Genetic Manipulation in Mouse (II)

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Early Mouse Development

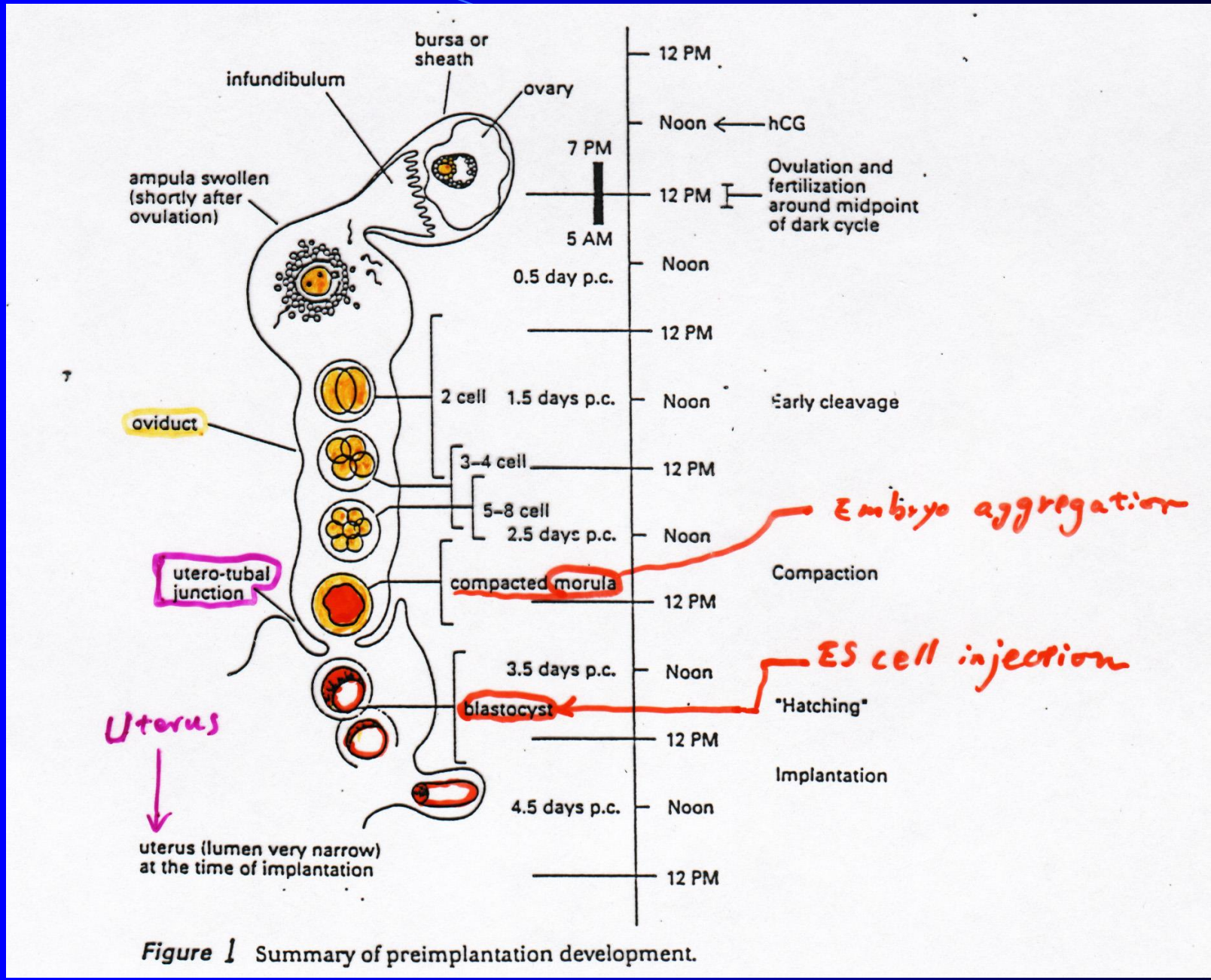
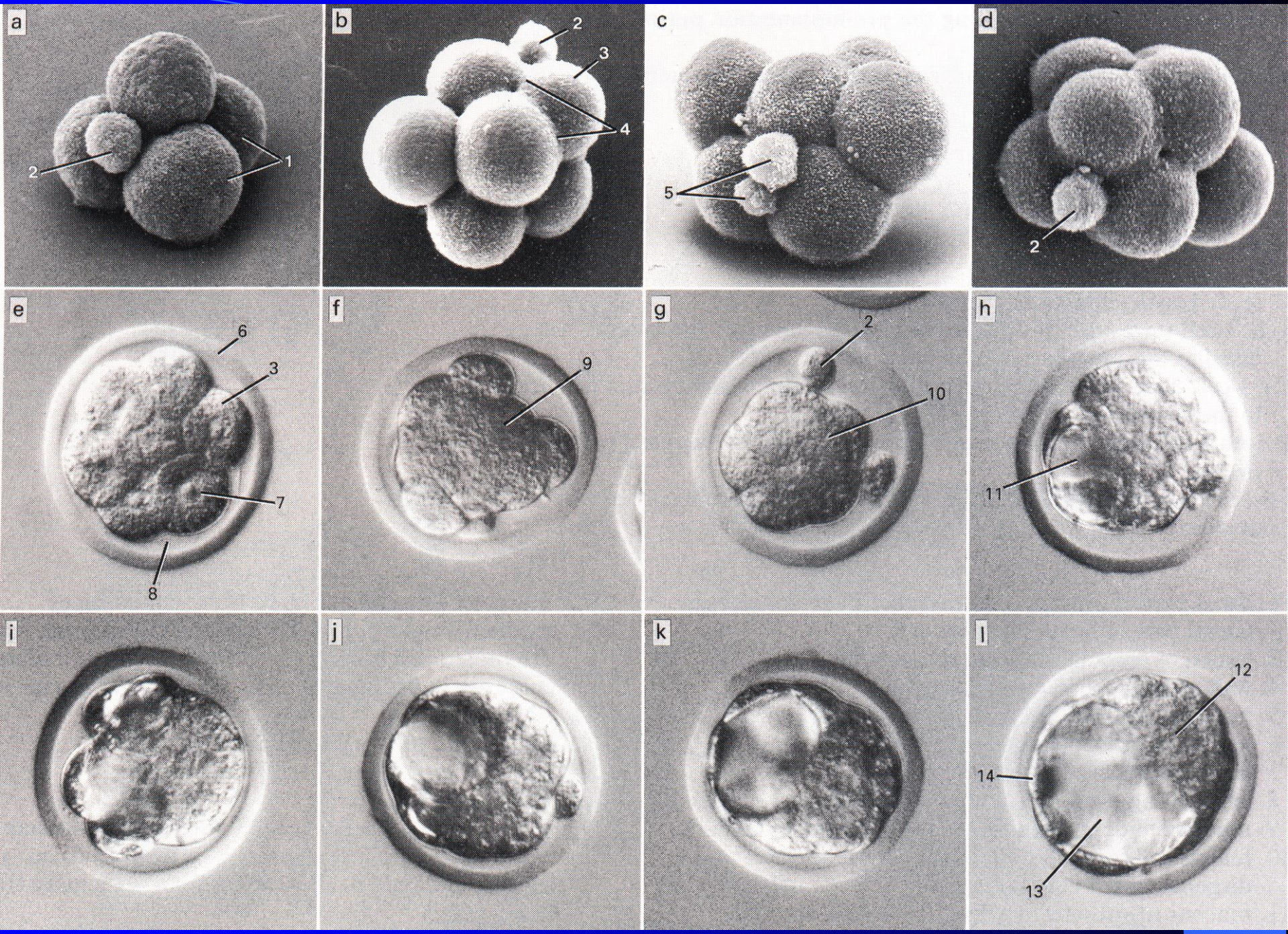


Figure 1 Summary of preimplantation development.


a
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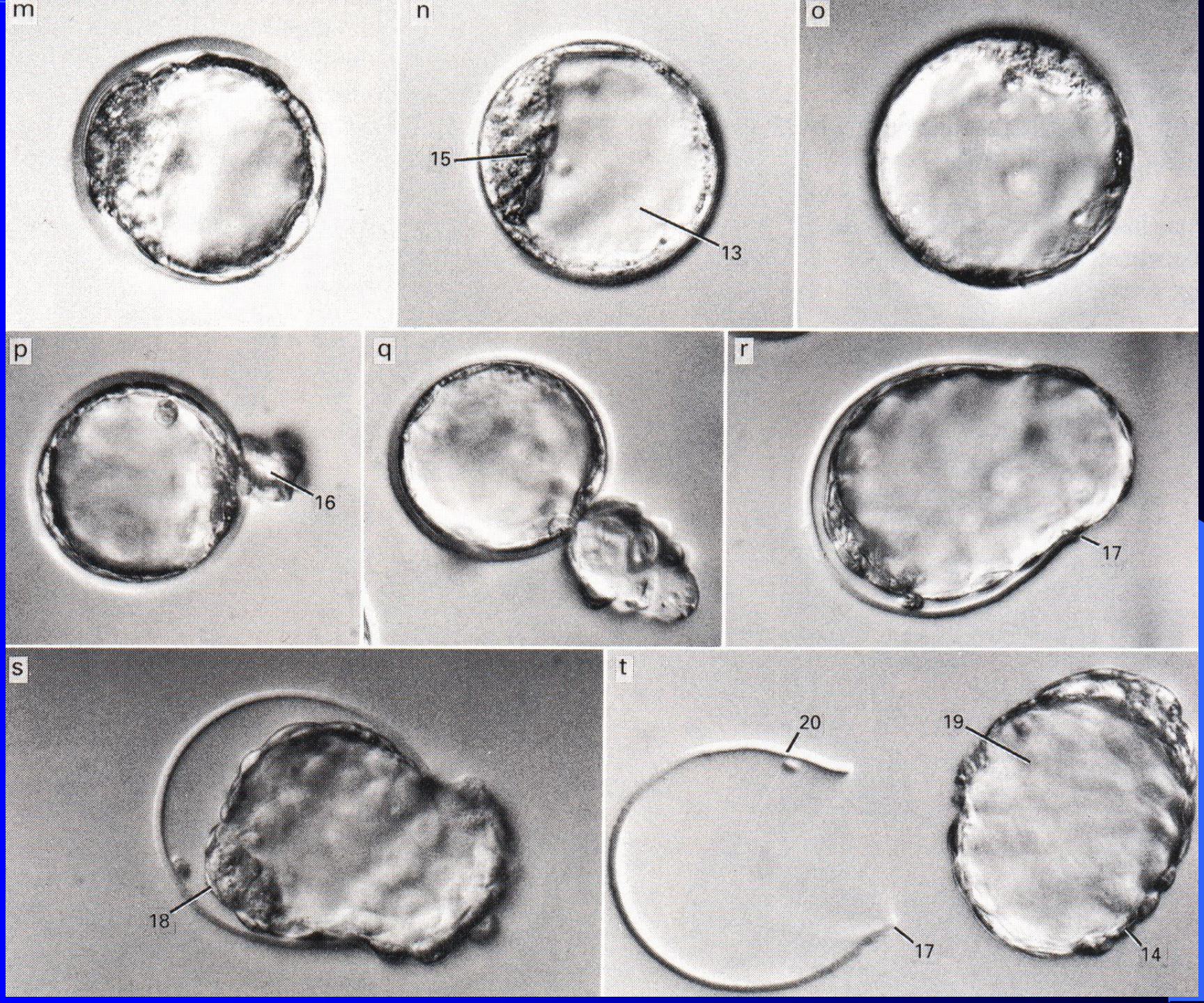
m
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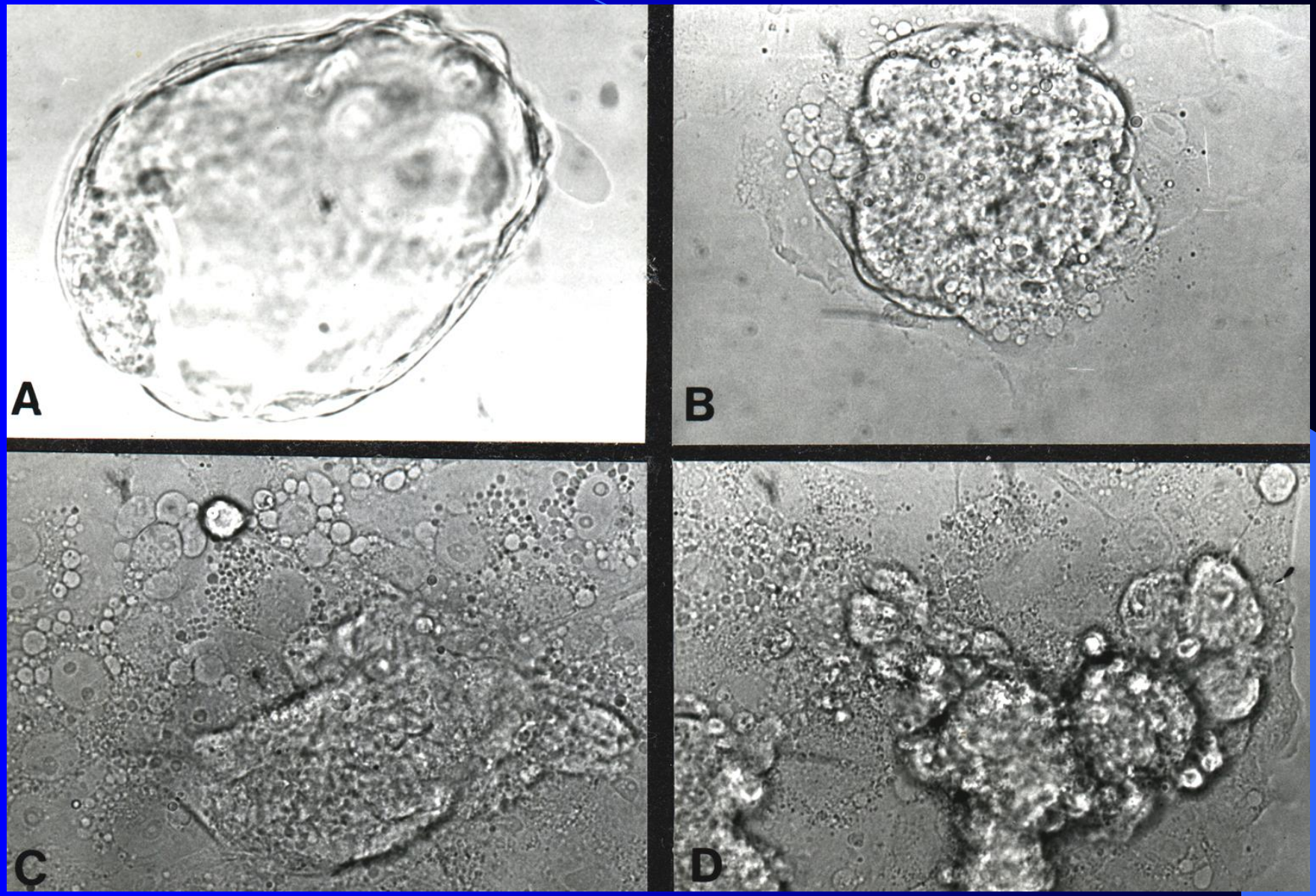
Blastocyst hatching out and implantation



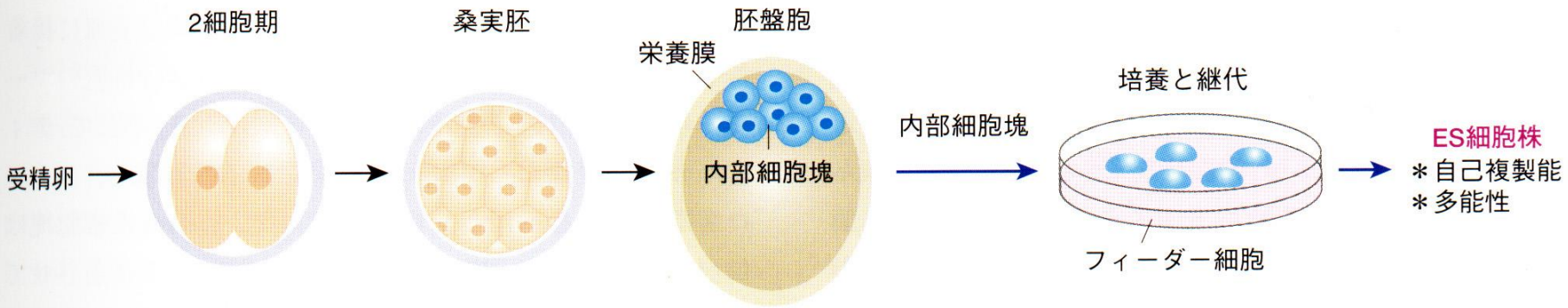
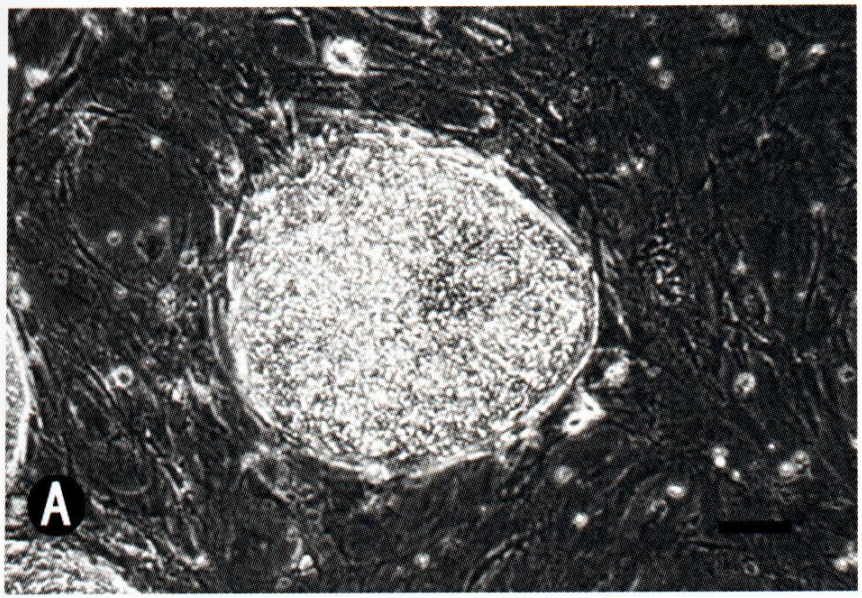
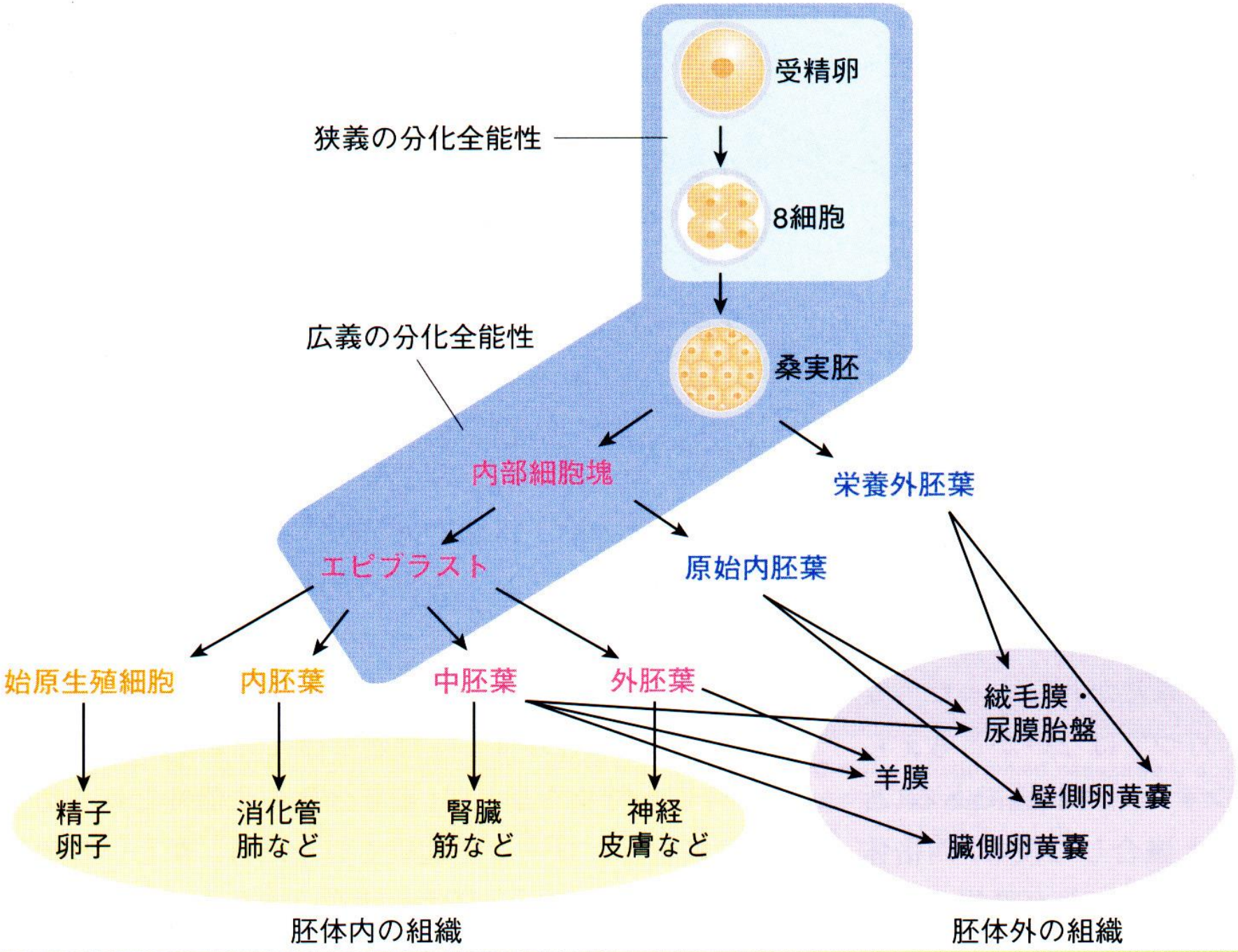
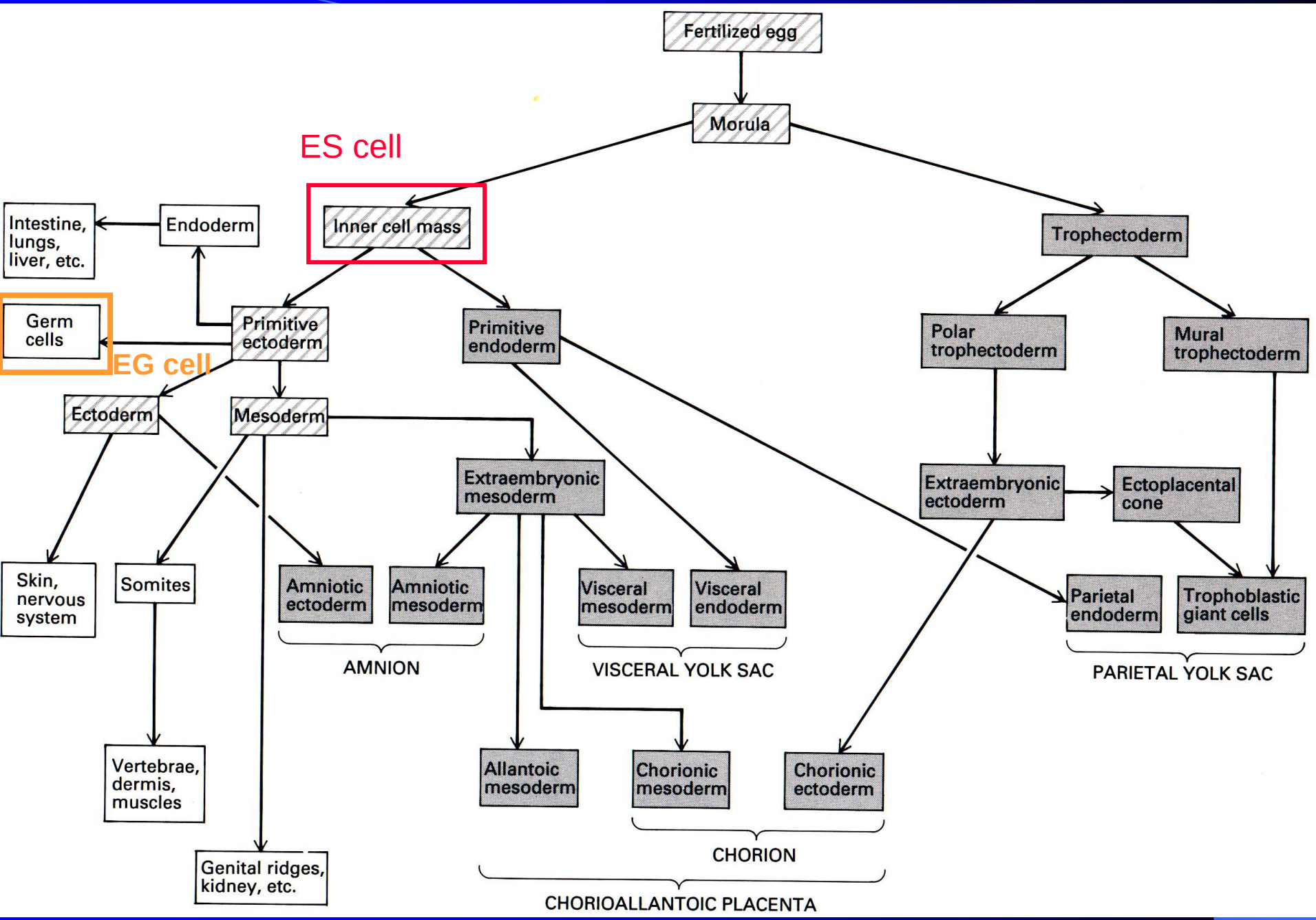


