# European Society of Human Reproduction and Embryology

## COURSE 3

## Towards a comprehensive approach of fertilization failure and how to make the best choice between IVF and ICSI

**Special Interest Group Embryology**

**18 June 2006**
**Prague - Czech Republic**

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## Course 3 - Pre-congress course organised by the

## Special Interest Group “Embryology”

## “Towards a comprehensive approach of fertilization failure and how to make the best choice between IVF and ICSI”

## PROGRAM

**Course co-ordinators**: D. Royere (F), K. Lundin (S), C. Magli (I), E. Van den Abbeel (B)

**Topic 1:** **Gamete Activation**

09.00 - 09.30 Oocyte activation – **J. Carroll (UK)**
09.30 - 10.00 Acquisition of sperm fertilizing capacity – **L. Fraser (UK)**

**Topic 2:** **Gamete interaction**

10.00 - 10.30 Current status on sperm-egg interaction – **J.P. Wolf (FR)**
10.30 - 11.00 Fertilisation failure: How and why? – **J. Van Blerkom (USA)**

*11.00 - 11.30* *Coffee break*

**Topic 3:** **How to make the right choice?**

11.30 - 12.00 Does looking to sperm morphology have any value? – **Ch. Barrat (UK)**
12.00 - 12.30 How to define the limits between IVF and ICSI? - **H. Tournaye (B)**

*12.30 - 13.30* *Lunch*

13.30-14.30 **Business Meeting of the Special Interest Group Embryology**

**Topic 4:** **Genomic activation**

14.30 - 15.00 Animal models - **V. Duranthon (F)**
15.00 - 15.30 Human embryo development, gene expression and genomic activation - **M. Monk (UK)**

*15.30 - 16.00* *Coffee break*

**Topic 5:** **Clinical and laboratory factors of fertilization failure?**

16.00 - 16.30 Egg sharing as a tool for discriminating egg vs sperm quality – **T. Tuuri (FIN)**
16.30 - 17.00 Laboratory conditions and fertilization failure - **R. Cutting (UK)**

## Acquisition of sperm fertilizing capacity:

### Insights into capacitation and possible causes of male infertility

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## Learning Objectives

To understand:

- the importance of capacitation
- possible links between capacitation defects and reduced fertility
- some of the mechanisms that promote and/or regulate capacitation, including signal transduction pathways involved
- application of basic research results to clinical practice that might enhance fertility *in vitro*

## Introduction

- Capacitation involves a physiological 'switching on' of sperm so that they can fertilize an egg.
- At the time of release, mammalian sperm will have a number of different molecules present on their plasma membrane that keep sperm uncapacitated.
- These are referred to as 'decapacitation factors' (DF). During capacitation, DF molecules are lost and this allows the sperm to 'switch on'. If DFs are added to capacitated suspensions, the sperm will be 'switched off' and revert to the uncapacitated state, hence the term decapacitation. Therefore, **capacitation is reversible**.

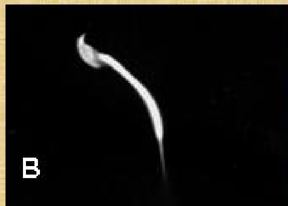
## Investigating capacitation

- **How can one analyze treated sperm suspensions for effects on capacitation?**
  - **spontaneous acrosome reaction**
  - **induction of acrosome reaction**
  - **chlortetracycline (CTC) patterns**
  - **biochemical parameters (e.g., cAMP production, protein tyrosine phosphorylation)**
  - ***in vitro* fertilization**

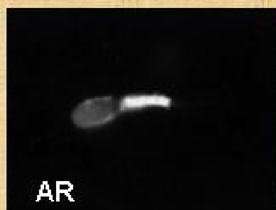
## CTC fluorescence patterns

| | Pattern | Functional state | Acrosome |
|---|---|---|---|
| **F** | uniform fluorescence | uncapacitated | present |
| **B** | fluorescence-free band in posterior head | capacitated (potentially fertile) | present |
| **AR** | dim/absent fluorescence | capacitated | absent |

### CTC fluorescence patterns in mouse sperm



### CTC fluorescence patterns in human sperm



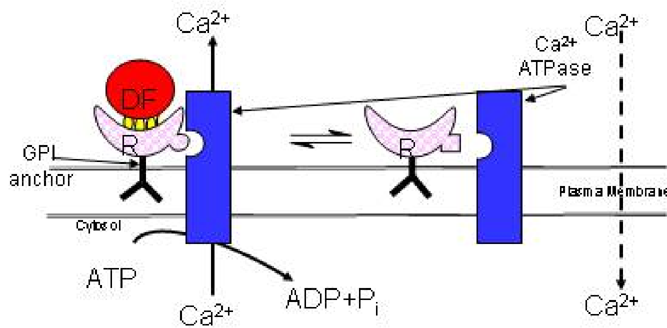

### I - Mouse sperm DF

- Mouse sperm have a DF with fucose residues that enable the DF to bind to a receptor (DF-R) with fucose binding sites. A GPI anchor is involved in attachment of DF-R to the membrane.

- DF binding to its receptor results in activation of a calcium ATPase, resulting in maintenance of low intracellular calcium .

- During capacitation, DF is lost and this allows intracellular calcium to rise. Centrifugation removes the DF, resulting in accelerated capacitation.

## Initial hypothesis for mechanism of action of DF




## Enhanced fertilizing ability in the presence of exogenous fucose which can displace DF molecules

Adding exogenous **fucose** to uncapacitated mouse sperm suspensions during the final 10 min of a 25 min preincubation period accelerates capacitation as evidenced by a significantly higher fertilization rate.

| Treatment | Fertilized oocytes/ total oocytes, | (range, %) | Polyspermic oocytes |
|---|---|---|---|
| Control | 95/189, 50.3% | (23-67) | 2/95, 2.1% |
| 5 mM fucose | 255/343, 74.3%** | (53-95) | 41/255, 16.1% |

** $P < 0.025$ compared with control suspensions; oocytes fixed 75 min after sperm addition and evaluated for the presence of one or more decondensing sperm heads (n=4).

## Human sperm appear to have a similar DF/DF-R system

- Mouse sperm DF can bind to capacitated human sperm and decapacitate them.
- Addition of exogenous fucose to human sperm suspensions accelerates capacitation.

## Recent experimental results

- DF-R has been purified, sequenced and found to have high homology with phosphatidylethanolamine-binding protein 1 (PEBP 1).

- An anti-peptide antiserum to PEBP 1 has been used for Western blotting and immunocytochemistry, using both **mouse and human sperm**.

## PEBP 1 localization - mouse

- **Intensity of PEBP 1/DF-R staining is capacitation state-dependent & reversible** (note that adding DF to DF-depleted or capacitated cells changes the staining pattern to that of uncapacitated cells).
- **PI-PLC treatment to remove GPI-anchored proteins essentially abolished staining.**

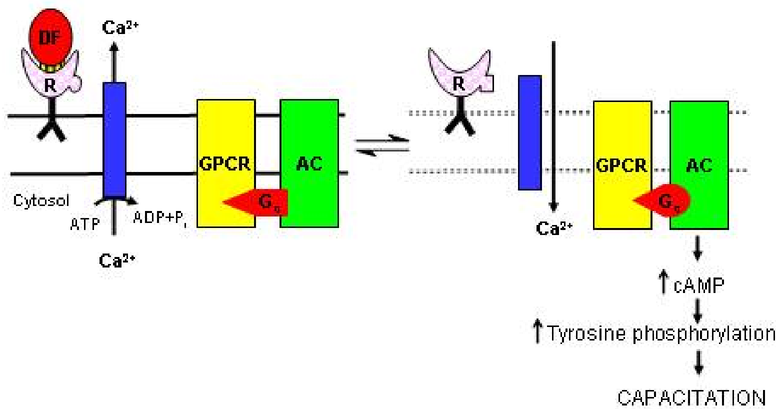
## PEBP 1 localization – human

- **PEBP 1/DF-R staining distribution is similar to that of mouse sperm but capacitation state-dependent changes are less obvious.**

- **PI-PLC treatment to remove GPI-anchored proteins again abolished staining.**

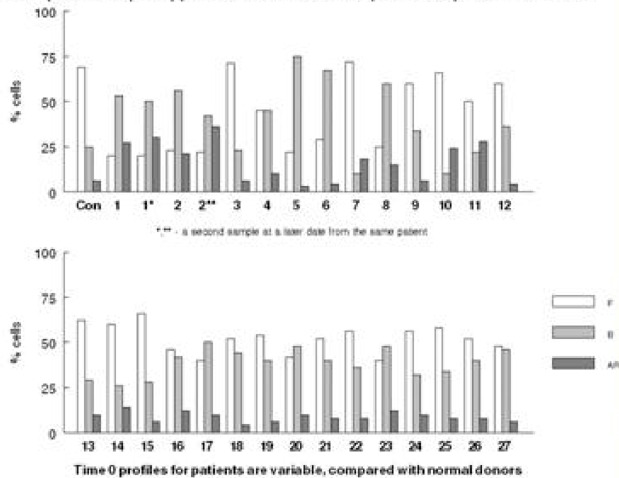
**Revised hypothetical mechanism of action:**

*DF↔DF-R affects the functionality of many membrane proteins (including several GPCRs), not just $Ca^{2+}$-ATPase*




## Possible links with infertility

- DF molecules might not be lost, resulting in sperm that would never switch on.
- Sperm might have no or little DF, resulting in precocious capacitation and early loss of the acrosome.
- Such defects would not be associated with reduced sperm numbers, so could account for 'unexplained' infertility – see next slide. All samples evaluated had reasonable sperm concentrations and motility, but many had atypical proportions of capacitated and/or acrosome-reacted cells.



## II - Regulation of capacitation

- When released into an appropriate environment, sperm usually begin to capacitate and then continue until some have 'over-capacitated' and undergo spontaneous acrosome reactions.

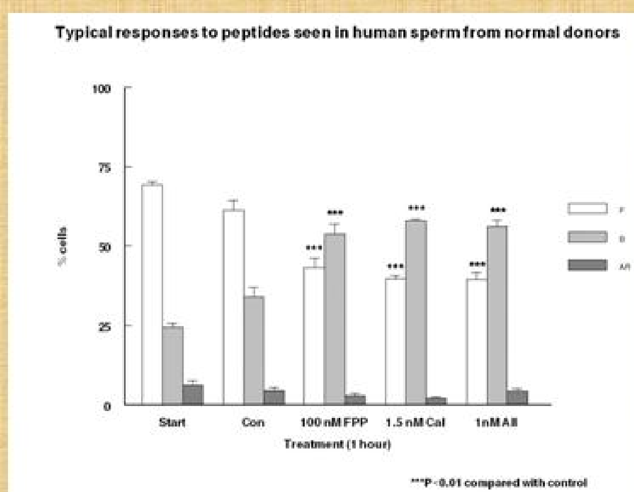
- Biologically, this is undesirable since these sperm will be non-fertilizing.

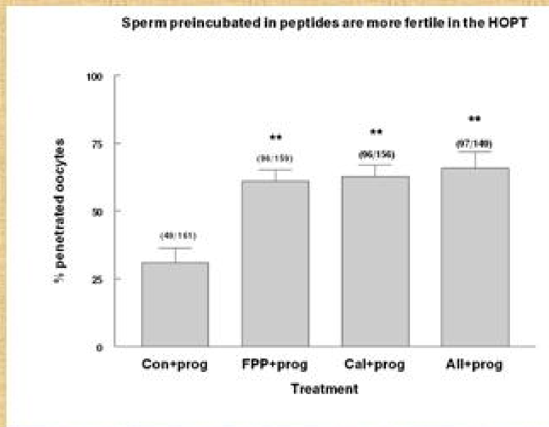
➢ **Regulation** of capacitation is needed to provide maintenance of fertilizing potential.

## Seminal plasma molecules of interest

- FPP (fertilization promoting peptide)
- Adenosine
- Calcitonin

➢ **All 3 *regulate* capacitation by regulating adenylyl cyclase(AC)/cAMP pathways, initially accelerating capacitation and then preventing 'over-capacitation' by inhibiting the spontaneous acrosome reaction**



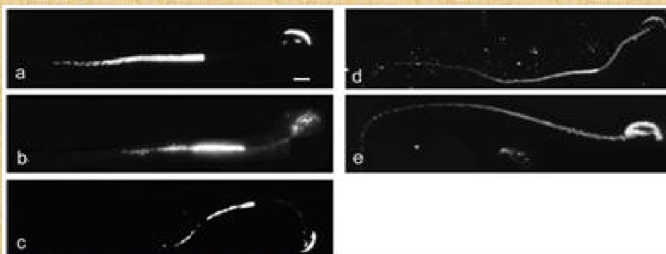



## Components of signal transduction pathways are present in the same regions of sperm

- **Receptors** for FPP, adenosine and calcitonin are all found in the acrosomal cap region and in the flagellum.

- Stimulatory and inhibitory **G proteins** and **mACs**, involved in pathways activated by FPP, calcitonin and adenosine, are also found there.

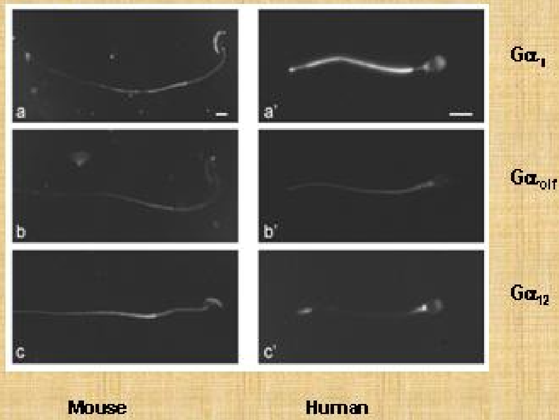
## Localization of adenosine receptors: ***note that staining pattern is capacitation state-dependent***

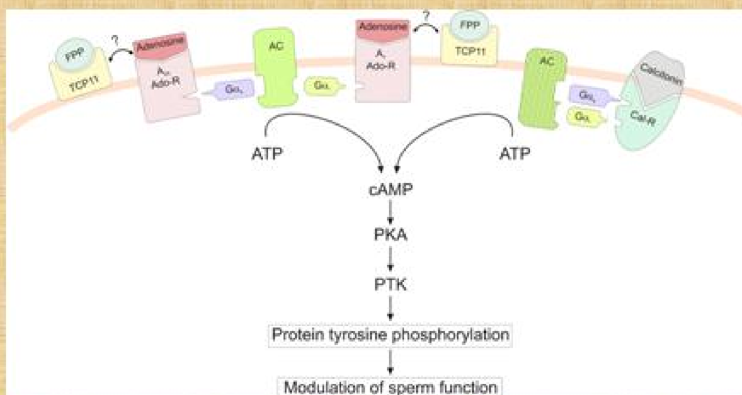



**$A_{2A}$ receptors in: (a) uncapacitated, (b) capacitated, (c) capacitated cells + DF – show localization seen in uncapacitated cells (a)**

**$A_1$ receptors in: (d) uncapacitated and (e) capacitated cells**

## Immunolocalization of Gα subunits



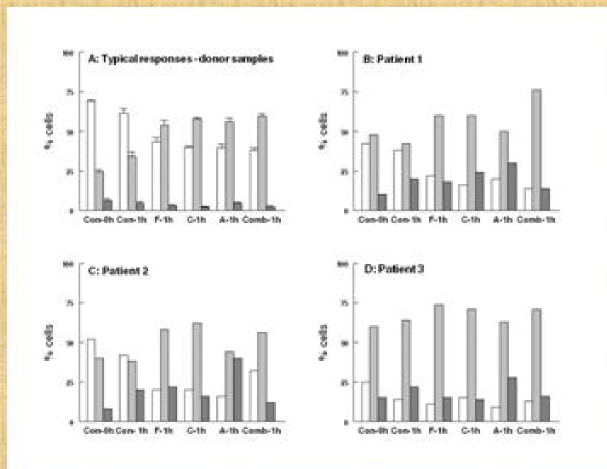

## Conclusion

- Since these first messengers present in seminal plasma and body fluids can regulate AC/cAMP pathways ***in vitro***, they might also regulate sperm function ***in vivo***. This would maximize the number of potentially fertilizing sperm in the vicinity of unfertilized oocytes.

- Sperm used for ART are removed from seminal plasma and so are no longer in contact with these peptides.

## Possible links with infertility

- Defective receptors might allow sperm to acrosome-react precociously and so become non-fertilizing.
- A lack of 'first messengers' might allow sperm to acrosome-react precociously.
- These molecules could be added to sperm suspensions used for IVF and/or IUI to accelerate capacitation and ensure early penetration.



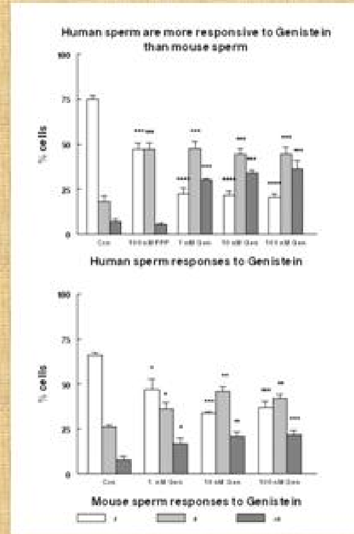

## III - Can exogenous compounds affect sperm function?

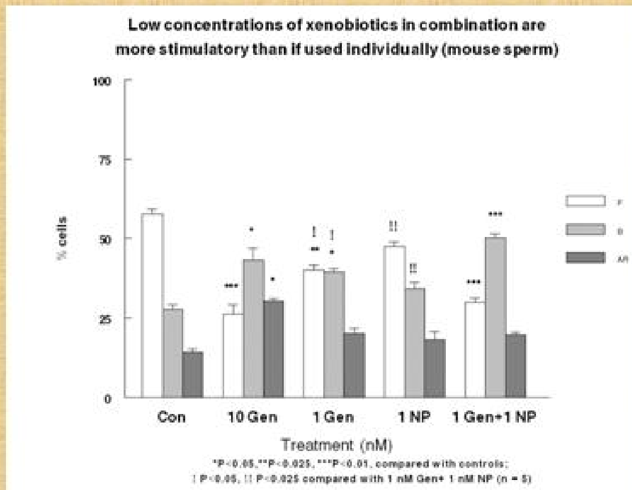
- Estrogenic xenobiotics, e.g., genistein (in soy)

- **Genistein** at low nM concentrations *in vitro* accelerates capacitation and then stimulates the acrosome reaction in both mouse and human sperm. *Human sperm appear to be even more responsive than mouse sperm.*

- These low concentrations are well within the range found in human females 3 h after drinking 25 g of soy milk.

