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

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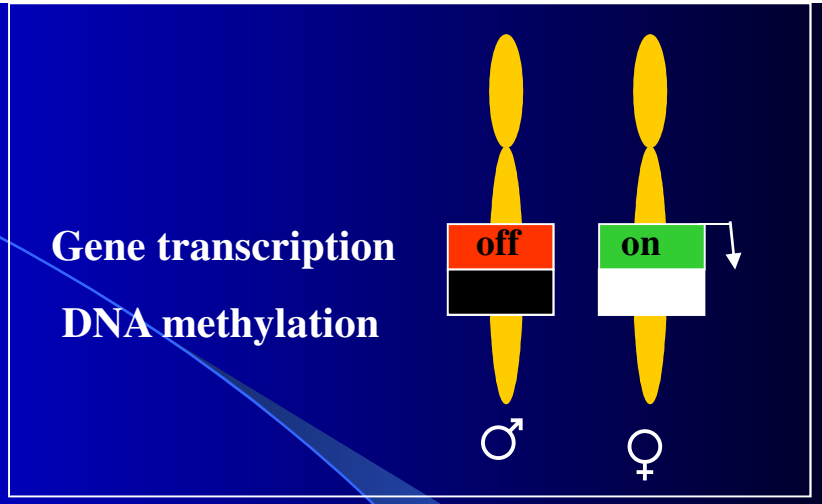
Genomic imprinting



