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From genes to gestation

Special Interest Groups Early Pregnancy
and Reproductive Genetics

3

3 July 2011
Stockholm, Sweden

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Organised by
Special Interest Groups Early Pregnancy and Reproductive Genetics

Contents

Course coordinators

Ole B. Christiansen (Denmark, SIG Early Pregnancy) and Stephane Viville (France, SIG Reproductive Genetics)

Course description

The course is basic to advanced.
The course will give an overview of which genes are known or believed to influence fertilization, embryo implantation and early embryo development before and after implantation. Potential pathophysiologic pathways linking genetics and abnormal fertilization, implantation and embryo development will be discussed. Consequences of the current knowledge in the management of infertility, implantation failure after ART and recurrent miscarriage will be reviewed

Target audience

Reproductive physicians, embryologists and basic scientists

Scientific programme

Genetics of embryo fertilization and implantation

09.00 - 09.30	Preparing embryonic development in male gametes – **Bradley Cairns (USA)**
09.30 - 09.45	Discussion
09.45 - 10.15	What do we know about genes affecting embryo implantation? – **Nick Macklon (United Kingdom)**
10.15 - 10.30	Discussion
10.30 - 11.00	Coffee break

Epigenetics and ART

11.00 - 11.30	What is epigenetics and how can it affect embryo development? **- Jorn Walter (Germany)**
11.30 - 11.45	Discussion
11.45 - 12.15	Small RNAs and control of retrotransposons during gametogenesis and early development - **Martin Matzuk (USA)**
12.15 - 12.30	Discussion
12.30 - 13.30	Lunch

Genetics and pregnancy

13.30 - 14.00	Chorionic gonadotropin beta-gene variants as a risk factor for recurrent miscarriages - **Maris Laan (Estonia)**
14.00 - 14.30	Genomic changes detected by array CGH in human embryos with developmental defects – **Evica Rajcan-Separovic (Canada)**
14.00 - 14.15	Discussion
14.15 - 14.45	Non invasive prenatal diagnosis using cell-free nucleic acids - **Diana Bianchi (USA)**
14.45 - 15.00	Discussion
15.00 - 15.30	Coffee break
15.30 - 16.00	Genetics of molar pregnancies – **Rosemary Fisher (United Kingdom)**
16.00 - 16.15	Discussion

Treatment of genetic abnormalities affecting reproduction

16.15 - 16.45	Gene therapy for the fetus: how far have we come? – **Donald Peebles (UK)**
16.45 - 17.00	Discussion

Contact

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Preparing Embryonic Development in Male Gametes'

Stockholm 2011

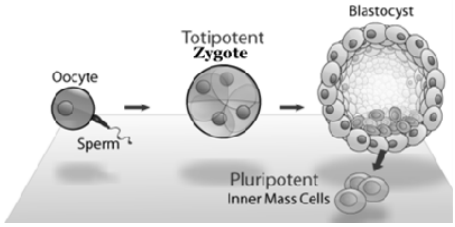
Brad Cairns PhD

Department of Oncological Sciences & HHMI
Huntsman Cancer Institute
University of Utah

Learning Objectives

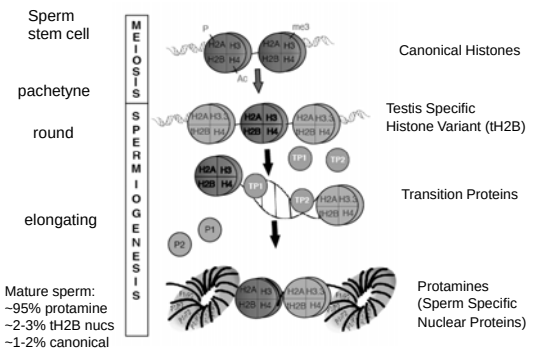
1. Understand the protein packaging, histone modifications, and DNA methylation patterns residing on the inherited sperm genome.
2. Discuss the implications of the packaging/modifications and DNA methylation for gene and locus poising – that the sperm genome poises the genes that drive the embryonic development program - and discuss how this might be the mechanism for transgenerational inheritance.
3. Recognize the conserved features of the sperm epigenome that are present in zebrafish – an experimental model where the implications of the work in human germ cells can be tested.
4. Discuss the major role that genomics will play in our understanding of genomes.

How is germ cell chromatin structured, and is it solely for the germ cell, or does it influence embryo totipotency or development?

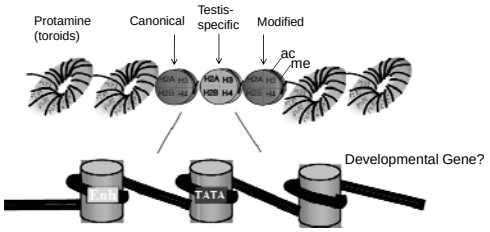


Are genes important for guiding embryo development poised by distinctive chromatin/DNAme in mature germ cells (sperm and eggs)?

Chromatin Reorganization During Spermiogenesis

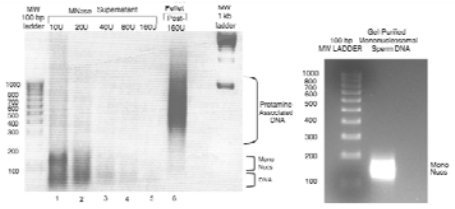


Where are the nucleosomes located: canonical, testis-specific, and covalently-modified?



The mature human sperm genome contains
~95% protamine and ~5% histone

Localizing nucleosomes and histone modifications in the sperm genome



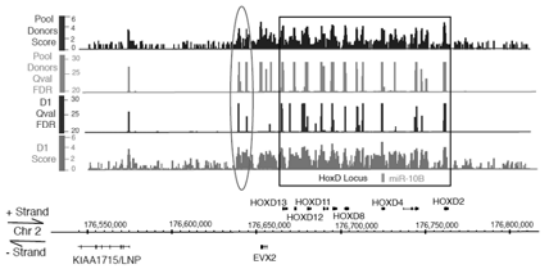
Samples : pooled from four men of known fertility, mature and purified

1- Sequence the mononucleosomal DNA (20 mil. reads): whole genome

2- Perform Chromatin IP from mononucleosomes, sequence (~10 mil.)

Hammoud et al., *Nature* 2009

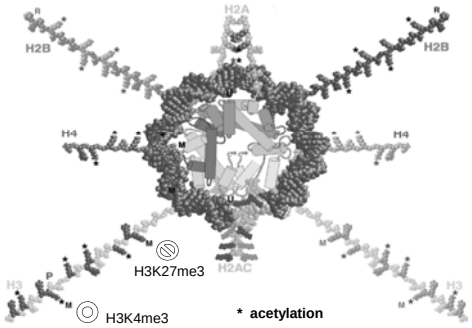
Nucleosomes are enriched at HOX loci in sperm



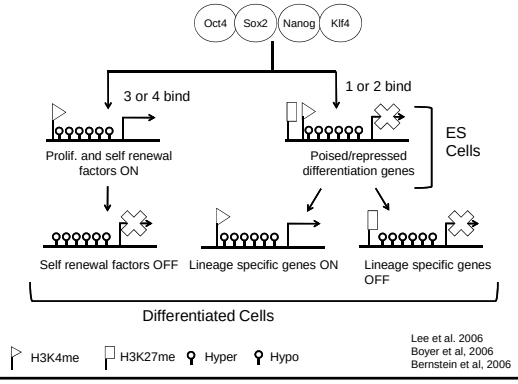
1) A nucleosome/protamine mixture exists at nucleosome-enriched regions.
2) Canonical H2B at developmental promoters, not tH2B

Hammoud et al., *Nature* 2009

Histone 'tails' are present on the amino termini of every histone, emanate every 20 bp from the nuc, and receive multiple modifications

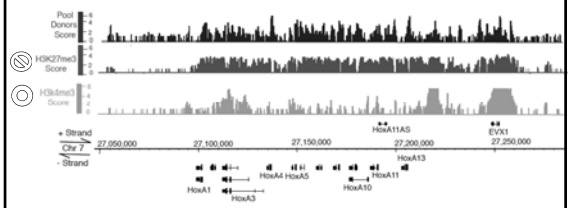


Txn factors and K4/K27 'bivalency' regulate pluripotency



Lee et al. 2006
Boyer et al, 2006
Bernstein et al, 2006

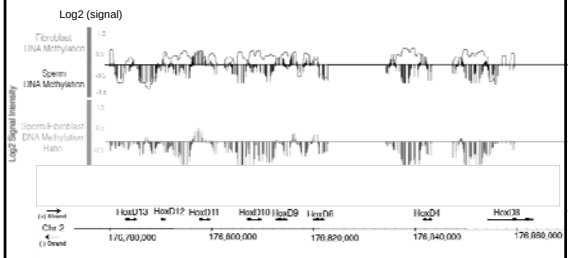
Nucleosomes and K4/K27 bivalency at the HoxA locus



H3K4me3 and H3K27me3 are often observed in large blocks in sperm
Rather than being restricted to the 5' ends of genes, which is typical in diff cells

Hammoud et al., *Nature* 2009

Developmental promoters are DNA hypomethylated in sperm, but acquire methylation following differentiation



Also, germline-specific genes become methylated.

Hammoud et al., *Nature* 2009

Factors important for embryogenesis are DNA demethylated in sperm

Performed MeDIP, analyzed with extended promoter arrays.

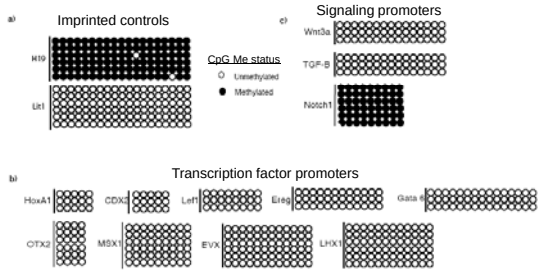
GO-STAT Analysis

- *Embryonic Development*
- *Transcription factors / TF regulators*
- *System development*
- *Anatomical Structural development*
- *Development Process*
- *Nervous System Development*
- *Pronucleus formation*
- *CNS development*
- Brain development
- Gastrulation

All: FDR <0.01

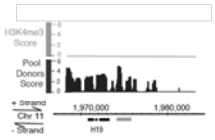
Consistent with work from W. Reik and T. Bestor labs on DNAme pattern in mice

The vast majority of developmental promoters lack DNA methylation in sperm

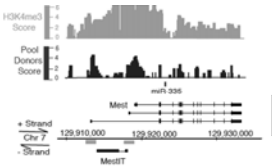


Hammoud et al., *Nature* 2009

Paternally DNA methylated loci lack H3K4me3
Paternally DNA demethylated loci bear H3K4me3



Paternally DNA methylated



Paternally DNA demethylated

H3K9me present on DNA methylated imprinted genes.
Raises the possibility that histone modifications may deter or encourage DNAme at imprinted genes, for establishing or maintaining DNAme patterns

Genes for embyro development are packaged with 'regional multivalent' chromatin in human sperm

The promoters of embryonic developmental transcription factors in sperm are enriched in nucleosomes with:

1. Positive histone modifications (H3K4me2/3)
2. A silencing histone modification (H3K27me3)
3. Profound DNA hypomethylation throughout the promoter.
4. Imprinted genes: DNAme have H3K9, hypometh have H3K4.

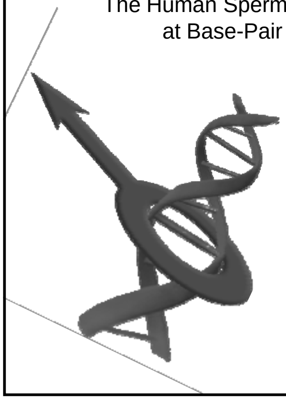
Hammoud et al. *Nature* 2009

Positive marks may promote open, transcriptionally-competent chromatin and deter DNA methylation.

Silencing mark may keep genes for embryo development off in the germline.

(related work in mice from A. Peters Lab, Brykczynska et al., NSMB, 2010)

The Human Sperm DNA Methylome at Base-Pair Resolution



Sue Hammoud
David Nix (HCI Bioinformatics)
Doug Carrell,
Dept. of Surgery, Phys. & Ob/Gyn
Utah Andrology and IVF Clinic

DNA Methylation Analysis by Whole-Genome Shotgun Bisulphite Sequencing of Two Fertile Donors

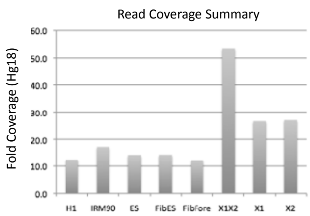
	ESCell H1	Fibroblast IRM90	ESCell WA09	Fibroblast from WA09	Fibroblast from Neonatal Foreskin	Sperm X1X2	Sperm X1	Sperm X2
	H1	IRM90	ES	FibES	FibFore	X1X2	X1	X2
Alignments	589,934,603	734,098,118	877,923,955	865,035,987	855,743,197	1,544,540,320	724,265,294	820,275,026
Alignments passing filters	541,534,702	673,526,855	764,619,389	753,681,573	713,323,669	1,329,101,478	654,425,981	674,675,497
Aligned Q30 bps passing filters	28,418,395,697	39,121,044,683	32,384,271,937	32,527,770,107	27,958,042,793	122,156,821,036	60,612,041,178	61,544,779,858
Coverage for Hg18 Sequence, no gaps, assuming 80% mapping	12.4	17.1	14.2	14.2	12.2	53.4	26.5	26.9

~1.54 billion 101-mer sequencing reactions

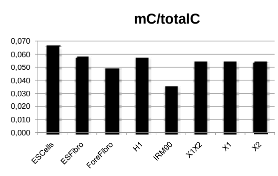
~1.32 billion mapped, including repeat elements. Conversion rates >99%

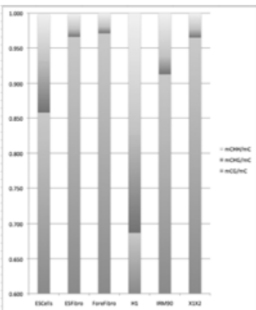
122 Billion base pairs of mapped filtered reads, giving ~53.4-fold genome coverage

Two donors nearly identical r^2 value >0.9.

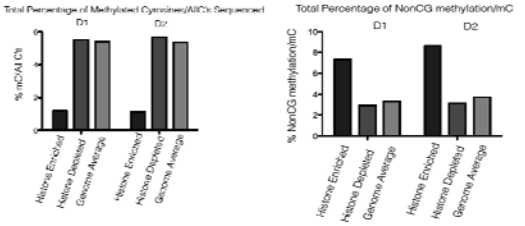


Bulk Cytosine Methylation: Sperm resemble differentiated somatic cells more than ES cells

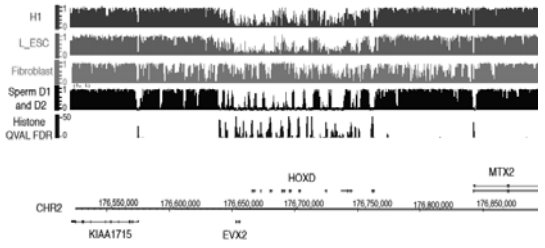




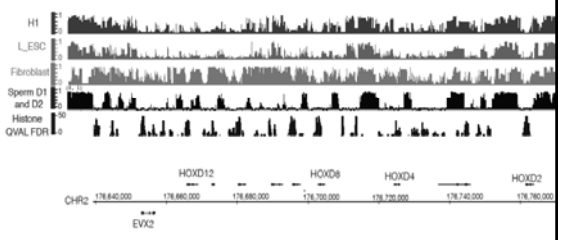
Histone–Enriched Loci Show Striking Reductions in Total Cytosine Methylation, Though are Higher in Relative non-CG Methylation



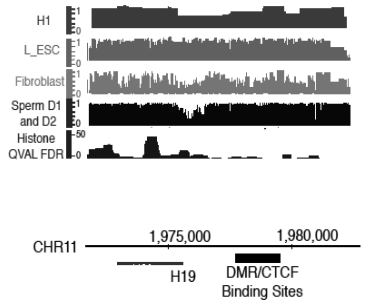
Loci with nucleosomes lack DNA methylation



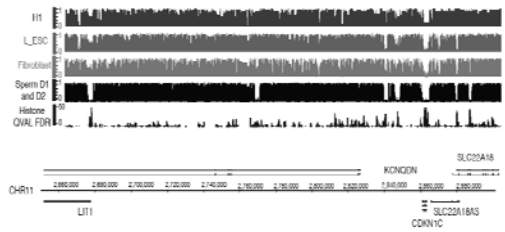
DNA Methylation Within the HOX Gene Clusters: Unmethylated Promoters, Methylated 3' UTRs/IG



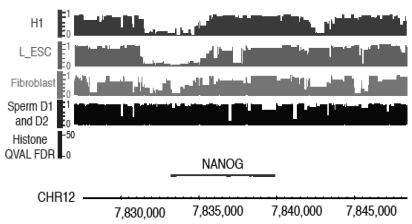
Methylation At Imprinted Genes



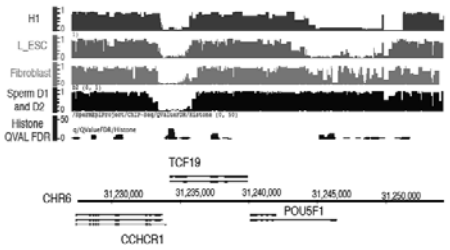
DNA methylation at Paternally-Expressed Imprinted Genes



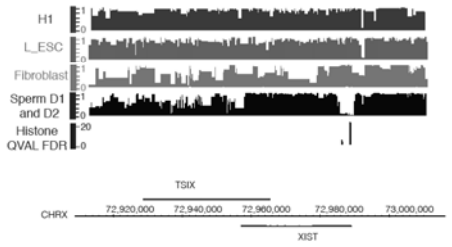
NANOG is Methylated in Sperm



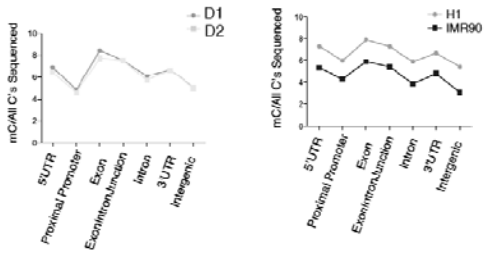
OCT4 is Methylated in Sperm



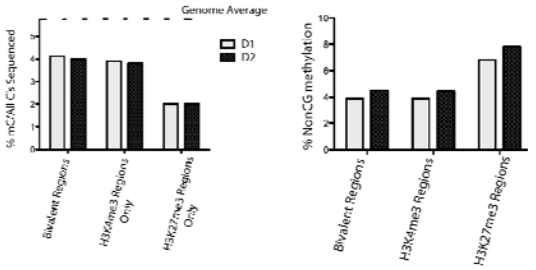
Possible Mechanism for Paternal X Imprinting in Humans



Distribution of Methylation Over Gene Bodies

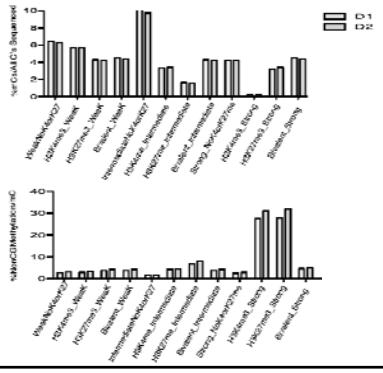


Methylation is reduced at regions bearing H3K4me3 or especially H3K27me3



This data considers the whole genome – next, CpG islands

Pronounced DNA demethylation at CpG Islands requires both 'strong' classification and H3K4me



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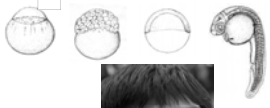
4. Imprinted genes: DNAme have H3K9, hypometh have H3K4.

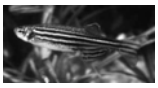
Hammoud et al. *Nature* 2009

Need: Developmental/Experimental model to understand these patterns.
What is the true role of these modifications: germline, embryo, or both?
What is the modification pattern of develpmental TFs in the egg?
Are these or other modifications/variants retained and truly instructive?

Part II: The Zebrafish model system

Question: Are genes important for embryo development packaged in a special manner in sperm?





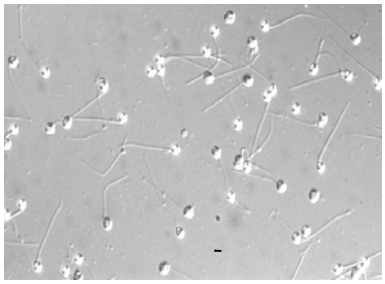
ZFIN

Shan-Fu Wu

Why Zebrafish?
1) easy access to germ cells (sperm and egg) and staged early embryos
2) manipulation of embryos available to address hypotheses
3) However – no known imprinting – no placenta, no Dnmt3L

Data analyzed on custom Agilent arrays, -9 to +2kb of most promoters.

Zebrafish sperm: ~10 uM 'round' spermatids at maturation

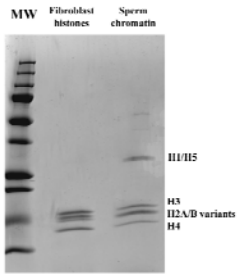


Scale bar: 10 uM

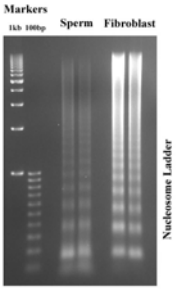
Wu et al., *Genome Research*, in press

Zebrafish sperm chromatin involves nucleosomes and H1/5 linker histones, not protamine

Chromatin composition



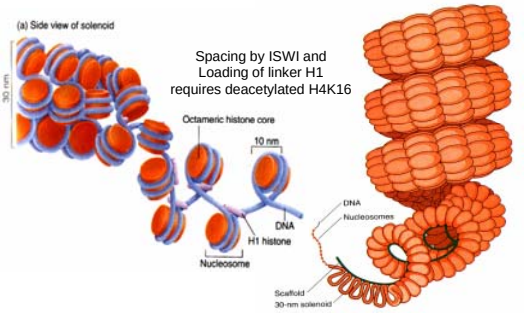
Nucleosome pattern



DNA compaction by Chromatin

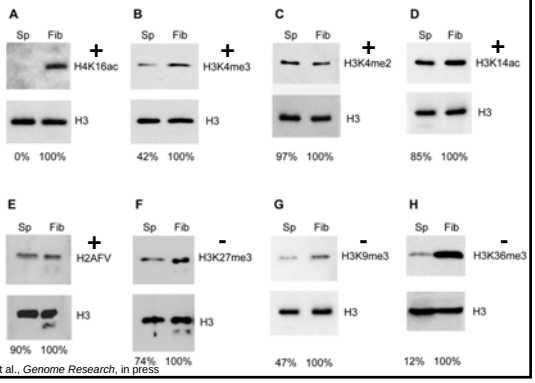
Radial Solenoid Model of 30nm Chromatin Fiber.

