



XNAs

NA = nucleic acids

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Overview

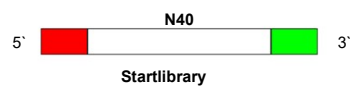
- RNA/DNA aptamers
- Synthetic modified mRNA
- siRNA
- miRNA

selection not easy, but chosen correctly can be used often

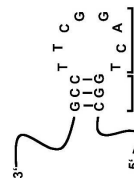
Aptamers

- The term is derived from the Latin word *aptus*, meaning to fit, and the Greek word *meros*, meaning piece)

- Short single-stranded DNA- or RNA-oligonucleotides (generally 25-90 nucleotides)
not that much

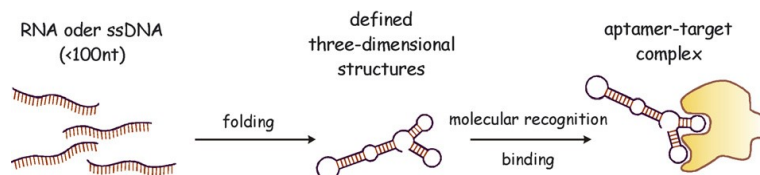


- They can be selected out of a huge oligonucleotide library consisting of approximately 10^{15} different sequences



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Aptamers



Stoltenburg R. et al., Biomolecular Engineering (2007)

Dissociation constants (K_d values) of aptamer-target complexes are in the picomolar to nanomolar range, which is comparable to and sometimes even better than those of some monoclonal antibodies

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Aptamers

Aptamers can be selected against a variety of targets

- Small molecules - like organic dyes, amino acids
- Antibiotics
- Peptides
- Proteins
- Viruses
- Bacteria
- Cells

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SELEX (Systematic Evolution of Ligands by Exponential Enrichment)

Aptamers are selected by a combinatorial chemistry process called SELEX

- Established independently by Ellington and Szostak, Tuerk and Gold, and Robertson and Joyce in 1990.

Tuerk and Gold: selected RNA aptamers specific for bacteriophage T4 DNA polymerase (gp43)

Ellington and Szostak: selected RNA aptamers specific for 6 different small organic dyes, e.g. Cibacron Blue 3GA, and Reactive Red 120

Robertson and Joyce: selected ribozymes (RNA molecules with a well defined tertiary structure that enables them to catalyze a chemical reaction) with improved enzymatic activity to cleave single-stranded DNA

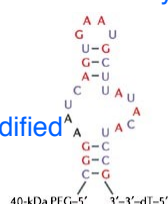
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Anti-VEGF RNA Aptamer for AMD therapy

The first representative of an aptamer drug is Macugen (Pegaptanib), a highly modified anti-VEGF RNA aptamer for treatment of age-related macular degeneration (AMD)

→ approved by FDA in 2004

only one for clinical use, but several in preclinical testing



only these 2 are not modified

2'-O-methylated purines are shown in red,
2'-fluoro-modified pyrimidines are shown in blue
Unmodified ribonucleotides are shown in black

At the 5'-end: Attachment of a 40-kDa polyethylene glycol moiety

increases the half life
At the 3'-end: thymidine cap

protects the RNA from exonucleases

Sequence and predicted secondary structure of pegaptanib.

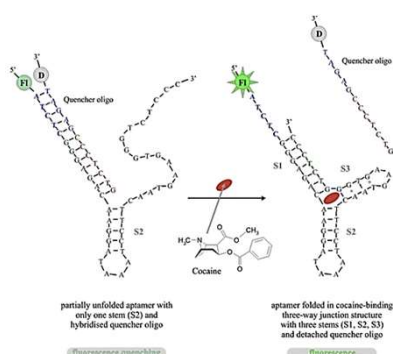
Ng E.W.M. et al., Nature Reviews Drug Discovery (2006)

aptamers are degraded, RNA more easily degraded than DNA

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Aptamer based assay to detect cocaine

Fluorescence resonance energy transfer (FRET)-based cocaine assay using a modified cocaine aptamer and a quencher oligonucleotide



In the absence of cocaine:

- the quencher oligomer hybridizes to the partially unfolded aptamer
- fluorophore and quencher come into close proximity and fluorescence is deleted

no cocaine no fluorescence signal

fluorescent assay to detect cocaine based on conformational changes of the anti-cocaine aptamer

F1 Fluorophore (quenched fluorescein)

 Fluorophore (dequenched fluorescein)

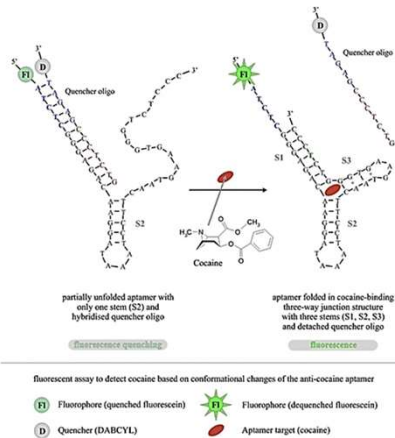
D Quencher (DABCYL)

Strehlitz B, Reinemann C, Linkorn S, Stoltenburg R - *Bioanal Rev* (2011)

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Aptamer based assay to detect cocaine

Fluorescence resonance energy transfer (FRET)-based cocaine assay using a modified cocaine aptamer and a quencher oligonucleotide



In the presence of cocaine:

