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ARTICLE

Variable imprinting of the *MEST* gene in human preimplantation embryos

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There is evidence that expression and methylation of the imprinted paternally expressed gene 1/mesoderm-specific transcript homologue (*PEG1/MEST*) gene may be affected by assisted reproductive technologies (ARTs) and infertility. In this study, we sought to assess the imprinting status of the *MEST* gene in a large cohort of *in vitro*-derived human preimplantation embryos, in order to characterise potentially adverse effects of ART and infertility on this locus in early human development. Embryonic genomic DNA from morula or blastocyst stage embryos was screened for a transcribed *Afl*III polymorphism in *MEST* and imprinting analysis was then performed in cDNA libraries derived from these embryos. In 10 heterozygous embryos, *MEST* expression was monoallelic in seven embryos, predominantly monoallelic in two embryos, and biallelic in one embryo. Screening of cDNA derived from 61 additional human preimplantation embryos, for which DNA for genotyping was unavailable, identified eight embryos with expression originating from both alleles (biallelic or predominantly monoallelic). In some embryos, therefore, the onset of imprinted *MEST* expression occurs during late preimplantation development. Variability in *MEST* imprinting was observed in both *in vitro* fertilization and intracytoplasmic sperm injection-derived embryos. Biallelic or predominantly monoallelic *MEST* expression was not associated with any one cause of infertility. Characterisation of the main *MEST* isoforms revealed that isoform 2 was detected in early development and was itself variably imprinted between embryos. To our knowledge, this report constitutes the largest expression study to date of genomic imprinting in human preimplantation embryos and reveals that for some imprinted genes, contrasting imprinting states exist between embryos.
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INTRODUCTION

Paternally expressed gene 1/mesoderm-specific transcript homologue (*PEG1/MEST*) encodes an α/β hydrolase fold family enzyme of unknown function. In mice, *Mest* maps to an imprinted region that affects growth[1] and disruption of the *Mest* gene leads to embryonic growth retardation and abnormal maternal nurturing behaviour.[2] *Mest* expression is upregulated in obese adipose tissue and may regulate adipocyte growth and lipid accumulation.[3–6] In humans, the imprinted *MEST* gene maps to chromosome 7q32,[7,8] and is a candidate gene for Silver-Russell syndrome (SRS), although no corresponding sequence or epigenetic mutations in *MEST* have yet been reported.[9–11] In mice, loss of imprinting (LOI) of *Mest* is associated with altered growth.[12] LOI of *MEST* in humans has been described in colorectal cancer[13] and lung-cancer cell lines.[14] LOI has also been reported in invasive breast cancer,[15] but the biallelic expression observed here is likely to be the result of a promoter usage switch between *MEST* isoforms.[16]

Assisted reproductive technology (ART) and infertility may be associated with epigenetic defects.[17] There is a suggestive evidence that methylation and expression of the *MEST* gene may be affected by various forms of ART in humans, mice and non-human primates. Hypermethylation at the *MEST* differentially methylated region (DMR), has been reported in a girl with SRS who was conceived by *in vitro* fertilisation (IVF),[18] although it is unclear whether this paternally inherited epimutation was responsible for the syndrome. Aberrant DNA methylation of *MEST* has also been observed in superovulated human oocytes.[19] Differential methylation at *MEST* has been observed between children conceived *in vitro* and *in vivo*[20] and has been observed to differ between children conceived through IVF as compared with intracytoplasmic sperm injection (ICSI) and naturally conceived children.[21] In mice, the *Mest* methylation imprint may be susceptible to the effects of *in vitro* culture or ageing of oocytes *in vitro*.[22] Significant reduction in DNA methylation at *Mest* has been reported in mouse oocytes generated after ovarian follicle culture under low methyl donor levels.[23] There may also be adverse effects imposed on the murine *Mest* gene induced by *in vitro* folliculogenesis, leading to a loss of methylation.[24] Finally, overexpression of the *MEST* gene has been described following *in vitro* maturation of rhesus monkey oocytes.[25]

Aberrant methylation at *MEST* is associated with certain forms of male infertility. Hypermethylation of *MEST* has been described in males with oligozoospermia,[26,27] and also idiopathic male infertility.[28] *MEST* DMR methylation errors were the most common defects observed in infertile men relative to several other imprinted genes.[29] Global sperm DNA methylation analysis has also revealed that elevated methylation of *MEST* is associated with poor sperm parameters.[30]

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Collectively, this data implies that the *MEST* locus may be susceptible to the effects of ART manipulation and aberrant epigenetic programming that occurs in infertility. In this study, we therefore sought to characterise the imprinting status of *MEST* in human preimplantation embryos derived by assisted reproduction.

MATERIALS AND METHODS

Oocytes and embryos

Human preimplantation embryos that were not selected for transfer, and which were therefore surplus to treatment needs were donated for research under informed consent by couples attending the Assisted Conception Unit (ACU) at Leeds General Infirmary. All tissues were donated under protocols that have been approved by the Leeds West Research Ethics Committee and licensed by the Human Fertilisation and Embryo Authority (HFEA). The procedures of ovarian stimulation, oocyte collection and embryo culture in Medi-Cult IVF medium (MediCult UK Ltd, Reigate, UK) and *in vitro* fertilisation were performed at the Leeds ACU according to published protocols.[31] Donated, fresh embryos were transported at day-2 post insemination to the University of York as previously described[32,33] and cultured to the blastocyst stage. Cryopreserved embryos that were surplus to treatment requirements were donated for research under informed consent by couples attending Bourn Hall Clinic, Cambridge and were thawed and cultured to the blastocyst stage in the HFEA licensed research laboratories in Leeds. All embryos were individually cultured in 4 μl droplets of Earle's Balanced Salt Solution, supplemented with 1 mM glucose, 5 mM lactate, 0.47 mM sodium pyruvate, 0.5% (v/v) human serum albumin (Zenalb 20; Bioproducts Lab, Elstree, UK) and amino acids at close-to-physiological concentrations, based on the data of Tay *et al* 1997,[34] under embryo tested mineral oil at 37 °C under 5% CO_2 in air. Embryos were transferred to fresh drops of culture medium after each 24 h culture period. The morphological grade of each morula or blastocyst was recorded at the end of culture as described previously.[32] Only embryos of grades 1–3 were used. At the end of culture, embryos were allowed to perish by cooling to room temperature over 10 minutes in ambient atmospheric conditions, washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline at 4 °C (Life Technologies, Paisley, UK), before being snap frozen in lysis buffer (Dynabeads, Life Technologies). For embryo sample numbers 9 and 10 (Figure 2b) at the end of culture, the zona pellucidae were removed by exposure to Acid Tyrode's solution (Sigma-Aldrich, Gillingham, UK).

