PRE-CONGRESS COURSE 10

SIG Andrology

"Paternal inheritance – sperm and epigenetics"

CONTENTS

PRE-CONGRESS COURSE 10 - PROGRAMME

SIG Andrology

Paternal inheritance – sperm and epigenetics

Course co-ordinators: J. Antonio Castilla (E) & L. Björndahl (S)

Course description: From an overview of basic concepts of epigenetic control of the inheritance to prospects of clinical applications.

Target audience: Clinicians, paramedicals and laboratory staff with an interest to widen the knowledge about basic and clinical andrology

Programme

09.00 - 09.30:	Welcome and introduction – Course coordinators
09.30 - 10.15:	DNA methylation in sperm: patterns, regulation and inheritance -***M. Benchaib (F)***
10.15 - 10.30:	*Discussion*
10.30 - 11.00:	***Coffee break***
11.00 - 11.30:	RNA in sperm and epigenetics: RNA and chromatin dynamics in human spermatozoa -***D. Miller (UK)***
11.30 - 11.45:	*Discussion*
11.45 - 12.15:	Sperm proteomic and epigenetics -***R. Oliva (E)***
12.15 - 12.30:	*Discussion*
12.30 - 13.30:	***Lunch***
13.30 - 14.00:	Imprinting in sperm of men with abnormal semen parameters -***M Sousa (P)***
14.00 - 14.15:	*Discussion*
14.15 - 14.45:	Epigenetic transgenerational actions of endocrine disruptors on reproduction and disease: the ghost in your genes -***M.K. Skinner (USA)***
14.45 - 15.00:	*Discussion*
15.00 - 15.30:	***Coffee break***
15.30 - 16.00:	RNA-mediated hereditary epigenetic variations (paramutations) in the mouse - ***M. Rassoulzadegan (F)***
16.00 - 16.15:	*Discussion*
16.15 - 16.45:	Sperm-Mediated Gene Transfer: mechanism and implications - ***C. Spadafora (E)***
16.45 - 17.00:	*Discussion*
17.00 - 17.30:	Concluding panel discussion – all speakers, co-ordinators
17.30 - 18.30:	SIGA Business Meeting

The author have disclosed all commercial relationship or other activities that might be perceived as a potential conflict of interest.

DNA methylation in sperm: patterns, regulation and inheritance.

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INTRODUCTION

Components of DNA

DNA is a polymer. The monomer units of DNA are nucleotides, and the polymer is known as a "polynucleotide." Each nucleotide consists of a 5-carbon sugar (deoxyribose), a nitrogen containing base attached to the sugar, and a phosphate group. There are four different types of nucleotides found in DNA, differing only in the nitrogenous base. The four nucleotides are given one letter abbreviations as shorthand for the four bases.

Purine Bases

- A is for adenine
- G is for guanine

Pyrimidine Bases

- C is for cytosine
- T is for thymine

A fifth base could be added the **5 methylcytosine.**

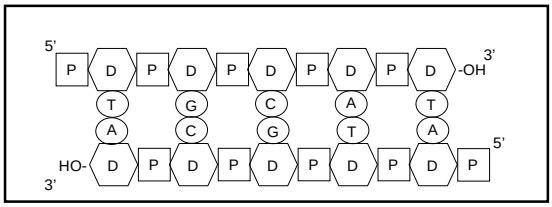
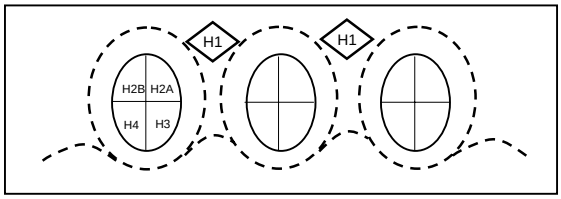


Figure 1 : Structure of DNA polynucleotide

P Phosphate group
HO- Hydroxyl
D Deoxyribose
G Guanine
A Adenine
C Cytosine
T Thymine

This bases are the primary constituents of genes, these one are transcript in RNA in order to give the protein.

The histone proteins H2A, H2B, H3, and H4 form octamers that constitute the nucleosome core particles in all eukaryotes. The histone H1 constitute the "linker" (figure 2).



DNA
Histone Core

Figure 2 : histones organisation.

Definition of epigenetic

The epigenetic phenomena are chemical modifications of the components of chromatin that are transmissible through mitoses and meiosis. All the components can be modified, by adding something for example by DNA methylation, or by acetylation or phosphorylation of the histones.
The primary structure of the DNA molecule is not modified

The N-terminal tails of histones are subject to post-translational modifications such as acetylation, phosphorylation, methylation, ubiquitination, glycosylation, and ADP ribosylation.

- Acetylation : acetyl group added by acetyl transferase on Lysine

- Phosphorylation : phosphate group added by Kinase on Serine/Tyrosine

- Methylation : methyl added by "Histone Methyl Transferase" on Lysine and Arginine.

In spermatozoa the histones have been replaced by protamines, but some of them remain (about 15 to 20% in human spermatozoa). These histones could constitute a part of the genome memory.

One change in chromatin structure that often precedes the activation of tissue-specific gene expression is DNA methylation.

DNA methylation is implied in various processes such as :

- parental imprinting,
- genome expression,
- X chromosome inactivation,
- differential gene expression.

This presentation will focus on the establishment of global sperm DNA methylation.

PATTERN

Definition of CpG sites

CpG sites are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases along its length.

"CpG" stands for cytosine and guanine separated by a phosphate, which links the two nucleosides together in DNA. The "CpG" notation is used to distinguish a cytosine followed by guanine from a cytosine base paired to a guanine.

Definition of CpG islands

There are regions of the DNA that have a higher concentration of CpG sites, known as CpG islands.

Many genes in mammalian genomes have CpG islands associated with their promotor. Because of this, the presence of a CpG islands is helpful for the prediction of gene behavior.

PATTERN

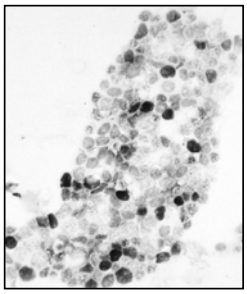
The methylation takes place on CpG dinucleotids: approximately 70% are methylated.

The CpG dinuclelotids are grouped in the regulating areas of genes and are implied in their expression.

The hypomethylation of a gene allows its expression because chromatin is not compacted (euchromatine) and allows the transcription.

The hypermethylation compacts chromatin (heterochromatine) and prevents the transcription.

Pattern of 5 methylcytosine (5mc)



Immunostaining of 5 methylcytosine on spermatids, pachytene cells of fragment of rat testis (DAB revelation, x 400).

Immunostaining of 5 methylcytosine on human spermatozoa (DAB revelation, x 1000).

The methylation is gradually set up at the time of the spermatogenesis. However DNA methylation modifications also occur in the epididymal tract, so epigenetic modifications could occur very late in sperm maturation. (figure 3).

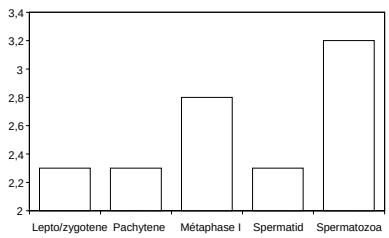


Figure 3 : DNA global methylation evolution during mice spermatogenesis (from Narayan et al, 1995).

Studies examining spermatogenesis-specific genes have shown that the acquisition of the appropriate pattern of DNA methylation by the sperm genome may represent a critical facet of sperm maturation.

This DNA methylation occurs during spermatogenesis but also during epididymal transit. Indeed, mammalian sperm DNA is reported to have a 5-methylcytosine (5mc) content lower than that in somatic cells from the same species, but higher than that in premeiotic germ cells.

During gametogenesis :

1. : the DNA methylation is deleted (spermatogonia)
2. : De novo methylation
3. : Even during the epididymal transit the methylation pattern could be modified (table 1).

Genes	Gonie A	Gonie B	Pachytene	Round cell	Epidim.	Authors
	Site-5'	*Site-5'*	*Site-5'*	*Site-5'*	*Site-5'& 3'*	
Pgk-2	--	--	--	--	+++	**Ariel et al, 1991**
MTP1	+--	++-	+--	+--	+++	**Trasler et al, 1990**
MP1	+++	+--	++-	+++	++++	**Trasleret al, 1990**
MP2	+++	+--	++-	+++	++++	**Trasler et al, 1990**
Oct-3/4	--	--	--	--	+++	**Ariel et al, 1994**
ApoA1	--	--	--	--	+++	**Ariel et al, 1994**
ß-Actine	--	--	--	--	--	**Trasler et al, 1990**

Table 1 : some examples of DNA methylation evolution.

REGULATION

This DNA methylation is supported by the adjunction of a methyl group from the S adenolsyl-1methionine on the 5 carbon of the cytosine by the action of a DNA Methyl Transferase (DNMTs) to obtain the 5 methylcytosine residue (5mc) in CpG dinucleotide (figure 4).

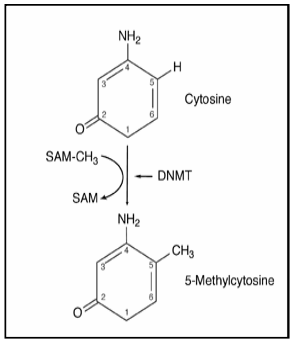


Figure 4 : the obtention of 5 methylcytosine

During spermatogenesis, changes occur in DNA methylation level, two important modifications could be pointed :

(1) A DNA demethylation during the first meiosis, after DNA replication (pachytene stage).

(2) An active methylation during epididymary transit.

INHERITANCE

Any type of cells have their own methylation pattern so that a unique set of proteins may be expressed to perform specific functions.

Thus, during cell division, the methylation pattern should also pass over daughter cell.

This is achieved by specific enzymes called maintenance methylase. This enzyme only methylate CG sequence paired with methylated CG.

Methylation reprogramming during gametogenesis involves the erasure and reestablishment of methylation of imprinted genes and other nonimprinted genes.

This process allows :

- to ensure that both gametes acquire the appropriate sex-specific epigenetic states and establish the epigenetic states required for early embryonic development and toti- or pluripotency
- the erasure of epimutations that adult germ cells may have inherited or developed during their lifetime.

The DNA methylation is carried out on carbon 5 of cytosine base by DNA methyl transferase (DNMTs), which transfers a methyl group from S-adenosyl-1-methionine to carbon 5 of cytosine base.

The family of DNMTs is composed of 5 known members: DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L.

Only DNMT1, DNMT3A and DNMT3B showed their catalytic activity.

- DNMT1 is known to be responsible for the maintenance of the DNA methylation in the somatic cells.
- DNMT3A and DNMT3B is involved in the de novo methylation which establishes the new models of methylation of the embryonic cells.

DNMT 1

DNMT1 is the most abundant DNA methyltransferase in mammalian cells, and considered to be the key maintenance methyltransferase in mammals. It predominantly methylates hemimethylated CpG di-nucleotides in the mammalian genome. This enzyme is 7–20 fold more active on hemimethylated DNA as compared with unmethylated substrate in vitro, but it is still more active at de novo methylation than other DNMTs.

DNMT1 has several isoforms, the somatic DNMT1, a splice variant (DNMT1b) and an oocyte specific isoform (DNMT1o). DNMT1o is synthesized and stored in the cytoplasm of the oocyte and translocated to the cell nucleus during early embryonic development, while the somatic DNMT1 is always found in the nucleus of somatic tissue.

DNMT 2

Although DNMT2 has strong sequence similarities with 5-methylcytosine methyltransferases of both prokaryotes and eukaryotes, the enzyme was shown to methylate position 38 in Aspartic acid transfer RNA and does not methylate DNA.

To reflect this different function, the name for this methyltransferase has been changed to TRDMT1 (tRNA aspartic acid methyltransferase 1) to better reflect its biological function. TRDMT1 is the first RNA cytosine methyltransferase to be identified in a vertebrate.

DNMT 3

DNMT3 is a family of DNA methyltransferases that could methylate hemimethylated and unmethylated CpG at the same rate. There are three known members of the DNMT3 family: DNMT3a, 3b and 3L.

DNMT3a and DNMT3b can mediate methylation-independent gene repression. DNMT3a can interact with DNMT1, which might be a co-operative event during DNA methylation. DNMT3a methylates CpG sites at a rate much slower than DNMT1, but greater than DNMT3b.

DNMT3L contains DNA methyltransferase motifs and is required for establishing maternal genomic imprints, despite being catalytically inactive. DNMT3L is expressed during gametogenesis when genomic imprinting takes place. The loss of DNMT3L lead to bi-allelic expression of genes normally not expressed by the maternal allele. DNMT3L interacts with DNMT3a and DNMT3b and co-localized in the nucleus. Though DNMT3L appears incapable of methylation, it may participate in transcriptional repression.

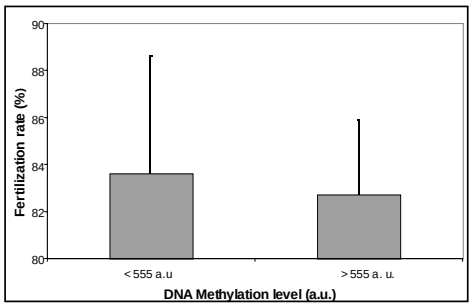
CLINICAL APPLICATION

A prospective study was undertaken, ejaculates were obtained from men (n=63) undergoing an ART procedure.

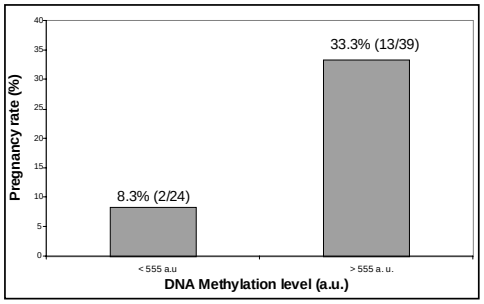
The 5mc is quantified in the same sperm used for IVF : spermatozoids were selected with a discontinuous gradient.

The 5mc was immunostained with a polyclonal antibody and revealed by FITC.
The DNA methylation level was then quantified by flow cytometry.

Fertilization rate & Methylation



Pregnancy rate & Methylation



Our data show that the global status of sperm DNA methylation :

- does not influence the fertilisation rate
- influences embryo development (impaired, if global DNA methylation level is below a cut-off value).

CONCLUSION

- Sperm DNA methylation could be used as a sperm maturation parameter.

- Is there any relationship between sperm DNA methylation and sperm maturation (as protamine ratio, DNA fragmentation…) ?

- Sperm DNA methylation defect could influence the sperm fertilizing capacity and/or the embryos development.

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RNA in Sperm and Epigenetics:
RNA and chromatin dynamics in human spermatozoa

UNIVERSITY OF LEEDS

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1 in 6 couples experience infertility problems

Estimates of male involvement range from 30-50%

Obstructive azoospermia < 5%

Non obstructive azoospermia / severe oligozoospermia < 5%

- **Structural and numerical chromosomal abnormalities ~ 5%**
- **Microdeletions of the Y ~ 15%**
- **Rare metabolic disorders (Spino-Bulbar, PAI etc) < 5%**
- **Unknown others > 50%**

All other infertility / subfertility > 90%

- **Abnormal semen profiles ~ 40%**
- **Apparently normal semen profiles ~ 60%**

Locating genes affecting male fertility

Traditional gene cloning strategies?

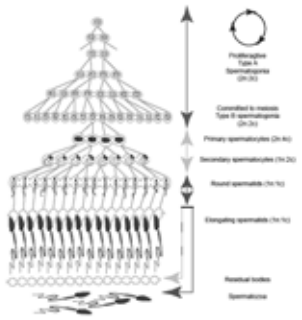
Because different mutations may cause similar effects, TGCS's are unsuitable.