- aptamer folds in stable cocaine-binding structure
- the quencher oligomer is displaced from the aptamer and released into the surrounding solution
- the fluorescence intensity increases in dependence of the cocaine concentration in the measuring solution

Strehlitz B, Reinemann C, Linkorn S, Stollenburg R - *Bioanal Rev* (2011) 9

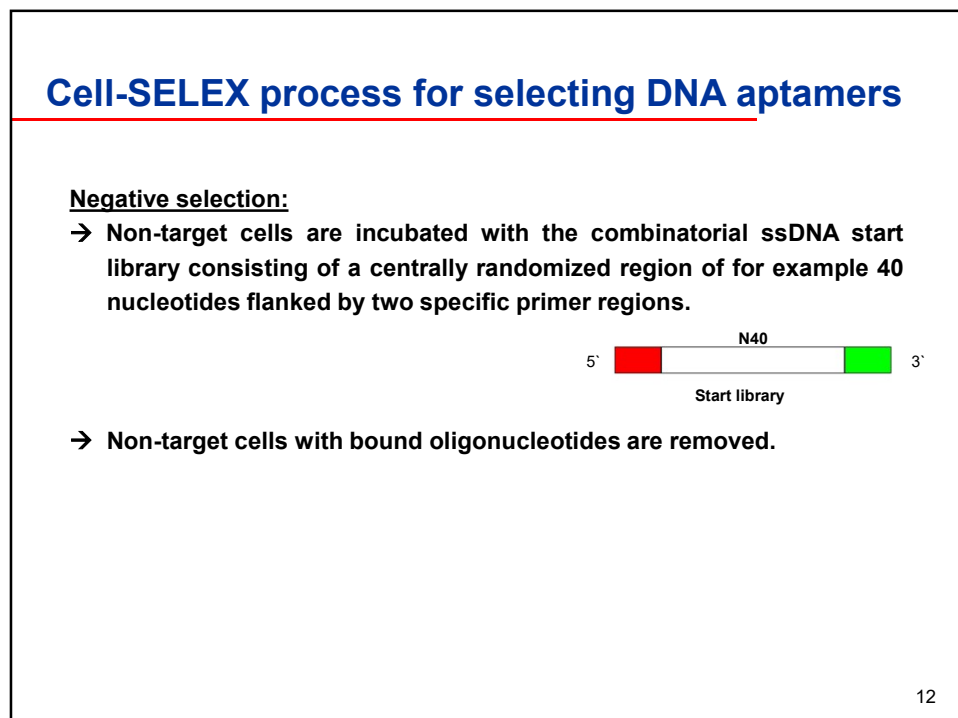
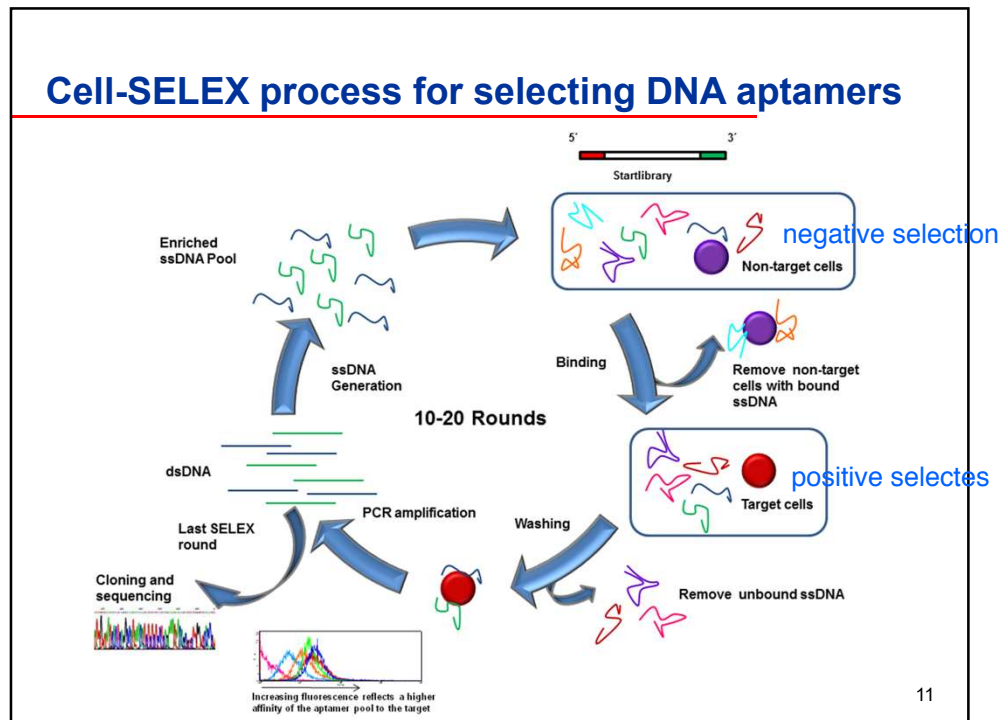
Cell-specific aptamers

Some aptamers obtained against whole cells:

- **Immunoglobulin Heavy Mu Chain**
(on Ramos cells, a B-cell Burkitt's lymphoma cell line)
- **Tyrosine-Protein Kinase-Like 7 Enzyme (PTK7)**
(on human T cell lymphoblast-like cell line, CCRF-CEM cells)
- **Interleukin 17 receptor A (IL-17RA)**
(on mouse embryonic fibroblast cell line, NIH3T3 cells)
- **Tenascin C**
(on human U251 glioma cells)
- **Human epidermal growth factor receptor 2 (HER-2)**
(on SKBR3 human breast cancer cell line)
- **Transforming growth factor-beta type III receptor (TbRIII)**
(on Chinese hamster ovary cells expressing TbRIII, CHO-TbRIII cells)

