Eukaryotic Gene Expression: Basics & Benefits

P N RANGARAJAN

Lecture 35

Transgenic animals

Eukaryotic protein expression systems-II (lecture 31)

Protein expression in mammalian cells (non viral vectors)

Cell-free protein expression systems

Eukaryotic protein expression systems-III (lecture 32)

Protein expression in mammalian cells (viral vectors)

Human gene therapy (lecture 33)

DNA vaccines (lecture 34)

Transgenic animals (lecture 35)

Integration of foreign genes into genome of animals and their transmission to progeny (Germline gene transfer)

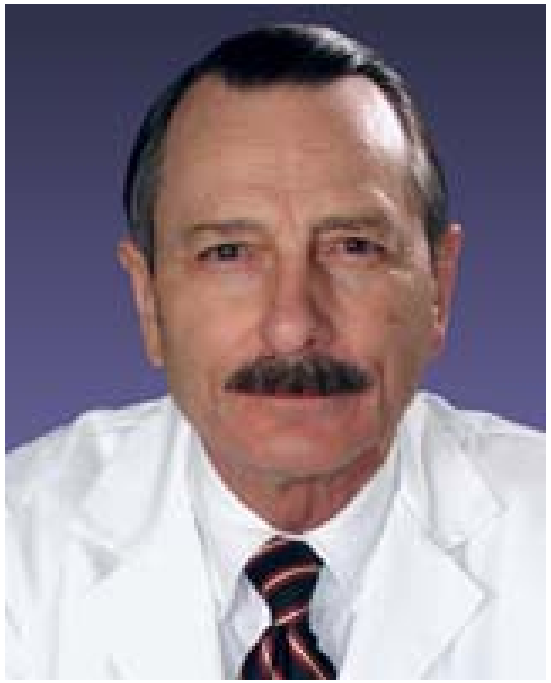
Transgenic technology

Transgenic technology led to the development of fish and livestock with an altered genetic profile that enabled them to grow faster (salmon), to reduce waste (pig) or fight diseases (prion-free cows resistant to bovine spongiform encephalopathy, known as mad cow disease).

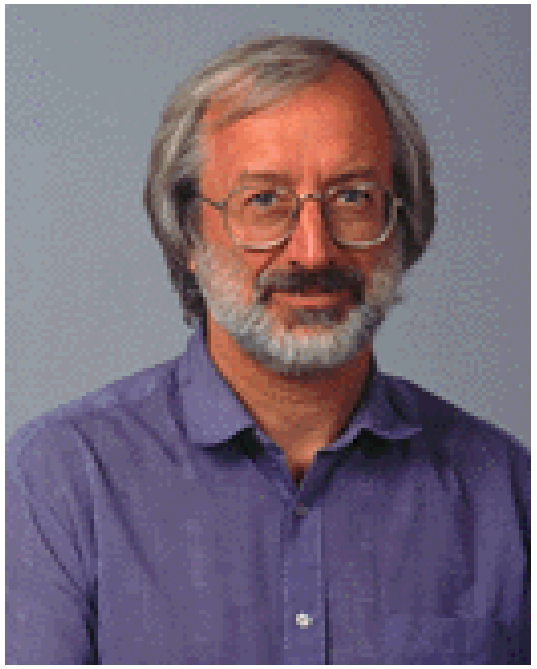
Thanks to the transgenic technology, today we have mouse models for several types of cancer and of human genetic disorders including chronic hepatitis, sickle cell disease, diabetes, Alzheimer's disease, Lou Gehrig's disease etc.

Transgenic Animal

Animal has one or more foreign genes inserted into its chromosomes



Ralph Brinster
U Pennsylvania



Richard Palmiter
University of Washington

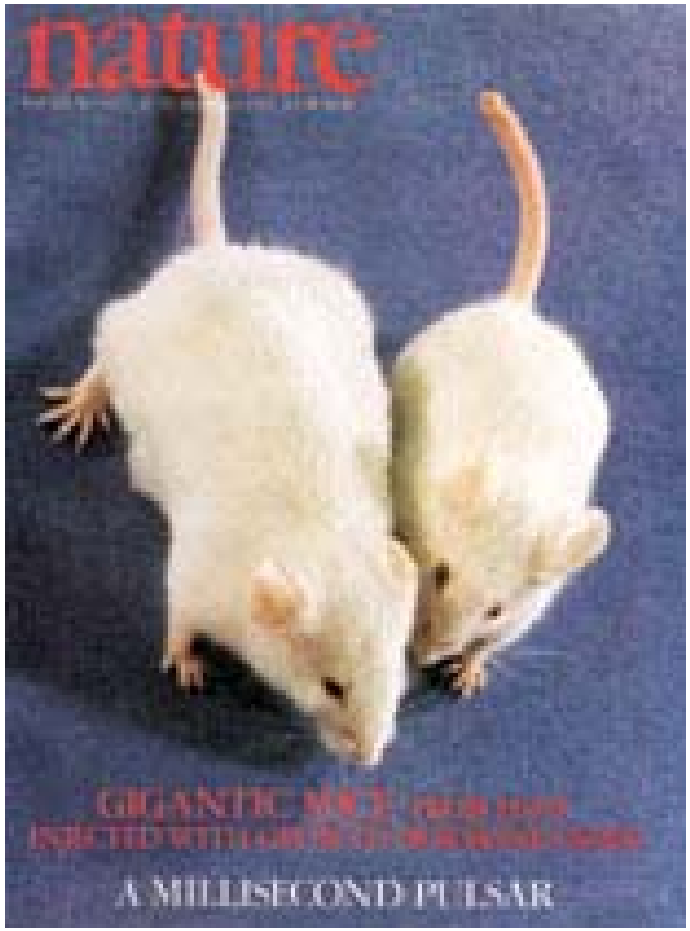
1982
First transgenic mouse with a phenotype

