Totipotency
Cell loss during preimplantation development

Hilde Van de Velde, PhD

Universitair Ziekenhuis Brussel
Vrije Universiteit Brussel
Centrum voor Reproductieve Geneeskunde

Tours, April 11, 2008

Learning objectives

- Definitions
  - → Totipotency
  - → Plasticity
- Cell loss during preimplantation development
  - → Fragmentation
  - → Cryodamage
  - → Biopsy for PGD/PGS

Tours, April 11, 2008

The totipotent cell

is able to develop into
normal fertile offspring

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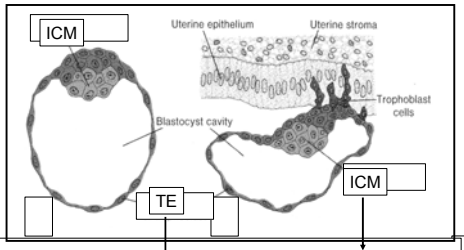
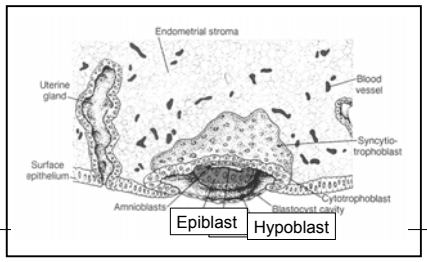
The totipotent cell in the blastocyst (day 6)



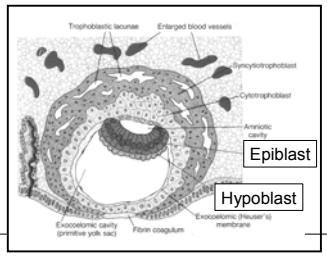
The invading blastocyst (day 8)

ICM → bilaminar germ disc: epiblast and hypoblast



The embedded blastocyst (day 15)

ICM → → trilaminar germ disc: gastrulation



Extra embryonic
- Endoderm: yolk
- Ectoderm: amnion
- Mesoderm: cord

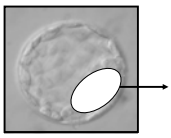
Embryonic
- Endoderm
- Ectoderm
- Mesoderm

} Germ layers

- PGC

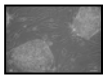
~~Trophoblast~~

Pluripotent human embryonic stem cells



ICM

ESC



Trophoblast

Extra embryonic
- Endoderm
- Ectoderm
- Mesoderm

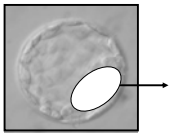
Embryonic
- Endoderm
- Ectoderm
- Mesoderm
- PGC

} Germ layers

Thomson Science 1998,282:1145-1147
Reubinoff Nature Biotechnology 2000,18:399-404

Xu Nature 2002,20:1261-1264
Hyslop STEM CELLS 2005,23:1035-1043

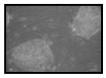
Pluripotent human embryonic stem cells



ICM

ESC

Pluripotent



Trophoblast

Extra embryonic
- Endoderm
- Ectoderm
- Mesoderm

Embryonic
- Endoderm
- Ectoderm
- Mesoderm
- PGC

} Germ layers

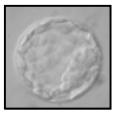
Thomson Science 1998,282:1145-1147
Reubinoff Nature Biotechnology 2000,18:399-404

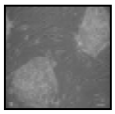
Xu Nature 2002,20:1261-1264
Hyslop STEM CELS 2005,23:1035-1043

Legally: no hybrids

Expression of 'stemness' markers in human preimplantation embryos and ESC

Cauffman Greet





POU5F1_iA	+
SOX2	+
SALL4	+
NANOG	+

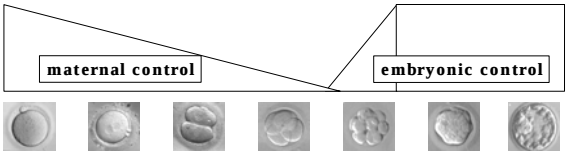
Expression of 'stemness' markers in human preimplantation embryos and ESC

