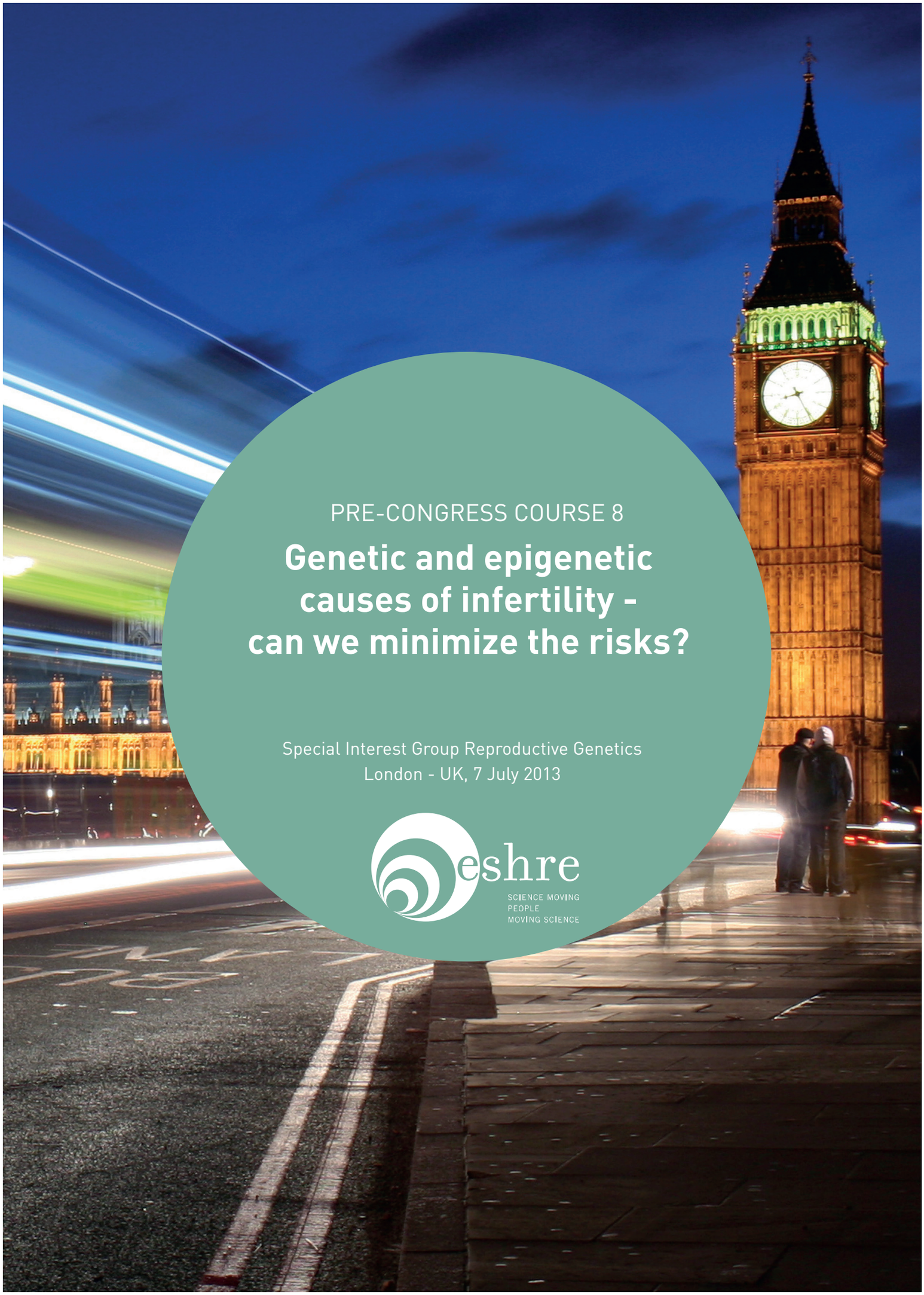

PRE-CONGRESS COURSE 8

Genetic and epigenetic causes of infertility - can we minimize the risks?

Special Interest Group Reproductive Genetics
London - UK, 7 July 2013

eshre
SCIENCE MOVING
PEOPLE
MOVING SCIENCE

Genetic and epigenetic causes of infertility - can we minimize the risks?

London, United Kingdom
7 July 2013

Organised by
The ESHRE Special Interest Group Reproductive Genetics

Contents

Course coordinators

Ursula Eichenlaub-Ritter (Germany), Joyce Harper (United Kingdom), Wendy Dean (United Kingdom) and Tania Milachich (Bulgaria)

Course description

The link between reproduction and genetics has been studied extensively, having benefitted immensely from the human genome project. What is now apparent is that epigenetics may play an equally important role in reproductive potential. In the post genomic era, whole genome scanning may become routine practice before couples try to conceive. This will be an exciting time but not without ethically difficult issues to resolve. This workshop is designed to update delegates on our current knowledge of genetic testing and epigenetics in relation to fertility. The course will cover some of the latest findings relating to the female, the male and the embryo. One of the questions will be – can we minimise genetic and epigenetic risks? This is an advanced course and so a basic knowledge in genetics and embryology is necessary.

Target audience

Scientist, embryologists and medics interested in genetics, PGD specialists, geneticists

Scientific programme

Chairman: Joyce Harper - United Kingdom

Introduction

09:00 - 09:30	Genes and genetic testing – where are we today? *Alan H. Handyside - United Kingdom*
09:30 - 09:45	Discussion
09:45 - 10:15	Epigenetics and fertility *Wendy Dean - United Kingdom*
10:15 - 10:30	Discussion
10:30 - 11:00	Coffee break

Female

11:00 - 11:30	Genome scanning to identify genes in PCOS and early onset menopause *Joop S.E. Laven - The Netherlands*
11:30 - 11:45	Discussion
11:45 - 12:15	Epigenetics in the oocyte *Thomas Haaf - Germany*
12:15 - 12:30	Discussion
12:30 - 13:30	Lunch

Male

13:30 - 14:00	Genetic factors for male infertility *Stephane Viville - France*
14:00 - 14:15	Discussion
14:15 - 14:45	Paternal DNA packaging in sperm – more than the sum of its parts? DNA, histones, protamines, and epigenetics *David Miller - United Kingdom*
14:45 - 15:00	Discussion
15:00 - 15:30	Coffee break

Embryos

15:30 - 16:00	Epigenetic mechanisms in the preimplantation embryo *Robert Feil - France*
16:00 - 16:15	Discussion

Pregnancy and minimizing the risks

16:15 - 16:45	Links between the genome and the epigenome in utero *Gudrun Moore - United Kingdom*
16:45 - 17:00	Discussion

Genetic and epigenetic causes of infertility –
can we minimize the risks?
Pre-congress Course 8
ESHRE Annual Meeting
London, UK 7th July 2013

Genes and genetic testing – where are we today?

Alan H Handyside

BlueGnome

Conflict of interest statement

Prof Alan H Handyside is

Head of Preimplantation Genetics

Bluegnome, an Illumina Company

BlueGnome

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1. Array CGH
2. SNP arrays for cytogenetics
3. SNP genotyping and Karyomapping
4. Next-generation sequencing

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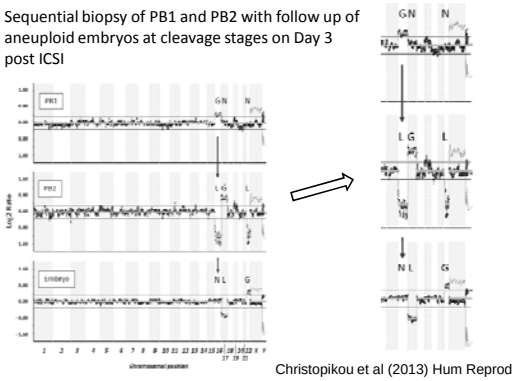
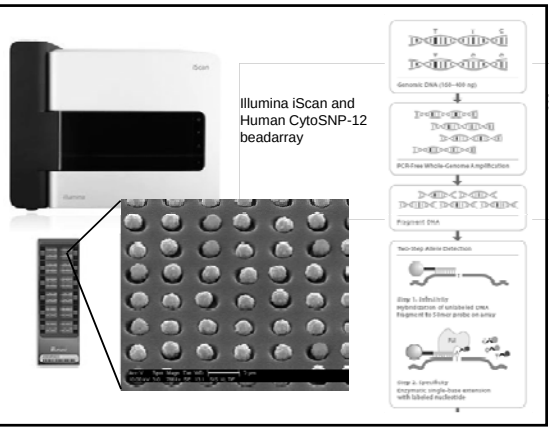
Sequential biopsy of PB1 and PB2 with follow up of aneuploid embryos at cleavage stages on Day 3 post ICSI

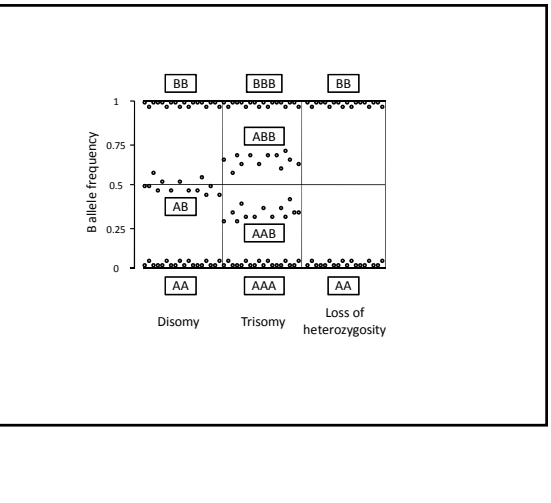


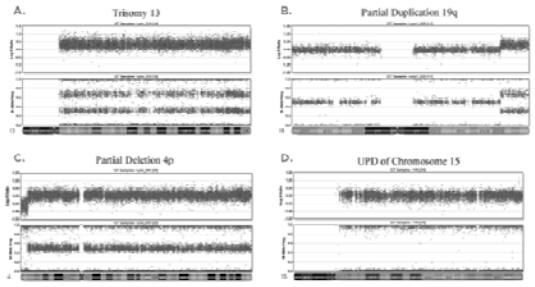
Christopikou et al (2013) Hum Reprod

BlueGnome



Illumina iScan and Human CytoSNP-12 beadarray





Original article

Karyomapping: a universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes

Alan H Handyside,[1,2] Gary L Harton,[3] Brian Mariani,[3] Alan R Thornhill,[1,4] Nabeel Affara,[5] Marie-Anne Shaw,[7] Darren K Griffin[4]

Handyside et al (2010) J Med Genet 47, 651-658

BlueGnome

Karyomapping for detection of reciprocal translocation chromosome imbalance

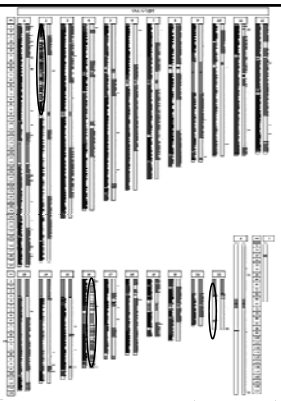
46, XY t(2;22)(p12;q11.2)

Embryo 1

Paternal 2 and der 22

Unbalanced

Adjacent-1 segregation



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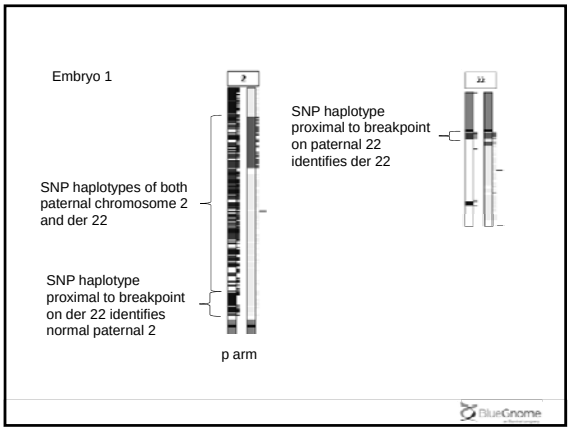
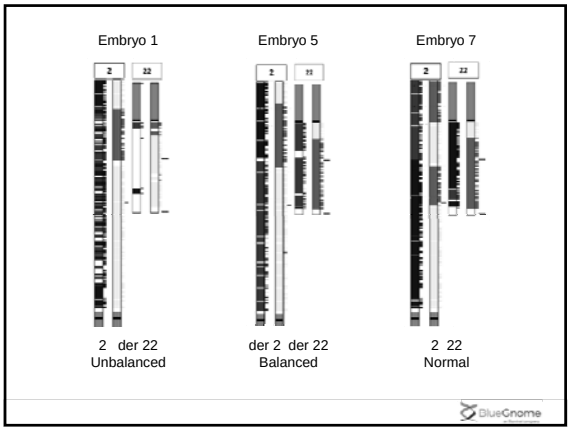


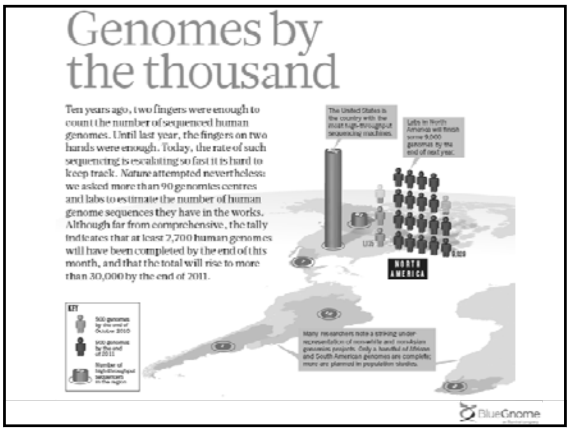


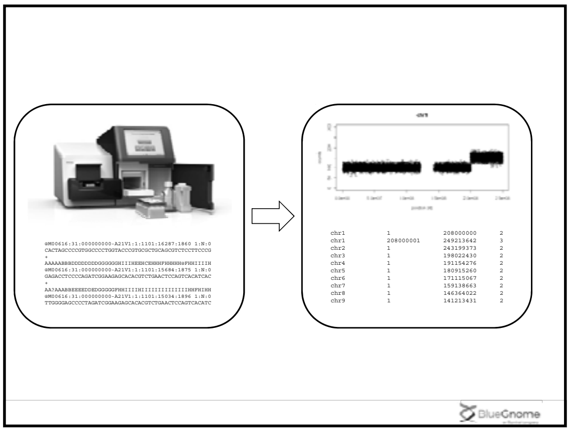
James D Watson

May 31st, 2007

Next-Generation Sequencing

- The dawn of the era of personal genomics with the prospect of personalised medicine
- Complete sequencing of the genomes of James D Watson and J Craig Venter by massively parallel sequencing


Genomes by the thousand
Ten years ago, two fingers were enough to count the number of sequenced human genomes. Until last year, the fingers on two hands were enough. Today, the rate of such sequencing is escalating so fast it is hard to keep track. Nature attempted nevertheless: we asked more than 90 genomics centres and labs to estimate the number of human genome sequences they have in the works. Although far from comprehensive, the tally indicates that at least 2,700 human genomes will have been completed by the end of this month, and that the total will rise to more than 30,000 by the end of 2011.
NORTH AMERICA
BlueGnome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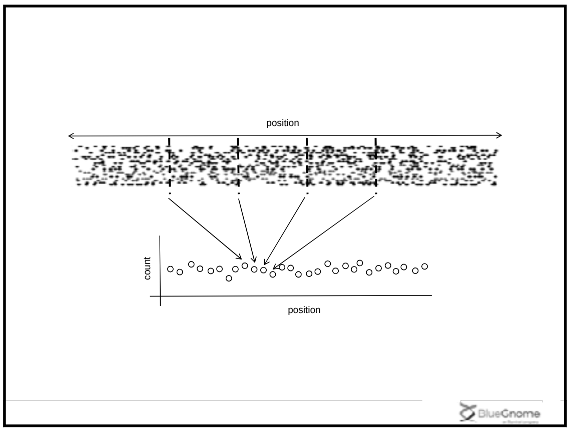
position
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BlueGnome

Massively Parallel Sequencing for Chromosomal Abnormality Testing in Trophectoderm Cells of Human Blastocysts[1]

XuYang Yin,[2,6] Ke Tan,[2,10] Gábor Vajta,[2,6,8] Hui Jiang,[2,6,9] YueQiu Tan,[7,10,11] ChunLei Zhang,[6] Fang Chen,[6,9] ShengPei Chen,[6,13] ChunSheng Zhang,[6] XiaoYu Pan,[6,12] Chun Gong,[6] XuChao Li,[6] ChuYu Lin,[6] Ya Cao,[6] Yu Liang,[6] Xin Yi,[6] Feng Mu,[6] HJian Zhao,[6] HuanHuan Peng,[6] Bo Xiong,[11] ShuoPing Zhang,[7,11,12] DeHua Cheng,[14] GuangXiu Lu,[7,10,11,12] XiuQing Zhang,[2,6] Ge Lin,[6,7,10,11,12] and Wei Wang[2,6]

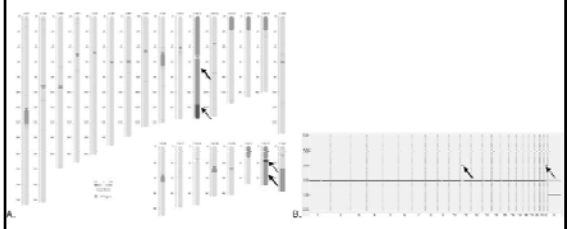
[1]BGI-Shenzhen, Shenzhen, China
[2]Institute of Reproductive and Stem Cell Engineering, Central South University, Changsha, China
[3]Institute for Resource Industries and Sustainability (IRIS), Central Queensland University, Rockhampton, Queensland, Australia
[4]Department of Biology, University of Copenhagen, Copenhagen, Denmark
[5]National Engineering and Research Center of Human Stem Cell, Changsha, China
[6]CITIC Xiangya Reproductive & Genetic Hospital, Changsha, China
[7]Key Laboratory of Stem Cell and Reproductive Engineering, Ministry of Health, Changsha, China
[8]State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Nanjing, China
[9]School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, China

Yin et al (2013) Biol Reprod 88, 69

BlueGnome

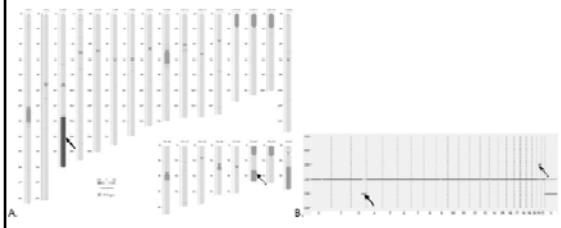
- Trophectoderm cells were biopsied from 38 blastocysts in 16 IVF cycles
- 13 couples had structural chromosomal abnormalities including 4 Robertsonian and 9 reciprocal translocations and one inversion
- Illumina HiSeq2000 used to sequence whole genome amplification products at 0.07x depth with average 5.5% coverage
- 26 (68%) blastocysts euploid, 6 (16%) aneuploid, 4 (11%) unbalanced only, 2 (5%) unbalanced and aneuploid
- Highly concordant with SNP array results

BlueGnome



46,XY,t(11;22)(q23;q11)

BlueGnome



46, XY, t(3;21)(q21;q22)

BlueGnome

Evaluation of targeted next-generation sequencing–based preimplantation genetic diagnosis of monogenic disease

Nathan R. Treff, Ph.D.,[a,b,c] Anastasia Fedick, B.S.,[a,b] Xin Tao, M.S.,[a] Batsal Devkota, Ph.D.,[a] Deanna Taylor, Ph.D.,[a,c] and Richard T. Scott Jr., M.D.[a,c]

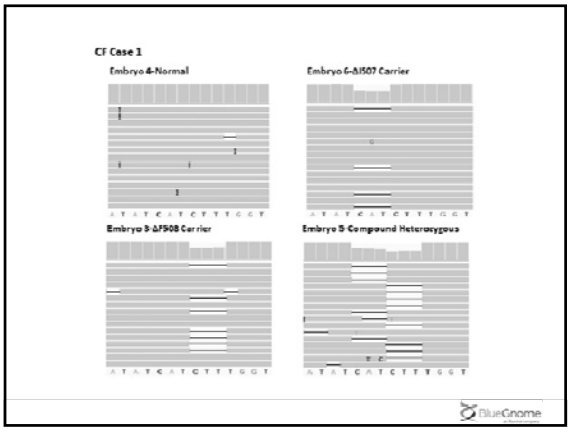
[a] Reproductive Medicine Associates of New Jersey, Morristown, New Jersey; [b] Molecular Genetics, Microbiology and Immunology, and [c] Obstetrics, Gynecology, and Reproductive Sciences, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, New Brunswick, New Jersey

Treff et al (2013) Fertility and Sterility 99, 1377-1384

BlueGnome

- Trophectoderm cells were biopsied from 21 blastocysts in 3 PGD cycles in two couples at risk of cystic fibrosis and one of Walker-Warburg syndrome
- Whole genome amplification was followed by targeted Taqman amplification of mutation site was followed by in depth sequencing (Ion Torrent) with 8 barcoded samples per chip
- Real time qPCR used for 24 chromosome aneuploidy testing
- 17 (81%) blastocysts euploid, 4 (19%) aneuploid
- 100% concordance of mutation status with STR and minisequencing

BlueGnome



- Microarray-based technologies remain the most cost effective and validated methods for routine clinical use for preimplantation genetics
- NGS costs rapidly decreasing and samples can be multiplexed at low read depth
- Whole genome amplification from single or a few cells introduces artefactual copy number and sequence variants which are difficult to distinguish from true de novo variants
- Beyond aneuploidy and segmental chromosome imbalance, the development of powerful bioinformatics filters will be needed for accurate interpretation
- NGS definitely on the horizon!