図1. ヒトES細胞の樹立法

胚盤胞から分離した内部細胞塊をフィーダー細胞（マイトマイシンCやγ線などで不活性化したマウス胎仔線維芽細胞）上で培養し，未分化細胞を選抜しながらフィーダー細胞上で継代培養を続けることにより樹立される．




受精卵
狭義の分化全能性
8細胞
広義の分化全能性
桑実胚
内部細胞塊
栄養外胚葉
エピブラスト
原始内胚葉
始原生殖細胞
内胚葉
中胚葉
外胚葉
絨毛膜・
尿膜胎盤
羊膜
壁側卵黄嚢
臓側卵黄嚢
精子
卵子
消化管
肺など
腎臓
筋など
神経
皮膚など
胚体内の組織
胚体外の組織


Fertilized egg
Morula
ES cell
Inner cell mass
Endoderm
Intestine, lungs, liver, etc.
Trophectoderm
Germ cells
EG cell
Primitive ectoderm
Primitive endoderm
Polar trophectoderm
Mural trophectoderm
Ectoderm
Mesoderm
Extraembryonic mesoderm
Extraembryonic ectoderm
Ectoplacental cone
Skin, nervous system
Somites
Amniotic ectoderm
Amniotic mesoderm
Visceral mesoderm
Visceral endoderm
Parietal endoderm
Trophoblastic giant cells
AMNION
VISCERAL YOLK SAC
PARIETAL YOLK SAC
Vertebrae, dermis, muscles
Allantoic mesoderm
Chorionic mesoderm
Chorionic ectoderm
CHORION
Genital ridges, kidney, etc.
CHORIOALLANTOIC PLACENTA

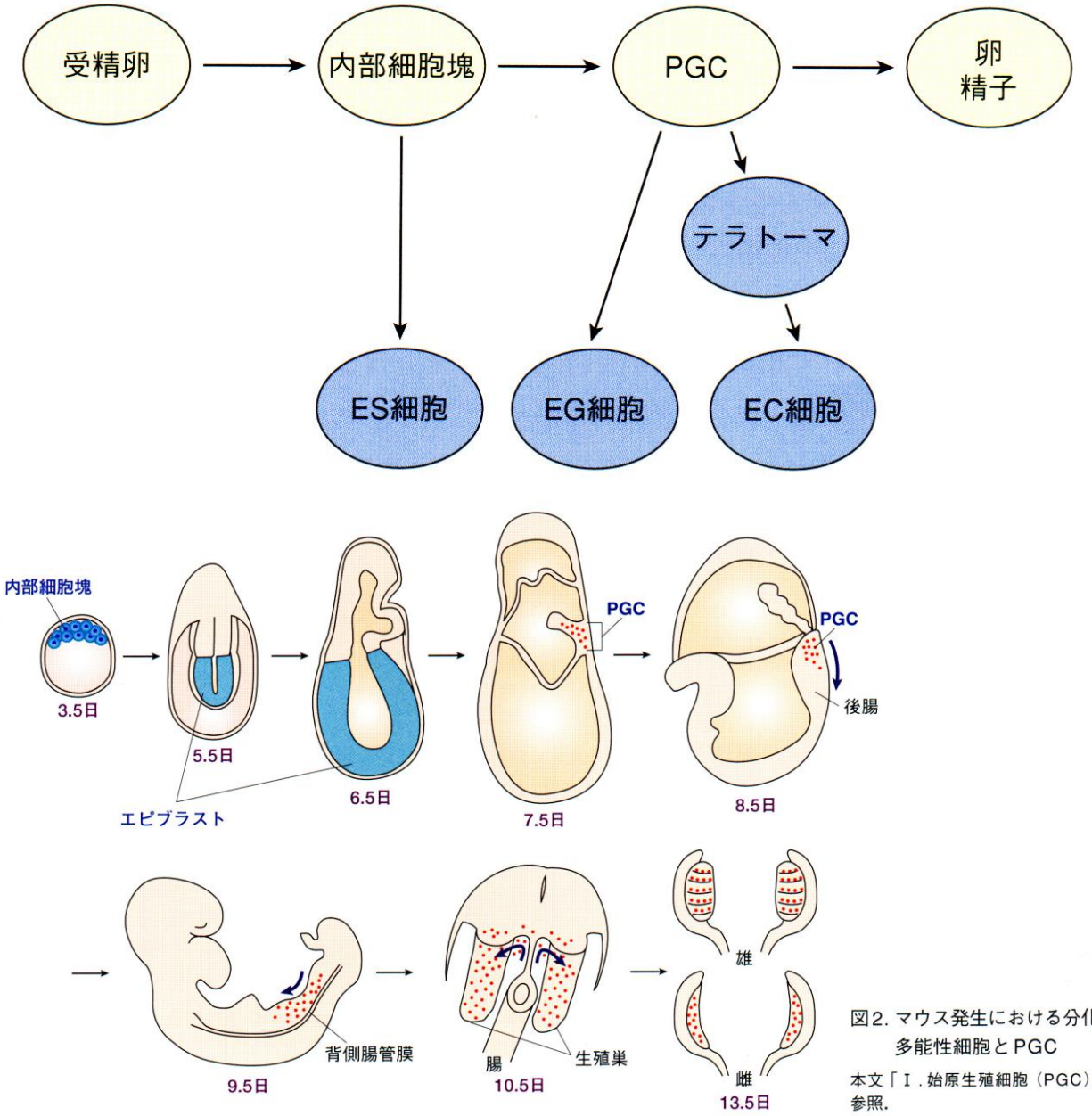
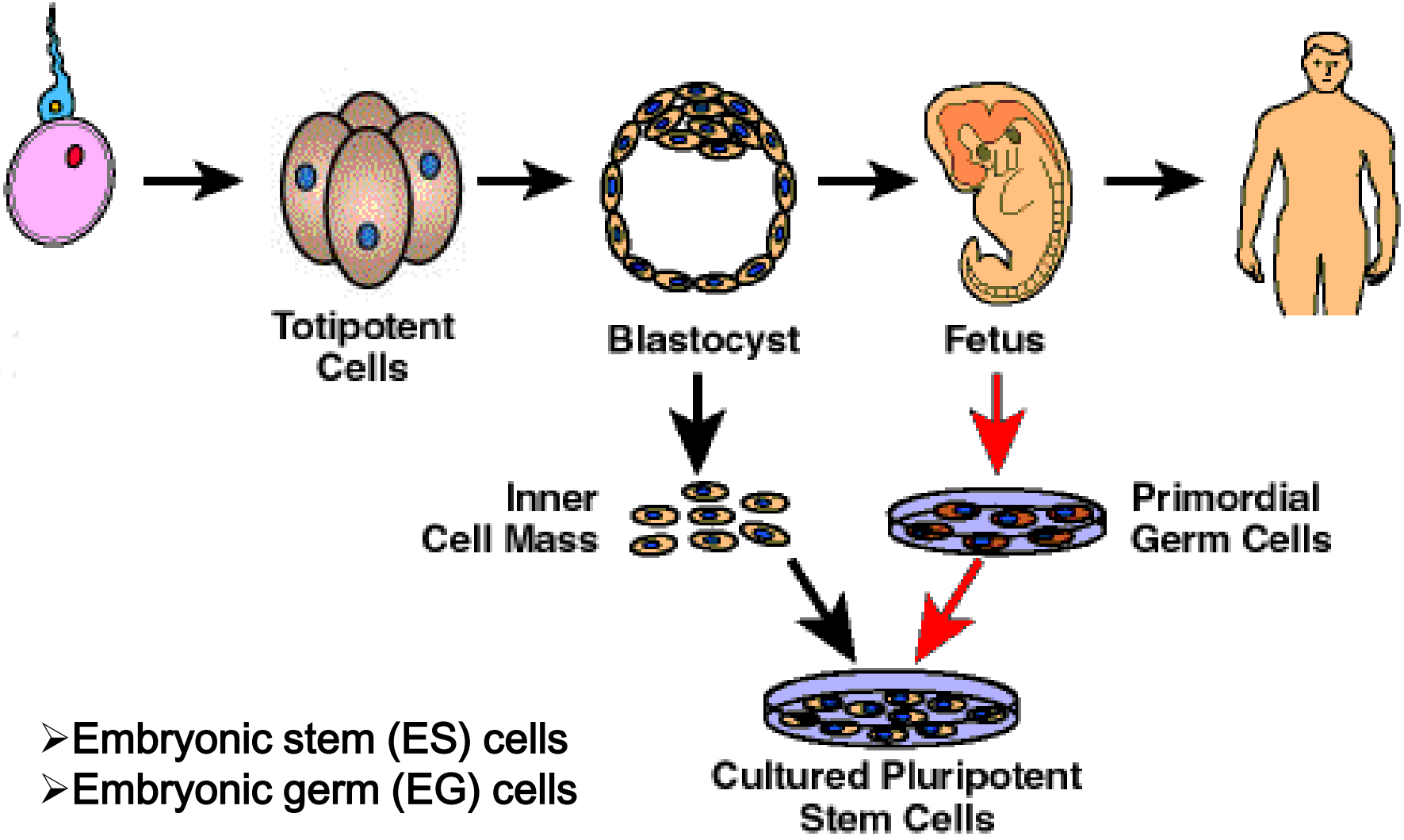


図2. マウス発生における分化多能性細胞とPGC

本文「Ⅰ. 始原生殖細胞（PGC）」参照.

動し，そこで雌雄それぞれの生殖細胞として分化を開始する.

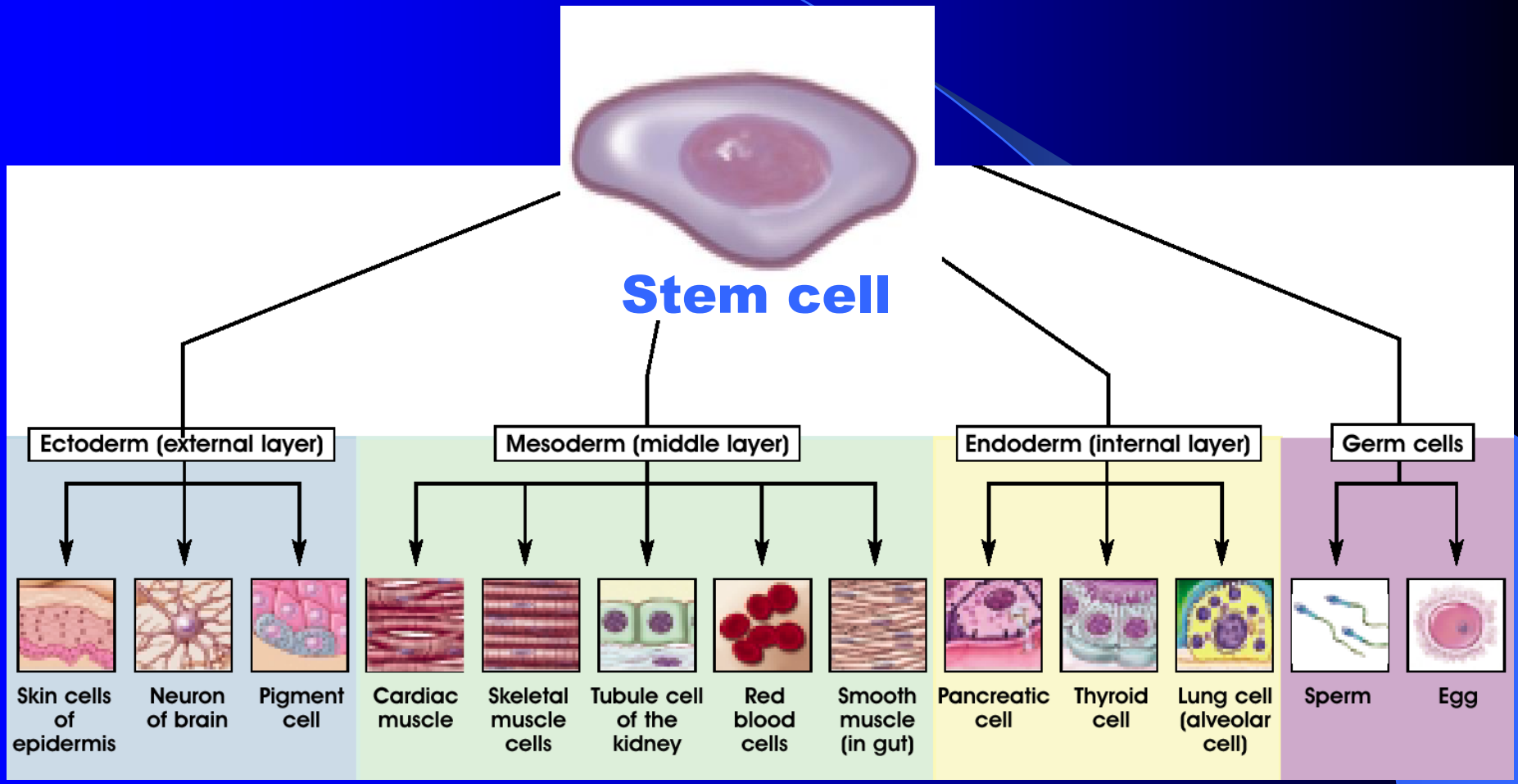
Pluripotent stem cells

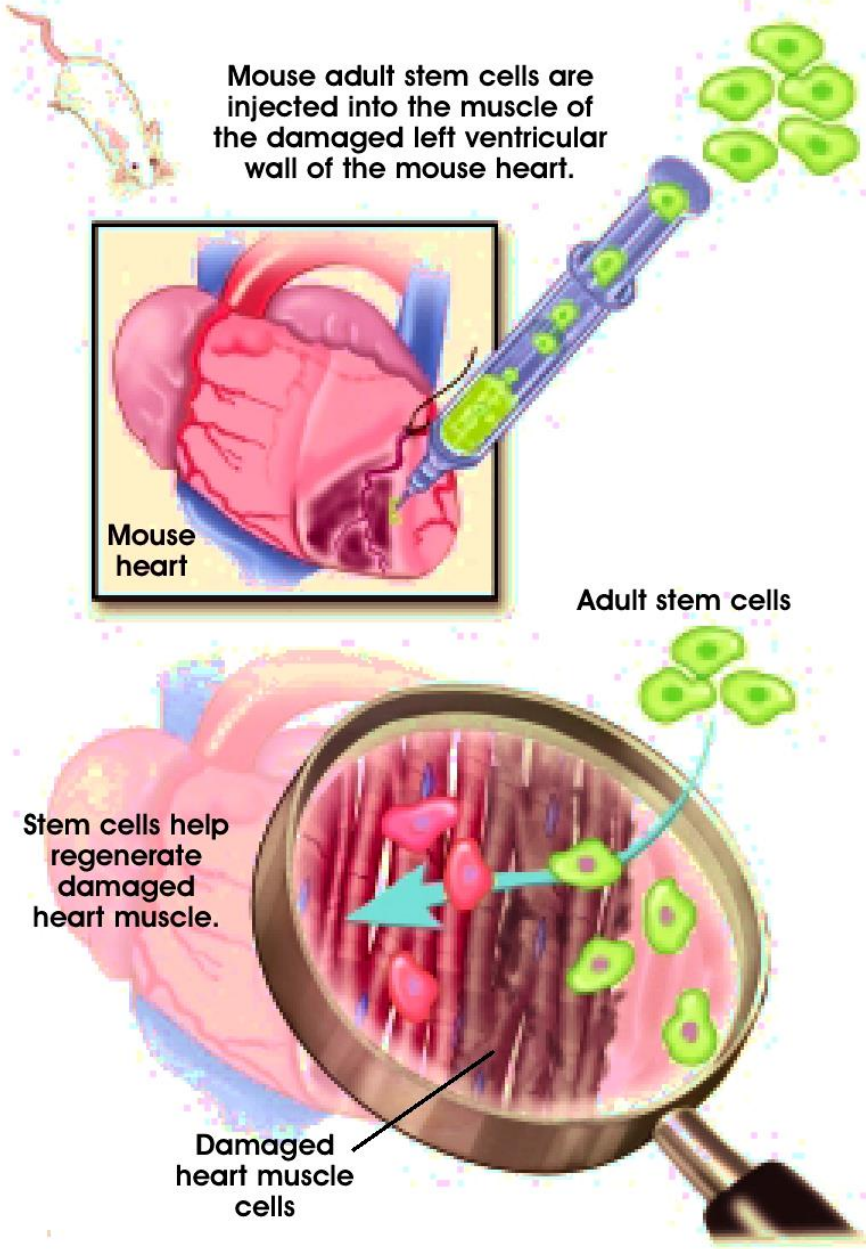


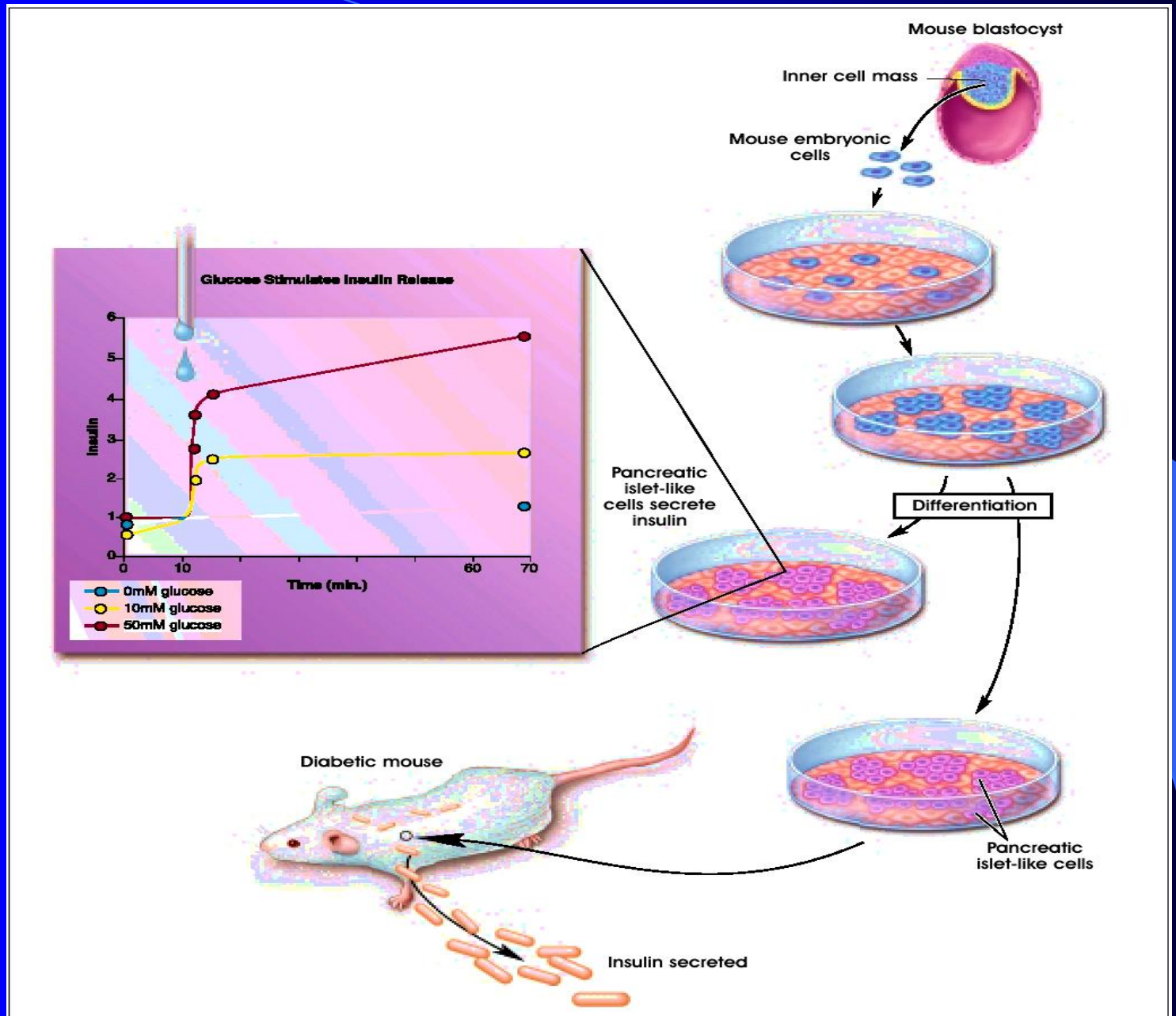
- Embryonic stem (ES) cells
- Embryonic germ (EG) cells

