Cytokines and IVF Embryo Culture –
The GM-CSF Experience

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first days of life are critical
peri-conceptual determinants of implantation and fetal growth

- genetics and epigenetics
- nutrition and obesity
- smoking & lifestyle factors
- environmental stressors

CYTOKINES
establishing pregnancy… a partnership
establishing pregnancy… a partnership

- Both the embryo and the endometrium must be healthy and adequately prepared
- Disruption $\rightarrow$ infertility, miscarriage, IUGR, preeclampsia
epithelial cells → embryo communication
epithelial cells → leukocyte communication

cytokines

UEA1
two-way communication \(\rightarrow\) optimal outcome

EMBRYO DEVELOPMENT

cytokines

IMMUNE RESPONSE
peri-conceptual cytokines are a key factor in pregnancy success

infertility
miscarriage
IUGR
preeclampsia

healthy pregnancy
cytokine regulation of pre-implantation embryos

GM-CSF
LIF
PAF
GH
IGF-I
IGF-II
EGF
TGFα
TGFβ
TNFα
IFNγ

autocrine
paracrine
many (but not all) cytokines are autocrine
effects of cytokines on pre-implantation embryos

<table>
<thead>
<tr>
<th>Effect</th>
<th>Mo Hu</th>
</tr>
</thead>
<tbody>
<tr>
<td>% zygote development to blastocyst</td>
<td>?</td>
</tr>
<tr>
<td>Speed of development to blastocyst</td>
<td>✅</td>
</tr>
<tr>
<td>Cell number &amp; allocation to ICM and TE</td>
<td>✅</td>
</tr>
<tr>
<td>Cell viability and apoptosis</td>
<td>✅</td>
</tr>
<tr>
<td>Gene expression profile</td>
<td>✅</td>
</tr>
<tr>
<td>Metabolism</td>
<td>✅</td>
</tr>
<tr>
<td>Stress response</td>
<td>✅</td>
</tr>
<tr>
<td>Implantation &amp; developmental competence</td>
<td>✅</td>
</tr>
<tr>
<td>Developmental programming in fetus</td>
<td>?</td>
</tr>
</tbody>
</table>
GM-CSF – a pivotal cytokine in early pregnancy
Pathway from laboratory to clinic

- Investigate fundamental reproductive biology
- Devise rational, evidence-based clinical intervention
- Rigorously evaluate and prove safety and efficacy
GM-CSF (CSF2) = granulocyte-macrophage colony-stimulating factor

- 23 kD glycoprotein secreted / ECM-associated
- binds GM-CSF Rα / βc to signal via JAK/STAT & MAPK
- monocyte/macrophages, dendritic cells, granulocytes
- proliferation of progenitors, cell survival, differentiation
- endothelial cells, trophoblasts
GM-CSF expression in mouse uterus

(Robertson et al. 1992, 1995)
Expression of GM-CSF receptors in embryos

(Robertson et al. Biol Reprod 2001)
Effect of GM-CSF in culture medium on blastocyst and post-blastocyst development

<table>
<thead>
<tr>
<th></th>
<th>Control (N)</th>
<th>Control %</th>
<th>+ rGM-CSF (N)</th>
<th>+ rGM-CSF %</th>
</tr>
</thead>
<tbody>
<tr>
<td>% 8-cell → Bφ</td>
<td>(696)</td>
<td>92</td>
<td>(538)</td>
<td>89</td>
</tr>
<tr>
<td>% Bφ Hatch</td>
<td>(330)</td>
<td>78</td>
<td>(294)</td>
<td>82</td>
</tr>
<tr>
<td>% Bφ Attach</td>
<td>(492)</td>
<td>79</td>
<td>(267)</td>
<td>92*</td>
</tr>
</tbody>
</table>

*p < 0.001

(Robertson et al. Biol Reprod 2001)
Effect of GM-CSF on cell number in blastocysts

- **Total**
- **ICM**
- **TE**

(63) vs. (75)contro/(23) vs. (37)GM-CSF

* (*Robertson et al. *Biol Reprod* 2001)
GM-CSF deficiency & apoptosis in blastocysts

