Cell Reports

Cellular Heterogeneity in the Level of mtDNA Heteroplasmy in Mouse Embryonic Stem Cells

Graphical Abstract

Highlights

- mtDNA heteroplasmy is compared in ESCs, trophectoderm (TE), and second polar bodies (PB2)
- mtDNA heteroplasmy in ESCs is more closely associated with TE cells than PB2
- Cellular heterogeneity of homoplasmic mtDNA haplotypes occurs at the single-cell level
- The level of mtDNA heteroplasmy increases with progressive passage numbers

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In Brief

Neupane et al. show that, in comparison to second polar bodies, mtDNA heteroplasmy in trophectoderm (TE) cells is more closely correlated with the corresponding ESCs, indicating that TE biopsy is preferable to polar body biopsies for pre-implantation genetic diagnosis in mtDNA disorders. Wider variability in mtDNA heteroplasmy in stem cells could explain the existence of tissues with different heteroplasmic loads in individuals with heteroplasmic mtDNA conditions.
Summary

Variation in the level of mtDNA heteroplasmy in adult tissues is commonly seen in patients with a mixture of wild-type and mutant mtDNA. A mixture of different mtDNA variants may influence such variation and cause mtDNA segregation bias. We analyzed cellular heterogeneity in embryonic stem cells (ESCs) derived from a polymorphic mouse model containing NZB and BALB mtDNA genotypes. In ESCs, inter-colony heterogeneity varied up to 61%, whereas intra-colony heterogeneity varied up to 100%. Three out of five cell lines displayed nearly homoplasmic BALB and NZB mtDNA haplotypes in differentiated single cells. The proportion of NZB mtDNA genotype increased with progressive passaging (0.39%; p = 0.002). These results demonstrate the bimodal segregation of mtDNA haplotypes, indicating the occurrence of tissues with variable levels of heteroplasmy in individuals with mtDNA mutations. Furthermore, proliferation of one mtDNA genotype over another may pose the risk of accumulating mutant mtDNAs during subsequent cell divisions (Jenuth et al., 1996). Unlike nuclear DNA, mtDNAs are inherited uniquely from the maternal line of origin (Al Rawi et al., 2011; Birky, 2001). During oogenesis in the maternal germline, there is a 1,000-fold increase in mtDNA copy numbers from the primordial germ cells (PGCs) to the mature metaphase II (MII) oocytes (Cao et al., 2007, 2009; Cree et al., 2008; Monnot et al., 2013; Pikó and Taylor, 1987; Wai et al., 2008). If mtDNA is present in a heteroplasmic form in the PGCs, then either of the mtDNA variants can be selected for or against (St John, 2014), leading to intercellular variability. In the absence of a therapeutic remedy, pre-implantation genetic diagnosis (PGD) and germline genome transfer are the only possible approaches to avoid the transmission of heritable mtDNA disorders. However, in both cases, the potential risk of mtDNA carryover to the offspring may result in a heteroplasmic condition, resulting in the unbalanced segregation of mtDNA variants.

Introduction

Normally, all copies of mtDNA in a cell are identical (“homoplasmic”). However, more than one type of mtDNA variant may exist within a cell (“heteroplasmic”). Mutation, deletion, or depletion of mtDNA results in mtDNA disorders, which can be lethal (Wallace, 1999). In cases of mtDNA mutation disorders, a mixture of wild-type (WT) and mutated mtDNA copies are present. Intercellular variation in the level of mtDNA heteroplasmy is frequently seen in individuals with heteroplasmic mtDNA mutations. The mtDNA bottleneck followed by random genetic drift is thought to be responsible for the unbalanced segregation of heteroplasmic mtDNAs in the subsequent cell divisions (Jenuth et al., 1996). Unlike nuclear DNA, mtDNAs are inherited uniquely from the maternal line of origin (Al Rawi et al., 2011; Birky, 2001). During oogenesis in the maternal germline, there is a 1,000-fold increase in mtDNA copy numbers from the primordial germ cells (PGCs) to the mature metaphase II (MII) oocytes (Cao et al., 2007, 2009; Cree et al., 2008; Monnot et al., 2013; Pikó and Taylor, 1987; Wai et al., 2008). If mtDNA is present in a heteroplasmic form in the PGCs, then either of the mtDNA variants can be selected for or against (St John, 2014), leading to intercellular variability. In the absence of a therapeutic remedy, pre-implantation genetic diagnosis (PGD) and germline genome transfer are the only possible approaches to avoid the transmission of heritable mtDNA disorders. However, in both cases, the potential risk of mtDNA carryover to the offspring may result in a heteroplasmic condition, resulting in the unbalanced segregation of mtDNA variants.
RESULTS

