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Imprinting of *PEG1*/*MEST* Isoform 2 in Human Placenta

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The *PEG1* gene (a.k.a. *MEST*) is expressed in human placental trophoblast and endothelium, and data from knockout mice show that this gene regulates placental and fetal growth. Isoform 1 of *PEG1* mRNA initiates from exon 1c and produces the long form of the MEST protein. This isoform is imprinted, with expression only from the paternal allele in many human and mouse organs, including placenta. In contrast, *PEG1* isoform 2, initiating from exon 1a and producing the short form of MEST protein, is biallelically expressed (non-imprinted) in several non-placental organs. Here we show that *PEG1* isoform 2 is in fact imprinted in a large subset of human placentae. A CpG island overlapping *PEG1* exon 1a is unmethylated in various fetal and adult non-placental tissues, but is often substantially methylated in the placenta, with the extent of methylation in a large series approximating a normal distribution. Bisulfite conversion/sequencing indicates that the inter-individual differences reflect the relative representation of heavily methylated vs. unmethylated alleles, and RT-PCR/RFLP analysis shows strongly biased allelic expression of *PEG1* isoform 2 mRNA in a majority of placentae with a high proportion of methylated alleles. These data highlight *PEG1* isoform 2 as a marker for future studies of inter-individual epigenetic variation and its relation to placental and fetal growth in humans.

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INTRODUCTION

Paternally Expressed Gene-1 (*PEG1*, a.k.a. *MEST*) is an imprinted gene that is highly expressed in human placental tissues, including villous cytotrophoblast and vascular endothelium [1]. This gene encodes a presumptive hydrolase enzyme, with unknown substrates. The location and timing of *PEG1* mRNA expression in the human placenta suggest a role for this gene in placental and decidual angiogenesis [1], and genetic experiments have shown that the mouse orthologue, *Peg1*, controls prenatal growth, with growth retardation of the fetus and the placenta in *Peg1*-deficient conceptuses [2].

Defects in functional imprinting underlie certain rare human developmental disorders, but our understanding of epigenetic variation and its impact on phenotypes in the general population is limited. With this background, we initiated a project to evaluate inter-individual variation in DNA methylation at imprinted loci in human placentae. Here we describe results for the human *PEG1* gene that provide a clear example of variable imprinting in human placentae, both at the level of DNA methylation and allele-specific mRNA expression.

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MATERIALS AND METHODS

Placentae and parental DNA

Biopsies of chorionic villous tissue from human placentae, and biopsies of non-placental control tissues were obtained as anonymous samples from the Columbia University Cancer Center Tissue Bank, under an exempt Institutional Review Board (IRB)-approved protocol. Peripheral blood leukocyte DNA from parents was obtained with informed consent, under a separate protocol approved by the IRB. Placentae for trophoblast enrichment were obtained from Washington University St. Louis, as anonymous tissues under an IRB-approved protocol.

Analysis of DNA methylation by Southern blotting and Phosphorimaging

For Southern analysis, genomic DNAs (4 μg) from human tissue were digested with *Pst*I or *Pst*I plus *Sma*I restriction enzymes, resolved in 1% agarose gels, and transferred to nylon membranes. The blots were hybridized with ^{32}P-labeled probes that were generated by PCR of genomic DNAs using primers

Table 1. Sequences of PCR primers used in this study

Primer name	Sequence	Product	No. in Figure 1
KvDMR1-F	5′-CAGGCAGCAGAAAACAAAACAGAG-3′	KvDMR1 probe	na
KvDMR1-R	5′-TTAGAGGTCTCAGTGGGGTATGGG-3′		
PEG1-1a-RTPCR-F	5′-AGGCAAGGTCTTACCTGAATC-3′	1a transcript	1,4
PEG1-1a/1c-RTPCR-R	5′-GCTCATAGGTCTTTCAGAGTTTG-3′	(short isoform; isoform 2), RT-PCR	
PEG1-1c-RTPCR-F	5′-GATAACGCGGCCATGGTG-3′	1c transcript	2,4
PEG1-1a/1c-RTPCR-R	5′-GCTCATAGGTCTTTCAGAGTTTG-3′	(long isoform; isoform 1), RT-PCR	
PEG1-gDNA-F	5′-GGTCCTGGCCATCAAACATA-3′	Genomic PCR	3,5
PEG1-RTPCR-R	5′-CAGCACCATTTGCTCATAGG-3′		
PEG1-1a-F	5′-GCCTGCCATTTCATACCCTTTG-3′	Exon 1a CpG island probe	na
PEG1-1a-R	5′-TGCCTGCGACCTTTCCAAC-3′		
PEG1-1c-F2	5′-TCAGGGGTCTGCTGTTTTTGC-3′	Exon 1c CpG island probe	na
PEG1-1c-R	5′-GAAATCCTAAATCTCACCACGACG-3′		
PEG1-1a-bs-F	5′-CACCAGTCCTAGGGGCAGGTAAGTG-3′	PCR for bisulfite sequencing	na
PEG1-1a-bs-R	5′-CTGGCTGTCCCACAAGTAGGAAGGC-3′		

