PRECONGRESS COURSE 14

Is the oocyte the main determinant of embryo quality? Strategies for the selection of the most competent oocyte

Middle East Fertility Society Exchange course
Geneva – Switzerland, 2 July 2017
Is the oocyte the main determinant of embryo quality? Strategies for the selection of the most competent oocyte

Geneva, Switzerland
2 July 2017

Organised by
the Middle East Fertility Society (MEFS)
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Course coordination
Johnny Awwad (Lebanon) and Mohammad Aboulghar (Egypt)

Course type
Advanced

Course description
The oocyte is the key player in the sperm-egg interaction and the major determinant of embryo developmental potential. In addition to transmitting the maternal chromosomal complement, it also contributes the mitochondrial genome to the developing embryo. Surprisingly little research have focused on studying the oocyte contribution to a successful implantation. Determining oocyte quality remains restricted to a morphological analysis, a well-proven inaccurate science. Alternative innovative strategies, the outcome of extensive research, could prove useful in enhancing the ability of the treating team to select the most competent oocytes for fertilization and subsequent embryo transfer. In addition to advancing our ability to alter reproductive pathways, such technologies have also greatly expanded our understanding of the biology of reproduction. Oocyte competence could hence be better identified on the basis of minimally invasive enhanced diagnostic modalities, such as distribution pattern and function of mitochondria, polar body genomic analysis, cumulus cell molecular signature and many others. Some of these developments have also led to focused interventions designed to improve oocyte reproductive performance, namely mitochondrial enhancement and androgen priming. This pre-congress course discusses biologic pathways which influence oocyte competence and evaluates diagnostic and therapeutic interventions designed to promote oocytes with the highest reproductive potential.

Target audience
Fertility Specialists and Reproductive Endocrinologists

Educational needs and expected outcomes
At the completion of this pre-congress course, participants should be able to:
Describe the biologic pathways which determine oocyte competence
Evaluate the merits of minimally invasive diagnostic modalities in enhancing the selection of the most competent oocyte for fertilization
Develop an evidence-based assessment of the value of proposed interventions in improving the reproductive capability of women
Scientific programme

Chairman: Mohamed A. Aboulghar, Egypt

09:00 - 09:30 Oocyte competence: The mitochondria hypothesis
Dagan Wells, United Kingdom

09:30 - 09:45 Discussion

09:45 - 10:15 Does oocyte mitochondrial injection improve outcomes in women with multiple IVF failures? An assessment of biological rational and clinical data
Kutluk H. Oktay, U.S.A.

10:15 - 10:30 Discussion

10:30 - 11:00 Coffee break

Chairman: Michel Abou Abdallah, Switzerland

11:00 - 11:25 Oocyte competence: The hypoxia hypothesis
Jeremy G. Thompson, Australia

11:25 - 11:50 Oocyte competence: The androgen hypothesis
Wiebke Arlt, United Kingdom

11:50 - 12:15 Androgen priming of antral follicles prior to assisted reproduction: An oocyte rejuvenating therapy?
Johnny Awwad, Lebanon

12:15 - 12:30 Discussion

12:30 - 13:30 Lunch break

Chairman: Johnny Awwad, Lebanon

13:30 - 14:00 Oocyte competence: The aneuploidy hypothesis
Elpida Fragouli, United Kingdom

14:00 - 14:15 Discussion

14:15 - 14:45 Does polar body analysis accurately predict the aneuploidy status of the developing embryo?
Alan H. Handyside, United Kingdom

14:45 - 15:00 Discussion

15:00 - 15:30 Coffee break

Chairman: Mohamed A. Aboulghar, Egypt

15:30 - 16:00 Oocyte competence: The follicle environment hypothesis
Jeremy G. Thompson, Australia

16:00 - 16:15 Discussion

16:15 - 16:45 Human cumulus cells molecular signature: Does it predict oocyte competence and embryo implantation potential?
Samir Hamamah, France

16:45 - 17:00 Discussion
Oocyte competence: The mitochondria hypothesis

Dagan Wells, United Kingdom

Contribution not submitted by the speaker
Does oocyte mitochondrial injection improve outcomes in women with multiple IVF failures? An assessment of biological, translational and clinical data

Kutluk Oktay, MD, PhD, FACOG
Professor of Obstetrics & Gynecology, Medicine, Cell Biology & Anatomy, and Pathology
Vice Chair, Department of Obstetrics & Gynecology
New York Medical College
Director, Division of Reproductive Medicine and Innovation Institute for Fertility Preservation and IVF

Disclosures

• Nothing to disclose (No conflicts pertinent to this presentation.)
Objectives

• To update the audience on mitochondria physiology
• Discuss whether mitochondrial dysfunction is relevant to reproduction
• Review proposed treatments for mitochondrial dysfunction

Mitochondria: Battery for the Living

• Multicopy genome, circular ds-DNA molecule
  Anderson et al., 1981
• Codes for 13 essential subunits of the respiratory chain complexes
  Wallace, 1992
• Maternally-inherited
• 10x more prone to DNA damage
Striking Differences Between Mitochondrial and Nuclear Genome

Comparison between the human nuclear and mitochondrial genomes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nuclear genome</th>
<th>Mitochondrial genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>~3.3 x 10^9 bp</td>
<td>16,369 bp</td>
</tr>
<tr>
<td>Number of DNA molecules per cell</td>
<td>23 in haploid cells; 46 in diploid cells</td>
<td>Several thousand copies per cell (polyplody)</td>
</tr>
<tr>
<td>Number of genes encoded</td>
<td>~20,000–30,000</td>
<td>37 (13 polypeptides, 22 tRNAs and 2 rRNAs)</td>
</tr>
<tr>
<td>Gene density</td>
<td>~1 per 40,000 bp</td>
<td>1 per 450 bp</td>
</tr>
<tr>
<td>Intense</td>
<td>Frequently found in most genes</td>
<td>Absent</td>
</tr>
<tr>
<td>Percentage of coding DNA</td>
<td>~3%</td>
<td>~55%</td>
</tr>
<tr>
<td>Codon usage</td>
<td>The universal genetic code</td>
<td>AUA codes for methionine; TCA codes for tryptophan; AGA and AGG specify stop codons</td>
</tr>
<tr>
<td>Associated proteins</td>
<td>Nucleosomes-associated histone protein and non-histone proteins</td>
<td>No histones, but associated with several proteins (for example, TFAM) that form nucleoids</td>
</tr>
<tr>
<td>Mode of Inheritance</td>
<td>Mendelian inheritance for autosomes and the X chromosome; paternal inheritance for the Y chromosome</td>
<td>Exclusively maternal</td>
</tr>
<tr>
<td>Replication</td>
<td>Strand-coupled mechanism that uses DNA polymerases α and β</td>
<td>Strand-coupled and strand-displacement models; only uses DNA polymerase γ</td>
</tr>
<tr>
<td>Transcription</td>
<td>Most genes are transcribed individually</td>
<td>All genes on both strands are transcribed as large polycistrons</td>
</tr>
<tr>
<td>Recombination</td>
<td>Each pair of homologues recombines during the prophase of meiosis</td>
<td>There is evidence that recombination occurs at a cellular level but little evidence that it occurs at a population level</td>
</tr>
</tbody>
</table>

Taylor and Trumbull, Nature Review Genetics 2005

Peculiarities of Mitochondria

- Most genes needed for mitochondrial function are coded by the nucleus; traverse mitochondrial membrane to function
- Mitochondrial numbers can change depending on energy needs:
  - High energy need: grow and divide
  - Low energy need: destroyed and become inactive

Wallace, 1992
Mitochondrial Function not Limited to Just Being an “Energy Plant”

- Redox functions
- Oxygen sensing
- Fatty-acid oxidation (B-oxidation)
- Calcium hemostasis
- Cell Signaling
- Programmed Cell Death

Van Blerkom J Mitochondrion 2011

When mitochondria do not function...

• Normally O₂ is reduced to H₂O through redox reactions
• ~ 0.1–2% of electrons passing through the chain oxygen is prematurely and incompletely reduced to give rise to superoxide radical (*O₂⁻)
• The pronated form hydroxyperoxyl (HO*) inactivates enzymes or initiate lipid peroxidation


Mitochondrial Theory of Aging

ROS Generation during Mitochondrial Function → Induces mtDNA Mutations → Accumulation of more mtDNA Mutations → Impaired RedOx Function → Reduced Energy & Signaling Functions → Apoptosis and Cell Senescence → Loss of Cellular and Tissue Functions
Mitochondrial Function and Oocyte Quality: Is there a Connection?

Mitochondria in Oocytes

- Large endowment of mitochondria: 100K->600K
- Round/few cristae in oocytes vs. elongated/many cristae in morula/blastocyst
- Localization changes based on stage:
  - GV/M-I perinuclear (to support meiotic activities, spindle formation, chromosomal segregation?)
  - More peripheral in M-II
  - Perinuclear at 2-PN stage
  - Transient microzonation
Mitochondrial Threshold for Viable Embryo Development in Mice

Mitochondria directly influence fertilisation outcome in the pig

Table 5 Fertilisation rates after IVF or ICSI on oocytes with (supplemented) and without (sham injection) mitochondrial supplementation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IVF fertilisation rate (%)</th>
<th>ICSI fertilisation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCB⁺</td>
<td>37.5⁴</td>
<td>40.4⁹</td>
</tr>
<tr>
<td>BCB⁻</td>
<td>17.6⁵</td>
<td>19.8⁹</td>
</tr>
<tr>
<td>BCB⁻ supplemented</td>
<td>31.0⁴</td>
<td>34.0⁹</td>
</tr>
<tr>
<td>BCB⁻ sham injected</td>
<td>17.0⁵</td>
<td>10.0⁹</td>
</tr>
</tbody>
</table>

Values in the same column with different superscripts differ (P < 0.002); values in the same column with different superscripts differ (P < 0.001).
Mitochondrial dysfunction leads to telomere attrition and genomic instability

Mitochondrial dysfunction and oxidative stress have been implicated in cellular senescence, apoptosis, aging and aging-associated pathologies. Telomere shortening and genomic instability have also been associated with replicative senescence, aging and cancer. Here we show that mitochondrial dysfunction leads to telomere attrition, telomere loss, and chromosome fusion and breakage, accompanied by apoptosis. An antioxidant prevented telomere loss and genomic instability in cells with dysfunctional mitochondria, suggesting that reactive oxygen species are mediators linking mitochondrial dysfunction and genomic instability. Further, nuclear transfer protected genomes from telomere dysfunction and promoted cell survival by reconstitution with functional mitochondria. This work links mitochondrial dysfunction and genomic instability and may provide new therapeutic strategies to combat certain mitochondrial and aging-associated pathologies.

Shift in Mitochondria Function in Bovine Oocyte?

