Come si studia il ruolo di una proteina?

Approcci positivi o negativi:
- trasfezioni stabili e transienti
- siRNA
- animali transgenici
- animali knock-out
- animali knock-in
- tecnica cre-lox per knock-out condizionali

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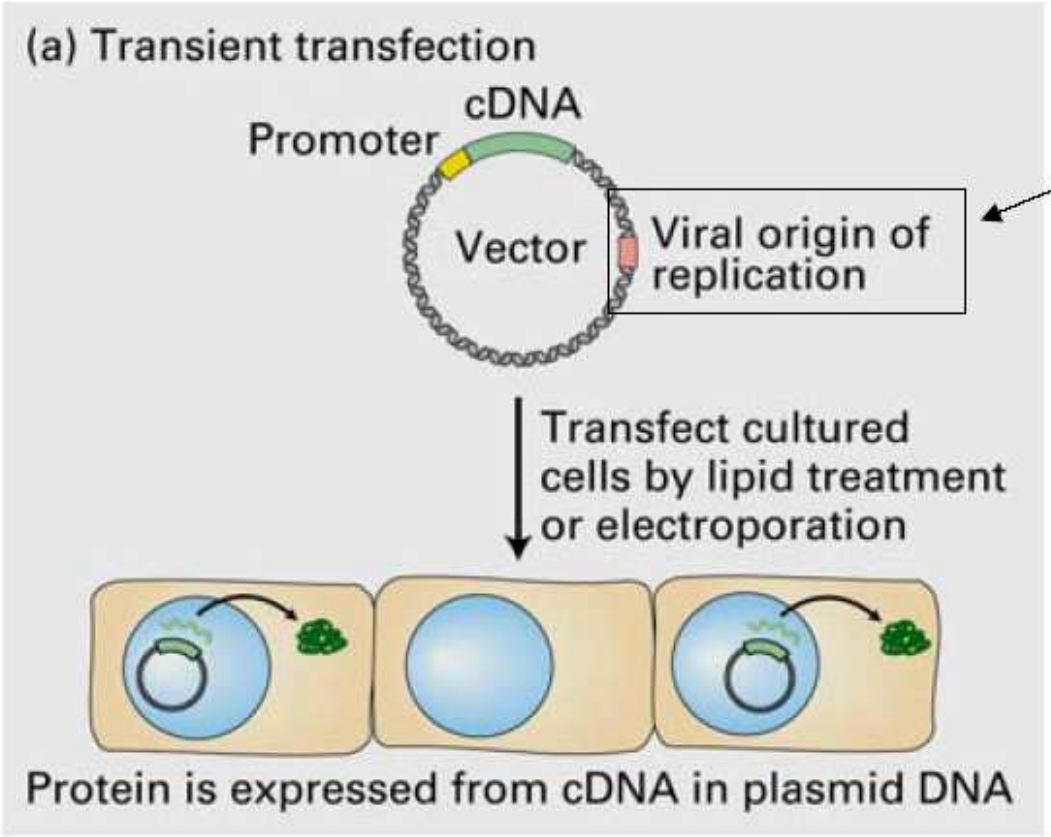
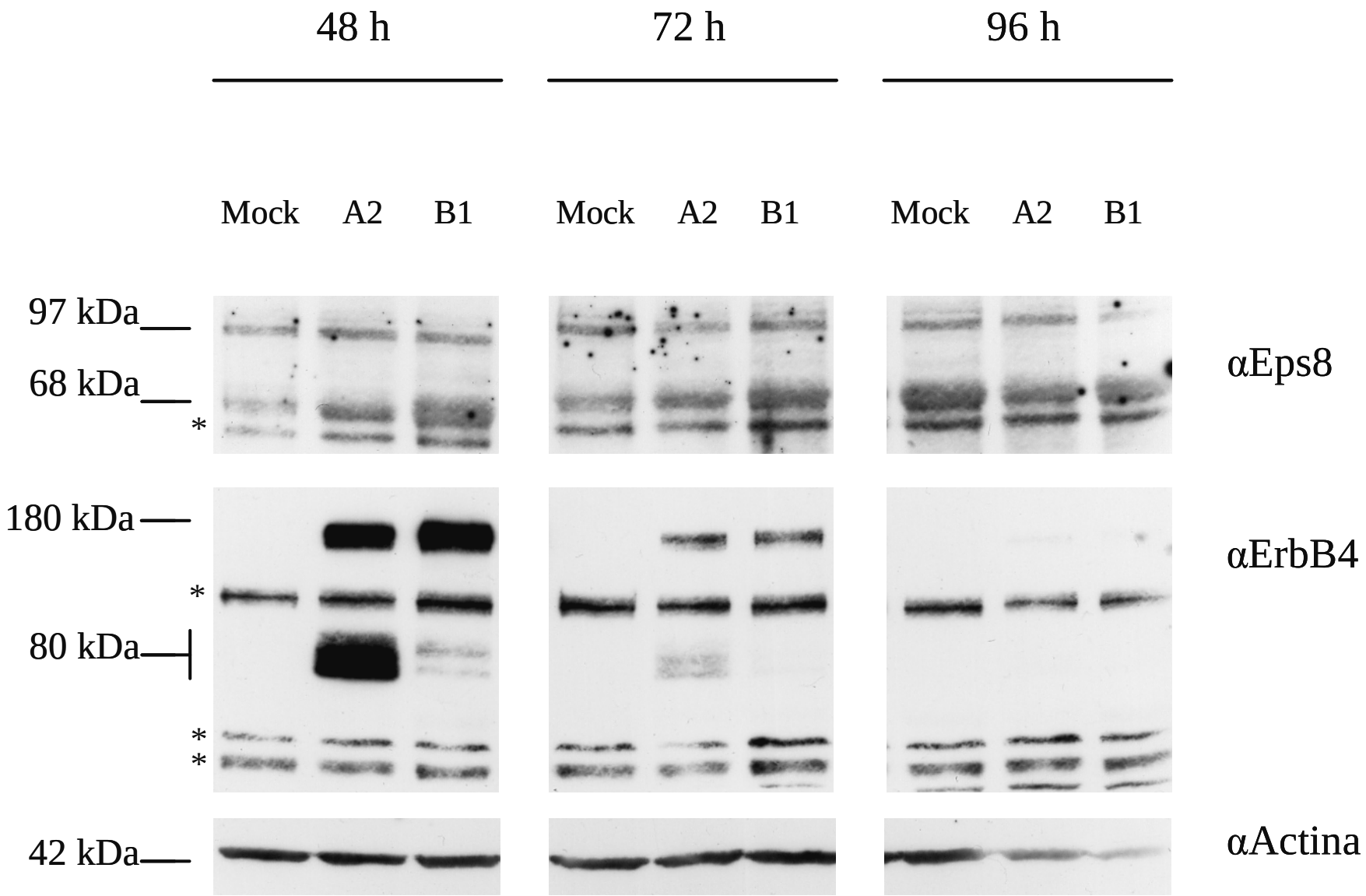
TRANSIENT TRANSFECTION



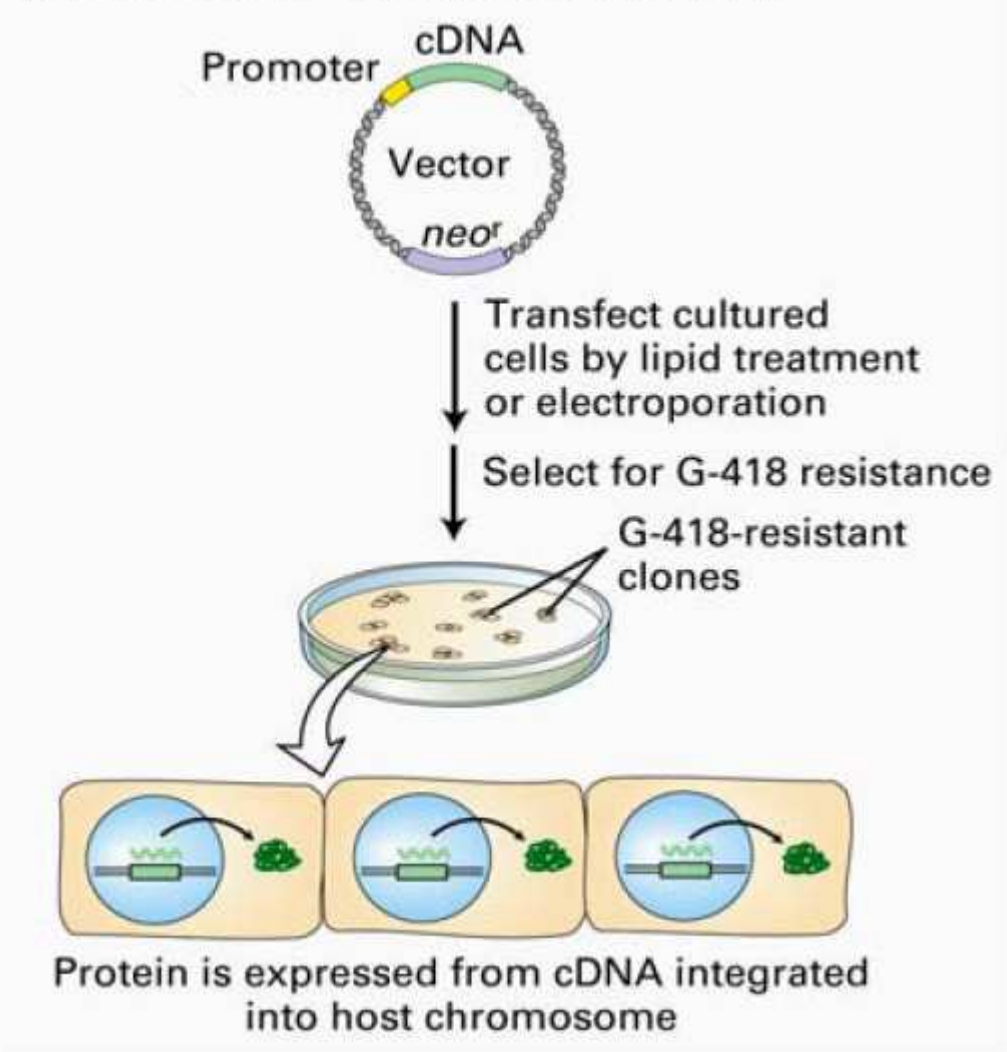
This allows replication if cells are competent for viral replication.
E.g. if SV40 origin, *cos* cells can be used, which express the LT viral antigen, needed for viral replication.

Trasfezione transiente: quale proteina è stata trasfettata?



(Rua et al., 2008)

