Nobel Lecture in Physiology or Medicine

Embryonic Stem Cells: The Mouse Source – vehicle for Mammalian Genetics

Martin Evans

CARDIFF UNIVERSITY

PRIFYSGOL CAERDYDD

School of Biosciences

- In this presentation I wish to introduce mouse embryonic stem cells and to tell you
- where the ideas came from
- the story of their isolation and development
- their use as a vehicle for genetic manipulation
- some of our latest work which indicates exactly where in the early mouse embryo these embryonic stem cells come from.

Lineages of cells and stability of differentiated state

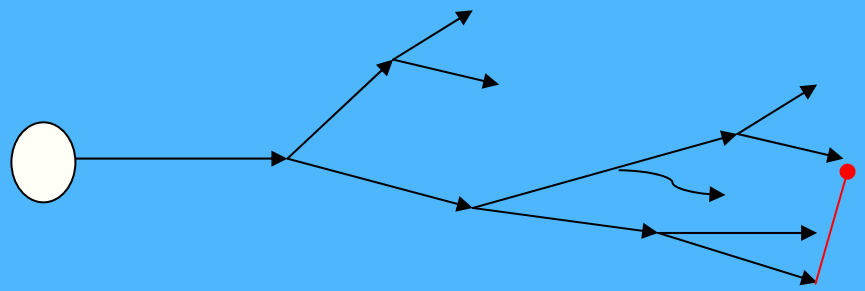
- Structure and the function of the body depends upon the autonomous but integrated action of a large number of diversely functioning specialised (that is, differentiated) cells that are organised into specific tissues (eg the cornea of the eye, skin, blood) and organs (eg liver, kidneys).

Lineages of cells and stability of differentiated state

- These cells have all developed from the single cell of a fertilised egg by cell division. This proliferation and differentiation is accompanied by progressive restriction of the potential fate of the cell's progeny.

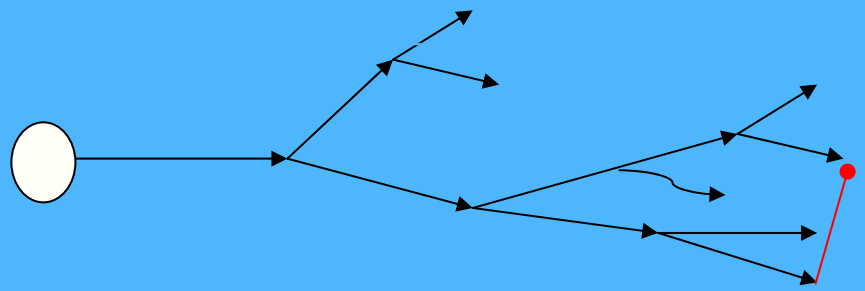
Lineages of cells and stability of differentiated state

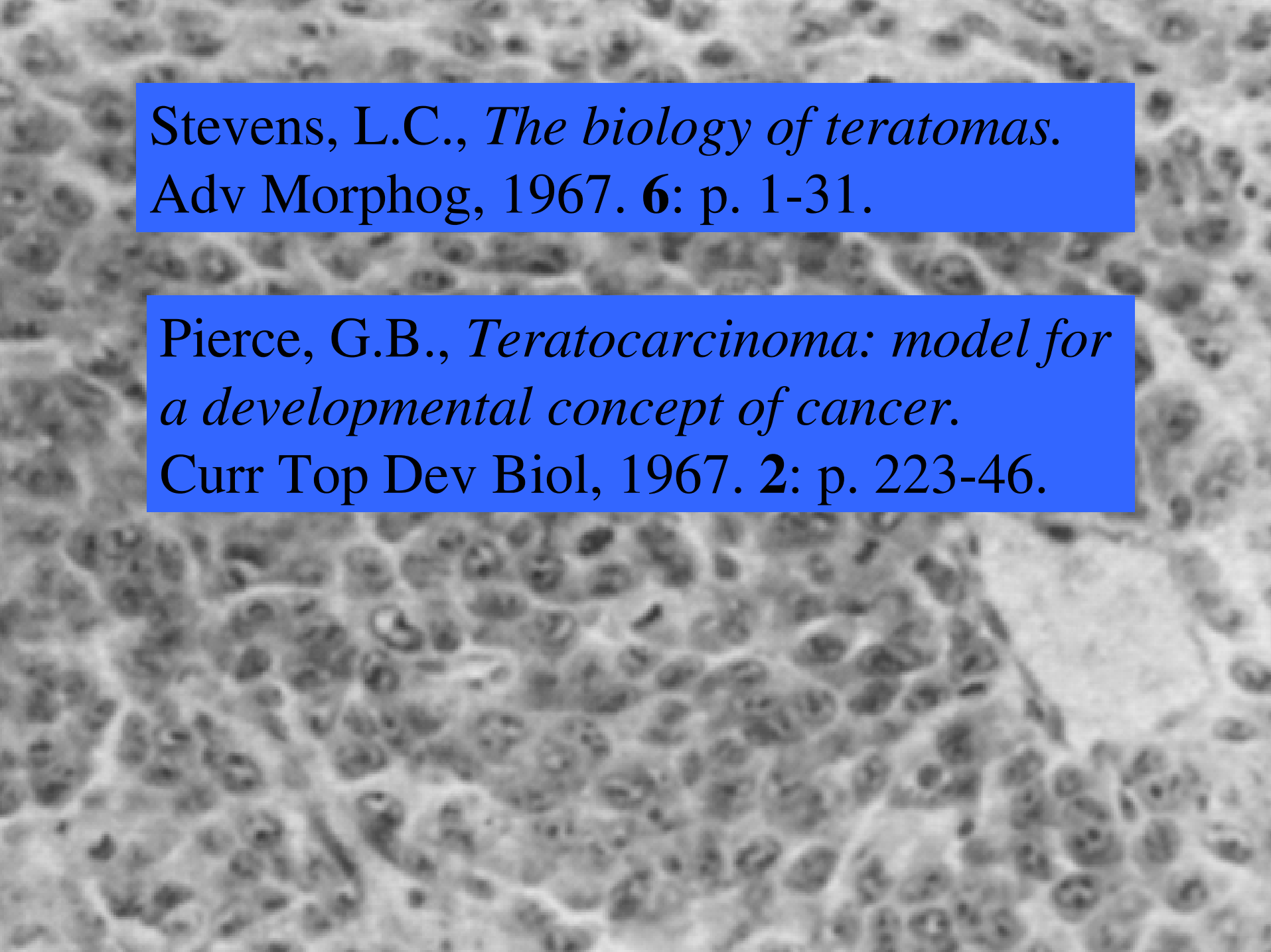
- Cells, both during development and in the adult do not, typically, change from one type to another.



Lineages of cells and stability of differentiated state

•At the very early stages of development, therefore, there must be cells from which the entire organism is derived. What is not necessarily self-evident, however, is that a replicating population of such cells may exist. Evidence for such pluripotential stem cell populations came from studies of the biology of mouse teratocarcinomas.

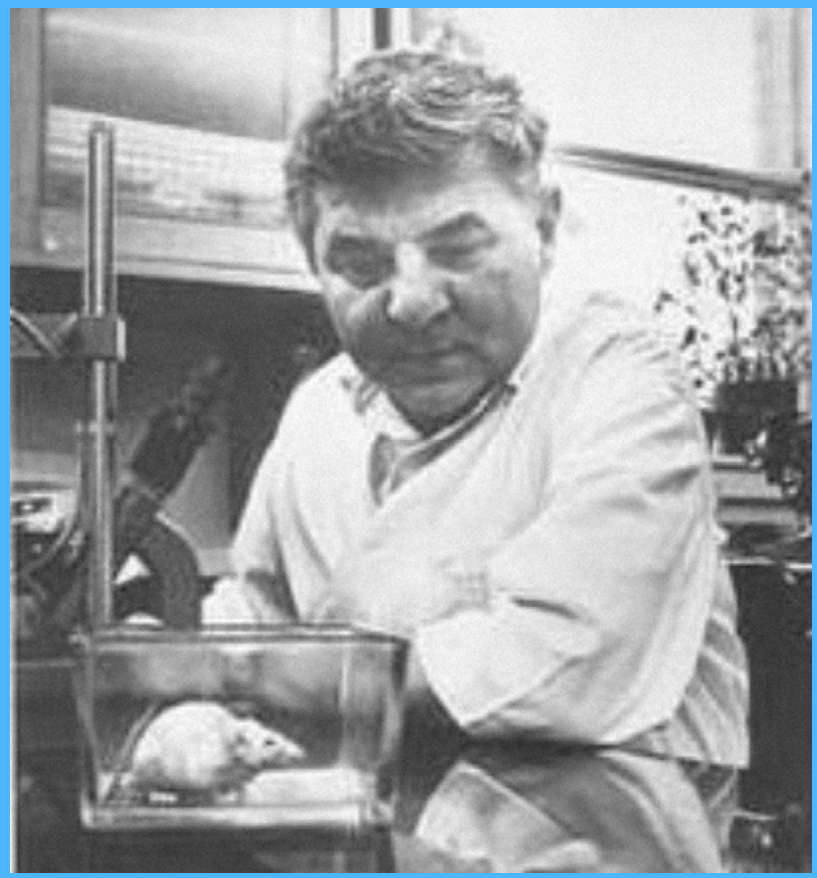




Stevens, L.C., *The biology of teratomas.* Adv Morphog, 1967. **6**: p. 1-31.

Pierce, G.B., *Teratocarcinoma: model for a developmental concept of cancer.* Curr Top Dev Biol, 1967. **2**: p. 223-46.

