European Society of Human Reproduction and Embryology

COURSE 6

"(Epi)genetic aspects of human embryonic and germ stem cells"

Special Interest Groups
Stem Cells
Reproductive Genetics

1 July 2007
Lyon, France

PRE-CONGRESS COURSE 6

Special Interest Groups Stem Cells and Reproductive Genetics
"(Epi)genetic aspects of human embryonic and germ stem cells"

CONTENTS

PRE-CONGRESS COURSE 6 - PROGRAM

Joint SIG Stem Cells and SIG Reproductive Genetics

(Epi)genetic aspects of human embryonic and germ stem cells

Course coordinators: A. Veiga (E) & K. Sermon (B)

Course description:

To focus on more fundamental aspects of human stem cell research:

- Differences between hES and hEG
- Epigenetic aspects of hES research
- Genetic changes in hES

To give an update on more clinically oriented stem cell research:

- Update on hES carrying genetic diseases
- Production of male and female germ cells
- Other potential uses of hES

Target audience: Scientists working in the field of human embryonic and germ stem cells; Scientists working on the differentiation of hES to different cell types; Geneticists with an interest in hES as a model of human genetic disease; Geneticists with an interest in epigenetics

Program

09.00 - 09.10: Welcome - ***A. Veiga (E) and K. Sermon (B)***

Session 1 - Chair: A. Veiga (E) / K. Sermon (B)

09.10 - 09.20: What is an embryonic stem cell? – ***A. Veiga (ES)***
09.20 - 10.00: Update on hES lines carrying genetic abnormalities – ***K. Sermon (B)***
10.00 - 10.40: Genetic changes at chromosomal and DNA level during long term cultivation of hES cells – ***J. Inzunza (S)***

10.40 – 11.10: Coffee break

Session 2 - Chair: S. Viville (F) / P. Vogt (D)

11.10 - 11.50: Exploring germline epigenetic reprogramming *in vitro* – ***N. Geijsen (USA)***
11.50 - 12.30: Discussion on morning topics

12.30 – 13.30: Lunch break

Session 3 - Chair: A. Veiga (E) / S. Viville (F)

13.30 - 14.15: Nuclear reprogramming – ***K. Campbell (UK)***

14.15 - 15.00: Nuclear transfer for Stem Cell derivation – ***M Stojkovic (ES)***

15.00 - 15.30: Coffee break

Session 4 - Chair: A. Veiga (E) and J. Geraedts (NL)

15.30 – 16.15: The potential use of hES cells other than in cell therapy - ***M. Peschanski (F)***

16.15 – 17.00: General discussion and closing remarks

What is an embryonic stem cell?

Anna Veiga [1,2]
Begoña Aran [1]
Juan Carlos Izpisua Belmonte [1,3]

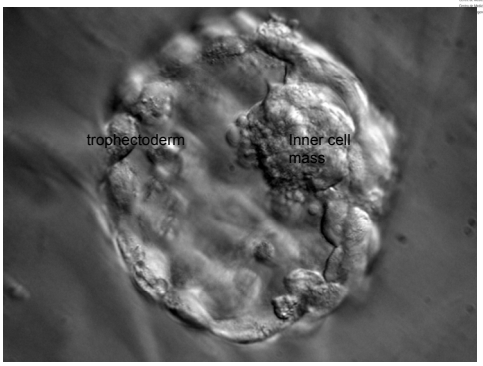
1.- Barcelona Stem Cell Bank. Centre for Regenerative Medicine. Barcelona
2.- Servei de Medicina de la Reproducció. Institut Universitari Dexeus. Barcelona.
3.- The Salk Institute for Biological Studies. La Jolla. USA

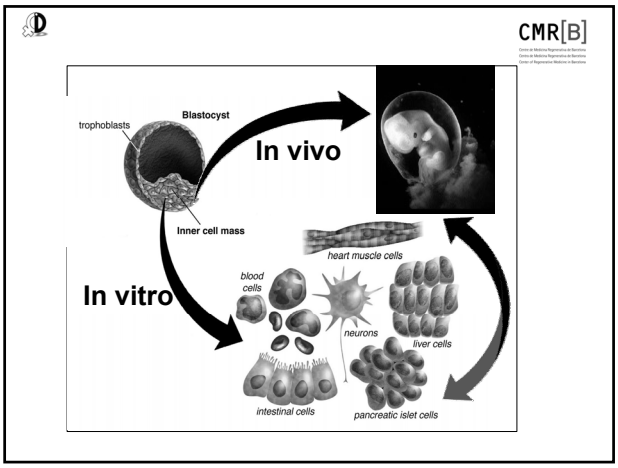
REPORTS

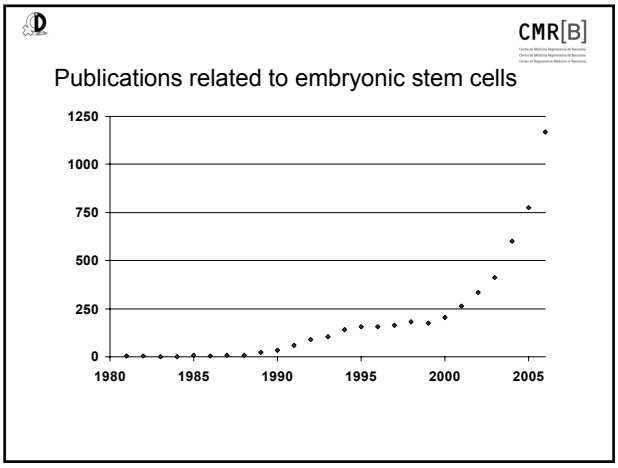
Embryonic Stem Cell Lines Derived from Human Blastocysts

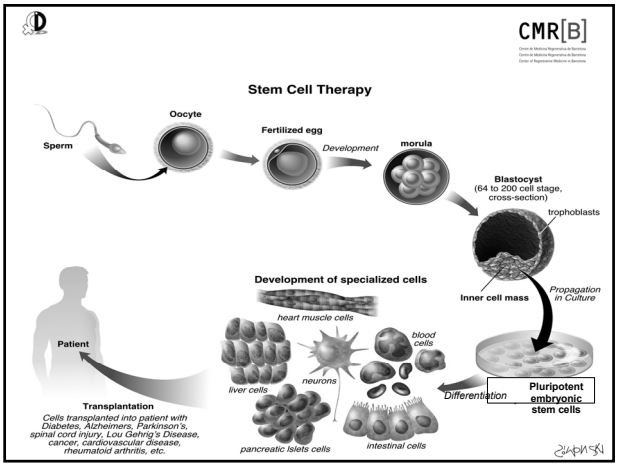
James A. Thomson,* Joseph Itskovitz-Eldor, Sander S. Shapiro, Michelle A. Waknitz, Jennifer J. Swiergiel, Vivienne S. Marshall, Jeffrey M. Jones

www.sciencemag.org SCIENCE VOL 282 6 NOVEMBER 1998 1145




CMR[B]
Blastocyst
trophoblasts
In vivo
Inner cell mass
heart muscle cells
blood cells
In vitro
neurons
liver cells
intestinal cells
pancreatic islet cells


CMR[B]
Publications related to embryonic stem cells
1250
1000
750
500
250
0
1980
1985
1990
1995
2000
2005


CMR[B]
Stem Cell Therapy
Oocyte
Sperm
Fertilized egg
Development
morula
Blastocyst
(64 to 200 cell stage, cross-section)
trophoblasts
Inner cell mass
Propagation in Culture
Pluripotent embryonic stem cells
Differentiation
Development of specialized cells
heart muscle cells
blood cells
neurons
liver cells
pancreatic islets cells
intestinal cells
Patient
Transplantation
Cells transplanted into patient with Diabetes, Alzheimers, Parkinson's, spinal cord injury, Lou Gehrig's Disease, cancer, cardiovascular disease, rheumatoid arthritis, etc.

Derivation Methodology

Embryo thawing and culture

- Embryo (day 1, day 2 , day 3, day 5-6) thawing and culture to the blastocyst stage.
- Zona pellucida removal with pronase (5mg/ml)
- ICM isolation (optional)

Feeder cell culture

- Inactivated (mouse/human) fibroblasts in gelatine coated dishes/ Matrixes.

Embryo seeding – hESC culture

- ICMs or zona pellucida free blastocysts are transferred to feeder/ matrix dishes.

Shortcomings of embryonic stem cell therapy

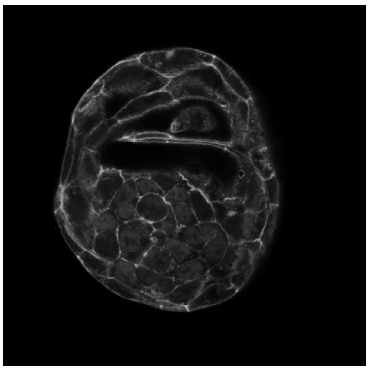
Derivation of hES cell lines
- Clinical-grade lines
- Source of lines

Protocols of directed differentiation
- Selection protocols
- Directed differentiation

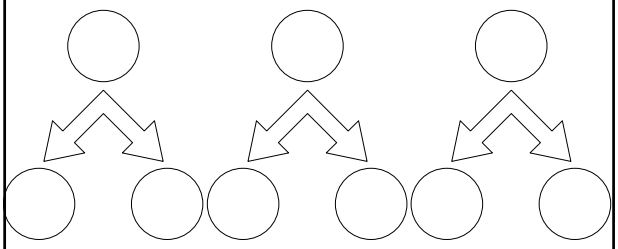
Immune rejection
- SCNT
- Parthenogenetic lines

Control of cell proliferation (teratoma formation)
- Bases of self-renewal
- Bases of pluripotency

The source of mouse ES cells



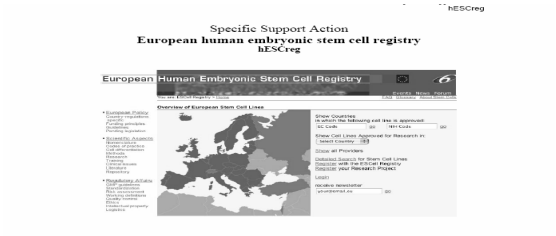
Embryonic Stem Cells: Self Renewal and Differentiation



Asymmetric division | Self-renewal | Differentiation

European Human Embryonic Stem Cell Registry (EU hESC Reg)

Coordinators: Anna Veiga CMRB Barcelona
Joeri Borstlap Charité Berlin



EU hESC Registry (VI FP - EC).

- The objective is to put in place a website to provide information on existing hESC lines (European and world-wide) available to the scientific community
- Start: 1/03/2007

14 participants

Coordinators: CMRB (Barcelona)
BCRT (Berlin)

UK
Spain
France
Belgium
Israel
Sweden
The Netherlands
Czech Republik
Finland
Denmark
Switzerland

- EMBRYO INFORMATION
- DERIVATION METHODOLOGY
- CULTURE CONDITIONS
- CHARACTERISATION

EMBRYO INFORMATION

- NUMBER OF EMBRYOS DONATED
- NUMBER OF EMBRYOS USED
- SURVIVAL RATE
- BLASTOCYST RATE
- ICM/BLASTOCYSTS USED
- ICM/BLASTOCYSTS ATTACHED
- INITIAL OUTGROWTHS
- ESTABLISHED LINES
- EFFICIENCY

EMBRYO INFORMATION

- EMBRYO CHARACTERISTICS

EMBRYO ORIGIN
- FRESH/FROZEN
- DONATED SURPLUS EMBRYOS
- DONATED GAMETES
- DISCARDED POOR QUALITY EMBRYOS (NO TRANSFER, NO FREEZING)
- NO WISH FOR CRYOPRESERVATION
- PGD

1. PGS
2. CHROMOSOMAL ABNORMALITIES
3. MONOGENIC DISEASES

EMBRYO QUALITY
1. EARLY CLEAVAGE/MORULA/BLASTOCYSTS
2. EMBRYO QUALITY (EARLY CLEAVAGE)
3. BLASTOCYST QUALITY

- EXPANSION STATUS
- ICM MORPHOLOGY
- TROPHECTODERM MORPHOLOGY
- CULTURE CONDITIONS
- CULTURE MEDIUM
- GAS PHASE

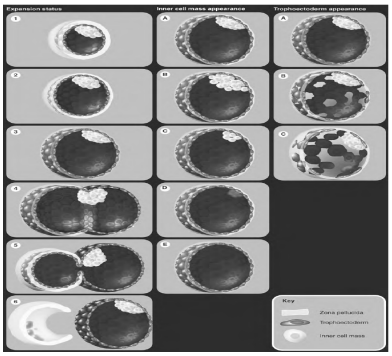
Proposal for a universal minimum information convention for the reporting on the derivation of human embryonic stem cell lines.

Emma Stephenson[1,2], Peter Braude[2,3], Chris Mason[1]
[1]Advanced Centre for Biochemical Engineering, University College London
[2]Department of Women's Health, School of Medicine, King's College London
[3]Assisted Conception Unit, Guy's and St Thomas' Foundation Trust, London

Essential	Desirable
Whether fresh or frozen embryos used	Source if fresh: • donated gametes • embryos deemed of too poor quality to cryopreserve • no wish for cryopreservation • PGD or PGS
Total number of embryos transferred to research	
Number developing to blastocyst	
Number of blastocysts suitable for use	
Grade of all blastocysts used	
Number of ICMs obtained (if applicable)	
Number of ICMs that attach to feeder layer/ECM	Day of development when blastocysts used
Number of stem cell like colonies	Hatched naturally or zona removed (non-PGD)
Number of hESC lines	Number of days after plating until appearance of stem cell like colony
Known disease (if PGD)	Sex (useful for tracking cells)
	Media used
	Gas phase employed

Table 2. Data deemed essential or desirable in publications using human embryos for stem cell research.

