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SCIENCE MOVING
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The blastocyst: perpetuating life

Special Interest Groups Embryology and Stem Cells

2

3 July 2011
Stockholm, Sweden

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Organised by
Special Interest Groups Embryology and Stem Cells

Contents

Course coordinators

Cristina Magli (Italy, SIG Embryology), Karen Sermon (Belgium, SIG Stem Cells)

Course description

This advanced course aims at providing insight into the cellular and molecular similarities and differences between the blastocyst and its famous by-product, the embryonic stem cell. Although the topics mainly discuss basic scientific findings, each of them have potential repercussions for IVF or regenerative medicine. We will look into the origins of pluripotency and the roles played by eg mitochondria, intercellular connections, and microRNAs

Target audience

Fundamental embryologists, clinical embryologists with an interest in fundamental embryology, stem cell biologists

Scientific programme

09.00 - 09.30	Embryo development and blastocyst formation: timing and synchronization of events -**Thorir Hardarsson (Sweden)**
09.30 - 09.45	*Discussion*
09.45 - 10.15	Cell (pre)-destiny in the human preimplantation embryo and implications for IVF and PGD - **Hilde Van De Velde (Belgium)**
10.15 - 10.30	*Discussion*
10.30 - 11.00	*Coffee break*
11.00 - 11.30	Metabolic requirements of embryo growth and viability - **Henry Leese (United Kingdom)**
11.30 - 11.45	*Discussion*
11.45 - 12.15	The relationship between pluripotency and mitochondrial DNA proliferation during early embryo development and embryonic stem cell differentiation – **Justin St John (United Kingdom)**
12.15 - 12.30	*Discussion*
12.30 - 13.30	*Lunch*
13.30 - 14.00	Developmental stages of blastocysts: intercellular junctions and cell polarity – **Takashi Hiiragi (Germany)**
14.00 - 14.15	Discussion
14.15 - 14.45	The role of microRNA in embryos and hESC – **Gustavo Tiscornia (Spain)**
14.45 - 15.00	*Discussion*
15.00 - 15.30	*Coffee break*
15.30 - 16.00	Pluripotency and stem cell states – **Ewart Kuijk (The Netherlands)**
16.00 - 16.15	Discussion
16.15 - 16.45	Blastocyst cryopreservation: maximizing survival and development – **Etienne Van den Abbeel (Belgium)**
16.45 - 17.00	*Discussion*
17.00 – 17.30	SIG Embryology Business Meeting

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ESHRE – European Society of Human Reproduction and Embryology

What is ESHRE?

ESHRE was founded in 1985 and its **Mission Statement** is to:

- promote interest in, and understanding of, reproductive science
- facilitate research and dissemination of research findings in human reproduction and embryology to the general public, scientists, clinicians and patient associations.
- inform policy makers in Europe
- promote improvements in clinical practice through educational activities
- develop and maintain data registries
- implement methods to improve safety and quality assurance

Executive Committee 2009/2011

Chairman	• Luca Gianaroli	Italy
Chairman Elect	• Anna Veiga	Spain
Past Chairman	• Joep Geraedts	Netherlands
	• Jean François Guérin	France
	• Timur Gürgan	Turkey
	• Ursula Eichenlaub-Ritter	Germany
	• Antonis Makrigiannakis	Greece
	• Miodrag Stojkovic	Serbia
	• Anne-Maria Suikkari	Finland
	• Carlos Plancha	Portugal
	• Françoise Shenfield	United Kingdom
	• Etienne Van den Abbeel	Belgium
	• Jolieneke Schoonenberg-Pomper	Netherlands
	• Veljko Vlaisavljevic	Slovenia
	• Søren Ziebe	Denmark

ESHRE Organisation

- General Assembly of Members
  - Executive Committee
  - Committee of Nat. Representatives
  - Central Office
  - ESHRE Consortia
    - EIM Consortium
    - PGD Consortium
  - Sub-Committees
    - Finance Sub-Committee
    - Comm. Sub-Committee
    - Publ. Sub-Committee
      - Editorial Office
      - Publisher
      - Editors-in-Chief
    - Int'l Scientific Committee
    - SIG Sub-Committee
      - SIG Coordinators
  - Task Forces

ESHRE Journals

Human Reproduction with impact factor 3.859

Human Reproduction Update with impact factor 7.042

Molecular Human Reproduction with impact factor 3.005

Campus Activities and Data Collection

Campus / Workshops

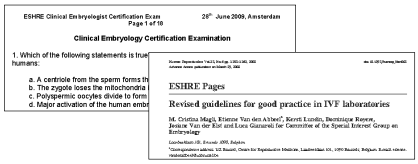
- Meetings are organised across Europe by Special Interest Groups and Task Forces
- Visit www.eshre.eu under CALENDAR

Data collection and monitoring

- European IVF Monitoring Group data collection
- PGD Consortium data collection

ESHRE Activities

- Embryology Certification
- Guidelines
- Position papers
- News magazine “Focus on Reproduction”


ESHRE Clinical Embryologist Certification Exam 20th June 2009, Amsterdam
Page 1 of 10

Clinical Embryology Certification Examination

1. Which of the following statements is true
humans:

a. A centriole from the sperm forms th
b. The zygote loses the mitochondria
c. Polyspermic oocytes divide to form
d. Major activation of the human embr

ESHRE Pages

Revised guidelines for good practice in IVF laboratories

M. Cristina Magli, Etienne Van den Abbeel, Kevrli Lundin, Dominique Royere, Jessica Van der Elst and Luca Gianaroli for Committee of the Special Interest Group on Embryology

ESHRE COMMUNITY

RSS feeds for news in reproductive medicine

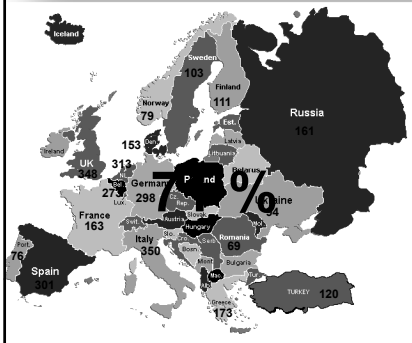
Since launch 12/2009: **1,360 Fans**

Since launch 12/2009: **190 followers** (journalists, scientific organisations, patient societies, governmental bodies)

Retweets to MHR

Find a member

ESHRE Membership (1/3)



4,017	Europe
476	Asia
372	North America
332	Middle East
221	Africa
142	Oceania
99	South America

TOTAL MEMBERSHIP*: 5 659 members

* as of July 2010

ESHRE Membership (2/3)

	1 yr	3 yrs
Ordinary Member	€ 60	€ 180
Paramedical Member*	€ 30	€ 90
Student Member**	€ 30	N.A.

*Paramedical membership applies to support personnel working in a routine environment such as nurses and lab technicians.
**Student membership applies to undergraduate, graduate and medical students, residents and post-doctoral research trainees.

ESHRE Membership – Benefits (3/3)

1) Reduced registration fees for all ESHRE activities:

Annual Meeting	Ordinary	€ 480	(€ 720)
	Students/Paramedicals	€ 240	(€ 360)
Workshops*	All members	€150	(€ 250)

2) Reduced subscription fees to all ESHRE journals – e.g. for Human Reproduction €191 (€ 573!)

3) ESHRE monthly e-newsletter

4) News Magazine "Focus on Reproduction" (3 issues p.a.)

5) Active participation in the Society's policy-making

*workshop fees may vary

Special Interest Groups (SIGs)

The SIGs reflect the scientific interests of the Society's membership and bring together members of the Society in sub-fields of common interest

- Andrology
- Early Pregnancy
- Embryology
- Endometriosis / Endometrium
- Ethics & Law
- Safety & Quality in ART
- Psychology & Counselling
- Reproductive Genetics
- Reproductive Surgery
- Stem Cells
- Reproductive Endocrinology

Task Forces

A task force is a unit established to work on a single defined task / activity

- Fertility Preservation in Severe Diseases
- Developing Countries and Infertility
- Cross Border Reproductive Care
- Reproduction and Society
- Basic Reproductive Science
- Fertility and Viral Diseases
- Management of Infertility Units
- PGS
- EU Tissues and Cells Directive

ESHRE – Annual Meeting

- One of the most important events in reproductive science
- Steady increase in terms of attendance and of scientific recognition

Track record:

ESHRE 2010 – Rome: 9,204 participants
ESHRE 2009 – Amsterdam: 8,055 participants
ESHRE 2008 – Barcelona: 7,559 participants

Future meetings:

ESHRE 2011 – Stockholm, 3-6 July 2011
ESHRE 2012 – Istanbul, 1-4 July 2012

ESHRE 2011, Stockholm, Sweden

When: **3 - 6 July 2011**
Where: **Stockholmsmässan, Mässvägen 1, Älvsjö, Sweden**
www.stockholmsmassan.se

Chair of conference: **Kersti Lundin**

Hotel and Travel:
MCI - Stockholm Office
Phone: +46 (0)8 54651500
E-mail: eshre@mci-group.com

For updates visit www.eshre.eu

ESHRE 2011, Stockholm

Keynote Lectures

Aneuploidy in humans: what we know and we wish we knew – Terry Hassold (USA)

Historical Lecture

A brave new world with a brave old humankind; quo vadimus – E. Diczfalusy (SE)

MHR Symposium – The paternal genome

Sperm chromatin packaging – B. Robaire (CDN)

The human sperm epigenome – B. Cairns (USA)

ESHRE 2011, Stockholm: Debates

This house believes that obese women should not receive treatment until they have lost weight

- **Yes: Mark Hamilton *(UK)***
- **No: Guido de Wert *(NL) - TBC***

Paramedical invited session: Should we pay donors?

- **Yes: Herman Tournaye (BE)**
- **No: Laura Witjens (UK)**

Annual Meeting – Pre-Congress Courses

- PCC 1: The challenges of embryo transfer (Paramedical Group)
- PCC 2: The blastocyst: perpetuating life (SIG Embryology and SIG Stem Cells)
- PCC 3: From genes to gestation (SIG Early Pregnancy and SIG Reproductive Genetics)
- PCC 4: Lifestyle and male reproduction (SIG Andrology)
- PCC 5: Ovarian ageing (SIG Reproductive Endocrinology)
- PCC 6: The impact of the reproductive tract environment on implantation success (SIG Endometriosis/Endometrium)
- PCC 7: Adhesion prevention in reproductive surgery (SIG Reproductive Surgery)

Annual Meeting – Pre-congress Courses

- PCC 8: Theory and practice update in third party reproduction
  (SIG Psychology and Counselling)
- PCC 9: Ethical aspects of non-invasive prenatal diagnosis
  (SIG Ethics & Law)
- PCC 10: Patient-centered fertility services
  (SIG SQUART)
- PCC 11: Clinical management planning for fertility preservation in female cancer patients
  (TF Basic Science and TF Preservation in Severe Disease in collaboration with the US OncoFertility Consortium)
- PCC 12: Opportunities for research in female germ cell biology
  (TF Basic Science)

Annual Meeting – Pre-congress courses

- PCC 13: Assisted reproduction in couples with HIV
  (TF Fertility and Viral Diseases)
- PCC 14: Prevention of infertility – from preconception to post-menopause
  (TF Reproduction and Society)
- PCC 15: Hot topics in male and female reproduction
  (ASRM exchange course)
- PCC 16: Academic Authorship programme
  (Associate Editors ESHRE journals)
- PCC 17: Science and the media, an introduction to effective communication with the media
  (Communications SubCommittee ESHRE)

Certificate of attendance

1/ Please fill out the evaluation form during the campus

2/ After the campus you can retrieve your certificate of attendance at www.eshre.eu

3/ You need to enter the results of the evaluation form online

4/ Once the results are entered, you can print the certificate of attendance from the ESHRE website

5/ After the campus you will receive an email from ESHRE with the instructions

6/ You will have TWO WEEKS to print your certificate of attendance

Contact

ESHRE Central Office
Tel: +32 (0)2 269 09 69
info@eshre.eu / www.eshre.eu

Embryo development and blastocyst formation: timing and synchronization of events

Thorir Hardarson, PhD.

Fertilitetscentrum Göteborg

Potential conflict of interest

None

Fertilitetscentrum Göteborg

Learning objectives

Embryo development and blastocyst formation: timing and synchronization of events

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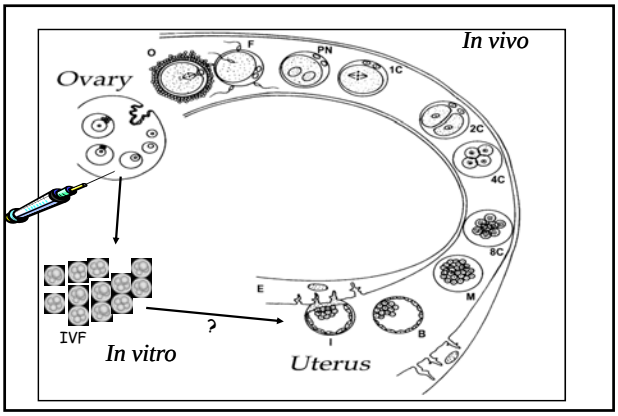


"Creation of hES cells"

Where does it start?

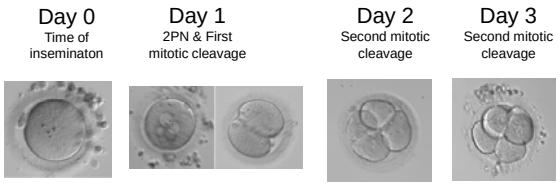
Ovulation

Where does it start?

The sperms route
to
the Oocyte

From the Zygote
to the
4-cell stage

Timing of the first cell divisions



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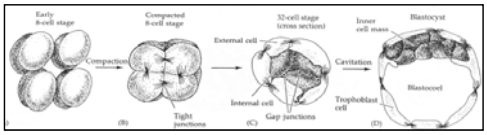
Blastulation

First morphological differentiation of embryonic cells

Creation of ICM and TC cells

DAY 3 DAY 4 DAY 5-6



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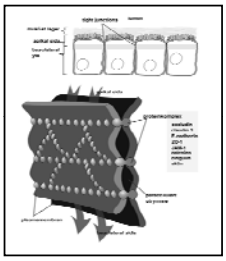
Tight junctions



Main functions:

- Bind the cells together
- Prevent ions and water to freely pass through between the cells

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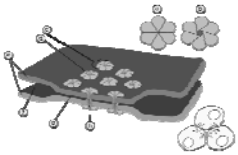
Gap junctions



Main functions:

- Allow exchange of ions between cells

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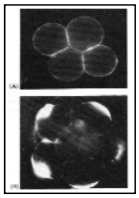
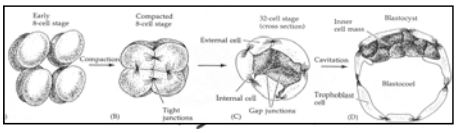
Compaction

- Begins at 8-16 cell stage
- Membrane polarization
- Glycoproteins (E-cadherine)
  - *Dependent on cell-cell interactions*
- Tight- and gap junctions
- Role of cytoskeletal actin filaments
  - *Actin filaments (microvilli), stretch between cells and shorten and flattening the outer cells*

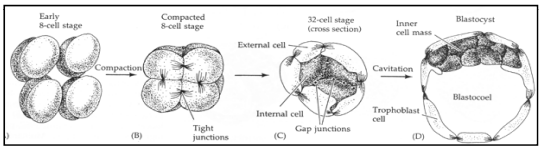




Blastocoel formation

Starts with: ”Secretion of fluid from the ”outer cells” forming a small cavity”

Day 3 Day 4 Day 5



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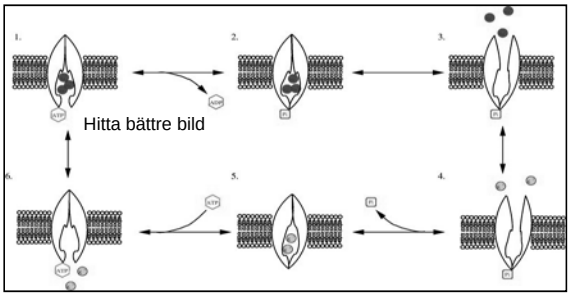
Na^+/K^+ ATPase



Hitta bättre bild

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From the Morula to the Blastocyst stage

How many cells become the fetus?

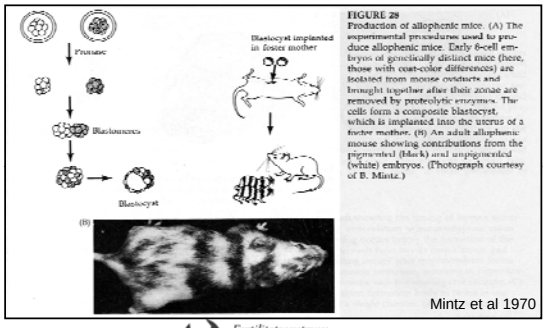

FIGURE 28
Production of allophenic mice. (A) The experimental procedures used to produce allophenic mice. Early 8-cell embryos of genetically distinct mice (here, those with coat-color differences) are isolated from mouse oviducts and brought together after their zonae are removed by proteolytic enzymes. The cells form a composite blastocyst, which is implanted into the uterus of a foster mother. (B) An adult allophenic mouse showing contributions from the pigmented (black) and unpigmented (white) embryos. (Photograph courtesy of B. Mintz.)

Mintz et al 1970

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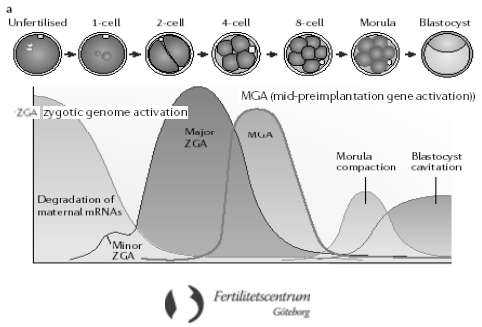
Blastocysts – gene activation



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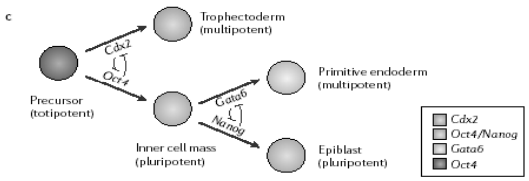
Specific genes and their impact on cell linage determination



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Cell Therapeutics Scandinavia

Beating hES-cells

Thorir Hardarson
Mikael Englund
Petter Björquist

List of citations:

Gilberts
Developmental Biology, 7th Ed

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Thank you for your attention!

