Different genetic modifications or GMO methods using in developmental, cellular and molecular biology

LMR05.001 – 20
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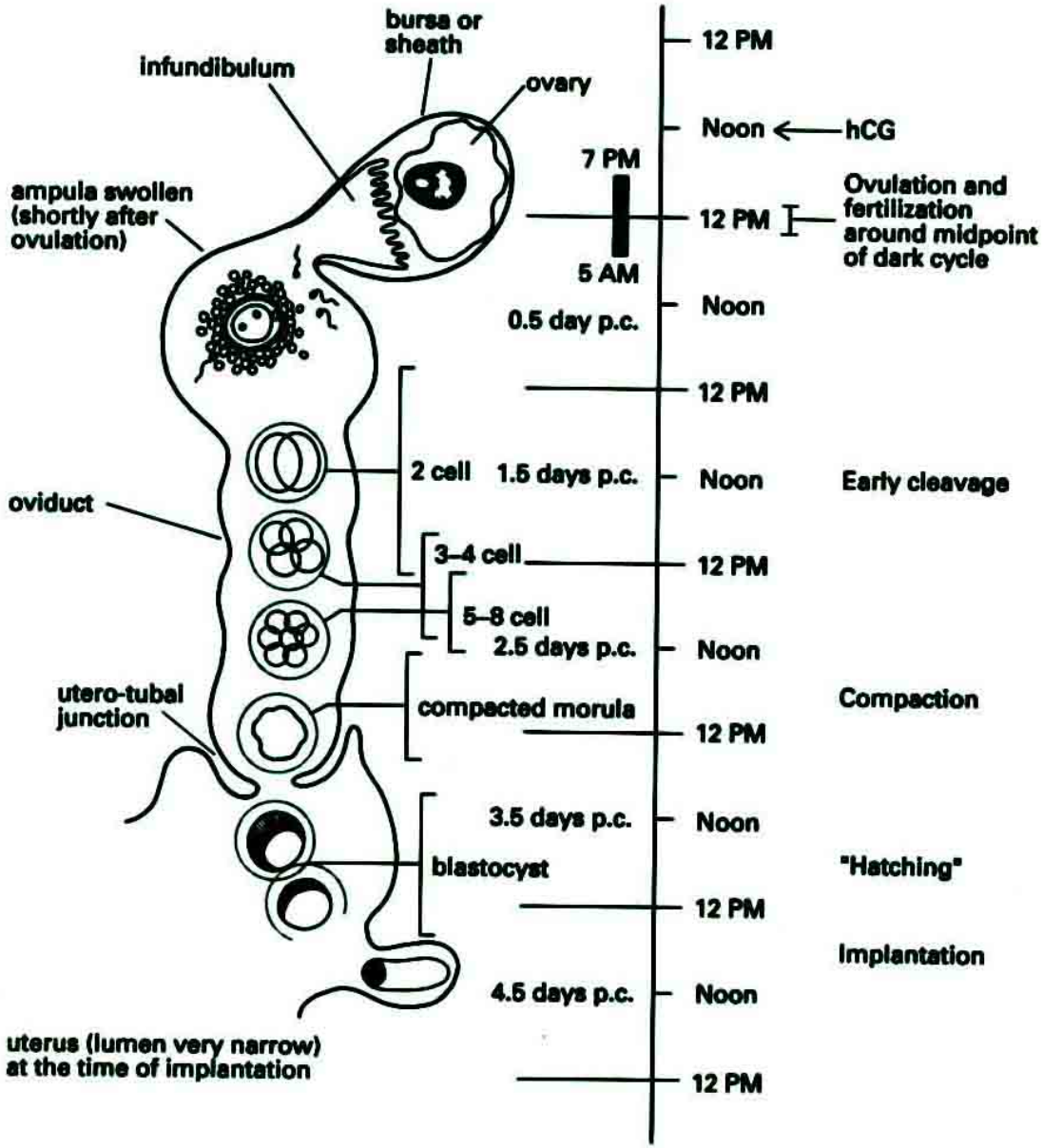
Inst Mol & Cell Biol

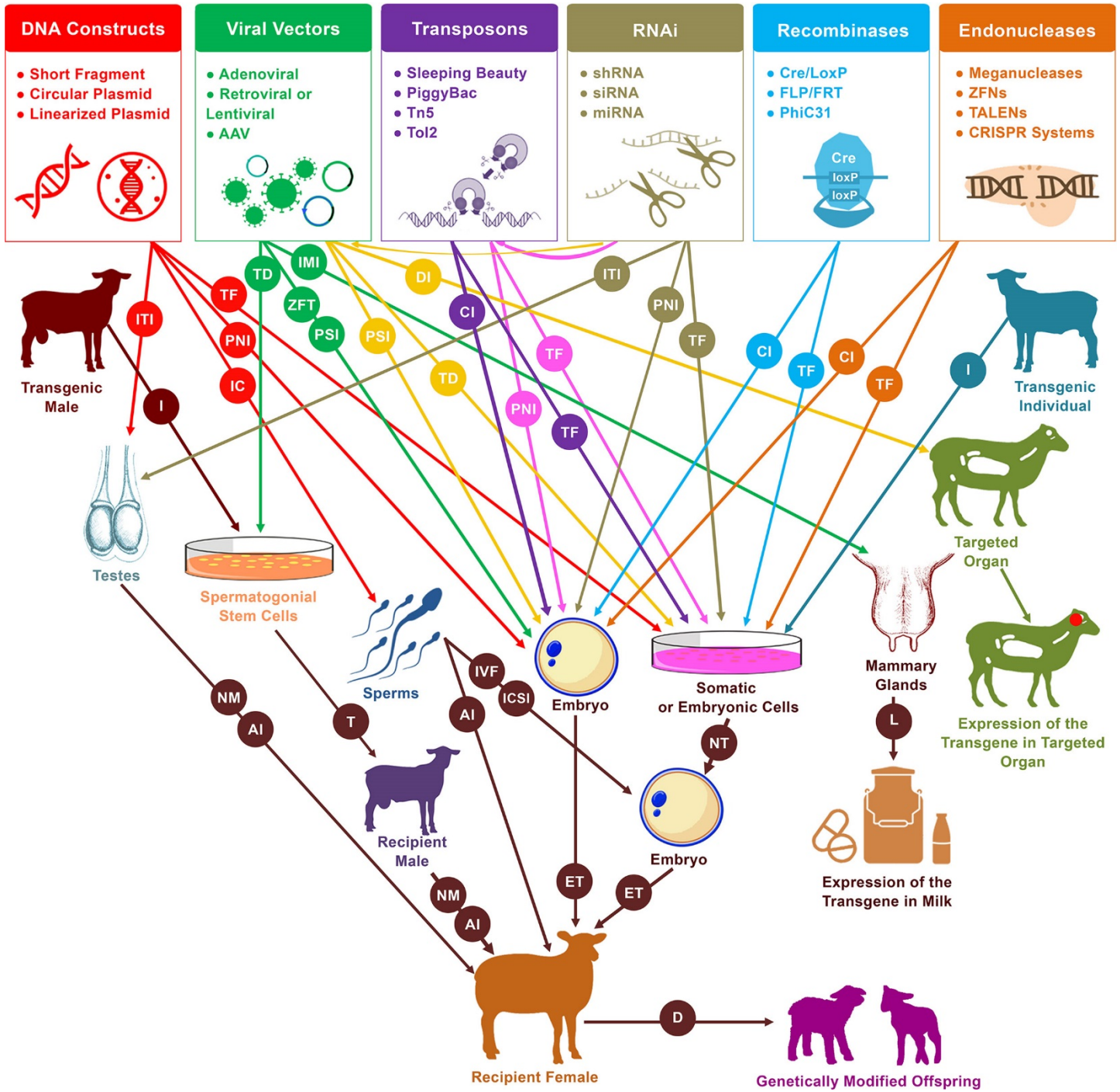
In all animal experiments or experiments with living organisms we must observe carfully about wellness of of used laboratory animals.

Refinement
Reduction
Research

3R principles in animal experiments

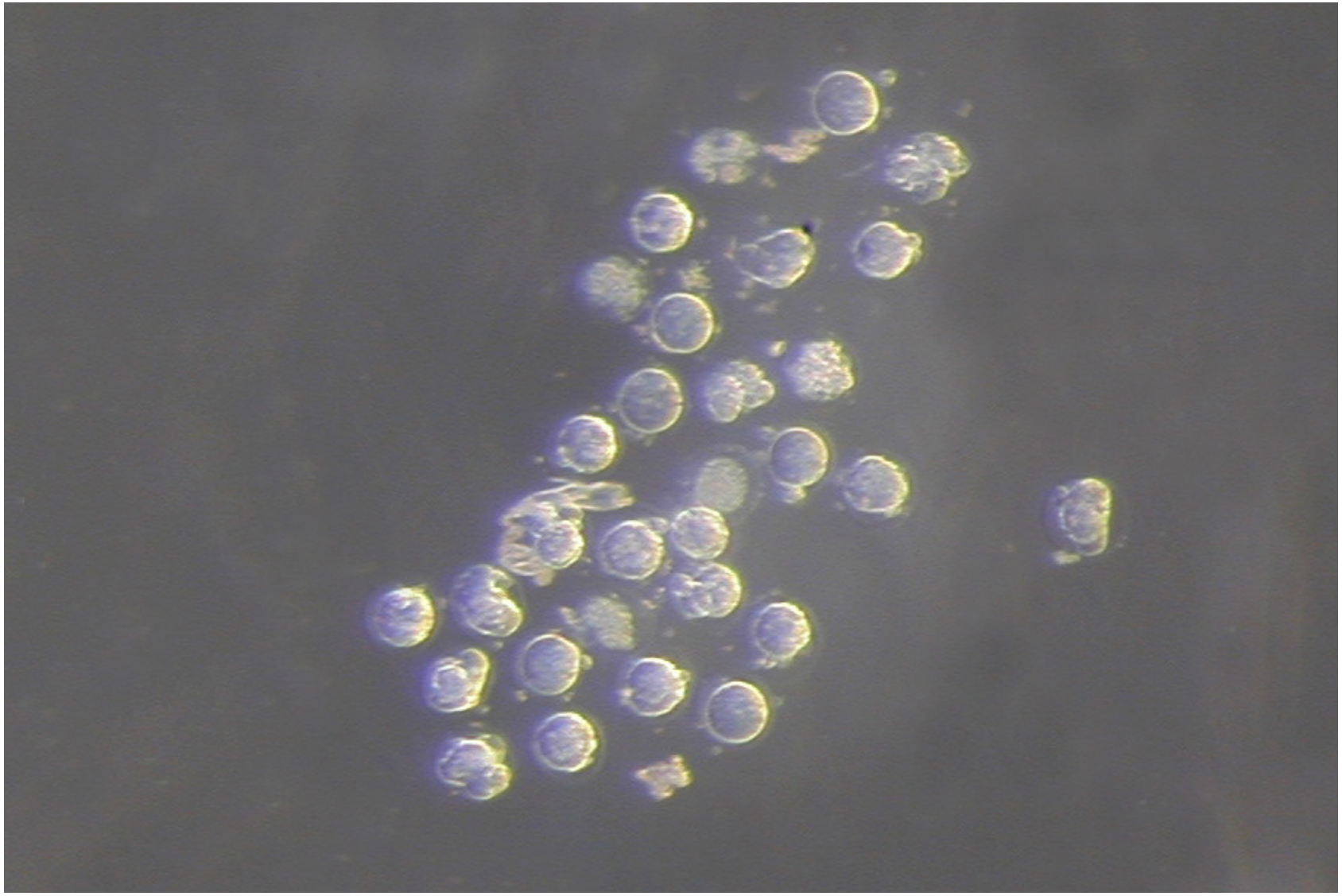
The moving of fertilized egg-cell in the Fallopian tube and in the uterus after the fertilization in the infundibulum (the example is mouse development time-table).





Different GMO-methods

Fertilized egg-cells



Aggregation

Microinjection

Cell nucleus replacement (cloning)

Blastocyst injection

Lentivirus-method

Embryonic stem cell method

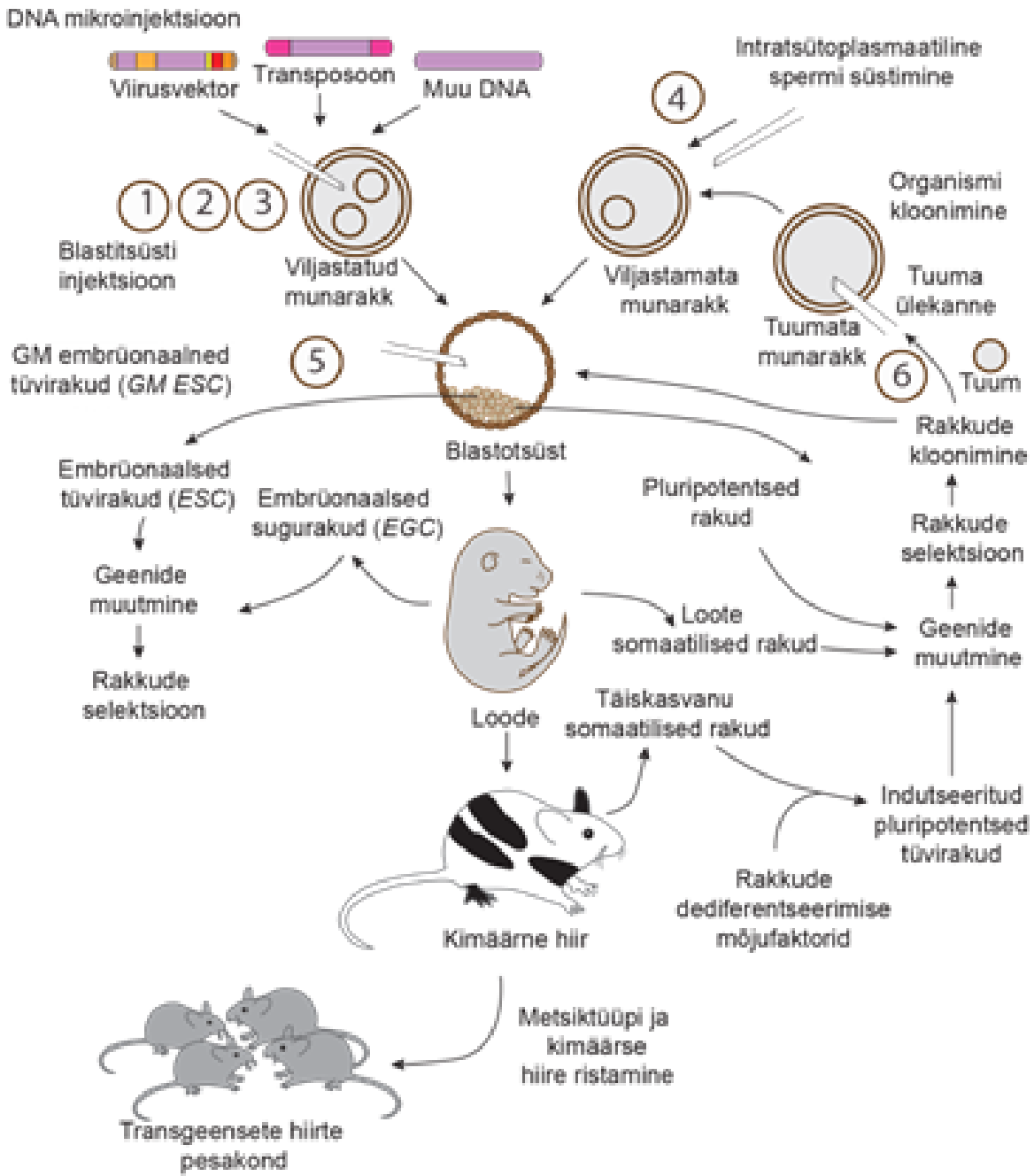
Induced pluripotent cell method

Transpson method

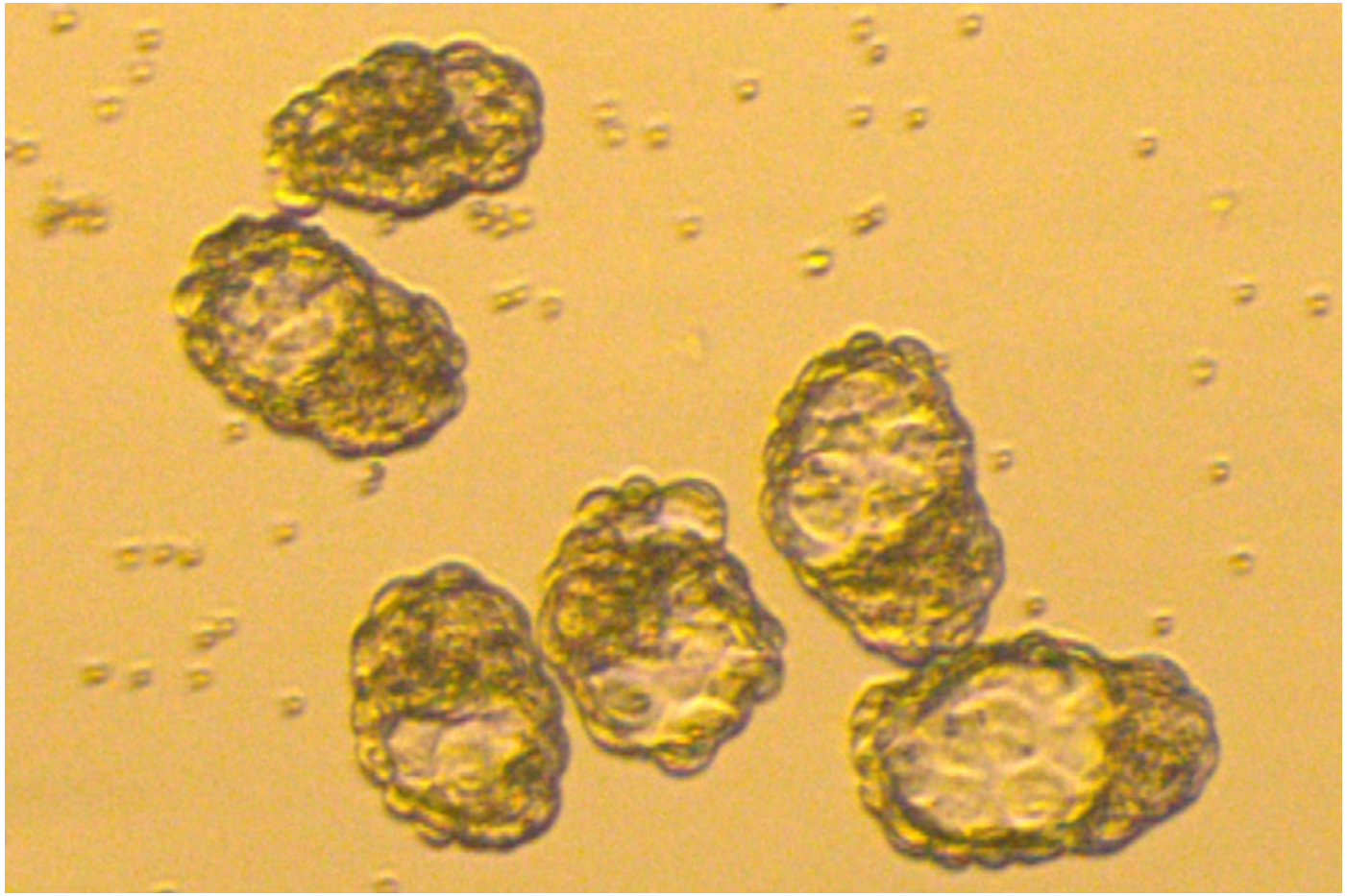
CRISPR-Cas method

Talen-method

etc

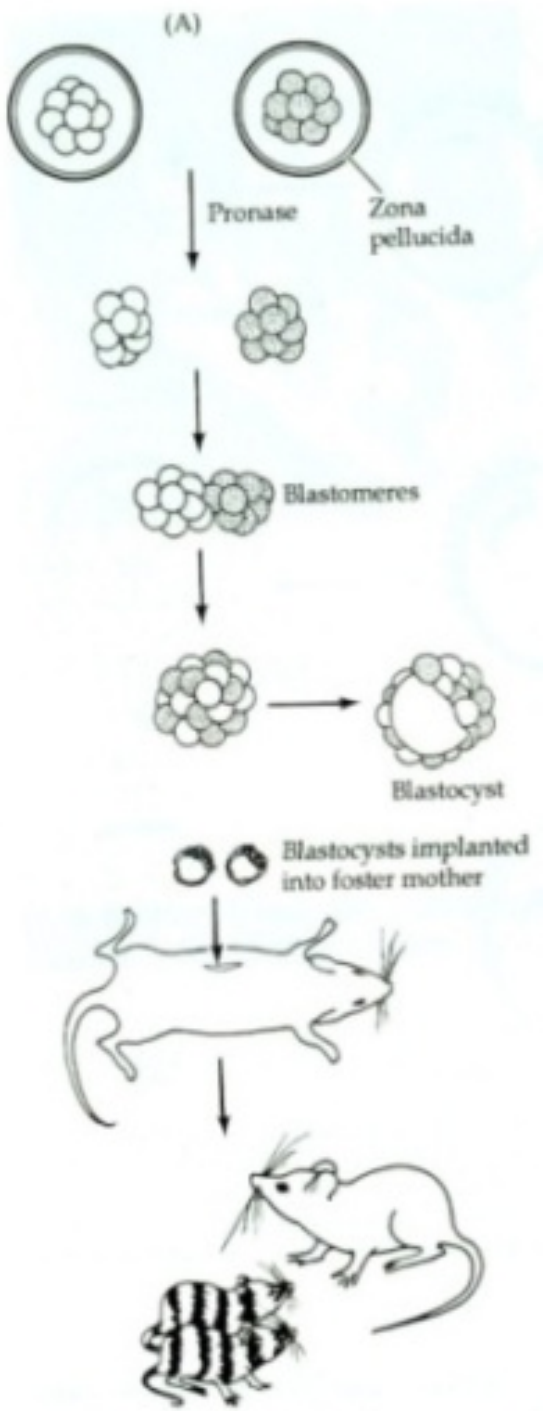

DNA mikroinjektsioon
Viirusvektor
Transposoon
Muu DNA
1
2
3
Blastitsüsti injektsioon
Viljastatud munarakk
4
Intratsütoplasmaatiline spermi süstimine
Viljastamata munarakk
Organismi kloonimine
Tuumata munarakk
Tuuma ülekanne
6
Tuum
Rakkude kloonimine
5
GM embrüonaalned tüvirakud (GM ESC)
Blastotsüst
Embrüonaalsed tüvirakud (ESC)
Embrüonaalsed sugurakud (EGC)
Pluripotentsed rakud
Rakkude selektsioon
Geenide muutmine
Rakkude selektsioon
Loote somaatilised rakud
Geenide muutmine
Loode
Täiskasvanu somaatilised rakud
Indutseeritud pluripotentsed tüvirakud
Kimäärne hiir
Rakkude dediferentseerimise mõjufaktorid
Metsiktüüpi ja kimäärse hiire ristamine
Transgeensete hiirte pesakond