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negative selection step + positive selection cells



Cell-SELEX for selection of DNA aptamers

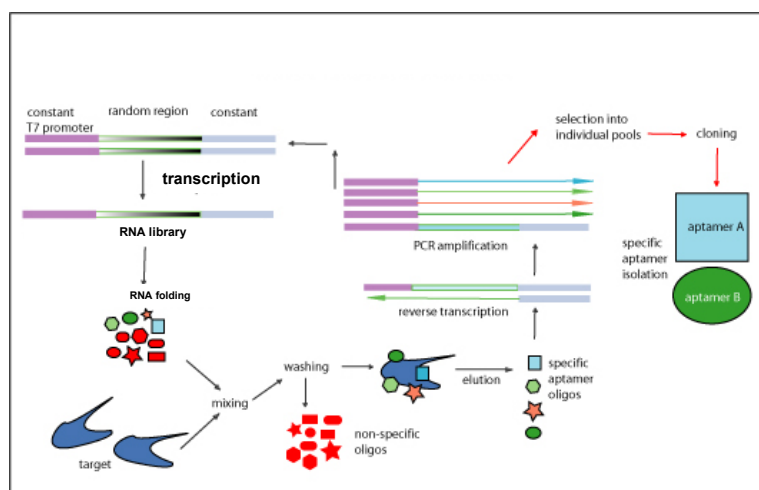
Positive selection:

The remaining ssDNA pool is incubated with the target cells.

- Unbound oligonucleotides are removed by washing steps.
- ssDNA bound to the cells is amplified by PCR using fluorescein and phosphate modified primers.
- PCR product is purified and ssDNA is generated by lambda exonuclease digestion of phosphorylated undesired strand.
- The resulting enriched ssDNA pool is used in the next SELEX round for the incubation with cells.
- The enrichment of target specific aptamers is monitored by flow cytometry.
- In the last SELEX round, the selected ssDNA molecules are cloned in *E.coli* and sequenced for aptamer identification.

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RNA-SELEX Process



http://www.genelink.com/newsite/products/images/aptamer_sellex.jpg

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DNA more cheaper,
RNA more sensitive
(exonucleases,...)

The advantage of cell-SELEX compared to protein SELEX

All cell surface molecules are

- presented in their natural surrounding and native conformation
- and
- contain possible posttranslational modifications

whole cells or select surface protein to perform protein SELEX,
cells -> in natural surrounding!

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Advantages of aptamers

- Aptamers can be easily and chemically synthesized with high reproducibility and accuracy.
- They remain stable in long-term storage and undergo reversible denaturation.
- In vitro selections can be performed without prior knowledge of the target structure on the cells.
- The target can be identified after the selection of aptamers.
 - New, previously undiscovered marker on cells can be identified.
- Negative selections with non-target cells can be performed to remove unspecific binders and to obtain aptamers with very discrete specificities.

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Advantages of aptamers

- Aptamers can be precisely adapted during their in vitro selection phase concerning their physicochemical properties to the later application.
- The aptamer stability and half-life in the body can be increased for example by chemical modification of the nucleotides at the 2'-position of the ribose using 2'-fluoro or 2'-amino modification or coupling to polyethylene glycol (PEG).
- Reporter molecules such as fluorescent dyes or biotin can be site-specifically inserted at defined sites of the aptamer.
- The smaller size of aptamers compared with that of antibodies (<15 kDa versus 150 kDa) facilitates their in vivo delivery by promoting better tissue penetration.

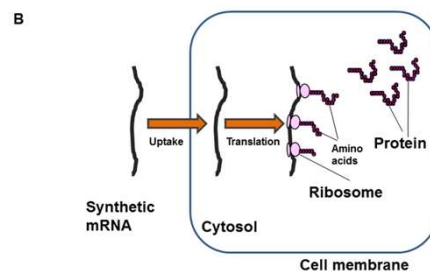
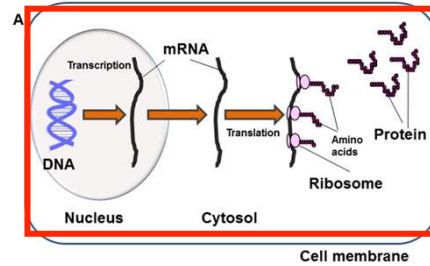
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Applications of cell-specific aptamers

- Sensors
- Diagnostics
- Imaging
- Therapeutics
 - Inhibition
 - Stimulation
 - Delivery of cargoes to cells
 - siRNA
 - Toxins
 - Small molecule drugs
- Regenerative Medicine

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Exogenous delivery of modified synthetic messenger RNA (mRNA)



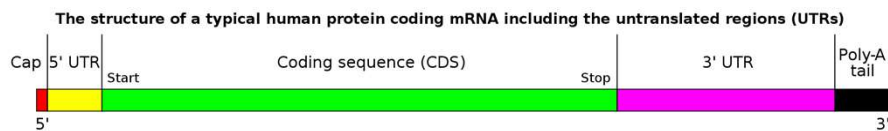
DNA not influenced

Avci-Adali M., et al. (2014) *J Biol Eng.* 8(1):8

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retroviral vectors go into the DNA and is incorporated into the DNA and protein is produced all the time but it is randomly, cancer can be caused

