Effects of low methyl donor levels during mouse follicle culture on follicle development, oocyte maturation and oocyte imprinting establishment.

Ellen Anckaert, Sergio Romero, Tom Adriaenssens, Johan Smitz

Follicle Biology Laboratory

UZ Brussel, Brussels
Genomic imprinting

- Phenomenon causing parent-of-origin specific monoallelic expression of genes

- Important role in: embryo development and growth, placental differentiation, behaviour, tumor growth and human genetic syndromes

- Gene imprinting: regulatory DNA sequences are differentially methylated during gametogenesis (DMR); and this differential DNA methylation should be maintained after fertilization
Genome imprinting and ART

- Studies in relatively small cohorts suggest that human ART could be associated with rare imprinting-related disorders
- In vitro embryo culture is associated with aberrant imprinting in different animal species
- A few studies suggest aberrant imprinting in oocytes after IVM and superovulation
Genome imprinting and ART

- The underlying mechanism of aberrant imprinting after ART is not known.

- **Human** imprinting disorders after ART
  - are more frequently associated with a hypomethylation of the maternal allele than sporadic cases
  - the underlying infertility of the couple may play a role

- In vitro embryo culture in **animal species**
  - is associated with both hyper- and hypo-methylation of DNA at imprinted genes
  - responsible factor is unknown
Genome imprinting and ART

- To identify a possible association between ART and imprinting disorders in children born after ART very large studies are necessary.

- Therefore, in vitro studies in animal models are necessary to study the association between ART and aberrant imprinting.
In-vitro follicle culture system in mouse

C57BL/6J x CBA/Ca; α-MEM supplemented with 5% HIA FBS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium and 10 IU/l r-FSH
During oogenesis: methylation is acquired asynchronously in a gene-specific manner

Snrpn = small nuclear ribonucleoprotein N; Peg = paternally expressed gene; IAP = non-imprinted intracisternal A particle (repetitive retroviral-like sequence)
In MII oocytes obtained after prolonged in-vitro follicle culture, we found the expected DNA methylation patterns at DMRs of key imprinted genes H19, Snrpn, Igf2r and Peg3.

Supraphysiological doses of r-FSH during did not alter imprinting establishment at H19, Snrpn, Igf2r.

*Anckaert, Int J dev Biol 2009*
In MII oocytes obtained after prolonged in-vitro follicle culture, high levels of ammonium and mineral oil overlay did not alter imprinting establishment at H19, Snrpn, Igf2r.

Anckaert, Biol Reprod 2009
The methionine cycle plays an important role in DNA methylation processes. Methionine is actively transported into oocytes, and converted into S-adenosylmethionine (SAM), the methyl donor for DNA methylation reactions (Menezo 1989).

Vitamin B12, folic acid, choline and vitamin B6 may also influence DNA methylation levels through their involvement in the methionine cycle.
Background of the study (2)

- In mouse, supraphysiologic maternal dietary methyl group supplementation (before and during pregnancy) induced a DNA hypermethylation at the viable yellow agouti and at the axin fused metastabile epialleles in the offspring (Waterland R and Jirtle 2003; Waterland RA, Genesis 2006).

- Clinically relevant reductions in dietary inputs to the methionine/folate cycles during periconception in mouse can lead to widespread alterations in DNA methylation and a modified phenotype in offspring (Sinclair, PNAS 2007).

- Loss of imprinting at the Igf2-H19 locus (and global loss of DNA methylation) in human adults with hyperhomocysteinaemia can be ameliorated by oral folate therapy (Ingrosso, Lancet 2003)
Aim of the study

- To study the influence of reduced methyl donor levels in culture medium on follicle development, oocyte maturation capacity and oocyte imprinting establishment.
Materials and methods

- α-MEM without methionine, vitamin B12, folic acid, choline chloride and vitamin B6 was used.

- Add back experiments with these 5 components were performed (n=713 follicles) to determine the influence of methyl donor levels on
  - follicle survival
  - follicle development
  - MII oocyte rate

- The methylation status of DMRs of 4 key imprinted genes was studied in oocytes cultured under low methyl donor levels
  - 2 independent cultures (involving 4 mice per culture) were performed
  - approximately 100 MII and 100 GVBD-oocytes per culture were pooled
  - bisulphite sequencing was performed on the four oocyte pools for the analysis of DMRs of Snrpn, Igf2r, Peg1 and H19.
Results

α-MEM with vitamin B12, folic acid, choline chloride, pyridoxal + 5%FBS

α-MEM w/o vitamin B12, folic acid, choline chloride, pyridoxal + 5%FBS

# p<0.01; $ p<0.05; * p<0.001
Oocytes grown and matured during in vitro follicle culture, collected 18h post hCG/EGF: (a) in control conditions; (b-i) under low methyl donor levels
DNA methylation of *Igf2r* DMR2 (A) in MII oocytes after in vitro follicle culture in control conditions; and (B) in MII and (C) in GVBD oocytes after in vitro follicle culture under reduced methyl donor levels.
DNA methylation of *Snrpn* DMR1 (A) in MII oocytes after in vitro follicle culture in control conditions; and (B) in MII and (C) in GVBD oocytes after in vitro follicle culture under reduced methyl donor levels.
DNA methylation of *Mest (Peg1)* Promotor & exon 1 (A) in MII oocytes after in vitro follicle culture in control conditions; and (B) in MII and (C) in GVBD oocytes after in vitro follicle culture under reduced methyl donor levels.
DNA methylation of *H19* (DMR containing the CTCF 1-2 region) (A) in MII oocytes after in vitro follicle culture in control conditions; and (B) in MII and (C) in GVBD oocytes after in vitro follicle culture under reduced methyl donor levels.
Methyl donor levels in culture media

<table>
<thead>
<tr>
<th></th>
<th>Exp α-MEM + 5% FBS¹</th>
<th>Control α-MEM ²</th>
<th>Culture media³</th>
<th>Rodent plasma/serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>7 µM</td>
<td>100 µM</td>
<td>0-200 µM</td>
<td>48-75 µM (mouse)</td>
</tr>
<tr>
<td>Vit B12</td>
<td>207 pM</td>
<td>1 µM</td>
<td>0-1 µM</td>
<td>812 pM (rat)</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1.4 nM</td>
<td>2.3 µM</td>
<td>0-6 µM</td>
<td>240 nM (mouse)</td>
</tr>
<tr>
<td>Vit B6</td>
<td>19 nM</td>
<td>4.9 µM</td>
<td>0-12 µM</td>
<td>700 nM</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1 mg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Measured; ² Manufacturer data; ³ Commercially available embryo culture media (Steele, RBM online 2006)
Conclusion

In the current culture set-up, low concentrations of methyl donors during follicle culture led to:
- a decrease in follicle development up to the antral stage
- a dramatic decrease in MII oocyte rate
- without however inducing aberrant imprinting establishment at the studied regulatory sequences in MII or in GVBD-arrested oocytes (preliminary).