Running title

Differential parental *BRCA1* expression in human embryos

Title

Differential expression of parental alleles of *BRCA1* in human preimplantation embryos

Author names and affiliations

Tulay, P., Ph.D.[1*], Doshi; A., M.Sc. [2], Serhal, P., MD. [2], SenGupta, SB., Ph.D. [1]

[1] UCL Preimplantation Genetics Group, Institute for Women's Health, University College London, UK

[2] The Centre for Reproductive and Genetic Health, University College Hospital, The New Wing Eastman Dental Hospital, 256 Gray's Inn Road, London WC1X 8LD, UK

***Corresponding author (Present Address)**

Pinar Tulay

Yakin Dogu University, Faculty of Medicine, Department of Medical Genetics, Nicosia, Cyprus

pintulay@gmail.com

Abstract

Gene expression from both parental genomes is required for completion of embryogenesis. Differential methylation of each parental genome has been observed in mouse and human preimplantation embryos. It is possible that these differences in methylation affect the level of gene transcripts from each parental genome in early developing embryos. The aim of this study was to investigate if there is a parent specific pattern of *BRCA1* expression in human embryos and to examine if this affects embryo development when the embryo carries a *BRCA1* or *BRCA2* pathogenic variant.

Differential parental expression of *ACTB*, *SNRPN*, *H19* and *BRCA1* was semi-quantitatively analysed by mini-sequencing in 95 human preimplantation embryos obtained from 15 couples undergoing preimplantation genetic diagnosis (PGD).

BRCA1 was shown to be differentially expressed favouring the paternal transcript in early developing embryos. Methylation specific PCR showed a variable methylation profile of *BRCA1* promoter region at different stages of embryonic development. Embryos carrying paternally inherited *BRCA1* or *2* pathogenic variants were shown to develop more slowly compared to the embryos with maternally inherited *BRCA1* or *2* pathogenic variants.

This study suggests that differential demethylation of the parental genomes can influence the early development of preimplantation embryos.

Keywords

BRCA1, human preimplantation embryo, methylation, imprinting

Summary

Expression of maternal and paternal genes is required for the completion of embryogenesis. The differential methylation of the parental genomes observed in human preimplantation embryos may lead to differential expression of parental genes. In case of the transmission of any parental pathogenic variant to the embryo, this differential gene expression may cause embryonic developmental delays.

This study has shown that the parental alleles of *BRCA1* are differentially expressed depending on the embryonic development stage. Differential *BRCA1* expression is associated with the differential methylation status of *BRCA1*. Furthermore, embryos carrying paternally inherited *BRCA1* or *2* pathogenic variants were shown to develop slower compared to the embryos with maternally inherited *BRCA1* or *2* pathogenic variants. Hence, differential gene expression can influence the early development of preimplantation embryos, depending on the parental origin of the *BRCA1* or *2* pathogenic variant. Further extrapolation of this data suggests that paternally inherited *BRCA1* or *2* pathogenic variants leads to embryos with poor viability compared to embryos with maternally inherited pathogenic variants.

Introduction

Preimplantation embryo development follows a series of critical events, deprogramming of the genomes of sperm and egg and remarkable reprogramming of gene expression occurs to activate the embryonic genome. With the exception of imprinted loci, the expression of both maternal and paternal copies of genes is required for the completion of embryogenesis [1]. In mice, the genome of the oocyte is markedly undermethylated compared to the sperm [2]. Upon fertilization, mammalian zygotes (including humans) undergo genome-wide demethylation to establish the pluripotency of the newly developing embryo [2]. Selective demethylation of the male pronucleus occurs upon fertilization [3-9]. In contrast to the male pronucleus, demethylation of the female mouse pronucleus starts with the first cleavage divisions [2, 3, 7-10]. Recent genome-wide DNA methylation studies have reported a wave of demethylation in early preimplantation embryos. Throughout early embryonic development a differential methylation pattern is maintained in the majority of the differentially methylated regions in imprinted genes, although some show stage-specific changes [11]. Repetitive elements, housekeeping genes and genes controlling pluripotency or differentiation have been reported to have specific methylation patterns during embryo development [12, 13].

We hypothesize that, during the transition of demethylation and deprogramming in preimplantation embryo development, there is differential expression of parental alleles in certain genes that are not imprinted genes. The differential expression of parental alleles arises due to variation in the timing of demethylation and the level of methylation of each parental genome. Changes in the methylation patterns of *BRCA1* have been reported in early developing preimplantation embryos [14]. If there is differential demethylation and

remethylation of non-imprinted genes of maternal and paternal genomes during early development, then the level of transcription from each parental genome may also be different. Thus, when a pathogenic variant is present, the differential level of mutant and normal transcripts available for translation in the early embryo will be determined by the parental origin of the variant in the embryo. This in turn suggests that the effect of inheritance of a variant may vary in the early embryo, depending on whether it was transmitted from the maternal or paternal genome.

We therefore sought to characterize differential parental gene expression in human preimplantation embryos obtained from patients undergoing preimplantation genetic diagnosis (PGD) and to investigate the possible effect of a pathogenic variant on embryo development depending on the parental inheritance of the variant.

Methods

Sample collection and processing

This study was licensed by the Human Fertilization and Embryology Authority (Reference: RO113) and ethical approval was granted by the National Research Ethics Service, Research Ethics Committee (Reference: 10/H0709/26). Whole blood and surplus embryos were collected from couples who had given informed consent following PGD treatments for a variety of monogenic disorders.

Ovarian reserve tests and gonadotrophin stimulation were performed as described previously [15]. Briefly, immature oocytes were matured in G-IVF Plus medium (Vitrolife) within 4 hours of collection. Intracytoplasmic sperm injection was performed approximately 40 hours post hCGH injection. Fertilisation was assessed at 16-20 hours post insemination

and the presence of two pronuclei and polar bodies indicated normally fertilized oocytes [16]. Embryos were cultured in G-1/G-2 PLUS media (Vitrolife, UK). Preimplantation embryos were graded according to Bolton and colleagues [17]. Those embryos diagnosed as affected following preimplantation genetic diagnosis (PGD) or not suitable for transfer were collected on day 6 post fertilisation, in order to be used for this project. Each embryo was washed and transferred in phosphate buffered saline with 0.1% polyvinyl alcohol solution (PBS/PVA, Sigma, USA) and 0.3U/µl RNasin plus RNAse inhibitor (Promega, UK).

