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Culture media: the best environment for gametes and embryos

Special Interest Group Embryology

3

1 July 2012
Turkey, Istanbul

Culture media: the best environment for gametes and embryos

Istanbul, Turkey
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Organised by
the Special Interest Group Embryology

Contents

Course coordinators

Kersti Lundin (Sweden), M. Cristina Magli (Italy), M. José de los Santos (Spain), Josephine Lemmen (Denmark)

Course description

Culture media formulations have been modified significantly throughout the last years and the resulting embryo viability is significantly enhanced in comparison with the historical use of simple salt solutions. Complex media have been designed to support embryo growth based on the assumption that the embryo experiences changing energy requirements during its development. However, the tenet that a sequence of media is required to efficiently produce viable embryo has been challenged with data from optimized single media.

In order to increase media enrichment, several growth factors, antioxidants, cytokines and vitamins are added at concentrations that, quite often, are far from physiological. Can they be used with effectiveness and safety in the clinical IVF setting?

Several formulations are now commercially available, but their composition is usually not released and a general concern arises on the non physiological concentrations of additives.

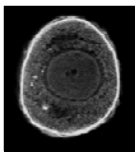
There is a need to understand whether promoting embryo growth so aggressively in vitro might affect later development. This basic course is aimed at providing the best knowledge for clinical embryologists to perform an aware and justified selection of the culture medium to be used in their laboratory.

Target audience

Clinical embryologists and clinicians involved in reproductive medicine and biology.

Scientific programme

09.00 - 09.30	Oogenesis: acquisition of oocyte competence – **Carlos Plancha (Portugal)**
09.30 - 09.45	Discussion
09.45 - 10.15	The impact of growth factors in culture medium on early embryo development – **Daniel Brison (United Kingdom)**
10.15 - 10.30	Discussion
10.30 - 11.00	Coffee break
11.00 - 11.30	Mitochondrial activity during oocyte and embryo development – **John Carroll (United Kingdom)**
11.30 - 11.45	Discussion
11.45 - 12.15	Development of culture media: impact on embryo viability – **Nadir Ciray (Turkey)**
12.15 - 12.30	Discussion
12.30 - 13.30	Lunch
13.30 - 14.00	Nutritional requirements from the oocyte to the blastocyst: implications for embryo culture – **Henry Leese (United Kingdom)**
14.00 - 14.15	Discussion
14.15 - 14.45	Ways to support in vitro oocyte maturation – **Julius Hreinsson (Sweden)**
14.45 - 15.00	Discussion
15.00 - 15.30	Coffee break
15.30 - 16.00	Epigenetic events in gametes and early embryos – **Martine De Rycke (Belgium)**
16.00 - 16.15	Discussion
16.15 - 16.45	The top quality embryos – **Kersti Lundin (Sweden)**
16.45 - 17.00	Discussion



Oogenesis: acquisition of oocyte competences

Carlos E. Plancha[1,2]

[1] Unidade de Biologia da Reprodução, Inst. Histologia e Biologia do Desenvolvimento, Faculdade de Medicina de Lisboa, Portugal

[2] CEMEARE – Centro Médico de Assistência à Reprodução, Lisboa, Portugal

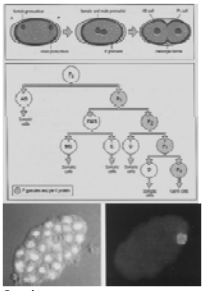
DISCLOSURE

CE Plancha does not have any commercial and/or financial relationship with manufacturers of pharmaceuticals, laboratory supplies and/or medical devices.

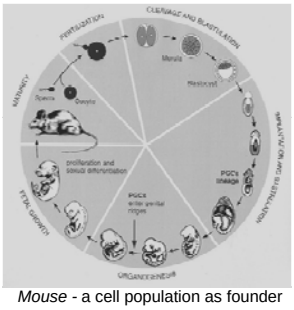
Presentation outline and learning objectives:

1. Germ cell lineage establishment
2. Structural features of Oocyte Growth and Maturation
3. Functional features of Oogenesis: acquisition of competences
4. Future chalenges in this area of Clinical Embryology

Germ cell lineage

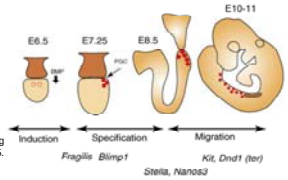


C. elegans - one cell as founder

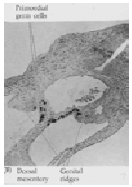


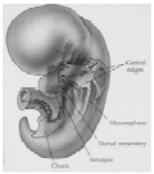
Mouse - a cell population as founder

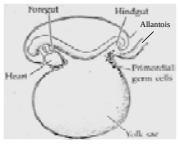
Signalling pathways involved in **Induction, Specification and Migration** of Primordial Germ Cells in mammals



Y Saga (**2008**) Mouse germ cell development during embryogenesis.Curr Opin Genet & Develop 18:1–5.





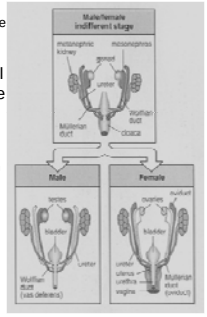


Development of the **Somatic Sexual Phenotype** in mammals

Initial **indifferent** / **bipotential** stage

Germ cells arrive to genital ridge at this stage

Subsequent differentiation of **gonads** and other **reproductive system** organs

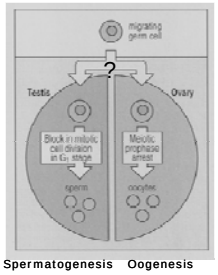


Mouse:
12,5 days p.f.

Human:
7th week p.f.

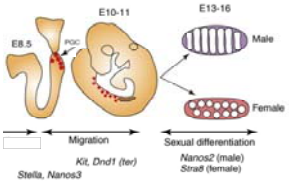
MA Edson et al.. (2009) The Mammalian Ovary from Genesis to Revelation. Endocrine Reviews, 30:624-712.

Signalling pathways involved in **Germ Cell Sex Differentiation** in mammals

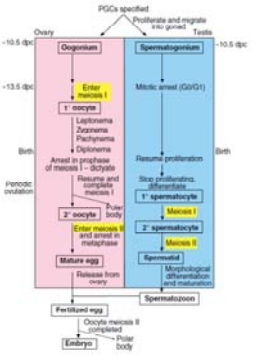


Spermatogenesis Oogenesis

Y Saga (**2008**) Mouse germ cell development during embryogenesis. Curr Opin Genet & Develop 18:1–5.



Gametogenesis

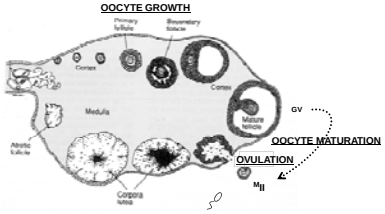


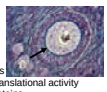
Oogenesis

Spermatogenesis

J Bowles and **P Koopman** (**2007**) Retinoic acid, meiosis and germ cell fate in mammals. Development 134:3401-3411.

Oogenesis occurs inside the ovarian follicle and involves both **Growth** and **Maturation** phases



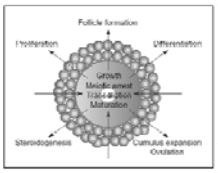


◆**Oocyte growth phase**:
- Oocyte diameter increases
- High transcriptional and translational activity
- Accumulation of RNA / proteins
- Building of new structures
. (zona pellucida, cortical granules)

Maturation

◆**Oocyte maturation phase**:
- Nuclear / cytoplasmic events with resumption of meiosis and arrest at M_{II} shortly before ovulation
- Organelle redistribution
- Cell polarity and asymmetric division

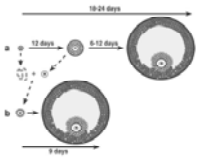
The Oocyte secretes dominant paracrine effectors (GDF-9, BMP-15)



Dong et al. (**1996**) **Nature** 383: 531.
Galloway et al. (**2000**) **Nature Genetics** 25: 279.
Juengel et al. (**2002**) **Biol Reprod** 67: 1777.

and

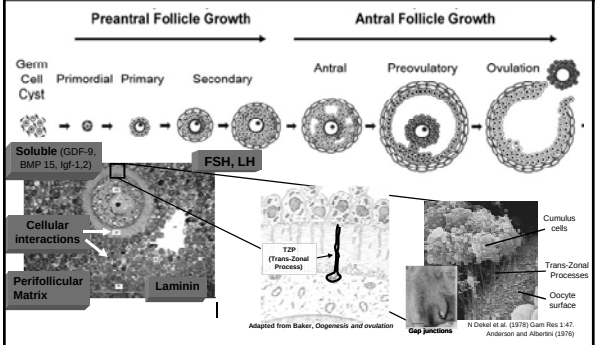
dictates the rate of follicle development



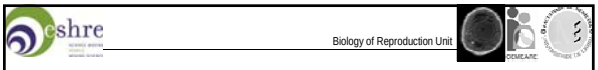
JJ Eppig (**2001**) **Reproduction** 122: 829.

JJ Eppig, K Wigglesworth, FL Pendola (**2002**) The mammalian oocyte orchestrates the rate of ovarian follicular development. **PNAS** 99:2890–2894.

Successful oogenesis and folliculogenesis require **complex paracrine connectivity**

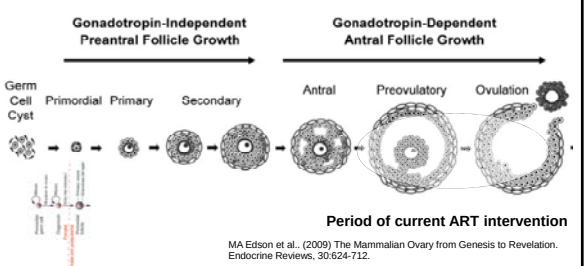


MA Edson et al. (2009) The Mammalian Ovary from Genesis to Revelation. Endocrine Reviews, 30:624-712.
SG Hillier (2009) Paracrine support of ovarian stimulation. Mol Hum Reprod 15: 843–850.



Ovarian follicle development in humans

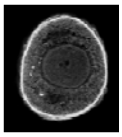
Primordial → ~120-180 days → Preantral → ~90 days → ovulation
Poorly understood (except Gonadotropin-dependent last stages)



Period of current ART intervention

MA Edson et al.. (2009) The Mammalian Ovary from Genesis to Revelation. Endocrine Reviews, 30:624-712.

During **Oogenesis** the oocyte acquires **critical competences** for initial **Development**



1. Meiotic
a. competence to reach the metaphase II arrest
b. competence to allow correct meiotic chromosome segregation

2. Activation
competence to fuse with sperm, finish meiosis,
block polyspermy, and form pronuclei

3. Developmental
competence to trigger and support embryonic development

Meiotic competence: Oocyte ability to leave prophase I and to reach metaphase II

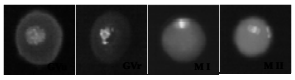
Mouse model

⇒ During the oocyte growth phase (to about 60-65µm: 80% of full size)

⇒ Sequential acquisition, first of the capacity to re-initiate meiosis, than to reach metaphase I and finally to reach metaphase II

⇒ Associates with chromatin and microtubule configuration modifications and with centrosome phosphorylation during the prophase I arrest

⇒ Associates with differential accumulation / localization of several cell cycle related molecules (p34^{cdc2}, cyclin B1, cdc25C, wee1)

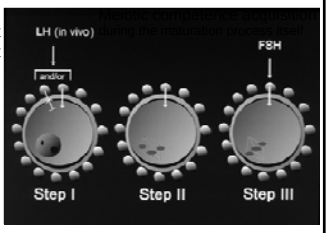


Sorensen and Wassarman (1976) Dev Biol 50:531-536.
Mattson and Albertini (1990) Mol Reprod Dev 25: 374-383.
Wickramasinghe and Albertini (1992) Dev Biol 152: 62-74.
Eppig et al. (1994) Dev Biol 164: 1-9.
Kanatsu-Shinohara et al. (2000) Biol Reprod 63: 1610-1616.
MA Edson et al. (2009) Endocrine Reviews, 30:624-712.

Gonadotropin impact upon meiotic competence: Lessons from the hamster

Hamster model

Meiotic cell cycle progression requires gonadotropin-dependent and -independent somatic cell input



CE Plancha, DF Albertini (1994)
Hormonal regulation of meiotic maturation in the hamster oocyte involves a cytoskeleton mediated process.
Biol Reprod 51:852-864.

Ovine, Bovine, Human models

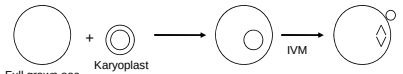
⇒ After the oocyte growth phase
⇒ Human primary oocytes recovered from antral follicles ›3mm already acquired meiotic competence and can reach metaphase II *in vitro*

Trounson et al. (1998) Hum Reprod 13 (Suppl. 3): 52-62.
MA Edson et al. (2009) Endocrine Reviews 30:624-712.

Meiotic competence acquisition

Mouse

⇒ Although the visible expression of this competence is the nuclear compartment, **it is the cytoplasm that confers meiotic competence**



Full grown ooc. (enucleated GV) (**competent**)

Karyoplast (non-growing ooc.) (**incompetent**)

Kono et al. (1996) Nat Genet 13: 91-94.

The Oocyte is the major source of chromosome aneuploidies in the Human

Maternal age strongly impacts on Chromosome Anomalies in Miscarriages

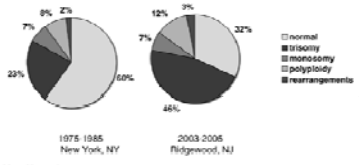


Figure 1. Effect of Maternal Age on the Distribution of Chromosome Anomalies in Miscarriages
Distribution of chromosome abnormalities among consecutive karyotyped miscarriages with developmental age < 18 weeks, from (left) New York City from 1974 to 1985 and (right) Ridgewood, New Jersey, from 2003 to 2005 (Warburton, Kline, and Kinney, unpublished data).

Scrambling Eggs in Plastic Bottles
Editorial by R S Hawley, D Warburton
In PLoS Genetics, Jan 2007, Vol 3, 1

Factors influencing human oocyte aneuploidies (chromosome anomalies)

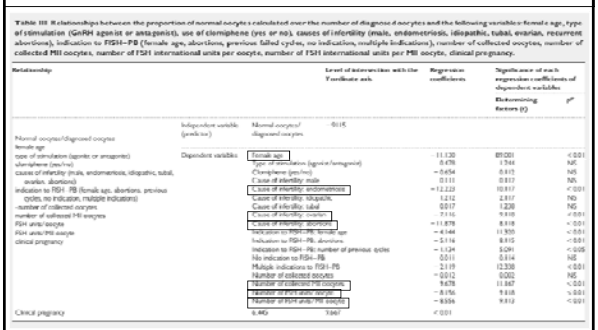
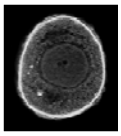


Table III Relationships between the proportion of normal oocytes calculated over the number of diagnosed oocytes and the following variables: female age, type of stimulation (GnRH agonist or antagonist), use of clomiphene (yes or no), causes of infertility (male, endometriosis, idiopathic, tubal, ovarian, recurrent abortions), indication to FISH–PB (female age, abortions, previous failed cycles, no indication, multiple indications), number of collected oocytes, number of collected MII oocytes, number of FSH international units per oocyte, number of FSH international units per MII oocyte, clinical pregnancy.

