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Imprinted Gene Expression Analysis in Human Preimplantation Embryos : Biomarkers for Assessing Epigenetic Disruption

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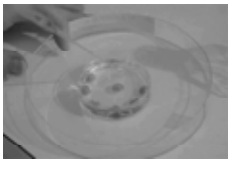
Aberrant Imprinted Gene (IG) Expression Associated With ART

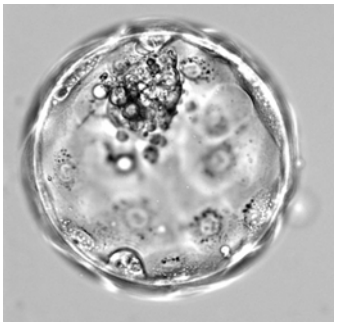
Cell Type	ART Technique/ conditions	Species	Affected Gene or Epigenetic Mark	Comments	Reference
PREIMPLANTATION EMBRYOS	Culture Media	Mouse	*H19, IgF*	Aberrant expression due to presence of FCS in M16 culture medium.	Khosla *et al.*, 2001
	Culture Media	Mouse	*H19*	Loss of *H19* methylation upon culture in Whitten's medium.	Doherty *et al.*, 2000
	Culture Media	Mouse	*IgF*	Aberrant expression bias to maternal allele in preimplantation embryo	Ohno *et al.*, 2001
	Culture Media	Mouse	*H19*	High levels of ammonium causes aberrant expression of *H19*	Lane and Gardner 2003
	Culture Media	Mouse	*IgF, Meg1 and Peg1*	Reduced expression of three imprinted genes after culture with FCS	Fernandez-Gonzalez *et al.*, 2004
	Culture Media	Mouse	*H19, IgF*	Quinn's medium causes aberrant *H19* expression in embryos, aberrant *H19* and *IgF* in ES cells	Li *et al.*, 2005
	In vitro development	Sheep	*IgF*	Aberrant expression and methylation of IgF in a Large Offspring Syndrome model	Young *et al.*, 2001
	In vitro development	Cow	*IgF, IgF*	Reduced expression in *in vitro* produced embryos compared to in vivo embryos	Gutierrez-Adan *et al.*, 2004
	In vitro culture	Mouse	*Dnmt1*	Increased *Dnmt1* expression in *in vitro* produced blastocysts	Wang *et al.*, 2005
	In vitro development	Cow	*Dnmt1, Mash2*	Increased *Dnmt1* expression, decreased *Mash2* in *in vitro* produced blastocysts	Wrenzycki *et al.*, 2001
	In vitro development	Mouse, rat	DNA methylation	Increased DNA methylation compared to *in vivo* embryos	Zaitseva *et al.*, 2007
	Culture Media	Cow	*Dnmt3a, IgF*	Upregulated expression of *Dnmt3a* and *IgF* in CR1aa and KSOMaa respectively	Sagirkaya *et al.* 2006
OOCYTES	*In vitro* growth of follicles	Mouse	*IgF, Peg1, H19*	Loss of methylation at *IgF* and *Peg1*. Gain of methylation at *H19*	Kerjean A. *et al.*, 2003
	In vitro maturation	Mouse	*Peg1*	*In vitro* culture for 8 h, *Peg1* Mat DMR becomes fully methylated. Demethylation may occur after culture for 28 hrs *in vitro*	Imamura *et al.*, 2005
	In vitro maturation	Human	*H19*	Abnormal methylation at *H19* locus	Borghol *et al.*, 2006
	Superovulation	Mouse	DNA methylation	Global methylation abnormalities	Shi and Haaf., 2002
	Superovulation	Human	*H19, PEG1*	Aberrant gain of methylation at *H19*, loss of methylation at the *PEG1* gene	Sato *et al.*, 2006
	Cause not identified	Human	*KvDMR1*	Failure to establish methylation imprint at *KvDMR1* in a MII oocyte	Geuns *et al.*, 2007
	Synthetic serum substitute in media during *in vitro* maturation	Cow	*Dnmt3a*	Significantly reduced *Dnmt3a* expression during IVM with media containing synthetic serum substitute	Sagirkaya *et al.*, 2007

Studies on Human Oocytes & Preimplantation Embryos

- 1. Establish which epigenetic regulatory factors are expressed during human oogenesis + preimplantation development
- 2. Understand **imprint** establishment and maintenance
- 3. To analyse expression of imprinted genes during normal oogenesis + preimplantation development
- 4. Compare epigenetic marks and imprinted gene expression in embryos derived from various ART treatments (IVF vs ICSI)
- All this to serve as a framework for understanding epigenetics in normal development and also ART-induced epigenetic disease