Helical Ribbon Model of Chromosome Scaffold



Adapted from M. Grunstein, *Scientific Am.* 267:68(1992). From A.J.F Griffiths et al., An Intro. to Genet.Anal. (1993)

Chromatin modifications and H2A.Z in zebrafish sperm



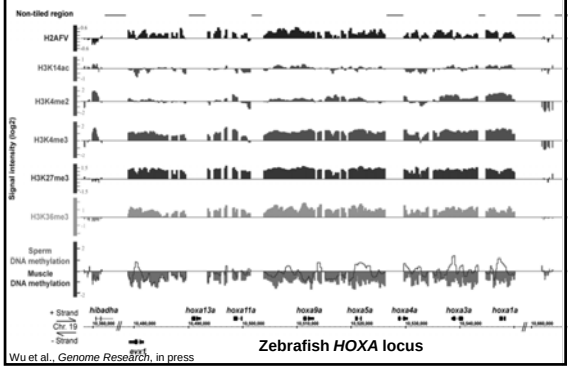
Wu et al., *Genome Research*, in press

H3K27me3 is enriched in embryonic transcription factors & developmental genes in zebrafish sperm

HYPERLINKED GO CATEGORY	ENRICH	FDR
GO:0045449_regulation_of_transcription	4.614	0
GO:0006350_transcription	4.49	0
GO:0016070_RNA_metabolic_process	4.042	0
GO:0010467_gene_expression	3.432	0
GO:0006139_nucleobase__nucleoside__nucleotide_and_nucleic	3.278	0
GO:0007275_multicellular_organismal_development	3.784	0
GO:0032501_multicellular_organismal_process	3.408	0
GO:0032502_developmental_process	3.264	0
GO:0048513_organ_development	4.097	0
GO:0007399_nervous_system_development	5.391	0
GO:0007420_brain_development	7.289	0
GO:0008152_metabolic_process	1.538	0
GO:0009790_embryonic_development	4.106	0
GO:0030154_cell_differentiation	2.792	0
GO:0048869_cellular_developmental_process	2.792	0
GO:0009952_anterior_posterior_pattern_formation	5.589	0
GO:0048598_embryonic_morphogenesis	5.269	0

Of the top 250 genes with H3K27me3, 90% are developmental TFs

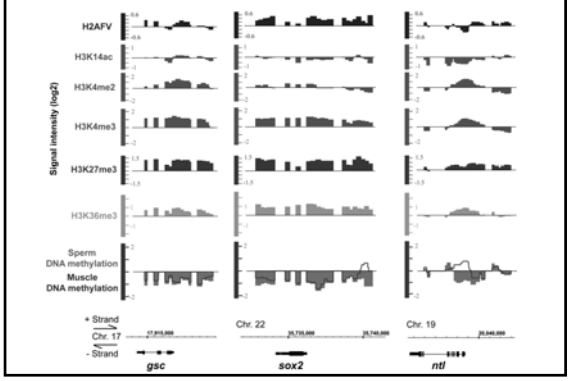
HOX loci in zebrafish sperm have large blocks of 'multivalent' chromatin



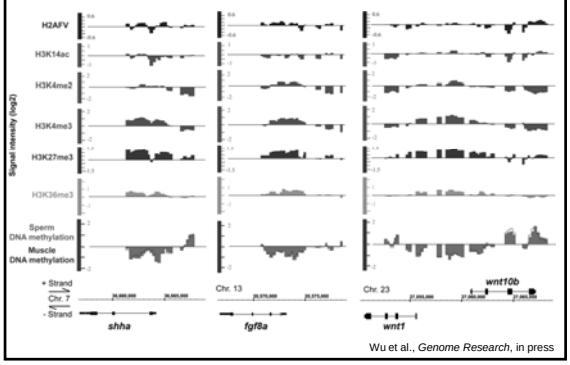
Zebrafish *HOXA* locus

Wu et al., *Genome Research*, in press

Regional multivalent chromatin at stand-alone developmental TFs

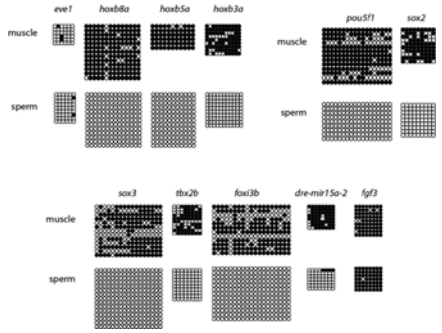


Regional multivalent chromatin at key developmental signaling genes in zebrafish



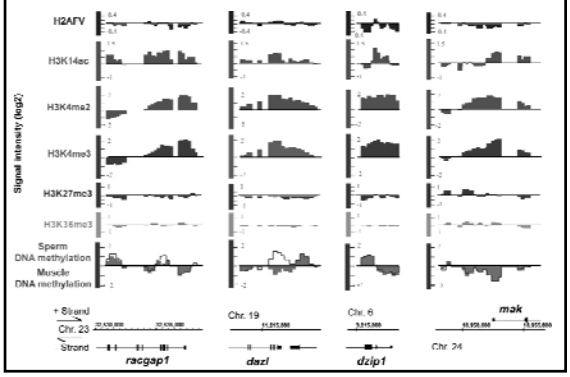
Wu et al., *Genome Research*, in press

An absence of DNA methylation at almost all developmental promoters in zebrafish sperm

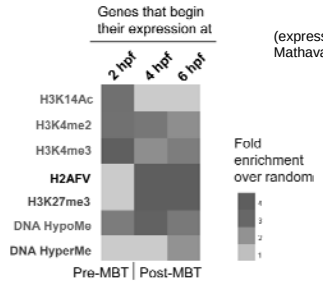


Wu et al., *Genome Research*, in press

Genes highly transcribed during sperm development have high H3K4me and H3K14ac



Correlations between the few genes activated prior to/early in MBT and the presence of 'positive' marks in sperm



(expression data from Mathavan et al, 2006)

Fold enrichment over random

P-value < 0.009

Wu et al., *Genome Research*, in press

Summary

Human sperm (Hammoud et al., *Nature* 2009; and in preparation)

- Histones package ~4% of the human sperm genome, and are enriched at the promoters of developmental transcription factors.
- Bivalency and DNA hypomethylation at developmental factors – poising?
- Unpublished: DNA methylome at >50-fold coverage.
- Severe hypomethylation of histone-associated regions, and also of transcription factor genes (homeodomain, others) important for development, and miRNAs.
- Striking hypermethylation of certain loci, such as UCEs, piRNA clusters, and rRNA.

Zebrafish sperm (Wu, Zhang and Cairns, *Genome Research*, 2011)

- Zebrafish sperm genome is fully histone, with high levels of H1/5 linker histone and ISWI, and lacks H4K16ac – which are tools for promoting condensation.
- Developmental transcription and signaling factors have regional multivalent chromatin, with extensive DNA hypomethylation.

Questions:
Is 'multivalent' chromatin for the germline, the embryo (poising), or both.
How are the histone mod's and DNA methylation status established and bounded?
Which sperm chromatin marks survive in the early embryo, and are they instructive?
What is the epigenetic status of the egg genome – similar to sperm or very different?
How does aging, health status, environment etc. alter the sperm epigenome (fertility)?

Acknowledgements

Huntsman Cancer Institute, University of Utah

Zebrafish
Shan-Fu Wu
Haiying Zhang

Human
Sue Hammoud
David Nix
Jahnvi Purwar

Doug Carrell

HHMI
HCI

What do we know about genes affecting embryo implantation?

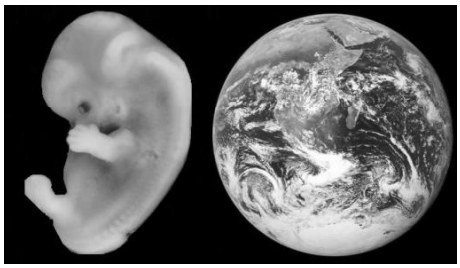
Nick Macklon
Professor of Obstetrics and Gynaecology, University of Southampton
Director, Complete Fertility Centre, Southampton

Declaration of interests

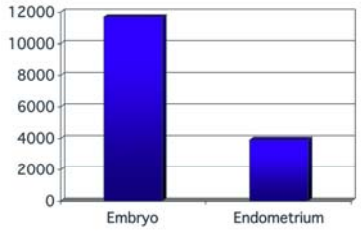
- I have received grant funding, consultation and speakers fees from:

  Merck Serono, MSD, Ferring, Anecova

An embryocentric world..

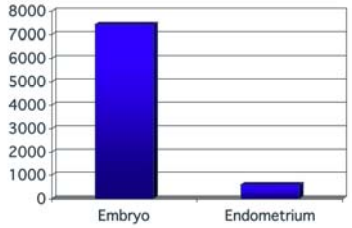


PUB MED: Implantation papers



Papers published

PUB MED: IVF papers

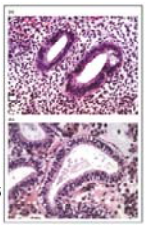


Papers published

Learning aims

- Recent studies indicating gene markers of receptivity
- The limits of genomics
- The role of secretomics
- Understanding embryo-endometerial interactions
- The embryo selection window concept

Noyes Criteria



- **Architecture preserved**
- **Widely used**
- **Subjective interpretation**
- **Glandular-stromal dyssynchrony**
- **Provides "a rough idea of quantitative progeste**

Noyes et al. Fertil Steril, 1950

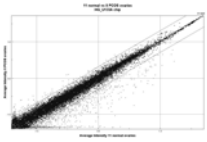
- **Does not correlate well with implantation**

Murray et al. Fertil Steril, 20

Human Peri-implantation Events

Endometri-*omics*

Endometrial genomic studies



•Proliferative versus secretory	*Kao et al 2002, Borthwick et al 2*
•Mid versus late secretory	*Martin et al 2002, Reisew*
•hMG and GnRH agonist versus natural cycle	*Horjacas*
•recFSH and GnRH antagonist/agonist and P4 versus natural cycle	*Mirkin*
•recFSH and GnRH antagonist only versus natural cycle	*Macklon*
•GnRH antagonist versus GnRH agonist	*Haouzi e*

Endocrine Research

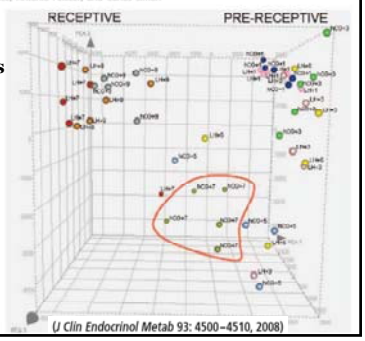
Controlled Ovarian Stimulation Induces a Functional Genomic Delay of the Endometrium with Potential Clinical Implications

José A. Horcajadas, Pablo Minguez, Joaquín Dopazo, Francisco J. Esteban, Francisco Domínguez, Linda C. Giudice, Antonio Pellicer, and Carlos Simón

25 natural cycles

25 stimulated cycles

Biopsies at
On day 1
3
5
7
after LH rise/hCG



J Clin Endocrinol Metab 93: 4500–4510, 2008)

Is Recurrent Implantation Failure associated with dysregulated endometrial gene expression?

University of Southampton School of Medicine

25 women with RIF (>3 ETs with top embryos)
- normal responders
- regular ovulatory cycles
- no thrombophilia

25 controls (live birth after first ICSI cycle for male factor)

- Endometrial biopsy in spontaneous cycle LH + 6
- Samples compared against a commercial reference
- Quiagen Human Array-21329 genes
- Genes with a *p*-value <0.05 after Benjami-Hochberg multiple testing correction were considered significantly differentially expressed.

Recurrent Implantation Failure: altered genes.

University of Southampton School of Medicine

FOXO 1:regulates decidualization

ADAMTS8: disrupts angiogenesis

MUC 16: Regulates embryo adherence

Gene dysregulation is similar to that caused by IUD.

Boomsma et al, 2008

Boomsma et al 2009

Genomics

- Large variation in genes identified between studies
- Housekeeping genes variably expressed
- To work, genes must be transcribed, producing a variable set of mRNA molecules.

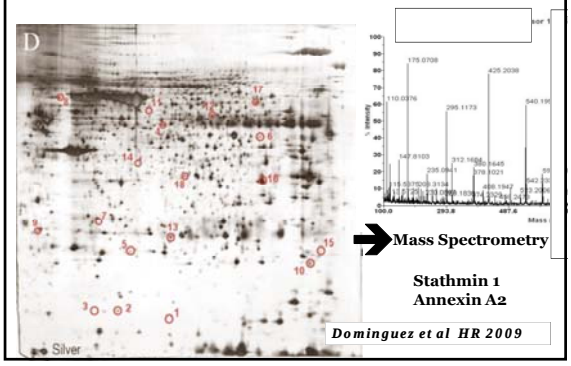
Every gene transcribes at least three proteins....

...and then post-translational modifications occur

.... and protein interaction...

Gene products, not genes, define the phenotype

From genomics to proteomics



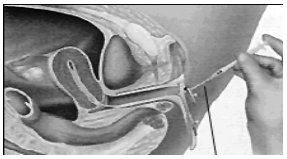
Stathmin 1
Annexin A2

Dominguez et al HR 2009

Limitations of tissue based analyses

- Biopsy material required
- Cannot be carried out during the window of implantation
- Implantation itself is, therefore, not the endpoint

The endometrial secretome



proteins, amino acids, electrolytes, glucose, urea, cytokines, growth factors, metalloproteinases and their inhibitors, immunoglobulins, alpha-1 antitrypsin precursor, haptoglobin and transferrin...

Endometrial secretions: initial questions

- **Safe to aspirate?**
- **Can protein profiles be measured?**
- **Do they correlate with dating?**

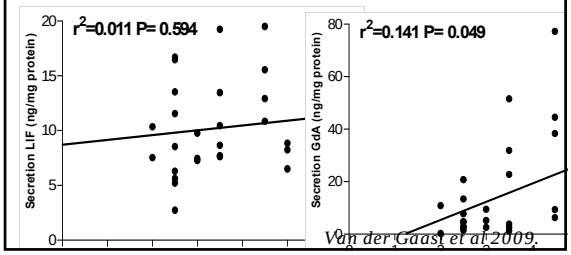
Does aspiration disrupt implantation?

Treatment results	Study group n=210	Controls n=210	p-value
Age (years)	34.9 ± 4.1	35.0 ± 3.9	0.5
Embryos transferred *(%)* Single ET Double ET	110 *(52.4)* 100 *(47.6)*	110 *(52.4)* 100 *(47.6)*	0.3
Pregnancy rate / ET(%)	68 *(32.4)*	62 *(29.5)*	0.6

Boomsma *et al*. HR 2009

Endometrial secretion glycodelin, but not LIF levels correlated to dating by histology and Ki67 expression

Secretion vs biopsy (26 ovulatory volounteers)



Van der Gaast et al 2009.

Does the technique work?

- Well tolerated by patients
- Sufficient material in 99.5% of cases
- Almost all markers quantifiable

Next Questions...

Can multiple markers be quantified in endometrial secretions?

Does cervical mucus contaminate aspirations?

Can a 'receptive' molecular fingerprint predictive of pregnancy be identified in endometrial secretions?

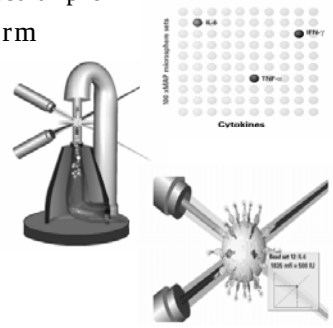
What is the impact of ovarian stimulation on endometrial secretions?

What is the impact of bacterial vaginosis on endometrial secretions?

Endometrial molecular profiling

Luminex platform

- Bead-based multiplex
- Each microsphere coated with specific reagent for specific bioassay
- Requires 50 μL

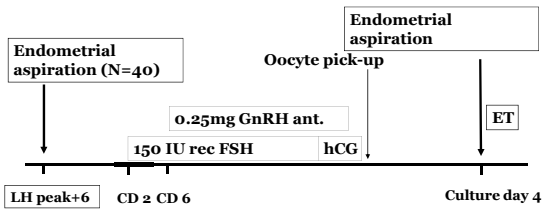


An 'Endometrial Fingerprint'

Pro-inflammatory cytokines	IFN-γ IL-1, IL-12, IL-15, IL-17,TNFα
Anti-inflammatory cytokines	IL-5, IL-6, IL-10
Chemokines	CXCL 10, MCP-1, MIF, Eotaxin
Growth factors	VEGF, HB-EGF
Signaling factors	DKK-1

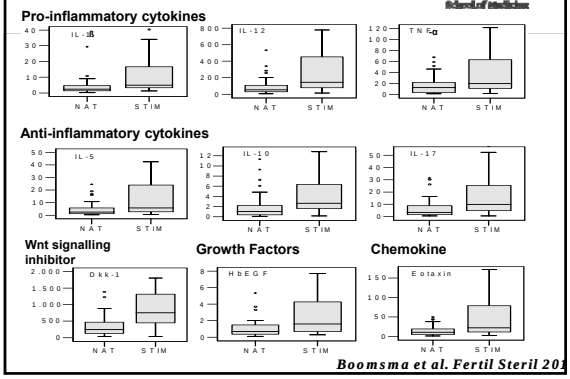
Impact of ovarian stimulation?

- **240 women undergoing first cycle IVF**
- **Exclusion criteria: >41 years of age**



Boomsma et al Fertil Steril 2010

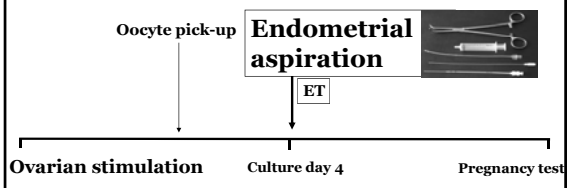
Impact of Ovarian stimulation on intra-uterine environment



Boomsma et al. Fertil Steril 201

Study design

- **240 women undergoing first cycle IVF**
- **Exclusion criteria: >41 years of age**



A secretion profile of receptive endometrium

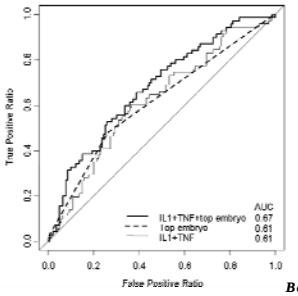
↓IL-1ß

↑TNF-α

↑MIF

Boomsma et al, HR 2009

Both endometrium and embryo quality predict implantation

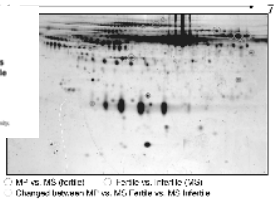


Boomsma et al, HR 2009

Boomsma et al, Hum Reprod 200

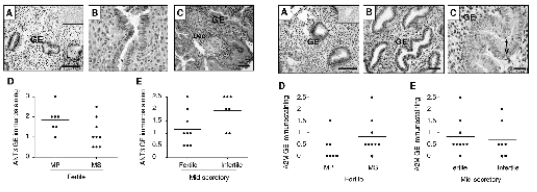
research articles Journal of proteome research

2D-DIGE Analysis of the Human Endometrial Secretome Reveals Differences between Receptive and Nonreceptive States in Fertile and Infertile Women



Changes in Abundance of Endometrial Secreted Proteins

research articles



Modeling Human Endometrial Decidualization from the Interaction between Proteome and Secretome

Tamara Garrido-Gomez, Francisco Dominguez, Juan Antonio Lopez, Emilio Camafeita, Alicia Quiñonero, Jose Antonio Martinez-Conejero, Antonio Pellicer, Ana Conesa, and Carlos Simón

J Clin Endocrinol Metab 96: 706–716, 2011)

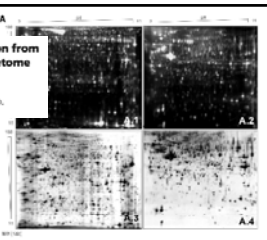
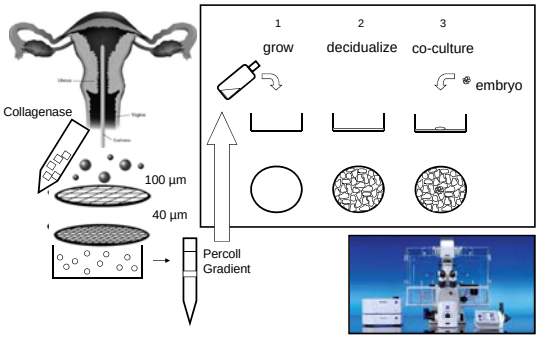


TABLE 2. List of the secreted proteins modified by the decidualization effect

Protein name[a]	Fold change[a]	*P* value (%)[a]	Description[a]
MPIF-1	7.02	0.001	C-C motif chemokine 23
IGFBP-1	6.51	0.001	Insulin-like growth factor-binding protein 1
IL-10Rβ	4.98	0.005	Interleukin-10 receptor subunit β
IL-21R	4.49	0.0002	Interleukin-21 receptor
PECAM-1	4.20	0.0014	Platelet endothelial cell adhesion molecule 1
IL-1RI	3.39	0.001	Interleukin-1 receptor I
B7-1(CD80)	2.93	0.002	Activation B7-1 antigen
Leptin R	2.74	0.001	Leptin receptor
IL-5Rα	2.73	0.0001	Interleukin-5 receptor α
PRL	2.44	0.0004	Prolactin
IL-1R4ST2	2.39	0.002	Interleukin-1 receptor 4
IL-13Rα2	1.85	0.00	Interleukin-13 receptor α 2
Fas Ligand	1.81	0.00	Tumor necrosis factor ligand superfamily member 6
L-Selectin	1.80	0.002	Leukocyte-endothelial cell adhesion molecule 1
IL-18BPa	1.64	0.00	Interleukin-18 bind protein a
ICAM-2	1.40	0.003	Intercellular adhesion molecule 2
IL-6	−2.31	0.00	Interleukin-6
MCP-3	−3.17	0.001	Chemokine ligand 7

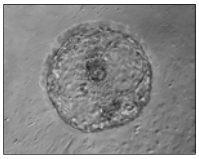
An in vitro model for embryo endometrium interactions



Embryo-endometrial cross talk: in vitro model

Day 4 — Thawing cryo-preserved embryos

Day 5 — in vitro extended culture



Day 8 Human Embryo

Day 8 — Assessment of Embryo quality ⟷ Cytokine profile

G. Teklenberg et al, 2009

Decidual cell response to arresting vs developing embryos

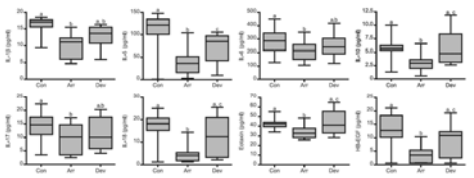


Figure 3. Developmentally impaired human embryos inhibit the secretion of selective implantation modulators by decidualizing ESCs. Primary ESCs were first decidualized for 5 days and then co-cultured with human embryos or not (control cultures, Con). Over the 72-hour co-culture period, 30 embryos arrested (Arr) whereas 11 continued to develop normally (Dev). Analysis of the culture supernatants revealed that the presence of an arresting embryo inhibited the secretion of the indicated factors. The letters above the box plots indicate significant differences between groups. $P<0.01$ for all comparisons except for IL-6 and IL-17 ($P<0.05$).
doi:10.1371/journal.pone.0010258.g003

The non-decidualised endomterium:no response

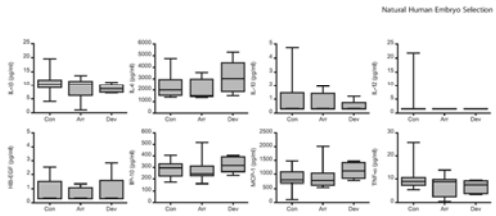
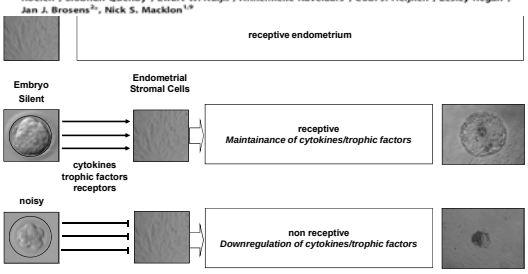


Figure 4. The human embryo does not elicit a secretory response in undifferentiated endometrium. Undifferentiated primary ESCs were co-cultured with embryos or not (control cultures, Con). Over the 72-hour co-culture period, 15 embryos arrested (Arr) whereas 6 continued to develop normally (Dev). Co-culture with either an arrested or developing embryo had no impact on the secreted levels of the indicated factors ($P>0.05$). The concentrations of IL-5, -12, -15, -17, -18, and eotaxin in culture supernatants of undifferentiated ESCs were below the level of detection.
doi:10.1371/journal.pone.0010258.g004

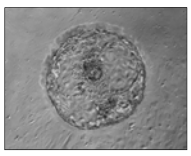
April 2010 | Volume 5 | Issue 4 | e10258
OPEN ACCESS Freely available online
PLoS one

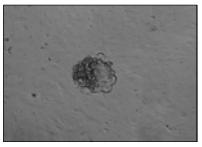
Natural Selection of Human Embryos: Decidualizing Endometrial Stromal Cells Serve as Sensors of Embryo Quality upon Implantation

Gijs Teklenburg[1,*], Madhuri Salker[2], Mariam Molokhia[3], Stuart Lavery[3], Geoffrey Trew[2], Tepchongchit Aojanepong[2], Helen J. Mardon[4], Amali U. Lokugamage[3], Raj Rai[2], Christian Landles[2], Bernard A. J. Roelen[6], Siobhan Quenby[7], Ewart W. Kuijk[1], Annemieke Kavelaars[8], Cobi J. Heijnen[8], Lesley Regan[2], Jan J. Brosens[2,*], Nick S. Macklon[1,9]



The poor embryo has to work harder





The viable embryo:
Transcriptome and proteome OK

↓

Growth
Development

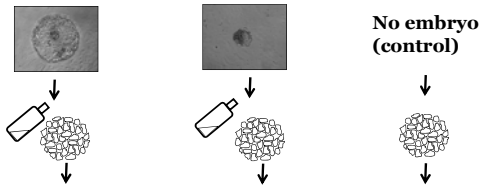
Less viable embryo:
Transcriptome and proteome not OK
compensates

↓

Repair
Rescue
Apoptosis

Does the embryo signal affect gene expression?