- Similar responses *in vivo* could result in <u>sperm losing fertilizing ability</u> before contacting an egg: already acrosome-reacted sperm cannot bind to the zona.



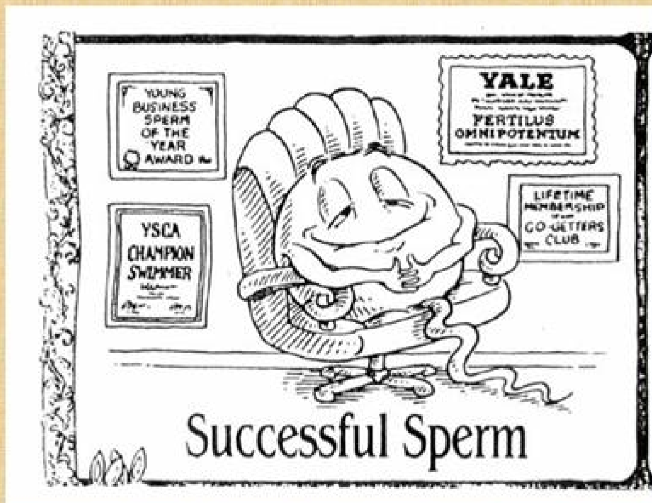

## Mechanism of action

- These estrogenic xenobiotics appear to <u>act at the sperm cell surface</u> rather than via classical estrogen receptors.

- These xenobiotics appear to stimulate capacitation and acrosome loss by causing <u>unregulated cAMP production</u>.

## Conclusions

- These effects observed *in vitro*, especially the occurrence of the acrosome reaction, might have negative implications for fertility *in vivo*, especially since humans are likely to be exposed to more than one xenobiotic at any given time.
- Since August 2005, I have been told by 3 women (in Canada, UK, Israel) that they have become pregnant within a short time of ceasing to eat and drink soya-containing products! This could just be coincidence but...........




## Recent research colleagues

**Susan Adeoya-Osiguwa**
**Rhona Baxendale**
**Ergin Beyret**
**Rachel Gibbons**
**Stella Markoulaki**
**Stuart Milligan**
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**Acquisition of sperm fertilizing capacity**

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Fraser LR, Adeoya-Osiguwa SA and Baxendale RW (2003) First messenger regulation of capacitation via G protein-coupled mechanisms: a tale of serendipity and discovery. Mol Hum Reprod 9,739-748. **Review of signal transduction pathways that are present in mammalian sperm and appear to regulate capacitation.**

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*pdfs available upon request

## Current status on sperm-egg interaction

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## Learning objectives

This lecture will focus on the sperm-egg interaction at their membrane level upon fertilization. Fertilization results of the sperm and oocyte's membranes fusion which allow the incorporation of the sperm within the ooplasme. In order to achieve fertilization, gametes have to bind first and then to fuse. This lecture will summarise the current status on this gamete interaction process and reviewed the possible future of the understanding.

## Lecture summary

*The model of fusion mediated by sperm ADAM proteins and the egg integrin α6β1.*
Almeida et al. had discovered the sperm receptor on the oolemma and its ligand of the sperm membrane [1]. Indeed, Fertilin α and Fertilin β form a heterodimer and were initially discovered through the generation of a mAb to Fertilin β that strongly inhibited sperm-egg fusion [2]. Fertilin β is located to the equatorial region of the sperm[3] and the soluble extracellular domains of Fertilin α and Fertilin . could bind to the microvillar region of mouse eggs [4,5]. Moreover, fertilin β peptides were shown to inhibit the binding of sperm to oocytes and fusion [1,3]. Several laboratories have also demonstrated that isolated soluble fertilin β proteins, or particular domains, inhibited the binding of sperm to the oocyte [4-6]. In some studies, this resulted in inhibition of sperm-egg fusion [5]. These studies have pointed at a critical role for the disintegrin domain of fertilin β [1,5-7]. Both fertilin and fertilin (also known as ADAM-1 and ADAM-2 respectively) belong to the ADAM (A Disintegrin And Metalloprotease) family of plasma membrane proteins [8]. Male mice with invalidation of fertilin β and cyritestin (ADAM3) genes were infertile [9,10]. Both fertilin $\beta^{-/-}$ and cyritestin$^{-/-}$ sperm yielded a dramatic reduction in the sperm ability to bind to the zona pellucida and to zona-free oocytes. Sperm with knock-out of fertilin β had a 50% decreased ability to fuse with zona-free oocytes [9] but sperm lacking cyritestin fused at normal rates [10,11]. Fertilin $\beta^{-/-}$ cyritestin$^{-/-}$ double-knockout sperm are similar to Fertilin $\beta^{-/-}$ sperm. Disintegrins were originally identified in snake venom proteins and interact with integrins through a domain containing tripeptide sequences that mimic the Arg–Gly–Asp (RGD) tripeptide recognition sequence of various integrin ligands [12]. This further supported the proposal that the egg

receptor for fertilin β could be an integrin. In support of this hypothesis, a mAb to α6β1 integrin was shown to inhibit sperm-egg fusion and binding of fertilin β peptides and recombinant fertilin β proteins to the oocyte [1,13-15]. The α6β1 integrin was also reported to mediate the binding of cyritestin disintegrin domain to the egg surface [13].. RGD peptide was actually shown to inhibit sperm egg interaction reducing the number of fused spermatozoa when co-incubated with both gametes [18]. Since the integrin α6β1 is not RGD sensitive, we have to conclude that another integrin is involved in the gamete binding process. Fibronectine and vitronectin, glycoproteins that contain functional RGD sequences are both present on human spermatozoa [19,20] and integrin αvβ3, that recognize these ligands, have been detected on both spermatozoa and eggs in human [21,22]. GdRGDSP which blocks both fibronectine and vitronectin receptors significantly inhibits the binding of human sperm to oolemma of zona pellucida free hamster eggs [23]. But recently the understanding of this process was questioned by several experiments. Firstly, some data concerning integrins were not confirmed by others [4,16,17]. Secondly, integrins were found to be dispensable for sperm-egg fusion. Oocytes lacking the α6 integrin subunit could be fertilized in vitro at normal rates [17]. To determine whether other integrins could functionally compensate for the lack of the integrin α6β1, He *et al.* generated mice producing oocytes lacking β1 integrin using the Cre-loxP system. These females were completely fertile and the oocytes collected in these mice had normal binding and fusion with sperm in an *in vitro* fertilization assay. Moreover, blocking antibodies to the β3 or αv integrin did not inhibit sperm-egg fusion *in vitro*, using β1-integrin null eggs. Thus, it was concluded that integrins do not play an essential role during sperm-egg fusion [24]. Whether integrins play an accessory role remains to be determined. In this regard, the α6β1 integrin clusters at the site of sperm contact [25] is subjected to a major redistribution upon fertilization [26].

*Other molecules on the egg side*

<u>*CD9 and the tetraspanin web:*</u> the role of CD9 in sperm-oocyte fusion was firmly established in 2000, when mice with deletions in this gene were generated in three laboratories [27-29]. The major defect of these mice was a severely reduced fertility of the females. Indeed, in vitro fertilization assays demonstrated a defect in the fusion process. However, the binding of sperm to zona-free $Cd9^{-/-}$ eggs was not normal. Sperm attached normally to the surface of mutant eggs but they id not detach when washed gently 90 minutes after insemination [29]. Sperm in the perivitelline space did not attach to the membrane but instead moved actively. Additionally mAbs to CD9 were shown to inhibit in vitro fertilization[27,30]. How CD9 functions to regulate the process of sperm-egg fusion is so far unknown. The assembly of membrane microdomains by tetraspanins suggests that CD9 could regulate the function of a molecule to which it directly associates.

<u>*GPI-anchored proteins:*</u> GPI-anchored proteins possess a covalently linked glycosylated phosphatidylinositol moiety which serves to attach the protein portion of the molecule to the cell surface lipid bilayer [31]. GPI-anchored proteins can be released from the cell surface by treatment of cells with the highly specific enzyme phosphatidylinositol specific phospholipase C (PI-PLC) [32]. Coonrod et al. demonstrated that treatment of eggs with PI-PLC significantly reduced sperm–egg binding and fusion, indicating a role for GPI-anchored proteins from the oolemma in sperm-egg fusion [33]. Two molecular-weight protein clusters were released from the oolemma as a result of PI-PLC treatment.

*Molecules on the sperm side*

The Izumo protein was identified through the generation of a mAb that inhibits sperm-egg fusion [34]. The expression of Izumo was found to be testis-specific. However, Izumo was not

detectable on the surface of fresh sperm but become exposed only after the acrosome reaction had occurred. The size of the Izumo protein is 56 kDa in the mouse and 37 kDa in humans. The localization of Izumo is not restricted to the equatorial segment. The Izumo gene encodes a novel immunoglobulin superfamily (IgSF), type I transmembrane protein with an extracellular immunoglobulin domain that contains one putative glycosylation site. *Izumo*$^{-/-}$ females had a normal fecundity. *Izumo*$^{-/-}$ males were sterile despite normal mating behaviour and ejaculation. Many sperm were found in the perivitelline space of oocytes collected in females mated with Izumo -/- males, indicating a defect in fusion but not of migration. This defect in fusion was confirmed by in vitro fertilization assays since no egg could be fertilized in vitro with Izumo -/- sperm. Izumo is likely to play a role in the fusion process in human since a polyclonal antibody blocked binding and fusion of human sperm with hamster oocytes [35].

***Conclusions***

Every thing is much more complicated than expected. There is not a single molecule acting as a receptor but a multimolecular complex which affinity for its ligand is controlled by a subset of molecules. We recently described a reorganisation of the α6β1 integrin localisation that occurs upon fertilization under the control of the tetraspanine web, namely CD9, CD151 and CD81. On the oocyte membrane, this complex includes probably several integrins such as α6β1 and αvβ3. We also have just discovered that the α6β1 integrin is also present on the sperm side. This finding linked to recent discovery of the Izumo protein suggests that a membrane adhesion complex is also present on the sperm side. As the sperm does not bind to other cell within the female genital track, it means that the membrane complex probably induced by the onset of the acrosome reaction has its avidity and affinity up regulated during the interaction with the oocyte membrane component. The analysis of the shear force that are induced during gamete interaction becomes an essential step for the understanding of the molecular mechanism of the gamete fusion process as well as the common fusion mechanism of other types of cells. We have several mutant mice bearing specific molecules deletion which will allow us to further analyse the dynamic of both sperm and oocyte adhesions complex.

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**Fertilization and early developmental failure: how and why**

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In the broadest view, fertilization is generally assumed to encompass a progressive series of molecular and cellular events that begins with the attachment of spermatozoa to the zona pellucida, followed by its binding to the oolemma and in normal circumstances, penetration of a single gamete into the ooplasm. Penetration is followed by 'activation' of meiosis that was arrested at metaphase II, abstriction of the second polar body, movement of the sperm nucleus within the cytoplasm, the evolution of the male and female pronuclei, DNA replication and the condensation of chromatin into chromosomes. Fertilization can be considered to end with pronuclear membrane dissolution and the mixing of maternal and paternal chromosomes prior to the first cleavage division.

In the relatively early days of clinical IVF, fertilization failure for some or all of the meiotically mature oocytes obtained in stimulated cycles was not unexpected and in instances of insemination with normospermic samples, was largely attributed to defects intrinsic to the female gamete, such as a zona pellucida that was refractory to sperm attachment or penetration. In the latter instance, an impenetrable zona was suspected of having undergone 'hardening' because of premature cortical granule exocytosis. Common cytoplasmic abnormalities observed by light microscopy were described by Van Blerkom and Henry (1992) under the rubric, 'cytoplasmic dysmorphism', and some were associated with abnormalities in the fertilization process, early developmental arrest, or high frequencies of chromosomal aneuploidy. More recently, this descriptor has been applied to a wide range of cytoplasmic characteristics observed in human oocytes, some of which may be more apparent than real with respect to their impact on fertilization and embryo competence.

Static and time-lapse imaging of the cellular elements of fertilization in human oocytes free of apparent dysmorphisms has revealed temporal and spatial aspects of this process that lead to the development of a normal embryo, as well as specific defects associated with failure at each phase (Ash et al, 1995; Van Blerkom et al, 1995; 2000). When correlated with molecular and biochemical studies, morphodynamic imaging can provide important insights into subtle causes of fertilization failure that involve both paternal and maternal gametes. For example, subtle differences and defects in the spatial organization of the smooth-

surfaced endoplasmic reticulum in the human oocyte may be associated with perturbed calcium signaling and disorders in the cell cycle, cytokinesis and genomic imprinting (Otsuki et al, 2004). In this respect, contemporary research in early human development has made the necessarily transition from subjective microscopic studies of gametes to the objective molecular and cellular analyses that can define specific defects whose consequences include fertilization failure and early embryo demise.

As laboratory-based methods to assess oocyte competence and procedures for sperm analysis, selection and preparation have steadily improved, the frequency with which fertilization failure occurs for most or all MII stage oocytes within cohorts has declined markedly. Indeed, in current clinical IVF, insemination options that include ICSI have resulted in fertilization failure *per se* becoming increasingly rare. For example, failure with conventional insemination is often followed by ICSI when no signs of fertilization are evident within the first 16-20 hours. In our experience, an earlier re-insemination protocol largely avoids the developmentally compromising effects of spontaneous oocyte aging (e.g., loss of meiotic spindle integrity), is rarely accompanied by dispermy, and if performed when pronuclei are first detectable (between 8-to-10 hours after conventional IVF), has outcomes similar to time-appropriate fertilizations (i.e., ICSI or IVF within a few hours after ovum retrieval).

The increased ability of the clinical IVF laboratory to avoid significant or complete fertilization failure has shifted emphasis to understanding the etiology of early embryo demise and developmental abnormality in general, and in particular, the extent to which the causes may originate in the gametes; i.e., fertilization and early embryo failure may be expressions of common defects in the sperm or oocyte. While manipulations of the fertilization process, such as insemination or re-insemination by ICSI combined with calcium ionophore activation, may enhance the probability of obtaining embryos (in what could otherwise be failed cycles), whether they are developmentally normal remains a central question. In this context, while certain aspects of the fertilization process can be accomplished by physical or chemical manipulations, the underlying defect(s) that required their use may not be understood or may not be recognized as potentially problematic for normal development. This is particularly relevant in circumstances where a clinical IVF program's imperative is to achieve fertilization 'by whatever methods' in order to avoid presenting the often emotionally devastating news of failure to an infertile couple; the occurrence of apparent cytoplasmic defects that have the potential for demise after embryo transfer is weighed against a programs experience with normal outcomes in these situations. Decisions concerning which oocytes to fertilize or embryos to transfer are the most difficult when they occur against a background of evident dysmorphisms that *may* produce embryos with

high frequencies of implantation failure, post-implantation demise, or most recently, the possibility they contain disorders in genomic imprinting (Otsuki et al, 2004).

**AN AWARENESS OF THE SUBTLER MOLECULAR AND CELLULAR ASPECTS OF HUMAN OOCYTES AND SPERM THAT CONTRIBUTE TO FERTILIZATION AND EARLY DEVELOPMENTAL FAILURE IS THE PRINCIPAL LEANING OBJECTIVE OF THIS PRESENTATION.**

Sperm analyses that examine the extent of chromatin breakage by flow cytometry, or in individual cells by the TUNEL or Comet assays, have recently entered the clinical IVF field as evaluative methods to determine the following: (i) the extent to which breakage may have contributed to unexpected fertilization and early embryo failure, and as a predictive means to (ii) assess relative sperm DNA integrity prior to IVF. Competent sperm contain a low frequency of chromatin breaks that arise naturally during the condensation, remodeling and nuclear packaging stages of spermatogenesis. Normally, these breaks are repaired by ligating enzymes during the unfolding, decondensation and replication phase of male pronuclear evolution that precedes the first cell division. Studies (to be described) which used an experimental in vitro assay to determine the ability of the human ooplasm to repair breaks in both maternal and paternal DNA show that this enzymatic activity varies widely between normal-appearing metaphase II oocytes, both within and between cohorts; for some oocytes, this capacity was undetectable. Of particular note are findings showing that immature oocytes (GV and early MI stage) obtained in gonadotropin-stimulated cycles which matured in vitro to MII may be largely deficient in this capacity; this defect may contribute to the high frequencies of structural chromosomal abnormalities reported after their fertilization. The inability of the ooplasm to adequately repair normal levels of DNA breakage, beginning at the pronuclear stage, may be an occult and proximate factor in competence determination for the embryo that likely increases with maternal age. The extent to which DNA ligating activity is associated with specific cytoplasmic dysmorphisms will be discussed. In instances of sperm chromatin breakage beyond baseline levels, oocyte-specific differences in ligating ability may be a significant determinant of embryo chromosomal normality, and when severe, likely exceeds the normal repair capacity of the oocyte.

Evaluations of the pattern and rate of sperm motility prior to insemination are often used to decide whether insemination should involve conventional IVF or ICSI. Although infrequent, an abrupt drop-off and premature cessation of motility during insemination are observed during the insemination phase of IVF. The etiology of this phenomenon is especially puzzling when initial count, morphology and motility pattern are normal, and similar samples were used

previously in multiple IUI procedures (and recoded as normal), although without a successful outcome. With respect to outcome in these IVF cycles, this phenomenon is particularly problematic because most or all of the presumably competent MII oocytes are unpenetrated and unfertilized when inspected for pronuclear formation (usually between 16 and 20 hours after insemination). Typically. it is at this time that the cessation of motility for virtually all of the sperm is first noted. The results of follow-up studies (to be presented) which examined subsequent ejaculates from affected individuals, show that a precipitous cessation of sperm motility is accompanied by a sudden depolarization of the inner mitochondrial membrane potential, the electrochemical force that drives the conversion of ADP to ATP.