Structure of modified synthetic mRNA



http://upload.wikimedia.org/wikipedia/commons/thumb/b/ba/MRNA_structure.svg/2000px-MRNA_structure.svg.png

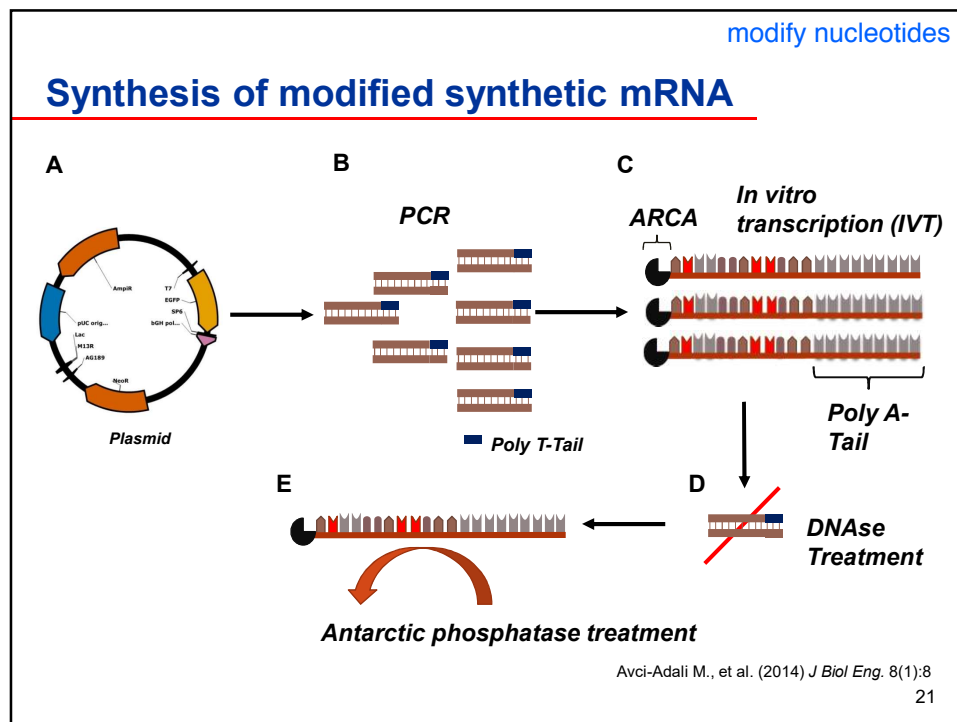
codes protein

UTR = untranslated region, important for translation and location of mRNA

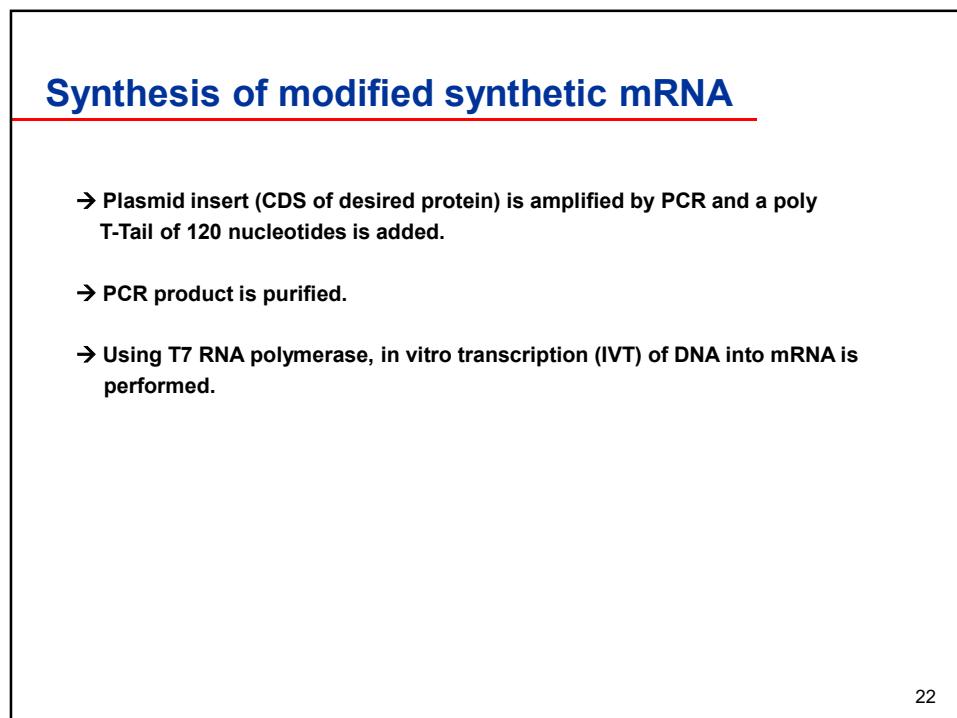
Cap = protection against exonucleases

Poly-A tail = small

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Disadvantages:
stability,
degradation,
translation (modified
nucleotides),
immune reaction