Working with Single Human Preimplantation Embryos



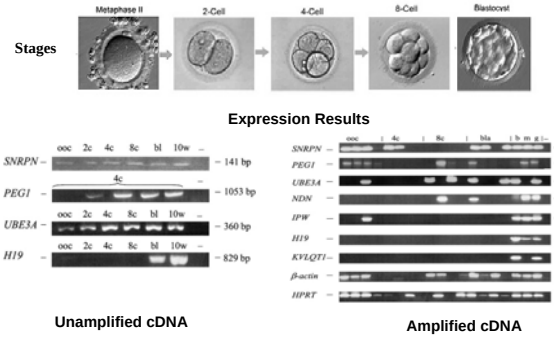
Human Blastocyst

Challenges and Restrictions
- Ethical
- Consent from patient for research use
- Quality of samples
- Sample Numbers low
- Small amount of mRNA , DNA
- Little/no functional work can be done

From Single Embryo Can Analyse:

1. Genotype
2. Methylation
3. Gene expression
4. Secretome +uptake from media

Earlier Work: Human Imprinted Gene Expression From Oocyte to Blastocyst



Salpekar A, Huntriss J, Bolton V, Monk 2001
The use of amplified cDNA to investigate the expression of seven imprinted genes in human oocytes and preimplantation embryos. Mol Hum Reprod. 7(9):839-44.

Limitations of Analysis in Human Oocytes Preimplantation Embryos

- **Limitations of non-array methods for expression**
- Non-array methods allow analysis of only ~4 genes per embryo/oocyte
- Hard to control across individual experiments
- Replicate experiments?

Focussed Microarray Analysis of Imprinted Gene Expression in Human Preimplantation Embryos

- 1. To design, develop & validate a bespoke gene expression microarray containing all human IGs
- 2. To develop gene expression microarray technology to single oocyte/embryo level to detect potential disruption of IG expression in human blastocysts.
- 3. Use to map IG expression in *in vitro* derived embryos
- 4. To use this system to test the 'safety' of existing and emerging human ART procedures including IVF, ICSI and oocyte IVM (*in vitro* maturation).

Justification of a Focussed Gene Expression Approach to Assessing Epigenetic Disruption in Human Embryos and ES cells.

- Imprinted Genes are susceptible to disruption during *in vitro* culture
- Use Imprinted Genes themselves as **Biomarkers**
- Focussed array-based methods allows analysis of all known imprinted genes (n=70) per single embryo/oocyte
- Can repeat experiments from each embryo, pool or use individually
- Gives a 'Global' idea of epigenetic disruption for imprinted genes across all chromosomes
- In contrast, alternative such as bisulphite genomic sequencing analysis limited to one gene/region.
- Controls included (sample 'quality', sexing)
- Limitation to 100 genes (total) reduces complexity of bioinformatics

Focussed Human Imprinted Gene Array Design Features

Chromosome 1 Imprinted genes: *ARH1/NOEY2, TP73*

Chromosome 6 Imprinted genes: *PLAGL1, HYMAI, IGF2R,SLC22A2, SLC22A3*

Chromosome 7 Imprinted genes :- *GRB10, PEG10, ASB4 SGCE, DLX5, calcR Neuralin / PPP1R9A, MEST(PEG1), PEG1-AS (MEST(T1)) ,COPG2,CPA4 Controls BPGM, ASNS*

Chromosome 11 Imprinted genes:- *WT1, AWT1, WT1-AS, H19, IGF2 IGF2AS, INSIrnotin, ASCL3/HASH2, PHEMX, CD81, TSSC4, TRIM5, KCNQ1/KvLQT1, KCNQ1OT1/ LIT1/ KvLQT1-AS/ KvDMR1, KCNQ1CN/BWR1F, CDKN1C/p57KIP2, SLC22A1L/IMPT1, BWR1A/ORCTL3/ITM1/TSSC5/HET, SLC22A1LS/ORCTL3AS, PHLDA2/TSSC3/IPL/BWR1C, NAP1L4/SBP1/GSBPL8, ZNF215,SDHD Control APOA2*

Chromosome 12 Imprinted genes *ATA3/SLC38A4 , DCN*

Chromosome 13 Imprinted genes *HTR2A*

Chromosome 14 Imprinted genes *MEG3, GTL2 , DLK1, PEG9 , DIO3*

Chromosome 15 Imprinted genes:- *MKRN3/ZNF127, ZNF127AS, MAGEL2, NDN , SNURF, PAR-SN, PAR5, SNRPN , IPW , PAR1, UBE3A, E6-AP ATP10A , GABRB3 , GABRA5, GABRG3, OCA2, RAS/GRF1 Control FBN fibrillin*