Testicular teratocarcinomas



Dr Leroy Stevens

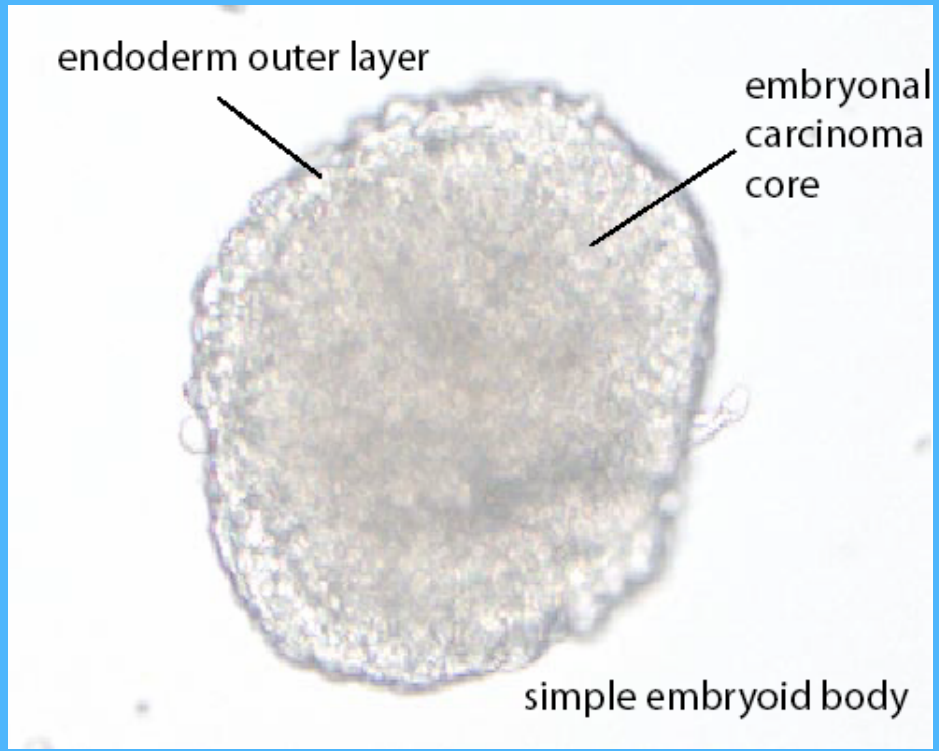
Spontaneous Testicular Teratomas in an Inbred Strain of Mice
Leroy C. Stevens, Jr. and C. C. Little
Proc Natl Acad Sci U S A. **40** 1080–1087 (1954)

- Inbred strain of mice which spontaneously develop Teratomas in testis
- These are from primordial germ cells
- also from ectopic embryos

"Following repeated serial transplantations, these tumors have retained their pleomorphic character. ***Pluripotent embryonic cells*** *appear to give rise to both rapidly differentiating cells and others which, like themselves, remain undifferentiated."*

Dr G. Barry Pierce

Kleinsmith L J and Pierce GB
MULTIPOTENTIALITY OF SINGLE EMBRYONAL CARCINOMA CELLS.
Cancer Res. 1964 Oct;24:1544-51



Two models for source of multiplicity of cell types in teratoma

a) Multiple precursor lines

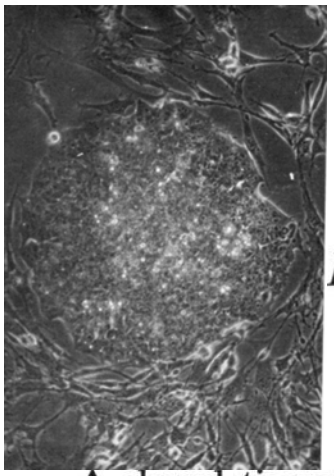
b) Single pluripotential stem cell line

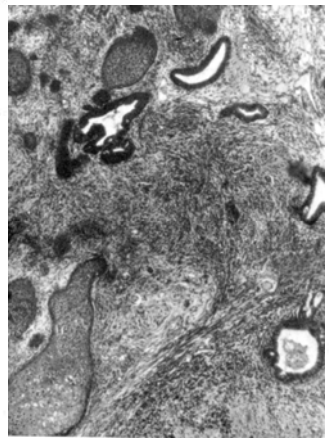
J. Embryol. exp. Morph. Vol. 28, 1, pp. 163–176, 1972
Printed in Great Britain

Clone of EC cells

The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratoma cells

Teratoma in vivo





By MARTIN J. EVANS[1]

From the Department of Anatomy and Embryology, University College London

SUMMARY

A clonal tissue culture strain of pluripotent cells has been isolated from a transplantable teratoma of inbred strain of mice 129 Sv-Sl^{j} CP. This cell strain SIKR when re-inoculated into mice produces teratomas containing at least ten types of tissue. Sub-clones have been isolated and two types distinguished.

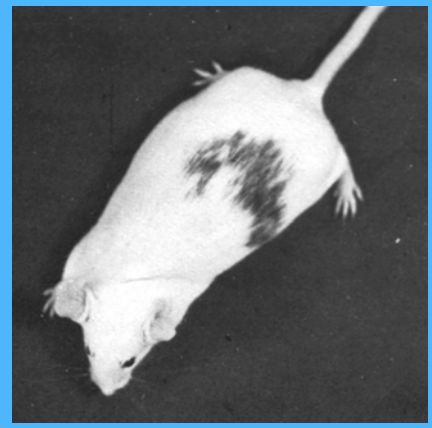
(1) 'C-type' with a densely-piled *in vitro* growth. These are tumourigenetic and pluripotent displaying a comparable range of differentiation to the original SIKR.

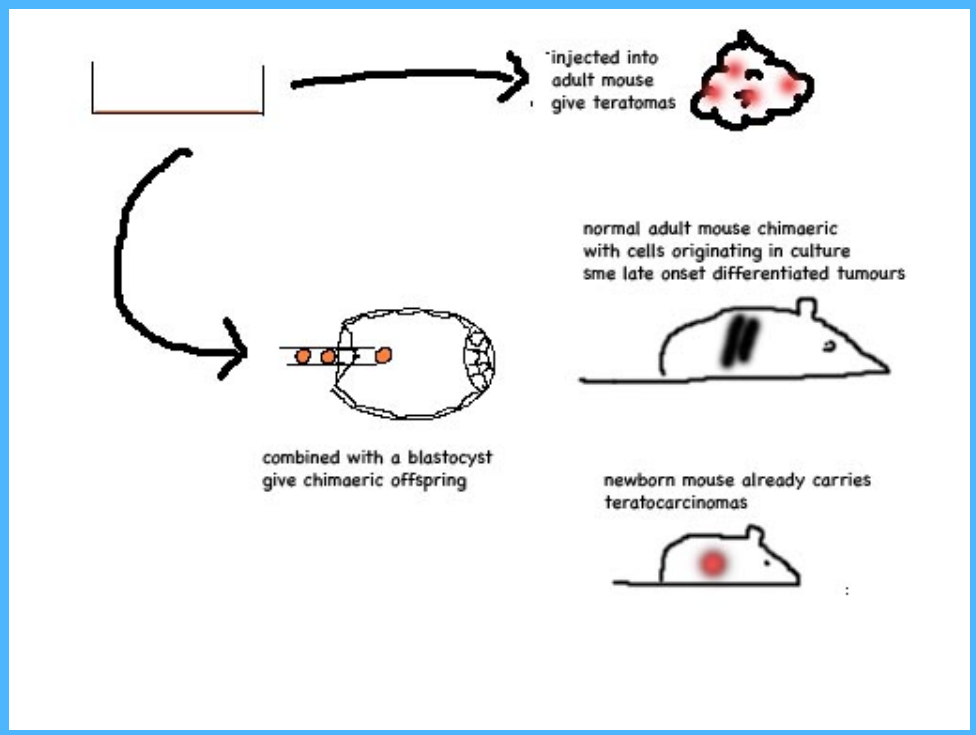
(2) 'E-type' spreading, often epithelioid growth. These grow to a lower density in culture than 'C-type'. Mostly non-tumourigenetic; in those cases where a tumour has been obtained it did not display multiple differentiations.

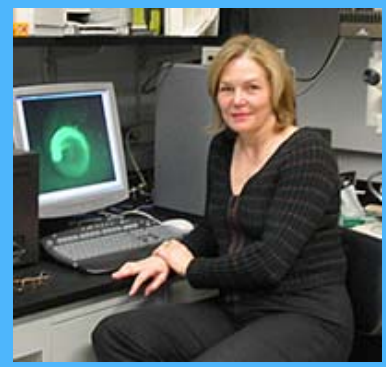
The results are interpreted as demonstrating that the culture consists of equivalently pluripotent cells which may become determined and differentiate spontaneously *in vitro* into slower growing cell types which are continuously overgrown by the culture.