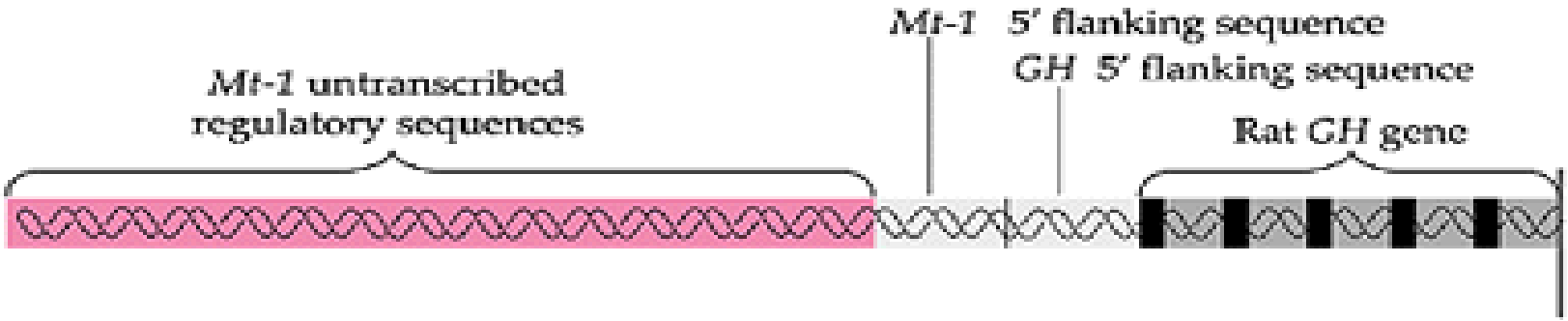
A team led by Richard Palmiter and Ralph Brinster made a construct in which the rat growth-hormone gene was placed under the control of zinc-inducible metallothionin promoter.

This construct was injected into fertilized mouse embryos and the resulting transgenic offspring, were fed with extra zinc, which turned on the metallothionin promoter.

This resulted in the expression of growth hormone gene and the resulting high levels of circulating rat growth hormone dramatically changed the phenotype of the transgenic mice by stimulating them to grow twice as large as normal.

The giant mice instilled major excitement in the scientific and public communities, markedly enhancing attention on the transgenic mouse system.




Mt-1 5′ flanking sequence
GH 5′ flanking sequence
Mt-1 untranscribed
regulatory sequences
Rat GH gene

PALMITER, R.D., BRINSTER, R.L., HAMMER, R.E., TRUMBAUER, M.E., ROSENFELD, M.G., BIRNBERG, N.C. and EVANS, R.M.

Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes.

Nature (1982) 300: 611-615.

Ralph L. Brinster and Richard Palmiter thus pioneered the development of methods to transfer foreign genes into the germline of animals, and their seminal experiments showed that new genes could be introduced into the mammalian genome.

They extended the transgenic technology to domestic livestock, thereby demonstrating the potential to enhance growth, modify resistance to disease, and produce milk containing human proteins of medical importance, such as blood clotting factors for hemophiliacs and growth hormone.

The impact of transgenesis is emphasized by the huge number of research groups and corporations that utilized the transgenic technology to study important fields of embryonic and adult physiology.

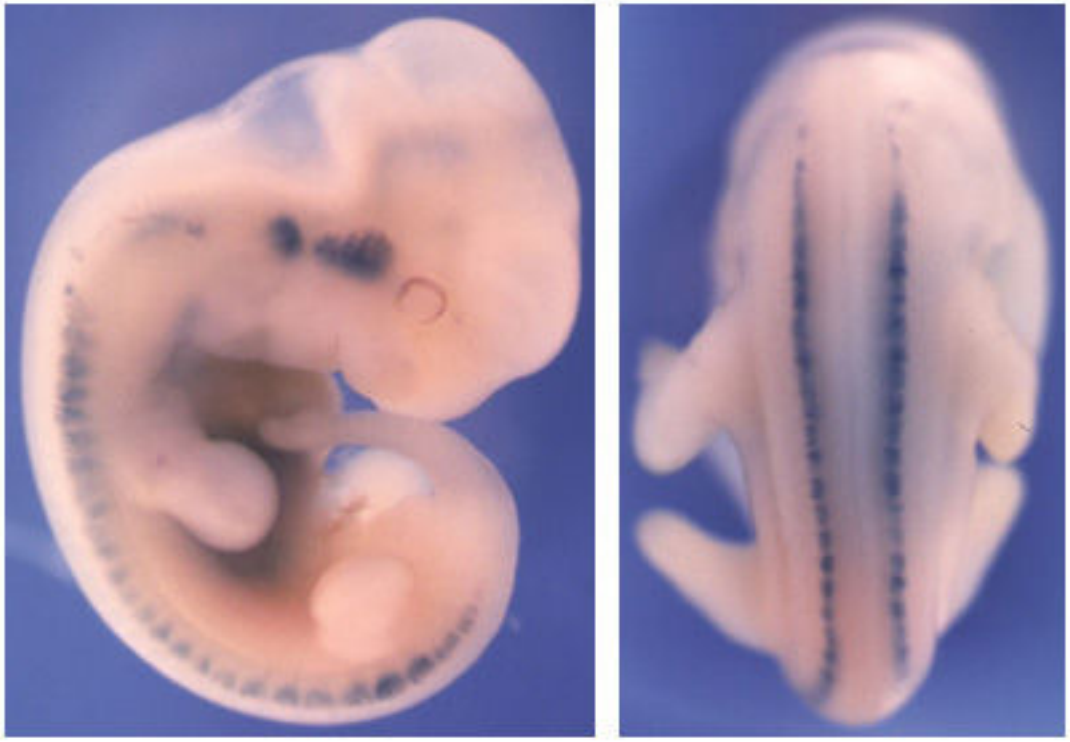
The transgenic technology also became an excellent tool in basic research for understanding the functions of a number of mammalian genes as well as their regulation.

