Lecture 7

Viral vectors,
continued…

21st November 2011

Zakład Biotechnologii Medycznej Wydziału Biochemii, Biofizyki i Biotechnologii UJ
oraz Oddziały Krakowskie Polskiego Towarzystwa Biologii Komórki i Polskiego Towarzystwa Biochemicznego

serdecznie zapraszają na wykład, który wygłosi

Prof. Dr Jürgen Hescheler

Institute of Neurophysiology, University of Cologne, Germany

President of the German Society for Stem Cell research

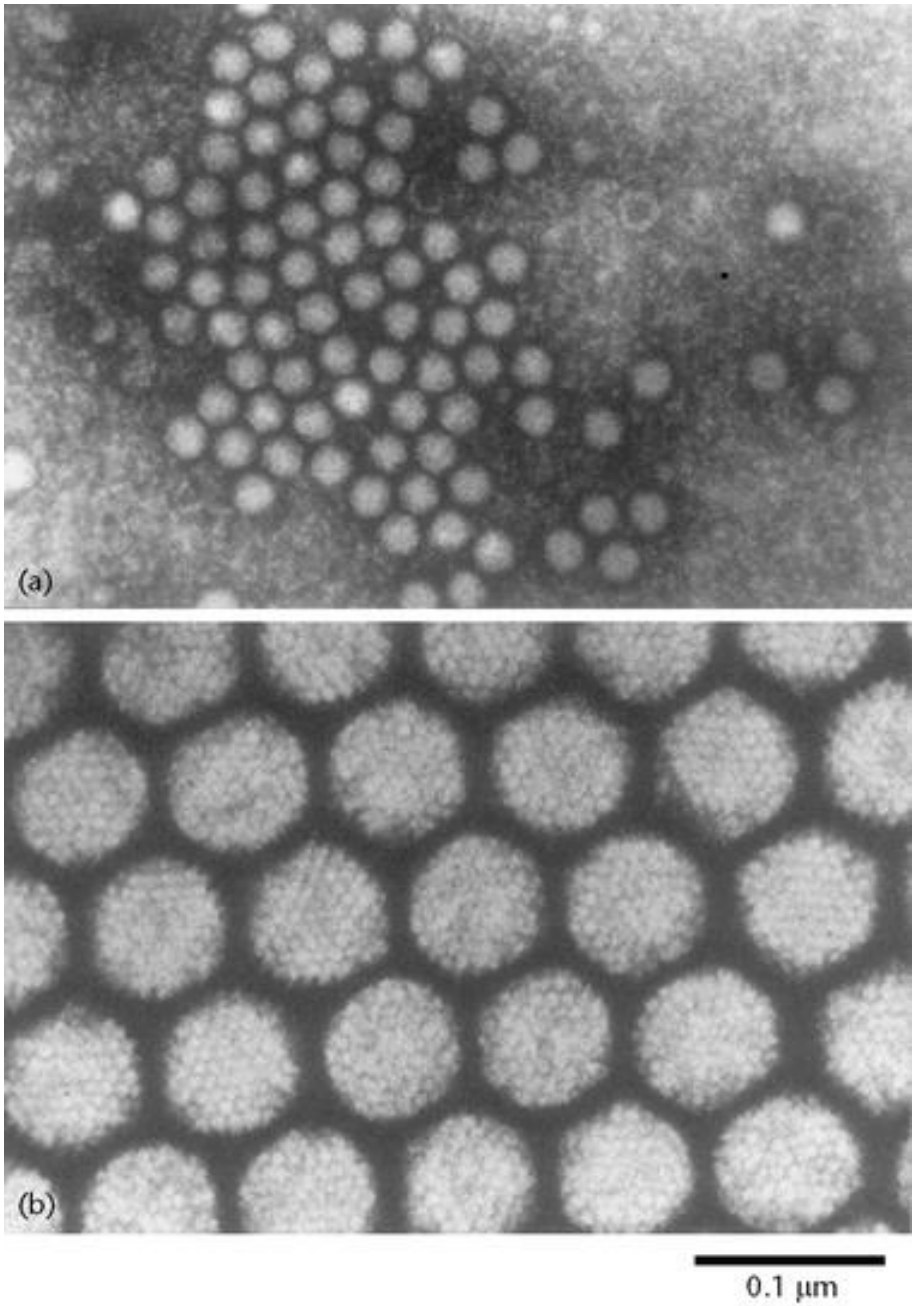
Induced Pluripotent Stem Cells for Basic Research and Clinical Application

Wykład odbędzie się **25 listopada** (piątek) o godzinie **10.00** w sali **posiedzeń Rady Wydziału** budynku Wydziału Biochemii, Biofizyki i Biotechnologii UJ

Serdecznie zapraszamy!

AAV vectors

adeno-associated viral vectors



AAV

adenovirus

Adeno-associated viruses – AAV

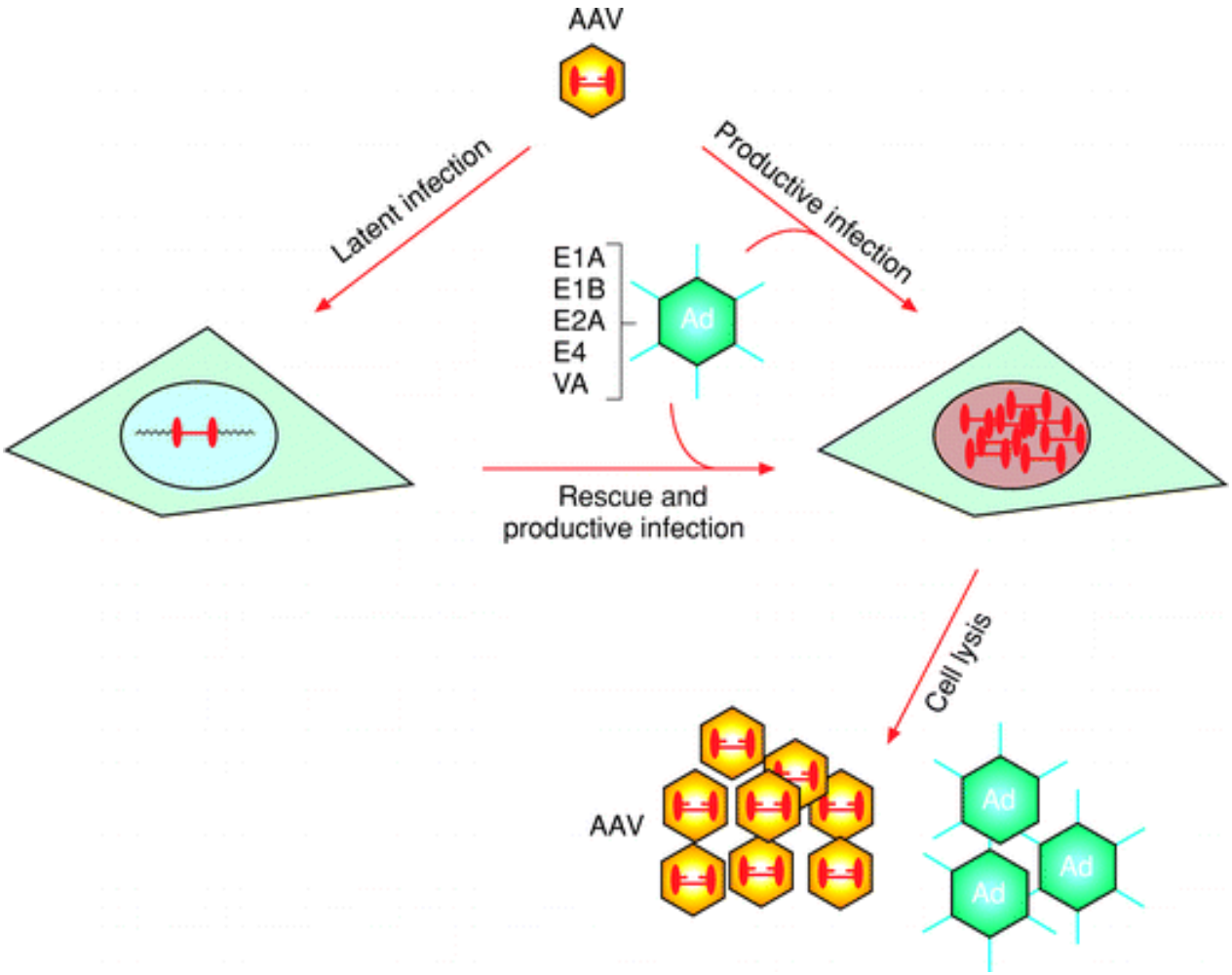
Small, non-pathogenic single stranded DNA viruses

For replication require additional genes delivered by other viruses (adenoviruses or herpes simplex viruses)

Genome AAV – 4681 nucleotides, at both ends there are 145 nt-long ITR (*inverted terminal repeats*)

ITR – necessary *in cis* – initiation of replication
- packaging signal
- integration into genome

Infectious cycle of AAV



AAV vectors

removal of rap and cap genes
transgene insertion

ITR

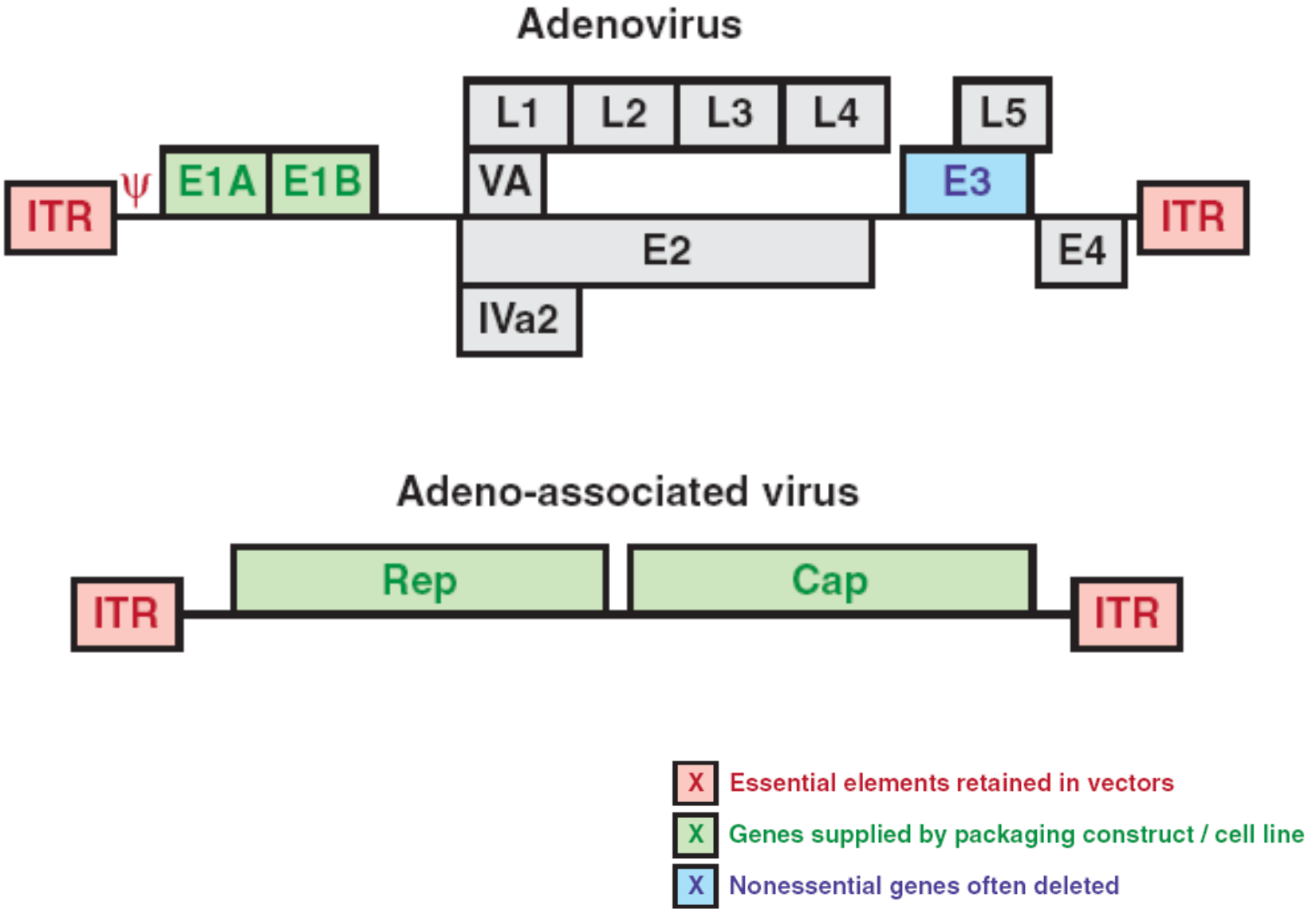
ITR

Ways of production of AAV vectors

- dependent on helper vector

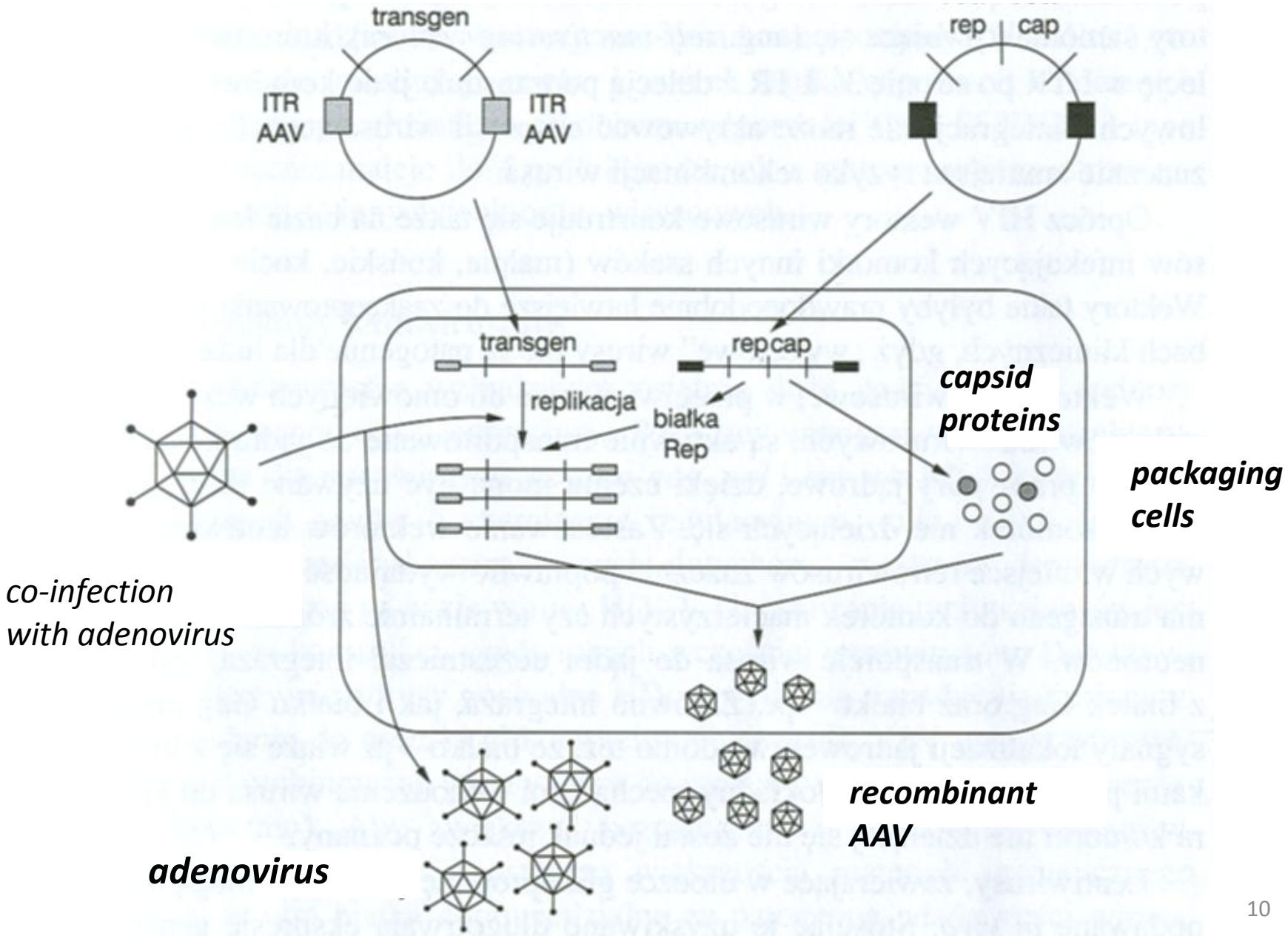
- helper-vectors independent

Essential and non-essential elements in different viral vectors

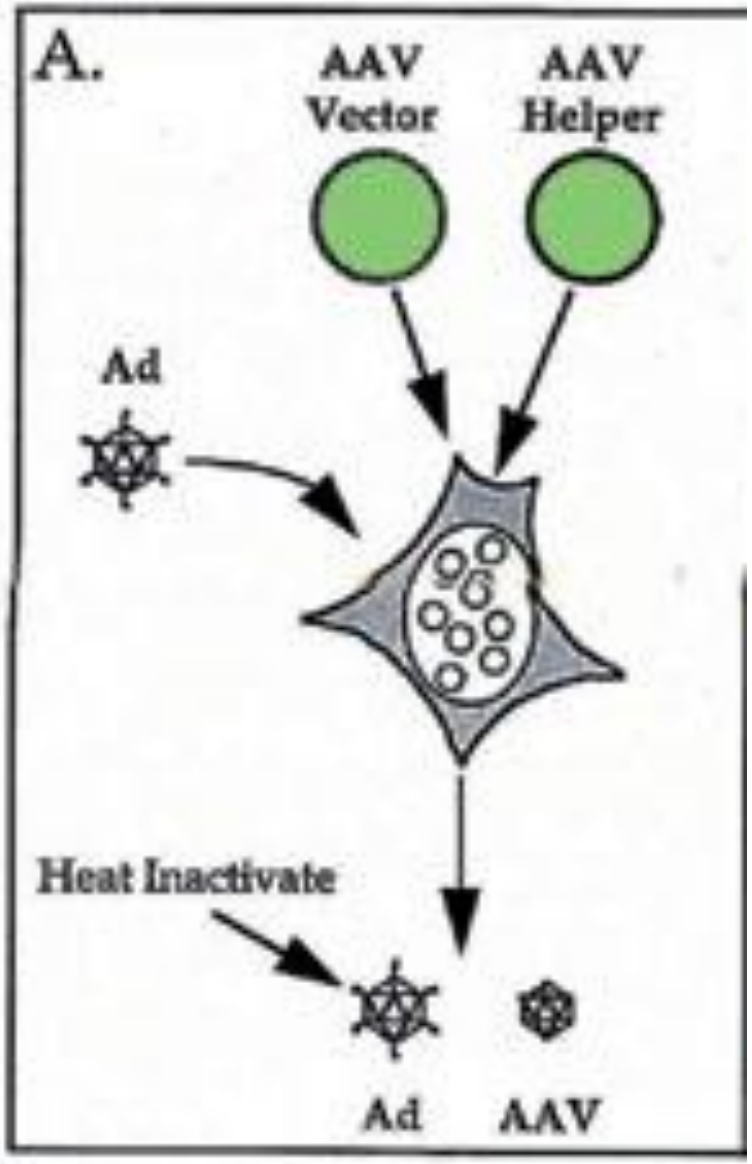


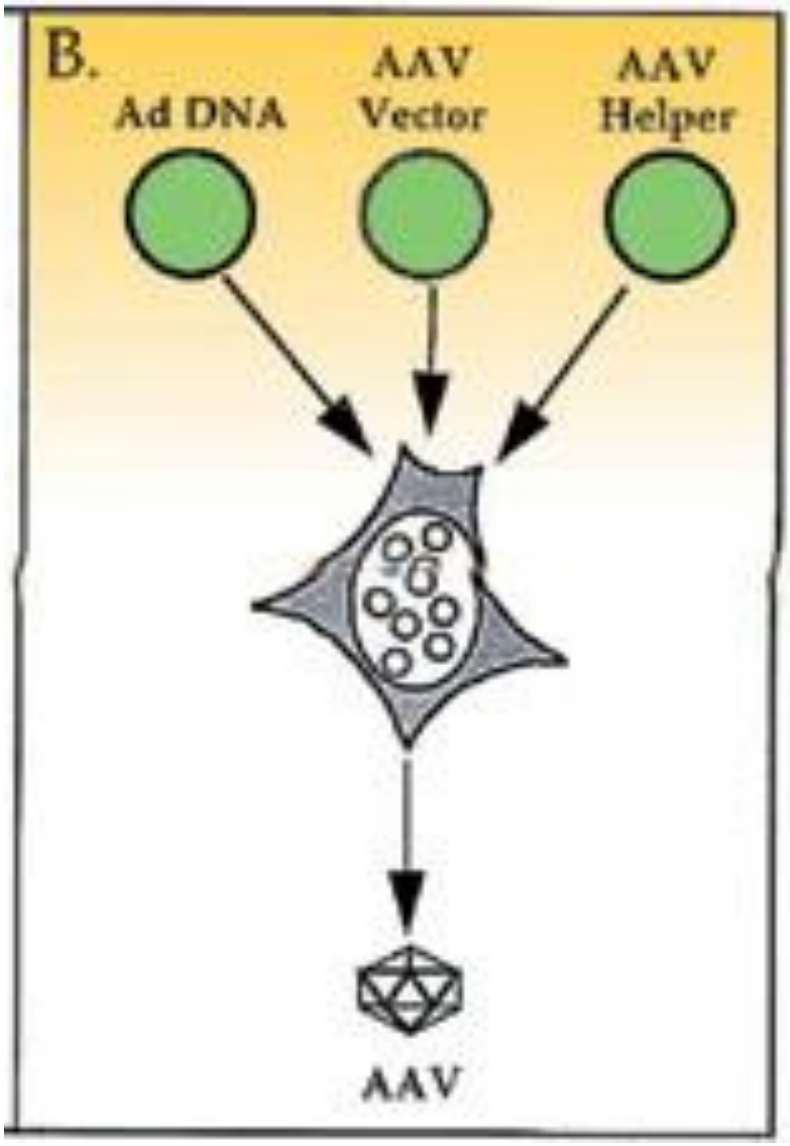
Verma & Weitzman, Ann Rev Biochem 2005

Construction of AAV vectors – system with helper adenoviral

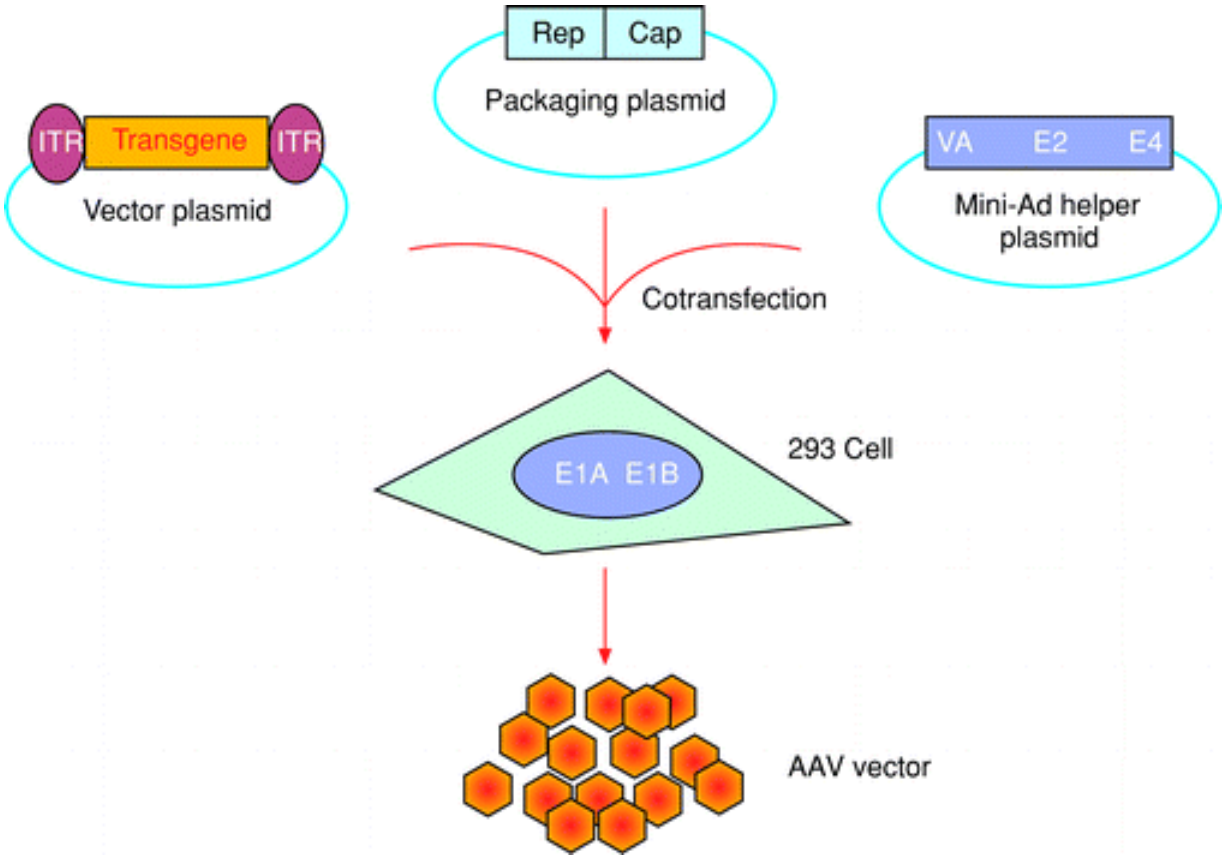


Production of AAV vectors – it is safer to omit helper adenovirus



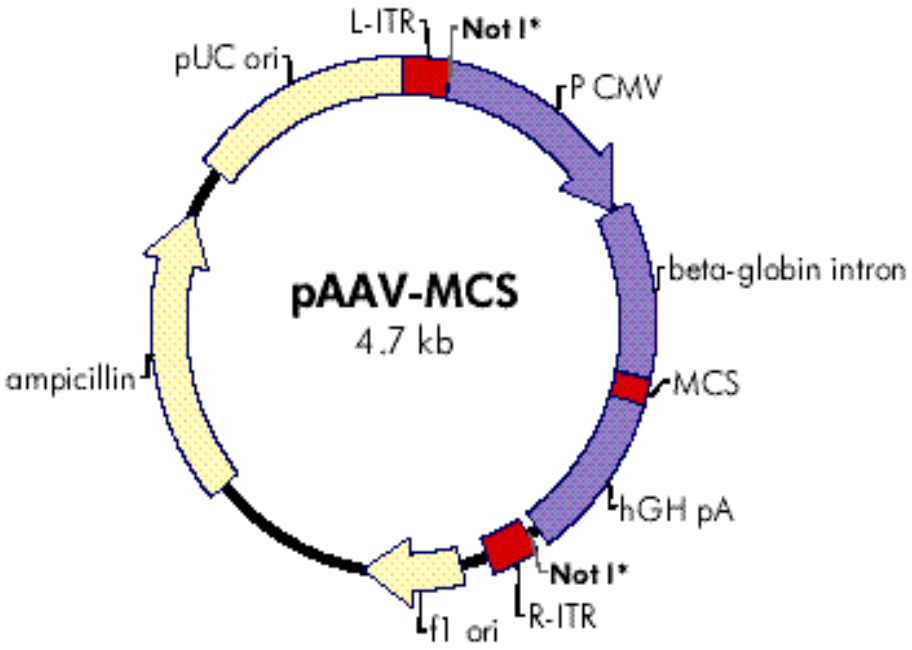


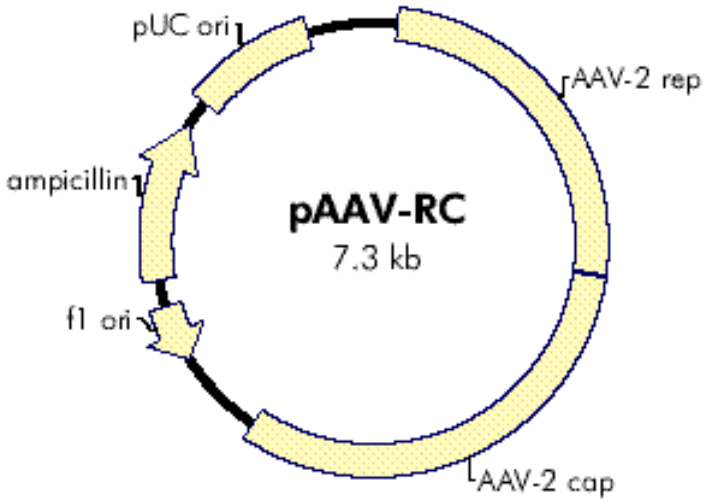
AAV Helper-Free System

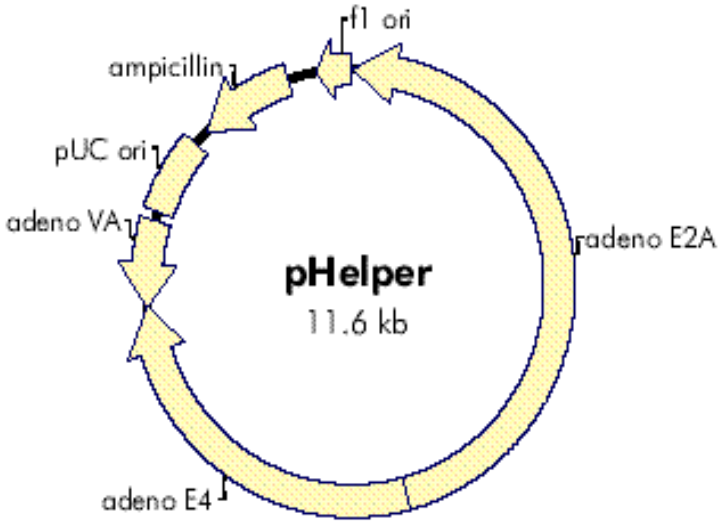


For production of AAV vectors only three sets of adenoviral genes are required: E1, E2A, E4 & VA