Stigma of male infertility makes recruitment of consanguineous subjects very difficult

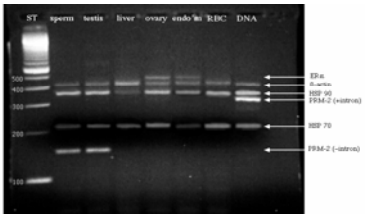
Testicular biopsy?

Only reasonable with clear phenotypes (azoospermia / severe oligozoospermia)

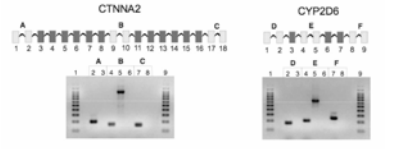
Spermatozoa as a proxy of the testis?



Full length processed transcripts persist in human spermatozoa



Miller et al, Gene, 1999

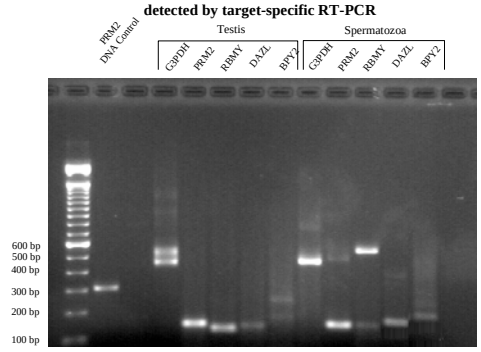


Ostermeier et al, J Androl, 2005

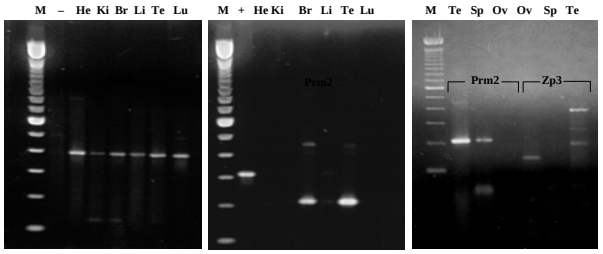
CTNN2 catenin (cadherin-associated protein)
CYP2D6 cytochrome P450, family 2

Historical context

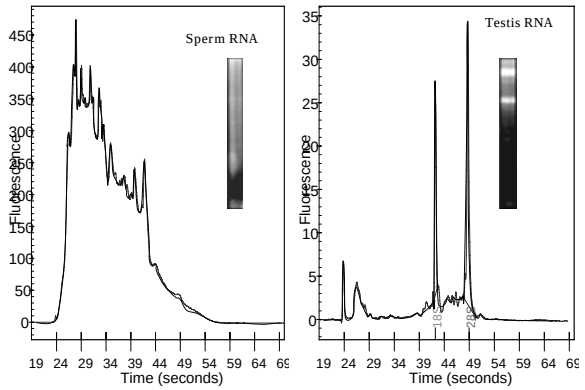
Azoospermia-associated gene transcripts in ejaculate spermatozoa, detected by target-specific RT-PCR



RT-PCR amplification of transcripts from murine tissues

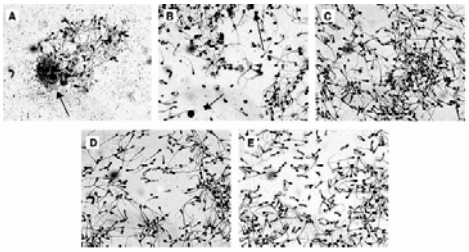


A **B** **C**



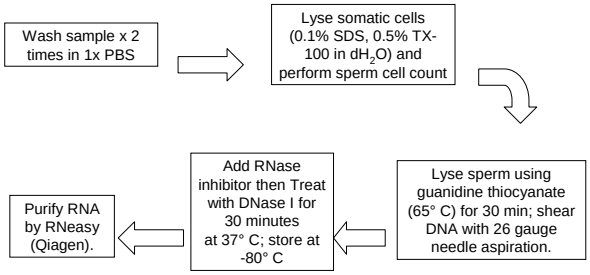
RNA profiles examined using an Agilent™ Bioanalyzer

Diagnostic potential of
of sperm RNA



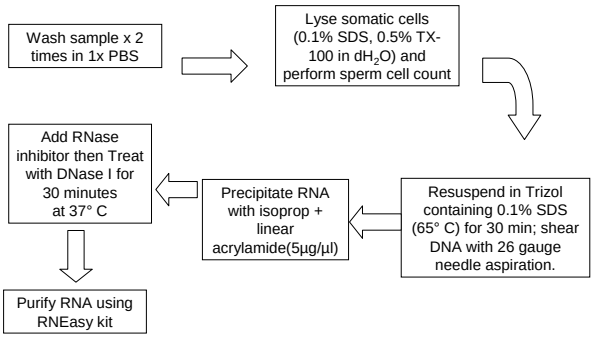
Purity of spermatozoa in crude semen (A), gradient interface (B), pellet fromfirst round of centrifugation (C), pellet after second round of centrifugation (D), and pellet after hypotonic treatment (E) Arrows show somatic-cell contaminants.

RNA Extraction From Sperm

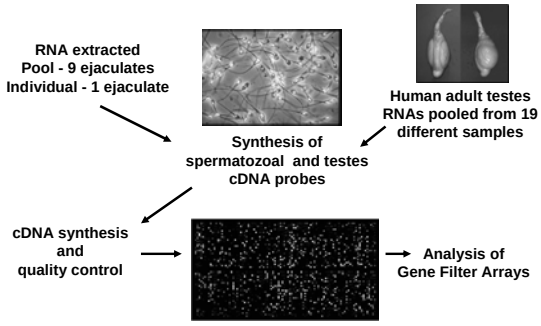


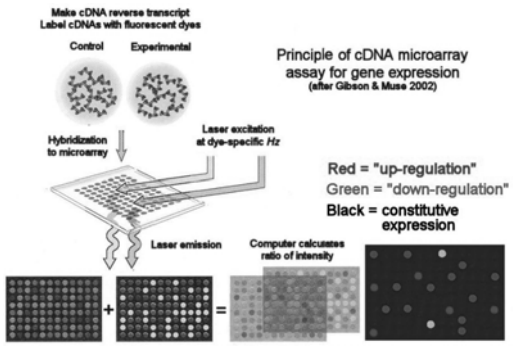
RNA Extraction From Sperm

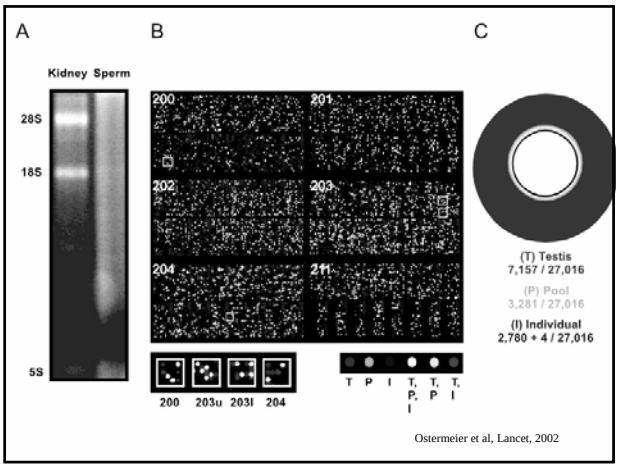
Alternative using Trizol



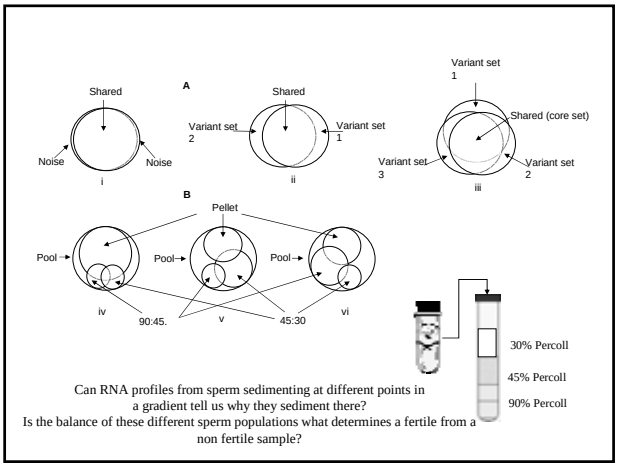
EXPERIMENTAL STRATEGY



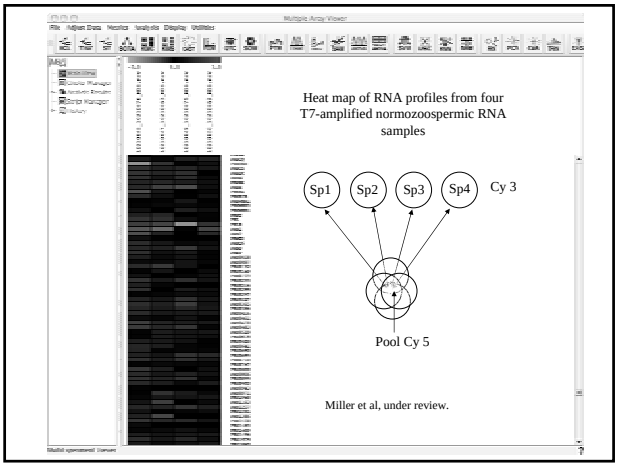




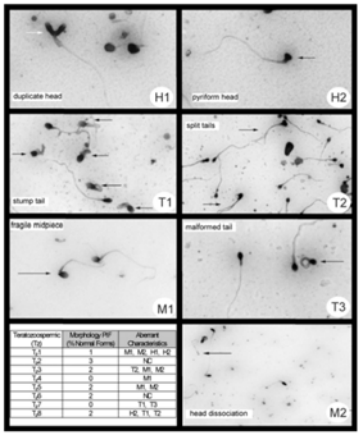
Ostermeier et al, Lancet, 2002



Can RNA profiles from sperm sedimenting at different points in a gradient tell us why they sediment there?
Is the balance of these different sperm populations what determines a fertile from a non fertile sample?

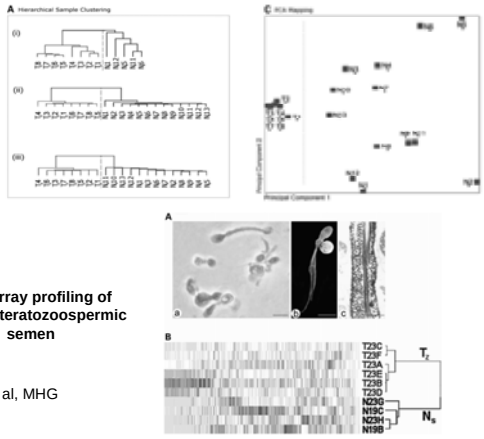


Miller et al, under review.



Microarray profiling of (human) teratozoospermic semen

Platts et al, MHG 2007



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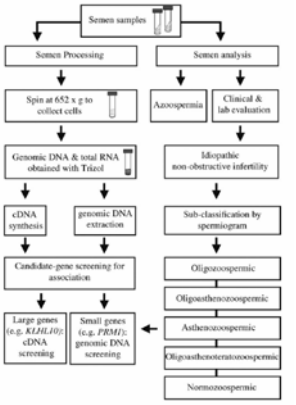
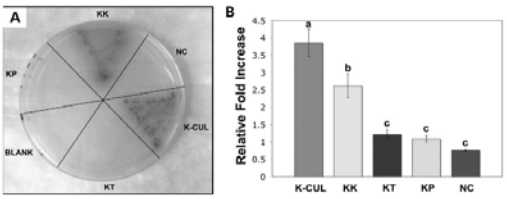


Non-invasive genetic diagnosis of male infertility using spermatozoal RNA: KLHL10 mutations in oligozoospermic patients impair homodimerization

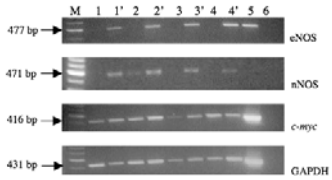
Yatsenko et al, 2006.

Yeast two hybrid assay for human spermatozoal *KLHL10* protein



Yatsenko et al, 2006.

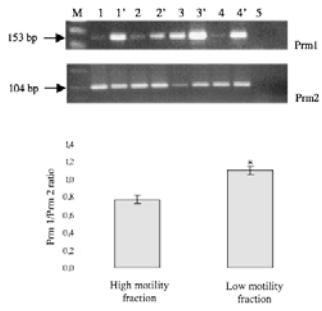
eNOS and nNOS levels in human spermatozoal sub-populations



1-4: highly motile spermatozoa
1' - 4': poorly motile spermatozoa
5 granulosa cell cDNA
6 water blank

Lambard et al, 2004

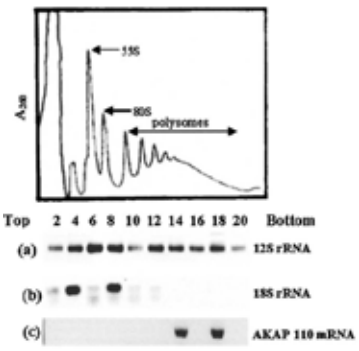
PRM1/2 ratios in human spermatozoal sub-populations



1-4: highly motile spermatozoa
1' - 4': poorly motile spermatozoa
5 water blank

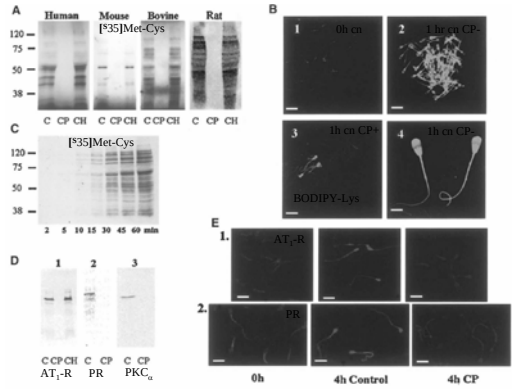
Lambard et al, 2004

Translation in bovine spermatozoa?



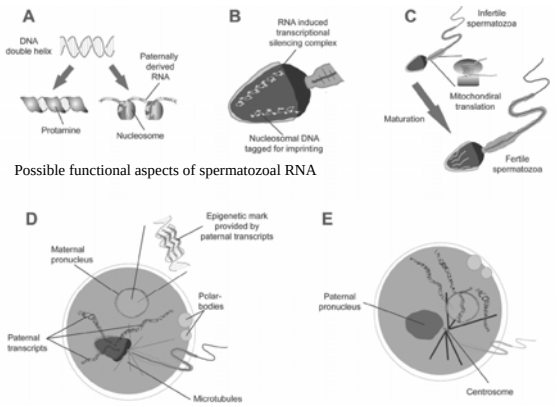
Gur & Breitbart
Genes Dev
2006

Translation in mature spermatozoa?



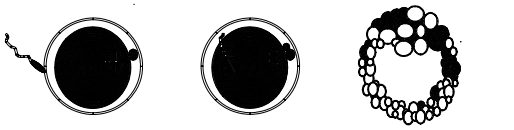
Gur and Breitbart, Genes Dev, 2006

Why does the sperm retain mRNA?



