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
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
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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

Conflict of interest statement
Prof Alan H Handyside is
Head of Preimplantation Genetics
Bluegnome, an Illumina Company

1. Array CGH
2. SNP arrays for cytogenetics
3. SNP genotyping and Karyomapping
4. Next-generation sequencing
Sequential biopsy of PB1 and PB2 with follow up of aneuploid embryos at cleavage stages on Day 3 post ICSI


Illumina iScan and Human CytoSNP-12 beadarray

0.5 allele frequency

Disomy Trisomy Loss of heterozygosity

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


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
SNP haplotypes of both paternal chromosome 2 and der 22

Embryo 1

SNP haplotype proximal to breakpoint on paternal 22 identifies der 22.

Embryo 1

SNP haplotype proximal to breakpoint on der 22 identifies normal paternal 2.

Embryo 5

Embryo 7

2 der 22 Unbalanced

der 22 der 22 Balanced

2 22 Normal

James D Watson

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

May 31st, 2007

Page 12 of 111
Genomes by the thousand

Two years ago, the Human Genome Project announced it had completed sequencing the human genome. They had almost succeeded. They had read it enough. Today, analysis of each chromosome is now complete. However, the data structure is not yet final. These data are the result of the human genome project. The project was completed with the help of billions of dollars and millions of hours of work. Although the project was successful, the fully sequenced chromosome at about 22 million base pairs and 10 times the length of the 22nd chromosome at the end of 2003, and then the final 2007 was more.
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.
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.
- 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!
Genetic and epigenetic causes of infertility

Can we minimize the risks?

Epigenetics & Fertility

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 germ line?

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

Do novo methylation results in an asymmetric distribution of DNA methylation

Characterisation of DNA methylation in germine reprogramming using next generation sequencing
Profound DNA methylation losses leave male and female gametes hypomethylated at mitotic and meiotic arrest.

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

>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.
Both groups shared the same spectrum of abnormalities. Development and the 'normal' epigenotype are mechanistically linked. These results suggested that the problem may be underlying and not a consequence of treatment for infertility. Systematic prospective studies have reached similar conclusions.

A review of known imprinting syndromes and their association with assisted reproduction technologies:

To date, reports have identified nine imprint 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.
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.

Lessons from embryonic stem cells

Culture conditions profoundly alter quantitative DNA methylation.

Prdm14

Key factor involved in specification of germ cells

Negative regulator of de novo methylases

Prdm14 connects germ cell development and DNA methylation erasure and may serve as a biosensor 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 Kräger
Laura Biggin

Wellcome Trust Sanger Institute
Sophie Messager
David Jackson
Pre-Congress Course Genetics:
Genome scanning to identify genes in PCOS and Early Menopause

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Senior Consultant OBGYN,
Professor Reproductive Medicine,
Div. Reproductive Medicine, Dept Obstetrics and Gynecology,
Erasmus Medical Center, Rotterdam,
University Medical Center Utrecht, The Netherlands

Disclosure
- 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
- 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)
**Genetic approaches in PCOS**

- 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
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- Isolated populations: Linkage or association, Transmission Disequilibrium Test (TDT)

**ESHRE / ASRM endorsed, PCOS consensus meeting 2003, Rotterdam, The Netherlands**

**PCOS is Complex Genetic Trait**

**Table 1: Phenotype groups: NIH / Rotterdam / AES**

<table>
<thead>
<tr>
<th>Features</th>
<th>NIH</th>
<th>Rotterdam</th>
<th>AES</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PCO+ OD+ HA</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>B</td>
<td>OD+ HA</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>C</td>
<td>CO+ OD</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>PCO+ HA</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

HA = Clinically or biochemically proven hyperandrogenemia, OD = Ovulatory dysfunction, PCO = Poly cystic ovaries

*Phenotype groups according to Azziz, 2006*

**Genetic Basis of PCOS**

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

**Table 1:** Distribution and prevalence of polymorphisms in woman.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Non affected</th>
<th>affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>8</td>
<td>11</td>
</tr>
</tbody>
</table>