Preliminary results show that mitochondrial depolarization is preceded by a sudden efflux of calcium from these energy-generating organelles, which is associated with the presence of an instability in the mid-piece portion of the sperm plasma membrane. This abnormal activity occurs in a wide variety of culture media and atmospheric conditions, and its restriction to the midpiece is suggested by the absence of unusual levels of DNA breakage or discontinuities in the plasma membrane overlying the acrosome. To date, we have detected acute mitochondrial depolarization only in sperm isolated from the seminal fluid, as is commonly done in preparation for IVF. However, its frequency is significantly reduced when isolated sperm are incubated at room temperature, or below. For some men, this focal plasma membrane instability may be associated with the elimination of seminal proteins and other bioactive molecules, but it's apparent inhibition at subnormal temperatures is inconsistent with conventional fertilization in vitro, owing to disruption of the meiotic spindle in the oocyte at reduced temperature. We are examining the dynamics of this process in affected individuals by investigating whether sperm isolation increases mitochondrial reactive oxygen species generation to levels that can destabilize mitochondrial membranes (inner and outer) or effect the integrity of overlying plasma membrane; damage at either level can lead to profound changes in mitochondrial polarity and calcium homeostasis which in turn, will have toxic effects on fertilizability. For clinical purposes, our laboratory protocol now includes the inspection of control (oocyte-free) insemination dishes during the first 3 hours of culture and immediate ICSI if a precipitous motility drop-off is detected and no sperm are observed within the zona pellucida after complete denudation by mechanical or enzymatic treatment of the oocytes. Recognition of this phenomenon from previous IVF attempts, or timely treatment by ICSI with the remaining few motile sperm, has resulted in successful outcomes where fertilization failure would have normally occurred.

While conventional analyses of the male gamete in infertility evaluation and treatment examine grossly detectable numerical and morphological characteristics, the above findings illustrate how subtle defects that may be relatively common (DNA breakage), or comparatively rare (acute global mitochondrial depolarization), can adversely affect the ability of apparently normal sperm to penetrate a competent oocyte and perhaps, establish a chromosomally normal embryo. Because of the limited number of human oocytes obtained in stimulated cycles and their intended use for IVF, studies of fertilization failure that may involve the female gamete have been largely confined to light microscopic observations; the primary intent of these studies has been to identify cellular and extracellular features that may be inconsistent with normal competence. In this respect, experimental studies of the 'natural' causes of fertilization and embryo developmental failure that originate with the oocyte can be more difficult to design and undertake that those using sperm.

Recent evidence suggests that subplasmalemmal cytoplasm is a site of regulatory activities in the oocyte and early embryo that can determine the normality of fertilization and subsequent development. This notion derives from the detection of regulatory molecules (reviewed by Edwards, 2005), mRNA (for transcription factors; Deb et al, 2006), and domains of high-polarized mitochondria (reviewed by Van Blerkom, 2004) in the subplasmalemmal cytoplasm of mouse and human oocytes and early embryos. We have reported that high-polarized mitochondria that are complexed with discrete arrays of smooth endoplasmic reticulum occur in stable subplasmalemmal domains in human oocytes; their absence in the intact oocyte, or focal elimination into small cytoplasts as a result of certain types of limited, spontaneous (minor) fragmentation, can have adverse effects during fertilization and for the early embryo, the effects are blastomere-specific (Van Blerkom and Davis, 2006). A relatively small number of high-polarized mitochondria are localized in subplasmalemmal domains and as a result of position, we have proposed they represent 'vanguard' mitochondria involved in ionic homeostasis and signal transduction; downstream developmental consequences may result if their polarity is perturbed, and if spontaneously eliminated from the cell (oocyte, pronuclear embryo, blastomere), they are not replaced nor are the domains re-established.

Several studies have shown that a low mitochondrial (mt) DNA copy number is associated with fertilization failure and demise for the early human embryo (see review by Van Blerkom, 2004). Although reduced ATP production is generally assumed to be the developmentally toxic factor, preliminary findings suggest that such oocytes may also be unable to establish a normal complement of 'vanguard' mitochondria and as a result, may be deficient in their ability to establish regulatory processes that may normally reside in subplasmalemmal domains;

evidence to be presented indicates that a subplasmalemmal deficit, rather than a metabolic insufficiency, may be a proximate cause of certain developmental defects observed during human preimplantation embryogenesis.

Spatial perturbations of polarized subplasmalemmal domains of regulatory proteins, mRNA, and organelles, such as high-polarized mitochondria, likely represent another class of spontaneous activities with negative effects on human oocyte and early embryo developmental competence. Preliminary observations from time-lapse imaging of human MII oocytes and pronuclear embryos show that small fragments elaborated from the animal pole (peri-polar body region) can move within the perivitelline space and are often reabsorbed at the 'vegetal' pole, and vice versa. If confirmed by experimental manipulations with 'tagged' fragments, as will be discussed, it could indicate that domains of regulatory molecules and organelles may spontaneously transpose. This in turn could effect cell-specific inheritance patterns during cleavage and although speculative at present, one consequence of their elimination or transposition could be to alter cell-type specification and function at the blastocyst stage (Antczak and Van Blerkom, 1999; Edwards, 2005; Deb et al, 2006).

The 'occult' disturbances described above are part of an emerging picture of early human embryogenesis that offers new insight into how their expression in the gametes and nascent embryo may determine the normality of fertilization and subsequent development, respectively. In clinical IVF, information of this type may lead to the identification of common origins for developmental abnormalities exhibited by embryos during culture, as well as those that may adversely influence implantation and post-implantation competence. The present challenge is to relate these specific molecular and cellular findings to oocyte and embryo phenotypes that clearly indicate their occurrence.

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**Sperm morphology is of limited clinical use – so what else is on the horizon? The - omic revolution?**

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**Learning objectives**

1. Understand the development (and limitations) of current techniques of sperm morphology assessment as a part of semen analysis.
2. Understand the sterilizing defect globozoospermia as a clinical example of isolated teratozoospermia.
3. Understand, and have an appreciation for, the new methods of diagnosing sperm function, their importance and potential applications. The use of - omics.

Background

There is a clear need to significantly improve our understanding of the cellular basis of normal sperm function. This knowledge is fundamental for two key developments in male fertility: firstly, to provide the basis for effective diagnostic tools and, secondly, to facilitate the study of the physiology of abnormal/dysfunctional cells which is central to developing rational, non-ART therapy. Epidemiological data shows that 1:7 couples are classed as sub-fertile. Sperm dysfunction is the single most common cause of infertility and affects approximately 1:15 men. Studies using semen assessment as the criteria for sub fertility (sperm concentration $<20X10^6$/ml) show that 1:5 18 year olds are classed as sub-fertile. This is a high proportion of the population compared with other prevalent diseases such as diabetes. Thus, male sub-fertility is a very significant global problem and, what is most worrying, are the recent reports suggesting that its prevalence is increasing.

Sperm morphology : Is it of value?

"*We have come to the end of something, surely someone will want to carve a headstone for traditional sperm analysis or perhaps a mausoleum would be more fitting*" Paul G McDonough 1997

The value of traditional semen parameters (concentration, motility and morphology) in the diagnosis and prognosis of male infertility has been debated for over 60 years and, perhaps not surprisingly, the debate continues. Suffice it to say traditional semen parameters do provide some degree of prognostic and diagnostic information for the infertile couple. However, it is only at the lower ranges of the spectrum that these parameters are most useful and, even then they can only be used as guidance for couples and do not represent absolute values. Traditional semen analysis will therefore only be a limited first line tool in the diagnosis of male infertility. Consequently, the emphasis in reproductive medicine has been on developing simple, robust and effective tests of sperm function. Yet despite the plethora of potential assays available, results have been very disappointing and this is one reason why we are still debating the value of traditional semen parameters.

There are several guiding principles before any conclusions relating to sperm morphology can be presented:

1. Our expectations of the usefulness (value) of sperm morphology in the reproductive medicine are unrealistic. The % 'normal' forms are significantly correlated with sperm concentration and motility. Morphology is only one aspect of a semen assessment-reflecting the quality of a semen sample - and as such should only rarely be considered on its own.
2. Morphology is a subjective assessment and there is, unfortunately, no internationally accepted universal standard.
3. Consequently, methods of assessment, quality control and assurance, reporting values, reference ranges etc are often personalised (regionalized) and thus rarely can be used/tested in other environments (e.g. clinical settings).
4. Many studies have used inadequate numbers of subjects, inappropriate end points, and minimal, if any, quality control. Such studies often conclude that morphology is of no importance.
5. With the use of high standards, consistent assessments, large numbers and appropriate end points morphology has been shown to have limited clinical value.

Bearing this in mind what do we conclude?

Conclusion 1

An accurate assessment of sperm morphology shows that the % 'normal' forms is related to progress of the sperm through the female tract (e.g. penetration of cervical mucus, transport to the oviduct and binding to the zona). Thus, it is of little surprise that, in appropriately controlled studies, using *in vivo* conception (birth) as an endpoint sperm morphology has some predictive value. Of course what is often forgotten (or not reported/hidden in the small print) is the large confidence limits associated with morphology values in such studies. In fact, although morphology has some predictive value (pregnant/not pregnant or time to pregnancy) very often, female factors (e.g. age)

and length of infertility have a far greater influence on the outcome. Thus, sperm morphology assessment has a limited value in the diagnosis/prognosis of future (natural) fertility in the infertile couple.

Conclusion 2

When assessing the predictive/diagnostic value of sperm morphology for assisted reproduction (IUI, IVF) things become more challenging. This is simply because we are selecting cells and placing them closer to the egg. Additionally, there have been significant improvements in IUI and IVF techniques with time and thus studies from 20 years ago based on IVF techniques have limited relevance today.

Conclusion 3

When selecting cells for ICSI we are asking different/more refined questions to those in normal semen assessment. Consequently the overall morphology of a semen sample has little relevance. However, when individual cells are selected based on their morphology it has some value

Conclusion 4

Although the correlation is relatively weak there is a significant association between the shape of a cell (normal/abnormal) and it’s genetic material – often represented as chromosome number.

Conclusion 5

There are specific morphology profiles that provide very clear diagnostic information. For example, large headed sperm, two heads, multiflagellar sperm, and distinct midpiece abnormalities. However, because the majority of spermatozoa in a human semen sample are ‘abnormal’ the proportions of these stated abnormalities need to be significant. Some sterilizing defects do occur e.g. globozoospermia. These conditions are rare and, unbelievably, often missed by the laboratory.

Overall conclusion on sperm morphology

Sperm morphology is usually performed poorly (as is semen analysis) and as such will have no value. However, it is relatively easy to do well and all effort should be made to understand the nature of the test and maintain an effective training programme. Even when performed to the highest standards, it should only be used in conjunction with other surrogate markers of fertility assessment (including female factors).

Globozoospermia - isolated teratozoospermia.

Globozoospermia is manifested as a severe form of teratozoospermia, where 100% of the spermatozoa have a rounded globular (spherical) head (fig 1, 2). It is an uncommon condition and the literature is confined to a few case reports. Consequently, little is known about its incidence, cause and pathogenesis. It was first described by in 1971. Before ICSI, men with globozoospermia were regarded as sterile. Preliminary *in vitro* sperm function studies suggested the failure of round headed spermatozoa to bind to zona free hamster oocytes and penetrate the human zona pellucida. The first successful pregnancy after intracytoplasmic injection was described as a case report 1994. Although

several successful pregnancies have subsequently been reported with ICSI, with and without oocyte activation (it's not clear if it is needed), the fertilisation rates remain variable and low. Interestingly, the majority of men have almost normal sperm concentrations with a relatively high proportion of motile spermatozoa.

New tools in assessing sperm physiology.

1. Understanding the calcium toolkit as a basic function of the spermatozoon-signalsome.

As the functioning of a spermatozoon is critically dependent upon the tight regulation of calcium which, when disrupted, results in fertilisation failure there is a clear need to understand its regulation. However, in contrast to somatic cells an understanding of $Ca^{2+}$-signalling in the sperm cell is only now developing. In our laboratories we have developed a complex model for sperm $Ca^{2+}$-homeostasis involving several types of $Ca^{2+}$ permeable channel in the plasma membrane and at least two stores (fig 3). It is clear that these toolkit components are distributed to allow localisation of $[Ca^{2+}]_i$ signals. It appears that the acrosome functions as an $IP_3$-releasable store, activated by agonists linked to phospholipase C. Recent studies suggest mobilisation of acrosomal $Ca^{2+}$ is intimately involved in activation of acrosome reaction. A separate store, probably the RNE/calreticulin store, exists in the neck. We can only speculate as to the function of the latter store but we believe it may be controlling calcium oscillations in the sperm which are related to significant flagellar movement patterns.

2.. The Sperm and Testis Transcriptome

Following considerable debate, it has now been established that human sperm contain complex populations of RNA. Using cDNA micro arrays, the possibility of the sperm RNA profile as an indication of male fertility has been tested. A rigorous study by Ostermeier and colleagues documented sperm RNA profiles of fertile men. Comparisons were made with gene expression in the testis and lymphocyte controls. Intriguingly, there was minimal overlap with the lymphocyte profile suggesting an essentially pure population of sperm RNA. Additionally, one individual ejaculate contained almost all the unique ESTs (expressed sequence tags) as were detected in the pooled fertile controls. Concordance between testicular and the sperm profiles supported the view that sperm RNA can be used to monitor past events during spermatogenesis. This study paves the way for the more detailed molecular examination of sperm dysfunction and may represent a powerful non-biased systematic approach.

There have been a large number of publications using microarray analysis to study the transcriptome of the animal testis. A variety of approaches have been developed ranging from relatively simple methods examining a very limited gene set, to more sophisticated techniques developing subtracted cDNA libraries. The widespread use of gene expression array technology to assess global transcription represents a very powerful tool to study a plethora of exciting developments in sperm production such as profiling the specific stages of genes expression in spermatogenesis, examining the effect of androgens on gene expression obtaining expression profiles of isolated germ cells and in reproductive toxicology.

An example of the power of this technology is given by the study by Schultz and colleagues. They used an Affymetrix mouse [U74 v2] oligonucleotide array set analyzing approximately 20,000 genes to study the germ cell-enriched gene expression profile from post-partum day 1 to adult mice. There were a significant number of testis-specific transcripts identified coincident with or after meiosis - approximating to 4% of the mouse genome. These data provided a large number of candidate genes for contraceptive targeting as well as a number of proteins that may be involved in fertilization. Comparison of specific post-meiotic gene transcripts to the literature database showed approximately 50% of the genes led to complete loss of fertility in knock out (KO) animals. These results encourage examination of late/post meiotic transcripts to assess their potential as contraceptive targets.

The advent of microarrays to scan the genome makes it now possible to study human spermatogenesis in normal and sub fertile populations. In conjunction with non-invasive fine needle aspirations of the testis we are likely to see a number of studies of human expression profiles associated with male fertility.

3. <u>The sperm proteome: understanding the sperm cell and diagnosis of sperm dysfunction</u>

Comprehensive and systematic identification and quantification of proteins expressed in cells and tissues are providing important and fascinating insights into the dynamics of cell function. Spermatozoa are ideally suited for proteomic analysis as they have no transcription or translation machinery and thus are solely dependent on post translational protein modification for signaling. Additionally, spermatozoa are relatively simple cells having jettisoned a number of their organelles and much of their cytoplasm at the late stages of spermiogenesis in order to develop a hydrodynamically efficient specialized cell capable of swimming in the female tract, locating the egg and delivering their DNA. . Comprehensive studies are now being reported of the human sperm proteome. For example, Johnston and colleagues have performed an initial proteomic analysis of sperm from one normal man. 1,760 proteins were identified with high confidence. Many originated from the accessory glands and a large number were either novel proteins or ones where no gene ontology was yet available. Proteomics are also being used to examine sperm specific processes, for example proteins that are tyrosine phosphorylated during capacitation. Interestingly, it is over 10 years since the discovery of tyrosine phosphorylation as a putative marker of capacitation yet the role of the proteins and their sequence of activation is very sketchy and, with the exception of the AKAPs (AKAP3 and AKAP4), only a small number of candidate proteins have been identified. We are still a long way from obtaining even a minimal ‘picture’ of events.

In our laboratory we have been using proteomic strategies to identify defects in sperm function responsible for fertilization. Specifically we are interested in identifying differences in sperm protein expression between control (fertile) men and patients with spermatozoa that failed to fertilize oocytes in vitro. Our initial studies have focused on a 2D gel-based approach and developing a series of fertile controls (with several ejaculates) in order to determine if any differences observed in the patient samples are real. Initial results are interesting. To our surprise, there was relatively little intra-donor and inter-

donor variation (1.4 % and 1.8 % of the total number of spots identified, respectively). However, differences between gels do occur and when accounting for this, we have categorized one man where we have identified 20 differences from the control that we are confident represent true differences.

Summary
There is an urgent need to develop a more detailed understanding of the physiological, biochemical and molecular functioning of the human sperm cell. We can use this knowledge as a platform to improve the diagnosis of male infertility and importantly to develop potential non ART based therapies. The tools at our disposal have never been more sophisticated and it is likely that rapid progress will be made in this area within the next 5 years. Perhaps then we will see a decrease in the use of inappropriate ART treatment.

