New Evidence Confirms That the Mitochondrial Bottleneck Is Generated without Reduction of Mitochondrial DNA Content in Early Primordial Germ Cells of Mice

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Abstract

In mammals, observations of rapid shifts in mitochondrial DNA (mtDNA) variants between generations have led to the creation of the bottleneck theory for the transmission of mtDNA. The bottleneck could be attributed to a marked decline of mtDNA content in germ cells giving rise to the next generation, to a small effective number of mtDNA segregation units resulting from homoplasmic nucleoids rather than the single mtDNA molecule serving as the units of segregation, or to the selective transmission of a subgroup of the mtDNA population to the progeny. We have previously determined mtDNA copy number in single germ cells and shown that the bottleneck occurs without the reduction in germline mtDNA content. Recently one study suggested that the bottleneck is driven by a remarkable decline of mtDNA copies in early primordial germ cells (PGCs), while another study reported that the mtDNA genetic bottleneck results from replication of a subpopulation of the mtDNA genome during postnatal oocyte maturation and not during embryonic oogenesis, despite a detected a reduction in mtDNA content in early PGCs. To clarify these contradictory results, we examined the mtDNA copy number in PGCs isolated from transgenic mice expressing fluorescent proteins specifically in PGCs as in the aforementioned two studies. We provide clear evidence to confirm that no remarkable reduction in mtDNA content occurs in PGCs and reinforce that the bottleneck is generated without reduction of mtDNA content in germ cells.

Introduction

Mammalian mitochondrial genome shows a 5 to 10 times greater mutation rate than the nuclear genome [1,2]. This elevated mutation rate coupled with clonal maternal transmission leads to the high mtDNA polymorphism in populations. However, despite the prevalence of genetic variance within a species, most individuals possess only a single mtDNA variant. Pedigree analyses of heteroplasmic individuals in cattle, mice and humans revealed that mtDNA genotypes shift rapidly among offspring and return to homoplasm in some progeny within a few generations [3–8], suggesting that a mtDNA bottleneck accounts for the rapid segregation. Early studies have proposed that the bottleneck occurs in embryonic development in consequence of a drastic reduction of mtDNA content in PGCs [9,10]. In mice, the size of the bottleneck is estimated as to be ~200 mtDNA segregation units [11].

To test these hypotheses, three independent research groups have attempted to quantify mtDNA copy number in single germ cells at different developmental stages in mice. Cao et al. [12] made the first direct measurements of mtDNA copy number in single PGCs (identified by endogenous alkaline phosphatase (ALP) activity) in wild-type mice using quantitative real-time PCR (qPCR) and found that PGCs contained consistent amounts of mtDNA with a mean of ~1350–3600 copies per cell between 7.5 days post coitum (dpc) and 13.5 dpc, indicating that the bottleneck occurs without a marked reduction of mtDNA copies in PGCs. Recently using Stella-GFP transgenic mice to isolate PGCs, a study determined a mean of ~450 mtDNA copies per PGC at 7.5 dpc (median ~200) and a mean of ~1100–2200 copies between 8.5 dpc and 14.5 dpc. The drastic reduction in PGC mtDNA content at 7.5 dpc was suggested to be the cause of the bottleneck [13]. Taking advantage of using Oct4EGFP mice heteroplasmic for two mtDNA sequence variants, Wai et al. [14] measured both mtDNA copy number and heteroplasm in single germ cells. They detected that PGCs possessed a mean of ~280 mtDNA copies...
Mutations of mtDNA are responsible for many types of mitochondrial diseases in humans, including myopathy and neurological disorders. Females carrying a mixture of mutant and wild-type mtDNA variants transmit a variable amount of mutant mtDNA to each offspring. The proportion of mutated mtDNA inherited from the mother determines the onset and severity of diseases. Studies have suggested that the mtDNA genome is transmitted through a bottleneck, but the underlying mechanism remains controversial. By detecting mtDNA copy number in single cells, we previously showed that the bottleneck occurs without reduction of mtDNA content in germline cells. However, recently a study reported a marked decline of mtDNA copies in embryonic germ cells and attributed this reduction to the creation of the bottleneck. Yet another study concluded that the bottleneck occurs during postnatal oocyte maturation and not during embryonic oogenesis. To resolve these controversies, we examined mtDNA copies in embryonic germ cells identified using the same methodology as in the other two studies. We show solid evidence to confirm our previous findings. This confirmation is important because the understanding of mtDNA content in female germ cells will facilitate the development of therapeutic strategies preventing the transmission of mitochondrial diseases from mother to offspring.