Genetic approaches include: Chromosomal abnormalities, Linkage analysis, Association analysis.
**WHO II Anovulation & PCOS Phenotype**


- **No OCP**
  - PCO: 85%
  - Hirsutism: 44%
  - Androgens: ↑

- **OCP**
  - PCO: 93%
  - Hirsutism: 41%
  - Androgens: ↑

---

**Insulin Resistance & Metabolic Syndrome in PCOS**

(Mulders et al., RBM online, 2004)

- **BMI Groups**
  - <25
  - 25-30
  - >30

---

**Ethnic Differences between PCOS Patients**

(Valkenburg et al., JCEM, 2011)

- **Phenotype / Ethnic group**
- **Definition / Ethnic group**
Problems with GWAS in PCOS

- Populations stratification
  - Founding
  - Ethnic differences
  - Population differences
  - Genetic drift

- Phenotype problems
  - Definitions used
  - Treatment differences
  - Age related aspects

- Control Groups
  - Super controls
  - Normal controls
  - Population controls

- Reproducibility
  - Hits are not consistent
  - Number of hits is low
  - Ingenuity analyses are not very elusive

- Platform issues
  - Illumina® vs. Affymetrix®
  - Hardware and software problems
  - Quality control
  - Significance levels
  - Imputation
  - TIME !!!!

- Genetic issues
  - Differences between patients
  - Environment (obesity, MBS)
  - Genetic predisposition (t2DM, CVD)

Genetics of Population Variation: SNP’s
(Uitterlinden et al., Gene 2004)

GWAS PCOS
(Chen et al., Nature genetics, 2011)
GWAS PCOS
(Chen et al., Nature genetics, 2011)

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 until now)
- FSHR located downstream nearby
  - (replicated in the second Chinese GWAS)

At the 2p 21. locus
- ZFP36L2 and LOC100129726
- 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 can bind to endoplasmatic reticulum protein 1 (ERAP1)
- Elevated serum levels of ERAP1 have been associated with PCOS
  - (Replicated in three different studies)
### Meta Analysis GWAS data in PCOS patients from European descent

(Louwers et al., unpublished data)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13405728</td>
<td>LHCGR</td>
<td>0.92 (0.70-1.22)</td>
<td>0.58</td>
</tr>
<tr>
<td>rs12468394</td>
<td>THADA</td>
<td>0.86 (0.76-0.97)</td>
<td>0.02</td>
</tr>
<tr>
<td>rs13429458</td>
<td>THADA</td>
<td>0.86 (0.70-1.05)</td>
<td>0.15</td>
</tr>
<tr>
<td>rs12478601</td>
<td>THADA</td>
<td>0.88 (0.78-0.99)</td>
<td>0.04</td>
</tr>
<tr>
<td>rs10818854</td>
<td>DENND1A</td>
<td>1.15 (0.87-1.52)</td>
<td>0.32</td>
</tr>
<tr>
<td>rs2479106</td>
<td>DENND1A</td>
<td>0.97 (0.85-1.11)</td>
<td>0.68</td>
</tr>
<tr>
<td>rs10986105</td>
<td>DENND1A</td>
<td>1.45 (1.08-1.94)</td>
<td>0.01</td>
</tr>
<tr>
<td>rs2268362</td>
<td>FSHR</td>
<td>0.87 (0.77-1.00)</td>
<td>0.05</td>
</tr>
<tr>
<td>rs2349415</td>
<td>FSHR</td>
<td>0.94 (0.77-1.15)</td>
<td>0.05</td>
</tr>
<tr>
<td>rs4385527</td>
<td>c9orf3</td>
<td>0.84 (0.77-0.99)</td>
<td>0.04</td>
</tr>
<tr>
<td>rs3802457</td>
<td>c9orf3</td>
<td>0.77 (0.45-1.81)</td>
<td>0.77</td>
</tr>
<tr>
<td>rs1894116</td>
<td>YAP1</td>
<td>1.27 (1.13-1.67)</td>
<td>0.04</td>
</tr>
<tr>
<td>rs705702</td>
<td>RAB5B, SUOX</td>
<td>1.27 (1.06-1.48)</td>
<td>0.02</td>
</tr>
<tr>
<td>rs2272046</td>
<td>HMGA2</td>
<td>0.70 (0.83-1.71)</td>
<td>0.36</td>
</tr>
<tr>
<td>rs4784165</td>
<td>TOX3</td>
<td>1.15 (0.95-1.25)</td>
<td>0.27</td>
</tr>
<tr>
<td>rs2059807</td>
<td>INSR</td>
<td>1.14 (0.93-1.05)</td>
<td>0.27</td>
</tr>
<tr>
<td>rs6022786</td>
<td>SUMO1P1</td>
<td>1.13 (0.92-1.21)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