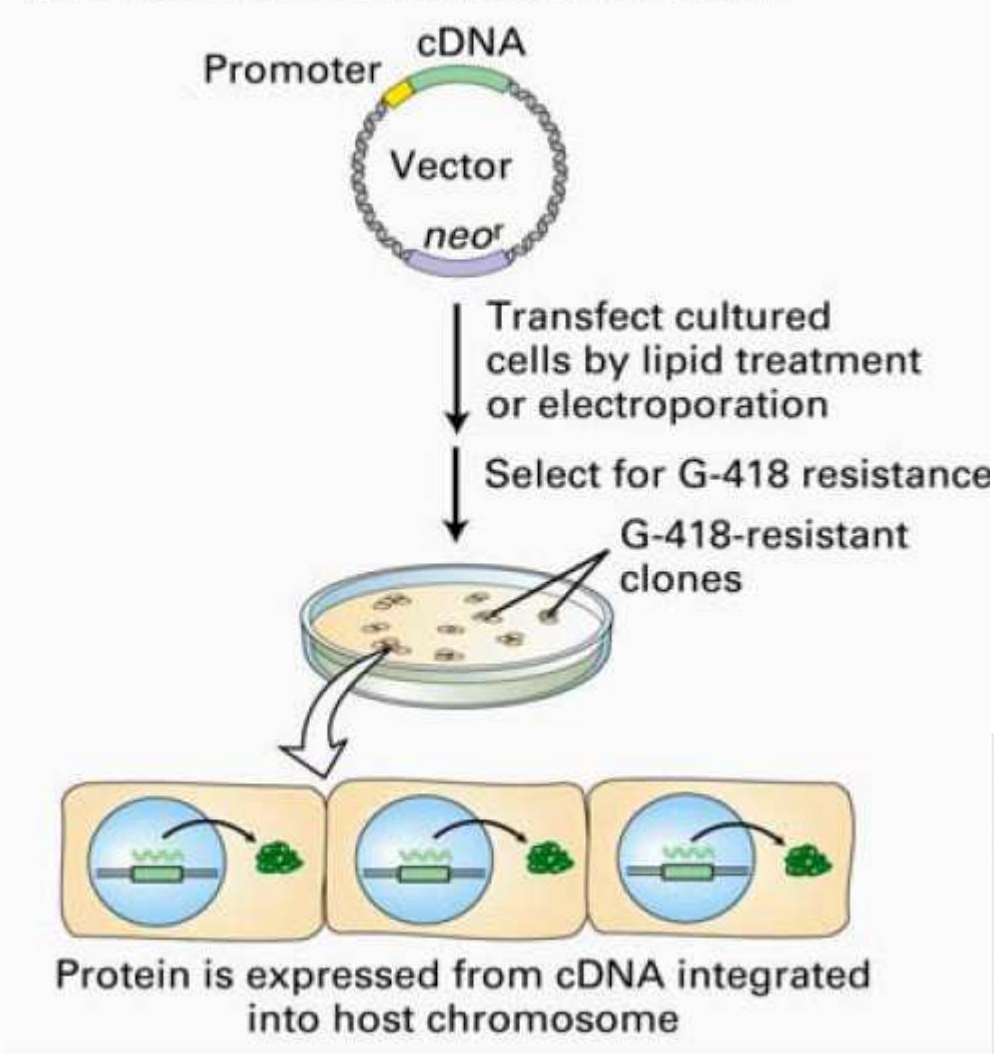
TRASFEZIONE STABILE

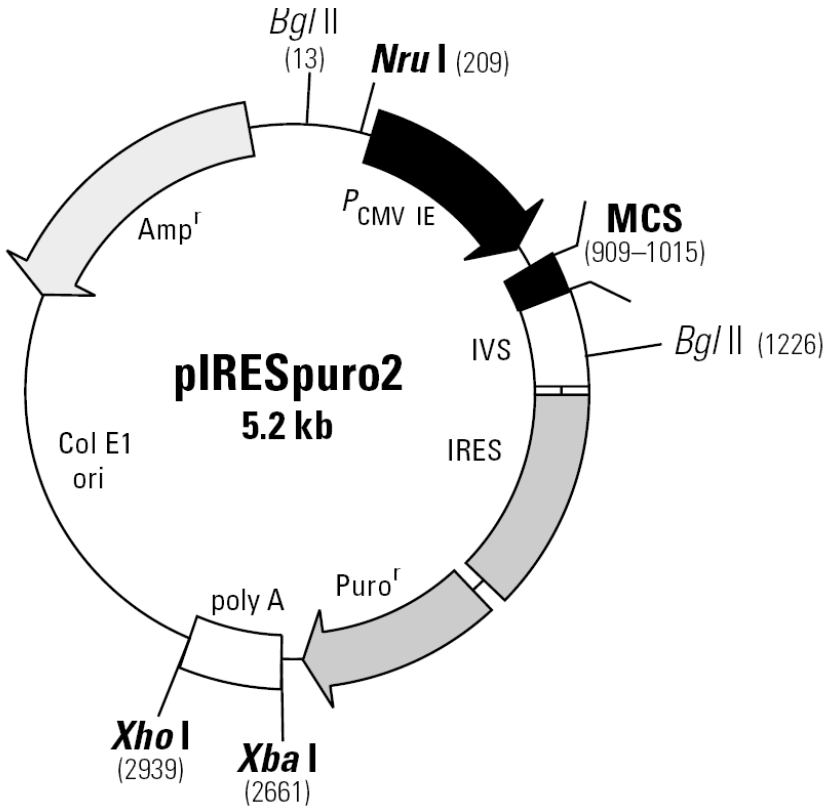


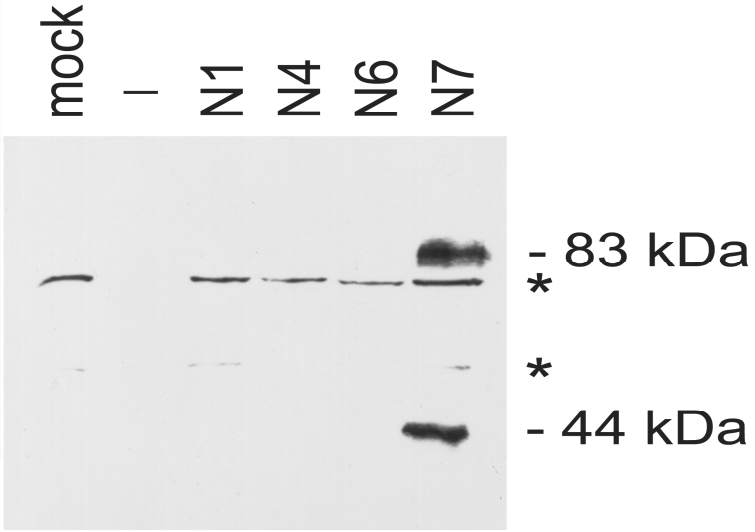
For stable transfection, a selection marker is needed (e.g. the *neo* resistance gene or *tk* etc.)

The selection marker gene can be either in the same vector as YFG or in a different vector: animal cells co-integrate exogenous DNA, most commonly at the same locus.

TRASFEZIONE STABILE



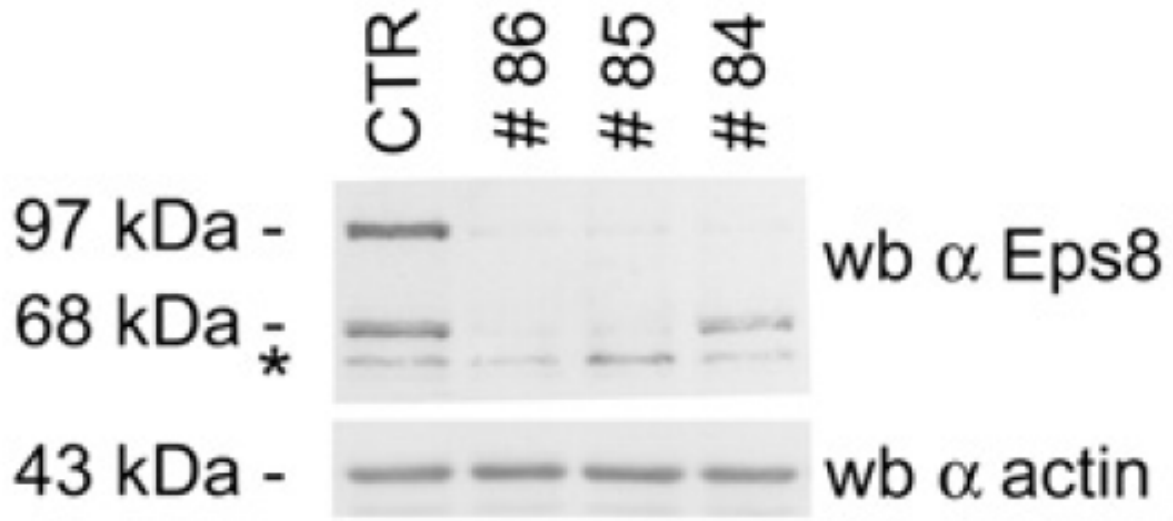




Come si studia il ruolo di una proteina?

Approcci positivi o negativi:
- trasfezioni stabili e transienti
- **siRNA**
- animali transgenici
- animali knock-out
- animali knock-in
- knock-out condizionali

- http://www.youtube.com/watch?v=cK-GB1_ELE



NATURE METHODS | VOL.3 NO.10 | OCTOBER 2006 |

Minimizing the risk of reporting false positives in large-scale RNAi screens

Christophe J Echeverri[1], Philip A Beachy[2], Buzz Baum[3], Michael Boutros[4], Frank Buchholz[5], Sumit K Chanda[6], Julian Downward[7], Jan Ellenberg[8], Andrew G Fraser[9], Nir Hacohen[10,11], William C Hahn[10,12], Aimee L Jackson[13], Amy Kiger[14], Peter S Linsley[13], Lawrence Lum[15], Yong Ma[2], Bernard Mathey-Prévôt[16], David E Root[8], David M Sabatini[8,17], Jussi Taipale[18], Norbert Perrimon[16,19] & René Bernards[20]

Large-scale RNA interference (RNAi)-based analyses, very much as other 'omic' approaches, have inherent rates of false positives and negatives. The variability in the standards of care applied to validate results from these studies, if left unchecked, could eventually begin to undermine the credibility of RNAi as a powerful functional approach. This Commentary is an invitation to an open discussion started among various users of RNAi to set forth accepted standards that would insure the quality and accuracy of information in the large datasets coming out of genome-scale screens.

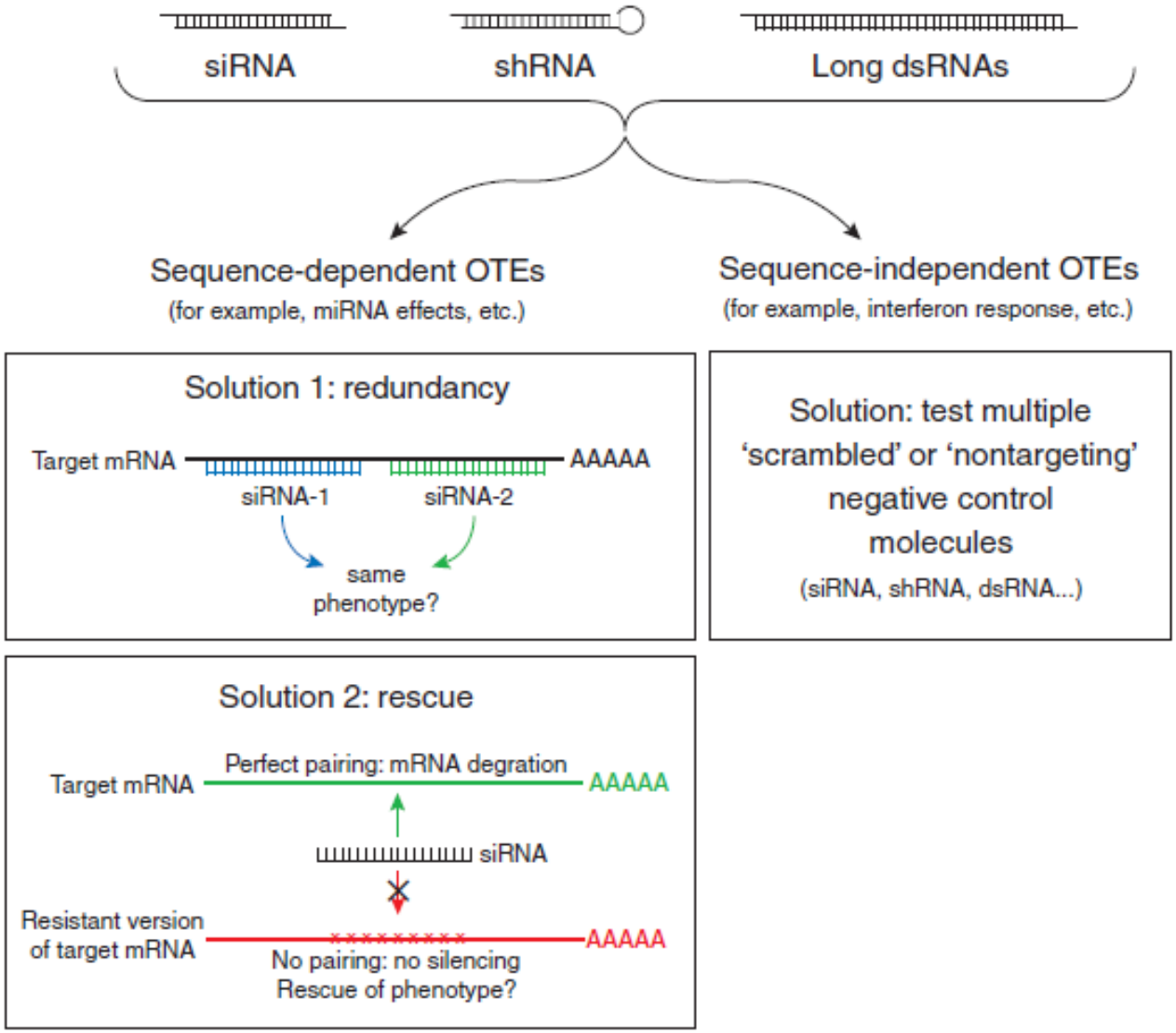


Figure 1 | Appropriate experimental controls to minimize risks of misinterpretation of RNAi data due to off-target effects (OTEs). siRNA-like molecules, vector-based shRNAs and long dsRNAs trigger detectable off-target effects in all major systems studied to date, from mammalian cells to *D. melanogaster* and *C. elegans*. Simple solutions are available to minimize the risk that an observed phenotype may arise from an off-target effect rather than the targeted gene's loss of function.

