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Evidence from Human Oocytes for a Genetic Bottleneck in an mtDNA Disease

D. R. Marchington,[1] V. Macaulay,[2] G. M. Hartshorne,[3,*] D. Barlow,[3] and J. Poulton[1]

Departments of [1]Paediatrics and [2]Cellular Science and [3]Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford

Summary

We have examined oocytes from a patient with Kearn-Sayre syndrome caused by mtDNA rearrangements. In mtDNA diseases, mutant and wild-type mtDNA frequently coexist in affected individuals (the condition of heteroplasmy). The proportion of mutant mtDNA transmitted from mother to offspring is variable because of a genetic bottleneck, and the "dose" of mutant mtDNA received influences the severity of the phenotype. The feasibility of prenatal diagnosis is critically dependent on the nature and timing of this bottleneck. Significant levels of rearranged mtDNA were detectable in the majority of the patient's oocytes, by use of multiplex PCR, with wide variation, in the levels of mutant and wild-type molecules, between individual oocytes. We also used length variation in a homopolymeric C tract, which is often heteroplasmic in normal controls, to identify founder subpopulations of mtDNAs in this patient's oocytes. We present direct evidence that the number of segregating units (*n*) is three to five orders of magnitude less than the number of mitochondria in the human female oocyte. In some cases, the best estimate of *n* may correspond to a single mitochondrion, if it is assumed that intergenerational transmission of mtDNA can be treated as a single sampling event. The bottleneck appears to contribute a major component of the variable transmission from mother to oocyte, in this patient and in a control. That this bottleneck had occurred by the time that oocytes were mature advances the prospects for prenatal diagnosis of mtDNA diseases.

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Address for correspondence and reprints: Dr. Joanna Poulton, Department of Paediatrics, University of Oxford, Level 4, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom. E-mail: joanna.poulton@paediatrics.ox.ac.uk

* Present affiliation: Department of Biological Sciences, University of Warwick, Coventry, United Kingdom.

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Introduction

The transmission of human mtDNA variants is poorly understood (Lightowlers et al. 1997). There are thousands of copies of mtDNA in a cell, and yet, usually, the overwhelming majority of mtDNAs in an individual human are identical (homoplasmy). When there is a point-mutation difference between a mother and her offspring, there may be a complete switch of mtDNA type within a single generation (a bottleneck). This implies that a small number of founder mtDNAs populate the organism.

The wide variation in the level of mutant mtDNAs transmitted to the heteroplasmic offspring impedes genetic counseling and prenatal diagnosis in families with heteroplasmic mtDNA point mutations. For example, the level of 8344G:C mutant mtDNA ranged from 0% to 73% in the blood of the offspring of an asymptomatic mother with 14% mutant mtDNA in muscle (Larsson et al. 1992). This variation in proportion of mutant transmitted could arise from two sources: random segregation of a specific number of founder mtDNAs (i.e., a bottleneck) or nonrandom proliferation of a subpopulation because of some selective advantage that appears to be rare before birth (Suomalainen et al. 1993; Matthews et al. 1994). Hauswirth and Laipis (1985; also see Laipis et al. 1988) investigated a herd of Holstein cows for a common polymorphism. When there was a point-mutation difference between a mother and her offspring, each could be homoplasmic with respect to that base. Because oocytes contain ~100,000 mtDNAs, Hauswirth and Laipis suggested that only a small number (*n*) of mtDNAs ultimately populated the organism (in a "single selection" model, in which it is assumed that there is a single restriction/amplification event or bottleneck). By analysis of the individuals in which the switching was not complete, they were able to estimate that $n = 1–6$ and that the segregating unit might be a single mitochondrion (Hauswirth and Laipis 1985). This bottleneck appears to be necessary for maintenance of homoplasmy.

We have shown elsewhere that individual human oocytes can be heteroplasmic for length variants in a homopolymeric C tract in the vicinity of bp 310 in the major

noncoding region of mtDNA. Tissues from normal individuals possessed one major length variant (>95%), but among somatic tissues in any control individual there was no difference in the pattern of the length variants when bulk samples were taken. In two normal controls, the major length variants differed among oocytes from the same donor, suggesting that segregation of founder mtDNA molecules had occurred by the time that the oocytes were mature (Marchington et al. 1997). This result is consistent with animal data (Jenuth et al. 1996). In this report, we have analyzed the variance in individual oocytes from a patient with rearrangements of mtDNA, compared with that in a control, to estimate the most likely size of the genetic bottleneck, and we have found that the number of segregating units (n) could be as low as a single mitochondrion.

