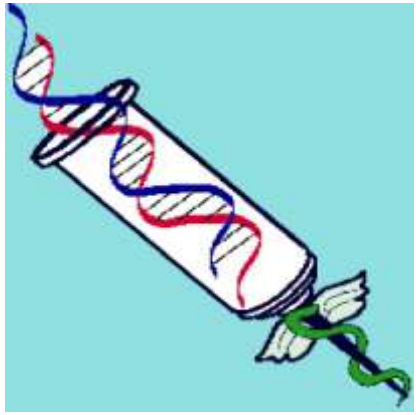
Basics of recombinant DNA medical biotechnology

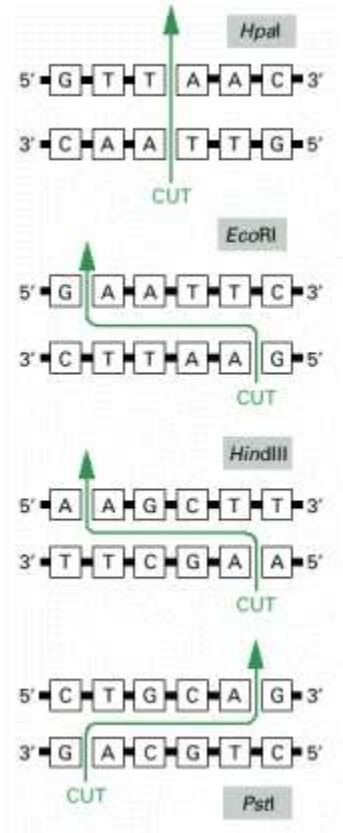
Lecture VI

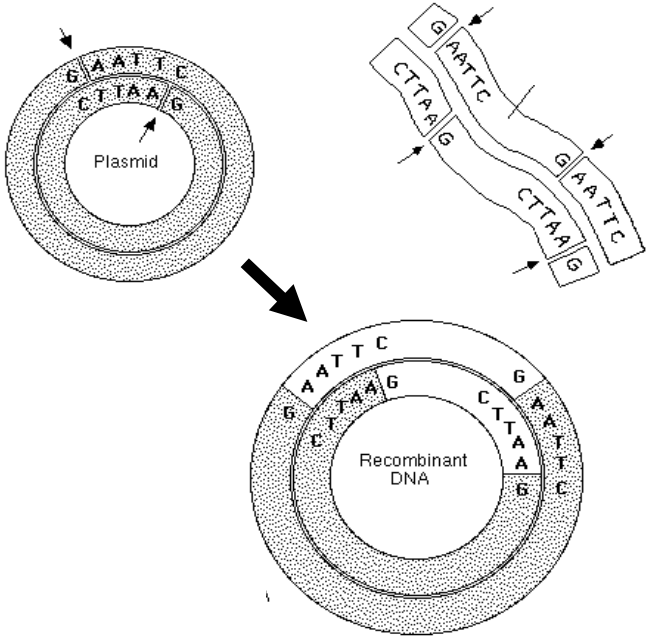


14.05.2013

Genetic engineering

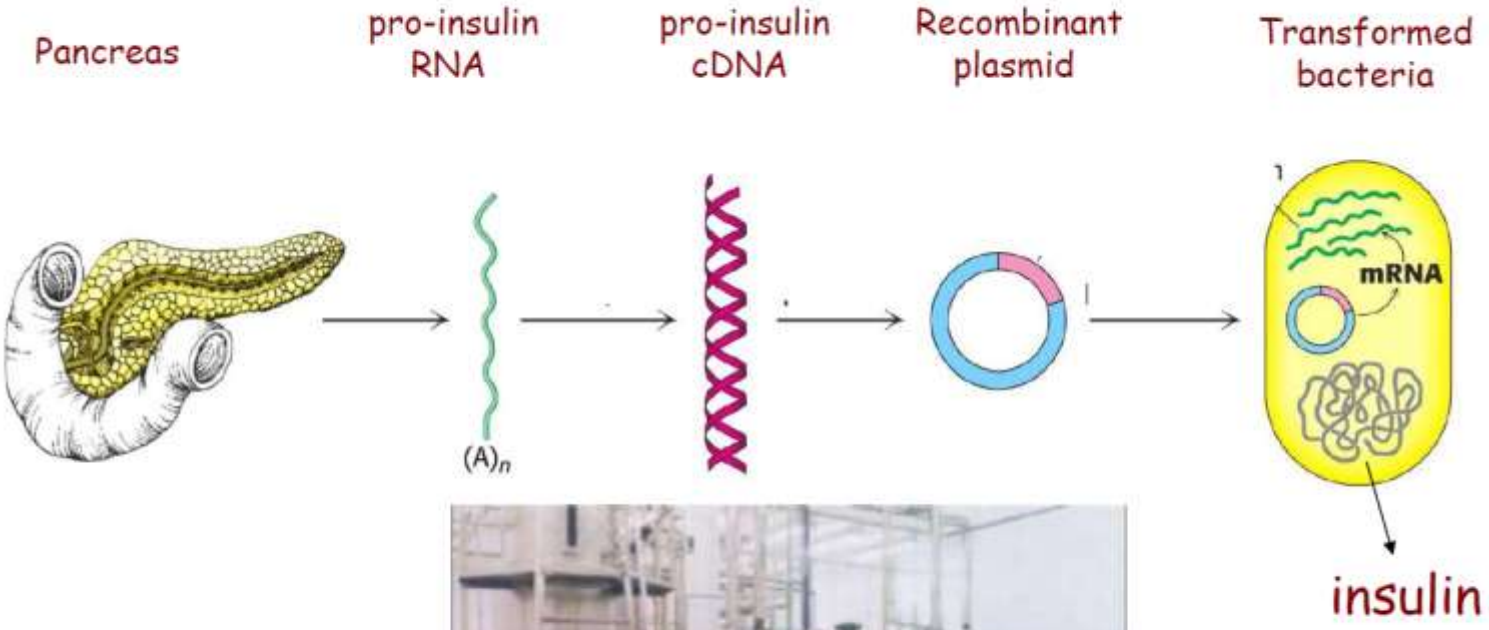
Modification of genetic material of different organisms in a designed and controlled way, by means of techniques of DNA recombination





Recombinant DNA technology

Genetically modified bacteria produce insulin



The manufacturing of insulin

From research to industry

The first steps of biotechnology industry:

- The biotechnology industry originated in the 1970s, based largely on a new recombinant DNA technique whose details were published in 1973 by Stanley Cohen from Stanford University and Herbert Boyer from the University of California, San Francisco. Herbert Boyer went on to co-found **Genentech**,

Herbert Boyer *Stanley Cohen*

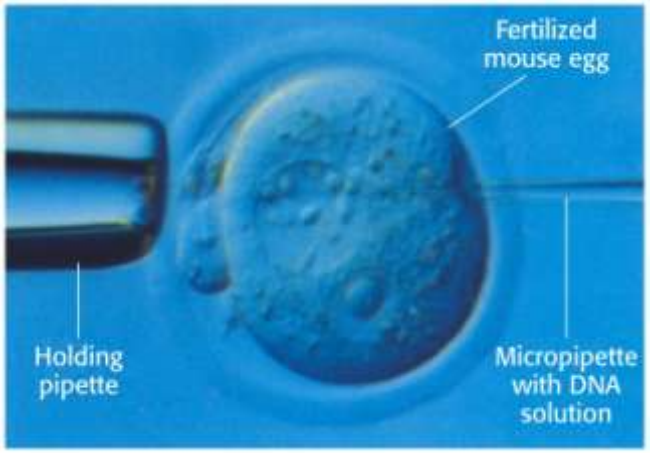
- In 1982, recombinant human insulin became the first biotech therapy to earn FDA approval. The product was developed by Genentech and Eli Lilly and Co.

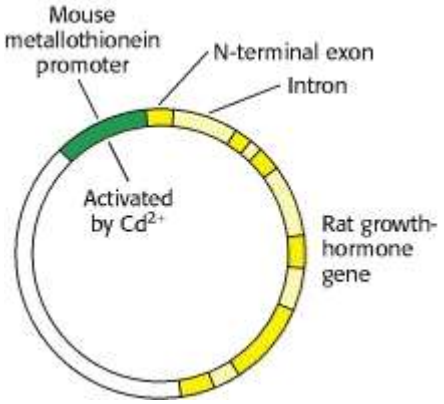
Medicines produced by genetic engineering

Name	Therapeutic application
Insulin	diabetes
Clotting factor	haemophilia
Growth hormone	dwarfism
Interferons	cancers, infections
Interleukins	cancers
tPA (tissue plasminogen activator)	myocardial infarction stroke

Recombinant DNA technology for understanding the mechanisms of diseases and development of innovative therapies

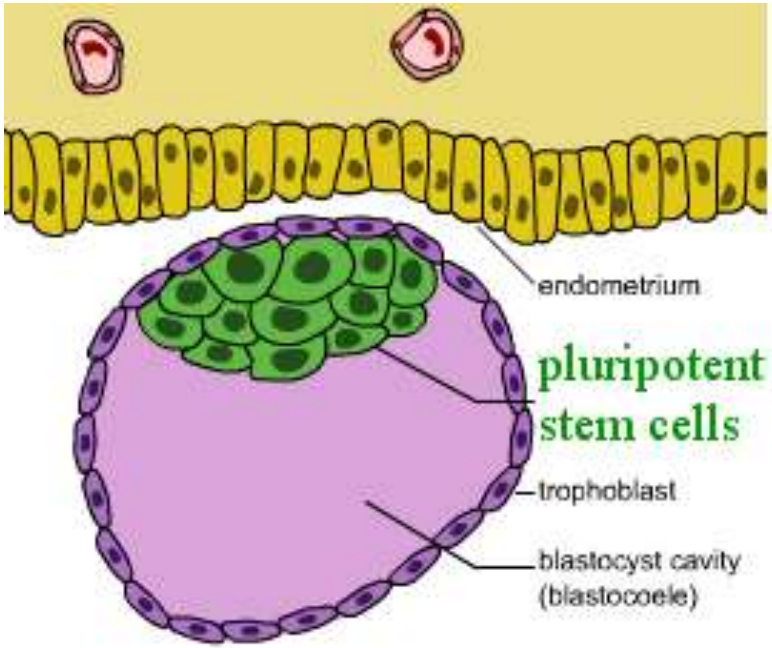
First transgenic mice



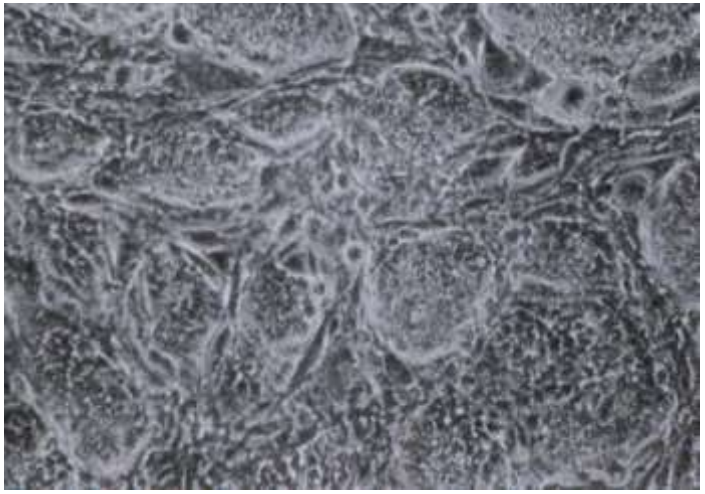


Biochemistry. 5th edition.
Berg JM, Tymoczko JL, Stryer L.
New York: W H Freeman; 2002.

