IGF2 and *H19* methylation in sperm of infertile men

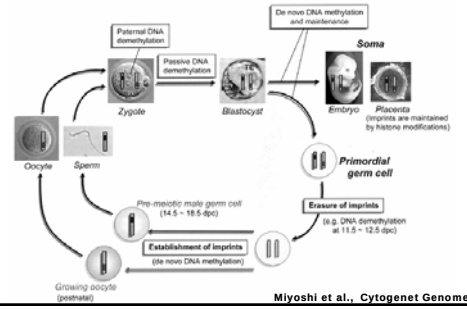
Pierre Jouannet, Céline Chalas, Hélène Jammes

Biologie de la Reproduction, Hôpital Cochin, Université Paris5
INSERM Institut Cochin U 567, CNRS UMR 8104

Epigenetics in ART, ESHRE Campus, LISBON 2008

IGF2 and *H19* locus, a paradigme of imprinted genes

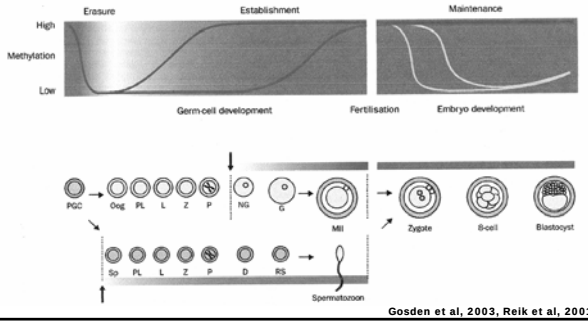
Imprinted genes are expressed only from one allele in a parent-of-origin-dependent manner. The most prominent form of imprinting is the allele-specific differential DNA methylation within Differentially Methylated Regions (DMRs) occuring predominantly at CpG dinucleotide position



Miyoshi et al., Cytogenet Genome Res 2006

Programmed demethylation and methylation of genomes of developing oocytes, spermatozoa, and embryos

After global demethylation occuring at early stages of germ cell development, new imprints should be established according to the sex of the germ line



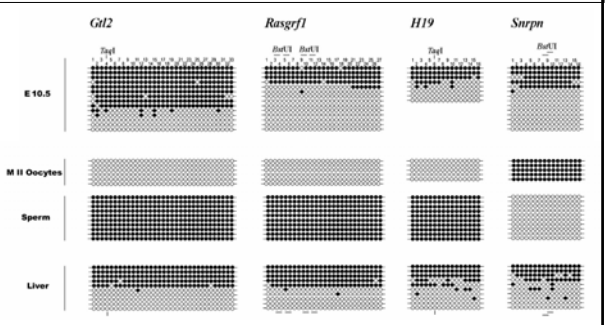
Gosden et al, 2003, Reik et al, 2001

Paternal methylation imprinting of *H19 gene*
during human spermatogenesis

Fetal spermatogonia (24 weeks)	**100%**	**unmethylated**
Adult spermatogonia	**25%**	**unmethylated**
	6%	**hypomethylated**
	69%	**methylated**
Adult spermatocytes	**5%**	**hypomethylated**
	95%	**methylated**
Adult spermatids	**100%**	**methylated**

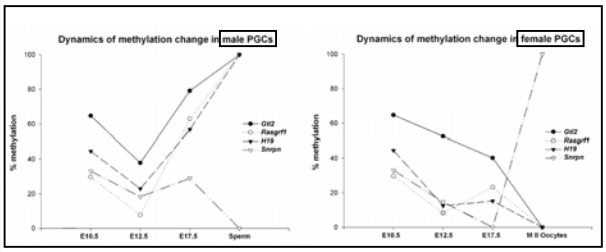
Kerjean et al, Hum Mol Gen, 2000

Establishment of paternal methylation imprints in the mouse



Li et al, Genomics, 2004

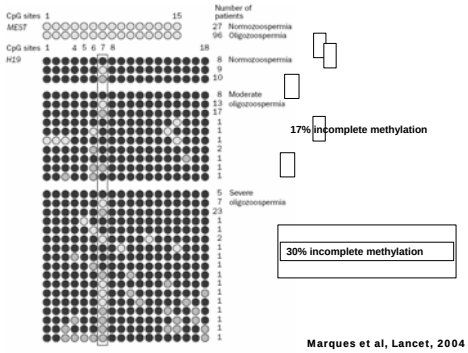
Establishment of paternal methylation imprints in the mouse



Li et al, Genomics, 2004

Are there disruptions of imprinting gene methylation in human sperm, mainly in case of spermatogenetic failure?