Mouse embryos – the blastocyst stage



Aggregation

– the first method (historically) of genetically modified organism



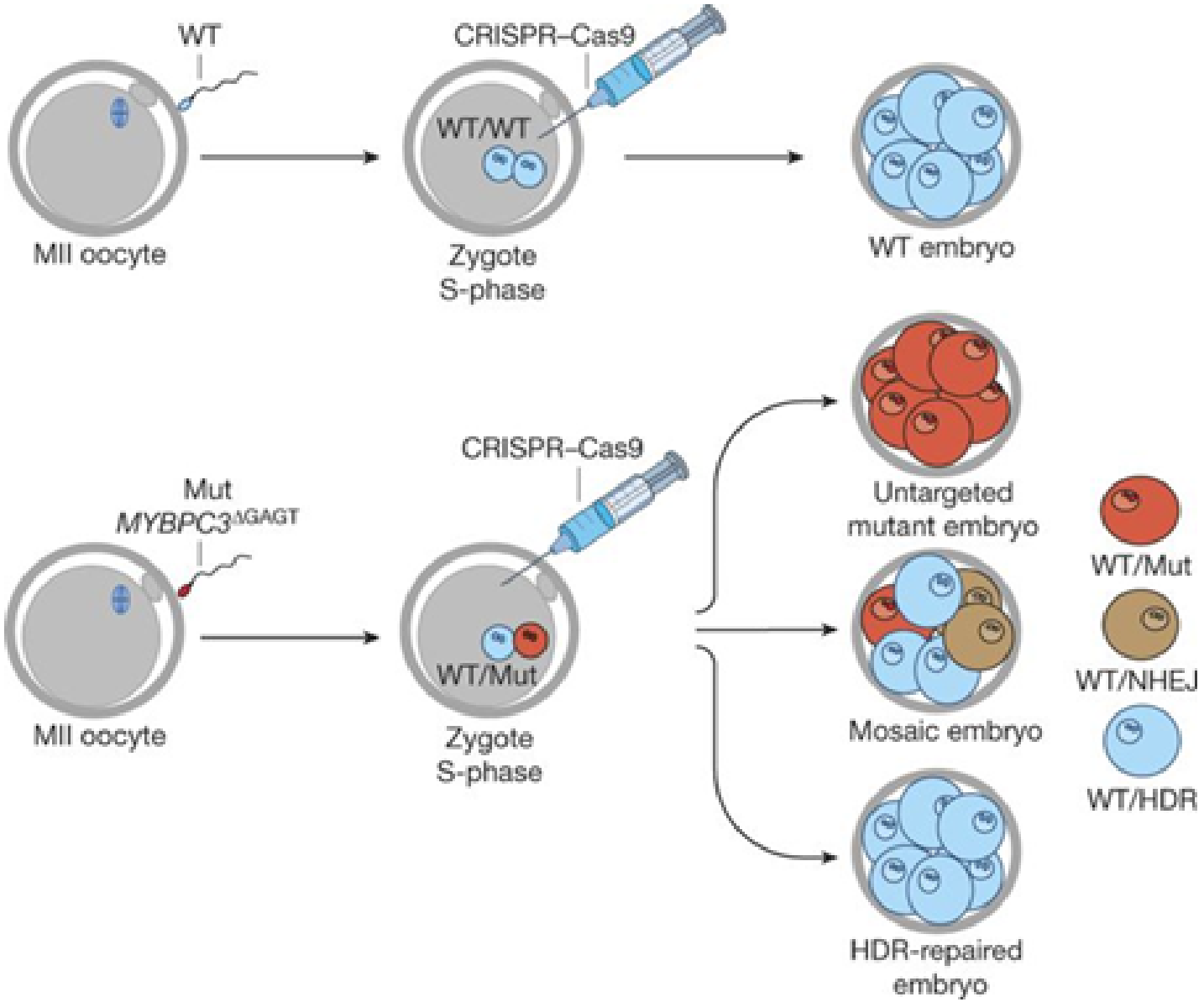
Morula staadium

Dissociation of early embryo cells

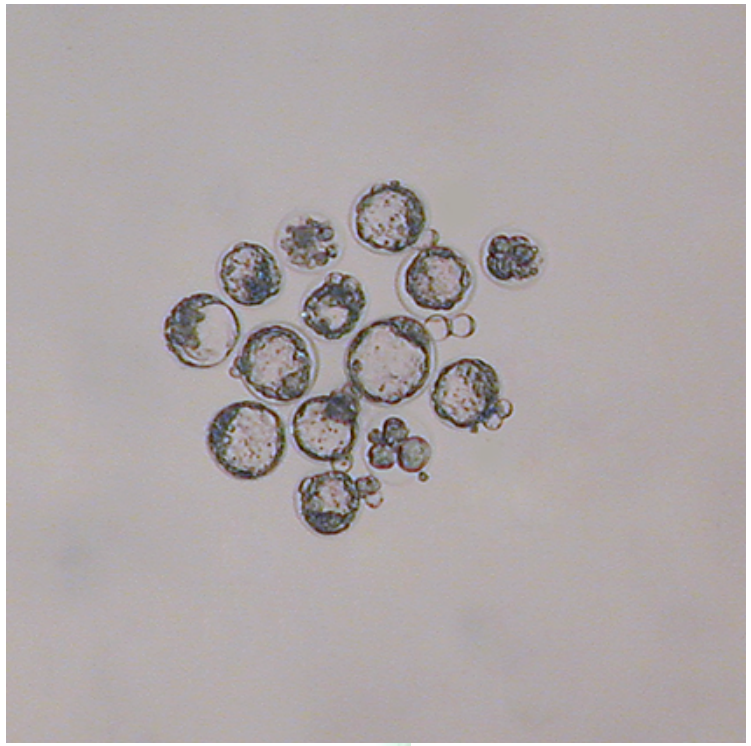
Co-existing of two embryo cells in one Petri dish and reaggregation *de novo of them in vitro* condition

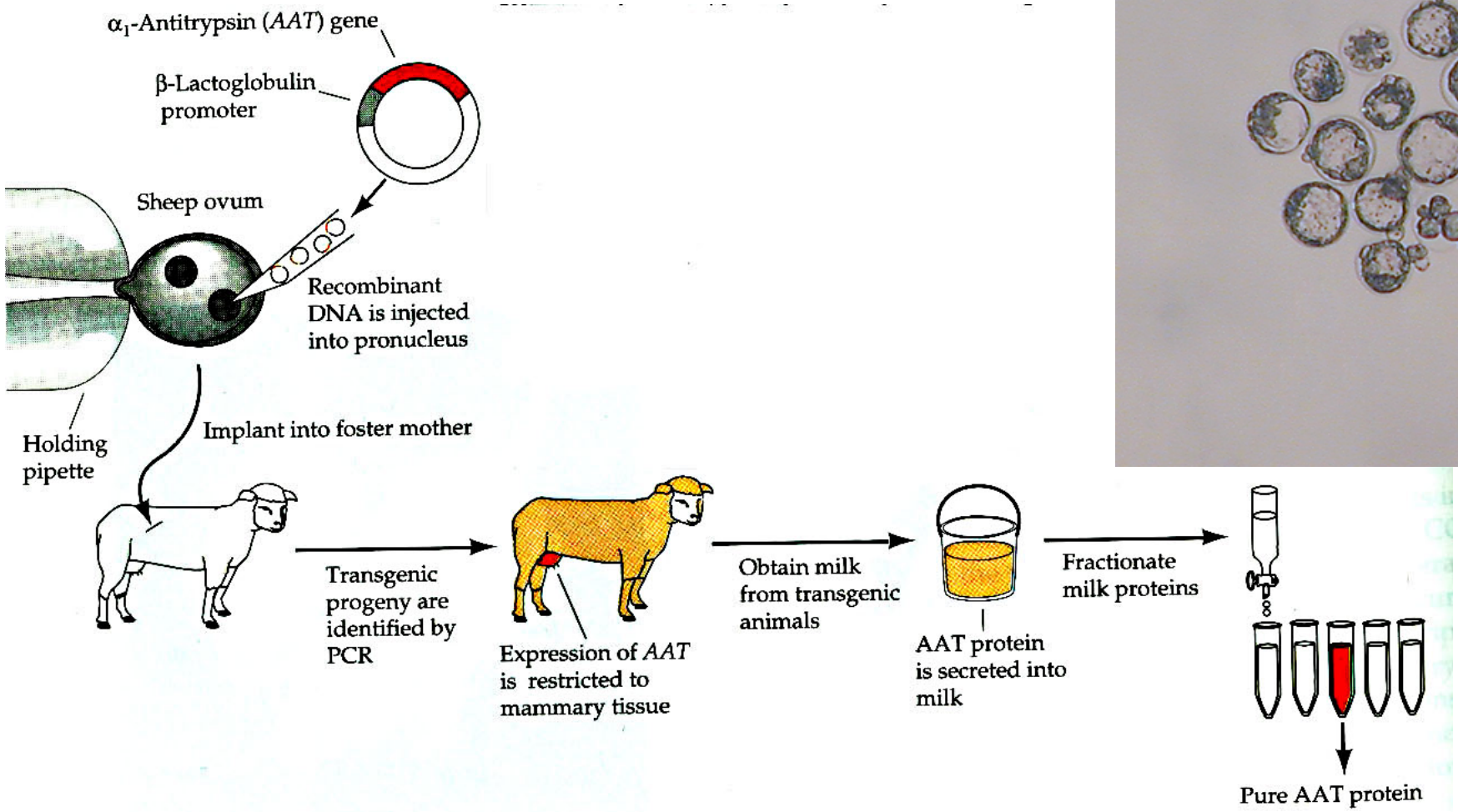
Blastocysts reimplantation to the foster pseudopreganant mouse uterus

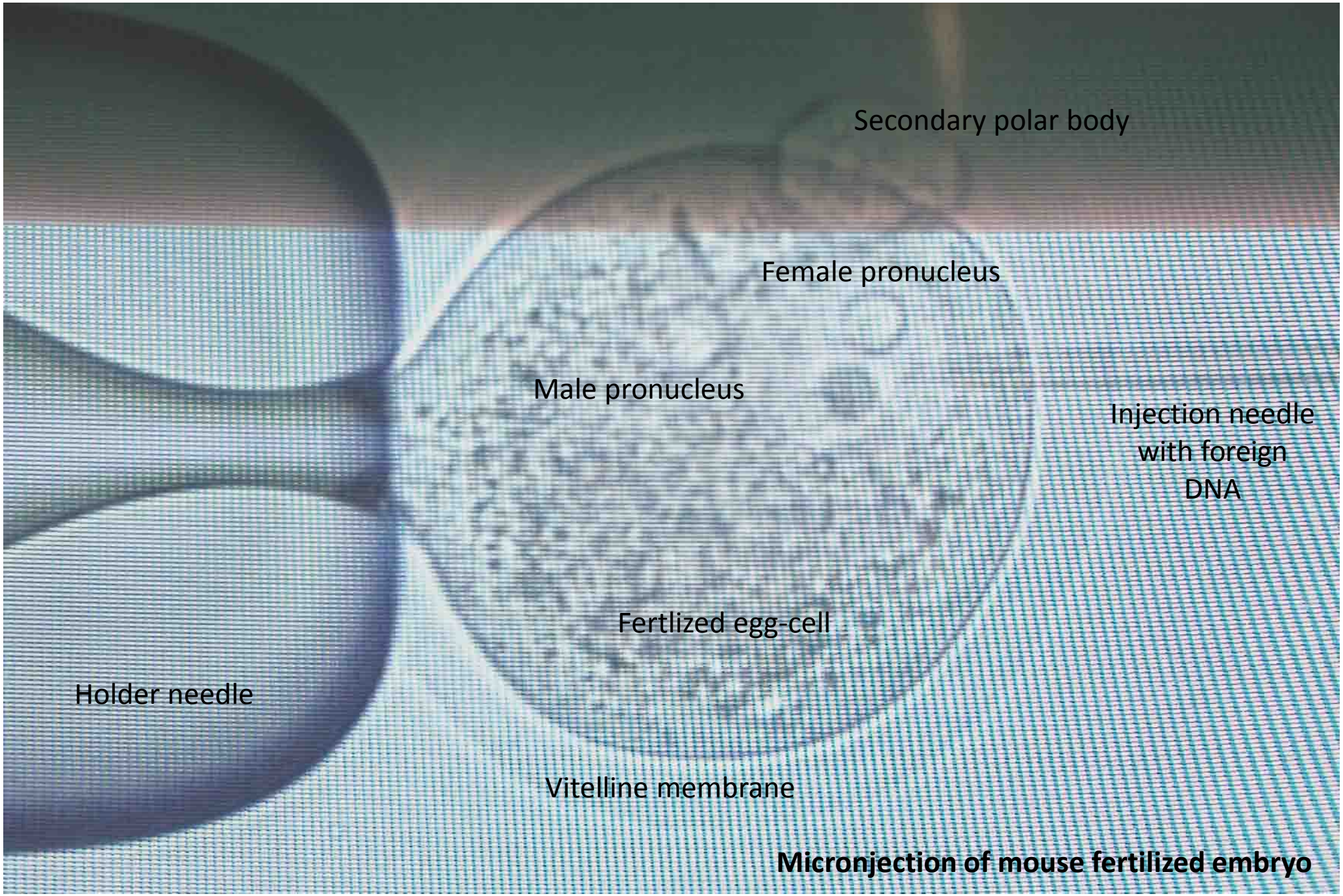
Ca 21 days after aggregation birth of chimeric mice


WT
CRISPR–Cas9
WT/WT
MII oocyte
Zygote
S-phase
WT embryo
Mut
MYBPC3ΔGAGT
CRISPR–Cas9
Untargeted
mutant embryo
WT/Mut
WT/Mut
MII oocyte
Zygote
S-phase
Mosaic embryo
WT/NHEJ
WT/HDR
HDR-repaired
embryo

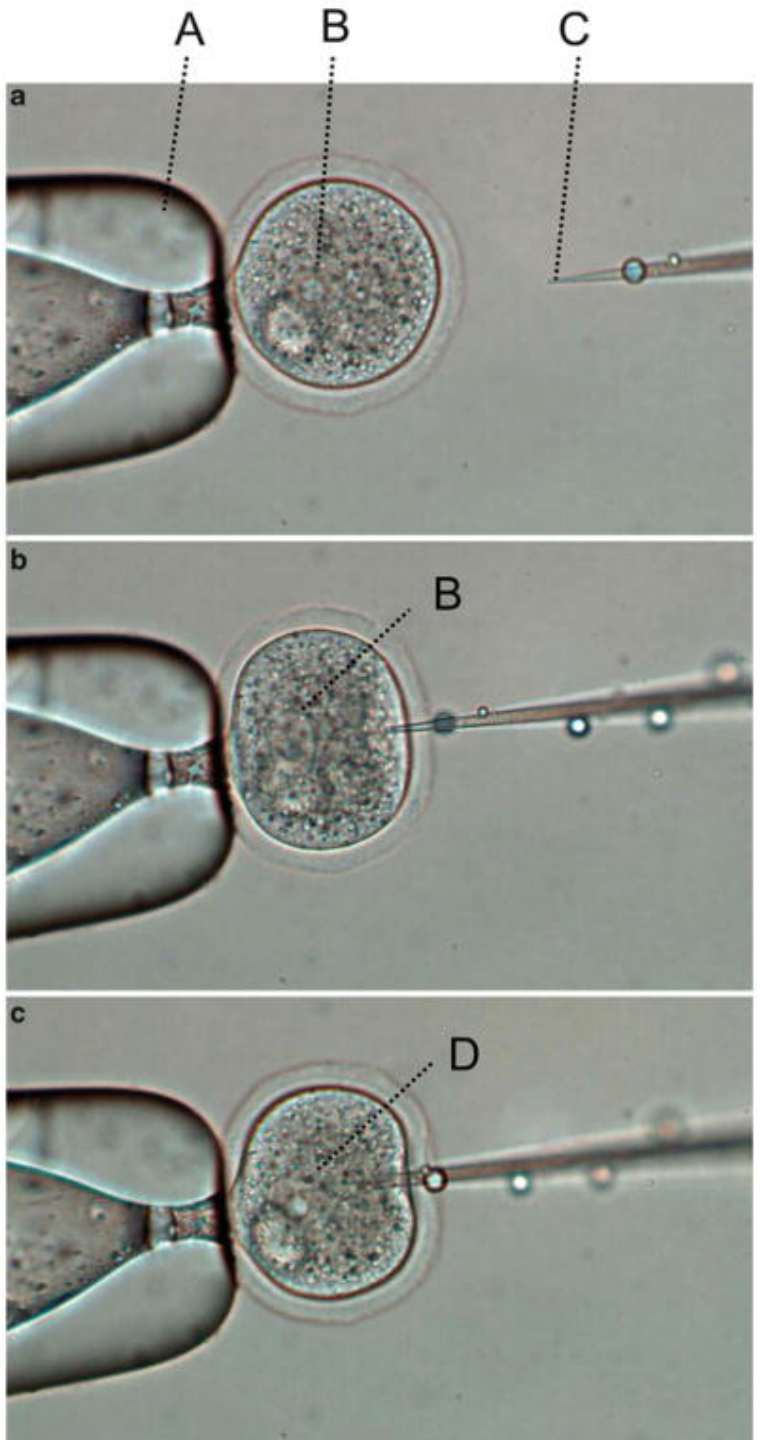
The second method of GMO – zygote injection or microinjection or injection of fertilized embryo or pronuclear microinjection







Micronjection of mouse fertilized embryo

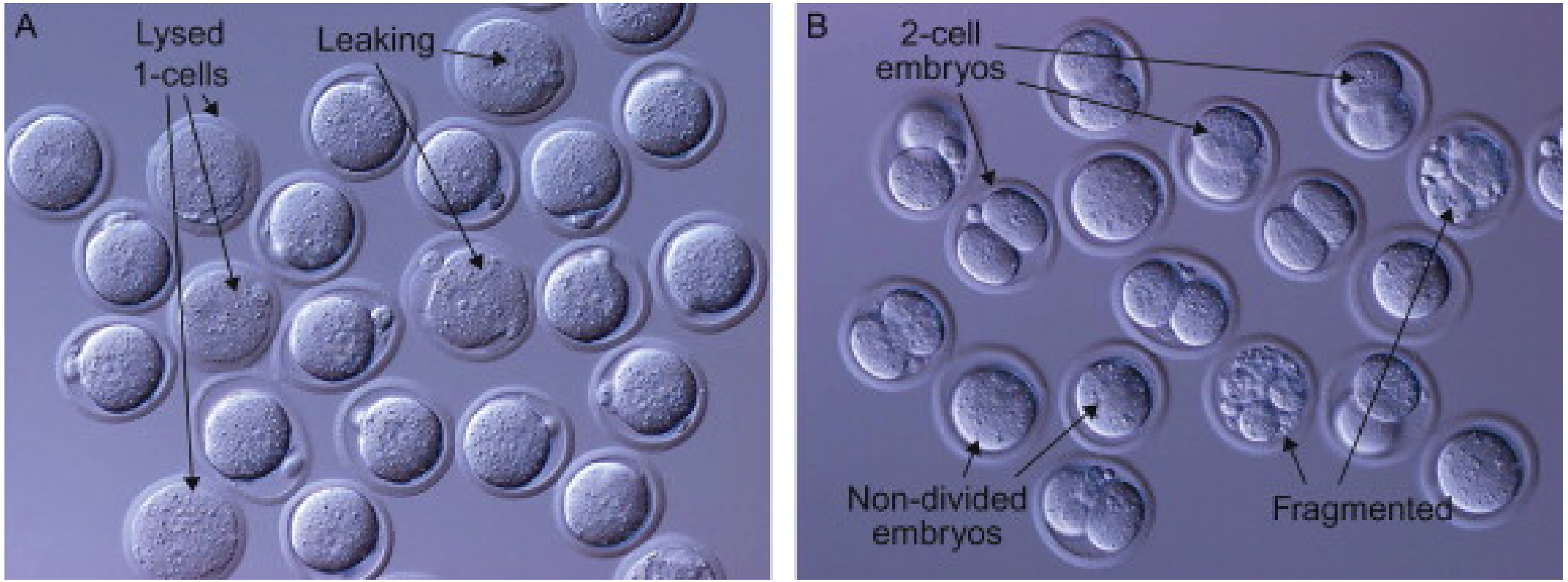


Pronuclear microinjection.

(a) A zygote is first grabbed by the holding pipette (A). The focus of the microscope is adjusted so that one of the two pronuclei (B) is in sharp focus. Next, the micromanipulator is adjusted to raise or lower the microinjection needle so that the tip of the needle (C) is in the same focal plan as the pronucleus.

(b) The needle is inserted into the zygote with a quick motion.

(c) When the needle tip is inside the pronucleus, the foot peddle of the Transjector is pushed to deliver DNA solution into the nucleus. A sudden expansion of the nuclear envelope (D) is an indication that the embryo has been successfully micro-injected. Then, the needle is gently but quickly withdrawn



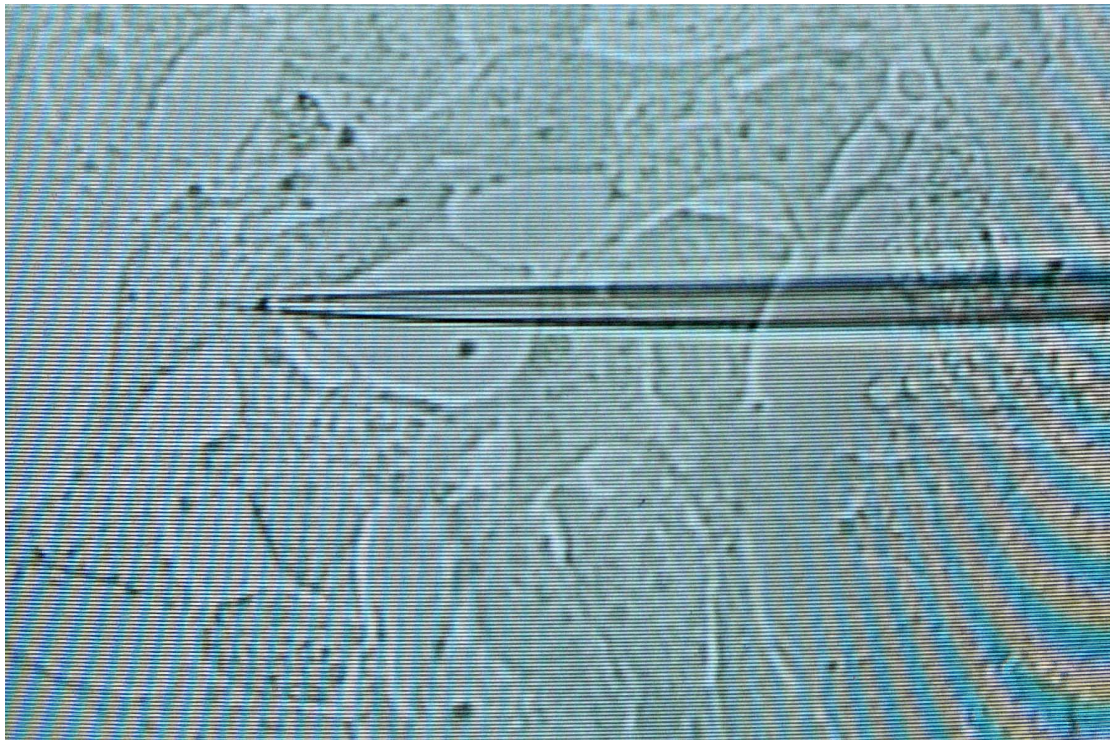
Mouse embryos following microinjection.

(A) Embryos immediately after microinjection. While most embryos survive the microinjection process, some are irreversibly damaged and do not survive. The arrows show both completely lysed one-cell embryos and those that have been lysed and are slowly leaking. (B) Mouse embryos following overnight incubation. Photograph shows healthy embryos that have divided to the two-cell stage: nondivided embryos arrested at the one-cell stage, and fragmented nondeveloping embryos. Only the two-cell embryos are viable and suitable for transplantation. Magnification, 80×.