Differentiation of EC cells

1) *in vivo* in tumour
2) *in vivo* in chimaeric embryo
3) *in vitro* in tissue culture





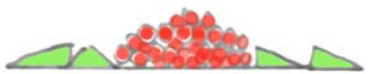


Papaioannou VE, McBurney MW, Gardner RL, Evans MJ. Fate of teratocarcinoma cells injected into early mouse embryos. Nature. 1975 258:70-73

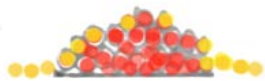
Differentiation of EC cells

1) *in vivo* in tumour
2) *in vivo* in chimaeric embryo
3) *in vitro* in tissue culture

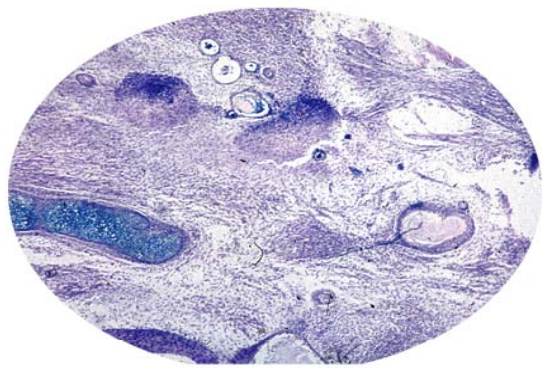
1)



Clone grows as colony on feeders

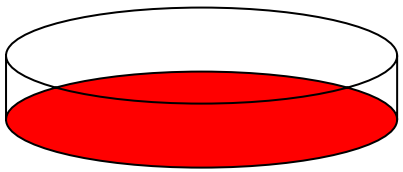


Feeders die and outer cells differentiate to embryonic endoderm

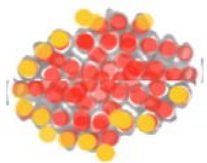


Further growth on a surface gives extensive differentiation

2)

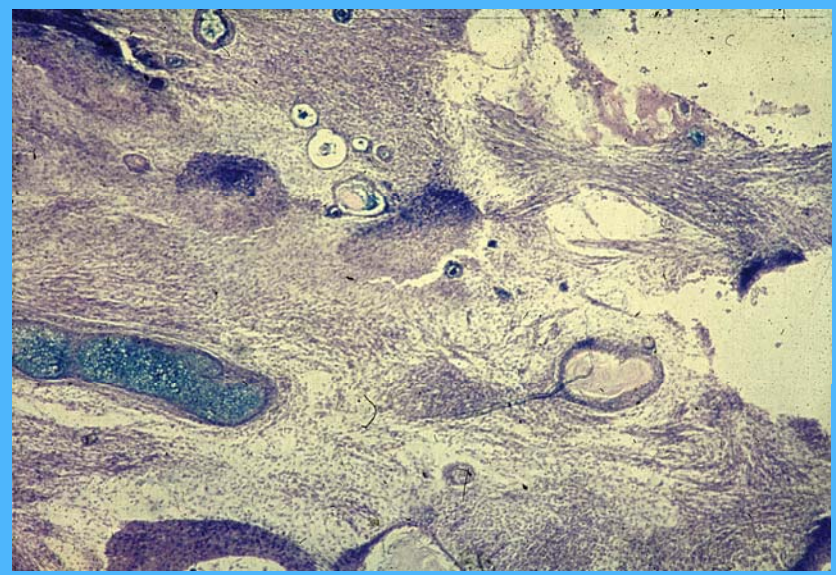


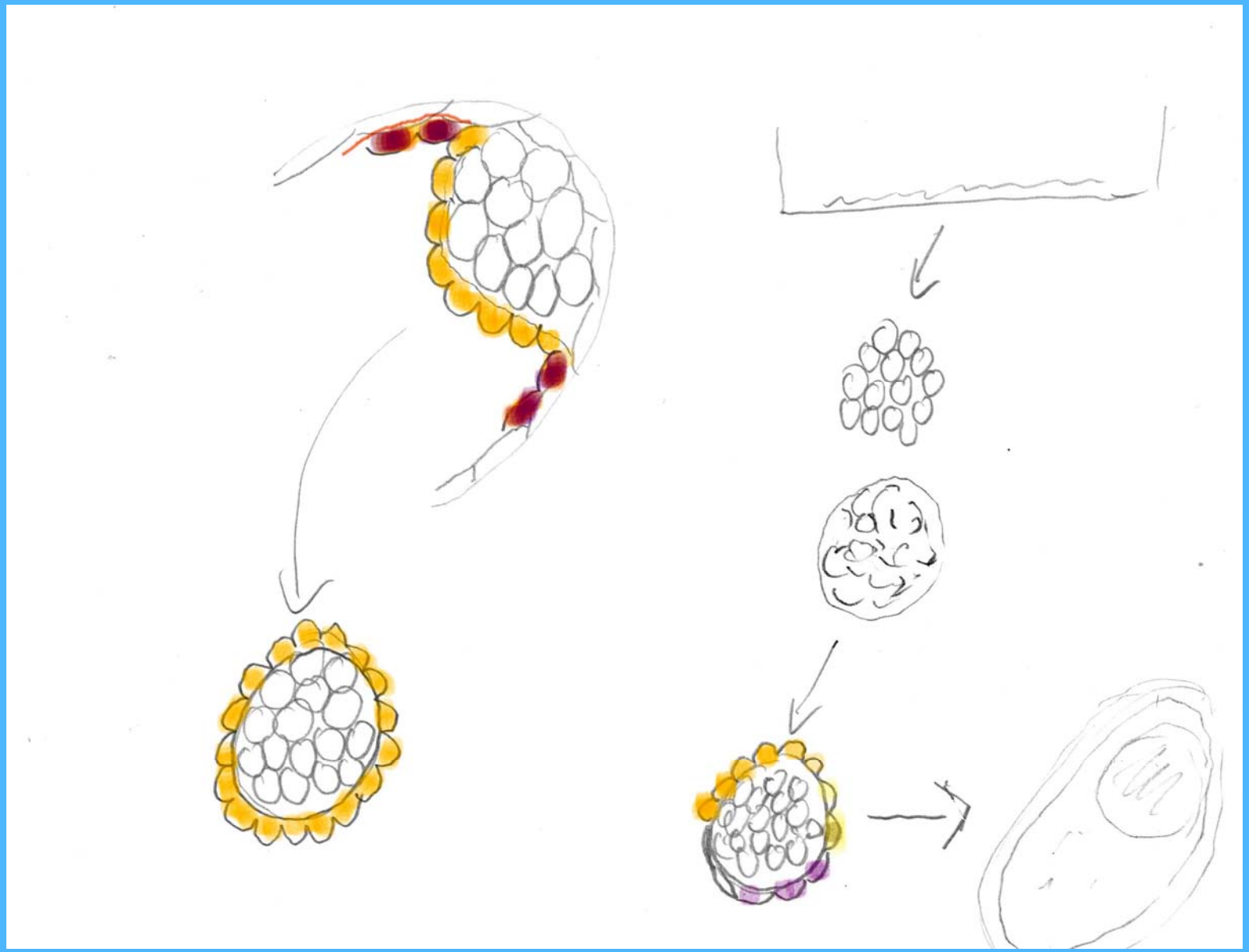
Mass culture allowed to overgrow

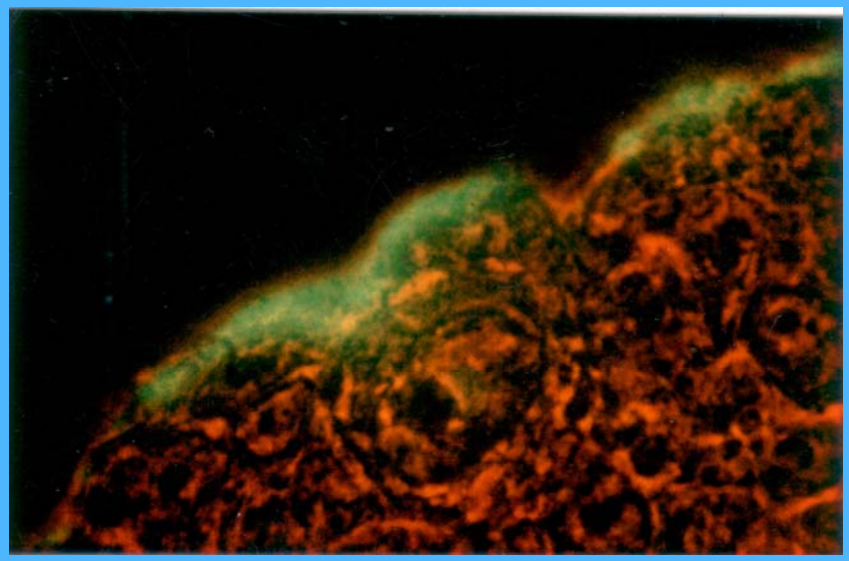


Clumps float off and form endoderm on outer surface -- Embryoid Body

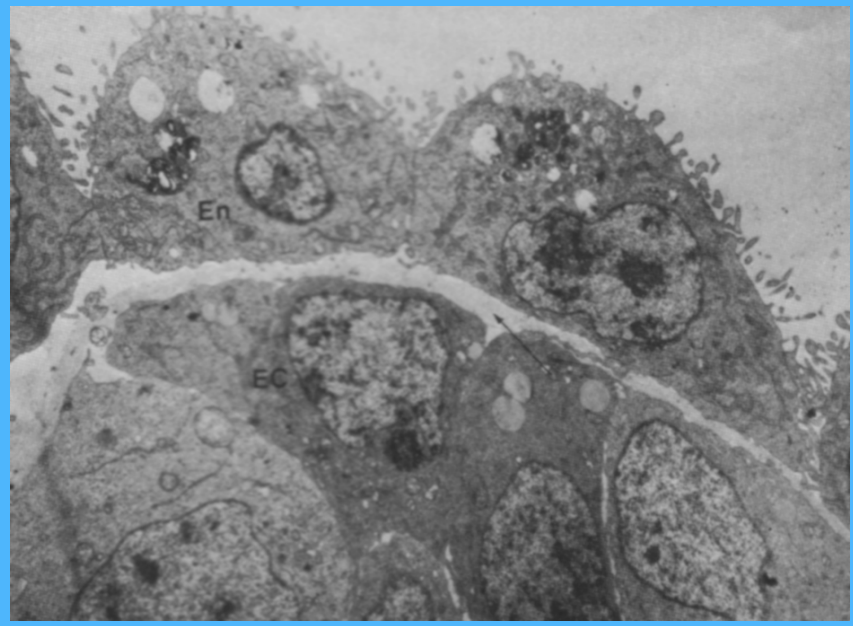
- One of the conceptual breakthroughs on the road to ES cells was the realisation that their differentiation was not abnormal, disorganised, random or stochastic but followed the normal pathways of early embryonic development.