Genotyping couples

DNA extracted from whole blood from the couple was sequenced (BigDye® Terminator v3.1 Cycle Sequencing, ABI, UK) for exonic regions of *GAPDH*, *ACTB*, *UBE3A*, *SNRPN*, *IGF2*, *H19* and *BRCA1* to identify informative single nucleotide polymorphisms (SNPs) between the partners (Supplemental table I). A couple was defined as fully-informative for an SNP when each partner was homozygous for different alleles, whereas a couple was defined as semi-informative when one partner was homozygous and the other was heterozygous with one shared allele at the SNP.

DNA and RNA extraction from embryos

DNA and RNA from embryos were extracted using the AllPrep DNA/RNA micro kit (Qiagen, UK). The quality of RNA was assessed using the RNA 6000 Pico kit (Agilent, UK) on a Eukaryote Total RNA Pico Series II chip using the Agilent 2100 Bioanalyzer (Agilent, USA).

Analysis of differential gene expression

Reverse transcription of RNA obtained from the embryos was performed using the SuperScript™ III first-strand synthesis system for RT-PCR (Invitrogen, UK). DNA

contamination was identified by multiplex PCR (Qiagen, UK) using two markers (Eurogentec, UK) D19S112 and APOC2. These markers were selected to detect DNA contamination since they amplify an exonic as well as an intronic region. Additionally, these markers were used due to the high heterozygosity.

SNaPshot Minisequencing assay sensitivity

Differential expression of parental transcripts using RNA samples was analysed semi-quantitatively on the previously identified informative and semi-informative SNPs by SNaPshot minisequencing assay (Applied Biosystems, UK) (Supplemental table II). Differential expression of one parental transcript relative to the other was defined as an allele peak height ratio greater than 1:2. Monoallelic or preferential allelic expression of a transcript was only considered in embryos where both parental alleles could be identified in the analysed SNP. If an allele shared by each parent was expressed in the embryo, the sample was excluded from the analysis. Statistical analysis was performed by Student's T-test using GraphPad prism v6 software. The quantitative difference between each parental allele in the embryos identified by mini-sequencing was validated by real time PCR with subsequent high resolution melting analysis (Roche, UK).

Chromosomal copy number analysis

The chromosome copy number of the genes (chromosomes7, 11, 15 and 17) was analysed using DNA to ensure that the differential expression detected is not due to an aneuploidy in the embryo. The copy number of chromosomes 7, 11, 15 and 17 was determined by haplotype analysis. Polymorphic markers that are on the same chromosomes with the genes analysed, *ACTB*, *SNRPN*, *H19* and *BRCA1*, and that were available in our laboratory were used to determine the chromosome copy number. Analysis of the copy number of chromosome 7

were linked to *CFTR* (D7S2420, D7S2459, D7S486), chromosome 11 markers were linked to *HBB* (D11S1338, D11S1997, D11S4147), chromosome 15 to *FBN1* (D15S992, D15S123, D15S94) and chromosome 17 to *BRCA1* (D17S579, D17S1789, D17S1353, D17S841). The copy number of the chromosomes was scored only if the origin of the parental alleles at that locus could be distinguished. In these cases, if only one parental allele was detected, the embryo was considered to have lost the copy of the chromosome harbouring the missing allele. Embryos were considered to have gained a chromosome when three alleles were detected. An isodisomy of the chromosome could not be detected in the embryo using this method.

A subset of embryos was also analysed by array comparative genomic hybridization (aCGH) using the 24Sure system, following whole genome amplification (BlueGnome, UK). The slides were scanned using ScanArray Express (Perkin Elmer, USA) and the arrays were analysed using Bluefuse Multi analysis software v.2.6 (BlueGnome, UK). The cut-off of the log2 ratio fluorescent test signal over the control DNA was set as +0.3 for the gain and -0.3 for the loss of a chromosome by the software.

Methylation studies

The methylation status of *ACTB*, *H19* and *BRCA1* was analysed in a subset of embryos. The EpiTect Bisulfite conversion kit (Qiagen, UK) was used for DNA treatment. A set of outer primers with no CpG dinucleotides was designed for *ACTB*, *H19* and *BRCA1* (Supplemental Table III). Two sets of inner primers resulting in PCR products of different sizes directed to the methylated and unmethylated sequences were used for the promoter regions of *ACTB*, *H19* and *BRCA1* [18].

Statistical Analyses: Embryos with *BRCA1* or *2* pathogenic variants and preimplantation embryo development

The developmental stage of all the embryos with *BRCA1* or *2* pathogenic variants was examined on day 5/6 post fertilization and related to the parental inheritance of the variant. Statistical analysis was performed to investigate the difference between the developmental stage of embryos with paternally inherited *BRCA1* or *2* pathogenic variants and the maternally inherited *BRCA1* or *2* pathogenic variants by Chi-square test using GraphPad prism software v6.

Results

Genotyping analysis

Parental genotyping by sequencing of seven genes identified informative SNPs between 15 partners that enabled detection of heterozygous embryos in four genes (Table I).

Haplotyping analysis was performed to determine the copy number of chromosomes 7, 11, 15 and 17 in embryos. The detailed results are listed in table II. Of these embryos, 26 were also analysed by aCGH. Twelve embryos were shown to be euploid and the rest of the embryos showed various aneuploidies. Only two of these embryos (embryo 69 and 72) showed aneuploidies for the chromosomes of interest (gain of chromosome 17) and these embryos were excluded from the analysis (Table II).

Minisequencing assay sensitivity for allelic imbalance

The sensitivity of the mini-sequencing analysis was validated by real time PCR. Amplification of cDNA from the embryos showed that the mean Cq values for the start of the exponential phase of amplification were 36 for *ACTB*, 34 for *SNRPN*, 41 for *H19* and 35 for *BRCA1*.

Therefore, PCR prior to mini-sequencing analysis was stopped before the exponential phase was reached. High resolution melting analysis of all the PCR products from embryos confirmed the allelic imbalances identified by minisequencing.