Reverse transcription and cDNA amplification

Single embryos and oocytes were collected and lysed at 80 °C in lysis buffer (Dynabeads mRNA DIRECT Micro Kit, Life Technologies). Following RNA extraction with oligo-dT Dynabeads, cDNA was generated and amplified using an adaptation of existing cDNA amplification protocols[35,36] using 1 μg each of the cDNA amplification primers, primer 1: 5′-AAACGACGGCCAGTGAATTG TAATACGACTCACTATAGGGCGCT$_{24}$-3′ and primer 2: 5′-AAGCAGTGGTAT CAACGCAGAGTACGCGGG-3′ using superscript II RNAseH-reverse transcriptase (Invitrogen) and associated reagents with incubation for 2 h at 42 °C. The cDNA was amplified by PCR using an additional 1 μg of each primer, 2 μl 50 × advantage 2 polymerase (Clontech, Mountain View, CA, USA), in a thermal cycler for 32 cycles of 95 °C for 45 s, 65 °C for 6 min 45 s. The embryonic cDNA libraries are used across multiple experiments.

Genotyping embryos

Genomic DNA was extracted from embryos using the Qiagen DNA Micro kit (Crawley, UK) according to protocol, utilising the cell lysate that was residual from the mRNA extraction process. Two rounds of PCR were performed on 2.5 μl of extracted genomic DNA using the primer PEG1-gDNA-F GGTCCTGGCCATCAAACATA[37] and R1 5′-CCTCTACTGTAGCTCAAGA G-3′[38] in the first round, and PEG1-gDNA-F and R2 5′-GGAGTCTCAGACT GTTATTTGC-3′[38] in the second round (Figure 1a). PCR conditions were 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min 30 s. Second round PCR products were split and one-half treated with three units of *Afl*III and digested at 37 °C overnight, followed by a subsequent addition of two further units *Afl*III for a further 3 h to completion. Products were run on a 1.5% agarose gel with reference to 100 bp ladder and enzyme digestion controls. Alternatively, embryos were genotyped by sequencing of *MEST* genomic PCR products (Biomolecular Analysis Facility, University of Leeds, Leeds, UK).

Imprinting analysis and isoform 2-specific PCR

For heterozygous (genotyped) embryos and the screening cDNAs of embryos of unknown genotype, the expressed alleles of the *MEST* gene were analysed by PCR from 50 ng amplified embryonic cDNA using the *MEST* primers F1 5′-GCAACAATGACGGGAACTTAGT-3′ and R1 5′-CCTCTACTGTAGCTCAA GAG-3′ in the first round, and F2 5′-TCAGAGGAAGAAGTTCAGAAGG-3′ and R2 5′-GGAGTCTCAGACTGTTATTTGC-3′ in the second round.[38] Half of the PCR product was digested with *Afl*III as described above. Appropriate enzyme digestion controls that were amplified from limiting quantities of somatic tissue mRNA were run in each experiment. Alternatively, *MEST* PCR products were sequenced. For isoform-specific PCR (not spanning the *Afl*III polymorphism), exon A (5′-CCTGTAGGCAAGGTCTTACCTG-3′) and exon 1 (5′-GCGGCGGGCGGCATGGGATA-3′) specific primers[16] were used in conjunction with EXON 2 R1 5′-GAAGACTTCCATGAGTGAAGGGC-3′ in the first round and the forward primers were then hemi-nested with EXON 2 R2 5′-ATGTGCAGGTACGCAGCAAG-3′ in the second round. For isoform 2-specific PCR spanning the *Afl*III polymorphism, Exon A and R1 were used in the first round, with primer F1 and primer R2 in second.[38] RT-PCR product identity from selected embryos was confirmed by direct sequencing using primers F1 and R1. Allele ratios were quantified using the average of three peak readings per band using Image J (http://rsbweb.nih.gov/ij/). Predominantly monoallelic expression was defined as an allele ratio greater than 1:3, as described previously.[39,40] PCR products obtained using isoform-specific primers were sequenced by cloning into TOPO-TA (Invitrogen) using standard M13 primers. Other primers described here are: *GAPDH* forward 5′-TTGTCAAGCTCATTTCCTGGTAT-3′, *GAPDH* reverse 5′-TCTCTCTTCCT CTTGTGCTCTTG-3′, ZP2 forward 5′-GACCTGCCCTGTGTCCTCTA-3′, ZP2 reverse 5′-AGATCAGATGAGCCGACAC-3′, ZP3 forward 5′-GATATACATCAC CTGCCACC-3′, ZP3 reverse 5′-TCACTTCATGGTCACCACTC-3′.

