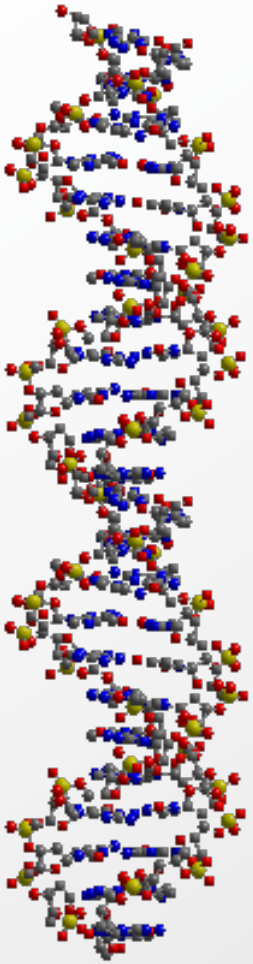
SISTEMAS DE EXPRESSÃO GENÉTICA EM CÉLULAS ANIMAIS



Dr. Carlos Frederico Martins Menck

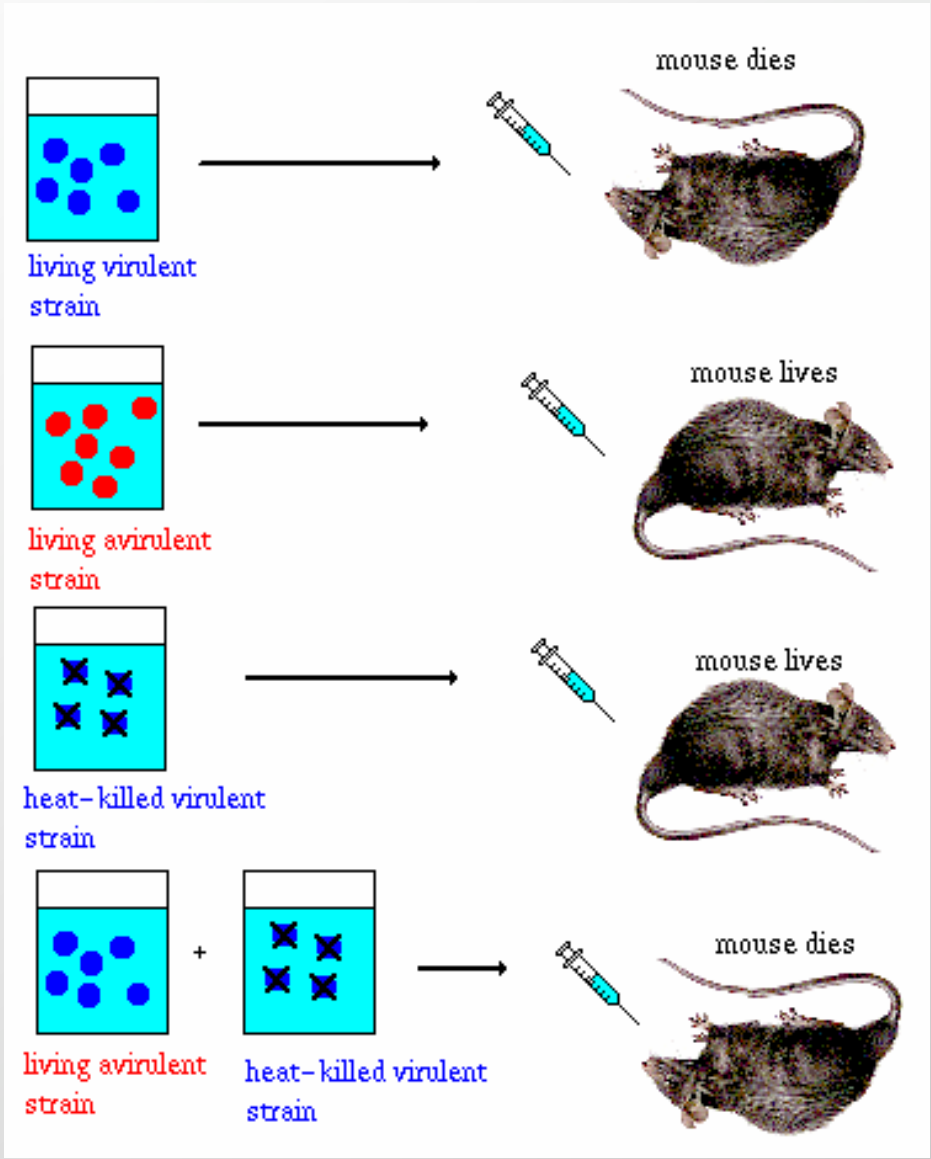
ICB-USP

E tudo começou há muito tempo atrás:

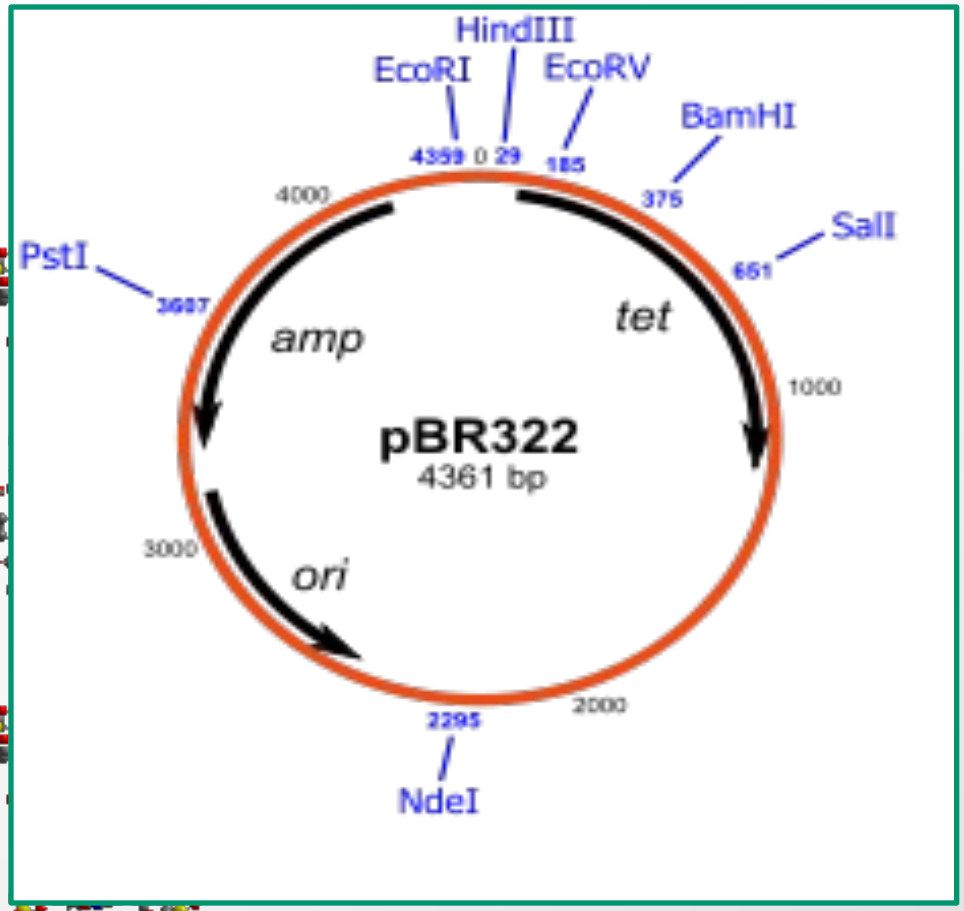
Griffith, 1928

Avery, McLeod, McCarty, 1944

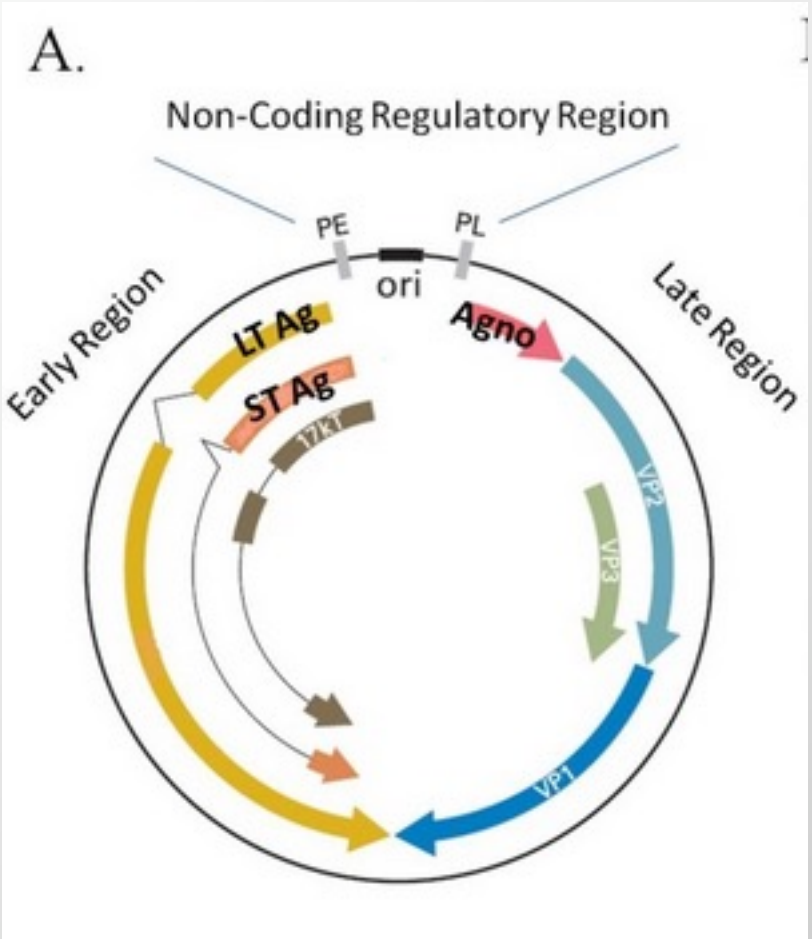
Demonstração que o DNA é o material genético



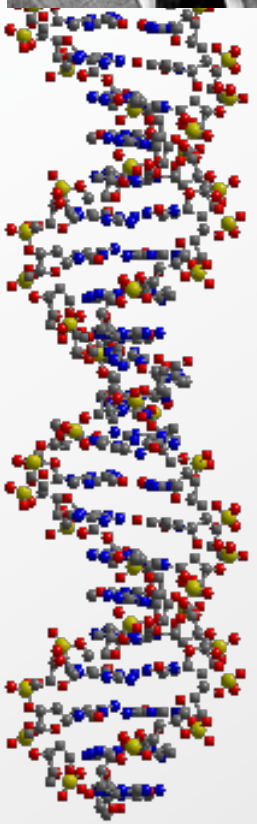
pBR322
(plasmídeo Bolivar e Rodrigues)

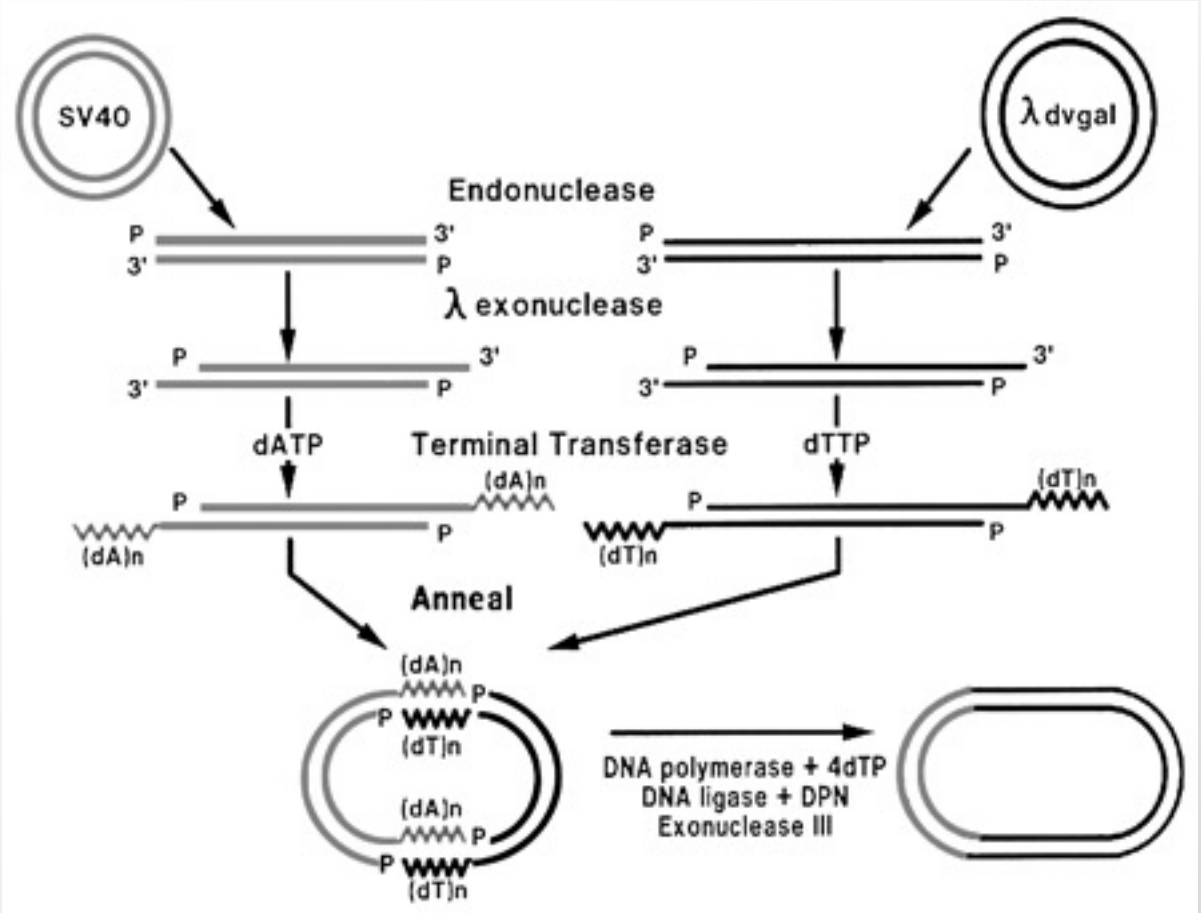


SV40
~5200 bp



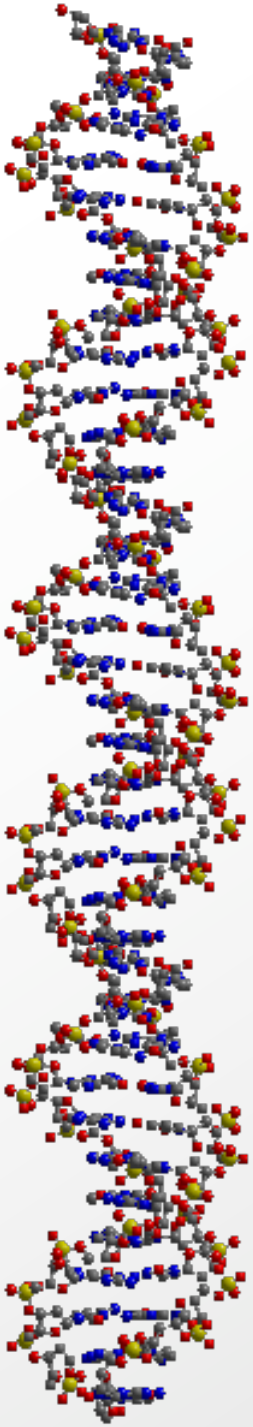
Jackson, D.A., Symons, R.H. and Berg, P., "Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA containing lambda phage genes and the galactose operon of Escherichia coli," Proc. Nat. Acad. Sci. USA 69, pp. 2904-2909 (1972).





Paul Berg- Nobel prize Chemistry 1980

"for his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant-DNA"



Mas como precisa ser um vetor para Expressar em células humanas:

-precisa ter introns?

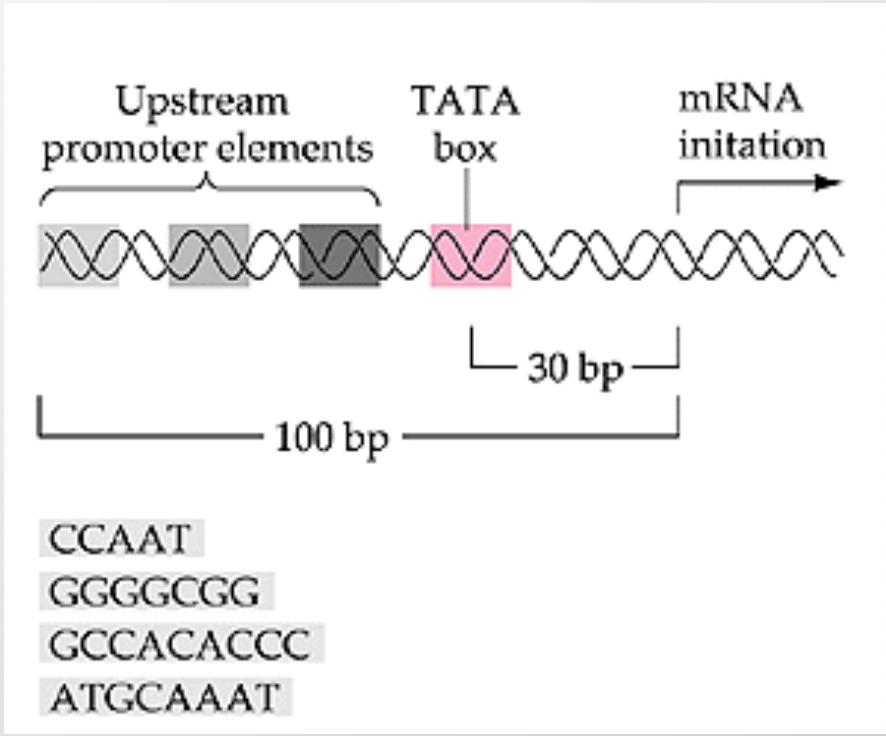
-O que é um cassete de expressão genética?

-Como fazer DNA entrar nas células?

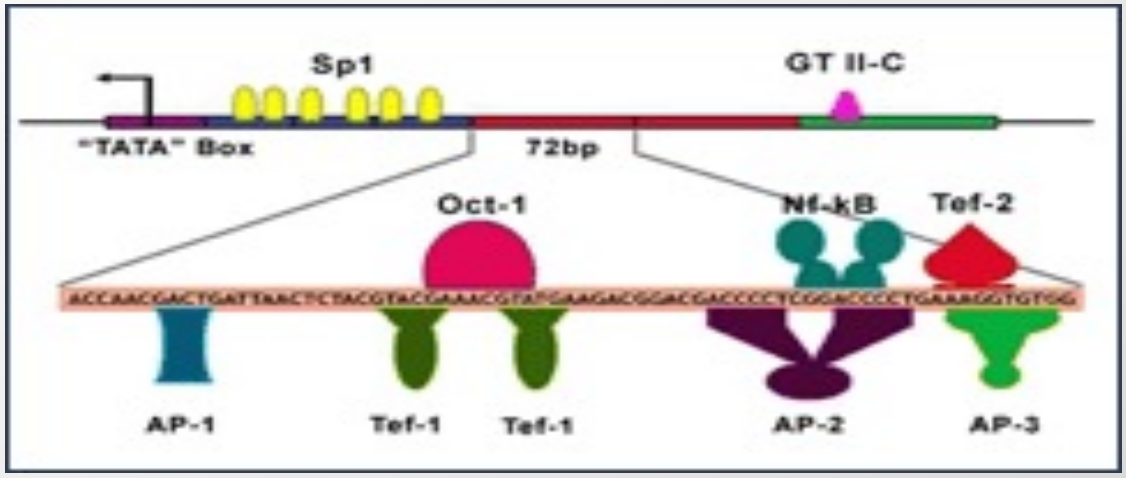
-Quanto tempo essa expressão vai durar?

Sequências necessárias para expressão gênica:

Região 5´ upstream do gene, promotor: qual a função da TATA box?

