Julius-Maximilians-
UNIVERSITÄT
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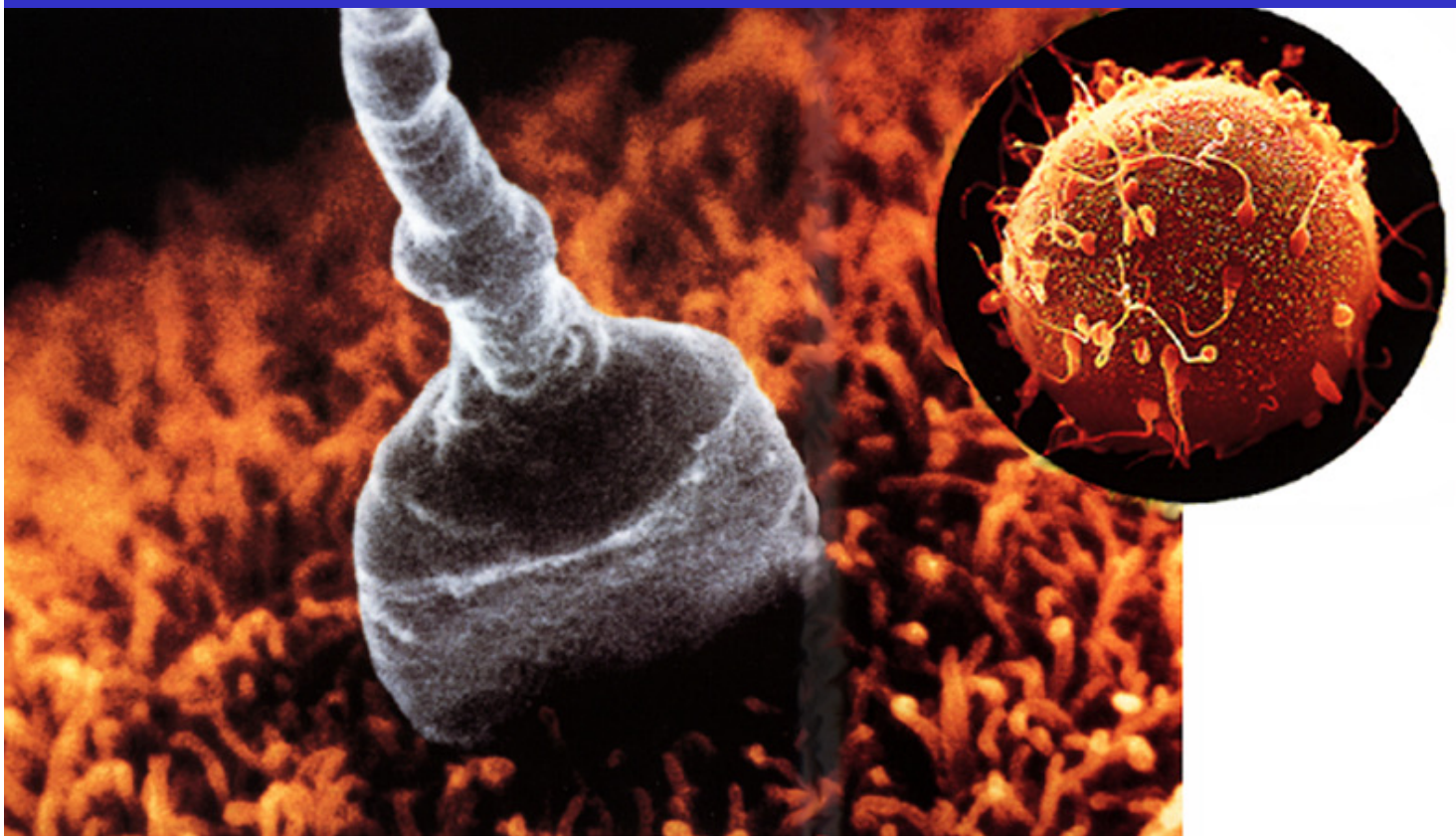
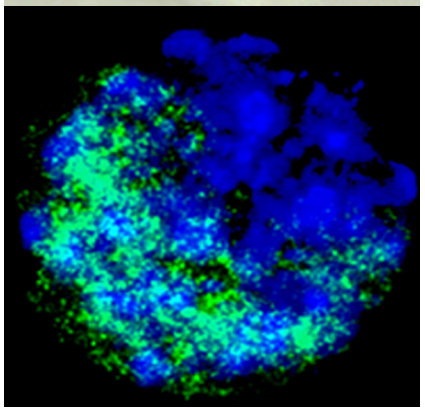
Univ.-Prof. Dr. Thomas Haaf

Institut für Humangenetik

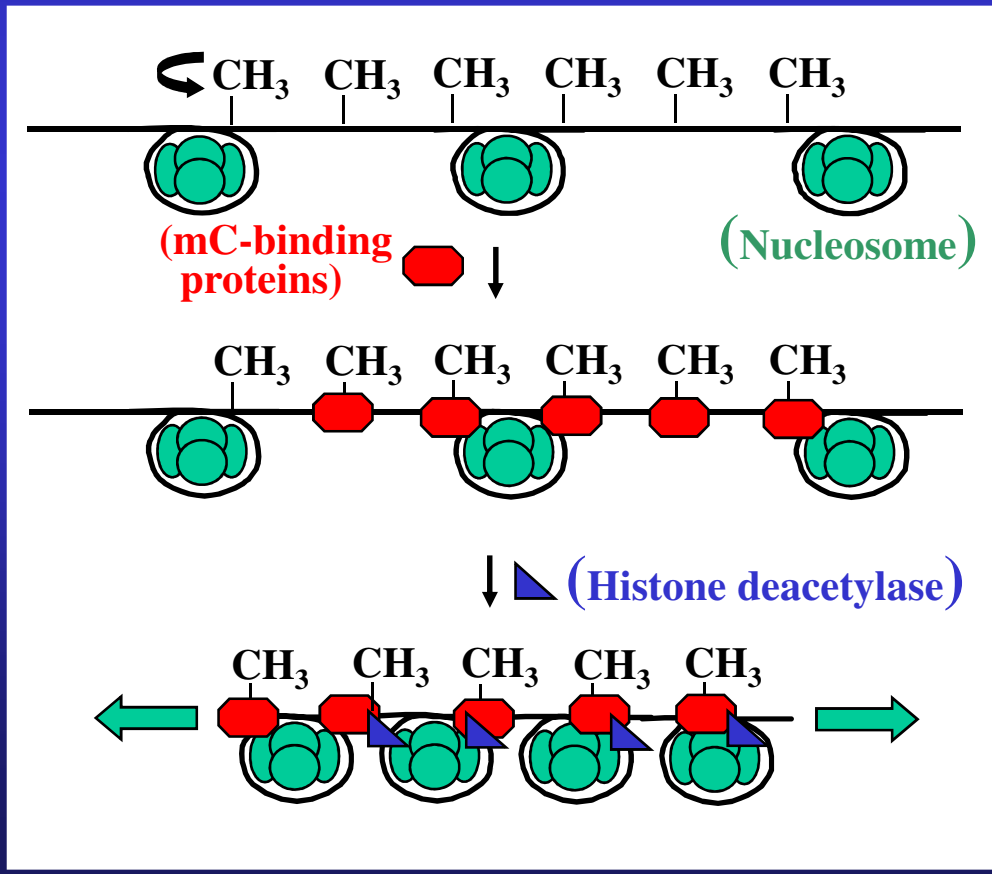
Methylation reprogramming dynamics and defects in gametogenesis and embryogenesis: implications for reproductive medicine





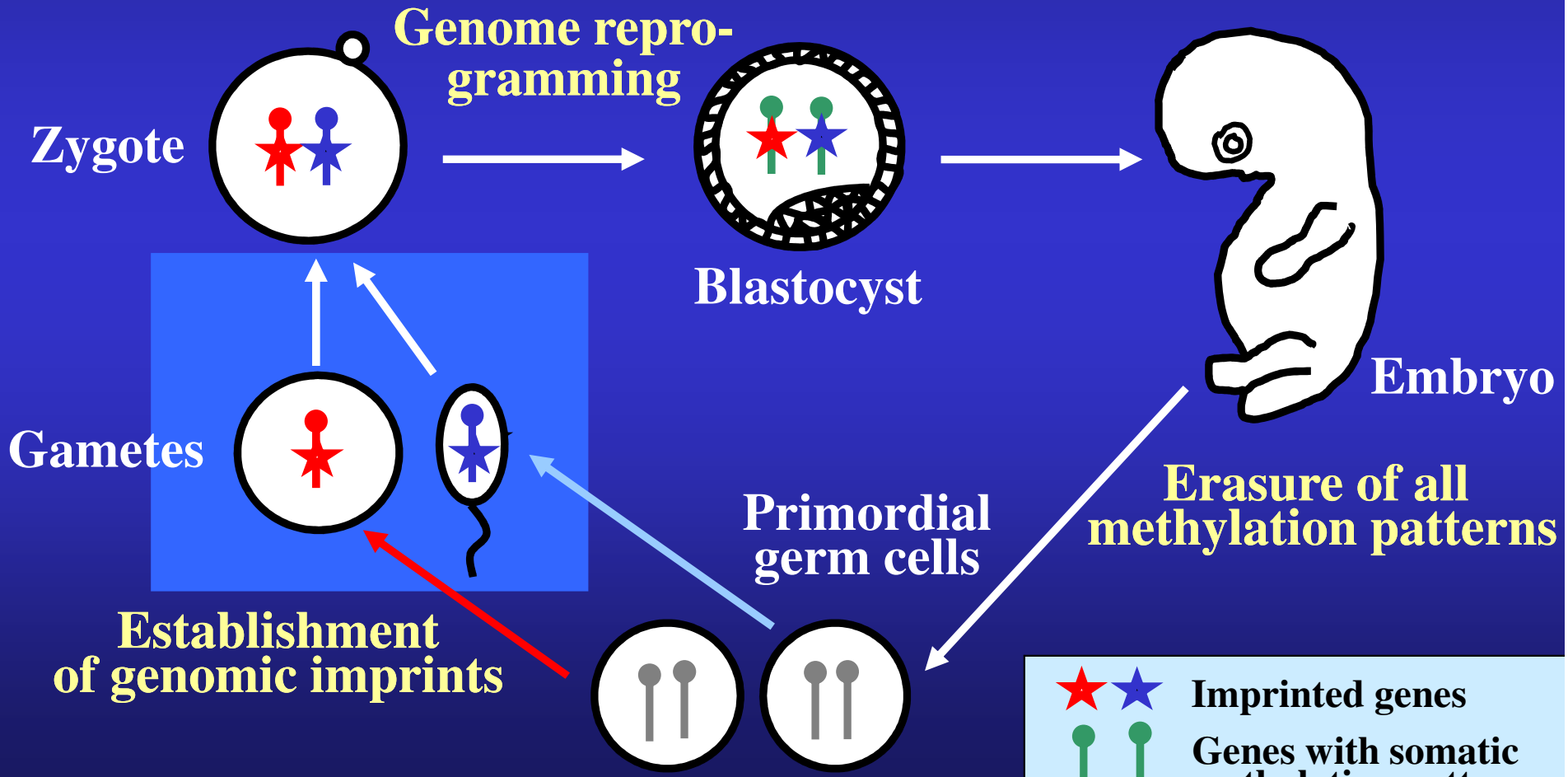
Epigenetics and DNA methylation

- Heritable change of the genetic information, which is not produced by a change in the DNA sequence itself (mutation), but by reversible modification of the DNA and chromatin structure.
- Methylation at CpG dinucleotides is the only epigenetic mark that targets DNA directly.
- It suppresses transcription by recruiting methylcytosine-binding proteins and chromatin remodelling complexes to cis-regulatory regions.



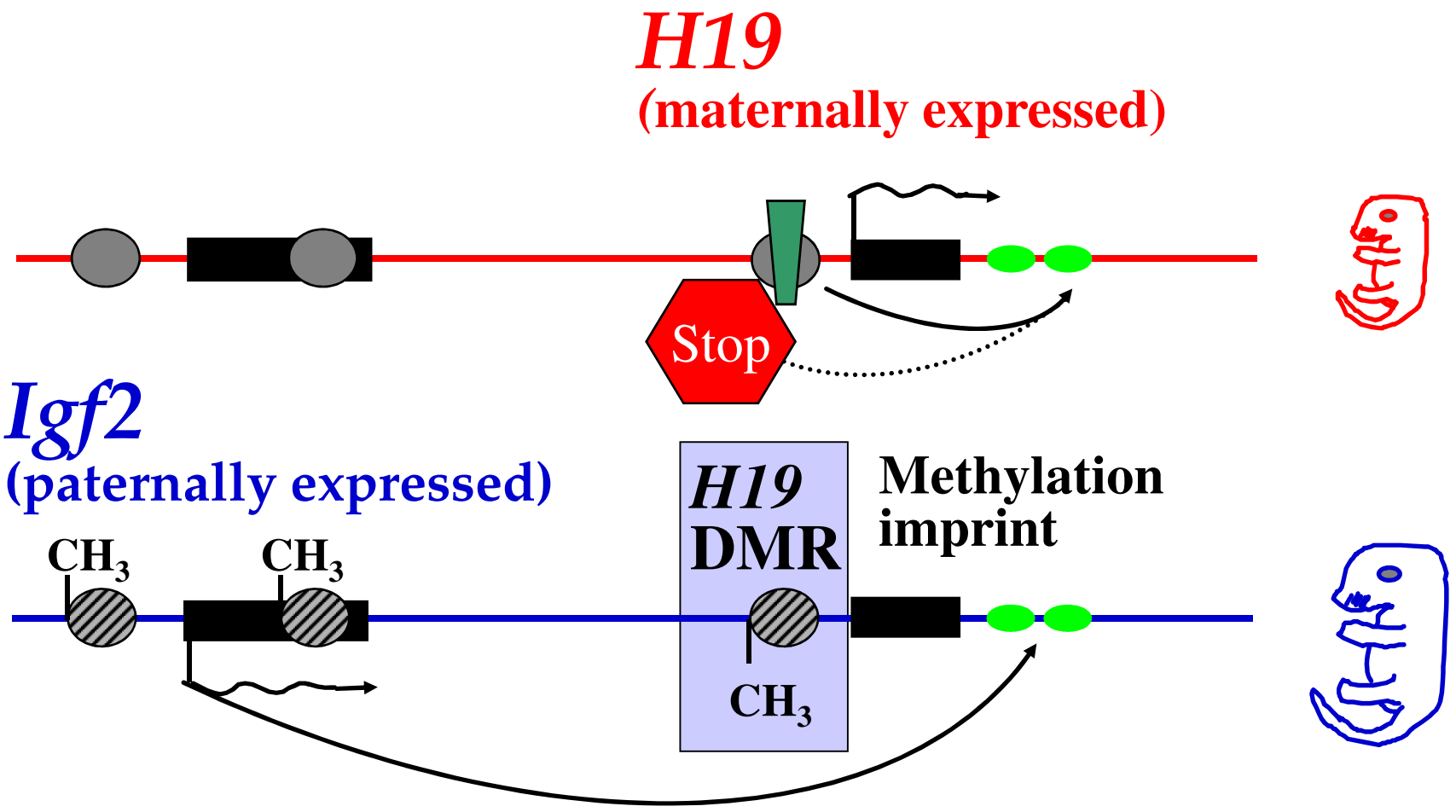
Genomic imprinting and genome reprogramming

During gametogenesis the two germline genomes acquire parent-specific epigenetic modifications in form of DNA methylation patterns.