MEST and *H19* methylation in infertile men spermatozoa



Marques et al, Lancet, 2004

Methylation status of *H19* in human sperm

18 CpG of *H19* DMR
including the 4 informative CpGs of the 6th CTCF binding site

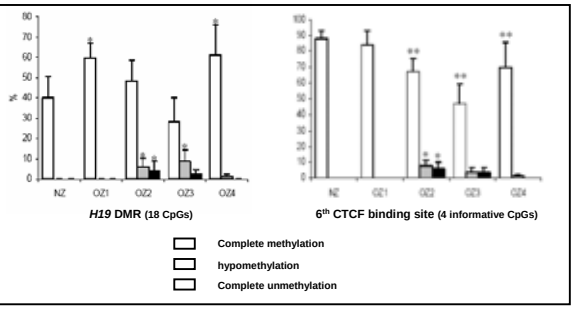
Supplementary Table 1. Methylation status of *H19* in human sperm

Groups	Clones	Number of unmethylated CpGs													
	N	0	1	2	3	4	5	6	9	10	14	15	16	17	≥9
Controls	72	28	30	10	2	2									
Oligozoospermia															
Mild	76	44	25	5	2										
Moderate	101	48	29	12	3		2						1	6	7
Severe	84	26	35	10	4	1		1	3	1		1		2	7
Very severe	86	51	24	32	7						1				1

Number of unmethylated CpGs

Marques et al, Mol Hum Reprod, 2008

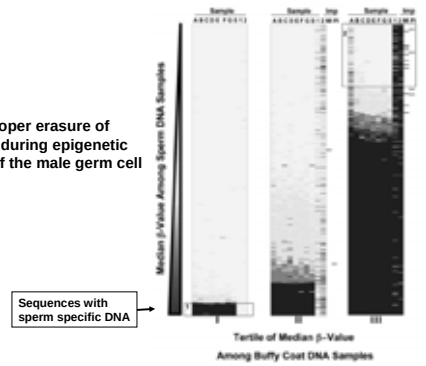
H19 DMR and 6th CTCF binding site methylation according to human sperm concentration



Marques et al, Mol Hum Reprod, 2008

Broad epigenetic defects associated with abnormal semen parameters

Hypothesis: Improper erasure of DNA methylation during epigenetic reprogramming of the male germ cell line



Houshdaran et al., PLoS ONE, 2007

Alterations of methylation state in human sperm

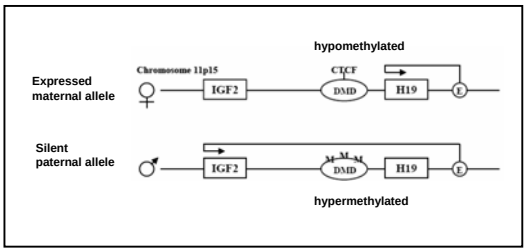
	Global / Gene	Sperm	Methylation imprinting Maternal (MI) - Paternal (PI)
Manning et *al.*, 2001	*SNRPN*	mixed	57-63% abnormal MI
Benchaib et *al.*, 2003	Global	mixed	in teratozoospermia
Marques et *al.*, 2004	*MEST*	normal	Unmethylated, normal MI
	H19	normal	Methylated, normal PI
		oligo	17-30% incomplete PI
Houshdaran et *al.*, 2007	*NTF3, MT1A*	mixed	Hypermethylation
	PAX8, PLAGL1		
Kobayashi et *al.*, 2007	*H19, GTL2*	oligo	14 % abnormal PI
	PEG1, LIT1, ZAC		21 % abnormal MI
	PEG3, SNRPN		
Marques et *al.*, *2008*	*MEST*	oligo	14 % abnormal MI
	H19	oligo	47% abnormal PI
	*H19*DMR6thCTCF	oligo	