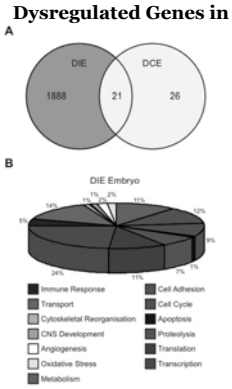
- Incubated decidualized HESCs with pooled culture supernatants from poorly developing embryos (n=30) and from embryos of ongoing pregnancies (n=30).
- Control cultures :decidualized HESCs incubated with unconditioned embryo culture medium.



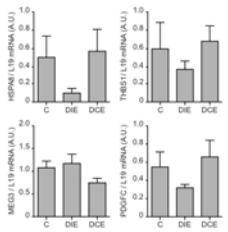
•Total RNA was harvested after 12 hours of incubation

•Subjected to genome-wide expression profiling.

Dysregulated Genes in Stromal Cells

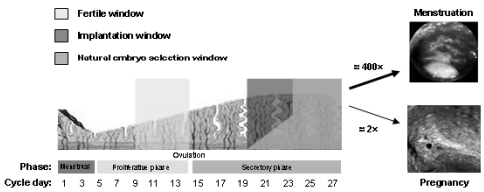


Real time PCR validation



Teklenburg et al, Submitted

From Implantation window to Selection window



Teklenburg et al 2011

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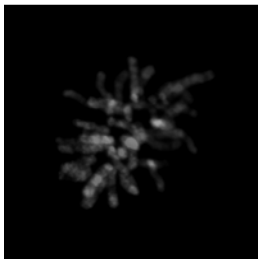
F. Holstege	J. Brosens (London)
C. Heijnen	L. Salamonsen (Melbourne)
B. Fauser	

What is epigenetics and how can it affect embryo development?



JÖRN WALTER
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UNIVERSITÄT DES SAARLANDES

Definition of Epigenetics

„Heritable“ and reversible changes of the chromatin structure which influence the functional state of the genome

- Gene expression (control of regulatory elements)
- Genomic stability (recombination & repair)
- Replication (timing, coordination and segregation)

Epigenetic control is important for:

Genome structure and function: Chromosome organisation/compaction, maintenance of nuclear integrity and identity (mitosis/meiosis)

Transcriptional memory and control: long term control of developmental processes, e.g. silencing of developmental regulators

Genome defence: silencing of retroviral/transposable elements, „Taming of transposable elements“

Epigenetic control

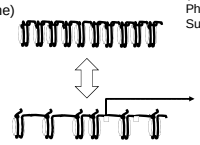
i. A combination of covalent DNA- and histone modifications

ii. A combination of proteins/enzymes setting and reading these modifications

Epigenetic modifications

DNA-methylation
(5-methylcytosine,
5-Hydroxymethylcytosine)

Histone modifications
(Methylation, Acetylation, Phosphorylation, Ubiquitination, Sumoylation, Isomerisation,...)



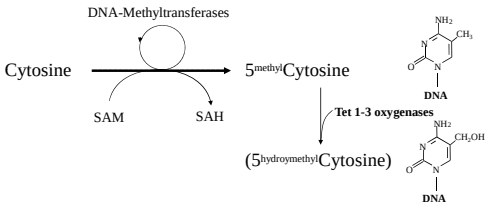
DNA-sequence + structure
(repeats,gene structure, length, base content, CpG islands...)

Histone variants
(H1.1, H2AX, H2AZ,H3.3, CenpA..)

RNAs
(smallRNAs, ncRNAs,..)

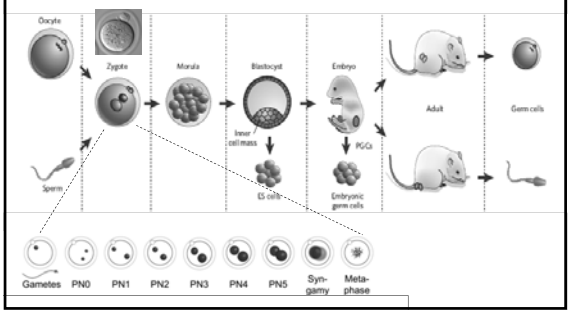
None-histone proteins
TF's, Repressors, Chromatin-Remodellers, Chromatin-associated proteins, ...

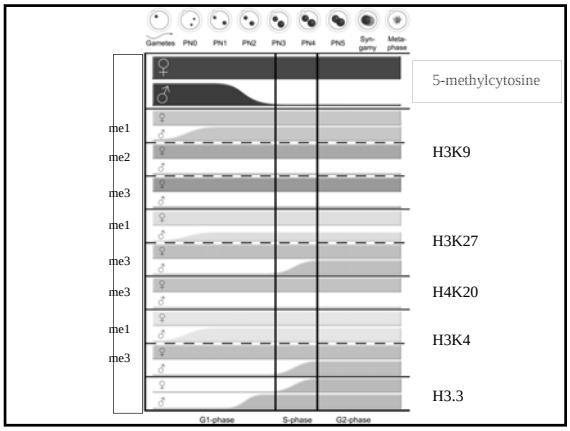
DNA-Methylation



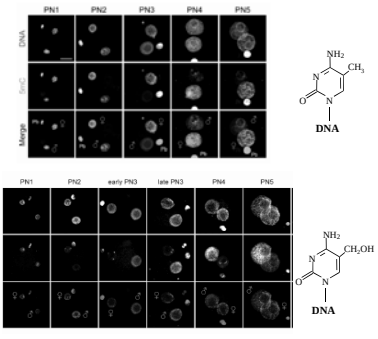
Tahiliani et al 2009
Kriaucionis and Heintz 2009

Epigenetic programs and development

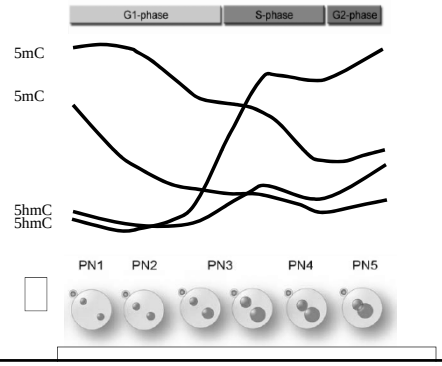




DNA-Methylation in the zygotic

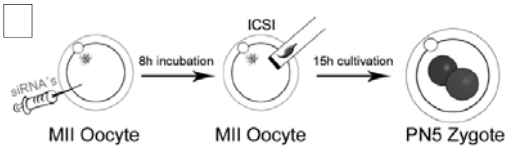


Dynamic change of 5mC and 5hmC the zygote

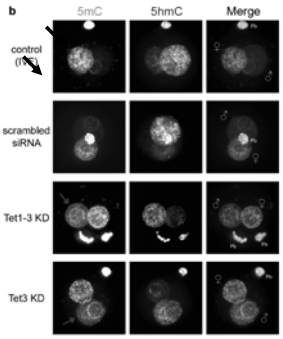


Conversion of 5mC to 5hmC is mediated by Tet dioxygenases:

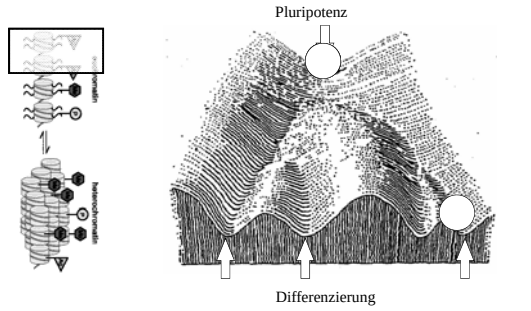
Reduction of Tet3 activity changes the DNA-methylation reprogramming



Reduction of Tet3 activity changes the DNA-methylation reprogramming



Epigenetic control is important for development



Conrad Waddington: Epigenetic landscape & "Canalization"
Modified: Timo C. Dinger & Albrecht Müller

Why epigenetic memory is important for development：

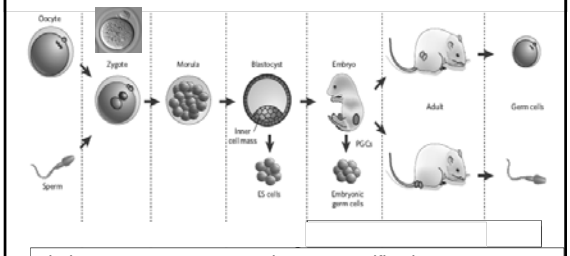
Stem cell - genes ready for action
ES cells (Zygote)

genetic + epigenetic programs

some genes on, some off

some genes on, some off

Epigenetic programs and development



Pluriopotency genes Lineage specification genes

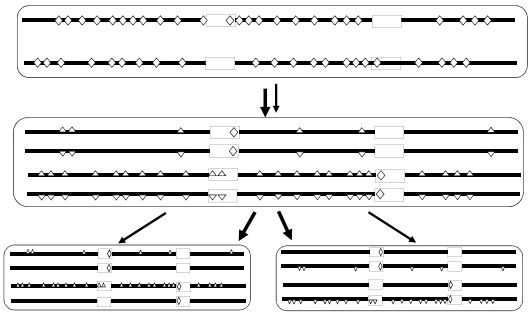
Key Observations:

Epigenetic changes lead to the activation of pluripotency genes

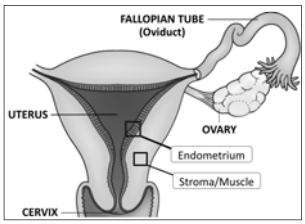
Changes in epigenetic modification are specific and necessary to Induce the development of inner cell mass cells and to establish ES and TS (Epiblast stem) cells

Manipulations of embryos (e.g. “cloning” SCNT) lead to Abnormal reprogramming which results in increased failure of development.

Supplementary figure: How is DNA methylation copied and lost during replication



Small RNAs and control of retrotransposons during gametogenesis and early development



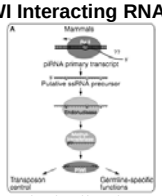
Focus of Today's Talk

- piRNAs in male reproduction
- SiRNAs & microRNAs in female reproduction

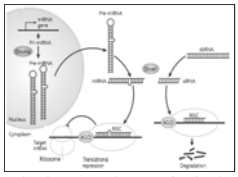
BCM

Comparison of Small RNAs

PIWI Interacting RNAs **vs** **MicroRNAs/siRNAs**



Farazi, Juranek, and Tuschl, Development, 2008



Kotaja and Sassone-Corsi, Nature Reviews (2007)

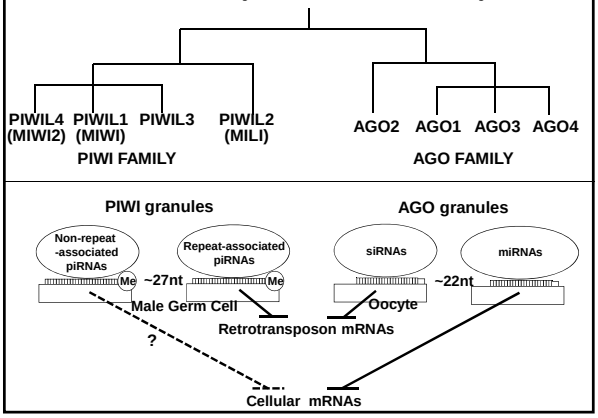
- ~28 nt noncoding ssRNAs
- Dicer-independent
- Probably >100,000 in mammals
- Suppress retrotransposons in male germ cells through interactions with PIWI family members such as MILI

- ~22 nt noncoding ssRNAs
- Dicer-dependent
- >1000 in humans
- Target complementary transcripts for translational repression and mRNA cleavage

Table: Small RNA characteristics and tissue(s) of function in mammals.

	piRNA	siRNA	miRNA
Approximate sizes	25-30nt	18-24nt	18-24nt
Major cell type	Male germ cell	Oocyte	Multiple
DICER-dependent	No	Yes	Yes
Drosha-dependent	No	No	Yes
DGCR8-dependent	No	No	Yes
Major function	Suppression of transposon synthesis	Cleavage of transposon mRNAs	Cleavage of target mRNA and suppression of translation
Estimated number	>10,000	>10,000	600-1000

PIWI Family Versus AGO Family



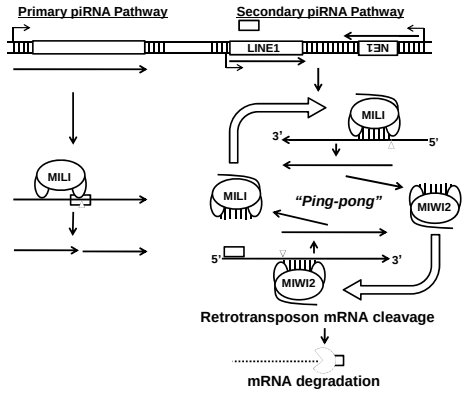
Topic 1

- piRNAs in male reproduction

piRNA History

- piRNAs were originally identified through their association with *Drosophila* PIWI (P-element-induced wimpy testis) family members, and studies in the fruit fly have revealed many of their properties
- In 2006, several reports identified piRNAs for the first time in the germlines of mice and rats
- piRNAs (and their synthesis pathways) are deeply rooted among the animalia kingdom from sponges to humans, have recently been discovered in *Tetrahymena* and *Paramecium*, but are absent in plantae and fungi which employ siRNAs instead
- The functions of piRNAs are nearly exclusive to gametogenesis and are essential to spermatogenesis in mammals through their ability to maintain the integrity of the germline.

Pathways for synthesis of piRNAs



piRNA structural features

- The signature of piRNA synthesis is an enrichment for A at the 10th position of secondary piRNAs due to its base-pairing with the corresponding initial 5' U on the primary piRNA.
- The terminal event of biogenesis is methylation of the 3' end of the mature piRNA by HEN1 2'-O-methylase, allowing for the preferential binding of the 2'-O-methylated piRNA within the PAZ domain pocket of PIWI proteins but not the AGO subfamily, presumably making it resistant to the action of uridylation.

Tudor family members

- The assembly of all mammalian germ granules depends upon the association between structural TUDOR domain containing proteins (reviewed in Siomi, M.C., Mannen, T. and Siomi, H. 2010 How does the royal family of Tudor rule the PIWI-interacting RNA pathway? Genes Dev 24:636-646)
- TUDOR domains are selective for symmetrical dimethylarginines, and the "writer" of this post-translational mark is PRMT5 (Protein arginine methyl-transferase 5) in association with its adaptor protein WDR77 (WD containing region 77)

TDRD:PIWI interactions

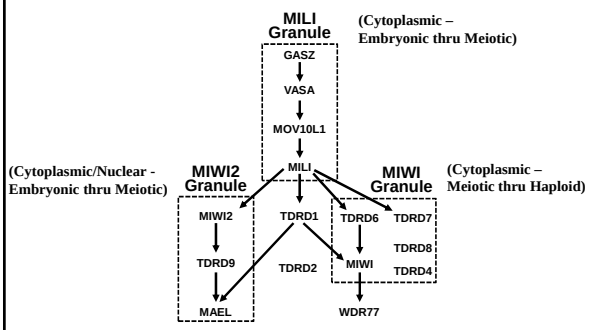
Table 2. *Interaction between PIWI and TDRD proteins in mice*

PIWI protein	TDRD protein	References
Adult testis		
MILI	TDRD1/MTR-1	Chen et al. 2009, Kojima et al. 2009, Reuter et al. 2009, Vagin et al. 2009, Wang et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009
	TDRD6	Vagin et al. 2009
MIWI	TDRD1/MTR-1	Chen et al. 2009, Kojima et al. 2009, Vagin et al. 2009
	TDRD2/TDRKH	Chen et al. 2009, Vagin et al. 2009
	TDRD4/RNF17	Vagin et al. 2009
	TDRD6	Chen et al. 2009, Vagin et al. 2009, Vasileva et al. 2009, Kirino et al. 2010
	TDRD7/TRAP	Chen et al. 2009
	TDRD8/STK31	Chen et al. 2009
Transgenic mouse		
Adult testis MIWI (sDMA)	TDRD1/MTR-1	Vagin et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009
	TDRD4/RNF17	Vagin et al. 2009
	TDRD6	Vagin et al. 2009
	TDRD7/TRAP	Vagin et al. 2009
	TDRD9	Vagin et al. 2009
Embryonic testis MILI	TDRD1/MTR-1	Vagin et al. 2009
Embryonic testis MIWI2	TDRD1/MTR-1	Vagin et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009
	TDRD9	Vagin et al. 2009
HEK293T/HEK293		
MILI (sDMA [Reuter et al. 2009, Vagin et al. 2009])	TDRD1/MTR-1	Kojima et al. 2009, Reuter et al. 2009, Vagin et al. 2009, Wang et al. 2009
	TDRD2/TDRKH	Vagin et al. 2009, Wang et al. 2009
	TDRD9	Vagin et al. 2009
MIWI (sDMA [Chen et al. 2009, Vagin et al. 2009])	TDRD1/MTR-1	Kojima et al. 2009, Wang et al. 2009, Vagin et al. 2009
	TDRD2/TDRKH	Chen et al. 2009, Vagin et al. 2009
	TDRD9	Vagin et al. 2009
MIWI2	TDRD1/MTR-1	Kojima et al. 2009, Wang et al. 2009
	TDRD9	Shoji et al. 2009
Rabbit reticulocyte lysate system		
MILI	TDRD6	Vasileva et al. 2009
MIWI	TDRD6	Vasileva et al. 2009

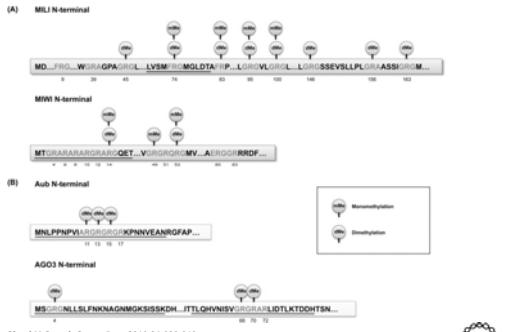
Summary of protein–protein interactions observed between PIWI and TDRD proteins in mice.

Siomi et al. Genes Dev 24:636-646

piRNA granules and genetic association



Arginine methylation status of PIWI proteins



Siomi M C et al. Genes Dev. 2010;24:636-646

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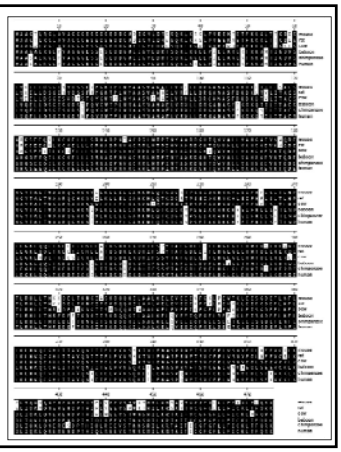
Table: Conservation of piRNA pathway proteins and functions of piRNA pathway proteins in mice.

Drosophila	*M. musculus*	Mouse knockout phenotype	Knockout Ref.
PIWI	PIWIL1 (MIWI)	**Sterile; spermatid block**	(13)
AUBERGINE	PIWIL2 (MIL1)	**Sterile; spermatocyte block**	(10, 12)
AGO3	PIWIL4 (MIWI2)	**Sterile; spermatocyte block**	(12, 14)
-	GASZ	**Sterile; spermatocyte block**	(17)
MAELSTROM	MAEL	**Sterile; spermatocyte block**	(28)
VASA	DDX4 (VASA)	**Sterile; spermatocyte block**	(24)
-	DDX25	**Sterile; spermatid block**	(202)
ARMITAGE	MOV10L1	**Sterile; spermatocyte block**	(25, 26)
TUDOR	TDRD1	**Sterile; spermatid block**	(32)
CG7802	TDRD2	-	
-	TDRD4 (RNF17)	**Sterile; spermatid block**	(33)
TEJAS	TDRD5	-	
KRIMPER	TDRD6	**Sterile; spermatid block** (miRNA pathway)	(34)
-	TDRD7	-	
-	TDRD8 (STK31)	-	
SPN-E	TDRD9	**Sterile; spermatocyte block**	(27)
	DNMT3L	**Sterile; spermatocyte block and spermatogonia loss**	(30, 203)
HEN1/PIMET	HEN1	-	
CAPSULEEN	PRMT5	**Early embryonic lethality**	(204)
VALOIS	WDR77	**Early embryonic lethality**	(205)
SQUASH	-	-	
ZUCCHINI	-	-	
	RANBP9	-	

Example: GASZ, piRNAs, and male reproduction

GASZ is a 475aa Germ cell-specific evolutionarily-conserved protein with 4 Ankyrin (Ank) repeats, a Sterile alpha motif (SAM), and a basic leucine Zipper domain

GASZ orthologs share high amino acid identity in mammals, fish, and frogs



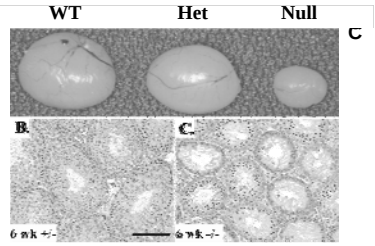
Yan, W., Rajkovic, A., Viveiros, M.M., Burns, K.H., Eppig, J.J., and Matzuk, M.M. Molecular Endocrinology 16, 1168-1184 (2002).

Yan, W., Ma, L., and Matzuk, M.M. BOR 70, 1619-1625 (2004).