### Second GWAS in Chinese PCOS patients

(Shi et al. Nature Genetics 2012)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs13405728</td>
<td>G</td>
<td>0.71 (7.55x10^-21)</td>
<td>0.58</td>
</tr>
<tr>
<td>rs12468394</td>
<td>A</td>
<td>0.72 (1.59x10^-20)</td>
<td>0.02</td>
</tr>
<tr>
<td>rs13429458</td>
<td>C</td>
<td>0.67 (1.73x10^-23)</td>
<td>0.15</td>
</tr>
<tr>
<td>rs12478601</td>
<td>T</td>
<td>0.72 (3.48x10^-23)</td>
<td>0.04</td>
</tr>
<tr>
<td>rs10818854</td>
<td>A</td>
<td>1.51 (9.40x10^-18)</td>
<td>0.32</td>
</tr>
<tr>
<td>rs2479106</td>
<td>G</td>
<td>1.34 (8.12x10^-19)</td>
<td>0.68</td>
</tr>
<tr>
<td>rs10986105</td>
<td>C</td>
<td>1.47 (6.90x10^-15)</td>
<td>0.01</td>
</tr>
<tr>
<td>rs2268362</td>
<td>T</td>
<td>0.87 (9.89x10^-13)</td>
<td>0.83</td>
</tr>
<tr>
<td>rs2349415</td>
<td>T</td>
<td>1.19 (2.35x10^-12)</td>
<td>0.05</td>
</tr>
<tr>
<td>rs4385527</td>
<td>A</td>
<td>0.84 (5.87x10^-09)</td>
<td>0.04</td>
</tr>
<tr>
<td>rs3802457</td>
<td>A</td>
<td>0.77 (5.28x10^-14)</td>
<td>0.77</td>
</tr>
<tr>
<td>rs1894116</td>
<td>G</td>
<td>1.27 (1.08x10^-22)</td>
<td>0.01</td>
</tr>
<tr>
<td>rs705702</td>
<td>G</td>
<td>1.27 (8.64x10^-26)</td>
<td>0.02</td>
</tr>
<tr>
<td>rs2272046</td>
<td>C</td>
<td>0.70 (1.95x10^-21)</td>
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<td>A</td>
<td>1.13 (1.83x10^-09)</td>
<td>0.38</td>
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</table>

### Replication second GWAS in PCOS patients from European descent

(Louwers et al., unpublished data)
FSHR Polymorphisms and FSH and LH levels
(Laven et al., Fertil Steril 2003; Valkenburg et al., Human Reprod, 2009)

FSHR Polymorphisms and of PCOS
(Mutharasan et al., JCEM 2013)

Northern European population
Chinese population
rs2268361
**Conclusions**

- 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)
Genetic Variation and Age of Menopause

(He et al., Nature Genetics, 2011)

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

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.

SYCP2L is required for protein synthesis in the synaptonemal complex which zips together homologue chromosomes 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.