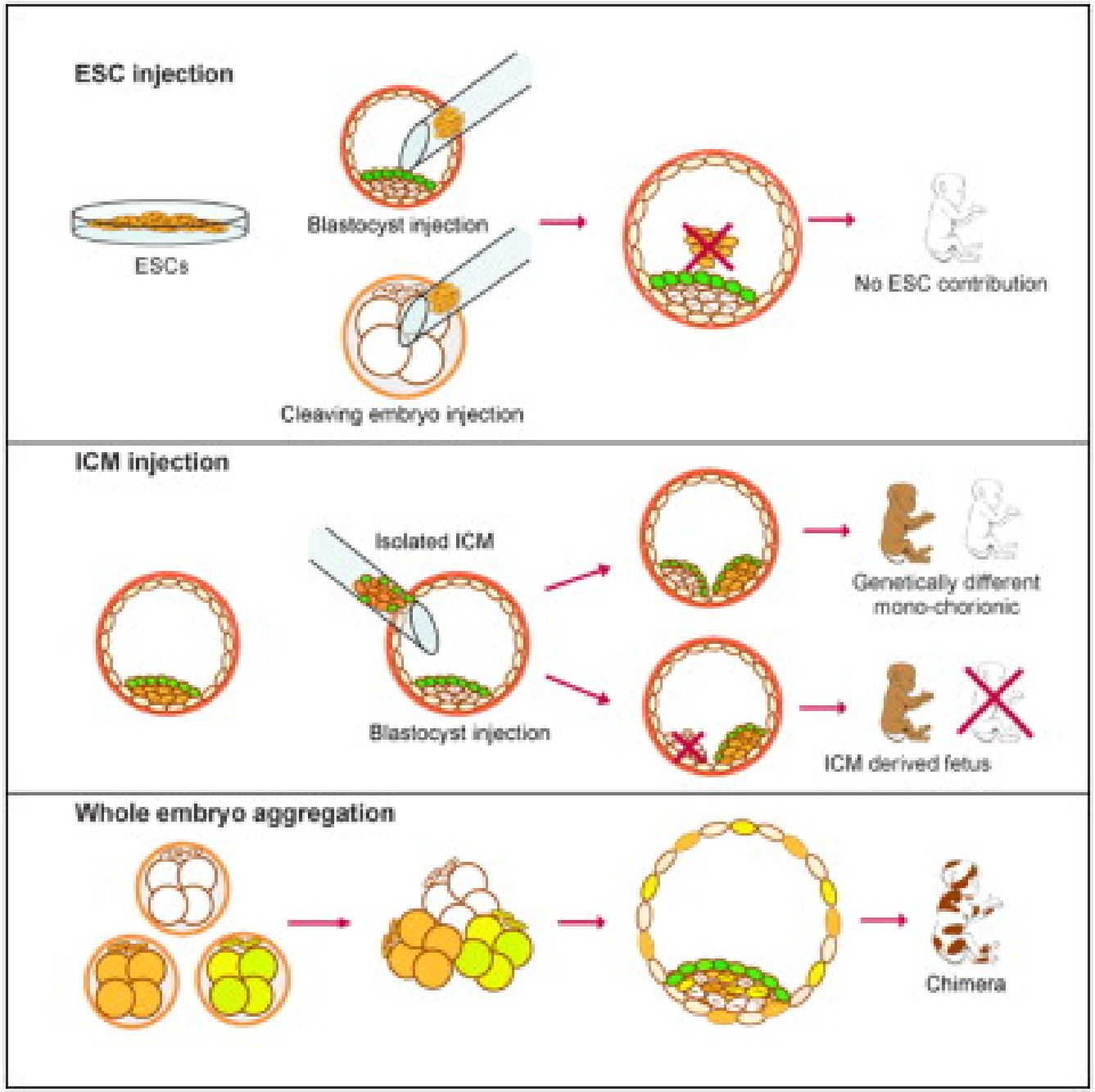
HeLa is an immortal cell line used in scientific research. It is the oldest and most commonly used human cell line.
The line is named after and derived from cervical cancer cells taken on February 8, 1951, from Henrietta Lacks, a 31-year-old African-American mother of five, who died of cancer on October 4, 1951.

The cells from Lacks's cancerous **cervical tumor** were taken without her knowledge or consent, which was common practice in the United States at the time.Cell biologist George Otto Gey found that they could be kept alive, and developed a cell line. Gey's lab assistant Mary Kubicek used the roller-tube technique to place the cells into culture.
It was observed that the cells grew robustly, doubling every 20–24 hours unlike previous specimens that died out.

Microinjection of somatic cells on the Petri dish. Here cancer cell line (HeLa, *in vitro*).



HeLa cells were the first human cells to be successfully cloned in 1953 by Theodore Puck and Philip I. Marcus at the University of Colorado, Denver. They are "immortal cells"



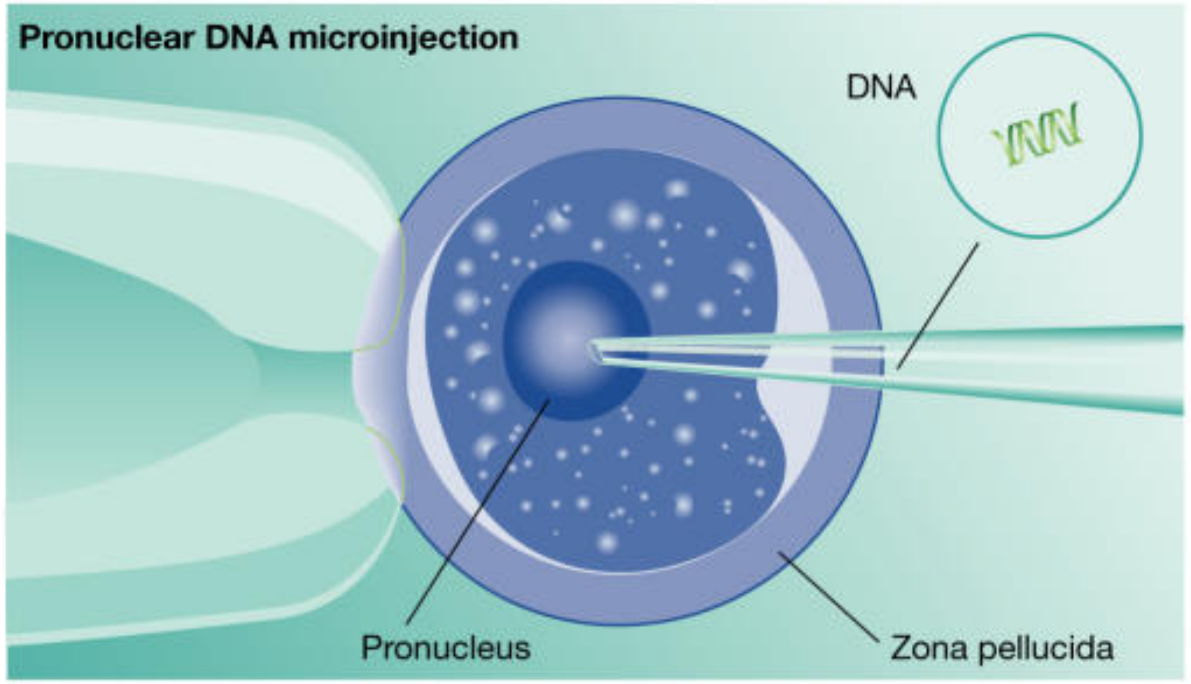
AGGREGATION

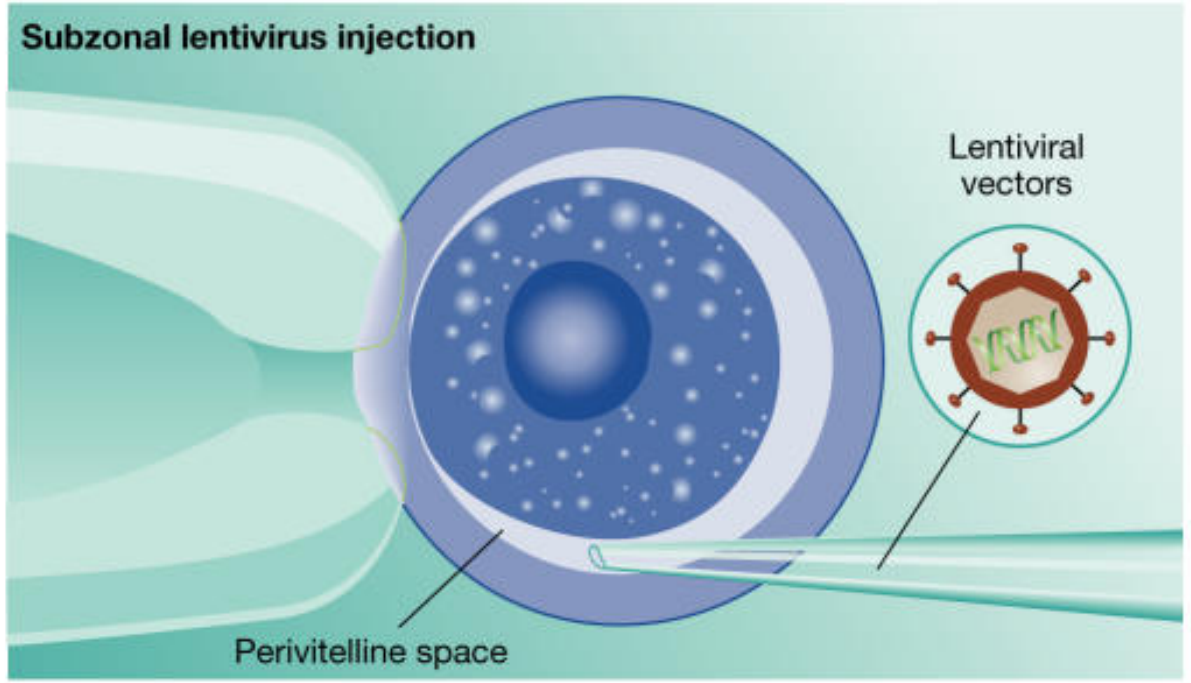
Mitalipov et al., ca 2005

Rhesus macaque Embryonic cell injection and intracytoplasmic injection experiments.

He and his fellows got the chimaric monkey.
They used the whole embryo aggregation method also – see the last section.

ESC – embryonic stem cell
ICM – inner cell mass

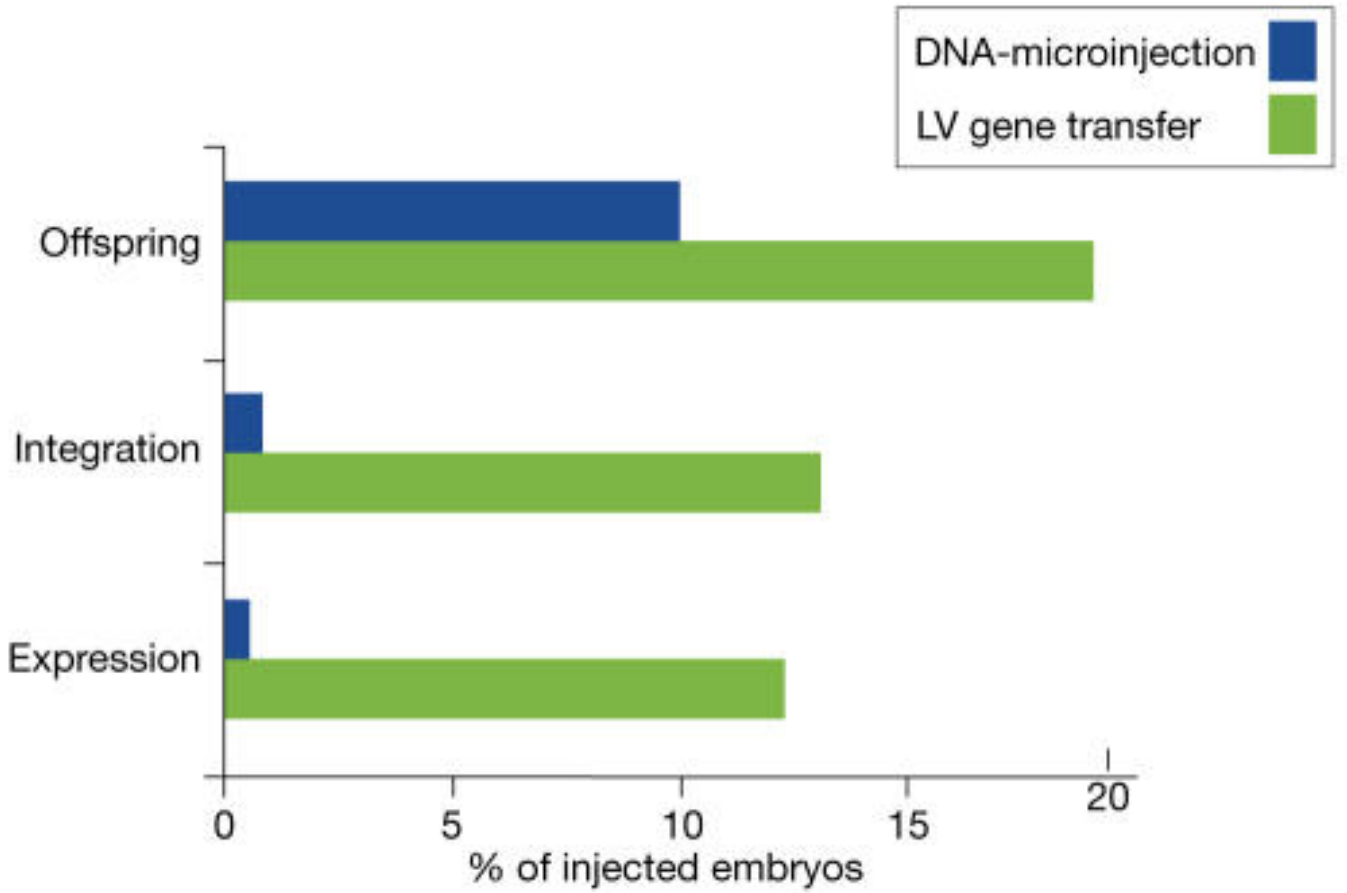




Gene transfer using lenitiviral transformation or co-injection of interested (modified) gene to the fertilized egg

It's the third method

EMBO, Jan, 2004

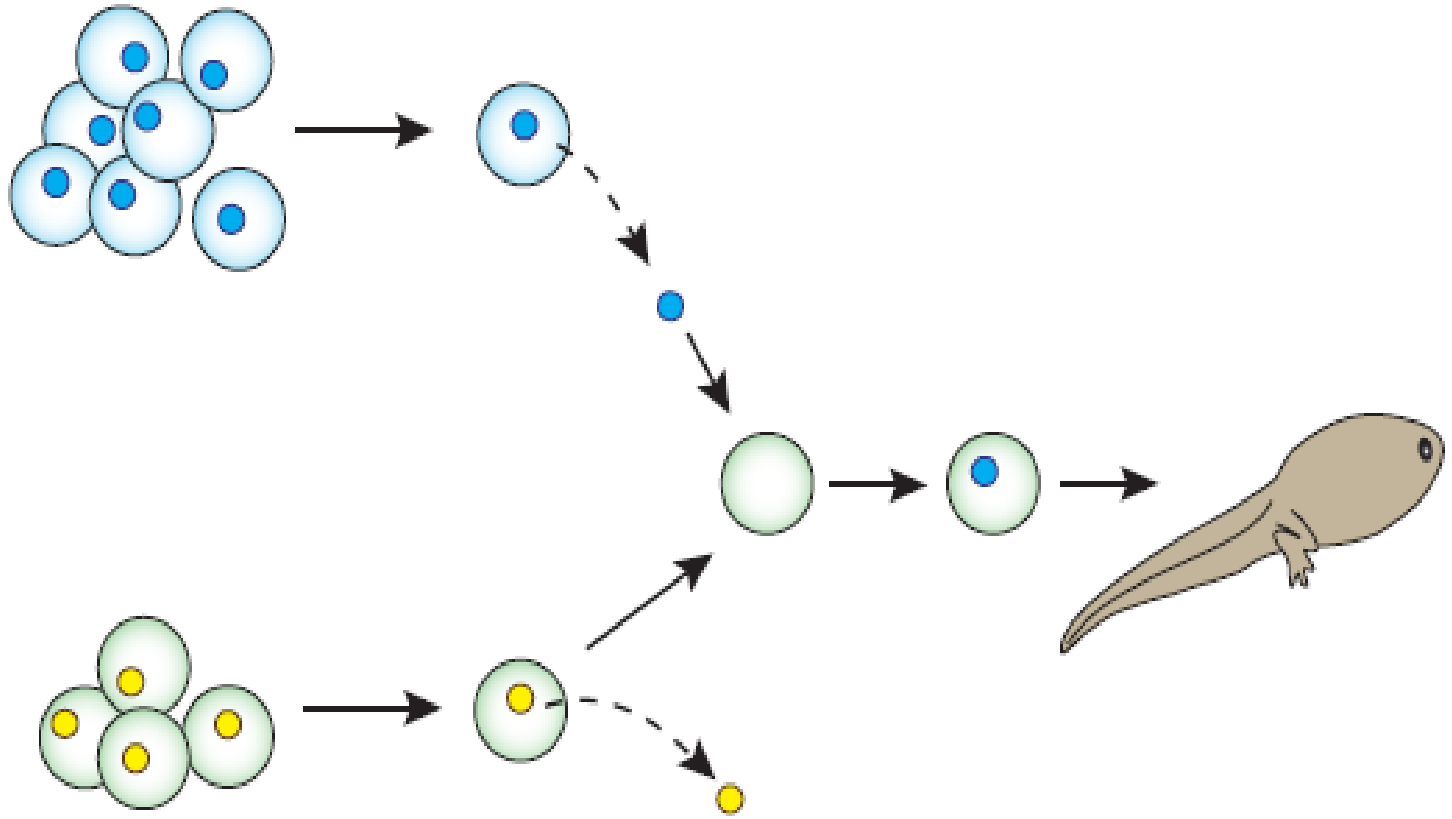


DNA microinjection is reviewed by Wall (1996);
the data on lentiviral (LV) transgenesis are from Hofmann *et al* *(2003)*

Microinjection or injection of egg-cell

(injection of recombinant DNA into the pronuclear area of egg-cell)

1963. British biologist **John B.S. Haldane** used first after J.B. Gurdoni experiments term clone (kr. k. κλών - klōn, “ratoon or twig”).

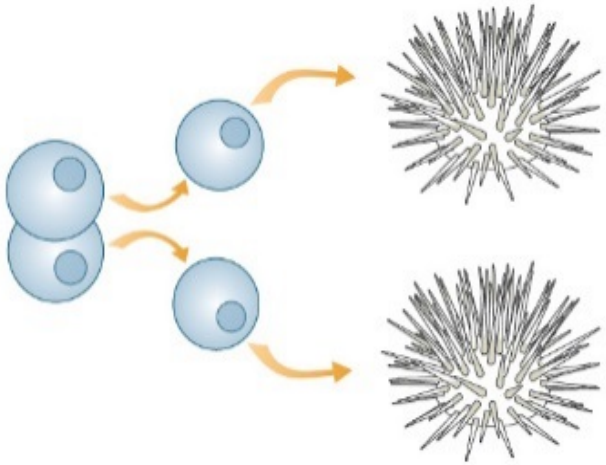


T.J. King and R. Briggs, 1952 Norhern Leopard frog (*Rana pipiens*) development from egg-cell to tadpole

Cloning –

this method is not GMO-technique but it is the simple nuclear transfer of nucleus from one embryonic or somatic cell to the enucleated egg-cell from another species.

J.B.S. Haldane – termin “clone”

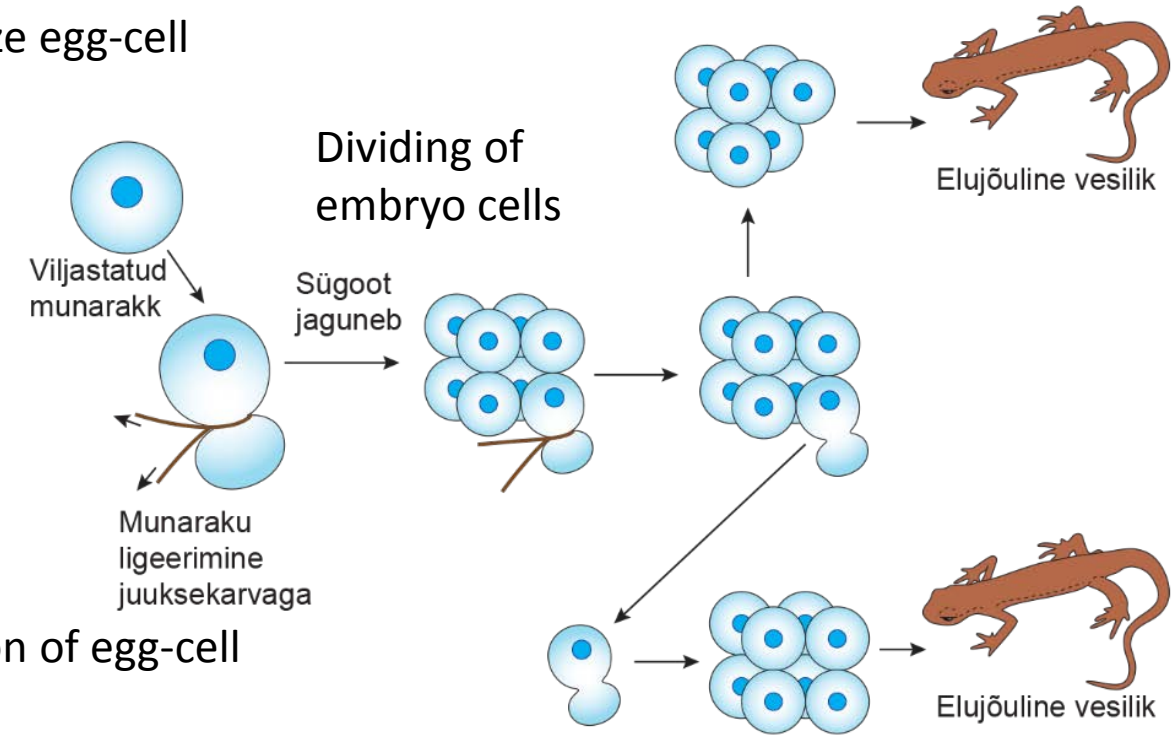


1885.a. **Hans Adolf Edward Driesch** (1867 – 1941),
Sea urchin 2-cell stadium embryo