Relationship			Level of interaction with the Fertilizate axis	Regression coefficients	Significance of each regression coefficients of dependent variables: Backcrossing factors (r)	R^2
Normal oocytes/Diagnosed oocytes	Independent variable (predictor)	Normal oocytes/ diagnosed oocytes	0.015			
female age	Dependent variables	Female age		−11.130	89.001	< 0.01
type of stimulation (agonist or antagonist)		Type of stimulation (agonist/antagonist)		0.478	1.744	NS
clomiphene (yes/no)		Clomiphene (yes/no)		−0.454	0.615	NS
causes of infertility (male, endometriosis, idiopathic, tubal, ovarian, abortions)		Cause of infertility: male		0.111	0.817	NS
indication to FISH–PB (female age, abortions, previous cycles, no indication, multiple indications)		Cause of infertility: endometriosis		−12.223	10.817	< 0.01
number of collected MII oocytes		Cause of infertility: idiopathic		1.212	2.817	NS
FSH units/oocyte		Cause of infertility: tubal		0.017	1.230	NS
FSH units/MII oocyte		Cause of infertility: ovarian		−7.116	9.918	< 0.01
clinical pregnancy		Cause of infertility: abortions		−11.878	8.918	< 0.01
		Indication to FISH–PB: female age		−4.144	11.930	< 0.01
		Indication to FISH–PB: abortions		−5.116	8.915	< 0.01
		Indication to FISH–PB: number of previous cycles		−1.124	5.091	< 0.05
		No indication to FISH–PB		0.011	0.614	NS
		Multiple indications to FISH–PB		2.119	12.330	< 0.01
		Number of collected oocytes		−0.012	0.002	NS
		Number of collected MII oocytes		9.678	11.847	< 0.01
		Number of FSH units/ml/cycle		−8.156	9.818	< 0.01
		Number of FSH units/ml/MII oocyte		−8.996	9.813	< 0.01
Clinical pregnancy		0.440	1.967	< 0.01		

Luca Gianaroli et al. **(2010)** Predicting aneuploidy in human oocytes: key factors which affect the meiotic process. **Hum Reprod 25(9): 2374–2386.**

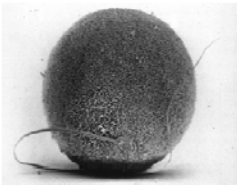
Oogenesis as acquisition of functional competencies



1. **Meiotic**
   a. competence to reach the metaphase II arrest
   b. competence to allow correct meiotic chromosome segregation
2. **Activation**
   competence to fuse with sperm, finish meiosis, block polyspermy, and form pronuclei
3. **Developmental**
   competence to trigger and support embryonic development

Activation involves:

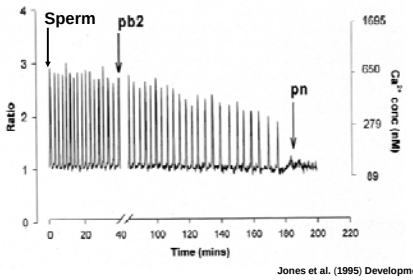
- Induction of oocyte intracelular [Ca^{2+}] oscilations
- Cortical reaction and block to polyspermy
- Conclusion of meiosis
- Decondensation of sperm chromatin
- Pronuclei formation



Oocyte aquisition of mechanisms of **Ca^{2+} signalling** are essential for activation competence

Intracellular [Ca^{2+}] oscillations at fertilisation

Mouse model

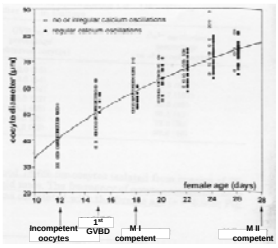


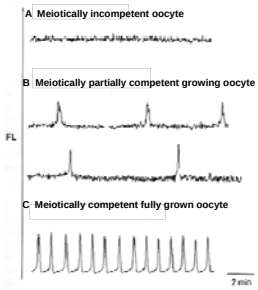
An oocyte property

Jones et al. (1995) Development 121:3259-3266.

Mechanisms of Ca^{2+} signalling become functional with female age and oocyte growth

Mouse model



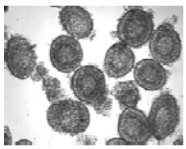


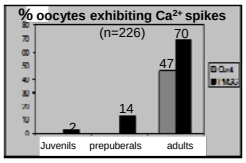
Lefèvre et al. (**1997**) **Exp Cell Res** 236:193-200.

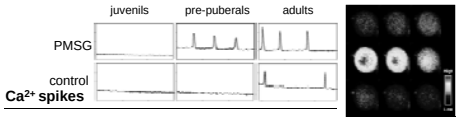
Carroll et al. (**1994**) **Development** 120:3507-3517.

Gonadotropins and **female age** increase activation competence acquisition in oocytes from a defined preantral follicle population

Mouse model







Gomes et al (**1999**) Int J Dev Biol 43: 839-842.

The cytoplasmic reorganization during oocyte maturation confers **competence for activation** in the mouse model

- Involves the calcium stores (SER) and the cortical granules
- Corresponds to part of the classic cytoplasmatic component of oocyte maturation
- Mechanisms proposed:
  1. changes in the regulation and an increase in levels of InsP3 receptor
  2. changes in the structure of the calcium stores (SER)
  3. changes in the size of the calcium store itself

Ducibella (**1996**) The cortical reaction and developmental of activation competence in mammalian oocytes. **Hum Reprod Update** 2:29-42.

Cheung et al (2000) Hum Reprod 15: 1389-1395.

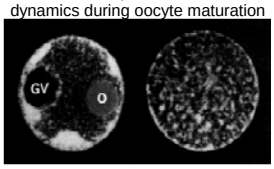
The maximal sensibility to the mechanisms of calcium release seems to occur at the end of oocyte maturation, just before ovulation in the human model

- That sensibility increase seems be due to the redistribution of the intracellular calcium deposits in the oocyte.

MA Edson et al. (**2009**) The Mammalian Ovary from Genesis to Revelation. **Endocrine Reviews**, 30:624-712.

Interestingly, an element of the cytoskeleton may be involved in the **competence for activation** in the hamster model

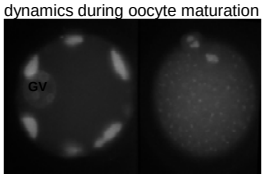
Smooth Endoplasmic Reticulum dynamics during oocyte maturation



Prophase I Metaphase II

Shiraishi et al. (**1995**) Developmental changes in the distribution of the endoplasmic reticulum and inositol 1,4,5-triphosphate receptors and the spatial pattern of Ca^{2+} release during maturation of hamster oocytes. **Dev Biol** 170:594-606.

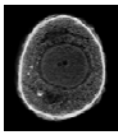
Cytokeratin could be involved in SER dynamics during oocyte maturation



Prophase I Metaphase II

Plancha (**1996**) Cytokeratin dynamics during oocyte maturation in the hamster requires reaching of metaphase I. **Differentiation** 60:87-98.

Oogenesis as acquisition of functional competencies



1. Meiotic
a. competence to reach the metaphase II arrest
b. competence to allow correct meiotic chromosome segregation

2. Activation
competence to fuse with sperm, finish meiosis, block polyspermy, and form pronuclei

3. Developmental
competence to trigger and support embryonic development

Developmental competence: Oocyte capacity to support embryonic development

Mouse model

Acquisition

⇒ During oocyte growth, (to full size ±80μm), before oocyte maturation

⇒ Sequential acquisition, first of the capacity to reach the 2-cell stage, and than to reach the blastocyst stage

⇒ Importance of animal age, follicular growth, gonadotropins and germ-somatic cell interactions

Eppig and Schroeder (**1989**) Biol Reprod 41: 268-276.

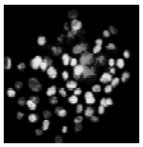
Eppig (**1993**) Serono Symposia, USA Series. (Barry Bavister, ed). Springer-Verlag New York, Inc., pp. 43-53.

Developmental competence acquisition: mechanisms of long-term effects

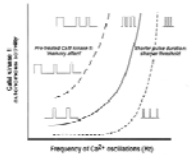
Mouse model

⇒Different patterns of the Ca^{2+} oscillations at fertilization and during the first cell cycle influence the ratio of ICM to TE cells in blastocysts

⇒The frequency of Ca^{2+} oscilations at activation influences the rate of implantation after embryo transfer



Stachecki and Armant (1996) Development 122:2485-2496.
Bos-Mikich et al. (1997) Dev Biol 182:172-179.
Swann and Ozil (1994) J Physiol 483: 331-346.



How could the frequency of Ca^{2+} oscilations be decoded by the oocyte?

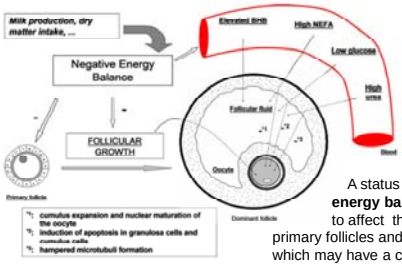
Ca^{2+} / Calmodulin kinase II could function as a frequency decoder of Ca^{2+} oscilations inside the oocyte

CaM kinase II activity ↑↑ exponentially with the frequency of Ca^{2+} spikes

Dupont and Goldbeter (1998) BioEssays 20: 607-610.
De Koninck and Schulman (1998) Science 279: 227-230.

Metabolic mechanisms linking negative energy balance and **Oocyte Developmental Competence**

Bovine model

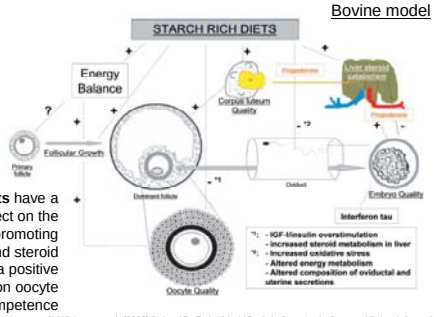


A status of **negative energy balance** is thought to affect the health of the primary follicles and of follicle growth which may have a carry-over effect on oocyte developmental competence

JLMR Leroy et al. **(2008)** Reduced Fertility in High-yielding Dairy Cows: Are the Oocyte and Embryo in Danger? Part I - The Importance of Negative Energy Balance and Altered Corpus Luteum Function to the Reduction of Oocyte and Embryo Quality in High-yielding Dairy Cows. **Reprod Dom Anim 43: 612–622.**

Major mechanisms how starch-rich diets can affect **Oocyte Developmental Competence**

Bovine model

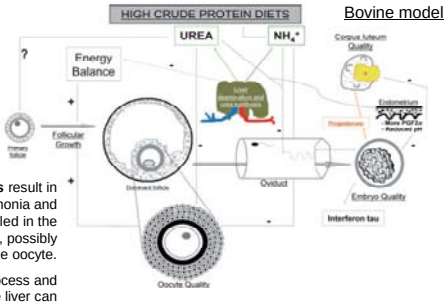


Starch-rich diets have a glucogenic effect on the energy balance, promoting follicular growth and steroid production, with a positive effect on oocyte developmental competence

JLMR Leroy et al. **(2008)** Reduced Fertility in High-yielding Dairy Cows: Are the Oocyte and Embryo in Danger? Part II - Mechanisms Linking Nutrition and Reduced Oocyte and Embryo Quality in High-yielding Dairy Cows. **Reprod Dom Anim 43: 623–632.**

Mechanisms how high protein diets can affect **Oocyte Developmental Competence**

Bovine model



Protein-rich diets result in elevated blood ammonia and urea, paraleled in the follicular fluid, possibly harming the oocyte.

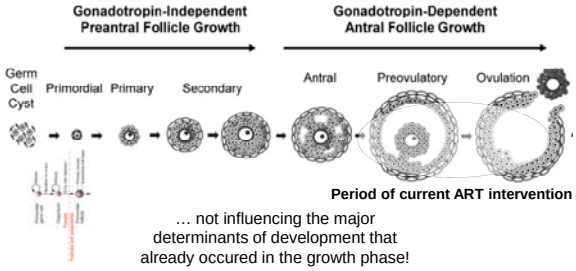
The deamination process and urea synthesis in the liver can exacerbate the negative energy balance.

JLMR Leroy et al. **(2008)** Reduced Fertility in High-yielding Dairy Cows: Are the Oocyte and Embryo in Danger? Part II - Mechanisms Linking Nutrition and Reduced Oocyte and Embryo Quality in High-yielding Dairy Cows. **Reprod Dom Anim 43: 623–632.**

Current ART intervention occurs at late stages of the **Oogenesis** process …

Ovarian follicle development in humans - poorly understood (except Gonadotropin-dependent last stages)

Primordial → ~120-180 days → Preantral → ~90 days → ovulation



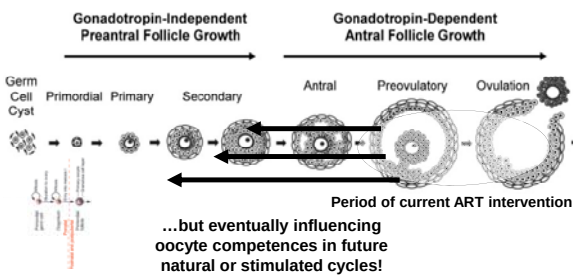
Period of current ART intervention

… not influencing the major determinants of development that already occured in the growth phase!

Current ART intervention occurs at late stages of the **Oogenesis** process …

Ovarian follicle development in humans - poorly understood (except Gonadotropin-dependent last stages)

Primordial → ~120-180 days → Preantral → ~90 days → ovulation



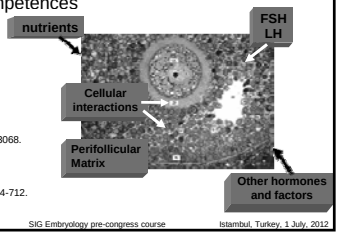
Period of current ART intervention

…but eventually influencing oocyte competences in future natural or stimulated cycles!

Chalenges of research today

• To fully understand the biological mechanisms underlying oocyte competence acquisition during oogenesis

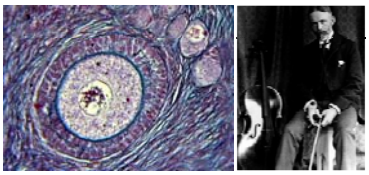
• To identify hormonal, nutritional, culture conditions, pathological situations and other factors able to modify *in vivo* and/or *in vitro* the acquisition of oocyte competences



Eppig et al. (1998) Biol Reprod 59: 1445-1453.
Rabindranath et al. (1999) Hum Reprod 14: 3060-3068.
Merriman et al. (1998) Hum Reprod 13: 690-695.
Ikeda et al. (2000) Biol Reprod 63: 1067-1074.
MA Edson et al.. (2009) Endocrine Reviews, 30:624-712.

During **Oogenesis** the oocyte acquires **critical functionalities** for initial **Development**

"(…) embryogenesis begins during oogenesis"



Edmund B. Wilson (**1925**) The Cell in Inheritance and Development. 3rd edition. Macmillan, New York.

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JJ Eppig (1993) Serono Symposia, USA Series. (Barry Bavister, ed). Springer-Verlag New York, Inc., pp. 43-53.
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Ikeda et al. (2000) Biol Reprod 63: 1067-1074.

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Juengel et al. (2002) Biol Reprod 67:1777.

Kanatsu-Shinohara et al. (2000) Biol Reprod 63:1610-1616.

De Koninck, Schulman (1998) Science 279: 227-230.

Kono et al. (1996) Nat Genet 13: 91-94.

B Lefèvre et al. (1997) Exp Cell Res 236:193-200.

JLMR Leroy et al. (2008) Reprod Dom Anim 43: 612–622.

JLMR Leroy et al. (2008) Reprod Dom Anim 43: 623–632.

B Mattson, DF Albertini (1990) Mol Reprod Dev 25: 374-383.

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CE Plancha (1996) Differentiation 60:87-98.

CE Plancha, DF Albertini (1994) Biol Reprod 51:852-864.

Rabindranath et al. (1999) Hum Reprod 14: 3060-3068.

Y Saga (2008) Curr Opin Genet & Develop 18:1–5.

Shiraishi et al. (1995) Dev Biol 170:594-606.

Sorensen, Wassarman (1976) Dev Biol 50:531-536.

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A Trounson et al. (1998) Hum Reprod 13 (Suppl. 3):52-62.

D Wickramasinghe, DF Albertini (1992) Dev Biol 152:62-74.

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Past / present staff at ART Unit CEMEARE, Lisbon

MJ Carvalho (Head), D Rodrigues, T Rocha, SC Correia, P Sá e Melo, L Vicente, M Rato, PR Carvalho, G Pinto, I Reis, S Pimentel, PN Costa

Other past / present colaborators

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A Neves, S Jorge, S Correia (Unid. Medicina da Reprodução, MAC, Lisboa, Portugal)

DF Albertini (Kansas City Medical Center, USA) J Smitz (Dutch-Speaking Brussels Free University)

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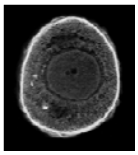
SCIENCE MOVING PEOPLE MOVING SCIENCE



Oogenesis: acquisition of oocyte competences

Carlos E. Plancha

Thank You !

The impact of growth factors in culture medium on early embryo development?

Professor Daniel R Brison PhD, FRCPath
Department of Reproductive Medicine
St Mary's Hospital, Manchester
University of Manchester, UK

Disclosure

D R Brison is a shareholder in Novocellus Ltd, a company which is developing methods for diagnosing embryo health

Learning objectives

- Describe the rationale for adding growth factors or cytokines to human embryo culture media.
- Be able to discuss candidate growth factors and their impact on embryo development.
- Be aware of potential impacts on future offspring.

Overview

- Growth factors and cytokines in early embryo development
- Growth factors and cytokines as culture media supplements
- Impact of growth factors and cytokines on embryo development
- Long term impacts/concerns

In vitro veritas…

Mimic the in vivo environment

Key questions

- How good is our human embryo culture system?
- How closely does it mimic in vivo?
- Endpoints:
  - embryo development
  - live birth success rate
  - long term health outcomes
- Is our medium lacking anything?