Vectors in AAV helper-free system

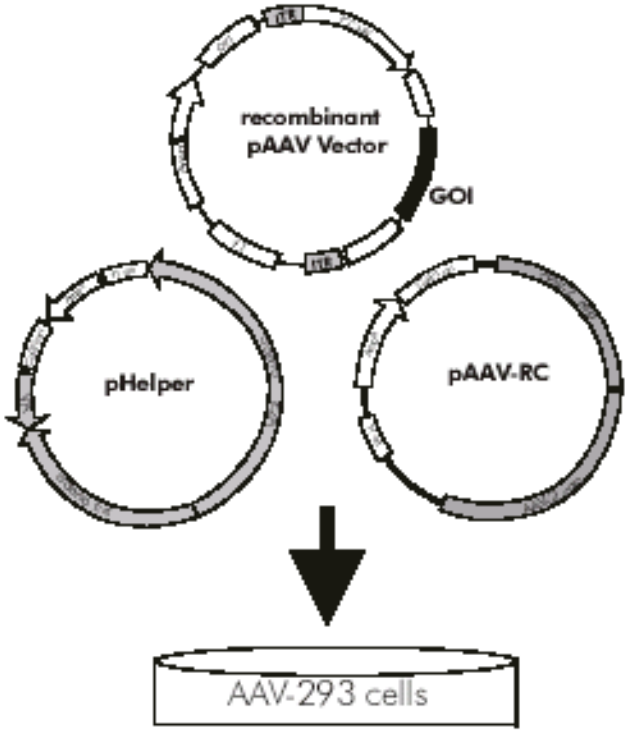






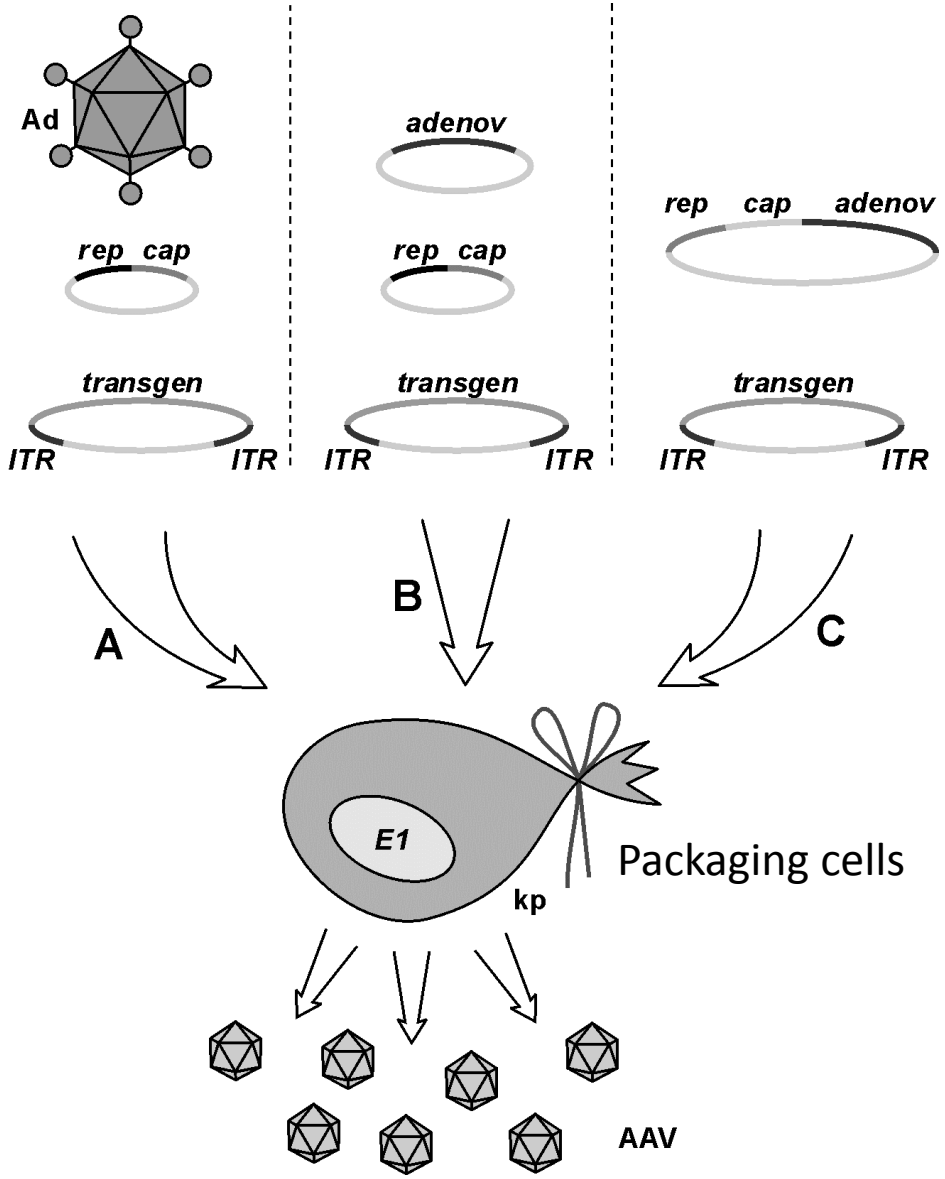
Helper-free production of AAV vectors (2)

Co-transfect
AAV-293 cells with:
Recombinant pAAV Vector
pAAV-RC
pHelper



Produce
AAV Particles
in AAV-293 cells

Strategies of production of AAV vectors



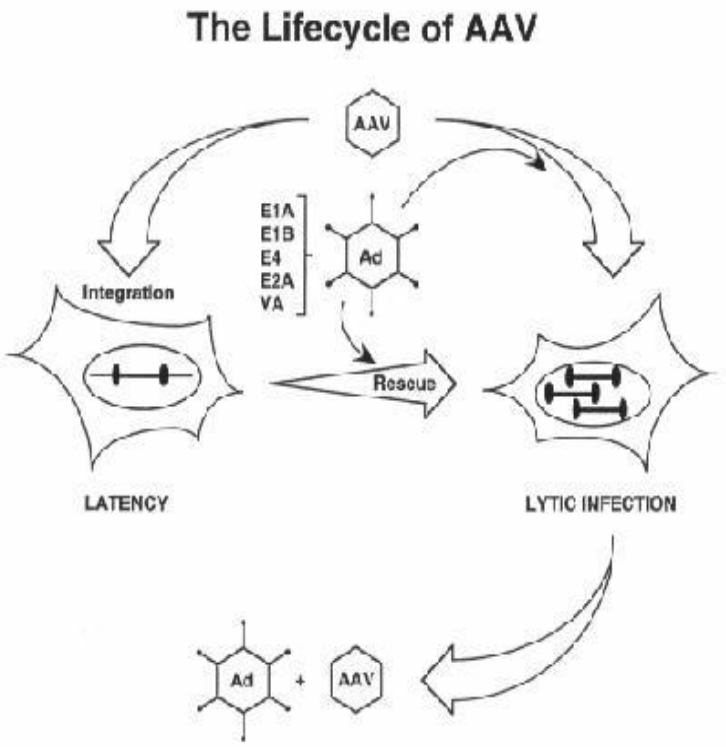
Three vs two plasmid systems

Rutkowski et al., Biotechnologia 2007

AAV and genomic integration

Site-specific integration

- AAV integrates usually stably into a specific site on chromosome 19q13.3 (AAVS1)
- Integration region- AAVS1 (RBS,TRS)
- Rep78 and Rep68 bind to a 109 bp DNA fragment near AAVS1 and can mediate complex formation (DNA of chromosome 19 and AAV harpin DNA)
- Viral DNA replication within AAVS1 are likely involved in site-specific integration;



AAV vectors features

- due to the lack of Rep68 and Rep78 the specific integration into chromosome 19 is lost

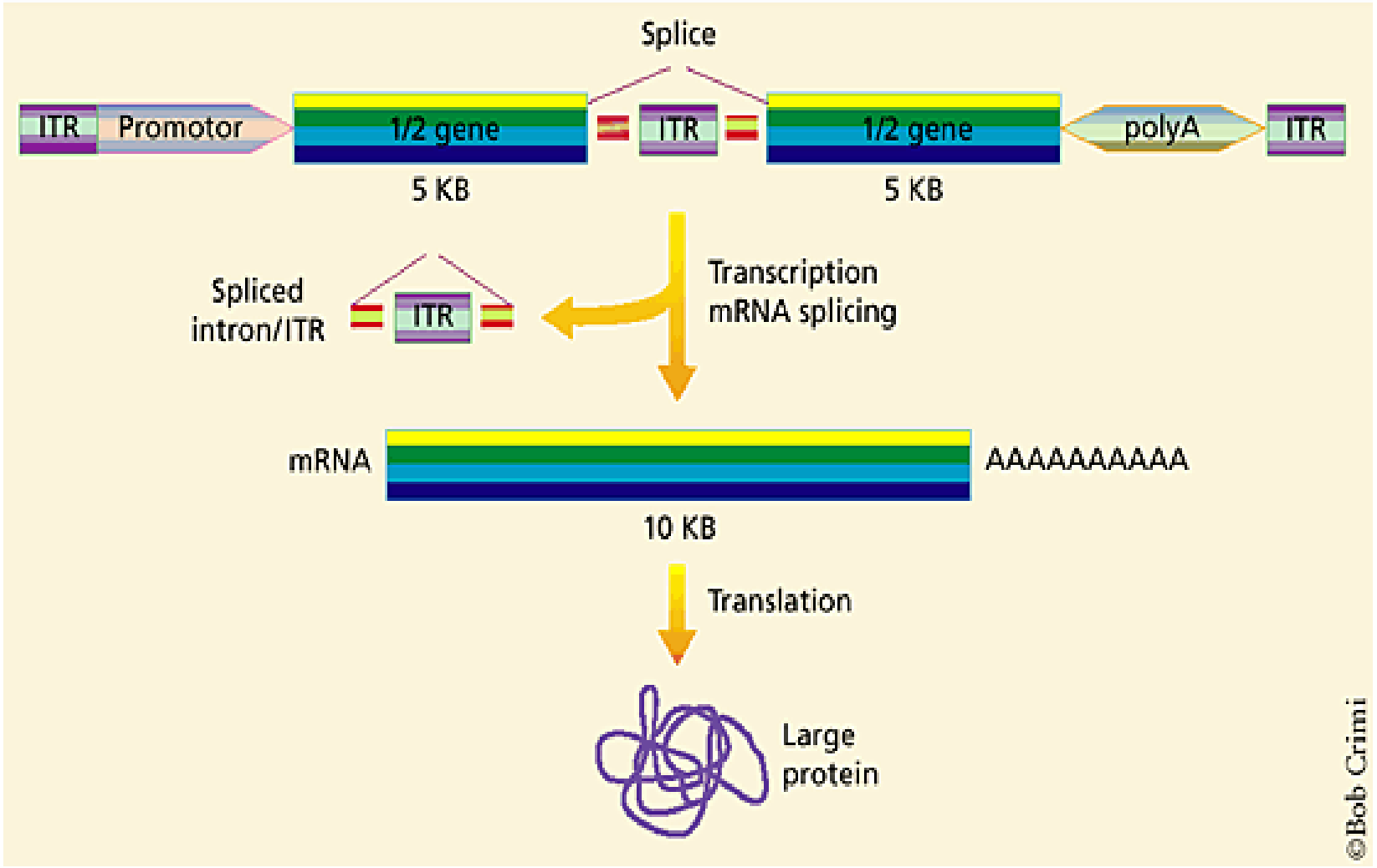
- **unspecific integration** (low efficacy, about 5-10%)

- **episomal expression**

- because of non-immunogenic nature the episomal expression in non-dividing cells can be long-term

How to deal with a small capacity of AAV vectors?

AAV- concatamerisation



AAV serotypes

11 serotypes are known

AAV-2 serotype is the most commonly used

Different serotypes can employ various receptors to enter the cells

- **AAV-2**: heparan sulphate
- **AAV-1** & **AAV-5** – sialic acid
- **AAV-5** co-receptor: PDGF-B receptor

AAV2 vector trafficking

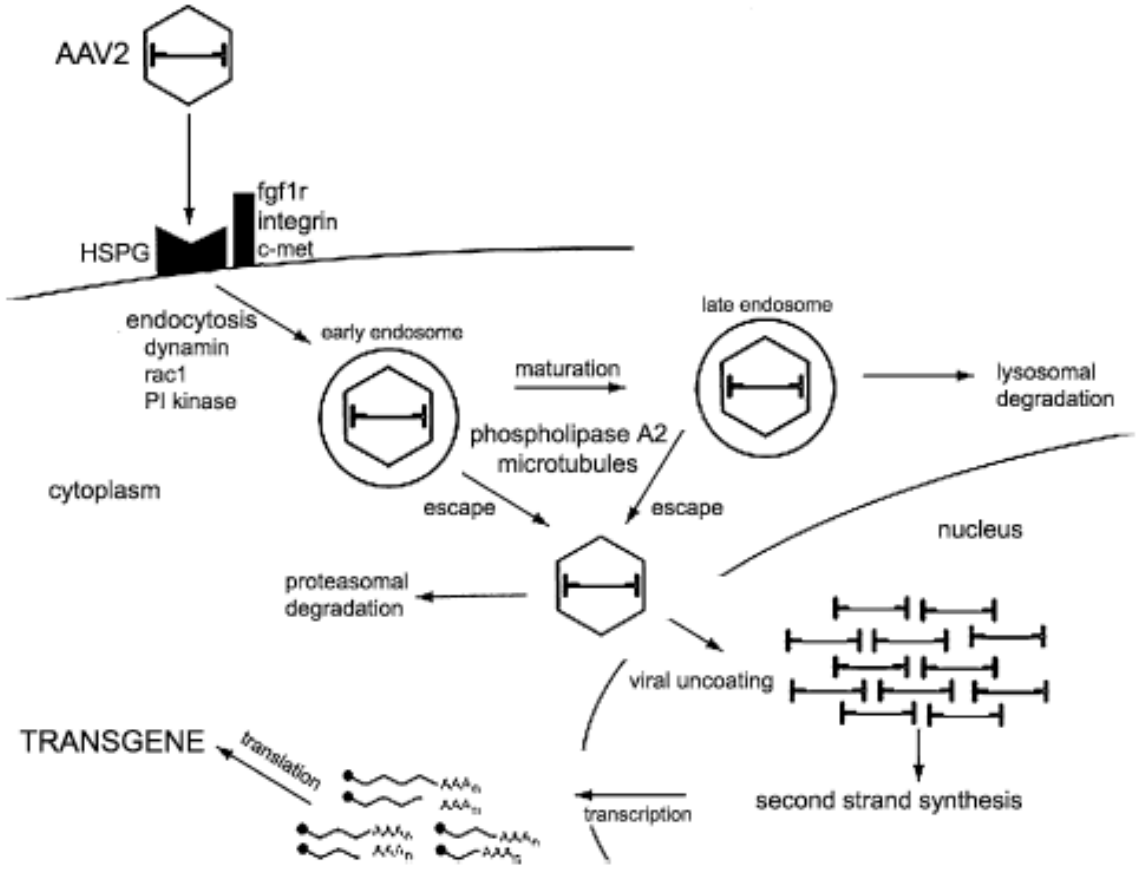
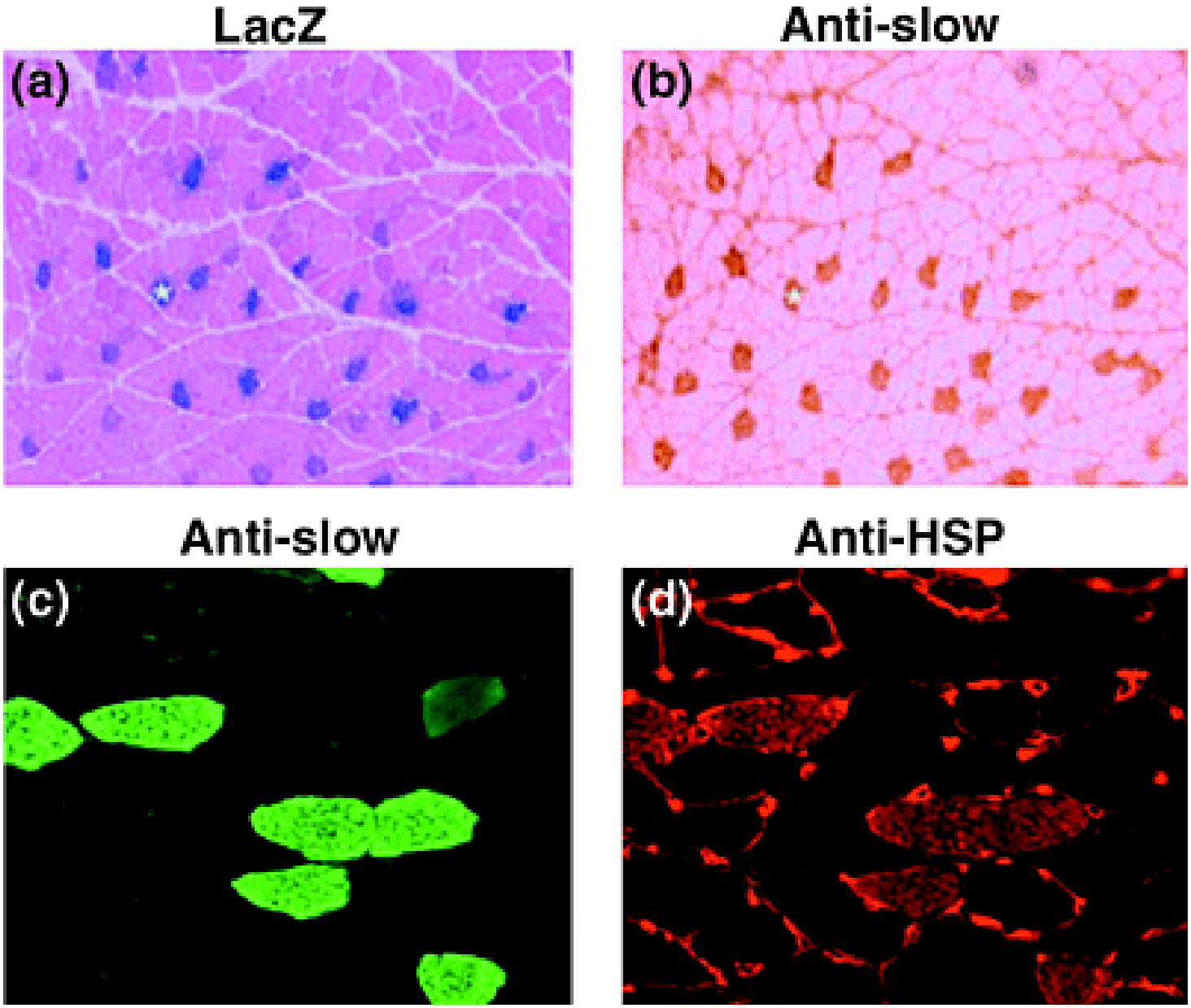


Fig. 2 AAV vector trafficking. AAV-2 binds cell surface heparan sulfate proteoglycan (HSPG) and is endocytosed via three potential co-receptors (fgf1r, integrin, and c-met receptors). Endocytosis of AAV-2 is dependent on dynamin, rac1, and PI kinase activities. Vector particles escape early and late endosomal compartments using an inherent phospholipase A2 activity and translocate to the nucleus in a microtubule-dependent fashion. A significant portion of input vector may be degraded by the proteasome or lysosomal proteases. While it is still unclear whether intact AAV vectors enter the nucleus, upon uncoating of the viral genome, second-strand synthesis provides a transcriptionally active template leading to expression of the delivered transgene

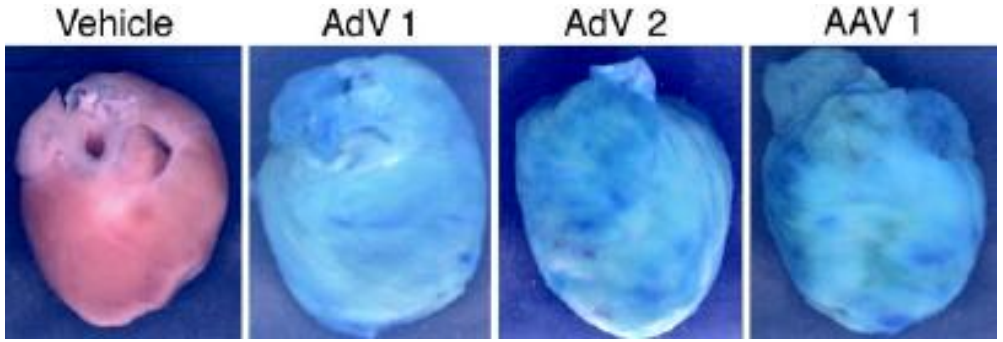
Receptor – through heparan sulphate binding

co-receptors: $\alpha_V\beta_5$, FGF receptor (FGF1r), c-met

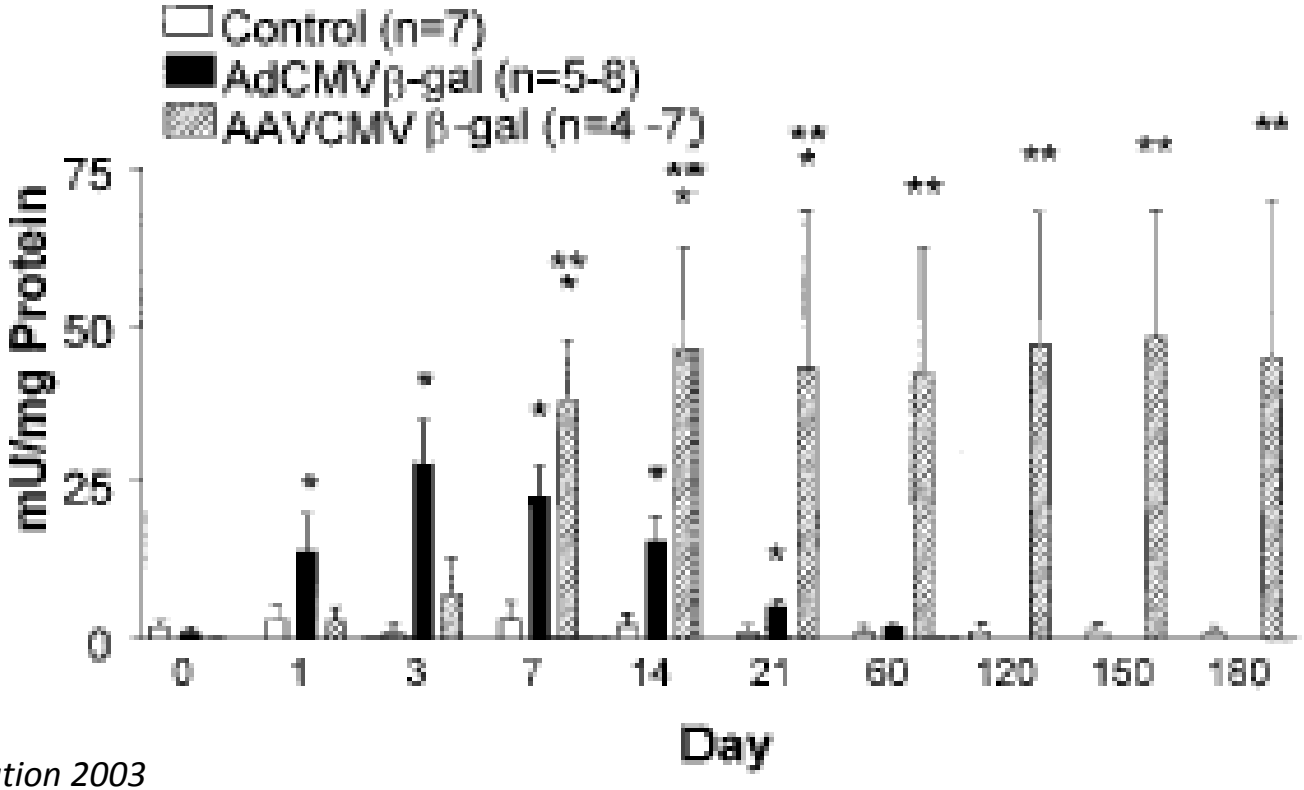
AAV-2 vectors tropism to skeletal muscles



