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"From gamete to heartbeat: the missing link"

SPECIAL INTEREST GROUPS

EMBRYOLOGY & EARLY PREGNANCY

2

28 June 2009
Amsterdam
The Netherlands

PRE-CONGRESS COURSE 2

Organised by the Special Interest Groups Embryology and Early Pregnancy

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PRE-CONGRESS COURSE 2 - PROGRAM

From gamete to heartbeat: the missing link

Organised by the Special Interest Groups Embryology and Early Pregnancy

Course co-ordinators: Etienne Van den Abbeel (Belgium), Cristina Magli (Italy), Dominique Royère (France) and Kersti Lundin (Sweden) (Embryology) and Roy Farquharson (United Kingdom) (Early Pregnancy)

Course description: A joint pre-congress course between Embryology and Early Pregnancy Special Interest Groups which will focus on shared topics of relevant modern significance

Target audience: All doctors and scientists interested in basic science and clinical aspects of modern day embryology and early pregnancy

Session 1 - Basics

09:00 - 09:30	How to select the optimal gamete and the impact on fertilisation and implantation? - ***Arne Sunde (Norway)***
09:30 - 09:45	Discussion
09:45 - 10:15	Placental development ***- Larry Chamley (New Zealand)***
10:15 - 10:30	Discussion
10:30 - 11:00	**Coffee break**

Session 2 - Genomics

11:00 - 11:30	Genomics of Gametes and Embryo Development - ***Gayle Jones (Australia)***
11:30 - 11:45	Discussion

11:45 - 12:15 Genomics of Endometrial Implantation - ***José Antonio Horcajadas (Spain)***

12:15 - 12:30 Discussion

12:30 - 13:30 Lunch

Session 3 - Understanding Implantation

13:30 - 14:00 Soluble HLA-G and embryo implantation - ***Philippe Le Bouteiller (France)***

14:00 - 14:15 Discussion

14:15 - 14:45 Research Models: in vitro co-cultures - ***Judith Cartwright (United Kingdom)***

14:45 - 15:00 Discussion

15:00 - 15:30 Coffee break

Session 4 - Unpredictable Implantation

15:30 - 16:00 Non-implantation of the ‘right’ embryo. The embryonic view – ***Sören Ziebe (Denmark)***

16:00 - 16:15 Discussion

16:15 - 16:45 Implantation of the ‘wrong’ embryo. The endometrial view – ***Siobhan Quenby (United Kingdom)***

16:45 - 17:00 Discussion

How to select the optimal gamete and the impact on fertilisation and implantation?

Arne Sunde, PhD
Fertility Clinic, St. Olav's University Hospital of Trondheim
Department of Laboratory Medicine, Children's and Women's Health
Norwegian University of science and technology

Commercial interest:
Shareholder and adviser to CellCura A/S, Norway

Learning objectives

- Selection strategies in general
  - Selection objectives
  - Selection strategies
  - Strengths and limitations
- Methods for selection of human sperm cells
  - Selection of sperm populations with desired properties
  - Selection of individual sperm cells with desired properties
- Methods for selection of human oocytes

Scientist or engineer ?

- Different approaches if you are:
  - A basic scientist
    - Understanding is everything
      - Complex approaches
  - An engineer
    - understand just enough to be able to act
      - Reductionistic approaches
- Clinical embryologists are in this contexts primarily engineers ☺..
  - And this lecture will be an engineers approach

Why select gametes ?

- A high fertilization rate ?
- Obtain maximum number of good embryos ?
- A high pregnancy rate from fresh transfers?
- A low miscarriage rate
- A high cumulative delivery rate (fresh + frozen)
- A low rate of multiple pregnancies/deliveries
- Or....?

- Your success criteria will to a large extent define the selection parameters that will be used.

Statistics or causality

- Often we only have information about a correlation between:
  - the characteristics of a group of cells (i.e. sperm cells)
  - and the performance of another group of cells (the embryo)
  - and the way this group of cells interact with a tissue (endometrium), an organ (the uterus) and a body (the woman)
- Complex relationships, to put it mildly..

Basis for selection of gametes

- General points in evaluating a selection parameter
  - Dependent or independent variable(s)?
  - Relative weight?
  - Predictive power?
  - Robustness?
    - Objective/subjective?
  - Practical in a routine setting?
    - Expensive?
    - Laborious?
    - Time consuming?

Basis for selection of gametes

- Dependent or independent variable (s)?
  - Do NOT waste time, money and energy on collecting redundant information.
  - The selection variable should provide independent predictive power

Basis for selection of gametes

- General points in evaluating a selection parameter
  - Can it only be used to predict the quality of a cohort of gametes?
  - Or
  - Can it be used to select a single gamete to be used for fertilization?

Sperm cells

Sperm cells

- Methods that can give a correlate between a group of cells and the outcome
  - Semen sample
  - Processed semen sample
  - Inseminate
- Methods that enable “one-to-one” knowledge?
  - One sperm-one embryo-one child

Sperm cells

- Methods that can give a correlate between a group of cells and the outcome
  - Semen sample
  - Processed semen sample
  - Inseminate
- **It is fairly well established that it is a correlation between the characteristics of a semen sample and the likelihood for conception *in vivo***

- Bonde, JP (1998)
- Larsen L (2000)
- Spiessens C *(* 2003)
- Aitken, RJ (2005)

Semen characteristics and conception in vivo

- 430 first pregnancy planners recruited from 50 000 trade union workers
- Included consecutively as they discontinued contraception
- Semen sample analyses at enrolment
- Followed up to six menstrual cycles (or to pregnancy)
- Female partner kept daily records of menstrual bleeding and intercourse

Bonde, JP et al. (1998)

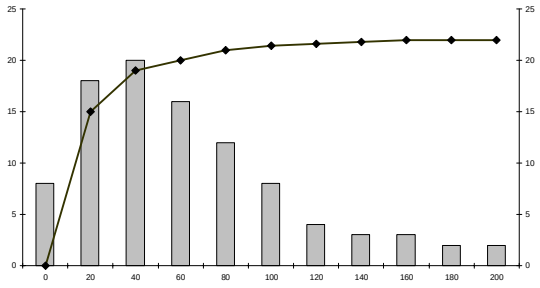
Semen parameters and natural conception

- Levels where fecundity started to drop

• Seminal volume	< 2ml
• Concentration	< 40 mill/ml
• Motility	<50%
• Morphology	< 40% normal

Bonde, JP., at al., 1998

Frequency distribution of Sperm concentration and fecundability (1024 men)



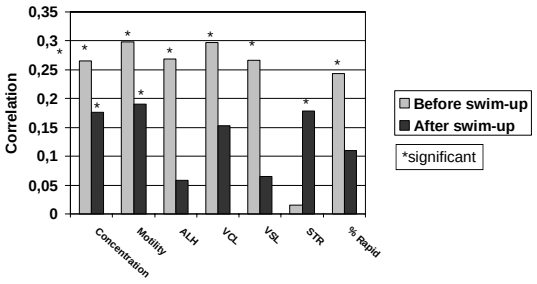
Bonde JP, et al. 1999

IVF and sperm characteristics

- CASA parameters
  - weak correlation
- Sperm penetration assays
  - Not a good predictor
- Acrosome reaction
- Hemi-zona assay
  - Predictors of fertilization rate

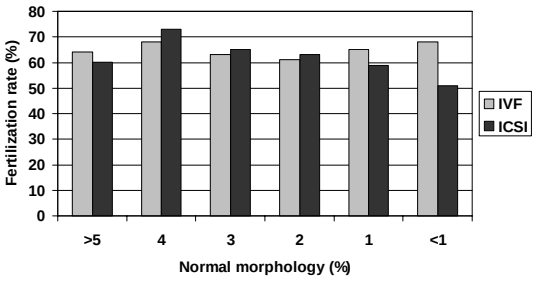
Oehninger et al. 2000, Arslan et al. 2006

Predictability of the IVF Fertilization Outcome by the Semen Characteristics Before and After Swim-Up



Hirano et al. (2001)

Isolated teratozoospermia and fertilization in vitro



Keegan BR et al. 2007

Assays of sperm chromatin

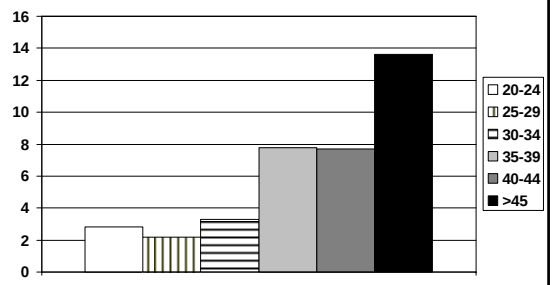
- Chromosome number
  - Aneupliody?
- DNA –integrity tests
  - DNA strand breaks?

Damage to sperm DNA
A two step model

- Defective spermiogenesis
  - Impaired chromatin remodelling
  - Inefficient protamination
  - Vulnerability to stress
- Oxidative stress
  - Apoptosis
  - Oxidative damage

- Aitken RJ 2008

Incidence of chromosomal structural abnormalities in spermatozoa in different age groups of men



Adapted from Plas E et al, 2000

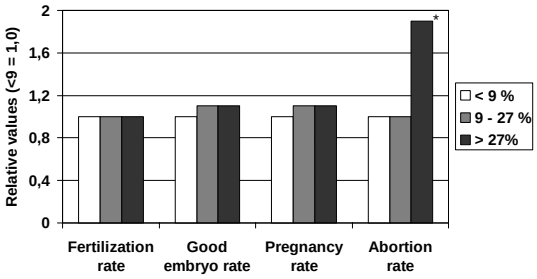
Sperm chromatin assays

- Methods used:
  - TUNEL-assay
    - Terminal Deoxynucleotidyl Transferase (dUTP) nick end labelling
      - "free 3'-OH strands of DNA"
  - Flow cytometry
    - Acridine orange fluorescence
      - single vs. double strand DNA

Damage to DNA in sperm. Clinical relevance?

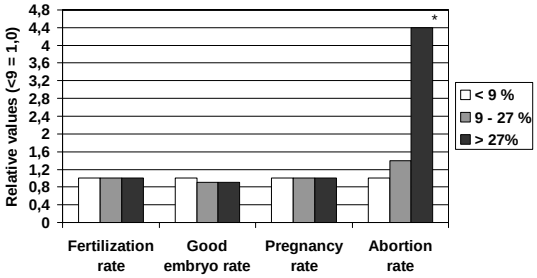
- High fragmentation index poorer outcome in IUI
  - Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome
    - Bungum M., et al. 2007
- Review:
  - DNA integrity tests does not predict pregnancy after in vitro fertilization?
    - Collins J et al. 2008
- Meta analysis:
  - Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI
  - OR for pregnancy loss: 2,37 (1,45-3,88)
    - Zini A. et al,, 2008
- Review
  - Sperm DNA damage: clinical significance in the era of assisted reproduction
    - Zini A & Libermann J 2006

DNA fragmentation index and IVF



Adapted from Lin MH et al. 2008

DNA fragmentation index ICSI



Adapted from Lin MH et al. 2008

Sperm DNA integrity assays Complicating factor?

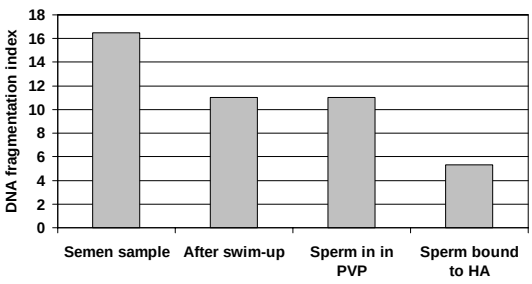
- **Reparable versus non-reparable damage ?**
  - Single or double strand breaks?
  - Oocyte competence for repair of DNA-damage?
- **This will influence the relationship between the Sperm chromatin integrity assays and the clinical outcome.**

Binding of spermatozoa to Hyaluronic acid

- Sperm cells have a receptor for Hyaluronic acid
  - A Correlation between sperm maturity, normal morphology, euploidy and binding to hyaluran-coated surfaces

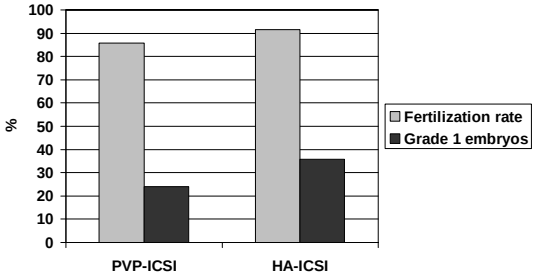
– Jakab A et al. 2005
– Huszar G et al. 2006
– Nasr-Esfahani MH et al. 2008
– Parmegiani L et al. 2009

”Physiologic ICSI’’: Hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus



Parmegiani L et al. 2009

”Physiologic ICSI’’: Hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus

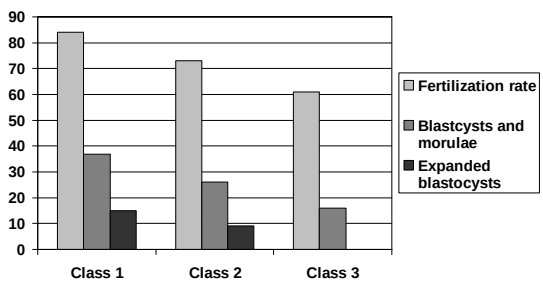


Parmegiani L. et al. 2008

High performance microscopy during ICSI – real-time selection

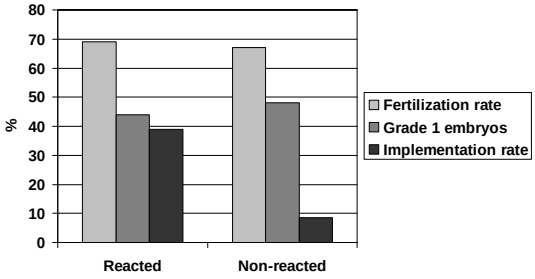
- Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection
  - Bartoov B. et al. 2003
- A new real-time morphology classification for human spermatozoa: a link for fertilization and improved embryo quality.
  - Classification:
    - Class 1: Spermatozoa with normal head and maximum of two other abnormalities (not located in the head).
    - Class 2: Spermatozoa with more than two abnormalities.
    - Class 3: Spermatozoa with several head defects.
  - Cassuto NG et al. 2008

A new real-time morphology classification for human spermatozoa: a link for fertilization and improved embryo quality



Cassuto NG et al. 2008

Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection



Gianaroli L. et al. 2009

Oocytes

The oocyte- cumulus compelex

- There is not a good correlation between the oocyte corona-cumulus complex morphology and nuclear maturity of oocytes
    - Rattanachaiyanont M et al. 1999

The oocyte- cumulus compelex

- One can study the gene expression in cumulus cells.
  - A large number og genes can be analysed simultaneously using DNA-array technology
  - Might correlate with oocyte, embryo quality
  - Early days yet ☺

– Assou S et al. 2008

Zona pellucida

- Data has been presented suggesting a correlation between the appearance of the Zona Pellucuda and the apparent quality of the oocyte (embryo).
  - Zona Thickness variation
    - Gabrielsen A et al. 2001
    - Sun YP et al (2005)
  - Polarized light microspopy
    - Pelletier C et al 2004
  - Light retardation (absorpsion)
    - Shen Y et al., 2005
    - Shen Y et al. (2008)
- Dependent or independent variables ?