Autonomy/adaptability of the embryo

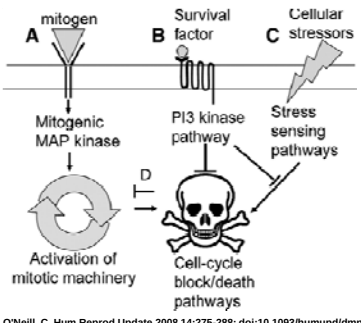
- "Eggs and embryos are relatively autonomous and have astonishing regulatory powers"
  - Anne McLaren (1976)
- "(and can) adapt to the artificial environments that are inevitably imposed on them when placed in culture"
  - Lawitts & Biggers (1993)
- "They are capable of developing in media ranging from simple balanced salt solutions to complex systems involving serum and somatic cells... …however, embryos are sensitive to environmental conditions that can affect future developmental potential both pre-and post-natally"
  - Lonergan et al (2006)

Slide courtesy of Henry Leese

Is anything lacking in our culture system ?

- Preimplantation embryos develop autonomously in defined media
- In vitro development is compromised compared to in vivo:
  - Animal: Bowman and McLaren (1970), Paria and Dey (1990), Brison and Schultz (1997)
  - Human?
- Need for exogenous factors?
- Growth factors and cytokines (GF/CKs)?

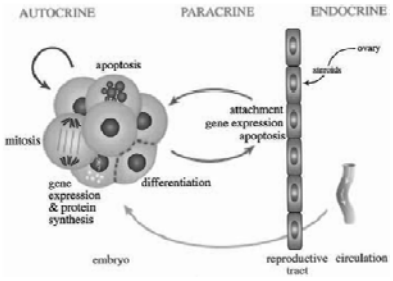
Canonical actions of trophic Ligands



O'Neill, C. Hum Reprod Update 2008 14:275-288; doi:10.1093/humupd/dmn002

Human Reproduction Update

Growth factors in vivo



Hardy and Spanos (2002)

Evidence for need for GFs in human embryo development

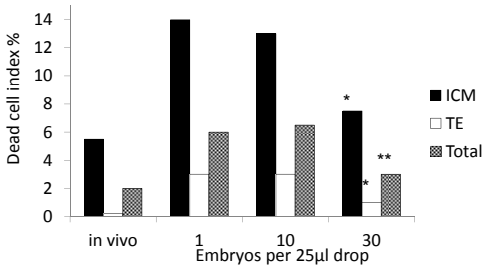
- In vitro development compromised (?)
- Maternal tract expresses GFs
- Embryo expresses GFs and receptors
- Embryo group culture/low volume medium improves development
- Co-culture with somatic cells can improve development

Kane 1997

How is development compromised in vitro?

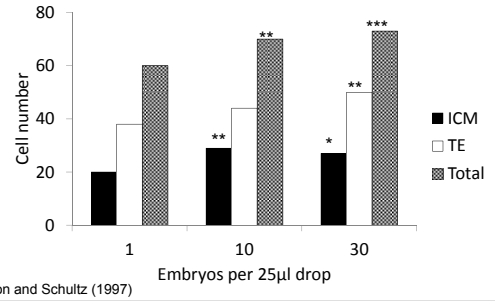
- Mouse embryos cleave more slowly in vitro than in vivo
  - (Bowman and McLaren, 1970)
- Reduced blastocyst formation, cell number and increased apoptosis
  - (Jurisicova et al., 1996, 1998; Brison and Schultz, 1997)
- Partially reversed by group or low volume culture
  - (Wiley, 1986; Paria and Dey, 1990; Lane and Gardner, 1992; Brison and Schultz, 1997)

Effect of embryo culture and density on mouse blastocyst apoptosis



Brison and Schultz (1997)

Effect of embryo culture density on cell proliferation and allocation



Brison and Schultz (1997)

Evidence for autocrine/paracrine factors in early embryos:

A culture distance of 81-160µm is optimal for the culture of in vitro produced porcine/bovine embryos providing evidence for embryo cross-talk in vitro

Stokes et al Developmental Biology 284: 62: 2005
Gopichandran and Leese Reproduction 131: 269-277: 2006

Slide courtesy of Henry Leese

Animal embryo co-culture with somatic cells - evidence for a role for GFs?

- Co-culture improves development in animals
- ? In humans (Guerin and Menezo, 2010)
- Effect mediated metabolically, or via GFs?
- Interestingly, co-culture on feeder cells (fibroblasts) is routine in culture of human embryonic stem cells. Can be replaced by GF supplementation...

Evidence for GF role in human embryos

Growth Factor	Maternal tract ligand	Embryo receptor	Culture effect
EGF	✓	✓	
TGFα	✓	✓	
HB-EGF	✓	✓	✓
IGF1	✓	✓	✓
IGF2	✓	✓	
GM-CSF	✓	✓	✓
LIF	✓	✓	✓

From Richter 2008

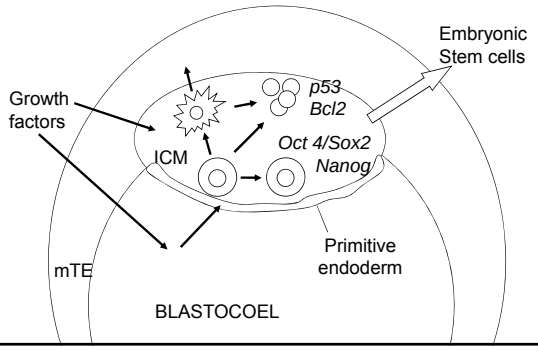
"Embryos are naturally exposed to a **complex mixture of growth factors** that play an important role in preimplantation embryo development and that are likely to be of substantial benefit if added to in-vitro culture media."

Richter 2008

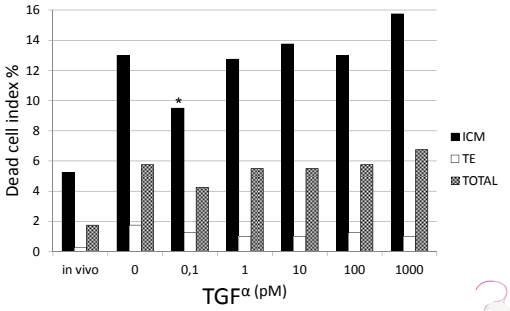
Functions of GFs

- Pleiotropic effects on the embryo
  - blastocyst formation (LIF, HBEGF, PAF)
  - cell division (IGF1, FGF4)
  - cell differentiation (TGFα)
  - embryo metabolism (insulin, IGF1)
  - ion transport (TGF α)
  - gene expression (TGFα)
  - implantation (LIF)
  - apoptosis (TNFα, TGFα, IGF1)

Cell fate in the mammalian blastocyst

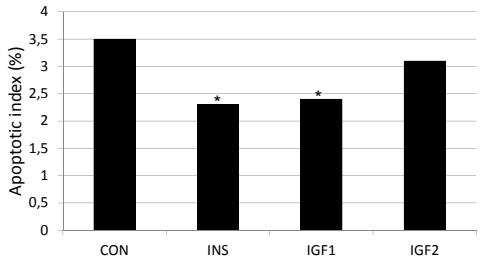


Effect of TGFα on blastocyst apoptosis



Brison and Schultz, 1997

Effect of IGFs on apoptosis



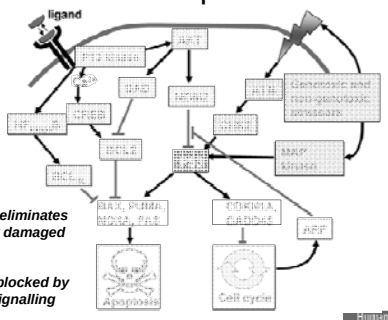
Brison, 2000; Byrne et al, 2002; Spanos et al., 2000

The effect of culture in GM-CSF on apoptosis in blastocysts

	Control	GM-CSF
n	29	32
Number total cells	111 ± 17	134 ± 20
Number ICM cells (%)	36 ± 9 (32)	51 ± 8 (39)
Number TE cells (%)	75 ± 9 (68)	82 ± 15 (61)
Total apoptotic cells (%)	5.6 ± 3.0 (4.9)	2.8 ± 1.4 (2.1)
Apoptotic ICM cells (%)	2.4 ± 1.5 (6.3)	0.75 ± 0.76 (1.5)
Apoptotic TE cells	3.2 ± 2.2 (4.2)	2.1 ± 1.5 (2.6)
Mitoses (%)	3.3 ± 1.3 (3.1)	3.5 ± 1.7 (2.6)

Sjöblom et al., 2002

P53 is a critical guardian of cell survival and proliferation

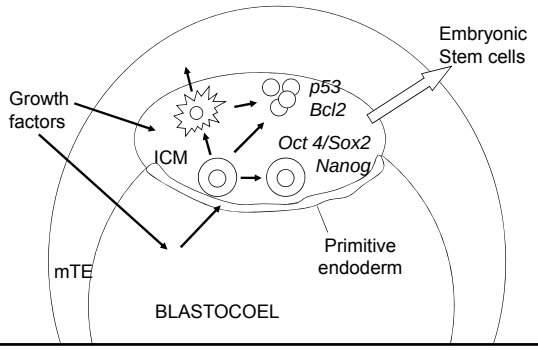


Apoptosis eliminates genetically damaged cells

Pathways blocked by GF- PI3K signalling

D'Neill, C. Hum Reprod Update 2008 14:275-288; doi:10.1093/humupd/dmn002

Cell fate in the mammalian blastocyst



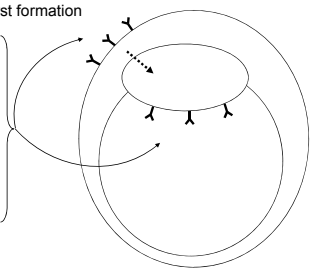
Growth factor regulation of human embryo development and cell fate

GFs promote human blastocyst formation

HBEGF
Martin et al., 1998

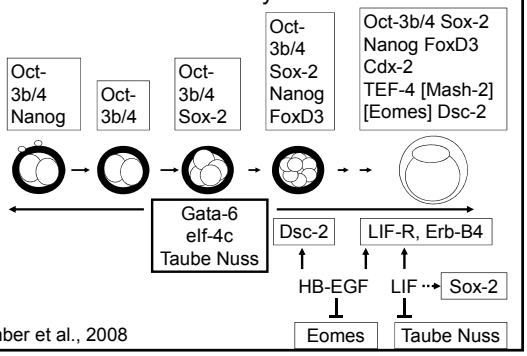
LIF
Dunglison et al., 1996

IGF1
Lighten et al., 1998



Culture from day 2-5 in physiological concs of LIF, IGF1, HBEGF

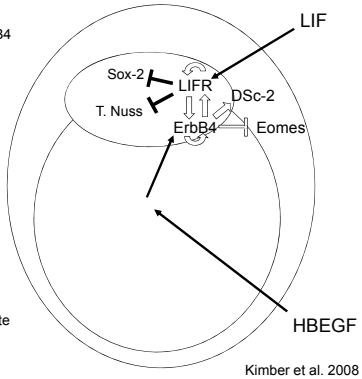
Cell fate genes and their regulation by growth factors in the human embryo



Kimber et al., 2008

LIF and HBEGF both upregulate LIFR and EbrB4

?cross talk between pluripotency and self-renewal pathways in ICM

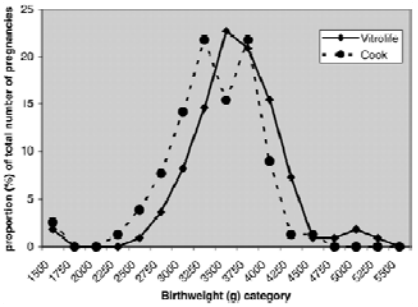


LIF may regulate ICM pluripotency via Taube Nuss and Sox2

HB-EGF may block TE fate by blocking Eomes not Cdx2

Kimber et al. 2008

Birthweight distributions of live born singletons resulting from embryo culture in either Vitrolife or Cook sequential media



Dumoulin, J. C. et al. Hum. Reprod. 2010 25:605-612; doi:10.1093/humrep/dep456

Human Reproduction

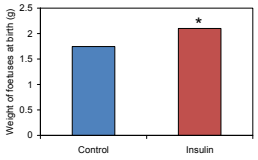
GM-CSF alleviates adverse effect of embryo culture on fetal growth

	Medium	GM-CSF	In vivo
Total BΦ transferred to all recipients	317	316	280
Pregnant recipients/total recipients (%)	29 of 32 (91)	29 of 32 (91)	29 of 29 (100)
Total BΦ transferred to pregnant recipients	288	286	280
BΦ implanted in pregnant recipients (%)	218 (76)	214 (75)	190 (68)
Total implantation sites	7.5 ± 0.2	7.3 ± 0.2	6.4 ± 0.2
Viable implants/BΦ transferred (%)	205 of 288 (71)	207 of 286 (72)	172 of 280 (61)
Viable implants/total implants (%)	205 of 218 (94)	207 of 214 (97)	172 of 190 (91)
Resorptions/total implants (%)	13 of 218 (6.0)	7 of 214 (3.3)	18 of 190 (9.5)
Fetal weight (mg)	1160 ± 10	1206 ± 9	1291 ± 13
Placental weight (mg)	123 ± 2	124 ± 2	123 ± 2
Fetal to placental weight ratio	9.7 ± 0.1	10.0 ± 0.1	10.9 ± 0.2

Sjöblom et al., 2005

Effect of pre-implantation exposure to insulin

- Increases foetal weight
- Increases expression of imprinted genes H19, igf2
- Decreases DNA methylation



Shao et al., 2007

Summary

- Need for GFs in human embryo culture media?
- Candidate factors: IGF1, EGFs, GM-CSF
- Effects on embryo development: mitogenic, apoptosis, cell pluripotency, + unknowns
- Post-implantation effects on fetal development, birthweight, imprinting

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Novocellus Ltd
ANGLE plc
Origio

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Mitochondrial dynamics and energy production in the oocyte to embryo transition

John Carroll

Laboratory for Oocyte and Embryo Development

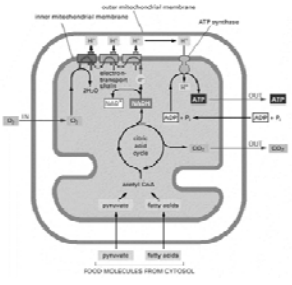
Biosciences

UCL

Learning Objectives

1. To understand the role of mitochondria in oocytes and embryos
1. That mitochondria are dynamic organelles and are actively localized during oocyte maturation.
1. That mitochondria are responsible for ATP generation in oocytes and embryos and that ATP turnover is modulated during maturation.
1. That ATP can be measured in living single oocytes using a FRET-based fluorescent probe.
1. Mitochondrial distribution and activity is modulated by intrinsic factors in the oocyte and extrinsic factors including the presence of cumulus cells and even the maternal environment.
1. Culture media can also be expected to influence mitochondrial activity and ATP measurement may provide a means of assessing culture media quality.

Mitochondria are special!



Mol Biol Cell

All mitochondria in an organism are inherited from those present in the oocyte.

Mitochondria generate most ATP in oocytes and embryos

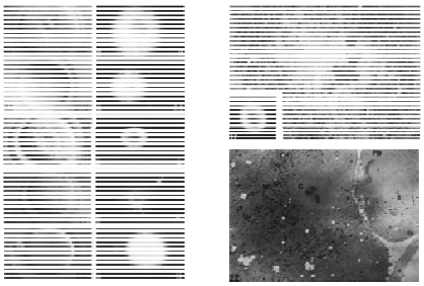
Dramatic expansion of mitochondrial numbers during oocyte growth.