RESULTS

Embryonic genomic DNA and cDNA screening

A nested PCR around the *Afl*III polymorphism in the 3′-untranslated region of the *MEST* gene was used for the purpose of genotyping embryos and imprinting analysis (Figure 1a). For the present study, parental DNA samples were unavailable for genotyping and thus we were unable to identify informative heterozygous embryos in this way. Therefore, we developed a technique that allows extraction of embryonic genomic DNA from the residual wash buffer that is remaining after the mRNA isolation procedure, to allow direct genotyping of the embryonic DNA (Figure 1b). While sperm may remain adhered to the zona on IVF-derived stage embryos, the observations from this study suggest that sperm DNA was degraded by the morula or blastocyst stage and did not therefore amplify in the genomic screening PCRs. Furthermore, ICSI embryos (no adherent sperm) and zona-stripped embryos were also included in the study. Details regarding all embryos used in the study are presented in Table 1. We identified 10 late preimplantation stage embryos that were heterozygous for the *Afl*III polymorphism in the *MEST* gene.

MEST imprinting analysis in heterozygous embryos

For *MEST* imprinting analysis, cDNA libraries that were generated from human preimplantation embryos were validated by screening for transcripts of housekeeping genes and appropriate developmental marker genes including β-actin (*ACTB*), *GAPDH*, *HPRT* and *OCT4* (Figure 1c). Allelic expression was assessed in the 10 heterozygous embryos using a nested-PCR scheme that amplifies all known *MEST* isoforms. In Figure 2a, PCR amplification of *MEST* transcripts was performed from the cDNA of five of the heterozygous embryos.

PCR products were digested with *Afl*III alongside appropriate enzyme digestion controls derived from cDNA libraries that were amplified from limiting quantities of somatic tissue mRNA (0.5 μg), which were either homozygous for each allele (A or G) or heterozygous. Allele ratios for all embryos are shown in Table 1. Imprinting analysis for sample 1, a grade-2, day-6 IVF morula demonstrated predominantly monoallelic expression of *MEST* (Figure 2a, sample 1) with 93% to 7% expression of the A and G alleles, respectively. Thus, there was weak expression detectable from the minor allele (the inferred maternal allele). In contrast, in four further late preimplantation stage embryos, expression was strictly monoallelic (Figure 2a, samples 2–5). These samples included a grade-2 IVF blastocyst (sample 2), a grade-2, day-7 IVF blastocyst (sample 3), a grade-2, day-7 IVF blastocyst (sample 4) and a grade-2, day-6 ICSI morula (sample 5), respectively. *MEST* imprinting analysis was also performed in a second set of heterozygous embryos, where the embryonic genomic DNA was genotyped by sequencing the *Afl*III polymorphism (Figure 2b, sample numbers from 6 to 10). *MEST* imprinting analysis was performed in their corresponding cDNAs by sequencing and/or *Afl*III restriction digest. For sample 6 (a day-6 expanded ICSI blastocyst), expression was biallelic. Monoallelic *MEST* expression was observed in three additional blastocysts (Figure 2b, samples 7–9). Predominantly monoallelic expression was observed in a day-6 expanded IVF blastocyst (sample 10).

MEST imprinting in blastocyst and morula cDNA libraries

The observation of variable *MEST* imprinting in the heterozygous embryos prompted a more extensive screening of embryonic *MEST* imprinting using a large series of cDNA libraries ($n = 61$) derived from human blastocysts and a smaller number of morulae (Figure 3). Genomic DNA was not available for these samples. As the genotype of these embryos is unknown, these experiments are informative only if expression from both alleles is detected. However, assessing a large cohort of embryos may yield useful information about the variability of imprinting between embryos. Eight embryos were identified with expression detectable from both alleles (Figure 3). Relaxed imprinting was observed in IVF-derived embryos (Figure 3, samples 11, 12, 29, 57 and 58) and ICSI-derived embryos (samples 10, 39 and 41). Two sibling ICSI embryos obtained following treatment for male factor infertility exhibited biallelic and predominantly monoallelic expression (Figure 3, samples 39 and 41, respectively). *MEST* imprinting was discordant between two sibling IVF embryos that exhibited predominantly monoallelic expression and monoallelic expression (Figure 3, sample 11 and Figure 2a, sample 2, respectively).

Expression of MEST isoforms in human oocytes and preimplantation embryos

Two main isoforms of *MEST* are expressed in humans, isoform 1 and isoform 2, that arise from promoters P2 and P1, respectively.[41]