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, NEJM 2009)
The trichothiodystrophy (TTD) phenotype includes:
- prematurely 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.
HRT and CVD risk in Postmenopausal women
(Sanchez et al. Cochrane Reviews, 2005)

- No protective effect of HRT was seen for any of the cardiovascular outcomes assessed: all cause mortality, cardiovascular death, non-fatal MI, venous thromboembolism or stroke.
- Higher risks of various 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 1.85) 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)

Pre-eclampsia & Ovarian Function
(Woldringh et al, Human Reprod, 2006)

Pre-eclampsia & Ovarian Function
(Woldringh et al, Human Reprod, 2006)
## Female Fertility and Longevity


<table>
<thead>
<tr>
<th>Table 1</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Helle et al. (2006)</td>
<td>Determined sex-ratio at birth, fertilization, and survival to calculate maternal age at birth.</td>
</tr>
<tr>
<td>Smith et al. (2005)</td>
<td>Estimated sex-ratio at birth, fertilization, and survival to calculate maternal age at birth.</td>
</tr>
</tbody>
</table>

**Best Predictor for Longevity is Age at last Reproductive Event!!!!**
Possible Confounders:
- Lactation
- Different lifestyle, less sedentary, obesity
- Contraception
- Weight gain or loss during pregnancy and lactation
- # of Children and sex of children
- Longer E2 exposure
- More Breast, Endometrial and Ovarian Cancer
- More Colon Cancer

Conclusions
- PCOS is a heterogeneous phenotype indicating a complex genetic background which 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 hyperandrogenism
- 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
Acknowledgements

Netherlands
- Evert van Santbrink
- Jite Schipper
- Olivier Valkenburg
- Sharon de Koning
- Yvonne Comans
- Wendy van Daalen
- Sani Schouten-Molema
- Andre Jelkens-Vosmaer
- Frank de Jong
- Anneke Verhagen
- Jenny van der Steeg
- Wilma van der Leer
- Ralph Cooijmans

International
- ENGAGE
- DeCode
- Dutch PCOS Consortium
- Stephen Franks
- Sandro Granelli
- Paul Garcia
- James Allen
- C.H. Melampy
- Uzi Szulman
- Carine Tack
- Bill Tunney
Learning Objectives

- Epigenetic genome reprogramming in the female germ line.
- Imprinted genes as a model to study epigenetic effects of different ART.
- 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.
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. 
  

State of the ART: IVF/ICSI of in vivo 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. 


  Denomme and Mann, Reproduction 144, 393-409, 2012

  El Hajj et al., Epigenetics 6, 1176-1188, 2011


Methylation patterns of IGF 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 unmethylated CpGs and filled circles methylated CpGs. Abnormally methylated alleles are indicated by an *.
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.

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 large antral follicles,
- in vitro grown (for 10-12 days) GV oocytes isolated from fresh preantral follicles.
- in vitro grown (for 10-12 days) GV oocytes isolated from vitrified 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
- DNA contamination
  - 1:1
  - 5:1
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).

In vitro grown (10 days) oocytes from vitrified preantral follicles

Snrpn DMR1

Negative controls

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.

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).
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 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.
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.

Endocrine disruptors are synthetic chemicals that resemble natural hormones. Exposure of Agouti (4/4) 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. Guida 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)
References


Genetic factors for male infertility

Pr. STéphane Viville
viville@igbmc.fr

Disclosing slide

I declare that I have no potential conflict of interest

Spermatogenesis, where can it go 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 dygenesis

• Gonadotrope axe (hormons and receptors)
  ex: Kallmann syndrom (RX, RA, DA)

• Gametogenesis
  ex: Y microdeletion

• Organs malformations
  ex: cystic fibrosis (CBAVD and CFTR)

• Sexual behaviour

McLachlan Rl and O’Ryan MK. J. Clin. Endo. Metab. 2010

Contents of lecture

• What is known:
  ➢ Chromosomal anomalies
    • Numerical
    • Translocations/chromosomal rearrangements
    • Yq microdeletions
Chromosomal anomalies

**Numerical**

XXY Klinefelter's syndrome (KS)

XXY
XX male
XY female

Chromosomal anomalies

**Translocations/chromosomal rearrangements**

Robertsonian translocations

46 chro. 45 chro.