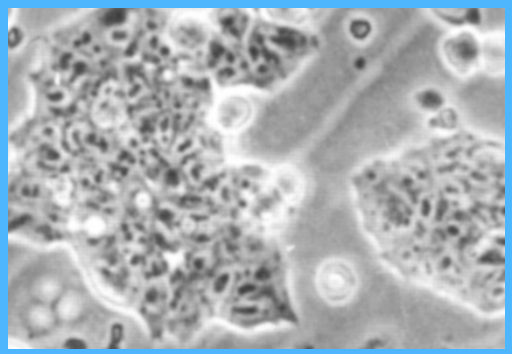




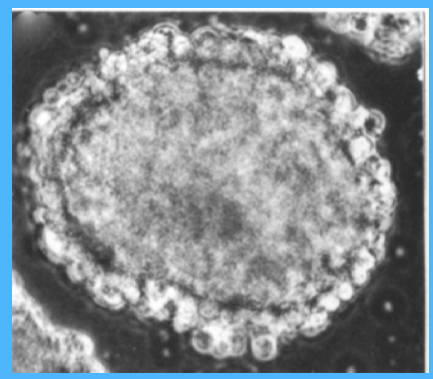
Embryoid body stained for alphafoetoprotein (green) in some of the endoderm cells



Electron Microscope section of edge of embryoid body



Embryonal Carcinoma cells in culture



Embryoid body

Origin of mouse embryonal carcinoma cells and the possibility of their direct isolation into tissue culture

Martin Evans *J. Reprod. Fert.* (1981) **62**, 625–631

- In this review I presented the evidence that EC cells should be able to be isolated into tissue culture directly from normal early embryos.

- I surmised that maybe there were three explanations for failure up until now:

  - **NUMBER** The number of pluripotential cells in the embryo at any one time may be very low; sufficient in vivo but insufficient in vitro where there is greater cell mortality.
  - **TIME** There may be a short time window - in vivo this is extended by growth of the embryo up to this point or regression of some of the cells of a later embryo following damage of transplantation.
  - **TOO GOOD!** EC cells which differentiate readily are more difficult to maintain in tissue culture than those which are more culture adapted and differentiate less well. "..the genuine embryonic cell counterpart may differentiate and lose its pluripotency and rapid growth characteristics all too readily under culture conditions. .."

Matt Kaufman

- Haploid (parthenogenetic) embryos grown to egg cylinder
- I could grow cell lines from ICM's -e.g. ICME
- Had refined media in particular in growing human teratocarcinoma cells
- Genetic opportunity ! Haploid cells in culture

Isolation of Embryonic Stem Cells

Notebook page June-July 1980

"Giant blastocysts from 129 mice put into delay by ovariectomy and depo provera"

Embryo Cells

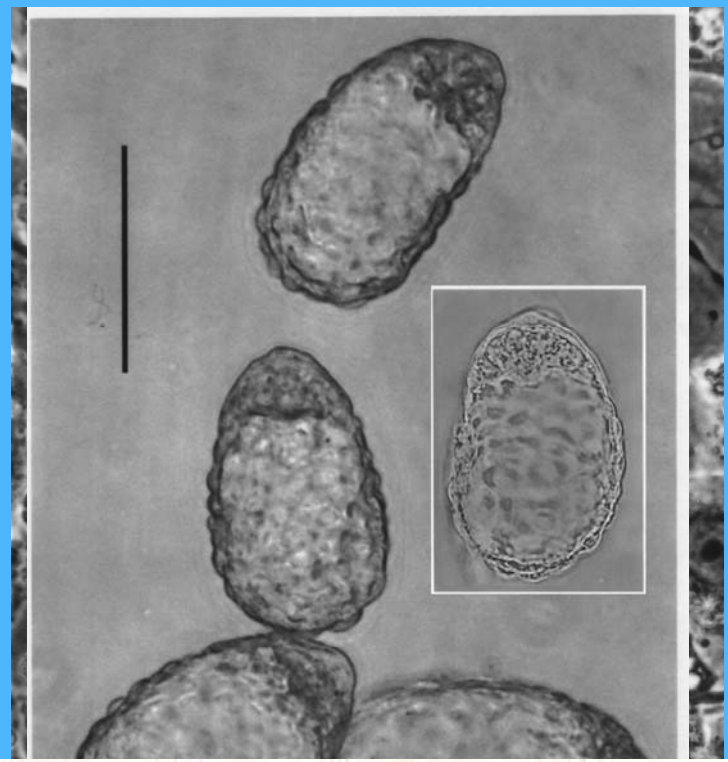
Giant blastocysts formed from 129 mice put into delay by ovarectomy + depot provera.

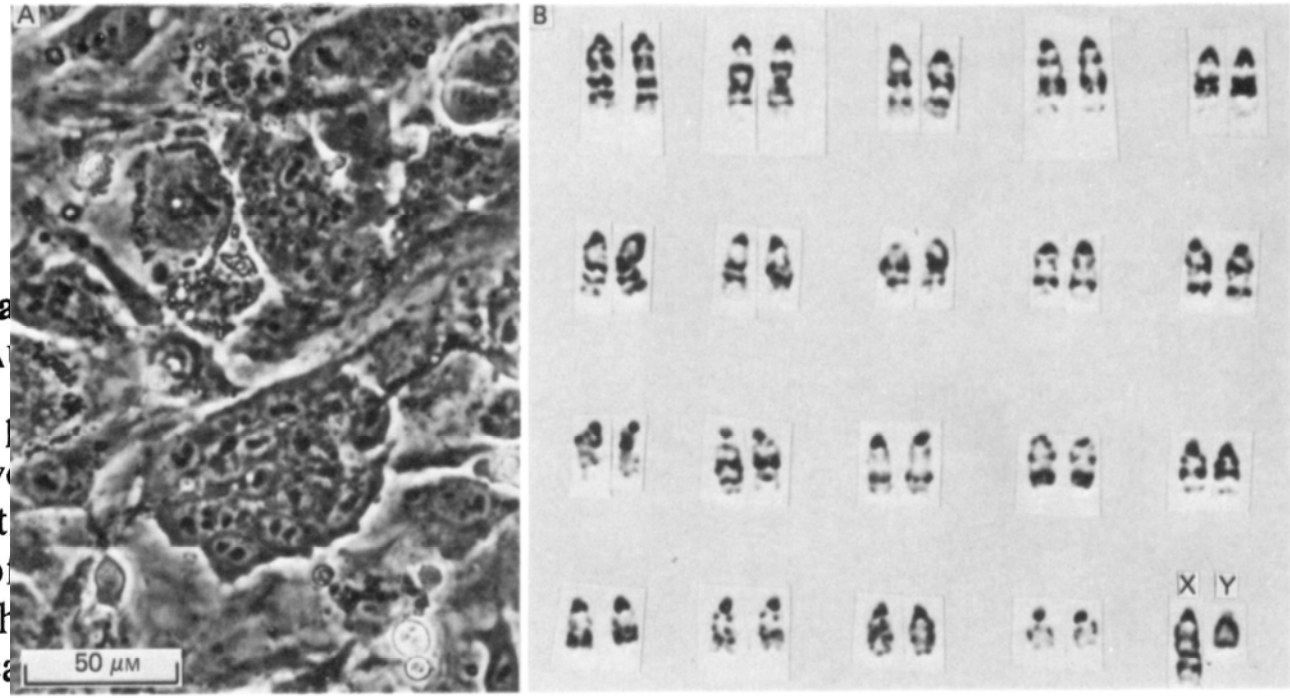
3 groups used A
B
C - 1.7.80

All passaged onto G STOM in MMM* after they had been allowed to attach & start to outgrow in above.

The time of psg for C was just when the ICM's became extended but before end. A & B slightly later.

A looked promising from the first with obvious embryo lumps. 4th passage grew up a number of good EC like colonies. These were all picked into micropipette and piped to 5th on G STOM on 9/7/80 11/7 - many more colonies but were less well attached - fed / 12/7 Psged to 4 x 6 cm feeder plates.