No GM-CSF + GM-CSF
GM-CSF null mutant mice

(Stanley et al. 1994)
Summary: effects of GM-CSF deficiency

- litter sizes are 25% smaller at weaning due to late gestation and early postnatal loss
- miscarriage is increased 2-fold
- fetal malformation is increased 2-fold
- IUGR is increased 9-fold
- males are more adversely affected
- placental structure is altered

(Robertson et al. Biol Reprod 1999)
Microarray to analyse GM-CSF regulation of blastocyst gene expression

Affymetrix microarray

→ candidate gene families / genes

A. in vitro control

B. in vitro + 2 ng/ml GM-CSF
## Microarray Results: Pathway Express

<table>
<thead>
<tr>
<th>KEGG Pathway name</th>
<th>Impact</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal adhesion</td>
<td>35.6</td>
<td>0.04</td>
</tr>
<tr>
<td>MAPK signalling pathway</td>
<td>31.8</td>
<td>0.14</td>
</tr>
<tr>
<td>Adherens junction</td>
<td>17.7</td>
<td>0.006</td>
</tr>
<tr>
<td>Tight junction</td>
<td>15.3</td>
<td>0.25</td>
</tr>
<tr>
<td>Calcium signalling pathway</td>
<td>12.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Wnt signalling pathway</td>
<td>12.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>9.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Toll-like receptor signalling pathway</td>
<td>5.6</td>
<td>0.28</td>
</tr>
<tr>
<td>Phosphatidylinositol signalling</td>
<td>5.7</td>
<td>0.08</td>
</tr>
<tr>
<td>Notch signalling pathway</td>
<td>4.9</td>
<td>0.08</td>
</tr>
</tbody>
</table>

(Chin et al *Human Reproduction* 2009)
## Microarray Results: GM-CSF-regulated genes

### Apoptosis and cell survival:
- **Cbl** casitas B-lineage lymphoma: -20.8
- **Ccar1** cell division cycle & apoptosis regulator 1: -4.13
- **Gas 5** growth arrest specific 5: -3.92
- **Pik3c2a** phosphatidylinositol 3-kinase, alpha: -2.77

### Heat shock proteins:
- **Hspa5** heat shock 70kD protein 5: -6.37
- **Hsp105** heat shock protein 105: -3.26
- **Hspa4** heat shock protein 4: -2.60

### Stress response genes:
- **Hif1a** hypoxia inducible factor 1, alpha: -2.76

(Chin et al *Human Reproduction* 2009)
qRT-PCR analysis of stress response genes

Mann-Whitney, * P < 0.05, **P < 0.005, #P = 0.091
Effect of GM-CSF on HSPA1A/1B in blastocysts

GM-CSF control n=28
rmGM-CSF n=27

independent t-test, * P ≤ 0.03
(Chin et al Human Reproduction 2009)
Effect of GM-CSF on Bcl2 protein in blastocysts

GM-CSF

control

irrelevant 1° Ab

Total Mean Fluorescence + SEM

control n=14
rmGM-CSF n=18

independent t-test, * P ≤ 0.05
(Chin et al Human Reproduction 2009)
Gene pathways influenced by GM-CSF

GM-CSF signalling → autocrine GF signalling

↑ embryo integrity
↓ CELL STRESS
↓ APOPTOSIS

↑ developmental competence
How important is early embryo exposure to GM-CSF in later fetal development? Could GM-CSF act to ‘program’ the embryo for late fetal and post-natal health?
Effect of embryo exposure to GM-CSF on later fetal and placental development

1. **A. In vitro:** control medium (n=415)
2. **B. In vitro:** + GM-CSF (n=483)
3. **C. In vivo** (n=383)

1. Embryo transfer
2. Embryo transfer
3. Late gestation outcome E18

(Sjöblom et al. *Endocrinology* 2005)
Effect of GM-CSF on fetal and placental weights

(Sjöblom et al. *Endocrinology* 2005)

