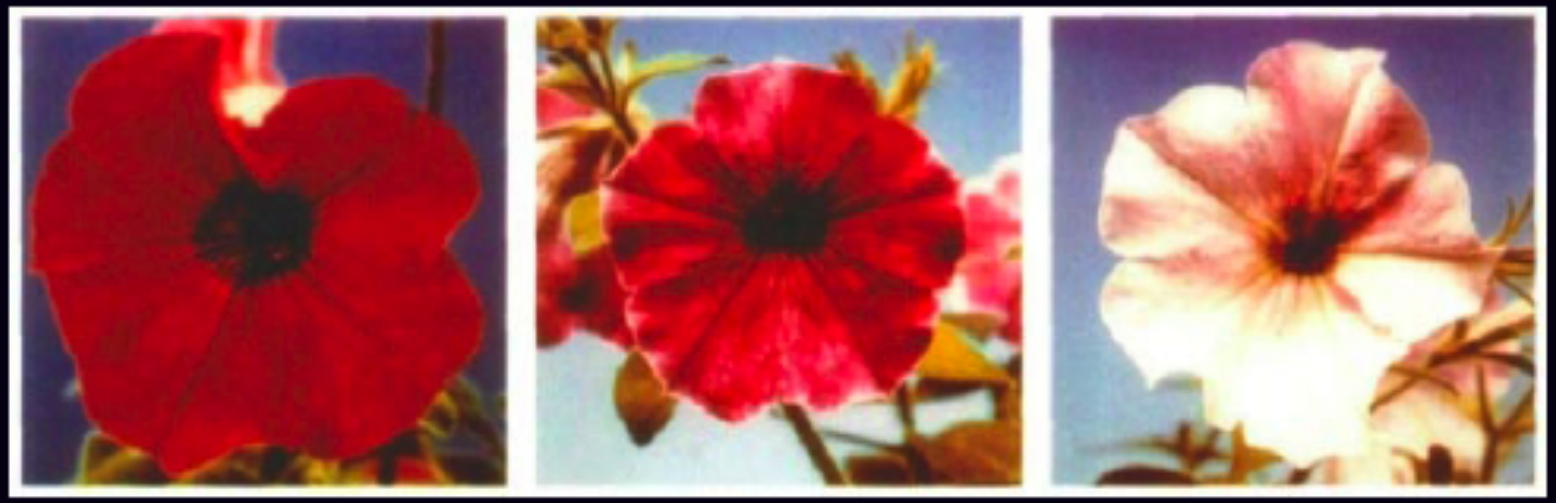
- Cytokinesis, apoptosis, insulin signaling and cell differentiation.
- To identify novel pathways
- Validating targets for a number of cellular processes and diseases including cancer, HIV infection and hepatitis
- Animal disease models

Controls for siRNA Experiments

- Positive control: to ensure the delivery method is sufficient to achieve effective silencing
- Negative control: to separate sequence-specific effects from the effects of experimental conditions on cellular responses.
- Untreated control: a useful baseline reference for cell phenotypes and gene expression levels.

History of RNA-mediated suppression

Curious findings in plants led the way…



Adding a pigment gene to petunias made them less pigmented! Biology works in mysterious ways…

Jorgensen 1990
van der Krol 1990

Gene injection (pigmentation Enzyme-petunias)
Expectation: more red color
Co-suppression of transgene and endogenous gene.

Bill Douherty and Lindbo 1993

Gene injection with a complete tobacco etch virus particle.
Expectation: virus expression
Co-suppression of transgene and virus particles via RNA.

Hamilton and Baulcombe 1998

Identification of short antisense RNA sequences
dsRNA?
How?

Ambros 1993 (2000)

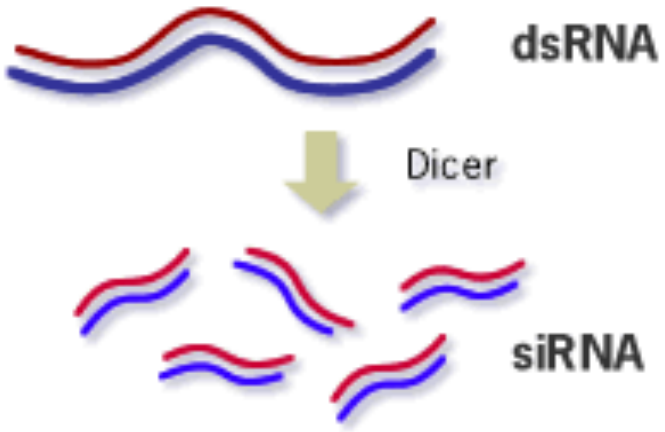
Identification of small RNA in C. elegans (micro RNA)

Fire and Mello 1998

Injection of dsRNA into C. elegans
RNA interference (RNAi) or silencing

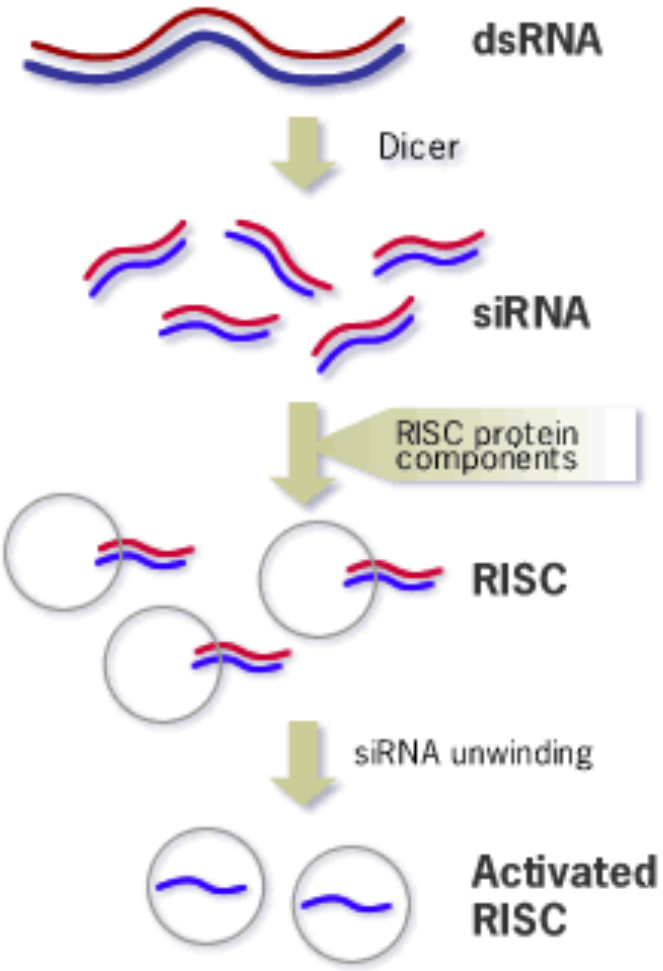
Step 1

- dsRNA is processed
- 21-25 nucleotides in length
  - have 2-3 nt 3' overhanging ends
  - Done by *Dicer* (an RNase III-type enzyme)