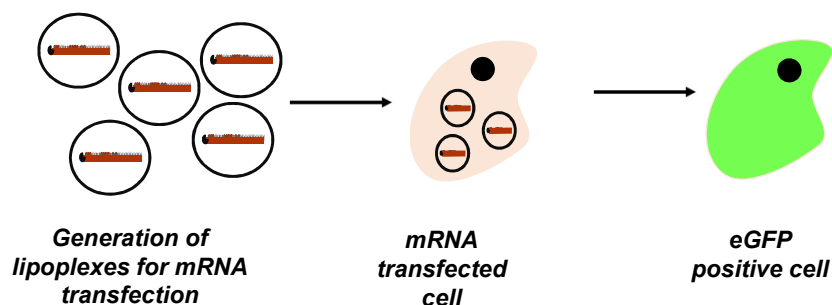
Synthesis of modified synthetic mRNA

- During IVT, anti reverse cap analog (ARCA), ATP, GTP, 5-methylcytidine-5'-triphosphate (5-Methyl-CTP), and pseudouridine-5'-triphosphate (Ψ) are used.
- Next, the template DNA is removed by DNase treatment of the IVT reaction mixture.
- After purification, mRNA is treated with Antarctic phosphatase to remove 5'-triphosphates. Thereby, mRNA is modified to reduce immune activation.
- The quality of the mRNA is analyzed by agarose gel electrophoresis.

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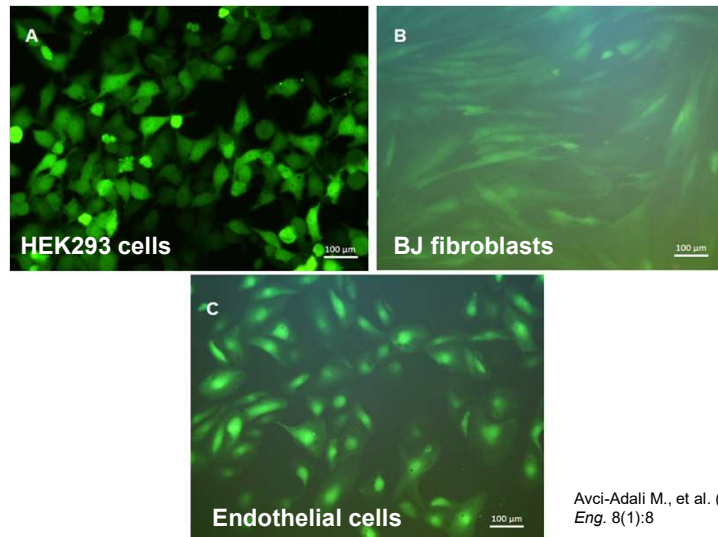
Transfection of cells with modified synthetic mRNA

nucleic acids have a negative charge,
surface of cells negative charge,
NA won't go into the cells -> delivery
system needed!



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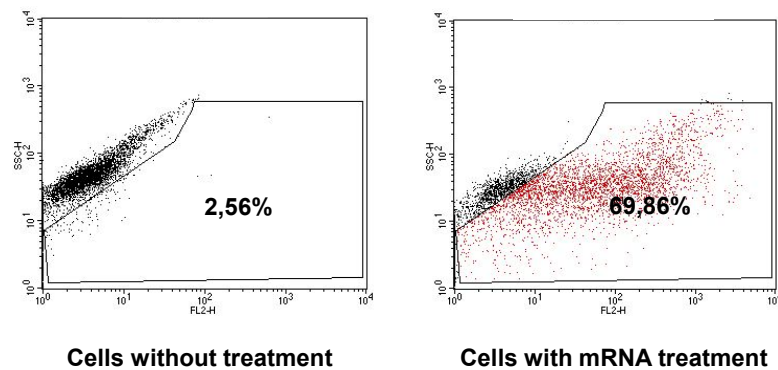
Transfection of cells with modified synthetic mRNA



Avci-Adali M., et al. (2014) *J Biol Eng.* 8(1):8 25

Transient expression of receptors on cells

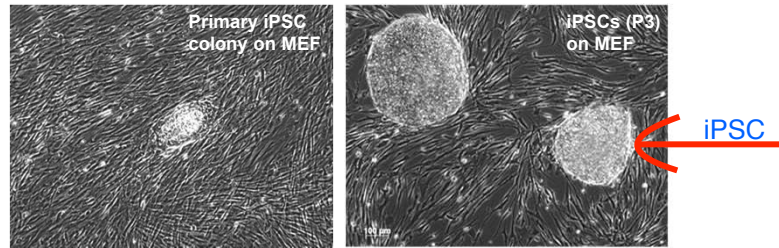
Treatment of cells with an mRNA coding a cell surface receptor



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Generation of iPSCs

Treatment of fibroblasts with Yamanaka factors (OCT4, KLF4, cMYC, LIN28, and SOX2) coding mRNAs for the generation of iPSCs (induced pluripotent stem cells). [used retroviral](#)

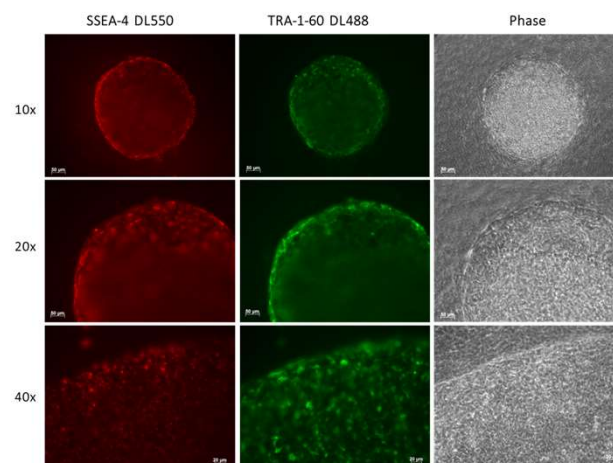


The first colonies appear after 13 days of treatment
with [synthetic mRNA](#)