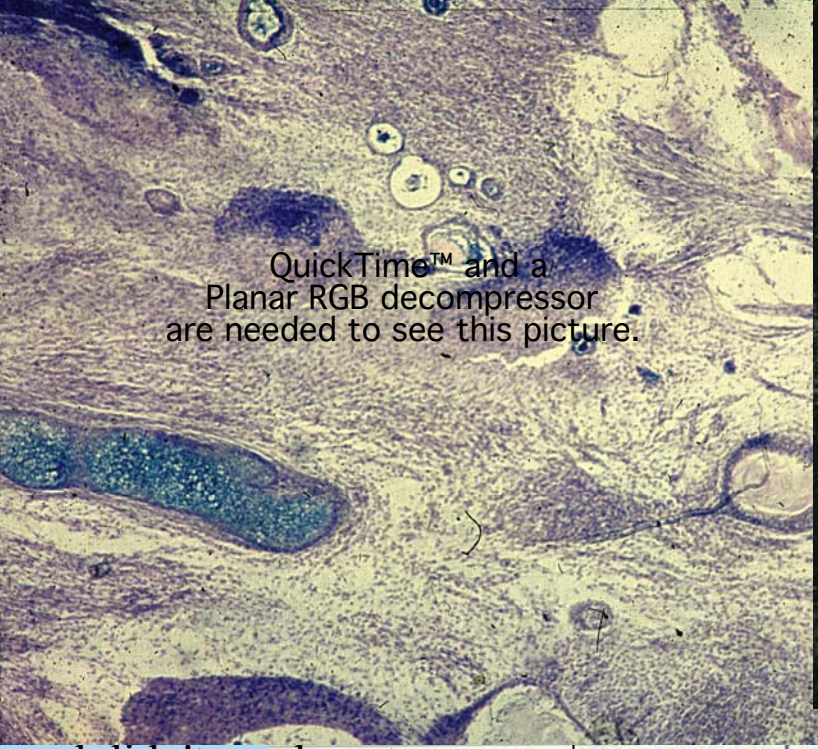


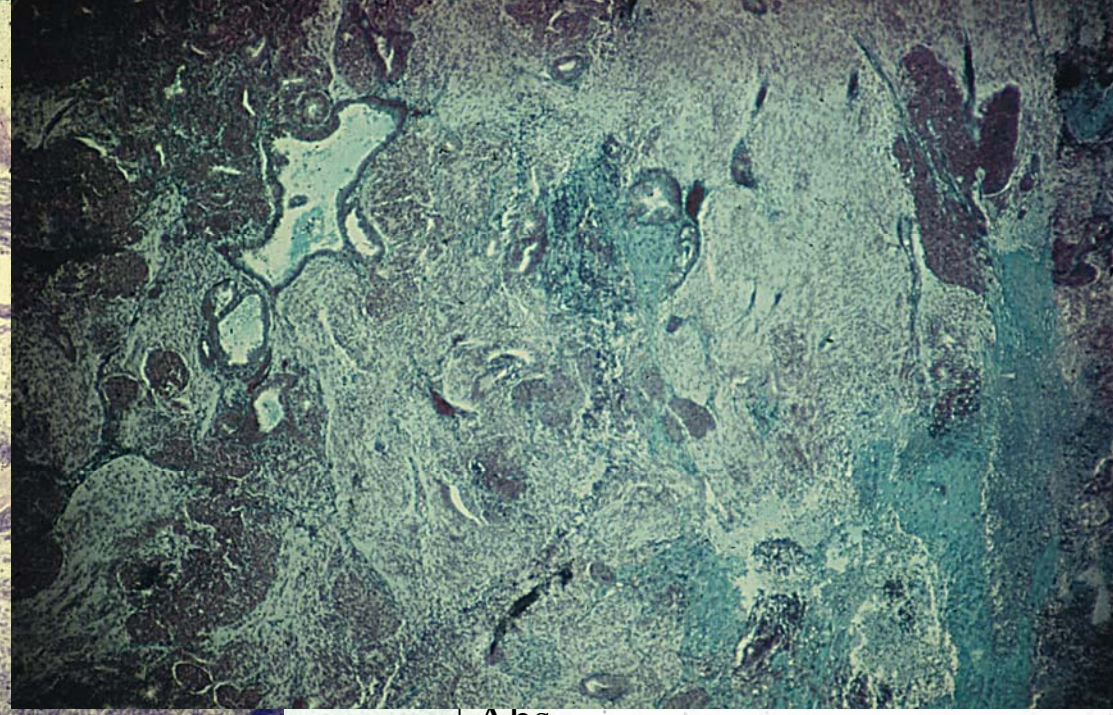
3. Isolation a J. EVANS and
M. H. KA *ridge* (Fig. 2)

Although from mouse
embryos hav the stage of
isolation of t pression and
the pattern o st that neither
the cells of th omology with
embryonal ca n embryo at
5–5½ days po e epiblast in
experimental just prior to
implantation.

Mice of th day of preg-
nancy and ' ransferred to
dishes contai EM medium
supplemented cultures were
examined dai vely prolifer-
ating colonies from an early
stage (Fig. 2A

Cell culture ome analysis
has revealed XX and XY
(Fig. 2B) cell has induced
the formation oluripotential
cells are calle





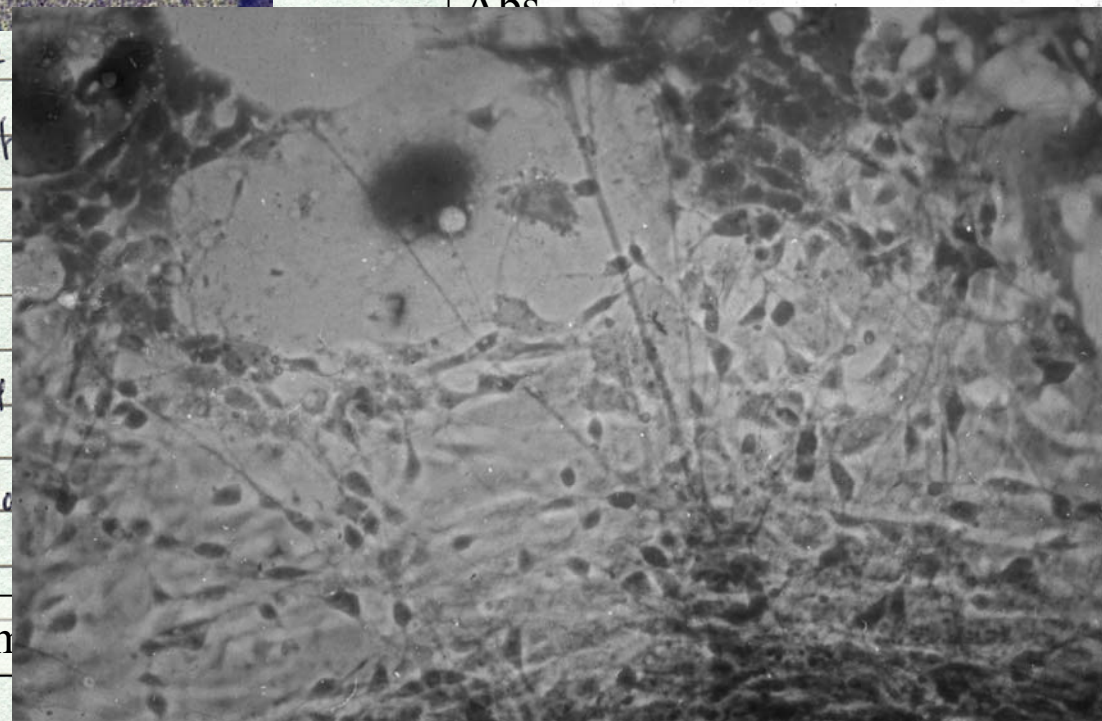
and didn't produce tumours.

- Single
- In vit

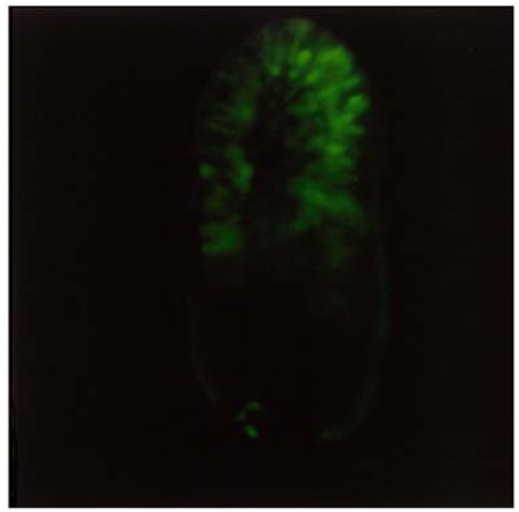
As the B series
spontaneously - th
B_1, B_2, B_3, B_4