Future Medicine, Reg. Medicine 1(6), 739-750



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Expansion Status

1. No expansion in overall size, zona pellucida still thick
2. Some expansion in overall size, zona pellucida beginning to thin
3. Full expansion, zona pellucida very thin
4. Partially hatched, trophectoderm beginning to herniate through zona pellucida. Full expansion status
5. Partially hatched, no expansion in overall size – seen following pre-implantation genetic diagnosis
6. Fully hatched blastocyst, zona pellucida may contain cells rejected during blastocyst formation

ICM Appearance (see Figure 2 for photographic examples)

A. Cells compacted, tightly adhered together and indistinguishable as individual cells
B. Cells less compacted so larger in size, loosely adhered together, some visible as individual cells
C. Very few cells visible, either compacted or loose, may be difficult to completely distinguish from trophectoderm
D. Cells of ICM appear degenerate
E. No ICM cells visible in any plane

Trophectoderm Appearance

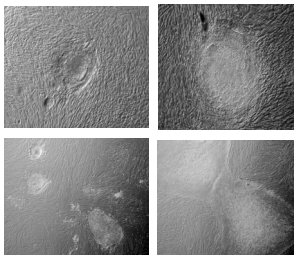
A. Many small identical cells forming a continuous trophectoderm layer
B. Fewer, larger cells, may not form completely continuous layer
C. Sparse cells, may be very large, very flat or appear degenerate

Future Medicine, Reg. Medicine 1(6), 739-750

DERIVATION METHODOLOGY

- BLASTOCYST HATCHING
1. SPONTANEOUS/ZONA PELLUCIDA REMOVAL
   - ENZYMATIC
   - CHEMICHAL
   - MECHANICAL

- ICM ISOLATION
1. WHOLE BLASTOCYST SEEDING
2. IMMUNOSURGERY
3. MECHANICAL
4. LASER
- SUBSTRATE
1. FEEDER CELL COCULTURE
   - MURINE
   - HUMAN
2. PROTEIN MATRIX
3. DEFINED MATRIX
4. NO MATRIX
- CULTURE MEDIUM
1. PROTEIN SOURCE
2. GROWTH FACTORS



DERIVATION METHODOLOGY

- GAS PHASE
- PASSAGING
1. MECHANICAL
2. ENZYMATIC
3. PASSAGE NUMBER
- MICROBIOLOGY
1. HUMAN PATHOGENS
2. MURINE PATHOGENS
- GROWTH RATE
- DOUBLING TIME
- FREEZING/THAWING
1. METHODOLOGY
2. SURVIVAL RATE
- CLONING EFFICIENCY

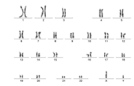
* XENO FREE CONDITIONS
* DEFINED CONDITIONS
* GMP CONDITIONS

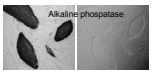
DERIVATION METHODOLOGY CULTURE CONDITIONS

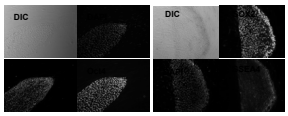
- hESC derived without feeder cells. Klimanskaya, I. Lancet 365: 1636-1641; 2005
- The derivation of clinical grade hESC lines. Skottman, H. FEBS letters 580: 2875-2878; 2006.
- Derivation of a xeno-free human ES cell line. Ellestrom, C. Stem Cells 24: 2170-2176; 2006.
- Defined culture conditions of hESC. Lu, N. PNAS 103: 5688-5693; 2006.
- Testing of nine different xeno-free culture media for hESC cultures. Rajala, K. Hum Reprod 22: 1231-1238; 2007.

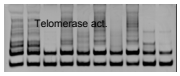
CHARACTERISATION

- KARYOTYPE
1. PASSAGE NUMBER
- ALKALINE PHOSPHATASE
- TELOMERASE ACTIVITY
- PLURIPOTENCY MARKERS
- RT PCR
- IMMUNOFLUORESCENCE STAINING
- MEMBRANE SURFACE MARKERS
  * SSEA-3, SSEA-4, TRA 1-60, TRA1-81….
- TRANSCRIPTION FACTORS
  * OCT4, NANOG, REX1, SOX



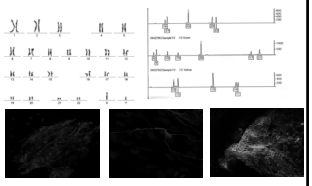


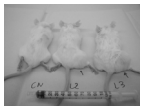


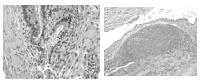


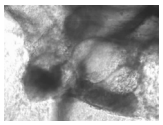
CHARACTERISATION

- HLA TYPING
- MICROSATELITES (FINGERPRINTING)
- IN VIVO DIFFERENTIATION
1. TERATOMAS
2. CHIMERAS
- IN VITRO DIFFERENTIATION (EB)
1. ECTODERM MARKERS
2. ENDODERM MARKERS
3. MESODERM MARKERS









DOCUMENTS

- CONSENT FORM
- DONOR EMBRYO SCREENING
- STANDARD OPERATING PROCEDURES (SOP)
1. EMBRYO CULTURE
2. EMBRYO THAWING
3. FEEDER CELL CULTURE, PASSAGING, FREEZING
4. ZONA PELLUCIDA REMOVAL
5. ICM ISOLATION
6. ICM/WHOLE BLASTOCYST SEEDING
7. DERIVATION METHODOLOGY
8. HESC CULTURE, PASSAGING, FREEZING

- INSTITUTIONAL REVIEW BOARD APPROVAL
- ETHICS COMMITTEE APPROVAL

- COMPETENT AUTHORITY APPROVAL

*TRACEABILITY
*QUALITY CONTROL

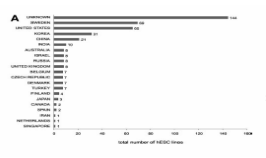
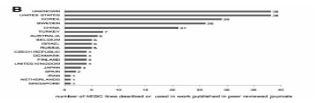


Figure 1. Derived hESC lines (as of January 1, 2006).



Current State of Human Embryonic Stem Cell Research: An Overview of Cell Lines and Their Use in Experimental Work
Anke Guhr, Andreas Kurtz, Kelley Friedgen and Peter Löser
Stem Cells 2006;24:2187-2191; originally published online Jun 15, 2006;

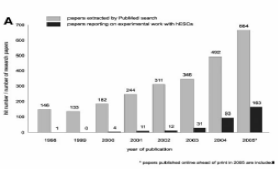


Figure 2. Overview of published work reporting on experimental use of hESCs.

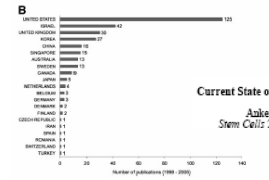


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Table 3. Most frequently used human embryonic stem cell (hESC) lines

Top hESC lines	NIH code	Use in published work	Provider[a]
Work published 1998–2005			
H9[b]	WA09	110	WiCell Research Institute
H1[c]	WA01	93	WiCell Research Institute
H7	WA07	37	WiCell Research Institute
BG01	BG01	26	BresaGen, Inc.
HES-3	ES03	23	ES Cell International
HES-2	ES02	18	ES Cell International
HSF-6	UC06	16	University of California, San Francisco
BG02	BG02	16	BresaGen, Inc.
HES-1	ES01	13	ES Cell International
Miz- hES1	MI01	12	MizMedi Hospital, Seoul National University
H13	WA13	11	WiCell Research Institute
H14	WA14	11	WiCell Research Institute
HES-4	ES04	11	ES Cell International
Work published in 2004			
H9[b]	WA09	34	WiCell Research Institute
H1[c]	WA01	27	WiCell Research Institute
BG01	BG01	11	BresaGen, Inc.
H7	WA07	9	WiCell Research Institute
HSF-6	UC06	7	University of California, San Francisco
HES-3	ES03	6	ES Cell International
HES-4	ES04	5	ES Cell International
H13	WA13	5	WiCell Research Institute
BG02	BG02	5	BresaGen, Inc., Athens, Georgia
Work published in 2005[d]			
H1[c]	WA01	51	WiCell Research Institute
H9[b]	WA09	44	WiCell Research Institute
H7	WA07	19	WiCell Research Institute
HES-3	ES03	14	ES Cell International
BG01	BG01	13	BresaGen, Inc., Athens, Georgia
HES-2	ES02	10	ES Cell International
HSF-6	UC06	9	University of California, San Francisco
BG02	BG02	9	BresaGen, Inc., Athens, Georgia
Miz-hES1	MI01	8	MizMedi Hospital, Seoul National University
SNUhES3	Not listed	8	Seoul National University
SA002[e]	SA02	8	Cellartis AB
AS034[e]	Not listed	6	Cellartis AB
Miz-hES4	Not listed	5	MizMedi Hospital, Seoul National University

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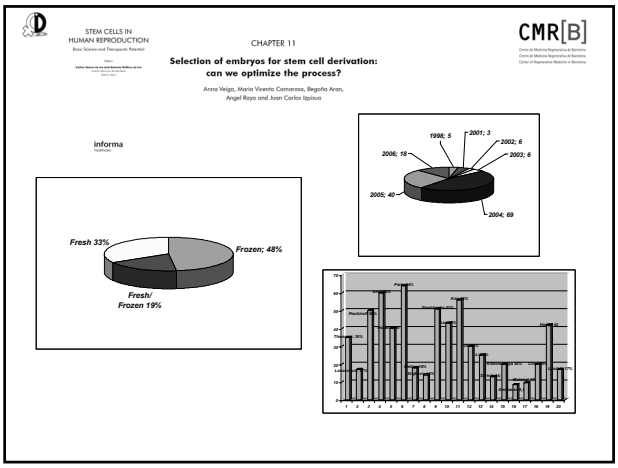
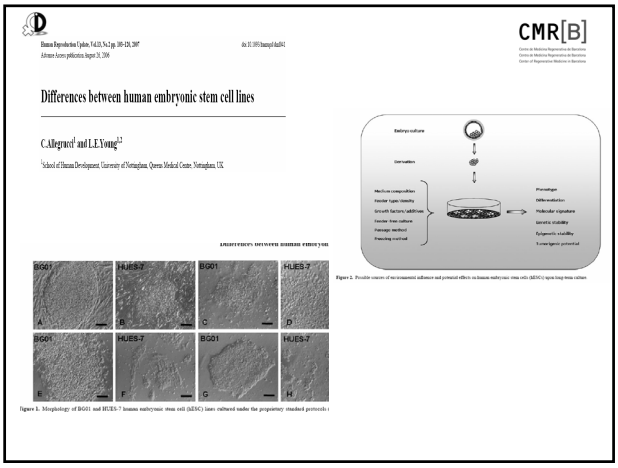


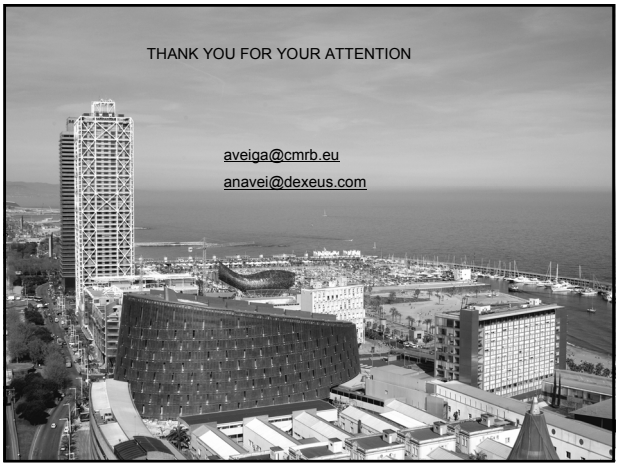
PI	Centre/Country	N° embryos Fresh/FroZen	Feeders	Embryo culture medium	ICM culture	ICM Isolation	N° and %Cell lines from ICM	initial split(Mec. Coll. Disp-Day)	Cell lines
Thomson 1998	WiCell - USA	36 F/FZ	Irrad MEF		20% FBS	IS	5 35%		H1,H7,H9, H13,H14
Reubinoff 2000	Monash Inst Aust/Singap.	NA FZ	MitoC MEF		20%FBS+LIF	IS	2 50%	MD	HES-1, HES2
Lanzendorf 2001	Jones Inst for Reproductive Medicine- USA	110 F	Irrad MEF		20%FBS+LIF	IS	3 17%	M4-11	ES-76, ES-78-1, ES-78-2
Amit 2002	Rambam Medical Center- Israel	5 FZ	MEF		N/A	IS	3 60%		I-3, I-4, I-6
Richards 2002	Dept. Obstetrics and Gynaecology-Singapore	1 FZ	MitoC HF (FetMusl)	Vitrolife	20%HS	IS	1	MD 10	1
Hovatta 2003	Karolinska Inst-Sweden	NA F	Irrad postnatHFF	Vitrolife	20%FBS+LIF	IS	2 40%	D9-19	HS181, HS207
Mitalipova 2003	BresaGen- USA	19 FZ	Irrad MEF		20%FBS+LIF +bFGF	IS	4 NA	M7-10	BG01, BG02, BG03, BG04
Park 2003	MizMedi Hosp Seoul- Korea	NA FZ	MitoC MEF	Vitrolife	20%FBS+LIF	IS	3 NA		3
Pickering 2003	St Thomas LND-UK	58 F					3 NA		3
Baharvand 2004	Royan Inst- Iran	NA NA	MitoC MEF		20%FBS+LIF	No IS	1	M1	Royan H11
Heins 2004	Cellartis AB-Sweden	NA F/FZ	MitoC MEF	Vitrolife	VitroES+BFG F	IS	6 NA	M7	SA002,FC0 18 AS034,AS0 38 SA121 SA181
Mellon 2004	Howard Hughes Medical Inst- USA	286 embryos+ 58 blasts FZ	MitoC MEF		8-10%SR, 8-10% Plasmanate, 5% FBS+LIF+bF GF	IS	17 18%	M	HUES1-17
Park 2004	Maria Infertility Hospital- Korea	20blasts 20Pronuclei FZ	MitoC STO	Vitrolife	20%FBS+LIF +bFGF	IS	9 64%	M5-8	MB01-07 MB08-09