The Adenosine Salvage Pathway as an Alternative to Mitochondrial Production of ATP in Maturing Mammalian Oocytes

The Adenosine Salvage Pathway as an Alternative to Mitochondrial Production of ATP in Maturing Mammalian Oocytes

Sara Scantland, et al.

BIOLOGY OF REPRODUCTION (2014) 91(3):75, 1–11
Oocyte Mitochondrial DNA Copy Number is Reduced in Ovarian Insufficiency

Low oocyte mitochondrial DNA content in ovarian insufficiency

Human Reproduction Vol.20, No.3 pp. 593–597, 2005

P.May-Panloup1,2, M.F.Chrétien3, C

3Hôpital de la Reproduction–Laboratoires HY, 3INC Gynécologie Obstétrique–UPF Médecine de la Repro
F–49033 Angers cedex 01, France

1To whom correspondence should be addressed. E-n

BACKGROUND: Mitochondrial biogenesis–embryo development. We have investigated lack of oocyte maturity observed during IVI
an insufficiency. METHODS: We used 116 oocytes obtained from 47 women underg
from women with a normal ovarian profile insufficiency. RESULTS: We found an aver
mtDNA copy number was not significantly t
ificantly lower in oocytes from women with

Our results suggest that low mtDNA content

Mitochondrial DNA content affects the fertilizability of human oocytes


P.Reynier1,3, P.May-Panloup2, M.F.Chrétien2, C.J.Morgan1, M.Jean3, E.Savagner1, P.Barrière3 and Y.Malthiery1

mtDNA

Normal

Male Factor    Idiopathic Fert. Failure

Group 1 Group 2

0 100 200 300 400 500

Group 2 Group 3
Premature Ovarian Failure in Women with Mitochondrial Mutations


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Clues from Women with Mitochondrial Diseases

Table 1. Fertility Rates in Women with Inherited Pathogenic Mitochondrial DNA Mutations, as Compared with Rates in the General Population.

<table>
<thead>
<tr>
<th>Age Range</th>
<th>Women with Mitochondrial DNA Mutations</th>
<th>General Population</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Live Births</td>
<td>No. of Person-Years</td>
<td>Live Birth Rate (95% CI)</td>
<td>Live Birth Rate</td>
</tr>
<tr>
<td>15–19 yr</td>
<td>17</td>
<td>761</td>
<td>22.3 (13.0–35.8)</td>
</tr>
<tr>
<td>20–24 yr</td>
<td>69</td>
<td>713</td>
<td>96.8 (75.3–122.5)</td>
</tr>
<tr>
<td>25–29 yr</td>
<td>91</td>
<td>651</td>
<td>139.8 (112.5–171.6)</td>
</tr>
<tr>
<td>30–34 yr</td>
<td>41</td>
<td>588</td>
<td>69.7 (50.0–94.6)</td>
</tr>
<tr>
<td>35–39 yr</td>
<td>12</td>
<td>516</td>
<td>23.3 (12.0–40.6)</td>
</tr>
<tr>
<td>40–44 yr</td>
<td>2</td>
<td>446</td>
<td>4.5 (0.5–16.2)</td>
</tr>
<tr>
<td>45–44 yr</td>
<td>222</td>
<td>3674</td>
<td>63.1 (55.2–71.8)</td>
</tr>
</tbody>
</table>

* Values for women with inherited pathogenic mitochondrial DNA mutations are compared with the equivalent age- and era-weighted fertility rates (live births per 1000 women) and for the general population obtained from the U.K. Office for National Statistics.

Menopause Timing is Tied to Mitochondrial Dysfunction

- Meta-analysis of 22 genome-wide association studies (GWAS) in 38,968 women of European descent, with replication in up to 14,435 women.
- Gene-set enrichment pathway analyses using the full GWAS data set identified exoDNase, NF-κB signaling and mitochondrial dysfunction as biological processes related to timing of menopause.
Summary

• Indirect evidence in human and some direct evidence in animals support essential role for intact mitochondrial function in oocyte health.
• Because mitochondria plays a multitude of functions in cell viability, the impact of mitochondrial dysfunction may not just be through reduced energy production.
• Direct evidence that mitochondrial function declines with age in human oocytes is missing.

qRT-PCR Analysis Indicates Age-Induced Increase in Mitochondrial DNA Damage in Mouse Oocytes

Bahar Kartal, PhD, Thesis Work, Oktay Lab
Library preparation for Next generation sequencing

MtDNA Damage in Human Primordial Follicles: RNA Sequencing

FPKM: Fragments per kilobase of transcript per million mapped reads

Results are the average of 3 biological replicates
Proposed ART Treatments for Mitochondrial Disorders

- Donor cytoplasmic transfer
- Pronuclei transfer
- Spindle transfer
- Autologous mitochondria injections

Abandoned

Egg Quality Treatment?

Autologous Mitochondria from Oogonial Precursor Cells (OPCs)

- Described in cortical tissue of the ovary
- Unipotent, germline cells
- Because of slow self-renewing nature, proposed to be less predisposed to age-related mitochondrial damage

Nature Med. 2004
**Neo-oogenesis Has Been Refuted**

Experimental evidence showing that no mitotically active female germline progenitors exist in postnatal mouse ovaries.

**Fidelity of DDX4 Ab Questioned**

Characterization of extracellular DDX4- or Ddx4-positive ovarian cells

Silvia F. Hernandez,1,6,5 Nina A. Valdés,4,6 Sofi Pesh,2,6 Patrick Wilczek5, John Tindale5, Bev R. Ronald5,6,7 & Erin F. Wolff5,6,7

To the Editor:

A few groups1–8 have now reported that ovarian-derived stem cells (OSC; also known as oogonial stem cells or oocyte precursor cells) have been isolated from adult mouse2 and rat ovaries; these cells are able to undergo meiosis after transplantation back into recipient ovaries and give rise to offspring. These cells have also been isolated from human adult ovaries, where they can give rise to oocytes after transplanted.7 The marker used to isolate viable cells with germ cell characteristics, ITF444.4 in humans or ITF44.6 in mice and rats (hereafter referred to collectively as DDX4/ITF44) is controversial because it was historically considered to be exclusively an intracellular protein distributed in the cytoplasm of germ cells. However, DDX4/ITF44 was reported to have a C-terminal domain that is expressed extracellularly, whereas the N terminus is expressed intracellularly1,2,7. DDX4/ITF44 expression was reported in freshly isolated OSCs and after propagation for 18 months (mice) and 4 months (humans) in defined cultures by immunostaining, reverse transcription PCR (RT-PCR), and fluorescence-activated cell sorting (FACS). Here we further characterize the expression of DDX4/ITF44 in mouse, rhesus macaque, and human ovarian cells using a polyclonal antibody specific to DDX4/ITF44 (shb 1400; Abcam; generously provided by Jonathan Tilly and purchased from Abcam).

By immunohistochemical analysis of paraform-embedded tissue, we observed staining for DDX4/ITF44 in the expected locations in a...
Debate on DDX4

Zhang et al.\(^4\) state that they were unable to repeat findings presented in their 2012 publication in Nature Medicine regarding the characterization of ovarian stem cells (OSCs) in mouse and human ovaries\(^5\) using methods further detailed a year later\(^6\). Separately, Hernandez et al.\(^7\) question the specificity of antibodies that target the C terminus of DDX4 (DEAD box polypeptide 4) to visibly sort OSCs from adult mouse, monkey and human ovaries, as we reported\(^8,9\). Although these two correspondences focus on our work from 2012, DDX4-specific antibody-based sorting of OSCs was first published in 2009 by another laboratory\(^9\). A year before this publication, Richards et al.\(^8\) reported isolation of viable germ cells from cultures of human embryonic stem cells using fluorescence-activated cell sorting (FACS) coupled with DDX4-specific antibodies. Our 2012 study therefore represents independent methodological verification of these two earlier reports.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample</th>
<th>Mass spectrometry, DDX4 aromologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Total ovarian cell lysate</td>
<td>Not detected</td>
</tr>
<tr>
<td>Mouse</td>
<td>COO enriched from total ovarian cell lysate</td>
<td>Not detected</td>
</tr>
<tr>
<td>Mouse</td>
<td>iP-ovarian cell lysate</td>
<td>Detected</td>
</tr>
<tr>
<td>Mouse</td>
<td>iP-ovarian cell lysate cultured</td>
<td>Detected</td>
</tr>
</tbody>
</table>

Prepared by DDX4 antibody-based sorting. Because, however, iPS cells sorted and used by Zhang et al.\(^4\) differ from the OSCs we have isolated and described, it is not surprising that their downstream endpoint analyses would not reproduce what has been already reported using purified OSCs as starting material.

With that said, apparent differences between our findings and those of Zhang et al.\(^4\) and Hernandez et al.\(^7\) regarding the ability of DDX4-specific antibodies to isolate OSCs highlight a fundamental issue raised by both sets of authors. Namely, is DDX4 expressed in all germ cells at all stages of differentiation or do OSCs differ from other germ cells in their membrane localization of DDX4, thus making the protein available to be targeted in purification schemes involving magnetic-assisted cell sorting or FACS? If the latter case is true, why have some groups been able to repeat the DDX4-specific antibody-based approach to isolate OSCs while others have failed?

Indirect Evidence Supporting OPC May Have Healthy Functional Mitochondria

- **Human Oocyte**
- **OPC**

- **Table:**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass spectrometry, DDX4 aromologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ovarian cell lysate</td>
<td>Not detected</td>
</tr>
<tr>
<td>COO enriched from total ovarian cell lysate</td>
<td>Not detected</td>
</tr>
<tr>
<td>iP-ovarian cell lysate</td>
<td>Detected</td>
</tr>
<tr>
<td>iP-ovarian cell lysate cultured</td>
<td>Detected</td>
</tr>
</tbody>
</table>
**Autologous Germline Mitochondrial Energy Transfer**

Poor in vitro fertilization (IVF) success rates in women of advanced maternal age reflect unmet energy needs in the egg.