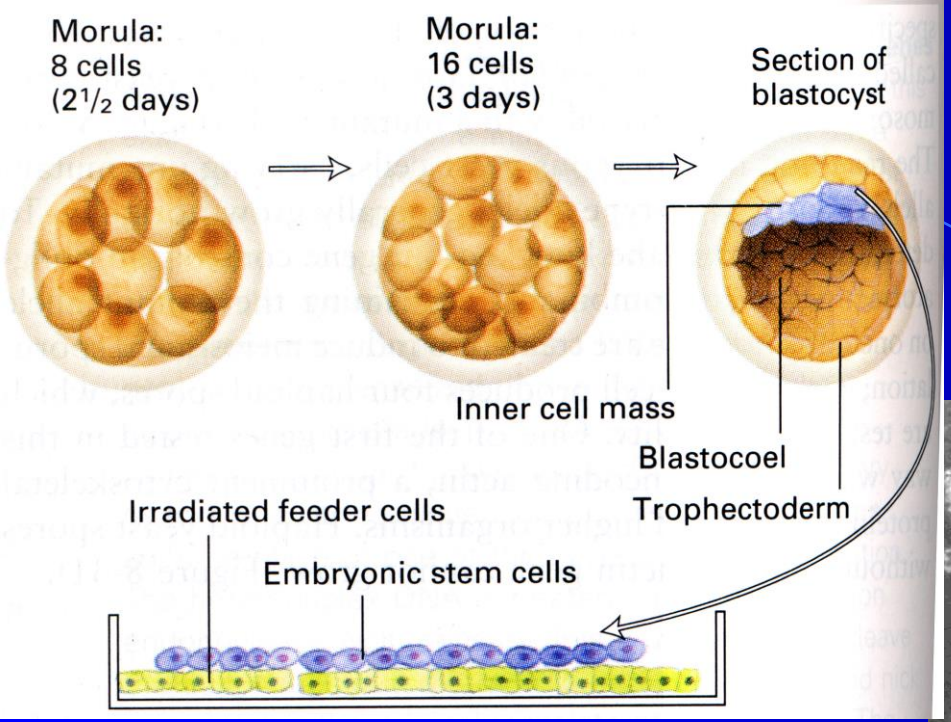
Cell differerentiation

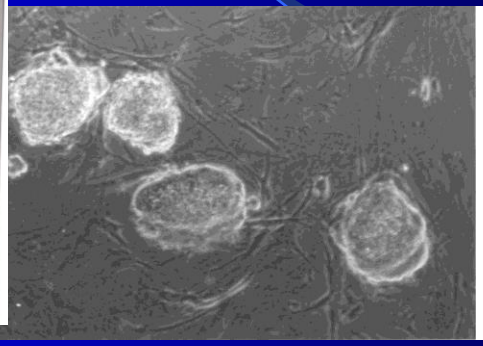
With combination of growth differentiation factors

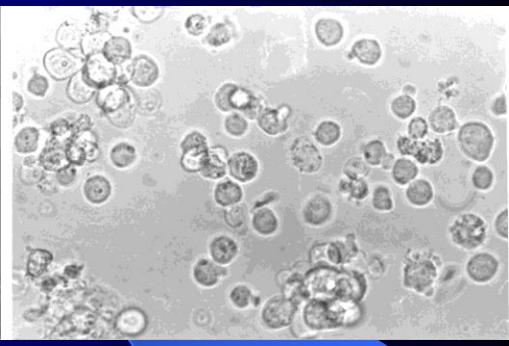



Mouse adult stem cells are injected into the muscle of the damaged left ventricular wall of the mouse heart.
Mouse heart
Adult stem cells
Stem cells help regenerate damaged heart muscle.
Damaged heart muscle cells
Human adult bone marrow stem cells are injected into the tail vasculature of a rat.
The stem cells induce new blood vessel formation in the damaged heart muscle and proliferation of existing vasculature.
Adult stem cells
New blood vessels
Damaged heart muscle cells

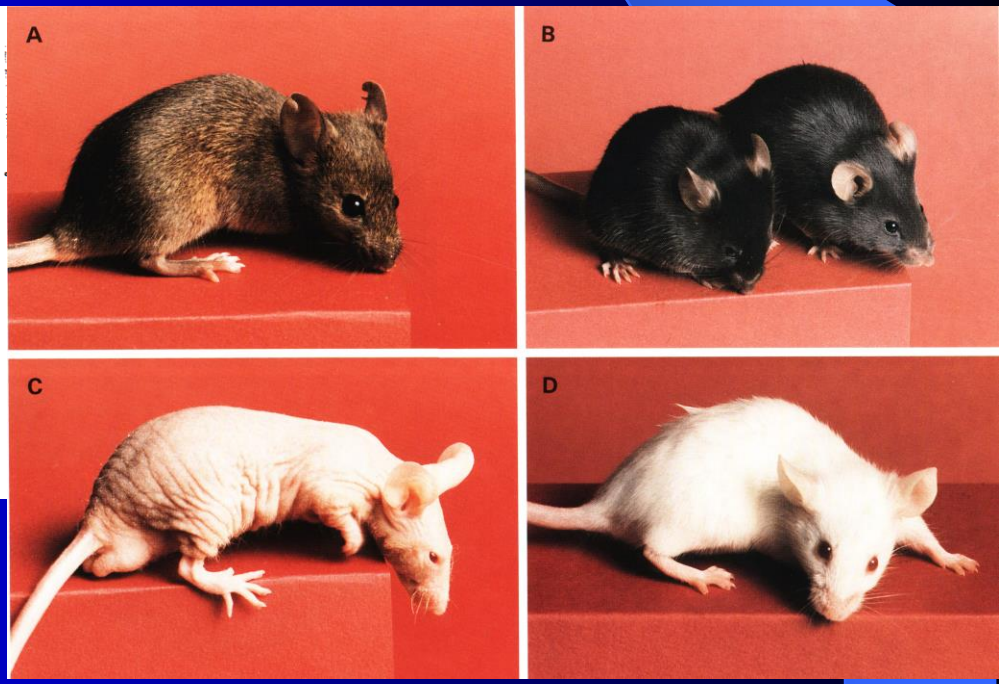

Mouse blastocyst
Inner cell mass
Mouse embryonic cells
Glucose Stimulates Insulin Release
Insulin
Time (min.)
0mM glucose
10mM glucose
50mM glucose
Pancreatic islet-like cells secrete insulin
Differentiation
Diabetic mouse
Pancreatic islet-like cells
Insulin secreted







Substrain	Locus				ES cell lines
	Agouti	Albino	Pink-eyed dilution	GPI-1	
129/J	A^w	c, c^{ch}	p	Gpi-1^a	
129/SvJ	A^w	c, c^{ch}	p	Gpi-1^a	PJ1-5
129/Sv	A^w	$+^c$	$+^p$	Gpi-1^a	D3
129/Sv//Ev	A^w, A	$+^c$	$+^p$	Gpi-1^a or Gpi-1^c	AB-1, CP-1 CCE,CC1.2
(129/Sv x 129/SvJ)F_1	A^w	$c/+^c$	$p/+^p$	Gpi-1^a	R1
129/Ola	A^w	c^{ch}	p	Gpi-1^a	E14
C57BL/6J	a	$+^c$	$+^p$	Gpi-1^b	ES632
BALB/c	A	c	$+^p$	Gpi-1^a	



Homologous Recombination

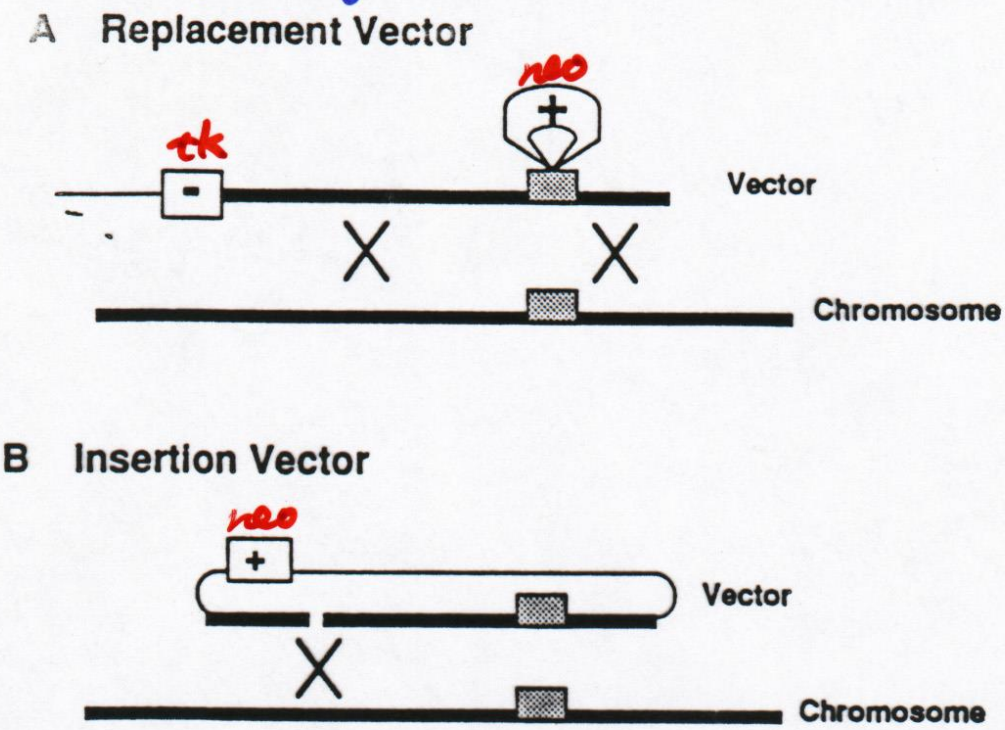


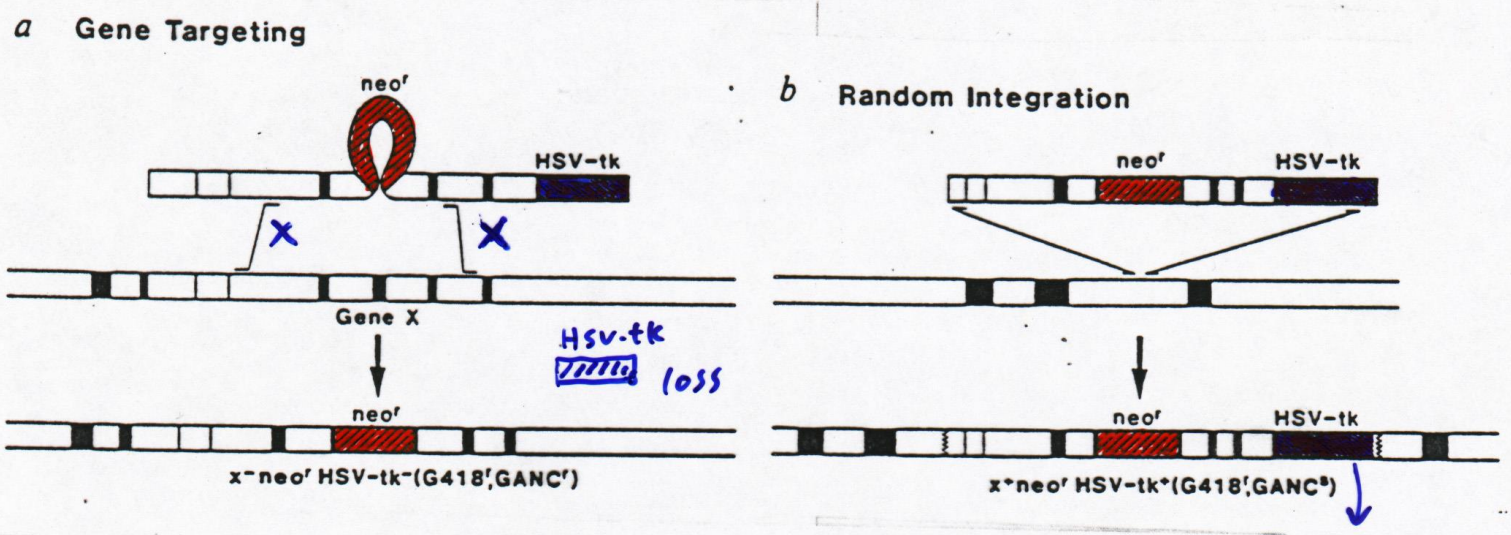
Figure 5. Diagram of a replacement and insertion vector. The thick line represents the vector homology to the target locus; the thin line represents bacterial plasmid. The stippled rectangle represents an exon. The positive selection marker is shown as a box that contains a +. (A) The replacement vector. The positive selection marker interrupts the target homology. This is required for a replacement vector. The negative selection marker is shown as a rectangle that contains a −. The replacement vector is linearized outside the target homology prior to transfection. (B) An insertion vector. A positive selectable marker may be cloned into the homologous sequences or the vector backbone. A double strand break is generated in the target homology prior to transfection.

Positive selection marker neo^r

neomycin phosphotransfera

negative selection marker Hsv-tk

thymidine kinase (herpes simplex virus)



Hsv-tk loss

active thymidine kinase

Fig. 4 The PNS procedure used to enrich for ES cells containing a targeted disruption of gene *X*. *a*, A gene *X*-replacement vector, that contains an insertion of the *neo*^{r} gene in an exon of gene *X* and a linked HSV-*tk* gene, is shown pairing with a chromosomal copy of gene *X*. Homologous recombination between the targeting vector and genomic *X* DNA results in the disruption of one copy of gene *X* and the loss of HSV-*tk* sequences. Such cells will be X^-, *neo*^{r} and HSV-*tk*$^-$ and will be resistant to both G418 and GANC. *b*, Because non-homologous insertion of exogenous DNA into the genome occurs through the ends of the linearized DNA[9-11], the HSV-*tk* gene remains linked to the *neo*^{r} gene. Such cells will be X^+, *neo*^{r} and HSV-*tk*$^-$ and therefore resistant to G418 but sensitive to GANC. Open boxes denote introns or flanking DNA sequences, closed boxes denote exons and cross-hatch boxes denote the *neo*^{r} or HSV-*tk* genes.

HSV-tk → GANC → GANC-p

GANC: anti-viral drug gancyclovir.

↓ pick up by DNA polymerase

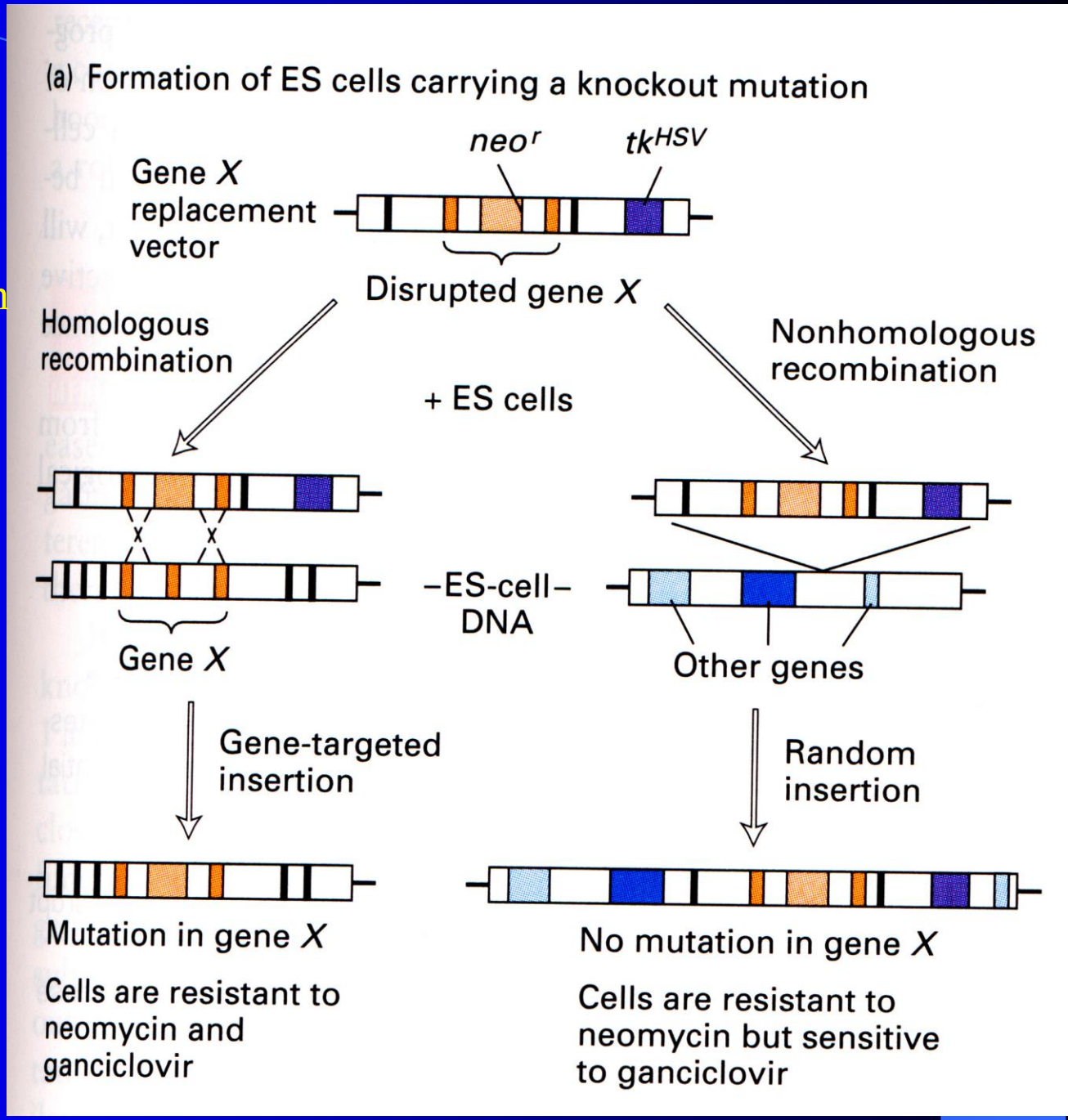
⇓ Inhibition Enzyme activity

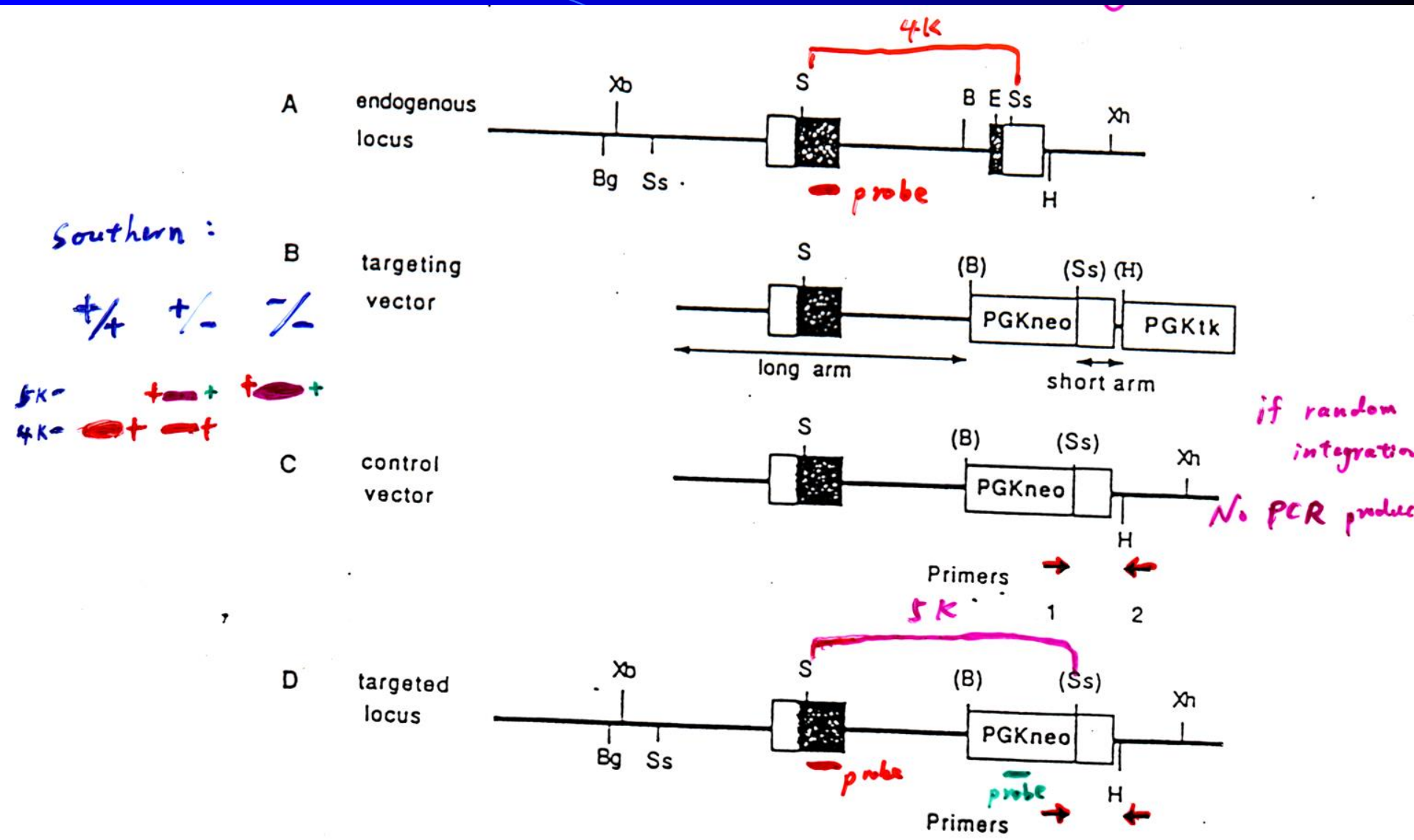
⇓ cell can't proliferate

⇓ death

Gene Targeting:

Knockout a gene via homologous recombination after a Positive-Negative Selection (PNS)




4k
A
endogenous
locus
Xb
S
B E Ss
Xh
Bg
Ss
probe
H
Southern :
+/+ +/- -/-
5K-
4K-
B
targeting
vector
S
(B)
(Ss) (H)
PGKneo
PGKtk
long arm
short arm
if random
integration
C
control
vector
S
(B)
(Ss)
Xh
PGKneo
H
No PCR produc
Primers
1
2
5K
D
targeted
locus
Xb
S
(B)
(Ss)
Xh
Bg
Ss
PGKneo
probe
probe
H
Primers

After Positive - Negative Selection

ES cell colonies still needed to be screening by PCR or Southern.

e.g.

