European Society of Human Reproduction and Embryology

COURSE 8

Update in stem cell derivation and cell therapy

Special Interest Group Stem Cells

18 June 2006
Prague - Czech Republic

Contents

Course 8 - Pre-congress course organised by the Special Interest Group Stem Cells

"Update in stem cell derivation and cell therapy"

PROGRAM

Course co-ordinators: A. Veiga (E) and L. Gianaroli (I)

Course description: The aim of the course is to provide the attendants with updated information in stem cell research.Recent improvements in human embryonic stem cell derivation and culture will be described together with therapeutic approaches involving the use of both embryonic and adult stem cells. Banking, registry organisation as well as legislation will also be treated. Leaders in research in the field of stem cells have been selected as speakers.

09.00 - 09.30	Feeder free hESC derivation and culture - **O. Hovatta (FIN)**
09.30 - 09.45	Discussion
09.45 - 10.15	Use of PGD embryos for stem cell derivation - **S.Minger (UK)**
10.15 - 10.30	Discussion
10.30 - 11.00	*Coffee break*
11.00 - 11.30	Trophoblastic stem cell lines: implantation model - **H. Moore (UK)**
11.30 - 11.45	Discussion
11.45 - 12.15	hESC for neural repair - **B. Reubinoff (IL)**
12.15 - 12.30	Discussion
12.30 - 13.30	*Lunch*
13.30 - 14.00	Stem cells for cardiac repair - **A. Bayes-Genis (E)**
14.00 - 14.15	Discussion
14.15 - 14.45	Stem cell banks and registries: the UK Stem Cell Bank – **G. Stacey (UK)**
14.45 - 15.00	Discussion
15.00 - 15.30	Coffee break
15.30 - 16.00	The future of stem cell research - **A. Trounson (AUS)**
16.00 - 16.15	Discussion
16.15 - 16.45:	EU legislation, guidelines and funding for hESC research – **G. Joliff-Botrel (EU)**
16.45 - 17.00:	Discussion

Feeder free culture and derivation of clinical quality human embryonic stem cell lines

Outi Hovatta
Karolinska Institutet, Karolinska Universtetssjukhuset Huddinge
Stockholm, Sweden

ESHRE 2006, Prague

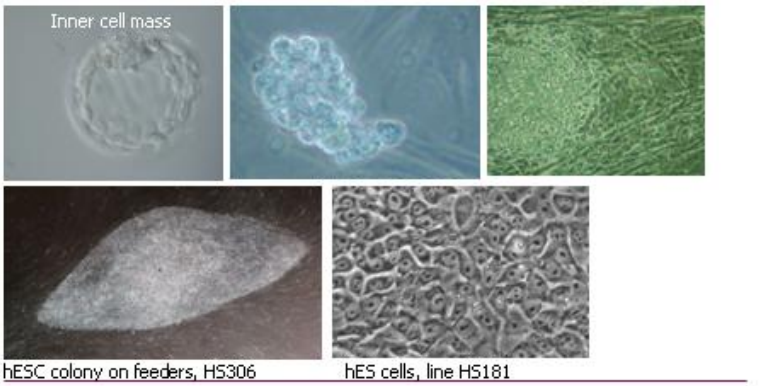
Learning objectives

- Derivation procedure of human embryonic stem (hES) cells
- Requirements of clinical quality stem cell lines, GMP
- Feeder cells
- Feeder-free cultures
- Feeder free derivation
- How a safe feeder-free derivation procedure might be achieved
- Immunological problems, SCNT
- An existing feeder-cell containing system for derivation of clinical quality hES cells

Derivation of permanent human embryonic stem cell (ES) lines

- Outgrowth of cells from the inner cell mass (Fishel, Edwards, Evans, Science 1984, see also Edwards Nature 2001, RBMonline 2002, Bongso et al.1994)
- Nine permanent lines cultured as foetal mouse fibroblasts as feeder cells (Thomson et al. Science 1998)
- Six lines (Reubinoff et al. Nature Biotechnol 2000), also on mouse derived feeder cells
- In 2006, some 250-300 hES lines existed in the world

Derivation of hESC lines



Human embryonic stem cell lines at Karolinska University Hospital Huddinge

- A total of 25 permanent lines 2002-2006
  → HS181, HS207, HS235, HS237, HS293, HS306, HS346, HS351, HS360, HS361, HS362, HS363, HS364, HS366, HS368, HS380, HS382, HS386, HS401, HS402, HS415, HS420, HS421, HS426, HS429
- 17 additional early lines which stopped growing during the period
- 139 blastocysts have been obtained 2002-2006
- Success dependent on embryo quality
  → The good embryos are always used for patients' infertility treatment

- All the lines have been derived using serum replacement instead of fetal calf serum in the medium Hovatta et al. Hum Reprod 2003
- The lines HS293-429 (n=22) have been derived on postnatal human skin fibroblasts using serum replacement containing medium Inzunza et al.Stem cells 2005
- The lines HS415-429 (n=5) have been derived after mechanical isolation of the Inner cell mass

Cell IQ system

- Machine vision + artificial intelligence, a controlled culture system (Chipman Technologies, Tampere, Finland)
- Enables detailed follow-up of the developing cells in long-term culture
- Records all the events in culture
  →Cell divisions, apoptosis, morphological changes etc
- Recognizes single cells, 24 wells can be followed at a time
- Allows comparison of culture constituents

GMP

- The EU commission directive 2003/94/EC, of October 2003, presents the principles and guidelines for good manufacturing practice (GMP)
  → Cell transplantation under this directive
- The EU commission directive 2004/23/EC, of March 2004
  → Human cells and tissues, quality and safety requirements, comes to effect 1 April, 2006, includes all stem cells
- A quality approach to manufacturing
  → Enables the manufacturers to eliminate or minimise instances of contamination, mixups and errors.
  → Protects the consumer from purchasing products which are not effective or even dangerous.
- GMP regulations address record keeping, personnel qualification, sanitation, cleanliness, equipment verification, validation of the processes, and complaint handling

Animal proteins in cultures are immunogenic

- Animal protein containing culture components are absorbed by the growing cells
  → Mouse feeder cells
  → Fetal bovine serum
  → Serum replacements
- The cells contain non-human sialoproteins
- Most human individuals have antibodies against these mouse- and bovine proteins, and the cells may be rejected

Risk of infections with animal derived culture components

- Mouse cells and antibodies
  - →Viruses in mice: Hantaan, LCMV, reovirus 3
  - →EMEA guidelines contains a long list of possible microbes
- Bovine serum and serum albumin
  - →Prions etc
  - →Not considered suitable for human use, even though used for the time being

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Other types of human feeder cells also feasible

- Foetal skin and mucscle, tissue from adult Fallopian tubes Richards et al Nature Biotechnol 2002
- Human placental fibroblasts Genbajev et al. Fertil Steril 2005, Simon et al. Fertil Steril 2005
- Adult bone marrow stroma cells Lee et al. Biol Reprod 2005
- Human endometrial cells Cheng et al. Stem Cells 2003
- hESC derived fibroblasts Wang et al. Stem Cells 2005

Culture medium

- Knockout D-MEM + Glutamax, β-mercaptoethanol
- In the first five derivations foetal calf serum
- from 2003, Knockout Serum Replacement 15% (Invitrogen) + rhFGF-basic (R&H Systems) 8 ng/ml
  - →this SR contains animal proteins
- a systematic comparison between FCS, human serum and SR was made, best non-differentiated growth with SR (Koivisto et al RBMonline 2004)
- a completely animal-protein free medium would be better because of immunogenicity of and risk of infectious agents
  - →systematic comparisons going on, several human protein media function, but not very well
  - →A defined medium, Ludwig et al. 2006

Feeder-free derivation successful in mouse

- Feeder-free nondifferentiated culture of mouse feeder cells is possible by adding leukemia inhibitory factor (LIF) to the culture medium (Smith et al. Nature 1988)
- Feeder-free derivation also possible (Nichols et al. Development 1990)

LIF does not keep human ES cells undifferentiated

-The expression of LIF and its receptors (LIFR and gp130) is low in undifferentiated hESCs, but increases during differentiation.
-Added LIF does not prevent spontaneous differentiation.
-SOCS-1 may inhibit LIF signaling in hESCs

* Aghajanova L, *Skottman H, Strömberg A, Inzunza J, Lahesmaa R, Hovatta O: The expression of leukemia inhibitory factor and its receptors is increased during differentiation of human embryonic stem cells. Fertil Steril 2006, in press

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Feeder-free culture of existing hES cell lines

- Culture of a line on Matrigel using conditioned medium from mouse foetal fibroblasts, Xu et al. Nature Biotechnol 2001, Carpenter et al. Cloning Stem Cells 2003, several articles since then
- Culture of a line on human laminin and fibronectin, using a medium containing FGF, LIF and TGFbeta, Amit et al. Biol Reprod 2004
- BMP signalling inhibition by very high concentration of bFGF (100ng/ml) or combination of Noggin and bFGF (40ng/ml) (Xu et al. Nature Methods 2005)
  - These unphysiologically high concentration of growth factors may cause epigenetic changes in the ES cells (Steele et al. 2005)
- Chromosomal changes may be promoted in feeder-free cultures (Draper et al. Nat Biotechnol 2004)

Feeder-free derivation

- Would be optimal in many respects
- A successful derivation reported in 2005, Klimanskaya et al. Lancet
  - → An ECM manufactured from mouse tissues was used
  - → not suitable for transplantable human cells
- Two lines derived using high concentrations of bFGF and noggin in the derivation medium, culture on matrigel and laminin-collagen
  - → Ludwig et al. Nature Biotechnol 2006
  - → One line had karyotype 47 XXY, one had gained an extra chromosome 17 at passage level 40
  - → Probably not optimal conditions to derive transplantation quality cells

Human matrix

- From human cells ?
- Or chemically defined culture medium after identifying the mechanisms of self renewal
  - →genomics
  - →proteomics

Mechanism of self-renewal of hES cells

- Gene expression profling
- Proteomics

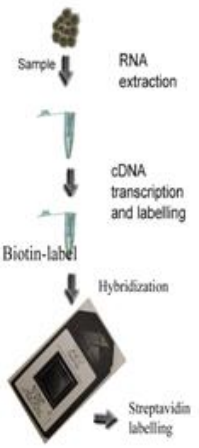
Affymetrix microarrays

HG-U133A and HG-U133B:
39 000 transcripts and variants, including over 33 000 human genes (>45 000 probe sets)

Affymetrix small sample protocol:
Starting with 100 ng of tRNA (linear IVT trancription, 2 cycles, non-PCR based amplification)

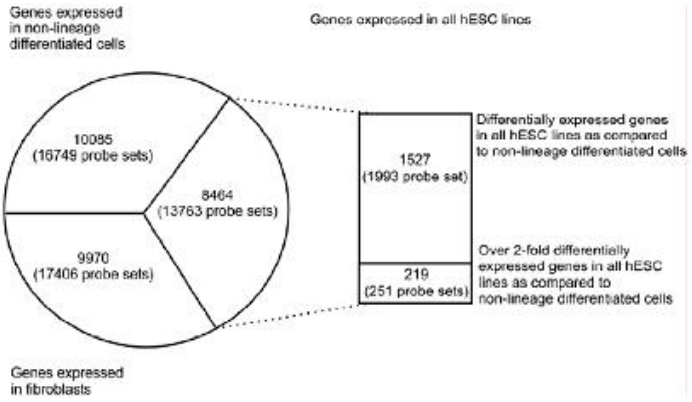
2 Biological replicates from each line + from fibroblasts and differentiated cells

Data from 36 arrays



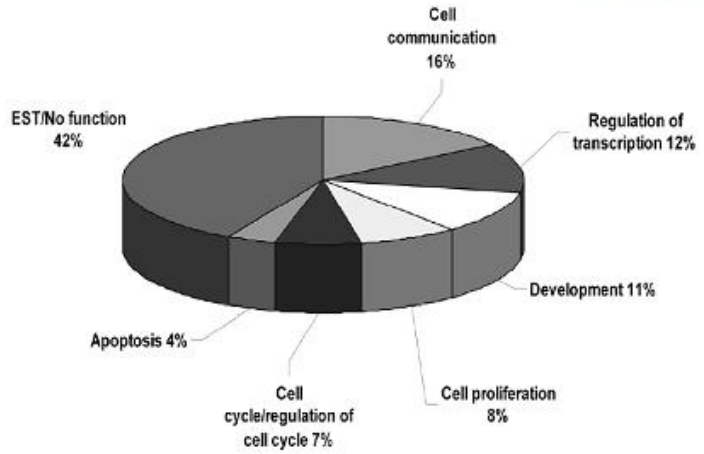
Helsinki: Timo Tuuri/Tinom Otonkoski, Tukholma: Heli Skottman/Outi HovattaTurku:Riitta Lahesmaa

Genes expressed in seven hES cell lines

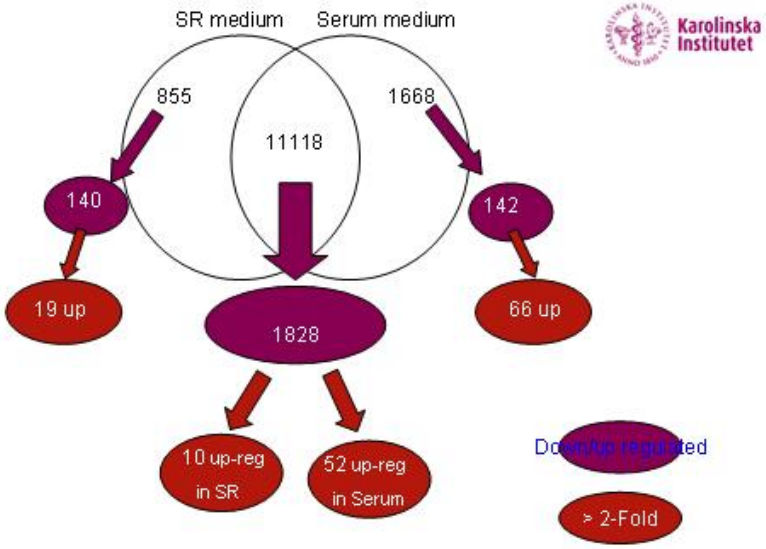


Skottman et al. Stem cells 2005

Functions of genes expressed in 7 hES cell lines



Skottman et al. Stem cells, Aug 4, 2005



Heli Skottman et al. Stem Cells Aug 11, 2005

Genetic and epigenetic stability

- Repeated karyotypes
- Comparative genomic hybridisation, microarrays
- Gene expression profiling
- Epigenetic profiling

Human Embryonic Stem Cells induced immunity when transplanted to immunologically competent mice

they express:

- HLA class I
- low levels of HLA class II
- low levels of co-stimulatory molecules
- no expression of immunosuppressive cytokines
- when transplanted into an immunologically competent host, these stem cells induce rejection and immunity
- this immunological response is dependent on the same signalling pathways as other allogeneic and xenogeneic cell transplants.

Grinnemo et al. 2006

How to avoid rejection in embryonic stem cell transplantation

1. Somatic cell nuclear transfer

2. A large number of lines in an international registry to obtain donated cells to minimize the need of immunosuppression

   a challenge to all IVF units

3. Immunosuppression

Somatic cell nuclear transfer

- A method to avoid rejection in stem cell transplantation
- Ethics approval June 2005
- Immature oocytes from ICSI women treated by intracytoplasmic sperm injection, usually discarded
- In vitro maturation = IVM
- MII oocytes if more that 20 obtained in retrieval (1-2)
- Enucleation
- Nuclear transfer from granulosa cells or skin fibroblasts
- Fusion and activativation using electric pulse
- Blastocyst culture
- Derivation of stem cell lines

Team: Jose Inzunza, Julius Hreinsson, Lev Levkov, Susanne Ström, Ami Strömberg, Eija Matilainen, Outi Hovatta

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GMP-quality feeder cells

- Human skin fibroblast lines can be cultured in GMP conditions
- In initial derivation, the cells attach poorly in serum replacement medium
- Human serum works well
  - →Human AB-serum obtained from GMP-quality blood bank in intial derivation
  - →Then human protein-containing serum replacement for propagation
- A safe option if many infections can be excluded (RNA and DNA measurements), EMEA's list

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Clinical quality

- **GMP** quality process and laboratory
- **Animal protein free** derivation and propagation
- **Mechanical isolation** of the inner cell mass
- Derivation using GMP-quality **human skin fibroblasts as feeders or**
- **Feeder-free derivation**
  → Human matrix would be optimal, but to obtain safe cells, it would be necessary to know more about the mechanisms of self-renewal
- GMP quality **serum replacement which only contains human proteins**, none of the present commercially available ones functions perfectly
- **Mechanical splitting** safe, and easy in our hands

Conclusions

- Most of the existing hES cell lines have been derived using feeder cells, first mouse and then human feeders
- Feeder-free cultures of hES cells possible using conditioned medium from mouse feeders, or combinations of growth factors
- Feeder-free derivation reported on mouse-derived extracellular matrix, and on human matrix udsing high concentrations of bFGF and noggin
- Feeder-free culture and derivation may drive hES cells to chromosomal and epigenetic changes and mutations
- Derivation of GMP-quality hES cell lines feasible using human feeder cells

Low oxygen good for stem cell renewal

Chimborazo, Ecuador, January 2006

Use of PGD Embryos for Stem Cell Derivation

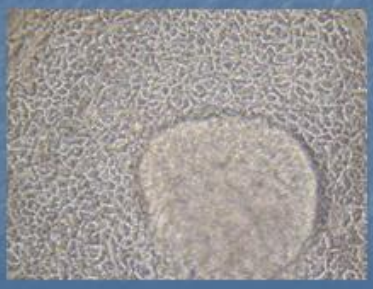
Dr Stephen L Minger
Director, Stem Cell Biology Laboratory
Wolfson Centre for Age-Related Diseases
King's College London

Lecture Objectives

- Understand the rationale for the use of PGD embryos for human embryonic stem (hES) cell derivation
- Outline of the characterisation process of hES cell lines
- Outline of the differentiation protocols relevant to disease-specific hES cell lines

New regulations give UK the lead in stem cell work
Nature Medicine, April 2002

UK human embryonic stem cell first
Human embryonic stem cells have been grown in the UK for the first time, a team at King's College London announced on Wednesday.
BBC, August 13, 2003



Pickering and Minger, (2003) Reprod BioMed

Human Embryo Research in UK

- Human Fertilisation and Embryology Authority Created in 1990
- All Reproductive Medicine & Human Embryo Research must be licensed by HFEA
- HFEA-Licensed Research Areas
  - Treatment of Infertility
  - Causes of Congenital Diseases
  - Causes of Miscarriage
  - Development of more Effective Conception
  - Improvements in Preimplantation Genetic Diagnosis

License for Derivation of Human ES cells from UK Human Fertilisation and Embryology Authority

- Awarded to Drs Susan Pickering, Stephen Minger & Professor Peter Braude in May 2002 – renewed in 2005 for three additional years

- Derivation from donated embryos with informed consent
- No financial inducement for donation
- Cannot create embryos for stem cell derivation
- All cell lines must be deposited in UK Stem Cell Bank and made freely available to other research groups
- Cell Nuclear Replacement (Therapeutic) permitted with license
- Cell Nuclear Replacement (Reproductive) banned with criminal penalties

Early human development in vitro

How do we get embryos for stem cell research ethically?