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**Figure 1**

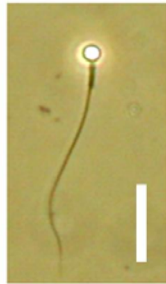

**Figure 2**

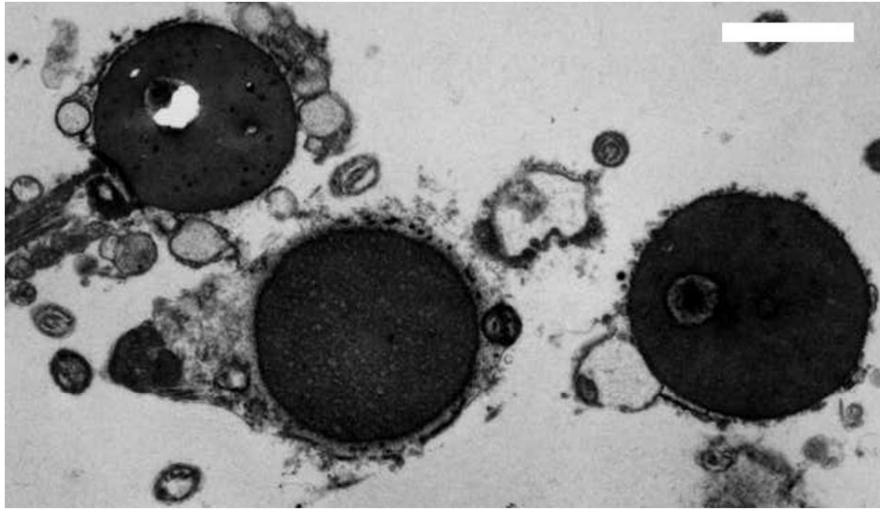

**Figure 3**

Current thinking - calcium regulation in sperm

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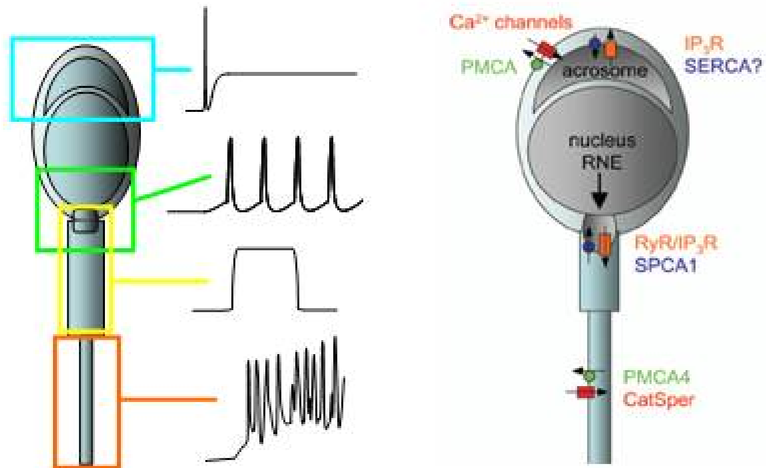

## How to define the limits between IVF and ICSI?

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### Learning objectives

• the main aim of ART should always be to use the simplests and least expensive procedure, with the greatest long-term chance of a healthy child

• whenever performing IVF for borderline male infertility corrective measures towards insemination concentration are to be taken

• there is currently no evidence that a split insemination strategy (ICSI vs IVF) on sibling oocytes has any added value when high insemination concentrations are used for IVF.

### Lecture summary

Defining the limits between IVF-ICSI is an important issue because it has not only a repercussion on the success rate of an IVF program, but may have an important psychological impact on the couple. Complete fertilisation failure is an unwanted event because it has an important negative psychological impact on the couple. The prevalence of fertilisation failure in conventional in-vitro fertilisation is reported to occur for non-male indications in 5 to 15% of cycles, when IVF is performed for male indications, the complete fertilisation failure rate can be as high as 50% (*Molloy et al 1991, Ben-Schlomo et al 1992, Coates et al 1992, Lipitz et al 1993, Lipitz et al 1994*). The causes of complete fertilisation failure after IVF are related to either oocytes factors or sperm factors (especially in male indications) or eventually lab factors. Furthermore, complete fertilisation failure after IVF has been reported to be repetitive in 30 to 50% of cycles. In contrast, after ICSI, complete fertilisation failure occurs in less then 3% of started cycles *(Liu et al 1995, Flaherty et al 1998)* and can be present in subsequent cycles in up to 25%. Although ICSI has been proposed as the most robust technique for achieving fertilisation in an IVF program, the aim of any method of assisted reproduction should always be to use the simplest and least expensive procedure with the greatest long-term chance of healthy children *(Tucker et al 1993).* But even apart from these considerations, the question remains whether ICSI is the method of choice for all IVF indications. The Cochrane Library of Systematic Reviews includes one meta analysis on IVF versus ICSI *(Van Rumste et al 2000).* The last update of 2003 dealing with non-male indications only comprised 10 randomised control trials (RCTs) all, except for one *(Bhattacharya et al 2001),* had a sibling oocyte set-up design. In this set-up, oocytes obtained after ovum pick up are randomly assigned to either conventional IVF or ICSI. Because the 9 RCTs on sibling oocytes do not allow any conclusion towards implantation and pregnancy

rates, the Cochrane review only selects the study by *Bhattacharya* as best evidence. This study is a large multicentre RCT powered to detect a 10% difference in implantation rate. It comprises 206 conventional IVF cycles versus 209 ICSI cycles. Although there was no difference in complete fertilisation failure between IVF or ICSI (5% versus 2% respectively), the fertilisation rate per oocyte was significantly higher after IVF then after ICSI (58% versus 47% respectively). Furthermore, the implantation rate per embryo after IVF was also higher then after ICSI (30% versus 22%, p=0.03). The lower implantation rate after ICSI may be attributed to the denuding step in ICSI, which may alter the implantation potential of the eventual embryo. Given the results of this adequately powered multicentre study, there is obviously no benefit in performing ICSI for non-male indications. The choice between IVF or ICSI for these indications is thus easy.

**But what about male indications?**

Although clinical evidence is lacking for many typical ICSI indications, there are strict male indications for ICSI: use of surgically retrieved sperm, use of spermatozoa with flagellar dyskinesia (immotile cilia syndromes), use of round-headed spermatozoa (globozoospermia). Although good evidence is lacking, the prevalence of relevant titers of antisperm antibodies may also be an indication for performing ICSI (*Nagy et al 1995, Lahteenmaki et al 1995, Vasquez-Levin et al 1995*). The same goes for cryopreserved sperm from cancer patients. Again, no prospective comparative studies are available in the literature, however, based on retrospective case series it may be assumed that for most of these patients, given the poor quality of sperm cryopreserved, the post-thaw sperm damage and the limited numbers of spermatozoa frozen, ICSI is the method of choice when assisted reproduction is indicated (*Kellehers et al 2001, Tournaye et al 2003*).
But what about oligoasthenoteratozoospermia? The cut-off values used for conventional IVF are mostly experience-based and extrapolated from small, older studies not always using the same laboratory standards as of today. Apart from the motile count after sperm preparation, motile count in the native semen sample as well as morphology according to Tijgerberg criteria has been used for accepting couples for conventional IVF treatment. *Kastrop et al* proposed a motile count of at least 1 million spermatozoa in the native semen sample (K*astrop et al 1999*). When the motile progressive count after sperm preparation is taken as a criterion, numbers vary from 1 million *(Fisch et al 1991)* to 0.5 million progressive motile spermatozoa *(Verheyen et al 1999)* or even 0.2 million motile progressive spermatozoa *(Payne et al 1994)*! When morphology is taken as a criterion, in general 5% normal morphology is the cut-off value below which a low fertilisation after conventional IVF is anticipated *(Kruger et al 1988, Grow et al 1994).* Exceptionally, a combination of morphology and motile count is used: *Plachot et al* proposed 0.5 million normal motile progressive count in the ejaculate for accepting couples for conventional IVF *(Plachot et al 2002).* Nowadays, strategies for defining the limits between IVF and ICSI are either based on these experience-based preset cut-off values or are based on the assumption that ICSI is the more robust insemination technique. Then, a split IVF-ICSI set up is also proposed as a strategy *(Plachot et al 2002).*
But even when the preset cut-off values for conventional IVF are met, as in "border-line male infertility", the choice between conventional IVF and ICSI may be difficult. In our

meta-analysis, dealing only with border-line oligoasthenoteratozoospermia, we concluded that the fertilisation and fertilisation failure rate after IVF can be highly depended on the insemination protocol used for conventional IVF. Although corrective measures have been proposed many years ago *(Oehninger et al*, 1988) still today, many IVF programs use suboptimal insemination concentrations for couples with border-line oligoasthenoteratozoospermia*).* In our own study *(Tournaye et al 2003)* fertilisation rate after conventional IVF was 37.5% per oocyte versus 64.2% after ICSI when an insemination concentration of 0.2 million progressively motile spermatozoa per ml were used for conventional IVF. However, after correcting the insemination concentration to the 0.8 million progressively motile spermatozoa per ml, the fertilisation rate after IVF was 59.7% versus 67.6% (difference not significant). With the suboptimal insemination concentration complete failure of fertilisation occurred in 25.7% of cycles while with the optimised protocol total fertilisation failure only occurred in 5.26% of cycles. The results of the meta-analysis corrobrate these findings: RCTs a suboptimal insemination concentration report a significant higher fertilisation rate per oocyte after ICSI than after conventional IVF. However, subanalysis of the 3 RCTs in which a high insemination concentration was used shows no significant benefit of ICSI over IVF. Although the current evidence is limited, the result of this meta-analysis calls for caution when promoting ICSI for border-line oligoasthenoteratozoospermia.

**Is a split IVF-ICSI set up the way to go?**

Performing a split IVF-ICSI strategy in a clinical setting as performed in the RCTs mentioned above, has been proposed as the method of choice when attempting a first IVF cycle in couples with border-line male infertility. Eventually embryos transferred are derived both from conventional IVF and ICSI, and therfore this approach is not allowed in all countries. But even then, it is far from clear whether this approach is indeed the first choice. One study concluded that "this strategy enabled ... to avoid 32.8% of complete fertilisation failures after IVF" (*Plachot et al 2002*). However, in another study with a similar set-up, the complete fertilisation failure after conventional IVF was only 7.1% *(Elizur et al 2004).* Strikingly, the main methodological difference between both studies was that in the former study an insemination concentration of only 0.06 million motile spermatozoa was used. Another study also failed to show any benefit of a split set up in patients with moderate oligoasthenoteratozoospermia *(Hershlag et al 2002).*

From the above may be concluded that we need better methods to define the limits between IVF and ICSI and that the outcome of the two methods are closely related to the methodology used in the IVF laboratory. Preferentially, each IVF program should try to define its own limits based on a prediction of fertilisation failure after conventional IVF in its own setting. Rhemrev et al. published a perfect example of this approach *(Rhemrev et al 2001).* In their study, first they constructed a multiple regression model based on a set of 2366 couples undergoing IVF with a complete fertilisation failure rate of 25% as being acceptable. Then, they validated their model on a subsequent set of 917 other couples. They used a high insemination concentration of 1 million motile spermatozoa per ml. They showed that apart from the post-preparation motile count, the number of follicles is an important predictor for complete fertilisation failures. For example, if a

pick up is scheduled in a couple in which the wife shows less then 5 follicles, at least 1.11 million motile spermatozoa are to be obtained after sperm preparation for accepting the couple for conventional IVF with an anticipated risk for complete fertilisation failure of 25%. On the other hand, when more than 15 follicles are present, the post preparation motile count can be as slow as 0.35 million before the risk for complete fertilisation failure after conventional IVF exceeds 25%. They also calculated, based on the number of follicles, probabilities of a total fertilisation failure according to the postwash total progressive motile count. For example, when the number of follicles in their model is between 10 and 15, the probability of a complete fertilisation failure is 23% when the post wash total progressive motile count was 1 million. But the probability drops to 14% when this count is 3 million. The approach as proposed by *Rhemrev* seems to be very rational and hopefully more studies will be performed with centre-specific adaptations proving that it is a valuable strategy. Depending on the local situation towards reimbursement and costs of IVF versus ICSI, a predefined and acceptable total fertilisation failure rate can be introduced in the predictive model. When complete fertilisation failure is totally unacceptable, a split IVF-ICSI set up can be proposed although clinical evidence for the superiority of this approach is currently lacking.

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# Genomic activation : animal models

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**Learning objectives :**

1. Nucleo-cytoplasmic interactions at genome activation : interspecific differences.
2. Molecular identification of the earliest functions expressed by the embryonic genome.

**Introduction: Transcriptional activation and epigenetic reprogramming.**

Fertilization brings together two haploid genomes that are transcriptionally silent. These two genomes come from highly differentiated cells, namely the gametes. The epigenetic histories of these genomes are quite different and the molecular reasons resulting in their transcriptional silence also differ. The molecular support of oocyte genome silencing remains poorly understood, whereas high compaction of paternal genome mediated by the histone-protamine replacement at the end of spermatogenesis is responsible for sperm genome silencing. At fertilization the zygotic cell is totipotent which means that it can give rise to a complete, normal and fertile individual. This totipotent state is quite transient : it only spans few cell cycles, and as soon as the blastocyst stage, the embryo only contains two cell categories regarding their potency : inner cell mass pluripotent cells and trophectoderm differentiated cells, the first differentiated cells of the whole organism. Pluripotency is defined as the cell ability to participate to all tissues of the embryo itself (excluding the extraembryonic tissues) when introduced in a normal embryo, thus giving rise to a chimeric individual.

The first function of oocyte cytoplasm at fertilization (Fig.1), is to assume both the transcriptional activation of the newly formed embryonic genome and the epigenetic reprogramming in order to restore nuclear totipotency. These molecular processes occur concomitantly and are tightly linked. They require precisely regulated nucleo-cytoplasmic interactions.

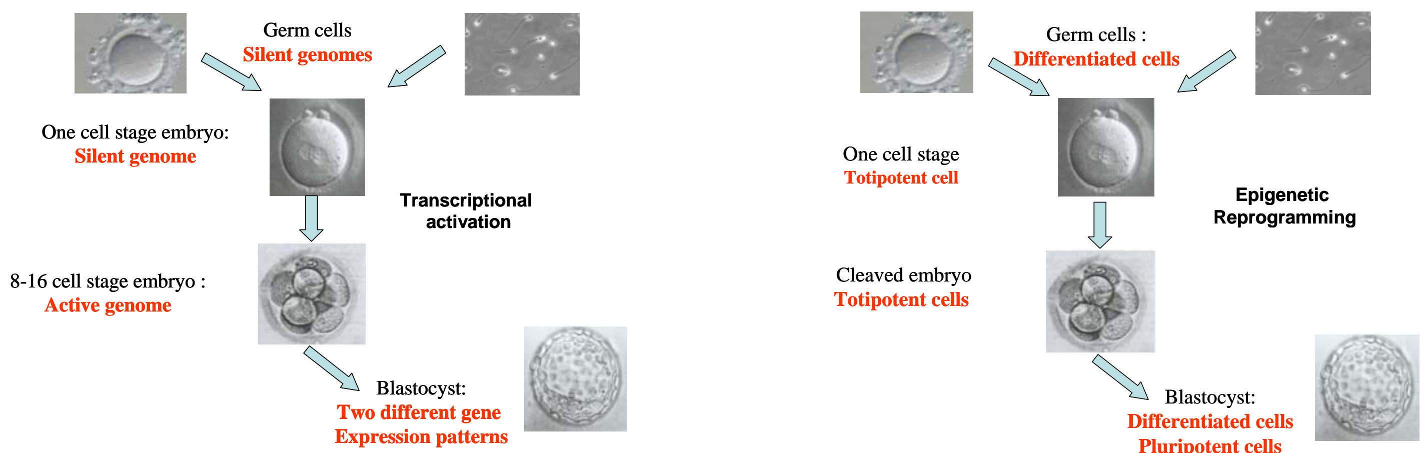


*Fig 1: First function of the oocyte cytoplasm at fertilization : transcriptional activation and epigenetic reprogramming of the newly formed embryonic genome.*

Epigenetic modifications of the genome ensure proper gene activation during development. They involve genomic methylation changes, assembly and post-translational modifications of histones and remodelling of other chromatin-associated proteins. Modifications of DNA methylation have been extensively studied in the mouse model where an active and rapid demethylation of paternal DNA occur during the one cell stage, followed by a passive demethylation of the whole genome during cleavage (1). This situation however is not representative of DNA methylation modifications in all other species. In the sheep for example, paternal DNA is not demethylated after fertilization and the passive demethylation over cleavages is only partial (2). Analyzing alternative models is thus absolutely required before concluding on the functional significance of such modifications. Histone post-translational modifications during genome transcriptional activation period are being precisely characterized by numerous studies, until now, nearly all of them use the mouse model.

**I Genome transcriptional activation: a progressive event.**

Early development relies on maternal information synthesized and stored all over oogenesis as transcripts and proteins. The time and developmental stage when embryonic transcripts are absolutely required for further development varies according to species (Table1). This stage can be experimentally defined as the stage where embryo development stops in culture media containing RNA polymerase II inhibitors (usually α amanitin). It often corresponds to a blockage stage in non optimal culture conditions and coincides with a significant increase in transcription level and to major changes in the protein synthesis pattern. It corresponds to major transcriptional activation.

| Species | Elapsed time between fertilization and Major transcriptional activation | Major transcriptional activation Developmental stage |
|---|---|---|
| Mouse | 24 hours | 2 cell |
| Rat | ND | 4 cell |
| Rabbit | 36 hours | 8-16 cell |
| Cattle | 60-70 hours | 8-16 cell |
| Sheep | 45-60 hours | 8-16 cell |
| Goat | 48-60 hours | 8-16 cell |
| Horse | 72 hours | 8-16 cell |
| Pig | 72-96 hours | 4 cell |

*Table 1 : Genome major transcriptional activation in different mammalian species*

Embryo is however able to transcribe before this stage. In all tested species, transcriptional activity is detected as early as the end of the one cell stage. The period when the embryo is transcriptionally active but does not depend on its own transcripts to develop further is called the minor transcriptional activation period. This period spans one (mouse) to several cell cycles (four in cattle and rabbit for example). It is experimentally evidenced by incorporation of labelled ribonucleotides, transcription of injected plasmidic constructions and even transgene expression. During this period, the transcriptional activity remains weak. The functions encoded by the embryonic genome are not well known.