AAV vectors, in contrast to adenoviral, can provide long-term expression

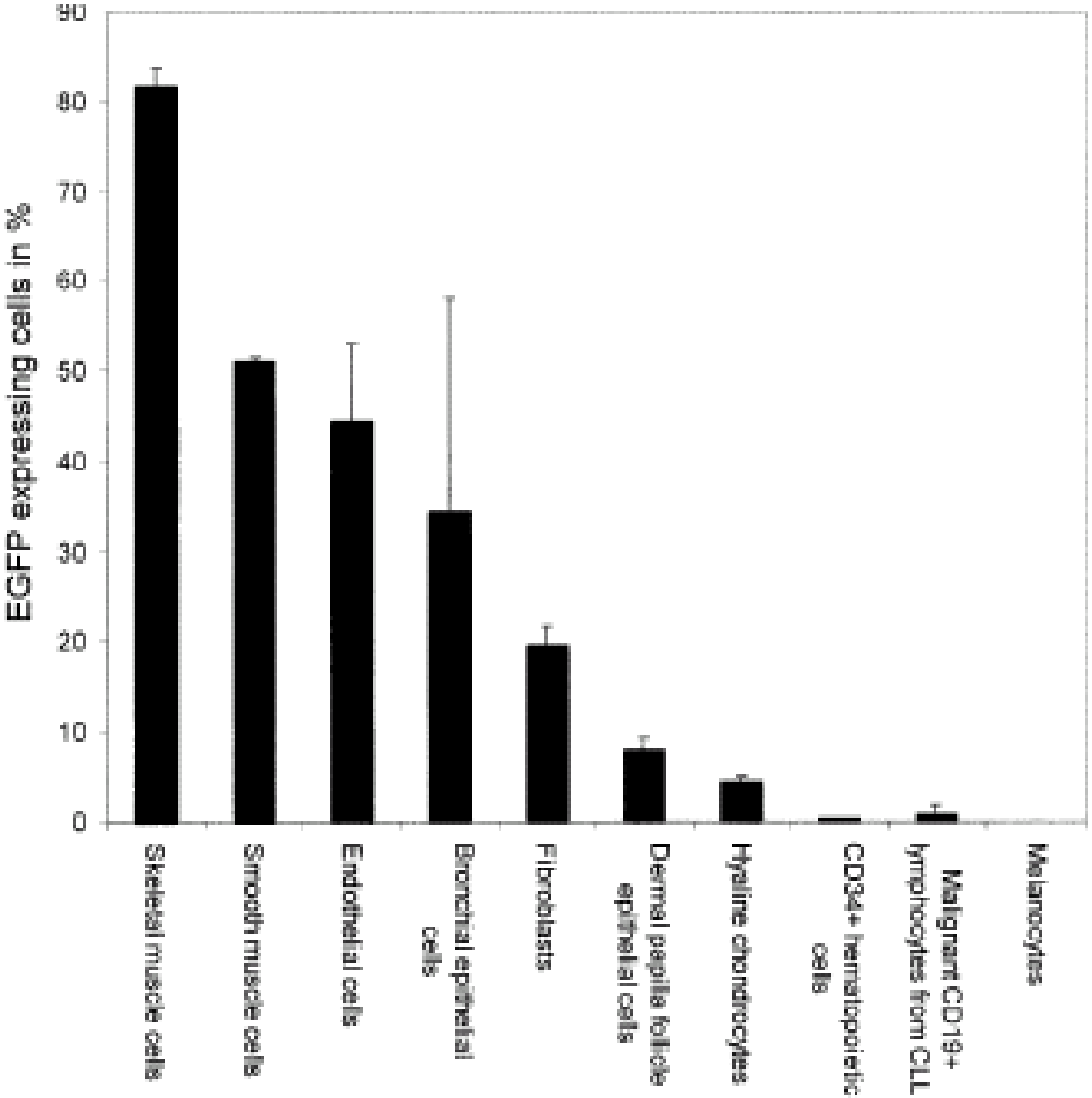


Mouse heart

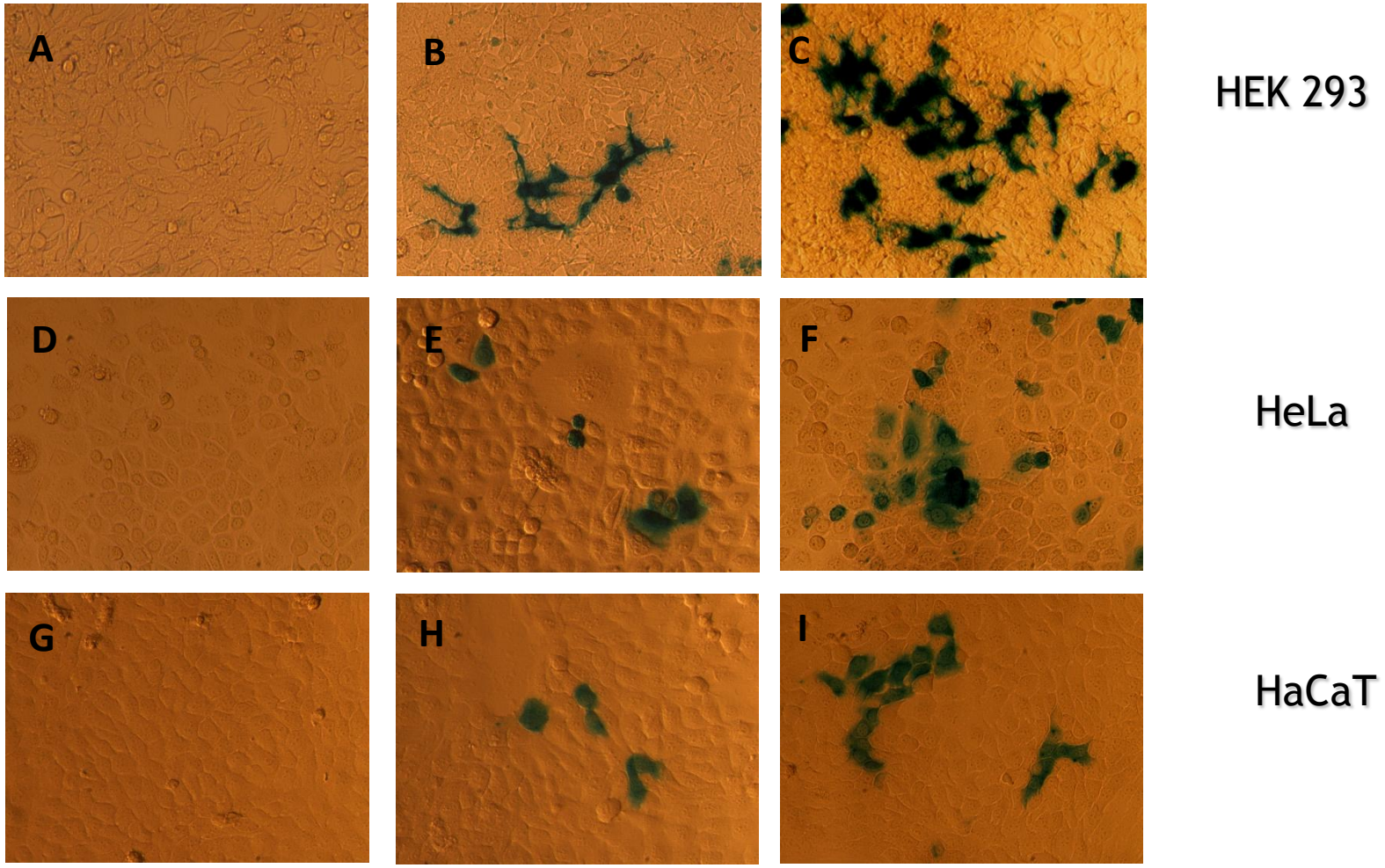


Champion et al., Circulation 2003

Different transduction efficiency of AAV-2 viral vectors

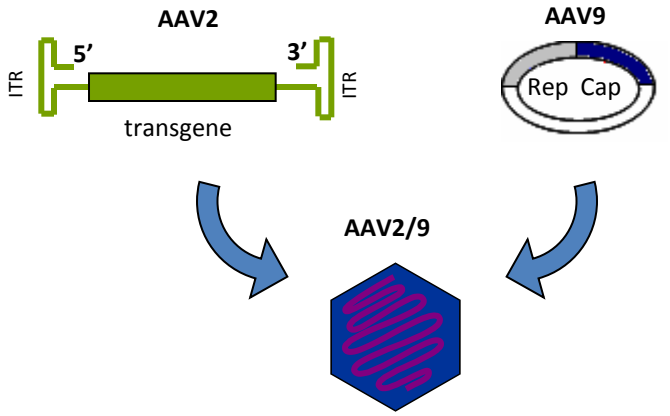


Transduction efficacy of AAV2 vectors depends on cell type

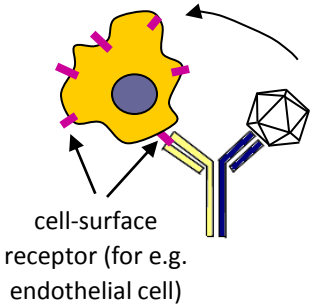


Methods enhancing the effectiveness of AAV vectors

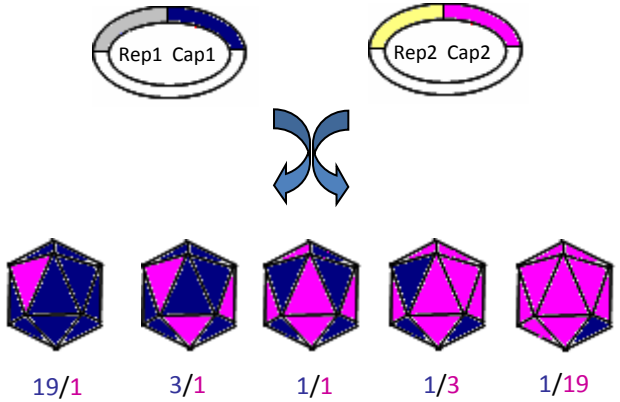
A) transcapsidation



B) bi-specific antibody

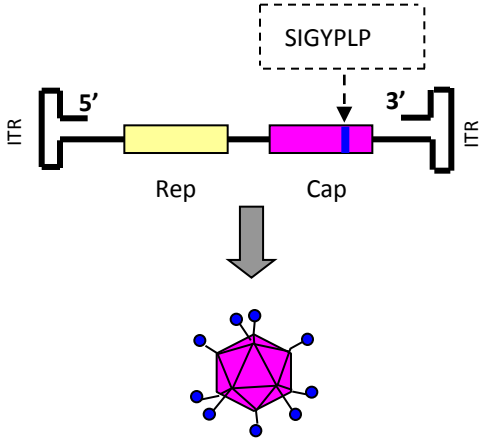


C) mosaic capsid



Different ratios of plasmids

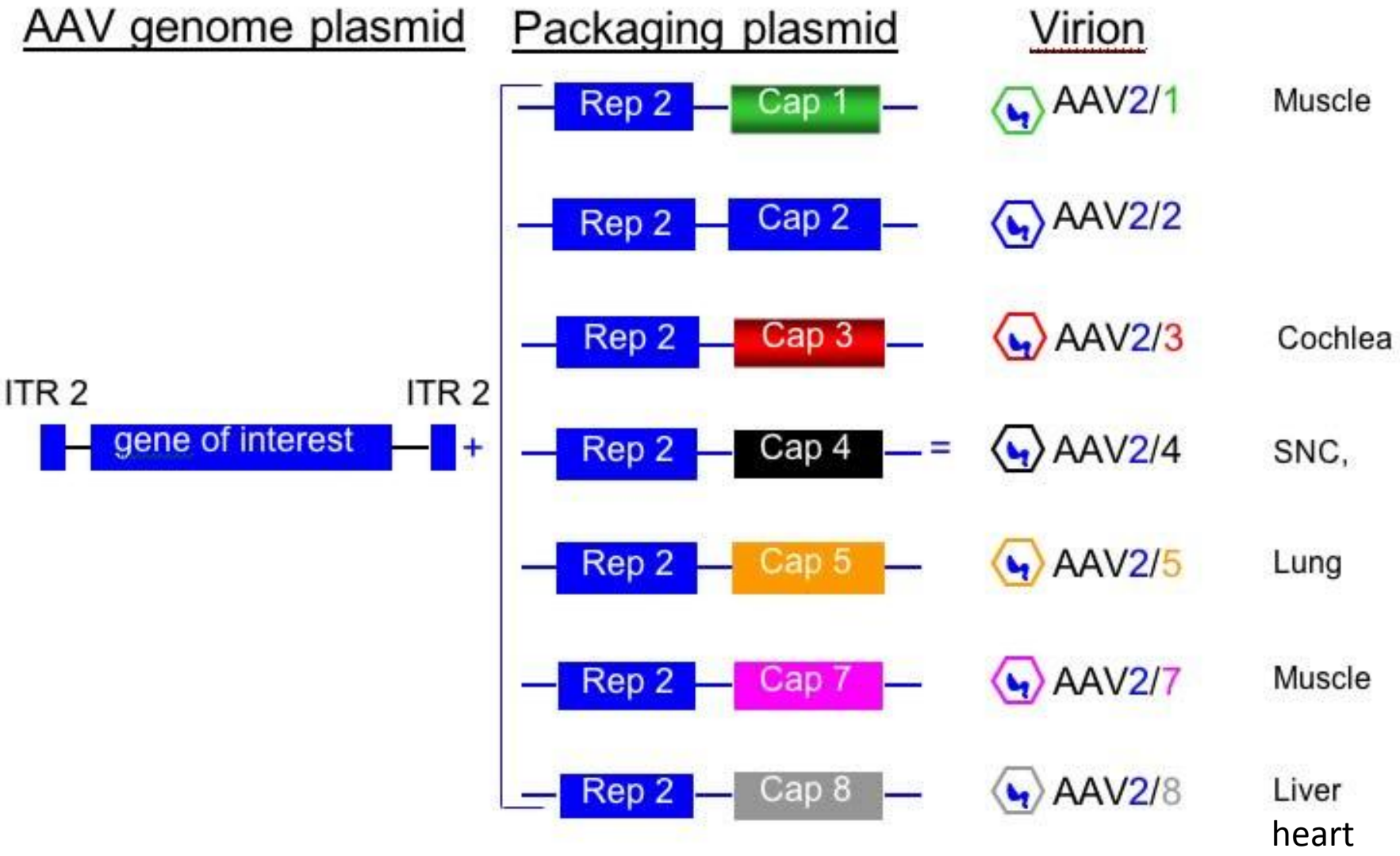
D) chimeric capsid



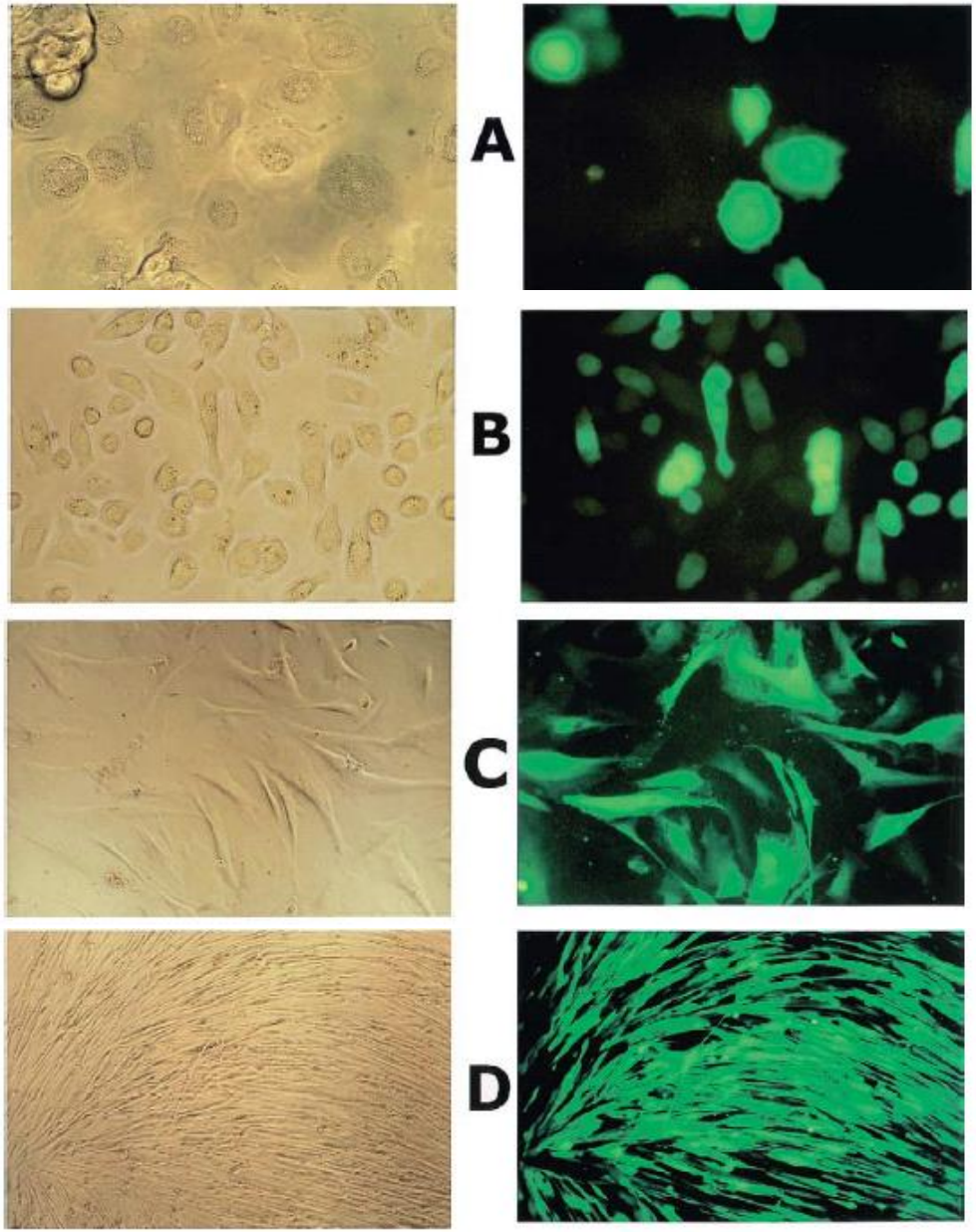
AAVsig

Jazwa et al.,, Curr Gene Therapy, 2007

Targeting of AAV vectors – usage of different serotypes



Different transduction efficiency of AAV-2 viral vectors



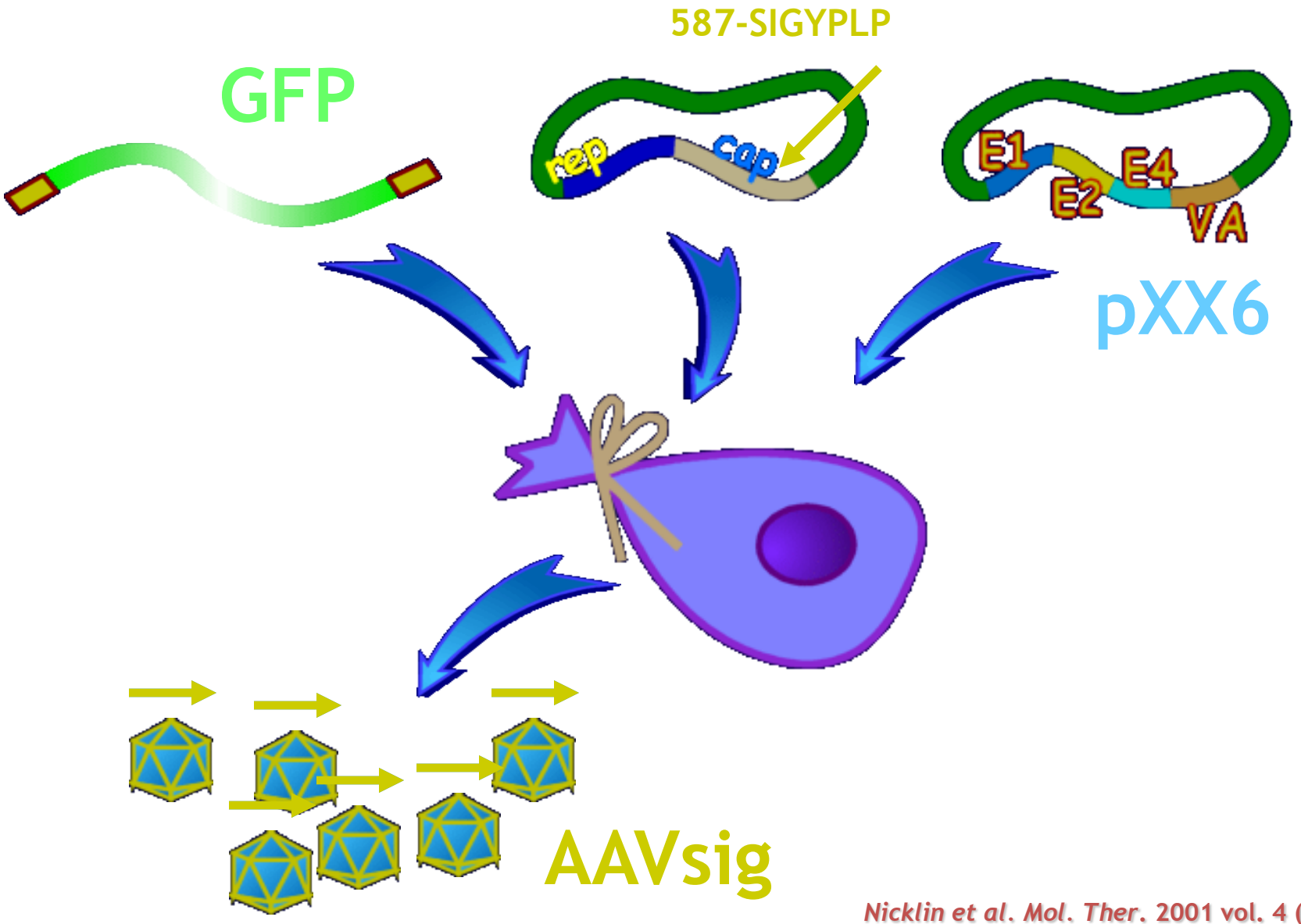
Endothelial

Bronchial epithelial

Vascular smooth muscle

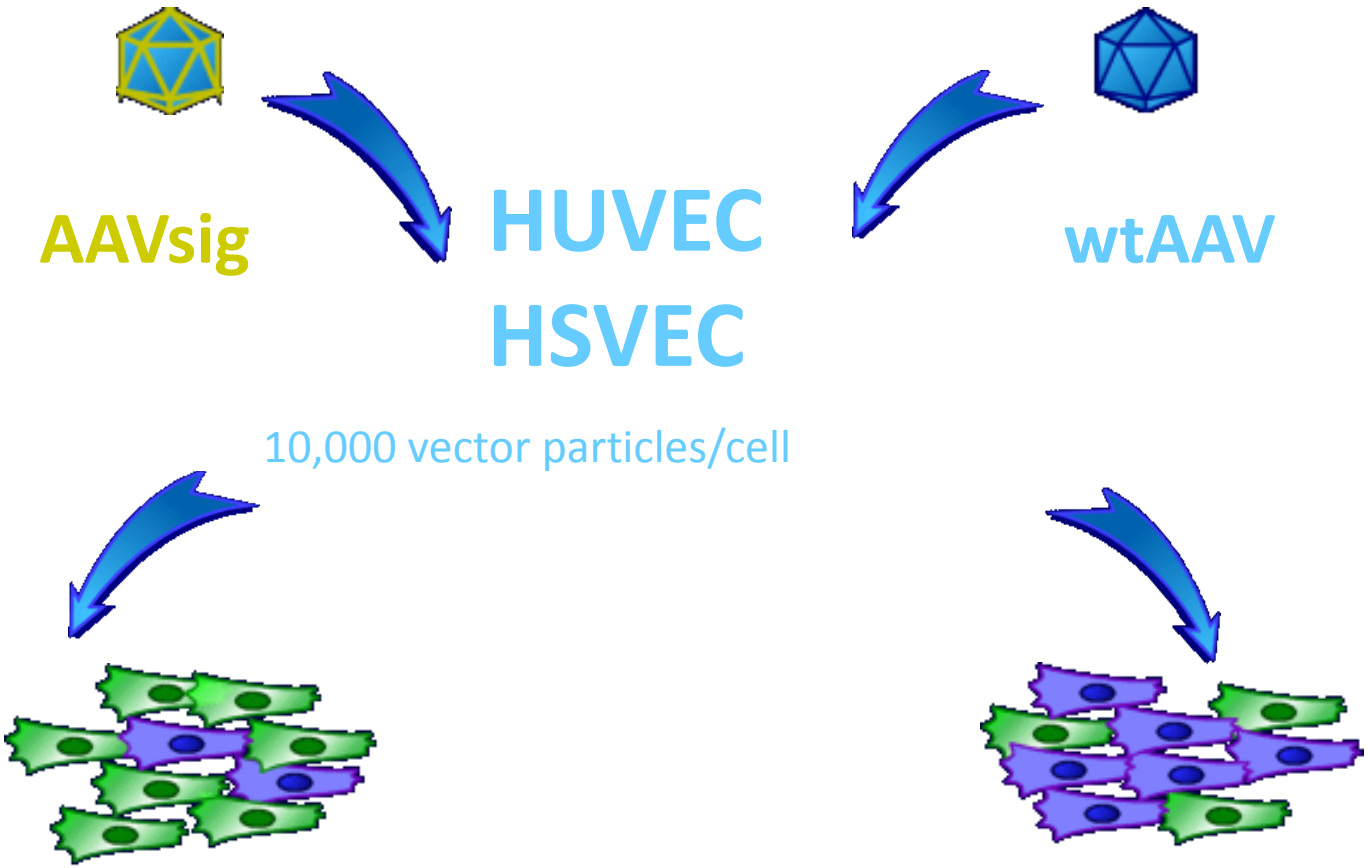
Skeletal muscle

Targeting of AAV vectors to endothelial cells



Nicklin et al. Mol. Ther. 2001 vol. 4 (2)

Targeting of AAV vectors to endothelial cells



5.9-fold HUVEC

28.2-fold HSVEC

Mol. Ther. 2001 vol. 4 (2)

Features of AAV vectors

Advantages

1. Long term expression

2. High efficiency of transduction of many cell types

3. Non-pathogenic viruses. Low risk of cellular immune response, which is additionaly limited by removal of viral sequences

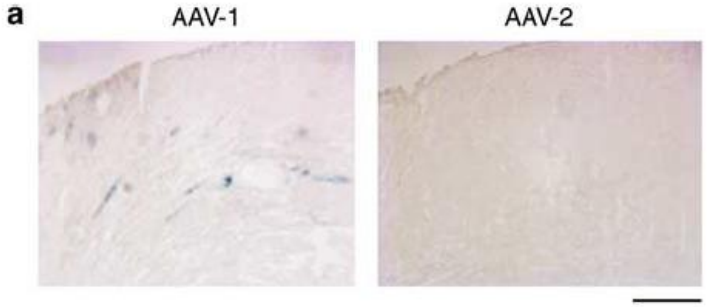
Limitations

1. Unspecific integration
2. Small capacity – max. 4 kbp
3. Low efficiency of transduction of certain cell types – targeting might be required
4. Difficulty of production in sufficient titer for in vivo work
5. Risk of humoral immunity: antibodies detect capsid proteins