**NO fertilization or POOR embryo development = FAILED pregnancy**

**SUCCESSFUL fertilization and embryo development = HEALTHY pregnancy**

**Tilly and Sinclair, Cell Metabolism 2013**

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**Laparoscopic ovarian biopsy** (Three 5x5 mm)

- **Cell sorting**
- **Oogonial precursor cells**
- **Mitochondria isolation**
- **ICSI**

**Ovarian Stimulation**

**Oocyte retrieval**
Ovarian Tissue Harvesting for AMI/Augment

**Oogonia Cell-Derived Autologous Mitochondria Injection to Improve Outcomes in Women With Multiple IVF Failures Due to Low Oocyte Quality: A Clinical Translation**

Kutluq Oktay, MD, FACOG1,2,3, Volkan Baltaci, MD,3, Murat Sonmezler, MD7, Volkan Turan, MD1,3, Evrim Unsal, PhD3, Ayşen Baltaci, MD1, Suleyman Aktuna, PhD1, and Fred Moy, PhD1,2

**Abstract**

Background: Mitochondrial dysfunction has been suggested as a major cause of age-induced decline in oocyte quality. In the past, donor oocyte cytoplasmic transfer showed some success but was abandoned due to concerns with heteroplasmy. Recent studies indicated presence of oogonial precursor cells (OPCs) in the human ovary, which could be an autologous source of “healthy mitochondria.” We sought to investigate the clinical efficacy of OPC-derived autologous mitochondrial injection (AMI) to improve oocyte quality in women with multiple in vitro fertilization (IVF) failures. Methods: The OPCs were isolated from laparoscopy-obtained ovarian cortical pieces by cell sorting using a monoclonal anti-DOX antibody. They were then disrupted and mitochondria were isolated. Reconstructed mitochondria were injected into each oocyte during intracytoplasmic sperm injection. Paired comparisons were made between the first failed cycles and the post-AMI cycles. Results: Of the 15 women undergoing ovarian stimulation, 2 were canceled and 3 decided to pool oocytes for laser AMI. In remaining 10 (mean age 34.7 ± 4.1), AMI significantly improved fertilization rates (49.7 ± 11.3 vs 26.3 ± 18.9; P < 0.003) with a trend for better embryo grades (2.3 ± 0.3 vs 1.1 ± 0.7; P < 0.08). Four of 10 women conceived after single-frozen embryo transfer and 3 after confirmation of diploidy via CGH (clinical pregnancy/embryo transfer = 4/10). Conclusion: These data show encouraging results for AMI in comparison to previous failed IVF cycles and a historical control group.
17 with $2 \leq$ IVF failure

15 underwent COH

2 excluded due to lack of prior IVF outcomes

10 underwent ET

• 3 oocyte pooling
• 2 cancellation (1 arrest at 2pn, 1 aneuploidy after PGS)

4 with PGS

6 without PGS

Characteristics of Patients

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>D2-3 FSH (IU/ml)</th>
<th>D2-3 E2 (pg/ml)</th>
<th>AMH (ng/ml)</th>
<th>N of IVF failures</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>6.1</td>
<td>57</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td>31</td>
<td>7.2</td>
<td>39</td>
<td>3.5</td>
<td>3</td>
</tr>
<tr>
<td>32</td>
<td>5.9</td>
<td>36</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>34</td>
<td>15.2</td>
<td>100</td>
<td>1.4</td>
<td>7</td>
</tr>
<tr>
<td>35</td>
<td>7.4</td>
<td>47</td>
<td>1.8</td>
<td>5</td>
</tr>
<tr>
<td>35</td>
<td>6.9</td>
<td>NA</td>
<td>NA</td>
<td>4</td>
</tr>
<tr>
<td>36</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>36</td>
<td>5.5</td>
<td>134</td>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>40</td>
<td>9</td>
<td>NA</td>
<td>NA</td>
<td>7</td>
</tr>
<tr>
<td>41</td>
<td>5.6</td>
<td>39</td>
<td>0.7</td>
<td>3</td>
</tr>
</tbody>
</table>
AMI Impact on Oocyte Quality

### Pregnancy outcomes

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Fresh/ Frozen</th>
<th>PGS (N of embryos)</th>
<th>N of embryos transferred</th>
<th>Pregnancy outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>Fresh</td>
<td>NA</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>Frozen-thawed</td>
<td>2 normal out of 7</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>Frozen-thawed</td>
<td>4 normal out of 8</td>
<td>1</td>
<td>Pregnancy loss</td>
</tr>
<tr>
<td>34</td>
<td>Frozen-thawed</td>
<td>1 normal out of 3</td>
<td>1</td>
<td>Live birth</td>
</tr>
<tr>
<td>35</td>
<td>Fresh</td>
<td>NA</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>35</td>
<td>Fresh</td>
<td>NA</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>Frozen-thawed</td>
<td>1 normal out of 7</td>
<td>1</td>
<td>Ongoing Pregnancy</td>
</tr>
<tr>
<td>36</td>
<td>Frozen-thawed</td>
<td>NA</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>Fresh</td>
<td>NA</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>Frozen-thawed</td>
<td>NA</td>
<td>1</td>
<td>Pregnancy Loss</td>
</tr>
</tbody>
</table>

Error bars: 95% CI
17 patients with 2≤ IVF failure
15 underwent COH
2 excluded due to lack of prior IVF outcomes

Clinical pregnancy rate: 40%
Live/On-going pregnancy rate: 20%

4 with PGS
6 without PGS

1 live birth
1 on-going pregnancy
1 pregnancy loss

2 cancellation (1 arrest at 2pn, 1 aneuploidy after PGS)

Oktay et al Reprod Sciences, 2015;22(12):1612-7

Pregnancies After OPC-Derived AMI

<table>
<thead>
<tr>
<th>PRE-AMI</th>
<th>POST-AMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 yo, 7 IVF Failures Single FET Normal PGS</td>
<td>41 yo, 3 IVF Failures Single FET</td>
</tr>
</tbody>
</table>

Pre (a,c) and post-AMI (b,d) embryo development of pregnant patients. First (b) has delivered and the second (d) resulted in a first trimester pregnancy loss.
**“Augment” Baby: Elanur**

**Comparison Of Augment with Age Matched Historical Control Group**

<table>
<thead>
<tr>
<th></th>
<th>Augment (n=10)</th>
<th>Control (n=20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age</td>
<td>34.7 ± 4.1</td>
<td>35.1 ± 3.6</td>
<td>0.91</td>
</tr>
<tr>
<td># Previous IVF Failures</td>
<td>4.3 ± 2.0</td>
<td>3.4 ± 0.5</td>
<td>0.53</td>
</tr>
<tr>
<td># Embryos Transferred</td>
<td>1.6 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>1.00</td>
</tr>
<tr>
<td>Clin. Pregnancy Rate (%)</td>
<td>4/10 (40)</td>
<td>3/20 (15)</td>
<td>0.18</td>
</tr>
<tr>
<td>Live/Ong. Birth Rate (%)</td>
<td>2/10 (20)</td>
<td>1/20 (5)</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Data From other Augment Centers

### The AUGMENT Experience

<table>
<thead>
<tr>
<th></th>
<th>TCART</th>
<th>FAKIH IVF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total AUGMENT cycles initiated</td>
<td>34</td>
<td>60</td>
</tr>
<tr>
<td>Average cycles per patient</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total embryo transfers</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td>Clinical pregnancy rate:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- per cycle initiated</td>
<td>12/34 (35%)</td>
<td>13/80 (22%)</td>
</tr>
<tr>
<td>- per embryo transfer</td>
<td>12/26 (46%)</td>
<td>13/34 (38%)</td>
</tr>
<tr>
<td>Ongoing clinical pregnancy and live birth rate:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- per cycle initiated</td>
<td>9/34 (26%)*</td>
<td>11/60 (18%)**</td>
</tr>
<tr>
<td>- per embryo transfer</td>
<td>9/20 (35%)*</td>
<td>11/34 (32%)**</td>
</tr>
</tbody>
</table>

*Includes 1 live birth
**Includes 2 live births (two sets of twins)

Note: All on-going clinical pregnancies reported here were continuously on-going pregnancies at the time of publication submission


---

Data From other Augment Centers

### Patient History vs. Clinical Pregnancy Rate

<table>
<thead>
<tr>
<th></th>
<th>Clinical Pregnancy Rate per Initiated AUGMENT Cycle</th>
<th>Clinical Pregnancy Rate per AUGMENT Embryo Transfer</th>
<th>Ongoing Clinical Pregnancy Rate/ Live Birth Rate per Initiated AUGMENT Cycle</th>
<th>Ongoing Clinical Pregnancy/ Live Birth Rate per AUGMENT Embryo Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average age:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36.0</td>
<td>35%</td>
<td>48%</td>
<td>26%</td>
<td>35%</td>
</tr>
<tr>
<td>1-5 prior IVF cycles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>United Arab Emirates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average age:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37.3</td>
<td>22%</td>
<td>38%</td>
<td>18%</td>
<td>32%</td>
</tr>
<tr>
<td>1-16 prior IVF cycles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- No Control group
- Patients with single IVF failure treated

Augment: Lack of Livebirths > Age 40

<table>
<thead>
<tr>
<th>Age (years)</th>
<th># of Patients</th>
<th># bHCG +</th>
<th># Clinical Pregnancies</th>
<th># Ongoing Clinical Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-30</td>
<td>5</td>
<td>3*</td>
<td>3*</td>
<td>2*</td>
</tr>
<tr>
<td>31-35</td>
<td>10</td>
<td>7*</td>
<td>6*</td>
<td>8*</td>
</tr>
<tr>
<td>36-40</td>
<td>14</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>41-45</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>46-48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Totals</td>
<td>34</td>
<td>15</td>
<td>12</td>
<td>9</td>
</tr>
</tbody>
</table>

*Includes one pregnancy each from a subsequent frozen embryo transfer

Data From other Augment Centers

- Aug group received 1.7x oocytes
- Non-blinded or randomized
- No IRB; “clinical experience”

Sumary & Conclusions

• Initial non-randomized studies suggest some improvement in fertilization, embryo quality and possibly pregnancy rates < age 40 with OPC-derived mitochondria injection.
• Specificity of these improvements cannot be proven from the current data
• Further prospective-randomized data are needed before this treatment can be considered effective and safe.

Future Work

• Comparative data on mitochondrial health: OPC-derived vs. aged oocyte
• Better quantification and correlation with outcome of mitochondria numbers placed in the oocyte
• Randomized-blinded study:
  – Straight randomization of oocytes vs. patients
  – Cross-over design?
• A registry of children born from “Augment”
Mitochondrial Treatments: There is a Responsible Way

Oocyte Aging

Mitochondrial Treatments

- Data Safety Boards & Public Transparency
- IRB Oversight
- Long-term Follow Up
- Rigorous Case Selection

Healthy baby

- Laboratory of Molecular Reproduction & Fertility Preservation
  - Shiny Titus, PhD
  - Fred Moy, PhD (Biostat)
  - Enes Taylan, MD
  - Yodo Sugishita, MD, PhD
  - Robert Stobezki, PhD
  - Tai Kawahara, MD

- Innovation Institute for Fertility Preservation
  - Kutluk Oktay, MD, PhD
  - Enes Taylan, MD
  - Giuliano Bedoschi, MD
  - Allison Rosen, PhD
  - Anitra Miraglia

- Extra-Mural Collaborators:
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  - Maura Dickler, MD & Mark Robson, MD, (Memorial Sloan Kettering Cancer Center)

Supported by R01 HD053112 and R21 HD061259

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- Kyungah Jeong, MD
- Volkan Turan, MD
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- Sinan Ozkavukcu, MD, PhD
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  – Carmen Dabao

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  PhD, Yeni Yuzyl Univ, Istanbul, TR

Supported by R01 HD053112 and R21 HD061259

References
Oocyte competence: The hypoxia hypothesis

Jeremy Thompson

Conflicts of Interest Statement

The University of Adelaide receives consultation funds from Cook Medical LLC for use by Jeremy Thompson for research expenditure.
Learning objectives

• Have an understanding of the paradox of antral follicle oxygenation
• Brief overview of Hypoxia Inducible Factors (HIFs) and how they are activated
• Examine the evidence for the potential role of HIFs in the follicle
• Examine the evidence that haemoglobin is a follicular and oocyte protein
• Explore the role for HIF and haemoglobin in regulating oocyte competence
Is the antral follicle hypoxic?