Hans A.E. Driesch'i +**Wilhelm Roux + Ernst Heinrich Philipp August Haeckel**

1928. Hans Spemann + Hilde Mangold – idea about the real cloning

Fertilize egg-cell



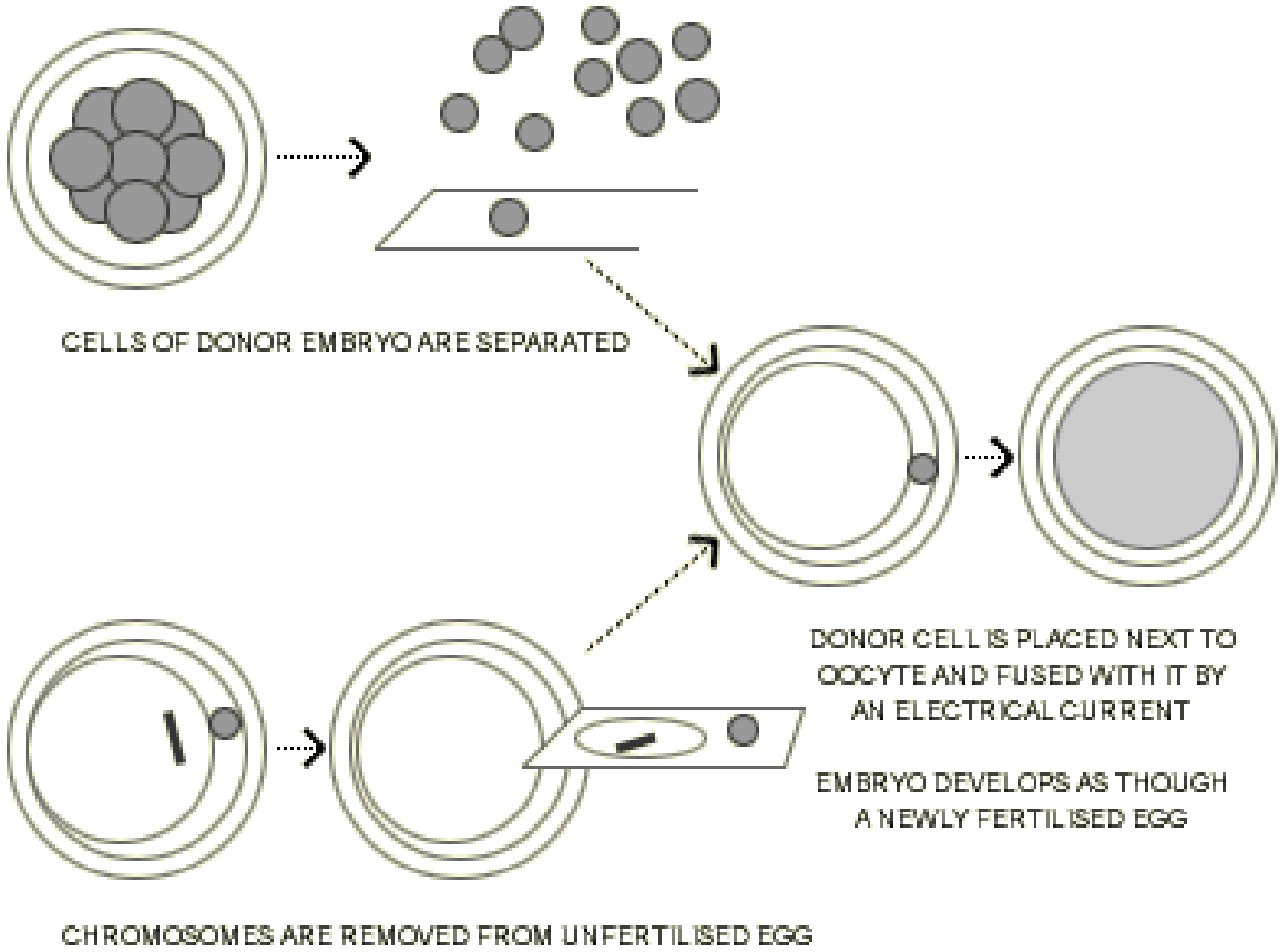
Dividing of embryo cells

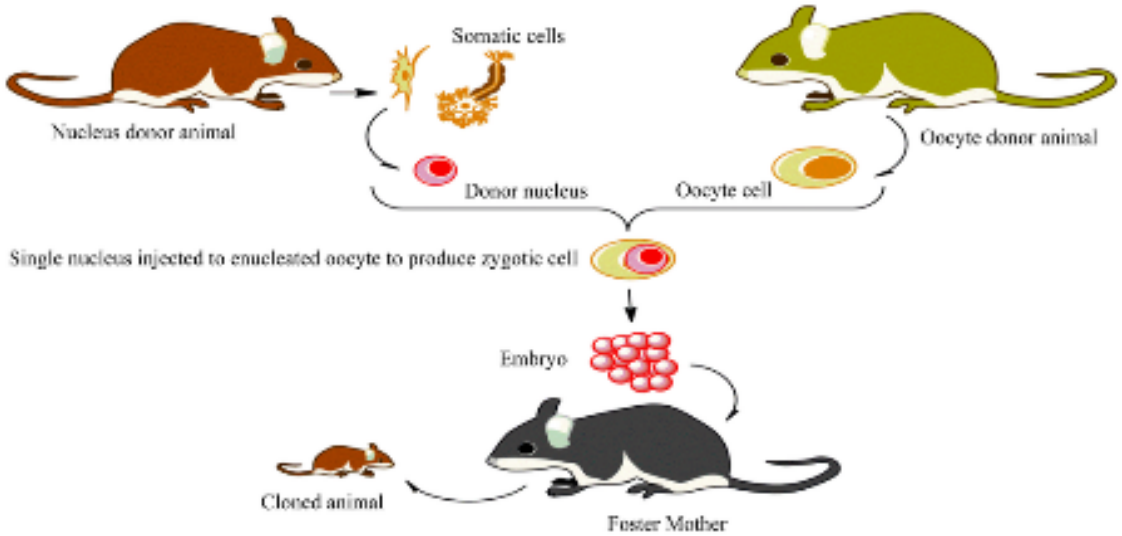
Normal lizard

Ligation of egg-cell

Normal lizard

(method of nuclear transfer in Livestock)

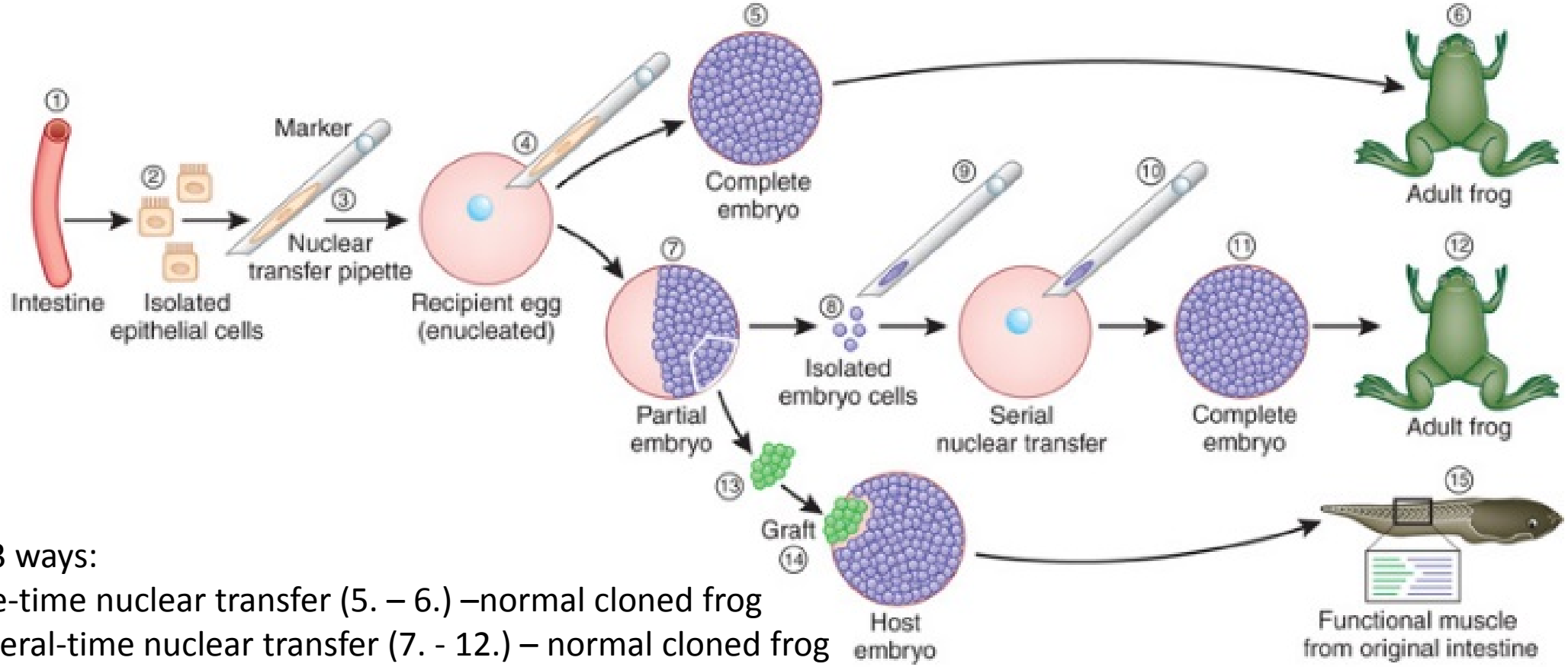




Cloning of mice; from egg cells it will be removed the nucleus and from another egg-cell or from embryo cell or from functionally active adult somatic cell from ohter close relative organism we can catch the nucleus. Donor nucleus will be transplanted into the acceptor cell which is without own nucleus. We can so get cloned diploid egg-cell which is able to divide and from it we can get new „cloned“ organims.

Xenopus sp. cloning

At first the cell from intstine were isolated and after growing them *in vitro* the nucleus from intestine cell was transferred to the enucleated recipient egg-cell.



Then 3 ways:

- one-time nuclear transfer (5. – 6.) –normal cloned frog
- several-time nuclear transfer (7. - 12.) – normal cloned frog
- graft-experiments where only from the part of the cells of early blastocyst are implanted to another host-embryo – normal cloned frog tadpole with functional muscle from original intestine

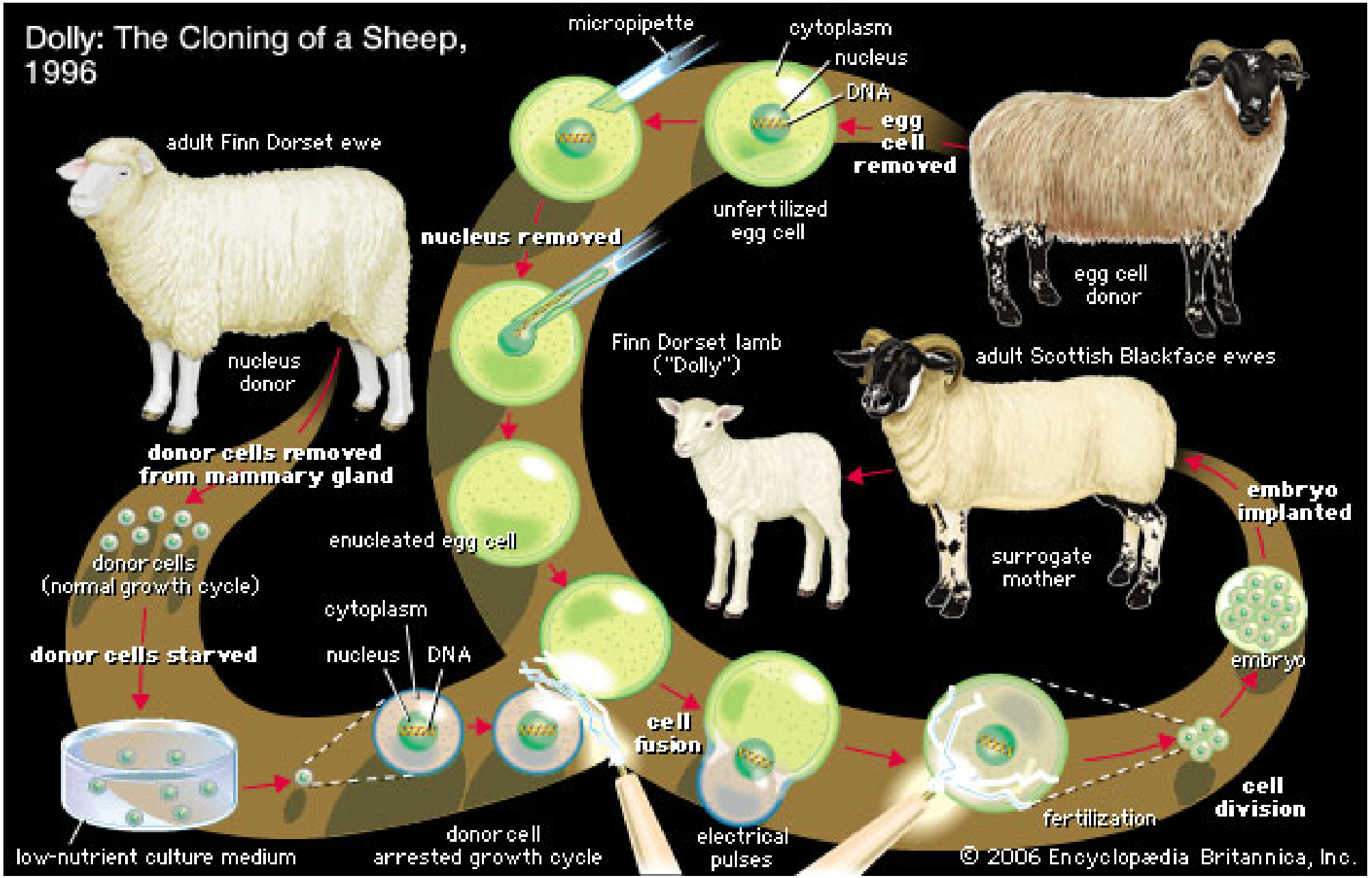
Cloning:

Robert Briggs ja Thomas King, 1952
John Gurdon, 1962 - amphibians
First cloned Fish was carp
Tong Dizhou
First mammalian cloning– *in vitro* rabbit egg-cell nucleus was replaced with embryonic cell nucleus (J. Derek Bromhall, 1975)

Steen Wiladsen – sheep cloning, 8-cell embryo one nucleus was replaced with egg-cell nucleus (1984) – birth of 3 lambs

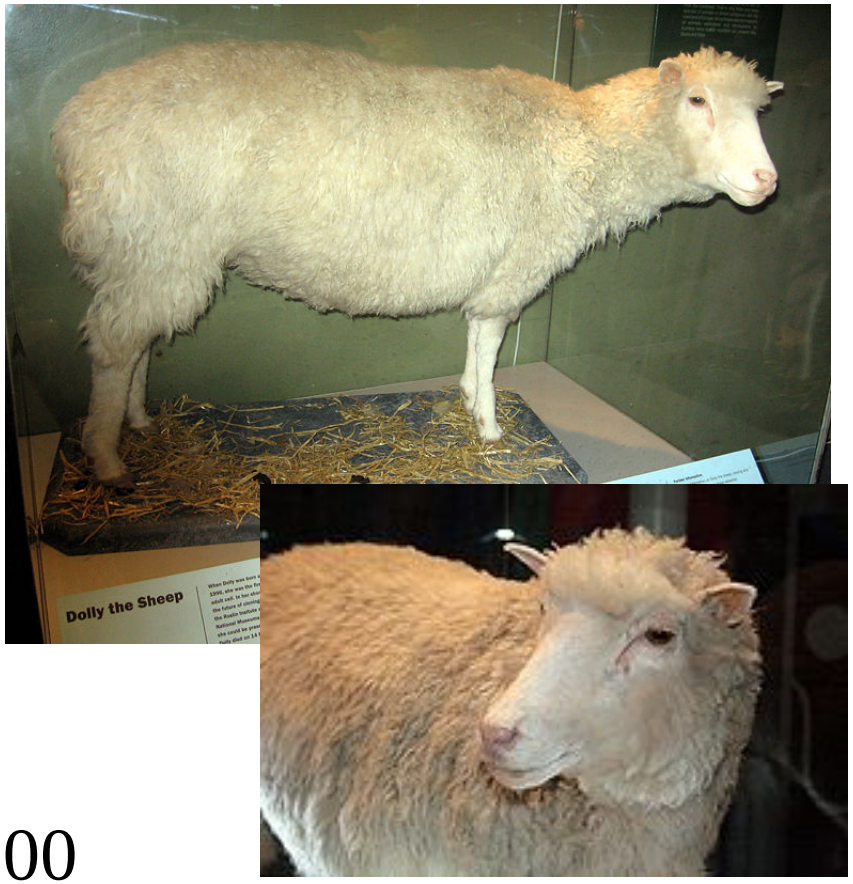
1987 (Randall S. Prather, W. Eyestone) 2 first newborn calfs (Fusion ja Copy)

1996 Edinburgh, Megan ja Morag - injetion of 244 egg-celss, development of 34 and borning 5 of them, development until adult age - only Megan ja Morag
Donor: *in vitro* blastocyst (I. Wilmut, K. Campell)
1997 Dolly the lamb


Dolly: The Cloning of a Sheep, 1996
micropipette
cytoplasm
nucleus
DNA
egg cell removed
adult Finn Dorset ewe
unfertilized egg cell
nucleus removed
egg cell donor
nucleus donor
Finn Dorset lamb ("Dolly")
adult Scottish Blackface ewes
donor cells removed from mammary gland
enucleated egg cell
embryo implanted
donor cells (normal growth cycle)
surrogate mother
cytoplasm
nucleus
DNA
donor cells starved
cell fusion
embryo
cell division
fertilization
low-nutrient culture medium
donor cell arrested growth cycle
electrical pulses
© 2006 Encyclopædia Britannica, Inc.

Dolly descendants:

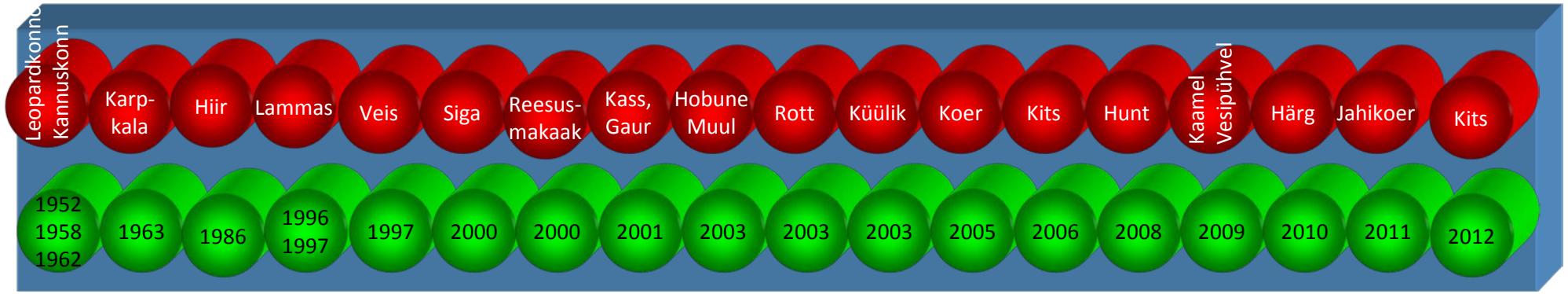
- Bonnie – 1998
- Twins Sally ja Rosie – 1999
- Triplets: Lucy, Darcy ja Cotton - 2000



Dates of cloning of some species

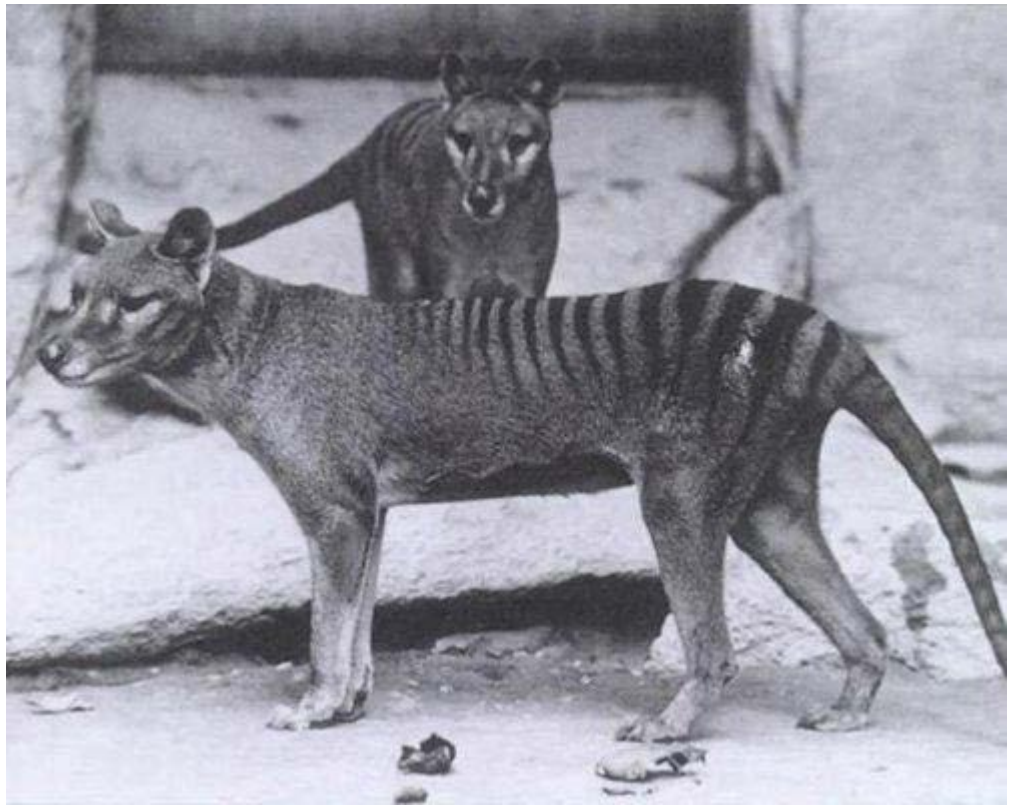
Frog Carp Mouse Sheep Calf Pig

Wolf Camel Ox Hound dog Ibex



Macaque Cat Horse Rat Rabbit Dog Goat

For succesfull cloning:



- Extinction must be as late as it possible
- Very close relatives are alive
- The DNA of extincted animals must be founded

Tasmanian tiger (*Thylacinus cynocephalus*), ca 1930 was hunted the last specimen

What You think – is it possible to clone this specimen or mammoth for example?
Can we do it?

C.p. victoriae e gredos

-The last Pyrene ibex or bucardo female(Celia) died on2000; from she had collected fibroblasts and they had frozen (1999.a.)

- Egg-cells collected from home coat by superovulation
- To the egg-cells without the nucleus were implanted by elecrical shock the nucleus of fibroblast
- Embryos were developed 36 h or 7 days in cell culture and then implanted to the foster mummies (spanich ibex or hybrid: spanish ibex male x simple (home) she-coat)
- one cloned bucardo was born but it lived only 17 minutes

- 2013. a. Dr Alberto Fernandez-Arias:

Cloning the bucardo

Crispr/Cas-method for bucardo rebirth

This plan is also on the table with Tasmanian devil because they have several facial tumors – this is connected with high rate of inbreeding *Devil Facial Tumour Disease – DFTD*).

Asia elephant – genome editing – get the mammoth....!? (in Japan)

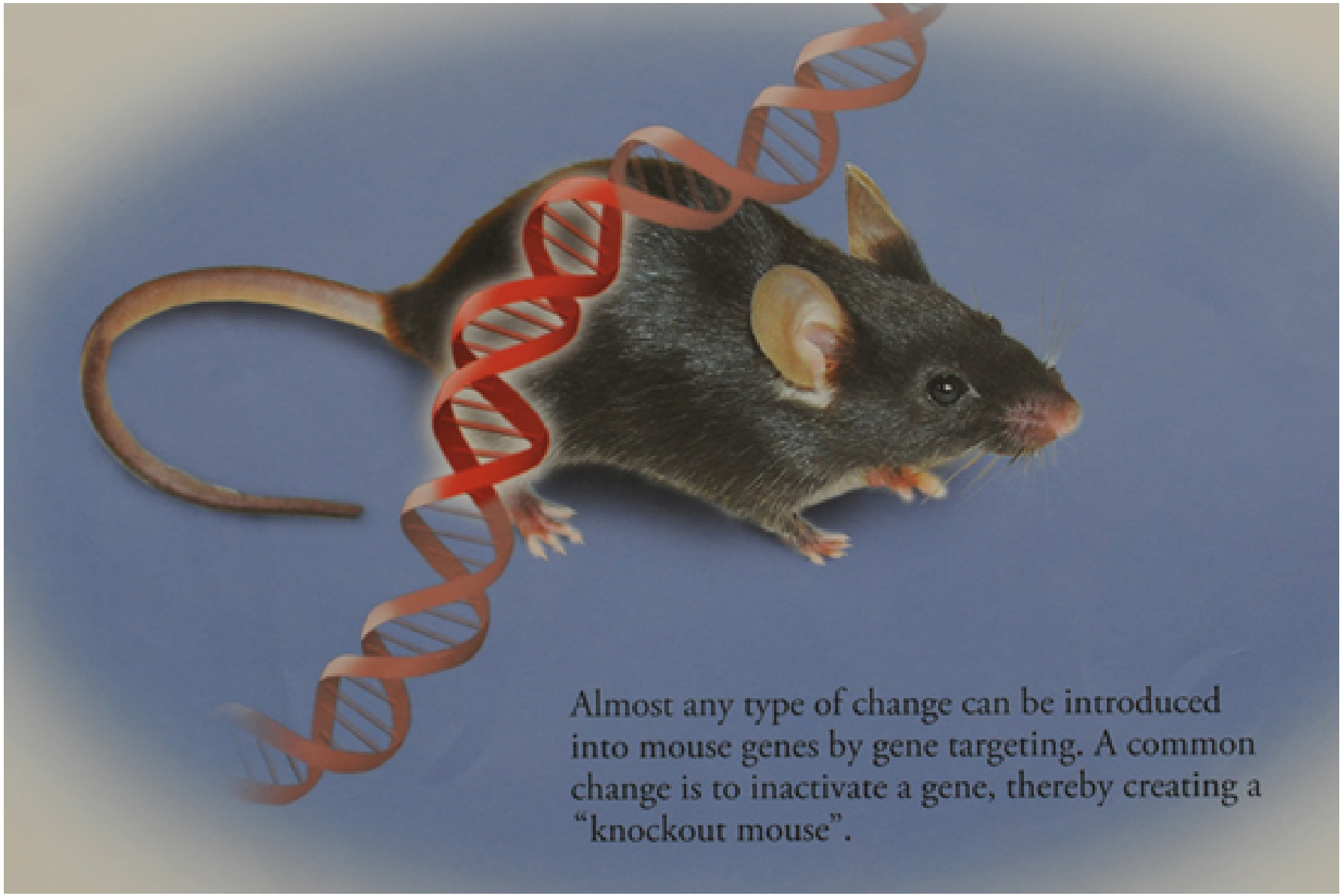
Juuni (June)– Estonian newborn calf
2013.a.

growth hormone (GH)
no transgene

19.08.2015 born of Augustina – the second cloned calf in Estonia

FSH (Follicle-Stimulating Hormone) -calf; no transgene

They have had cloned succesfully but
their genome consists no forign DNA –
they were normal off-springs without transgenes.