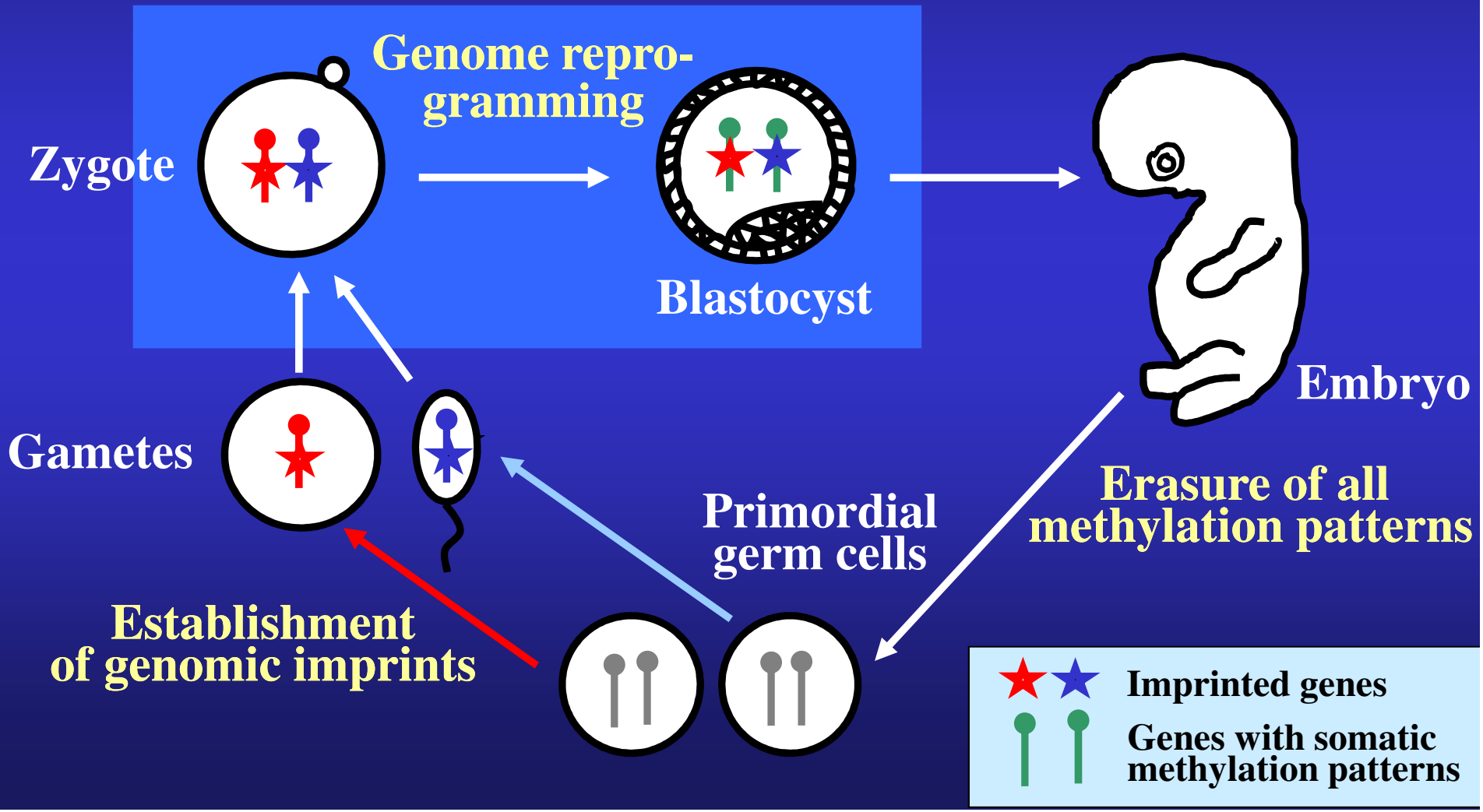
Parent-offspring conflict hypothesis

… postulates a conflict over the allocation of maternal resources during pregnancy.

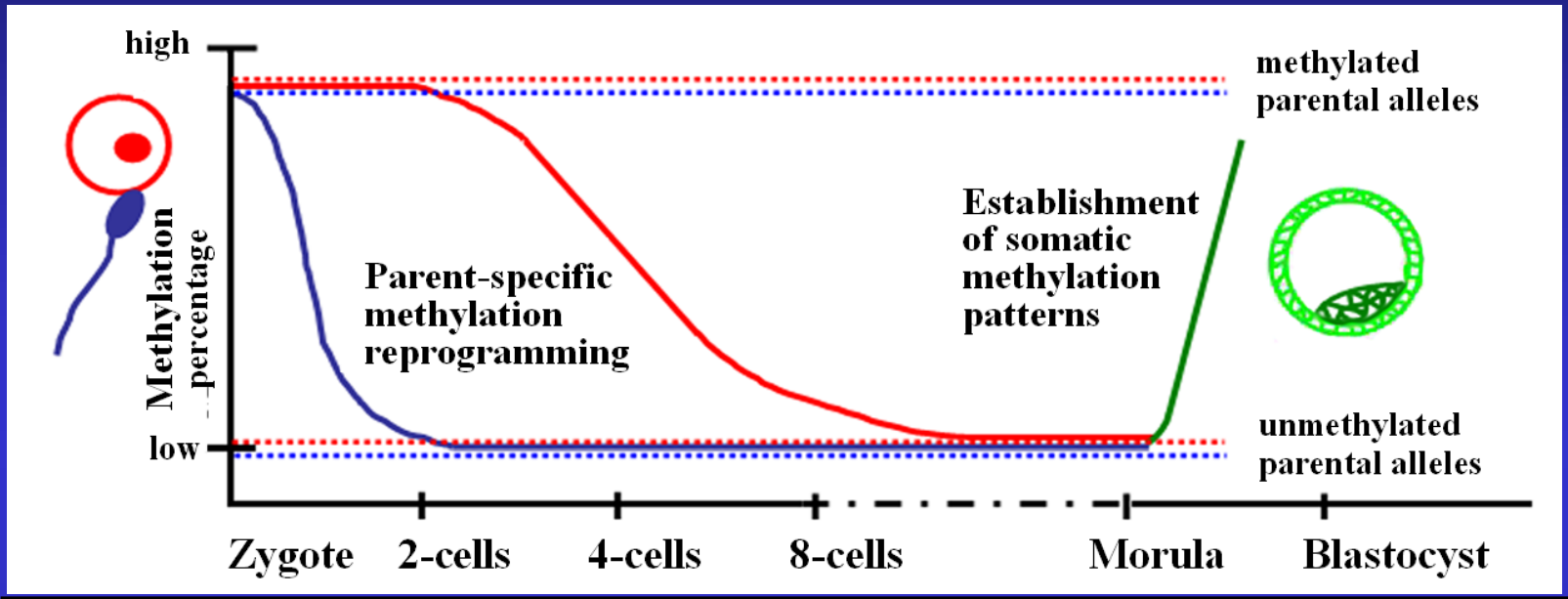


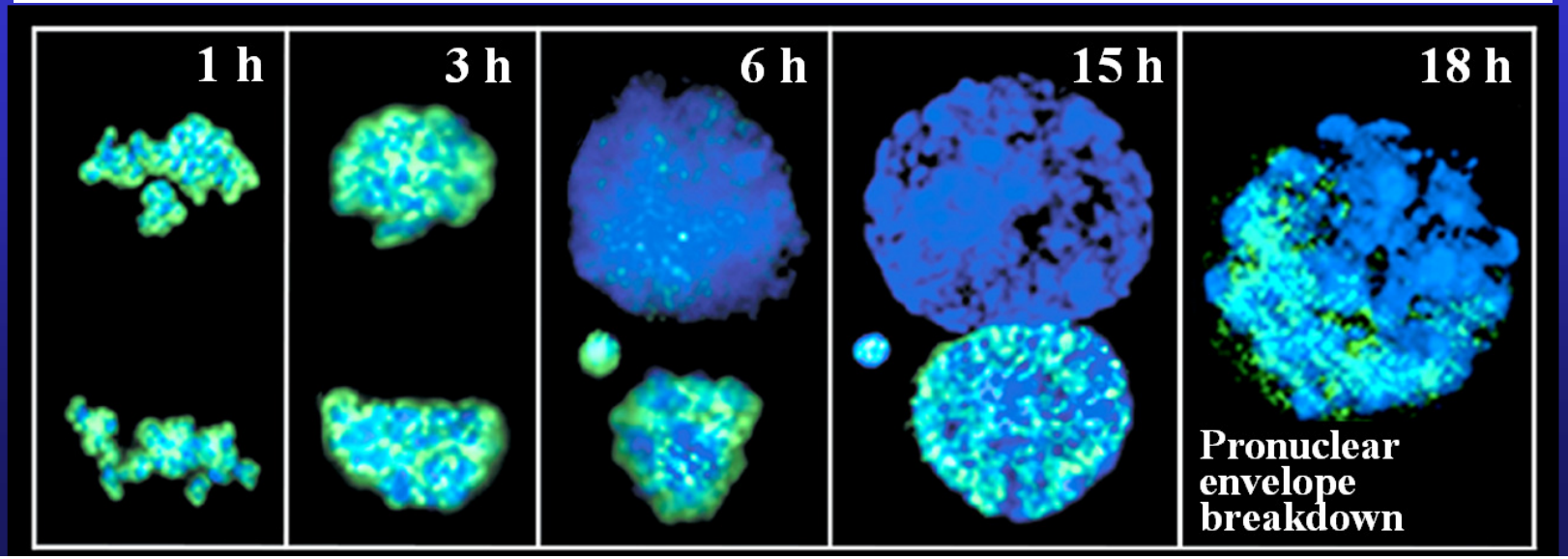
Genomic imprinting and genome reprogramming

The two complementary sets of chromatin must be reprogrammed after fertilization for somatic development of the new organism.

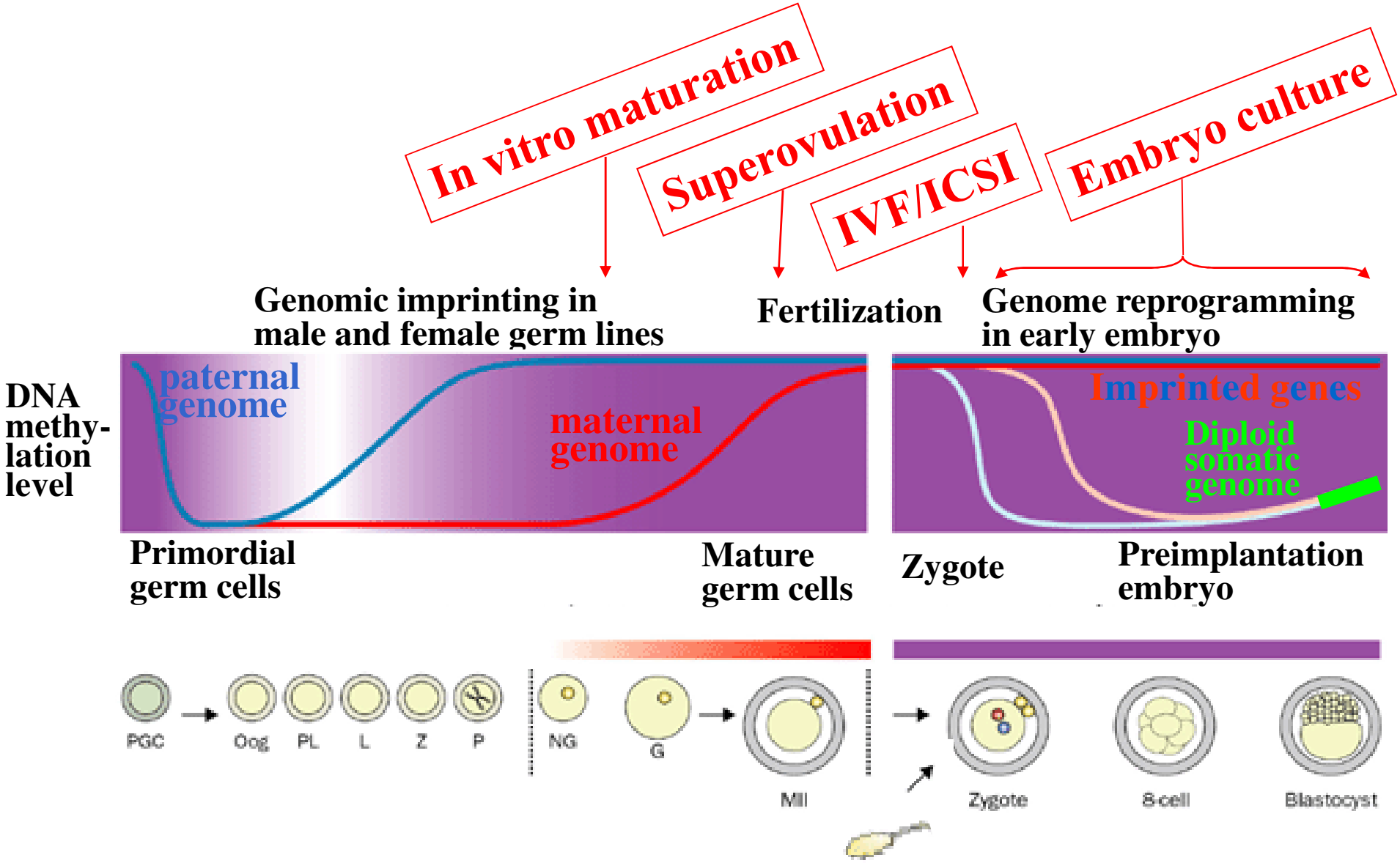


The maternal and paternal genomes are reprogrammed at different time points and by different mechanisms during preimplantation development