8-10% \( pO_2 \)?

Oocytes need \( O_2 \)

• Totally dependent on oxidative phosphorylation – little glycolytic capacity
  – Biggers et al (1967) PNAS

• Low follicular \( O_2 \) associated with poor development
Follicular pO₂

↓pO₂ <2.5% O₂ associated with:
↓developmental competence
↑meiotic spindle defects


Intrafollicular pO₂ determines developmental competency

Berg et al (unpublished)
Mathematical modelling of oxygen transport-limited follicle growth

G P Redding¹, J E Bronlund¹ and A L Hart²

¹Institute of Technology and Engineering, Massey University, Private Bag 11222, Palmerston North 4440, New Zealand ²Food and Health, AgResearch Grasslands, Private Bag 11008, Palmerston North 4440, New Zealand

Mathematical modelling

Cumulus cells consume relatively small amounts of oxygen.

The follicular fluid oxygen concentration is close to what the oocyte sees.

2 – 20% O₂ - 0.5 %
Metabolism of the Cumulus-oocyte Complex

Spatial Characterization of Bioenergetics and Metabolism of Primordial to Proovulatory Follicles in Whole Ex Vivo Murine Ovary

Rachel Cincio, Michelle A. Dignam, Enrica Gratton, and Ulrike Ladewig

Bound NADH = Oxidative metabolism
Free NADH = Glycolytic metabolism

Oxygen-regulated gene expression

- Hypoxia inducible factors (HIFs)
- bHLH-PAS transcription factors
  - Heterodimeric proteins
  - HIF-1, -2, -3α
  - HIF-1β (ARNT)
  - Binding to HRE’s (5’-RCGTG)
- Activates transcription of genes involved in response to low oxygen
HIF Regulation

Oxygen Sensor

Ubiquitination → Proteasomal Degradation

HIF-1α

O2 → Hypoxia

HIF-2α

HIF-1α (SGST)

Stabilisation

Vascular function genes
Angiogenesis genes
Glycolysis genes

Hypoxia induction of oxygen regulated genes

HIF-1

HIF-2

mRNA expression of HIF 1α and 1β

HIF-1α

Arbitrary units Corrected for 18S

Mil 2-cell 8-cell Morula Blast

HIF-2α

Arbitrary units Corrected for 18S

Mil 2-cell 8-cell Morula Blast

HIF-1β

Arbitrary units Corrected for 18S

Mil 2-cell 8-cell Morula Blast

Kind et al unpublished
HIF-1α

Stabilisation

CYTOPLASM

GFP Reporter

HIF-1α (ARNT)

HIF-1α

HRE

Hypoxia-inducible factor

Hypoxic induction of oxygen regulated genes

NUCLEUS


Thompson et al, unpublished

Epididymis

Page 40 of 135
HIF1α protein is found in both follicle and CL, but....

Is HIF regulated by gonadotrophins?

- FSH
  - No evidence that FSH influences HIF1
- LH/hCG
  - Combination of hCG and low O2 stabilises HIF1 protein
  - Temporally regulated by LH in vivo

GFP increases following LH surge

![GFP increase following LH surge](image)


The paradox of HIF activity in the ovary

- Large antral ovarian follicles are supposed to be hypoxic, or at least close to hypoxia?
  - No measurable HIF-induced response in both pre-antral and antral
- The developing corpus luteum is HIF active
  - Does this mean the developing CL is hypoxic?
  - CL formation is dependent on VEGF
- Is there a further regulatory mechanism that could help explain this paradox?
Low O₂ IVM turns on HIF

• Low O₂ IVM increases mRNA of many classic HIF1/2 regulated genes in cumulus cells, but not in vivo

Banwell et al unpublished
Kind et al (2014) RFD

Table 2. List of selected genes differentially regulated, as detected by microarray analysis, in cumulus cells derived from in vitro compared with in vivo matured of cumulus-oocyte complexes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession no.</th>
<th>M-value</th>
<th>P-value</th>
<th>Gene function (biological process)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphodiesters (ATPase)</td>
<td>NM_013873</td>
<td>7.5</td>
<td>0.00016</td>
<td>cAMP phosphodiesterase activity</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase 1 (Mapk1)</td>
<td>NM_001858</td>
<td>6.6</td>
<td>0.00013</td>
<td>ATP binding, kinase activity</td>
</tr>
<tr>
<td>Hyaluronan-like growth-factor-binding protein 1</td>
<td>NM_010538</td>
<td>6.5</td>
<td>0.0003</td>
<td>Growth factor binding</td>
</tr>
<tr>
<td>Bone morphogenetic protein 4 (Bmp4)</td>
<td>BC073459</td>
<td>5.7</td>
<td>0.0102</td>
<td>BMP receptor binding, growth factor activity</td>
</tr>
<tr>
<td>Growth arrest specific 5 (Gas5)</td>
<td>NM_134547</td>
<td>4.8</td>
<td>0.00042</td>
<td>Calcium binding, metal ion binding</td>
</tr>
<tr>
<td>A disintegrin-like and metalloproteinase (Adam17)</td>
<td>NM_019527</td>
<td>-4.1</td>
<td>0.00046</td>
<td>Receptor binding</td>
</tr>
<tr>
<td>Lithium-sensing category 2 (Lil2)</td>
<td>NM_001598</td>
<td>-4.9</td>
<td>0.00004</td>
<td>ATPase binding, Lili receptor activity</td>
</tr>
<tr>
<td>Pentosan-related gene (Zra3)</td>
<td>NM_081975</td>
<td>-5.2</td>
<td>0.00019</td>
<td>Inflammatory response</td>
</tr>
<tr>
<td>Hyaluronan synthease 2 (Has2)</td>
<td>NM_080266</td>
<td>-5.2</td>
<td>0.00006</td>
<td>Hyaluronan synthase activity</td>
</tr>
<tr>
<td>Interleukin-6 (Il6)</td>
<td>NM_031108</td>
<td>-7.8</td>
<td>0.000067</td>
<td>Cytokine activity, growth factor activity</td>
</tr>
<tr>
<td>Beta-2-adrenergic (B2AR)</td>
<td>NM_073785</td>
<td>-7.2</td>
<td>0.00026</td>
<td>Growth factor activity</td>
</tr>
<tr>
<td>Amyloidogenic (Amy)</td>
<td>NM_005754</td>
<td>-5.7</td>
<td>0.000066</td>
<td>Growth factor activity, cytokine activity</td>
</tr>
<tr>
<td>Serotonin (5-HT2 (5HT2C)</td>
<td>NM_0035754</td>
<td>-10.6</td>
<td>0.000016</td>
<td>G protein transport</td>
</tr>
<tr>
<td>Serotonin (5-HT2 (5HT4C)</td>
<td>NM_082238</td>
<td>-11.6</td>
<td>0.000017</td>
<td>G protein transport</td>
</tr>
</tbody>
</table>

Kind et al (2013) RFD
Haemoglobin mRNA regulated in the mouse follicle

**GRANULOSA CELLS**

- Pooled human granulosa and cumulus cells collected from women undergoing ART.

**Human granulosa and cumulus cells**

- Pooled human granulosa and cumulus cells collected from women undergoing ART.
Haemoglobin lost during IVM

![Image]

Ovulated (hCG 16 h)

IVM (17 h)

Protein aberrantly located in cumulus cells and lost from oocyte

Conclusions

- Follicular O₂ is:
  - Correlated to oocyte health
  - Difficult to measure
  - Oocyte most likely in a “hypoxic” range – but not hypoxic
- Hypoxia Inducible Factors in antral follicles
  - Do not appear to regulate pre-LH surge follicle growth
  - Regulates post-LH surge differentiation
- Haemoglobin present in follicular cells
  - Gas transport? – but which gas? Or other function?
- HIF and Haemoglobin
  - How does Hb interact with HIF, if at all?
  - Not present at all during IVM - what does this mean for oocyte in vitro maturation?
  - Present during embryo development? Yes!
Many people need to be thanked!

- Early Development Group
  - PhD students
    - Alex Harvey
    - Laura Frank
    - Mel Sutton-McDowall
    - Kim Tam
    - Kelly Banwell
  - Post Docs
    - Karen Kind
    - Hannah Brown
    - Mel Sutton-McDowall

- Murray Whitelaw & Dan Peet (U of A)

- Robinson Research Institute colleagues
  - Darryl Russell
  - Claire Roberts

---

Current hypothesis

Antral follicle

12 h differentiation

O2 O2 HIF Hb LH
Androgen Priming of Antral Follicles Prior to Assisted Reproduction: An Oocyte Rejuvenating Therapy?

Johnny Awwad, MD
Professor of Obstetrics and Gynecology
Head, Division of Reproductive Endocrinology and Infertility
American University of Beirut Medical Center

Non Disclosure Statement

NO CONFLICT OF INTEREST
ADJUVANT THERAPY

STUDY QUESTION

ANDROGENS in the ovarian micro milieu potentiates FSH action

INTERVENTIONS

Do Androgens and Androgen-modulating Agents benefit POR

- Follicle number
- Oocyte quality
- Live births

Live births
A 38 year old woman G0P0 was referred to you for primary infertility of 8 years duration.
AMH 0.9 ng/dl. CD3 FSH 13.0 IU/l. E2 45pg/ml. AFC 5.

She reports a previous ART cycle failure in which she received 300 IU daily dose of rec-FSH, developed 2 pre-ovulatory follicles and produced 2 oocytes.

She is planning her third ART cycle, and has heard about androgen therapy. To maximize benefit, you propose testosterone transdermal patches should be started:

A. On the first day of ovarian stimulation and until hCG.
B. Two weeks prior to ovarian stimulation and until hCG.
C. Six weeks prior to ovarian stimulation and until hCG.
D. Twelve weeks prior to ovarian stimulation and until hCG.
Testosterone-treated women achieved significantly higher live birth rate, clinical pregnancy rate and required significantly lower doses of FSH.

Testosterone-treated women achieved significantly higher live birth rate, clinical pregnancy rate and required significantly lower doses of FSH.

Testosterone-treated women achieved significantly higher live birth rate, clinical pregnancy rate and required significantly lower doses of FSH.
When the clinical pregnancy rate was adjusted per embryo transferred, differences among the two groups were not statistically significant.
When compared with placebo or no treatment, pre-treatment with DHEA was associated with higher live birth rates.