Transgenic mice are often generated to :

1. characterize the ability of a promoter to direct tissue-specific gene expression
   - e.g. a promoter can be attached to a reporter gene such as LacZ or GFP

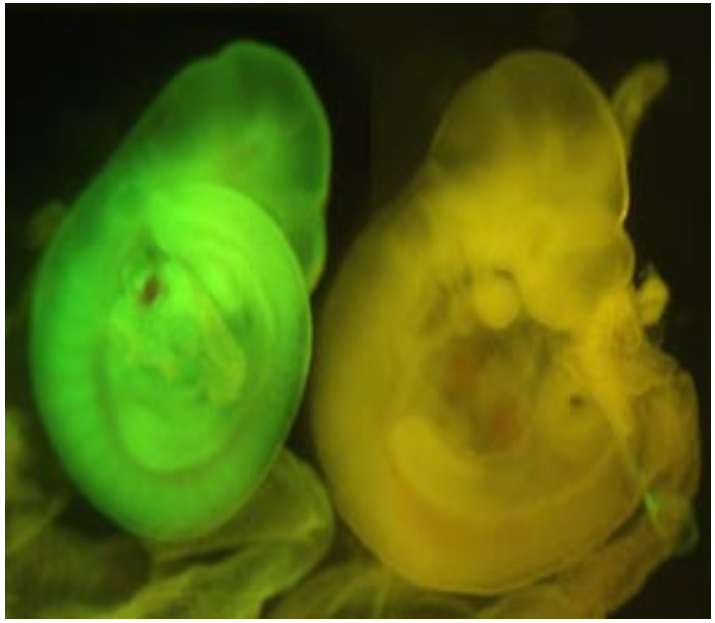
2. examine the effects of overexpressing and misexpressing endogenous or foreign genes at specific times and locations in the animals

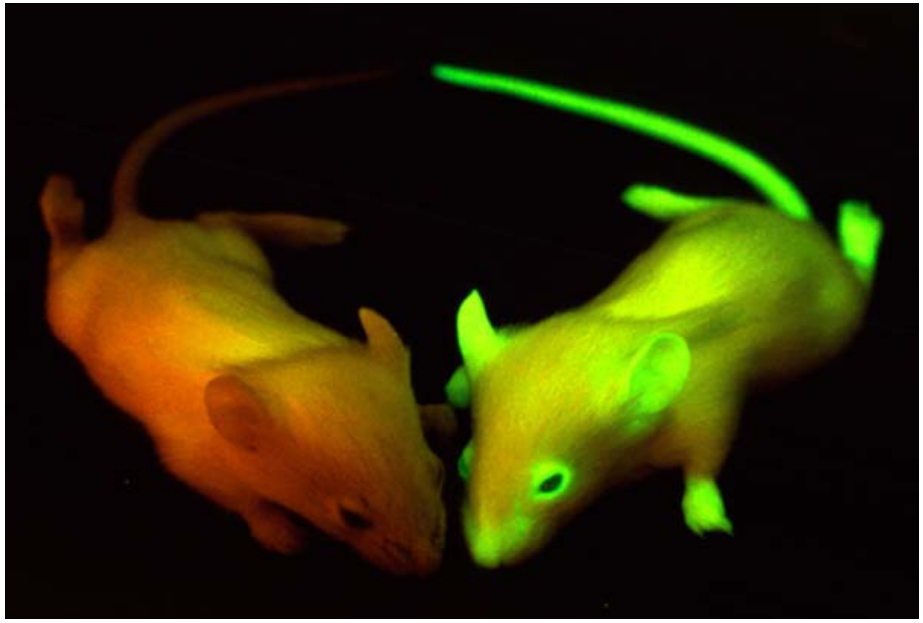
To study developmentally regulated genes



Trangenic mouse embryo expressing beta-galactosidse
under the control of neurogenin 1 promoter.

http://www.ucihs.uci.edu/anatomy/calofpix1b.html





Transgenic mice expressing GFP

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12644–12648, December 1994
Developmental Biology

Homeotic transformation of cervical vertebrae in *Hoxa-4* mutant mice

(pattern formation/skeleton)

GERALD S. B. HORAN*, KE WU†, DEBRA J. WOLGEMUTH†, AND RICHARD R. BEHRINGER*

Hoxa-4 plays a role in conferring positional information along the anteroposterior axis to specify the identity of the third and the seventh cervical vertebrae.

Okubo and Hogan made transgenic mice in which the Wnt signaling pathway was constitutively activated in the lungs of the late embryo .

In the resulting transgenic mice the alveoli of the lungs are quite abnormal, being composed of large air spaces lined with highly proliferative cuboidal epithelium.

Remarkably, this epithelium contains cells resembling differentiated types normally found in the intestine rather than the lung.

Okubo T, Hogan BLM: **Hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm.** *J Biol* 2004 , **3:**11.

Humanized Mouse To Become Basic Tool To Test Drug-Drug Interactions

July 27, 2000

A genetically engineered mouse, equipped with a human gene that senses potentially toxic substances in the body, including drugs, has been created by scientists at The Salk Institute.