Fertilitetscentrum
Göteborg

Cell (pre)-destiny in the human preimplantation embryo and implications for IVF and PGD

Prof. Hilde Van de Velde

Universitair Ziekenhuis Brussel
Vrije Universiteit Brussel

Centre for Reproductive Medicine
Centre for Medical Genetics
Department of Embryology and Genetics

REGE Reproduction and Genetics

ESHRE2011, July 3, 2011

No disclosure

Outline

- Reproductive Biology
  - → Lessons from animal models
    - Totipotency
    - Pre-patterning and destiny
  - → Lessons from the human embryo
    - Totipotency and differentiation
- Reproductive Medicine
  - → Fragmentation
  - → Cryodamage
  - → Pre-implantation genetic diagnosis
- Conclusions

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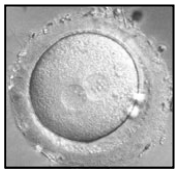
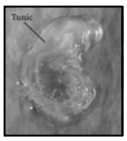
Totipotency

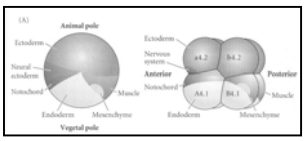
The totipotent cell is ...



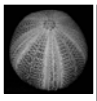
- able to develop into fertile offspring
- the zygote
- able to produce all lineages of differentiation

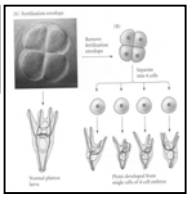
Lessons from invertebrata





Tunicata
Destiny
Irreversible

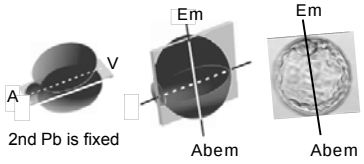




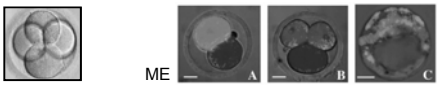
Sea urchin
Totipotent
Regulative development
Reversible

Lessons from the mouse embryo

- Pre-patterning
  → Gardner et al. 2001

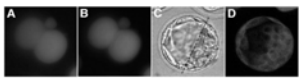


  → Piotrowska et al. 2001; Torres-Padilla et al. 2006



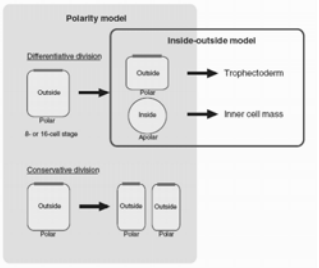
Lessons from the mouse embryo

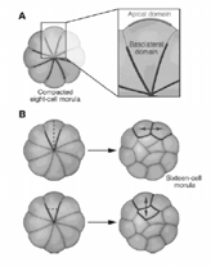
- Regulative development
  →The dance of the embryo (time lapse)
  Kurotaki et al. 2007
  - ZP (extrinsic factor) induced cavity
  - 2nd Pb is not fixed, embryo rotates in the ZP
  - In vivo experiments: photoconversion
  - No pre-patterning



Lessons from the mouse embryo

- Polarization (Johnson and McConnel, 2004)
- Position: inside-outside (Tarkowsky and Wroblewska, 1967)





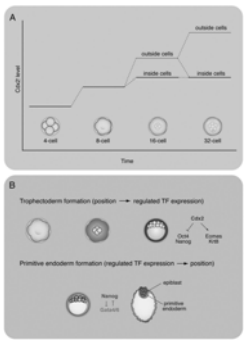
Cockburn and Rossant, 2010

Lessons from the mouse embryo

- Lineage segregation
  Ralston and Rossant, 2008

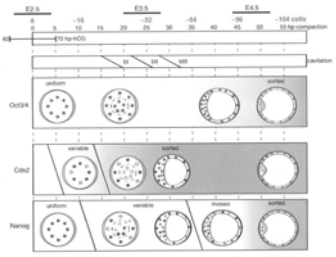
  1st lineage
  TE: Cdx2
  ICM: Pou5F1/Nanog

  2nd lineage
  EPI: Nanog
  PE: Gata4/6



Lessons from the mouse embryo

- Stochastic model = regulative development
  Dietrich and Hiiragi, 2007

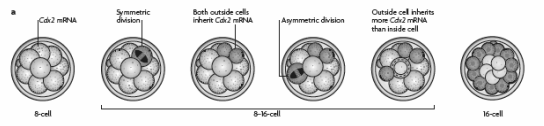


Oct-4: uniform and sorted

Cdx2 and Nanog: 2 phases
(1)Variable
(2)Sorted

Lessons from the mouse embryo

- 1st lineage segregation
  → Ralston and Rossant, 2008
  Cdx2 downstream of polarization
  → Jedrusik et al. 2008; Jedrusik et al. 2010
  Cdx2 mRNA is polarized in outside cells



Lessons from the mouse embryo

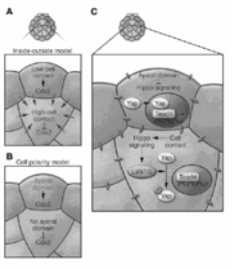
- 1st lineage segregation
  Nishioka et al. 2009; Cockburn and Rossant, 2010

Different cell contact

Apical domain

Lat1/2

Hippo

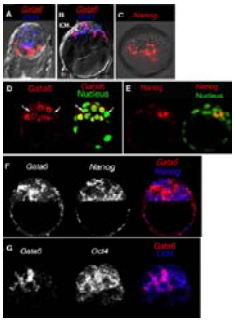


Lessons from the mouse embryo

- 2nd lineage segregation
  Chazaud et al. 2006

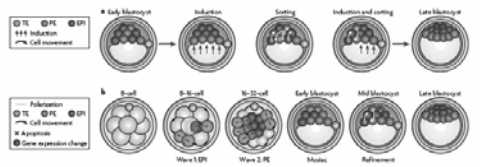
  Pepper-and salt-distribution
  Nanog and Gata6

  Sorting
  EPI: Nanog
  PE: Gata4/6



Lessons from mouse embryo

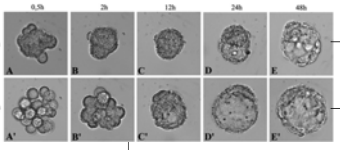
- Zernicka-Goetz et al. 2009
  → Cell position
  - Cell movement according to gene expression pattern
  - Changes in gene expression
  - Apoptosis



Lessons from the mouse embryo

- Potency of 'IN' and 'OUT' blastomeres
  Suwinska et al. 2008

16-cell stage
Compacted

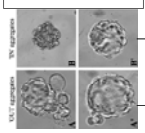


puppies

IA

pups

32-cell stage
Early blastocyst



ICM+TE, no pups

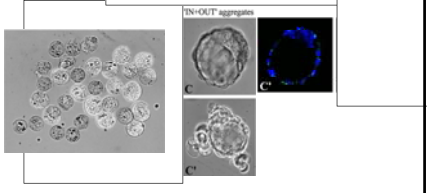
TE

→ direction is reversible at compaction but irreversible at blastulation

Lessons from mouse embryo

- Regulative development
  Suwinska et al. 2008

32-cell stage
Early blastocyst
→ sorting
→ pups

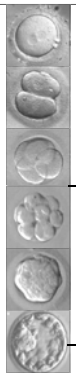


→ outer cells early blastocyst have reached their DESTINY

Outline

- Reproductive Biology
  - → Lessons from animal models
    - Totipotency
    - Pre-patterning and destiny
  - → Lessons from the human embryo
    - Totipotency and differentiation
- Reproductive Medicine
  - → Fragmentation
  - → Cryodamage
  - → Pre-implantation genetic diagnosis
- Conclusions

Lessons from the human embryo



→ Embryonic genome activation (day 2/3)
(Braude et al. 1988; Dobson et al. 2004;
Cauffman et al. 2005; Cauffman et al. 2006)

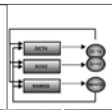
→ 1st differentiation (day 5)
- → TE: differentiated
- → ICM: pluripotent
  - → Extra-embryonic endoderm, mesoderm and ectoderm
  - → 3 germ layers
  - → PGC
  - → embryonic stem cells (hESC)

Lessons from the human embryo

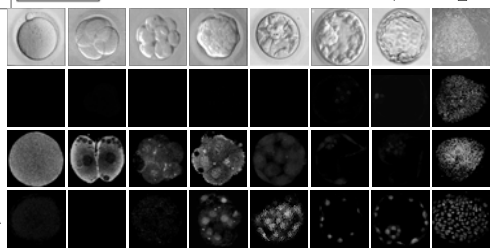
- Totipotency
  - → Marker for totipotency?
  - → When is totipotency lost?
    - When is the 1st differentiation irreversible?
    - Regulative development

Totipotency ↔ Differentiation

No 'stemness' marker to identify totipotent cells

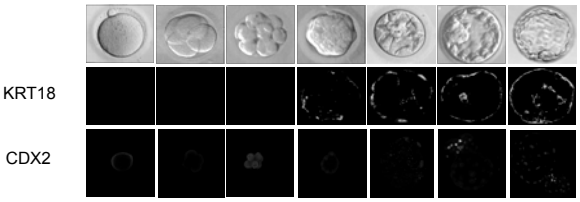


Boyer et al. 2005
hESC



Cauffman et al. 2005; Cauffman et al. 2006; Cauffman et al. 2009

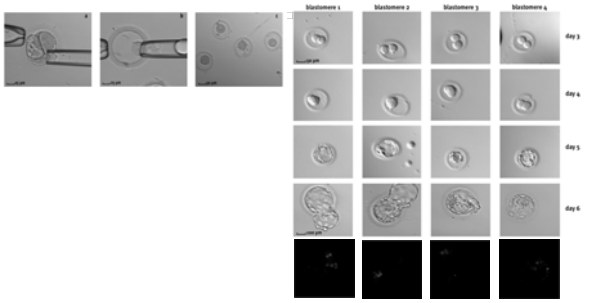
Markers of differentiation



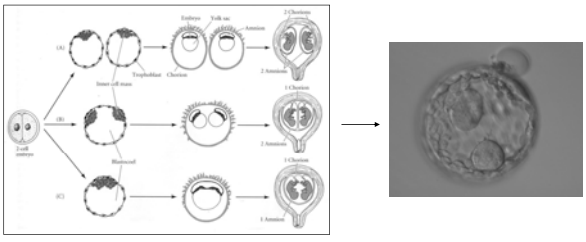
Cauffman et al. 2009; De Paepe (unpublished)

The sister 4-cell stage blastomeres are potentially totipotent

- Embryo splitting (Van de Velde et al. 2008)

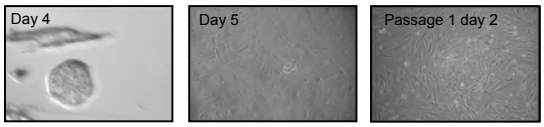


Developmental Biology: human monozygotic twinning



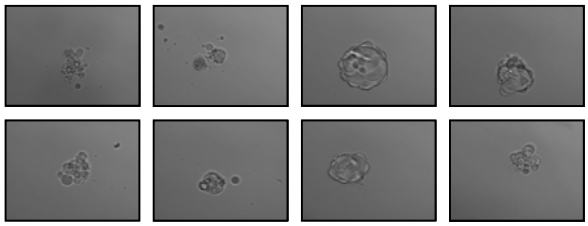
At least one blastomere is pluripotent at the 4-cell stage

- Feki et al. 2009: frozen-thawed embryo, chromosomally abnormal hESC line, teratocarcinoma
- Geens et al. 2009: two hESC lines of distinct embryos
  - → VUB_26Quatro: 46 XX mosaic dup(7)(q33qter), del(18)(q23qter)
  - → VUB_27Patru: 46 XY normal



The sister blastomeres of 8-cell stage embryos

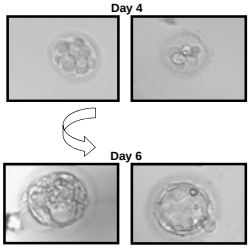
- Embryo splitting



No totipotent capacity
or not enough cells to form an inner cell population?

Outer cells (5-7) of a compacted embryo

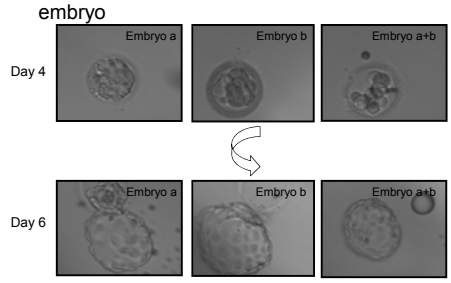
- When is the first differentiation (TE or ICM) irreversible?
  - → Investigate capacity of outer cells compacted embryo (micromanipulation)



No ICM
Cell mass?

Lessons from the human embryo

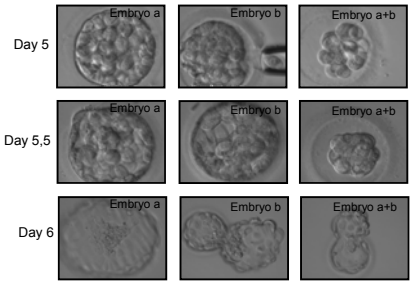
- When is the first differentiation (TE or ICM) irreversible?
  → Investigate capacity outer cells (10-14) compacted embryo



ICM + TE

Lessons from the human embryo

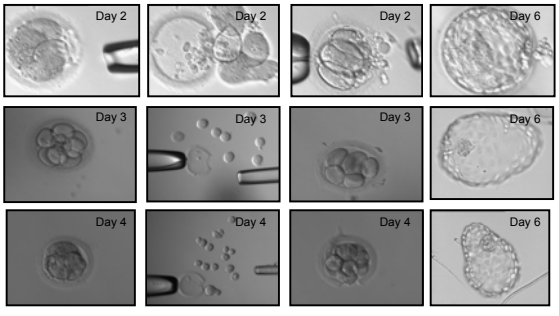
- When is the first differentiation (TE or ICM) irreversible?
  → Investigate capacity TE cells



ICM+TE

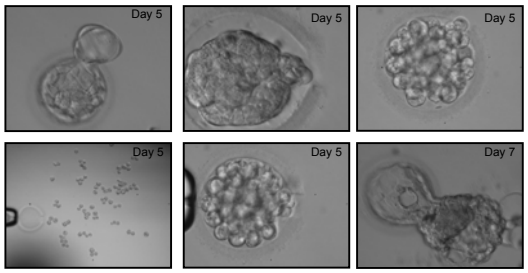
Lessons from the human embryo

- Regulative development: change position blastomeres



Lessons from the human embryo

- Regulative development: change position blastomeres



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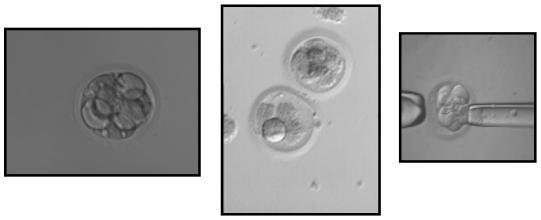
Cell loss during preimplantation development

- Reduction in cell mass
- Allocation TE or ICM
- Obstruction (compaction, cavitation)
- Toxic

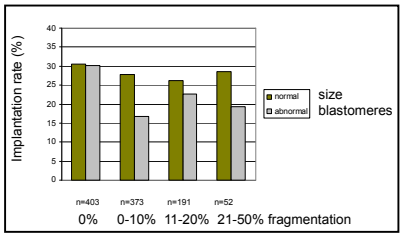


Cell loss during preimplantation development
Fragmentation

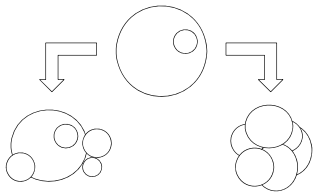
- Alikani et al. (1999) Fertil Steril 71:836
  - → Fragment removal improves implantation rate
  - → No randomized controlled study
- Hardarson et al. (2002) RBMOnline 5:36-38
  - → Small scattered fragments are generated during divisions
  - → Fragments can be reabsorbed
- Van Blerkom (2008) RBMOnline 16: 553
  - → Fragments move between blastomeres and fuse
  - → Resorption of mitochondria, regulatory proteins

Cell loss during preimplantation development
Fragmentation degree and blastomere size

- UZ Brussel, SET day 3, 8-cell stage embryos



Cell loss during preimplantation development
Fragmentation pattern and blastomere size



Loss of cytoplasm
Small scattered fragments
Smaller cells

Loss of cells: nucleus + cytoplasm
Localized fragments: 1 cell
Large fragments: ≥ 2 cells

8-cell stage embryo

? = 7-cell stage with 12.5% fragmentation
? = 6-cell stage with 25% fragmentation

Cell loss during preimplantation development
Cryodamage

Edgar et al. (2007)
RBMOnline 14: 718

ET day 2	IR sFRET
4/4	26.0%
3/4	27.5%
2/4	9.4%

Zheng et al. (2008)
J Assist Reprod Genet 25: 281

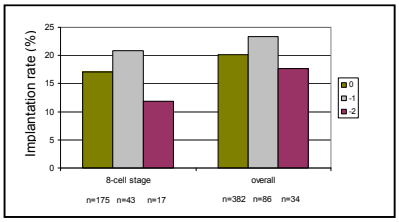
ET day 3	IR FRET
7/8 - 0	26.3%
7/8 - 1/2	26.3%

Removal of damaged cells:
Nagy et al. (2005) Fertil Steril 84: 1606
Rienzi et al. (2005) Fertil Steril 84: 888