Cauffman Greet

		TE	ICM	
POU5F1_iA	-	+	+	+
SOX2	-	+ → -	+	+
SALL4	-	+	+	+
NANOG	-	-	+	+

The zygote does not express 'stemness' markers

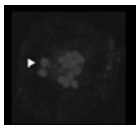
Embryonic genome switch



day 0 | 16 hours | 22 hours | **day 2** | **day 3** | day 4 | day 5

Braude Nature 1988,13:1461-1470
Monk Oncogene 2001, 20, 8085-8091
Dobson Hum Mol Genet 2004, 13:1461-1470
Cauffman Mol Hum Reprod 2005,11:173-181
Cauffman Mol Hum Reprod 2005,11:405-411

NANOG expression in human preimplantation embryos



NANOG expression in some ICM cells?
- → = totipotent cells
- → = good quality ICM cells
- → = ICM cells from which hESC can be derived
- → = cells committed to epiblast
- → = future primordial germ cells

Are ESC an in vitro artefact?

Totipotency

- Conclusions
  - → The zygote is the ultimate human totipotent cell
  - → There is no marker for totipotency, there are 'stemness' markers
  - → NANOG is the best marker for ICM cells
- Questions
  - → What is an ESC? What is the origin of ESC?
  - → Where is the totipotent cell in the embryo?

Learning objectives

- Definitions
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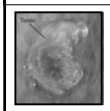
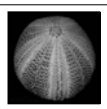
  - → Biopsy for PGD/PGS

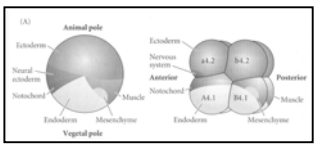
Potency in invertebrates

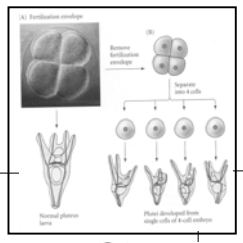


Tunicata
Fate
Irreversible

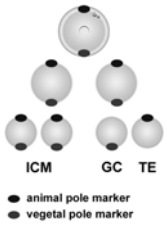


Sea urchin
Plasticity
Reversible





Edwards



Edwards Mol Hum Reprod 1997,3:863-905
Edwards RBMOnline 2005,11:206-218

Where is the totipotent cell?

Splitting experiments cleavage stage embryos

- Mouse 2-cell stage blastomeres
- Some mouse 4-cell stage blastomeres in chimeras
- Some rabbit and cow 4- and 8-cell stage blastomeres
- Human: legal and ethical objections

Where is the totipotent cell?

→ Splitting experiment human 4-cell stage embryo
→ Approval Ethical Commity UZ Brussel May 11, 2006
→ Informed consent
  - > 8 mature oocytes: 1 for research
  - Fresh mature oocytes if no sperm found in TESE
→ ICSI with donor sperm

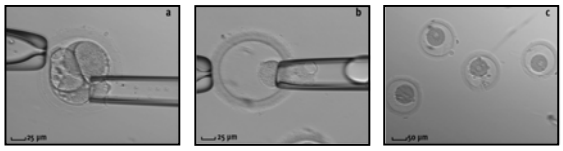
→ Day 2: biopsy of 4 cells
→ Each cell is manipulated and put into empty ZP
→ In vitro culture day 2-day 6

Where is the totipotent cell?

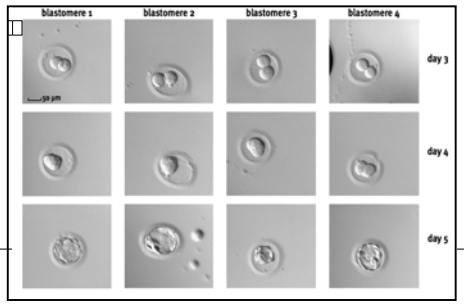
- Can each cell of the 4-cell stage human embryo develop into blastocyst with ICM and TE?
- Do the blastomere-derived blastocysts express 'stemness marker' NANOG?
- Can we derive pluripotent embryonic stem cells from a 4-cell stage human embryo?