Preimplantation genetic diagnosis

Transfer only unaffected embryos to the patient

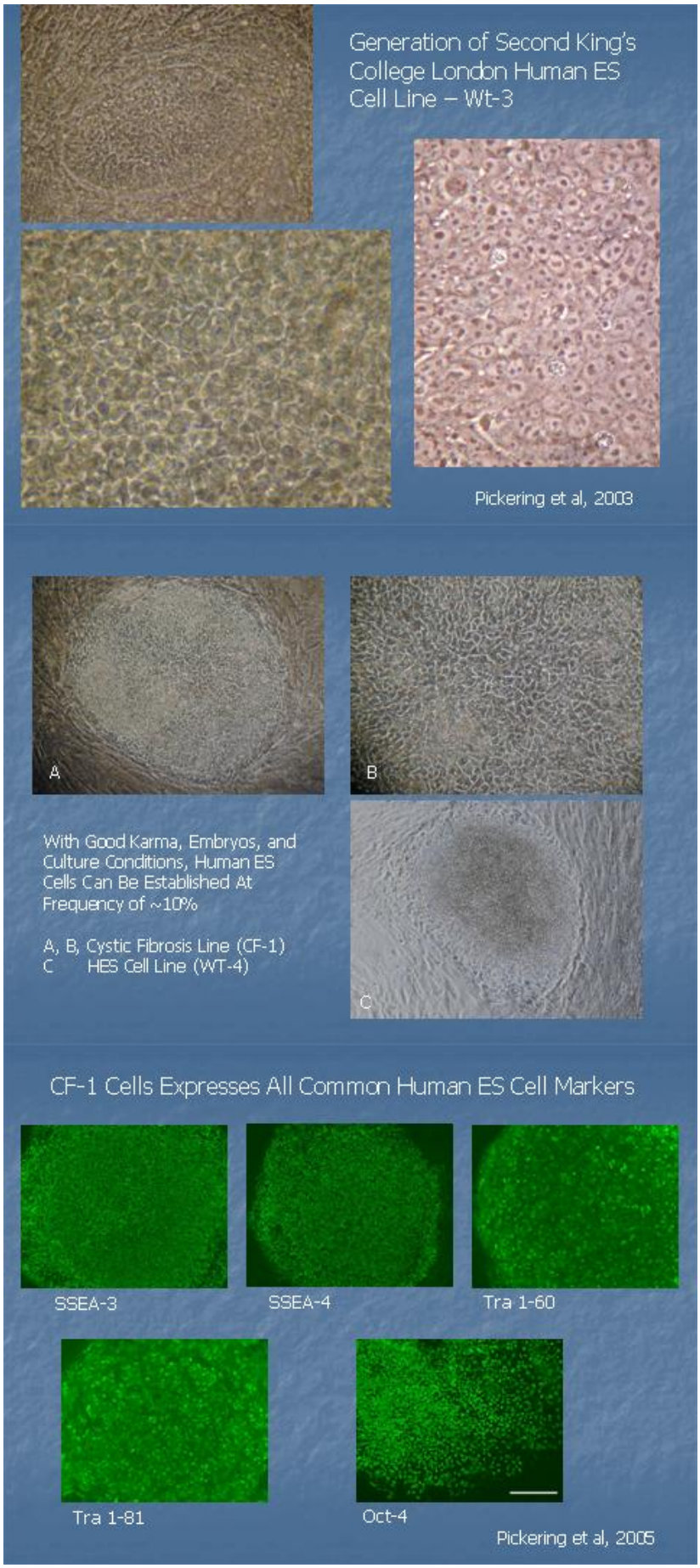
In the UK a Licence from the HFEA is required for each specific condition for PGD

Sexing for X-linked disorders
Incontinentia Pigmenti
Duchenne Muscular Dystrophy
Haemophilia A
Anderson Fabry Disease
Wiskott-Aldrich Syndrome
Adrenoleukodystrophy
Hunter's Syndrome
X-linked Mental Retardation
Ornithine Carbamoyl Transferase Deficiency
Pelizaeus Merzbacher Disease
Choroideraemia (OMIM® 303200)
Alport syndrome
Becker's Muscular dystrophy

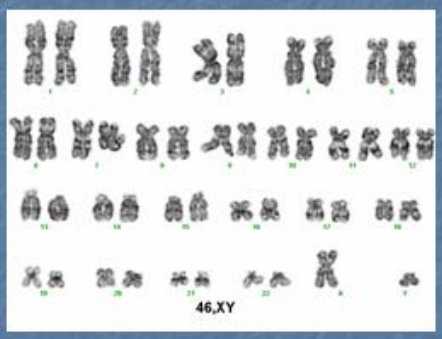
Specific Diagnosis (Molecular tests)
Sickle cell disease
Spinal Muscular Atrophy
Cystic Fibrosis
Epidermolysis Bullosa
Compound heterozygous mutations leading to ablation of Plakophilin 1 (PKP1) resulting in skin-fragility ectodermal dysplasia syndrome
Huntington's disease

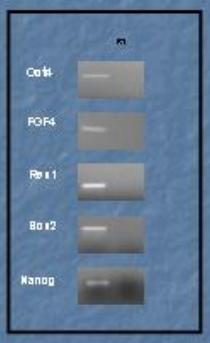
Specific Diagnosis (FISH)
Robertsonian Translocation Karyotype 45,XX,der(14;21)(q10;q10)
Robertsonian Translocation Karyotype 45,XX,der(13;14)(q10;q10)
Robertsonian Translocation Karyotype 45,XX,der(13;21)(q10;q10)

Chromosomal Translocation Karyotype 46,XX,t(1;19)(q32.1;q13.1)
Chromosomal Translocation Karyotype 46,XX,t(12;17)(p13;p13)
Chromosomal Translocation Karyotype 46,XX,t(11;22)(q23.3;q11.2)
Chromosomal Translocation Karyotype 46,XX,t(14;22)(q11.2;q13.3)
Chromosomal Translocation Karyotype 46,XY,t(7;12)(q22;q13.1)
Chromosomal Translocation Karyotype 46,XX,t(8;18)(p21.1;q21.1)
Chromosomal Translocation Karyotype 46,XX,t(3;5)(p12;q14.2)
Chromosomal Translocation Karyotype 46,XX,t(4;15)(q21.1;q13)
Chromosomal Inversion Karyotype 46,XX,inv(13)(p11.2;q21.2)
Chromosomal Inversion Karyotype 46,XY,inv(14)(p11.2;q24.1)

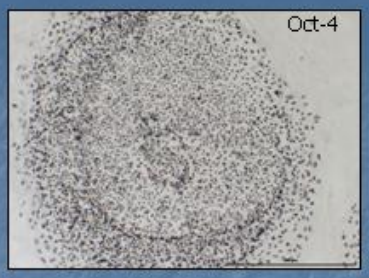

Generation of Second King's College London Human ES Cell Line – Wt-3
Pickering et al, 2008
A
B
With Good Karma, Embryos, and Culture Conditions, Human ES Cells Can Be Established At Frequency of ~10%
A, B, Cystic Fibrosis Line (CF-1)
C HES Cell Line (WT-4)
C
CF-1 Cells Expresses All Common Human ES Cell Markers
SSEA-3
SSEA-4
Tra 1-60
Tra 1-81
Oct-4
Pickering et al, 2005

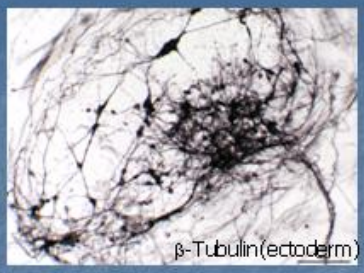
CF-1 Human ES Cell Line is Karyotypically Normal (p30) and Expresses Usual ES Cell Genes

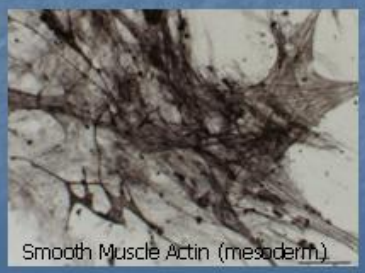


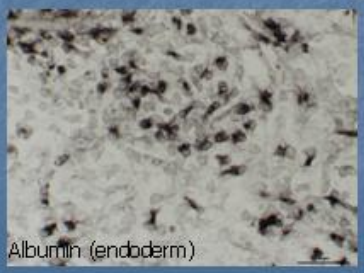


Pickering et al, 2005









Summary of ES Derivation 2002-2004

KING'S College LONDON

	Embryos donated	Proportion reaching blastocyst	Proportion from which intact ICM was recovered	Proportion of ICMs which outgrew	Proportion from which stem cell like cells were isolated
Fresh (from PGD)	77	50 (65%)	36 (46%)	21 (27%)	4 (5%)
Cryo-preserved	28	12 (43%)	9 (32%)	4 (14%)	1 (4%)
Total	105	62 (59%)	44 (42%)	25 (24%)	5 (5%)


Strengths of UK Stem Cell Research
World-class academic research centres
Tight regulation on Assisted Reproduction & Human Embryo Research by HFEA
Government Commitment to Stem Cell Research (~£45 million – 2003-2004); £350 million 10-year strategy from Chancellor's Office announced Dec 2005
Government Funded UK Stem Cell Bank for foetal, adult & embryonic human stem cell lines
UK Human ES Cell Forum – 3 research mtgs/year
London Regenerative Medicine Network – drive towards clinical applications
Stem Cell Biology Laboratory and Collaborators
Dr Stephen Minger
Minal Patel
Hannah Taylor
Sarah Pringle
Jessica Cooke
Zhenling Luo
Dr Antigoni Ekonomou
Dr SH Cedar
Dr Chetan Shatapathy
Professor Paul Sharpe
Dr Jonathan Corcoran
Dr Sukhi Bansal
Professor Tony Ng
UCL Bioprocessing
Chris Mason
Aalborg/Aarhus
Dr Helle Andersen
Dr Anette Gabrielsen
Prof Peter Braude
Dr Sue Pickering
Sara Hall
Emma Stephenson
Prof Constantino Pitzalis
Prof Adrian Hayday
Dr Cosimo De Bari
Dr Francesco Dell'Accio
GlaxoSmithKline
Dr Aaron Chuang
Dr Jules Ellis
Prof Peter Jones
Dr Shanta Persaud
Dr Chris Burns
Dr Yue Wu
Norwich/Vienna
Prof Brian Salter
Prof Herbert Gottweis
Mayo Clinic
Dr Cindy Wetmore
Dr Susan Duty
Prof Clive Ballard
Prof Elaine Perry
Prof Steve McMahon
Dr Liz Bradbury
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Birmingham
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Uppsala Univ.
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UBC/Vancouver
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Novartis
Dr Alan Jackson
Dr Phil Kemp
Baylor/Houston
Dr Karen Hirschi
Oxford University
Dr Paul Fairchild
Karl Karlsson
MRC/Hammersmith
Dr Huseyin Mehmet
Dr Jan Brosens
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Trophoblast stem cell lines: implantation model

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Learning objectives

1. List requirements for trophoblast cell lines for studying human implantation
2. Describe mechanisms of early differentiation of human embryonic stem cells
3. Outline methods for haracterisation of human trophoblast and differentiation strategies
4. Describe in vitro implantation models

Summary
Background: An effective embryonic–maternal interaction is crucial for successful human pregnancy. Failure of this process is a major cause of infertility and can lead to placental dysfunction resulting in recurrent miscarriage, foetal retardation and pre-eclampsia. Current research is severely constrained by ethical and practical considerations. It is now possible to generate cytotrophoblast stem (CTBS) cell lines from human embryonic stem cells (HESCs) as tools to investigate early placentation.
Method: Human chorionic gonadotrophin (hCG) is used as a marker of trophoblast. In defined culture, embryoid bodies are generated from HESCs and selected for trophoblast enrichment by rounds of cellular aggregation and disaggregation. Distinct cytotrophoblast stem cell (CTBS) lines can be isolated and characterised. Spheroid CTBS aggregates are formed and their interaction with luteal–phase endometrial stroma analysed by real-time image analysis.
Findings: CTBS lines were derived which could be maintained in the absence of residual HESCs, fibroblast feeder cells or extra-cellular matrix. CTBS cells displayed typical cyto- and syncytio-trophoblast characteristics and exhibited further differentiation to the invasive endovascular phenotype involved in uterine blood vessel remodelling. Spheroid CTBS cells mimicked closely the early invasive stages of implantation when incubated with human endometrial stroma *in vitro*.
Interpretation: These human CTBS cell lines are a significant new model for investigating human placentation and have considerable potential in cell therapy applications.

Introduction
During human implantation the continuous proliferation of cytotrophoblast stem cells (CTB) enables the embryo to rapidly invade the endometrial stroma and establish a haemochorial placenta. The early differentiation of cytotrophoblast to an invasive endovascular phenotype is critical for promoting feto-maternal immune tolerance and for remodelling uterine blood vessels for continuation of pregnancy. Aberrant development of trophoblast is associated with serious complications, including recurrent miscarriage, pre-eclampsia (maternal high blood pressure) and restricted fetal growth

(1,2). The process of trophoblast differentiation is poorly understood in women as investigations are severely constrained by ethical and practical considerations.

In the mouse, trophoblast stem cells isolated from the pre- and post-implantation embryo can be maintained indefinitely in culture and have the capacity to differentiate along the trophoblast lineage (3). However, until recently, the derivation of human trophoblast stem cells from pre-implantation blastocysts has been achieved, highlighting the differences in early embryo and placental development between these species (figure 1). Therefore we used human embryonic stem cells (HESCs ,4) as a route to obtaining a trophoblast stem cell population (5). While HESCs differentiate spontaneously to trophoblast-like cells in cultures when supplemented with bone morphogenetic protein 4 or when Oct 4 is down regulated, these cells are terminally differentiated and display a limited proliferative capacity (6). Trophoblast differentiation can develop further when HESCs are aggregated to form embryoid bodies (EBs) but residual HESCs and other cell types in the culture resulting from spontaneous differentiation confound the findings from these preparations (7). We surmised that the proportion of trophoblast cells that developed in human EBs *in vitro* would vary and sought to identify viable EBs containing trophoblast cells undergoing syncytium by measuring secretion of the β subunit of hCG (hCGβ); a hormone essential for maintaining early pregnancy. Such EBs might potentially contain a trophoblast stem cell population for enrichment and purification.

Methods and Results

Details of the method have been reported (5). First, we generated trophoblast - containing EBs, using HESCs (H7 and H14) of normal karyotype, which were maintained and passaged by standard protocols using serum-replacement medium. EBs were prepared by aggregation of single HESCs (dissociated with 1 mg/ml collagenase IV) in ES medium without basic fibroblast growth factor in Petri dishes in 5% CO_2 in air. On day 5, EBs were then transferred singly to wells of a 96-well culture plate and cultured for a further three days before aliquots of medium were subjected to ELISA assay. HCGβ was detected in most wells (4 x 96 well plates) but only 3.8% of wells had concentration of hormone greater than 500 m I.U./ ml (figure 2A). The EBs in these wells were of equivalent size and morphology, indicating that any increase in hCGβ was likely to be due mainly to the proportion of trophoblast cells rather than a greater overall number of cells.