Oocyte morphology and fertilization rate

- Extracytoplasmatic parameters
  - Fragmented 1.st polar body
  - **Abnormal 1.st polar body**
  - Abnormal zona pellucida
  - **Large perivitelline space**
  - Abnormal shape
- Cytoplasmic evaluation
  - Granular cytoplasm
  - Centrally located granular area
  - **Vacuoles**
  - Smooth endoplasmic reticulum clusters
  - Refractile body

• Rienzi L et al., 2008

Oocyte morphology and Pronuclear morphology

- Extracytoplasmatic
  - Fragmented 1.st polar body
  - Abnormal 1.st polar body
  - Abnormal zona pellucida
  - **Large perivitelline space**
  - Abnormal shape
- Cytoplasmic evaluation
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• Rienzi L et al., 2008

Oocyte morphology and Embryo morphology

- Extracytoplasmatic
  - Fragmented 1.st polar body
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- Cytoplasmic evaluation
  - Granular cytoplasm
  - **Centrally located granular area**
  - Vacuoles
  - Smooth endoplasmic reticulum clusters
  - Refractile body

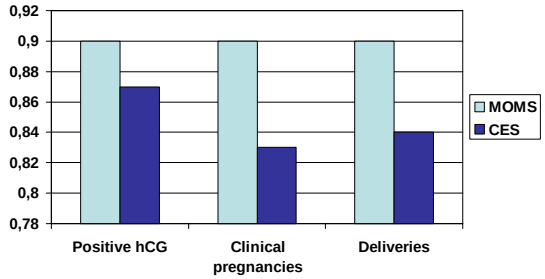
• Rienzi L et al., 2008

Oocyte morphology and Embryo morphology

- Metaphase II oocyte morphological scoring (MOMS)
- Extracytoplasmic features
  - Abnormal 1st polar body 2,0
  - Large perivitelline space 1,4
- Cytoplasmic features
  - Granular cytoplasm 1,4
  - Centrally located granular area 2,7
  - Vacuoles 2,1

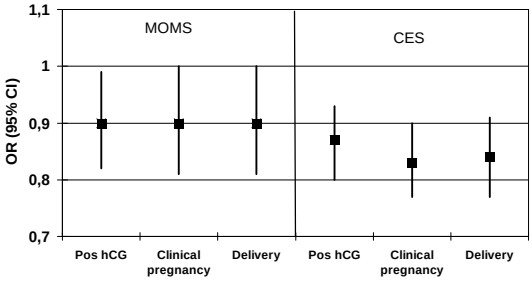
• Rienzi L et al., 2008

OR versus oocyte and embryo score



Rienzi L et al., 2008
MOMS: Metaphase II oocyte morphological scoring
CES: Cumulative embryo scoring

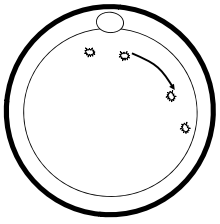
Odds ratio versus oocyte and embryo score



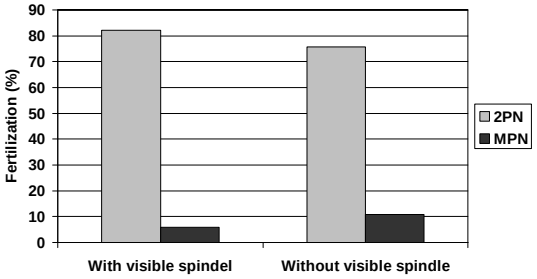
Rienzi L., et al., 2008
MOMS: Metaphase II oocyte morphological scoring
CES: Cumulative embryo scoring

The meiotic spindle

- Presence ?
- Location ?
  - Close to the polar body after completion of meiosis.
  - May migrate more centrally.
    - "Post MII- ageing"

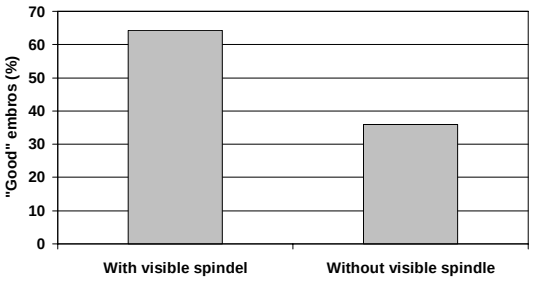


Visual meiotic spindle and abnormal fertilization



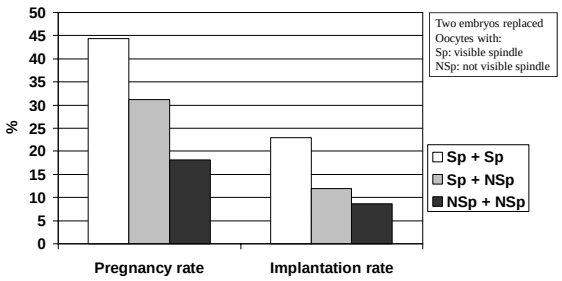
Moon JH et al. 2003

Visible meiotic spindle and ”good” embryos



Moon JH et al. 2003

Visible meiotic spindle ICSI and implantation



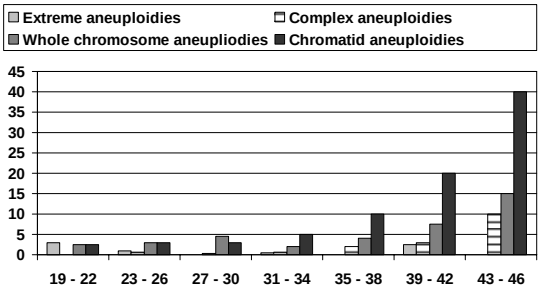
Madaschi C et al., 2008

Polar body analysis

- Relationship between the morphology and relative positions of
  - 1st. polar body
  - 2nd. Polar body
  - Pronuclei
- and implantation rates

– Gianaroli et al. 2007

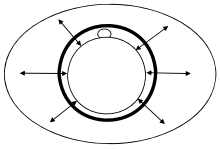
Effect of maternal age on the frequency of cytogenetic abnormalities in human oocytes



Adapted from Pellestor F et al. 2005

Cells interact with the surrounding medium take up – metabolise - secrete

- Embryos may be selected based on their uptake, secretion and metabolism of molecules involved in basic metabolism
- Unclear whether this may be extended to oocytes or sperm
  - Follicular fluid
  - Seminal plasma
  - Culture media



- Leese JH et al. 2008
- Butros L et al. 2008
- Singh & Sinclair 2007
- Scott R et al. 2008

Metabolic profiling - metabolomics

- Techniques that may be used:
  - Nuclear magnetic resonance (NMR)
  - structural information – spatial information 10-6
- Infra red spectroscopy
  - functional groups (C=O,N-H, C-N) 10-6
- Chromatography – sensitive detectors
  - GC/LC combined with UV, MS Fluorescence
  - variety of relatively small molecules 10-12 – 10-23
- Direct MS
  - variety of relatively small molecules 10-15

– Singh & Sinclair 2007

General summary

- Human sperm cells and human oocytes may be selected based on characteristics that correlates to fertilization rate, development rate and in some cases to pregnancy/delivery rates
- When choosing selection strategies, always do a cost/benefit analysis.
  - Money and time versus increase in quality or efficiency
  - Choose parameters that have independent predictive power
  - Do not evaluate many variables simultaneously

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Placental development

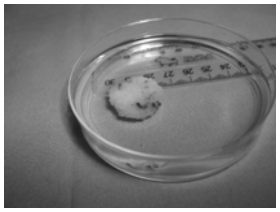
Associate Professor Larry Chamley, PhD,
Department of Obstetrics and Gynaecology, School of Medicine,
Faculty of Medical and Health Sciences
University of Auckland
l.chamley@auckland.ac.nz

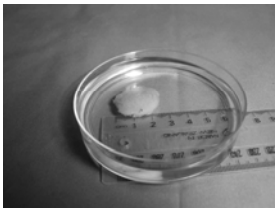
I have no commercial/financial or other conflicts of interest

Learning Objectives

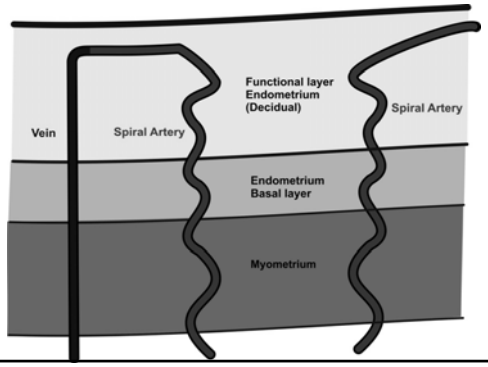
1. To understand the structure of the early gestation placenta
2. To understand the functions of extravillous trophoblasts especially in transforming the uterine spiral arteries
3. To understand the consequences of failed regulation of implantation/placentation
4. To be aware of trophoblast deportation and its potential roles in pregnancy

Six Week Placenta





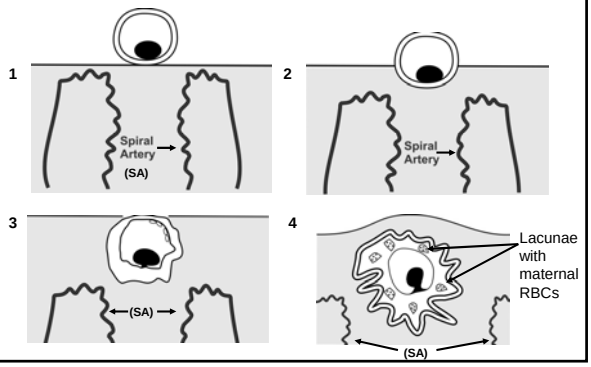
Endometrium/decidua



The Placenta: Implantation

- During the lacunar stage days 8-12 (post fertilisation)
  - At approximately day 7 post ovulation the blastocyst attaches to the uterine epithelium.
  - The embryo burrows into the decidua
  - Digests the decidua forming gaps in the maternal tissue called lacunae
  - the former trophectoderm of the blastocyst is now called trophoblast, protrusions of which (called trabeculae) extend into the lacunae

Implanting embryo

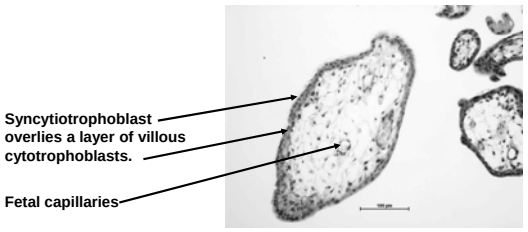


The villous period

- From about day 12 the villous period begins the real placenta!!!
  - Cytotrophoblasts proliferate and invade the trabeculae – these become primary villi
  - The lacunar system is now called the intervillous space
  - At about day 14, cells of the extra-embryonic mesenchyme invade the primary villi forming secondary villi

The Villous period

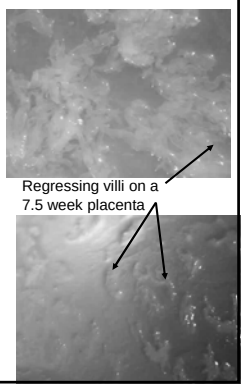
- About 18-20 days capillaries form in the villi tertiary villi
  - Form this point on, almost all villi are tertiary villi



Floating villi from an 11.2 wk placenta

Villi Regress to form the smooth chorion

- **The placenta forms essentially as a sphere surrounding the embryo but as gestation progresses**
  - **villi to the sides and luminal aspect regress to form the smooth chorion**
  - **Only villi basal to the implantation site remain as the definitive placenta**



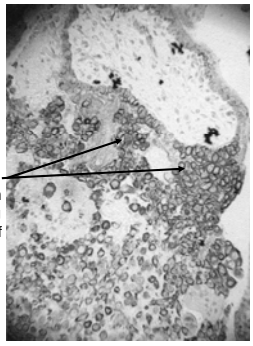
Regressing villi on a 7.5 week placenta

The Placenta

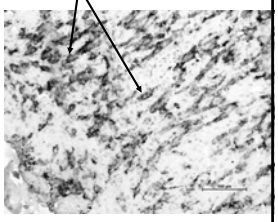
- Floating villi
  - During most of pregnancy the majority of villi do not have contact with the maternal tissues but are suspended in maternal blood in the intervillous space
    - these are called floating villi
  - Floating villi are covered by a continuous layer of syncytiotrophoblast and are responsible for the exchange and barrier functions of the placenta

Extravillous trophoblasts.

Anchoring villus of first trimester placenta stained for cytokeratin 7 showing columns of EVTs



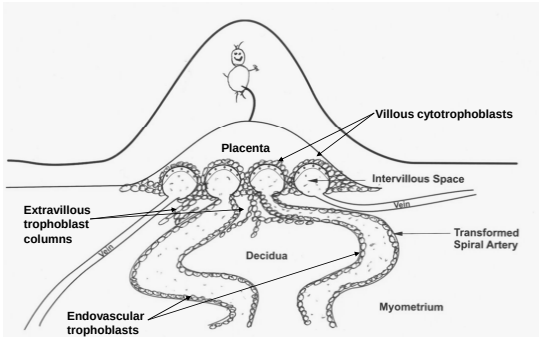
Implantation site 10 weeks of gestation. Stained for HLA-G demonstrating extravillous trophoblasts migrating in the decidua.



Physiological Changes of Pregnancy

- In fully transformed vessels trophoblasts migrate 1/3 into the myometrial segments of the spiral arteries.
  - The transformed vessels have an enlarged bore and can not respond to maternal vasoconstricting signals guaranteeing a good maternal blood supply for fetal growth.

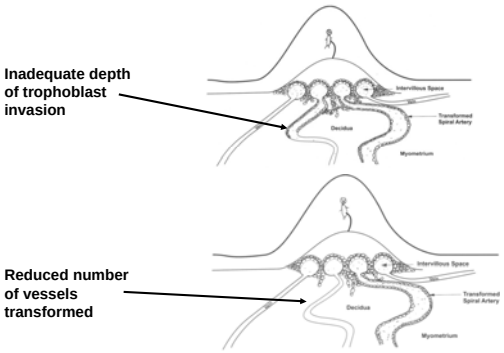
Normal “physiological changes”
(mid gestation)



Inadequate Physiological Changes

- Failure to transform the spiral arteries is associated with preeclampsia and intrauterine growth restriction (Brosens *et al*., 1967; Robertson *et al*., 1967; Khong *et al*., 1986).
- The failure may be either in the depth of invasion of the trophoblasts or in the number of vessels transformed.
  - Both lead to a reduced maternal blood supply to the placenta/fetus with consequences in later gestation

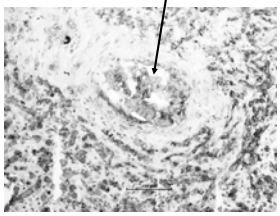
Inadequate “physiological changes”

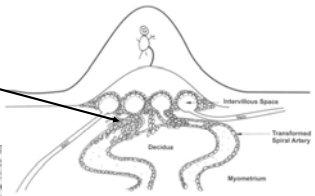


Trophoblast plugs

- There is good evidence that until 10-12 weeks of gestation there is limited flow of maternal blood into the intervillous space.
  - This is because endovascular trophoblasts form plugs in the lumens of the spiral arteries
  - Between 10-12 weeks these plugs dissipate allowing maternal blood flow.

Endovascular trophoblast plugs





Spiral artery with a trophoblast plug and interstitial trophoblasts. 9 weeks gestation, stained for HLA-G, counter stained with haematoxylin.

Trophoblast plugs

- Multiple lines of evidence support occlusion by trophoblasts plugs (see review by Jaffe *et al*., 1997)
  - Histological evidence of trophoblast plugs
  - Direct observation of the intervillous space shows clear liquid
  - Chorionic villous sampling is a blood-less procedure in the first trimester
  - Direct measurement of oxygen levels in the placenta and decidua prior to termination show much lower O_2 in the placenta
  - Doppler U/S shows little/no flow in the intervillous space before 11 weeks

Contrary evidence

- The “traditional” view is maternal blood flow is established at about day 29 of gestation (Carnegie collection)(Ramsey and Donner, 1980)
- 3-dimensional Power Doppler ultrasound suggests flow in the intervillous space which increases from 6 weeks of gestation (Mercé *et al.*, 2009)
- Some histological studies suggest the plugs are not in all vessels (Meekins *et al.*, 1997)
  - Of 232 decidual spiral arteries from 25 first trimester pregnancies,
    - 20% had plugs of trophoblast partially occluding the vessel and
    - 17% had plugs totally filling the vessel lumen
    - **63% were not plugged**

Trophoblast plugs

- These plugs mean that the oxygen levels at the placenta for most of the first trimester are low.
- “Physiological hypoxia”
- Placental oxygen levels
  - 8 weeks < 20mm Hg
  - 12 weeks > 50 mm Hg
  - The change in oxygenation is accompanied by increases in expression of placental antioxidant systems at the end of the first trimester (Jauniaux *et al.*, 2000)

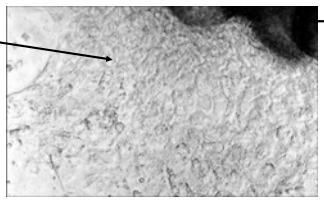
Oxygen regulates trophoblast behaviour

- Low oxygen (1.5% cf 8%) reduced the frequency and size of outgrowths from first trimester placental explants (James *et al.*, 2006a)

Outgrowth of EVTs from a 9 week placental explant

Grown on 1:10 diluted Matrigel

(very thin layer)



Explant

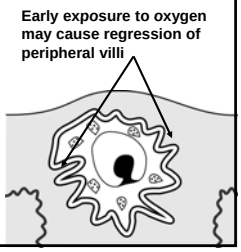
Oxygen regulates trophoblast behaviour

Conversely,

- low oxygen (2-3% cf 20%) increased EVT outgrowth (Genbacev *et al.*, 1997, Cannigia *et al.*, 2000)
- Although the exact action of low oxygen on first trimester trophoblast is not yet agreed there is general agreement that oxygen **critically** regulates trophoblast behaviour with the potential to reduce the maternal blood supply to the placenta.
- See James *et al*., 2006b for review of oxygen-responsive regulating factors

Progressive oxygenation starts at the periphery of the placenta

- Normally the placenta becomes oxygenated at the periphery first.
  - This may account for the formation of the smooth chorion via "physiological placental oxidative stress" (Burton, 2008)



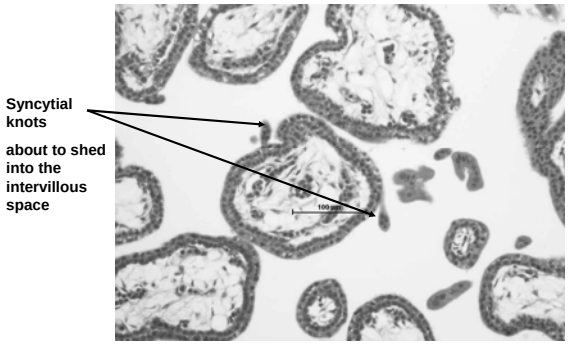
Premature blood flow and miscarriage

- Doppler ultrasound demonstrated an increased flow of maternal blood to the placenta in missed miscarriage at 7-9 or 10-11 weeks in missed miscarriages compared to normal controls (Jauniaux et al., 2003)
- There was no difference in blood flow at 12-13 weeks gestation between missed miscarriage and controls (Jauniaux et al., 2003)
- The premature maternal blood flow was distributed centrally and across the placenta whereas, in normal pregnancies, the maternal blood flow was more likely to be observed at the periphery of the placenta.