Figure 4. Forest plot of comparison: 1 DHEA or testosterone versus placebo/no treatment, outcome: 1.1 Live birth/ongoing pregnancy rate.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Odds Ratio M.H. (Fixed, 95% CI)</th>
<th>Substrata only</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Live birth rate by length of T administration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3 up to 7 days</td>
<td>1</td>
<td>Odds Ratio (M.H., Fixed, 95% CI)</td>
</tr>
<tr>
<td>4.3 up to 28 days</td>
<td>2</td>
<td>Odds Ratio (M.H., Fixed, 95% CI)</td>
</tr>
</tbody>
</table>

When compared with placebo or no treatment, pre-treatment with testosterone was associated with higher rates of live birth.
Androgens Stimulate Early Stages of Follicular Growth in the Primate Ovary

Keith A. Vendola, Juan Zhou, Oluyemisi O. Adebayo, Stacie A. Weil, and Carolyn A. Eddy

Developmental Endocrinology Branch, National Institute of Child Health and Development, National Institutes of Health, Bethesda, Maryland 20892

The Journal of Clinical Investigation
Volume 101, Number 12, June 1998, 2622–2629
http://www.jci.org

Experimental animals. Female rhesus monkeys (Macaca mulatta), 6–13 yr of age, from the NIH Pockesville colony were used in accordance with a protocol approved by the NICHD animal care and use committee. Animals had pellets (Innovative Research of America, Toledo, OH) inserted subcutaneously between their shoulder blades under ketamine anesthesia. In the first set of experiments, groups (n = 4–6) received pellets containing vehicle, high-dose testosterone (4 mg/kg per day for 3 d), or testosterone (400 μg/kg per day for 10 d). In a subsequent set of experiments, animals received pellets with placebo, low-dose testosterone (20 μg/kg per day for 3 d), or dihydrotestosterone (DHT; 145 μg/kg per day for 5 d). At the end of the closing periods, ovariec- toonies were performed on the monkeys under ketamine anesthesia via a midventral laparotomy.

Testosterone treatment increased
- Ovarian size
- Follicular number
Androgen treatment stimulated early stages of primate ovarian follicular growth, independently of cycle stage or gonadotropin effect.

Testosterone treatment
- Significantly increased the numbers of small follicles (primary to small antral)
- Did not increase the abundance of large antral follicles (pre-ovulatory)

Androgen and Follicle-Stimulating Hormone Interactions in Primate Ovarian Follicle Development

STACIE WEIL, KEITH VENDOLA, JIAN ZHOU, AND CAROLYN A. BONDY
Developmental Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

Female Rhesus monkeys, 6–13 yr of age (from the NIH Poolesville, MD, colony) were studied under a protocol approved by the NICHD Animal Care and Use Committee. Monkeys were treated with sc pellets (Innovative Research of America, Toledo, OH) containing vehicle (n = 8) or sustained release T (4 mg/kg for 3 days, n = 4; or 0.4 mg/kg for 10 days, n = 4), as previously described (3). Another group (n = 4) received sc injections of recombinant FSH (Metrodin, Serono, Norwell, MA, 35 IU) for 2 days. Ovariectomies were performed under ketamine anesthesia via a ventral laparotomy.
Androgens promoted follicular growth indirectly by amplifying FSH effect.

FSHR mRNA expression was
- Significantly increased following testosterone treatment
- Only modestly increased following FSH treatment

Androstenedione induces abnormalities in morphology and function of developing oocytes, which impairs oocyte meiotic competence

Setting: St. Maryana University School of Medicine.
Animals: Prepubertal 6-day-old (HEF) female mice.
Intervention: Early secondary follicles were isolated from the ovaries and were cultured individually in vitro with or without androstenedione (10⁻⁷M) to 10⁻¹M for 12 days. Thereafter, the follicles were treated with hCG and epidermal growth factor (EGF).
Equivalent to ovarian follicular fluid concentrations:
- PCOS - $10^{-5} \text{ M}$
- Healthy women - $10^{-9} \text{ M}$

In androstenedione-treated follicles,
- Survival rate of follicles decreased in a dose-dependent manner
- Rate of follicles with abnormal morphology higher
Excess androgen induced abnormalities in the morphology and function of developing oocytes, which impairs oocyte meiotic competence.

- Failure of spindle assembly higher
- Misaligned chromosomes more frequent

Excess androgen induced abnormalities in the morphology and function of developing oocytes, which impairs oocyte meiotic competence.
Does clinical research follow principles of ovarian physiology

The transition from preantral to antral follicular stage = 70 days

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample Size</th>
<th>Duration, days</th>
<th>Dose (per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boisdou et al.</td>
<td>50</td>
<td>21</td>
<td>10 mg</td>
</tr>
<tr>
<td>Kien et al.</td>
<td>110</td>
<td>21</td>
<td>12.5 mg</td>
</tr>
<tr>
<td>Fabregues et al.</td>
<td>62</td>
<td>5</td>
<td>0.02 mg/kg</td>
</tr>
<tr>
<td>Massin et al.</td>
<td>49</td>
<td>15</td>
<td>10 mg</td>
</tr>
</tbody>
</table>

Testosterone in excess to 5.5 mg/d may be detrimental to follicle development
Serum testosterone and DHEA-S levels were statistically significantly higher after DHEA supplementation.
Median follicular DHEA-S level, but not testosterone, was statistically significantly higher in the DHEA group.

No significant difference in the oocytes obtained, clinical pregnancy, ongoing pregnancy, live birth or miscarriage was observed.
Transdermal testosterone pretreatment in poor responders undergoing ICSI: a randomized clinical trial


STUDY DESIGN, SIZE, DURATION: The present RCT was designed to detect a difference of 1.5 COCs (sample size required = 48 patients). From 02/2014 until 04/2015, 50 poor responders fulfilling the Bologna criteria have been randomized (using a randomization list) to either testosterone pretreatment for 21 days (n = 26) or no pretreatment (n = 24).

All patients underwent a long follicular protocol with GnRH agonist triptorelin (Arvekap, Ipsen Ltd, France) 3.75 mg depot, starting on the first day of the menstrual cycle, followed by daily injections of triptorelin (Arvekap, Ipsen Ltd, France) 0.1 mg, if necessary. In the testosterone group, a daily dose of 10 mg of testosterone gel (Tostoran 2% Gel, ProStrakan) was applied transdermally onto the inner thigh daily, for 21 days as suggested by Kim et al. (2011), starting from the GnRH agonist initiation. Testosterone was supplied in a canister with a closing pumping mechanism, which delivered one half gram of gel containing 10 mg of testosterone each time the piston was depressed.

Table IV Clinical outcome between the testosterone pretreatment group and the no pretreatment group.

<table>
<thead>
<tr>
<th></th>
<th>Testosterone pretreatment (n = 26)</th>
<th>No pretreatment (n = 24)</th>
<th>Difference % 95% CI* Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of patients with at least one top quality embryo</td>
<td>20.0 (4)</td>
<td>12.8 (5)</td>
<td>-3.8 (-28.2 to +21.5)</td>
</tr>
<tr>
<td>Patients with embryo transfer</td>
<td>83.3 (20)</td>
<td>91.3 (21)</td>
<td>-8.0 (-28.2 to +12.2)</td>
</tr>
<tr>
<td>Cancellation rate</td>
<td>1.7 (2)</td>
<td>9.2 (2)</td>
<td>-7.5 (-13.5 to +0.3)</td>
</tr>
</tbody>
</table>

The non-significant increase in the number of COCs, following transdermal testosterone pretreatment was not associated with the probability of embryo transfer.
Does ANDROGEN supplementation benefit POR

**Likely**

**How**
- Dose threshold
  - T 5.5mg/d

**Which**
- Duration threshold
  - > 72 days

**When**
- T vs A adjuvant
Oocyte competence: The aneuploidy hypothesis

Elpida Fragouli, PhD, FRSB
Scientific Director, Reprogenetics UK

Disclosure

Employee (scientific director) of Reprogenetics UK, a PGD service provider
Learning objectives

• Origin of aneuploidy & relevance to reproductive failure
• Oogenesis & meiosis
• Mechanisms leading to aneuploidy of female meiotic origin:
  1. Whole chromosome non-disjunction
  2. Unbalanced chromatid pre-division
• Methods employed for oocyte/ PB analysis:
  Advantages & disadvantages
• Oocyte analysis data:
  1. Karyotyping & FISH
  2. Comprehensive molecular cytogenetic methods (CGH & aCGH)
• Why is female meiosis so error prone?
  Recombination

The impact of aneuploidy in reproductive success & failure

• Numerical chromosome abnormalities: common & clinically significant
• 5% of clinically recognised pregnancies carry a trisomy or monosomy
• Aneuploid pregnancies mostly miscarry
• A few trisomies & sex chromosome abnormalities lead to live births
• Relevance of aneuploidy to IVF
  1. Embryonic arrest
  2. Implantation failure
  3. Spontaneous abortion

Hassold and Hunt, 2001: Hassold et al., 2007
Origin of aneuploidy

- Aneuploidies arise during oogenesis (mostly) & post-fertilization
- Meiotic aneuploidy principally influenced by female age

Reprogenetics UK data from > 2000 oocytes

Meiosis

- Specialised cell division taking place in reproductive tissue
- Reduces diploid chromosome number by half- haploid gametes created
Oogenesis

- Females born with complete oocyte set
- Oogenesis starts during fetal development
- Mitotic divisions form primordial follicles containing diploid primary oocytes
- MI begins on 12th week of fetal development
- Diplotene prophase I arrest:
  - homologous chromosome recombination
  - germinal vesicle (GV) formation
- Puberty & menstrual cycle:
  - one oocyte released each month
  - uterus prepares for implantation

Whole chromosome non-disjunction

- Takes place during MI or MII
- Homologous chromosomes move towards same meiotic spindle pole
- Oocyte with extra chromosome & 1st or 2nd PB with missing chromosome or vice versa
  
  ![Diagram of whole chromosome non-disjunction](image)

- Due to reduced or no recombination & position of chiasmata
- Female age affects recombination patterns

Jones, 2008

Delhanty, 2005; Hassold et al., 2007
Unbalanced chromatid predivision

- Takes place during MI
- Sister chromatids divide prematurely & segregate randomly
- Lead to aneuploidy in 50% of cases

- Due to reduced or no recombination & female age

Angell et al., 1993

Cytogenetic analysis of oocytes & polar bodies
Oocyte & polar body fixation on a slide

- Required for karyotyping or FISH analysis
- Involves hypotonic treatment followed by fixation on slide
- Enables visualisation of chromosomes & chromatids
- Risks artefactual chromosome loss
- Accurate hyperhaploidy scoring only

