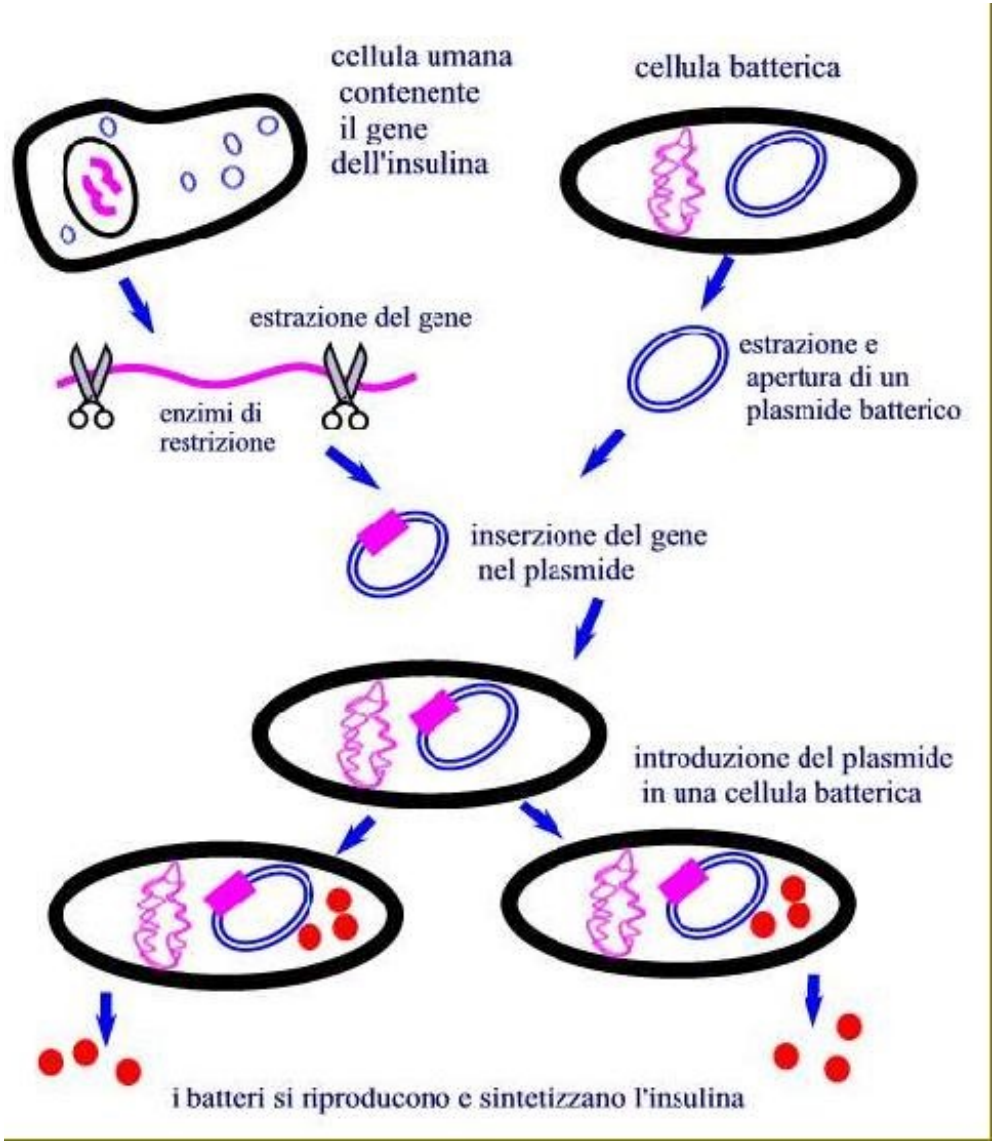
"Traditional" Genetic Engineering

Modern genetic engineering: "Recombinant DNA"



Fundamental steps for the development of the recombinant DNA technique

1967:
DNA ligases

1968:
Restiction enzymes
discovered

1972:
Plasmidial DNA

Transgenic anim als and
plant available

Transgenic animals, what are they?

Animals in whose genome an exogenous gene has been inserted, or depleted / modified an endogenous

Why?

Production of biologically active peptides

Induction

Organi per xenotrapianti

Organs for xenotransplantation

Basic science

Organisms used as transgenic models

Arabadopsis

C. Elegans

Drosofila

Xenopus

Zebrafish

Mouse

Rat

Sheep

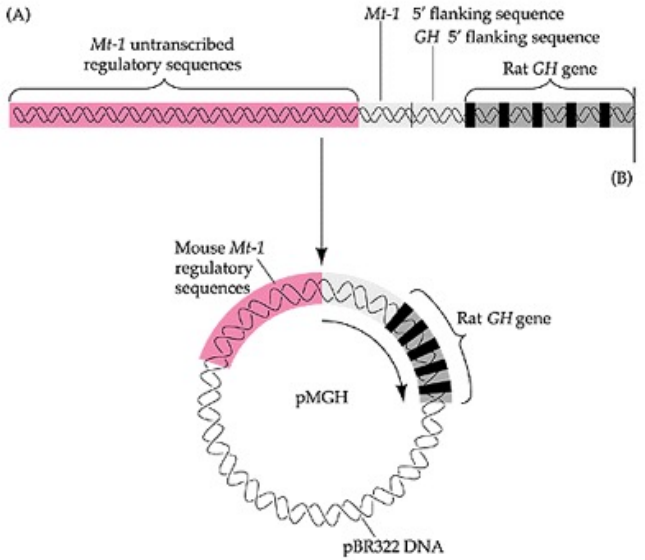
Bovine

Goat

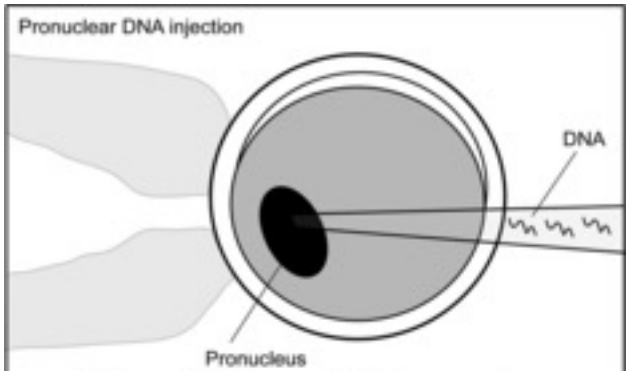
Pig

Rabbit

Palmister & Brinster, 1982



Pioneers



Phase I: synthesis and preparation of the transgenic vector

Costruzione del transgene

Amplificazione del transgene

Purificazione del transgene

Production of a transgenic mouse: Methodology

Injecting female mice with PMSG (pregnant Mare Serum Gonadotrophin)
HCG (induces ovulation)

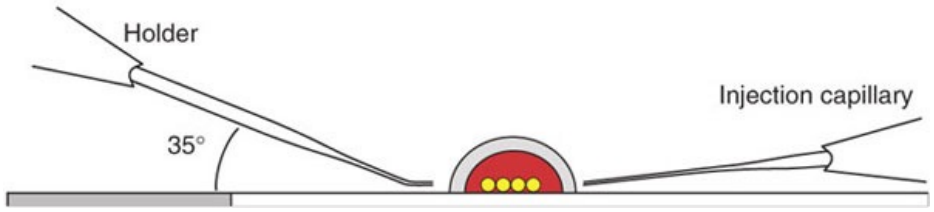
Check the coupling (vaginal plug)

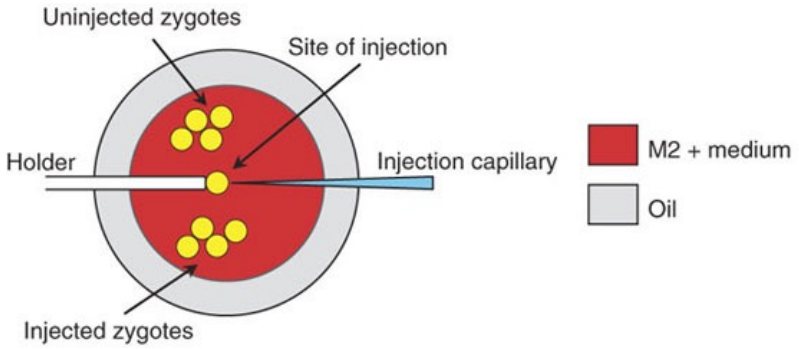
Recover the zygotes at the pronuclear stage

Injection (capillaries with internal filament) male pronuclear:
(1/2 picolitre linearized DNA: 100/200 copies of the gene –Eppendorf Injectman)

Transplantation into the recipient (oviduct) coupled with a vasectomized male!

Birth





https://www.youtube.com/watch?v=h-Bfc1GPWpE

Molecular bases

Insertion of random exogenous DNA

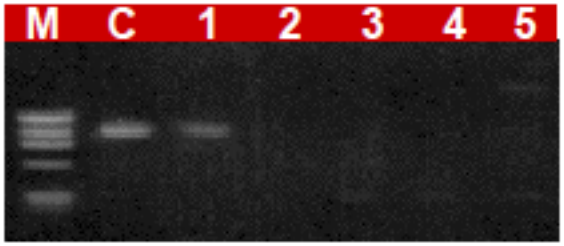
DNA breakage due to mechanical injection action

Plasmid with exogenous DNA inserted by ligase during DNA repair

Screening of offspring

Estrazione DNA dalle code della progenie

Analisi tramite PCR



Analisi tramite Southern-blot

Efficiency

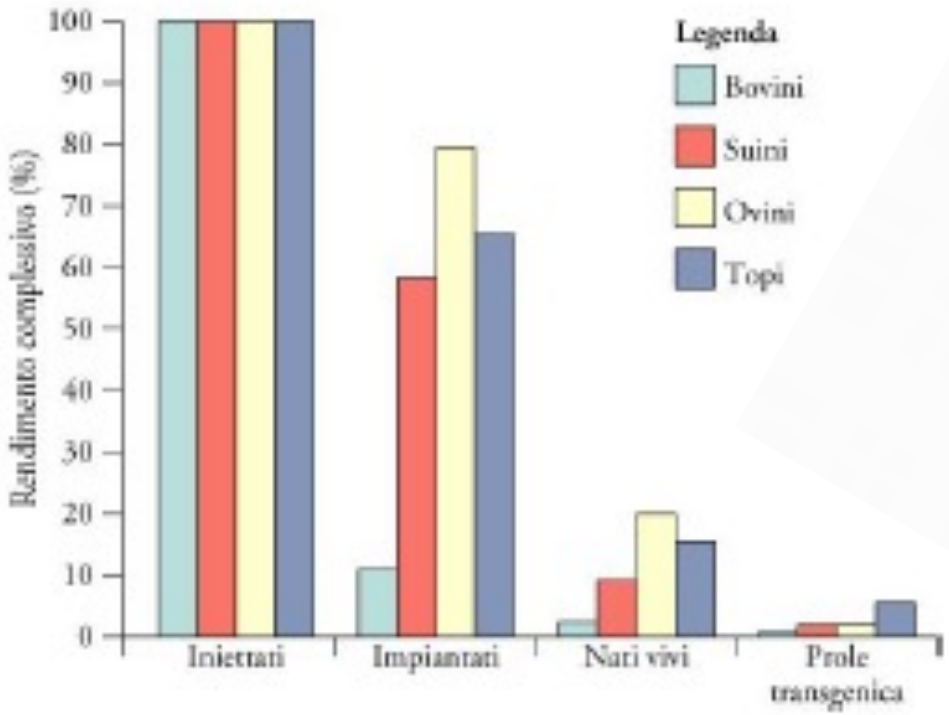
7-10% animals born are transgenic (Southern blot genomic DNA)

1-3% transgenic animals express the exogenous gene (?)

1) Insertion of transgene in unexpressed loci

2) Methylation of the transgene

General framework for pronuclear injection efficiency



Transgenesis by pronuclear injection

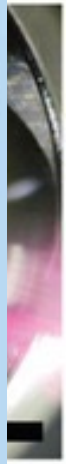


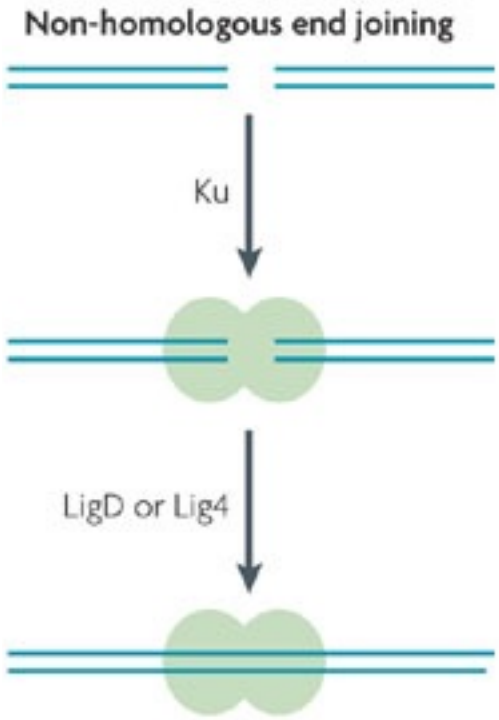
TABLE 1. Examples of transgenes introduced into fish that cause significant phenotypic effects*

Phenotype targeted	Species	Transgene
Growth (> twofold)	Atlantic salmon Tilapia Rainbow trout Coho salmon Chinook salmon Rohu Loach	Growth hormone
Freeze tolerance	Atlantic salmon	Antifreeze protein
Disease resistance	Catfish Carp Medaka	Cecropin Lactoferrin Cecropin
Carbohydrate metabolism	Rainbow trout Rainbow trout	Glucose transporter Hexokinase
Reproduction	Rainbow trout	Antisense GnRH
Lipid metabolism	Zebrafish	D6-desaturase
Phosphorus metabolism	Zebrafish	Phytase
Vitamin C metabolism	Rainbow trout	L-gulono-gamma-lactone oxidase

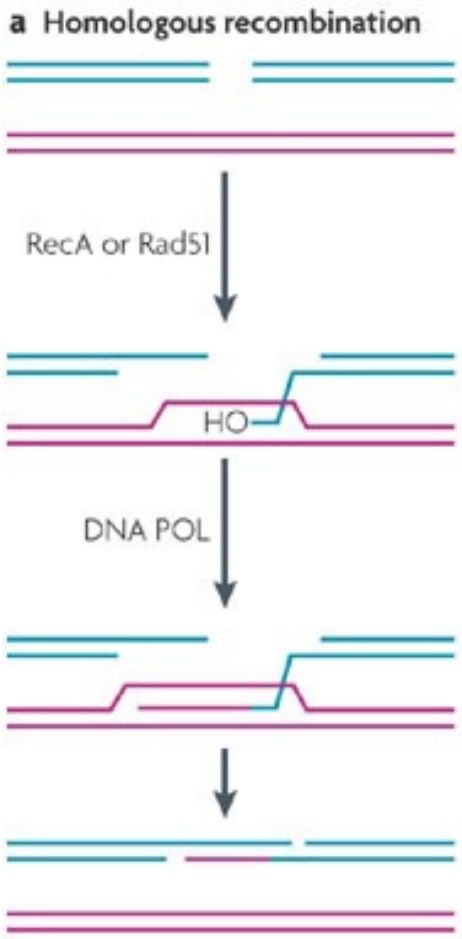
* Changes in physical or chemical traits.

Source: Reprinted from Trends in Biotechnology, Vol. 24, Devlin RH, Sundstrom LF, Muir WM, Interface of biotechnology and ecology for environmental risk assessments of transgenic fish, p 89–97 (2006), with permission from Elsevier.