**Results/Discussion**

In this study we have focused on our reassessment of mtDNA copy number in PGCs at 7.5 and 13.5 dpc. These two stages were chosen because 7.5 dpc was the only stage showing significantly different PGC mtDNA copies between the studies using either ALP staining or PGC-specific reporter transgenic mice for PGC identification, and the mean mtDNA copy numbers detected in PGCs at other stages did not differ very much [12,13]. PGCs of 13.5 dpc would serve to compare the mtDNA copy numbers in PGCs between the two developmental stages and between genders. 7.5 and 13.5 dpc PGCs were isolated from mice expressing: i) mRFP protein under the control of a Blimp1 genomic fragment [16]; and ii) GFP protein under the control of an Oct4 genomic fragment with deletion of the proximal enhancer, respectively [17,18]. Blimp1-mRFP and Oct4AP-E-GFP are reliable PGC markers at the corresponding developmental stages [18,19]. Our construct of Oct4AP-E-GFP is identical to that in Yoshimizu et al. [18].

Due to subtle variations in the time of conception, there is a variation in the developmental stage of individual mouse embryos at any given time point [20]. To examine whether PGC mtDNA copy number differs among 7.5 dpc embryos of different developmental stages we have identified and divided 7.5 dpc embryos into two groups having morphology of early bud (EB) stage and late bud (LB) stage, respectively.

**Validation of Blimp1-mRFP as a reliable marker to identify PGCs at EB and LB stages of 7.5 dpc embryos**

Blimp1 expression marks nascent PGCs as well as precursors of PGCs in early developing mouse embryos. In the restricted posterior region (after removal of visceral endoderm) of 7.5 dpc embryos, Blimp1 has been proved to express specifically in PGCs [19]. To facilitate the isolation of PGCs without any staining steps at 7.5 dpc, we used bacterial artificial chromosome (BAC) transgenic mice in which monomeric red fluorescent protein gene (mRFP) was inserted into the Blimp1 locus [16]. First we determined the Blimp1-mRFP expression profile. At both EB and LB stages mRFP expression was observed at the posterior end of the embryonic ectoderm and visceral endoderm in embryos (Figure 1B and 1J), consistent with the endogenous Blimp1 expression [19]. To further characterize Blimp1-mRFP positive cells, embryos were immuostained for Stella (PGC7), a PGC-specific marker, with the anti-Stella antibody whose specificity for PGCs has been proved [21]. Stella was detected exclusively in PGCs located at the posterior region of the embryos. All Stella positive cells were Blimp1-mRFP positive. At EB and LB stages Stella protein was expressed in 62.5% and 90% of Blimp1-mRFP positive cells in the posterior region, respectively (embryo number = 2 at both stages) (Figure 2), which is comparable with the results of Seki et al. [22]. As a second assay, we stained cells isolated from the posterior fragments (visceral endoderm removed) of Blimp1-mRFP embryos for alkaline phosphatase, another classical marker of PGCs. Cells were divided into Blimp1-mRFP positive and negative two groups prior to staining. At the EB stage, Blimp1-mRFP positive and negative cells were 79.4% (n = 34) and 0% (n = 28) positive for ALP staining, while at the LB stage, 92.9% (n = 28) and 0% (n = 40), respectively (Figure 3). The results agree with that Blimp1 expression precedes that of Stella and alkaline phosphatase [19]. The cells positive for Blimp1-mRFP but negative for Stella are either precursors of PGC or PGCs with weak Stella expression beyond the detection limit of anti-Stella antibody. Taken together, the Blimp1-mRFP expression profile of our Blimp1-mRFP line is highly similar to that of Blimp1 transgenic.
mice reported [19,22]. Our Blimp1-mRFP, therefore, can be used as a reliable marker to identify PGCs in the posterior region of the embryo at EB and LB stages.