Trophoblast Deportation

- Aged regions of the syncytiotrophoblast form multinucleated clusters called **syncytial knots** which are shed into the intervillous space and deported into the maternal circulation
- Normally, syncytial knots are formed by an apoptosis-like process

Trophoblast deportation



Trophoblast Deportation

- A mechanism for inducing maternal immune tollerance?
- The fetus/placenta is an allograft
- Phagocytosis of apoptotic cells leads to tolerising/anti-inflammatory immune responses
- Is the shedding of apoptotic trophoblasts a mechanism for feeding apoptotic fetal cells to the maternal immune system?

Induction of Maternal Tolerance

- Exposing macrophages to apoptotic syncytial knots *in vitro* results in
  - Increased secretion of anti-inflammatory IL-10
  - Increased expression of the (immunosuppressing) enzyme indoleamine 2,3 dioxygenase (IDO)
  - Decreased secretion of proinflammatory IL-1β
    - Abumaree *et al*., 2006.

Trophoblast deportation

- In pathological conditions, especially preeclampsia
  - Excess syncytial knots are formed
  - The syncytial knots may be formed by a non-apoptotic process
  - Abnormal syncytial knots may contribute to the pathogenesis of preeclampsia

Conclusions

1. The early human placenta is most likely to develop in a state of physiologically low oxygen
2. Excess oxygenation in the first trimester may be associated with miscarriage
3. Oxygen appears to be crucial in regulating trophoblast invasion of the spiral arteries
4. Trophoblast deportation may be a mechanism for tolerising the maternal immune system to the fetal allograft.

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Genomics of Gametes and Embryo Development

Pre-Congress Symposium
"From Gamete to Heartbeat"
25th Annual Meeting of ESHRE
Amsterdam, The Netherlands
June 28th, 2009

Dr Gayle M. Jones, Ph.D.
Director of Research
Centre for Human Reproduction,
Genesis Athens Clinic, Athens, Greece

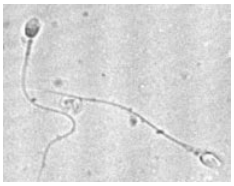
Learning Objectives

- Definition and current knowledge of the molecular network covered by the term 'omics'
- When is maternal message transcribed in the oocyte and when is this replaced by the embryonic genome
- How is maternal message stabilized in the oocyte
- Understanding that incorrect accumulation or temporal utilization of molecular message can result in pathologies such as cleavage failure or loss of viability
- Application of modern 'omics' technologies to evaluate the molecular health of an oocyte/embryo in order to define/predict viability

Omics

Gen**omics** = Study of the Genome
- Transcript**omics**
  - mRNA and microRNA
- Prote**omics**
  - Protein - >1mill. proteins
    - Metabol**omics**
      - small molecule biomarkers <1kDa
      - reflects metabolic status in health & 'disease' states
    - Secret**omics**
      - Proteins produced by the embryo and secreted into the culture medium

Spermatozoan



Male Infertility Genomics

- an example of a complex disease with a substantial genetic basis
- 10-15% severe male infertility
  - Chromosomal aberrations
    - Sex chromosome aneuploidies and translocations
    - Y chromosome microdeletions
    - Robertsonian or reciprocal autosomal translocations
  - Single gene mutations
    - *CFTR*
    - *AR*
    - *INSL3*
    - *LGR8*

Ferlin et al., 2007

Male Infertility Transcriptomics Expression Profiling

- Human testis global gene expression profiling
  - He *et al.,* 2006
  - Sha *et al.*, 2002
  - Cheng *et al.,* 2002; 2003
  - Xu *et al.,* 2003
  - Fang *et al.,* 2004
  - Zheng *et al.,* 2005
  - Bayne *et al.,* 2008
- Normal versus pathological testis
  - Fox *et al.,* 2003
  - Yang *et al.,* 2004
  - Lin *et al.,* 2006
- Normal versus pathological sperm
  - Wang *et al.,* 2004
  - Platts *et al.,* 2007

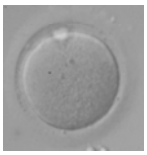
Spermatozoa Proteomics

- Complex array of receptors present on sperm surface
  - Tyrosine kinase/phosphatase receptors
    - Insulin receptor
    - Isoform 1 of prolactin receptor precursor
    - Isoform 1 of G-CSF receptor precursor
  - Seven-pass transmembrane receptors
  - Glutamate-gated ion channel family of neurotransmitter receptors
  - Progesterone receptor
  - Transient receptor family
  - Putative zona receptor glycoprotein

Aitken & Baker, 2007
Oliva et al., 2008

Oocyte

GV



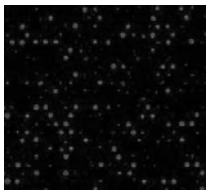
MII

Oocyte Genomics

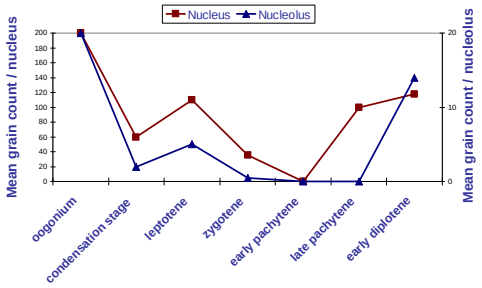
- Invasive – Metaphase spreads
- Non-invasive diagnosis following interpretation of results from biopsied polar body
  - FISH
  - CGH
- Aneuploidy levels high in oocytes and increases with maternal age
- Origin of aneuploidy is in meiosis I and to a lesser degree meiosis II
- Can only analyse maternal contribution

Martin, 2008
Wells et al., 2008

Oocyte Transcriptomics



Transcription During Prophase I of Meiosis Pre-natal Development



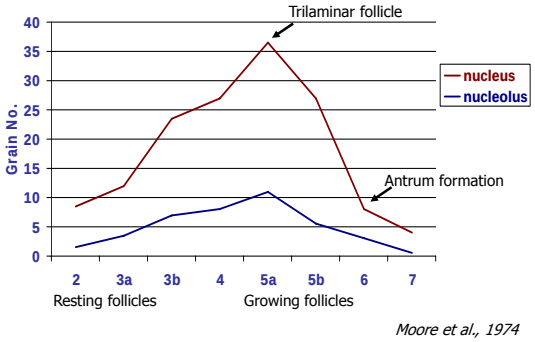
Hartung & Stahl, 1978

Folliculogenesis

Primordial Follicle to Ovulation > 6.5 months

Gougeon, 1996

Transcription During the Growth Phase



Moore et al., 1974

Transcription During Meiotic Maturation

- Once the maximal oocyte diameter is reached there is a sharp decline in transcription but RNA synthesis continues to within 2hours of GVBD
- Transcription virtually ceases once the germinal vesicle breaks down and meiosis is reinitiated
- 20% of total RNA is degraded during meiotic maturation
- Total degradation or deadenylation of one half of the accumulated Poly(A) RNA during meiotic maturation

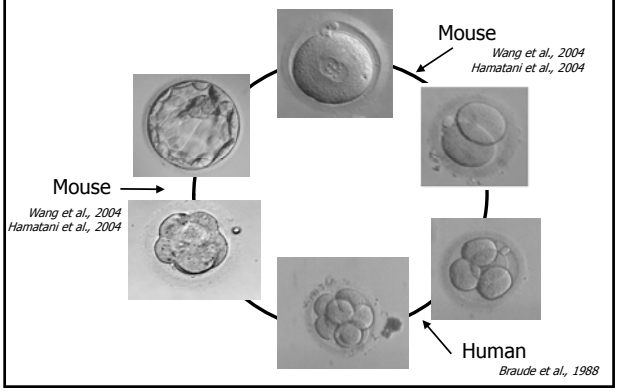
Transcripts Acquired During the Human Oocyte Growth Phase

- Completion of meiosis
- Entry into and completion of first 2-3 mitotic cell cycles
- Modification of chromatin structure and epigenetic properties
- Creation of an embryonic genome
- Initiation of transcription of the correct array of genes to begin the developmental program
- Basic homeostatic and metabolic processes

Oocyte Maternal mRNA's

- Stored in inactive, masked form and recruited for translation in a stage-specific manner during oocyte maturation and early embryogenesis
- Relative abundance differs between species and may account for difference in timing of zygotic genome activation between species
- Failure to accumulate and regulate the maternal message acquired during oogenesis may result in incorrect temporal utilization of message and is likely to cause delays or failure in progression through preimplantation development

Embryonic Genome Transcription



Maternal mRNA Expression & Regulation Rhesus Monkey Oocytes & Embryos

Zheng et al., 2005

- Oocytes from 3 sources were used
  - In vivo matured oocytes following FSH + hCG stimulation = **high** developmental competence
  - In vitro matured oocytes from large follicles primed with FSH = **moderate** developmental competence
  - In vitro matured oocytes from small follicles in the absence of stimulation = **low** developmental competence

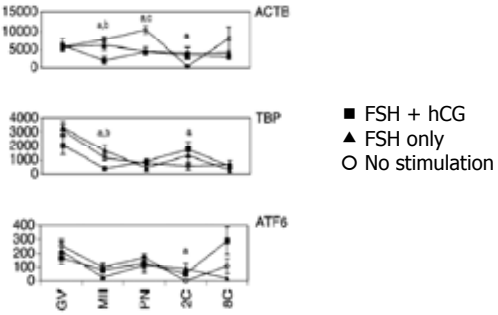
Maternal mRNA Expression & Regulation Rhesus Monkey Oocytes & Embryos

Zheng et al., 2005

- Non-stimulated oocytes showed aberrant accumulation of a number of maternal mRNAs with precocious loss by 2-cell stage
- FSH primed oocytes also showed aberrant gene expression relative to FSH + hCG stimulated oocyte but much less severe

Maternal mRNA Expression & Regulation Rhesus Monkey Oocytes & Embryos

Zheng et al., 2005



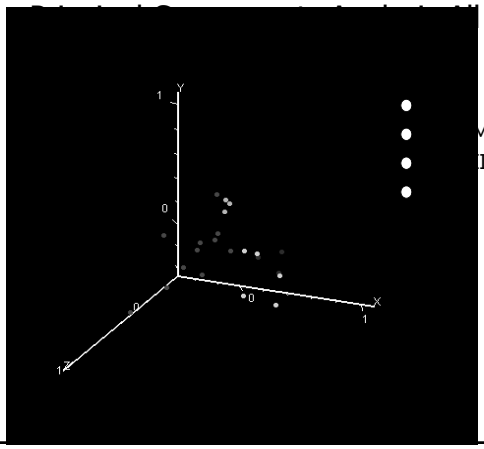
Human Oocyte Transcriptomics

- Neilson *et al.,* 2000
- Stanton *et al.,* 2002
- Bermudez *et al.,* 2004
- Dobson *et al.,* 2004
- Assou *et al.,* 2006
- Kocabas *et al.,* 2006
- Li *et al.,* 2006
- Zhang *et al.,* 2006
- Gasca *et al.,* 2006
- Wood *et al.,* 2006
- Jones *et al.,* 2008a
- Wells *& Patrizio, 2008*

In Vivo vs In Vitro Oocyte Maturation

Jones et al., 2008a

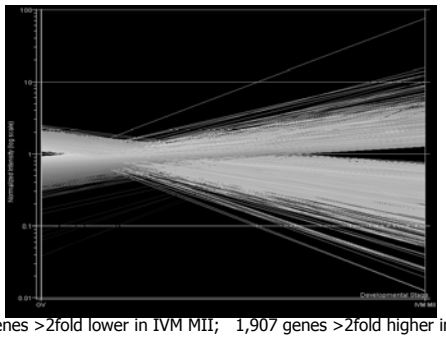
- Oocytes donated from women undergoing superovulation for assisted reproduction
- Each oocyte carefully assessed for developmental stage
  - GV, GVBD/MI, MII
- Some immature oocytes artificially matured in vitro
  - IVM MII
- All oocytes pooled in groups of 5
- Arrayed on Codelink Whole Human Genome Arrays – 54,840 Discovery Probes



Summary of Microarray Results

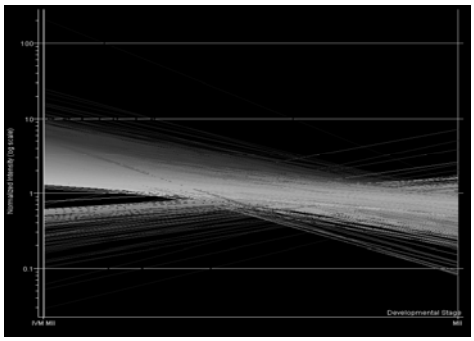
Developmental Stage	Number of independent replicates	Number of genes
GV	5	10,962
MI	3	12,329
MII (in vivo)	11	7,546
MII (in vitro)	3	9,479

GV vs IVM MII Gene Expression



802 genes >2fold lower in IVM MII; 1,907 genes >2fold higher in IVM MII

IVM MII vs MII Gene Expression



42 genes >2fold lower in IVM MII; 2,348 genes >2fold higher in IVM MII

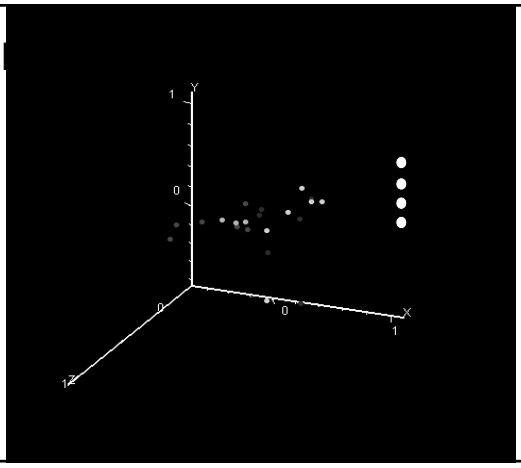
Rank	GO: Biological Process	No.transcripts
1	Nucleobase, nucleoside,nucleotide & nucleic acid metabolism **A.** Transcription **B.** DNA metabolism **C.** RNA metabolism	469 324 71 109
2	Cell cycle	135
3	Transport	254
4	Cell division	49
5	Cellular protein metabolism	382
6	Response to stress **A.** Response to DNA damage stimulus **B.** Response to oxidative stress	74 57 2
7	Cell death	78
8	Signal transduction	259
9	Cell proliferation	61
10	Generation of precursor metabolites and energy	22
11	Cell organization and biogenesis A. Cytoskeleton organization and biogenesis B. Chromosome organization and biogenesis	60 22 10
12	Biological process unkown	67
13	Reproduction	28
14	Cellular lipid metabolism	35
15	Development	72

Summary – In Vitro vs In Vivo Maturation Superovulated Cycles

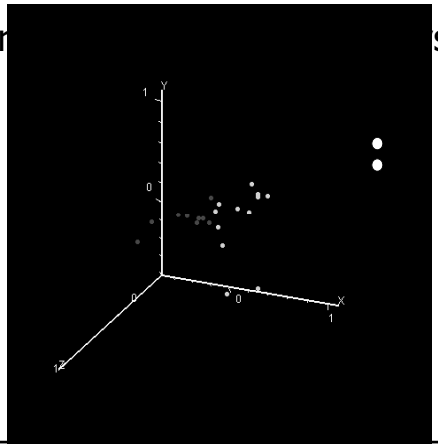
- In vitro matured MII oocytes have a large number of genes expressed at significantly higher levels than in vivo matured MII oocytes
- Many of these genes are involved in transcription, the cell cycle and its regulation, transport and cellular protein metabolism
- The over-abundance of genes observed for in vitro matured oocytes is likely due to dysregulation of transcription or post-transcriptional modification of transcribed genes

Human Oocyte Gene Expression Profiles & Maternal Age

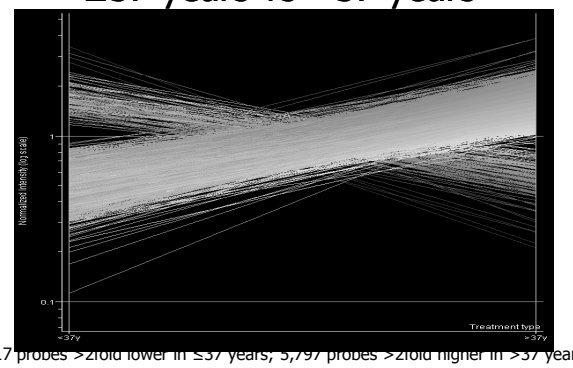
- All mature MII oocytes from gonadotrophin stimulated cycles
- 9 replicates (45 oocytes) from women aged between 28-37
  - 3 replicates 28-34 years
  - 6 replicates 35-37 years
- 12 replicates (60 oocytes) from women aged 38-43
  - 6 replicates 38-40 years
  - 6 replicates >40 years



Pr sis



≤37 years vs >37 years

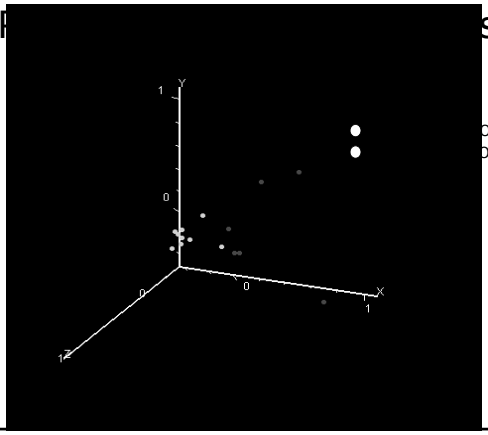


117 probes >2fold lower in ≤37 years; 3,797 probes >2fold higher in >37 years

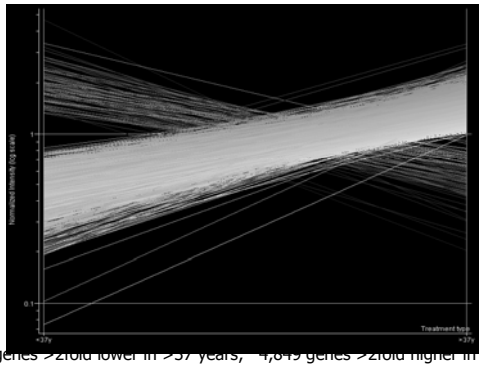
Gene Expression in Aneuploid Oocytes & Maternal Age