AAV
investigations on the new serotypes

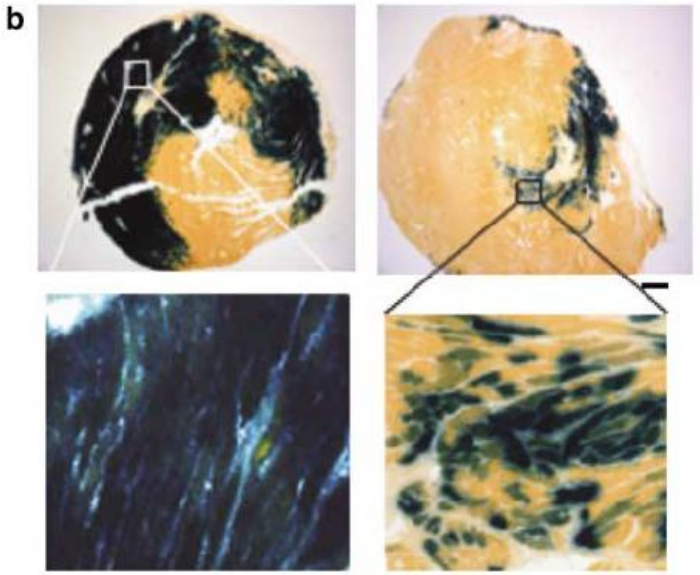
AAV1 provides earlier and higher expression in the heart than AAV2

1 day

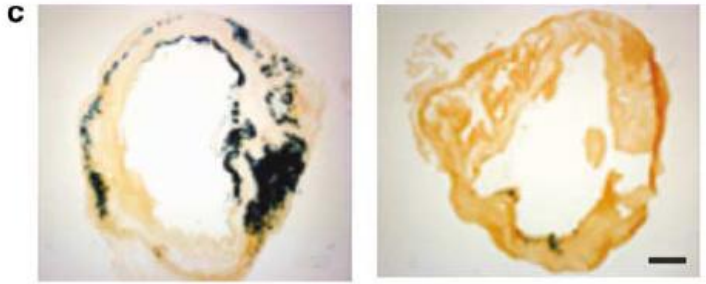


Normal heart

14 days



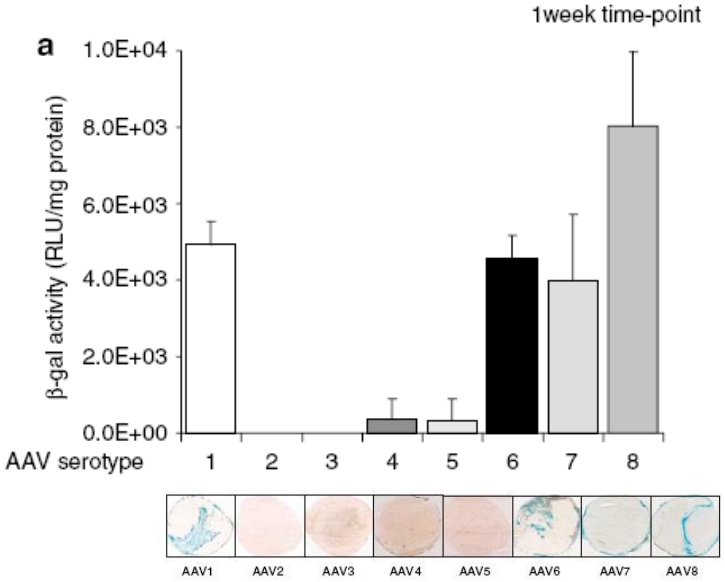
14 days

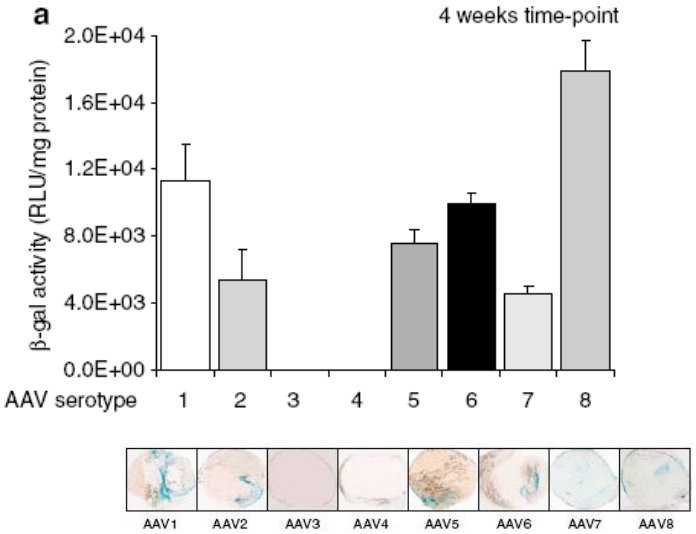


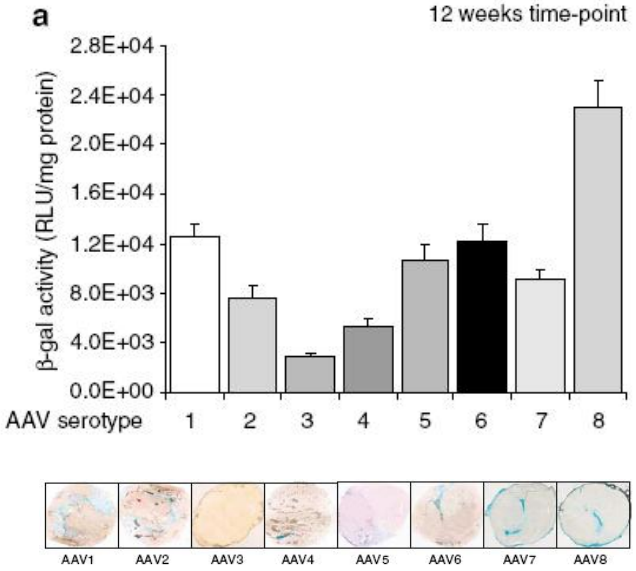
Ischemic heart
-expression in myocytes around the scar

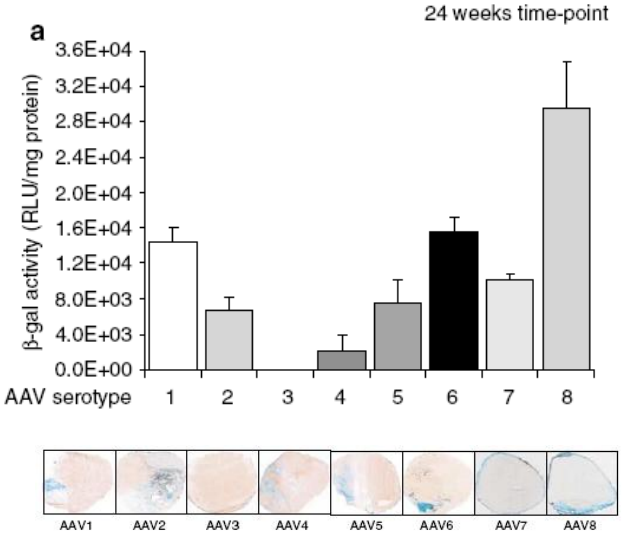
Su et al., Gene Therapy 2006

AAV8 provides higher expression in the rat heart









Palomeque et al.. Gene Therapy 2007

AAV8 provides higher expression in the rat heart

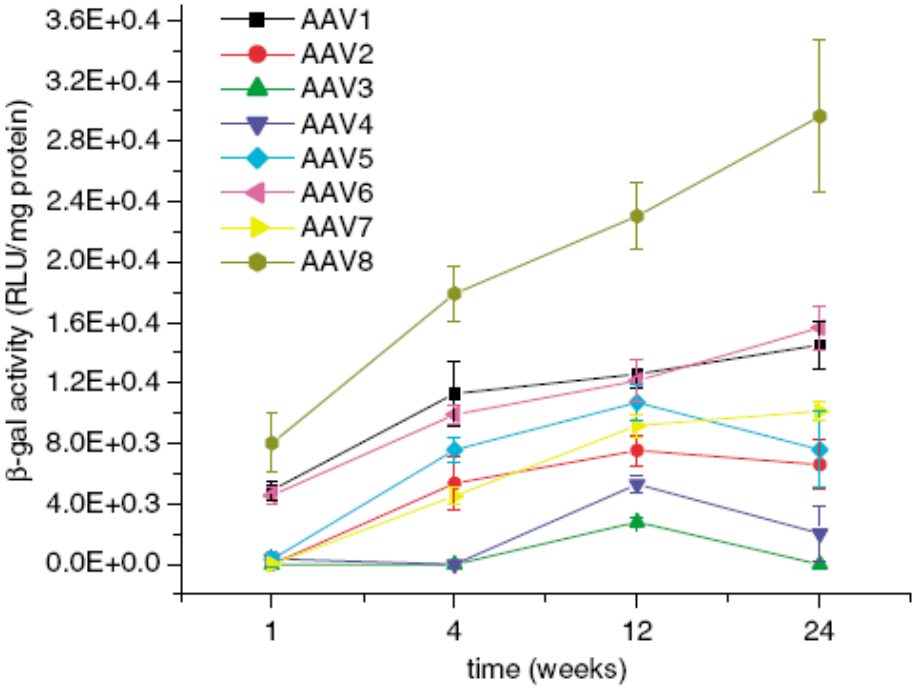
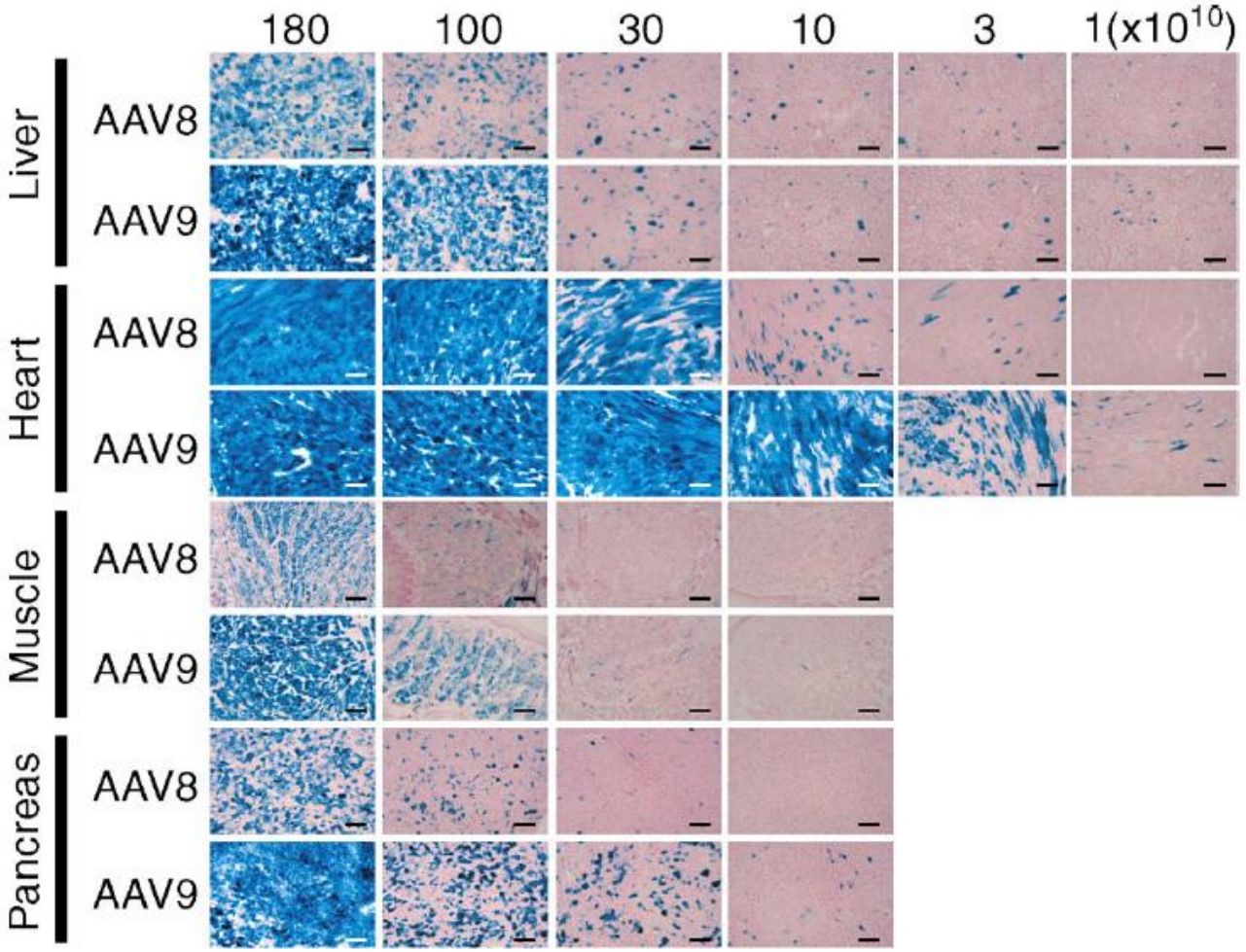


Figure 6 AAV serotypes time course followed during 6 months. Overall results of β-gal activity indicating the time course followed by each AAV serotype. AAV8 was clearly the serotype that reaches the quickest and highest value during the time frame of the study. AAV1 and 6 developed a significant β-gal activity as soon as 1 week after the gene transfer, and maintain this high performance during 6 months. AAV2, 5 and 7 achieved β-gal activity values comparable to AAV1 and 6 at 12 weeks post-injection, however, at the last time point the enzymatic activity decreased. AAV3 and 4 only attained significant β-gal activity increase 12 weeks after the gene delivery. Values from Figures 2–5 were placed here for visual comparison.

Palomeque et al.. Gene Therapy 2007

AAV9 is even more effective than AAV8 even at lower doses !

systemic delivery

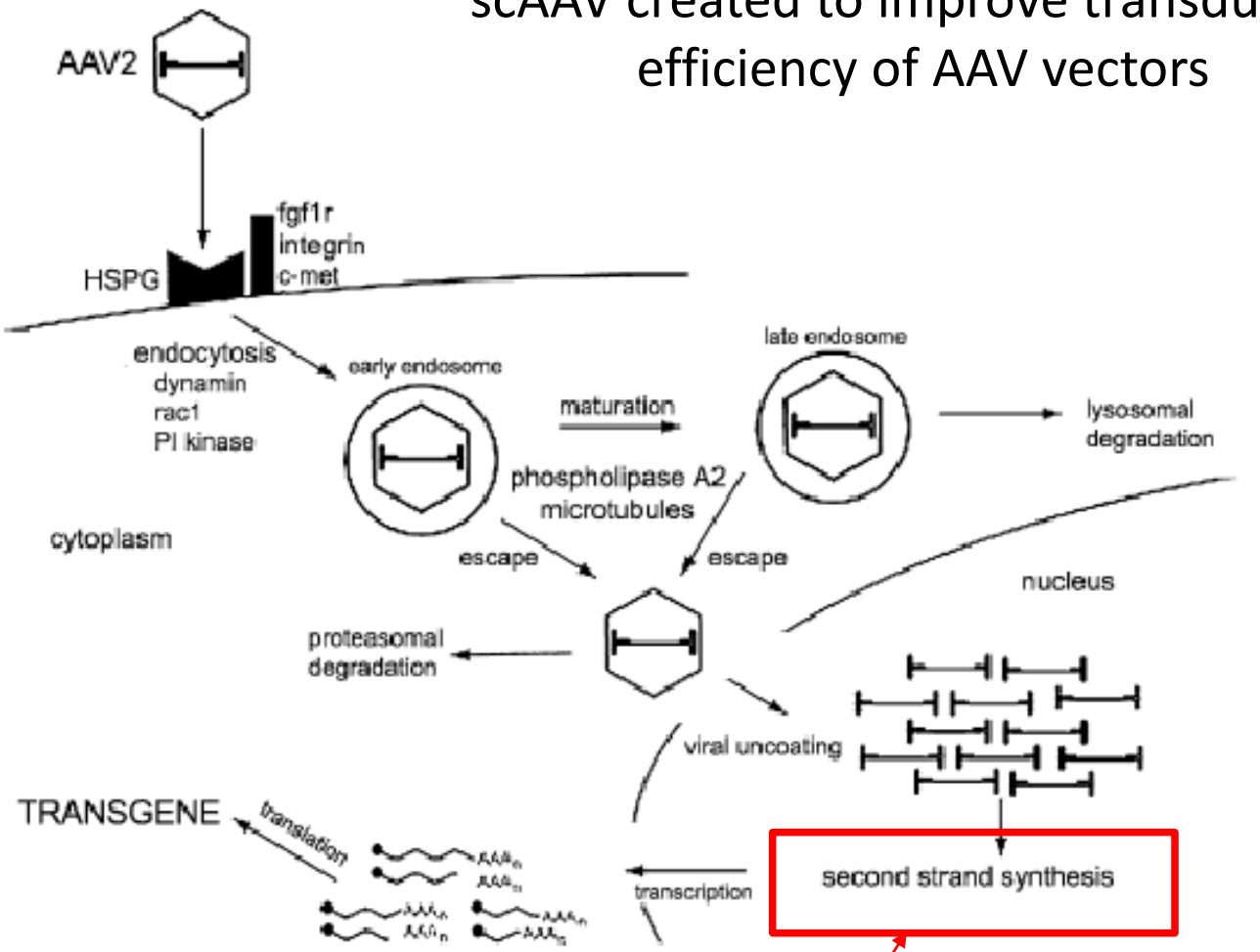


Ianagaki et al., Mol Therapy 2006

Self complementary AAV

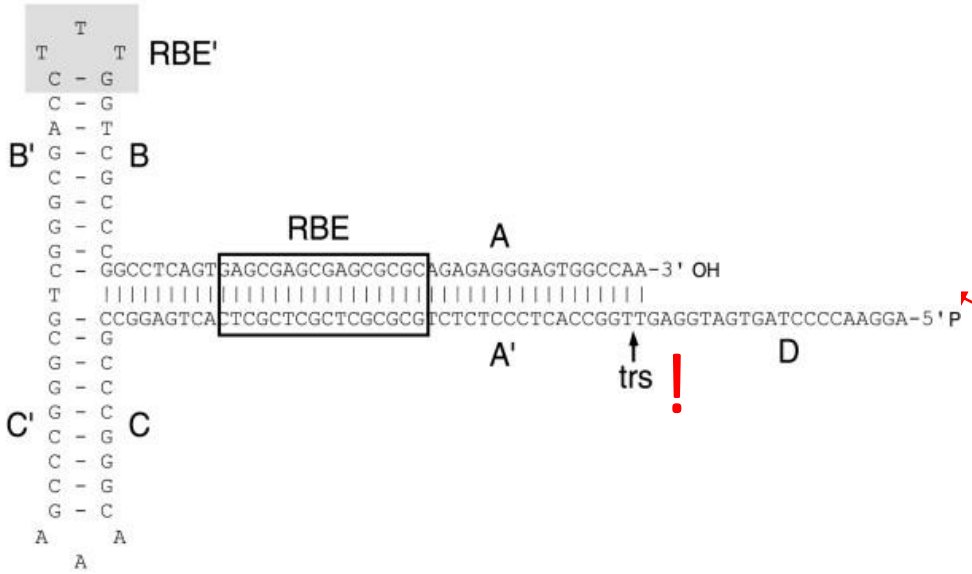
scAAV – short introduction

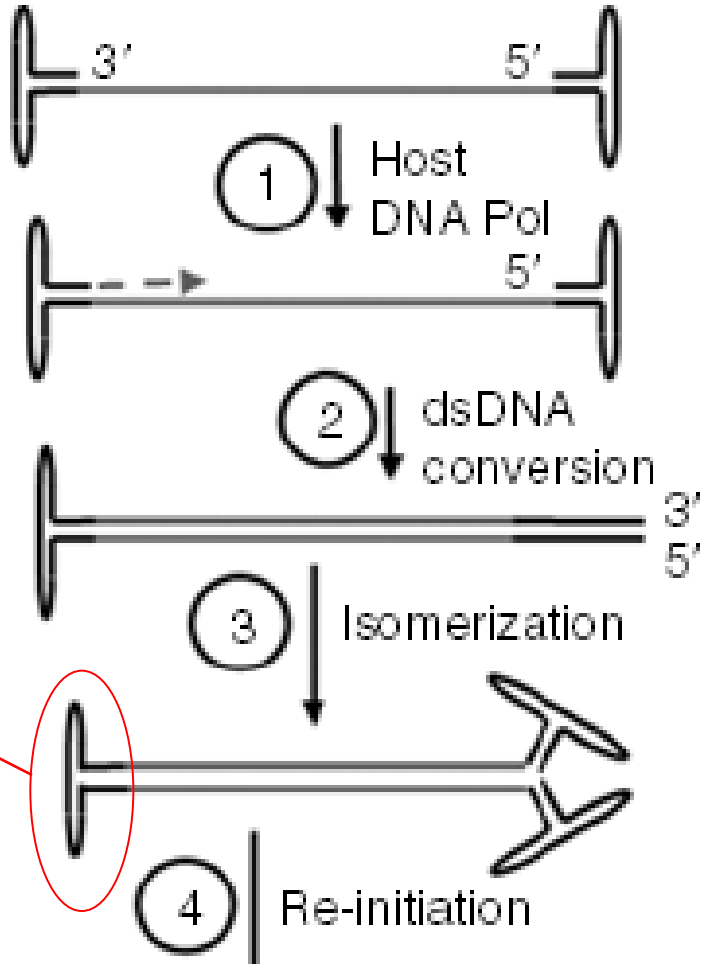
scAAV created to improve transduction efficiency of AAV vectors



Important rate-limiting step for transduction

Self complementary AAV

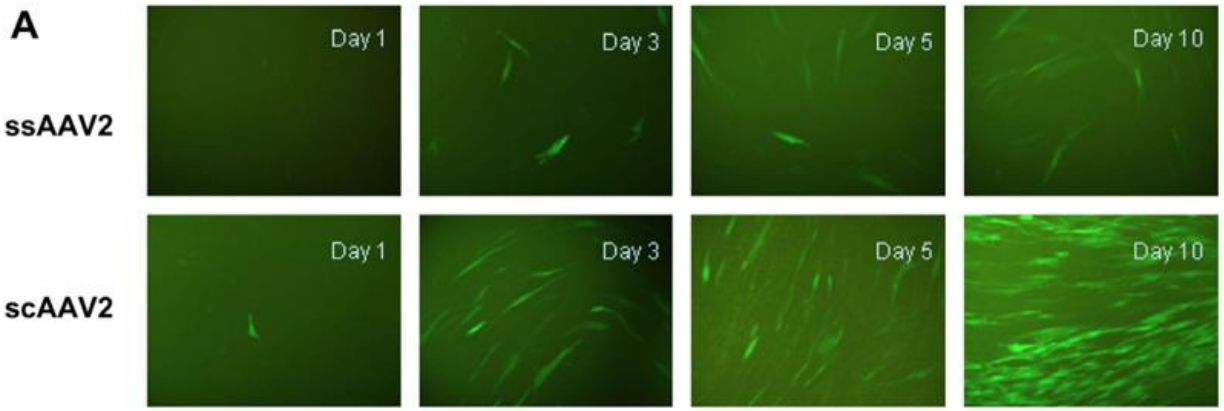


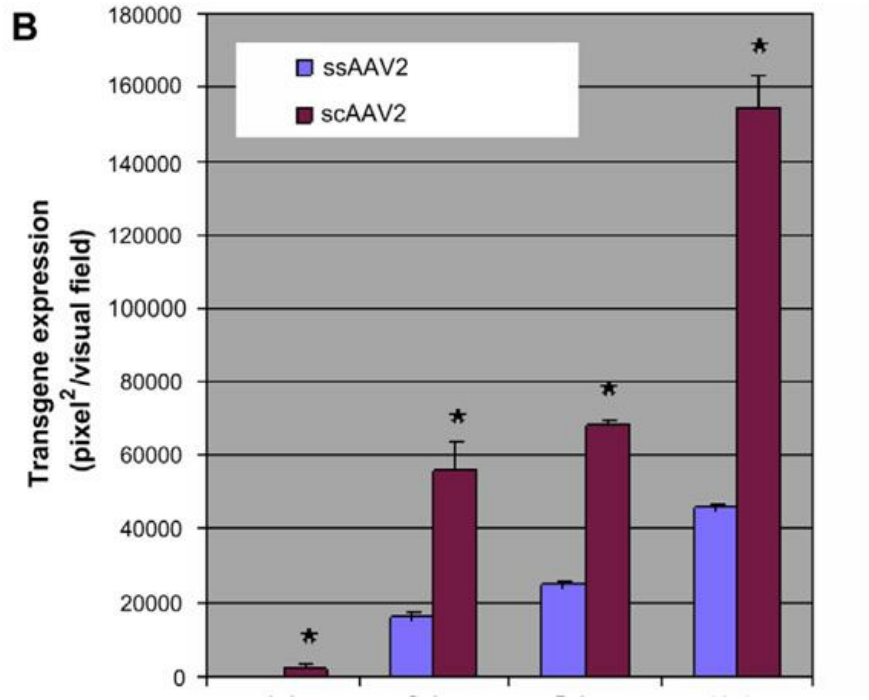


RBE – Rep-binding element

trs – terminal resolution site

Self complementary AAV





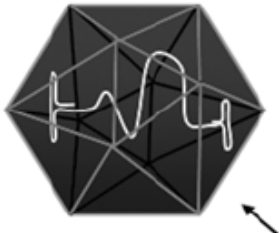
Transduction efficiency of ssAAV and scAAV vectors in human fibroblast cells (10000 vetor particles/cell)

Han Z. et al. Mol Genet Metab. 2008 April 93(4): 381-387

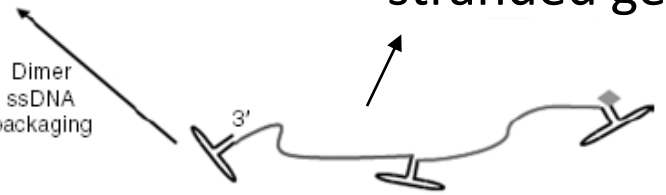
Self complementary AAV

First publication: August 2001, McCarty DM *et al.*

Demonstrated to be an improved transduction vector with a fast onset of gene expression



Lead to enhanced formation of transcription-competent double-stranded genomes



Production and purification of scAAV vectors is the same as conventional ssAAV

Production of scAAV constructs with one mutated ITR typically yields > 90% dimeric genoms

scAAV vs ssAAV

✓ Transgene expression evenly distributed among hepatocytes throughout the liver
✓ Transduce muscle cells 10 – 15 times more efficiently
✓ Greater saturation of transduced neuronal cells within limited area
✓ Transduce 2500-fold more retinal cells per particle at 5 weeks after infection
✓ Threefold increase in transduction after single infection in bone marrow-derived dendritic cells
✓

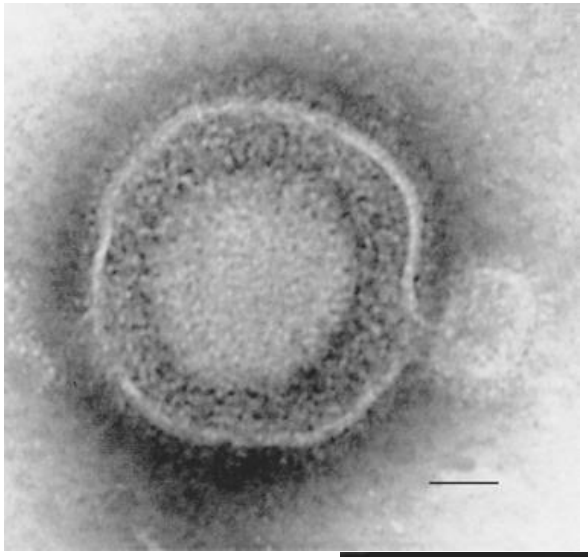
✗ Cell types that do not show improved transduction: polarized airway epithelial cell, primary B-cell chronic lypmhocytic leukemia cells
✗ Smaller capacity: only ~2,2 kb

