Letters to the Editor

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Isoform-Specific Imprinting of the Human *PEG1/MEST* Gene

To the Editor:

Mouse *Peg1/Mest* encodes a protein with sequence homology to the alpha/beta-hydrolase (Sado et al. 1993). The gene maps to an imprinted region of mouse chromosome 6 and is expressed monoallelically from the paternal allele (Kaneko-Ishino et al. 1995). When the null allele is paternally transmitted, the offspring exhibit severe intrauterine growth retardation (Lefebvre et al. 1998). Uniparental disomy of mouse chromosome 6 is associated with a similar phenotype, presumably as a result of lack of expression of *Peg1/Mest* (Ferguson-Smith et al. 1991). The human homologue, *PEG1/MEST,* has been mapped to 7q31.3, within a region of conserved synteny corresponding to mouse chromosome 6, and is monoallelically expressed from the paternal allele in a wide variety of tissues during prenatal and postnatal development. Uniparental disomy of chromosome 7 in humans is associated with phenotypic features of Russell-Silver syndrome (MIM 180860), characterized by intrauterine growth retardation with dysmorphic features such as triangular facies. *PEG1/MEST,* as the only known imprinted gene on chromosome 7, has been considered a candidate gene for the syndrome (Kobayashi et al. 1997; Lefebvre et al. 1997; Riesewijk et al. 1997).

Imprinting of *PEG1/MEST* is apparently lost in lymphocytes and transformed lymphoblastoid cell lines. In these tissues, *PEG1/MEST* is apparently expressed from both the paternal allele and the maternal allele (Riesewijk et al. 1997). Furthermore, *PEG1/MEST* is transcribed in lymphoblastoid cell lines from patients with maternal uniparental disomy of chromosome 7, or "upd(7)mat" (Cuisset et al. 1997; Riesewijk et al. 1997). Because upd(7)mat cells lack a paternal allele of *PEG1/MEST,* the transcript must derive from the maternal allele.

The purpose of this report is to delineate the underlying mechanism of apparent loss of imprinting in lymphocytes,to better understand the control of imprinting of the human *PEG1/MEST* gene. In general, loss of imprinting may be accounted for by several mechanisms. First, imprinting can be regulated in a tissue-specific way. Relaxation of imprinting or biallelic expression of imprinted genes is observed in some tissues. Examples include insulin (*Ins*) *1* and *Ins2* (Giddings et al. 1994; Deltour et al. 1995), and *Ube3a* (Albrecht et al. 1997). Second, imprinting may be controlled in a promoter-specific manner. Such promoter-specific imprinting was first identified in the *IGF2* gene (Vu and Hoffman 1994; Ekstroem et al. 1995). In liver and chondrocytes, the *IGF2* transcript from the P1 promoter is always derived from both the paternal allele and the maternal allele, whereas transcripts from other promoters (P2–P4) are expressed solely from the paternal allele. This finding demonstrated that both imprinting and a lack of imprinting could occur within a single gene in a single tissue, suggesting that regional imprinting factors might be important. Third, imprinting can be governed in an isoform-specific way when a single transcription unit encodes different proteins. Maternally derived (e.g., from *NESP55*), paternally derived (e.g., from *XLALPHAS*), and biallelically derived (e.g., from *GSALPHA*) proteins are produced by different patterns of promoter use and alternative splicing of a single transcription unit, *GNAS1* (Hayward et al. 1998; Peters et al. 1999).

Comparison of the 5′ end of the expressed-sequence tag (EST) sequences assembled as the *PEG1/MEST* UniGene cluster (Hs. 79284) revealed that six EST clones—AA305098 and AA305289 (colon carcinoma), AA337069 (endometrial tumor), R18211 (infant brain), AA095601 (8-wk fetal heart), and AA092738 (10-wk fetal heart)—share a novel sequence joined to exon 2 of *PEG1/MEST* (Cuisset et al. 1997), suggesting transcription of an alternative isoform (fig. 1). We first characterized the alternative isoform of *PEG1/MEST* and examined expression of each of the original and novel isoforms independently. In the following discussion, the original isoform and the alternative one will be referred to as "isoform 1" and "isoform 2," respectively. To delineate the genomic structure of the *PEG1/MEST* transcription unit containing the two isoforms, finished genomic-sequence contigs of 7q31.3, deposited at the University of Washington Genome Center were surveyed and aligned against the isoform 1–specific and the isoform 2–specific cDNA sequences, by means of Sequencher software (Gene Codes). Because a mapping

