Letters to the Editor

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Genetic Basis of Peroxisome-Assembly Mutants of Humans, Chinese Hamster Ovary Cells, and Yeast: Identification of a New Complementation Group of Peroxisome-Biogenesis Disorders Apparently Lacking Peroxisomal-Membrane Ghosts

To the Editor:

Complementation analysis has been used to study the genetic basis of peroxisome-biogenesis disorders (PBDs; MIM 601539) at the Academic Medical Centre (AMC) in the Netherlands (Brul et al. 1988), Kennedy Krieger Institute (KKI) in the United States (Roscher et al. 1989), and Gifu University in Japan (Yajima et al. 1992). These initial studies led to identification of 15 complementation groups. When we standardized these complementation groupings to establish the true number of different complementation groups, we found a total of 9 independent groups (Shimozawa et al. 1993). In only 5 years, the molecular study of PBDs has advanced rapidly: (1) Several peroxisome-deficient mutants of Chinese hamster ovary (CHO) cells and yeast were isolated, and these mutants were used to clone *PEX* genes, by functional complementation, that are required for peroxisome assembly. (2) Five *PEX* genes involved in peroxisome biogenesis—*PEX1, -2, -5, -6,* and *-12*—have been identified as apparently responsible for PBD groups E (group 1 at KKI), F (group 10 at KKI), 2, C (group 4 at KKI), and 3, respectively (Shimozawa et al. 1992*b;* Dodt et al. 1995; Fukuda et al. 1996; Yahraus et al. 1996; Chang et al. 1997; Okumoto and Fujiki 1997; Portsteffen et al. 1997; Reuber et al. 1997); and *PEX7* was found to be responsible for rhizomelic chondrodysplasia punctata (RCDP) (Braverman et al. 1997; Motley et al. 1997; Purdue et al. 1997). (3) The role of these six *PEX* genes may be importing peroxisomal-matrix protein, since empty peroxisomal-membrane structures (peroxisomal ghosts) were seen in fibroblasts from PBD groups C (4 at KKI), E (1 at KKI), 2, and 3 (Santos et al. 1988; Wendland and Subramani 1993).

We have now identified a new complementation group of PBDs, group J (we are leaving out "I" to avoid confusion with group 1 at KKI), which is genetically different from the 11 currently known groups, including complementation groups G (Poulos et al. 1995) and H (Shimozawa et al. 1998). Complementation tests on human fibroblasts from various PBD patients were performed by restoration of peroxisomes by means of immunocytochemical staining of catalase in fused cells (Yajima et al. 1992). Formation of peroxisomes in the majority of multinucleated cells was detected after fusion between fibroblasts from the patient and fibroblasts from the 11 complementation groups (A–H, 2, 3, and 6) of PBD (data not shown). These observations mean that this patient can be regarded as representing a new complementation group, J (table 1). Interestingly, careful immunofluorescence-microscopy studies of fibroblasts from a patient belonging to the newly identified group J, performed with an antibody directed against human 70-kD peroxisomal-membrane protein (PMP [PMP70]) (Imanaka et al. 1996), revealed the absence of empty peroxisomal-membrane structures (ghosts) (fig. 1*a* and *b*), as well as, when performed with anti–human catalase antibody, catalase-containing particles—that is, peroxisomes (fig. 2*a* and *b*). Furthermore, among the 11 complementation groups so far tested, fibroblasts from all patients belonging to group D very rarely have peroxisomal ghosts (fig. 1*c*) and those from group G have none (fig. 1*d*), whereas peroxisomal ghosts were detected in the fibroblasts from PBD groups A–C, E, F, H, 2, 3, and 6 (fig. 1*e–m*). In fibroblasts from a patient with RCDP, both catalase-containing (fig. 2*c*) and PMP70-containing particles were seen (fig. 1*n*). In addition, we performed immunofluorescent staining with an anti–adrenoleukodystrophy protein (ALDP; 75-kD PMP) antibody. As in the case of PMP70, ALDP-positive particles were not detected in fibroblasts from PBD complementation groups G and J, and ALDP-positive particles were rarely found in those from group D. In contrast, ALDP-positive particles that were larger and fewer than those in control fibroblasts were detected in fibroblasts from the other nine complementation groups (data not shown). These results suggest that the primary defect in PBD groups D, G, and J may not be matrix-protein import but, rather, synthesis or maintenance of PMP (Santos et al. 1988; Wendland and Subramani 1993; Baerends et al. 1996; Dodt and Gould 1996; Wiemer et al. 1996).

The patient from the newly identified complementation group J had the phenotype of classic Zellweger syndrome (ZS; MIM 214100). Dihydroxyacetone phosphate acyltransferase activity was severely diminished in fibroblasts from the patient (0.11 nmol/120 min per mg protein), in comparison with findings in control fibroblasts (1.55 nmol/120 min per mg protein) (Shimozawa et al. 1988). β-Oxidation activity of lignoceric acid rel-

Table 1

Complementation Groups of PBDs

Complementation Group								
Gifu	KKI[a]	AMC	Phenotype(s)[b]	Peroxisomal-Membrane Ghosts[c]	CHO Mutant(s)	Human Gene	Mapping	Yeast Gene
A	8		ZS, NALD, IRD	+				
B	7 (5)		ZS, NALD	+				
C	4	3	ZS, NALD	+	ZP92	*PEX6* (*PAF2*)	6p21.1	*Pex6*
D	9		ZS	−				
E	1	2	ZS, NALD, IRD	+	Z24, ZP101	*PEX1*	7q21-22	*Pex1*
F	10	5	ZS, IRD	+	Z65	*PEX2* (*PAF1*)	8q21.1	*Pex2*
G			ZS	−				
H			NALD	+				
J			ZS	−	ZP119[d]			
	2	4	ZS, NALD	+	ZP102	*PEX5*	12p13.3	*Pex5*
	3		ZS	+	ZP104, ZP109	*PEX12*		*Pex12*
	6		NALD	+				
					ZP110, ZP 111 ZP114			
R	11	1	RCDP			*PEX7*	6q22-24	*Pex7*

[a] The numbering listed under KKI is based on the study by Moser et al. (1995).
[b] NALD denotes neonatal adrenoleukodystrophy, and IRD denotes infantile Refsum disease.
[c] A plus sign (+) indicates presence, and a minus sign (−) indicates absence.
[d] Kinoshita et al. (1998).

ative to that of palmitic acid in this patient's fibroblasts was also decreased (0.038), in comparison with findings in the control cells (0.58), determined as described by Suzuki et al. (1991). In addition, all of the patients from PBD groups D, G, and J had only the severe phenotype of ZS (Shimozawa et al. 1993; Poulos et al. 1995), whereas some patients from the other nine PBD groups had the severe phenotype but others had milder phenotypes, such as neonatal adrenoleukodystrophy and infantile Refsum disease.

We then performed cell fusion between fibroblasts from group J and CHO mutants ZP110 (Tateishi et al. 1997), ZP114 (Tateishi et al. 1997), and ZP119, the CHO mutant newly isolated by Kinoshita et al. (1998), which were found to belong to complementation groups other than the known PBD groups (A-H, 2, 3, 6, and RCDP) (Shimozawa et al. 1998). Numerous peroxisomes were detected after fusion by use of methods reported elsewhere (Shimozawa et al. 1992*a*), between fibroblasts and CHO mutants ZP110 (fig. 2*d*) and ZP114, whereas no peroxisome was detected after fusion between fibroblasts and ZP119 (fig. 2*e*). These observations imply that the newly identified CHO mutant ZP119 represents ZS fibroblasts from group J. Furthermore, this CHO mutant, like group J, had no peroxisomal ghosts (Kinoshita et al.; 1998), whereas large but fewer particles immunoreactive with anti-PMP70 antibody were detected in CHO mutants Z24, Z65, and ZP92, which belong to the same complementation groups as E, F, and C, respectively (Shimozawa et al. 1992*a*).