Subjects, Material, and Methods

Human Subjects

The patient in the present study had Kearn-Sayre syndrome (KSS) and rearranged mtDNA, comprising duplications, deletions, and deletion dimers, in addition to wild-type molecules (Poulton et al. 1995). The control was attending an infertility clinic. The study was approved by the Central Oxfordshire Ethics Committee.

Three-Way PCR to Quantitate mtDNA Rearrangements

All three primers (tRNA Y, corresponding to bp 5864–5883; CO1/5R, reverse and complementary to bp 6154–6135; and Cytb3, reverse and complementary to bp 15151–15132) were added to each reaction. Amplification conditions were 94°C for 5 min, 55°C for 1 min, and 72°C for 1 min (cycle 1) and then 94°C for 1 min; 55°C for 1 min and 72°C for 1 min (39 cycles); and then 72°C for 9 min, after the last cycle. PCR was performed on DNA from different tissues (brain cortex, heart, ovary, liver, cerebellum, kidney, pituitary, and pancreas) from patient 1, and the products were labeled by last-cycle labeling with $\alpha[^{32}P]$-dCTP. The products were run on 6% acrylamide gels and were vacuum dried, and the ratio of the two bands was quantitated on a Phosphorimager. Interassay variability typically was $\pm 1\%$.

Three-Way PCR of Single Oocytes from Patient 1

Five-millimeter sections of frozen ovary were placed in sterile PBS and were allowed to come to room temperature. The sections were washed in sterile PBS, and the tissue was teased apart by use of sterile scalpel blades, under a dissecting microscope. The oocytes released were placed in fresh PBS and were examined by light microscopy. Any that were damaged or that had adherent cells were discarded. Oocytes were lysed by being boiled for 10 min in 20 μl of PBS, and 1/10 of the lysate was taken for PCR. Three-way PCR analysis was performed on 15 oocytes and the bulk ovarian tissue from which they were derived. The relationship of three-way PCR versus Southern blot data from tissues (fig. 1*B*) was used to derive the relative proportions of rearranged and wild-type molecules in individual oocytes, by use of a curve derived from standards in a range of dilutions (spanning the template concentration in oocytes) that were amplified in the same run. Duplicate determinations on single oocytes varied by $\pm 0.75\%$. Because the three-way PCR did not distinguish between the different rearrangements, three ratios were calculated for each oocyte: the percentage of mutant when it was assumed that all rearrangements were (1) deletion monomers (shown in table 1), (2) deletion dimers (see Statistical Analysis subsection), and (3) duplications (see Statistical Analysis subsection).

Statistical Analysis

The data obtained were analyzed as described elsewhere (Bendall et al. 1996), to infer the size of the intergenerational bottleneck (n), on the assumption that it represents a one-time sampling (i.e., single selection) of a small number of mtDNAs from a large pool. In this approach, Bayes's theorem is used to evaluate the posterior probability of n, given the observed proportions of the heteroplasmic variants in ovary/blood and oocytes, and the estimated experimental error in these proportions. The patient's ovary contained 84% wild-type, 3% deletion-monomer, 5% deletion-dimer, and 8% duplicated molecules (by Southern blot). For the purposes of the calculations, we chose to assume that all the mutant molecules were of the same rearrangement. On the basis of the ratio of the PCR products for ovary and oocytes, the plot in figure 1*B* was used to estimate the proportion of mutant molecules, on the assumption that all rearrangements were deletion monomers. Similar results were obtained if it was assumed that they were deletion dimers or duplications. In order to apply the method of Bendall et al. (1996) in the case of the length variation, variants were pooled into two classes, consisting of the major length variant in the ovary and of the rest. From the posterior distribution, we quote the most probable n and the central 95% confidence interval (95% CI) region. This analysis assumes that, once generated, the rearrangements and length variants are stable. Our results represent maximum estimates of n, since a significant rate of intermolecular conversion (homologous recombination and generation of new length variants [Marchington et al. 1997], respectively) would result in overestimates of n (Bendall and Sykes 1995).