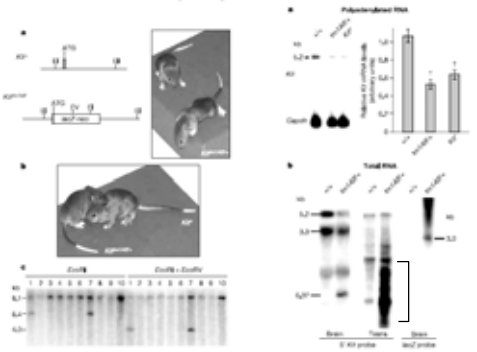
Possible functional aspects of spermatozoal RNA

Paternal Derived Transcripts



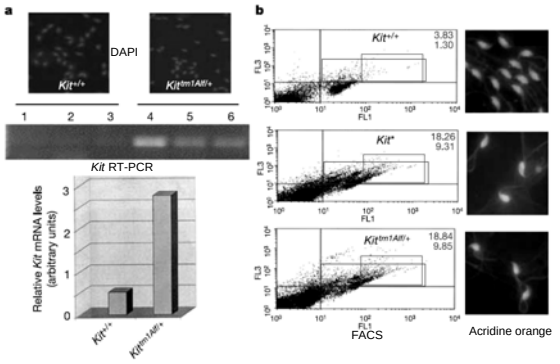
Fertilization	Stress Response	Embryogenesis Morphogenesis
Clusterin	HSF2	MID1
Calmegin	HSPA1L	NLVCF
AKAP4	DNAJB1	CYR61
Oscillin	HSBP1	EYA3
PRM2	DUSP5	FOXG1B
		WNT5A
		WHSC1
		SOX13

Heritable epigenetic modification of coat phenotype by sperm delivered RNA



Lanes 2-6, 8, 9. Kit* paramutated genomic DNA
Lanes 1, 7. Kit ^{hetero} mutated DNA.

Rassoulzadegan et al, Nature, 2006



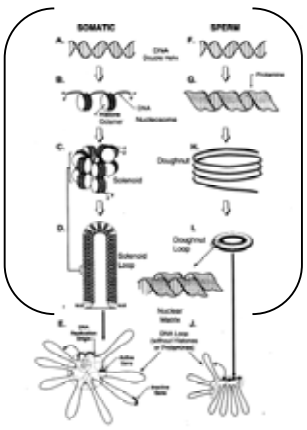
Rassoulzadegan et al, Nature, 2006

Evidence for a global epigenetic signature in sperm chromatin

Control of gene expression in spermatogenesis

Chromatin packaging in somatic and sperm cells. Sperm achieve a reduction in nuclear volume of over 90% compared with a typical somatic cell nucleus.

Stripping out histones and /or protamines from cell nuclei shows that the DNA is attached to the nuclear matrix where DNA/RNA is thought to occur. The loops are thought to contain inactive genes.



Fractionation of sperm chromatin

Wash spermatozoa in 100mM NaCl, 10mM Tris, 1mM EDTA and 0.1mM PMSF

10 mM DTT in 50 mM Tris pH 8.0 → Decondense chromatin

1% CTAB → Check for tail detachment

1% CTAB, 0.05% Digitonin washes → Retain supernatent

TBS- Digitonin wash

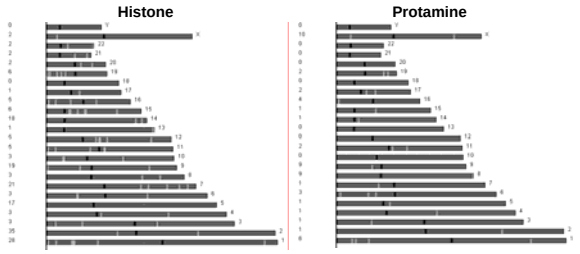
0.65M Nacl, 10mM Tris-Cl pH 8.0, 0.05% digitonin → Retain supernatent; Histones released

Digest pellet with Bam H1 and EcoR1 → Retain supernatent; Histone-bound DNA

Isolate DNA from pellet and digest with Bam H1 and EcoR1 → Protamine-bound DNA

Adapted from Gardiner-Garden et al 1998

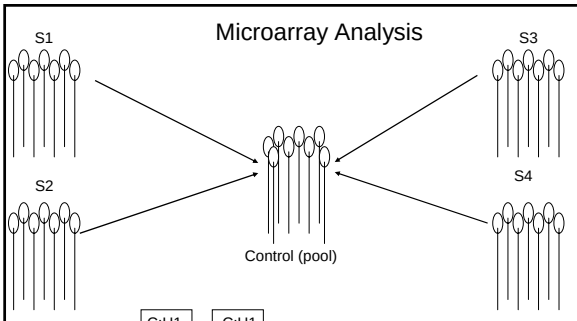
Chromosomal distribution of genes



1 = 15%
2 = 19%
14 = 10%
X = 1%

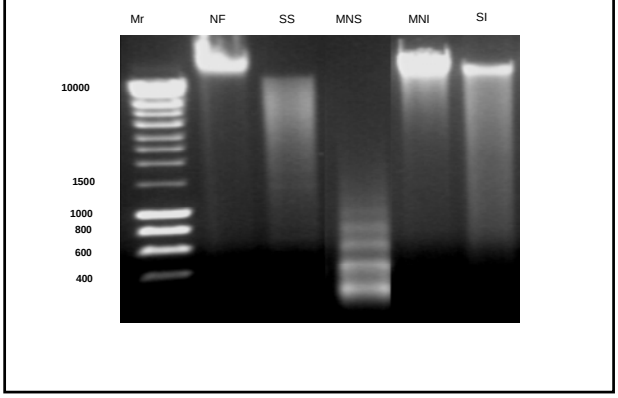
1 = 3%
2 = 1.8%
14 = 1.8 %
X = 17.8%

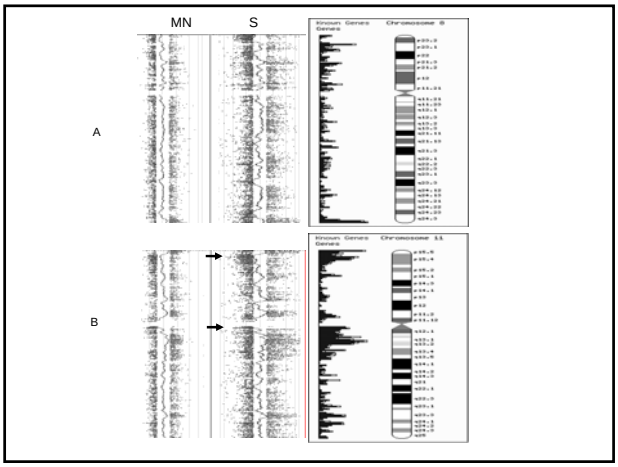
Microarray Analysis

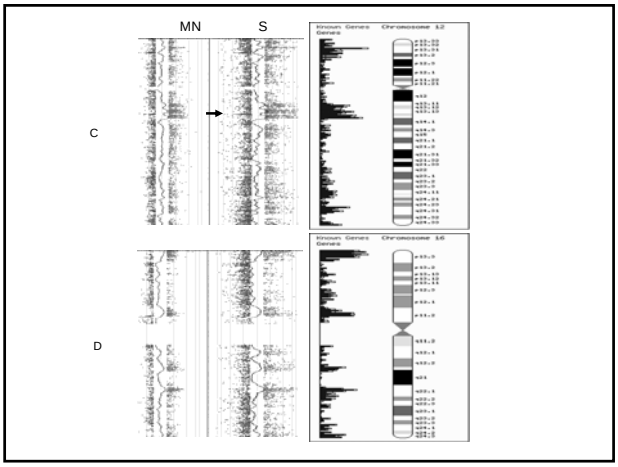


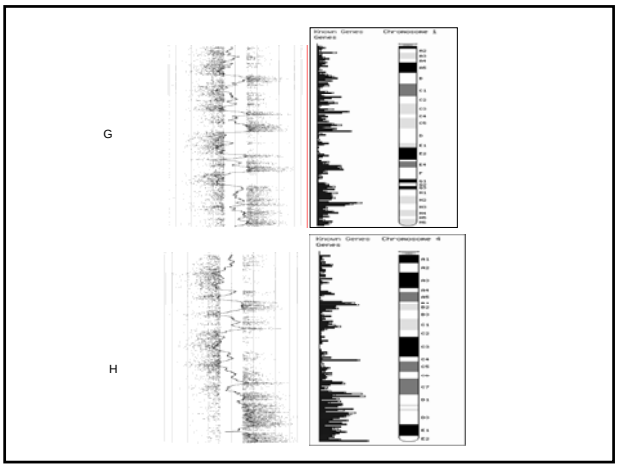
C:H1 C:H1
C:P1 C:P1
H2:P2 H2:P2
H3:P3 H3:P3
H4:P4 H4:P4

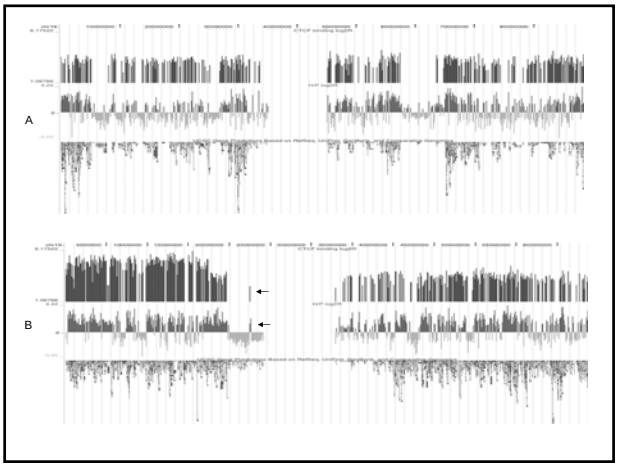
→ Scan and feature extract


Mr
NF
SS
MNS
MNI
SI
10000
1500
1000
800
600
400


MN
S
A
B

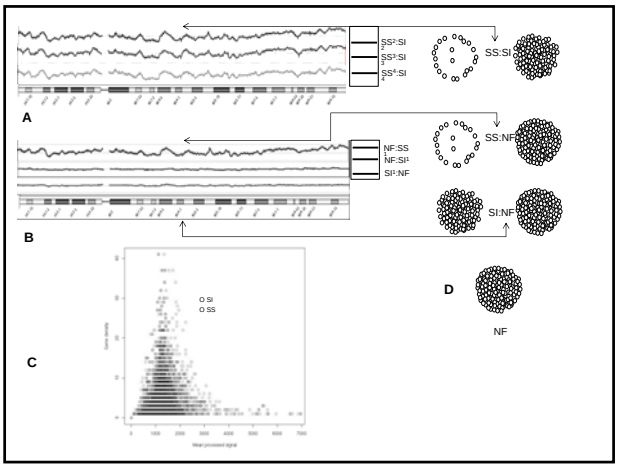

MN
S
C
D

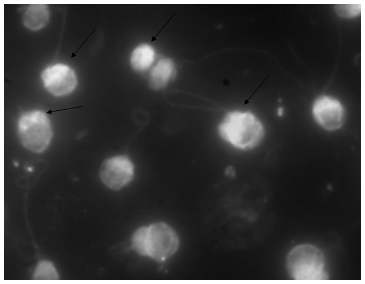


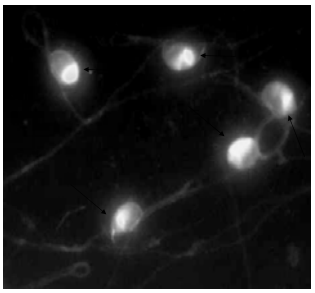


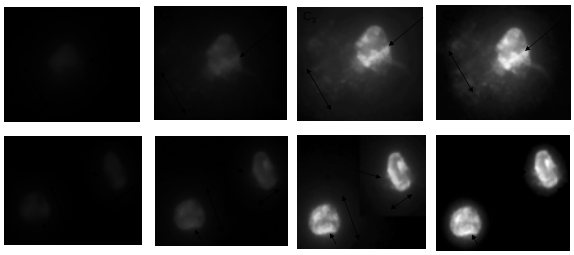
Bioprocess

Count H:P	Term	Part:Cntr	Hist or Prot
348:234	Cell Comm	14:3	Hist
225:141	Development	17:3	Hist
165:179	Trnscr Regln	-10:-5	Prot
127:156	Prot Mod	-16:0	Prot
163:91	Morphogns	23:4	Hist
132:66	Organogns	28:2	Hist
127:71	Signl Transdcn	23:4	Hist
88:47	Ion Transpt	25:-2	Hist
31:68	Ubiquitin Cycle	-42:-9	Prot


SS²:SI
SS³:SI
SS⁴:SI
SS:SI
A
SS:NF
NF:SS
NF:SI
SI:NF
SI:NF
B
C
O SI
O SS
D
NF







- Sperm RNA is a reality and will be diagnostically useful.
- The RNA may be required by both the spermatozoon itself and by the zygote.
- Both the above statements justify further research.
- Sperm DNA is organisationally more complex than we thought.
- The minor histone component of both human and sperm chromatin appears to package most of the genes!
- It is possible that the template for differential repackaging is laid down earlier in spermatogenesis.
- More research is needed to determine the function of differential packaging and how its disruption impacts on male fertility.

Sperm Proteomics and Epigenetics

Rafael Oliva
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University of Barcelona, Barcelona, Spain e-roliva@ub.edu

Pre-congress Course "Paternal inheritance - sperm and epigenetics", 2008 ESHRE Annual Meeting. Barcelona

IDIBAPS UNIVERSITAT DE BARCELONA CLÍNIC BARCELONA Hospital Universitari

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  - 2D and MALDI-TOF
  - LC-MS/MS
- Sperm nuclear anomalies and reproductive outcome
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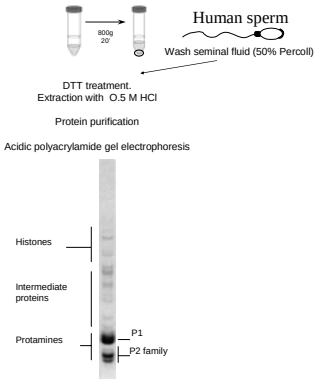

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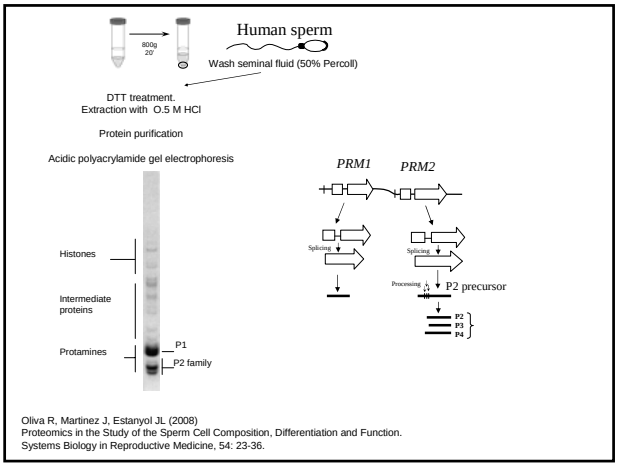

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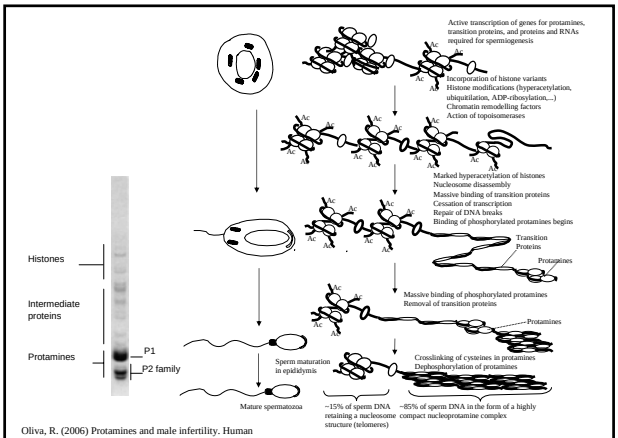

Oliva, R. (2006) Protamines and male infertility. Human Reproduction Update. 12: 417-435.