1×10^6 X 10 plates

↓ After PN Selection

1,000 colonies

↓ screening by PCR

1 ~ 10 target clones.

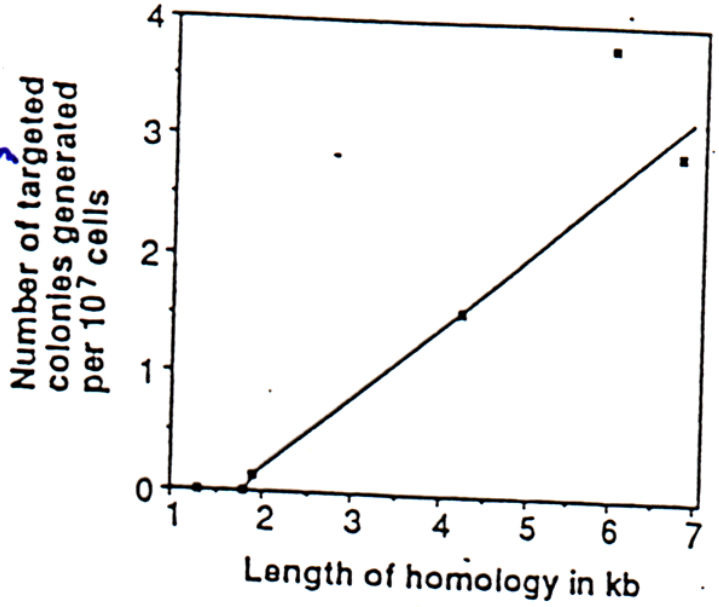


Figure 6. The relationship between the targeting frequency and the length of homology in a replacement vector (12).

longer is better!

only YFG expressed in the ES cells !!

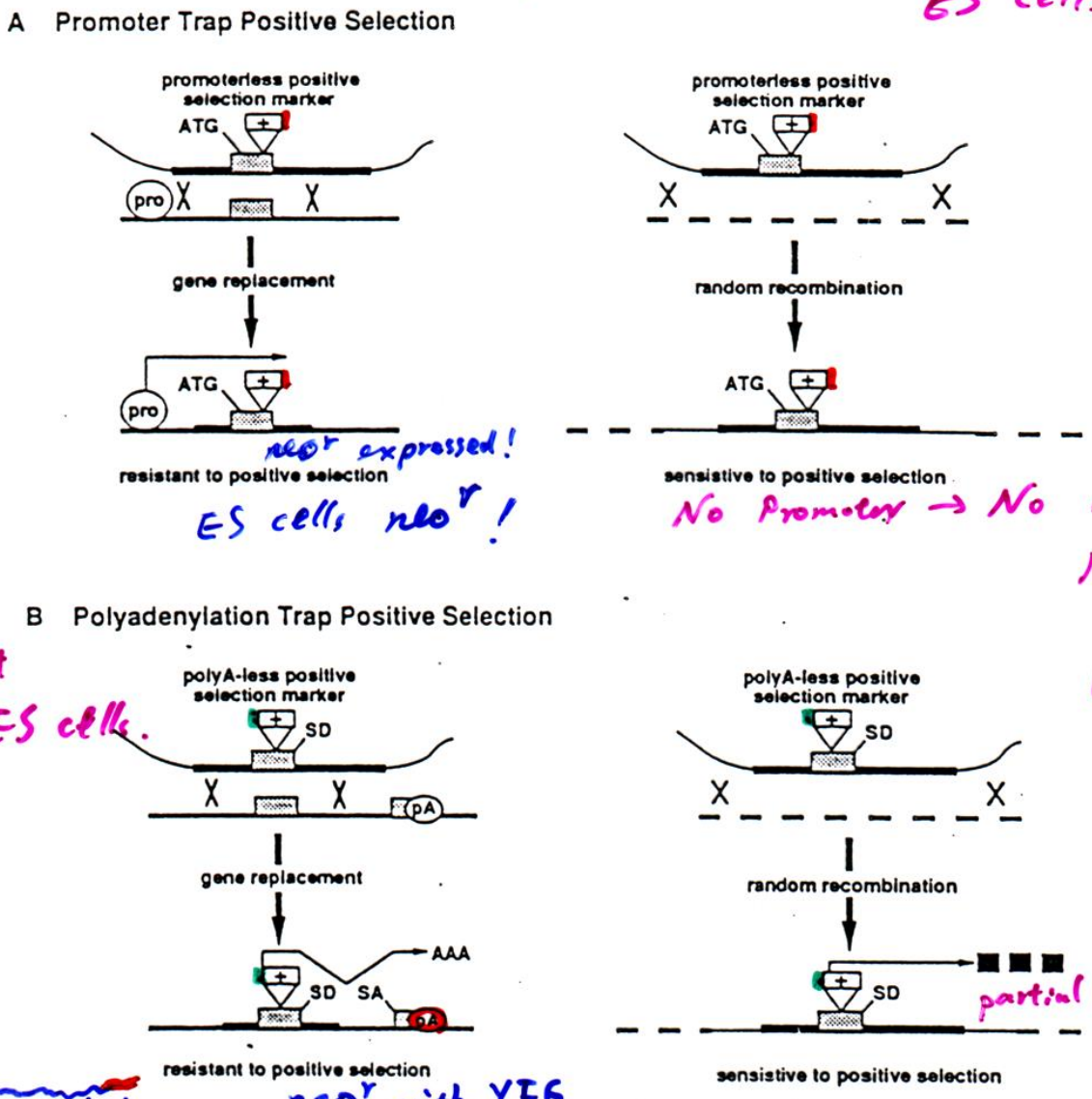
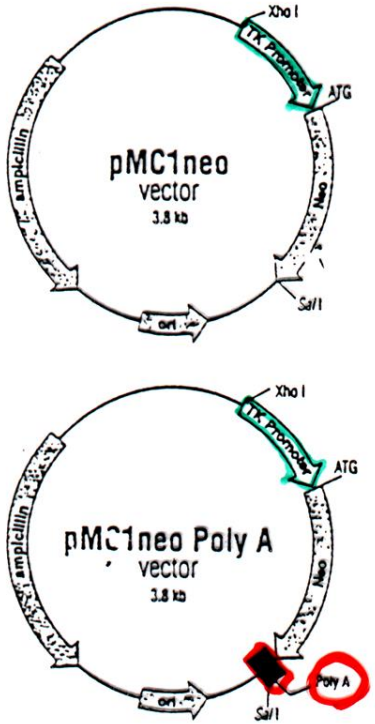
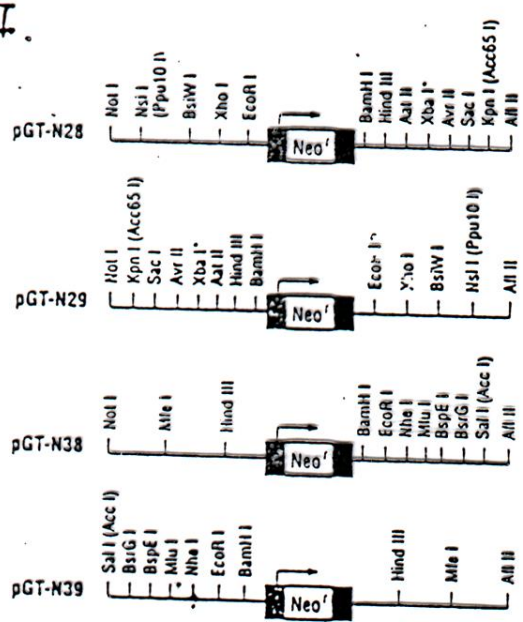


Figure 8. Enrichment strategies for gene targeting events. The replacement vector is

I.



II.



III.

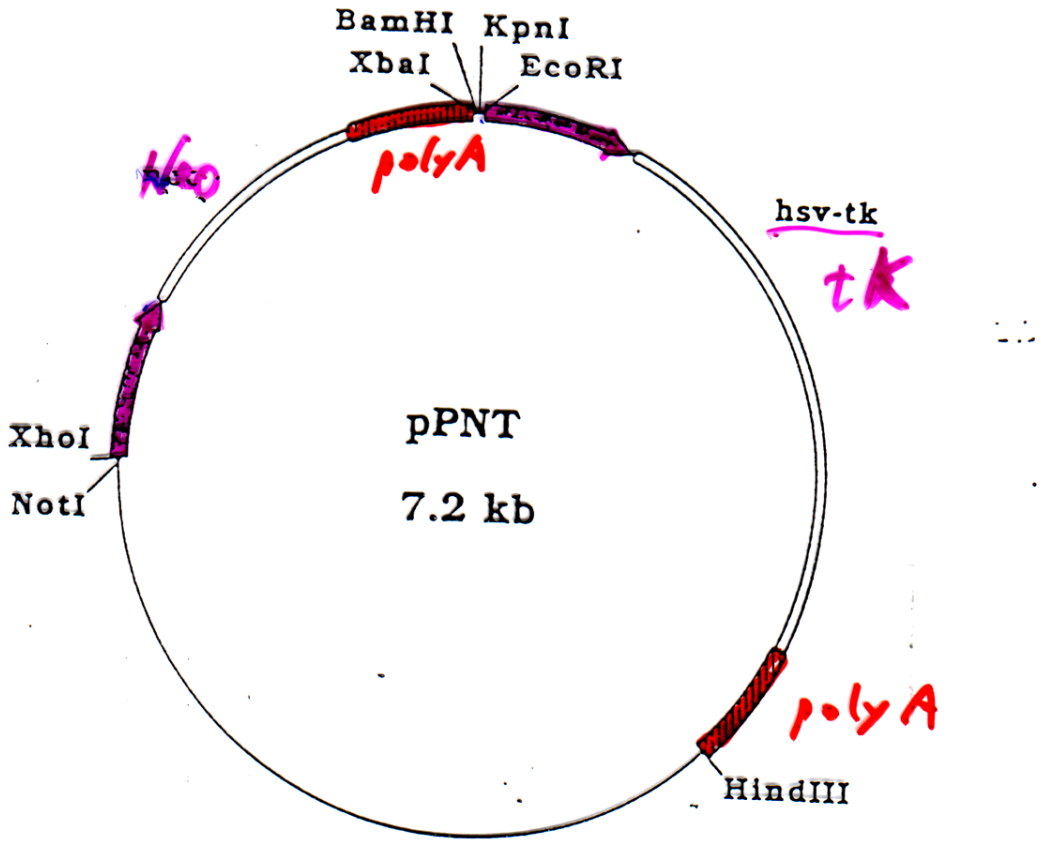


Figure 1. Structure of pPNT

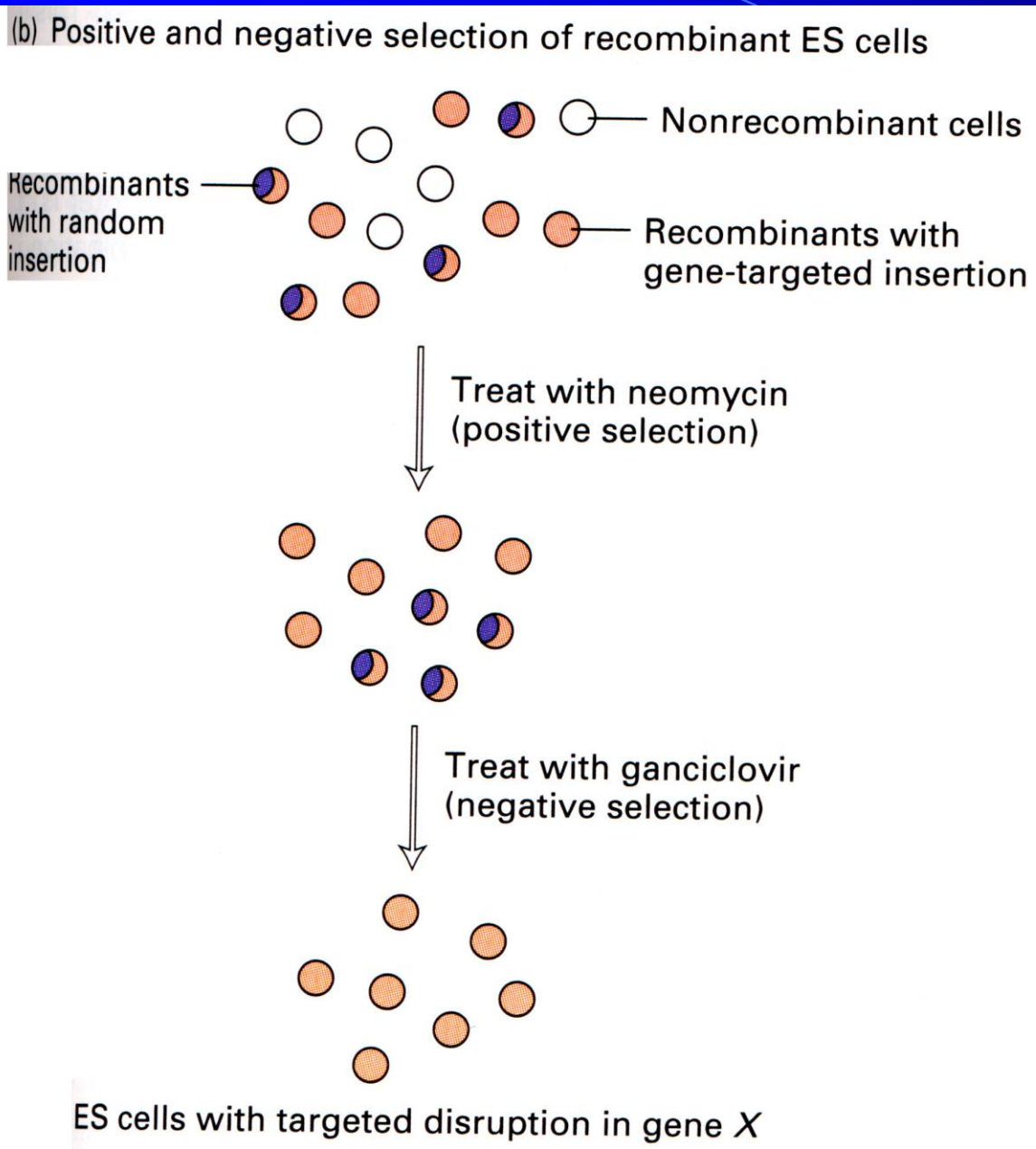
The shaded arrows represent the PGK-1 promoter, the hatched boxes represent the PGK-1 poly(A) addition sequences, the open boxes are the *neo* and HSV-*tk* genes as labeled, and the line represents the plasmid backbone. Unique restriction sites are indicated. The precise nature of each of the fragments is described in Experimental Procedures.

PGK promoter is better!