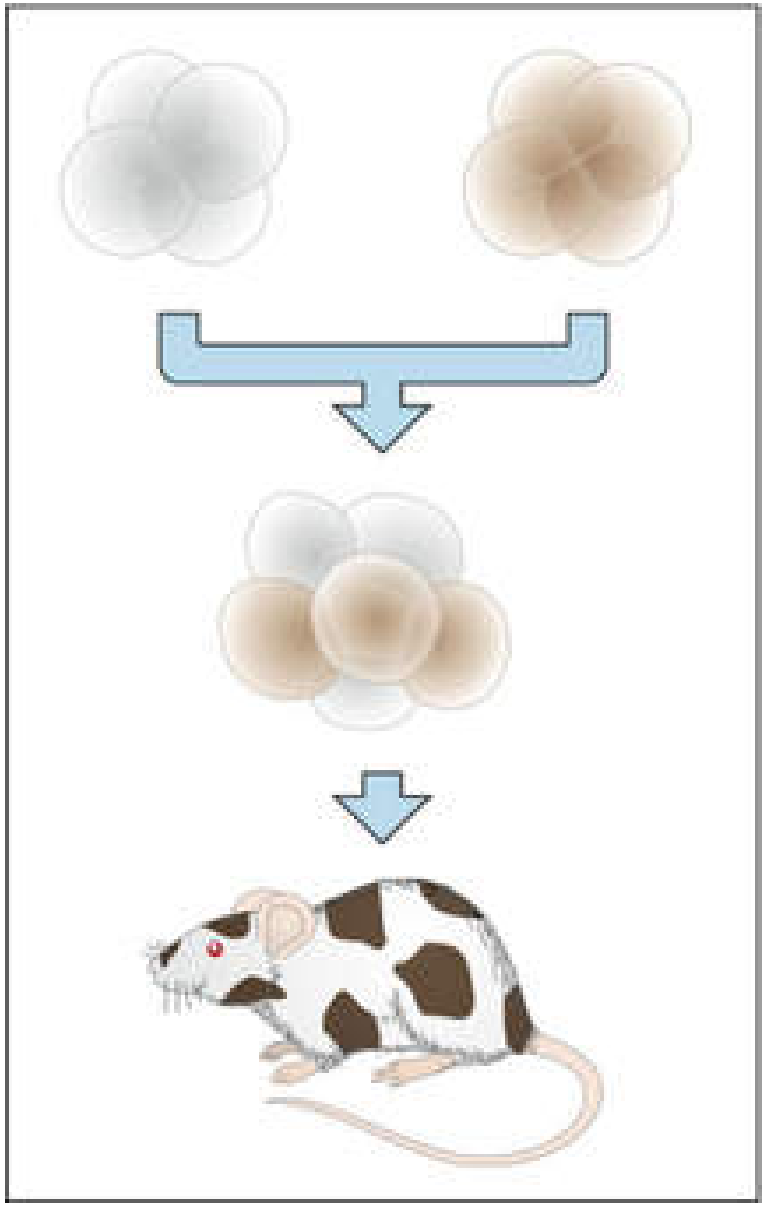

Almost any type of change can be introduced into mouse genes by gene targeting. A common change is to inactivate a gene, thereby creating a "knockout mouse".

Blastocyst injection –

the fourth method of getting GMOs

We can get the chimaric mouse, some
of the cell have one set og genetic material
but the others have some forigen trangenes.
From that the name - transgenic mice.

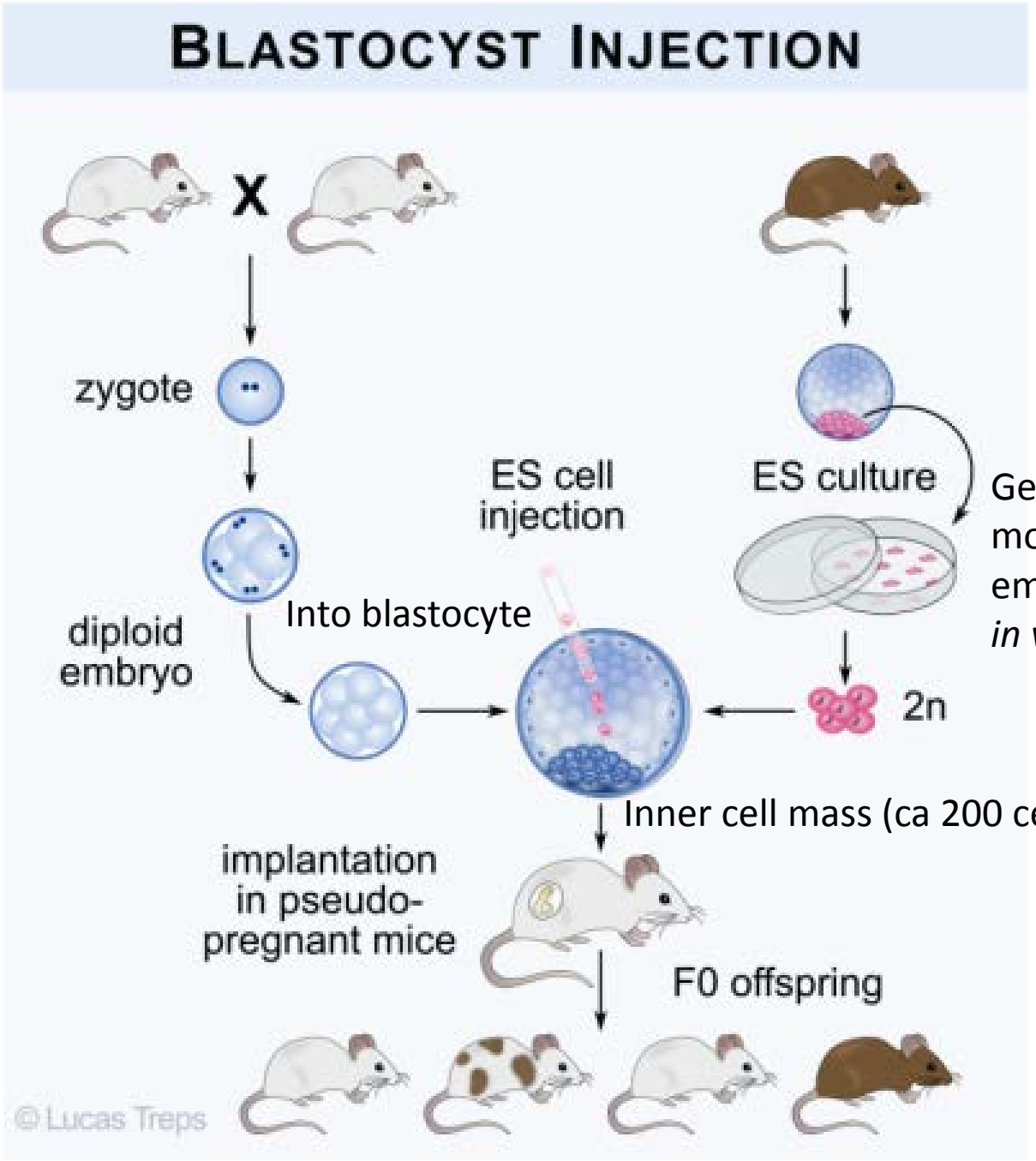
We can get:
Knock-in mice,
Knock-out mice,
Knock-down mice.
What theese names denote?

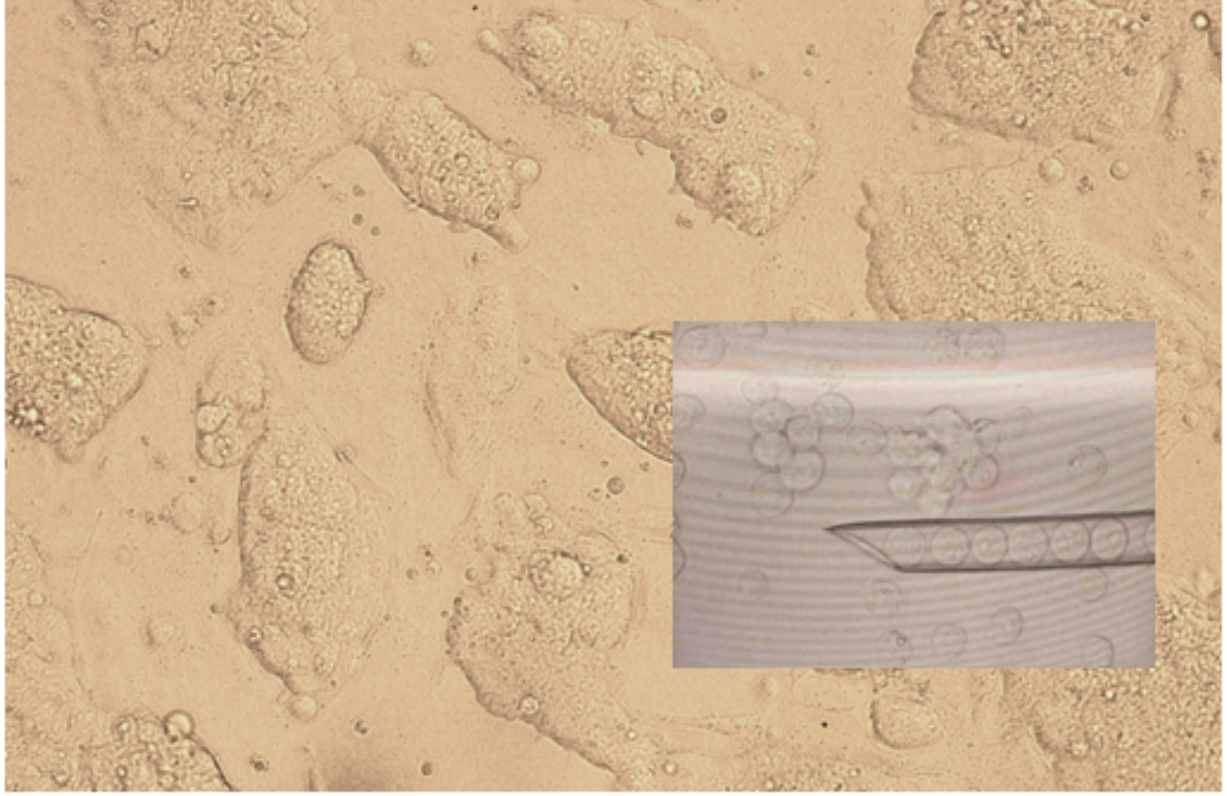


Blastocyst injection

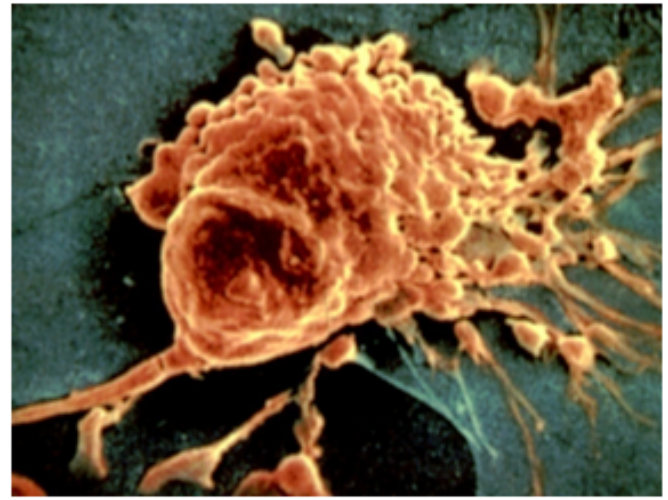
Blastocyst stadium is very early developmental stadium –
Mouse about 3...3,5 *days post coitum (dpc)*
Human about 10 *dpc*

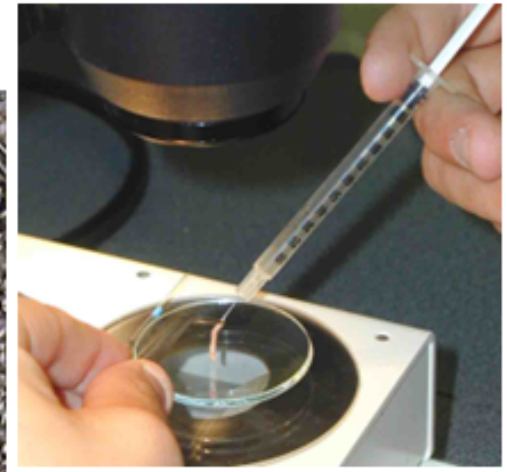
This method means that the GM ESC or iPSC will be injected into the early embryo at the blastocyst stadium (into the blastocoel) where they can integrate freely to inner cell mass of this embryo.
When theese cells integrate successfully to the foster embryo we can talk about genetically modified embryo or transgenic embryo.