Murine embryonic stem cells



Wikipedia

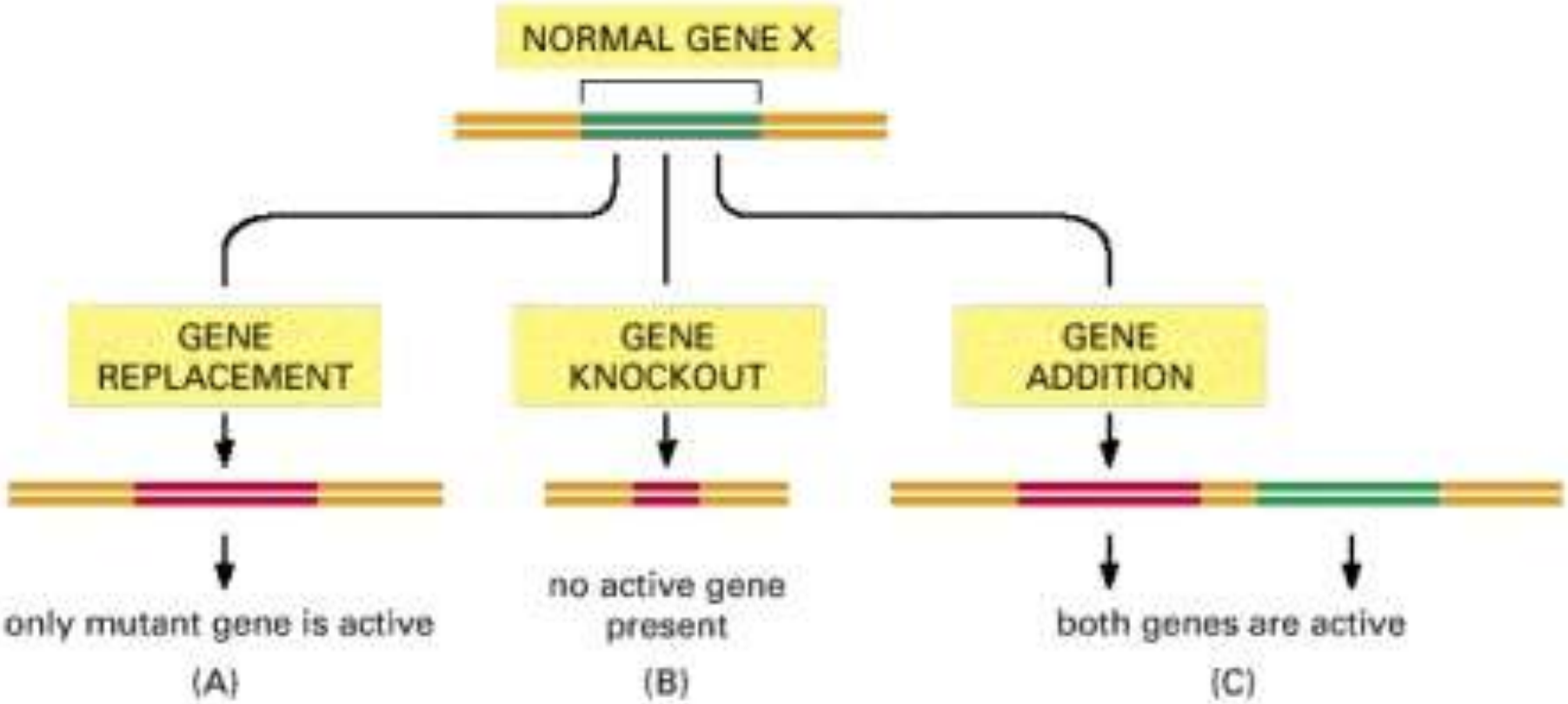


Sir Martin Evans, Mario Capecchi, Olivier Smithies

for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells

Nobel Prize 2007

Gene replacement, gene knockout, and gene addition



Recombinant DNA technology – gene knockout

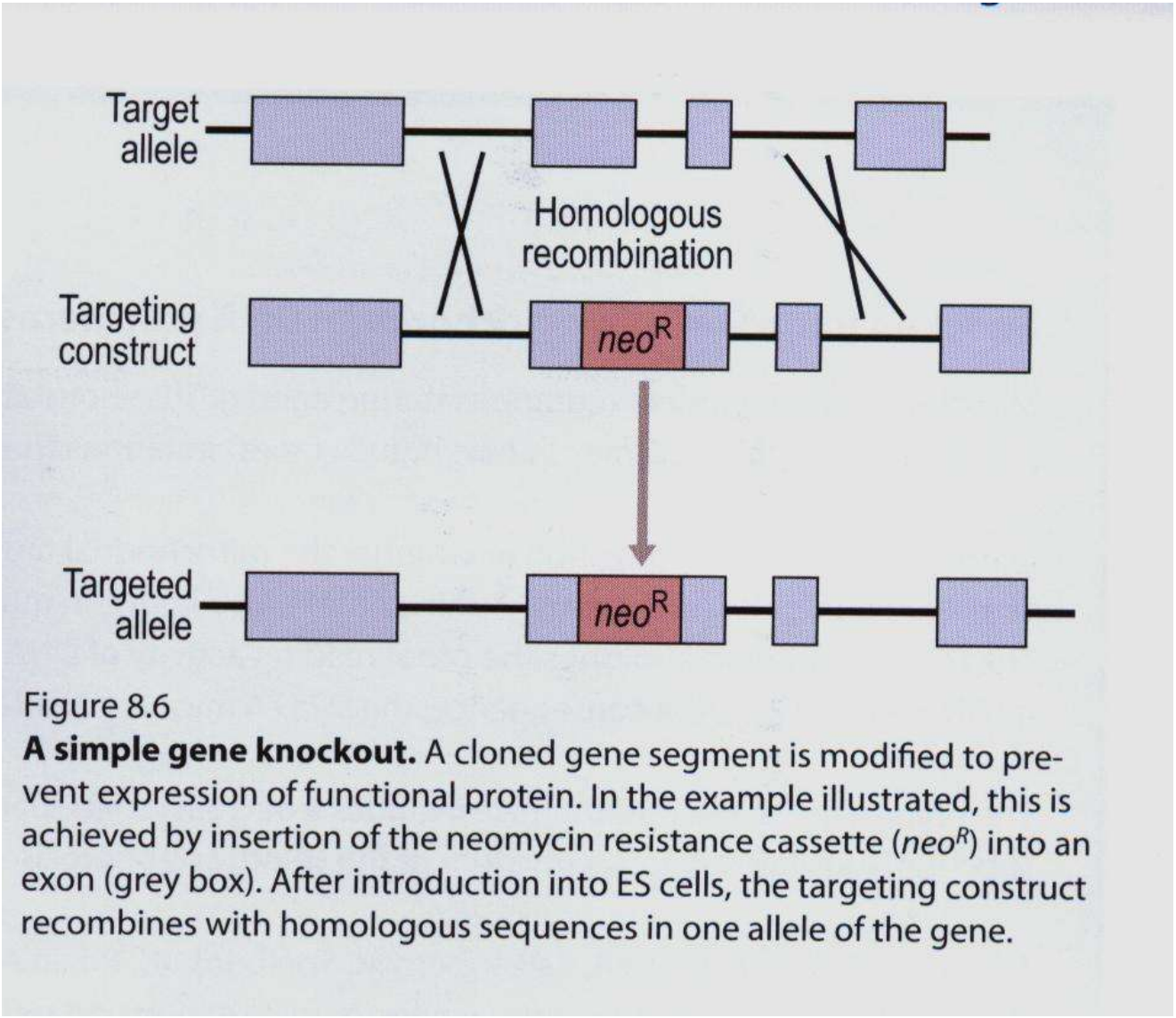
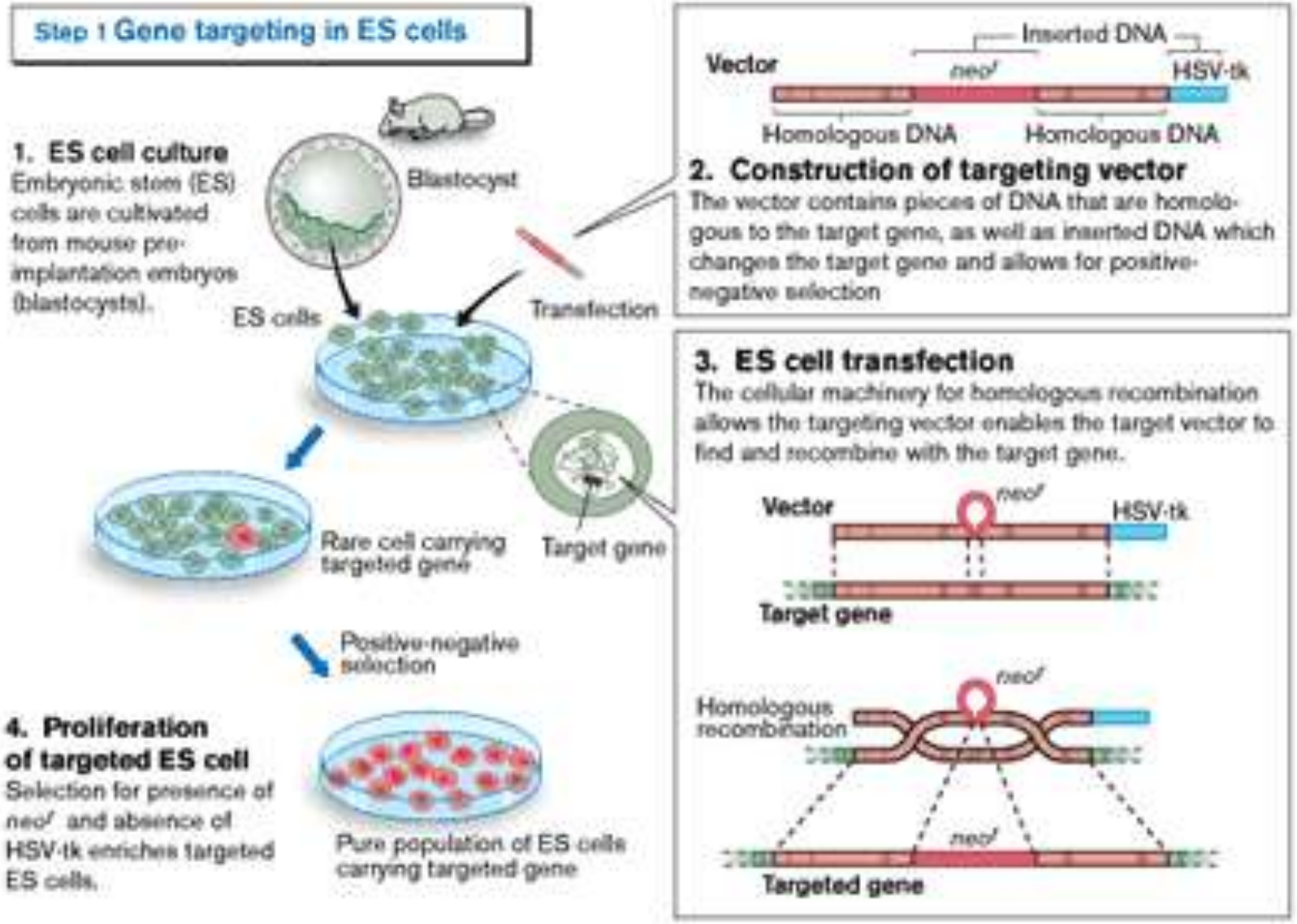


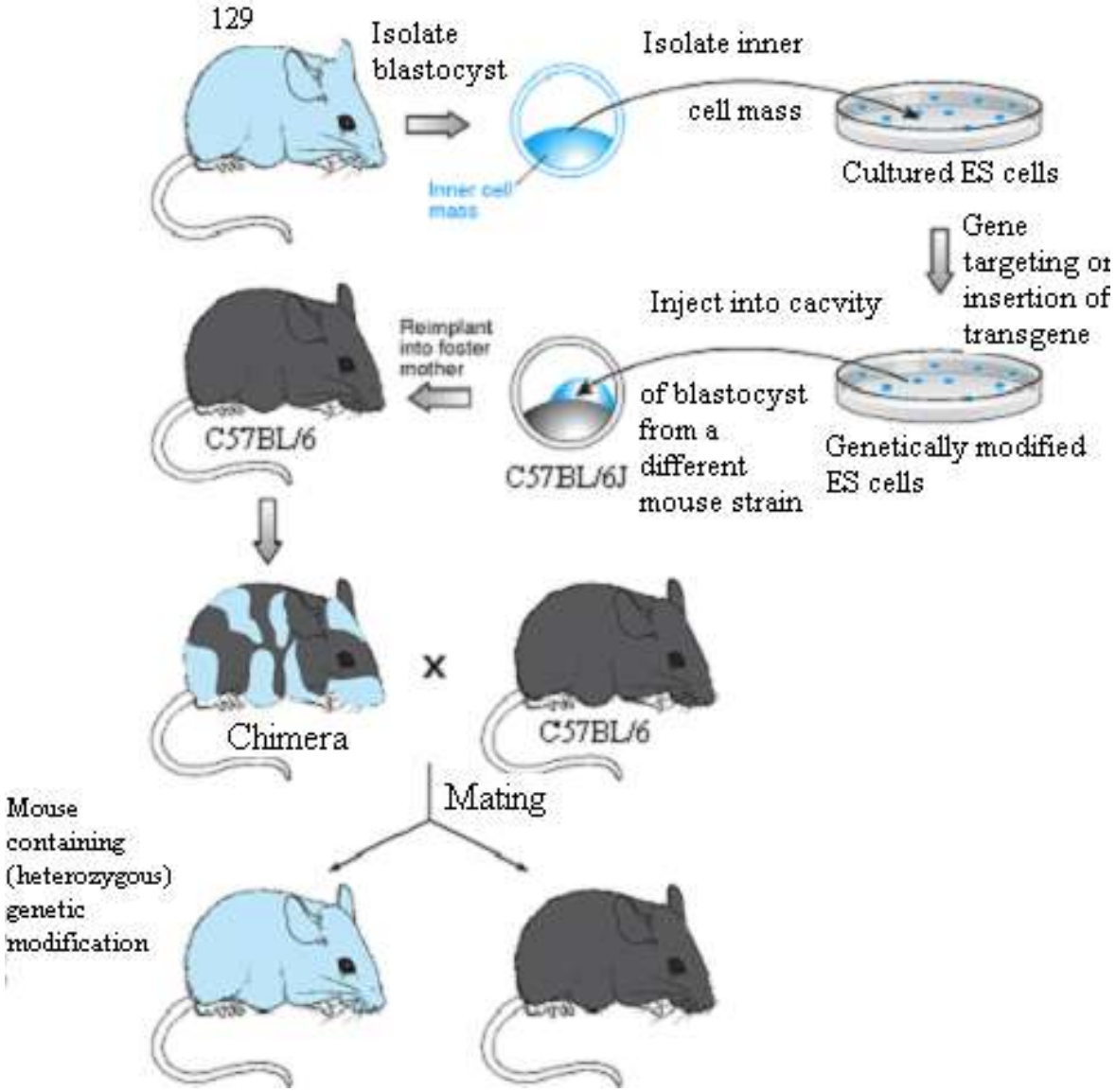
Figure 8.6
A simple gene knockout. A cloned gene segment is modified to prevent expression of functional protein. In the example illustrated, this is achieved by insertion of the neomycin resistance cassette (*neo*^{R}) into an exon (grey box). After introduction into ES cells, the targeting construct recombines with homologous sequences in one allele of the gene.

J Pongracz, M. Keen - Medical Biotechnology, 2009

Gene knockout technology

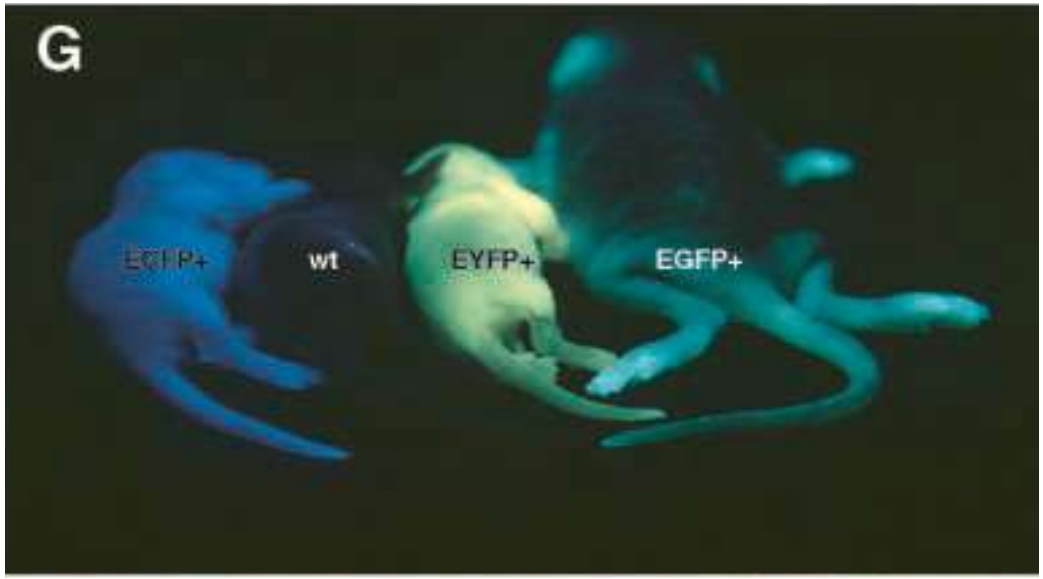


Knockout and transgenic animals



Transgenic animals

Pigs expressing GFP



1. Investigation of the mechanisms of disease
2. Testing new therapies
3. Producing new drugs -

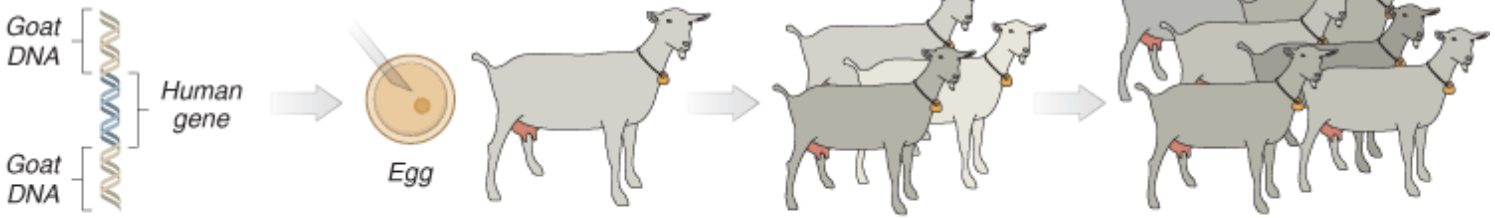
Orphan diseases and recombinant DNA technology

Atryn

Trasgenic animals as drug factories

Bioengineering on the Farm

The Food and Drug Administration has approved the first drug produced in the milk of genetically engineered animals.



MODIFYING THE DNA
A human gene that produces the blood protein antithrombin is inserted into a short strand of goat DNA.

IMPLANTING THE DNA
The modified DNA is injected into the nucleus of a fertilized goat egg, which is then implanted into a female.

TESTING THE OFFSPRING
Kids born from the modified eggs are tested for the presence of antithrombin in their milk. Promising kids are bred normally to create a herd of modified goats.

EXTRACTING THE PROTEIN
Milk from the herd is filtered and purified. Annually, each goat can produce as much antithrombin as 90,000 human blood donations.

Sources: GTC Biotherapeutics

- **ATryn** – first drug produced by transgenic goats, which has been registered by the European Commission in July 2006

Human anti-thrombin, a natural serum protein with anti-thrombotic and anti-inflammatory properties. It is used in obstetrics, treatment of deep vein thrombosis

One GM goat can produce the same amount of antithrombin in a year as 90,000 blood donations

Other examples

Transgenic animals

Researchers are developing transgenic animals, including cows, goats and sheep, that produce milk containing therapeutic proteins. Some interesting ongoing projects include:

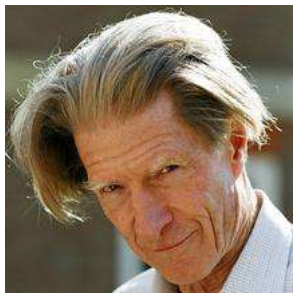
* **Atryn** - The first drug product for humans produced by a transgenic animal was in July 2006 approved by the European Commission. This protein is human anti-thrombin, a naturally occurring plasma protein that has both anti-coagulant and anti-inflammatory properties. The protein is produced by transgenic goats whose milk contains human anti-thrombin.

* Growth hormon - In 2005 in Argentina, cows were improved with biotechnology to produce human growth hormone. Scientists estimate that just 15 of these Jersey cows could produce enough human growth hormone to meet the current world demand.

* Dutch researchers are working with biotech rabbits that secrete a potential drug for Pompe's disease (acid maltase deficiency) in their milk. Pompe's disease is an extremely rare genetic disorder that can result in crippled muscles, breathing problems and sometimes death.