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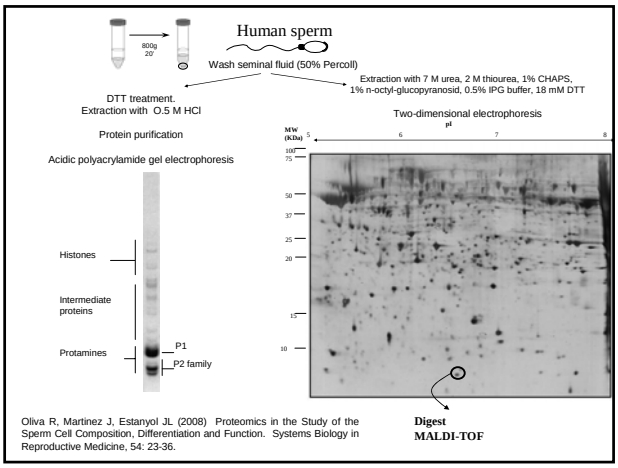

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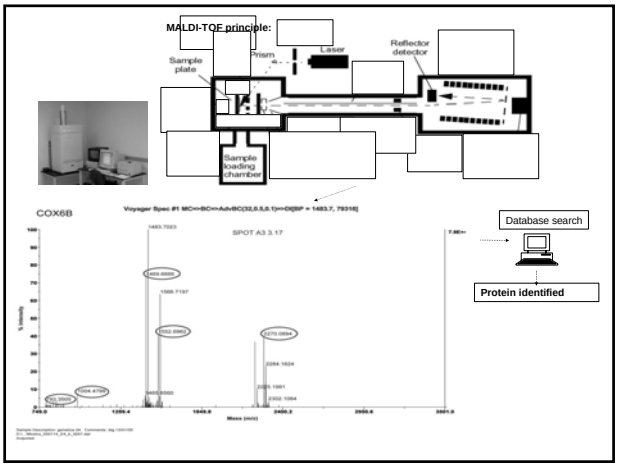
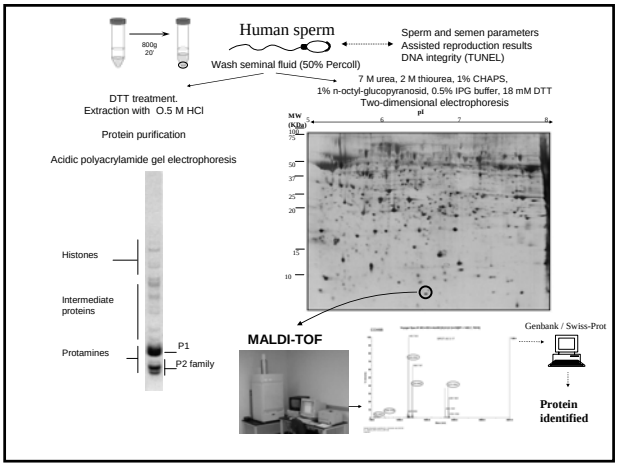
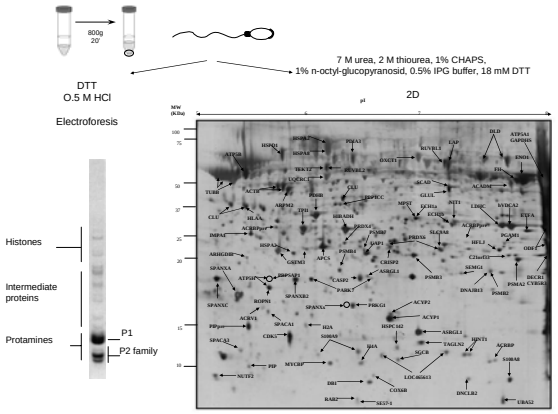
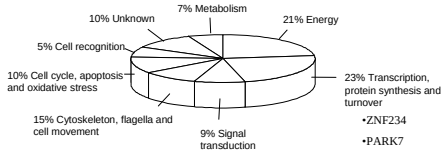






Martínez-Heredia J, Estanyol JM, Ballescà JL and Oliva R (2006) Proteomic identification of human sperm proteins. Proteomics 6, 4356-4369.

132 different proteins identified out of 203 spots excissed:



- ZNF234
- PARK7
- MYCBP
- RuvB like 1

Martínez-Heredia J, Estanyol JM, Ballescà JL and Oliva R (2006) Proteomic identification of human sperm proteins. Proteomics 6, 4356-4369.

de Mateo S, Martínez-Heredia J, Estanyol JM, Domíguez-Fandos D, Vidal-Taboada JM, Ballescà JL and Oliva R (2007) Marked correlations in protein expression identified by proteomic analysis of human spermatozoa. Proteomics 2007;7:4264-77.

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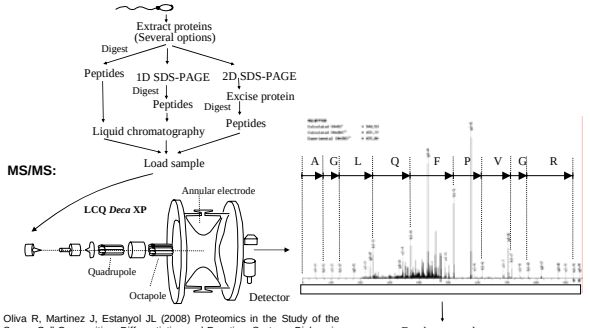

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Baker MA, Reeves G, Hetherington L, Müller J, Baur I and Aitken RJ (2007) Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. Proteomics Clin. Appl 1: 524–532.

Database search

Protein identified as Histone **H2A.m**

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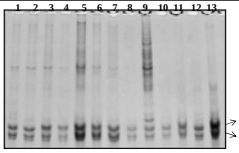
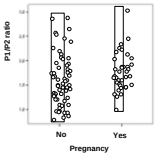


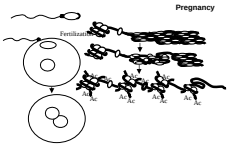
Altered P1/P2 ratio in infertile patients
Silvestroni et al., 1976; Chevalier et al., 1987; Balhorn et al., 1988; Bach et al., 1990; Blanchard et al., 1990; Belokopytova et al., 1993; Oliva and Dixon, 1991; de Yebra et al., 1993; Colleu et al., 1996;Mengual et al., 2003; Aoki et al., 2005; Oliva, R. (2006) Protamines and male infertility. Human Reproduction Update. 12: 417-435.

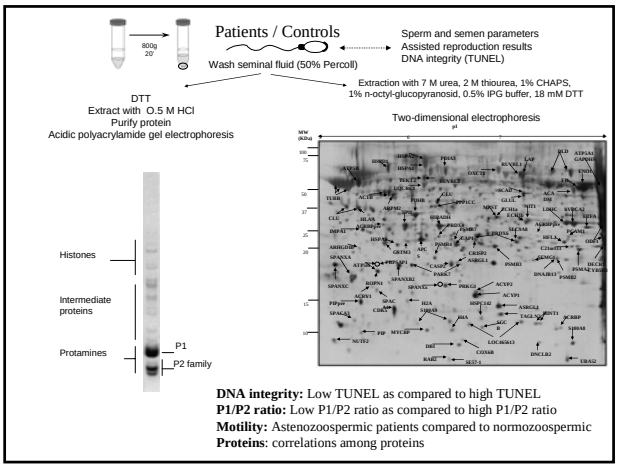
	Low P1/P2	Medium P1/P2	High P1/P2
IVF % zygotes/treated MII	34.9 *	68.2	48.8
ICSI % zygotes/punctioned MII	60.7	67.9	65.6
% Pregnancy per cycle	7.7 **	44	30



de Mateo S, Gázquez C, Guimerà M, Balasch J, Meistrich ML, Ballescà JL, Oliva R. Protamine 2 precursors (Pre-P2), protamine 1 to protamine 2 ratio (P1/P2), and assisted reproduction outcome. Fertil Steril. 2008 Mar 1.

Oliva, R. (2006) Protamines and male infertility. Human Reproduction Update. 12: 417-435.

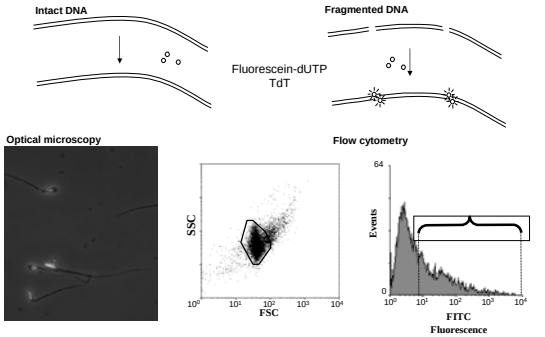




DNA integrity: Low TUNEL as compared to high TUNEL
P1/P2 ratio: Low P1/P2 ratio as compared to high P1/P2 ratio
Motility: Astenozoospermic patients compared to normozoospermic
Proteins: correlations among proteins

TUNEL assay

(Terminal deoxynucleotidyl transferase [TdT]-mediated dUTP [deoxyuridine triphosphate] Nick End Labelling)



Domínguez-Fandos D, Camejo MI, Ballescà JL, Oliva R (2007) Human Sperm DNA Fragmentation: Correlation of TUNEL Results as Assessed by Flow Cytometry and Optical Microscopy. Cytometry A. 2007;71:1011-8.

Proteins correlating with TUNEL results:

Protein	Average density of spot		Mann-Whitney
	Low TUNEL Group	*High TUNEL Group*	*P*
CLU-1	10655	25606	0,028
HSPA2	4375	8747	0,044
LOC465613	15773	6313	0,006
PARK7-1	1073	3552	0,028
PHB	7154	8394	0,009
PSMA6	10346	14378	0,044
SEMG-1	3552	6265	0,028
SPANXC	6394	11999	0,047

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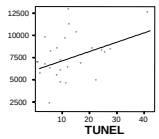
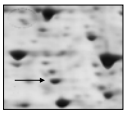
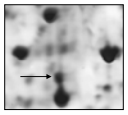
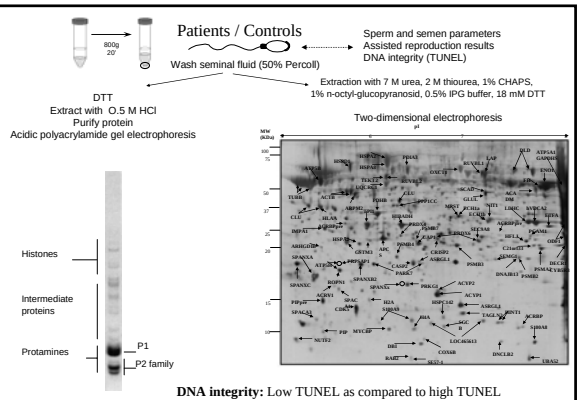
TUNEL

Prohibitin









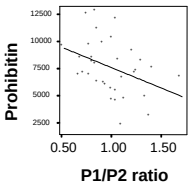
DNA integrity: Low TUNEL as compared to high TUNEL
P1/P2 ratio: Low P1/P2 ratio as compared to high P1/P2 ratio
Motility: Astenozoospermic patients compared to normozoospermic
Proteins: correlations among proteins

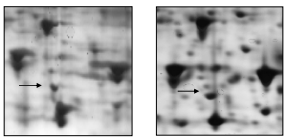
Proteins correlating with the P1/P2 ratio:

P1/P2 ratio	Protein	Average density of spot			Mann-Whitney
		Low P1/P2	*Normal P1/P2*	*High P1/P2*	*P*
Low vs Normo	ATP5B	38843	21308	42717	0,030
	HSPDI	20840	13430	19131	0,042
	PHB	9226	7063	4818	0,035
	PRKAR1A	7643	4683	5258	0,046
	SGCB	765	2524	1782	0,036
Normo vs High	ATP5B	38843	21308	42717	0,082
Low vs High	HINT1-2	2379	2959	5060	0,036
	PHB	9226	7063	4818	0,014
	RUVBL1	5286	9560	14995	0,040

de Mateo S, Martínez-Heredia J, Estanyol JM, Domíguez-Fandos D, Vidal-Taboada JM, Ballescà JL and Oliva R (2007) Marked correlations in protein expression identified by proteomic analysis of human spermatozoa. Proteomics 2007;7:4264-77.

P1/P2





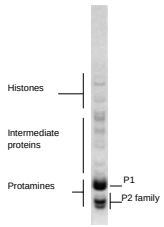
Patients / Controls

Wash seminal fluid (50% Percoll)

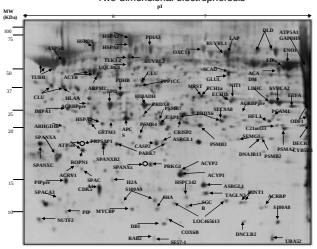
Sperm and semen parameters
Assisted reproduction results
DNA integrity (TUNEL)

Extraction with 7 M urea, 2 M thiourea, 1% CHAPS, 1% n-octyl-glucopyranosid, 0.5% IPG buffer, 18 mM DTT

DTT
Extract with O.5 M HCl
Purify protein
Acidic polyacrylamide gel electrophoresis



Two-dimensional electrophoresis



DNA integrity: Low TUNEL as compared to high TUNEL
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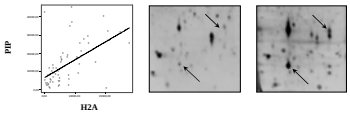
Motility: Astenozoospermic patients compared to normozoospermic
Proteins: correlations among proteins

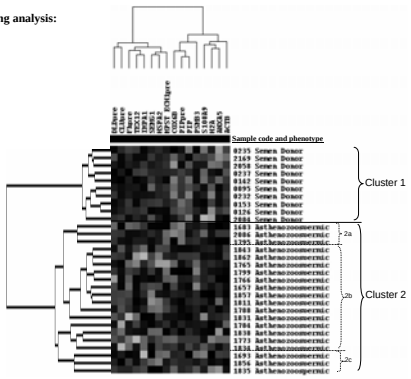
Protein-protein correlations



58 proteins – 67 correlations (P < 0.001 y r > 0.5)
- 22 proteins correlating with onother protein
- 18 correlating with 2 proteins
- 18 correlating with 3 proteins or more proteins

de Mateo S, Martínez-Heredia J, Estanyol JM, Domíguez-Fandos D, Vidal-Taboada JM, Ballescà JL and Oliva R (2007) Marked correlations in protein expression identified by proteomic analysis of human spermatozoa. Proteomics 2007;7:4264-77.

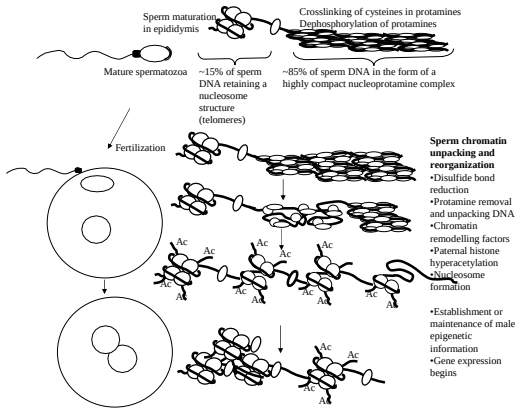
Non supervised clustering analysis:



Martínez-Heredia J, de Mateo S, Vidal-Taboada JM, Estanyol JM, Ballescà JL and Oliva R (2007) Proteomic expression differences between asthenozoospermic and normozoospermic sperm samples. Human Reproduction 23:783-91.

Sperm Proteomics and Epigenetics

- Analysis of the sperm nuclear proteome
  - 1D
  - 2D and MALDI-TOF
  - LC-MS/MS
- Sperm nuclear anomalies and reproductive outcome
- **Proteomic contribution to zygotic chromatin**
- Conclusions



Oliva, R. (2006) Protamines and male infertility. Human Reproduction Update. 12: 417-435.