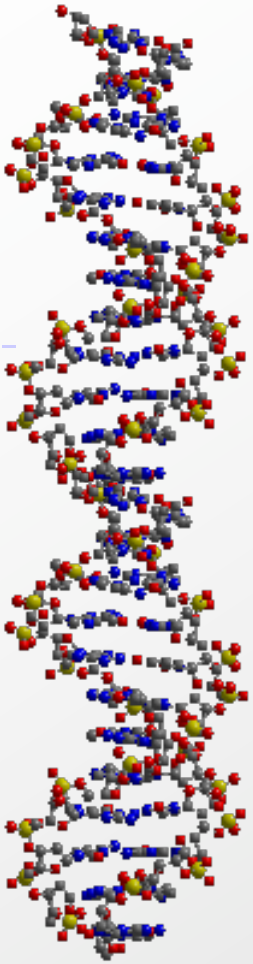


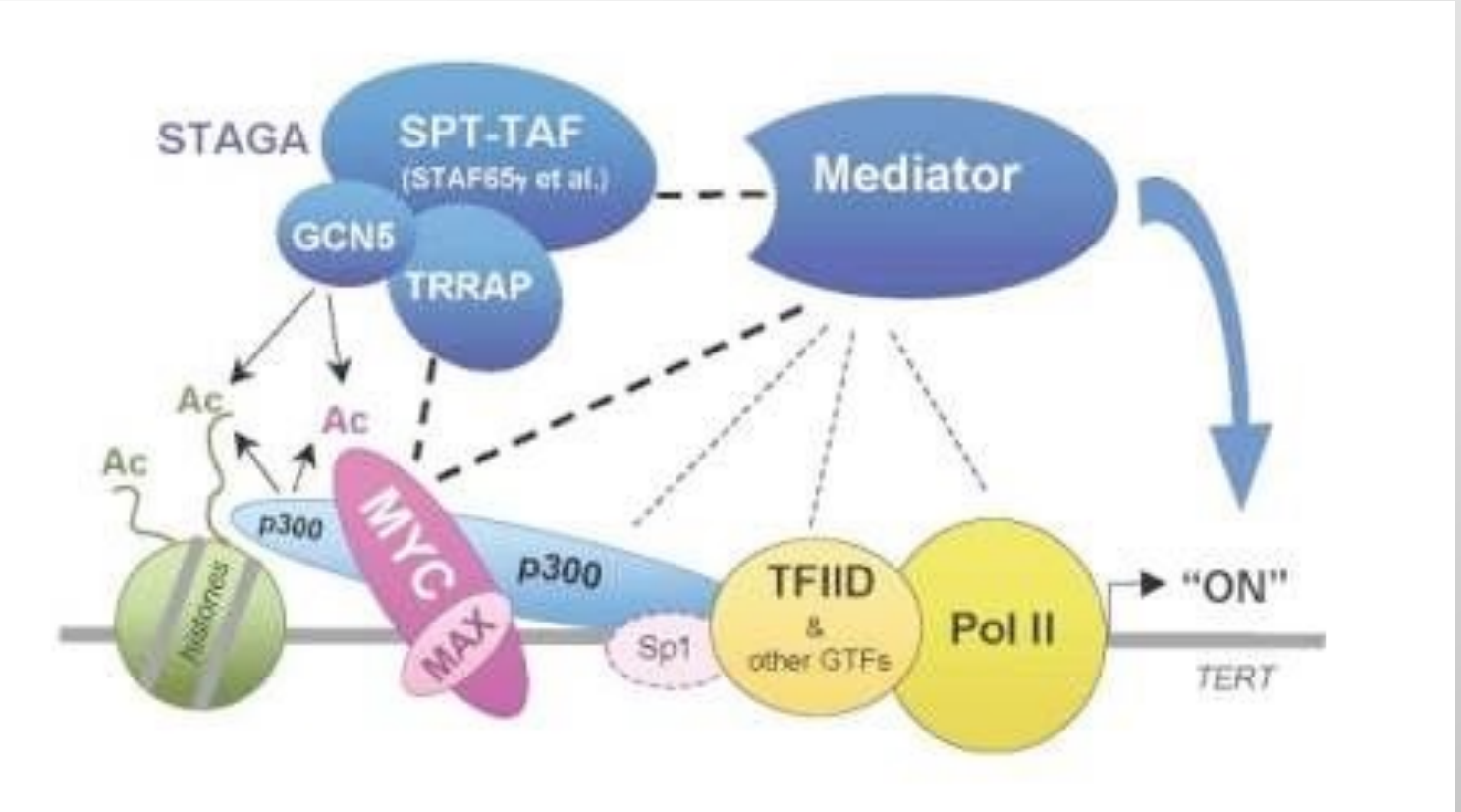
Promotor do SV40- bilateral.....

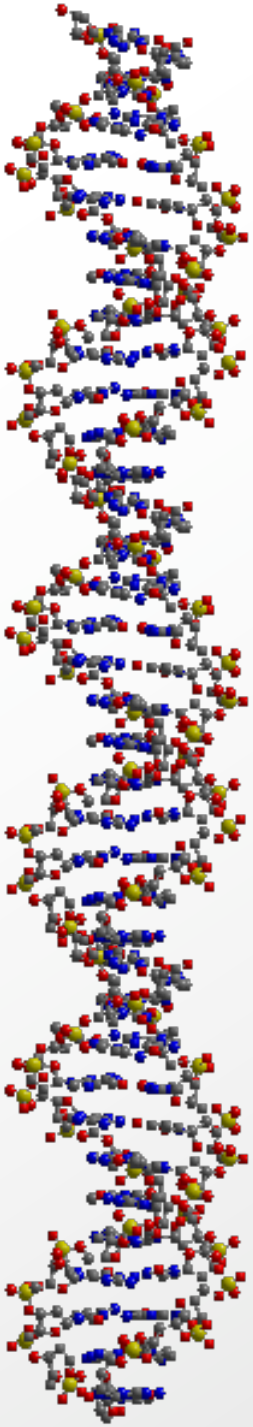


Sequências necessárias para expressão gênica:

Região 5´ upstream do gene, promotor....
tudo para o acesso da RNA polimerase II:







Outros promotores promíscuos:

-MLP adenovirus
-LTR de retrovírus (o que é LTR?)

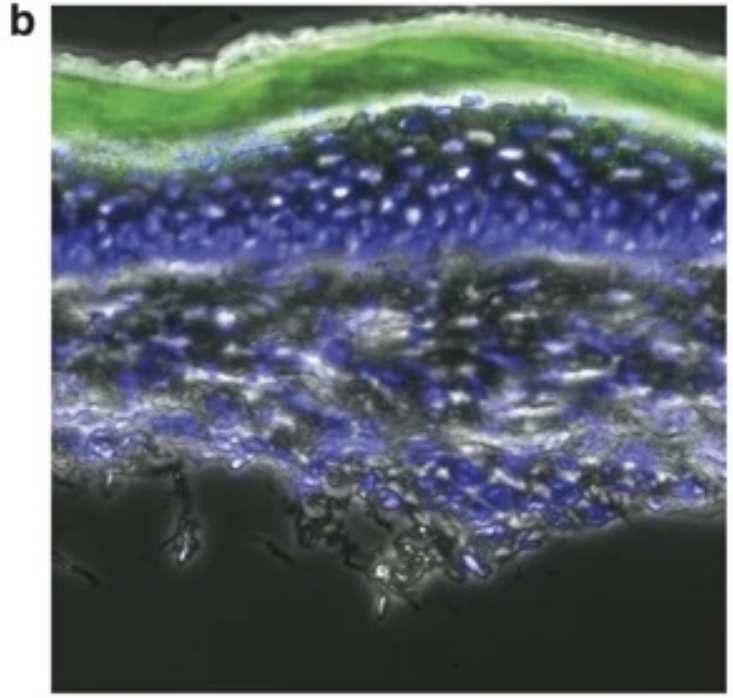
-Late promoter de CMV (muito forte).

-Quais as vantagens desses promotores?

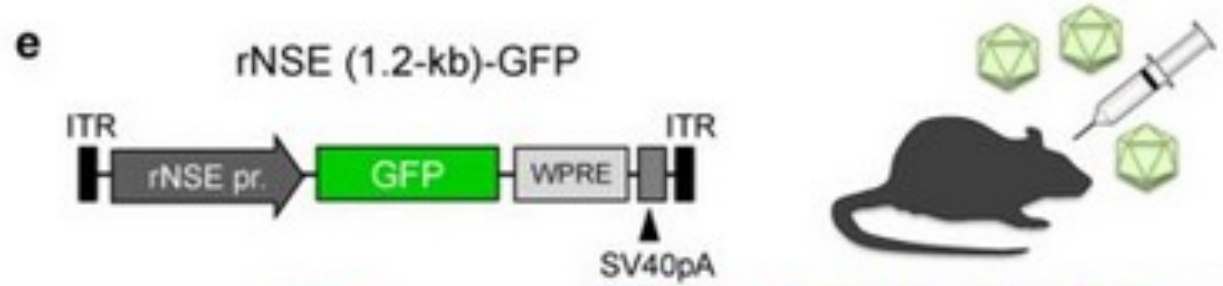
-Quais as desvantagens?

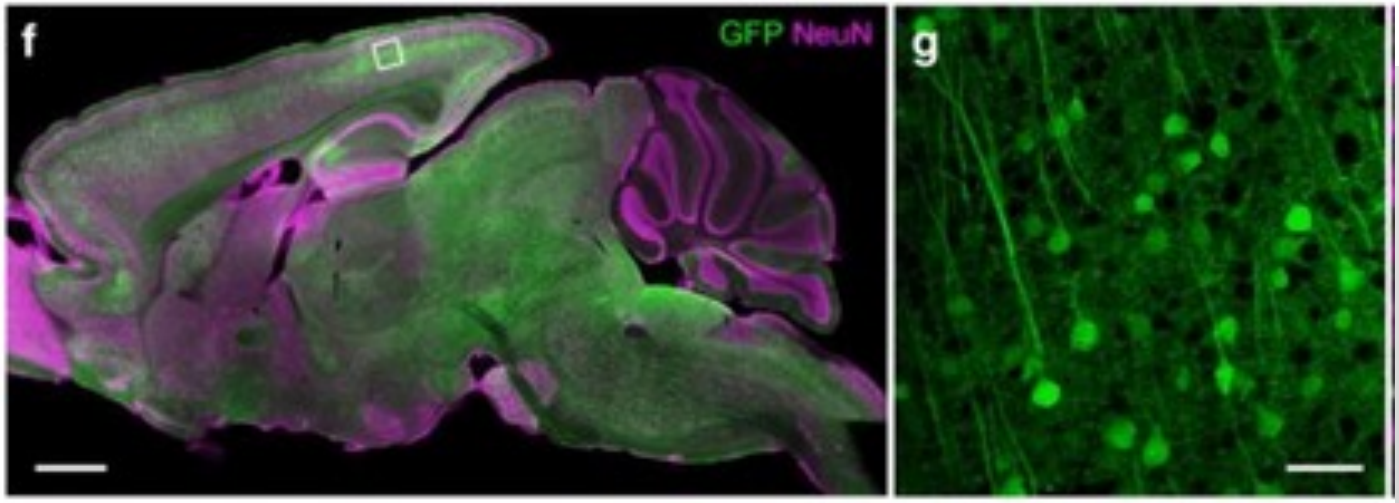
- **Promotores tecido específico!**

- Queratinócitos
(promotor do gene K14)



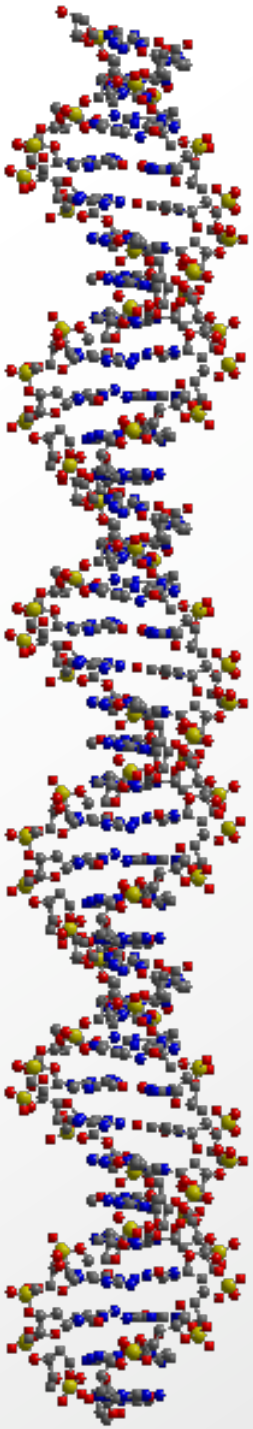
- Neurônios





Gonzalez-Gonzalez et al,
Gene Therapy, 2009

Shinohara et al,
Molecular Neurology, 2019

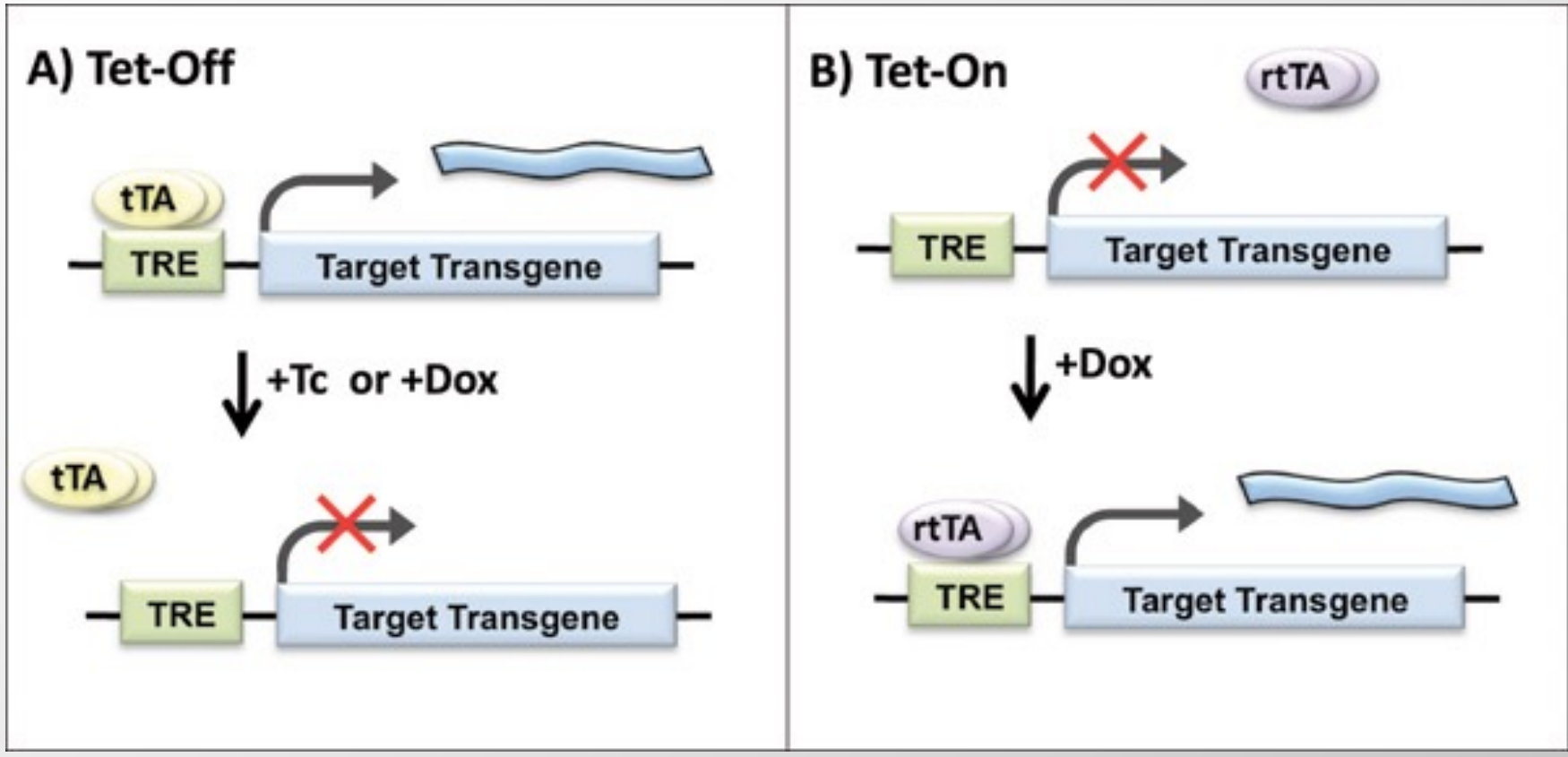


-Promotores regulaveis:

pMT (metalotioneína, indutível com Cd e Zn)

-Promotores Tet on and Tet off

(uso de tetraciclina ou doxiciclina)



https://www.youtube.com/watch?v=42FPB2joEHE

Sequencia para início de tradução em células humanas:

-não tem Shine-Dalgarno

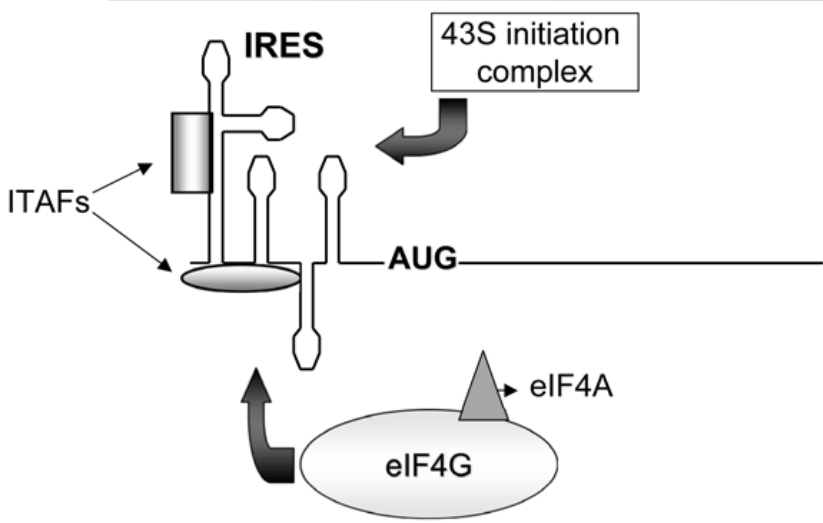
-1o AUG

- sequencia consenso:

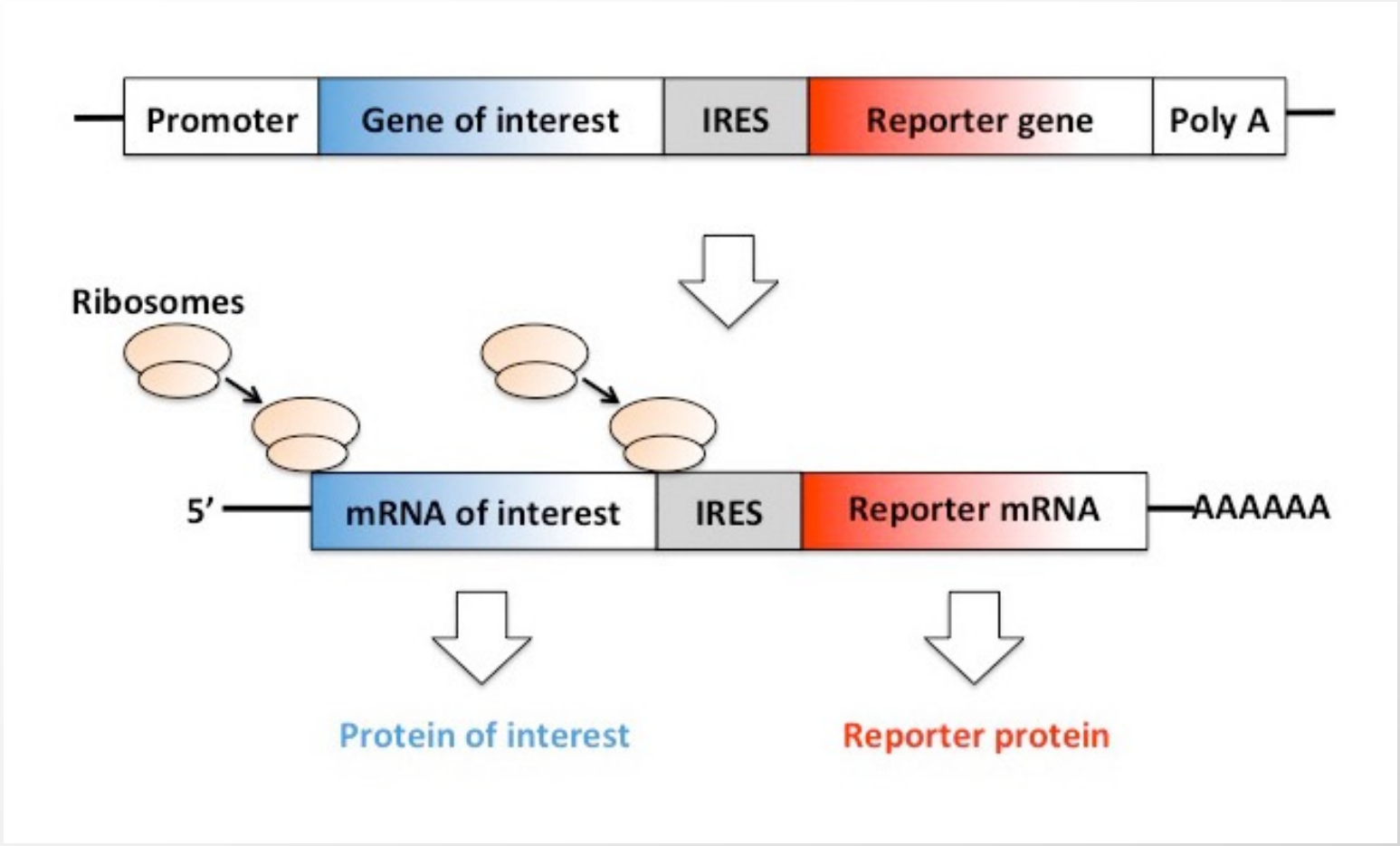
-5´GCCGCCAGCCAUGG-3´

-EXCETO IRES (poliovirus):

internal ribosome entry



-Como pode ser usado o IRES?



-Permite expressões simultâneas!

-Para que?

- o que são gene repórters?

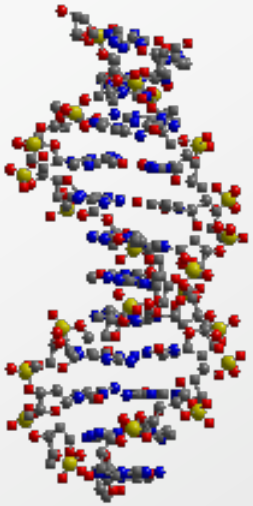
Outros sinais necessários no gene:

Sítios de splicing (cDNA ou genômico).