Moderate copy number (mean >1,000) of mtDNA in PGCs and low copy number of mtDNA in embryonic somatic cells

The mean numbers of mtDNA molecules in single 7.5 dpc EB and LB, 13.5 dpc female and 13.5 dpc male PGCs were 1396, 1479, 1747 and 2039, respectively. Of note, no extremely low mtDNA copy number was detected in any PGCs, and the minimum number of mtDNA copies determined in single PGCs was 767. The variation in mtDNA copy number per cell was similar for each group of PGCs (CV = 0.25–0.45, Table 1, Figure 4). At 13.5 dpc no significant difference in the average mtDNA copy number between female and male PGCs was observed (t-test, P = 0.06). In contrast, somatic cells from the gonad were found to contain less than half the number of mtDNA copies found in PGCs at 13.5 dpc (mean mtDNA copy number in somatic cells = 702) (Table 1, Figure 4). All these results are, therefore, in very good agreement with previous estimates obtained from PGCs identified by alkaline phosphatase activity [12], taking into account that the cell samples in the present study were collected randomly without cell size classification. The present data confirm our previous findings [12]: i) There is no occurrence of remarkable reduction of mtDNA copies in early PGCs; ii) The amount of mtDNA molecules in PGCs is moderate (mean >1000 copies, comparable with that in adult somatic cells [23]) and consistent across stages; and iii) Embryonic somatic cells possess much lower mtDNA amount than PGCs.

How can we explain the discrepant findings among Cree et al. [13], Wai et al. [14] and ours? Cree et al. [13] detected median, ≥200 mtDNA copies (mean 451) in PGCs at 7.5 dpc and more than 1000 copies at later stages. This result may be associated with the aspects of their methodology for PGC sorting. Cree et al. [13] identified and sorted PGCs by flow cytometry. Isolation of PGCs using flow cytometry was shown to be inaccurate for early developmental stage embryos [14,15]. Szabo et al. [15] carried out PGC sorting from Oct4APE-EGFP transgenic mice that were generated using the construct identical to that in Yoshimizu et al. [18]. This Oct4APE-EGFP were reported to be specifically expressed in PGCs from 8.5 dpc onwards [17,18]. At 8.5 dpc (five somites) only 62% EGFP+ cells sorted by flow cytometry were positive for the PGC marker, whereas 96% or more were positive at 9.5 dpc and later stages [15], indicating the inaccuracy of flow cytometry in identifying GFP cells expressing a PGC marker at early developmental stages when PGCs are small in number relative to non-PGC cells in the sample. The number of PGCs in single embryos at 7.5 dpc is even smaller than at 8.5 dpc.

Figure 1. Isolation of single PGCs from 7.5 dpc early bud (A–H) and late bud (I–N) stage Blimp1-mRFP embryos. (A,C,E,G,I,K,M) Light microscopy images. (B,D,F,H,J,L,N) Fluorescent microscopy images. At both EB and LB stages mRFP was detected as a cluster in the posterior part of the embryo (dashed rectangle) and in the visceral endoderm (arrowheads). For isolating PGCs, the posterior regions bearing PGCs were cut off carefully to remove the visceral endoderm (E,F,K,L). The resulting tissue fragments were disaggregated through trypsinization, and single PGCs with fluorescence were collected unambiguously using micromanipulators under a fluorescent microscope (G,H,M,N).
Figure 2. Stella expression in the posterior region of Blimp1-mRFP transgenic mouse embryos. Blimp1-mRFP (red, left column), Stella (green, middle column), and overlapped images (right column) at the EB (top row) and LB (bottom row) stages.
doi:10.1371/journal.pgen.1000756.g002

Figure 3. Alkaline phosphatase staining pattern of cells from the posterior region of Blimp1-mRFP embryos. (A,B) Cells from EB stage. (C,D) Cells from LB stage. (A,C) Blimp1-mRFP negative cells. (B,D) Blimp1-mRFP positive cells. Scale bar, 20 μm.
doi:10.1371/journal.pgen.1000756.g003
Therefore it is possible that more than 38% of GFP+ cells sorted and analyzed by Cree et al. [13] at 7.5 dpc were not PGCs but somatic cells. Embryonic somatic cells have been shown to contain significantly lower amounts of mtDNA than PGCs [12,24]. By studying whole embryos, Aiken et al. [24] reported that the mean of mtDNA copies per somatic cell was \(300\) between 6.5 dpc and 13.5 dpc.

