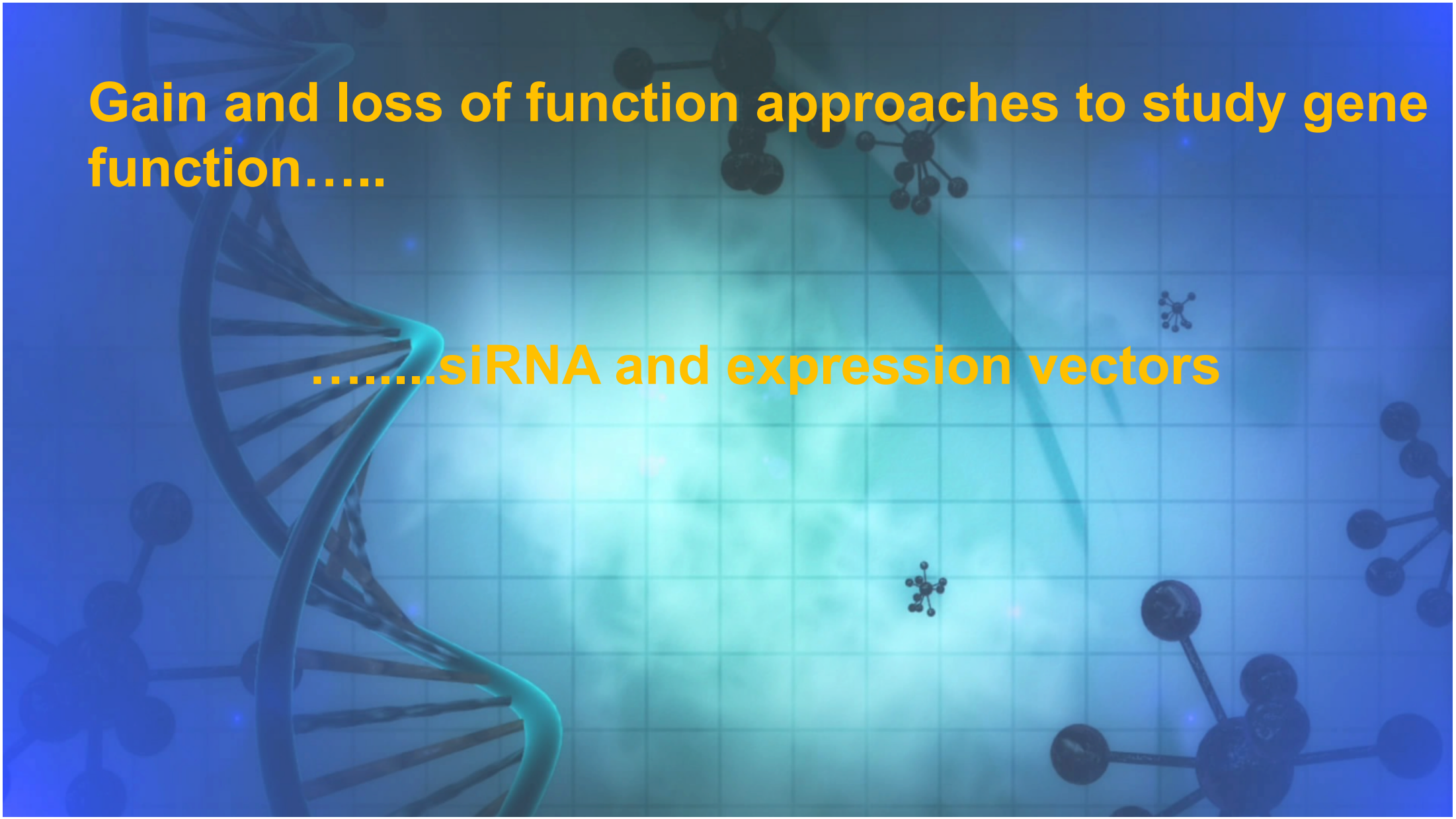
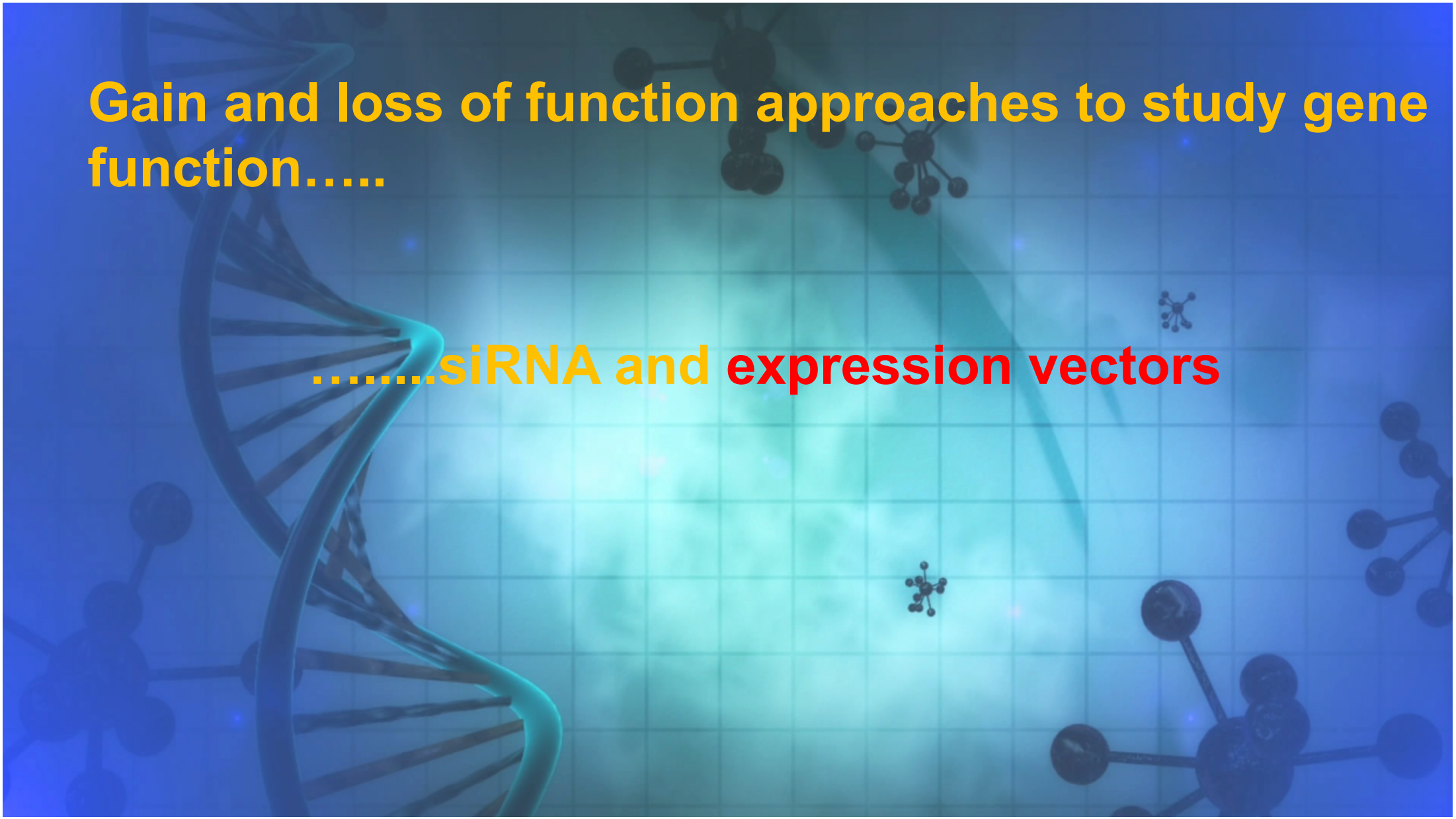
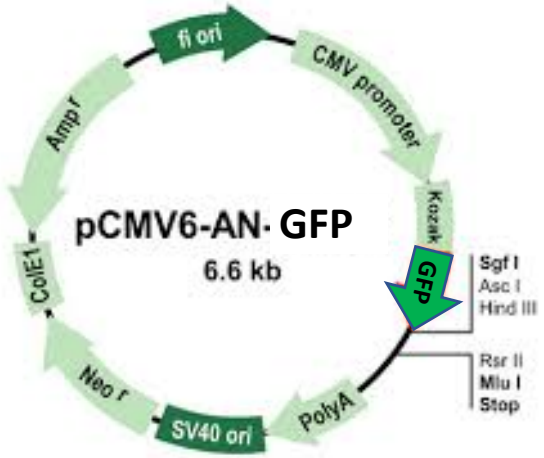
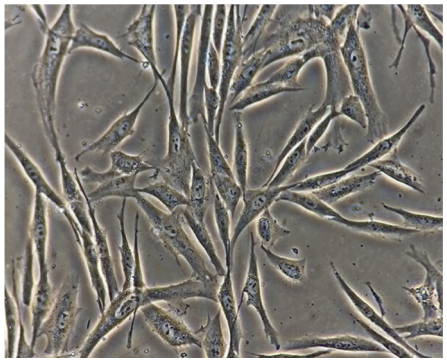