To select for CTBS cells, EBs exhibiting high hCGβ secretion were subjected to several rounds of selective enrichment by growth in 'TS' medium comprising conditioned medium from fibroblast feeders supplemented with fibroblast growth factor 4 (FGF4) and heparin. TS medium promotes differentiation of murine trophoblast stem cells from extra-embryonic ectoderm. Those EBs maintaining a high secretion of hCGβ were pooled, disaggregated and allowed to form new EBs and this enrichment protocol repeated consecutively for three rounds until all EBs displayed consistently high hCGβ secretion. EBs were disaggregated (0.25% trypsin-EDTA) and then single cells allowed to proliferate in adherent culture in TS medium without feeders. Control cultures of EBs in HES medium without bFGF exhibited only basal hCGβ levels indicating poor trophoblast differentiation. Initially, four cell lines were generated with variable proliferation, two of which have maintained persistent proliferative capacity for more

than 30 passages (CTBS1 from H7 HESC and CTBS2 from H14 HESC) and form epithelial-like cell colonies with single and multinucleated cells (figure 2B). An additional CTBS cell line (CTBS-GFP1) was generated by the same methods but from H7 HESCs with constitutive expression of enhanced green fluorescent protein, eGFP (figure 2H and 2I). Continuous proliferation of each cell line was related to the persistence of a mononuclear cytotrophoblast population (relative to syncytium formation) as determined by immunostaining for cytokeratin and hCGβ (figure 2D-G). Cell proliferation was maintained by regular cell passage every 5 days as this inhibited terminal differentiation. When CTBS cells were aggregated and returned to mouse embryonic fibroblast feeders with HES medium they failed to revert to or generate either HESC colonies or EBs with pluripotent developmental capacity other than trophoblast. This indicated the absence of residual HESCs in the cell lines and the likely restricted developmental capacity of CTBS cells as has been shown for the mouse.

The trophoblast phenotype of the cell lines was confirmed by immunolocalisation of the pan trophoblast marker cytokeratin 7, Stage-Specific Embryonic Antigen 1 (SSEA1, 8), and human placental lactogen (hPL). Additionally, reverse transcription and the polymerase chain reaction (RT-PCR) were performed with primer sequences for marker genes of HESCs and trophoblast. Compared with HESCs, mRNA expression for Oct 4, Sox2, FGF4, Nanog in the derived cell lines was absent while trophoblast- related mRNAs for Cdx2 (*caudal*-related homeobox), Ck7, HLA- G, and Cd9 and were up regulated or maintained (figure 3A). The latter two are known markers for extravillous cytotrophoblast, which invades the uterine stroma (deciduas) during placentation (9). Surprisingly, eomesodermin (eomes), a marker of mouse early postimplantation trophoblast, was expressed strongly in HESCs but was weak or absent in the CTBS cell lines (figure 3A). Several reports have highlighted differences between mouse and human ESCs including eomesodermin expression in HESCs but not mouse ES cells (10). Furthermore, the expression of some trophoblast markers in stock cultures of HESCs may relate to spontaneous differentiation to trophoblast lineage. We had previously shown that expression of trophectodermal markers in such cultures occurred predominantly in the SSEA (-) and SSEA1(+) subsets of cells, consistent with their expression in the differentiated derivatives of the HESCs rather than in the HESCs themselves.

To further assess the subtype of trophoblast cells, the comparative cell surface expression of histocompatibility HLA class I (pan HLA antibody W6/32) and HLA-G (antibody G233) antigens was determined by fluorescent activated cell sorting (FACS) and immunolocalisation. The majority of cells (~90%) expressed HLA- class I histocompatibility antigens consistent with extravillous trophoblast (figure 3B). The expression of HLA-G (11), the non-classical HLA-class I antigen also specifically expressed in anchoring extravillous cytotrophoblast of first trimester placentae was relatively weak in most cells, but a small proportion (~10%) of cells displayed strong immunoreactivity (figure 3B and C). Some cells expressed vimentin, possibly indicating interstitial CTB. Following extended culture for one week or more in T25 flasks, the proportion of HLA-G^+ cells increased considerably (>90%). These cells exhibited distinct endothelial cell morphology similar to cultures of differentiating cytotrophoblast from first trimester human placental tissue and resembled endothelial

morphological differentiation from embryonic stem cells (12). Significantly, however, the cells coexpressed HLA-G and the platelet endothelial cell adhesion molecule-1 (PECAM-1), both markers of invasive endovascular (endothelial-like) CTB (figure 4). VE –cadherin and E-cadherin immunolocalisation was weak or absent on endovascular cells but strong on a relatively small proportion (<5%) of multinucleated cells also present at this stage and most likely equivalent to the syncytial giant cells found in stroma of the developing placenta. As determined by RT-PCR, adherent endovascular trophoblast in culture exhibited PECAM –1 mRNA expression but neither vascular endothelial growth factor receptor 1 (flt -1) or VE-cadherin in comparison with EBs again distinguishing these cells from a true endothelial phenotype (Figure 4i).

To determine the functional capacity of CTB cells, we first investigated the formation of non-proliferative, syncytiotrophoblast by cell-cell fusion of villous cytotrophoblast (1). The spontaneous generation of syncytium in adherent cell cultures of CTBS1 was monitoring cells under an inverted microscope for up to 3 days in a chamber at 37 ^{o}C in 5% CO_2 in air by continuous time-lapse recording. Adherent cells displayed progressive migration across the culture dish promoted by pseudopodial-like extension of cells. When cells occasionally converged they fused to form multi-cellular syncytiotrophoblast cells (figure 2G) that were hCGβ, and Ck7 positive but HLA class 1 negative. This cell fusion was captured unequivocally by digital time-lapse microscopy.

Next, we examined the invasive implantation potential of the CTBS cell lines by adopting a three- dimensional spheroid culture. This technique has been shown to maintain extra villous CTB phenotype of first trimester placental tissue (13). Aggregates of CTBS cells were generated from confluent monolayers following brief trypsinisation and incubation in non-adherent culture for 5-10 days. When cultured in extracellular matrix (Matrigel) drops, these CTBS spheroid aggregates developed characteristic outgrowths, which expressed hCGβ and cytokeratin (figure 5Ai and ii). The hCG receptor is expressed on invasive cytotrophoblast and similar observations have been reported for EB differentiation to trophoblast. On further culture with primary human endometrial tissue (luteal phase) prepared using well-characterised protocols (14), CTBS aggregrates attached to both epithelial cells and stromal cells. Significantly, as shown by time-lapse microscopy (figure 5B) CTBS spheroids with stromal cell cultures displayed a characteristic circular migratory movement and exhibited polar outgrowths from which endovascular cells streamed After about 24-36 hours in co-culture, these trophoblast outgrowths were the site of an erosion of the extracellular matrix of the stroma. This was identified by the rapid retraction of the trophoblast vesicle due to the dissolution of underlying extracellular matrix of the stromal cells (figure 5B, 2-5). A similar process of trophoblast invasion has been observed for human blastocyst co-culture with stromal cells *in vitro* (15). The erosion site was characterised by extravillous (HLA $–G^{+}$) trophoblast that expressed matrix metalloproteinase 2 (gelatinase A, figure 5b4, i and ii), identified recently as a key enzyme correlated with first trimester invasive capacity of human cytotrophoblast and whose activity is altered in cytotrophoblast in women with pre-eclampsia. Single GFP- trophoblast cells with endometrial stroma in culture displayed a similar response.

Discussion

These CTBS cell lines are the first distinct set of multipotent progenitor stem cell lines to be derived from HESCs and maintained independently. The method of selecting individual viable EBs with an appropriate secretory marker, followed by rounds of enrichment by the regeneration of EBs, could be applied in principle to derive a range of other cell types. It has been shown previously that clonally derived HESCs maintain full pluripotency and proliferation suggesting that CTBS cells develop from a homogeneous HESC population rather than multiple (i.e. ES and TS) precursors. Hence, our findings differ from the mouse where trophoblast cells may be derived from extra-embryonic ectoderm but not from murine ESCs without conditional gene expression.

Human cytotrophoblast stem cell lines differ from immortalised placental lines in their capacity to differentiation into several cytotrophoblast subtypes including terminal differentiation of endovascular cells. Cell cultures therefore closely mimic the implantation process *in vitro* and represent an important new model of placental dysfunctions such as pre-eclampsia which causes 15% of pregnancy associated morbidity. The efficient generation of endovascular cytotrophoblast also offers the prospect of using these cells for regenerative medicine. Their pseudo-vasculogenic and invasive characteristics might be utilised in a variety of cell therapies remote from the uterus but related to angiogenesis and vessel remodelling, especially as expression of HLA-G (16) and indoeamine 2,3, -dioxygenase (17) render the cells naturally refractory to immune rejection.

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General methods

Reverse transcription and polymerase chain reaction (RT-PCR).

Polymerase chain reaction (PCR) technique is used to identify genetic markers that are characteristic to cell type. Total RNA (2 µg) was reverse transcribed using 1 µg oligo-dT primer with MMLV Reverse-Transcriptase (Promega) in a 40 µl reaction volume containing 1.25 mM dNTPs at 37^{o}C. PCR was performed using 1µl of cDNA in 50 µl reaction volume containing 15 pmol of each primer, 0.2 mM dNTPs and 1 unit *Taq* polymerase (Promega). Primer sequences used and conditions of these reactions were as follows:

β-actin-F: 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3';
β-actin-R:5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3' (60°C annealing, x23 cycles).
Oct4-F: 5'-CGACCATCTGCCGCTTTGAG-3';
Oct4-R: 5'-CCCCCTGTCCCCCATTCCTA-3' (60°C annealing, x23 cycles).
Sox2-F: 5'-CCCCCGGCGGCAATAGCA-3';

Sox2-R: 5'-TCGGCGCCGGGGAGATACAT-3' (60°C annealing, x38 cycles).
FGF4-F: 5'-CTACAACGCCTACGAGTCCTACA-3';
FGF4-R: 5'-GTTGCACCAGAAAAGTCAGAGTTG -3' (56°C annealing, x40 cycles).
Nanog-F: 5'-GCCTCAGCACCTACCTACCC-3'
Nanog-R: 5'-GGTTGCATGTTCATGGAGTAG-3' (60 annealing and x30 cycles).
Eomes-F: 5'-TCACCCCAACAGAGCGAAGAGG-3';
Eomes-R: 5'- AGAGATTTGATGGAAGGGGGTGTC-3' (57°C annealing, x35 cycles).
Cdx2-F: 5'-CCTCCGCTGGGCTTCATTCC-3';
Cdx2-R: 5'-TGGGGGTTCTGCAGTCTTTGGTC-3' (60°C annealing, x35 cycles);
HLA-G-F: 5'-GCGGCTACTACAACCAGAGC-3';
HLA-G-R: 5'-GCACATGGCACGTGTATCTC-3' (55°C annealing, x26 cycles).
CD9-F: 5'- TTGGACTATGGCTCCGATTC-3';
CD9-R: 5'-GATGGCATCAGGACAGGACT-3' (55°C annealing, x26 cycles).
CK7-F: 5'-ACAGAGCTGCAGTCCCAGAT-3';
CK7-R: 5'-GTAGGTGGCGATCTCGATGT-3' (55°C annealing, x26 cycles).

Fluorescence activated cell sorting (FACS)

Trophoblast cells were prepared for cell sorting by dissociating CTBS cells into single cells with Trypsin-EDTA. Cells were resuspended at 5 $x10^6$/ml in FACS buffer with 40% normal goat serum to block on ice for 20 minutes. 90 µl of the cell suspension were aliquoted into FACS tube and 10 µl of G233 (TS marker for HLA-G) and W6/32 HLA-Class I control was added. G233 supernatant with NaN_3 (mouse IgG_{2a}) was kindly given by Dr Ashley King, University of Cambridge. The cells were incubated on ice for 30 minutes. After incubation, the cells were washed twice before being labelled with anti-mouse polyvalent immunoglobulin FITC conjugate for 30 minutes on ice. The cells were washed again and resuspended in 300 µl buffer.

Determination of hCGβ concentration in cell cultures.

Concentrations of hCGβ were determined using a sandwich enzyme immunoassay kit (Cat. # EIA-1469, DRG Diagnostics). The standards and the samples were incubated with 100 µl anti-hCG enzyme-conjugate for 30 minutes at room temperature followed by a five times washing procedure. A second incubation with 100 µl substrate solution for 10 minutes was stopped with the addition of 50 µl stop solution. Absorbance was read at 450 ± 10 nm with a microtitre plate reader. The concentration of hCGβ in the samples was determined from the standard curve as m I.U./ml.

Constitutive expression of eGFP in HESCs

A pCAG-GFP expression vector was constructed by excision of eGFP from pEGFP-1 (Clontech) with XhoI and NotI and insertion into the pCAG vector. H7 cells were seeded at the equivalent of $2x10^5$ per well of a 6-well plate on Matrigel. The next day they were transfected using ExGen 500 (Fermentas) according to the manufacturer's instructions. The DNA/NaCl Exgen mixture was then added directly to the normal growth medium in the well. The plate was centrifuged at 280 g for 5 minutes and placed back in the incubator. The medium was exchanged the next day with hES growth medium containing puromycin (at 1ug/ml). Viable colonies were picked after 2-3 weeks.

Endometrial – CTBS spheroid co-culture.
Luteal phase endometrial biopsies were obtained from women undergoing hysterectomy under full ethical approval and patient consent. Endometrial epithelial and stromal cells were isolated using a method described previously. Preparations were embedded in Matrigel on membrane inserts and primed with progesterone for 24 hours before the start of co-culture with CTBS. In monolayer co-culture, CTBS spheroids were cultured on a confluent layer of stromal or epithelial cells in culture wells. The co-cultures were maintained in 500 μl of either conditioned TS medium or serum-free HES medium up to six days.

Time-lapse Microscopy
Adherent CTBS cultures or CTBS–endometrial co-cultures were continuously monitored under an inverted microscope in a humidified physiological chamber at 37°C in 5% CO_2 in air (DigitalPixel Ltd) for up to three days. Preliminary experiments indicated no difference in the viability of cells maintained under these conditions compared to a standard incubator. Regions of interest (ROI) were identified and programmed for analysis using Simple PCI software (C-Imaging) with control over xyz scan, transmitted light, and image capture. Routinely 20 ROIs were identified with image capture every 15 minutes.

1. Movie of adherent multinuclear TS cells exhibiting cell fusion
2. Movie of TS vesicle attached and migrating on endometrial stromal cells in co-culture and displaying erosion site.

Figure 1. A comparison in the pathways of early differentiation of mouse and human embryos to trophoblast stem cells.

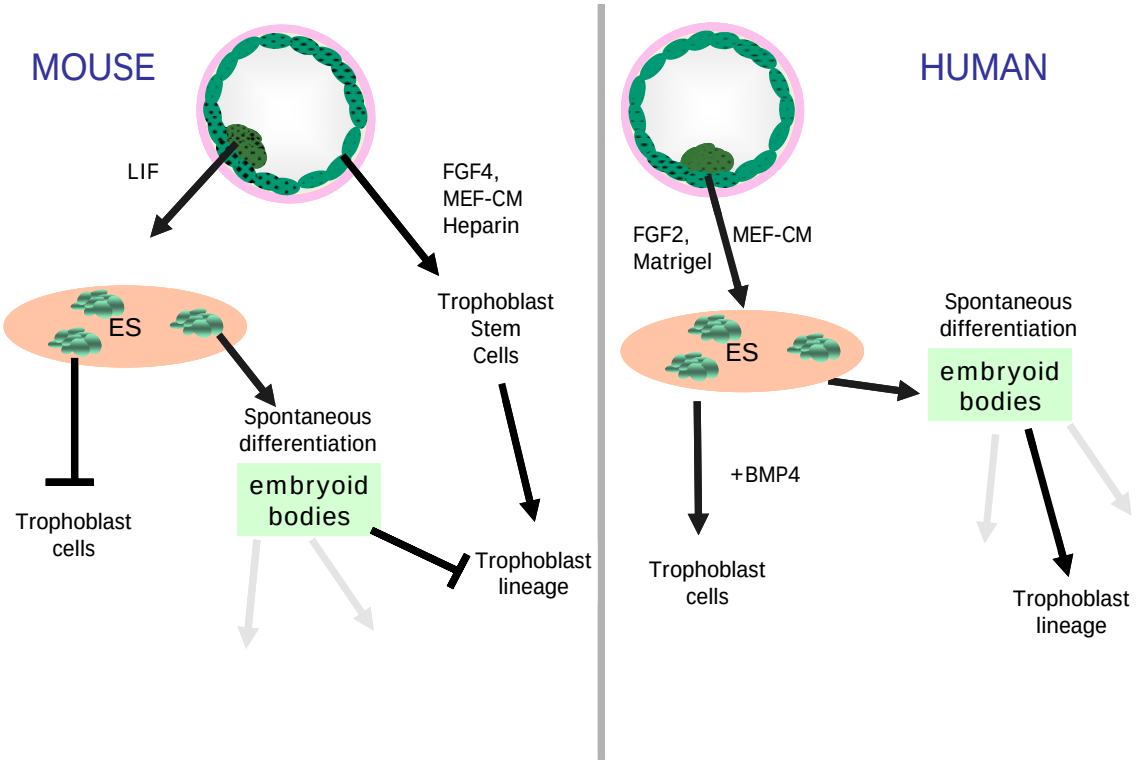
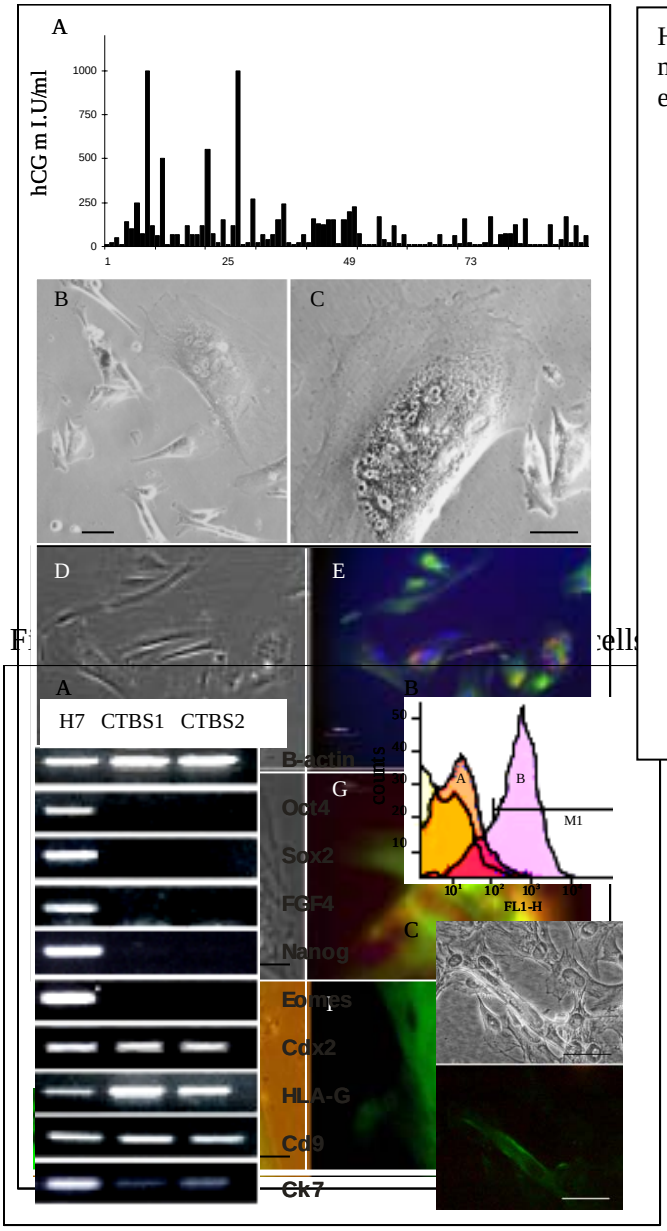


Figure 2. Derivation and initial characteracterisation of human CTBS cell lines



Histogram of hCGβ concentration in culture medium in 96-wells containing single embryoid bodies.