Application of AAV in clinical gene therapy

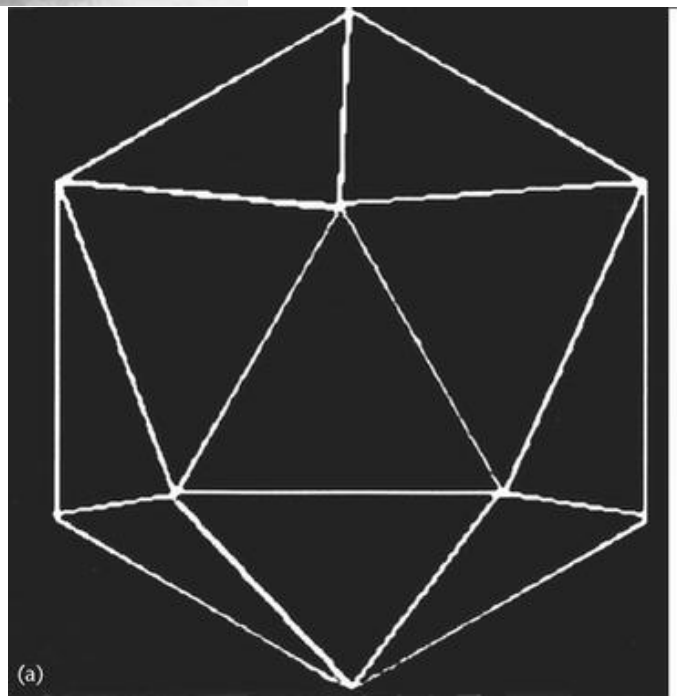
1. Nervous system diseases – Canvan disease
2. Cystic fibrosis
3. Haemophilia – transfer of factor IX
4. Muscular dystrophy
5. Leber's congenital amaurosis (blindness)
6. Cardiovascular diseases

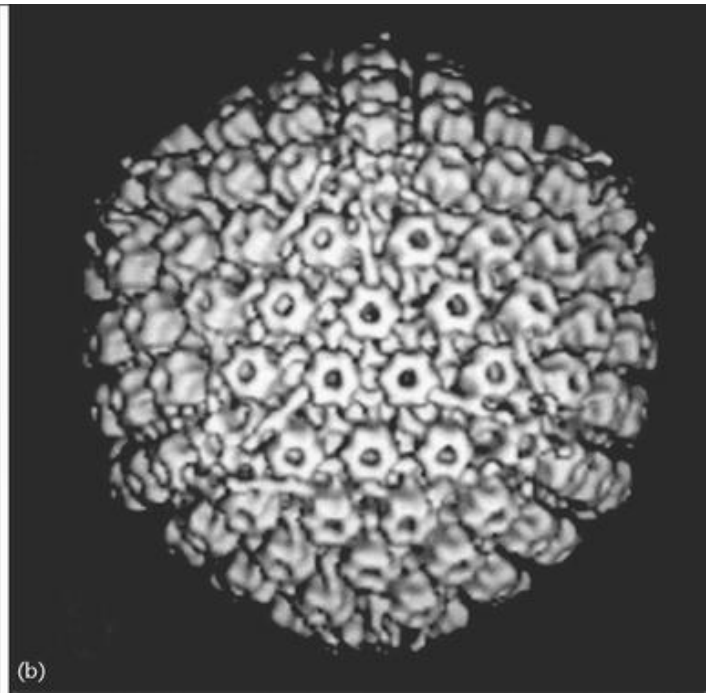
Other vectors

Herpes simplex viruses



HSV





Herpes Simplex Virus

- causes recurrent oropharyngeal cold sores
- initially establishes a productive infection in epithelial cells, gains access to the sensory nerve endings supplying the infected area, and travels by retrograde axonal flow to neuronal cell bodies within the respective dorsal root ganglia
- can establish a latent infection within sensory neurons and lytic replication in the nervous system is generally limited
- reactivates periodically in a fraction of the latently infected neuronal cells
- the newly replicated virus is transported anterograde, usually to a site at or near the portal of entry into the body, where it may cause a localized lesion

Herpes virsus - 1

Advantages

Very large genome – 152 kb – linear, double strand DNA, contains at least 84 genes, of which around half is not required for the virus replication

Large transgene can be introduced – up to 30 kb

Large number of viral copies can be produced

Virus is not toxic, may stay in latent form for a long time

Transduce numerous cell types

Limitations

Lack of data concerning the application of recombinant herpesviruses in patients

Problems with targeting to specific cell type

Applications of HSV-1 vectors

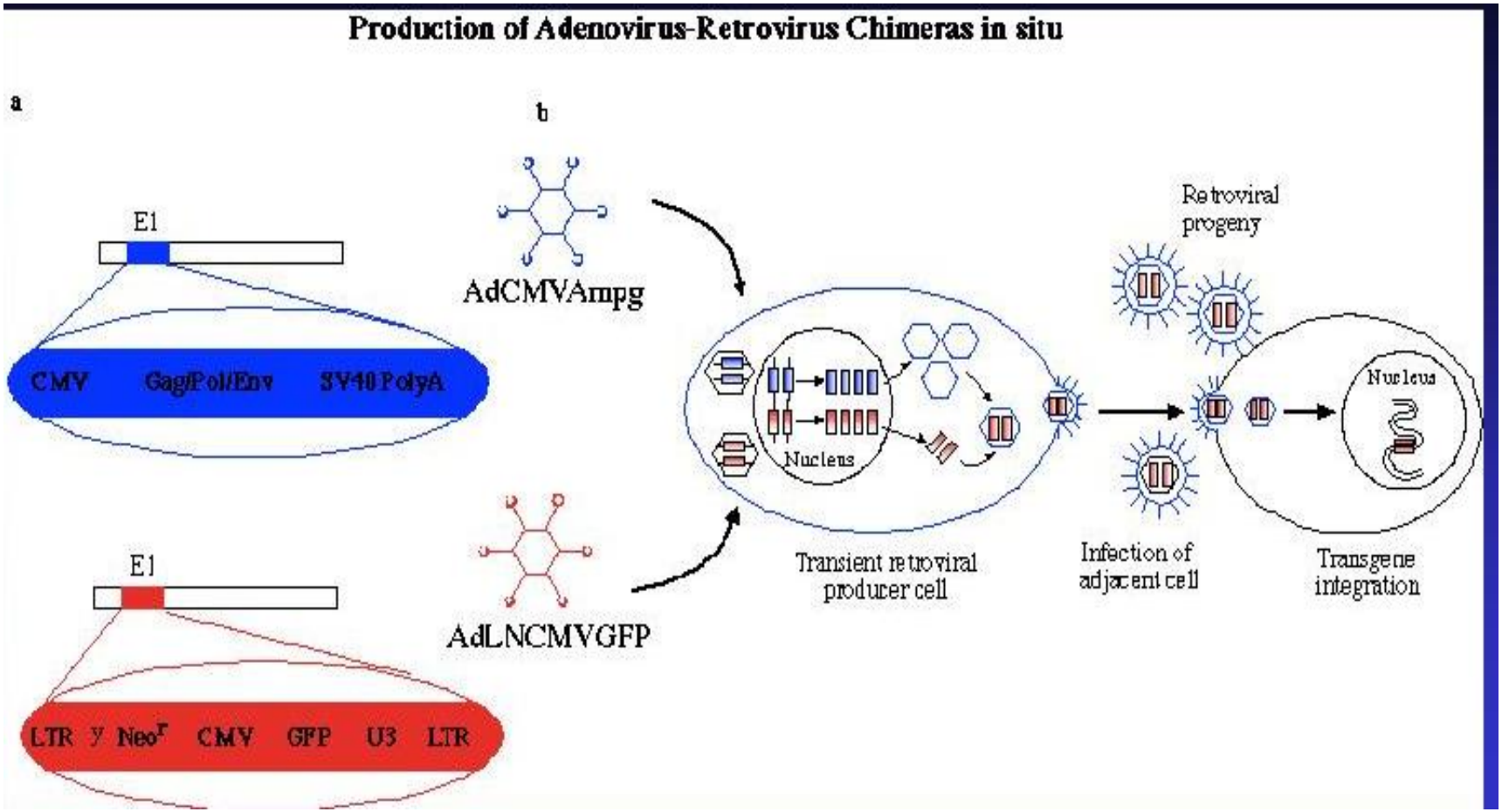
Experimental studies

1. Tumors, including nervous systems
2. Diseases of the central and peripheral nervous systems
3. Injury to spinal cord
4. Treatment of pain – delivery to sensory nerves looks particulalry promising

Adeno-retroviral Chimeric Vectors

- incorporate the favorable attributes of different vectors
- combines the high *in vivo* efficiency of gene delivery of recombinant adenoviral vectors with integrative capacities derived from retroviruses

Adeno-retroviral chimeric vectors



Other viral vectors

1.retro-adenoviral vectors: contains LTR of retrovirus,
capsid and other genome features of adenovirus

2. Polio virus, hepatitis A virus

3. Ebola virus

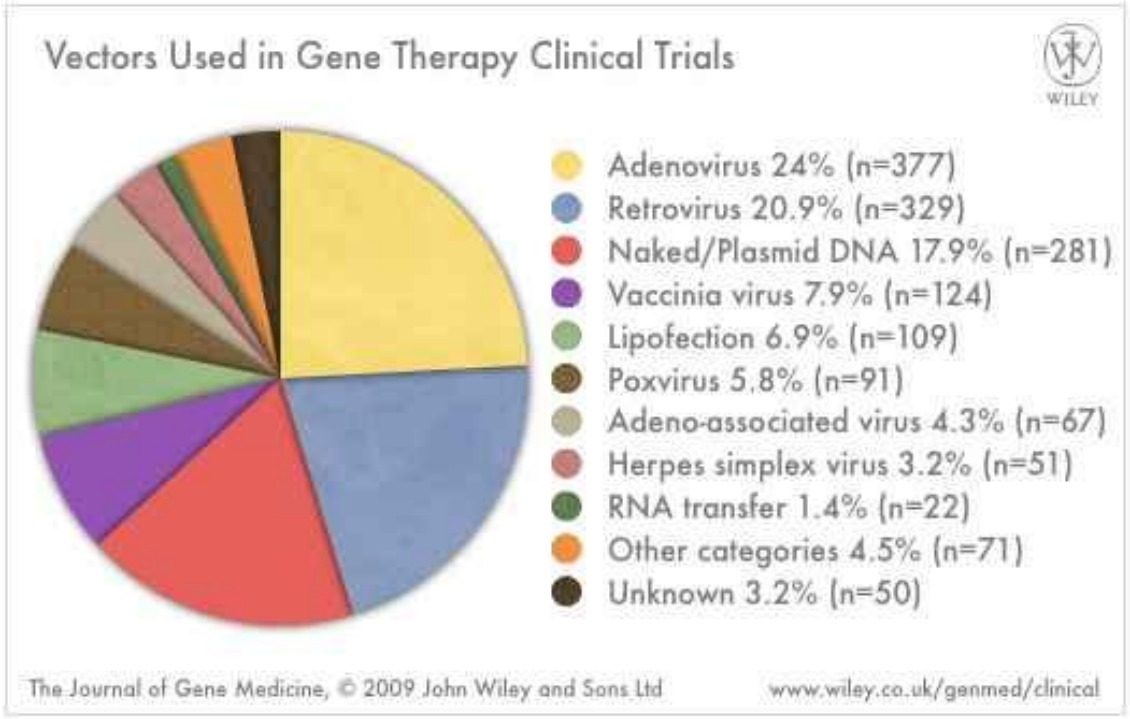
lentiviral vectors containing proteins of Ebola virus capsid
- infects cells of respiratory epithelium

4. Baculoviruses

5. Alpha viruses

Types of vectors used in clinical trials of gene therapy

DMB



six years ago...

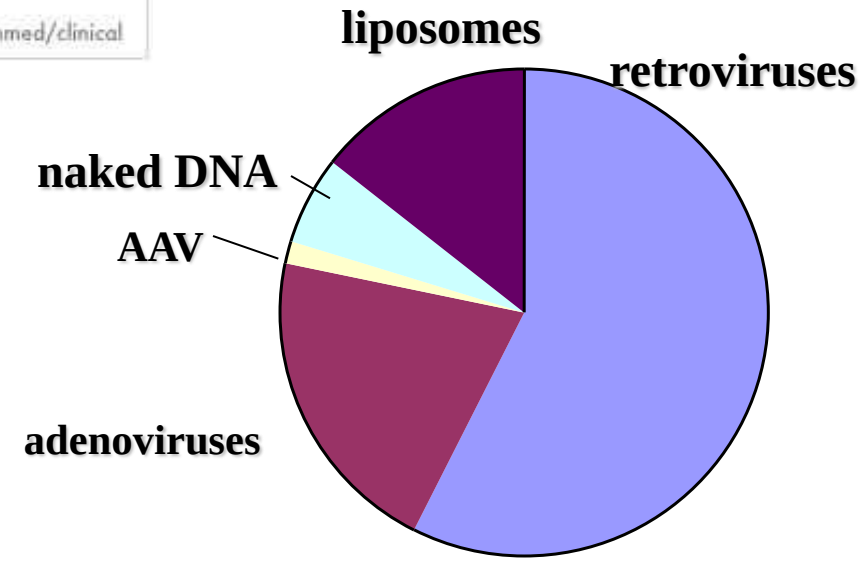


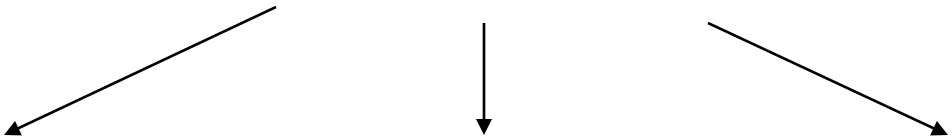
Table 1 | **Key features of viral vectors**

Feature	Adenoviral vector	Helper-dependent adenoviral vector	AAV vector	Retroviral vector	Lentiviral vector
Particle size (nm)	70–100	70–100	20–25	100	100
Cloning capacity (kb)	8–10	~30	4.9 (10 after heterodimerization of two AAV virions)	8	9
Chromosomal integration	No	No	No (yes if *rep* gene is included)	Yes	Yes
Vector yield (transducing units/ml)	High (10^{12})	High (10^{12})	High (10^{12})	Moderate (10^{10})	Moderate (10^{10})
Entry mechanism	Receptor (CAR)-mediated endocytosis, endosomal escape and microtubule transport to the nucleus		Receptor-mediated endocytosis, endosomal escape and transport to the nucleus	Receptor binding, conformational change of Env, membrane fusion, internalization, uncoating, nuclear entry of reverse-transcribed DNA	
Transgene expression and practical application	Weeks to months; highly efficient short-term expression (e.g. for cancer or in acute cardiovascular diseases)	>1 year; highly efficient medium- to long-term expression	>1 year; medium- to long-term gene expression for non-acute diseases (onset of transgene expression after ~3 weeks)	Long-term correction of genetic defects	
Oncolytic potential?	Yes	No	No	No (but has potential to spread through the tumour without lysis, thereby spreading a suicide gene that encodes a pro-drug-converting enzyme)	
Emergence of replication-competent vector *in vivo*?	Possible but not a major concern	Negligible, low risk	Possible but not a major concern	Risk is a concern	Risk is a concern
Infects quiescent cells?	Yes	Yes	Yes	No	Yes
Transcriptional targeting affected by chromosomal integration site?	No	No	No	Yes	Yes
Risk of oncogene activation by the vector?	No	No	No	Yes	Yes

AAV, adeno-associated virus; CAR, coxsackie and adenovirus receptor; Env, viral envelope protein.

Inhibition of gene expression by means of nucleic acids

Gene therapy



Enhancing

enhancement
of gene expression

Acquired diseases

Substituting

delivery of
the missing gene

Inherited diseases

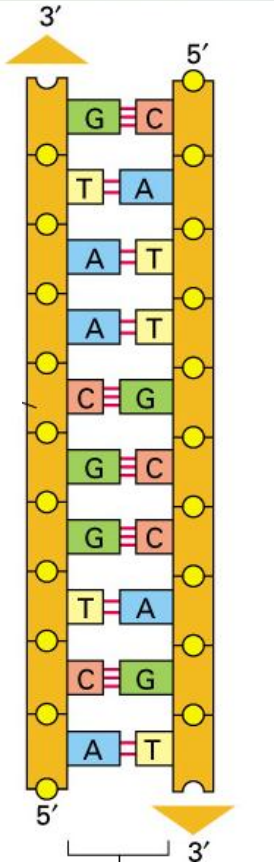
Suppresive

inhibition of
gene expression

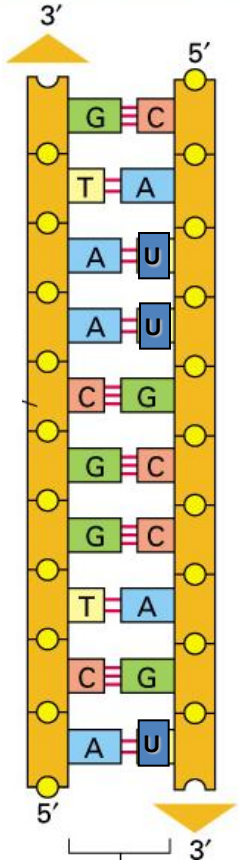
Acquired diseases

Hybridisation of nucleic acids as a way to inhibit gene expression

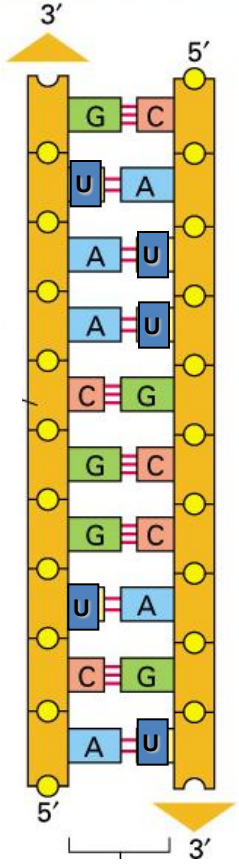
DNA hybridizes with DNA



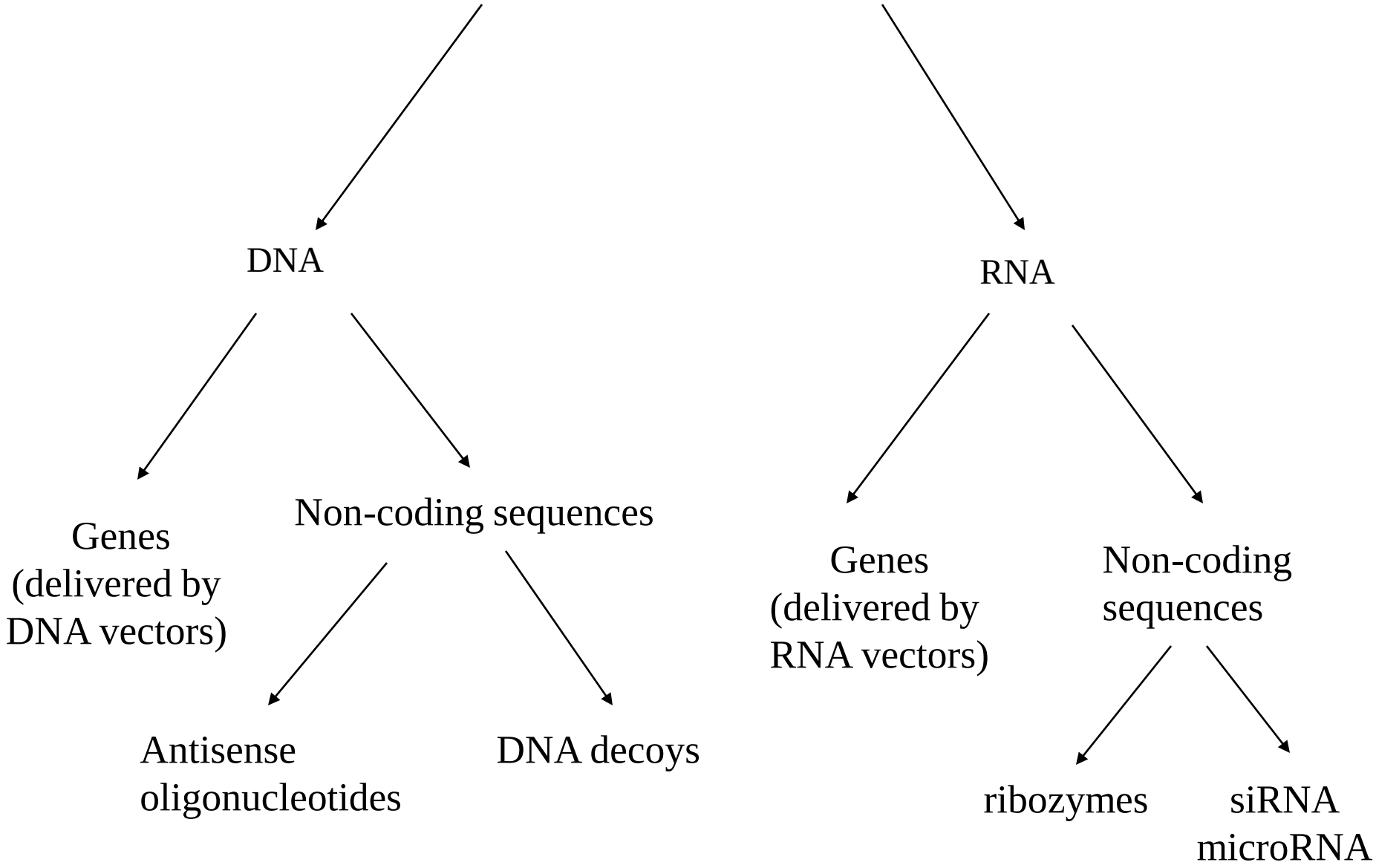
DNA hybridizes with RNA



RNA hybridizes with RNA



Therapeutic nucleic acids



Inhibitory nucleic acids

1. **Antisense oligonucleotides**: short, 12-20 nts.

2. **Triple helix-forming oligonucleotides (TFO)**

pyrmidine oligodeoxynucleotides that specifically bind to a major groove of polypurine region of dsDNA via the formation of triple helices according to recognition rules established by Hoogsten

3. **Ribozymes** – short catalytically active RNSs

4. **Deoxyribozymes (DNAzymes) –** short catalytic DNA that cleave sequence-specifically ratget RNA.

More stable than RNA, it is easier to synthesize and to modify them.

5. siRNA/microRNA

Aptamers

Nucleic acids that bind proteins

Exist naturally – produced by viruses (HIV, adenoviruses)

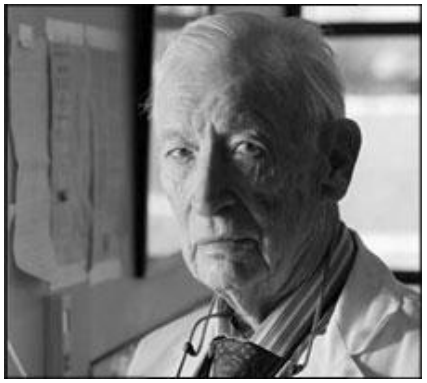
1. **DNA decoys** – bind transcription factors

2. **Pegaptinib sodium** – a pegylated oligonucleotide binding $VEGF_{165}$

Antisense oligonucleotides

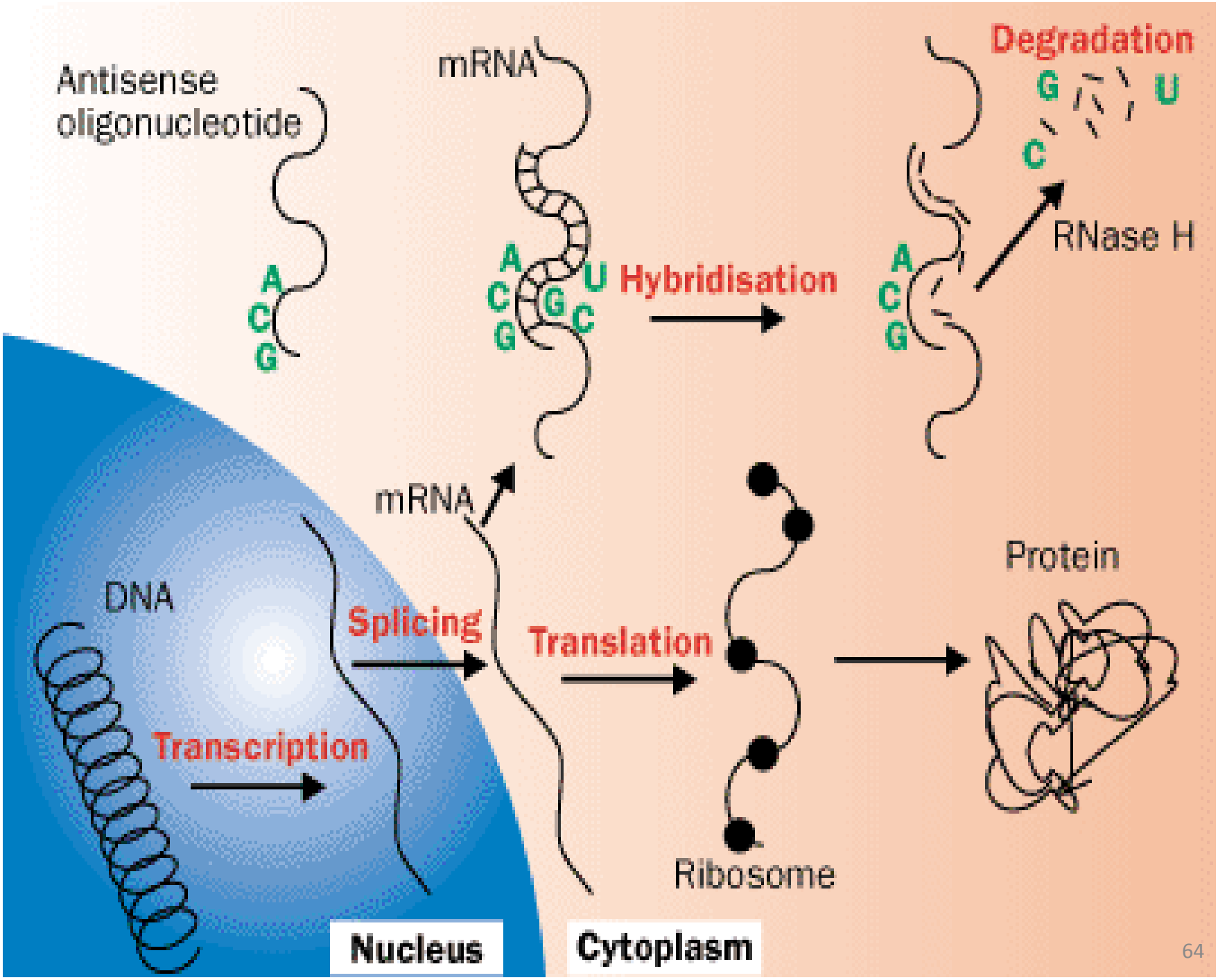
Antisense oligonucleotides

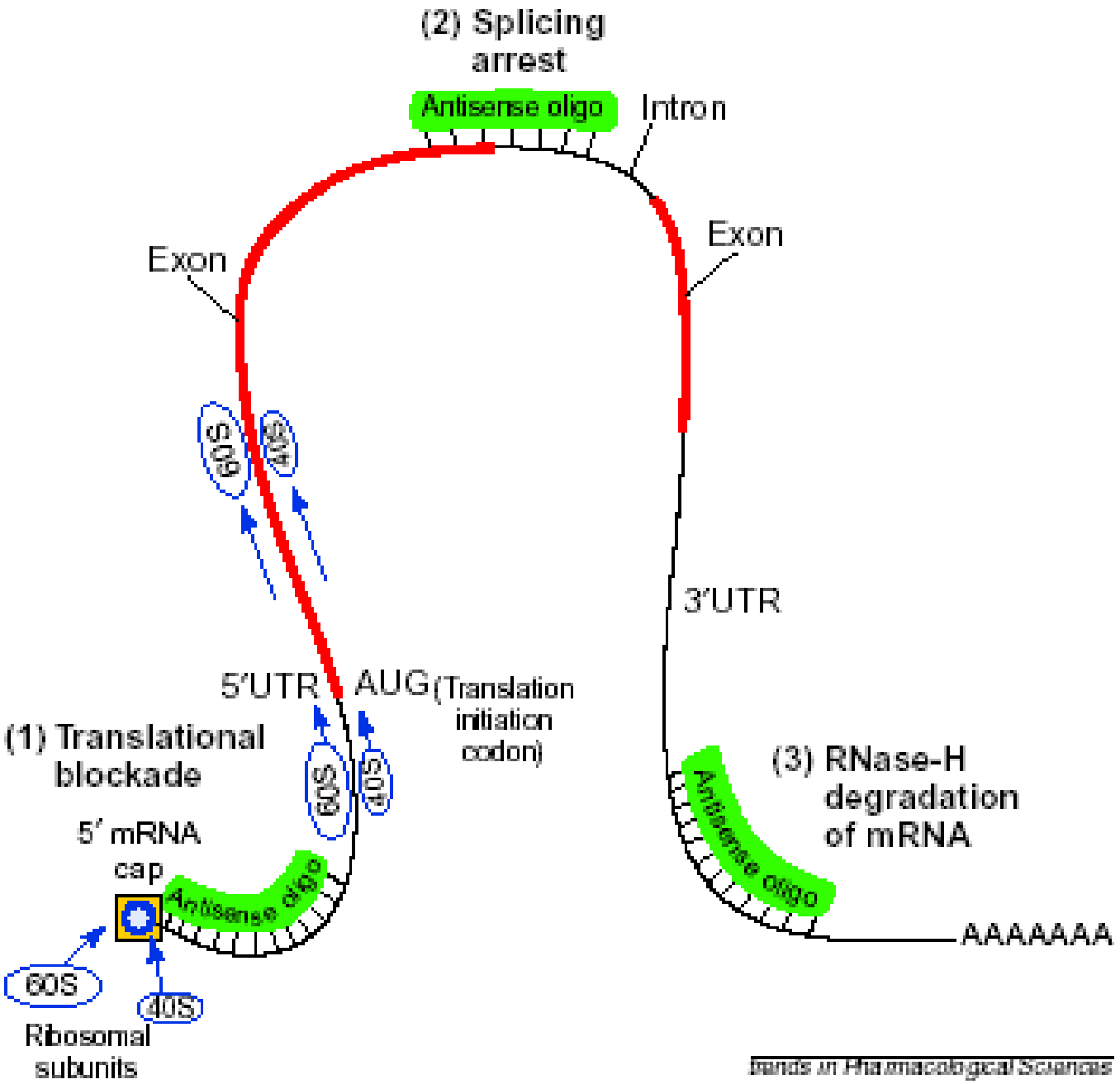
Short fragments of single strand, chemically modified DNA nucleotides (oligonucleotides), complementary to a given mRNA.