BlueGnome

29th Annual Meeting – London 7th July 2013
The European Society of Human Reproduction and Embryology
(ESHRE)

Genetic and epigenetic causes of infertility

Can we minimize the risks?

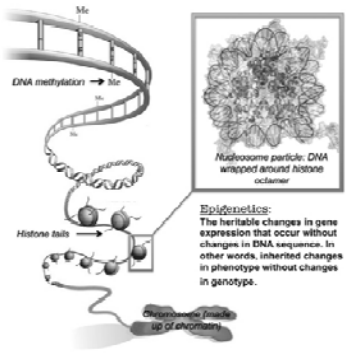
WENDY DEAN
EPIGENETICS PROGRAM
THE BABRAHAM INSTITUTE
CAMBRIDGE UK

29th Annual Meeting – London 7th July 2013
The European Society of Human Reproduction and Embryology
(ESHRE)

Epigenetics & Fertility

WENDY DEAN
EPIGENETICS PROGRAM
THE BABRAHAM INSTITUTE
CAMBRIDGE UK

Epigenetics is the study of…….



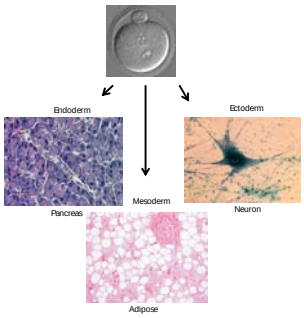
Epigenetics is important because …….

These modifications , marks or molecules define transcriptional states and specify and reinforce lineage decisions

During key stages of gametogenesis and during development epigenetic marks are reprogrammed in order to establish and lock in cellular fate

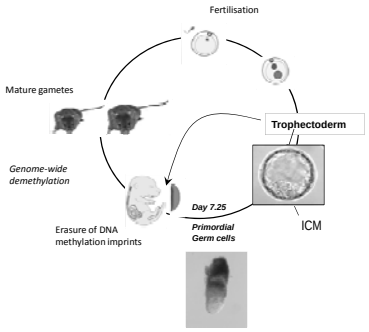
Establishment of epigenetic states is essential for reproductive success

These modifications , marks or molecules define transcriptional states and specify and reinforce lineage decisions

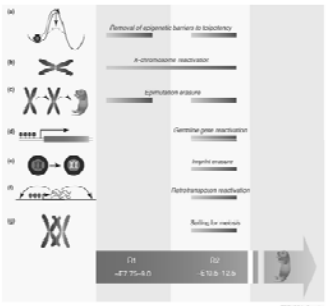


One genotype – many phenotypes

During key stages of gametogenesis and during development epigenetic marks are reprogrammed in order to establish and lock in cellular fate



Why reprogram the germline?



Parallel mechanisms of epigenetic reprogramming in the germline

Three significant developmental windows are likely to be sensitive to exogenous signals that may alter epigenetic profiles with impact on reproduction and fertility

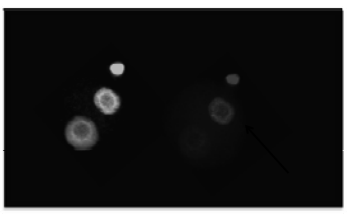
Reprogramming of the germ line during PGC erasure- 

DNA methylation establishment during oocyte growth and maturation 

Reprogramming in first cell cycle – meiosis and mitosis in transition 

These windows all involve dramatic changes in DNA methylation

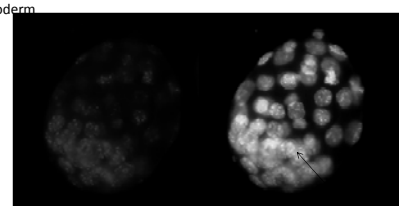
Epigenetic regulation in the zygote



Active loss of methylation from the male pronucleus results in an asymmetric distribution

Epigenetic regulation of lineage establishment

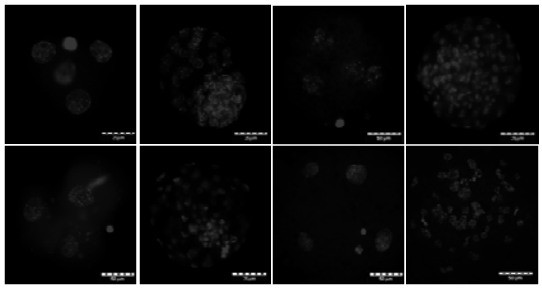
Hypomethylated trophectoderm

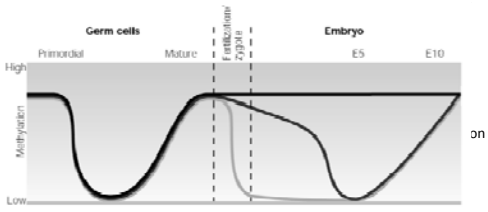


Hypermethylated ICM

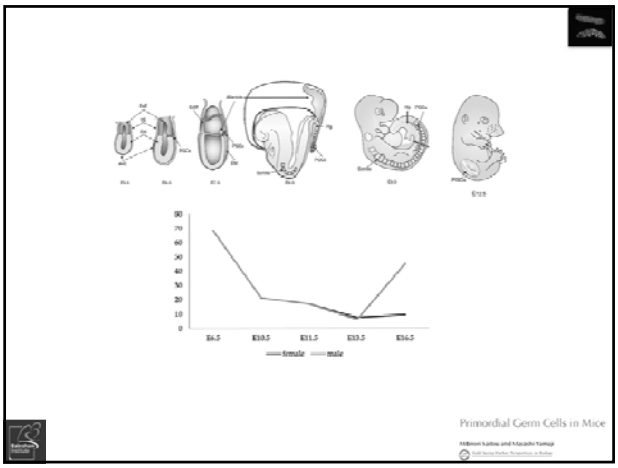
Do novo methylation results in an asymmetric distribution of DNA methylation

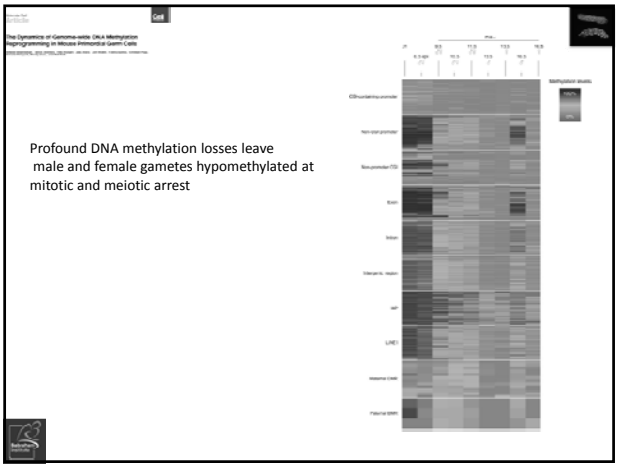
Evaluation of epigenetic marks in human embryos derived from IVF and ICSI

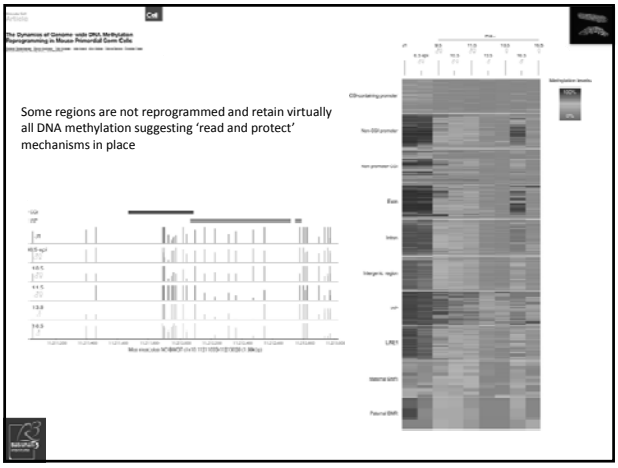


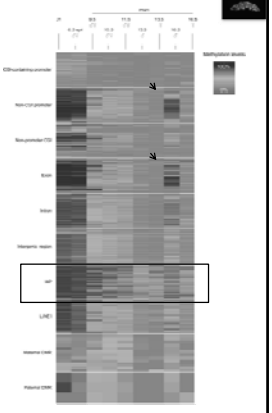


Characterisation of DNA methylation in germline reprogramming using next generation sequencing


E6.5
E10.5
E11.5
E13.5
E16.5
female
male
Primordial Germ Cells in Mice


Cell
The Dynamics of Genome-wide DNA Methylation Reprogramming in Mouse Primordial Germ Cells
Profound DNA methylation losses leave male and female gametes hypomethylated at mitotic and meiotic arrest

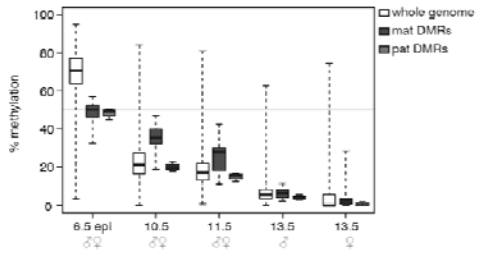

Cell
The Dynamics of Genome-wide DNA Methylation Reprogramming in Mouse Primordial Germ Cells
Some regions are not reprogrammed and retain virtually all DNA methylation suggesting 'read and protect' mechanisms in place



IAPs are a family of active retrotransposons

Transgenerational epigenetic inheritance

Genomic targets ordinarily subject to germline erasure which may elude this erasure event and consequently transmit epigenetic information into the next generation that may result in a heritable phenotype

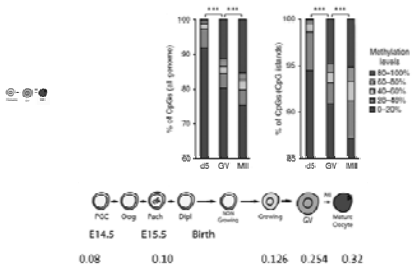


Programming of DNA methylation during Oocyte growth and maturation

High resolution reduced bisulphite sequencing (RRBS)

Establishment of the oocyte methylome …more than just imprinted genes

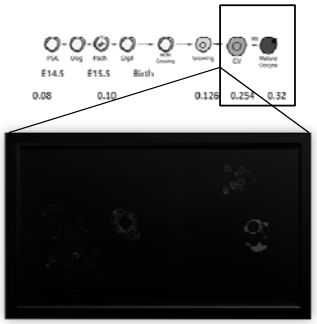
Programming of DNA methylation during Oocyte growth and maturation



Establishment of the oocyte methylome …more than just imprinted genes

Smallwood et al., Nature 2011

Programming of DNA methylation during Oocyte growth and maturation

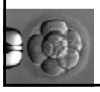


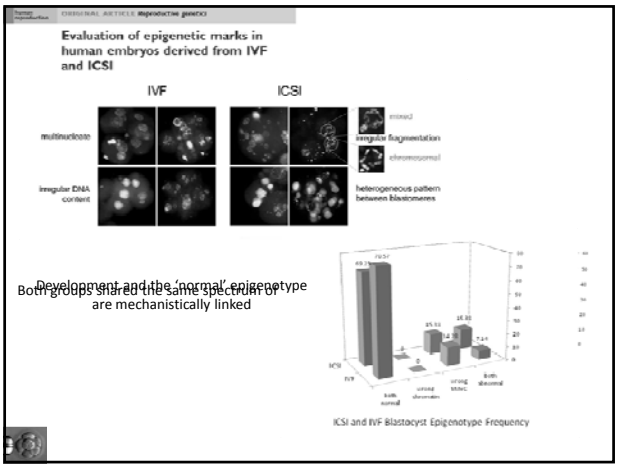
>1000 oocyte DMRs are included in the MII methylome with 10% added between GV and MII

ART is reported to have causal association with imprinted disease frequency

Over a decade ago a series of reports triggered concern that children born as a result of ART were found to have increased frequencies of a number of diseases known to have an epigenetic aetiology (DeBaun et al. 2003; Gicquel et al. 2003; Maher et al. 2003; Moll et al. 2003; Halliday et al. 2004).

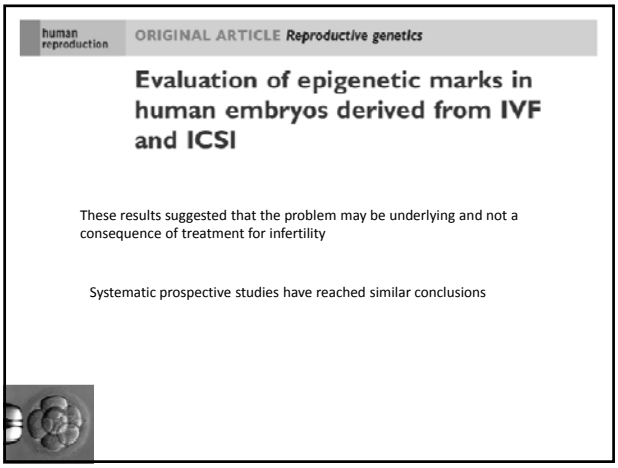
Moreover, some reports hinted that ICSI procedures were more detrimental than IVF





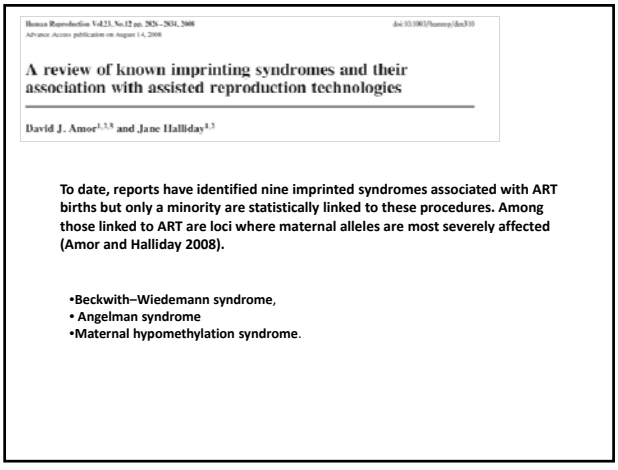
Development and the 'normal' epigenotype are mechanistically linked

Both groups shared the same spectrum of



These results suggested that the problem may be underlying and not a consequence of treatment for infertility

Systematic prospective studies have reached similar conclusions

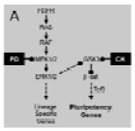


To date, reports have identified nine imprinted syndromes associated with ART births but only a minority are statistically linked to these procedures. Among those linked to ART are loci where maternal alleles are most severely affected (Amor and Halliday 2008).

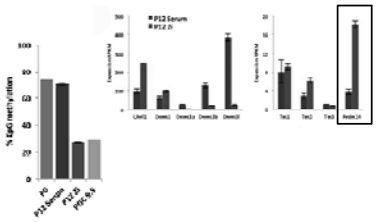
- **Beckwith–Wiedemann syndrome**,
- **Angelman syndrome**
- **Maternal hypomethylation syndrome**.

Lessons from embryonic stem cells

Recent results from pluripotent ES cells indicate that regulation of the DNA methylation machinery is remarkably sensitive to environmental signals

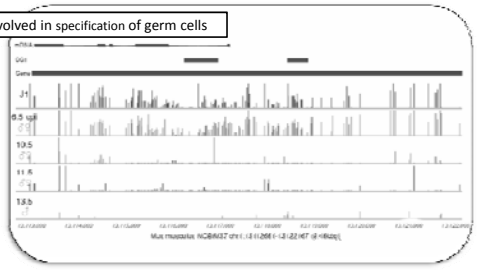


Culture conditions profoundly alter quantitative DNA methylation



Prdm14

Key factor involved in specification of germ cells



Negative regulator of Do novo methylases

Prmd14 connects germ cell development and DNA methylation erasure and may serve as biosenor to environmental change mediated by key signalling pathways

Conclusion and Outlook

Maternal reproductive health is a reflection of events over generations with multifactorial, environmentally sensitive, read out involving genes undergoing reprogramming during the critical period of gametogenesis

The fidelity of the epigenotype ensures the perpetuation of both beneficial and deleterious epimutations

Underlying infertility may well be established and neither caused nor enhanced by most ART procedures commonly in use in the treatment of infertility

Thank you

Babraham Institute &
University of Cambridge
Reik Lab

Steffi Seisenberger
Fatima Santos
Gabi Ficz
Tim Hore
Miguel Branco

Babraham Bioinformatics

Simon Andrews
Felix Krüger
Laura Biggins

Wellcome Trust Sanger Institute

Sophie Messager
David Jackson

Erasmus MC
Universitair Medisch Centrum Rotterdam

ESHRE 2013 London
Pre-Congress Course Genetics:
Genome scanning to identify genes in PCOS and Early Menopause

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Erasmus Medical Center, Rotterdam,
University Medical Center Utrecht, The Netherlands

Disclosure

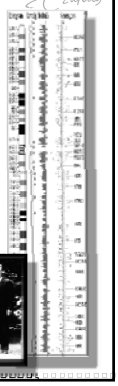
Erasmus MC

- Past President of the Dutch Society for Reproductive Medicine
- Past Chairman of the Task force Reproductive Endocrinology of the RDCOG
- Board member of Genovum, company for valorisation of genetic findings
- Received unrestricted research grants from Ferring®, Merck Serono®, MSD®, Organon®, Serono®
- Received grants from the Erasmus Trust Fund and the Netherlands Genomics Initiative

Genetic approaches in PCOS

Erasmus MC

- Chromosomal abnormalities: Structural or numerical
- Family studies: Linkage analysis in monogenic disorders (mode of inheritance AD, AR, X-linked)
- Affected Sib-pair or Affected relative-pair studies: Association analysis (mode of inheritance unknown, complex disorders)
- Positional or functional Candidate genes: Direct sequencing, SNPs, Micro-arrays.
- Animal & Human models: Knock outs and experiments of nature
- Complete genome searches: Microsatellite markers or SNP's
- Isolated populations: Linkage or association, Transmission Disequilibrium Test (TDT)



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ESHRE / ASRM endorsed, PCOS concensus meeting 2003, Rotterdam, The Netherlands

Erasmus MC

Oligo- or Amenorrhea (cycle length > 35 days)
Hyperandrogenism (hirsutism and / or FAI > 4.5)
PCO (> 12 follicles, 2 – 9 mm, in one or both ovaries and /or ovarian volume > 10 mL)

2 out of 3 !!!!
Oligo- or Amenorrhea
Hyperandrogenism (Clinical or Biochemical)
PCO

PCOS

Wild
Tan
Dunaif
Devoto
Straus

Rule out:
Hyperprolactimemia
NCAH
Cushing's syndrome
Androgen Secreting Neoplasm
Acromegaly

PCOS is Complex Genetic Trait

Table 1 Phenotype groups: NIH / Rotterdam / AES

	Features	Rotterdam	AES	NIH
A	PCO+ OD+ HA	X	X	X
B	OD+ HA	X	X	X
C	PCO+ OD	X		
D	PCO+ HA	X	X	

HA= Clinically or biochemically proven hyperandrogenemia, OD= Ovulatory dysfunction, PCO= Poly cystic ovaries * Phenotype groups according to Azziz, 2006

Fauser Tarlatzis

Genetic Basis of PCOS

(Legro et al. Proc N Acad Science, 1998; Vink et al., JCEM, 2006)

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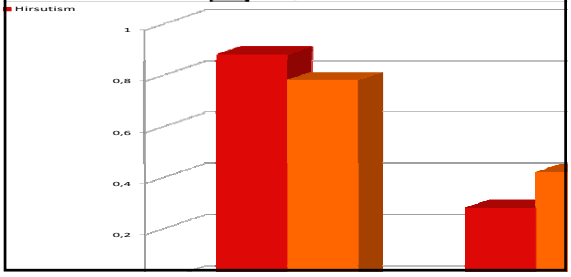
Table 1. Definition and prevalence of phenotypes in sisters

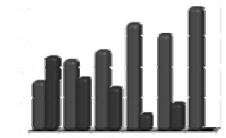
Phenotype of sister	No. affected (total, n = 115)	% affected	Summary
PCOS	17		Premenopausal, ≥6 menses per yr, and elevated T and/or aT levels
Hyperandrogenemia alone	19		Premenopausal, menses 27-35 d, elevated T and/or aT levels
Unaffected	43		Premenopausal, menses 27-35 d, normal T, aT, and DHEAS levels

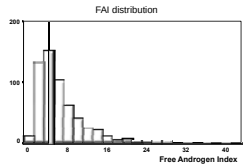


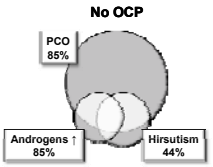
WHO II Anovulation & PCOS Phenotype

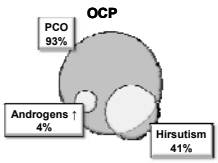
Erasmus MC Academy

(van Santbrink et al., Fertil Steril, 1997; Laven et al Gyn Obstet Survey, 2002)





