Exogen markers
in human embryo culture

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EMBRYO ASSESSMENT FOR TRANSFER

Reducing the risk of multiple gestation

Maximizing the probability of pregnancy

The selection of embryos with higher implantation potential is one of the major challenges in assisted reproduction technology.
FETAL COMPLICATIONS OF MULTIPLE PREGNANCIES

Relative increase

Preterm delivery  Cerebral palsy  Infant death

Twin and higher order multiple gestations are associated with significantly increased risks of infant morbidity and mortality compared to singletons. ☐ Singleton; ☐ Twin; ☐ Higher order.

WHICH EMBRYO TO SELECT FOR SET?
Embryo selection is based on:
- Embryo morphology
- Rate of embryo development
An improvement over current embryo assessment strategies is needed.

Novel invasive (genomics: biopsy of blastomeres, transcriptomic analysis of blastomeres) and non-invasive (genomics: transcriptomic analysis of cumulus & granulosa cells; proteomics & metabolomics) approaches are investigated.

For clinical application, a technology should:
- Not damage the embryo
- Be easy
- Be rapid
- Be inexpensive
- Require a small sample
THE FUTURE FOR EMBRYO SELECTION

METABOLOMENE: the complete array of small molecule metabolites that are found in a biological system and reflects the functional phenotype (SECRETOME)

METABOLOMICS: studies the dynamic inventory of metabolites
TECHNOLOGY PLATFORMS FOR METABOLOMICS

- Capillary electrophoresis (CE)
- ELISA
- Mass spectrometry (MS)
- Nuclear magnetic resonance (NMR)
- Gas chromatography (GC)
- Liquid chromatography (LC)
- Raman spectroscopy
- Near-infrared spectroscopy
- Proton NMR
- Protein microarrays

Goodacre et al. Trends in Biotechnology, 2004
SECRETOME ANALYSIS TO ASSESS HUMAN OOCYTE AND EMBRYO QUALITY

<table>
<thead>
<tr>
<th>Target Molecule</th>
<th>Method of analysis</th>
<th>Embryonic stage tested</th>
<th>Clinical practicality</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>Ultramicrofluorescence</td>
<td>Day 1–5</td>
<td>High technicality, Less practical.</td>
<td>Contrasting results</td>
</tr>
<tr>
<td>Glucose</td>
<td>Ultramicrofluorescence</td>
<td>Oocytes, Day 1–5 embryos</td>
<td>High technicality, Less practical.</td>
<td>Contrasting results</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Microspectrophotometry</td>
<td>Oocytes, blastocysts</td>
<td>High technicality, Less practical.</td>
<td>Acquired oxygen consumption rates for oocytes and blastocysts</td>
</tr>
<tr>
<td></td>
<td>Respirometry</td>
<td>Oocytes</td>
<td>Expensive equipment.</td>
<td>Respiration rates correlated to maturation and viability of oocytes</td>
</tr>
<tr>
<td>Amino acids</td>
<td>Reverse-phase high performance liquid chromatography</td>
<td>Day 2–5 embryos</td>
<td>High technicality, Practicality still to be demonstrated</td>
<td>Predict blastocyst formation, pregnancy and live birth.</td>
</tr>
<tr>
<td></td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
<td>Day 3 embryos</td>
<td>High technicality, impractical.</td>
<td>Viability index correlated with pregnancy outcome.</td>
</tr>
<tr>
<td>HLA-G</td>
<td>Enzyme-linked immunoabsorbent assay</td>
<td>Follicular fluid, Day 0–5</td>
<td>High technicality, Less practical</td>
<td>Contrasting findings.</td>
</tr>
<tr>
<td>Leptin</td>
<td>Enzyme-linked immunoabsorbent assay</td>
<td>Day 5 embryos</td>
<td>High technicality, Less practical</td>
<td>Positive correlation between leptin secretion and blastocyst development</td>
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**Groups of molecules targeted**