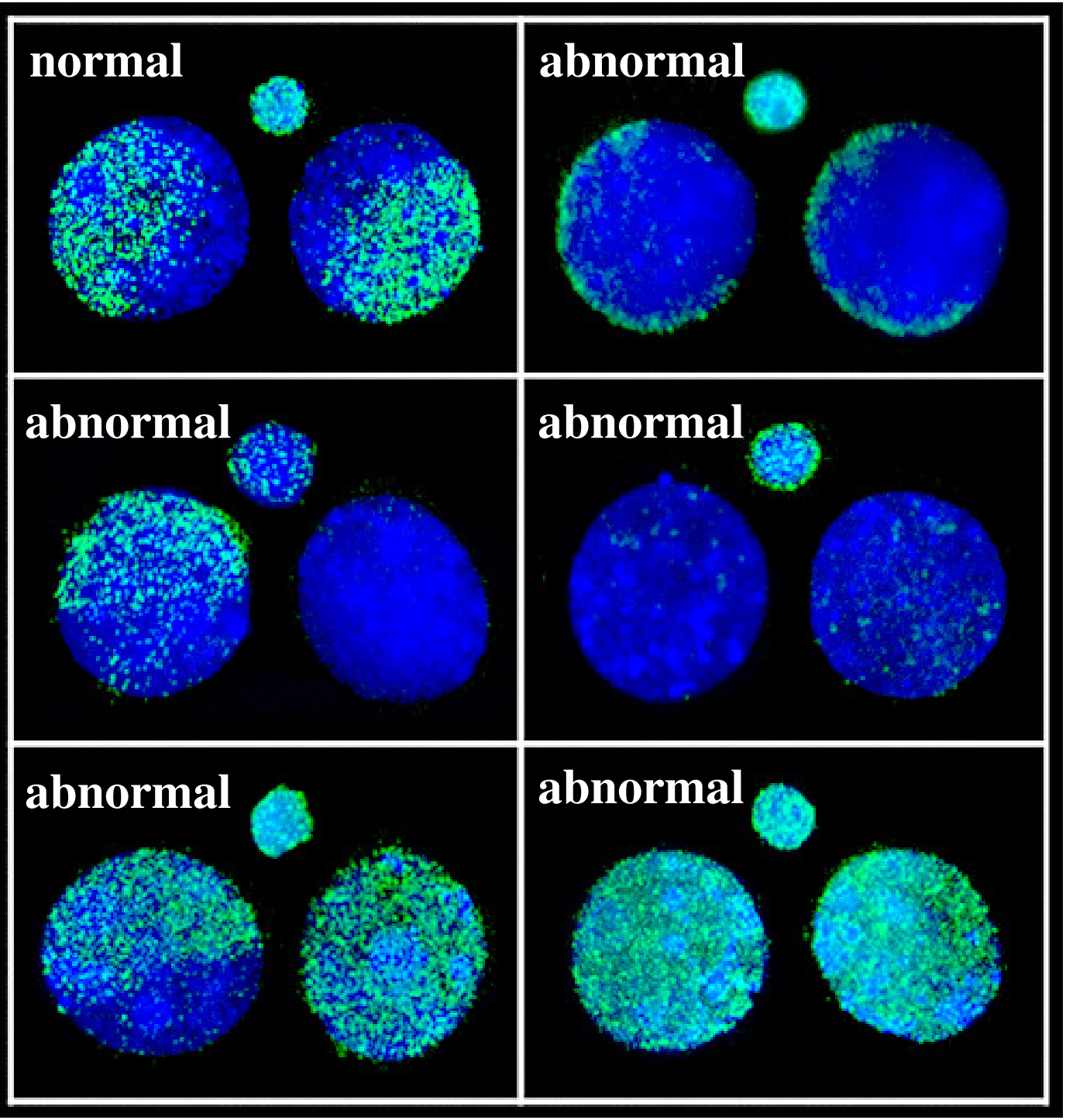
Assisted reproductive technologies interfere with very sensitive time periods for epigenetic genome reprogramming in the germline and early embryo



Genome-wide methylation reprogramming defects in mouse two-cell embryos

Excess demethylation (diandric patterns)

Failed demethylation (digynic patterns)



Effects of epigenetic factors: ovarian stimulation

Embryos	Treatment/ Medium	In vitro development: Number analyzed	In vitro development: % not developing to blastocyst	MeC staining: Number analyzed	MeC staining: % with abnormal methylation pattern
B6C3F1	IVF / HH[a]	20	80%	7	71%
B6C3F1	IVF / M199[b]	28	25%	22	32%
B6C3F1	IVF / M16[c]	29	17%	12	9%
B6C3F1	Direct preparation from non-superovulated females	40	→ 5%	100	→ 10%
B6C3F1	Direct preparation from superovulated females	50	→ 14%	30	→ 20%
B6C3F1	M16	45	20%	18	22%
B6C3F1	M16-[d]	47	29%	17	29%
B6C3F1	Acetaldehyde / M16	30	87%	18	61%
NMRI	M16	56	28%	35	40%
NMRI	M16-	57	63%	36	58%
NMRI	Acetaldehyde / M16	25	100%	28	71%

Superovulation increases the rate of abnormal methylation patterns and embryo loss during preimplantation development.

- **Environmental factors such as superovulation, embryo culture, and toxic compounds (alcohol, endocrine disruptors, etc.) can affect the regulation of methylation reprogramming.**
- **Genome-wide disturbances of methylation reprogramming lead to early embryo loss. („All or Nothing“ rule)**
- **Methylation reprogramming defects of individual genes may be compatible with further development and then contribute to phenotypic variation and disease.**

Important mouse studies

- **Ovarian stimulation decreases the oocyte and embryo quality in mice.**
  Ertzeid and Storeng, J. Reprod. Fertil. 96, 649-655, 1992
- **Mouse embryo culture affects fetal development, methylation patterns and expression of imprinted genes.**
  Doherty et al., Biol. Reprod. 62, 1526-1535, 2000
  Koshla et al., Biol. Reprod. 64, 918-926, 2001
  Mann et al., Development 131, 3728-3735, 2004
- **Embryo culture affects the behaviour of adult mice.**
  Ecker et al.,
  PNAS 101, 1595-1600, 2004

Long-term effects of culture of preimplantation mouse embryos on behavior

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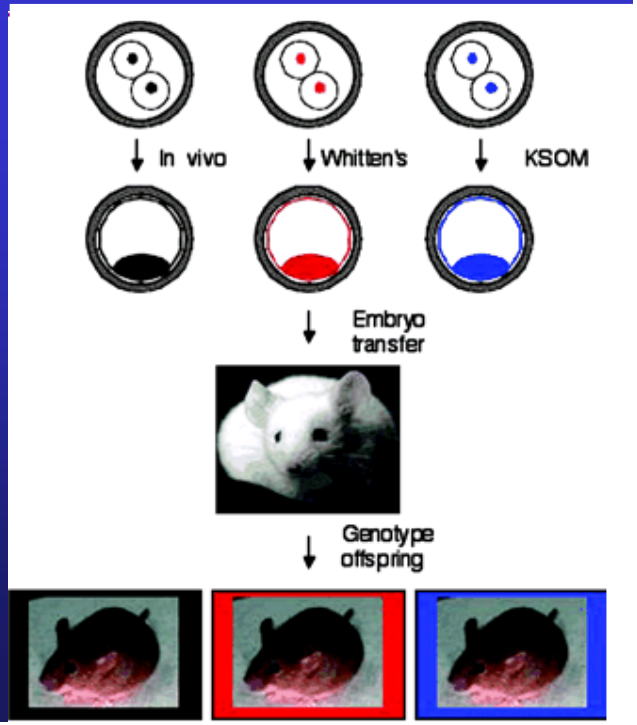
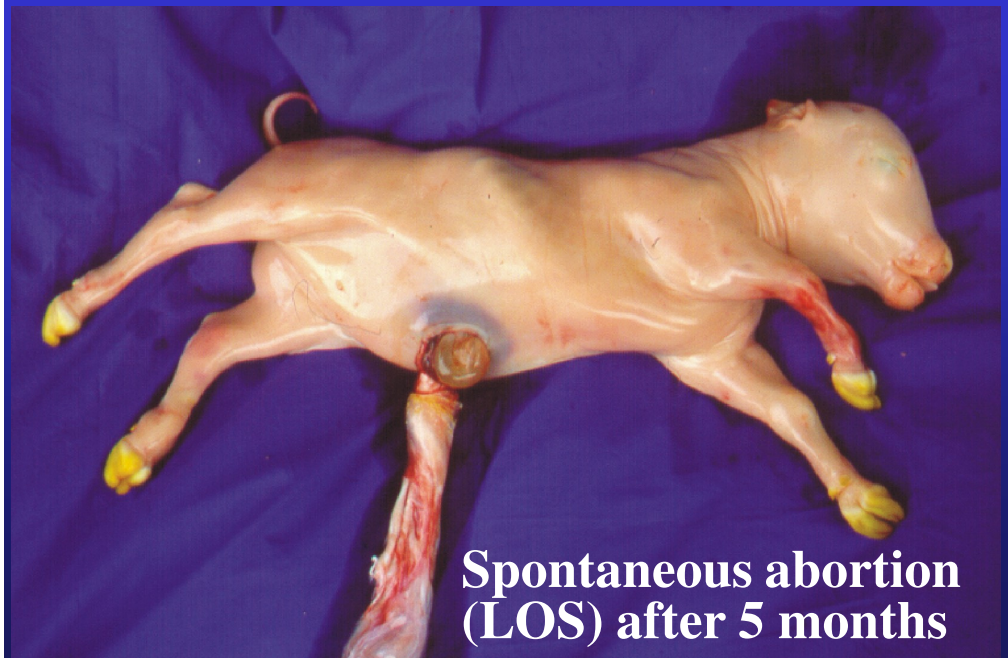
Departments of *Biology and ¶Biostatistics and Epidemiology and ‡Center for Research on Reproduction and Women's Health, University of Pennsylvania, Philadelphia, PA 19104



Large offspring syndrom (LOS)

- In bovine and ovine in vitro production.
- In vitro maturation of oocytes and embryo culture are associated with epigenetic changes in *IGF2R* and fetal overgrowth.

(Young et al., Nature Genet. 27, 153-154, 2001)



Our specific goals

- We want to study the epigenetic effects of different in vitro maturation (IVM), cryopreservation, and superovulation conditions on oocytes and the resulting embryos.
- Comparative aspects in mouse, bovine, and human oocyte and embryo development and potential.
- One important prerequisite for such studies is the development of efficient techniques for the methylation analysis of multiple genes in a few cells.

The analysis of DNA methylation patterns in single cells or small pools of cells is a challenging technological problem

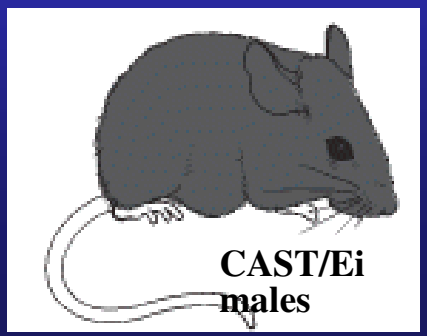
- High signal-to-noise ratio.
- Competition of background DNA for primer annealing.
- Bisulfite-converted DNA is highly degraded, depleted of C (sense) and G (antisense) nucleotides, and endowed with long T stretches.
- No reliable methods for whole genome amplification (WGA) of small amounts of bisulfite-converted DNA.
- **Amplification bias** that is the preferential amplification of a single or a few DNA molecules from the starting material (bisulfite-converted DNA).

Effects of superovulation on methylation imprints in early mouse embryos

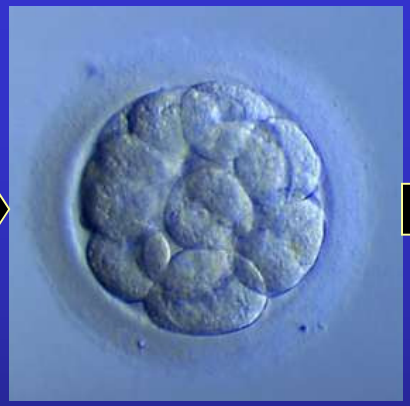
- Bisulfite sequencing of differentially methylated regions (DMRs) of representative imprinted genes.
- Comparative analysis of methylation patterns in individual mouse morula-stage embryos from superovulated and non-superovulated C57BL/6J x CAST/Ei matings.

C57BL/6J females

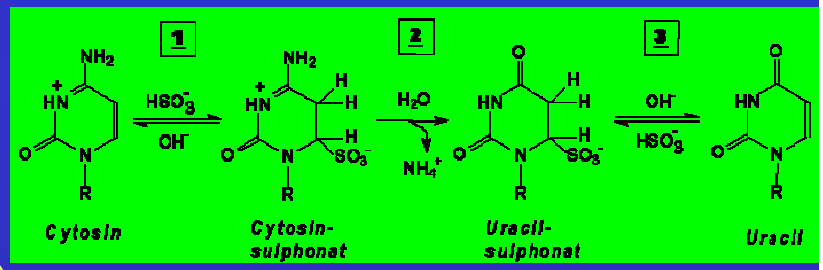
X

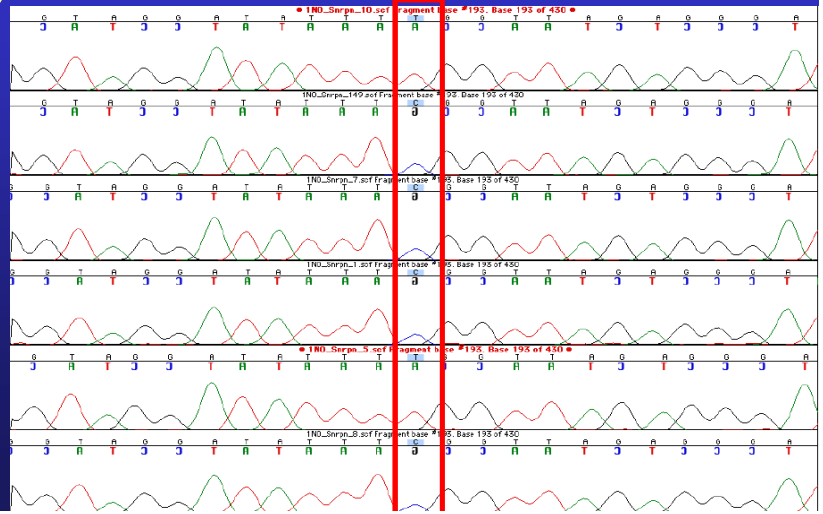


CAST/Ei males

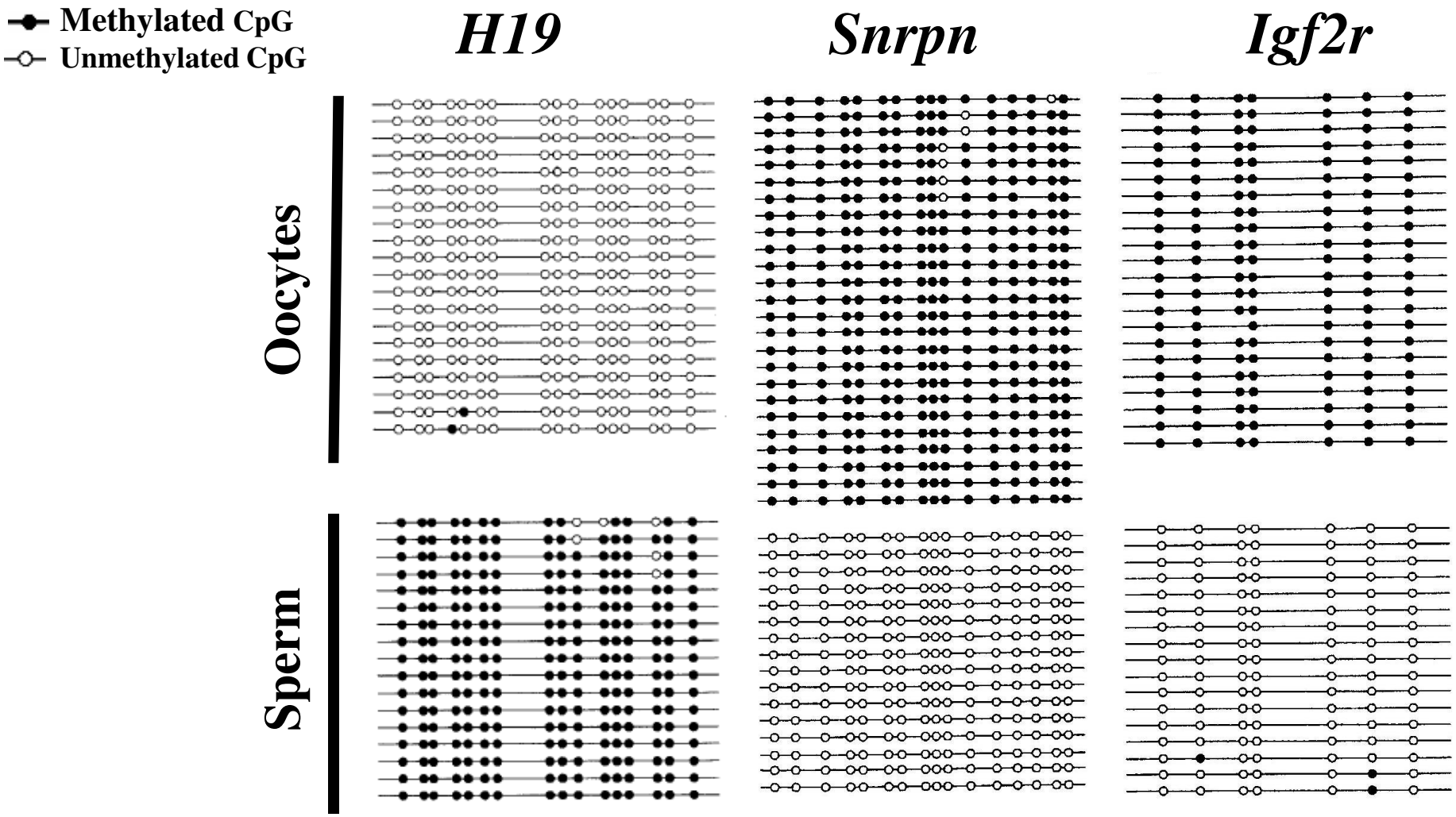


60 h after fertilization.