Gain and loss of function approaches to study gene function…..
……..siRNA and expression vectors


Gain and loss of function approaches to study gene function…..
……..siRNA and expression vectors

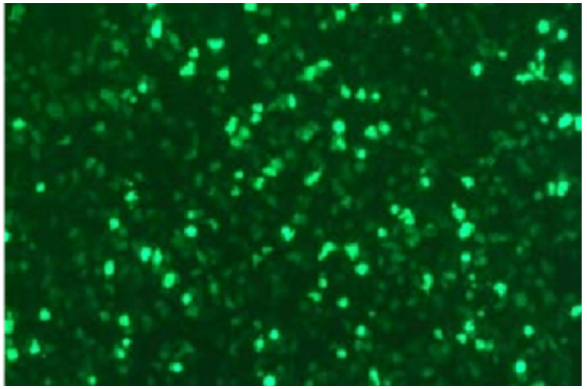
Ectopic expression of genes using gene expression vectors



insert



Transcription and translation of GFP



microscope

GFP: green fluorescent protein

Bacteria:

- ColE1 origin (replication in E.coli)
- Ampicilin resistance

Vertebrate cells:

- vertebrate promoter: active in vertebrate cells (i.e CMV)
- Kozak sequence: upstream of start codon of gene of interest (for eukaryotic initiation of translation
- Gene of interest (open reading frame for GFP)
- Poly A site for termination of transcription
- Resistance marker for selection in vertebrate cells (NeoR, Neomycin inibits translation by binding to the 35S ribsome subunit)

Optional: SV40 ori, allows replication using SV40 replication system

→ **Shuttle vector**: replicates in E-coli and other species (vertebrate)

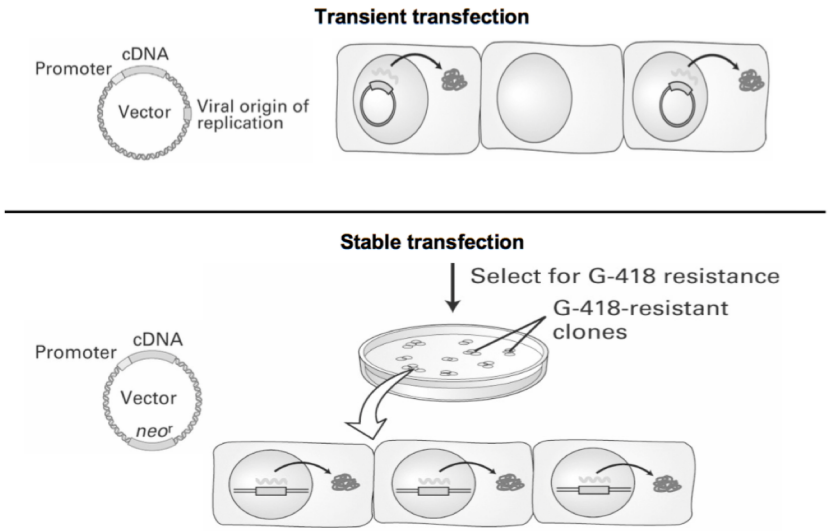
Introduction of vector DNA into mammalian cells

Ectopic expression of genes

Introduction of foreign DNA in cells = TRANSFECTION

- **INCUBAZIONE CON DNA CO-PRECIPITATO CON FOSFATO DI CALCIO O DIETILAMINOETIL-DESTRANO (DEA)**
- **ELETTROPORAZIONE**
- **LIPOSOMI**

- **Transduction = VEICOLAZIONE DI DNA DA PARTE DI UN VIRUS (Retrovirus, Lentivirus)**



Transient transfection

High expression over limited time (2-3 days)
Plasmid is not segregated by mechanism and will get lost in a few days

- → **Electroporation**
- → **Lipofection**
- → **Calciumphosphate**

Stable transfection

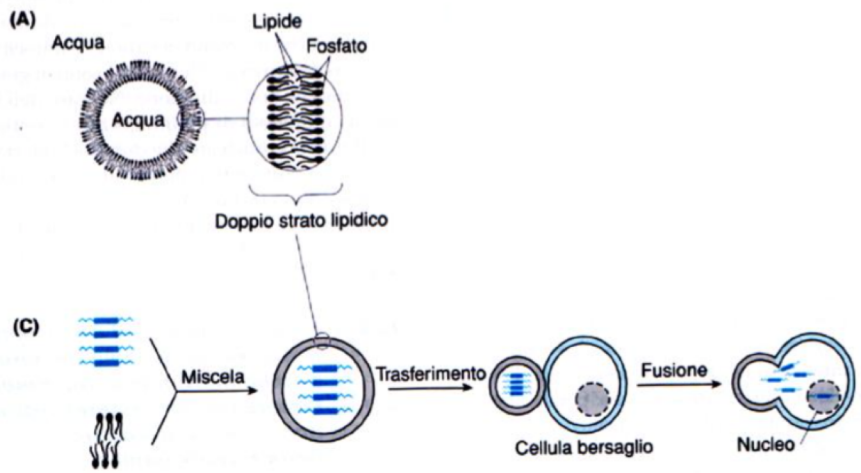
Continuous expression; requires stable integration of vector into genomic DNA; vector must contain a selectable marker (i.e. resistance to neomycin)