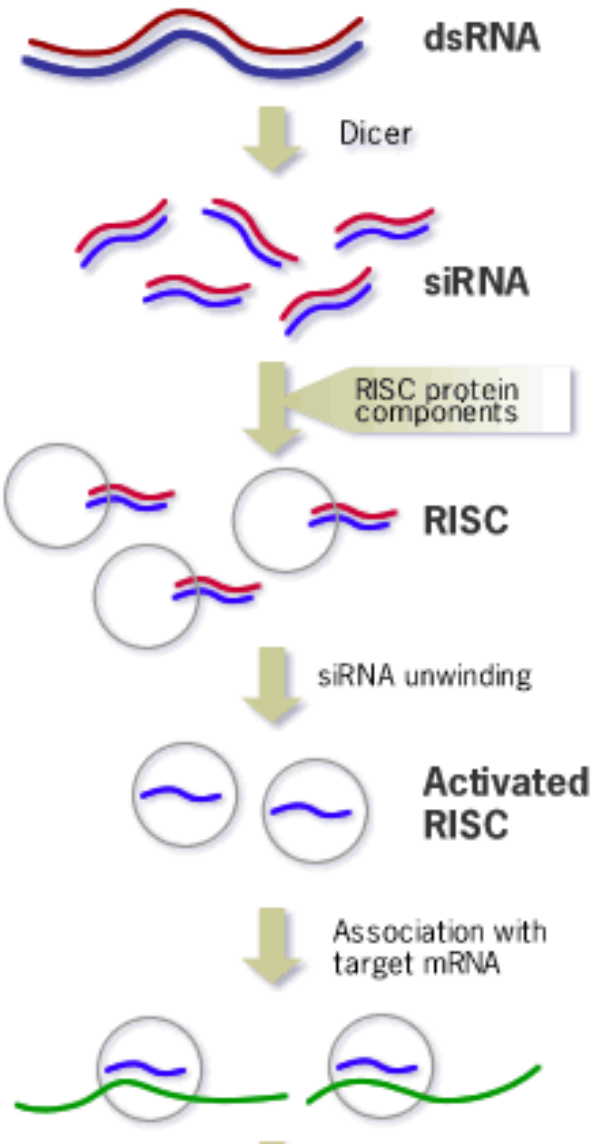
Step 2

- The siRNAs associate with *RISC* (RNA-induced silencing complex) and unwind



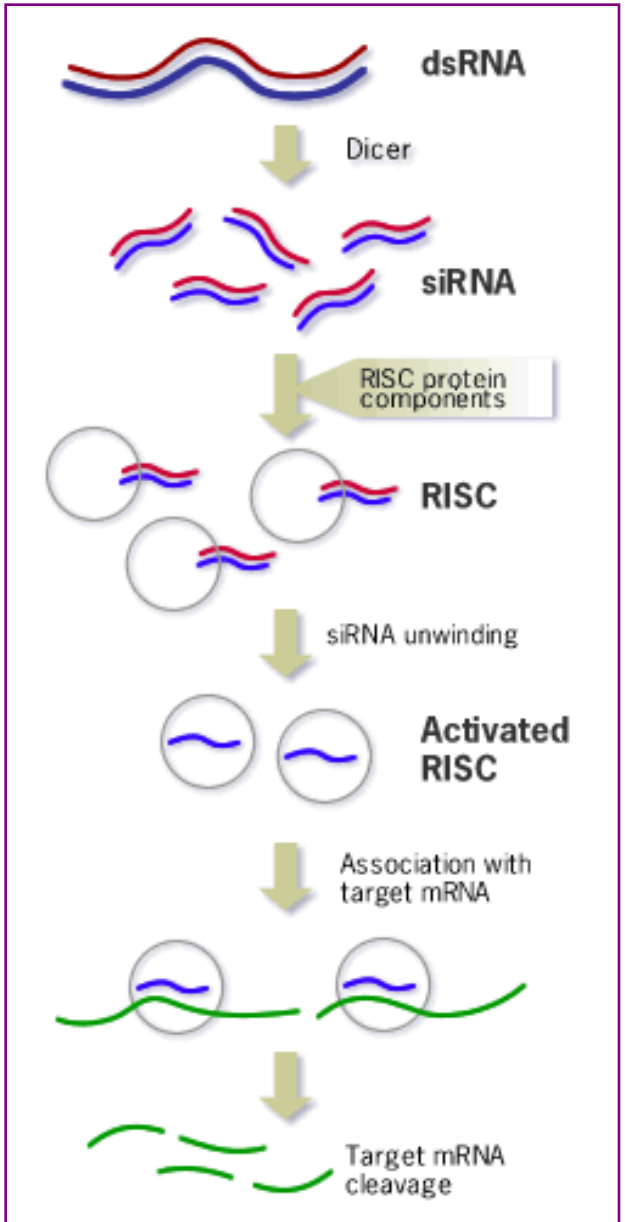
Step 3

- the antisense siRNAs act as guides for *RISC* to associate with complimentary single-stranded mRNAs.



Step 4

- *RISC* cuts the mRNA approximately in the middle of the region paired with the siRNA
- The mRNA is degraded further