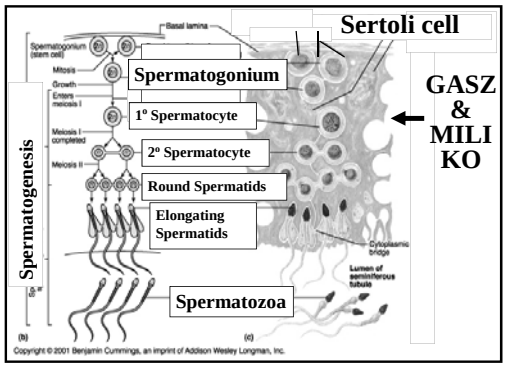
Example: GASZ, piRNAs, and male reproduction

GASZ is a 475aa Germ cell-specific evolutionarily-conserved protein with 4 Ankyrin (Ank) repeats, a Sterile alpha motif (SAM), and a basic leucine Zipper domain

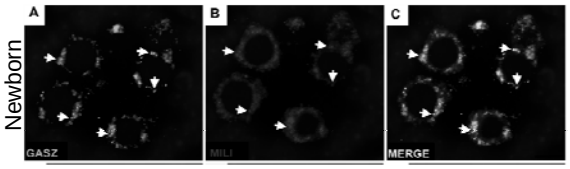
- GASZ null males are sterile
- GASZ null males have a block at the pachytene stage of meiosis



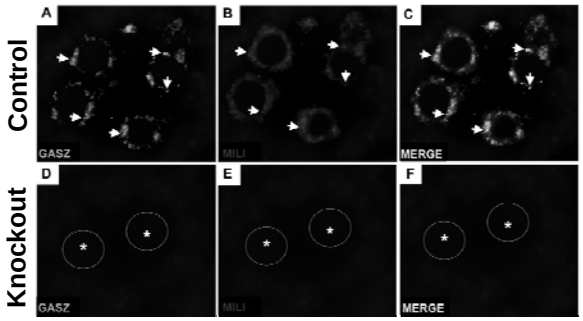
GASZ null testes show a meiotic block identical to KO of the PIWI family member, MILI



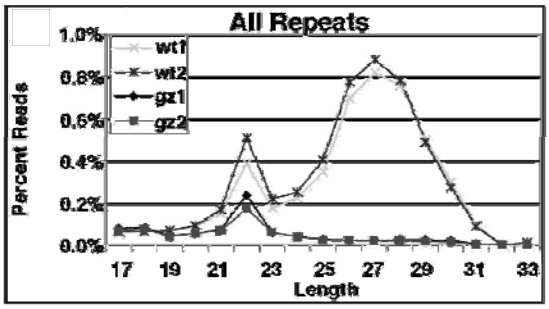
GASZ and MILI are expressed in perinuclear cytoplasmic granules in spermatogonia and spermatocytes



Knockout of GASZ abolishes MILI Expression



Using Next Generation Sequencing, we discovered that GASZ KO testes have a decrease in piRNAs that map to repeat sequences

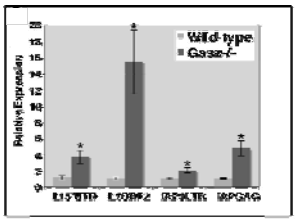


GASZ KO testes have a significant decrease in piRNAs that map to Line 1 and LTR repeats

	gz1	gz2	wt1	wt2	
Total Reads	4,416,053	4,760,967	3,909,858	3,272,500	
Repeat	Number of Reads	Number of Reads	Number of Reads	Number of Reads	Fold Reduction in GASZ vs WT
Total	44,710	43,710	190,887	174,124	5.3-fold
LINE L1	325	363	33,600	25,055	108-fold
LTR ERVK IAP	338	368	30,681	29,623	109-fold
LTR MaLR MTA	35	22	15,961	12,693	634-fold

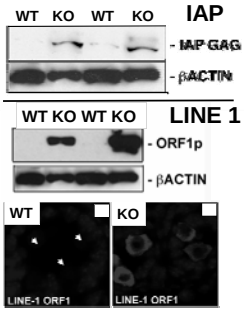
Absence of GASZ (similar to MILI KO) leads to increased expression of retrotransposon mRNAs and encoded proteins

RT-PCR

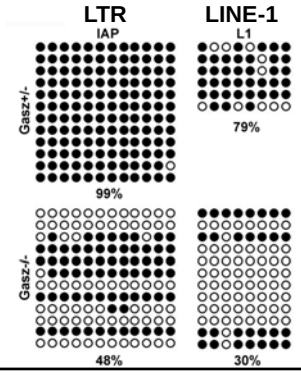


LINE 1 IAP LTR

Western blot



Absence of GASZ leads to hypomethylation of retrotransposon promoters



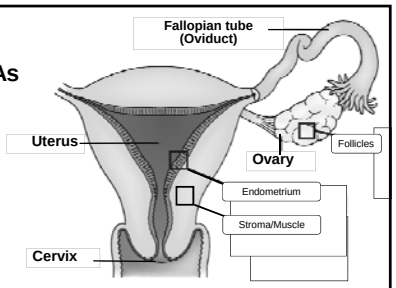
Summary

- ❖ **KO of GASZ causes male-specific sterility due to a zygotene-pachytene stage meiotic block**
- ❖ **GASZ is an essential structural component of nuage**
- ❖ **GASZ -/- testes show a dramatic reduction in novel and retrotransposon-associated piRNAs, leading to increased retrotransposon synthesis**
- ❖ **The Illumina sequencing platform is a sensitive means to evaluate small RNA populations and identify novel small RNAs**
- ❖ **GASZ and its interacting partners are novel testis-specific contraceptive targets**

Ma, L., Buchold, G.M., Greenbaum, M.P., Roy, A., Burns, K.H., Zhu, H., Han, D.Y., Harris, R.A., Coarfa, C., Gunaratne, P.H., Yan, W. and Matzuk, M.M. 2009 GASZ Is Essential for Male Meiosis and Suppression of Retrotransposon Expression in the Male Germline. PLoS Genet 5:e1000635

Topic 2

siRNAs & miRNAs in Female Reproduction



miRNA and siRNA synthesis

- MicroRNAs (miRNAs) are synthesized using a pathway that requires Drosha and DGCR8 in the nucleus and DICER in the cytoplasm
- Small interfering RNAs (siRNAs) only require the RNAse III activity of DICER
- Mice lacking DICER die at the gastrula stage secondary to defects in embryonic stem cell development

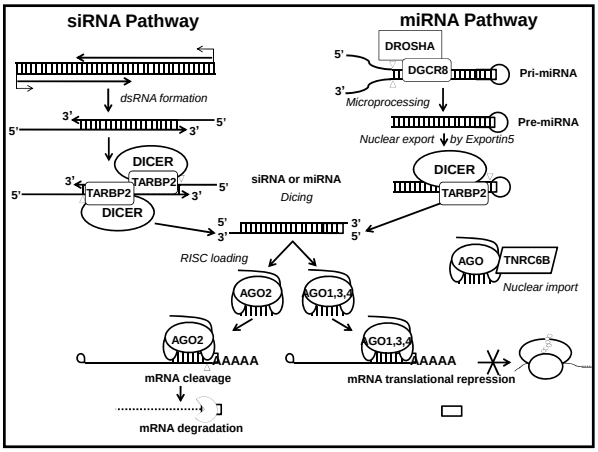


Table: Mouse models created to study siRNA and miRNA function in reproduction.

Mutant allele	Pathway altered	Phenotype	Reference
Argonaute 2 (null)	miRNA	E9.5 lethality; embryonic defects including neural tube and cardiac defects	(45)
Argonaute 2 (flox) *Zp3-Cre*	siRNA	Female sterility; oocyte meiosis I block	(50)
Argonaute 2 (flox) *Tnap-Cre*	miRNA	Normal male fertility	(172)
DICER (null)	miRNA (siRNA?)	E7.5 lethality; defects in ES cells	(44)
DICER (hypomorph)	miRNA	Female sterility; defects in vasculature leading to ovarian corpus luteum defects	(206)
DICER (flox) *Amhr2-Cre*	miRNA	Female sterility; oviductal diverticuli and uterine implantation defects	(75-78)
DICER (flox) *Amh-Cre*	miRNA	Male sterility due to defective Sertoli cell differentiation and spermatid loss	(164-166)
DICER (flox) *Tnap-Cre*	miRNA	Male sterility due to impaired spermatogonial proliferation and possible stem cell defects	(172, 173)
DICER (flox) Nr5a1-Cre	miRNA	Male sterility due to germ cell apoptosis secondary to altered somatic gonadal cells	(207)
DICER (flox) *Pitx2-Cre*	miRNA	reduced GH, prolactin, and TSHβ; normal proopiomelanocortin and LHβ	(68)
DICER (flox) *Zp3-Cre*	siRNA	Sterile; disorganized spindles, defects in chromosome alignment, and a block at meiosis I	(45, 46)
DICER (flox) Aarr2pb-Cre	miRNA	Prostate atrophy due to reduced prostatic stem cell proliferation	(186)
DGCR8 (flox) *Zp3-Cre*	miRNA	Normal fertility; confirms that miRNAs are not required in oocytes	(53)

SiRNA history

- Small interfering RNAs (siRNAs) were first discovered by David Baulcombe's group at the Sainsbury Laboratory in Norwich, England as part of post-transcriptional gene silencing in plants
- The Nobel Prize in Physiology or Medicine 2006 was awarded jointly to Andrew Z. Fire and Craig C. Mello *"for their discovery of RNA interference - gene silencing by double-stranded RNA"* (work that was performed in animals)

Dicer function in oocytes

- Initial studies to decipher the roles of DICER in oocyte biology demonstrated that the DICER deletion (using zona pellucida 3 (Zp3)-Cre) results in infertility
- The major defects in DICER-deficient oocytes were disorganized spindles, defects in alignment of the chromosomes, and arrest at metaphase of meiosis I
- Phenotypically similar oocyte meiotic arrest and spindle and chromosome defects are observed in oocytes with AGO2 deletion

Murchison et al. (2007) Critical roles for Dicer in the female germline. Genes Dev 21:682-693

Tang et al. (2007) Maternal microRNAs are essential for mouse zygotic development. Genes Dev 21:644-648

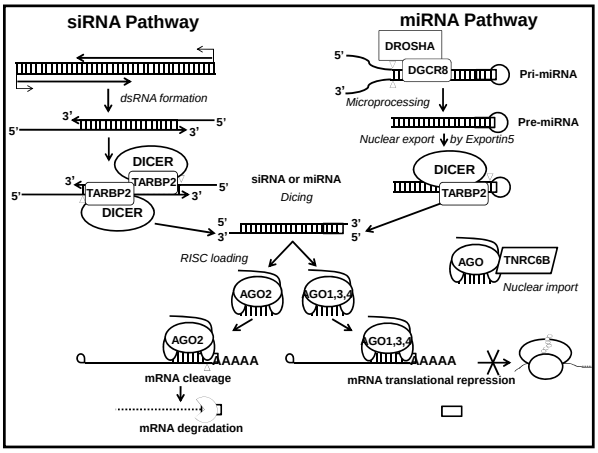
Kaneda et al. (2009) Essential role for Argonaute2 protein in mouse oogenesis. Epigenetics & Chromatin 2:9

Dicer function to suppress repetitive elements in oocytes

- Tam et al. and Watanabe et al. discovered that siRNAs are made in oocytes, these siRNAs are depleted in the DICER null oocytes, and specific mRNA targets of the siRNAs are upregulated in the absence of DICER.
- mRNAs encoding some repetitive elements (mouse transposon (MT) including the MaLR family and SINE) were upregulated in the absence of DICER
- Unlike the findings with absence of the piRNA machinery, LINE1 sequences were not increased.
- The MT and RLTR10 retrotransposon mRNAs were also upregulated in the absence of AGO2

Tam et al. (2008) Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* 453:534-538

Watanabe et al. (2008) Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* 453:539-543



Proof that siRNAs alone function in oocytes

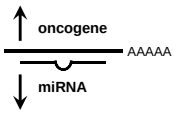
- Despite an abundance of miRNAs in oocytes, Ma et al. showed that these small RNAs were incapable of mediating mRNA cleavage or repressing translation
- These findings were genetically confirmed by Suh et al. who showed that absence of DGCR8 in oocytes leads to normal oocyte maturation, fertilization, and offspring
- These and additional experiments in these reports indicate that DGCR8-independent, DICER-dependent production of siRNAs is required for oocyte maturation whereas miRNAs and many additional DICER-derived miRNAs are dispensable for oocyte function and fertility

Ma et al. (2010) MicroRNA activity is suppressed in mouse oocytes. Curr Biol 20:265-270

Suh et al. (2010) MicroRNA function is globally suppressed in mouse oocytes and early embryos. Curr Biol 20:271-277

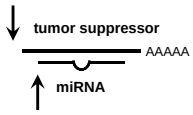
MicroRNAs function as tumor suppressors and oncogenes

Tumor suppressor



Decreased miRNA activity increases levels of target oncogene

Oncogene

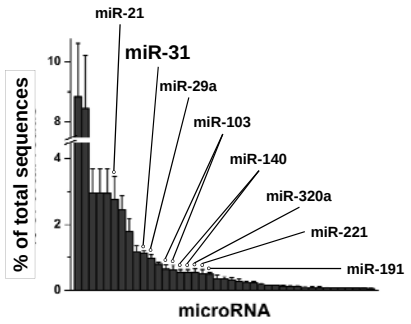


Increased miRNA activity decreases levels of target tumor suppressor

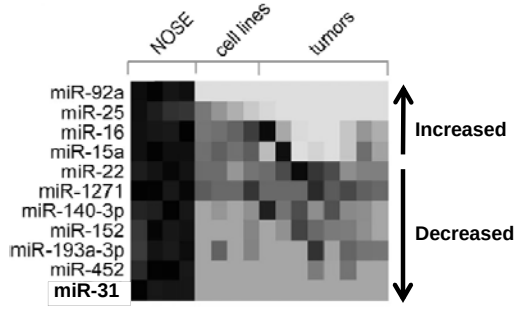
Table: miRNAs dysregulated or functional in serous ovarian cancer.

miRNA/family	Potential role	Reference
Let-7 family	Targets KRAS, HRAS, MYC, HMGA2; promotes tumorigenesis	(208, 209)
miR-9	Targets NFKB1; downregulated in cancer and suppresses cell growth	(210, 211)
miR-15a/miR-16	Target Bmi1; reduces proliferation	(212)
miR-22	Inhibits cell migration and invasion	(213)
miR-29b	Downregulated and correlated with survival	(214)
miR-31	Targets E2F2 and cell cycle; most downregulated miRNA in serous cancers	(215)
miR-34 family	Targets cell cycle genes; loss of p53 suppresses miR-34	(216)
miR-182	Amplified in 28.9% of ovarian cancers; promotes tumor growth *in vivo* (i.e., putative oncomir)	(217)
miR-185	Targets Six1; suppresses anchorage-independent growth and cell migration	(218)
miR-199a-5p	Targets IKb; fosters pro-tumor environment	(219)
miR-200 family	Represses epithelial-mesenchymal transition	(220)
miR-214	Targets PTEN; overexpression promotes chemoresistance	(221)

Use Illumina sequencing to profile miRNAs in ovarian surface epithelium (versus ovarian cancer)



miR-31 was universally downregulated >30-fold in our human serous ovarian cancers and cell lines



Hypothesis: miR-31 is an ovarian tumor suppressor

miR-31 overexpression in OVCAR8 serous ovarian cancer cell line halts proliferation mainly by inducing apoptosis

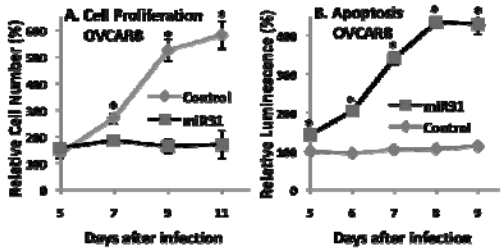
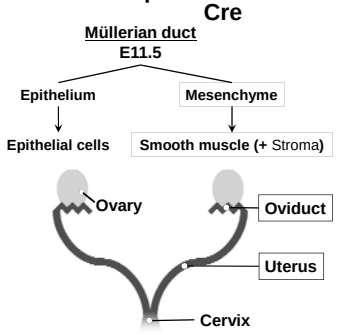


Table: Tumor models with altered miRNA synthesis or individual miRNA(s)

Altered gene or miRNA	Mouse model	Cancer type	miRNA function	Reference
Dicer deletion (with $Kras^{G12D}$ expression)	Conditional: *Dicer* deletion & $Kras^{G12D}$ expression	Lung cancer	Tumor suppressor	(45,60)
Dicer deletion (with *Rb* deletion)	Conditional: *Dicer* and *Rb* deletion	Retinoblastoma	Tumor suppressor	(61)
miRNA cluster (*mir15a* and *mir16-1*) deletion	Targeted deletion of a miRNA cluster	Leukemia	Tumor suppressor	(62)
Overexpression of *miR-31*	Xenograft	Breast cancer	Anti-metastatic factor	(64,65)
Overexpression of *miR-21*	Transgenic	B-cell lymphoma	Oncogene	(66)
Overexpression of *miR-21* (with $Kras^{G12D}$ expression)	Transgenic	Lung cancer	Oncogene	(67)
mir-21 deletion (with $Kras^{G12D}$ expression)	Targeted deletion of *mir-21*	Lung cancer	Oncogene	(67)
Overexpression of *mir-155*	Transgenic	B-cell malignancy	Oncogene	(68)
Overexpression of *miR-9*	Xenograft	Breast cancer	Pro-metastatic factor	(69)
Overexpression of *miR-10b*	Xenograft	Breast cancer	Pro-metastatic factor	(70,71)

Conditional knockout of Dicer in somatic cell of the female reproductive tract using Amhr2-Cre



Amhr2-Cre

Anti-Müllerian hormone receptor type 2 (Dr. Richard Behringer)

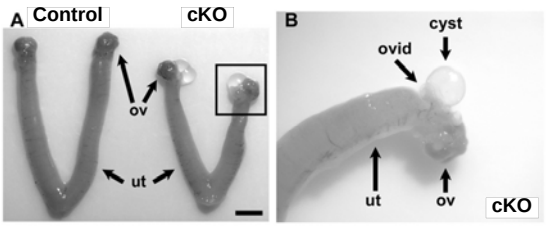
Dicer Floxed (Dr. Alexander Tarakhovsky)

Dicer1 conditional knockout (cKO) females are sterile

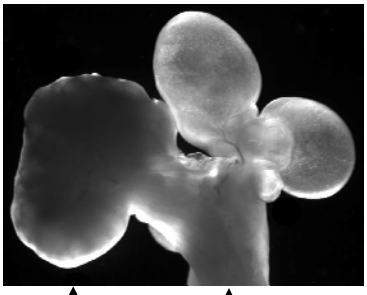
Table 1. Fertility testing of *Dicer1*$^{flox/-}$ and *Dicer1* cKO females. Six week-old *Dicer1*$^{flox/-}$ and *Dicer1*$^{flox/-}$ *Amhr2*$^{cre/+}$ females were mated to wild type males for 13-30 weeks. Data are shown as the mean ± SEM.

Genotype	n	Litters	Total pups	Pups/litter	Litters/month
Dicer1$^{flox/-}$	10	62	575	9.2 ± 0.4	1.2 ± 0.04
Dicer cKO	**10**	**0**	**0**	--	**0**

Dicer cKO females have shorter uteri and oviducts contain bilateral diverticuli



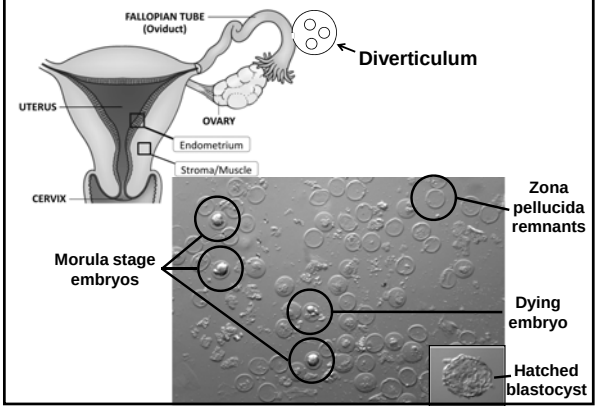
First mouse model with diverticuli in the oviduct



Ovary **Uterus**

Diverticulum (Out-pouching form because of defects in smooth muscle formation)

The oviductal diverticuli trap oocytes and embryos



DICER/MicroRNA Conclusions

- ❖ MicroRNAs including miR-31 are implicated as tumor suppressors in serous ovarian cancer
- ❖ Dicer expression in the somatic cells of the female reproductive tract is essential for fertility
  - ➢ Ovarian granulosa cells
    - – Limited effects in ovulation and early embryonic development
  - ➢ Uterus
    - – **Absence of Dicer in stroma and muscle results in smaller uterus that is not receptive to embryos (decidualization)**
  - ➢ **Oviduct**
    - – **Diverticuli block embryos from reaching the uterus**

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Chorionic gonadotropin beta-gene variants as a risk factor for recurrent miscarriages

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ESHRE 2011, course 3 "From genes to gestation"
July 3rd 2011, Stockholm

Research support: Wellcome Trust, Howard Hughes Medical Institute, Estonian Science Foundation, Estonian Ministry of Education and Science, Alexander-von-Humboldt Foundation

Learning Objectives:

1. One of the first proteins produced by the conceptus is human chorionic gonadotropin (HCG), also known as "the pregnancy hormone".

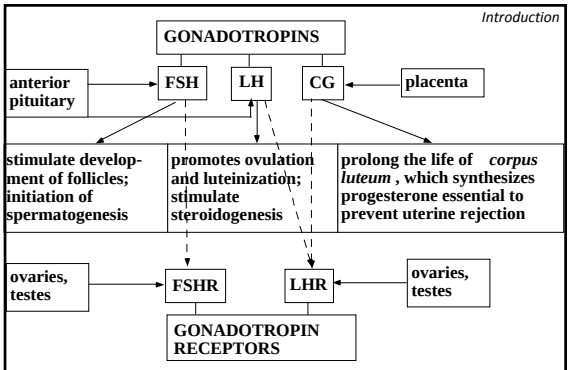
2. The main function of HCG is to delay the apoptosis of the *corpus luteum* during the first trimester of pregnancy.

3. Low level and non-exponential increase of HCG in maternal serum during the first trimester of the pregnancy is a clinically accepted risk factor for miscarriage (Buyalos et al 1992; Dumps et al 2002; Tong et al 2006)

4. The hormone-specific hCG beta-subunit is expressed by placental syncytiotrophoblasts and is encoded by four duplicated *Chorionic Gonadotropin Beta* genes (*CGB, CGB5, CGB7* and *CGB8*)

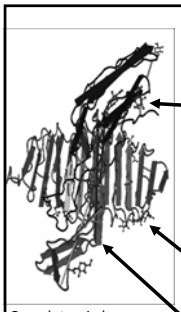
5. An increased prevalence of miscarriage among first-degree relatives of the women suffering from RM suggests genetic contribution in recurrent pregnancy loss (Christiansen, 1996; Kolte et al 2011).

6. The main topic of this presentation is to explore whether particular variants in *hCG beta coding* genes may contribute to pregnancy failure.