Picture from Mahmood et al., 2000; Fragouli et al., 2011

Oocyte analysis via classical cytogenetic methods

- Pellestor et al. (2003) analysed 1397 oocytes via R banding
- 792 women of average age 34 years
- Modified fixation minimised artefactual chromosome loss
- 10.8% oocytes abnormal
- Aneuploidy increased with advancing female age
- Unbalanced chromatid pre-division affected by advancing female age
- IVF indication did not influence aneuploidy rates

Pellestor et al., 2003
### Karyotyping results conclusions:

1. Determined how advancing female age affects whole chromosome non-disjunction & unbalanced chromatid pre-division
2. Smaller chromosome groups D-G malsegregated more frequently

- Poor oocyte metaphase morphology meant that specific chromosomes could not be identified

---

### Oocyte analysis via classical cytogenetic methods

- FISH probes targeted smaller chromosomes
- FISH provided results for ALL types of oocyte metaphase spreads
- FISH enabled assessment of polar bodies
- Limited number of chromosomes assessed

---

*Picture from Mahmood et al., 2000*
Oocyte analysis via fluorescent in situ hybridisation

- 124 women of average age 32.5 years
- Chromosomes 1, 9, 12, 13, 16, 18, 21, X assessed
- Chromosome gains assessed only
- Hyperploidy rate: 4%
- Chromosomes 13, 16, 18 and 21 malsegregated more frequently
- Chromosome non-disjunction, chromatid predivision, germinal/gonadal mosaicism identified

Oocyte analysis via FISH/ clinical results

- Verlinsky et al. assessed first & second PBs for PGS purposes
- Chromosomes 13, 16, 18, 21, 22 targeted
- Data from 7,103 first and second PB pairs:
  1. Chromatid errors more frequent than whole chromosome errors in older women (27.1% vs. 2.4%)
  2. MI and MII abnormality rates similar in older women (42% vs. 38%)
  3. Malsegregation affecting chromosome 16 taking place mostly in MII
  4. Malsegregation affecting chromosome 18 taking place mostly in MI
  5. MII correction of MI chromatid error for 33% of oocytes
**Oocyte analysis via comprehensive molecular cytogenetic methods**

- **Karyotyping and FISH technical issues**
  1. Artefactual loss of chromosomes due to slide spreading - inability to determine global oocyte aneuploidy rate
  2. Inability to examine entire chromosome complement
- **Combination of whole genome amplification & comparative genomic hybridisation overcame issues**

Wells et al., 1999; Wells and Delhanty, 2000

---

**Oocyte & 1st PB analysis via CGH**

- Fragouli et al. (2006) analysed 107 oocyte-PB complexes via CGH
- 46 women of average age 32.5 years
- Reciprocal chromosome & chromatid errors identified in oocyte-PB pairs
- Aneuploidy rate: 22%
- Malsegregation affected all chromosome groups
- Smaller chromosomes (13-22 & X) more frequently abnormal
- Larger chromosomes (1-12) affected by whole chromosome non-disjunction only
- Smaller chromosomes (13-22 & X) affected by whole chromosome non-disjunction & unbalanced chromatid pre-division
- Structural abnormalities identified

Fragouli et al., 2006
Oocyte analysis via CGH/ clinical results

- Fragouli et al. (2011) analysed 308 first & second PB pairs via CGH for PGS purposes
- 70 women of average age 41 years
- Total aneuploidy rate: 70%
- MI aneuploidy rate: 40% vs. MII aneuploidy rate: 50%
- Unbalanced chromatid pre-division more frequent than whole chromosome non-disjunction during MI (62% vs. 38%)
- Chromosome losses more frequent than chromosome gains (MI 68% losses vs. 32% gains; MII 60% losses vs. 40% gains)
- Malsegregation affected all chromosome groups
- Smaller chromosomes (13-22 & X) more frequently abnormal
- Advancing female age affected MII more

Is analysis of both PBs predictive of embryo’s chromosome complement?

- Christopikou et al. (2013) analysed 34 first & second PB pairs & corresponding cleavage stage embryos via aCGH
- Aim: determine predictive ability of PB1 & PB2 analysis for corresponding embryo’s chromosome status
- 30/34 cleavage stage embryos confirmed as aneuploid - 100% concordant with PB1 & PB2 results
- 12% PB copy number changes were not detected in corresponding embryos
- False positive copy number changes were more common in PB1
Why is female meiosis so error prone?

• Female meiosis stops & starts during foetal & adult life
• Strict regulation of oocyte nuclear & cytoplasmic maturation essential
• Advancing female age & aberrant genetic recombination affect accurate oocyte chromosome segregation
• Recombination patterns predisposing to aneuploidy:
  1. Chiasmata formation absence
  2. Chiasmata formation too close or too far from chromosome centromere

Fisher et al., 1995; Hassold et al., 1995; Nicolaides and Petersen, 1998; Sherman et al., 2005
Recombination rates in oocytes

- Ottolini et al., (2015) mapped 2,032 female & 1342 male crossovers to infer 529 chromosome pair segregations
- 23 sets of PB1, PB2 & corresponding oocytes/embryos & 29 embryos analysed by karyomapping
- > 4 million SNPs genotyped after sample WGA
- 39 instances of whole chromosome aneuploidy & 3 segmental errors identified
- Unbalanced chromatid pre-division as main MI aneuploidy causing mechanism
- New “reverse” segregation detected
- Normal oocytes/embryos with ~6x more recombination events than aneuploid
- Higher global recombination rates protect again chromosome malsegregation

Ottolini et al., 2015

Crossover maturation in oocytes

- Wang et al. (2017) examined male and female meiosis via computer modelling approach
- Oocyte & sperm crossover patterns simulation analysis
- Female recombination affected by crossover maturation inefficiency
- Phenomenon not observed for male recombination
- Phenomenon creates vulnerable chromosome configurations
- Phenomenon contributes significantly to oocyte aneuploidy
- Is aneuploidy an evolutionarily favoured trait?

Wang et al., 2017
Conclusions

- Chromosome abnormalities of female meiotic origin contribute significantly to reproductive failure
- Large numbers of oocytes/PBs examined with various cytogenetic methods
- Main mechanisms of female meiotic aneuploidy:
  1. Whole chromosome non-disjunction
  2. Unbalanced chromatid pre-division
  3. Germinal/gonadal mosaicism (?)
- All chromosomes affected by aneuploidy, but smaller groups (D-G) more frequently abnormal
- Advancing female age affects both meiotic divisions, but MII especially
- Crossover frequency & maturity influence meiotic chromosome segregation
- Is aneuploidy an evolutionarily favoured trait?
- Does the use of PB1 & PB2 provide accurate representation of embryo?

Reading list

Readings list


Hassold T, Hall H, Hunt P: The origin of human aneuploidy: where we have been, where we are going. Hum Mol Genet 16:R203–R208 (2007).


Reading list


Thank you for your attention

elpida@reprogenetics.co.uk
elpida.frugouli@obs-gyn.ox.ac.uk
Does polar body analysis accurately predict the aneuploidy status of the developing embryo?

Alan Handyside
The Bridge Centre, London, University of Kent,
Canterbury and Illumina, Cambridge, UK

Disclosure
I am a part time employee of, and have share options in, Illumina, San Diego, CA, USA, based in Cambridge, UK, which manufactures equipment and reagents for DNA sequencing, diagnostics and preimplantation genetic testing
Learning objectives

• Normal and abnormal patterns of chromosome segregation in female meiosis
• Basis of polar body analysis, advantages and disadvantages
• Principles of copy number analysis by array comparative genomic hybridisation (array CGH) or next generation sequencing (NGS)
• Principles of genome-wide single nucleotide polymorphism (SNP) and meiomapping for polar body analysis
• Accuracy of polar body analysis for maternal aneuploidies arising in female meiosis

Chromosome segregation in female meiosis

- Prophase I arrest: Fetal ovary
- Metaphase II arrest: Ovulation post puberty

Maiosis I (homologs segregate)
- Primary oocyte
- Bivalent
- Crossover
- Homologs

Maiosis II (sisters segregate)
- Mature oocyte
- Fertilization (ICSI)
- Zygote
- PB1
- PB2
Polar body testing for detection of female meiotic errors

**Advantages**
- Relatively non-invasive
- Early diagnostic results
- Direct detection of female meiotic errors
- Female meiotic errors 10x > paternal meiotic errors
- Highly likely to affect whole embryo
- No confusion with mitotic (mosaic) errors

**Disadvantages**
- Need to test first (PB1) and second (PB2) polar bodies
- Copy number analysis by array CGH less reliable
Single cell genomics

Single cell (or 3-10 cells)
~ pg DNA

Whole genome amplification (WGA)

>ug DNA

PGD
SNP genotyping arrays and karyomapping

PGS
BAC arrays for Array CGH
Low read depth NGS

NGS based solutions for combined PGD and PGS

24 chromosome copy number analysis by array comparative genomic hybridisation (array CGH)
THE TIMES

September 2, 2009

New IVF test—Array CGH

Produces baby Oliver, offering hope to infertile

- 13 previous failed IVF cycles
- 7/9 first polar bodies aneuploid

PGS for 24 chromosomes by array CGH of the first (PB1) and second polar bodies (PB2) in advanced maternal age

- 41 patients, 42 ICSI cycles
- Mean maternal age 40 years
- 226 oocytes/zygotes biopsied
- Array CGH of both polar bodies and the corresponding zygote analysed blind to confirm the diagnosis
- 55 (28%) euploid, 140 (72%) aneuploid
- All aneuploid in 19/42 cycles (42%)
- 8 clinical pregnancies, 1 livebirth, 7 ongoing
- 19% per cycle, 33% per ET

Segregation patterns of copy number gains and losses in the first and second polar bodies and corresponding zygotes (PB1/PB2/Zygote)

<table>
<thead>
<tr>
<th>Zygote</th>
<th>Origin</th>
<th>Pattern</th>
<th>Caused by</th>
<th>No with different patterns per chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td>1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19 20 21 22 XY Total %*</td>
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<td></td>
<td>NNN</td>
<td>MI</td>
<td>LGG</td>
<td>1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19 20 21 22 XY Total %*</td>
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Segregation patterns of copy number gains and losses in the first and second polar bodies and corresponding zygotes (PB1/PB2/Zygote)

Chromosome position (left to right: 1 to 22, X, Y)

Segregation patterns of copy number gains and losses in the first and second polar bodies and corresponding zygotes (PB1/PB2/Zygote)

<table>
<thead>
<tr>
<th>Zygote</th>
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</table>

Segregation patterns of copy number gains and losses in the first and second polar bodies and corresponding zygotes (PB1/PB2/Zygote)

Chromosome position (left to right: 1 to 22, X, Y)
Polar Body Biopsy With Follow Up at Cleavage Stages on Day 3

Christopikou et al. (2013) *Hum Reprod* 28, 1426
• 30/30 (100% concordant) embryos predicted to be aneuploid by array CGH of both polar bodies confirmed in day 3 embryos
• 69/73 (93%) of aneuploidies associated with copy number changes in polar bodies
• 68/69 (98.5%) of aneuploidies correctly predicted
• 19/20 reciprocal copy number changes in the first and second polar bodies resulted in normal copy number in the embryo
• 17 (12%) false positive copy number changes in polar bodies not associated with aneuploidy in the embryo
• Only 12/17 of these predicted aneuploidy