Random vs targeted transgene insertion: homologous recombination



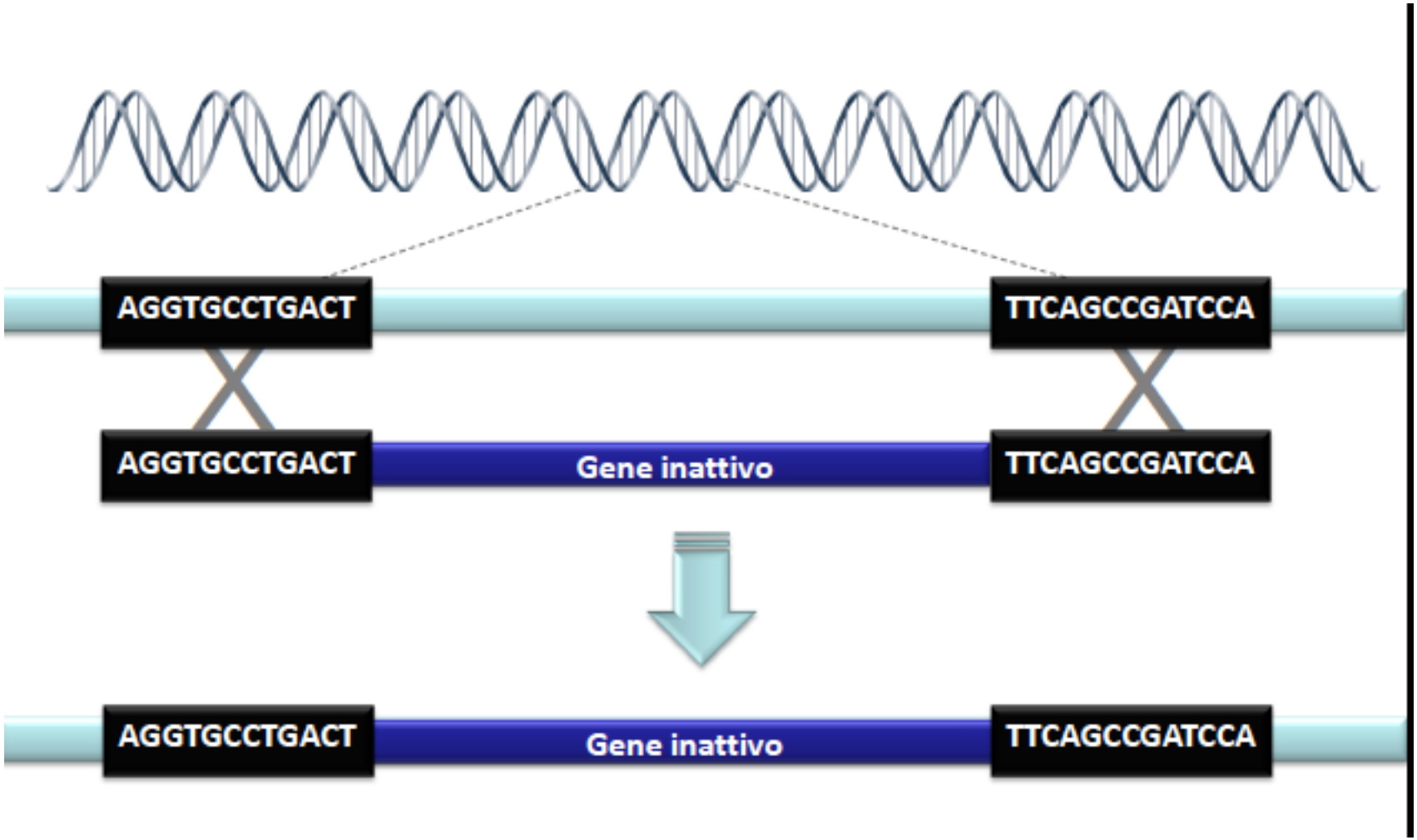
Pronuclear injection



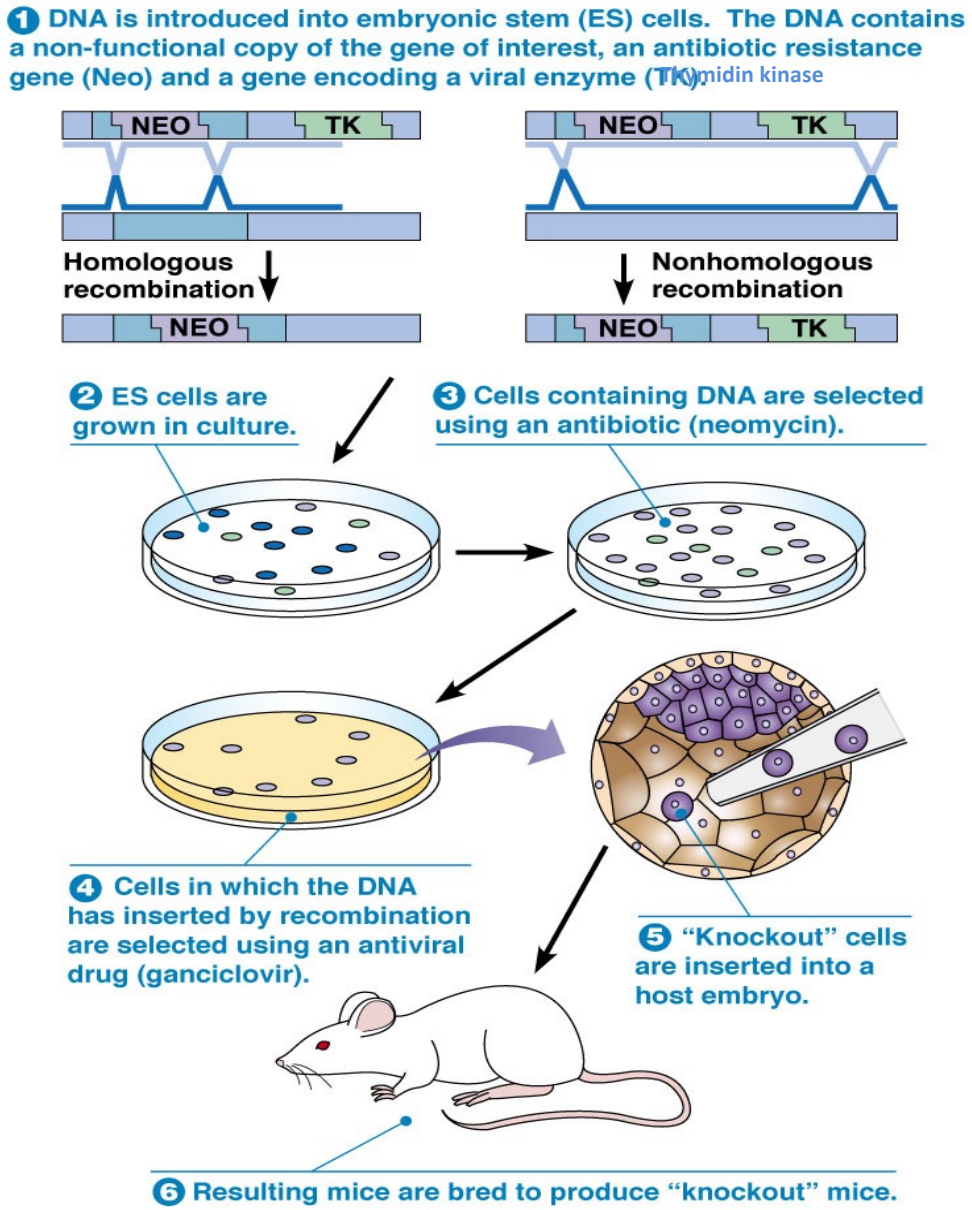
Strategy:

Support the transgene in 3 ’and 5’ with sequences Complementary to the genomic region of interest

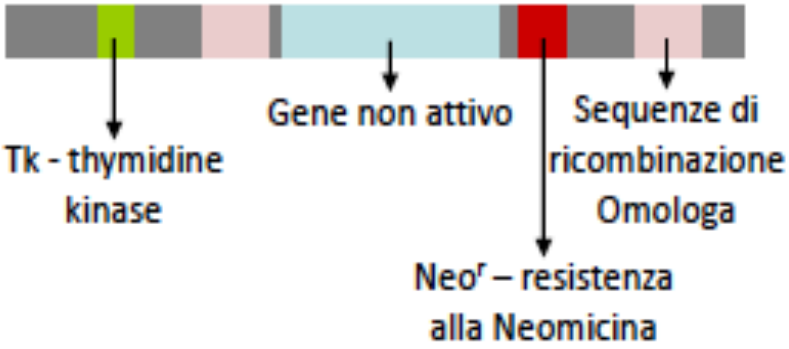
Scheme

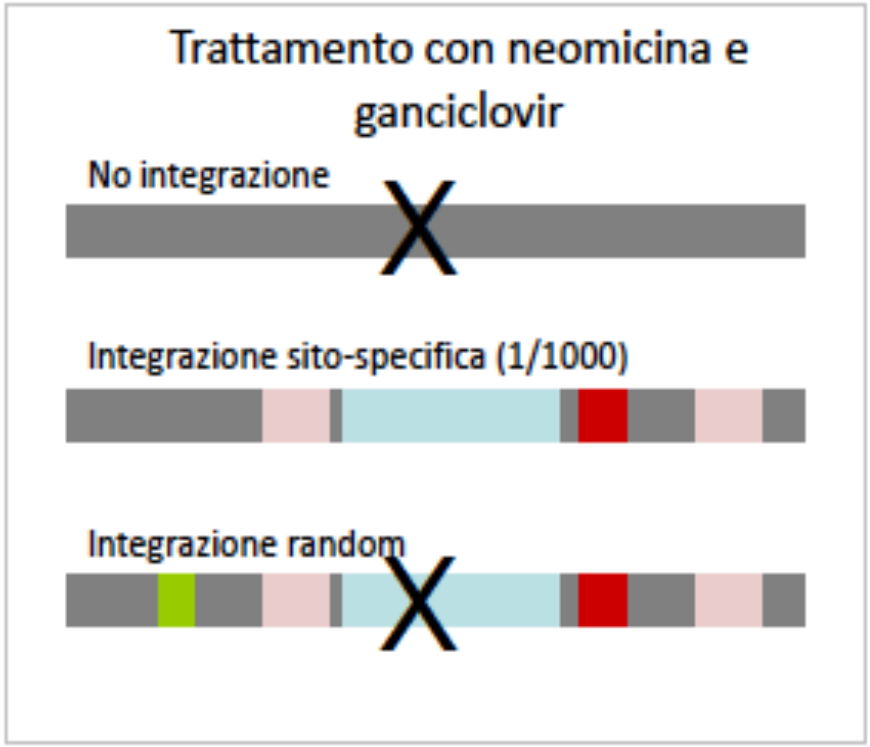

AGGTGCCTGACT
TTCAGCCGATCCA
AGGTGCCTGACT
Gene inattivo
TTCAGCCGATCCA
AGGTGCCTGACT
Gene inattivo
TTCAGCCGATCCA

Homolous recombination in stem cells

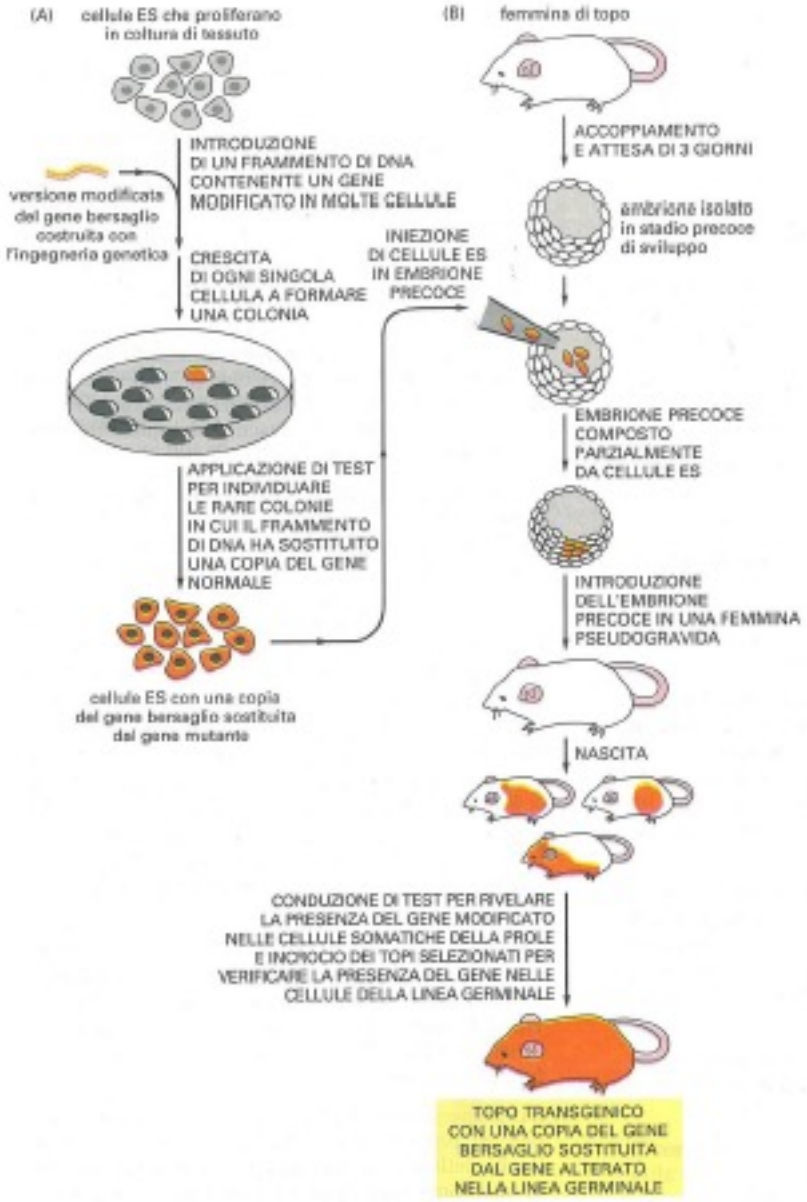


Selection of cells with transgene in the correct position





Transgenic mouse : Workflow

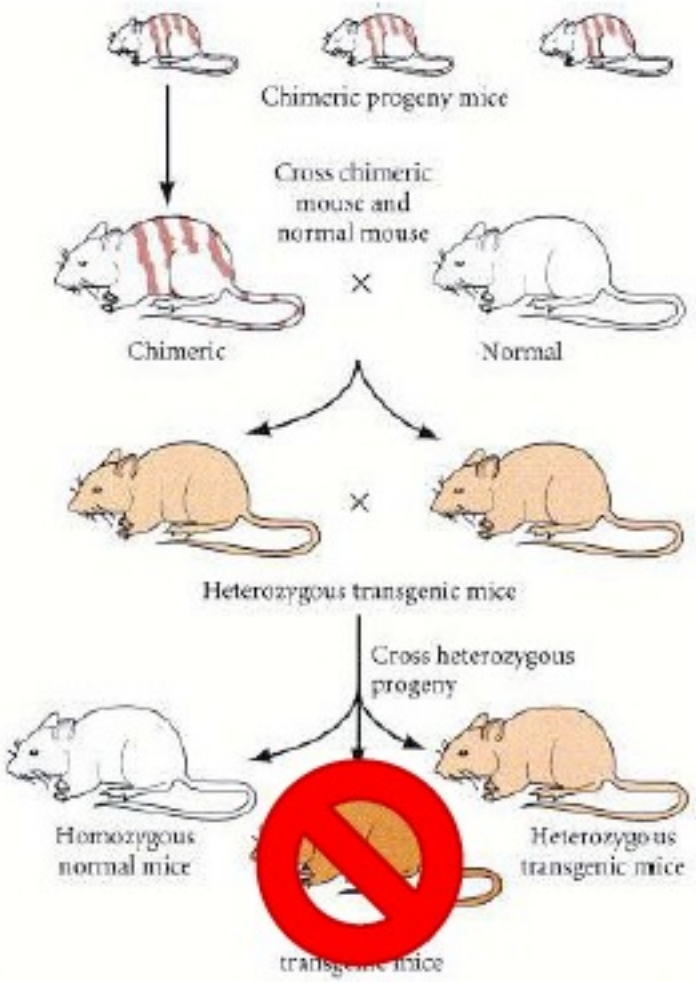


Targeted insertion of a transgene serves to:

- **KNOCK-OUT**: INATTIVAZIONE dell'espressione di un gene
- **KNOCK-IN:** INSERZIONE di un gene difettivo/selvatico (modelli di patologie con mutazioni puntiformi; geni reporter)

Limite KO costitutivi:
possibile mortalità degli omozigoti durante lo sviluppo.