Translocations/chromosomal rearrangements

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
Meiosis abnormalities
Chromosome rearrangements

- Schiasma and segregation perturbations
- Higher frequency in cases of oligozoospermia or dysovulation
- Higher frequency in case of spontaneous abortions

Chromosomal abnormalities

Yq microdeletions
Prevalence ~ 7%

- AZFa → SCOS
- AZFb → Germ cell arrest
- AZFc → oligo-azoospermia (92% sperm recovery by TESE)

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


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 spermatozoa
  - Very low to non pregnancy rate


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Globozoospermia: DPY19L2

67% of the patients are mutated for DPY19L2

Elkadi et al, HMG 2012

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**Clinical studies**

<table>
<thead>
<tr>
<th>Sperm deficiency</th>
<th>Before AOA</th>
<th>After AOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total globozoospermia (n = 9)</td>
<td>0 (4/9, 5 cycles)</td>
<td>0 (17/225, 19 cycles)</td>
</tr>
<tr>
<td>Partial globozoospermia (n = 1)</td>
<td>0 (1/2, 3 cycles)</td>
<td>0 (3/3, 2 cycles)</td>
</tr>
</tbody>
</table>

AOA: assisted oocyte activation

Adapted from Holotycka et al. 2008

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

**DPY19L2**<sup>mut</sup> and AOA+ (n = 15) vs DPY19L2<sup>mut</sup> and AOA- (n = 14)

<table>
<thead>
<tr>
<th>DPY19L2&lt;sup&gt;mut&lt;/sup&gt; patients</th>
<th>Conventional ICSI</th>
<th>ICSI + AOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilization rate (%; 2pn/MII)</td>
<td>33.3 % (107/324)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.4 % (222/334)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>hCG rate per ET</td>
<td>15.8 % (6/38)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.6 % (13/31)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ongoing pregnancy rate per ET</td>
<td>15.8 % (6/38)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.3 % (10/31)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Live birth rate per ET</td>
<td>13.8 % (6/38)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.3 % (10/31)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>p < 0.001;  <sup>b</sup>p < 0.05;  <sup>c</sup>p < 0.107, NS;  <sup>d</sup>p < 0.056, NS

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

Kuentz et al. II 2013

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**Macrozoospermia**

- AURORA C gene mutation, founder effect
- AURORA C implicated in the meiotic fusion formation
- Phenotype: macrozoospermia, with tetraploid content of DNA, multiple flagella
- Impossibility to offer ART

Dirickx 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 azoosperma, ended with SCO

Dnmt3L WT 30dpp Dnmt3L KO 5months old

Stage IV: apoptosis at pachytene
After stage IV: no germ cells

Bourc'his D. and Bestor TH Nature 2004

Contents of lecture

• What the future:
  ➢ si/mi/piRNA
    ✓ Small RNA of 18 to 30 nucleotides
    ✓ Involved 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 rearrangements 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.

Conclusions

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

Except that……….
The solution

Defects in several steps have been correlated with sperm genome dysfunction

Transition Proteins

Infertility

Embryonic Lethality

Courtesy of Rod Balhorn

Lower DFI in successful preg’s

Simon et al, Fert Steril, 2012

SRD/MND based Microarray Analysis

S1

S2

S3

S4

Scan and feature extract

Simulate tissue / Extract RNA

BamH1/EcoR1

S (0.65M NaCl) or direct MNase digestion. Residue ≥ 1
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.
Lane 6: MNase insoluble (MNDI) DNA.

Arpanahi et al., 2009

ChIP-chip based Microarray Analysis

Arpanahi et al., 2009

Differential composition of human sperm chromatin.

Arpanahi et al., 2009
Differential composition of mouse sperm chromatin.