Consenso: seq invariável (GU...AG)

5´-AGGU(A)AGU....INTRON....(U/C)n....CAGG-3´

Mas é necessário um intron????



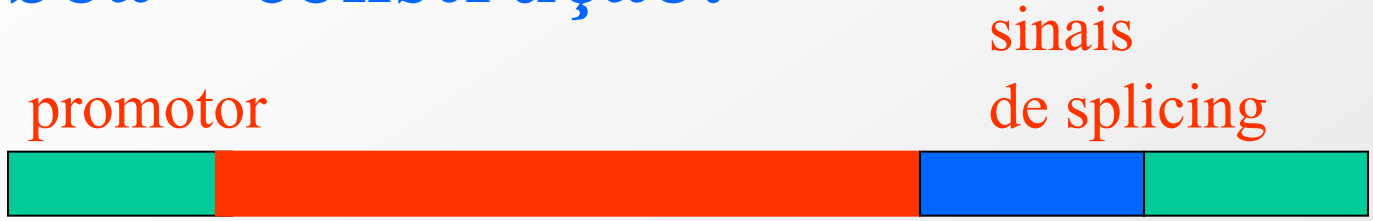
-- 3´do gene- terminação

-GU ou U rich...

-sinal de poliA= AAUAAA

-Estabilidade do transcrito.

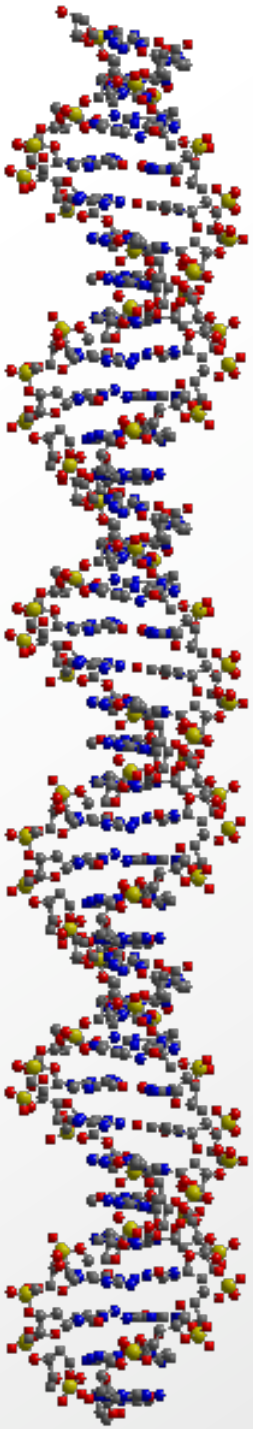
A "boa" construção:



Em que estará esta construção?

1. Plasmídeos pro ou eucariontes- cuidado com sequências veneno!!

2. Replicar ou não replicar, eis a questão? (replicons virais).



Como introduzir o DNA dentro das células?

1. **Fosfato de cálcio**
   - **-forma precipitado**
   - **-usa muito DNA (10 ug DNA/placa)**
   - **-simples mas pouco eficiente.**
   - **-shock de glicerol (tóxico)**

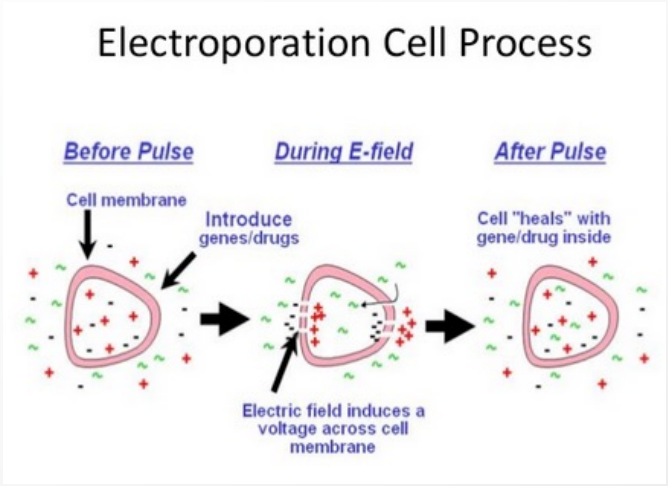
2. **DEAE-dextran (pioneiro- 1968)**
   - **- eficiente para expressão transiente.**
   - **- só para algumas células**
   - **- pouco DNA é necessário (1 ug DNA/placa).**

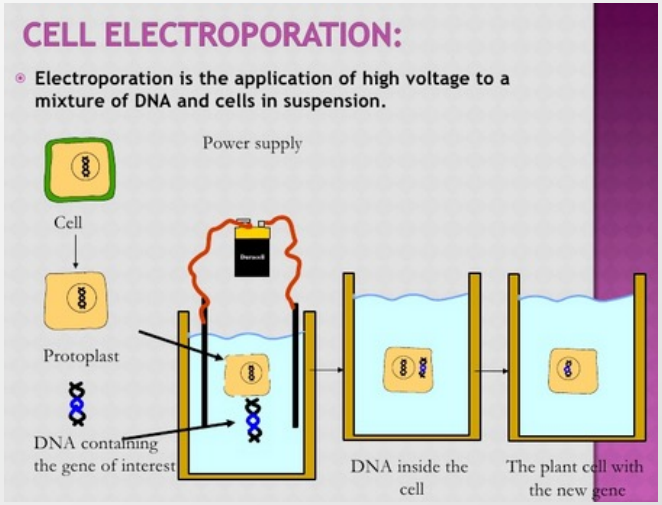
3. **Lipossomos (evolução clara com aumento de eficiência de transfecção)**

Como introduzir o DNA dentro das células?

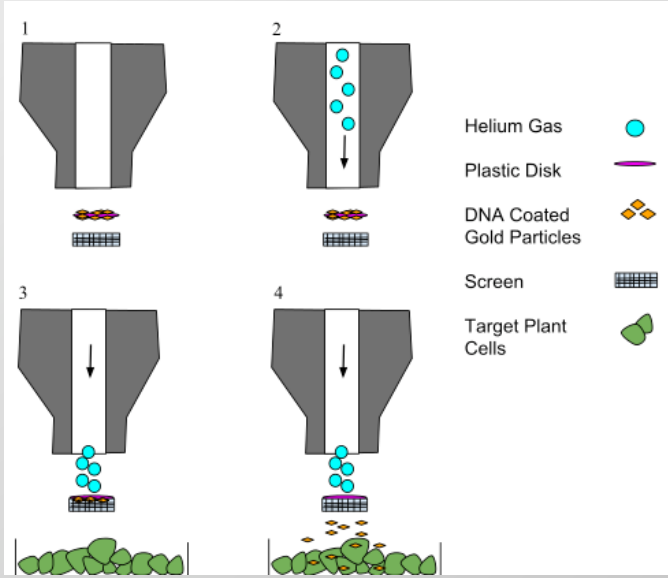
Métodos físicos:

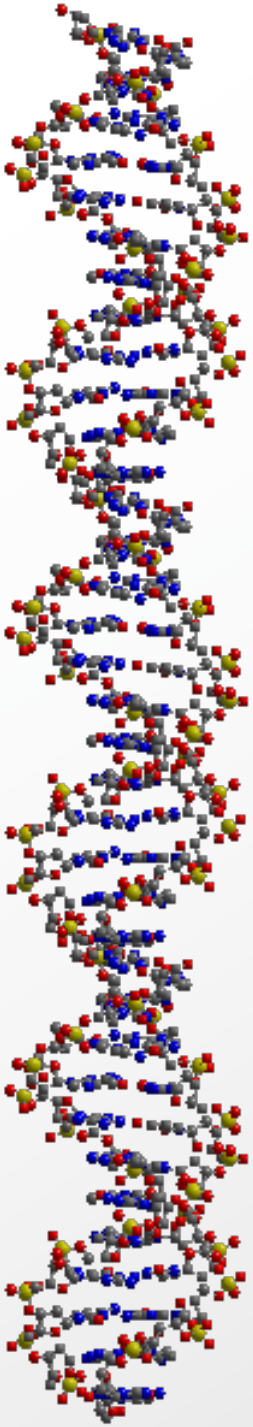
4. Eletroporação: pouco DNA





5. Microprojéteis (gene gun)





O que ocorre com o DNA?

Ataque de nucleases!

Integrar (permanente) ou não integrar (transiente):
essa não é a questão!

ONDE INTEGRAR??????

Frequencia de entrada na célula- 20%,

Mas frequencia de integração: 10^{-4} a 10^{-6}!!!

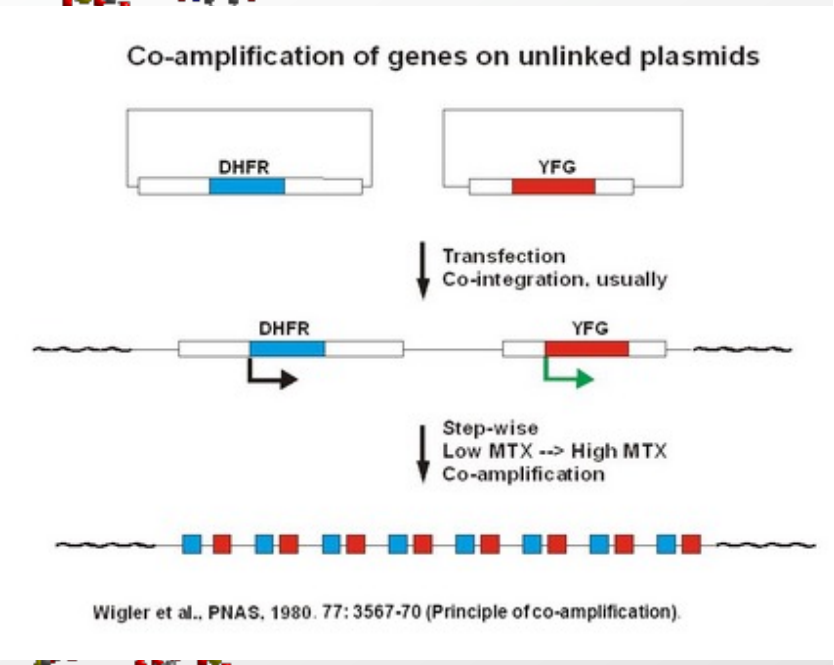
Como encontrar uma em um milhão de células??

Marcadores genéticos:

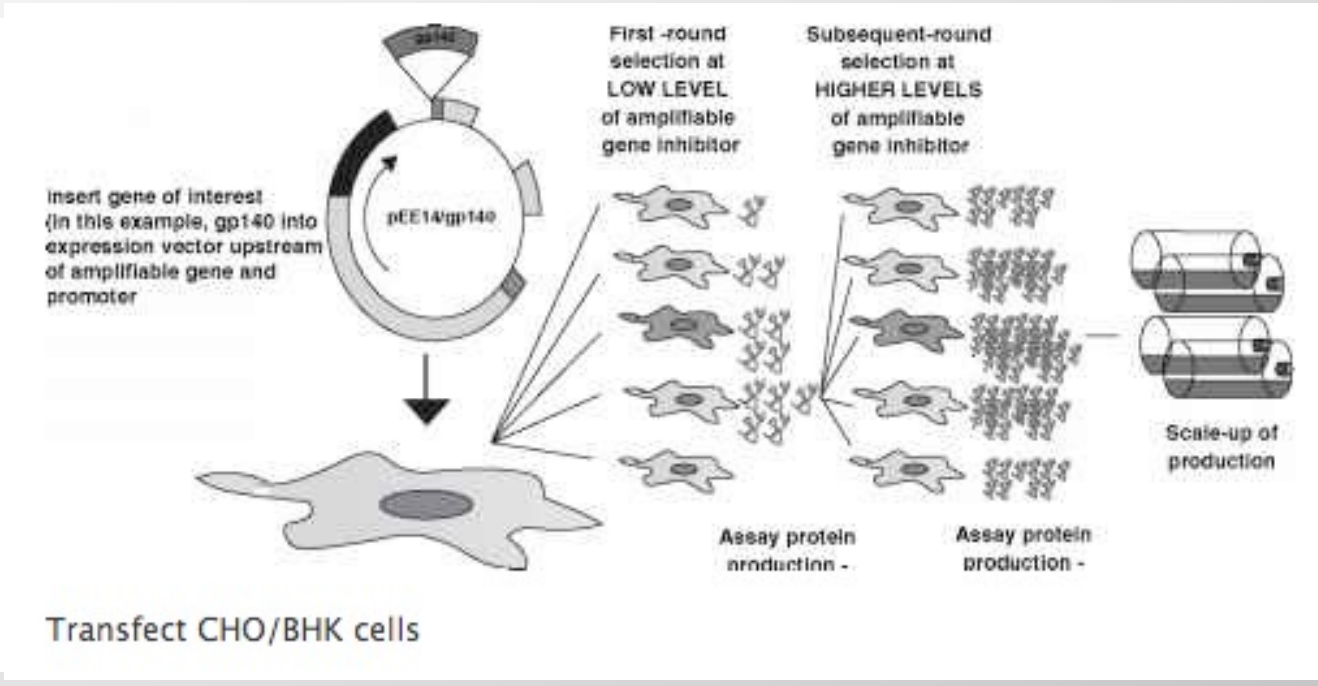
-DHFR- participa na formação de folato

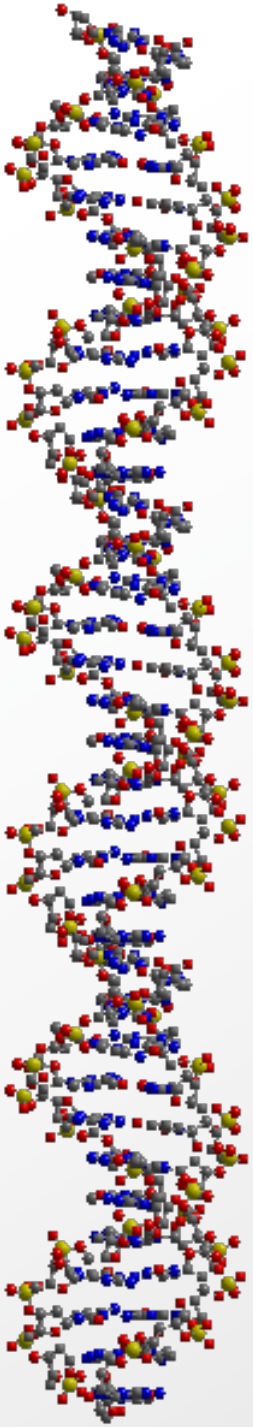
células DHFR-/-- síntese de nucleotídeos comprometida!

methotrexato e a amplificação gênica!!



Wigler et al., PNAS, 1980. 77: 3567-70 (Principle of co-amplification).





Marcadores genéticos:

Marcadores dominantes

Resistência a antibióticos:

-Neo- geneticina (=G418~ kanamicina)

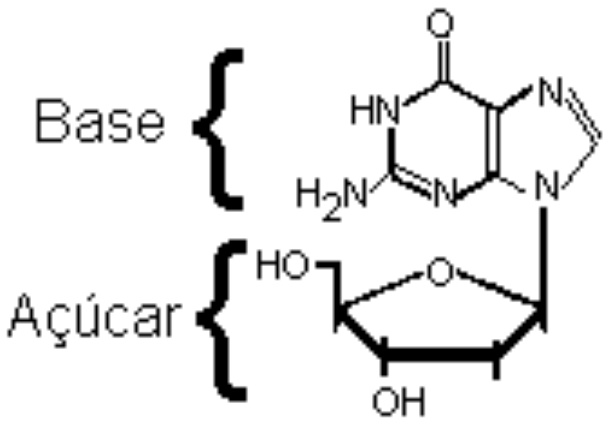
-Hyg- higromicina.

-Puromicina

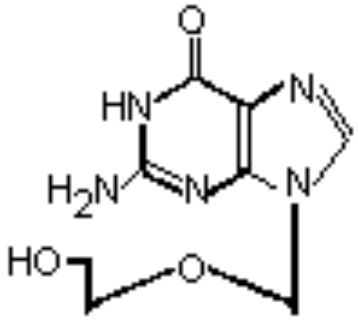
-blasticidina

Seleção Negativa (por que usar?):

Timidina quinase de Herpes:



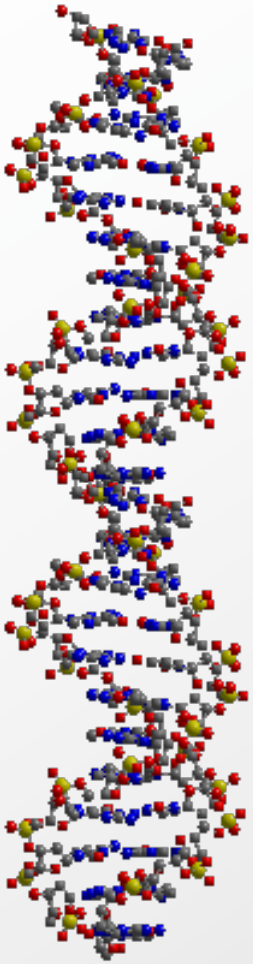
Deoxiguanosina

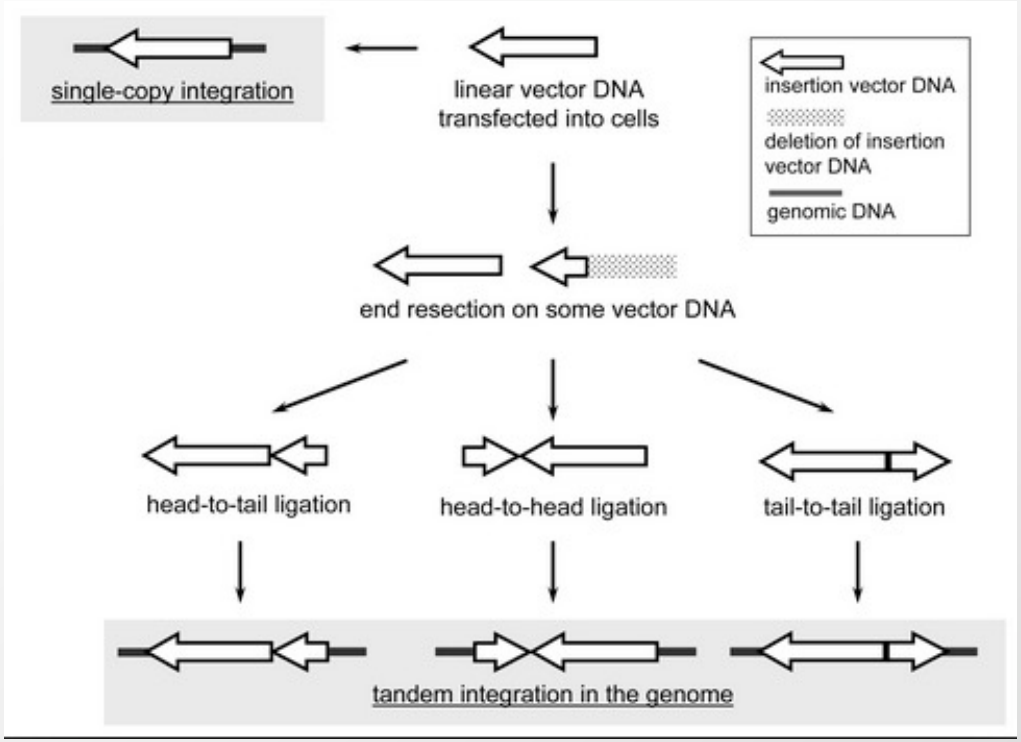


Aciclovir

Mas como usar os marcadores para introduzir Genes nas células?

- integração de DNA na célula em tandem! (qual a vantagem?)



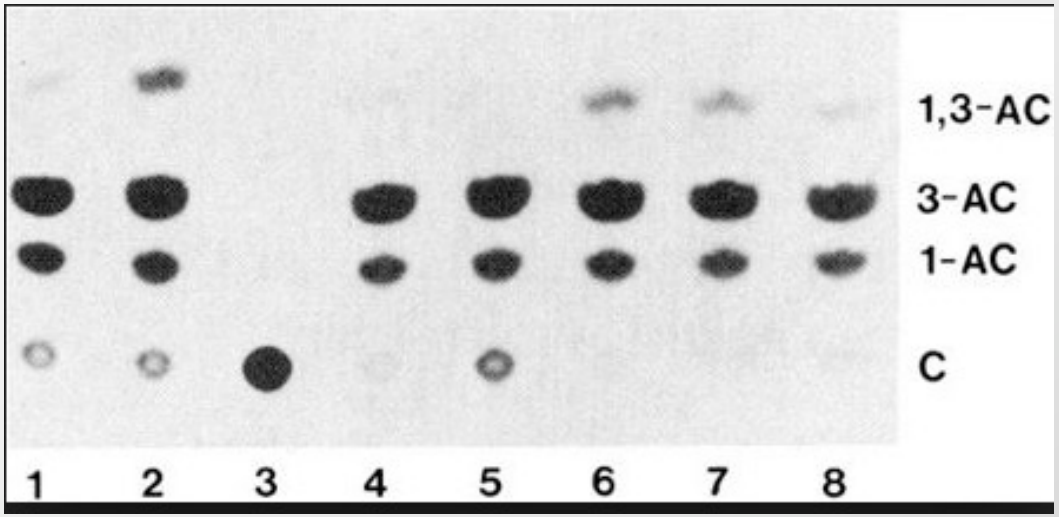


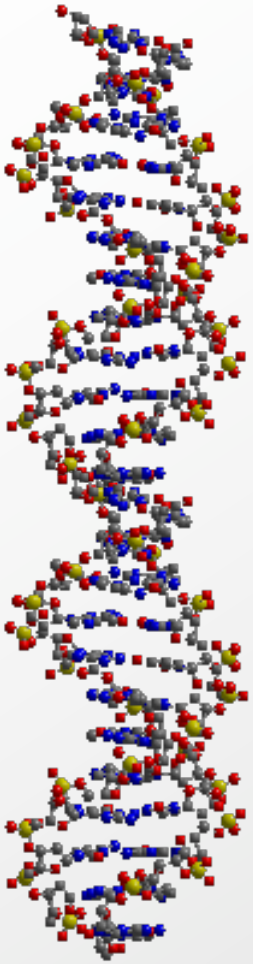
- clivar o DNA (linear X super coil).