ClinicalTrials.gov
A service of the U.S. National Institutes of Health

siRNA and Clinical Trials

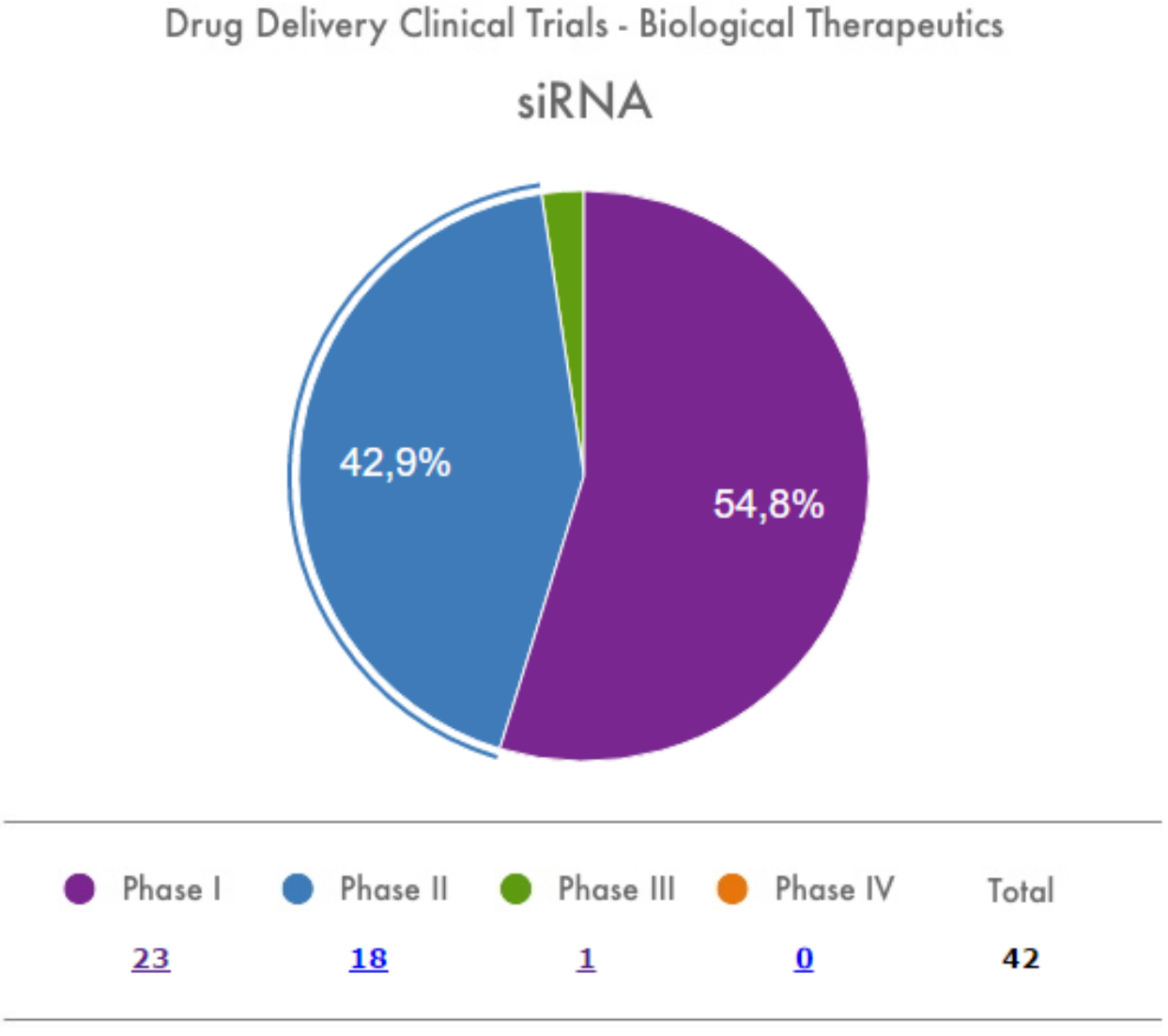


Table 1: RNAi-based drugs in clinical trials.

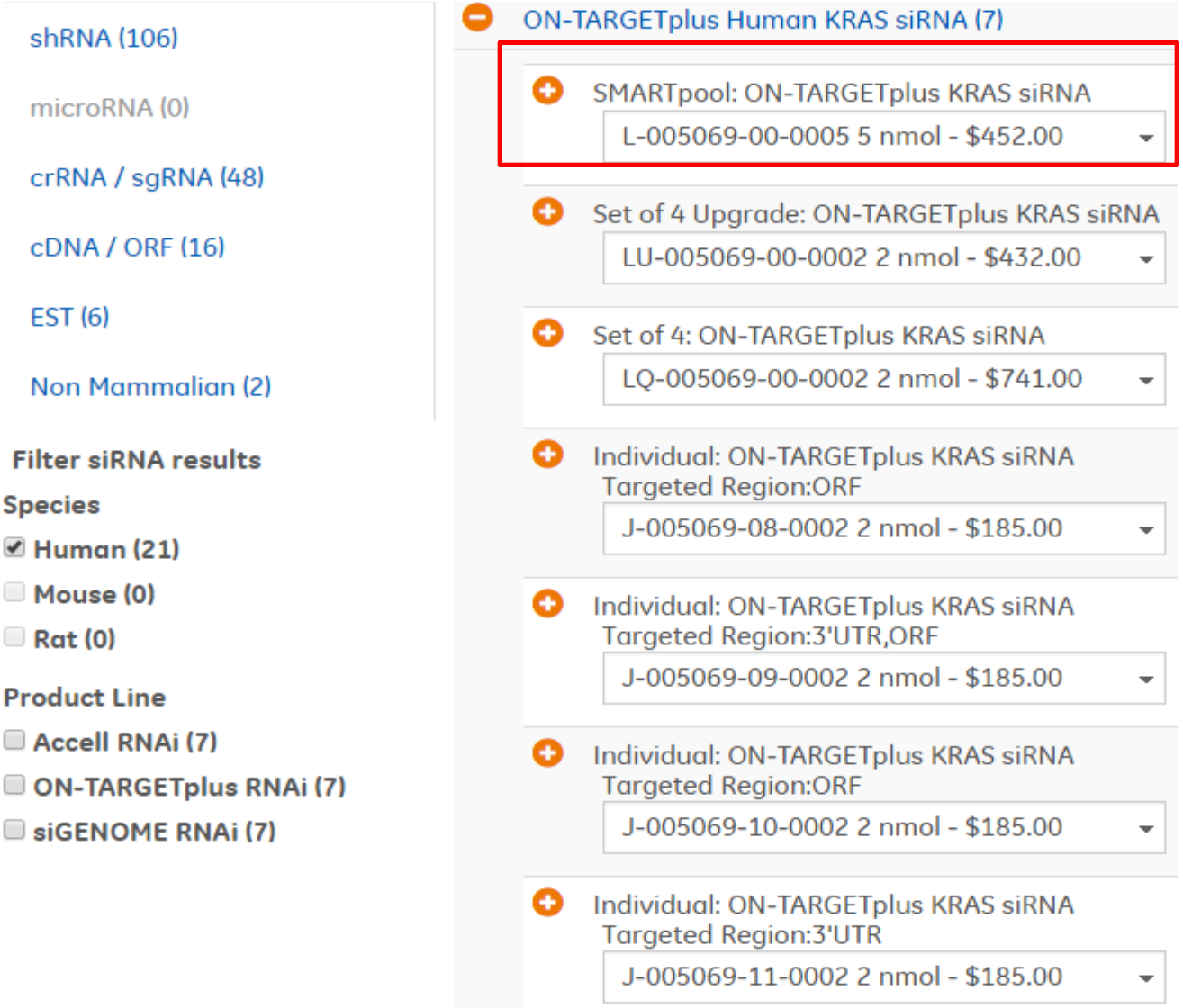
Drug	Target	Delivery system	Disease	Phase	Status	Company	ClinicalTrials.gov identifier
ALN–VSP02	KSP and VEGF	LNP	Solid tumours	I	Completed	Alnylam Pharmaceuticals	NCT01158079
siRNA–EphA2–DOPC	EphA2	LNP	Advanced cancers	I	Recruiting	MD Anderson Cancer Center	NCT01591356
Atu027	PKN3	LNP	Solid tumours	I	Completed	Silence Therapeutics	NCT00938574
TKM–080301	PLK1	LNP	Cancer	I	Recruiting	Tekmira Pharmaceutical	NCT01262235
TKM–100201	VP24, VP35, Zaire Ebola L-polymerase	LNP	Ebola-virus infection	I	Recruiting	Tekmira Pharmaceutical	NCT01518881
ALN–RSV01	RSV nucleocapsid	Naked siRNA	Respiratory syncytial virus infections	II	Completed	Alnylam Pharmaceuticals	NCT00658086
PRO-040201	ApoB	LNP	Hypercholesterolaemia	I	Terminated	Tekmira Pharmaceutical	NCT00927459
ALN–PCS02	PCSK9	LNP	Hypercholesterolaemia	I	Completed	Alnylam Pharmaceuticals	NCT01437059