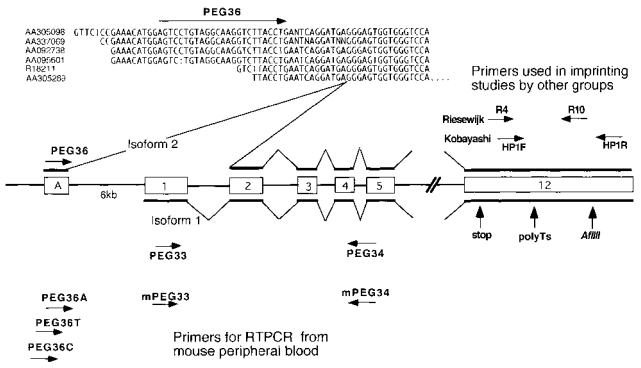

Figure 1 Alternative splicing of human *PEG1/MEST*. Arrows indicate primers used for expression studies. RT was followed by PCR amplification by use of exon specific primers. *Top,* Human EST sequences that matched with the first exon of the alternative isoform. *Middle,* Exon organization of the *PEG1/MEST* transcription unit that transcribes two isoforms. In the present study, RT-PCR was done either with PEG36 and PEG34 or with PEG33-PEG34, to detect isoform 2 and isoform 1, respectively. Poly T's and *Afl*II correspond to polymorphic sites in the 3′ UTR that have been described elsewhere. Riesewijk et al. (1997) and Kobayashi et al. (1997) used primer pair R4 and R10 and primer pair HP1F and HP1R, respectively. *Bottom,* Primers used for RT-PCR, from mouse peripheral blood.

study had indicated that genetic distance between *PEG1/MEST* and D7S649 (also known as "sWSS1203") was <1 cM (Kobayashi et al. 1997), sequence contigs flanking the PAC clone djs213 containing D7S649 were analyzed (GenBank accession number AC007938). Comparison of the genomic sequence of PAC djs201 and cDNA sequences of isoform 1 and isoform 2 revealed the following: (1) the two isoforms have distinctive first exons (the first exon of isoform 2 will be referred to as "exon A," the first exon of isoform 1 as "exon 1"); and (2) exon A is located 6 kb upstream of exon 1. Exon A contains a stop codon only 6 bases 5′ of the exon-intron boundary. It is likely that the start codon of isoform 2 is within exon 2 and that exon A comprises the 5′ UTR of isoform 2. Exon A is ⩾57 bp in length. Expression of isoform 1 and isoform 2 in lymphoblastoid cells was detected by means of reverse transcription–coupled PCR (RT-PCR) assay. Either the forward PCR primer (PEG36 [5′-agtcctgtaggcaaggtcttacctg]), based on the isoform 2–specific sequence in exon A, or the forward primer specific for the exon 1 of the isoform 1 (PEG33 [5′-atgggataacgcggccatggtg-3′]), was used with the reverse primer that anneals to the portion of the cDNA sequence shared between the two isoforms (PEG34 [5′-atagtgatgtggtctcggtttgtcactg-3′]) (fig. 1). A upd(7)mat lymphoblastoid cell line (GM11496) (Spence et al. 1988) and a paternal uniparental disomy of chromosome 7, or "upd(7)pat," lymphoblastoid cell line (Pan et al. 1998) were obtained from the National Institute of General Medical Sciences (NIGMS Coriell Cell Repositories) and from the tissue culture core at Baylor College of Medicine, respectively. The cells were cultured under standard conditions, and total RNA was extracted by means of an RNA purification kit (QIAGEN). One microgram of total RNA was used to synthesize cDNA with the Superscript preamplification system (GIBCO/BRL), and 1% of the resulting material was used for RT-PCR. The cycling conditions were 94°C for 10 min (1 cycle); 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min (40 cycles); and 72°C for 10 min (1 cycle). RT-PCR revealed that the upd(7)mat cell line expressed isoform 2 but not isoform 1, whereas normal lymphocytes and the upd(7)pat cell line expressed both isoform 1 and isoform 2 (fig. 2).