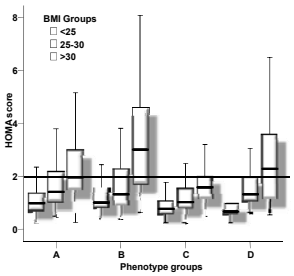


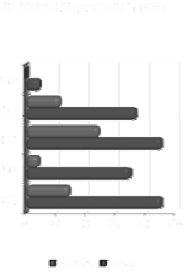


Insulin Resistance & Metabolic Syndrome in PCOS

Erasmus MC Academy

(Mulders et al., RBM online, 2004)

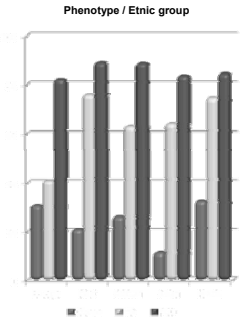


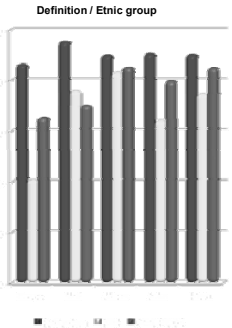


Ethnic Differences between PCOS Patients

Erasmus MC Academy

(Valkenburg et al., JCEM, 2011)





Problems with GWAS in PCOS

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- ***Populations stratification***
  - Founding
  - Ethnic differences
  - Population differences
  - Genetic drift
- ***Phenotype problems***
  - Definitions used
  - Treatment differences
  - Age related aspects
- ***Platform issues***
  - Illumina® vs. Affymetrix®
  - Hardware and software problems
  - Quality control
  - Significance levels
  - Imputation
  - TIME !!!!
- ***Control Groups***
  - Super controls
  - Normal controls
  - Population controls
- ***Reproducibility***
  - Hits are not constant
  - Number of hits is low
  - Ingenuity analyses are not very elusive
  - SNP's are markers for gens involved
  - Biological role
- ***Genetic issues***
  - Differences between patients
  - Environment (obesity, MBS)
  - Genetic predisposition (t2DM, CVD)

Genetics of Population Variation: SNP's

(Uitterlinden et al., Gene 2004)

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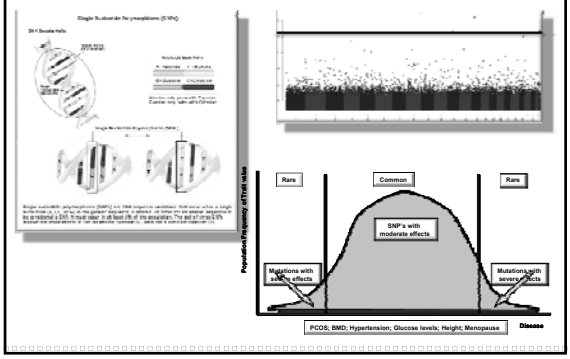
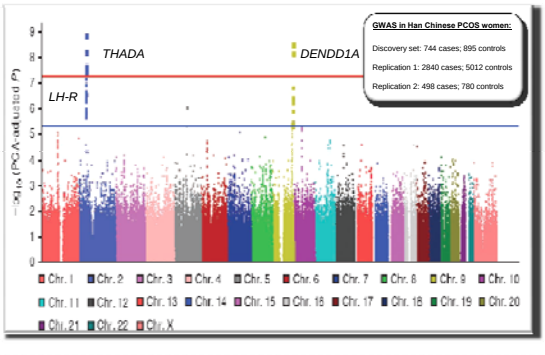


GWAS PCOS

(Chen et al., Nature genetics, 2011)

Erasmus MC



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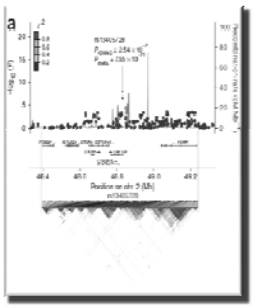

- *At the 2p 16.3 locus*
  - *GTF2A1L is germ cell line specific and involved in spermatogenesis*
  - *LHCGR is the LH and hCG receptor*
  - *(not replicated untill now)*
  - *FSHR located downstream nearby*
  - *(replicated in the second Chinese GWAS)*
- *At the 2p 21. locus*
  - *ZFP36L2 and LOC100129726 and*
  - *THADA is associated with thyroid adenomas*
  - *THADA recently identified in a GWAS for T2D*
  - *(Replicated in three different studies)*
- *At the locus 9q33.3*
  - *DENND1A encodes DENN which cab bind to endoplasmatic reticulum protein 1 (ERAP1)*
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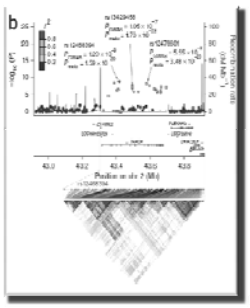

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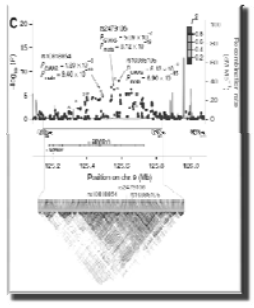

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Meta Analysis GWAS data in PCOS patients from European descent

Erasmus MC

(Louwers et al., unpublished data)

	NORTHERN EUROPEAN sample cases n=725 controls n=2184		Sample GOODARZI et al cases n=1474 controls n= 1802		Sample WELT et al cases n=1144 controls n=17,619		META ANALYSIS	
SNP *nearby gene*	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	**OR (95% CI)**	**P**
rs13405728 *LHCGR*	0.92 (0.70-1.22)	0.58	0.83 (0.67-1.01)	0.10	0.87 (0.66-1.14)	0.34	0.86 (0.75-0.99)	0.04
rs12468394 *THADA*	0.86 (0.76-0.97)	0.02	0.84 (0.76-0.93)	6.0×10^{-4}	0.91 (0.82-1.00)	0.077	**0.87 (0.82-0.93)**	**1.01×10^{-6}**
rs13429458 *THADA*	0.86 (0.70-1.05)	0.15	0.93 (0.80-1.09)	0.39	0.95 (0.79-1.15)	0.60	0.91 (0.83-1.02)	0.10
rs12478601 *THADA*	0.88 (0.78-0.99)	0.04	0.89 (0.80-0.98)	0.02	0.92 (0.82-1.04)	0.18	**0.88 (0.83-0.94)**	**1.77×10^{-4}**
rs10818854 *DENND1A*	1.15 (0.87-1.52)	0.32	1.87 (1.48-2.35)	9.8×10^{-8}	1.53 (1.17-2.00)	1.9×10^{-3}	**1.53 (1.32-1.78)**	**1.08×10^{-8}**
rs2479106 *DENND1A*	0.97 (0.85-1.11)	0.68	1.04 (0.93-1.16)	0.51	1.05 (0.93-1.18)	0.45	1.02 (0.96-1.10)	0.50
rs10986105 *DENND1A*	1.45 (1.08-1.94)	0.01	-	-	1.68 (1.27-2.23)	3.3×10^{-4}	**1.57 (1.28-1.92)**	**1.63×10^{-5}**

Second GWAS in Chinese PCOS PAtients

Erasmus MC

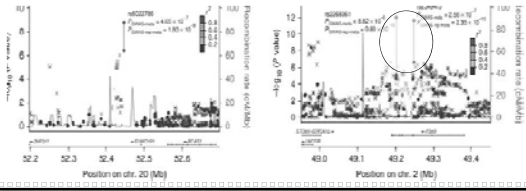
(Shi et al.Natutre Gentics 2012)

Second GWAS in Chinese PCOS patients:

total sample: 10,485 cases and 7,910 controls

PCOS according to the Rotterdam consensus

All sub-phenotypes (OD-HA, OD-PCOM and HA-PCOM along with OD-HA and PCOM were included



Replication second GWAS in PCOS patients from European descent

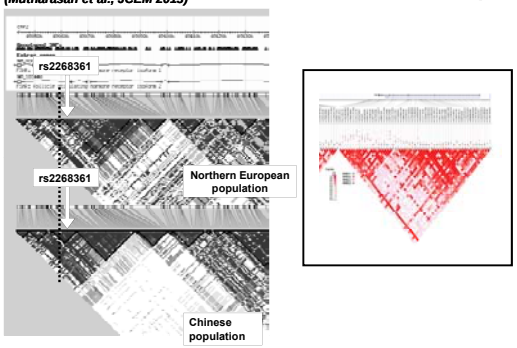
Erasmus MC

(Louwers et al., unpublished data)

SNP	nearby genes	Allele	CHINESE sample		NORTHERN EUROPEAN sample	
			OR	P-value	OR (95% CI)	P-value
rs13405728	*LHCGR*	G	0.71	7.55×10^{-21}	0.92 (0.70-1.22)	0.58
rs12468394	*THADA*	A	0.72	1.59×10^{-20}	**0.86 (0.76-0.97)**	**0.02**
rs13429458	*THADA*	C	0.67	1.73×10^{-23}	0.86 (0.70-1.05)	0.15
rs12478601	*THADA*	T	0.72	3.48×10^{-23}	**0.88 (0.78-0.99)**	**0.04**
rs10818854	*DENND1A*	A	1.51	9.40×10^{-18}	1.15 (0.87-1.52)	0.32
rs2479106	*DENND1A*	G	1.34	8.12×10^{-19}	0.97 (0.85-1.11)	0.68
rs10986105	*DENND1A*	C	1.47	6.90×10^{-15}	**1.45 (1.08-1.94)**	**0.01**
rs2268362	*FSHR*	T	0.87	9.89×10^{-13}	0.94 (0.77-1.15)	0.83
rs2349415	*FSHR*	T	1.19	2.35×10^{-12}	**1.15 (1.00-1.32)**	**0.05**
rs4385527	*c9orf3*	A	0.84	5.87×10^{-24}	**0.87 (0.77-0.99)**	**0.04**
rs3802457	*c9orf3*	A	0.77	5.28×10^{-14}	0.90 (0.45-1.81)	0.77
rs1894118	*YAP1*	G	1.27	1.08×10^{-11}	**1.37 (1.13-1.67)**	**1.89×10^{-3}**
rs705702	*RAB5B,SUOX*	G	1.27	8.64×10^{-25}	**1.21 (1.06-1.38)**	**4.31×10^{-3}**
rs2272046	*HMGA2*	C	0.70	1.95×10^{-21}	1.19 (0.83-1.71)	0.36
rs4784165	*TOX3*	G	1.15	3.64×10^{-11}	1.09 (0.95-1.25)	0.27
rs2059807	*INSR*	G	1.14	1.09×10^{-08}	0.93 (0.82-1.05)	0.27
rs6022786	*SUMO1P1*	A	1.13	1.83×10^{-09}	1.06 (0.92-1.21)	0.38

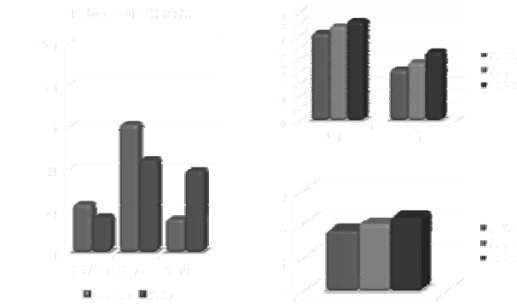
FSH-R Polymorphisms and of PCOS

(Mutharasan et al., JCEM 2013)



FSHR Polymorphisms and FSH and LH levels

(Laven et al.,Fertil Steril 2003; Valkenburg et al., Human Reprod, 2009)



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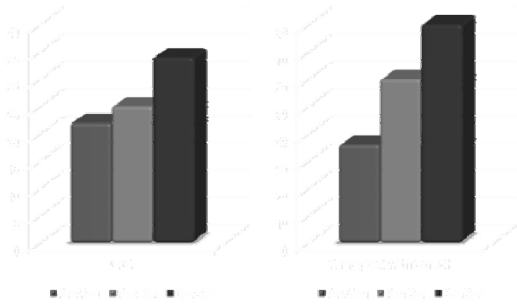

FSHR Polymorphisms and OI Outcome

(Valkenburg et al., To be Published soon, 2012)

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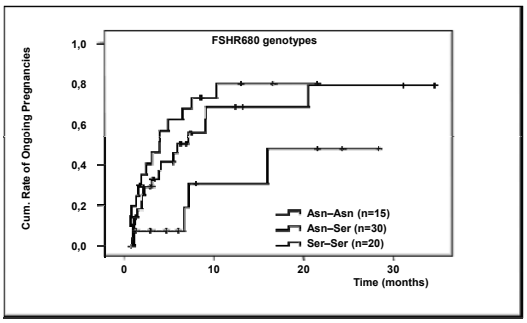

Conclusions

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- Arrays nowadays do identify the more common genetic variants that play a role in normal complex traits or diseases (The Low Hanging Fruit)
- Power and numbers do improve sensitivity of these techniques, therefore consortia are important to collaborate in (The Higher Hanging Fruit)
- Menopause have a high degree of heritability and genetic variants may explain variation to a certain extent however, some more rare variants might also play a role
- Only a very limited number of genetic variants can be associated with known processes that are important during folliculogenesis and ovulation as well as for ovarian (dys)function. However, most SNP's are referring to genes involved in ageing, DNA repair, DNA replication, Telomere length control etc.
- Menopause is related to reproductive success which in turn is associated with longevity
- Ageing of the soma might be the predominant driver for loss of ovarian function
- In case the soma becomes too old it is of no use to invest in the germ cell line and therefore you are not allowed to reproduce anymore !!!! Hence you switch your ovary off.

Genetic Variation and Age of Menopause

(Stolk et al., Nature Genetics, 2012)

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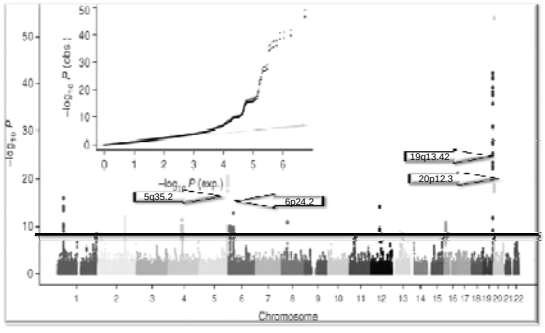

Genetic Variation and Age of Menopause

(He et al., Nature Genetics, 2011)

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SNP	Total N	Major, Minor Alleles	Common Homozygote N	Common Homozygote Mean	Heterozygote N	Heterozygote Mean	Variant Homozygote N	Variant Homozygote Mean
Age at Natural Menopause								
rs16891832	9,811	G, A	5,850	50.5	3850	51.7	55	
rs1172822	9,096	C, T	3,715	51.0	4,115	50.6	1,266	
rs2304287	9,087	T, C	3,422	51.0	4,333	50.6	1,433	
rs897798	9,084	A, G	2,926	51.0	5,132	50.7	2,026	
rs365132	8,007	C, T	2,366	50.3	4,210	50.7	2,241	
rs7788674	9,811	A, G	2,954	50.3	4,509	50.7	2,348	
rs4909611	9,054	C, T	2,354	50.3	4,490	50.7	2,340	
rs7246479	9,102	G, T	2,476	50.2	4,442	50.8	2,186	
rs1555152	9,011	A, C	5,360	50.0	3,117	50.5	526	
rs691141	9,088	C, T	2,742	50.4	4,443	50.7	1,903	
rs12611021	8,882	T, C	2,826	50.3	4,418	50.7	2,088	
rs7151157	9,046	C, T	7,575	50.6	4,448	50.7	1,025	
rs822/0193	9,100	G, A	1,023	50.0	4,070	50.6	1,007	

- 13 SNP's genome wide significant for Age at Menopause all located in or nearby known genes
- 4 different regions on chromosomes 5q32.2, 6p24.2, 19q13.42 and 20p12.3
- After adjustment for the most significant SNP in each region none of the others was still significant
- Together the four significant SNP's explained 2.69% of the age of Menopause

Genetic Variation and Age of Menopause

(He et al., Nature Genetics, 2011)

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- Genes identified are either involved in DNA repair, or immune function and very few are affecting the neuro-endocrine pathways and ovarian function indicating the process of ageing as a shared player in both somatic and germ line ageing.
- Only SYCP2L is required for protein synthesis in the synaptonemal complex which zips together homologue chomosomes during the first meiotic division !!!!
- All the other SNP's are referring to genes involved in ageing, DNA repair, DNA maintenance and replication, Telomere length control etc.
- Hence, only ONE gene might be involved in folliculogenesis
- Could it be that ageing of the soma is the primary driver for the loss of ovarian function in women instead of the old dogma which implies that loss of ovarian function initiates ageing of the soma?
- Time for a Paradigm Shift?

DNA Damage, Ageing and Cancer

(Hoeijmakers , N.E.J, Nat . Med 2009)

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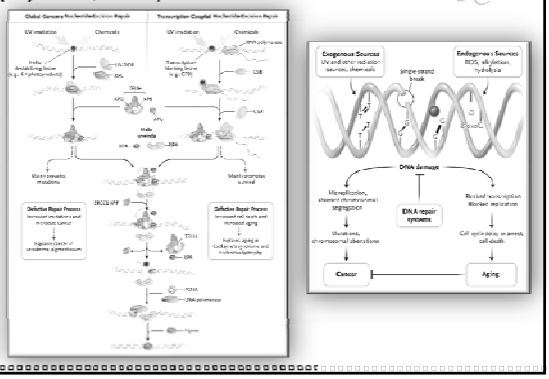

DNA Damage, Ageing and Cancer

(Hoeijmakers JHJ, NEJM 2009)

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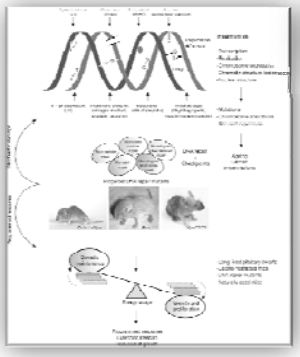

The trichothiodystrophia (TTD) Phenotype

- premature aging
- osteoporosis and kyphosis
- osteosclerosis
- early greying
- cachexia
- infertility
- reduced life-span

TTD mice carrying an additional mutation in XPA, which enhances the DNA repair defect, showed a greatly accelerated aging phenotype, which correlated with an increased cellular sensitivity to oxidative DNA damage.

DNA Damage, Ageing and Cancer

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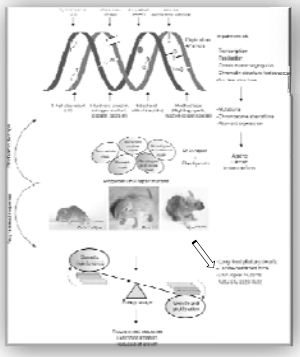

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CVD Risk profile and Age at Menopause

(Gast et al., Maturitas 2010, Menopause 2011)

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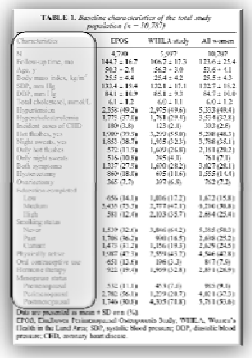

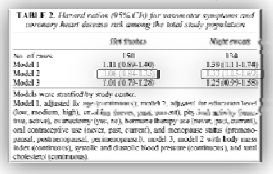

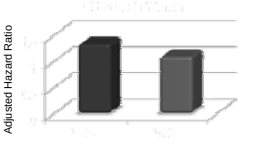

HRT and CVD risk in Postmenpausal women

(Sanchez et al. Cochrane Reviews, 2005)

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- No protective effect of HRT was seen for any of the cardiovascular outcomes assessed: all cause mortality, cardiovascular death, non-fatal MI, venous thromboemboli or stroke.
- Higher risks of venous thromboembolic events (Relative risk (RR) 2.15, 95% CI 1.61 to 2.86), pulmonary embolus (RR 2.15, 95% CI 1.41 to 3.28), and stroke (RR 1.44, 95% CI 1.10 to 1.89) was found in those randomised to HRT compared with placebo.
- No substantial heterogeneity (p <0.1) was detected in any of the outcomes studied.
- At present, a recommendation for initiating HRT for the reason of preventing cardiovascular events in post-menopausal women (with or without cardiovascular disease) should not be made.
- Women with other risk factors for venous thromboembolic events should be discouraged from using HRT if the sole goal is to prevent cardiovascular events.

Pre-eclampsia & Ovarian Function

(Woldringh et al, Human Reprod, 2006)

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Table I. Demographic characteristics of the study groups

	Pre eclampsia ($n = 41$)	Controls ($n = 82$)	*P* value
Maternal age at delivery (years)	33.6 [26.3–40.9]	33.6 [26.6–39.8]	0.760
Pre-pregnant BMI (kg/m²)	22.9 [18.8–31.6]	22.6 [17.8–32.8]	0.408
Maternal smoking			
No smoking (%)	75.6	81.7	0.479
Smoking, but not during pregnancy (%)	9.8	7.3	0.730
Smoking, also during pregnancy (%)	14.6	11.0	0.569
Nulliparous (%)	87.8	87.8	1.000
Singleton pregnancies (%)	53.7	53.7	1.000
Assisted reproductive technologies			
IVF (%)	41.5	41.5	1.000
ICSI (%)	58.5	58.5	1.000
Duration of subfertility (years)	4.2 [1.2–11.7]	3.9 [0.7–14.3]	0.953
Indication for assisted reproductive technologies			
Tubal factor (%)	7.3	9.8	0.750
Endometriosis (%)	9.8	9.8	1.000
Male subfertility (%)	58.5	61.0	0.846
Unexplained subfertility (%)	19.5	17.1	0.805
Cervical hostility (%)	4.9	2.4	0.600

Data are expressed as median [range] or percentage.
P value is calculated non-parametrically (Mann–Whitney *U*-test or Fisher's Exact test).