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**Table 1.** mtDNA copy number in single cells of early developing mouse embryos.

| Stage     | Sorting marker | Gender | Cell size | n  | Mean mtDNA copy number \((x10^3)\) | Median mtDNA copy number \((x10^3)\) | Range \((x10^3)\) | CV   |
|-----------|----------------|--------|-----------|----|-----------------------------------|--------------------------------------|-----------------|------|
| **Primordial germ cells** | | | | | | | | |
| 7.5 dpc   | ALP staining (+) | L       | 19        | 23.16 | 21.54 | 16.88–33.35 | 0.23 |
|           | ALP staining (+) | M       | 19        | 20.01 | 18.37 | 12.44–31.18 | 0.29 |
|           | ALP staining (+) | S       | 15        | 17.30 | 16.36 | 12.58–31.43 | 0.26 |
| EB        | Blimp1-mRFP (+)  |        |           | 25   | 13.96 | 13.34 | 7.67–23.27 | 0.25 |
| LB        | Blimp1-mRFP (+)  |        |           | 83   | 14.79 | 13.86 | 8.18–40.75 | 0.35 |
| 13.5 dpc  | ALP staining (+) | Female  | L     | 15   | 36.56 | 27.49 | 18.78–94.50 | 0.65 |
|           | ALP staining (+) | Female  | M     | 25   | 18.43 | 18.16 | 11.44–32.04 | 0.25 |
|           | ALP staining (+) | Female  | S     | 31   | 15.32 | 14.04 | 9.95–32.35 | 0.32 |
|           | Oct4::GFP (+)    | Female  |        | 35   | 17.47 | 17.03 | 9.23–55.98 | 0.45 |
|           | ALP staining (+) | Male    | L     | 16   | 30.53 | 27.61 | 12.9–87.28 | 0.56 |
|           | ALP staining (+) | Male    | M     | 28   | 18.95 | 19.60 | 3.78–34.52 | 0.33 |
|           | ALP staining (+) | Male    | S     | 26   | 13.50 | 12.88 | 3.26–25.06 | 0.37 |
|           | Oct4::GFP (+)    | Male    |        | 65   | 20.39 | 19.68 | 11.90–40.79 | 0.29 |
| **Somatic cells** | | | | | | | | |
| 13.5 dpc  | ALP staining (−) | Female  | L     | 10   | 6.45  | 6.07  | 3.44–8.90  | 0.25 |
|           | ALP staining (−) | Female  | S     | 9    | 4.86  | 4.50  | 0.39–9.55  | 0.63 |
|           | Oct4::GFP (−)    | Female  |        | 35   | 7.02  | 6.54  | 0.74–16.29 | 0.58 |

Range, range in mtDNA copy number of cells. CV, coefficient of variation in mtDNA copy. EB, early bud stage. LB, late bud stage. number. (+) and (−) stand for positive and negative, respectively. Data are from Cao et al. [12]. L, large size cell. M, medium size cell. S, small size cell. Somatic cells are from 13.5 dpc female gonads.

doi:10.1371/journal.pgen.1000756.t001

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**Figure 4.** mtDNA copy number in single cells of 7.5 and 13.5 dpc mouse embryos (logarithmic scale). Open black circles, primordial germ cells (PGCs); Open grey circles, somatic cells. Each circle represents a single cell. Horizontal lines indicate mean values. EB, early bud stage. LB, late bud stage. F, M, and Soma denote samples from female PGCs, male PGCs, and gonadal somatic cells, respectively.

doi:10.1371/journal.pgen.1000756.g004
18.5 dpc, comparable with the 451 copies in the 7.5 dpc PGC sample of Cree et al. [13]. In contrast, our isolation of 7.5 dpc PGCs was performed manually under a fluorescent microscope using micromanipulators. Hence the purity of PGCs was ensured (Figure 1). Wai et al. [14] found a median of 145 mtDNA copies (mean ~200) per PGC at 8.5 dpc and more than 1000 copies at other stages. It is currently unclear why the study of Wai et al. [14] gave the result significantly different from that of Cree et al. [13] and ours for 8.5 dpc PGCs. However, the fact that both Cree et al. [13] and Cao et al. [12] detected a mean of mtDNA copies more than 1000 per 8.5 dpc PGC weakens the possibility of extremely lower mtDNA copy number (mean ~280) in 8.5 dpc PGCs.