Preceding the onset of the minor transcriptional activation period, the embryo is unable to transcribe. This inability is probably due to biochemical properties of the egg cytoplasm since transcriptionally active nuclei transferred in such egg cytoplasm become silent.

Embryonic genome transcriptional activation is thus a progressive event. The kinetics of this activation relative to morphological events such as cell cleavage varies according to the species (Fig 2) and the mouse kinetics is not representative of most mammalian species. In the mouse, major transcriptional activation occurs as soon as the G2 phase of the two cell stage, only after one DNA replication cycle (3). Whereas in all other species, embryonic transcriptional activation spans over several cell cycles (4).

As a consequence, most mechanistic studies concerning embryonic genome transcriptional activation use the mouse model, but have to be either confirmed or completed using alternative models that may be more relevant because more representative of most species.

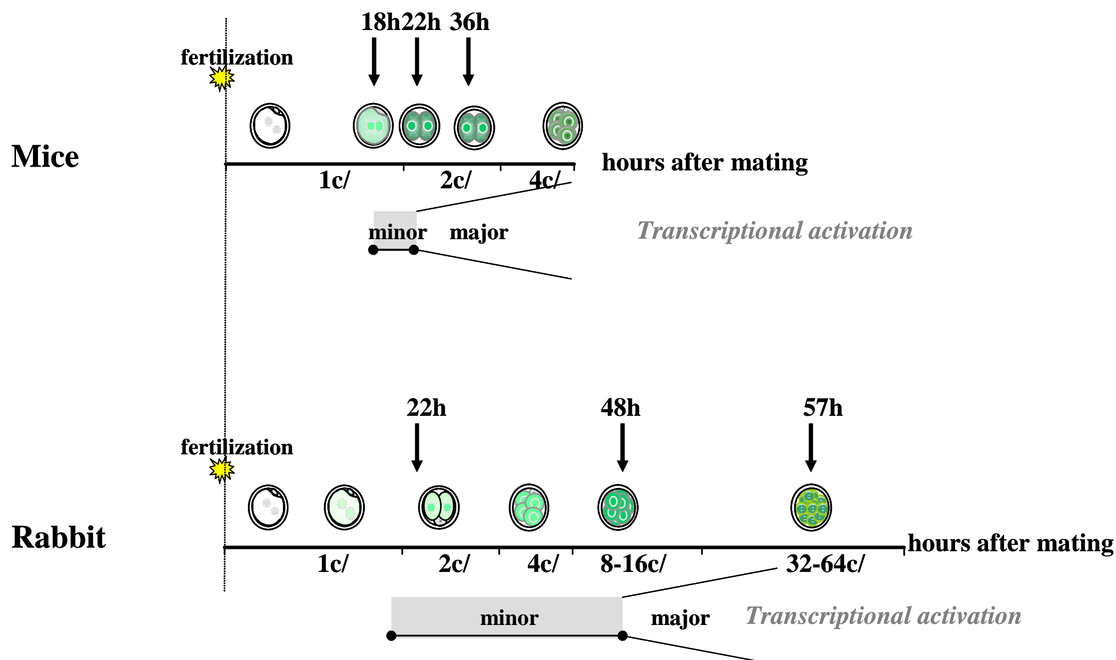
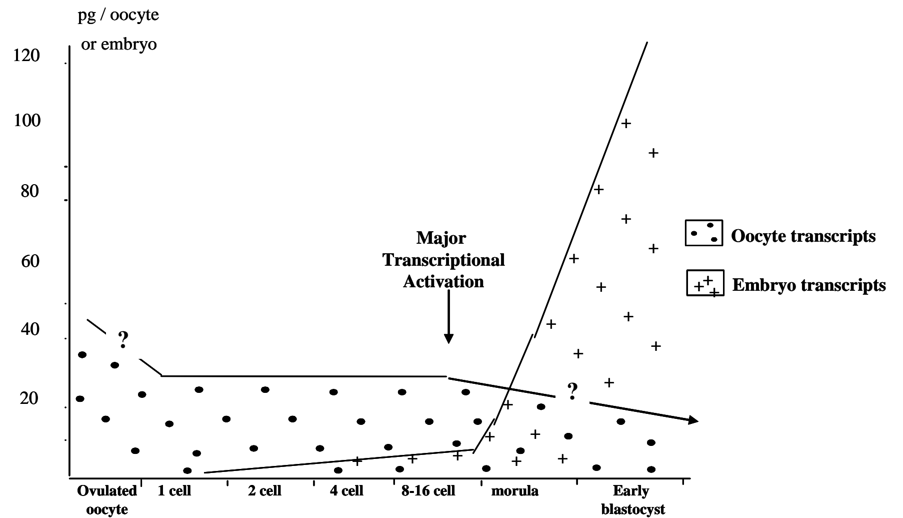



*Fig 2: Kinetics of genome transcriptional activation : comparison between mouse and rabbit embryo.*

## II Preimplantation development : a specific period governed by two distinct genetic information regulated at two distinct levels.

All over this progressive transcriptional activation, two different genetic information co-exist in the embryo (Fig 3): embryonic transcripts progressively accumulate whereas maternal transcripts are progressively degraded with distinct kinetics.

*Fig 3 : Messenger RNA quantification in rabbit embryo.*




Maternal and embryonic information are encoded by two distinct genomes and are regulated at two very distinct levels: maternal information is post-transcriptionally regulated whereas embryonic information is mainly regulated at the transcriptional level. These two distinct information interact very tightly all over the preimplantation period. Maternal information regulates embryonic genome activation and complex epigenetic events responsible for genome reprogramming, but embryonic encoded products may also interfere with maternal information regulation. So that nucleo-cytoplasmic interactions are decisive for the onset of embryonic development (Fig 4). These nucleo-cytoplasmic interactions are also decisive for long term development due to their involvement in the epigenetic reprogramming of the embryonic genome (5).

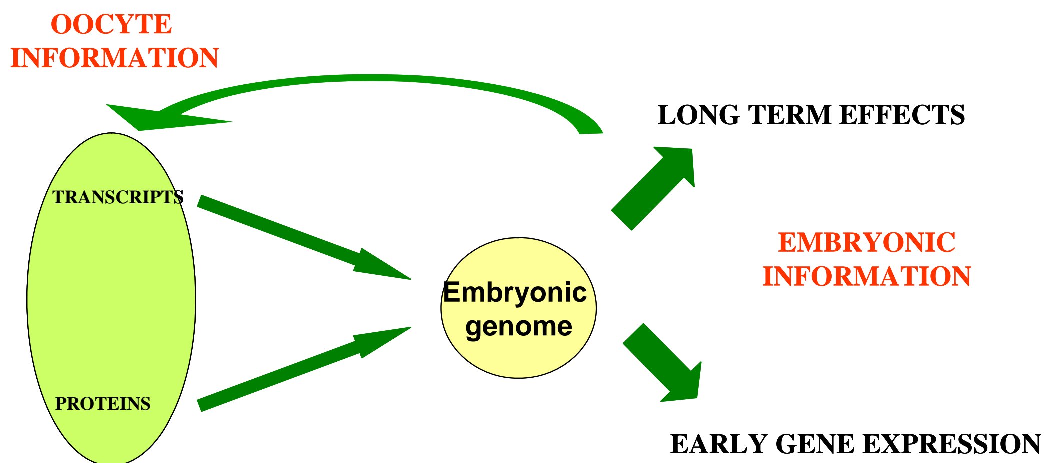



*Fig 4: Determinant interactions between maternal and embryonic information at embryonic genome activation.*

## III Transcripts profiling at major transcriptional activation.

Large scale genomic analyses have been developed mainly in the mouse species. They have demonstrated accurate and dynamic patterns of gene expression during early development, and have predicted relevant biological themes and signal pathways at each stage including genomic activation. They confirm previous data obtained from studies dealing only with few genes but also give some new insight into early regulation of genome expression.

First of all, early embryonic transcriptome is very complex and contain both ubiquitously expressed and very specific genes. The complexity at that stage is illustrated by the proportion of genes whose transcript levels are above a threshold (the so called Present call) when analysing a 36 000 gene Affimetrix array, this proportion reaches 47% (6). Sequencing the early expressed EST results in the identification of still unknown genes, whatever the species, and even in the mouse. Analysing the genes represented by ESTs from a two cell stage mouse embryo cDNA library, Evsikov et al. (7) have identified 2 771 known genes and 1 155 unknown genes. In the pig, a very recent study (8), sequencing and assembling ESTs from preimplantation stages libraries obtained 1114 unknown clusters from a total of 2489 clusters. Our own unpublished observations show that sequencing and assembling EST expressed at the major transcriptional activation in cattle embryo provides about 20% new contigs corresponding to unknown genes.

Using a transcriptomic array dedicated to early developmental stages, Hamatani et al (9) have provided a very interesting large scale analysis of preimplantation gene expression patterns. It points to global waves of gene activation at different stages, the earliest taking place at major transcriptional activation of the genome. This wave concerns 1700 genes (among 12 000) mainly involved in basic cellular machinery. In the mouse model, expression of genes involved in morphological events such as compaction and first differentiation is delayed to a second wave of transcription that begins at the 8 cell stage onward and spans the morula and blastocyst stages. These observations however have to be confirmed in mammalian species where major transcriptional activation occurs closer to the first differentiation events (rabbit or cattle).

In an independent study, Zeng and Schultz (10) detected 2600 genes whose expression is $\alpha$ amanitin sensitive at major transcriptional activation in the mouse. These 2600 genes represent 17% of the transcripts detected at that stage and are mainly involved in ribosome biogenesis, protein biosynthesis, nucleic acid metabolism, RNA processing and transcription.
The restricted number of functions encoded by early transcribed genes shows that transcriptional activation of the genome is probably not as promiscuous and global as previously proposed.

Clustering the genes according to their patterns of expression (k-means clustering) confirms the three main aspects of embryonic genome activation involvement in the maternal – embryonic transition : elimination of an exclusively maternal program, activation of an embryonic program of gene expression, and pursuing the expression of genes present as maternal transcript and also necessary for further embryo development (9).

The expression of retrotransposons and retroviral sequences has been recently reported at embryonic genome activation in the mouse (7). The first transcribed gene detected in the mouse embryo as soon as 8 hours post fertilization encodes for an endogenous retrovirus like gene MuERV-L (11). But also chimeric transcripts containing retrotransposon 5' sequence and an endogenous gene coding sequence are expressed at that stage. Indeed, expression of

such retrotransposon sequences alone or involved in chimeric transcripts was shown to be tightly regulated during oogenesis and early development (12).

This regulated expression raises questions about its functional significance and its evolutionary meaning. Only few observations are available to answer the first question : these sequences may be necessary to the embryo since inhibiting their expression impairs preimplantation development (13,14). Moreover, RNA interference processes are involved in the regulation of their expression level (13).

**IV Identification of minor transcriptional activation encoded functions.**

Only few genes transcribed during the minor transcriptional activation period have been identified in the mouse. As mentioned above, Mu-ERVL is the earliest expressed gene. This transcript doesn't contain the env gene: its function, if any, is thus restricted to inside the cell.

Few other genes have been shown to be transcribed either at the end of the one cell stage (Hsp70-1 (15), TRC transcripts) or during the G1 phase of the two cell stage in the mouse (eFIα (16)), U2afbp-rs (17).

Two functions have been shown to be encoded by the embryonic genome before the major transcriptional activation. Maternal pronucleus encodes some function involved in the inhibition of blastomere fragmentation as soon as the one cell stage in the mouse (18).
In the rabbit embryo, we have shown (our unpublished data) that early embryonically transcribed function (at the four cell stage) regulates some maternal transcripts stability.

**Conclusion:** Embryonic genome transcriptional activation results from fine tuned nucleo-cytoplasmic interactions. It is concomitant with an extended epigenetic remodelling of the newly formed genome, so that some expressions may be promiscuous and only progressively regulated. Large scale analysis however point to accurate and dynamic patterns of gene expression at the beginning of development. The robustness of such patterns remains to be checked: does the embryo come back (and how it comes back) to an essential pattern of gene expression when perturbed by micromanipulations or micro-environmental changes such as in vitro culture (19, 20)? Are early modifications of this pattern compatible with preimplantation and long term development ? These puzzling questions still remain to be answered.

**Readings :**

**Interspecies differences in methylation patterns :**
Young LE, Beaujean N. 2004 DNA methylation in the preimplantation embryo: the differing stories of the mouse and sheep. *Anim Reprod S*ci.;82-83:61-78. Review.

**Retroviral and transposon expression in oocyte and early embryo:**
Shapiro JA 2005 Retrotransposons and regulatory suites.*Bioessays*;27(2):122-5. Review.

**Global gene expression patterns in preimplantaion development :**
Hamatani T, Carter MG, Sharov AA, Ko MS. 2004 Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell.*;6(1):117-31.

**Global gene expression patterns at embryonic genome transcriptional activation:**
Zeng F, Schultz RM. 2005. RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. *Dev Biol.* 1;283(1):40-57.

**Alteration of gene expression patterns by in vitro culture:**
Wang S, Cowan CA, Chipperfield H, Powers RD. 2005 Gene expression in the preimplantation embryo: in-vitro developmental changes. *Reprod Biomed Online*.;10(5):607-16.

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9.Hamatani T, Carter MG, Sharov AA, Ko MS. 2004 Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell*.;6(1):117-31.

10.Zeng F, Schultz RM. 2005 RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. Dev Biol. 1;283(1):40-57.

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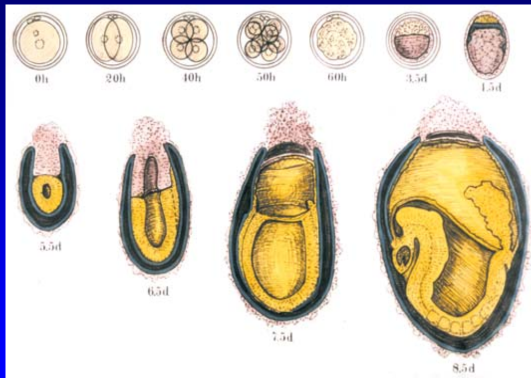
## Gene Expression and its Regulation in Early Mammalian Development

**Mouse** **Human**

**Professor Marilyn Monk**
**Institute of Child Health**
**London**

## Gene Expression in Development
## Clinical Relevance

- Progress and evaluation of assisted reproduction
  - IVF ICSI IVM
  - Imprinting
  - Contraception
- Pre-implantation diagnosis
- ES cell research and tissue transplantation
- Cancer genes and cancer vaccines



Mouse Early Development - Rosa Beddington

## Sensitive Single Cell Molecular Assays
## Gene transcription, mutation, modification

**X linked enzyme activity**

Hprt/Aprt $^{3}$H inosine → $^{3}$H IMP / $^{14}$C adenine → $^{14}$C AMP
Pgk-1A and Pgk-1B
G6PD
etc

**PCR specific genes**

Gene expression (RT-PCR), gene mutation, CpG methylation

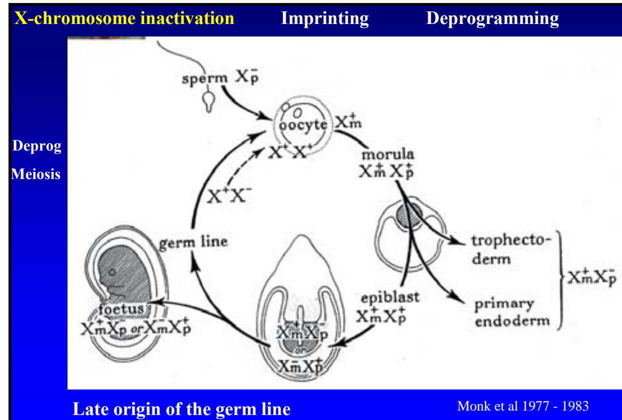
**Single embryo amplified cDNA and libraries**

## Gene expression and its regulation in development

- **X-inactivation** - timing / imprinted paternal XCI.
- **Regulation** - genome deprogramming, specific gene CpG methylation, methylation dependent DNA binding proteins, reconstruction by transgenesis.
- **Pre-implantation diagnosis.**
- **Imprinting** - Onset mono-allelic expression.
- **Gene expression** in human oogenesis, preimplantation embryos and primordial germ cells.
- **Embryo / cancer genes**. Deprogramming genes

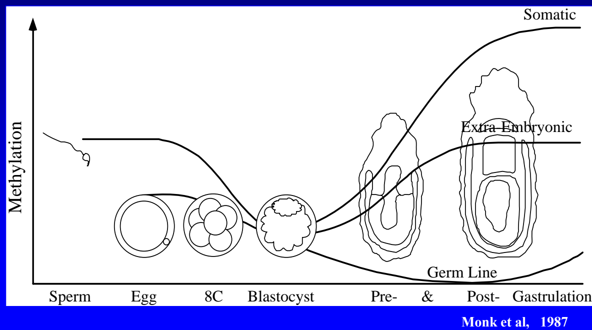
**X-chromosome inactivation** **Imprinting** **Deprogramming**

**Deprog**
**Meiosis**




**Late origin of the germ line** Monk et al 1977 - 1983

## Demethylation deprogramming in early development

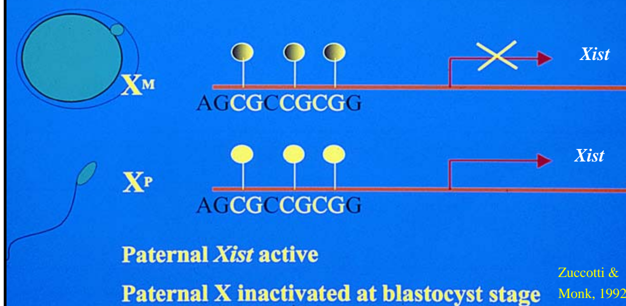



Monk et al, 1987

# Imprinting

- Differential modification of sperm and egg.
- Differential expression of paternal and maternal genes. (The failure to erase gametic modification differences prior to delineation of extra-embryonic and embryonic tissues.)
- XCI in soma is regulated by *Xist* gene methylation.
- Imprint may be difference in methylation of sperm and egg *Xist* promoter.