Modified histones in HOXD locus

Deregulated histone deposition in HOXD locus of infertile men
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)

Saida et al, 2011

Saida et al, 2011

Saida et al, 2011
Spatial organization of repetitive DNA sequences in the bovine sperm nucleus

powell et al. JCS 1990

Alu - µsat - rDNA

CENP-A (Palmer et al., 1990)

Salt wash → Endonuclease digestion

Non-random chromosome positioning

Mudrak et al., 2005

Foster et al., 2005
Ab-FITC

DNA-DAPI

Modified histones in bovine sperm (H3K27.Me3)

Merge

Soluble DNA

Insoluble DNA

DNA-DAPI Merge

Transition from histone to protamine packaged chromatin in fly

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

Histones in decondensed Drosophila melanogaster sperm

ChIP-FISH: Fly sperm nucleosomal chromatin is organised in distinctive regions
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?
Nucleosomes including modified histones

Nuclear envelope

Acrosomal cap

Nucleoprotamine

H4K12Ac in gametes and embryos

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 interspecific hybrids between closely related species.
Learning objectives of the course

* DNA methylation in the pre-implantation embryo
* Genomic imprinting and its somatic maintenance in the early embryo
* Perturbation 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)

Imprinted genes influence development, nutrient transfer and behaviour

Placental development and function
Foetal growth control
Postnatal fitness
Postnatal behaviour
Two imprinted domains involved in fetal growth

ICR ICR ICR ICR ICR ICR
CDKN1C IGF2 H19

Maternal Paternal

ICR = 'Imprinting Control Region'

= DNA methylation

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

ICR = DNA methylation

ncRNA = non-coding RNA

KCNQ1 domain

Imprinting Control Regions (ICRs)

CH3 CH3 CH3 CH3 CH3 CH3

Parental allele WITH DNA methylation

Parental allele WITHOUT DNA methylation

Remi Terranova et al. Dev Cell 2008
David Monk et al. PNAS USA 2006
David Umlauf et al. Nature Genet. 2004
ICRs: Somatic maintenance of allelic DNA methylation throughout development

Parental allele WITH DNA methylation

Parental allele WITHOUT DNA methylation

For further discussion, please see:
Kelsey and Feil, 2013

DNA-methylation

protection against methylation

HP1α
H4R3me2s
H4K20me3
H3K9me3
specific proteins

CH3 CH3 CH3

H3K4me2/3

CH3 CH3 CH3 CH3

DNMT3A and DNMT3L

Katia Delaval & Feil 2004
Curr Opin Genet Dev
Satya Kota & Feil,
Dev Cell 2010

SOMATIC MAINTENANCE: DNMT1

Parental allele WITH DNA methylation

Parental allele WITHOUT DNA methylation

DNMT1

H4R3me2s
H4K20me3
H3K9me3
specific proteins

DNA-methylation

protection against methylation

HP1α

CH3 CH3 CH3

DNMT3A and DNMT3L

Katia Delaval & Feil 2004
Curr Opin Genet Dev
Satya Kota & Feil,
Dev Cell 2010
H4R3me2s marks the methylated allele of ICRs

Michael Girardot et al., in review.

Pre-implantation epigenetic maintenance and disease?

Examples of genomic imprinting
Intrinsic and environmental perturbation of DNA methylation patterns

Silver-Russell Syndrome (SRS)

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

Silver-Russell Syndrome (40% of cases)
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 & Pseudohyopoparathyroidism-1B.

- Mackay DJ et al. 2008. *Nature Genetics*
- Azzi S et al. 2010. *Epigenetics*
- Court F et al. 2013. *Hum. Mutation*
Frequent perturbation of imprints \textit{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  
  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

• \textit{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 \textit{in vitro} embryo culture in sheep: Aberrant \textit{IGF2R} imprinting, but unaltered \textit{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

MINOR nutritional effects on imprinted DNA methylation

- Dutch Hunger Winter, periconceptional exposure to famine:
  - Decreased DNA methylation at imprinted genes in children.