- All oocytes diagnosed as aneuploid by FISH following PB biopsy and staining for chromosomes X, 13, 15, 16, 18, 21, 22
- 5 oocytes per microarray sample
- Group 1 ≤37 years
  - 28-34y (n=1)
  - 35-37y (n=5)
- Group 2 >37 years
  - 38-40y (n=5)
  - > 40y (n=5)

[illegible]s



≤37 years vs >37 years Aneuploid Oocytes



81 genes >2fold lower in >37 years, [illegible] genes >2fold higher in >37 years

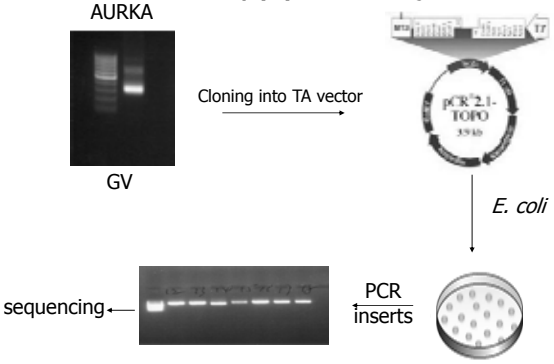
Summary – Maternal Ageing

- Oocytes from **older women** that are physiologically less developmentally competent are associated with **higher** expression of a significant number of genes compared to the oocytes of **young women**
- Over-representation of genes involved in mitochondrial function and energy production and genes involved in translation and RNA processing
- Aneuploidy is usually implicated as the major factor responsible for the reduced developmental competence of oocytes however there are other contributors as large gene expression differences were detected in aneuploid oocytes from young women compared to older women

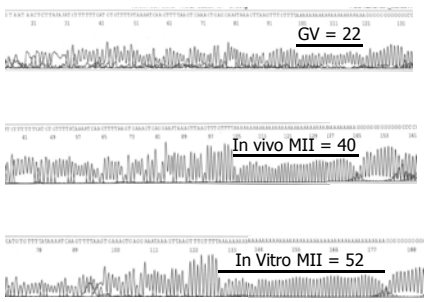
Conclusion – Oocyte Microarray Studies

- We propose that the developmental incompetence associated with oocytes matured in vitro or oocytes from women of advanced maternal age is a consequence of:
  - precocious polyadenylation of transcripts normally required later in development
  - pathologic new transcription
  - failure of the normal deadenylation and/or degradation processes that occur during maturation in vivo

Cloning & Sequencing to Determine Exact Poly(A) Tail Lengths



Differential Poly(A) Tail Lengths for *MAD2L1*



Observed gene trends during oocyte maturation: In Vitro MII versus GV

Genes	Trend Microarray	Trend Poly(A)	Interpretation
BUB1B; CENPE	↑	↑	de novo transcription & polyadenylation
TBPL1; PAIP1	↑	=	de novo transcription
MAD2L1, CPEB2	=	↑	polyadenylation
TCEB1; AURKA; PUM1	=	=	no post-transcriptional modification
CCNK; AURKB	=	↓	deadenylation

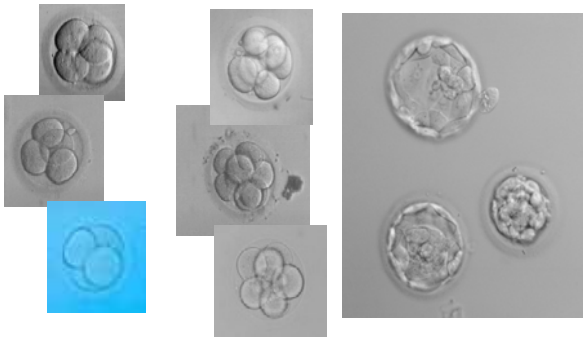
Observed gene trends during oocyte maturation: In Vitro MII versus In Vivo MII

Genes	Trend Microarray	Trend Poly(A)	Interpretation
TBPL1; BUB1B; CENPE; MAD2L1; AURKA	↑	↑	de novo transcription & polyadenylation
PAIP1	↑	↓	de novo transcription & no microarray bias to longer Poly (A) tail lengths
CCNK; AURKB; PUM1	=	=	not different

Summary

- Differences in microarray expression levels are not reflected in differences in Poly(A) tail length indicating no tail length bias in dT amplification during RNA preparation for microarrays
- Upregulation of transcripts from GV to In Vitro MII for selected genes is indicative of aberrant de novo transcription during maturation in vitro
- In addition increases in Poly(A) tail length from GV to In Vitro MII is indicative of the post-transcriptional modification of polyadenylation which prepares transcripts for translation
- Some transcripts are deadenylated during maturation in vitro similar to that which normally occurs during maturation in vivo
- Differences in expression level and Poly(A) tail length depending on maturation conditions indicates a pathology in de novo transcription and post-transcriptional modification when oocytes are matured in vitro which may explain the observed differences in developmental competence

Embryo Genomics



Embryo Genomics

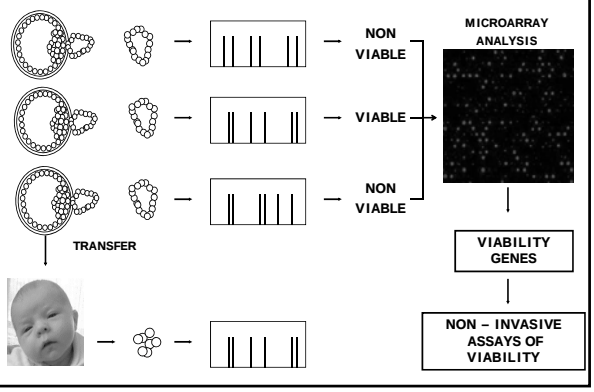
- Diagnosis following interpretation of results from biopsied single cell from D3 embryos or TE from blastocyst stage embryos
  - Aneuploidy
    - FISH
    - CGH
    - PCR
  - Single gene disorders
    - PCR
- Advantage is that maternal and paternal contribution can be evaluated
- Aneuploidy levels high in embryos and increases with maternal age
- Accurate interpretation complicated by the high incidence of mosaicism

Kuliev & Verlinsky, 2008
Wells et al., 2008

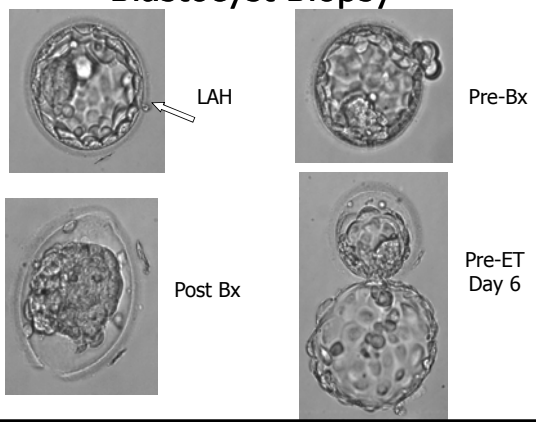
Embryo Transcriptomics

- Adjaye *et al.,* 1997; 1998; 1999
- Dobson *et al.,* 2004
- Adjaye *et al.,* 2005
- Li *et al.,* 2006
- Jones *et al.,* 2008b

Strategy to Identify Viable Embryos



Blastocyst Biopsy



Blastocyst Biopsy Outcomes

Type of Implantation	No. Patients	No. Biopsied Blastocysts Implanted	No. Biopsied Blastocysts that Failed to Implant	No. Babies Born
Group 1 All Implanted	7	18	0	11
Group 2 Some Implanted	18	34	27	26
Group 3 None Implanted	23	0	70	NA

Samples for Microarray

Viable TE sample

- Biopsied TE from 8 blastocysts known to have implanted (Linear x 1; Exponential x 1)

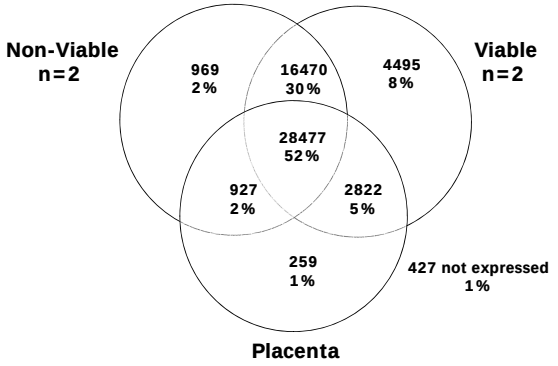
Non Viable TE sample

- Biopsied TE from 8 blastocysts that failed to implant in young women (<35) with male factor or tubal disease as the aetiology of infertility (Linear x 1; Exponential x 1)

Placenta

- Results kindly provided by Amersham Biosciences/ GE Healthcare

Raw Data – Present in any sample Codelink Discovery Genes 54,840 probes

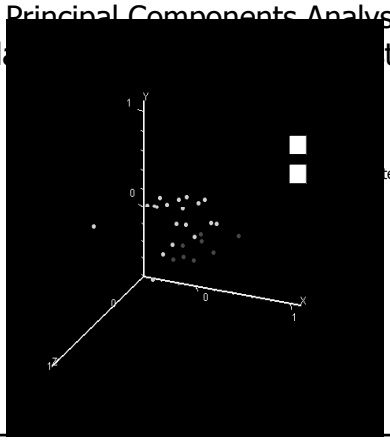


Major Themes in 7317 Genes Unique to Viable TE

	Subcategory	P value
Cell adhesion	Homophilic cell adhesion	1.2×10^{-4}
	Calcium-independent cell adhesion	1.3×10^{-2}
	Neuron adhesion	3.5×10^{-2}
	Calcium-dependent cell adhesion	4.1×10^{-2}
Cell communication	Cell–Cell signalling	1.7×10^{-4}
	Synaptic transmission	1.4×10^{-2}
	Nerve ensheathment	1. x 10^{-2}
	Signal transduction	1.4×10^{-2}
	Adenylate cyclase activation	1.9×10^{-2}
	G-protein signalling	1.9×10^{-2}
	Transmembrane receptor protein tyrosine kinase activation	3.0×10^{-2}
		3.3×10^{-2}
	Acetylcholine receptor signalling	4.1×10^{-2}
	Glutamate signalling pathway	
	Cell surface receptor linked signal transduction	
	Activation of MAPK activity	
Cellular metabolic process	Positive regulation of interleukin-13 biosynthesis	2.4×10^{-3}
	Positive regulation of interleukin-6 biosynthesis	8.5×10^{-3}
	Alanyl-tRNA aminoacylation	1.9×10^{-2}
	Cyclic nucleotide metabolism	3.5×10^{-2}
Response to stimuli	Defense response to bacteria	5.6×10^{-3}

Principal Components Analysis

Impl tocysts



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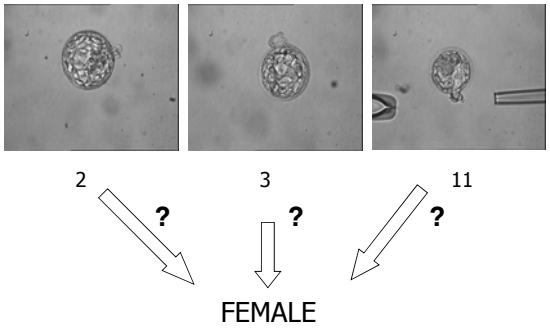
Genomics Applications to Improve Prediction of Viability

Group 2 – Some Implanted

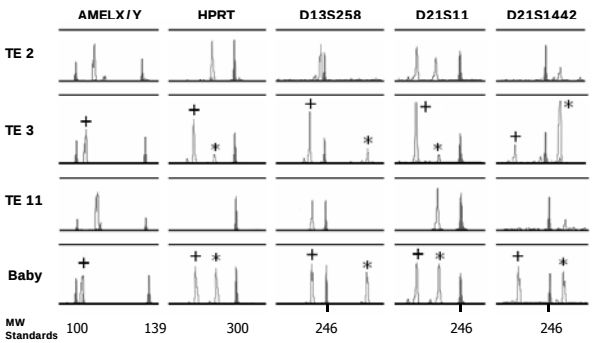
Genomic DNA Fingerprinting

- Half the lysate was used for this purpose leaving half the lysate to generate cDNA libraries for gene expression
- Whole genome amplification followed by analysis of microsatellite markers
- Sibling embryo fingerprint compared to fingerprint from baby

Blastocyst Research Protocol (BRP) 33



BRP 33 Genomic Fingerprint



Summary

- It is possible to generate informative transcriptomes of blastocysts using TE biopsy samples without compromising blastocyst viability following transfer
  - Transcriptomes of viable embryos reveal genes involved in cell adhesion and communication important to the early events of implantation
  - Potential to prospectively screen for a select number of viability associated genes in blastocysts prior to transfer to identify the single most viable blastocyst within the cohort
- DNA fingerprints can be generated from biopsied TE cells to positively identify the origin of any resultant offspring
  - Useful technology for application to many research applications in ART
  - Fingerprint diagnosis of viability can be used to improve existing non-invasive pregnancy predictive markers such as morphology, metabolic markers, spectroscopic analysis of spent culture medium

Oocyte/Embryo Metabolomics

O_2 consumption

- *Van Blerkom et al., 1997*

Carbohydrate consumption

- Hardy *et al.,* 1989
- Gott *et al.,* 1990
- Jones *et al.,* 2001
- Gardner *et al.,* 2001

Amino Acid Metabolism

- Houghton *et al.,* 2002
- Brison *et al.,* 2004
- Sturmey *et al.,* 2008

HLA-G

- Noci *et al.,* 2005
- Sher *et al.,* 2005
- Warner *et al., 2008*

Oocyte/Embryo Metabolomics/Secretomics

Chip Technology
- Dominguez *et al.,* 2008

Mass spectrometry
- Katz-Jaffe *et al.,* 2006a & b

Proton Nuclear Magnetic Resonance
- Seli *et al.,* 2008

Raman /FT-IR & NIR spectroscopy
- Seli *et al.,* 2007
- Scott *et al.,* 2008
- Nagy *et al.,* 2008
- Vergouw *et al.,* 2008
- Botros et al., 2008

Summary

- Embryos with a high implantation potential alter their culture environment differently to embryos with no implantation potential
- These differences can be detected by metabolomic profiling using sophisticated biospectroscopy techniques
- Relies on complex mathematical algorithms
- Advantage is that it is non-invasive
- Preliminary evidence suggests that the information is independent of morphology and therefore an adjunct to existing embryo selection criteria

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Cristina Magli
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Genomics of Endometrial Implantation

25th Annual Meeting of ESHRE
Amsterdam – 2008
Pre-congress course Embryology and Early Pregnancy

Course title: From Gamete to Heartbeat: the Missing Link

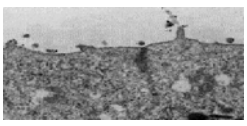
Dr. José A. Horcajadas, PhD
Molecular Biology Group Leader
Fundación IVI (FIVI)-Instituto Universitario IVI (IUIVI)
and University of Valencia, Spain
Chief Scientific Officer, iGenomix, Valencia, Spain

LEARNING OBJECTIVES

- (1) To define endometrial receptivity.
- (2) To describe the different gene expression profiles between the window of implantation (WOI) in natural and non natural conditions.
- (3) To understand the application of the new technologies for the study of endometrial receptivity.

Receptive endometrium features

» Morphological markers
» Biochemical markers
» Gene expression pattern



GENE EXPRESSION PROFILING OF HUMAN ENDOMETRIAL RECEPTIVITY ON DAYS LH+2 VS LH+7 BY MICROARRAYS

EXPERIMENTAL DESIGN

FIVE WOMEN

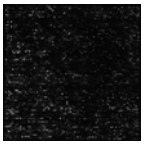
TWO ENDOMETRIAL BYOPSIES

LH+2

LH+7

RNA ISOLATION

AFFIMETRIX HG-U95A 12,686 genes



MICROARRAY

Caucasian
Fertile women with normal cycles
23–39 years
body mass index:19-25 kg/m2

Riesewijk et al. Molec Human Reprod 9, 253-64, 2003

Genes regulated during human endometrial receptivity

	Up at LH+7	Down at LH+7
Strong (>10)	22	5
Medium (5-10)	47	12
Weak (3-5)	84	41
	153	58

Results (>3.0 fc in 4 out of 5)

EFFECT OF AN INTRAUTERINE DEVICE (IUD) ON THE GENE EXPRESSION PROFILE OF THE ENDOMETRIUM

INTRAUTERINE DEVICE HISTORY

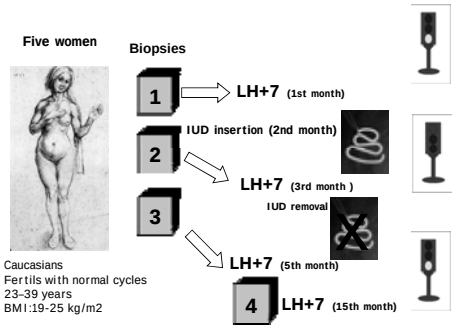
-One of the most effective interceptive methods with a typical Pearl index around 0.5 (pregnancies per 100 women per year). Mosher WD 1990 National Center for Health Statistics.