PI	centre/country	N° embryos	Feeders	Embryo culture medium	ICM culture medium	ICM Isolation	N° and %Cell lines from ICM	initial split* (Mec. Coll. Disp - day)	Cell lines
Sjögren 2004	Dept. Obstetrics and Gynaecology Sweden	748 F/FZ	MEF	Vitrolife/ ISM-2	VitroES+BF GF	IS	22 19%		22
Stojkovic 2004	Inst of Human Genetics UK	11 day-2 embryos F	Irrad MEF		FCS, then SR+bFGF	IS	1 14%	M17	HES-NCL1
Strelchenko 2004	Reproductive Genetic Inst Chic USA	117 (46 morula, 71 blastocyst)	MitoC MEF or BRL		10-20%FBS or SR+bFGF	IS	20 62.5%		**15, 18, 21, 24, 27 28, 31, 33** 53 60,62,63, 79, 80, 81 93, 94, 95,96,97
Suss-toby 2004	Rambam Medical Center Israel	60 F	Inact MEF		N/A	IS	1 16.6%	M	I9
Verlinsky 2004	RGI Chicago USA	72 F PGD	MitoC MEF or BRL				18 NA	ED8-14	18 abn
Chen 2005	Tongi Hospital China	130 F	MitoC MEF	Vitrolife	20%FBS+b FGF	IS	2 20%	M5-8	2
Findikli 2005	Istambul Memorial Hospital Turkey	31 F	MitoC MEF		15% FBS+LIF 12 ng/ml+ITS	15IS, 12 direct	7 NA	7-10	7
Genbacev2005	UCSF USA	192 FZ	Human Plancental fibroblasts		20%SR+bF GF	No IS	1 7%	M	UCSF-2
Genbacev 2005	UCSF USA	321 F	Human Plancental fibroblasts		20%SR+bF GF	No IS	1 10%		UCSF-1
Inzunza 2005	Karolinska Inst Sweden	10 F/FZ	Irrad HFF		20%FCS, then SR+ bFGF+ITS	IS	2 NA	M12	HS293, HS206
Kim/Moon 2005	MRC Seoul Nat. Univ Korea	? FZ	MitoC STO	Vitrolife	20%SR+ 4 bFGF+LIF	IS/Mechanic al	13 NA	M7	13
Kim/Yoon 2005	MRC MizMedi Hosp Korea	FZ	MitoC MEF	Vitrolife	20%SR+ bFGF	IS	9 56%	M	Miz-Hes4-8, 10-13
Klimanskaya 2005	ACT USA	FZ	MitoC Lysed MEFs		8%SR+8% plasmanate + LIF+bFGF	IS	1 20%		ACT-14

PI	Centre/Country	Nº embryos	Feeders	embryo culture medium	ICM culture medium	ICM Isolation	Nº and %Cell lines from ICM	initial split (Mec. Coll. Disp - day)	Cell lines
Lee 2005	Medical Research Center Korea	8 PN-stage Embryos FZ	MitoC Human uterine endometrial cells		N/A	IS	3 43%	M6-8	Miz-endol.-2,-3
Li 2005	Hospital of Sun Yat-sen University China	N/A FZ	Irrad MEF		20%FBS+LI F	IS	1 25%		CHES-1
Mateizel 2005	Research Center for Reproduction and Genetics Belgium	69 F/FZ	Irrad/MitoC MEF	Vitrolife	20%FBS+LI F+ bFGF	IS	5 9,6%		VUB1,2, 3,4,5 abn
Mummery 2005	Hubrecht Lab.	22 F	MitoC MEF		20%FCS	IS	1 6%	MD8	NL-HESC1
Oh 2005	Medical Research Center Korea	73 FZ	MitoC STO	Vitrolife	20%SR+ bFGF	IS/ whole	3 30%	M7-8	SNUhES1-3
Pickering 2005	St Thomas LND UK	F PGD					1 NA	M17	CF1 abn
Simon 2005	Valencia Stem Cell Bank Spain	40 FZ	Human Plancental fibroblasts		20%SR+ bFGF	No IS	2 13%	M15 M21	VAL-1, VAL-2
Wang 2005	Xinhua Hodpital China	NA	Irrad MEF			IS	2 NA		SH1, SH2
Wang 2005	Xinhua Hodpital China	NA	55Gy EDF				1 NA	M/C 10-14	SH7
Baharvand 2006	Royan Inst Iran	NA	MitoC MEF	Vitrolife	20% FCS+LIF + ITS	IS	5 NA	MD 10	Royan H2-3-4-5-6
Hampl 2006	Inst Expel Medicine Academy	98 FZ	MEF			IS	6 42%		CCTL8,9,6, 10, 12,14
Khanna 2006	Reliance Life Sciences	NA					1 NA		ReliCell hES S1
Lysdahl 2006	Stem Cell Research Lab Denmark	198 F	Irrad HFF	ISM2	20%SR+ bFGF	IS	4 17%	M10-15	CLS1, CLS2, CLS3, CLS4
Ludwig 2006	WiCell USA	FZ		Vitrolife		IS	2 NA		WA15, WA16

Total 194





Update on hESC lines carrying genetic abnormalities

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Why hESC with genetic abnormalities?

- Chromosomal abnormalities:
  - Dissection of syndromes (eg trisomy 21)
  - Cells have growth advantage
- Monogenic abnormalities:
  - Study of pathogenesis
  - Development of therapies

Origin of genetically abnormal embryos

- Acquired: culture artefact
- Present in the embryo
  - After Preimplantation Genetic Diagnosis
    - 25-50 % of embryos with monogenic disease
    - Up to 80% with translocation
    - About 50% with aneuploidy
  - Surplus to IVF
    - About 50% with aneuploidy

Preimplantation genetic diagnosis

- Early form of prenatal diagnosis on preimplantation embryos
- For high-risk situations: monogenic diseases, chromosomal aberrations
- For low-risk situations: aneuploidy screening

Embryo biopsy

HESC lines with chromosomal abnormalities

- Heins et al., 2004:
  - SA002: 47,XX,+13 and FC018: 69,XXY
- Ludwig et al., 2006:
  - line WA16: 47,XXY
- VUB06:
  - 47,XX,+17
- Kim et al, 2005 (retracted):
  - Line Miz-hES13: 47,XX,+3
- All discovered at first karyotype and early passage: acquired or embryonic?

HESC lines with chromosomal abnormalities

- Verlinsky et al., 2006
  - Stem cell repository with 166 lines
  - All after PGD
  - Minimal characterisation, low passages
  - Eight with chromosomal abnormalities:
    - Translocations: 46,XX,der(4)t(4:13), 46,XX,t(10:22)(q25;q13)
    - 47,XX,+14
    - Sex chromosome abnormalities: 69,XXY, 47,XXY (X2), 45,XO, 47,XXX

Why 50% abnormal embryos and so few abnormal hESC lines?

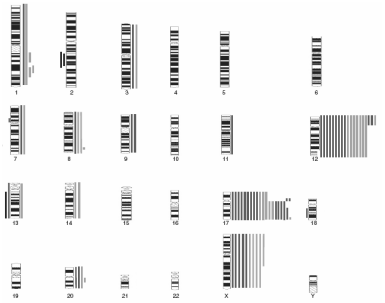
- Baart et al., 2005
  - Comparison PGS D3 vs D5 in women <38yrs
  - Only 50% confirmation rate (24/48)
  - Blastocysts: 14/33 (42%) abnl at D3 ⇏ nl at D5
- Munné et al., 2005
  - Comparison PGS D3, D6 and D12 after plating
  - 50 blastocysts, 34 attached, 24 FISH
    - 7 totally normal
    - 6 mostly abnormal
    - 11 partial chromosomal normalisation (21-88% normal cells)

Acquired chromosomal abnormalities

- 18 reported acquired chromosomal abnormalities
  - Chromosomes 12 (12x), 17 (11x), X (5x) and combinations
- 30 cultures, 17 abnormalities
  - Mostly 17 (15x), 12 (10x) and X (5x)
  - X mostly in combination with one of the other chromosomes

Baker et al., 2007, Nature Biotech

Acquired chromosomal abnormalities



Baker et al., 2007, Nature Biotech

Acquired chromosomal abnormalities

- Chromosome 17:
  - *BIRC5*: anti-apoptotic gene
- Chromosome 12
  - *NANOG, DPPA3, GDF3*: stemness markers
  - *CCND2*: cell cycle regulator
  - *KRAS*: oncogene
  - *SOX5*: cell fate regulator
- Chromosome X:
  - Androgen receptor (*AR*) and interacting protein *NONO*
- "Adaptation" of hESC similar to testicular germ cell tumours

Baker et al., 2007, Nature Biotech

hESC carrying monogenic diseases: literature

- Pickering et al. (2005): KCL-003-CF1
- Mateizel et al (2006): VUB03_DM1, VUB04_CF1, VUB05_HD
- Verlinsky et al. (2006): 14 AD, 8 AR, 10 X-linked

hESC carrying monogenic diseases: literature

Verlinsky et al. (2006):

- 14 AD: HD (4), Marfan (1) DM1 (2), NF1 (6), torsion dystonia (1)
- 8 AR: β-thal affected (2), β-thal carrier (2), CF (1), sickle cell (1), SMA (1), Fanconi anemia (1)
- 10 X-linked: ALD (1), BMD (1), DMD (1), DMD carrier (2), Emery-Dreyfus (1), Emery-Dreyfus carrier (1), FRAXA (1), FRAXA carrier (1), ocular albinism (1)
- Minimal characterisation, low passage
- Eight not in repository

hESC carrying monogenic diseases: not published

- KCL, Peter Braude: HD line
- IVI Valencia, Carlos Simon: DM1 line
- Starting up:
  - IGBMC Strasbourg, Stéphane Viville
  - CMRB Barcelona, Anna Veiga

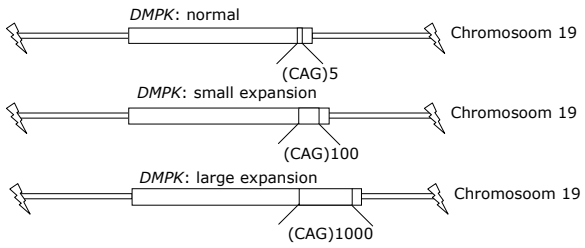
hESC carrying monogenic diseases at the VUB

- VUB08_MF: Marfan syndrome
- VUB09_FSHD: facio-scapulo-humeral dystrophy
- VUB10_SCA7: spinocerebellar ataxia type 7
- VUB11_FRAXA and VUB13_FRAXA: Fragile X syndrome carriers
- VUB14_BLS: Bare lymphocyte syndrome

Research with HESC lines with monogenic disease

- Myotonic dystrophy (DM1)
- Caused by a CTG expansion in the 3' end of the DMPK gene
  - Normal individuals: 5-37 repeats
  - Mildly affected individuals: > 50
  - Severely affected individuals: > 500
  - Congenital form: several thousands

Molecular biology of *DMPK*



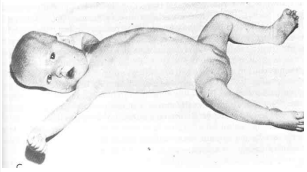
Mosaicism: (CAG)800-(CAG)2000 in one individual

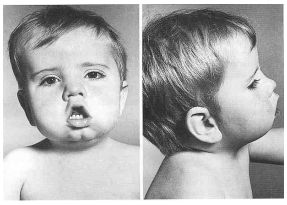
Clinical features of DM1

- Muscle weakness
- Myotonia
- Difficult swallowing, spastic colon
- Sudden death through heart rhythm disturbances
- Cataracts, male infertility
- Congenital form: floppy infant, tented upper lip, breathing difficulties, early death
- Anticipation: worsening of the symptoms over generations

Clinical features of DM1

Clinical features of congenital DM1



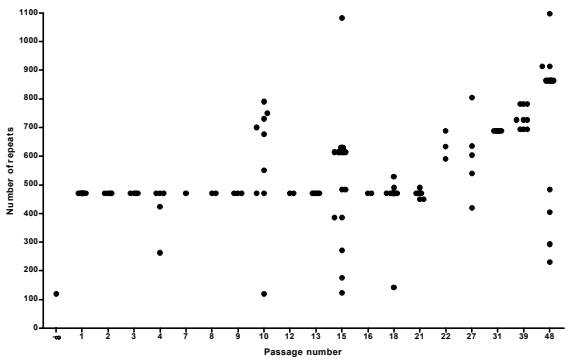


Mechanism of anticipation in DM1

- Expansion of triplet repeat during meiosis
- Somatic instability of triplet repeat
- Repeat is stable in preimplantation embryo and foetus up to week 16 of pregnancy
- DNA repair and replication is involved
- DM1 stem cells: good model?

Experiments on DM1 hESC

- Manipulation of genes for DNA repair and replication
- Instability in different tissues (gametes, somatic)
- Condition: repeat is stable in hESC (cfr embryos and fetuses)



Conclusions

- VUB03_DM1 has low doubling time (24 h)
- Instability during DNA replication? Special cell cycle of hESC
- hESC comparable to PGC?
- In vitro effect?

Collaborators

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Prof. H. Tournaye
Prof. Dr. Em. A. Van Steirteghem
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Nele De Temmerman
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Mieke Geens
Lindsey Van Haute

Genetic changes at chromosomal and DNA level during long term cultivation of hES cells

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- *Department of Clinical Sciences Division of Obstetrics and Gynecology Karolinska University Hospital*

Sources of Stem Cells

- ❑Blastocyst embryos - pluripotent
- ❑Foetal tissues - pluripotent or multipotent
- ❑Umbilical cord blood - multipotent
- ❑Adult tissues – multipotent or unipotent

Types of Stem Cells

Pluripotent Stem Cells: Can give rise to all different cell types *in vitro*.

Multipotent Stem Cells: Can give rise to several cell types of a tissue or organ.

Progenitor Stem Cells: Progeny consist of a single cell type only.

Human Embryonic Stem Cells

- Thomson et al 1998
- Reubinoff et al 2000
- Embrynic carcinoma, teratocarcinomas
- Bongso et al 1994 (successful separation of ICM)
- Karolinska Institutet 1998- 2000 2007

What are embryonic stem cells?