Pre-eclampsia & Ovarian Function

(Woldringh et al, Human Reprod, 2006)

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Table II. Baseline, stimulation and response variables of the study groups

	Pre-eclampsia ($n = 41$)	Controls ($n = 82$)	*P* value
Baseline			
FSH (IU/l)	5.7 [2.9–11.7]	6.3 [2.8–13.0]	0.304
Estradiol (E_2) (pmol/l)	110 [66–250]	135 [38–340]	0.462
Stimulation			
Administered FSH in dose/day (IU/d)			
Days of administration			
Total administered FSH (U)			
Response			
Maximal E_2 (pmol/l)	7700 [440–15 000]	7000 [1100–14 000]	0.931
Maximal endometrial thickness (mm)	11.5 [5.0–18.2]	12.0 [7.3–18.5]	0.329
Number of follicles (5–9 mm)			
Number of obtained oocytes			
Number of usable embryos	5 [2–17]	6 [1–18]	0.101
Indexes			
Administered FSH/follicle (U)			
Administered FSH/obtained oocyte (U)			

Data are expressed as median [range].
P value is calculated non-parametrically (Mann–Whitney *U*-test).

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Confounders of Age at Menopause
(Kaszmarek et al, Maturitas, 2007)

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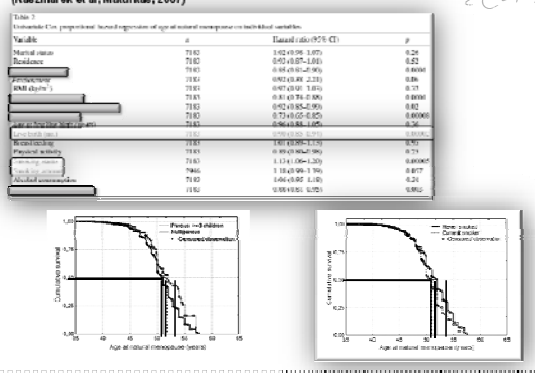

Female Fertility and Fitness after Menopause
(Lahdenpera et al., Nature 2011)

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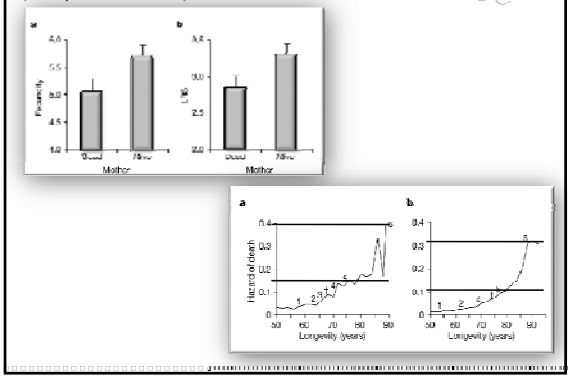

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Female Fertility and Longevity
(Larke and Crews, J Physiol Anthropol 2006)

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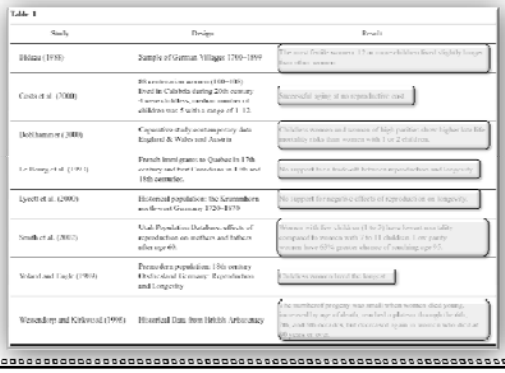

Female Fertility and Longevity
(Larke and Crews, J Physiol Anthropol 2006; Helle et. al., Proc Biol Science 2004)

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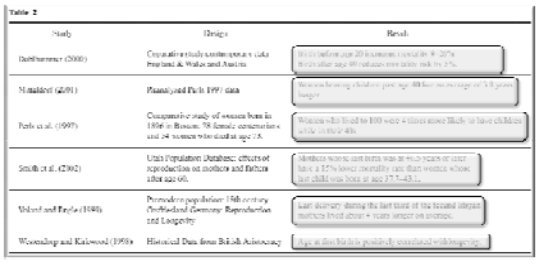

Best Predictor for Longevity is Age at last Reproductive Event !!!!!

Fecundity, Age at Menopause and Longevity

(Helle et. al., Proc Biol Science 2004)

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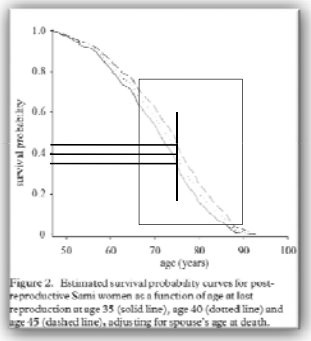

Figure 2. Estimated survival probability curves for post-reproductive Sami women as a function of age at last reproduction at age 35 (solid line), age 40 (domed line) and age 45 (dashed line), adjusting for spouse's age at death.

Possible Confounders:

- Lactation
- Different Life style, less sedentary, obesity
- Contraception
- Weight gain or loss during pregnancy and lactation
- # of Children and sex of children
- Longer E_2 exposure
- More Breast, Endometrial and Ovarian Cancer
- More Colon Cancer
- Women seem to be different from mice and other domestic animals

Fecundity, Age at Menopause and Longevity

(Faddy, Mol Cell Endocrinol, 2000)

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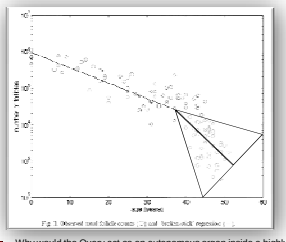

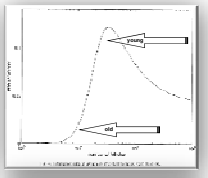

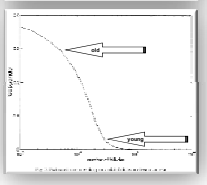

- Why would the Ovary act as an autonomous organ inside a highly well organised and centrally regulated organism?
- The fact that menopause as well as the fact that the rate at which follicles are lost is different between women suggest an extra-ovarian regulatory mechanism
- The aging soma alone or in conjunction with the CNS might constitute such a mechanism

Conclusions

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- PCOS is a heterogeneous phenotype indicating a complex genetic background which in turn might be altered by the environment
- The Phenotype of PCOS is not constant neither within individuals nor in time
- Phenotyping includes not only whether women are oligo- or amenorrheic, suffer from hyperandrogenaemia or hirsutism, have PCOM but should also include treatment response, short- and long-term health risks
- Conventional genetic tools are less effective in deciphering the genetic background
- Complex diseases need a more sophisticated approach using GWAS, expression arrays, metabolomics and proteomics
- Some 3 – 5 common SNP's have been identified yet and to a certain extent they have been replicated
- Some SNP's do also correlate with phenotypic features of PCOS such as Hyper-androgenism
- GWAS resolution seems to be hampered by numbers, power, population stratification, ethnic differences and environmental factors
- Future research should be aiming at consortia and meta analysis as well as on models assessing the role of identified genes in PCOS

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- Nils Lambalk

International

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- DeCode
- Dutch PCOS Consortium
- Stephen Franks
- Mark McCarthy
- Adam Balen
- Ricardo Azziz
- Mark Goodarzi
- Unnur Styrkadottir
- Corinne Welt
- Bil Crowley

ESHRE 2013, London – Precongress course 8

Genetic and epigenetic causes of infertility – can we minimize the risks

Epigenetics in the oocyte

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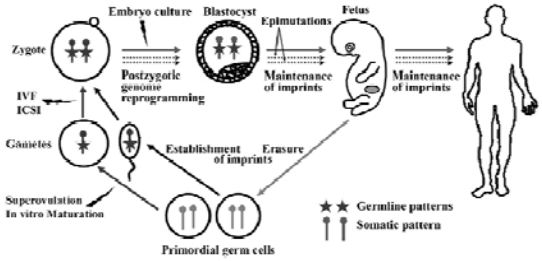
The author has declared that no competing interests exist.

Learning Objectives

- **Epigenetic genome reprogramming in the female germ line.**
- **Imprinted genes as a model to study epigenetic effects of different ARTs.**
- **Sensitivity of oocyte and embryo epigenome to environmental cues.**
- **Epigenetic risks associated with ovarian stimulation.**
- **Epigenetic risks associated with in vitro culture and maturation of oocytes.**
- **Limitations of mouse oocyte and embryo assays for assessing the safety of human ART.**
- **Little is known about the long-term epigenetic and phenotypic consequences of human ART.**

Imprinted genes are a convenient model to study the epigenetic effects of different ART.

Since they escape postzygotic reprogramming, aberrant oocyte imprints cannot be corrected after fertilization and, thus, may directly interfere with development.



Haaf, Adv. Mol. Biol. Med. Vol.1, 601-628, 2012

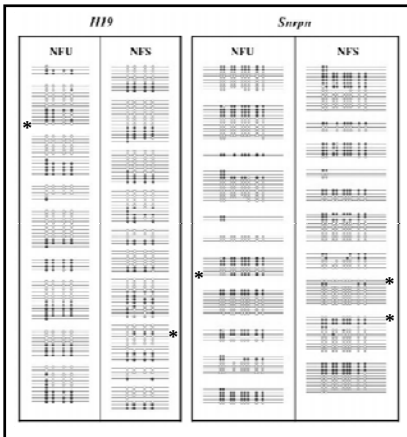
Epigenetic reprogramming in the female germline

- **All parental methylation patterns (at imprinted and non-imprinted loci) are erased in primordial germ cells, by the time they have migrated to the genital ridge.**
  Guilbert et al., Genome Res. 22, 633-641, 2012
- **There are very low methylation levels prior to oocyte growth.**
  The major phase of de novo methylation occurs after birth during oocyte growth.
  Smallwood et al., Nat. Genet. 43, 811-814, 2011
- **Adverse environmental factors during late stage of oocyte development, when the oocyte epigenome is still very plastic, may interfere with the establishment and/or maintenance of oocyte methylation patterns.**
  El Hajj and Haaf, Fertil. Steril. 99, 632-641, 2013

State of the ART: IVF/ICSI of <u>in vivo</u> matured oocytes following ovarian stimulation.

- **Numerous studies in various animal models and limited evidence in humans suggest that superovulation can affect the epigenome of the oocyte as well as the resulting embryo, fetus and placenta.**
- **Most epimutations may occur after fertilization due to impaired maintenance of maternal imprints.**

Fortier et al., Hum. Mol. Genet. 17, 1653-1665, 2008
Denomme and Mann, Reproduction 144, 393-409, 2012
El Hajj et al., Epigenetics 6, 1176-1188, 2011
Fauque, Fertil. Steril. 99, 616-623, 2013



Methylation patterns of *H19* and *Snrpn* in in vivo produced mouse (*M. musculus* x *M. castaneus*) 16-cell embryos from unstimulated (NFU group) versus superovulated matings (NFS group). Each line indicates an individual allele. Maternal alleles (highlighted in red) and paternal alleles (blue) from the same embryo are grouped together. Open circles represent unmethyl-ated CpGs and filled circles methylated CpGs. Abnormally methylated alleles are indicated by an *.

Cytogenetic and Genome Research

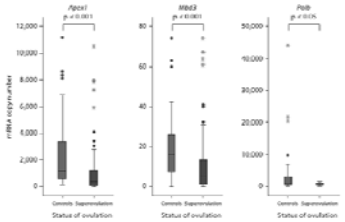
Cytogenet Genome Res 2013;139:71–79
DOI: 10.1159/000343755

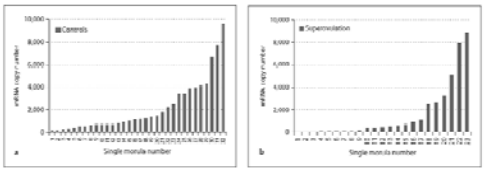
The Impact of Ovarian Stimulation on the Expression of Candidate Reprogramming Genes in Mouse Preimplantation Embryos

M. Linke[a] A. May[a] K. Reifenberg[b] T. Haaf[c] U. Zechner[a]

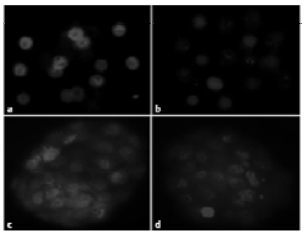
[a]Institute of Human Genetics and [b]Central Laboratory Animal Facility, Johannes Gutenberg University, Mainz, and [c]Institute of Human Genetics, Julius Maximilian University, Wurzburg, Germany

Using absolute quantification of mRNA by quantitative real-time PCR, we observed an association of ovarian stimulation with a downregulation of mRNAs encoding the base excision repair proteins APEX1 and POLB as well as the 5-methyl-CpG-binding domain protein MBD3 in individual morula embryos.





APEX immunofluorescence staining of early 16-cell (a,b) and late 32-cell (c,d) morula stage blastomeres from spontaneously ovulated control females (a,c) and superovulated females (b,d).

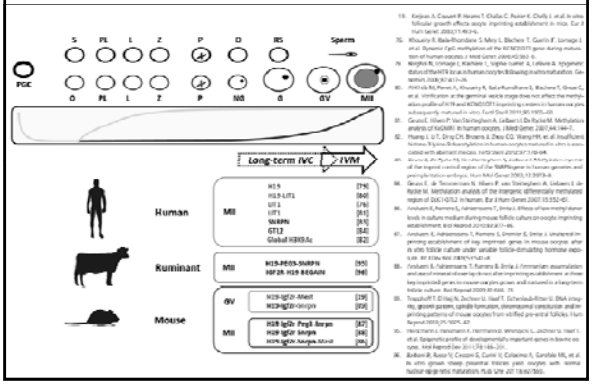


In vitro growth and maturation of oocytes

- **In vitro growth (IVC) and maturation (IVM) of oocytes from primordial or early preantral follicles and subsequent fertilization and normal embryo development was only achieved in the mouse (long-term IVM).**
- **For the in vitro production of cattle and sheep, oocytes are usually retrieved in the germinal vesicle stage and then cultured to complete the final steps of maturation to obtain fertilizable metaphase II oocytes (short-term IVM).**
- **So far short-term IVM has limited clinical utility in humans.**

Anckaert et al., Hum. Reprod. Update 19, 52-66, 2013
El Hajj and Haaf, Fertil. Steril. 99, 632-641, 2013

Methylation analyses of IVC/IVM oocytes



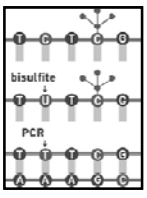
Effects of vitrification and preantral follicle culture on methylation imprints in mouse oocytes

LD bisulphite pyrosequencing of cis-regulatory regions of two maternally imprinted (*Igf2r* and *Snrpn*) and one paternally imprinted (*H19*) gene(s) in

- **in vivo grown GV-stage oocytes isolated from from large antral follicles.**
- **in vitro grown (for 10-12 days) GV oocytes isolated from <u>fresh</u> preantral follicles.**
- **in vitro grown (for 10-12 days) GV oocytes isolated from <u>vitrified</u> preantral follicles.**

Methylation analysis of a few cells is a challenging problem.

Bisulfite conversion (to distinguish between methylated and unmethylated cytosines) heavily degrades DNA.



Amplification bias

1:1

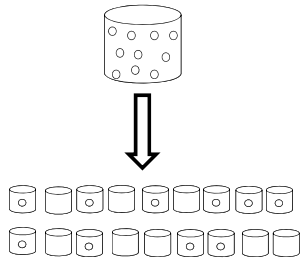
C **mC**

5:1

DNA contamination

Methylation analysis of single DNA molecules using „Limiting Dilution (LD)“

Limiting Dilution

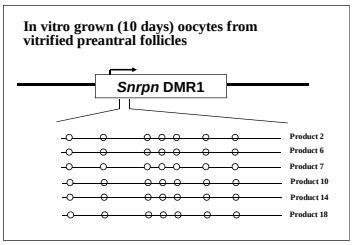


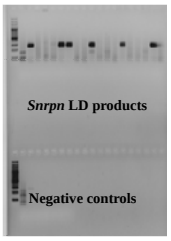
Can detect rare events

Needle in a haystack!

Bisulfite pyrosequencing of LD products from 10 oocytes (multiplex with *H19*, *Igf2r* and *Snrpn*):

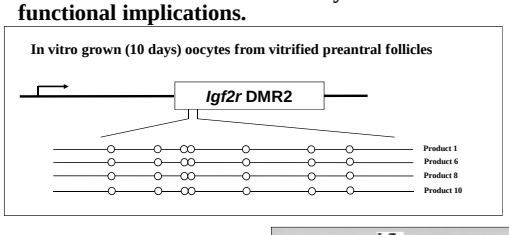
Abnormal methylation of all CpGs in a given allele indicates an imprinting mutation (epimutation).

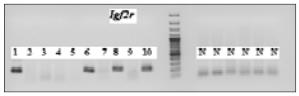




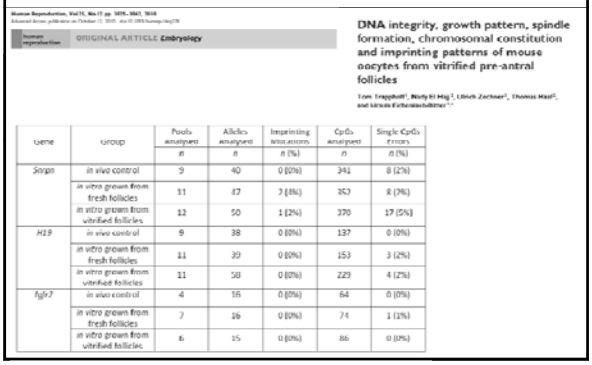
Bisulfite pyrosequencing of LD products from 10 oocytes (multiplex with *H19*, *Igf2r* and *Snrpn*):

Abnormal methylation of individual CpGs in a given allele indicates a stochastic methylation error without functional implications.





The rate of imprinting mutations and stochastic methylation errors is not dramatically increased by in vitro culture of mouse oocytes from fresh or vitrified preantral follicles.



ORIGINAL ARTICLE Embryology

DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles

Gene	Group	Pools analysed	Alleles analysed	Imprinting mutations	CpGs analysed	Single CpG errors
		n	*n*	*n* (%)	*n*	*n* (%)
Snrpn	*in vivo* control	9	40	0 (0%)	341	8 (2%)
	in vitro grown from fresh follicles	11	47	2 (4%)	353	8 (2%)
	in vitro grown from vitrified follicles	12	50	1 (2%)	370	17 (5%)
H19	*in vivo* control	9	38	0 (0%)	137	0 (0%)
	in vitro grown from fresh follicles	11	39	0 (0%)	153	3 (2%)
	in vitro grown from vitrified follicles	11	58	0 (0%)	229	4 (2%)
Igf2r	*in vivo* control	4	16	0 (0%)	64	0 (0%)
	in vitro grown from fresh follicles	7	26	0 (0%)	74	1 (1%)
	in vitro grown from vitrified follicles	6	25	0 (0%)	86	0 (0%)

RESEARCH ARTICLE Epigenetic Profile of Developmentally Important Genes in Bovine Oocytes

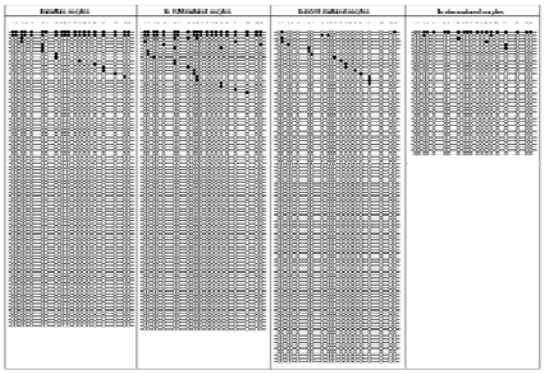
Molecular Reproduction & Development 78:888–901 (2011)

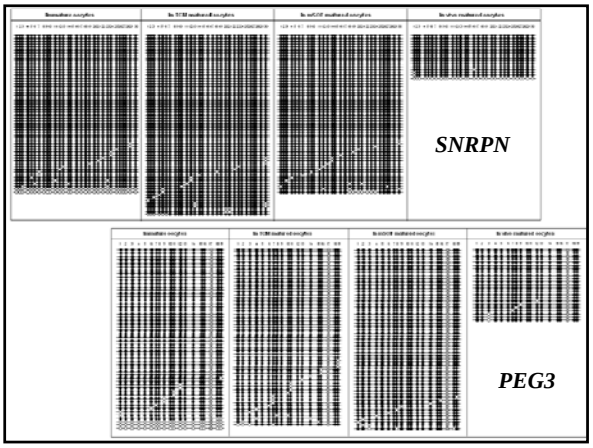
Similarly, short-term IVM appears to have only marginal effects on bovine oocytes.