Mutations in *Gonadotropin Hormone and Receptor genes* alter sexual development and lead to reproductive failure

Introduction



Gonadotropin hormone and receptor complex

Protein Data Bank, www.rcsb.org/pdb/

Gonadoptropin hormone structure is highly conserved:

hormone specific beta subunits:
FSH beta coded by the *FSHB gene* at chr. 11p13
LH beta coded by the *LHB gene* at chr. 19q13
HCG beta coded by FOUR *copies of hCG beta (CGB)* genes at chr. 19q13

beta subunit binds to the gonadotropin receptor and is responsible for HORMONE-specific signaling

all hormones share identical alpha subunit coded by *CGA gene* at *chr.6q12.21*

Outline of today's presentation:

Part I: Genetics of *Chorionic Gonadotropin Beta (CGB)* genes

1. Genomic and evolutionary context of *LHB/CGB gene region*
2. Human *CGB* genes – genetic diversity patterns
3. Human *CGB* genes – expression profile in normal and complicated pregnancy

Part II: hCG beta coding *CGB* genes and recurrent miscarriage (RM)

4. Polymorphisms in *CGB5* and *CGB8* genes is association with RM
5. Novel type of genetic mutation – methylation allele polymorphism in *CGB5* gene and RM
6. Functional consequences of amino acid changing mutations in *CGB5* and *CGB8* genes, identified in RM patients

Genomic context

mammals
teleost fish
Xenopus laevis (frog)

1 Lutenizing Hormone Beta (LHB) gene with conserved function

GENE DUPLICATIONS in primate lineage

New World monkeys

LHB & *1CGB*

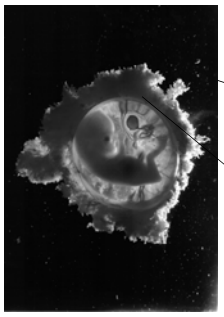
LHB & $(CGB)_{2\text{-}n}$

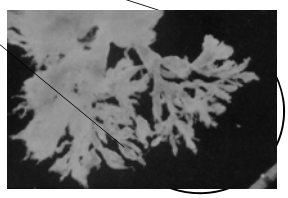
Old World monkeys, apes& humans

– *CGB* gene coding for beta-subunit of Chorionic Gonadotropin is PRIMATE-SPECIFIC
– hCG hormone has evolved to be essential in early pregnancy in monkeys, apes and humans

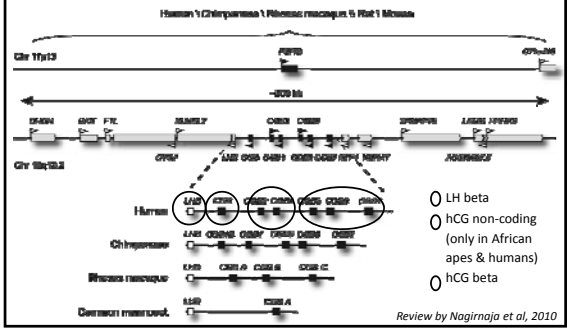
HCG is secreted by the syncytiotrophoblasts of the placenta:

- support *corpus luteum* function
- prepare endometrium for the implantation
- improve the maternal blood supply
- ensure uterine quiescence
- modify the local immunoreactivity in endometrium



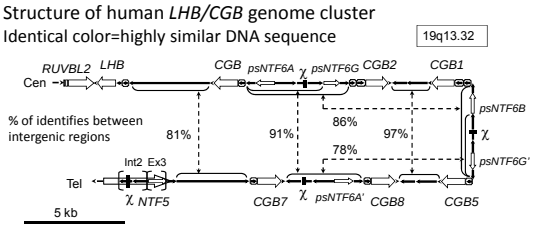


LHB/CGB genes: tandem duplicated, highly similar loci in a gene-rich region and species-specific evolutionary young gene cluster arrangement
In contrast: *FSH beta coding FSHB* gene: a single evolutionarily conserved gene in a gene-poor environment



Review by Nagirnaja et al, 2010

Structure of human *LHB/CGB* genome cluster
Identical color=highly similar DNA sequence



Duplicate genes are very similar to each other:

hCG beta coding *CGB, CGB5, CGB7, CGB8* – 97-99% DNA homolgy

hCG beta non-coding *CGB1 &CGB2* genes – 97-99% DNA homolgy

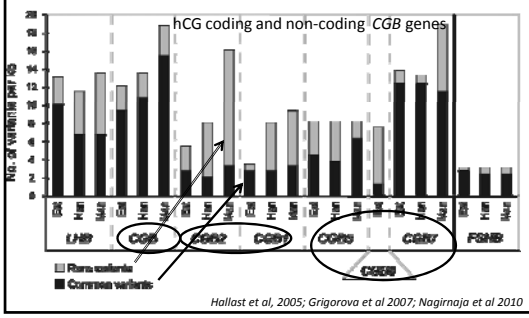
hCG beta coding and non-coding 85%

LHB and hCG beta coding – 92-93%

Hallast et al, 2005

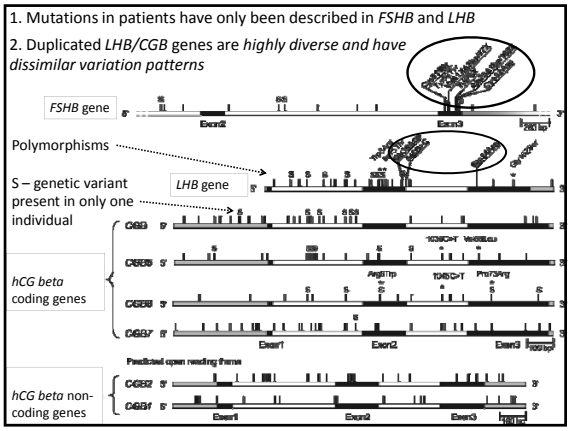
Gene diversity

1. *Gonadotropin beta* genes differ highly in genetic variation patterns
1. *LHB/CGB* genes are AMONG THE MOST DIVERSE GENES in humans: Estonians (Est), Han Chinese (Han), African Mandenka (Man)
2. 20-50 polymorphisms in total per gene (the length of a gene is 1.5 kb)



Hallast et al, 2005; Grigorova et al 2007; Nagirnaja et al 2010

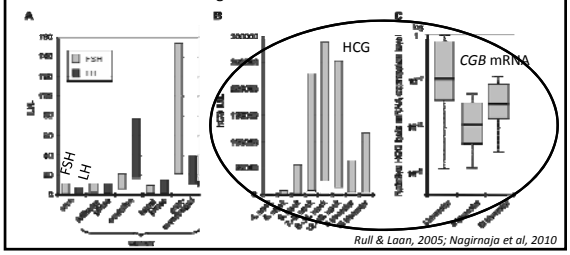
1. Mutations in patients have only been described in *FSHB* and *LHB*
2. Duplicated *LHB/CGB* genes are *highly diverse and have dissimilar variation patterns*



Gene diversity and gene expression

Gonadotropin beta genetic variation: reflection in hormone levels?

1. FSH and LH: in female follicular and luteal phase and in men narrow range of hormone concentration
2. FSH and LH: rise in female during ovulation and post-menopausal period;
3. hCG: large fluctuations in production consistent with large inter-individual differences in *CGB* gene expression and may probably reflect high genetic variation in the *HCG beta* genes



Rull & Laan, 2005; Nagirnaja et al, 2010

Relative contribution of *CGB, CGB5, CGB7* and *CGB8* into total *HCG beta* mRNA production in placenta : despite high gene homology, there are manifold inter-individual and intergenic differences in expression

	Contribution (%±s.d.) of an individual gene expression in total HCG beta mRNA							
	CGB8		*CGB5*		*CGB*		*CGB7*	
	mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD	range
I trimester	39.3±1.8	29-63	25.5±2.7	6-55	27.1±1.5	11-39	8.1±1.2	0-21
II trimester	48.1±2.8***	28-86	25.7±4.2	9-45	20.0±2.3***	1-29	6.2±1.8	0-16
III trimester	39.2±2.5	24-71	36.0±3.8**	7-62	20.1±2.1***	2-32	4.7±1.6**	0-19
ectopic pregnancy	48.0±3.2**	31-62	18.7±4.7	7-28	25.3±2.6	14-54	8.0±2.1	0-17
recurrent miscarriage	47.1±3.7**	28-56	23.3±4.9	13-34	22.0±2.8*	17-27	7.6±2.2	0-13

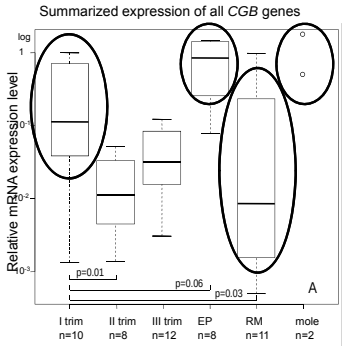
***p<0.005; **p<0.05; *p<0.08; reference: I trimester of normal pregnancy

CGB8 – the "master" gene, providing most of hCG beta mRNA the lowest no of polymorphisms

CGB7 – minor transcribed gene the highest number of polymorphisms

Rull & Laan, 2005

Gene expression in normal and complicated pregnancy



1. *CGB* transcription is activated in blatocyst stage

2. hCG β genes – highly expressed throughout the pregnancy

4. Recurrent miscarriages: reduced transcription of CGB genes compared to uncomplicated I trimester

5. Ectopic and molar pregnancy - high expression of CGB genes due to failure of a normal negative autoregulatory feedback?

Rull & Laan, 2005; Rull et al, 2008a

Intermediate summary:

1.Human Chorionic gonadotropin (*CGB*) genes are young genes, evolved by duplication events form the ancestral *LHB* gene in primate lineages.

2.hCG beta is coded by four highly similar genes (*CGB, CGB5, CGB7, CGB8*).

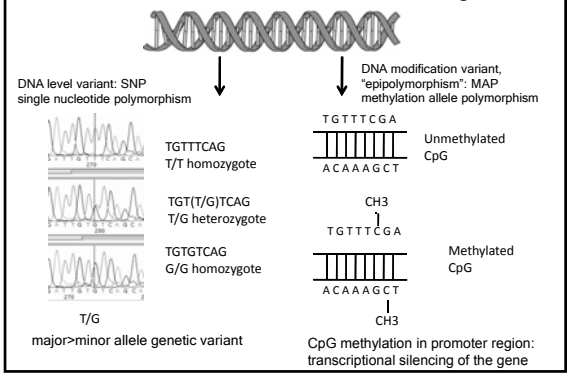
3.hCG beta coding genes are highly polymorphic and among the most diverse genes in humans

4.There are vast differences in hCG beta expression profiles among individuals and between gene copies coding for hCG beta subunit.

5.High inter-individual variation in gene expression is accompanied by hgh inter-individual variation in hormone levels.

6.hCG beta gene expression is significantly reduced in cases of recurrent miscarriage and increased in cases of ectopic and molar pregnacy.

Are there any genetic or epigenetic variants that are associated with increased or decreased risk of recurrent miscarriages?



STUDY DESIGN: *hCG beta* gene variants and recurrent miscarriage (RM)

A)DISCOVERY ASSOCIATION STUDY in Estonian & Finnish (*Rull et al 2008b*)
Dr. K. Rull, Tartu University Hospital Women's Clinic
Dr. V.-M. Ulander, prof. K. Aittomäki; Helsinki University Central Hospital
184 RM patients with ≥ 3 consecutive miscarriages
195 fertile women, no miscarriage in their reproductive history

Targeted genes: *CGB5, CGB8*, which contribute 2/3 of total HCG beta mRNA
Experiment: Resequencing of full gene + gene regulatory promoter in all individuals

B) REPLICATION of the ASSOCIATION STUDY in Danish samples (*unpublished*)
Prof. O.B. Christiansen, Copenhagen Rigshospitalet
451 RM patients with ≥ 3 consecutive miscarriages
237 fertile controls, no miscarriage in their reproductive history

C) EPIGENETICS of *CGB5* & *CGB8* promoter methylation in RM (*Uusküla et al 2011*)

D) FUNCTIONAL STUDY to test the consequence of identified aminoacid mutations in *CGB5* & *CGB8* genes on hCG hormone assembly and function (*unpublished*)
Collaboration with Dr. H.Peltoketo, prof. I. Huhtaniemi, Imperial College London, and Česlovas Venclovas, Vilnius University, Lithuania

Polymorphism study: discovery

Results I. High variation and low allelic association in *CGB5* and *CGB8*

CGB5: **49 SNP, in promoter 18**
CGB8: **22 SNP, in promoter 3**

} 71 SNP, of which 48 (68%) novel

CGB5

CGB8

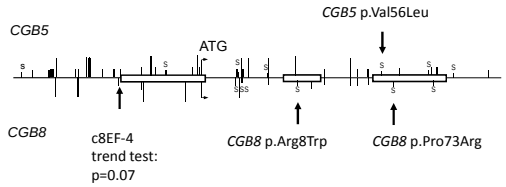
*** 41 (58%) of SNPs were shared by the Estonian and Finns**
*** In both samples sets:**
15 population-specific SNPs, mainly rare variants
***Low population stratification: allele frequences differed between Estonians and Finns for 8 of 71 SNPs**
*** Joint analysis of two population samples possible**

Rull et al, 2008b

Polymorphism study: discovery

Results II. Rare variants identified ONLY AMONG RECURRENT MISCARRIAGE PATIENTS:

non-synonymous amino acid changes in single patients and a proximal promoter SNP in three patients



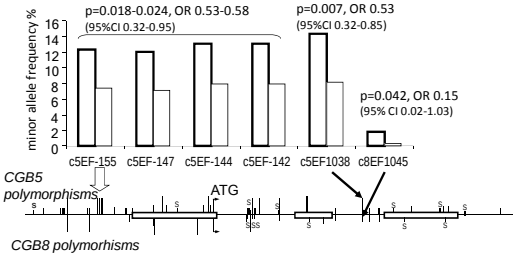
SNP c8EF-4 flanks the transcription initiator element

Rull et al, 2008b

Polymorphism study: discovery

Results III: *CGB5* Promoter and *CGB5/CGB8* intron 2 harbor SNPs with protective effect towards recurrent pregnancy loss

(statistical analysis by Cochran-Armitage trend test)

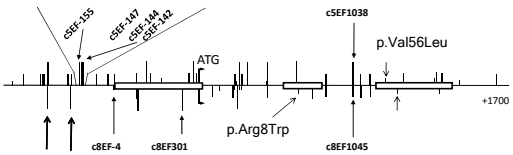


MAF - minor allele frequency

Rull et al, 2008b

Intermediate summary

1. The minor alleles of six identified promoter and intronic variants of *CGB5&CGB8* genes reduce significantly ~1.7-fold the risk for recurrent pregnancy loss.
Decision: FOLLOW-UP REPLICATION STUDY in DANISH study samples



2. Only RM patients carried rare aminoacid changes in *CGB5* & *CGB8:* p.Arg8Trp, p.Pro73Arg, p.Val56Leu
Decision: FOLLOW-UP FUNCTIONAL EXPERIMENTS using *in vitro* assays to understand the role these aminoacid mutations on hCG hormone function and in pregnancy succes

Polymorphism study: follow-up

Follow-up study: joint association analysis of the discovery and replication sample to test association recurrent miscarriage

Logistic regression analysis adjusted to recruitment centre

	Est N=216		Finn N=185		Danish N=569		All N=870	
SNP	Fertile controls	RM	Fertile controls	RM	Fertile controls	RM	p-value	OR (95%CI)
c5-155	13.16	9.17	11.50	6.55	7.14	5.62	**0.002**	**0.59 (0.42-0.83)**
c5-142	13.16	9.17	13.00	7.74	7.14	5.62	**0.001**	**0.57 (0.41-0.81)**

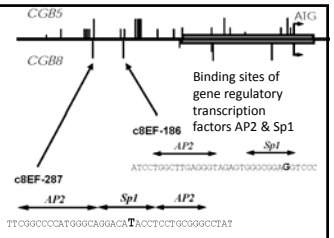
- Two linked polymorphisms in *CGB5* gene promoter were present with higher frequency among fertile controls

-Joint analysis of all study samples confirmed statistically significant association with reduced risk for recurrent miscarriage

- *CGB5* promoter segment carrying the minor alleles of these SNPs originates from the "master" *CGB8* gene by meiotic gene conversion event

K. Rull, O.B. Christiansen et al, unpublished

Unexplained phenomenon in the *CGB8* gene missing in all populations the carriers with the genotype combination including minor allele homozygosty of either of the two promoter polymorphisms (c8EF-186 and c8EF-287)



observed

c8EF-186 Chi^2-test p= 2.28E^{-26} expected

c8EF-186

c8EF-287

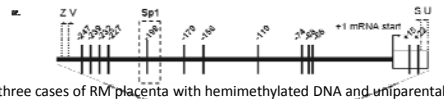
	GG	GT	TT	
TT	137	225	96	458
TC	192	154		346
CC	75			75
	404	379	96	879

c8EF-287

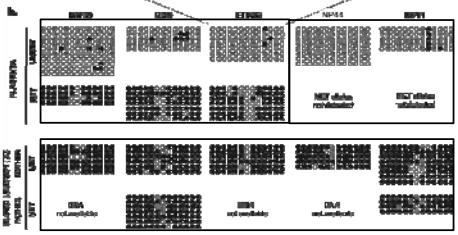
	CC	CA	AA	
AA	210.5	197.5	50	458
AG	159	149.2	37.8	346
GG	34.6	32.3	8.2	75
	404	379	96	879

K. Rull, O.B. Christiansen et al, unpublished

Additionally to DNA variants, *CGB* gene expression may be also affected by *epigenetic* polymorphic promoter methylation silencing the transcription of one parental allele



CGB5 promoter region three cases of RM placenta with hemimethylated DNA and uniparental expression



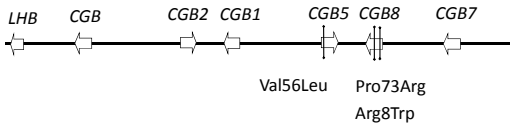
Normal pregnancy (NP) in placenta: unmethylated DNA and biparental expression

Positive control in blood leucocytes: fully methylated DNA and no transcription

Uusküla et al 2011

Functional study: hCG beta mutations

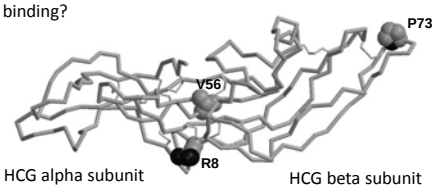
Functional consequences of non-synonymous amino acid mutations in the *CGB5* and *CGB8* genes



Heterozygous cases:
Val56Leu – one male partner of Finnish RM couple
Arg8Trp – one male partner of Estonian RM couple
Pro73Arg – on Estonian and one Danish RM patient, two Danish partners of fertile women

Rull et al. 2008b; K.Rull, O.B. Christiansen et al, unpublished; L. Nagirnaja, C. Venclovas et al, unpublished

Positions of mutated residues in the context of three-dimensional structure of hCG hormone:
All mutations are located next to aminoacids forming disulfide-bonds in intact hormone affecting either
(i) protein folding, (ii) heterodimer assembly or (iii) gonadotropin receptor binding?



PDB id: 1HCN

L. Nagirnaja, C. Venclovas, H. Peltoketo et al, unpublished

Design of functional studies:
Collaboration: Dr. H. Peltoketo, Prof. I. Huhtaniemi Institute of Reproductive and Developmental Biology, Imperial College London
Česlovas Venclovas, Vilnius University, Lithuania

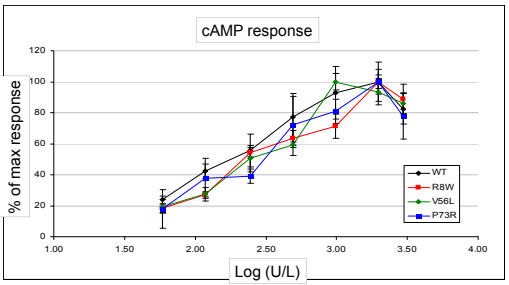
1.Co-expression of hCG α-subunit with mutant hCGβ-subunits in cell culture: R8W, V56L and P73R mutations carrying genes compared to wild-type *CGB8* gene transcript variant
- Collection of cell culture media for the analysis of secreted recombinant hormones

2. Comparative analysis of mutant and wildtype recombinant hormones
(A)Effect on gonadotropin receptor binding and downstream signalling?
- Bioassay measuring cAMP signalling molecule
(B) Effect of hCG hormone assembly?
-Analysis of precipitated hormone using co-immunoprecipitation and Western Blot (via α- and β-subunits)

L. Nagirnaja, C. Venclovas, H. Peltoketo et al, unpublished

Bioassay:
- Stimulation of hCG/LHR with different hCG mutants in cell culture
- Measurement of down-stream signalling molecule – cAMP

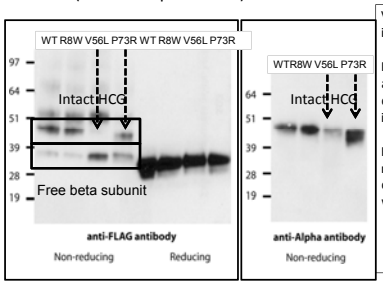
Result: No effect of mutant molecules on binding efficiency with hCG/LHR receptor



L. Nagirnaja, C. Venclovas, H. Peltoketo et al, unpublished

Effect on hormone assembly:

Analysis of precipitated hormone using co-immunoprecipitation and Western Blot (via α- and β-subunits)



Detection via β-subunit

Detection via α-subunit

V56L – intact hormone is almost missing

P73R – effect on assembly dynamics, detection of intermediate products

R8W –applied methods did not show difference from wildtype variant

L. Nagirnaja, C. Venclovas, H. Peltoketo et al, unpublished

Summary of the hCG beta R8W, V56L and P73R mutations:

1. hCG/LHR Receptor binding unaffected
2. hCG Glycosylation unaffected
3. Mutation-specific changes in the structure of hCG:

V56L – identified in *CGB5* in one individual

Positioned in the cystein knot, assembly-deficient but biologically active

P73R – identified in *CGB8* in five individuals

Positioned in the loop, potentially affects kinetics of the assembly

3. R8W – identified in *CGB8* in one individual,

Positioned on the surfice of the hormone in the cystein knot; other studies have shown that mutation is this position potentially affects kinetics of the assembly (Wilken&Bedows 2007)

L. Nagirnaja, C. Venclovas, H. Peltoketo et al, unpublished

Take home messages:

1. Four duplicate copies of hCG beta subunit genes coding for IDENTICAL PROTEIN guarantee sufficient hCG beta production in implantation.

2. Human hCG beta coding genes are highly polymorphic and large fluctuations in gene expression are tolerated during pregnancy.

3. Genetic variants affecting the expression of one duplicate *CGB* gene is predicted NOT result in strong phenotypic effect due to expressional compensation by the rest of gene copies.

4. Among four genes coding for hCG beta, *CGB8* seems to be the "master gene":

(i) it provides most of the mRNA transcripts and its seems to carry the most optimal promoter sequence; (ii) gene conversion of this sequence to *CGB5* promoter is associated with reduced risk to recurrent miscarriage (RM).

5. *hCG beta* gene expression may also be affected by polymorphic methylation of gene promoter leading to silencing the transcription of one parental allele

6. Despite there are eight functioning *hCG beta* genes per genome, mutations causing amino acid changes in the beta subunit are not tolerated: these mutations are rare (single carriers among screened 1000 Europeans), affect production of intact hCG and thus, increase the risk for recurrent miscarriages.