KO condizionali:
l'induzione della mutazione **tempo** e **tessuto** specifica



Conditional transgenesis: The Cre-LoxP system

Eliminate / activate a given gene in a given tissue, organ, or In a particular phase of development

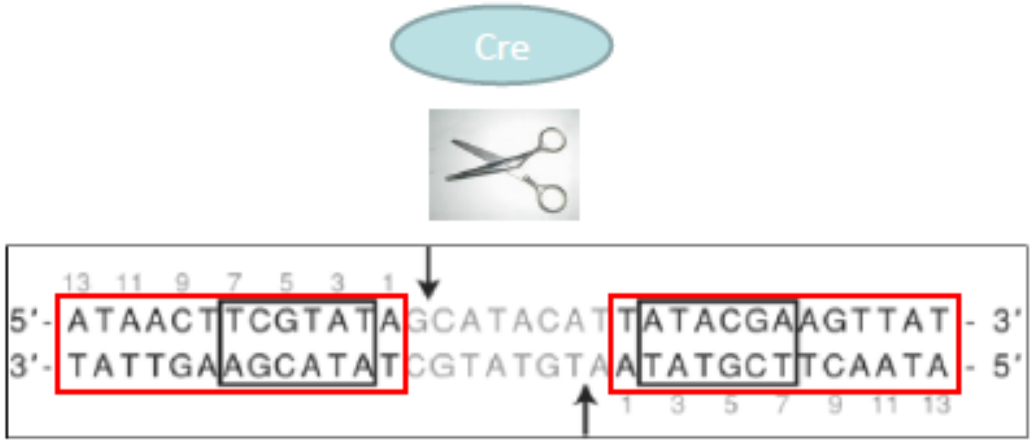
Sistema di RICOMBINAZIONE del fago P1

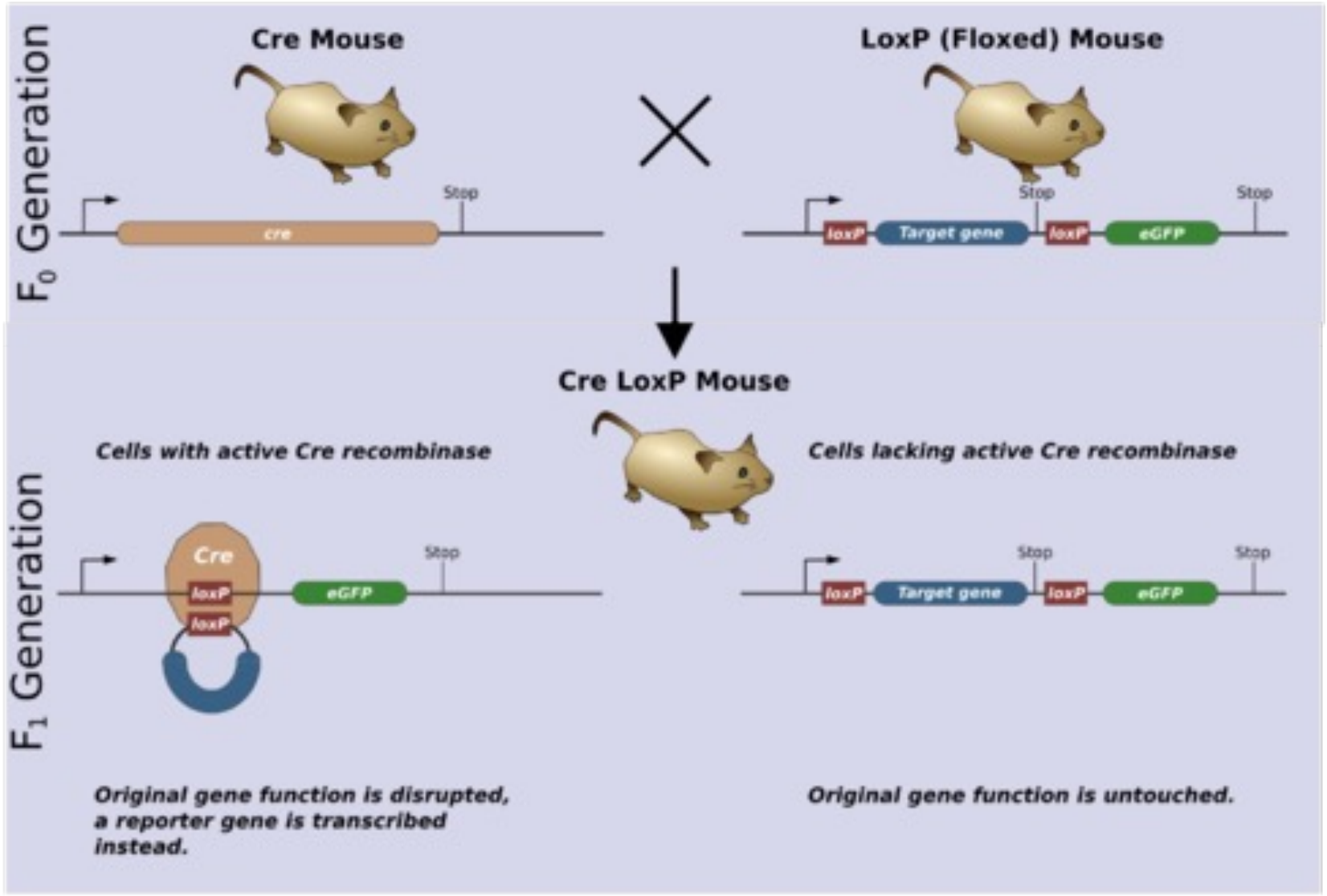
"**C**auses **re**combination"

CRE (cyclization **re**combination**), ricombinasi specifica**

LoxP (locus of **X**-over **P1**), **locus di crossover**

(2 seq palindrome di 13bp + regione centrale di 8nt)




F0 Generation
Cre Mouse
LoxP (Floxed) Mouse
Stop
cre
Stop
loxP
Target gene
loxP
eGFP
Stop
Cre LoxP Mouse
F1 Generation
Cells with active Cre recombinase
Cells lacking active Cre recombinase
Cre
loxP
loxP
eGFP
Stop
Stop
loxP
Target gene
loxP
eGFP
Stop
Original gene function is disrupted, a reporter gene is transcribed instead.
Original gene function is untouched.

The CRE-Lox system
Conditional transgenesis

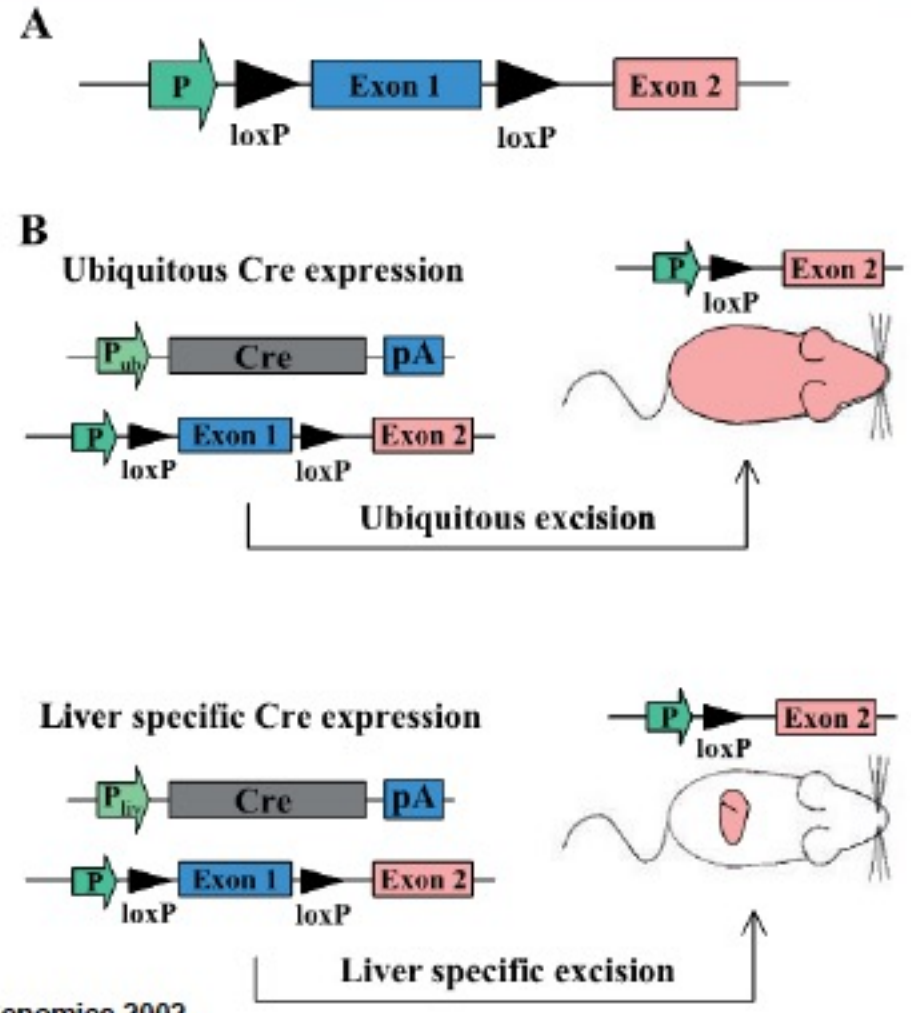
Controllo dell'espressione genica:

- **nello SPAZIO**: utilizzo di un PROMOTORE TESSUTO SPECIFICO

- **nel TEMPO**: utilizzo di un PROMOTORE
  - dipendente dallo STADIO di SVILUPPO
  - INDUCIBILE

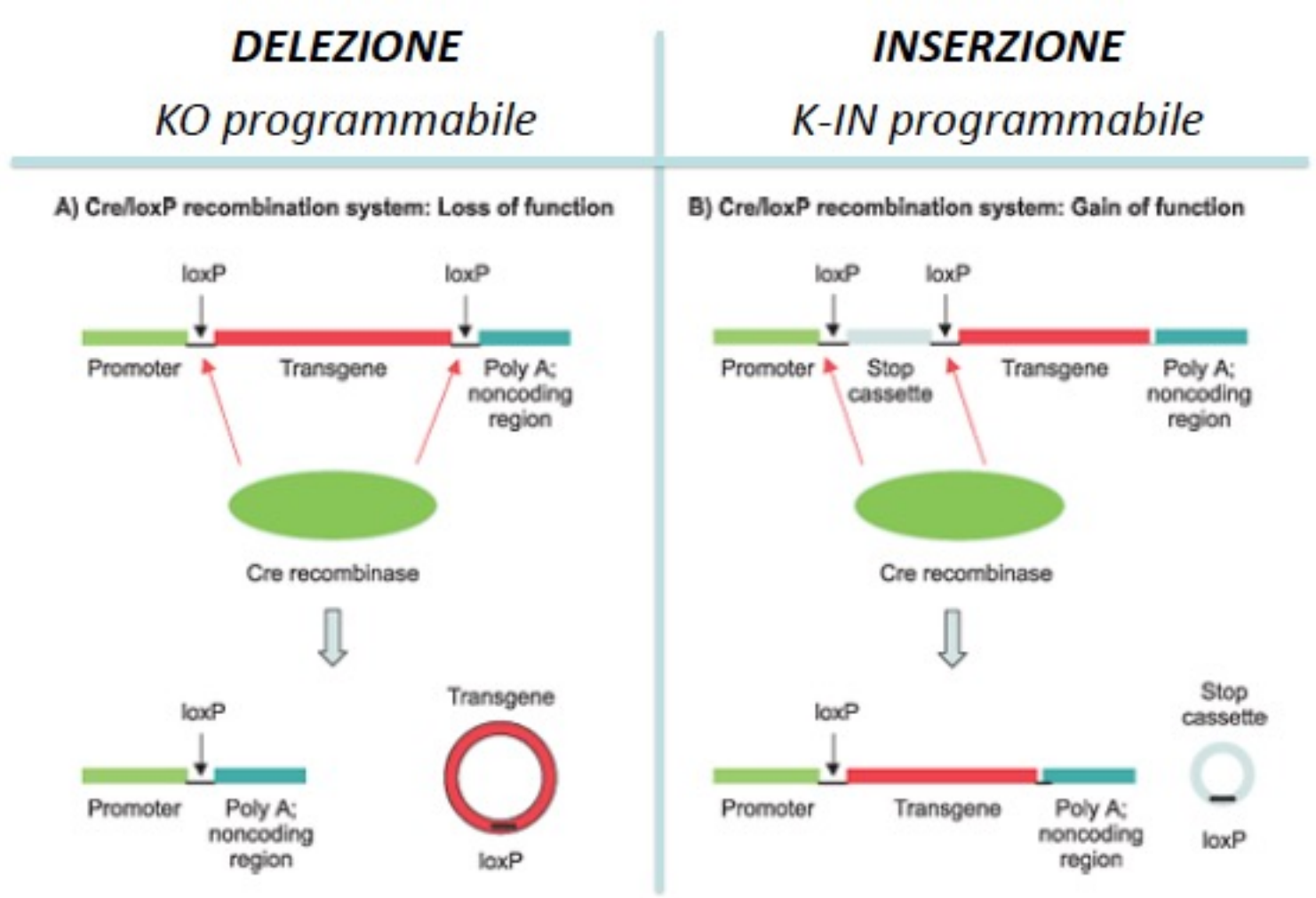
3 Il sistema Cre-loxP

Esempio di delezione tessuto specifica



Bockamp E et al., Physiol. Genomics 2002

Systema Cre-LoxP

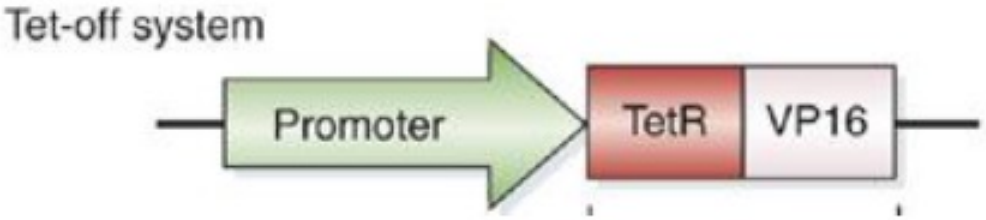


Conditional transgenesis, the TET OFF / TET ON system

Sistema a 3 elementi:

- **TeT-Repressor**: repressore della trascrizione
- **Tc/Dox**: tetraciclina/doxyciclina
- **TRE**: tetracycline response element

a TET-OFF



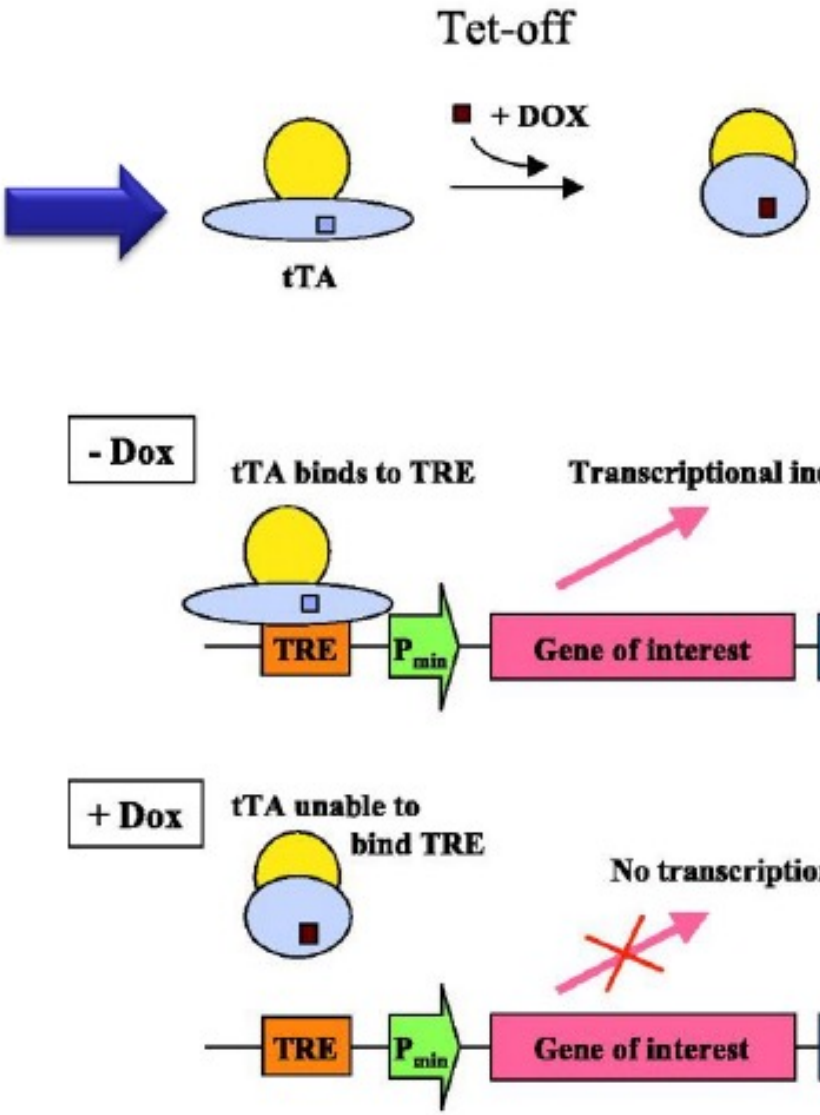
TetR: tet repressor
VP16: transactivation domain of herpes simplex virus

tTA

Tetraycline controlled transactivator

Sistema TET-OFF

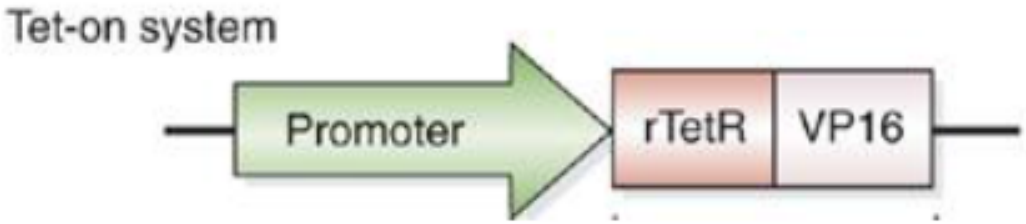
tTA: tetraycline controlled transactivator



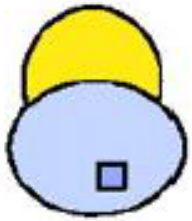
Tet-off system (tTA) will activate expression in the absence of its ligand doxycycline.