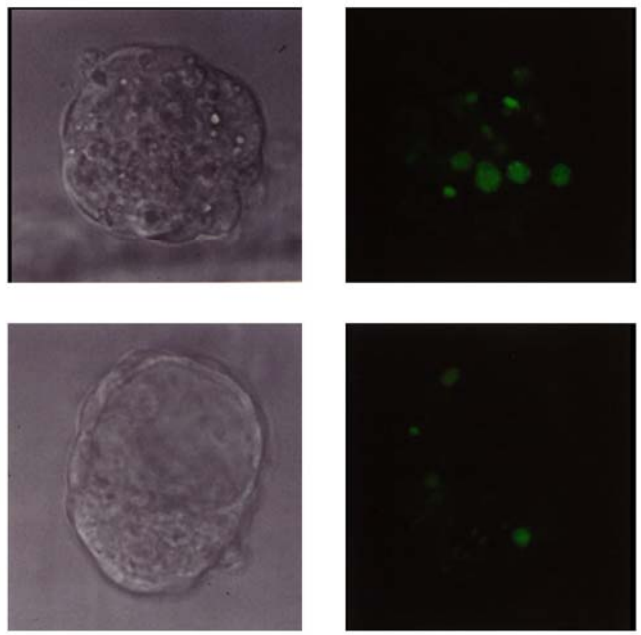
I could inject B_1 & B_7

I still have som

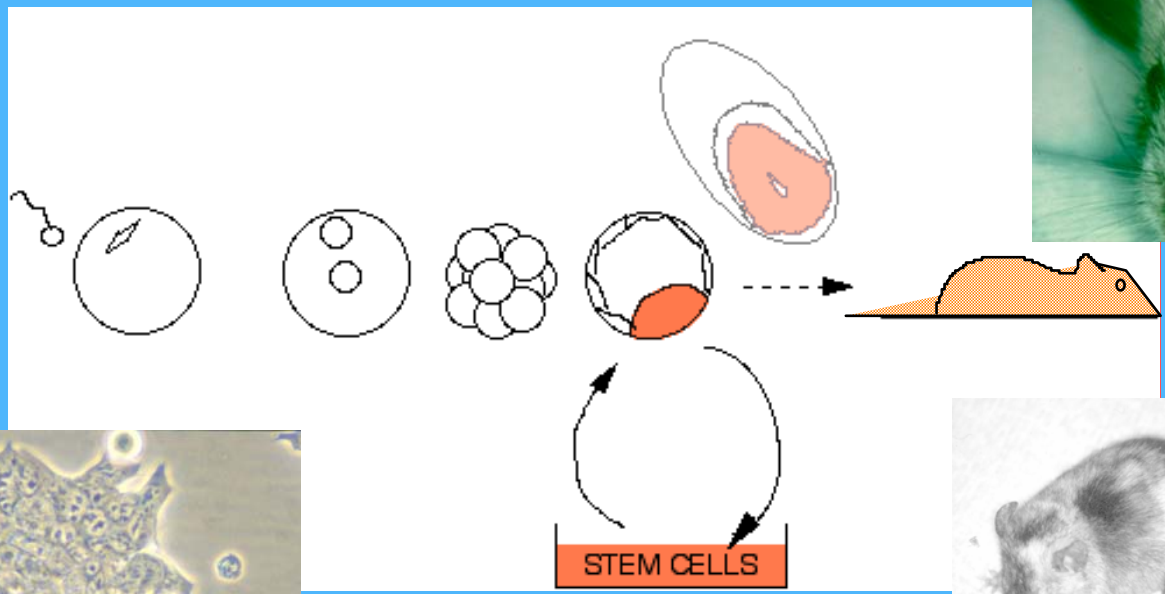


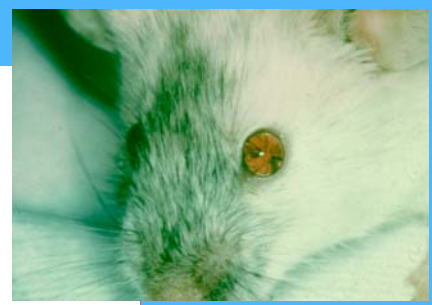
ES Cells expressing a green fluorescent marker (GFP) when inserted into a blastocyst are traced to the Embryonic Epiblast. Showing that ES cells can become embryo cells.

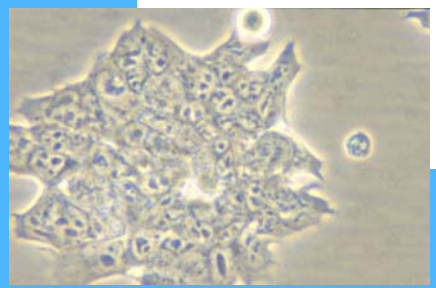




Experimental Mammalian Genetics
ES cells are a vector to the whole animal genome







Experimental Mammalian Genetics ES cells are a vector to the whole animal genome

- Test function of gene
- Illuminate understanding of genetic disease process
- Allow experimental approaches to therapy

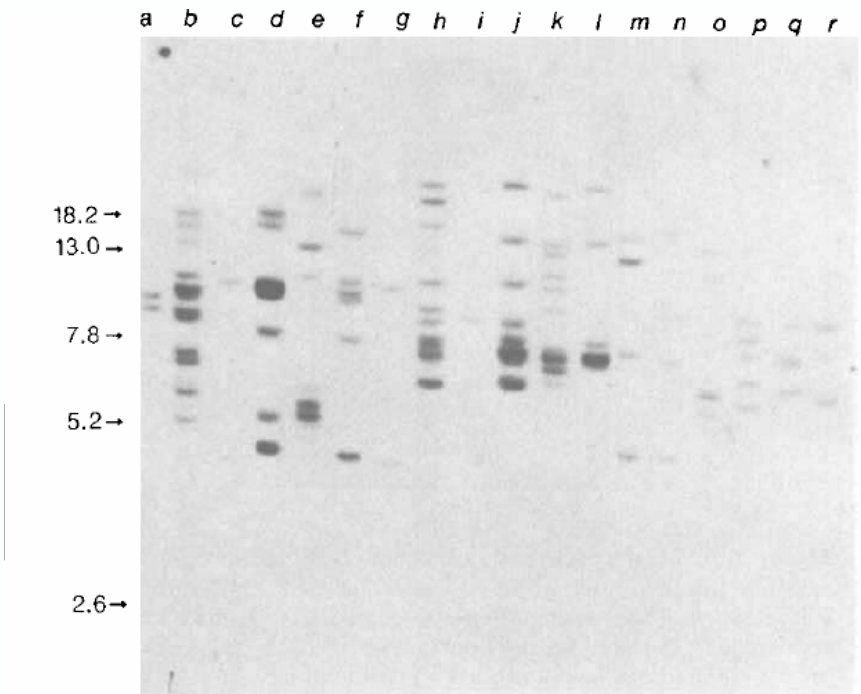
- Mutate, Trap, Target, Manipulate

1: Nature. 1986 Oct 2-8;323(6087):445-8.

Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector.

Robertson E, Bradley A, Kuehn M, Evans M.

Embryonic stem cells isolated directly from mouse embryos can be cultured for long periods in vitro and subsequently repopulate the germ line in chimaeric mice. During the culture period these embryonic cells are accessible for experimental genetic manipulation. Here we report the use of retroviral vectors to introduce exogenous DNA sequences into a stem-cell line and show that these modified cells contribute extensively to the somatic and germ-cell lineages in chimaeric mice. Compared with current methods for manipulation of the mouse genome, this approach has the advantage that powerful somatic-cell genetic techniques can be used to modify and to select cells with germ-line potential, allowing the derivation of transgenic strains with pre-determined genetic changes. We have by this means inserted many proviral vector sequences that provide new chromosomal molecular markers for linkage studies in the mouse and that also may cause insertional mutations.



Embryonic lethal

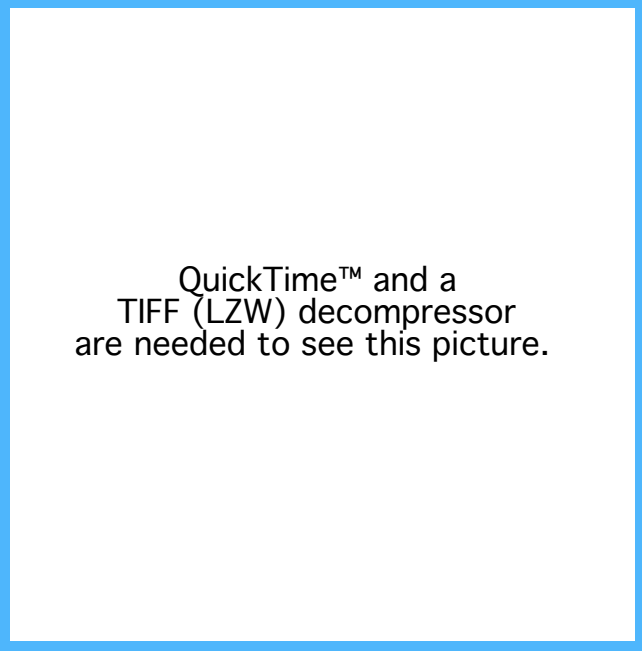
Table 1. *Identification of a homozygous lethal mutation in pedigrees derived from male 413*

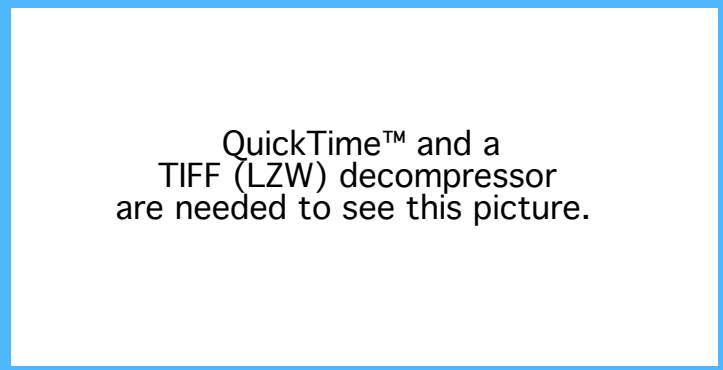
Proviral band tested	Number of progeny genotyped	Wild type	Heterozygous	Homozygous
413.a	27	6 (22%)	12 (45%)	9 (33%)
413.b	42	12 (28.5%)	18 (43%)	12 (28.5%)
413.c	44	7 (16%)	21 (48%)	16 (36%)
413.d	79	26 (33%)	53 (67%)	–

A total of 106 F_2 progeny were genotyped. F_1 parents shared 1 or 2 bands.