We then transfected human PMP70 cDNA (Kamijo et al. 1992) into fibroblasts lacking peroxisomal ghosts, from groups D, G, and J, according to methods reported elsewhere (Shimozawa et al. 1996). In all these transfectants, peroxisomes were not detected when we performed immunostaining with an anti–human catalase antibody (fig. 2*f–h*), and the same held true for transfectants of PMP70 into fibroblasts from groups A–C, E, F, H, 2, 3, and 6 (data not shown). Therefore, human PBD groups caused by defects in the PMP70 gene have heretofore not been identified. Furthermore, when we transfected, into the fibroblasts from the group J patient, human *PEX13* cDNA, which encodes an SH3 protein of the peroxisomal membrane (Gould et al. 1996). Peroxisomes were not evident in the transfectants (fig. 2*i*). In summary, (1) in mammalian cell lines there are 15 known peroxisomal-deficient complementation groups, including RCDP and CHO mutants; (2) abnormalities of PMP synthesis, not matrix-protein import, may be the primary defect, at least in PBD groups D, G, and J, and all patients from these groups manifested only the severe phenotype of ZS, whereas the other groups included various phenotypes; and (3) there were no PBD groups complemented by human PMP70.

It was first reported that in ZS fibroblasts from complementation group 4 at KKI (group C at Gifu [*PEX6* defect]) the PMPs were located in unusual empty membrane structures (peroxisomal ghosts) of a larger size—a finding determined mainly by use of an anti-PMP70 antibody (Santos et al. 1988).Later, ghost size and abundance were noted in seven ZS fibroblasts belonging to five complementation groups (Santos et al. 1992), and detectable PMP70 in vesicles was noted in

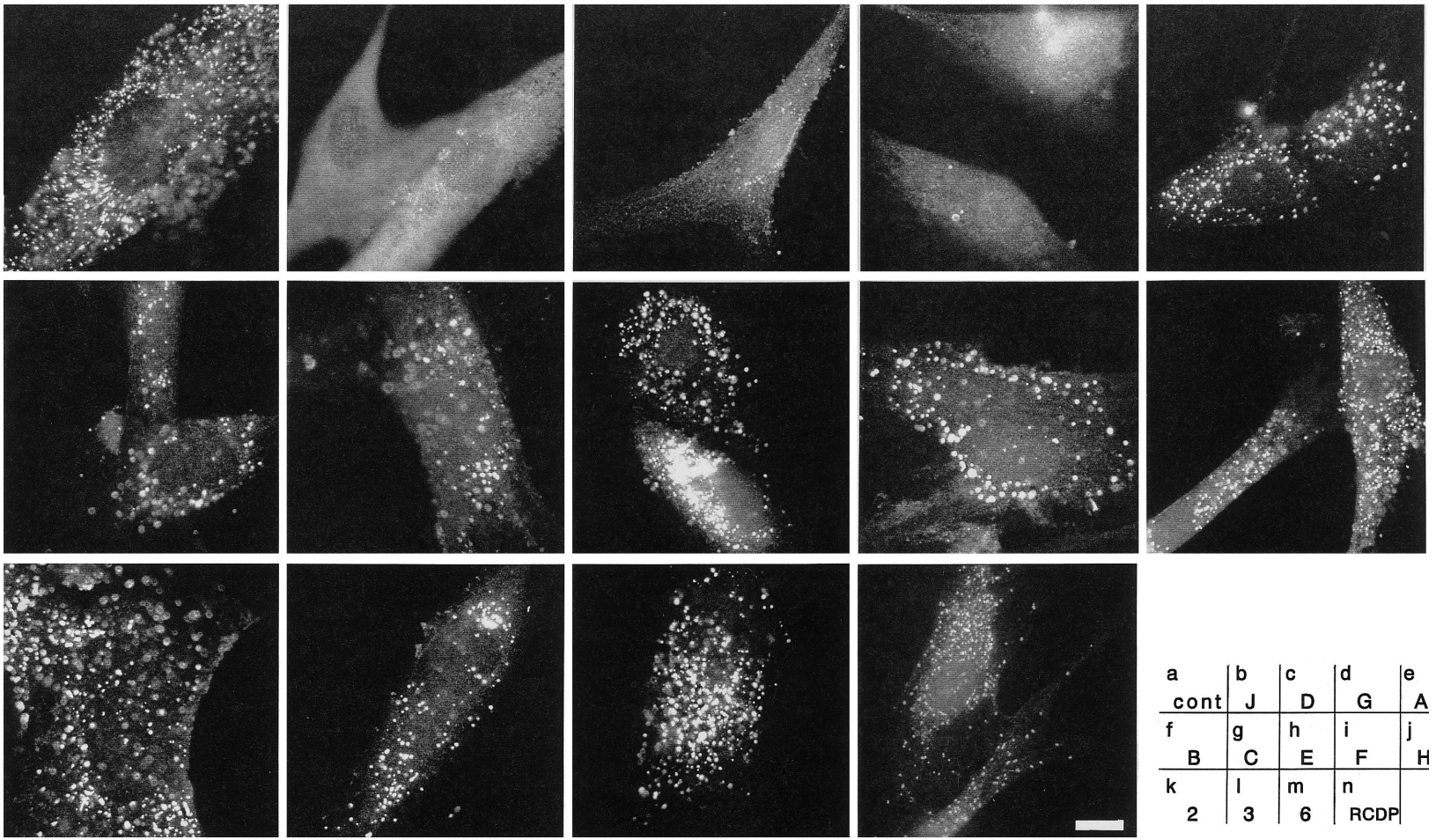

Figure 1 Immunofluorescent staining with anti–human PMP70 antibody. *a,* Control fibroblasts. *b,* Fibroblasts from group J patient. *c–m,* Fibroblasts from patients from groups D, G, A–C, E, F, H, 2, 3, and 6. *n,* Fibroblasts from RCDP patient. (Bar = 15 μm). In the fibroblasts from groups J and G, no PMP70-positive particles are visualized, except for the nonspecific puncta, and the cytosol is stained strongly and diffusely. Very few and various PMP70-positive particles are detected in the fibroblasts from group D and the other nine groups (A, B, C, E, F, H, 2, 3, and 6).