In this study, we have demonstrated that (1) an alternative isoform of *PEG1/MEST* is expressed concurrently with the original isoform in adult lymphocytes and lymphoblastoid cell lines and (2) isoform 1 (the original isoform) is expressed only from the paternal allele, whereas isoform 2 (the alternative isoform) is expressed from both the paternal allele and the maternal allele. These results are discordant with the results of previous studies, which support biallelic expression of the *PEG1/MEST* in lymphocytes. In retrospect, it is understandable why the previous studies failed to identify such differential imprinting: the primers used for RT-PCR in other studies would not have allowed discrimination between the imprinted isoform and the nonimprinted isoform (fig. 1). In lymphocytes, recognition of an imprinted isoform (isoform 1) was masked by the presence of the nonimprinted form (isoform 2).

Other studies have demonstrated that, in upd(7)mat lymphocytes, only the methylated allele is present at the

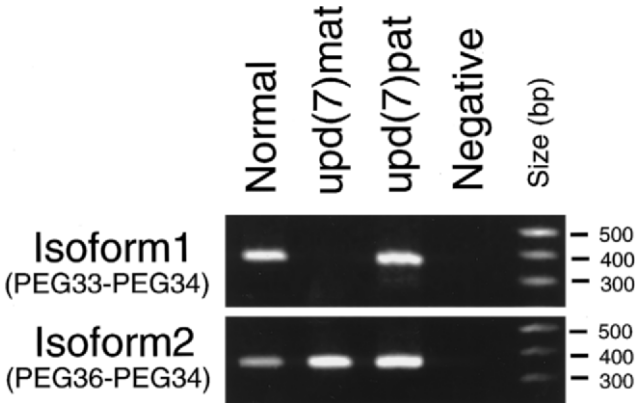

Figure 2 Expression patterns of the *PEG1/MEST* isoforms. Expression of isoforms 1 and 2 in normal, upd(7)mat, and upd(7)pat lymphoblastoid cell lines were analyzed by means of RT-PCR. Isoform 1 is expressed in upd(7)pat but not in upd(7)mat. Isoform 2 is expressed in both cell lines. Hence, isoform 1 is imprinted, whereas isoform 2 is not.

promoter of the isoform 1 of *PEG1/MEST,* whereas both methylated and unmethylated alleles are present in normal lymphocytes (Riesewijk et al. 1997). We now conclude, on the basis of findings from the present study, that parental-of-origin–specific loss of isoform 1 expression is strictly correlated with the methylation of the promoter of isoform 1. Documentation of this tight correlation validates the use of methylation analysis of *PEG1/MEST* gene in lymphocytes as a diagnostic assay for upd(7)mat.

Identification of isoform-specific imprinting illustrates several important issues with respect to imprinting studies in general. First, effort should be made to identify isoforms when one is evaluating new potentially imprinted genes. A potentially imprinted gene could be mistakenly disregarded if isoform-specific imprinting is overlooked. As shown in this study, use of the EST database can be very helpful in the identification of alternative isoforms. Second, imprinted genes that are allegedly subject to tissue-specific imprinting may need further evaluation. As shown with *PEG1/MEST* and *GNAS1,* nonimprinted or reciprocally imprinted isoforms may be expressed in tissues in which imprinting is apparently lost (Hayward et al. 1998; Peters et al. 1999). Third, the concept of leaky expression needs to be challenged. Examples of leaky expression include *p57kip2* (Reik and Maher 1997) and *IMPT1/ORCTL2* (Cooper et al. 1998; Dao et al. 1998). With respect to *PEG1/MEST,* a minimal but detectable level of expression from the maternal allele was observed in early (6–9 wk) human embryos, and this was considered to be leaky expression from the imprinted inactive maternal allele (Kobayashi et al. 1997). It is probable that these leaky transcripts from the maternal allele represent isoform 2, in light of the fact that isoform 2 is expressed as early as 8–10 wk in fetal heart (EST sequences AA092738 and AA095601). Similarly, the concept of interspecific imprinting differences may need revision. In contrast to the human *PEG1/MEST* gene, the mouse gene is not expressed from the paternal allele in lymphocytes (Riesewijk et al. 1997), nor is leaky expression from the maternal allele observed in mouse embryos (Kaneko-Ishino et al. 1995). Hence, a difference, in imprinting patterns, between mice and humans may simply reflect absence of isoform 2 in the mice. In fact, evaluation of the mouse *Peg1/Mest* UniGene cluster (Mm. 1089), consisting of 181 mouse EST sequences, revealed no evidence of alternative splicing: all 40 ESTs that contained exon 2 were flanked by exon 1 sequence, not by exon A–like sequence. Furthermore, RT-PCR, done with cDNA obtained from mouse peripheral blood by means of mouse-specific primer positioned within exon 4/5 of mouse *Peg1/Mest* gene (mPEG34 [5′-atgtggtctcggcttgtcactg-3′]), in combination with any of the four human-specific primers positioned within exon A (PEG36, PEG36A [5′-agtcctgtaggcaaggtcttacctga-3′], PEG36T [5′-gagtcctgtaggcaaggtcttacct-3′], and PEG36C [5′-gagtcctgtaggcaaggtcttacc-3′]) failed to amplify, whereas primer mPEG33 (5′-gggataatgcggccatggtg-3′), designed on the basis of mouse exon 1 sequence (the exon unique to isoform 1), yielded a specific PCR product when used with mPEG34 (fig. 1). These observations support the contention that isoform 2 may not be expressed in mouse peripheral blood and/or lymphocytes. In summary, human *PEG1/MEST* is imprinted in an isoform-specific manner rather than in a tissue-specific manner in lymphocytes.