Nat Biotechnol. 2009 June ; 27(6): 549–555. doi:10.1038/nbt.1543.

siRNA

Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs

Aly A. Khan, Doron Betel, Martin L. Miller, Chris Sander, Christina S. Leslie[*], and **Debora S. Marks**[*]

Abstract

Transfection of small RNAs (si/miRNAs) into cells typically lowers expression of many genes. Unexpectedly, increased expression of genes also occurs. We investigated whether this upregulation results from a saturation effect, i.e. competition for intracellular small RNA processing machinery between the transfected si/miRNAs and the endogenous pool of microRNAs (miRNAs). To test this hypothesis, we analyzed genome-wide transcript responses from more than 150 published transfection experiments in 7 different cell types. We show that endogenous miRNA targets have significantly higher expression levels following transfection, consistent with an impaired effectiveness of endogenous miRNA repression. Further confirmation comes from concentration and temporal dependence. Strikingly, the profile of endogenous miRNAs can largely be inferred by correlating miRNA sites with gene expression changes after transfections. The saturation and competition effects present practical implications for miRNA target prediction, the design of si/shRNA genomic screens and siRNA therapeutics.

Come si studia il ruolo di una proteina?

Approcci positivi o negativi:
- trasfezioni stabili e transienti
- siRNA
- **animali transgenici**
- animali knock-out
- animali knock-in
- tecnica cre-lox per knock-out condizionali

TRANSGENIC MICE

Introduction of foreign genes or altered forms of an endogenous gene into an organism (mice)

The introduced genes are called TRANSGENES

The organisms carrying this gene are called TRANSGENICS

TRANSGENIC MICE

Useful to study:

- Normal mammalian biology
- Disease processes (i.e. oncogenes)
- Development

TOPI TRANSGENICI

per produrre topi transgenici è necessario:

- preparare un costrutto

- inserirlo nell’animale

Basic Transgenic Construct

Promoter - to drive cell-specific, tissue-specific, or time-specific expression

Intron - often provides essential regulatory elements

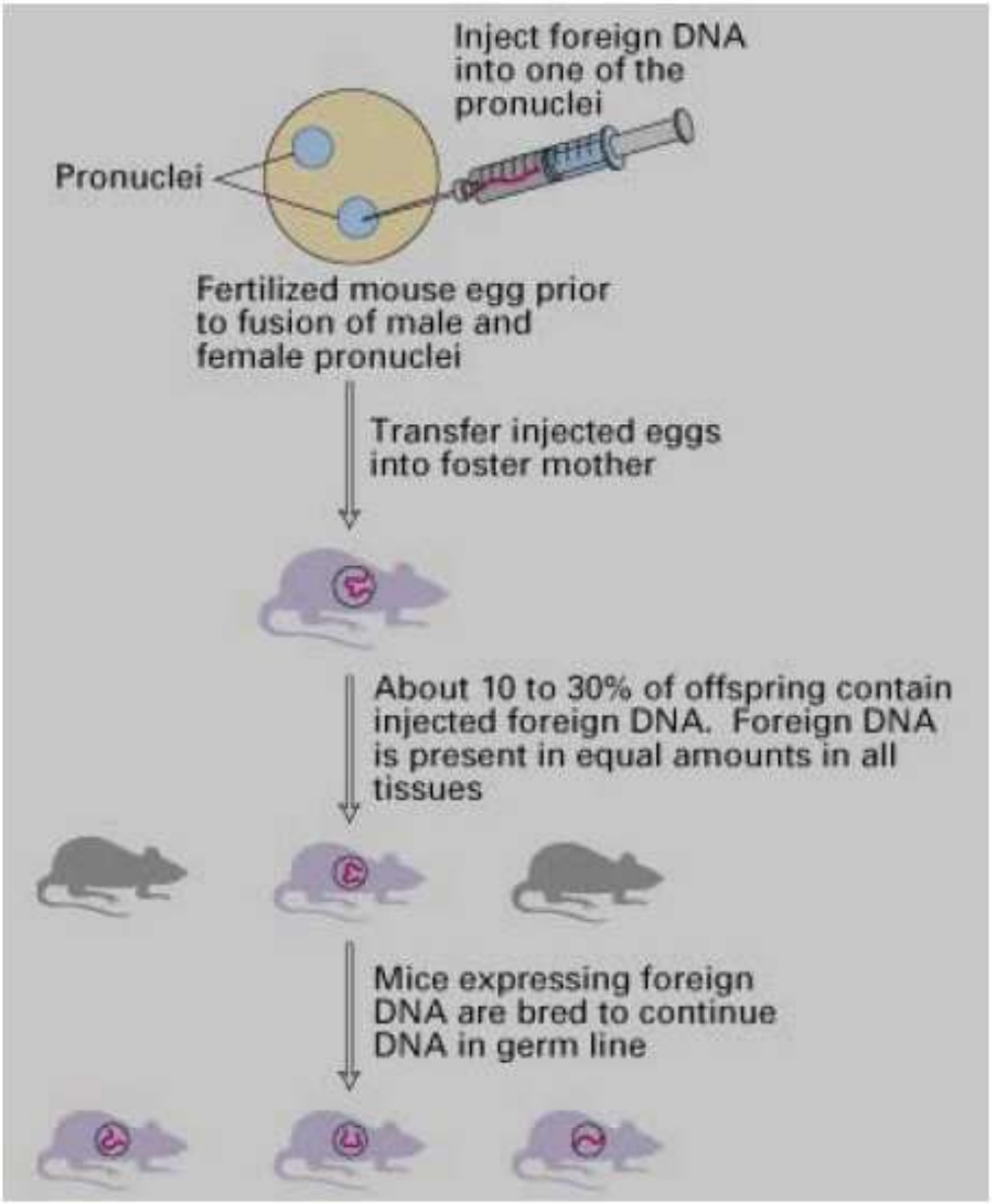
Transgene - expressing cDNA for gene of interest , **PolyA** with or without mutations, or containing sequences from other organisms etc...

Useful for:

- Overexpression
- Dominant negative, insertional/point mutants
- Complementation

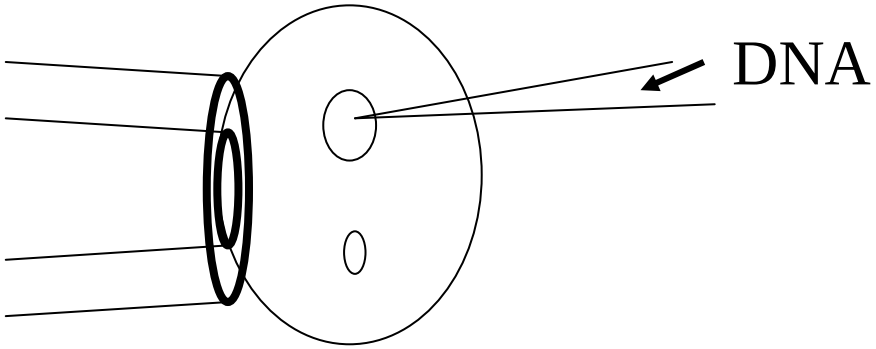
Dove inietto questo costrutto?

Production of transgenic mice



-Dove si integra il DNA?
-In quale cromosoma?
-Quali cellule o tessuti esprimono la proteina d'interesse?
-Da cosa dipende l'eventuale specificità di espressione?

Microinjection in fertilized eggs



The transgene is injected into the male pronucleus of a fertilized egg

The DNA is inserted in the genome RANDOMLY by non-homologous recombination

G0 offsprings from surrogate mothers contain transgene in ALL cells

G0 crossed with non-transgenics. Offsprings called FOUNDERS

Come si studia il ruolo di una proteina?

Approcci positivi o negativi:
- trasfezioni stabili e transienti
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- **animali knock-in**
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TOPI KNOCK-OUT & TOPI KNOCK-IN

Inattivazione selettiva di un gene che viene rimpiazzato da un altro gene (knock-out)
o da una copia mutata o modificata dello stesso gene (knock-in)

TOPI KNOCK-OUT & TOPI KNOCK-IN

- per produrre un topo knock-out/in è necessario:

- preparare un costrutto

- inserirlo nell’animale

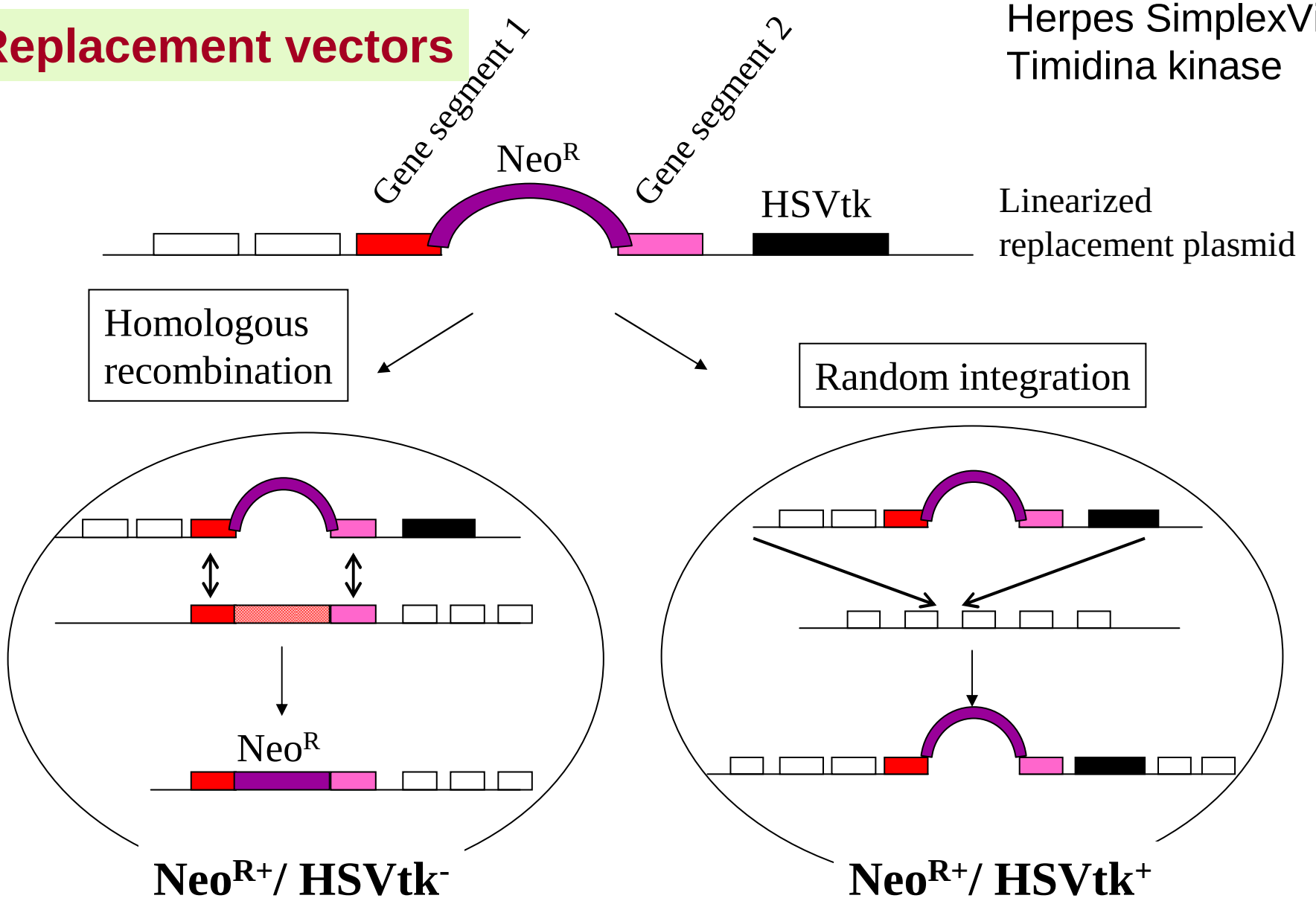
KNOCK-OUT MICE

Useful to study:

- Development
- Behavior
- Physiology
- Human genetic diseases

Replacement vectors

Herpes SimplexVirus
Timidina kinase



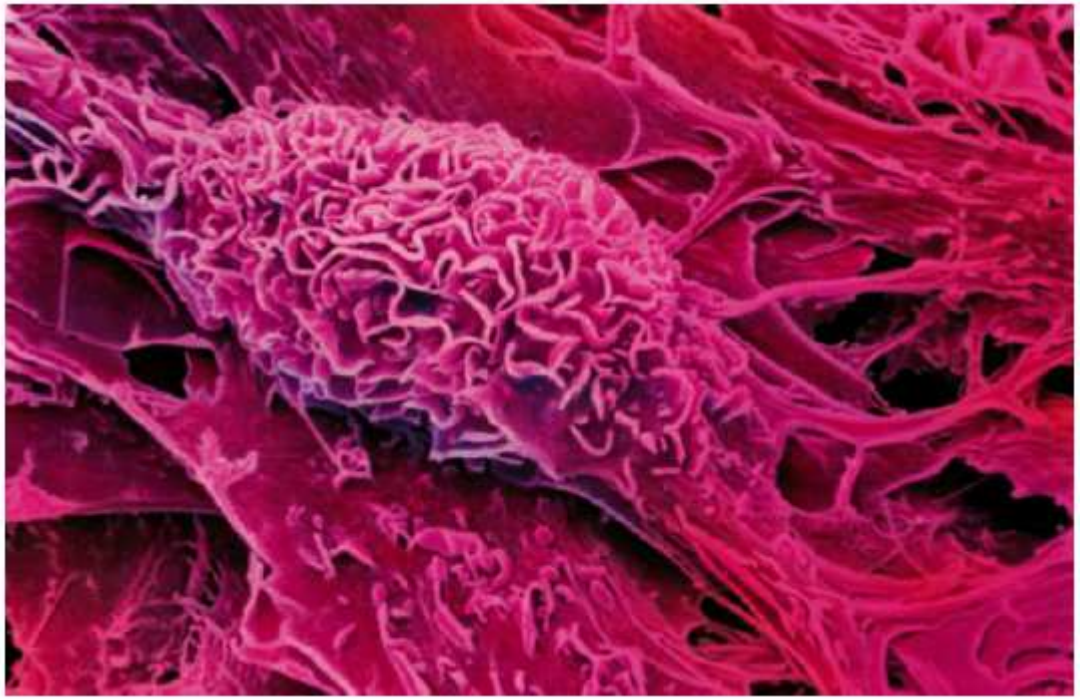
HSVtk will convert gancyclovir into a toxic drug and kill HSVtk+ cells

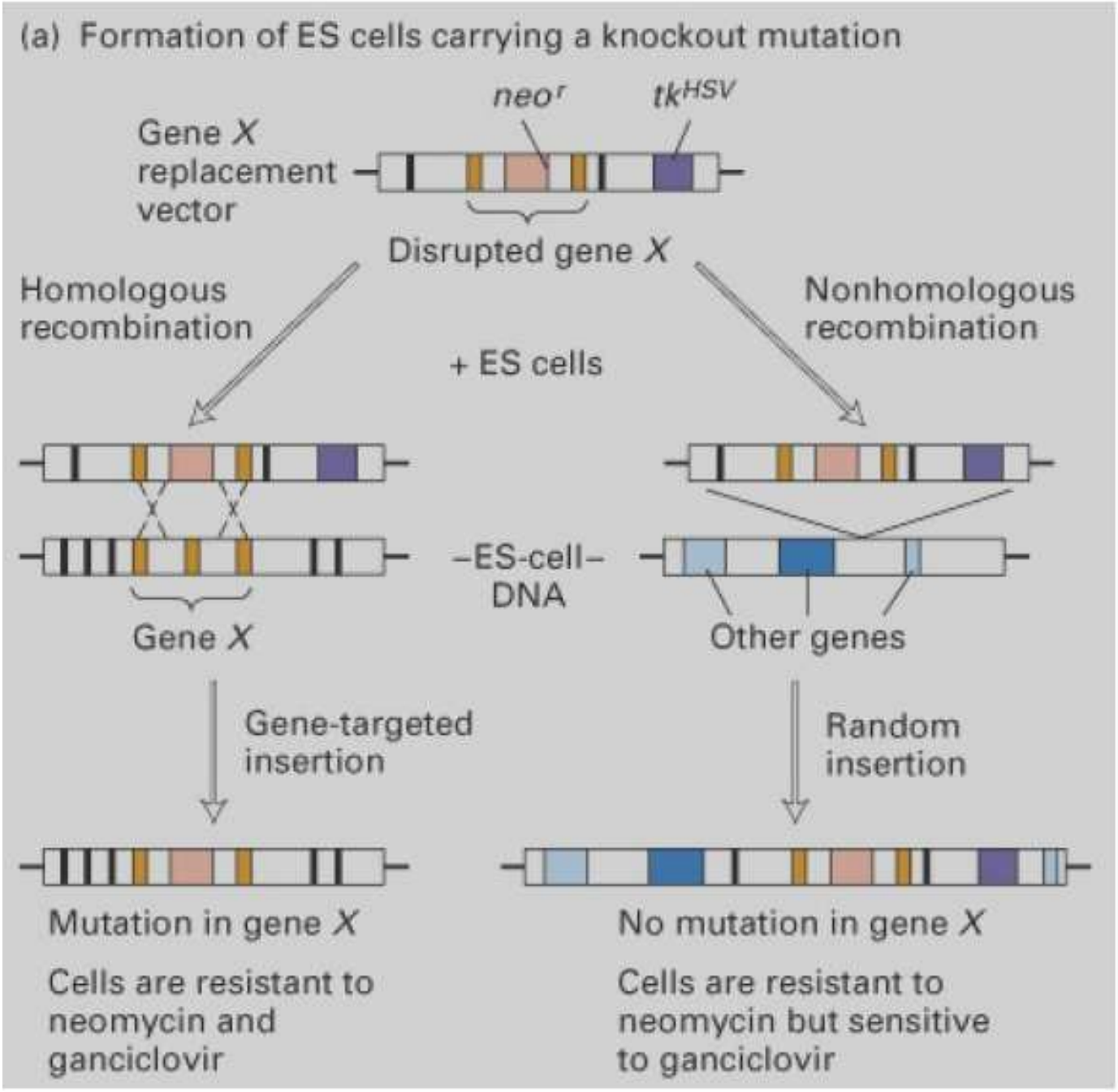
- Dove inietto questo costrutto?

Embryonic stem (ES) cells

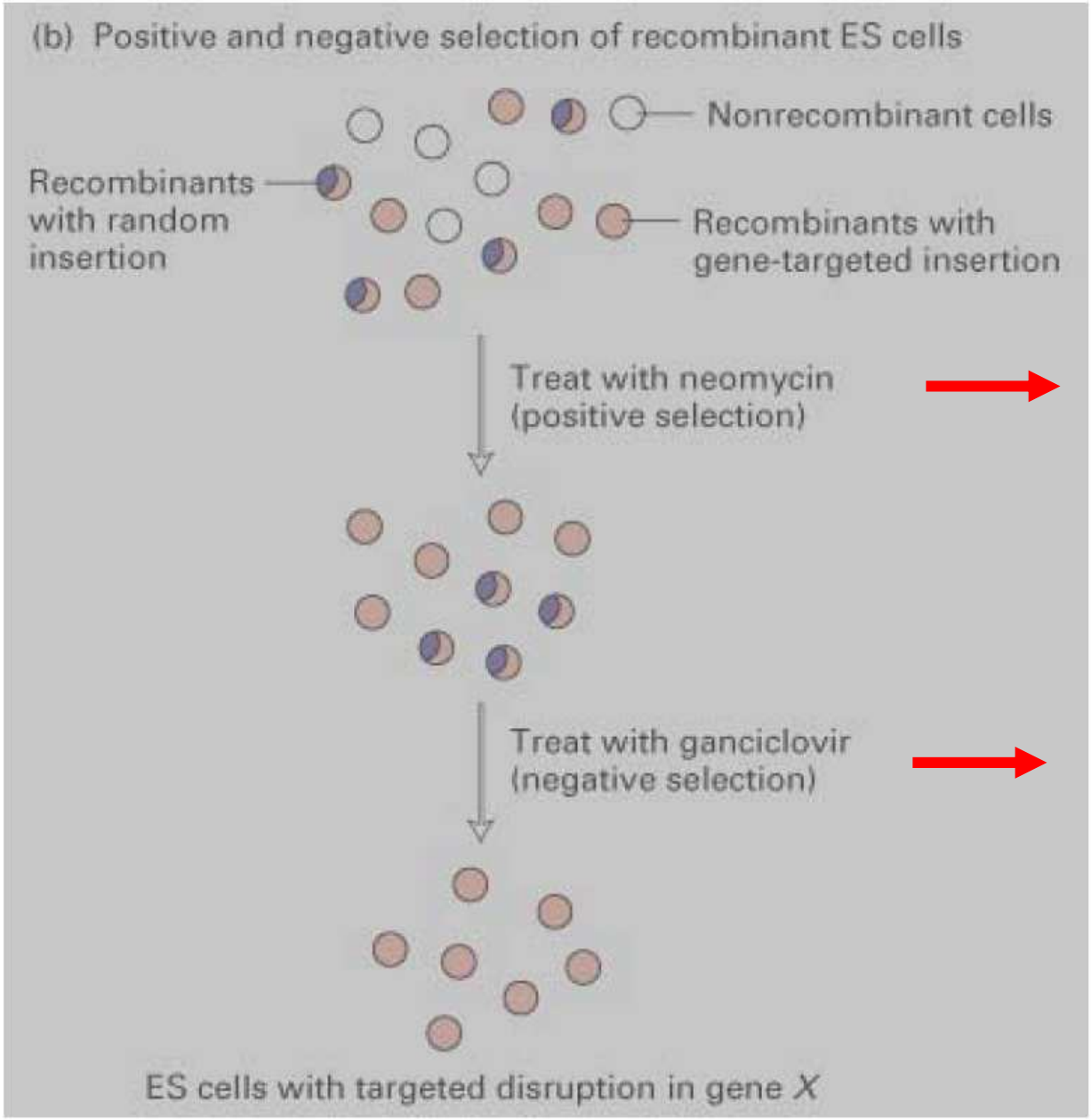
Pluripotent stem cells derived from the inner cell mass of the blastocyst

Can be cultured, manipulated and then reinjected into blastocysts, where they can go on to contribute to all parts of embryo.





ES cells selection



Quali cellule muoiono?
Quali cellule sopravvivono?