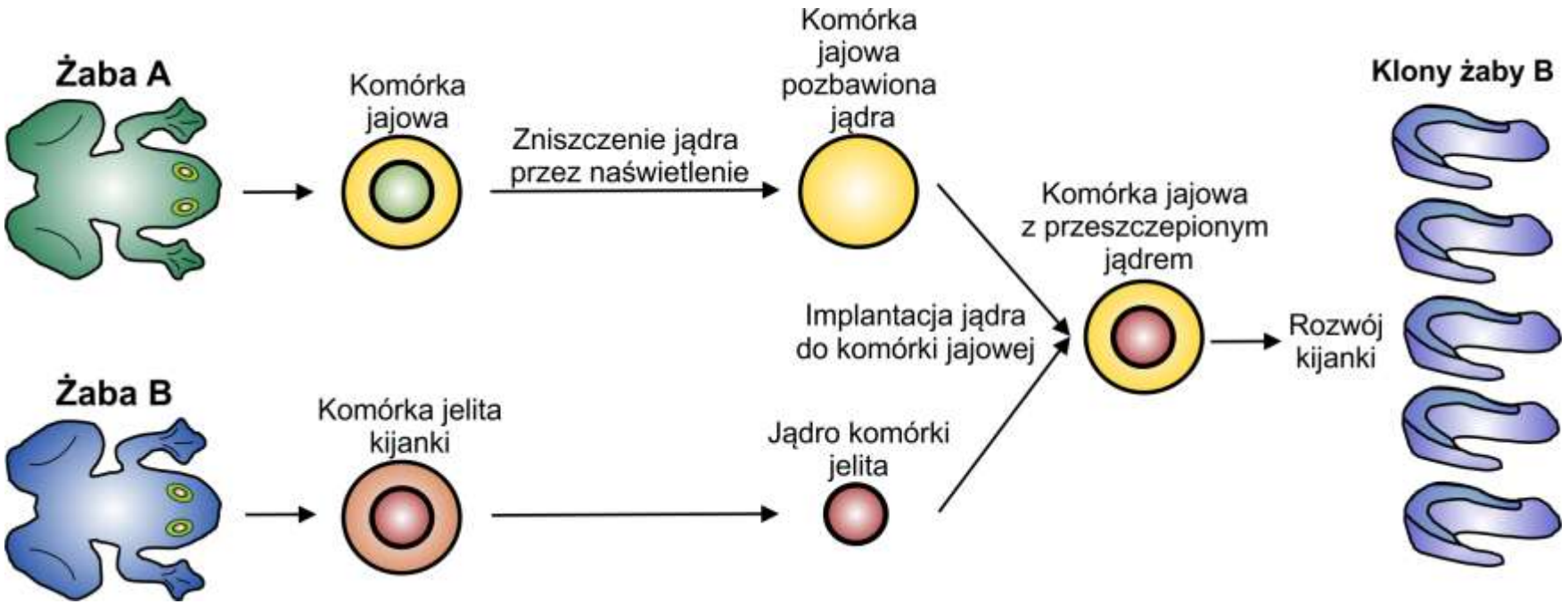
* Biotech cows can now produce the human milk protein lactoferrin, which is an antibacterial protein that can be used to treat immunosupressed patients or incorporated into infant formula.

Reprogramming of the somatic nuclei – cloning



Sir John Gurdon, 1962

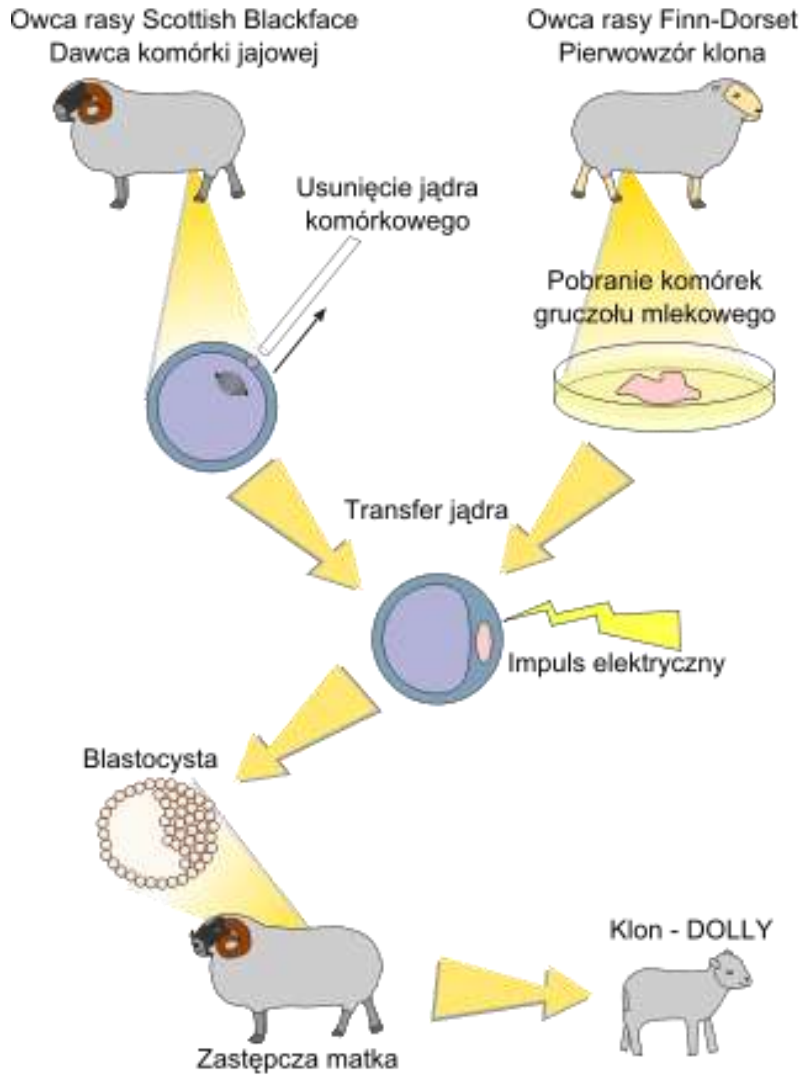
Nobel prize – 2012 (together with Shinya Yamanaka)



Krzysztof Szade

Dulak J, Wszechswiat, 12/2012

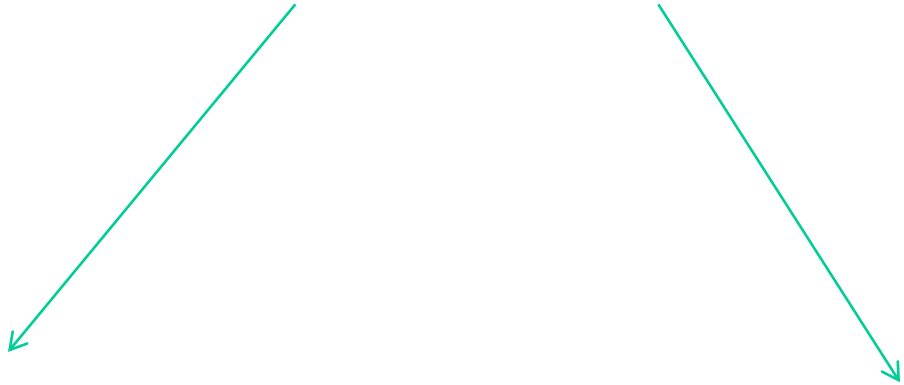
From gene cloning to animal cloning



Dolly

5 July 1996 – 14 February 2003

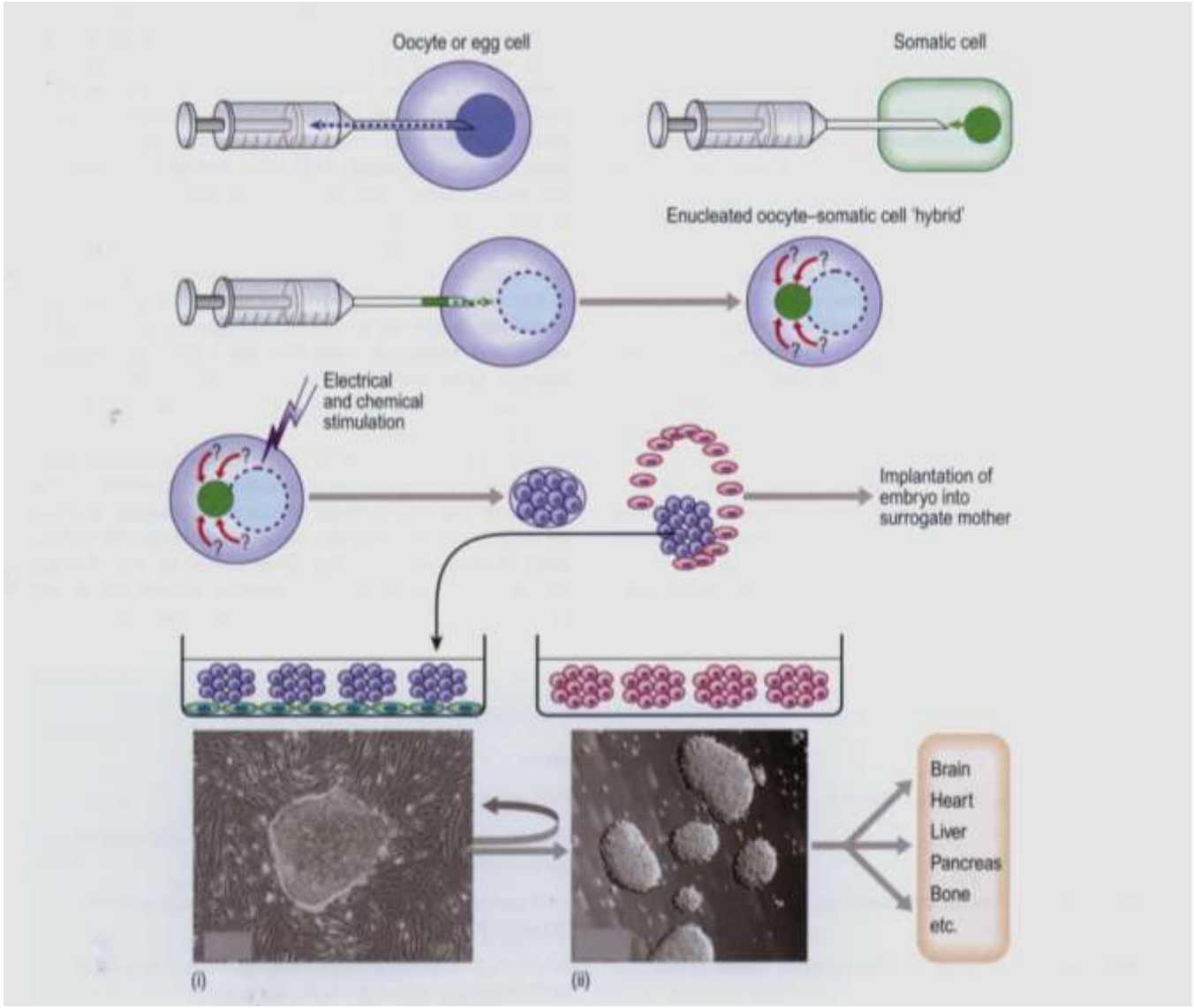
Cloning



reproductive

Therapeutic (SCNT – *Somatic cell nuclear transfer*)

Somatic cell nuclear transfer (SNCT)



J. Pongracz, M. Keen –Medical Biotechnology, Churchill Livingstone, 2009

Biotechnology - achievements

Biotechnology therapeutics approved by the U.S. Food and Drug Administration (FDA) to date are used to treat many diseases, including leukemia and other cancers, anemia, cystic fibrosis, growth deficiency, rheumatoid arthritis, hemophilia, hepatitis, genital warts, and transplant rejection.

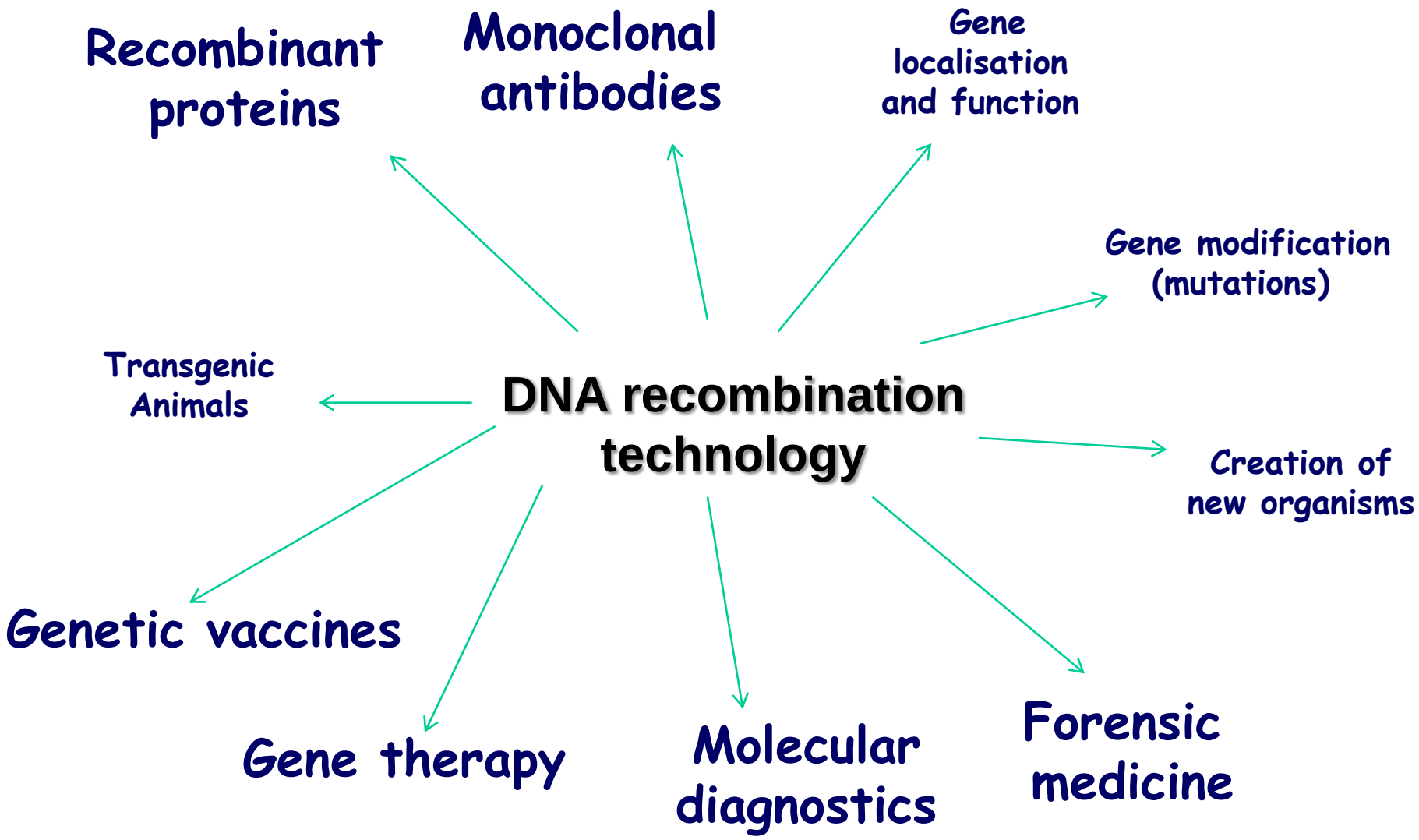
Biotechnology has created:

* more than 200 new therapies and vaccines, including products to treat cancer, diabetes, AIDS and autoimmune disorders.

* more than 400 drug products and vaccines currently in clinical trials targeting more than 200 diseases, including various cancers, Alzheimer's disease, heart disease, diabetes, multiple sclerosis, AIDS and arthritis.

* hundreds of medical diagnostic tests for early detection of diseases, for keeping the blood supply safe, or for detection of pregnancy at home.

* DNA fingerprinting, which has dramatically improved criminal investigation and forensic medicine.