Why do PGCs contain more than 1000 mtDNA copies in sharp contrast with consistent ~300 per embryonic somatic cell across stages between 6.5 dpc and 18.5 dpc? One explanation is that the moderate mtDNA copy number in PGCs allows the cell to have an elevated tolerance for less deleterious mtDNA mutations and serves as a device to maintain the adaptive potential of mtDNA genome, which for a nuclear genome is achieved via diplody by means of sexual reproduction. On the other hand, a tight physical bottleneck in somatic lineages during embryonic development enables pathogenic mtDNA mutations (both severe and less deleterious variants) to rapidly segregate and be eliminated more efficiently. The mtDNA copy number in gonadal somatic cells at 13.5 dpc was much higher (mean 702) than the average mtDNA level per cell of the whole embryo (mean ~300) [24], suggesting that the physical bottleneck in embryonic somatic cells appears lineage specific which might be associated with tissue-specific cell function and bioenergetic demands. Alternatively, the moderate mtDNA copy number may be entailed to meet the energetic demand for PGC migration, mitotic replication and cell function. Recent findings from two research groups have provided new insight into the mtDNA segregation between generations. Wai et al. [14] showed that the mtDNA genetic bottleneck occurs not during embryonic oogenesis but during postnatal oocyte maturat...
Isolation of single PGCs and single somatic cells

(C57BL/6N×DBA/2J)F1 female mice were mated with Blimp1-mRFP and Oct4APE-GFP males. 7.5 and 13.5 dpc embryos were collected in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen) supplemented with 10% FCS. The restricted posterior parts of 7.5 dpc EB and LB Blimp1-mRFP embryos bearing PGCs (visceral endoderm removed, Figure 1) and the gonads of 13.5 dpc Oct4APE-GFP embryos were isolated using fine needles, and incubated in Trypsin-EDTA solution (Sigma) at 37°C for 10 min. Cells were dissociated in DMEM supplemented with 10% FCS by pipetting, were pelleted and washed twice in PBS and were resuspended in modified Dulbecco’s phosphate-buffered medium (PBI). Single PGCs expressing fluorescent proteins and the gonad somatic cells were randomly collected under a fluorescent microscope using micromanipulators (Leica). PGCs from female and male gonads were isolated separately at 13.5 dpc, at which time the gender of the fetus can be identified microscopically.

References

1. Brown WM, George M, Jr., Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76: 1967-1971.
2. Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18: 225-239.

Estimation of mtDNA copy number by quantitative real-time PCR (qrt–PCR)

Single cells were extracted and used directly for qrt–PCR analysis as previously described [12,36]. Quantification of absolute mtDNA copy number per cell was carried out by the standard curve method. Details of the probe, primers and standard DNA are as in Cao et al. [12]. To evaluate whether pure DNA of plasmids containing mouse mtDNA fragment is suitable to serve as standard DNA for the measurement of mtDNA copy number in single cells, we performed qrt–PCR on samples containing the plasmid DNA in the presence of a single mtDNA-less pB6B2 cell line was derived from L cells [37,38] whose mtDNAs encompass the same mtDNA fragment integrated into the plasmid. Mixing the plasmid DNA with a pB6B2 cell creates sample conditions closer to that of single cell DNA for PCR amplification. It showed that the estimated copy values highly correlated with their true copy values (r²>0.99) over a range of mtDNA concentrations that comprise the values detected in our single cell studies (Figure S1), indicating that a systematic underestimation or overestimation of actual plasmid copies did not occur. Therefore the pure plasmid DNA is suitable for using as standard DNA for our determination of mtDNA copies in single cells. This qrt–PCR system could detect as few as 10 copies of standard DNA template reliably. The linear regression analysis of all standard curves for samples with copy numbers between 10 and 10⁶ showed a high correlation (r²>0.99).

Supporting Information

Figure S1 Correlation between plasmid copy values measured by qrt–PCR and their corresponding true copy values in samples containing plasmid DNA in the presence of a pB6B2 cell. Plasmid DNA was quantified and serially diluted. Each dilution was added with a single pB6B2 cell and underwent qrt–PCR amplification in triplicate using PCR conditions referred to in the manuscript text. The graphs represent the results of two independent assays. A tight correlation between the measured copy number values and their true copy number values was found in both assays (R²>0.99).