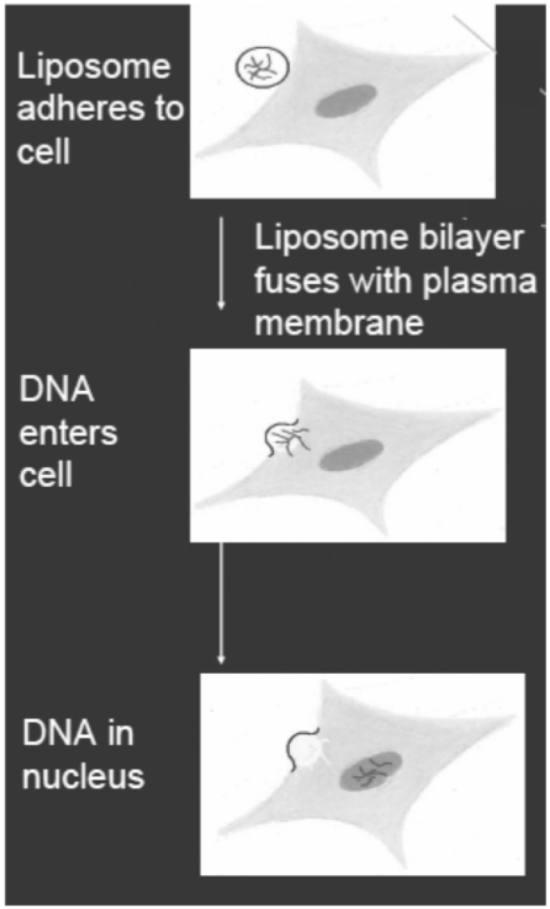
- → **Electroporation**
- → **Lipofection**
- → **Calciumphosphate**
- → **Viral transduction (stable and episomal)**

After introduction of vector→apply selection (i.e. Neomycin for genome integrants

Alternative: SV40 system allows episomal maintenance of plasmid

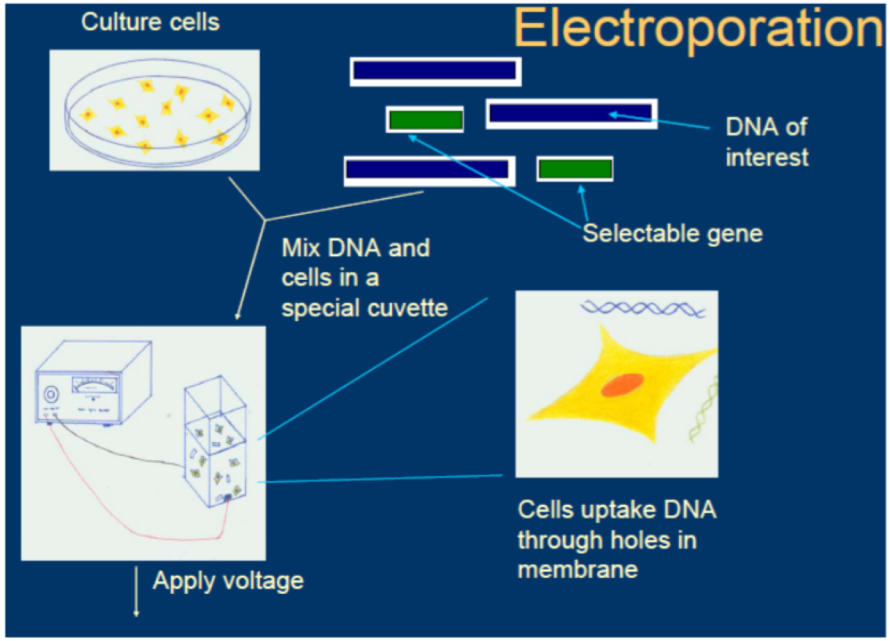
LIPOFECTION OF MAMMALIAN CELLS





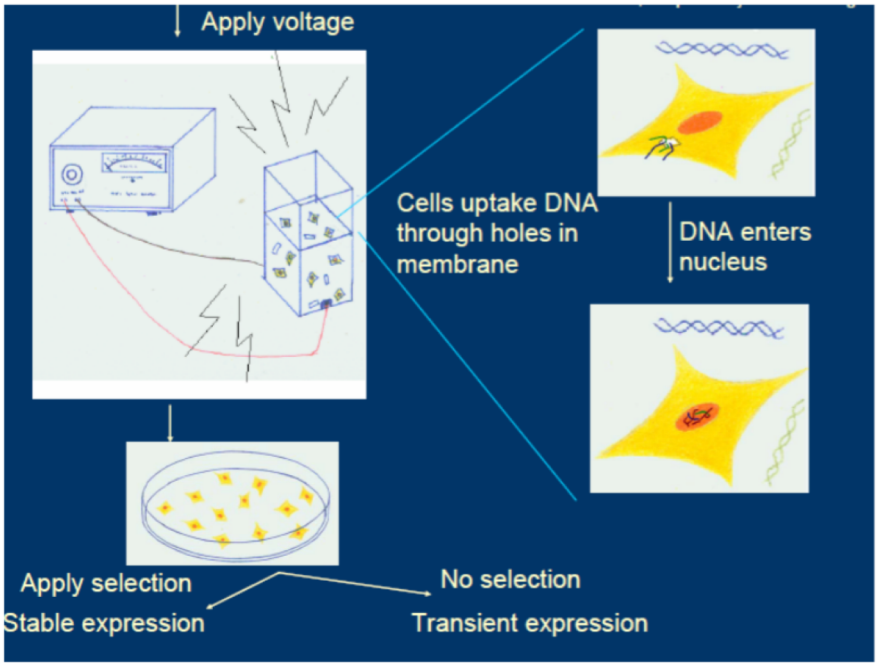
Lipids form spheres and integrate DNA
Lipids fuse with nuclear membrane, DNA enters cell
DNA (vector) ready for transcription