Differential gene expression in preimplantation embryos

A total of 95 embryos were analysed to establish the parental expression profiles of *ACTB*, *SNRPN*, *H19* and *BRCA1*. A summary of the results is shown in table I and figure I. The expression level of maternal and paternal transcripts of *ACTB* was similar for all eleven embryos analysed for this gene.

Minisequencing analysis of imprinted gene transcripts showed that paternal transcript of *SNRPN* predominated in more than half of the embryos regardless of their developmental stage (*p=0.01; 56.5%, 13/23, figures I and II). Monoallelic expression of paternal *SNRPN* transcript was observed in 69% (9/13) of these embryos. As the embryos reached later stages of development, differential expression favouring the paternal *SNRPN* transcript increased from 61% (8/13) at cleavage and morula stages to 70% (7/10) for the blastocyst stage embryos.

Unlike *SNRPN*, *H19* was not readily detected in human preimplantation embryos (31%, 15/48). One embryo was excluded from the analysis since it was shown to have only the maternal copy of chromosome 11 by haplotyping. This accounted for the detection of only the maternal *H19* transcript by the SNapShot assay. Overall, 60% (9/14) of the embryos expressed predominantly the maternal *H19* transcript with 78% (7/9) being strictly monoallelic for the maternal transcript. Preferential expression of the maternal *H19* transcript was observed to be 50% (2/4) at cleavage, 66% (4/6) in morula and 75% (3/4) in blastocyst stage embryos.

Two SNPs, located in exon 11 and exon 12, were analysed to investigate differential expression of parental *BRCA1* transcripts. Thirteen per cent (10/75) of the embryos analysed were excluded from the analysis since the differential expression was not concordant at these two SNPs. Two embryos were excluded from the analysis since they showed a gain of chromosome 17, reflecting the preferential *BRCA1* expression by the SNaPshot assay. Differential *BRCA1* expression in the embryos was 66% (10/15) at cleavage, 55% (10/18) at morula and 50% (7/14) at the blastocyst stage. Overall, there were significantly more embryos with elevated expression of paternal *BRCA1* transcripts compared to embryos with increased expression of maternal transcripts, regardless of the developmental stage (*p=0.03, Figures I and II).

Differential methylation analysis

Methylation analysis was performed by bisulfite conversion, followed by methylation-specific PCR. Due to insufficient starting material, the amount of DNA obtained from a single embryo and the bisulfite treatment being deleterious for DNA, sequencing analysis on the converted DNA could not be performed. The promoter regions of three genes, *ACTB*, *H19* and *BRCA1*, were amplified by nested PCR. When monoallelic expression of one parental transcript was observed by SNaPshot analysis, a hemi-methylated profile was expected to be observed since only one parental transcript is present. When both parental transcripts were expressed at similar levels by SNaPshot analysis, an unmethylated profile was expected to be observed, representing both parental transcripts at similar levels. Since allele specific methylation was not studied, it is not possible to draw a definite conclusion as to whether the methylation status was the reason for differential gene expression.

Methylation analysis of *ACTB* in five embryos showed that these embryos were unmethylated, which supports the expression profile observed by SNaPshot analysis (Figure III). Methylation analysis of 3/8 embryos confirmed the differential *H19* expression. Of these embryos, two were hemi-methylated. These embryos also showed monoallelic expression of *H19* transcript. One embryo was unmethylated, and this supported the biallelic expression of *H19* transcripts by the minisequencing assay. In 5/8 embryos differential expression of the parental transcripts did not relate to the methylation status. The hemi-methylated profile of *BRCA1* confirmed differential expression of parental transcripts in 58% (18/31) of the embryos.

Development of embryos with *BRCA1* and *BRCA2* variants

Following PGD for *BRCA1* and *BRCA2* in six couples, 31 embryos were identified with a *BRCA1* or *BRCA2* pathogenic variants. Half of the embryos with paternally-transmitted *BRCA1* germline pathogenic variants (8/16) were arrested at the cleavage stage. Only 12.5% (2/16) of these embryos reached the blastocyst stage 5 days post fertilization. Embryos with maternally-inherited *BRCA1* variants developed at a significantly faster rate (8/15, 53% at blastocyst stage, p=0.01) compared to the embryos with paternally inherited *BRCA1* and *BRCA2* pathogenic variants (Figure IV).

Discussion

Differential expression

This study showed that similar levels of parental *ACTB* transcripts were expressed in human embryos. The paternally imprinted gene *H19* was not always detected in embryos. Some studies have reported the detection of both parental alleles in slow growing embryos or

morphologically poor embryos [19], whereas some did not detect *H19* at all [20]. In this study, *H19* was detected in 31% (15/48) of the embryos. Although biallelic expression of the *H19* transcript (7/14) was detected in half of the embryos confirming previously published reports [21], preferential expression of maternal *H19* transcripts was observed in the majority of the embryos. Although it is well accepted that *H19* is a paternally imprinted gene, expression of both parental *H19* alleles has been reported in the human oocytes and preimplantation embryos. Studies have suggested that developmentally delayed embryos show an unexpected expression and methylation profile [19]. In this study, embryos with similar parental expression of *H19* developed at a slower rate where seven embryos were between 5-10 cell stage and two were at morula stage on day 5/6 post fertilisation. Additionally, all of the embryos that reached blastocyst stage showed opposing patterns of parental expression for these two imprinted genes. This observation supports the finding of Khoueiry and colleagues (2012) who reported that slow developing embryos had a balanced pattern of methylated and unmethylated strands of H19DMR [21].

The imprinting of *SNRPN* was not completed in the early developing preimplantation embryos, such that the maternal *SNRPN* transcript was detected in 60% (9/15) of the embryos, as reported previously [20, 22]. The unexpected expression of maternal *SNRPN* alleles could be due to the on-going maternal mRNA degradation in these embryos, whereas the paternal *H19* transcripts could be caused by partial resetting of *H19* in the sperm [19]. The onset of the monoallelic expression of these genes might be at a later stage in human embryos or the time of the monoallelic expression could be variable among embryos. It has been reported that the time of the monoallelic expression of the imprinted genes, such as *IGF2*, *SNPRN* and *MEST*, varies in mouse embryos [22, 23]. Developmentally delayed

276 embryos were shown to have unusual expression and methylation profiles [19, 21]. This was
277 also observed in our study, where 20% (3/15) of slow developing embryos had similar
278 expression levels of *H19* and 39% (9/23) of slow developing embryos had similar parental
279 levels of *SNRPN* transcripts. None of these embryos reached the blastocyst stage. It is also
280 possible that assisted reproductive technology techniques lower the level of methylation for
281 the imprinted genes [19, 24-26], causing the unexpected expression of transcripts [27].