(A) Adherent epithelial CTBS cells in 'TS' culture without feeder cells. Bars = 20 μm throughout

(B) Adherent multinucleated syncytiotrophoblast in same culture as (B).

(C) & (E) adherent CTBS cells under phase contrast and UV light displaying cytokeratin (green) and hCGβ (red/orange) immunolocalisation (nucleus, blue).

(F) & (G) Single CTBS cells fusing to adherent multinucleated syncytium under phase contrast and UV. Single cells mainly cytokeratin $^{+}$ and syncytium mainly hCGβ^{+}.

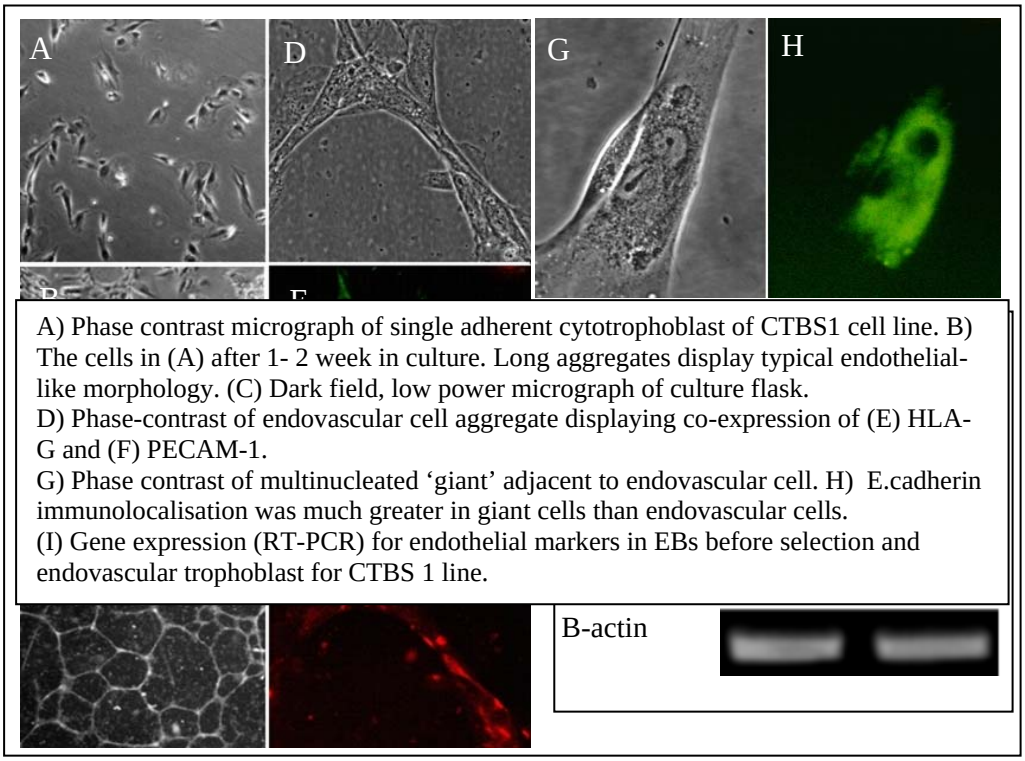
(H) Adherent GFP-syncytial trophoblast (phase contrast and UV) and (I) UV

(A) Gene expression (RT-PCR) for undifferentiated HESCs (H7) and CTBS 1 and 2 cell lines.

(B) FACS analysis profile for CTBS2 (similar data for CTBS1 not shown) in early culture. A proportion of cells express non classical HLA-G (peak A) while the majority express all forms of HLA class 1.

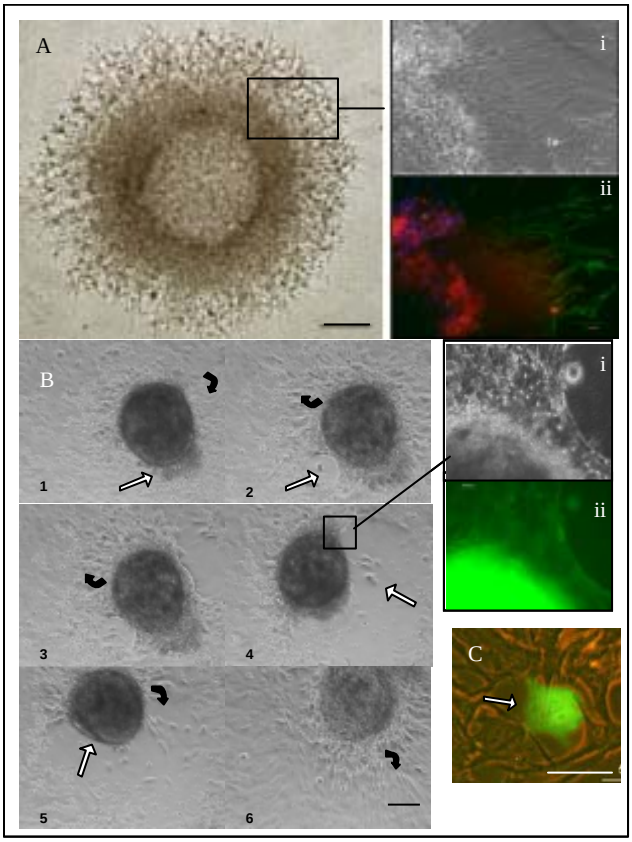
(C) Phase-contrast and immunofluorescent labelling of cells used for FACs analysis indicating HLA-G staining. Bar = 20 μm

Figure 4. Differentiation of CTBS cell line to endovascular cells in 'TS' conditioned medium.



A) Phase contrast micrograph of single adherent cytotrophoblast of CTBS1 cell line. B) The cells in (A) after 1- 2 week in culture. Long aggregates display typical endothelial-like morphology. (C) Dark field, low power micrograph of culture flask.
D) Phase-contrast of endovascular cell aggregate displaying co-expression of (E) HLA-G and (F) PECAM-1.
G) Phase contrast of multinucleated 'giant' adjacent to endovascular cell. H) E.cadherin immunolocalisation was much greater in giant cells than endovascular cells.
(I) Gene expression (RT-PCR) for endothelial markers in EBs before selection and endovascular trophoblast for CTBS 1 line.

Figure 5. CTBS spheroids in extracellular matrix and endometrial co-culture.

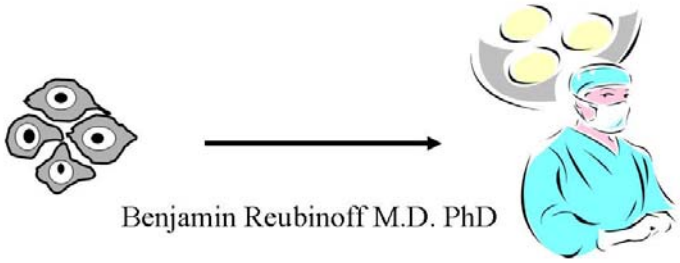


(A) CTBS spheroid (CTBS1 cell line) in Matrigel culture for 5 days with long microvilli protrusions of syncytium. Inset (i) and (ii): higher magnification phase contrast and immunostaining displaying cytokeratin(green) and hCGβ (red) in syncytial bed. Bar = 100 μm

(B) Images from time-lapse movie (see supplementary information) of CTBS1 aggregate in co-culture with endometrial stromal cells; bar = 150 μm. Black arrow throughout indicates direction of migration of vesicle. (1), white arrow indicates invasive cytotrophoblast outgrowth; (4 and 5), white arrow indicates stromal erosion site; (4) inset (i) and (ii), higher magnification of margin at erosion site showing phase contrast and MMP-2 immunolocalisation.

(C) CTBS - GFP cells in co-culture with endometrial stroma; bar =20 μm.

HUMAN EMBRYONIC STEM CELLS- FOR NEURAL REPAIR



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The Hadassah Human Embryonic Stem Cell Research Center
The Goldyne Savad Institute of Gene Therapy &
The Obstetrics & Gynecology Department
Hadassah University Hospital

hESCs for neural repair

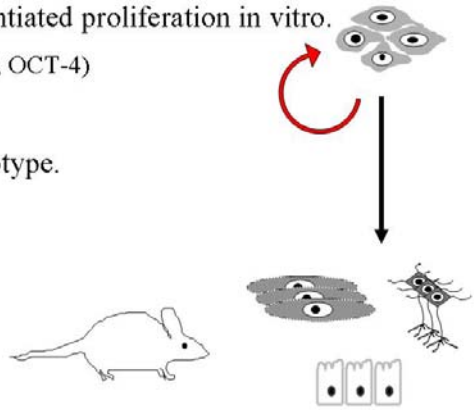
Learning Objectives:

To understand:

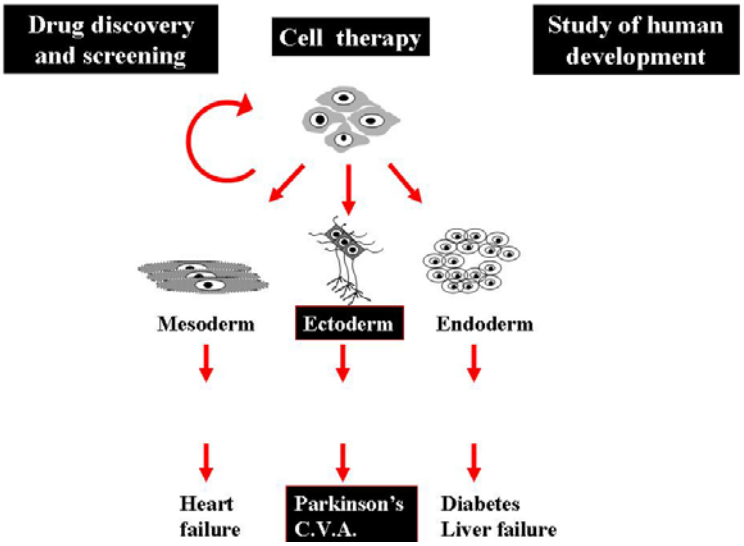
- The potential of hESC for neural repair
- The obstacles that need to be overcome
- Current status with regard to potential solutions

THE KEY PROPERTIES OF EMBRYONIC STEM CELL LINES

- Indefinite undifferentiated proliferation in vitro.
  (stem cell markers, AP, OCT-4)
- Stable normal karyotype.
- Pluripotency.



HUMAN EMBRYONIC STEM CELLS – THE POTENTIAL!!



POTENTIAL APPLICATIONS OF HUMAN ES CELLS THERAPY:

Parkinson's disease
Huntington's disease
Alzheimer's disease

Multiple sclerosis

Stroke

Spinal cord injuries
ALS

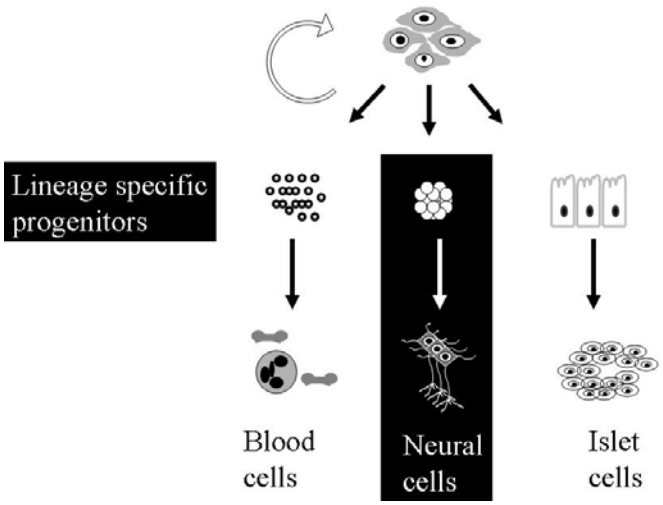
Macular degeneration

POTENTIAL APPLICATIONS OF HUMAN ES CELLS THERAPY:

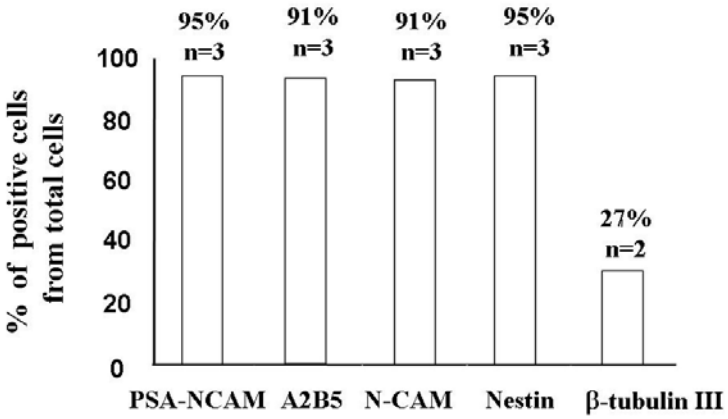
- Over 16,000,000 patients worldwide suffer from neurodegenerative disorders.

- Over 1,000,000 patients in the USA suffer from Parkinson's disease.

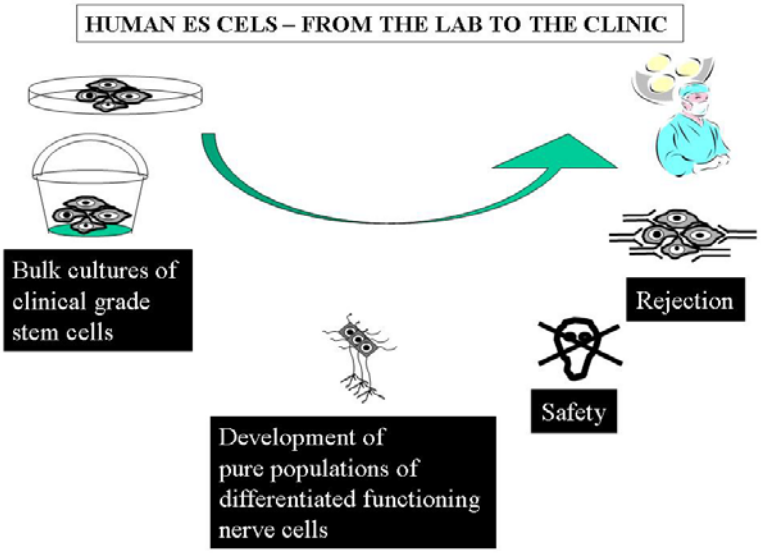
Generation of lineage specific progenitors



Immuno phenotype of spheres



Neural progenitor markers



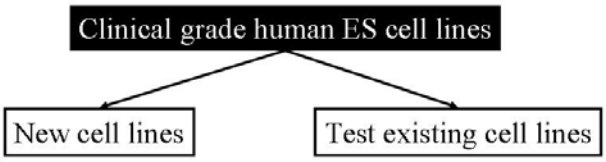
Clinical grade human ES cell lines

Current cell lines are not suitable for clinical trials

1. Mouse feeders –
   xenotransplantation

2. Inappropriate documentation of derivation processes

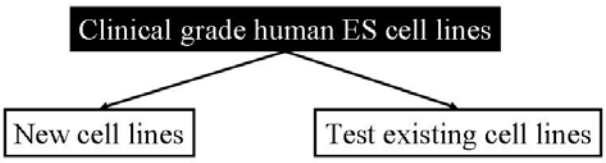
3. Inappropriate culture system



1. Human feeders / feeder free

2. Appropriate animal-free
   reagents

1. GMP facility

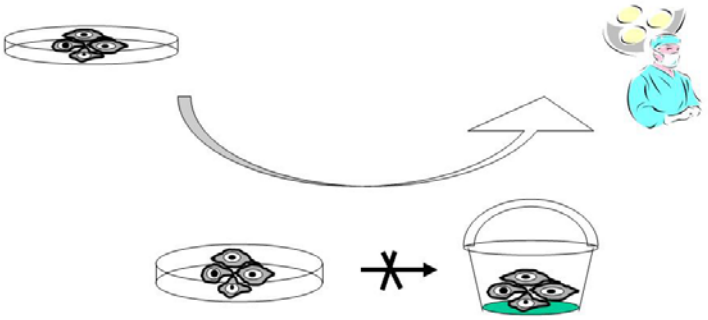


1. Human feeders / feeder free

2. Appropriate animal-free
   reagents

1. GMP facility

HUMAN ES CELS – FROM THE LAB TO FUTURISTIC MEDICINE



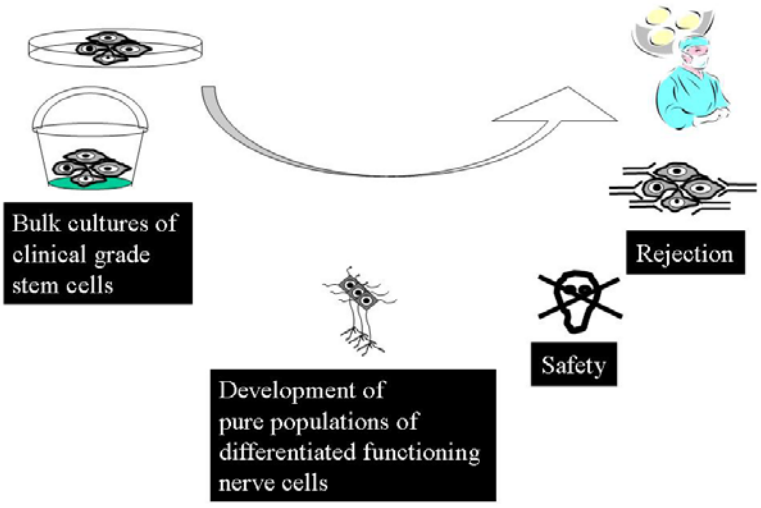
Limitations of the culture system.