-1964. First cytological and histological studies. Ishihama et al. *Yok. Med Bull 15:201-5.*

- Genomic Studies:
  - 1998 Expression of c-JUN, oestrogen receptors, progesterone receptors and Ki-67. Salmi et al. *Mol Hum Reprod. 4:1110-5*
  - 2000 IGF and IGFB proteins. Rutanen. *Hum Reprod. 3:173-81*
  - 2003 Androgen receptor and 17beta-hydroxysteroil dehydrogenase type 2 Burton et al. *Hum Reprod. 18:2610-7.*

  - No Wide Genomic Analysis

EXPERIMENTAL DESIGN



Horcajadas et al., 2006 J Clinical Endocrinol Metabolism 91:3199-3207

EFFECTS OF AN INERT IUD ON THE GENE EXPRESSION PROFILE OF THE ENDOMETRIUM AT THE TIME OF IMPLANTATION

Comparison results (>2.0 fc in 4 out of 5)

		LH7 / LH2 FC>2.0	
		Up 894	down 505
IUD/PreIUD	Up 78	6	12
IUD/PreIUD	Down 69	33	1

35%

Horcajadas et al., 2006 J Clinical Endocrinol Metabolism 91:3199-3207

GRAPHS INDICATING MEDIAN RATIO ACROSS EACH EXPERIMENTAL GROUP

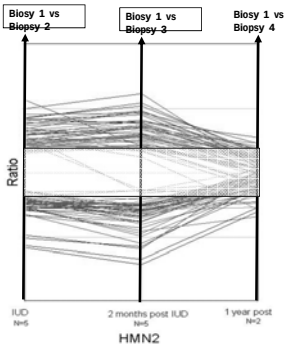
-Cohen J 1981 Fecundity following IUD use. Contracept Fertil Sex 9:465-468.

-Belhadj et al 1986 Recovery of fertility after use of the levonorgestrel 20 mcg/d or Copper T 380 Ag intrauterine device. Contraception 34:261-267.

- Gupta et al 1989 Return of fertility in various types of IUD users. Int J Fertil 34:123-125.

- Delbarge et al 2002 Return to fertility in nulliparous and parous women after removal of the GyneFix intrauterine contraceptive system. Eur J Contracept Reprod Health 7:24-30.

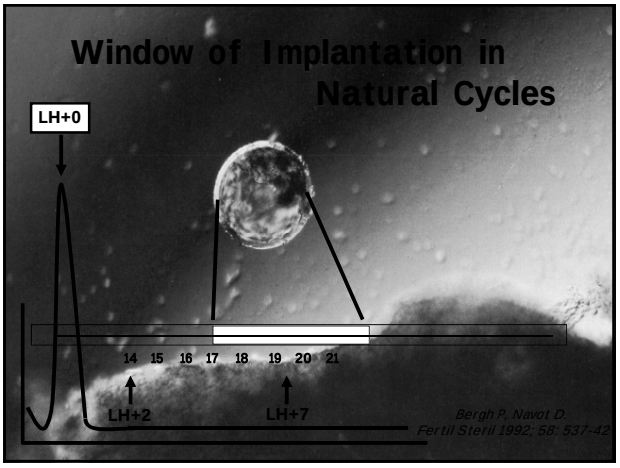
- Hata et al 1969 The effect of long-term use of intrauterine devices Int J Fert 14:241-249.

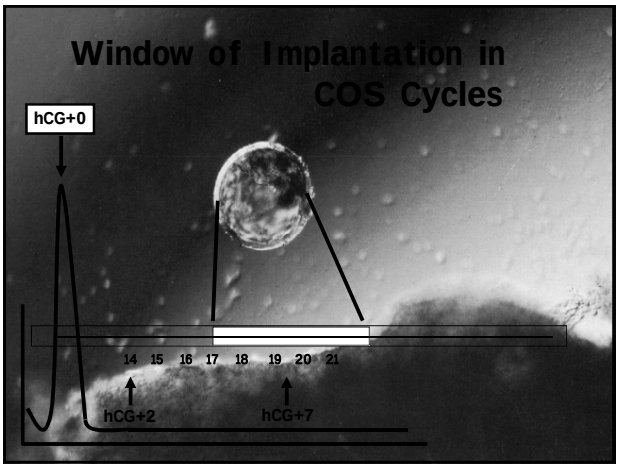


Horcajadas et al., 2006 J Clinical Endocrinol Metabolism 91:3199-3207

THE IMPACT OF COS IN ENDOMETRIAL RECEPTIVITY

- In high responders to gonadotrophins, supraphysiological levels of E2 on the day of hCG administration, are deleterious to embryonic implantation (Simón et al., 1995, 1998, 2003; Pellicer et al., 1996)
- Low doses of E2 maintain the uterus in a receptive state, high doses cause it to become refractory in mice (Ma et al., 2003, PNAS).
- Uterine receptivity is diminished during COS used for IVF compared to natural cycles (Paulson et al., 2000). The endometrium is histologically advanced.





STUDIES OF THE GENE EXPRESSION PROFILE OF THE ENDOMETRIUM UNDER COS

- **Gene expression profile of the endometrium during the WOI in women under treatment with agonists and different doses of antagonist and in comparison to natural cycle**

0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(11):5742–5752

doi: 10.1210/jc.2004-0605

Gene Expression Profiles and Structural/Functional Features of the Peri-Implantation Endometrium in Natural and Gonadotropin-Stimulated Cycles

SEBASTIAN MIRKIN, GEORGE NIKAS, JENG-GWANG HSIU, JOSÉ DÍAZ, AND SERGIO OEHNINGER

STUDIES OF THE GENE EXPRESSION PROFILE OF THE ENDOMETRIUM UNDER COS

- **Gene expression profile of the endometrium during the WOI in women under treatment with agonists in comparison to natural cycle**

Molecular Human Reproduction Vol.11, No.3 pp.195–205, 2005
Advance Access publication February 4, 2005
doi:10.1093/molehr/gah150

Effect of controlled ovarian hyperstimulation in IVF on endometrial gene expression profiles

José Antonio Horcajadas[1], Anne Riesewijk[2], Jan Polman[2], Roselinde van Os[2], Antonio Pellicer[1], Sietse Mosselman[2] and Carlos Simón[1,3]

STUDIES OF THE GENE EXPRESSION PROFILE OF THE ENDOMETRIUM UNDER COS

- **Gene expression profile of the endometrium during the WOI in women under treatment with agonists and different doses of antagonist and in comparison to natural cycle**

Human Reproduction Vol.20, No.12 pp. 3319–3327, 2005 doi:10.1093/humrep/dei243
Advance Access publication August 5, 2005.

Similar endometrial development in oocyte donors treated with either high- or standard-dose GnRH antagonist compared to treatment with a GnRH agonist or in natural cycles

C.Simon[1,2,6], J.Oberyé[3], J.Bellver[2], C.Vidal[2], E.Bosch[2], J.A.Horcajadas[1], C.Murphy[5], S.Adams[5], A.Riesewijk[4], B.Mannaerts[3] and A.Pellicer[1,2]

COMPARISON OF THE DIFFERENT STIMULATION PROTOCOLS

			Window of implantation genes	
	Regimen/direction of regulation†	N° of genes	Typically upregulated (n = 894)	Typically downregulated (n = 504)
	Leuprolide (agonist)			
	Up	281	9	115
	Down	277	227	0
	Ganirelix 0.25 mg/day (antagonist)			
	Up	22	0	4
	Down	69	46	0
	Ganirelix 2 mg/day (antagonist)			
	Up	88	0	7
	Down	24	15	1
	Buserelin long protocol (agonist)			
	Up	22	3	4
	Down	100	76	2

GENE EXPRESSION PROFILING OF WINDOW OF IMPLANTATION IN NATURAL AND STIMULATED CYCLES

EXPERIMENTAL DESIGN

50 WOMEN

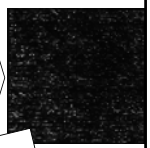
Caucasian
Fertile women with normal cycles
23-39 years
body mass index:19-25 kg/m2

FIVE ENDOMETRIAL BYOPSIES AT EACH TIME POINT

LH Day 1 2 3 4 5 6 7 8 9

hCG Day 1 2 3 4 5 6 7 8 9

AFFIMETRIX HG-133A >22,000 genes



GeneSpring 7.0
GEPAS

MICROARRAY

HISTOLOGICAL CONTROL: BLIND DATING BY NOYES CRITERIA

PCA OF THE ENDOMETRIAL BIOPSIES FROM LH+1 TO LH+9 AND hCG+1 TO hCG+9

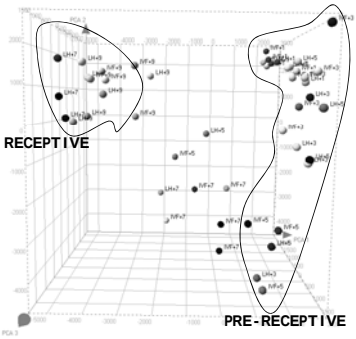
- Principal Component Analysis (PCA) integrates the gene expression data of thousand of genes randomly selected to establish relationships between samples.

- This analysis allows to distribute the endometrial samples in a three dimensional space according to their gene expression profile.

- Those samples with similar gene expression patterns cluster together in this type of analysis.

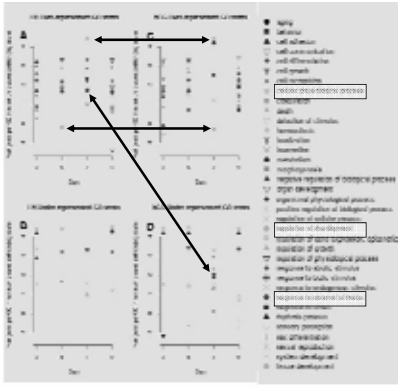
PRINCIPAL COMPONENT ANALYSIS (PCA)

Natural/LH vs IVF across the WOI

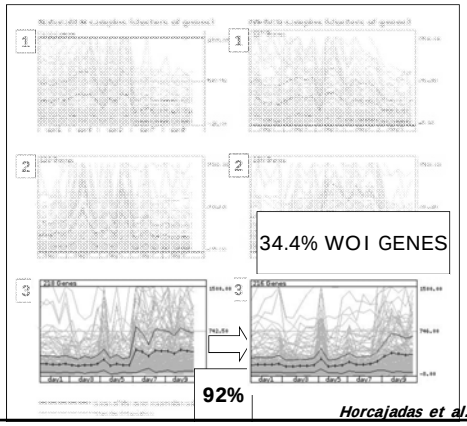


Horcajadas et al., 2008

BIOLOGICAL PROCESSES FROM DAY LH+1 TO LH+9 COMPARED TO COS CYCLES



GENE CLUSTERING IN NATURAL AND COS CYCLES

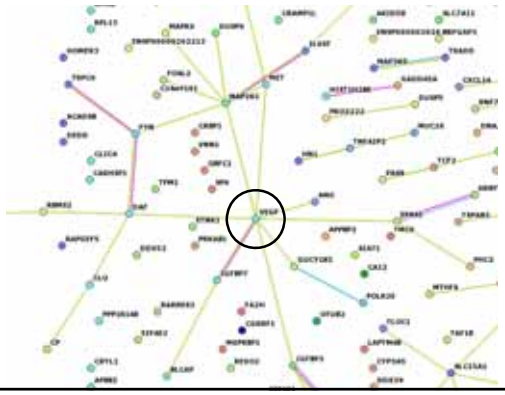


Horcajadas et al., 2008

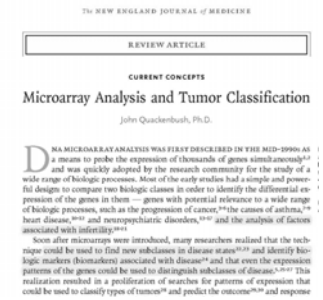
BIOLOGICAL PROCESSES OF THE 203 DELAYED GENES

BIOLOGICAL TERM	Count	%	PValue
taxis	5	2,67%	0,0437137
cell motility	7	3,74%	0,04103273
blood vessel development	4	2,14%	0,03996548
negative regulation of physiological process	12	6,42%	0,09769689
transport	41	21,93%	0,02625948
positive regulation of apoptosis	6	3,21%	0,02985379
locomotory behavior	5	2,67%	0,04919509
phosphate metabolism	16	8,56%	0,07933242
negative regulation of biological process	15	8,02%	0,03312411
locomotion	7	3,74%	0,04103273
cell death	11	5,88%	0,09003195
localization of cell	7	3,74%	0,04103273
localization	48	25,67%	0,00371126
fructose 6-phosphate metabolism	2	1,07%	0,04132375
organic acid metabolism	10	5,35%	0,08394103
carboxylic acid metabolism	10	5,35%	0,08237153
chemotaxis	5	2,67%	0,0437137
behavior	6	3,21%	0,05876496
positive regulation of programmed cell death	6	3,21%	0,03071029
negative regulation of cellular process	13	6,95%	0,07576561
phosphorus metabolism	16	8,56%	0,07933242
negative regulation of cellular physiological	12	6,42%	0,08059537
cellular physiological process	126	67,38%	0,05501213
development	27	14,44%	0,0787654
angiogenesis	4	2,14%	0,03590782
vasculature development	4	2,14%	0,03996548
response to stress	19	10,16%	0,0442493
negative regulation of cell proliferation	5	2,67%	0,080454
death	11	5,88%	0,09312635
response to chemical stimulus	9	4,81%	0,05742527
cell proliferation	13	6,95%	0,01235572
establishment of localization	47	25,13%	0,00571022

BIOLOGICAL CONECTIONS AMONG THE 203 DELAYED GENES



New England Journal of Medicine 2006;354:2463-72.



The NEW ENGLAND JOURNAL of MEDICINE

REVIEW ARTICLE

CURRENT CONCEPTS

Microarray Analysis and Tumor Classification

John Quackenbush, Ph.D.

DNA MICROARRAY ANALYSIS WAS FIRST DESCRIBED IN THE MID-1990S AS a means to probe the expression of thousands of genes simultaneously[1,2] and was quickly adopted by the research community for the study of a wide range of biologic processes. Most of the early studies had a simple and powerful design to compare two biologic classes in order to identify the differential expression of the genes in them — genes with potential relevance to a wide range of biologic processes, such as the progression of cancer,[3-6] the causes of asthma,[7-9] heart disease,[10-12] and neuropsychiatric disorders,[13-17] and the analysis of factors associated with infertility.[18-21]

Soon after microarrays were introduced, many researchers realized that the technique could be used to find new subclasses in disease states[22,23] and identify biologic markers (biomarkers) associated with disease[24] and that even the expression patterns of the genes could be used to distinguish subclasses of disease.[5,25-27] This realization resulted in a proliferation of searches for patterns of expression that could be used to classify types of tumors[28] and predict the outcome[29,30] and response

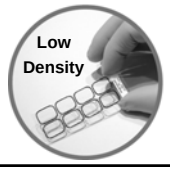
INTEGRATING THE AVAILABLE INFORMATION

INTO A MOLECULAR TOOL FOR ENDOMETRIAL EVALUATION

NATIONAL INSTITUTE OF BIOINFORMATIC

INB

GENE EXPRESSION DATA



Low Density

BIOINFORMATICS FOR FUNTIONAL GENOMICS

Endometrial Evaluation Predictor

EEP

Endometrial Receptivity Array (ERA)

Probe group: 206 + 28 + 76: 310 genes – 17 genes =

293 genes / 738 probes

Número total de spots:15.744 spots

Control Agilent: 536 spots

Spots útiles: 15.208 spots

8 replicates = 738 x 8 = 5.904 spots

Empty sponts = 9.304

8x15K

Endometrial Receptivity Array (ERA)

Microarray Data Analysis

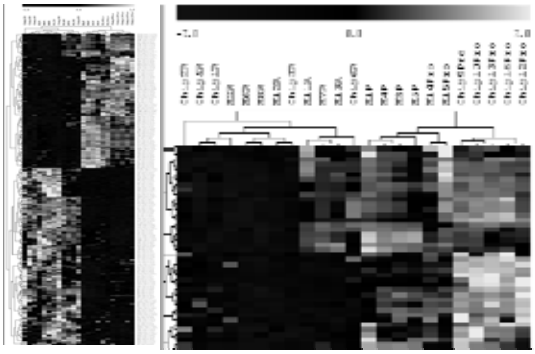
Prophet

Class prediction with Prophet

Ignacio Medina
imedina@cipf.es

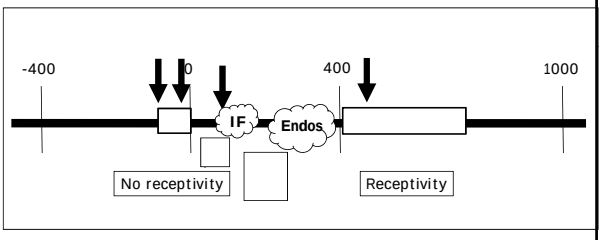
Bioinformatics Department
Centro de Investigacion Principe Felipe
(Valencia)

Endometrial Receptivity Array (ERA)



Endometrial Receptivity Array (ERA)

Scoring



CONCLUSIONS (I)

-There is a high number of genes, with a define pattern, involved in endometrial receptivity (WOI genes)

-There is a high number of WOI genes that are aberrantly expressed in stimulated cycles at the time of implantation (LH+7 in natural cycles and hCG+7 in COS cycles) and in contraceptives conditions

- Microarray technology is a good tool for analyzing gene expression profile of the endometrium at the time of implantation to compare optimal versus non optimal conditions (infertility or subfertility)

CONCLUSIONS (II)

-These data are useful for both, to improve the stimulated cycles in IVF and also to increase our knowledge in the physiology of the implantation process

-Endometrial Receptivity Array (ERA) based on microarray technology can be useful for endometrial evaluation

Soluble HLA-G and embryo implantation

Philippe Le Bouteiller, PhD
INSERM U563, Toulouse, France

Figures and Tables presented here are reproduced from Tabiasco *et al.* (2009) RBMOnline Vol 18 (3) 374-381, with permission.