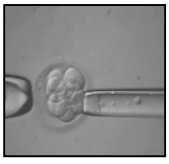
No prospective randomized controlled study

Cell loss during preimplantation development
Cryodamage

- Van Landuyt et al. ASRM2008
  → 2004-2007: 530 sFRET cycles, cryo day 3, ET day 4
  → 2 children born 4/8



Cell loss during preimplantation development
Preimplantation genetic diagnosis



1 cell ↔ 2 cells

Safety ↔ Viability

Cell loss during preimplantation development Preimplantation genetic diagnosis

- De Vos et al. 2009
  Cohort of day 5 SET, 1-cell versus 2-cell biopsy PGD and PGS
  All embryos resulted from 8-cell stage embryos on day 3

	8 – 1 n=182	**8 – 2 n=259**	**8 – 0 (control) n=702**	**P value**
hCG per ET	46.7%	36.3%	48.6%	0.028
LBR per ET	37.4%	22.4%	35.0%	0.006

	8 – 1	**8 – 2**
Only 1 GTE	25.9%	18.2%
More than 1 GTE	46.5%	27.6%
Non-elective SET	34.1%	22.1%
Elective SET	55.0%	35.4%

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Conclusions

- So far, there is no marker for totipotent cells, no marker for allocation to ICM
- 4-cell stage blastomeres are potentially totipotent
- KRT18 and CDX2 are expressed in outer cells of compacted embryo before any visible sign of differentiation
- The decision of the outer cells of a blastocyst to become TE is still reversible
- Preimplantation development is highly regulative
- Cell loss: mass reduction > allocation

Thanks

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eshre
SCIENCE MOVING PEOPLE MOVING SCIENCE

Metabolic requirements of embryo growth and viability

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H·Y·M·S
THE HULL YORK MEDICAL SCHOOL

LEARNING OBJECTIVES

At the conclusion of this presentation, Participants should be able to:

(i) Describe various approaches, their strengths and limitations, for determining early embryo metabolic requirements including those based on:
- **Physiological and biochemical knowledge**
- **Measurements of nutrient utilisation**
- **Culture without exogenous nutrients**

(ii) Be able to describe the general metabolic requirements of early embryos and the relationship between nutrient turnover and subsequent viability including the possible role of 'quiet metabolism'

(iii) Be familiar with the metabolism of Inner Cell Mass, Trophectoderm and Embryonic Stem cells

DISCLOSURE

HJ Leese is a Scientific Adviser and Shareholder in *Novocellus Ltd*, a company which is developing methods for diagnosing embryo health

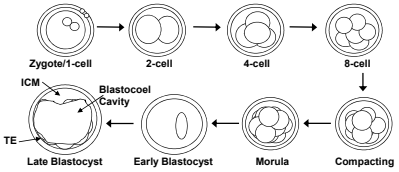
Defining requirements for early embryos

- **Physiological and biochemical knowledge**
- **Utilisation of nutrients**
- **Oxygen consumption: global marker of energy metabolism**
- **Nutrient consumption: amino acids: pyruvate: glucose**
- **Culture in absence of exogenous nutrients**
- **Relationship between metabolism and viability**

Defining nutrient requirements from first principles :

What are the likely metabolic needs of the major cellular processes which occur during preimplantation development ?

Characteristics of preimplantation embryo development:



Strategy
Large egg: largest volume in female mammal: **large energy store**
Successive 'cleavage' divisions until adult cell size reached
Duplication of nuclear material
No duplication of cytoplasm
Diameter of early blastocyst = diameter of egg
Growth (net increase in protein) begins at blastocyst stage
Maternal control prior to activation of embryonic genome
Eggs and embryos are relatively autonomous *and have astonishing regulative powers* (Anne McLaren, 1976)

What is ATP used for?

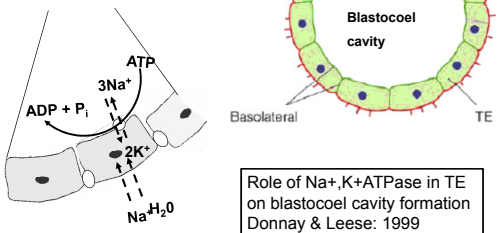
- Protein synthesis (~30-40 %)*
- Ion pumps: notably, Na^+K^+ATPase:(~30-40 %)**
- DNA/RNA synthesis (~10%)
- Other ATPases
- Substrate cycling

*Net growth, requiring increased protein synthesis, begins at the blastocyst stage

** Required for blastocoel formation

Buttgereit and Brand *Biochem. J.* **312**: 163-167
Wieser and Krumschnabel *Biochem. J.* **355**: 389-395
Leese et al in Gardner & Lane (eds) *ART and the Human Blastocyst* Serono Symposia 2001: Springer-Verlag, New York

Mammalian blastocyst
ICM = Inner Cell Mass
TE = Trophectoderm



Role of Na+,K+ATPase in TE on blastocoel cavity formation
Donnay & Leese: 1999
Molec Reprod Dev **53**: 171

Predictions:

1 Embryo requirements for energy substrates and amino acids will be relatively low during early preimplantation development and then increase with blastocyst formation

2 Eggs and early embryos have high endogenous reserves which potentially may provide energy

3 Trophectoderm cells will have a higher metabolism than those of the Inner Cell Mass

4 Stem cells will have a relatively quiescent metabolism prior to differentiation

Defining requirements for early embryos

Physiological and biochemical knowledge

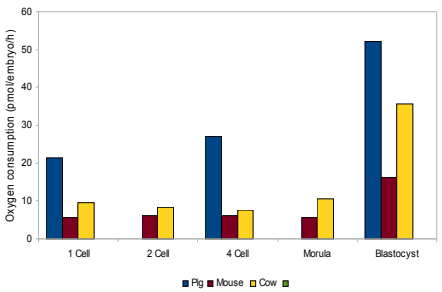
Utilisation of nutrients

Oxygen consumption: global marker of energy metabolism

Nutrient consumption: amino acids: pyruvate: glucose

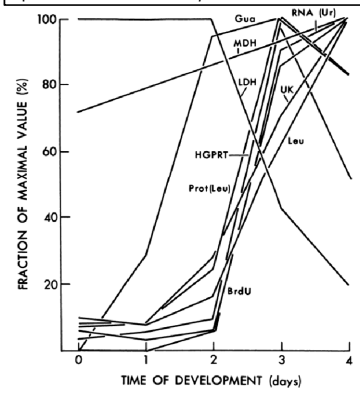
Culture in absence of exogenous nutrients

Relationship between metabolism and viability



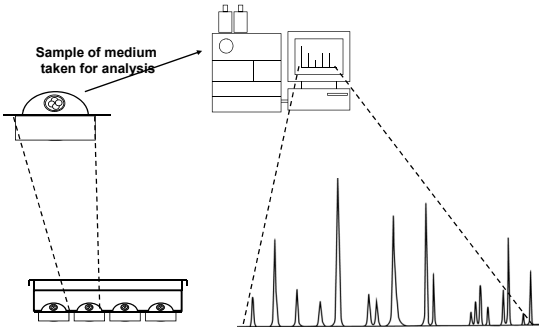
Oxygen consumption rates of preimplantation embryos of three mammalian species.
Based on data of Sturmey and Leese (2003: pig) Houghton et al (1996: mouse) and Thompson et al (1996: cow)

Biochemical changes in mouse preimplantation embryos
Epstein CJ. 1975 *Biol Reprod*: 12:82-105



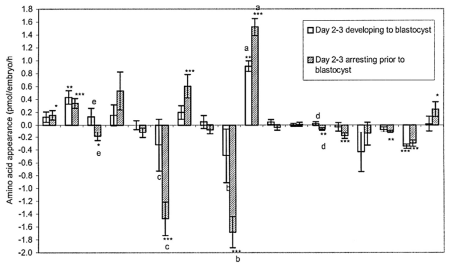
Increase in uptake of biosynthetic precursors in order to form a blastocyst

Amino acid analysis by high performance liquid chromatography (HPLC)



Non-invasive Amino Acid Profiling

Amino Acid metabolism of single human embryos
Houghton et al (2003) *Human Reproduction* 18: 1756
Developing and arresting embryos had the same appearance
Developing had a lower amino acid turnover than arrested

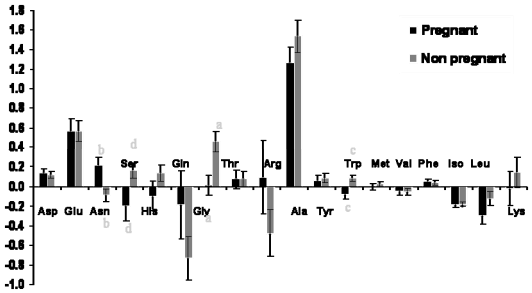


Houghton, F.D. et al. Hum. Reprod. 2003 18:1756-1757; doi:10.1093/humrep/deg389

Human Reproduction

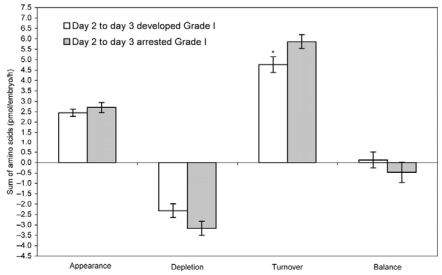
Stage	Amino acids consumed by the embryo		Amino acids produced by the embryo	
	Developing embryos	Arresting embryos	Developing embryos	Arresting embryos
Day 2 to 3	Leu ^{E}	Asn ^{E} Gln ^{NE} Arg ^{C} Met ^{E} Val ^{E} Iso ^{E} Leu ^{E}	Glu ^{NE} Ala ^{NE}	Asp ^{NE} Glu ^{NE} Gly ^{C} Ala ^{NE} Lys ^{E}
Compact 8-cell to morula	Ser ^{C} Arg ^{C} Leu ^{E}	Asn ^{E} Gln ^{NE} Arg ^{C} Val ^{E} Iso ^{E} Leu ^{E}	Asp ^{NE} Glu ^{NE} Ala ^{NE} Trp ^{E}	Asp ^{NE} Glu ^{NE} Gly ^{C} Ala ^{NE}
Morula to blastocyst	Ser ^{C} Arg ^{C} Met ^{E} Val ^{E} Leu ^{E}		Asp ^{NE} Glu ^{NE} Ala ^{NE}	

Amino acid profiles and IVF pregnancy outcome



Cleavage-stage embryos with the potential to give a pregnancy have a lower turnover of amino acids:
Re-drawn from original data in Brison et al (2004) *Hum Reprod* 19: 2319

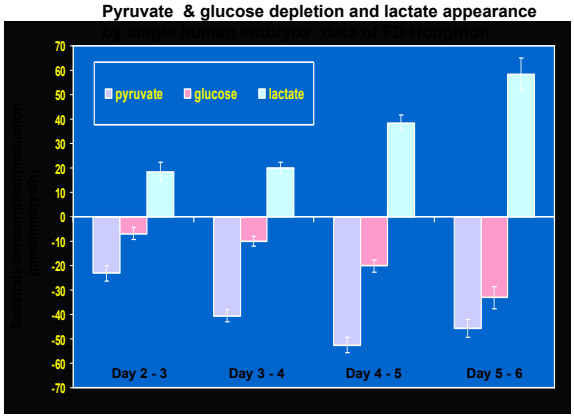
Total amino acid depletion, appearance turnover and balance by Grade I thawed human embryos from day 2 to day 3 of development



Stokes, P. J. et al. Hum. Reprod. 2007 22:829-835

Human Reproduction

Pyruvate & glucose depletion and lactate appearance



Summary: Ralph Brinster (1973) Nutrition and metabolism of the ovum, zygote and blastocyst. Handbook of Physiology (ed Greep)

Studies on embryo requirements in vitro have contributed considerably to our knowledge of embryo metabolism and development. In general they have indicated that the embryos need an environment similar to the environment found necessary for other mammalian cells grown in vivo

Pyruvate appears to be the central energy substrate in those species (mouse, rabbit and monkey) in which energy source requirements of the embryo have been examined. During the first day or two of the embryo's life, the Embden-Meyerhoff pathway (glycolysis) has a very low capability, but after blastocyst formation there is a sharp increase in glycolytic ability.

The Krebs cycle is the main source of energy throughout the preimplantation period. Large increases in oxygen consumption and uptake and incorporation of carbon occur at about the time of blastocyst formation.

The embryo goes from a relatively inactive metabolic tissue at ovulation to a rapidly metabolizing tissue at implantation.

Ralph Brinster working in the laboratory at the Lippincott Building, School of Veterinary Medicine, University of Pennsylvania c. 1963.

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

Defining *requirements* for eggs and embryos

- **Physiological and biochemical knowledge**
- **Utilisation of nutrients**
- **Oxygen consumption: global marker of energy metabolism**
- **Nutrient consumption: amino acids: pyruvate: glucose**
- **Culture in absence of exogenous nutrients**
- **Relationship between metabolism and viability**

Culture without exogenous nutrients

Rabbit* ***1-cell rabbit embryo has sufficient endogenous energy sources to allow up to 3 or more cleavage divisions in the absence of any added energy substrates***

Mouse Zygotes cultured in KSOM or KSOM without nutrients 10.5 hours: *'no nutrients'* group all degenerating; *'plus nutrients'* healthy**

Cow* Control zygotes cultured in SOFaaBSA 80 hours: 65% cleaved to 2-cell of which 80% reached 8-16 cell by 80 hours**
'*No nutrients*' cultured in SOF-PVA 45% cleaved to 2-cell, of which 30% reached 8/16 cell by 80 hours

Further evidence for a role for fatty acids during oocyte maturation/early embryo development: Sturmey, Reis, Leese and McEvoy (2009) *Reprod Dom Anim* 44 (Suppl 3) 50-58

***Kane *Biol Reprod* 37: 775: 1987 **Manser & Leese (unpublished)**
*****Leese & Ferguson (1999) *Towards Reproductive Certainty*: Jansen & Mortimer (eds): Parthenon Publishing, New York, p 360**

Endogenous lipid: a potential source of energy in early embryos

Species	Amount of Fat (ng)	Reference
Mouse	4	Lowenstein & Cohen, 1964
Cow	58	Ferguson & Leese, 1999
Pig	156	McEvoy *et al.* 2000
Sheep	89	Coull *et al.* 1997

TG is metabolised during oocyte maturation *in vitro*

TG levels fall during oocyte maturation (*cow and pig)*

Concomitant change in oxygen consumption *(pig)*

Inhibition of TG metabolism during oocyte maturation reduces viability post- fertilisation *(cow and pig)*

Mitochondria and TG droplets co-localise during oocyte maturation *(pig)*

Conclusions:
Metabolic requirements of the early embryo:

Nutritional needs relatively simple:

Cleavage stages quiescent metabolically

Metabolic activity increases dramatically with blastocyst formation

Pyruvate required by eggs and cleavage stage embryos

Glucose consumed in greater amounts during the later stages with a major proportion converted to lactate *in vitro*

Amino acids required throughout

High endogenous energy store in domestic animals/human: potential energy-buffering capacity

Leese (2003) *Human Fertility* 6: 180-185
Summer and Biggers (2003) *Hum Reprod Update.* 9 :557-82

Defining *requirements* for eggs and embryos

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Amino Acid metabolism of single human embryos
Houghton et al (2002) *Human Reproduction* 17: 999-1005
Developing and arresting embryos had the same appearance
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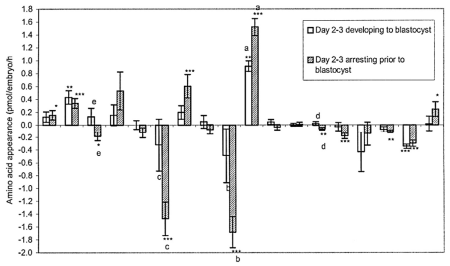
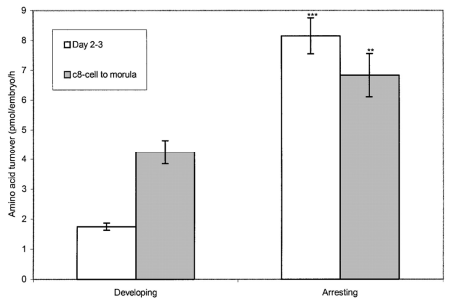


Houghton, F.D. et al. Hum. Reprod. 2003 18:1756-1757; doi:10.1093/humrep/deg389

Human Reproduction

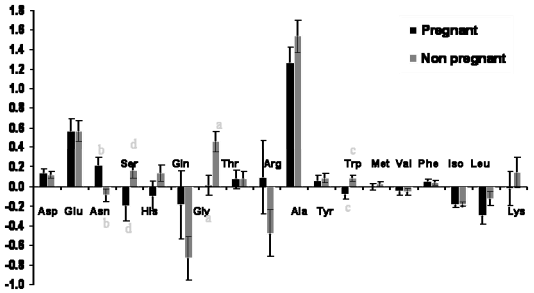
Total amino acid turnover **lower** in developing vs arrested human embryos



Houghton, F.D. et al. Hum. Reprod. 2003 18:1756-1757; doi:10.1093/humrep/deg389

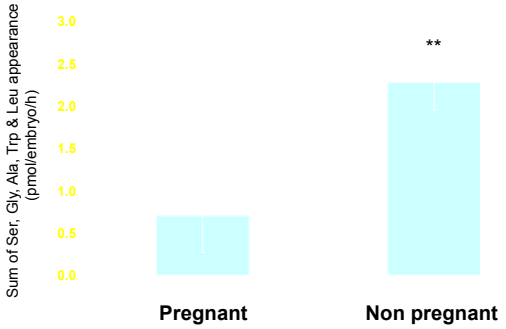
Human Reproduction

Amino acid profiles and IVF pregnancy outcome



Cleavage-stage embryos with the potential to give a pregnancy have a lower turnover of amino acids:
Re-drawn from original data in Brison et al (2004) *Hum Reprod* 19: 2319

Amino acid turnover is lower in embryos which give rise to a pregnancy



Conclusion:

Amino acid turnover (sum of depletion and appearance) is reduced in cleavage-stage human embryos which have the potential to develop to the blastocyst stage in culture and to give rise to a pregnancy following transfer

Hypothesis:

Quiet please, do not disturb:
a hypothesis of embryo metabolism and viability

Leese: *Bioessays* 24, 845-849 (2002)

What is a viable embryo?