Factors suggested to support feeder free cultures

1. **TGFβ-1+ LIF + FGF2** on fibronectin
   (Amit et al., 2004)
2. Activation of **WINT** signaling
   (Sato et al., 2004)
3. High **FGF2 +/- noggin** on matrigell
   (Xu et al., 2005)
4. **Activin** and **FGF2**
   (Vallier et al 2005)
5. **TGFβ-1 + FGF2+ LiCl+GABA+pipecolic acid**
   (Ludwig et al., 2006)

Bulk culture

HUMAN ES CELS – FROM THE LAB TO THE CLINIC

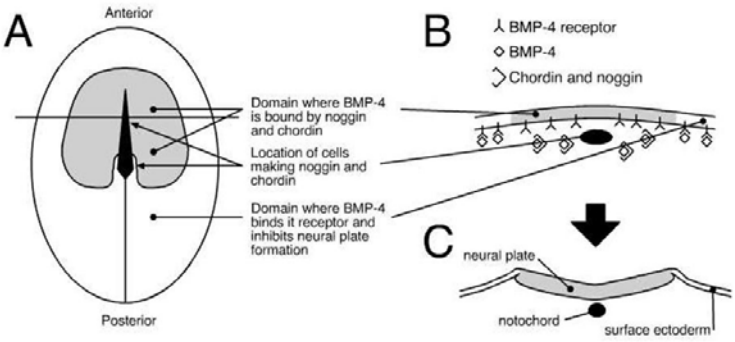


Human ESCs for neuronal repair

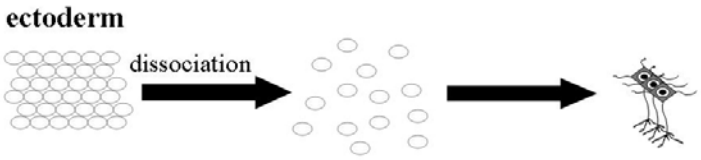
Objective:

To develop a strategy for the controlled conversion of hESCs into neural precursors in defined culture conditions.

Embryonic neural induction

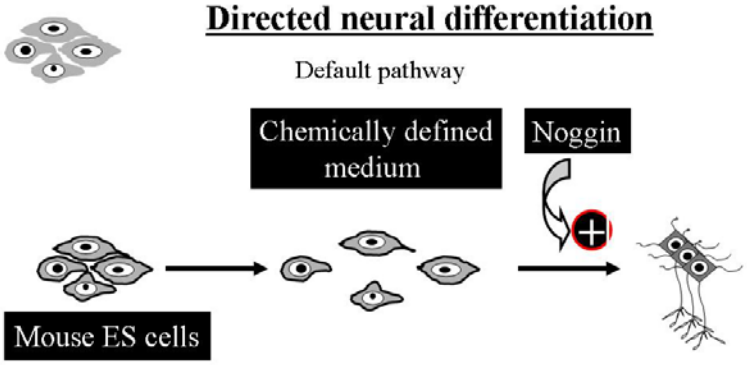


The default model of neural induction *(Xenopus)*



Dissociated cells avoid paracrine endogenous BMP-like activity

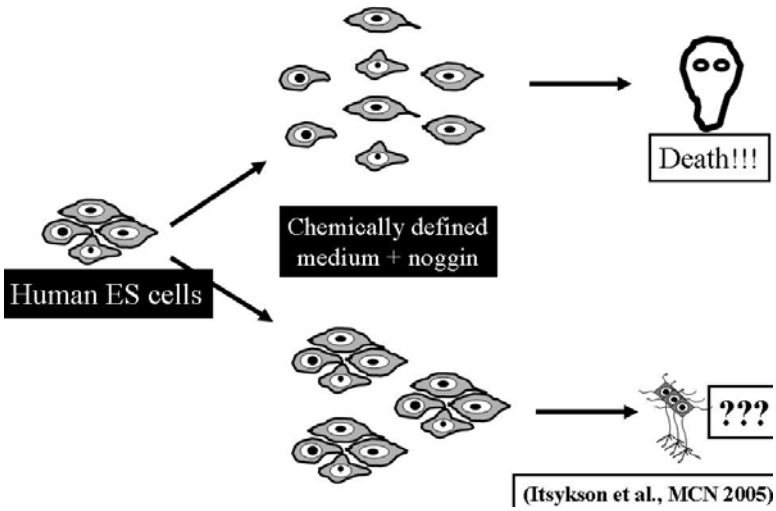
Directed neural differentiation



Dissociated mouse ES cells avoid paracrine endogenous BMP-like activity

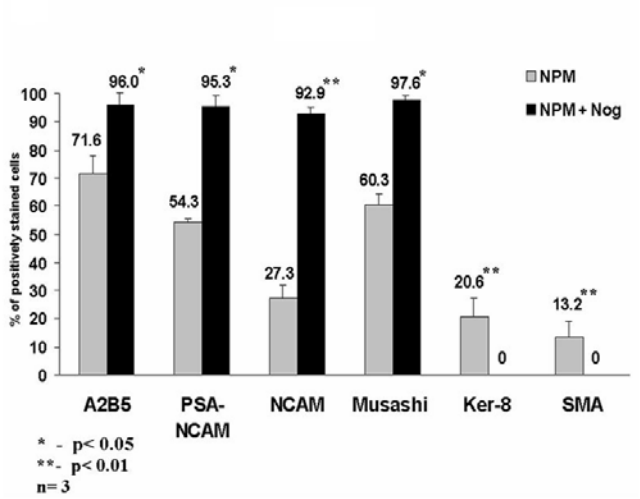
(Tropepe et al Neuron 2001)

Controlled conversion of hESCs into neural precursors



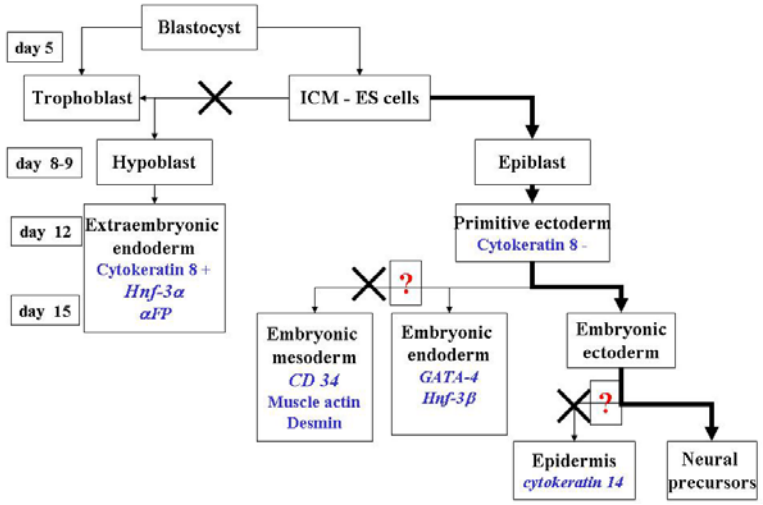
(Itsykson et al., MCN 2005)

Controlled conversion of hESCs into neural precursors

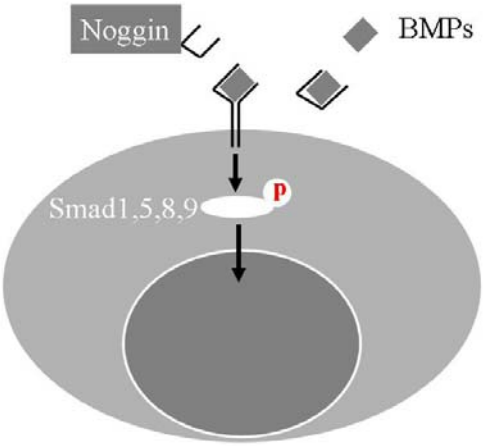


(Itsykson et al., MCN 2005)

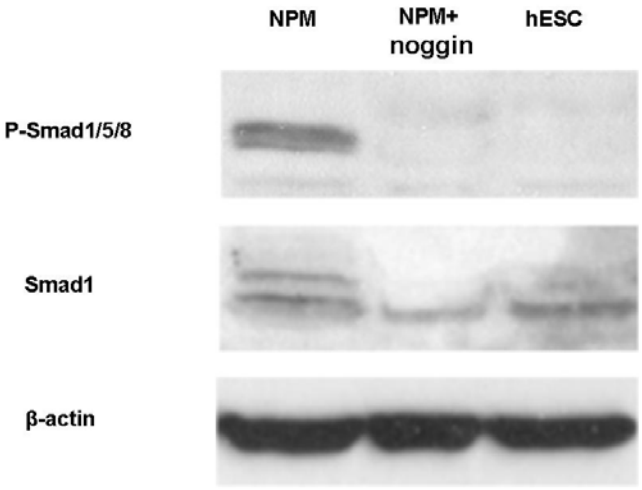
Controlled conversion of hES cells into neural precursors



BMPs signal transduction pathway

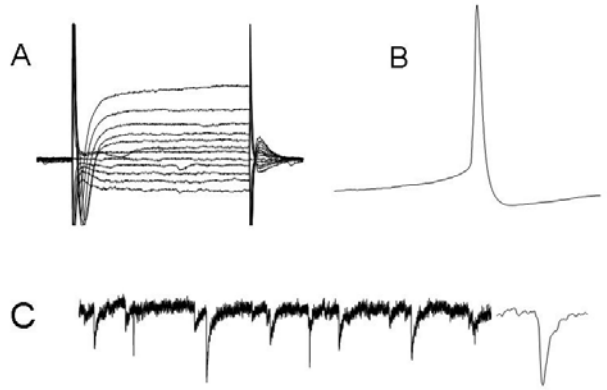


Noggin prevents the phosphorilation of Smad1/5/8



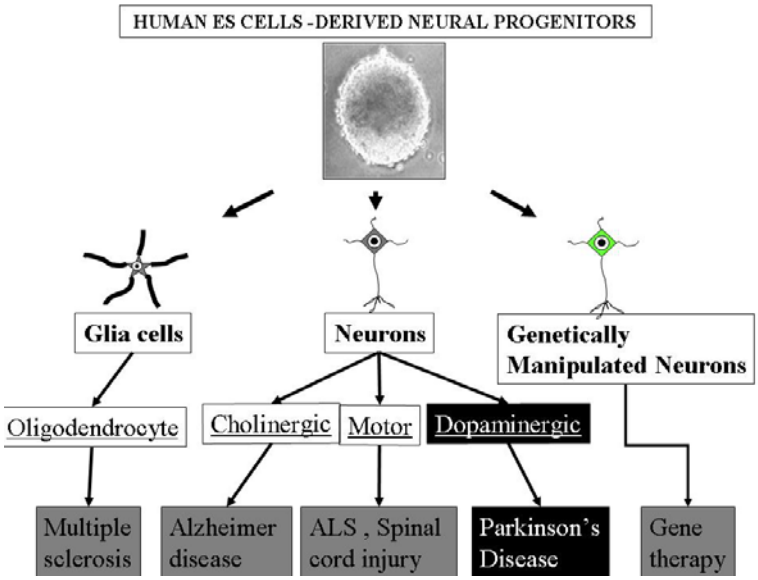
(Itsykson et al., MCN 2005)

Differentiation into mature functional neurons



A. IV curve
B. Action potential discharge
C. Spontaneous synaptic activity

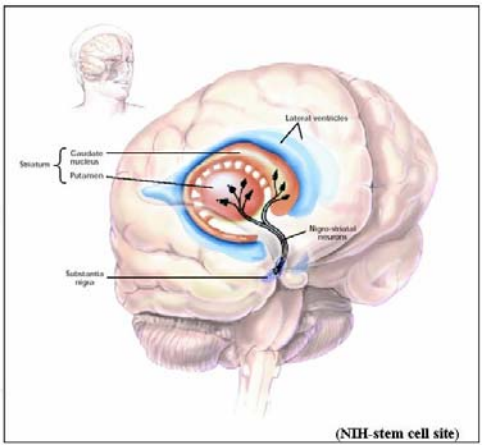
(Itsykson et al., MCN 2005)



Parkinson's disease

The second most common neurodegenerative disorder

Results from:
Degeneration of dopaminergic neurons in the midbrain.



Parkinson's disease

Characterized by:

- Tremor at rest
- Muscle rigidity
- Postural instability
- Inability to perform routine movements

No cure!!!

L-DOPA – Transient effect
Side effects

Transplantation of dopaminergic neurons from aborted fetuses:
Clinical benefit in some patients
Impractical
Ethical problems

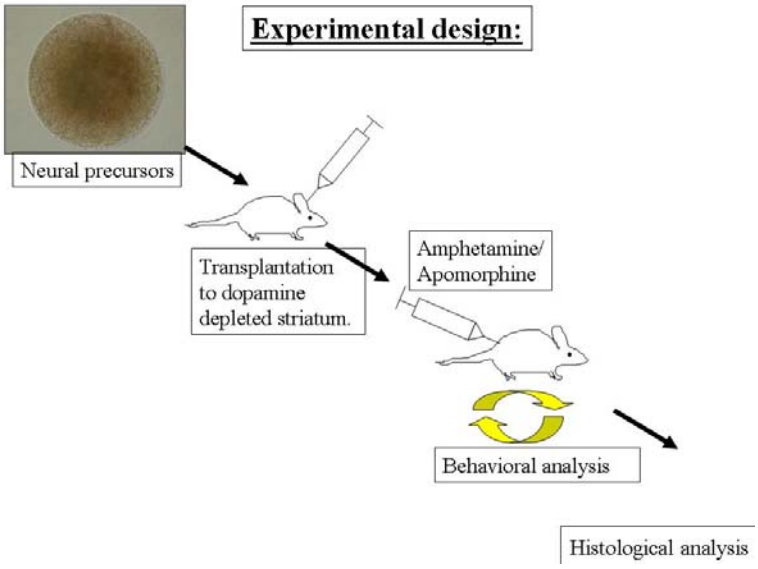
Research question

To test following transplantation of the neural precursors to the midbrain of Parkinsonian rats:

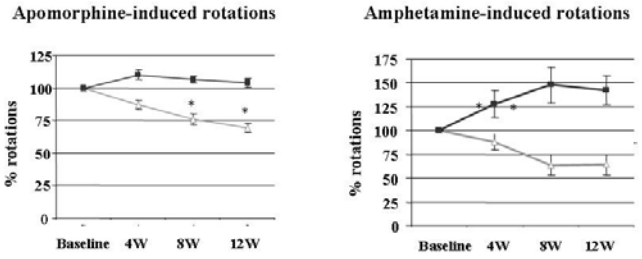
1. Whether the striatal micro-environment will promote the differentiation of the transplanted neural precursors towards a dopaminergic fate.

2. Whether the transplanted neural precursors will induce functional recovery of the rats.

Experimental design:



Pharmacological behavioral tests



▪ Controls
△ NPs transplanted

(Ben-Hur et al., 2004)

Conclusions:

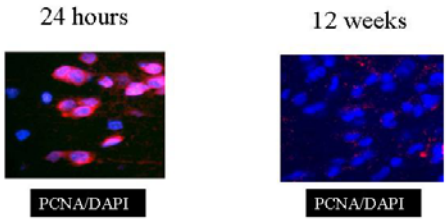
The host lesioned striatum could not direct the transplanted neural precursors to acquire a dopaminergic fate.

Induction of differentiation towards a midbrain fate prior to transplantation is probably required for complete correction of behavioral deficit.

Safety!!!!