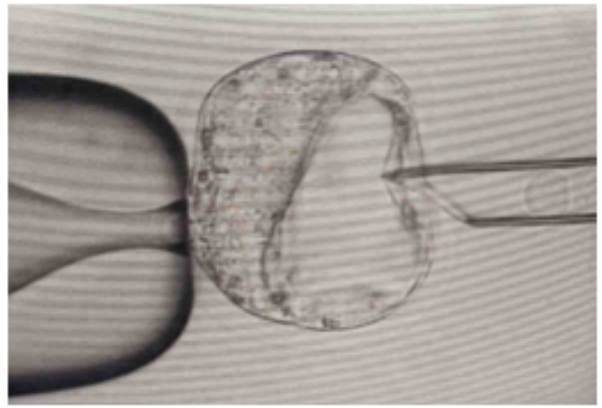


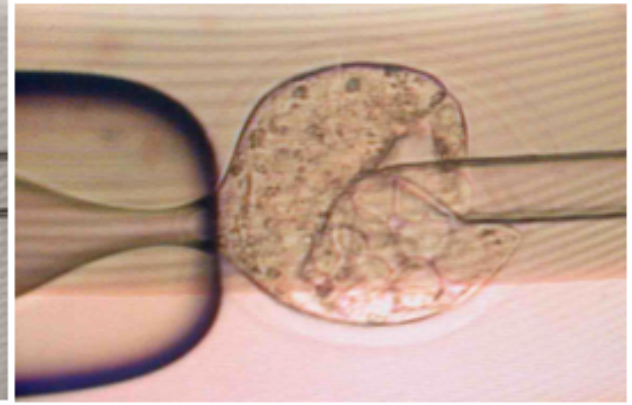
129 w4 embryonic stem cells

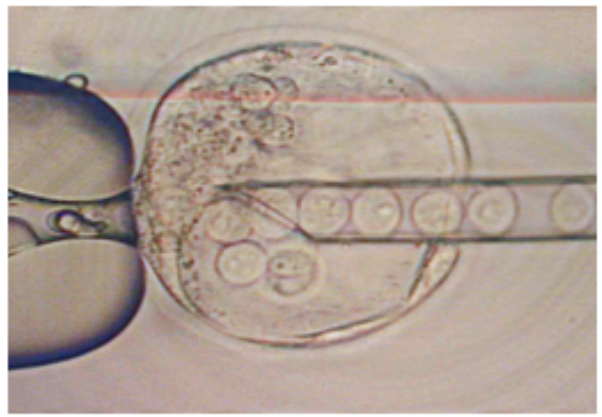


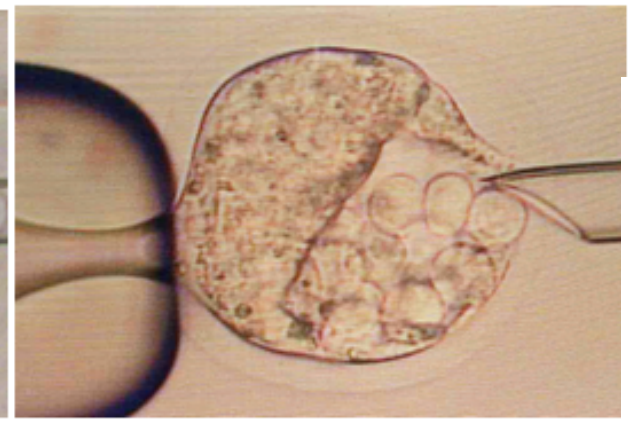


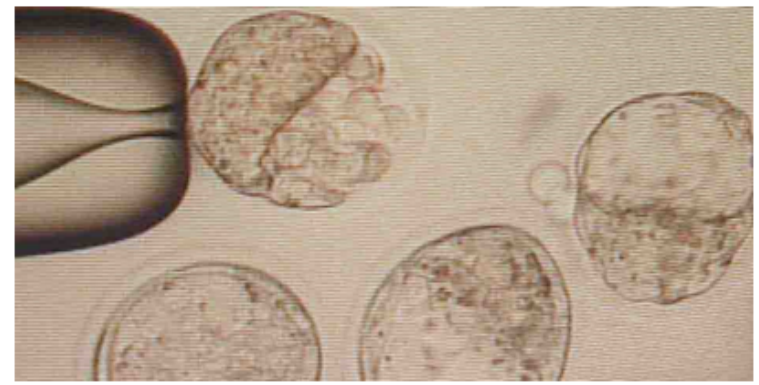
ESC injection to the embryo

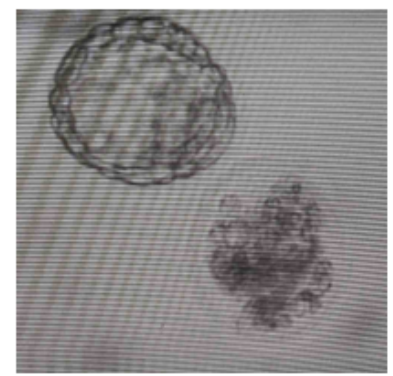




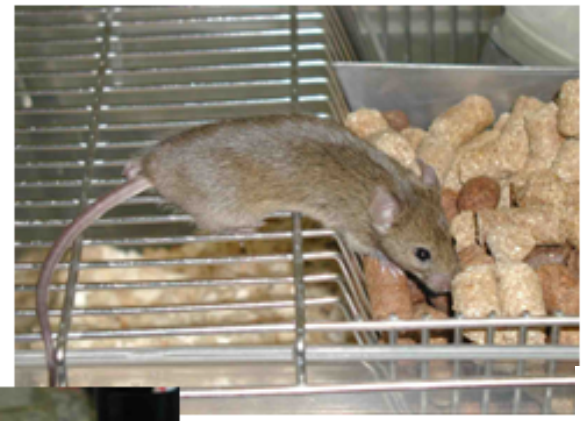




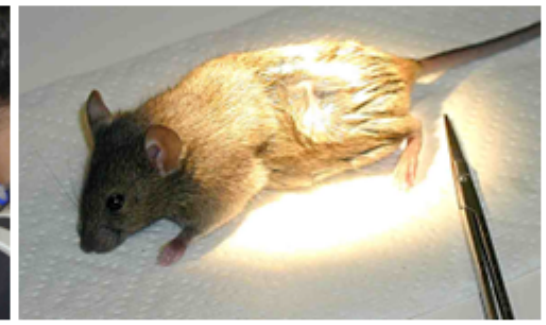


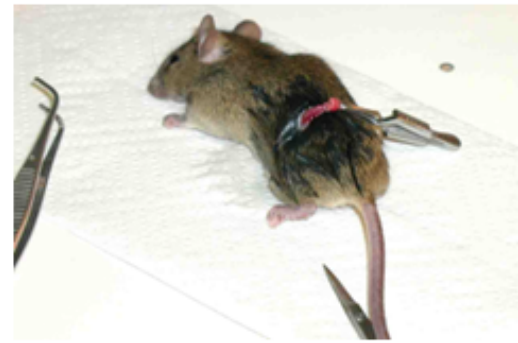


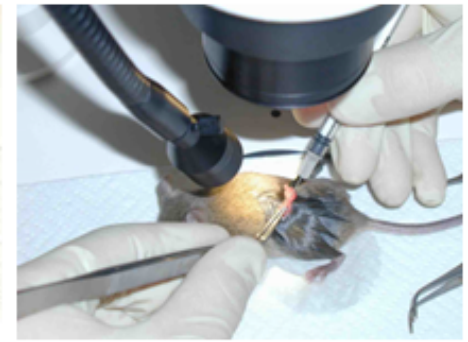
Foster mother = pseudopregnanat mouse



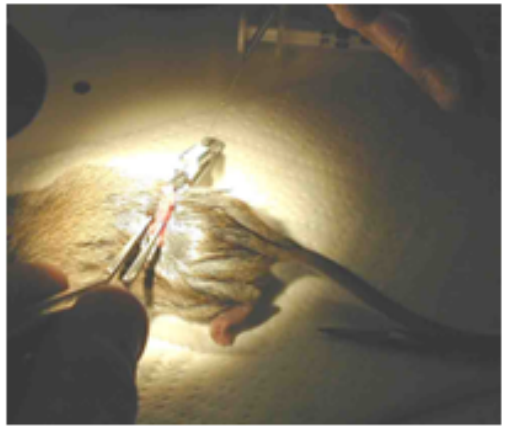
Retransfer



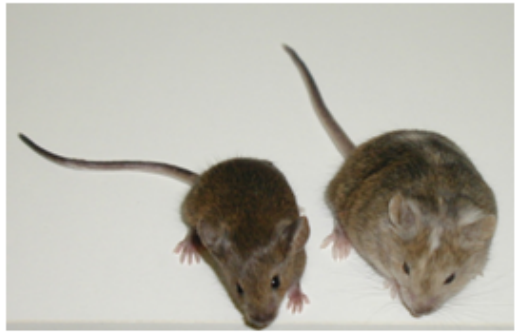


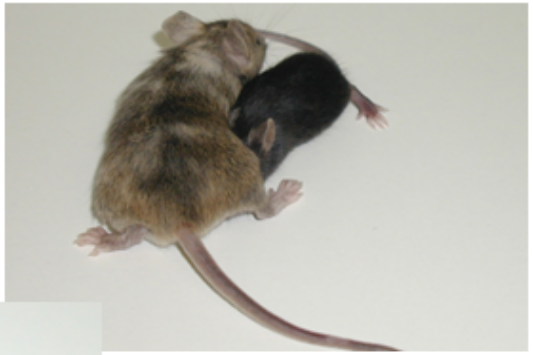


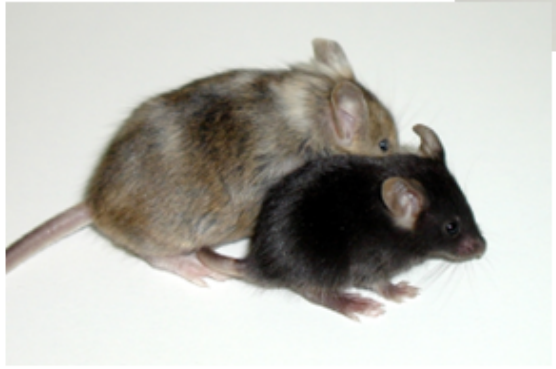
Retransfer

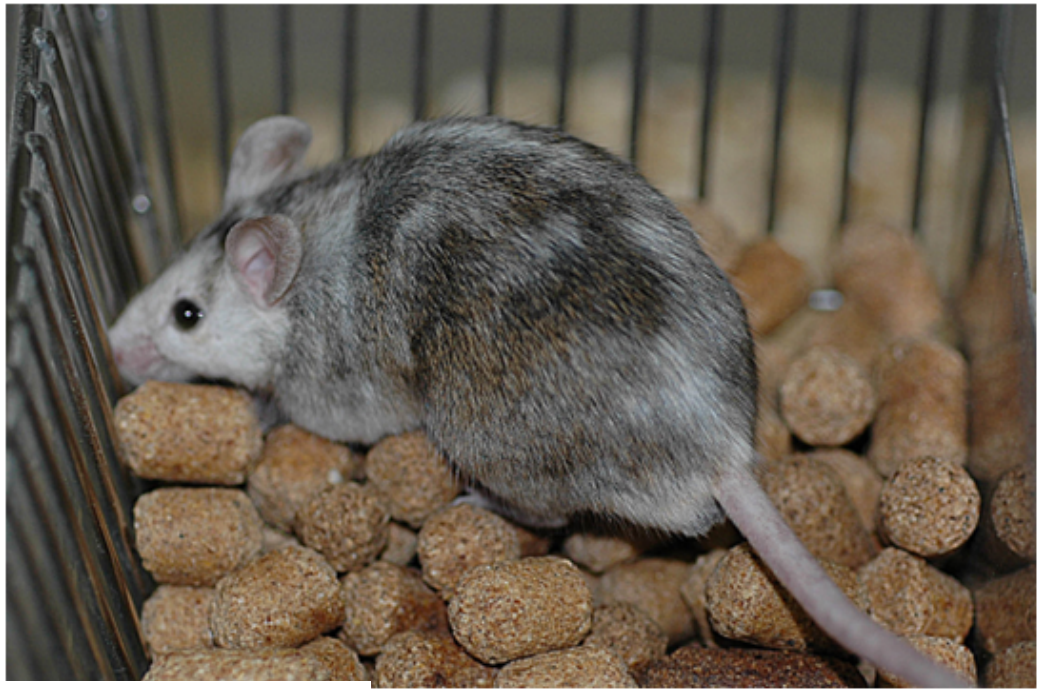


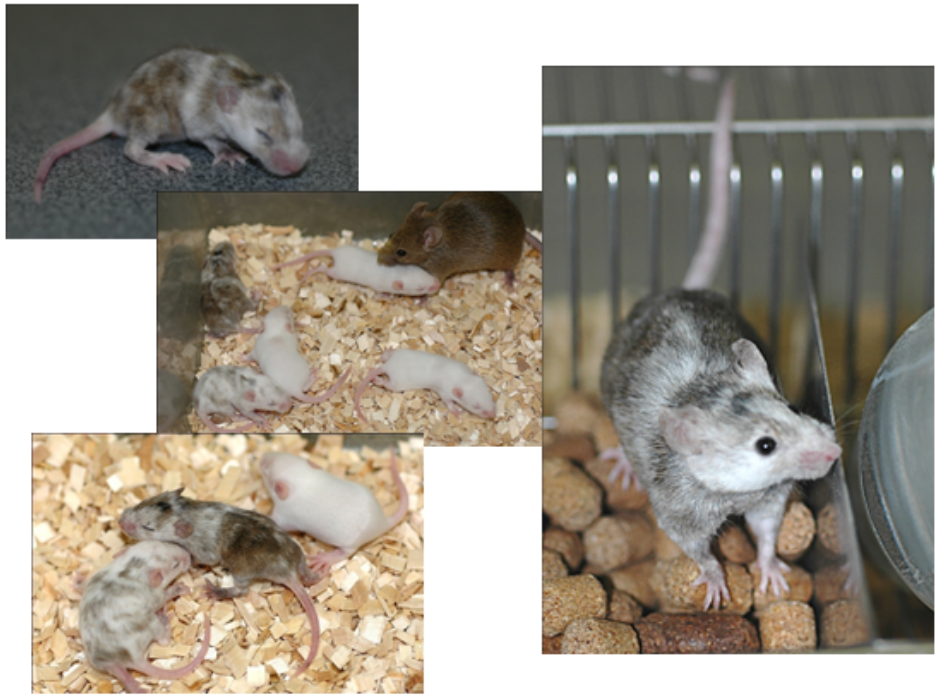
Offspring

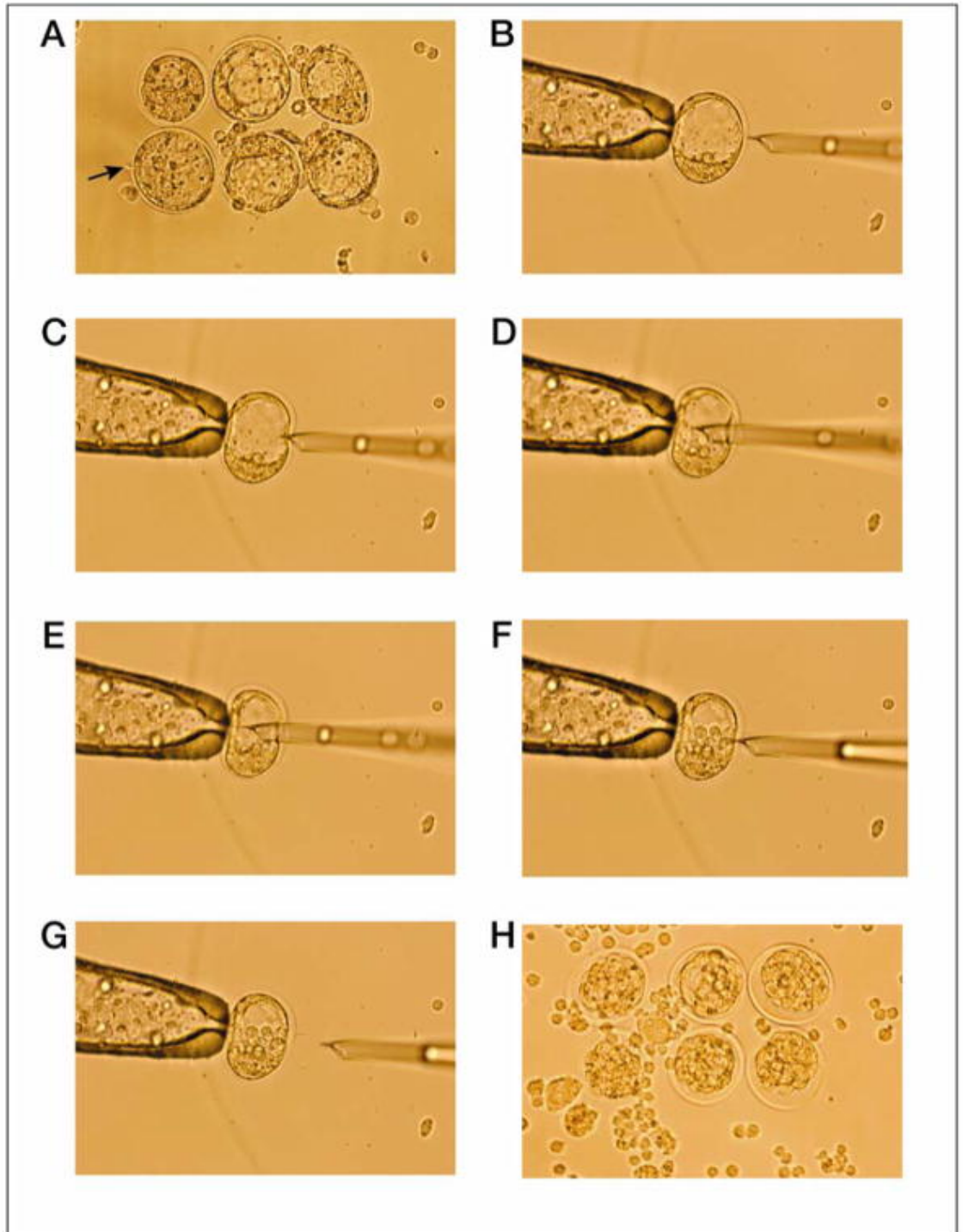








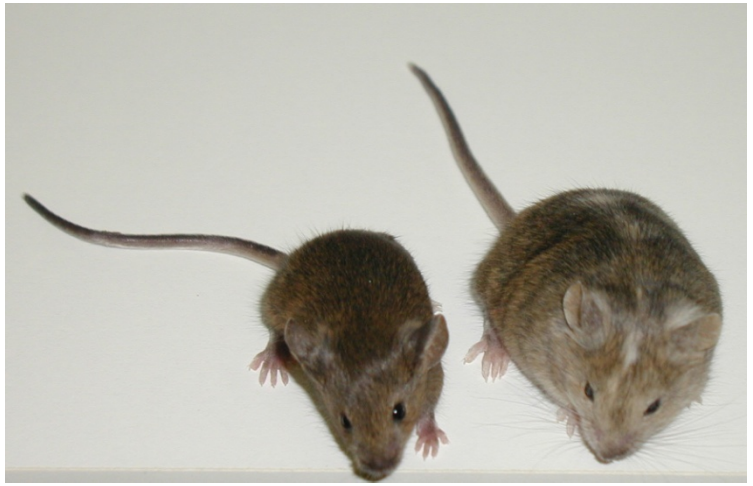




Injection of mouse blastocytes (3,5 day old after fertilization) with embryonic stem cells (small cells – see H)

Some chimaric mice (spotted and brown-white colored) at the University of Tartu (IMCB). The two black and one brown mice are wild-type mice from the same litter – it means that they are all at the same age.

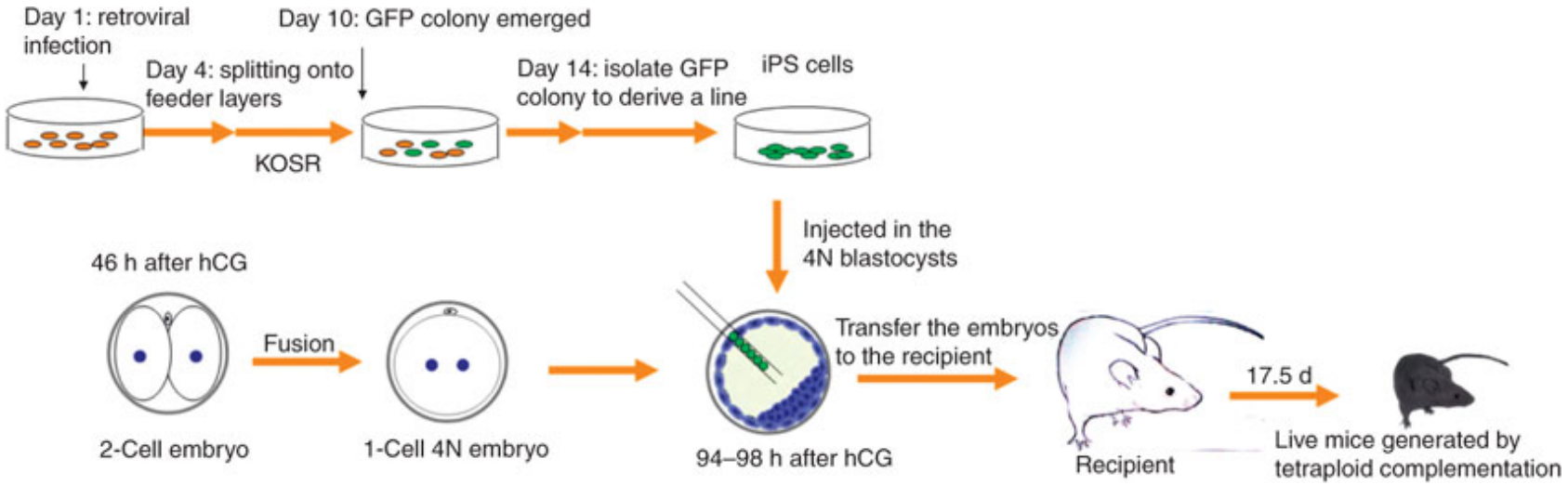
Why the big one have so called spotted pelage?
Whay they have in different size?
Your explanation!



Zeiss Axiovert
Eppendorf

In 2008, Science named induced pluripotent stem cells (iPS) the breakthrough of the year.1 In 2009, Nature Methods called the process of generating iPS cells the "Method of the Year."

- 2009 Tetraploid complementation method - see what it menas?



KOSR – knockout seerum replacement, its as FCS for differentiation of embryonic cells

The tetraploid complementation assay is a technique in biology in which cells of two mammalian embryos are combined to form a new embryo. It is used to construct genetically modified organisms, to study the consequences of certain mutations on embryonal development, and in the study of pluripotent stem cells.

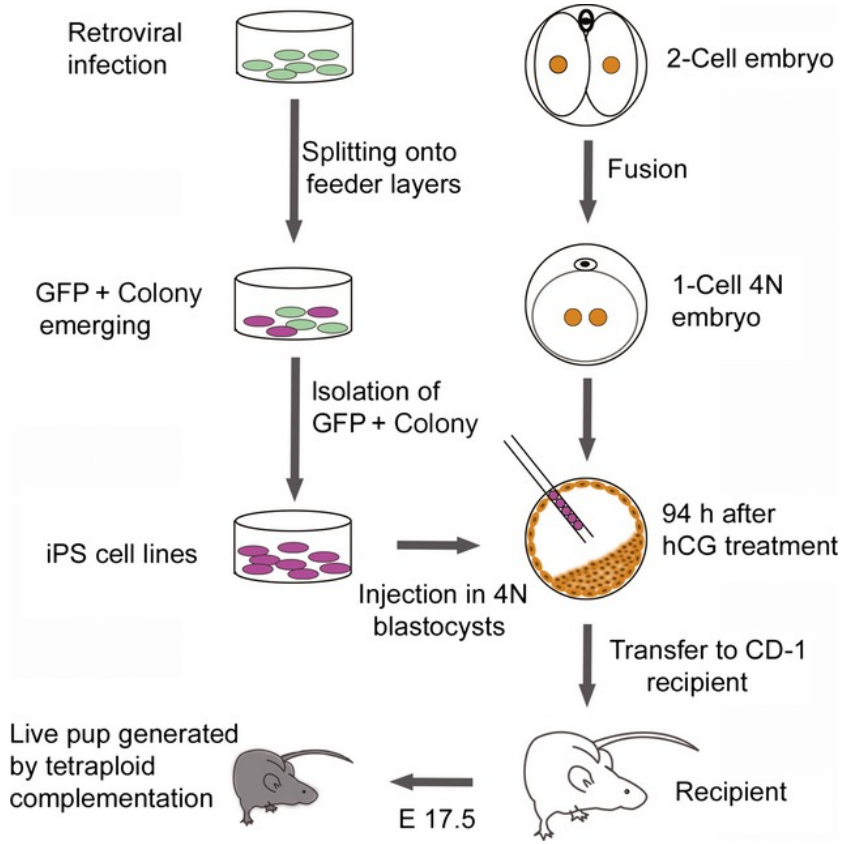
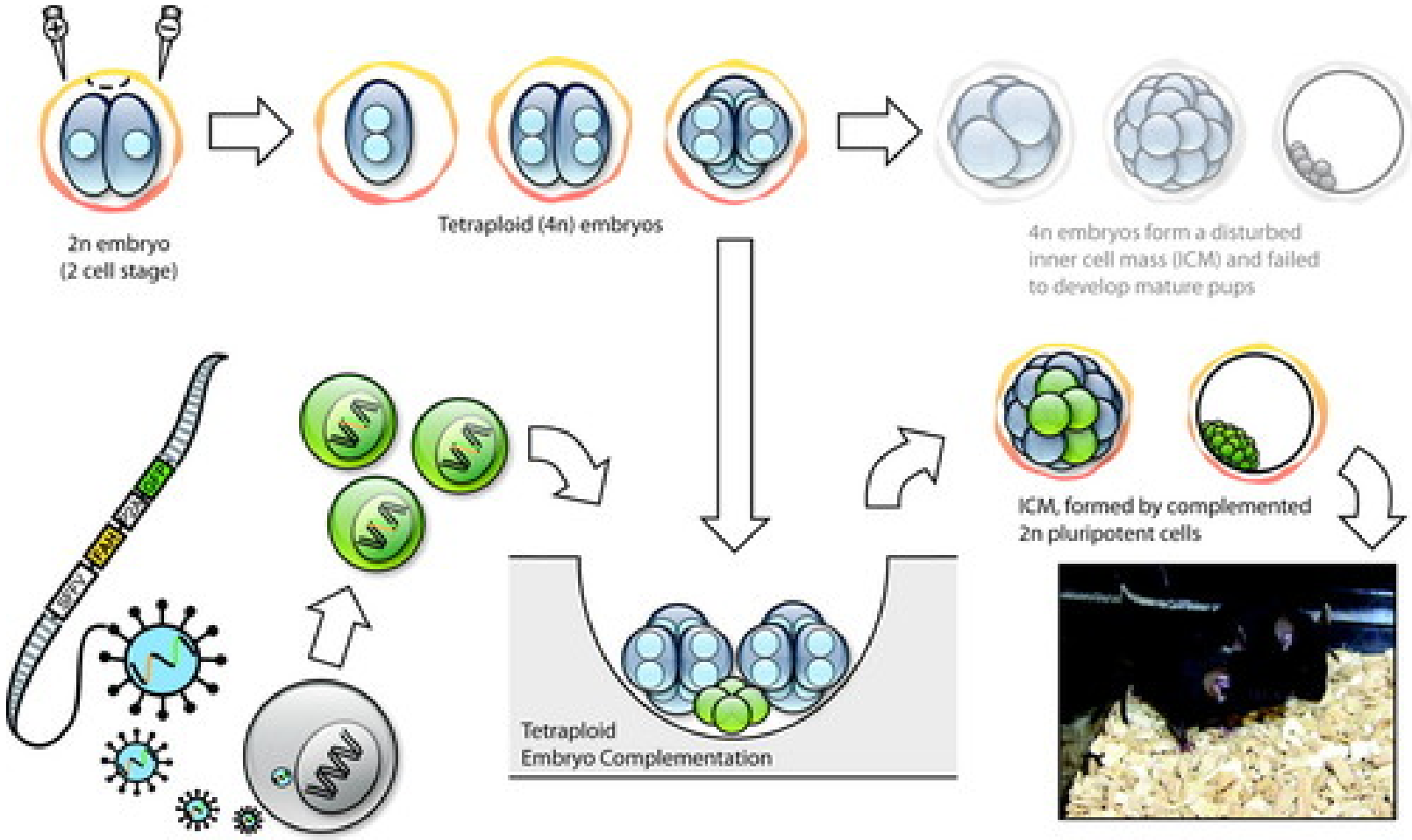


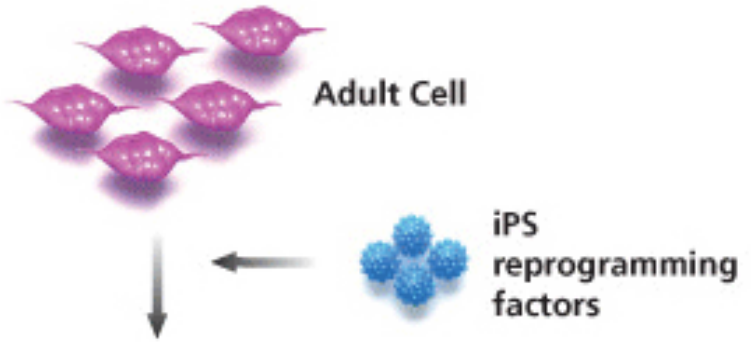
Diagram demonstrating the process described by Wu et al for creating mice from gene-corrected disease-specific iPS cells. (Illustration credit: Cosmocyte/Ben Smith, adapted from an image provided by Tobias Cantz).



At 2011 a team led by Tobias Cantz, at the Max-Planck-Institute for Molecular Biomedicine, published a proof-of-principle paper in PLoS Biology demonstrating that iPS cells remained pluripotent after correction of genetic defects

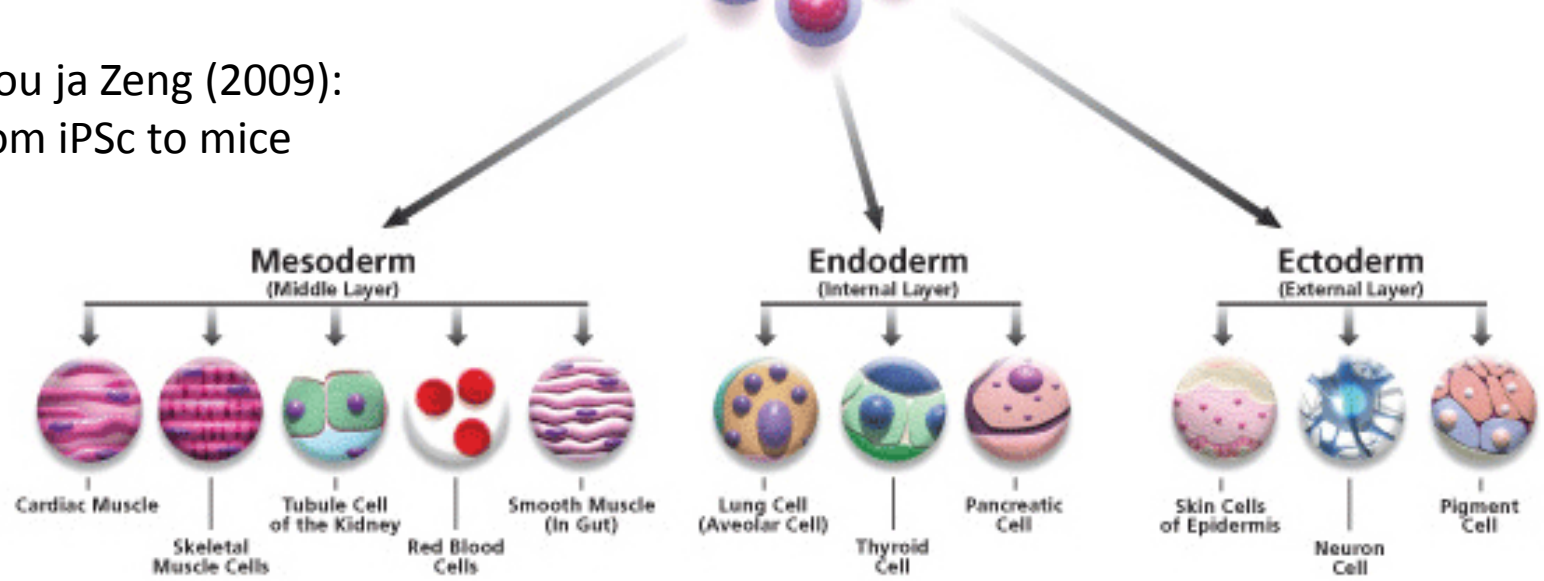
PLURIPOTENCY

Today is more common to use for injection or for getting the transgenic animals **induced pluripotentic stem cells and we are able to change genetic information of them.**