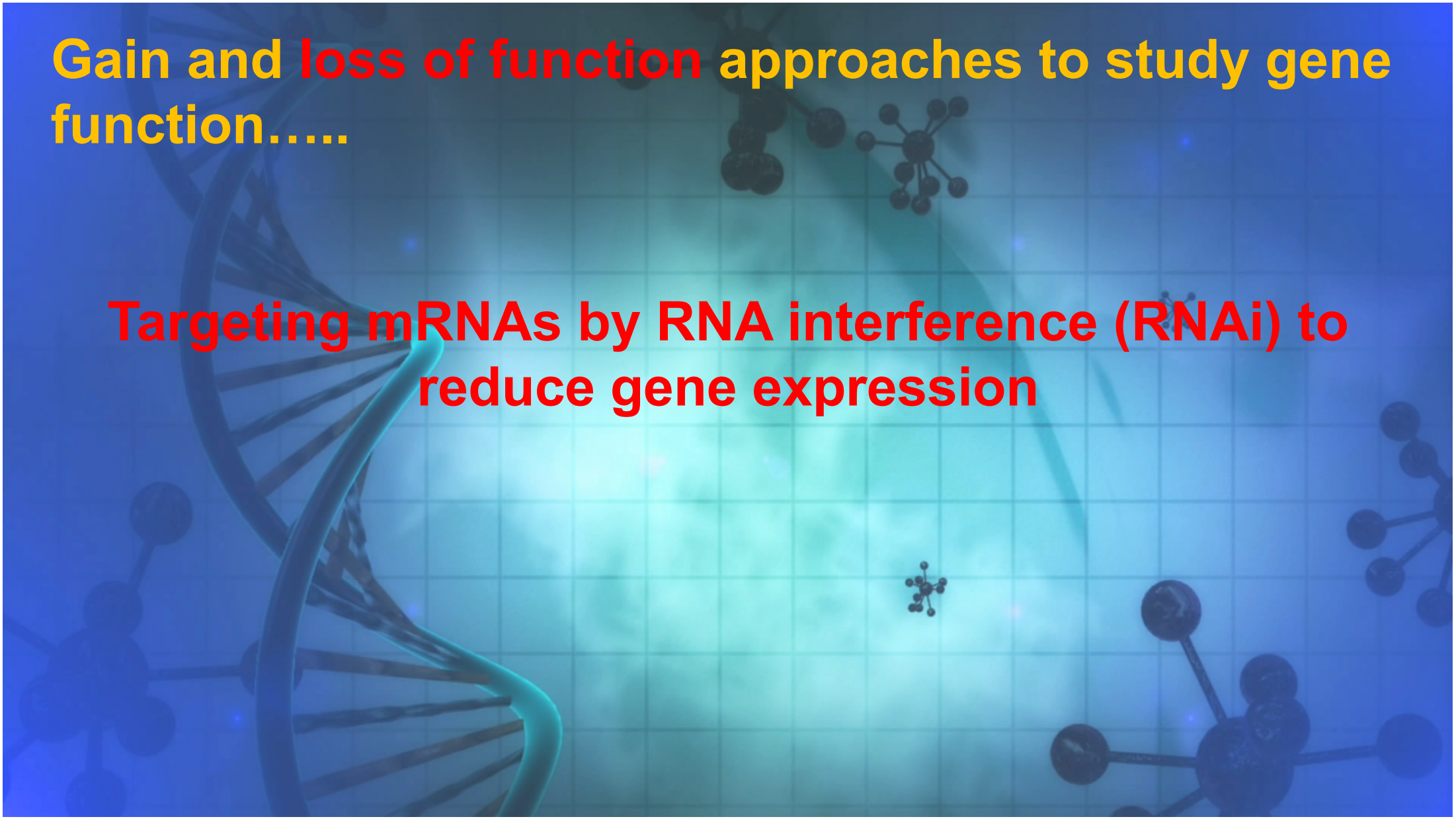
ELECTORPORATION OF MAMMALIAN CELLS



DNA of interest: linear or cicular

Application of current cause transient perforation of cell membrane and DNA can enter cell.

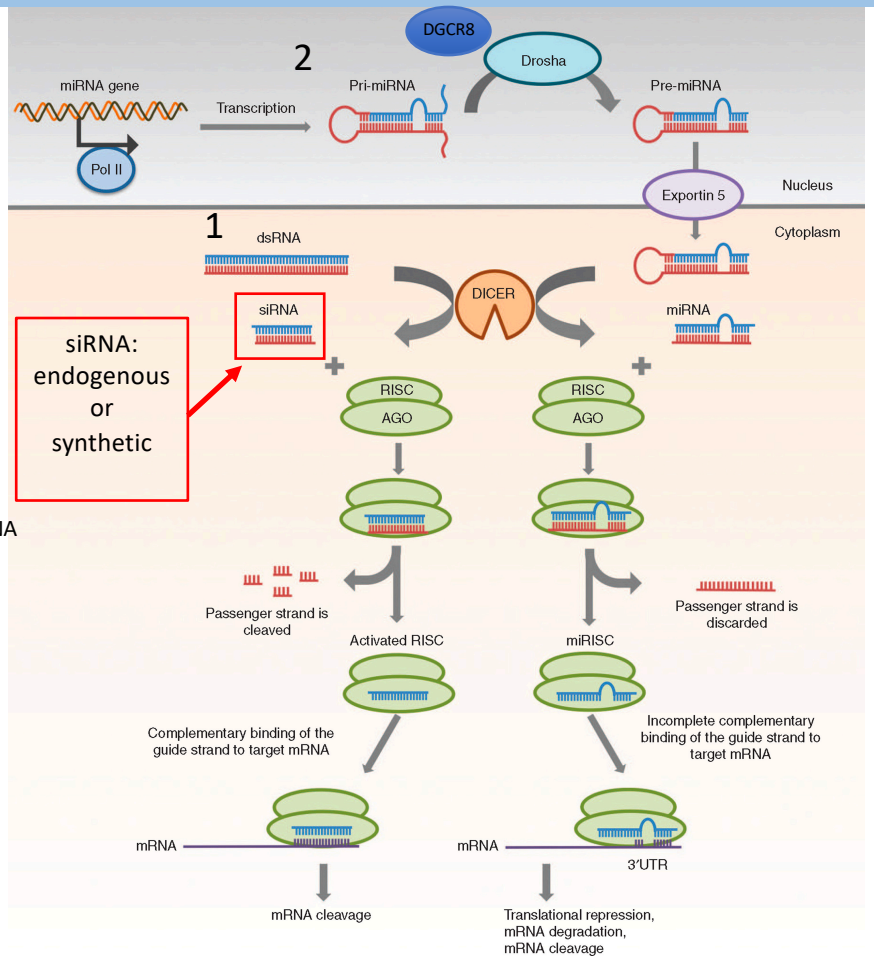



Gain and loss of function approaches to study gene function…..
Targeting mRNAs by RNA interference (RNAi) to reduce gene expression

siRNA and miRNA biogenesis and gene regulation

1. siRNA biogenesis

1. dsRNA production and transfer to cytoplasma
2. RNaseIII family enzyme Dicer processes pre-miRNA generating a 20-25 base dsRNA with overhang at the 3'end (2 bases)
3. Transfer of dsRNA to RISC complex (RNA induced silencing complex)
4. Selection of guide RNA → regulatory RNA passenger RNA → will be eliminated
7. RISC complex+guide RNA → regulatory function
   A. Perfect target RNA matching → RNA degradation = siRNA effect (cutting = "slicing")



2. miRNA biogenesis

1. Long, unprocessed precursor dsRNA or stem loop RNA **(pri-miRNA)** produced in a independent and controlled manner from miRNA hosting gene
2. Processing in the nucleus by the RNaseIII family protein Drosha generates a stem-loop RNA with characteristic length of 65-70 nucleotides. Drosha is in complex with DGCR8 that is important for Drosha activity
3. Exportin 5-RanGTP transports pre-miRNA in ternary complex thought nuclear pore to cytoplasm. RanGAP stimulates GTP; pre-miRNA released from Exportin.
4. RNaseII family enzyme Dicer processes pre-miRNA generating a 20-25 base dsRNA with overhang at the 3'end (2 bases)
5. Transfer of dsRNA to RISC complex (RNA induced silencing complex)
6. Selection of guide RNA → regulatory RNA passenger RNA → will be eliminated
7. RISC complex+guide RNA → regulatory function
   A. RNA degradation = siRNA effect (cutting = "slicing"
   B. inhibition of mRNA translation =mRNA effect
   C. transfer to nucleus and chromatin regulation = siRNA mediated silencing

Transfection of cells with synthetic siRNAs that pair to a target RNA can be used to degrade RNA target

Generation of artificial siRNAs to alter gene expression

It is difficult to introduce long dsRNA strands into mammalian cells due to the interferon response, the use of siRNA mimics has been more successful.