TE Cells Are Informative of the Level of mtDNA Heteroplasmy in ESCs

The survival rate of the embryos, following two biopsies, was 100% (n = 14); PB2 biopsy was done at the zygote stage and TE biopsy on same embryo at the blastocyst stage. Heteroplasmic ESC lines were derived from eight embryos (~57%), five of which were used for further analysis. First, we investigated the association between the level of mtDNA heteroplasmy in the TE cells and that in the corresponding PB2, and we found no evidence of association between them (R = 0.14; p = 0.64) (Table S2). As TE cells have been shown to represent the inner cell mass (ICM) (Treff et al., 2012) and whole blastocysts (Heindryckx et al., 2014; Neupane et al., 2014b), the poor correlation between the PB2 and their corresponding TE cells confirmed that polar bodies were not reliable samples for biopsy during PGD to avoid the transmission of pathogenic mitochondrial diseases. In terms of coefficient of determination ($R^2$), 4.7% of the variability in ESCs could be attributed to a difference in PB2, whereas 19.1% was due to the difference in TE cells, indicating that the level of mtDNA heteroplasmy in TE cells was more closely associated with that in ESCs than in PB2 (Tables S1 and S2).

mtDNA Heteroplasmy Increases with Progressive Passaging

Next, we analyzed the level of mtDNA heteroplasmy in undifferentiated ESC colonies at different passages (n = 137) (Figure 2; Table S1) and also in differentiated colonies (p30; n = 42) (Table S1). Heterogeneity in the level of mtDNA heteroplasmy was compared between individual colonies (inter-colony heterogeneity) within the same line in five cell lines with variable heteroplasmic loads. For measuring inter-colony heterogeneity, five to ten individual colonies were randomly selected from each line. Inter-colony heterogeneity in the proportion of NZB mtDNA haplotype in pluripotent ESC colonies ranged from 12.2% to 21.5% in line 1, 14.0% to 49.8% in line 2, 14.7% to 54.5% in line 3, 19.3% to 61% in line 4, and 13.1% to 39.9% in line 5 (Table S1). Interestingly, the proportion of mean NZB mtDNAs increased from 65.8% to 88.7% in line 1, 45.8% to 54.9% in line 2, 47.0% to 64.1% in line 3, 79.2% to 86.9% in line 4, and 90.1% to 92.4% in line 5 (Figure 2; Table S1). The statistical analysis revealed a strong evidence of an increase in the NZB mtDNA haplotype with each passage (mean increase per passage 0.39%, p = 0.002) (Figure 2; Table S1).

In order to investigate if the ESCs exhibit segregation bias after differentiation, we analyzed the level of mtDNA heteroplasmy in differentiated colonies (RA-mediated differentiation on p30) from each ESC line. In differentiated cells, inter-colony heterogeneity varied by 7.6% in line 1, 45.3% in line 2, 27.4% in line 3, 1.5% in line 4, and 45.2% in line 5 (Table S1). However, no evidence of the difference in the level of heteroplasmy between differentiated and undifferentiated cells (mean difference −0.72, p = 0.74) in the ESC colonies was observed.

Single-Cell Heterogeneity Displayed a Homoplasmic State for mtDNA Haplotypes

Given that each ESC colony comprises hundreds of single cells, we aimed to investigate if individual cells within a colony exhibit difference in the proportion of mtDNA haplotypes. For this, heterogeneity at the single-cell level in pluripotent (n = 79) and differentiated (n = 107) ESCs was studied. We analyzed 15–22 single
cells randomly selected from one colony from each cell line, before and after differentiation, originating from the same colony. The proportion of NZB mtDNA haplotype in pluripotent single cells varied from 59.9% to 100% in line 1, 30.8% to 79.9% in line 2, 16.7% to 96% in line 3, 69.4% to 100% in line 4, and 59.8% to 100% in line 5 (Table S1). In pluripotent single cells, the level of heteroplasmy varied by 36% on average (Figure 3; Table S1). A schematic diagram showing the heteroplasmic load measurement is shown in Figure S2.