Mitochondrial generate ROS

Mitochondrial dysfunction leads to developmental arrest

Mitochondria can initiate apoptosis

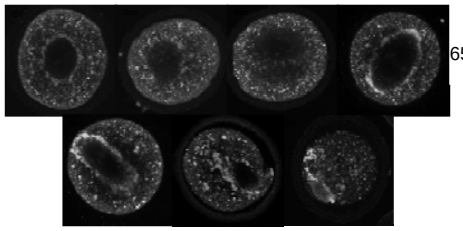
Distribution of mitochondria in oocytes



Mitochondrial Reorganization During Resumption of Arrested Meiosis in the Mouse Oocyte
Van Blerkom and Runner, The American Journal of Anatomy, 1984

65%

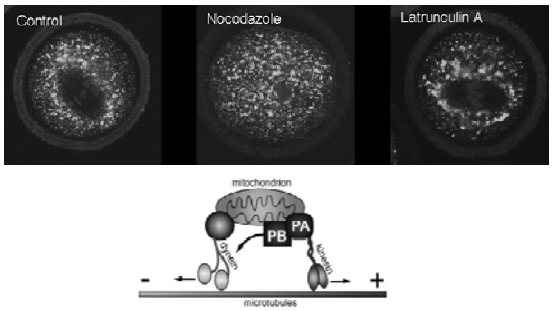
Distribution of mitochondria during oocyte maturation



65%

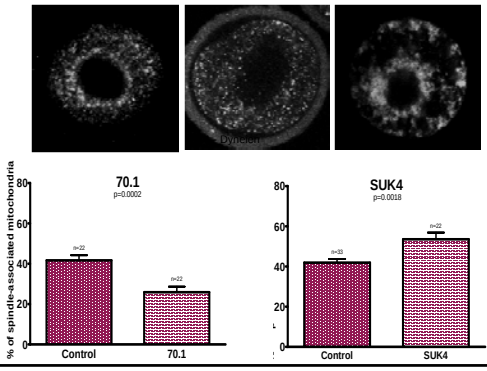
Caroline Dalton

Mitochondrial aggregation requires microtubules not microfilaments

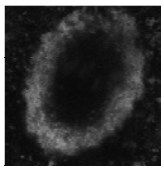


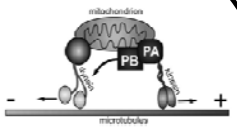
Milton controls the early acquisition of mitochondria by *Drosophila* oocytes,
Cox and Spradling, Development, 2006

Motor proteins actively distribute mitochondria



Mitochondrial dynamics during oocyte maturation



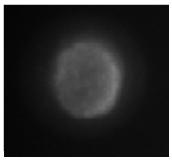


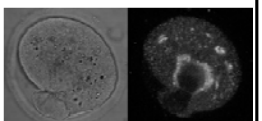
During maturation the balance favours dynein mediated trafficking.

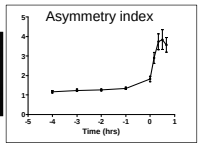
.....But what happens at Pb formation.....

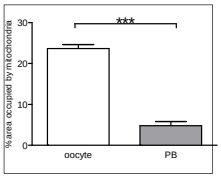
ry index

Mitochondria are actively retained in the oocyte

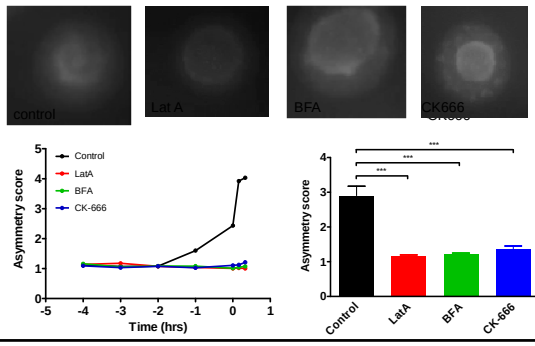




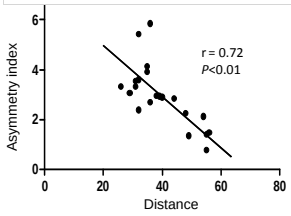




Spindle migration is necessary for asymmetrical mitochondrial inheritance

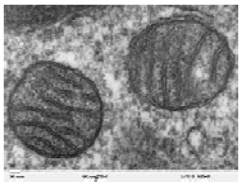


Asymmetry of mitochondrial inheritance is related to distance from the cortex



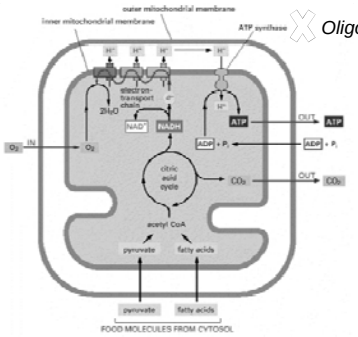
Spindle-cortical interactions appear to facilitate the asymmetric inheritance of mitochondria

ENERGY

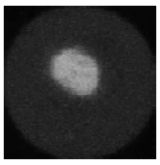


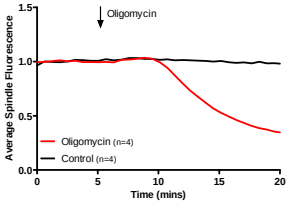
ATP

ATP production by mitochondria

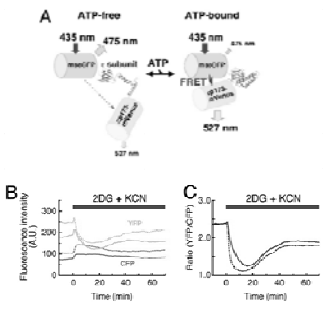


ATP depletion leads to spindle disassembly



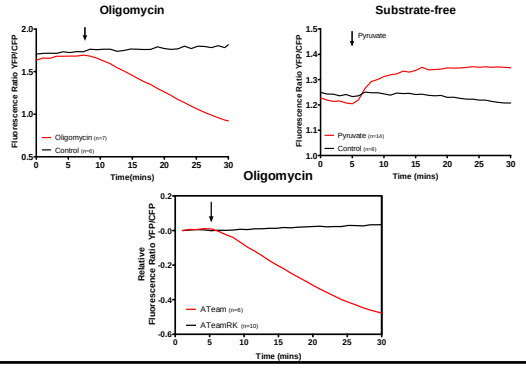


Measuring ATP in living oocytes

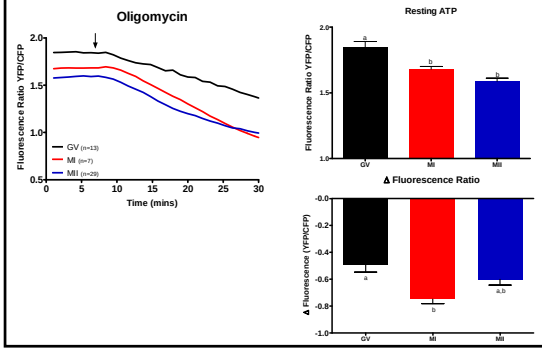


Imamura H et al. PNAS 2009;106:15651-15656

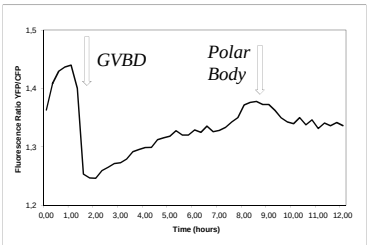
Measuring ATP in living oocytes



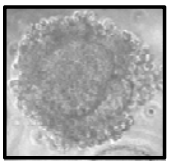
ATP levels and ATP consumption are modulated during oocyte maturation

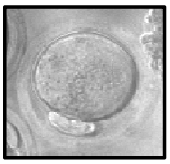


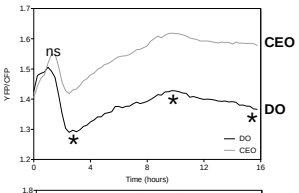
ATP levels change during the course of oocyte maturation

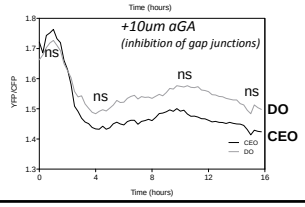


Impact of cumulus cells on ATP in the oocyte









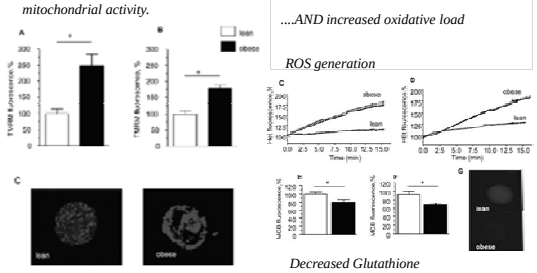
Maternal diet impacts on oocyte mitochondria

Igosheva et al., PLOS One, 2010.

Obese mice have increased mitochondrial activity.

....AND increased oxidative load

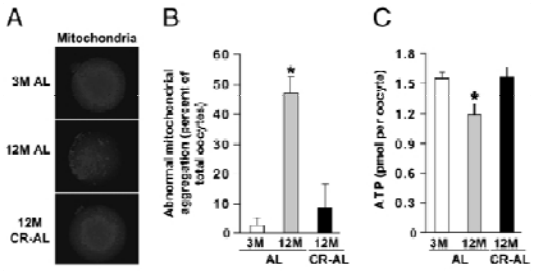
ROS generation



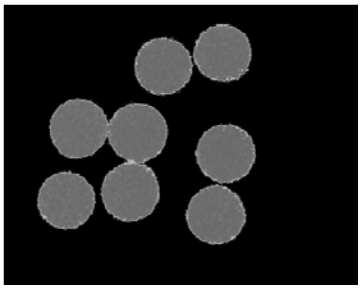
Decreased Glutathione

Ageing-dependent effects on mitochondrial function: reversed by caloric restriction.

Selesniemi et al., PNAS, 2011

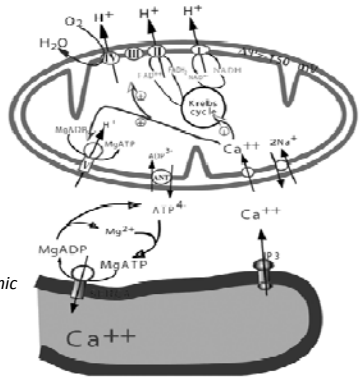


Fertilization stimulates Ca^{2+}oscillations

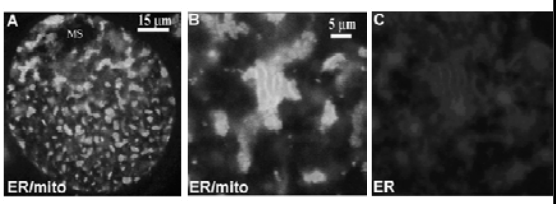


MITOCHONDRIA

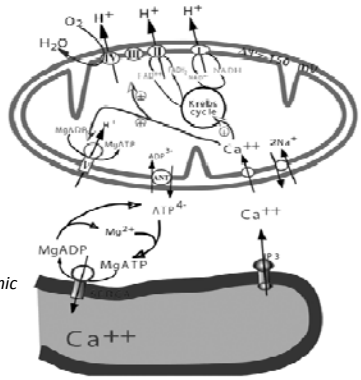
Endoplasmic Reticulum



Endoplasmic reticulum and mitochondria are in close proximity

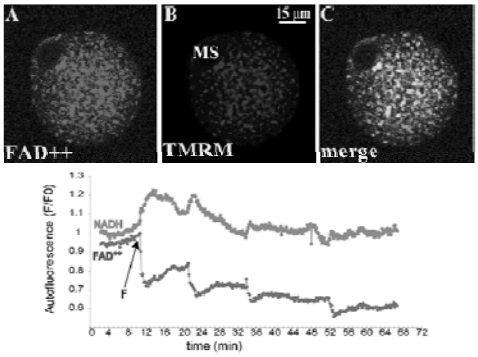


MITOCHONDRIA

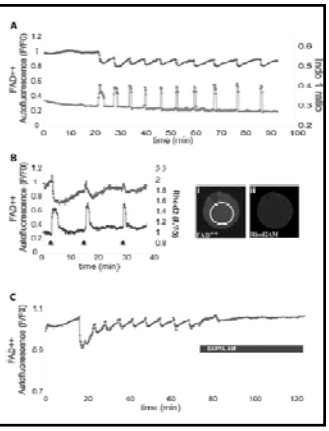


Endoplasmic Reticulum

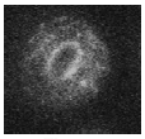
Fertilization stimulates mitochondrial activity

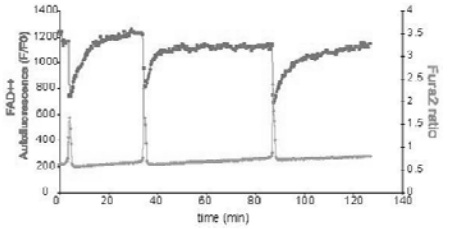


Calcium is necessary and sufficient for mitochondrial activation at fertilization



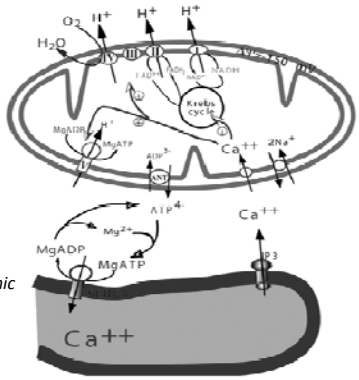
Mitotic Ca^{2+}transients stimulate mitochondrial activity



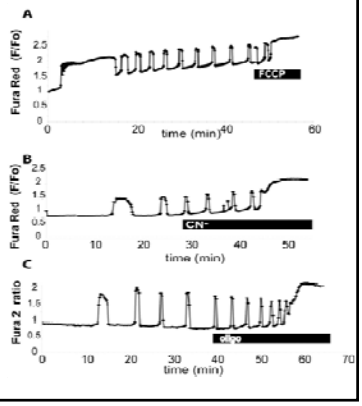


MITOCHONDRIA

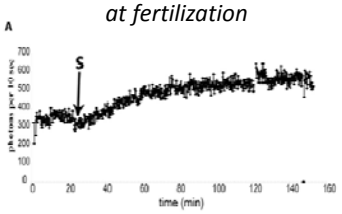
Endoplasmic Reticulum



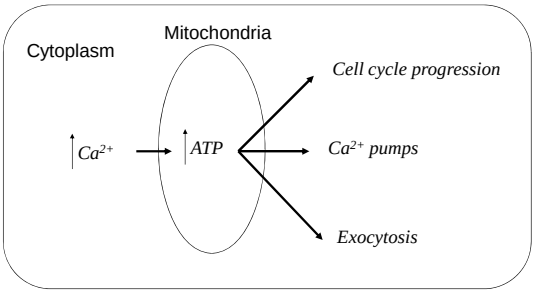
ATP production at fertilization is necessary to maintain calcium oscillations



ATP levels increase at fertilization



ATP – Ca^{2+} may ensure a match in supply and demand



Allows mitochondrial respiration to be set at a minimum level thereby minimizing risk of free radical production to which mitochondria are susceptible.

Conclusions

1. Mitochondria are dynamically organized during maturation via motor proteins, dynein and kinesin.
1. Mitochondria undergo two phases of reorganization. One at the time of spindle formation and another at the time Pb polar body formation.
1. That ATP production and consumption are modulated during maturation.
2. Cumulus cells modulate ATP content in the oocyte.
3. Maternal metabolic state can influence mitochondrial activity.
1. Fertilization stimulates ATP production, providing a mechanism of matching ATP supply with demand.

Development of Culture Media: Impact on Embryo Viability

H. Nadir CIRAY, M.D., PhD.
Associate Professor
BAHCECI Health Group
Embryology Director

Learning Objectives

- To review the knowledge of risks and limitations associated with utilization of human embryo culture media,
- To evaluate the media ingredients and culture strategies that may have a significant impact on embryo development and viability,
- To discuss the impact of these strategies on clinical parameters,

At the end of this presentation

- Audience will be able to make decisions on the type of culture systems appropriate for their clinical requirements

DISCLOSURE

- Share holder/scientific advisor of GONAGEN company
  - Distributor in Turkey, some Middle East, Balkan and FIS countries whole range of IVF products , including
    - Irvine Scientific
    - EmbryoScope

PRESENTATION SCHEME

- Embryo viability and vulnerability
- Ingredients of culture media
- Various strategies of embryo culture
- Is there a difference in embryology/clinical parameters among various embryo culture strategies?

Embryo viability
-Quantification-

1. Utility
2. Cryo-tolerance
3. Live-births
4. *Fetal health?*

Embryo viability
-limitations/determining factor-

- ….. the number of blastocysts that develop in culture reflect the qualities of gametes from which they were derived…

Behr *et al.,* 1998

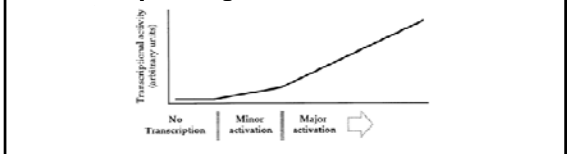
Culture Media is not the whole story!

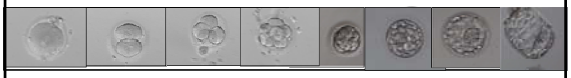
Embryo viability is compromised under in-vitro conditions

- Culture media component does not fully mimic in vivo conditions.