LEARNING OBJECTIVES:

Assessing the implantation potential of the embryo(s) to be transferred is crucial

- To increase the success rates of IVF-ICSI cycles while reducing the risk of multiple pregnancies.
- To promote the “single embryo transfer” (SET) policy
- To decrease the maternal and foetal morbidity and mortality associated with assisted reproductive technologies (ART)
- The analysis of the morphology of the pre-implantation embryo, although important, is generally not sufficiently informative .

Non-invasive Biomarker of implantation

Soluble HLA-G in Day-2/3 embryo culture supernatants?

Could soluble HLA-G produced by some human IVF/ICSI-derived embryo be a predictive marker of embryo implantation potential?

- A considerable interest has been aroused and sevral ART groups have performed similar kinds of studies: Fuzzi *et al*., 2002, Criscuoli *et al*., 2005; Desai *et al*., 2006; Fisch *et al*., 2007; Noci *et al*., 2005;Rebmann *et al*., 2007; Sageshima *et al*., 2007; Shaikly *et al*., 2008; Sher *et al*., 2004; Sher *et al*., 2005 a; Sher *et al*., 2005 b;Yie *et al*., 2005; Vercammen *et al.,* 2008
- Not all investigators agree with their conclusions
- Technical differences including IVF/ICSI culture conditions, duration of embryo culture, number of embryos transferred, ELISA methods used and their sensitivity to detect sHLA-G in embryo culture supernatants (ES) are the most likely explanations for such discrepancies

To understand and unravel these differences requires collaborations

- ❑ We conducted collaborations to collect samples and set-up standards and technique for validation
- ❑ A total of 1405 ES were collected from 355 cycles including 87 IVF and 268 ICSI from 3 ART Centres: Poissy, Toulouse, Liège

Patient characteristics and assisted reproductive technique data

Number of women/cycles	*POISSY CENTRE* *(n= 77)*	*TOULOUSE CENTRE* *(n = 196)*	*LIEGE CENTRE* *(n = 82)*
Age (years)	32.6	32.3	35.8
Range	[24-41]	[21-39]	[21-45]
No. of attempts	1.8 +/- 0.12	2.0 +/- 0.15	1.7 +/- 0.11
ART types			
IVF	0	74	13
ICSI	77	122	69
Mean No. of oocytes retrieved	9.2	8.8	10.6
Mean No. of embryos obtained	4.9	3.8	6.5
Mean No. of embryos transferred			
Fresh transfer	2.1	2.1	1.7
Freeze-Thaw transfer			1.5
Clinical pregnancy rate	38.5%	30.6%	31.7%[a]
Multiple pregnancies	32%	23.3%	15%
Implantation rate[b]			
Fresh transfer	23%	18%	27.8%
Freeze-Thaw transfer	-	-	22.7%

Characteristics of the collected samples

	POISSY CENTRE	*TOULOUSE CENTRE*	*LIEGE CENTRE*
Number of women/cycles	77	196	82
ART procedure	ICSI	IVF or ICSI	IVF or ICSI
Analyzed samples	360 embryo supernatants 197 corresponding follicular fluids	450 embryo supernatants	595 embryo supernatants and 40 unfertilized oocyte supernatants
No. of embryos transferred:			
Fresh	146	404	132
Freeze-Thaw			44
Day of transfer or freezing	Day-2	Day-2	Day-3

Media used

	POISSY CENTRE	*TOULOUSE CENTRE*	*LIEGE CENTRE* **Medium 1** Irvine Scientific	**Medium 2** COOK
Medium for oocytes (washing, rem oval of cumulus, ICSI …)	Ferticult Hepes (JCD, France)	G fert (Vitro life)	mHTF modified Human Tubal Fluid	OWB Oocyte Wash Buffer
Medium for sperm washing	Fertipro (JCD, France)	Spermafix (Eurobio, France)	SWM Sperm Washing Medium	SB Sperm Buffer
Fertilization medium	ISM1 (Medicult, France)	G (Vitrolife)	HTF Human Tubal Fluid	FM Fertilization Medium
Cleavage medium	ISM1 (Medicult, France)	G (Vitrolife)	ECM Early cleavage Medium	CM Cleavage Medium

Tabiasco *et al.*, 2009

Would soluble HLA-G detection be informative of the outcome ?

- Individual embryo supernatants were stored at --80°C at the time of embryo transfer, freezing or destruction
- Analysis of sHLA-G concentrations in Toulouse
  - Coating Antibody: anti-HLA-G MEMG/9
  - Capture Antibody anti-MHC I: W6/32

- Chemioluminescent Elisa Assay to improve the sensibility of detection in 40 µl samples (automated light luminescence counter for microplate application)

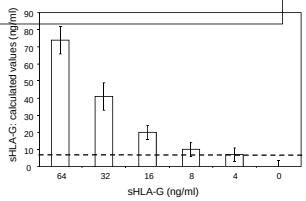
Soluble HLA-G Detection

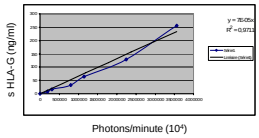
❖ **Chemioluminescent Elisa Assay**
(anti-HLA-G MEM-G/9 + W6/32 mAb)

- Detection limit was established by spike recovery

- Purified standard recombinant sHLA-G (rsHLA-G)
- Standard Curves for each plate
- Positive controls of known rsHLA-G concentration scattered in each plate

Fournel *et al.*, 1999
Fournel *et al.*, 2000





Limit of detection of soluble HLA-G

In each experiment:

❖ 60 individual blank wells scattered troughout each plates were analyzed simultaneously in each experiment with samples

❖ Blanks: culture medium without embryo

❖ A mean of 1 false positive for 60 blanks

❖ Detection limit of sHLA-G:~ 7.7 ng/ml

❖ Intraplate coefficient of variation: <1%

3 blanks are at the limit of positivity and 4 blanks are false positive

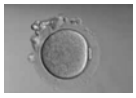
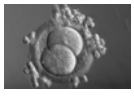
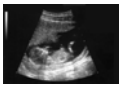
Signals below the detection limit were considered as negative

Would soluble HLA-G detection be informative of the outcome ?

TRACEABILITY of 726 samples analyzed

Outcome:

- Clinical implantation rate for each sample
  Number of gestational sac/number of embryos transferred
- Documentation of the corresponding embryos

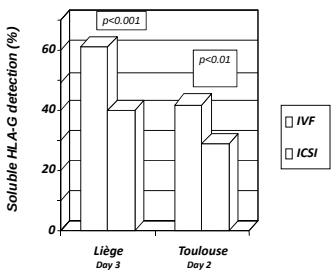
The proportions of sHLA-G-positive embryo culture supernatants and concentrations substantially vary among the different ART Centres

	POISSY CENTRE Day-2 *(n=360)*	*TOULOUSE CENTRE* Day-2 *(n=450)*	*LIEGE CENTRE* Day-3 *(n=595)*
% of sHLA-G positive ES	19%	34%	44%
Mean sHLA-G concentration (ng/ml) in sHLA-G positive ES	17.74 ±11.2	53.7 ±32	34.57 ±28
Mean sHLA-G concentration (ng/ml) in all sHLA-G positive and negative ES tested	3.3 ± 0.45	18 ± 1.5	16.2 ± 1

Soluble HLA-G in embryo culture supernatants is influenced by ART procedures

- Comparison of the percentage of sHLA-G detection in IVF (281 ES) and ICSI (718 ES) embryo culture supernatants in Liège and Toulouse

Soluble HLA-G in embryo culture supernatants is influenced by ART procedures



Soluble HLA-G in embryo culture supernatants and clinical implantation rates: differences between ART Centres

	Implantation rate (%)	*Implantation rate (%)*	*p*
	sHLA-G positive ES	sHLA-G negative ES	
Poissy Centre Day-2 *(n=146)*	34%	19%	*0.0379
Toulouse Centre Day-2 *(n=404)*	17%	18%	NS
Liège Centre Day-3 *(n=176)*	17%	18%	NS

→ ***The presence of sHLA-G in ES is not always associated with higher implantation potential***

Embryo quality is independent of soluble HLA-G in embryo culture supernatants

	POISSY CENTRE *(n=349)*		***LIEGE CENTRE*** *(n=595)*	
Embryo quality	High quality	Medium-low quality	High quality	Medium-low quality
No. of embryos observed	166	183	147	448
HLA-G positive ES	18%	20%	39%	46%

Soluble HLA-G detection in embryo culture supernatants is influenced by culture media

	LIEGE CENTER *595 embryo supernatants*		*p* values*
Media	Culture medium 1 (Irvine Scientific)	Culture medium 2 (Cook)	
sHLA-G positive ES	23.4 % (77/328)	70 % (187/267)	< 0.0001
sHLA-G positive ES ong embryos which were transferred	27.8% (34/122)	61 % (33/54)	< 0.0001
Implantation rate	15.6 %	23.1 %	0.18

Multicentre study on 1405 embryo cultured supernatants

- The presence of soluble HLA-G in ES does not always correlate with clinical pregnancy, depending on ART procedures.
- Variability of percentages and concentrations of soluble HLA-G positive ES among different Centres

Concentration of sHLA-G in ES

- Variability of sHLA-G concentration in ES in the three centres ranging from 3.3 to 18 ng/ml
- In 50µl of medium: 55 to 300 pg of sHLA-G secreted per day per embryo, much lower than previously reported (Menezo *et al.,* 2006).
- To rule out an artefact of detection: centralised Elisa was used for the thre Centres
  - Standard curve
  - Addition of many blanks to reduce intraplate coefficient of variation
  - And the most effective MEM-G9 and W6/32 monoclonal antibody combination reported to detect sHLA-G (Fournel *et al,* 2000)

Soluble HLA-G detection among ICSI-generated embryos

- The only centre with a statistical significant association between sHLA-G positive ES and implantation rates , even if % of detection was the lower (19%)
  ONLY included ICSI
  - Rebmann *et al.* (2007) already reported similar association
  - Proportion of sHLA-G positive ES vary between IVF and ICSI: Less positive in ICSI cultured embryos

Clinical application may be a matter of debate

- Similar implantation rates (18-19%) in the three centres among sHLA-G negative embryo supernatants
- Higher percentage of detection in Day-3 ES
- Investigation on Day-5 blastocyst stage embryo culture supernatants should bring additional informations

sHLA-G is likely to be related to the ART conditions

- Soluble HLA-G secretion in ES could be a useful tool to set optimal *in vitro* embryo culture conditions
- Identification of ICSI-cultured embryos with enhanced developmental potential
- But not suitable for a negative selection in a cohort of embryos

Tabiasco *et al.*, 2009

GENERAL CONCLUSION:

Soluble HLA-G in IVF/ICSI embryo culture supernatants does not always predict implantation success

Tabiasco *et al.*, 2009

EMBIC collaborative study

Toulouse: INSERM U563
P de Viguier Hospital
- Julie Tabiasco
- Jean Parinaud, Roger Léandri
- Noemi Kozma, Maryse Aguerre
- Philippe le Bouteiller

CHR Liège
- Sophie Perrier d'Hauterive
- Fabienne Thonon
- Carine Munaut
- Jean-Michel Foidart

Clamart: **INSERM U782**
- Sylvie Dubanchet
- Marie Petitbarat
- Gérard Chaouat

CHI Poissy-St Germain/UVSQ
- Marianne Bergère
- Ibrahim Hammoud
- Jacqueline Selva
- Yves Ville
- Raoul Lombroso
- Marc Bailly
- Robert Wainer
- Pierre Oger
- Nathalie Lédée

Tabiasco *et al.,* 2009

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25th Annual Meeting of ESHRE
Amsterdam 28/06/09
Pre-congress course: Embryology/Early Pregnancy
Course title: From Gamete to Heartbeat: the missing link

Research models: *in vitro* co-cultures

Judith Cartwright PhD
Division of Basic Medical Sciences,
St. George's, University of London,
Cranmer Terrace, London, SW17 ORE
jcartwri@sgul.ac.uk

Learning Objectives

- Describe the cellular interactions that can be modelled *in vitro* during implantation and trophoblast invasion.
- Be aware of the advantages and limitations of each of the models.
- Give examples of research findings made using these *in vitro* models.

What stages can be modelled in vitro?

- Embryo implantation
- Trophoblast invasion of the placental bed
- Trophoblast interaction with uterine spiral arteries

Types of *in vitro* models

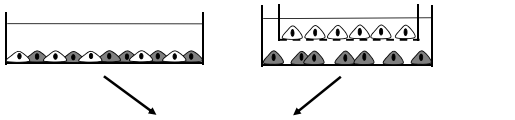
Simple co-culture of 2 cell types

Simple explant cultures

3D co-cultures

Complex explant cultures

Simple co-cultures



Biological effect

Which cell type is which?

Cell sorting

Fluorescent tagging

Staining for distinguishing markers

Which cell type is responsible for the biological effect?

Cell-targeted knockout

- simple co-culture is amenable to using many different cell types
- doesn't need large cell numbers - useful for primary cell cultures
- easy to manipulate the system with blocking abs, siRNA etc.

Endometrial cells
Primary epithelial
Primary stromal
Cell lines

Trophoblast
Primary 1st trimester EVT
EVT lines

Immune cells
Primary 1st trimester uNK
NK/T cell lines

Endothelium
Primary 1st trimester decidual EC
Primary HUVEC
Endothelial cell lines

Vascular smooth muscle
Human smooth muscle cell lines

- assumes that cell monolayers reflect the physiology of real tissues

Why is it important to incorporate 3D models?

2D versus 3D

Cell shape	Loss of polarity and altered shape in 2D
Gene expression	Cells in 2D vs. 3D often have different patterns of gene expression
Growth	3D-matrix dependent regulation of cell growth
Morphogenesis	3D-matrix induced vessel sprouting, invasion
Motility	Altered single and collective cell motility patterns in 3D matrices
Differentiation	3D-matrix induced cell differentiation

Pampaloni *et al.* 2007

Differing behaviour of cells in 2D vs. 3D

Re-establishes cell-cell and cell-ECM interactions

Example: LaMarca *et al.* 2005. Three-dimensional growth of extravillous cytotrophoblasts promotes differentiation and invasion.

Protein expression

	2D	3D
MMP-3	-	+
MMP-9	-	+

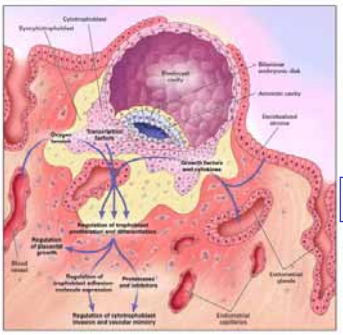
Gene expression

VCAM-1	x4
PECAM-1	x6
L-selectin	x9
MMP-3	x25
MMP-11	x3
MMP-13	x5
MMP-24	x35
uPA	x15

What stages can be modelled in vitro?

- Embryo implantation
- Trophoblast invasion of the placental bed
- Trophoblast interaction with uterine spiral arteries

Modelling implantation



What cells are interacting?

Blastocyst cells

Endometrial epithelial cell

Endometrial stromal cell

Endometrial cell cultures

- Endometrial cells from women undergoing IVF or hysterectomy
- Endometrial cell lines

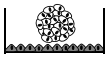
+ embryo

Examples:
Meseguer *et al.* 2001
Endometrial epithelial cells

Carver *et al.* 2003
Endometrial stromal cells

+ trophoblast spheroids

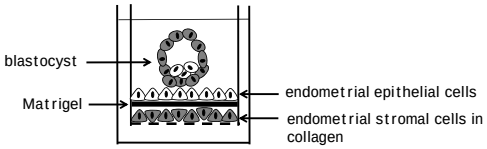
Example: Tinel *et al.* 2000



Access to tissue, complexity of the endometrium

Can be manipulated with siRNA, inhibitors, blocking abs etc

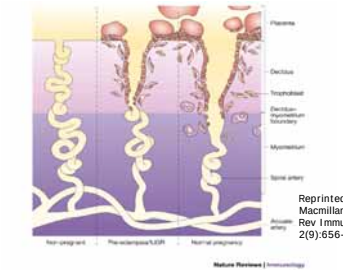
3D endometrial - blastocyst cultures



Example: Bentin-Ley *et al.* 1994.

Implantation models reviewed by Mardon *et al.* 2007.

Modelling invasion and artery remodelling



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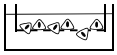
Difficulties

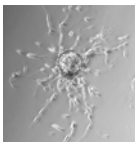
- First trimester tissue
- 3D environment
- Events occur slowly over weeks
- Most studies have looked at a snapshot of events
- How to compare normal to pre-eclamptic pregnancies

Trophoblast invasion

Invasion through Matrigel coated porous inserts

Example: Librach *et al.* 1991.

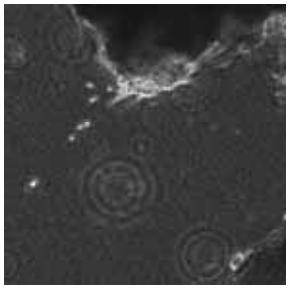




Trophoblast grown on microcarrier beads embedded in a fibrin gel

Example: Cartwright *et al.* 1999.