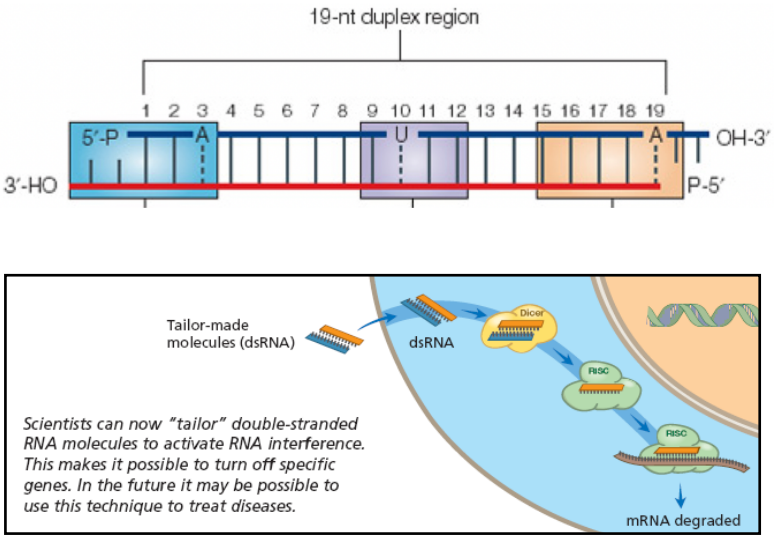
First applications to reach clinical trials were: the treatment of macular degeneration and respiratory syncytial virus,

RNAi has also been shown to be effective in the reversal of induced liver failure in mouse models.

Other proposed clinical uses center on antiviral therapies:

- HSV type 2
- knockdown of host HIV receptors
- silencing of HIV, HAV,HBV and flu genes
- inhibition of measles viral replication.

Synthetic siRNAs mimic the product by Dicer processing and are optimized for mRNA targeting



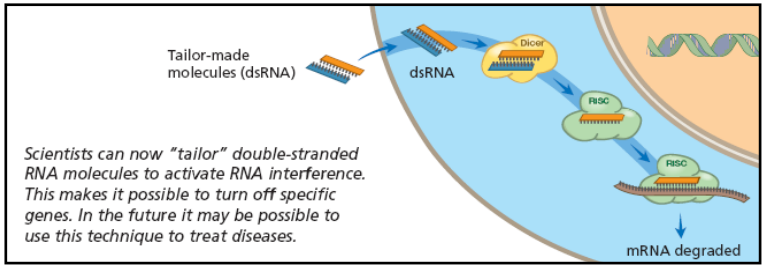
Viruses like HIV-1 are particularly difficult targets for RNAi-attack because they are escape-prone, which requires combinatorial RNAi strategies to prevent viral escape.

RNAi
dalla teoria alla pratica di laboratorio

convertire un meccanismo biologico in uno strumento per eliminare l'espressione di un gene di interesse in modo semplice --> siRNA sintetici

Come disegnare un siRNA in lab

Trasfezione transiente → liposomes



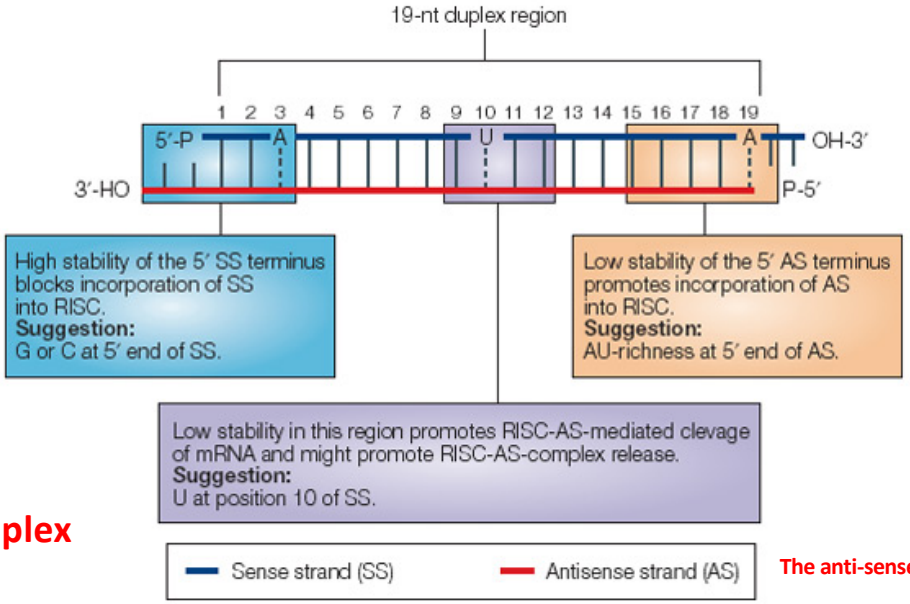
siRNA PROFILING TECHNOLOGIES

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' overhang
- Distribuzione nucleotidica
- Controllo della specificità



- siRNAs are chemically synthesized
- target RNA specific
- short dsRNAs
- Transfected into cells
- siRNAs are processed by the RISC complex
- target RNA and induce cleavage

The anti-sense strand is complementary to the target RNA

Come disegnare un siRNA in lab

Preventing Off-Target Effects

siRNA sequences are short:
Risk that one siRNA targets more than one gene
Check unique targeting *in silico*

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.

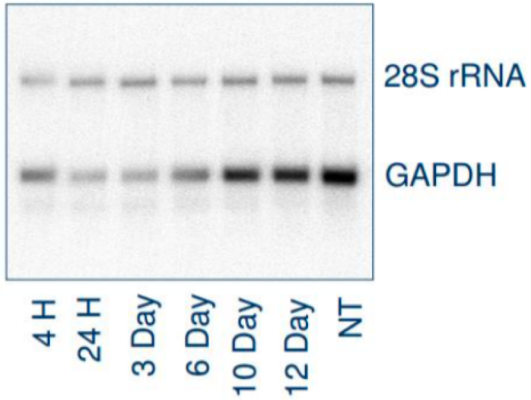
Verify specificity of RNAi effect by testing independent siRNAs to the same target

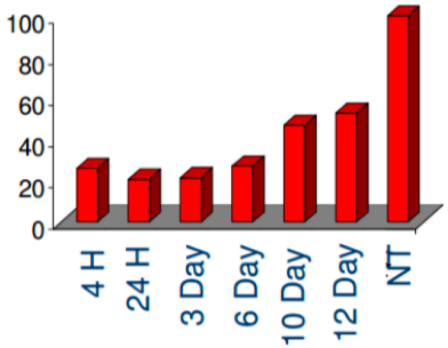
Durata del silenziamento transiente

Trasfezione transiente → **liposomes or electroporation(see expression vectors)**