EMBRYO IS STRESSED

Embryonic genome activation





V U L N E R A B L E

Primitive mechanisms for regulation of homeostasis
Low metabolic and biosynthetic activity
Oxidative stress

Increase in biosynthetic activity

Adaptation to stress at early stages

Consequences are evident at later stages

- Increased cell death, diminished embryo development, implantation, fetal growth (Bowman *et al.,* 1970; Brison and Schultz, 1997; Halliday *et al.,* 2010)
- Expression and imprinting of key genes (Doherty *et al.,* 2000; Fauque *et al*., 2007)
  - Cleavage stages are common (Gosden *et al.,* 2003; Halliday *et al.,* 2004; Li *et al.,* 2005)

Impact on fetal development and birth-weight

Dumoulin *et al*., 2010; Nelissen *et al*., 2011

- Singletons conceived by IVF
  - Two commercially available culture media
- 12 weeks; higher levels of fβ-hCG in one medium
- 20 weeks; significantly larger fetuses in the same group
- Babies: significantly higher birth weights

INITIATIVES

- ESHRE
  - 'Task Force?' in 2012?
    - Issues related to development of culture media
    - Research on culture media and epigenetics
- EpiHealth (EU-FP7 Funded)
  - ART child health outcomes
    - Epigenetics, imprinting of genes
      - Consulting experts in UK
      - Work-package to be approved by HFEA
        - HFEA database 1990-2009 to be assessed

Embryo Culture Media

- Simple media
  BSS+ energy substrates
  - Development of zygotes from inbred strains of mice and their F1 hybrids
- Complex media
  BSS + aminoacids, fatty acids, vitamins, nucleotide bases, macromolecules, antibiotics etc.
  - Growth of somatic cells in culture

Ions (BSS)

- Osmolarity higher in vivo than culture media
- Culture media osmolarity range tolerated by many ingredients
  - Glycine and some other aminoacids
  - Osmolytes such as betaine
- The ratios of ions matter

Energy

- Carboxylic acids (pyruvate/lactate/aspartate) and aminoacids prior to genomic activation (lipids?)
- Glucose after compaction
- Glucose has many functions even before compaction
  - Synthesis of triaclylglycerols/phospholipids
  - Precursor for complex sugars/glycoproteins
  - Generates ribose (nucleic acid synthesis)
- Phosphate beneficial at later stages when embryo acts like somatic cells
- Glucose and phosphate combined only in complex media (with aminoacids and antioxidants) otherwise decrease respiratory/mitochondrial activity/ATP production
  - Only glucose no phosphate
  - No glucose no phosphate

Aminoacids

- Very important for embryo viability/blastocyst development
  - 5 min.s absence impairs subsequent development
    - Media for handling oocytes/embryos
- Turnover described by H. Leese & coll.
- Their ratio may be even more important than their concentrations
  - Competition for membrane transport systems
- Chelators
- Osmolytes
- pHi buffer
  - Glycine (osmoregulator, pH regulator, precursor for proteins and nucleic acids)
- Antioxidant
- Energy metabolism regulator
- Biosynthetic precursor
- Energy substrate

Aminoacids

- Absence disrupts normal imprinted expression of H19
  - Bi-allelic expression
  - S-aminoacids (methionine)=methylation of nucleic acids
  - Cysteine to taurine= neutralize toxic aldehyde by-products
  - Cysteine to glutathione= maintain redox potential
  - Homocysteine to Methionine pathway poorly expressed in human oocyte / not existent (Benkhalifa *et al*., 2010)
  - Protein synthesis requires methionyl t-RNA, cysteine and methionine important
- Pre-compaction= non-essential+***glutamine*** *(carbon+energy)*
- Post-compaction= + essential (for ICM)

(L)- Glutamine

- Unstable
  - Produce ammonium at 37° C
    - alter blastocyst development/ gene expression
    - cellular health of blastocysts may be affected
      - Animal models
        - Reduced implantation
        - Increased fetal loss
- Alanyl-glutamine
- Glycyl-glutamine
- Media renewal every 24 hours

L- Glutamine

- Weakly degraded in slightly alkaline conditions (culture media)
- Conjugation does not help degradation

Antioxidants

light, elevated oxygen, transitional metals, etc.

- Pyruvate
  - Ammonium to alanine
- EDTA + aminoacids
- Lipoate
- Glutathione
- Taurine
- Vitamins + aminoacids

Chelators (EDTA/Transferrin?)

- Binds metal ions for them to remain in solution but exhibit diminished activity
- Presence in during fertilization may chelate calcium required from sperm motility, capacitation, acrosome reaction
- Should be removed after compaction
  - Inhibits glycolysis and may cause reduced ICM development
- Should not be removed after compaction
  - Reduced concentration from 0.1 mmol/L to 0.005-0.01 mmol/L have no effect/stimulatory?

Macromolecules

- Patient serum (no longer acceptable!)
  - Metabolic alterations
  - Ultrastructural changes
  - Methylation disorders in imprinted genes
  - Fetal overgrowth
- Human Serum Albumin
  - Cell membrane stabilization
  - Trace/chelate toxic components
  - Capillary membrane permeability
  - Osmotic regulation
  - Easy manipulation
  - Significant differences between sources and even from the same source
  - Recombinant available
- Hyaluronan
  - Can substitute albumin
  - In synergy with albumin
    - Increased development
    - Improved cryosurvival

Antibiotics

- Faster cleavage in antibiotic-free media
  - Risk of contamination

Growth factors

- Usually very costly
- Transition from morula to blastocyst
- Blastocysts express ligands and receptors for several growth factors
  - Cross-reaction possible
    - Difficult to interpret effect of a single factor

Addition of Peptide Growth Factors in Culture Media

(Sjoblom *et al.*, 2005; Harper *et al.*, 2011)

- IGF-I (insulin-like growth factor)
- HF-EGF (heparin-binding epidermal growth factor)
  - Increased cell proliferation,
  - Decreased apoptosis,
    - Elimination of cells that are chromosomally defective, structurally defective, or genetically abnormal ?
  - Promote blastocyst formation

Nucleic Acid Precursors

- Not required (Gardner *et al.*, 2007)
  - De-novo synthesis for DNA repair
- Required (Menezo *et al.*, 2011)
  - ROS can decay bases to cause genetic instability
  - Should be removed to avoid reintroduction into DNA
  - Human oocyte can sanitize but needs energy
  - The embryo can transport bases from the surrounding requiring less energy

Embryo Culture Systems

- BACK TO NATURE

Sequential

- Media change at day3
- Media change every day

- LET THE EMBRYO CHOOSE

Monoculture

- Media change at day3
- No change of media until day5

BACK TO NATURE

(Leese & Hardy, Bavister, Quinn, Gardner)

- The changing needs of the developing zygote is mimicked
- The concentrations should approximate to what the embryo is exposed to
- Embryo physiology
  - Pre- and post compaction stages

LET THE EMBRYO CHOOSE (Biggers)

- Simplex Optimization Media (SOM)
  - Eventually mKSOM-AA
  - Determines optimal concentration of each component
- Simultaneous use of all the concentrations in a mixture
  - Effects of each component may depend on the concentrations of the other components
- As long as the concentrations are within the tolerable range, the embryo will adapt itself and will utilize whatever it requires

Sequential is better because;

- End-point is NOT blastocyst development
  - Embryo viability
  - Implantation
- Post-compaction stage; through inhibition of glycolysis, EDTA has a negative impact on ICM/fetal development

Monoculture is better because;

- Measurements of the components of oviduct and uterine fluids are highly variable and are subject to changes
- These measurements only reflect the overall composition of the tract fluids, not the micro-environment around the embryo
- The physical and chemical environment of the embryo in vivo is completely different from its environment in vitro
  - There is no pool of fluid (except pathology)
    - Large pool of fluid in vitro (accumulating waste products, consuming nutrients)
  - Embryo is surrounded by thin layer of fluid
  - Close apposition to maternal tissues; rapid exchange of nutrients, gases, wastes, effectors..
- Stress is different!

Clinical studies

Biggers&Racowsky, 2002

- KSOM-AA versus P1+CCM
- Blastocyst yield was similar

Clinical studies

Macklon *et al*., 2002

- Randomization of patients
- Several manufacturers
- Outcome parameters;
  - Embryo development
  - Clinical
- No difference

Clinical studies

Sepulveda *et al*., 2009

- Randomization of patients (donors)
- Several manufacturers
- Monoculture
  - More compacted embryo at day3
  - More morula at day4
  - Higher blastocyst yield at day5
  - Higher implantation rate

Clinical studies

Reed *et al*., 2009

- Randomization of fertilized oocytes
- Several manufacturers
- Similar embryo quality at day3
- More blastocysts at day5 in monoculture
- Clinical data inconclusive
  - Most transfers included both groups

Clinical studies

Paternot *et al*., 2010

- Randomization of patients
- Several manufacturers
- Outcome parameter
  - Utilization rate
- Similar early cleavage rates
- Higher cell number at days 2 and 3 in monoculture
- Higher utilization rate in monoculture

Conclusions

- Ingredients of media for in vitro culture of human embryos are important
- Embryo culture media are different
- However, embryos adapt themselves
- The consequences of adaptation are not clear
- Sub-optimal conditions affect dominantly cleavage-stages but the impact may not be evident until long-term observation of embryos, even fetuses

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Nutrient requirements from the oocyte to the blastocyst: implications for embryo culture

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LEARNING OBJECTIVES

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

Describe various approaches, their strengths and limitations, for determining egg and early embryo nutrient requirements including those based on:

- **Physiological and biochemical knowledge**
- **Measurements of nutrient utilisation**
- **Quantitative studies: transport kinetics**
- **Culture without exogenous nutrients**
- **Consideration of the relationship between nutrient turnover and subsequent viability: the possible role of 'quiet metabolism'**

DISCLOSURE

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

Defining nutritional requirements from oocytes to blastocysts

- **Basis of nutritional requirements in man**
- Basis of nutritional requirements for eggs and embryos

  Physiological and biochemical knowledge

  Utilisation of nutrients including transport kinetics

  Composition of oviduct/uterine fluid (second lecture)
- Relationship between nutrient turnover and subsequent development: role of *quiet metabolism* (?)

Dietary Reference Values for Food Energy and Nutrients for the UK
Basis of nutritional requirements in man:

Energy: based on **basal metabolic rates** plus increments for physical activity

Protein: based on principles of **nitrogen balance**

Vitamins and minerals:
- deprivation studies to **define minimum**
- measurement of levels in tissues
- biochemical markers (e.g. riboflavin)
- biological markers (e.g. iron)
- animal experiments

Calculation of protein requirement (WHO/FAO)

Obligatory losses on protein-free diet	mg N/kg body wt
Urine	37
Faeces	12
Skin + miscellaneous	5
Total	54

	per kg body wt
Requirement to replace obligatory losses	54
Additional amount to maintain N balance (+30%)	66
Additional amount to cover individual variation (+30%)	86
Expressed at protein (x6.25)	0.54g
Allow for protein quality (75%)	0.72g
Intake for 65kg man	47g/d
% of energy intake	~ 8%

Average protein intake in UK = 70g/day

Urinary N metabolites

urea	**10 – 35 g /day**
ammonium ions	**340 – 1200 mg /day**
amino acids	**1.3 – 3.2 g /day**
uric acid	**250 – 750 mg /day**
creatinine	**women 1.2, men 1.8 g /day**
creatine	**< 50 mg /day**

From DA Bender: Nutrition and Metabolism: 4th Edition CRC Press (2008)

Calculation of protein requirement (WHO/FAO)

Obligatory losses on protein-free diet	**mg N/kg body wt**
Urine	37
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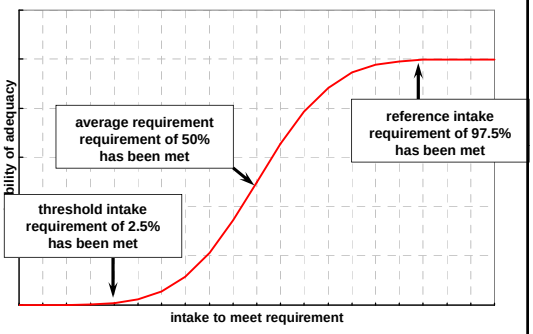
Allow for protein quality (75%)	0.72g
Intake for 65kg man	**47g/d**
% of energy intake	~ 8%

Average protein intake in UK = **70g/day**

Determination of reference intakes

Plotting the same data as a cumulative graph of those whose requirements have been met at each level of intake



Defining requirements

Basis of calculation of nutritional requirements in man :
Defines minimum but adequate level
Takes account of individual variation

Basis of nutritional requirements for eggs and embryos

- Physiological and biochemical knowledge
- Utilisation of nutrients
- Quantitative requirements: transport kinetics
- Culture in absence of exogenous nutrients

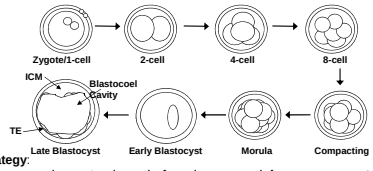
Defining requirements for eggs and embryos

- **Physiological and biochemical knowledge**
- Utilisation of nutrients
  - Oxygen consumption: global marker of energy metabolism
  - Nutrient consumption: amino acids: pyruvate: glucose
  - Quantitative requirements: transport kinetics
  - Culture in absence of exogenous nutrients

Defining nutrient requirements from first principles :

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

Characteristics of preimplantation embryo development:



Strategy:
Large egg: largest volume in female mammal: **large energy store**
Successive 'cleavage' divisions until adult cell size reached
Duplication of nuclear material
No duplication of cytoplasm but membrane proliferation
Diameter of early blastocyst = diameter of egg
Growth (net increase in protein) begins at blastocyst stage
Maternal control prior to activation of embryonic genome

Eggs and embryos are relatively autonomous *and have astonishing regulative powers* (Anne McLaren, 1976)

What is ATP used for?

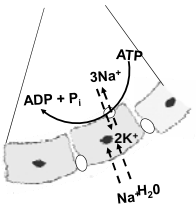
- Protein synthesis (~30-40 %)*
- Na^+K^+ATPase: sodium pump (~30-40 %)**
- DNA/RNA synthesis (~10%)
- Other ATPases
- Substrate cycling

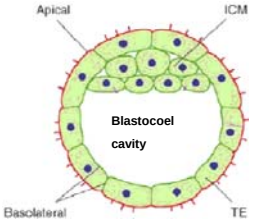
*Net growth, requiring increased protein synthesis, begins at the blastocyst stage

** Required for blastocoel formation

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

Mammalian blastocyst
ICM = Inner Cell Mass
TE = Trophectoderm





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

Predictions:

1. *Embryo requirements for energy substrates and amino acids will be relatively low during early preimplantation development and then increase with blastocyst formation*
2. *Eggs and early embryos have high endogenous reserves which potentially may provide energy*

Defining requirements for eggs and embryos

Physiological and biochemical knowledge

Utilisation of nutrients

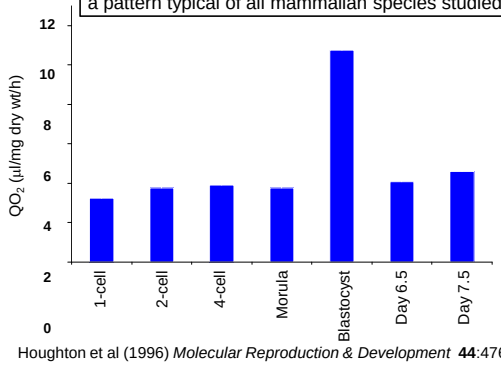
Oxygen consumption: global marker of energy metabolism

Nutrient consumption: amino acids: pyruvate: glucose

Quantitative requirements: transport kinetics

Culture in absence of exogenous nutrients

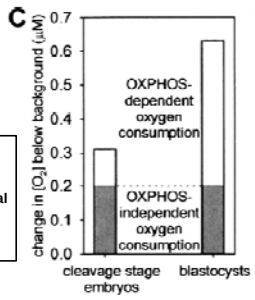
Oxygen consumption by early mouse embryos: a pattern typical of all mammalian species studied



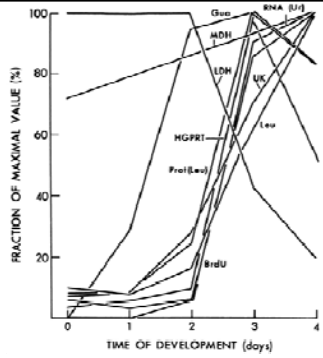
Houghton et al (1996) *Molecular Reproduction & Development* **44**:476-85.