Reference

1. Tybulewicz et al. (1991) *Cell* 65: 1153-1163.

Selection of Targeted ES Clones



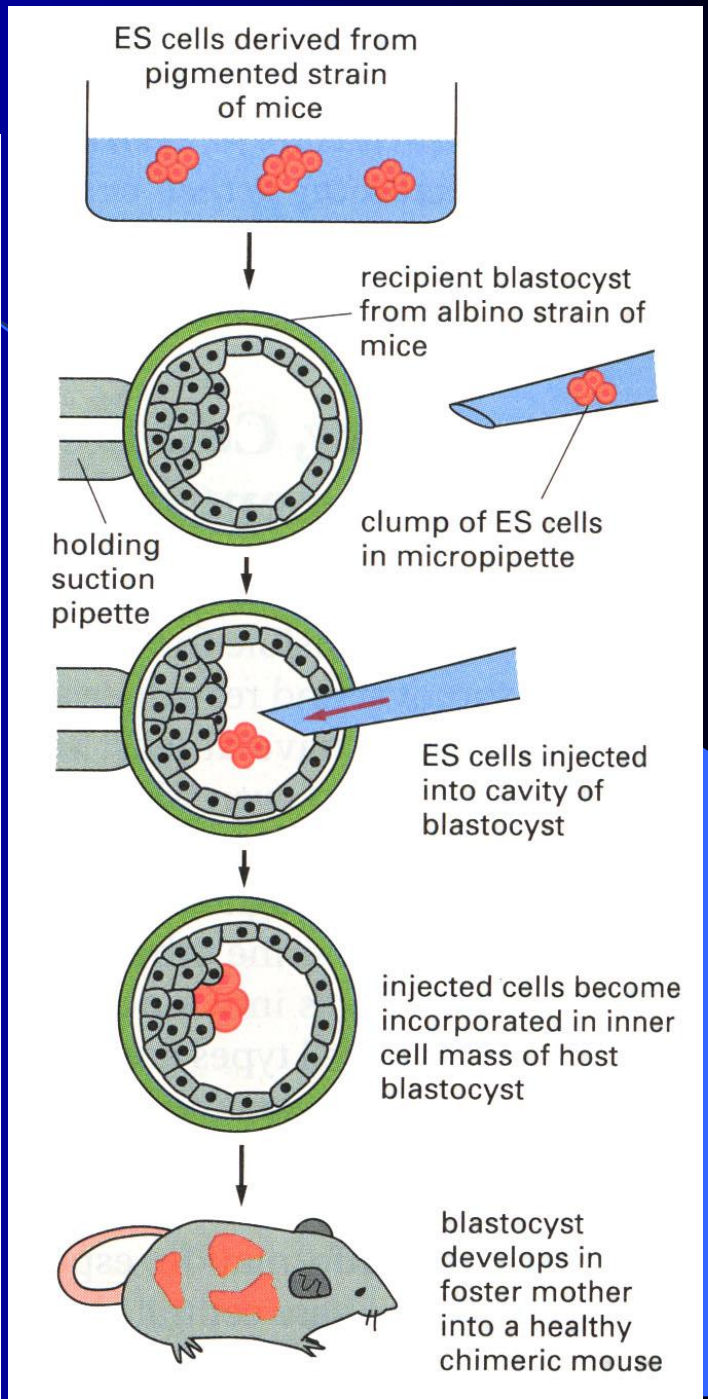
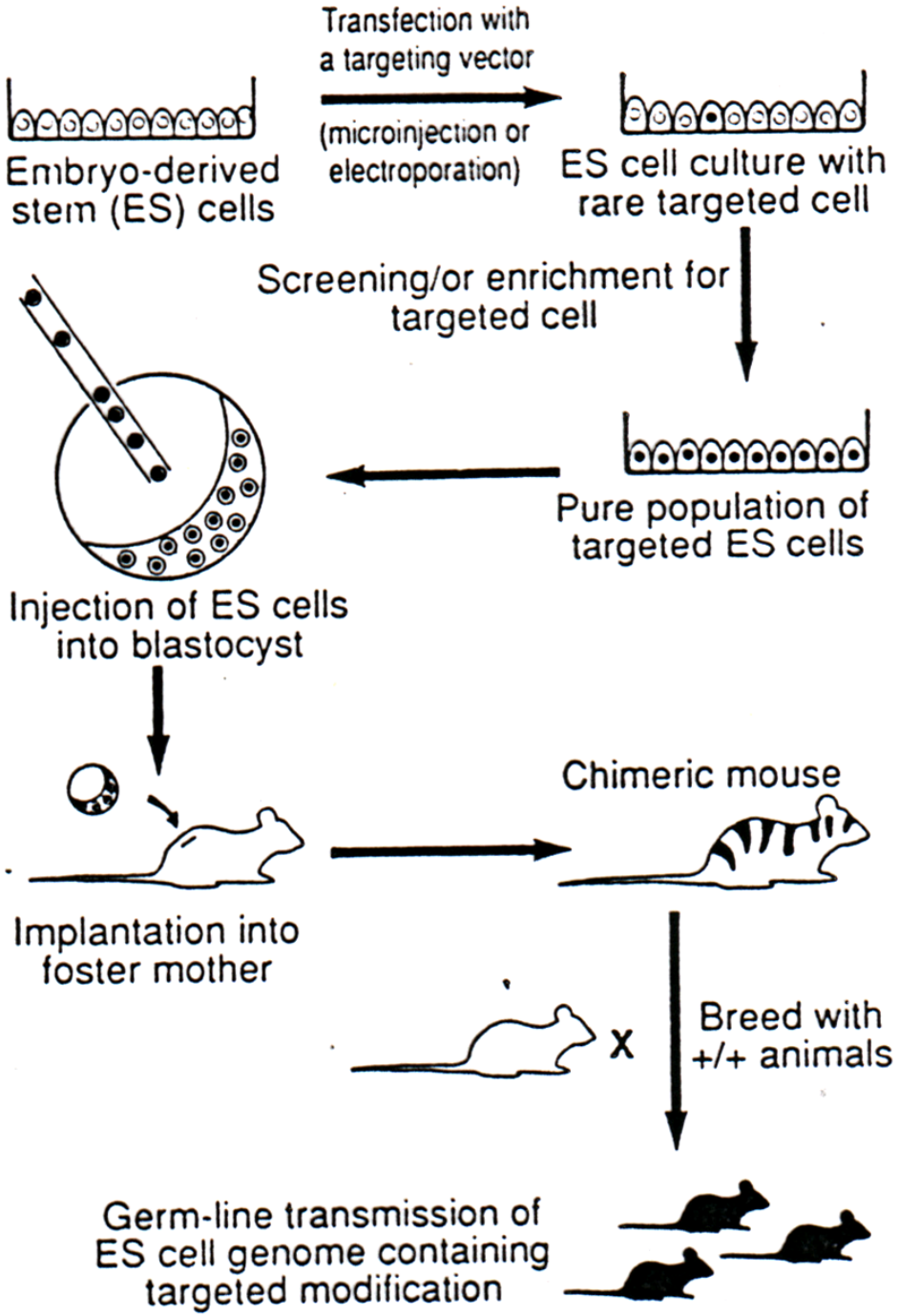
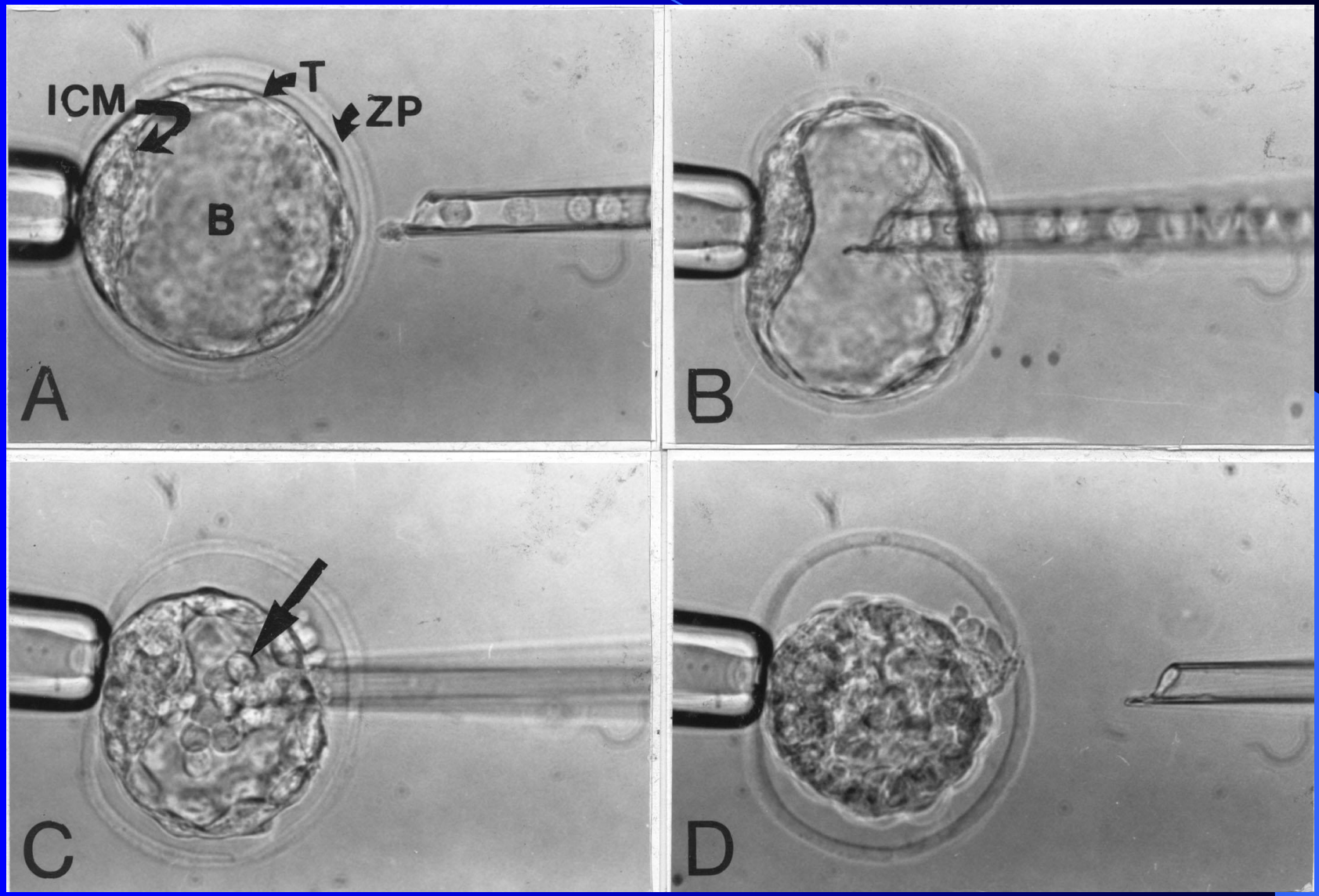
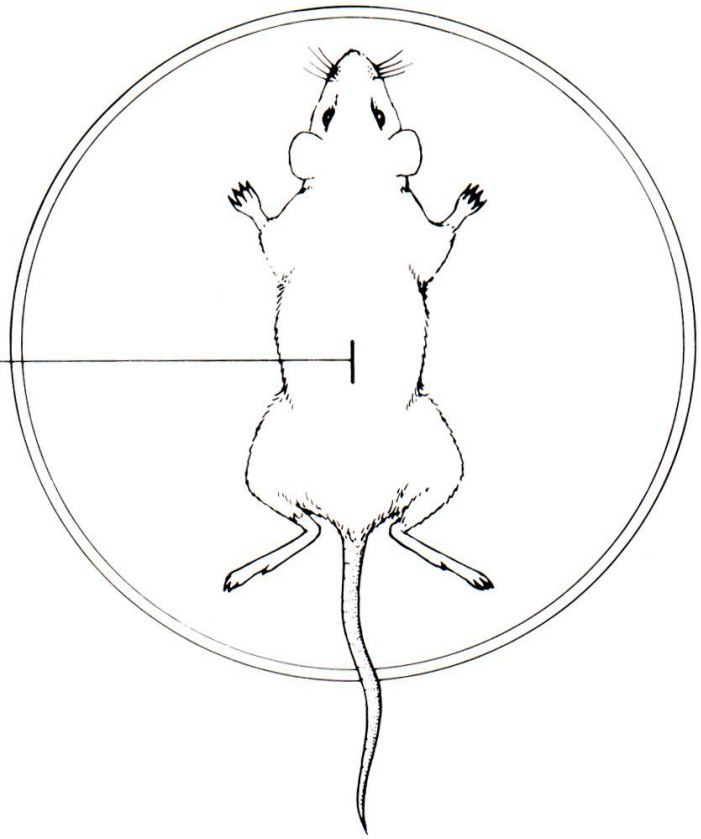


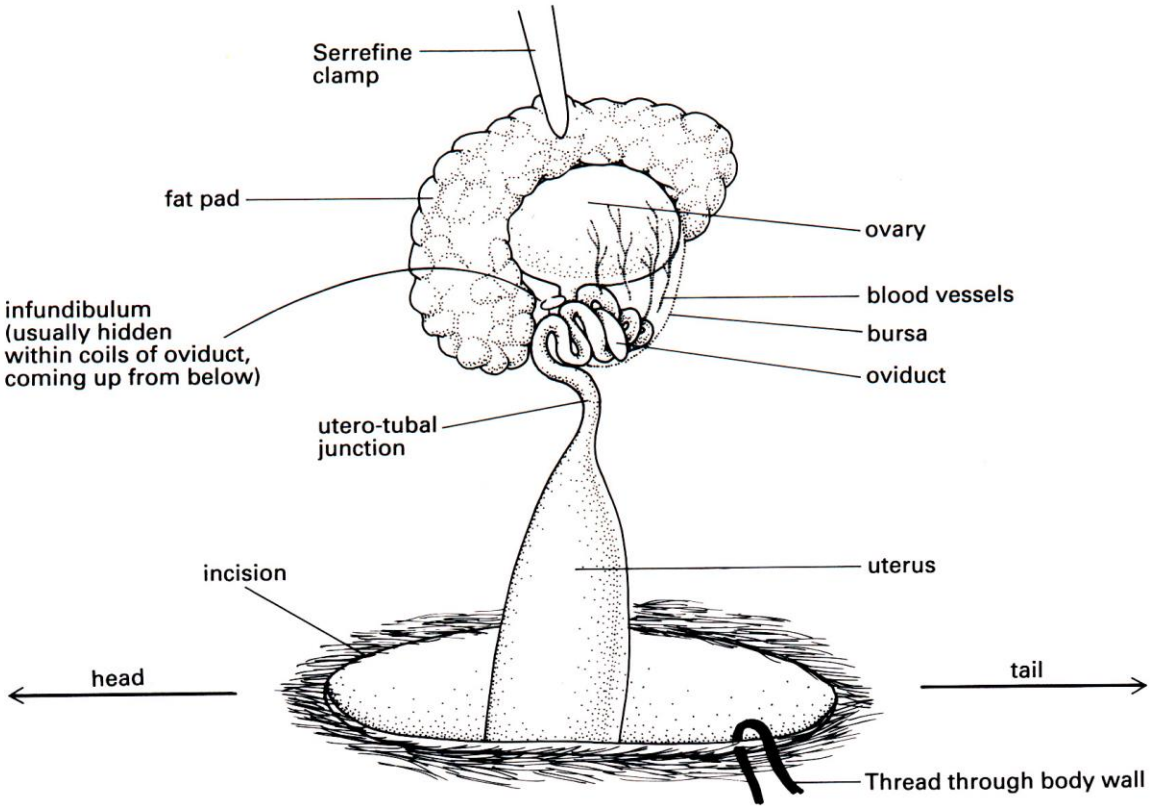
Fig. 9. Generation of mouse germ line chimeras from embryo-derived stem (ES) cells containing a targeted gene disruption.



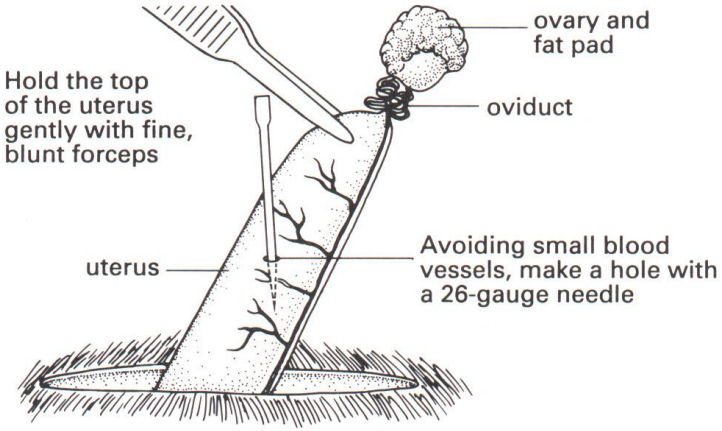
ES cells microinjected into blastocyst

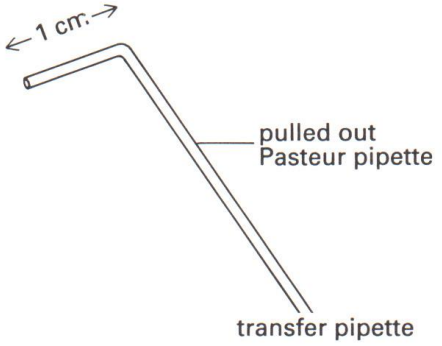


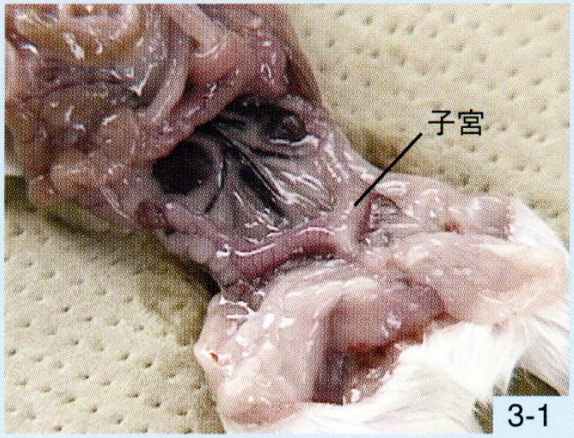


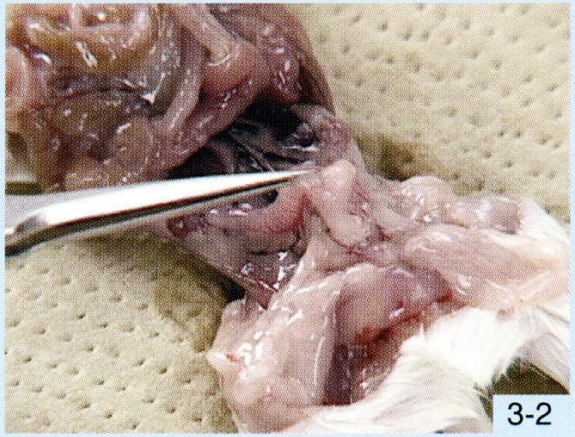


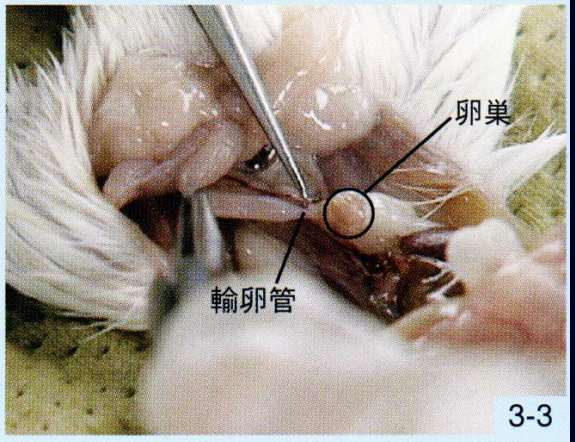
n of incision for uterine transfers.

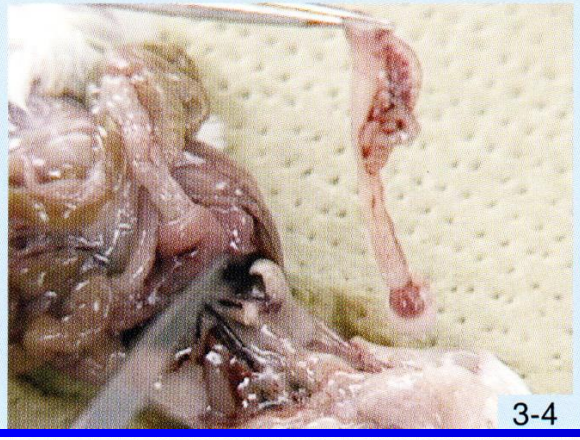












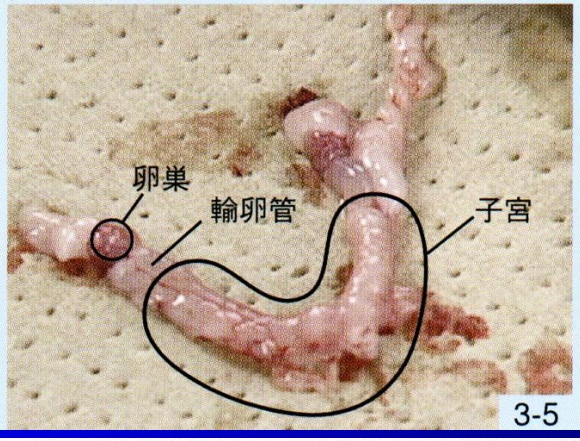


図3　子宮，卵巣，輸卵管の摘出

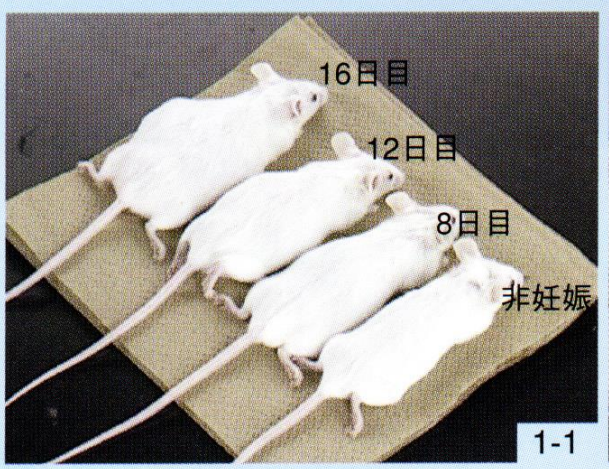
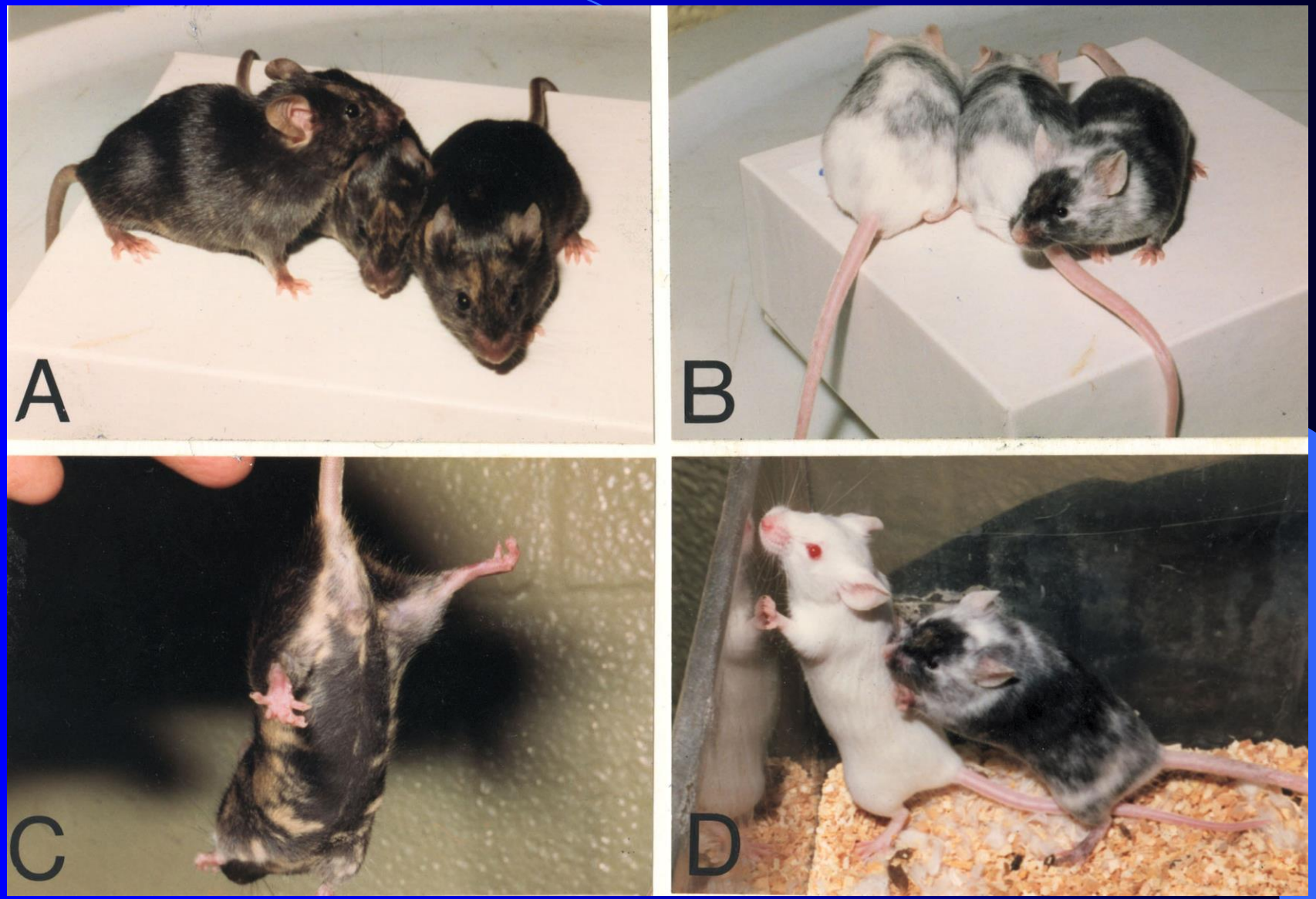
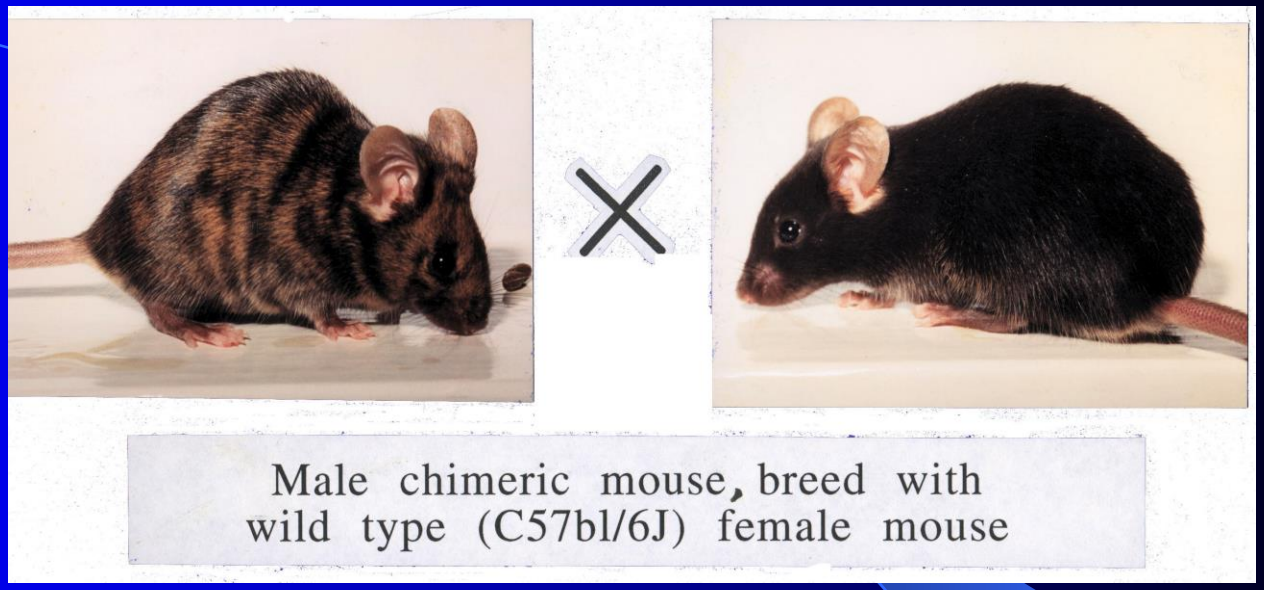


図1　妊娠日齢による変化

Chimeric mice generated by the ES cell injection



Germ-line transmission analysis



Aa x Aa:

- ¼ AA (wild type)
- ½ Aa (heterozygote)
- ¼ aa (homozygote)

Cell, Vol. 62, 1073–1085, September 21, 1990.