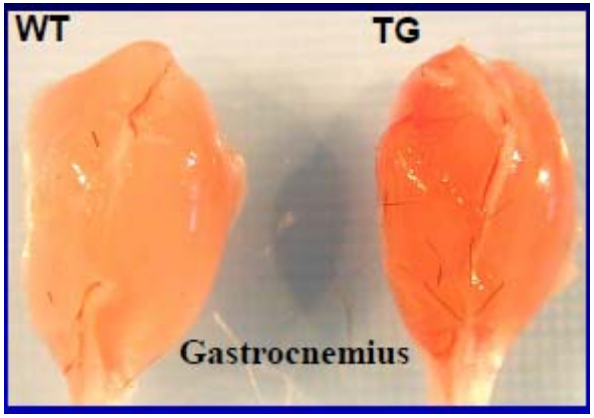
Species differences in drug metabolism and responsiveness between mice and humans mean that it is difficult to extrapolate results obtained in animal trials to humans. Therefore humanised mice for the key drug metabolising enzymes, transporters and the transcription factors that regulate them can provide new insights into the drug development process.

Xie W et al., Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature.* 2000; 406: 435–439

http://www.salk.edu/news/pressrelease_details.php?press_id=26

PPARδand the Creation of the Marathon Mouse

A strain of mice was engineered to express an activated PPARδ transgene(termed VP-PPARδ) in their skeletal muscle.



Red Muscle Increased in Transgenic Mice

http://www.wada-ama.org/Documents/Science_Medicine/Scientific%20Events/2005/WADA_Symposium_2005_2_6_Evans_Declaration.pdf

Y Wang & al, Plos Biology, 294, 2004

Mighty mice

➔ Transgenic mice with a truncated form of myostatin

➔ Increasing muscle mass and strenght

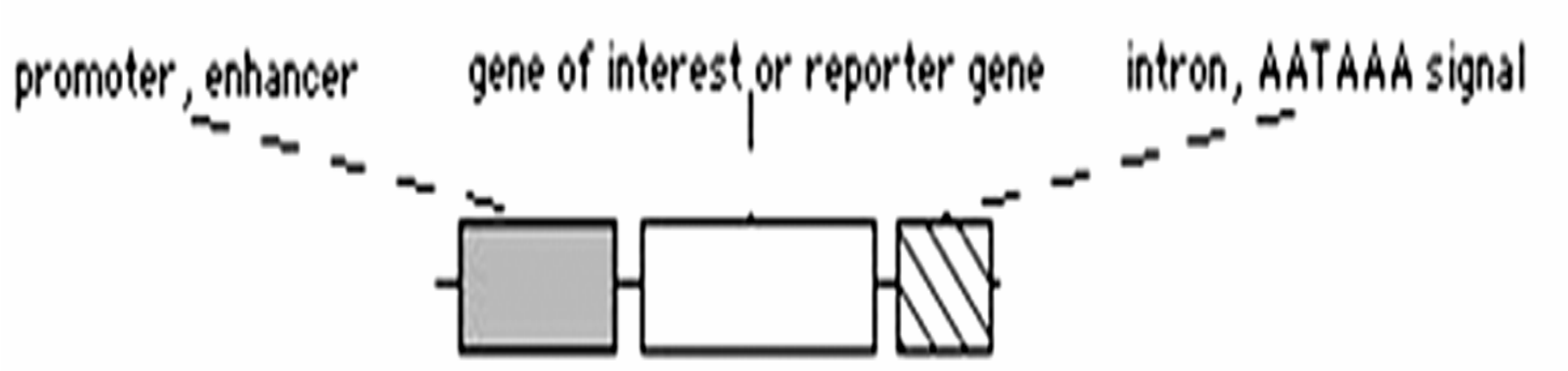
➔ Controlled animal models

➔ ST Lee & al, PNAS, 98, 2002

PRODUCTION OF TRANSGENIC ANIMALS – THE METHODOLOGY

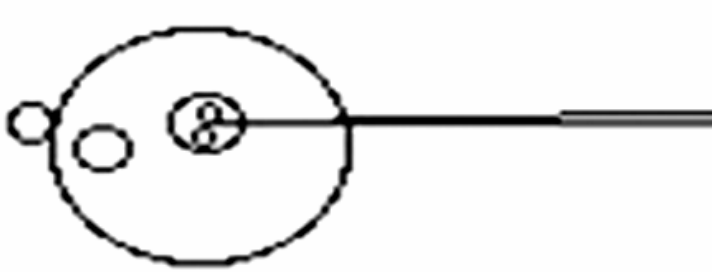
There are many ways to produce transgenic animals:

Microinjection
blastocyst injection
and using a retrovirus
Nuclear transfers
Artificial chromosomes for gene transfer


promoter, enhancer
gene of interest or reporter gene
intron, AATAAA signal

Generation of transgenic mice by microinjection

microinject purified
DNA
into male pronucleus of
fertilized mouse egg



Most eggs do not survive or do not have the transgene, but between 1% and 30% of the eggs injected can produce a live transgenic animal.

Generation of sufficient number of eggs for microinjection

A fertile male is mated with a superovulated female

Superovulated female = immature female induced to superovulate

- Pregnant mare's serum (=FSH) on day 1
- Human Chorionic Gonadotropin (=LH) on day 3

Mated on day 3

Fertilized oocytes microinjected on day 4 with foreign DNA construct.