Upon addition of DOX, transcription of the gene of interest is extinguished.

Sistema TET-ON



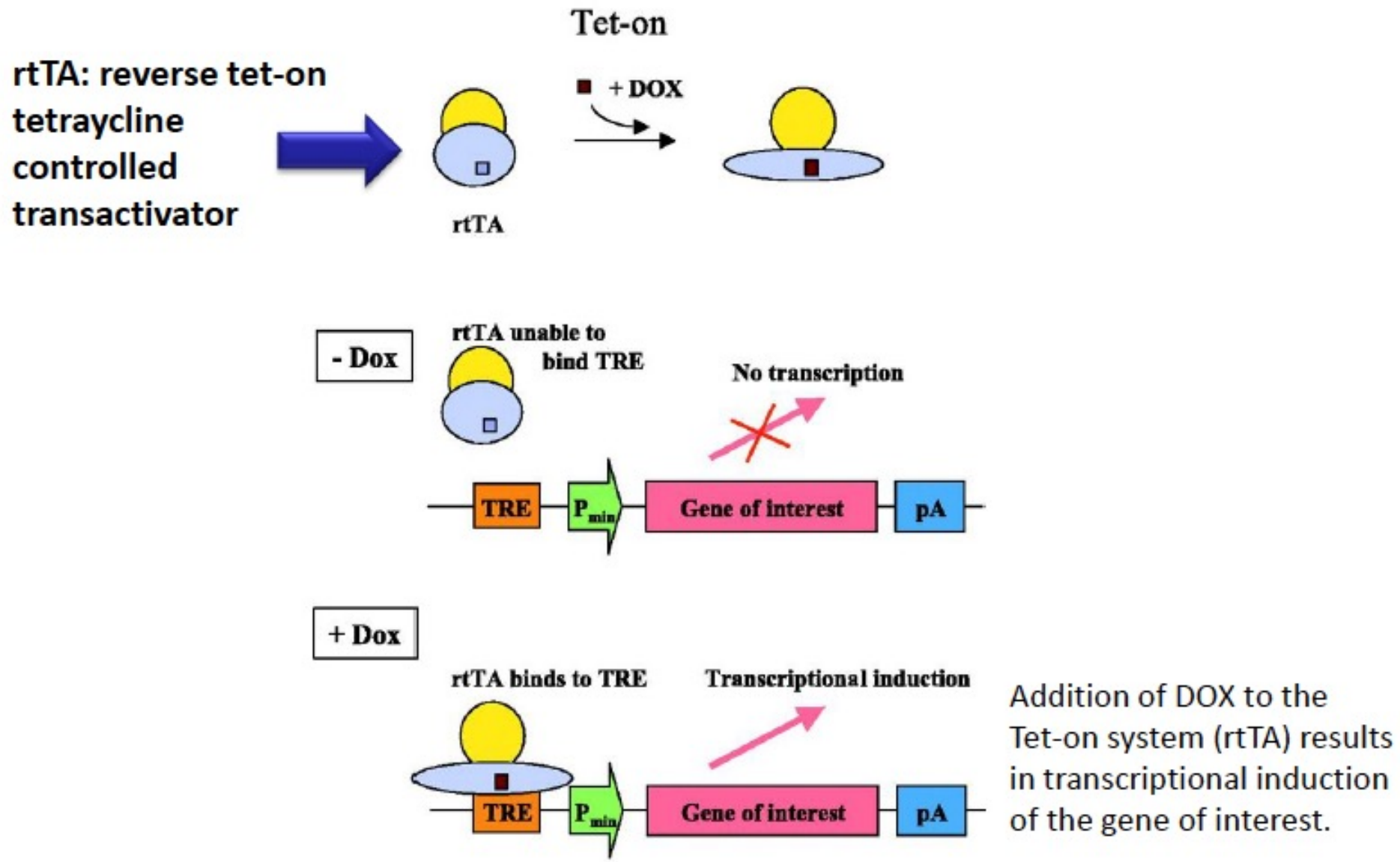
rTetR: reverse tet repressor
VP16: transactivation domain of herpes simplex virus



rtTA

Reverse tet-on Tetraycline controlled transactivator

Systema TET-ON



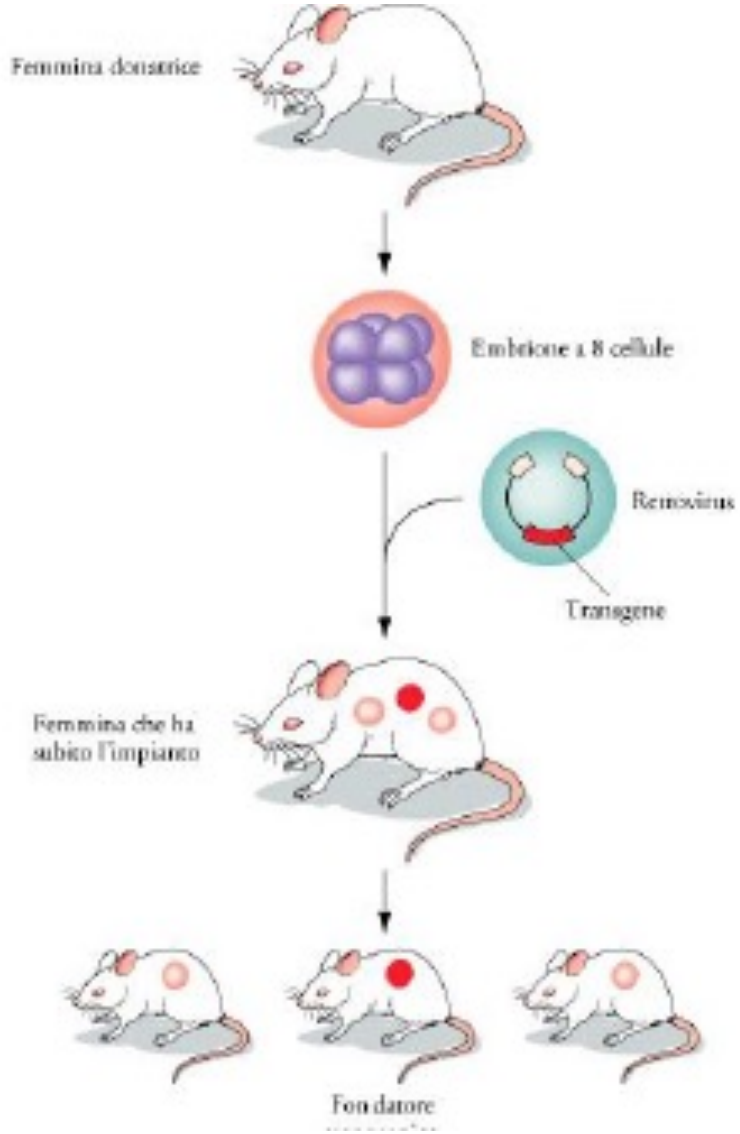
Addition of DOX to the Tet-on system (rtTA) results in transcriptional induction of the gene of interest.

p E et al., Physiol. Genomics 2002

Principali tipi di vettore virale: caratteristiche

Vettore virale	Vantaggi	Svantaggi
Retrovirus	**Capacità d'inserimento del gene, integrazione stabile nel DNA dell'ospite, elevati titoli di virus ricombinante, ampio tropismo d'infettività, relativa facilità di manipolazione del genoma virale**	**Difficoltà nel controllare l'infezione virale, mancata infezione delle cellule non in divisione, integrazione a caso nel genoma dell'ospite**
Lentivirus	Infezione delle cellule in divisione o meno, espressione stabile del gene, elevata capacità d'inserimento	Mutagenesi potenziale presenza di sequenze proteiche regolatrici e accessorie
Adenovirus	Elevati titoli di virus, alta espressione genica, grande capacità d'inserimento, infezione di cellule in divisione e non in divisione	Risposte immuni alle proteine virali, nessuna integrazione nel genoma dell'ospite, espressione genica transitoria
Virus adeno-associati	Infettano le cellule in divisione o meno, ampio tropismo cellulare, potenziale d'integrazione, bassa immunogenicità e non patogenicità	Limitate capacità per i transgeni, difficile generazione di alti titoli virali, presenza di adenoviruds o herpevirus per la moltiplicazione dei virus adeno-associati
Herpesvirus	Infettano un'ampia varietà di tipi cellulari, alta capacità d'inserzione, tropismo naturale per le cellule neuronali, seguita dalla produzione di alti titoli virali	Possibile tossicità, rischio di ricombinazione, nessuna integrazione virale nel DNA dell'ospite
Poxvirus	Alta capacità d'inserzione, possibile inserzione di grandi framenti di DNA, alti livelli di di espressione transgenica,, seguita da virus vivo ricombinante	Possibile effetto citopatico
Virus di Epstein-Barr	Infetta cellule in divisione o meno con prevalenza per i linfociti B, alta capacità d'inserimento	Difficoltà di controllare le linee cellulari

Transgenesis induced with retroviral vectors. workflow



Advantages and disadvantages of transgenesis with viral vectors

Advantages:

high transfection efficiency

Problems:

Possitility to generate new viruses
May induce mutations
Small DNA can be inserted
Immunolgical complications
High costs

Yeast Artificial Chromosome – YAC

Possibility to insert large DNA sequences
Full artificial chromosomes,
origin or duplication, telomeres, etc

Yeast Artificial Chromosome - YAC

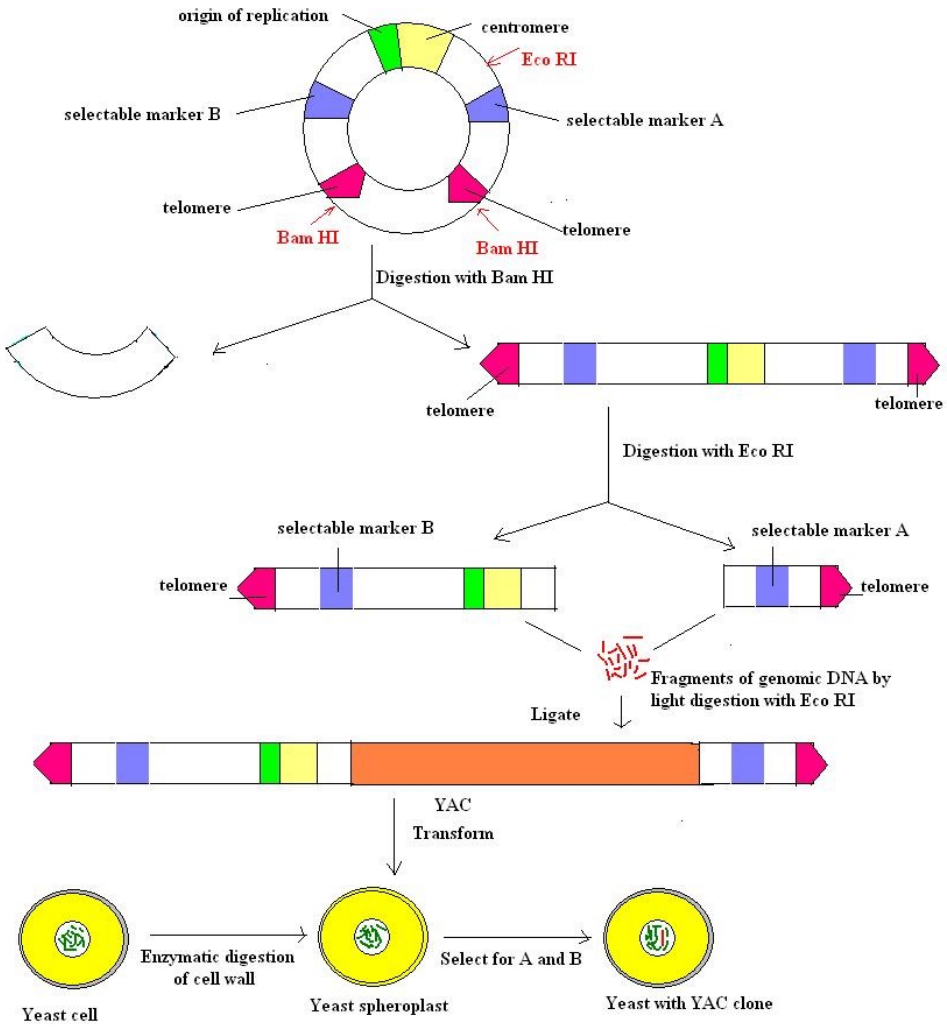
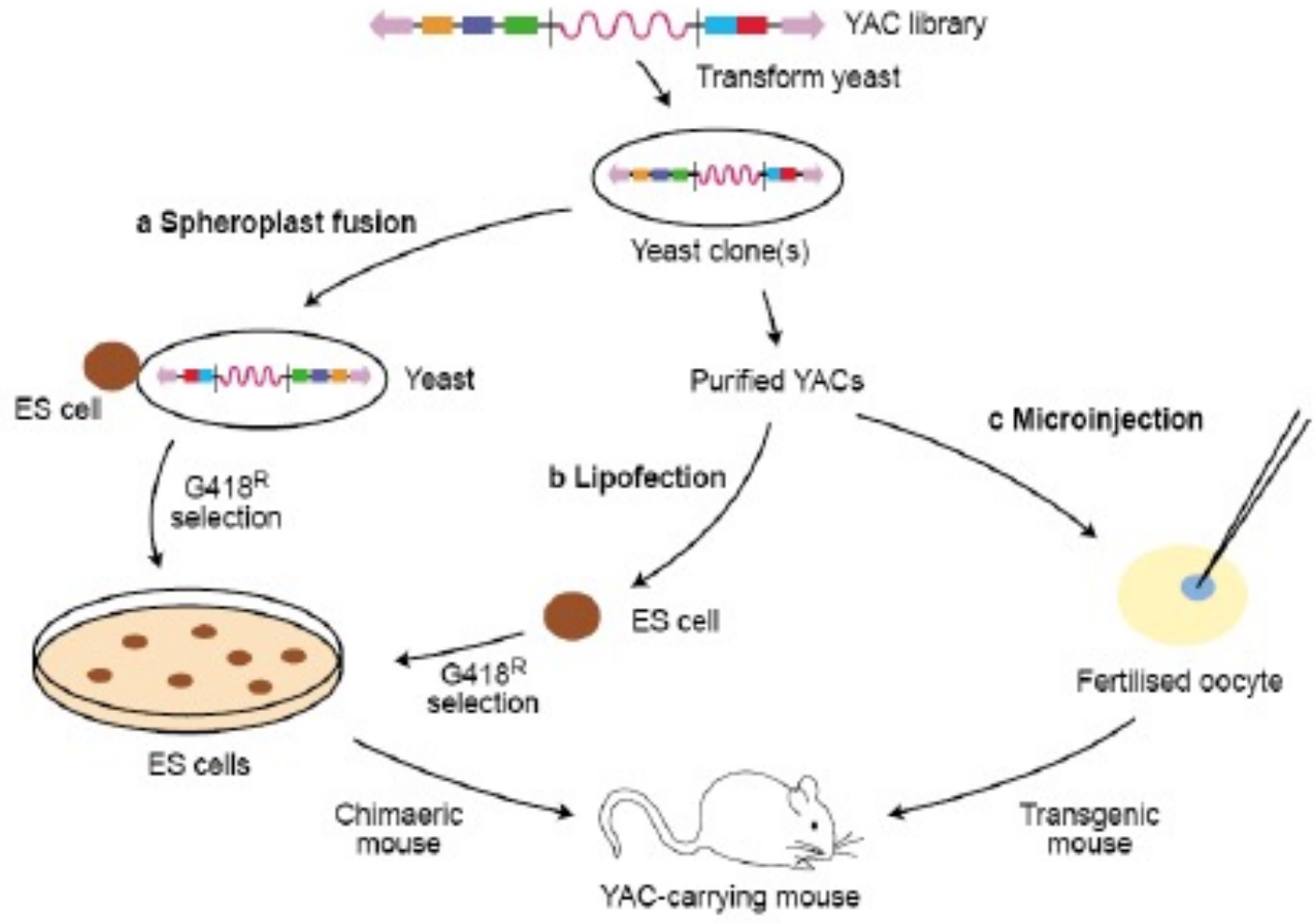


Figure: Construction of a yeast artificial chromosome (YAC)

Yeast Artificial Chromosome – YAC

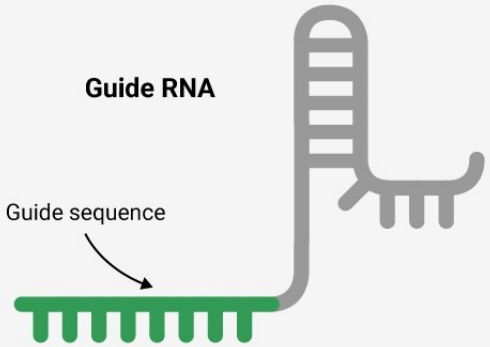
SCHEME -



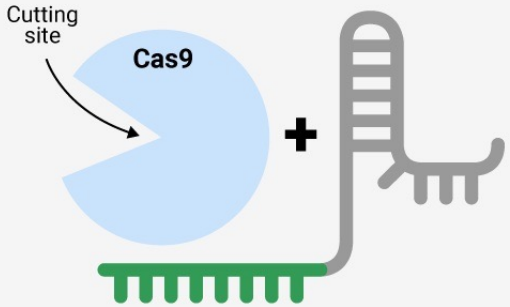
Generation of YAC-transqenic mice

EDITING A GENE USING THE CRISPR/CAS9 TECHNIQUE

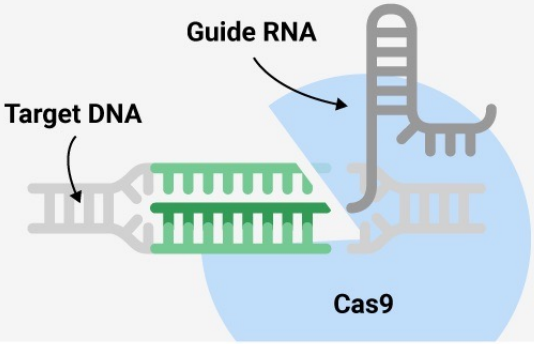
1 Scientists create a genetic sequence, called a "guide RNA," that matches the piece of DNA **they want to modify.**



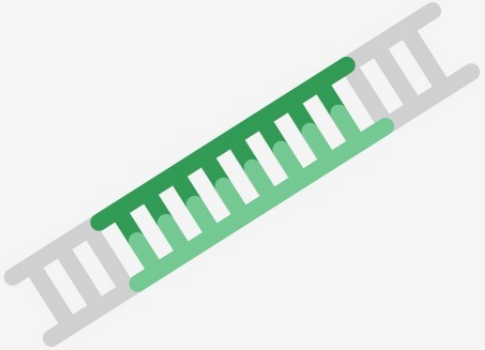
2 This sequence is added to a cell along with a protein called Cas9, which **acts like a pair of scissors** that cut DNA.