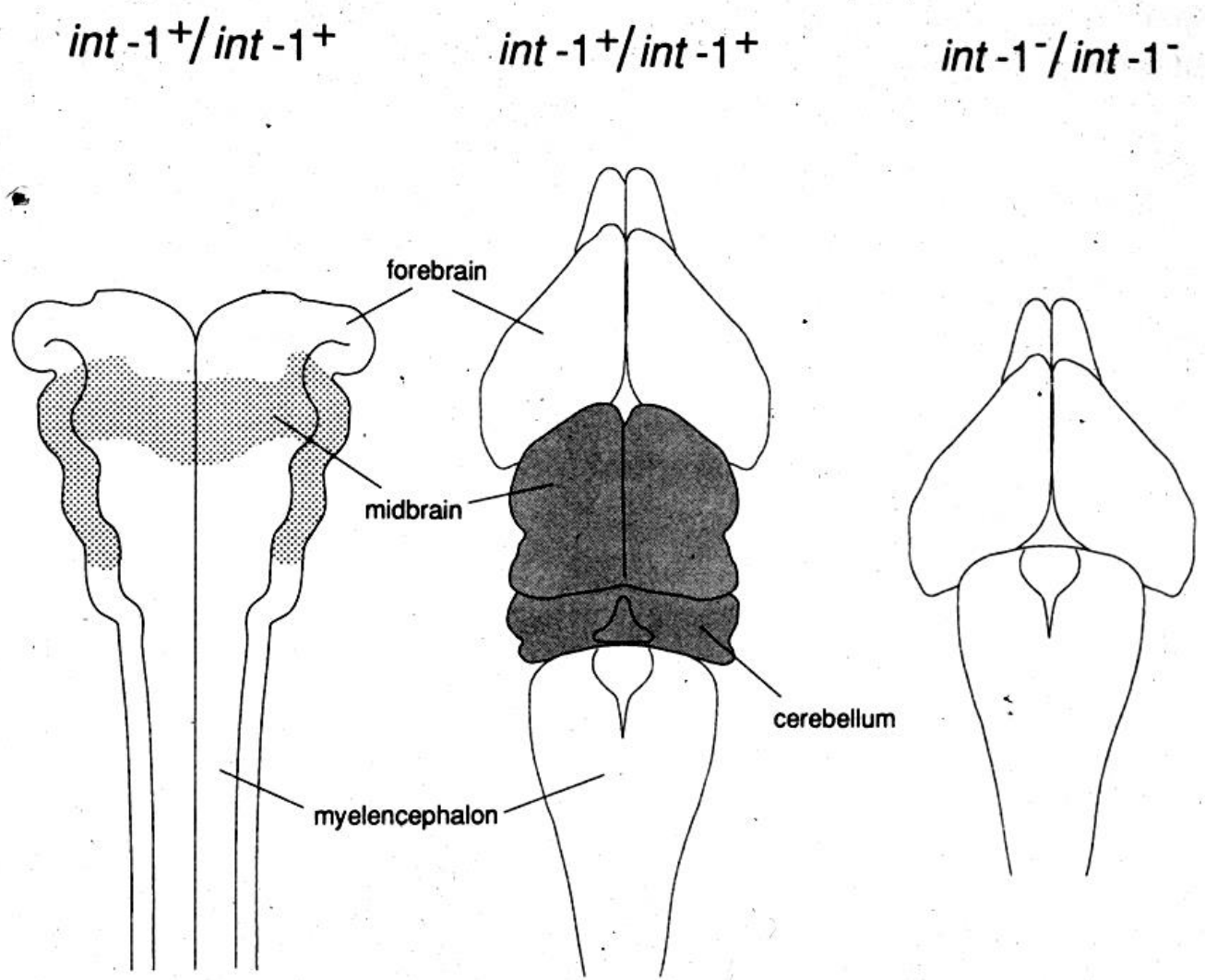
14

The *Wnt*-1 (*int*-1) Proto-Oncogene Is Required for Development of a Large Region of the Mouse Brain

Andrew P. McMahon* and All
* Department of Cell and Deve
Roche Institute of Molecular Bi
Roche Research Center
Nutley, New Jersey 07110
† Institute for Molecular Genetic
Baylor College of Medicine
Houston, Texas 77030

Summary

**The *Wnt*-1 (*int*-1) proto-onco
putative signaling molecule, i
in the developing central ne
testes. To examine the role of
independent embryonic stem
tion of a *neo*^{R} gene by homolo
activated a *Wnt*-1 allele. G**



Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member

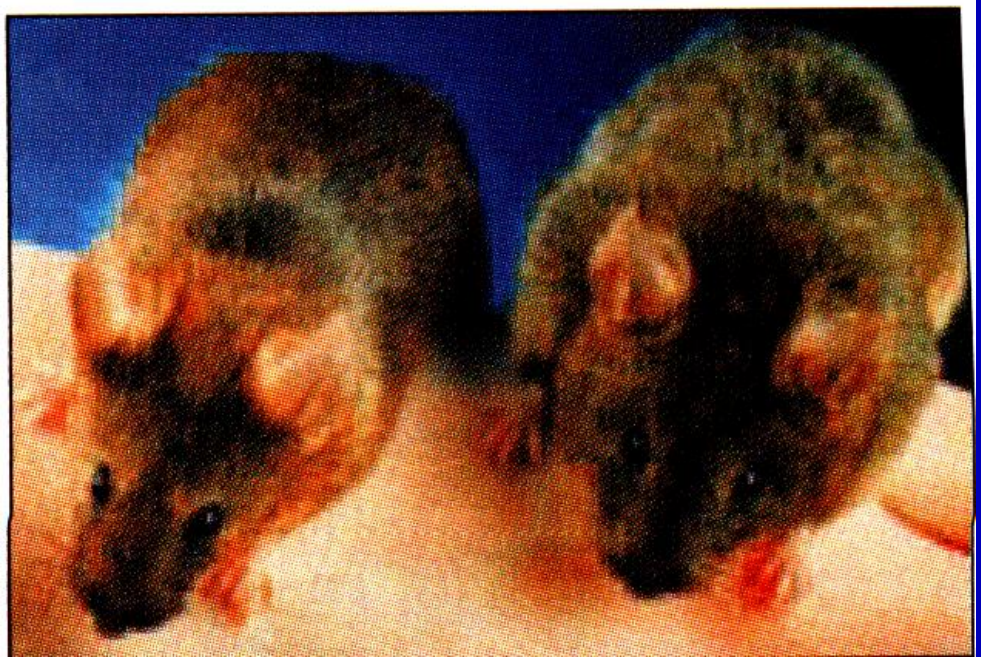
Alexandra C. McPherron*, Ann M. Lawler† & Se-Jin Lee

* *Department of Molecular Biology and Genetics, and † Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA*

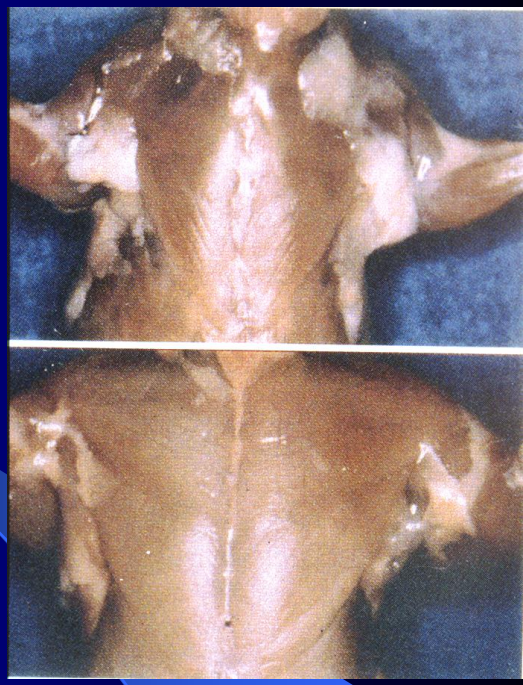
The transforming growth factor-β (TGF-β) superfamily encom
passes a large group of growth and differentiation factors playin
important roles in regulating embryonic development and i
maintaining tissue homeostasis in adult animals[1]. Using deger

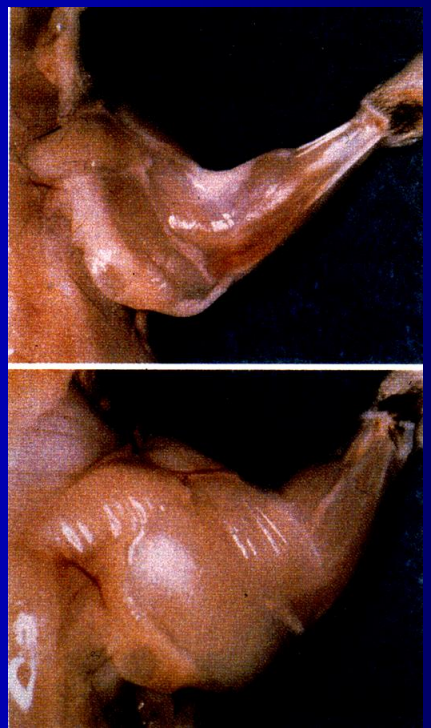
wild-type mouse

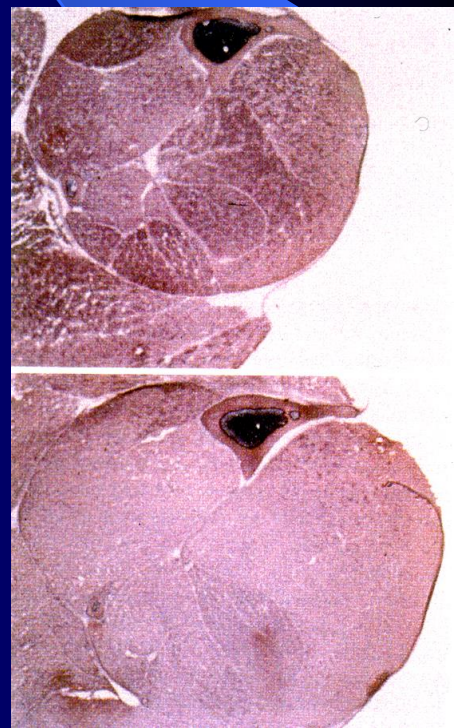
myostatin mutant



Myostatin gene KO (Nature 387:83-90, 1997)







Belgian Blue Mutation at the myostatin gene

Nature knockout mutants: in the Belgian Blue

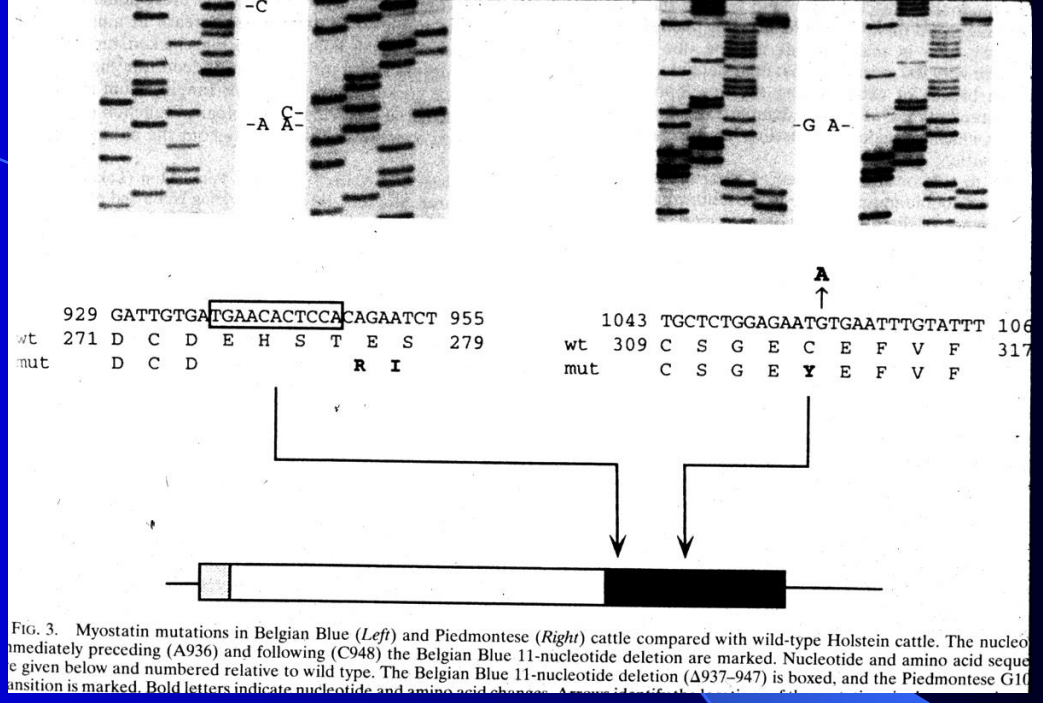
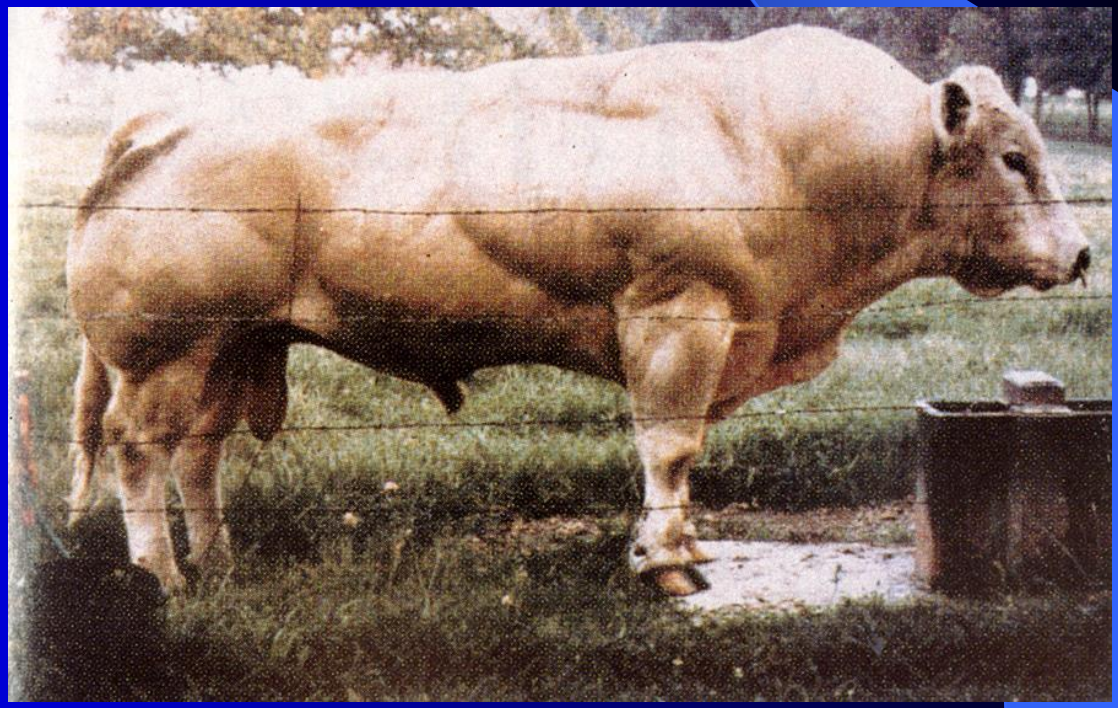


FIG. 3. Myostatin mutations in Belgian Blue (*Left*) and Piedmontese (*Right*) cattle compared with wild-type Holstein cattle. The nucleo
mediately preceding (A936) and following (C948) the Belgian Blue 11-nucleotide deletion are marked. Nucleotide and amino acid seque
e given below and numbered relative to wild type. The Belgian Blue 11-nucleotide deletion (Δ937–947) is boxed, and the Piedmontese G1
ansition is marked. Bold letters indicate nucleotide and amino acid changes.



腫瘤抑制基因(Rb gene) 基因敲毀轉殖動物模式

基因轉殖突變促使胚胎生長缺陷 (Nature 359: 288-294, 1992)

ARTICLES

Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis

Eva Y.-H. P. Lee*†, Chi-Yao Chang*, Nanpin Hu*, Yi-Chun J. Wang*, Chen-Ching Lai*‡, Karl Herrup§, Wen-Hwa Lee* & Allan Bradley‖

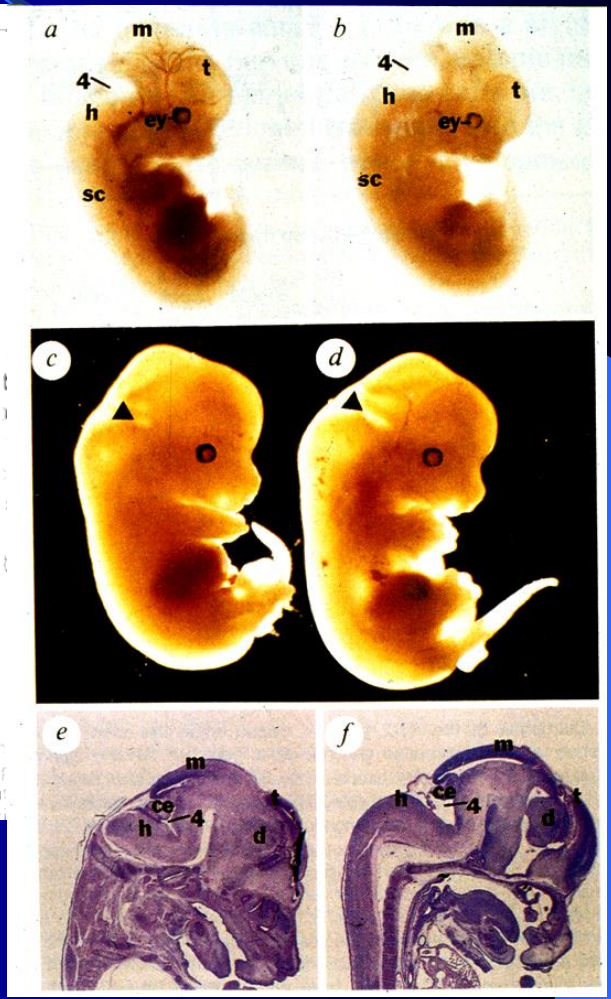
* Center for Molecular Medicine and Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, Texas 78284, USA
§ Alzheimer Research Lab, Case Western Reserve Medical School, Cleveland, Ohio 44106, USA
‖ Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030, USA

The retinoblastoma gene, a prototypic tumour-suppressor gene, encodes a nuclear phosphopr (Rb). To understand better the role of Rb in development and in tumorigenesis, mice with an insert mutation in exon 20 of the *Rb-1* locus were generated. Homozygous mutants die before the embryonic day with multiple defects. The haematopoietic system is abnormal; there is a signif increase in the number of immature nucleated erythrocytes. In the nervous system, ectopic mit and massive cell death are found, particularly in the hindbrain. All spinal ganglion cells die, bu neural retina is unaffected. Transfer of the human retinoblastoma (*RB*) mini-transgene into the mu mice corrects the developmental defects. Thus, Rb is essential for normal mouse development.

RETINOBLASTOMA, an ocular childhood tumour, has been a model for studies of the role of tumour suppressor genes in cancer predisposition[1,2]. The hereditary form of the disease is an autosomal dominant trait[3]. But a recessive nature of the mutant gene was proposed in Knudson's 'two-hit' hypothesis[4], and later substantiated[5–9]. Although the eye is usually the first site of tumour formation, patients with hereditary retinoblas-

This region seems to be important for Rb function bc carboxy-terminal truncations of Rb deleting the T/E1A-bi domains are nonfunctional[16,38].