- Undifferentiated
- Continuously self-renewing
- High levels of telomerase activity up to 300 passages

- Ability to form any adult cell (higher plasticity than ASCs)
- Unlimited source of specific cell
- Provide a tool for studying the molecular mechanisms
  - → Early embryionic developmental pathways
  - → The pathological basis of genetic disorders
  - → Provide a source of transplantable cells and tissue

Phenotype and Molecular Characteristics of hESC

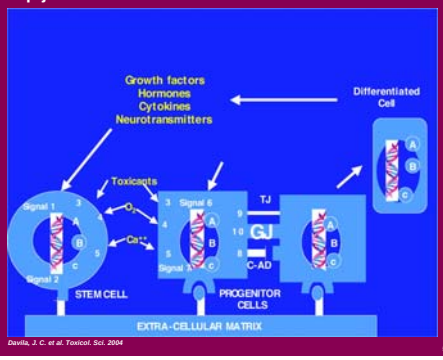
- hESC form relatively flat and compact colonies
- Grow more slowly than mESC
- A population doubling period take 36 h
- Express:

Transcription factors (Control of the Pluripotence)
- Oct4
- Sox1
- Nanog
- Rex1
- Foxd3

Signaling molecules
- STAT3
- FGF4
- LIF

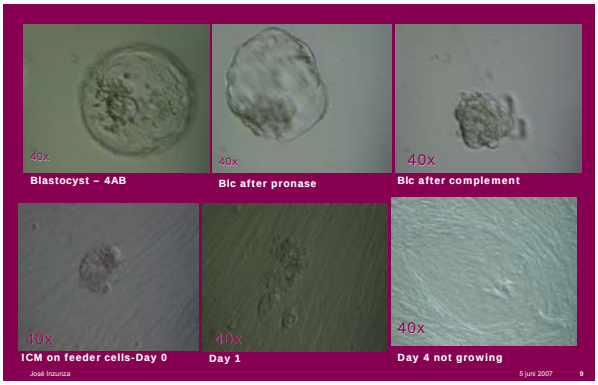
Cell surface
- SSEA3, SSEA4, TRA1-60, TRA1-81, GTCM2, TGT343

Gap-junctional intercellular communication mechanisms



Davila, J. C. et al. Toxicol. Sci. 2004

Where do embryonic stem cells come from?



From ICM to colonies

ICM on feeder cell day 5

Growing ICM day 6

Growing ICM day 9

Growing ICM day 11

P1: 1 day after splitting

P1: 3 days after splitting

Colonies


Differentiating area


Stem cells growing under feeder cells

hES cells culture medium

- Knockout Dulbecco's modified Eagle's medium
- Supplemented with SR medium
- 2mM L-glutamine
- 1% penecillin-streptomycin
- 1% nonessential amino acids
- 0.5 mM 2-mercaptoethanol
- 1% insulin-selenium-transferrin
- 8ng/ml bFGF

Feeder cells

Weekly preparation of feeder cells:

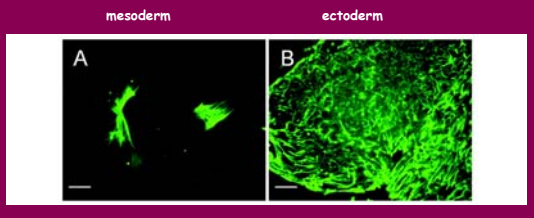
- Thawing feeder cells
- Cell expansion: growing in culture bottles
- Trypsinization
- Irradiation
- Seeding cells in Petri dishes until confluence

Feeder cell type:

- Fetal mouse fibroblast (not allow for further transplant)
- In Karolinska: Human adult foreskin fibroblast commercially available (Hum Reprod 2003, Hovatta)

Spontaneous differentiation on colonies (Fat cells)

Spontaneous differentiation

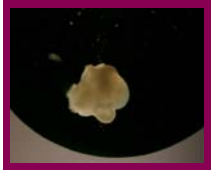


Smooth muscle
Actin antibody

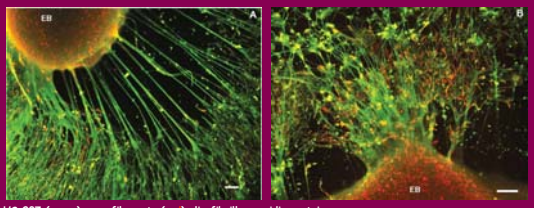
Neuronal cells
Nestin antibody

Embryoid bodies

Retinoid Acid
w/out serum



Neurofilaments and radial glia



HS 237 (green) neurofilaments (red) glia fibrillary acidic protein

Nat R. and Hovatta O 2004

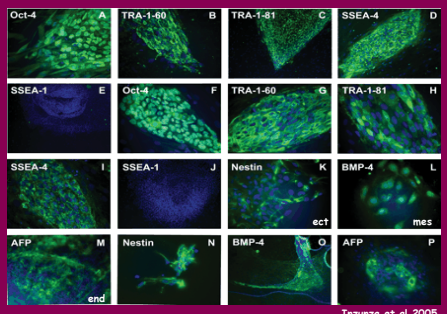
Alkaline phosphatase

Fully characterized hES

- UNDIFFERENTIATION
  - Alkaline phosphatase
  - Cell surface (SSEA3, SSEA4, TRA1-60, TRA1-81, GTCM2, TGT343)
  - Intracellular markers (Nanog, OCT4, Rex1)
  - Express high levels of telomerase activity
  - Normal karyotype
- CAPABILITY TO DIFFERENTIATE
  - Embryoid Bodies (in vitro)
  - Teratomas (in vivo)

Undifferentiated/Differentiated markers

hESC
HS293 p51
HS306 p37

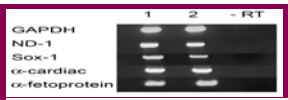


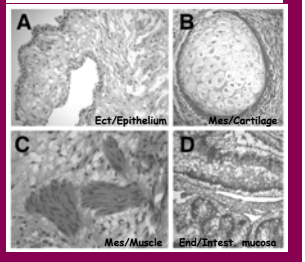
Inzunza et al 2005

Pluripotency

- Ectodermal (Sox-1)
- Mesodermal (alfa-cardisc actin)
- Endodermal (alfa-fetoprotein)

- Teratomas





Inzunza et al. 2005

Obstacles hindering the development of therapies using hESC

- Ethical and legal aspects
- Purification and Lineage Selection
- Tumorigenesis
- Tissue-Specific Integration and Function
- Immunogenicity and Graft Rejection
- Genetic and Epigenetic Concerns
  (hESC for therapeutic and research purposes has to be genetically and epigenetically normal)

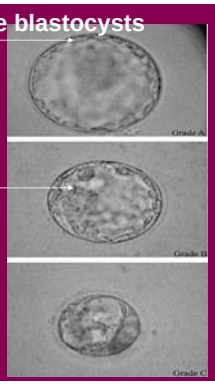
DNA and Chromosome changes?

- Derivation
- Culture — The microenvironment ("niche")
- Characterization
- Differentiation (protocols) Challenging to direct differentiation of hESC into specific lineage of fully functional cell
- Applications (varied biotechnological practices referred to generally as tissue engineering)

Different causes that may produce DNA or chromosomal changes in hESC.

- Blastocysts selection
- Derivation procedure
- Feeder cells
- Splitting to passages
- Cultivation long/short
- Cryopreservation
- Thawing
- Transportation
- Inducing differentiation and diverse

Selection of the blastocysts

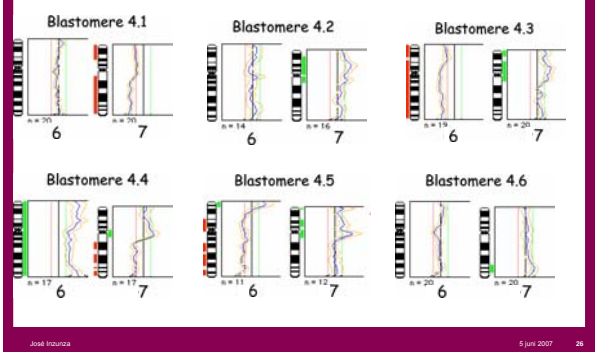


1
2
3AB
4AA
4AB
4BB
4CB
5
6

1,2 ,3,4, 5,6

Garner et al 1999

Patient 14 embryo 4 t(6;7)(p25;q11.2)



Derivation procedure

IMMUNOSURGERY (trophoblast cells lyses)

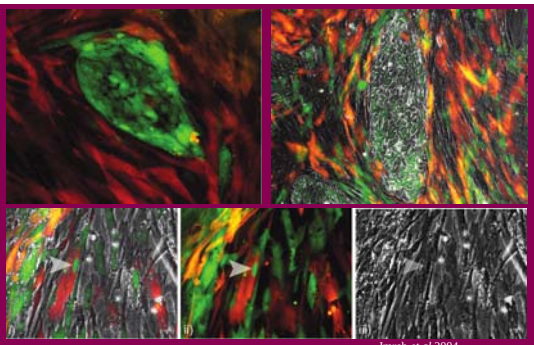
- Transfer of Blastocyst from IVF lab to Stem cells lab (as near as possible)
- Dissolve ZP with Pronase 0.5% (<1min)
- ZP-free Blc incubated with Anti-human serum antibody (30 min)
- Blc incubated with Guinea pig complement (45 min), and removing of lysed trophoblast cells by pipetting
- ICM placed into dish containing feeder cells
- ➢ Method not allow in the future for transplant purposes (because of complement)

MICROSURGERY: without Ab and complement, using micropipettes

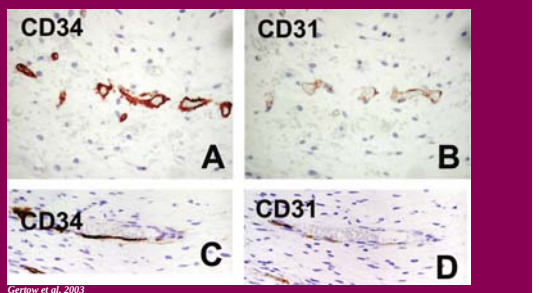
Feeder cells

- MEF
- STO
- Fetal muscle
- Skin
- Human foreskin fibroblasts cells
- Adult fallopian epithelial cells
- Adult marrow cells
- Matrigel Matrix
- Dishes coated with animal-based ingredients

hES Cells i a double-color system

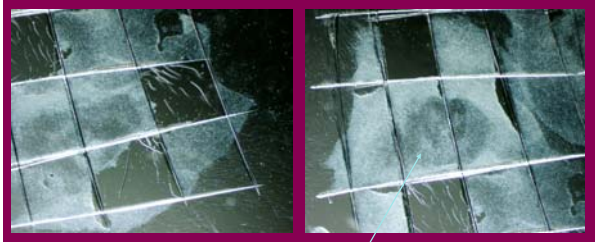


Imreh *et al* 2004



Gertow et al. 2003

Mechanical splitting: scalpel



Ø 2,000µm

Differentiated area

hES cell colonies

Passage 2: Mechanical splitting

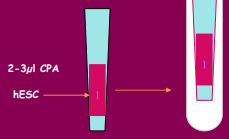
Passage 81: Dispase splitting

Cryopreservation

- Vitrification in pulled open straws (Reuninoff et al.2001)

  20 % Ethylene glycol
  20% dimethylsulphoxide
  1 M Sucrose

- Thawing



Stem cells Laboratory

Directed Differentiation of hESC

- Neural tube FGF2, Laminin; *In vivo* able to respond to signals from neighboring cells- environment (neuronal stem cells, progenitors, glial cells, motors and dopaminergic neurons cells) dopaminergic neurons PAX 2 and PAX5

- Cardiomyocytes were differentiated in media containing ascorbic acid into beating cardiomyocytes, purified using a Percoll gradient
- Endothelial differentiation
- Hematopoitic differentiation
- Osteogenic differentiation (osteocalcin) mineralization of culture medium, component of bone matrix (hydroxyapatite)

- Hepatic differentiation
- Insulin-producing islet-like cells by culturing them in the presence of nicotinamide, ITSFn, and N2 medium.

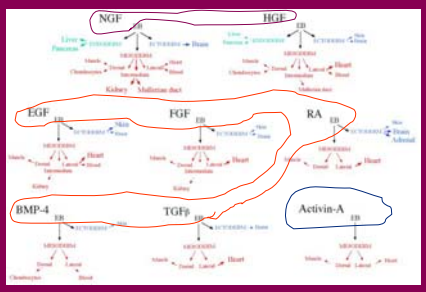
- Extraembryonic differentiation: hESC can spontaneously dif to trophoblast or in the presence of BMP-4

Growth of hESC and their induced differentiation

- LIF
- Fibroblast growth factor (FGF)
- TGFβ
- Bone morphogenetic protein (BMP)
- IGFBP 4
- Pigment epithelium-derived factor
- Secreted protein, acidic and rich in cysteine (SPARC)
- Nidogen-2
- Galectin 1
- Investigate the proteome of hESC and feeders

An interpretation of the effects of varius growth factors

- β nerve growth factor
- Hepatocyte growth factor
- Epidermal growth factor
- Basic fibroblast growth factor
- Retinoid acid
- Bone morphogenic protein 4
- Transforming growth factor β1
- Activin A



Chromosome alteration and culture adaptation

- There is clear indication that hESC culture in feeder-free conditions may gain chromosomal changes
- Reflect the progressive adaptation of self-renewing cells to their culture condition
- Genetic change that increases the capacity of cells to proliferate has obvious parallels with malignant transformation
- A number of karyotypic abnormalities have been identified in culture hESC
- A gently new passaging method without dissociation of single cells might preventing abnormalities
- Immunoreactions cause by animal substances in media
- Pathogens may change the property of the cells.
- The use of feeder cells sets limitations for researcher, results in a combination
- Variation in culture protocol
- It is important to study the properties of several hESC lines in similar conditions
- A real long-term studies are missing

Optimization and standardization of culture methods

- Many properties of the cells are alteres
- Animal- free substrate
- Mechanical isolation of ICM
- Derivation and culture in defined and animal-free culture media
- Passing of cells mechanically or with animal-free chemicals
- Characterization of undifferentiated hESC
- In vitro and in vivo differentiation of hESC
- Quality of hESC, Karyotype, contaminations
- Toxicity of the media

Most common genetic changes or chromosomal abnormalities of hES cells

- Summersgill *et al* (2001), showed that gains of material from chromosomes 12, 17 and X are particulary frequent in embryonal carcinoma (EC) cells
  - → The genes that are responsible are unknown
    - But likely affect:
      - → Cell cycle
      - → Differentiation
      - → Control of apoptosis
- These genetic changes are mostly non-random, and show a striking similarity to those noted in EC cells of GCT.
  - → Baker *et al*, 2007 consider that this cells are culture adapted cells.
  - → Suggesting that the genetic alteration have imparted a growth advantage in vitro.