(DNA com pontas livres integra no genoma mais eficientemente)

Noções de gene repórter:

- CAT (cloranfenicol acetil transferase)



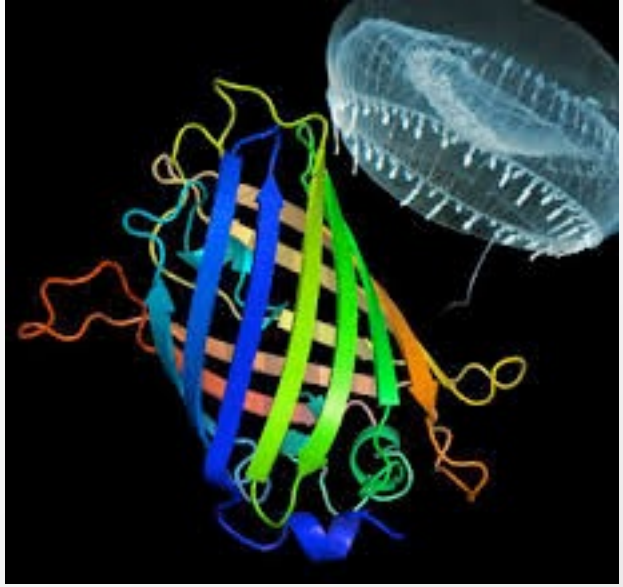


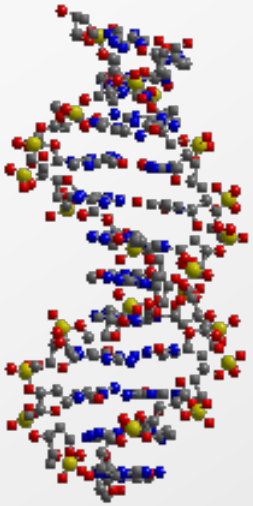
- beta-galactosidase
- EGFP
- luciferase

EGFP:

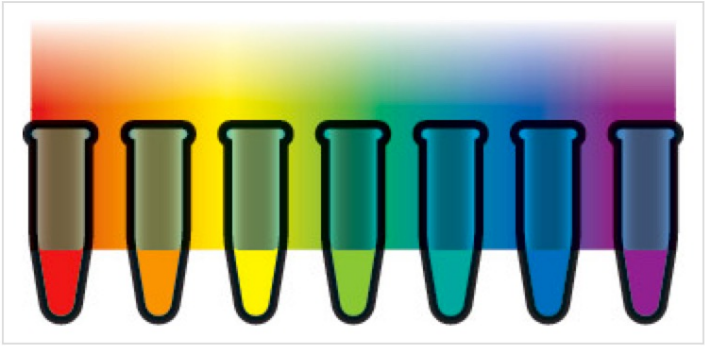
Jelly fish protein

Green Fluorescent protein





Outras cores:



In 1999, Russian scientists isolated a red fluorescent protein, DsRED, from a coral. This protein was larger and more cumbersome than GFP. Tsien, however, managed to decrease the size of DsRED. From DsRED, Tsien also developed proteins with mouth-watering names like mPlum, mCherry, mStrawberry, mOrange and mCitrine.

The researchers called this experiment “the brainbow”.

Researchers at Harvard University in the USA have coloured the nerve cells in a mouse’s brain so that it fluoresces in all the colours of the rainbow. The nerve cells produce different amounts of three GFP-like proteins that fluoresce yellow, cyan and red, mimicking the colours used in a printer. This enables researchers to see how individual nerve cells in the brain are woven together in a network. Photo: Livet et al (2007) Nature 450 56-63.

EGFP: Green Fluorescent protein NOBEL Química 2008

"for the discovery and development of the green fluorescent protein, GFP"

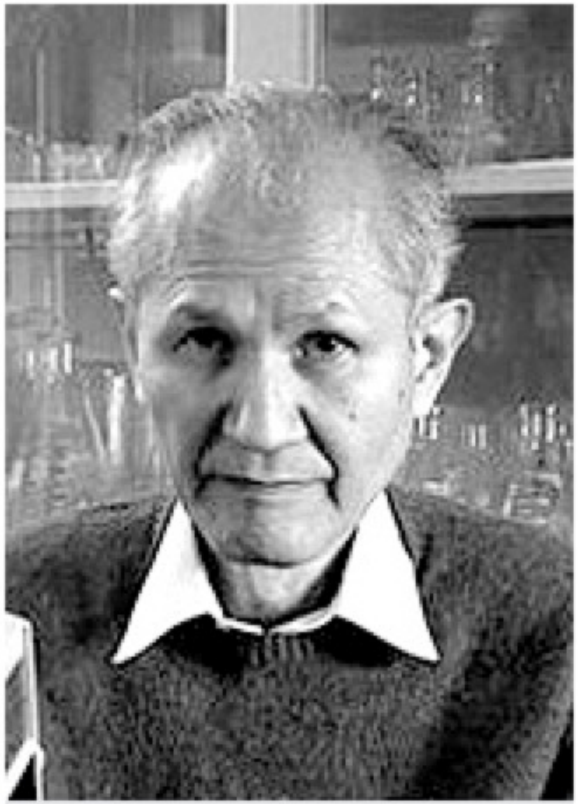


Photo: J. Henriksson/SCANPIX

Osamu Shimomura

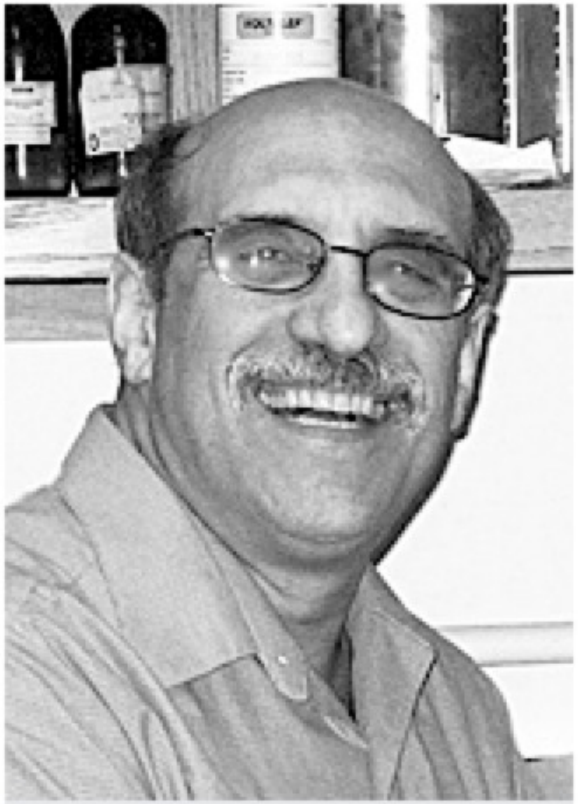
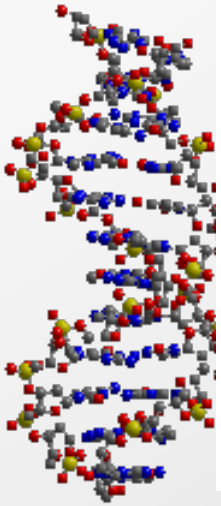


Photo: J. Henriksson/SCANPIX

Martin Chalfie

Photo: UCSD

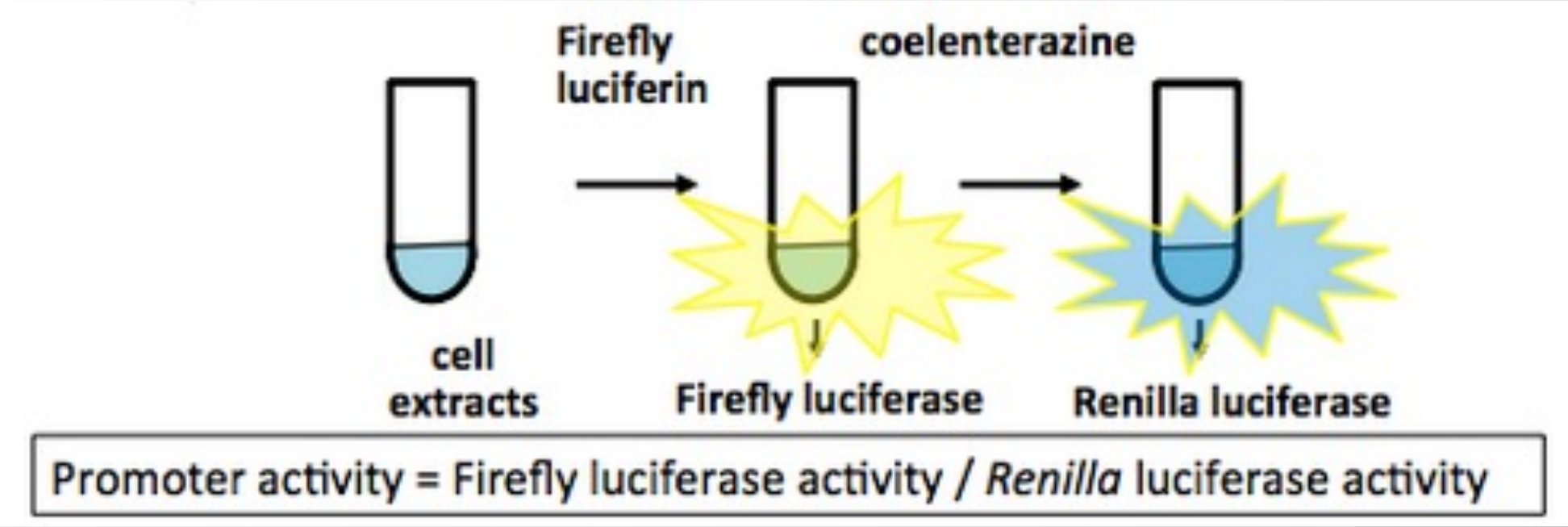
Roger Y. Tsien



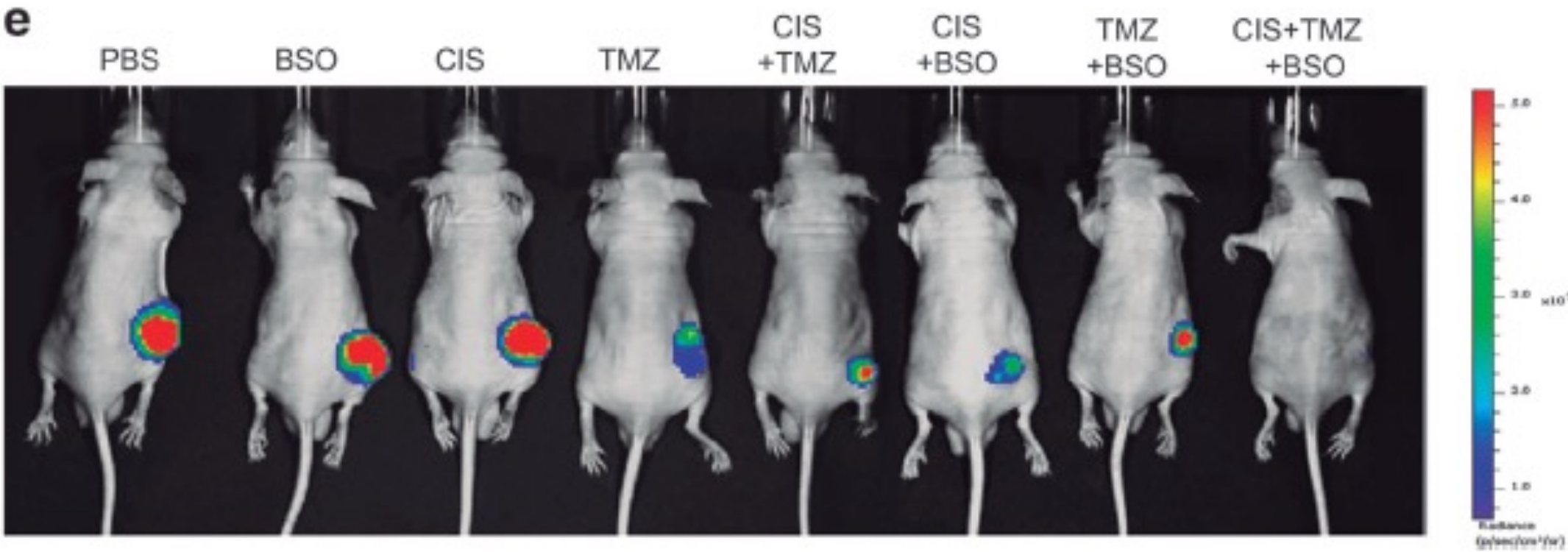
Outro gene repórter importante: luciferase!!

Por que? Qual a vantagem em relação ao GFP?

A luciferase precisa de substrato, o GFP não!!!!



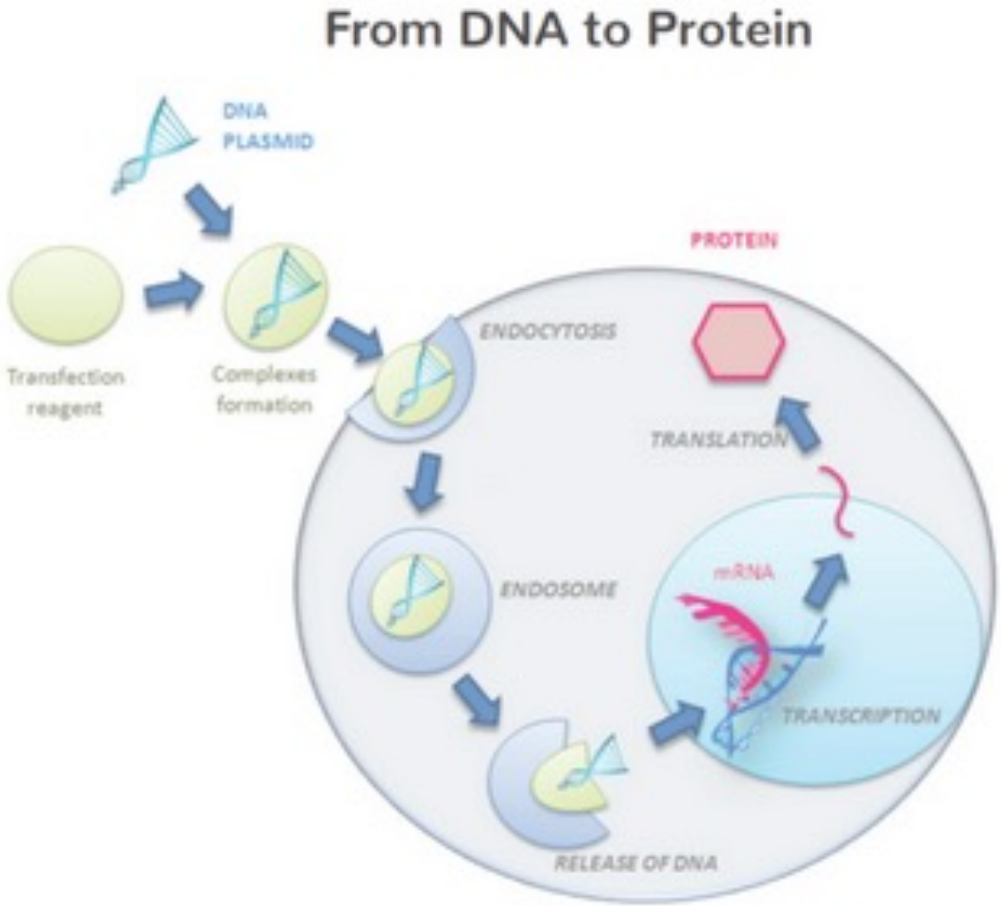
Visualização in vivo em IVIS!!!

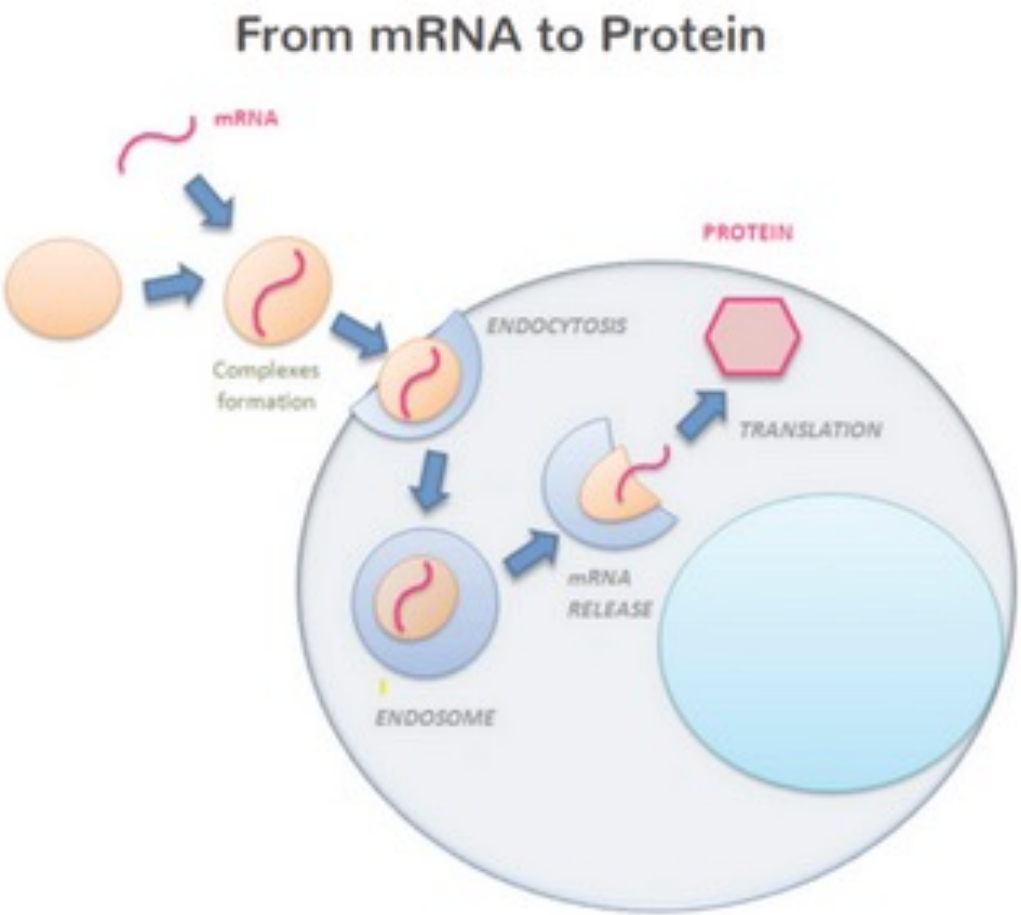


Clarissa Rocha

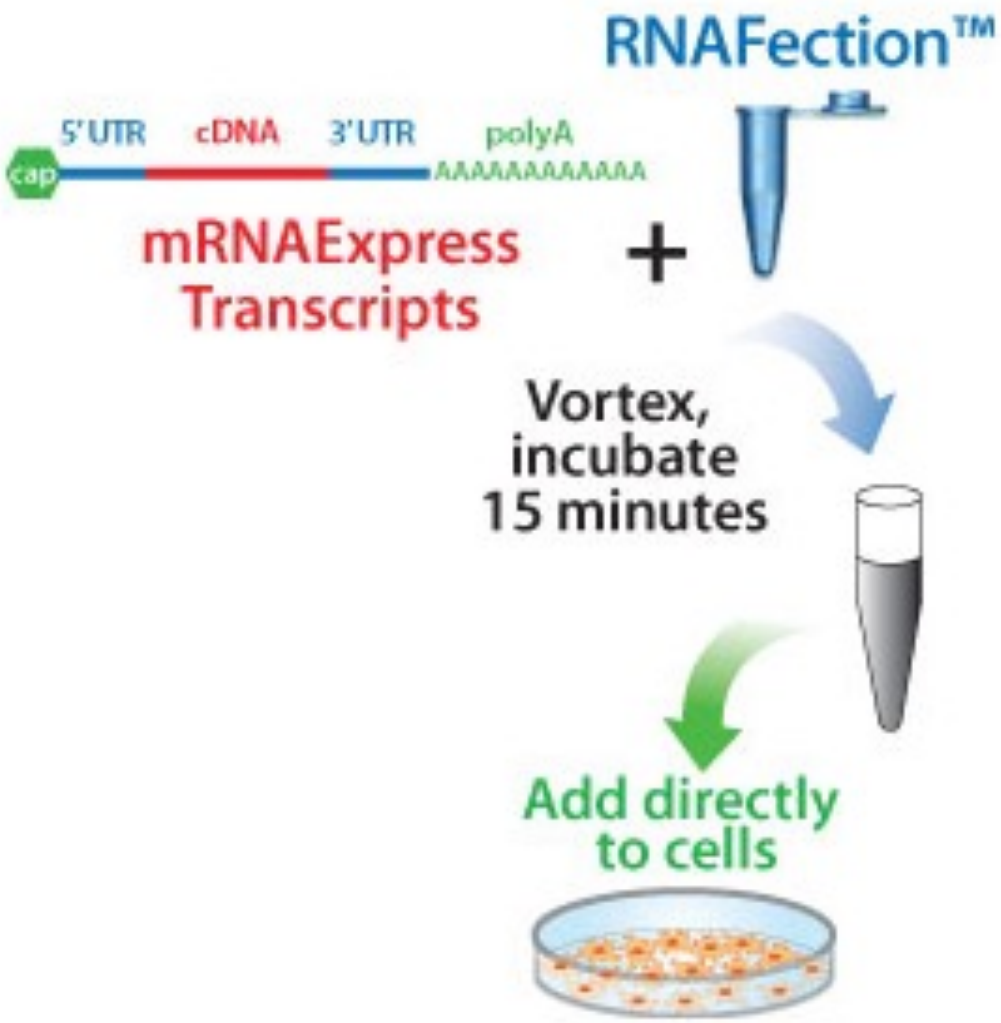
A revolução da transfecção de RNA!!!