Simple explant cultures – 2D/3D



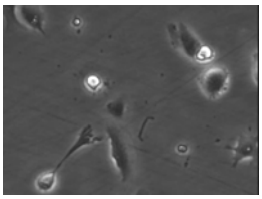
- Pure EVT
- Can add another cell type eg. uNK from same pregnancy
- First trimester

Example: Whitley *et al.* 2007.

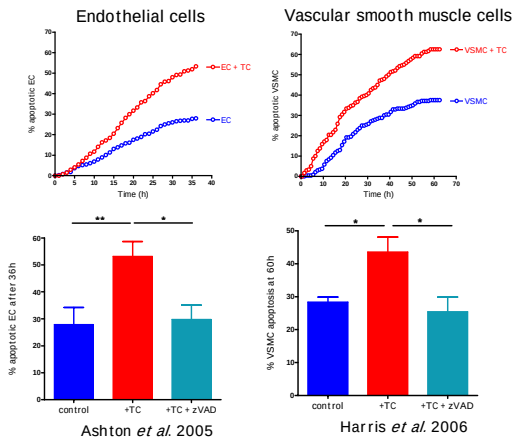
Trophoblast interaction with vascular cells

Hypothesis: Trophoblast induce changes in vascular cells that lead to their loss and subsequent vessel remodelling

- Primary first trimester trophoblast co-cultured with human aortic vascular smooth muscle cell line
- Cell tracker dye to distinguish cell type
- Morphology of cells monitored by time-lapse microscopy



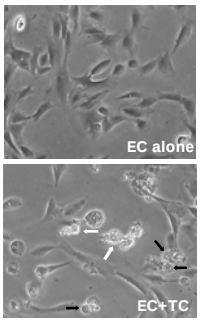
Total duration: 39.5 h
Cells in contact: 2.5 h
Phase bright: 8.5 h
Blister forms:12 h

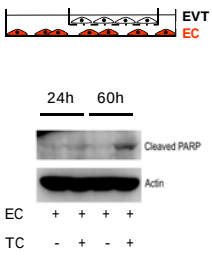


Ashton *et al.* 2005

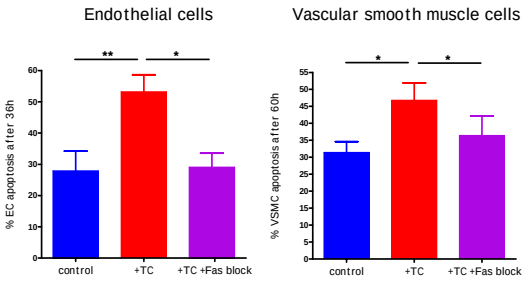
Harris *et al.* 2006

Identification of a soluble apoptotic factor





Trophoblast use Fas/FasL to induce apoptosis in EC and VSMC



What other information can be gained from simple co-cultures?

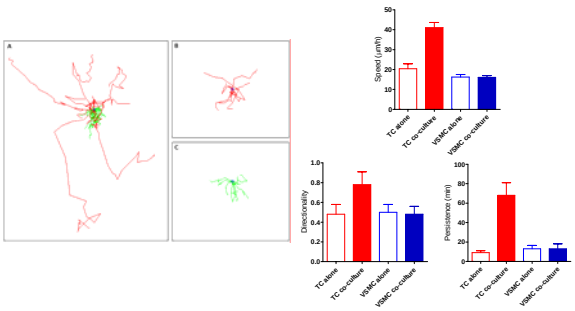
Which adhesion molecules are involved in TC interacting with vascular cells?

- Vitronectin receptors (Douglas *et al.* 1999).
- β1-integrin expression (Thirkill *et al.* 2004).
- VCAM-1 and α4β1 (Cartwright & Balarajah 2005).
- MUC1 expression (Thirkill *et al.* 2007).

What is the effect of flow?

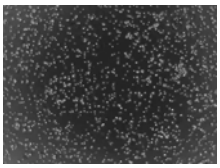
- Soghomonians *et al.* 2005.

Is trophoblast migration towards vascular cells directional?

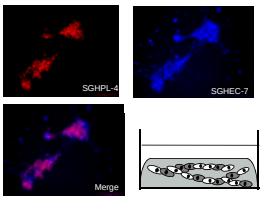


Hamzic *et al.* 2008

Mimicking vessels
Are trophoblast recruited to *in vitro* vessels?



Endothelial cells grown on Matrigel forming tubes/vessels



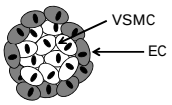
EVT recruited to *in vitro* capillary-like structures

- Add other cells
- Image with time-lapse microscopy
- Recover cells

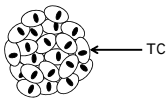
- Immunostaining difficult
- Doesn't have VSMC layer

Mimicking vessels - spheroids

Example: Korff et al 2001



VSMC/EC co-cultured in non-adherent plates



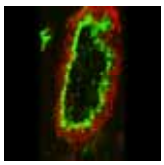
Addition of trophoblast spheroids

3D co-culture models

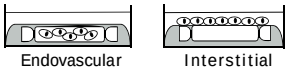
Spiral artery explant model

Dissect spiral arteries

Perfuse with fluorescently-labelled TC or TC-conditioned medium



Incubate to mimic *in vivo* events



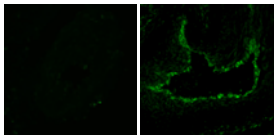
Endovascular Interstitial

Cartwright *et al* 2002

Trophoblasts induce apoptosis in spiral artery vascular cells

SA endothelial cells

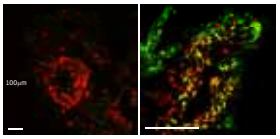
control | TC in lumen for 20h



Ashton *et al.* 2005
green = cleaved PARP

SA vascular smooth muscle cells

control | TC-conditioned medium in lumen for 24h

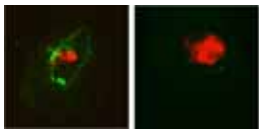


Harris *et al.* 2006
green = TUNEL staining
red = propidium iodide

Trophoblast induced spiral artery vascular cell apoptosis involves Fas/FasL and TRAIL

SA endothelial cells

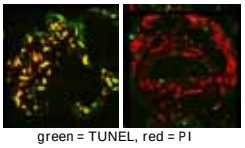
TC in lumen for 20h
+ FasL blocking Ab



green = cleaved PARP, red = fluorescent TC

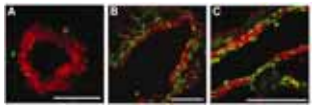
SA vascular smooth muscle cells

TC-conditioned medium in lumen for 24h
+ FasL blocking Ab



green = TUNEL, red = PI

+ rhTRAIL (0.5μg/ml)



green = TUNEL, red = smooth muscle actin

Ashton *et al.* 2005
Harris *et al.* 2006
Keogh *et al* 2007

- Unmodified vessels by location
- Can compare normal vs. pre-eclamptic
- Comparison of the role of interstitial vs. endovascular invasion
- Can use manipulated cells, blocking abs etc

- Term vessels
- Dissection difficult
- Requires non-placental bed biopsy tissue

Complex explant cultures

Dunk *et al.* 2003
First trimester villous explants cultured at low oxygen tension in contact with sections of decidua parietalis from the same patient.

Reprinted by permission from Macmillan Publishers Ltd: Lab. Invest. Dunk et al 83:1821-1828, copyright 2003.

- First trimester tissue
- All relevant cells present in decidua
- From same patient

- Have to carefully identify decidua parietalis
- Difficult to manipulate to identify mechanisms

Conclusions

- Start simple and build up complexity
- Consider the endpoint and choose an appropriate model to address the question
- Know the limitations of the culture models used

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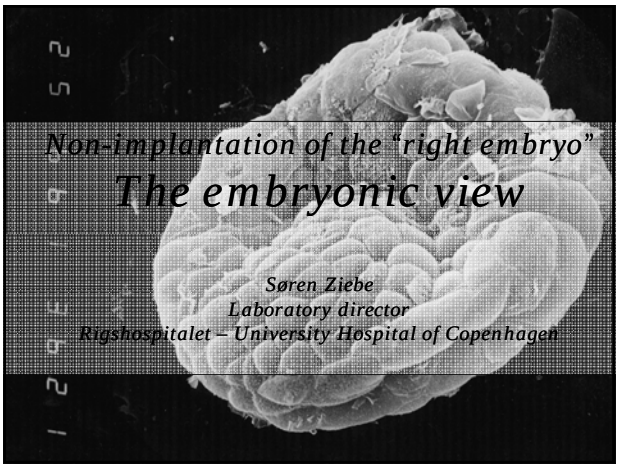


Non-implantation of the "right embryo"

The embryonic view

Søren Ziebe
Laboratory director
Rigshospitalet – University Hospital of Copenhagen

Non-implantation of the "right embryo" The embryonic view

Learning objectives

-Some in-vitro produced embryos are not supposed to implant …but do it anyhow

-More than morphology characterizes embryo quality

-Communication is an integral part of implantation

-We need information on the embryonic physiology to improve selection

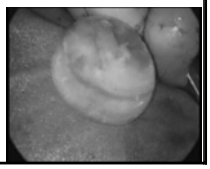
Non-implantation of the "right embryo" The embryonic view

The C -lecture

- Communication
- Control
- Correction
- Compensation
- CO_2
- Chromosomes
- Cell Cycle Checkpoints
- Culture conditions

Non-implantation of the "right embryo" The embryonic view

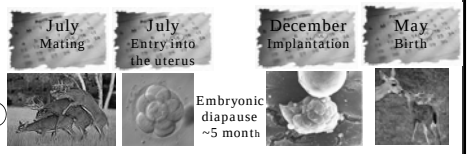
- Implantation of the right embryo
  ⟹ *everybody is happy*
- Non-implantation of the right embryo
  ⟹ *everybody is frustrated*
- Implantation of the wrong embryo
  ⟹ *everybody is sad*
- Non-implantation of the wrong embryo
  ⟹ *we should be happy*
- Implantation of the right embryo – the wrong place
  ⟹ *really bad luck*
- Controlled non-implantation
  ⟹ *optimization ?*



Non-implantation of the "right embryo" The embryonic view

Controlled non-implantation

delayed implantation or embryonic diapause



Non-implantation of the "right embryo" The embryonic view

Two statements:

"*The spontaneous abortion is not the problem – it is the solution ! The problem is the occurrence of an implantation that shouldn't have happened*"

"*Non-implantation is the correct response to avoid this problem*"

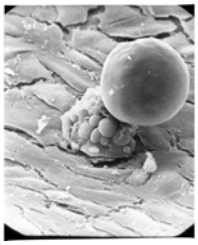
Non-implantation of the “right embryo” The embryonic view

Two more statements:

“*The **right embryo** is an embryo capable of communicating with the surrounding organism in a precise and timely manner*”

“*The **right embryo** is probably an embryo that possesses the ability to perform corrective measures when something is wrong*“

Non-implantation of the “right embryo” The embryonic view



QUESTIONS:

By what scale is it the right embryo ?

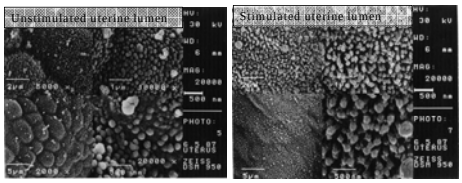
Is it the right endometrium?

Is the embryo located at the right place at the right time ?

Is communication synchronized between embryo and endometrium?

Photo by T.Høst and S.Ziebe

Non-implantation of the “right embryo” The embryonic view



- and then there is the **surrounding organism** including the uterus, endometrium, endocrine status etc.

THIS WILL BE ADRESSED IN THE NEXT LECTURE

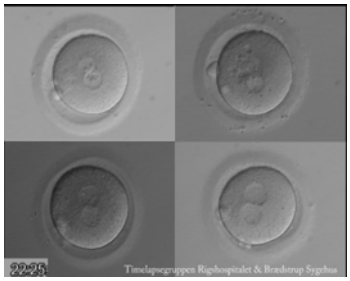
Photo by T.Høst and S.Ziebe

Non-implantation of the “right embryo”
The embryonic view

Morphology and Kinetics

Non-implantation of the “right embryo”
The embryonic view

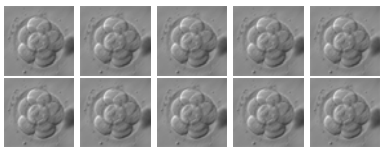
Differences in embryonic development



Non-implantation of the “right embryo”
The embryonic view

From morphology to physiology

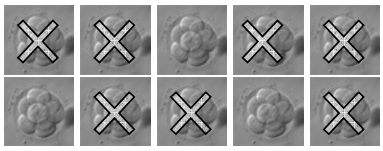
What is the right embryo ?



Non-implantation of the “right embryo”
The embryonic view

From morphology to physiology

What is the right embryo ?



WHY ? - Morphology apparently is not enough…….

Non-implantation of the “right embryo”
The embryonic view

Implantation is also about communication

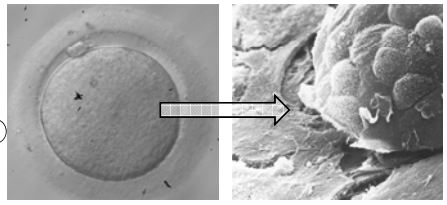


Photo by T.Høst and S.Ziebe

Non-implantation of the “right embryo”
The embryonic view

Paracrine factors

EXAMPLE

Non-implantation of the “right embryo”
The embryonic view

From morphology to physiology
Signals in the early phases

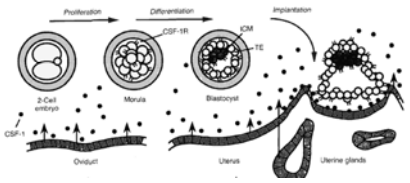


Illustration by T.Høst

EXAMPLE

Non-implantation of the “right embryo”
The embryonic view

From morphology to physiology

GM-CSF

The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to:

- Be present in the female reproductive organs during early pregnancy in mice, sheep, cows and humans.
- That the level of GM-CSF rises in the female after exposure to sperm, in both mice and humans (Robertson 1990 and 1992).

EXAMPLE

Non-implantation of the “right embryo”
The embryonic view

From morphology to physiology

GM-CSF

Embryo development during *in vitro* culturing in the presence of GM-CSF is associated with:

- Increased uptake of nutrients (*mice:* Robertson 2001)
- Reduced incidence of cell death/apoptosis (*mice:* Robertson 2001)
- Generally increased number of cells (*humans:* Sjöblom 1999)
- Increased inner cell mass (ICM) in the blastocyst (*mice:* Robertson 2001; *humans:* Sjöblom 1999)
- Accelerated embryo development (*humans:* Sjöblom 1999)
- An increased proportion of early cleavage embryos that develop to the blastocyst stage (*humans:* Sjöblom 1999)
- Increased *in vitro* hatching (zona pellucida dissolution) and embryo adhesion to extracellular matrix-coated culture dish (*humans*: Sjöblom 1999)

EXAMPLE

Non-implantation of the “right embryo”
The embryonic view
From morphology to physiology

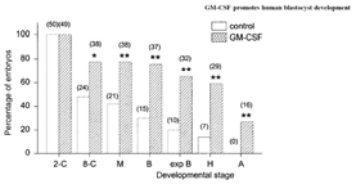
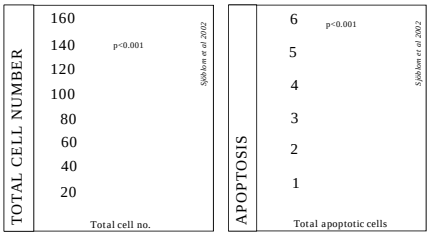


Figure 3. The effect of recombinant human granulocyte–macrophage colony–stimulating factor (rhGM-CSF) on the development of embryos to blastocyst, hatching and attachment stages. Data are the number of embryos developed to or beyond each stage, from experiments 1, 2 and 3 combined, expressed as a percentage of the initial number of cleaved (2–4-cell) embryos. 2-C = 2-cell embryos; 8-C = 8-cell embryos; M = morula; B = blastocyst; exp B = expanded blastocyst; H = hatching; A = attached with trophectoderm outgrowth. The number of embryos in each group is given in parentheses. Data were analysed by Fisher's exact test. *$P < 0.004$; **$P < 0.001$ denote significant difference from control group.