A viable embryo functions with a high degree of efficiency; it is better equipped to contend with damage to the genome transcriptome and proteome or may possess less damage than its less viable counterparts and need only consume the minimum quantity of nutrients to correct such damage, i.e., it exhibits a quiet metabolism

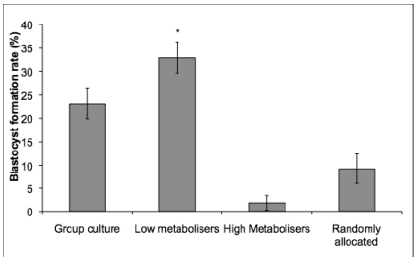
Baumann CG, Morris DG, Sreenan JM and Leese HJ. (2007)
The quiet embryo hypothesis: molecular characteristics favoring viability:
Molecular Reproduction and Development. **74**, 1345-1353.

Leese HJ, Sturmey RG, Baumann CG and McEvoy TG (2007)
Embryo viability and metabolism: obeying the quiet rules.
Human Reproduction **22**: 3047-3050

Leese, HJ, Baumann CG, Brison DR, McEvoy TG and Sturmey RG (2008)
Metabolism of the viable mammalian embryo: quietness revisited
Molecular Human Reproduction **14**: 667-672

Sturmey RG, Hawkhead J, Barker EA and Leese HJ (2009)
DNA damage and metabolic activity in the preimplantation embryo
Human Reproduction **24**: 81-91

Prospective determination of bovine zygote developmental potential on the basis of Amino Acid Profiling



- Zygotes fell into 2 groups based on Amino Acid Profiling (AAP) '***low metabolisers***' (LM AAP < 3 pmol/embryo/h) and '***high metabolisers*** (HM AAP > 3 pmol/embryo/h) - assigned into groups on this basis
- Blastocyst rates collected from LM, HM and compared with randomly allocated and conventional group culture

Metabolism of:

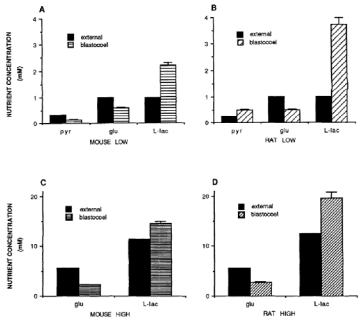
Inner cell mass
Trophectoderm

Embryonic Stem (ES) cells

TE and ICM metabolism: conclusions:

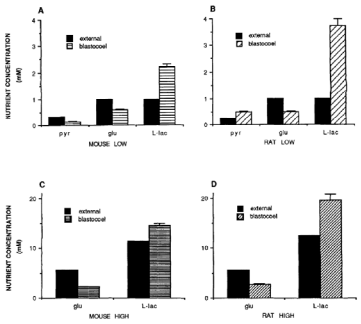
- **Blastocoel cavity nutrient concentrations reflect, to a large extent, external concentrations.**
- **TE more active than ICM – which is relatively quiescent**
- **Isolated ICM give reliable data**
- **Isolated TE – not a reflection of TE in intact blastocyst**
- **Metabolic integrity of TE lost on isolation**
- **More reliable data on TE by subtracting ICM data from intact blastocyst**

Glucose, pyruvate and lactate concentrations in the blastocoel cavity of rat and mouse embryos
Brison, Hewitson & Leese (1993)
Molecular Reproduction & Development **35**: 227-232



Glucose, pyruvate and lactate concentrations in the blastocoel cavity of rat and mouse embryos
Brison, Hewitson & Leese (1993)
Molecular Reproduction & Development **35**: 227-232

Blastocoel nutrients reflect external concentrations – in rat, mouse and bovine



Metabolic characterization of the bovine blastocyst, inner cell mass, trophectoderm and blastocoel fluid.
Gopichandran & Leese (2003)
Reproduction **126**: 299-308

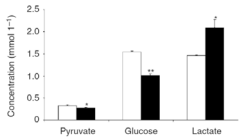


Fig. 4. Composition of blastocoel fluid in day 8 expanded bovine blastocysts (■, $n = 8$) and culture medium (□, $n = 8$, Hepes-buffered synthetic oviductal fluid (SOF) supplemented with minimum essential and non-essential medium amino acids, 1 mmol glutamine l^{-1} and 4 mg BSA ml^{-1}). Asterisks denote significant differences between blastocoel fluid and culture medium (* $P < 0.05$ and ** $P < 0.001$).

Glucose, pyruvate and lactate concentrations in the blastocoel cavity of rat and mouse embryos
Brison, Hewitson & Leese (1993)
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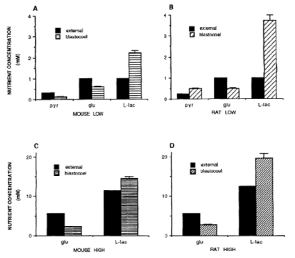

- **Isolated ICM give reliable data**
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Gopichandran & Leese (2003)
***Reproduction* 126: 299-308**

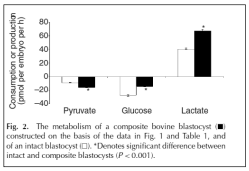


Fig. 2. The metabolism of a composite bovine blastocyst (■) constructed on the basis of the data in Fig. 1 and Table 1, and of an intact blastocyst (□). *Denotes significant difference between intact and composite blastocysts ($P < 0.001$).

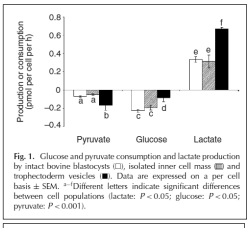


Fig. 1. Glucose and pyruvate consumption and lactate production by intact bovine blastocysts (□), isolated inner cell mass (▨) and trophectoderm vesicles (■). Data are expressed on a per cell basis ± SEM. a–fDifferent letters indicate significant differences between cell populations (lactate: $P < 0.05$; glucose: $P < 0.05$; pyruvate: $P < 0.001$).

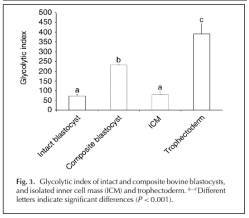
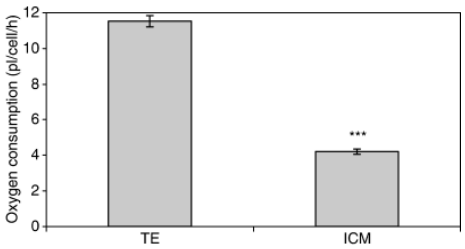


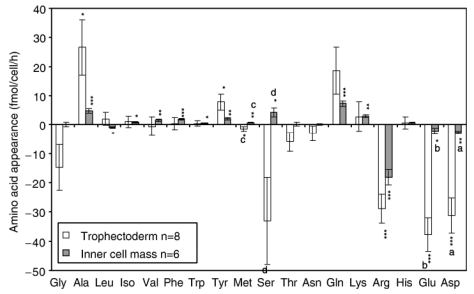
Fig. 3. Glycolytic index of intact and composite bovine blastocysts, and isolated inner cell mass (ICM) and trophectoderm. a–cDifferent letters indicate significant differences ($P < 0.001$).

Houghton (2006) *Oxygen consumption by mouse isolated ICM and TE (values are intact blastocyst - ICM)*



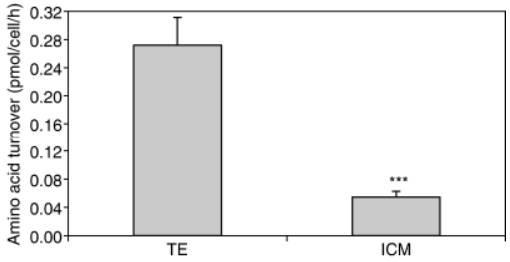
Differentiation. 2006 **74**:11-18.

Houghton: *Amino acid depletion and appearance by mouse isolated ICM and TE (values are intact blastocyst - ICM)*



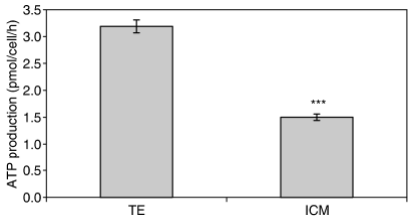
Differentiation. 2006 **74**:11-18.

Houghton: *Total amino acid turnover by mouse isolated ICM and TE (values are intact blastocyst - ICM)*



Differentiation. 2006 **74**:11-18.

Houghton: ATP production by *mouse isolated ICM and TE (values are intact blastocyst - ICM)*



Average lactate production was 8.7±5.8 pmol/blastocyst/h and 3.9±1.1 pmol/ICM/h.
From these values ATP production in the blastocyst were
121.3±12.5 pmol/blastocyst/h and 22.5±1.9 pmol/ICM/h in the ICM.
When cell number was taken into account ATP production by the two lineages was
3.19±0.06 and 1.5±0.12 pmol/cell/h for the TE and ICM, ($p<0.001$).

Evidence for triglyceride utilisation by isolated mouse ICM
Hewitson, Martin & Leese (1996)
***Molecular Reproduction & Development* 43: 323-330**

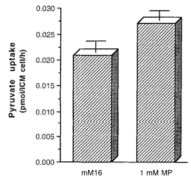


Fig. 2. Pyruvate uptake by mouse inner cell mass (ICM) cells in modified M16 (mM16) and in medium supplemented with 1 mM methyl palmoxirate (MP).

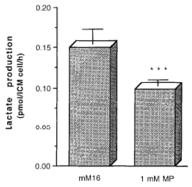
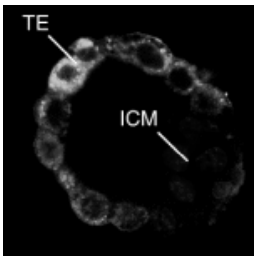


Fig. 3. Lactate production by mouse inner cell mass (ICM) cells in modified M16 (mM16) and in medium supplemented with 1 mM methyl palmoxirate (MP). Significantly different from control: ***$P < 0.001$.

Evidence for flexibility in fuel use depending on availability

Cells of the inner cell mass are quieter than those of trophectoderm



Laser scanning confocal midplane section through a mouse blastocyst displaying mitochondrial localization (green) and cell nuclei (blue).

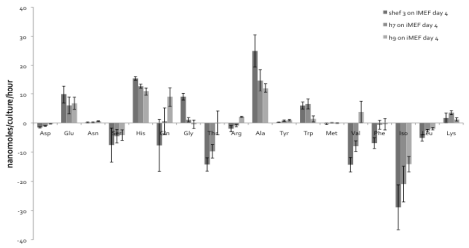
Stem cells are also thought to have a a quiet metabolism

FD Houghton
Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst
***Differentiation*: 74: 11-19 (2006)**

Stem Cells

H·Y·M·S
THE HULL YORK MEDICAL SCHOOL

Stem cell amino acid turnover

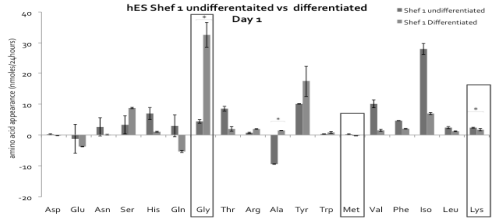


Sachamitr, Coles, Leese & Sturmey (unpublished)

Stem Cells

H·Y·M·S
THE HULL YORK MEDICAL SCHOOL

Stem cell amino acid turnover increases upon differentiation



Sachamitr, Coles, Leese & Sturmey (unpublished)

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Daniel Brison
Tom McEvoy
Roger Sturmey

UK Medical Research Council
UK Biotechnology and Biological Sciences Research Council
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Scottish Agricultural College Roslin BioCentre, Scotland
The Leverhulme Trust
Novocellus Ltd

Metabolic requirements of embryo growth and viability

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Baumann CG, Morris DG, Sreenan JM, Leese HJ. The quiet embryo hypothesis: molecular characteristics favoring viability. Molec Reprod Dev. 2007; 74: 1345-53.

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The relationship between pluripotency and mitochondrial DNA proliferation during early embryo development and embryonic stem cell differentiation.

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I declare that I have no competing commercial or financial interests.

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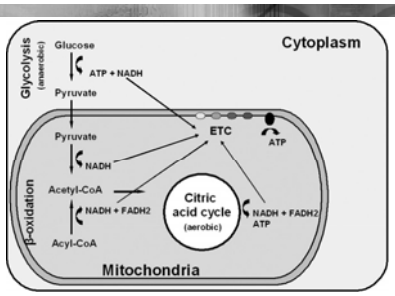
Learning objectives

- Define the role of mitochondrial DNA
- Understand how and why mtDNA copy number increases during oogenesis
- Understand why mtDNA copy number is important to fertilisation outcome
- Understand how mtDNA copy number is regulated during preimplantation development
- Understand why it is important to have low mtDNA copy number during pluripotency – establishment of the mtDNA set point.
- Understand how mtDNA replication is regulated during early and later stages of differentiation
- Understand why the loss of mtDNA regulation during preimplantation development is detrimental to development
- Understand the relationship between nuclear and mtDNA compatibility for the generation of embryonic stem cells.

Cellular Energy



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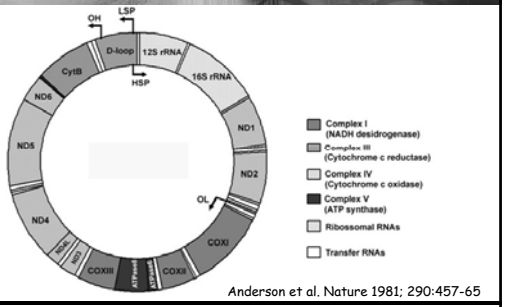
Mitochondrial Genome



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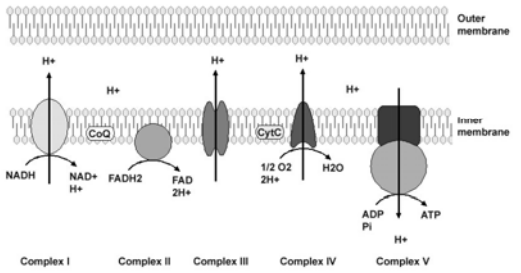
Electron Transfer Chain



Transmission of mtDNA

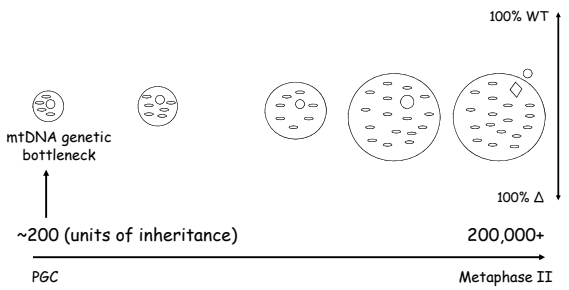
100% WT
or
100% Δ (lethal)

WT + Δ
% Δ:WT = onset of disease

- Mutations
  - Coding genes - LHON, NARP
  - tRNAs and rRNAs - MELAS, deafness
- Deletions
  - Single large-scale - KSS
  - Multiple - Aging and somatic
- Depletion
  - Large scale deletions – CPEO
  - Associated with mtDNA replication factors

Wallace, Science 1999; 283:1482-8

Oogenesis and clonal expansion of mtDNA



Smith & Alcivar J Reprod Fertil Suppl 1993; 48:31-43

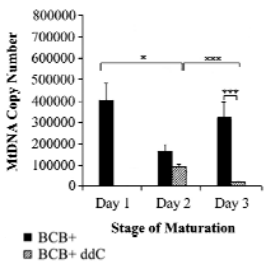
Fertilisation and copy number (Porcine)

Treatment	No Oocytes inseminated	Mean oocyte complex volume (μm^3)	Fertilisation rate (%)	Mean Oocyte mtDNA copy no. (SD)
BCB+	360	1.55×10^{6} [a]	46.6 [a]	222446 ± 217250 [a] (*n* = 41)
BCB-	291	1.35×10^{6} [b]	22.7 [b]	115352 ± 117052 [b] (*n* = 39)
Control	257	1.45×10^{6} [c]	32.3 [c]	138022 ± 153841 [c] (*n* = 46)

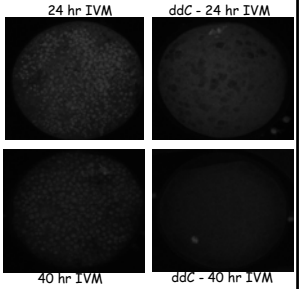
a,b,c in the same column ($P < 0.001$)

El Shourbagy et al. *Reprod* 2006; 131:233-45

Depletion of mtDNA during IVM



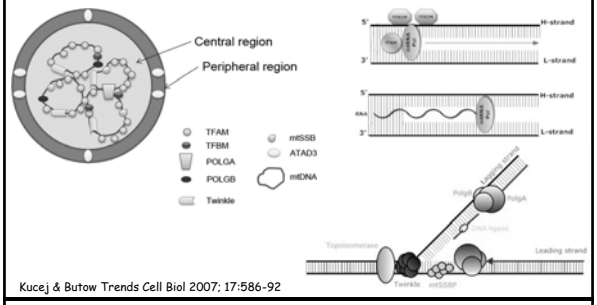
ddC = 2',3'-dideoxycytidine



Red: MitoTracker Red; Blue: DAPI

Spikings et al. *Biol Reprod* 2007; 76:327-35

How is mtDNA replicated?