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Generation of iPSCs

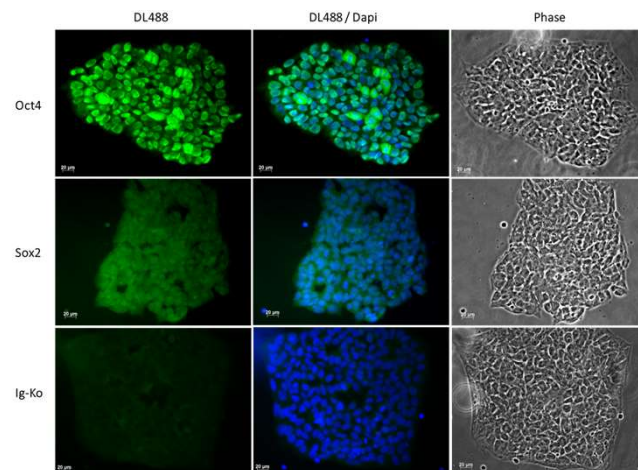
Characterization of iPSCs by fluorescence microscopy



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Generation of iPSCs

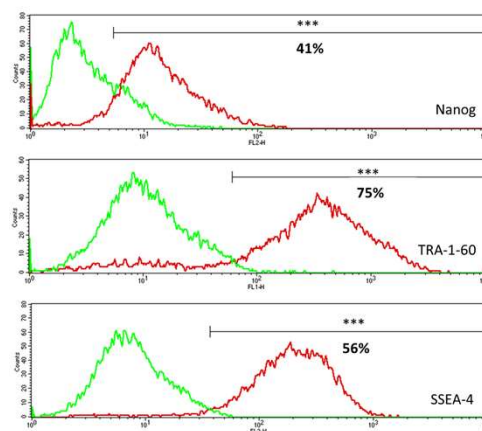
Characterization of iPSCs by fluorescence microscopy



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Generation of iPSCs

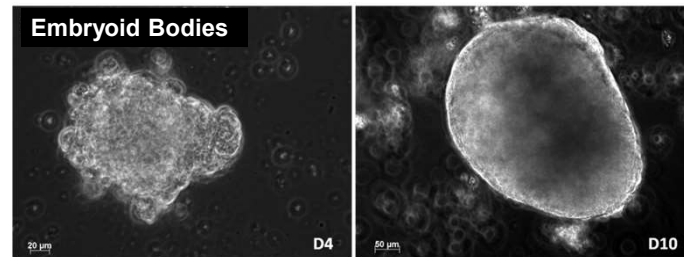
Characterization of iPSCs by flow cytometry



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Generation of iPSCs

Characterization of iPSCs

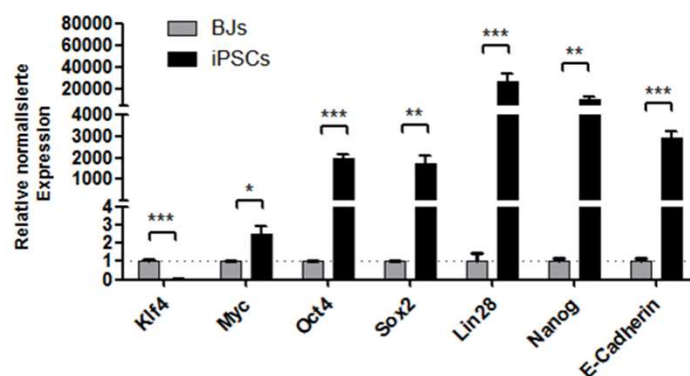


→ Formation of Embryoid Bodies

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Generation of iPSCs

Characterization of iPSCs using real-time RT-PCR



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Generation of iPSCs

Generation of stem cells from patient-derived somatic cells



Generation of desired autologous cells

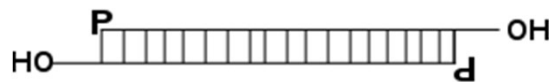
- tissue regeneration
- no rejection reaction

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mRNA:
iPSC,
to express proteins
that are normally
expressed but are not
due to a disease

siRNA (Small interfering RNA)

- 21-25 nt long
- double stranded RNA (dsRNA) with phosphorylated 5' ends (P) and hydroxylated 3' ends (OH) with two overhanging nucleotides
- Mostly exogenous origin

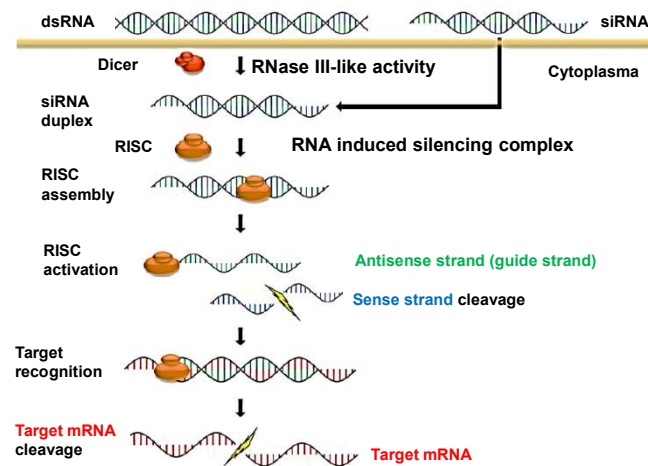


siRNA plays an important role in the RNA interference (RNAi) pathway.