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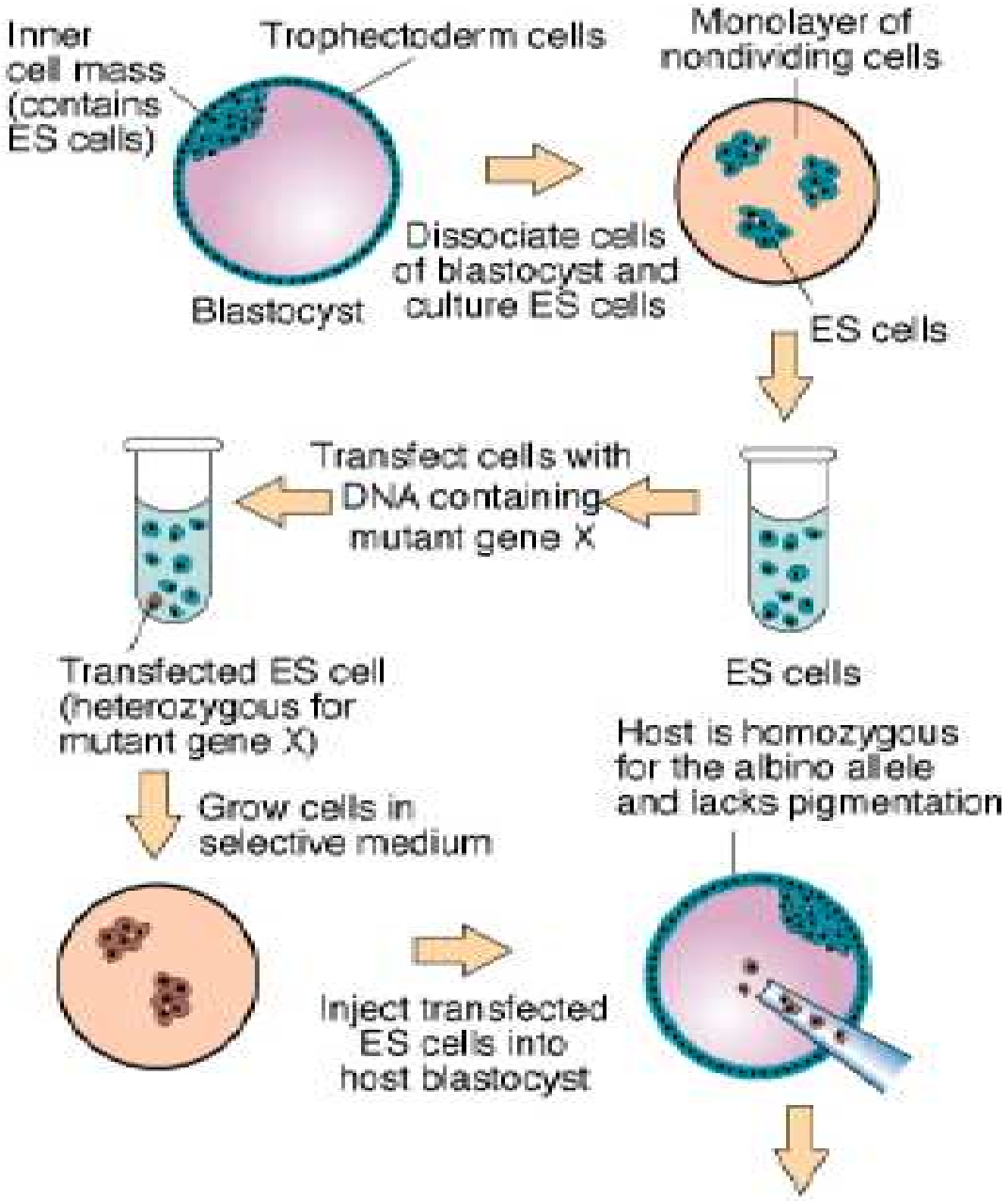
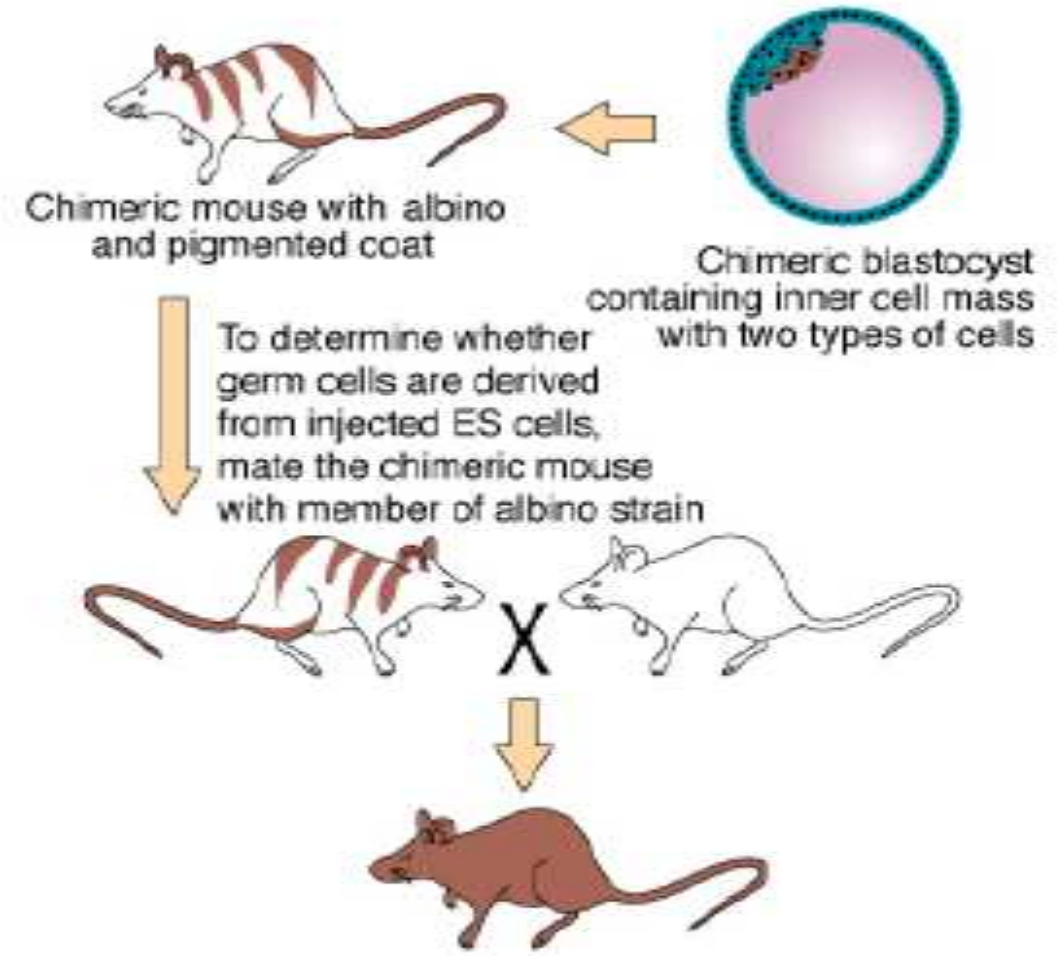
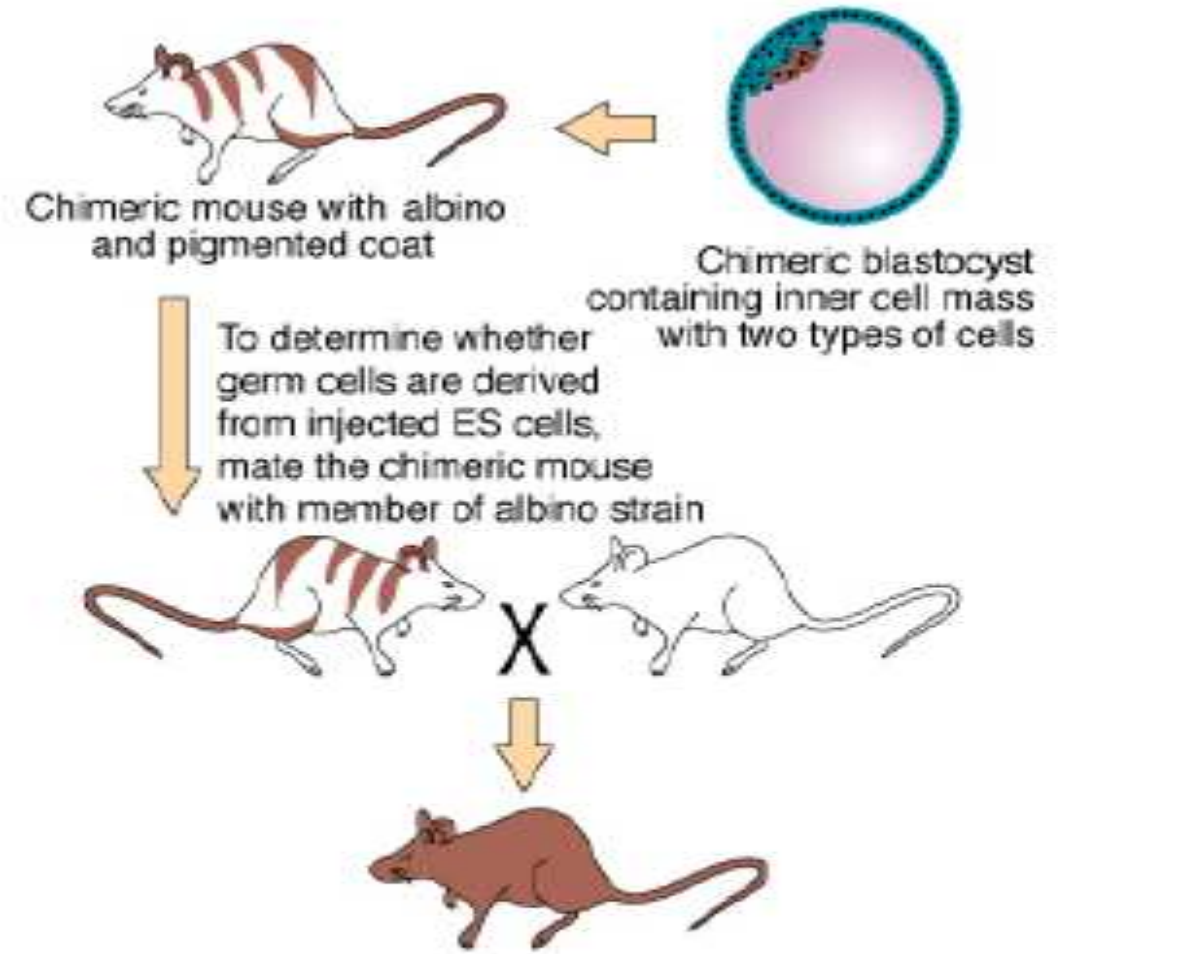
Quali cellule sopravvivono?

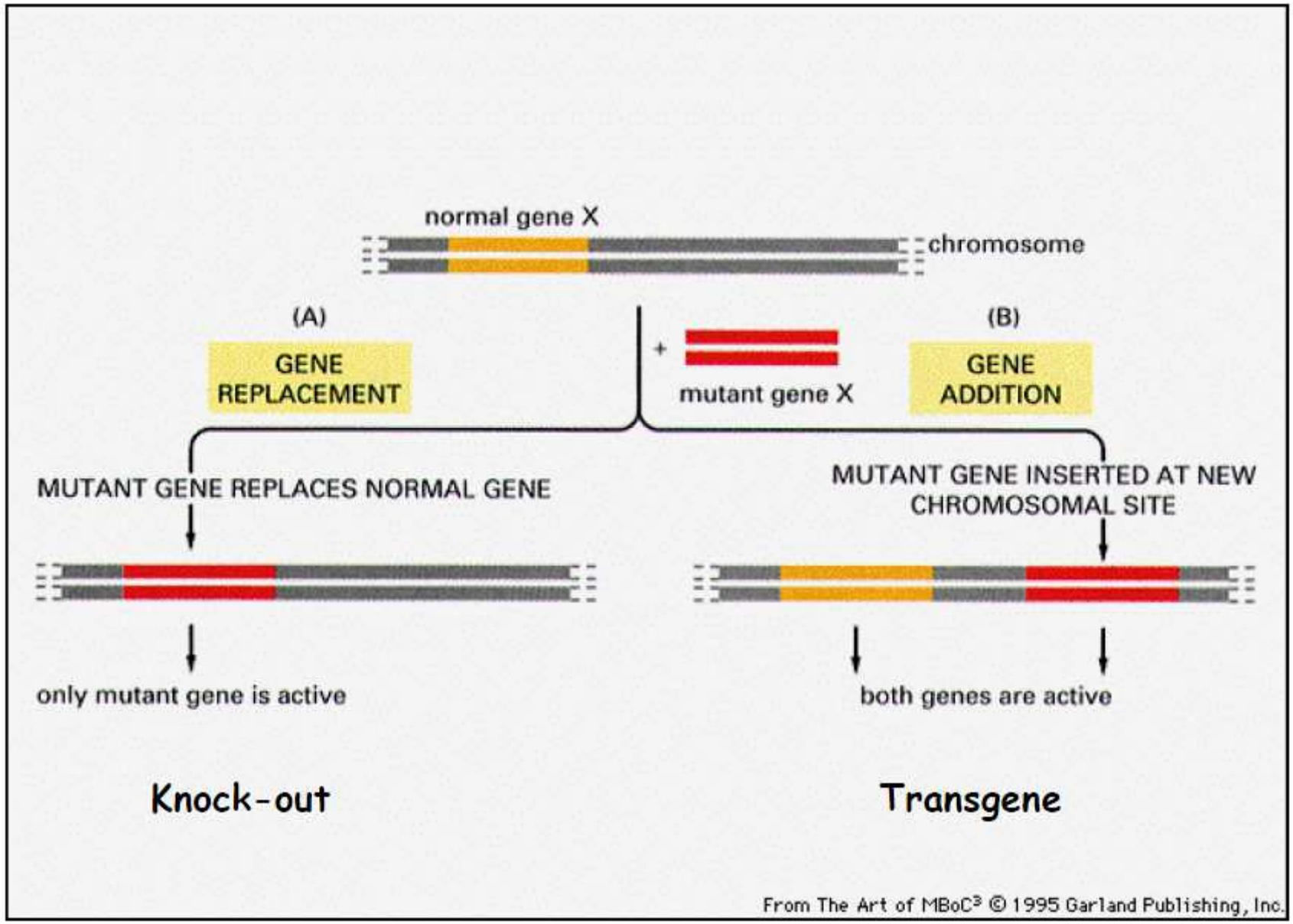




Quello che ottengo è un topo knockout omozigote?