Sperm Proteomics and Epigenetics

- Analysis of the sperm nuclear proteome
  - 1D
  - 2D and MALDI-TOF
  - LC-MS/MS
- Sperm nuclear anomalies and reproductive outcome
- Proteomic contribution to zygotic chromatin
- **Conclusions**

Our Laboratory:
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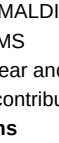
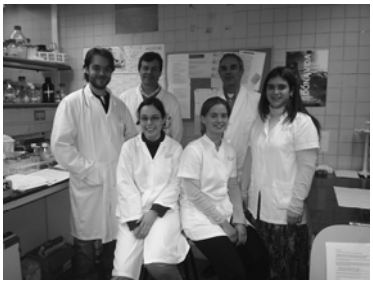
Josep Maria Estanyol

Assisted reproduction Unit:
José Luís Ballescà





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Project supported by grant BMC006-03479

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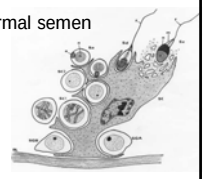
IDIBAPS

Imprinting in sperm of men with abnormal semen parameters



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Department of Genetics, Faculty of Medicine; Centre for Reproductive Genetics A. Barros;
Lab Cell Biology, ICBAS, University of Porto, Portugal
6 July 2008

We declare no conflict of interest

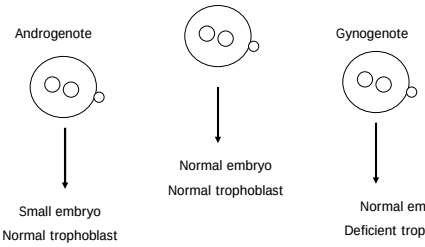
Learning objectives

- Are imprinting marks correctly established in sperm from oligozoospermic patients?
- Are imprinting errors found in oligozoospermia present in all sperm cells or affecting only a fraction of cells?
- Are imprinting marks established in elongated spermatids/spermatozoa retrieved from testicular biopsies of azoospermic patients?

Methylation imprinting marks of *H19* and *MEST* imprinted genes in:

- population of human sperm from normozoospermic individuals and oligozoospermic patients (moderate and severe) : population study (direct sequencing)
- individual spermatozoa from normozoospermic individuals and oligozoospermic patients (mild, moderate, severe and very severe) : cloning analysis
- individual testicular late spermatids/spermatozoa from azoospermic patients, due to anejaculation, obstructive azoospermia (inflammatory and CBAVD), and secretory azoospermia (hypospermatogenesis)

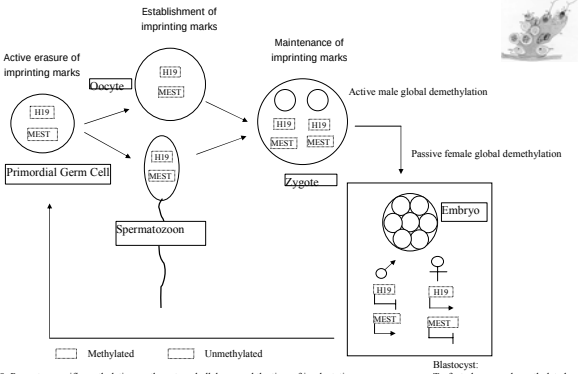
Genomic imprinting



- Surani, 1984. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, 308, 548
- McGrath and Solter, 1984. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, 37, 179
- Cattanach, 1985. Differential activity of maternally and paternally derived chromosome regions in mice. *Nature*, 315, 496.

• Genomic imprinting – mechanism that regulates gene expression leading to monoallelic, parental-dependent expression of imprinted genes.

• Imprinting marks – consist of methylation of CpGs in the DMRs (Differentially Methylated Regions) of imprinted genes.

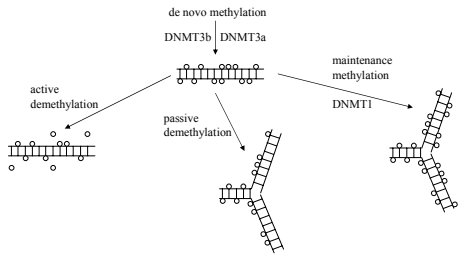


H19: Promotor specific methylation on the paternal allele around the time of implantation

Blastocyst:
Trofectoderm - undermethylated
Inner cell mass - hypermethylated

Reik & Walter, 2001,Nat Rev Genet 2:21-32

DNA methyltransferases



de novo methylation – gametogenesis - DNMT3A, DNMT3B e DNMT3L

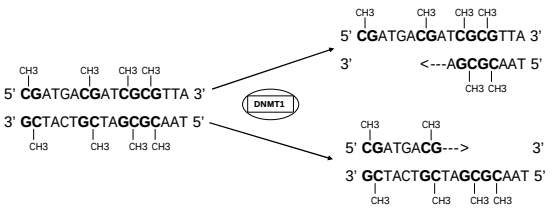
Maintenance of methylation patterns – somatic cells (during replication) - DNMT1

DNA demethylation – passive (during replication) and active (Zygote-paternal; PGC); linked to DNA repair ?

Reik & Walter, 2001,Nat Rev Genet 2:21-32

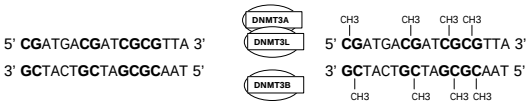
DNA methyltransferases

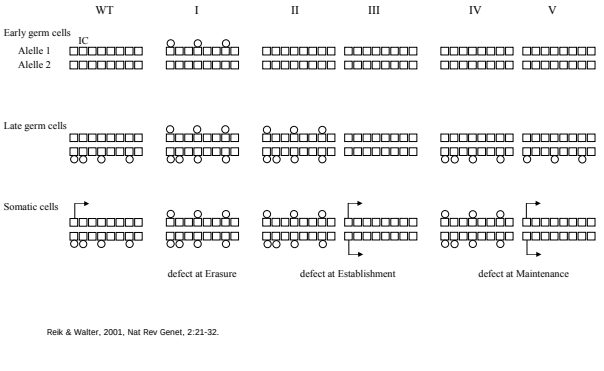
maintenance



DNA methyltransferases

de novo





Reik & Walter, 2001, Nat Rev Genet, 2:21-32.

Imprinting syndromes

• Prader-Willi syndrome - 15q11-13

SNRPN: maternal-methylated (silenced)

Deletions on the paternal chromosome; mUPD; Absence of *SNRPN* expression (type II)

• Angelman Syndrome – 15q11-13

SNRPN: paternal-unmethylated → antisense mRNA to UBE3A (silenced)

Deletions on the maternal chromosome; pUPD; Absence of *UBE3A* expression

• Beckwith-Wiedemann – 11p15.5

H19: paternal-methylated (silenced) → IGF2 expressed

pUPD; biparental methylation of *H19* -DMR; maternal microdeletions on *H19* -DMR; biallelic expression of *IGF2*.

• Silver-Russell syndrome – 11p15.5

Loss of H19 paternal methylation → H19 biallelic expression and IGF2 inactivation (2005, 2006)

Raised in ART babies?

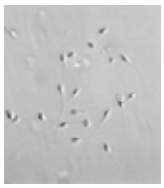
– Yes (Ludwig et al., 2005, J Med Genet 42:289-91; Sutcliffe et al., 2006, Hum Reprod 21:1009-11; Chang et al., 2005, Fertil Steril 83:349-54; Halliday et al., 2004, Am J Hum Genet 75:526-8; DeBaun et al., 2003, Am J Hum Genet 72:156-60; Gicquel et al., 2003, Am J Hum Genet 72:1338-41; Maher et al., 2003, J Med Genet 40:62-4; Orstavik et al., 2003, Am J Hum Genet 72:218-9; Cox et al., 2002, Am J Hum Genet 71:162-4.

- No (Bowdin et al., 2007, Hum Reprod 22:3237-40; Lidegaard et al., 2005, Hum Reprod, 20:950-4)

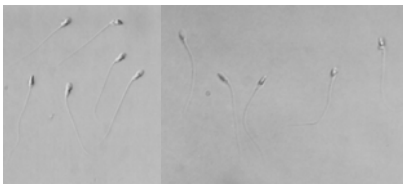
Learning Objective I

⇨ • Are imprinting marks correctly established in sperm from oligozoospermic patients?

- population of human sperm from normozoospermic individuals and oligozoospermic patients (moderate and severe) : population study (direct sequencing)



Swim-up



Micromanipulation

RESEARCH LETTERS

Genomic imprinting in disruptive spermatogenesis

Cristina Joana Marques, Filipa Carvalho, Mário Sousa, Alberto Barros

The possibility of imprinting disease transmission by assisted reproductive technologies has been raised after births of children with Angelman's and Beckwith-Wiedemann's syndromes. To investigate whether imprinting defects were associated with disturbed spermatogenesis, we studied two oppositely imprinted genes in spermatozoan DNA from normozoospermic and oligozoospermic patients. In the mesodermal specific transcript gene (MEST), bisulphite genomic sequencing showed that maternal imprinting was correctly erased in all 123 patients. However, methylation of the H19 gene did not change in any of 27 normozoospermic individuals (0%, 95% CI 0–13%), compared with methylation changes in eight moderate (17%, 6–31%, p=0·026) and 15 severe (30%, 18–45%, p=0·002) oligozoospermic patients. Our data suggest an association between abnormal genomic imprinting and hypospermatogenesis, and that spermatozoa from oligozoospermic patients carry a raised risk of transmitting imprinting errors.

Lancet 2004; **363**: 1700–02

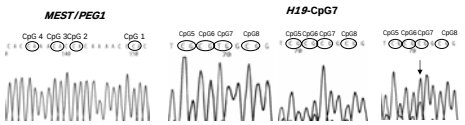
Materials and Methods

N = 123 individuals	Normozoospermic (≥20x10^6 Sz/ml) N= 27 cases
	Moderate oligozoospermia (≥5<20x10^6 Sz/ml) N= 46 cases
	Severe oligozoospermia (<5x10^6 Sz/ml) N= 50 cases

Marques *et al*, 2004, Lancet, 363, 1700-1702

Materials and Methods

- Sperm isolation by swim-up technique
- DNA extraction by alkaline lysis buffer
- Sodium bisulphite modification (CpGenome Modification kit, Chemicon)
- H19 and MEST PCR amplification
- Direct sequencing of PCR products

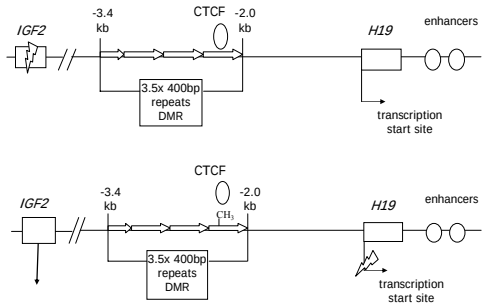


Marques *et al*, 2004, Lancet, 363, 1700-1702

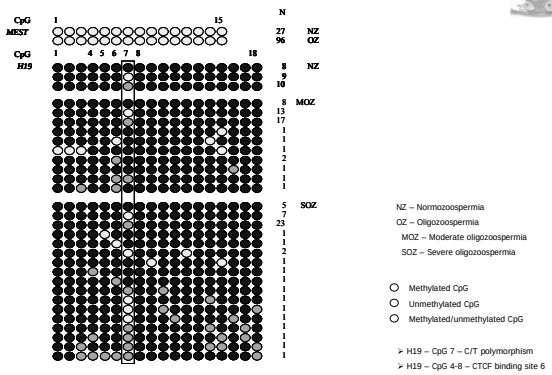
Imprinted genes

- *MEST/PEG1* 7q32
  - Unmethylated on the paternal allele → expressed
  - Erasure of methylation
  - Regulates embryonic development and controls adult behaviour
- *H19* 11p15.5
  - Methylated on the paternal allele → inactive
  - Establishment of methylation
  - Regulates the expression of *IGF2* (Insulin-like growth factor 2)

H19 and *IGF2* imprinted genes



Results



Marques *et al*, 2004, Lancet, 363, 1700-1702

Results

- MEST is completely unmethylated in all sperm samples analysed
- H19 is completely methylated in NZ samples
- however
  - 24% (23/96) of OZ samples present hypomethylation of H19
    - 17.4% (8/46) of MOZ samples (P=0.026 to NZ)
    - 30% (15/50) of SOZ samples (P=0.002 to NZ)

Marques *et al*, 2004, Lancet, 363, 1700-1702

Conclusions

MEST/PEG1

✓ Inherited maternal imprinting marks are correctly erased in sperm from NZ and OZ

H19

✓ Methylation is correctly established in NZ

✓ Hypomethylation occurs in OZ, more frequent in SOZ

✓ Hypomethylation of CTCF binding site occurs in 11.5% (11/96) of OZ

⇩ Risk of transmitting paternal IGF2 allele inactive ?

Marques *et al,* 2004, Lancet, 363, 1700-1702

Learning Objective II

•Different patterns of methylation are present in the sperm population of a patient. Are imprinting errors found in oligozoospermia present in all sperm cells or affecting only a fraction of cells?

- individual spermatozoa from normozoospermic individuals and oligozoospermic patients (mild, moderate, severe and very severe) : cloning analysis of methylation patterns

Materials and Methods

	N Patients	N H19 clones	N MEST clones
Normozoospermia >20x10^6 Sz/ml	5	72	79
Mild OZ 10-20x10^6 Sz/ml	5	76	62
Moderate OZ 5-10x10^6 Sz/ml	5	101	82
Severe OZ 1-5x10^6 Sz/ml	5	84	80
Very severe OZ <1x10^6 Sz/ml	5	86	66

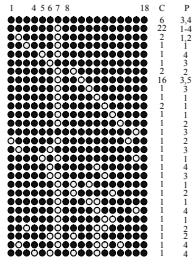
Marques *et al,* 2008, Mol Hum Reprod, 14, 67-73

Results

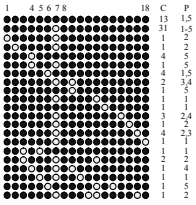
- Methylated CpG
- Unmethylated CpG

H19

Normozoospermia



Mild oligozoospermia



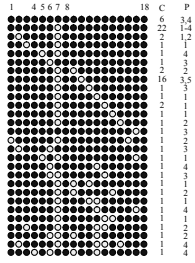
Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

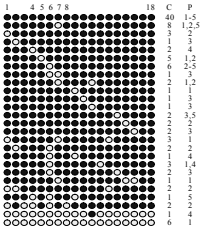
- Methylated CpG
- Unmethylated CpG

H19

Normozoospermia



Moderate oligozoospermia



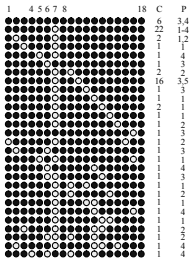
Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

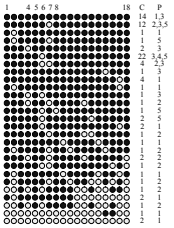
- Methylated CpG
- Unmethylated CpG

H19

Normozoospermia



Severe oligozoospermia



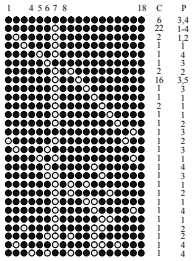
Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

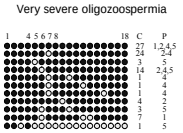
○ Methylated CpG
○ Unmethylated CpG

H19

Normozoospermia



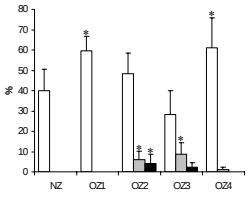
Very severe oligozoospermia



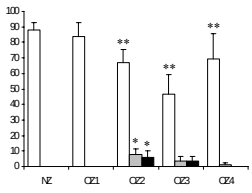
Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

H19



CTCF binding site 6



* P<0.05; ** P<0.01

☐ Methylated
☐ Hypomethylated
☐ Unmethylated

NZ – Normozoospermia
OZ1 – Mild OZ
OZ2 – Moderate OZ
OZ3 – Severe OZ
OZ4 – Very severe OZ

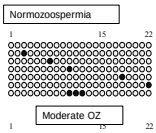
Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

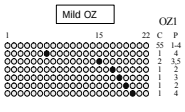
○ Methylated CpG
○ Unmethylated CpG

MEST

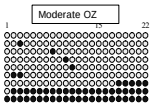
Normozoospermia



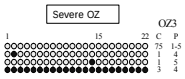
Mild OZ



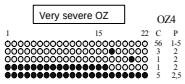
Moderate OZ



Severe OZ



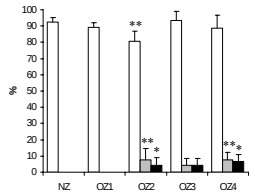
Very severe OZ



Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

MEST



* P<0.05; ** P<0.01

☐ Unmethylated
☐ Hypermethylated
☐ Methylated

NZ – Normozoospermia
OZ1 – Mild OZ
OZ2 – Moderate OZ
OZ3 – Severe OZ
OZ4 – Very severe OZ

Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

LINE 1

Methylation status of LINE-1 in human sperm. One patient from each group (the one with more unmethylated H19)

Groups	Clones N	Total CpGs N	Methylated CpGs N
NZ	11	195	153 (78.5%)
Oligozoospermia			
OZ1	19	340	264 (77.6%)
OZ2	17	315	264 (83.8%)
OZ3	20	280	194 (69.3%) *
OZ4	17	295	218 (73.9%)

Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Results

Defective methylation of imprinted genes occurs in sperm from patients with a sperm count below $10x10^6$ Sz/ml:

❑ H19

Hypomethylation - 5.5% (1.2-8.3%) of clones

Complete unmethylation - 2.95% (0-5.9%) of clones

❑ CTCF binding site

Hypomethylation – 4.8% (1.2-8.9%) of clones

Complete unmethylation – 3.7% (0-6.9%) of clones

❑ MEST

Hypermethylation – 8.3% (3.8-12.2%) of clones

Complete methylation – 6.1% (3.8-7.6%) of clones

Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Conclusions

✓ Imprinting errors occur in sperm of patients presenting less than $10x10^6$ Sz/ml

Association between abnormal spermatogenesis and the occurrence of imprinting errors ?