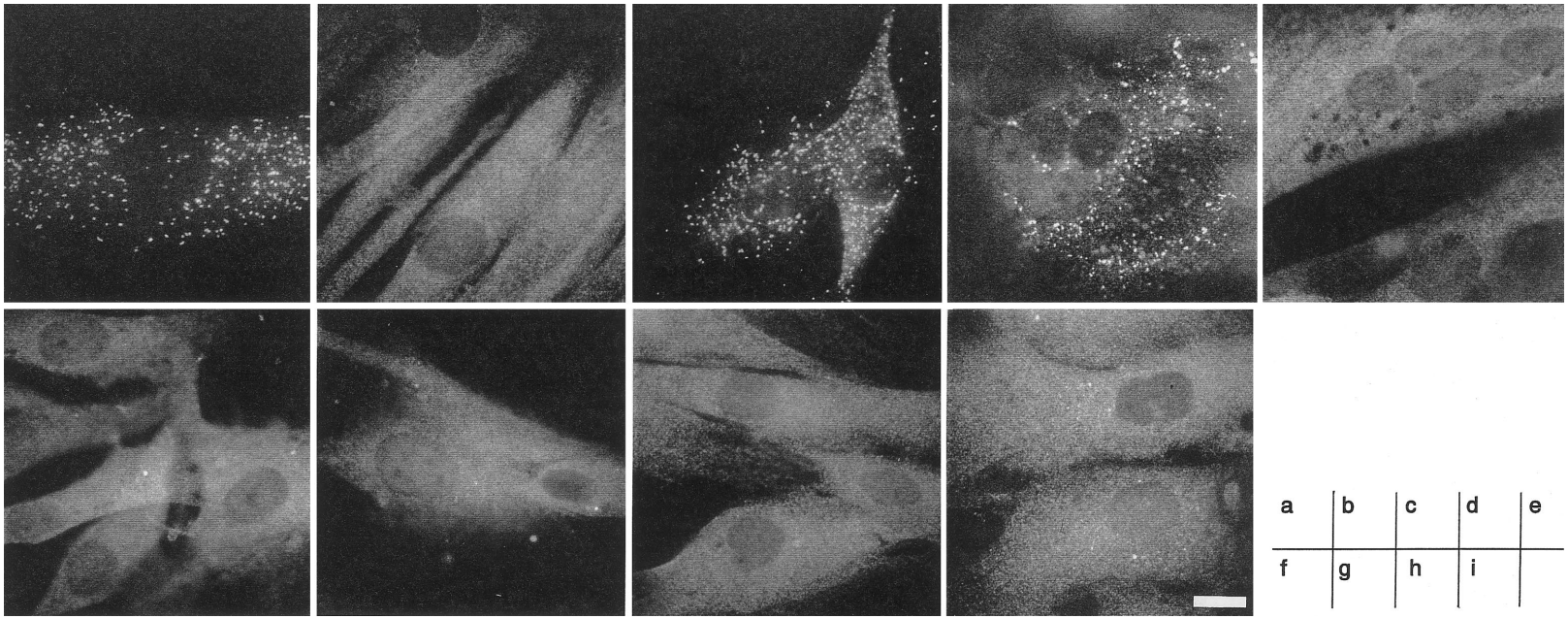

Figure 2 Immunofluorescent staining with anti–human catalase antibody. *a,* Control fibroblasts. *b,* Fibroblasts from group J patient. *c,* Fibroblasts from RCDP patient. *d* and *e,* Cell hybrids of fibroblasts from the group J patient with ZP110 and the group J patient with ZP119, respectively. *f–h,* Transfectants with human PMP70 cDNA into group D, G, and J fibroblasts, respectively. *i,* Transfectants with human *PEX13* cDNA into group J fibroblasts. (Bar = 15 μm)

those from KKI groups 1 (E at Gifu), 2, 3, 6, and 8 (A at Gifu) (Wendland and Subramani 1993). ALDP-positive particles were also detected in two PBD cell lines from group 1 but were rare in ZS fibroblasts from group D (Mosser et al. 1994). All these data support our findings of heterogeneity of peroxisomal ghosts in PBD complementation groups.

At least 18 yeast *PEX* genes have been identified, and several human genes have been considered to be human orthologues of these *PEX* genes. It has been suggested that there are yeast mutants without peroxisomal ghosts—for example, *Hansenula polymorpha per9* or *Pichia pastoris pas2* (*PEX3* gene defect) (Baerends et al. 1996; Wiemer et al. 1996)—and that these *PEX* genes may play roles of synthesis or maintenance of peroxisomal membrane. Therefore, any of these *PEX* genes may be primary defects of PBD groups D, G, and J. We are using western blot and pulse-chase experiments with some PMP antibodies to perform detailed analyses of ghosts in these three groups, and we are examining genes responsible for these PBD groups by identifying human orthologues of these *PEX* genes and by performing functional cloning of peroxisome-deficient CHO mutants.

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Evidence of Somatic and Germinal Mosaicism in Pseudo–Low-Penetrant Hereditary Retinoblastoma, by Constitutional and Single-Sperm Mutation Analysis

To the Editor:

Retinoblastoma is a pediatric cancer of the retina, initiated by two consecutive inactivating mutations at the

retinoblastoma locus (RB1; Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). Tumorigenesis may occur by two distinct pathways: in nonhereditary retinoblastoma (60% of patients), unifocal clonal expansion occurs after two somatic RB1 mutations in a single retinal precursor cell; however, in hereditary retinoblastoma (40% of patients), the first RB1 mutation is inherited classically and is present in both retinas, where uni- or multifocal tumorigenesis can be initiated in any cell by the somatic mutation of the remaining allele.

Typically, germ-line mutation may be passed dominantly by an affected genitor, or it can be transmitted as a prezygotic, mostly paternal neomutation from a healthy parent. However, postzygotic mutagenesis has long been suspected to contribute to the pool of transmittable mutations of RB1. Two main predictions characterize retinoblastoma patients with mutational mosaicism: (1) somatic mosaicism may significantly reduce tumor susceptibility, and (2) gonadal mosaicism may cause linkage-based analysis of inheritance to be biased toward apparent low penetrance, as a result of a non-Mendelian output of mutant versus wild-type gametes.

Several cases of retinoblastoma with mosaicism are mentioned in the literature (Greger et al. 1990; Huang et al. 1992; Shimizu et al. 1994; Thonney et al. 1996; Lohmann et al. 1997; Sippel et al. 1998). The incidence of mosaicism as well as its phenotypic influence on hereditary retinoblastoma remain to be determined, to improve genetic counseling and to shed light on the mechanisms underlying expression and penetrance of retinoblastoma.

To detect the presence of mosaicism in hereditary retinoblastoma, we selected pedigrees found through either healthy carriers or affected individuals in whom linkage analysis concomitantly documented an apparent low penetrance, from a series of 210 consecutive index patients referred for genetic counseling to the Retinoblastoma Clinics at the Jules Gonin Hospital during the period 1986–96. Among these patients, 147 (70%) had bilateral disease, and 34 (16.2%) had a familial history of retinoblastoma. All patients with familial retinoblastoma were investigated genetically by linkage analysis using intragenic DNA-sequence polymorphisms, and all were informative (Munier et al. 1992, 1996). Apparent low penetrance was present in eight pedigrees. After the exclusion of two families with fortuitous familial aggregation of independent retinoblastoma cases (Munier et al. 1993), the six remaining pedigrees were documented for reduced penetrance, as defined by the presence of healthy individuals >3 years of age in linkage phase with affected family members. A systematic search for the RB1 mutation in these six families was then performed in order to describe the molecular basis of the presumed low penetrance.

Among the six families (A–F) with apparent low penetrance, linkage analysis detected 21 unaffected carriers, of whom 6 were obligate retinoblastoma transmitters; 2 of the 6 obligate transmitters were founders (in families A and B; data not shown). The other four pedigrees were founded either by unilaterally (families C, D, and F) or bilaterally (family E) affected males, and attenuated expressivity or retinoma was present in three of these males: two in family E (the affected father had one eye enucleated for unilateral retinoblastoma and a unifocal flat chorioretinal scar, reminiscent of type IV regression, in his untreated eye; the affected son had bilateral multifocal retinomas) and one in family F (the affected grandfather had a unifocal staphylomic macular scar).

Two abnormal conformers were identified when SSCP analysis of exons 8 and 23 was performed for the two index patients of families D and E, respectively (fig. 1). Sequence analysis of these DNA fragments revealed C→T transitions at CpG dinucleotides of two arginine codons, at positions 251 and 787, in families D and E, respectively. These changes also abolished two *Taq*I restriction sites. When segregation of the mutations was analyzed in both families, the *Taq*I restriction digest showed a faint undigested band in affected fathers, in addition to the expected digested products, suggesting the presence of somatic mosaicism. Confirmation of mosaicism was obtained following analysis of the *Taq*I site in cloned PCR amplicons of exons 8 and 23. The mutant digestion pattern was recovered in only 8 (10.7%) of 75 inserts from family D and in 4 (12.1%) of 33 inserts from family E. In contrast with the results of the linkage analysis, nonpenetrance failed to be validated by the presence of mutations in the three unaffected sibs (individuals III-1 and III-2 in family D and individual III-2 in family E) of the probands, strongly suggesting germinal mosaicism in the founders.

Direct quantitative analysis of gonadal mosaicism was performed on semen from only the family E founder (fig. 2), by means of a *Taq*I restriction assay in amplicons of exon 23. Single sperms were isolated by fluorescence-activated cell sorting into 96-well microtiter plates (Cui et al. 1989; Li et al. 1991). We performed amplification reactions in 576 wells and obtained an amplification signal for 415 wells. No amplification was obtained for the 40 negative controls (wells without cells), which is consistent with absence of contamination. All 18 positive controls (10 spermatozoa/well) gave a signal on an agarose gel; two alleles were observed in 3 wells, indicating the presence of more than one spermatocyte. The remaining 394 wells had only one allele. The mutated and wild-type alleles were easily distinguished, after *Taq*I digestion, as one 255-bp amplicon or as products of 159 + 96 bp, respectively (fig. 2). Of 394 unicellular digested products, 365 (92.64%) revealed the presence of a normal allele, and 29 (7.36%) revealed the presence of a mutant allele.