Genomic alteration

- Spontaneous mutation at rate of 10-9 per nucleotide
- The overall biological properties of the cell culture are not altered
- Dominant mutant genotype may potentially affect cellular phonotype
- Alterations commonly observed.
  - → Aberrations in copy number (45%)
  - → Mitochondrial DNA sequence (22%)
  - → Gene promoter methylation (90%). Hypermethylation observed in cancer-related genes during tumorigenesis, during the differentiation/expansion
- in human cancer

Methods to reveal DNA sequence and chromosome mutations

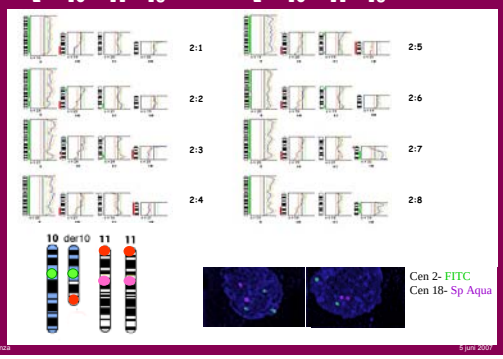
- Cytogenetic analysis
- Karyotyping (G or Q banding)
- Spectral karyotyping (SKY)
  - → DNA- mutations
    - Point mutations
    - Insertions
    - Deletions
  - → Chromosome mutations
    - Translocations
    - Aneuploidies
- Fluorescents in sito hybridization (FISH)
- Comparative genomic hybridization (CGH)

Inzunza et al. 2004

Methods difficulties

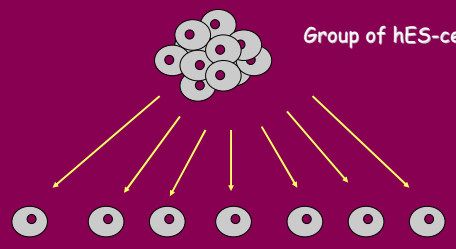
- Karyotyping with G or Q-banding
  - Difficult to obtain high quality metaphases
  - The number of cells possible to analyse will be limited
  - Clones of cells with chromosomal changes could easily be missed
- Interphase FISH analysis
  - Only a limited part of the genome
  - Few chromosomes may be included

Patient 18 Embryo 2 t(10;11)(q12;p13)



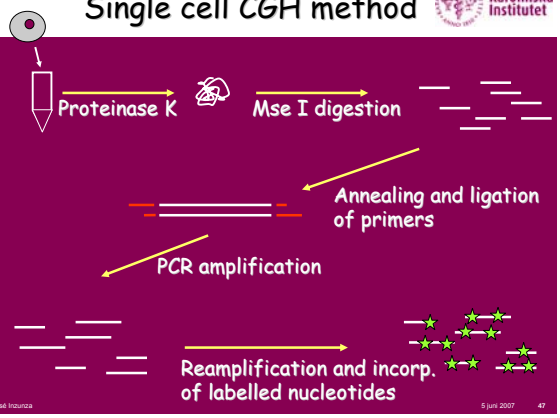
Cen 2- FITC
Cen 18- Sp Aqua

CGH of embryonic stem cells

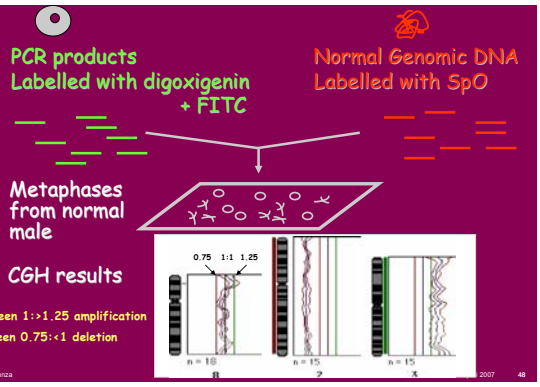


Individual hES cells, each analysed by single cell CGH

Single cell CGH method

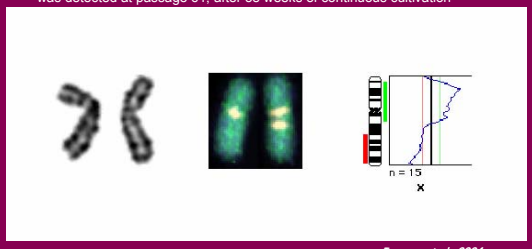


Single cell CGH method



Genetic and Epigenetic Concerns

- 46,XX,idic(X)q21HS 237 an aberrant X chromosome was detected at passage 61, after 58 weeks of continuous cultivation



Inzunza et al. 2004

- Aneuploidie (Chromosomes 12 and 17)

Discussions

- CGH proved to be a feasible technique
- CGH gives an overview of the whole genome
- Allows the detection of DNA copy number changes
- Neither smaller deletions or amplifications, nor balanced rearrangements are detected
- Resolution limits are 10-20Mb for CGH
- The number of cells possible to analyse each time is only limited
- CGH is a time demanding method 4-5 days

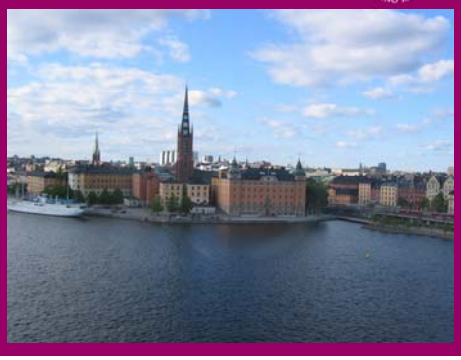
Why CGH?

- hES cells are important research tools
- Experience in embryo cultivation
- Karyotyping
  - →Difficult to culture without feeder
  - →Difficult to obtain metaphases
- FISH (Fluorescence In Situ Hybridization)
  - →using a set of chromosome specific probes
  - →a large number of cells may be screened for chromosomal aberrations
  - →Due to technical limitation, only a limited part of the genome can be analysed this way

Final conclusion

- CGH may be used for continuous analysis of the hES cell lines during cultivation, in order to reassure the chromosomal stability/constitution, which is crucial when considering transplantation of these cells in combination with karyotyping and interphase FISH analysis.
- A variety of biological mechanisms serve to eliminate aberrant or dysfunctional cells from the body
- Adult stem cells & ES cells
- Stem cell, like any other drugs, will have expiration dates

Thank you!



Exploring germline epigenetic reprogramming *in vitro*.

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Background

Over the next decade, the wealth of genetic information generated by the Human Genome Project will significantly advance the detection and treatment of genetic diseases. However, diseases caused by epigenetic defects such as improper DNA methylation or aberrant X-inactivation demonstrate that, in addition to the genetic sequence, epigenetic gene regulation plays an important role in human health and disease. Epigenetic gene regulation is tightly associated with the germline, since it is during germ cell development that epigenetic patterns, such as regulation of imprinted gene expression, are established. Imprinting, also called parent-of-origin-effect, is a mechanism by which certain genes are epigenetically marked in a parent-specific manner. The most prominent epigenetic mark on imprinted loci is DNA methylation and this imprinted methylation affects gene expression, resulting in either gene silencing or activation. As a result, imprinted genes are expressed mono-allelic and in a parental origin dependent fashion. Misregulation of imprinted gene expression can lead to a variety of diseases including Beckwith-Wiedemann syndrome, Angelmann syndrome and Präder-Willi syndrome and recent data demonstrate that aberrant imprinting can be a causative event in tumorigenesis. While the importance of DNA methylation in regulating imprinted gene expression has been well established and the genes mediating initiation and maintenance of DNA methylation have been identified, the mechanism by which imprints are reprogrammed and DNA methylation marks are erased is entirely unknown. Imprint erasure occurs during germ cell development when the imprinted methylation marks are first completely erased during early primordial germ cell formation and then re-established on the genome in a sex-specific manner. Therefore, the germline is the cell type of choice to investigate the molecular mechanism of imprint erasure. Unfortunately, the inaccessibility and small cell numbers of primordial germ cells in the mammalian embryo make it extremely difficult to study this process *in vivo*. A recent study from our lab as well as others demonstrated that embryonic stem cells (ES cells) can be coaxed to form germ cells *in vitro*[1-3]. In a groundbreaking paper, Hübner *et al* demonstrated that when ES cells are grown in monolayer culture in the absence of the anti-differentiation factor LIF, they form primordial germ cells that differentiate into aggregates resembling primordial follicles[2]. In studies presented by ourselves and Toyooka et al., ES cells were aggregated into three-dimensional structures called Embryoid Bodies (EBs). Differentiating EBs are composed of tissues of all three germ layers[4]. In the context of EB differentiation, the fate of numerous cell types is specified in a choreographed, stepwise process[5] and therefore EBs provides a means to investigate otherwise inaccessible cell populations of the early murine embryo such as the early germ cells.

Analysis of the *in vitro* developing germ cells revealed that these cells indeed undergo epigenetic reprogramming of imprinted genes between during their *in vitro* development, demonstrating that the *in vitro* EB differentiation system can be used to probe biological properties of PGCs developing *in vivo*. In this workshop we will outline how the EB differentiation system can be used to explore the molecular determinants of germline imprint erasure and DNA demethylation.

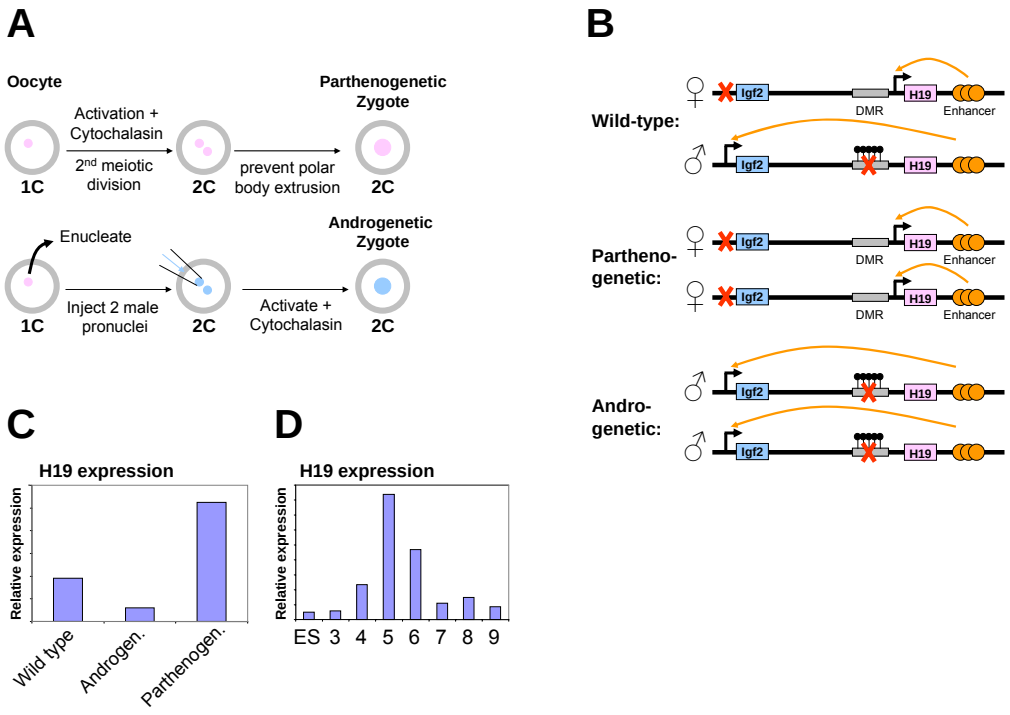


Figure 1: Using monoparental ES cell lines to monitor imprint erasure.
A. Schematic representation of the derivation of monoparental embryos. **B.** Schematic representation of the imprinted H19/Igf2 locus in wild-type, parthenogenetic and androgenetic ES cells. **C.** Q-PCR analysis of H19 expression in wild-type, parthenogenetic and androgenetic ES cells. **D**. Q-PCR analysis of H19 expression in germ cells generated *in vitro* from androgenetic ES cells.

Main Topics

Since the expression of imprinted genes is determined by their parental heritage, theoretically, imprinted gene expression can be used as a rapid and easy way of monitoring the imprint state of developing germ cells. In wild-type cells, imprinted loci such as the well known Igf2/H19 locus express H19 from the unmethylated maternal allele and Igf2 from the methylated paternal allele (Figure 1). Germline erasure of imprinted DNA methylation causes a doubling of H19 expression, as this gene is now expressed from both alleles, while Igf2 expression is downregulated. Thus, H19 expression levels can serve as readout for the imprinting state of a cell. Unfortunately, it

is challenging to robustly and reliably detect a two-fold increase in gene expression in a small cell population. To solve this problem we adapted our system monitored germline development and imprint erasure using monoparental ES cells. Monoparental ES cell lines are created from either entirely female or and entirely male genome (Figure 1A). Activation of an oocyte in the presence of the microfilament inhibitor cytochalasin prevents the extrusion of the second polar body and results in the creation of an early zygote with a completely maternal genome. While such embryos cannot give rise to viable offspring, they do develop to the blastocyst stage and can give rise to parthenogenetic ES cell lines. Likewise, injection of two male pronuclei into an enucleated oocyte results in the creation of a zygote with a completely paternal genome that can yield androgenetic ES cells. Androgenetic and parthenogenetic ES cell lines are pluripotent and can contribute to all germ layers as well as the germline in chimeric mice, (albeit at a reduced frequency compared to wild-type ES cells)[6-8]. Monoparental ES cell lines offer the advantage that their imprint status is either entirely paternal or entirely maternal (Figure 1B). This means that in parthenogenetic ES cells H19 is expressed from both alleles whereas H19 expression is silenced by the dual paternal imprints in androgenetic ES cells. Figure 1C demonstrates the Q-PCR comparison of H19 levels in wild-type, parthenogenetic and androgenetic ES cells. Since it was reported that androgenetic ES cells can form primordial germ cells *in vivo* that undergo normal imprint erasure, we analyzed the expression and imprint erasure of H19 in the androgenetic ES cell line LB4/8 during *in vitro* germline differentiation[7]. Figure 1D shows the Q-PCR analysis of H19 expression in undifferentiated androgenetic ES cells and their germ cell derivatives isolated from EBs at different time points. As expected, the paternal imprint status of the androgenetic ES cells prevented the expression of H19 in the undifferentiated ES cells (Figure 1D). However, in upon *in vitro* germline differentiation we observed an induction of H19 RNA expression starting at day 5 of EB differentiation, coinciding with a change in methylation status of the H19 DMR (not shown)
Thus, this modified system of germline differentiation from monoparental ES cells provides a rapid and easy method for the identification of molecular factors involved in germline imprint erasure.