listed in Table 1. High stringency hybridizations were performed at 42 °C in ULTRAHyb solution (Ambion, Inc., Austin, TX) and the blots were washed for 30 min at room temperature in 2 × SSC/0.1%SDS followed by a 64 °C wash in 0.1 × SSC/0.1% SDS. Band intensities were measured by Phosphorimaging (Storm PhosphorImager®, Amersham Biosciences Corporation, Piscataway, NJ). The methylation ratio was calculated by dividing the numerical value assigned to the methylated (upper) band in each lane by the value assigned to the unmethylated (bottom) band, after background subtraction.

Analysis of DNA methylation by bisulfite conversion and sequencing

The procedure for bisulfite-mediated conversion was similar to published protocols [3]. Briefly, genomic DNA (gDNA, 5 μg in 60 μL of water) was first denatured by adding 2.5 μL of 5 N NaOH and incubating at 37 °C for 10 min. Hydroquinone (36 μL of a 10 mM solution) was added, followed by addition of sodium bisulfite (622 μL of a 3 M solution) and incubation at 50 °C for 16 h. The bisulfite-converted DNA was then purified using Centricon YM-30 spin columns (Millipore, Bedford, MA), with elution in 50 μL of water. The DNA was desulphonated in 0.3 N NaOH at room temperature for 5 min, and then precipitated by adding an equal volume of 10 M ammonium acetate, followed by two volumes of ice-cold ethanol. The pellet was washed in 70% ethanol, dried briefly and dissolved in 50 μL of water. Each subsequent PCR reaction utilized 2 μL of the resulting DNA solution. Primers for amplifying a portion of the *PEG1* exon 1a CpG island from the bisulfite-converted DNA are in Table 1. PCR was performed using Platinum Taq (Invitrogen, Carlsbad, CA) with an initial denaturation at 94 °C for 2 min followed by 32 cycles of the following protocol: 94 °C for 45 s, 56 °C for 45 s, 72 °C for 45 s, with a final extension at 72 °C for 10 min. The PCR products were gel isolated, ligated into a TA cloning vector (pCR® 4-TOPO® vector, Invitrogen, Carlsbad, CA) and the resulting clones were sequenced.

Reverse-transcription PCR (RT-PCR) and analysis of allele-specific mRNA expression

Genotyping for a single-nucleotide polymorphism (SNP) in *PEG1* exon 12 (rs10863, NCBI) was done by restriction digestion and direct sequencing, essentially as previously described [4]. Briefly, PCR amplification of genomic DNAs using specific primers (Figure 1 and Table 1) was performed using an initial denaturation step at 94 °C for 2 min followed by 32 cycles of denaturation at 94 °C, annealing at 56 °C for 45 s and a 1 min extension and a final extension of 72 °C for 10 min. An aliquot of 2 μL from each sample was digested

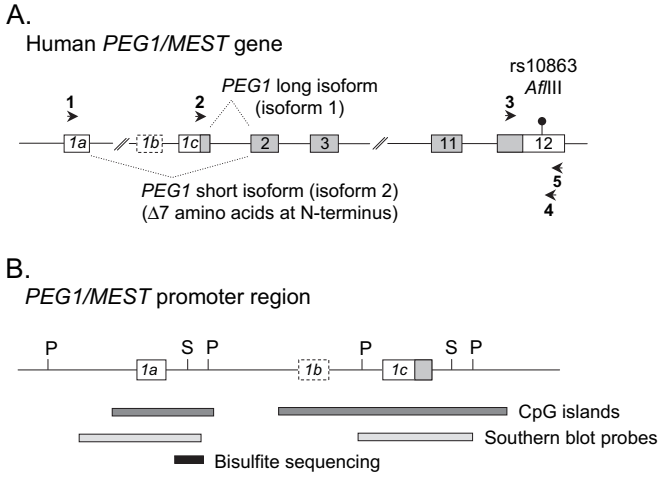

Figure 1. Map of the human *PEG1/MEST* gene. (A) The human *PEG1* gene, showing the major alternative mRNA isoforms and the PCR primers utilized in this study. Exons are rectangles, and the splicing patterns that distinguish *PEG1* isoforms 1 and 2 are indicated. Exon 1b (hatched lines) is represented by a single EST in Genbank, and therefore may be a rarely transcribed variant. PCR primers are numbered as in Table 1. (B) Magnified view and partial restriction map of the alternative upstream exons. CpG islands and probes utilized for Southern blotting are indicated by the darkly shaded and lightly shaded rectangles, respectively. The region subjected to bisulfite conversion/sequencing is indicated by the black rectangle. S, *Sma*I; P, *Pst*I.