DNA recombination technology
Recombinant proteins
Monoclonal antibodies
Gene localisation and function
Gene modification (mutations)
Transgenic Animals
Creation of new organisms
Genetic vaccines
Gene therapy
Molecular diagnostics
Forensic medicine

Gene therapy was born in... 1962

Prof.Wacław Szybalski

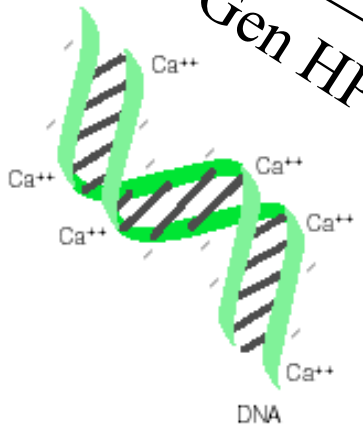
McArdle Laboratory
for Cancer Research,
Wisconsin, Madison,
USA

$HPRT^{-/-}$ cells

HAT medium

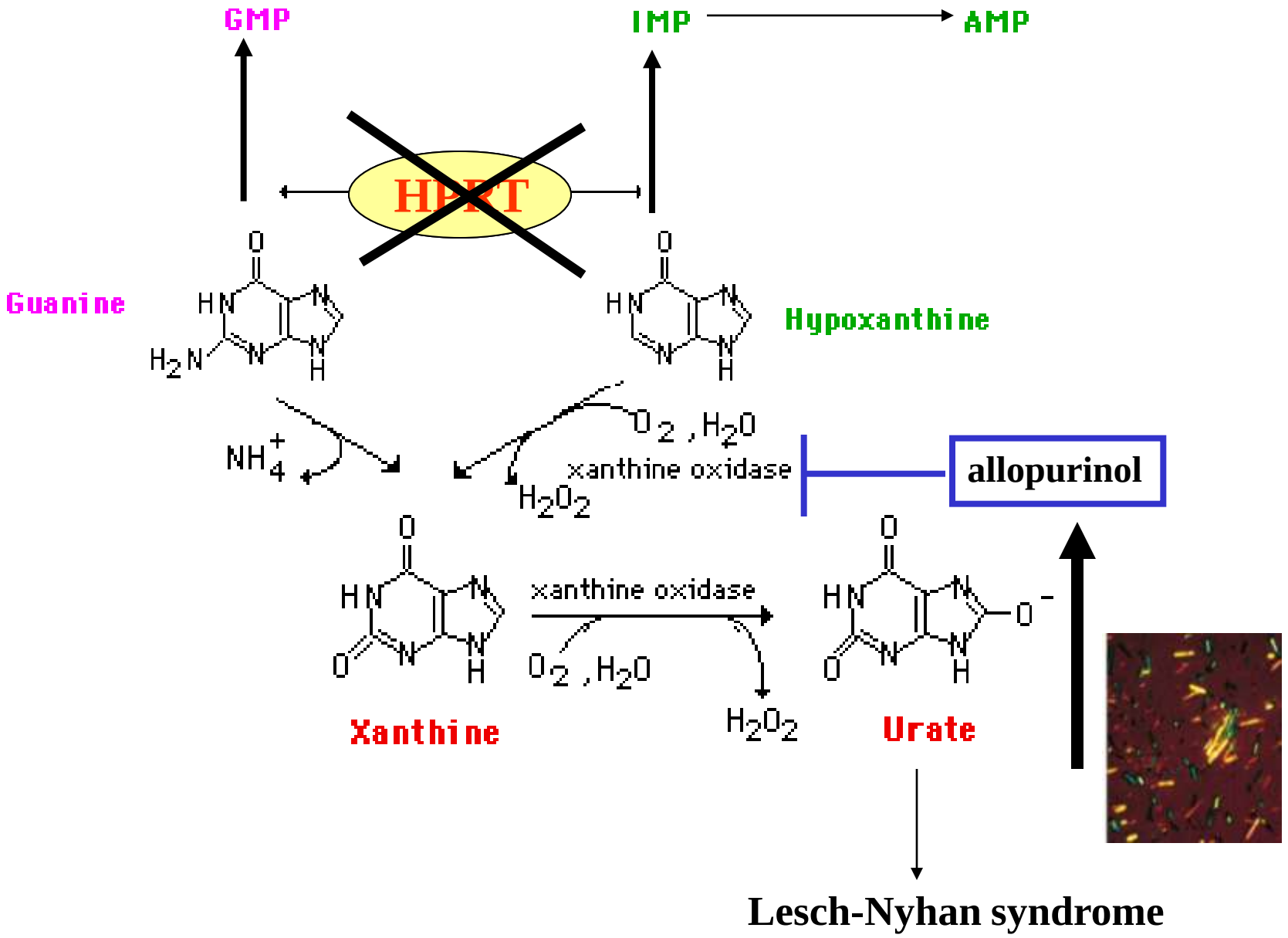
HAT medium

Gen HPRT



$HPRT^{+/+}$ cells

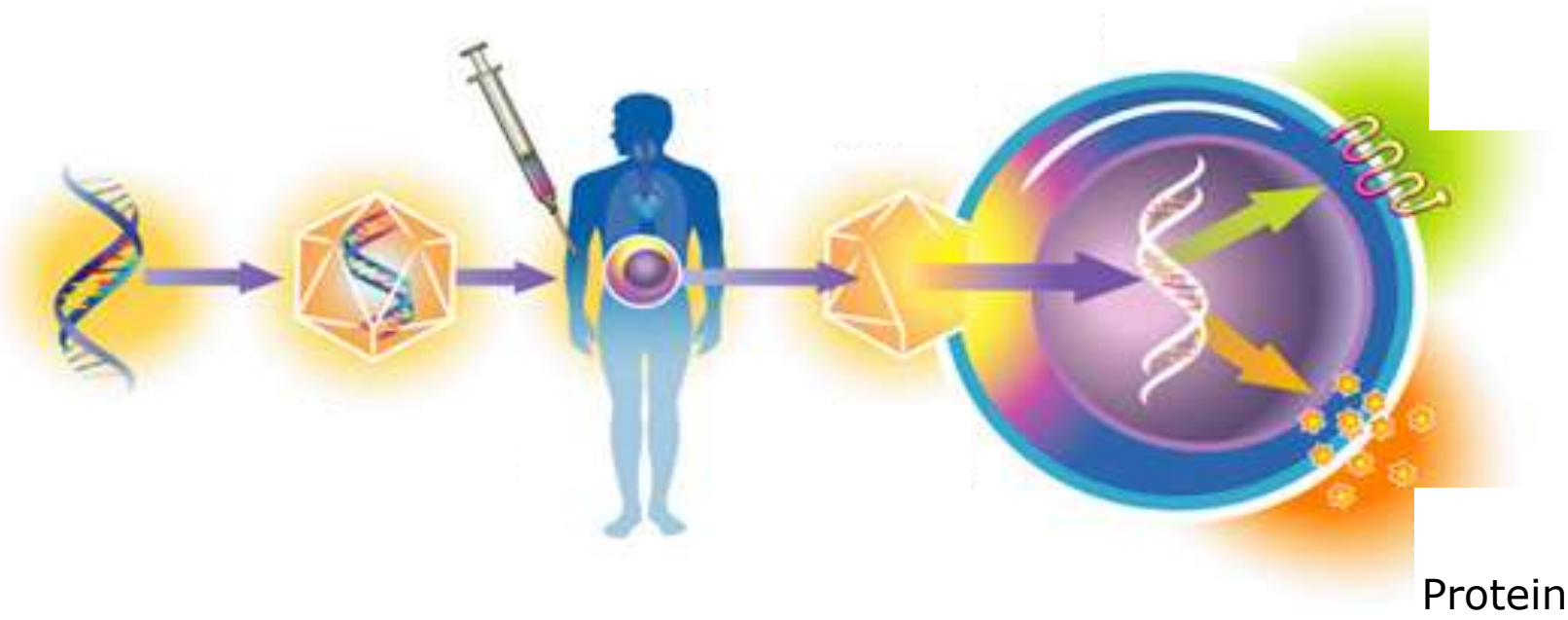
Inborn error of metabolism – deficiency of HPRT



Development of gene therapy

Development of tools (vehicles)

Gene therapy



Therapeutic gene (transgene)

Vector

Patient

Expression of therapeutic gene

Gene therapy is also silencing or replacing the bad genes or overexpression of good genes

Vectors

Carriers of the therapeutic nucleic acids

Vectors

Non-viral/plasmids

viral

„naked" DNA

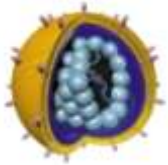
Lipoplexes/

Viroplexes
(lipoplexes enhanced
in proteins from viral
capsids)

Complexes
With chemical
vehicles

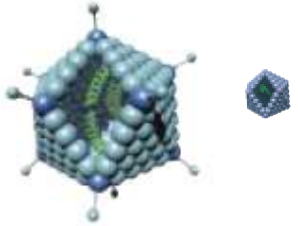
RNA

Retroviral
(including
Lentiviral)