In addition, a standard population-genetic equation for the variance in proportion on repeated sampling (Nei

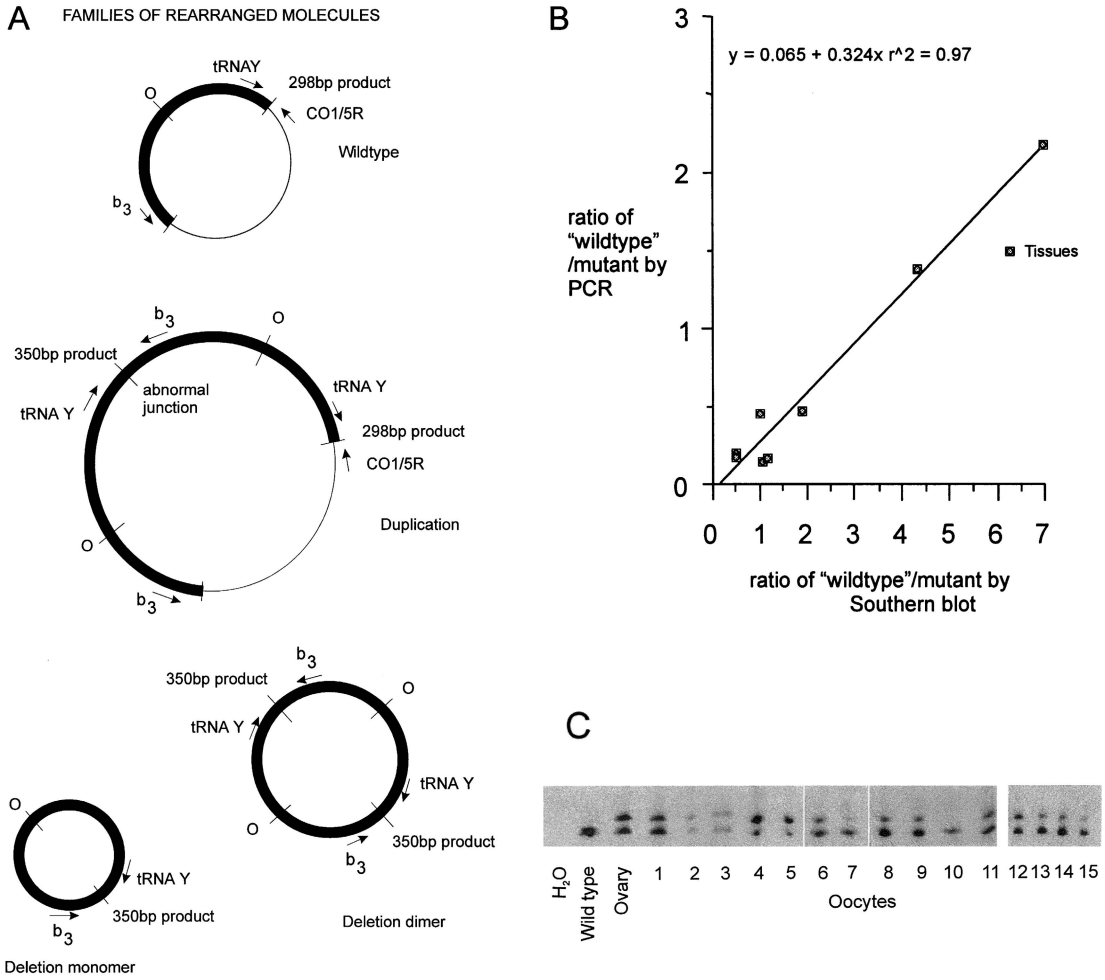

Figure 1 *A,* Location of primers on patient 1's rearranged mtDNA molecules. Patient 1 had three distinct but related types of rearrangement in addition to the wild type (*top*): a direct tandem duplication (*middle*), a deletion monomer (*bottom left*), and deletion dimers (*bottom right*). The breakpoint is at bp 6130–15056. The thinner line represents the deleted region, and the thicker line, which includes the large noncoding region and heavy-strand origin (O), represents the duplicated region (Poulton and Holt 1994). All the mutant molecules contain one or more abnormal junctions (one in duplicated and deletion monomer molecules and two in deletion dimers). Primers were designed to produce a product of 350 bp across the junction (tRNA Y and [Cyt]b_3) but no product in the wild type. A third primer (CO1/5R) gives a 298-bp product in wild-type and duplicated mt DNA (which contains a complete wild-type molecule). *B,* Quantitation of rearranged mtDNAs by PCR. The ratio of the bands that would be expected on the basis of the known quantities of the different mtDNA forms, as demonstrated by Southern blot analysis of eight tissues (i.e., wild-type+duplication, vs. duplication+deletion monomer+2 × deletion dimer), was calculated and plotted against the value obtained by the three-way PCR analysis of these tissues. The .97 correlation coefficient demonstrates that the PCR method reflects the relative quantities of the different molecules present. *C,* Three-way PCR of single oocytes from patient 1. Three-way PCR was used to quantitate the proportion of wild type to mutant, both in 15 single oocytes from patient 1 and in the bulk ovarian tissue from which they were derived, as well as in DNA from a wild-type control. The upper band represents the 350-bp product amplified from mutant molecules, and the lower band represents the 298-bp product amplified from wild-type or duplicated molecules.

1987, p. 359; Solignac et al. 1987) was used to estimate the bottleneck size, on the assumption that there were 23 resamplings (Jenuth et al. 1996) ("repeated selection"). This approach requires an assignment of p_0, the initial proportion of mutant DNA. We derived this from the proportion identified in ovary, 21.9% ± 0.61%, which was not significantly different from the mean proportion in the oocytes, 19.7% ± 3.05%.

Results

The patient in the present study had KSS and rearranged mtDNA comprising duplications, deletions, and deletion dimers in addition to wild-type molecules (Poulton et al. 1995) (see fig. 1*A*). Since, apart from rare exceptions (Bernes et al. 1993; Poulton and Holt 1994), the majority of mtDNA rearrangements are sporadic,

Table 1