Acknowledgments

We are grateful to Dr. Bruce Loveland for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: LC HS JIH KY.Performed the experiments: LC HS MS. Analyzed the data: LC HS MS. Contributed reagents/materials/analysis tools: MS JIH KA HY. Wrote the paper: LC HS JIH KA HY.

3. Ashley MV, Laipis PJ, Hauswirth WW (1989) Rapid segregation of heteroplasmic bovine mitochondria. Nucleic Acids Res 17: 7325-7331.
4. Bliok RB, Gook DA, Thorburn DR, Dahl HH (1997) Skewed segregation of the mtDNA at 8993 (T→G) mutation in human oocytes. Am J Hum Genet 60: 1405-1501.

2. Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18: 225-239.
5. Laios PJ, van de Walle MJ, Haaswirth WW (1988) Unequal partitioning of bovine mitochondrial genomes among siblings. Proc Natl Acad Sci USA 85: 8107–8110.

6. Larsson NG, Tullinius MH, Holme E, Oldfors A, Andersen O, et al. (1992) Segregation and manifestations of the mDNA-RNAj44, A–G(3474) mutation of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome. Am J Hum Genet 51: 1201–1212.

7. Meirelles FV, Smith LG (1997) Mitochondrial genotype segregation in a mouse heteroplasmic lineage produced by embryonic karyoplast transplantation. Genetics 145: 445–451.

8. Olivo PD, van de Walle MJ, Laios PJ, Haaswirth WW (1983) Nucleotide sequence evidence for rapid shifts in the bovine mitochondrial DNA D-loop. Nature 306: 400–402.

9. Jansen RP, de BK (1998) The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. Mol Cell Endocrinol 134: 81–88.

10. Krakauer DC, Mira A (1999) Mitochondria and germ-cell death. Nature 400: 125–126.

11. Jenuth JP, Peterson AC, Fu K, Shoubridge EA (1996) Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. Nat Genet 14: 146–151.

12. Cao L, Shitara H, Horii T, Nagao Y, Imai H, et al. (2007) The mitochondrial mitochondrial DNA in the mouse egg. Dev Biol 49: 1–10.

13. Cree LM, Samuels DC, de Sousa Lopes SC, Rajasimha HK, Wonnapinij P, et al. (2008) A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. Nat Genet 40: 249–254.

14. Wai T, Teoli D, Shoubridge EA (2008) The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. Nat Genet 40: 1484–1488.

15. Szabo PE, Hubner K, Scholer H, Mann JR (2002) Allele-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in specific expression of the Oct-4/green fluorescent protein transgene in primordial germ cells in mice. PLoS Genet 3: e116. doi:10.1371/journal.p-gen.0030116.

16. Shitara H, Kaneda H, Sato A, Inoue K, Ogura A, et al. (2002) Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. Genetics 156: 1277–1284.

17. Bernes SM, Bacino C, Prezant TR, Pearson MA, Wood TS, et al. (1993) Identical mitochondrial DNA deletion in mother with progressive external ophthalmoplegia and son with Pearson marrow-pancreas syndrome. J Pediatr 123: 596–602.

18. de Vries D, de Wijs L, Ruitersbeek W, Beeger J, Smit P, et al. (1994) Extreme variability of clinical symptoms among siblings in a MELAS family correlated with heteroplasmy for the mitochondrial A3243G mutation. J Neurol Sci 124: 77–82.

19. Puoti G, Carrara F, Sampaolo S, De Caro M, Vaccin C, et al. (2003) Identical large scale rearrangement of mitochondrial DNA causes Kearn-Sayre syndrome in a mother and her son. J Med Genet 40: 856–863.

20. White SL, Shanske S, McGill JJ, Mountain H, Geraghty MT, et al. (1999) Mitochondrial DNA mutations at nucleotide 8993 show a lack of tissue- or age-related variation. J Inherit Metab Dis 22: 899–914.

21. Wallace DC (2003) The mitochondrial genome in human adaptive radiation and disease: on the road to therapeutics and performance enhancement. Gene 354: 169–180.

22. Stewart JR, Frycer C, Elson JL, Larsson NG (2000) Purifying selection of mitochondrial DNA and its implications for understanding evolution and mitochondrial disease. Nat Rev Genet 9: 657–662.

23. Barthelemy C, Ogier de Baulny H, Diaz J, Cheval MA, Frachon P, et al. (2001) Late-onset mitochondrial DNA depletion: DNA copy number, multiple deletions, and compensation. Ann Neurol 49: 607–617.