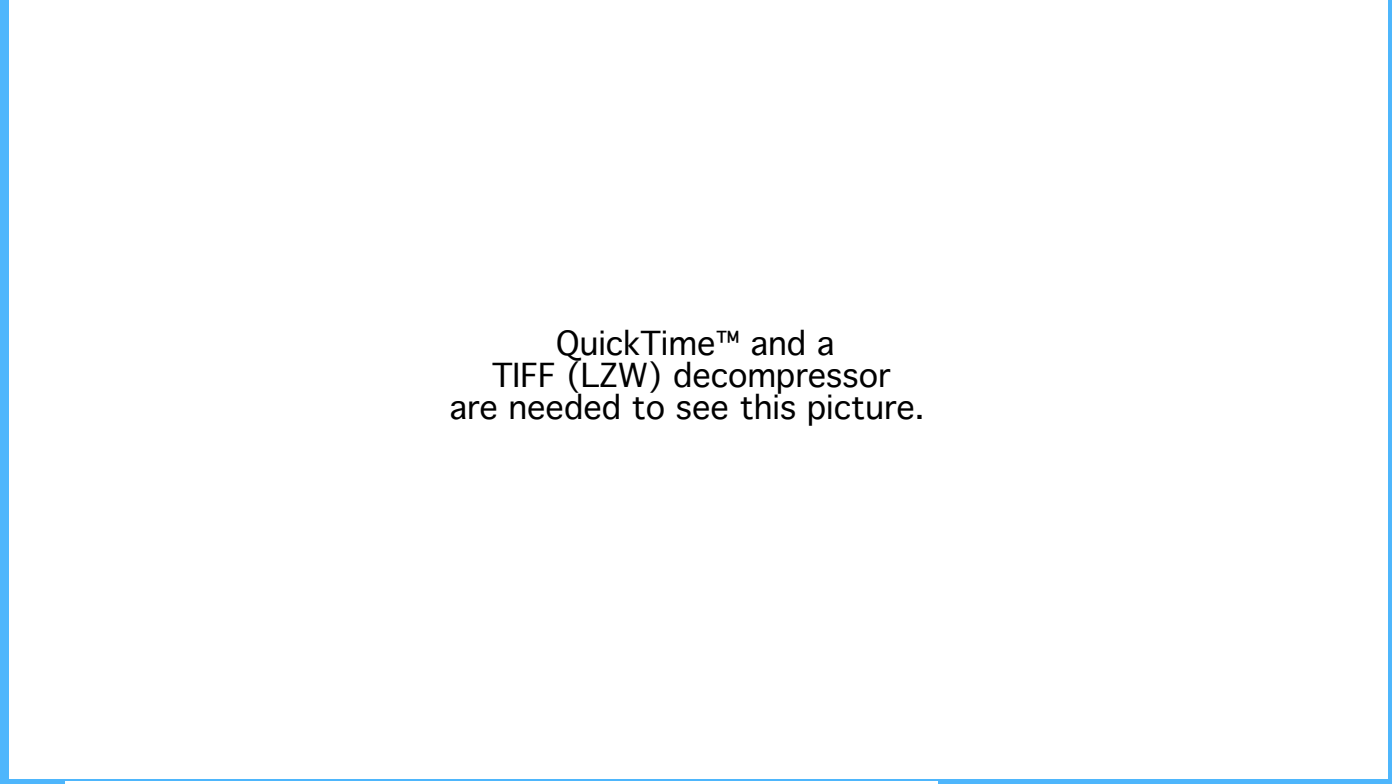
413d Conlon, Barth & Robertson
Development 111 969 (1991)

Phenotype

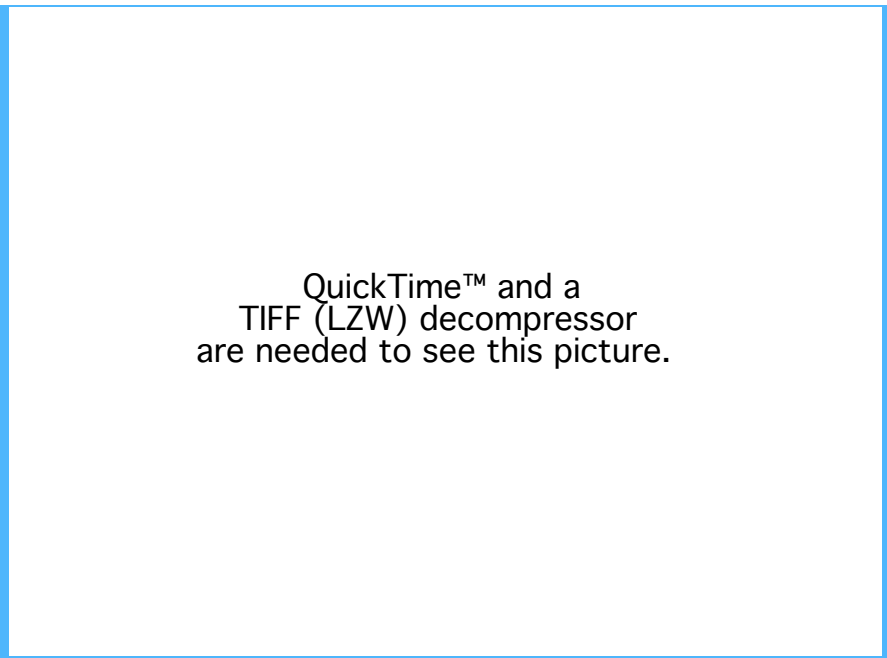




Carlton MB, Colledge WH, Evans MJ.
Crouzon-like craniofacial dysmorphology in the mouse is caused by an insertional mutation at the Fgf3/Fgf4 locus.
Dev Dyn **212**:242-9. (1998)



Hprt



A potential animal model for Lesch–Nyhan syndrome through introduction of HPRT mutations into mice

Michael R. Kuehn, Allan Bradley, Elizabeth J. Robertson & Martin J. Evans

Nature **326**, 295 - 298 (1987)

ROSA β-geo gene trap of H3.3A

- Gene trap inserts into position 42 in the1537bp first intron of H3.3A
- 4.1 kb lac-z transcript includes ~200 amino acids
- Severe reduction but not ablation of H3.3A m-RNA

A retroviral gene trap insertion into the histone 3.3A gene causes partial neonatal lethality, stunted growth, neuromuscular deficits and male sub-fertility in transgenic mice.

C. Couldrey, M. Carlton, P. Nolan, W. Colledge, and M. Evans. Human Molecular Genetics, 8(13): p. 2489-2495, (1999).

QuickTime™ and a TIFF (LZW) decompressor are needed to see this picture.

Three oncogenes

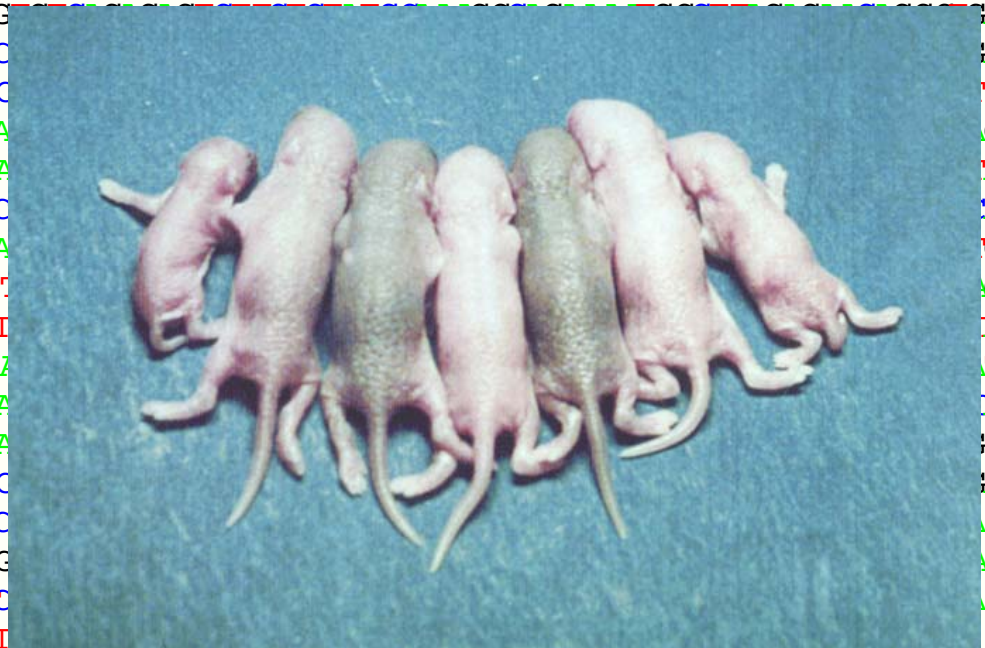
- brca2
- c-mos
- hox11

Identification of the breast cancer susceptibility gene BRCA2

Richard Wooster, Graham Bignell, Jonathan Lancaster, Sally Swift, Sheila Seal, Jonathan Mangion, Nadine Collins, Simon Gregory, Curtis Gumbs, Gos Micklem, Rita Barfoot, Rifat Hamoudi, Sandeep Patel, Catherine Rices, Patrick Biggs, Yasmin Hashim, Amanda Smith, Frances Connor, Adalgeir Arason, Julius Gudmundsson, David Ficenec, David Kelsell, Deborah Ford, D. Timothy Bishop, Nigel K. Spurr, Bruce A. J. Ponder, Rosalind Eeles, Julian Peto, Peter Devilee, Cees Cornelisse, Henry Lynch, Steven Narod, Gilbert Lenoir, Valdgardur Egilsson, Rosa Bjork Barkadottir, Douglas F. Easton, David R. Bentley, P. Andrew Futreal, Alan Ashworth, Michael R. Stratton

Nature **378**, 789 - 792 (28 Dec 1995) Letter

"The known sequence of 2,329 amino acids encoded by the BRCA2 gene does not show strong homology to sequences in the publicly available DNA or protein databases, and therefore we have no clues to its functions."