282 In this study, differential expression of paternal *BRCA1* transcript was observed in embryos.
283 This preferential expression was more prevalent in cleavage stage embryos. As the embryos
284 developed to the blastocyst stage, differential expression of *BRCA1* was reduced. In 13% of
285 the embryos, differential *BRCA1* expression could not be determined since the expression
286 profiles for the two SNPs analysed were not concordant. *BRCA1* is known to undergo
287 alternative splicing in a number of its exons, forming isoforms that skip exon 5, exons 2-10,
288 exons 9-11, exon 11 only, exons 14-17 and exons 14-18 [28, 29]. Alternative splicing of exon
289 11 yields a full-length isoform and also shorter isoforms either through the use of an
290 alternative intra-exonic splice donor site [29-31] or through complete skipping of exon 11.
291 Therefore, if one of these isoforms was present in the embryos analysed in this study,
292 differences in the expression profiles of *BRCA1* in exon 11 and exon 12 may have arisen.

293 The methylation analysis showed that preferential expression observed in *H19* and *BRCA1*
294 may be due to the methylation status of the parental transcripts. A gradual demethylation
295 was previously observed for *BRCA1* during cleavage divisions of human embryos where
296 approximately 30% of the methylated *BRCA1* residues remained up to the blastocyst stage
297 [14]. A recently published study also showed that monoallelic as well as biallelic expression
298 was detected in human primary fibroblasts [32]. They reported that each cell mostly

299 expressed one allele. The abundance of the cellular transcripts and the monoallelic
300 expression could account for phenotypic variability in humans including penetrance and
301 expressivity of a dominant developmental disorder, or cellular heterogeneity in cancers and
302 predisposition to a complex phenotype [32].

303 **Development of embryos with *BRCA1* and *BRCA2* pathogenic variants**

304 The development of the embryos with *BRCA1* or *2* pathogenic variants were investigated in
305 six couples undergoing PGD. Three males and three females with *BRCA1* or *2* pathogenic
306 variants opted for PGD. Two of the male partners had *BRCA1* pathogenic variants and one
307 had *BRCA2* pathogenic variant. The female partner of the rest of the couples undergoing
308 PGD had *BRCA1* pathogenic variants. The majority of the embryos with paternally inherited
309 *BRCA1* or *2* pathogenic variants (8 embryos with *BRCA1* and 8 embryos with *BRCA2*
310 pathogenic variants, respectively) were shown to arrest at the cleavage between 4 to 10 cell
311 stages (50%). Only 38% of the embryos developed to the morula stage and only 12.5% of the
312 embryos reached the blastocyst stage. Embryos with paternally *BRCA1* and *BRCA2* inherited
313 pathogenic variants (16 embryos) were shown to develop significantly slower compared to
314 embryos with maternally inherited variants (15 embryos, *p=0.01). Since the paternal
315 genome undergoes a rapid demethylation starting at the early stages of preimplantation
316 embryos, embryos with paternally inherited *BRCA1* and *BRCA2* pathogenic variants and
317 defective homologous recombination pathways may be prevented from developing to the
318 later stages of preimplantation development. However, embryos with maternally inherited
319 *BRCA1* pathogenic variants may compensate for the variant and initiate homologous
320 recombination repair through paternal transcripts that were free from the variant.
321 Therefore, when the embryos are carrying a paternally inherited *BRCA1* or *BRCA2*

pathogenic variants, it may be more prone to embryonic lethality during cleavage divisions. Thus we speculate that less viable embryos with paternally inherited *BRCA1* or *2* pathogenic variants are produced compared to the maternally inherited *BRCA1* or *2* pathogenic variants. Therefore, we assume that there are more adults with maternally inherited *BRCA1* or *2* pathogenic variants. The higher number of maternally inherited *BRCA1* or *2* variants was also reported previously [33, 34]. Although these articles were evaluating the risk of cancer and the onset of cancer depending on the parental origin of the *BRCA1* or *2* variants, their data showed that there were more patients with maternally inherited *BRCA1* or *2* variants [33, 34]. Once the genome wide demethylation of genes is completed during cleavage divisions, we assume that embryos with maternally and paternally inherited *BRCA1* or *2* variants have similar chances of implantation and pregnancy.

Conclusion and future perspectives

The main limitation of this study was the small number of embryos analysed due to the scarcity of the human embryos. However, even with this small number of embryos, the data shows significant outcome relating gene expression with development of preimplantation embryos. This study showed that *SNRPN*, *H19* and *BRCA1* transcripts were differentially expressed in human embryos. The presence of a *BRCA1* or *2* pathogenic variants inherited from the paternal genome had a greater detrimental effect on the development of the embryo to blastocyst compared to pathogenic variants inherited from the maternal genome. This may stem from differences in methylation patterns of the parental genomes in embryos. Therefore, the contribution of the paternal genome in the preimplantation embryo development may be vital, especially in the early stages. Further extrapolation of this data suggests that the risk of transmitting a *BRCA1* or *2* pathogenic variants may be altered by the

parental origin of the variant. Paternally transmitted *BRCA1* or *2* pathogenic mutations are more likely to result in embryos that fail to reach blastocyst thereby limiting the implantation potential of these embryos. Consequently this may lower the overall risk of males with BRCA1 or 2 mutations having children who have inherited their pathogenic mutation.

.For couples undergoing PGD for BRCA 1 or 2, where the male partner carries the pathogenic mutation

Author's roles:

Pinar Tulay: Substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content, and final approval of the version to be published.

Alpesh Doshi: substantial contributions to acquisition of data, revising it critically for important intellectual content, and final approval of the version to be published.

Paul Serhal: substantial contributions to acquisition of data, revising it critically for important intellectual content, and final approval of the version to be published.