3 The guide RNA homes in on the target DNA sequence, and Cas9 **cuts it out.** Once their job is complete, the guide RNA and Cas9 leave the scene.



4 Now, another piece of DNA is swapped into the place of the old DNA, and **enzymes repair the cuts.** Voilà, you've edited the DNA!



SOURCES: Nature News; Carl Zimmer

BUSINESS INSIDER

Transgenesis in large animals - livestock Advantages

TABLE 1: Comparison of the different systems used to produce recombinant pharmaceutical proteins.

Production level	
Investment cost	
Production cost	
Scaling-up ability	
Collection	
Purification	
Posttranslational modifications	
Glycosylation	
Stability of product	
Contaminant pathogens	
Products on the market	

Table adapted from [1].

Transgenesis in large animals - livestock
Advantages

Table 2: Comparative estimated production cost between cell culture and transgenics.

Production scale (Kg/year)	System	Cost (dollars/gram product)
50	Cell culture	147
	Transgenics	20
100	Cell culture	48
	Transgenics	6

Table adapted from [7].

Transgenesis in large animals - livestock Application

Milk:
Production increase
Production of biological action peptides
Compound elimination (-lactose)

Blood:
Peptides
drugs
Disease resistance

Hormones:
Pituitary release factors (gnrh)
Neuropeptidi

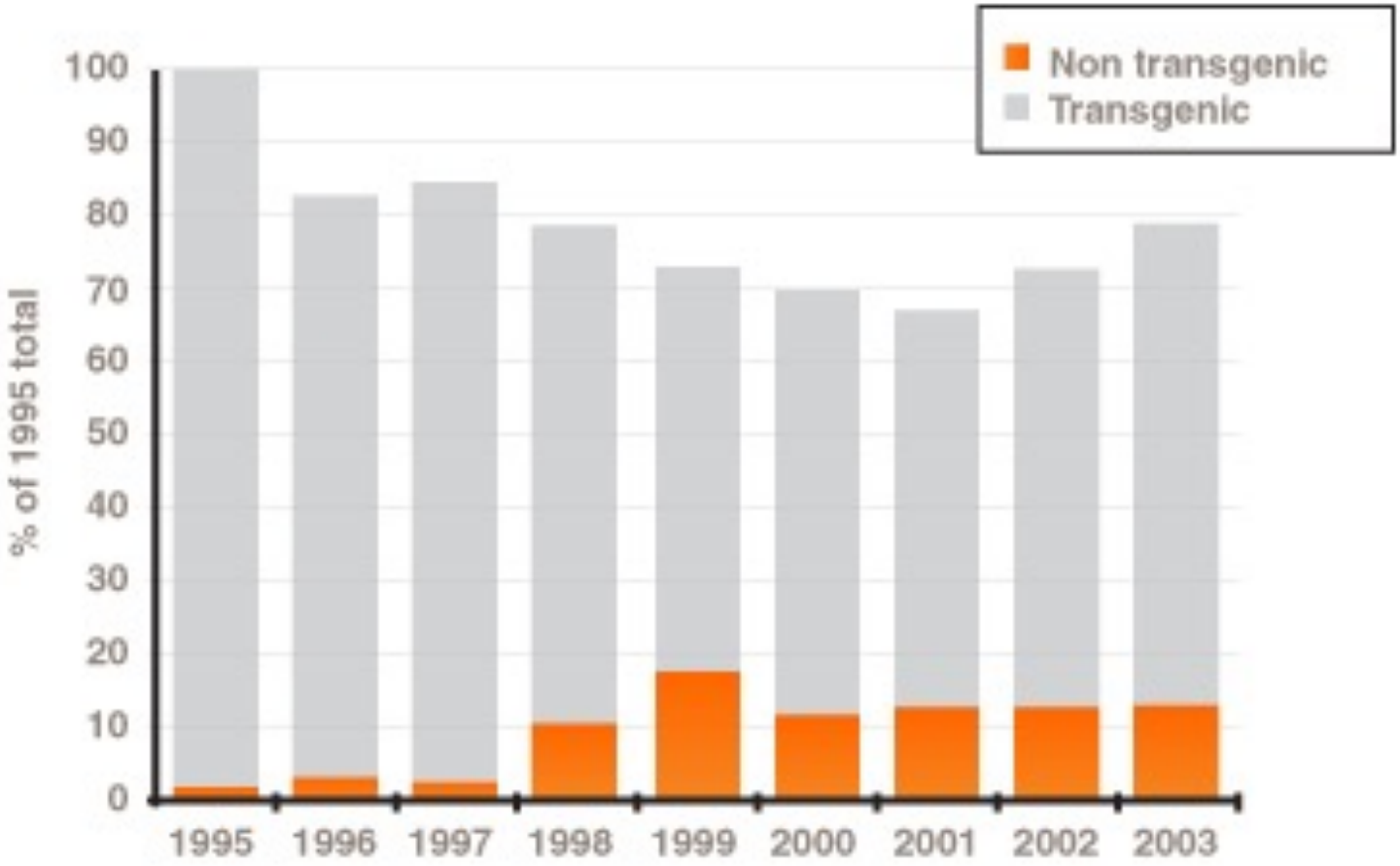
Improvement Productions of:
Meat
Wool,
leather

Transgenesis in large animals - livestock
Methods

Pronuclear injection
Retrovirus injection
Sperm transfection
Genetic modification of spermatogonia and transplantation into the testicle
Nuclear transplantation of genetically modified cells

Embryonic stem cells ideal tool for transgenic:
not isolated in the these species

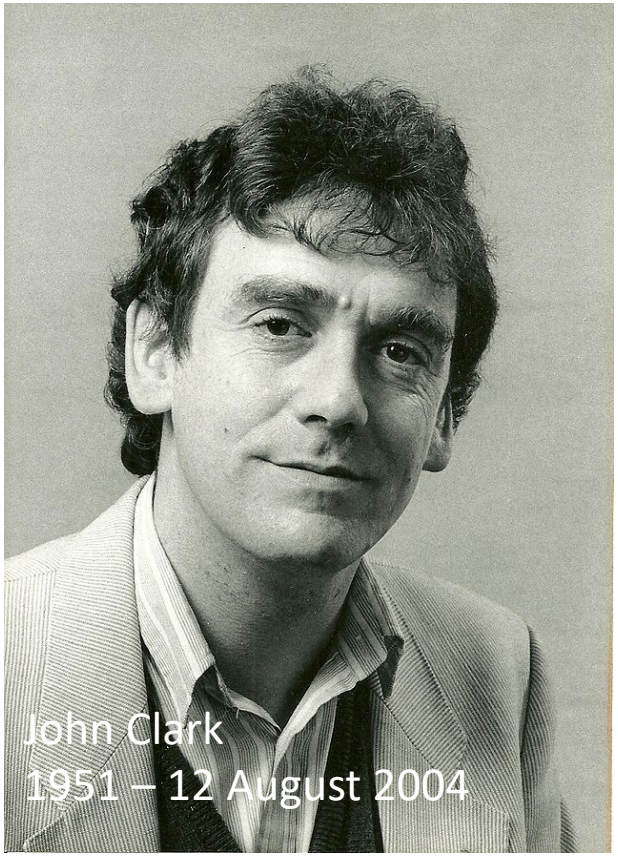
Number of continuously growing transgenic animals
USA, Asia, South Korea, Japan, Europe



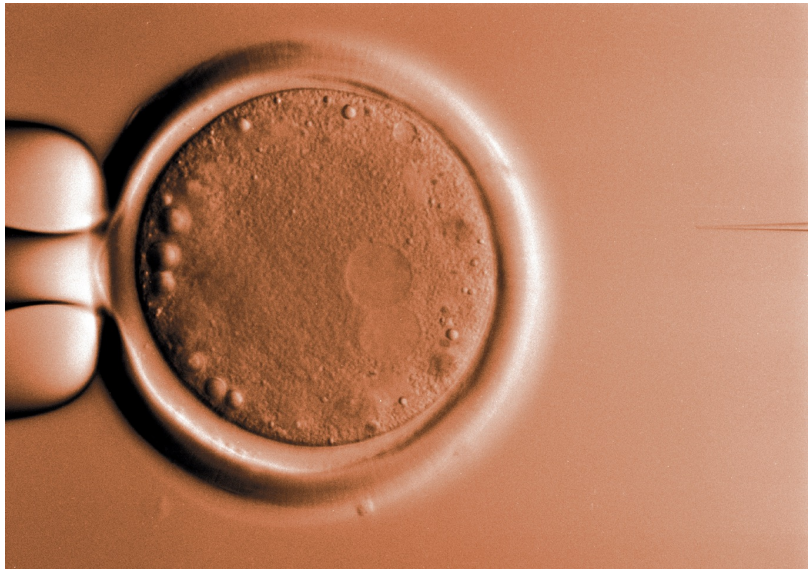
Pioneer: Helen Sang, first transgenic chicken by Nuclear Injection

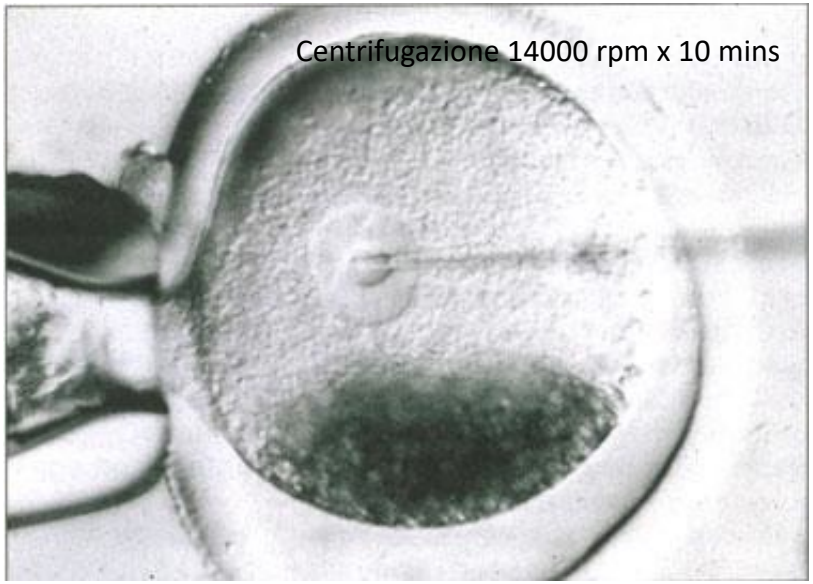
Animals as bioreactors: history

Tracy - first transgenic sheep produced by John Clark, Roslin Institute (produced 35 g alpha-1-antitrypsin per liter of milk 1991)



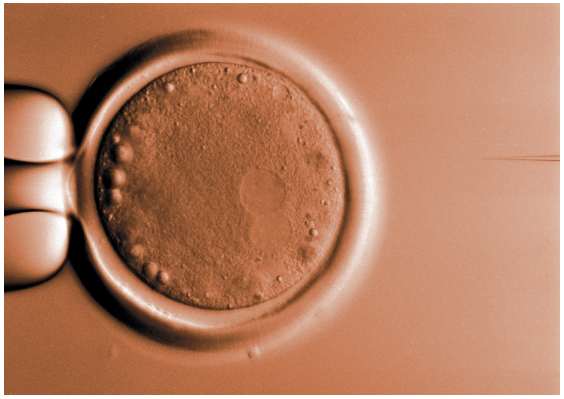
Pronuclear injection in sheep's zygote

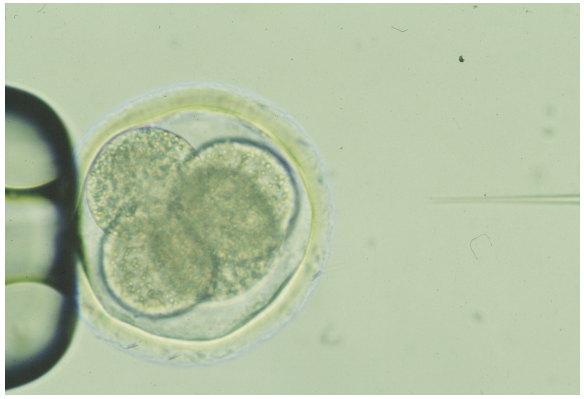




Main types of optics for micromanipulation

"Nomarski" interference contrast, DIC (Differential Contrast)
Indispensable glass petri dishes!





"Hoffman" interference contrast
The most used, plastic petri dishes

Bioreactors: purification of the peptide expressed in the milk of transgenic animals