PF-655 (PF-04523655)	RTP801 (Proprietary target)	Naked siRNA	Choroidal neovascularization, diabetic retinopathy, diabetic macular oedema	II	Active	Quark Pharmaceuticals	NCT01445899
siG12D LODER	KRAS	LODER polymer	Pancreatic cancer	II	Recruiting	Silenseed	NCT01676259
Bevasiranib	VEGF	Naked siRNA	Diabetic macular oedema, macular degeneration	II	Completed	Opko Health	NCT00306904
SYL1001	TRPV1	Naked siRNA	Ocular pain, dry-eye syndrome	I, II	Recruiting	Sylentis	NCT01776658
SYL040012	ADRB2	Naked siRNA	Ocular hypertension, open-angle glaucoma	II	Recruiting	Sylentis	NCT01739244
CEQ508	CTNNB1	*Escherichia coli*-carrying shRNA	Familial adenomatous polyposis	I, II	Recruiting	Marina Biotech	Unknown
RXi-109	CTGF	Self-delivering RNAi compound	Cicatrix scar prevention	I	Recruiting	RXi Pharmaceuticals	NCT01780077
ALN–TTRsc	TTR	siRNA–GalNAc conjugate	Transthyretin-mediated amyloidosis	I	Recruiting	Alnylam Pharmaceuticals	NCT01814839
ARC-520	Conserved regions of HBV	DPC	HBV	I	Recruiting	Arrowhead Research	NCT01872065

Faz III and siRNA

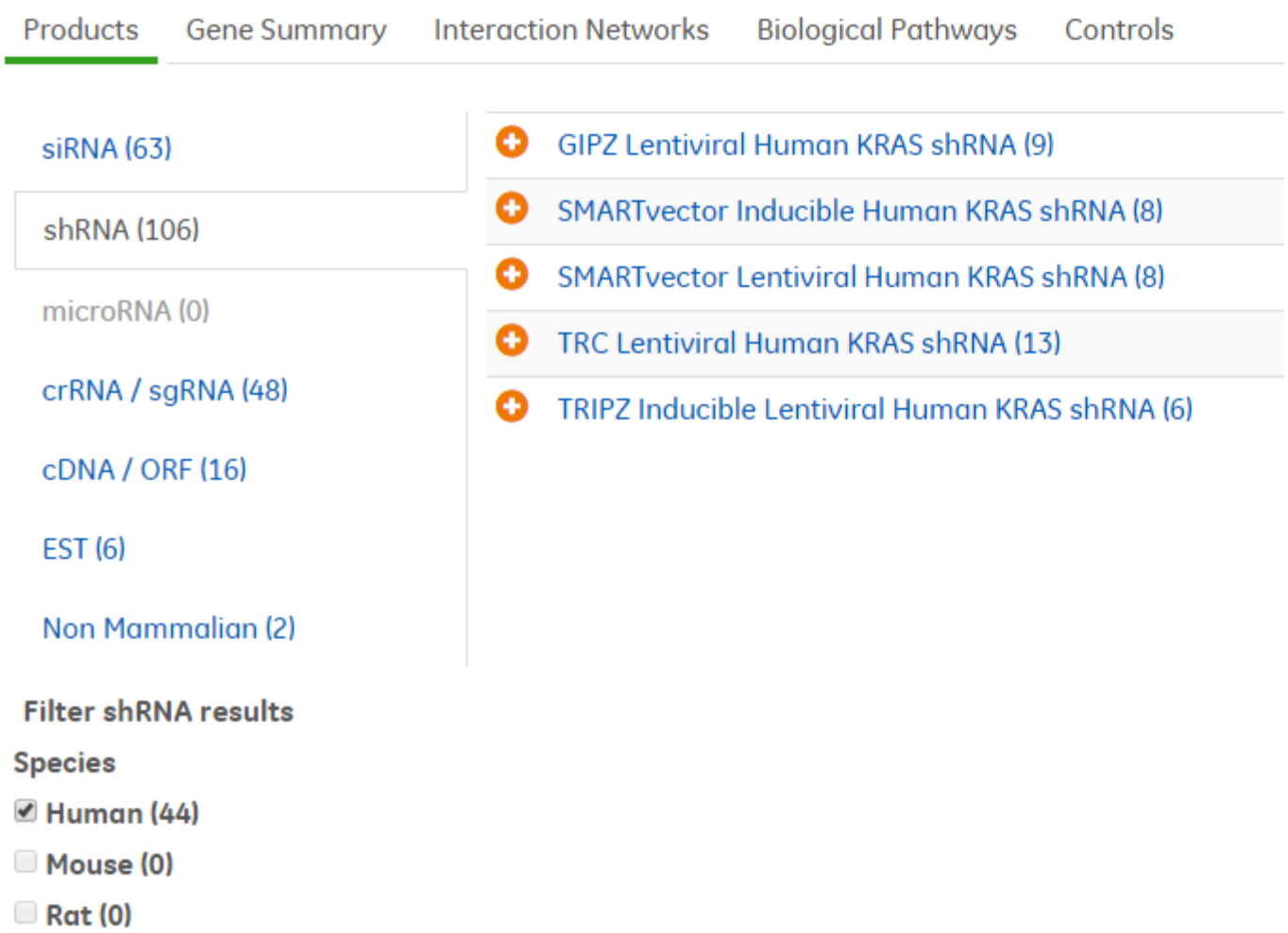
- Age-related macular degeneration
- Bevasiranib sodium
- RNA interference; Vascular endothelial growth factor A inhibitors

Ticari siRNA temin etme



http://dharmacon.gelifesciences.com/biology overview/?term=KRAS&sourceId=EG/3845

Ticari shRNA temin etme



http://dharmacon.gelifesciences.com/biology overview/?term=KRAS&sourceId=EG/3845

siRNA çalışmasında nasıl yapıyoruz?