However – not all oxygen consumed is used to make ATP

Trimarchi et al (2000)
Oxidative phosphorylation-dependent and independent oxygen consumption by individual preimplantation mouse embryos
Biology of Reproduction
62, 1866 - 1874



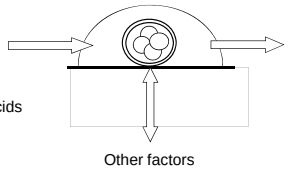
Biochemical changes in mouse preimplantation embryos
Epstein CJ. 1975 *Biol Reprod*: 12:82-105



Increase in uptake of biosynthetic precursors such as amino acids in order to form a blastocyst

Nutrition and metabolism of early embryos: non-invasive assessment

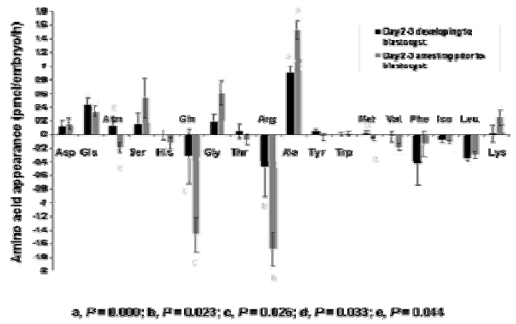
Depletion	Appearance
Pyruvate	H_20
Lactate	CO_2
Glucose	Lactate
Lipid	Amino acids
Amino acids	NH_4^+
Oxygen	Enzymes
	Cytokines
	Proteins



Other factors

Gardner DK. and Leese HJ. (1999). Assessment of embryo metabolism and viability. In: Handbook of In vitro Fertilization Trounson A, Gardner DK (eds). Pp 348-366. CRC Press.

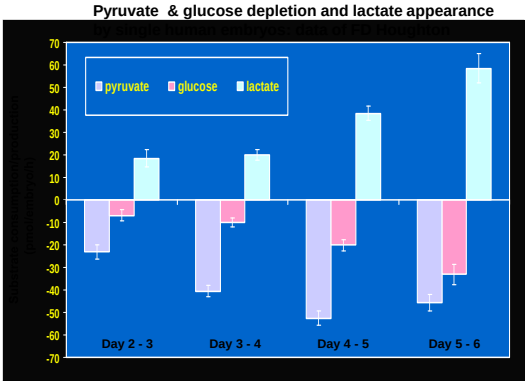
Amino Acid metabolism of single human embryos



Houghton et al (2002) *Human Reproduction* **17**: 999-1005

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

Pyruvate & glucose depletion and lactate appearance

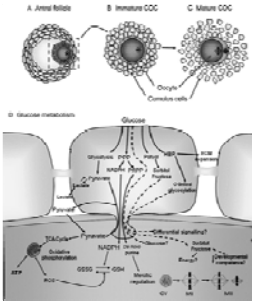


Oocyte metabolism: role of follicle and cumulus in supplying pyruvate and other metabolites

Sutton-McDowall, Gilchrist & Thompson
The pivotal role of glucose metabolism in determining oocyte developmental competence
Reproduction 2010: **139** 685-695

Harris, Leese, Gosden, Picton.
Pyruvate and oxygen consumption throughout the growth and development of murine oocytes
Mol Reprod Dev 2009:**76**:231-8

Harris, Adriaens, Leese, Gosden, Picton.
Carbohydrate metabolism by murine ovarian follicles and oocytes grown in vitro
Reproduction 2007:**134**:415-24.



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

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

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

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

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

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

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

Endogenous lipid: a potential source of energy in early embryos

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

TG is metabolised during oocyte maturation *in vitro*

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

Concomitant change in oxygen consumption *(pig)*

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

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

Culture without exogenous nutrients: a first approach to defining minimum requirements

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

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

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

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

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

- *The Krebs cycle and oxidative phosphorylation provide the main source of energy throughout the preimplantation period.*
- *Pyruvate is a central energy substrate during the first cleavage in those species in which energy source requirements of the embryo have been examined, although it is not obligatory for all species (e.g., porcine).*
- *Other substrates, notably, amino acids, lactate and endogenous fatty acids derived from triglyceride, combine with pyruvate to provide embryos with a range of potential energy sources through to, and including, the blastocyst stage.*
- *These nutrients have numerous, overlapping, metabolic roles.*
- *Prior to the morula stage, glucose consumption and metabolism is low, although some glucose is necessary for intracellular signalling purposes.*
- *With blastocyst formation, large increases in oxygen consumption and the uptake and incorporation of carbon occur and there is a sharp increase in glycolysis, at least in vitro.*
- *The embryo goes from a relatively inactive metabolic tissue at ovulation to a rapidly metabolizing tissue at implantation.*
- *Mitochondria play a pivotal role during early development, as well as providing a cellular focus for metabolic events.*
- *We are almost totally ignorant of the metabolism of preimplantation embryos in situ (in the oviduct and uterus) and understanding of signal transduction within the embryo is in its infancy as is the molecular dialogue between embryos in culture and with the maternal tract in vivo.*

Leese HJ (2012) Metabolism of the preimplantation embryo: 40 years on *Reproduction (in Press).*

Conclusions: Nutritional needs of the egg/early embryo:

Nutritional needs relatively simple:

Cleavage stages metabolically quiescent: activity increases with blastocyst formation

Pyruvate required by eggs/cleavage stage embryo: later stages more flexible: pyruvate, lactate, amino acids

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

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

Leese (2003) *Human Fertility* **6**: 180-185
Summer and Biggers (2003) *Hum Reprod Update*. **9** :557-82
Leese (2012) Reproduction

However: these conclusions provide information on <u>what</u> nutrients are required, not on <u>how much</u>.

Defining *requirements* for eggs and embryos

Physiological and biochemical knowledge

Utilisation of nutrients

Oxygen consumption: global marker of energy metabolism

Nutrient consumption: amino acids: pyruvate: glucose

Quantitative requirements: transport kinetics

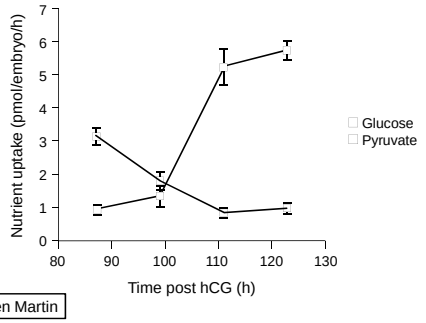
Culture in absence of exogenous nutrients

Can data on nutrient consumption indicate quantitative requirements?

Suppose an embryo consumes 5 pmol glucose/hour.
This value will be influenced by:

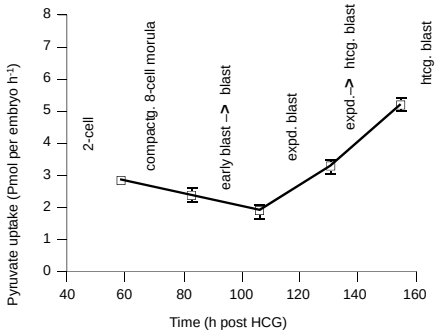
- Glucose concentration in the medium
- Concentration of other nutrients in the culture medium
- Endogenous nutrients
- Stage of development
- Kinetics of glucose disappearance:
  - number and type of GLUT transporters
  - rate of intracellular metabolism

Pyruvate and glucose consumption by preimplantation mouse embryos provided with pyruvate and glucose:

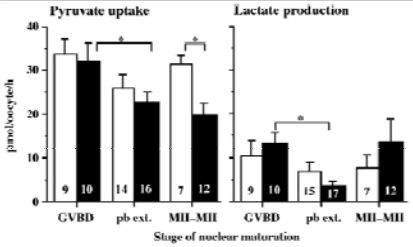


Data of Karen Martin

Pyruvate consumption by preimplantation mouse embryos provided with pyruvate alone: Martin & Leese: 1995 *Molec Reprod Dev* **40**: 436



Culture environment modulates maturation and metabolism of human oocytes: Roberts, Franks and Hardy



Roberts, R. et al. Hum. Reprod. 2002 17:2950-2956; doi:10.1093/humrep/17.11.2950

Effect of stage of nuclear maturation on pyruvate **uptake** (pmol/oocyte/h) or lactate production by oocytes cultured in TCM 199 () or MEME (). GVBD = germinal vesicle breakdown; pb ext. = polar body extrusion; Met II–Met II = oocytes which remain at the metaphase II stage during a single incubation period

Can transport kinetic data define quantitative requirements?

Kinetic approach:

Calculate Apparent K_t and J_{max} for glucose transport into the mouse blastocyst

Apparent K_t = 0.14 mM glucose
J_{max} = 3.53 pmol/embryo/hour

Gardner & Leese *Development* **104**, 423-429 (1988)

Gardner & Leese *Development* **104**, 423-429 (1988)

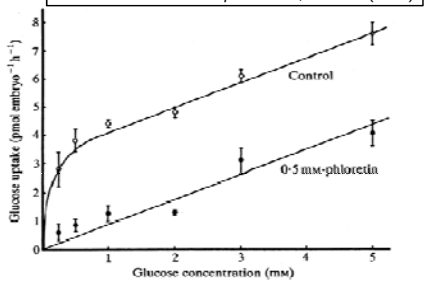


Fig. 1. The effect of increasing concentrations of glucose on glucose uptake by single mouse blastocysts in the presence and absence of 0·5 mM-phloretin. Values are mean (±S.E.) of at least six embryos.

Defining quantitative needs

- Can we use the Apparent K_t and J_{max} to arrive at a medium glucose concentration which *gives the embryo what it needs*?
- No – any value is arbitrary
- If it were an enzyme – give 10x the K_m to ensure V_{max} (maximal rate) – but this is still arbitrary

Conclusions:

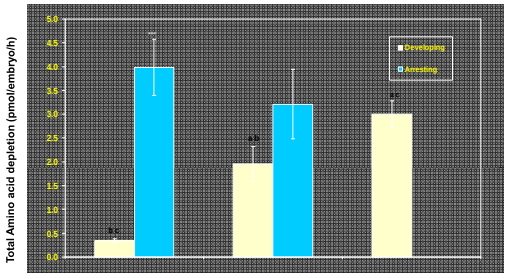
Defining quantitative requirements:

Nutrient transport and metabolism data of limited use

Defining nutritional requirements from oocytes to blastocysts

- Basis of nutritional requirements in man
- Basis of nutritional requirements for eggs and embryos
  - Physiological and biochemical knowledge
  - Utilisation of nutrients including kinetics
  - Composition of oviduct/uterine fluid
- **Relationship between nutrient turnover and subsequent development: possible role of quiet metabolism (?)**

Amino acid depletion by single human embryos

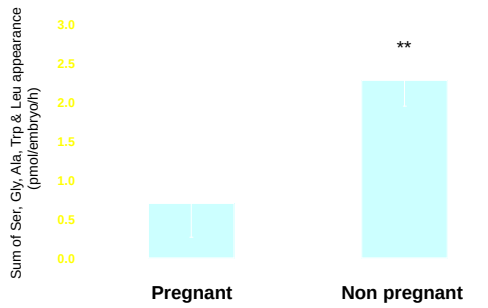


a, $P = 0.05$; b, $P = 0.01$; c, $P = 0.001$

Developing embryos have a lower amino acid turnover than arresting

Houghton et al (2002) *Human Reproduction* 17: 999-1005

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



Conclusion:

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

Hypothesis:

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

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

What is a viable embryo?

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

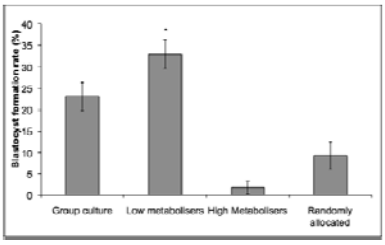
Baumann CG, Morris DG, Sreenan JM and Leese HJ. (2007)
The quiet embryo hypothesis: molecular characteristics favoring viability:
Molecular Reproduction and Development. **74**, 1345-1353.

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

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

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

Prospective embryo selection



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

Implications for nutrient requirements of promoting metabolism which is 'quiet' rather than 'active'

- ***Limit the concentrations of nutrients***
- ***Encourage utilisation of endogenous nutrients***
- ***Mimic nutrient concentrations in reproductive tract***

Defining nutrient requirements of eggs/early embryos

Conclusion: requires a variety of approaches:

- **Physiological and biochemical knowledge**
- **Utilisation of nutrients including transport kinetics**
  Depends on what nutrients are provided and developmental stage
- **Culture in absence of exogenous nutrients to define endogenous contribution**
- **Consideration of the relationship between nutrient turnover and subsequent viability: possible role of 'quiet metabolism'**
- **Female tract nutrient composition**

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ANGLE plc
Origio

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Ways to support in vitro oocyte maturation

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Laboratory Director

Centre for Reproduction
Uppsala University Hospital

AKADEMISKA SJUKHUSET

Conflict of interest statement

I have no commercial relationships in the context of this lecture and no other activities that might be considered a conflict of interest

AKADEMISKA SJUKHUSET

Overview of the lecture – learning objectives

- Events in the oocyte and follicle before and after ovulation
- IVM - What is it and why is it interesting
- Culture systems for supporting IVM
- Selection of oocytes in IVM
- Imprinting and oocyte mechanisms
- Outcome of IVM – current knowledge

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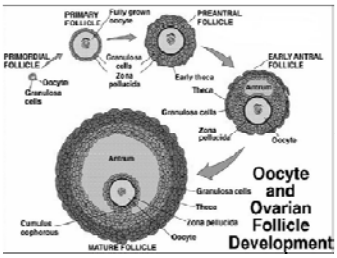
Follicle growth



Growth period is at least 3 months, probably more

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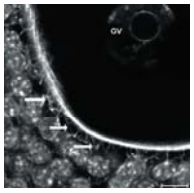
Follicle growth

- Early stages show oocyte growth, limited granulosa cell proliferation
- Later stages show granulosa cell proliferation and cytoplasmic maturation of the oocyte
- Trans-zonal projections between cumulus cells and oolemma



Hutt, Albertini, RBM Online, 2007

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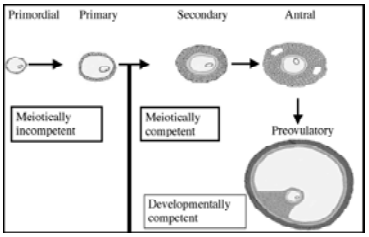
Follicle growth



Telfer& McLaughlin, RBM Online 2007

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Oocyte maturation

- Germinal vesicle (GV), the visible nucleus disappears, 1.st polar body is formed, granulosa cells withdraw from the cell membrane of the oocyte
- Flow of cAMP and nutrients to the oocyte stops, inhibition of maturation ceases

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Overview of IVM

- Small to medium sized antral follicles aspirated – usually largest follicle 10-12 mm
- Basically 3 types, depending on gonadotrophin priming
  - No FSH, no hCG
  - FSH only
  - FSH + hCG
- Efficacy of gonadotrophin priming is still not fully defined
- Culture of immature oocytes after COH and denudation does not constitute IVM
- Oocytes are matured in-vitro for 24-48 hrs before ICSI
  - GV → MI → MII, then embryo culture, ET, cryo etc.
- Spontaneous maturation when removed from the follicle – release of inhibition

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Why do IVM

- Treatment is possible with very low doses of gonadotrophins
  - Avoid OHSS
  - Cheap in terms of costs for medication
  - Advantageous for patients with PCOs
  - Fertility preservation, cryopreservation of oocytes or ovarian tissue
  - Younger women with good ovarian reserve
  - Male factor infertility

  - Increase understanding of maturation processes
  - Maximize utilization of available oocytes

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Clinical aspects

- Low-dose FSH priming or natural cycle monitoring
- Leading follicle approximately 10-12mm (avoid negative effect on sibling oocytes) and endometrium at least 5mm » oocyte aspiration
- Usually cycle day 9-11, sometimes later for anovulatory patients
- Aspiration with low-pressure, multiple needle punctures, more difficult then regular oocyte collection
- Estradiol + progesterone supplementation until pregnancy week 12
- Maximise FSH/hCG effect without risking OHSS

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Laboratory methods

- Immature oocytes have a small and compact cumulus, same colour as granulosa cells - difficult to find
- Aspirates are usually bloody - heparin 2-5 IU/ml in HEPES buffered medium in aspiration tubes
- Follicle aspirates filtrated through a 70µm filter to isolate the oocytes

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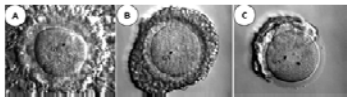
Laboratory methods

- In hCG cycles, oocytes with dispersed/expanded or multilayer cumulus cells may be found – already mature in some cases, greatest developmental potential
- In effect, in-vivo matured oocytes in IVM give best results



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Son, Tan Hum Reprod Update 2010

Selection of oocytes in IVM

- Partial cumulus cell coverage is a negative sign
- Oocytes with expanded cumulus may need only 24 hrs maturation time, otherwise they will be aged in culture
- MII at pick-up should be inseminated directly

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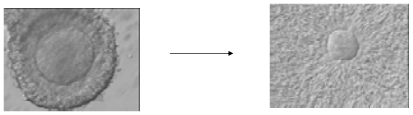
Laboratory methods

- IVM-culture for (24) 32-36 hours, then ICSI (IVF)
- Maturation medium with 10% patient serum, collected on the day of OPU
- FSH and hCG added to the culture medium



Immature oocyte at OPU

IVM for 32 hours

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IVM culture

- Culture media for IVM are usually developed for somatic cells (TCM 199)
- Lately commercialised media have been developed – MediCult and Sage
- Serum gives higher maturation rates then HSA – presence of EGF, inhibins & activins
- FSH and LH&hCG are important
- Important to identify mature oocytes as soon as possible – avoid ageing of already mature oocytes
- ICSI is the fertilisation method of choice – IVF may work but more difficult to assess maturation

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IVM culture

- If good quality embryos are available on day 3, then blastocyst culture/transfer may be beneficial
- Poor cryosurvival of IVM embryos
- Preliminary results show that vitrification may be a good alternative to controlled-rate cryopreservation

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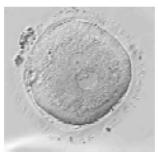
Results



50-80% of the oocytes mature to MII

Fertilization 30-90%

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Results

- Clinical pregnancy rates 20-40% per transfer, 50% in blastocyst transfer (Suikkari 2007, Son & Tan 2010)
- Cancellation rates are usually not reported
- In most publications somewhat lower results than after conventional IVF / ICSI
- Some groups/publications show comparable results to stimulated cycles
- Varying results may be explained partly by different patient groups and numbers of embryos for ET
- What about outcome?