Imprinting status analysis
of *H19* and *IGF2* Differentially Methylated Regions
in normal and infertile men

17 men with normal sperm parameters
19 men with isolated alteration of sperm mrphology
22 men with oligo-astheno-teratozoospermia

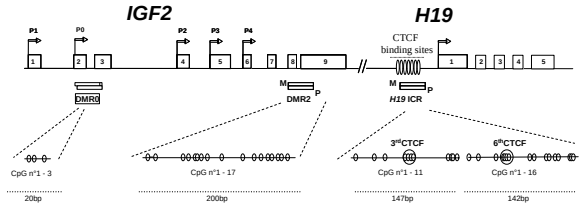
Methylation status of 47 CpGs arranged in 4 clusters localized in DMR0 and DMR2 of *IGF2* gene
and in *H19* DMR (3rd and 6th CTCF binding site)
after bisulfite conversion of genomic DNA by pyrosequencing

Genomic imprinting at the *IGF2*/*H19* locus



Biermann and Steger, J Androl 2007

Organization of human *IGF2-H19* locus



4 amplified regions
Methylation state analysis of 47 CpG by pyrosequencing

Chalas et al, to be published

Genomic DNA treatment by sodium Bisulfite

Genomic DNA Extraction

Methylated allele	Unmethylated allele
5'.TGTC [mCG] ACC.3'	5'.TGTC [CG] ACC.3'
3'.ACAG [GCm] TGG.5'	3'.ACAG [GC] TGG.5'

⇩

Sodium bisulphite conversion

5'.TGTU [mCG] AUU.3'	5'.TGTU [UG] AUU.3'

⇩

PCR amplification

5'.TGTT [CG] ATT.3'	5'.TGTT [TG] ATT.3'

⇩

Analysis by pyrosequencing

Epigenetic mark ⇩ Single Nucleotide Polymorphism

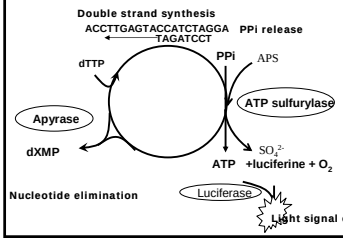
Chalas et al, to be published

Analysis of amplified bisulfite converted DNA by pyrosequencing

Automated pyrosequencing instrument, Biotage, Uppsala, Sweden

Pyrosequencing (Dupont et al., 2004; Tost et al., 2007)

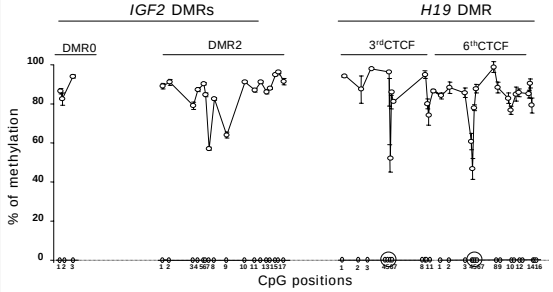
- is a realtime sequencing-synthesis technology
- using PCR products as template
- permits a quantification of incorporated nucleotide



- Specific nucleotide incorporation
- Light signal integration
- Nucleotide incorporation quantification
- Methylation quantification of each CpG (%)

Chalas et al, to be published

Quantitative methylation status analysis in Normozoospermia patients (NZ)

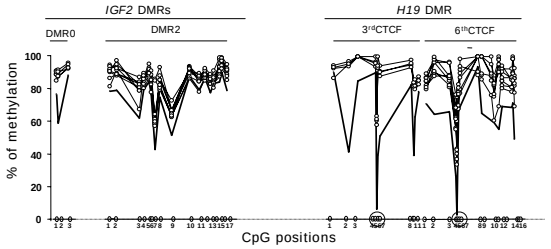


Results expressed as mean ± SD (n = 17)

Chalas et al, to be published

Quantitative methylation status analysis
in teratozoospermia patients (T)

42 % of Teratozoospermia patients exhibited a similar methylation pattern than in Normozoospermia patients.