Sioban SenGupta: substantial contributions to conception and design, interpretation of data, revising it critically for important intellectual content, and final approval of the version to be published

Funding:

There was no funding received for this study.

Conflict of interest:

The authors have no conflict of interest to declare.

References

1. McGrath J, Solter, D. *Completion of mouse embryogenesis requires both the maternal and paternal genomes.* Cell, 1984. **37**(1): p. 179-83.
2. Monk M, Boubelik M, Lehnert S. *Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development.* Development, 1987. **99**(3): p. 371-82.
3. Rougier N, Bourc'his D, Gomes D M, *et al*. *Chromosome methylation patterns during mammalian preimplantation development.* Genes Dev, 1998. **12**(14): p. 2108-13.
4. Dean W, Santos F, Stojkovic M, *et al*. *Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13734-8.
5. Swald J, Engemann S, Lane N, *et al*. *Active demethylation of the paternal genome in the mouse zygote.* Curr Biol, 2000. **10**(8): p. 475-8.
6. Mayer W, Niveleau A, Walter J, Fundele R, Haaf T. *Demethylation of the zygotic paternal genome.* Nature, 2000. **403**.
7. Beaujean N, Hartshorne G, Cavilla J, *et al*. *Non-conservation of mammalian preimplantation methylation dynamics.* Curr Biol, 2004. **14**(7): p. R266-7.
8. Santos F, Dean W. *Epigenetic reprogramming during early development in mammals.* Reproduction, 2004. **127**(6): p. 643-51.
9. Santos F, Hendrich B, Reik W, Dean W. *Dynamic reprogramming of DNA methylation in the early mouse embryo.* Dev Biol, 2002. **241**(1): p. 172-82.
10. Howlett SK, Reik W. *Methylation levels of maternal and paternal genomes during preimplantation development.* Development, 1991. **113**(1): p. 119-27.
11. Omizawa S, Kobayashi H, Watanabe T, *et al*. *Dynamic stage-specific changes in imprinted differentially methylated regions during early mammalian development and prevalence of non-CpG methylation in oocytes.* Development, 2011. **138**(5): p. 811-20.
12. Zhao MT, Rivera RM, Prather RS. *Locus-specific DNA methylation reprogramming during early porcine embryogenesis.* Biol Reprod, 2013. **88**(2): p. 48.
13. Paranjpe SS, Veenstra GJ. *Establishing pluripotency in early development.* Biochim Biophys Acta, 2015. **1849**(6): p. 626-636.
14. Magdinier F, D'estaing SG, Peinado C, *et al*. *Epigenetic marks at BRCA1 and p53 coding sequences in early human embryogenesis.* Mol Hum Reprod, 2002. **8**(7): p. 630-5.
15. Muttukrishna S, Mcgarrigle H, Wakim R, Khadum I, Ranieri DM, Serhal P. *Antral follicle count, anti-mullerian hormone and inhibin B: predictors of ovarian response in assisted reproductive technology?* BJOG, 2005. **112**(10): p. 1384-90.
16. Sahu B, Ozturk O, Deo N, Fordham K, Ranierri M, Serhal P. *Response to controlled ovarian stimulation and oocyte quality in women with myotonic dystrophy type I.* J Assist Reprod Genet, 2008. **25**(1): p. 1-5.
17. Bolton VN, Hawes SM, Taylor CT, Parsons JH. *Development of spare human preimplantation embryos in vitro: an analysis of the correlations among gross morphology, cleavage rates, and development to the blastocyst.* J In Vitro Fert Embryo Transf, 1989. **6**: p. 30-35.
18. Esteller M, Silva JM, Dominguez G, *et al*. *Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors.* J Natl Cancer Inst, 2000. **92**(7): p. 564-9.
19. Ibala-Romdhane S, Al-Khtib M, Khoueiry R, Blachere T, Guerin JF, Lefevre A. *Analysis of H19 methylation in control and abnormal human embryos, sperm and oocytes.* Eur J Hum Genet, 2011. **19**(11): p. 1138-43.
20. Monk M, Salpekar A. *Expression of imprinted genes in human preimplantation development.* Mol Cell Endocrinol, 2001. **183 Suppl 1**: p. S35-40.

21. Khoueiry R, Ibala-Romdhane S, Al-Khtib M. *Abnormal methylation of KCNQ1OT1 and differential methylation of H19 imprinting control regions in human ICSI embryos.* Zygote, 2012: p. 1-10.
22. Huntriss J, Daniels R, Bolton V, Monk M. *Imprinted expression of SNRPN in human preimplantation embryos.* Am J Hum Genet, 1998. **63**(4): p. 1009-14.
23. Lighten AD, Hardy K, Winston RM, Moore GE. *IGF2 is parentally imprinted in human preimplantation embryos.* Nat Genet, 1997. **15**(2): p. 122-3.
24. DeBaun MR, Niemitz EL, Feinberg AP. *Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19.* Am J Hum Genet, 2003. **72**(1): p. 156-60.
25. Orstavik KH, Eiklid K, Van Der Hagen CB, *Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection.* Am J Hum Genet, 2003. **72**(1): p. 218-9.
26. Gicquel C, Gaston V, Mandelbaum J, Siffroi JP. Flahault, A. & Le Bouc, Y. *In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene.* Am J Hum Genet, 2003. **72**(5): p. 1338-41.
27. Sato A, Otsu E, Negishi H, Utsunomiya T, Arima T. *Aberrant DNA methylation of imprinted loci in superovulated oocytes.* Hum Reprod, 2007. **22**(1): p. 26-35.
28. Orban TI, Olah E. *Emerging roles of BRCA1 alternative splicing.* Mol Pathol, 2003. **56**(4): p. 191-7.
29. Lixia M, Zhijian C, Chao S, Chaojiang G, Congyi Z. *Alternative splicing of breast cancer associated gene BRCA1 from breast cancer cell line.* J Biochem Mol Biol, 2007. **40**(1): p. 15-21.
30. Wilson CA, Payton MN, Elliott GS, *et al*. *Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-delta11b.* Oncogene, 1997. **14**(1): p. 1-16.
31. Tammaro C, Raponi M, Wilson DI, Baralle D. *BRCA1 exon 11 alternative splicing, multiple functions and the association with cancer.* Biochem Soc Trans, 2012. **40**(4): p. 768-72.
32. Borel C, Ferreira PG, Santoni F, *et al*., *Biased allelic expression in human primary fibroblast single cells.* Am J Hum Genet, 2015. **96**(1): p. 70-80.
33. Senst N, Llacuachaqui M, Lubinski J, *et al*. *Parental origin of mutation and the risk of breast cancer in a prospective study of women with a BRCA1 or BRCA2 mutation.* Clin Genet. **84**(1): p. 43-6.
34. Ellberg C, Jernström H, Broberg P, Borg Å, Olsson H. *Impact of a paternal origin of germline BRCA1/2 mutations on the age at breast and ovarian cancer diagnosis in a Southern Swedish cohort.* Genes Chromosomes Cancer. **54**(1): p. 39-50.