siGAPDH

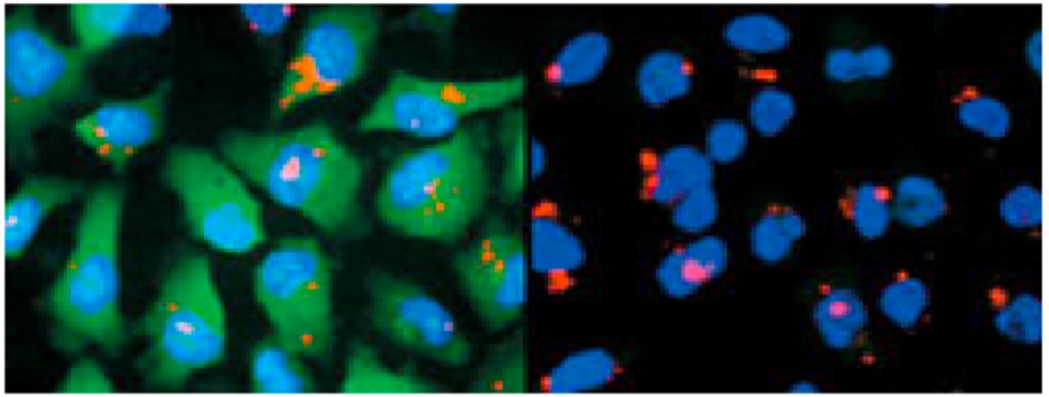
Northern blotting





CELLULE HeLa

In rosso: siRNA marcato
In blu: nuclei
In verde la proteina GAPDH



si RNA non specifico

si RNA contro GAPDH

Trattamento: 48h

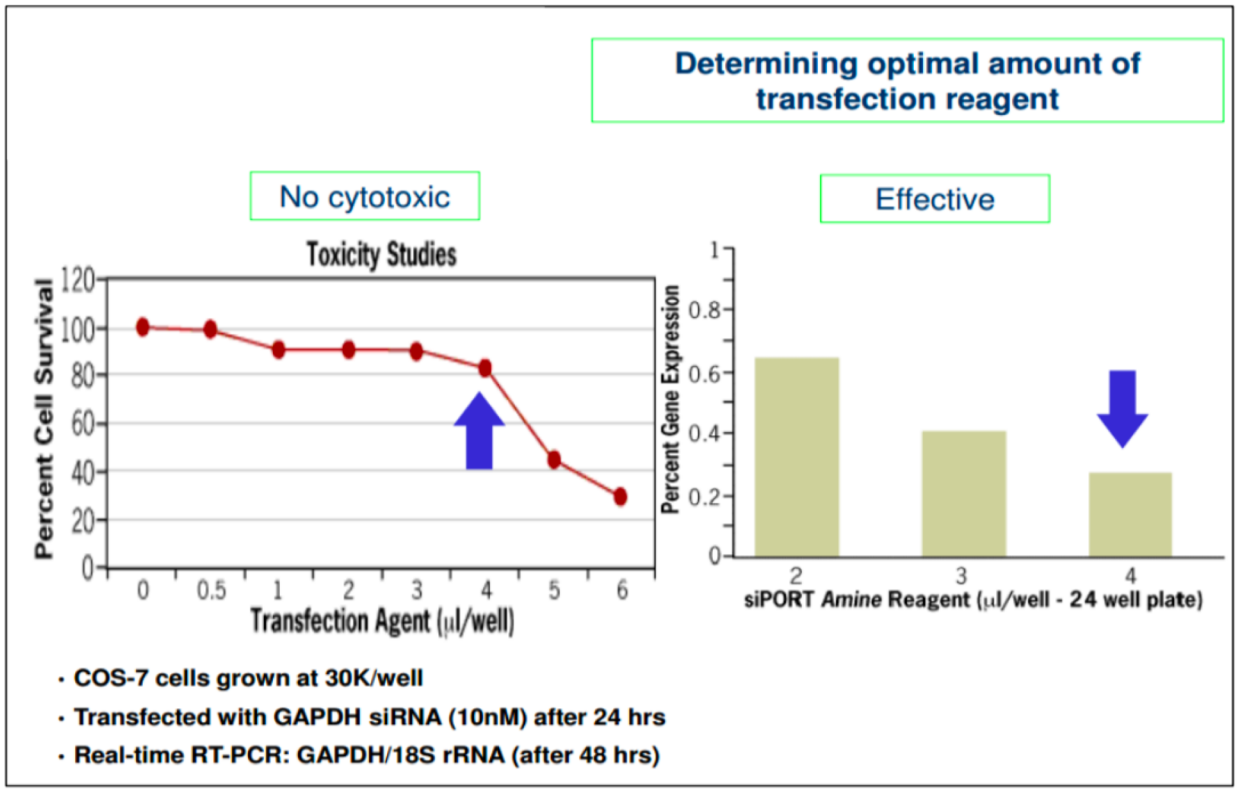
Trasfezione con il siRNA: ottimizzazione delle condizioni

Prevenire effetti di spegnimento del target:

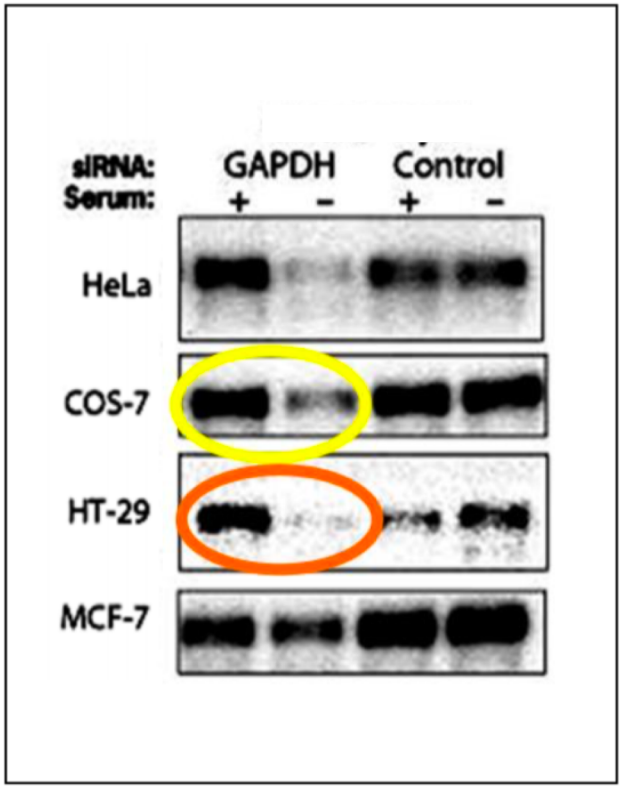
- **Basse concentrazioni** (~5-30 nM) di siRNA per minimizzare l'attivazione l'**interferon pathway** come risposta anti-virale
- E' preferibile usare un solo siRNA molto efficiente piuttosto che una miscela di siRNA meno potenti, la MIXTURE fa aumentare la concentrazione totale
- Usare RNAi specifici, dopo aver effettuato test di siRNA differenti sullo stesso mRNA bersaglio