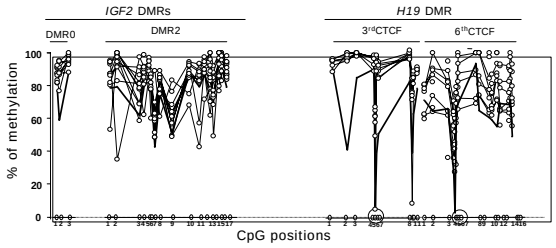
— **Threshold defined as methylation mean of NZ patients - 2 SD**

Chalas et al, to be published

Quantitative methylation status analysis
in teratozoospermia patients

58 % of Teratozoospermia patients exhibited

- a sporadic loss of methylation at specific CpG positions of the *IGF2*-DMR2 and the *H19* DMR 6th CTCF
- without modification of methylation pattern at *IGF2*-DMR0 and *H19* DMR 3rd CTCF

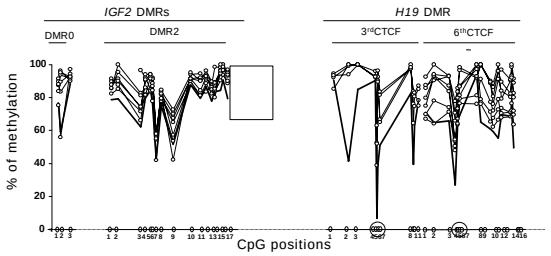


— **Threshold defined as methylation mean of NZ patients - 2 SD**

Chalas et al, to be published

Quantitative methylation status analysis
in oligo-astheno-teratozoospermia patients (OAT)

41 % of OAT patients exhibited a similar methylation pattern than in NZ patients.



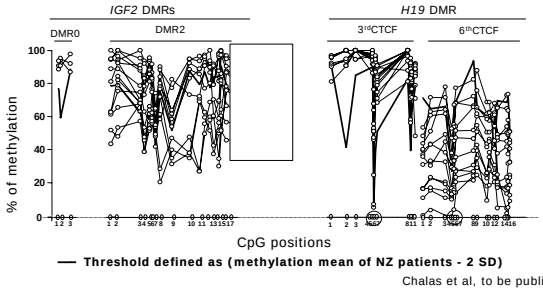
— **Threshold defined as methylation mean of NZ patients - 2 SD**

Chalas et al, to be published

Quantitative methylation status analysis in oligo-astheno-teratozoospermia patients (OAT)

59 % of OAT patients exhibited

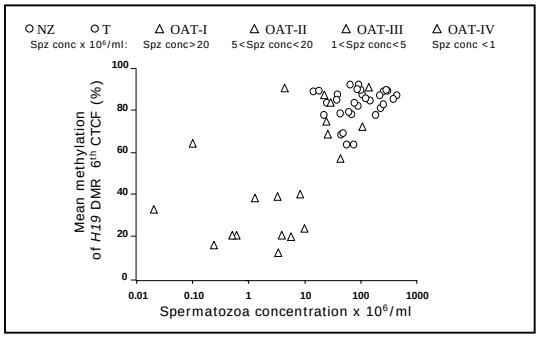
- A severe loss of methylation of the *H19* ICR 6th CTCF, sometimes associated with an *IGF2* DMR2 altered methylation pattern
- Without methylation alteration of DMR0 and *H19* ICR 3rd CTCF



— Threshold defined as (methylation mean of NZ patients - 2 SD)