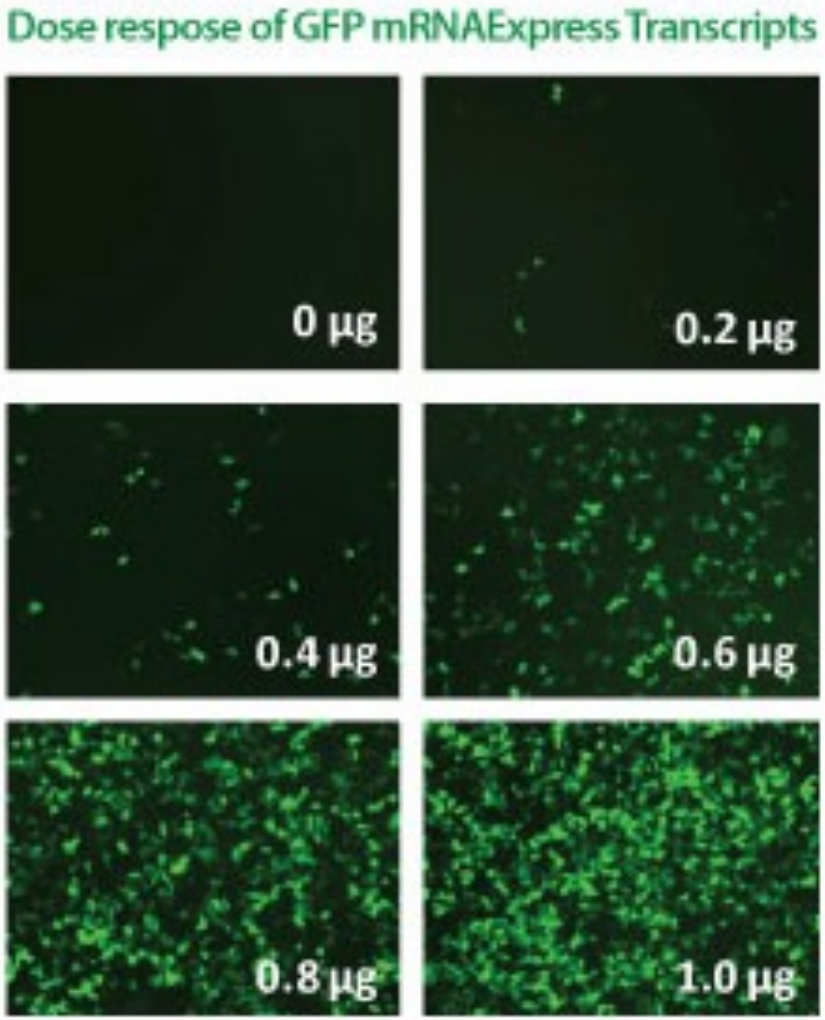
Kati Karikó



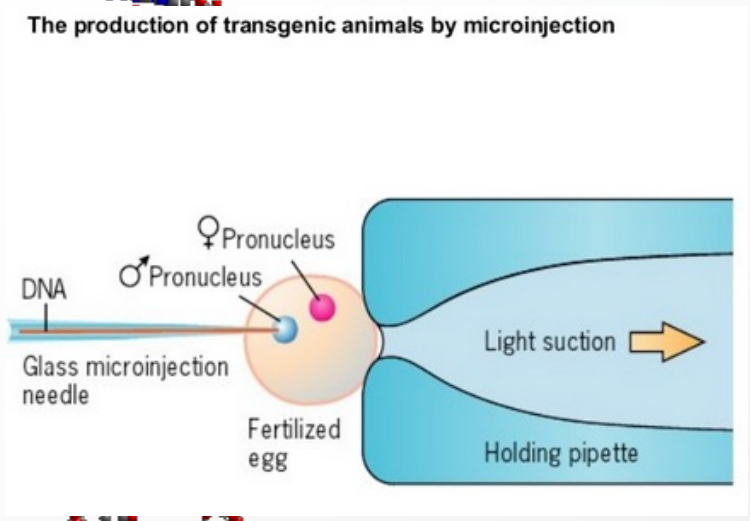


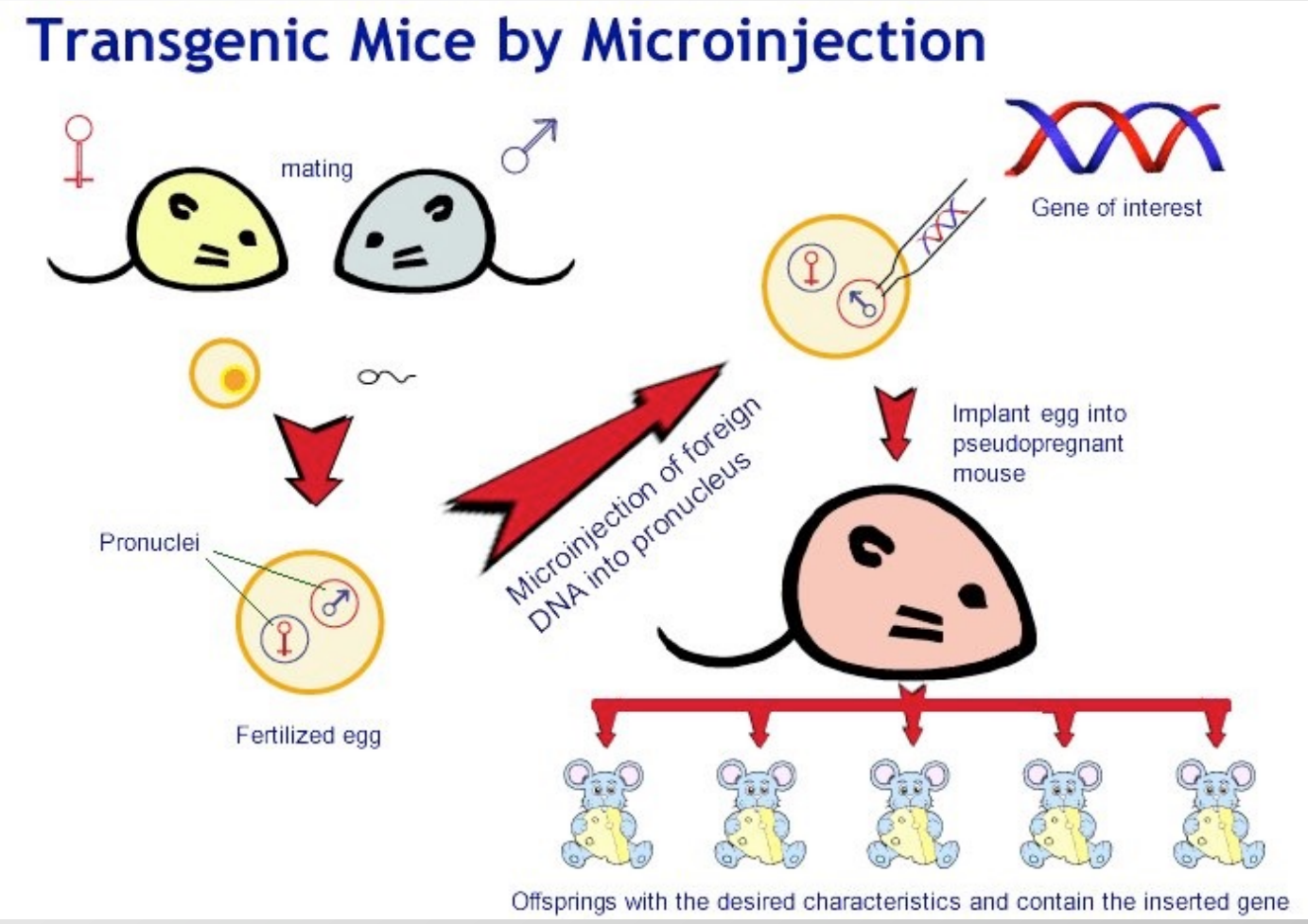
A revolução da transfecção de RNA!!!



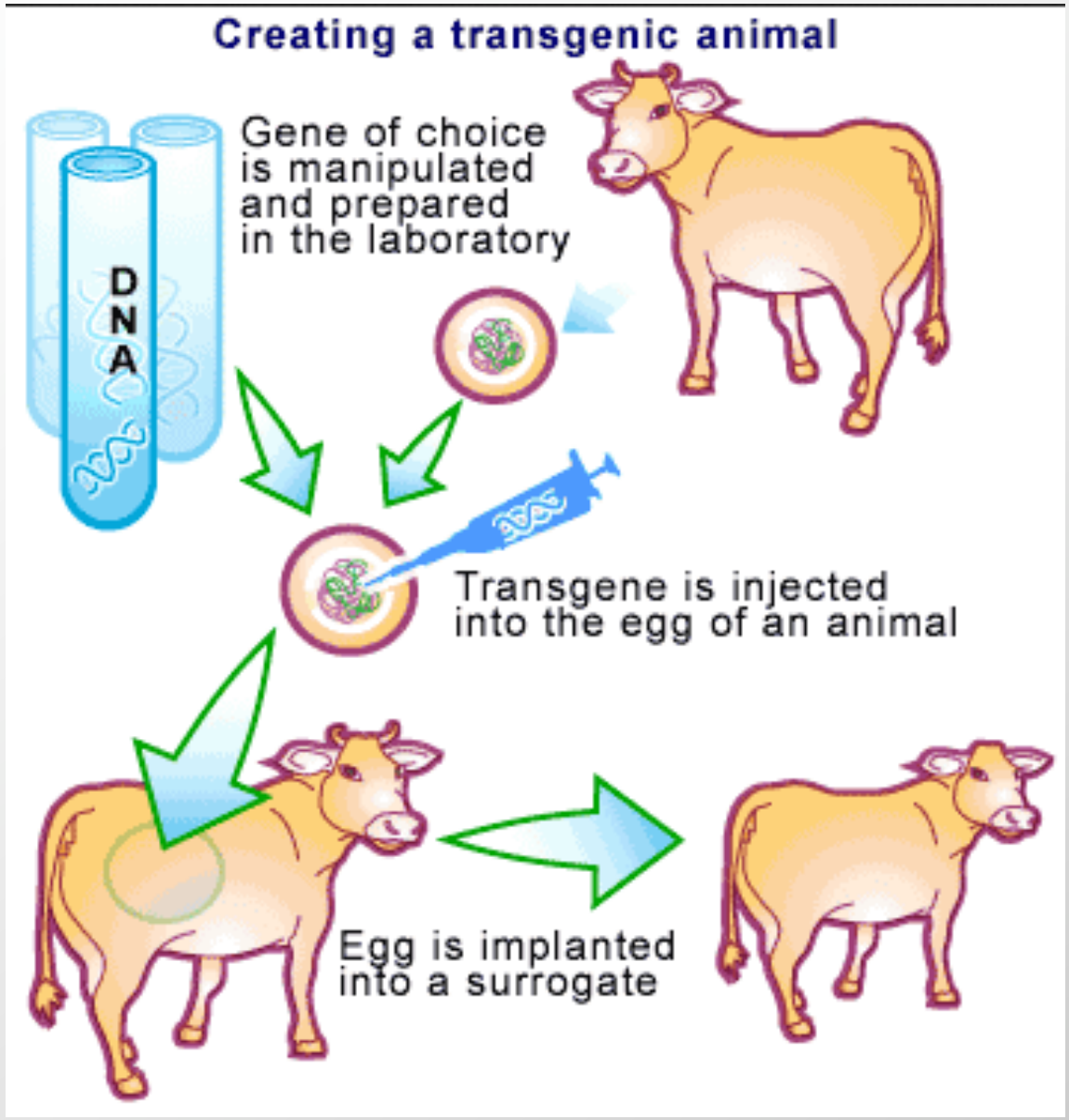


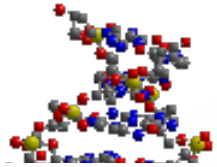
Camundongos transgênicos





Animais transgenicos: produção agropecuária!



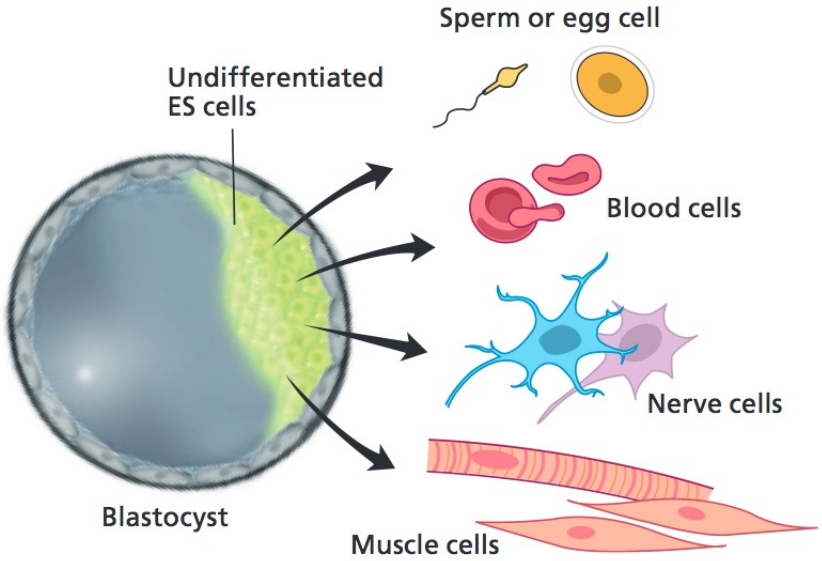


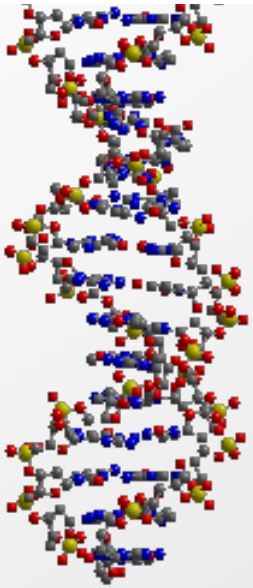
Células tronco embrionárias

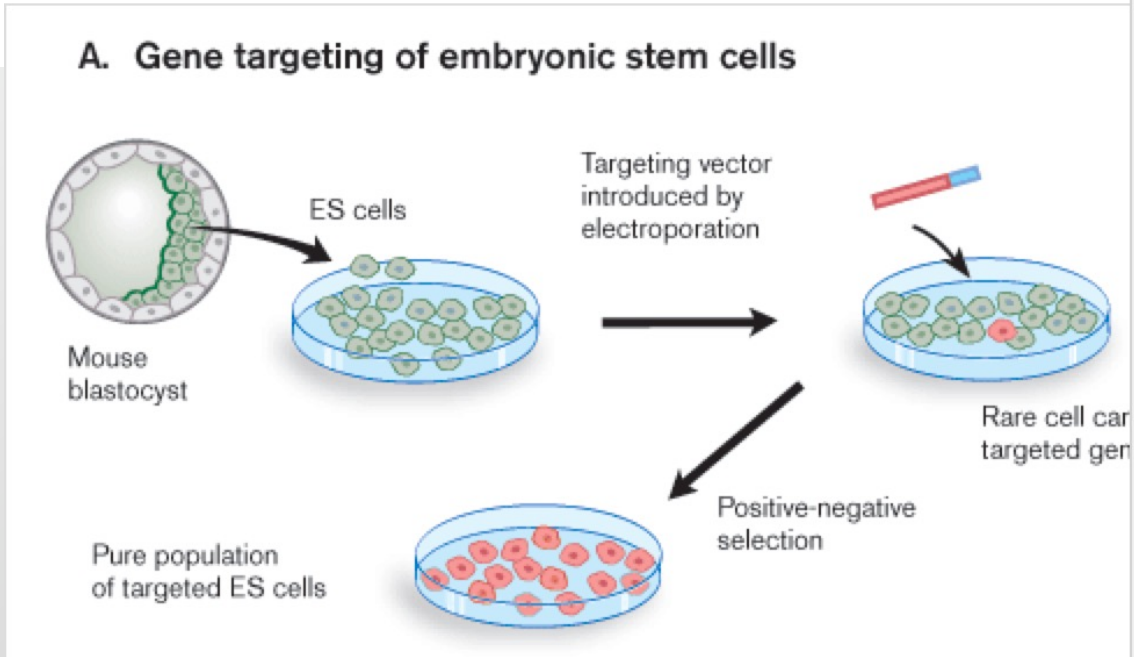
Embryonic stem cells

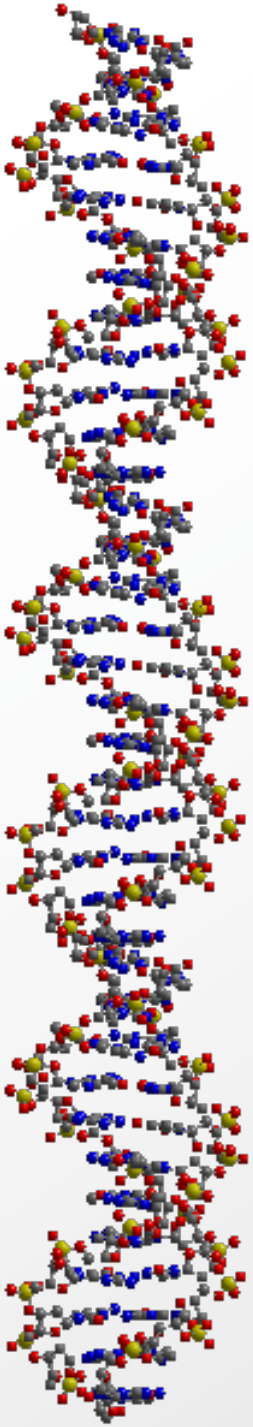
An early embryo is called a blastocyst. The cells in the outer layer (gray) will form the placenta. During a normal pregnancy, cells in the cell mass on the inside (green) would develop into an embryo.

These embryonic stem cells, ES cells, have not yet differentiated. This means they are still capable of developing into any of the cell types required to make up the new individual.