Bisulfite sequencing of cis-regulatory regions of two maternally imprinted (*PEG3* and *Snrpn*) and one paternally imprinted (*H19*) genes in

- **immature oocytes**
- **IVM oocytes (grown in tissue culture medium TCM199)**
- **IVM oocytes (grown in modified synthetic oviduct fluid mSOF)**
- **in vivo matured oocytes**

H19 methylation patterns in bovine oocytes

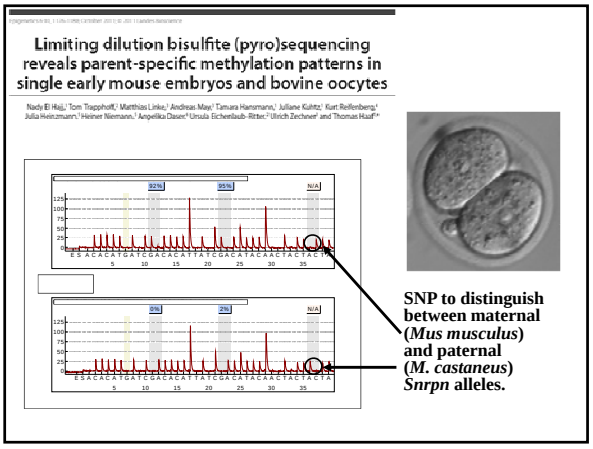




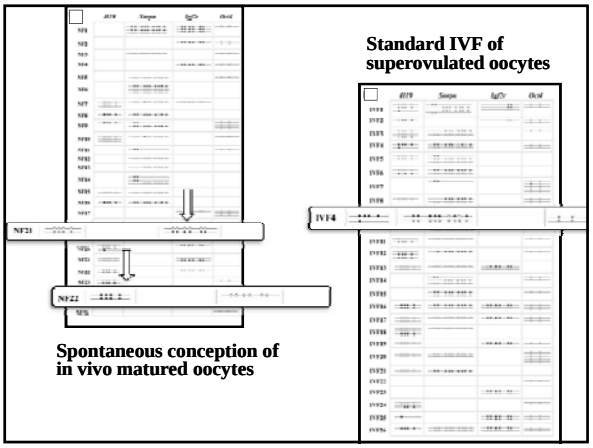
Effects of in vitro maturation and standard IVF on methylation imprints in early mouse (two-cell) embryos

Bisulfite sequencing of cis-regulatory regions of three representative imprinted genes (*H19*, *Igf2r* and *Snrpn*) and one pluripotency gene (*Oct4*) in

- **naturally fertilized in vivo produced embryos from in vivo matured oocytes in unstimulated cycles (NF group).**
- **in vitro fertilized embryos derived from in vivo matured superovulated oocytes (IVF group).**
- **in vitro fertilized embryos derived from preantral oocytes that were grown and matured in vitro during culture over 13 days (IVC group).**



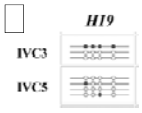
SNP to distinguish between maternal (*Mus musculus*) and paternal (*M. castaneus*) *Snrpn* alleles.



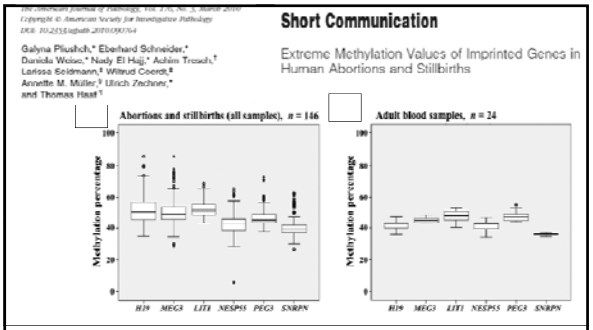
Embryo groups		*H19*	*Snrpn*	*IgDr*	*Oct4*
NFU	Number of embryos analyzed	26	26	26	26
	Number of recovered maternal alleles per embryo	0.6	0.4	0.4	0.4
	paternal alleles per embryo	0.1	0.4	0.1	0.4
	Number (percentage) of abnormal maternal alleles	2/16 (13%)	0/10 (0%)	1/10 (10%)	0/10.5 (0%)
	abnormal paternal alleles	0/0 (0%)	0/11 (0%)	0/3 (0%)	0/10.5 (0%)
	Number (percentage) of maternal single CpG errors[b]	0/56 (0%)	2/64 (3%)	0/54 (0%)	0/21 (0%)
	paternal single CpG errors[b]	0/29 (0%)	2/95 (2%)	0/18 (0%)	0/21 (0%)
IVF	Number of embryos analyzed	26	26	26	26
	Number of recovered maternal alleles per embryo	0.9	0.7	0.5	0.8
	paternal alleles per embryo	0.7	0.8	0.1	0.8
	Number (percentage) of abnormal maternal alleles	0/24 (0%)	0/18 (0%)	0/12 (0%)	0/19.5 (0%)
	abnormal paternal alleles	0/17 (0%)	0/20 (0%)	0/8 (0%)	0/19.5 (0%)
	Number (percentage) of maternal single CpG errors[b]	0/93 (0%)	6/117 (5%)	1/60 (2%)	1/39 (3%)
	paternal single CpG errors[b]	3/58 (5%)	0/173 (0%)	0/40 (0%)	1/39 (3%)
IVC	Number of embryos analyzed	18	18	18	18
	Number of recovered maternal alleles per embryo	1.2	0.8	0.4	0.4
	paternal alleles per embryo	0.3	0.1	-	0.4
	Number (percentage) of abnormal maternal alleles	2/22 (9%)	0/11 (0%)	1/8 (12.5%)	0/6.5 (0%)
	abnormal paternal alleles	0/5 (0%)	0/1 (0%)	-	0/6.5 (0%)
	Number (percentage) of maternal single CpG errors[b]	6/77 (8%)	0/57 (0%)	0/38 (0%)	0.5/13 (4%)
	paternal single CpG errors[b]	1/20 (5%)	0/9 (0%)	-	0.5/26 (1%)

Standard IVF of superovulated oocytes and the use of IVM oocytes were not associated with significantly increased rates of single CpG methylation errors and epimutations (allele methylation errors), when compared with the in vivo produced controls.

- **In the mouse and in the bovine model, standard IVF of superovulated oocytes and even the use of IVM oocytes were not associated with significantly increased rates of stochastic single CpG methylation errors and imprinting mutations, when compared with the in vivo produced controls.**
- **The observed epigenetic effects of ART in other studies may be mainly due to embryo culture conditions.**
  In most ART programs embryos are transferred at the blastocyst stage.
- **Most imprinting mutations may arise postzygotically and are observed in a mosaic state state in early embryos.**



- **Imprinting mutations are more frequent in early embryos (approximately 3% of the analyzed alleles) than later in life, suggesting a natural selection during embryogenesis and/or further pregnancy.**



Short Communication

Extreme Methylation Values of Imprinted Genes in Human Abortions and Stillbirths

Galyna Plushch,* Eberhard Schneider,* Daniela Weise,* Nady El Hajj,* Achim Trosch,† Larissa Eckmann,‡ Wilfried Coerdt,‡ Annette M. Müller,§ Ulrich Zechner,* and Thomas Haaf*

Abortions and stillbirths (all samples), $n = 146$

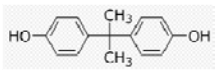
Adult blood samples, $n = 24$

Methylation percentage

H19 MEG3 L3T1 NESP55 PEG3 SNRPN

- If extreme methylation values in imprinted and/or other developmentally important genes exceed a critical threshold, spontaneous pregnancy loss may occur.
- Similar to other multifactorial diseases, additional genetic and environmental factors might also play a role.

Adverse effects of prenatal exposure to bisphenol A (BPA)

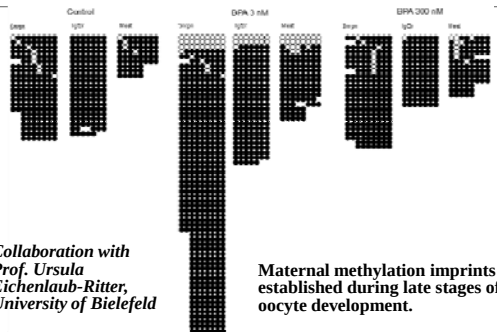


- Endocrine disruptors are synthetic chemicals that resemble natural hormones.
- Exposure of Agouti (A^{vy}/a) mouse mothers to BPA induces epigenetic changes in the offspring.
- Offspring of BPA-exposed mothers show increased rates for diabetes, obesity, cancer, neurological problems, infertility, …

Dolinoy, et. al., PNAS 104, 13056-13061, 2007

Low doses of BPA in mouse follicle culture

interfere with establishment and/or maintenance of maternal methylation imprints.



Collaboration with Prof. Ursula Eichenlaub-Ritter, University of Bielefeld

Maternal methylation imprints are established during late stages of oocyte development.

Summary

- **Superovulation of oocytes with gonadotropins, IVF/ICSI and embryo culture are widely used for human infertility treatment.**
  In vitro culture and maturation of oocytes are integral components of the in vitro production of cattle/sheep, but so far have only limited clinical utility in humans.
- **Imprint establishment in late oocyte stages and maintenance after fertilization are vulnerable to environmental cues.**
- **Despite accumulating evidence in animal models that superovulation as well as in vitro culture/maturation of oocytes can interfere with epigenetic genome reprogramming, there does not appear to be a dramatic increase of epimutations in the resultant offspring.**
- **Most embryos/fetuses with stochastic or ART-induced epimutations may not develop until birth.**

Caveats

- **Because gametogenesis and embryonic development differ considerably in rodents and humans, mouse oocyte and embryo assays do not necessarily allow one to extrapolate to the human situation.**
- **Due to the striking similarities with human development, bovine oocytes and embryos are increasingly used as models for human ART.**
- **For legal and ethical reasons, it is not possible to use large numbers of human oocytes and embryos to systematically study the epigenetic and phenotypic effects of different oocyte manipulations.**
- **Because it is problematic to assess the epigenetic safety of human ART using animal models, manipulation of oocyte and embryo should be restricted to a minimum or to the advantage of a specific technique and must outweigh possible negative epigenetic effects.**

Developmental origins of adult disease

- **It is now widely accepted that an adverse periconceptional and intrauterine environment is associated with epigenetic malprogramming of the fetal metabolism and predisposition to chronic, in particular metabolic disorders later in life (“Barker hypothesis”).**
- **The epigenome appears is most plastic in the late stages of oocyte and the early stages of embryo development.**
- **Suboptimal conditions during oocyte and embryo development may lead to persistent changes in the epigenome influencing disease susceptibilities later in life.**
- **Today a successful pregnancy is mainly defined by the outcome at birth, however we also have to consider the consequences of ART conditions for later life.**

Gluckman et al., Nat. Rev. Endocrinol. 5, 401-408, 2009
Lehnen et al., Mol. Hum. Reprod., 2013 (Epub ahead of print)

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Fauque P. Ovulation induction and epigenetic anomalies. Fertil Steril 2013;99:616-623.

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Pliushch G, Schneider E, Weise D, El Hajj N, Tresch A, Seidmann L, Coerdt W, Müller AM, Zechner U, Haaf T. Extreme methylation values of imprinted genes in human abortions and stillbirths. Am J Pathol 2010;176:1084-1090.

Smallwood SA, Tomizawa S, Krueger F, Ruf N, Carli N, Segonds-Pichon A, Sato S, Hata K, Andrews SR, Kelsey G. Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. Nat Genet 2011;43:811-814.

Trapphoff T, El Hajj N, Zechner U, Haaf T, Eichenlaub-Ritter U. DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of oocytes from vitrified pre-antral follicles. Hum Reprod 2010;25:3025-3042.

UNIVERSITÉ DE STRASBOURG

Les Hôpitaux Universitaires de STRASBOURG

Genetic factors for male infertility

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Disclosing slide

I declare that I have no potential conflict of interest

Spermatogenesis, where can it goes wrong?

Everywhere!

Matzuk *et al., Nat Med 2008*

Contents of lecture

- **Introduction**
- **What is known:**
  - Chromosomal anomalies
  - Genetic abnormalities
  - Genomic imprinting
- **What the future:**
  - Transposable elements
  - si/mi/piRNA
- **Clinical implications:**

Introduction

Where Genes can interfere with fertility

- **Gonads development (in utero life)**
  ex testicular dysgenesy
- **Gonadotrope axe** (hormons and receptors)
  ex: Kallmann syndrom (RX, RA, DA)
- **Gametogenesis**
  ex: Y microdeletion
- **Organs malformations**
  ex : cystic fibrosis (CBAVD andCFTR)
- **Sexual behaviour**

McLachlan RI and O'Bryan MK J. Clin. Endo. Metab 2010

Contents of lecture

- **What is known:**
  - Chromosomal anomalies
    - Numerical
    - Translocations/chromosomal rearrengments
    - Yq microdeletions

Chromosomal anomalies

Numerical

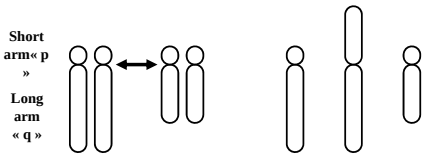
XXY Klinefelter's syndrome (KS)

XYY
XX male
XY female

Chromosomal anomalies

Translocations/chromosomal rearrengments

Robertsonian translocations

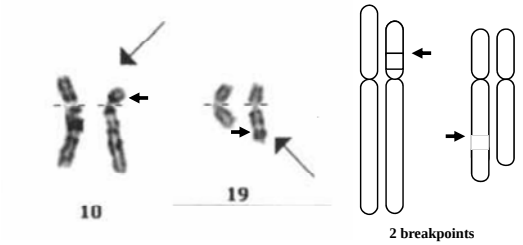


46 chro. **45 chro.**

Chromosomal anomalies

Translocations/chromosomal rearrengments

Reciprocal translocations



2 breakpoints

Chromosome abnormalities in ICSI patients

- Oligospermia
  - Abnormalities: 2 - 9 %
  - Mainly structural abnormalities
- Azoospermia
  - Abnormalities: 2 - 9 %
  - Mainly sex chromosomal abnormalities

Chromosomal abnormalities transmitted by ICSI (I)

- **1995 In 't Veld et al: extremely high incidence (33%) of sex-chromosome abnormalities**
- **1995 Liebaers et al: much lower (1%) but still higher incidence than in newborns (0.19%)**
- **1998 Bonduelle et al: increased incidence of structural abnormalities**

Chromosomal abnormalities transmitted by ICSI (II)

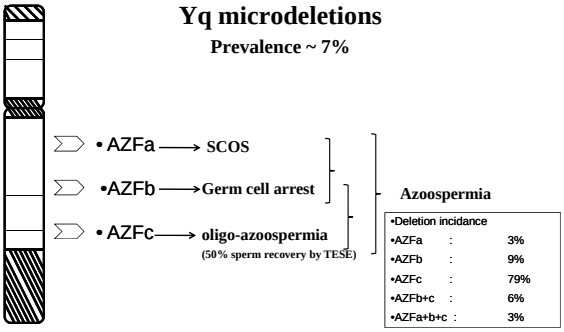
- Significantly increased number of de novo chromosome abnormalities (1.6 % instead of 0.56 %)
- About 3-fold increase of sex chromosome abnormalities
- Also increase of structural autosomal abnormalities

Bonduelle, et al 2003

Meiosis abnormalities
Chromosome rearrengments

- Schiasma and segregation perturbations
- Hihger frequence in cases of oligozoospermia or dysovulation
- Hihger frequence in case of spontaneous abortions

Chromosomal abnormalities



Contents of lecture

- **What is known:**
  - Genetic abnormalities
    - Syndromic
    - Non syndromic

Genetic abnormalities

Syndromic

~50 monogenic disorders associated with infertility

- **Cystic Fibrosis**
- **Myotonic dystrophy**
- **Noonan syndrome**
- **Kartagener syndrome**
- **Sickle cell disease**
- **Beta thalassemia**

Genetic abnormalities

Cystic Fibrosis (CF)

CFTR gene mutations can have large varieties of consequences:

- **CF more or less severe**
- **Congenital Bilateral Absence of Vas Deferens (CBAVD);**

↓

Obstructive azoospermia, with ~100% sperm recovery by TESE

~90% of the CBAVD patients carried at least one mutation on CFTR gene

Cuppens H, et al Int J Androl. 2004

Genetic abnormalities

Cystic Fibrosis (CF)

Genetic counseling in patients having CFTR mutation is complex and difficult because of the large number of mutations which render the prognostic difficult

The female partner of the CBAVD patient carrying CFTR mutation should be screened for the mutations in CFTR gene before ART

Genetic abnormalities

Non-Syndromic

Only few genes have been described affecting only the spermiogenesis

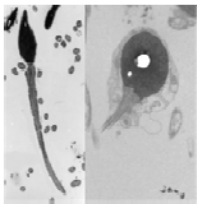
- **Globozoospermia or Round Head Syndrom**
  - ✓ **SPATA 16 gene**
  - ✓ **DPY19L2 gene**
- **Macrozoospermia**
  - ✓ **AURORA C gene**
- **Asthenozoospermic**
  - ✓ **CATSPER1 C gene**

Globozoospermia

- SPATA16 gene mutation, family study
- DPY19L2 gene deletion, family study

Both genes are implicated in acrosom formation

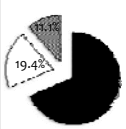
- Phenotype:
  - ✓ Globozoospermia or
  - ✓ Round headed spermatozooa
- Very low to non pregnancy rate



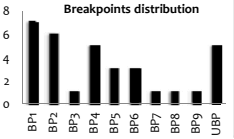
Dam *et al.*, 2007. Koscinski *et al* 2011. Harbuz R *et al* 2011

Globozoospermia: *DPY19L2*

67% of the patients are mutated for *DPY19L2*



■ Homozygous deletion
□ Heterozygous deletion
⊠ Homozygous mutation



Globozoospermia type I	Breakpoint zone	Origin	Spermoconcentration, million/ml
	BP1	Jordan	52
	BP1	France	71
	BP1	Iran	ND
	BP2	Turkey	ND
	BP2	France	ND
	BP2	Algeria	98
	BP2	Iran	ND
	BP1,BP2	France	79
	BP3	Turkey	10,25
	BP3	Saoudi Arabia	72,5
	BP3	Belgium	5,2,5
	BP5	Turkey	56
	BP5	Morocco	94
	BP5	Belgium	35
	BP5	Belgium	ND
	BP6	France	8,99
	BP6	Belgium	ND
	BP6	Iran	ND
	BP7	Morocco	176
	BP8	Morocco	69
	BP9	Turkey	ND

1.7 Kb — Hot spot 1 Alu seq

117 bp — Hot spot 2 THE1B

ElInati *et al*, HMG 2012

Perspectives: Globozoospermia

Clinical studies

Sperm deficiency	Before AOA		After AOA	
	Fertilization rate (%)	Pregnancy rate (%)	Fertilization rate (%)	Pregnancy rate (%)
Total globozoospermia (n = 9)	10 (5/49, 5 cycles)	0	76 (179/235, 19 cycles)	42 (8/19)
Partial globozoospermia (n = 1)	3 (1/32, 3 cycles)	0	76 (31/41, 2 cycles)	50 (1/2)

AOA: assisted oocyte activation

Adapted from Heindryckx et al, 2008

Globozoospermia: Clinical studies

ICSI + AOA influences the clinical outcome in patients with a known *DPY19L2* mutation ?