GONADOTROPIN GENE FAMILY TEAM at Maris Laan laboratory:

Pille Hallast | Marina Grigorova | Liina Nagirnaja | Liis Uusküla | Kristiina Rull

Collaborators in gonadotropin beta gene studies:

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Ilpo Huhtaniemi & Hellevi Peltoketo, Imperial College London, UK
Ole B. Christiansen, Rigshospitalet, Copenhagen University Hospital, Denmark
Česlovas Venclovas, Vilnius University, Lithuania
Jörg Gromoll & Frank Tüttlemann, University of Münster
Aarno Palotie, Wellcome Trust Sanger Center, UK
Margus Punab, University of Tartu Clinics & colleagues from Baltic Andrology Centres
Robert K. Campbell, Serono Reproductive Biology Institute, USA
Tõnu Margus, Department of Bioinformatics, IMBC, University of Tartu

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Hallast P, Laan M (2009) Evolution of the chorionic gonadotropin beta genes in primates. In: Encyclopedia of Life Science (ELS), John Wiley & Sons, Ltd: Chichester; p. 1-12; DOI: 10.1002/9780470015902.a0021966.
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Genomic changes detected by array CGH in human embryos with developmental defects

Dr Evica Rajcan-Separovic
University of British Columbia

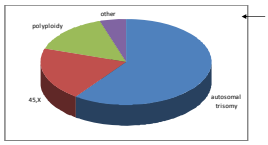
Learning objectives:

- Understand the principle of whole genome array analysis
- Become familiar with results of two whole genome array based studies of miscarriages (sporadic and recurrent miscarriages)
- Recognize the benefits and challenges of array analysis of miscarriages in clinical practice

Miscarriage

Significant health issue as 10-15% recognised pregnancies end in miscarriage

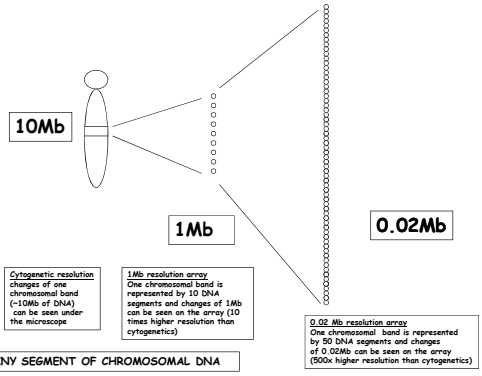
Cytogenetic analysis of miscarriage 60~ 70% have abnormal karyotype



Types of abnormalities

~30% of miscarriages have a normal karyotype and the cause of miscarriage remains unknown

GENOMIC ARRAYS IMPROVE CHROMOSOME RESOLUTION AND ALLOW DETECTION OF SUBMICROSCOPIC CHANGES



Whole genome arrays are widely applied to study submicroscopic chromosomal causes of intellectual disability (they are used as a routine clinical service).

Whole genome arrays are rarely used to study the genomic composition of miscarriages

Application of arrays to study miscarriages

Literature – <10 publications describing application of whole genome arrays to miscarriages (~1000 cases)

1-13% miscarriages have submicroscopic gains or losses (DNA copy number variants-CNVs)

HOWEVER,
Array studies of miscarriages are limited by:
-incomplete confirmation of miscarriage CNV
-no parental analysis (uncertain if miscarriage CNV de novo or parental in origin)
- no clinical information on miscarriage or couples
- Presence of miscarriage CNVs in controls not routinely checked (Database of Genomic Variants not fully developed at the time)

Goal of our work:

•Identify CNVs in idiopathic chromosomally normal miscarriages

•Confirm CNVs in miscarriage

•Follow them up in parents to determine origin of CNV or determine their presence in controls using DGV

•Obtain as much clinical information on the miscarriages and couples as possible

We have 2 whole genome (array CGH) studies:

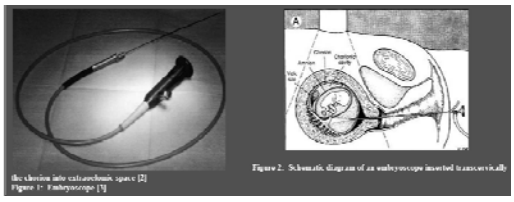
1) Array CGH of chromosomally normal sporadic miscarriages that showed morphologic abnormalities as determined by embryoscopy (in collaboration with Dr Tom Philipp, Vienna)

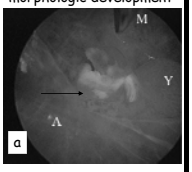
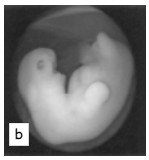
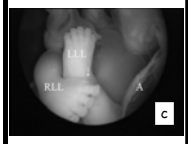
- 17 embryos studied

2) Array CGH of chromosomally normal miscarriages from couples with idiopathic Recurrent Pregnancy loss (RPL) (in collaboration with Dr Mary Stephenson, University of Chicago)

- 26 miscarriages from 20 couples with RPL studied

Study 1-Embryoscopy



GROWTH DISORGANIZED EMBRYOS early failure of embryo development; distortion of body shape; inconsistent morphologic development	MULTIFOCAL ABNORMALITIES	ISOLATED FOCAL ABNORMALITY
		
a) GD3 Embryo. The embryo showed two underdeveloped branchial arches, a tail with an abnormal kink, no upper and lower limb	b) Embryo with microcephaly, a dysplastic face, paddle-shaped limbs, retarded development relative to CRL	c) Early fetus with a missing toe

Study 1-cont.

EMBRYOSCOPY description available for 17 embryos:

- 7 embryos had growth disorganization (GD)
- 9 embryos had multiple external defects (8/9 embryos had abnormal head/brain development)
- 1 embryo had an isolated external defect

WHOLE GENOME ARRAY CGH ANALYSIS
- Whole genome Agilent 105k array used
- Custom array/qPCR used for confirming/refining unique CNVs and determining their origin (parental or de novo)

Results
Study 1-Genomic findings is sporadic miscarriages

- Frequency of unique CNVs:
30% miscarriages had **unique**, previously not described CNV
(6 unique CNVs in 5 cases)

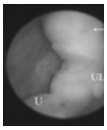
- Size:
all CNVs smaller than 250kb (~40 times less than a chromosome band)

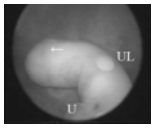
- origin of CNVs:
  - 1 (6%) de novo
  - 3 familial
  - 2 uncertain (insufficient DNA)

- Association of CNVs with morphological abnormalities: not obvious; type or number of CNVs can not be associated with severity in development (number of cases still small)

Examples:

1. De novo unique CNV





Embryo 1	Embryo had severe microcephaly, facial dysplasia, a short cord and severely retarded upper and lower limbs	Origin: de novo	Chromosome band: 13q32.1	Change: gain	Size 12.8kb	GENE: *GPR180*: role in vascular remodeling. Expressed in most adult tissues, predominantly in vascular smooth muscle cells. In the murine null model, the deletion of *GPR180* gene did not show any obvious phenotypic effect, except poor response to vascular injury.

Examples cnt.

2. Unique CNVs of parental origin
(example *WDR and* Syntaxin genes) :

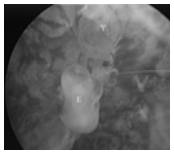
These genes are required for cell division and development and occurred in more than one studied embryo

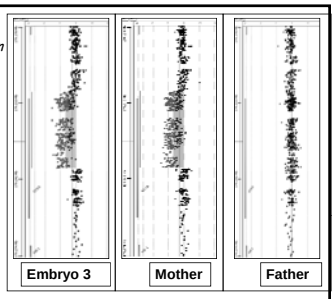
How do familial CNVs cause a different phenotype (e.g. carrier mother normal, her embryo carrying the CNV did not survive)

- Recessive mutation
- Epigenetic causes (imprinting)
- Variable expressivity
- Coincidental

- Environmental injury? Example Syntaxin6 deletion in miscarriage

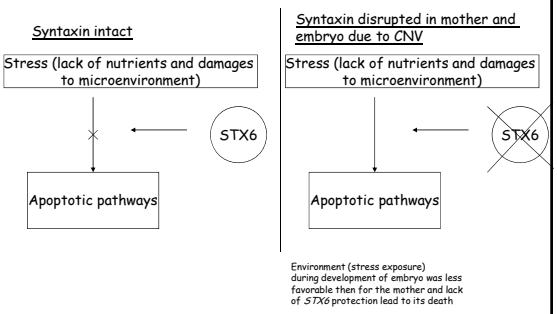
Familial Syntaxin 6 gene deletion





Embryo 3	GD1	Chromosomal band 1q25.3	Type of change: loss	Size:49kb (31)	Origin: maternal	Genes: STX6 KIAA1614; unknown function

Syntaxin 6 gene is associated with stress response as it counters the activation of cell death pathways caused by stress signals (e.g. lack of nutrients and damages to microenvironments; Zhang et al, 2008)



Environment (stress exposure) during development of embryo was less favorable then for the mother and lack of *STX6* protection lead to its death

Study 2: Genomics of recurrent pregnancy loss

(26 miscarriages studied from 20 couples)

Results- Genomic findings is recurrent miscarriages

•Frequency of CNVs:
8/20 couples (~50%) had miscarriages with unique CNVs
(a total of 13 CNVs detected)

•Size:
80% CNVs smaller than 250kb

•origin of CNVs:
all familial

Example 1

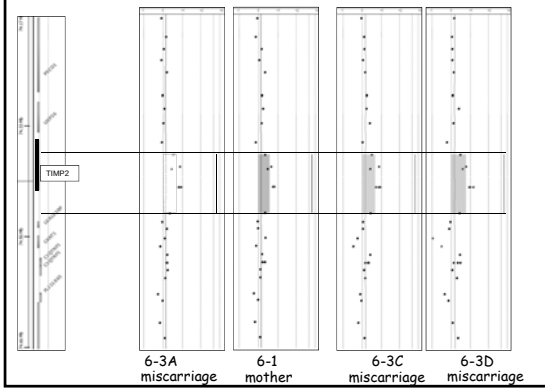
RPL Couple

10 miscarriages
5 studied by array

Fetal demise (34 yrs) *multiple placental infarcts;*
Fetal demise 46,XX (35 yrs) *multiple placental infarcts*
Fetal Demise (35 yrs);
Fetal demise 46,XY (36 yrs) *decidual infartcs 50%*

6-3D Yolk sac misc 47,XX,+16 (37 yrs);
Biochem misc (37 yrs)
→ **6-3A Emb misc** 46,XX (37 yrs) ***marked perivillous fibrin deposition in>90% of villi***
6-3B Emb misc 46,XY (38 yrs) ***marked perivillous fibrin involving 80% of villi;***
6-3C Emb misc 46,XX (38 yrs) ***extensive perivillous fibrin with villous fibrosis***
6-3E Emb misc 46,XY (39 yrs) ***multifocal perivillous fibrin with villous fibrosis***

CNV=Minute duplication of TIMP2 gene (results in TIMP2 disruption)



TIMP2 gene

Critical role in modulating invasion of the trophoblast into maternal decidua, endometrium, as well as in vascular remodeling and angiogenesis in the first trimester.

It is suspected to be expressed only from the maternal allele in placenta (based on finding that it is not expressed in complete moles, and shows an altered expression in mouse model of RPL)

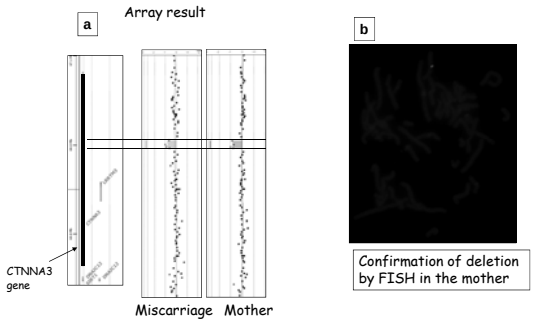
?Lack of functional maternal copy in the conceptus prevents placenta development/function and leads to miscarriage

Example 2

RPL Couple
13 miscarriages
one studied by array

Anemb misc (29 yrs);
Anemb misc (30 yrs);
Biochem misc (30 yrs);
Anemb misc (31 yrs);
Anemb misc (31 yrs);
Anemb misc (32 yrs);
Emb misc (32 yrs);
Biochem misc (32 yrs);
Yolk sac misc (33 yrs);
Emb misc (33 yrs);
Twins: anemb and yolk sac misc (33 yrs)
→**7-3A Emb misc 46,XX (34 yrs)**;
Gestational surrogacy term twins 3345, 3487 gm, both 46,XY (35 yrs)

CNV=deletion of catenin gene (alpha catenin)



Confirmation of deletion by FISH in the mother

Alpha catenin (*CTNNA3*)

•Function: Alpha catenin gene belongs to a family of catenin genes that control morphogenesis, differentiation and remodeling of the placenta

•Expression from the maternal copy of the gene in placenta

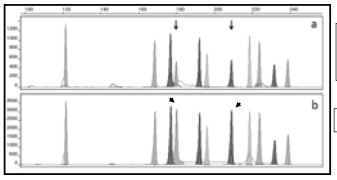
•?Lack of functional maternal copy in the conceptus prevents placenta development/function and leads to miscarriage

Recurrence of affected genes in additional females with RPL

Dr Wendy Robinson and Dr Dan Diego

Multiplex Fluorescent PCR of Short Fragments to screen for alpha catenin and TIMP2 copy number changes in 261 females with RPL and 60 control fertile females

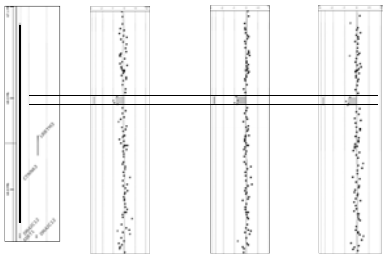
1 additional female with CTNNA3 deletion found in the RPL cohort and not in controls



Women with RPL with CTNNA3 deletion

Fertile control

Alpha catenin deletion



Miscarriage

Mother with RPL

Additional female from the RPL cohort screened specifically for catenin deletion

Results-summary of 2 studies

	Study 1 (Sporadic miscarriages, embryoscopy)	Study 2 Miscarriages from couples with RPL
Frequency of unique CNVs	30% miscarriages	50% of miscarriages
Origin of CNVs	6% de novo 24% familial	All familial
Size of CNV	All CNVs smaller than 250kb	>80% were smaller than 250kb

Conclusions

1. Array CGH detected *de novo* CNVs in 6% of sporadic miscarriages. This is less than the frequency of de novo CNVs in chromosomally normal subjects with developmental abnormalities observed postnatally (10-15%)

2. CNVs are small in miscarriages (>90% are smaller than 250kb). In comparison 25% of pathogenic CNV are small in subjects with developmental abnormalities observed postnatally (75% are large and >1Mb)

3.Whole genome array analysis has the potential to identify CNVs that contain new culprit "miscarriage" genes (e.g. those imprinted in placenta)

4. By further studying additional miscarriage specimens and parental DNA, we should be able to identify couples who have a genetic basis for their history of RPL.

Array CGH as routine clinical test in miscarriages

Pro	Con
Detects all large chromosomal imbalances as conventional cytogenetics (except ploidy changes);	Quality and amount of DNA from miscarriages poor (DNA degraded, DNA from paraffin embedded not optimal)
Arrays are quick (4 days) and DNA based so tissue culture failure or maternal contamination not an issue	Confirmation of CNV is more challenging-no chromosomes for FISH, confirmation has to be DNA based
Detects potentially pathogenic CNVs that cause or contribute to miscarriage	More parental investigations 30-40% of couples will have to be investigated for the presence of unique CNVs detected in miscarriages (currently ~5% miscarriages show structural chromosomal abnormalities that require parental follow-up)
Improves understanding of genetic and biological factors implicated in early human development	Increased genetic counseling necessary for uncertain findings

Key collaborators:
Dr Mary Stephenson, University of Chicago
Dr Tom Philipp, Donau Hospital, Vienna

Vancouver Laboratory:

Dr. Christine Tyson
Dr. Ying Qiao
Chansonette Harvard
Sally Martell
Celina Fawcett

Funding agency

CIHR IRSC

References:

1. Study 1: Rajcan-Separovic E, Qiao Y, Tyson C, Harvard C, Fawcett C, Kalousek D, Stephenson M, Philipp T. Mol Hum Reprod. 2010 Feb;16(2):125-34.

2. Study 2: Rajcan-Separovic E, Diego-Alvarez D, Robinson WP, Tyson C, Qiao Y, Harvard C, Fawcett C, Kalousek D, Philipp T, Somerville MJ, Stephenson MD. Hum Reprod. 2010 Nov;25(11):2913-22.

Non-invasive Prenatal Diagnosis Using Cell-Free Nucleic Acids

Diana W Bianchi, M.D.

ESHRE Pre Congress Course 3

"From Genes to Gestation"

Disclosure:

I am the Chair of the Clinical Advisory Board of Verinata Health, Inc. and I hold equity options in this company.

Learning Objectives

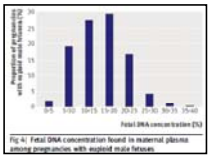
- **Understand why this talk is relevant: Case scenario**
- **Learn about cell-free DNA in maternal blood**
  - **Introduce its biology and metabolism**
- **Apply this technology to clinical medicine**
  - **Fetal sex determination**
  - **Fetal Rhesus D diagnosis**
  - **Can it be used in twin gestations?**
- **Understand potential future clinical applications**
  - **Aneuploidy**
  - **Single gene disorders**

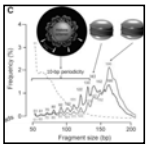
Case Scenario- Why Is NIPD Relevant in This Course?

- 40 year old G1P0 conceived after multiple IVF cycles
- First trimester prenatal screening shows risk of Down syndrome of 1 in 1500
- Second trimester anatomy scan shows soft marker (echogenic intracardiac focus) of Down syndrome
- Couples wants reassurance that the fetus has normal chromosomes but given "precious status" of this pregnancy they are unwilling to undergo invasive procedure and associated small but real risk of miscarriage
- Is NIPD for aneuploidy available to them?

Cell-free Fetal DNA in Maternal Serum/Plasma: Essential Facts

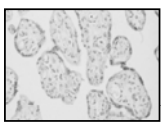
- Both the mother and fetus produce cell-free DNA
  - Maternal DNA originates in bone marrow
  - Fetal DNA originates in placenta
- A maternal plasma sample contains ~90% maternal and ~10% fetal cell-free DNA
- It circulates in nucleosomes that are likely from ruptured apoptotic bodies
- Fetal DNA fragments are shorter than maternal DNA fragments





From Lo et al. 2010

Evidence That the Cell-Free Fetal DNA in Maternal Blood Comes from the Placenta as Opposed to the Fetus



Cleaved caspase 3 immunohistochemical staining on villi showing areas of apoptosis

- **Detectable in maternal circulation before placental circulation is established** (Guibert et al. 2003)
- **Detectable in anembryonic gestations** (Alberry et al. 2007)
- **In cases of confined placental mosaicism, DNA sequences in maternal blood reflect the placental karyotype** (Masuzaki et al. 2004)

Diagnostic Applications of Cell-Free Fetal DNA in Maternal Plasma

- **Detection of fetal DNA in maternal plasma can be <u>quantitative</u>**
  - **Elevation in a complication of pregnancy**
    - ***Pre-eclampsia***
    - ***Unstoppable pre-term labor***
- **Y chromosome can be used as a fetal marker if male**
- **Epigenetic sequences (differentially-methylated in fetus or placenta versus mother) can serve as gender-independent DNA markers**
  - **These include hypomethylated *maspin* and hypermethylated *RASSF1A* in placenta**
- **Or, detection of fetal DNA can be <u>qualitative</u>**
  - **Unique fetal sequence inherited from father is present or absent**

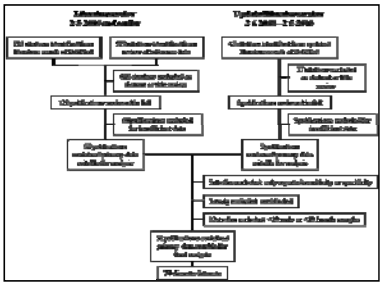
DTC Genetic Testing: The "Dark Side"

Noninvasive Prenatal Diagnosis of Fetal Sex: Is It Accurate?

(Devaney et al, 2011, in review)

- **Performed a meta-analysis of the existing medical literature to evaluate internet-based claims**
- **We asked the following questions:**
  - **How reliably can fetal gender be predicted by testing of cell-free fetal DNA using maternal blood?**
  - **How do analytic and clinical validity vary by testing methodology, sample type, amount of DNA available, GA at sampling, Y sequence amplified?**

Pub Med Literature Search for the Evidence Review



792 abstracts, 135 papers read in full

Results of Evidence Review
(Devaney et al. 2011, in review)

- Only studies with > 10 male and female subjects included
- 46 publications: 3352 male and 2825 female fetuses
- Overall sensitivity was 95.3% (CI 94.5-96.0%), specificity was 98.5% (CI 98.0-99.0%)
- Claims of accuracy < 7 weeks were unsubstantiated
- After 20 weeks' sensitivity and specificity was 100%
- RT-PCR performed better than conventional PCR
- No difference in type of sample: whole blood vs. plasma vs. serum
- Maternal urine unreliable

Current Clinical Status of Non-invasive Prenatal Diagnosis Using Cell-Free Nucleic Acids in Maternal Blood

Fetal Sex Determination

- Useful for fetuses at risk for X-linked conditions
  - *Can reduce the need for invasive testing*
- If fetus at risk for congenital adrenal hyperplasia, may help in decision to use maternal steroids
  - *(not needed if fetus is male)*
- May help in management of ambiguous genitalia detected by sonogram
  - *Fetuses with Y chromosome should be raised as males*

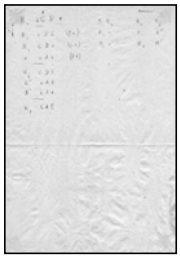
Effects of NIPD on Pregnancy Management

Data from Lyn Chitty, University College London, UK

- **X-linked Disorders (n=332)**
  - **No invasive test performed in 45% of cases**
- **Hemophilia (n=114)**
  - **Only 8% of pregnancies had invasive testing**
- **Congenital adrenal hyperplasia (n=123)**
  - **No invasive test performed in 45% of cases**
  - **Avoided steroid treatment in 18% of cases**
  - **Stopped steroids at <11 weeks in 36% of cases**

Noninvasive Prenatal Diagnosis of Rhesus D

- **15% of Caucasians, 3-5% of Africans, and very few Asians are *RhD* negative**
- **Noninvasive determination of *RhD* status is clinically useful because no further testing or therapeutic procedures are necessary if the fetus is *RhD* negative**
- **Most *RhD* negative pregnant women have a deletion of the gene on both copies of chromosome 1**
- **Detection of *RhD* in maternal plasma indicates an *RhD* positive fetus**



Sir Ronald Fisher Archive, U of Adelaide

Current Commentary

Noninvasive Prenatal Diagnosis of Fetal Rhesus D

Ready for Prime(r) Time

Diana W. Bianchi, MD, Neil D. Avent, PhD, Jean-Marc Costa, PhD, and C. Ellen van der Schoot, MD, PhD

- **Highly accurate (>95%) in large-scale clinical trials performed in the UK, the Netherlands, and France**
- **False-negative cases due to early gestation or insensitive methods to detect fetal DNA**
- **False-positive cases due to non-deletion genotypic variants (pseudogenes) in African individuals**
- **In 2005 we wrote that the US was ready for this testing-what is taking so long for routine incorporation into prenatal care?**

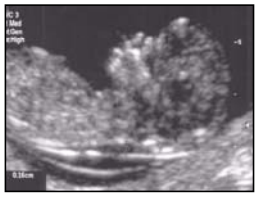
VOL. 106, NO. 4, OCTOBER 2005 OBSTETRICS & GYNECOLOGY 1

Current Clinical Status of Non-invasive Prenatal Diagnosis Using Cell-Free Nucleic Acids in Maternal Blood