Teratoma formation or non-neural tissues were not observed in any of the host brains.

With time, transplanted cells cease to proliferate.



Conclusions:

Human ESCs may be converted into developmentally competent neural precursors, in a controlled fashion and under defined culture conditions.

Human ESC-derived neural precursors can induce partial functional recovery in an experimental model of Parkinson's disease.

Human ESCs for neuronal repair

Conclusions:

This data set the stage for further developments

that may eventually allow the exploitation

of hESCs in cell and gene therapy of the CNS

as well as in basic neuroscience.

Human ESCs for Parkinson's Disease

Conclusions:

Nevertheless,
new cell lines eligible for clinical trials,
improved control of the growth and differentiation,
rigorous evaluation of safety issues,
are required to allow the exploitation of hESs for therapeutic purposes.

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Stem cells for cardiac repair

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Spontaneous cardiac regeneration occurs in zebrafish after surgical resection of the left ventricle [1]. In rodents, the potential of bone marrow cells of becoming cardiomyocytes is yet controversial [2,3]. In humans, damaged myocardium is irreversibly replaced by a scar. Recent findings suggest, however, that cardiomyocytes may proliferate in end-stage cardiac failure [4] and after myocardial infarction [5].

Cardiac chimerism: 'proof of concept' of cardiac regeneration in humans
Experiments performed in recipients of organs donated by other humans (human chimerism) have shed light about the regenerative potential of the human heart. Investigators have followed the trace of the Y chromosome, by FISH and/or PCR, in sex-mismatch transplants. Quaini *et al* examined hearts from female donors that had been transplanted into male recipients and found that ~10% of the myocytes contained a Y chromosome [6]. This finding was only partially supported by other groups that found significantly lower post-transplant chimerism [7]. The reported differences in the magnitude of cardiac chimerism could be due either to intrinsic biological characteristics of the specimens examined or to differences in the technical approach. To assess whether the source of these cells was the bone marrow, Deb *et al* studied the heart of female patients who had received male donor bone marrow and died of non-cardiac causes [8]. After examination of 80,000 cardiomyocyte nuclei they found a mean percentage of Y chromosome-positive cardiac myocytes of 0.23±0.06%. Bayes-Genis *et al* examined the presence of cardiac chimerism after peripheral-blood stem cell transplantation (previously mobilized by G-CSF) by means of PCR of highly polymorphic short tandem (STR) markers [9]. Gender and non-gender STR donor alleles were identified in the recipient heart. These data suggest that adult human bone marrow may be a source of extracardiac progenitor cells contributing to cardiomyocyte formation.

Autologous adult stem cells for cardiac regeneration after myocardial infarction.
The revolutionary concept of human cardiac regeneration by bone marrow-derived cells was immediately attempted in patients to replace damaged myocardium after myocardial infarction (MI). Investigators obtained mononuclear cells from bone marrow aspirates and injected them intracoronarily in the acute and sub-acute phase of MI. Preliminary, pilot and non-randomized studies suggested a positive effect of these cells in left ventricular ejection fraction and reverse remodeling [10,11]. However, two recent randomized studies have shed serious concerns about this therapy [12,13]. Janssens *et al* did a randomized, double-blind, placebo-controlled study in 67 patients from whom they harvested bone marrow after successful percutaneous coronary intervention for ST-elevation MI. They assigned patients optimum medical treatment and infusion of

placebo (n=34) or bone marrow stem cells (n=33). After 4-months follow-up, the investigators concluded that intracoronary transfer of autologous bone marrow cells within 24 hours of optimum reperfusion therapy did not augment recovery of global left ventricular function after MI.
A different and apparently simple approach was the mobilization of bone marrow stem cells by circulating cytokines. However, the results of the REVIVAL-2 trial recently published indicate that stem cell mobilization by G-CSF therapy in patients with acute MI and successful mechanical reperfusion has no influence on infarct size, left ventricular function, or coronary restenosis [14]. Perhaps only a small fraction of mobilized stem cells homed the infarcted myocardium. Homing and engraftment of mobilized stem cells into the site of myocardial injury displays a fundamental step in cell-based regeneration of the injured myocardium [15].

Fetal and cord-blood somatic stem cells.
Cardiac chimerism after pregnancy of a male offspring (fetal cell microchimerism) has also been demonstrated indicating the existence of cells of fetal origin capable of differentiating into cardiomyocytes [16]. The search for human somatic stem cells from placental cord blood with such properties has yielded erncouraging preliminary results. A population of pluripotent, CD45-negative population of cells from human cord blood, termed unrestricted somatic stem cells (USSC) has been described [17]. This cord blood stem cell population differentiated into osteoblasts, chondrocytes, adipocytes and myocytes both *in vitro* and *in vivo* in various animal models. These cells, when tested for the major transplantation antigens, may serve as an allogeneic stem cell source for the future development of cellular therapy for tissue repair and tissue regeneration.
In conclusion, the human heart has limited regenerative capacity, only to maintain normal cardiac homeostasis, but it is unable to meet a long-term demand for cardiac repair after injury. We need to obtain extensive knowledge about adult (mesenchymal stem cells, endothelial progenitors, adipose-derived stem cells, MAPCs) and fetal-embryonic stem cells (USSCs) capable of restoring the human heart before we can propose such therapeutic option to our patients.

References

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monash immunology and stem cell laboratories

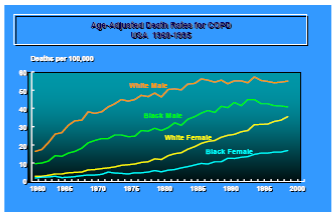
ESHRE The Future of Stem Cell Research

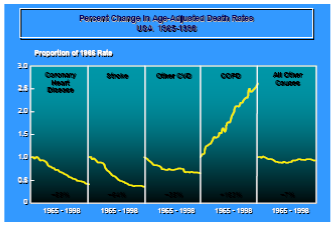
Alan Trounson

Australian Stem Cell Centre

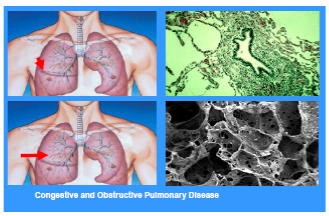
Stem Cells and Respiratory Disease

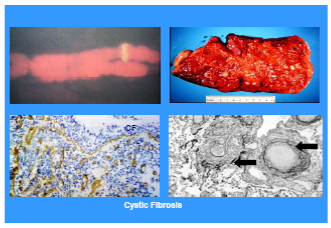
The Problem



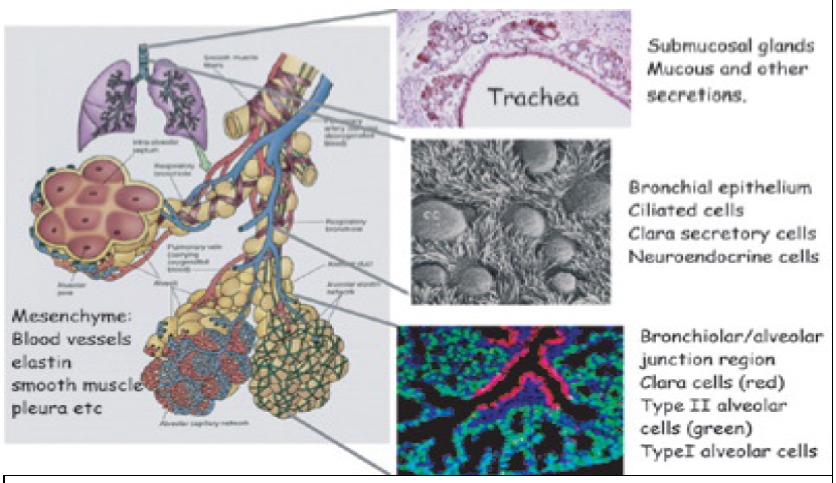


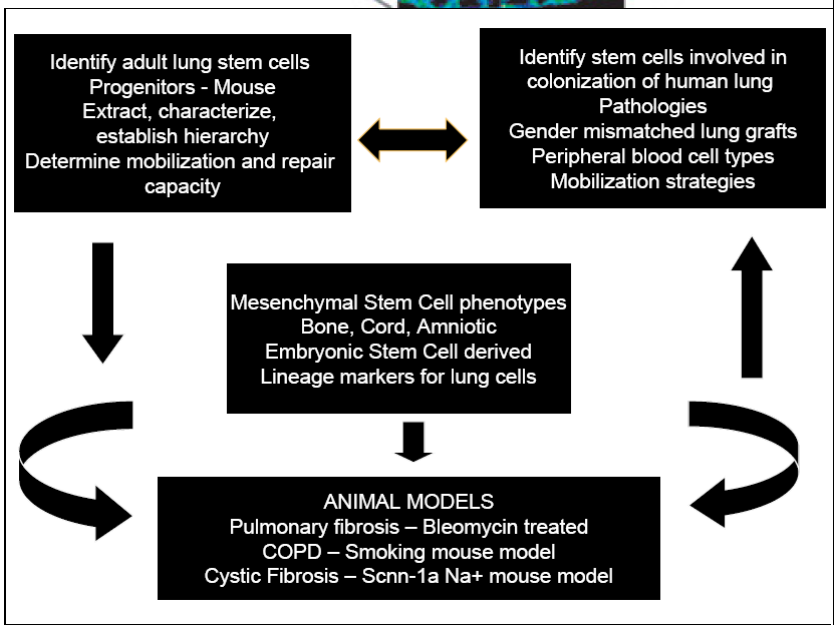
The Disease



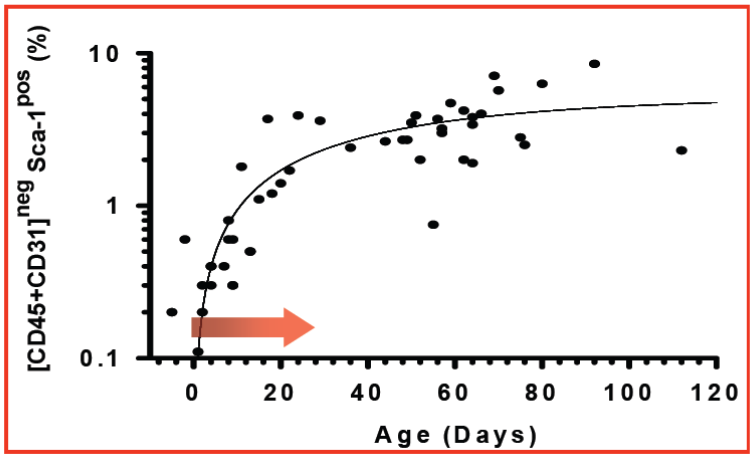


Lung Repair:
Key cell-types to be generated





Sca-1 antigen expression is a marker of alveolarization



Characterization of Stem Cells in the Adult Murine Lung

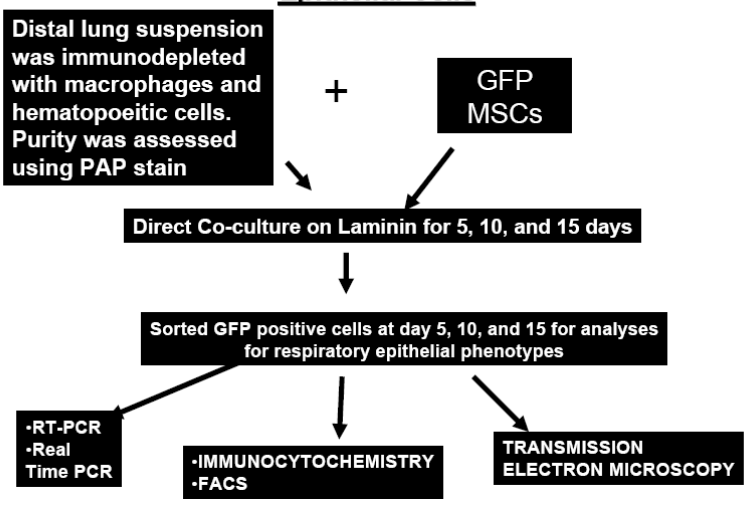
Summary:

- Sca-1 antigen expression is a marker of lung alveolarization
- [CD45+CD31]^{neg} - Sca-1^{pos} lung cells have a mesenchymal "signature" whereas the Sca-1^{neg} subset has a respiratory epithelial "signature"
- The majority of proliferative activity in culture resides in the [CD45+CD31]^{neg} - Sca-1^{pos} subset
  - growth patterns are heterogeneous
  - evidence of transplantation potential
- Developmental potential and hierarchical relationships remain to be determined.

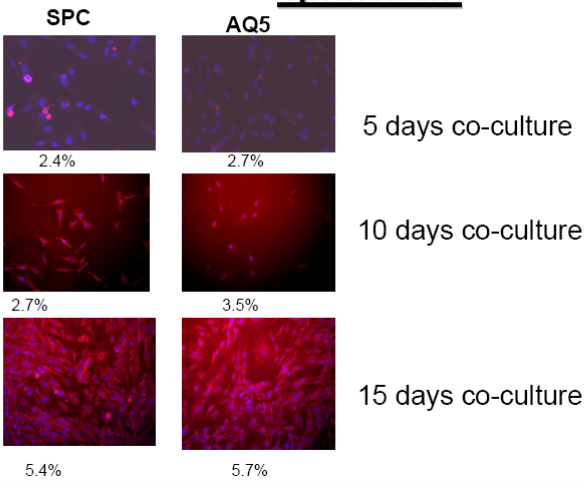
Mesenchymal Stem Cells

- Adult bone marrow stromal stem cells have been identified in damaged and grafted lung tissue
- The mouse MSC immuno-phenotype is similar to that of Sca+ putative stem cells of the adult lung
- BM MSCs may have significant trophic effects for lung function

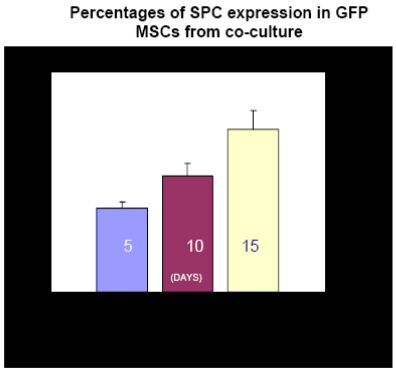
MSCs differentiation down the Respiratory Epithelial Lineage by co-culturing GFP MSCs with Respiratory Epithelial Cells

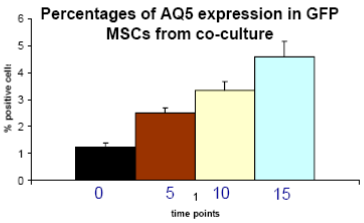


SPC and AQ5 immunocytochemistry MSCs in coculture with respiratory epithelium



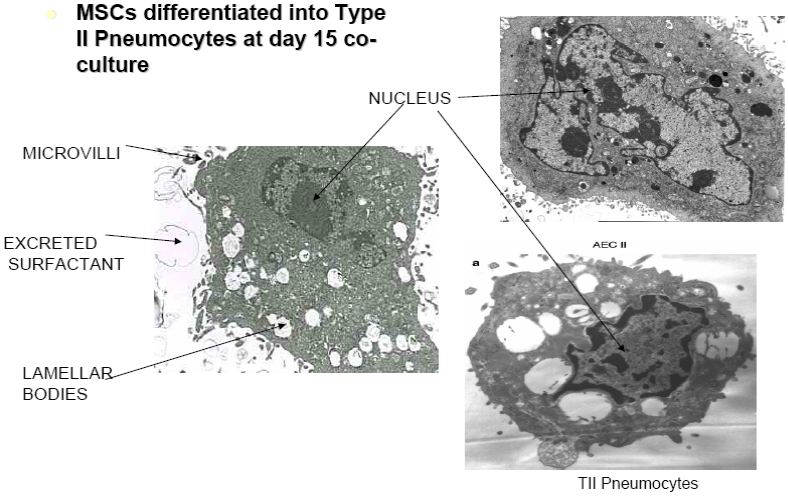
SPC and AQ5 expression in GFP MSCs from co-cultured with respiratory epithelium





Transmission Electron Microscopy

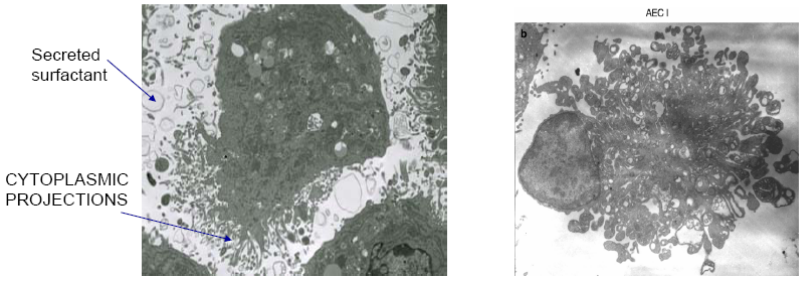
- **MSCs differentiated into Type II Pneumocytes at day 15 co-culture**