Titles and legends to tables and figures

Table I Summary of differential expression analysis.

The genes analysed for differential expression, percentage of informative haplotypes where each parental allele was distinguished and the percentage of embryos with differential gene expression favouring the paternal and maternal transcripts are listed. *BRCA1* was analysed at two loci and the results shown here were collective from both loci. In ten embryos differential expression of *BRCA1* at two loci did not agree and these were not included in the analysis. *Only the paternal expression of *SNRPN* was observed in 9/13 embryos and only the maternal expression of *H19* was observed in 7/9 embryos.

Genes analysed	Number of embryos included for the study	% of informative haplotypes (number of embryos)	% of embryos showing differential expression favouring: (number of embryos)		
			Paternal	Maternal	Similar
ACTB	30	36.7 (11)	0	0	100 (11)
SNRPN	34	67 (23)	56.5 (13)*	17 (4)	26 (6)
H19	48	33 (15)	13 (2)	60 (9)*	26 (4)
BRCA1	75	64 (49)	58 (29)	19 (9)	22 (11)

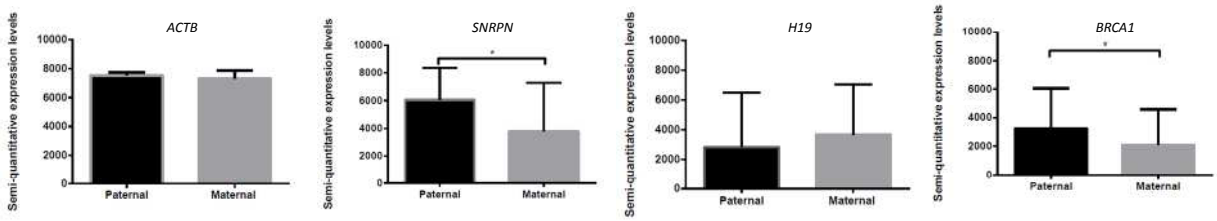
Table II Table summarising the embryos analysed for haplotyping analysis for the chromosomes of 7 (*CFTR*), 11 (*HBB*), 15 (*FBN1*) and 17 (*BRCA1*).

Number of embryos included in the haplotyping analysis with the percentage of informative haplotypes and heterozygote embryos are summarised. The percentage of inconclusive analysis due to amplification failure was also shown.

Chromosomes analysed	Number of embryos included for the study	% of informative haplotypes (number of embryos)	% of heterozygous embryos (number of embryo)	% of embryos with amplification failure (number of embryo)
CFTR	30	86.7 (26)	86.7 (26)	13 (4)
HBB	34	53 (18)	94 (17)	26 (9)
FBN1	48	25 (12)	91 (11)	4 (2)
BRCA1	75	68 (51)	94 (48)	19 (21)

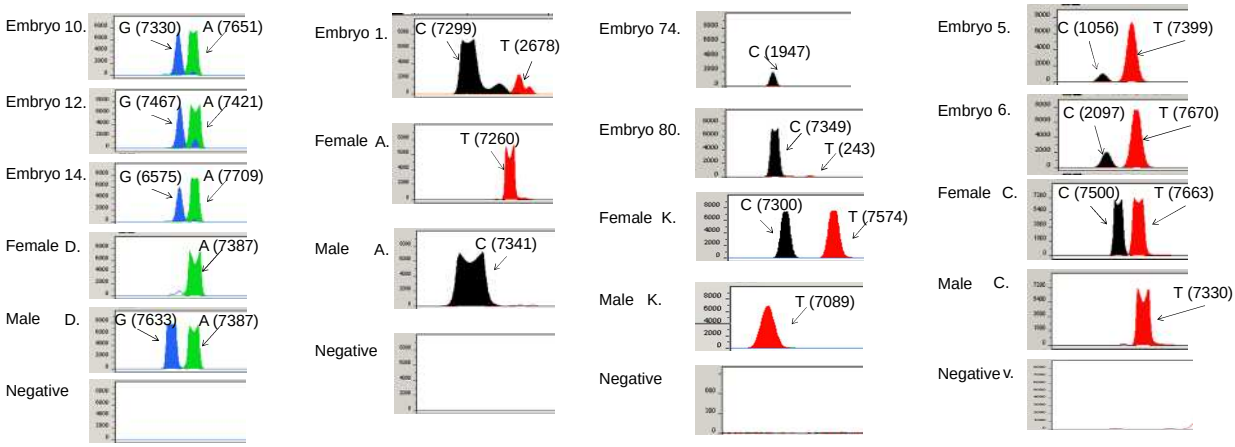
Titles and legends to figures

Figure I Overall differential expression of paternal and maternal transcripts for four genes in human embryos.