***DPY19L2*^{mt} and AOA+ (n = 15) vs *DPY19L2*^{mt} and AOA- (n = 14)**

DPY19L2^{mt} patients	Conventional ICSI	ICSI + AOA
Fertilization rate (%; 2pn/MII)	31.3 % (107/342)*	65.4 % (212/324)*
+hCG rate per ET	15.8 % (6/38)**	40.6 % (13/31)**
Ongoing pregnancy rate per ET	15.8 % (6/38)[a]	32.3 % (10/31)[a]
Live birth rate per ET	13.2 % (5/38)[b]	32.3 % (10/31)[b]

*p < 0.001; **p < 0.05; [a] p < 0.107, NS; [b]p < 0.056, NS

ICSI + AOA restores the fertilization rates & + hCG in mutated patients

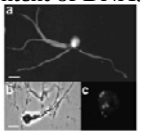
Kuentz *et al*, HR 2013

Macrozoospermia

- AURORA C gene mutation, founder effect
- AURORA C implicated in the meiotic fusion formation
- Phenotype:

macrozoospermia, with tetraploid content of DNA, multiple flagela

- Impossibility to offer ART



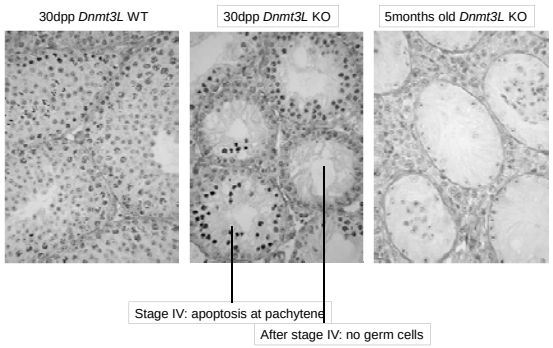
Dieterih et al., 2007

Contents of lecture

- **What the future:**
  - **Transposable elements**
    - ✓ Ancestral traces of retroviruses
    - ✓ ~50% of the human genome
    - ✓ They are reactivated during spermatogenesis and early development
    - ✓ They are tightly controlled to not jump anywhere in the genome
    - ✓ In mouse, mutations of proteins involved in their control is provoking a male infertility, most of the time with a blockage at the pachytene stage

Zamudio and Bourc'his Heredity 2010

Dnmt3l KO with age goes from severe oligo to azoospermia, ended with SCO



Bourc'his D. and Bestor TH Nature 2004

Contents of lecture

- **What the future:**
  - si/mi/piRNA
    - ✓ Small RNA of 18 to 30 nucleotides
    - ✓ Involve in many biological processes
    - ✓ Play a major role in male germ cells differentiation (piRNA)
    - ✓ Play a crucial role in the control of transposable elements
    - ✓ Involved in the control of gene expression
    - ✓ In mouse, mutations of proteins involved in their control is provoking a male infertility

Blumenstiel JP 2011 Trends in Genetics

Contents of lecture

- **Clinical implications:**

Candidate for ICSI can show:

– Increase of chromosome abnormalities including chromosomes rearrengments or deletion such as Y chromosome microdeletions resulting in severe oligospermia or azoospermia

– Things being always more complicated: it seems that some deletion of AZFc may increase the sperm count

(Noordam et al 2011)

Candidate for ICSI can show:

– Increase of chromosome abnormalities

– Mutation in genes involved in spermatogenesis (meiosis or spermiogenesis such as acrosome formation)

Candidate for ICSI can show:

– Increase of chromosome abnormalities

– Mutation in genes involved in spermatogenesis

– Mutation in genes involved in syndrome including fertility (CF mutations)

Candidate for ICSI can show:

– Increase of chromosome abnormalities

– Mutation in genes involved in spermatogenesis

– Mutation in genes involved in syndrome including fertility

– Genomic imprint defaults

Clinical implications

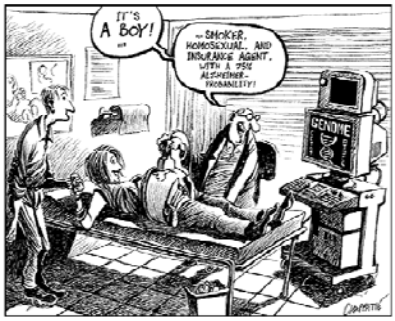
- Reproductive genetic counselling should be given by a genetic counselor with specialist knowledge in reproductive genetics
- Since ART and reproductive genetics are overlapping fields, a necessity for collaboration between genetic centers and ART centers has arisen.
- European Societies of Human Genetics and Human Reproduction and Embriology declared a common policy and published it.

✓ ***Sirpa S et al*** **EJHG (2006)**
✓ ***Recommendations of the European Societies of Human Genetics and Human Reproduction and Embryology*** **EJHG 2006**

Conclusions

- ART is a multidisciplinary team work
- Genetic Counseling Is Necessary for ART

Thank you


IT'S A BOY!
...SMOKER, HOMOSEXUAL, AND INSURANCE AGENT, WITH A 75% ALZHEIMER-PROBABILITY!
GENOME

Paternal DNA packaging in sperm – more than the sum of its parts? DNA, histones, protamines, and epigenetics

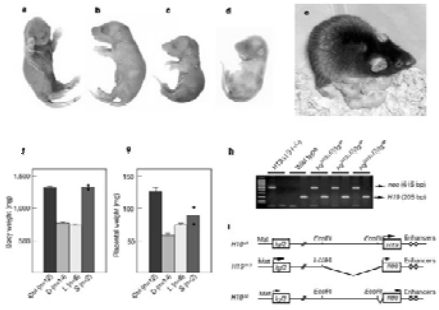
David Miller, BSc, PhD

University of Leeds

At the end of this lecture, you should be more aware of the following:

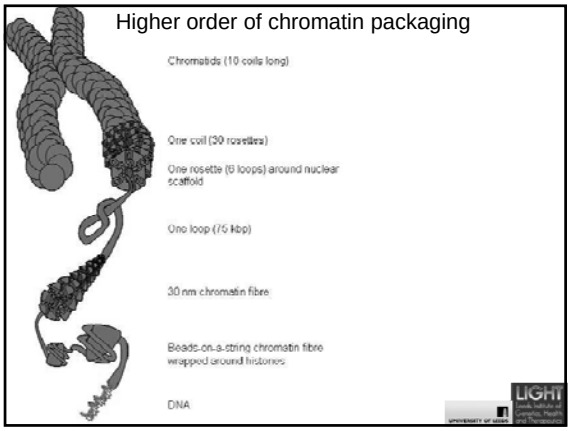
- Evidence showing that the paternal genome is dispensable even in mammals.
- The unique solution adopted by sperm to packaging the paternal genome.
- Evidence for sperm DNA damage contributing to pregnancy failure.
- The unexpected complexity of DNA packaging in sperm including evidence for non-random chromosome positioning.
- Evidence for disturbances in sperm chromatin configuration including epigenetic marking (modified histones) contributing to infertility.
- Evidence for similar packaging phenomena in other species including mice and (preliminary) flies.
- A theoretical consideration of measures that males may have taken to ensure continued transmission of the paternal genome.

HELP: males not needed! ☹



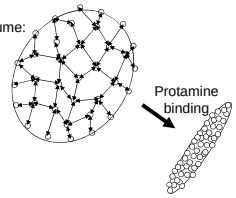
Kono et al, Nature, 2004

Higher order of chromatin packaging



The problem of sperm DNA packaging

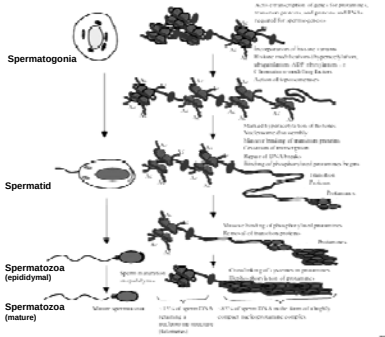
Somatic *chromatin* volume: histone binding ~15.5 µm^3



Sperm *nuclear* volume: 10-12 µm^3

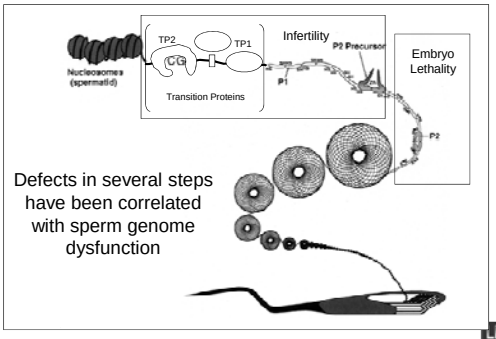
From Rod Balhorn

Except that……..



Oliva, Hum Rep 2006

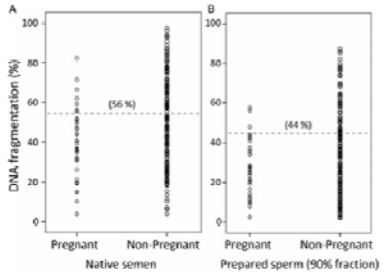
The solution



Defects in several steps have been correlated with sperm genome dysfunction

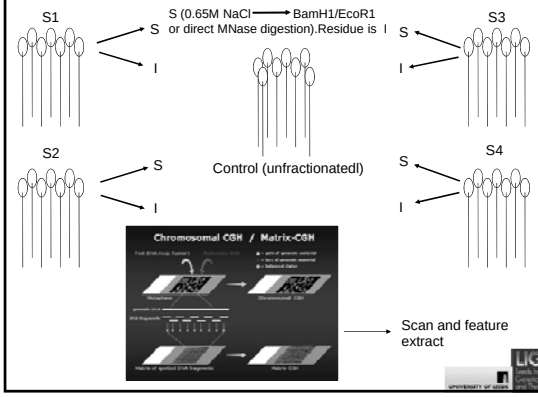
Courtesy of Rod Balhorn

Lower DFI in successful preg's

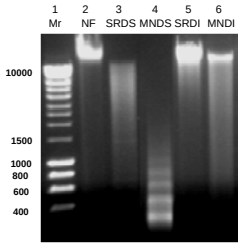


Simon et al, Fert Steril, 2012

SRD/MND based Microarray Analysis



AGE analysis of sperm DNA



Lane 1:- Mr ladder.

Lane 2: - Unfractionated sperm DNA (NF).

Lane 3: - Salt soluble (SRDS) DNA.

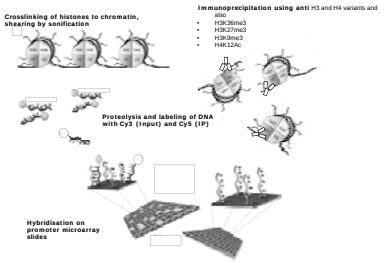
Lane 4: - MNase soluble (MNDS) DNA.

Lane 5: -Salt insoluble (SRDI) DNA.

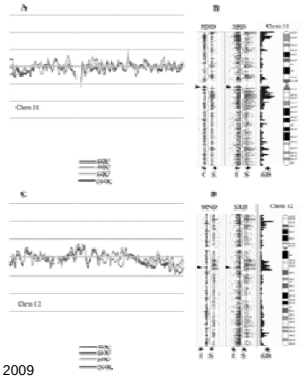
Lanes 6: - MNase insoluble (MNDI) DNA.

Arpanahi et al, 2009

ChIP-chip based Microarray Analysis

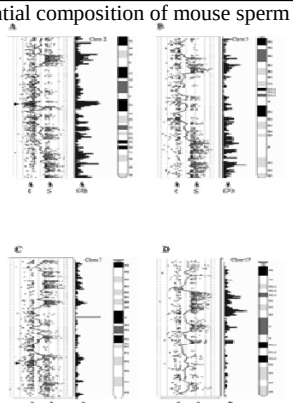


Differential composition of human sperm chromatin.



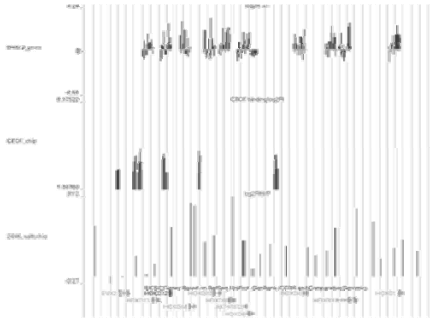
Arpanahi et al, 2009

Differential composition of mouse sperm chromatin.



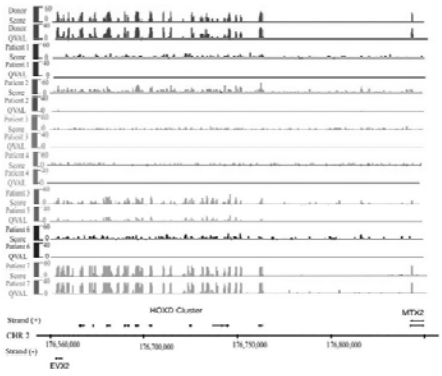
Arpanahi et al, 2009

Modified histones in *HOXD* locus



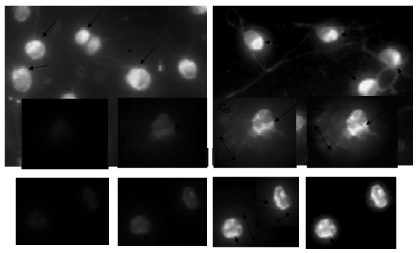
Arpanahi et al, Gen Res 2009

Deregulated histone deposition in *HOXD* locus of infertile men



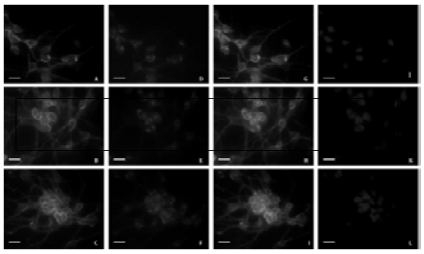
Hammoud et al, Hum Rep 2011

ICC and FISH analysis of human and murine sperm chromatin.



Miller et al, unpublished and Paradowska et al, 2012

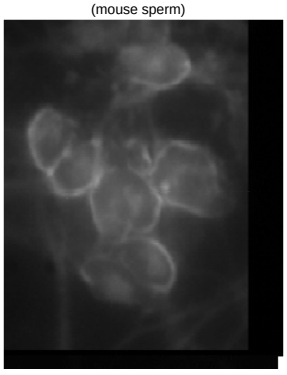
Nucleosomal chromatin is organised in distinctive regions
(mouse sperm)



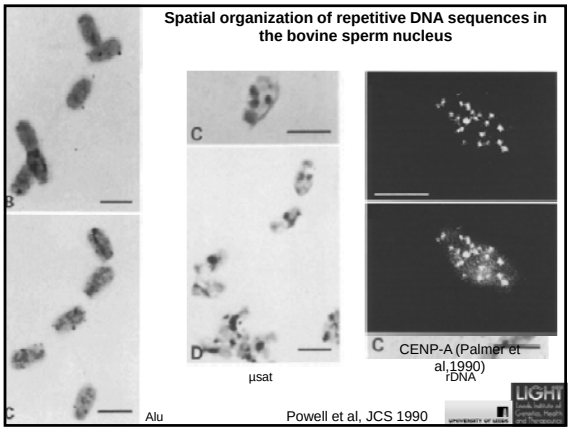
$MNDS_{FITC}$ $MNDI_{TRITC}$ MERGE $DAPI_{DNA}$

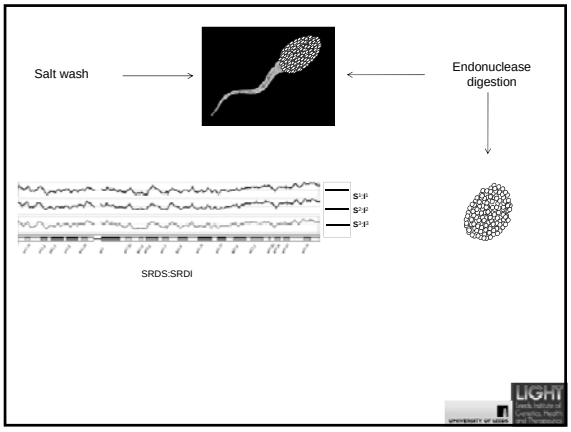
Saida et al 2011

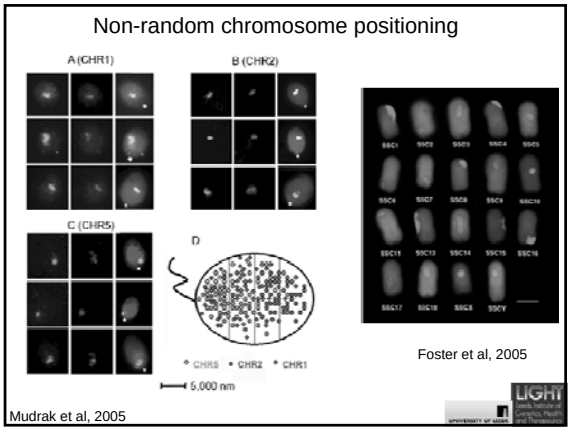
Nucleosomal chromatin is organised in distinctive regions
(mouse sperm)

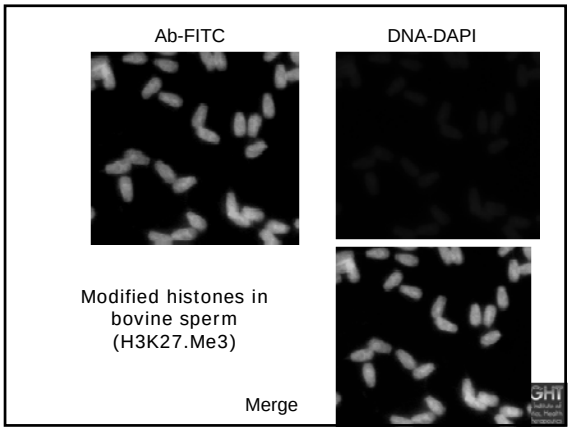


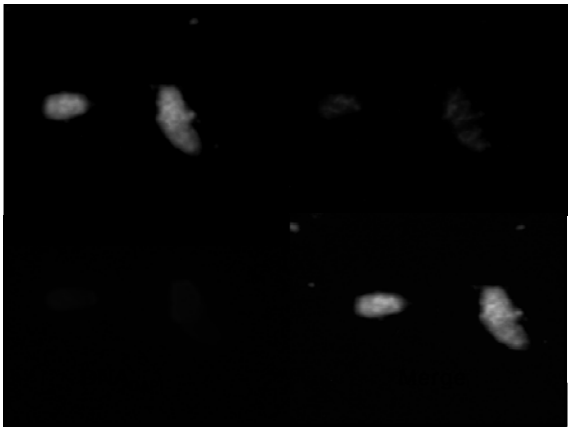
Saida et al 2011

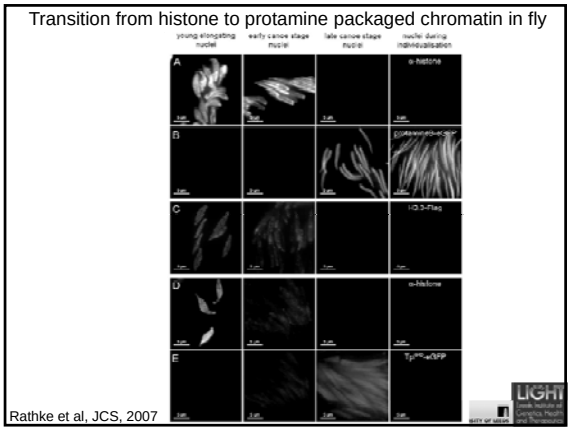

Spatial organization of repetitive DNA sequences in the bovine sperm nucleus
B
C
C
D
C
CENP-A (Palmer et al,1990)
µsat
rDNA
Alu
Powell et al, JCS 1990


Salt wash
Endonuclease digestion
SRDS:SRDI

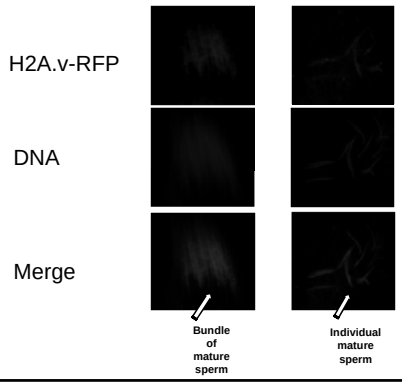

Non-random chromosome positioning
A (CHR1)
B (CHR2)
C (CHR5)
D
5,000 nm
Foster et al, 2005
Mudrak et al, 2005


Ab-FITC
DNA-DAPI
Modified histones in bovine sperm (H3K27.Me3)
Merge

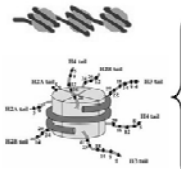



Transition from histone to protamine packaged chromatin in fly
young elongating nuclei
early canoe stage nuclei
late canoe stage nuclei
nuclei during individualization
A
α-histone
B
C
HA3-Flag
D
α-histone
E
Rathke et al, JCS, 2007

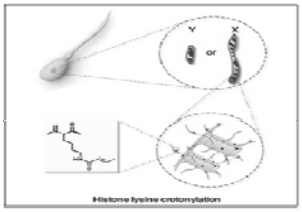
1- H2Av-RFP transgenic flies



Histone modifications

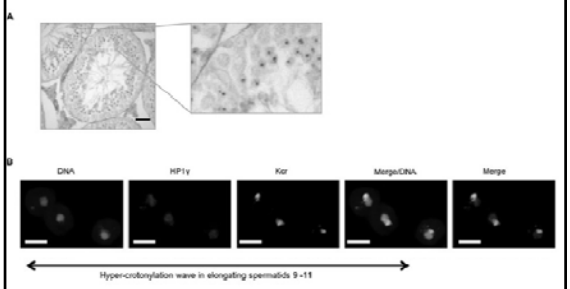


Histone modifications(cont'd)



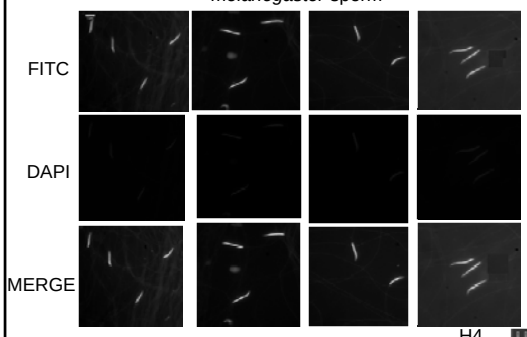
Tan et al, Cell 2011

Lysine crotonylation in (murine) spermatogenesis

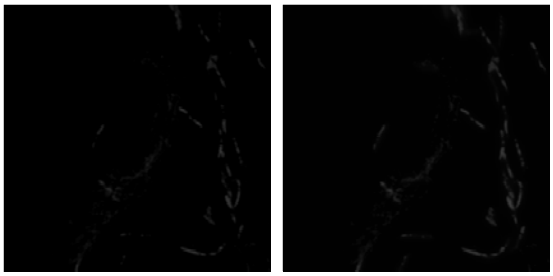


Tan et al, Cell 2011

Histones in decondensed *Drosophila melanogaster* sperm

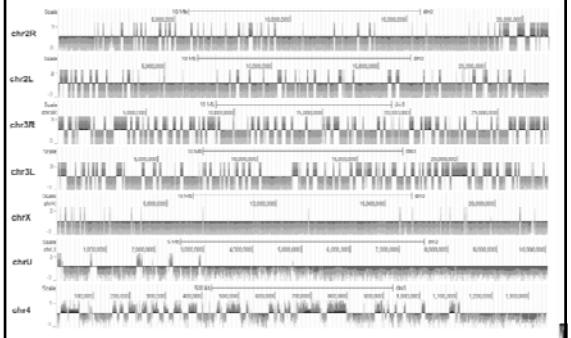


ChIP-FISH: Fly sperm nucleosomal chromatin is organised in distinctive regions

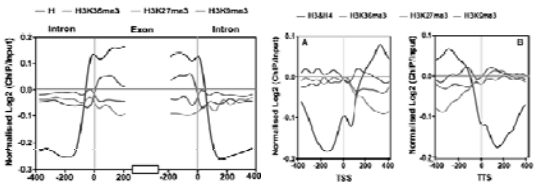


H3/H4 DNA $_{TRITC}$ DAPI $_{merge}$

Chromosomes packaging by nucleosomes in the sperm of *Drosophila melanogaster* .