Transgene vs Knock-out



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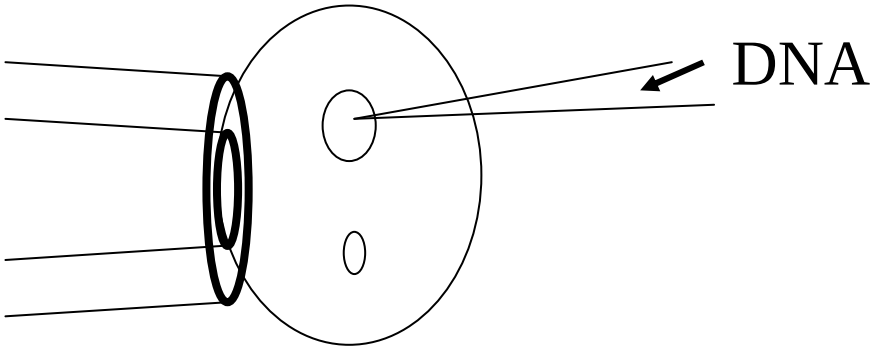

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Transformation of ES cells

ES cells are selected by Neomycin (Neo accompany Your Gene)

Transformed ES cells are injected into 3 day embryo (blastula)

Chimerae etc as for knocks

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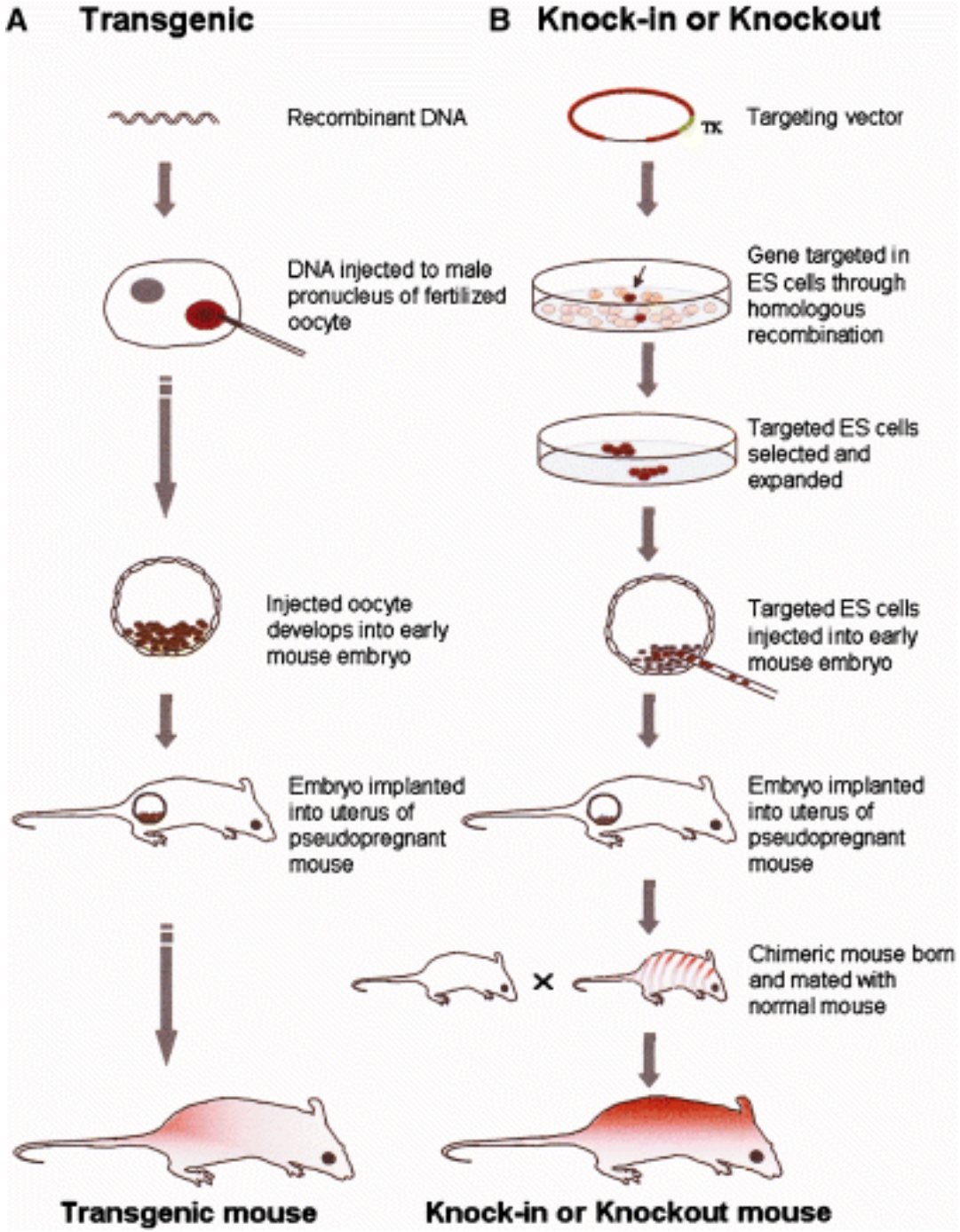
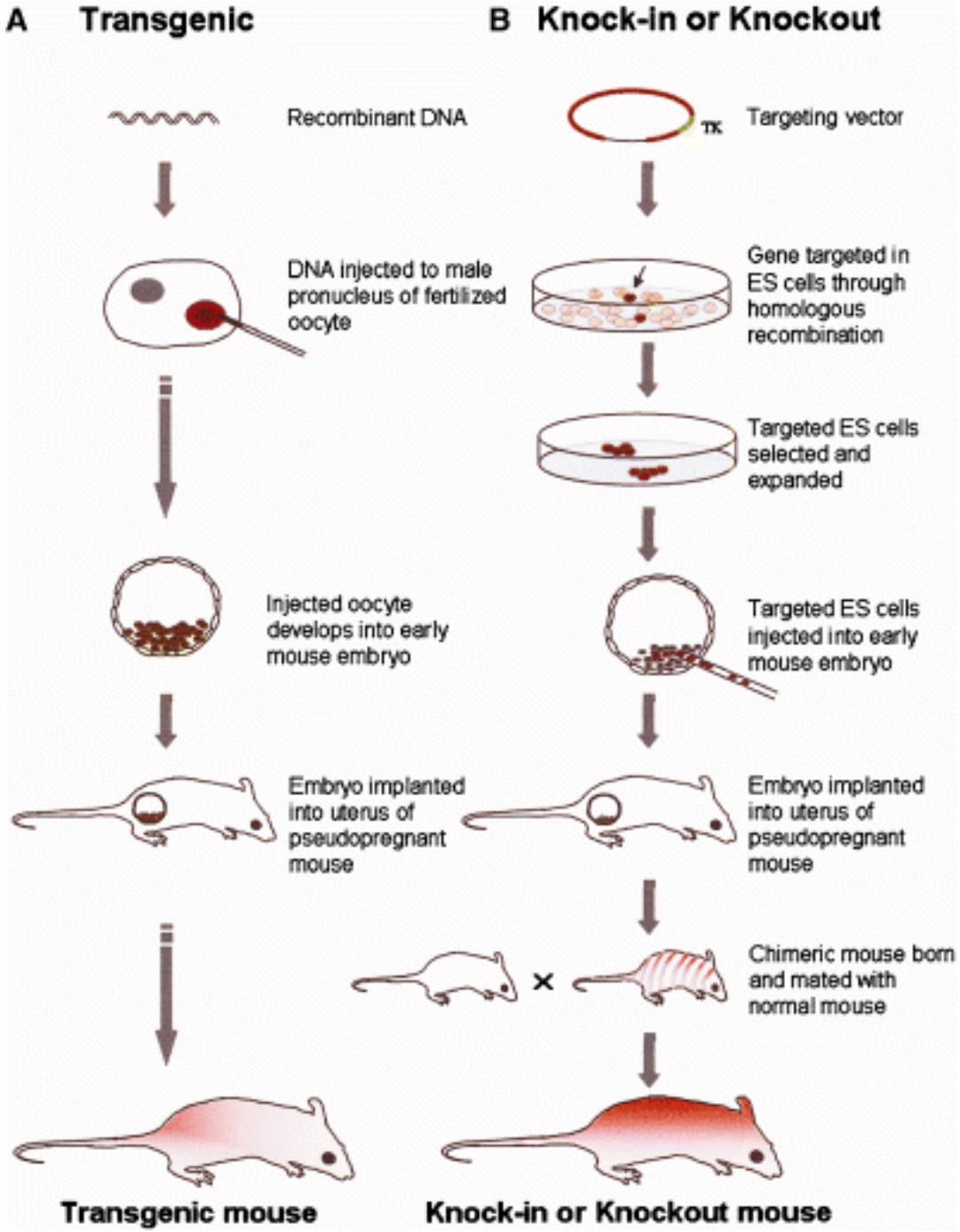

A Transgenic
B Knock-in or Knockout
Recombinant DNA
DNA injected to male pronucleus of fertilized oocyte
Injected oocyte develops into early mouse embryo
Embryo implanted into uterus of pseudopregnant mouse
Transgenic mouse
TK
Targeting vector
Gene targeted in ES cells through homologous recombination
Targeted ES cells selected and expanded
Targeted ES cells injected into early mouse embryo
Embryo implanted into uterus of pseudopregnant mouse
Chimeric mouse born and mated with normal mouse
Knock-in or Knockout mouse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- **knock-out condizionali**

nature 23 November 1995

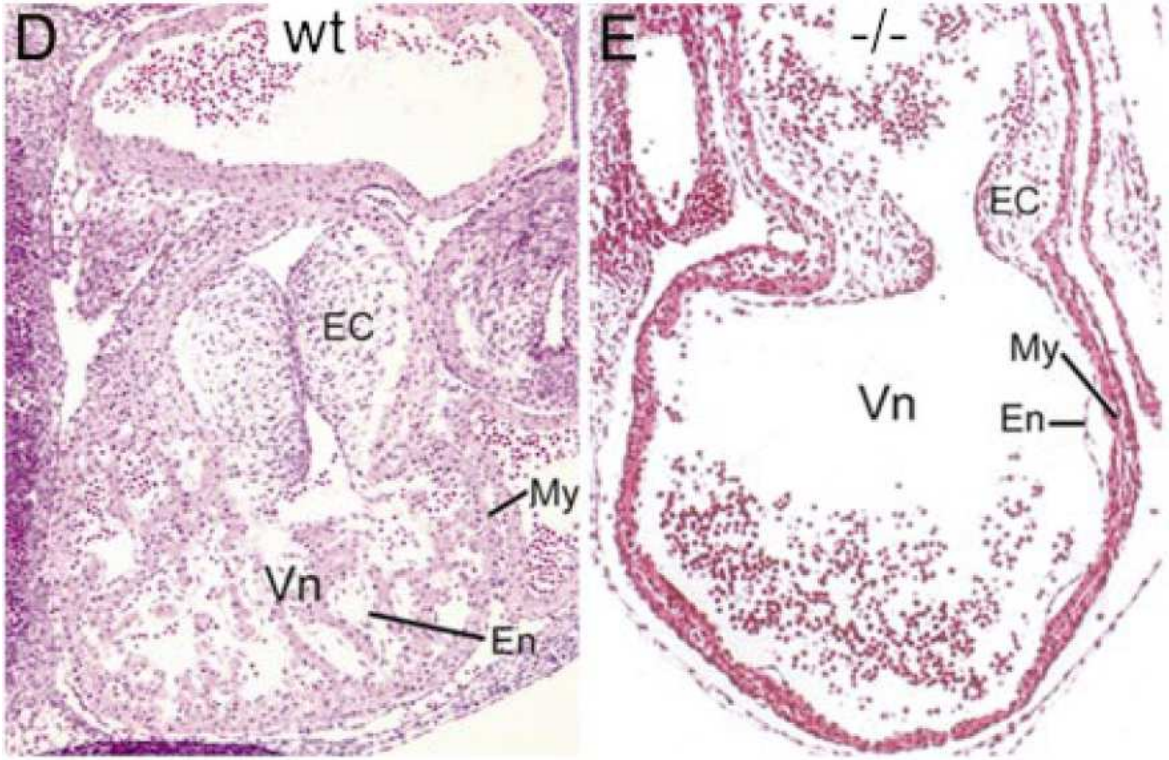
letters to nature

Nature **378**, 390 - 394 (1995); doi:10.1038/378390a0

ErbB4 KO

Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor

MARTIN GASSMANN, FRANCA CASAGRANDA, DONATA ORIOLI, HORST SIMON, CARY LAI, RÜDIGER KLEIN & GREG LEMKE



- histological examination revealed dramatic lack of trabeculation within the myocardium (My) of E10.5 **ErbB4^{-}/- mice**

My, myocardium; En, endocardium; Vn, ventricle; EC, endocardial cushion.