Trasfezione con il siRNA: ottimizzazione delle condizioni

Scelta del reagente trasfettante

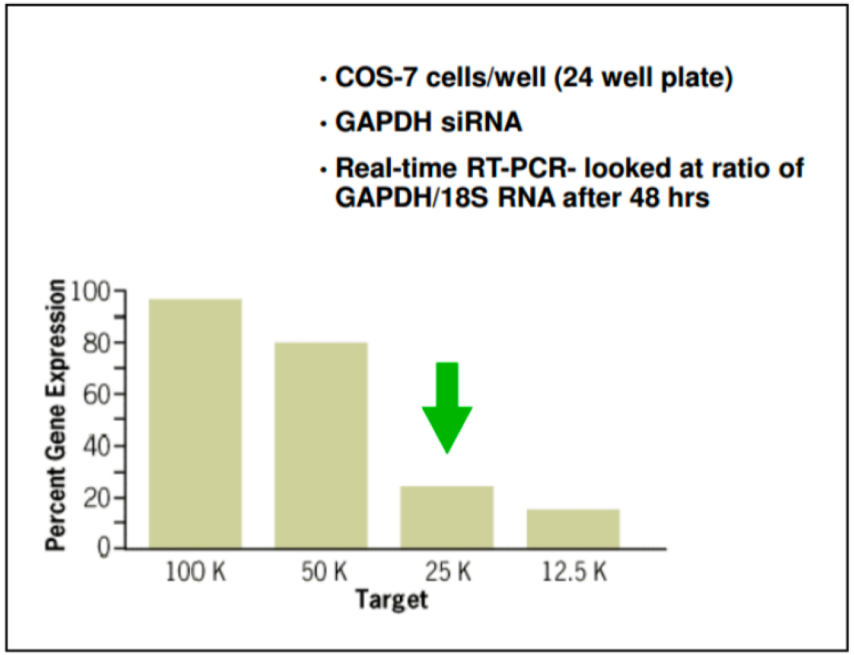


Presenza ed assenza di siero

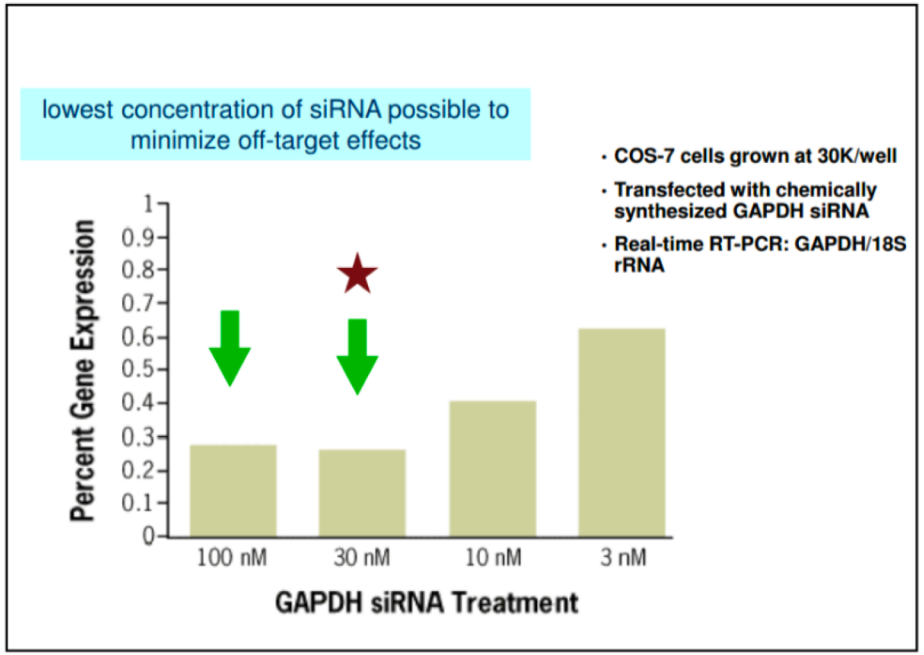


Trasfezione con il siRNA: ottimizzazione delle condizioni

Determinare la densità di semina delle cellule



Concentrazione di siRNA



Dal transiente alla trasfezione con vettori

PRO

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

CONTRO

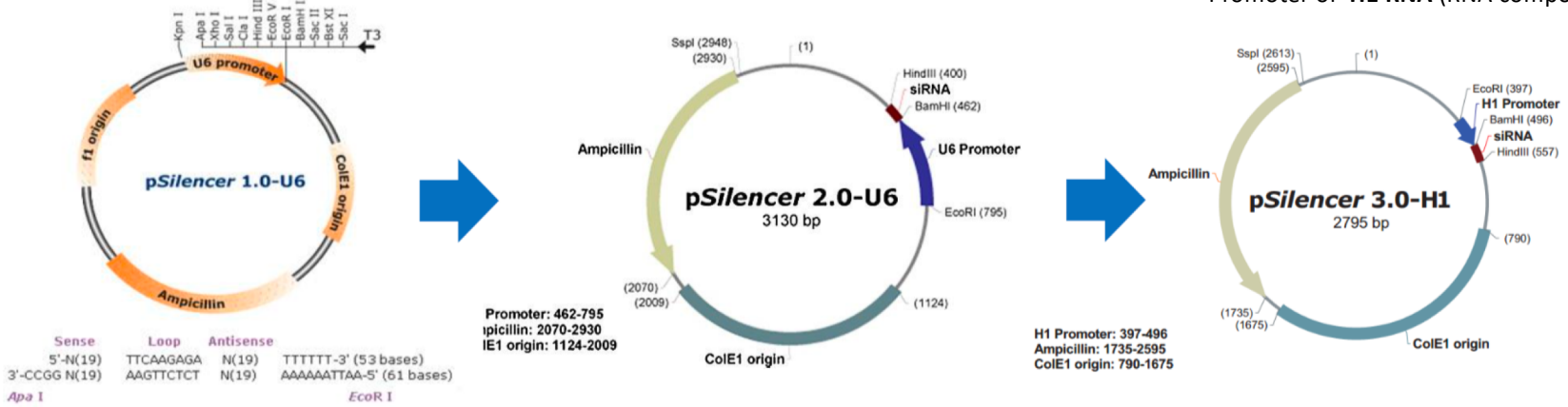
- Alcune cellule sono refrattarie alla trasfezione e la loro elettroporazione spesso causa morte cellulare
- I siRNA sono stabili, ma la trascrizione può risultare transiente se le cellule si duplicano molto in fretta diluendo il silenziamento e la vita media della proteina

Superamento del problema mediante……

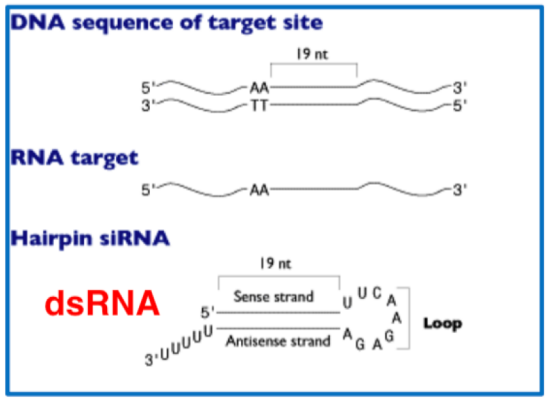
Dal transiente alla trasfezione con vettori