Table 1. Recommended volumes per well for transfecting siRNA (at 25 nM final concentration) in standard plate formats.

		Tube 1: diluted siRNA (µL/well)		Tube 2: diluted DharmaFECT (µL/well)			
Plating Format (wells/ plate)	Surface Area (cm_2/ well)	Volume of 5 µM siRNA (µL)	Serum-free Medium (µL)	Volume of DharmaFECT reagent (µL)*	Serum-free Medium (µL)	Complete Medium (µL/well)	Total Transfection Volume (µL/well)
96	0.3	0.5	9.5	0.05 –0.5	9.95 - 9.5	80	100
24	2	2.5	47.5	0.25-2.5	49.75 – 47.5	400	500
12	4	5	95	0.5-5.0	99.5 - 95.0	800	1000
6	10	10	190	1.0-10.0	199.0 - 190.0	1600	2000

Hücre tipi ve Dharmafect

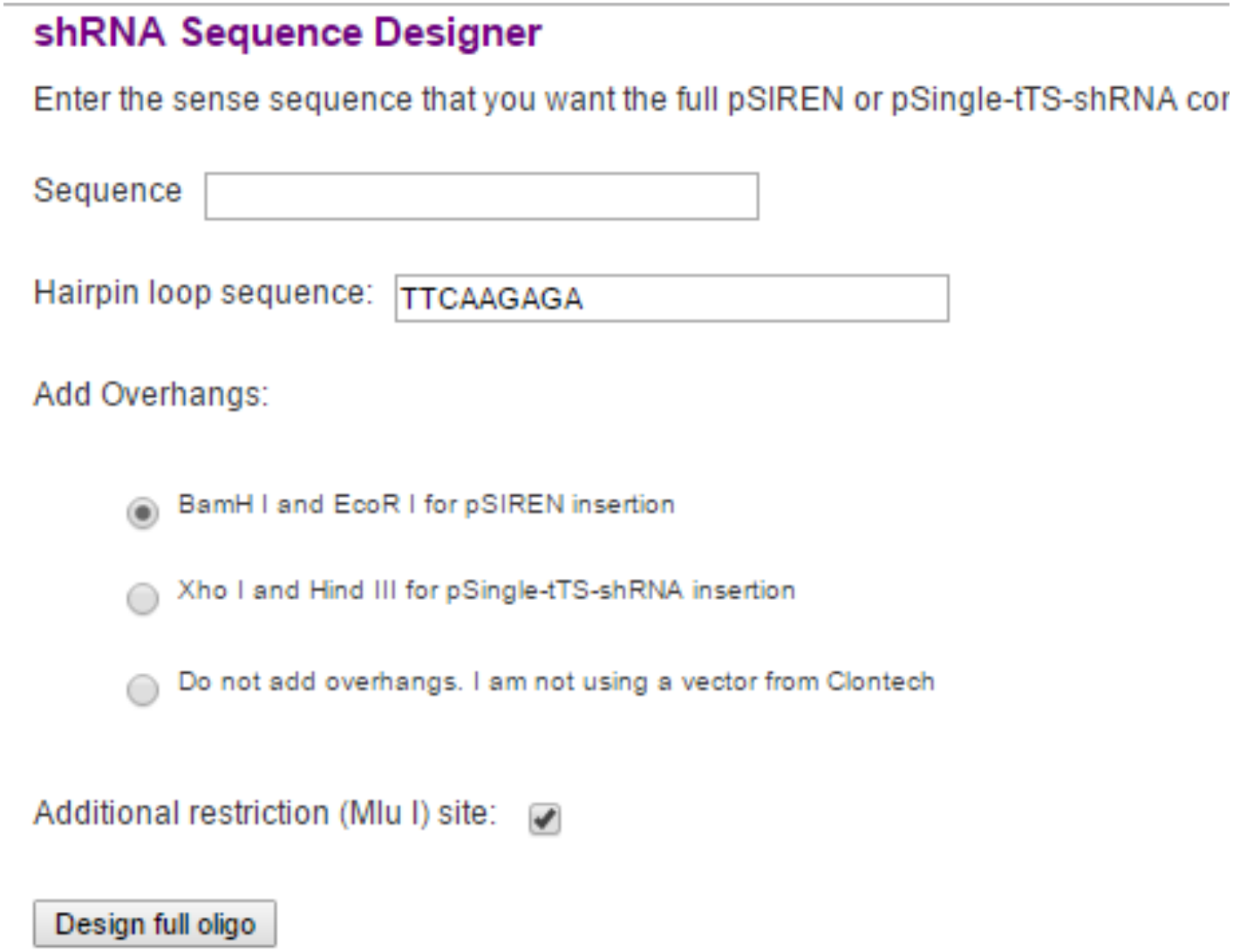
Cell line	Cell type	Recommended DharmaFECT formulation	DharmaFECT volume/well (µL)	Plating density	Other successful formulations
Human					
A549	Lung carcinoma	1	0.2	1×10^4	2, 3, 4
BxPC3	Pancreas; adenocarcinoma	2	0.2	5×10^3	1, 3, 4
DU 145	Prostate; metastatic: brain carcinoma	1	0.2	1×10^4	2, 3, 4
HEK293	Kidney transformed embryonic cells	1	0.2	1×10^4	2, 4
HeLa	Cervical epithelial adenocarcinoma	1	0.2	5×10^3	2, 3, 4
HeLa S3	Cervical epithelial adenocarcinoma	4	0.4	5×10^3	1, 2, 3
HepG2	Hepatocellular carcinoma	4	0.4	1×10^4	1, 2
H1299	Lung carcinoma	2	0.2	1×10^4	4
HT-1080	Fibrosarcoma	4	0.2	5×10^3	1, 2, 3
HT-29	Colorectal adenocarcinoma	1	0.2	5×10^3	1, 2, 3, 4
MCF-7	Breast adenocarcinoma	1	0.2	1×10^4	2, 4
MCF-10a	Breast adenocarcinoma	1	0.2	1×10^4	2
MDA-MB-453	Mammary gland; metastatic	2	0.2	1×10^4	1, 3, 4