ErbB4 KO è letale, ma se espresso nel cuore no

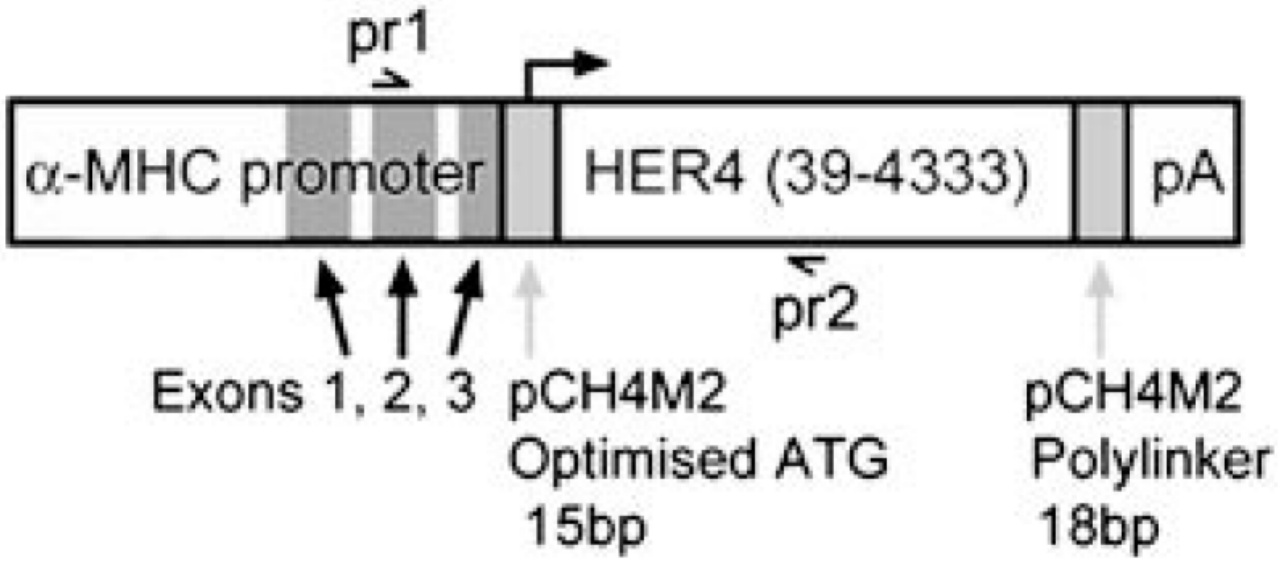
- **1**-Come posso ottenere un topo knock-out, che **esprime** il gene di interesse solo in un tessuto?
  (es: solo nel cuore)

- **2**-Come posso ottenere un topo knock-out che **non esprime** il gene di interesse solo in un tessuto, o in una certa fase dello sviluppo?
  (es: un knock-out che non esprime ErbB4 solo nei neuroni)

Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality

Hester Tidcombe*†, Amy Jackson-Fisher†‡, Kathleen Mathers§, David F. Stern‡, Martin Gassmann¶‖, and Jon P. Golding*

PNAS | July 8, 2003 | vol. 100 | no. 14 | 8281–8286



A cosa serve questo costrutto?
Lo userò per ottenere un topo knock-out o un transgenico?
Dove lo inietto?

Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality

Hester Tidcombe*†, Amy Jackson-Fisher†‡, Kathleen Mathers§, David F. Stern‡, Martin Gassmann¶‖, and Jon P. Golding*

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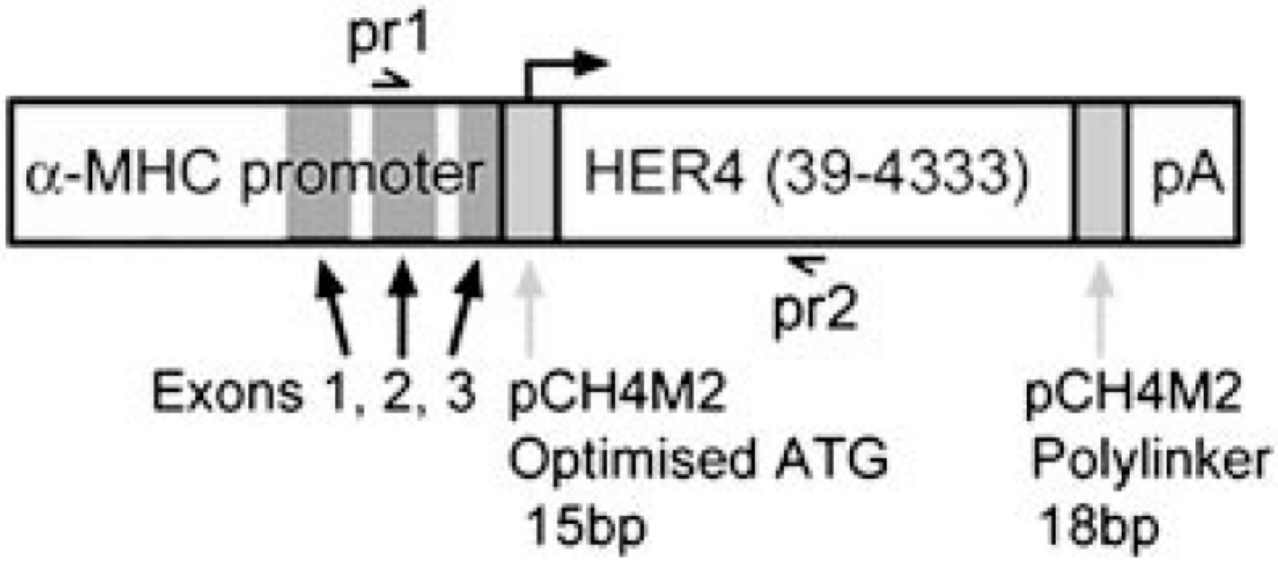
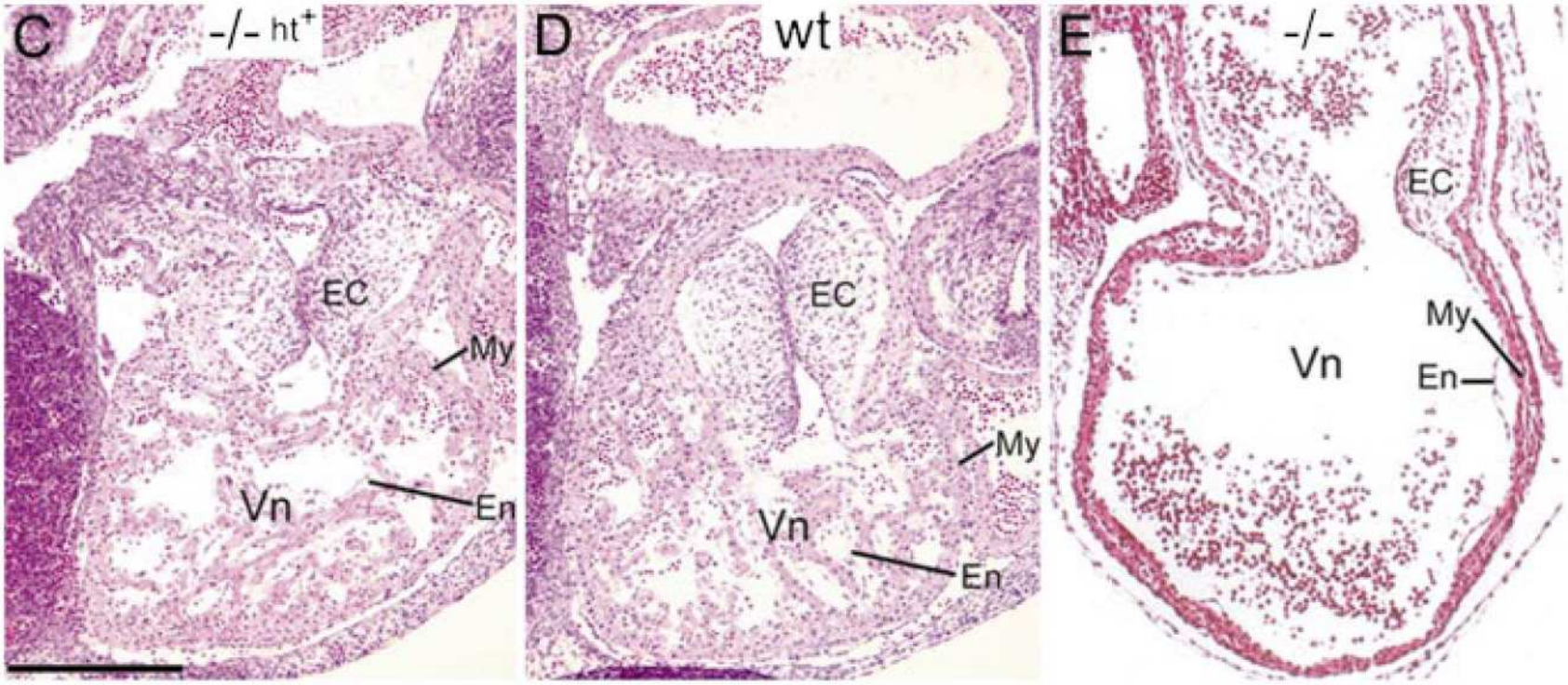


A DNA construct was made, consisting of human ErbB4 (HER4) cDNA under the control of the α-myosin heavy chain (**αMHC**) promoter and this was microinjected into fertilized mouse ova, which were placed into the oviducts of pseudopregnant females



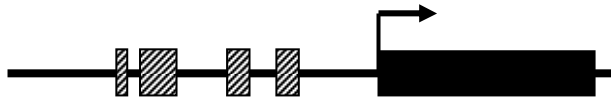
• the HER4^{heart} mouse line was crossed with an ErbB4+/- line to generate ErbB4-/- HER4^{heart} (***-/- ht+***) offspring in the F2 generation

• histological examination revealed no differences between E10.5 $^{-}$/- ht+ heart (*C*) and E10.5 WT heart (*D*). This finding contrasts with the dramatic lack of trabeculation within the myocardium (My) of E10.5 ErbB4^{-}/- mice (*E*)

My, myocardium; En, endocardium; Vn, ventricle; EC, endocardial cushion (Scale bar = 0.3 mm.)

Conditional knock-outs

inactivate a gene only in specific tissues
and at certain times during development and life.