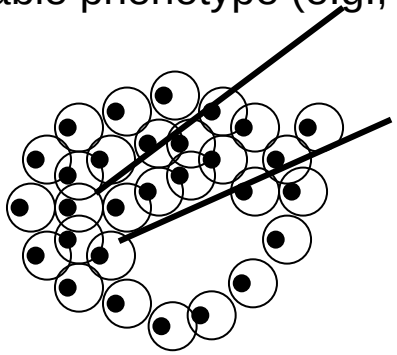
- The microinjected eggs are implanted the same day or are incubated overnight and implanted the next day
- Injected eggs are transferred to the oviduct of a 0.5 dpc pseudopregnant female

Generation of pseudopregnant females

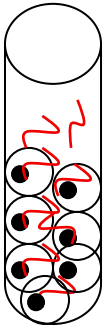
- Female mice are tricked into thinking they are pregnant
- A mouse in estrus is mated with a vasectomized male
- Pseudopregnancy
- If eggs (blastocysts) implanted will become truly pregnant and will give birth to live offspring

Generation of transgenic mice using ES cells

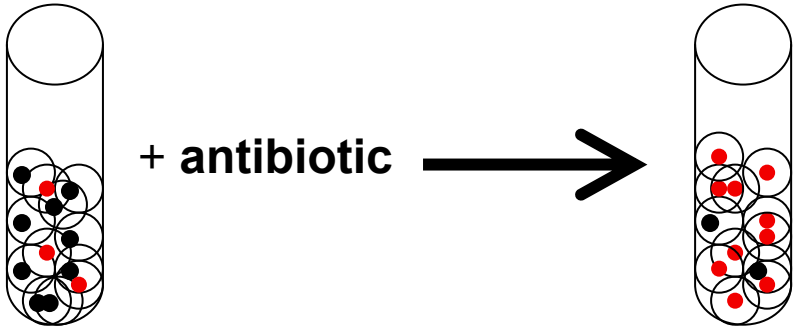
Micropipette ES cells from the inner cell mass of a blastocyst (i.e. early mouse embryo) in a strain with a physically recognizable phenotype (e.g., pigmented).



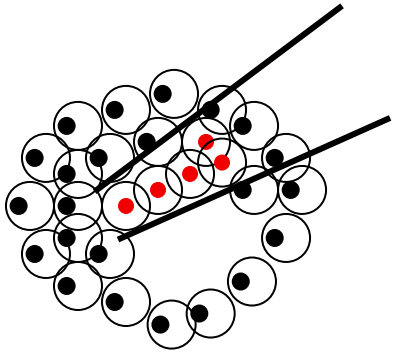
Introduce transgenic construct into ES cells by electroporation or viral vectors.



Culture the cells in presence of antibiotic. Cells which are not transgenic (black dots) will be killed, while those that have taken up the DNA and are transgenic (red dots) survice.



Insert the transgenic stem cells into the blatocyst of a mouse with a *different* genetic background trait (e.g., an albino if the original stem cells came from a pigmented mouse).



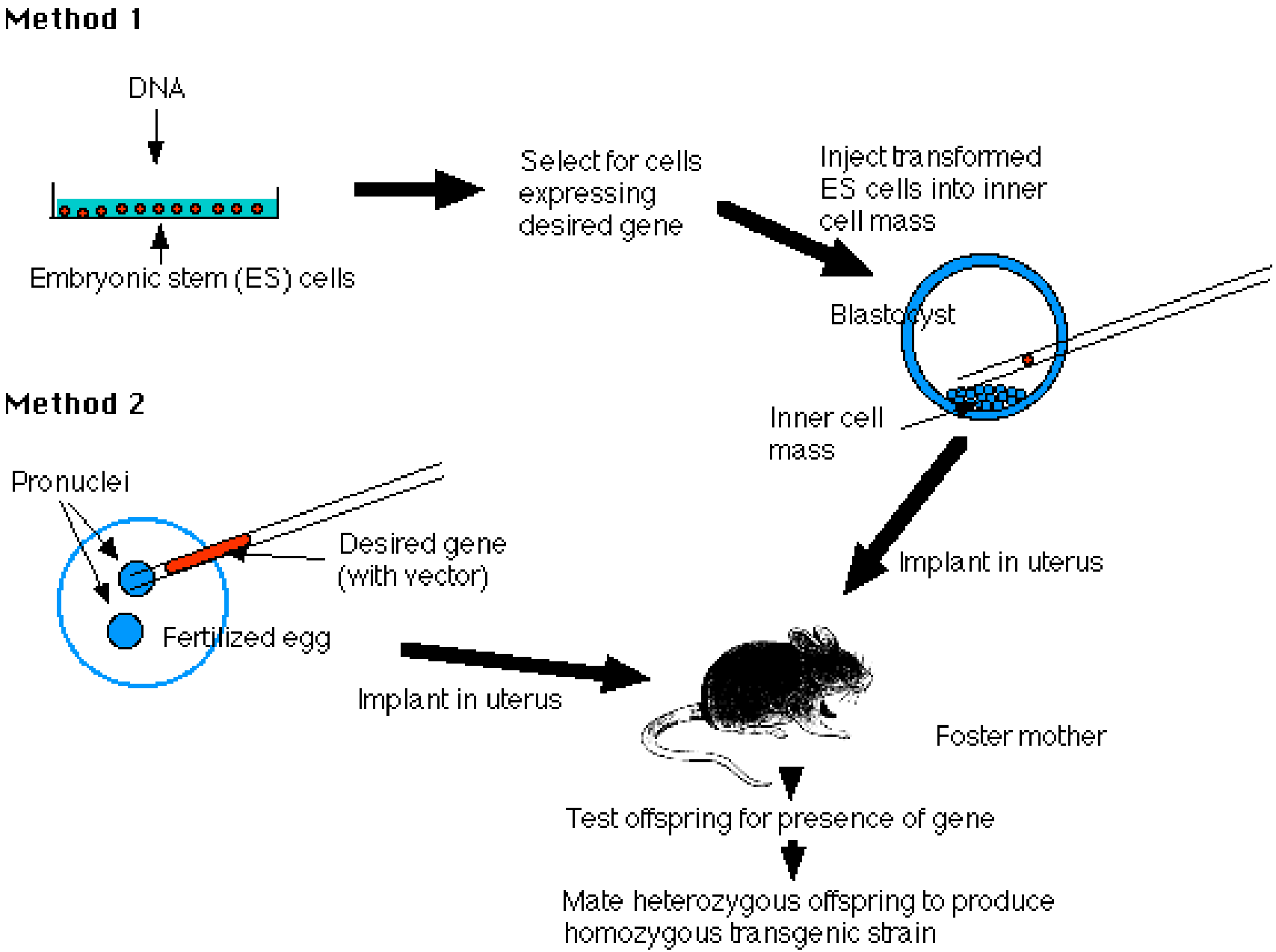
Implant the new blastocysts into a pseudopregnant female with a visible phenotype different from the blastocyst phenotype (e.g., albino if the blastocyst is pigmented).