DNA

Adenoviral
AAV
Herpes

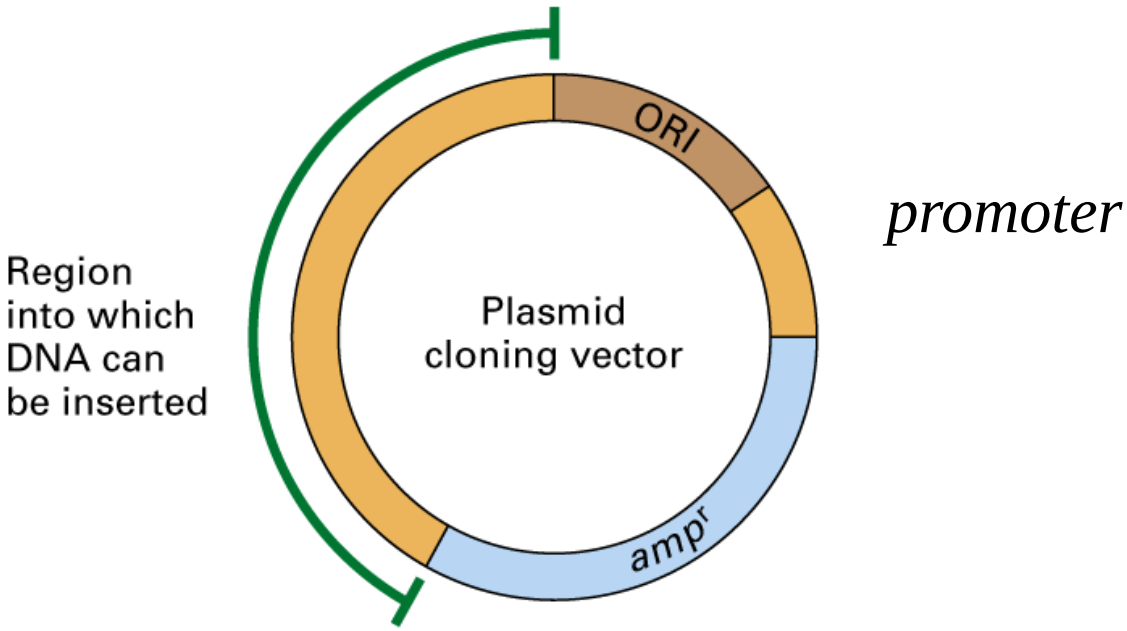


Plasmids

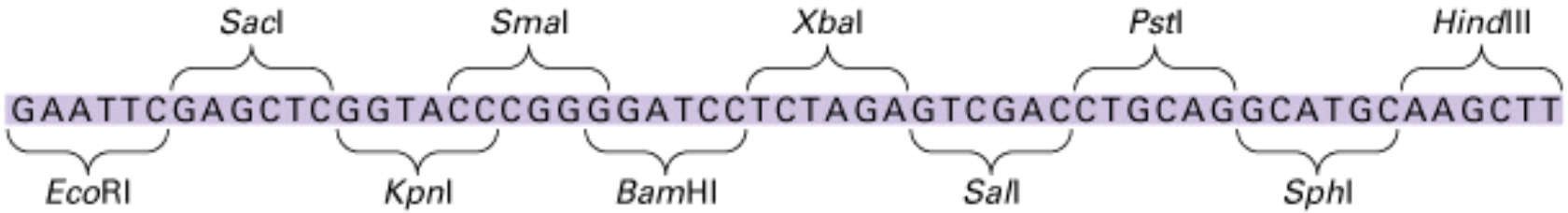
the main tools of gene therapy

Plasmids are always in the begining…

Organisation of a typical plasmid vector

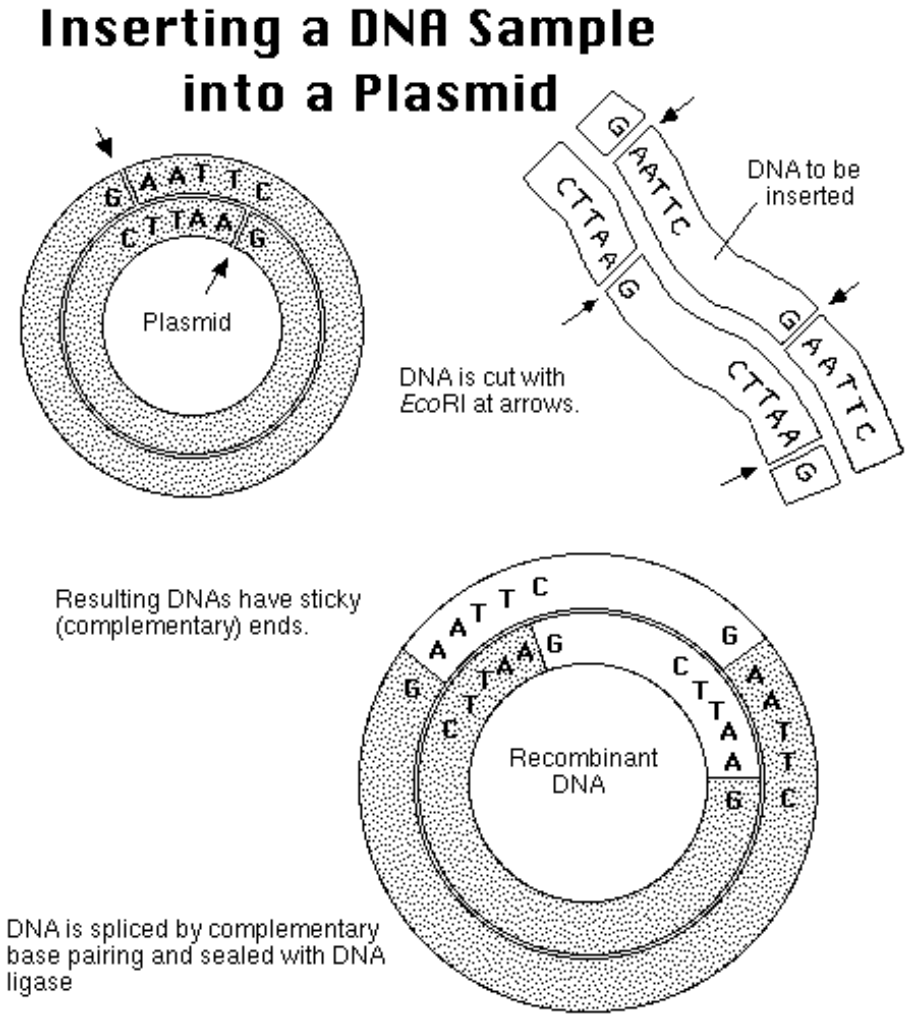


(a) Sequence of polylinker



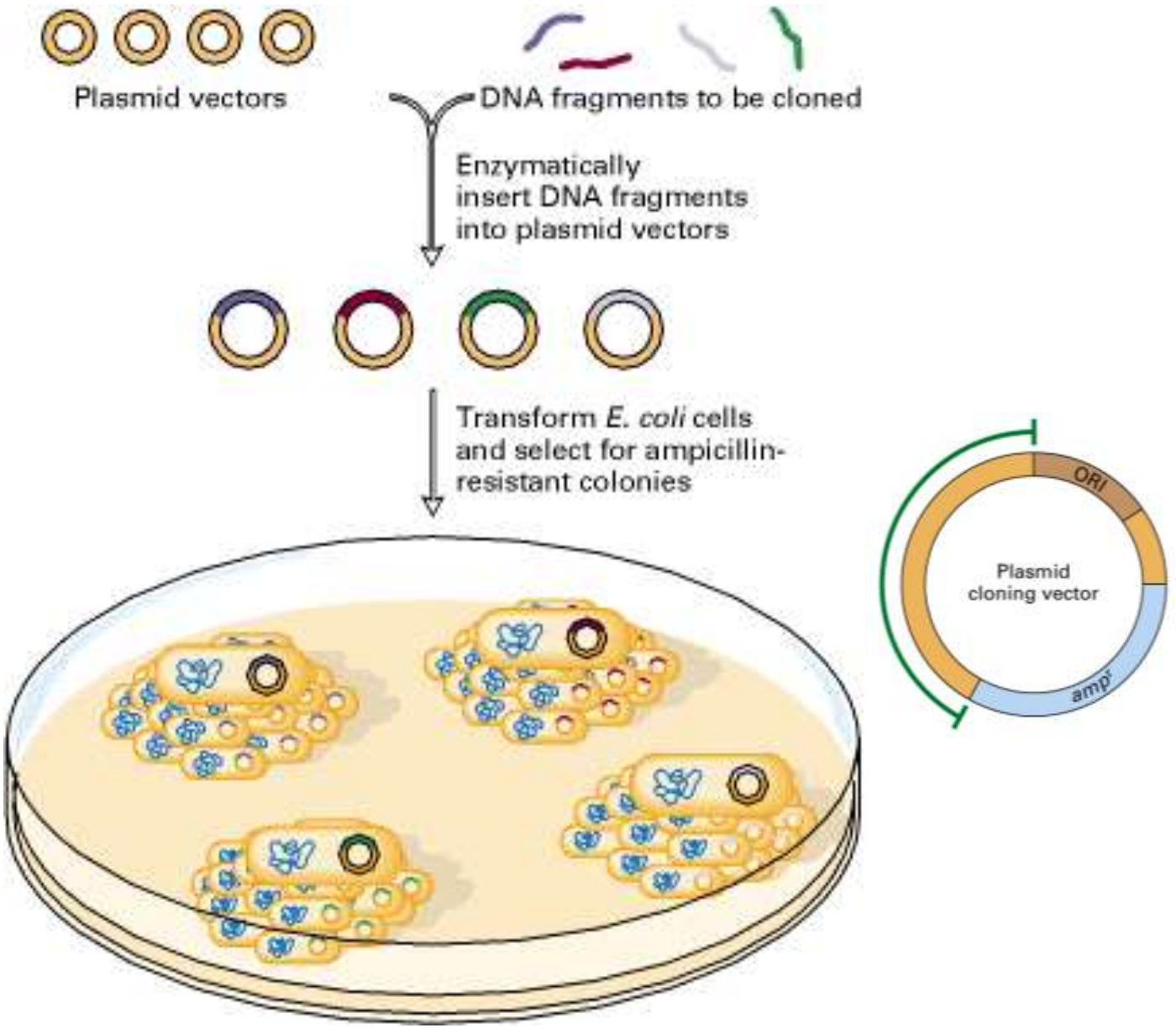
Plasmids are of bacterial origin, so they have to be modified to act in eucaryotic cells

DNA cloning



A piece of target DNA can be inserted into a plasmid if both the circular plasmid and the target DNA have been cleaved by the same restriction nuclease in such a way as to create sticky ends. The newly created recombinant molecule is stabilized with the DNA ligase enzyme which repairs nicks in the backbone of the DNA molecule.

Isolation of DNA fragments from a mixture by cloning in a plasmid vector



Transformation of bacteria

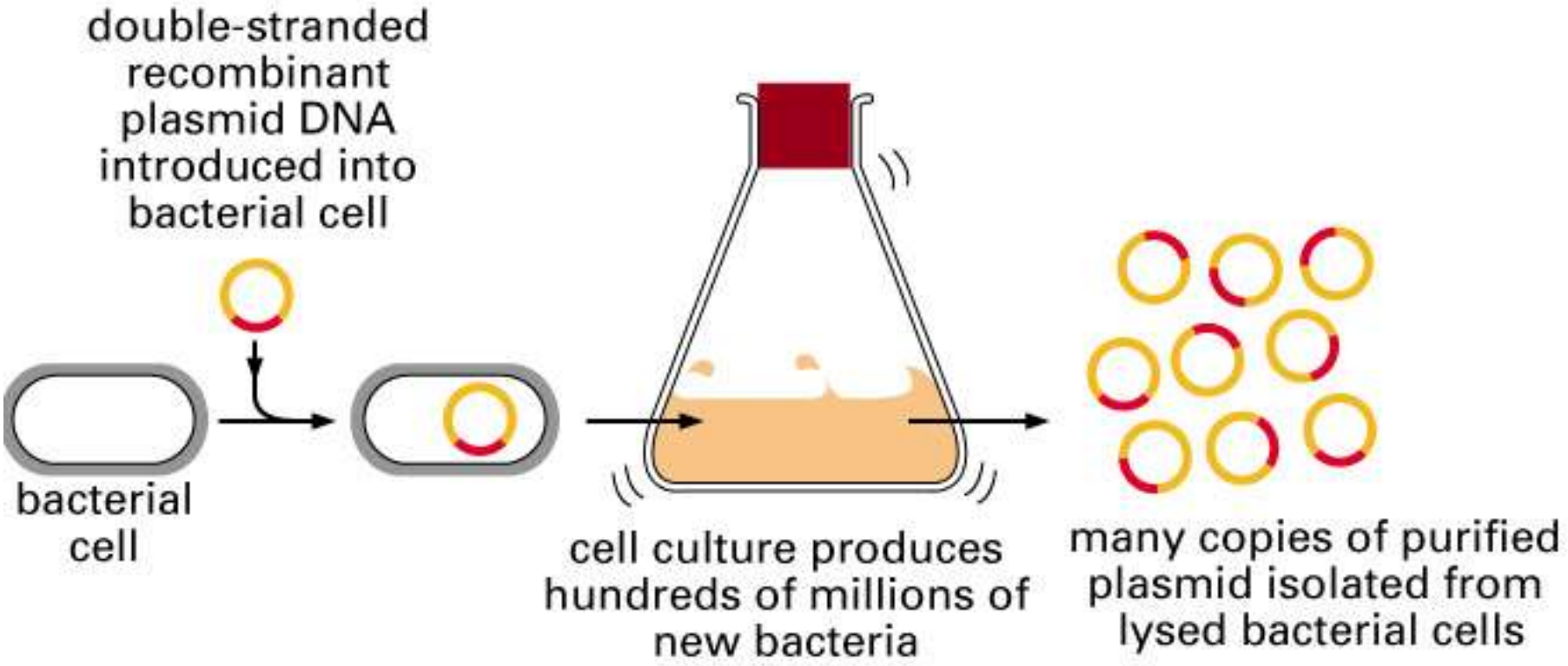


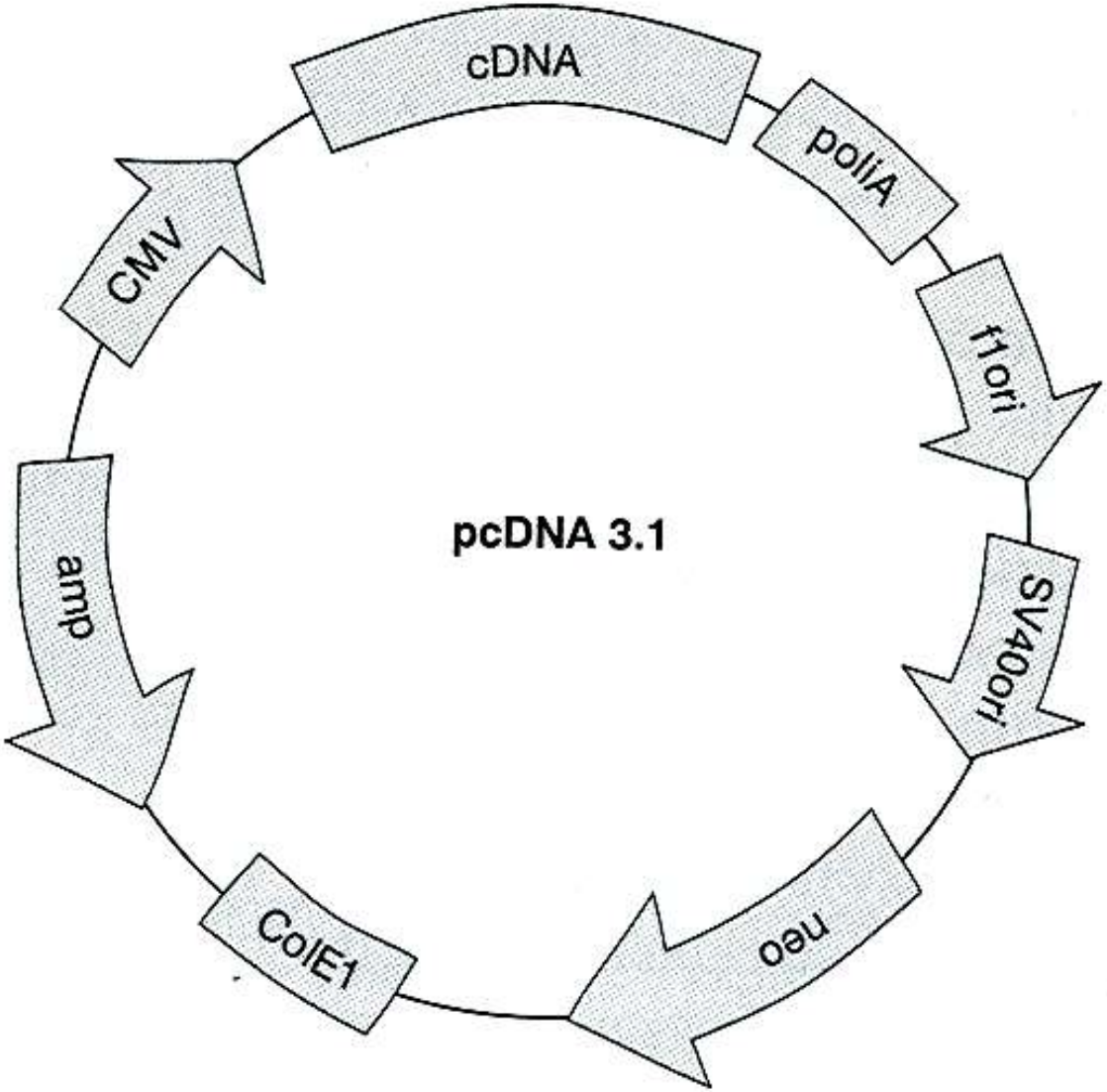
Figure 8–31. Molecular Biology of the Cell, 4th Edition.

How to make a plasmid working in mammalian cells?

Expression of foreign genes in eucaryotic cells requires the eucaryotic promoter in a plasmid vector

1. Viral promoters : CMV, SV40
2. Eucaryotic promoters
   - constitutive:
     - non-selective: β-globin
     - tissue specific:
   - inducible
3. Complex

Mammalian expression plasmid



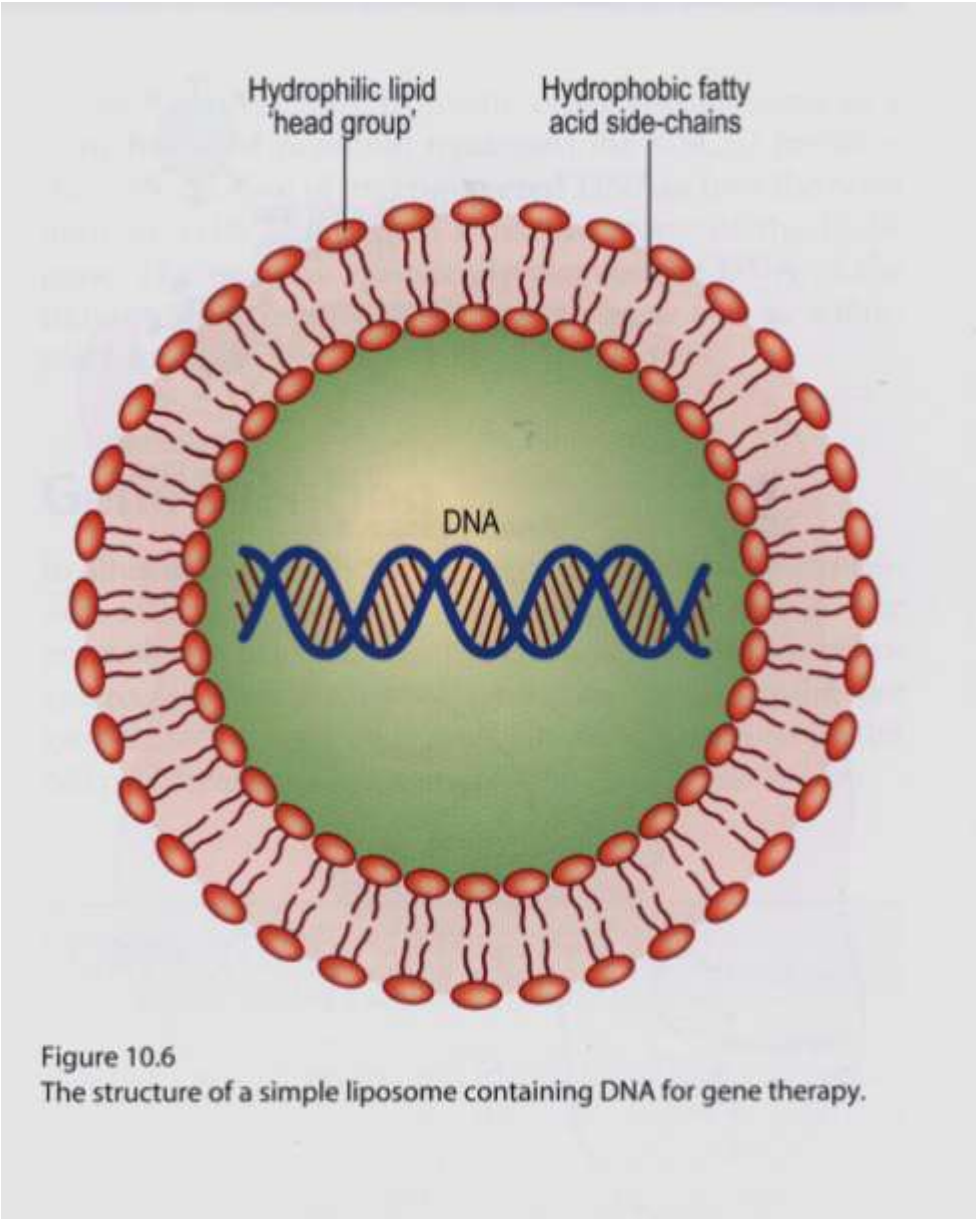
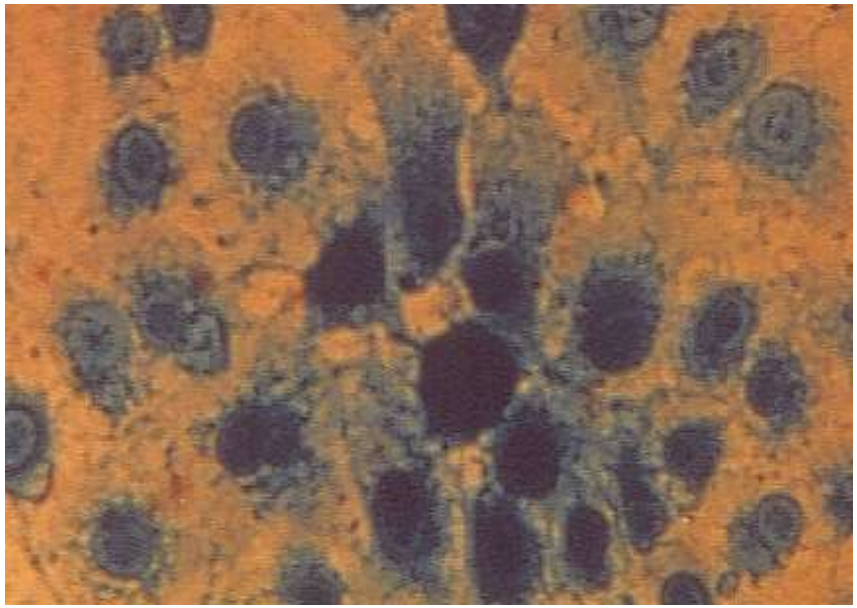


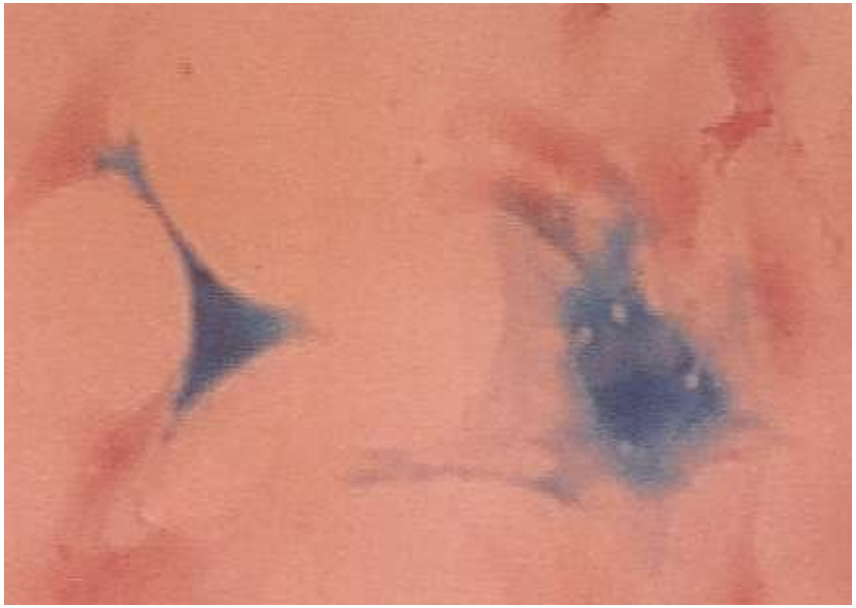
Figure 10.6
The structure of a simple liposome containing DNA for gene therapy.