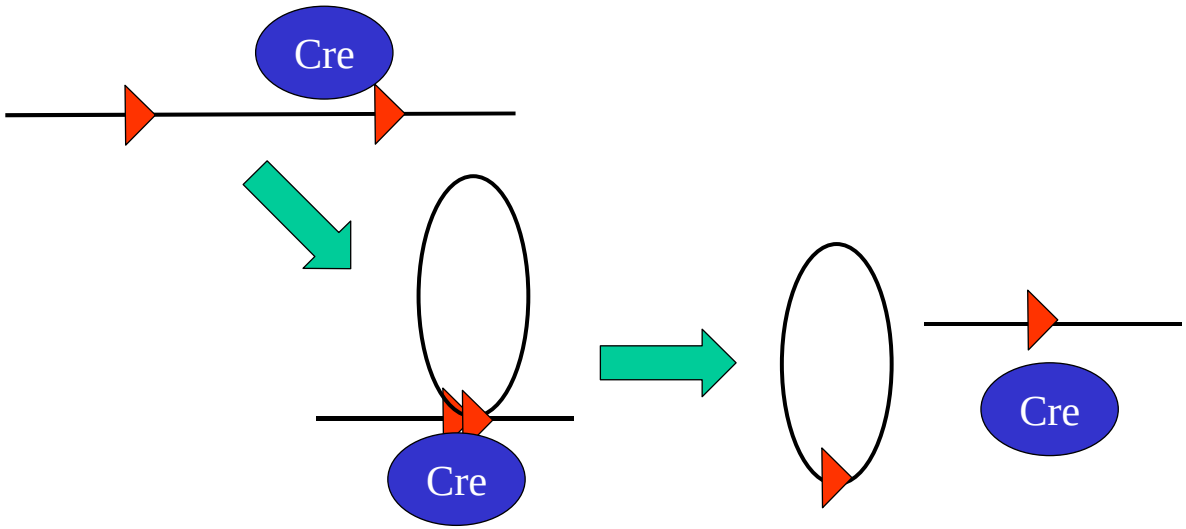


Promoter element Cre

Cre-lox technology

Cre – a site-specific recombinase enzyme from the P1 phage.

Recognises a 34bp DNA sequence *loxP* =

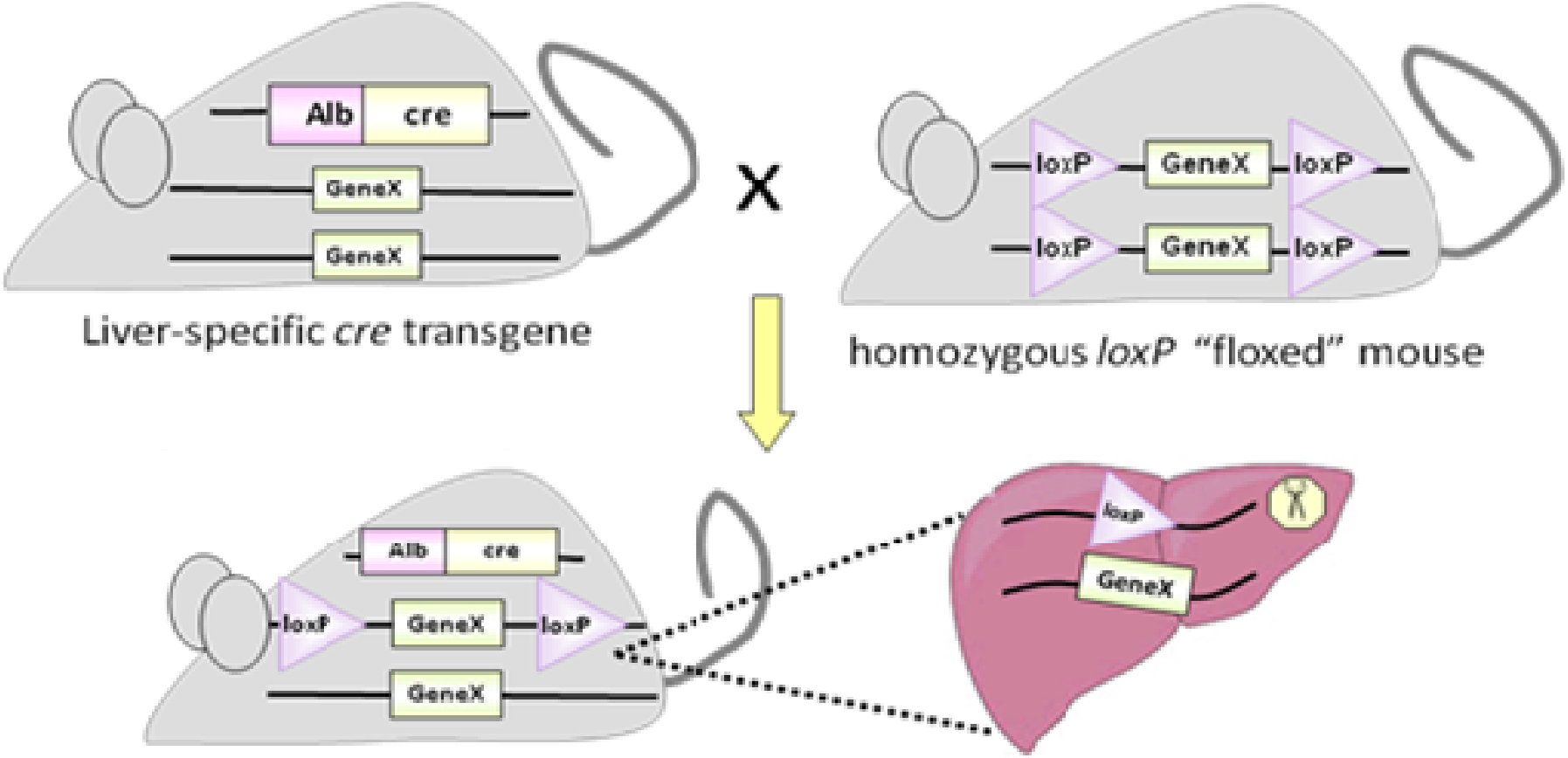


Your gene of interest is **flanked by 34 bp *loxP* sites (floxed).**

Where CRE recombinase is expressed at least once

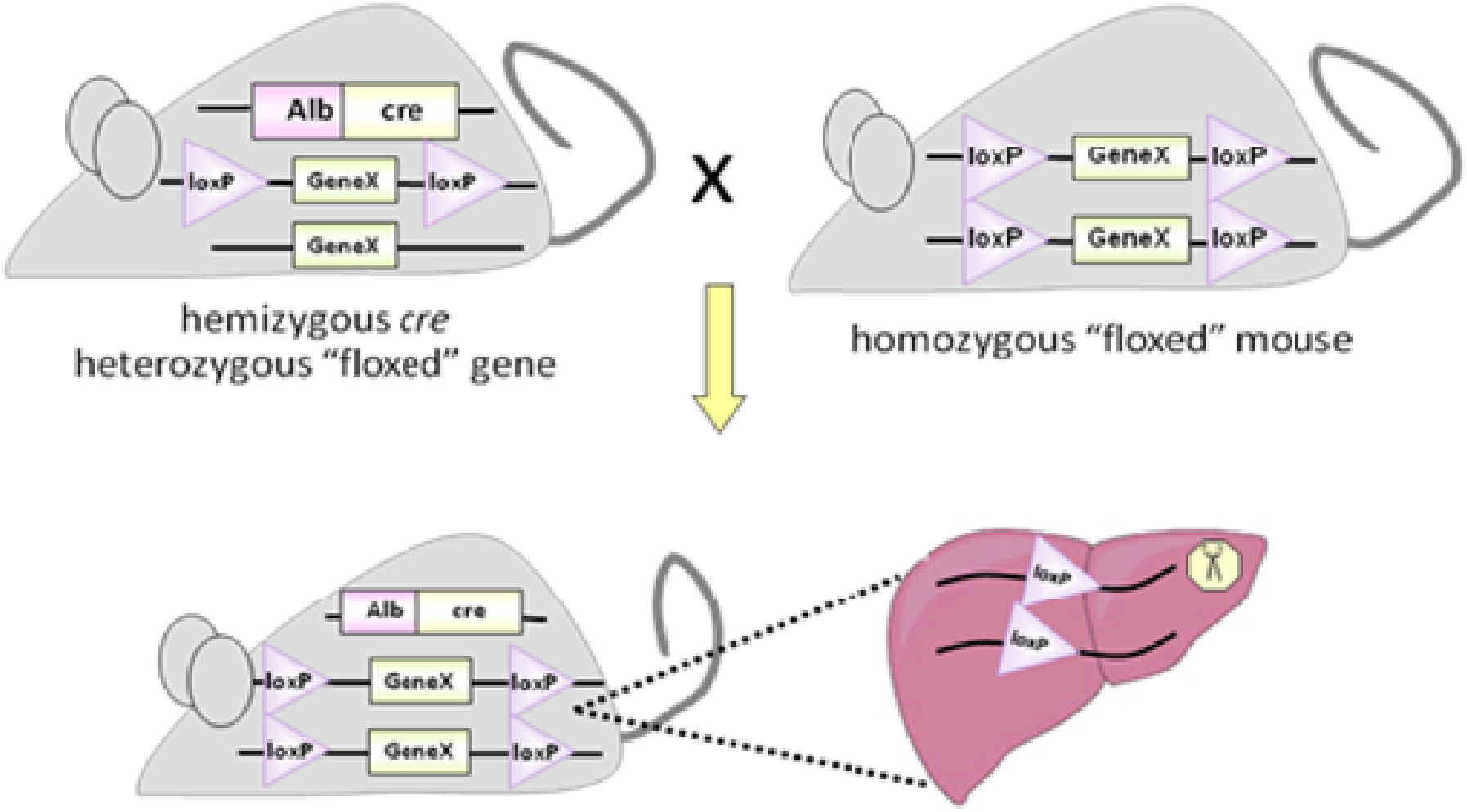
Gene between loxP sites is removed

Cre - lox *Tissue-Specific* Knockout



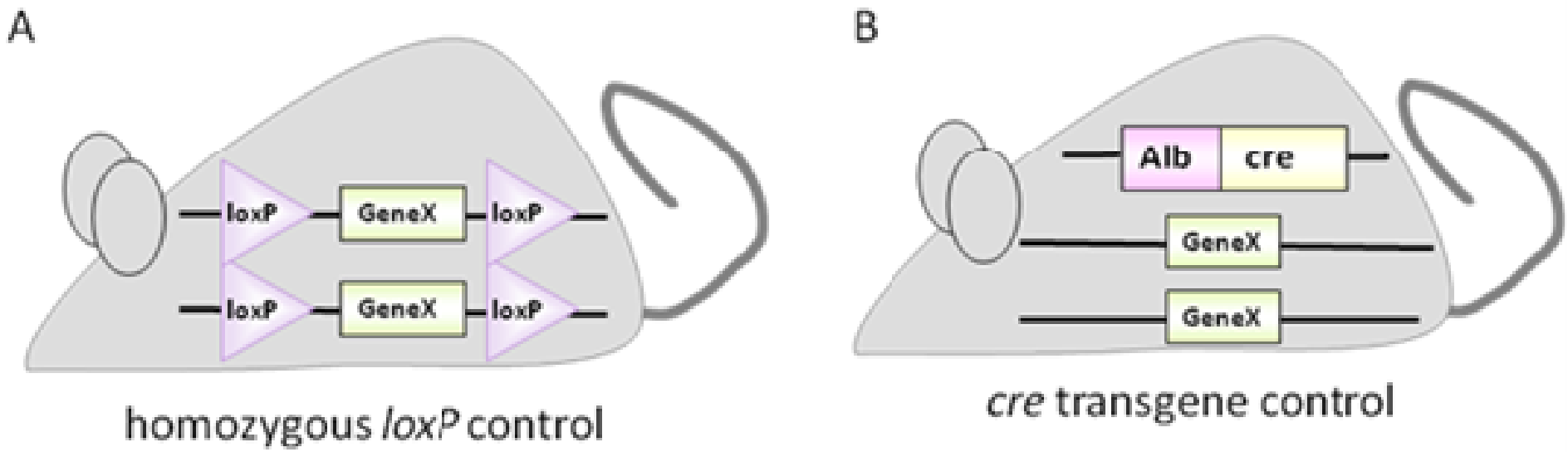
Cre-lox mouse:
heterozygous for GeneX conditional knockout after 1 generation

Cre - lox *Tissue-Specific* Knockout (cont.)



Quali saranno i controlli/mock?

Controls



Quale tipo di approccio devo utilizzare per ottenere il topo “loxato”?

Quale tipo di approccio devo utilizzare per ottenere un topo con una cre ricombinasi tessuto specifica?

Che tipo di costrutto dovrò preparare per il topo lox?

Che tipo di costrutto dovrò preparare per il topo cre?

Inietto il DNA nel pronucleo maschile dell’uovo fertilizzato o trasfetto le cellule ES?

Cosa succede quando incrociate un topo “CRE” con un topo “loxato”?

se incrocio un topo con la cre-ricombinasi regolata dal promotore di un gene espresso solo nella ghiandola mammaria con un topo che presenta ErbB4 “loxato”,

Quali cellule esprimeranno ErbB4?

Quali non lo esprimeranno?

Perché?

L’effetto è reversibile?