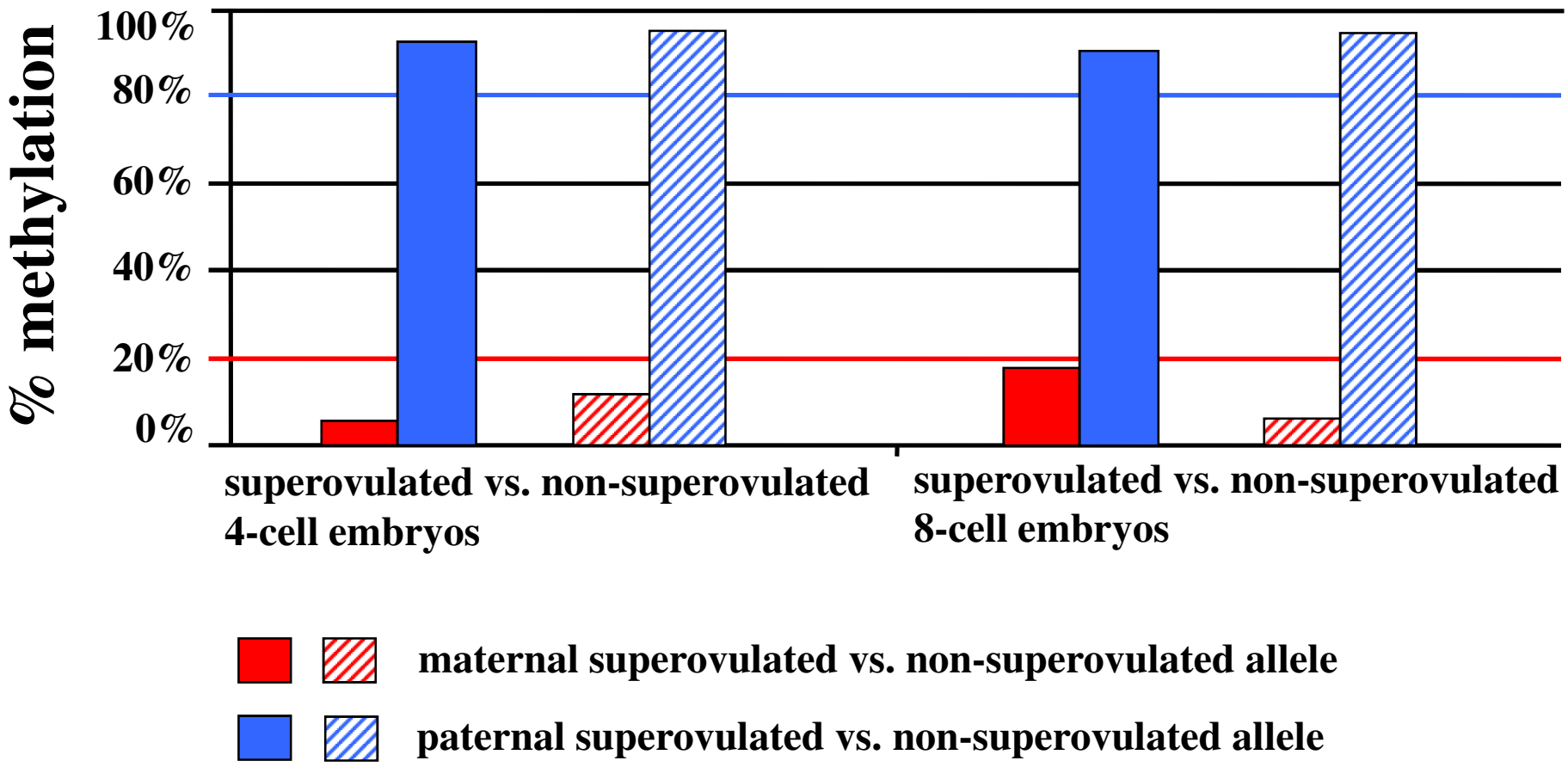




Methylation imprints on paternal (*H19*) and maternal (*Snrpn*, *Igf2r*) chromosomes



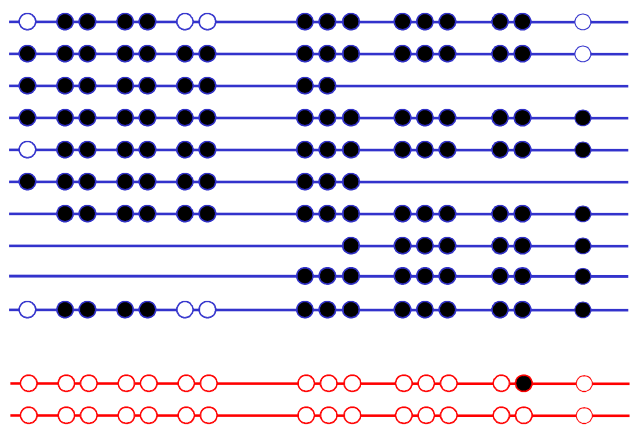
No detectable differences in *H19* methylation between pools of superovulated versus non-superovulated C57BL/6J x CAST/Ei embryos



Classic bisulfite (plasmid) sequencing of the *H19* DMR in individual mouse (B57BL6/J x CAST/Ei) hybrid morula-stage embryos

...
does not produce representative results on the epigenetic status of the embryo.

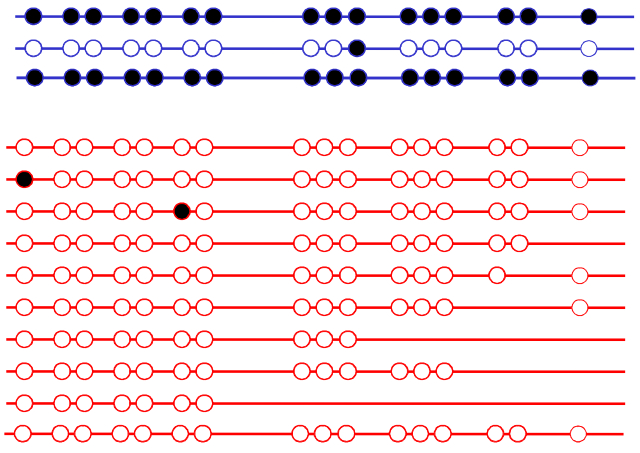
Amplification bias!!!



2 plasmids
24 plasmids
3 plasmids
2 plasmids, 2 clones

36 paternal plasmids
10 paternal alleles

3 maternal plasmids
3 maternal alleles



3 plasmids, 2 clones
9 plasmids, 4 clones
43 plasmids, 3 clones
2 plasmids, 2 clones
2 plasmids
2 plasmids

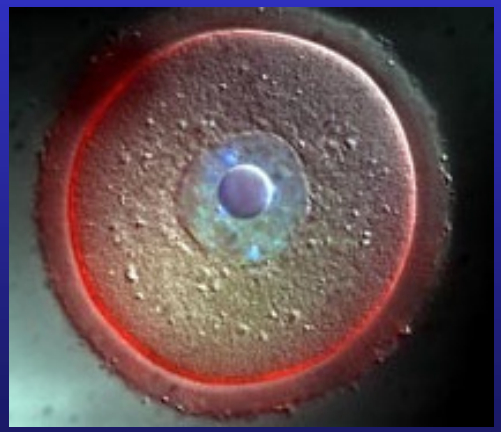
13 paternal plasmids
7 paternal alleles

55 maternal plasmids
13 maternal alleles

Limiting Dilution (LD): a novel strategy for methylation analysis of small amounts of DNA

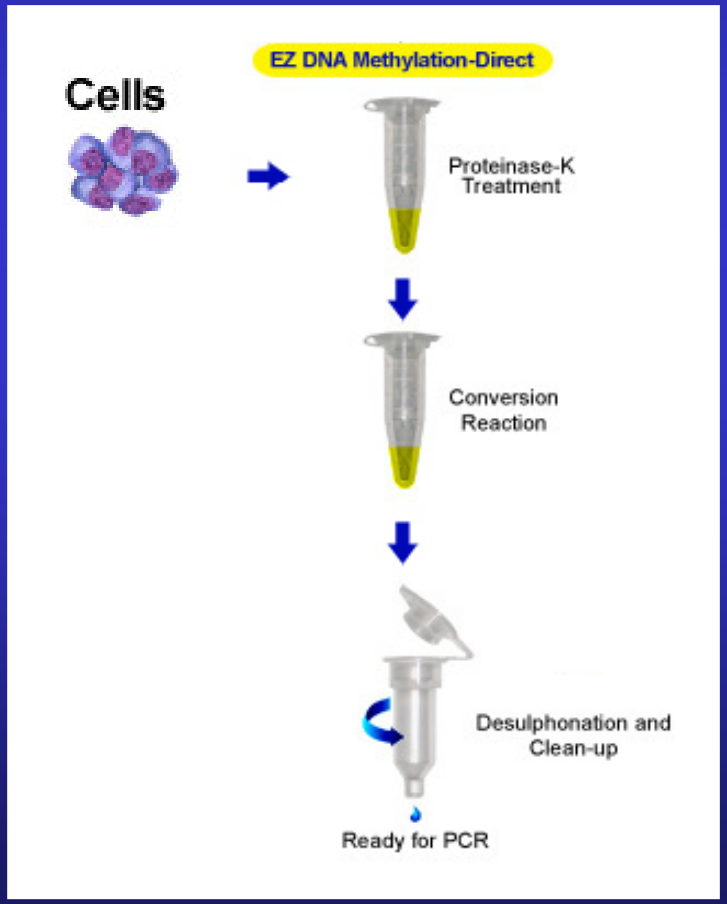
- Single DNA molecules (i.e. from a few bisulfite-treated cells) are distributed into separate wells/tubes and then amplified individually.
- The aim is to detect a binary state: signal / no signal. .
- The dilution of competing background molecules (distribution over several PCR tubes) increases template-to-background ratio).
- This avoids any amplification bias and allows one to determine the methylation patterns of single DNA molecules.
- Multiplex analyses after LD provide a way to increase the number of studied genes.

Sample Preparation → Bisulfite Conversion → 1st Round Multiplex PCR → 2nd Round Nested PCRs → (Pyro-) Sequencing



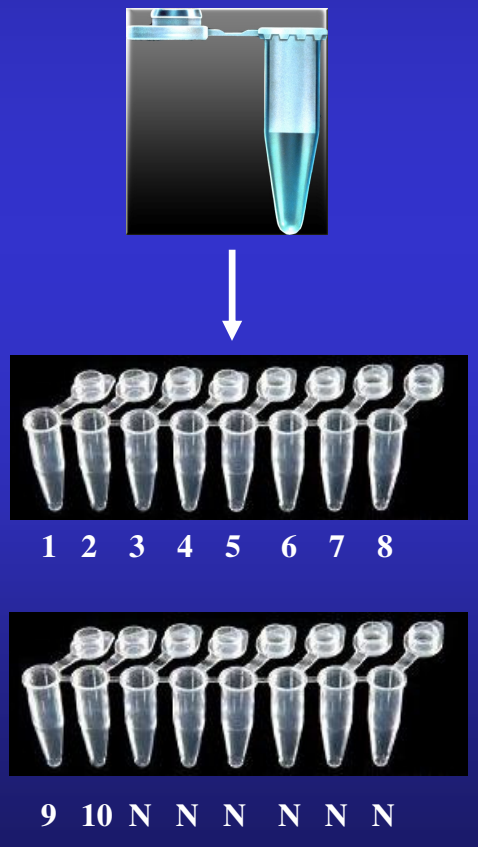
- Collecting oocytes from superovulated C57BL/6J mice.
- Hyaluronidase digestion to remove cumulus cells.
- Visual inspection of oocytes: are all cumulus cells removed?
- Collecting aliquots of 10 oocytes each in 10 µl PBS.