- Increased folate/alterd 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
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?
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

<table>
<thead>
<tr>
<th>40 week term baby</th>
<th>50cm</th>
<th>3,300g</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 week fetus</td>
<td>38cm</td>
<td>1,000g</td>
</tr>
<tr>
<td>16 week fetus</td>
<td>15cm</td>
<td>250g</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Normal</th>
<th>Syncytiotrophoblast</th>
<th>Amnion</th>
<th>Endoderm</th>
<th>Gastrointestinal</th>
<th>Neural plate</th>
<th>Neural tube</th>
<th>Ovarian teratoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mother’s genes only</td>
<td>Father’s genes only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hydatidiform Mole: Father’s genes only

Trophoblast: (parthenogenosis)
## Uniparental disomies

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>MatUPD7/hypometh 11</th>
<th>Beckwith-Wiedemann Syndrome PatUPD11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver-Russell Syndrome</td>
<td>FGR</td>
<td>OVERGROWTH</td>
</tr>
<tr>
<td>1 in 7,000</td>
<td>1 in 15,000</td>
<td></td>
</tr>
</tbody>
</table>

**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?
### 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc22a2 (17 A1)</td>
<td>M</td>
<td>cation transport</td>
</tr>
<tr>
<td>Slc22a3 (17 A1)</td>
<td>M</td>
<td>cation transport</td>
</tr>
<tr>
<td>Grb10 (11 A1)</td>
<td>P/M</td>
<td>GR/IUGR</td>
</tr>
<tr>
<td>Sgce (6 A1)</td>
<td>P</td>
<td>myoclonic</td>
</tr>
<tr>
<td>Peg10 (6 A1)</td>
<td>P</td>
<td>GR/embryonic lethal</td>
</tr>
<tr>
<td>Ppp1r9a (6 A1)</td>
<td>M</td>
<td>synapsis formation in neural tissue</td>
</tr>
<tr>
<td>Mest (6 A3)</td>
<td>P</td>
<td>GR/cardiac defects/abnormal maternal behaviour</td>
</tr>
<tr>
<td>Klf14 (6 A3)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Sfmbt2 (2 A1)</td>
<td>P</td>
<td>NO DATA</td>
</tr>
<tr>
<td>H19 (7 F5)</td>
<td>M</td>
<td>GR/lethal</td>
</tr>
<tr>
<td>Igf2 (7 F5)</td>
<td>P</td>
<td>GR</td>
</tr>
<tr>
<td>Igf2as (7 F5)</td>
<td>P</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Ins2 (7 F5)</td>
<td>P</td>
<td>GR</td>
</tr>
<tr>
<td>Ascl2 (7 F5)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Tssc4 (7 F5)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Kcnq1 (7 F5)</td>
<td>M</td>
<td>GR/deafness/behaviour</td>
</tr>
<tr>
<td>Kcnq1ot1 (7 F5)</td>
<td>P</td>
<td>GR/IUGR</td>
</tr>
<tr>
<td>Cdkn1c (7 F5)</td>
<td>M</td>
<td>GR/developmental defects</td>
</tr>
<tr>
<td>Slc22a18 (7 F5)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Phlda2 (7 F5)</td>
<td>M</td>
<td>GR</td>
</tr>
<tr>
<td>Nap1l4 (7 F5)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Osbpl5 (7 F5)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Ampd3 (7 E3)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Dhcr7 (7 F5)</td>
<td>M</td>
<td>multiple problems at birth</td>
</tr>
<tr>
<td>Slc38a4 (15 F1)</td>
<td>P</td>
<td>GR</td>
</tr>
<tr>
<td>Dlk1 (12 F1)</td>
<td>P</td>
<td>GR/skeletal abnormalities</td>
</tr>
<tr>
<td>Dlk1 downstream transcripts (12 F1)</td>
<td>P</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Meg3 (12 F1)</td>
<td>M</td>
<td>GR</td>
</tr>
<tr>
<td>Rian (12 F1)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Mirg (12 F1)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Dio3 (12 F1)</td>
<td>P</td>
<td>GR/severe</td>
</tr>
<tr>
<td>Magel2 (7c-5b)</td>
<td>P</td>
<td>GR</td>
</tr>
<tr>
<td>Gatm (2 E5)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Peg3 (7a2-b1)</td>
<td>P</td>
<td>GR/nervous system</td>
</tr>
<tr>
<td>H13 (2 H1)</td>
<td>M</td>
<td>NO DATA</td>
</tr>
<tr>
<td>Gnas (2 E1-H3)</td>
<td>M</td>
<td>GR</td>
</tr>
</tbody>
</table>