✓ Risk of transmitting H19 hypomethylated

Association with Silver-Russell syndrome in children born after ART ?

✓ Risk of transmitting paternal inactive IGF2

Abnormal pre-implantation embryo development and/or pregnancy loss ?

Low birth weight in children born after ART ?

Marques *et al*, 2008, Mol Hum Reprod, 14, 67-73.

Learning Objective III

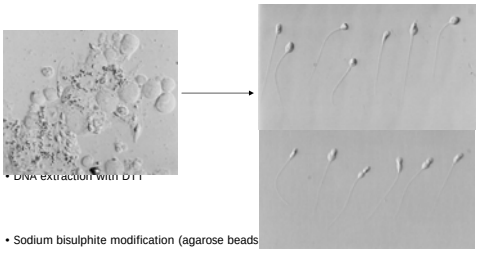
•Are imprinting marks established in elongated spermatids/spermatozoa retrieved from testicular biopsies of azoospermic patients?

Methylation imprinting marks of *H19* and *MEST* imprinted genes in:

- individual testicular late spermatids/spermatozoa retrieved from testicular biopsies of patients presenting azoospermia, due to anejaculation, obstructive azoospermia (inflammatory and CBAVD), and secretory azoospermia (hypospermatogenesis)

Materials and Methods

• Testicular sperm isolation by micromanipulation from testicular biopsies – Anejaculation (ANJ), Obstructive azoospermia (inflammatory, CBAVD) and Germinal Hypoplasia (HP)



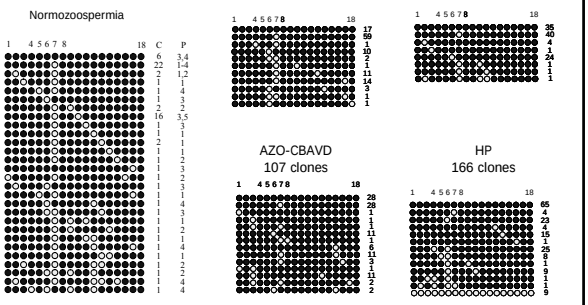
• DNA extraction with DTT

• Sodium bisulphite modification (agarose beads

Marques *et al*, 2008, submitted.

Results

H19



Marques *et al*, 2008, submitted.

Results

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

Groups	Clones	Number of unmethylated CpGs					
	N	0	1	2	3	17	≥9
ANJ	120	76 (63%) **ab**	39	5			
AZO-sec	107	75 (70%) **a**	31		1		
AZO-CBAVD	107	56 (52%) **bc**	35	16			
HP	166	69 (42%) **c**	43	43	2	9 (5%) **a**	9 (5%) **a**

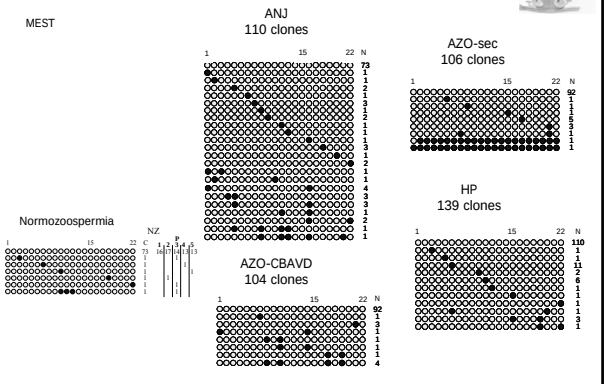
Table 2. Methylation status of CTCF binding site 6 in human testicular sperm

Groups	Clones	Number of unmethylated CpGs				
	N	0	1	2	4	≥3
ANJ	120	102 (85%) **a**	18			
AZO-sec	107	83 (78%) **a**	24			
AZO-CBAVD	107	93 (87%) **a**	14			
HP	166	89 (54%) **b**	33	35	9 (5%) **a**	9 (5%) **a**

Marques *et al*, 2008, submitted.

Results

MEST



Marques *et al*, 2008, submitted.

Results

Table 3. Methylation status of *MEST* in human testicular sperm

Groups	Clones	Number of methylated CpGs							
	N	0	1	2	4	5	21	22	>11
ANJ	110	73 (66%) **a**	20	15	1	1			
AZO-sec	106	92 (87%) **b**	11	1			1	1	2 (2%)
AZO-CBAVD	104	92 (89%) **b**	4	4	4				
HP	139	110 (79%) **b**	24	5					

Marques *et al*, 2008, submitted.

Conclusions

• Patients with secretory (non-obstructive) azoospermia are more prone to have unmethylation of H19 and CTCF binding site as oligozoospermic patients with less than 10^6 Sz/mL → the risk increases as spermatogenesis is more affected

• On the contrary, MEST gene seems to be correctly unmethylated except in ANJ group, where there is an increase in MEST methylation → loss of testicular innervation leads to erroneous methylation ??

The occurrence of imprinting errors is associated with abnormal spermatogenesis

Acknowledgements

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• Lab of Cell Biology, ICBAS, University of Porto, Portugal.

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Jorn Walter, Thomas Mikeska

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FCT Fundação para a Ciência e a Tecnologia

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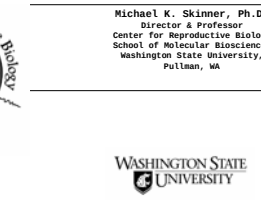
Epigenetic Transgenerational Actions of Endocrine Disruptors on Reproduction and Disease: The Ghosts in your Genes

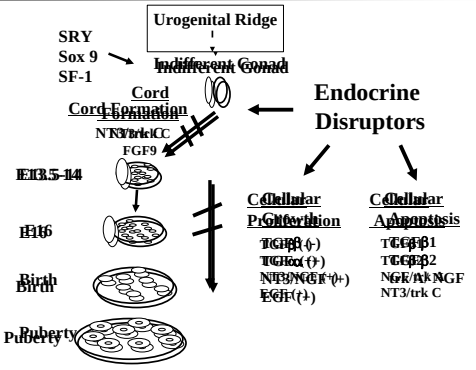
Michael K. Skinner, Ph.D.
Director & Professor
Center for Reproductive Biology
School of Molecular Biosciences
Washington State University,
Pullman, WA



Environmental Impact on Biology

- Regional Disease Frequencies
- Low Frequency of Genetic Component of Disease
- Increases In Disease Frequencies
- Identical Twins and Variable Disease Frequency
- Environmental Exposures and Disease
- Environment and Endangered Species
- Evolutionary Differences

Testis Development



Endocrine Disruptors

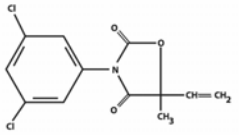
-Environmental compounds that bind to hormone receptors and alter hormone actions and endocrine system

-Examples include pesticides, fungicides and plastics such as DDT, BPA and phthalates

-Influence a number of different species from frogs to humans

-Promote disease states from reproductive defects to tumors

Model Endocrine Disruptor: Vinclozolin



- Vinclozolin is a systemic fungicide (e.g. Wine Industry)
- Two degradation products : Butenoic acid and enanilide
- Vinclozolin and its metabolites are anti-androgenic
- Late embryonic/early postnatal exposure causes abnormal reproductive tract development and gonadal function

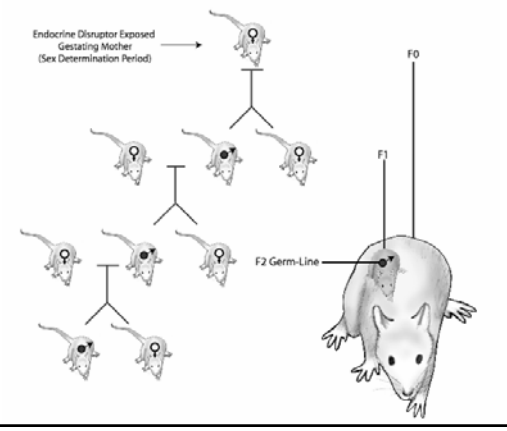
Transgeneration Transmission

Vinclozolin

F0 ⇨ F1 ⇨ F2 ⇨ F3 ⇨ F4

VOC − F2 ♂ + WT ♀

RVOC − F2 ♀ + WT ♂

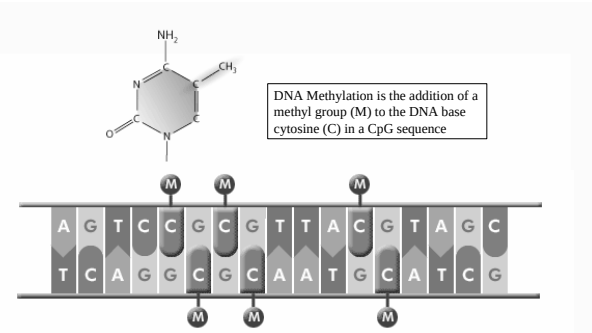


Transgenerational Phenotype

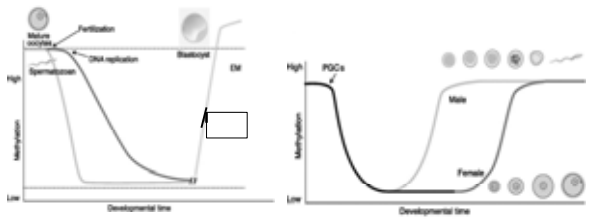
	DNA Mutation	Epigenetic Mutation
Frequency -	<0.01% (Hot Spot 1-5%)	High (30-100%)
Reproducible-	Random Event	Highly Reproducible
Genetics-	Mendelian (decline frequency generationally)	Non-Mendelian

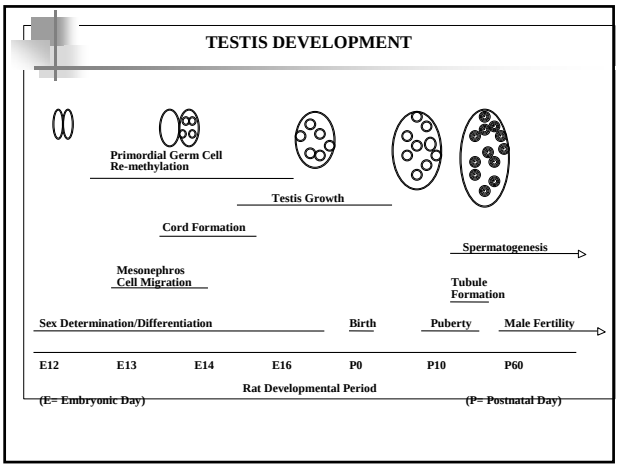
Epigenetic Mechanisms of Gene Regulation

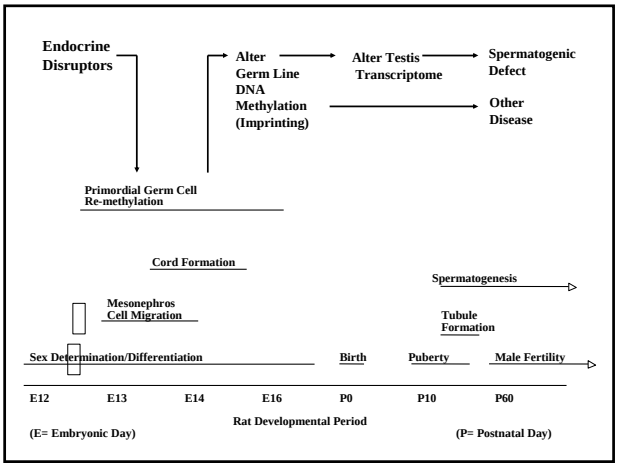
-DNA Methylation
-Histone Modification
-Chromatin Structure
-DNA Organization into Domains (eg Loops)
-Nuclear Compartmentalization (eg nuclear matrix)
-Replication Timing During S Phase
-Noncoding functional RNAs



The Process of Methylation

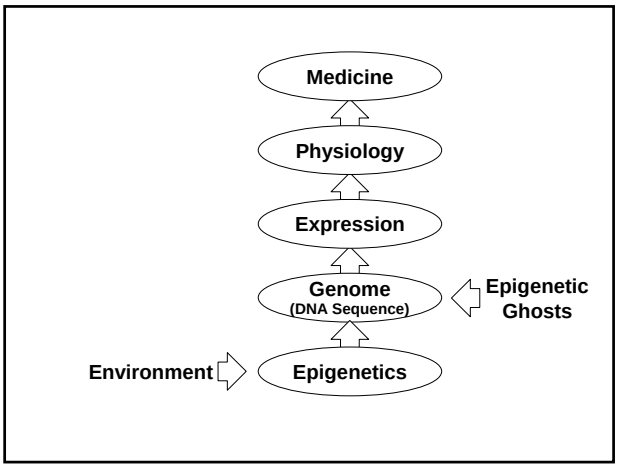






Summary

- Transient Embryonic Exposure Effects Adult
  - -Sex Determination Period
  - -Fetal Basis of Disease
- Spermatogenic Fertility Defect & Other Diseases
- Epigenetic Transgenerational Phenotype
  - -Toxicology Endocrine Disruptors
  - -Permanent Re-Program (Imprint) Germ-Line
  - -Disease Etiology
  - -Evolutionary Biology


Medicine
Physiology
Expression
Genome
(DNA Sequence)
Epigenetic
Ghosts
Environment
Epigenetics

ESHRE Barcelona July 2008

"Paternal inheritance - sperm and epigenetics"

Minoo Rassoulzadegan PhD (Molecular Biology)

Director of Inserm U636 laboratory:

Genetic of Normal and Pathological Development

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Tel: 33 4 92 07 6412, Fax: 33 4 92 07 64 02

RNA-mediated hereditary epigenetic variations (paramutation) in the mouse

ESHRE Barcelona July 2008

Inserm

ANR

Paramutation, a hereditary epigenetic modification

… discovered 50 years ago

R.A Brink, Genetics, 1956

A hereditary change in phenotype induced by "cross-talk"between defined pairs of alleles in heterozygotes.
A departure from the law of Mendel which states that allelic forms segregate unchanged from heterozygotes.

… extensively studied in plants...