Figure 2. Variation in the Level of mtDNA Haplotypes as a Function of Passage, Shown in Five Different Cell Lines
Level of mtDNA heteroplasmy (% of NZB mtDNA haplotype) was measured in five to ten pluripotent ESC colonies from cell lines 1 (black), 2 (red), 3 (green), 4 (blue), and 5 (cyan) on passages 8, 12, 30, and 40. The mean NZB haplotype proportion increased from 65.8% to 88.7% in line 1, 45.8% to 54.9% in line 2, 47.0% to 64.1% in line 3, 79.2% to 86.9% in line 4, and 90.1% to 92.4% in line 5. The proportion of NZB mtDNA haplotype in the pluripotent ESCs increased by 0.39% per passage during culture (p = 0.002). Data are presented in Table S1. A schematic diagram showing the heteroplasmic load measurement is shown in Figure S2.

We further investigated whether the difference in the proportion of NZB and BALB mtDNA haplotypes observed at the single-cell level in the pluripotent state varied after differentiation. For this purpose, we analyzed the proportion of mtDNA haplotypes present in single ESCs after spontaneous differentiation by forming embryoid bodies (EBs). Much wider variation in the proportion of mtDNA haplotypes was observed in differentiated single cells, demonstrating homoplasmic haplotypes for both NZB and BALB mtDNA (Figure 3; Table S1). The statistical analysis showed a significant difference in the level of mtDNA heteroplasmy between pluripotent and differentiated single cells (mean difference = −8.5%; p = 0.0014). The proportion of NZB mtDNA haplotype in differentiated single cells varied from 0% to 100% in line 1, 18.4% to 65.8% in line 2, 20.3% to 61.5% in line 3, 7% to 100% in line 4, and 0% to 100% in line 5 (Figure 3; Table S1). When these single cells were grouped into different categories as <30%, <30–70%, and >70%, more than half (53%) of the analyzed single cells showed segregation toward NZB haplotype (Table S1). Slightly more than one-third (34%) of the cells showed bidirectional segregation, and nearly one-eighth (13%) of the cells showed affinity toward the BALB mtDNA haplotype. However, no statistical difference was observed in mtDNA heteroplasmy levels between single cells and their corresponding colonies obtained from the same passage (p = 0.21) (Table S1).

DISCUSSION
In this study, first we compared the level of mtDNA heteroplasmy in PB2, TE cells, and their corresponding ESCs in heteroplasmic mouse models. We then analyzed cellular heterogeneity at the stem cell level, both in pluripotent and differentiated states. Paull et al. (2013) have investigated the mtDNA heterogeneity in pluripotent and differentiated stem cells to detect the re-emergence of heteroplasmic mtDNA after germline nuclear transfer, if any, in humans. While the transmitted mtDNA was detected at very low levels (<1%) initially, no detectable mtDNA heteroplasmy was observed in the isolated stem cell lines, even after extensive passaging or differentiation (Paull et al., 2013). They investigated heterogeneity in stem cell colonies derived from single cells and
also in each of the germ layers after differentiation, unlike our study, where we assessed the mtDNA heteroplasmy in single cells from pluripotent stem cell colonies and EBs in a heteroplasmic mouse model.

In line with the current study where we observed a poor correlation between PB2 and TE cells, previous studies have also reported a poor correlation between PBs and oocytes or blastomeres (Gigarel et al., 2011; Vandewoestyne et al., 2012) and a strong correlation between TE cells and inner cell mass (Treff et al., 2012) or blastocysts (Heindryckx et al., 2014). The poor correlation between PB2 and TE cells could be due to at least two factors: (1) random genetic drift may lead to unbalanced distribution of mtDNA haplotypes during successive cell divisions in the embryos (Jenuth et al., 1996), and (2) biased inheritance of mtDNA haplotypes during asymmetric cell division in the pre-implantation embryos may cause variation in the proportion of mtDNA haplotypes in the daughter cells (Dalton and Carroll, 2013).

Unlike previous studies that have shown unidirectional loss of mtDNA heteroplasmy in the pluripotent stem cell colonies and EBs in a heteroplasmic mouse model, observed in the current study, to either the BALB homoplasmic state or NZB homoplasmic state at the single-cell level could be due to asymmetric mtDNA transmission during subsequent cell divisions. In line with our study, Hämäläinen et al. (2013) demonstrated bidirectional segregation of mtDNAs in induced PSCs toward WT or mutant homoplasy, indicating no clear selection for or against mutant m.3243A > G mtDNA upon reprogramming (Hämäläinen et al., 2013).