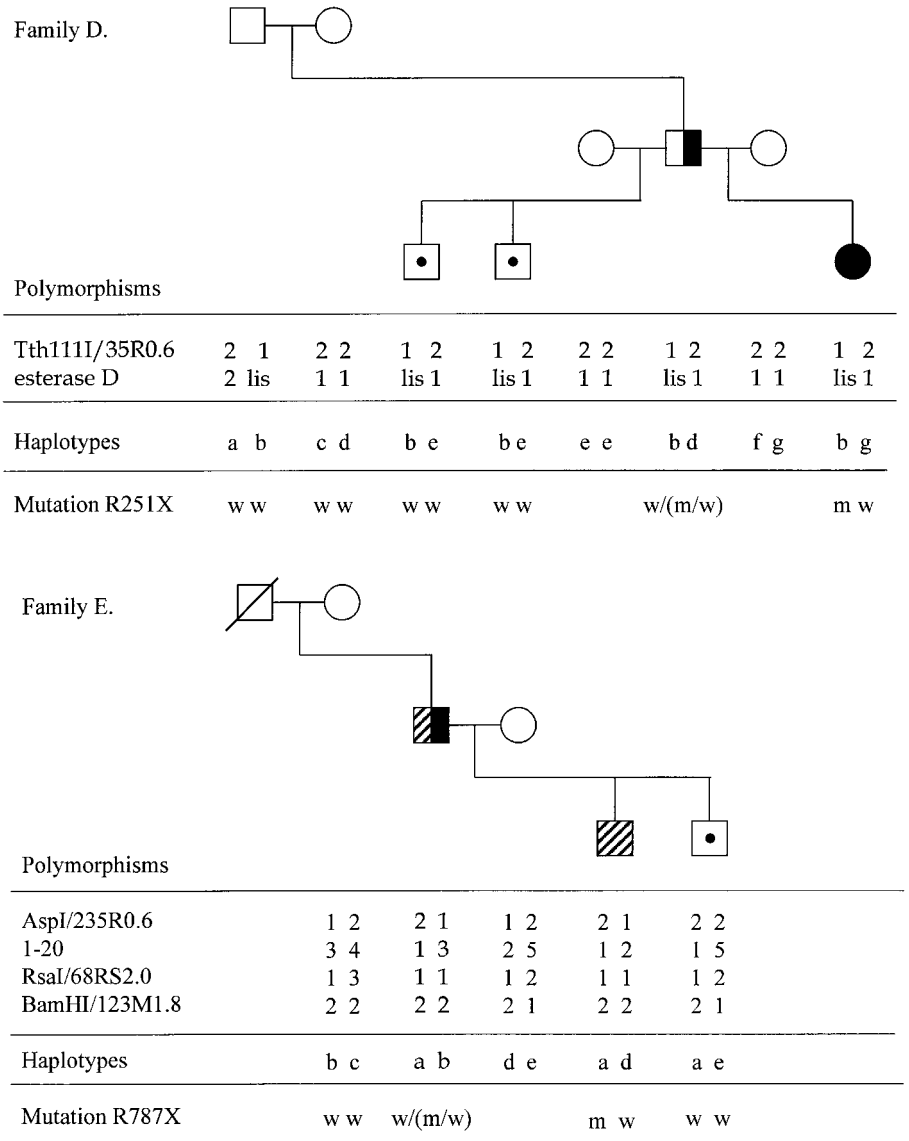

Figure 1 Pedigrees of two low-penetrant retinoblastoma families (D and E), and genotypes after linkage and/or mutation analysis at the RB1 locus. mw = heterozygote for the RB1 mutation, ww = homozygote for the RB1 wild type, w/(m/w) = mosaic, and lis = esterase D lisbon isoenzyme. The blackened circle represents a bilaterally affected female patient; half-blackened squares represent unilaterally affected males; hatched and half-hatched squares represent patients with bilateral and unilateral retinomas, respectively; and squares containing a black dot represent unaffected male carriers of the apparently disease-linked haplotype.

Intragenic linkage analysis of 34 familial retinoblastoma cases revealed a low-penetrance pattern of inheritance in eight families, of which four followed pseudo–low-penetrant mechanisms, including independent occurrence of RB1 mutations in two different sets of cousins (Munier et al. 1993) and, as shown in this study, germ-line mosaicism in two affected founders. For the four remaining families, the molecular basis of the apparent low penetrance could not be determined and will await identification of the disease-causing mutation(s). Given the reported sensitivity (26%–83%) of the various RB1 mutation-scanning methods for hereditary retinoblastoma (Blanquet et al. 1995; Liu et al. 1995; Lohmann et al. 1996), the observation of mutations in 33% of the cases was not surprising. One possible explanation is that several mutations can be missed by SSCP screening or may lie outside the scanned RB1 exons (exons 2–26). Analysis of incomplete penetrance in retinoblastoma has previously led to the identification of two major types of gene alterations, resulting in either transcription reduction via a promoter mutation (Sakai et al. 1991; Cowell et al. 1996) or partial protein in-

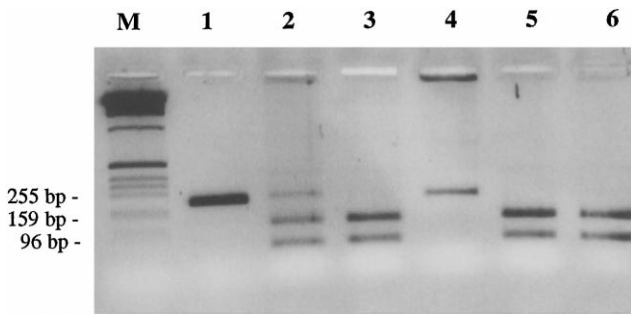

Figure 2 Determination of genotypes in single sperm from patient II-1 from family E, as detected by ethidium-bromide staining of *Taq*I-digested PCR products. Lane 1, Undigested PCR product. Lane 2, Digested PCR product from 10 spermatozoa (presence of the two alleles). Lanes 3–6, PCR product from single sperm with a mutant allele (lane 4) or a normal allele (lanes 3, 5, and 6). Lane M, Molecular-weight marker (1-kb ladder; Gibco BRL).

activation via missense mutations (Kratzke et al. 1994; Lohmann et al. 1994; Ahmad et al. 1997) and in-frame deletions (Lohmann et al. 1992; Dryja et al. 1993; Bremner et al. 1997; Schubert et al. 1997).

We report two affected patients with somatic and gonadal mosaicism for two RB1 nonsense mutations (R251X and R787X). The pathogenicity of these two mutations has been well established (Yandell et al. 1989; Cowell et al. 1994). For both affected patients, somatic mosaicism has been documented in peripheral lymphocytes and possibly involves the retina, as suggested by the nonpenetrance of retinoblastoma in one eye of the founder of family D. Mosaicism further extends to include the germ line, as proved by the segregation of three different chromosomes 13 in both families (fig. 1). In family D, indirect evidence of gonadal mosaicism in the father was provided by the analysis of three informative meiotic events in his progeny, after exclusion of nonpaternity, since his affected girl and two unaffected sons inherited the same paternal haplotype, including a rare esterase D polymorphism, ESD*Lis (Munier et al. 1988). In family E, direct evidence of germ-line mosaicism in the father's semen is based on the study of 394 meiotic events, from which 7.3% of the spermatozoa are mutant, which is not very different from the 12.1% observed in the peripheral leukocyte DNA.