Estimation of Bottleneck Size in Oocytes from Patient 1, Based on Mutant Level (Oocytes 1–15) and D310-Tract Length Variation (Oocytes 16–19)

Oocyte Number	Most Probable *n*	Central 95% CI	Mutant mtDNA[a] (%)
(Ovary)			21.9
1	26	>14 to >100	19.3
2	9	>8 to >100	22.2
3	4	>3 to >100	24.4
4	31	24 to 45	48.6
5	21	18 to 42	47.7
6	8	>6 to >100	12.3
7	16	>10 to >100	18.7
8	12	10 to 72	8.5
9	9	>7 to >100	11.3
10	1	1 to 15	.0
11	>100	>10 to >100	22.1
12	4	>3 to >100	25.0
13	8	>6 to >100	13.2
14	10	>7 to >100	9.4
15	8	>6 to >100	12.8
16	6	5 to 68	NA
17	6	5 to 68	NA
18	11	8 to 31	NA
19	10	2 to 23	NA

[a] All rearrangements are considered to be deletion monomers. NA= not available.

the patient had been counseled that the recurrence risk was low. Single oocytes were dissected from the frozen postmortem ovary of the patient. The size of the bottleneck was estimated by use of two independent methods. First, the differences in the levels of the different mutant molecules (Poulton et al. 1995) was used as the basis to develop a quantitative, PCR-based analysis of the level of mutant molecules in single oocytes (fig. 1*A* and *B*). Fifteen different oocytes were investigated for the presence of rearranged mtDNA molecules. Figure 1*C* demonstrates that there was a wide range in the level of mutant molecules, relative to that in bulk ovarian tissue. Rearranged mtDNA molecules were detectable by PCR in 14 of 15 oocytes from the patient with KSS. Of the 15 oocytes, 3 (20%) (oocytes 8, 10, and 14) contained <10% mutant mtDNA, whereas the other 12 contained proportions <50% (mean 23%). Had the oocytes been viable, this patient might have been able to transmit the disorder to her offspring. Patients with mtDNA duplications may have a propensity for germ-line transmission of mtDNA rearrangements (Poulton and Holt 1994).

In the second method, heteroplasmic length variation in a homopolymeric tract in the vicinity of bp 310 (i.e., the D310 tract) was investigated in the same patient. In contrast to what was observed for the homopolymeric C tract in the vicinity of bp 16189 (Bendall and Sykes 1995), individual length variants in this tract segregate stably with mtDNA subpopulations (Marchington et al. 1997). Four single oocytes were dissected from this patient and were amplified by trimmed-PCR, as described elsewhere (Marchington et al. 1997). The size of the $\alpha[^{32}P]$-labeled single-stranded product is representative of the length of the homopolymeric tract and was used to quantitate length variants after they were run on a sequencing gel and were subjected to Phosphorimager analysis. Figure 2 shows that all four oocytes were heteroplasmic for D310-tract length variation. In three of the oocytes (oocytes 16–18 in table 1) the major length variant was the same as that in the ovarian tissue from which they were obtained, but in the fourth oocyte (oocyte 19) the major length variant was different.