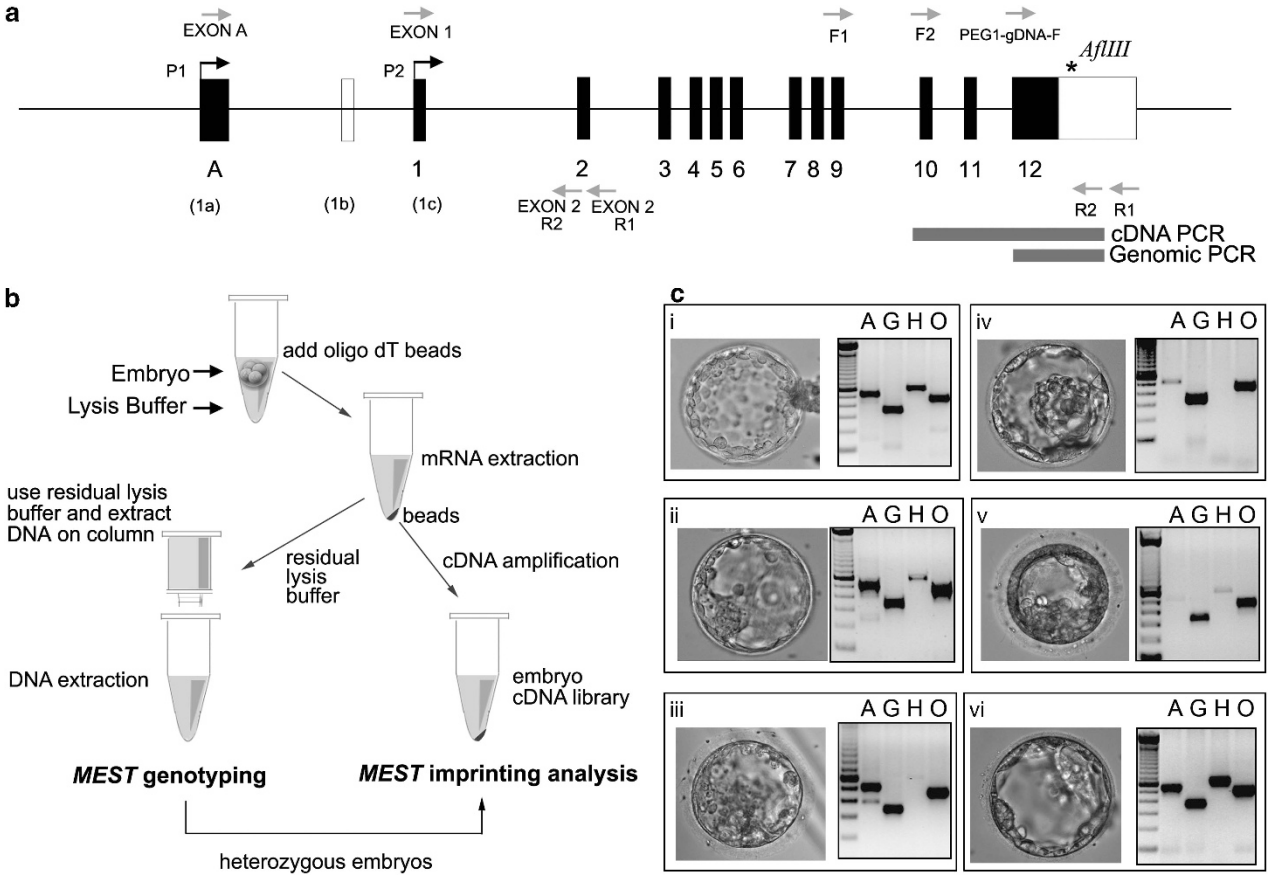

Figure 1 (**a**) Scheme for *MEST* genomic and imprinting analysis PCR assays showing PCR primer locations, PCR products and the *Afl*III polymorphism. (**b**) Scheme of protocol for the isolation of genomic DNA and cDNA from single preimplantation embryos, genotyping and imprinting analysis. (**c**) Examples of quality screening by PCR for housekeeping genes and developmental markers in embryonic cDNA libraries derived from six embryos (i–vi) that were used in the *MEST* imprinting study. Embryonic cDNAs were screened with β-actin (*ACTB*) gene (A), *GAPDH* (G), *HPRT* (H-variable between embryos), and *OCT4* (O).