In contrast to the ectodermal lineage of the retina, leukocytes and primordial germ cells have an extraembryonic origin and derive from the blastocyst endoderm. Since the didermic stage starts at ~8 d after conception, it is tempting to adopt this age as the upper limit for the occurrence of the postzygotic mutations R251X and R787X. On the other hand, the earliest mutational event leading to mosaicism can already have taken place in the postmeiotic gametes, as "half-chromatid mutations" (Carlson and Desnick 1979). The fact that the mutations in both mosaics occurred on the paternally derived chromosomes suggests that the well-known preferential prezygotic paternal mutagenesis (Dryja et al. 1989; Zhu et al. 1989) lasted in the zygote until the 8th d of development. Interestingly, these two mutations are C→T transitions at CpG dinucleotides, most likely occurring by spontaneous 5-methylcytosine deamination. In support of this mutational mechanism, the genome of haploid spermatozoa is known to have a higher methylation content than the undermethylated DNA of the oocyte and to be completely devoid of repair capabilities (Monk 1995). Hypermutability of the male-derived conceptus genome may be momentarily repressed following the massive demethylation that takes place at ~2 d after conception. De novo methylation of the unmethylated blastocyst genome occurs again, at ~6 d after conception, at the time of implantation (Dost and Lee 1995; Razin and Shemer 1995). The CpG of codon 251 in RB1 was recently proved to be constitutively methylated, whereas no information is yet available with regard to the methylation status of the CpG of codon 787 (Mancini et al. 1997). In summary, we tentatively can assign a mutational window spanning from shortly before fertilization to the 8th d of development. Since both observed mutations in the two mosaics occurred on the paternally derived chromosomes, most likely following a cytosine methylation–mediated mechanism, the mutational events involved likely happened no later than 2 d after conception, on the methylated paternal genome, before massive demethylation took place. Furthermore, we postulate that the different methylation status between sperm and oocyte genomes may temporarily persist in the zygote and, hence, may cause a preferential paternal origin of mosaicism.

This study indicates that hereditary retinoblastoma does not originate exclusively from gametic neomutations but also may result from embryonic mutagenesis. The relative contribution of gametic and embryonic neomutations in hereditary retinoblastoma remains unknown but may be determined by systematic screening for both somatic mosaicism in patients with presumed de novo mutations and cryptic mosaicism in their parents. The time interval between the end of meiosis and the differentiation of soma from the germ line is viewed by some as a significant source of transmittable dominant neomutations, with estimates ranging from 5% to >15% (Dost and Lee 1995).

The mosaic nature of the two mutations described in this study could not be detected in any of the other 10 germ-line mutations identified by us (data not shown). A search was performed in index patients and their healthy parents. On the basis of this small series, we estimated a mosaic prevalence of 16.6% (2/12). Interestingly, Lohmann et al. (1997) estimated a similar prev-

alence, with 1 (16.6%) in 6 mutations that cause isolated unilateral hereditary retinoblastoma found to be mosaic in nature. Such a frequent occurrence of mosaicism was also highlighted recently by Sippel et al. (1998), whose data indicate a 10% rate of mosaicism in a population of 156 retinoblastoma patients. Finally, in a review of 140 retinoblastoma cases associated with constitutional 13q14 chromosomal rearrangements, 25 (18%) had proved mosaicism (Munier et al. 1989).

Taken together, these data suggest that mosaicism may be a frequent phenomenon, often interfering with expression and transmission of retinoblastoma. Somatic mosaicism may cause attenuated expression, such as unilaterality or nonpenetrance of retinoblastoma. Likewise, germinal mosaicism may be associated with apparent reduced penetrance of retinoblastoma in linkage-based molecular assessments of inheritance. This fact should be included in the calculation of the recurrence risk of retinoblastoma, especially for families with unilaterally affected male founders.

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Genetic Counseling and Prenatal Diagnosis for mtDNA Disease

To the Editor:

Over the last decade, clinicians have seen an increasing number of patients who have been diagnosed with mtDNA disease (Chinnery and Turnbull 1997*b*). As a consequence, clinicians also have seen more and more women of childbearing age carrying a pathogenic mtDNA mutation who seek advice about the potential risks to future offspring (Chinnery and Turnbull 1997*a*). With this trend in mind, the recent editorial by Poulton et al. (1998) was timely, tackling the difficult but intriguing problem of the origin, segregation, and inheritance of heteroplasmic mtDNA mutations. The authors placed particular emphasis on the transmission of pathogenic mutations, and, on the basis of their interpretation of mechanisms, they suggested an approach to the counseling for and prenatal diagnosis of mtDNA disease that could be used in clinical practice. However, because our understanding of the mechanisms governing these processes is rudimentary at best, caution must be used when counseling families with mtDNA disease.

At the most general level, two processes contribute to the marked intrafamilial variation of genotype and phenotype that is the hallmark of mtDNA disease (Chinnery and Turnbull 1997*b*). The first process occurs during the early embryonic development of a female. Between the formation of the zygote and the maturation of the oocyte lineage, the cellular copy number of mtDNA is reduced and then amplified. This results in a high level of variability in the level of mutated mtDNA that is transmitted to the subsequent generation. Poulton et al. (1998) refer to this process as the bottleneck, but whether the variability results from selection events (e.g., see Hauswirth and Laipis 1985) or from random drift (Jenuth et al. 1996) is the subject of debate. In the second process, further diversity is generated as mutated genomes differentially replicate and segregate during histogenesis

and organ maturation (for a review, see Lightowlers et al. 1997).

These two processes are of intense interest to those scientists who seek to understand the mechanisms that determine the inheritance of mtDNA. Not surprisingly, their importance also has been recognized by clinicians who counsel women at risk of transmitting heteroplasmic pathogenic mtDNA mutations. At present, unfortunately, very little guidance can be offered to these women. There are three possible approaches to resolving the clinical problem: (1) application of our understanding of the mitochondrial genetic bottleneck; (2) empirical investigation of the relationship between the maternal mutation load and the mutation load and clinical phenotypes among offspring; and (3) the use of prenatal diagnostic tests.

Although our rudimentary understanding of the bottleneck might be tempting to use when counseling patients, it is, in fact, of limited practical use at present. There are a number of theoretical and experimental problems that beset the bottleneck phenomenon. First, differences in the number of mtDNA molecules and in the number of cell divisions during germ-line development may have a profound effect on the size of the bottleneck, thus weakening any simple extrapolation of data from animal studies to humans (Austin 1995; Strachan and Lindsay 1997). Second, undefined differences among embryos may result in bottlenecks of different sizes (Herbert et al. 1995); there is no evidence of a simple, one-size-fits-all bottleneck (Howell et al. 1992). Finally, and perhaps most importantly, although mathematical models of the bottleneck can be used to predict the range of possible levels of mutant mtDNA in an offspring, the resulting range is so wide as to be of limited value in counseling. For the specific example described by Poulton et al. (1998)—namely, a female with 21% mutant mtDNA in her blood—the 95% confidence interval of the mutation load in her offspring is 0%–50%. Furthermore, even if prediction of the precise level of mutant mtDNA in the blood of the offspring was possible, the clinical outcome from a particular mtDNA mutation load cannot be predicted with a high level of confidence, at the present time (Chinnery et al. 1997). For example, even for the A8344G mutation, there is considerable overlap between the levels of mutant mtDNA detected in the blood of clinically affected individuals and the levels in their unaffected relatives (fig. 1). Thus, the complex and multisystem clinical phenotypes are difficult to predict on the simple basis of blood levels of mutant mtDNA in an individual harboring an mtDNA mutation.

An alternative, more empirical approach would be to study the outcome of pregnancy in a large number of women with heteroplasmic mtDNA mutations, *without* making any assumptions about the mechanism of the bottleneck. Thus, even with the limitations of a retrospective multicenter study, the frequency of clinically affected offspring born to women who harbor the A3243G or the A8344G point mutation recently has been shown to be related to the level of mutant mtDNA in the mothers' blood (Chinnery et al., in press). However, the relationship clearly differed for the two pathogenic mutations, which indicates significant differences in the expression of the two mutations, for a given inherited mutation load. Although the use of this retrospective data to give precise estimates of the risks involved for a particular female is premature, these observations underscore the potential value of more-extensive longitudinal, tissue-distribution, and, especially, prospective analyses.