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>control</th>
<th>+ GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>280</td>
<td>317</td>
<td>316</td>
</tr>
<tr>
<td># pregnant</td>
<td>29/29</td>
<td>29/32</td>
<td>29/32</td>
</tr>
<tr>
<td>fetal weight</td>
<td>1291 ± 13</td>
<td>1160 ± 10†</td>
<td>1206 ± 9*</td>
</tr>
<tr>
<td>placental weight</td>
<td>123 ± 2</td>
<td>123 ± 2</td>
<td>124 ± 2</td>
</tr>
<tr>
<td>fetal:placental ratio</td>
<td>10.9 ± 0.2</td>
<td>9.7 ± 0.1†</td>
<td>10.0 ± 0.1*</td>
</tr>
</tbody>
</table>

† *p < 0.05 vs. in vivo group  * *p < 0.05 vs. medium only group

Data are mean ± SEM
Effect of GM-CSF on placental structure at E18

(Sjöblom et al. Endocrinology 2005)

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>control</th>
<th>+GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>junctional zone</td>
<td>49 ± 0.2</td>
<td>52 ± 1.4†</td>
<td>48 ± 0.6*</td>
</tr>
<tr>
<td>labyrinth</td>
<td>51 ± 0.2</td>
<td>48 ± 1.4†</td>
<td>52 ± 0.6*</td>
</tr>
<tr>
<td>La : Jz</td>
<td>1.04</td>
<td>0.95† (↓27%)</td>
<td>1.07 *</td>
</tr>
</tbody>
</table>

† p <0.05 vs. in vivo group, * p <0.05 vs. GM-CSF group
Effect of GM-CSF on placental exchange function

(Sjöblom et al. Endocrinology 2005)
GM-CSF and the immune response to pregnancy

Immune quality control of implantation
Effect of GM-CSF null mutation on antigen presentation and T cell activation

(Moldenhauer et al. *J Immunology* 2010)
(Robertson et al. *J Reprod Immunol* 1994)
Immune balance and implantation success

Th1 / Th17

Treg / Th2

Rejection  Tolerance
Conclusion

Peri-conceptual GM-CSF assists implantation success and pregnancy outcome through:

1. Promoting robust embryo development, reducing cellular stress and inhibiting apoptosis

2. Programming developmental trajectory, resulting in optimal placental development and function

3. Stimulating immune system to promote quality control to ensure only healthy embryos implant
GM-CSF expression in human uterus and oviduct

- GM-CSF is expressed in epithelial cells of oviduct - maximal in early secretory phase
  (Zhao and Chegini, JCEM 1994)

- GM-CSF is expressed in epithelial cells of uterus - maximal in mid-secretory phase
  (Giacomini et al., Hum Reprod 1995; Chegini et al., MHR 1999)

- GM-CSF is abundant in uterine luminal fluid
  (Paiva et al., Hum Reprod 2011)
GM-CSF (pg / 10^5 cells / 24 hr)

- GM-CSF is regulated by E and P & induced by seminal plasma and sperm (Sharkey et al., MHR 2007; Sharkey & Robertson, unpub)
- GM-CSF is induced by TLR ligands and suppressed by IFN (Sharkey & Robertson, unpub)
- GM-CSF is induced by hCG (Paiva et al., Hum Reprod 2011)
GM-CSF and reproductive dysfunction in women

• Serum GM-CSF in pregnancy is reduced in women with recurrent miscarriage

GM-CSF and reproductive dysfunction in women

- GM-CSF synthesis by endometrial epithelial cells is associated with IVF success (Spandorfer et al., Am J Reprod Immunol 2008)

- Follicular fluid GM-CSF is reduced in women experiencing unexplained infertility (Calogero et al., Cytokine 1998)

- Trend to reduced endometrial GM-CSF mRNA expression in cohort of women with recurrent miscarriage (Jasper et al., J Reprod Immunol 2007)
GM-CSF in human IVF?