- 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

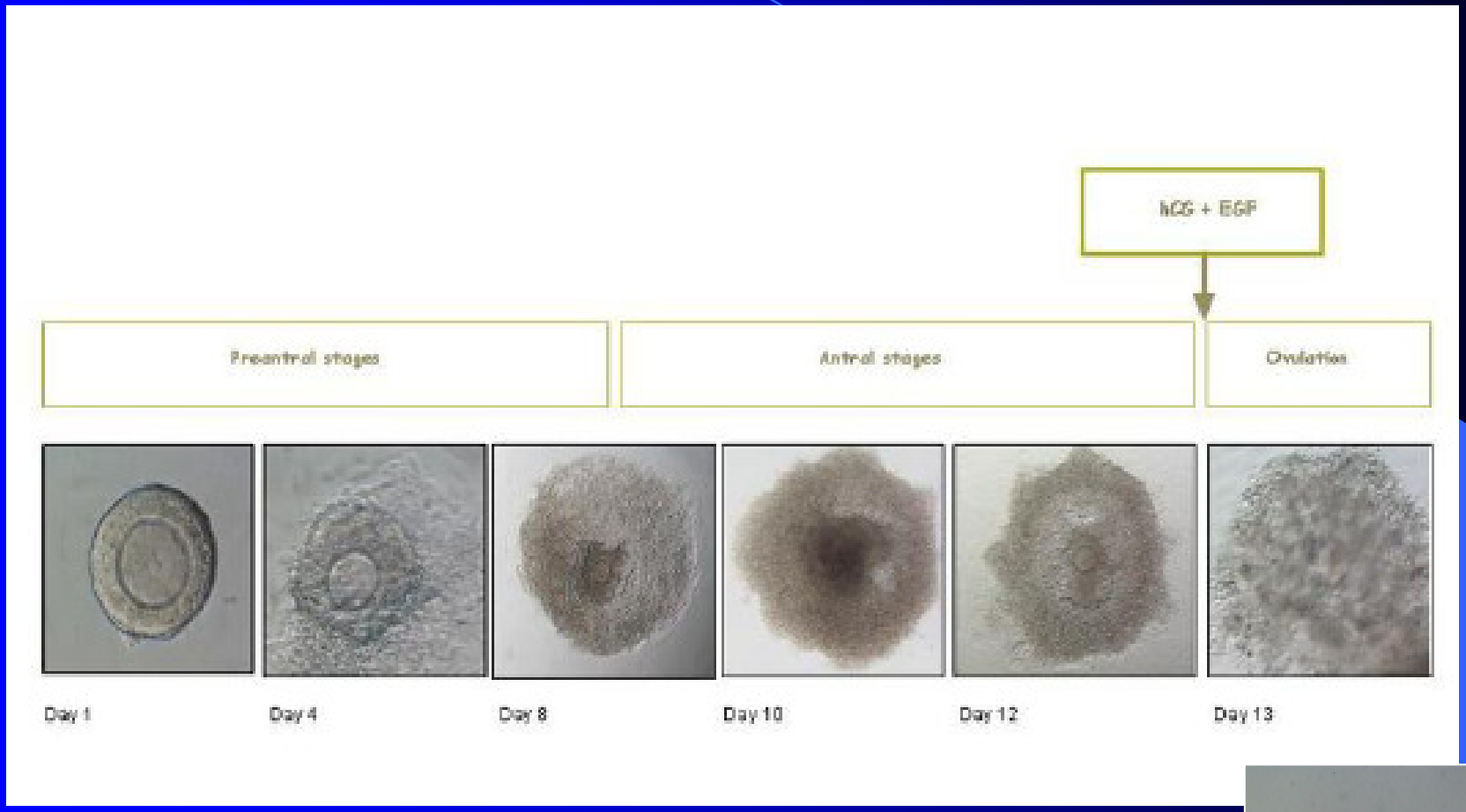
Genome imprinting and ART

- 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

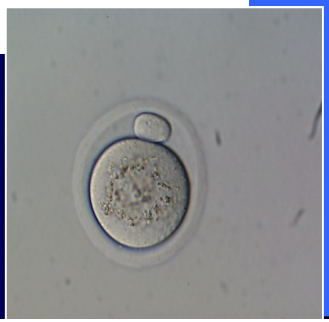
Genome imprinting and ART

- 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

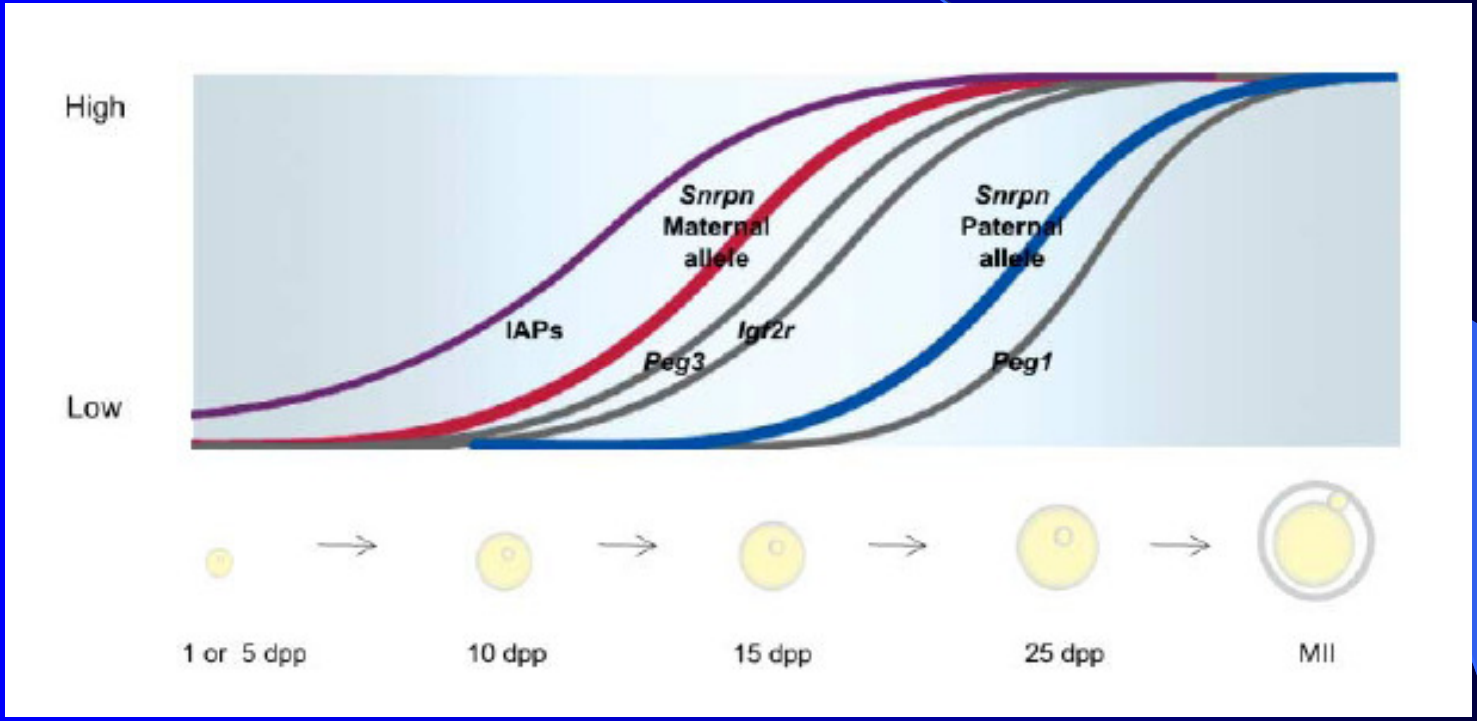
In-vitro follicle culture system in mouse