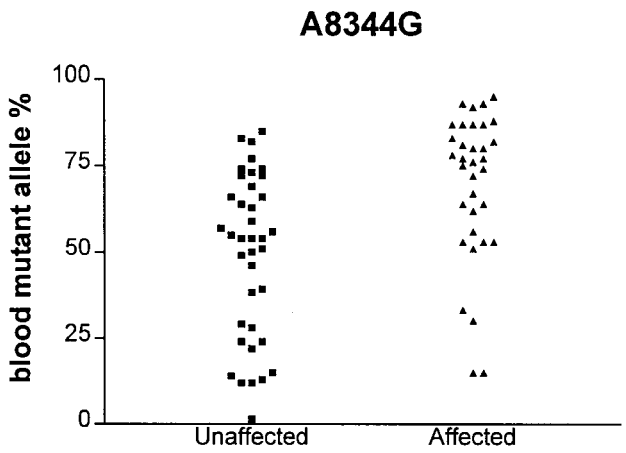

Figure 1 Percentage of mutant mtDNA in blood, for 72 individuals harboring the A3243G MERRF (myoclonic epilepsy with ragged red fibers) mutation. For the details of data acquisition, see the article by Chinnery et al. (1997).

Finally, preimplantation testing of a chorionic villus biopsy may prove useful for counseling; however, at present, there is very little data to support its use. If the level of mutant mtDNA is distributed evenly to all the tissues of a developing embryo and if the mutation load stays constant with time, then this technique may be reliable (as may be the case for the T8993G/C point mutations). However, it is already known that the level of mutant mtDNA is not distributed evenly in most patients with mtDNA disease and that this differential segregation probably occurs at the later stages of development. Even subtle variations in tissue mutation load may lead to a profound variation in the phenotype, and sampling of a single cell or chorionic villus may not reflect the load in clinically relevant organs such as the brain. Further studies are needed to establish the value of these potentially hazardous techniques in the counseling of patients with mitochondrial disease.

The prevalence of pathogenic mtDNA defects is at

least 1/10,000 in the general population of northern Europe (Majamaa et al. 1998; P.F.C. and D.M.T., unpublished data). Many of these individuals are women of childbearing age who urgently need genetic counseling and advice, with regard to both the prognosis for their children and the risk of disease in subsequent offspring. Poulton et al. (1998) have highlighted some of the difficulties encountered in counseling these patients, and their discussion has given us much food for thought. However, their recommended acceptance of a proposed simple bottleneck model and its application to prenatal mitochondrial diagnosis is premature.

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Reply to Chinnery et al.

To the Editor:

We thank Chinnery et al. (1998 [in this issue]) for their appreciation of our article (Poulton et al. 1998) and for their reiteration of its main points, particularly the need to gather further data prospectively. Our article is in full agreement with all four of their reservations about direct application of current knowledge to clinical practice, and their new data on the 8344 mutation are very similar to the example we cite (Hammans et al. 1993). A recent study by White et al. (1998) that uses an empirical approach generates advice that is very similar to the predictions of our model.

We would, however, like to correct two points. First, our figure 2 (Poulton et al. 1998) refers to levels of mutant mtDNA in *ovary* and *progeny,* not in *blood* (clearly stated in the figure). To clarify the validity of our predictions, we now display the figure, along with the measured levels of mutant mtDNA (fig. 1; Marchington et al. 1998). It is clear that such accurate estimates

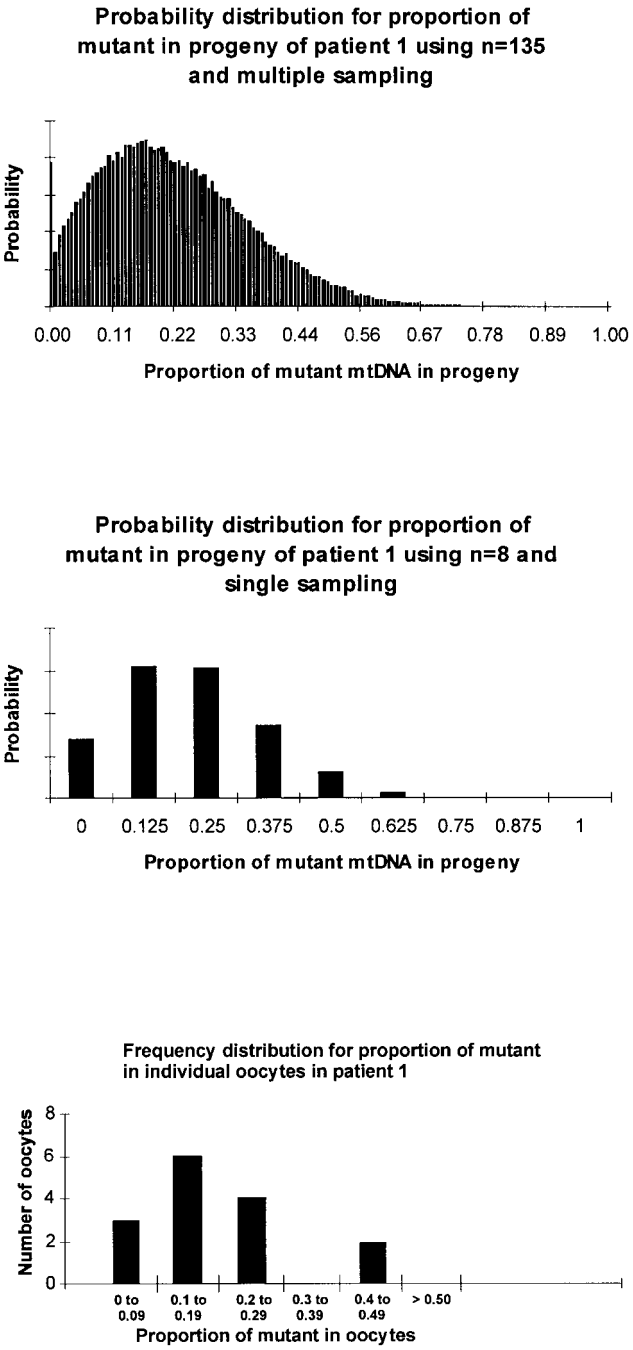

Figure 1 Fitting the repeated- and single-selection models to the data on mtDNA rearrangements: idealized plots for patient 1, for predicted percentage mutant in offspring, when 21% mutant mtDNA is in ovary, for repeated sampling ($g = 15$, $n = 135$; *top*) and for single selection ($g = 1$, $n = 8$; *middle*). Both reasonably fit the observed distribution (*bottom;* Marchington et al. 1998).

of the level of mutant mtDNA in ovary only rarely will be available to the genetic counselor; hence, we use the 8344 mutation as an example of a mutation that "generally exhibits less variation between tissues than is seen among some of the other, more common mtDNA mutations" (Poulton et al. 1998, pp. 755–56).

Second, our discussion in the section "Models Describing the Mitochondrial Bottleneck" (Poulton et al. 1998, pp. 754–55) is far from a "recommended acceptance of a proposed simple bottleneck model" or a premature "application to prenatal mitochondrial diagnosis" (Chinnery et al. 1998, p. 000). We did not recommend acceptance but suggested that "*once more data have been collected* [such as that described in White et al. 1998], such estimations will become usable in the medium term; reasonable fits may be more useful to patients than is the quality of information currently issued" (Poulton et al. 1998, p. 756). We also stated, "*Although most clinicians will feel that CVS* [chorionic villus sampling] *is not yet widely applicable to mtDNA disease,* there is clearly an urgent need to collect the human data needed to complete the picture" (Poulton et al. 1998, p. 756).