## Differential *Xist* methylation in egg and sperm



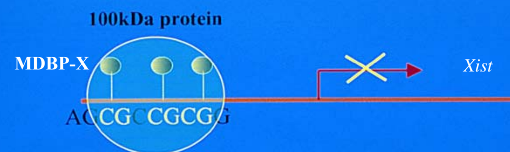

**Paternal *Xist* active**

**Paternal X inactivated at blastocyst stage**

Zuccotti & Monk, 1992

## Methylation-dependent binding protein Binds to methylated domain on *Xist* promoter

**100kDa protein**




**Binds to methylated promoter**
**Bandshift**
**Southwestern**
**Mutate CpGs to abolish binding**

Huntriss et al , 1997

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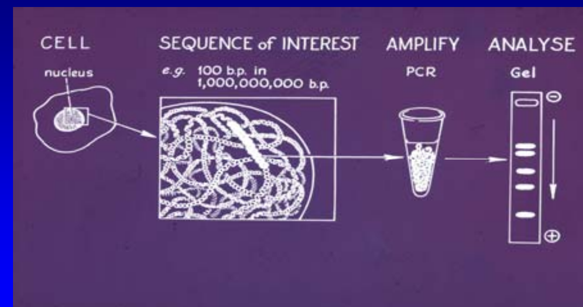
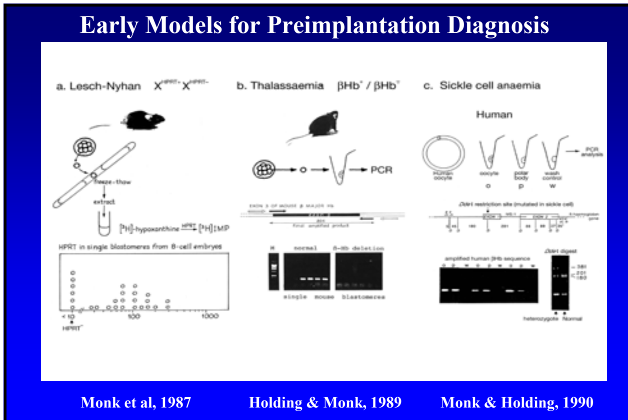
## Single Cell PCR




Monk & Holding 1985 -

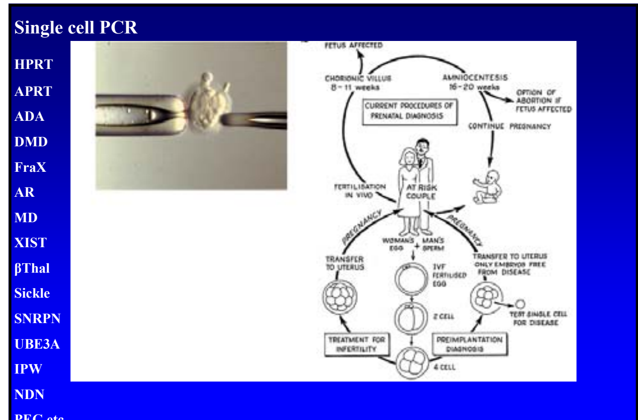
## Early Models for Preimplantation Diagnosis




Monk et al, 1987 Holding & Monk, 1989 Monk & Holding, 1990

## Single cell PCR

HPRT
APRT
ADA
DMD
FraX
AR
MD
XIST
βThal
Sickle
SNRPN
UBE3A
IPW
NDN
PEG etc

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## Human embryos and germ cells

- Very few cells
- Rare
- Ethical constraints

Need single cell sensitive techniques to study gene mutation/modification/expression.

Amplified cDNA and cDNA libraries from individual oocytes and embryos.
(Direct analysis still required for epigenetic analyses.)

## cDNA libraries from single embryos

**a limitless resource of all the expressed genes in**

egg 2 cell 4 cell 8 cell blastocyst PGCs

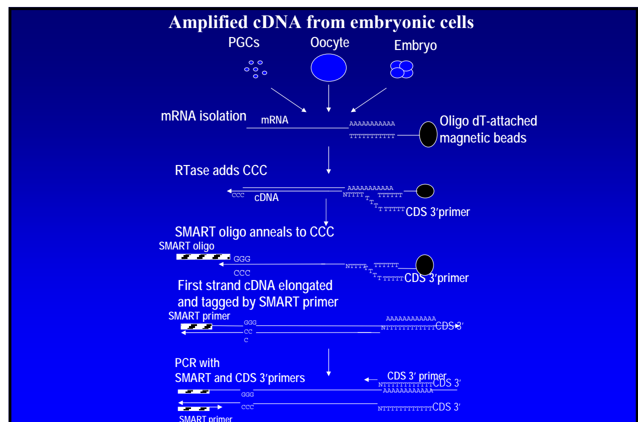
Human eggs and embryos, surplus to IVF requirement and donated for research.

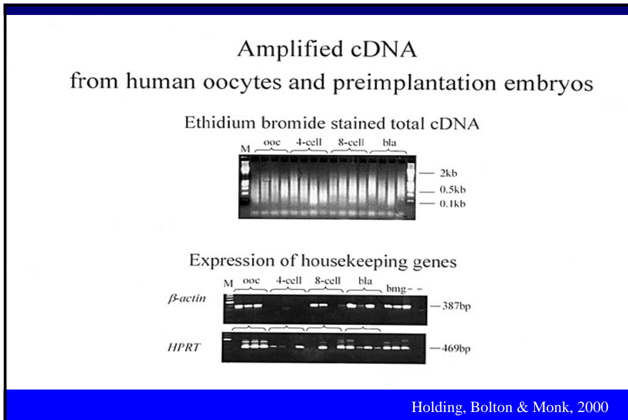
Stages of oogenesis, hormonally induced, naturally ovulated matured *in vivo* and *in vitro.*

Human fetal primordial germ cells, EC and ES cells.

Lysed to release mRNA which is copied and amplified as cDNA.
Amplified cDNA screened for known and novel embryonic genes

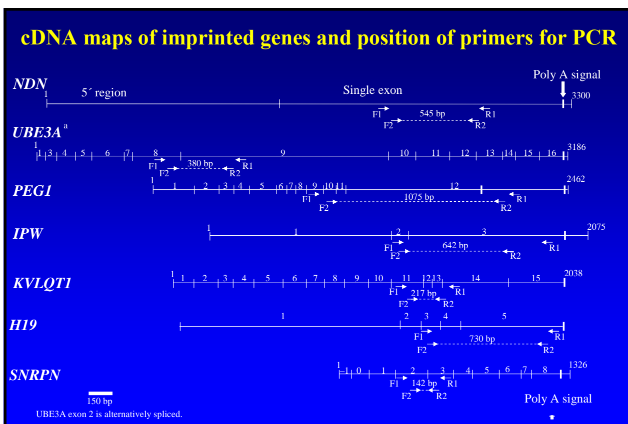
## Amplified cDNA from embryonic cells

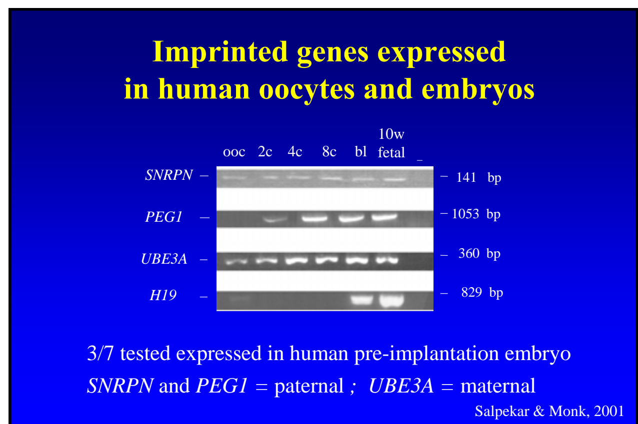


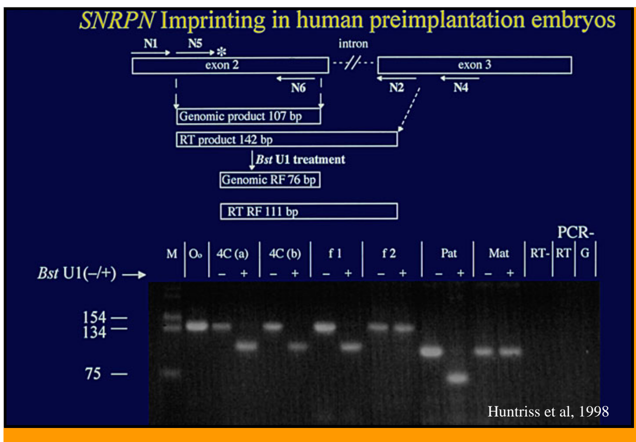



## Imprinted genes in human

- Mono-allelic expression due to differential modification of the gene in sperm and egg
- Disturbance of imprinting in development is associated with disease and cancer. Need to monitor imprinting with assisted reproduction procedures
- Determine onset of expression and onset of mono- allelic expression in pre-implantation development
- Studies on mechanisms.

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## Gene expression in oogenesis, embryogenesis and in PGCs

Oocyte-, embryo- and PGC-specific genes
Stage-specific, treatment-specific (ART)

*Examples -*

Differential expression of zona pellucida genes

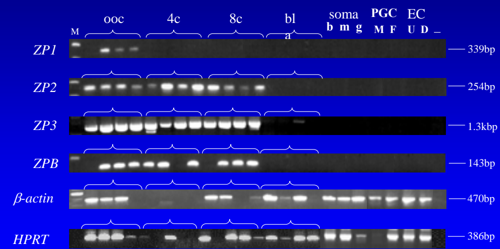
Isolation of PGC specific member of *OLF-R* family

Genes markers for oogenesis – GV, MII. IVM, S/O

Novel embryonic genes re-expressed in cancer cells

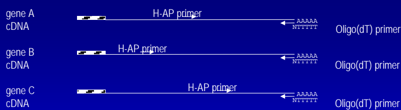
Deprogramming and maintenance of embryonic stem cells

## Expression of human zona pellucida genes in oocyte and embryo cDNAs




Salpekar & Monk, unpub.

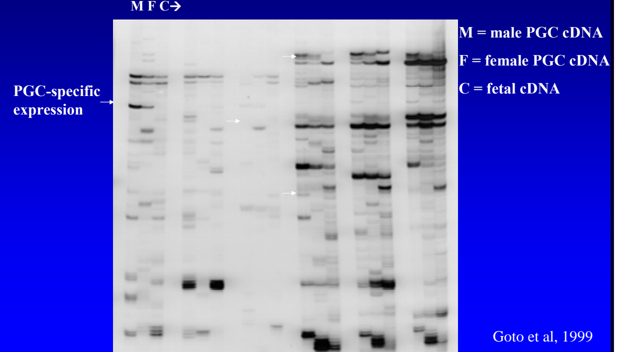
## Isolation of new embryonic genes - Differential Display






- Design primers within differential display sequence to isolate the full length cDNA by 5'RACE
- Database analysis
- Functional analysis

## Differential display of human primordial germ cell cDNA compared with fetal cDNA



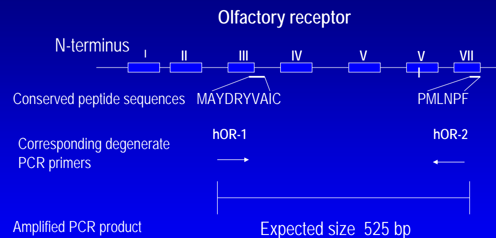

Goto et al, 1999

## cDNA fragments differentially expressed in fetal germ cells

| Frag | Male PGC | Female PGC | Whole Fetus | Database search |
|---|---|---|---|---|
| 1 | + | + | + | Polypep-assoc[d] alpha subunit |
| 2 | + | + | + | Tum[r] necrosis factor recep 1 |
| 3 | + | + | - | Hum olfactory receptor |
| 4 | + | + | - | aa460929 from 9 week fetus aa993606 from testis |
| 5 | + | + | - | As frag 4 but deletion 3' end |
| 6 | - | + | - | Novel (no match) |
| 7 | - | + | - | 3' end with L1 sequence |
| 8 | + | - | - | Novel |
| 9 | + | - | - | Calreticulin |
| 10 | + | - | - | RalBP1-assoc[d] EH domain |

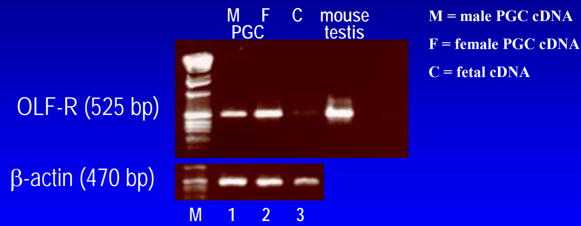
Goto et al, 1999

## Scheme for isolation of *OLF-R* sequences in PGC cDNA




Goto, Salpekar & Monk, 2001

## Expression of *OLF-R* in PGC cDNA




M = male PGC cDNA
F = female PGC cDNA
C = fetal cDNA

Goto, Salpekar & Monk, 2001

## Olfactory Receptor Gene Sequences in Human Primordial Germ Cells

**30 clones from PCR products sequenced**
(16 from male and 14 from female PGCs)

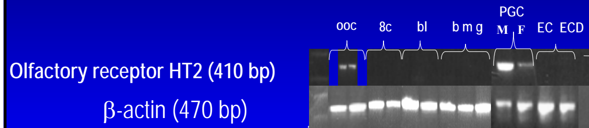
**There are 1000 members in the *OLF-R* family, yet 12 out of the 30 clones were the same sequence**

**This PGC-specific *OLF-R* sequence is identical to human testis-specific OLF-R gene – HT2**

**Use of testis-specific *OLF-R* gene primers confirmed the same sequence expressed in the PGCs**

**Germ cell *OLF-R* functions in migration of PGC and sperm?**

## Expression of human testis specific *OLF-R* in oocytes and PGCs



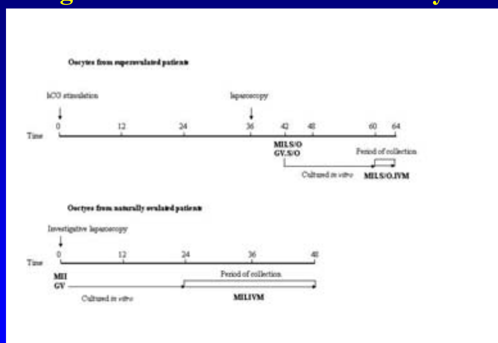

Goto, Salpekar & Monk, 200

## Stages of Oogenesis - GV and MII Stage specific and treatment specific gene expression

**Hormonally stimulated or naturally ovulated**

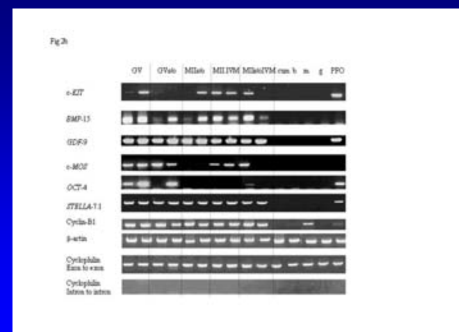
**MII from GV matured *in vivo* or *in vitro***

## Stage and treatment of human oocytes



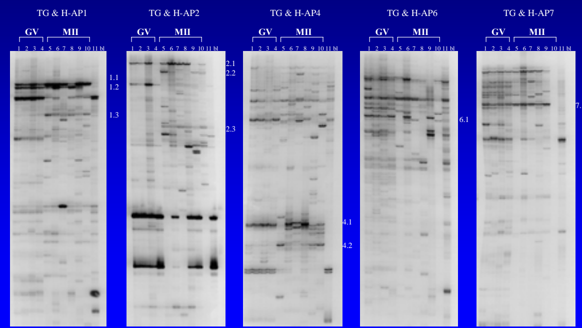

Monk, Hu. Jones and Trounson, unpub

## Expression of known genes in GV and MII oocytes

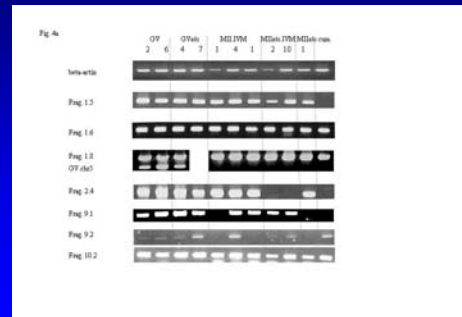



Monk, Hu, Jones, Goto, Trounson. unpub

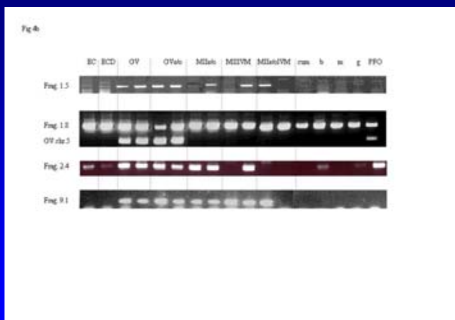
## Differential display of GV, MII and cumulus cDNAs




## Expression of new oocyte specific sequences



Monk, Hu, Jones, Goto, Trounson, unpub



## Genes specifically expressed in human embryonic cells?

*De-programming to proliferative stem cell - initiation*

Erasure gametic epigenetic programmes (demethylation)

*Immortality - maintenance*

Embryonic cells removed from developmental constraints grow indefinitely, e.g., ICM ➲ES PGC ➲EG

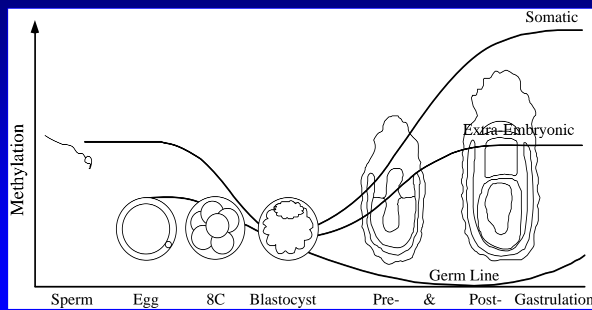
*Undifferentiated stem cell state*

Embryonic cells are the archetypal totipotent stem cell

*Invasiveness*

Embryonic cells are invasive,
e.g., trophectoderm, PGC migration (*OLF-R?)*

## Demethylation deprogramming in development




Monk et al, 1987

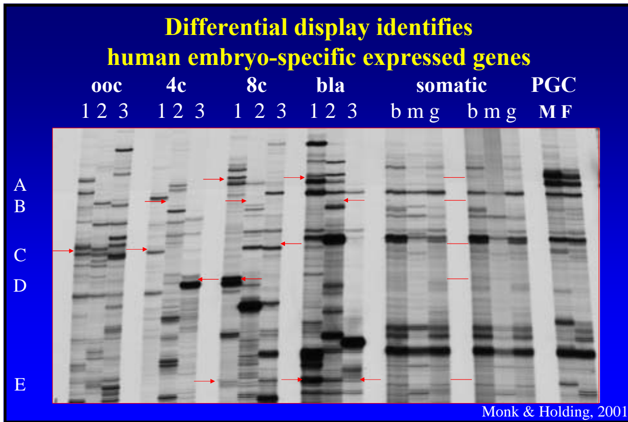
## Human embryonic genes re-expressed in cancer cells?