TII Pneumocytes
Chen et al, 2004

Transmission Electron Microscopy

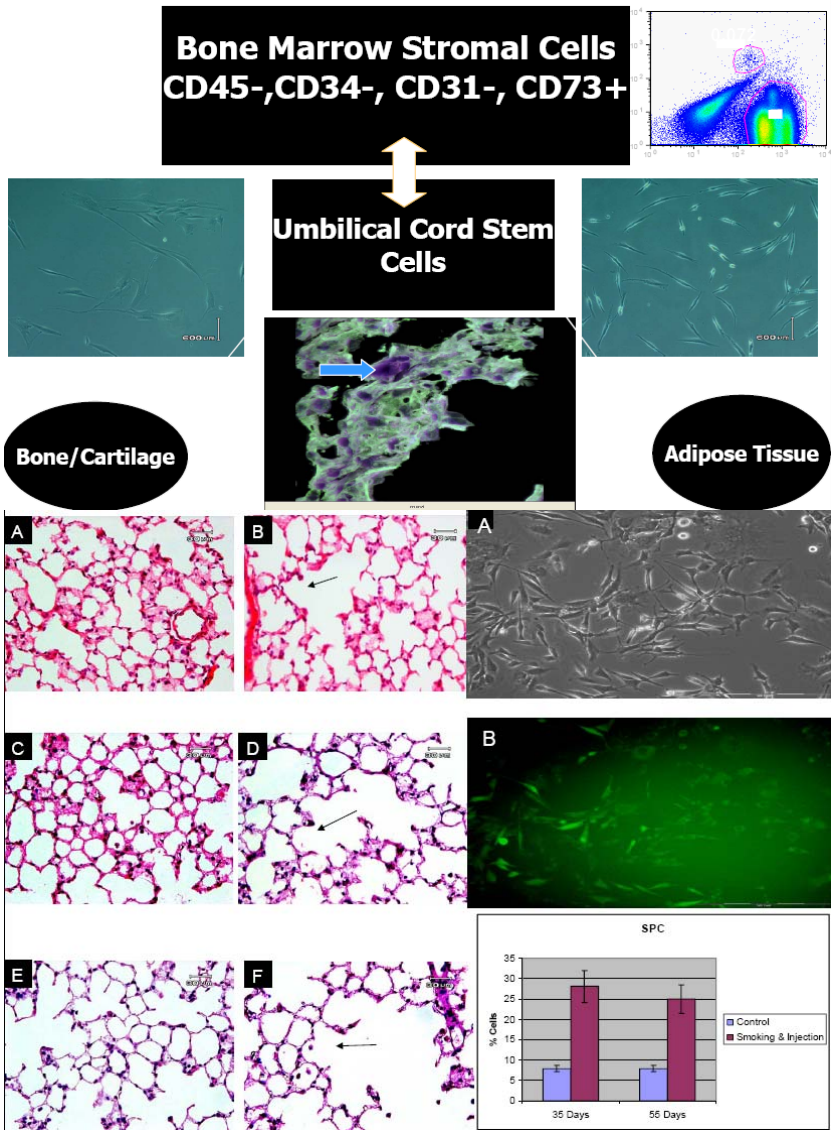
MSCs differentiate into Type I pneumocytes at day 15 of co-culture



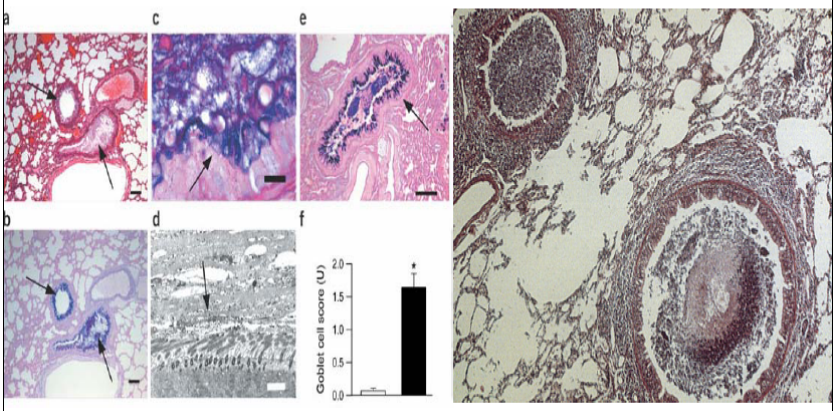
Type I Pneumocytes
Chen et al, 2004

•Electron Microscopy- 4-5% cells showed features according to the FACS data.

Mesenchymal stem cells



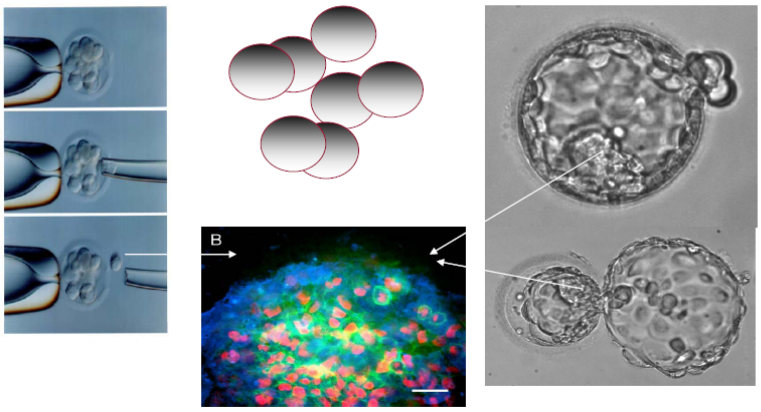
REPAIR OF UPPER AIRWAYS IN MODELS OF CYSTIC FIBROSIS



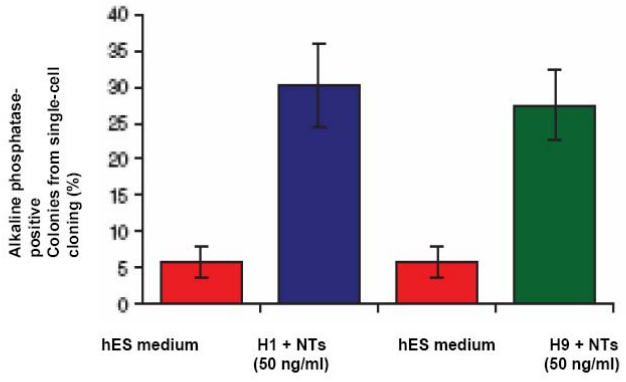
Scnn1b-transgenic Mice

Mall et al. 2004

Normal and Mutant Embryonic Stem Cells – **CF, HD, etc**



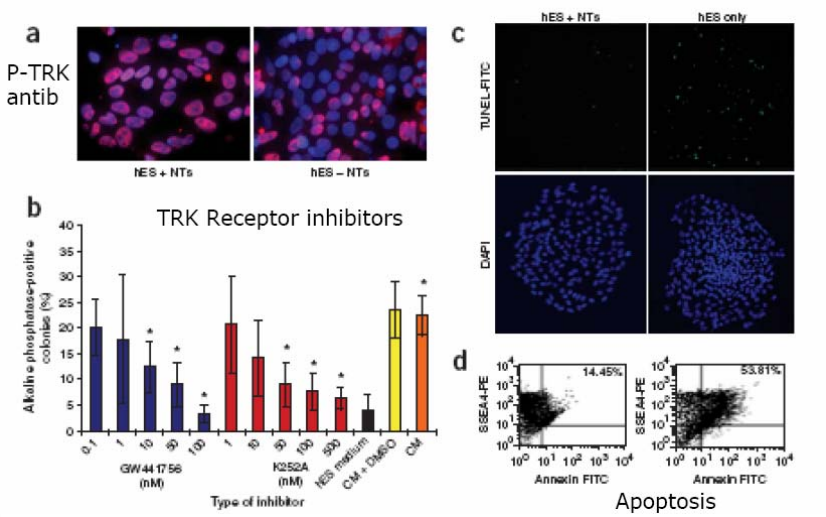
Neurotrophins and ES cell growth



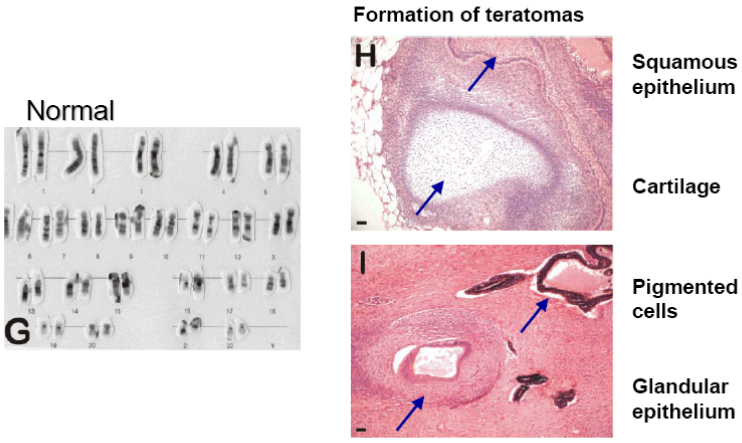
Pyle et al 2005

NTs-BDNF, NT3, NT4

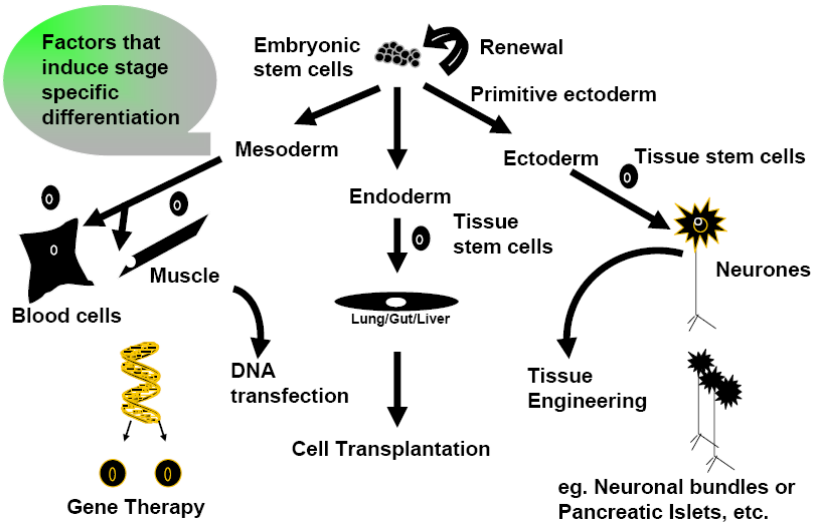
Inhibiting Neurotrophic Responses of hES Cells
Pyle et al (2005)



Long-term cultures retain normal karyotype and teratoma formation



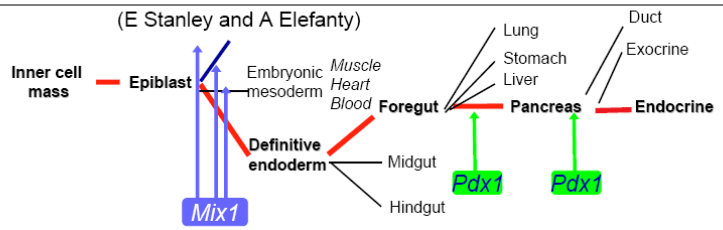
Stem Cell Pathway



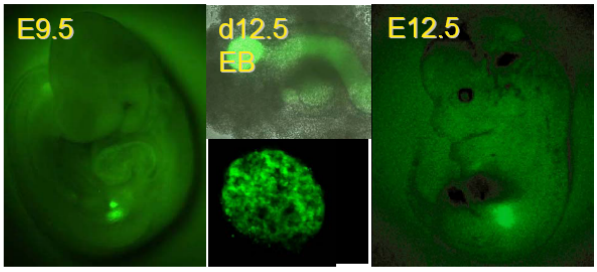
Replicating developmental pathways

- Knockin of fluorescent reporter genes into key endogenous development gene promoter regions
- Sequential expression of mesendoderm – definitive endoderm – patterning genes eg foregut regional indicator genes – tissue progenitors
- Full functional demonstration of endocrine capability and importantly regulation of hormone production

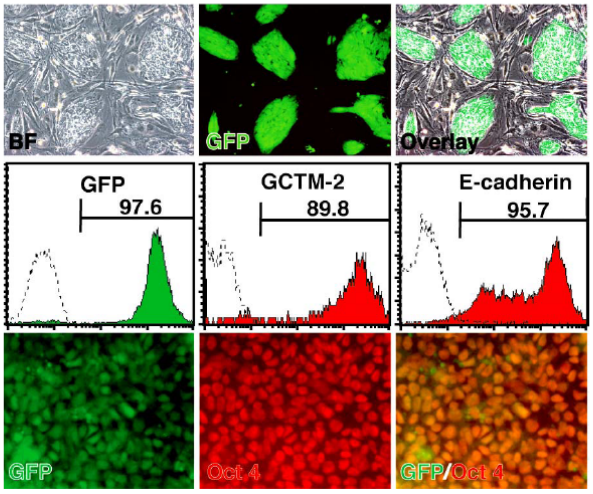
Mouse ES cells can be directed though pancreatic differentiation



Mouse ES cells differentiated in pancreatic cells express GFP from the pancreatic specific locus Pdx1.

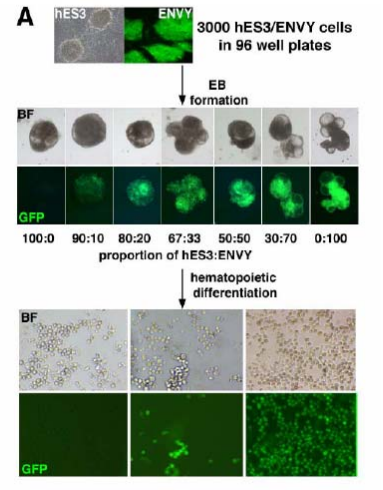


Tools for discovery – Green ESCs ENVY



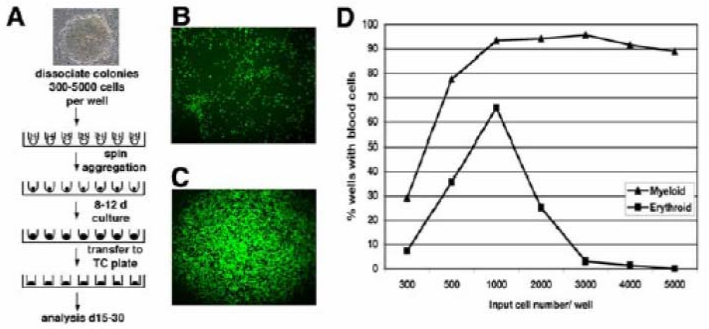
Differentiation of Human ES Cells

(Ng et al. 2005)



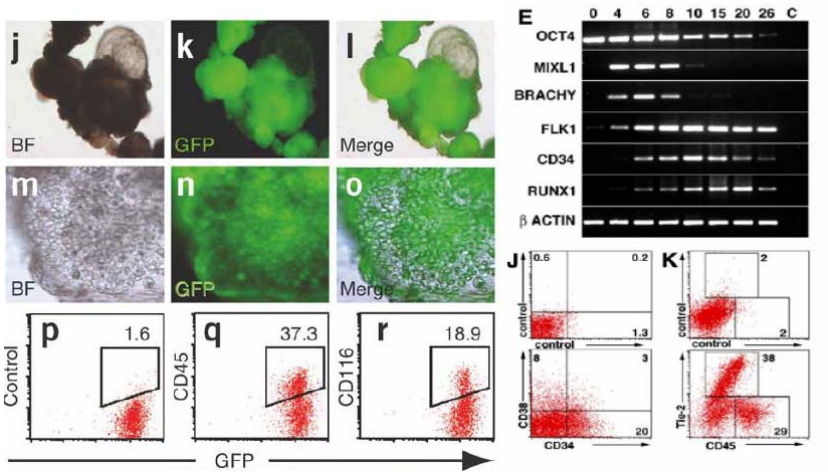
Formation of Spin-EBs and Differentiation into Blood Cells

(Ng et al. 2005)

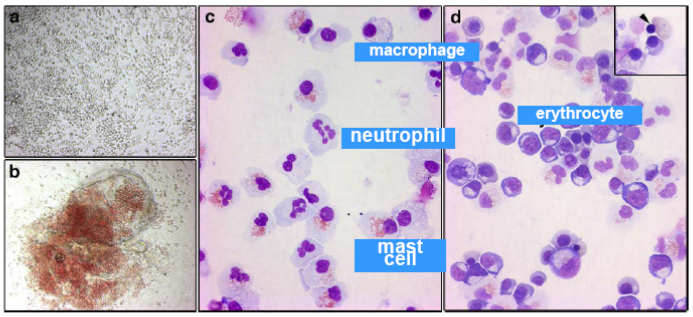


Blood Cell Differentiation of ENVY Cells

(Costa et al. 2005) (Ng et al. 2005)



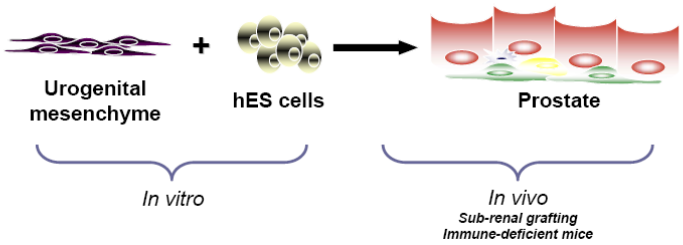
Generation of blood cells from human ES cells



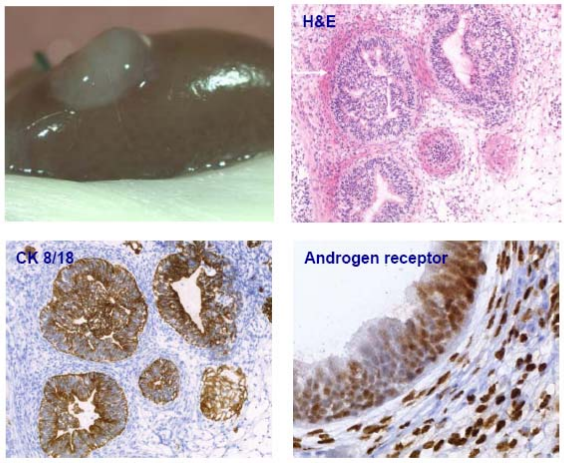
Multiple types of human of blood cells can be generated from human embryonic cells using directed differentiation