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<td>Protein compliment</td>
<td>Surface-enhanced laser desorption ionization time-of-flight mass spectrometry</td>
<td>Day 5 embryos</td>
<td>High technicality, Less practical.</td>
<td>Protein profiles are related to blastocyst morphology.</td>
</tr>
<tr>
<td></td>
<td>Protein microarray</td>
<td>Day 5 embryos</td>
<td>High technicality, Less practical.</td>
<td>Expensive equipment.</td>
</tr>
<tr>
<td>Metabolomic compliment</td>
<td>Non-optical spectroscopy</td>
<td>Day 3 embryos</td>
<td>High technicality, impractical.</td>
<td>Implantation potential corresponds to specific protein secretion levels.</td>
</tr>
<tr>
<td></td>
<td>(Proton nuclear magnetic resonance) Oocytes, Day 3–5 embryos</td>
<td>High technicality, impractical.</td>
<td>Expensive equipment.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vibrational spectroscopy (Near infrared: Raman)</td>
<td>Simple, rapid procedure, inexpensive, practicality still to be demonstrated.</td>
<td>Oocyte viability score correlates to developmental potential. Embryo viability score predicts pregnancy independent of morphology.</td>
<td></td>
</tr>
</tbody>
</table>
METABOLIC PARAMETERS OF EMBRYOS (1)

GLYCOLITIC ACTIVITY

Pre-compaction: carboxylic acid metabolism

Pyruvate and lactate are the embryo’s main sources of energy

Transition from morula to blastocyst

Post-compaction: glucose metabolism
Pyruvate uptake, closed bars; glucose uptake, open bars.
Statistically significant differences: a = P<0.05; b, c, d = P<0.01

Gardner et al. Fert & Ster, 2001
## PYRUVATE AND GLUCOSE METABOLISM

**Botros et al. Mol Human Repr, 2008**

Inconclusive whether pyruvate and glucose uptake is predictive for embryo development and viability

**Limiting factor:** stress on embryos due to a lack of essential nutrients in the culture media

<table>
<thead>
<tr>
<th>Study</th>
<th>Embryo stage examined</th>
<th>Altered metabolite associated with improved outcome</th>
<th>Technology used</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardy et al. 1989</td>
<td>Day 2-4</td>
<td>↑ pyruvate uptake</td>
<td>Ultramicrofluorescence assay</td>
<td>Blastocyst development</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>↑ pyruvate uptake</td>
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<td></td>
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<td>↑ glucose uptake</td>
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<td></td>
<td></td>
<td>↑ glucose uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ lactate production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conaghan et al., 1993</td>
<td>Day 2 – 3</td>
<td>↓ pyruvate uptake</td>
<td>Ultramicrofluorescence assay</td>
<td>Clinical pregnancy</td>
</tr>
<tr>
<td>Turner et al., 1994</td>
<td>Day 2</td>
<td>Intermediate pyruvate uptake</td>
<td>Ultramicrofluorescence assay</td>
<td>Clinical pregnancy</td>
</tr>
<tr>
<td>Gardner et al., 2001</td>
<td>Day 4</td>
<td>↑ pyruvate uptake</td>
<td>Ultramicrofluorescence assay</td>
<td>Blastocyst development</td>
</tr>
<tr>
<td>Seli et al.</td>
<td>Day 2-3</td>
<td>↑ glucose uptake</td>
<td>Proton NMR</td>
<td>Pregnancy and delivery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ pyruvate uptake</td>
<td></td>
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</tr>
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</table>

*Botros et al. Mol Human Repr, 2008*
# Metabolic Parameters of Embryos (2)

## Amino Acid Turnover

### Table: Amino Acid Uptake and Secretion by the Embryo as a Predictor of Embryo Development Viability—Human Studies

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</thead>
</table>
| Houghton et al., 2002 | Day 2 – 3             | ↓ amino acid turnover (sum of depletion and appearance)
|                 |                       | ↓ glutamine, arginine, methionine uptake
|                 |                       | ↓ alanine and asparagine release
|                 | 8 cell–Morula         | ↓ amino acid turnover (sum of depletion and appearance)
|                 |                       | ↓ serine uptake
|                 |                       | ↓ alanine and glycine release
| Brison et al., 2004 | Day 2                 | ↓ glycine and leucine in culture media
|                 |                       | ↑ asparagine levels in culture media
| Seli et al., 2008  | Day 3                 | ↑ glutamate levels in culture media

*Botros et al. Mol Human Repr, 2008*

Embryos with greater viability have a lower or quieter amino acid metabolism than those that arrest
Compositional changes as function of development:

Embryos which develop beyond the 4-cell stage have significantly higher concentrations of the unsaturated acids, particularly linoleic acid. Vice versa for saturated acids.