Taken as a whole, the data surrounding the behaviour present something of a paradox. Its ubiquitous expressio seeming involvement in cell-cycle regulation suggest that it a central role in essential cellular activity. But by con



腫瘤抑制基因(p53 gene)基因敲毀轉殖動物模式

P53基因轉殖敲毀後促使動物腫瘤發生(Nature 356: 215-221, 1992)

Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours

Lawrence A. Donehower*, Michele Harvey*, Betty L. Slagle*, Mark J. McArthur†, Charles A. Montgomery Jr†, Janet S. Butel* & Allan Bradley‡

* Division of Molecular Virology, † Center for Comparative Medicine, and ‡ Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030, USA

Mutations in the p53 tumour-suppressor gene are the most frequently observed genetic lesions in human cancers. To investigate the role of the p53 gene in mammalian development and tumorigenesis, a null mutation was introduced into the gene by homologous recombination in murine embryonic stem cells. Mice homozygous for the null allele appear normal but are prone to the spontaneous development of a variety of neoplasms by 6 months of age. These observations indicate that a normal p53 gene is dispensable for embryonic development, that its absence predisposes the animal to neoplastic disease, and that an oncogenic mutant form of p53 is not obligatory

***MyoD* KO**: normal in muscle development, yet leads to up-regulation of the myogenic gene *Myf*-5 (Cell 71:383-390, 1992)

***Myf*-5 KO:** abnormal rib development and perinatal death (Cell 71: 369-382, 1992)

Table 2. Identification of MyoD1 and some MyoD1 homologs

MyoD1 homolog	Animal	Homology
MyoD1	Mouse	MyoD1
Myf-3	Human	MyoD1
XMyoD	Frog (*X. laevis*)	MyoD1
CMD1	Chicken	MyoD1
qmf1	Quail	MyoD1
CeMyoD	Worm (*C. elegans*)	MyoD1
nau	Fly (*D. melanogaster*)	MyoD1
Myogenin	Rat/mouse	myogenin
Myf-4	Human	myogenin
qmf2	Quail	myogenin
myf-5	Human	myf-5
qmf3	Quail	myf-5
MRF-4	Rat	MRF-4
Herculin	Human	MRF-4

Cell, Vol. 71, 383–390, October 30, 1992, Copyright © 1992 by Cell Press

Inactivation of *MyoD* in Mice Leads to Up-Regulation of the Myogenic HLH Gene *Myf*-5 and Results in Apparently Normal Muscle Development

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*Whitehead Institute
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Cambridge, Massachusetts 02142
‡Department of Toxicology
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2000 Hamburg 13
Grindelallee 117
Germany

the skeletal myocyte lineage (Olson, 1990; Weintraub et al., 1991; Buckingham, 1992).

In vertebrates, skeletal muscle originates from a sma pool of progenitor cells that arise in the early somite (re viewed by Buckingham, 1992; Miller, 1991, 1992). Thes premyoblast stem cells become the dermamyotomal com partment of the maturing somite, from which myoblas expand into the developing embryo. In mice, skeletal mu cle development occurs in several phases. First, to diffe entiate in the fetus at 8.5 days of gestation, the myotom fiber precursors give rise to small spindle-like myotom fibers displaying the earliest expression of muscle-specif

Cell, Vol. 71, 369–382, October 30, 1992, Copyright © 1992 by Cell Press

Targeted Inactivation of the Muscle Regulatory Gene *Myf*-5 Results in Abnormal Rib Development and Perinatal Death

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University of Hamburg Medical School
2000 Hamburg 13
Grindelallee 117
Germany
†Whitehead Institute for Biomedical Research
and Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02142

Summary

The *Myf*-5 gene, a member of the myogenic basic HLH factor family, has been inactivated in mice after homologous recombination in ES cells. Mice lacking *Myf*-5 were unable to breathe and died immediately after

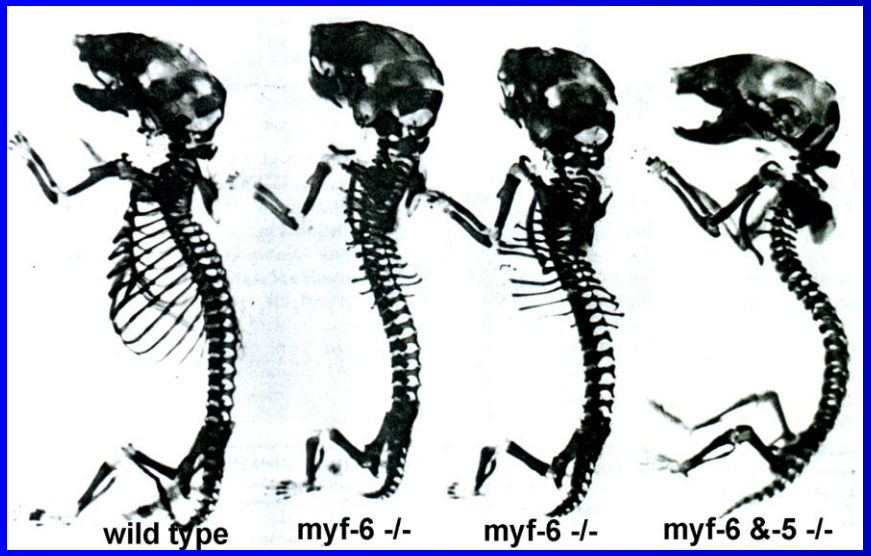
CANNTG. Detailed mutational analysis of MyoD (Davis et al., 1990), myogenin (Brennan et al., 1991), and Myf-5 (Winter et al., 1992) has demonstrated that the conserved basic and HLH domains are responsible for sequence-specific DNA binding and heterodimerization with the ubiquitously expressed HLH products of the E2A gene, respectively. Transcriptional activation is dependent on a transactivator domain located in the NH_2–terminus of MyoD (Weintraub et al., 1991b), and on two regions located upstream and downstream of the conserved HLH domain in myogenin and Myf-5 (Schwartz et al., 1992; Braun et al., 1990b; Winter et al., 1992).

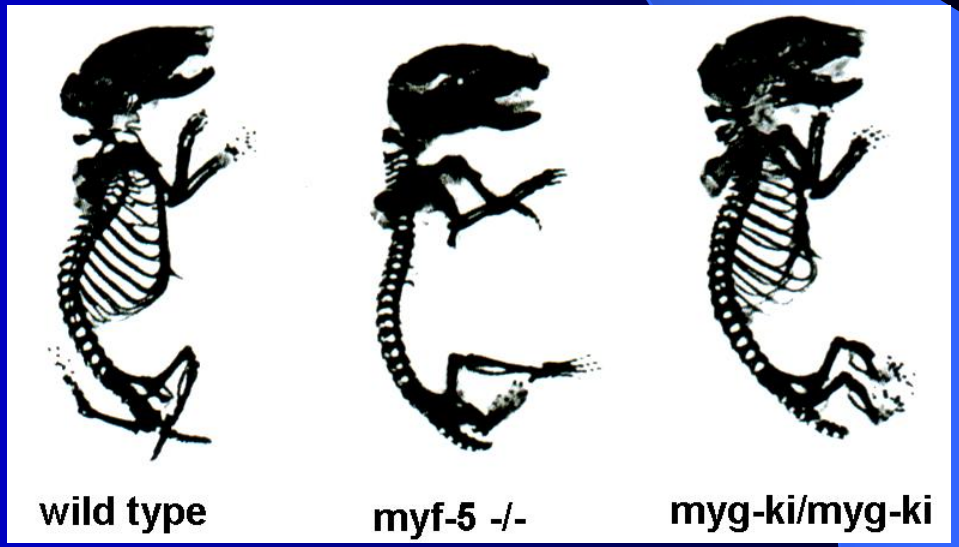
Despite suggestive evidence obtained from tissue culture experiments, the individual or collective role of myogenic factors during muscle development in vivo has not been determined to date. It has been difficult to ascribe specific functions to the individual myogenic HLH proteins, because each factor can influence its own expression as well as that of the other factors in most cell lines (Thayer

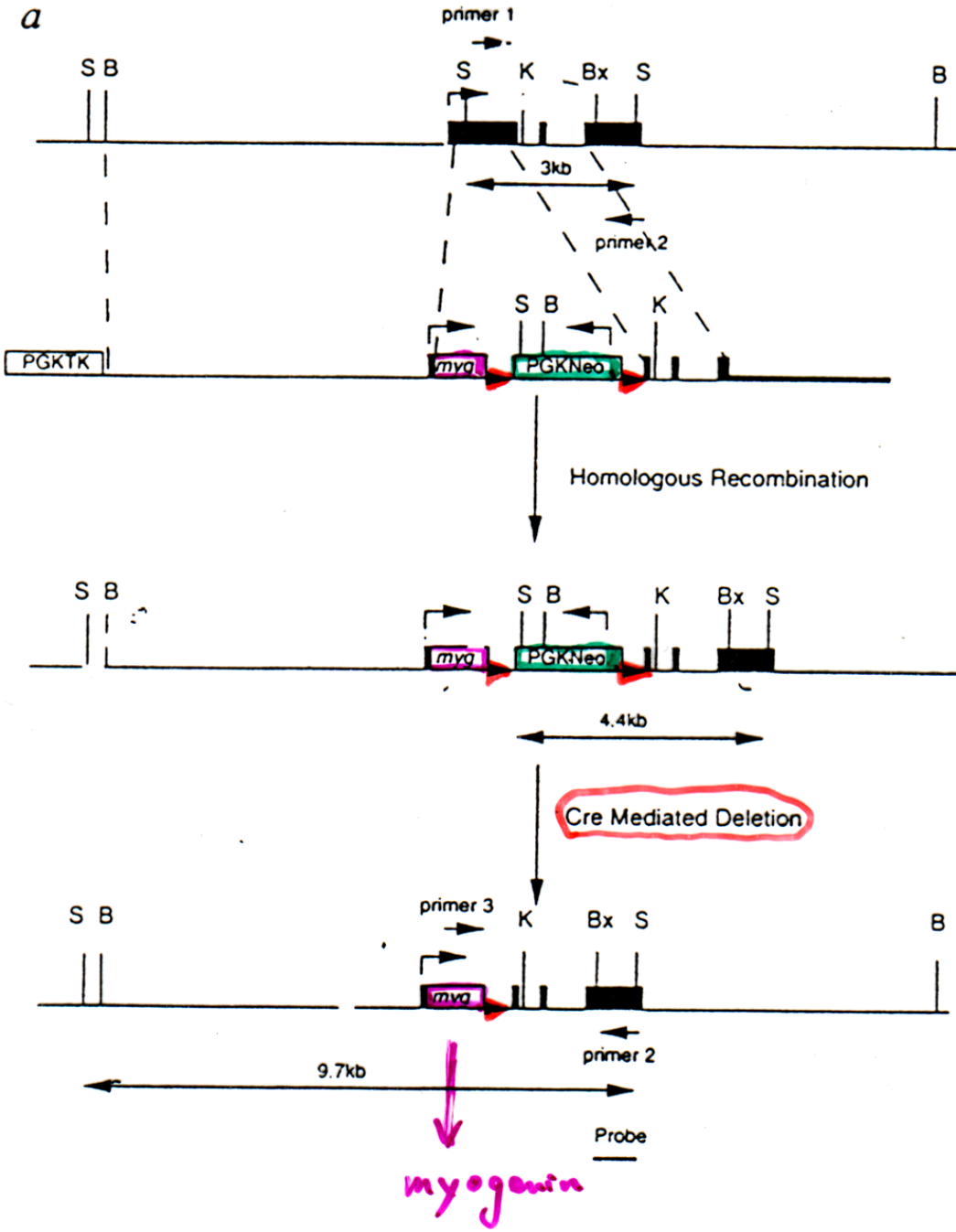
Myogenin KO: Muscle deficiency and neonatal death (Nature 364:501-506, 1993)

Myf-5 and Myf-6 double KO: alterations in skeletal muscle development (EMBO J. 14: 1176-1186, 1995)

Myogenin knock-in in myf-5 KO mice: Functional reduundancy of the muscle-specific transcription factors Myf5 and myogenin (Nature 379: 823-825, 1996)







Myf-5 loss

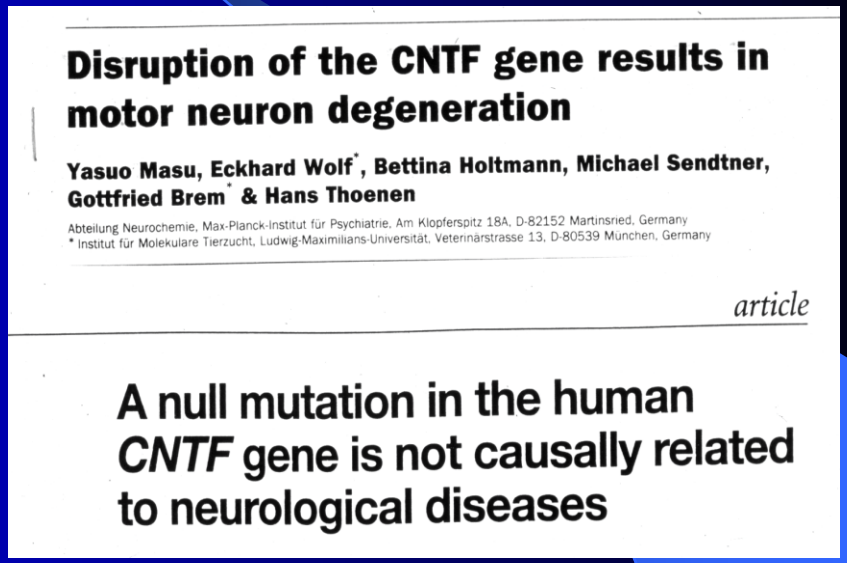
myf-5 knock out

myogenin Knock in

A knockout mouse model is a really good animal model for studying human genetic disease?

- *CNTF* (ciliary neurotrophic factor) KO mice: motor neuron degeneration (Nature 365:27-32, 1993)

- A null mutation in the human *CNTF* gene is not causally related to neurological diseases. (Nature Genetics 7:79-84, 1994).



Disruption of the CNTF gene results in motor neuron degeneration

Yasuo Masu, Eckhard Wolf*, Bettina Holtmann, Michael Sendtner, Gottfried Brem* & Hans Thoenen

Abteilung Neurochemie, Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18A, D-82152 Martinsried, Germany
* Institut für Molekulare Tierzucht, Ludwig-Maximilians-Universität, Veterinärstrasse 13, D-80539 München, Germany

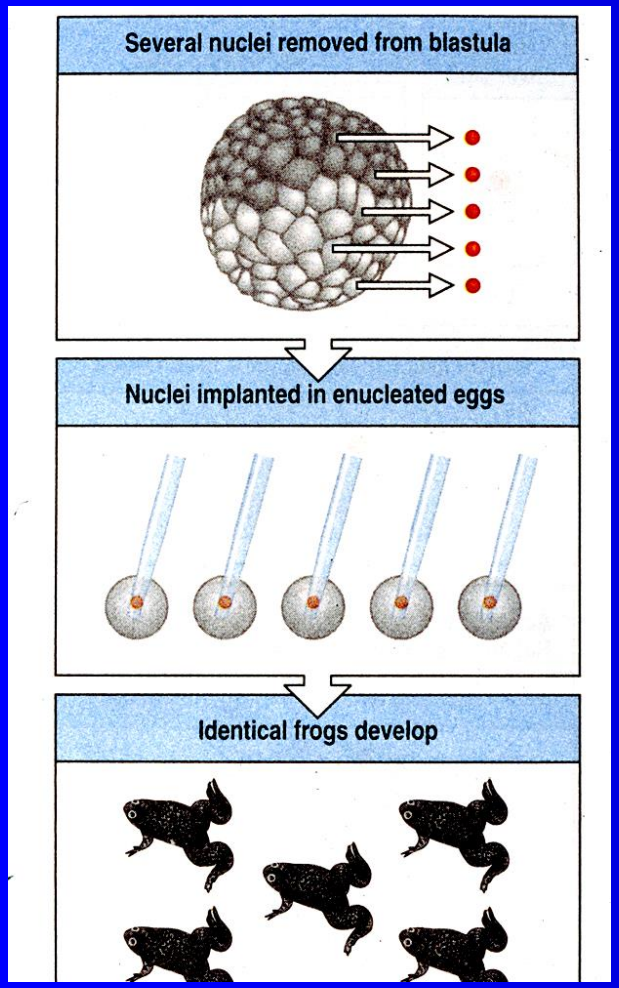
article

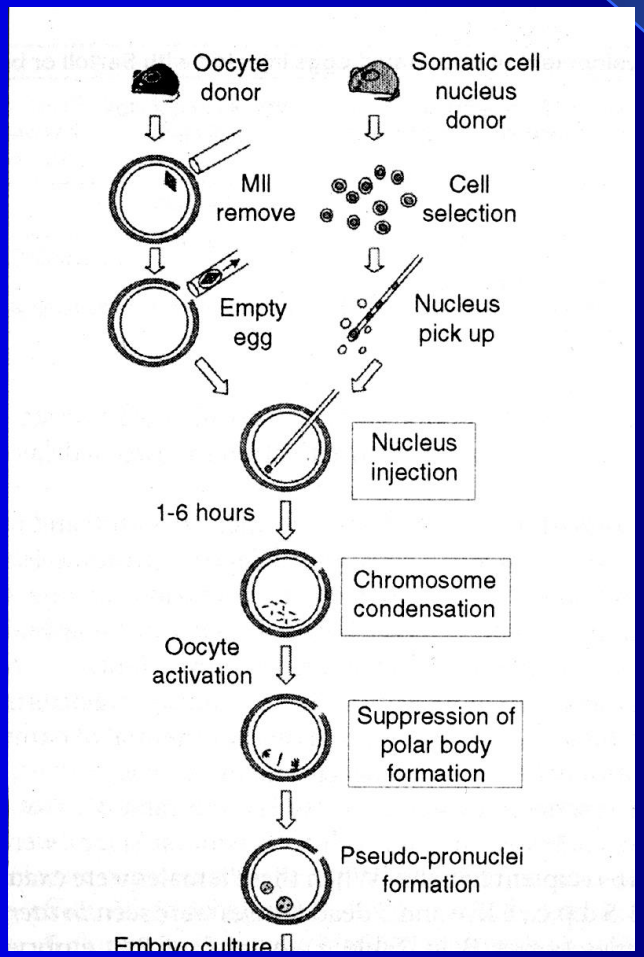
A null mutation in the human *CNTF* gene is not causally related to neurological diseases

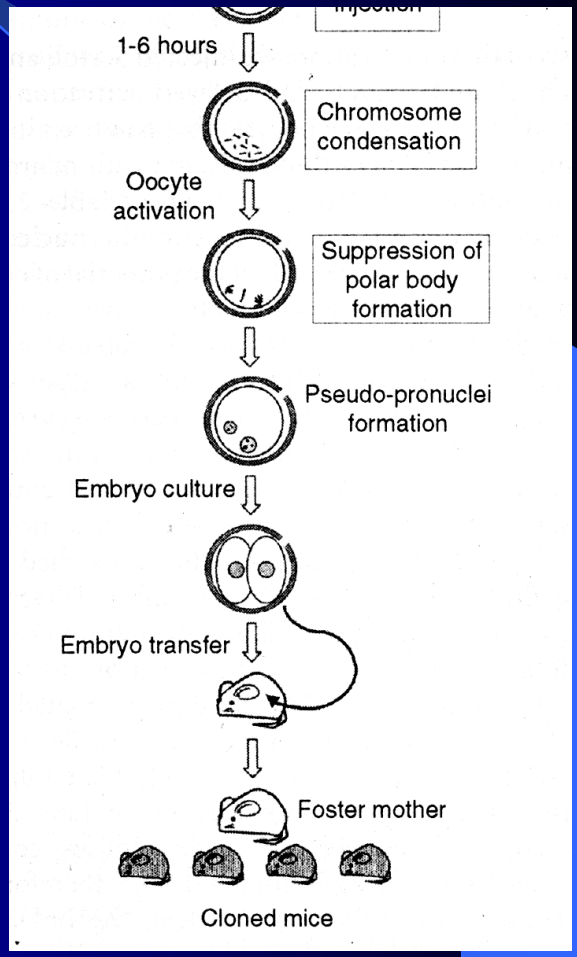
- *CNTFR* KO mice: die perinatally and display severe motor neuron deficits. (Cell 83:313-322, 1995)

細胞核轉殖與複製動物

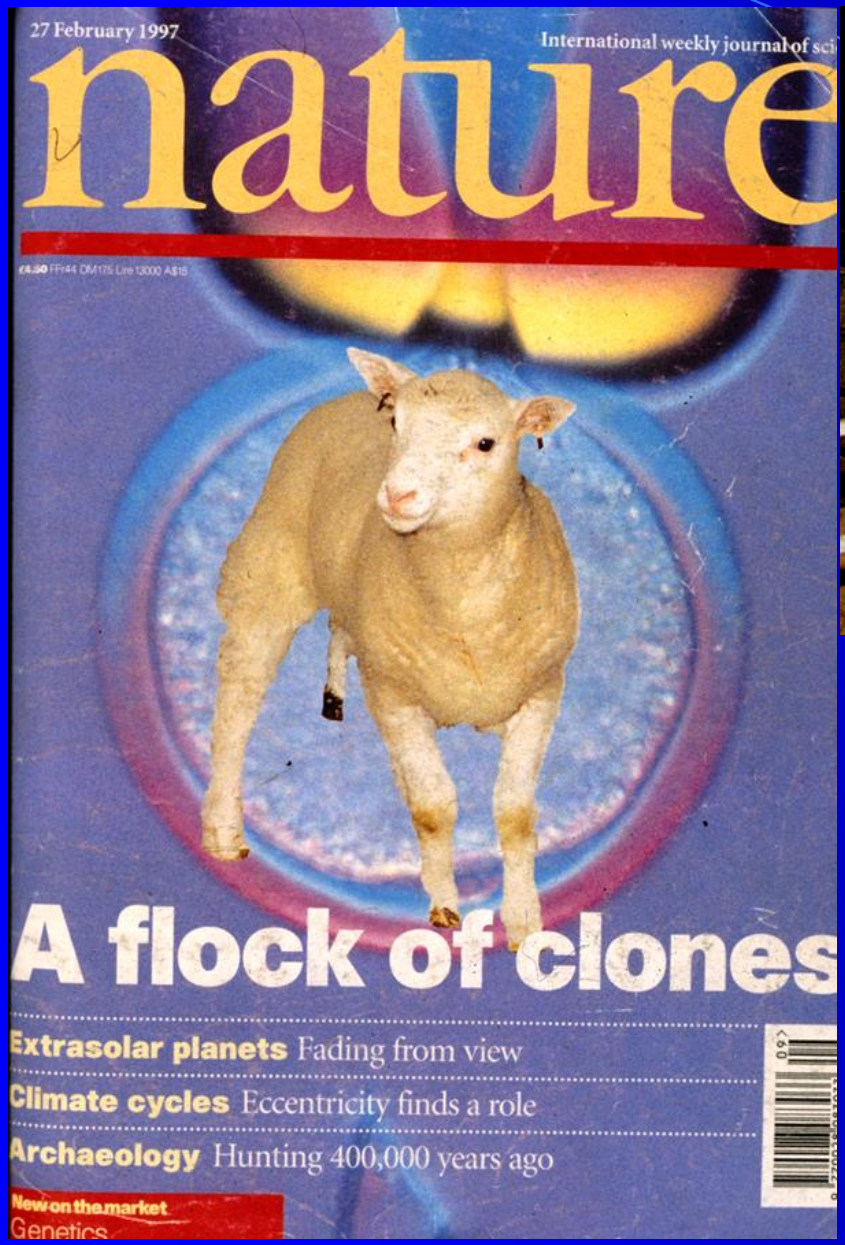
利用胚胎或成體細胞細胞核，置換未受精或已受精卵的細胞核，以製造出複製動物的技術。