Silenziamento tramite vettori a DNA

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

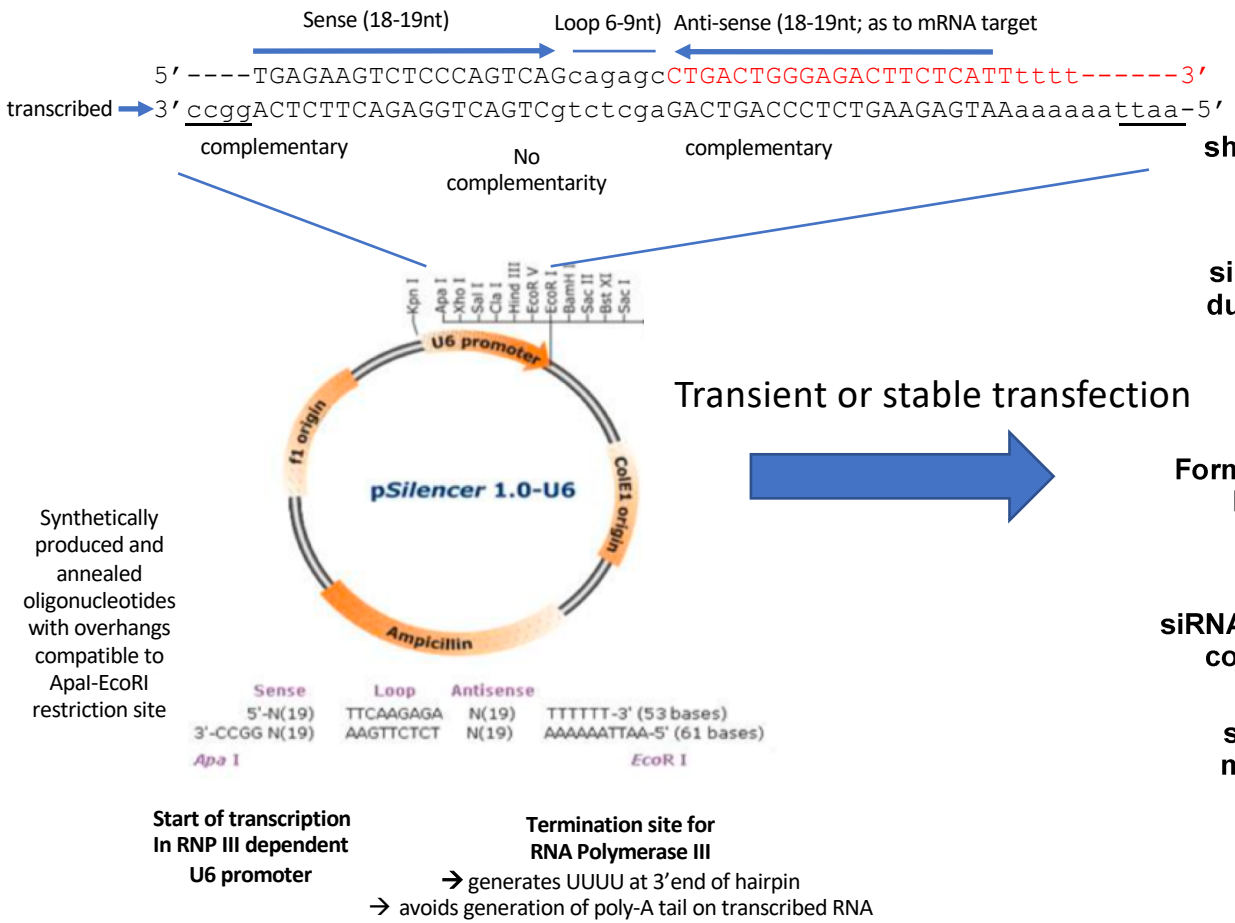


Mimicking miRNA production



- 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**

Dal transiente alla trasfezione con vettori



Synthetically produced and annealed oligonucleotides with overhangs compatible to ApaI-EcoRI restriction site

Start of transcription In RNP III dependent U6 promoter

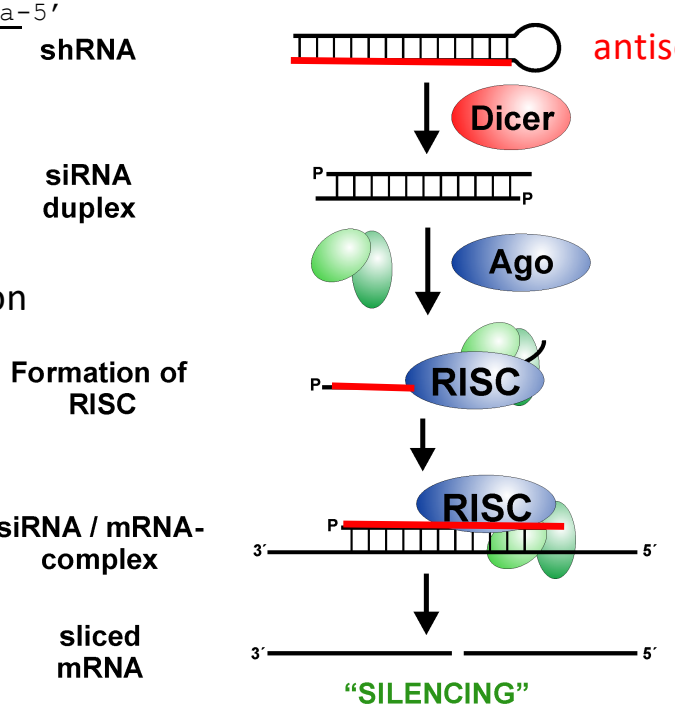
Termination site for RNA Polymerase III

→ generates UUUU at 3'end of hairpin

→ avoids generation of poly-A tail on transcribed RNA

Transient or stable transfection

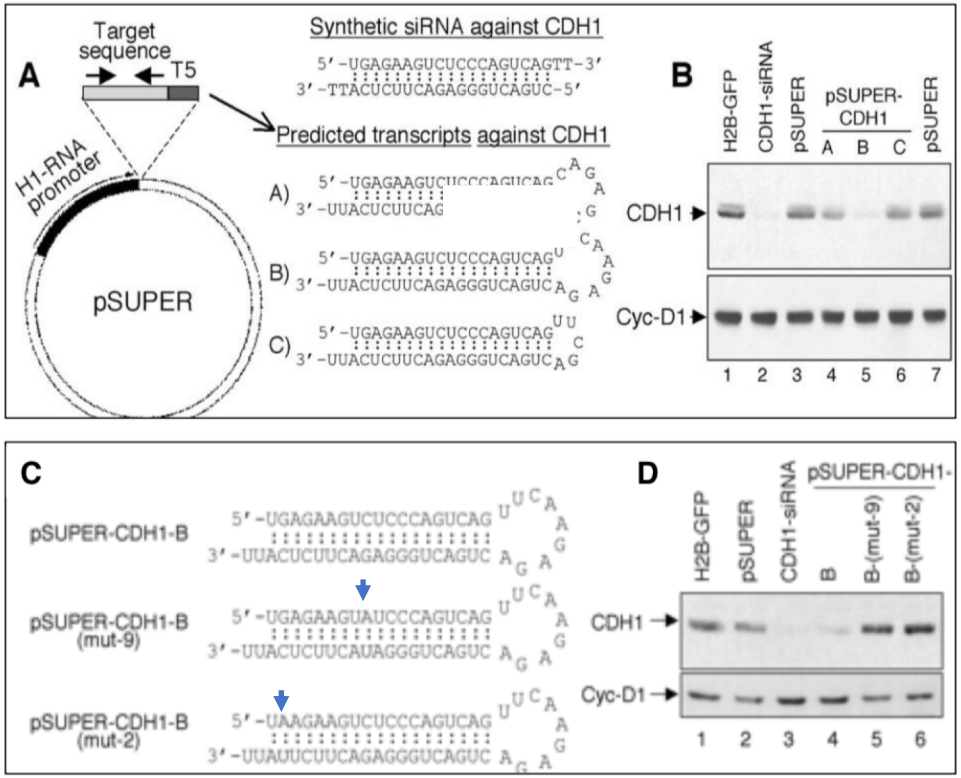
RNA Pol III dependent transcription generates an artificial type of pre-miRNA



In cytoplasma of transfected cell

Espressione stabile di shRNA

Un sistema per l'espressione stabile di *short interfering RNA* in cellule di mammifero: vettore plasmidico



Hairpin sequence and size can alter processing of shRNA by Dicer

Mutations inserted in stem destroys targeting of CDH1 mRNA targeting