Kucej & Butow Trends Cell Biol 2007; 17:586-92

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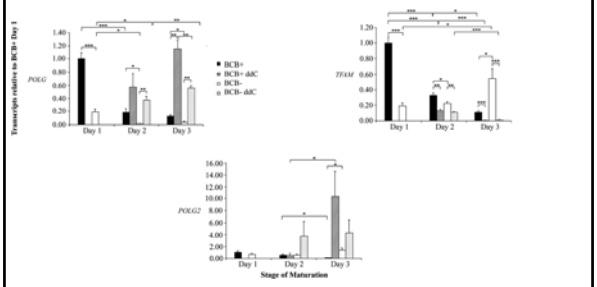
Expression of mtDNA replication factors during porcine IVM



Spikings et al. *Biol Reprod* 2007; 76:327-35

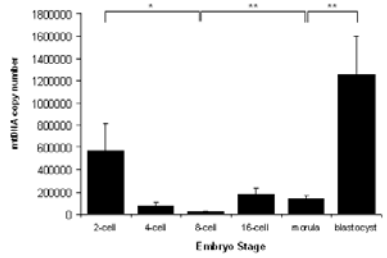
mtDNA Supplementation

Treatment	IVF fertilisation rate (%)	ICSI fertilisation rate (%)
BCB+	37.5[a]	40.4[c]
BCB–	17.6[b]	19.8[d]
BCB– supplemented	31.0[a]	34.0[c]
BCB– sham injected	17.0[b]	10.0[d]

[a,b] in the same column ($P < 0.002$); [c,d] in the same column ($P < 0.001$).

El Shourbagy et al. *Reprod* 2006; 131:233-45

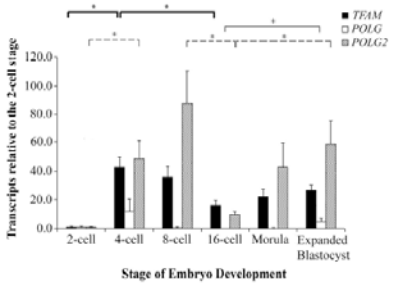
mtDNA copy number in porcine IVF-derived embryos



* $P \leq 0.01$, ** $P < 0.005$

Spikings et al. *Biol Reprod* 2007; 76:327-35

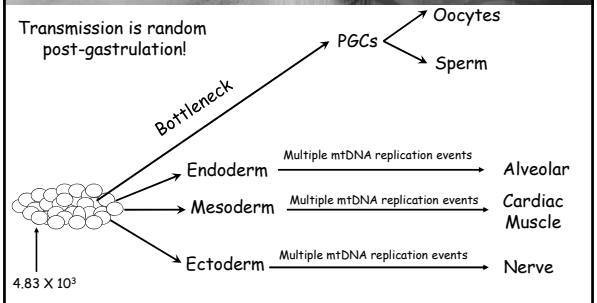
Expression of mtDNA Replication Factors in Porcine IVF Embryos



* $P \leq 0.02$

Spikings et al. *Biol Reprod* 2007; 76:327-35

Inner Cell Mass to adult cell



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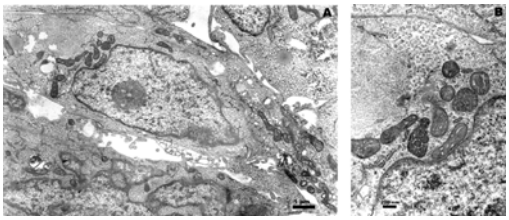
TEM of Undifferentiated hESCs



St. John et al. *Clon Stem Cell* 2005; 7:141-53

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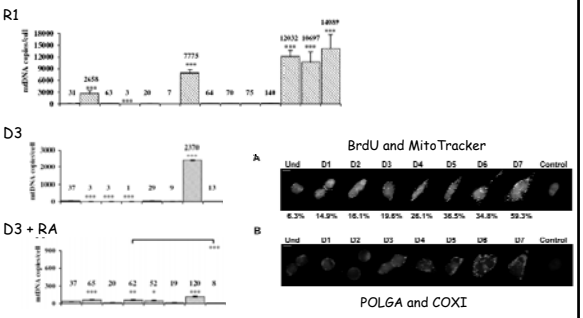
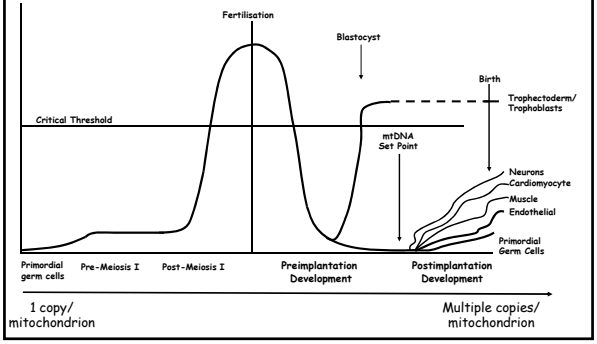
mtDNA copy number in mESCs

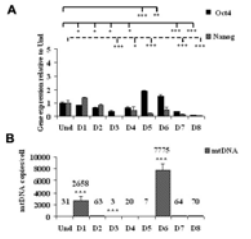


Facucho-Oliveira et al. *J Cell Sci* 2007; 120:4025-34

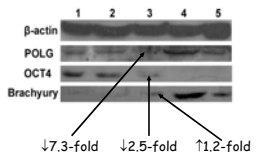
The continuous recycling of the mtDNA genome through the oocyte



Is there a relationship between pluripotency and replication of mtDNA?



Facucho-Oliveira & St. John, *Stem Cell Rev Rep.* 2009; 5:140-58.



↓7.3-fold ↓2.5-fold ↑1.2-fold

↓ POLG → ↓OCT4 + Differentiation

Facucho-Oliveira et al. *J Cell Sci* 2007; 120:4025-34

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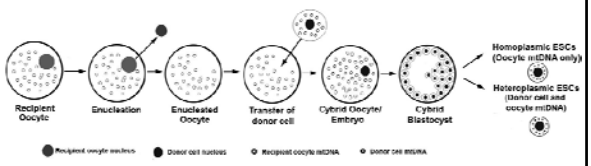
Does Somatic Cell Nuclear Transfer (SCNT) affect mtDNA replication?



- Sheep, mice, cattle, pigs, goats etc.
- Human SCNT blastocysts (Stojkovic et al. *RBM Online* 2005; 11:226–31)
  - Considerable shortage of oocyte donors

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mtDNA transmission following SCNT

Nuclear donor	Species	Analysed	% donor mtDNA	Authors
Somatic	Pig	Blood and hair root Progeny	0.1-1% 0-44%	Takeda et al. 2006
Somatic	Pig	5 Live offspring	0%; But heteroplasmy after double-NT	St. John et al. 2005
Somatic	Cow	11 tissues, blood offspring & foetuses	0-59%	Takeda et al. 2003
Somatic	Cow	11 tissues - offspring	0-12.7%	Hiendleder et al. 2003
Somatic	Cow	Several tissues from offspring	0.6-2.8%	Steinborn et al. 2002
Blastomere	Rhesus macaque	Blood from 2 live offspring	Recipient oocyte; sperm and oocyte)	St. John & Schatten, 2004
Somatic	Sheep	4 fetuses, 3 offspring	0.1 - 46.5%	Burgstaller et al. 2001
Somatic	Sheep	Several tissues from offspring	0%	Evans et al. 2001

Bowles et al. *Curr Top Dev Biol* 2007; 77:251-90
St. John et al. *Hum Reprod Update* 2010: 16:488-509

PDFF2 SCNT Embryos

Donor Cells	No. Oocytes	No. Fused embryos	No. Cleaved embryos	No. NT blastocysts	No. Cells/ blastocyst (mean ± SEM)
$MtDNA^{+}$	206	183 (88.3%)[a]	166 (90.7%)[a]	61 (33.3%)[a]	60.7 ± 8.6[a]
$MtDNA^{R}$	75	70 (93.3%)[a]	56 (80.0%)[b]	15 (21.4%)[a]	68.5 ± 7.8[a]
$MtDNA^{PD}$	130	95 (73.1 %)[b]	77 (81.1 %)[b]	30 (31.2%)[a]	ND

Fusion: $mtDNA^{PD}$ v $mtDNA^{+}$ = P<0.0003
$mtDNA^{PD}$ v $mtDNA^{R}$ = P<0.0004
Cleavage: $mtDNA^{+}$ v $mtDNA^{PD}$ v $mtDNA^{R}$ = P<0.03

Lloyd et al. *Genetics* 2006; 172:2515-27

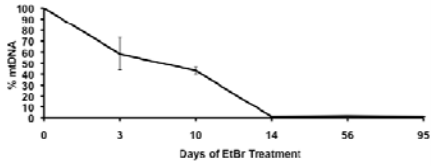
SFF1 SCNT Embryos

Groups	No. Oocytes	No. Fused Couplets (%)	No. Cleaved Embryos (%)	No. Blastocysts (%)	No. cells/ Blastocyst (Mean ± SEM)
$MtDNA^{+}$	106	94 (88.7)[a]	81 (86.2)[a]	15 (16.0)[a]	48.7 ± 4.5[a]
$MtDNA^{R}$	106	86 (81.1)[a]	65 (75.6)[a]	16 (18.6)[a]	69.8 ± 6.2[b]

a v b: P < 0.05

Lloyd et al. *Genetics* 2006; 172:2515-27

Homoplasmic Sheep



Lee et al. *Cell Reprogram* 2010; 12:247-255

mtDNA Analysis

Donor cell	2 cell	4 cell	8 cell	16 cell	32 cell	Blastocyst	Hatched Blastocyst	Mean ± SD
$MtDNA^{+}$	0.05	0.06	0.00	0.02	0.13	0.03 (n=2) 0.05 0.10 0.45 0.61 (n=2) 0.93 3.66 8.72	0.00 0.02 0.52 0.92 1.00	0.90 ± 2.02
$MtDNA^{Pb}$	0.86	0.30	0.27	0.84	1.68	0.08 0.48 0.23 0.39	ND	0.57 ± 0.49
$MtDNA^{R}$	0.00 (n=2)*	0.00	ND	0.00	ND	0.00 (n=4) 0.02	0.00 0.01 0.02 0.04 0.08	0.01 ± 0.02

Lloyd et al. *Genetics* 2006; 172:2515-27

Interspecies SCNT

- Human-bovine → blastocysts
  (Chang et al. Fertil Steril 2003; 80:1380-7)
- Sheep, pig and monkey nuclei into bovine → blastocysts
  (Dominko et al. Biol Reprod 1999; 60:1496-1502)
- Human-rabbit → hESC lines
  (Chen et al. *Cell Res* 2003; 13:251-63)

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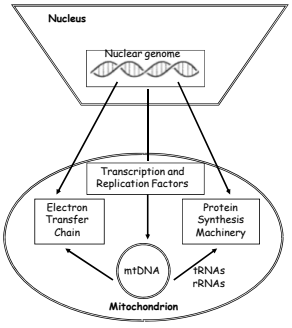
Nucleo-mitochondrial compatibility



Two-fold problem:

- Donor cell nuclear- and recipient oocyte mtDNA-encoded subunits of the ETC
- Nuclear-encoded T & R factors and the recipient oocyte mtDNA-encoded subunits
- Efficiency is likely to be function of mtDNA genetic distance & affects ATP output
  - Cybrids
    (McKenzie & Trounce, J Biol Chem 2000; 275:31514-9)

Caprine-Ovine SCNT

Donor Cells	Fused couplets (%)	Cleavage 2-cells (%)	>4-12 cells (%)	>16 cells (%)	M&BI (%)
$mtDNA^{+}$	95/116 (81.9)[a]	52/95 (54.7)[a]	28/41 (68.3)[a]	6/41 (14.6)[a]	0
$mtDNA^{R}$	111/119 (93.3)[b]	76/111 (68.4)[a]	57/76 (74)[a]	24/51 (47)[b]	0

[a] vs [b] $P<0.01$

Bowles et al. *Genetics* 2007; 176:1511-26

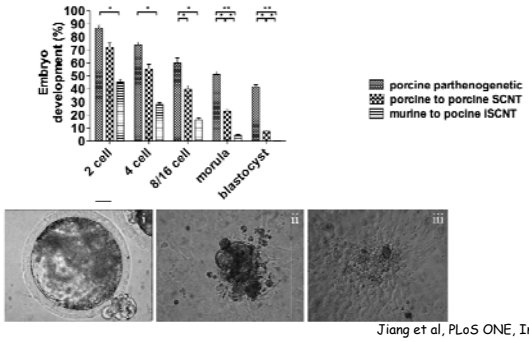
? Persistence of donor cell (Caprine) mtDNA

Donor	1 cell	2 cell	3 cell	4 cell	8 cell	12 cell	20 cell
$MtDNA^{+}$	0.00 (n=4) 0.03	0.00	0.00	0.00 (n=3)	0.00	0.00 (n=3) 19.99	ND
$MtDNA^{R}$	0.00 (n=9) 0.06	0.00 (n=3)	ND	0.00 (n=3)	ND	0.00 (n=3)	0.00 (n=2) 3.42

19.99% = x 23.2
3.42% = x 125.3

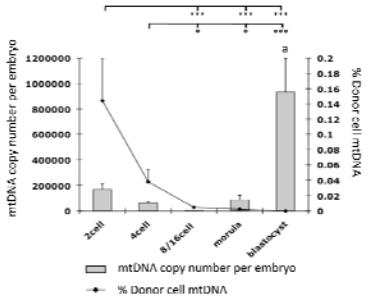
Bowles et al. *Genetics* 2007; 176:1511-26

Murine-Porcine SCNT



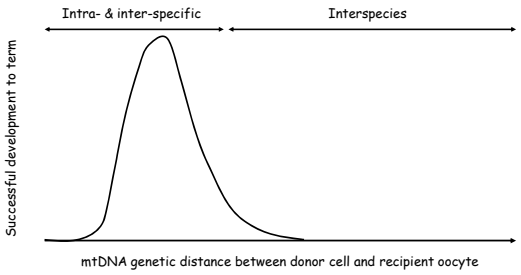
Jiang et al, PLoS ONE, In Press

mtDNA copy number - preimplantation development



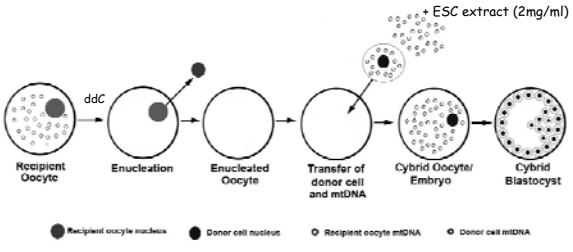
Jiang et al, PLoS ONE, In Press

MtDNA genetic distance

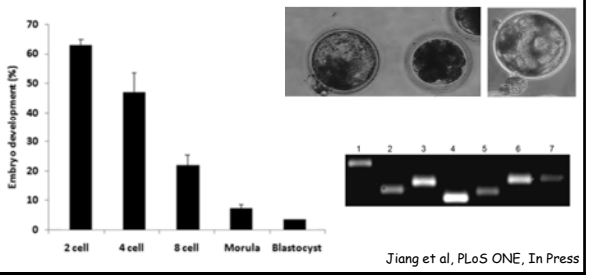


Bowles et al. *Genetics* 2007; 176:1511-26
Bowles et al. *Stem Cells* 2008; 26:775-82

Remodeling the oocyte's cytoplasm



Remodeling development rates



Jiang et al, PLoS ONE, In Press

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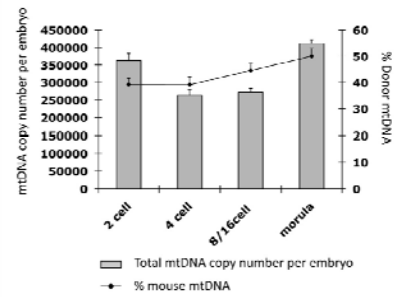
mtDNA copy number and reprogramming



Jiang et al, PLoS ONE, In Press

Conclusions

- The strict regulation of mtDNA copy number during the final stages of oocyte maturation; preimplantation development; and in pluripotent stem cells is an essential developmental process.
- The establishment of the mtDNA set point ensures that pluripotent cells have the potential to acquire the appropriate numbers of mtDNA copy to meet the specific requirements of the specialised cells they differentiate into.
- Nuclear and mtDNA compatibility are essential to embryo development and pluripotency.

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Acknowledgements

Mitochondrial Genetics Group

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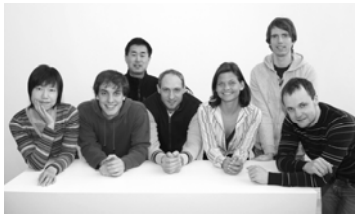
Developmental stages of blastocysts: intercellular junctions and cell polarity



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Disclosure

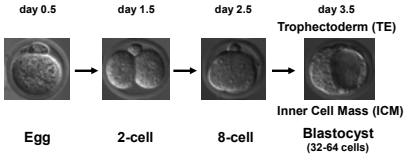
I declare to have no conflict of interest.

Learning Objectives

What are the ***principles*** of patterning mammalian embryos?

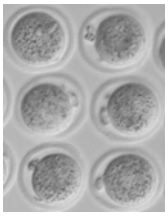
Is ***cell-cell junction*** or ***cell polarity*** a basis for patterning?

Mouse pre-implantation development



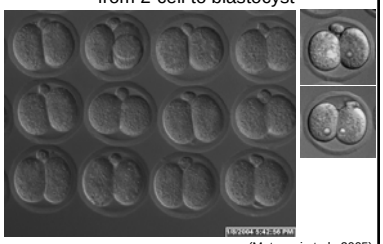
Principle 1. Dynamic processes

Mouse egg



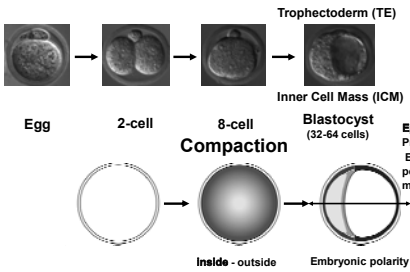
(Hiiragi and Solter, 2004)

Mouse pre-implantation development
- from 2-cell to blastocyst



(Motosugi et al., 2005)

Mouse pre-implantation development

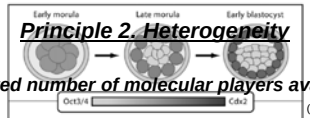


(Motosugi et al. 2005)

Molecular mechanism of the initial lineage specification

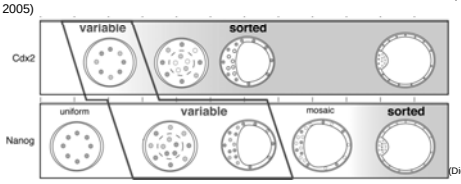
Principle 2. Heterogeneity

A limited number of molecular players available !