Células tronco embrionárias e disrupção gênica. NOBEL 2007

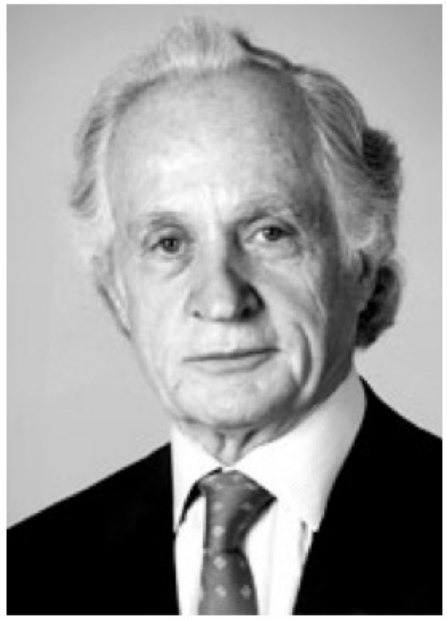


Photo: U. Montan

Mario R. Capecchi

Prize share: 1/3

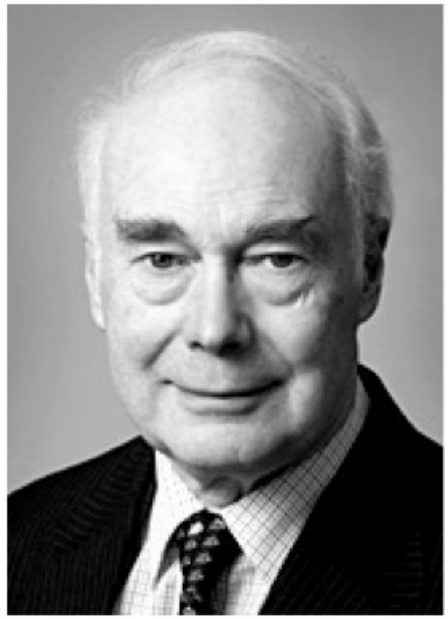


Photo: U. Montan

Sir Martin J. Evans

Prize share: 1/3

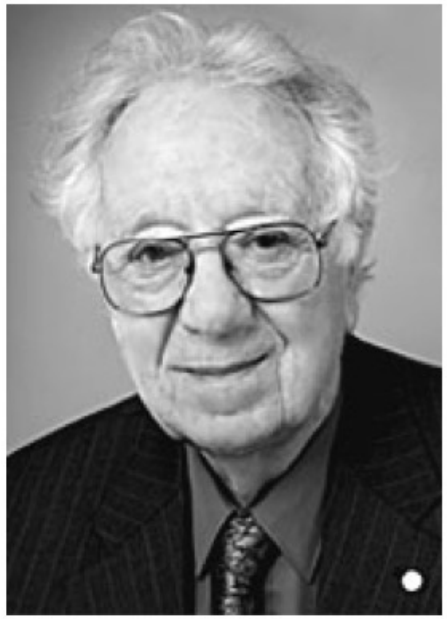


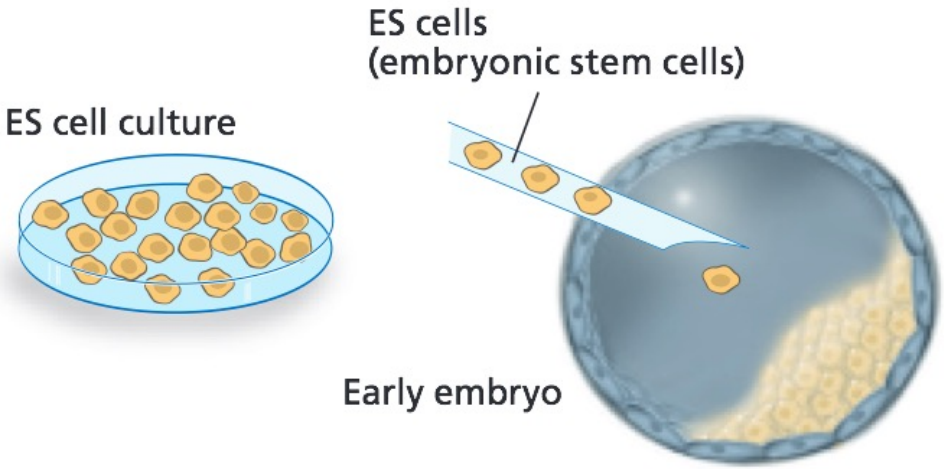
Photo: U. Montan

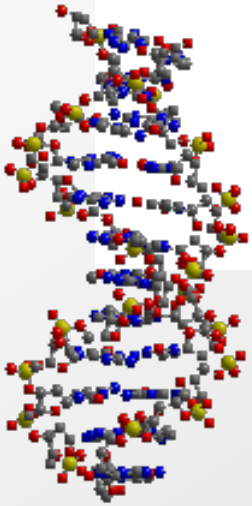
Oliver Smithies

Prize share: 1/3

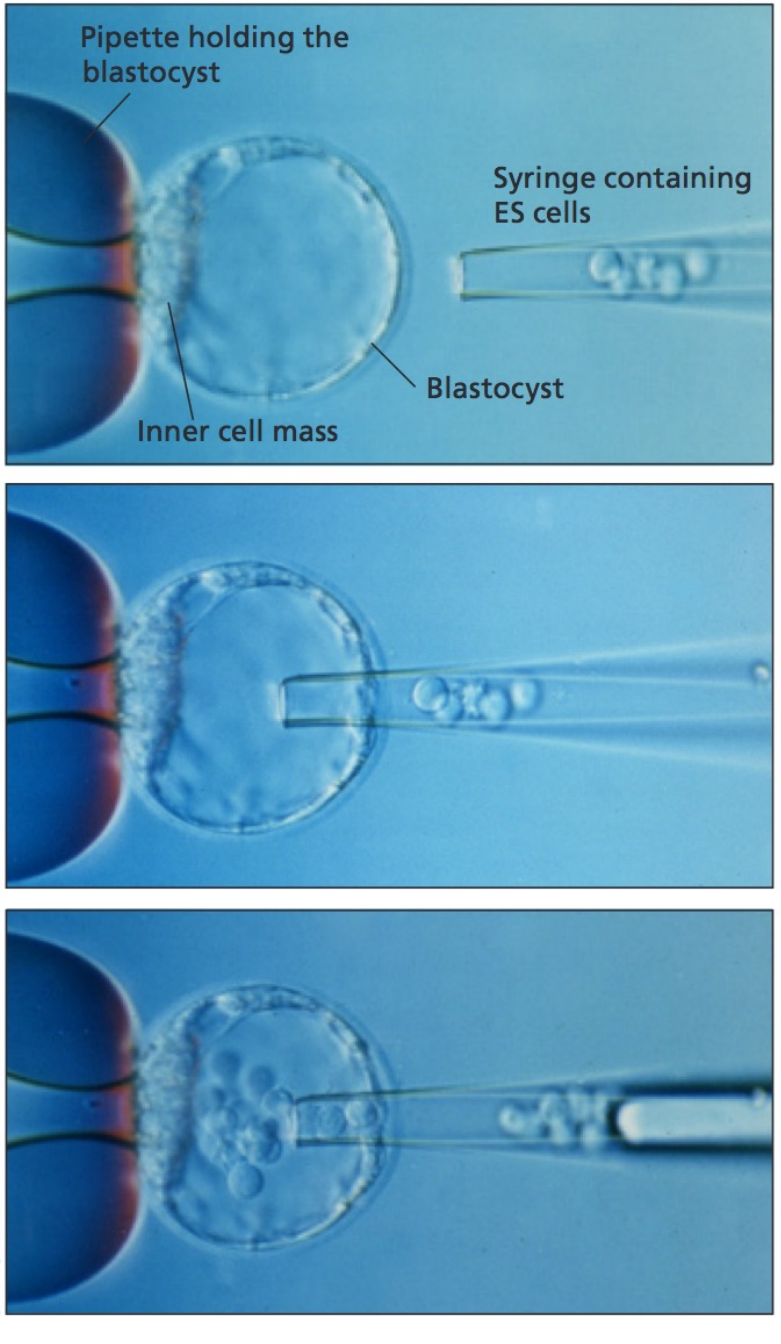
The Nobel Prize in Physiology or Medicine 2007 was awarded jointly to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies *"for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells"*.

Células tronco embrionárias



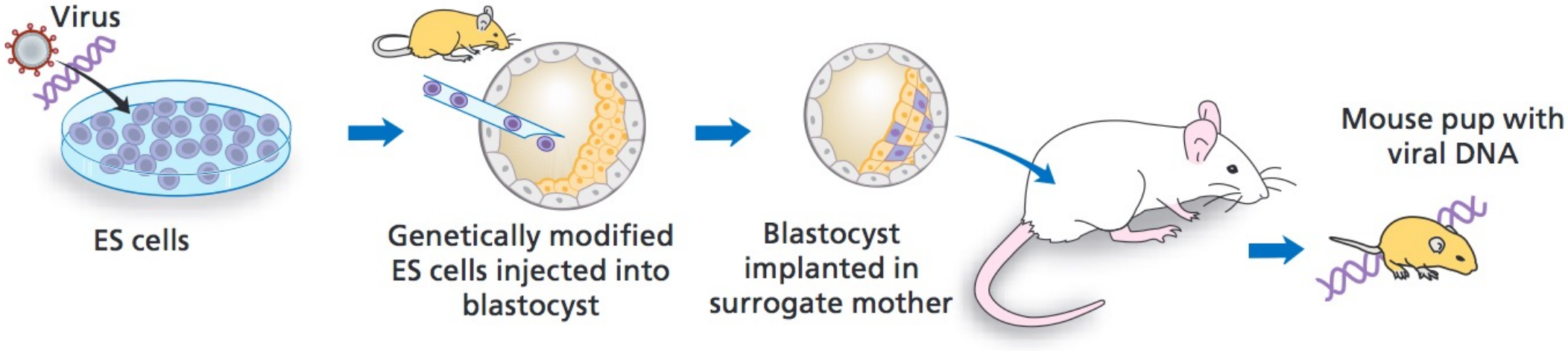


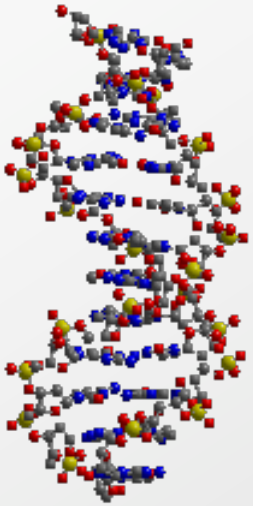
Evans showed that if a genetic modification is inserted into an ES cell that later gives rise to a new individual, the modification will be present in every cell of that new individual.

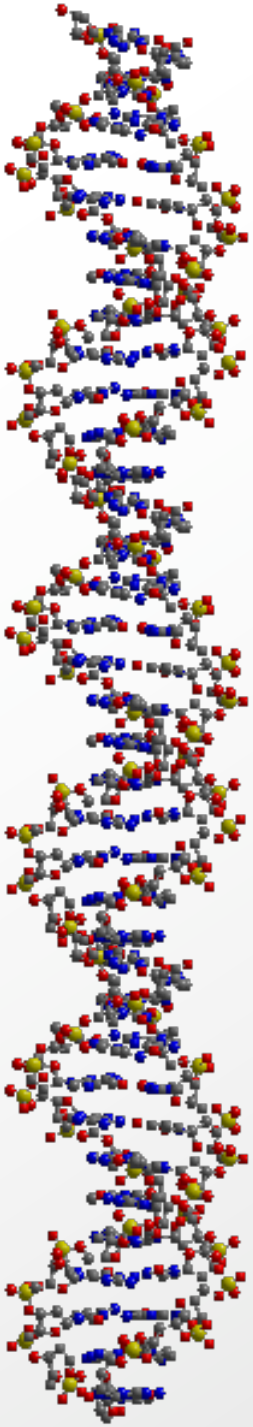


Células tronco embrionárias- elas podem passar de geração em geração!!!

CAMUNDONGOS OUIMERAS....







B. Generation of gene targeted mice

Targeted ES cells are injected into blastocysts...

...which are implanted into foster mothers

...which give birth to chimeric mice

Mating between chimeric mouse and normal mouse.

♀

♂

Egg

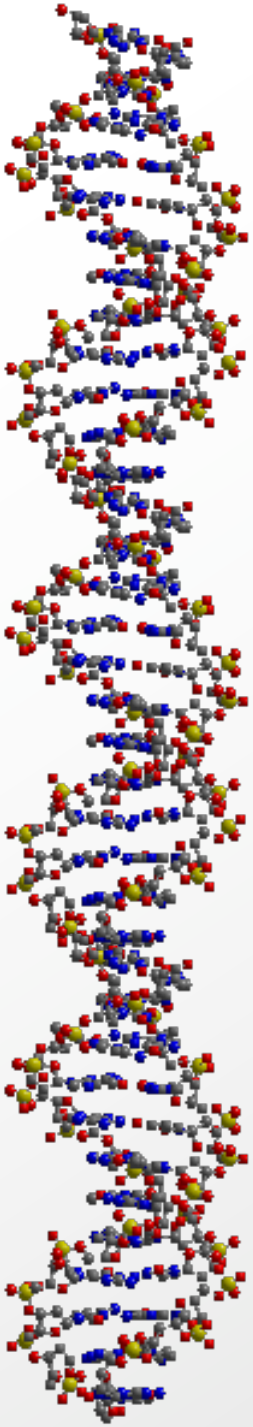
Sperm

Egg

Sperm

Gene targeted mice

Normal mice



Células tronco embrionárias: gene targeting

HGPRT gene (resistência a 6-tioguanina):

Lesch Nyhan syndrome

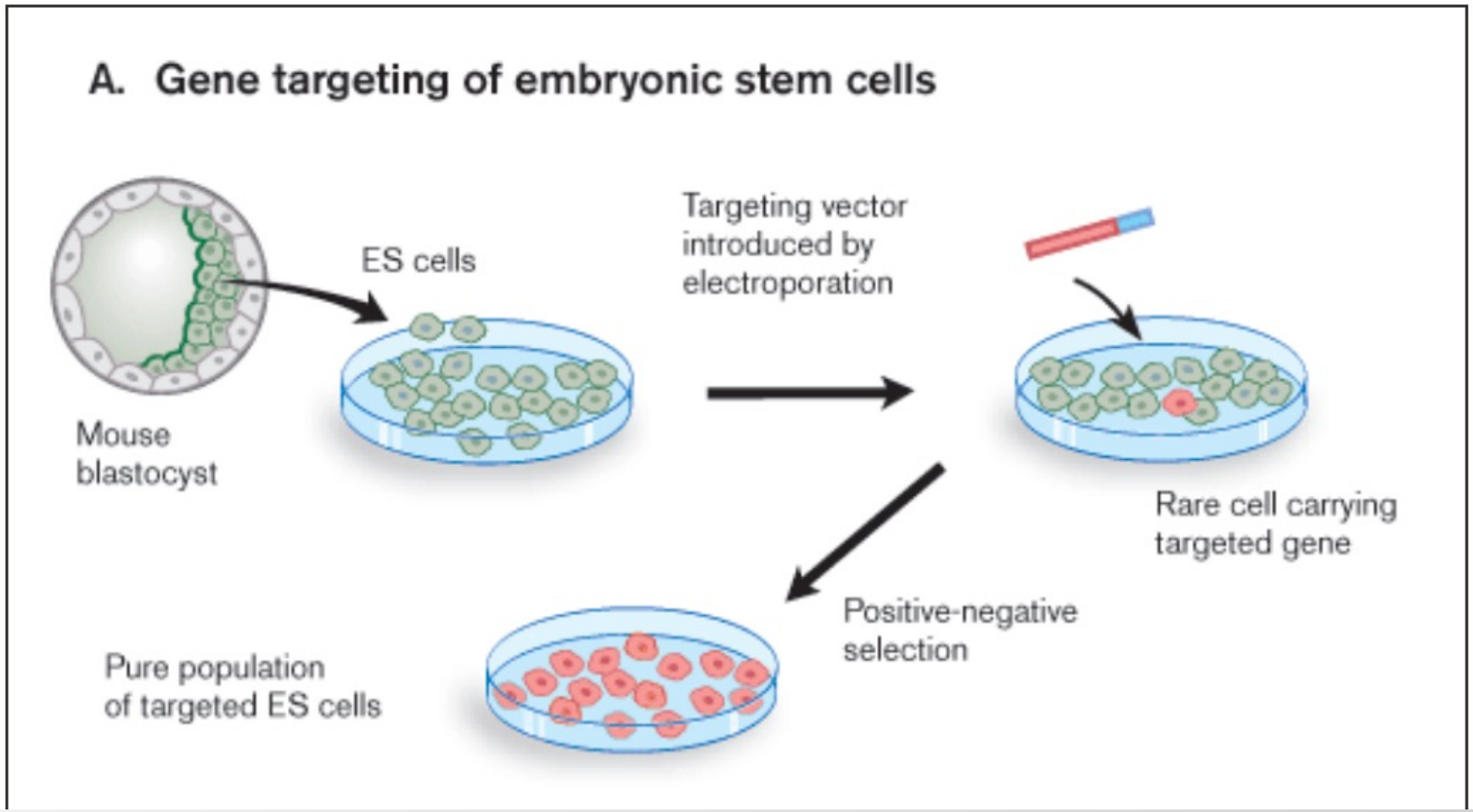
Síndrome ligada ao X_ afeta principalmente homens (uma cópia)

Capecchi e Smithies conseguiram gerar ES com a mutação em camundongos

Frequência de 1/1000:

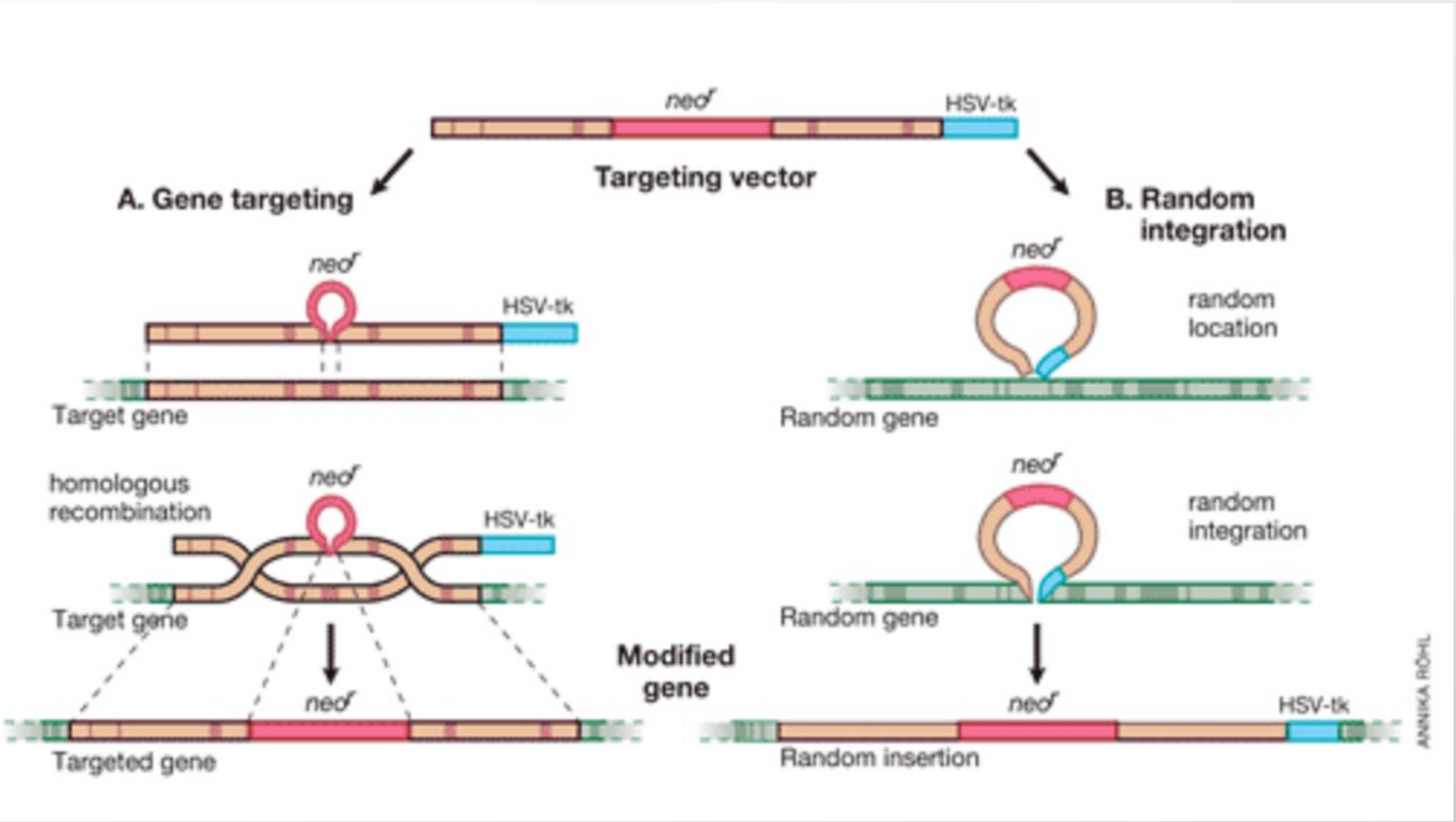
NÃO SIMULOU A SÍNDROME!

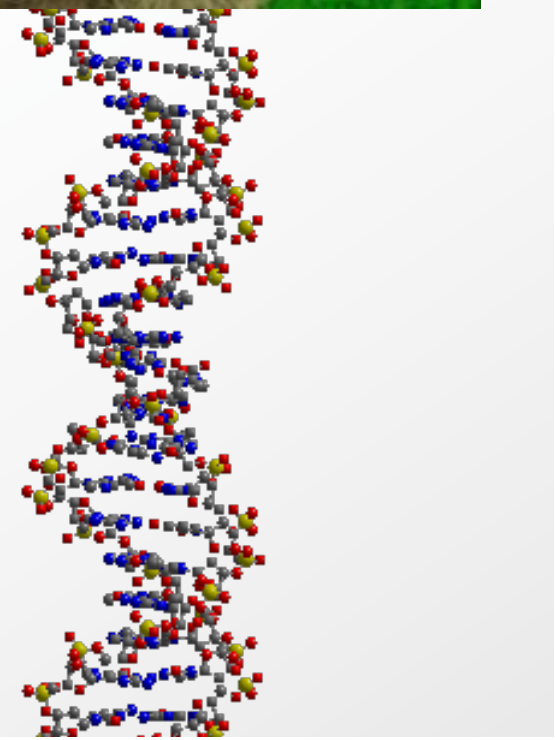
gene targeting por recombinação homóloga!



Por que precisa seleção positiva e negativa?