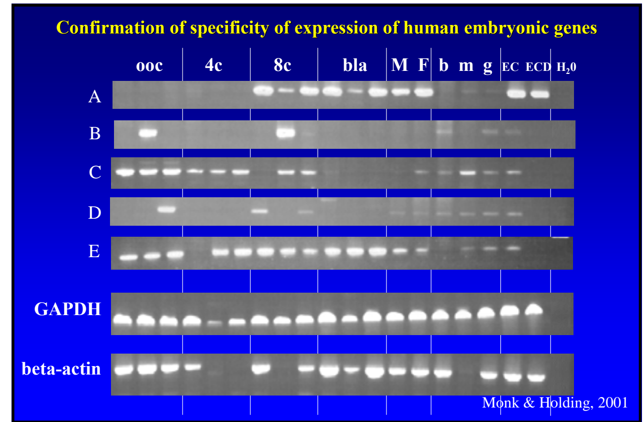
Human embryonic cells grow indefinitely, undifferentiated and invasive.

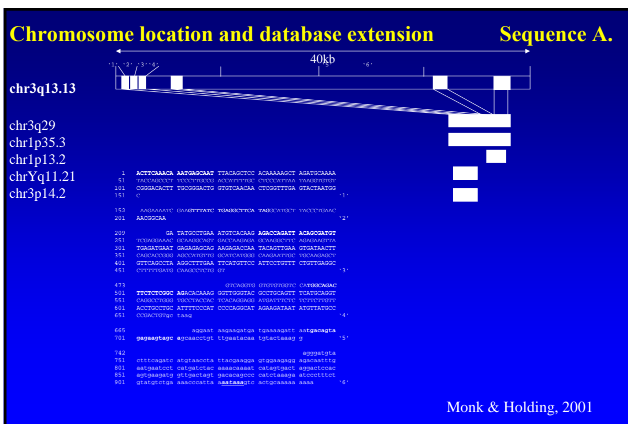
Cancer cells share these properties.

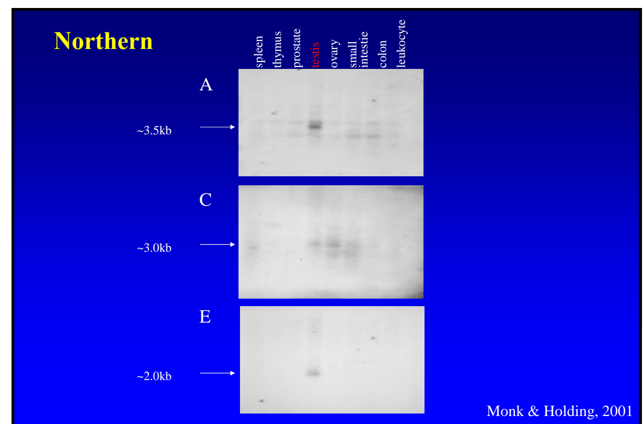
Test human embryonic genes isolated for their expression on cancer cells

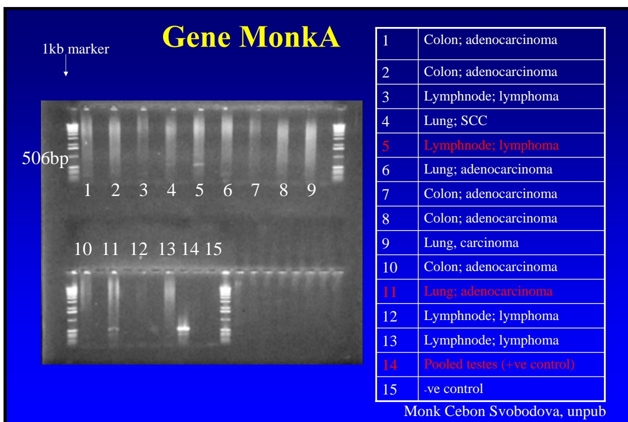
New cancer vaccines?

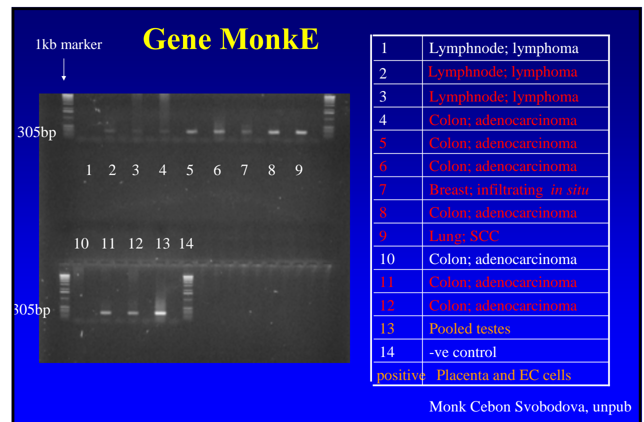

Differential display identifies
human embryo-specific expressed genes
ooc 4c 8c bla somatic PGC
1 2 3 1 2 3 1 2 3 1 2 3 b m g b m g M F
A
B
C
D
E
Monk & Holding, 2001


Confirmation of specificity of expression of human embryonic genes
ooc 4c 8c bla M F b m g EC ECD H20
A
B
C
D
E
GAPDH
beta-actin
Monk & Holding, 2001


Chromosome location and database extension
Sequence A.
40kb
chr3q13.13
chr3q29
chr1p35.3
chr1p13.2
chrYq11.21
chr3p14.2
Monk & Holding, 2001


Northern
spleen
thymus
prostate
testis
ovary
small intestie
colon
leukocyte
A
~3.5kb
C
~3.0kb
E
~2.0kb
Monk & Holding, 2001


Gene MonkA
1kb marker
506bp
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15
1 Colon; adenocarcinoma
2 Colon; adenocarcinoma
3 Lymphnode; lymphoma
4 Lung; SCC
5 Lymphnode; lymphoma
6 Lung; adenocarcinoma
7 Colon; adenocarcinoma
8 Colon; adenocarcinoma
9 Lung, carcinoma
10 Colon; adenocarcinoma
11 Lung; adenocarcinoma
12 Lymphnode; lymphoma
13 Lymphnode; lymphoma
14 Pooled testes (+ve control)
15 -ve control
Monk Cebon Svobodova, unpub


Gene MonkE
1kb marker
305bp
1 2 3 4 5 6 7 8 9
10 11 12 13 14
305bp
1 Lymphnode; lymphoma
2 Lymphnode; lymphoma
3 Lymphnode; lymphoma
4 Colon; adenocarcinoma
5 Colon; adenocarcinoma
6 Colon; adenocarcinoma
7 Breast; infiltrating in situ
8 Colon; adenocarcinoma
9 Lung; SCC
10 Colon; adenocarcinoma
11 Colon; adenocarcinoma
12 Colon; adenocarcinoma
13 Pooled testes
14 -ve control
positive Placenta and EC cells
Monk Cebon Svobodova, unpub

## Gene MonkA

| CANCER TISSUE | NUMBER |
|---|---|
| META. MELANOMA | 0/18 (0%) |
| **LUNG** | 7/21 (33%) |
| *(Lung adenocarcinoma* | *2/9 (22%))* |
| *(Lung SCC* | *3/6 (50%))* |
| **LYMPHNODE** | 2/32 (6%) |
| **COLON** | 2/34 (5%) |
| **BREAST** | 0/4 (0%) |
| **LIVER** | 0/1 (0%) |
| **TOTAL** | **11/110 (10%)** |
| **NORMALS** | **1/20 (Thymus)** |

Monk Cebon Svobodova, unpub

## Gene MonkE

| CANCER TISSUE | TYPE |
|---|---|
| **LUNG** | 8/12 (66%) |
| **LYMPHNODE** | 2/10 (20%) |
| **COLON** | 10/22 (45%) |
| **BREAST** | 3/3 (100%) |
| **Head and neck** | 0/1 (0%) |
| **Metastatic Melanoma** | 4/12 (33%) |
| **TOTAL** | **27 of 60 (45%)** |

Monk Cebon Svobodova, unpub

## Summary

**Gene expression and its regulation in embryonic development**

**Single cell molecular analysis – gene mutation, gene modification, gene expression**

**XCI, deprogramming. mechanisms of imprinting (mouse)**

**Preimplantation diagnosis**

**Human oocyte, embryo and PGC cDNAs.**
**Extensive comparative studies of gene expression within and between human oocytes, embryos and PGCs. Marker genes for oogenesis.**

**Onset mono-allelic expression *SNRPN &PEG* in human embryos**

***Known genes* - imprinted genes, zona pellucida genes, oogenesis stage specific genes**
***Novel genes* - new members of known gene families - *OLF-R* in PGC**
**new embryonic genes**
**embryo/ cancer genes**
**genes associated with initiation and maintenance of deprogramming**


## ACKNOWLEDGEMENTS

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**Ludwig Institute of Cancer Research Melbourne**

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## Egg sharing as a tool for discriminating egg vs sperm quality

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*Finland*

### Learning objectives

1) Understand the potential of ovum donation/egg sharing in discriminating egg and sperm quality

2) Understand the power of statistical analyses

3) Learn the theoretical sperm derived factors affecting early embryogenesis

Embryo quality is one of the most important factors affecting the outcome of IVF treatments. The quality of any given embryo is, in turn, the sum of various internal and external factors. The early development of human embryo is mostly quided by maternal and paternal proteins and mRNAs imported to zygote by oocyte and sperm cell. The embryo's own genome is activated at 4-8 cell stage where after the inluence of the inherited proteins and other factors on its development gradually decrease. The present article and presentation are focused on questions a) how to analyse the relative impact of germ cells on early developing embryos and b) what are the theoretical germ cell derived factors affecting the early embryonic growth.

### Evaluation of the embryo quality

Currently, embryo quality is usually evaluated based on its morphological appearance and blastomere cleavage rate. At zygote stage the pronulcear (PN) morphology and especially the number, size and location of nucleolar precursor bodies (NPB) in pronuclei has been thought to be predictive for embryo quality. Also, the appearance of the cytoplasmic halo at the periphery of zygote stage embryos has been associated to embryo implantation capacity (see review Rienzi et al. 2005).

The most important aspects evaluated at cleavage stage embryos (days 2-3 after aspiration) are the number (= cleavage rate) and size of the blastomeres and the amount of cellular fragmentation. According to general understanding the day 2 embryos should have 4-5 blastomeres of +/- equal size with minimal (< 5 % of total volume) fragmentation. Also, the timing of the first cleavage may be predictive for embryo quality: embryos that have finished their first cleavage within 25 hours after insemination (corresponding to 22-23 hours after fertilization) seem to have the highest implantation rates. The presence of anuclear fragments is inversely proportional to pregnancy rates but, on the other hand, fragmented embryos are not necessarily developmentally incompetent (Rienzi et al. 2005).

Although the above mentioned embryo parameters are unquestionably highly predictive for embryo quality, the only absolute parameter that definitively measures the quality of the embryo is (clinical) pregnancy rate. This, on the other hand, may be dependent on various other factors that cannot be controlled. For example, the receptivity of the uterus may be affected by the physiological condition of the woman, environmental factors (like cigarette smoke) or some unknown genetic factors. The hormonal stimulation, purity of the used hormones etc may affect the competence of the developing

oocytes (and hence the embryo quality) as well. In IVF/ICSI treatments also the cell culture system is of importance. For example, normoxic culture conditions (oxygen pressure about 20 %) that are generally applied in IVF clinics may be detrimental for developing embryos. Finally, the quality of the cell culture media plays a major role in supporting the early embryonic development and general viability of the (embryonic) cells.

**Ovum donation/Egg sharing as a model**

Probably the most important components determing the quality and viability of the early embryo are the germ cells that form it. Their roles, however, may be difficult to asses. To analyse the possible role of sperm cells on embryonic quality oocytes from a single source should be inseminated with various sperm samples. Alternatively, the impact of the oocytes could be analysed with similar kind of assay by fertilizing several "identical" oocyte groups by a single sperm sample and then comparing embryonic and clinical parameters. Ideally these experiments would necessitate some hundreds of oocytes divided to groups of 10-20, inseminated with studied sperm samples and transferred as single embryos as identical uteri as possible.

Of course, these kinds of study designs are non-applicable to human. However, of the every day clinical practises both ovum donation and egg sharing procedures can be used for dissecting the possible effects of oocytes and sperm cells. In ovum donation treatments healthy, fertile and preferable young females donate oocytes for infertile couples suffering lack of oocytes. Because of the constant shortage of the egg donors, oocytes from single donor are generally divided between two (or more) recipients. In egg sharings couples undergoing IVF treatment themselves donate part of their oocytes to other infertile couples. In both cases oocytes from single source are fertilized by two sperm samples which allow at least to some extent the comparison of the influence of the paternal factors on embryonic parameters and, with sophisticated mathematical analyses, also the influence of oocytes may be estimated. The major difference between the two methods is in the "quality" of the donors: in ovum donation treatments the donors are in most clinics proven to be fertile (i.e. they have conceived naturally), while in egg sharing procedures the reason for infertility may be linked to oocyte quality as well. It should be stressed that despite the oocytes in both cases are of common origin, oocytes are never identical. There may be major genetic and cytoplasmic variation within the oocytes of a given donor and this may also reflect on parameters measured.

**Ovum donation: Individual patients**

Tesarik et al. published 2002 a study where early embryos of a group of six oocyte recipients (study group) were compared to embryos from recipients receiving the sibling oocytes (sibling group) from the same donors. The study group patients repeatedly produced embryos with significantly lower zygote stage grade (based on NPB number and distribution) and implantation rates when compared to sibling group embryos. In study group, of the 92 formed embryos, only 16 % (15) were of good zygote morphology grade while in the sibling group 66 % (57 out of 87 embryos) were of top quality. Likewise, the implantation rates were 5 % and 29 % for study and the sibling groups, respectively. Although the study included only a small number of individuals the results suggest that paternal factors might contribute the early human development as soon as zygote stage, far before the actual genomic activation of the developing embryo occurs.

**Ovum donation: Statistical approach**

Ovum donation programme was initiated in our Infertility clinic in 1991. To asses the possible roles of oocytes and sperm cells on early embryo development we analysed retrospectively embryonic

parameters from 59 ovum donation cycles with 118 recipients performed during 1992-2001. Only normal IVF cycles with day 2 embryo transfer were included in this study.

The average number of oocytes retrieved/collection was 18.1 yielding an average of 5.5 embryos / recipient. To evaluate the influence of distinct sperm parameters, we classified patients into three groups according to the morphology of their sperm samples. Group 1 patients had less than 4 %, group 2 between 4 and 14 % and group 3 more than 14 % morphologically normal sperm cells. The main outcomes measured were fertility rates, morphological embryonic grade (sum of fragmentation and blastomere size) and blastomere cleavage rate.

Our approach was to analyse the data with two statistical models. With simple correlation analyses (Pearson's correlation analyses, SPSS 10.1.0) we could determine the influence of the oocytes on the investigated parameters. For this, the average blastomere number and embryo grade were calculated for both recipients of each donor. The values for recipient couples were then allocated between the x- and y-axes and the correlation was calculated. As in general correlation analyses, the closer the correlation coefficient (r) is to 1 the more identical the samples are. In this case the interpretation is that if the values are same in both recipients the sperm effect must be negligible.

To investigate all putative factors that might influence the embryo quality we carried out a mixed ANOVA test (SAS 8.1). The approach assumes that variability in embryos results from the variability of oocytes, sperm samples and some other residual (unknown) factors like differences between the oocytes of the same donor. The test estimates the contribution of each of the studied factors to the overall embryo variability and relates each studied factor to the probability. The factors are divided to random and fixed factors. Random factors are either unknown (residual) or those that cannot be specified (in this case general sperm and oocyte factors). Fixed factors were the known sperm parameters (motility, morphology and concentration) and they were further analysed by the corresponding regression analyses showing the relation between sperm characteristic and embryo parameter.

As a result we find direct correlations between germ cells and embryos. By Pearson's correlation analyses we were able to show that the average embryo morphology was strongly correlated between the recipients of the same donor ($p<0.001$) while weaker correlation was found in the average blastomere numbers between the recipients of the same oocyte donor ($p<0.005$). The results indicate that oocytes have major impact on overall morphology and less profound but significant on blastomere division rate as well. Also the mixed ANOVA test revealed similar influences of oocytes on embryo morphology ($p<0.001$) and blastomere cleavage rate ($p=0.01$) confirming the results of correlation analyses. Sperm factor didn't seem to influence on general morphology of the early embryos, but it was linked to blastomere cleavage rate ($p=0.015$). More specifically, sperm morphology was positively associated to blastomere division rate ($p=0.03$) while the influence of sperm count and motility (before and after semen preparation) were negligible.