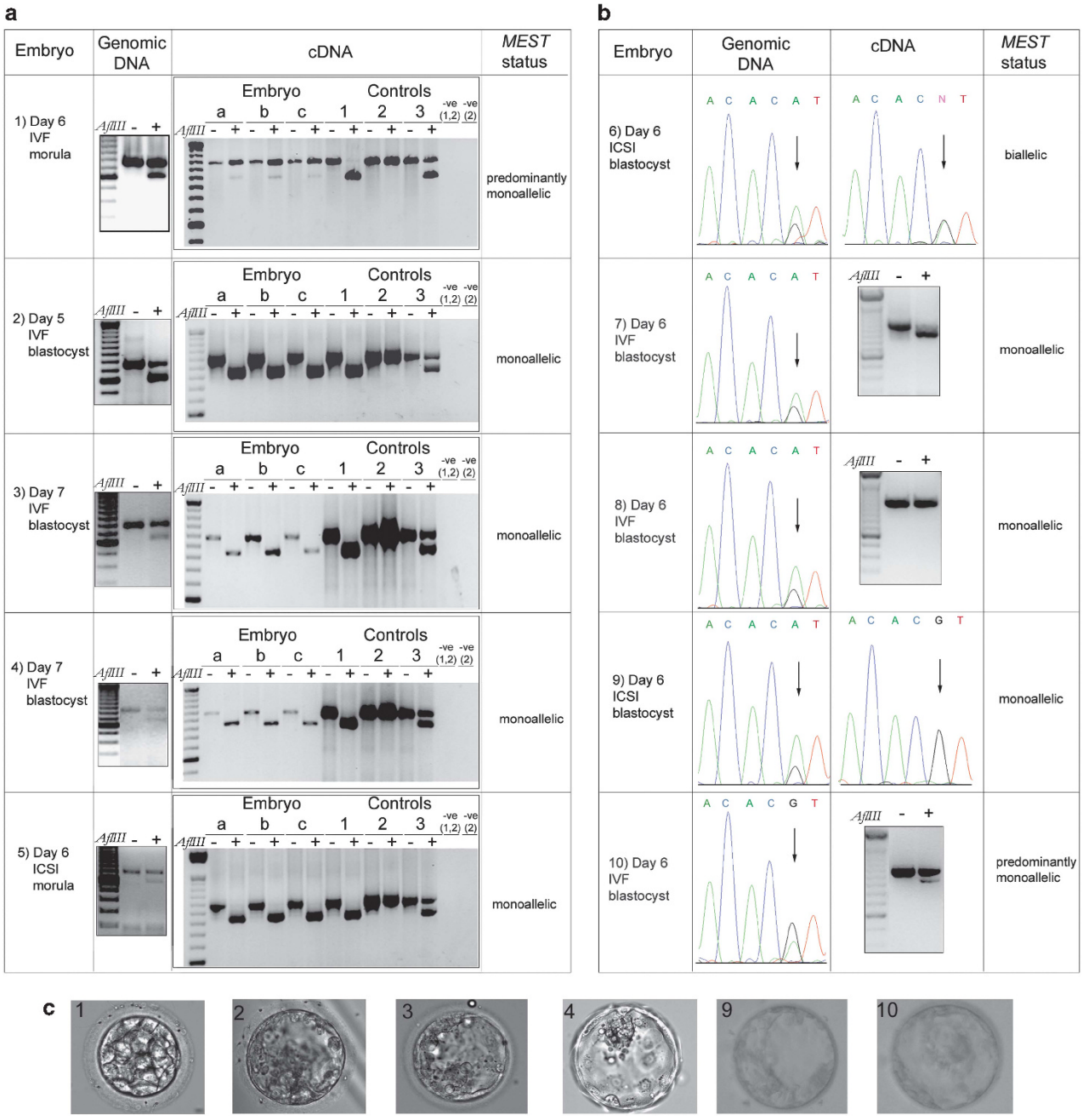

Figure 2 *MEST* imprinting analysis in 10 IVF and ICSI human preimplantation embryos that were heterozygous for the *MEST Afl*III polymorphism. The PCR scheme used for imprinting analysis from cDNA amplifies both *MEST* isoforms. Full sample details are given in Table 1. (**a**) *MEST* imprinting analysis by *Afl*III digestion in heterozygous blastocysts. Embryos were genotyped and then assessed by imprinting analysis. Nested genomic PCR and *Afl*III digestion of the second round PCR product was performed to identify heterozygous embryos (genomic DNA panels). Amplification of *PEG1/MEST* transcripts from amplified cDNA derived from each of these embryos was performed in triplicate (a–c) with undigested (−) and fully digested products (+) in adjacent lanes (cDNA panels). For each embryo, enzyme digestion controls of *MEST* PCR products were included from amplified cDNA libraries derived from limiting quantities of somatic tissue RNA of known genotype. These were either homozygous for each opposite allele (control lanes 1 and 2) or biallelic (control lane 3). The negative sample controls include a sample carried through both rounds of PCR (−ve 1, 2) and the second round only (−ve, 2). *MEST* imprinting in one morula (embryo 1) was predominantly monoallelic, while monoallelic *MEST* expression was observed for embryos 2–5. (**b**) *MEST* imprinting analysis in an additional set of heterozygous blastocysts achieved by sequencing of *MEST* PCR products from the genomic DNA of five human blastocysts and analysis of *MEST* imprinting in their corresponding cDNAs by sequencing or *Afl*III restriction digest. In one embryo (embryo 6) expression was biallelic (the peaks are overlapping in the cDNA sequence), while predominantly monoallelic expression was observed in a day-6 expanded IVF blastocyst (embryo 10). Monoallelic expression was observed in the three additional blastocysts (7, 8, 9). (**c**) Photographs of selected embryos used for imprinting analysis in this figure (embryos 1, 2, 3, 4, 9, 10).

We characterised the expression of *MEST* isoforms in human oocytes, and preimplantation embryos. In our assay, that used a hemi-nested-PCR protocol with isoform 1-specific primers spanning from exon 1 (also referred to as 1c) to exon 2, isoform 1 was not readily detected in oocytes, zygotes or morulae samples (Figures 4a–c). Expression of isoform 1 was weakly detected in only one out of a total of nine

Table 1 Summary of *MEST* imprinting data in 18 human preimplantation embryos. Descriptions of embryo stage and grade are given together with relative expression of each *MEST* allele, given as a percentage of total expression

Figure	Sample/lane number	Description	Grade	Embryo status	Cause of infertility	ART treatment	MEST imprinting	% of A allele	% of G allele
2a	1	Morula, day 6	2	Fresh	Idiopathic	IVF	Predominantly monoallelic	93.0	7.0
2a	2	Blastocyst	2	Fresh	Idiopathic	IVF	Monoallelic	0	100
2a	3	Blastocyst, day 7	2	Fresh	PCO	IVF	Monoallelic	0	100
2a	4	Blastocyst day 7	2	Fresh	PCO	IVF	Monoallelic	0	100
2a	5	Morula, day 6	2	Fresh	Male factor	ICSI	Monoallelic	0	100
2b	6	Expanded blastocyst, day 6	1	Fresh	Male factor + PCO	ICSI	Biallelic	50[a]	50[a]
2b	7	Expanded blastocyst, day 6	ND	Fresh	Idiopathic	IVF	Monoallelic	0	100
2b	8	Expanded blastocyst, day 6	2.5	Fresh	Idiopathic	IVF	Monoallelic	100	0
2b	9 (ZS)	Expanded blastocyst, day 6	ND	Frozen	Anovulation	ICSI	Monoallelic	0	100
2b	10 (ZS)	Expanded blastocyst, day 6	1	Frozen	Tubal + PCO	IVF	Predominantly monoallelic	87.5	12.5
3	10	Blastocyst	2	Fresh	Male factor + PCO	ICSI	Predominantly monoallelic	93.9	6.1
3	11	Blastocyst	2	Fresh	Idiopathic	IVF	Predominantly monoallelic	90.3	9.7
3	12	Blastocyst	2	Fresh	Idiopathic	IVF	Biallelic	47.5	52.5
3	29	Blastocyst, day 7	2	Fresh	Idiopathic	IVF	Predominantly monoallelic	13.1	86.9
3	39	Blastocyst, day 6	1	Fresh	Male factor + tubal	ICSI	Biallelic	54.1	45.9
3	41	Hatching blastocyst, day 6	1	Fresh	Male factor + tubal	ICSI	Predominantly monoallelic	80.1	19.9
3	57	Early blastocyst, day 6	ND	Fresh	Idiopathic	IVF	Predominantly monoallelic	69.7	30.3
3	58	Blastocyst, day 6	ND	Fresh	Idiopathic	IVF	Biallelic	55.8	44.2

Abbreviations: ART, assisted reproductive technologies; ICSI, intracytoplasmic sperm injection; IVF, *In vitro* fertilisation; MEST, mesoderm-specific transcript homologue; ND, no data; PCO, Polycystic ovaries; ZS, zona stripped.
[a]Approximate value from sequencing image.