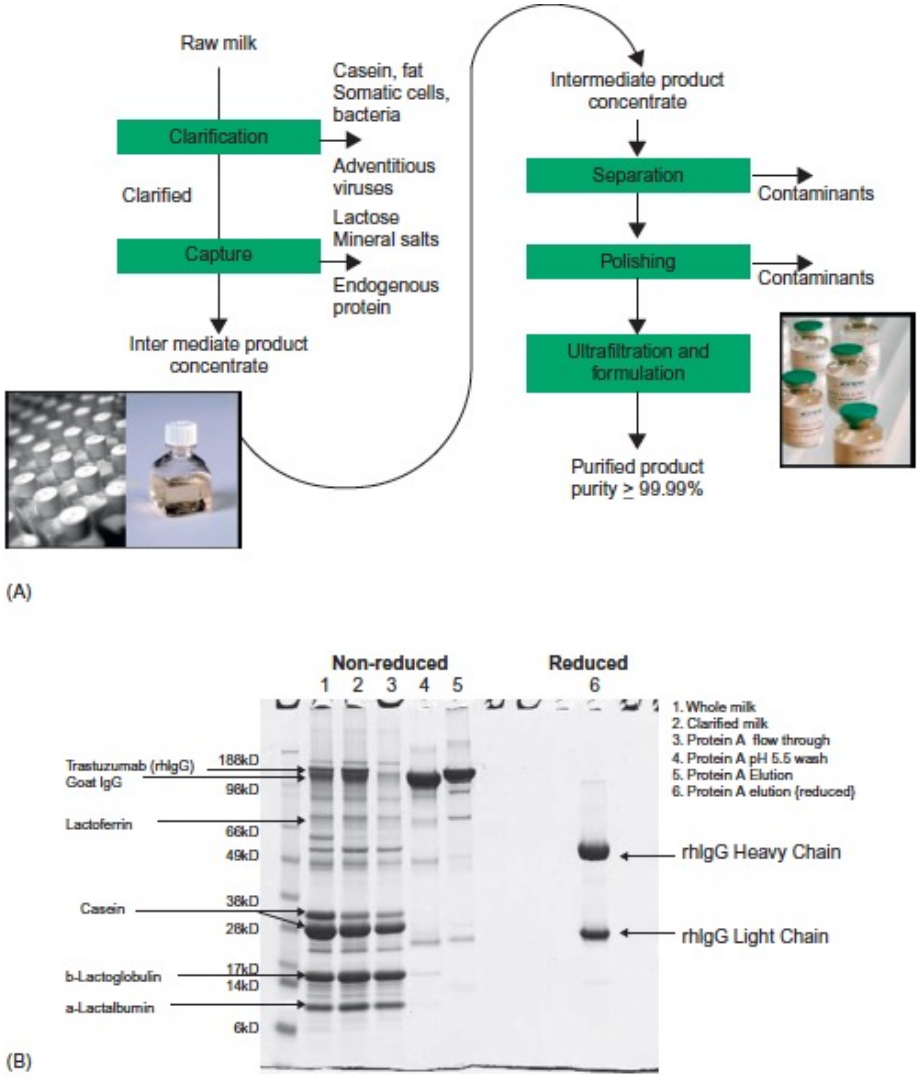
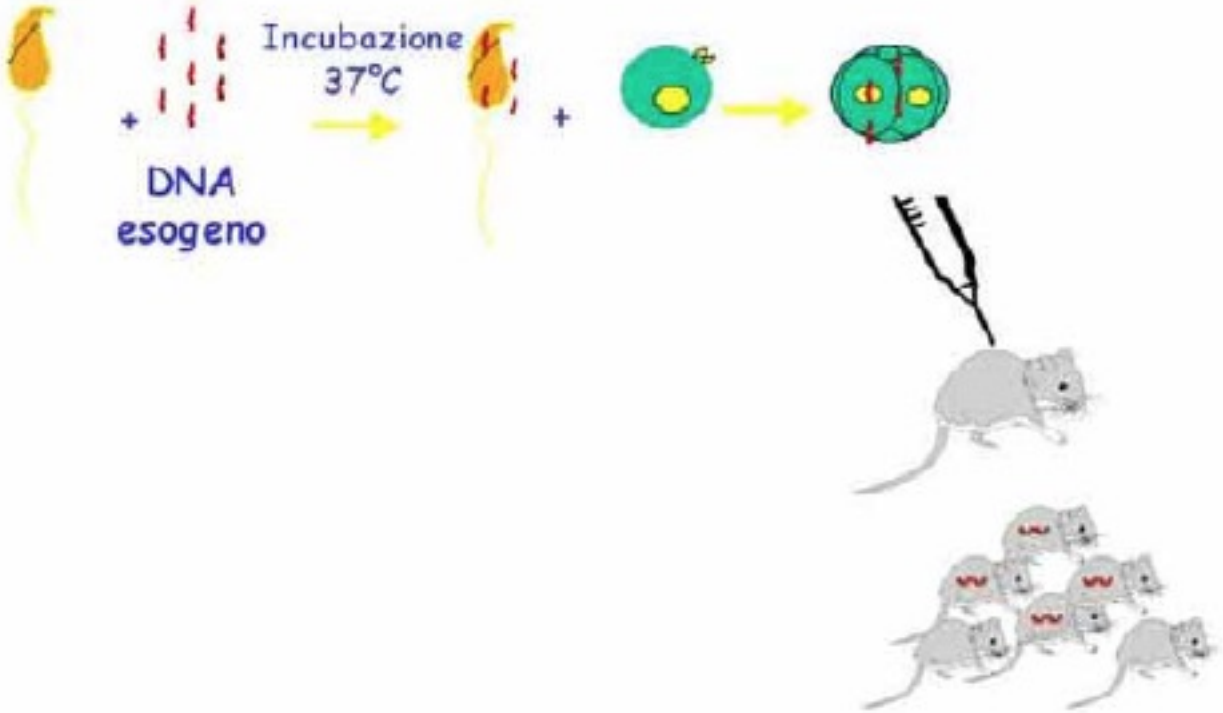


FIGURE 26.5 (A) Schematic representation of a typical purification process for the isolation of recombinant therapeutic proteins from the milk of transgenic goats: the clarification step can be accomplished by using techniques such as tangential flow filtration, depth filtration, or centrifugation. The capture, separation, and polishing steps are accomplished using standard chromatographic techniques. (B) Western blot of an antibody (trastuzumab) expressed in the milk of transgenic goats following the first purification column after clarification.

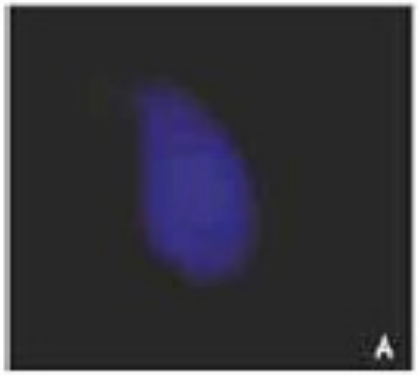
Transgenesi mediata da spermatozoi

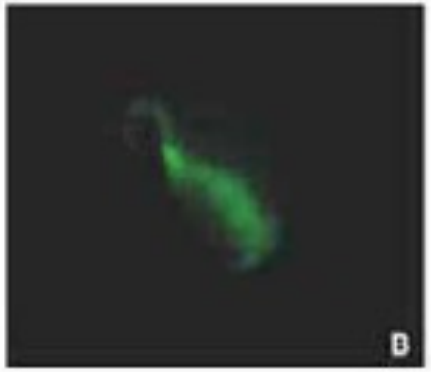


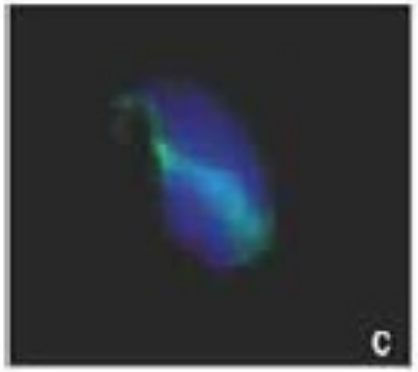
Spermatozoa "take" exogenous DNA

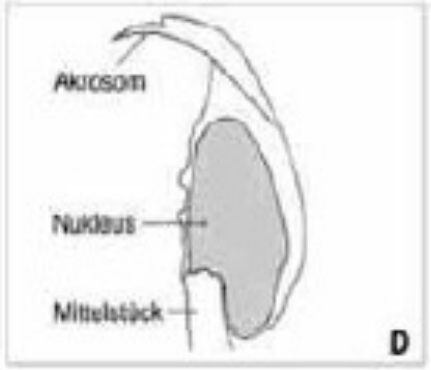
DAPI

FISH



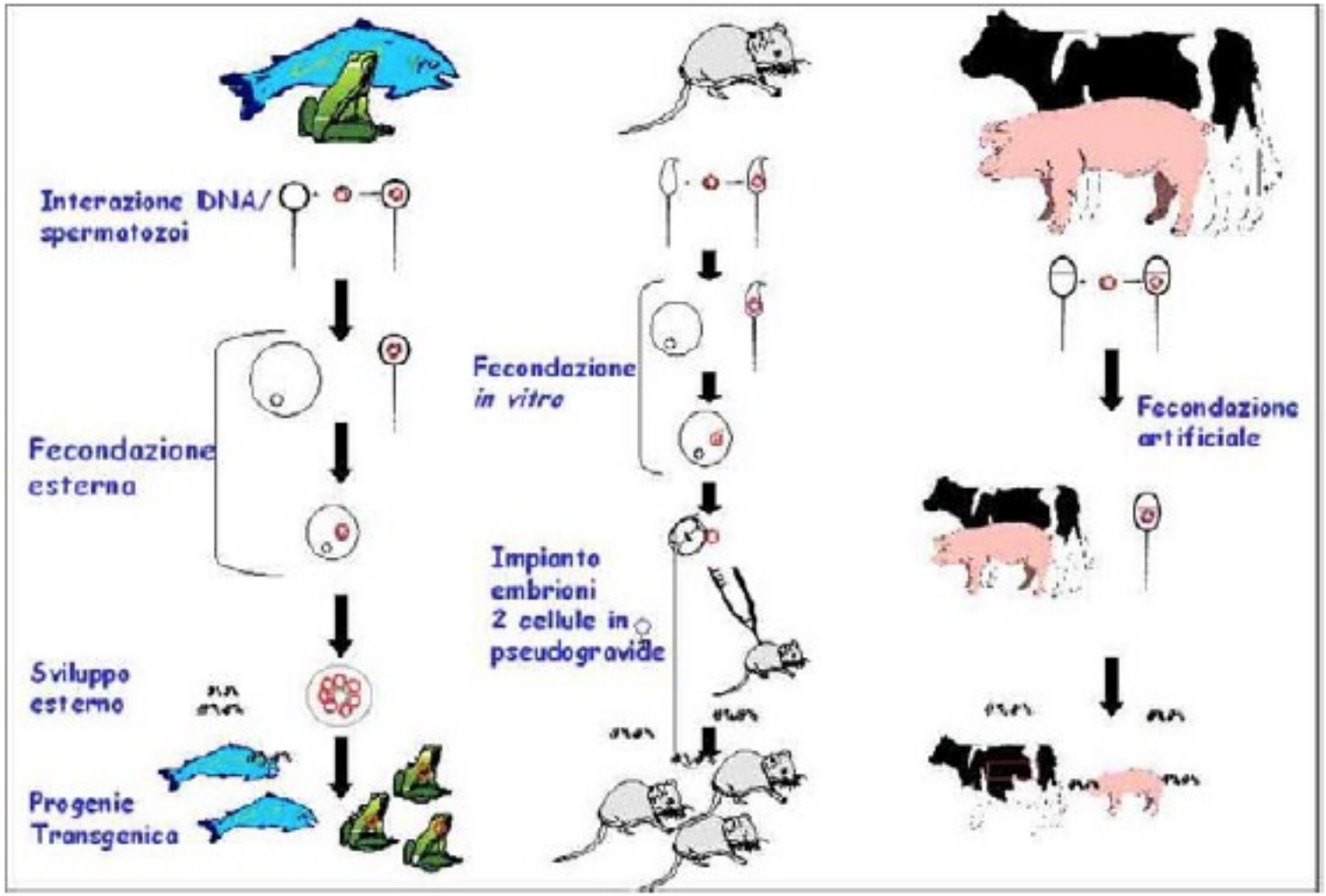




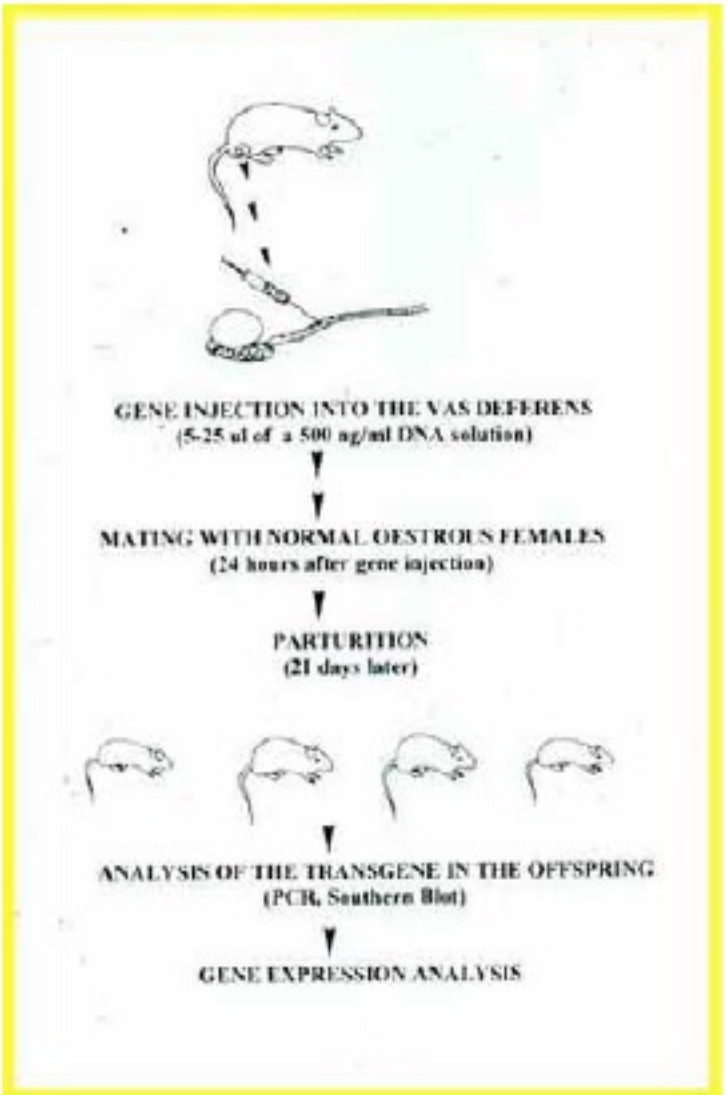


Merge

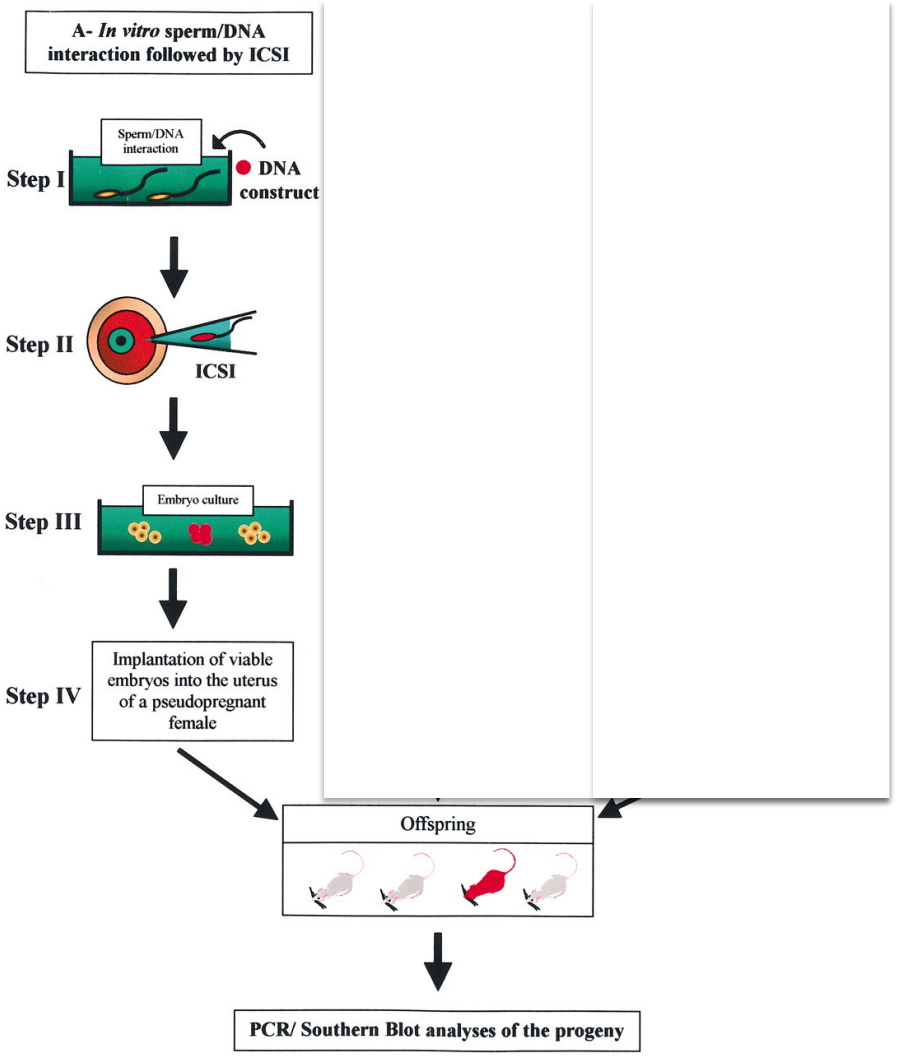
Transgenesi mediata da spermatozoi



Testis-mediated gene transfer



The three approaches to the production of transgenic animals modifying the male gametes



Celebi C et al. Biol Reprod 2003;68:1477-1483

The three approaches to the production of transgenic animals modifying the male gametes

Exposure of spermatozoa ejaculated with the DNA construct
(sometimes with pre-treatment - thermal shock - permeabilization - Electroporation - to facilitate DNA uptake)

Injection of the DNA construct by the endotesticular way (deferent-secret testis)

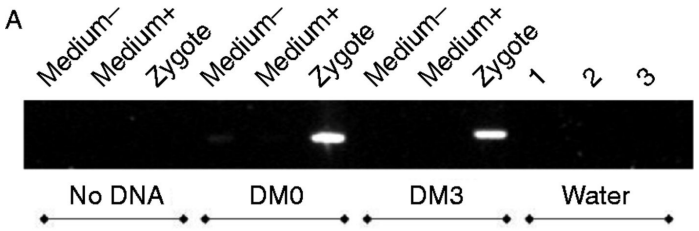
Intra-testicular injection of genetically modified germ cells
(it is necessary to eliminate the germ cells "resident" before)