J. Pongracz & M. Keen Medical Biotechnology, Churchill Livingstone, 2009

Expression of b-galactosidase in various cells after lipotransfection



Cells from kidney
of a monkey



Vascular smooth muscle cells
(rat)

Naked plasmid, containing lacZ gene has been injected into the leg muscle of a mouse.

Problems with transfection of naked DNA

1. Cell type dependent – only few cell types can be effectively transfected

2. Maximal expression – after 14 days in skeletal muscles

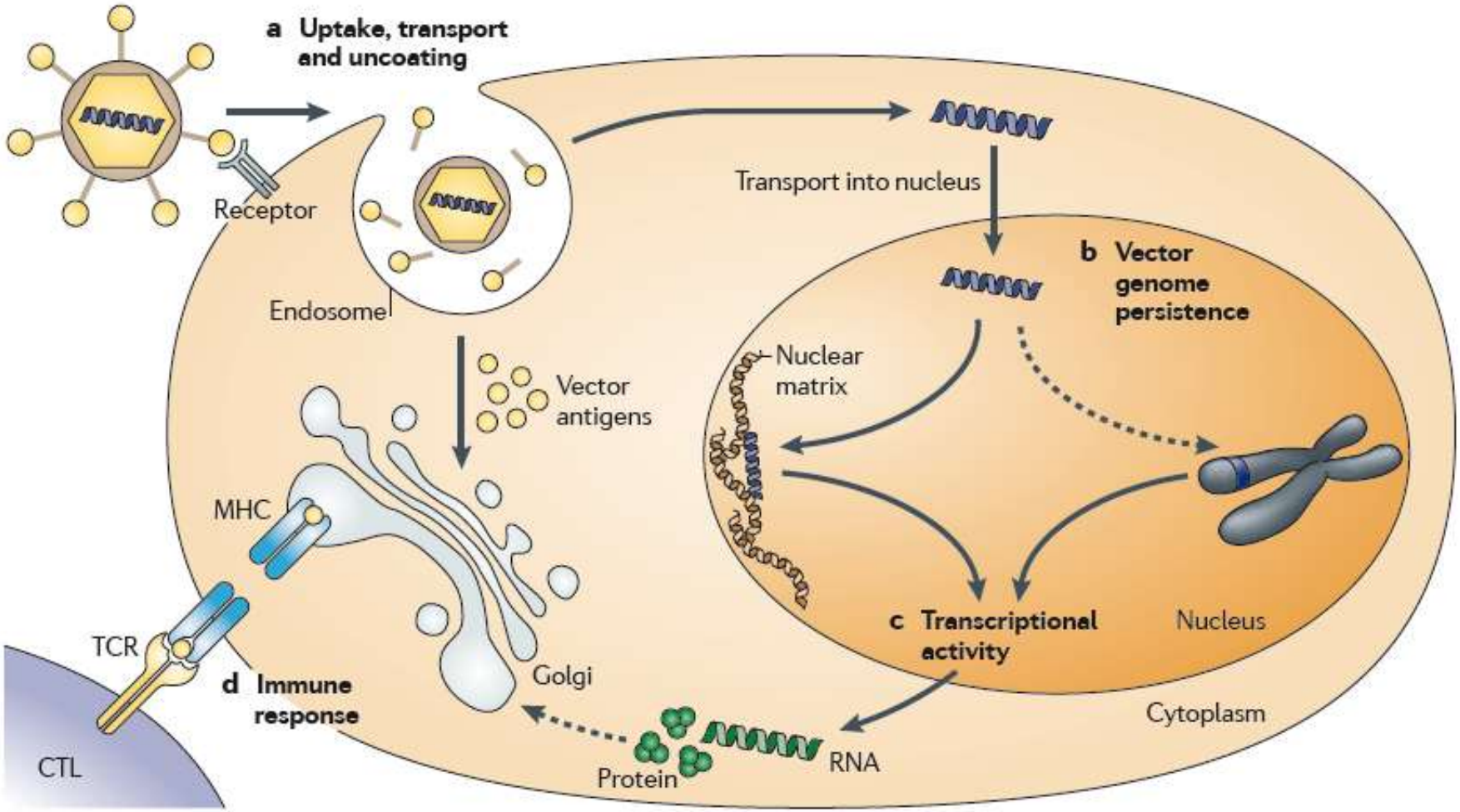
3. Long-term expression in skeletal muscle – episomal, even up to 2 years

4. Muscle regeneration enables higher expression

5. Promoters – better viral than cell specific ?

6. Efficiency is reversely dependent on the animal size...

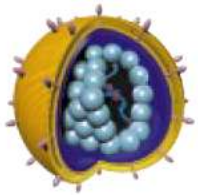
The fours barriers of successful gene therapy

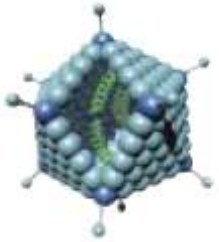


Kay M, Nature Rev Genetics, 2011

Viral vectors

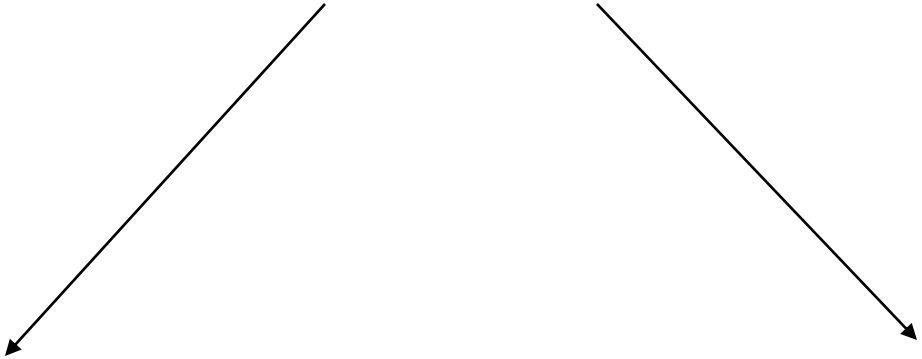
Vectors for gene therapy



	Plasmids	Retroviral	Lentiviral	AAV	Adenoviral 1st generation	„Gut-less" Adenoviral
Transfection efficacy	Very low	low	low	moderate	high	high
Capacity	unlimited	4-5 kb	9 kb	4 kb	7-10 kb	30 kb
Cytotoxicity	low	low	low	low	high	low
Expression	Days-weeks	Long-term	Long-term	Long-term	Weeks-months	Long-term

Viral vectors



Integrating

- retroviral
- lentiviral
- AAV

Non-integrating

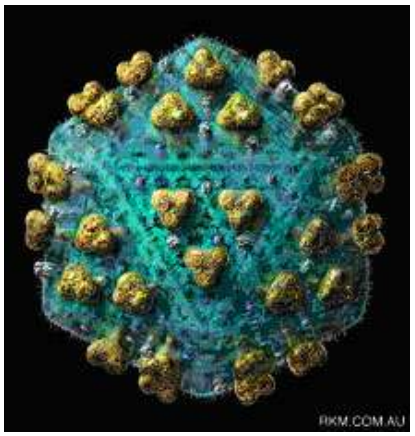
Adenoviral
HSV

Integration depends on:

-LTR sequences and integrase (retroviruses)

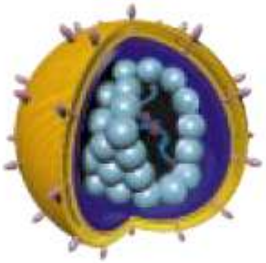
- ITR seqeuences and rep proteins (AAV)

Retroviral expression system



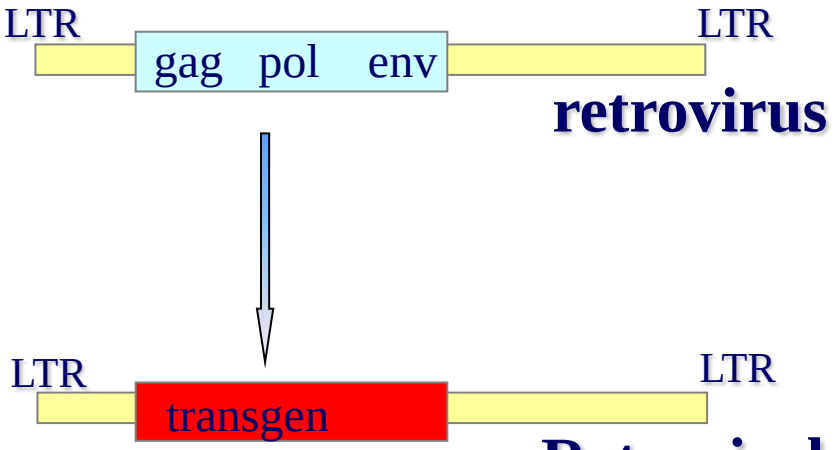
Gag – core proteins, matrix, nucleocapsid
Pol – reverse transcriptase and integrase
Env – envelope glycoproteins

Retroviral vectors

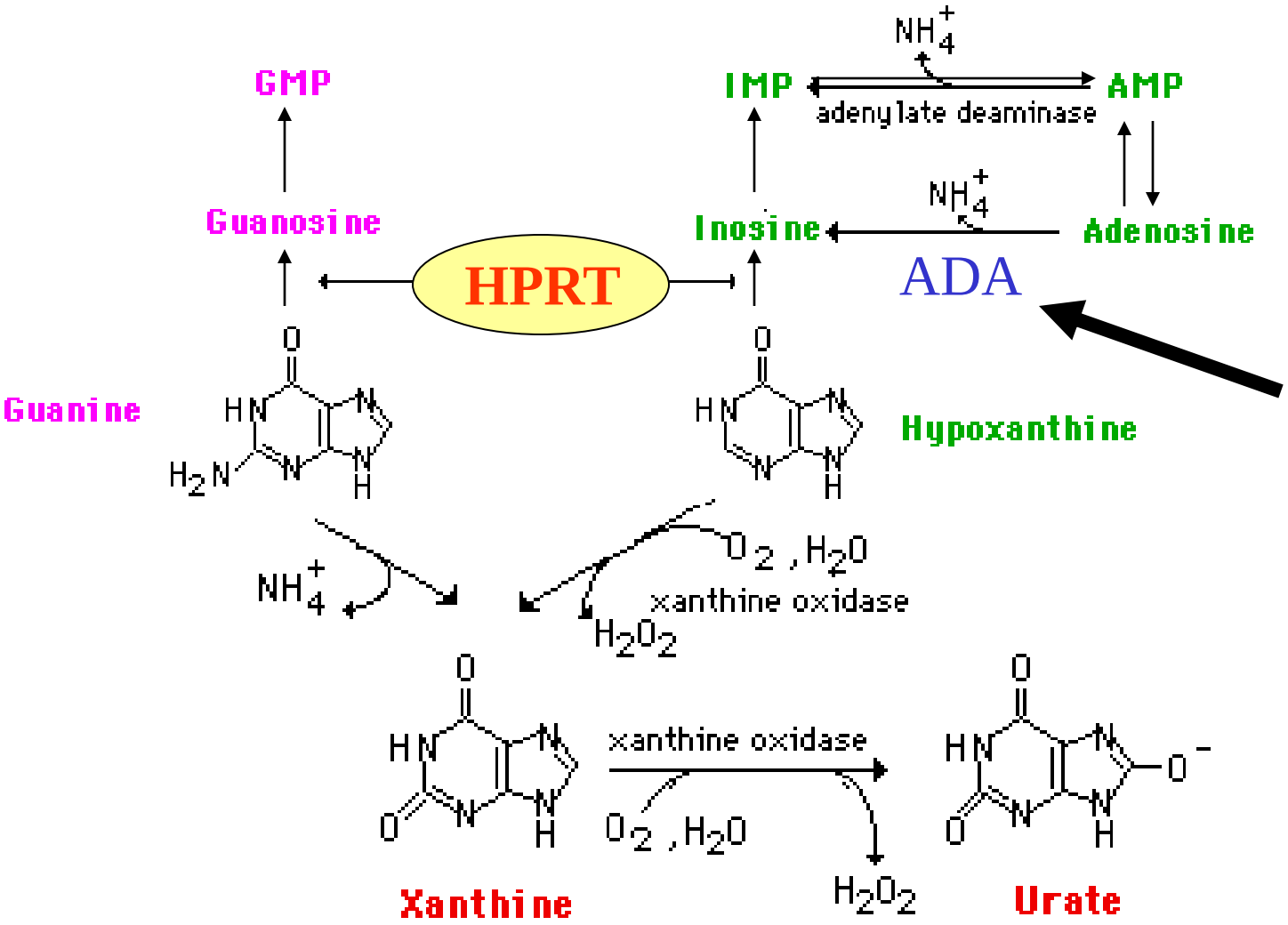


- *gag – structural proteins*
- *pol – reverse transcriptase*
- *env – envelope proteins*