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Molecular Cytogenetic Detection of Confined Gonadal Mosaicism in a Conceptus with Trisomy 16 Placental Mosaicism

To the Editor:

Confined placental mosaicism (CPM) is a dichotomy between the chromosomal constitution of the placental and embryonic/fetal tissues, observed in 1%–2% of all delivered pregnancies, and most commonly involves a trisomic clone confined to the placenta (Kalousek 1990). CPM has been shown to exist in three different forms (types I–III), depending on its origin and the placental cell lineages involved. In a diploid zygote, the trisomic cell line in CPM can arise from mitotic duplication of one chromosome in a specific placental cell lineage (either trophoblast or chorionic stroma), giving rise to type I or type II CPM. Rescue of a trisomic zygote, owing to chromosome loss by a postzygotic mitotic error in the embryonic progenitor cells, leads to trisomic cell-line expression in both placental lineages and is termed "type III CPM" (Kalousek et al. 1993; Robinson et al. 1997).

Embryological literature provides evidence that the chorionic stroma of the placenta and the primordial germ cells (PGCs) of the embryonic gonads share common progenitor cells (Buehr 1997), suggesting that conceptuses diagnosed with CPM involving the placental stroma may be at increased risk for gonadal mosaicism. We describe the conventional cytogenetic, molecular cytogenetic, and molecular genetic analyses of multiple fetal and placental tissues from a conceptus diagnosed with trisomy 16 placental mosaicism. Our results demonstrate the presence of trisomy 16 mosaicism in the placenta and disomy for chromosome 16 in all fetal tissues studied, except oocytes, which show mosaicism with a significant level of trisomy 16. This is the first published data documenting the existence of germ-cell mosaicism in an otherwise nonmosaic fetus, for a conceptus diagnosed with CPM.

Fresh fetal and placental tissues were obtained from a conceptus therapeutically aborted at 12 wk of gestational age and prenatally diagnosed with 100% trisomy 16 by means of cultured chorionic villus stroma. Conventional cytogenetic analysis, FISH, and microsatellite analysis were used to study the distribution of the trisomy 16 cell line in the conceptus. This study was approved by the Clinical Research Ethics Board of the University of British Columbia.

Trypsin G–banded metaphase chromosomes from cultured amnion and placental stroma were prepared in accordance with standard procedures. Trophoblast and stromal cell suspensions for FISH were obtained from chorionic villi, as described elsewhere (Henderson et al. 1996). Touch preparations of fetal lung and kidney and smears of umbilical cord blood and fetal brain were prepared by use of silanized slides. The fetal-cell preparations were fixed in 100% methanol for 5 min and then in 3:1 methanol/acetic acid for 5 min and were air dried. The fetal ovary was squashed onto silanized slides, as described by Blandau et al. (1963). By use of a chromosome 16–specific centromeric probe (D16Z2; Oncor), FISH was performed on all fresh tissues, except oocytes (see below), in accordance with the manufacturer's recommended protocol. By means of a Zeiss epifluorescence microscope, ~500 nuclei were scored, and the proportion of nuclei displaying one, two, three, or four or more hybridization signals was recorded for each sample. In addition, for each tissue type studied, FISH analysis was performed on identically processed disomic controls, to establish the cutoff values for significant levels of trisomy. From this tissue-specific control data, the lower level of trisomy detection was calculated for each tissue type, as described by Lomax et al. (1994).

For molecular cytogenetic analysis of the gonads, only meiotic prophase I oocytes were scored. Oocytes in the prophase of meiosis I can be identified by their large size and diffuse chromatin (Baker 1963) and are morphologically distinguishable from somatic cells (fig. 1). In

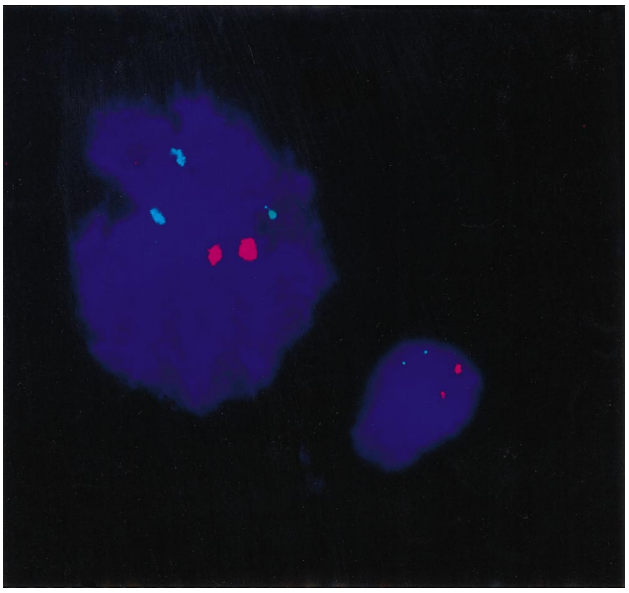

Figure 1 Two-color FISH analysis of nuclei from squash preparations of fetal ovary. The larger meiotic oocyte displays two red hybridization signals, corresponding to the centromeres of chromosome 18 (D18Z1), and three green hybridization signals, corresponding to the centromeres of chromosome 16 (D16Z2). The diffuse oocyte hybridization signals are due to the dispersed chromatin structure of an oocyte during meiosis. The smaller somatic cell shows two distinct hybridization signals, for the same probes, indicating disomy for both chromosomes 18 and 16.

order to select for oocytes with unpaired chromosomes and to eliminate erroneous results from paired chromosomes producing a single indiscriminate signal, two-color FISH was performed by use of both a chromosome 16–specific centromeric probe (D16Z2) and a chromosome 18–specific centromeric probe (D18Z1; Oncor). Only those oocytes in which the internal control (chromosome 18) exhibited two hybridization signals were scored for the chromosome 16 probe.

Conventional cytogenetic analysis of 15 metaphases from cultured placental stroma identified mosaicism, disomy, and trisomy for chromosome 16, whereas analysis of five metaphases from cultured amnion demonstrated only a diploid cell line. By use of FISH analysis, high levels of trisomy 16 were documented in trophoblast and chorionic stroma, whereas only disomy 16 was detected in fetal kidney, brain, lung, and cord blood (table 1). The finding that 26% of the oocytes displayed three hybridization signals corresponding to chromosome 16 indicates a significant level of trisomy in the germ cells of this ovary (fig. 1).

Microsatellite analysis at D16S423 demonstrated the presence of maternal uniparental disomy in fetal lung and adrenal gland (table 2). Results at D16S398 were consistent with a maternal meiotic origin of the extra chromosome 16 in placental tissues, including trophoblast, chorionic stroma, and amnion. A recombination event presumably occurred between D16S423 and D16S398, explaining reduction of maternal alleles to homozygosity for D16S423 and heterozygosity for D1S398.

This is the first published data documenting the existence of germ-cell mosaicism in an otherwise nonmosaic fetus, for a conceptus diagnosed with CPM. Our findings are consistent with trisomic zygote rescue resulting in diploid fetal somatic tissues, including blood, and placental mosaicism involving both the trophoblast and chorionic stroma. Although no mosaicism was detected in the fetal somatic tissues, the mosaicism observed in the germ cells was concordant with that found in the extraembryonic placental tissues. These results highlight the complex processes of origination and delineation of fetal and placental tissues.

In the developing human, the trophoblast is the first cell lineage to differentiate, forming the outer cells of the 16-cell morula. In the next developmental stage, blastogenesis, the trophoblast constitutes the outer layer, whereas the inner cell mass comprises multipotent cells, of which the majority become progenitors of the extraembryonic mesoderm and a smaller number give rise to the embryo/fetus proper (Markert and Petters 1978). During the 3d wk postconception and after, the progenitors of the extraembryonic mesoderm contribute to the formation of the placental stroma and the mesodermal layers of the amnion, the umbilical cord, and the secondary yolk sac (Vogler 1987). The secondary yolk sac is known to be the source of both the hematopoietic and PGC progenitors (Fujimoto et al. 1977; Vogler 1987).

Evidence for common progenitors of the PGCs and placental stroma is provided by animal models. For the mouse, studies following the development of embryonic cell lineages show that both the extraembryonic mesoderm and the PGCs originate from common progenitors in the epiblast of the pregastrulation embryo (Lawson and Hage 1994; Buehr 1997). These studies also provide evidence that the germ-cell line is not lineage restricted at 6–6.5 d postcoitum in mice. Alkaline phosphatase, used to identify early germ cells, is first detected at ~7.2 d postcoitum, when the PGC progenitor cells move into the extraembryonic region between the endoderm and the mesoderm of the ventral part of the amniotic fold.