Tissue-specific variability with bimodal segregation of polymorphic mtDNA variants was reported in the earlier studies in heteroplasmic mouse models (Jenuth et al., 1997; Sharpley et al., 2012). However, directional loss of mtDNA variants was seen in the mitotic tissues in these mice, resulting in tissue-specific variability. A recent study in heteroplasmic mouse models demonstrated mtDNA segregation bias in post-mitotic tissues within the same subspecies and showed a prenatal segregation pattern (Burgstaller et al., 2014). They showed that mtDNA segregation bias could occur in polymorphic mouse models with an admixture of mtDNA haplotypes. After analyzing intermediate to high heteroplasmic ESC lines in the present study, our results demonstrated the proliferation of the NZB mtDNA haplotype during progressive passage of pluripotent ESCs, irrespective of the original mtDNA proportion. The mechanism by which heteroplasmic mtDNA segregation bias occurs is unknown. It might be possible that one mtDNA haplotype is selected against another (Burgstaller et al., 2014); however, there is no proof to support this phenomenon, and it could vary in different models.

A recent study by our group demonstrated the divergent mutant load distribution in peripheral blood mononuclear cells (PBMCs), ranging from homoplasmic WT (0%) to nearly homoplasmic mutant (~95%) mtDNA variants in a patient with an m.3243A > G (MELAS) point mutation (Vandewoestyne et al., 2011). Implying such tremendous variability at the single-cell level in ESCs and PBMCs, we speculate that embryos with variable mutation loads could be screened during PGD in individuals with heteroplasmic mtDNA mutations. This could provide an opportunity for fertility clinics to search for mutation-free embryos to prevent the transmission of pathogenic mtDNA mutations, as was reported in our previous study (Heindryckx et al., 2014). From an ethical perspective, if an mtDNA mutation could be managed by a PGD approach by selecting and transferring mutation-free embryos (or those with undetectable mutant loads), it would avoid the use of donor oocytes/embryos, thereby avoiding the involvement of a third parent, which is required for germline nuclear transfer techniques (Craven et al., 2010; Neupane et al., 2014; Tachibana et al., 2013).

The heterogeneous distribution of mtDNA heteroplasmy in single cells is in concordance with the previous studies showing variability in mtDNA heteroplasmy in organs of adult mice toward either of the mtDNA genotypes, indicating that the segregation of polymorphic mtDNAs may not be neutral (Burgstaller et al., 2014; Jenuth et al., 1997; Sharpley et al., 2012). However, a striking difference may exist between germline and somatic cell mtDNA heteroplasmy segregation as reported by Sharpley and colleagues (2012). Furthermore, passage-associated increase in NZB mtDNA haplotype during in vitro cell culture was irrespective of the original mtDNA proportion, showing the unbalanced proliferation of a heteroplasmic mtDNA genotype. Proliferation of mtDNA genotypes has been associated with the difference in mtDNA genotypes, with proportional increment corresponding to distant genetic haplotypes (Burgstaller et al., 2014). Furthermore, proliferation of one mtDNA genotype could be linked with the selective advantage over another in some tissues, resulting in tissue-specific segregation (Takeda et al., 2000). However, such a selection might be influenced by the in vivo or in vitro state of the cells. Battersby and Shoubridge (2001) reported the selective advantage of NZB mtDNAs over BALB mtDNAs in mouse liver cells in vivo. However, isolation and in vitro culture of hepatocytes from the livers of same heteroplasmic mice reversed the segregation direction, enriching BALB mtDNAs (Battersby and Shoubridge, 2001), which indicates that growth medium might influence the segregation bias. An increase in level of NZB mtDNA in the current study might be due to the influence of in vitro culture, which needs further investigation in vivo. Nevertheless, the mechanism and direction of segregation might be due not to a difference in replication rate between two genotypes but to the maintenance of mitochondrial genome or mitochondrial turnover (Battersby and Shoubridge, 2001; Wallace and Chalkia, 2013).

The present study points out the risk of mtDNA segregation bias at the stem-cell level, which may occur if the embryo selected for PGD is not mutation-free or if the mtDNA carryover after meiotic nuclear transfer is not undetectable. However, results from animal models should be cautiously extrapolated to humans, since the species-specific difference in mtDNA segregation between mouse models and humans cannot be ignored. Moreover, polymorphic mtDNA may segregate differently from mutant mtDNA. Therefore, to avoid the concern of limited measurements and animal models, more studies should be conducted with bigger sample sizes to confirm these results in human.