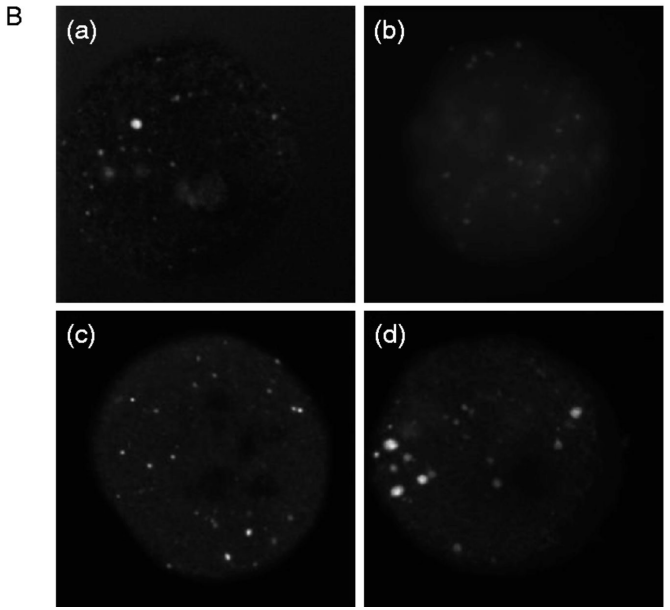
Application in the mouse; method still in experimental phase
So far low efficiency

Sperm – mediated transgensis controversial ……

Exposure to DNA is insufficient for *in vitro* transgenesis of live bovine sperm and embryos

Shahin Eghbalsaied[1,3], Kamran Ghaedi[2], Götz Laible[3], Sayed Morteza Hosseini[4], Mohsen Forouzanfar[5], Mehdi Hajian[4], Fleur Oback[3], Mohammad H Nasr-Esfahani[4] and Björn Oback[3]





Eghbalsaied S et al. Reproduction 2013;145:97-108

Gene Transferred	Objective	Achievements	Remarks
Mice and Swine			
Human hemoglobin and specific circulating immuno-globulins (antibodies)	Proteins from blood serum for blood transfusion and disease diagnosis	Genes expressed; proteins released in blood serum	Experimental stages
Rabbits			
Human genes: Interleukin 2, growth hormone, tissue plasminogen activator, α_1–antitrypsin, etc. Bovine gene: α-lactoglobulin	Molecular farming or gene farming	Genes expressed in mammary tissue; proteins harvested from milk	Experimental stage
Sheep			
Bacterial genes cys E and cys M (genes concerned with cysteine biosynthesis	Improved wool production/ quality	Genes achievement in transgenic animals	
Human growth hormone	Increased and desirable body growth	Improvements in body weight gain, feed efficiency. Fat composition, etc.	Produces joint pathology, skeletal defects, ulcers infertility, etc.
Cattle, Goat, Sheep and Swine			
Human genes α_1 antitrypsin, tissue plasminogen activator, blood clotting factor IX, and protein C.	Gene farming	Genes expressed in mammary tissue; protein secreted in milk in functional form	Experimental stages
Fish			
Salmon or rainbow trout growth hormone	Increased body growth	Upto 60% increase in size	Transgenes stably inherited; growth improve by selection.
Anti-freeze protein, α-globin gene, chicken δ-crystalline protein gene, etc.	Variable	Genes expressed in transgenic individuals	Transgenes are stably inherited.

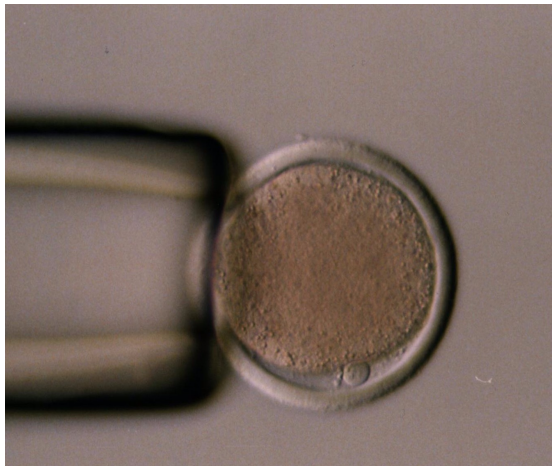
Increase the amount of milk with transgenesis (pronuclear injection)

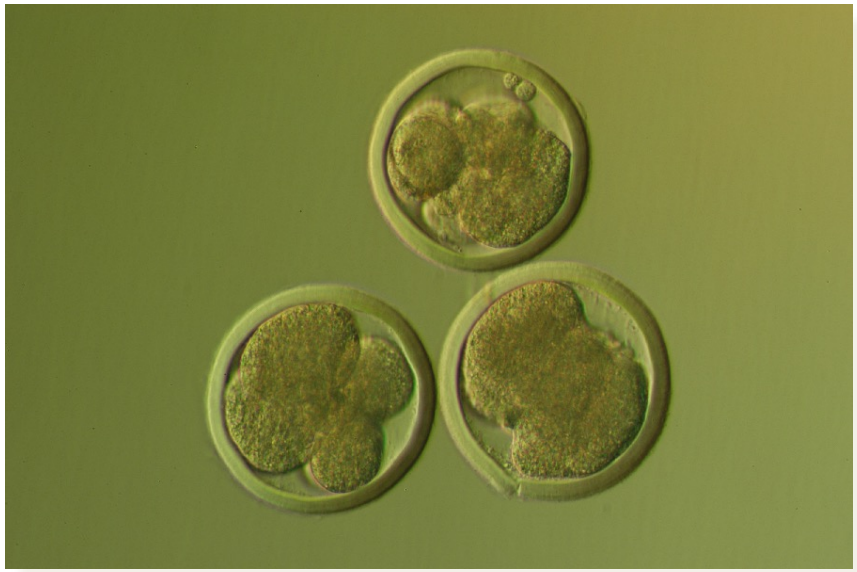
Figure 1: A litter of α-LA transgenic piglets.
Transgenic sows produce up to 70% more milk than control non-transgenic litter mates. Piglets grow up to 500 gm more during a 21d lactation (Noble **et al**. 2002).

Increase the amount of milk with transgenesis (pronuclear injection)

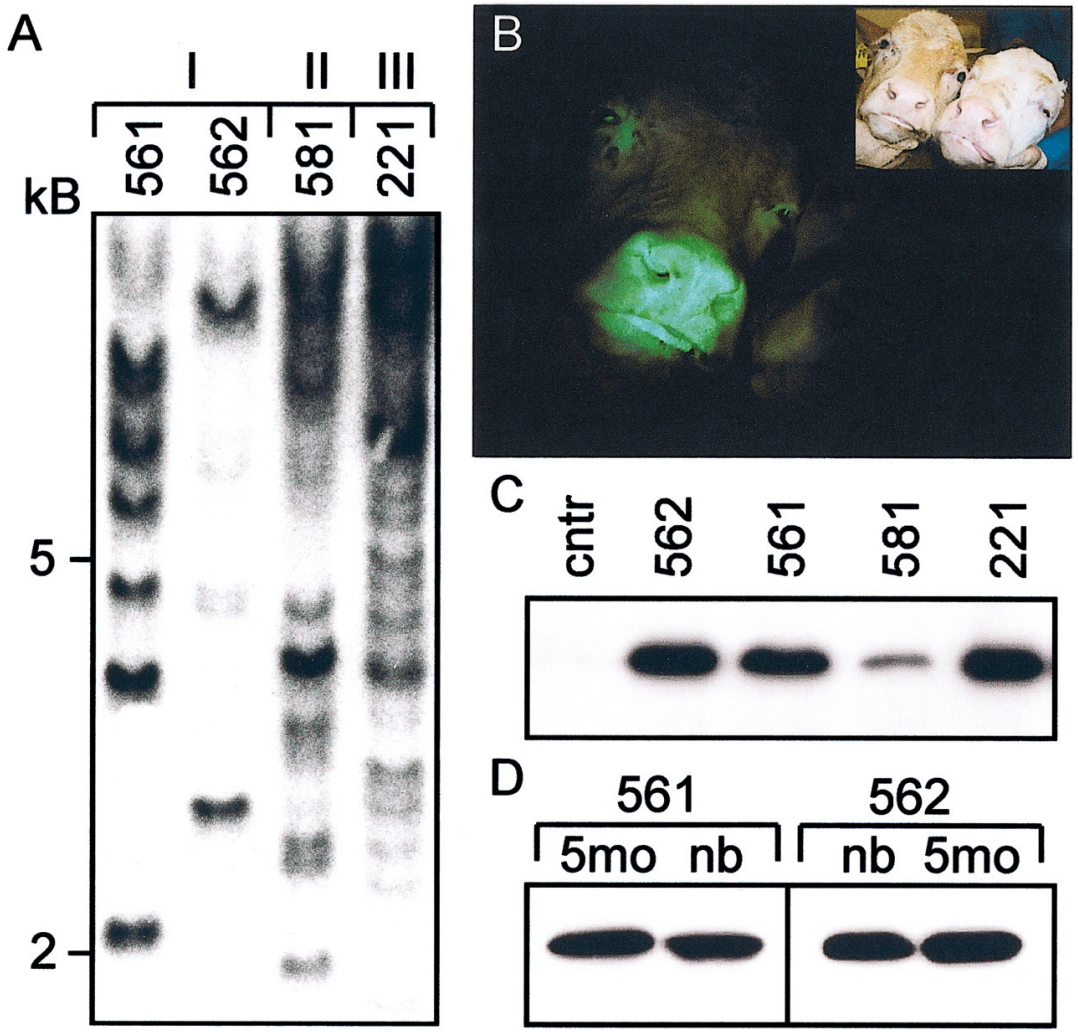
Figure 2: Can transgenic technology produce comparable milk volume?
Small improvements in milk volume in Guzerat cows (left) using genetic material from high-producing Holsteins (right) could have a significant impact on Brazilian beef production (Wheeler **et al**. 2010).

By simplifying transgenesis in farm animals:
Direct injection of lentiviral vectors into oocytes or embryo



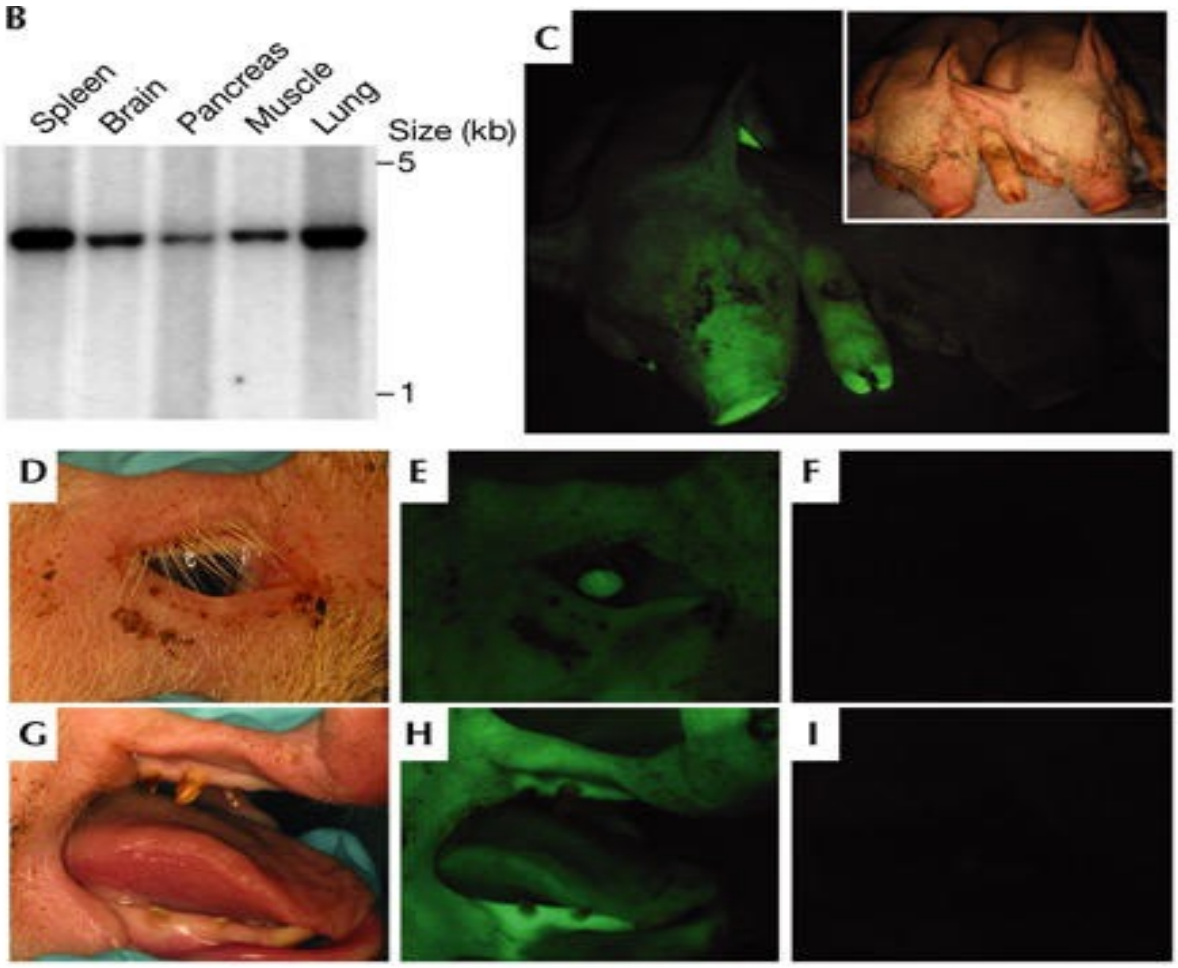


Generation of transgenic cattle (only GFP reporter gene)

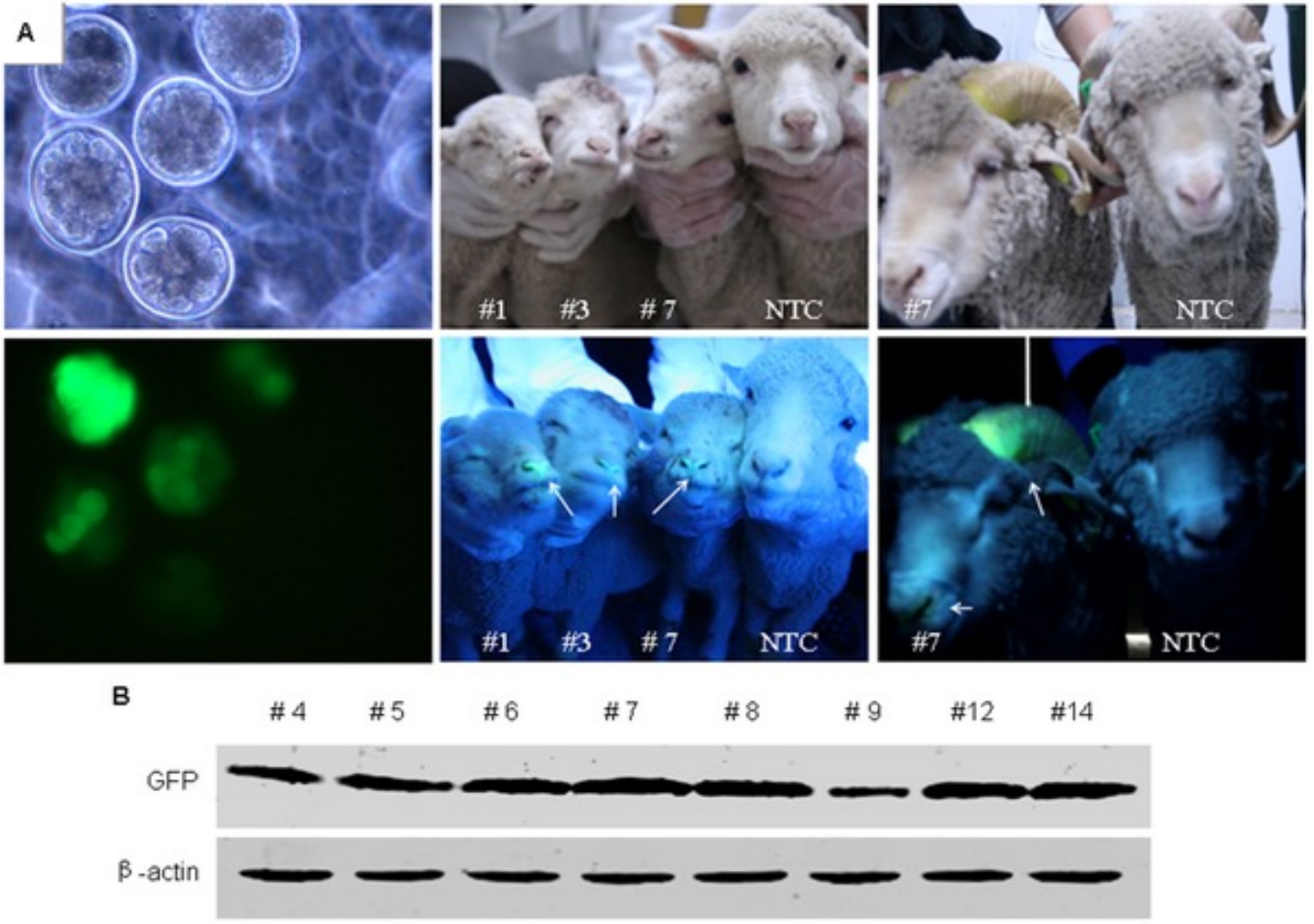


Hofmann A et al. Biol Reprod 2004;71:405-409

Generation of transgenic swine (only GFP reporter gene)