Chromosome 18 Imprinted genes *TCEB3C, IMPACT*

Chromosome 19 Imprinted genes *ZIM2, PEG3, USP29, ZIM3, ZNF264*

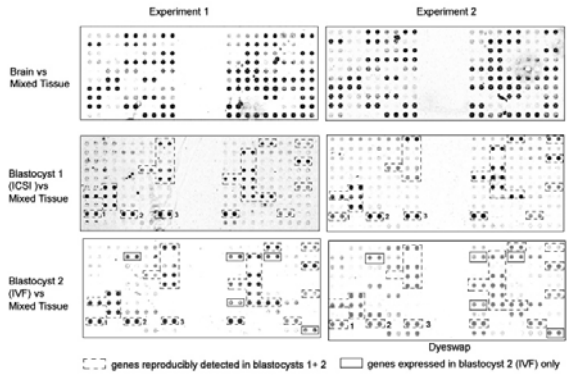
Chromosome 20 Imprinted genes *NNAT/.DM87L , GNAS-AS (GANG) NESP55, XLalpha s , GNAS , GSa (exon I)*

Epigenetic Regulators: *DNMT1, DNMT1o, DNMT2, DNMT3A, DNMT3A2, DNMT3B, DNMT3L*

Cell Sexing controls *ZFY, SRY, AFX, XIST*

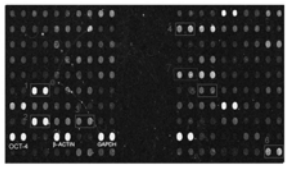
Blastocyst Marker Genes *OCT4, TERF1, DAB2, KRT18*

Replicate Array Experiments: Somatic Tissues and Single Blastocysts



Preliminary Microarray Data : Clinical Samples: IVF Derived Embryos vs ICSI Embryos

IVF blastocyst versus mixed tissue reference

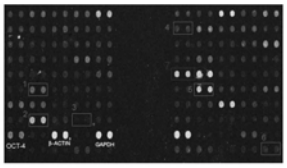


Screening human blastocysts on the imprinted gene microarray comparing a single IVF blastocyst vs. a human mixed tissue reference mRNA.

Differentially expressed genes between blastocysts 1 and 2 are shown in the red boxes.

Genes 2,3,6 are epigenetic regulators.
Genes 1,4,5 are imprinted.

ICSI blastocyst versus mixed tissue reference

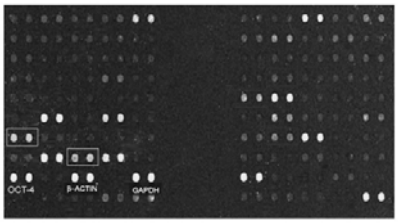


Preliminary data screening human blastocysts on the imprinted gene array comparing a single ICSI blastocyst vs. a human mixed tissue reference mRNA.

Differentially expressed genes between blastocysts 1 and 2 are shown in red boxes

Pooled IVF blastocyt cDNA versus stage & grade matched pooled ICSI blastocyst cDNA.

Pooled IVF versus pooled ICSI blastocysts



'Yellow' experiment

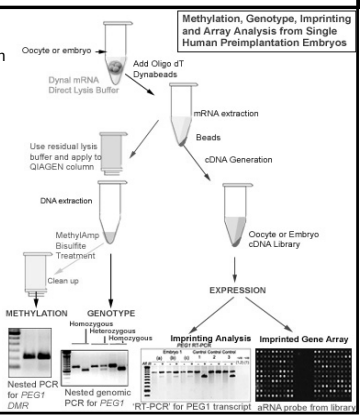
Differentially expressed genes in the red or green boxes.

Gene 7: chromosome 11 imprinted gene upregulated in ICSI (ratio 0.494)
Gene 8: chromosome 15 imprinted gene upregulated in ICSI (ratio 0.571)
Gene 9: chromosome 11 imprinted gene upregulated in ICSI (ratio 0.344)

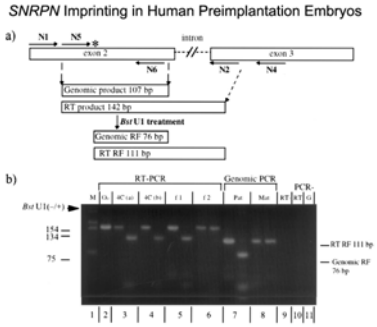
Gene 10: chromosome 11 imprinted gene upregulated in IVF (ratio 0.418)
Gene 11: blastocyst marker gene upregulated in IVF (ratio 0.526)