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

### Imprinting in Humans

- Maternal Homozygote
- Paternal expression
- Monoallelic
- Biallelic expression
- RNA extraction
- DNA
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**

- Placenta
- Fetal Brain
- Adult bone marrow
- Adult blood (PBL)
- Fetal Liver

**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

**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

Imprinting Status of \textit{PHLDA2}

- Maintained in placenta tissues irrespective of birth weight, (n= 41).
- The increased expression of \textit{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 \textit{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

\begin{tabular}{cccc}
\textbf{Ph}\textbf{e}\textbf{n} & \textbf{M} & \textbf{e}\textbf{n} & \textbf{e}\textbf{n} \\
\textbf{P} & \textbf{M} & \textbf{P} & \textbf{M} \\
\end{tabular}

Normal & Normal & Deletion effect & Deletion effect
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**

![Graph showing luciferase assay results](image)
**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

---

*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

---

**Sequencing analysis of CNV2/CNV1**
### Meta-analysis for PHLDA2 deletion effect on birth weight

<table>
<thead>
<tr>
<th>Study</th>
<th>Effect estimate (g) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moore UCL – FGS</td>
<td>122 (-43, 286)</td>
</tr>
<tr>
<td>ALSPAC</td>
<td>68 (-196, 332)</td>
</tr>
<tr>
<td>Overall</td>
<td>88 (6, 170)</td>
</tr>
</tbody>
</table>

*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
No molecular anomalies identified (48%)

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

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 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

H19 methylation

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

- **Hypomethylated**
- **Normal methylation level**

**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.

Acknowledgements

Post-docs
Sayeda Abu-Amero
Jenny Frost ICH/KCL
Will Paizy KCL/IHC
Anna Thomas
Andy Duncan
Sophia Apostolidou (UCL)
Mihu Ifsida
Caroline Delemarre
Ellie and Summer Students
Jaehan Chan, David Bell
Chris Hodgkinson
Ben Stanier/Mark Gabriel
Charalampos Demetriou

Collaborators
Philip Stanier, ICH
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Robert Feli, Montpellier
Dave Monk, Barcelona
Jerome Cavaille, Toulouse
John Todd, Cambridge
Chris Wallace, Cambridge

ALSPAC
Marcus Pembrey
Sue Ring
Karen Northstone

Institute of Child Health

References

You can now register for these upcoming ESHRE Campus events:

- Application and challenges of emerging technologies in preimplantation and prenatal diagnosis
  12-13 September 2013 - Prague, Czech Republic

- Female genital tract congenital malformations: new insights in an old problem
  27-28 September 2013 - Thessaloniki, Greece

- Introducing new techniques into the lab
  4-5 October 2013 - Barcelona, Spain

- Polycystic ovary syndrome: A new look at an old subject
  25-26 October 2013 - Rome, Italy

- Infections from conception to birth: role of ART
  7-8 November 2013 - Berlin, Germany

- Endoscopy in reproductive medicine
  20-22 November 2013 - Leuven, Belgium

- From early implantation to later in life
  28-29 November 2013 - Brussels, Belgium

Mark your calendar for:

- Premature ovarian insufficiency
  6-7 December 2013 - Utrecht, The Netherlands

www.eshre.eu
(see “Calendar”)

Contact us at info@eshre.eu