Reviews: Chandler & Stam, Nature Rev Genet, 2004, 5:532
Chandler, Cell, 2007, 128:641

… in the mouse

Only two instances of allele-dependent changes in DNA methylation patterns described as « paramutation-like » effects

Rassoulzadegan et al. EMBO J., 2002, 21:440
Herman, Soloway et al., Nat Genet., 2003, 34:199

Paramutation had been studied in plants and fungi for more than 50 years...

"Alexander Brink (1956, Genetics) coined the term "paramutation" to describe a violation of Mendel's first law which states that genetic factors segregate unchanged from a heterozygote"

Hollick et al., Trends Genet., 1997, 13:302

Still, its mode of inheritance remained mysterious...

Epigenetic modifications

- Changes in phenotype which result either from the activation or the repression of defined genes or genomic regions

⇒ without a change in the primary sequence (unlike mutations),

⇒ not associated with a unique differentiation process (unlike regulatory mechanisms),

⇒ mitotically stable, and in a number of instances, meiotically stable and inherited.

- Involve DNA methylation, histone modifications (methylation, acetylation,...).
- Our knowledge of the mechanisms inducing epigenetic changes remains rudimentary.
- Even more perplexing: the ways of inheritance...

Panthier

Development 122, 3023-3033 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2070

Spatial and temporal patterns of c-*kit*-expressing cells in $W^{lacZ}/+$ and W^{lacZ}/W^{lacZ} mouse embryos

Florence Bernex[1], Paulo De Sepulveda[1], Chantal Kress[2], Colette Elbaz[1], Claude Delouis[1] and Jean-Jacques Panthier[1,*]

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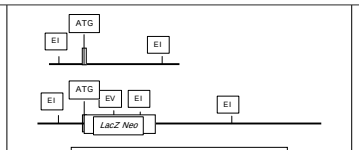


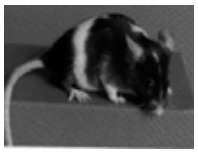
Officially:

W^{lacZ} is now $Kit^{tm1Alf/+}$

The tyrosine kinase Kit receptor

- Required in multiple developmental lineages
  - Hematopoiesis
  - Melanocyte differentiation
  - Germ line development
  - ...
- Oncogenic variants
- Null mutants are homozygous-lethal
- Heterozygotes show characteristic « white spotted » phenotypes

Kit mutants (mouse): characteristic « white-spotted » phenotypes

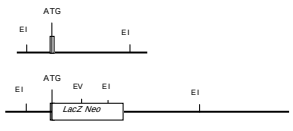


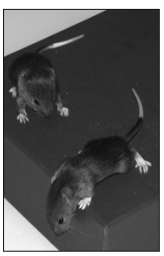
Paramutation at the tyrosine kinase Kit receptor locus

M. Rassoulzadegan, V. Granjean, P. Gounon et al. Nature, 2006, 441:469-474

The receptor is required in multiple developmental lineages
Null mutants are homozygous-lethal
Heterozygotes show characteristic « white spotted » phenotypes

The *Kit* $^{tm1Alf\ /\ +}$ heterozygotes



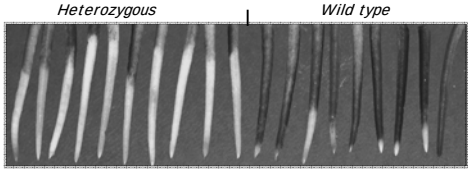


In other words…

The wild type Kit+ alleles transmitted by heterozygous parents maintain a "mark" resulting in the maintenance of the mutant phenotype

Kit^{tm1Alf} / Kit^{+} x Kit^{tm1Alf} / Kit^{+}

Heterozygous *Wild type*



Non-Mendelian segregation of phenotypes in heterozygote intercrosses

Offspring phenotype	Number of mice[1]	Genotype	
		Neo[2]	LacZ[3]
Full agouti coat	3	-	-
White tail and feet	30	+	+
Partially white tail and feet	24	-	-

[1] cumulated values of 8 litters

[2] PCR determination

[3] ß-galactosidase assay (X-Gal staining)

⇒ Wild type genotypes are generated as expected

⇒ Wild type phenotypes underrepresented: 3/57 instead of the Mendelian 1/3

Paternal and maternal transmission of paramutation independent of genetic backgrounds

Crosses[1]			Progeny[2]		
Male	Female	Genetic background[3]	Heterozygote	Paramutated	Wild type
Kit $^{tm1Alf/+}$	Wild type	129Sv	20	12	4
		C57BL/6	16	10	4
		B6D2	24	16	4
Wild type	*Kit* $^{tm1Alf/+}$	129Sv	21	11	5
		C57BL/6	15	10	4
		B6D2	22	14	5

[1] wild type and mutant partners of same genetic background in each cross

[2] cumulated values of 4 litters for each cross; genotypes and phenotypes of progenies as determined in Table 1

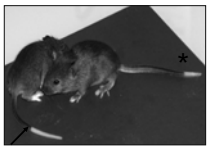
[3] 129Sv: the original *Kit* $^{tm1Alf/+}$ strain; other genotypes: at least 6 back-crosses of the mutant allele onto each genotype

Paramutation phenomena share three key features:

1- The newly established expression state is transmitted to subsequent generations even though the allele or sequences originally issuing the instructions is not transmitted;
2- The altered locus continues to issue similar instructions to homologous sequences;
3- There are no associated DNA sequence changes in the affected allele or sequences, indicating the memory and instructions are mediated through epigenetic mechanisms

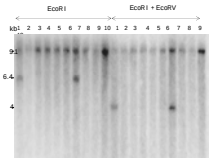
Viki Chandler, Review in Cell 2007

The Kit* paramutants:
mice with two structurally normal wild type alleles which maintain the white tails and feet of the heterozygote



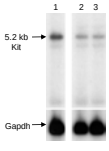
→ **heterozygote**

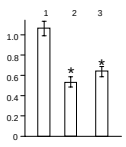
* **paramutated**



Southern blot analysis of
- one wild type control (lanes 10)
- two heterozygotes (lanes 1, 7)
- seven paramutated animals

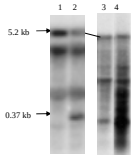
White-spotting of Kit* animals is the consequence of a reduced level of gene expression





Polyadenylated RNA in:
1: wild type
2: heterozygote
3: paramutated

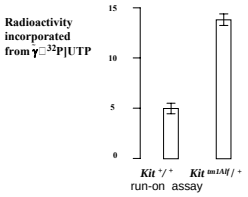
Modified post-transcriptional processing ?
Active RNA degradation ?



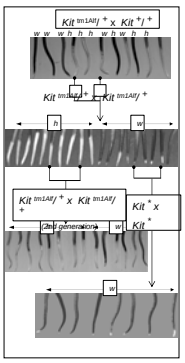
Total RNA of
1: wild type brain
2: heterozygote brain
3: wild type testis
4: heterozygote testis

Up-regulated transcription

The observed decrease in mature mRNA contrasts with an overall increase in the rate of transcription



Unlike the plant paramutation, none of the mouse epigenetic variations currently under analysis involves transcriptional silencing



Transgenerational maintenance:

the white-spotted phenotype is

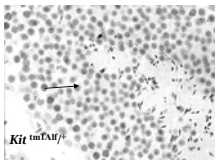
inherited for 2-3 generations in crosses with wild type partners

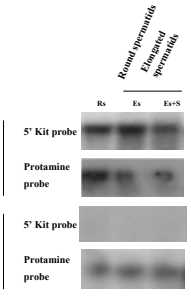
⇒ of either male

⇒ or female paramutants

Paternal inheritance: a signal associated with the compacted genome?

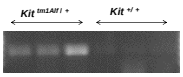
The increased transcriptional activity in the testis results in the accumulation of *Kit* RNA (and ß-galactosidase expression) at the late spermatogenic stages (round and elongated spermatids).



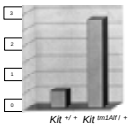


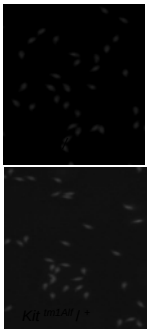
Kit RNA in sperm

RT-PCR amplification
(Kit 5' coding region)

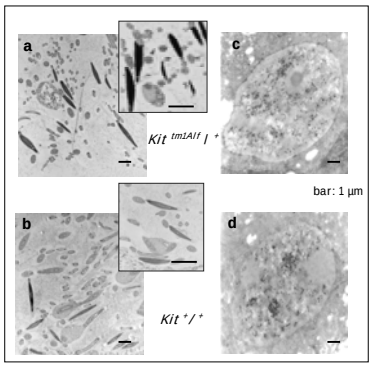


Real time qPCR: Kit relative to Gapdh





Kit RNA in sperm ? EM studies



EDTA regressive staining
(W. Bernhard, 1969, Biggiogera & Fakan, 1998):

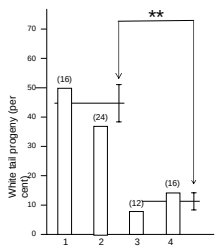
after staining with uranyl acetate, EDTA treatment removes stain in all cellular materials but RNA

a,c: *tm1Alf/+* heterozygote
b,d: wild type

a,b: epididymis section
c,d: spermatocytes

EM analysis: Pierre Gounon
Centre Commun de Microscopie
Université de Nice

RNA microinjection in fertilized eggs



⇒ Microinjection of heterozygote sperm or brain RNA induces the paramutated white-spotted phenotype

⇒ Low efficiency of wild type brain and unrelated RNAs

⇒ Induction by degradation product of Kit RNA?

Injected RNA
1: heterozygote sperm
2: heterozygote brain
3: wild type brain
4: LacZ

inherited white spotting
1st generation only

Cell, Vol. 115, 787–798, December 26, 2003, Copyright ©2003 by Cell Press

Prediction of Mammalian MicroRNA Targets

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Matthew W. Jones-Rhoades,[1,2] David P. Bartel,[1,2,*]
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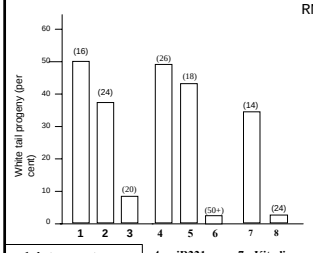
Table 1. Highly Cited Predicted Targets of Mammalian miRNAs

Category	Seed	miRNAs	Ensembl ID	Gene Name
Regulation of transcription/ DNA binding	AGUGCAA	miR-130,-130b	169057	Methyl-CPG-binding protein 2 (*MECP2*)
	GUGCAAA	miR-18a	169057	" "
	AAAGUGC	miR-20,-106	101412	Transcription factor *E2F1*
	GAGGUAG	*let*-7(a-g,i),miR-98	100823	DNA-(apurinic or apyrimidinic site) lyase (*APEN*)
	GGAAGAC	miR-7	136826	Kruppel-like factor 4 (*EZF*)
Signal transduction/ cell-cell signaling	UAAGGCA	miR-124a	166610	Signal transducer and act. of transcription 3 (*STAT3*)
	UGGUCCC	miR-133,-133b	010610	T cell surface glycoprotein CD4 precursor
	UCACAUU	miR-23a,-23b	107562	Stromal cell-derived factor 1 precursor (*SDF-1*)
	GCUACAU	miR-221,-222	157404	Mast/stem cell growth factor receptor precursor (*C-KIT*)
	GGAAUGU	miR-1,-206	176697	Brain-derived neurotrophic factor precursor (*BDNF*)
	UAAGGCA	miR-124a	154188	Angiopoietin-1 precursor (*ANG-1*)

RNA microinjection in fertilized eggs



1: heterozygote sperm
2: heterozygote brain
3: wild type brain

4: miR221
5: miR222
6: miR-1, miR-124, …

7: Kit oligonucleotide
8: unrelated (Cdk9) oligonucleotide

⇒ Microinjection in fertilized normal eggs of RNA prepared from sperm and somatic cells of heterozygotes induces the heritable white-spotted phenotype

⇒ MicroRNAs 221 and 222, which target *Kit* RNA, are also efficient inducers of the paramutated phenotype

⇒ OligoRnucleotides with randomly chosen sequences from the Kit coding sequences induce as well

Inheritance of the paramutated phenotype induced by RNA microinjection

Injected RNA	Mating partners (wild type)	White-spotted progeny / total	Transmission efficiency (per cent)
Paramutated brain RNA	female	25/32	78
	male	26/34	76
miR221	female	18/32	56
	male	18/31	58
miR222	female	20/36	56
	male	18/32	56

Accumulated results of four litters

Questions (some…) and elements of (preliminary…) answers

⇒Is the modification of the wild type *Kit*$^{+}$ allele in *Kit*$^{tm1Aff/+}$ heterozygotes a consequence of meiotic mispairing?

⇒ Could other epigenetic modifications/paramutation be induced by other miRs?

⇒ A function for human sperm RNA?

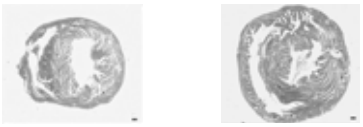
More heritable phenotypes induced by RNA microinjection in fertilized eggs using microRNAs to find target genes

- miR-1: cardiac hypertrophy
- miR-124: gigantism

The miR-1 paramutants

The miR-1 microRNA expressed in cardiac and skeletal muscle exerts crucial function(s) in heart development and physiology

Mice born after microinjection of miR-1 in the one-cell embryos show hypertrophic cardiomyopathy (HCM)



Microinjection random 20 nt ribo-oligonucleotide

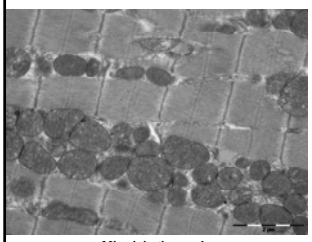
Microinjection miR-1

Adult heart (same enlargemt)

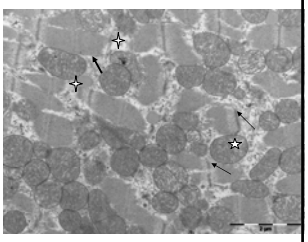
Human HCM: a life-threatening familial disease
Although several predisposition loci were identified, heredity not fully explained in mendelian terms

The miR-1paramutants

Morphology of a control mouse heart, disarranged morphology in the miR-1* heart with myofibrillar disorganization, sacromere shrinkage and disintegration, mitochondrial disorganization and abnormalities and the irregularity of z-discs.