- **Rhesus D**
  - **Science is solid**
  - **Clinically available in EU for 6 years, in US since Dec 2007**
  - **Little clinical uptake to date in US-why?**
    - ***Educational issues?***
    - ***Medico-legal issues?***
      - **What about false negatives due to too little DNA?**
    - ***Intellectual property issues?***
      - **May affect availability of testing sites in US**
      - **Not so much an issue in EU**

Current Clinical Status of Non-invasive Prenatal Diagnosis Using Cell-Free Nucleic Acids in Maternal Blood

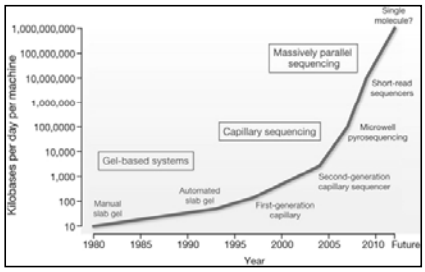
- **Trisomy 21**
  - **Technical problems have been largely solved**
  - **Coming soon?**



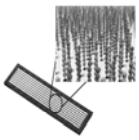
Multiple Approaches to NIPD of Aneuploidy

- **Cell-free DNA in maternal serum/plasma**
  - **Measure amount of fetal DNA: ~2-fold higher in trisomy 21 cases**
  - **Find differentially-methylated sequences on chromosome 21**
    - ***This reflects placental DNA***
    - ***Recent promising results using methylated DNA immunoprecipitation to examine fetal-specific DNA methylation ratios***
- **Cell-free RNA in maternal serum/plasma**
  - **Find gene sequences that map to chromosome 21, such as *PLAC4***
  - **Measure ratios of different alleles (SNPs) that reflect the number of chromosome 21s present**
    - ***Requires heterozygosity in DNA sequences from parental chromosomes***
- **Cell-free DNA in maternal serum/plasma**
  - **Measure amount of chromosome 21 DNA relative to a standard using next-generation sequencing**

Improvements in DNA Sequencing Technology: Implications for Prenatal Diagnosis

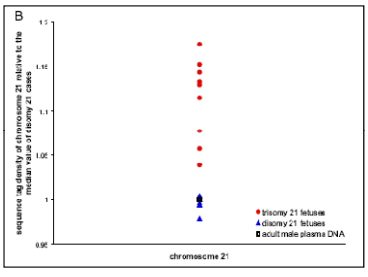


Advantages of high-throughput sequencing



1. **Entire process is automated**
2. **Multiple samples can be simultaneously analyzed**
3. **DNA is bound to a solid support, thousands of sequencing reactions can occur in parallel**

2008: Feasibility of Using Massively Parallel Sequencing Technology for NIPD of Trisomy 21 Shown



From Fan et al. Proc Natl Acad Sci USA 2008;105:16266

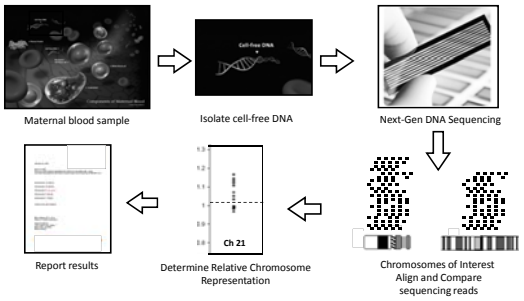
-Extremely sensitive

-Involves sequencing of 36 bp reads of DNA, mapping to chromosome of origin

-If extra 21 material is present it is readily apparent

-20-25 million sequence tags/sample

Diagnosis of Trisomy 21 by DNA Sequencing



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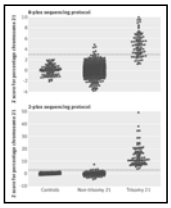
First Large-Scale Clinical Trial of NIPD of Trisomy 21 Using Sequencing
Chiu et al. BMJ 2011; 342:c7401

- **753 samples (prospective and retrospective)**
- **86 cases of trisomy 21 included**
- **8-plex approach 79% sensitivity, 99% specificity**
- **2-plex approach 100% sensitivity, 98% specificity**
- **Conceived of as a way to reduce invasive procedure rate (2nd tier screen)**
- **Could reduce from 573 to 11 procedures in high-risk population**



Chiu et al. BMJ 2011 study

- **Strengths**
- **Diagnostic performance compared against karyotype**
- **Largest clinical study to date of high throughput sequencing**
- **Largely first trimester samples**

- **Weaknesses**
- **Mix of prospective and retrospective samples**
- **100-fold increased prevalence of trisomy 21**
- **Positioned as 2nd tier screen, not diagnostic**
- **Cost=$700 per sequencing reaction, $6 million in equipment**
- **Could not dx trisomy 18**

Second study of NIPD of Trisomy 21


REPORTS OF MAJOR IMPACT www.AJOG.org

UNDER EMBARGO UNTIL FEBRUARY 10, 2011, 12:01 AM ET

Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: a study in a clinical setting

Mathias Ehrich, MD; Cosmin Deciu, MSc; Tricia Zwiefelhofer; John A. Tynan, DPhil; Lesley Cagasan, MSc; Roger Tim, DPhil; Vivian Lu; Ron McCullough, DPhil; Erin McCarthy; Anders O. H. Nygren, DPhil; Jarred Dean; Lin Tang, DPhil; Don Hutchison, MSc; Tim Lu, DPhil; Huiquan Wang, DPhil; Vach Angkachatchai, DPhil; Paul Oeth, MSc; Charles R. Cantor, DPhil; Allan Bombard, MD; Dirk van den Boom, DPhil

- Internal study performed at Sequenom
- 449 High-risk samples
- All 39 trisomy 21 cases identified (100% sensitivity)
- 409/410 euploid cases identified (99.7% specificity)
- Larger clinical validation study later this year

Use of Chromosome Ratios Allows Noninvasive Diagnosis of Trisomies 21 and 18

Sehnert et al. Clin Chem 2011; in press

- 1014 samples collected prospectively pre-invasive procedure
- Ethnically diverse population
- Preparation and sequencing performed blindly
- Training set: 26 abnl + 45 nl = 71 samples
- Test set: 27 abnl + 21 nl = 48 samples
- Single end 36 bp reads sequenced and aligned to human genome assembly 18 UC Santa Cruz
- Normalized sequence reads on chromosome of interest to another chromosome (21 to 9, 18 to 8, etc.)

The significance of normalizing chromosome ratios

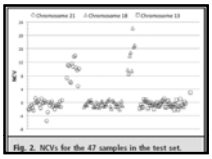


Fig. 2. NCVs for the 47 samples in the test set.

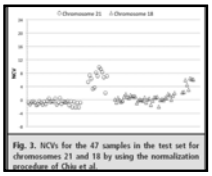


Fig. 3. NCVs for the 47 samples in the test set for chromosomes 21 and 18 by using the normalization procedure of Chiu et al.

Data from Sehnert et al. Clin Chem 2011

Table 3. Test set classification data.

	T21 classification		
	Classification		
Karyotype	Unaffected, n	T21, n	No call, n
Diploid Chr 21*	34		
47, XX, or XY, +21		13	
	T18 classification		
	Classification		
Karyotype	Unaffected, n	T18, n	No call, n
Diploid Chr 18	39		
47, XX, or XY, +18		8	

What About Twin Gestations?

- **Sehnert et al. study included 5 sets of twins (4 in training set, 1 in test set)**
- **Asked question whether different amounts of fetal DNA in twin gestation would confound results?**
- **All twin gestations were correctly classified**
  - **In 3 sets both twins were unaffected**
  - **In one set both twins were affected with trisomy 21**
  - **One set was fraternal with one affected fetus (sample was called affected)**

Noninvasive Prenatal Diagnosis of Aneuploidy: What is the Best Technique?

Current ultrasound/analyte approach	Future cell-free fetal DNA/approach
• Already in clinical practice	• Still in early stage trials
• Results validated in several large-scale clinical trials	• Unclear if existing IP will impede translation to practice
• First trimester scan gives additional information regarding CHD, other anomalies, single gene disorders	• Sequencing equipment, bioinformatics, data storage are expensive
• Less expensive, required equipment widely available	• Could be diagnostic (or an advanced screen)
• Not diagnostic	

Summary of My Talk Today-1

- **Cell-free DNA in maternal blood**
  - **Mainly originates from the placenta**
- **Current clinical uses**
  - **Fetal sex determination**
    - ***Accurate for medical indications in CLIA-certified labs***
    - ***Could reduce the rate of invasive procedures for X-linked conditions***
    - ***Could reduce steroid administration in CAH***
    - ***Beware of the "dark side" of direct to consumer testing!***
  - **Fetal Rhesus D diagnosis**
    - ***Accurate for medical indications in CLIA-certified labs***
    - ***Reduces the need for Rhesus D immune globulin if fetus is Rhesus D negative***

Summary of My Talk Today-2

- **Noninvasive Prenatal Diagnosis of Aneuploidy**
  - **Made possible by advances in high-throughput DNA sequencing**
    - ***Technique is fully automated***
    - ***Does not require genetic marker heterogeneity between the parents (no need for a paternal sample)***
    - ***Costs are still high***
  - **Larger-scale prospective blinded clinical trials are still needed to evaluate performance**
  - **These are ongoing (mainly organized by industry groups)**
  - **It is unclear at present whether test will be better utilized as a second tier screen or a noninvasive diagnostic test**

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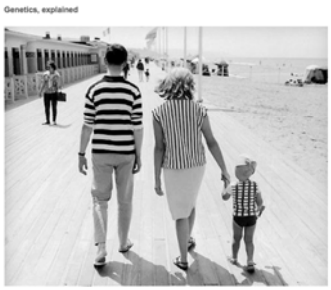
Thank you for your attention!



Genetics of Molar Pregnancies

Rosemary Fisher PhD, FRCPath

Department of Oncology, Imperial College Healthcare NHS, and Institute of Reproductive & Developmental Biology, Imperial College London, UK

The author has no commercial/financial or other conflicts of interest

Imperial College London

Learning Objectives

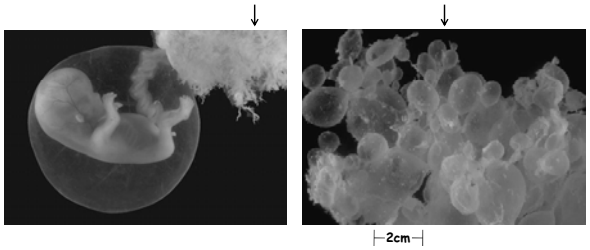
- Be able to describe the differences between complete and partial hydatidiform moles.
- Be able to describe the genetic origin of typical complete and partial hydatidiform moles.
- Be familiar with the characteristics of familial recurrent hydatidiform mole (FRHM) syndrome.
- Understand how genetic testing can be used to make a differential diagnosis between sporadic hydatidiform moles and FRHM syndrome.
- Understand the genetic basis of FRHM syndrome.

Lecture - Outline

- Molar pregnancies – clinical background
- Genetics of typical complete and partial hydatidiform moles
- Genetic diagnosis of molar pregnancies
  - Fluorescent microsatellite genotyping
- Genetics of recurrent molar pregnancies

Molar Pregnancy - Hydatidiform Mole

Normal placental villi ↓ Molar placental villi ↓



Normal Pregnancy | Complete Hydatidiform Mole

Hydatidiform Moles

Approx 1 in 600 viable conceptions are HMs – UK *

40%	60%
Complete mole	Partial mole
Marked cystic villi	Less marked placental abnormalities Range of villi from normal to cystic
No fetus	Fetus may be present - abnormal

** Savage et al; 2010*

Gestational Trophoblastic Diseases

Premalignant	Malignant
Hydatidiform Moles	Trophoblastic Tumours
• Complete mole	• Choriocarcinoma
• Partial mole	• Placental Site TT
	• Epithelioid TT

• Invasive mole

Gestational Trophoblastic Disease

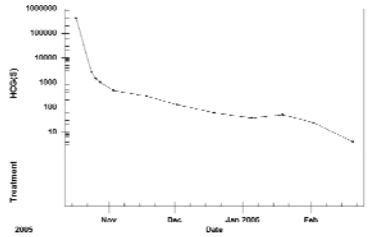
Incidence of gestational trophoblastic tumours

Normal pregnancy	1 in 40 - 50,000
Complete hydatidiform mole	1 in 8
Partial hydatidiform mole	1 in 100

Greatest risk factor for gestational trophoblastic neoplasia is a molar pregnancy

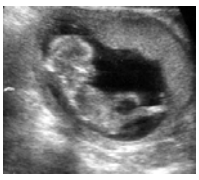
Hydatidiform Moles

Patients with a HM are registered and screened monitored using serial levels of human chorionic gonadotrophin

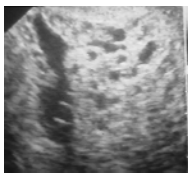


If hCG levels fail to fall or rise then chemotherapy is started

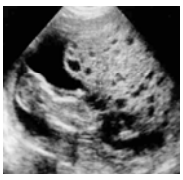
Diagnosis of Hydatidiform Moles - Ultrasound



Normal Pregnancy



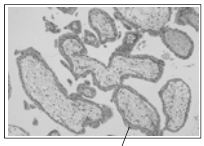
Complete hydatidiform mole



Partial hydatidiform mole

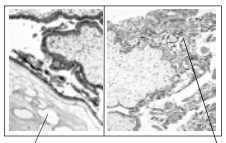
Pathology

Placenta



Placental villous surrounded by two layers of trophoblastic cells

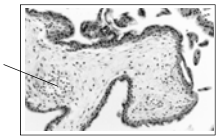
CHM



Hydropic villi

Trophoblastic hyperplasia

Presence of a fetus or fetal red blood cells



PHM

Genetics of Hydatidiform Moles

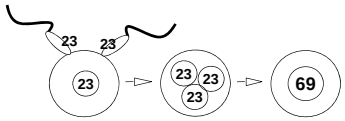
Partial Mole	Complete Mole
Triploid conceptus with 69 chromosomes	Diploid conceptus with 46 chromosomes
69,XXX 69,XXY 69,XYY	46,XX 46,XY

Szulman and Surti 1978

Genetic Origin of Hydatidiform Moles

Partial Mole

Two sperm fertilise an ovum



Triploid conceptus with 69 chromosomes

The additional chromosome set is paternal

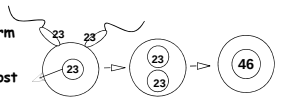
Two paternal contributions to the genome

Genetic Origin of Complete Moles

Dispermic Complete Mole - 20%

The egg is fertilised by two sperm

Maternal chromosomes are lost



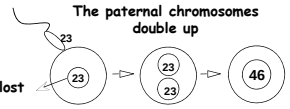
46,XX
46,XY

Monospermic Complete Mole - 80%

The egg is fertilised by a single sperm

The paternal chromosomes double up

Maternal chromosomes are lost



46,XX

Two paternal contributions to the genome

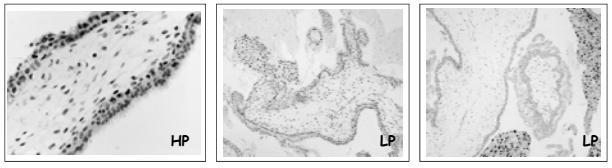
Expression of Imprinted Genes in HM

A small number of genes are expressed only from the maternally or the paternally inherited allele

PHM	CHM	
Overexpression of paternally expressed genes	Overexpression of paternally expressed genes	→ Trophoblastic hyperplasia
	+ Loss of maternally expressed genes	→ Loss of fetal development

$p57^{KIP2}$ Expression in HM

Placenta PHM CHM



$P57^{KIP2}$ - expressed only from the maternally derived allele in the villous trophoblast

Diagnostic Problems in Molar Pregnancies

- PHM mole or non-molar abortion
- Hydatidiform mole + fetus
- Women with recurrent hydatidiform moles

Fluorescent Microsatellite Genotyping

Blood

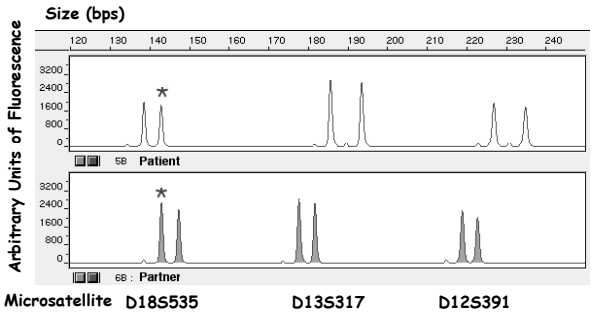
Sections from pathological blocks

DNA

Polymerase Chain Reaction
amplify polymorphic microsatellite sequences
on different chromosomes

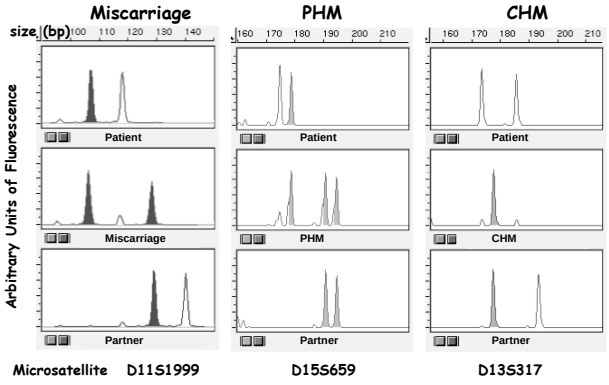
Separate by capillary electrophoresis in a 310 ABI PRISM Genetic Analyser

Microsatellite Polymorphisms



*Uninformative allele

Microsatellite Polymorphisms in PHM and CHM



Microsatellite D11S1999 D15S659 D13S317

HM with Co-existent Fetus

Case History

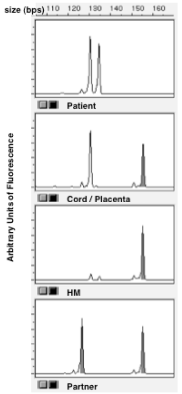
Apparently normal female infant

Single placenta

- areas of normal placental villi
- areas of CHM

Confined Placental Mosaisicm

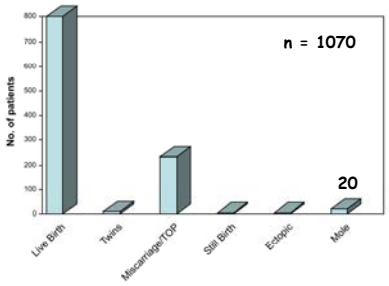
Makrydimas et al, 2002



D1S1656

Recurrent Hydatidiform Moles

Future Pregnancies in Women with HM

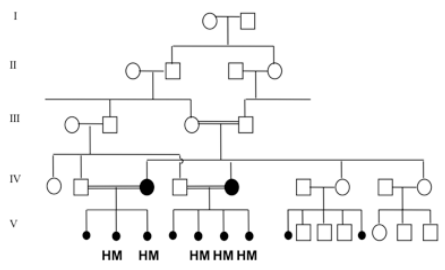


Outcome for women with a history of HM who became pregnant in 2007

Familial Recurrent HM Syndrome

- 2% of women with a HM have a second HM
- This includes a mall number of women who have an inherited predisposition to recurrent CHM
- Associated with families where one or more women are affected

Familial Recurrent HM Syndrome



Familial Recurrent HM Syndrome

- Women with this condition rarely have normal pregnancies

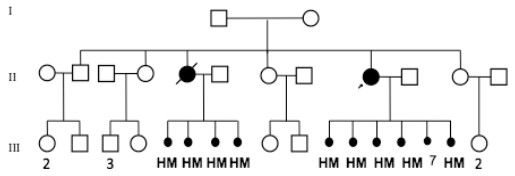
Familial recurrent HM - 14 families - 37 affected women

Normal pregnancies	- 7	(5%)
Pregnancy losses	- 26	(17%)
PHM	- 6	(78%)
CHM	- 113	

- Significant risk of developing GTT

Fisher et al 2004

Familial Recurrent HM Syndrome

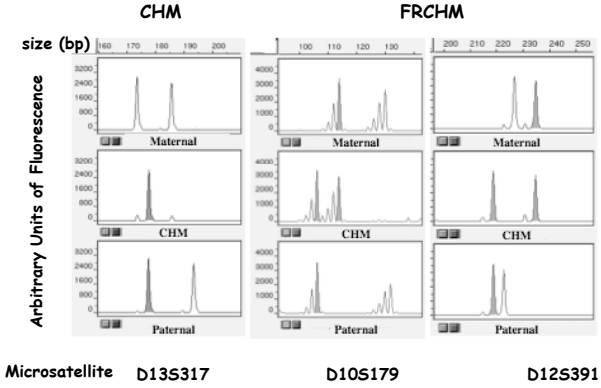


Zhao et al 2006

CHM in this condition are pathologically indistinguishable from typical androgenetic CHM

CHM in this condition are diploid but biparental in origin

Microsatellite Polymorphisms in CHM



FRHM is a Single Gene Disorder

nature genetics

Mutations in *NALP7* cause recurrent hydatidiform moles and reproductive wastage in humans

Sharlene Murdoch[1,2], Ugljesa Djuric[1,2], Batool Mazhar[3,9], Muheiddine Seoud[4,9], Rabia Khan[1,2], Rork Kuick[5], Rashmi Bagga[6], Renate Kircheisen[7], Asangla Ao[2], Bhawna Ratti[3], Samir Hanash[5], Guy A Rouleau[8] & Rima Slim[1,2]

Murdoch et al 2006

NLRP7 (NALP7)

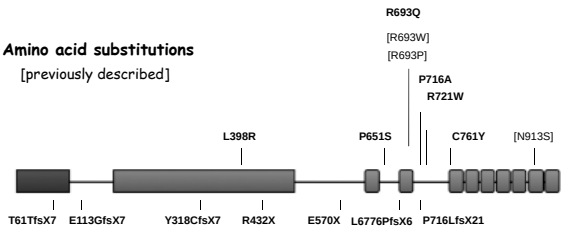
NACHT, leucine rich repeat and PYD containing 7

PYN (Pyrin-PAAD-Dapin) domain | **nucleotide binding site (NBS-NATCH subfamily)** | **leucine rich repeat region (LRR)**

NLRP7 mutations associated with FRHM

Amino acid substitutions

[previously described]

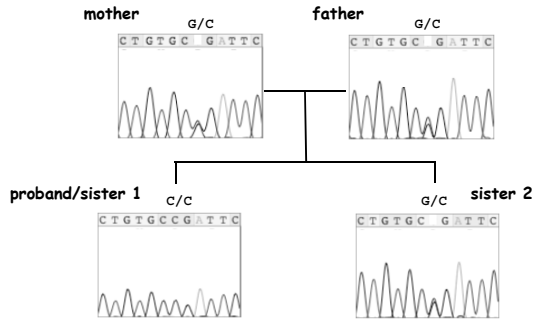


Mutations giving rise to a truncated protein

Wang et al 2009

Screening of families is feasible to identify affected individuals and carriers

Screening for Familial Recurrent HM



Mutation: c.2078G>C base change R693P protein change

NLRP7

- Approximately 50 families / individuals with recurrent BiCHM in the literature
- Almost all are homozygous or compound heterozygotes for mutations in *NLRP7*
- To date only one affected family with normal pregnancies
- Conventional IVF is unlikely to be successful for women with BiCHM

Role of *NLRP7* in pregnancy?

Males homozygous for the same mutations – no consequences *Qian et al 2007, Wang et al 2009*

Essential for normal reproduction in females

BiCHM and AnCHM have similar pathology and imprinting defects *Judson et al 2002, Fisher et al 2002*

Involved in setting the maternal imprint in the ovum?
IVF using oocyte donation?