Klimanskaya Nature 2006,444:512
Blastomeres from human 8 cell-stage embryos: 2% derivation rate

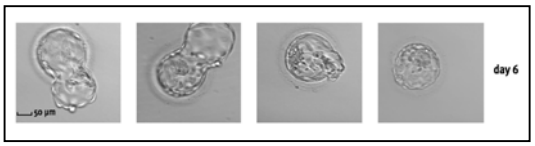
Where is the totipotent cell?
Human 4-cell stage preimplantation embryo



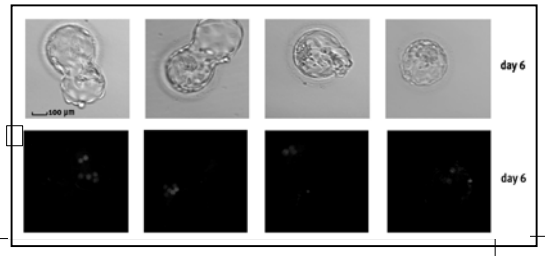
Where is the totipotent cell?
In vitro development day 3-day 5



Where is the totipotent cell?
In vitro development day 6



Where is the totipotent cell?
NANOG expression in ICM cells



Where is the totipotent cell?
In vitro development day 2-day 6

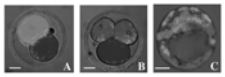
Embryo	Day 2 Survived	Day 3 2-cell	Day 4 Compaction	Day 5 Cavity	Day 6 Full/expanded blastocyst (ICM/NANOG)
1	4	4	3	3	2 (1/ND)
2	4	4	3	3	3 (2/2)
3	3	3	3	3	3 (1/1)
4	**4**	**4**	**4**	**4**	**4 (4/4)**
5	4	3	3	3	3 (3/2)
6	4	1	3	3	1 (1/0)
Total	23	19	19	19	16 (12/9)

ND: not done because lost during fixation

4 cells develop into blastocysts with ICM and TE
no allocation

Where is the murine totipotent cell? Prepatterning believers and non-believers

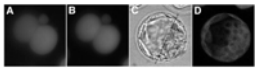
Believers
oocyte, zygote, 2-cell stage



Piotrowska 2001, Development 128: 3739-3748: dye

Torres-Padilla 2006 Nature 445: 214-218

Non-believers
inside-outside hypothesis
Tarkowsky 1967 J Em Exp Morph 18: 155-180



Kurotaki 2007 Science 316: 719-215: photoconversion in vitro and in vivo

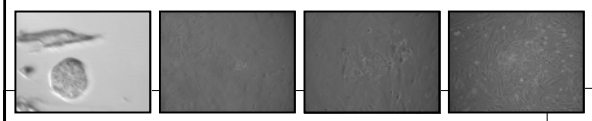
Gardner 1997 Development 124: 289-301
Gardner 2001 Development 128: 839-847
Gardner 2005 Hum. Reprod.
Piotrowska 2001 Nature 409: 517-521

Hiiragi 2006 Int. J. Dev. Biol. 50: 581-588
Motosugi 2006 PLoS 4: 799-804
Van de Velde Hum Reprod in press

25 Tours, April 11, 2008

Where is the pluripotent cell? hESC derivation from single blastomeres of 4-cell stage embryos

- → Splitting experiment human 4-cell stage embryo
- → Day 2: biopsy of 4 cells
- → Each cell is manipulated and <u>not</u> put into empty ZP
- → In vitro culture day 4 (small morula)
- → Mouse feeder layer



26 Tours, April 11, 2008

Where is the pluripotent cell? hESC derivation from single blastomeres of 4-cell stage embryos

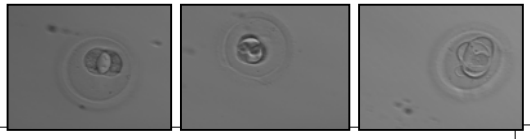
Geens Mieke
Mateizel Ileana
Spits Claudia

Derivation 2/16 cells from 4 embryos:
- VUB_26 QUATRO
  - passage 45
  - expresses stemness markers
  - teratoma, characterized (3 germ layers)
  - CGH: 46 XX, dup 7q33-ter, del 18q23-ter
- VUB_27 PATRU
  - passage 19
  - expresses stemness markers
  - no teratoma yet
  - CGH: 46 XY