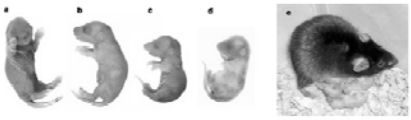


Nucleosomes occupancy within genes in the fly genome

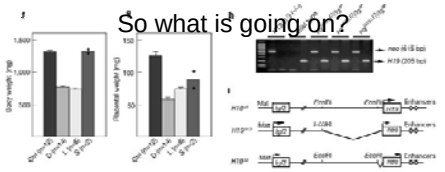


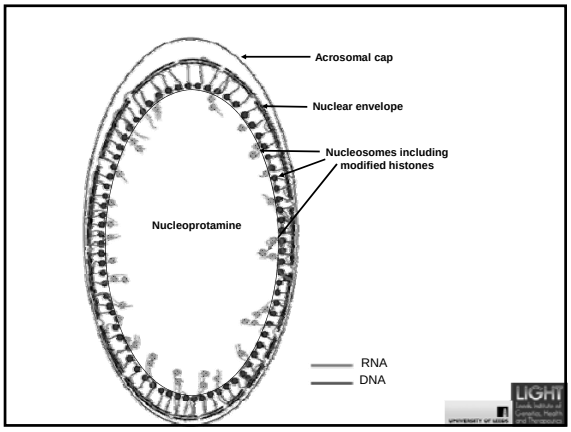
- Nucleosomal peaks are located at the intron/exon boundaries.
- Nucleosomes are preferentially located on exons rather than introns.
- Depletion of nucleosomes in both TSS and TTS regions.

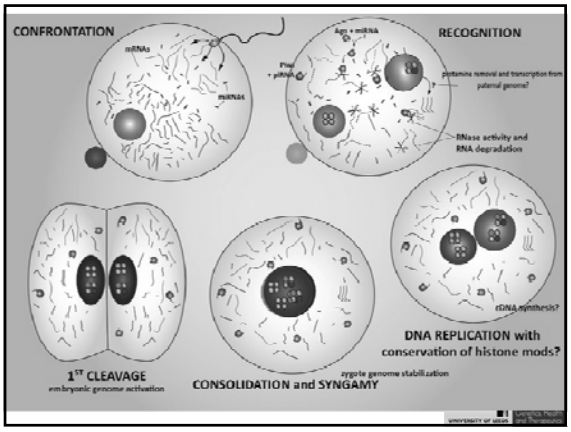
Males make themselves needed!

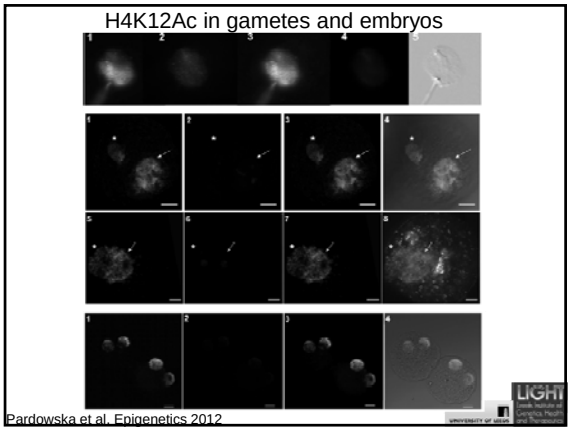


So what is going on?









Pardowska et al. Epigenetics 2012

- Even the paternal genome is dispensable.
- Sperm entry into the ooplasm poses a potential risk to the egg (entry of and hijacking by semi-autonomous elements).
- The paternal genome must be 'tolerated' and 'accepted' by the egg 'pre-syngamy check'.
- The paternal genome accommodates this requirement by having the correct epigenetic signature (DNA methylation and histone modifications) on board.
- Gynogenetic mammals can bypass this system by manipulation of imprinting control regions but quid pro quo, viable androgenetic mammals should be far more difficult to create.
- Somatic cell based clones have already gone through the pre-syngamy check and so only require pluripotency reprogramming.
- Pre-syngamy check helps reduce the incidence of inter-specific hybrids between closely related species.

With thanks to:-

Martin Brinkworth, University of Bradford.

Agnieszka Paradowska and Klaus Steger, University of Giessen, Germany.

David Iles, Abdul Elnfati, Ali Arpanahi, Myriam Saida, University of Leeds and the clinical embryologists at Seacroft Hospital, Leeds

With thanks to:-

« Epigenetic mechanisms in the pre-implantation embryo »

ESHRE-2013
Pre-Congress Course 8
London, 7th July 2013

Robert Feil
CNRS &
University of Montpellier

- **No commercial relationships with potential conflict of interest**
- **No other activities with potential conflict of interest**

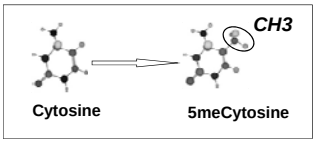
Dr. Robert Feil, Ir., Ph.D.,
Director of Research (DR1)

Institute of Molecular Genetics (IGMM),
CNRS UMR-5535
University of Montpellier I &II
Montpellier, France

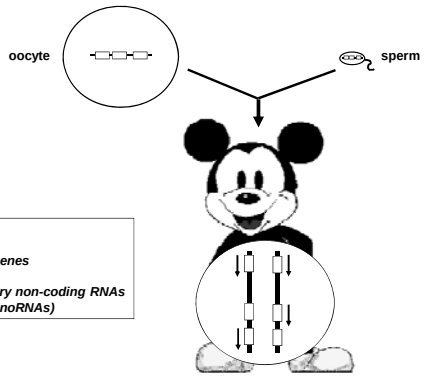
Learning objectives of the course

* DNA methylation in the pre-implantation embryo
* Genomic imprinting and its somatic maintenance in the early embryo
* Pertubation of DNA methylation imprints and its disease consequences
* Environmentally induced perturbation of DNA methylation imprints in the embryo
* Emerging questions for future research ?

Embryogenesis and DNA methylation



* Chromosome stability
* Repression of DNA elements of foreign origin
* Heritable, tissue-specific, repression of genes
* 'X-chromosome inactivation' in females
* **Genomic Imprinting**



Genomic imprinting:

~150 protein coding genes

-hundreds of regulatory non-coding RNAs (lncRNAs, miRNAs, snoRNAs)

genomic imprinting

Imprinted genes influence development, nutrient transfer and behaviour

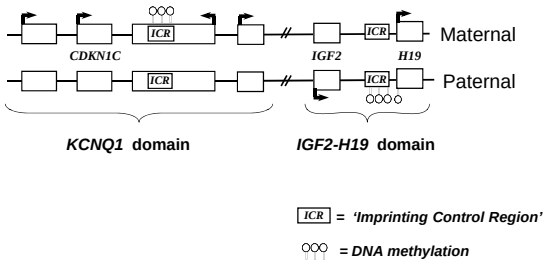
Placental development and function

Foetal growth control

Postnatal fitness

Postnatal behaviour

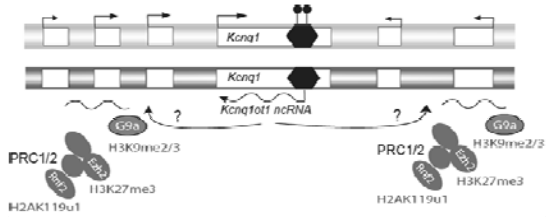
Two imprinted domains involved in fœtal growth



ICR = *'Imprinting Control Region'*

= *DNA methylation*

ncRNA-mediated histone methylation controls imprinted expression at the *KCNQ1* domain

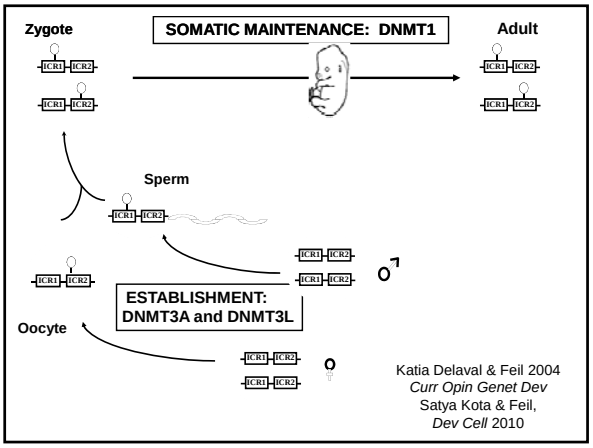


Alexandre Wagschal *et al. Mol. Cell. Biol.* 2008
Remi Terranova *et al. Dev Cell* 2008
David Monk *et al. PNAS USA 2006*
David Umlauf *et al. Nature Genet.* 2004

Imprinting Control Regions (ICRs)

CH3 CH3 CH3 CH3 CH3 CH3 — Parental allele WITH DNA methylation

Parental allele WITHOUT DNA methylation

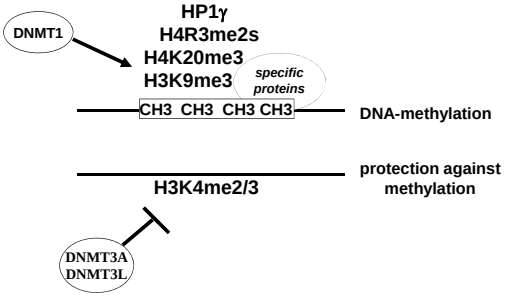


ICRs: Somatic maintenance of allelic DNA methylation throughout development

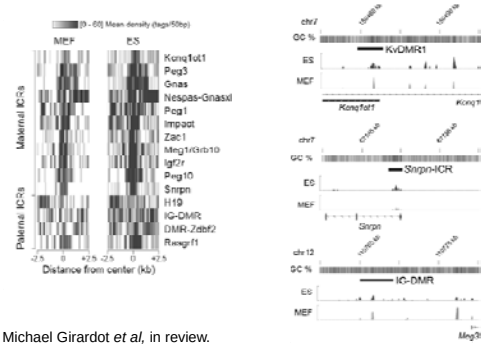
CH3 CH3 CH3 CH3 CH3 CH3 — Parental allele WITH DNA methylation

Parental allele WITHOUT DNA methylation

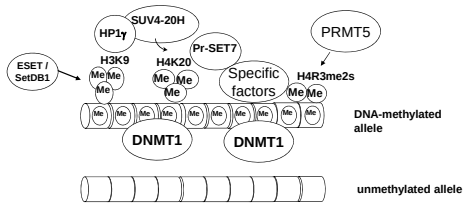
For further discussion, please see:
Kelsey and Feil, 2013
Philos Trans R Soc Lond B Biol Sci.



H4R3me2s marks the methylated allele of ICRs



Michael Girardot *et al,* in review.

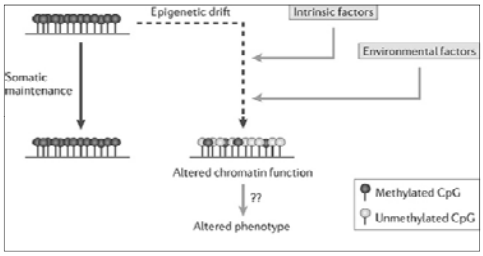


Pannetier *et al*., *EMBO Reports* 2008
Alexandre Wagschal *et al* MCB 2008
Yuan *et al. Genes Dev* 2010
Michael Girardot/Ryutaro Hirasawa *et al*., in review

Pre-implantation epigenetic maintenance and disease?

Examples of genomic imprinting

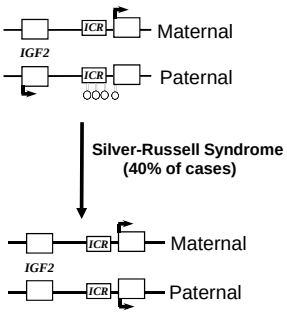
Intrinsic and environmental perturbation of DNA methylation patterns



Fraga and Feil,
Nature Rev Genet 2013

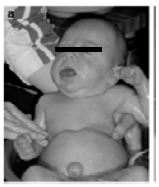
Silver-Russell Syndrome (SRS)

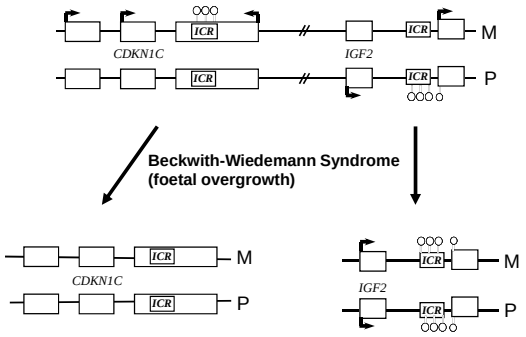
- Intra-uterine growth restriction (IUGR)
- Postnatal growth deficiency
- Learning disabilities
- Mostly sporadic



Beckwith-Wiedemann Syndrome (BWS)

- Foetal overgrowth
- Large internal organs, large tongue
- Predisposition to Wilms' tumour of the kidney
- Mostly sporadic





Hypomethylation occurs often in concert at multiple imprinted regions in BWS, SRS, TNDM & Pseudohypoparathyroidism-1B.

- Mackay DJ et al. 2008. *Nature Genetics*
- Bliek J et al. 2009. *Eur J Hum Genet*
- Hirasawa R and Feil 2010. *Essays Biochem.*
- Azzi S et al. 2010. *Epigenetics*
- Court F et al. 2013. *Hum. Mutation*

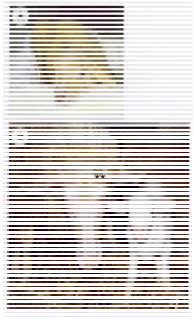
Frequent perturbation of imprints *in vitro*

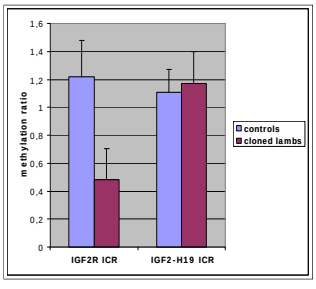
* **Derivation and culture of ES cells in certain media**	Dean *et al.* 1998; Humpherys *et al.* 2001
* **Pre-implantation embryo culture in certain media**	Khosla *et al.* 2001; Young *et al.* 2001
* **Reprogramming into induced pluripotent stem cells (iPS cells)**	Stadtfeld *et al.* 2010,
* **Somatic cell nuclear transfer**	Humpherys *et al.* 2001 Young *et al.* 2003
• **Assisted reproduction** (Humans)	Review: Denomme & Mann, *Reprod.* 2012 DeBaun *et al.* 2003; Cox et al. 2003) Maher *et al.* 2003; Örstavik *et al.* 2003; Halliday *et al.* 2004; Fortier et al. 2008

Imprinting is particularly labile in the extra-embryonic part of the embryo

- *In vitro* embryo culture often affects imprinting in the placenta (Mann *et al.,* 2004; Rivera *et al.,* 2008)
- Super-ovulation affects imprinted gene methylation in the placenta
  (Fortier *et al.,* 2008; Market-Velker et al., 2010)

'Cloning' and *in vitro* embryo culture in sheep : Aberrant *IGF2R* imprinting, but unaltered *H19-IGF2*





Young L *et al*. Mech Dev. 2003
Young L *et al*. *Nature Genet.* 2001

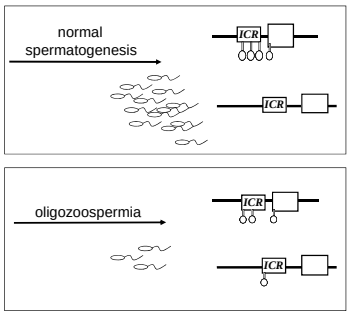
Endocrine disruptors: 'long-term' effects on DNA methylation imprints?

- Vinclozolin (50mg/kg) and methoxychlor (10 mg/kg) administration during pregnancy:

  --Sperm in F1, F2 & F3:
  --Slight reductions in DNA methylation at paternal ICRs
  --Gains in DNA methylation at maternal ICRs

Stouder *et al*, 2010, 2011;
Kang *et al,* 2011;
Somm *et al*., 2013

Perturbed sperm DNA methylation imprints in oligozoospermia



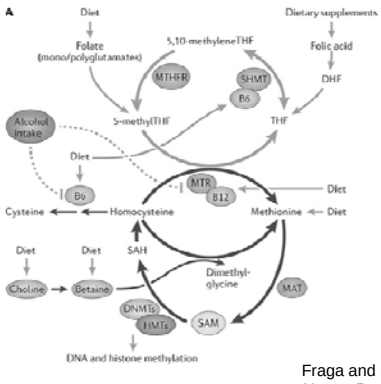
Marques *et al.* 2004, 2008
Kobayashi *et al.* 2007
Boissonnas *et al.* 2010

Endocrine Disruptors:
Stouder *et al.* 2010, 2011

MINOR nutritional effects on imprinted DNA methylation

- **Dutch Hunger Winter, periconceptional exposure to famine:**
  -Decreased DNA methylation at imprinted genes in children.
- **Increased folate/altered choline during pregnancy** (human, rat):
  Increased DNA methylation at *IGF2*.
- * **High-fat diet during gestation** (mouse):
  Altered DNA methylation at the *IGF2R* locus in placenta.
- **Alcohol consumption during pregnancy** (mouse):
  Decreased DNA methylation at *H19* ICR and *IGF2* in offspring.
- **Alcohol consumption in adult males** (mouse, human):
  Aberrant DNA methylation imprints in sperm (*H19* ICR, Ig-DMR)

Fraga and Feil,
Nature Rev Genet 2013



Fraga and Feil,
Nature Rev Genet 2013

remaining questions…..

- Why are certain loci more susceptible than others?
- Which mechanisms (recruiting factors) normally control DNA methylation at affected loci?
- Mechanistic link between environmental/toxic exposure and observed DNA methylation changes?
- What, if any, are the biological consequences of the observed epigenetic alterations?

Patricia Cavelier

Michael Girardot

Sébastien Lalevee (June 2013)

David Lières

Alice Marchand

Benoît Pignard

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Philippe Arnaud
Amandine Henckel
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Gudrun Moore	**London**
David Monk	**Barcelona**
Slimane Ait-Si-Ali	**Paris**
Laurent Journot	
Tristan Bouschet	**IGF, Montpellier**

Institute of Child Health

UCL

Links between the genome and the epigenome *in utero*

Professor Gudrun Moore, PhD, (hon) FRCPCH, FRCOG ad eundem
Clinical and Molecular Genetics
Institute of Child Health
University College London

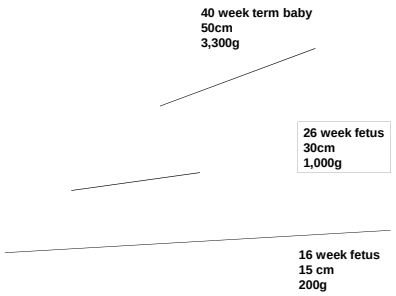
I have no commercial relationships, or other activities that might be perceived as a potential conflict of interest

Learning Objectives

- What is normal fetal growth?
- What is genomic imprinting?
- How can studying imprinted genes in humans help to understand growth?
- How can working on imprinted genes in placenta help?
- Two evidenced based examples (*PHLDA2* and *IGF2*) of the role of imprinted genes *in utero* and their effect on fetal growth linking the genome with the epigenome

What is normal fetal growth?