We now have analyzed these data through a Bayesian approach (Bendall et al. 1996), which assumes that a small sample from a large number of mtDNAs is withdrawn at random at one time point during oocyte maturation (i.e., the single-selection model). On the basis of the first approach (which uses the proportion of rearranged molecules), we estimated that the most probable bottleneck size is 1–31 segregating units (95% CI = 1–65 segregating units). The modal most probable *n* was 8 (in 3 of 15 oocytes), and the median was 9. This estimate was barely altered despite any assumptions made about the type of rearranged molecule in the oocyte (see the Subjects, Material, and Methods section). When the calculations were based on D310-tract length variation, the most probable bottleneck size was 6–11 (95% CI = 2–68). The magnitude of the bottleneck is similar in both patient and a control in whom the most probable *n* was 1–5 (95% CI = 1–6) (tables 1 and 2). By use of the same equation as was used by Jenuth et

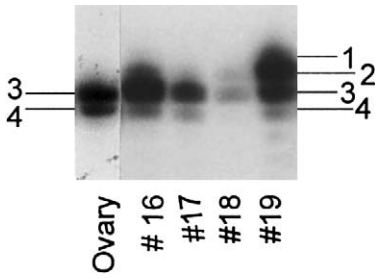

Figure 2 Quantitation of length variation in the D310-tract T-PCR, used to quantitate the proportion of different length variants in four oocytes (oocytes 16–19; see table 1) in the homopolymeric C tract in the vicinity of bp 310, as described elsewhere (Marchington et al. 1997). The intensity of signal in each band on the autoradiograph represents the proportion of the corresponding length variant. Three of the four oocytes (lanes 2–4) have the same modal length variant as is seen in ovarian tissue (i.e., variant 3; frequencies are 82.9%, 84.5%, and 91.2%, respectively, vs. 65.7% in ovarian tissue), whereas the fourth has a markedly different pattern of length variants.

Table 2

Estimation of Bottleneck Size in Oocytes from a Control (Subject 10 in Marchington et al. 1997), Based on D310-Tract Length Variation

Oocyte Number	Most Probable *n*	Central 95% CI
1	1	1–2
2	5	4–6
3	1	1–2

al. (1996), we calculate that, in the patient, the number of segregating units may be 9 (in single selection) or 210 (in repeated selection, on the basis of 23 cell divisions).

Discussion

The estimates of bottleneck size are similar whether rearrangements or the D310 tract is used. Taken together, these two approaches strongly suggest that a mtDNA bottleneck had occurred by the time that this patient's oocytes were mature, as we had observed earlier in human controls (Marchington et al. 1997). This is consistent with data on PCR of oocytes carrying the 8993 mutation (Blok et al. 1997). The occurrence of the major component of mtDNA bottleneck before fertilization suggests that the proportion of mutant mtDNA in a chorionic villus sample may be representative of the level in the rest of the conceptus. Although it is clear that segregation of mtDNA mutants occurs postnatally (Matthews et al. 1994; Poulton and Morten 1993) and in tissue culture (Dunbar et al. 1995), we consider that the present results improve the prospects for prenatal diagnosis.

Other investigators have estimated the number of segregating units either on the basis of indirect observations of intergenerational variance (Bendall et al. 1996) or, more directly, on the basis of the variance in heteroplasmic mouse oocytes (Jenuth et al. 1996). In a study of blood DNA from 180 twin pairs, Bendall et al. (1996) identified heteroplasmy in four families and demonstrated both very small and large swings in genotypic ratio, between generations (Bendall et al. 1996). Estimates of *n* varied for each mother-child pair but ranged from 2 to >100. Overall, the best estimate was ~3–20, consistent with the results from our study of control oocytes, by use of a single-selection model. However, others have derived higher estimates, by use of a repeated-selection model, which assumes that there may be multiple bottlenecks between generations (Howell et al. 1992).