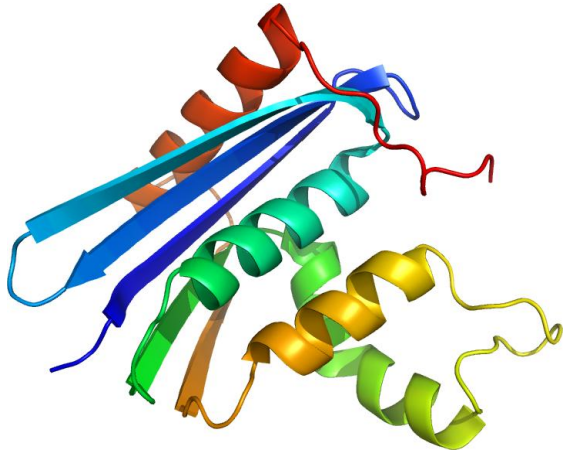
Paul Zamecnik
(b. 1912)
discoverer of tRNA
died October 2009

Later in his career, Zamecnik and Stephenson developed antisense technology, in which short, synthetic nucleotide sequences can be used to silence the activity of individual genes. They published their results, in which they used a 13-nucleotide sequence to halt production of Rous sarcoma virus in chicken embryos, in 1978. That paper, which appeared in *Proceedings of the National Academy of Sciences*, has been cited more than 900 times, according to ISI


Antisense
oligonucleotide
mRNA
Degradation
Hybridisation
RNase H
mRNA
DNA
Splicing
Translation
Protein
Transcription
Ribosome
Nucleus
Cytoplasm


(2) Splicing arrest
Antisense oligo
Intron
Exon
Exon
60S
40S
3′UTR
5′UTR
AUG (Translation initiation codon)
(1) Translational blockade
60S
40S
(3) RNase-H degradation of mRNA
5′ mRNA cap
Antisense oligo
Antisense oligo
AAAAAAA
60S
40S
Ribosomal subunits
Trends in Pharmacological Sciences

RNAse H



The enzyme **RNase H** (EC 3.1.26.4)is a ribonuclease that cleaves the 3'-O-P-bond of RNA in a DNA/RNA duplex to produce 3'-hydroxyl and 5'-phosphate terminated products. RNase H is a non-specific endonuclease and catalyzes the cleavage of RNA via an hydrolytic mechanism, aided by an enzyme-bound divalent metal ion.

Members of the RNase H family can be found in nearly all organisms, from archaea and prokaryota to eukaryota . In eukaryotic DNA replication RNase H is responsible for cutting out the RNA primer, allowing completion of the newly synthesized DNA

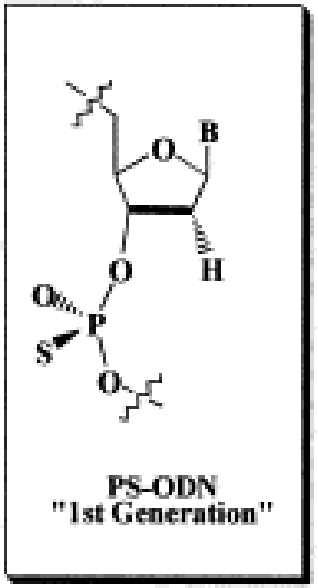
Antisense oligonucleotides

- 13-25 nucleotides long
- hybridize to corresponding RNA
- act by: a) modulation of splicing
  b) inhibition of protein translation by disruption of protein assembly
  c) utilise RNAse H enzymes

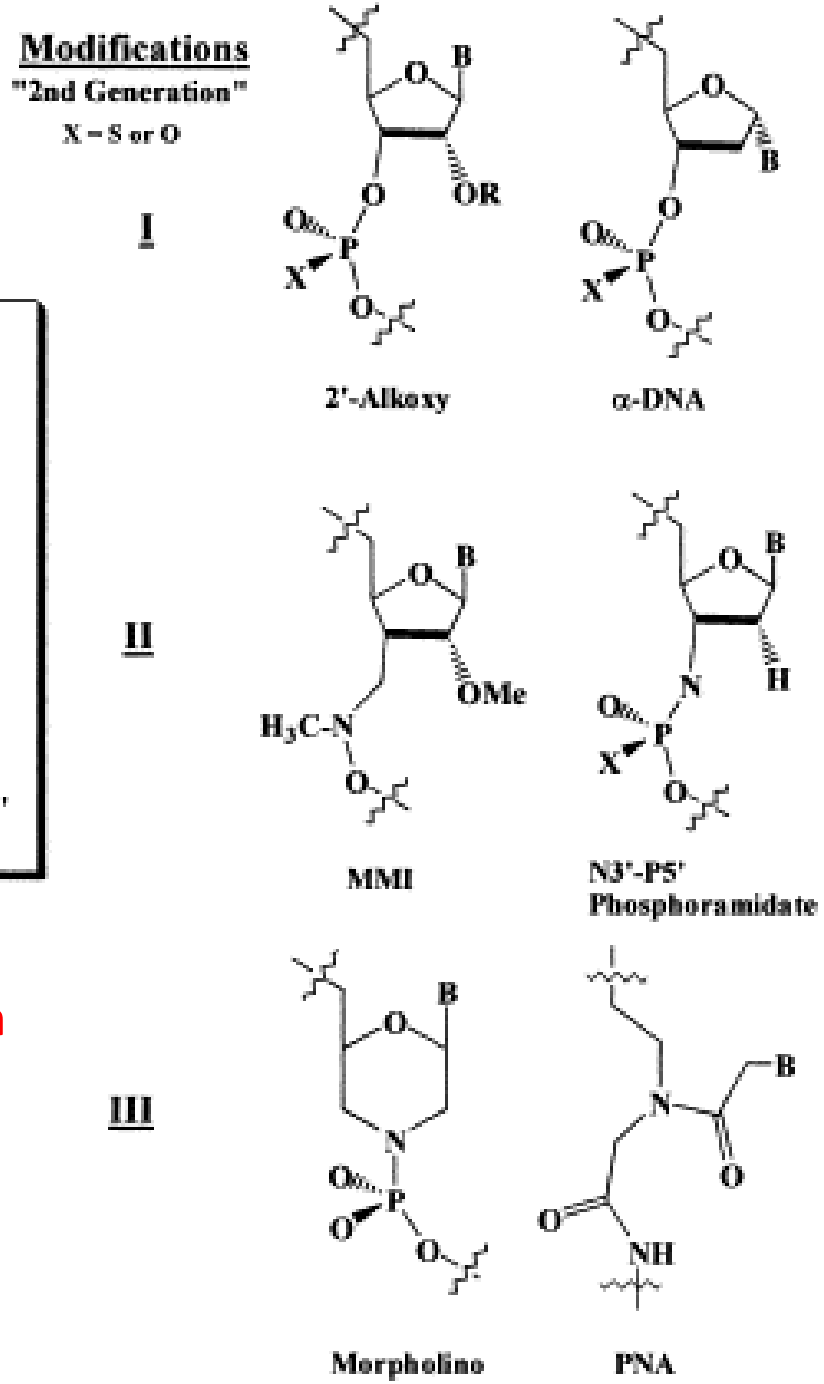
Oligonucleotides fate in vivo

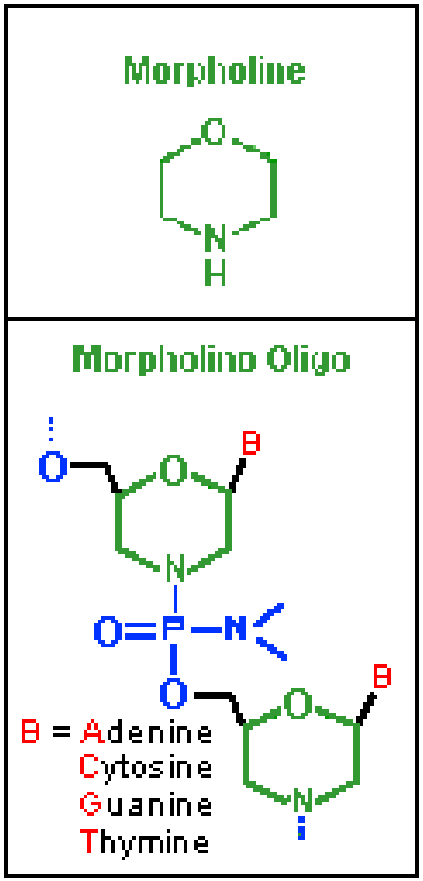
1.Degradation by serum nucleases
2. Degradation by liver cells (unspecific uptake)
3. Degradation by kidney cells and exctretion with urine

Modification of antisense oligonucleotide



1st generation





Antisense strategy - problems

1. Stability vs binding affinity
2. Delivery to target cells
3. Non-antisense effect
   a) immune stimulation - CpG motifs
   - phosphorotioate backbone

4. Well tolerated, side effects are dose dependent

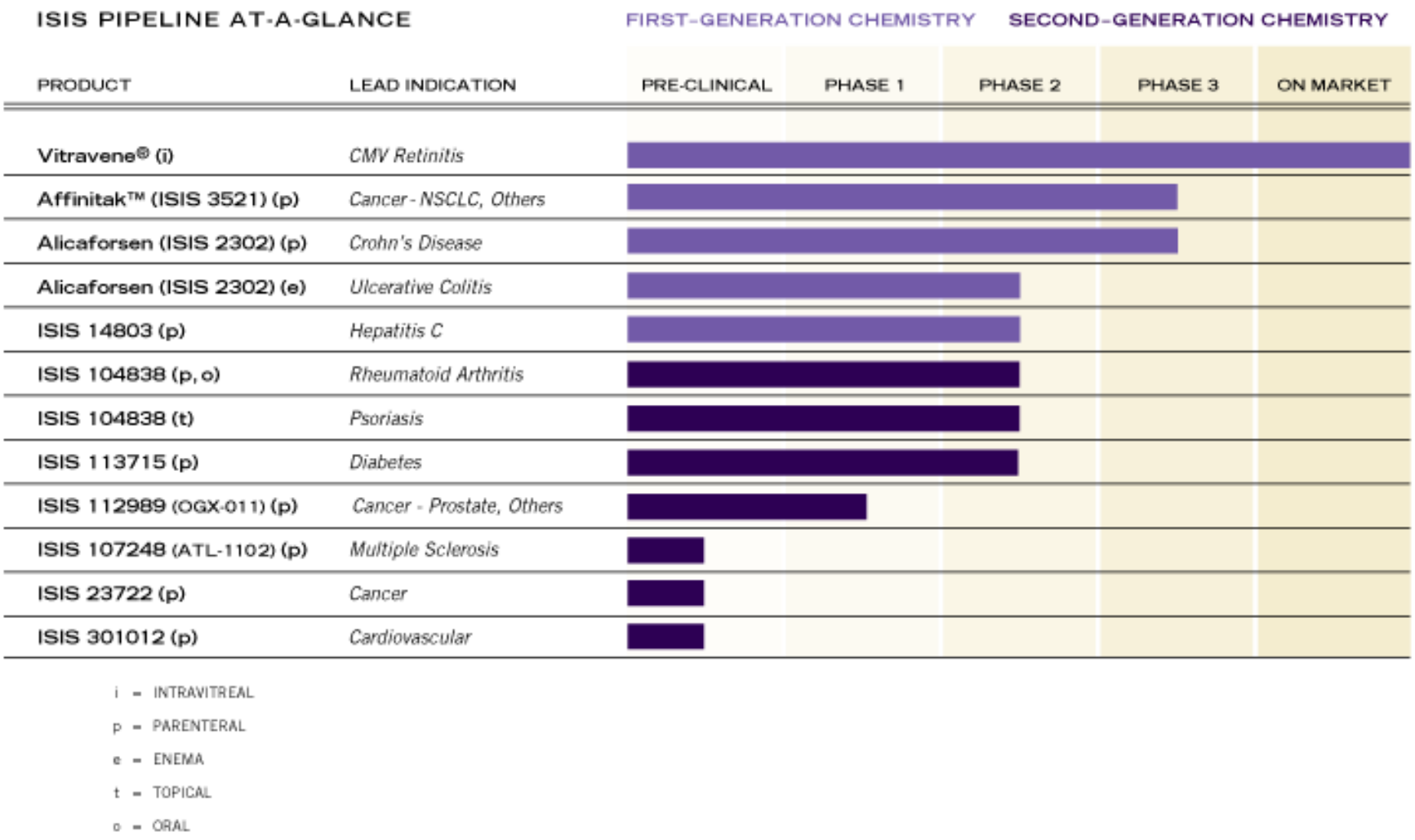
5. Side effects: thrombocytopenia
   hypotension
   fever
   asthenia
   increase liver enzymes
   complement activation

Clinical application of antisense oligonucleotides

Vitravene - CMV-induced retinitis

Bcl2 antisense - melanoma

Clinical trials with antisense oligonucleotides

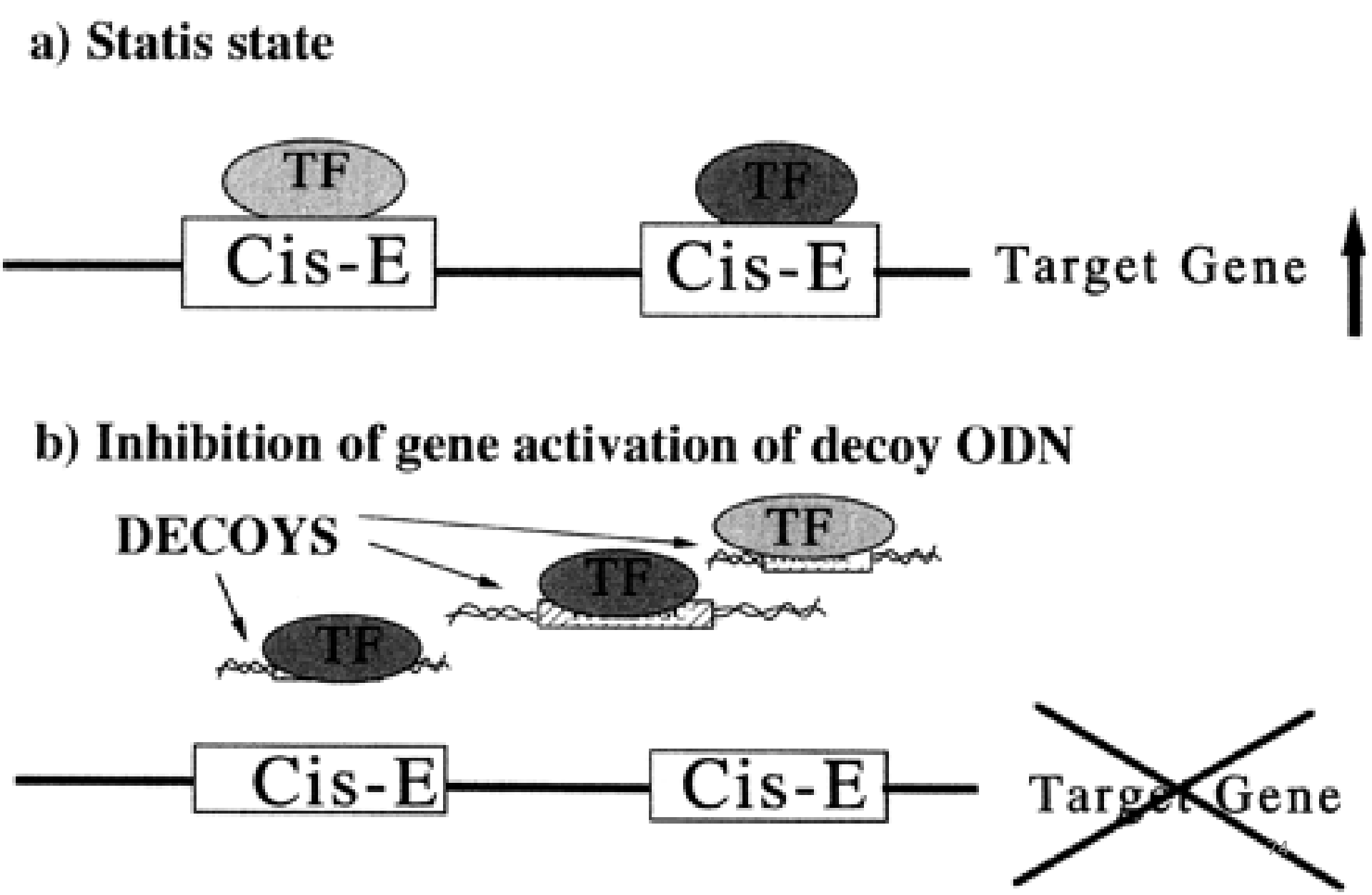


Clinical trials of antisense therapies was stopped

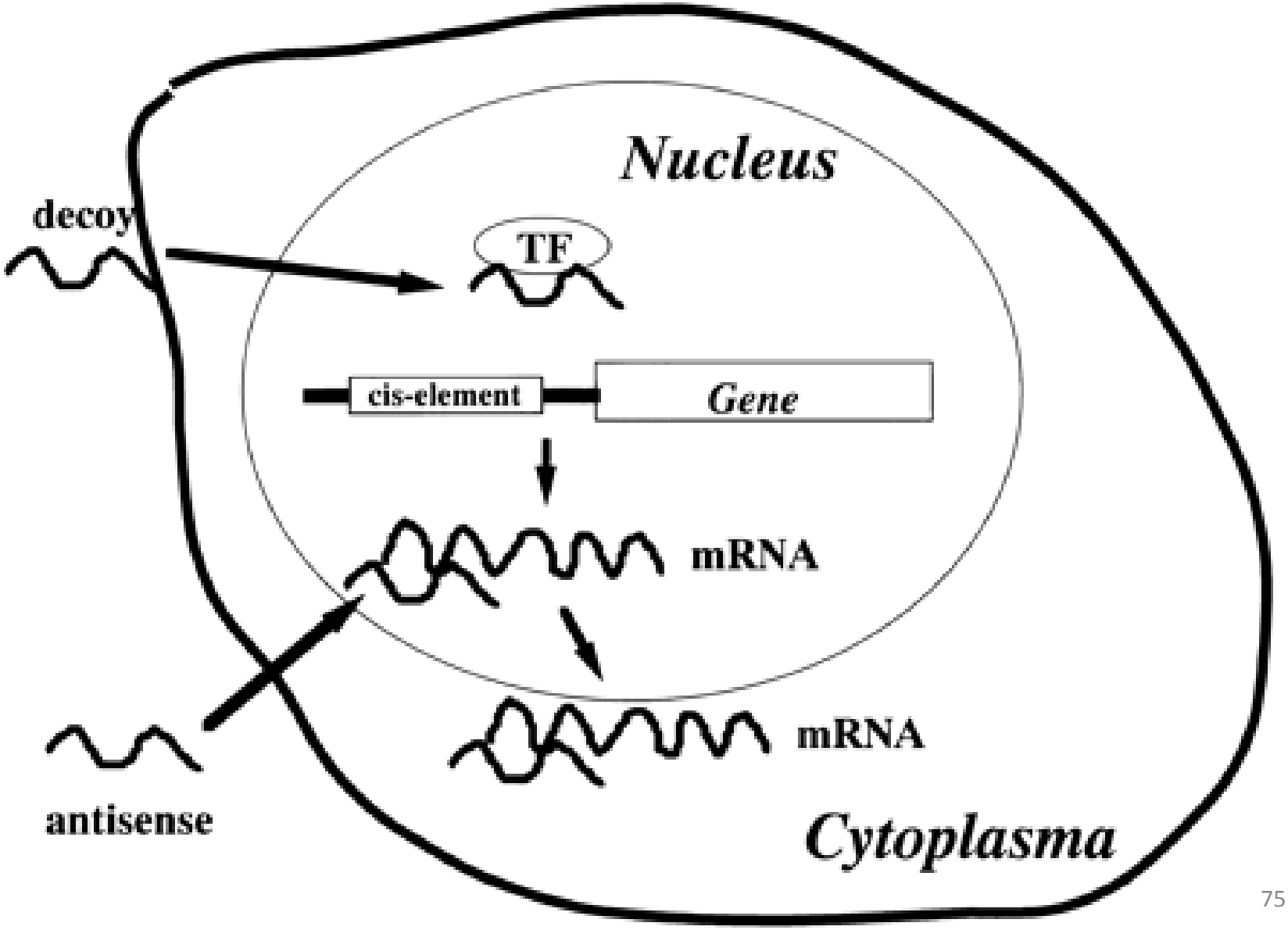
DNA decoys

Pułapki oligonukleotydowe

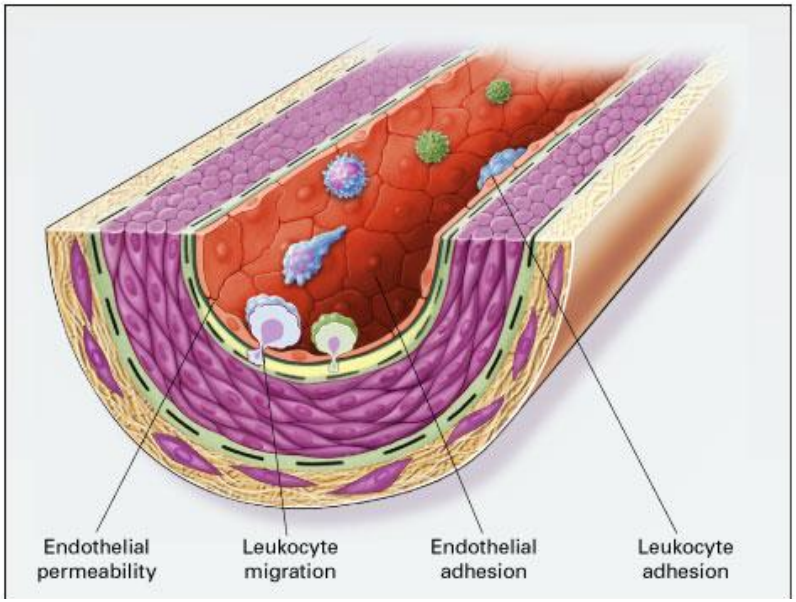
DNA decoys

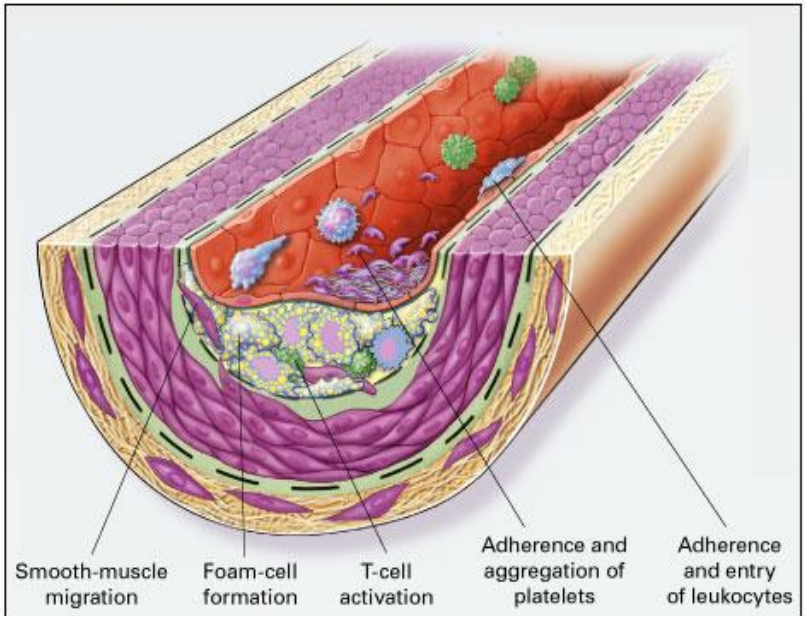

a) Statis state
TF
TF
Cis-E
Cis-E
Target Gene
b) Inhibition of gene activation of decoy ODN
DECOYS
TF
TF
TF
Cis-E
Cis-E
Target Gene