Chalas et al, to be published

Methylation status of the *H19* DMR 6thCTCF and the spermatozoa concentration



Chalas et al, to be published

Intra and interindividual epigenetic variation In human germ cells

Flanagan et al, Am J Hum Genet, 2006

- 46 healthy men

- BRCA1, BRCA2, HD, DM1, PSEN1, PSEN2

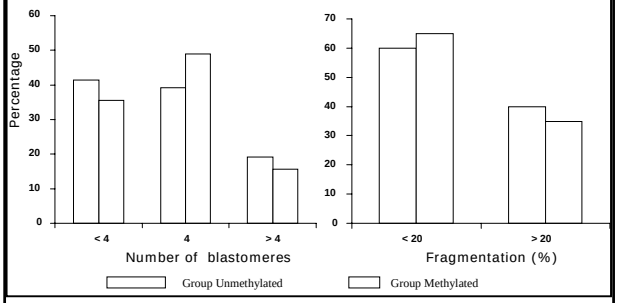
- Mapping of methylated cytosines
- Degree of epigenetic diversity in biosulphite modification-based experiments
- Micro array-based DNA methylation analysis

- Methylation dynamic with age

The male germ line exhibits locus-, cell- and age-dependent DNA methylation differences.
DNA methylation variation is signficant accross unrelated Individuals at a level that exceeds DNA sequence variation.

What are the consequences
on phenotypes and epigenetic inheritance?

Methylation status of the *H19* DMR 6thCTCF and Embryonic cleavage 2 days after IVF-ICSI



Chalas et al, to be published

Influence of the methylation status of *H19-IGF2* locus on IVF- ICSI outcome (treatment cycles = 43, 21 men)

	Methylation	
	normal (n=27)	**low (n=16)**
Pregnancy rate	**28%**	**15.8 %**
Delivery term (weeks)	**40.7 ± 0.58**	**40.3 ± 0.50**
Birth weight (g)	**3090 ± 394**	**3053 ± 91**

Chalas et al, to be published

Influence of global sperm DNA methylation on IVF results

Normal or subnormal sperm, anti - ^{5m}Cyt. antibody

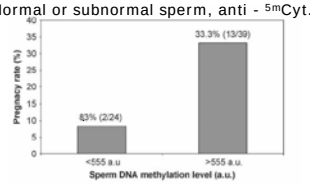


Table I. Odds ratio (OR) of prognostic parameters obtained with logistic regression analysis

	OR	CI	*P*
Oligoasthenospermia (yes/no)	3.52	0.60–20	NS
Asthenozoospermia (yes/no)	3.52	0.12–100	NS
Embryo quality: rate of (a + b) embryo grade (< 60%, > 80%)	6.33	1.59–25.0	< 0.01
Sperm DNA methylation (< 555, > 555 AU)	5.49	1.01–30.30	< 0.05

CI = confidence interval; NS = not significant; AU = arbitrary units.

Benchaib et al., Hum Reprod, 2005

I - Originality of this work

In normozoospermia patients:

1) we reported for the first time the methylation status of the two IGF2 DMRs (DMR0 and DMR2) and of two regions of /H19/ ICR containing the 3rd and 6th CTCF binding sites

2) The quantitative pyrosequencing analysis described with a high accuracy the methylation status of the 47 CpG included in these four regions and found a mean of 86.8% ±6.9 of methylation for these four regions

3) A high methylation level was found in /IGF2/ DMR0.suggesting the possibility of transmission of paternally methylated allele in somatic cells after fertilization.

In infertile patients

1) For the /IGF2/ DMR and /H19/ ICR 6th CTCF binding site, the quantitative pyrosequencing analysis of normal sperm samples lead us to define a threshold of methylation for all CpGs positions and classify the patients in different groups according their methylation status

2) Very specific perturbations of the /H19-IGF2/ locus methylation pattern have been frequently detected in human spermatozoa produced by abnormal spermatogenesis. Surprisingly, no isolated loss of methylation was observed on the IGF2 DMR0 and the H19 ICR 3rd CTCF binding site

II- Questions and perspectives

- Could studies of DNA methylation be informative to predict gamete imprinting defects associated with disturbed spermatogenesis?

- Are DNA methylation alterations observed in spermatozoa independent or not of chromatin remodelling and what are the consequences?

- Is the methylation profile of imprinted genes in spermatozoa stable in a given man?

- Even if using abnormally methylated sperm is probably not influencing ART outcome, consequences of imprint methylation errors on growth abnormalities and health of children born from ART need further studies.