Retained DNA from embryo can be used for methylation analysis or genotyping

- Is differential IG expression between embryos or ART treatments (array) associated with methylation differences?
- Use embryonic DNA & methylation analysis to investigate further.
- Use DNA for genotyping
- Use cDNA for imprinting analysis
- Use cDNA for array work
- Confirm array with Real Time PCR

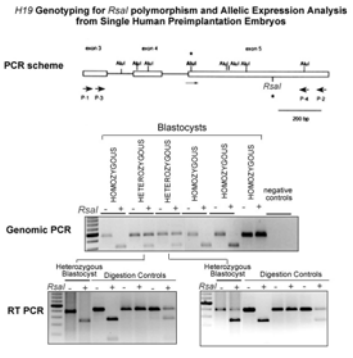


Allelic Expression of Human Imprinted Genes
When Do Genes Become Imprinted?



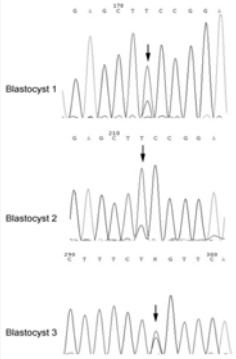
- PCR around a *BstU1* polymorphism
- 2 heterozygous 4-cell embryos identified
- Embryos were genotyped with DNA from parental samples
- *SNRPN* expression monoallelic In both embryos
- Huntriss et al., 1998 Am J Hum Genet. 63(4):1009-14.

Allelic Expression of Human Imprinted Genes
H19 Gene



- Genomic DNA + amplified cDNA obtained from same embryo
- 2 heterozygous blastocysts identified
- In one blastocyst, *H19* expression is strictly monoallelic (lower left)
- In a 2nd blastocyst, expression seen from both alleles, but allelic bias seen (lower right)
- 2 other blastocysts with expression from both alleles (not shown)

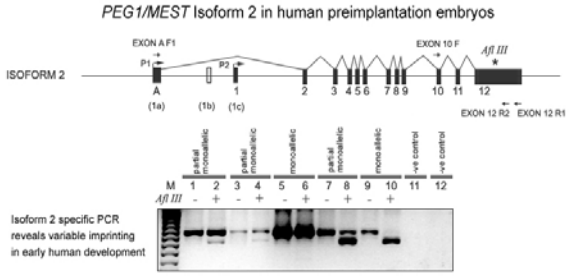
UBE3A Angelman Syndrome Gene



- Angelman syndrome due to lack of maternal *UBE3A* gene expression
- Are genes that are imprinted only in specific adult tissues, imprinted in early development?
- Is UBE3A imprinted in preimplantation embryo?
- Have 3 biallelic embryos out of approx. 20
- Studies ongoing

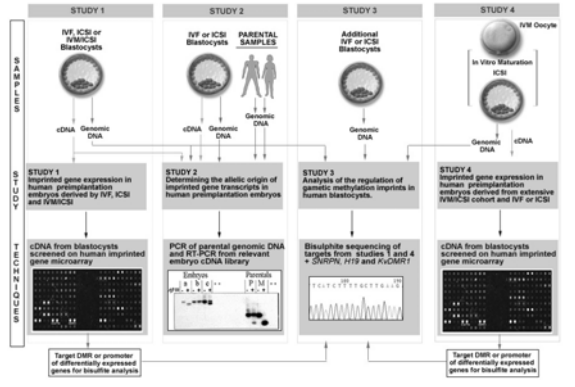
Allelic Origin of *PEG1* Isoform 2 in Human Embryos

Variable Imprinting of Isoform 2 in human preimplantation embryos



- 50 embryos tested: variable imprinting of *PEG1/MEST* **isoform 2** observed
- Inter-individual variation of isoform 2 imprinting reported in other tissues
- Submitted to ESHRE 2008 (Barcelona)

Experimental strategies for analysing the regulation of imprinting in human oocytes and preimplantation embryos.



Genomic DNA (green lines) , cDNA (orange lines)

Conclusions

•Aim to understand epigenetic biology of human gametogenesis & embryos to assess if/how ART may affect epigenetic regulation.

•Tools & methods described here to assess the effectiveness of using expression of imprinted gene as 'biomarkers' of epigenetic disruption.

•Where expression of IG is affected, follow up by bisulfite methylation analysis of imprinted gene DMRs in the same embryo.

•Tools such the IG array may become useful for the epigenetic safety testing of ART-derived embryos and other *in vitro* cultured cells like human ES cells.

•Allelic expression analysis suggests variable imprinting of *PEG1/MEST* and *H19* between human preimplantation embryos.

•Must establish cause of variability- may be natural inter-individual differences in imprinting or may be induced by ART and embryonic development *in vitro*

•Use this knowledge to adjust ART treatments accordingly

Acknowledgements

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