Jenuth et al. (1996) have constructed a heteroplasmic mouse model of mtDNA segregation by introducing donor cytoplasm into a fertilized recipient egg (Jenuth et al. 1996). They investigated the variance of the proportions of donor mtDNA at different developmental stages. They found that the variance increase between primordial germ cells and primary oocytes was greater than that at later stages of developing oocytes and progeny. Consistent with our findings, these data suggest that a major component of the mtDNA bottleneck has occurred by the time that oocytes are mature. However, Jenuth et al. (1996) have estimated that the number of segregating units is ~200, which is 7–200-fold greater than our estimates, in humans, on the basis of a single-selection model. Clearly, there may be a species difference, but the major difference relates to the mathematical model used. Thus, the model used by Jenuth et al. (1996) assumes that the variance in genotypic ratios of the progeny or developing oocytes is caused by an identical random-sampling event that occurs during each of the 15 or so cell divisions during the later stages of oogenesis (i.e., repeated selection), in contrast to a more dramatic reduction in segregating units during a briefer period (i.e., single selection). Substituting a single selection (rather than 15) in their equation, we obtain, on the basis of their data, a bottleneck size of 6–60, which overlaps with the 1–31 derived from our data. When subjected to the same equation as was used by Jenuth et al., our data suggest that, in the patient with mitochondrial disease, the number of segregating units may be 9 (in single selection) or 210 (in 23 selections, which is based on an estimate of the number of cell divisions in human oogenesis). Hence, the choice of model used generates an ~23-fold difference in the size of *n*.

Are there any additional data that favor either one of the models? Blok et al. (1997) have concluded that their data on skewing of oocytes to wild type or mutant was not compatible with a conventional bottleneck model (they calculated that *n* was >20, on the basis of a repeated-selection analysis). They do not present sufficient data to allow full recalculation with use of our model. However, if we consider their oocytes to be virtually homoplasmic, then the most likely bottleneck size is 1. Hence, a single-selection model is more suitable for the data of Blok et al. (1997). Theoretically, the shape of the distributions generated by the alternative models (i.e., the frequency of oocytes containing different percentages of mtDNA mutant) might allow one to determine which of the two models fits the data best (Poulton et al. 1998). The repeated-selection model generates distributions that are peaked at fixation for mutant and wild-type mtDNA (Preiss et al. 1995), whereas a single-selection model generates a binomial distribution. At low percentages of mutants, the shape of these two distributions is similar (Poulton et al. 1998), although at higher maternal levels of mutant it might be possible to distinguish between them.

Most of the 50-fold expansion in the number of mtDNAs—to 100,000/cell, in the development from primordial germ cells to mature oocyte—occurs after the final cell division. Hence, a repeated-selection model may be inappropriate, because it assumes 23 or so identical cell divisions. There is morphological evidence for distinct subpopulations of mitochondria during the development of human oocytes, and in *Xenopus* the mtDNA in one subgroup replicates faster than that in the other subgroup (Tourte et al. 1984). Similarly, it has been suggested that, in cultured human cells (Davis and Clayton 1996) and in early mouse embryos (Meirelles and Smith 1998), mtDNA in the mitochondria lying in close proximity to the nucleus may replicate faster than those at the periphery. Although there are no equivalent data on human oocytes, a small subpopulation of mtDNAs that replicate repeatedly might underlie the massive expansion in mtDNA during oocyte maturation. If so, this may fit the single-selection model more closely than it fits the repeated-selection model. In mtDNA diseases, the apparent bottleneck size may depend on the characteristics of the pathogenic mutation (Blok et al. 1997). It may be necessary to make a new estimate of the bottleneck size, in order to counsel each individual family. If so, a single-selection model is more usable (Poulton et al. 1998) and may describe the data more accurately (Blok et al. 1997), even if a repeated-selection model is more representative of the number of cell divisions during oogenesis. Bottleneck sizes could be used to improve calculations of personalized recurrence risks (Poulton et al. 1998).

Whichever model is used, it is clear that the number of segregating units in the female germ line is three to five orders of magnitude less than the number of mitochondria in the mature human ovum. Maintenance of homoplasmy may protect against the development of mtDNA disease. A tight mtDNA bottleneck thus may be a necessary solution to the accumulation of somatic mutations of mtDNA during the aging process (Cortopassi et al. 1992), an accumulation that also occurs in oocytes (Chen et al. 1995).

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