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



Lucifero D, 2004

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

Background of the study (1)

- The methionine cycle plays an important role in DNA methylation processes. Methionine is actively transported into oocytes, and converted into S-adenosylmethionine (SAM), the methyldonor 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, supraphysiological 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)*

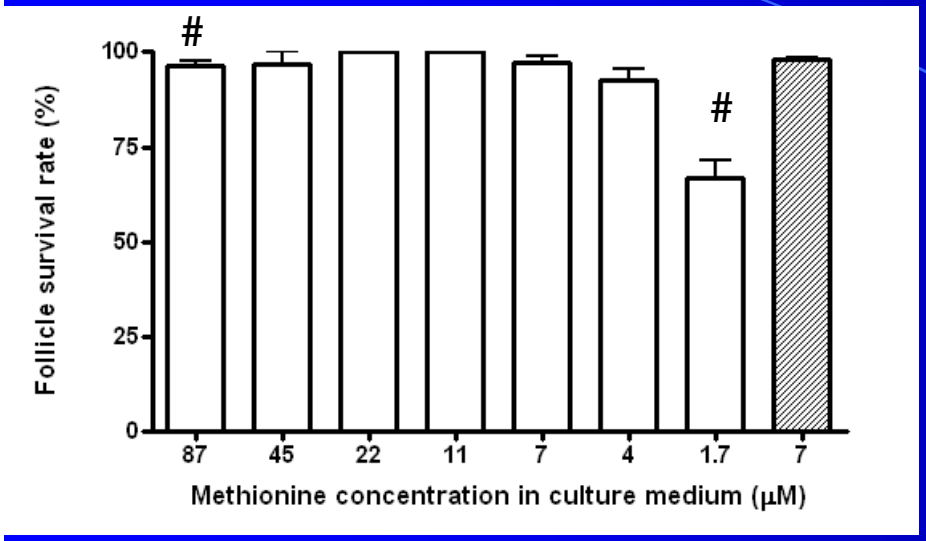
Aim of the study

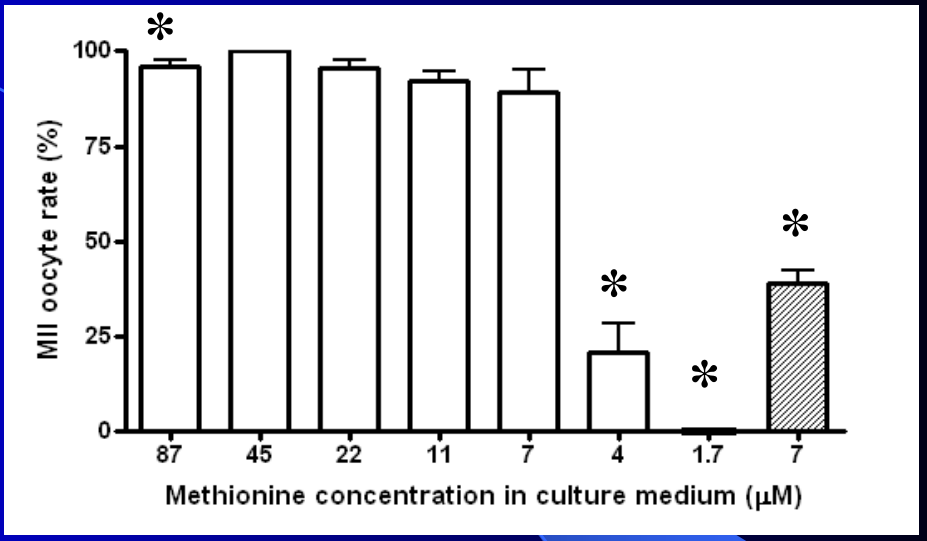
- To study the influence of reduced methyl donor levels in culture medium on follicle development, oocyte maturation capacity and oocyte imprinting establishment