24. Aiken CE, Cindrova-Davies T, Johnson MH (2006) Variations in mouse mitochondrial DNA copy number from fertilization to birth are associated with oxidative stress. Reprod Biomed Online 17: 806–813.

25. Fan W, Waymire KG, Narula N, Li P, Rocher C, et al. (2008) A mouse model of mitochondrial disease reveals germline selection against severe mtDNA Pediatr. Science 319: 930–962.

26. Sato A, Nakada K, Shiitara H, Kasahara A, Yonekawa H, et al. (2007) A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. Nat Genet 40: 249–254.

27. Barthelemy C, Ogier de Baulny H, Diaz J, Cheval MA, Frachon P, et al. (2001) Late-onset mitochondrial DNA deletion: DNA copy number, multiple deletions, and compensation. Ann Neurol 49: 607–617.

28. de Vries D, de Wijs L, Ruitersbeek W, Beeger J, Smit P, et al. (1994) Extreme variability of clinical symptoms among siblings in a MELAS family correlated with heteroplasmy for the mitochondrial A3243G mutation. J Neurol Sci 124: 77–82.

29. Puoti G, Carrara F, Sampaolo S, De Caro M, Vaccin C, et al. (2003) Identical large scale rearrangement of mitochondrial DNA causes Kearn-Sayre syndrome in a mother and her son. J Med Genet 40: 856–863.

30. White SL, Shanske S, McGill JJ, Mountain H, Geraghty MT, et al. (1999) Mitochondrial DNA mutations at nucleotide 8993 show a lack of tissue- or age-related variation. J Inherit Metab Dis 22: 899–914.

31. Wallace DC (2003) The mitochondrial genome in human adaptive radiation and disease: on the road to therapeutics and performance enhancement. Gene 354: 169–180.

32. Stewart JR, Frycer C, Elson JL, Larsson NG (2000) Purifying selection of mitochondrial DNA and its implications for understanding evolution and mitochondrial disease. Nat Rev Genet 9: 657–662.

33. Larsson NG, Holme E, Kristiansson B, Oldfors A, Tulinius M (1990) Progressive related variation. J Inherit Metab Dis 22: 899–914.

34. Simonetti S, Chen X, DiMauro S, Schon EA (1992) Accumulation of deletions in human mitochondrial DNA during normal aging: analysis by quantitative PCR. Biochim Biophys Acta 1180: 113–122.

35. Piko I, Matsuzaki I (1976) Number of mitochondria and some properties of mitochondrial DNA in the mouse egg. Dev Biol 49: 1–10.

36. Shiitara H, Kaneda H, Sato A, Inoue K, Ogura A, et al. (2000) Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. Genetics 156: 1277–1294.

37. Hayashi J, Gotoh O, Tagashira Y, Tosu M, Sekiguchi T, et al. (1982) Critical determinant of the germ cell lineage in mice. Nature 306: 400–402.

38. Inoue K, Takai D, Hosaka H, Ito S, Shitara H, et al. (1997) Isolation and sequence evidence for rapid genotypic shifts in the bovine mitochondrial DNA. Genet.0030116.

39. Hayashi J, Gotoh O, Tagashira Y, Tosu M, Sekiguchi T, et al. (1982) Critical determinant of the germ cell lineage in mice. Nature 306: 400–402.

40. Shitara H, Kaneda H, Sato A, Inoue K, Ogura A, et al. (2000) Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. Genetics 156: 1277–1294.

41. Hayashi J, Gotoh O, Tagashira Y, Tosu M, Sekiguchi T, et al. (1982) Critical determinant of the germ cell lineage in mice. Nature 306: 400–402.

42. Inoue K, Takai D, Hosaka H, Ito S, Shiitara H, et al. (1997) Isolation and sequence evidence for rapid genotypic shifts in the bovine mitochondrial DNA. Genet.0030116.

43. Hayashi J, Gotoh O, Tagashira Y, Tosu M, Sekiguchi T, et al. (1982) Critical determinant of the germ cell lineage in mice. Nature 306: 400–402.

44. Inoue K, Takai D, Hosaka H, Ito S, Shitara H, et al. (1997) Isolation and sequence evidence for rapid genotypic shifts in the bovine mitochondrial DNA. Genet.0030116.