Fertilitetcentrum, Gothenburg
Effect of GM-CSF on human embryo development

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>blastocyst</td>
<td>31%</td>
<td>76% *</td>
</tr>
<tr>
<td>hatch</td>
<td>47%</td>
<td>78% *</td>
</tr>
<tr>
<td>attach</td>
<td>0%</td>
<td>43% *</td>
</tr>
</tbody>
</table>

*P< 0.01

(Sjöblom et al. Hum Reprod 2000)
Effect of GM-CSF is not dependent on culture system

<table>
<thead>
<tr>
<th>Culture system</th>
<th>n</th>
<th>% blast</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scandinavian IVF Science</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVF-50 / S2</td>
<td>con</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 (37%)</td>
</tr>
<tr>
<td></td>
<td>+GM-CSF</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 (79%)(*3)</td>
</tr>
<tr>
<td>G1.2 / G2.2</td>
<td>con</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 (30%)</td>
</tr>
<tr>
<td></td>
<td>+GM-CSF</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 (71%)(*2)</td>
</tr>
<tr>
<td><strong>Cook IVF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sydney IVF cleavage / blastocyst medium</td>
<td>con</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29 (36%)</td>
</tr>
<tr>
<td></td>
<td>+GM-CSF</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58 (71%)(*3)</td>
</tr>
</tbody>
</table>

**p < 0.01, *** p< 0.005

(Sjöblom et al. *Hum Reprod* 1999)
(Sjöblom et al. *Biol Reprod* 2002)
Effect of GM-CSF on human embryo quality

(Sjöblom et al. Hum Reprod 2000)
**Effect of GM-CSF on apoptosis in human blastocysts**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>total apoptosis</td>
<td>4.9%</td>
<td>2.1%**</td>
</tr>
<tr>
<td>ICM apoptosis</td>
<td>6.3%</td>
<td>1.5%**</td>
</tr>
<tr>
<td>TE apoptosis</td>
<td>4.2%</td>
<td>2.6%*</td>
</tr>
</tbody>
</table>

**p < 0.01, * p < 0.05**

(Sjöblom et al. *Hum Reprod* 2000)
Summary: effects of culture with GM-CSF on human embryo development

- embryos express GM-CSF receptors
- GM-CSF doubles number of embryos reaching blastocyst stage, and increases hatching and attachment *in vitro*
- Effect is not dependent on culture media system
- GM-CSF accelerates blastocyst development by 14h
- GM-CSF increases cell number by 35%
- GM-CSF reduces apoptosis by 50%
Translation of GM-CSF to the IVF clinic

- GM-CSF is a necessary component of an ‘optimal’ environment for pre-implantation embryos
- Human trials using GM-CSF addition to IVF embryo culture media were warranted
- In 2005, we formed a commercial partnership with ORIGIO a/s (Denmark) to evaluate efficacy of GM-CSF in human IVF
GM-CSF does not adversely affect embryo karyotype

<table>
<thead>
<tr>
<th></th>
<th>medium</th>
<th>+GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>number embryos</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>number FISH</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>overall normal</td>
<td>50%</td>
<td>67%</td>
</tr>
<tr>
<td>uniformly normal</td>
<td>28%</td>
<td>33%</td>
</tr>
</tbody>
</table>

All chromosomes in all cells in all embryos assessed

Agerholm, Ziebe et al. *Reprod Biomed Online* 2010
Clinical trial to evaluate GM-CSF in human IVF

- Multicentre, placebo-controlled, randomised, double-blinded trial to evaluate effect of GM-CSF on IVF outcomes completed with ORIGIO a/s and Soren Ziebe (University Hospital of Copenhagen)
- 1332 IVF patients, 14 IVF clinics in Denmark and Sweden
- day 3 transfers, 1-2 embryos transferred
- 2 ng/ml GM-CSF in fertilisation, culture and transfer medium
- primary endpoint = ongoing implantation rate at week 7
A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization

Inge Agerholm, Ph.D., Michael Aasted, M.D., Anette Gabrielsen, M.Sc.,
Dorit P. Zobel, Ph.D., Bibi Munding, M.Sc., Susanne H. Bendz, Ph.D., and Sarah A. Robertson, Ph.D.

a Fertility Clinic, Rigshospitalet, University Hospital of Copenhagen, Copenhagen, Denmark; b Fertility Clinic, Skive Regional Hospital, Skive, Denmark; c Fertility Clinic, Odense University Hospital, Odense, Denmark; d Fertility Clinic, Braedstrup Hospital, Braedstrup, Denmark; e Fertility Clinic Dronninglund, Aalborg University Hospital, Dronninglund, Denmark; f Ciconia Aarhus Private Hospital, Aarhus, Denmark; g Fertility Clinic, Herlev University Hospital, Copenhagen, Denmark; h ORIGIO, Malmö, Denmark; and i Robinson Institute, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, South Australia, Australia
Randomized $n = 1332$