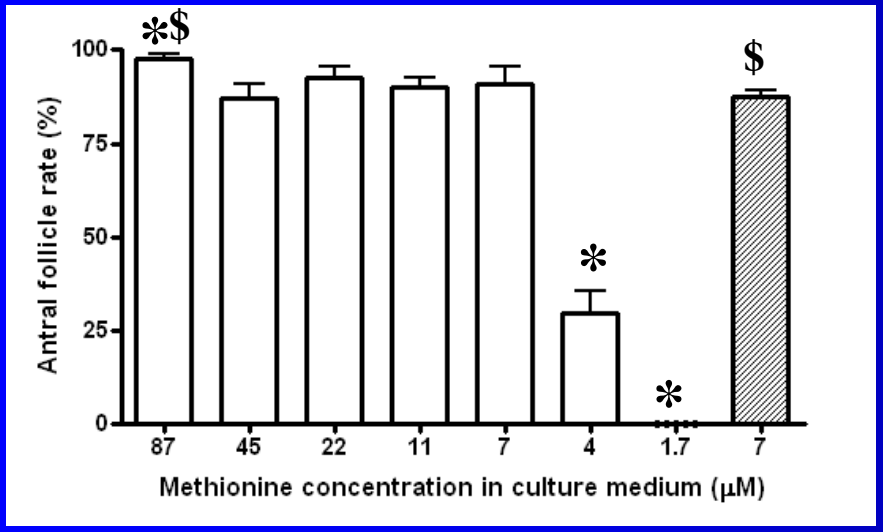
Materials and methods

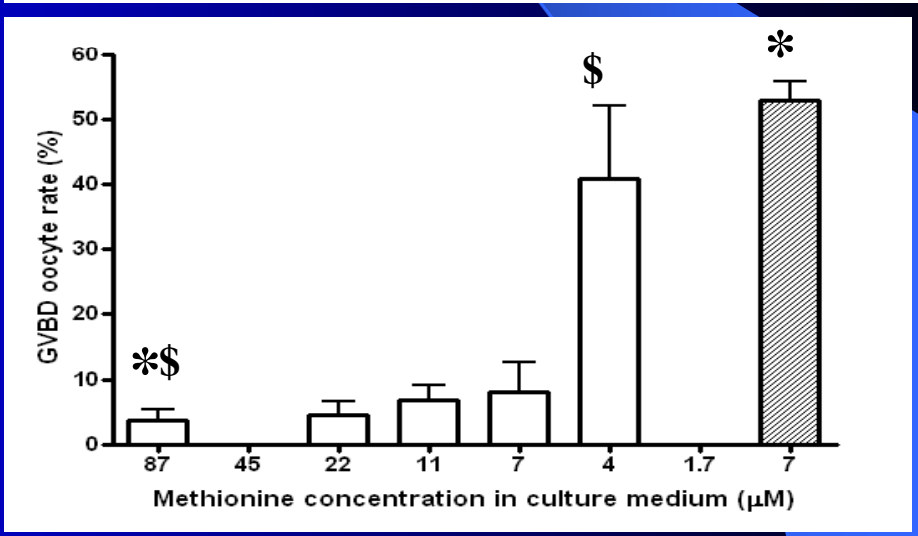
- **α-MEM without methionine, vitamin B12, folic acid, choline chloride and vitamin B6 was used.**
- **Add back experiments with these 5 components were performed (n=713 follicles) to determine the influence of methyl donor levels on**
  - **follicle survival**
  - **follicle development**
  - **MII 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