We report cellular heterogeneity in the ESCs in a mouse model with different mtDNA haplotypes, along with the relation between ESCs and their founder embryos (PB2 and TE). Selective proliferation of one mtDNA haplotype over another may result in
the potential amplification of mutant mtDNA with aging, as discussed recently (Ye et al., 2014). Furthermore, wider heterogeneity observed in differentiated single cells corresponds with the tissue-specific variability in the levels of heteroplasmies in individuals with mtDNA mutations. However, further studies are required to explore the germlayer specific segregation of mtDNA.

**EXPERIMENTAL PROCEDURES**

**Embryo Collection and Micromanipulation**

In-vitro-fertilized zygotes were collected from superovulated heteroplasmic BALB/cOlNaDt female mice, which contained the mtDNA mixture of BALB/cByJ and NZB/OlaHsd strains. PB2 were biopsied from zygotes, and the remaining zygote was cultured in potassium simplex oxidized medium (KSOM) until the morula stage and subsequently in Cook Blastocyst medium until the blastocyst stage. TE cells were biopsied from hatching blastocysts, and the remaining blastocyst was used for ESC derivation (Figure 1; Figure S1).

**ESC Derivation and Differentiation**

Mouse ESCs were derived as described elsewhere (Ghimire et al., 2015). For inducing differentiation, ESCs were cultured in N2B27 medium containing 1 mM retinoic acid (RA) for 7 days (without 2i and leukemia inhibitory factor [LIF]). For spontaneous differentiation, cells were plated in low-attachment 24-well plates in N2B27 medium (without 2i and LIF) for 15 days to form EBs.

**Polymerase Chain Reaction Restriction Fragment Length Polymorphism**

Isolated TE cells and ESCs were transferred to separately labeled micro-centrifuge tubes (Westburg) containing 10 ¼ l PicoPure DNA extraction buffer with proteinase K (PicoPure DNA extraction kit, Arcturus). All samples were incubated at 65°C for 3 hr, centrifuged briefly, and heated at 95°C for 10 min to inactivate proteinase K. PCR restriction fragment length polymorphism (RFLP) was used to measure the level of mtDNA heteroplasmies in the different stages of embryos and ESCs (Figures 1 and S2). Nested PCR was performed to amplify mtDNAs from single ESCs. First PCR amplification was performed using the forward primer NPMT9 (5'-ATATCAATTGTACCGAGACTTAC-3'), annealing the mitochondrial genome from nucleotides 3,493 to 3,515, and the reverse primer NPMT10 (5'-CTGTCACCTGAGGCTCCTAC-3'), annealing the mitochondrial genome from nucleotides 4,157 to 4,135. Second PCR amplification was performed using the 6-fluorescin amidite (6-FAM)-labeled forward primer M9 (5'-AGAGCTATCCATCCGCTTCCTA-3'), annealing the mitochondrial genome from nucleotides 3,571 to 3,591, and the reverse primer M10 (5’-CTGTTTACGAGGCTGCGGTT-3') (labeled with 1-naphthyl) ethylenediamine dithyrocchloride [NED]), annealing the mitochondrial genome from nucleotides 4,059 to 4,079 (Tables S4 and S6).

After restriction digestion, heteroplasmic amplicons were cleaved into a 339-bp and a 121-bp fragment, multiplied by 100. The areas of the 6-FAM labeled 339-bp fragment and the sum of the areas of the 6-FAM 339-bp fragment and the 6-FAM 121-bp fragment, multiplied by 100 (Figure S2). Heteroplasmic load below 2% was considered undetectable. Relation between input and output heteroplasmy has been shown in Figure S3 (Table S3).

**Statistical Analysis**

The analysis of the embryonic stem cells was based on linear mixed models to account for (random) line effects. The multivariate model included linear effects of differences between differentiated and undifferentiated cells. It moreover allowed for the variability in the level of heteroplasmy to change over passages. Marginal residual plots confirmed the adequacy of the final models. Statistical analyses were performed in RStudio version 0.97.320 and Microsoft Excel. p values below 0.05 were considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, three figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.019.

**AUTHOR CONTRIBUTIONS**

J.N. conceived, designed, and performed experiments, analyzed the data, and wrote the manuscript. S.G. derived and differentiated ESCs, performed immunostaining and qRT-PCR, and wrote the manuscript. Y.L. helped in animal handling and data collection. T.D. and M.V. supervised the experiment and reviewed the manuscript. T.D. conceived and helped in optimizing single-cell PCR experiments. S.V. interpreted the data, derived a statistical model, and wrote the manuscript. J.G. and R.V.C. reviewed the manuscript and provided critical suggestions. B.H., D.D., and P.D.S conceived and supervised the experiment and reviewed the manuscript.

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