Sample Preparation → **Bisulfite Conversion** → 1st Round Multiplex PCR → 2nd Round Nested PCRs → (Pyro-) Sequencing

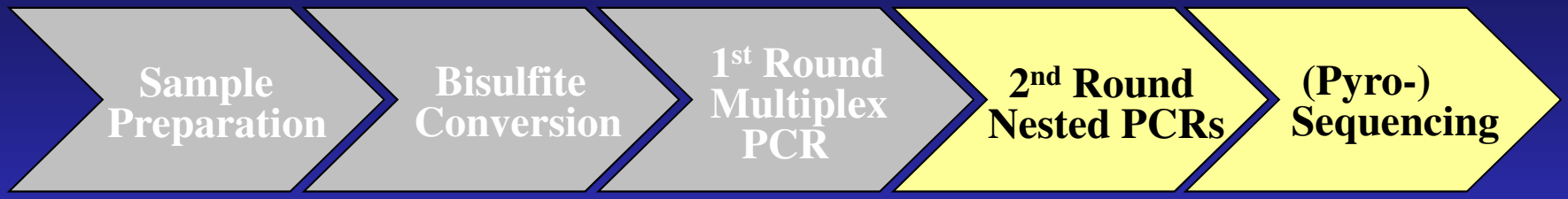


- **Bisulfite conversion with the EZ DNA Methylation Direct Kit (Zymo Research).**
- **Recovery rate ≥ 80%; Sensitivity 5-10 cells.**
- **Elution of bisulfite-converted DNA in 10 µl buffer.**
- **Dilution 1:10 with water to a final volume of 100 µl.**

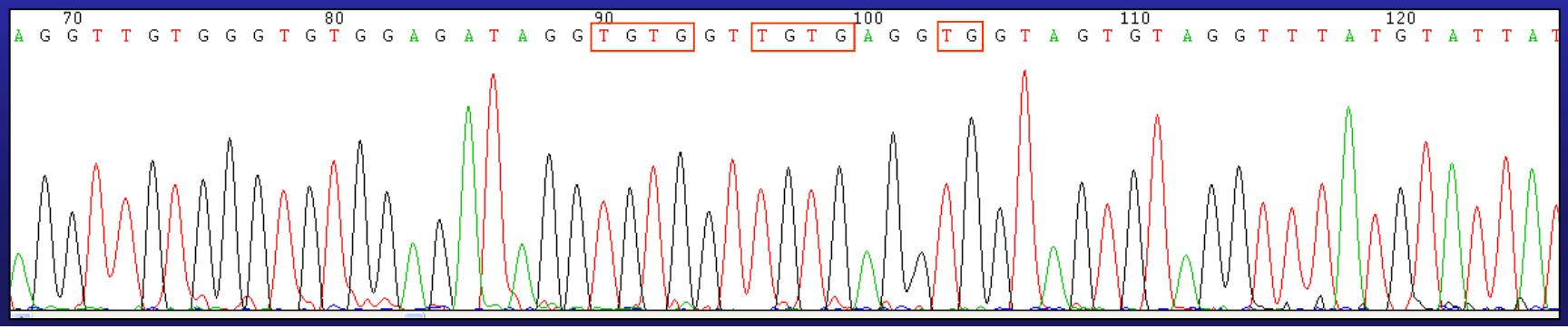
Sample Preparation → Bisulfite Conversion → **1st Round Multiplex PCR** → 2nd Round Nested PCRs → (Pyro-) Sequencing



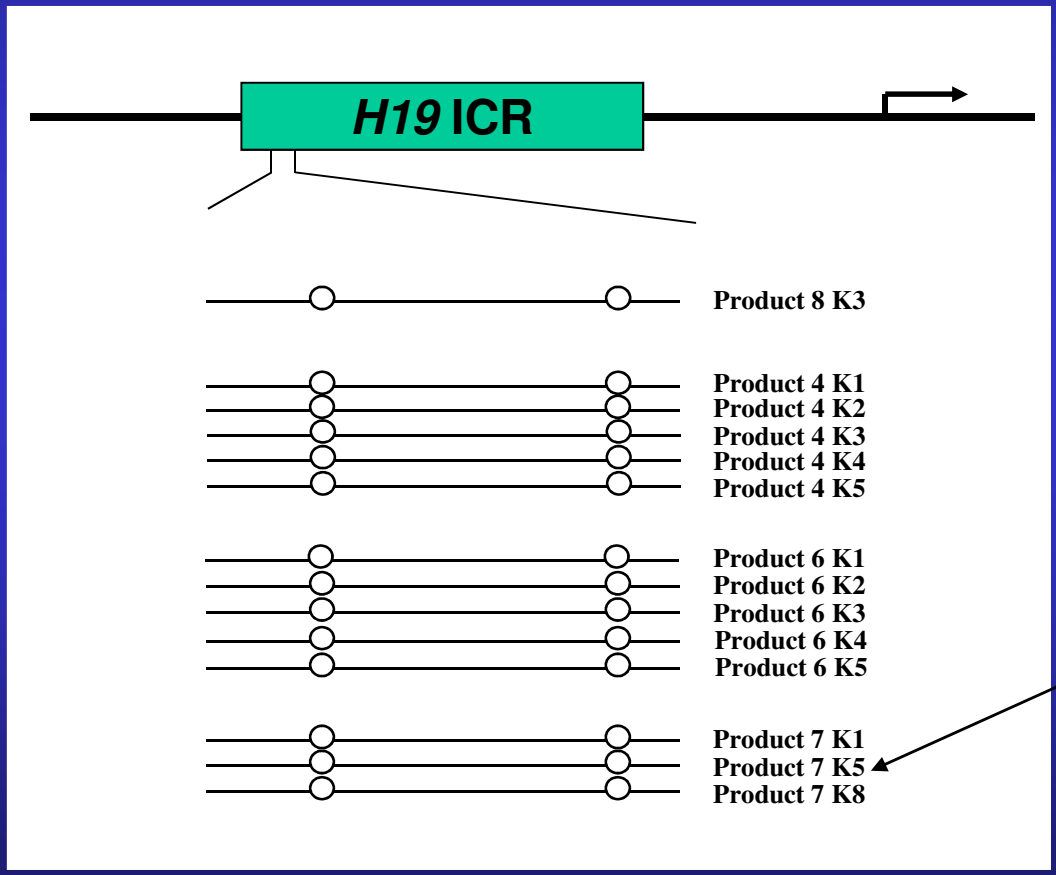
- 100 µl diluted bisulfite-converted DNA are distributed into 10 PCR tubes with 10 µl template each (equivalent to one oocyte).
- 6 control tubes without template DNA.
- 1st round Multiplex PCR with amplicons for three genes (*H19* , *Igf2r*, and *Snrpn*).
- AmpliTaq Gold (Applied Biosystems).

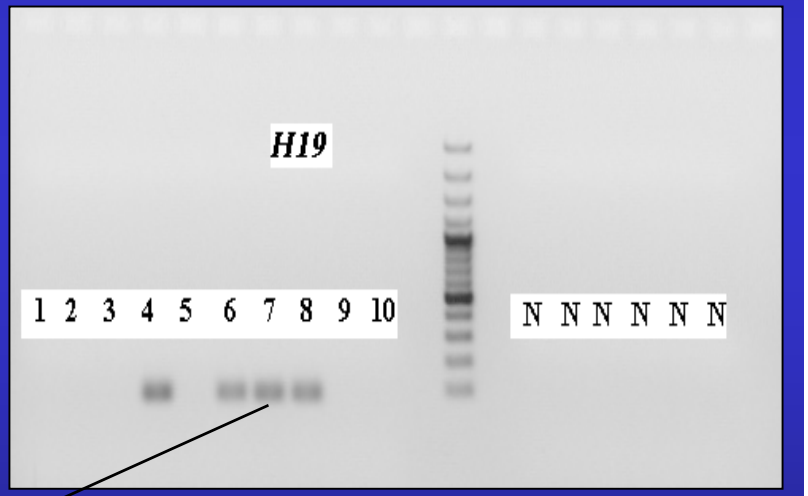


- **Optimized 2nd round nested PCR assays for the three studied genes (*H19*, *Igf2r*, and *Snrpn*) in Singleplex reactions.**
- **FastStart Taq system (Roche).**
- **Classic bisulfite (plasmid) sequencing.**
  Direct sequencing.
  Bisulfite pyrosequencing.



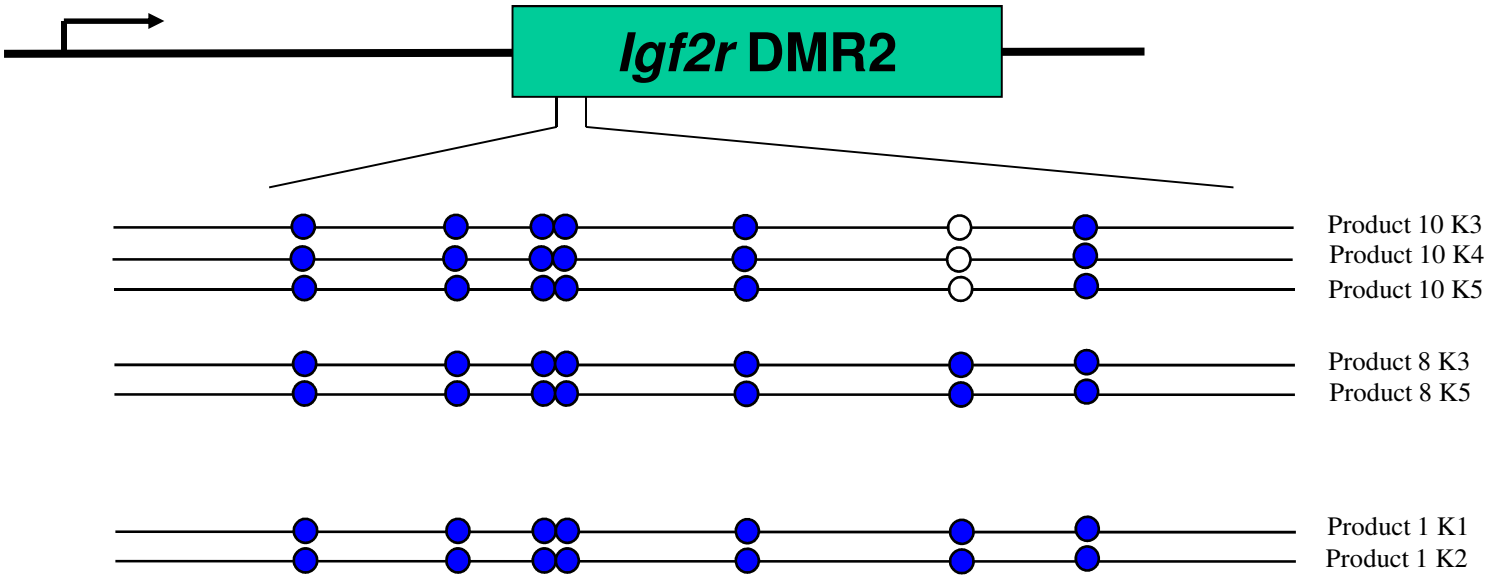
Classical bisulfite sequencing of LD products from 10 oocytes: *H19*

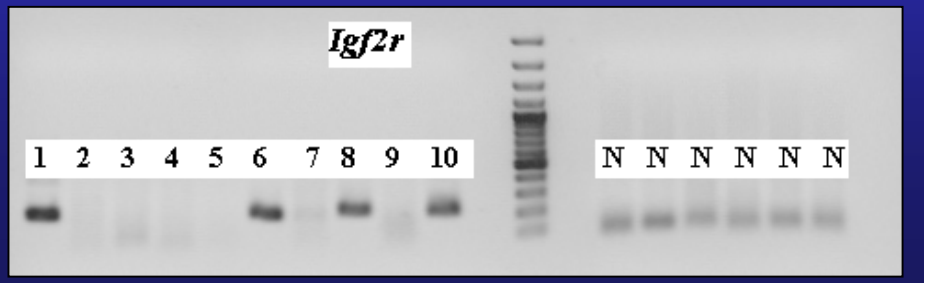




Nested PCR products of *H19* (100 bp ladder)

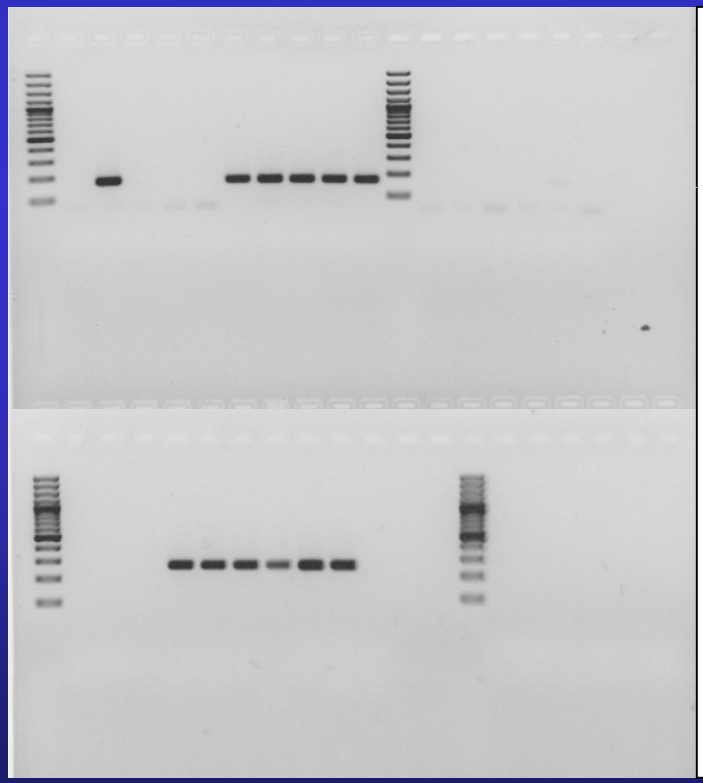
Classical bisulfite sequencing of LD products from 10 oocytes: *Igf2r*



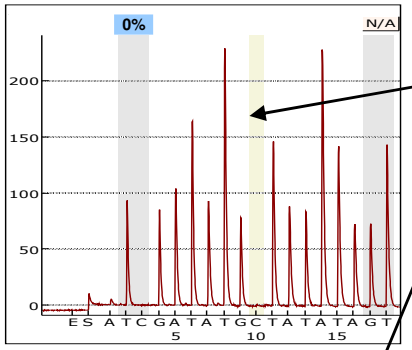


Bisulfite pyrosequencing of LD products from 10 oocytes (multiplex reaction):

H19 and *Snrpn*



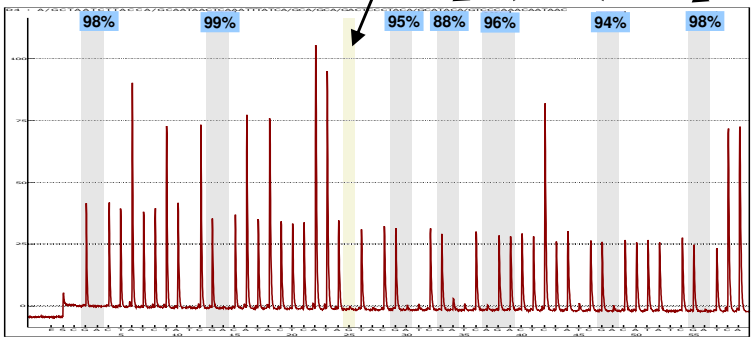
H19



Control for cytosine conversion

Methylation percentage of CpGs

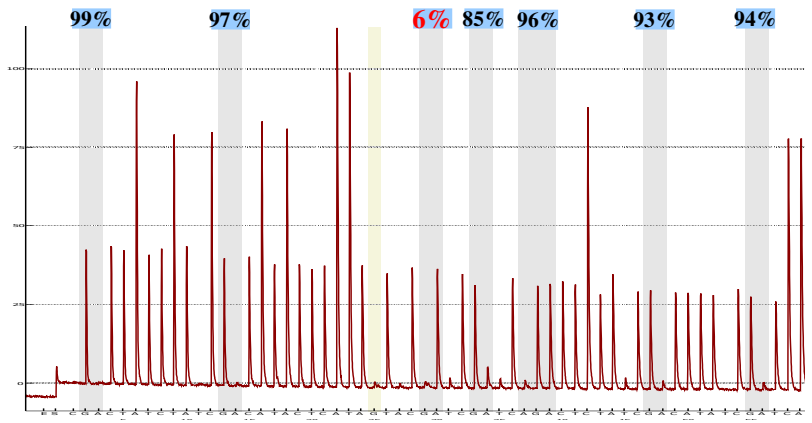
Snrpn

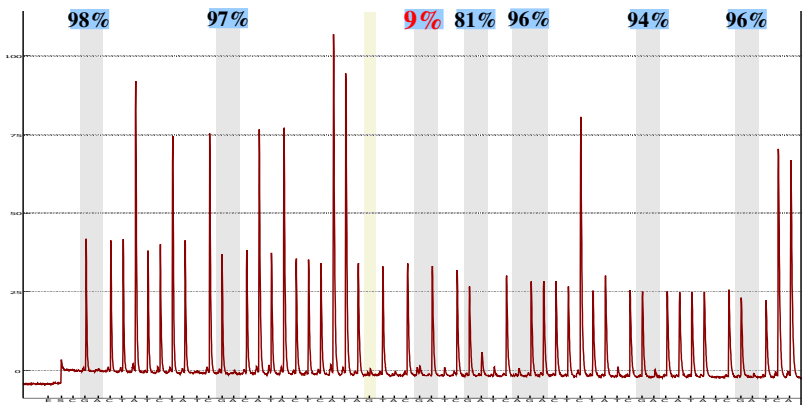


Bisulfite pyrosequencing of LD products reveals

Snrpn methylation errors at single CpG sites

- in vivo mouse oocytes — 8/241 CpGs (3%)
- in vitro matured oocytes — 8/246 CpGs (3%)
- vitrified IVM oocytes — 14/228 CpGs (6%)