Offspring that have pigmented sections are chimeras that have incorporated the transgenic sequence into their cell lines. Select them for further breeding.

Keep breeding the offspring of the chimeras until some fully pigmented mice are born. A fully pigmented mouse means that the transgenic germline generated one of the gametes that resulted in that mouse. Genotype the mouse to determine the genotype at the desired locus and the insertion point(s). (Most will be heterozygotes for the wild type allele and the transgenic allele).

Mate two heterozygotes and genotype their offspring. This will give all three genotypes--wild type homozygotes, heterozygotes, and transgenic homozygotes.

Compare the three genotypes on the phenotype of interest.


Method 1
DNA
Embryonic stem (ES) cells
Select for cells expressing desired gene
Inject transformed ES cells into inner cell mass
Blastocyst
Inner cell mass
Method 2
Pronuclei
Desired gene (with vector)
Fertilized egg
Implant in uterus
Implant in uterus
Foster mother
Test offspring for presence of gene
Mate heterozygous offspring to produce homozygous transgenic strain

Other Methodologies for Transgenic Animals:

Retroviral Vector Method

Sperm-mediated gene transfer

Nuclear Transfer

Yeast Artificial Chromosome Transgenesis

Sperm-mediated germline gene transfer

Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice.
Lavitrano M, Camaioni A, Fazio VM, Dolci S, Farace MG, Spadafora C. Cell. 1989 Jun 2;57(5):717-23. -

No simple solution for making transgenic mice.

Brinster RL, Sandgren EP, Behringer RR, Palmiter RD. Cell. 1989 Oct 20;59(2):239-41.

Fernandez, M A, Mani, S A, **Rangarajan P N** and Seshagiri P B. (1999) Sperm-mediated gene transfer into oocytes of Golden Hamster: assessment of sperm function. **Ind. J. Exp. Biol. 37** 1085-1092.

Problems

(a) Multiple insertions: too much protein.
(b) Insertion into an essential gene: lethality.
(c) Insertion into a gene leading to gene-silencing: no protein.
(d) Insertion in a different area can lead to differential gene regulation.

Applications for Transgenic Animals:

(I) Transgenic mice
(II) Transgenic Cattle
(III) Transgenic Goat and Pig
(IV) Transgenic Birds

Transgenic mice as tools

- Study gene function
  - Many human diseases can be modeled by introducing the same mutation into the mouse. Intact organism provides a more complete and physiologically relevant picture of a transgene's function than *in vitro* testing
- Drug testing

- Polio virus receptor

- Normal mice can't be infected with polio virus. They lack the cell-surface molecule that, in humans, serves as the receptor for the virus.

- Transgenic mice expressing the human gene for the receptor can be infected by polio virus and even develop paralysis and other pathological changes characteristic of the disease in humans

“Pharm” animals (transgenic livestock)

- Bioreactors whose cells have been engineered to synthesize marketable proteins
- DNA constructs contain desired gene and appropriate regulatory sequences (tissue-specific promoters)
- More economical than producing desired proteins in cell culture

Expressing recombinant Proteins in Animal Milk ?