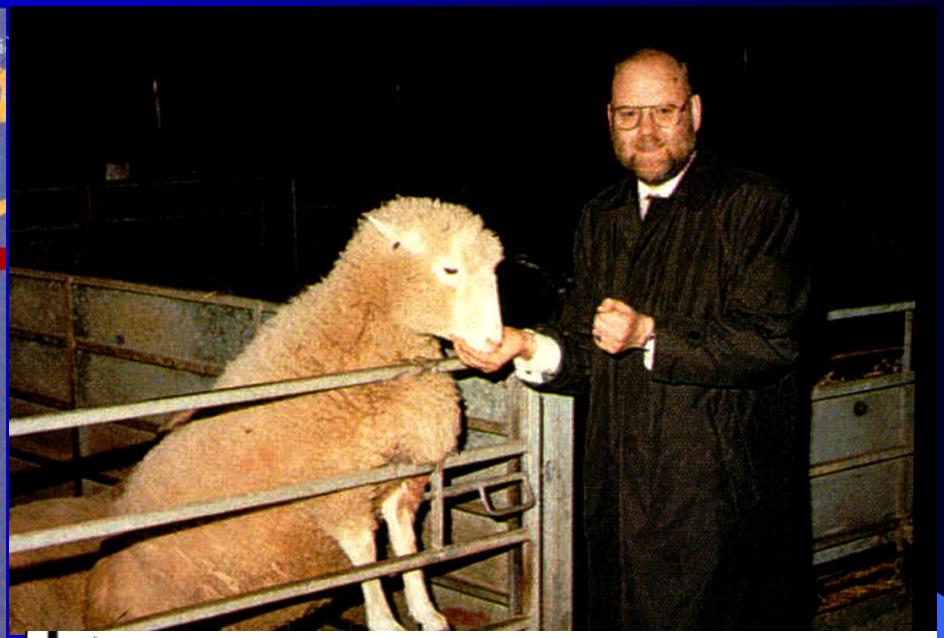


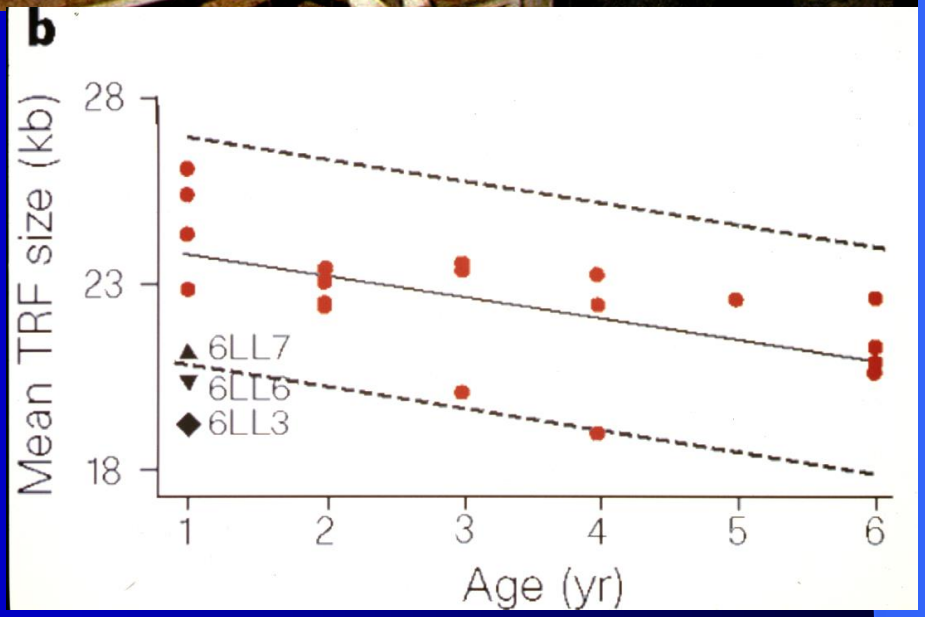




複製羊 "桃莉" (Nature 385: 810-813, 1997)







複製牛 (Science 280: 1256-1258, 1998)

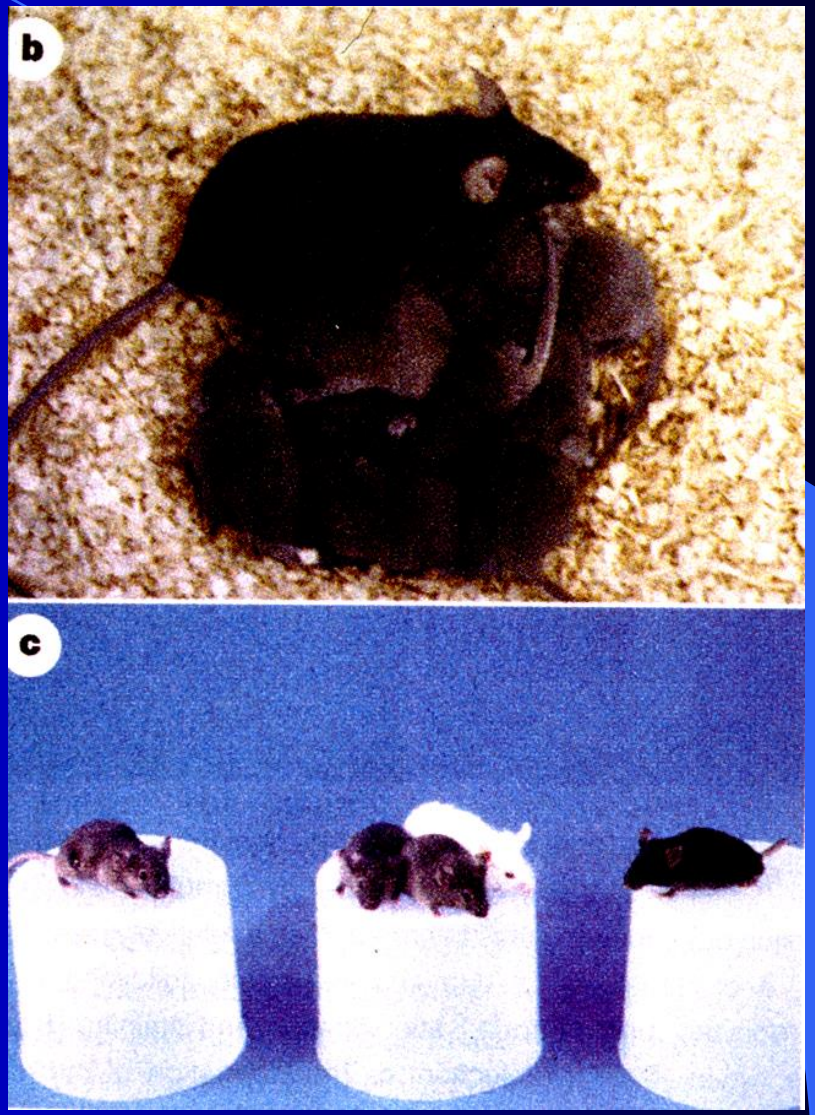
Cloned Transgenic Calves Produced from Nonquiescent Fetal Fibroblasts

Jose B. Cibelli, Steve L. Stice, Paul J. Golueke, Jeff J. Kane, Joseph Jerry, Cathy Blackwell, F. Abel Ponce de León, James M. Robl*

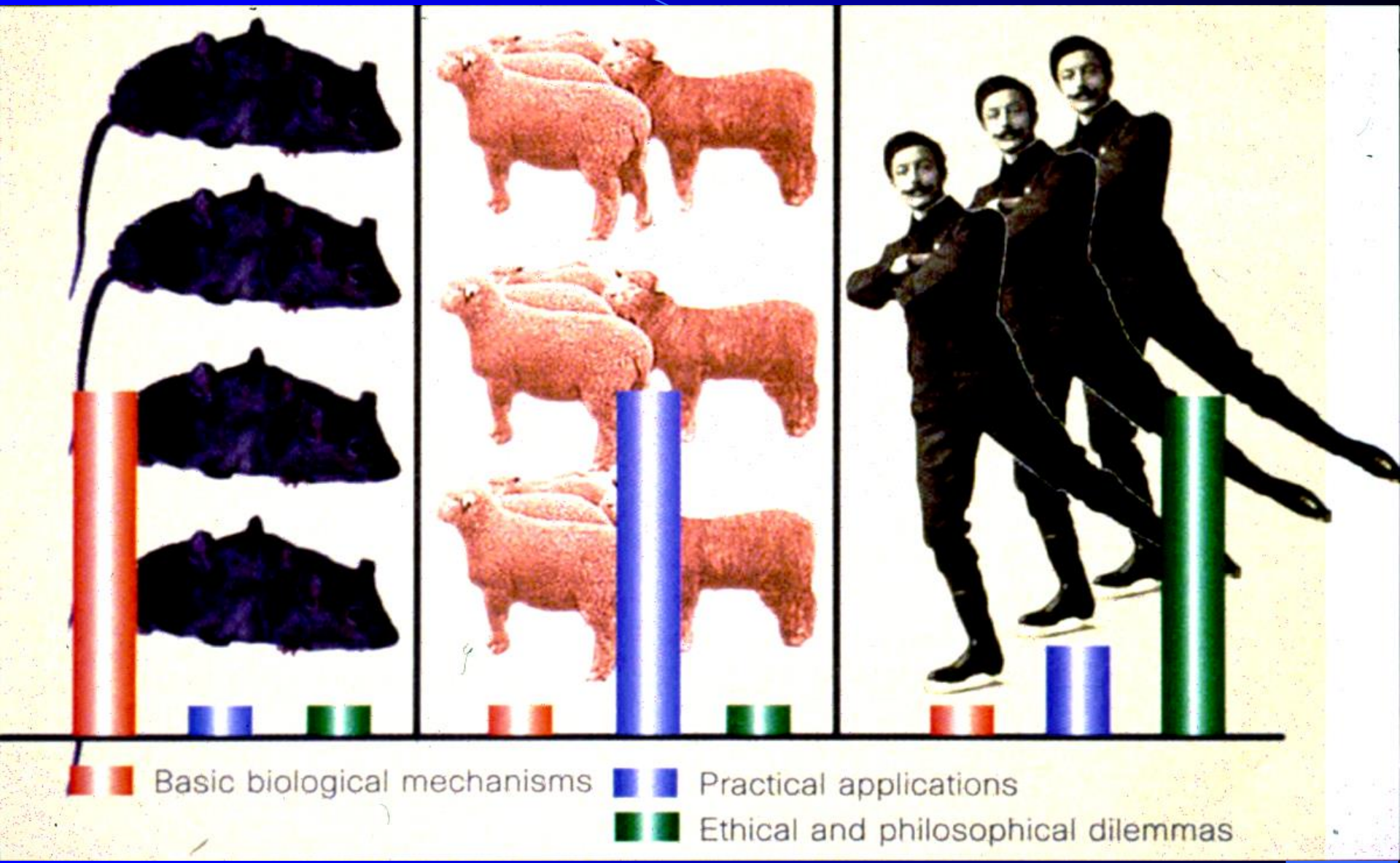
n efficient system for genetic modification and large-scale cloning of cattle is of im ortance for agriculture, biotechnology, and human medicine. Here, actively dividin tal fibroblasts were genetically modified with a marker gene, a clonal line was selected nd the cells were fused to enucleated mature oocytes. Out of 28 embryos transferre 11 recipient cows, three healthy, identical, transgenic calves were generated. Fur hermore, the life-span of near senescent fibroblasts could be extended by nuclea ansfer, as indicated by population doublings in fibroblast lines derived from a 40-day ld fetal clone. With the ability to extend the life-span of these primary cultured cells, thi ystem would be useful for inducing complex genetic modifications in cattle.

Fig. 2. Normal cloned calves ACT3, ACT4, and ACT5, at 3 weeks of age.

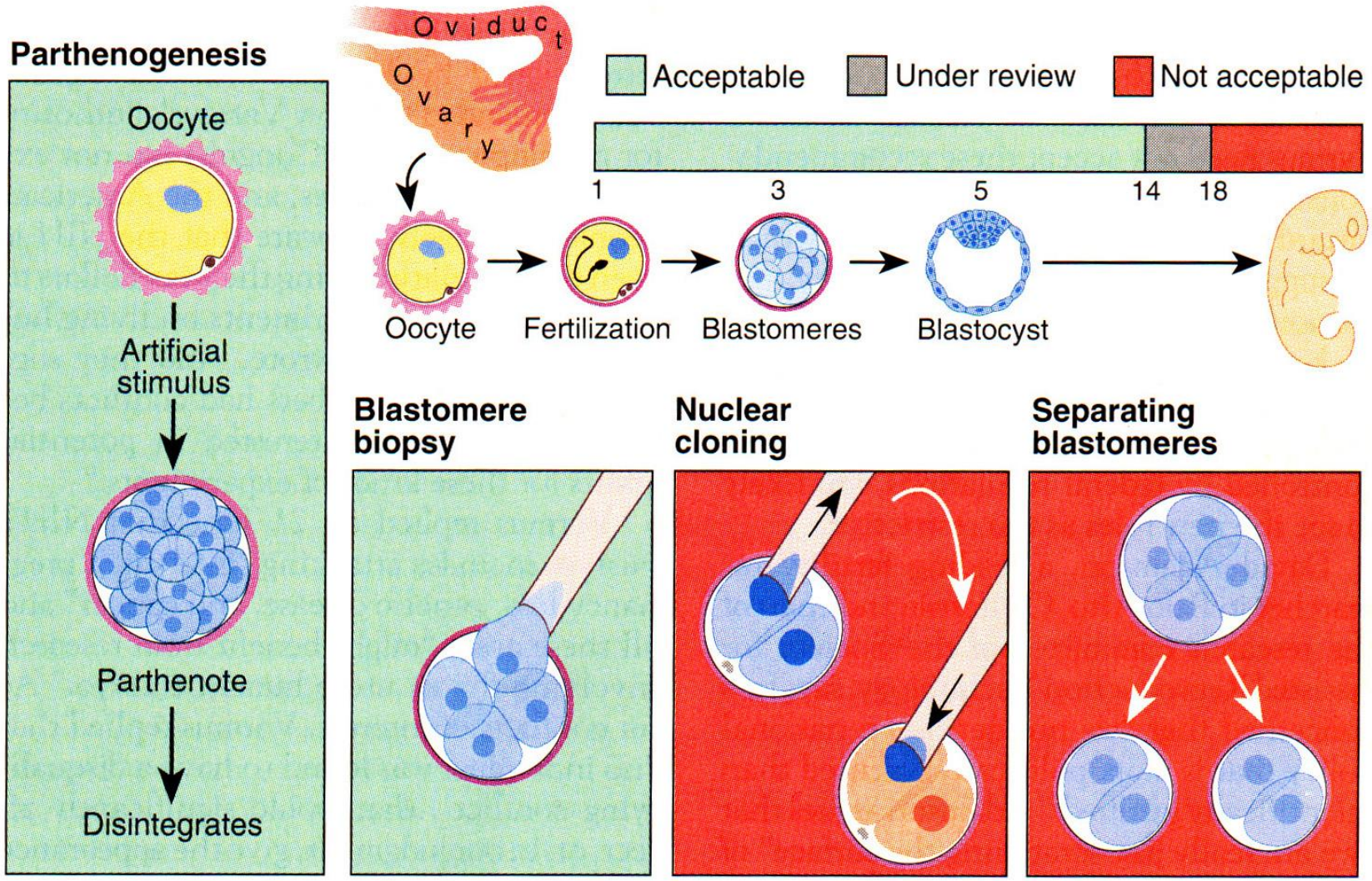
多代複製鼠 (Nature 394: 369-374, 1998)



人類基因與胚胎轉殖的可行性與相關道德倫理及法律問題

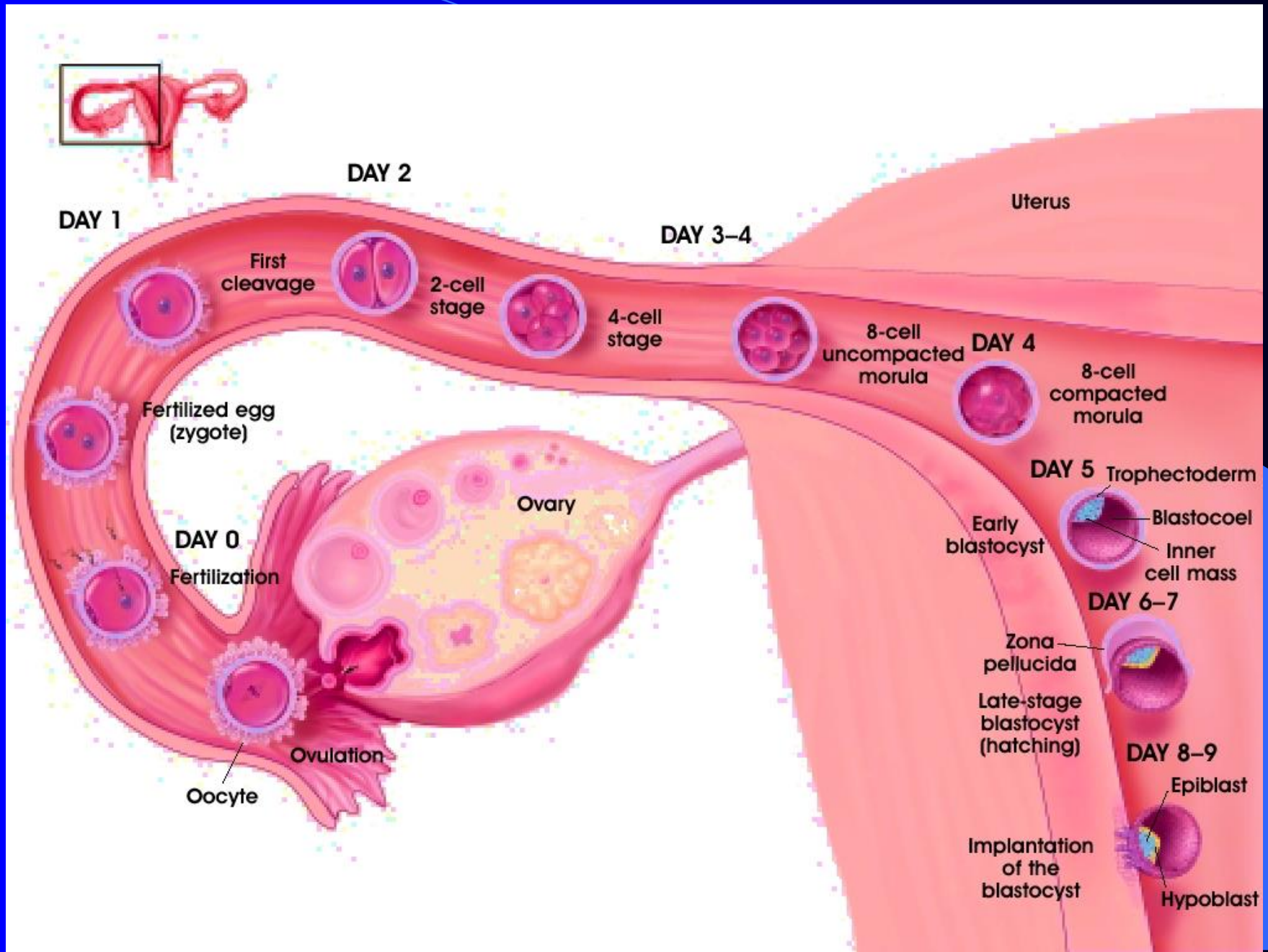


美國國家衛生研究院(NIH)對人類胚胎研究規範



Fertile grounds for debate. The NIH panel's decisions were based on timing with respect to fertilization *(above)* as well as on general ethical considerations *(below)*; as a result of the latter, "twinning" by separating blastomeres was ruled out along with some other procedures.

Human pre-implantation embryo (in oviduct)



MULLER PANEL'S GUIDELINES—IN GESTATION

FUND NOW, WITH NIH CASE-BY-CASE APPROVAL

- Research on existing, unused in vitro embryos, up to 14th day
- Limited creation of in vitro embryos for baseline data, but only for "compelling" research
- Cell extraction (blastomere biopsy) from embryos before implantation
- Derivation of cell lines from existing unused embryos
- Maturing unfertilized eggs (parthenotes) for research

NEEDING FURTHER CONSIDERATION

- Use of fetal oocytes to create embryos for research only
- Research on existing embryos beyond 14th day to neural tube closure
- Cloning by blastomere or blastocyst separation, research only
- Use of existing embryos for research when one progenitor was an anonymous gamete donor who received monetary compensation, or cannot be located to give explicit consent

NIH Muller Panel's Guidelines – in Gestation

NOT ACCEPTABLE

- Transfer of human embryos to animals for gestation
- Transfer of research embryos or parthenotes to humans
- Research on embryos beyond neural tube closure (18th day)
- Twinning (separation of blastomeres) for gestation
- Cloning of embryos by nuclear transplantation
- Creation of human-human or human-animal chimeras
- Creation of embryos strictly for research material, e.g., stem cells
- Cross species fertilization with human gametes, except clinical testing of sperm penetration (with hamster eggs)
- Transfer of embryos to cavity other than uterus
- Sex selection of embryos, except to prevent x-linked diseases
- Use of sperm, eggs, or embryos from donors who did not give explicit consent to research
- Use of sperm, eggs, or embryos for which donors received more than reasonable compensation