Sjöblom et al. 1999

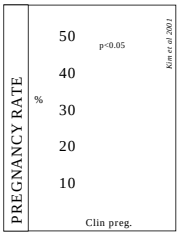
EXAMPLE

Non-implantation of the “right embryo”
The embryonic view
From morphology to physiology



EXAMPLE

Non-implantation of the “right embryo”
The embryonic view
From morphology to physiology



EXAMPLE

Non-implantation of the “right embryo” The embryonic view

From morphology to physiology

Primary outcome – GM-CSF Safety study

In a worst case scenario classifying all missing data as uniformly abnormal, the analyses showed

	Test	Control
Overall normal	67 %	50 %

	Test	Control
Uniformly normal	33 %	28 %

Unpublished data – publication in progress

Non-implantation of the “right embryo” The embryonic view

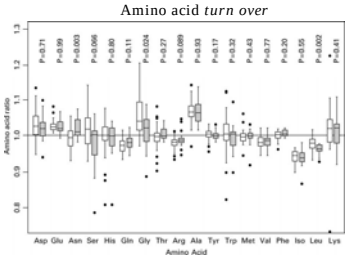
Metabolites

EXAMPLE

Non-implantation of the “right embryo” The embryonic view

From morphology to physiology

Amino acid *turn over*



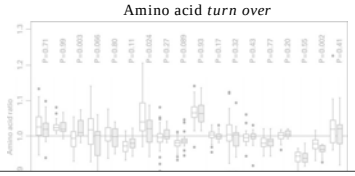
D.R.Brison et al. Human Reproduction 2004

EXAMPLE

Non-implantation of the “right embryo”
The embryonic view

From morphology to physiology

Amino acid *turn over*



“We have now shown that measurement of amino acid turnover could potentially increase significantly our ability to select the most viable embryo for transfer in clinical IVF, without the need for extended culture”

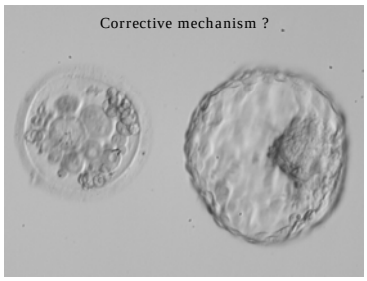
D.R.Brison et al. Human Reproduction 2004

Non-implantation of the “right embryo”
The embryonic view

Corrective measures

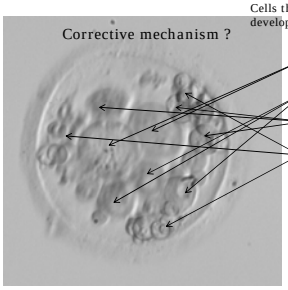
Non-implantation of the “right embryo”
The embryonic view

Corrective mechanism ?



Non-implantation of the “right embryo”
The embryonic view

Corrective mechanism ?



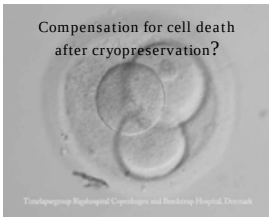
Cells that left embryonic development at:

- 8-cell stage
- 16-cell stage
- 32-cell stage
- 64-cell stage

Non-implantation of the “right embryo”
The embryonic view

Corrective mechanism ?

Compensation for cell death after cryopreservation?

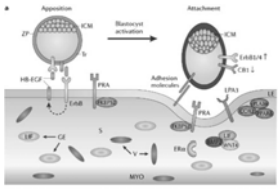


Non-implantation of the “right embryo”
The embryonic view

Chromosomes

Non-implantation of the “right embryo” The embryonic view

Many genes are involved in the different phases of implantation



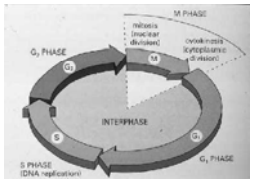
Involvement of genes in the implantation process

Competance in the different phases of implantation:
- -Apposition ?
- -Adhesion ?
- -Invasion ?

Wang & Dey 2006

Non-implantation of the “right embryo” The embryonic view

Chromosomes duplicates during cell cleavage



Cell cycle check point checks for errors

Non-implantation of the “right embryo” The embryonic view

- but mistakes happens..

Non-implantation of the “right embryo” The embryonic view

Design

Randomly selected – donated oocytes

Insemination using sperm from a single sperm donor

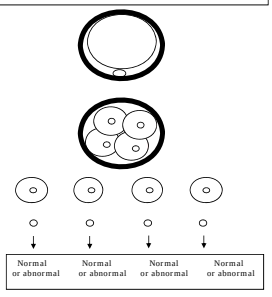
Morphological evaluation of the embryo and culture to day 3

Separation of the blastomeres and fixation of the nuclei

FISH analysis of each nuclei

Chromosomal classification of each nuclei for chromosome 13, 16, 18, 21, 22, X and Y

Overall classification of the embryos



Ziebe et al. Human Reproduction 18, 2003

Non-implantation of the “right embryo” The embryonic view

Chromosomes

Number of normal and abnormal blastomeres

Blastomeres displaying a normal diploid karyotype	242 (56%)
Blastomeres displaying abnormal karyotype	191 (44%)

Number of 100% normal embryos:

Normal embryos (%)	33 (31 %)
Abnormal embryos (%)	72 (69 %)

Number of embryos >50% normal

Normal embryos (%)	57 (54 %)
Abnormal embryos (%)	48 (46 %)

Non-implantation of the “right embryo” The embryonic view

Respiration

Non-implantation of the “right embryo”
The embryonic view
From morphology to physiology

Investigation of respiration of individual bovine embryos produced *in vivo* and *in vitro* and correlation with viability following transfer

A.S.Lopes[1,3], S.E.Madsen[2], N.B.Ramsing[4], P.Lovendahl[2], T.Greve[2] and H.Callesen[1]

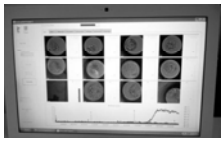
Table II. Pregnancy status according to respiratory category (high versus medium versus low) of bovine *in vivo*-produced embryos

Respiratory category	Pregnant	Non-pregnant
High (>1.10 nl/h)	25% ($n = 1$)	75% ($n = 3$)
Medium (0.78–1.10 nl/h)	100% ($n = 13$)	0% ($n = 0$)
Low (<0.78 nl/h)	48% ($n = 11$)	52% ($n = 12$)

Human Reproduction 2006

Non-implantation of the “right embryo”
The embryonic view
From morphology to physiology

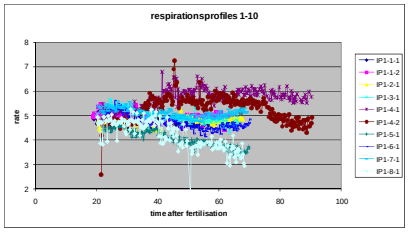
Respiration profiles Human embryos



Ziebe & Lemmen – Unpublished data

Non-implantation of the “right embryo”
The embryonic view
From morphology to physiology

Respiration profiles Human embryos



Ziebe & Lemmen – Unpublished data

Non-implantation of the “right embryo” The embryonic view

Conclusions

- Embryo competence is to a large extend reflected in the morphological appearance
- We need an improved understanding of the underlying physiological processes in the cytoplasm and organelles
- The kinetics is important
  - being at the right place at the right time
- A more detailed and objective knowledge of the embryos and their development may help improving selection and thus implantation
- Oocyte quality needs to be improved in order to obtain better embryos
- Oocyte and Embryo quality is initiated already during the stimulation phase

Non-implantation of the “right embryo” The embryonic view

Conclusions

- IMPLANTATION IS ALL ABOUT

COMMUNICATION

Non-implantation of the “right embryo” The embryonic view

Conclusions

Ps: DON'T FORGET
THE ENDOMETRIUM

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Implantation of the ‘wrong’ embryo. The endometrial view

Siobhan Quenby

Introduction / Learning Objectives

- Clinical evidence for selection of pregnancies and maternal-fetal interface
- Scientific evidence for selection
- How this selection could occur
- Clinical implications of this selection paradigm

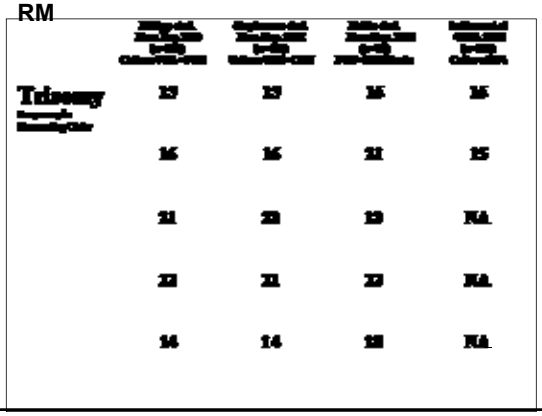
The decidua

- 90% karyotypically abnormal pregnancies miscarry in the first trimester
- 93% karyotypically normal pregnancies continue
  - McFadyen, 1989

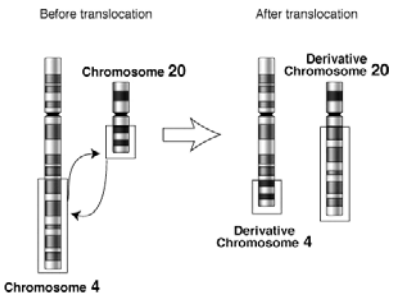
Karyotypical abnormality

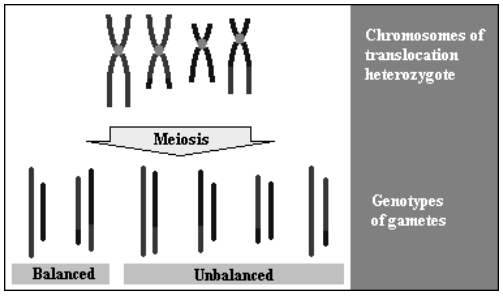
- High (29-57%) in RM population
  - Stern *et al.,* 1996,
  - Ogasawara *et al.,* 2000,
  - Carp *et al*., 2001,
  - Stephenson *et al.,* 2002
- Same rate recurrent and spontaneous miscarriage

Cytogenetic Analysis of Pregnancy Loss in RM



Balanced translocation





Balanced translocations in RM

- PGD
  - 70-80% embryos abnormal
    - Munne et al., 1998
- Natural conception
  - 1 infant unbalanced translocation n=95
    - Sugiura-Ogasawara 2004
  - 4 unbalanced translocations n= 550
  - No miscarriages not ≈ incidence of translocation
    - Goddijn 2006
- Where are the unbalanced translocations?
  - 38% of miscarriages unbalanced translocations
  - 0% of ongoing pregnancies
    - Stephenson and Sierra 2006

How does selection occur

- Abnormal trophoblast does not invade?
- Maternal endometrium can select?

Aneuploidy miscarriages

- RM women miscarried aneuploidy fetus found normal trophoblast invasion and plugging of spiral arteries
  - Sebire et al 2002

Poor invasion:

No plugs

↘

Infertility/Miscarriage

↗

No attachment

No spiral artery invasion

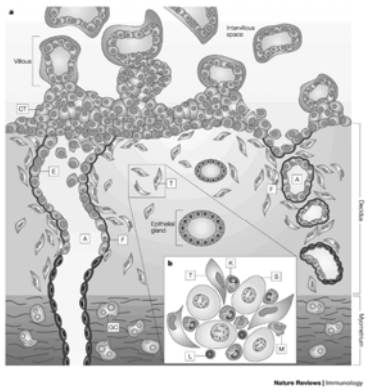
↓

Preclampsia

Too much invasion

↓↓

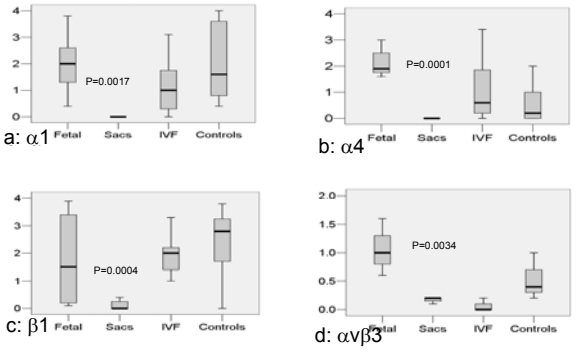
placenta accreta and moles



Adhesion molecules

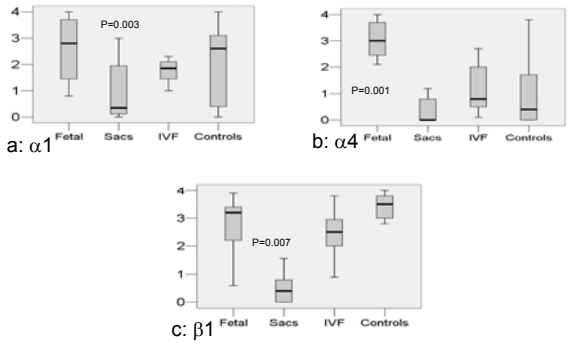
- Integrins help attachment of embryo to luminal surface endometrium
  - $\alpha 1\beta 1$, $\alpha 4\beta 1$, $\alpha v\beta 3$
- Maximal expressed in implantation window
- Lower in infertile women

Luminal epithelium



RBM online Quenby et al., 2007

Glandular epithelium



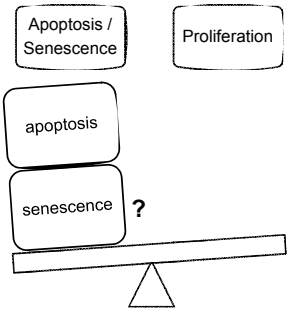
Barrier molecules

- MUC 1 glycoprotien barrier implantation
- Less in implantation window
- More expression in implantation failure
    - (Horne et al., 2005)
- Less when embryo reaches endometrial surface

Implantation and Endometrial Cell kinetics

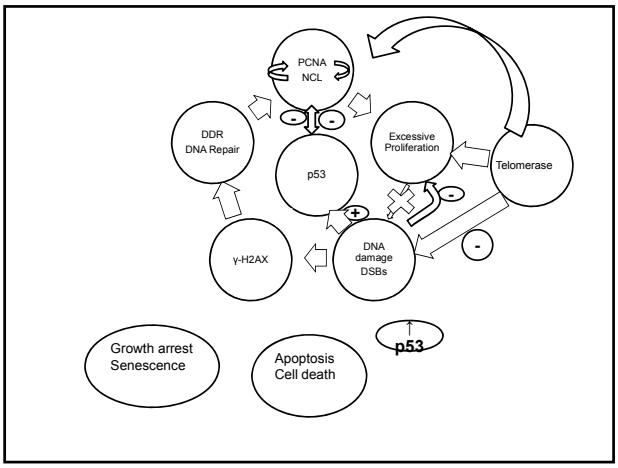
*The tree of **life and death** was painted as a symbol of how women are expected to provide nourishment for everything around them yet so often they don't nourish themselves and therefore wither and die.*

Normal Luteal phase endometrial cell kinetics

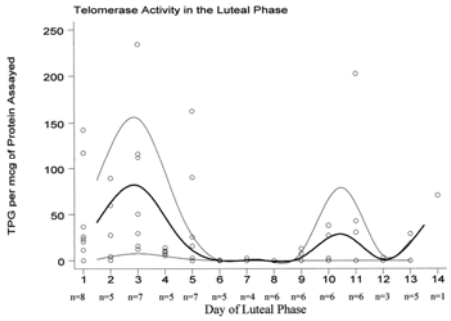


Markers of cell fate

- **Telomere**
  - Protect chromosome ends
  - Control the life span of somatic cells
  - Loose telomeric DNA with each cell division
  - May induce cellular apoptosis / senescence
- **Telomerase**
  - Maintains telomeres
  - Most cancer cells and germ cells express telomerase, but somatic cells do not
  - Activity provides unlimited proliferative potential to a cell
  - Evidence so far for endometrium
    - *Williams et al. 2001, Hapangama et al. 2008a & b*



Williams et al 2001



Williams, C. D. et al. J Clin Endocrinol Metab 2001;86:3912-3917

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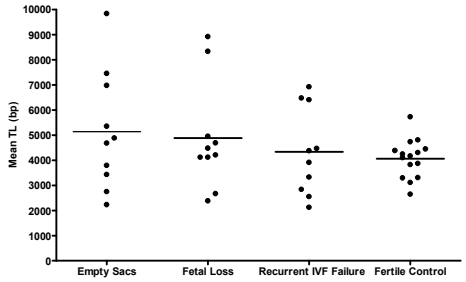
Telomeres- Recurrent pregnancy failure

Figure 4: Mean endometrial Telomere length



Hapangama et al. RBMO 2008

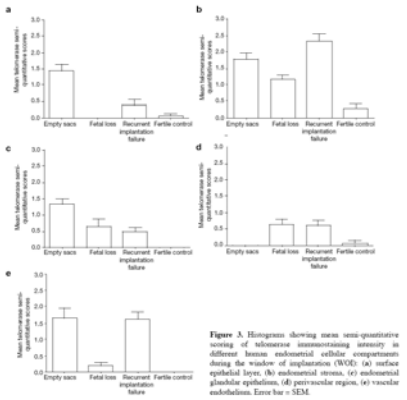


Figure 3. Histograms showing mean semi-quantitative scoring of telomerase immunostaining intensity in different human endometrial cellular compartments during the window of implantation (WOI): (a) surface epithelial layer, (b) endometrial stroma, (c) endometrial glandular epithelium, (d) perivascular region, (e) vascular endothelium. Error bar = SEM.

Cell kinetics in WOI

- Fertile control women
- Markers of cell fate suggest
  - Balance is towards senescence / apoptosis
- This may allow acceptance of invading embryo with minimum tissue disturbance
- Alterations
  - Maintain telomeres
  - Without evoking a DNA damage response
- This may have an impact on clinical presentation of sub-fertility, making endometrium resistant to apoptosis /senescence and more ‘hostile’

Embryo/endometrial dialogue

- Embryo –down regulated MUC
  - Aplin et al 1998
- HCG given to baboons upregulated
  - Embryo attachment genes
    - Sectreted frizzle related proteins
  - Endometrial remodelling
    - MMP7, 23 Serpin A
  - Antioxidant defence mechanism
    - SOD 2 FOX01
  - Immune response
    - LIF C3/C4
  - Sherwin et al., 2007

Clinical implications/Conclusions

- The endometrium has a role in selecting embryos
- Several functions to:
  - prevent bad embryos implanting
  - let in good embryos
  - respond to embryo signals including HCG
- Thus endometrium is a complex balance and concept of ideal endometrium we can assess may not be valid
- When we design treatments to improve endometrium need to be clear what we are trying to achieve
- Women with recurrent implantation failure want there receptivity assessed
- Recurrent miscarriage situation more complex.

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