Generation of transgenic sheep (only GFP reporter gene)



Liu C, Wang L, Li W, Zhang X, et al. (2013) Highly Efficient Generation of Transgenic Sheep by Lentivirus Accompanying the Alteration of Methylation Status. PLoS ONE 8(1): e54614. doi:10.1371/journal.pone.0054614
http://www.plosone.org/article/info:doi/10.1371/journal.pone.0054614

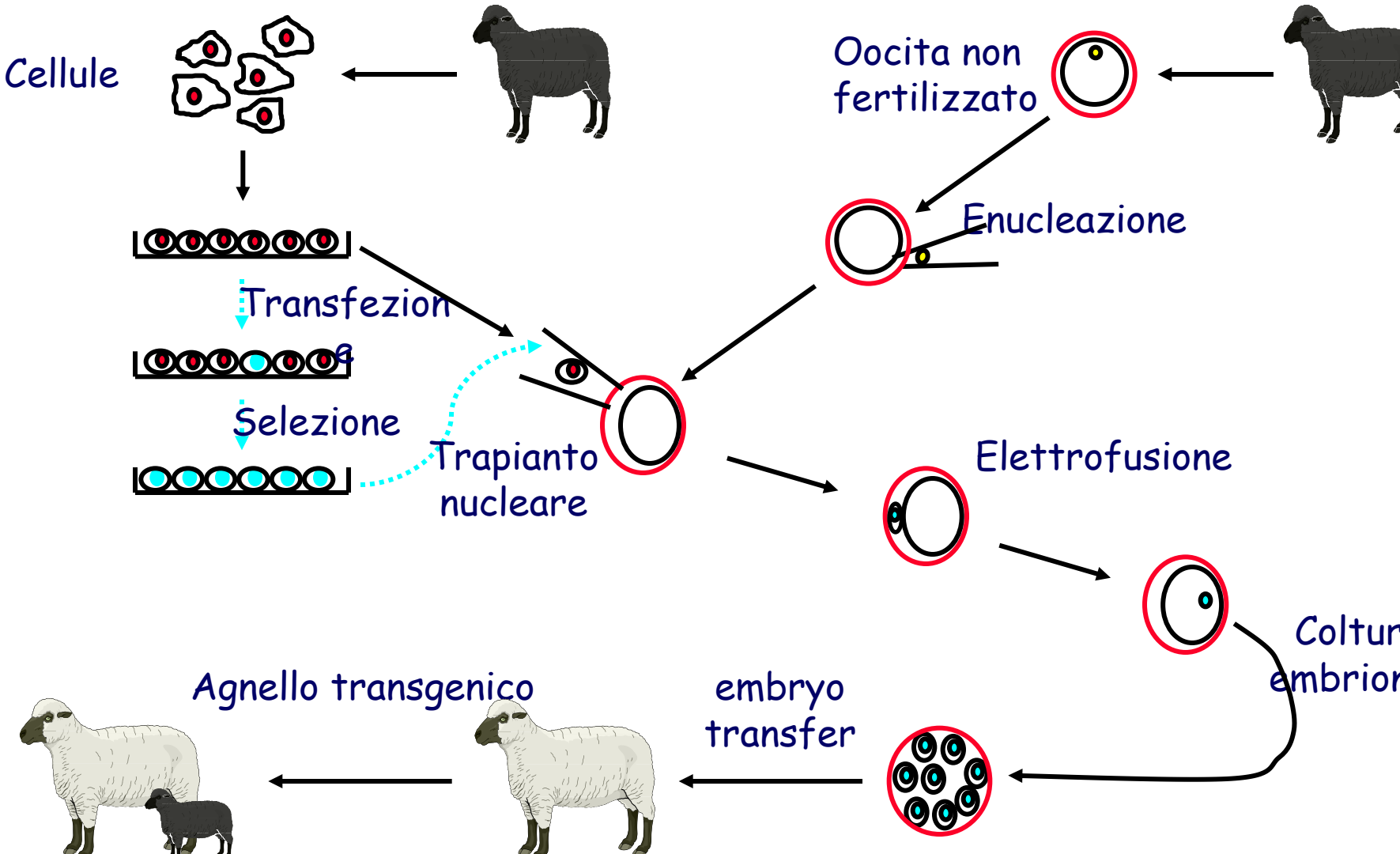
PLOS | ONE

Human proteins produced by transgenic animals obtained with pronuclear injection:

- Pigs: Factor VIII
- Sheep: Factor IX; human AAT (clinical trial phase III)
- cattle: Lactoferrin (anti-inflammatory / Immunomodulatory)
- goats: Anti-thrombin IIIa (clinical trial phase III)

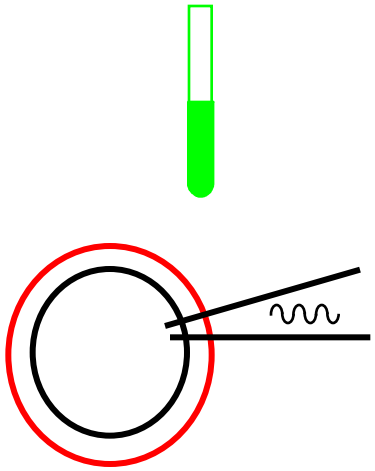
- Most reliable transgenic production technique at the moment
- Advantages:
- Large quantities (of the order g / L; ex. Factor Requirement IX = 2Kg / year)
- Production of any type of protein

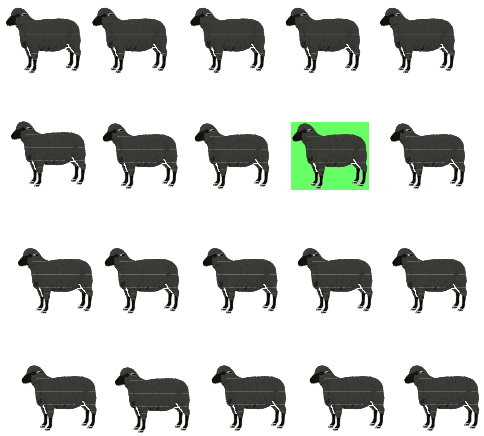
Transgenesis with nuclear transplantation (cloning) with somatic cells



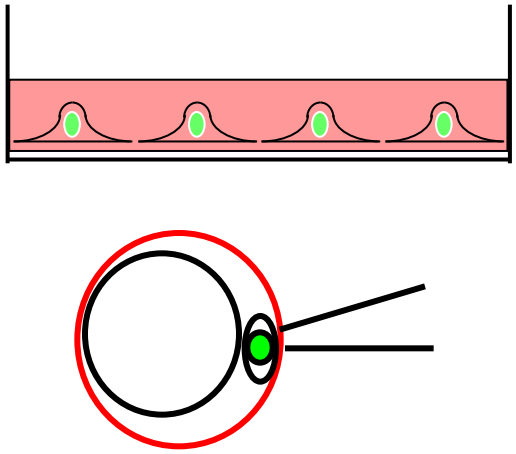
Nuclear Transfer Vs Microinjection

Microinjection





Nuclear trnasfer

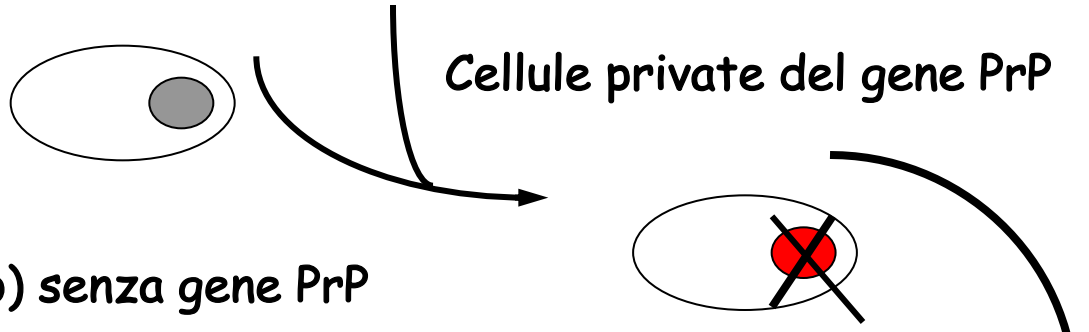


Cupid and Diana - transgenic products (nuclear transplant) with homologous recombination
They express the Alpha Anti-trypsin 1 (cystic fibrosis therapy).
Campbell, Nature 2003

Elimination of endogenous genes (Prion)

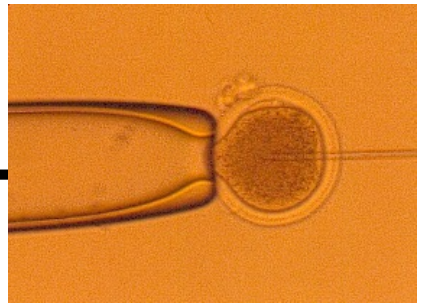
Cellule fetali → Cellule in coltura

Eliminazione del gene



Cellule private del gene PrP

Clonazione



Primo agnello (maschio) senza gene PrP (Denning, 2001)

FIGURE 27.1 ***PNRP* homozygous knockout cattle produced by BioDak, LLC.** This cohort of genetically engineered, cloned Holstein bull calves come from a single genetically modified cell line containing a functional knockout of both alleles of the *prion protein* gene locus.

FIGURE 27.2 Genetically engineered, cloned, crossbred dairy heifers produced from a single genetically modified cell line containing a human artificial chromosome (HAC) and functional knockouts of the four alleles of the bovine immunoglobulin-μ heavy chain loci. These cattle are known as transchromosomic (Tc) bovine and the HAC they possess contains the entire repertoire of the human antibody genes. After hyperimmunization with a target antigen, these animals undergo plasmapheresis to collect large volumes of plasma containing targeted polyclonal human antibodies. The antibodies are purified from the plasma to produce human antibody therapeutics.

Transgenic pig for "humanization" of organs pigs for xenotransplantation

- Lai et al & Dai et al., 2002 reported disruption of one allele of GGTA1

MEDICAL REVOLUTION THE ORGANS THAT COULD HELP

Trials of pig tissue transplanted into humans to treat diabetes, Parkinson's disease and blindness are "imminent" but solid organ transplants - hearts, kidneys, livers - are still "several years away".

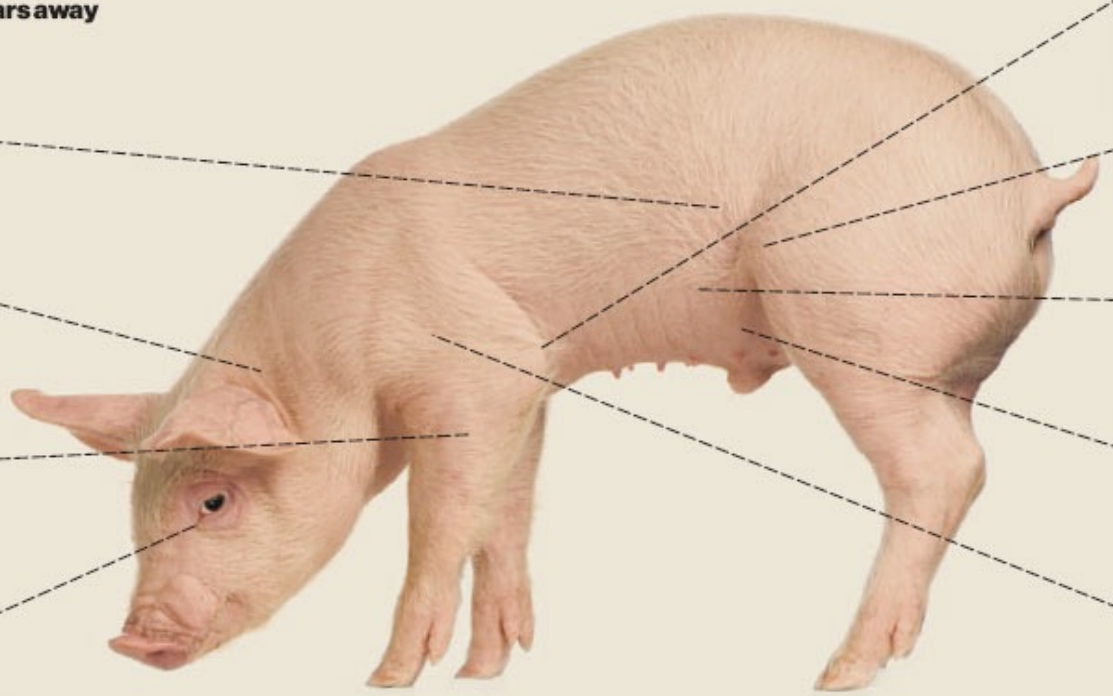
KEY **Imminent** **Years away**

Pancreatic islets to treat diabetes

Brain cells to treat Parkinson's and Huntington's disease

Red blood cells for transfusion

Eye tissues corneas etc.



Heart

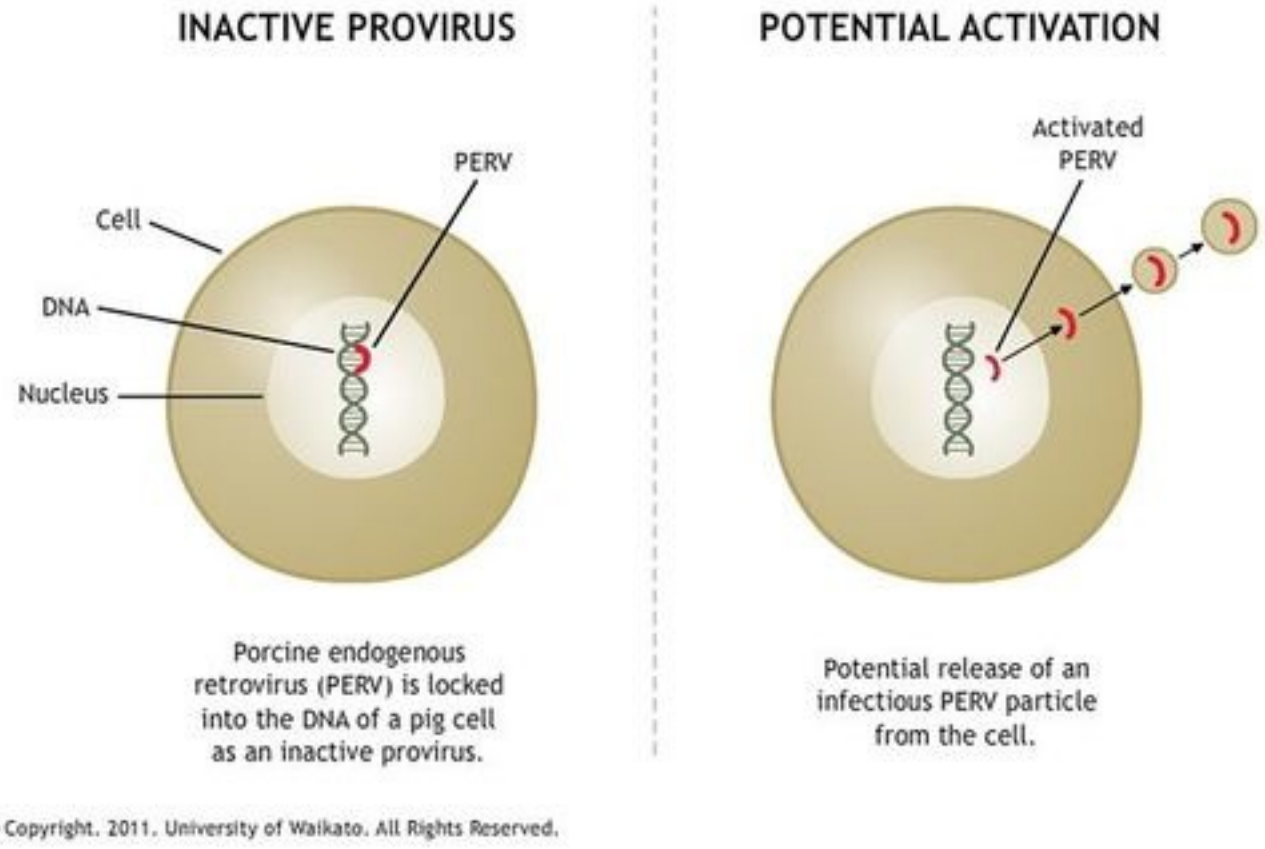
Kidney

Liver

Small bowel

Lungs

Xenograft risks:
Activation and passage of retroviruses (pigs) to humans



Eduardo Kac: "Edunia", a genetically engineered flower that is a hybrid of myself and Petunia. The Edunia expresses my DNA exclusively in its red veins.
Weisman Art Museum, Minneapolis, USA