shRNA kendi tecrübemiz

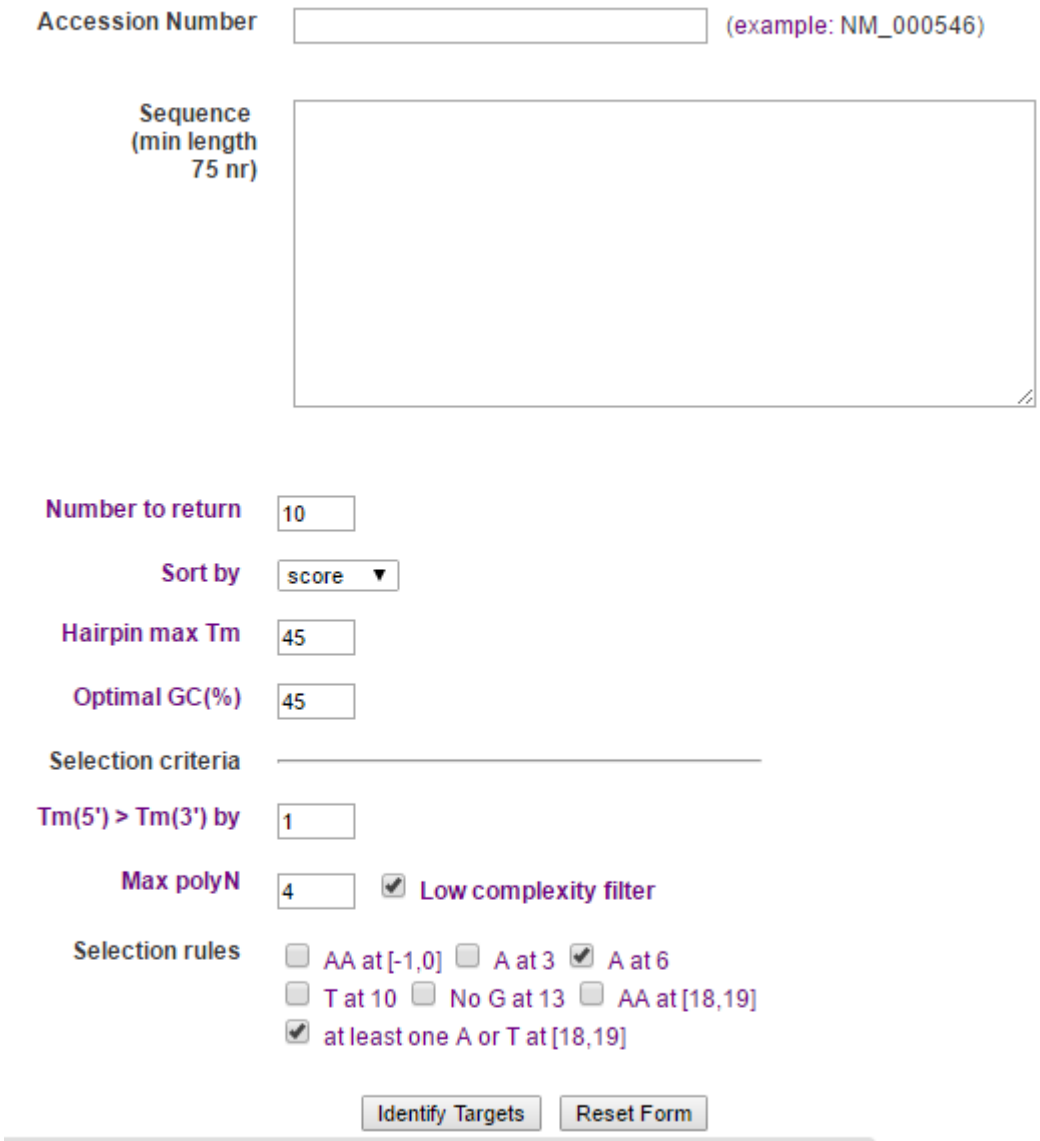
- http://bioinfo.clontech.com/rnaidesigner/sirnaSequenceDesignInit.do

- http://bioinfo.clontech.com/rnaidesigner/oligoDesigner.do?overhangs=on&restrictionSite=on

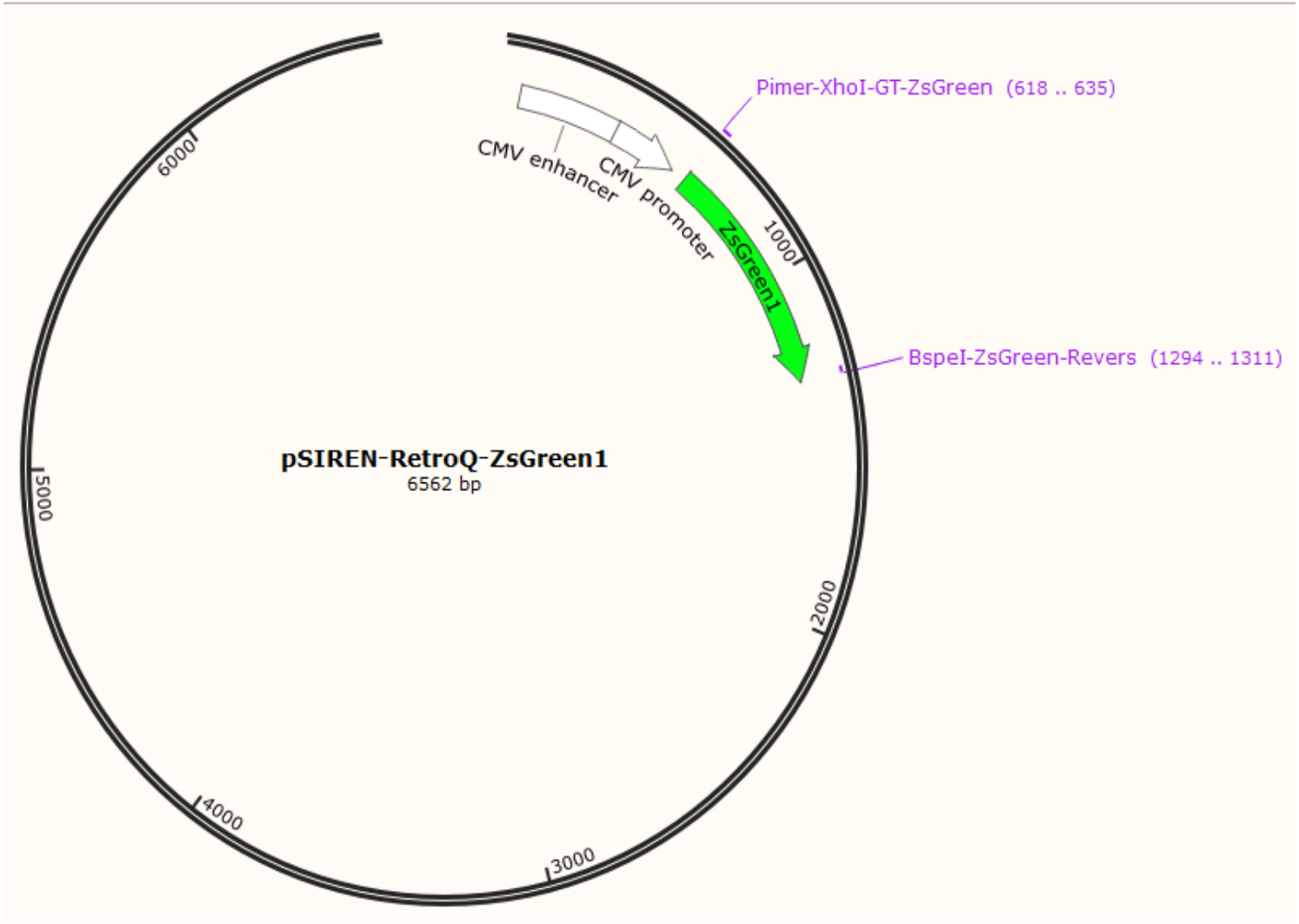
siRNA dizisini biliyorsanız!!!