Retrospective study of 351 patients

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PB aCGH</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>240</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>Maternal age</td>
<td>38.4</td>
<td>39.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Live birth per embryo</td>
<td>14.9</td>
<td>26.4</td>
<td>0.015</td>
</tr>
<tr>
<td>Live birth per patient</td>
<td>22.7</td>
<td>35.7</td>
<td>0.031</td>
</tr>
</tbody>
</table>


24 chromosome copy number analysis by low cost, low read depth (0.1x) next generation sequencing (NGS) and mapped fragment counting
Increased ratio change as relationship between mapped fragment counts and copy number is linear.
Analysis of all three products of meiosis: both polar bodies and oocyte (oocyte-PB trios)

- Sequential polar body and oocyte biopsy
- Whole genome amplification (WGA)
- SNP genotyping
- Phasing of SNPs using a haploid reference
**Typical MeioMap Visualisation**

- **p ter**
- **Centromere**
- **q ter**

Both haplotypes present in PB1

- Segregation – homologous chromosomes
- Recombination – PB1/PB2
- Recombination – PB1/Oocyte

**Maternal haplotype 1**

**Maternal haplotype 2**

**Maternal haplotype 1 and 2**

**PB1**  **PB2**  **Oocyte**

---

**Genome-wide Meiomap**

- Mendelian segregation of homologs
- Both non-sister and sister recombination events identified
- Harlequin pattern – unique oocyte/embryo and chromosome specific fingerprint
Examples of typical non-canonical segregation patterns in the pericentromeric region

- Non-disjunction (NDJ) relatively rare
- Premature separation of sister chromatids (PSSC) common
- Reverse segregation also common (not detectable by copy number analysis)

= MII NDJ
= Other errors
Summary

- Most aneuploidies in the human embryo arise as chromosome segregation errors in female meiosis
- Accurate diagnosis of maternal meiotic errors in the fertilised oocyte requires analysis of both the first and second polar bodies
- Copy number analysis by array CGH can result in false positives
- NGS based copy number analysis allows accurate discrimination of chromosome and chromatid gains and losses
- Meiomapping of all three products of meiosis allows accurate analysis of the mechanism of chromosome segregation errors

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University of Sussex
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The Bridge Centre
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Dr Vivienne Hall
Dr Rebecca Gould
Dr Jinjun Wang,
Ms Trina Shah and all
the lab staff

Illumina
Prof Alan Thornhill
Dr Senthil Natesan
Mr Robert Blanshard

University of Kent
Prof Darren Griffin

Genesis Genetics
Dr Tony Gordon
Oocyte competence: The follicle environment hypothesis

Jeremy Thompson
The University of Adelaide

Conflicts of Interest Statement

The University of Adelaide receives consultation funds from Cook Medical LLC for use by Jeremy Thompson for research expenditure.
Learning objectives

• Role of gap junctions in follicle communication with oocyte and its importance to oocyte competence
• Role of cAMP/cGMP in determining oocyte competence
• Role of oocyte secreted factors in determining oocyte competence
• Role of metabolism
• Question: is there such a thing as post-ovulatory follicular cell signalling?

Communication in the follicle is everywhere

Communication across
- Stroma
- Basal lamina
- Mural granulosa cells
- Antral fluid
- Cumulus
- Oocyte
Cumulus – oocyte communication  
It’s a conversation…..and both sides benefit!

- Gap junctions are key to oocyte competence
- cAMP/cGMP – keeping the junctions open during IVM
- Oocyte Secreted Factors - new insights on structure and function
- COC metabolism
- Something unknown - Post-ovulatory communication?

Cumulus cell – oocyte communication
Gap junctions are key
Gap junction communication can be measured

Li et al. (2016) Hum Reprod. 31:810-821

Gap junctions allow molecules to accumulate from cumulus to oocyte

Li et al. (2016) Hum Reprod. 31:810-821
Gap junction communication can be blocked

Campen et al. (2016)
Mol Cell Endo 420:46-56

More than small molecules from the cumulus to oocyte?

Macauley et al. (2014) Biol Reprod. 91: 90

Macauley et al. (2016) Biol Reprod. 94: 16
Keeping the gap junctions open during IVM


Keeping the gap junctions open during IVM

Romero et al. 2016 Biol Reprod 95:64
Oocyte Secreted Factors
- their structure is important

Maturation system

- **In vivo**
- **In vitro**
- **In vitro + OSFs**

<table>
<thead>
<tr>
<th>System</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>75%</td>
</tr>
<tr>
<td>In vitro</td>
<td>35%</td>
</tr>
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</table>

**Figure:**

- BMP15
- Time after hCG (h)
- IVM culture (h)
- %

References:
- **Bovine:**
  - Hussein TS (2011) RFD
  - Dey SR (2012)
  - Sugimura S (2014) MHR
- **Murine:**
  - Sudiman J (2014) JARG
- **Caprine:**
  - Romaguera R (2010)
- **Porcine:**
  - Gomez MNL (2012)
GDF9 & BMP15 secreted as pro-mature protein

All TGFβ family proteins secreted as pro-mature proteins

Simpson et al. (2012)
Endocrinology 153:1301–10
Mature proteins increase granulosa cell proliferation

![Graph showing mature proteins increase granulosa cell proliferation](image)


Mature homodimers of GDF9 and/or BMP15 do not improve oocyte quality

![Diagram showing mature homodimers of GDF9 and/or BMP15 do not improve oocyte quality](image)

But pro-proteins improve oocyte quality

<table>
<thead>
<tr>
<th>Form</th>
<th>Cleavage (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>94</td>
</tr>
<tr>
<td>GDF9 mature</td>
<td>89</td>
<td>36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP15 mature</td>
<td>92</td>
<td>50&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP15 pro-mature</td>
<td>89</td>
<td>58&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Bovine: Sudiman et al. (2014)
PLoS One 9(7):e103563

Low dose Pro-GDF9 and Pro-BMP15 synergism

J Biol Chem 290:24007–20
Pro-cumulin – A new potent OSF heterodimer


Pro-cumulin is a potent stimulator of gilt oocyte developmental competence

Pro-cumulin effective in other species:
Embryo development in cow and human

Is cumulin a natural GDF9 & BMP15 heterodimer?

- **Genetic evidence:**

- **Physical interactions of proteins:**
  - Liao WX (2003); McIntosh CJ (2008)

- **Functional studies:**
  - McNatty KP (2005a, 2005b); Mottershead DG (2012); Wigglesworth K (2013); Peng J (2014); Reader K (2016)

**HOWEVER**

A natural GDF9 and BMP15 form of a stable heterodimer, such as “Cumulin”, has not been isolated from in vivo as yet
The metabolic environment via FLIM

Spatial Characterization of Bioenergetics and Metabolism of Primordial to Preovulatory Follicles in Whole Ex Vivo Murine Ovary

Rachel Cino, Michelle A. Dignon, Enrico Grattarola, and Ulrike Jüderr

Bound NADH = Oxidative metabolism
Free NADH = Glycolytic metabolism

Post-ovulatory cumulus-oocyte communication?

Optical coherence tomography of mouse COCs in ampulla

Kindly provided by Irina Larina, Baylor College of Medicine, Houston, USA
Conclusions

• Cumulus-oocyte communication is key to optimal oocyte competence
• Factors that enable prolonged cumulus-oocyte communication are:
  – Managing cGMP/cAMP levels during maturation – Pre-maturation is an additional step to achieve this
  – Oocyte secreted factors enhance communication - for IVM “Cumulin” is a new, powerful OSF
• Metabolic health of the COC is important
  – Also regulated by OSFs
• What communication occurs between cumulus-oocyte post ovulation?

Many thanks to:
Human cumulus cells molecular signature: Does it predict oocyte competence and embryo implantation potential?

Pr. Samir Hamamah

Chair: Reproductive Biology/PGD Department
Head: ART/PGD Division
Director: INSERM U 1203

ART/PGD Department
Arnaud de Villeneuve hospital
University-hospital of Montpellier
INSERM U 1203 ‘Early embryo developement and pluripontency’
Montpellier-34295, France

Conflict of Interest

I declare that I have no commercial or financial interests in relation to the subject of this presentation or its content.
Learning objectives

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

- The interest of cumulus cells (CCs)
- The knowledge on human CCs
- The micro-RNA expression in CCs
- The impact of female aging on gene expression in human CCs
- The CCs as biomarkers of oocyte competence and pregnancy outcome

Concept to emerge today

Oocyte-cumulus complex

- Oocyte is not passive in ovarian follicle
- Fundamental regulator of somatic cell differentiation and function
- Oocyte-CC plays a central role in the regulation of folliculogenesis

Ovarian follicular microenvironment and maternal signals, mediated through GCs and CCs, are responsible for the gradual acquisition of oocyte competence
Why cumulus cells?

- Coordinates follicle development with oocyte maturation
- Provides energy substrate for oocyte meiotic resumption
- Regulates oocyte transcription
- Promotes nuclear and molecular maturation of the oocyte

Assou et al. 2010, MHR
Oocyte and embryo within their niche

**Somatic microenvironment**

- **Cumulus cells**
- **MII Oocyte**
- **Day 3 Embryo**
- **Blastocyst**
- **Endometrial tissue**

Cumulus cells vs. endometrial tissue

Both common and specific molecular signature between the two niches

**WHAT ARE THE CUMULUS CELLS?**

Where? Surrounding oocyte cells

When? During oocyte maturation and first embryo cleavage

What role? Bi-directional communication

The human-cumulus oocyte complex gene expression profile

CC mirror of oocyte quality and competence

Assou et al. 2006 HR
Knowledge on human CCs

MicroRNAs: regulators of cellular functions

Background
MicroRNAs: biosynthesis and function

MicroRNAs are non-coding sequences which are approximately 19-25 nucleotides in length

Joshi et al., 2011, modified
MicroRNA expression in cumulus cells and oocyte

Important facts

- 3 miRNAs in MII oocytes
  - MIR184 (1988 reads)
  - MIR10A (555 reads)
  - LET7b (51 reads)
- 32 miRNAs in cumulus cells
  - MIR21 (28 reads)

**Most abundant:**
- MIR184
- MIR10A
- LET7b
Potential gene targets of the miRNAs identified by sequencing

Identified microRNAs: 32 in CCs and 3 in the oocyte

GeneGo MetaCore software
568 predicted miRNA target genes are retrieved
538 for the cumulus cells and 30 for the oocyte

Among these genes, how many are differentially expressed between cumulus cells and the oocyte?