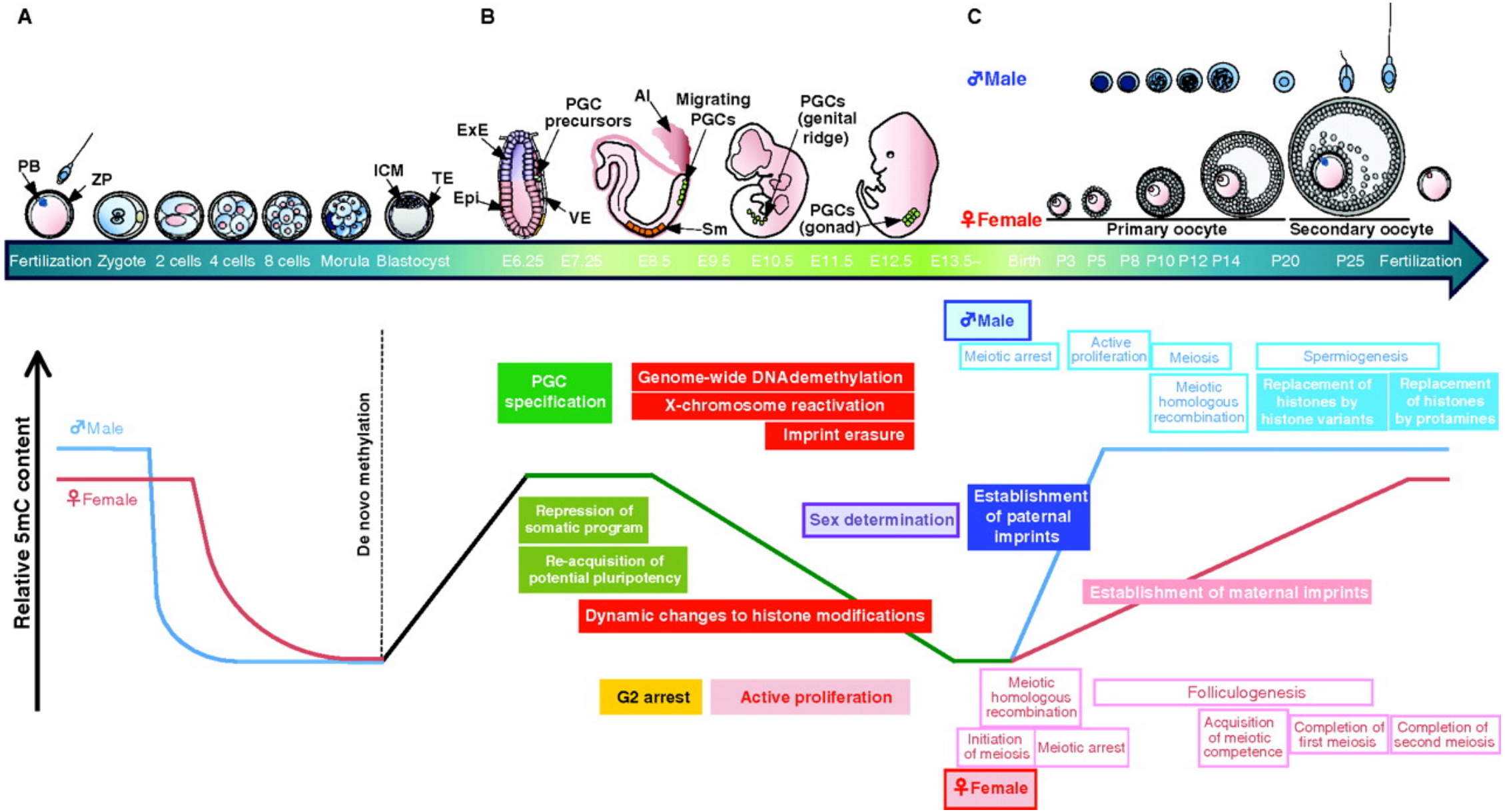


- iPSC - induced pluripotentic stem cells

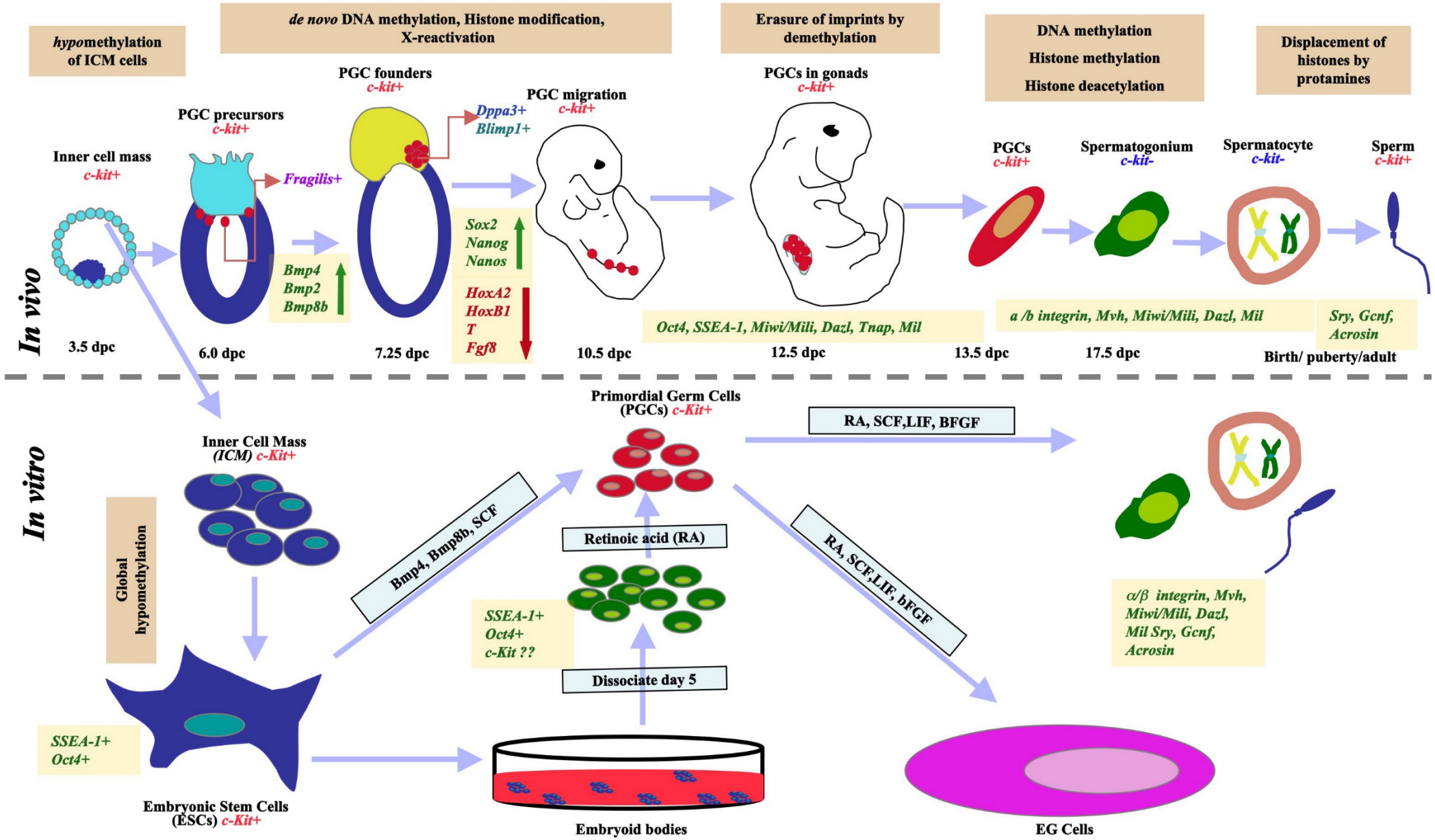
Zhou ja Zeng (2009): From iPSc to mice



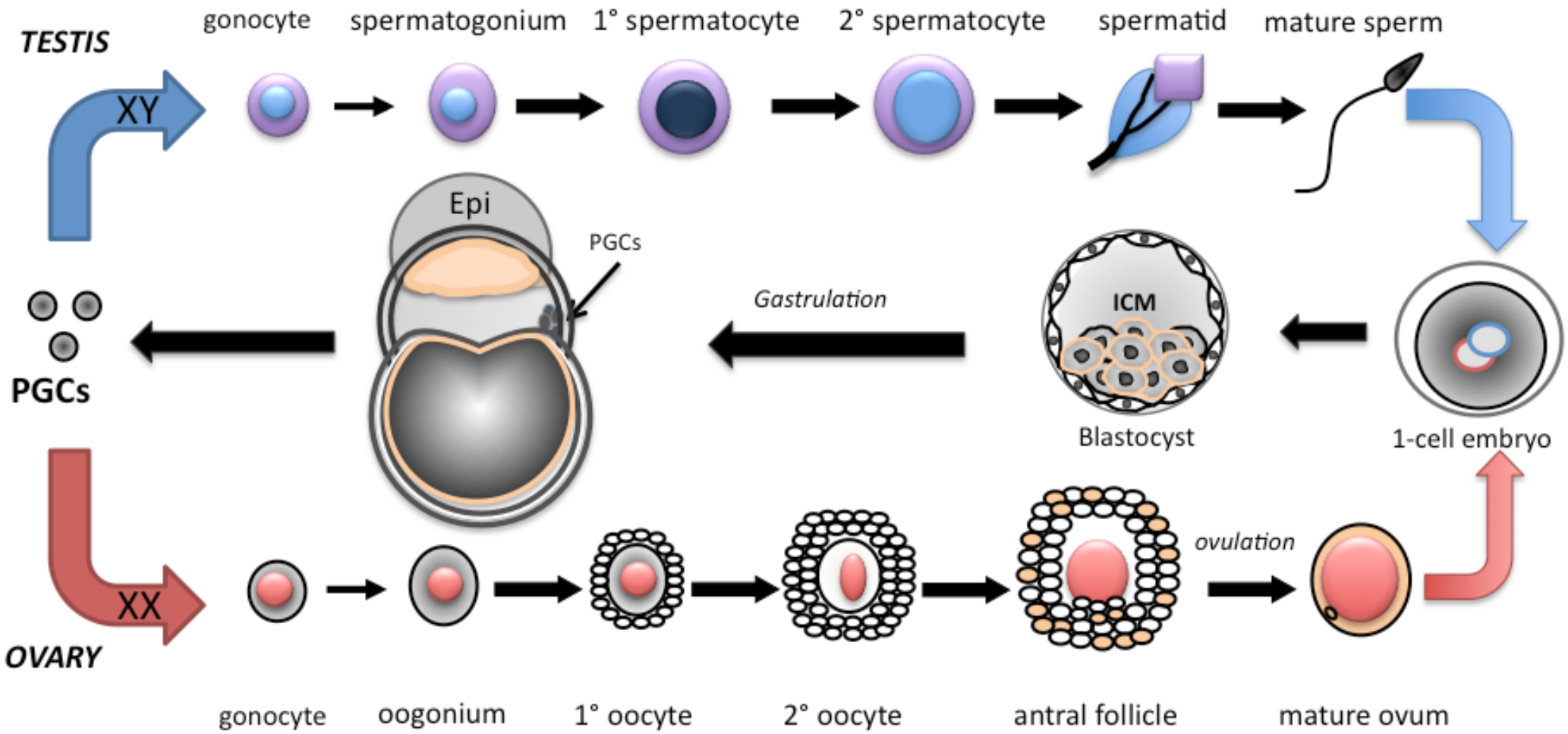
- Less etchical problems compare with embryonic stem cells
- iPSCs are fused with embryonic stem cells (ESC) in inner cell mass (ICM)
- Its the simplier method as nuclear transfer or traditional cloning of living organism.
- Negative aspect – we need living cells of the normal donor-organism



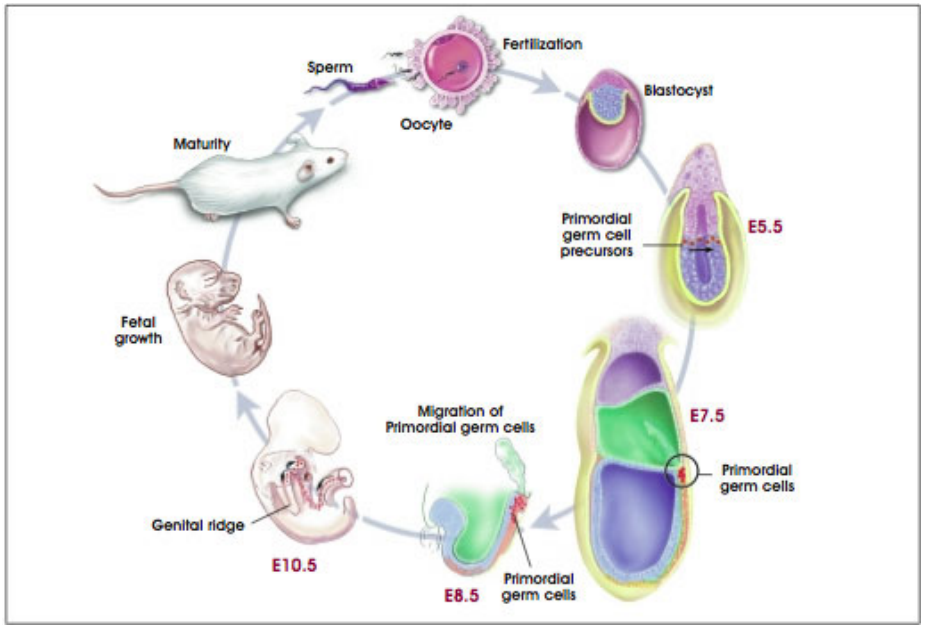
From fertilization until egg-cell development – see when happen DNA methylation, when histone modification, what it means maternal imprinting!?

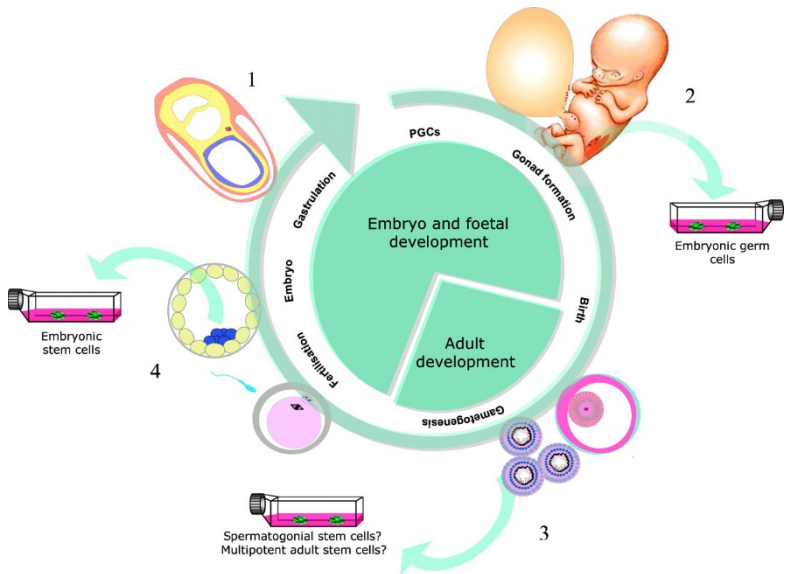


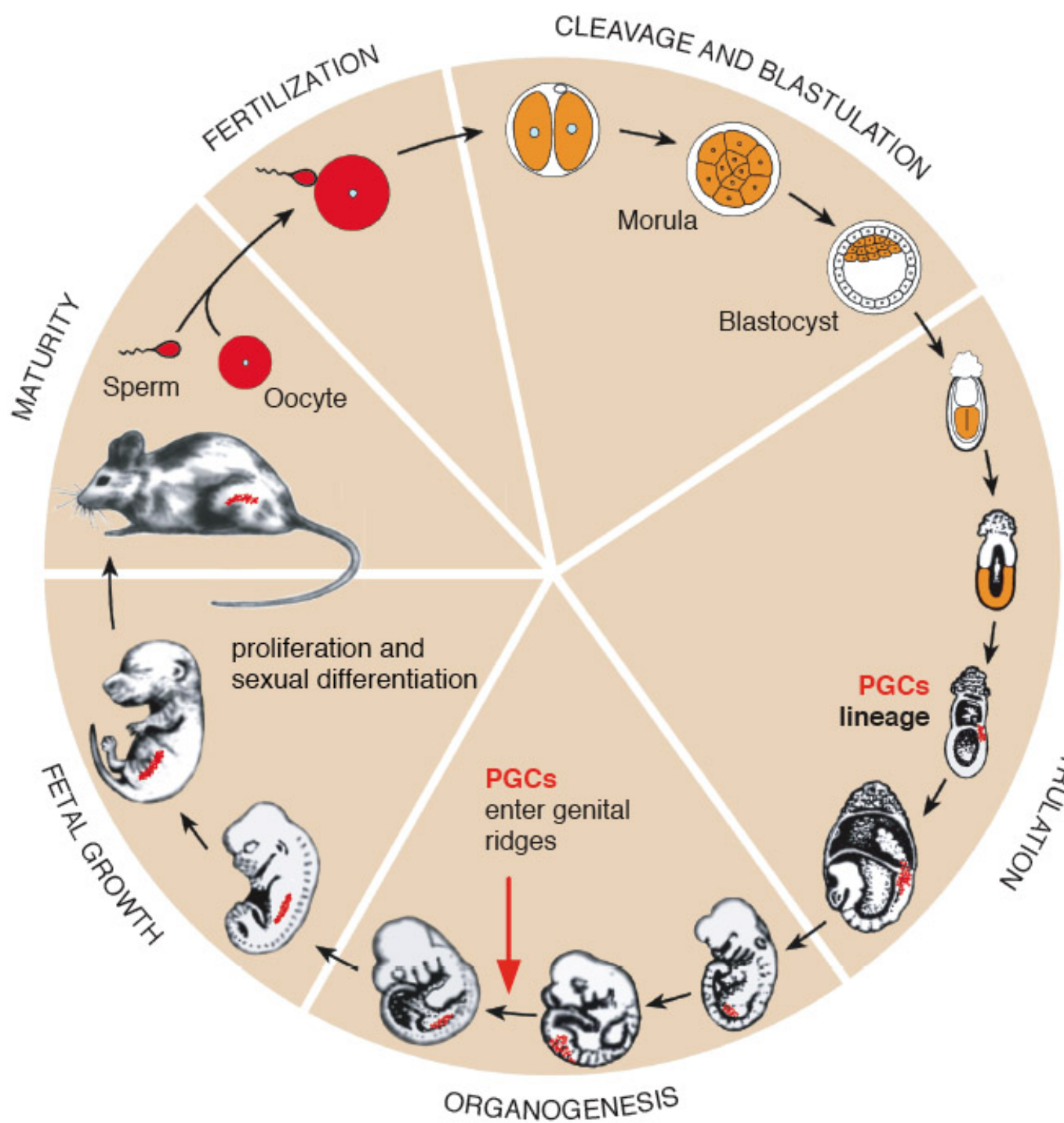
Primordial germ cel and embryonic stem cell

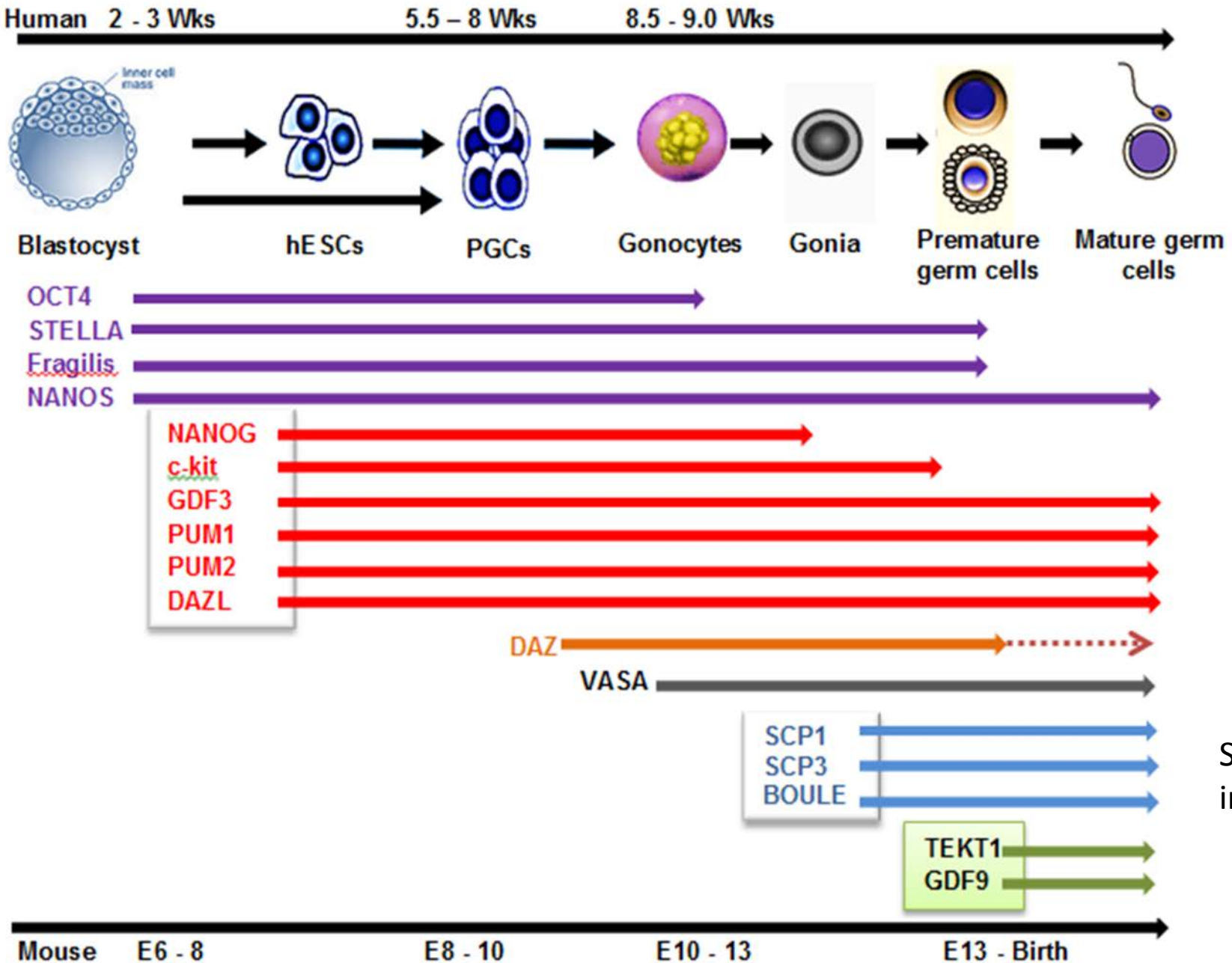


Differentiation of germ cells as *perpetum mobiile*


Fertilization
Sperm
Oocyte
Blastocyst
Maturity
Primordial germ cell precursors
E5.5
Fetal growth
E7.5
Migration of Primordial germ cells
Primordial germ cells
Genital ridge
E10.5
E8.5
Primordial germ cells


1
2
3
4
PGCs
Gonad formation
Birth
Gametogenesis
Fertilisation
Embryo
Gastrulation
Embryo and foetal development
Adult development
Embryonic germ cells
Embryonic stem cells
Spermatogonial stem cells?
Multipotent adult stem cells?