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siRNA: used to
knock down
expression of
proteins

RNAi pathway



Koenig O, et al. Pharmaceuticals (Basel). 2013 6(7):881-914.

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RNAi pathway

- Long dsRNA is cleaved by RNase III, which is called Dicer.
- siRNA is incorporated into a multinuclease complex, RISC (RNA induced silencing complex).
- An RNA helicase within the complex unwinds the siRNA and the RISC becomes activated.
- The sense strand of the siRNA is cleaved.
- The remaining anti-sense strand-RISC complex can then bind to target mRNAs and lead to its degradation.
- The translation and expression of target mRNA are stopped.

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siRNA applications

Gene knockdown (decrease in the expression of a targeted gene)

- to study the function of genes in vitro and in vivo in model organisms
- for therapeutic gene modulation (e.g. downregulation of receptors)
- for antiviral therapy (e.g. inhibition of viral gene expression, knockdown of host receptors)
- to treat cancers by silencing genes that are upregulated in tumor cells or genes involved in cell division.

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Challenges: avoiding nonspecific effects

Off-target effects can occur, when the siRNA has a nucleotide sequence that can pair with other mRNA sequences than the target

- Thereby, the expression of multiple genes can be reduced.

Therefore:

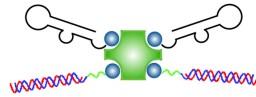
- multitude of software tools implementing algorithms for the design of specific siRNAs are used that automatically check possible cross-reactivity.

Furthermore, **negative control siRNAs (called scrambled siRNA)** should be used.
 shouldn't show any effect

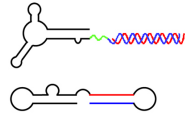
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Targeted delivery of siRNA

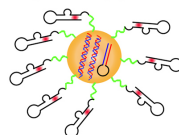
A Non-covalent aptamer-siRNA conjugates



B Covalent aptamer-siRNA or shRNA chimeras



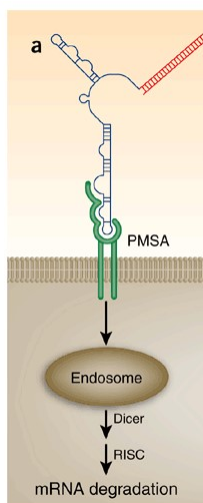
C Covalent aptamer-nanoparticle conjugates



Modified from Zhou et al. *Front Genet.* 2012

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Targeted delivery of siRNA



prostate cancer

RNA aptamer-siRNA chimeras

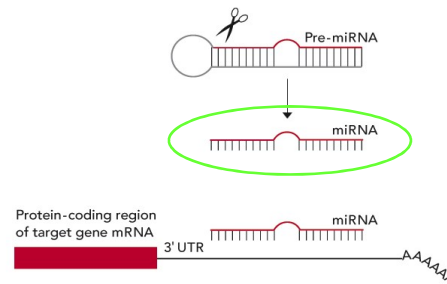
- Aptamer binds to the prostate-specific membrane protein (PSMA).
- After endosomal release, the chimeras are cleaved by Dicer, and the siRNAs enter the RNA-induced silencing complex (RISC) pathway.

Davidson, (2006) *Nature Biotechnology* 24, 951 - 952

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microRNA (miRNA)

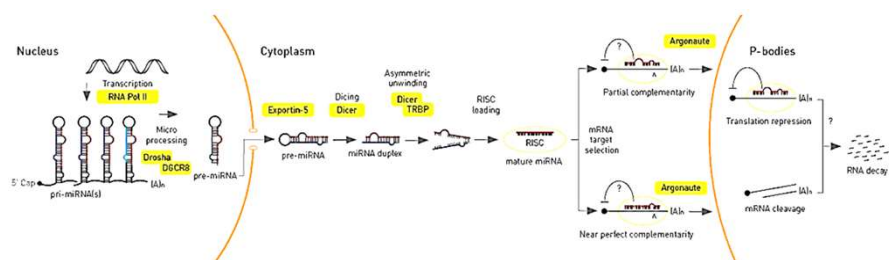
- Short, single-stranded RNA molecules approximately 22 nt in length
- Mostly endogenous origin
- miRNAs regulate the expression of genes affecting multiple cellular functions including growth and proliferation



Modified from
http://www.laskerfoundation.org/awards/images/2008basic_description.gif

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Transcription and processing of miRNA

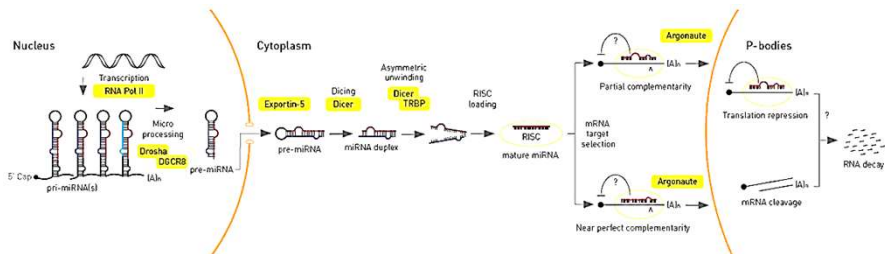


<http://www.exiqon.com/Is/PublishingImages/Figures/microRNA-biogenesis.htm>

- miRNA genes are transcribed by RNA polymerase II as large primary miRNA (pri-miRNA)
- pri-miRNA is processed by a protein complex containing the RNase III enzyme Drosha and dsRNA-binding protein, DGCR8, to form an approximately 70 nt precursor miRNA (pre-miRNA)
- Pre-miRNA is transported to the cytosol and processed by Dicer to generate a ds miRNA duplex of 22 nt