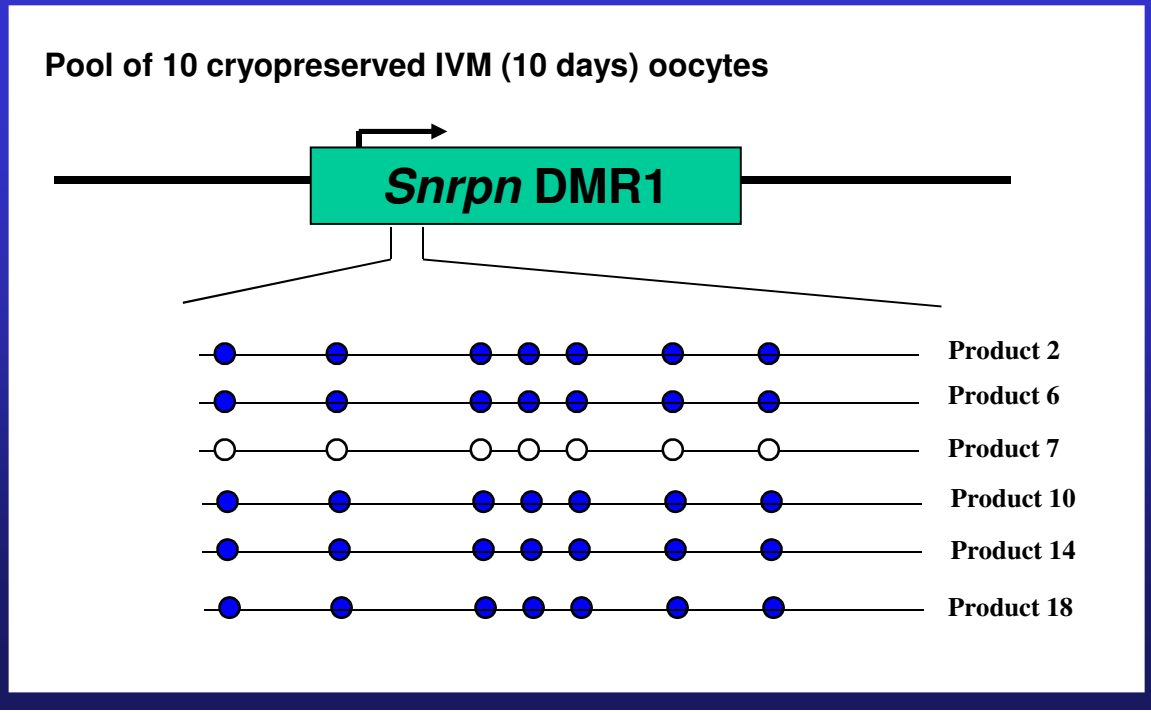




Stochastic errors
(most likely without functional implications)

Bisulfite pyrosequencing of LD products reveals abnormal *Snrpn* DMR methylation (imprinting mutation)

- in vivo mouse oocytes — 0/28 alleles (0%)
- in vitro matured oocytes — 2/32 alleles (6%)
- vitrified IVM oocytes — 1/30 alleles (3%)



Bisulfite pyrosequencing of LD products reveals

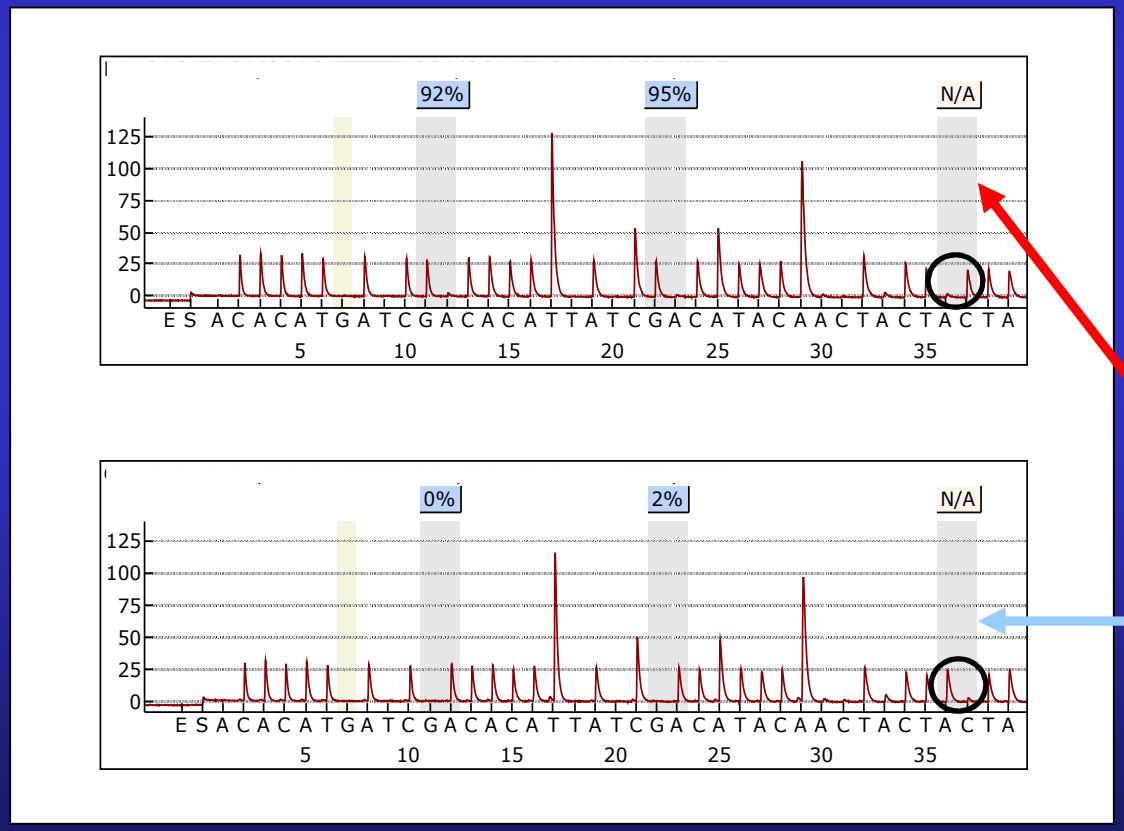
H19 methylation errors at single CpG sites

- in vivo mouse oocytes — 0/105 CpGs (0%)
- in vitro matured oocytes — 1/ 90 CpGs (1%)
- vitrified IVM oocytes — 3/127 CpGs (2%)

abnormal *H19* DMR methylation

- in vivo mouse oocytes — 0/24 alleles (0%)
- in vitro matured oocytes — 4/24 alleles (17%)
- vitrified IVM oocytes — 0/27 alleles (0%)

Bisulfite pyrosequencing of LD products from single morula-stage (C57BL/6J xCAST/Ei) hybrid embryo



SNP to distinguish between the maternal (*Mus musculus*) and the paternal (*M. castaneus*) alleles

Snrpn

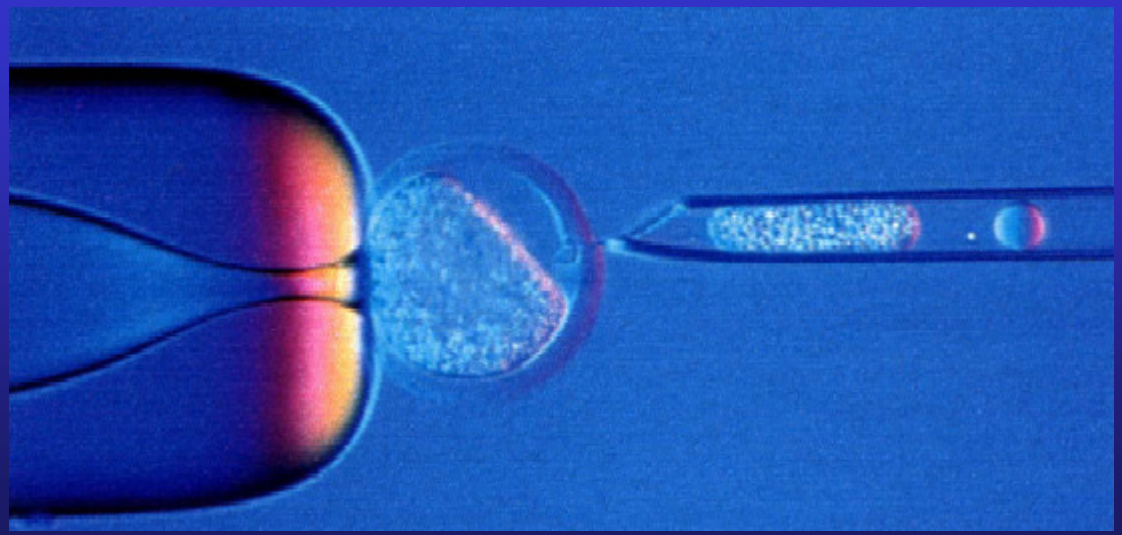
- **Limiting dilution combined with bisulfite sequencing or bisulfite pyrosequencing allows the methylation analysis of 3-5 genes from pools of 10 mouse oocytes or single morula stage embryos.**
- **Mouse hybrids allow one to distinguish between paternal and maternal alleles by analyzing SNP positions.**
- **Amplicon lengths up to 300 bp give reliable results. Targets larger than 300 bp are more likely to be fragmented during the bisulfite conversion step.**
- **Methylation errors occur at the level of individual CpG sites (stochastic errors) and at the level of the entire allele (imprinting mutations).**

ART is associated with an increased risk for

... congenital malformations (factor 1.3-1.4)

... low and very low birth weight (factor 2-3)

... some rare imprinting disorders (factor 3-12)



So far it is not clear

- whether epigenetic changes during gametogenesis and early embryogenesis and/or
- parental factors associated with infertility

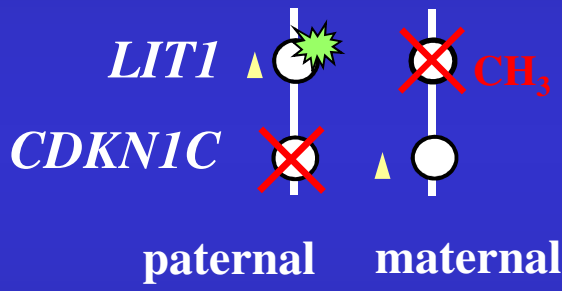
are responsible for these medical problems.

Beckwith-Wiedemann syndrome

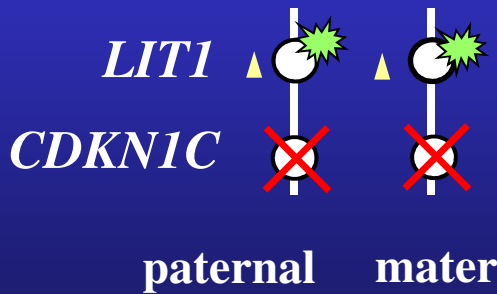
(Exomphalos, Macroglossia, Gigantism)

Despite a 3-9fold increased prevalence, the absolute risk of receiving an IVF/ICSI child with BWS remains low (1 in 5.000)´.

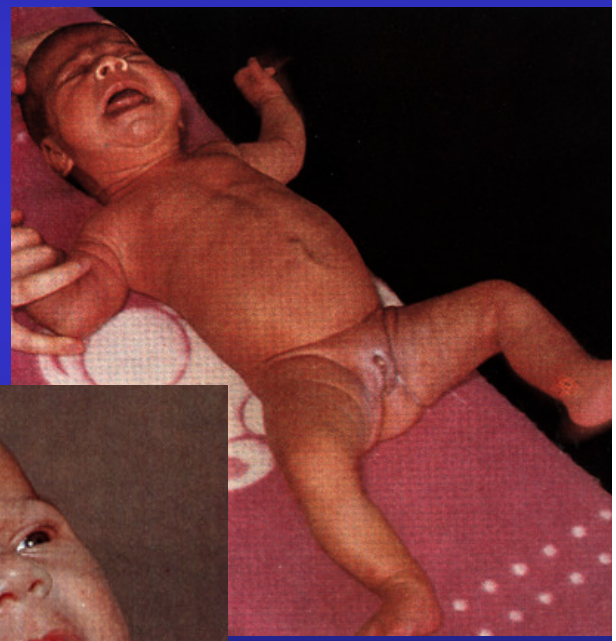
Loss *LIT1* imprinting on chromosome 11p15