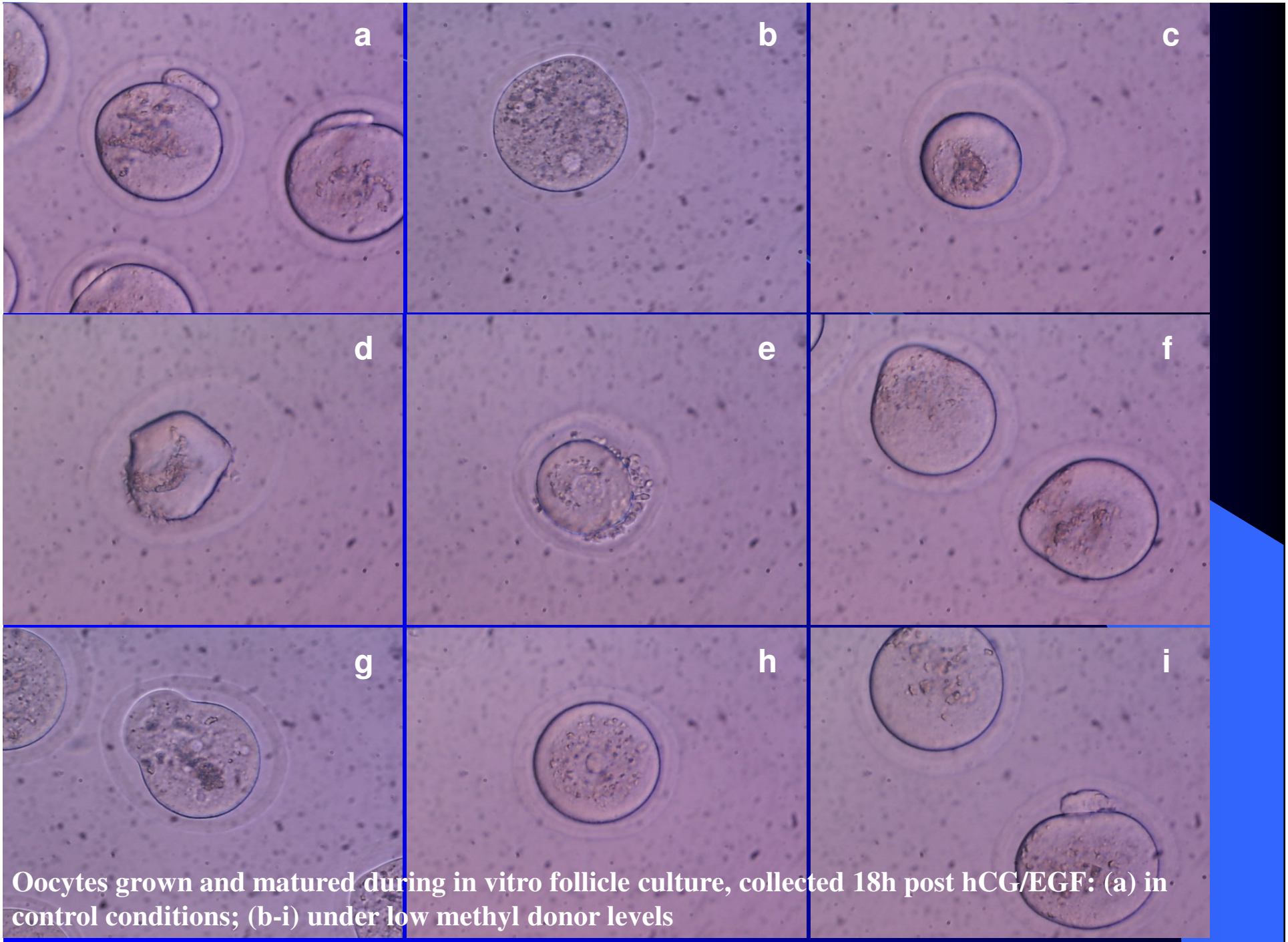




# p<0.01; $ p<0.05; * p<0.001

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

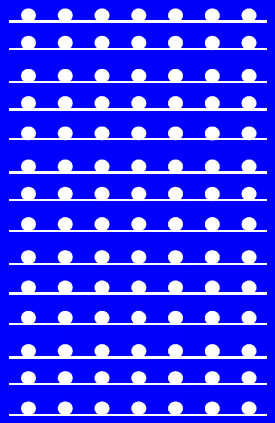
α-MEM w/o vitamin B12, folic acid, choline chloride, pyridoxal + 5%FBS



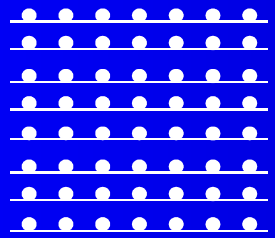
Oocytes grown and matured during in vitro follicle culture, collected 18h post hCG/EGF: (a) in control conditions; (b-i) under low methyl donor levels