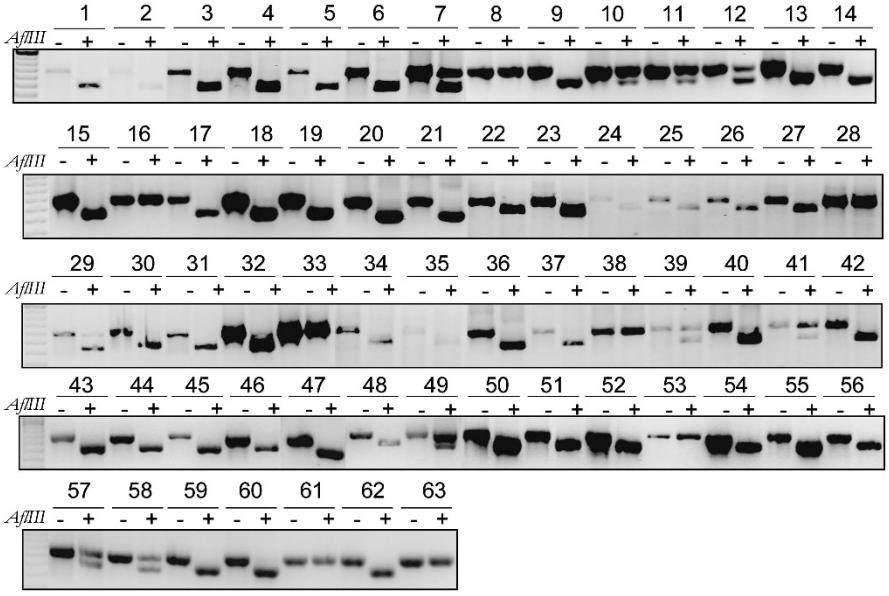

Figure 3 *MEST* imprinting analysis in 61 human preimplantation embryos of unknown genotype. The PCR assay amplifies both *MEST* isoforms. Undigested (−) and fully *Afl*III digested products (+) are shown in adjacent lanes. Control biallelic or predominantly monoallelic samples that were derived from mixed somatic tissues and liver are included (sample 7 and 49, respectively). Both *MEST* alleles were detected in embryo samples 10, 11, 12, 29, 39, 41, 57, 58. Embryo details and *MEST* imprinting status are summarised in Table 1. In this figure, Sample 39 and 41 are sibling ICSI-derived embryos. Sample 11 (Figure 3) is a sibling of sample 2 in Figure 2a, both are IVF-derived.

blastocysts, but was readily detected in control cDNAs derived from mixed human tissue, ovary and placenta (Figure 4c). In contrast, isoform 2 was detected in oocytes, zygotes, morulae and blastocyst stage embryos in addition to the control tissues using isoform 2-specific primers spanning from exon A (also referred to as 1a) to exon 2 (Figures 4a–c). The identity of the isoform-specific PCR products was confirmed by sequencing. *MEST* isoform 2 is the predominant *MEST* isoform that we detected in human oocytes and preimplantation embryos.

Alternative splicing of isoform 2

Alternative splicing within exon A of isoform 2 has been previously reported.[42] The shorter P1a transcript of isoform 2 was the major splice variant expressed in human oocytes, zygotes and all preimplantation stages tested here (Figure 4b). The larger P1b transcript was detected as a weaker band in two oocytes. The identity of PCR products was confirmed by sequencing.

Direct imprinting analysis of isoform 2 in morula and blastocyst stage embryos

Isoform 2-specific imprinting analysis was performed in three embryos (Figure 4d), to ascertain whether the variable *MEST* imprinting observed between embryos was attributable to variable imprinting of isoform 2. In Figure 4d, sample 1 was an ICSI blastocyst that had been previously been shown to have predominantly monoallelic *MEST* expression when analysed by the PCR assay that