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Transcription and processing of miRNA

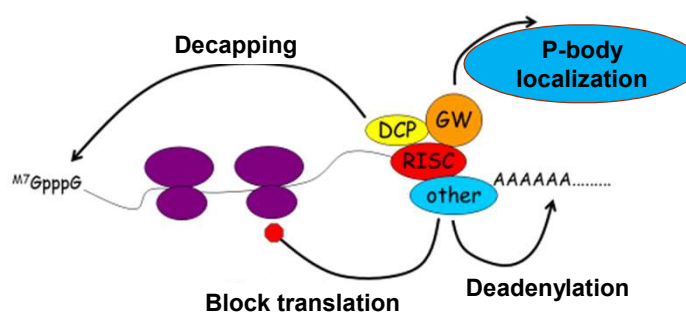


<http://www.exiqon.com/Is/PublishingImages/Figures/microRNA-biogenesis.htm>

- Following Dicer processing the miRNA duplex is rapidly unwound as it associates with Argonaute (Ago) proteins.
- One strand is retained to become the mature miRNA and is loaded into RISC.
- Depending on the level of complementarity between the miRNA and the target sequence, the mRNA can either be translationally repressed (partial complementarity) or cleaved (perfect complementarity).

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Mechanisms for translational repression



Modified from http://www.powershow.com/view/41352-MTJmN/RNAi_Mechanism_powerpoint_ppt_presentation

P-bodies (processing bodies) are cytoplasmic granules containing protein-RNA complexes
 → In P-bodies, miRNA-targeted mRNA can be sequestered from the translational machinery and degraded or stored for subsequent use.

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miRNA affects gene expression

- Due to partial base complementarity, individual miRNAs may target as many as 100 different mRNAs one miRNA is possible to do this
- Also, individual mRNAs may contain multiple binding sites for different miRNAs, resulting in a complex regulatory network

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Function of miRNAs

- Cell cycle control
- Apoptosis
- Stem cell differentiation
- Hematopoiesis
- Cardiac and skeletal muscle development
- Neurogenesis
- Insulin secretion
- Cholesterol metabolism
- Aging
- Immune responses

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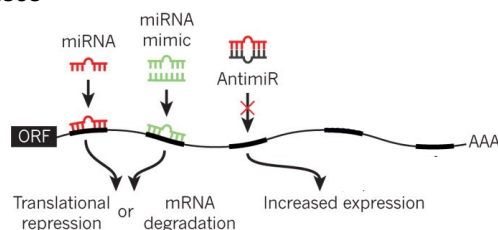
Function of miRNAs

- Highly tissue specific expression and distinct temporal expression patterns during embryogenesis → miRNAs play a key role in the differentiation and maintenance of tissue identity.
- miRNAs are involved in a number of diseases, such as cancer, heart disease and neurological diseases.

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Application of miRNAs

- miRNA mimics are used to restore a loss of function (miRNA replacement therapy)
They comprise the same nucleotide sequence as an endogenous miRNA.
→ aims to re-introduce miRNAs into diseased cells that are normally expressed in healthy cells, such as tumour suppressor miRNAs
- miRNA antagonists (antimiR) are complementary to an endogenous miRNA and can be used to inhibit endogenous miRNAs in diseased tissues
- miRNAs can be also used as diagnostic and prognostic biomarkers for diseases



Modified from Eric M. Small & Eric N. Olson, Nature (2011)

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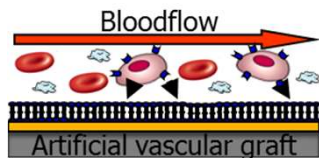
New strategies for vascular medical engineering

DNA- / RNA-technology platform

(modified and stabilized DNA / RNA)

DNA aptamers

Capturing molecules
for in vivo
endothelialization



siRNA / mRNA

Gene Silencing
or
Induction of protein
synthesis



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Summary

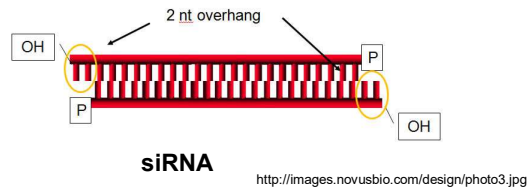
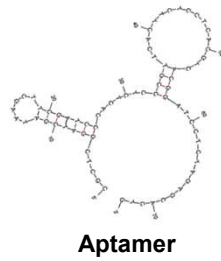
Different nucleic acid classes can be used for targeting, gene knockdown, gene regulation, or induction of protein synthesis.

EXAM!!!!

- How can be DNA aptamers selected against cells (SELEX)?
- How can be modified mRNAs synthesized?
- Structure of siRNA and miRNA? The difference?
structure, length, double/single strand
- What is RNAi?

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Thank you for your attention!



<https://www.youtube.com/watch?v=tzIGU5EI9rU> <https://www.youtube.com/watch?v=t5jroSCBBwk>

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