Igf2r

A



B



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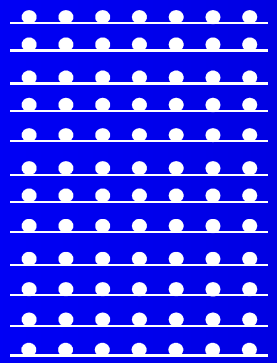
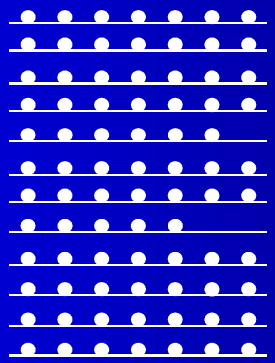
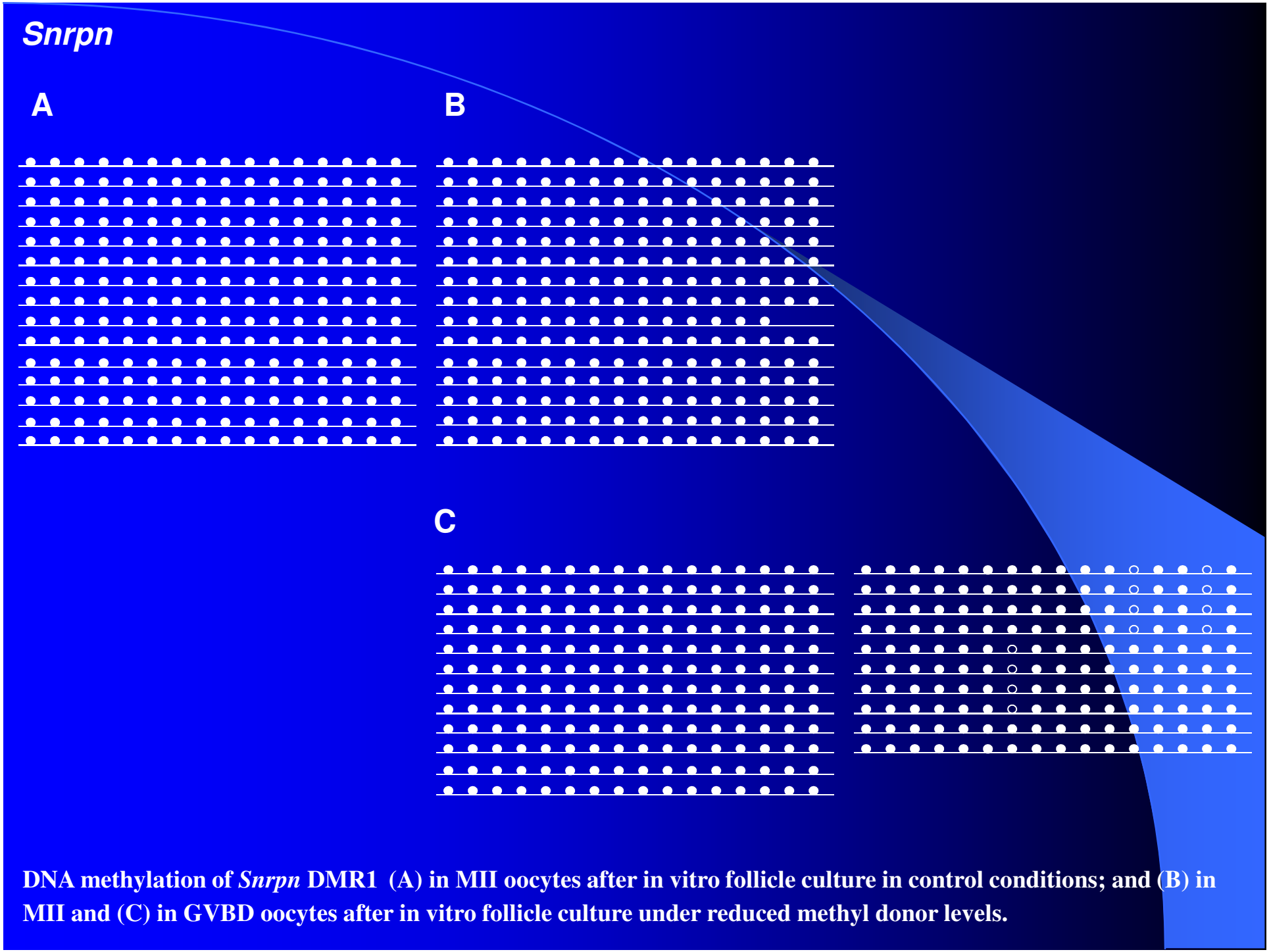