Directed differentiation of hESCs using tissue recombination

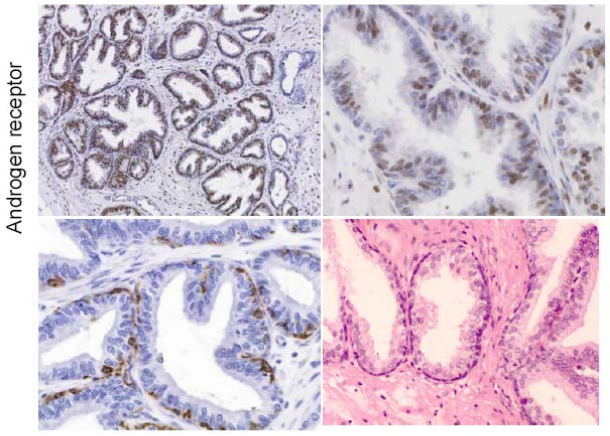
- Tissue recombination is based on the inductive/instructive potential of urogenital mesenchyme
- Kidney grafting provides a highly vascular site for grafts to grow for an extended period of time
- Cell-cell interactions are maintained and differentiation is dictated



Prostate grafts – 2 weeks in vivo

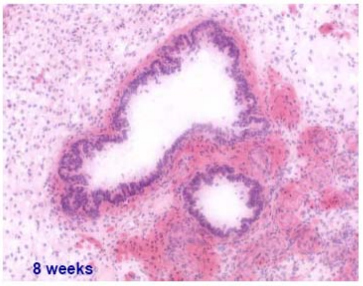


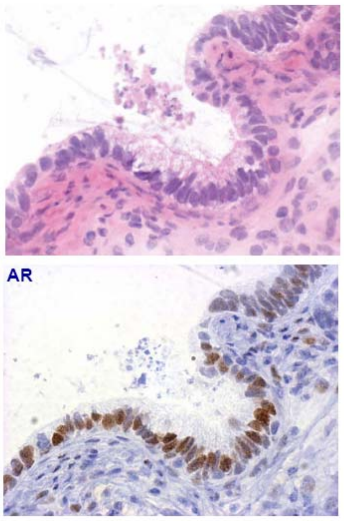
hES cell-derived prostate structures



- Ductal glandular structures
- Androgen receptor positive
- Cytodifferentiation of the epithelium
- Characteristic of normal human prostate

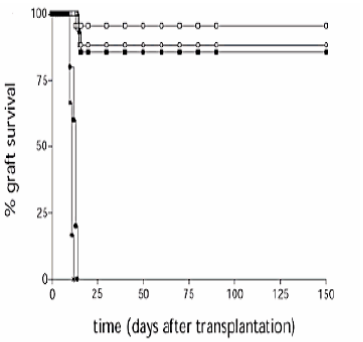
Prostate grafts

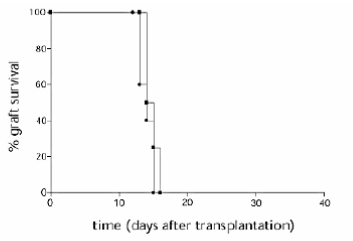


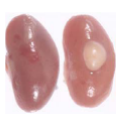


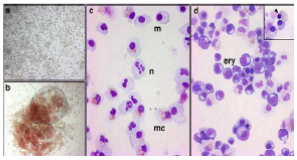
LONG-TERM ALLOGENEIC GRAFT ACCEPTANCE USING EMBRYONIC STEM CELL-LIKE CELLS

(Fandrich et al. Nature Medicine 8: 171 2002)



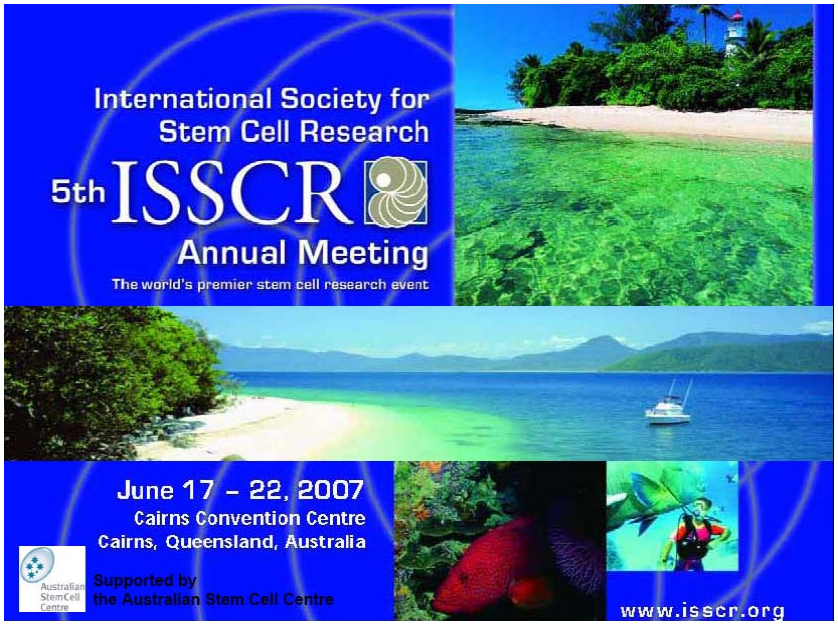






Acknowledgements

- Yuben Moodley, Ivan Bertoncello
- Tayfur Tecirlioglu
- Andrew Elefanty, Ed Stanley, Liz Ng, Magdaline Costa
- Renea Taylor
- Richard Boyd and colleagues



Stem Cell Research: EU Policy and Programme

Dr. Gwennaël Joliff-Botrel

Health Research Directorate
European Commission

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- **Stem Cell (SC) Research funded at the EU level**
- **The case of human embryonic stem cell (hESC) research / EU picture / worldwide picture & EU policy**
- **The future ⇒ FP7**

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SC research funded at EU level

FP5 (1998-2002)

- **~ 53 projects with at least one component of SC research :**
- **EC contribution ~ € 86 million** (not necessarily the SC research part).
- **Over 90% of projects involved somatic SC.**
- **2 projects also involved hESC.**

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SC research funded at EU level

FP5 (1998-2002)

- **Fundamental research on differentiation - neural SCs, mesodermal SCs, insulin-producing islets.**
- **Tissue engineering - cartilage, bone, skin.**
- **Haematopoietic SC therapy - bone marrow & cord blood transplantation.**
- **Ethical, legal & social research aspects**

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SC research funded at EU level

FP6 (2002-2006)

One call missing (~ ¼ of the projects)

- **more than 80 projects with at least one component of SC research :**
- **EC contribution ~ € 400 million** (not necessarily the SC research part).
- **about 90% of projects only involved somatic SC.**
- **9 projects also involved hESC.**

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SC research funded at EU level

FP6 (2002-2006)

~same research fields than in FP5, but more new developments:

- in Fundamental research (functional genomics, from animal ESC to hESC, epigenetics)
- in Tissue engineering (nanotechnologies)
- towards clinical applications
- 2 new topics: drug discovery & *in vitro* toxicology

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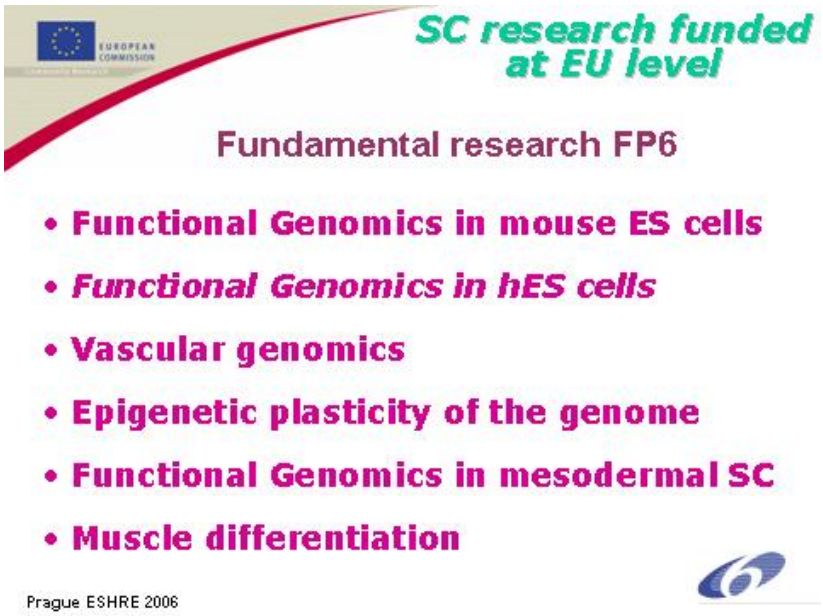
SC research funded at EU level
Fundamental research FP6
• Functional Genomics in mouse ES cells
• Functional Genomics in hES cells
• Vascular genomics
• Epigenetic plasticity of the genome
• Functional Genomics in mesodermal SC
• Muscle differentiation
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SC research funded at EU level
New therapies FP6
• Diabetes
• Spinal cord
• Heart repair
• Stroke
• Comparative research on SC for neuro/muscular/skin
• Umbilical Cord Blood
• Skin
• Immunotolerance
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SC research funded at EU level
Tissue engineering FP6
•Human corneas
•Intelligent biomaterial for cardiovascular repair
•Liver
•Bone and cartilage
•Bioartificial pancreas for diabetes
•New systems approach to tissue engineering
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SC research funded at EU level

Haematopoietic SC therapy

- **Haematological and neoplastic diseases**
- **Leukemia**
- **Improvement of radiotherapy of cancer**

In vitro technologies

- **Reproductive toxicity**
- **Short-term in vitro assays for long-term toxicity**

Ethical, legal & societal aspects

- **European patents law & ethics**

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SC research funded at EU level

Changes between FP5 & FP6

Change in size

- **significant increase of SC research funded at EU level (~ X 5)**

Change in nature

FP6 new instruments (~25 partners / ~ €10 million /i.e. ~ €400 000 per partner) help to:

- **Comparison of SC from different origins**
- **Translational research**

⇒ Stem cell research is a field where EU added value is clear

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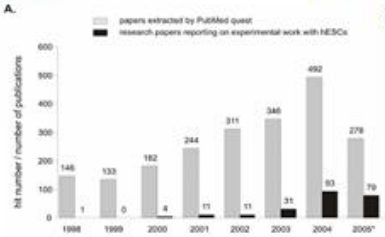
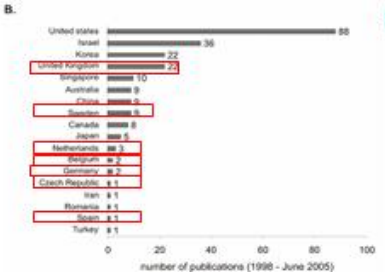
hESC research EU picture



Ref. Anke Guhr, Andreas Kurtz & Peter Löser



hES cell research: the point of discussion

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hESC research EU picture

hESC research: diversity

- Debate in most of the EU-15 countries
- Since 2001 ⇒ Boom of new specific legislations in this sector in EU-15 *(with one exception, in all cases to allow this research)*
- No debate in the new EU-10; no specific legislation

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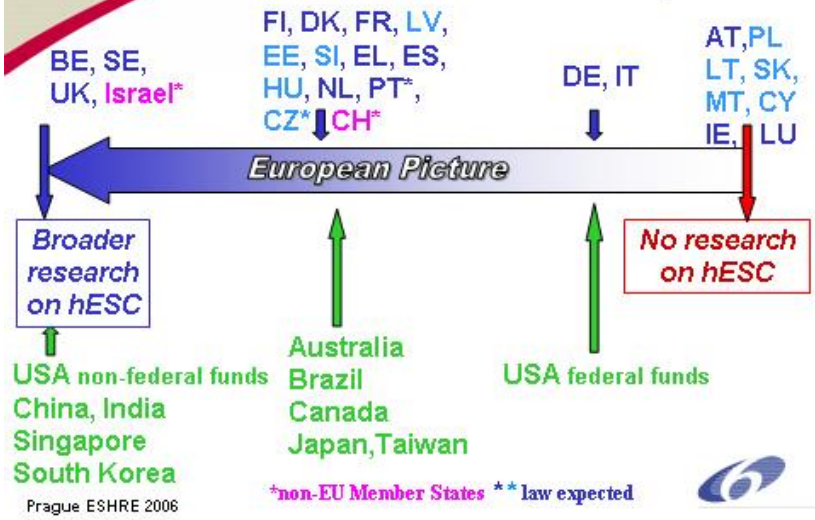
hESC research Worldwide picture



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hESC research EU policy

- EU has no legal competency to regulate in this sector of ethics
- The Commission has the responsibility to implement the EU research programs even where some areas of research raise important ethical issues.
  - Respect of national rules is a fundamental principle ⇒ no research forbidden in a Member State supported by EU funds there

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Future
New Commission:
Main goal
⇒ the «Lisbon agenda»: the blueprint for growth, competitiveness, employment and sustainable development.
• research is at the core of the Lisbon agenda
• stem cell research, including hESC research, is among the promising areas in life sciences & its biomedical applications
• FP7 is for 7 years instead of 4 years for the previous FP
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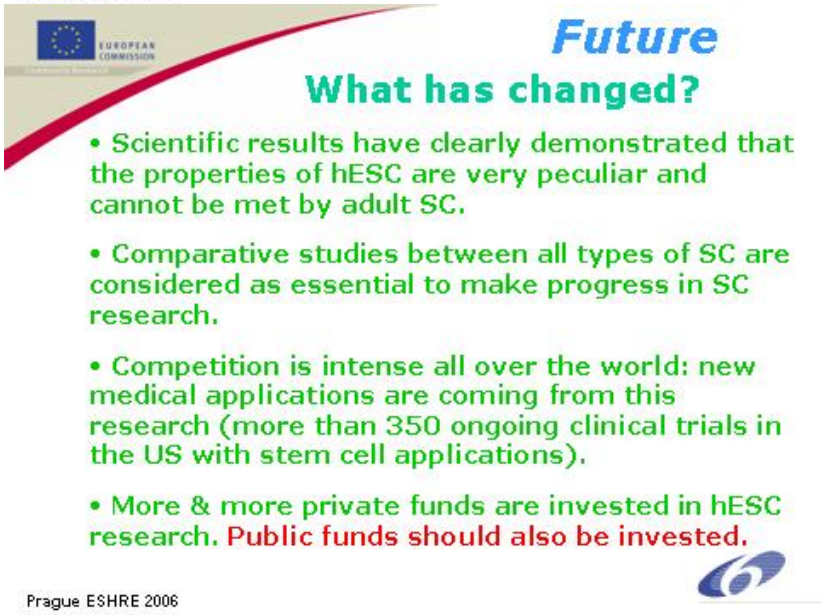
Future
What has changed?
• Scientific results have clearly demonstrated that the properties of hESC are very peculiar and cannot be met by adult SC.
• Comparative studies between all types of SC are considered as essential to make progress in SC research.
• Competition is intense all over the world: new medical applications are coming from this research (more than 350 ongoing clinical trials in the US with stem cell applications).
• More & more private funds are invested in hESC research. Public funds should also be invested.
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Future
Commission proposal
for FP7 (2006-2013)
⇒ The same ethical framework than in FP6 / same process
i.e. to allow hESC research on a case by case basis because opportunities outweigh risks & cooperative research in this sector is essential for Europe to be competitive
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Future

Member States What has changed?

Preparation of FP6 (2002-2006):

- in 2001 within the EU-15: only 3 countries with a law (UK, FIN, SE).

Preparation of FP7 (2007-2013):

- in 2005 within the EU-15: 10 countries have a law that allows at least some research on hESC
- configuration of EU-25
- Nov. 2005: declaration of 6 Member States against the use of EU funds for *"destruction of human embryos"*: AT, DE, IT, MT, PL, SK. In March 2006 Council 2 MS joined: LU, LT.

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Future

New EU Parliament

6th term 2004 – 2009: 730 Members:

- PPE-DE: Group of Christian & European Democrats (265)
- PSE: Socialist Group (201)
- ALDE: Group of Liberals & Democrats (89)
- Verts/ALE: Greens (42)
- GUE/NGL: European United Left (41)
- IND/DEM: Independence-Democracy Group (35)
- UEN: Union for Europe of the Nations (27)
- NI: non-attached Members (29)

Since Sept 2004, several resolutions voted to exclude human embryo & hESC research from FP

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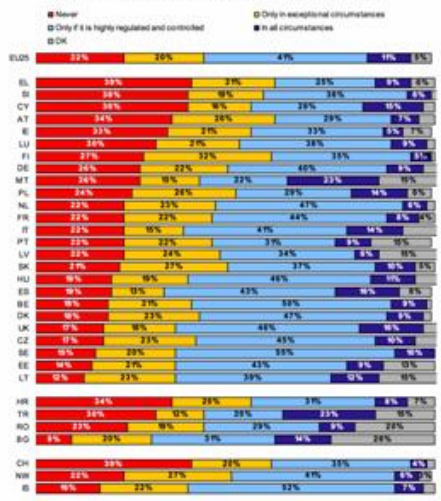
Citizens' Opinion

...please tell me to what extend, if at all, you approve of its use.

Cloning human stem cells from embryos to make cells and organs that can be transplanted into people with diseases



Extracted from Eurobarometer 2005 *"Social values, Science & technologies"*

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