siRNA dizisini bilmiyorsanız!!!



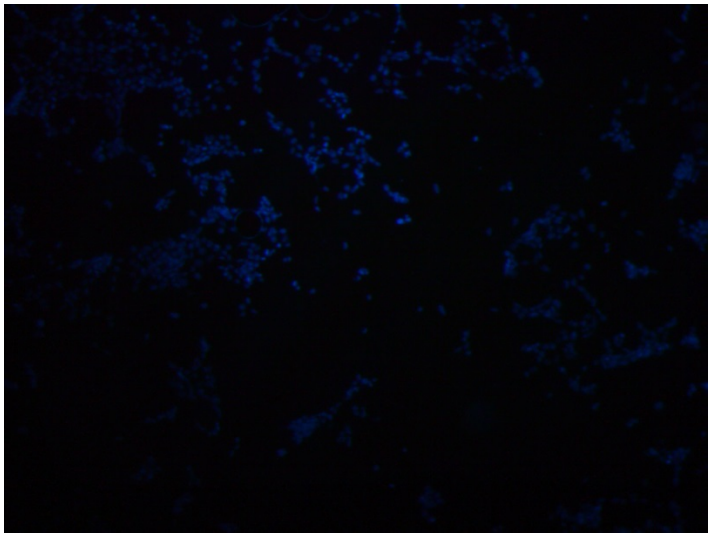
Tasarlanan shRNA'lerin içine atıldığı pSIREN vektörü

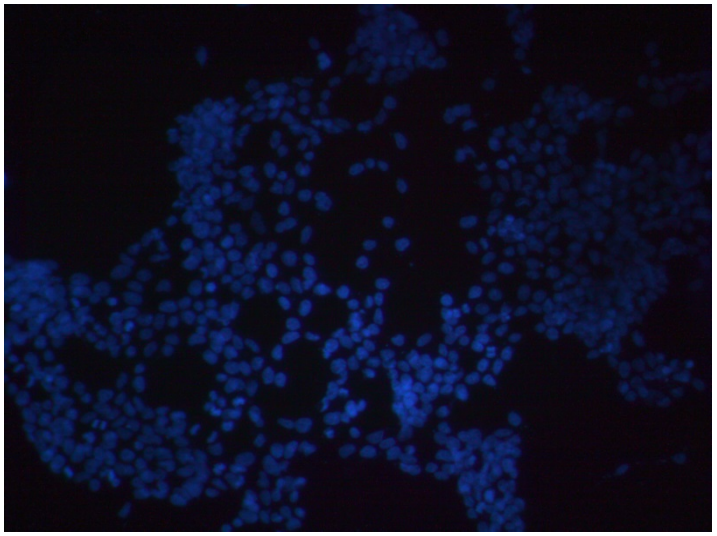


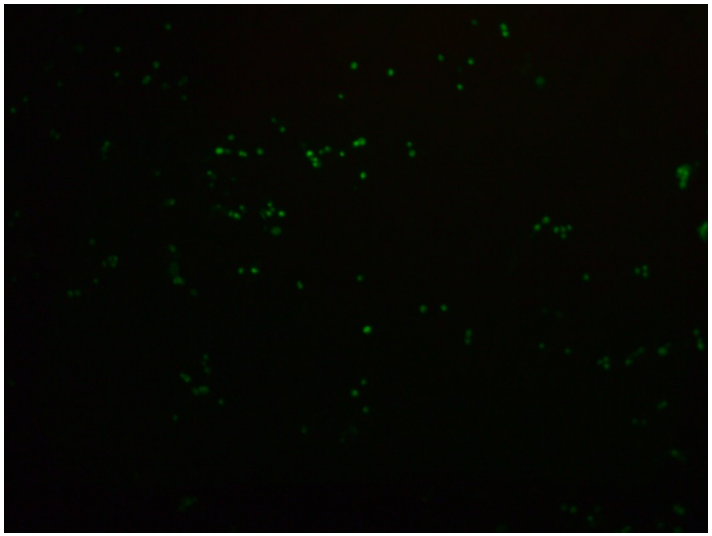
KRAS geni için tasarlanmış shRNA'lar

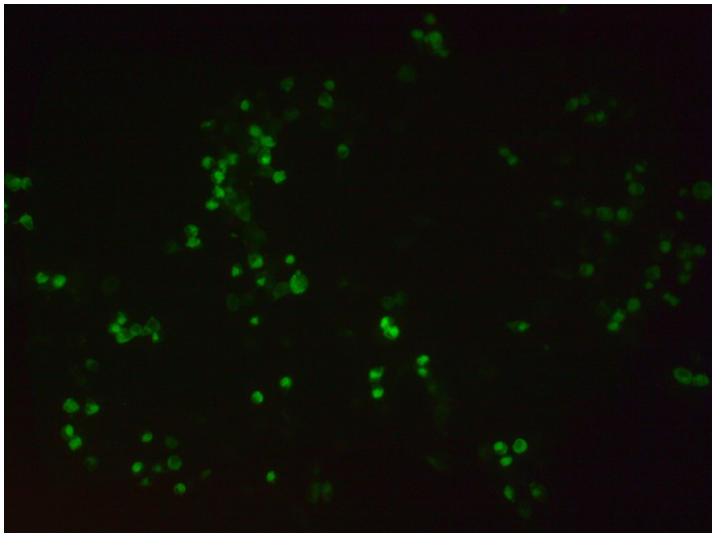
Oligo Adı	5' – 3' Sekans	Uzunluk (
KRAS shRNA-1F	gatccGGAGGGCTTTCTTTGTGTATTCAAGAGATACACAAAGAAAGCCCTCCTTTTTTACGCGTg	65
KRAS shRNA-1R	aattcACGCGTAAAAAAGGAGGGCTTTCTTTGTGTATCTCTTGAATACACAAAGAAAGCCCTCCg	65
KRAS shRNA-2F	gatccGTCAAAGACAAAGTGTGTAATTCAAGAGATTACACACTTTGTCTTTGATTTTTTACGCGTg	66
KRAS shRNA-2R	aattcACGCGTAAAAAATCAAAGACAAAGTGTGTAATCTCTTGAATTACACACTTTGTCTTTGACg	66
KRAS shRNA-3F	gatccGAAGTTATGGAATTCCTTTTTCAAGAGAAAAGGAATTCCATAACTTCTTTTTTACGCGTg	65
KRAS shRNA-3R	aattcACGCGTAAAAAAGAAGTTATGGAATTCCTTTTCTCTTGAAAAAGGAATTCCATAACTTCg	65
KRAS shRNA-4F	gatccGAGATAACACGATGCGTATTTCAAGAGAATACGCATCGTGTTATCTCTTTTTTACGCGTg	65
KRAS shRNA-4R	aattcACGCGTAAAAAAGAGATAACACGATGCGTATTCTCTTGAAATACGCATCGTGTTATCTCg	65