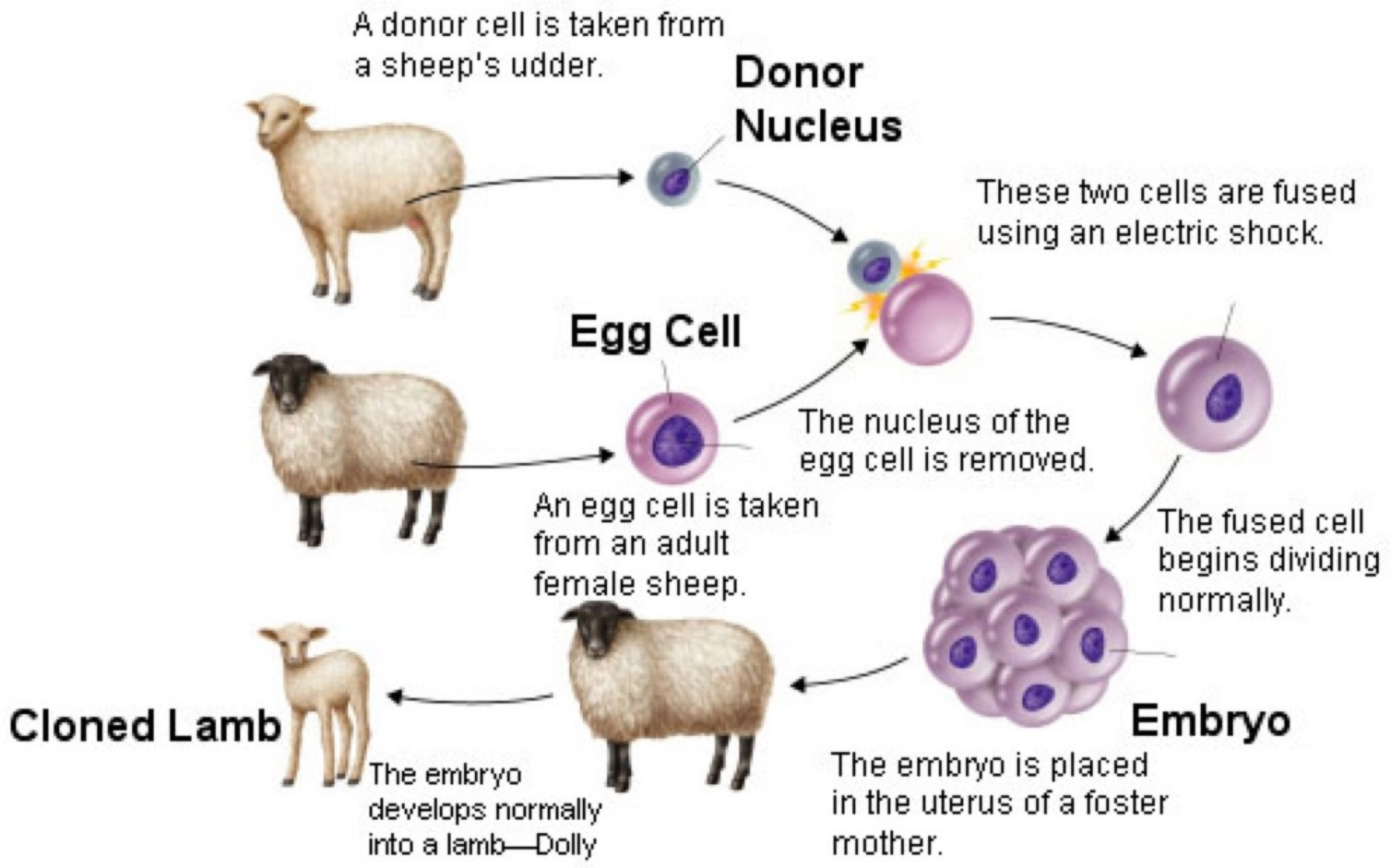
Gene targeting: seleção positiva e negativa!



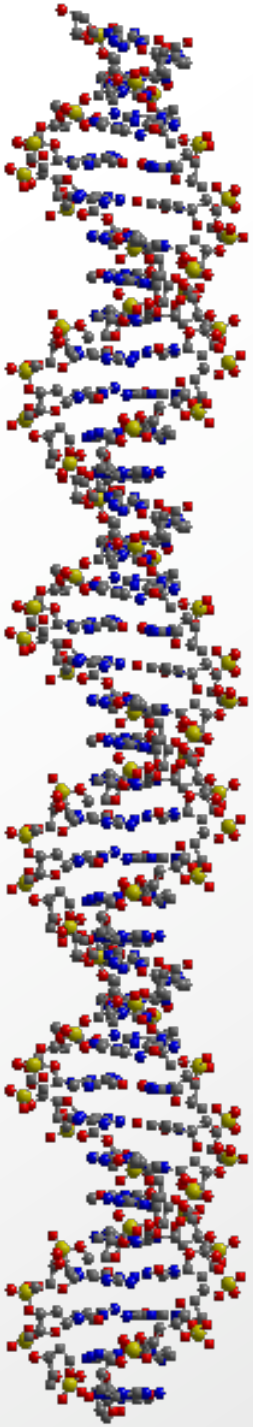


Reprogramação nuclear: clonagem

Dolly, a ovelha!!!!



Keith Campbell, Ian Wilmut, Edinburg, Escócia



Reprogramando células adultas em Células embrionárias: prêmio nobel 2012

Photo: U. Montan

Sir John B. Gurdon

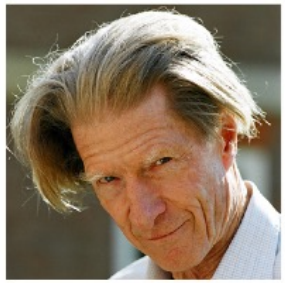
Prize share: 1/2

Photo: U. Montan

Shinya Yamanaka

Prize share: 1/2

Reprogramando células adultas em 1962!



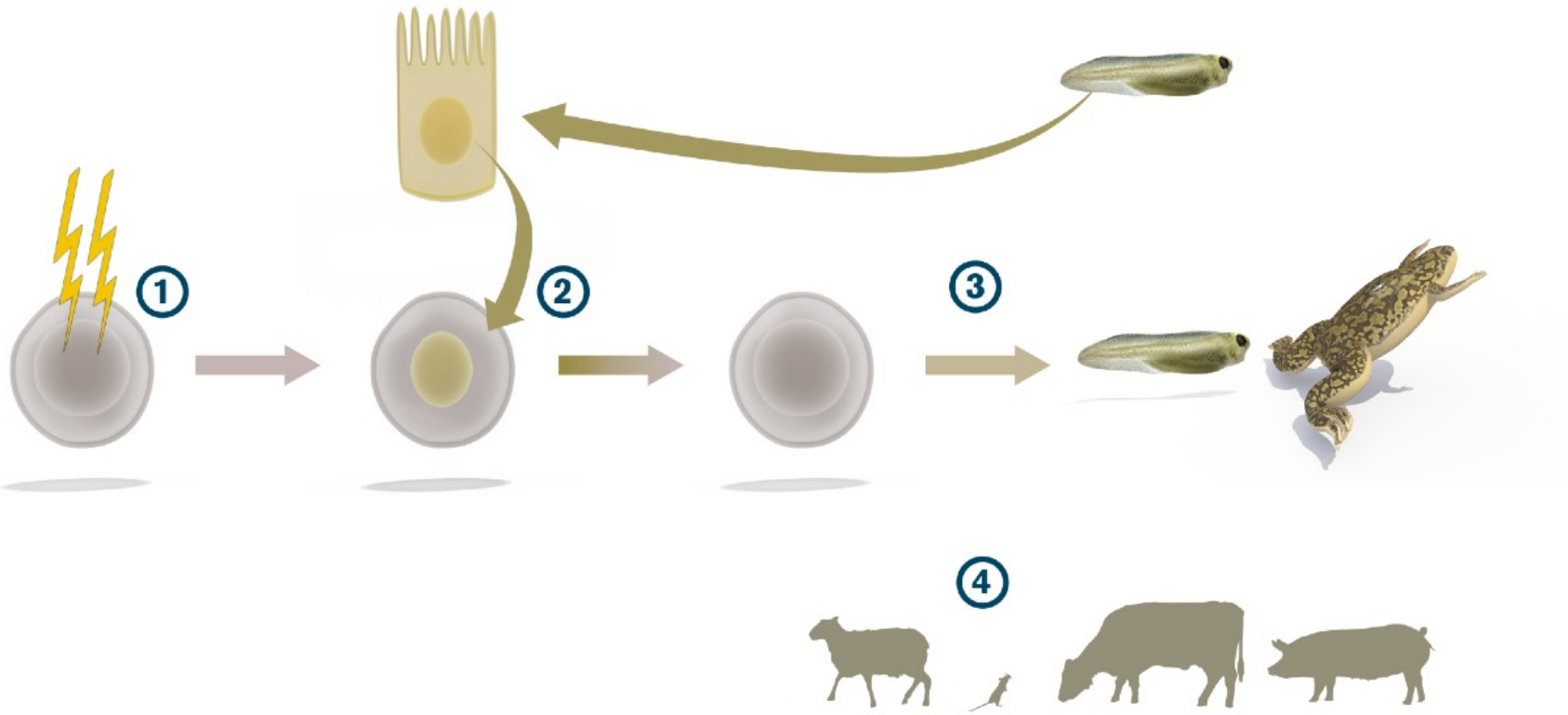
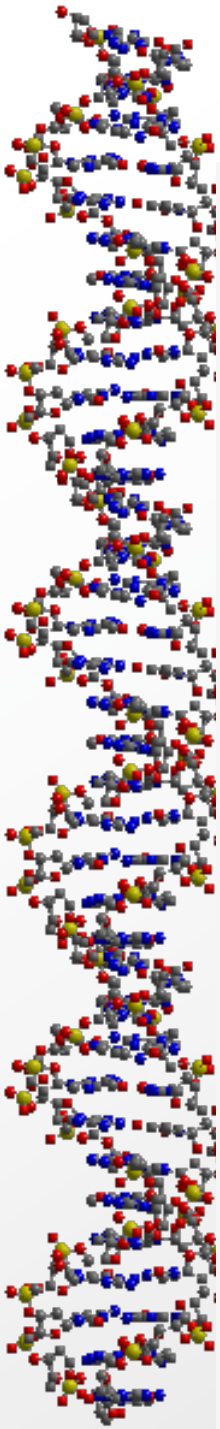
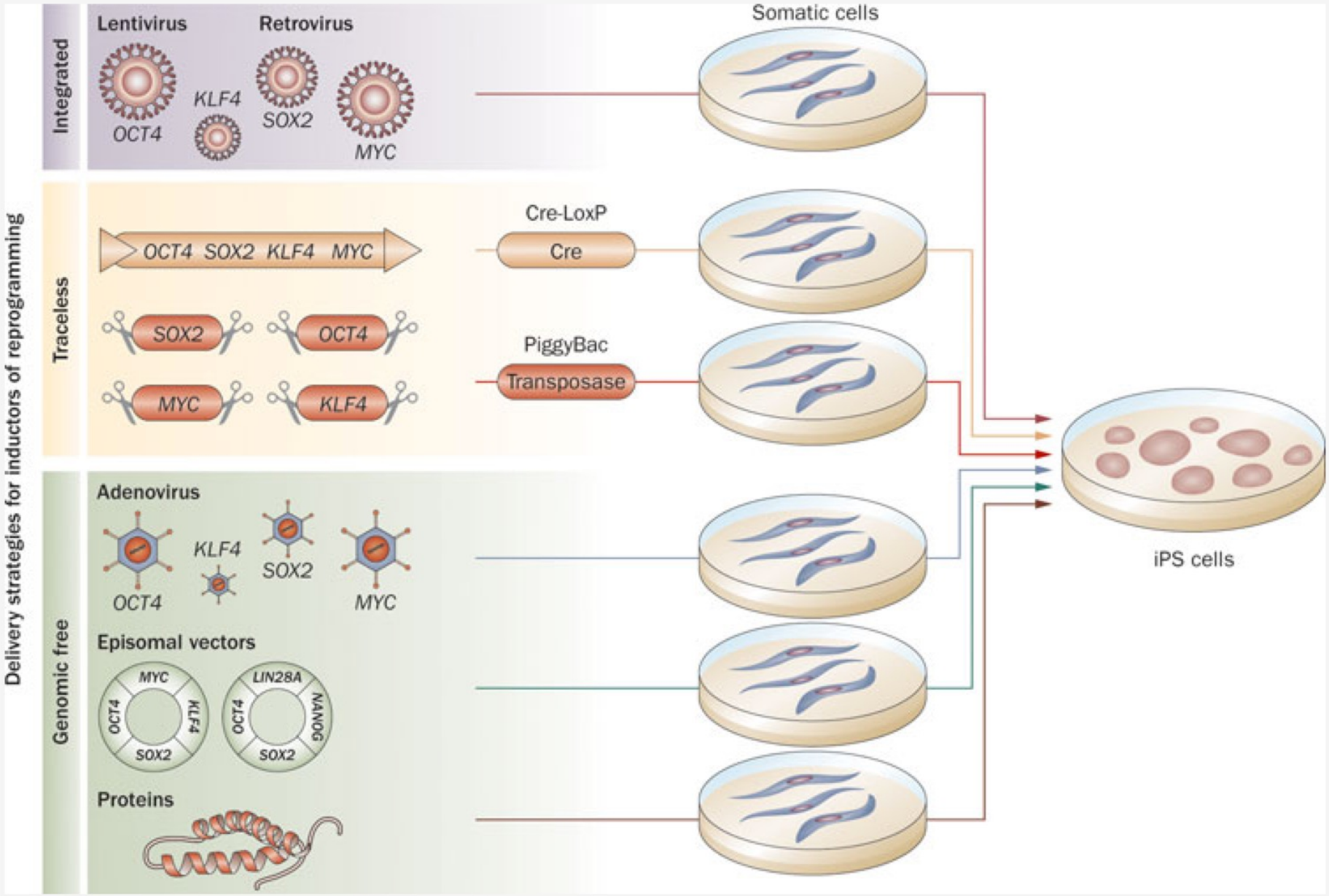


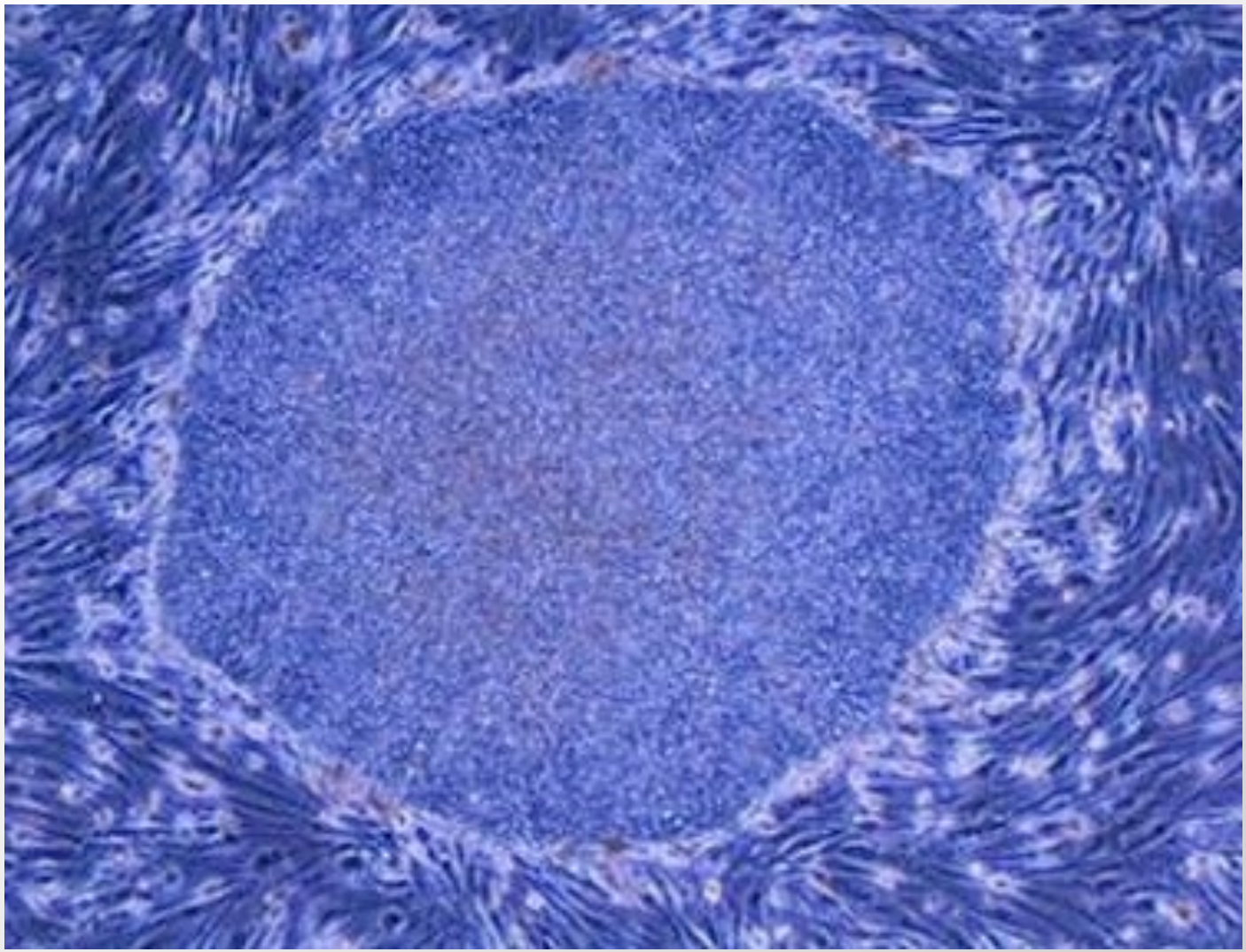
Figure 2 John Gurdon used UV light (1) to destroy the cell nucleus in a frog egg. He then replaced the egg nucleus with a cell nucleus from a differentiated intestinal epithelial cell from a tadpole (2). Many manipulated eggs did not develop but in several cases normal swimming tadpoles were generated (3). This showed that the genetic information required to generate the differentiated cells in a tadpole remained intact in the donor cell nucleus. Later studies have shown that also mammals can be cloned by this technique (4).

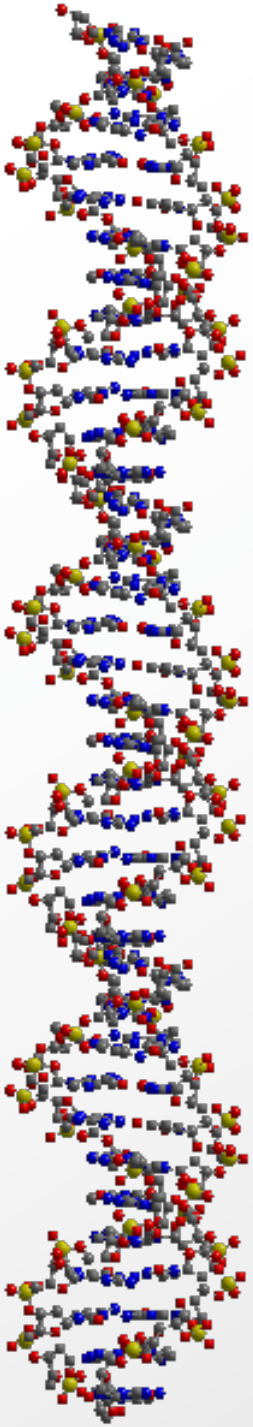
Reprogramando in vitro: células iPS!





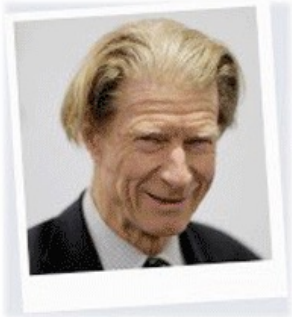
Reprogramando in vitro: células iPS!