- **long-term expression due to integration into cellular genome**

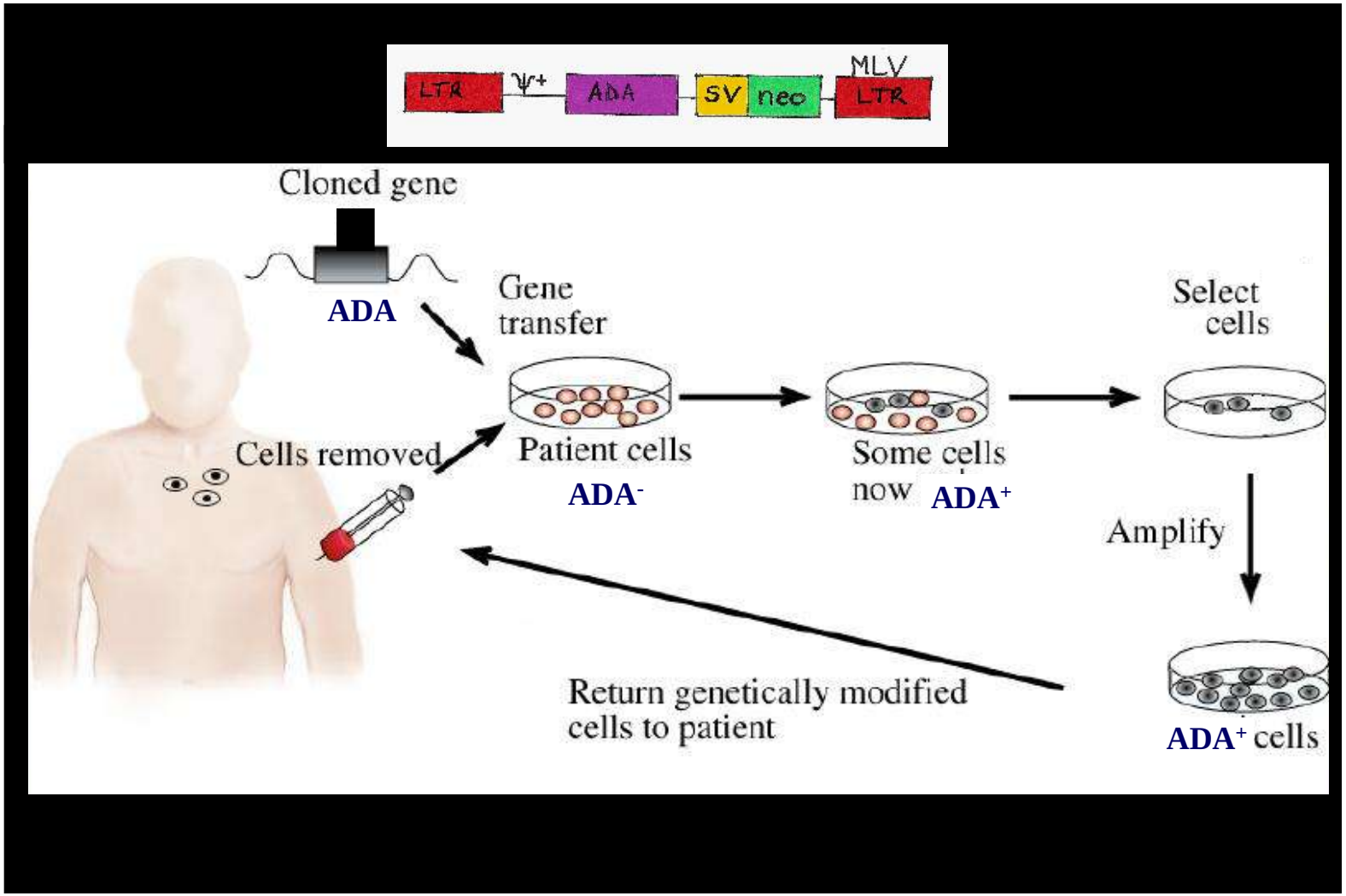


First controlled trial of gene therapy - 1990

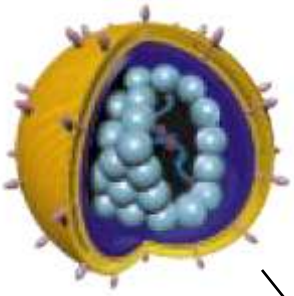


ADA deficiency– results in severe immunodeficiency syndrome

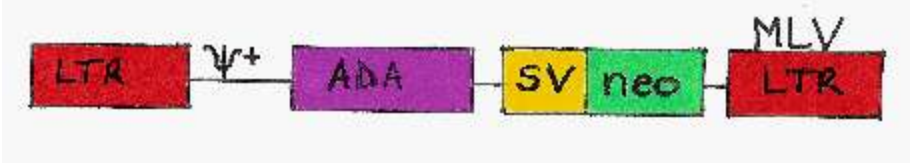
Gene therapy of ADA deficiency



First clinical trial of gene therapy - 1990



Retroviral vector containing correct ADA gene (cDNA) has been transduced into blood lymphocytes



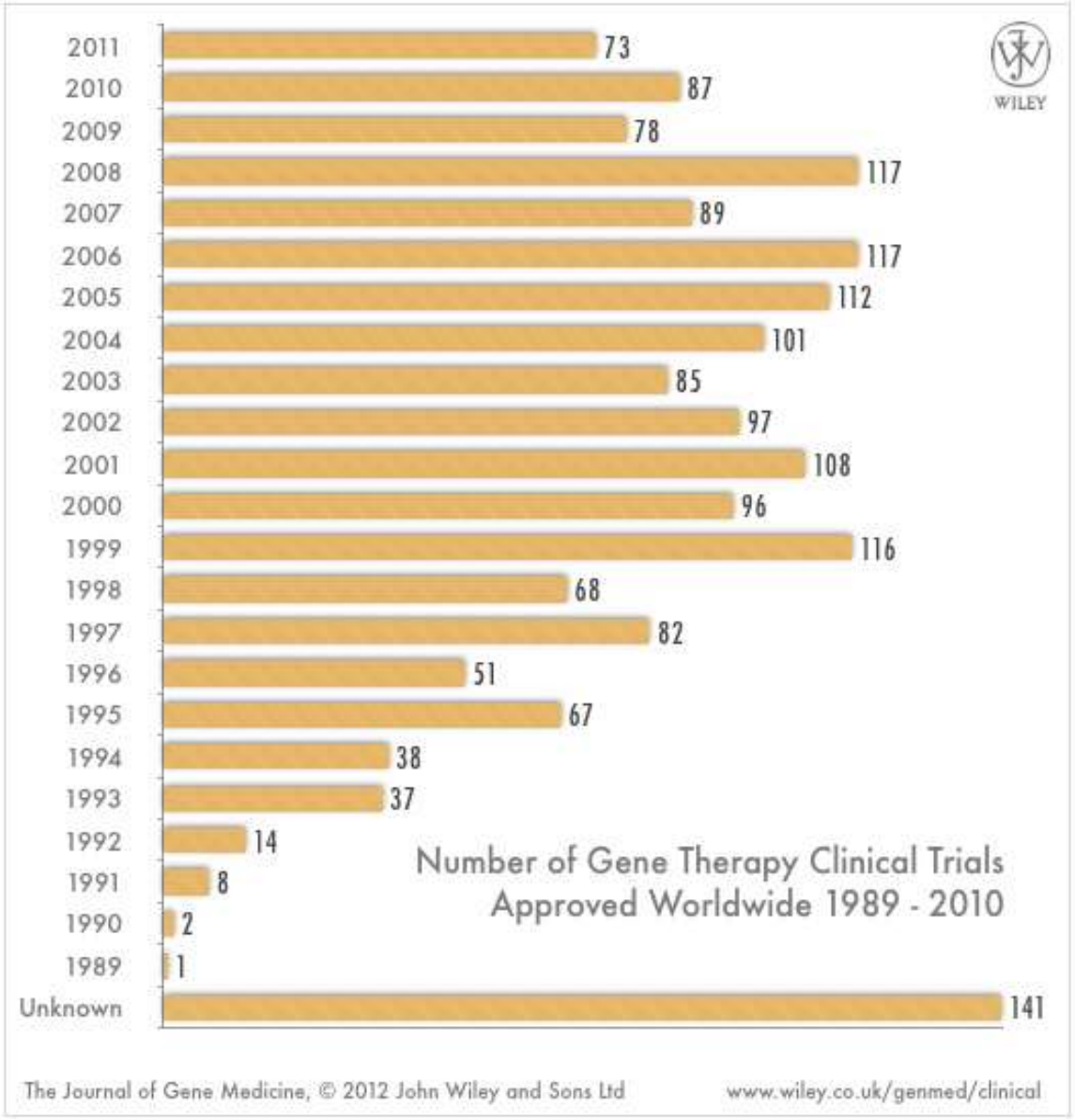
Ashanti De Silva (patient)

This first clinical trial was not „pure" from the methodological point of view.

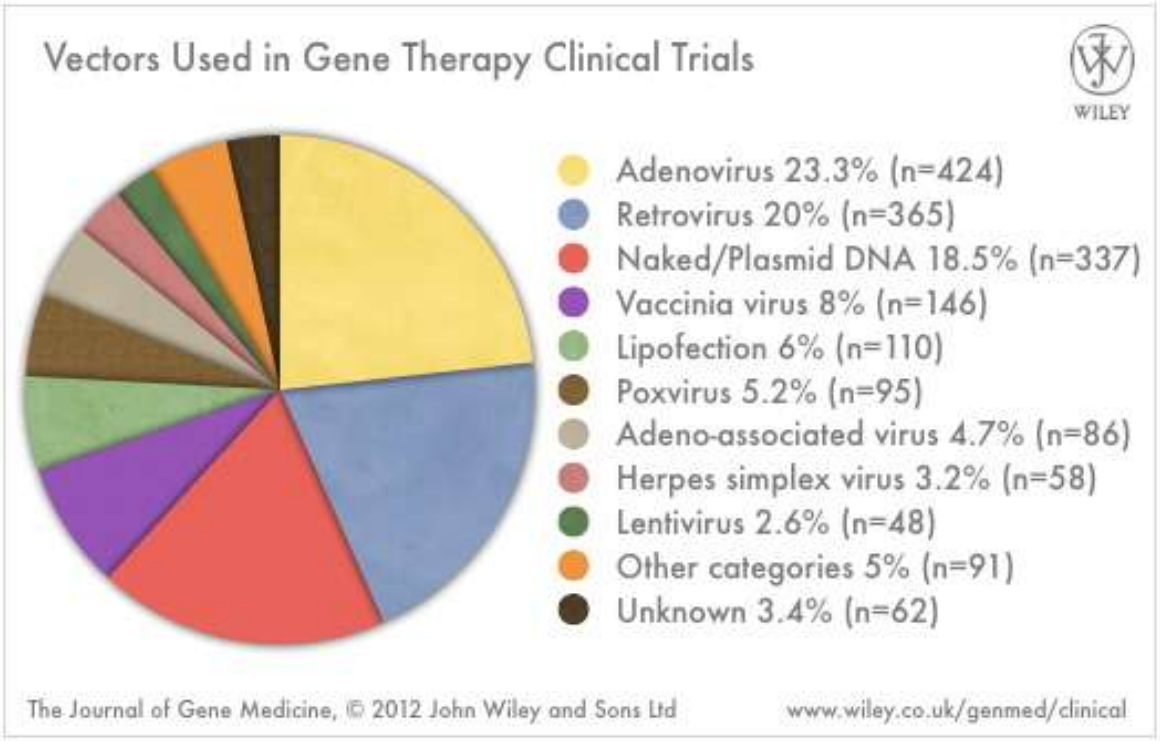
The patients have been treated concomitantly with enzyme injections – ADA-PEG.

Nevertheless, the marker transgene (neo) could be detected in the blood cells of the patients even more than 5 years after injection of modified cells.

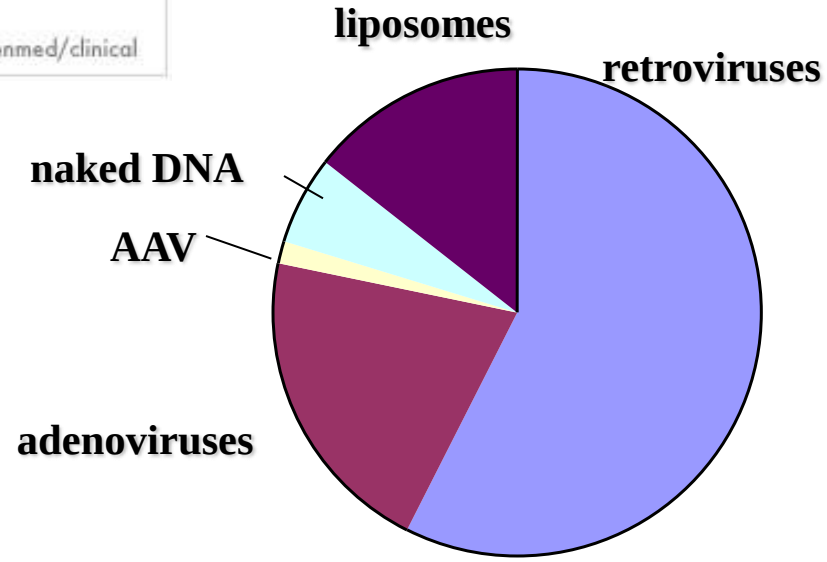
Gene therapy clinical trials



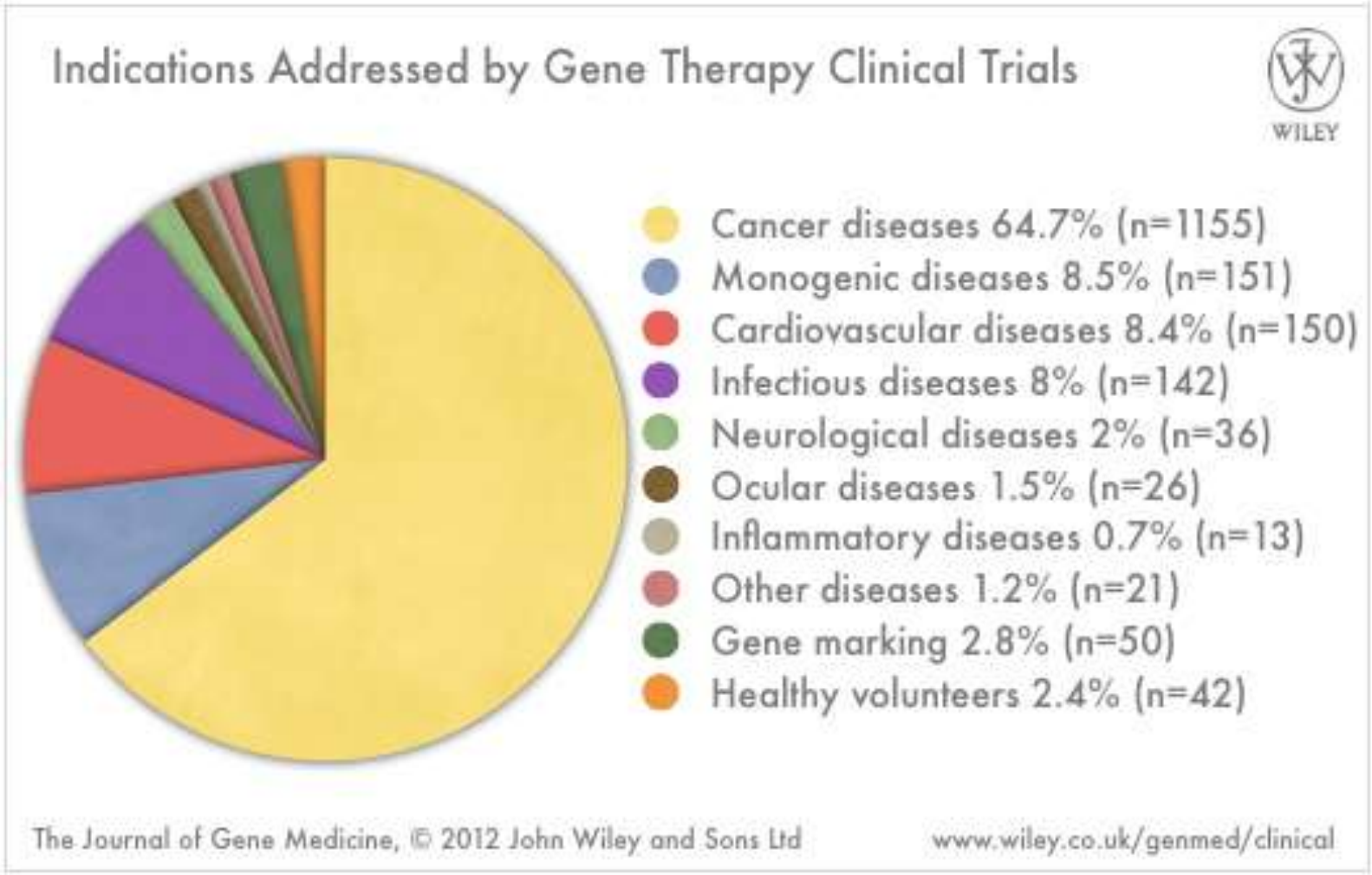
Types of vectors used in clinical trials of gene therapy



10 years ago...