(Smith, 2005)

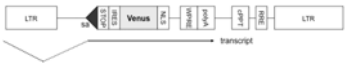
Interaction between Oct3/4 and Cdx2 determines ICM vs. TE differentiation. (Niwa et al., 2005)



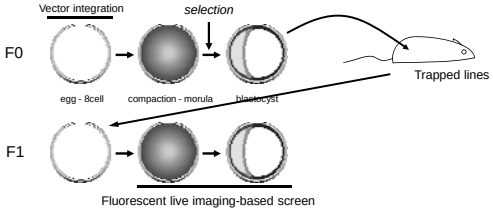
(Dietrich and Hiiragi, 2007

Two patterning phases: 1. heterogeneity
2. sorting

Promoter Venus-trap screen



Lentiviral vector



Fluorescent live imaging-based screen

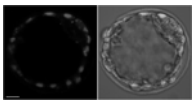
- Characterize dynamic patterning process
- Identify novel players

(Dietrich and Wennekamp; in collaboration with Trono lab)

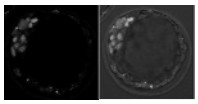
Venus-trap screen

Screen efficiency	
Transduced embryos	2451
Pre-screen positive	582 (24%)
Live founders	104 (18%)
Positive integrations	22 (21%)

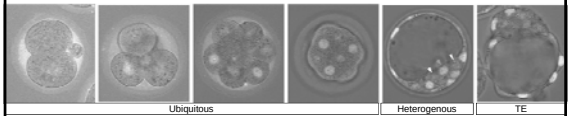
Expression type	
Ubiquitous	13
ICM	3
TE	3
TE and PE	2
heterogeneous	1



TE-specific fJAV 17-B



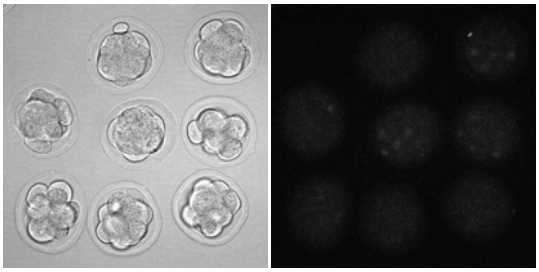
ICM-specific JAV 53-A



TE-specific fJAV 6-C

(Dietrich and Wennekamp; in collaboration with Trono lab)

Molecular dynamics revealed by Venus-trap mice



ICM-specific JAV 53-A: RP23-162H3.2

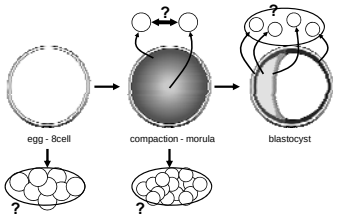
Molecular dynamics identified by Venus-trap mice

		2-4 cell	8-16 cell	Blastocyst e.d. 3.5	Blastocyst e.d. 4.5
fJAV2-D	Senp2				
fJAV3-A	Ctnna1				
fJAV5-A	Supt6h				
fJAV6-C	Rbm9				
fJAV12-A*	Lass6				
fJAV13-A	Hjurp				
fJAV17-B	Tmem50b				
JAV22-A	Samp				
JAV25-A	Polg				
JAV33-B	Plekhg3				
JAV36-C	Cd2ap				
JAV41-A	Cdk11b				
JAV50-A	Itpkc				
JAV53-A	RP23-162H3.2				
JAV53-C	n.d.				

Ubiquitous
TE
ICM
TE/ PE
Heterogenous

Single-blastomere gene expression profile

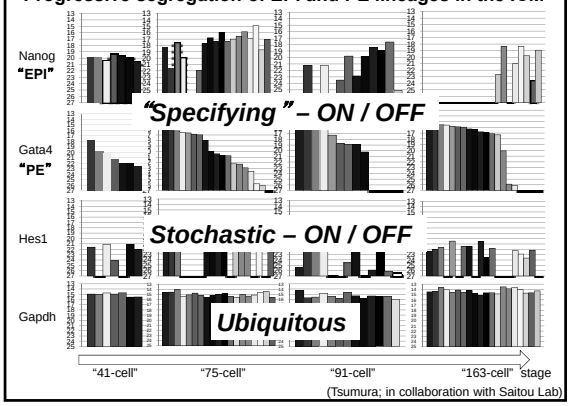
Are the cells "same" or "different"?



How many distinct populations?

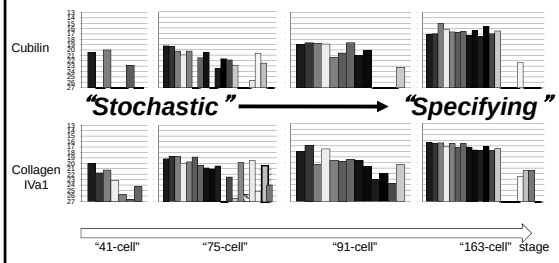
(Tsumura; in collaboration with Saitou lab)

Progressive segregation of EPI and PE lineages in the ICM

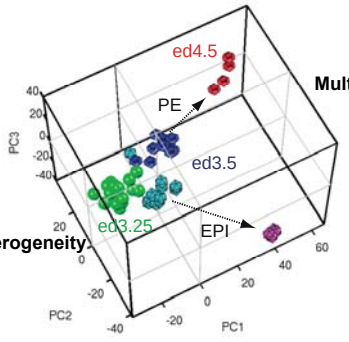


(Tsumura; in collaboration with Saitou Lab)

Progressive segregation of EPI and PE lineages in the ICM



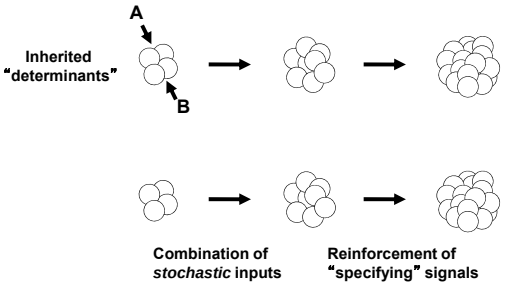
EPI vs PE lineage segregation based on molecular signature of every single cells

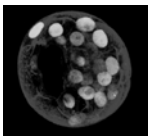


Multiple paths

Higher heterogeneity

Emergence of asymmetry

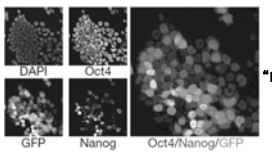




Two patterning phases: 1. heterogeneity
2. sorting

Cdx2 – outside TE
Nanog – inside ICM

Principle 2. Dynamic Heterogeneity – Pluripotency



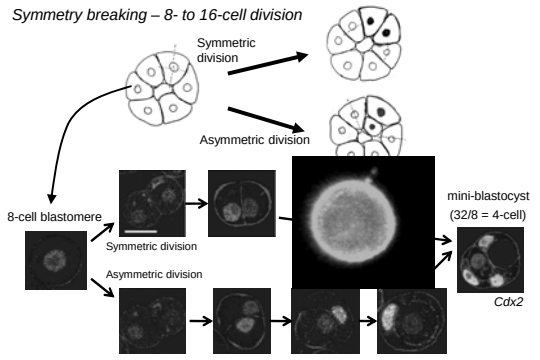
"Nanog fluctuates in mouse ES cells"
(Chambers et al. 2007)

"Dynamic equilibrium and heterogeneity"
Stella (Hayashi et al. 2008)
Rex1 (Toyooka et al. 2008)

Principle 3. Mechanical context plays a key role.

Lineage specification in relation to Cell Adhesion and Polarity

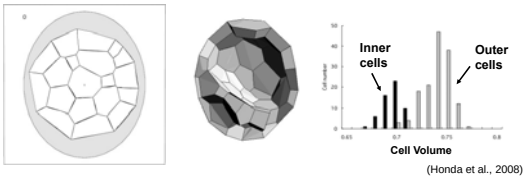
Symmetry breaking – 8- to 16-cell division



Cellular structural and mechanical context

Computer simulation of the blastocyst morphogenesis

- 40 equivalent cell aggregate
- expanding one blastocyst cavity



(Honda et al., 2008)

In-Out difference may emerge autonomously in the equivalent population.
− no need for *intrinsic* bias (localized *determinants)*

What are the principles of patterning mammalian embryos?

1. Dynamicity

2. Heterogeneity

3. Mechanical context

Principle underlying embryonic patterning

Spatial information

e.g. localized determinants

Asymmetry → Embryonic patterning

e.g. differential gene expression

Unique principles in early mammalian development

Spatial information

Extrinsic cues

Embryonic patterning

Self-organization

Asymmetry

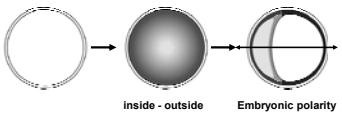
Intrinsic cues

Stochastic and autonomous emergence

Robustness = Regulative capacity

Random process → ? Self-organization

In a certain physical and temporal context



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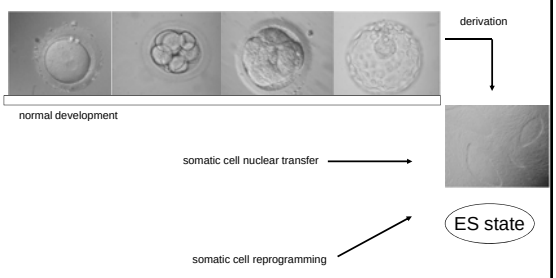
The Role of microRNAs in Embryos and Human ES cells

ESRHE 2011 Pre-Congress Course
Gustavo Tiscornia, PhD
Centre of Regenerative Medicine
Barcelona, Spain
(Conflict of Interests: none)

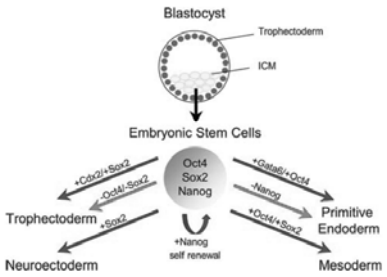
Learning Objectives

- To understand what a microRNA is,
  how it is formed,
  what it does
  and why it is important for cell regulation.
- To review how the role of microRNAS in embryonic stem cells was discovered.
- To examine how microRNAs are integrated into cell regulatory arquitecture.
- To conceptualize how microRNAs are involved in achieving, maintaining and abandoning the embryonic stem cell state.

Of pre-implantation embryos,ICM's, ES cells, iPS cells, their promises and what role microRNAs play

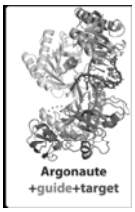


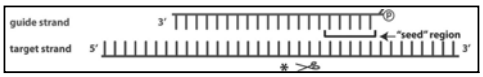
Expression levels of key regulatory factors in ES cells impact on their developmental potential



Bosnali et al, Cell. Mol. Life Sci. 2009

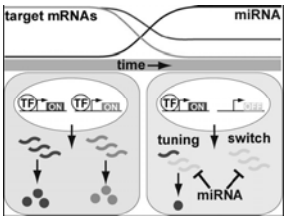
Mechanism of miR induced mRNA downregulation





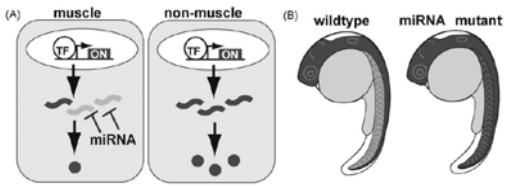
Pratt et al, J. Biol.Chem. 2009

miRNAs regulate developmental transitions



Takacs et al, Semin Cell Dev Biol 2010

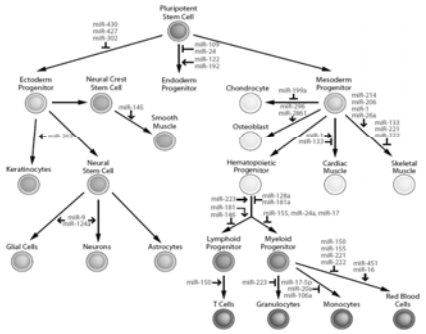
miRNAs play instructive roles in shaping gene expression



Takacs et al, Semin Cell Dev Biol 2010

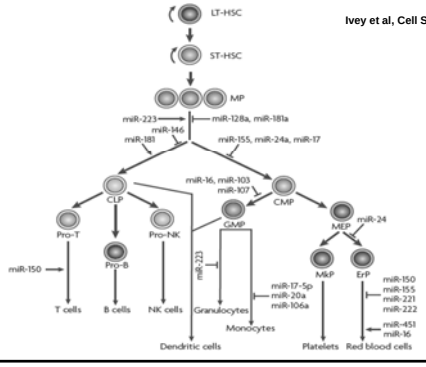
miRNAs Can Regulate the Differentiation of Numerous Cell Types

Gangaraju et al, Nature 2009

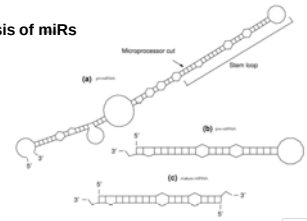


Mir involvement in differentation of the haematopoietic system

Ivey et al, Cell Stem cell 2010



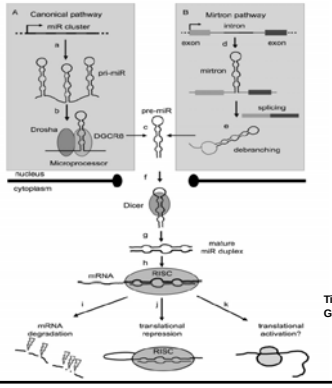
Biogenesis of miRs



Snøve and Rossi *Genome Biology* 2006

Neuman et al, Gen & Dev 2010

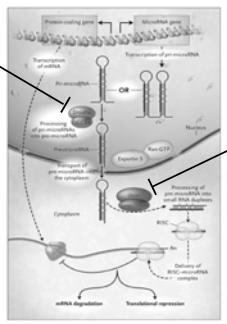
MiR biogenesis and main modes of action



Tiscornia et al, Gen & Dev 2010

Analysis of the role of miRs in ES cells

DGCR8 KO



DICER KO

Milder phenotype than Dicer

Proliferation defect

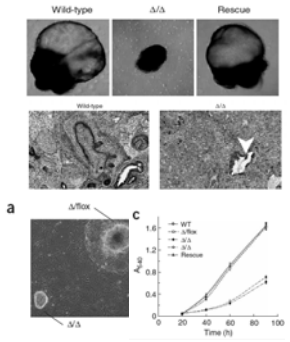
Fail to differentiate

Severe proliferation defect

Severe differentiation defect

Not only implicated in miRNAs biogenesis but also on dsRNAs

DGCR8 is essential for microRNA Biognenesis

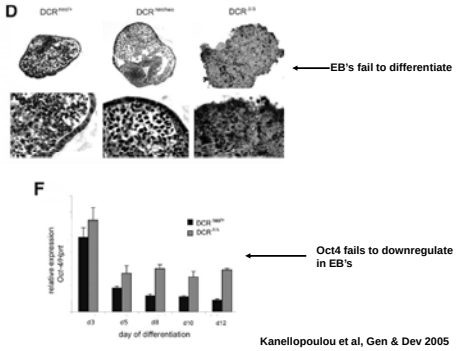


Conclusions:

- **Proliferation defect (accumulation in G1; no apoptosis)**
- **Differentiation defect (failure to fully downregulate pluripotency markers; weak, delayed or absent upregulation of differentiation markers)**
- **Phenotype distinct from Dicer -/- cells (Proliferation defect is more severe and unstable; differentiation defect also more severe**

Wang et al, Nature 2007

Dicer KO ES cells show similar phenotype

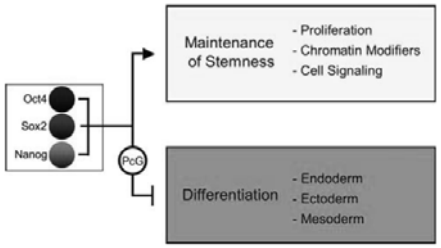


EB's fail to differentiate

Oct4 fails to downregulate in EB's

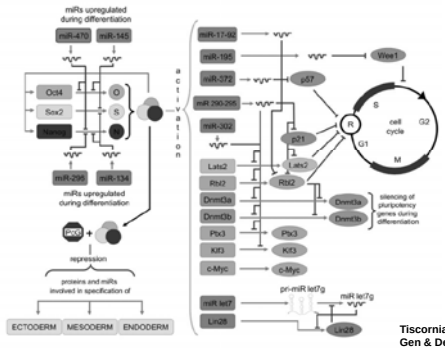
Kanellopoulou et al, Gen & Dev 2005

Oct4/Sox2/Nanog form a core transcriptional network regulating the stem cell machinery.



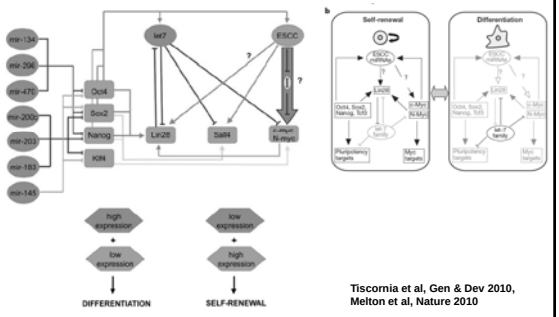
Bosnali et al, Cell. Mol. Life Sci. 2009

Oct4–Sox2–Nanog trio of TFs control a regulatory network of miRs and proteins that control pluripotency vs differentiation and cell cycle profile.



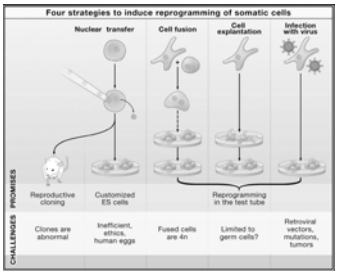
Tiscornia et al, Gen & Dev 2010

Regulatory network illustrating the role of miRs in ESC differentiation.