Methods

ES cell culture

ES culture medium:

425	ml	DME (Invitrogen: 11965)
75	ml	ES qualified serum (Invitrogen: 26140-095 (1L))
5	ml	non-essential amino acids (Invitrogen: 11140-050)
5	ml	Pen/Strep (100x solution, Invitrogen: 15140-122)
5	ml	L-Glutamine (100x solution, Invitrogen: 25030-081)
5	µg	LIF (Peprotech: 250-02)
4	µl	β-mercapto-ethanol (Sigma M7522-100ml)

When ES cells are thawed from a cryopreserved stock, we typically wash the cells twice with ES cells medium before plating to remove all DMSO present in the freezing-media.

ES cells are maintained in the ES culture medium outlined above. We grow our ES cells on gelatinized tissue culture dishes (regular tissue culture plastic precoated with 0.2% Gelatin in dH_2O for 15 min. @ 37°C) on top of a confluent monolayer of murine embryonic feeder cells (MEFs).

Generation of embryoid bodies

EB media

425	ml	IMDM (Invitrogen 12440-053)
75	ml	ES cell grade Serum (Invitrogen: 26140-095 (1L))
5	ml	L-Glutamine (Invitrogen: 25030-081)
5	ml	Pen/Strep (Invitrogen: 15140-122)
18.9	µl	Monothioglycerol (Sigma M-6145)
2	ml	FE-saturated transferring (Sigma, T1283, 50 mg/ml stock in H_2O)
250	µl	Ascorbic Acid (Sigma, A4403) 50 mg/ml Stock)
5	ml	Non-essential amino acids (Invitrogen: 11140-050)
5	ml	Sodium Pyruvate (Invitrogen 11360-070, 100 mM Stock)

Trypsinize ES cells, resuspend in a small volume (10 ml) of EB media

Put the cells back into an untreated 10 cm tissue culture dish and let the fibroblast feeders (MEFs) adhere for about 20 minutes. Repeat this step if necessary.

Take supernatant containing the ES cells and count cell density

Resuspend the cells @ 400 cells/ 25 µl in EB medium. 50 ml of medium will cover approximately ten 15-cm petridishes of EBs

Make 25 µl drops onto 15 cm petridish using 8-well multichannel pipette
It is **essential** that **petridishes** be used in this procedure. ES cells and EBs will stick to regular tissue culture plastic and prevent proper EB formation.

Carefully turn the dish over so that the drops hang down.

Incubate for maximally 3 days.
If longer differentiation is required, EBs need to be washed from the plate and pooled into 10 cm petridishes. Place the petridishes on a shaker and shake very slowly so that the EBs do not attach to the plate or each other. Also put a dish with water below and on top of each stack of EB containing petridishes.

We feed our EBs regularly; every other day the first few days, every day after day 10. By rotating the dish you can concentrate the EBs in the center, which allows you to aspirate the media from the side.

Isolation of SSEA1+ cells from embryoid bodies

Wash the EBs twice with PBS and finally resuspend into 1 ml PBS. EBs will settle quickly by gravity, but can be centrifuged @ 1200 rpm for 2 minutes to speed things up if

needed. Add 500 µL Collagenase (Type IV collagenase, 10 mg/ml stock, Sigma C5138) and put on ice for 15 minutes. This step allows the Collagenase to penetrate the tissue without starting to digest the connective tissue yet, avoiding over-digestion of the cells on the outside of the EB.

After this penetration step, Incubate at 37°C for 10-15 min. While the EBs will not dissociate to single cells, they will become more "fuzzy" in appearance. Dissociate the EBs by adding 10 ml cell dissociation buffer (Invitrogen 13151-014) and pipette up and down vigorously.

Pellet the cells for 3 min at 1400 rpm and resuspend the pellet into 10 ml EB media. To avoid cell clumps in proceeding steps, strain the cells using a 70 µM cell strainer (BD Falcon 352350). Pellet the cells again for 3 min at 1400 rpm Resuspend cells in approximately 300 µl ice-cold DME/1% Bovine serum albumin (BSA, Fisher, Cat# BP1600-100, 0.5 gram into 50 ml PBS) and add 100 µl anti-SSEA-1 (Developmental Studies Hybridoma Bank, University of Iowa, MC-480). Incubate for 60 min on ice. Wash twice with 10 mL ice-cold DME/1% BSA and resuspend cells in 1 ml ice-cold DME/1% BSA. Transfer the cells to a screw-cap eppendorf tube and add anti-IgM magnetic beads (DYNAL, Rat-anti-Mouse IgM, Cat# 110.39 (5ml)) according to the manufacturers specifications. Rotate the cell-magnetic bead suspension for 30 min at 4°C. Using a magnet stand (Promega, Magnasphere Magnetic Separation stand, 1.5mL tubes, Z5332), separate the cells that have bound the magnetic beads from the other contaminating cells. Discard the unbound fraction and wash the beads 3 times with ice-cold DME/1% BSA, repeating the above magnetic separation procedure.

To isolate RNA from the slected cells, after the final wash, take off all supernatant and resuspend the beads with the attached SSEA1-positive cells in 500 µL Trizol (Invitrogen 15596-018) using a 1 ml syringe with 23G needle. Pass the cells including the magnetic beads through the needle a few times to assure complete lysis. Proceed with RNA purification according to the manufacturers protocol.

Selected readings:

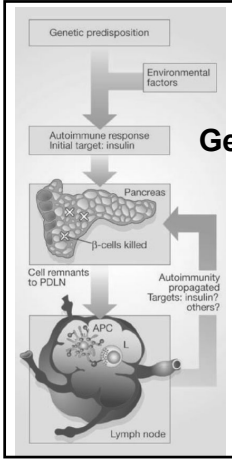
1. Geijsen, N. et al. Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* 427, 148-54 (2004).
2. Hubner, K. et al. Derivation of oocytes from mouse embryonic stem cells. *Science* 300, 1251-6. (2003).
3. Toyooka, Y., Tsunekawa, N., Akasu, R. & Noce, T. Embryonic stem cells can form germ cells in vitro. *Proc Natl Acad Sci U S A* 100, 11457-62 (2003).
4. Martin, G. R. & Evans, M. J. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proc Natl Acad Sci U S A* 72, 1441-5 (1975).
5. Leahy, A., Xiong, J. W., Kuhnert, F. & Stuhlmann, H. Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. *J Exp Zool* 284, 67-81. (1999).
6. Tada, T. et al. Epigenotype switching of imprintable loci in embryonic germ cells. *Dev Genes Evol* 207, 551-61 (1998).

7. Mann, J. R., Gadi, I., Harbison, M. L., Abbondanzo, S. J. & Stewart, C. L. Androgenetic mouse embryonic stem cells are pluripotent and cause skeletal defects in chimeras: implications for genetic imprinting. *Cell* 62, 251-60 (1990).
8. Surani, M. A. et al. Genome imprinting and development in the mouse. *Dev Suppl*, 89-98 (1990).

Derivation of NTSC

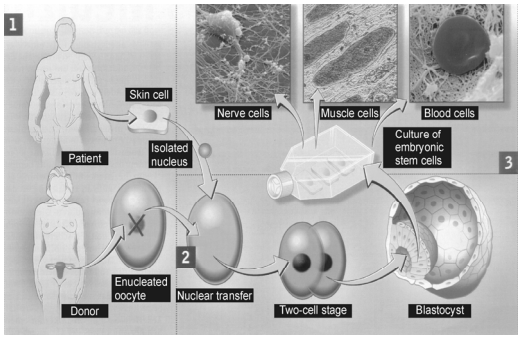
Nuclear transfer in human

- To avoid immunological rejection after transplantation of differentiated stem cells
- To learn more about mtDNA/epigenetic/reprogramming
- To bring disease to the plastic dish



Genetic predisposition and Type 1 diabetes

Nuclear transfer stem cells



Donor cells for human NT

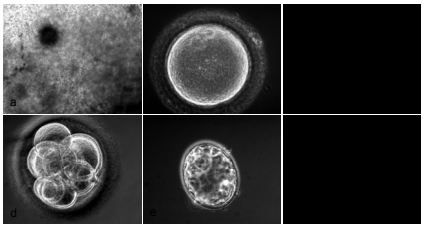
- Undifferentiated hESC
- Fibroblast cells from healthy female person
- Donor cells from patient with inherited disease

Developmental potential of human oocytes

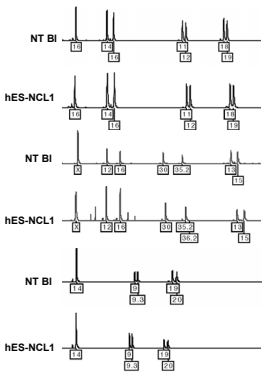
Table 1. Effects of different sources of human oocytes on cleavage and blastocyst rates after nuclear transfer with undifferentiated human embryonic stem cells. PB = polar body.

Sources of oocytes	*No. patients*	*No. oocytes*	*PB (%)*	*Cleaved (%) Day 3*	*Blastocyst (%) Day 5*
Failed fertilization	4	10	7 (70.0)	0	0
Follicle reduction	4	10	8 (80.0)	3 (30.0)	1 (10.0)
Failed to inseminate	1	12	10 (83.3)	0	0
Cysterectomy	2	4	2 (50.0)	0	0
Total	11	36	27 (75.0)	3 (8.3)	1 (2.8)

NT in human



DNA fingerprinting



Human nuclear transfer

Table II. Human nuclear transfer efficiencies using aged, failed-to-fertilize IVF (f-IVF), failed-to-fertilize ICSI (f-ICSI) oocytes and fresh ovulation-induced oocytes obtained from women undergoing infertility treatment

Oocyte source	Donor cell	MII oocytes (*n*)	Enucleated (%)*	Fused (%)*	Cultured (%)*	Cleaved (%)**	Blastocysts (*n*)
f-IVF	FIB-LIKE	26	14 (54)[a]	5 (19)[a]	5 (19)[a]	0	–
f-ICSI	FIB-LIKE	22	6 (27)[a]	1 (5)[a]	1 (5)[a]	0	–
Fresh	FIB-LIKE	9	9 (100)[b]	6 (67)[b]	6 (67)[b]	1 (17)[a]	0
Fresh	ESC	14	14 (100)[b]	8 (57)[b]	8 (57)[b]	1 (12.5)[a]	0
Fresh	FIB	25	23 (92)[b]	22 (88)[b]	18 (72)[b]	7 (39)[b]	0

Donor cells used were either a fibroblast-like cell line (FIB-LIKE), human embryonic stem cell (hESC) line (ESC) derived from NCL-1 or human skin fibroblasts (FIB).
*Percentage from total metaphase II (MII) oocytes.
**Percentage from cultured reconstructed embryos, includes fragmented embryos, not fragmented oocytes.
[a]and [b]indicate significant difference between column treatments using a standard two-tailed *t*-test ($P < 0.05$).

NT embryos

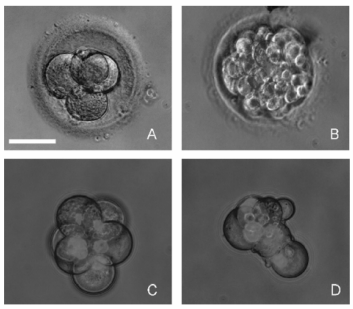


Figure 1. Nuclear transfer (NT) embryos reconstructed using fresh ovulation-induced oocytes and varying donor cell nuclei. (**A**) A 4-cell NT embryo produced from fibroblast-like (**FIB-LIKE**) donor cell. (**B**) A fragmented embryo produced from embryonic stem cell (ESC) donor cell. (**C**) A 6-cell and (**D**) fragmented 5-cell embryo produced from fibroblast (**FIB**) cell. Zona pellucida digested using protease following developmental arrest and before Hoechst staining (C and D). Scale bar = 30 μm.

Localisation of NuMA and α-tubulin

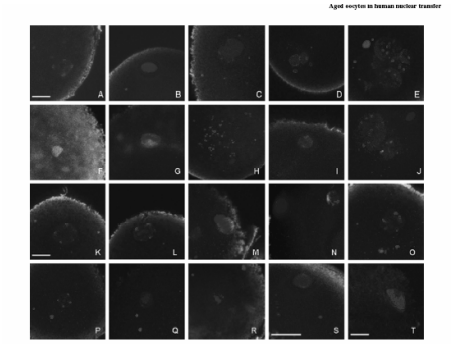


Figure 4. Double labelling of 20 ($n = 10$ f-IVF and $n = 10$ f-ICSI) failed-to-fertilize oocytes for microtubule markers, nuclear mitotic apparatus protein (NuMA) and α-tubulin. Localization of NuMA (red) and α-Tubulin (green) in f-IVF (A–J) or f-ICSI (K–T) oocytes. Chromatin was counterstained with Hoechst 33342 (blue). Tetrapolar spindles in A, K, L, P; unusual patterns of cytoplasmic NuMA in oocytes D, E, J; NuMA not co-localized to chromatin in oocytes H and M; distinct germinal vesicle-like expression of NuMA in oocyte T were observed. Scale bar in A, K, S and T indicates 10 μM for (A–J), (K–R), S and T, respectively.