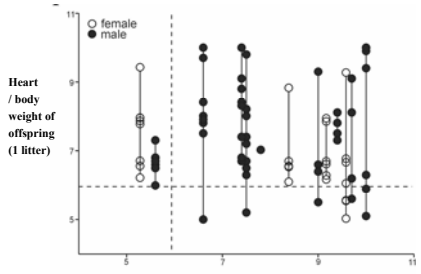


Microinjection random
20 nt ribo-oligonucleotide



Microinjection miR-1

Heredity of miR-1* HCM: 1. high efficiency of transmission with « rheostat » effect

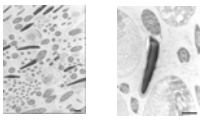


Heart / body weight of mir-1* parent mated with wild type partner

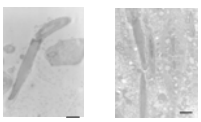
Dotted line: average wild type

Heredity of miR-1* HCM: 2. RNA load in sperm head, includes miR-1

EM: reverse EDTA staining

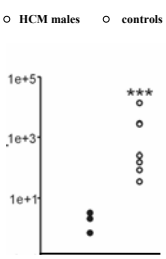


miR-1*



WT mouse miR-1* RNase

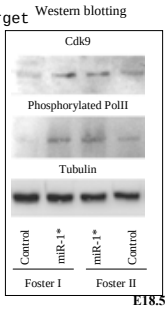
qPCR detects miR-1 sequences in sperm RNA

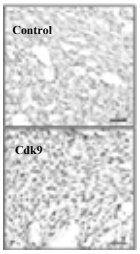


Cdk9-Cyclin T1 control of cardiomyocyte growth

- In normal heart development as in clinical/experimental HCM, phosphorylation of RNA PolII by the Cdk9-Cyclin T1 kinase is the critical element of cardiomyocyte growth
- Cdk9 is a potential target of miR-1 microRNA

Both Cdk9 expression and RNA PolII phosphorylaltion are increased in miR-1 HCM

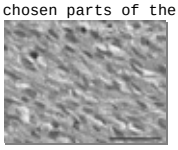




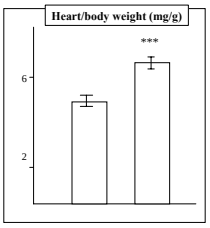
Epigenetic HCM is induced by Cdk9 RNA oligonucleotides microinjected in one-cell embryos

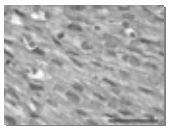
As the Kit* paramutation, epigenetic HCM is induced both by the homologous microRNA and by cleaved fragments of the target mRNA

(21-25 oligoribonucleotide sequences corresponding to randomly chosen parts of the coding sequence)



Control

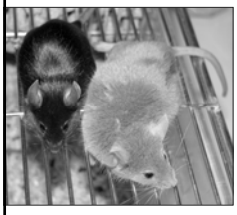


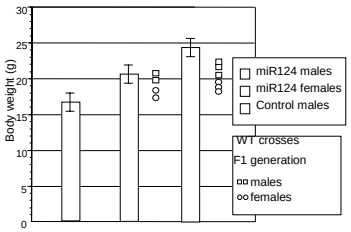


Cdk9

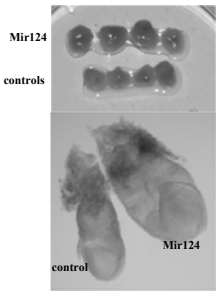
The giant miR-124 mouse

- miR-124 is expressed at high levels in brain
- microinjection in fertilized eggs results in accelerated growth



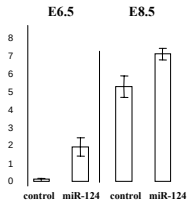


Accelerated development at a very early developmental stage

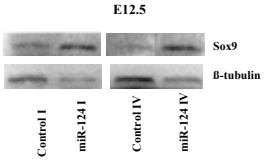


(E7.5 embryos)

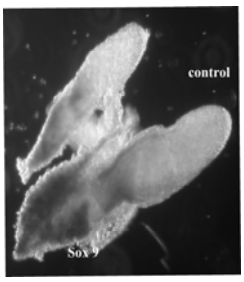
Sox9, target of the miR-124 paramutation?



Sox9 / Gapdh



Accelerated early development after microinjection of a *Sox 9* expression vector



(E7.5 embryos)

The mechanism of induction of paramutation?
(i) a control of RNA profiles in the early embryo?

Paramutation is initiated under three sets of conditions

⇒ a structurally modified allele

⇒ zygotic transfer of coding RNA fragments

⇒ zygotic transfer of microRNAs

⇒ a quality check of zygote RNAs based on sequence homologies

i.e. a zygotic surveillance mechanism ?

DNMT2 does not seem to methylate exclusively tRNA molecules

Methylation of *Kit* / or other mRNAs?

Methylation of *Kit* RNA in heterozygotes?

RNA methylation and the control of epigenetic states?

Paramutation in humans???

Would it explain:

⇒ familial distribution of cancers and other diseases (hypertrophic cardiomyopathy!!) with no Mendelian determinant identified so far ??

⇒ paternal inheritance of disease and mortality over several generations (epidemiological studies, Pembrey et al.) ??

But …
genetics is complicated in the human species by our extensively outbred reproduction and genome diversity

Human sperm contains relatively large amounts of RNA (Krawetz, Miller et al.)

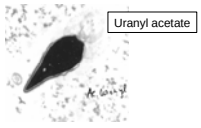
PATERNAL CONTRIBUTION: NEW INSIGHTS AND FUTURE CHALLENGES

Stephen A. Krawetz

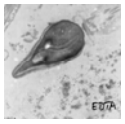
NEWS FEATURE

THE SECRET LIFE OF SPERM

Far from being mere DNA delivery boys, it's now becoming clear that sperm also ship a complex cargo of RNA and proteins that may be crucial for an embryo's early development. Claire Ainsworth reports.

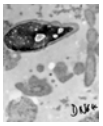


Uranyl acetate

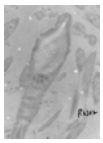


EDTA reverse

RNA in human sperm



DNase control



RNase control

Variation Charles Darwin

An individual organism placed under new conditions (often) sometimes varies in a small degree and in very trifling respects such as stature, fatness, sometimes colour, health, habits in animals and probably disposition.

With the amount of food man can produced he may have arrived at limit of fatness or size, or thickness of wool, but these are the most trivial points, but even in these I conclude it is impossible to say we know the limit of variation.

And therefore with the adapting selecting power of nature, infinitely wise compared to those of man, I conclude that it is impossible to say we know the limit of races, which would be true to their kind; if of different constitutions would probably be infertile one with another, and which might be adapted in the most singular and admirable manner, according to their wants, to external nature and to other surrounding organisms such races would be species.

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The "Paramutation group"

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- Valérie Grandjean
- Luc Martin
- Christine Vannetti
- Hossein Ghanbarian
- Jafar Kiani

Collaborations

- Pierre Gounon
  Centre Commun
  de Microscopie

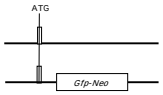
The Schedl group

- Nicole Wagner
- Kay Wagner

Induced by meiotic mispairing?

as suggested by analysis of gene silencing in *Neurospora*
(Shiu et al., Cell, 2001, 107:905)

Non-Mendelian inhheritance of the large insertion mutant $Kit^{GFP\text{-}Neo}$



Crosses		Progeny (4 litters)		
Male	Female	$Kit^{GFP\text{-}Neo/+}$	Kit *	$Kit^{+/+}$
$Kit^{GFP\text{-}Neo/+}$	$Kit^{+/+}$	23	13	3
$Kit^{+/+}$	$Kit^{GFP\text{-}Neo/+}$	21	12	6

Mendelian inheritance of the point mutant $Kit^{W\text{-}V}$

Crosses		Progeny (4 litters)		
Male	Female	$Kit^{W\text{-}v/+}$	Kit *	$Kit^{+/+}$
$Kit^{W\text{-}v/+}$	$Kit^{+/+}$	12	0	15
$Kit^{+/+}$	$Kit^{W\text{-}v/+}$	15	0	14

Kit^{Wv} strain

W^v is a missense mutation in the kinase domain of the *c-kit* coding sequence
(*T to M position 660/975aa*).

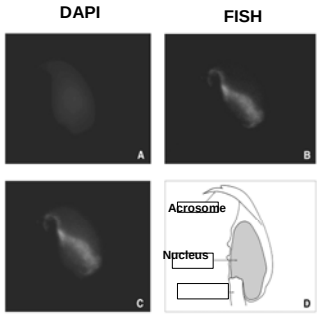
Kit^{Wv} / Kit^{+} x Kit^{Wv} / Kit^{+}

Sperm mediated gene transfer:
mechanism and implications

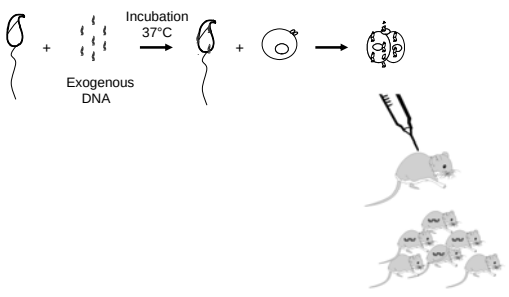
Corrado Spadafora

Italian National Institute of Health
Rome, Italy

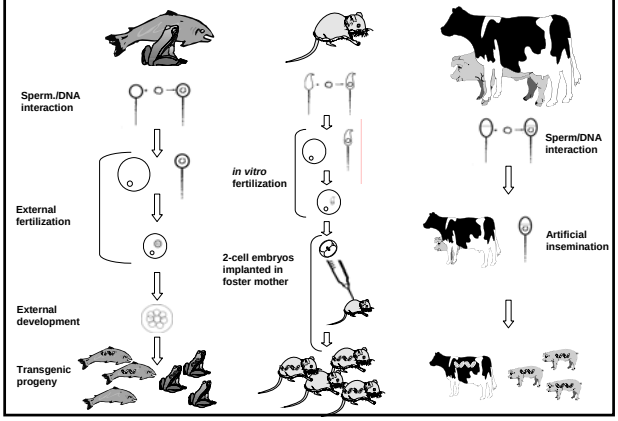
Mouse sperm cells internalize exogenous DNA molecules



Sperm-mediated gene transfer



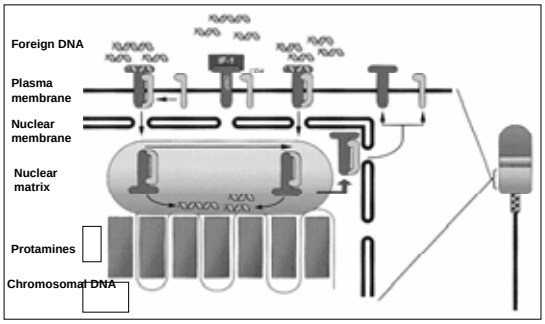
Sperm-mediated Gene Transfer applications



The controversy about Sperm-mediated Gene Transfer

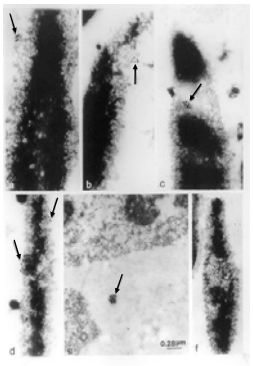
- Difficulty to reproduce our original results (Cell, 1989) in the mouse system
- Low efficiency in generating transgenic mice
- Generation of animals harboring heterogeneous, unstable patterns
- Evidence that transgenes remain as non-integrated episomal structures

A network of factors mediate the interaction of exogenous DNA molecules with sperm cells

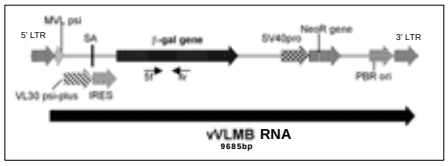


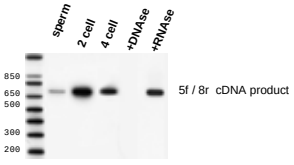
- ✓ The binding and nuclear internalization of DNA by sperm cells is not a random event, but is a well regulated process mediated by specific factors
- ✓ Exogenous DNA binding activates sperm nuclear functions that are otherwise repressed in spermatozoa
- ✓ One of these activities is an endogenous Reverse Transcriptase

Immunogold-EM localization of endogenous RT molecules on scaffolds of murine spermatozoa



β-gal cDNA is detected in sperm cells and embryos





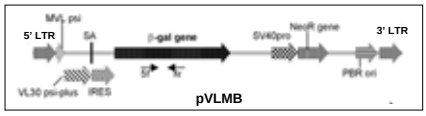
β-gal cDNA sequences in tissues of F0 and F1 mice

β-gal cDNA copies are :

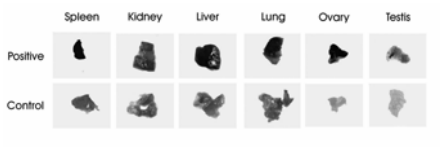
- ✓ mosaic distributed in tissues of positive F0 mice
- ✓ maintained at low copy number (< 1 copy / genome)
- ✓ sexually transferred from F0 to F1 offspring
- ✓ low copy number and mosaic distributed in tissues of F1 mice

Retrotranscribed sequences may be propagated as extrachromosomal structures

Sperm-mediated "Reverse" Gene Transfer

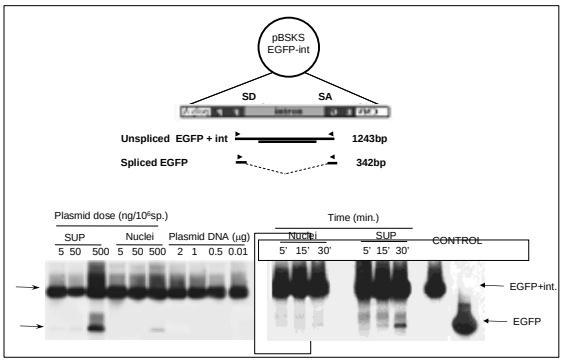


β-gal is expressed in organs of F0 and F1 animals from pVLMB RNA-transformed founders

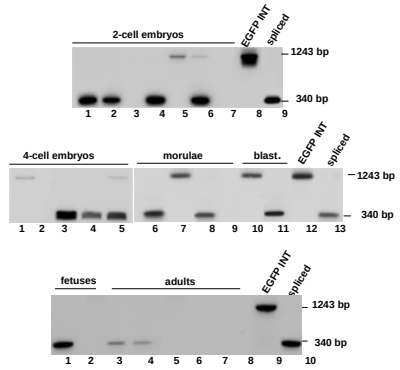


- ✓ **Exogenous RNA molecules internalizatized in sperm cells are reverse-transcribed in cDNA copies and delivered to oocytes at fertlization**
- ✓ **cDNAs are mosaic propagated in tissues of adult animals and transferred to the next generation as extrachromosomal structures**
- ✓ **cDNAs are transcriptionally competent and are expressed in tissues of both F0 and F1 animals**

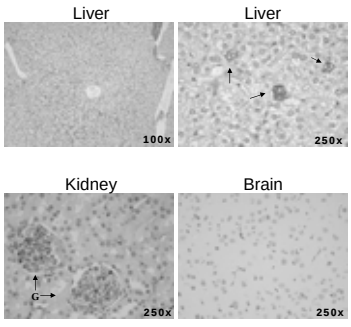
Reverse-transcribed spliced EGFP copies can be PCR-amplified after sperm cell incubation with pBSKS-EGFP-INT



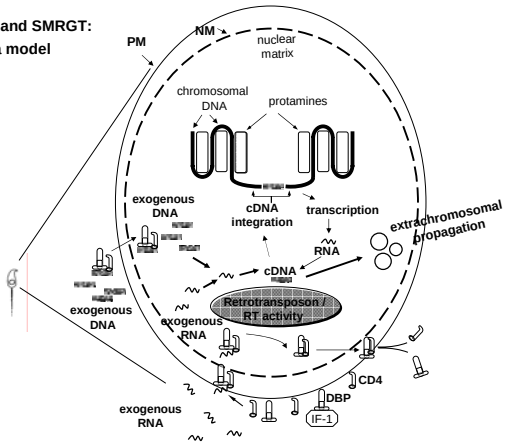
Retrotranscribed and spliced EGFP sequences are propagated in embryos and born animals



EGFP cDNA is expressed in tissues of PCR-positive mice



SMGT and SMRGT: a model



Conclusions - 1

1. Sperm cells can spontaneously take up exogenous DNA or RNA molecules and to internalize them in nuclei
2. An endogenous RT is active in spermatozoa and early mouse embryos
3. The sperm RT is implicated in the genesis of reverse-transcribed-new genetic information from exogenous RNA or DNA templates
4. Reverse transcribed sequences are maintained at low copy number, propagated to embryos and adult animals and transmitted to the next generation mostly as extrachromosomal structures
5. Reverse transcribed sequences are transcriptionally competent and are expressed in various tissues of adult F0 and F1 animals.

On these grounds, Sperm-Mediated Gene Transfer can be regarded as a retrotransposon-mediated phenomenon

Conclusions - 2

These results suggest that a sperm RT-mediated mechanism is responsible for the genesis and propagation of newly reverse-transcribed genetic information, besides that carried by chromosomes

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