Tiscornia et al, Gen & Dev 2010, Melton et al, Nature 2010

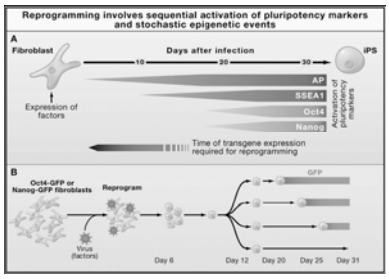
Direct Reprogramming to Pluripotency



miRs?

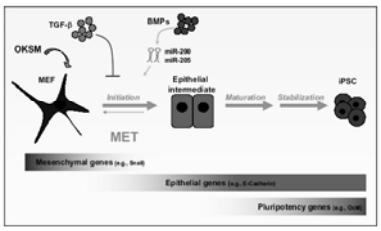
Jaenisch et al, Cell 2008

Dynamics of Reprogramming



Jaenisch et al, Cell 2008

Fibroblast Reprogramming into iPSCs Entails a Mesenchymal-to-Epithelial Transition



Polo et al, Cell Stem Cell 2010

Summary:

- miRs are a fundamental component of gene regulatory networks.
- miRs are involved in many if not all biological processes.
- miRs fine tune the proteome at the post-transcriptional level.
- miRs are heavily involved in differentiation and development.
- miRs can determine tissue-specific gene expression.
- ES cells can survive without miRs, but cease to be ES cells.
- miRs are responsible for the fast cycling of ES cells.
- miRs accelerate the cell cycle by inhibiting cell cycle inhibitors.
- Esc need miRs to turn off the pluripotency program.
- Opposing miR families regulate self-renewal vs differentiation.
- miRs are suspected to be involved in direct reprogramming.

THANK YOU!

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Pluripotency and stem cell states

The different shades of
mammalian pluripotent stem cells

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Author disclosure statement

- No commercial relationships or other activities that might be perceived as a potential conflict of interest to declare

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Learning objectives

- The various origins of pluripotent stem cells
- The molecular mechanism of pluripotency
- The different pluripotent stem cell states
- Reprogramming of cells
- The origin of the pluripotent stem cell population in mammalian development

3

Two key features of stem cells

- Self-renewal:
  - the ability to produce new daughter stem cells that are equal to the original stem cell
- Differentiation:
  - the capacity to give rise to differentiated cell types

NOTE: The oocyte and blastomeres of cleavage stage embryos are not stem cells because they lack self-renewal capacity.

4

(Thomson et al., 1998)

Stem cell types and differentiation potential

- Unipotent stem cells:
  - differentiation limited to 1 cell type
    - spermatogonial stem cells
- Multipotent stem cells:
  - differentiation to multiple cell types generally within the same lineage as the original stem cell
    - haematopoietic stem cells
    - hair follicle stem cells
    - intestinal stem cells
- Pluripotent stem cells:
  - potential to differentiate to cells of all three embryonic germ layers as well as germ cells

5

(Kuijk et al., 2011)

Pluripotent stem cells

- Embryonic stem (ES) cells
- Epiblast derived stem cells (EpiSCs)
- Embryonic germ cells (EG) cells
- Embryonal carcinoma (EC) cells
- Testis derived ES-like cells
- Induced pluripotent stem (iPS) cells

6

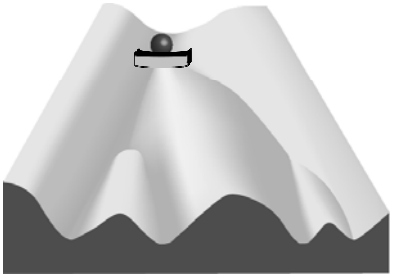
Embryonic stem cells

- Derived from ICM of blastocysts or from cleavage stage embryos
- Self renewal
- Pluripotent: 3 germ layers

7

(Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998)

Waddington's epigenetic landscape



8

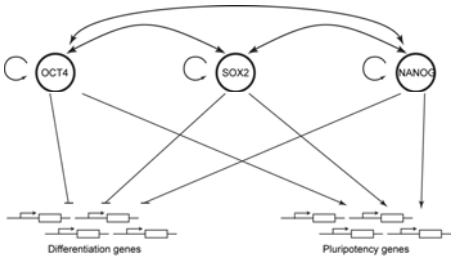
(Waddington, 1940)

Stable stem cell state

- The undifferentiated state of ES cells is determined by
  - a core network of pluripotency factors
  - epigenetic mechanisms
  - cell signaling

9

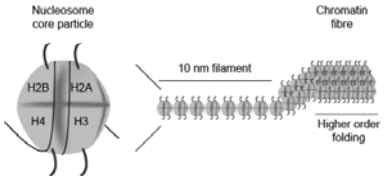
Core network of pluripotency



10

(Boyer et al., 2005; Chen et al., 2008; Kuijk et al., 2011; Loh et al., 2006; Sharov et al., 2008)

Epigenetic mechanism of pluripotency



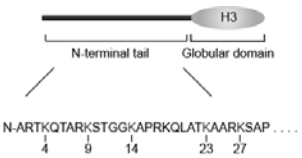
Chromatin fibre

11

(Luger et al., 1997; McGhee and Felsenfeld, 1980; Oudet et al., 1975)

Histone modifications

- Histone tails are carriers of epigenetic marks: heritable non-genetic modifications that affect transcriptional activity. Possible modifications include:
  - methylation, acetylation, phosphorylation, sumoylation, ubiquitination



12

(Goldberg et al., 2007)

Bivalent domains

- Combination of active and repressive marks at lysine residues of histone 3
- Silence developmental genes in ES cells while keeping them poised for activation

13

(Azuara et al., 2006; Bernstein et al., 2006)

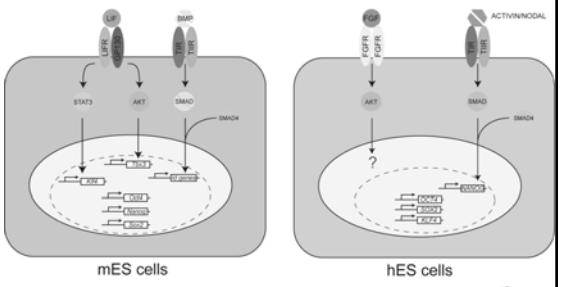
Differences between mouse and human ES cells

- Mouse ES cells
  - fast growing
  - dome shaped morphology
  - LIF/BMP4-dependent
  - No differentiation towards trophectoderm
  - Germline transmission
  - Efficient gene targeting
- Human ES cells
  - slow growing
  - flattened epithelial morphology
  - Activin/Nodal and FGF-dependent
  - Trophectoderm potential
  - Germline potential unknown
  - Inefficient gene targeting

14

(Buecker and Geijsen, 2010; Kuijk et al., 2011; Pera and Tam, 2010)

Signalling pathways and the pluripotency network



15

(Kuijk et al., 2011)

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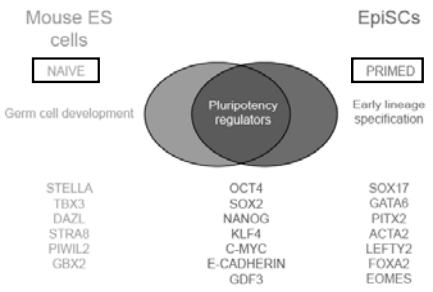
Epiblast derived stem cells

- Derived from epiblast of postimplantation mouse/rat embryos
- Self renewal
- Pluripotent: 3 germ layers
- No germline potential

17

(Brons et al., 2007; Tesar et al., 2007)

Differences between EpiSCs and mouse ES cells



18

(Brons et al., 2007; Tesar et al., 2007)

Similarities between EpiSCs human ES cells

- Slow growing
- Flattened epithelial morphology
- Activin/Nodal and FGF-dependent
- Trophectoderm differentiation potential

HYPOTHESIS:
Human ES cells are considered to be in an EpiSC-like primed state of pluripotency

19

(Brons et al., 2007; Tesar et al., 2007)

Further comparison between human ES cells and EpiSCs

MARKER	Human ES	EpiSC
OCT4	√	√
NANOG	√	√
SOX2	√	√
KLF4	√	X
DPPA3	√	X
REX1	√	X
GBX2	√	X
FGF5	X	√
SSEA1	X	√
SSEA3,SSEA4	√	X
ALKALINE PHOSPHATASE	√	X

20

(Brons et al., 2007; Pera and Tam, 2010; Tesar et al., 2007)

Are hES cells the in vitro counterparts of the postimplantation epiblast?

- Differences between human ES cells and EpiSCs might be species specific:
  - The human postimplantation epiblast is difficult to study. Studies on in vitro implantation models could help to better understand the nature of human pluripotency

21

(Carver et al., 2003; Grewal et al., 2008; Teklenburg and Macklon, 2009)

Pluripotent stem cells

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- Embryonal carcinoma (EC) cells
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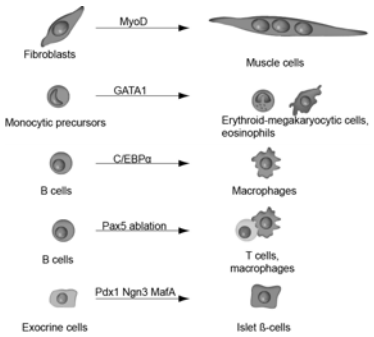
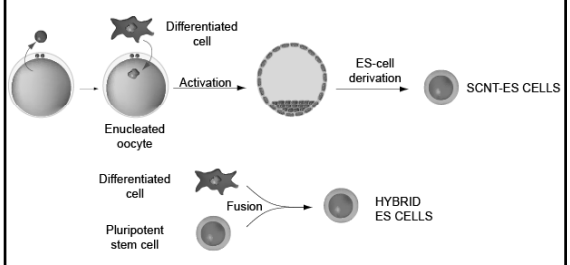
TF induced differentiation



(Graf and Enver, 2009)

23

Reprogramming of somatic cells to pluripotency



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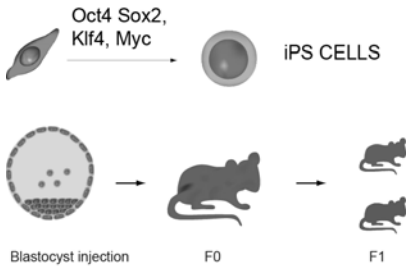
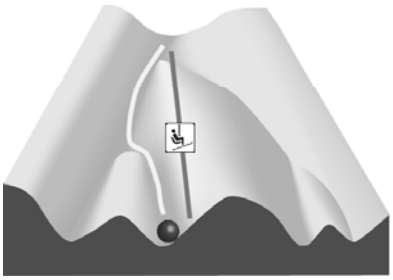
Induction of pluripotency



25

(Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007)

New epigenetic landscape



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Striking features of reprogramming

- miPS cells resemble mES cells
- hiPS cells resemble hES cells
- mouse genes can reprogram human cells
- it takes ~3 weeks to reprogram human somatic cells and ~2 weeks to reprogram mouse cells
  - reprogramming is much faster upon SCNT → what can we learn from the oocyte

27

(Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007)

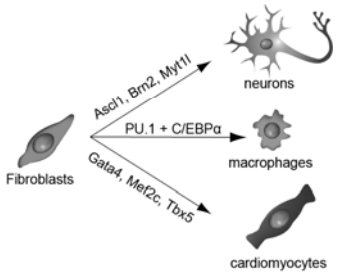
Are iPS similar to ES cells?

- Tetraploid complementation assay indicates that iPS cells are very similar to ES cells however:
  - iPS cells retain epigenetic “footprint” of origin
  - aberrant silencing of imprinted genes
  - genetic instability: reprogrammed cell lines carry somatic point mutations

28

(Blasco et al., 2011; Ghosh et al., ; Kang et al., 2009; Marchetto et al., 2009; Zhao et al., 2009)

Direct conversion of cells using cell type specific transcription factors



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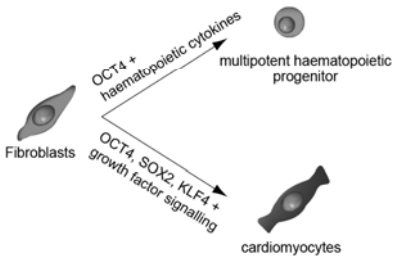
(Feng et al., 2009; Ieda et al., 2010; Vierbuchen et al., 2010)

Cell conversion with pluripotency factors and cell signalling



30

(Efe et al., 2011; Szabo et al., 2010)

Reprogramming

- Can we reprogram any cell type to become any other cell type? If so are all cells pluripotent?

31

Naive pluripotency in human?

- Murine EpiSCs resemble human ES cells
- Is it also possible to generate human pluripotent stem cell lines that resemble the mouse pluripotent state?
  - → homologous recombination

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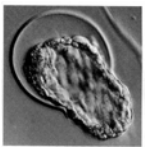
Naive pluripotency in human?

- Human iPS cells in mES cell culture conditions supplemented with inhibitors of cell signalling can acquire mouse ES cell-like properties including:
  - LIF dependence, gene expression pattern, morphology, growth characteristics
- However, no stable human naive pluripotent stem cell lines have been established yet → differences in development!

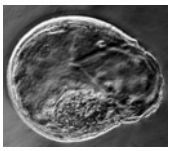
33

(Buecker et al., 2010; Hanna et al., 2010)

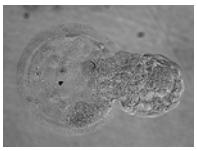
Differences in development?



Mouse hatching blastocyst



Bovine hatching blastocyst



Human hatching blastocyst

Is the mechanism by which the pluripotent epiblast is established conserved between species?

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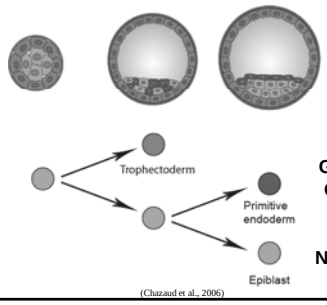
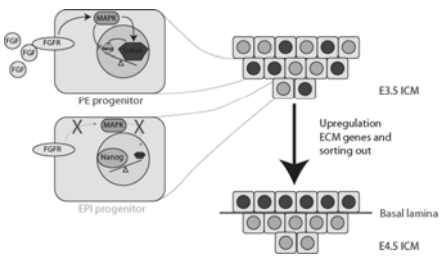
Early lineage segregation in mouse development



35

(Chazaud et al., 2006)

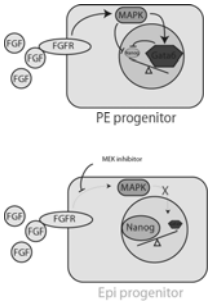
The role of FGF-signaling in lineage segregation



36

(Chazaud et al., 2006)

Inhibit FGF-signaling in early development



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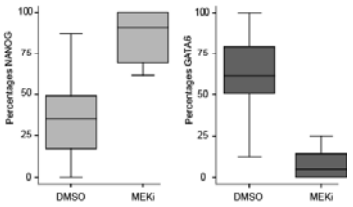
Effect of MEK-inhibition on bovine development

From morula to blastocyst



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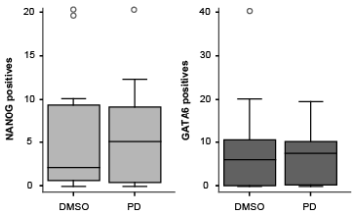
E.Kuijk, unpublished data

Role of MAPK-signaling in human development?



- MAPK signaling does not play a role in the segregation of hypoblast precursors from epiblast precursors in human embryos

39

E.Kuijk, unpublished data

Conclusions I

- Pluripotency is established by a network of transacting transcription factors
- Pluripotency factors can cooperate to impose a pluripotent state onto a somatic cell
- There are various shades of pluripotency, some more pluripotent than others

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Conclusions II

- Pluripotency is important in development: species differences in early development could account for species differences in pluripotency AND studies on early human development are essential to better understand human pluripotency

Embryologists are in a unique position to study human development and contribute to our knowledge on pluripotency

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Blastocyst cryopreservation: maximizing survival and development

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Disclosure

I declare to have no conflict of interest

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Outline of the presentation/Learning objectives

- **Introduction**
- **Discuss the status of the cryopreservation of human blastocysts**
- **Understand basic principles of vitrification**
- **Discuss closed vitrification of human blastocysts in clinical practice**
- **Future considerations**

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History

- **Benefits of blastocyst culture and ET**
  - Improve synchronization of embryo and uterine development
  - Provide a mechanism of self-selection of the viable embryos
    - after activation of the embryonic genome
    - in human: between 4 – 8 cell stage

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Introduction

- **ART success: birth of one healthy child**

Transfer of one embryo: SET and sFRET

The Road for Single Embryo Transfer = blastocyst transfer?