Initiated the study $n = 1322$

GM-CSF $n = 651$
- Withdrawn consent $n = 2$
  - Analysis of embryos $n = 649$
    - No embryo transfer $n = 83$
    - Analysis of implantation $n = 566$

Control $n = 671$
- Withdrawn consent $n = 1$
  - Analysis of embryos $n = 670$
    - Analysis of implantation $n = 585$
    - No embryo transfer $n = 85$
Effect of GM-CSF on implantation rate (all women)

OIR = ongoing implantation rate
(viable embryos / embryos transferred)

<table>
<thead>
<tr>
<th></th>
<th>OIR wk 7</th>
<th>OIR wk 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.9</td>
<td>18.7</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>23.4</td>
<td>22.8</td>
</tr>
</tbody>
</table>

↑17.6% p=0.10

↑21.9% p=0.02
Effect of GM-CSF on implantation rate (women with previous miscarriage)

OIR = ongoing implantation rate
(viable embryos / embryos transferred)

OIR wk 7:
- Control: 17.0
- GM-CSF: 24.5
- Increase: 44.1%
- p = 0.001

OIR wk 12:
- Control: 16.5
- GM-CSF: 23.2
- Increase: 40.6%
- p = 0.003
Effect of GM-CSF on perinatal endpoints

- 18.5% increase in children born (p=0.042)
- no effect on gestational age at delivery, perinatal death
- no effect on fetal abnormality
- no effect on rate of multiple pregnancies
- no effect on fetal weight
## Effect of GM-CSF on pregnancy progression

<table>
<thead>
<tr>
<th></th>
<th>GM-CSF</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of women with transfer</td>
<td>564</td>
<td>585</td>
<td></td>
</tr>
<tr>
<td>Positive hCG (N, % cycles)</td>
<td>214 (37.9)</td>
<td>218 (37.3)</td>
<td>0.46</td>
</tr>
<tr>
<td>Early pregnancy loss ≤12wk</td>
<td>49 (22.9)</td>
<td>73 (33.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>(N, % positive hCG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biochemical pregnancy</td>
<td>29 (13.6)</td>
<td>44 (20.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>Ectopic pregnancy</td>
<td>4 (1.9)</td>
<td>2 (0.9)</td>
<td>0.45</td>
</tr>
<tr>
<td>Miscarriage (wk 7→12)</td>
<td>16 (7.5)</td>
<td>27 (12.4)</td>
<td>0.11</td>
</tr>
<tr>
<td>Live birth</td>
<td>163</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Children born</td>
<td>194</td>
<td>164</td>
<td></td>
</tr>
</tbody>
</table>
GM-CSF is present in human reproductive tract and may be dysregulated in fertility disorders.

GM-CSF is essential for embryo protection from stress and optimal development.

GM-CSF promotes implantation success and developmental competence in embryos.

Providing GM-CSF to embryos supports robust placental development and fetal health.

**Take-home message**
Embryogen: Product Launch at ESHRE 2011

• New treatment option for women with previous miscarriage (IVF or natural conception)
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeremy Thompson</td>
<td>Robinson Institute</td>
<td>AUSTRALIA</td>
</tr>
<tr>
<td>Claire Roberts</td>
<td>University of Adelaide</td>
<td></td>
</tr>
<tr>
<td>Michelle Lane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anne Macpherson</td>
<td></td>
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<tr>
<td>Loretta Chin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecilia Sjoblom</td>
<td>Fertilitetscentrum, Goteborg</td>
<td>SWEDEN</td>
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<tr>
<td>Mats Wikland</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soren Ziebe</td>
<td>University Hospital of Copenhagen</td>
<td></td>
</tr>
<tr>
<td>Sussi Bendz</td>
<td>ORIGIO a/s, Måløv, DENMARK</td>
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</tbody>
</table>

**National Health and Medical Research Council**