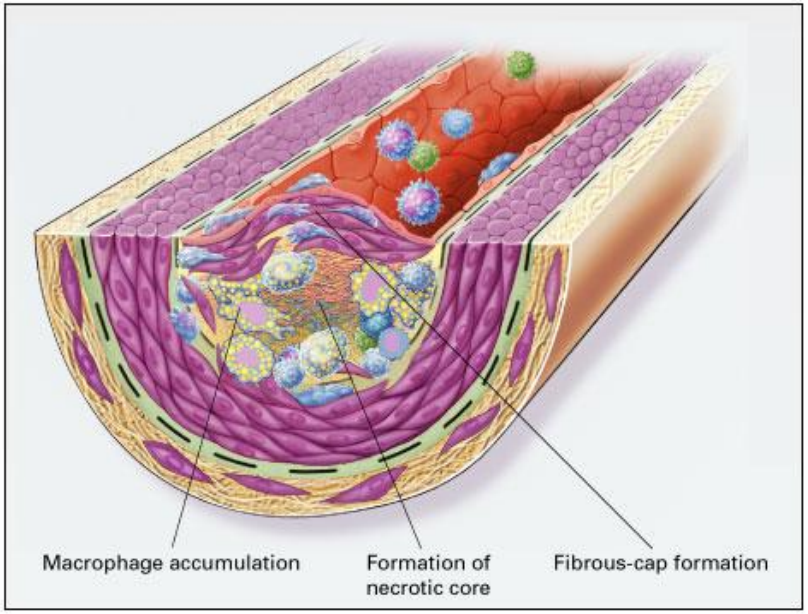
DNA decoys

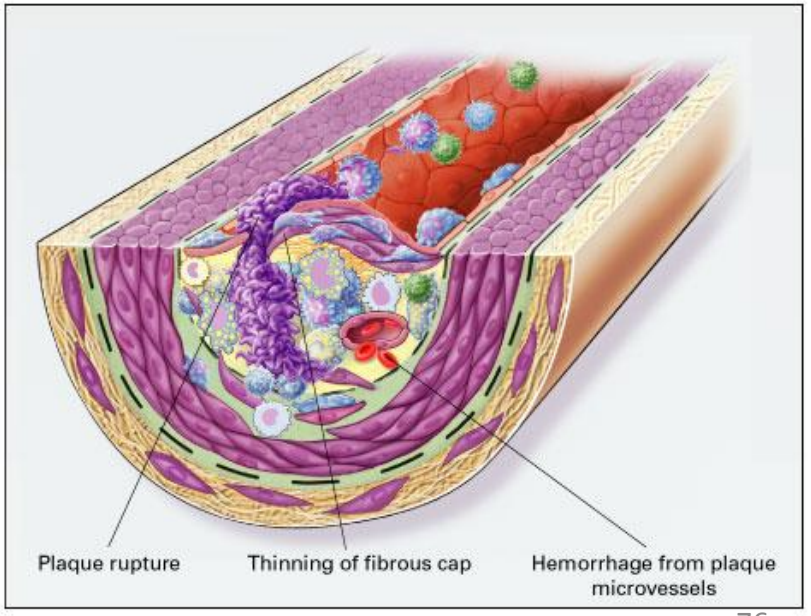

Nucleus
decoy
TF
cis-element
Gene
mRNA
mRNA
antisense
Cytoplasma

Progression of atherosclerosis

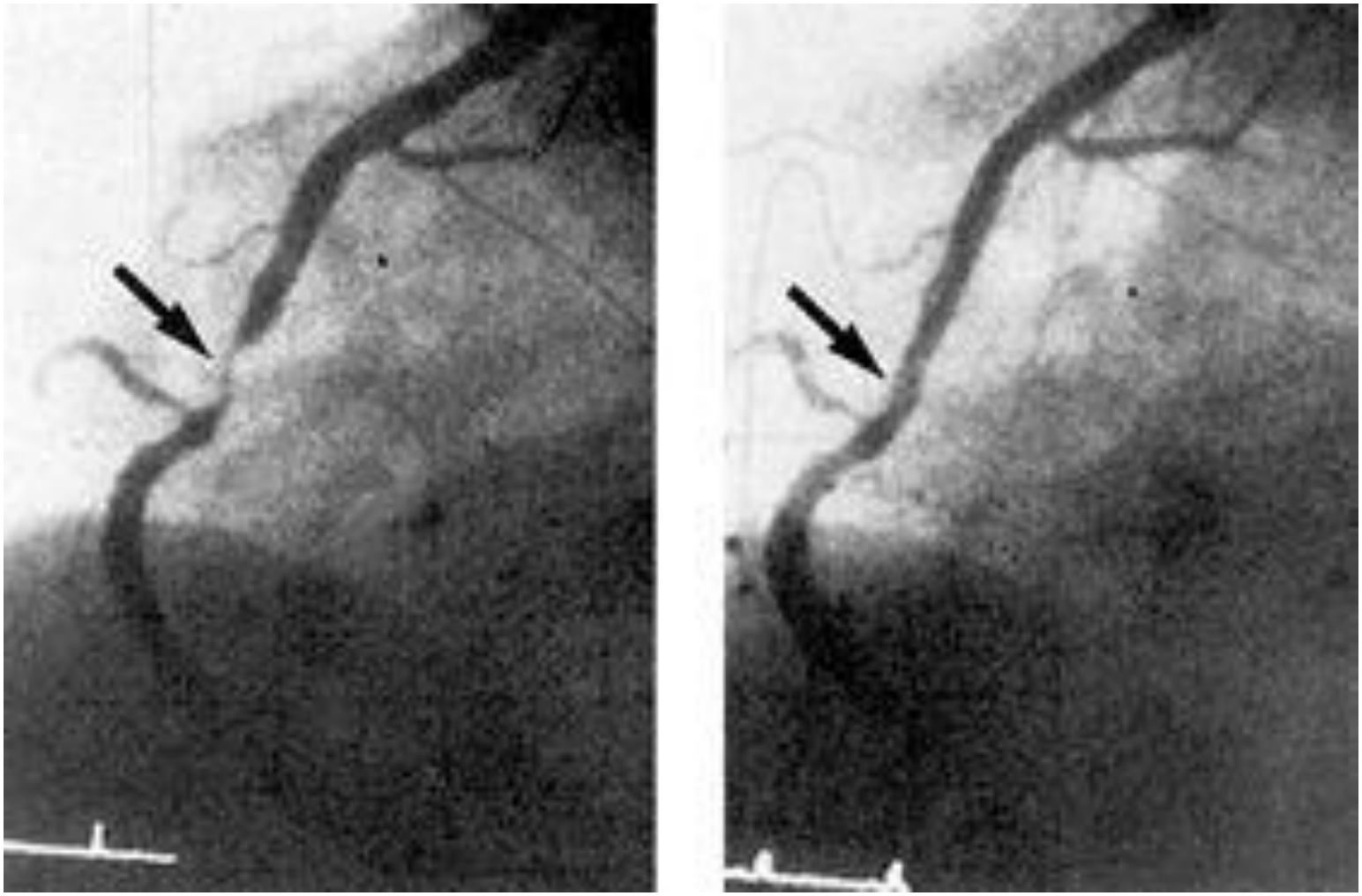




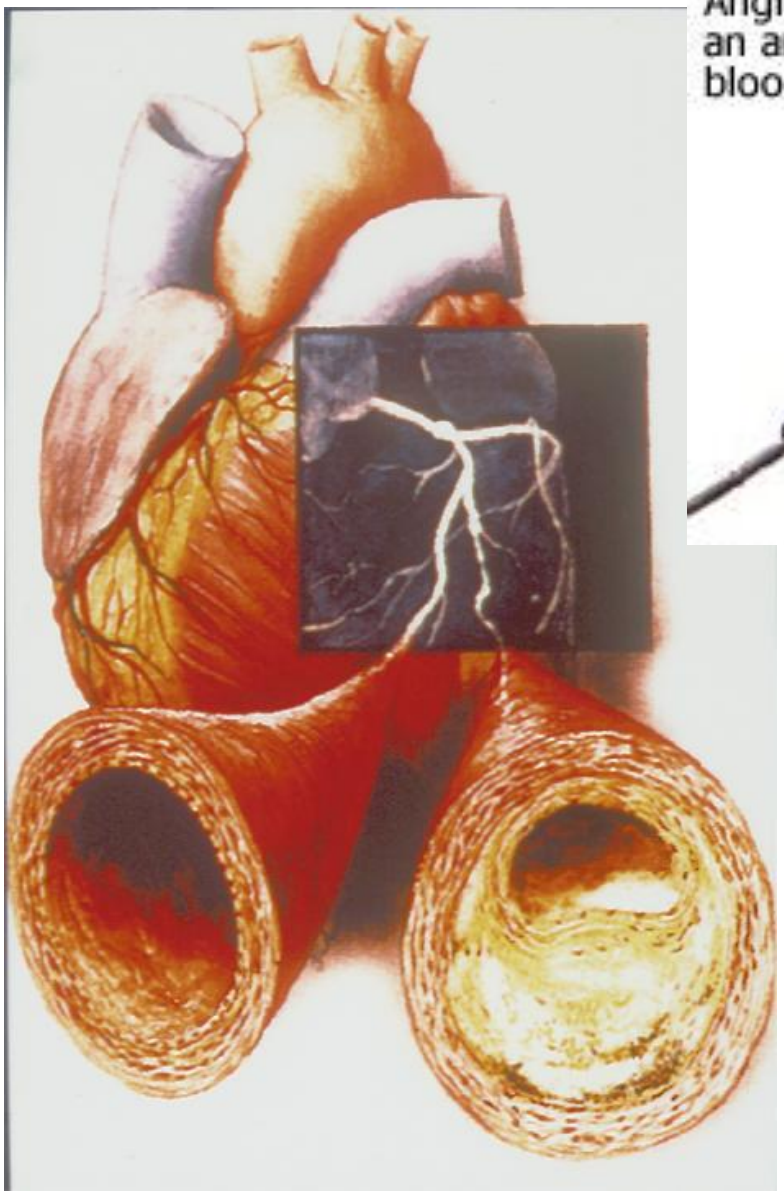


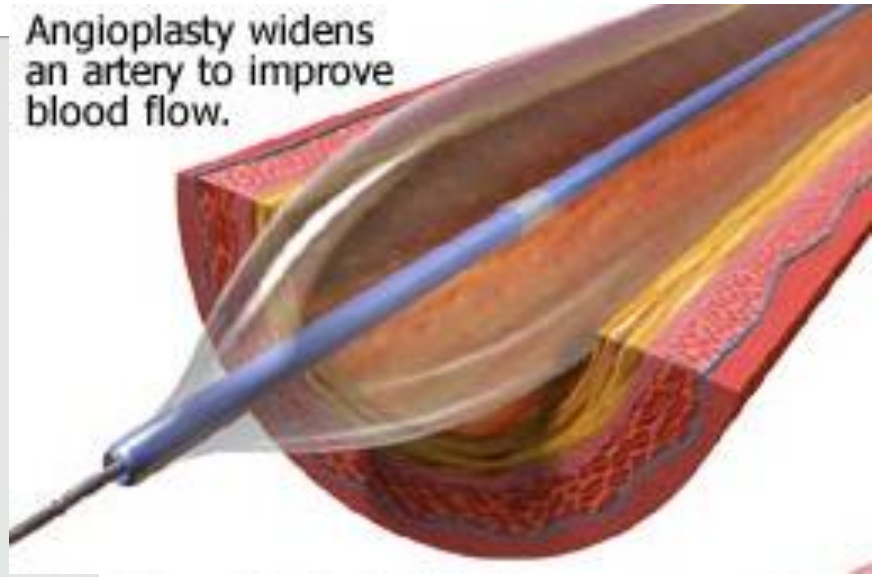


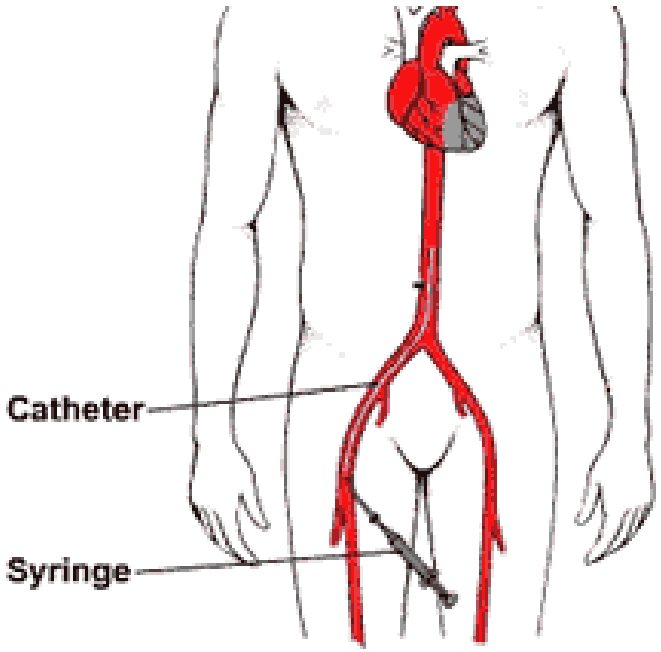
Ross, NEJM, 1999



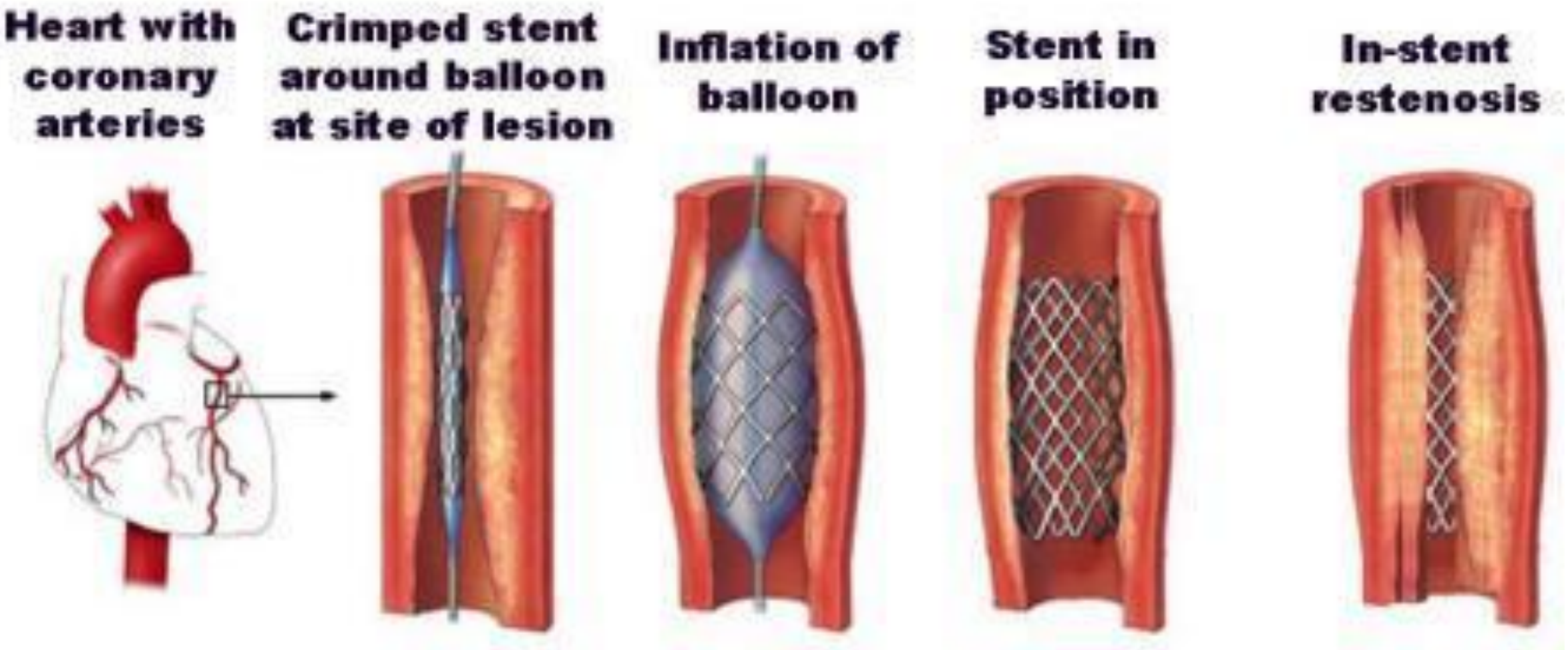
Balloon angioplasty for prevention of vessel narrowing



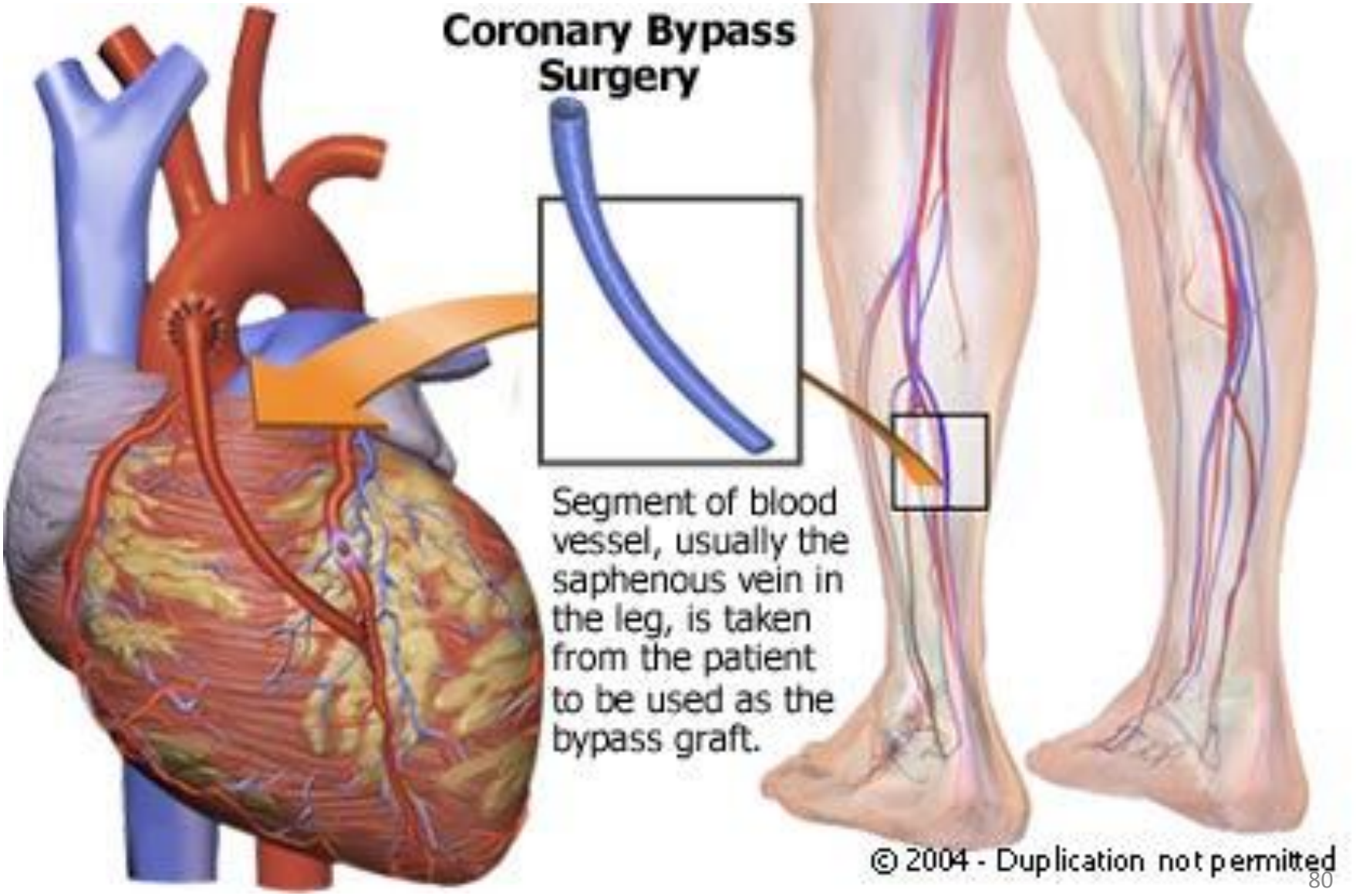




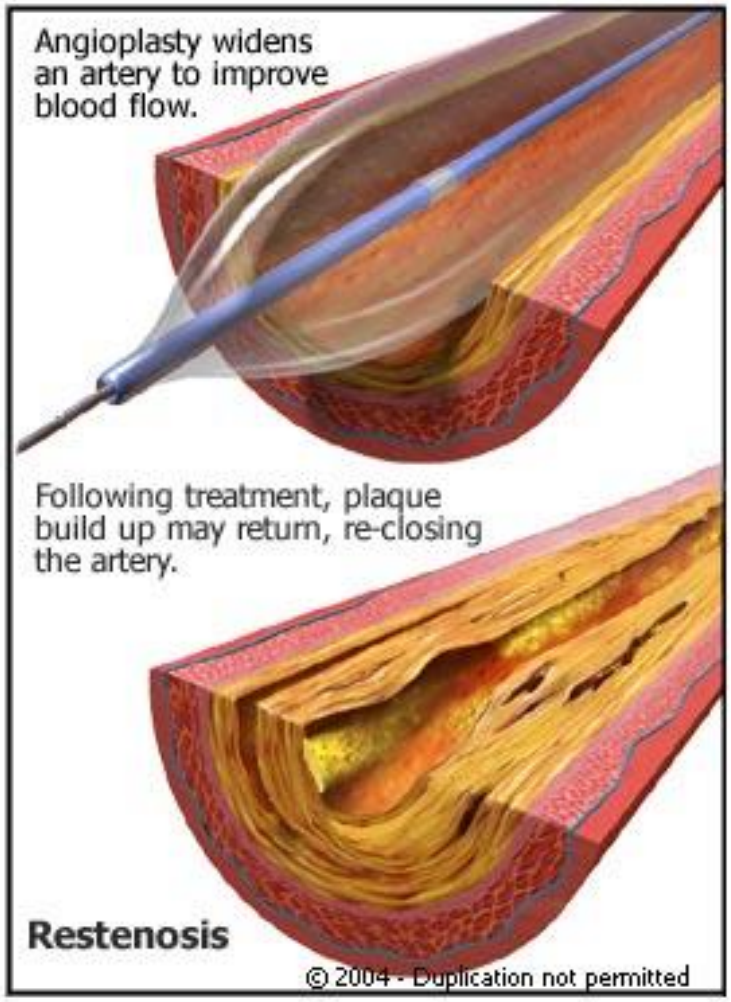
Treatmen of Atherosclerosis
STENT placement



Coronary bypass grafting (CABG)



Narrowing of blood vessels after angioplasty or CABG

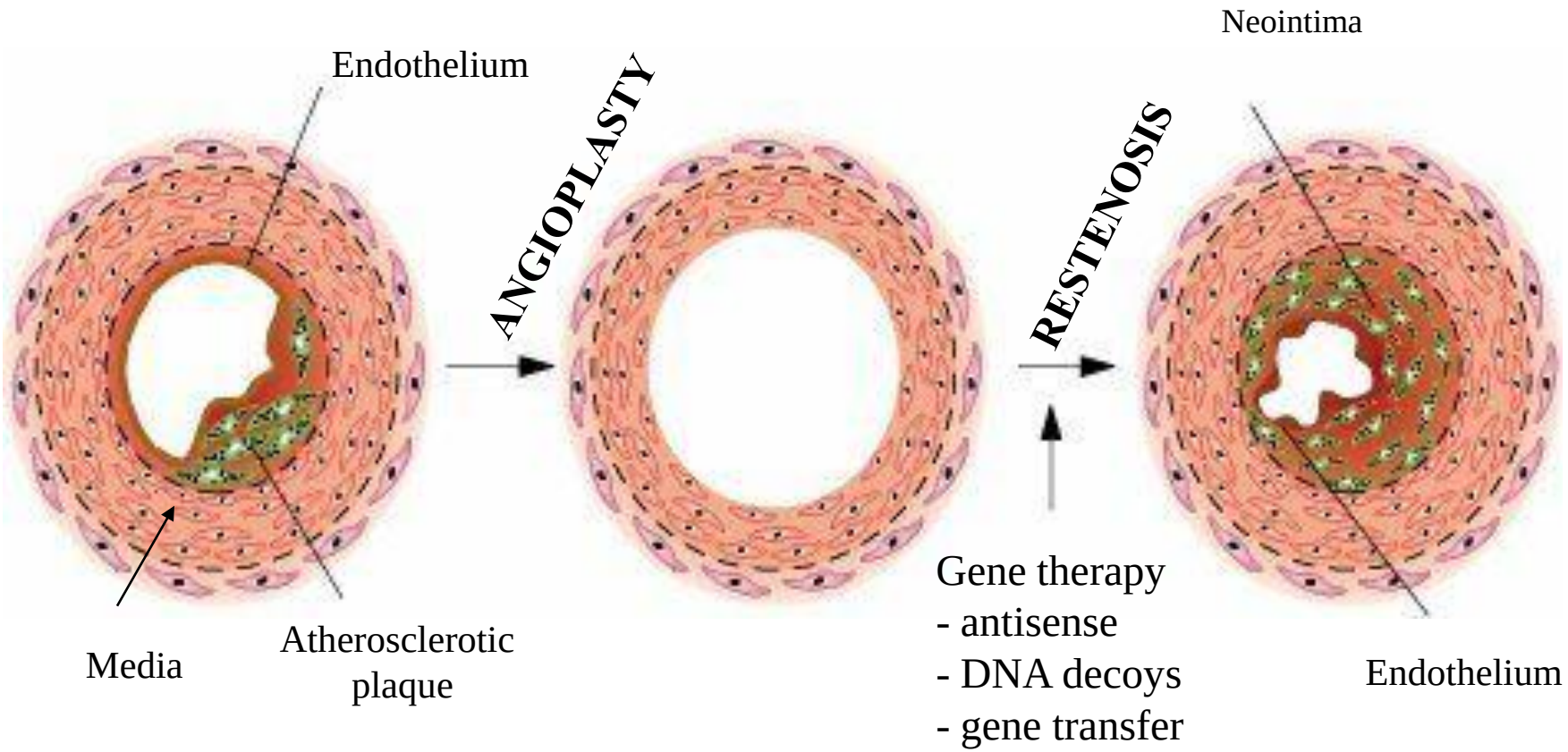


RESTENOSIS

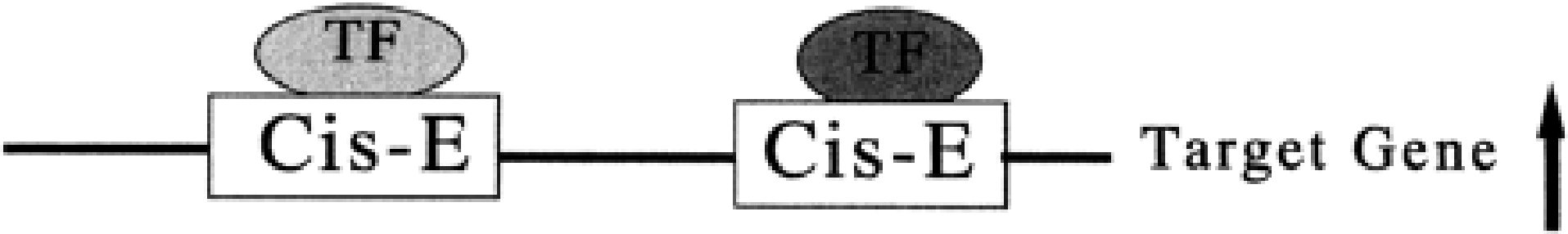
narrowing occurs also in vessels used for by-pass grafting