### Egg sharing as a model

There are only a few publications of the use of egg-share as a model to investigate the possible paternal effects on early embryo development. Sakkas et al published 2004 their analyses about a total of 111 egg-sharing cycles in Birmingham, UK. Their egg-share procedure includes random and equal distribution of oocytes from one patient to herself and to one recipient. Mean number of shared eggs was about 9 leading to mean zygote number of 6,5 for both donor and recipient. The results of the analysed data indicate, again, that approximately 30 % of the compared couples (donor vs. recipient) have clear differences in their overall embryo quality regardless of way of

fertilization (IVF or ICSI). Sperm characteristics didn't seem to affect embryo score which was a sum of the blastomere number and morphological appearance of the embryo. The conclusion is that sperm factor affecting the early development of human embryos exists.

**Theoretical sperm cell derived factors**

The fact that reproductive cloning by somatic nuclear transfer is possible (although with low frequency) indicates that oocytes can provide all the necessary support needed to early developing embryos. Studies with several animal species have revealed various proteins that are exclusively expressed in oocytes and are absolutely required for normal fertilization or development of the early embryos. This does not, however, mean that the contribution of sperm cells would be restricted to bringing the haploid genome. From above mentioned studies we can learn that sperm cells indeed regulate the development of the early embryos even before embryo's own genomic contribution can be detected. The following section shortly describes what is thought about these poorly characterized sperm derived factors and what are their possible targets in oocytes/embryos.

*1) Oocyte activation*

During oocyte activation large number of biochemical signalling pathways is activated resulting for example in modification of Zona Pellucida, the sperm nuclear envelope break down and chromatin remodelling. Many of the early processes are triggered by $Ca^{2+}$ oscillations starting within a few minutes after sperm-egg fusion and lasting up to few hours until the pronuclei are formed. The duration and amplitude of oocyte $Ca^{2+}$ oscillations seem to be critical for mammalian oocyte activation and early embryogenesis. Abnormal oscillation may lead to failure in completing the second meiotic division and pronuclear formation. Notably, also the developmental capacity of mammalian embryos has been shown to be dependent on $Ca^{2+}$ oscillation patterns. The factor that most likely triggers the $Ca^{2+}$ oscillations in fertilized oocytes has been recently identified as a novel PLC isoform called PLCζ brought to zygote by sperm cell. Injection of purified recombinant PLCζ or its cRNA into unfertilized oocytes is able to induce pulsatile $Ca^{2+}$ oscillations resembling those occurring after natural fertilization and oocytes activated by it show reasonable developmental capacity up to blastocyst stage without any contact to sperm cells. PLCζ has been cloned from human as well and it appears to induce $Ca^{2+}$ oscillations also in human oocytes (see review by Malcuit et al. 2006). PLCζ would be an ideal sperm factor candidate that could explain at least part of the early developmental variation linked to sperm cells that we and others have found in ovum donation and egg-sharing studies.

*2) Sperm DNA damage*

Recent studies have indicated that male germ cells are vulnerable to DNA damages occurring at testicular, epididymal or post-ejaculatory levels. Detailed analyses suggested that sperm samples with poor morphological quality and low motility include high numbers of sperm cells with aberrant DNA content. DNA damage is not necessarily associated to fertilization capacity of the given sperm cells (especially if ICSI is used) but it seems to be linked to lower cleavage rate of early embryos and also, to decreased pregnancy rates (see review by Lewis et al. 2005). Hence, one component of the sperm effect on embryonic cleavage rate may be related to the integrity of the sperm DNA.

*3) Dysfunctional centrosome*

In most mammals (including human) the centriole brought by sperm cell is essential for the formation of functional centrosomes needed for pronuclear migration and further cell cleavage. Indeed, oocyte centrioles are actively degenerated during oogenesis in several animal species from flies to human (Manadhar et al. 2005). It is possible that dysfunctional (or total lack of) centrioles could affect the early embryonic development. A recent case-report article showed that pathology of the sperm centriole might be responsible for abnormal sperm aster formation, syngamy and blastomere cleavage (Rawe et al. 2002). Dysfunctional centrioles could also explain the variation in statistical sperm factor analyses and it might explain male linked impaired embryogenesis at least in some individual cases.

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# Laboratory conditions and failed fertilisation

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## Aim of lecture

- To understand the relationship between laboratory conditions and fertilisation failure

## Objective 1

- Define failed fertilisation
- Discuss the incidence of failed fertilisation with IVF and ICSI
- Define the mean fertilisation rate
- Express expected fertilisation rates and define trigger criteria for identifying potential problems

## Objective 2

- Identify potential factors which may affect fertilisation
- Outline the role and mechanism by which the factors may affect fertilisation
- Discuss preventative measures to reduce problems caused by sub optimal laboratory conditions

## Failed fertilisation

- The failure of 2 pronuclei to form after insemination
- Incidence of complete failed fertilisation ACU (2001 – 2006)
  - IVF: 3%
  - ICSI: 0.6%

## Causes of failed fertilisation

- Sperm quality
- Oocyte quality / maturity
- Reduced oocyte numbers retrieved

## Laboratory Factors

- Drastic to cause total failed fertilisation
- May reduce fertilisation rate

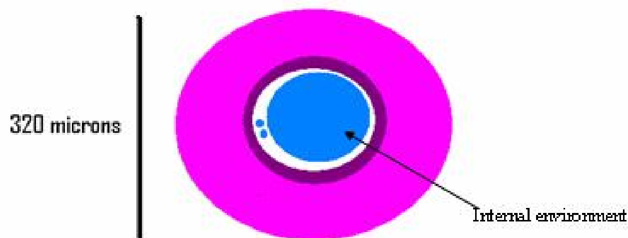
## Fertilisation rates

- Mean fertilisation rate (2001-2006)
  - IVF: 71%
  - ICSI: 66%

## Control of Environment

- To optimise viability
  - understand
    - interactions that occur between the oocytes intracellular and extracellular environment, particularly how these interactions are controlled by the oocyte.
- We can then compare this knowledge with our understanding of what happens in our lab with the aim of creating a more "natural" environment

## The Oocyte's Environment




## The Intracellular Environment

- Is a function of competent follicular maturation
- Is a pool of reserves used until the embryo develops the ability to manufacture proteins, enzymes, metabolites
- Is under maternal control until genomic activation

## Extra cellular environment Laboratory conditions which may affect fertilisation

- Temperature
- pH (CO2)
- Incubator environment
- ROS
- Bacteria
- Environmental contaminates
- Light
- Chemical factors (media):

## Temperature

- Oocytes
  - Meiotic spindles
    - essential for normal chromosomal alignment and separation of chromosomes during meiosis and fertilisation
    - Disruption of the spindles may cause failed fertilisation, or abnormal fertilisation

## Effects of cooling

Wang et al
Human Reprod.
2001 16, 11
2374 – 2378

**❖Exp 1:**

- Oocytes were cooled from 37°C to room temperature and then returned to 37°C
- Oocytes were imaged (polscope) every 30 secs

**Exp 2:**

- Cooled from 37°C to 33, 38 and 25°C for 10 minutes and then rewarmed
- Oocytes imaged before cooling, 5 and 10 minutes after cooing and 10 and 20 mins after rewarming.

**Results**

**Exp 1**

- At 1.5 mins after cooling, temp 31.9°C – spindle began to disassemble
- Complete disassembly after 5 minutes
- After 20 mins after warming spindles recovered

Maximum retardance (Microtubule density measurements):

- Before cooling 5.78nm
- At 27.1 = 2.44nm
- After 20 mins of warming = 5.01nm

**Results**

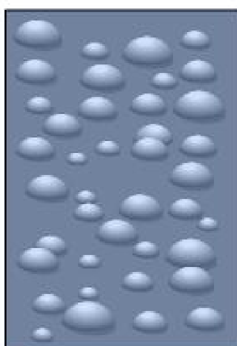
**Exp 2**

- Spindle disruption occurred at 33°C, but oocytes demonstrated full recovery.
- Fewer oocytes recovered after 10 mins cooling to 28°C and none recovered after cooling to room temp.

## Conclusions of study

- Spindle disassembly is temperature and time dependent
- The decrease in microtubule density after re warming suggests that the spindles did not re-polymerise completely.
- Essential to keep controlled temperature and work quickly to avoid problems

## pH



- The pH of bicarbonate buffered media and buffers increases overtime when outside the incubator
- Main cause: $CO_2$ escaping from solution
- $CO_2$ loss increases with temperature

# pH STRESS

- Constant neutral pH is preferred (basic vs. acidic)
- Regulation requires presence of $HCO_3^-$ and $CO_2$
- Long term exposure to HEPES buffers to be AVOIDED due to low $HCO_3^-$ levels and embryo toxicity of HEPES (Bavister Biology of Reproduction, Vol 31, 109-114))


# pH STRESS

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# MEDIA pH

- pH stability important for optimal growth
- Most manufacturers recommend using 5% - 6% $CO_2$ with 25 mM $NaHCO_3$ to give a pH of 7.2-7.4

# pH Summary – Minimise Stress

- Choosing correct buffer for procedure is important
- Culture dishes – Choose carefully
  Micro v. Macro?
- Prepare dishes early (night before)
- Oil prevents rapid pH changes BUT is SLOW to recover
- BE QUICK
  - Plan the procedure

# Incubator environment

❖ Catt and Henman Hum. Reprod. 2000 15, 2, 199-206

**Role of oxygen**

- Oxygen is consumed in oxidative phosphorylation, free radicals are generated from leakage of high energy electrons as they proceed down the electron transport chain
- Damage to mitochrondrial DNA, proteins, lipids, cytoplasmic compounds
- A lack of elasticity in the plasmalemmae of sperm and oocytes may affect fertilisation
- Reducing the oxygen tension may reduce the production of free radicals

# ROS (Reactive oxygen species) – sperm function

❖ Preparation method for sperm samples may increase production of ROS (Twig et al Mol Hum. Reprod. 1998, 4, 5 439-445)

- High levels ROS associated with swim up from pellet compared with swim up from semen (due to unfractionated centrifugation step)
- Washing without a gradient associated with increased ROS production

# ROS

- **Detrimental effects of ROS – differing opinions**
  - The exposure to iatrogenically induced ROS significantly increases DNA fragmentation levels.
  - DNA damage reduces fertilising potential of sperm and IVF fertilisation rates are significantly improved when use methods to reduce the production of ROS. (Aitkin and Clarkson, 1998, J. Androl. 9, 367-376)
  - However, paper by Morris et al shows that DNA damaged sperm do fertilise normally but embryo development compromised

# Bacteria

❖ **Hosseinzadeh et al Hum. Reprod. 2001, 16, 2, 293-299**

- Culture of sperm samples with the elementary bodies of Chlamydia trachomatis serovars E and LGV
- After 6 hours of incubation significant decrease in the percentage of motile sperm and increase in the proportion of dead sperm
- If either partner is infected and exposed to serovars in the culture environment sperm could rapidly loose their motility and viability and cause failed fertilisation

# Environmental contaminates

❖ Younglai et al. Arch Environ Contam Toxicol 2002 Jul; 43 (1) 121-6

- Serum, follicular fluid and semen analysed for variety of chemicals
- P,p'-DDE most frequently detected and was associated with failed fertilisation

## Chemical air contamination

- Cohen et al Human reprod 1972 12 8 1742-1749

- Volatile organic compounds
- Isopropyl alcohol

## Light?

- Various studies
  - Wavelength <480nm affects meiosis after fertilisation
  - Standard lighting impairs cell proliferation
  - UV light (360nm) impairs replication, cell division
  - Exact mechanisms unclear and most studies not from human oocytes / embryos

## Controlling the environment

- Validation

  ACU lab upgrade in 2005 to clean room grade B

  Full validation of all equipment and the facility (VALIDAIR)
  - Equipment
    - Installation qualification (IQ)
    - Operational qualification (OQ)

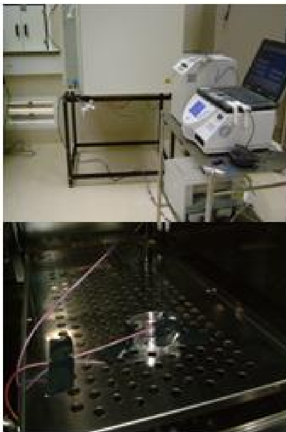
ACU lab upgrade in 2005 to clean room grade B

Full validation of all equipment and the facility by independent company

## Validation – what is involved

- **E.g. Incubator**
  - Confirm supporting documentation, service records, calibration certificates, manuals
  - Confirm correct installation (power and gas)
  - Confirm correct functioning of all components such as CO2 mixing device, temperature controller, alarms
  - Thermal mapping of chamber to detect temperature distribution
  - Determine the constitution of the 6% environment inside the incubator

## Incubator thermal mapping



Mixture of 6 different culture dishes on each shelf in incubator

- Thermocouple in medium in each dish
- Continually monitored by data logger
  - Over 24 hours
  - Opening and shutting doors under normal working conditions
  - Door left open for 10 minutes
  - Simulate a power failure.

## Heated stages

- **IOQ performed**
- **Individual heated stages (also performed for tube warmers, portable incubators, water bath)**
  - Confirm supporting documentation, service records, calibration certificates, manuals
  - Confirm correct installation (power)
  - Confirm correct functioning of all components such as switches, lights, alarms
  - Review commissioning and service reports if appropriate
  - Thermal mapping to detect temperature distribution

## Heated stages – thermal mapping

- **Temperature taken with calibrated surface thermometer in at least 6 points on surface and medium in dishes**
- **Temperature mapped by data loggers**
  - Uneven distribution observed, did not correspond to actual digital readout on stage controls
  - Manufacturer rectified problem
  - Stages fully checked on monthly basis

## Maintaining the environment

- Clean room environment
- Hepa filtered air
- Activated carbon filters

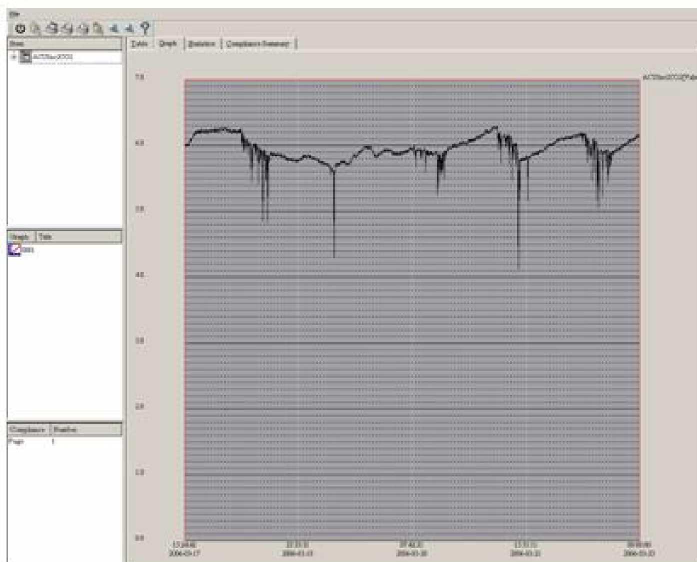
## Sheffield

- Clean room installation completed Oct 2005
- Grade B
- Early results good

- Grade B laboratories (currently working to grade c with respect to clothing)
- Grade C theatres

# Continual monitoring

- Facility monitoring system (FMS)
- Independent probes, independently calibrated and validated
- Computer based system which constantly monitors and stores data
  - Incubators, temperature and CO2
  - Fridge and freezer temperature
  - Dewar LN2 levels
  - Room pressures
  - Particle counts in flow hoods

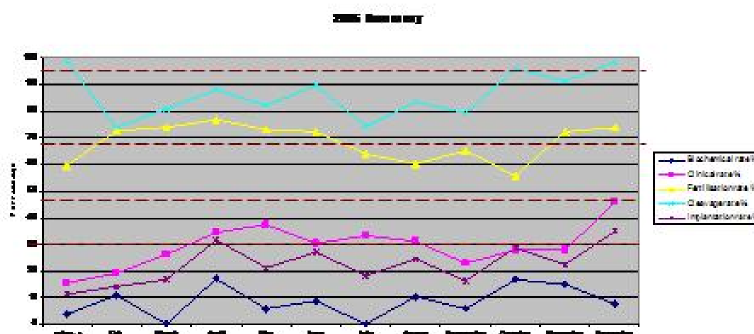


# Analysing results

- ISO 9001
- Quality manual
- Performance measures
  - Fertilisation rate
  - Cleavage rate
  - Implantation rate
  - Biochemical pregnancy rate
  - Clinical pregnancy rate
- Define limits to highlight when further investigation is required

# Defining limits

- E.g. Fertilisation
  - Mean fertilisation rate between 2001 and 2004
    - 69.7
  - Standard Deviation
    - 23.2%
- Use this information to compare prospective data



| Parameter | Value to trigger action |
|---|---|
| Implantation rates | <10% |
| Clinical Pregnancy rate | <15% |
| Biochemical rate | >20% |
| Fertilisation rates | <45% |
| Cleavage rates | <75% |
| Damage rate | >15% |

# Practical tips!

- Limit time oocytes exposed to ambient temperatures / air
- Use optimum concentration of sperm for insemination with IVF
  - Higher concentrations result in higher incidence of polyspermia
- Work quickly and efficiently
- Robust quality system in place
- Review procedures
  - Is theatre adjacent to lab, if not and oocytes are transported, has the incubator been validated
  - Check all heated stages on regular basis

# Choice of Media

- **Commercial media**
  - Produced to GMP standards
  - Rigorous QC programme,
    - pH, osmolarity, endotoxin, bioburden, filter integrity test, mouse embryo assay
- **Sequential system**
  - Use correct product
    - Follicle flush buffer, oocyte wash buffer and fertilisation media contain non essential amino acids to assist the maintenance of homeostasis within oocyte and cumulus complex.

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