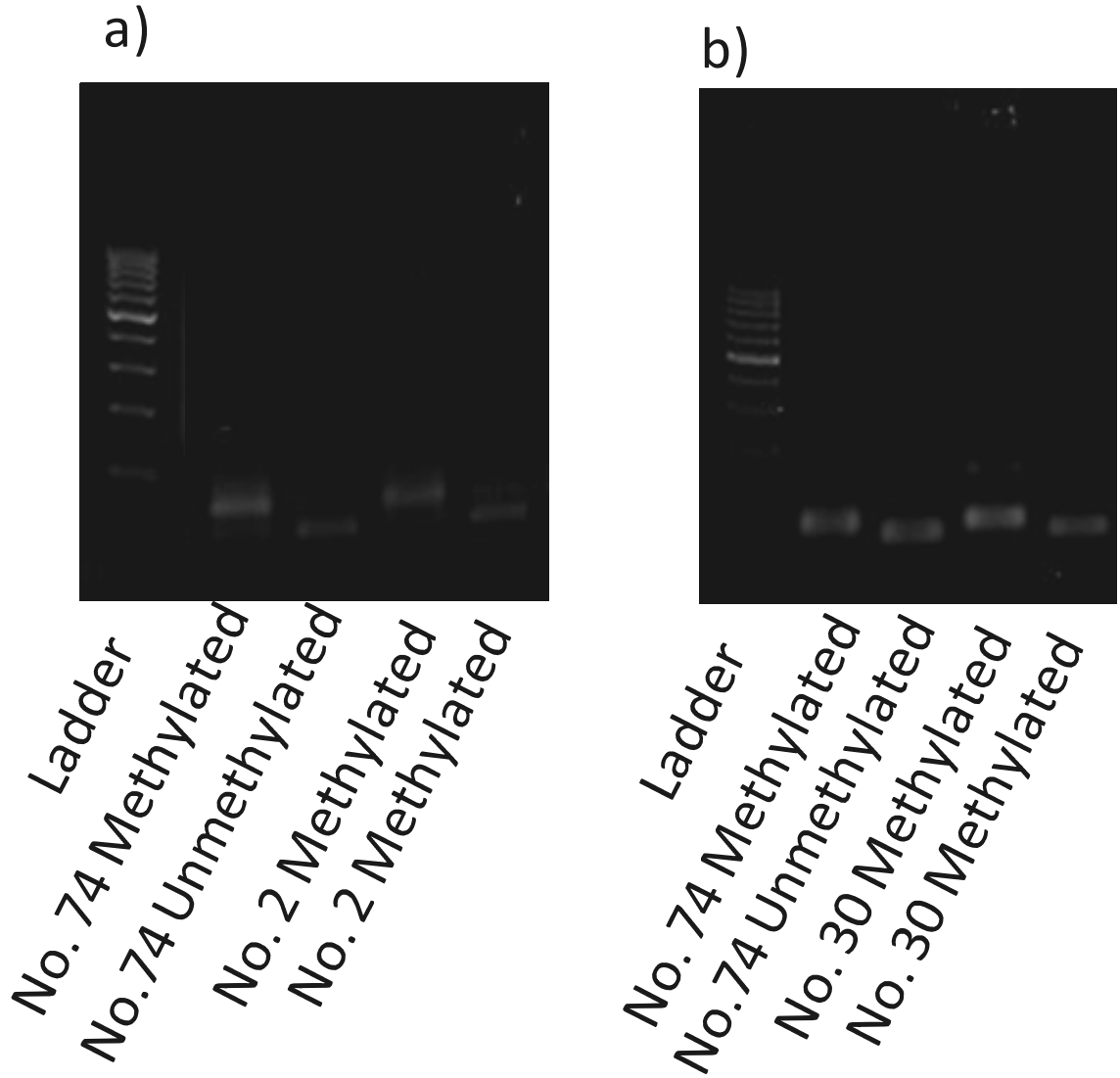
It was shown that there was no significance in the differential expression of parental transcripts for *ACTB* (p=0.2). Paternal *SNRPN* transcripts were expressed at significantly higher levels relative to the maternal transcripts (*p= 0.01) in embryos. Although higher levels of maternal *H19* transcripts were detected in embryos, this was not significant (p=0.4). The differential expression of paternal *BRCA1* expression was shown to be significant relative to the maternal transcripts (*p= 0.03) in the embryos.

Figure II GeneScan™ fragment size analysis result panels showing differential gene expression of: a) *ACTB* from couple D. The panel shows the embryo number 10, 12 and 14 (all heterozygote for G and A) with similar expression levels of the parental copies of the SNP analysed, female partner (homozygote for A), male partner (heterozygote G and A) of couple A and negative control with no DNA. **b) *SNRPN* from couple A.** The panel shows the embryo number 1 (heterozygote for G and A) with increased expression of the paternal copy of the SNP analysed, female partner (heterozygote for G and A), male partner (homozygote for G) of couple A and negative control with no DNA. **c) *H19* from couple K.** The panel shows the embryo number 74 (maternally expressed allele only, homozygote for C) and 80 (heterozygote for C and T) with increased expression of the maternal copy of the SNP analysed, female partner (heterozygote for C and T), male partner (homozygote for T) and negative control with no DNA. **d) *BRCA1* from couple C.** The panel shows the embryo number 5 and 6 (both heterozygote for C and T) with considerably increased expression of the paternal copy of the SNP analysed, female partner (heterozygote for C and T), male partner (homozygote for T) and negative control with no DNA. The panels show the alleles expressed in the embryo and the allele of the SNP of the female and male partner of the couple. The peak heights of the alleles are shown in parentheses.



507 **Figure III Agarose gel electrophoresis of embryos showing partial methylation for a) *H19* and**