Easy to purify - few other proteins in milk

Doesn’t harm transgenic animal- no change to physiology

Recombinant protein is authentically modified post-translationally

Large quantities

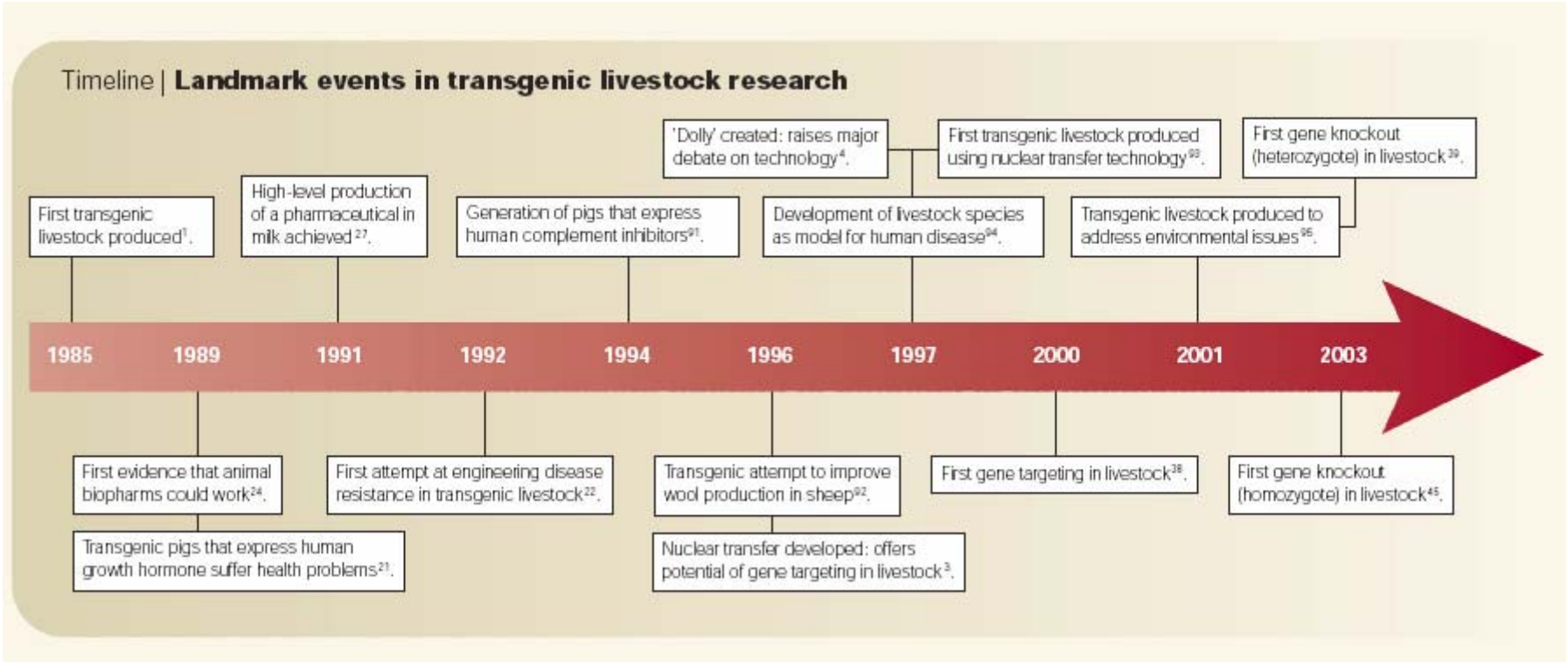
Renewable source

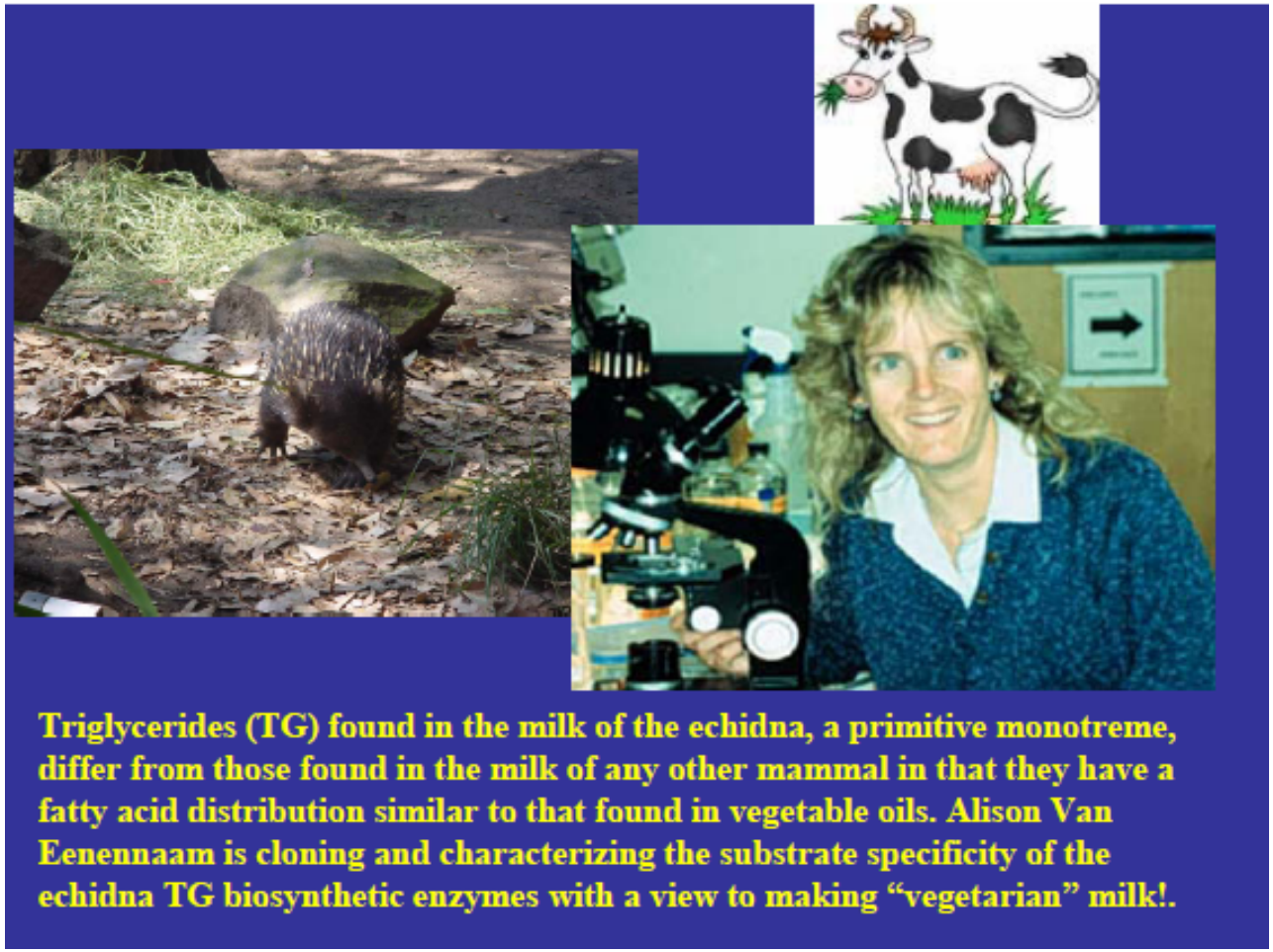
Major proteins in cattle milk

Proteins	Cattle
Casein	
α_{s1}-Casein	10.0
α_{s2}-Casein	3.4
κ-Casein	3.9
β-Casein	10.0
Major whey proteins	
α-Lactalbumin	1.0
β-Lactalbumin	3.0
Other proteins	
Serum albumin	0.4
Lysozyme	Trace
Lactoferrin	0.1
Immunoglobulins	0.7