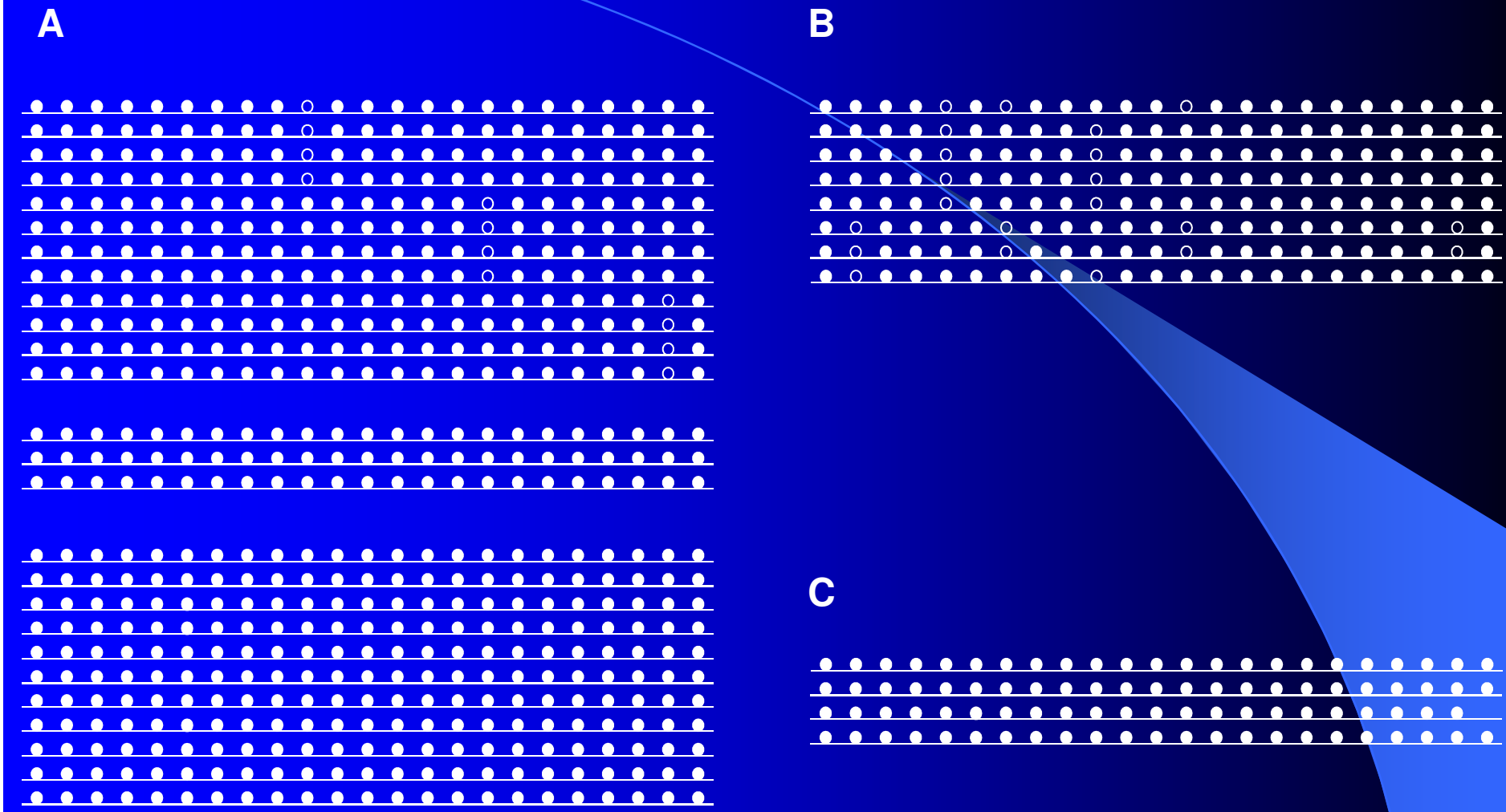


DNA methylation of *Igf2r* DMR2 (A) in MII oocytes after in vitro follicle culture in control conditions; and (B) in MII and (C) in GVBD oocytes after in vitro follicle culture under reduced methyl donor levels.