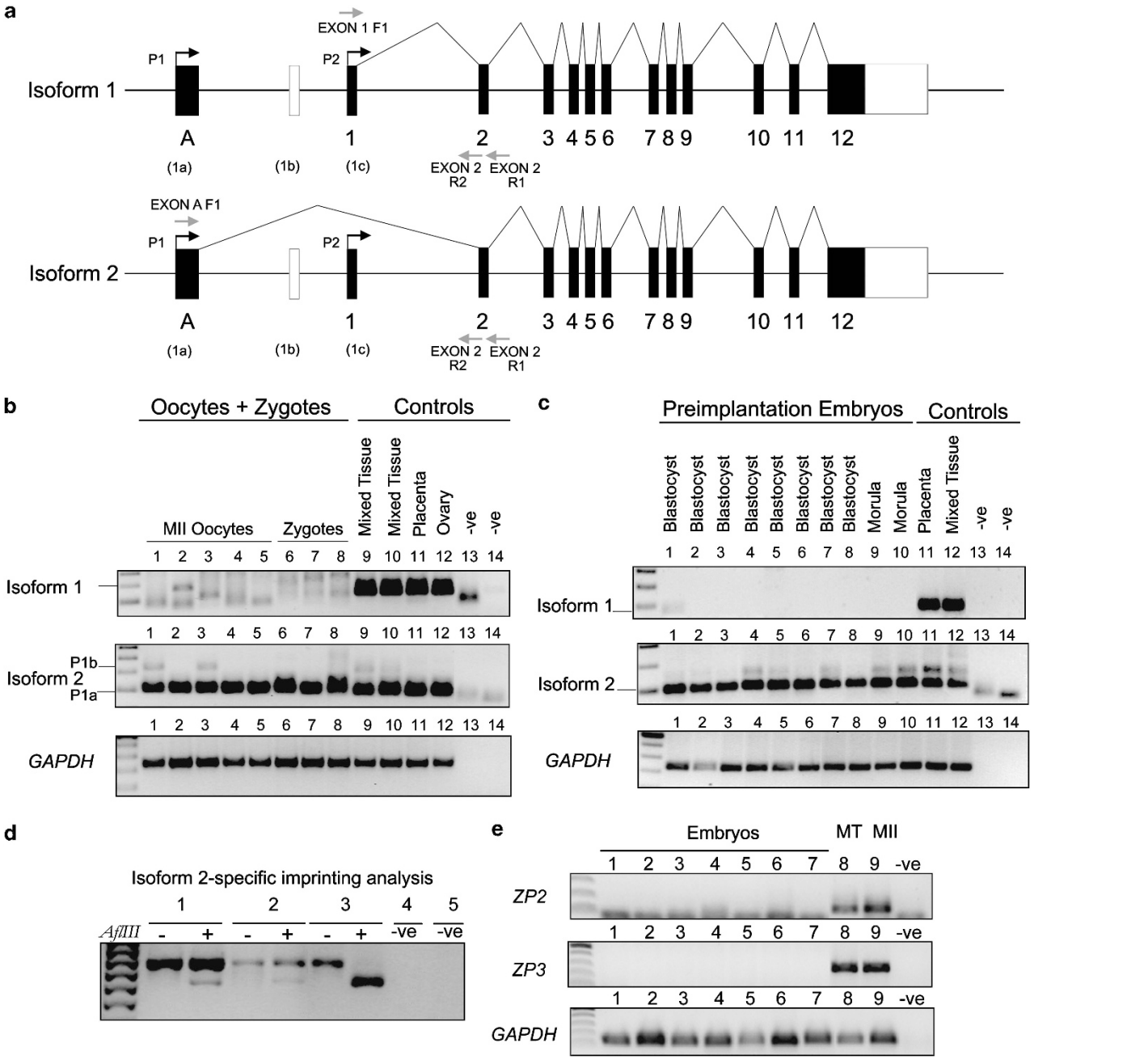

Figure 4 Expression of *MEST* isoforms in human oocytes, zygotes and preimplantation embryos. (**a**) Expression of isoform 1 was assessed using a hemi-nested-PCR protocol with isoform 1-specific primers spanning from exon 1 (also referred to as 1c) to exon 2. Expression of isoform 2 was detected using primers spanning from exon A (also referred to as 1a) through to exon 2. (**b**) Expression of Isoforms 1 and 2 in human oocytes (lanes 1–5) and zygotes (lanes 6–8). Isoform 1 was weakly detected in only one oocyte (Isoform 1, lane 2) but was detected in control samples comprising of mixed somatic tissues (Isoform 1 experiment, lanes 9 and 10), and placenta and ovary (lanes 11 and 12) in the same assay. Isoform 2 was detected in all cDNAs from oocytes and zygotes (Isoform 2 experiment, lanes 1–8) and controls (lanes 9–12). Isoform 2 may be alternatively spliced: the shorter P1a transcript of isoform 2 was the major splice variant expressed in the oocyte, zygote and all preimplantation stages. The larger P1b transcript was only detected as a weaker band in two oocytes as indicated (Isoform 2 experiment, lanes 1 and 3). (**c**) Expression of Isoforms 1 and 2 in human morulae and blastocysts. Isoform 1 was weakly detected in one blastocyst (Isoform 1, lane 1, as indicated). In contrast, isoform 2 was readily detected in all morula or blastocyst stage embryos (lanes 1 to 10) and control tissues (lanes 11, 12). (**d**) Isoform 2-specific imprinting analysis in three embryos using first round PCR primers located in exon A and the 3′-UTR (R1) that select for isoform 2, and second round primers in exon 10 and 3′-UTR (R2) that surround the *Afl*III polymorphism. Sample 1: ICSI blastocyst that had been previously been shown to have predominantly monoallelic expression in the PCR assay that amplifies both isoforms (see Figure 3, sample 10), sample 2: day-6 IVF morula that had been previously been shown to have predominantly monoallelic *MEST* expression (see Figure 2a, sample 1), sample 3: day-6 ICSI morula, that was previously confirmed to have monoallelic *MEST* expression (see Figure 2a, sample 5). Lanes 4 and 5 are negative controls for the first and second round PCRs, respectively. **(e)** Expression of maternal (oocyte-derived) zona pellucida gene transcripts (*ZP2*, *ZP3*) and *GAPDH* in embryos with biallelic or predominantly monoallelic *MEST* expression. (Lanes 1–7) Embryos with biallelic or predominantly monallelic *MEST* expression. (Lane 8) Mixed human somatic tissue control (MT), (Lane 9) Pooled MII oocyte control cDNA samples (MII), −ve control (−ve).

amplifies both isoforms (see Figure 3, sample 10). Sample 2 was a heterozygous day-6 IVF morula that had been previously been shown to have predominantly monoallelic expression (see Figure 2a, sample 1). Sample 3 was a day-6 ICSI morula that was previously confirmed as being heterozygous and imprinted (see Figure 2a, sample 5). The results confirm variability of imprinting for isoform 2 between embryos. The lack of detection of maternal transcripts (*ZP2*, *ZP3*) in the blastocysts that were identified to have monoallelic or biallelic *MEST* expression (Figure 4e) indicates that oocyte-derived transcripts are likely to be absent in these blastocysts. Therefore, *MEST* transcripts amplified in our assays are likely to be embryonic in origin.