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Miscarriage rates

- Reported rates of miscarriage are between 22-57%
  - Lin et al. 2003 22%
  - Chian et al. 2000 25%
  - Buckett et al. 2007 25%
  - Le Du et al. 2005 33%
  - Söderström Anttila et al. 2005 36%
  - Cha et al. 2005 37%
  - Child et al. 2001 40%
  - Mikkelsen, Lindenberg 2001 57%
- Not possible to calculate the mean, but clearly elevated

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Caesarean section

- Miscarriage rates have been proposed to be PCO-related (Buckett et al. 2007), however other groups with even higher rates of miscarriage have not had a majority of PCO patients
- Rates of caesarean section are also elevated
  - Söderström Anttila et al. 2005 35%
  - Buckett et al. 2007 39%
- Higher birth weight after IVM compared to IVF or national average - IVF is usually under the national average except for cryopreserved embryos

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Birth weight

- Cha et al. 2005 3252g vs. 3165g (Korean average)
- Mikkelsen et al. 2005 3720g vs. 3532g (Danish average) vs. 3457g (IVF/ICSI, Pinborg)
- Buckett et al. 2007 3482g vs. 3260g (controls) vs. 3189g (IVF/ICSI)
- Söderström Anttila 2006 3550g vs. 3541g (controls) vs. 3364g (IVF/ICSI)

- The differences are not great, but the pattern is clear and the number of children born is relatively high
- Same pattern is seen after IVM in farm animals

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Explanations?

- How can we explain these observations?
- What mechanism might cause higher miscarriage rates, higher rates of pregnancy interventions and higher birth weight
- Large offspring syndrome?
- What critical functions are operating in the follicle and oocyte in the final stages of follicle growth and oocyte maturation?
- Is it the endometrium? Is it imprinting defects? Growth factors? cAMP regulation?
- Lower developmental competence of oocytes from small antral follicles has been shown previously (CY Andersen 1993, C Bergh et al. 1998)

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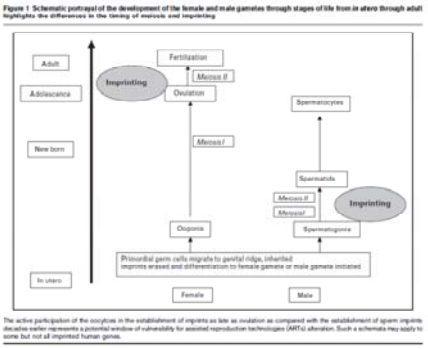
Imprinting



Wilkins-Haug 2009

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What is happening in the oocyte in the antral follicle?

- At ovulation the oocyte has normally completed all maturation steps – before that, this process may not be completed
- Incidentally, this may also be true of COH
- Condensation of chromatin
- The oocyte is the only cell which does not die during transmission to the next generation
- Several cell divisions without transcription
  - RNA is accumulated and stored
  - Normally 2-3 hrs in somatic cells, here up to 5 days, demands stability

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What is happening in the oocyte in the antral follicle?

- Cooperation with cumulus/granulosa cells for accumulation of components
- Oocyte must be synchronised with ovulation to maximise survival chances
- Removal before ovulation leads to spontaneous maturation (Pincus 1939, Edwards 1965)
- Removal of inhibition – default mechanism
- A competent oocyte may be a threat to the ovary, ovarian cancer

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What is happening in the oocyte in the antral follicle?

- Chromatin status, diffused → condensed
- Nucleolus, non-surrounded → surrounded
- Slow shut-down of transcription machinery
- IVM is facilitated by spontaneous maturation, but complicated by difficulties to induce proper cytoplasmic and molecular maturation for developmental competence
- How to maintain meiotic arrest outside of follicle?

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How to ensure in-vitro maturation

- One very interesting approach is to work with the cAMP, cGMP, phosphodiesterase system (Albuz et al 2010)
- cAMP modulators increase levels 100x
- To prevent maturation and maintain high levels, oocyte specific diesterase inhibitor is added + FSH
- Extended IVM interval which increases developmental potential
- In addition, culture in extracellular matrix before IVM may increase developmental potential (Vanhoutte et al 2009)
- Culture in extracellular matrix also shown to be important for culture of primordial follicles (Hornick et al 2012)

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Conclusion

- Oocyte competence is crucial for success in IVM
- Reflects on how we consider oocyte competence in stimulated cycles with follicles of various sizes
- The oocyte cytoplasm is competent at ovulation
- hCG primed cycles with in-vivo maturation work best in IVM
- Meiotic arrest is important until cytoplasmic competence of the oocyte is achieved

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Thank you for your attention

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Epigenetic events in gametes and embryos

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The author reports no conflicts of interest.

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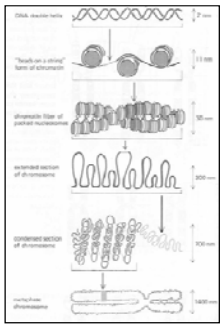
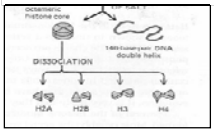
Objectives

- basics of epigenetics
- genomic imprinting
- epigenetic reprogramming during lifecycle
- data on epigenetic defects linked to *in vitro* culture systems and ART

What is epigenetics?

chromatin structure: compaction of DNA with histone proteins

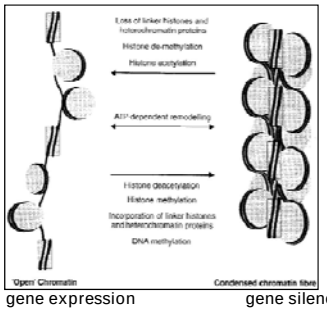




higher-order structures ← basic unit: nucleosome

Basic epigenetic mechanisms

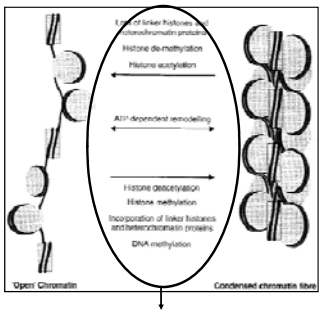
euchromatin heterochromatin



gene expression gene silencing

Basic epigenetic mechanisms

euchromatin heterochromatin



gene expression

gene silencing

epigenetics mechanisms and modifications

Basic epigenetic mechanisms

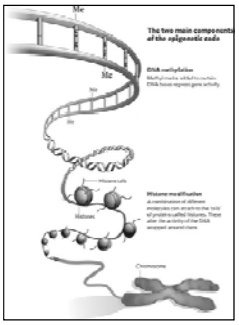
- regulate chromatin structure and gene expression

- occur without changes in the DNA code

- inheritable (mitosis and meiosis)

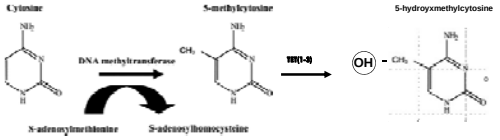
- influenced by environmental factors

- best studied are:
  - DNA methylation
  - histone modifications



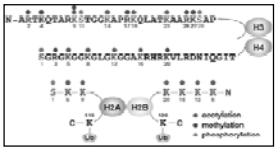
Qiu et al., 2006

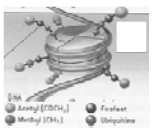
DNA (de)methylation



repetitive sequences transposons	CpG islands of house-keeping genes	embryonic stem cells
DNA hypermethylation	DNA hypomethylation	DNA hydroxymethylation
gene silencing	gene activation	gene activation

Histone modifications



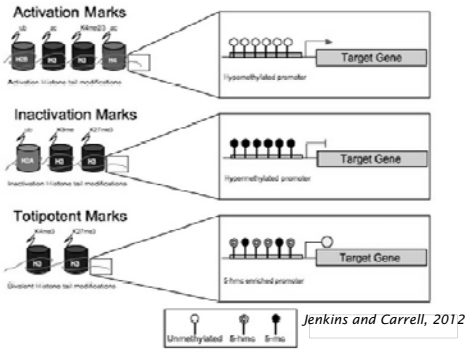


May affect chromatin structure

- directly via interaction between histones and DNA
- Indirectly by recruiting chromatin-remodelling complexes

=> influence the accessibility of DNA
=> critical for transcription, repair, replication

Histone and DNA modifications



Jenkins and Carrell, 2012

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Genomic imprinting

most genes:
expression of maternal ***and*** paternal alleles

imprinted genes:
expression of maternal ***or*** paternal alleles

Maternal copy is expressed Paternal copy is expressed

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Genomic imprinting

- > 80 imprinted genes (0.1-1% of all genes)
- eutherian mammals, marsupials, higher plants
- key role in embryonic growth and placental function, cognition and maternal behaviour
- defective imprinting involved in carcinogenesis and in human diseases

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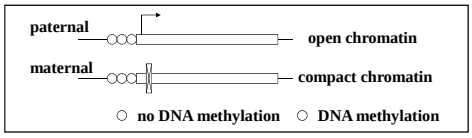
Genomic imprinting

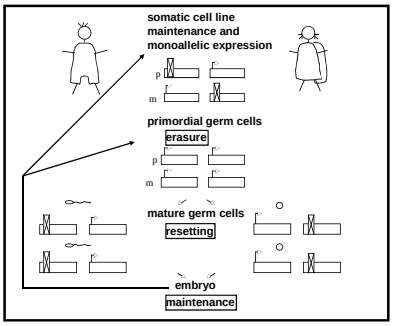
- maternal and paternal alleles carry different epigenetic modifications (“DMR”)



- imprinted genes: vulnerable
  - functionally haploid
  - differential epigenetic modifications
  - role in embryonic and placental development

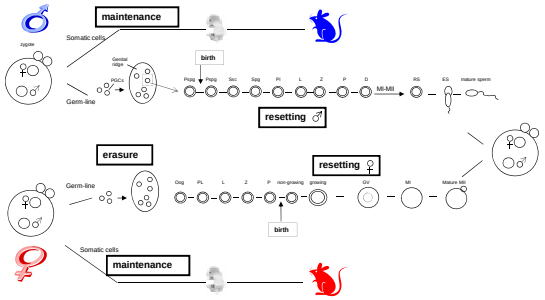
Epigenetic reprogramming of imprinting

gametogenesis
imprint resetting

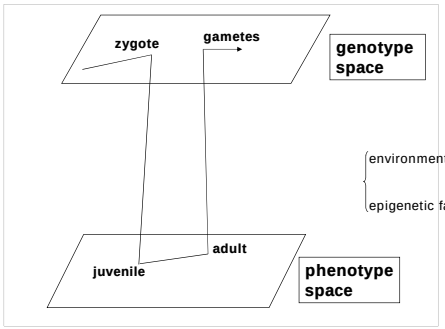


early embryonic development
imprint maintenance

Epigenetic reprogramming of imprinting



Epigenetic reprogramming during lifecycle



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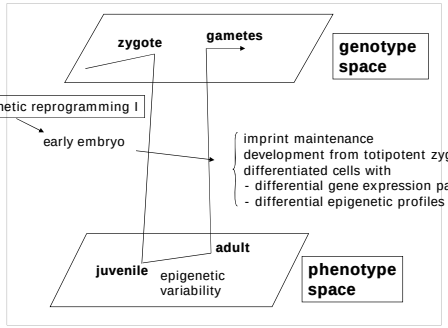
Epigenetic reprogramming during lifecycle



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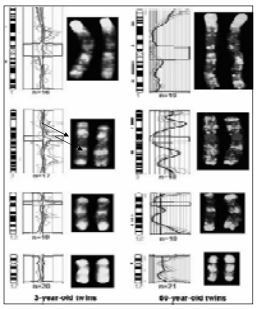
Epigenetic reprogramming during lifecycle



epigenetic profiles of twins:
- similar at young age
- differ at old age by 20%

Fraga et al., 2005

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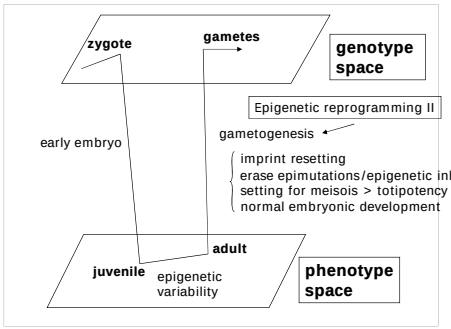
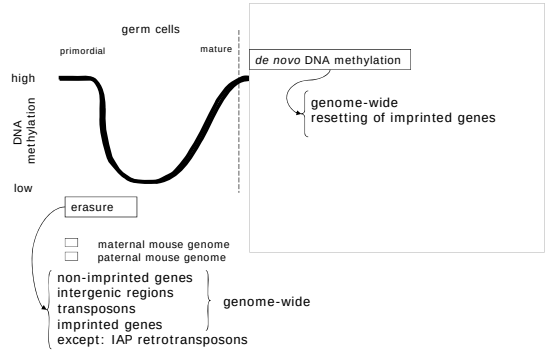
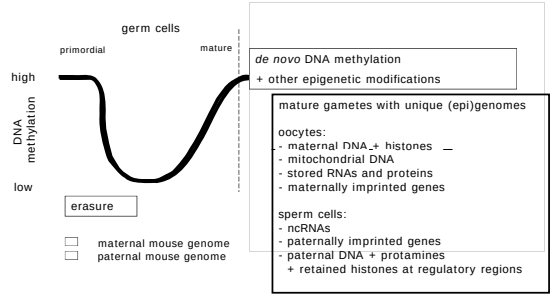
Epigenetic reprogramming during lifecycle



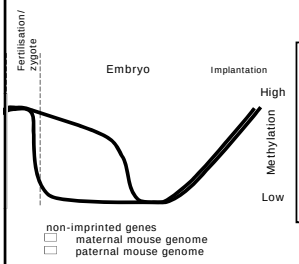
DNA methylation reprogramming during gametogenesis



DNA methylation reprogramming during gametogenesis



DNA methylation reprogramming in the early embryo



- exchange protamines > histones
- maintenance at imprinted genes, IAP retrotransposons
- active paternal demethylation via hmC
- passive maternal demethylation
- de novo methylation at implantation

Epigenetic defects linked to ART

Does ART interfere with

- epigenetic reprogramming (imprint maintenance) in the early embryo?
- epigenetic reprogramming (imprint resetting) during gametogenesis?