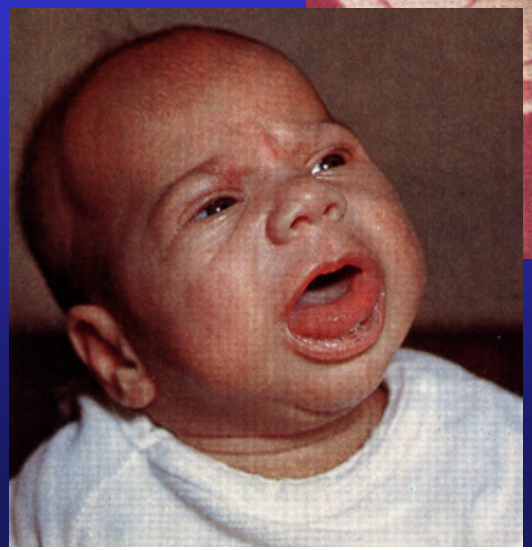


Normal situation



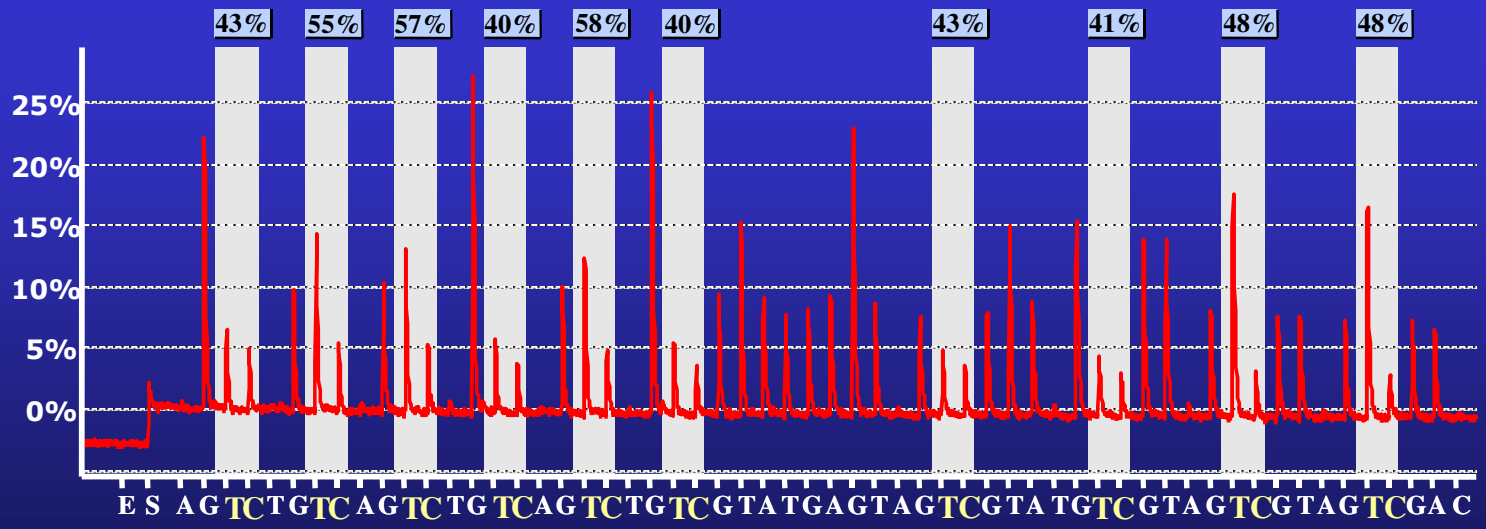
Imprinting defect in IVF/ICSI children





Are rare imprinting disorders in IVF/ICSI children only the tip of an iceberg ???

- **Systematic studies of the association between human ART and methylation reprogramming defects are warranted.**
- **We perform methylation analyses of developmentally important genes in pregnancy losses after IVF/ICSI and of spontaneous conceptions.**
- **Genomic DNA from chorionic villi and fetal muscles is analyzed by bisulfite pyrosequencing.**

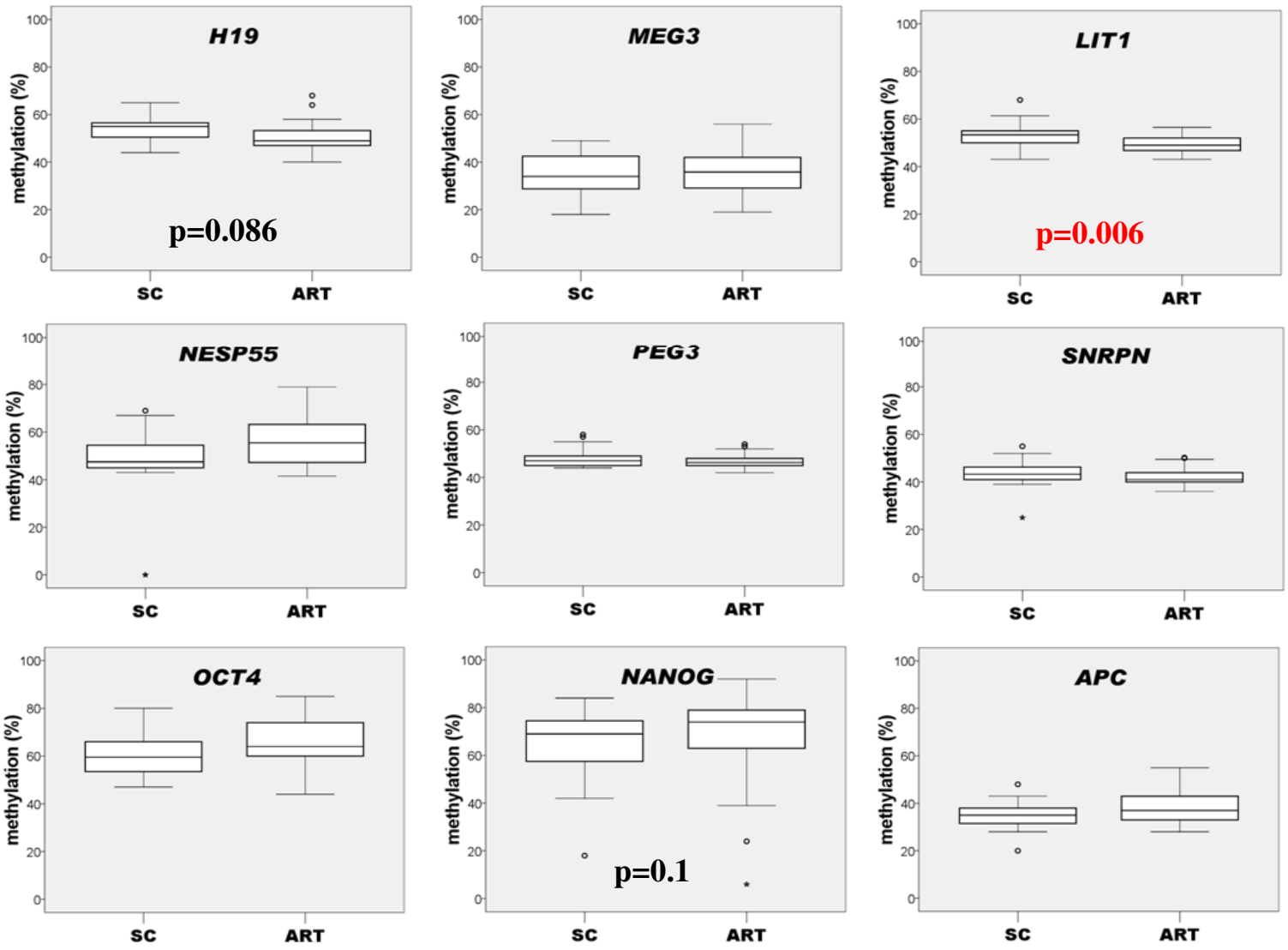


PEG3
(47% methylation)

Methylation analyses of CVS from abortions and stillbirths after assisted reproduction (ART) and of spontaneous conception (SC)

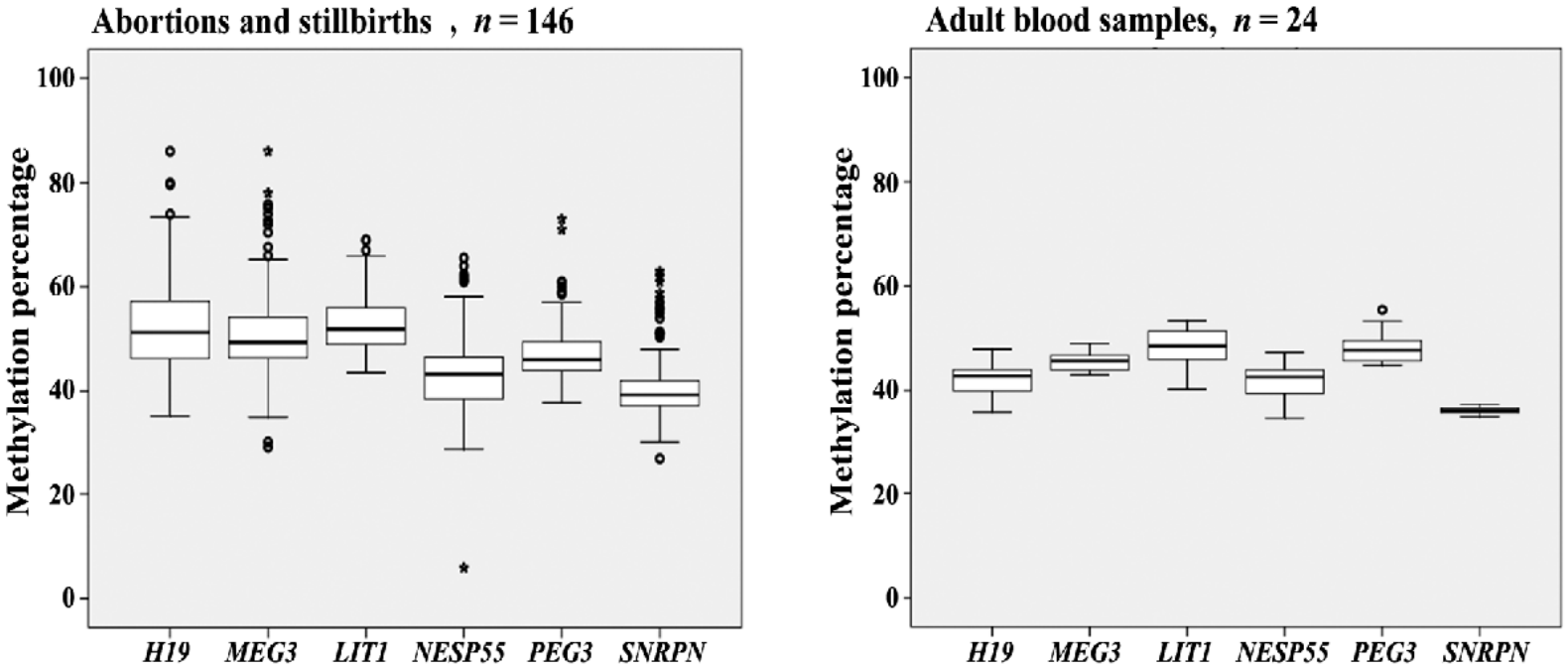
30 CVS after spontaneous conception (SC)

40 CVS after ART



- **We did not find an increased rate of extreme (potentially abnormal) DMR or promoter methylation values after ART, but significant and trend differences in the methylation levels of *LIT1*, *H19*, and *NANOG* between ART and non-ART samples.**
- **It is plausible to assume that the observed methylation differences at 3 of 9 loci studied are an indicator for more profound epigenetic differences.**
- **ART or factors associated with parental infertility can affect the epigenome of the next generation.**
- **The role of stochastic and/or environmentally induced epimutations for human phenotopic variation and disease is likely to be largely underestimated.**

Methylation analysis of fetal muscles from abortions and stillbirths of spontaneous conception



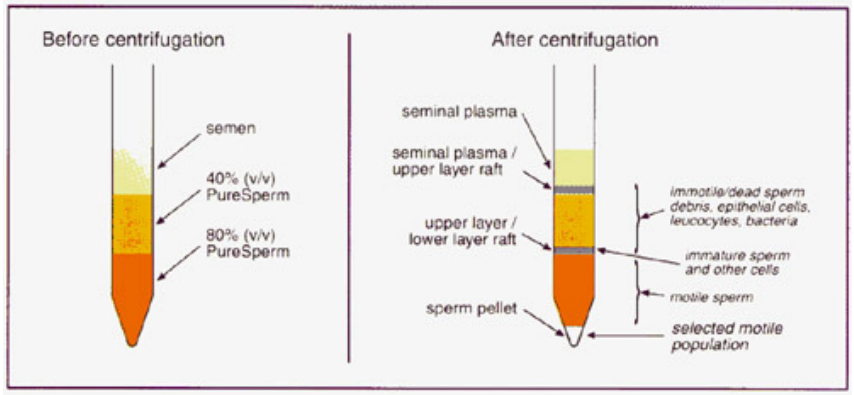
- Compared to adult blood samples, fetal muscles from abortions and stillbirths exhibit a much broader range of methylation variation and a higher proportion of extreme methylation values.

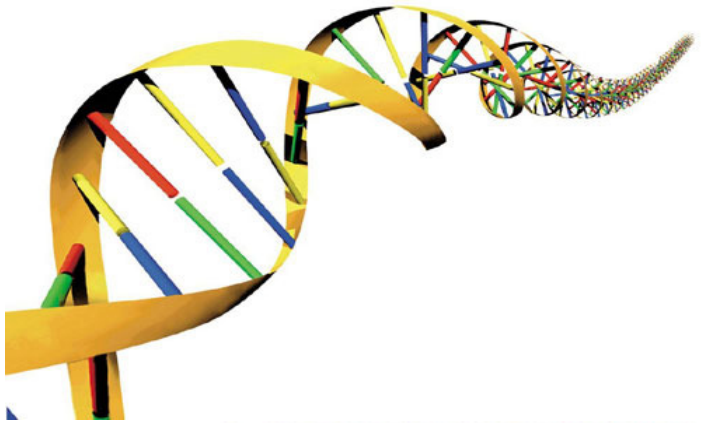
- **The vast majority (>90%) of outliers are hypermethylated DMRs. This points to stochastic failures of the mechanism which normally protects the hypomethylated allele from de novo methylation after fertilization.**
- **The observed methylation abnormalities are consistent with a mosaic state that is the presence of hypermethylated and normally methylated cells in the same sample.**
- **Multifactorial threshold model for epigenetic pregnancy loss: If extreme methylation values due to primary epimutations in imprinted and/or other developmentally important genes exceed a critical threshold, the phenotype becomes manifest. Similar to other multifactorial diseases, additional genetic and environmental factors might also play a role.**

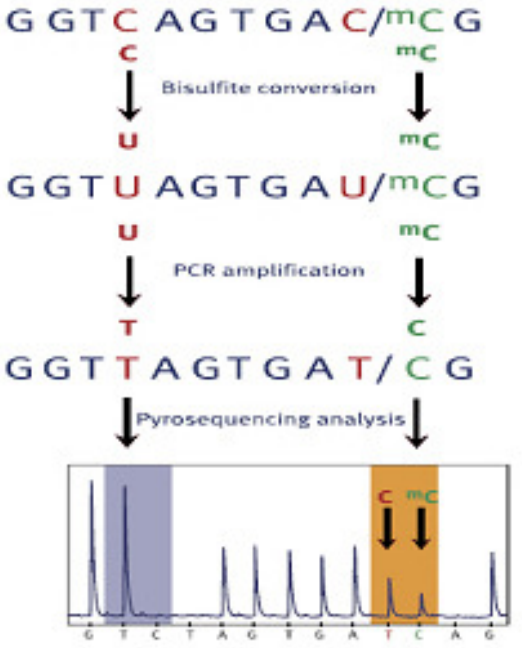
Subgroup Sample no.	Weeks gestation	Methylation value						No of outliers	Phenotype		Karyotype/ Genital sex
		H19	*MEG3*	*LIT1*	*NESP55*	*PEG3*	*SNRPN*		Placenta	Fetus	
Spontaneous abortions											
2	17	↔	↔	↑	↔	↑	↔	2	CHA	NAD	46,XY
118	20	↔	↑↑	↔	↔	↑↑	↑	3	RC	NAD	46,XY
Stillbirths											
1	35	↑	↑↑	↔	↑	↔	↑↑	4	VMI	NAD	male
6	37	↑	↑	↔	↑	↑	↑	5	VMI	NAD	46,XY
7	32	↔	↔	↔	↑	↔	↑	2	VMI	NAD	46,XY
16	22	↔	↑	↑	↔	↑	↑	4	VMI	NAD	female
30	36	↑	↑↑	↔	↑	↑↑	↑↑	5	VMI	NAD	46,XX
53	28	↑	↑↑	↔	↔	↑	↑↑	4	VMI	NAD	46,XY
93	22	↔	↑	↔	↔	↔	↑	2	VMI	NAD	46,XY
102	37	↔	↑	↔	↑	↔	↑↑	3	VMI	NAD	46,XY
104	26	↔	↑	↔	↑	↑	↑↑	4	CHA	NAD	46,XY
134	24	↔	↔	↑	↔	↑	↔	2	NAD	Lymphangioma	male