DISCUSSION

The assessment of the imprinting status of imprinted genes in the human preimplantation embryo can reveal how imprinted gene regulation may be affected by ART and infertility, and potentially, how can this be avoided. These data also provide control information against which the origins of epigenetic instability of human-embryonic stem cells can be better understood. In addition to analysing epigenetic marks such as DNA methylation in early development, it is also pertinent to study the expression of imprinted genes, as their expression status is governed by the collective influence of these epigenetic marks, irrespective of their nature. To date, the imprinting status of only two genes has been established for human preimplantation development revealing that *IGF2* and *SNRPN* are imprinted.[43,44]

MEST gene transcripts are known to be expressed in human oocytes and preimplantation embryos.[38] However, earlier attempts at characterising the imprinting status of *MEST* in human cleavage stage embryos were inconclusive (described in Monk and Salpekar (2001)[45]). In the current study, we have shown that in a large cohort of human preimplantation embryos that were mostly of the blastocyst stage, imprinting of the *MEST* gene was variable. Importantly, our data shows that the onset of imprinted *MEST* expression can indeed occur during late preimplantation development and this is consistent with the observation of a significant reduction of *MEST* expression at the blastocyst stage in human parthenogenetic preimplantation embryos.[46]

Across our whole study, we observed a total of 11 embryos in which transcripts from both alleles were detected, being biallelic (four embryos) or predominantly monoallelic (seven embryos). Indeed it is possible therefore that some aspects of the ART treatment or infertility *per se* may have influenced *MEST* imprinting in these cases. Most of the reports of disturbance of *MEST* imprinting in male factor infertility refer to hypermethylation of the paternal (expressed) *MEST* allele.[18,26,27] In the preimplantation embryo, it is unlikely that this abnormal methylation state would directly cause relaxed imprinting, namely, the onset of transcription from the normally silenced maternal allele. Therefore, the cause of relaxed *MEST* imprinting as observed in these embryos is likely to be maternal or embryonic in origin, such as a failure to establish correct imprinting in the oocyte and/or a failure to maintain the imprint in preimplantation development. In agreement, relaxed *MEST* imprinting was observed in both IVF and ICSI-derived embryos from couples presenting with different causes of infertility, and therefore can occur independently of an effect associated exclusively with male infertility (Table 1). We note that relaxed *MEST* imprinting was observed in embryos that were graded as 1 and 2, therefore implying that embryo morphological grade is not a useful indicator for the assessment of embryo epigenetic status, at least for *MEST*. Two sibling IVF blastocysts (sample 2, Figure 2a and sample 11, Figure 3) demonstrated contrasting states for *MEST* imprinting, with monoallelic and predominantly monoallelic expression, respectively. As these sibling embryos were fertilised and cultured together, it is not likely that the culture environment or an effect associated with female infertility is the causative factor in the embryo with relaxed imprinting; however, developmental differences between the embryos must be considered. Other possible causes of variability in *MEST* imprinting between embryos may be differing responses to the superovulation regimen.[19] Other studies have reported lower than average methylation of the *MEST* gene in children conceived through ART.[20] These observations concur with our findings reported here and could conceivably result in a loss of *MEST* imprinting in the early embryo. Our study cohort included embryos that were fertilised in two different fertility clinics, suggesting that variable *MEST* imprinting may be common in *in vitro*-derived embryos, but may also occur *in vivo*. Due to insufficient material (genomic DNA), we were unable to establish whether LOI was present at other loci, that would imply a global imprinting defect. Development up to the blastocyst stage can however clearly be achieved in the embryos with relaxed *MEST* imprinting.

These concerns must however be balanced against current knowledge of the imprinting status of isoforms that arise from the *MEST* locus. Isoform 1 appears to be expressed in a monoallelic fashion in numerous tissues.[7] Initial reports described isoform 2 as being biallelic in blood lymphocytes.[41] Subsequent reports have indicated that in certain tissues including breast, placenta and colon, isoform 2 may in fact be imprinted and, moreover, that the imprinting status (whether monoallelic or biallelic) may differ between individuals.[13,16,37,47] Isoform 2 has been observed to be preferentially paternally expressed in foetal placenta in other studies, and also in kidney and fibroblast lines, with polymorphic imprinting observed between different fibroblast lines.[48] Variable imprinting of *MEST*, including that of isoform 2, has also been reported between human-embryonic stem cell (hESCs) lines, raising concerns of the epigenetic stability of these cells.[40,49,50]

In view of the observations of aberrant *MEST* methylation and imprinting with disease,[13,14,16] we considered it important to further characterise expression at the *MEST* locus in the embryos with expression arising from both alleles. Our data demonstrates that isoform 2 was the predominant *MEST* isoform detected in preimplantation embryos and show directly that imprinting of isoform 2 itself is variable between embryos (Figure 4d). We were, however, unable to test biallelic embryos in the isoform 2-imprinting assay. We acknowledge that technical restrictions, or differential poly-A tail length usage between isoforms, may have limited our ability to detect isoform 1 expression in oocytes and embryos (Figures 4b and c, respectively). Significantly, we found no evidence for *MEST* imprinting states that are observed in disease, that is, either relaxation of imprinting of isoform 1, or of promoter shifting from imprinted isoform 1 to the variably imprinted isoform 2. The variability in *MEST* imprinting between hESC lines and also the placenta may therefore reflect an epigenetic programme that was intrinsic to the founder blastocyst, within the inner cell mass and trophectoderm, respectively. We detected expression of the P1b *MEST* splice variant in a small number of oocytes (Figure 4b) and this is in agreement with the observation of expression of P1b in the ovary.[42]

In conclusion, while infertility or ART-induced effects both remain as possible causes for the variable imprinting of *MEST* observed among the large cohort of blastocysts tested, our considered opinion is that effects due to inter-individual variation in imprinting of isoform 2 must also be considered. These *MEST* imprinting

differences may also exist in naturally conceived human preimplantation embryos. Regardless of the cause, the developmental consequences of the variable imprinting of *MEST* in early human embryos *in vitro* and *in vivo* remain unclear. In mice, elevated expression of *Mest* has been associated with fat mass expansion [4,51,52] and obese phenotypes with LOI of *Mest* have been obtained from epigenetically immature oocytes.[53] Finally, it remains possible that uncharacterized *MEST* transcripts, such as human equivalents of the longer *MestXL* variants that were recently described in the mouse,[54] may have been amplified in our non-isoform-specific PCR assays. These observations suggest that further characterisation of the causes and effects of contrasting embryonic imprinting states during *in vitro* conception are required.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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