Day of embryo transfer
Benefit of blastocyst transfer
=
Subject of debate

- **Blastocyst cryopreservation and PGD**
- **Blastocysts: an important source of human embryonic stem cells**

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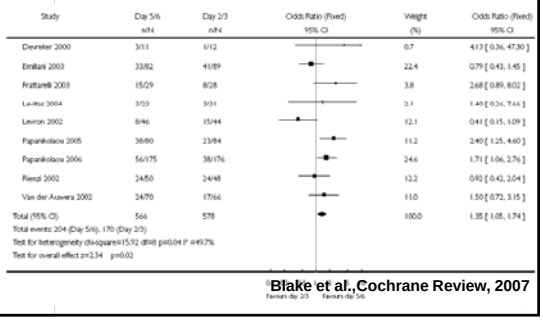
Live birth rate per couple: Cleavage&Blastocyst transfer



Blake et al., Cochrane Review, 2007

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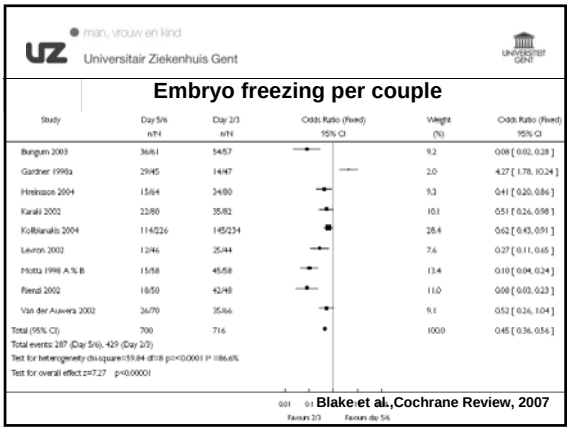
Embryo freezing per couple



Blake et al.,Cochrane Review, 2007

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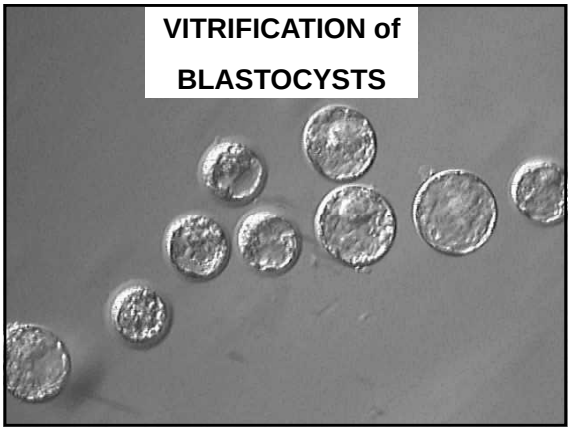
Considerations

- Higher cost **related to blastocyst culture – transfer**
  - Additional incubators
  - Additional culture media
  - More laboratory staff members
  - Expertise in blastocyst quality assessment
  - **Expertise in blastocyst cryopreservation protocols**
  - Optimal laboratory conditions

VITRIFICATION of BLASTOCYSTS



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Literature

- Blastocysts: more complex structure than embryos

1) **TE/ICM cells**: smaller volume of cells compared to cleavage stage embryos
   - Expect better survival because of rapid increase of CP in smaller cells
   - Smaller cells are less sensitive to osmotic stress during removal of CP

2) **Advanced blastocysts: large fluid filled cavity/TE barrier**
   - Later blastocyst stages have an insufficient permeation of the CPA inside the cavity during cooling
     lower viscosity results in **higher** risk of ice crystal formation
   - The larger the cavity, the higher the risk of ice crystal formation

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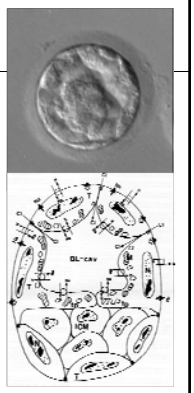
Mechanism of cavitation

- **Na/K ATPase ensures ionic gradient on basolateral membrane.**
- **Osmotic presure - water fills blastocoel.**
- **Tight junctions and desmosomes control leakage of fluid from blastocoel.**
- **Growth factors regulate the mechanism of cavitation.**



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Literature: morphological survival after warming

Vitrification versus slow freezing **(Kolibianakis et al. 2009).**

	Slow freezing	Vitrification	P value
Huang et al. (2005) RCT	41/72 (56.9%)	68/81 (84%)	< 0.0001
Kuwayama et al. (2005) PA	131/156 (84%)	5695/6328 (90%)	< 0.05
Liebermann et al. (2006)	525/570 (92.1%)	528/547 (96.5%)	NS
Bernal et al. (2008) RCT	110/145 (76%)	159/171 (93%)	< 0.0001

Vitrification of blastocysts appears to be better, as compared to slow freezing, in terms of post-thawing survival rates

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Literature: pregnancy and implantation rates

Vitrification versus slow freezing **(Kolibianakis et al. 2009)**

	Implantation rate/ embryo transferred	
	Slow freezing	Vitrification
Liebermann (2006)	152/518 (28.9)	160/523 (30.6)
Bernal (2008) RCT	37/110 (33.6)	56/159 (35.2)
	Clinical pregnancy rates only!	
Kuwayama (2005)	50/98 (51)	2516/4745 (53)

No difference in clinical pregnancy rates per transfer was observed by transferring blastocysts cryopreserved using vitrification versus slow freezing

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Open vitrification of human blastocysts

Choi (2000), Yokota (2001), Cho (2002), Reed (2002), Hiraoka (2004), Hu (2004), Stehlik (2005), Huang (2005), Kuwayama (2005), Mukaida (2007), Liebermann (2007), Son (2007), Hiraoka (2008), Ebner (2009) Liebermann (2009), Rama Raju (2009)

Clin P / ET	Impl /E Transferred	Impl / E Warmed
4974/10197	3124/11117	3124/13629
(48.8%)	(28.1%)	(22.9%)

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Literature: interpretation of results

No uniform data reporting

- Fresh cycle characteristics?
  - Patient selection
  - Number of transferred and vitrified blastocysts
- Vitrification and warming policy?
  - Open vitrification
  - Quality of vitrified blastocysts
  - Number of warmed blastocysts
  - Selection for transfer
  - Number of blastocysts transferred

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Results from literature: blastocysts (closed vitrification)

Stachecki (2008), Van der Zwalmen (2009), Liebermann (2009)

Clin P / ET	Impl /E Transferred	Impl / E Warmed
229/435	263/854	263/1004
(52.6%)	(30.8%)	(26.2%)

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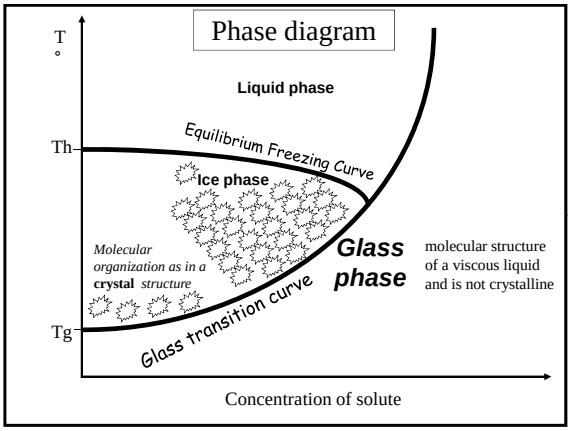
Vitrification
Maximizing safety, survival and development

Development of a safe, robust and efficient vitrification method for human blastocysts



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Basic principles of vitrification

Probability of vitrification

$$\frac{\text{Cooling/warming rates x [CPA]}}{\text{Sample Volume}}$$

Equilibrium vitrification: high [CPA], cooling rate independent, vol >100µl

Non-equilibrium vitrification (minimal volume vitrification): low [CPA], high cooling rates, very high warming rates, vol < 1µl

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Basic principles of vitrification

Principle variables of vitrification

- The effect of cooling and warming rates
- Permeability of cells to water and CPA
- CPA toxicity
- Osmotic responses in CPA solutions

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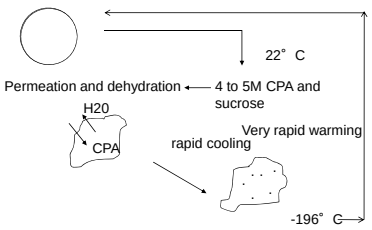
The effect of cooling and warming rates

Non-equilibrium vitrification (minimal volume (<1µl) vitrification)t



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Basic principles of vitrification

Principle variables of vitrification

- Permeability of cells to water and CPA
  - Glyc<EG<DMSO<PG
  - Variability amongst oocytes and embryos
  - Variability amongst blastomeres within one embryo
  - Oocytes<zygotes<embryos<blastocysts
  - TE cells versus ICM cells

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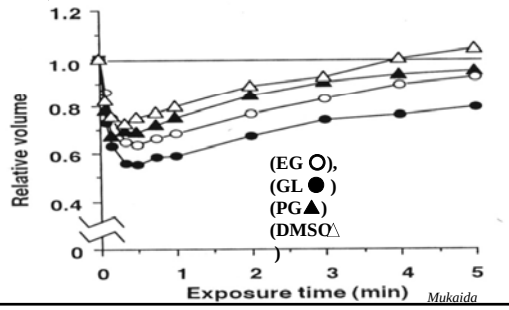
Volume change of bovine 8-cell



Mukaida

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Basic principles of vitrification

Principle variables of vitrification

- CPA toxicity
  - Type and concentration of CPA
    - PG, EG, DMSO, Glyc ….
  - Temperature of exposure
  - Genotoxicity – Ca homeostasis

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Basic principles of vitrification

Principle variables of vitrification

- Osmotic responses to CPA solutions

  Osmotic tolerance limits of cells to be vitrified

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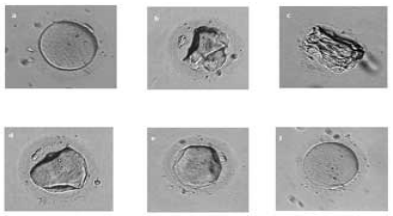
Osmotic responses to CPA solutions



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Conclusions on vitrification

Variables of vitrification that can profoundly influence its effectiveness:

- Technical proficiency of the embryologist
- Concentration and type of CPA and the temperature of exposure
- Risk of re-crystallization during storage or warming
- The device that is used for vitrification
  - Direct contact of the LN2 and the vitrification solution can be a source of contamination

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Closed Blastocyst vitrification: study (Van Landuyt et al, 2011, Hum Reprod)

- **March 2008: Implementation blastocyst vitrification instead of slow freezing for patients with fresh day 5 transfer**
- **To optimize day 5 cryopreservation program (low post-thaw survival rates after slow freezing)**

- **Closed system vitrification to prevent potential disease transmission**

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Blastocyst vitrification

- **Preclinical validation of media/devices for vitrification**
  - Vitrification of blastocysts derived from abnormally fertilized oocytes
  - No difference between open/closed devices (Cryotop vs Cryotip)
  - No difference between 2 closed devices (Cryotip vs HS CBS Vit)
  - DMSO based media performed better than PG-based media in the closed HS CBS Vit device

  (Guns et al. , abstract nr O-134 ESHRE 2008)

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Closed Blastocyst vitrification

- Observational study on blastocyst vitrification
- Aim:
  1. To assess the survival and transfer rate of vitrified day 5 and day 6 blastocysts in relation to blastocyst quality at the moment of cryopreservation
  2. To assess the efficacy of a closed vitrification system by analysing implantation rates in single frozen embryo transfers
  3. To assess the survival and implantation rates of blastocysts derived from biopsied embryos from patients with preimplantation genetic diagnosis treatment

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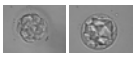
Closed Blastocyst vitrification



- **Vitrification criteria**
  - **Day 5 vitrification:**
    - early cavitating blastocysts (Bl1 and Bl2)
    - advanced blastocysts with full or expanded blastocoele (BL3 and Bl4)
      - ICM type A and B
      - Trophectoderm type A and B
  - **Day 6 vitrification:**
    - only advanced blastocysts
      - ICM type A
      - Trophectoderm type A and B

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Closed Blastocyst vitrification

- **Outcome parameters**
  - Survival rate:
    - Immediate morphological survival after warming
      - % fully intact and moderately damaged blastocysts on the number of warmed blastocysts
  - Transfer rate:
    - % blastocysts transferred on the number of warmed blastocysts
- **Clinical outcome**
  - Clinical pregnancy rate (gestational sac at 7W) per transfer and per warmed blastocyst
  - Implantation rate with FHB

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Results

Morphological survival and transfer rate

- **759 FRET warming cycles**
- **1185 blastocysts warmed**
- **Survival rate:** 77.8% **(921/1185)**
  - Fully intact: 55.9%
  - Moderate damage: 21.9%

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Results
Survival and transfer rate

Table I. Survival and transfer rates according to the day of vitrification and blastocyst quality

	N warmed	N survived (%)	N transferred (%)
Day 5 VIT	**864**	**696 (80.6)c**	**639 (74.0)d**
Day 6 VIT	**321**	**225 (70.1)c**	**199(62.0)d**
Day 5 Early	**384**	**333** (86.7)[a]	**314** (81.8)[b]
Day 5 Advanced	**480**	**363** (75.6)[a]	**325** (67.7)[b]
Day 5 ICM A	**267**	**204 (76.4)**	**184 (68.9)**
Day 5 ICM B	**213**	**159 (74.6)**	**139 (65.3)**

a) p < 0.05 b) p < 0.01 c,d) p < 0.001

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Closed Blastocyst vitrification

- Observational study on blastocyst vitrification
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Results: clinical outcome

Table II. Clinical outcome according to the day of vitrification and blastocyst quality in 530 frozen SET

	% Clinical P/ transfer	% Implantation (FHB)/ transferred embryo
Day 5 VIT	16.1 (67/406)	14.3 (58/406)
Day 6 VIT	16.1 (20/124)	13.7 (17/124)
Day 5 Early	12.2 (23/189)	10.6(20/189)
Day 5 Advanced	20.3 (44/217)	17.5 (38/217)
Day 5 ICM A	20.7 (25/121)	19.0 (23/121)
Day 5 ICM B	20.8 (20/96)	15.6 (15/96)

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Closed Blastocyst vitrification

- Observational study on blastocyst vitrification
- Aim:
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Results: PGD blastocysts

Outcome of PGD warming cycles

- **100 warming cycles analysed**
- **131 blastocysts warmed**
- **Morphological survival:** 83.2 % **(109/131)**
  - Day 5 blastocysts: 90.4 %
  - Day 6 blastocysts: 70.8 %
- **Clinical pregnancy rate per transfer:** 22.0% **(20/91)**
- **Implantation rate (with FHB) per embryo transferred:** 15.4 % **(12/78)**

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Closed Blastocyst vitrification: Discussion

- **Morphological survival rate immediately after warming was** 77.8% **for vitrified blastocysts after *random warming of all types of blastocysts***
- **In** 93.0% **of warming cycles, the patient had a transfer**
- **Early blastocysts survived better than advanced day 5 blastocysts**
  - 75.6% acceptable
  - Performing AS?
- **The ongoing implantation rate per transfer for all day 5 vitrified blastocysts was** 17.3%

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Closed Blastocyst vitrification: Discussion

- **The highest implantation rates were obtained with advanced day 5 vitrified blastocysts with ICM type A** (20.7%) **~ literature**
- **Day 6 vitrification: similar survival rates, poor implantation rates**
  - Reflection of impaired intrinsic quality, originated from day 5 embryos of doubtful quality
  - Day 6 vitrification is questioned in this particular freezing strategy

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Closed blastocys vitrification: Conclusions

- **This evaluation of blastocyst vitrification with a closed system shows promising results in both routine IVF/ICSI and PGD program**
  (Warming the best quality blastocyst first would increase the additive value of the first frozen transfer cycle)
- **Vitrification with the closed HS-VIT system is a feasible method in a large IVF setting**
- **Continue to opt for single vitrified blastocyst transfer for patients who received day 5 SET in the fresh cycle**
- **Policy maximizing the results obtained?**

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How can we further maximize blastocyst vitrification?

1. Higher CPA concentrations
   Longer exposure to CPA
   Exposure to CPA at higher temperature
   => More toxic conditions
   Smaller sample volumes → technically less feasible

2. **Artificial shrinkage of the blastocoele**

3. **Assisted hatching before cryopreservation**

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Artificial shrinkage = mechanical reduction of the blastocoele before vitrification

- By puncturing the trophectoderm cells with a glass micro-needle (Vanderzwalmen et al. 2002)
- By puncturing with a 29-gauge needle (Son et al. 2003)
- By repeated micropipetting with a hand-drawn Pasteur pipette (Hiraoka et al. 2004): less invasive, it takes longer
- By applying 1 single laser pulse on the TE cells (Mukaida et al. 2006)

⇒ to create a hole in the TE cells to induce immediate collapsing of the blastocoelic cavity

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Literature: morphological survival after warming

Survival rates for expanded blastocysts with and without AS

	-AS	+ AS
Vanderzwalmen et al. (2002) (BI3-4)	14/71 (20.3%)	53/75 (70.6%)
Son et al. (2003) - preliminary (poorQ) - clinical	37/52 (71.2%) /	48/53 (90.6%) 81/90 (90%)
Hiraoka et al. (2004) - preliminary (poorQ) - clinical	4/10 (40%) /	9/10 (90%) 48/49 (98%)
Mukaida et al. (2003a) Mukaida et al. (2006)	288/339 (85%) /	/ 488/502 (97.2%)

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Assisted hatching before vitrification

- **High survival rate in early and advanced PGD blastocysts where artificial hatching was observed**
  Van Landuyt et al, 2011
- **Zech et al. 2005:**
  - Investigated effect of artificial hatching of the ZP before vitrification on the survival rates
  - Expanded blastocysts survived better when they had partially or completely hatched (81% vs 55%)
  - How to explain beneficial effect of hatching?
    - Opening in ZP allows better contact with the CP solution
    - more pronounced and uniform decrease in the volume of the blastocoele (few or no TE cells attach to ZP)
    - faster expulsion of water out of the cavity

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General Conclusion

Closed vitrification is a safe, validated and efficient method for the cryopreservation of human blastocysts

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Mark your calendar for the upcoming ESHRE campus workshops!

- Early pregnancy disorders: integrating clinical, immunological and epidemiological aspects
  23-26 August 2011 - Copenhagen, Denmark
- The management of infertility – training workshop for junior doctors, paramedicals and embryologists
  7-8 September 2011 - St. Petersburg, Russia
- Basic genetics for ART practitioners
  9 September 2011 - Bucharest, Romania
- The whole man
  22-23 September 2011 - Sevilla, Spain
- Accreditation of a Preimplantation Genetic Diagnosis Laboratory
  3-4 October 2011 - Athens, Greece
- Human reproductive tissues, gametes and embryos: Innovations by science-driven culture and preservation systems
  9 October 2011 - Cairns, Australia
- Comprehensive preimplantation screening: dynamics and ethics
  13-14 October 2011 - Maastricht, The Netherlands
- Endometriosis and IVF
  28-29 October 2011 - Rome, Italy
- Endoscopy in reproductive medicine
  23-25 November 2011 - Leuven, Belgium
- What you always wanted to know about polycystic ovary syndrome
  8-10 December 2011 - Sofia, Bulgaria

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