Oocyte specific genes

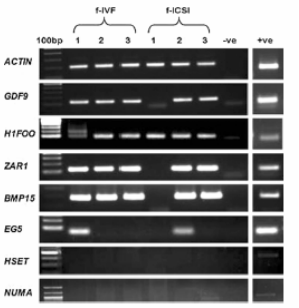
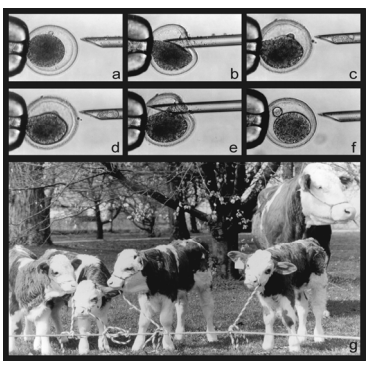


Figure 7. Messenger RNA (mRNA) expression of the house-keeping gene (*ACTIN*), oocyte-specific genes: growth differentiation factor 9 (*GDF9*), oocyte-specific histone 1 (*H1FOO*), zygote arrest 1 (*ZAR1*), bone morphogenetic protein 15 (*BMP15*) and microtubule markers: *EG5*, *HSET* and *NuMA* in single failed-to-fertilize, aged oocytes (n = 3 f-IVF and n = 3 f-ICSI). One f-ICSI oocyte aberrantly expressed *GDF9*, *ZAR1*, *BMP15* and *EG5*. Two of the six oocytes analysed correctly expressed microtubule marker *EG5*. The microtubule markers *HSET* and *NuMA* were not detectable at the RT-PCR level in any oocytes. One f-IVF oocyte expressed a double band of *H1FOO*, possibly indicative of a splice variant.

Clones are not clones

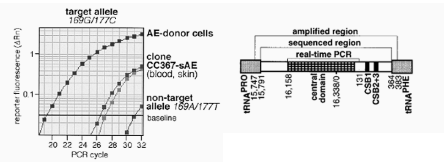


Mitochondrial DNA heteroplasmy in cloned cattle produced by fetal and adult cell cloning

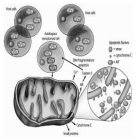
Table 1 • mtDNA heteroplasmy generated by fetal and adult cell cloning

Donor cell	Cloned animals	% donor mtDNA	Gestation in days	Tissues analysed	Abnormalities[10,11]
FF	CFI198-sFF	0.4, 0.8	60	liver, leg	no
FF	CFI324-sFF	≤0.3[a]	165	liver, kidney, heart, skin, lung, muscle	h
FF	CFI324-sFF	≤0.2[a]	165	liver, kidney, heart, skin, muscle	h, l
FF	CFI325-sFF	1–4	165	liver, kidney, heart, skin, cerebellum	h
FF	CCI328-nsFF	1.1, 0.6	281	blood, spermatozoa	no
FF	CCI328-nsFF	1.4	281	blood	l, o
AE	CF362-sAE	0[b]	155	liver	h, o
AE	CC367-sAE	0.4, 0.5	287	blood, skin	no
AF	CF267-sAF	2	212	skin	h, o
AF	CC042-sAF	0.6	286	blood	l, o

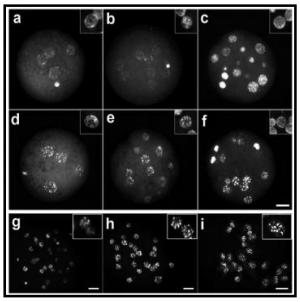
h, hydroallantois; l, 'large offspring syndrome'; o, others. Donor mtDNA ratios for CFI324-sFF: 0.3% in muscle; 0.2% in heart and lung; 0.1% in skin; <0.1% in kidney and liver. Donor mtDNA ratios for CFI324-sFF: 0.2% in liver, lung, heart and skin; 0.1% in kidney; and <0.1% in muscle. Assay sensitivity: [a]<0.1%; [b]0.1%.



Possible mechanism of acute graft rejection due to mitochondrial heteroplasmy following cell transplantation

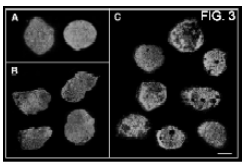


DNA-methylation in NT-bovine and rabbit embryos

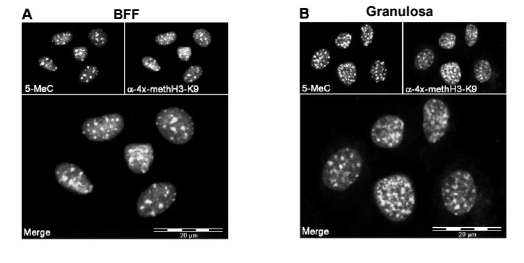


IVF

NT



Epigenetic modifications in donor nuclei used in NT



Efficiency of NT using different donor-cells

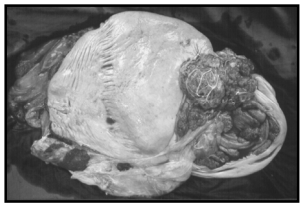
Table 1

Bovine granulosa cells vs. fetal fibroblasts as donors for nuclear transfer

Donor cells	Reconstructed (%)	Cleaved (%)	Blastocysts (%)[1]
Granulosa cells	99/107 (93)	68 (69)	30 (30)[a]
BFF 116	158/164 (96)	96 (61)	26 (16)[b]

[1]Blastocyst rates are based on the number of reconstructed (fused) karyoplast-cytoplast complexes. a:b: $P < 0.01$.

Consequences of reproductive NT in bovine

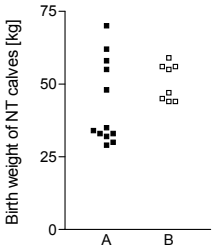


Aborted bovine fetus, 5. month

Placenta

V Zakhartchenko, M Stojkovic, H Wenigerkind, E Wolf
Munich, Germany

Large offspring syndrome

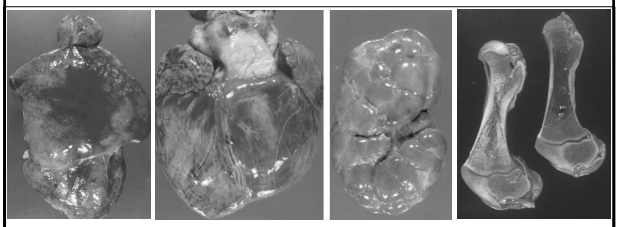


A: Munich; B:ACT

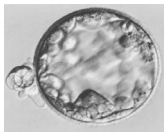
V Zakhartchenko, M Stojkovic, H Wenigerkind, E Wolf
Lehrstuhl für Molekulare Tierzucht und Biotechnologie

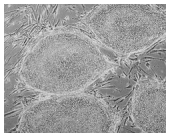
NT-calves using granulosa cells (14 years old Swiss brown cow)

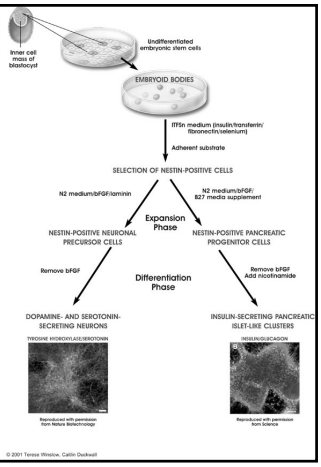
Organs of NT-calves



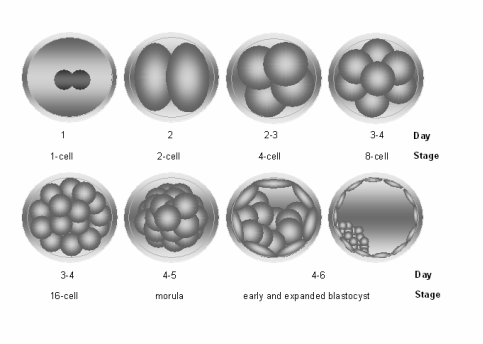
Liver Heart Kidney Bones



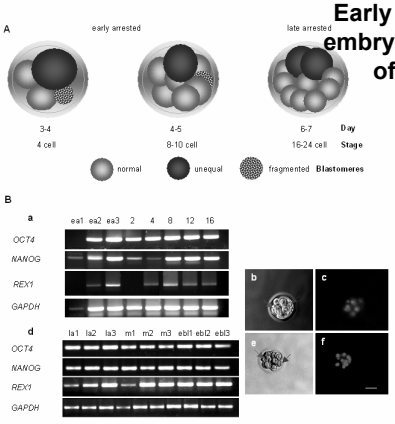




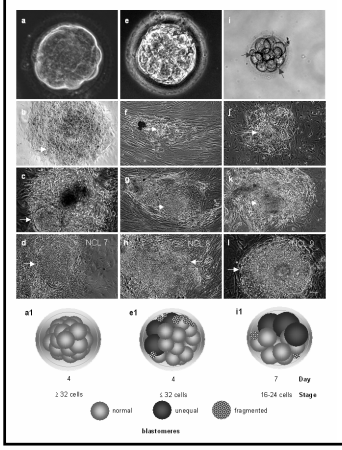
Normal development of early human embryos

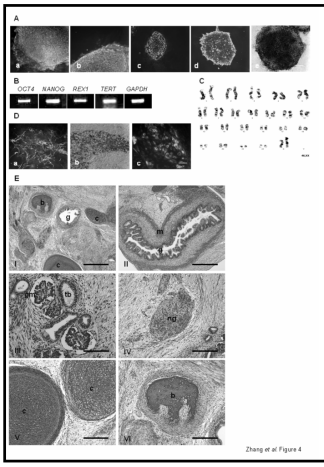


Early and arrested IVF embryos express genes of pluripotency



Chronology of hESC derivation





Characterisation of new line derived from arrested IVF embryo

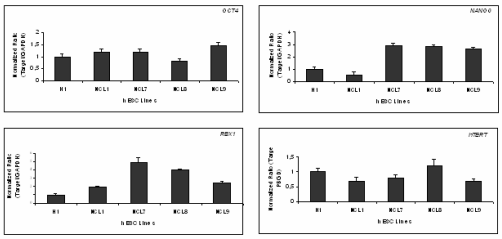
New hESC lines derived from IVF morulae, blastocysts and arrested embryos

Table 2: Characterisation of new hESC lines derived from developing and arrested embryos

Line	Staining				Intracellular markers				Karyotype	IVD	Teratoma
	TRA-1-60	TRA-1-81	SSEA4	AP	*OCT4*	*NANOG*	*REX1*	*TERT*			
hES-NCL2	+	+	+	+	+	+	+	+	46, XX	+++	nd
hES-NCL3	+	+	+	nd	+	+	+	+	46, XY	+++	+++
hES-NCL4	+	+	+	nd	+	+	+	+	46, XY	+++	+++
hES-NCL5	+	+	+	+	+	+	+	+	46, XX	+++	nd
hES-NCL6	+	+	+	+	+	+	+	+	46, XX	+++	nd
hES-NCL7	+	+	+	+	+	+	+	+	46, XY	+++	+++
hES-NCL8	+	+	+	+	+	+	+	+	46, XY	+++	+++
hES-NCL9	+	+	+	+	+	+	+	+	46, XX	+++	+++

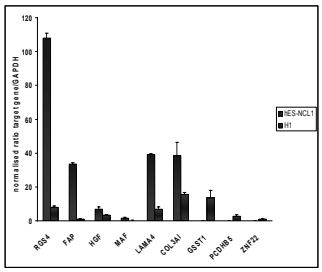
All hESC lines have been propagated for more than 23 passages and subsequently frozen. AP: alkaline phosphatase; nd: not determined; IVD: *in vitro* differentiation; +++: ectoderm, mesoderm, endoderm.

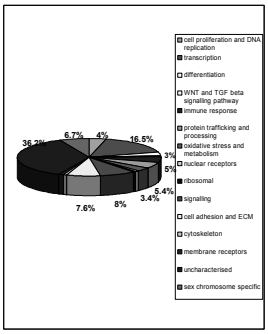
Real Time RT-PCR



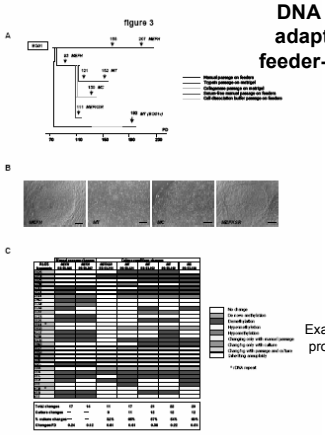
Supplementary Figure 4: Quantitative expression ± SD (by real-time RT-PCR) of pluripotency genes in old (H1 and hES-NCL1) and new (hES-NCL7-9) hESC lines. New hESC lines derived from morulae or arrested embryo expressed similar or higher level of different pluripotency genes (*OCT4*, *NANOG*, *REX1*, *TERT*) when compared with H1 or hES-NCL1 lines derived from blastocysts. Primer sequences and their PCR conditions are provided upon request.

Molecular characterisation of hESC





DNA methylation changes adapted to serum-free and feeder-free culture conditions

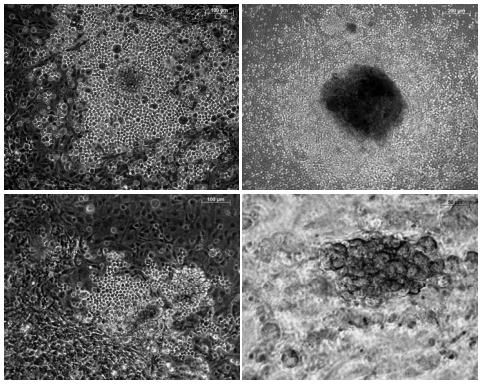


Examination of genome-wide DNA methylation profiles of *NotI* sites in gene-rich CpG islands

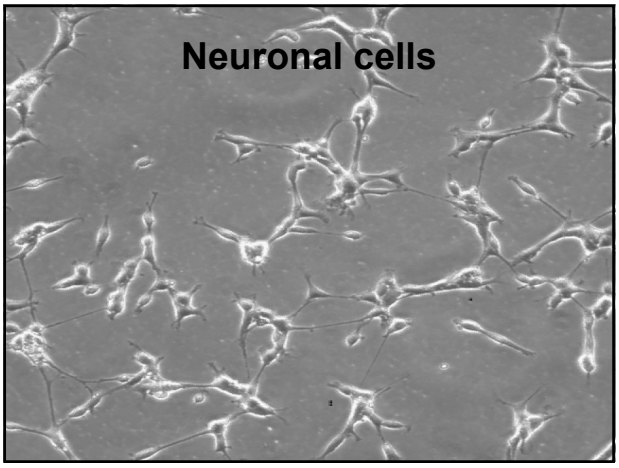
Epigenetic profile of hESC

- Examination of the DNA methylation profiles of >2000 genomic loci by Restriction Landmark Genome Scanning.
- We identified substantial interline epigenetic distance between four independently derived hESC lines.
- Lines were found to inherit further epigenetic changes over time in culture, with most changes arising in the earliest stages post-derivation.
- The majority of culture-induced changes (81.2-83.3%) were stably inherited both within the undifferentiated cells and post-differentiation.
- Adapting a line to a serum-free culture system resulted in additional epigenetic instability.