- **Two of 55 (4%) spontaneous abortions and 10 of 57 (18%) stillbirths displayed extreme methylation values in multiple genes.**
- **All but one abortion/stillbirth with multiple methylation abnormalities were male, indicating that the male embryo may be more vulnerable to epigenetic reprogramming defects.**

Detection of primary epimutations (faulty methylation patterns) in sperm

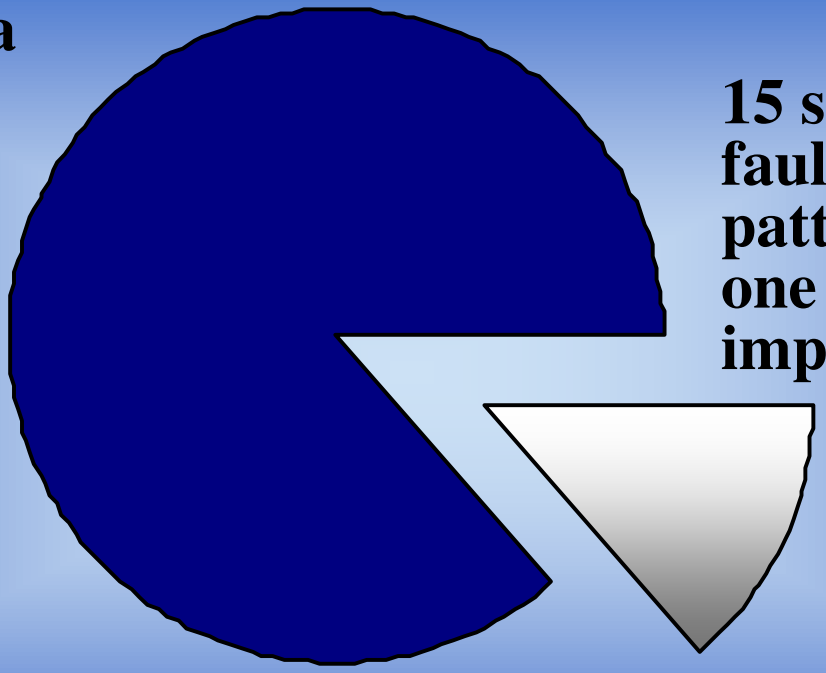






High prevalence of germline reprogramming defects in ICSI sperm

100 sperm samples of males attending a fertility center

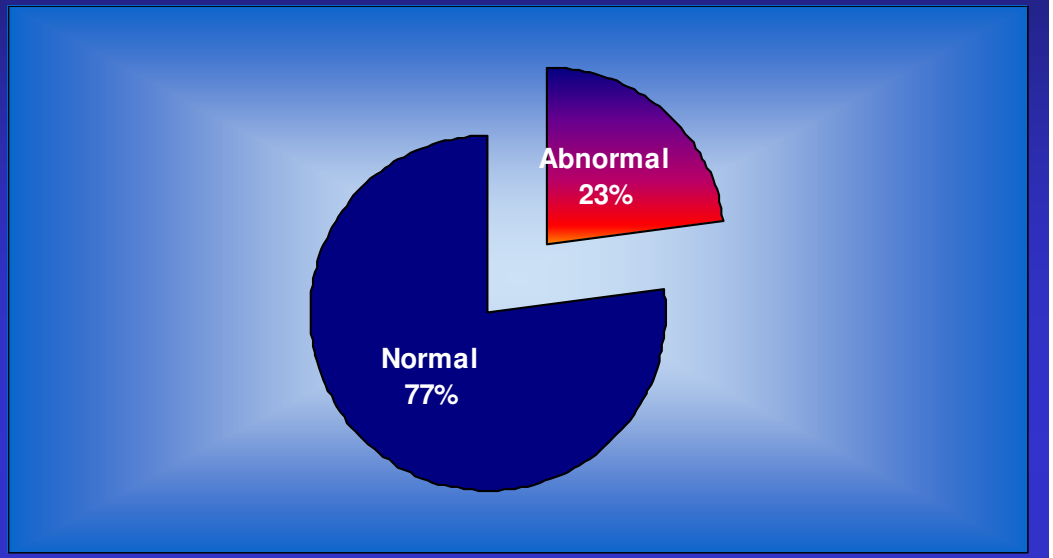


15 samples exhibited faulty methylation patterns in at least one the 7 studied imprinted genes

Abnormal methylation patterns and sperm quality

<20% sperms with normal morphology

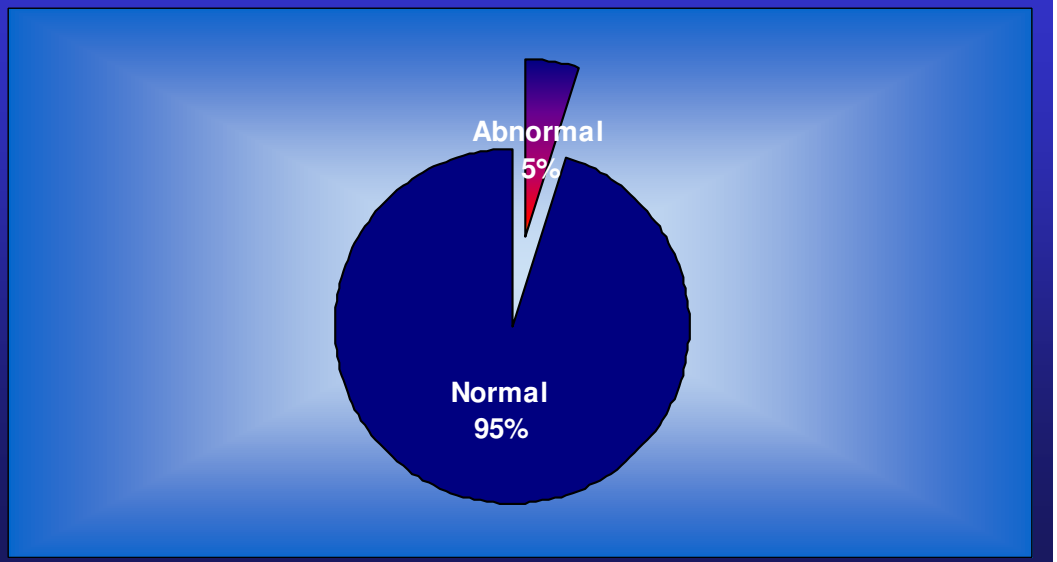
13/57 = 23%



p<0.05

>20% sperms with normal morphology

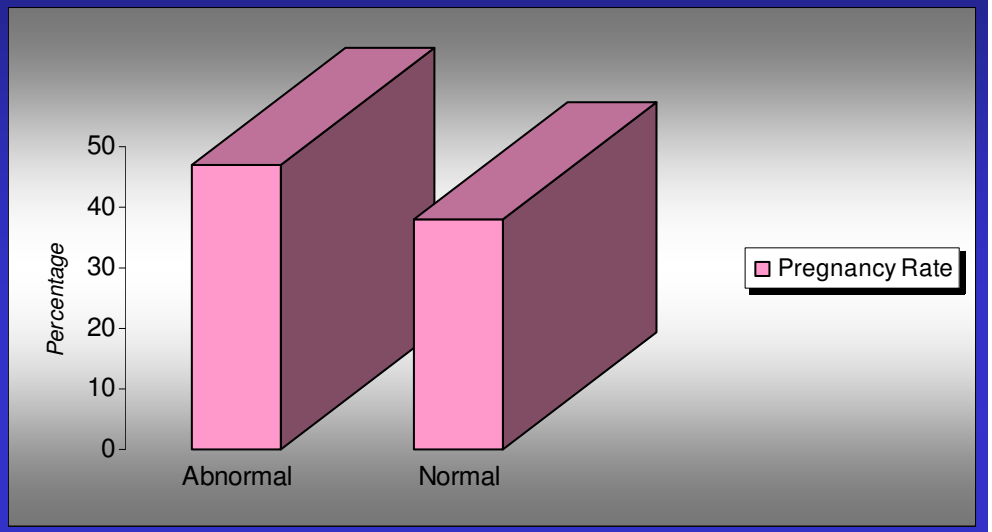
2/39 = 5%



Abnormal methylation patterns and ART outcome

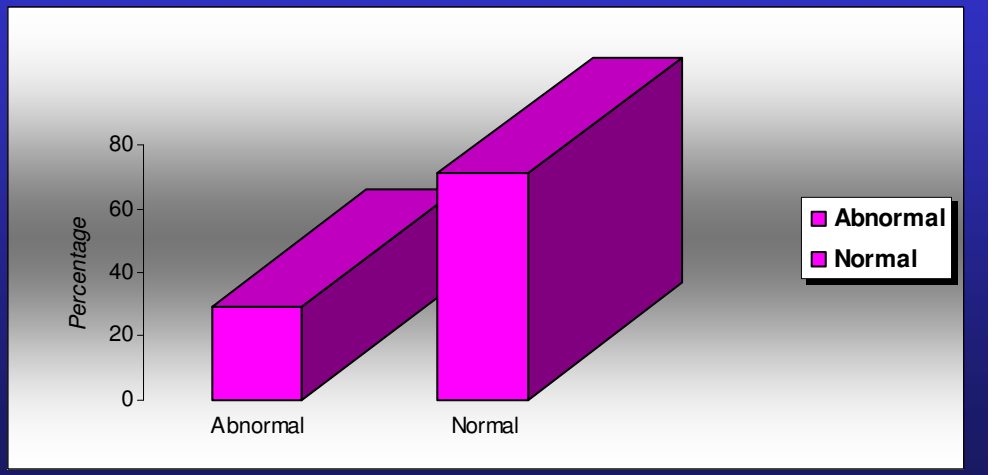
All sperm samples (n=96)

Methylation	Pregnancy Rate
Abnormal	7/15 = 47%
Normal	31/81 = 38%

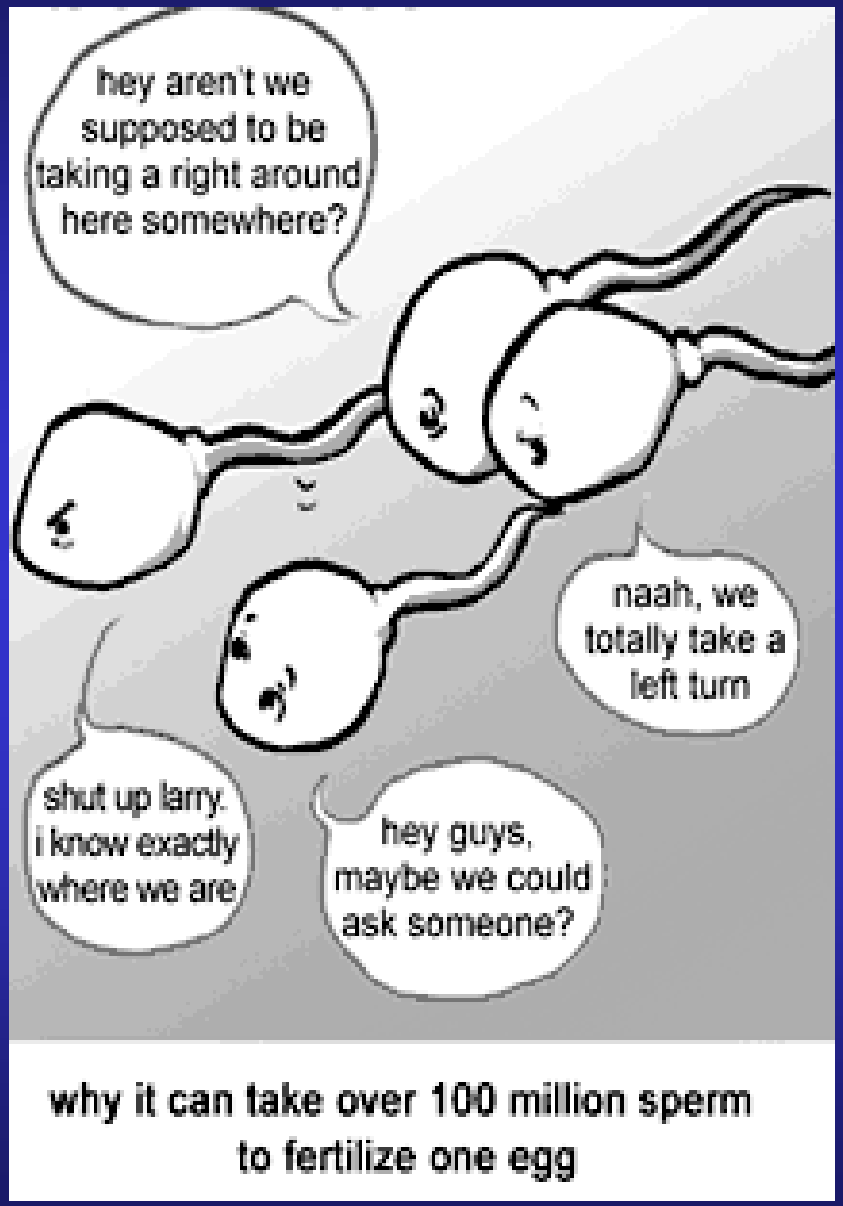


Oligospermic samples (n=14)

Methylation	Pregnancy Rate
Abnormal	2/7 = 29%
Normal	5/7 = 71%



- Our results suggest a role for germline reprogramming defects in male infertility.
- Transmission of epimutations by the sperm (and/or the oocyte) into the zygote may account for at least some of the mediacl problems that have been associated with ART



Conclusions

- The male and female germline-derived epigenomes must be reprogrammed after fertilization for somatic development.
- Disturbances in this highly coordinated process may contribute to developmental failures and defects.
- Much of the existing DNA methylation variation and abnormalities may result from stochastic events during epigenetic reprogramming in gametogenesis and early embryogenesis.
- The spontaneous epimutation rate is modified by genetic and environmental factors.
- Normal conception and assisted reproduction appear to be susceptible to similar dysregulation of epigenetic components.

- **Imprinting defects that can be linked to a defined syndrome(s)/disease(s) appear to be rare in human ART.**
- **Significant differences in the methylation levels of some genes (within the range of normal variation) between ART and non-ART conceptions indicate that ART-associated factors can affect the epigenome of the next generation.**
- **A better understanding of epigenetic reprogramming in gametogenesis and early embryogenesis and of possible genetic modifiers and environmental factors is crucial for improving human infertility treatment.**

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