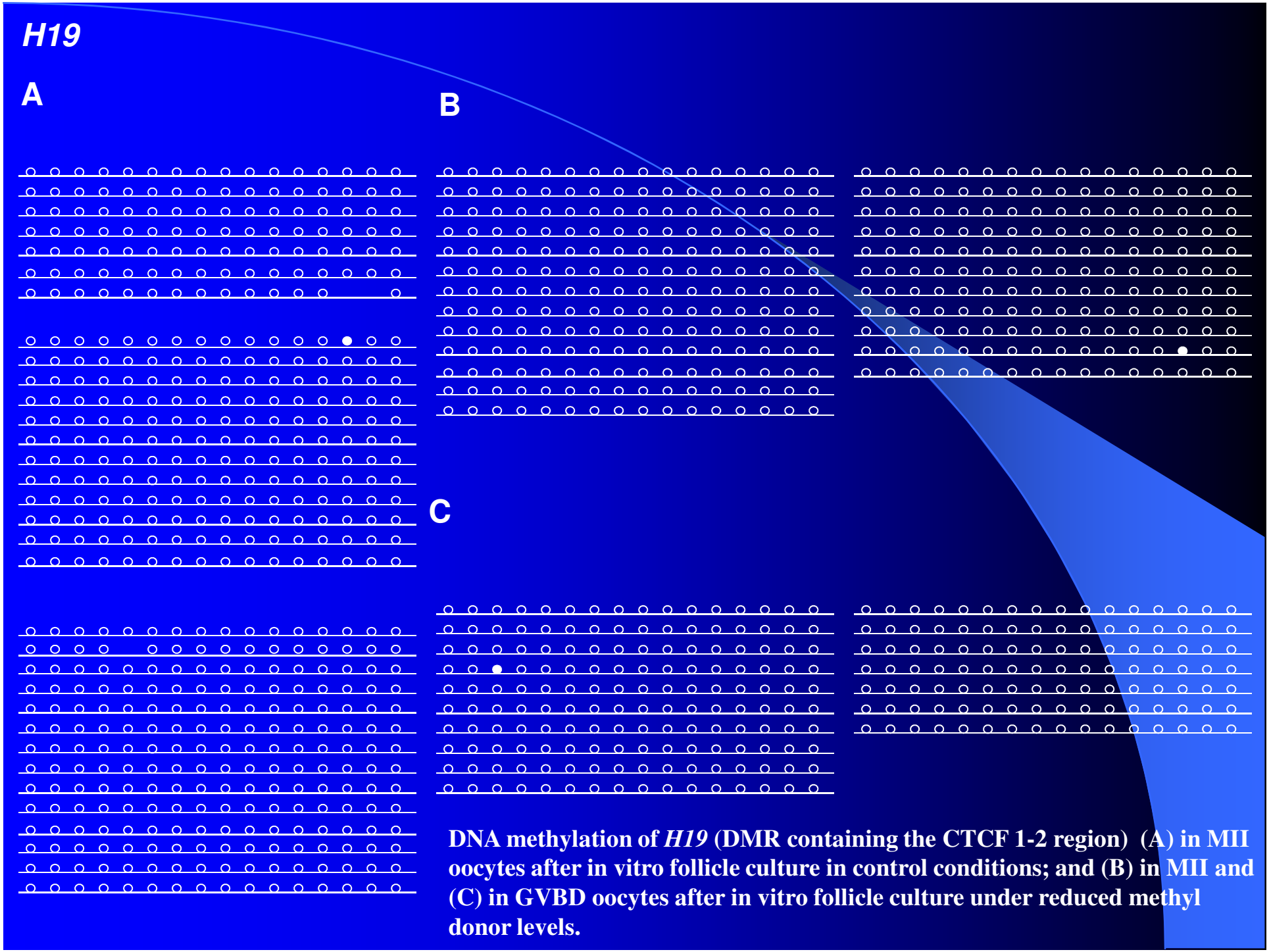


DNA methylation of *Snrpn* DMR1 (A) in MII oocytes after in vitro follicle culture in control conditions; and (B) in MII and (C) in GVBD oocytes after in vitro follicle culture under reduced methyl donor levels.

Mest (Peg1)



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

	Exp α-MEM + 5% FBS[1]	Control α-MEM [2]	Culture media[3]	Rodent plasma/serum
Methionine	7 µM	100 µM	0-200 µM	48-75 µM (mouse)
Vit B12	207 pM	1 µM	0-1 µM	812 pM (rat)
Folic acid	1.4 nM	2.3 µM	0-6 µM	240 nM (mouse)
Vit B6	19 nM	4.9 µM	0-12 µM	700 nM
Choline chloride		1 mg/L		

[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).