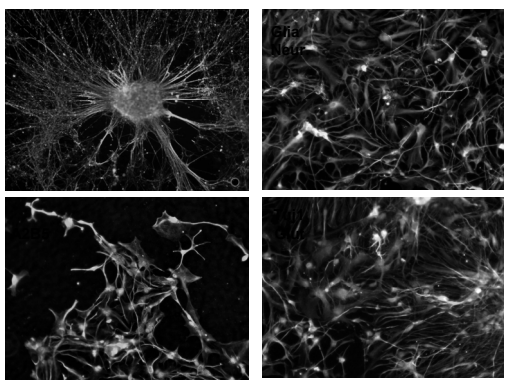
Cobblestone Areas and Blood-12 Days Colony Assays



Neuronal cells



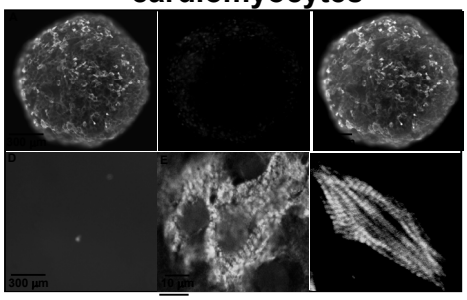
Neuronal characteristics



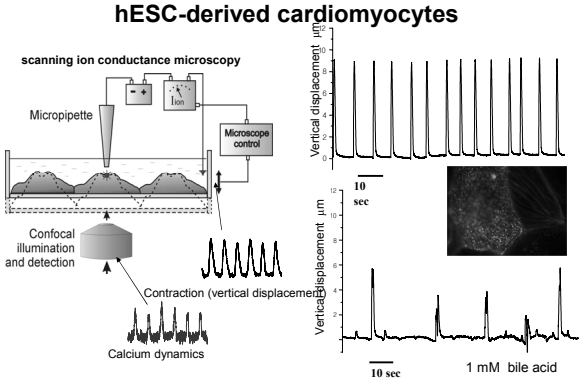
hESC treatment

hESC treatment

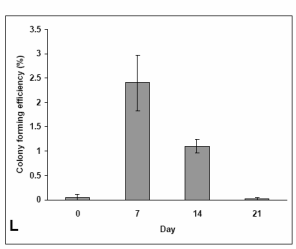
Targeted differentiation-cardiomyocytes

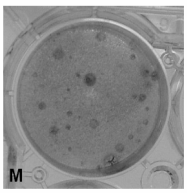


Influence of bile acid on contraction of hESC-derived cardiomyocytes



Colony forming efficiency of differentiating hESC

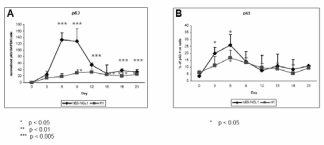


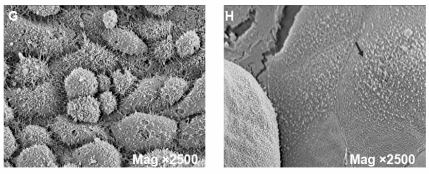


hESC into corneal

Sajjad *et al.* (i2007)

SEM of the differentiating hES-NCL1 cells on collagen IV coated plates





Conclusions

- NTSC offer enormous potential medical and basic biology benefits
- NT offers excellent posibilities to study physiology of human oocytes/embryos, mtDNA, epigenetic and gene (mis)expressions
- Improvement of derivation and growth conditions necessary
- Targeted differentiation
- Develop new treatments for genetic diseases, diseases of old age, serious injuries and to screen diseases

Acknowledgement:

P. Stojkovic
S. Erceg
C. Xiong
M-C. Escobedo
V. Blanca Benito
N. Martí Gutiérrez
S. Prado López
M. Pérez Aragó
R. Pilar
C. Menchon
D. López
M. Ronchi
...
M. Lako
L. Armstrong
V. Hall
L. Young
C. Allegrucci
J. Gorelik
W. Dean

- **Area: 32 000 m²**
- **Staff: >300**
- ***Stem cell bank***
- ***Animal facilities***
- ***Surgical unit***
- ***Proteomics***
- ***Bioinformatics***
- ***Drug development***
- ***Cancer research***
- ***Adult stem cells***
- ***Neurobiology***
- ***Molecular genetics***
- ***Immunology***
- ...

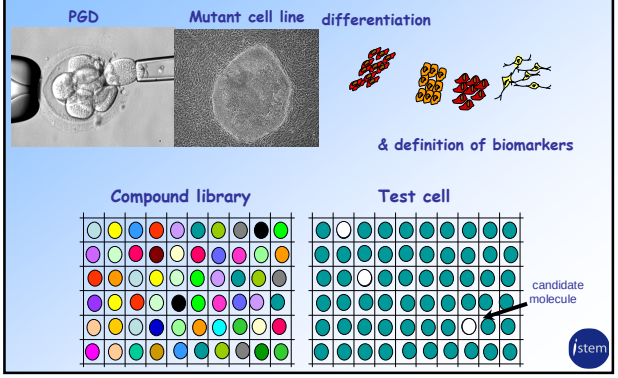
I-STEM

Institute for Stem cell Therapy and Exploration of mechanisms in Monogenic diseases (Evry France)

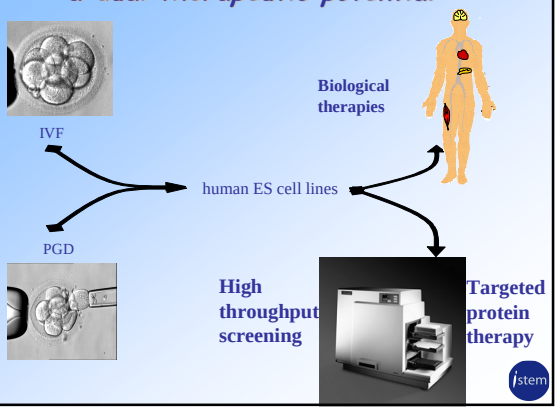
The potential use of hES cells other than in cell therapy

mpeschanski@istem.genethon.fr

General principles of the « drug discovery » program of I-Stem

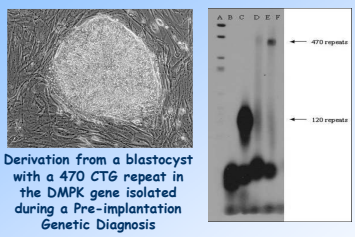


a dual therapeutic potential



ES cell lines derived from genetically characterized embryos

DM1-mutant hES cell line
VUBO3_DM1 (XX)



Derivation from a blastocyst with a 470 CTG repeat in the DMPK gene isolated during a Pre-implantation Genetic Diagnosis

Three normal hES cell lines

- VUB01 (XY)
- VUB06 (XX)

Derivation in the same conditions

- SA-01 (XY)

Cellartis

istem

Steinert disease or Myotonic dystrophy type 1 (DM1)

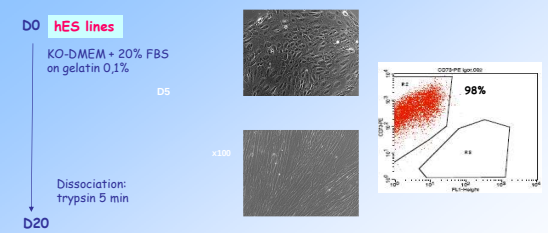
Genetic
Incidence of 13,5/ 100.000 newborns

Clinical symptoms
Myotonia : delayed muscle relaxation after a voluntary contraction
Muscle Dystrophy

Molecular bases
Mutation in the DMPK gene
Instable CTG repeats in the 3'-untranslated region
Mutant DMPK transcripts accumulate in the nucleus and effect the splicing of other mRNAs

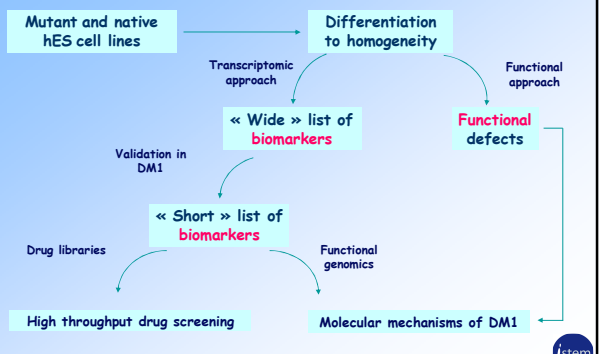
➡ This indirect effect leads to a wide diversity of cell types affected in DM1

Obtaining homogenous populations of cells example: mesenchymal precursors

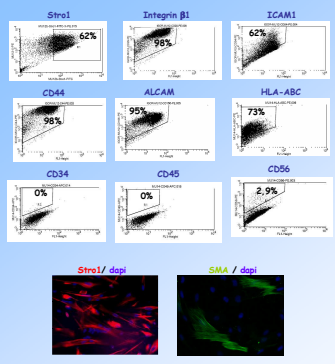


Obtention of a homogenous population of "MSC-like"

PERT chart of the project



Characterization of MSC-like population



CD105	99%
VCAM	0%
CD90 (Thy-1)	61%
Vimentine	86%
HLA-DR	0%
CD14 (hemato)	0%
CD31 (endo)	0%
Forse-1 (neuro)	0%

	MSCs
CD13	+
CD34	-
CD44	+
CD45	-
CD71 transferrin rec	+
CD73 SH-3	+
CD105 endoglin, SH2	+
CD166	+
HLA ABC	+
SSEA-4	+

(Olivier et al)

Nuclear foci revealed using a probe for the CTG repeats

Neural Precursor Cells (NPC)

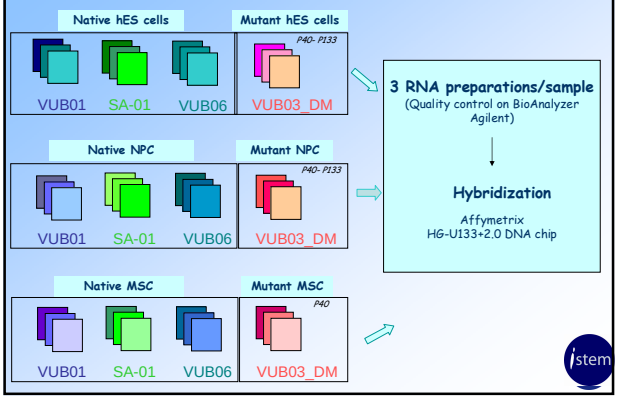
VUB01-P42 | VUB03_DM1-P50 | VUB03_DM1-P137

Candidate biomarkers for DM1

A few examples from mesenchymal precursors

Genes	Biological role	Decrease in expression	Possible test	Validation : Known as affected in DM1?	Chromosome
A	modulates muscle creatine kinase	12 times	Dosage CKM	no	1
B	Muscle contraction & relaxation	2.2 times	Dosage ATP	yes	12
C	development of the nervous system	22 times	ICC	no	4
D	modulation of cell growth	16 times	Cell growth	yes	17

Search for differential gene expression in normal vs mutant hES cells by transcriptome analysis



Validation of the potential biomarkers for DM1

Q-PCR
Validation of the modulations of gene expression in one mutant cell line (VUB03_DM1) vs 3 native

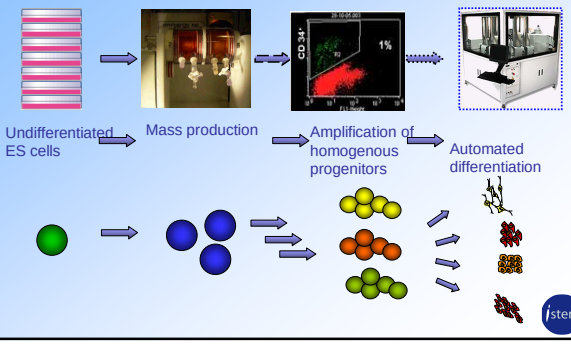
Extinction of the DMPK gene expression
Validation of the effect of the mutant DMPK gene on the modulations of gene expression

DM1 models
Validation of the modulated genes associated to the mutant phenotype

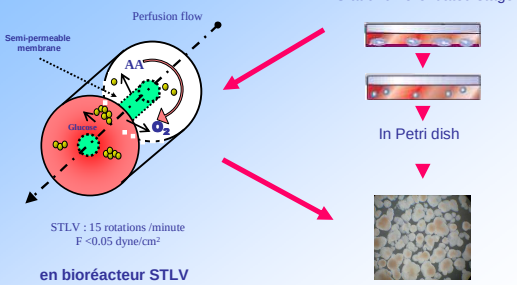
DM1 consortium
DM1 skin biopsies
Guillaume Bassez
Fetal and adult DM1 muscle cells
Denis Furling
Patient blood cells, muscle cells
Jacques Puymirat
Transgenic mouse
Geneviève Gourdon
Human neural cells tranfected by mutant DMPK
Nicolas Sergeant

VALIDATED BIOMARKERS

The cell factory



Amplification of embryoid bodies

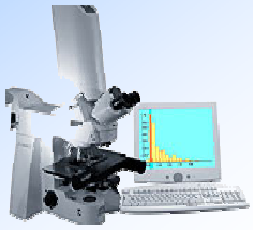


A fully automated cell culture system

A dedicated HTS platform

for compound libraries

and functional genomic tools



Organisation of a PGD-derived ES cell bank (collaboration with Stéphane Viville)

Goal : to create a mutant ES cell bank to provide new pathological models of monogenic diseases to all interested users