FERTILIZATION
CLEAVAGE AND BLASTULATION
IMPLANTATION AND GASTRULATION
ORGANOGENESIS
FETAL GROWTH
MATURITY
Sperm
Oocyte
Morula
Blastocyst
PGCs
lineage
PGCs
enter genital
ridges
proliferation and
sexual differentiation

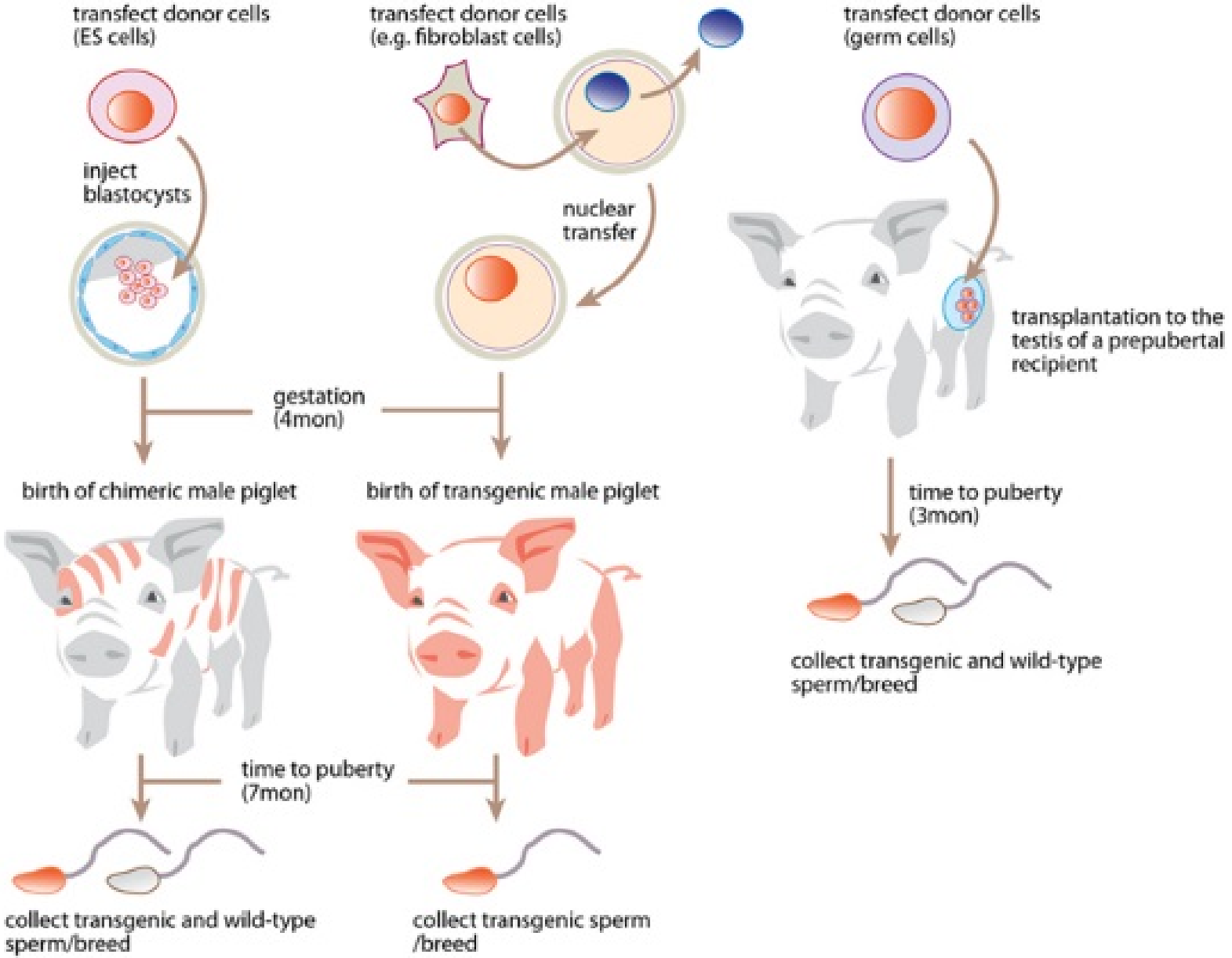


Developmental age in weeks

Several important steps
in human and mouse development

Developmental age in days

Germ Cell Transplantation & Transgenesis



Animal models of diferent human diseases

Chemical lesions

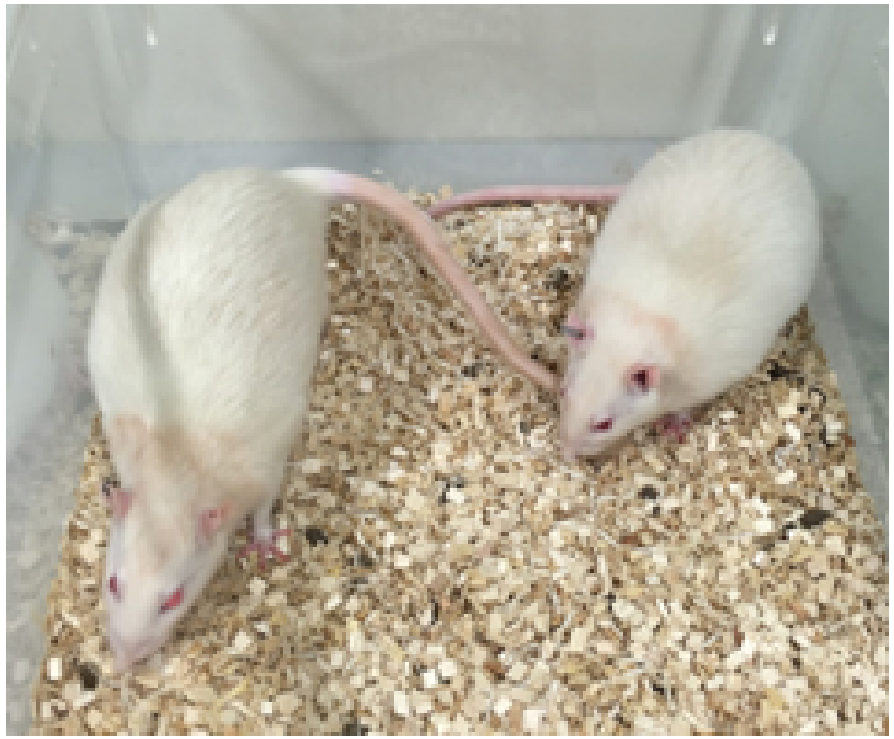
Surgical

Behavioural

Genetical

Phenotypically selected

Etc…



Shadow-land of animal models

1. Ethical considerations
2. High cost
3. Time consuming
4. Low throughput

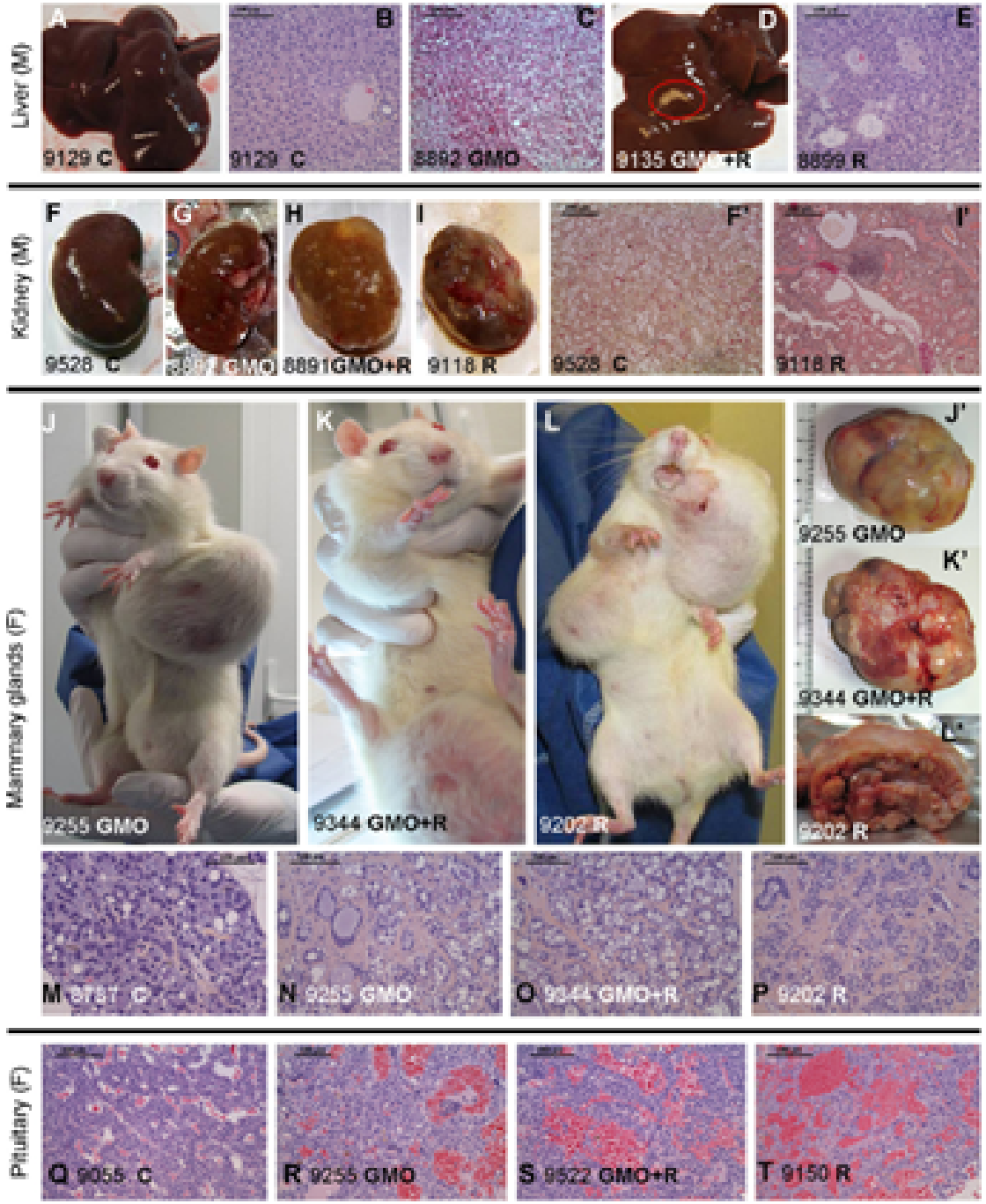
The three Rs (four Rs)

1. Replacement
2. Reduction
3. Refinement

+ Research

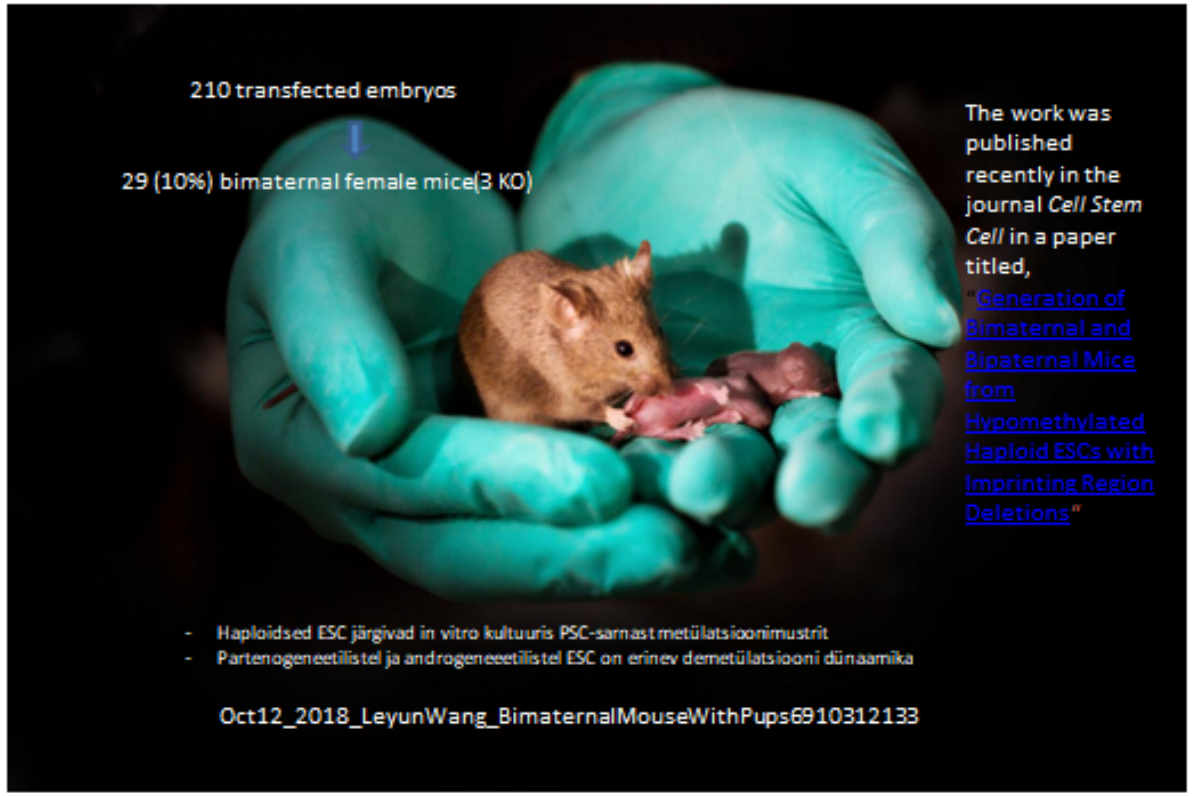
In vitro artificcialy fertilized mice.
To the egg cells were injected nucleusesfrom skin cells.

2004 Nature Medicine

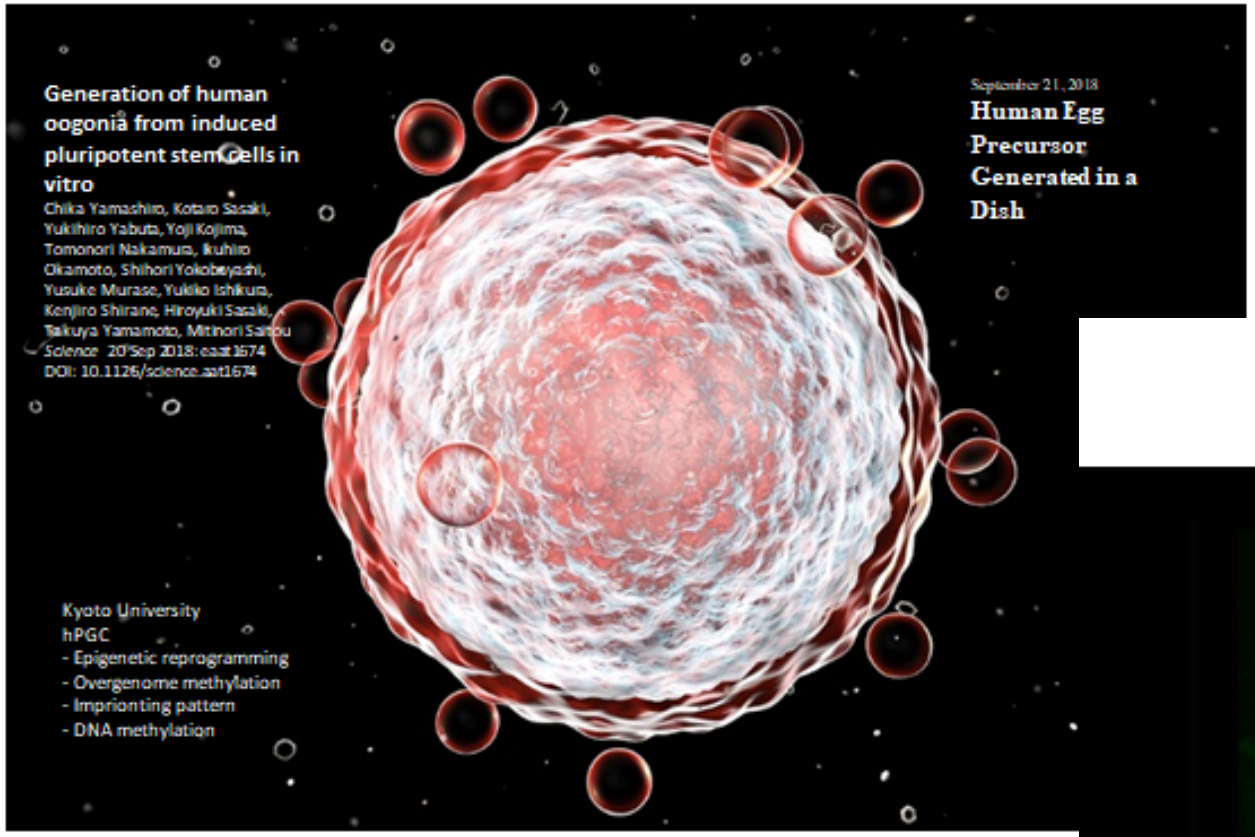


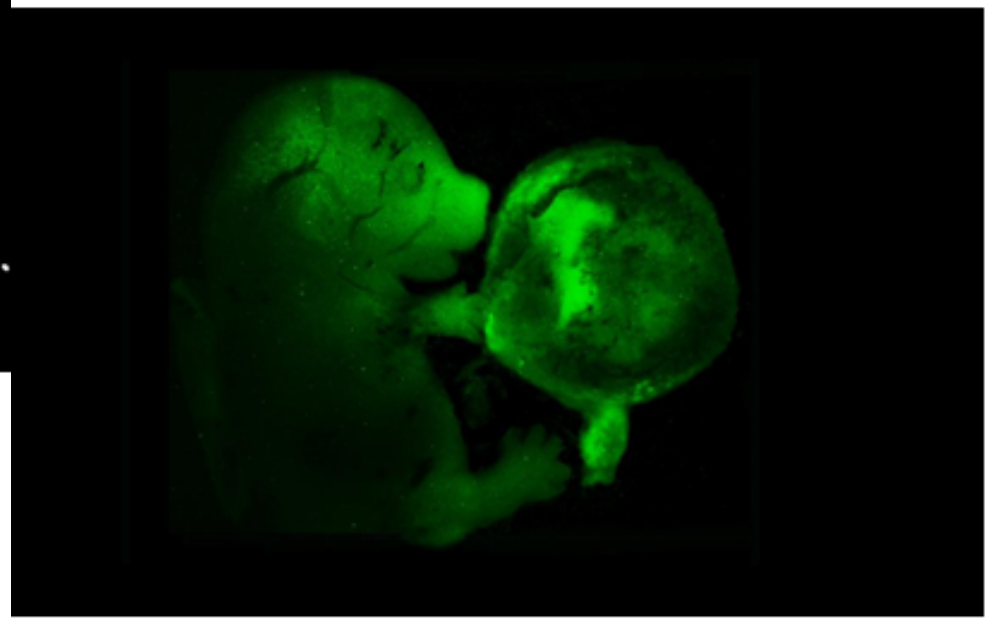
???? !!!! ????

Fig. 3. Anatomopathological observations in rats fed GMO treated or not by Roundup, and effects of Roundup alone. Macroscopic and microscopic photographs show male livers (A–E) and left kidneys (F–I0), female mammary glands (J–P) and pituitaries (Q–T), according to Table 2. The number of each animal and its treatment is specified. Macroscopic pale spots (D) and microscopic necrotic foci in liver (C clear-cell focus, E basophilic focus with atypia), and marked or severe chronic progressive nephropathies, are illustrated. In females, mammary tumors (J,J0,N adenocarcinoma and K,K0,L,L0,O,P fibroadenomas) and pituitary adenomas (R–T) are shown and compared to controls (C after the rat number).


210 transfected embryos
29 (10%) bimaternal female mice(3 KO)
The work was published recently in the journal *Cell Stem Cell* in a paper titled, "Generation of Bimaternal and Bipaternal Mice from Hypomethylated Haploid ESCs with Imprinting Region Deletions"
- Haploidsed ESC järgivad in vitro kultuuris PSC-sarnast metülatsioonimustrit
- Partenogeneetilistel ja androgeneeetilistel ESC on erinev demetülatsiooni dünaamika
Oct12_2018_LeyunWang_BimaternalMouseWithPups6910312133


477 tetraploidic blastocysts are implanted
12 (2,5%) bipaternal male mice (7 KO)
This image shows a bipaternal mouse pup (born to two fathers). [Leyun Wang]



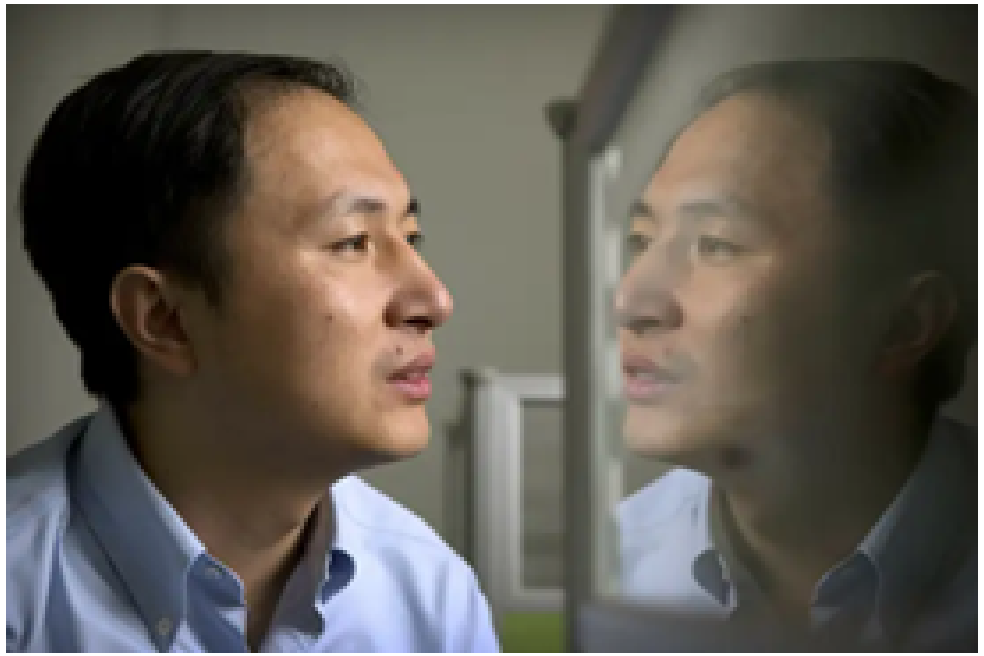


Ddx4 is also found on the surface of only a small population of ovarian cells, and that these rare cells have many properties of primitive precursors to oocytes, EOSC.
NIH Research Matters, March 5, 2012

Egg producing stem cells isolated from an adult human ovary can generate an oocyte (above) in culture. White et al., courtesy of Nature.

The stem cells in both cases (embryonic and placenta or body tisuse cells) are embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. The former are taken from embryos and the latter are adult tissue cells that are reprogrammed to act like stem cells.

The He Jiankui affair is a scientific and bioethical circumstance concerning the use of gene-editing in human cases following the first use by Chinese scientist He Jiankui, who made the first genome-edited human babies in 2018. The affair led to legal and ethical controversies with an indictment of He and his two collaborators, Zhang Renli and Qin Jinzhou. Gene *CCR5* knock-out.



2 + 1?

2009 . HIV receptor CCR5

IVF
22 embryos –1-cell-PCR

Modified 16 embryos

11 embryos implantated

1 pregnancy– double-twins

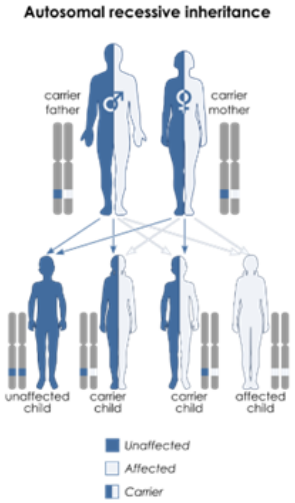
1 of them was against of CCR5-receptor negatiive-knockout – no disease!!!

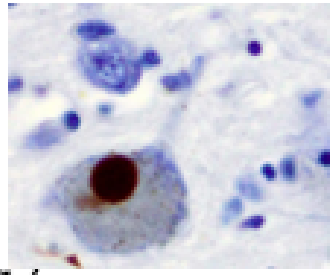
ETHICS!!!

He Jiankui says two babies have been born as a result of his CRISPR experiment
Read more: https://www.newscientist.com/article/2186504-worlds-first-gene-edited-babies-announced-by-a-scientist-in-china/#ixzz6edJ8PQBC

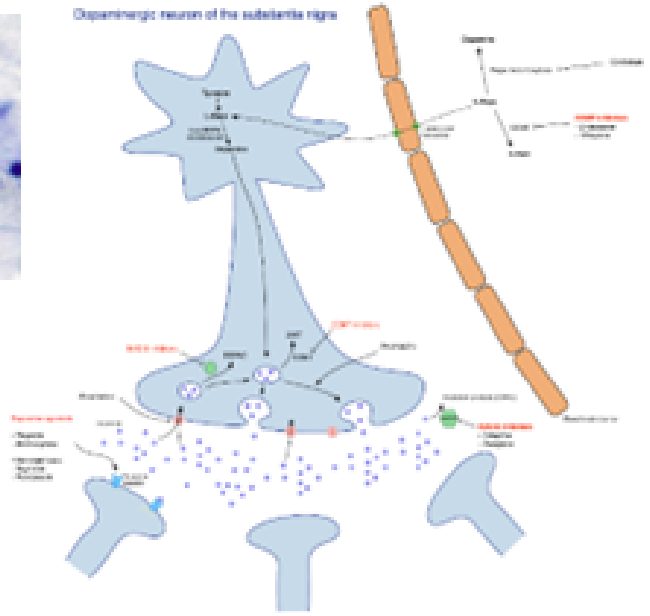
- On 30 December 2018, the Shenzhen Nanshan District People's Court sentenced
- He Jiankui to three years in prison and with a fine of 3 million RMB (US$430,000).
- Among the collaborators, only two were indicted – Zhang Renli of the Guangdong Academy of Medical Sciences and Guangdong General Hospital, received a two-year prison sentence and a 1-million RMB fine, and
- Qin Jinzhou of the Southern University of Science and Technology received an 18-month prison sentence and a 500,000 RMB fine.
- The three were found guilty of having "forged ethical review documents and misled doctors into unknowingly implanting gene-edited embryos into two women."

- Parkinson disease– genetic heredity
- sporadic disease – vibration, shaking, uncontrolled movement of muscles, neuron-death in the area of *substantia nigra*.





Catherine Metzger
13 Octobre 1869



Lewy bodies
In neurons, astrocytes, microgliia (alfa-synuclein bodies)

James Parkinson, 1817

The purpose of the model

...there are known knowns; there are things we know

...there are known unknowns; that is to say we know there are some things we do not know.

...there are also unknown unknowns—the ones we don't know we don't know.

Donald Henry Rumsfeld, 2002

(1932 – 2021)

USA defence minister 1975 – 1977 (during the tiome of G. Ford) ja 2001 – 2006 (the time of president G.W. Bush)