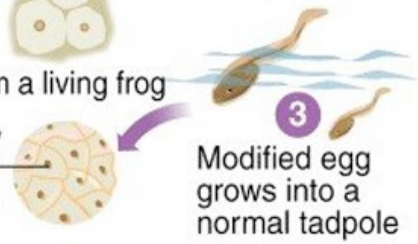
The Nobel Prize for Medicine

Shinya Yamanaka of Japan and John Gurdon of Britain honoured for the their work on cell programming



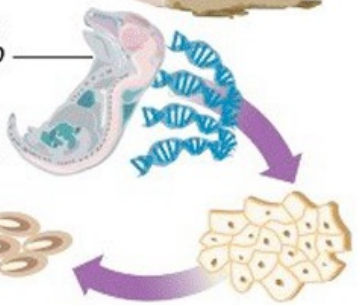
1962 **Gurdon** discovered that an adult frog cell could be **reprogrammed**

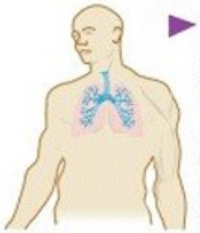
1. Nucleus of a cell of a frog egg removed from a living frog
2. Replaced by the nucleus of *a tadpole cell*
3. Modified egg grows into a normal tadpole



2006 **Yamanaka** discovered how mice cells could be **returned to their youthful state**, becoming 'pluripotent' stem cells

1. Four genes from *a mouse embryo* were transferred into cells taken from mouse skin
2. Reprogrammed, they became '**pluripotent**' stem cells which could be developed into all cell types of an adult mouse





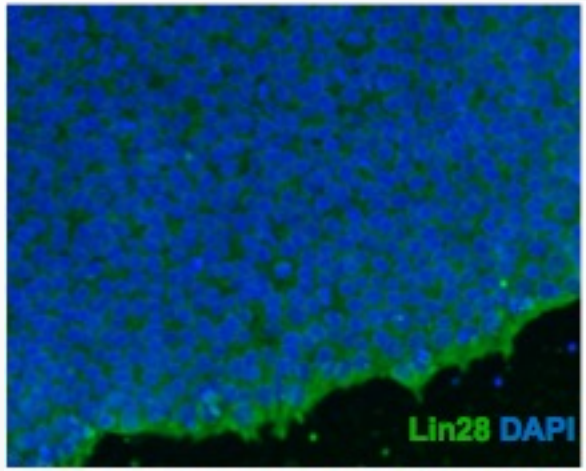
- '**Pluripotent**' cells, including nerve, heart and liver cells, could now be generated from humans
- The discoveries have raised hopes that replacement tissue may in the future be grown to fight disease in humans
- The process avoids the need to use stem cells from early-stage embryos for research

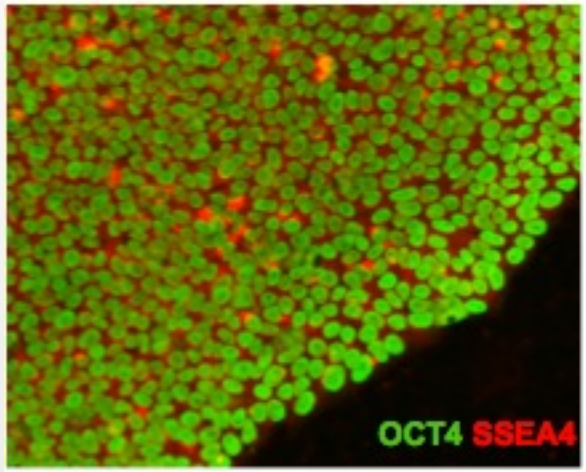
Source: Nobelprize.org

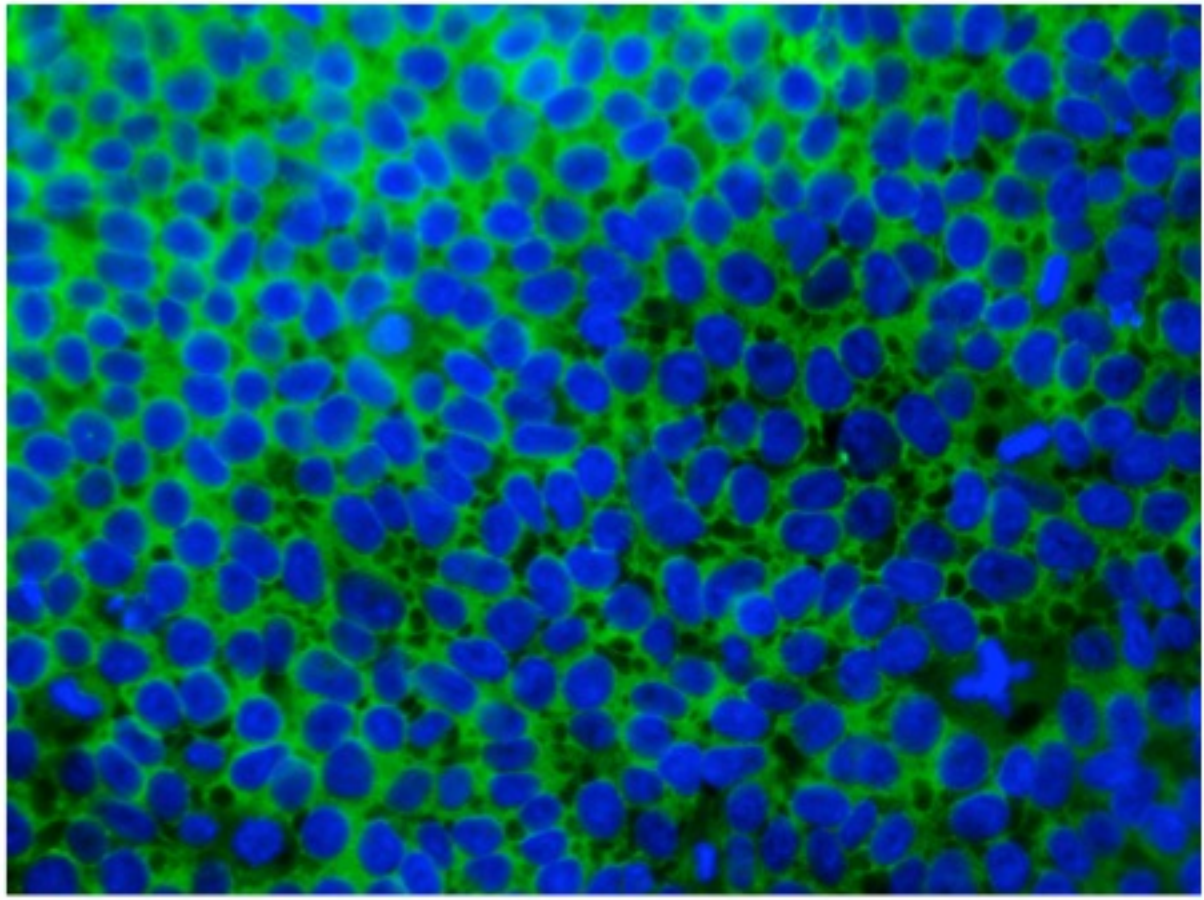
AFP

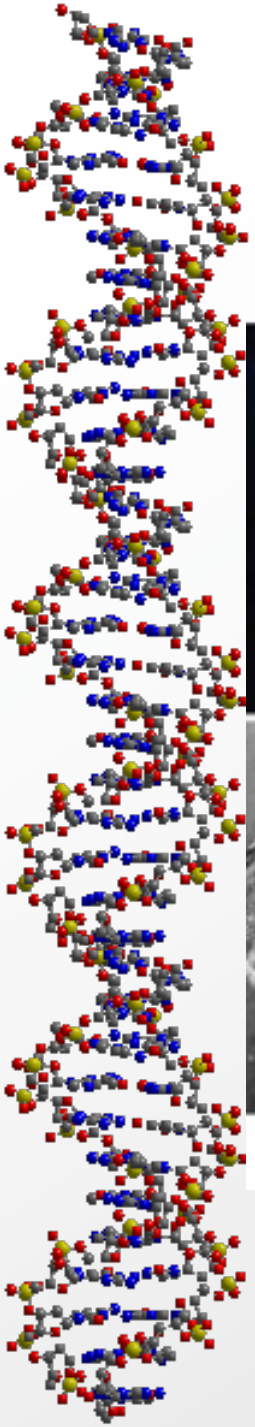
Colônia de iPSCs

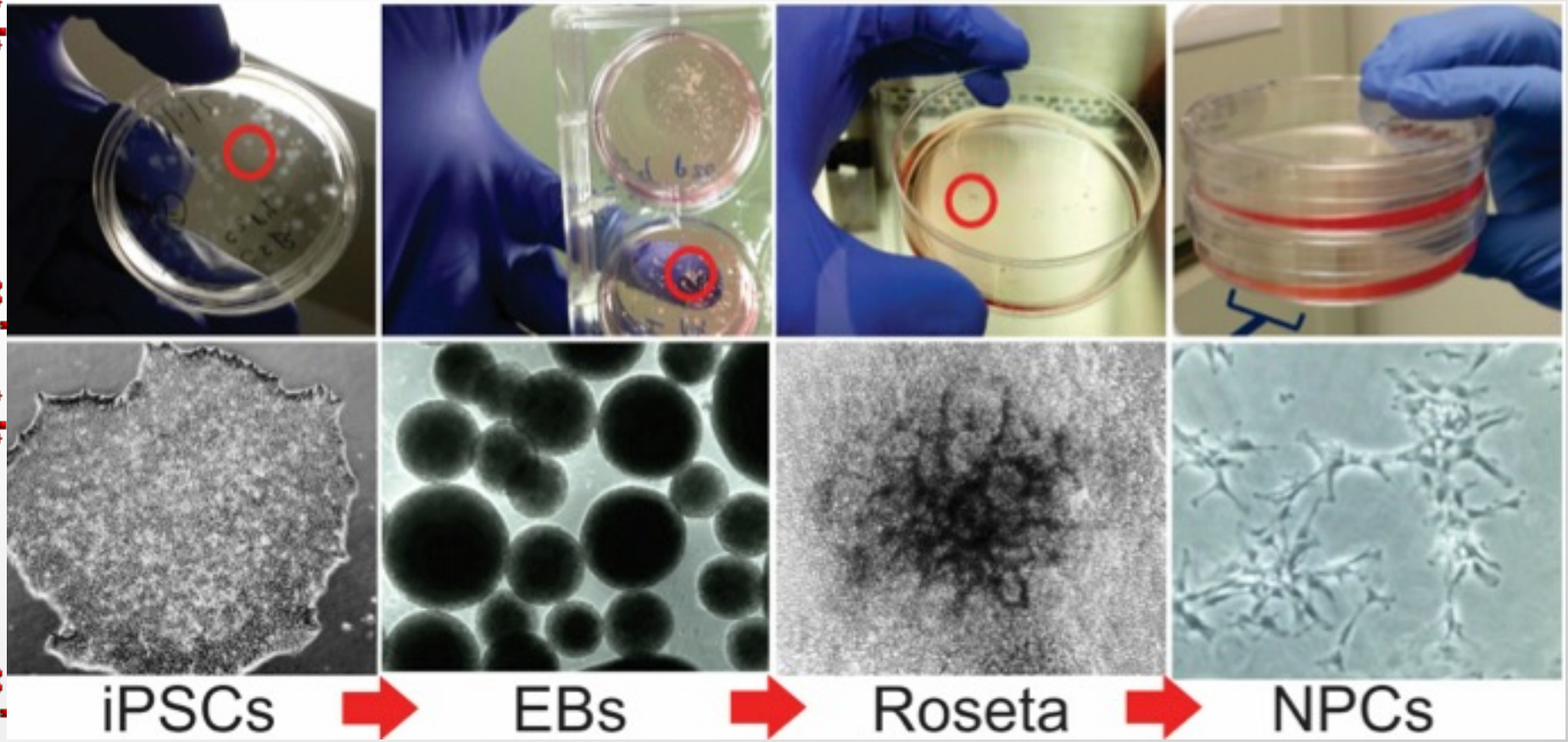
Verde: Lin28
Azul: DAPI







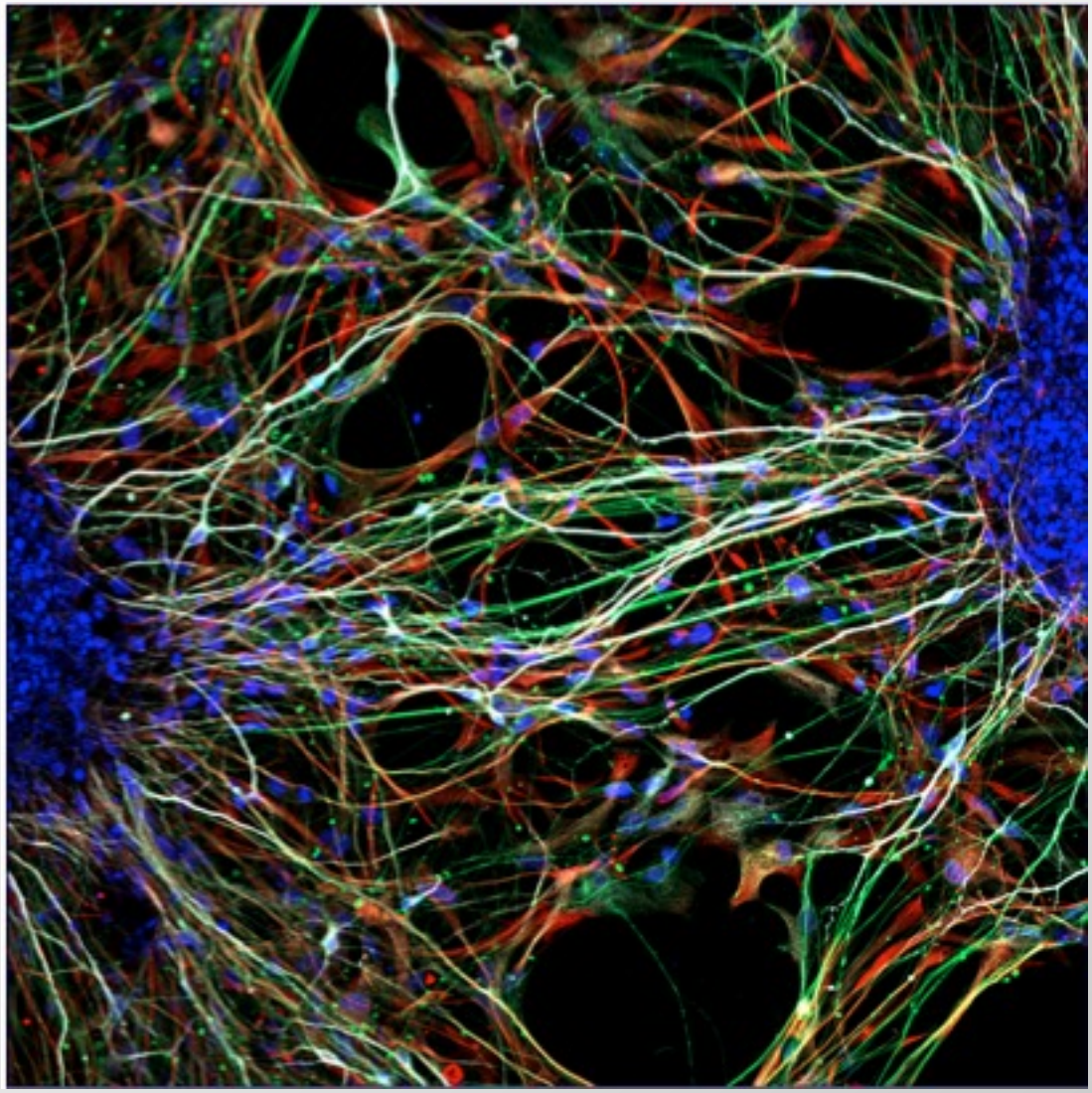



iPSCs
EBs
Roseta
NPCs

<u>Neurônios</u>
Verde: Tuj1
Branco:
MAP2

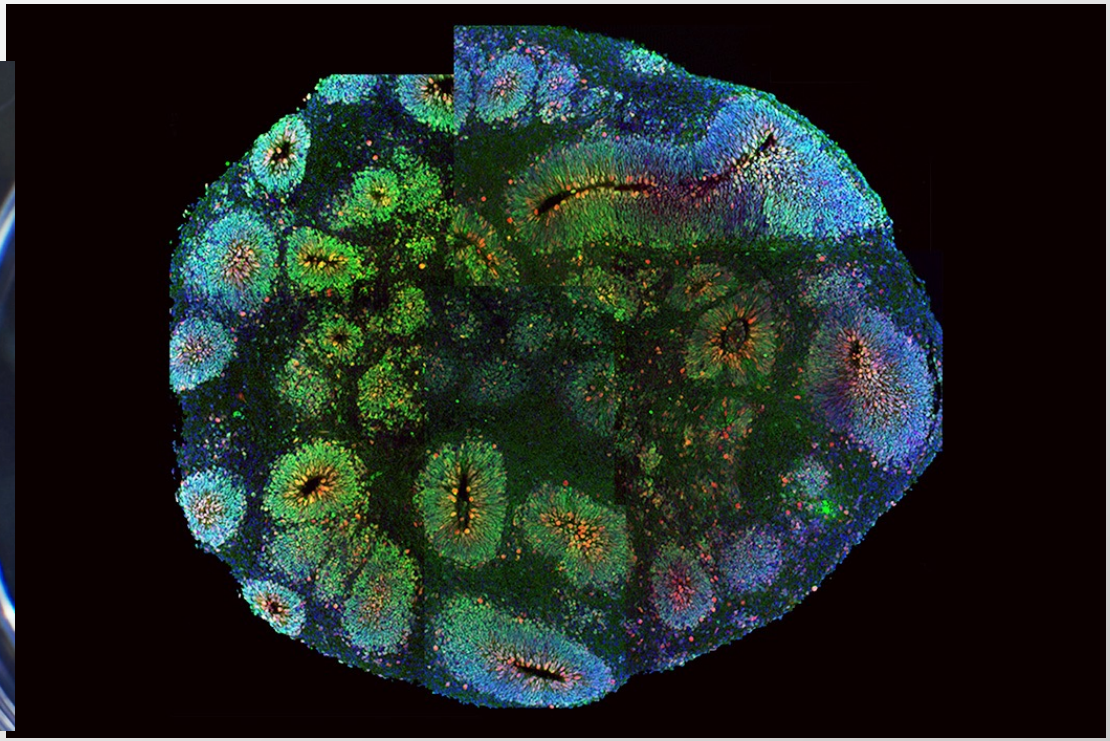
<u>Astrócitos</u>
Vermelho:
GFAP

<u>Núcleo</u>
Azul: DAPI

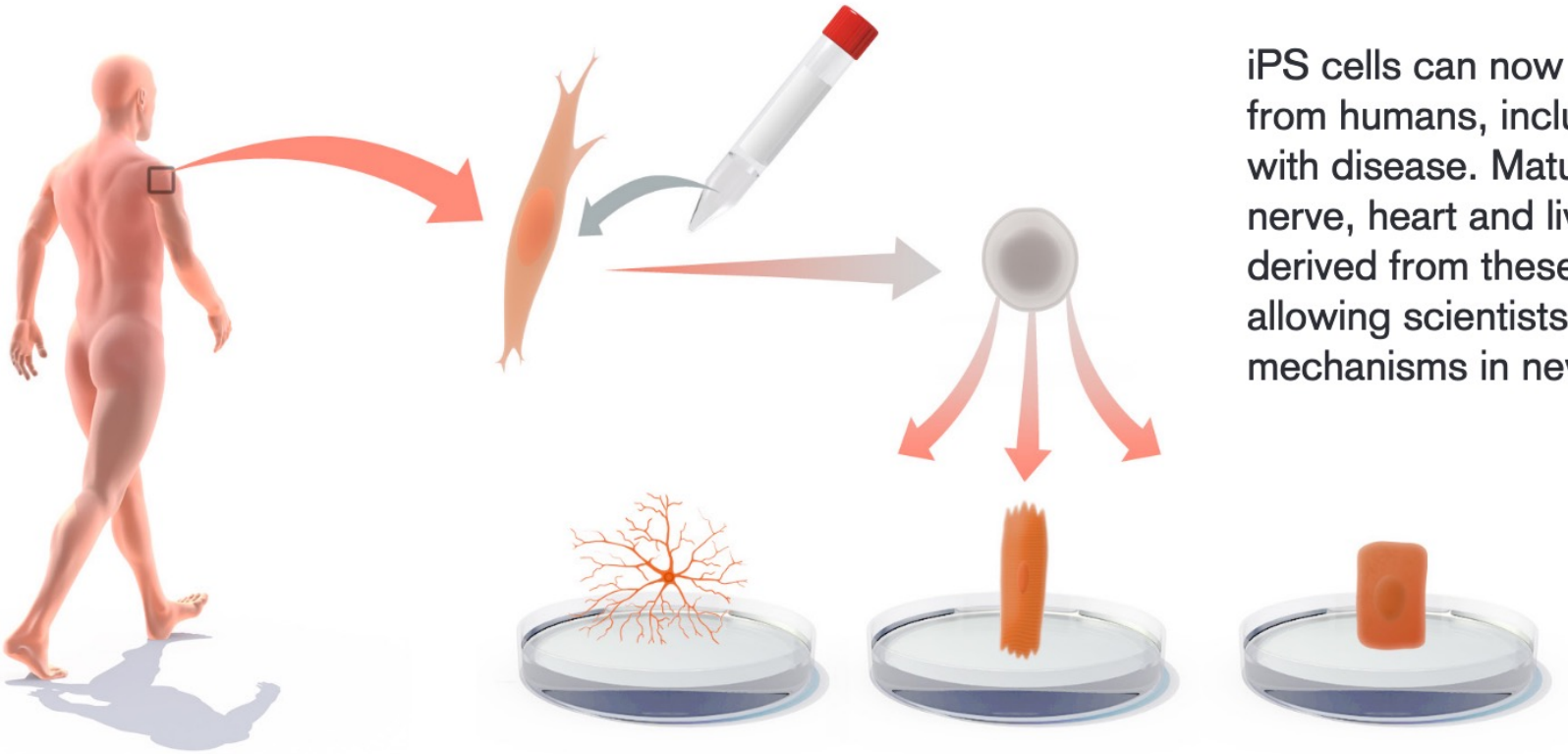


A partir de células iPS é possível

Cultivar organóides cerebrais!



Quanta Magazine



iPS cells can now be generated from humans, including patients with disease. Mature cells including nerve, heart and liver cells can be derived from these iPS cells, thereby allowing scientists to study disease mechanisms in new ways.

Illustration and layout: Mattias Karlén


OBAMA'S DECISION TO END THE BAN ON STEM CELL RESEARCH MAKES ME HOPPING MAD!!!
WISCONSIN RIGHT TO LIFE
REALLY?? WHAT'S HOPPING LIKE?
WISCONSIN RIGHT TO LIFE


SCIENCE
ARE THESE PICTURES OF STEM CELLS?
THOSE ARE MY KIDS.
PLEASE CHECK YOUR MORALS & ETHICS AT THE DOOR
Lisa©2009


STEM CELL RESEARCH
VETO PARTY
CLINK!
PARKIN-SONS
CANCER
AIDS
ALZ HEIM
SICKL CELL
SPINAL