508 **b) *BRCA1*.**

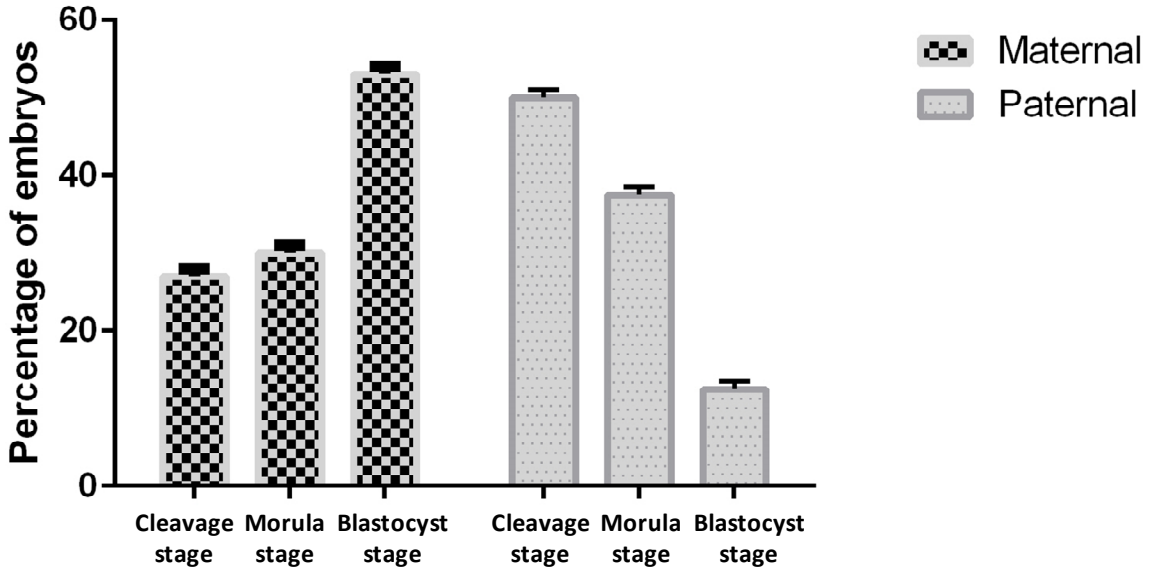


509

510 Lanes 1 for images a) and b) represent 100 base pair ladder and the rest of the lanes represent the
511 methylation PCR product results of DNA obtained from embryos following bisulfite conversion. Embryo
512 numbers are labelled for each lane and the PCR directed towards the methylated DNA is represented
513 as “methylated” and the unmethylated DNA as “unmethylated”.

514

Figure VI Developmental rate of embryos carrying paternally inherited *BRCA1* or *2* mutations compared to maternally inherited *BRCA1* or *2* mutations.



Significantly fewer embryos developed to the later stages of preimplantation development (morula and blastocyst stages) compared to the embryos carrying maternally inherited mutations (*p=0.01).

Supplemental Tables

Supplemental Table 1 Primer details used for differential gene expression analysis in embryos.

a) Primer names, sequences, chromosomal locations and PCR product sizes are listed. Sequences were obtained from Ensembl; *ACTB* (ENSG00000075624, Ensembl release 60), *SNRPN* (ENSG00000128739, Ensembl release 60), *H19* (ENSG00000130600, Ensembl release 60) and *BRCA1* (ENSG00000012048, Ensembl release 60) on the Ensembl genome browser.

Primer	Primer sequence	Locus	Product size (bp)
ACTBex7 F	5'-AACACTGGCTCGTGTGACAA-3'	7:5568239:5568860	236
ACTBex7 R	5'-GGGGTGTTGAAGGTCTCAAA-3'		
*BRCA1*ex11 F	5'-TCAAAGGAGGCTCTAGGTTTTG-3'	17:41244039:41244860	373
*BRCA1*ex11 R	5'-GCTTGAATGTTTTCATCACTGG-3'		
*BRCA1*ex12 F	5'-TCATTTAATGGAAAGCTTCTCAAAG-3'	17:41234330:41234954	290
*BRCA1*ex12R	5'-AAAGGGGAAGGAAAGAATTTTG-3'		
*BRCA1*ex12 RNA only F	5'-AGCAGGAAATGGCTGAACTA-3'	17:41234126:41234745	130
*BRCA1*ex12 RNA only R	5'-TCTGATGTGCTTTGTTCTGG-3'		

SNRPNex12 F	5'- CCTCTGCAGGCTCCATCTAC-3'	15:25219149:25219768	151
SNRPNex12 R	5'- ATTGCTGTTCCACCAAATCC-3'		
H19 F	5'-TTACTTCCTCCACGGAGTCG-3'	11:2016950:2017675	340
H19 R	5'-GACACGTGGGTGGGATGG-3'		

531

Supplemental Table 2 Sequences of primers used in minisequencing for differential gene expression analyses.

Primer name	Primer sequence
MS_BRCAex11_ rs16941	5'-CATTAGAGAAAATGTTTTTAAAAG-3'
MS_BRCAex12_ rs1060915	5'-CCCTTCCATCATAAGTGACTC-3'
MS_ACTBex7_rs852423	5'-CATTGTTTCTAGGAGAACC-3'
MS_SNRPNex12_rs75184959	5'-ATGATCTGTAAGGCAGAGAT-3'
MS_H19_rs2839701	5'-ACTCAGGAATCGGCTCT-3'

Supplemental table 3 Sequences of primers used in methylation specific PCR.

Primer name	Primer sequence	Expected product size (bp)
ACTB_promoter_outer_F	GATTTGATTGATTATT TTATGAAGAT TTTT	210
ACTB_promoter_outer_R	CTCATTACCAATAATAGATAACCTA	
ACTB_promoter_methylated_F	CGCGGTTATAGTTTTATTATTACGGTCGAG	96
ACTB_promoter_methylated_R	ACCATCTCTTACTCGAAATCCAAAACGACG	
ACTB_promoter_unmethylated_F	TGTGGTTATA GTTTTATTAT TATGGTTGAG	120
ACTB_promoter_unmethylated_R	ACCATCAAACAACTCATAACTCTTCTCCAA	
H19_promoter_outer_F	GGTTTTTAGATAGGAAAGTGGT	185
H19_promoter_outer_R	AATAAAATACTAAAAAACAAAAAAAAATAC	
H19_promoter_ unmethylated_F	TTGTGAATGGGATTGGGGTGTTTAGTGGTT	124
H19_promoter_ unmethylated_R	CACAAACCCCCTAATAAACACAATACC	
H19_promoter_methylated_F	GATCGGGGTGTTTAGCGGTTGTGGGGATTT	134
H19_promoter_ methylated_R	CGCAAACCCCCTAATAAACGCGATACC	
***BRCA1*_promoter_outer_F**	TTTTTTTATTTTTTGATTGTATTTTGATTT	184
***BRCA1*_promoter_R**	TTATCTAAAAAACCCCACAACCTATCCCCC	
***BRCA1*_promoter_unmethylated_F**	TTGGTTTTTGTGGTAATGGAAAAGTGT	86
***BRCA1*_promoter_unmethylated_R**	CAAAAAATCTCAACAAACTCACACCA	

***BRCA1*_promoter_methylated_F**	TCGTGGTAACGGAAAAGCGCGGGAATTA	75
***BRCA1*_promoter_methylated_R**	AAATCTCAACGAACTCACGCCGCGCAATCG	

537