Study design

Transcriptomic analysis

Bioinformatic data analysis

SAM software

RNA Extraction
Amplification & labelling
Hybridization on chip microarray

5962 genes up-regulated in the CCs
4207 genes up-regulated in the MII oocytes

Comparison with the 568 predicted miRNA target genes retrieved by GeneGo MetaCore software

224 differential genes that are potential targets of the identified miRNAs

Hierarchical Clustering
Important genes for chromosome or chromatin functions are upregulated in the MII oocyte and are predicted targets of CC-miRNAs. This suggests a dialogue between the cumulus cells and the oocyte through microRNA action.

Genes involved in a crosstalk between cumulus cells and oocyte are predicted targets of CC-miRNAs.

*GDF9* is known as a regulator of *PTGS2, CTGF, BMPR1B* and to be essential in oocyte-cumulus cells crosstalk.
Conclusions

- We have reported the first sequencing data of small non-coding RNAs in the human cumulus cells and oocyte.

- Our results illustrate the cellular specificity of the microRNAs. Some miRNAs are highly abundant in the oocyte and not even detected in the cumulus cells.

- CC-miRNA may be regulators of mRNAs over-expressed in the oocyte, illustrating the dialogue between cumulus cells and oocyte through miRNA action.

The impact of female aging on gene expression in human cumulus cells

- Molecular signature according to female age
- Pathways significantly affected by female aging
- Predicted miRNAs that target genes impacted by female age

Female aging alters expression of human cumulus cells genes that are essential for oocyte quality.

Al-Edani, et al, 2014
Impact of female aging on oocyte quality

Decrease of proteins stored in the oocyte
mtDNA damage, reduction of ATP production
Reduction of oocyte metabolic function
Increase of oxidative stress and apoptosis
Increase of oocyte aneuploidy

these modifications affect oocyte competence and quality

Bentov et al., 2011; Fragouli et al., 2010

The question is: which molecular changes account for these physiological changes?

Impact of aging on gene expression in the cumulus cells

Bentov et al., 2011; Fragouli et al., 2010

Molecular signature of cumulus cells according to female age

Identification of genes differentially expressed in the 3 age groups of cumulus cells

Hierarchical Clustering

20 genes of each class with highest expression

Blue: Up-regulated genes
Pink: Down-regulated genes

Molecular signatures reveal that CC >37 years are distantly located from the other groups

Al-Edani, et al, 2014
A molecular change occurs at age of 37 for essential biological processes

**Inflammatory genes**
- SERPINA1
- BAGALT1
- C15
- PIK3R1
- IGFBP5
- IGFBP3
- ANGPTL4
- TGFB
- LEPR

The 35/36 group behaves as the 31-34 group illustrating a molecular change at 37

Genes that play an essential role in the cumulus-oocyte dialogue and oocyte quality are down-regulated in older cumulus cells

Many genes of the TGF-b pathway are down-regulated (green), a few of them are up-regulated (red)
Conclusions

Transcriptomic data show that:

◆ aging widely impacts gene expression
◆ the decrease in fertility that occurs at 37 is supported by a dramatic molecular change after the age of 36
◆ the physiological impact of aging is underlied by an alteration of expression of genes and pathways that are critical for oocyte quality and competence (insulin,TGF-beta etc)
◆ Genes that are essential to buffer the effect of hypoxia (angiogenic genes), which is linked with aging, are up-regulated in older CCs

Genes impacted by aging in relation to CC-miRNAs

CC-miRNAs are validated regulators of the genes involved in pathways altered with aging
These miRNAs are potential biomarker candidates of follicle aging
Diagnostic tool in cIVF/ICSI

Cumulus cells predictive value

Cumulus cells (CC)

As biomarker of oocyte potential and pregnancy outcome

Oocyte

Diagnostic tool in cIVF/ICSI

Expression of several genes in cumulus cells

COX2: cyclooxygenase 2

indictive of oocyte and embryo quality

GREM1: gremlin 1,
HAS2: hyaluronic acid synthase 2,
STAR: steroidogenic acute regulatory protein,
SCD1, 5: stearoyl-co-enzyme A desaturase 1 and 5,
AREG: amphiregulin,
PTX3: pentraxin 3

positively correlated with embryo quality

GPX3: glutathione peroxidase 3,
CXCR4: chemokine receptor 4,
CCND2: cyclin D2,
CTNND1: catenin delta 1

inversely correlated with embryo quality

McKenzie et al. Hum Reprod 2004
Zhang et al. Fertil Steril 2005
Feuerstein et al., 2007
van Montfoort et al., 2008
During IVM
According to female age
Under in vitro or in vivo conditions
In PCOS patients

Gene expression profiling of human oocyte

<table>
<thead>
<tr>
<th>Technique</th>
<th>Samples</th>
<th>Number of identified genes</th>
<th>Targets</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope oil method</td>
<td>Oocytes</td>
<td>36 genes</td>
<td>Oocyte maturation</td>
<td>—</td>
</tr>
<tr>
<td>IVM (8 h, 20°C)</td>
<td>Oocytes</td>
<td>1,553</td>
<td>Understanding the factors regulating oocyte maturation</td>
<td>—</td>
</tr>
<tr>
<td>IVM (8 h, 37°C)</td>
<td>Oocytes</td>
<td>366</td>
<td>Compartmentalization of splicing during oogenesis</td>
<td>—</td>
</tr>
<tr>
<td>IVM (8 h, 20°C)</td>
<td>Oocytes</td>
<td>22</td>
<td>Development of germinal vesicle in oocytes</td>
<td>—</td>
</tr>
<tr>
<td>IVM (20°C)</td>
<td>Oocytes</td>
<td>82 genes</td>
<td>Study of gene expression during oocyte maturation</td>
<td>—</td>
</tr>
<tr>
<td>IVM (20°C)</td>
<td>Oocytes</td>
<td>22 genes</td>
<td>Development of germinal vesicle</td>
<td>—</td>
</tr>
</tbody>
</table>

Assou et al. 2010, HRU

Studies analysing the genomics of CC or granulosa cells to identify reliable biomarkers for oocyte quality and competence, and for embryo development predictors

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Name</th>
<th>Function</th>
<th>Sample (individual or pooled)</th>
<th>Approaches</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMP, ACE, C1S, PTGS, SC5D and SC85</td>
<td>Neutrophilic acute regulatory protein, enolase, angiotensin, coagulation factor</td>
<td></td>
<td>RT-PCR</td>
<td></td>
<td></td>
<td>Assou et al. 2010, HRU</td>
</tr>
<tr>
<td>HA2, PTG11, OX111</td>
<td>Hyaluronic acid synthase 2, proangiogenic and endoendothelial protein 2, gene 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BKNF, OX111</td>
<td>Brain-derived neurotrophic factor, gene 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKH, PCC, and RKG, CCK11, COX4I1, HCN1, WNK4, KCC4</td>
<td>Prostaglandin receptor 1, signal transduction, 2 and 3, cell division cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKC, PDC, and AKT, GRK1</td>
<td>Proteinase-activated receptor 3, actin cytoskeleton, cell adhesion, and gene 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Assou et al. 2010, HRU
A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study

S. Assou1,2,3, D. Haouzi1,2,3, K. Mahrous3, A. Annacheria1, Y. GUILLENIA1, Y. Pavis1,A
T. Récé1, H. Decaud1, J. De Vos1,2,3 and S. Hamann1,2,3

: non-invasive approach for competent embryo selection

Molecular Human Reproduction, Vol.16, No.9, pp. 531-538, 2010
Advance Access publication on April 29, 2010
doi:10.1093/molehr/gaq031

NEW RESEARCH HORIZON Review

Human cumulus cells as biomarkers for embryo and pregnancy outcomes

Said Assou1,2, Delphine Haouzi1,2, John De Vos1,2,3,
and Samir Hamann1,2,3,*

Genomic TEST
NON INVASIVE TEST FOR EMBRYO SELECTION
Cumulus cells biomarkers reflect oocyte and embryo developmental competence

- Identify potential biomarkers of oocyte competence and pregnancy outcome that are expressed in CC

G-TEST:
A NON INVASIVE TEST TO SELECT EMBRYO(S) WITH THE BEST POTENTIAL

Embryo scoring (1 to 100) rank embryo according its competence for transfer

What is the best embryo?

RT-qPCR

45 biomarkers

Algorithm prediction

45 biomarkers

Embryo Quality

Pregnancy

No pregnancy

Grade 1

Grade 3

PREDICTIVE MODEL

Sensitivity = 78%
Specificity = 95%

No relationship between gene expression of CCR7 and embryo morphological aspects
REAL TIME PCR TECHNOLOGY

Patient X

RNA extraction

RT-qPCR

ALGORITHM

EMBRYO SCORING

<table>
<thead>
<tr>
<th>Cumulus cells</th>
<th>Score</th>
<th>Error</th>
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</thead>
<tbody>
<tr>
<td>COC n° 5</td>
<td>90</td>
<td>0.18 %</td>
</tr>
<tr>
<td>COC n° 3</td>
<td>80</td>
<td>0.22 %</td>
</tr>
<tr>
<td>COC n° 1</td>
<td>60</td>
<td>0.19 %</td>
</tr>
<tr>
<td>COC n° 4</td>
<td>56</td>
<td>0.30 %</td>
</tr>
</tbody>
</table>

45 genes
Internal control

STATE OF THE ART

OOCYTE MATURATION → 13 STUDIES

EMBRYO DEVELOPMENT → 15 STUDIES

PREGNANCY → 15 STUDIES

LIVE BIRTH → 4 STUDIES
META-ANALYSIS

Comparaison of 29 studies

No common gene in microarray analysis
11 genes in common in RT-qPCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oocyte maturation</th>
<th>Embryo competence</th>
<th>Pregnancy</th>
<th>Live birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAN</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PTGS2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PTX3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ALCAM</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GREM1</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAS2</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>RGS2</td>
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<td>+</td>
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<tr>
<td>BMP15</td>
<td>+</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>GDF9</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STC2</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>SERPIN2</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reasons for this discrepancy between studies

**Technical aspects**

- Number of samples
- Type of DNA microarrays
- Fold change thresholds
- Statistical methodologies
- Variability inter-platform
- Variability inter-laboratory
- Microarrays vs. selected candidates

**Heterogeneous populations**

- Patient characteristics (age, BMI, diagnosis...)
- Multiple stimulation types
Conclusions

Omens provides us with the opportunity to analyse human oocytes and CCs expression profiles on a genome scale and permitted significant progress in the understanding of the molecular events involved in the processes governing oocyte maturation.

Many of the genes described here are biomarkers to monitor health, viability and competence of oocytes.

Analysis of CC surrounding the oocyte can be a non-invasive approach for oocyte, embryo selection and pregnancy outcome

G-test: is a novel concept, providing a new potential strategy for competent oocyte and embryo selection
Conclusions

From basic discoveries into clinical applications

To improve efficiency of IVF (higher pregnancy rates, lower cost per child born), by the establishment of SET and improved cIVF/ICSI results