27 Tours, April 11, 2008

Where is the totipotent cell?
Human 8-cell stage preimplantation embryo

4 embryos
32 cells
14/32 little blastocysts
4/14 small ICM



Learning objectives

- The cells of the 4-cell stage human embryo are
  - → potentially totipotent
  - → equivalent: individually able to develop into blastocysts with ICM and TE
  - → no allocation to ICM or TE: plasticity
- At least one cell of a 4-cell stage human embryo can develop into ESC
  - → potentially pluripotent

Learning objectives

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- Cell loss during preimplantation development
  - → Fragmentation
  - → Cryodamage
  - → Biopsy for PGD/PGS

Implantation rate after SET

- Van Royen 1999 Hum Rep 14,2345-2349
  Van Royen 2001 Hum Rep 16,326-332
  → SET: IR 100% versus 0%
  → Top quality on day 3: theoretical IR 47%
    - 4 or 5 blastomeres on day 2
    - at least 7 cells on day 3
    - ≤ 20% fragmentation
    - no multinucleation
- Gerris 2002 Hum Rep 17,2626-2631
  → Top quality on day 3: IR 35.1%

Fragmentation degree and pattern

Fragmentation day 3	BI rate	SET IR	Fragment removal
no	27.5%	48%	
0-5% minimal	19.9%	25%	38%
6-15% localized, small 1 blastomere	13.9%	29%	33%
6-15% scattered, small	8.8%	11%	31%
15-25% whole blastomeres	0%	0%	18%
>35% necrotic	0%	0%	

~~Alikani 1999~~
Fertil Steril 71: 836-842

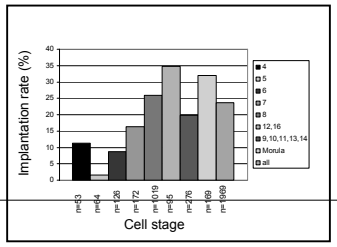
Stone Am J Obst Gynecol 2005,192:2014-2020

Fragmentation: degree and pattern

- Alikani Fertil Steril 1999,71:836-842
  → Large localized fragments:
  loss of cells is correlated with poor implantation rate
  → Fragment removal improves implantation rate
  → No prospective randomized controlled study
- Hardarson RBMOnline 2002,5:36-38
  → Small scattered fragments are generated during divisions
  → Fragments can be reabsorbed

UZ Brussel
Day 3 implantation rate (IR) according to cell stage

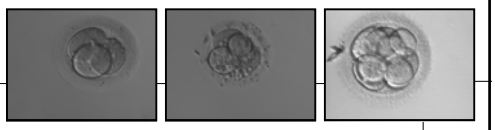
2004-2007 IR=FHB/ET
1969 cycles Overall 23.7%
SET 8-cell stage 26.0%



UZ Brussel
Size of the blastomeres

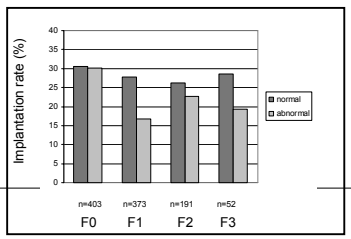
normal

abnormal

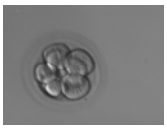


UZ Brussel
Day 3 IR according to cell stage, fragmentation and size of the blastomeres

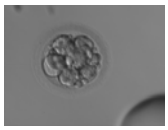
8 cell-stage
Fragmentation
Size of the blastomeres: normal or abnormal



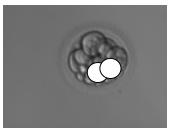
UZ Brussel
Fragmentation and size of the blastomeres