- ***STENOSIS***

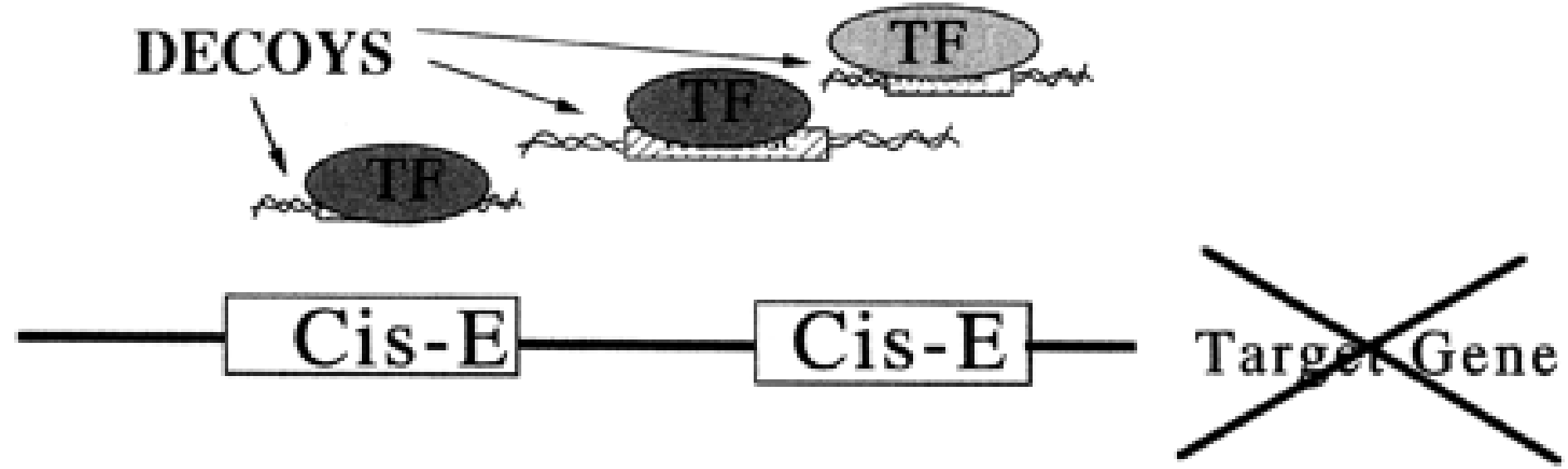
Gene therapy for treatment of neointima formation after balloon angioplasty



a) Statis state

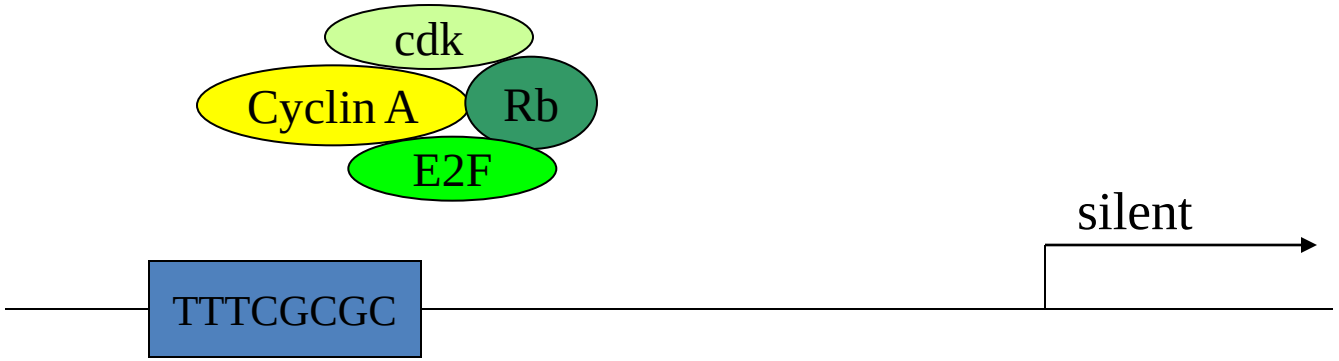


b) Inhibition of gene activation of decoy ODN

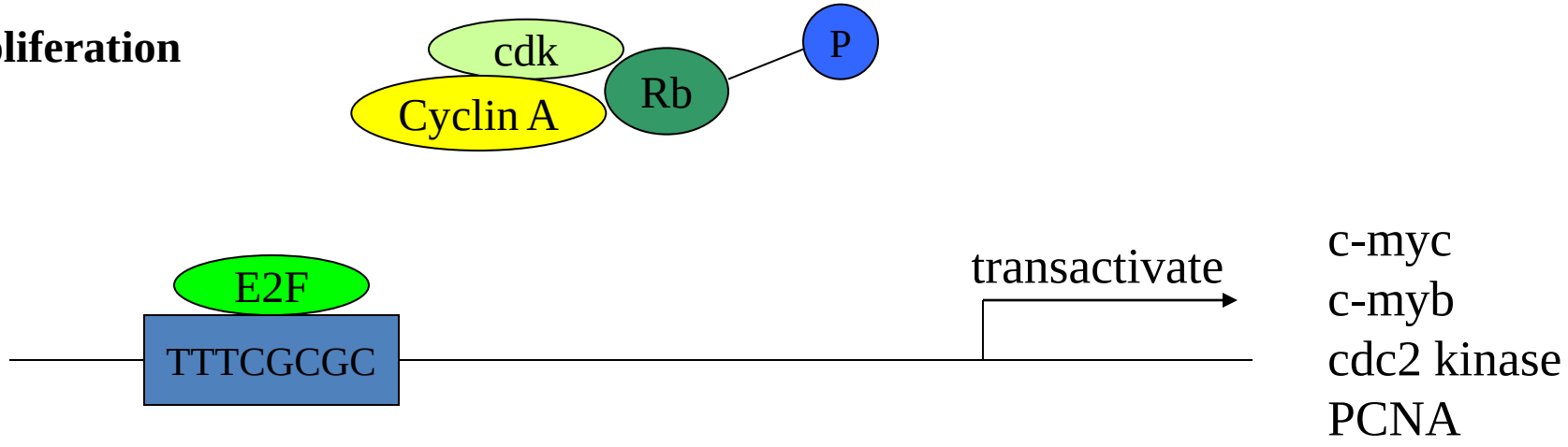


Transcription factor E2F

Quiescence



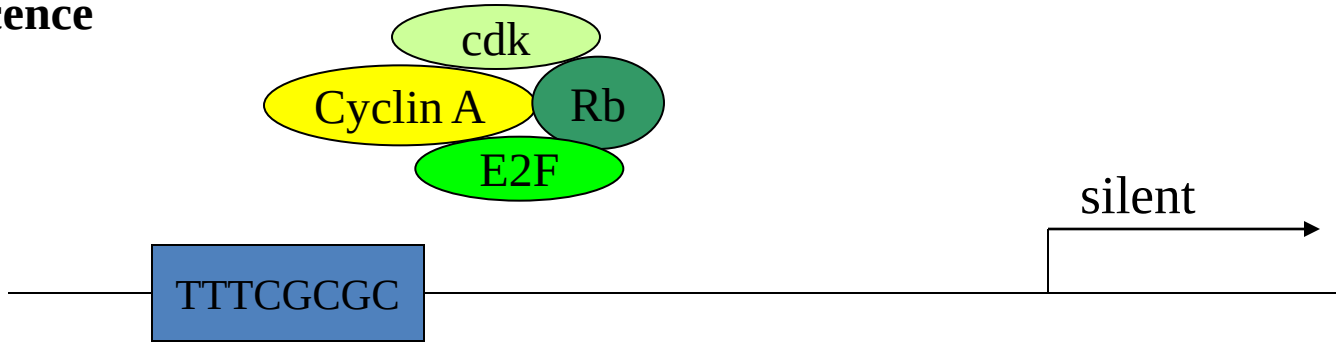
Proliferation



Mann MJ, Mol Med. Today 2000

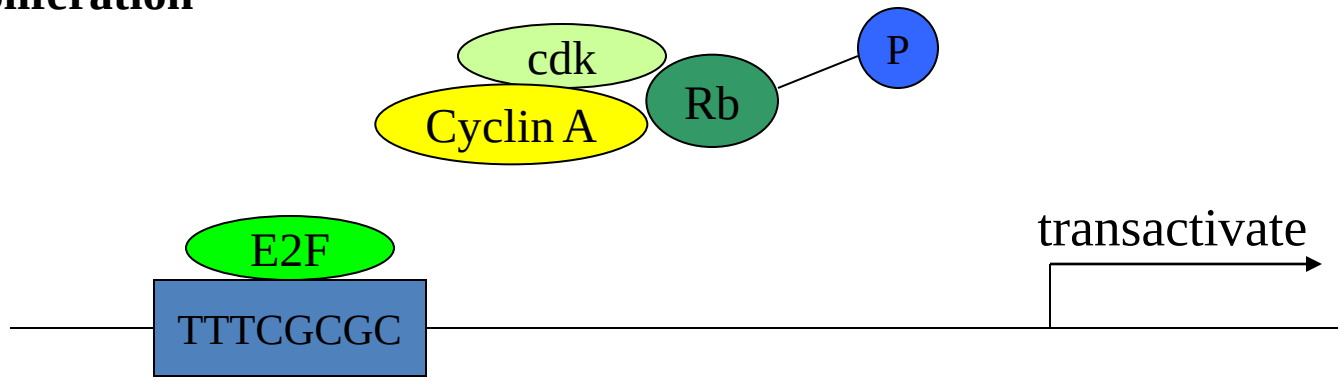
Inhibition of E2F dependent cell proliferation by antisense oligonucleotides against E2F-downstream genes

Quiescence



c-myc
c-myb
cdc2 kinase
PCNA

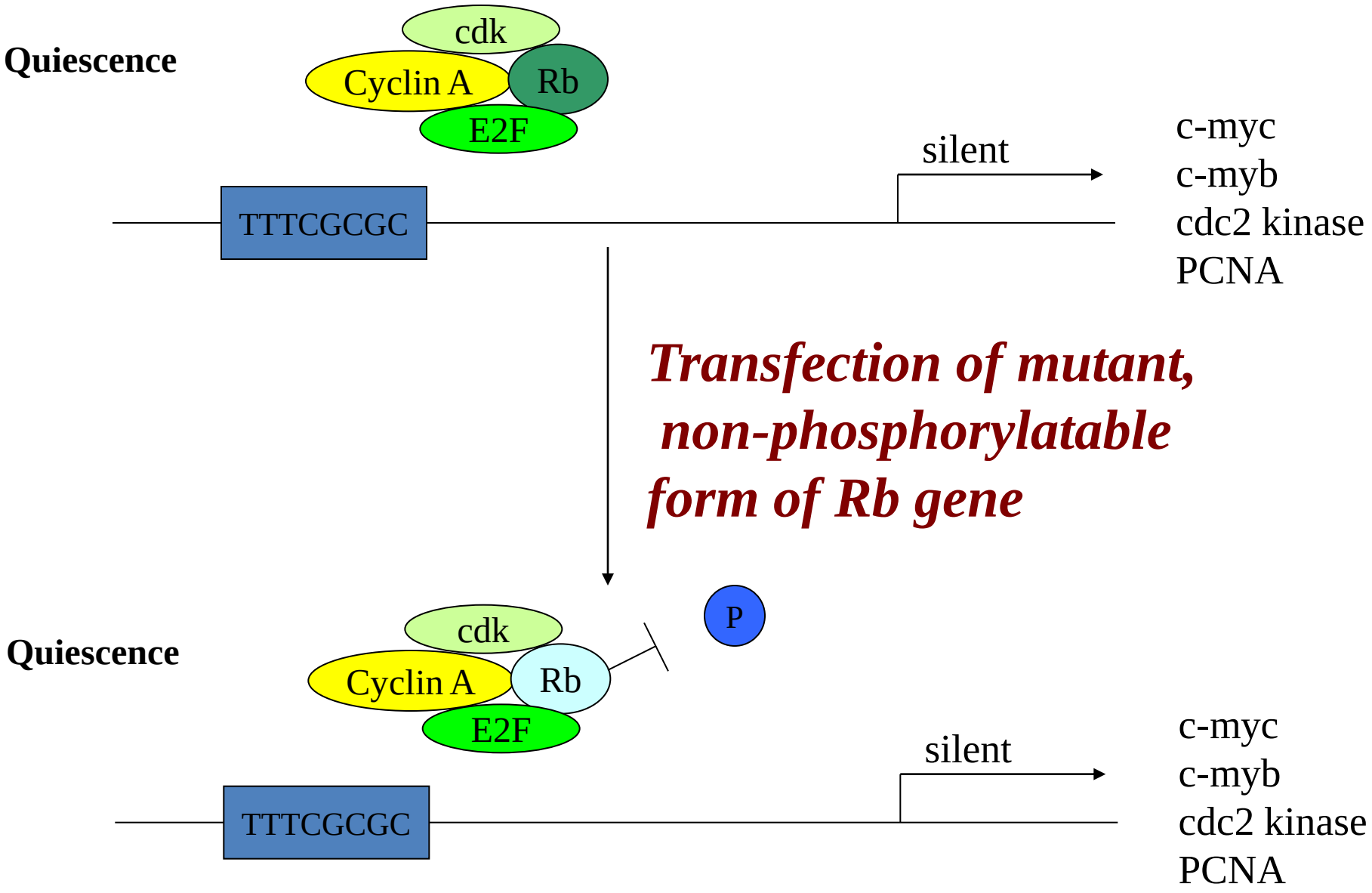
Proliferation



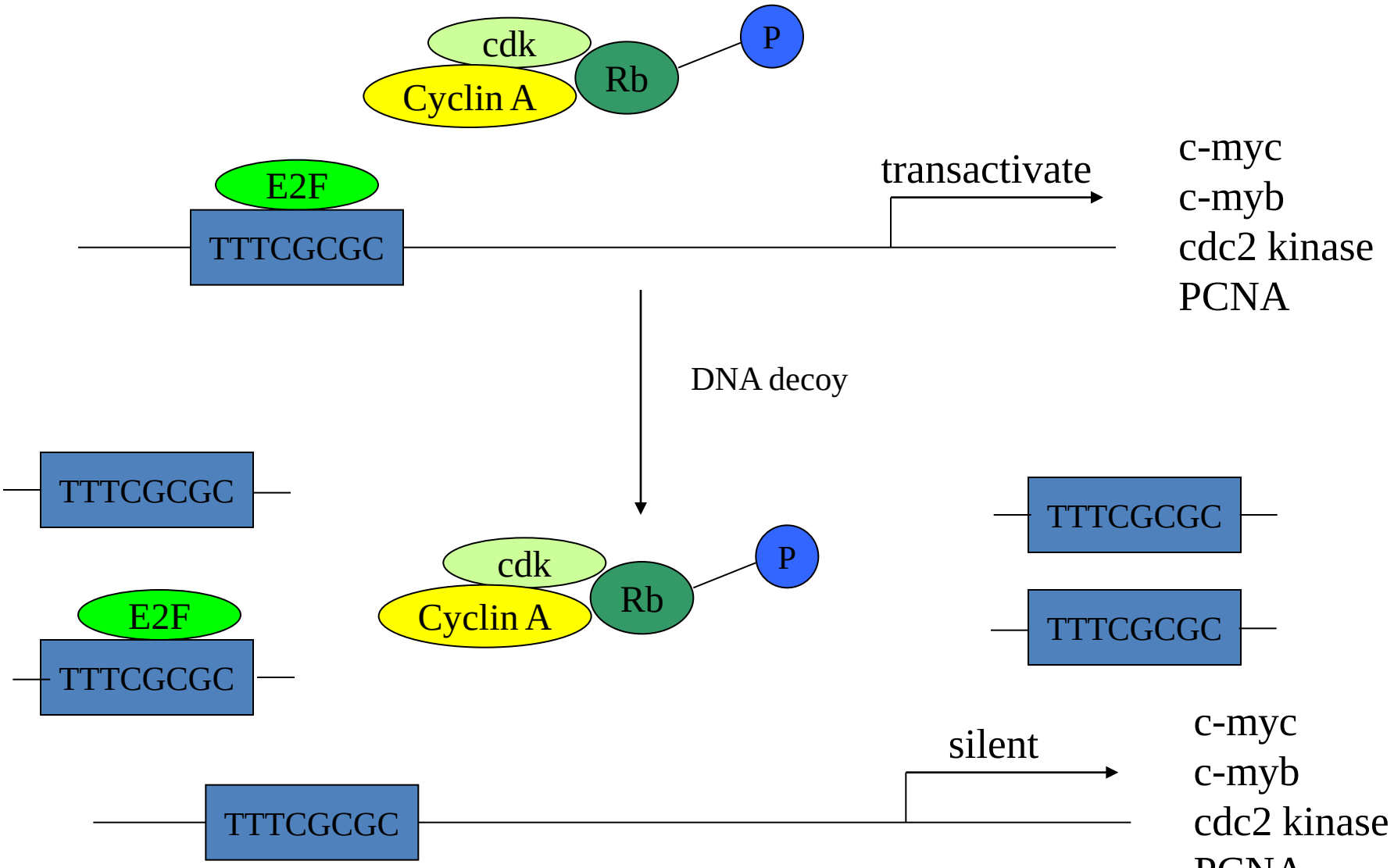
c-myc
c-myb
cdc2 kinase
PCNA

antisense inhibition

Inhibition of cell proliferation by overexpression of Rb mutant gene



Inhibition of cell proliferation by E2F decoys



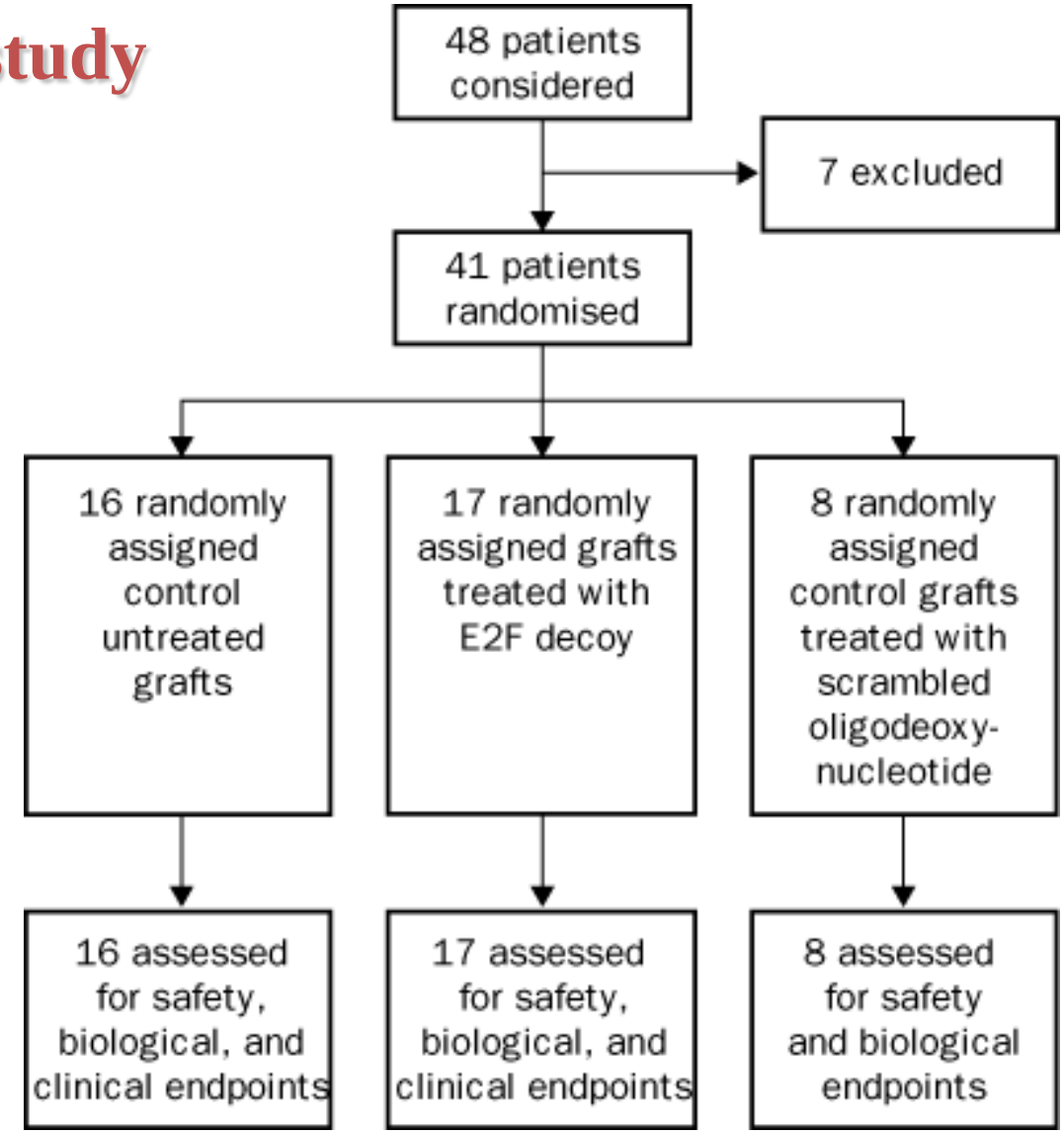
Mann MJ, Mol Med. Today 2000

Edifoligide

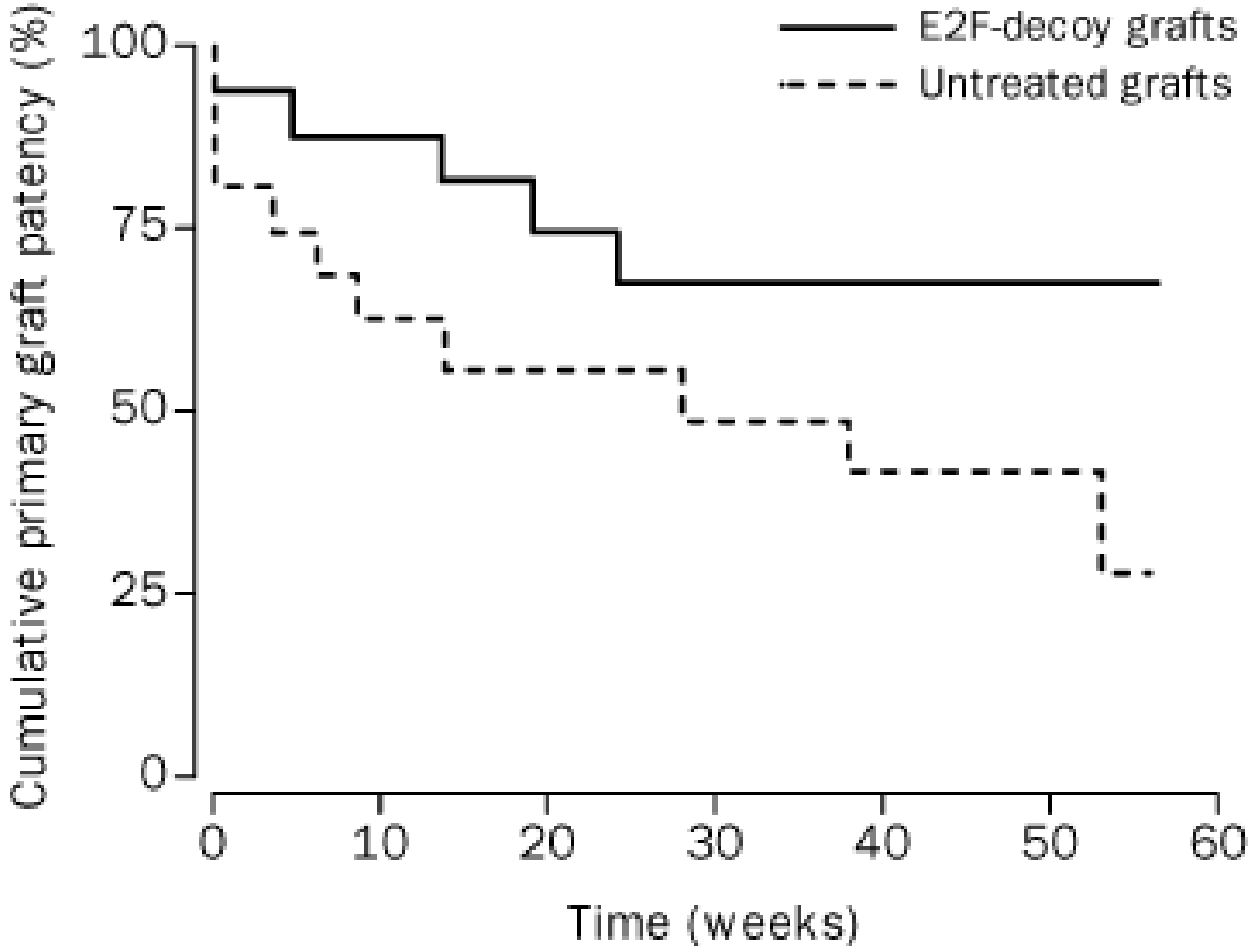
A commercial E2F decoy oligonucleotide

Corgentech

PREVENT I study



Lancet 1999 Oct 30;354(9189):1493-8



Patients at risk

Untreated	16	10	8	7	6	5	4
Treated	17	14	10	8	7	6	6

PRVENT Clinical Trials

In the phase I, or **PREVENT I, trial**, 33 patients who were undergoing lower-extremity bypass with an autologous vein received grafts treated with either edifoligide or saline. The randomized, double-blind trial showed that about 90% of the cells in small segments of the vein treated with edifoligide took in the decoy molecule. Edifoligide also inhibited specific cell-cycle genes and reduced the proliferation of smooth muscle cells in the graft *(Lancet 1999;354:1493-8*

The drug continued to show potential in **PREVENT II**, a double-blind, randomized, phase II study of 200 patients who were undergoing a cardiac artery bypass graft. Grafts treated with edifoligide had a 30% lower rate of vessel lumen occlusion of 75% or more, compared with those treated with saline, according to results from coronary angiography. In intravascular ultrasound images, total wall volume of the grafts treated with edifoligide declined by a significant 30%, compared with those that received saline.

Based on the results of those trials and the significant unmet clinical need, the Food and Drug Administration gave a fast-track designation for phase III trials to test edifoligide for the prevention of vein graft failure

Prevent III & IV

Efficacy and safety of edifoligide, an E2F transcription factor decoy, for prevention of vein graft failure following coronary artery bypass graft surgery: PREVENT IV: a randomized controlled trial.

Alexander JH et al., PREVENT IV Investigators

JAMA 2005, November, 294: 2495-2497

Failure of phase III trials with E2F decoys...

edifoligide failed to show any benefit for primary and secondary end points in two phase III trials...

In the **PREVENT III** trial of 1,404 patients with critical limb ischemia who needed peripheral artery bypass graft surgery, there was no difference between edifoligide and placebo on the primary end point of limb amputation. No differences were seen in secondary end points of critical graft stenosis, recurrent limb ischemia, or quality of life.

Similar results were reported in the **PREVENT IV trial**, which tested edifoligide against placebo in 3,014 patients for the prevention of vein graft failure after coronary artery bypass surgery.

Reasons?

Multiple isoforms of E2F exist, and the drug may not have inhibited them all. Edifoligide's pharmacokinetics may not have allowed it to inhibit E2F adequately