ART and imprinting disorders

- studies presented (limited) evidence for increased relative risk of Angelman syndrome and Beckwith-Wiedemann syndrome after ART, absolute risk is low (Cox et al., 2002, Orstavik et al., 2003, Ludwig et al.,2005, Sutcliffe et al.,2006, Doornbos *et al.,* 2007; DeBaun et al., 2003, Maher et al., 2003, Gicquel et al., 2003, Halliday et al., 2004, Lim et al., 2009)
- an epigenetic defect (loss of maternal methylation) was found in nearly all AS and BWS patients after ART vs 50% of BWS and 5% of AS cases in general population
- Insufficient evidence for association between ART and other imprinting disorders (SR, PWS, RB)

ART and epigenetic defects

* use of hormonal ovarian stimulation

- mouse: comparison of methylation in 4 imprinted genes in normal vs superovulated GVs: no differences for maternally methyl. genes, but gain of methylation for H19 after superovulation *Sato et al, 2007*
- human: superovulated GV & MI oocytes show gain of *H19* methylation (2/6) and loss of maternal methylation at *PEG1* (6/16) *Sato et al, 2007*
- human: superovulated GV, MI & MII oocytes show loss of *KvDMR1* methylation (1/16) *Geuns et al., 2007*

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ART and epigenetic defects

* parental infertility

subfertile couples are predisposed to epigenetic defects

- AS cohort study: subfertile couples have an increased risk of conceiving a child with an imprinting defect
TTP> 2 years, no therapy RR 6.25
TTP> 2 years, treatment RR 12.5 *Ludwig et al., 2005*

- similar link between fertility problems and epigenetic defects, no effect from ART *Doornbos et al., 2007*

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ART and epigenetic defects

* parental infertility

link between imprinting errors and disruptive spermatogenesis

sperm concentration correlates positively with *H19* methylation and negatively with *MEST* methylation
Marques et al., 2004 & 2008 & 2009, Kobayashi et al., 2007, Boissonnas et al., 2010, Poplinski et al., 2010

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ART and epigenetic defects

* parental infertility

aberrant DNA methylation in sperm > offspring
safeguard because of imprint resetting, selection of normal sperm for ICSI, embryonic developmental arrest

- no difference in *H19* DNA methylation for ART children (n=61) vs (n=30 NC) children *Shi et al., 2011*

- no difference in *H19* DNA methylation in placentas of IVF (n=32), ICSI (n=45) and NC (n=12) children *Wong et al., 2011*

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ART and epigenetic defects

* in-vitro culture systems: animal models

- Khosla et al. 2001: culture (serum) of preimplantation mouse embryos reduced fetal development (lower birthweight) and expression of imprinted genes
- Young et al. 1998: after exposure *in vitro* unusually large offspring syndrome (LOS) in relation with imprinted genes
- Young et al. 2000: epigenetic changes in *IGF2R* are associated with fetal overgrowth (LOS) after sheep embryo culture

The mammalian preimplantation embryo is very sensitive to culture conditions; epigenetic changes induced at the early stages may lead to altered phenotypes at later stages

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ART and epigenetic defects

* in-vitro culture systems: human

- 19% (Chen et al., 2010) or 38% (*Ibala-Romdhane et al.,2011)* of arrested low-quality embryos showed H19 DNA hypomethylation (normal corresponding sperm samples)

- the birthweight of IVF children derived from embryos cultured in two different commercial media was significantly different *Dumoulin et al., 2010*

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ART and epigenetic defects

* in-vitro culture systems: human

- *Katari et al., 2009:* changes in genome-wide DNA methylation and gene expression patterns of imprinted and non-imprinted genes in cord blood and placenta of ART children (n=10) versus spontaneously conceived children (n = 13)
- *Tierling et al., 2010*: no differences in DNA methylation (only 1/9 imprinted regions show slight hypermethylation) in cord blood and maternal blood of IVF compared with control conceptions

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ART and epigenetic defects

- *Ceelen et al., 2008*: follow-up of IVF children vs controls born to subfertile couples
  →IVF children show higher blood pressure levels
  →confirmed by *Sakka et al., 2010*
- ART is associated with an increased incidence of low birth weight
  maybe as a consequence of embryonic/fetal epigenetic programming in response to early adverse environmental factors and stress???

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Conclusion and perspectives

- evidence for epigenetic effect of ART: mouse > human
- full clinical effect to be determined
- need for further genome-wide and locus-specific epigenetic profiling
- relate epigenome with transcriptome
- need for long-term follow up of ART children

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The top quality embryos

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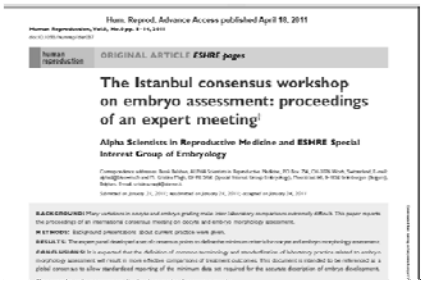
Sweden

Disclosure

I have no commercial and/or financial relationships with manufacturers of pharmaceuticals, laboratory supplies and/or medical devices.

What is top quality ?

- Is top always top?
- Is one top same as another top?
- Assessment variables
- Relation to the environment / culture media
- Early cleavage vs. Blastocyst
- Timing and Time-lapse
- Any news….?



Hum. Reprod. Advance Access published April 18, 2011

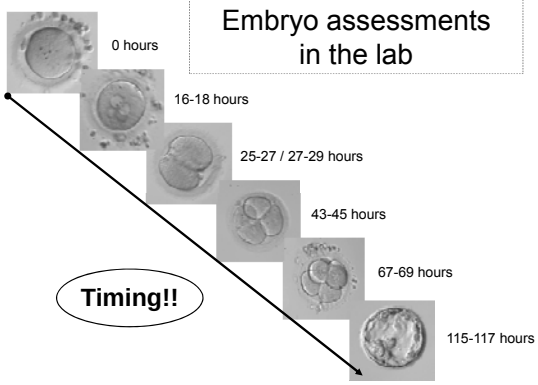
human reproduction ORIGINAL ARTICLE ESHRE pages

The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting†

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology

Hum Rep 2011, RBM online 2011

Embryo assessments in the lab

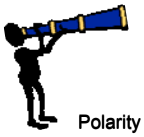


End points

Embryo development and morphology

Implantation

Live birth



Polarity / symmetry ⇕ Timing / Synchronisation

Nuclear status / cytoplasmic status / metabolic status / environment / chromosomal status

How to become a top quality embryo – Traditional scoring variables

- (Gamete and zygote quality ?) vs. end product?
- Cell numbers
- Cleavage timing
- Fragmentation
- Cell size
- Number of nuclei (total and per cell)
- ICM
- Trophectoderm
- Expansion

Is this a top quality embryo?

Or this?

Probably not this…

What about aneuploidy?

Mosaicism?

And the metabolism?

Can an embryo be **too** good-looking
- more on the outside = less on the inside..???

Environment *in vitro*

- Can we IMPROVE embryo quality? 
- Or can we only make it worse ? 

Taking care of gametes and embryos – Handling *in vitro* and environmental factors

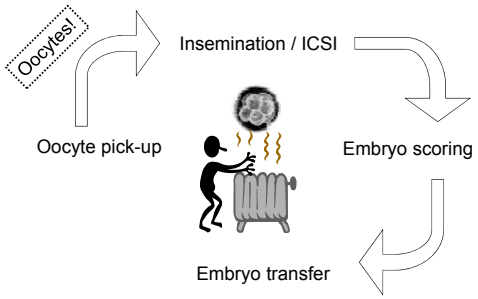
- pH
- Temperature
- Culture media
- Oxygen levels
- Quality control ……

Maintenance - Incubators, heating plates, cryo machines, etc….

- Air quality
- Temperature
- CO_2 / pH
- Incubators
- Displays vs. actual values

- Register and document!!

Keeping the temperature / pH ”chain”

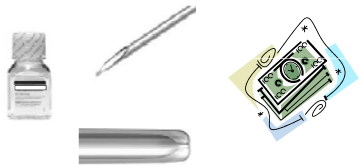


Work towards continuous improvement of quality…

IVF / ICSI - then ”homemade”

- Pipettes
- PVP
- Medium - serum

IVF / ICSI - today
”Ready to use”



Development of culture media

- From ”simple” cell culture media to complex media

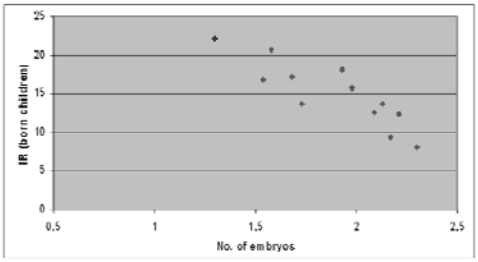
- From a single fertilisation /culture medium to sequential media

⟹ And back again…???

Selection

- Can we IMPROVE individual embryo quality?
- Or can we only improve our methods of selection?

Single embryo culture and single embryo transfer can get you far…



Countries with > 4000 transfers; **Belgium, Denmark, Finland, France, Germany, Greece, Italy, Norway, Russia, Spain, Sweden, UK**

ESHRE data, Hum Rep 2010

Morphology…..

What other variables could we use for selection?

- Prolonged culture
- Genetic/chromosomal ”normality”?
- Metabolic ”normality”?
- Improved morphology scoring (time-lapse)

All or nothing? – or degrees?

Predictors of blastocyst development

- Number of oocytes retrieved/fertilised
- PN size symmetry
- Early cleavage
- Number of 4/8-cell embryos on day 2/3

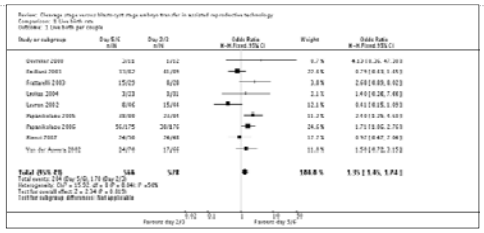
Only about 40-50% of blastocysts were preselected on day 3

E.g. Neuber et al 2003, Ebner et al 2003, Fenwick et al. 2002, Guerif et al 2007

Blastocyst vs. Cleavage stage (selected patients….)

- eSET day 2 (top quality embryo) – 50% IR
  (all embryos) - 36% IR
  (Salumets et al 2003)
- eSET day 3 (top quality embryo) – 47% IR
  (all embryos) - 37% IR
  (Gerris et al 1999)
- eSET day 5 41 – 60% IR
  (Gardner et al 2004, Papanikolaou et al 2006, Zech et al 2007)

Blastocyst vs. Cleavage stage



- This review provides evidence that there is a significant difference in pregnancy and live birth rates in favour of blastocyst transfer, with good prognosis patients with high numbers of eight-cell embryos on Day three being the most favoured in subgroup for whom there is no difference in cycle cancellation.

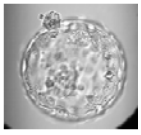
Blake et al 2009

Blastocyst versus cleavage stage transfer in in vitro fertilization: differences in neonatal outcome?

Källén B, Finnström O, Lindam A, Nilsson E, Nygren KG, Olausson PO.
Fertil Steril. 2010 Oct;94(5):1680-3

MAIN OUTCOME MEASURE(S):
Some neonatal characteristics were compared in 1,311 infants born after blastocyst-stage transfer and 12,562 infants born after cleavage-stage transfer. Comparisons were also made with all births, 2002-2007 (n = 598,687).

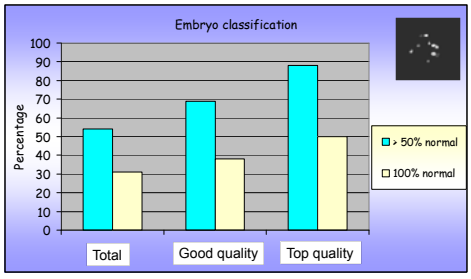
RESULT(S):
After adjusting for year of birth, maternal age, parity, smoking habits, and body mass index, the risk of preterm birth among singletons was significantly greater after blastocyst-stage transfer than after cleavage-stage transfer. The risk of congenital malformations was also significantly higher.



Short to prolonged culture

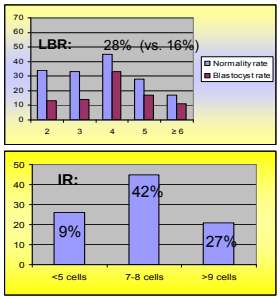
⟹ And back again…???

Chromosomal normality and embryo selection (n=144 embryos)



Ziebe et al 2003,

Chromosomal normality and cleavage rate day 2 and day 3



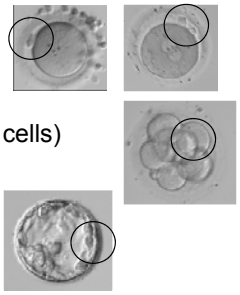
Magli et al 2001,
van Royen et al 2001,
De los Santos et al ESHRE 2006,
Thurin et al 2005

PGS – preimplantatory genetic *screening*

- Complement to morphologic assessment
- Efficiency not proven

Biopsy techniques
(development stage)

- Polar body biopsy
- Biopsy day 3 (~ 8 cells)
- Blastocyst biopsy



PGS - FISH

- 11 randomised control trials (embryos) so far (age, poor/good prognosis patients)
- Show no improvement in delivery rates

- Limited number of analysed chromosomes
- High rates of embryo mosaicism
- Poor correlation between results and implantation (*M. Hughes*)
- Invasive

Mastenbroek et al 2011, Harper et al 2011

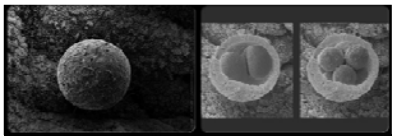
CGH - microarray

- Allows analysis of all chromosomes
- Complex technique
- Needs DNA amplification
- Longer time for preparation/analysis (combined with cryopreservation?)

- Prospective trial showing increased live birth rates for CGH cycles
- No RCTs performed, needs to be validated
- Same problems with mosaicism and invasiveness

Wells et al 2008, Fragouli et al 2008

Metabolically normal?

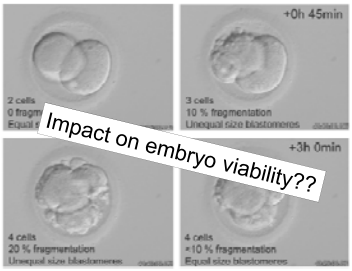


Analysis of the culture medium

- Amino acid consumption
- hCG
- Glucose uptake
- Oxygen consumption (embryo respiration)
- The ”omics”

- Provides a snap-shot of the current status
- Should preferably **not** correlate fully with development and/or morphology assessment
- Not validated in RCT:s

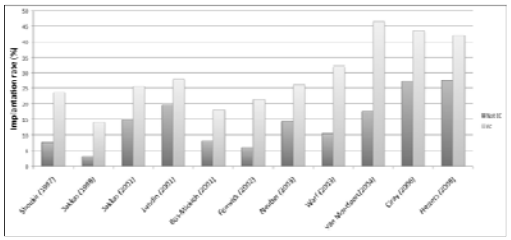
Fragmentation and cell size during cleavage



Lemmen et al 2008

Syngamy / Early Cleavage

20-22h, 25-27h, 27-29h



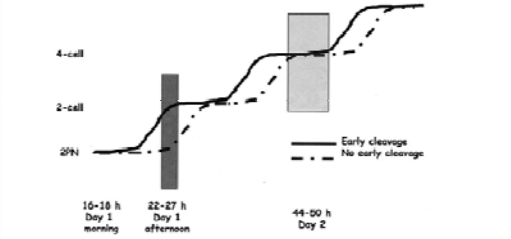
Hesters et al 2008

Early cleavage

- Snapshot image….

- When is too early ?
- When is too late ?

Establish ”timing windows” instead of snapshots



Sakkas et al, Fertil Steril 2001

A continuous observation

- Possible to document exact timing of different events
- Possible to analyse time intervals between events

AND…..

- Correlations with embryo morphology ?
- Correlations with implantation and birth rates ?

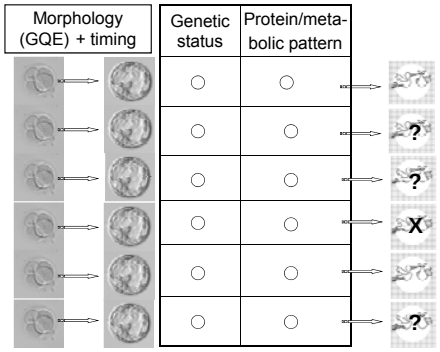
nature biotechnology

Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage

Connie C Wong[1,2,7], Kevin E Loewke[1,3,6,7], Nancy L Bossert[4], Barry Behr[2], Christopher J De Jonge[4], Thomas M Baer[5] & Renee A Reijo Pera[1,2]

- Blastocyst development can be predicted by measuring three imaging parameters in the early embryo stage (before embryonic genomic activation)
- Time of development events correlates to gene expression patterns

Wong et al 2010



Laboratory workstations with:

- Time-lapse incubators
- Automatic morphology assessments
- Continous monitoring systems
- Automatic culture media flow systems
- Automatic sampling and metabolite measurements

The future embryologist

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Mark your calendar for the upcoming ESHRE Campus events

- Basic Semen Analysis Course in Greek Language
  4-7 September 2012 - Athens, Greece
- Basic Genetics for ART practitioners
  7 September 2012 - Rome, Italy
- Regulation of quality and safety in ART – the EU Tissues and Cells Directive perspective
  14-15 September 2012 - Dublin, Ireland
- Basic Semen Analysis Course in Spanish language
  18-21 September 2012 - Galdakano,Vizcaya
- GnRH-antagonists in ovarian stimulation
  28 September 2012 - Hamburg, Germany
- The best sperm for the best oocyte
  6-7 October 2012 - Athens, Greece
- Basic Semen Analysis Course in Italian language
  8-11 October 2012 - Rome, Italy
- Accreditation of a preimplantation genetic diagnosis laboratory
  11-12 October 2012 - Istanbul, Turkey
- Endoscopy in reproductive medicine
  21-23 November 2012 - Leuven, Belgium
- Evidence based early pregnancy care
  29-30 November 2012 - Amsterdam, The Netherlands

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