8 cells
F0
Irregular size blastomeres



8 cells
F2, scattered fragmentation
Regular size blastomeres



6 cells
F2, localized fragmentation
Regular size blastomeres

Loss of cytoplasm
(smaller cells)
Scattered fragments

⇔

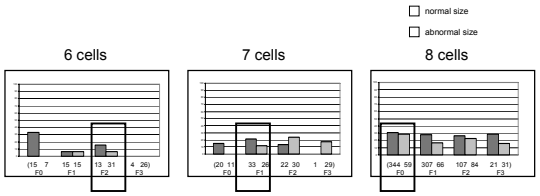
Loss of cells
(cytoplasm+nucleus)
Localized fragments

Cell loss and fragmentation

1	0%	F0
2	12.5%	F1
3	25%	F2
4	37.5%	F3
5	50%	F4
6	62.5%	
7	75%	
8	87.5%	

Loss of cells (cytoplasm+nuclei)
Localized fragments
6F2 ≤ 7F1 ≤ 8F0
6F3 ≤ 7F2 ≤ 8F1
6F4 ≤ 7F3 ≤ 8F2
7F4 ≤ 8F3

UZ Brussel: Day 3 implantation rate (%) according to cell stage, fragmentation and size of the blastomeres



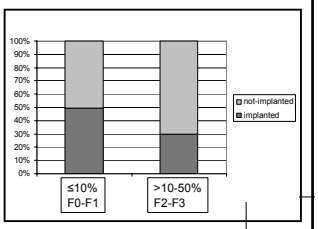
6F2 ≤ 7F1 ≤ 8F0
6F3 ≤ 7F2 ≤ 8F1
6F4 ≤ 7F3 ≤ 8F2
7F4 ≤ 8F3

UZ Brussel
Blastocyst development and fragmentation

Della Ragione, Verheyen
Rep Biol Endocrinol 2007,5:2

Fragmentation on day 3
P=0.03

stage	IR
Top Bl3AA and Bl3AB	28.3%
Top Bl4AA and Bl4AB	52.4%



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- Definitions
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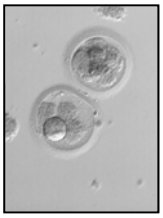
Damage after cryopreservation



- Veiga Hum Reprod 1987,2:321-323
  - → Case report 1/4, pregnancy
- Van den Abbeel Hum Reprod 1997,12:2006-2010
  - → Case report 3/4, born
  - → Case report 1/4, biochemical pregnancy
  - → obstructive (and/or toxic) effect damaged cell
- Van Landuyt and Van den Abbeel, in preparation
  - → Case report 4/8, born
  - → Case report 4/8, clinical pregnancy

Damage after cryopreservation

Edgar 2007 RBMOnline 14: 718-723

ET Day 2	IR sFET
4/4	26.0%
3/4	27.5%
2/4	9.4%

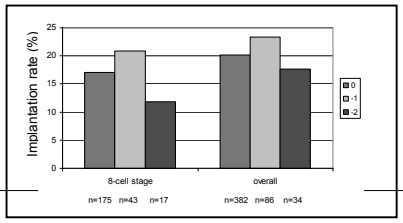
Tang 2006 Hum reprod 21: 1179-1183

ET Day 3	IR sFET
8/8, 7/7, 6/6	10.3%
7/8, 6/7, 5/6	11.1%
6/8, 5/7, 4/6	4.2%

Resumption of mitosis

Damage after cryopreservation

Van Landuyt and Van den Abbeel
2004-2007: 530 sFET cycles,
cryo day 3, ET day 4



Resumption of mitosis

Damage after cryopreservation

Removal of lysed blastomeres on day 3
no prospective randomized study
→ Nagy Fertil Steril 2005,84:1606-1612

→ Rienzi Fertil Steril 2005,84:888-894

	0 lost	1-2 lost	3-4 lost	P value
Cleavage rate	81.3%	76.8%	67.5%	NS
IR	21.6%	21.4%	17.2%	NS
LBR	34.4%	34.0%	29.4%	NS

Learning objectives

- Definitions
  - → Totipotency
  - → Plasticity
- Cell loss during preimplantation development
  - → Fragmentation
  - → Cryodamage
  - → Biopsy for PGD/PGS