Clinical trials in gene therapy



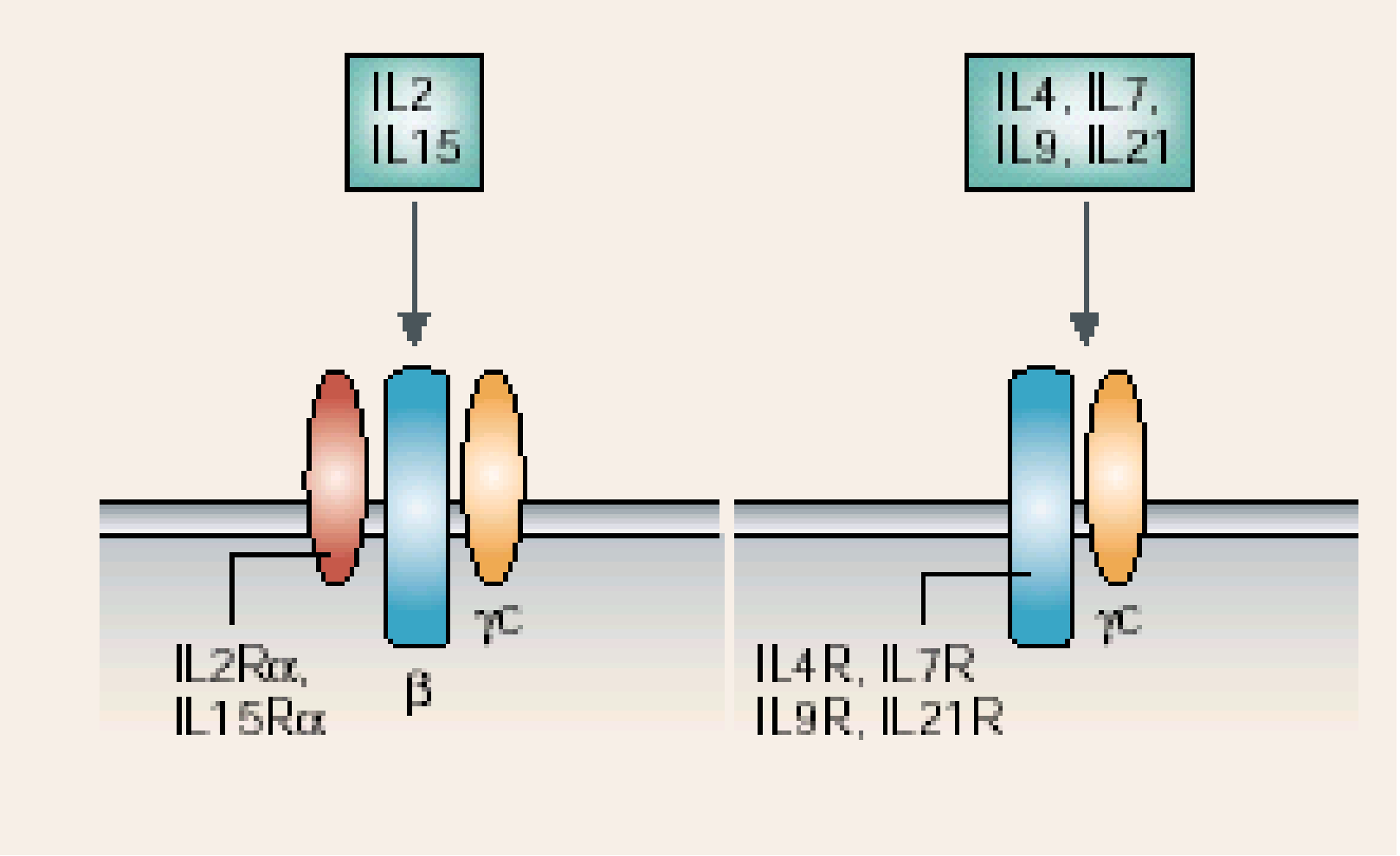
Succesful gene therapy

David Vetter - „Bubble Boy”

David has spent 12 years in a foil-protected environment. Finally he has received the bone marrow transplantation from his sister, but unfortunately died due to Epstein-Barr virus infection

X-SCID deficiency

Cytokines receptors



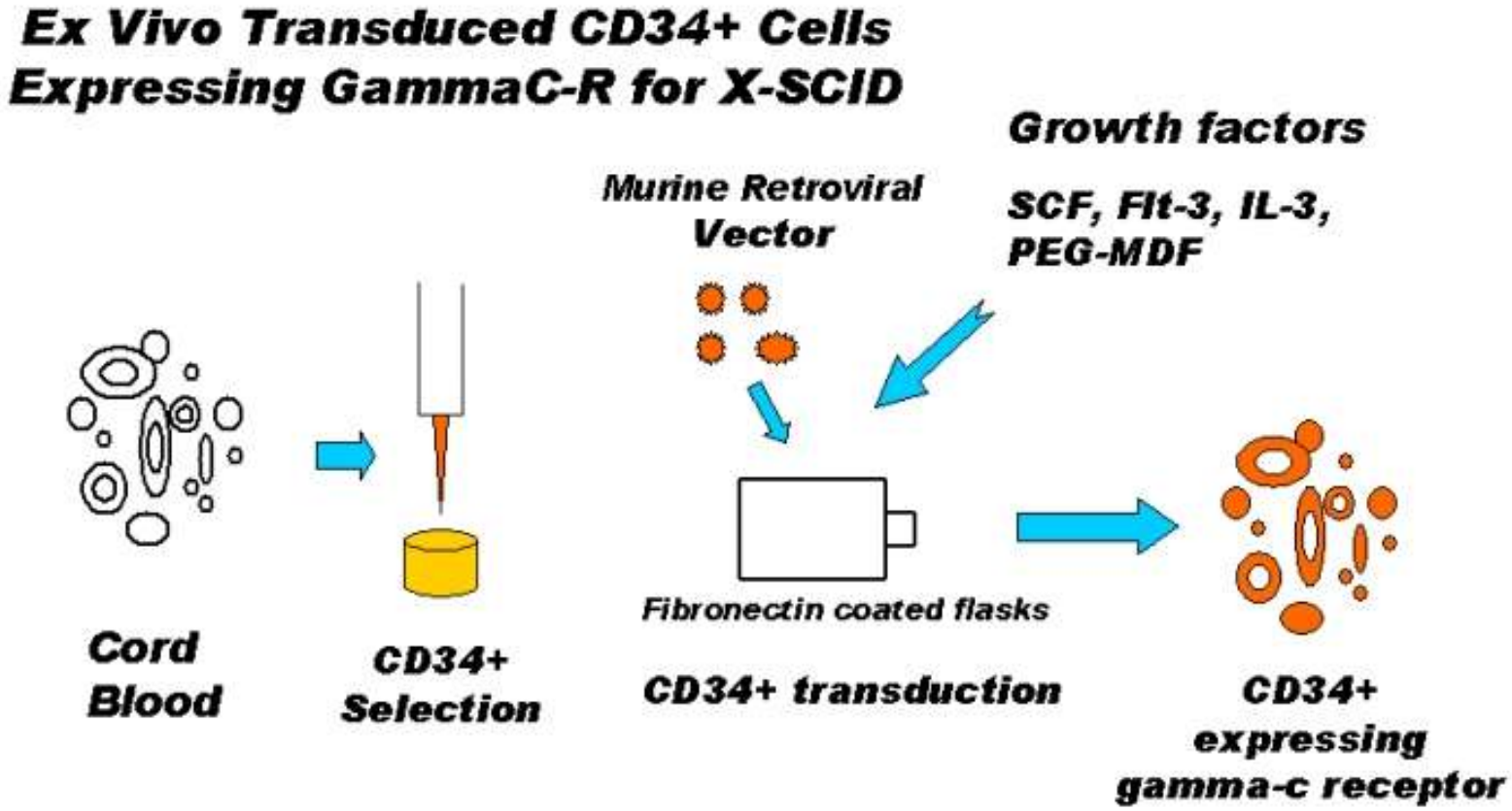
D. Kohn et al., Nature Rev Cancer July 2003

Cavazzana-Calvo M et al .

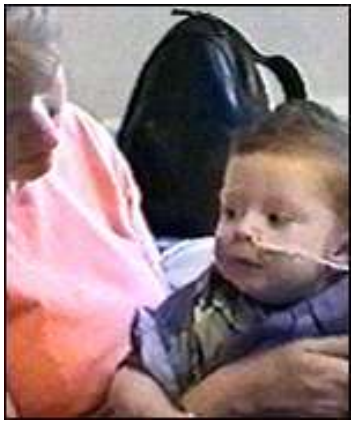
Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease

Science 2000: 28 April: 288: 669-672

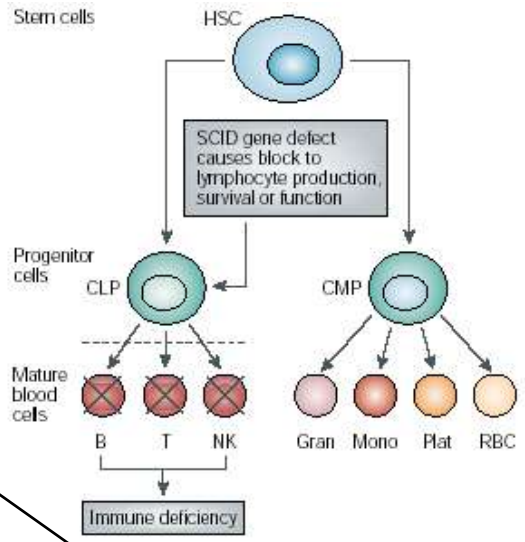
Gene therapy is efficient in treatment of X-SCID



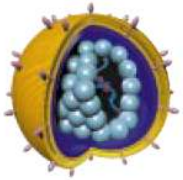
Gene therapy is efficient in treatment of X-SCID



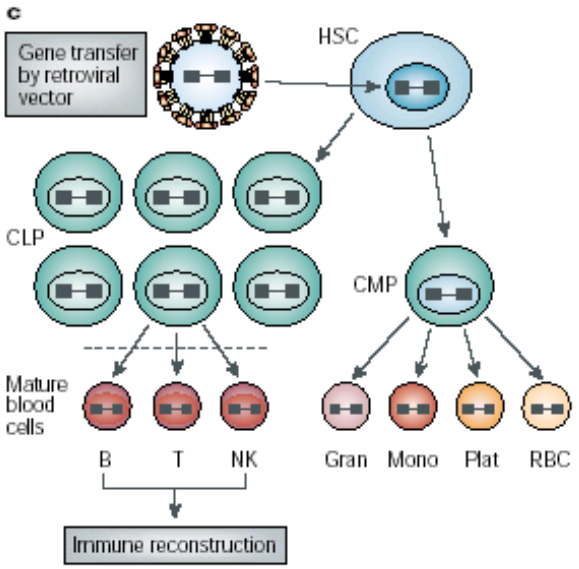
Stem cells without correct γc gene



Gene therapy



Retroviral vector with a correct γc gene

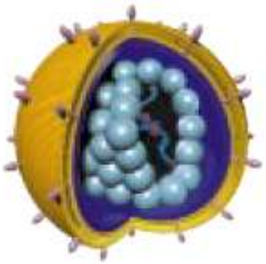


Gene therapy has been beneficial to most treated SCID-X1 patients!!!

- they can now cope with environment microorganisms and have a normal life in the absence of any specific therapy

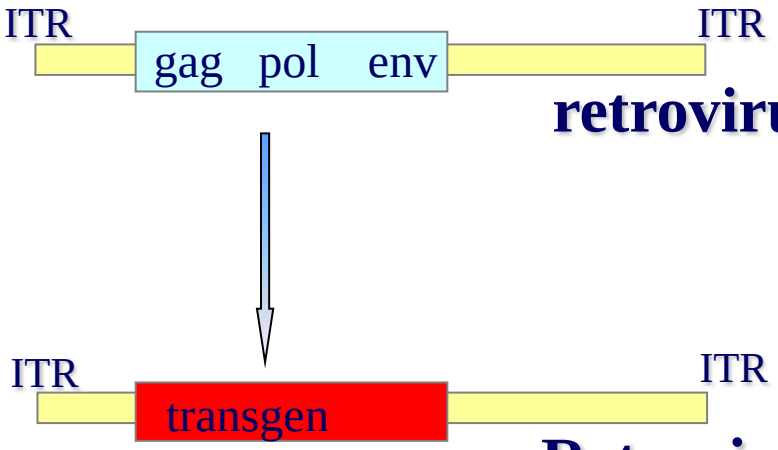
- no evidence for γc transgene silencing has been observed

Potential risk of application of retroviral vectors



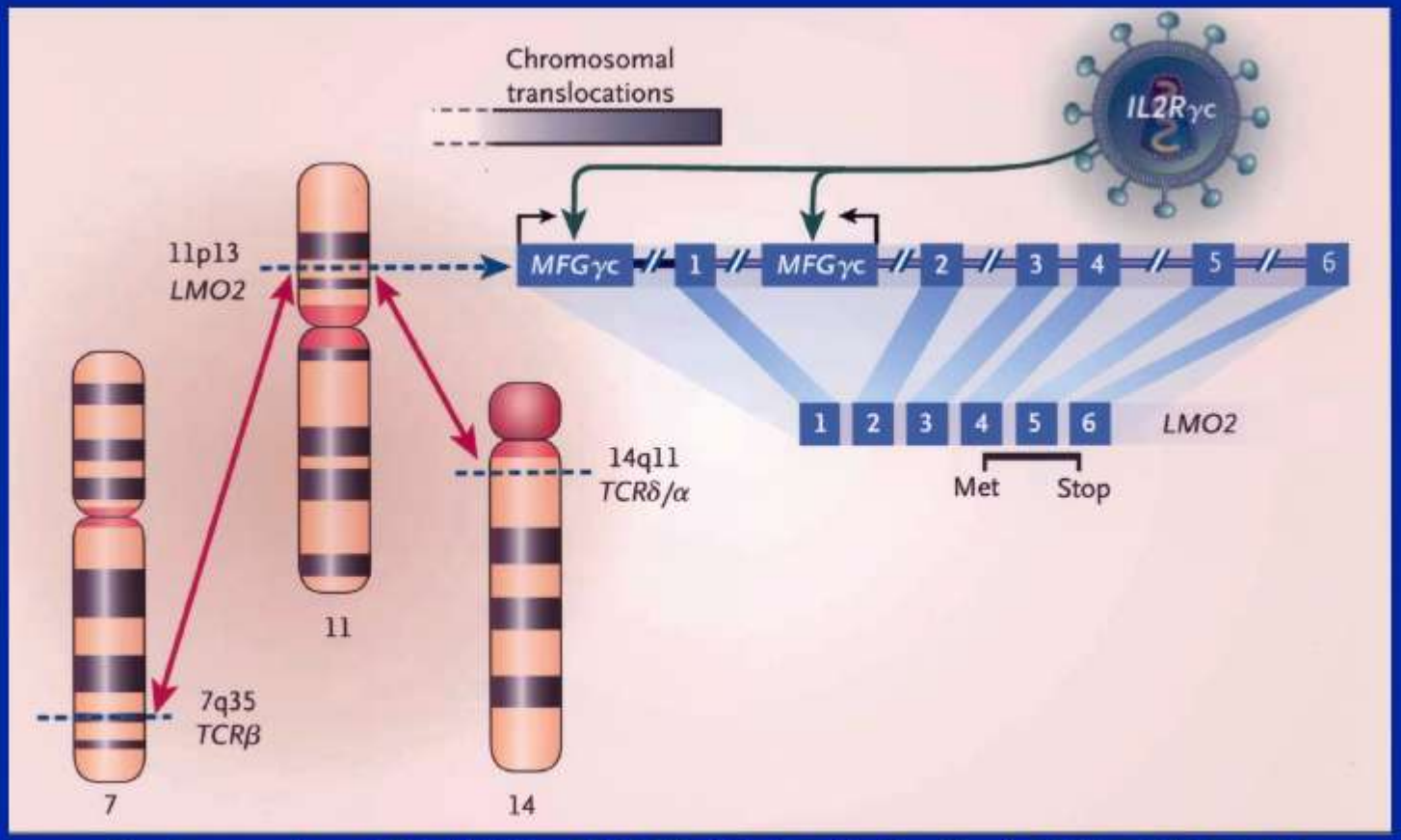
- *gag – structural proteins*
- *pol – reverse transcriptase*
- *env – envelope proteins*

- **long-term expression & integration into cellular genome**



random integration – risk of insertional mutagenesis

Integration of retroviral vector into the promoter of LMO2 gene



McCormack and Rabbitts (2004) *N. Engl. J. Med.* 350, 913-922

Gene therapy has been beneficial to most treated SCID-X1 and ADA patients!!!

SCID-X1:

1. French trial – 10 treated, 9 benefited. Unfortunately, four of those who benefited in the begining developed leukemia and one boy died this year because of leukemia.

2. British trial – 10 treated, 10 benefited –one developed leukemia

Gene therapy of ADA deficiency

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Gene Therapy for Immunodeficiency Due to Adenosine Deaminase Deficiency

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Among 10 patients with ADA-deficiency, gene therapy restored the immune functions in 9 !

During follow up observations (more than 7 years now) no side effects such as in case of X-SCID have been observed

Gene therapy is succesful in treatment of diseases

Some Gene Therapy Successes

Disorder	Disease type	Patients benefiting	First publication
X-SCID	Immunodeficiency	17/20	2000
ADA-SCID	Immunodeficiency	26/37	2002
Adrenoleukodystrophy	Neurologic	2/4*	2009
Leber's congenital amaurosis	Blindness	28/30	2008
Wiskott-Aldrich syndrome	Immunodeficiency	8/10	2010
β-thalassemia	Hemoglobinopathy	1/1	2010
Hemophilia	Coagulation	6/6	2011?

*Includes a patient treated too recently to see benefit

Science, 7th October 2011