Expressed in tissues other than oocytes – negative regulator of IL1B *Kinoshita et al 2005*

Involved in immune responses in early pregnancy?

Heterozygosity for rare *NLRP7* variants may be associated with reproductive loss *Deveault et al 2009*

NLRP7 may have a role in other types of reproductive loss ?

Summary

- Most PHM are triploid with two paternal contributions to the genome
- Most CHM are diploid and are androgenetic
- A small number of CHM (and PHM) are diploid and biparental
  - associated with a predisposition to recurrent HM
  - often identified in families / can occur in individuals
  - caused by mutations in *NLRP7*
- Not all individuals with recurrent CHM have BiCHM

Buyukkurt et al 2010

References 1:

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Diagnosis of molar pregnancies:

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Recurrrent molar pregnancies:

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Zhao J, Moss J, Sebire NJ, Cui QC; Seckl MJ, Xiang Y, Fisher RA. Analysis of the chromosomal region 19q13.4 in two Chinese families with recurrent hydatidiform mole. Hum Reprod 2006; 21: 536-41

Murdoch S, Djuric U, Mazhar B, Seoud M, Khan R, Kuick R, Bagga R, Kircheisen R, Ao A, Ratti B, Hanash S, Rouleau GA, Slim R. Mutations in NALP7 cause recurrent hydatidiform moles and reproductive wastage in humans. Nat Genet 2006; 38: 300-302

Wang C, Dixon P, Decordova S, Hodges M, Sebire N, Ozalp S, Fallahian M, Sensi A, Ashrafi F, Repiska V, Zhao J, Xiang Y, Savage P, Seckl M, Fisher R. Identification of 13 novel NLRP7 mutations in 20 families with recurrent hydatidiform mole; missense mutations cluster in the leucine rich region. J Med Genet. 2009; 46: 569-75

References 3:

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Judson H, Hayward BE, Sheridan E, Bonthron DT. A global disorder of imprinting in the human female germ line. Nature 2002; 416: 539-542

Kinoshita T, Wang Y, Hasegawa M, Imamura R, Suda T. PYPAF3, a PYRIN-containing APAF-1-like protein, is a feedback regulator of caspase-1-dependent interleukin-1beta secretion. J Biol Chem 2005; 280: 21720-5

Reubinoff BE, Lewin A, Verner M, Safran A, Schenker JG, Abeliovich D. Intracytoplasmic sperm injection combined with preimplantation genetic diagnosis for the prevention of recurrent gestational trophoblastic disease. Hum Reprod 1997; 12: 805-808

Qian J, Deveault C, Bagga R, Xie X, Slim R. Women heterozygous for NALP7/NLRP7 mutations are at risk for reproductive wastage: report of two novel mutations. Hum Mutat 2007; 28: 741.

Deveault C, Qian JH, Chebaro W, Ao A, Gilbert L, Mehio A, Khan R, Tan SL, Wischmeijer A, Coullin P, Xie X, Slim R. NLRP7 mutations in women with diploid androgenetic and triploid moles: a proposed mechanism for mole formation. Hum Mol Genet 2009; 18: 888-97

Buyukkurt S, Fisher RA, Vardar MA, Evruke C. Heterogeneity in Recurrent Complete Hydatidiform Mole: Presentation of Two New Turkish Families With Different Genetic Characteristics. Placenta 2010; 31: 1023-5.

Gene therapy for the fetus: how far have we come?

Prof Donald Peebles MA MD FRCOG
Professor of Obstetrics and Honorary Consultant in Maternal/Fetal Medicine
UCL Institute for Women's Health

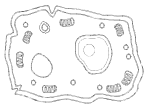
Some vectors used in this research supplied by ARK Therapeutics

Objectives

- Rationale for fetal gene therapy
- Explore which diseases are best candidates
- Choice of vector systems
- Examples of successful pre-clinical studies
- Targeting therapy to right organ
- Factors effecting length of transgene expression
- Safety issues
- Ethics
- Regulatory challenges and human studies

Gene therapy……

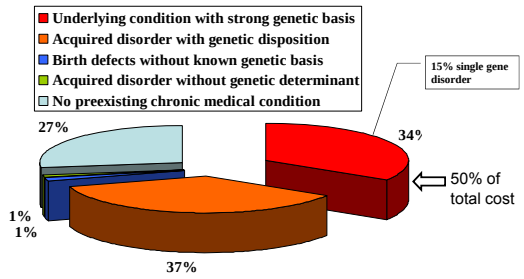
….uses genetic material as a drug delivery vehicle to facilitate the expression of therapeutic proteins



A "tool" for treating/preventing disease

The Burden of Genetic Conditions

5,747 children's admissions to a paediatric hospitals

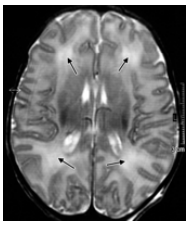


McCandless et al Am J Hum Genet 2004

Acquired conditions

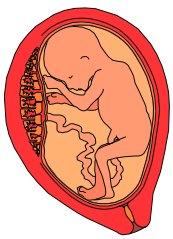
Severe fetal growth restriction

Hypoxic ischaemic encephalopathy

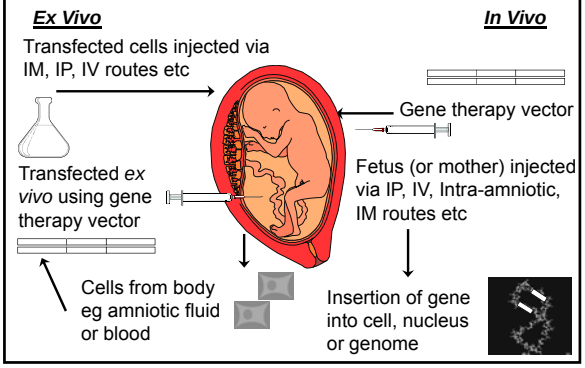


Are there advantages to giving therapy prenatally?

- Treat before irreversible damage by disease
- Target cells inaccessible in adult life
- Stem cell populations
- More efficient gene transfer
- Functional immaturity of immune system
- Fetal size: vector ratio



Gene therapy treatment



Which diseases?

NIH Recombinant DNA advisory committee.

Initial application of prenatal gene therapy should be limited to diseases where:

- Serious morbidity and mortality risks for the fetus exist either *in utero* or postnatally,
- No effective postnatal therapy is available
- Associated abnormalities can be corrected by the transferred gene
- Prenatal diagnosis is possible and there is a well defined genotype/phenotype relationship
- An animal model for the disease is available.

Human Gene Therapy 2000

Candidate diseases

Disease	Gene
Haemophilia: Factor VII deficiency	clotting factor VII
Haemoglobinopathy: α^0 thalassaemia	α globin chain
Cystic fibrosis	CFTR
Metabolic disorders: Crigler-Najjar type 1 syndrome	UDP glucuronyl–transferase
Storage diseases: Mucopolysaccharidosis type VII	β-glucuronidase
Muscular dystrophy: Duchenne	dystrophin
CNS: spinal muscular atrophy	survival motor neuron protein
Skin: dystrophic epidermolysis bullosa	type VII collagen
Hypoxic ischaemic encephalopathy	neurotrophic factors
Severe fetal growth restriction	Vascular Endothelial Growth Factor

The aim of therapy

- Expression of gene from single delivery
  - For the duration of the disease
    - Long term preferably for the lifetime of the individual
    - Short term for perinatal acquired conditions
  - Safe
  - Regulated expression
  - Therapeutic levels
- Does not interfere with development of fetal organs
- No adverse outcome for mother or future progeny

Integrating vectors eg retrovirus

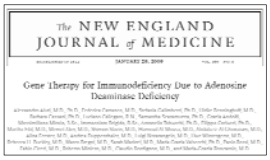
Reverse transcription

Integrate into genome

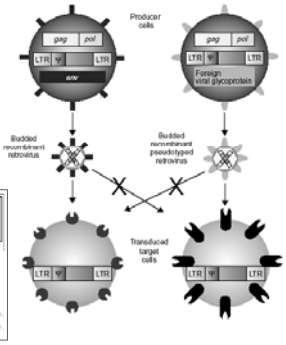
Insertional mutagenesis

Pseudotyping

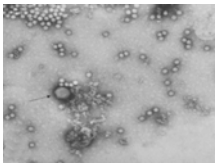
Self-inactivating lentivirus


The NEW ENGLAND JOURNAL of MEDICINE

Gene Therapy for Immunodeficiency Due to Adenosine Deaminase Deficiency



Adeno-associated virus vectors: AAV



AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B

Blood 2003

Catherine S. Manno, Amy J. Chew, Sylvia Hutchison, Peter J. Larson, Roland W. Herzog, Valder R. Arruda, Shing Jen Tai, Margaret V. Ragni, Arthur Thompson, Margareth Ozelo, Linda B. Couto, Debra G. B. Leonard, Frederick A. Johnson, Alan McClelland, Ciaran Scallan, Erik Skarsgard, Alan W. Flake, Mark A. Kay, Katherine A. High, and Bertil Glader

Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response

Nature Medicine 2006

Catherine S Manno[1,2,15], Glenn F Pierce[3,15], Valder R Arruda[1,2,15], Bertil Glader[4,15], Margaret Ragni[5], John J E Rasko[6], Margareth C Ozelo[7], Keith Hoots[8], Philip Blatt[9], Barbara Konkle[10], Michael Dake[4], Robin Kaye[1,2], Mahmood Razavi[4], Albert Zajko[10], James Zehnder[4], Pradip K Rustagi[11], Hiroyuki Nakai[4], Amy Chew[1,2], Debra Leonard[2,12], J Fraser Wright[3], Ruth R Lessard[3], Jürg M Sommer[3], Michael Tigges[3], Denise Sabatino[1], Alvin Luk[3], Haiyan Jiang[3], Federico Mingozzi[3], Linda Couto[3], Hildegund C Ertl[1,13], Katherine A High[1,2,14] & Mark A Kay[4]

Non-viral vectors

Naked DNA

Physical methods can improve gene transfer:

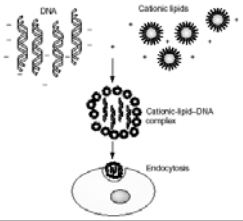
Electroporation

Magnetic gene gun

Ultrasound

Hyperdynamic injection

DNA complexes eg liposomes



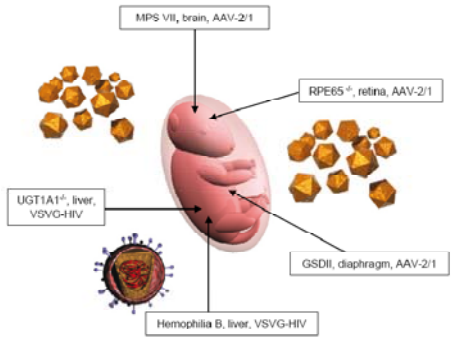
Sleeping Beauty Transposon

Journal of Gene Medicine 2007

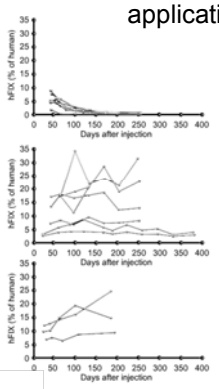
Prolonged expression of a lysosomal enzyme in mouse liver after *Sleeping Beauty* transposon-mediated gene delivery: implications for non-viral gene therapy of mucopolysaccharidoses

Elena L. Aronovich[1,4,*], Jason B. Bell[1], Lalitha R. Belur[1,4], Roland Gunther[2], Brenda Koniar[2], David C. C. Erickson[3,4], Patricia A. Schachern[4], Ilze Matise[3], R. Scott McIvor[1,4], Chester B. Whitley[3,4], Perry B. Hackett[1,4]

The evidence so far: proof of principle studies in rodents



Cure of haemophilia using prenatal application of lentivirus



Adenovirus: loss of expression in mice injected as adults

Lentivirus: permanent expression in normal mice injected as fetuses

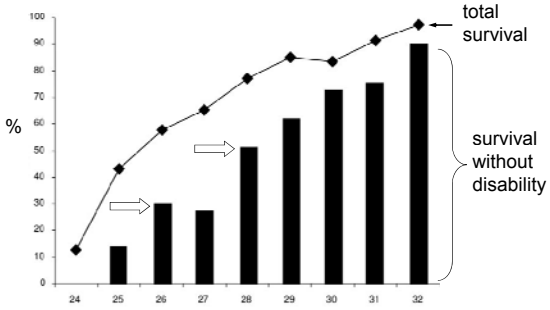
Lentivirus: safe permanent cure of haemophiliac mice after fetal injection

Waddington et al, Blood 2004

Issues facing prenatal gene therapy

- Choice of disease
- Targeting therapy to the correct organ(s)
  - Targeted delivery to the fetus
  - The best gestational age to deliver therapy
  - Manipulating the vector
  - Germline transmission
- Length of expression
  - Growth of the vector recipient
  - Vector silencing
  - Integrating vectors and insertional mutagenesis
  - Pre-existing maternal immunity
- Fetal and maternal immune response to vector and transgene
- Reversion to wild type vector
- Safety of fetus, mother and her future progeny
- Ethical concerns
- Going into humans

Neonatal outcomes in fetal growth restriction



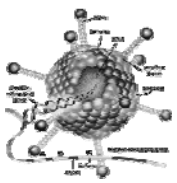
Baschat et al 2007

VEGF

- grows new vessels
- dilates vessels
- protects vessels

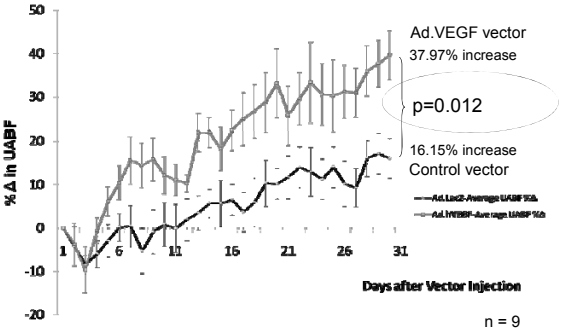
VEGF levels are abnormal in fetal growth restriction

Sustained levels of VEGF will treat fetal growth restriction



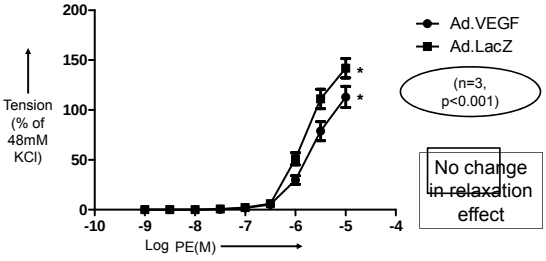
Adenovirus vector

Uterine artery blood flow increases after adenovirus VEGF vector injection



n = 9

Contractile response of transduced uterine arteries

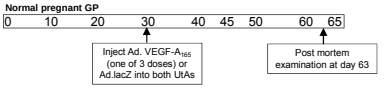


E_{max} = 155.7±13.65(Ad.VEGF side) v/s 180.7±10.39(Ad.LacZ side)

EC_{50}=3.48x10^{-6}M (Ad.VEGF side) v/s 2.33x10^{-6}M (Ad.LacZ side)

Animal models of FGR

- Human placentation is unique
- FGR guinea pig – nutrient restriction

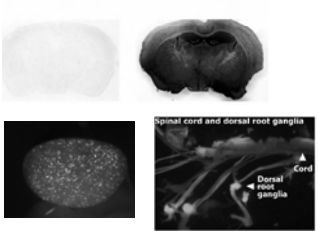


- FGR sheep – adolescent overfed ewe
  Rowett Institute, Aberdeen

UNIVERSITY OF ABERDEEN

Repairing brain injury

- Rahim et al (2009) Gene Therapy – prolonged transgene expression throughout CNS following direct injection into CNS of non integrating pseudotyped lentivirus



GFP expression 1 month following intravascular injection AAV2/9 in D16 fetal mice

Issues facing prenatal gene therapy

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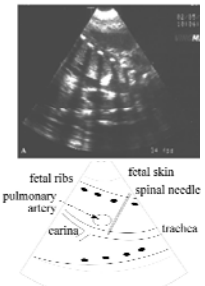
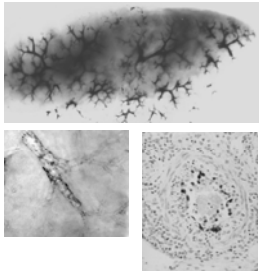
Targeted delivery to the fetus

Transthoracic injection of the fetal trachea

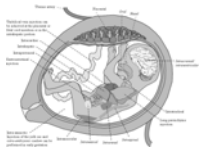


David et al, *Fetal Diagnosis & Therapy* 2003



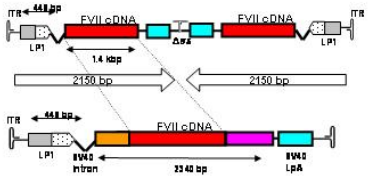
Peebles et al, *Gene Therapy* 2003

The best gestational age to deliver therapy depends on disease, access to organ and transduction efficiency



Route	Gestational age at application		
	Sheep fetus	Equivalent gestational age in the human fetus	
Amniotic	From D33	From W10	Skin, fetal membranes, airways
Peritoneal	From D50	From W14	Systemic delivery
Hepatic	From D50	From W14	Liver, haematopoietic system
Muscular	From D50	From W14	Muscle, some systemic
Umbilical vein	From D70	From W20	Systemic delivery
Pleural	From D60	From W16	Intercostal and diaphragm muscles
Cardiac	From D100	From W20	Systemic delivery
Tracheal	D80 – 115	W22 - 32	Airways
Gastric	From D60	From W16	Stomach, bowel, liver
Cerebral ventricles	D55 – 65	W15 - 17	Choroid plexus, lateral ventricle and neurocortex

Manipulating the vector:
AAV self-complementary vector



SC vector

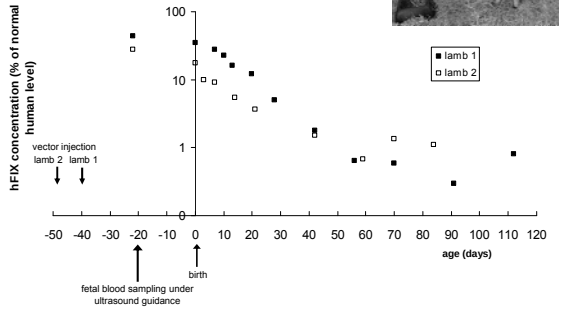
SS vector

Mini hFIX, liver-specific expression cassette packaged as complementary dimers within a single AAV virion

scAAV produces significantly higher hFIX level in macaques compared to ssAAV

Nathwani et al, Blood 2006

Growth of the vector recipient:
Plasma hFIX levels in lambs born after late gestation intraperitoneal fetal scAAV8.hFIX injection



Germline transmission

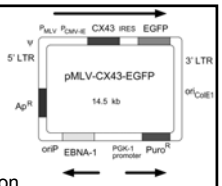
Male Germ-Line Cells Are at Risk Following Direct-Injection Retroviral-Mediated Gene Transfer *in Utero*

Christopher D. Porada,* Paul J. Park, Joe Tellez, Ferhat Ozturk, Hudson A. Glimp, Graça Almeida-Porada, and Esmail D. Zanjani

Molecular Therapy 2005

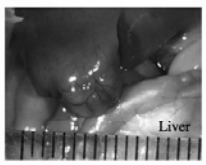
- 1st trimester injected rams estimated to have a testicular germ-cell transduction frequency of 1 in 6250 germ cells
- Compare with
  - 1 in 50 – 100 calculated frequency of naturally occurring endogenous insertions Kazazian et al Nature Genetics 1999
  - 1 in 6000 upper limit for exogenous insertions in human gene therapy trials

Vector silencing

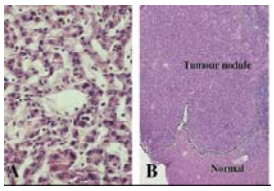


- Retrovirus MLV in particular
  - transcriptional silencing - methylation
  - variegation
  - extinction – occurs as cells differentiate
- Fetal cells and stem cells may be particularly susceptible
- Solutions
  - Insulator elements
  - Remove silencing elements in LTR
  - Lentivirus vectors

Hepatocellular carcinoma in mice injected with lentivirus as fetuses or neonates



SMART 3NZ Fetal



Vector	Tumour rate
Buffer	1 out of 20
HIV	1 out of 20
EIAV	15 out of 17

Themis et al, Mol Ther 2005

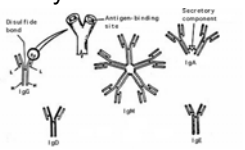
Immune response to prenatal gene therapy

Gestational age (term = 145 days) Sheep	Fetal antibodies		Maternal antibodies	
	Transgenic protein	Vector backbone	Transgenic protein	Vector backbone
≤ 65 days	-	+/-	+	+
> 65 days	+	+	+	+

David et al, *Human Gene Therapy* 2003

Pre-existing maternal immunity

- IgG can cross placenta
- May limit fetal expression
- AAV hFIX mouse experiments



Reversion to wild type vector

- Theoretical risk
- Need stringent manufacturing guidelines
  - Assays for replication competent viruses

Safety of transgenic proteins

- Developmental aspects
  - Adenovirus mediated CFTR expression in fetal rats alters lung development Larson et al, AJP 2000

Vector safety

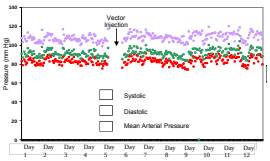
No significant changes in:

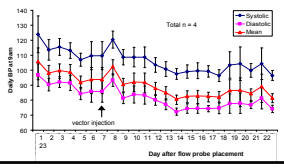
Maternal heart rate, blood pressure

Fetal heart rate, blood pressure

No fatalities

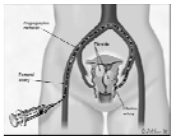
No fetal abnormalities





Going into the clinic

Injecting the uterine arteries is a well recognised clinical procedure



Growth restricted animal models

↓

Mechanism of action, safety & efficacy

↓

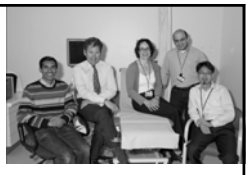
Toxicology studies
Ark Therapeutics Plc

→ Phase I/II trial

Gene therapy in prevention of perinatal conditions

- Proof of concept has been demonstrated
- Choosing the best vector, route of delivery and animal model is key
- Translation into man will be complex
- But for some diseases prenatal therapy may be the only opton

Acknowledgements



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Dr. Berrin Tezcan
Ms Beth Laverick
Ms. Laura Milross
Mr. Neil Smith
Mr. Michael Boyd

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- Basic genetics for ART practitioners
  9 September 2011 - Bucharest, Romania
- The whole man
  22-23 September 2011 - Sevilla, Spain
- Accreditation of a Preimplantation Genetic Diagnosis Laboratory
  3-4 October 2011 - Athens, Greece
- Human reproductive tissues, gametes and embryos: Innovations by science-driven culture and preservation systems
  9 October 2011 - Cairns, Australia
- Comprehensive preimplantation screening: dynamics and ethics
  13-14 October 2011 - Maastricht, The Netherlands
- Endometriosis and IVF
  28-29 October 2011 - Rome, Italy
- Endoscopy in reproductive medicine
  23-25 November 2011 - Leuven, Belgium
- What you always wanted to know about polycystic ovary syndrome
  8-10 December 2011 - Sofia, Bulgaria

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