In the human embryo, PGC progenitors are first observed in an extraembryonic location within the secondary yolk sac, together with hematopoietic progenitors (Fujimoto et al. 1977). The fate of these two extraembryonically located progenitors is different. Recent evidence from studies of the mouse show that the contribution, to embryonic hematopoiesis, of the hematopoietic progenitor cells from the secondary yolk sac

Table 1

Results of FISH Analysis with Probe D16Z2

Tissue	Two Signals[a]	Three Signals[a]	n[b]	Cutoff Values (No. of Controls)[c]
Trophoblast	21	76	1,004	6.6 (6)
Stroma	17	59.3	1,648	9.7 (8)
Oocyte	64.8	26	227	7.1 (8)
Brain	85.6	6.6	501	7.7 (8)
Lung	89.4	1.2	500	8.8 (5)
Kidney	93.6	2.2	500	6.2 (4)
Cord blood	87.4	5.8	501	5.9 (9)

[a] Nuclei displaying two or three hybridization signals (zero, one, and four signals not shown).

[b] Total no. of nuclei scored.

[c] Cutoff values for significant levels of trisomy, as calculated from hybridization results from control samples.

Table 2

Results of Microsatellite Analysis of Loci on Chromosome 16

Tissue	D16S423	D16S398
Maternal blood	ab	ab
Paternal blood	cd	…
Trophoblast	…	abc
Chorionic stroma	…	abc
Amnion	…	abc
Fetal lung	aa	ab
Fetal adrenal gland	aa	…

is transient and that definitive hematopoiesis is autonomously initiated later in the aorta-gonad-mesonephrous region of the embryo (Medvinsky and Dvierzak 1996). Thus, the PGCs represent the only permanent contribution from the secondary yolk sac, to the makeup of the embryo/fetus. The temporary sequestration of the germ line into extraembryonic regions (e.g., the secondary yolk sac in humans) has been described in many vertebrates, but the reasons for it are not understood. It has been suggested that the germ cells may be withdrawn from embryonic tissues, to escape the widespread tissue-specific methylation that occurs around the time of gastrulation (Buehr 1997).

The technical advances provided by molecular cytogenetic techniques permit accurate cytogenetic analysis of placental and fetal tissues in pregnancies with CPM and provide a unique opportunity to study the origin and interrelationship of various embryonic and extraembryonic cell lineages in mosaic conceptuses. However, since termination of a pregnancy with CPM is rare, opportunities to obtain further morphological data from human embryos or fetuses demonstrating the presence of aneuploid clones in both PGCs and chorionic stroma are infrequent. The consequences of germ-cell mosaicism likely will be specific for individual trisomic chromosomes involved in CPM. For example, some young mothers who give birth to offspring with trisomy 21 were born to mothers of advanced maternal age (Aagesen et al. 1984). It is possible that these young mothers originated as a trisomy 21 zygote that was rescued, leading to CPM 21 and trisomy 21 germ-cell mosaicism in their gonads. Further long-term prospective studies of individuals born from pregnancies with CPM are required, to document the effect of various placental aneuploidies on gonadal development and human fertility in both males and females. A diagnosis of CPM involving the chorionic stroma may represent an increased risk of chromosomal mosaicism in the germ cells and may have reproductive consequences later in life.

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The Sib Transmission/Disequilibrium Test is a Mantel-Haenszel Test

To the Editor:

Spielman and Ewens's (1998) proposed extension of the transmission/disequilibrium test (TDT), using discordant sibships, provides a simple and elegant way to apply the TDT in instances in which parents are not available. It is easy to see that the adapted test, called the "sib TDT" (S-TDT), is numerically equivalent to a Mantel-Haenszel test of trend, also known as the "Mantel extension test" (Rosner 1995).

The original Mantel-Haenszel test is used routinely in matched case-control studies, to test for association between disease and exposure. When exposure is expressed as a quantitative risk factor with C levels, the Mantel extension test allows the investigator to obtain a 1-df test against the alternative of a monotone trend. For each matched set, a 2 × C table classifying subjects according to disease and exposure status is formed. The statistic is determined by assigning the columns quantitative values corresponding to exposure level. The statistic also may be derived as a score test for no association within each matched set, by use of a model for the log odds of disease, which is linear in exposure level.

To obtain the S-TDT by use of the Mantel extension test, sibship is used as the stratifying variable, and for each sibship a 2 × 3 table cross-classifying sibs on the basis of disease status and genotype is formed. The quantitative value of exposure that yields the S-TDT assigns to each genotype the number of putative disease-associated alleles that a sib has (i.e., 2, 1, or 0) for genotypes AA, AB, or BB.

The advantages of viewing the S-TDT as a Mantel extension test are threefold. First, the test is already widely available on commercial software. For example, SAS currently implements the Mantel extension test as part of its Cochran-Mantel-Haenszel procedure. This version of the test allows user-specified scores for the levels of the quantitative variable but does not provide a continuity correction. Another program, StatXact, provides an exact *P* value for the Mantel extension test, as well as the asymptotic *P* value.

Second, it immediately is obvious how to use the test with other genetic models. For example, for an arbitrary genetic model, an investigator may want to use the 2-df Mantel-Haenszel test, which makes no assumption about how risk varies with number of A alleles. To test a dominant model, a value of 1 would be assigned to genotype AA or AB and a value of 0 to genotype BB; to test a recessive model, exposure values of 1 for AA and of 0 for all other genotypes would be used.

Third, if the marker is actually a candidate gene, the investigator may wish to estimate risk ratios. Collapsing over sibships and estimating risk ratios by use of the 2 × 3 margin results in biased estimates, which may be confounded because sibships may come from different populations with different disease risks and allele distributions. Instead, to estimate risk ratios, a Mantel-Haenszel estimate of odds ratio—or, in the general case, conditional logistic regression (Breslow and Day 1980)—should be used.

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Reply to Laird et al.

To the Editors:

Laird et al. (1998 [in this issue]) point out that the sib transmission/disequilibrium test (S-TDT) is identical in principle to the Mantel extension test for trend in multiple strata. We were not aware of this test and independently developed the S-TDT. Despite identity in theory, however, our test differs in practice from the Mantel extension test in two respects.

First, unlike the Mantel extension test described by Rosner (1995) and its implementation in SAS programs as described by Laird et al. (1998), our approach makes a continuity correction in the z score calculation and thus provides a somewhat more accurate approximation of *P* values. The difference is substantial when the data consist of only a small number of families. For instance, in our numerical example of three families (Spielman

and Ewens 1998), the use of a continuity correction led us to a z score of 1.9839, which results in a two-sided P value of .0473. The SAS program computes a two-sided P value of .016, which is also the value computed by use of the formula of Rosner (1995). In this example, the exact P value can be found by exhaustive enumeration of all permutations of the data from the three families: it is 3/70 = .0429, which is close to our value and is more than twice the value computed by the SAS program.

Laird et al. (1998) comment that the SAS calculation does not include a continuity correction but imply that the StatXact computer program can compute exact P values for the S-TDT. In practice, no procedure can do this when the sample consists of data from a large number of families, since in such cases an exact calculation would involve an astronomically large number of permutations. For such cases, StatXact resorts to Monte Carlo methods by using samples taken at random from the large number of permutations possible. We ourselves used this procedure for the S-TDT (Spielman and Ewens 1998).

Second, instead of the square of the z statistic, which the Mantel extension test applies, we prefer to use the unsquared z statistic, because one of our aims is to provide a combined test for data from some families that are suitable for the S-TDT test and from some that are suitable for the TDT (Spielman and Ewens 1998). The combined test is easily performed by use of the unsquared statistic but not by use of the squared statistic. A computer program that incorporates the continuity correction and that allows for the combination of the S-TDT and TDT procedures is now available at http://spielman07.med.upenn.edu/TDT.htm.

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