Transgene	Promoter	Transgenic species
Longer-acting tissue plasminogen activator	Whey acidic protein	Goat
α_1-Antitrypsin	β-Lactoglobulin	Sheep
Clotting factor IX	β-Lactoglobulin	Sheep
Soluble CD4 protein	Whey acidic protein	Mouse
Lactoferrin	α_{s1}-Casein	Cattle
Urokinase	α_{s1}-Casein	Mouse
CFTR	β-Casein	Mouse
Interleukin-2	β-Casein	Rabbit

Nature 371: 209; 1994.


Timeline | Landmark events in transgenic livestock research
First transgenic livestock produced[1].
High-level production of a pharmaceutical in milk achieved[27].
Generation of pigs that express human complement inhibitors[91].
'Dolly' created: raises major debate on technology[4].
First transgenic livestock produced using nuclear transfer technology[93].
Development of livestock species as model for human disease[94].
First gene knockout (heterozygote) in livestock[39].
Transgenic livestock produced to address environmental issues[95].
1985
1989
1991
1992
1994
1996
1997
2000
2001
2003
First evidence that animal biopharms could work[24].
Transgenic pigs that express human growth hormone suffer health problems[21].
First attempt at engineering disease resistance in transgenic livestock[22].
Transgenic attempt to improve wool production in sheep[92].
Nuclear transfer developed: offers potential of gene targeting in livestock[3].
First gene targeting in livestock[38].
First gene knockout (homozygote) in livestock[45].



B. T. Kao, Edward J. DePeters, and Alison L. Van Eenennaam. 2006. Mice Raised on Milk Transgenically-Enriched with n-3 PUFA have Increased Brain Docosahexaenoic Acid. Lipids. 41(6):543-9.

B. T. Kao, K. A. Lewis, E. J. DePeters, and A. L. Van Eenennaam. 2006. Endogenous Production and Elevated Levels of Long-Chain n-3 Fatty Acids in the Milk of Transgenic Mice. Journal of Dairy Science. 89:3195-3201.27.

GTC Biotherapeutics

ATryn® - RECOMBINANT HUMAN ANTITHROMBIN

February 6, 2009 – GTC Biotherapeutics ("GTC", NASDAQ: GTCB) and OVATION Pharmaceuticals, Inc. announced today that the U.S. Food and Drug Administration (FDA) approved ATryn® (Antithrombin [Recombinant]) for the prevention of peri-operative and peri-partum thromboembolic events in hereditary antithrombin deficient patients.

ATryn®, GTC's recombinant human antithrombin, has been approved for use in the United States and Europe.

ATryn® is the first therapeutic product produced in transgenic animals to be approved anywhere in the world.

http://www.gtc-bio.com/products/atryn.html

Coagulation Factors - Factor VIIa, Factor IX and Factor VIII

GTC in collaboration with LFB Biotechnologies is developing selected recombinant plasma proteins and monoclonal antibodies using GTC's transgenic production platform.

GTC, together with its collaboration partner LFB Biotechnologies, has established the transgenic rabbit production system for its recombinant form of human coagulation factor VIIa (rhFVIIa) which is being developed for the treatment of patients with hemophilia.

GTC has established a transgenic goat production system for the production of TG20, a MAb that targets CD20.

Other companies using Transgenic Biotechnology

Revivicor

Alexion Pharmaceuticals Inc

Sygen International

BresaGen

Transgenic animals

- Human Hb from pigs
- Human lactoferrin in cows' milk
- Alpha-1-antitrypsin in sheep
- HGH in mouse urine (uroplakin promoters)
- Human antibodies in mice (H and L chain tgenics → hybridomas)
- CfTCR in goats
- Tissue plasminogen activator (TPA) in goats
- Human antithrombin III in goats
- Malaria antigens in goats (vaccine)
- Alpha-glucosidase in rabbits (Pompe's disease)

Selected publications from Brinster and Palmiter's lab

Palmiter, R. D., Chen, H. Y. and Brinster, R. L., Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. Cell 29:701-710, 1982.

Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C. and Evans, R. M., Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300:611-615, 1982.

Brinster, R. L., Chen, H. Y., Warren R., Sarthy, A. and Palmiter, R. D., Regulation of metallothionein-thymidine kinase fusion plasmids injected into mouse eggs. Nature 296:39-42, 1982.

Brinster, R. L. and Palmiter, R. D., Induction of foreign genes in animals. Trends Biochem. Sci. 7:438-440, 1982.

Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer, R. E. and Brinster, R. L., Metallothionein-human growth hormone fusion genes stimulate growth of mice. Science 222:809-814, 1983.

Swanson, L. W., Simmons, D. M., Arriza, J., Hammer, R. E., Brinster, R. L., Rosenfeld, M. G. and Evans, R. M., Novel developmental specificity in the nervous system of transgenic animals expressing growth hormone fusion genes. Nature 317:363-366, 1985.

Embryo culture, stem cells and experimental modification of the embryonic genome

An interview with Professor Ralph Brinster

JUAN ARÉCHAGA*

Int. J. Dev. Biol. 42: 861-877 (1998)

Intestine in the lung
Jonathan MW Slack

Journal of Biology 2004, **3:**10doi:10.1186/jbiol8

Maggy A. and Ciana P.: Reporter Mice in Drug Discovery and Development. Nature reviews Drug Discovery 4, 249-255 (2005).