They support developmental processes in the embryos:
- Regulate gene expression
- Cell growth and differentiation by protein kinase C
- Essential components of membrane lipids which increase with each cell division

The fatty acid composition of embryos which fail to develop is similar to that of oocytes which fail to fertilize

Haggarty et al., Hum Reprod, 2006
FATTY ACID METABOLISM

Fatty acid uptake in relation to the stage of development:

The uptake of both fatty acid was very low prior to the 8-cell stage and increased with the stage of development for linoleic in particular.

Increasing uptake of linoleic acid and decreasing uptake of the saturated fatty acid with stage of development.

Haggarty et al., Hum Reprod, 2006
METABOLIC PARAMETERS OF EMBRYOS (4)

OXYGEN

Invasive techniques

- Microspectrophotometry
- Scanning electrochemical microscopy
- Loop-mediated isothermal amplification

Non-invasive techniques

- NanoRespirometer (individual measurement of embryonic respiration rates at a fixed time)
- Embryo Respirometer (individual, continuous measurement of respiration rates + images of each embryo)

- Oxygen consumption is higher among embryos of superior morphological quality
- Positive correlation between oxygen consumption pregnancy rate
- Reduced respiration rates in oocyte cohorts with low or no fertilization

but not used for clinical applications (most on bovine embryos), not significant test

Lopes et al. Theriogenology, 2007
SINGLE PROTEIN ANALYSIS IN SECRETOME

PAF
- Autocrine trophic/survival factor
- Influences maternal physiology alterations (immune function and platelet activation)

Leptin
- During the window of implantation, leptin establishes a molecular dialogue with leptin receptors on the maternal side
- Competent human blastocysts secrete higher leptin concentrations than arrested embryos

ApoA1
Increased levels of ApoA1 are associated with blastocyst of higher morphological grade

Ubiquitin
It is increased in the secretome of developing blastocysts

sHLA-G
- It is believed to play a role in immunotolerance during pregnancy
- Its presence correlates with the improved embryo cleavage rate and pregnancy potential

HOXA10 regulator
Another protein involved in the reciprocal embryo-endometrial interaction. HOXA10 expression by epithelial endometrial cells is regulated by an unknown soluble molecule secreted by human blastocysts.
**PROTEIN SECRETOME AS POTENTIAL MARKERS OF EMBRYO ANEUPLOIDY**

*i.e., Lipocalin-1*
- Overproduced under conditions of stress, inflammation, infection
- Increased secretion of lipocalin-1 from aneuploid blastocysts
- LCN-1 inhibits cysteine proteinases
- Cysteine proteinases are important in embryo hatching and implantation

*Katz-Jaffe & McReynolds. Fertil Steril, 2013*
METABOLOMIX PROFILING

Raman and NIR spectroscopic analysis of functional groups:

-CH
-NH
-OH

Oxidative stress

Viability score

Seli et al. Fertility and Sterility, 2007
Morphology grading and metabolomic Viability Score on predicting implantation outcome

- retrospective studies -

They failed to support the concept that NIR techniques can improve implantation rate in SET (day 2, 3 and 5)
CHALLENGES OF USING METABOLOMICS IN A CLINICAL SETTING

Limited template, low protein expression and lack of sensitivity of current proteomics platform

The overwhelming presence of albumin and other serum proteins in the culture media makes it difficult to identify the low expressed secreted embryonic proteins

Proteomic technologies require installation and implementation of a system into a lab that does not specialize in the technologies

Sources of variability, i.e., experimental design, data interpretation, lack of standardized sample collection and storage

To date there is no non-invasive platform that has been proven to be of true clinical predictive value or been examined in prospective randomized control trials to be better than current morphology-based selections methods
Thank you!