Biopsy for PGD/PGS

Hardy 1990 Hum Reprod 5,708-714
ICM/TE ratio is not altered

Goossens, De Rycke and De Vos 2007
Hum Reprod in press
Prospective randomized controlled study
n=592
1-cell versus 2-cell biopsy
Biopsy day 3, ET day 5
Life birth rate
no diagnosis, misdiagnosis

Biopsy for PGD/PGS
Randomized controlled study

Goossens, De Rycke and De Vos
Prospective randomized controlled study, 1-cell versus 2-cell biopsy

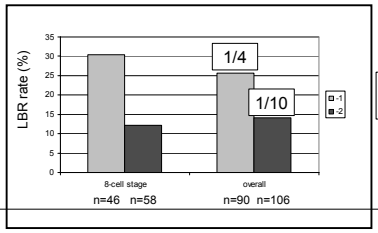
	1 cell	2 cells	P value
Cycles	288	304	
Transfer rate	77.1%	75.3%	NS
Implantation rate	23.5%	17.3%	NS
Miscarriage rate	34.0%	28.0%	NS
LBR	26.2%	22.8%	NS

1 less delivery following 2-cell biopsy every 33 cycles

In vitro development (BI rate):
1-cell biopsy > 2-cell biopsy (p=0.007)
Embryo quality on day 3 (p<0.0001)

Biopsy for PGD
Sub-analysis SET

De Vos Anick
ICSI, PGD/PGS, 1-cell versus 2-cell biopsy



eSET
= cryo ≥1

Case report 8-4: born

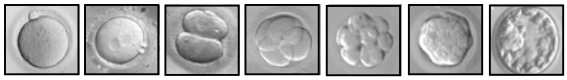
Conclusions

- The cells of the 4-cell stage human embryo are
  - → Potentially totipotent
  - → Equivalent (plasticity)
- Severe cell loss (>25%) is detrimental for implantation
  - → Fragmentation: scattered versus localized
  - → Cryodamage: 8/8 = 7/8 ≥ 6/8
  - → Biopsy for PGD/PGS: 8-1 ≥ 8-2

Cell loss during preimplantation development

- Two hypothesis on cell loss:
  - → Loss of large volume (nuclei and/or cytoplasm)
  - → Abnormal cell-cell contact (obstruction and/or toxic):
    compaction
    cavitation
    blastulation

Cell loss during preimplantation development



- Clinical experiments
  - → Look carefully and score
  - → Time lapse
  - → Randomized controlled studies removing
    - fragments
    - cryodamaged cells
  - → Combinations (fragmentation and biospy, fragmentation and cryodamage)

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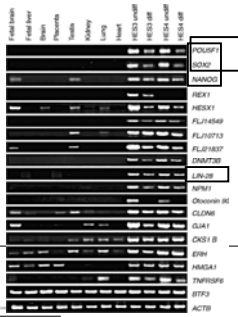
Stemness markers in hESC lines

Richards 2004 STEM CELLS 22: 51-64

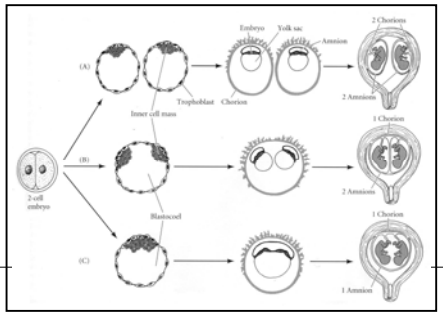


+ KLF4 + c-MYC
Takahashi 2007 Cell 131:1-12

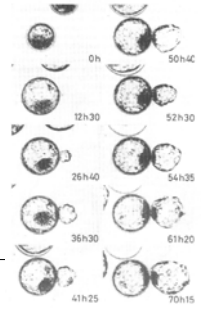
Yu 2007 Science

= reprogramming differentiated somatic cells into pluripotent state

Proof of the model?
Monozygotic bichorionic biamniotic twinning



Monozygotic bichorionic biamniotic twinning and ART



Massip et al. 1983
Abnormal ZP thinning

Alikani Hum Reprod 2003,18:1937-1943
Hall Lancet 2003,362:735-743
Allegra J Ass Reprod Genet 2005,22:437-441