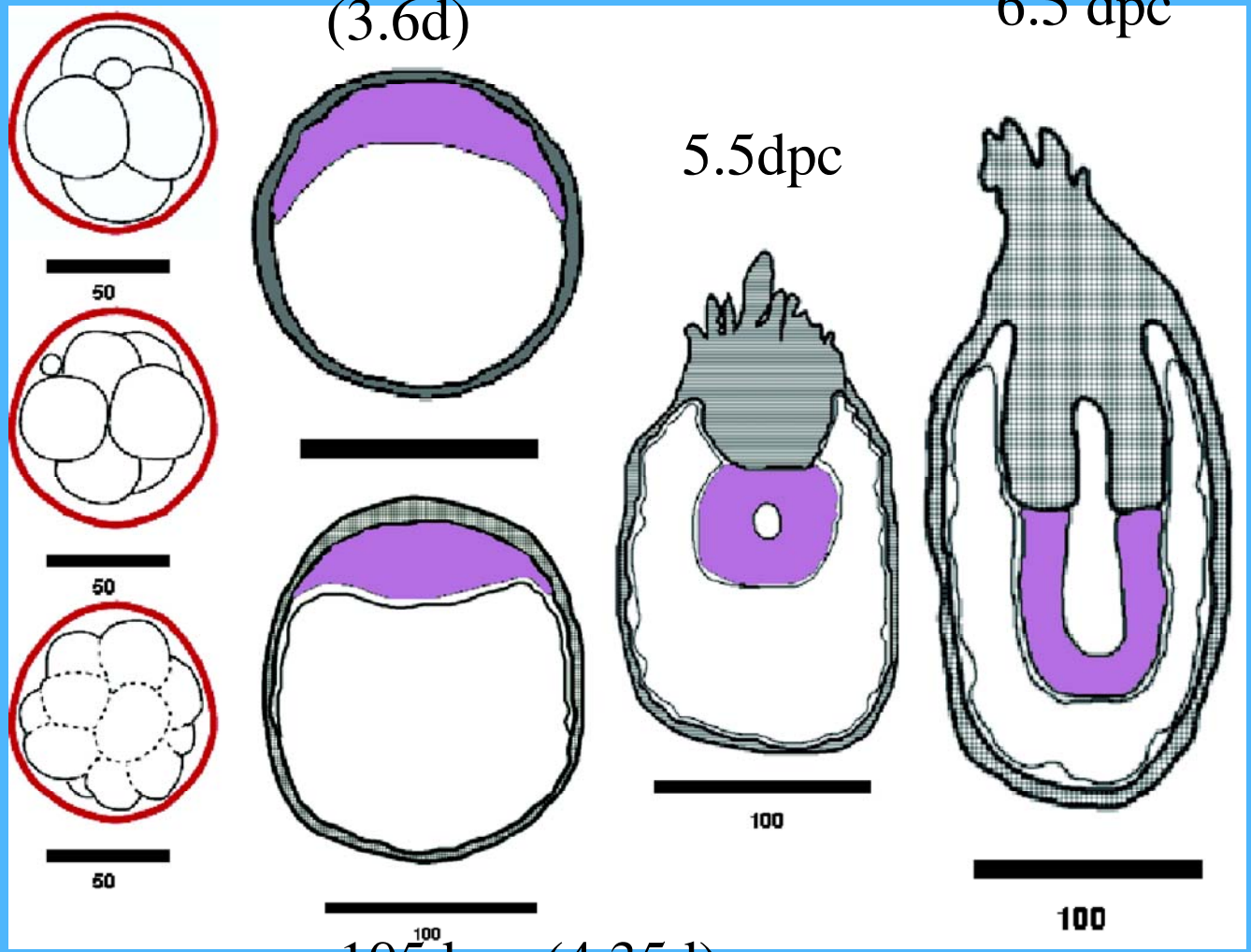


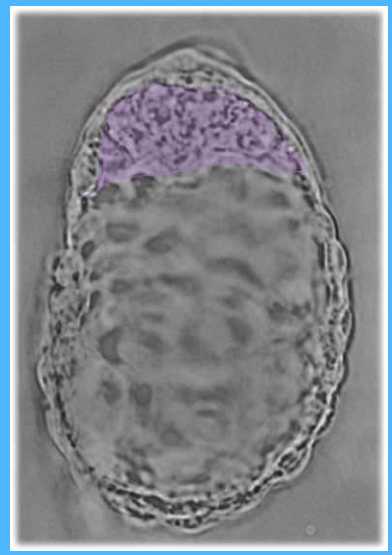
What are they?

- Are mouse ES cells a cell type normally found in the early embryo or are they effectively an artefact of culture?
- Lines of evidence
  - 2d protein separations
  - Microarray expressionomics

Stages used

Thieler stages from EMAP





Delayed
5.6d and 7.5d

ES microarray phenotyping

- 20 ICM's (~500 cells)
- Two roundsT7 amplification
- Amino-allyl labelling
- NIA 15k probes

Stepped aside or from normal pathway?

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

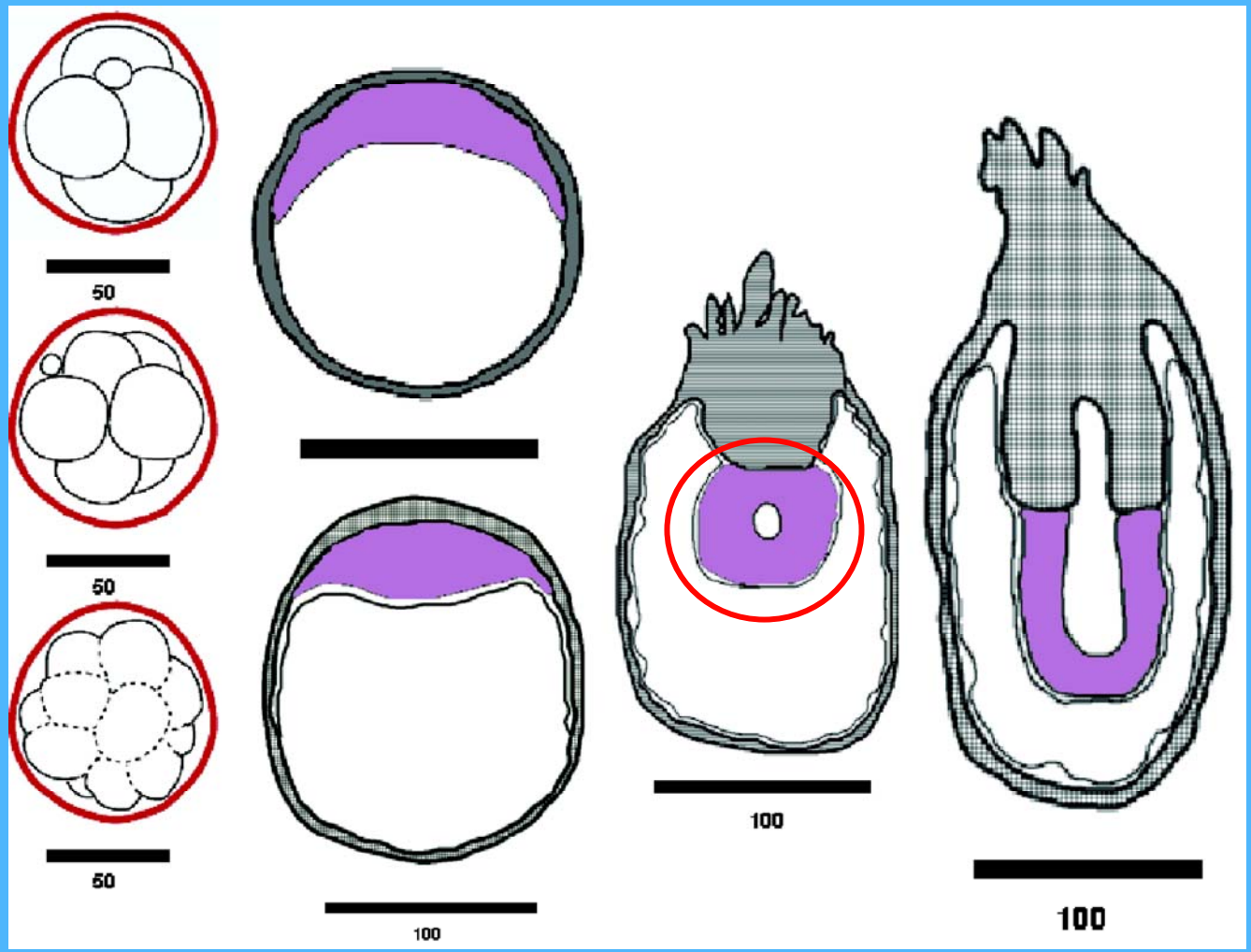
QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

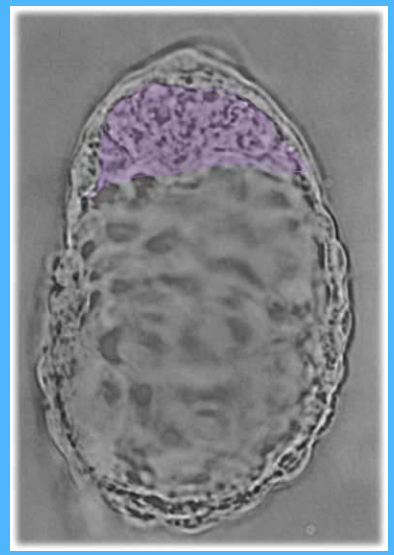
QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Where ES cells come from!





Two platform technologies

- Use of germ line chimaerism
  - vector to whole animal genetics and animal models of disease (mouse) understanding and drug discovery
- Wide range of developmental studies; *in vitro* differentiation
  - fundamental understanding of cell developmental biology
  - therapeutic scenario of damaged tissue being repaired by appropriate tissue specific stem and precursor cells possibly derived by specific differentiation of human ES cells. Moreover the possibility of using histcompatible cells either from a large pre-prepared bank or by dedifferentiation of other cells self-donated by the patient has done much to power interest in the field.

Future

- Whole animal genetics
- Analysis of differentiation
- Embryo surrogate and source of specific cells
- Understanding control of mammalian developmental cell biology & genetic readout in differentiation
- Practical medical applications

Nobel Lecture in Physiology or Medicine

Embryonic Stem Cells: The Mouse Source – vehicle for Mammalian Genetics

Martin Evans

CARDIFF UNIVERSITY

PRIFYSGOL CAERDYDD

School of Biosciences