Growth Chart



Fetal Growth Restriction (FGR)

Definition: Born <2.5kg with serial ultra-sound showing reduced fetal growth

Medical problems:
•major contributor to perinatal morbidity and mortality
•*120 IUGR perinatal deaths* in SE England/annum
•many that survive have severe brain damage = irreparable neurological delay

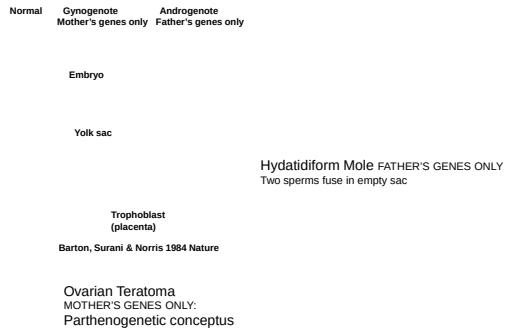
What is genomic imprinting?

Mouse experiments on blastocysts

Naturally occurring human examples?

Disomic mouse models that link to human Syndromes

Imprinting in Mice and Human



Hydatidiform Mole FATHER'S GENES ONLY
Two sperms fuse in empty sac

Ovarian Teratoma
MOTHER'S GENES ONLY:
Parthenogenetic conceptus

Uniparental disomies

Silver-Russell Syndrome
MatUPD7/hypometh 11
FGR
1 in 7,000

Beckwith-Wiedemann Syndrome PatUPD11
OVERGROWTH
1 in 15,000

VERY RARE SYNDROMES

How can studying imprinted genes in humans help to understand growth?

Paternal expressing imprinted genes = enhance fetal growth

Maternal expressing imprinted genes = restrict fetal growth

How can working on imprinted genes in placenta help?

What genes are key players in fetal growth?

Are they imprinted and important in the placenta?

Why are they imprinted?

Can their expression be regulated to reverse growth restriction?

Imprinted Gene In Mouse Placenta (Chr)	Expressed Allele	Mouse Phenotype (Growth)
Plagl1 (10 A1)	P	apoptosis
Igf2r (17 A1)	M	GR/overgrowth
Slc22a2 (17 A1)	M	cation transport
Slc22a3 (17 A1)	M	cation transport
Grb10 (11 A1)	P/M	GR/IUGR
Sgce (6 A1)	P	myoclonic
Peg 10 (6 A1)	P	GR/embryonic lethal
Ppp1r9a (6 A1)	M	synapsis formation in neural tissue
Mest (6 A3)	P	GR/cardiac defect/abnormal maternal behaviour
Klf14 (6 A3)	M	NO DATA
Sfmbt2 (2 A1)	P	NO DATA
H19 (7 F5)	M	GR/lethal
Igf2 (7 F5)	P	GR
Igf2as (7 F5)	P	NO DATA
Ins2 (7 F5)	P	GR
Ascl2 (7 F5)	M	NO DATA
Tssc4 (7 F5)	M	NO DATA
Kcnq1 (7 F5)	M	GR/deafness/behavioural
Kcnq1ot1 (7 F5)	P	GR
Cdkn1c (7 F5))	M	GR/developmental defects
Slc22a18 (7 F5)	M	NO DATA
Phlda2 (7 F5)	M	GR
Nap1l4 (7 F5)	M	NO DATA
Osbpl5 (7 F5)	M	NO DATA
Ampd3 (7 E3)	M	NO DATA
Dhcr7 (7 F5)	M	multiple problems at birth
Slc38a4 (15 F1)	P	GR
Dlk1 (12 F1)	P	GR/skeletal abnormalities
Dlk1 downstream transcripts (12 F1)	P	NO DATA
Meg 3 (12 f1)	M	GR
Rian (12 F1)	M	NO DATA
Mirg (12 F1)	M	NO DATA
Dio3 (12 F1)	P	GR/severe
Magel2 (7C-5B)	P	GR
Gatm (2 E5)	M	NO DATA
Peg3 (7A2-B1)	P	GR/nervous system
H13 (2 H1)	M	NO DATA
Gnas (2 E1-H3)	M	GR

SUMMARY:
IN MOUSE

38/85 imprinted in placenta (50%)

16 paternal
23 maternal

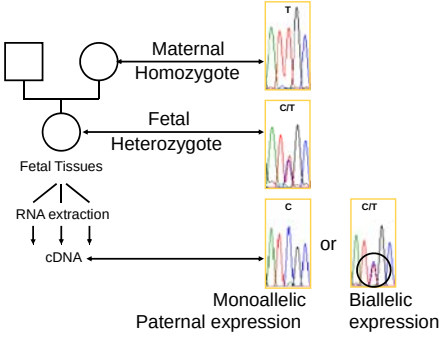
18 with known growth effects

20% growth effects of imprinting in mice

Location	Imprinted Gene In Human Placenta	Expressed Allele	Human Phenotype (Growth)
6q24	*PLAGL1*	P	GR/IUGR/TNDM
7p12	*GRB10*	P/M	GR/SRS?
7q21.3	*SGCE*	P	myoclonus dystonia
7q21.3	*PEG10*	P	NO DATA
7q21.3	*PPP1R9A*	M	synapsis formation in neural tissue
7q32.2	*CPA4*	M	carboxypeptidase
7q32.2	*MEST*	P	GR?/SRS?TNDM?
7q32.2	*MESTIT1*	P	yes
7q32.2	*KLF14*	M	NO DATA
11p15	*H19*	M	GR/SRS/BWS
11p15	*IGF2*	P	GR/SRS/BWS
11p15	*IGF2AS*	P	NO DATA
11p15	*KCNQ1* (temporal)	M	GR/BWS/LQT1/JLNS1
11p15	*KCNQ1OT1* (temporal)	P	GR/BWS
11p15	*CDKN1C*	M	GR/BWS
11p15	*SLC22A18*	M	NO DATA
11p15	*PHLDA2*	M	GR
11p15	*OSBPL5*	M	NO DATA
14q32	*DLK1*	P	GR
14q32	*MEG3*	M	GR
15q11-q12	*SNURF-SNRPN*	P	GR/PWS
16p13	*ZNF597*	M	NO DATA
19q13.41	*ZNF331*	M	NO DATA
19q13.43	*PEG3*	P	NO DATA
19q13.43	*ZIM2*	P(M)	NO DATA
20q13	*GNAS (NESP55)*	M	AHO

26/55 human imprinted and expressed in placenta (50%)
13 with growth phenotype = 20% involved in growth

Imprinting in Humans



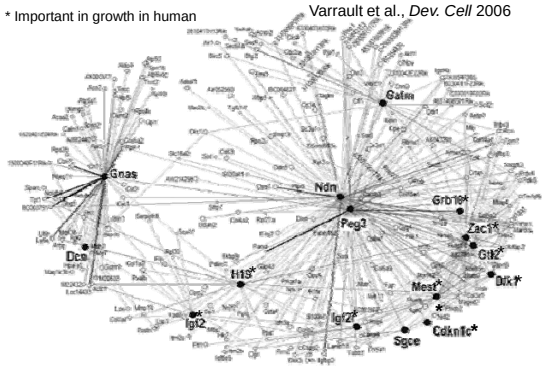
Monoallelic
Paternal expression

Biallelic
expression

Imprinted gene Networks:

* Important in growth in human

Varrault et al., *Dev. Cell* 2006



Imprinted Genes in the Human Placenta

Aim:

• To study the expression of imprinted genes in a white European population (Moore cohort >300 trios;UCL-FGS Cohort > 250 trios)

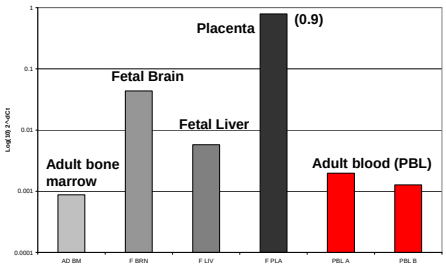
• Correlate the expression with birth weight and other clinical parameters

• Follow up promoter variants in ALSPAC cohort >10,000 baby and mother DNA

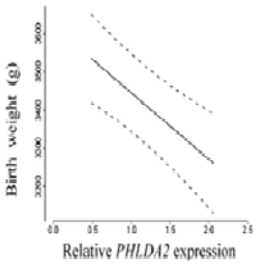
PHLDA2 = Pleckstrin Homology-Like Domain, Family A, Member 2

- Maternally expressed
- Chromosome 11p15.5 imprinted region controlled by ICR2
- Putative growth suppressor

PHLDA2 expression in fetal tissues and adult bone marrow and blood



PHLDA2 expression data in Moore Cohort

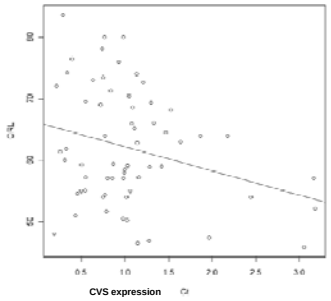


- *PHLDA2* expression level negatively associated with Birth weight

  (p = 0.0001)
- 200 normal human term placenta
- Real-Time PCR

Apostolidou S et al: 2007 J Med Mol

PHLDA2 expression versus Crown Rump length in Chorion Villus samples



Corrected for fetal sex and parity; gestational age 12 weeks, n=62 p=0.03*

PHLDA2 expression level negatively associated with Birth weight

Imprinting Status of *PHLDA2*

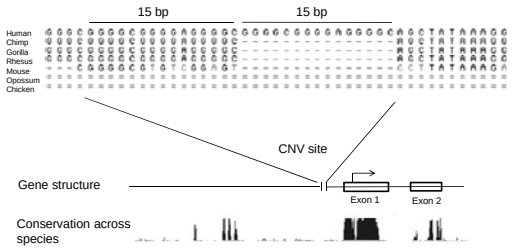
• Maintained in placenta tissues irrespective of birth weight, (n= 41).

• the increased expression of *PHLDA2* in the low birth weight babies was not due to Loss of Imprinting (LOI).

• In addition the methylation status of the KvDMR1 was normal.

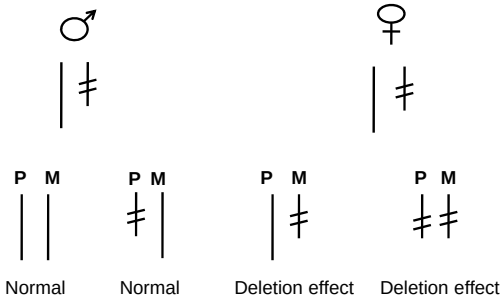
Therefore, the *PHLDA2* promoter itself must be influencing expression levels from the maternal allele.

Promoter region of PHLDA2

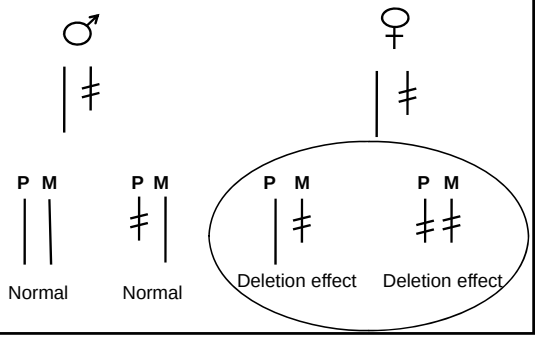


Having two CNVs is unique to Humans.

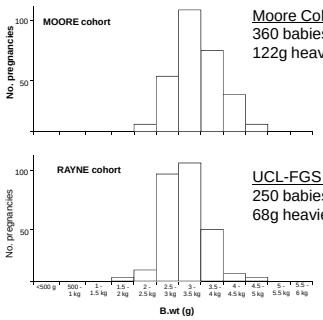
PCR based genotyping



PCR based genotyping



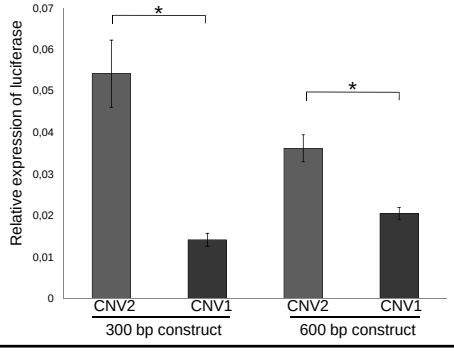
To see whether the *PHLDA2* promoter CNV1 influenced expression levels and therefore birth weight, we genotyped two separate trio cohorts (mother, father and baby) of normally distributed samples.



Moore Cohort:
360 babies
122g heavier (n=28)(p= 0.15)

UCL-FGS Cohort:
250 babies
68g heavier (n= 16) (p= 0.61)

Luciferase assay of CNV1 versus CNV2 in 293T cells



PHLDA2 is a putative growth suppressor

⇩

CNV1 reduces the *PHLDA2* promoter efficiency

⇩

CNV1 may act as growth enhancer

⇩

Correlate the CNV1 genotype and birth weight

Aims and Hypothesis

Hypothesis:

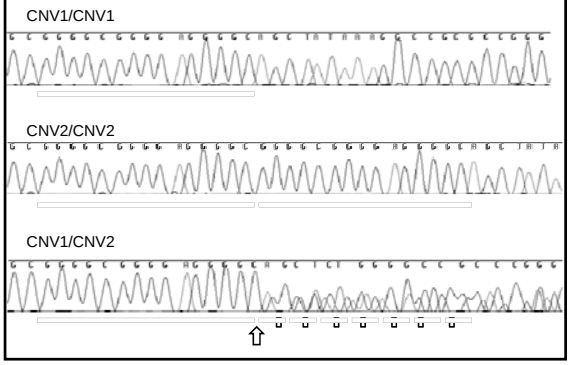
- The *PHLDA2* promoter deletion is a predictor of birth weight

Aim:

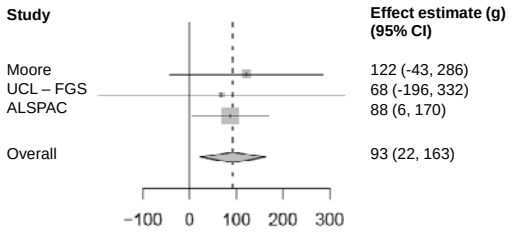
- Genotype sufficient samples to achieve statistical significance using ALSPAC cohort (~10,000 samples)

  (ALSPAC: the Avon Longitudinal Study of Parents and Children) at Bristol University

Sequencing analysis of CNV2/CNV1



Meta-analysis for *PHLDA2* deletion effect on birth weight



+155g with p=0.036* with mother homozygote (maternally deleted) and baby (maternally deleted)

Ishida M et al AJHG 2012

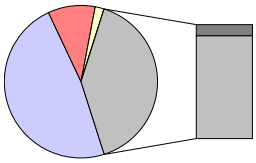
Summary on *PHLDA2*

- *PHLDA2* expression is significantly associated with smaller babies in CVS p=0.03 and term placenta p=0.0001
- Maternal expression maintained (no LOI)
- *PHLDA2* promoter copy number variant (CNV1) reduces expression therefore increasing birth weight
- *PHLDA2* CNV1 is found in heavier babies p=0.01 (93g heavier opposite to smoking 20 cigarettes less/day)
- Combination of imprinting and inheritance through the maternal allele to balance birth weight
- = Maternal control of growth up and down?

IGF2
Insulin-like growth factor -2
and *H19*
and Silver-Russell Syndrome

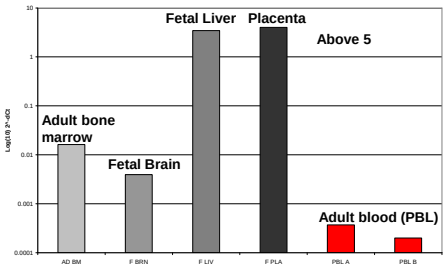
Silver-Russell Syndrome – associated molecular anomalies

IUGR; Typical Pointed face; Clinodactyly; Asymmetry



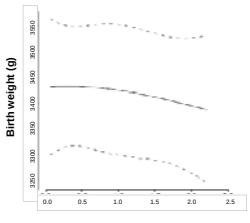
- No molecular anomalies identified (48 %)
- Sub-microscopic chromosomal aberrations (2 %)
- mUPD7 (10 %)
- H19 DMD hypomethylation (40 %)
- Changes in methylation at multiple imprinted loci (7 % of H19 HOs)

IGF2 expression in fetal tissues and adult bone marrow and blood

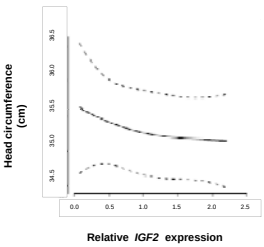


IGF2, birth weight & head circumference

The effect of relative *IGF2* expression on birth weight

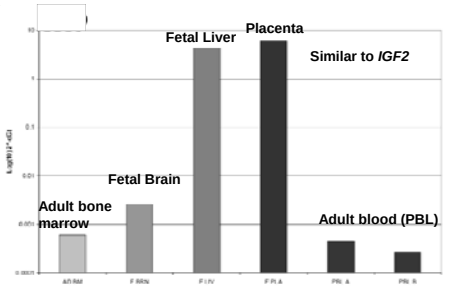


The effect of relative *IGF2* expression on head circumference



- No statistical significance was found between the relative *IGF2* expression and birth weight or head circumference

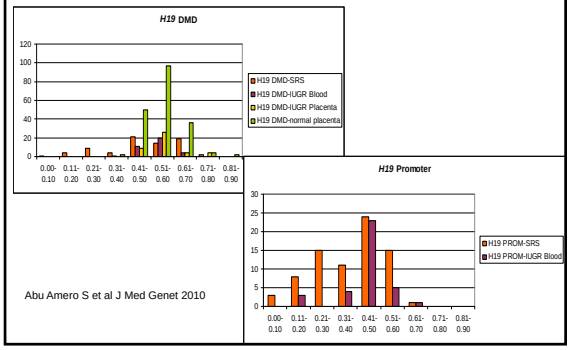
H19 expression in fetal tissues and adult bone marrow and blood



H19 methylation

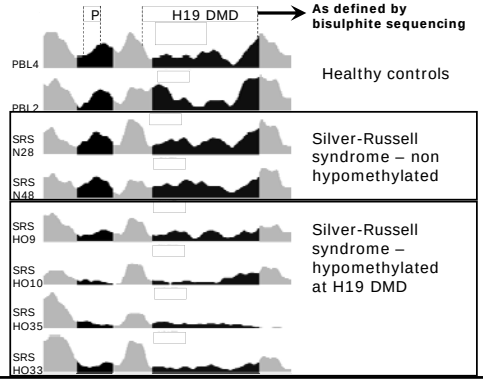
- *H19* is situated next to *IGF2*
- Maternally expressed non-translated RNA and controls the level of *IGF2* by suppressing *the* maternal *IGF2 gene*
- It expressed in the fetus and placenta in similar places to *IGF2*
- *H19* knockout mice are 40% larger
- In 5/9 SRS patients without mUPD7 loss of methylation at *H19* leading to *its* biallelic expression and decrease of *IGF2 (*Gicquel et al *Nat Gen* Sept 2005)
- Our 64 SRS DNAs; 45% hypomethylated

Methylation of the *H19* promoter and DMD in SRS and FGR

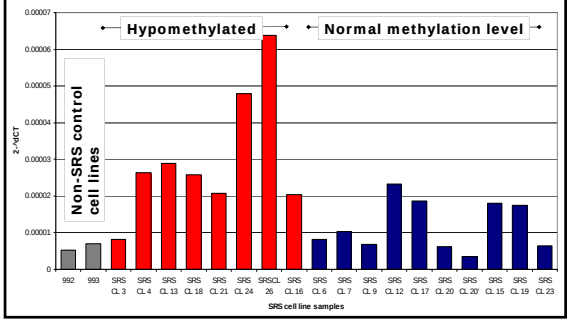


Abu Amero S et al J Med Genet 2010

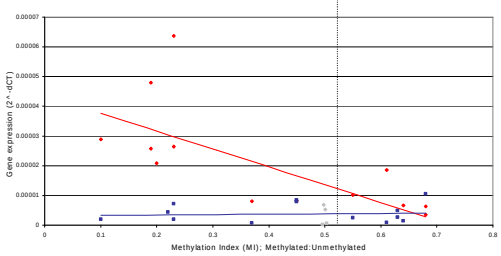
MeDIP SEQ – validation at H19 DMD (10M reads/sample)



H19 expression (by SYBR green Q-PCR) in SRS patients with and without hypomethylation at the H19 DMD



IGF2 and H19 expression versus methylation index value at H19 DMD in SRS cell lines



***H19* expression; NR_002196**
***IGF2* expression; NM_000612**
Normal control lymphoblastoid cell samples
Normal MI at the H19 DMD (0.5)

What genes are key players in fetal growth?

There are likely to be hundreds with small additive effects but by using genetic models that have growth restriction as a phenotype some of the key genes are being elucidated

Or: very large populations
GWAS 10,000 plus

Are they imprinted and important in the placenta?

There are several well characterised imprinted genes that are important in early fetal growth but the mouse *placental specific* imprinted genes are *not* all conserved in the human placenta

The best examples to date are still *PHLDA2*, *IGF2* modulated by *H19*.

Why are they imprinted?
What about litter size between species?

Can their expression be regulated to reverse growth restriction?

We are studying the levels of *PHLDA2* in chorion villous samples (CVS) and pregnant maternal blood and correlating this with birth weight and FGR to assess as a biomarker for growth *in utero*

Institute of Child Health

UCL

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  20-22 November 2013 - Leuven, Belgium
- From early implantation to later in life
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