HEK293T Hücre Hattı Transfeksiyon Sonrası İmmün-boyama görüntüleri

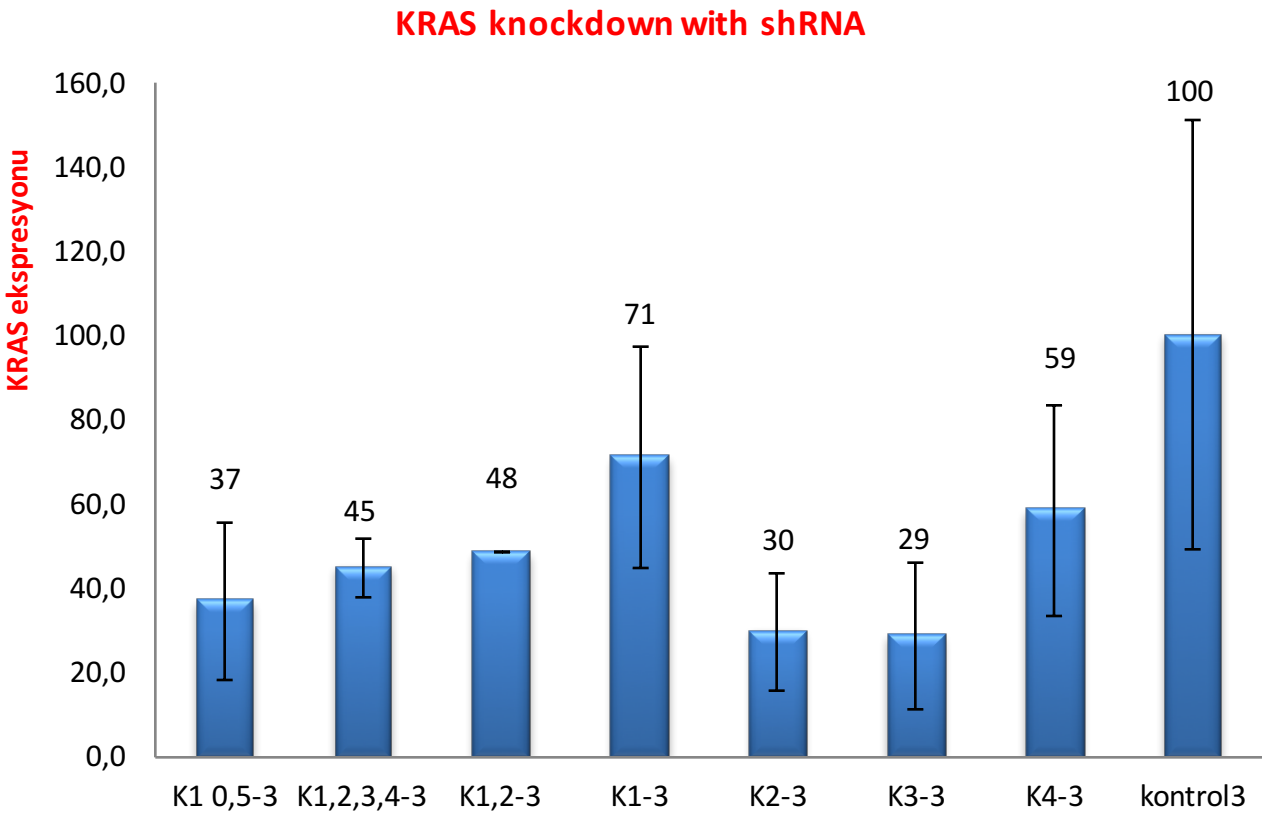








KRAS knockdown with shRNA



siRNA & shRNA

Criterion	siRNA	shRNA
Nomenclature	Small Interfering RNA	Short Hairpin RNA
Source	Laboratory synthesis	Nuclear expression
Delivery to the cell	Via synthetic/natural polymers and lipids to the cytoplasm	Via viral and other gene therapy vectors to the nucleus.
Persistence	99% degraded after 48 hours	Expressed for up to 3 years.
Administration	Local or limited systemic injection	Local and systemic injection
Dosage	High (low nM)	Low (5 copies)
Likelihood of specific 'off target' effects	Higher than shRNA	Lower than siRNA
Likelihood of non-specific 'off targets' effects	Higher immune activation, inflammation and toxicity	Lower immune activation, inflammation and toxicity
Application	Acute disease conditions; Where high doses are tolerable	Chronic, life threatening diseases or disorders; Where low doses are desirable

siRNA & miRNA

	siRNA	miRNA
Prior to Dicer processing	Double-stranded RNA that contains 30 to over 100 nucleotides	Precursor miRNA (pre-miRNA) that contains 70–100 nucleotides with interspersed mismatches and hairpin structure
Structure	21–23 nucleotide RNA duplex with 2 nucleotides 3'overhang	19–25 nucleotide RNA duplex with 2 nucleotides 3'overhang
Complementary	Fully complementary to mRNA	Partially complementary to mRNA, typically targeting the 3' untranslated region of mRNA
mRNA target	One	Multiple (could be over 100 at the same time)
Mechanism of gene regulation	Endonucleolytic cleavage of mRNA	Translational repression
		Degradation of mRNA
		Endonucleolytic cleavage of mRNA (rare, only when there is a high level of complementary between miRNA and mRNA)
Clinical applications	Therapeutic agent	Drug target
		Therapeutic agent
		Diagnostic and biomarker tool

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