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Kenjiro Kosaki,[1,2] Rika Kosaki,[1,3] William J. Craigen,[4] and Nobutake Matsuo[1]
[1]*Department of Pediatrics and* [2]*Pharmacia-Upjohn Fund for Growth & Development Research, Keio University School of Medicine, and* [3]*Keio University Health Center, Tokyo; and* [4]*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston*

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UniGene, http://www.ncbi.nlm.nih.gov/UniGene/
University of Washington Genome Center, http://www.genome.washington.edu/UWGC/chr-7/c7project.htm

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Address for correspondence and reprints: Dr. Kenjiro Kosaki, Division of Medical Genetics, Department of Pediatrics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: kkosaki@med.keio.ac.jp

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Involvement of the HLXB9 Homeobox Gene in Currarino Syndrome

To the Editor:

Anorectal malformations (ARMs) are among the most common congenital anomalies, accounting for 25% of digestive malformations that require neonatal surgery. ARMs have been found associated with sacral anomalies ~29% of the time (Rich et al. 1988). When ARMs are combined with lumbosacral anomalies, they fall into the spectrum of the caudal regression syndrome (CRS), which can also exhibit additional features such as partial or total sacrococcygeal agenesis, neural changes, and urogenital malformations (Lerone et al. 1997). The incidence of CRS is ~1 in 7,500 (Kallen et al. 1974). A detailed clinical characterization of patients affected by ARMs with partial or total sacrococcygeal agenesis revealed significant differences in the phenotypes, leading to the differentiation of five specific categories (Kalitzki 1965; Cama et al. 1996): (1) total sacral agenesis with normal or short transverse pelvic diameter and some lumbar vertebrae possibly missing (fig. 1*a* and *b*), (2) total sacral agenesis without involvement of lumbar vertebrae, (3) subtotal sacral agenesis or sacral hypodevelopment (with S1 present), (4) hemisacrum (fig. 1*c*), and (5) coccygeal agenesis.

In 1981, Guido Currarino described a form of CRS with hemisacrum (type IV sacral malformation), anorectal malformation, and presacral mass (anterior meningocele, teratoma, and/or rectal duplication) (fig. 1*d;* Currarino et al. 1981). The Currarino syndrome (CS; also called "Currarino triad") was observed to segregate in an autosomal dominant manner that often displayed phenotypic variability. As defined in the original reports, patients affected by true CS always exhibit the typical hemisacrum, with intact first sacral vertebra (sickle-shaped sacrum), which makes this specific sacral anomaly distinct to this syndrome.

Genetic studies suggested that a locus involved in normal sacral and anorectal development mapped to the terminal end (q36) of human chromosome 7 (Lynch et al. 1995; Seri et al. 1999). Mutations within the HLXB9 gene were identified in six cases, collectively grouped as