Review, Revised version

Mutations in mitochondrial DNA regulate mitochondrial diseases and metastasis but do not regulate aging

Jun-Ichi Hayashi[1]*, Osamu Hashizume[1], Kaori Ishikawa[1,2], and Akinori Shimizu[2]

[1]Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

[2]Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

*Corresponding author: Jun-Ichi Hayashi (jih45@biol.tsukuba.ac.jp)

ABSTRACT

The mitochondria theory of aging proposes that accumulation of mitochondrial DNA (mtDNA) with pathogenic mutations, and the resultant respiration defects, are responsible not only for mitochondrial diseases but also for aging and age-associated disorders, including tumor development. This theory is partly supported by results obtained from our transmitochondrial mice (mito-mice), which harbor mtDNA with mutations that are orthologous to those found in patients with mitochondrial diseases: mito-mice express disease phenotypes only when they express respiration defects caused by accumulation of mutated mtDNA. With regard to tumor development, specific mtDNA mutations that induce reactive oxygen species (ROS) enhance malignant transformation of lung carcinoma cells to cells with high metastatic potential. However, age-associated respiration defects in elderly human fibroblasts are due not to mtDNA mutations but to epigenetic regulation of nuclear-coded genes, as indicated by the fact that normal respiratory function is restored by reprogramming of elderly fibroblasts.

Introduction

Because mitochondria are highly oxygenic organelles that produce ROS and because many chemical carcinogens have been shown to accumulate in mitochondria, mtDNA in mitochondria experiences stress of both endogenous ROS and exogenous chemical carcinogens. Therefore, the mitochondria theory of aging holds that mtDNA accumulates mutations much faster than does nuclear DNA, and the resultant respiration defects are responsible for the expression of mitochondrial diseases, aging, and age-associated

disorders [1–6]. For example, the three most prevalent mitochondrial diseases (chronic progressive external ophthalmoplegia [CPEO]); myoclonic epilepsy with ragged-red fibers [MERRF]; and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes [MELAS]) are believed to be caused by mtDNA harboring a large-scale deletion (ΔmtDNA), a single point mutation in the $tRNA^{Lys}$ gene, and a single point mutation in the $tRNA^{Leu(UUR)}$ gene, respectively [1–3]. Moreover, in addition to their associations with mitochondrial diseases, these mtDNA mutations are hypothesized to be associated with human aging and age-associated disorders, including oncogenic transformation of normal cells to develop tumors. However, no convincing evidence for this hypothesis has yet been discovered.

Mito-mice as models for mitochondrial diseases and for gene therapy

Mitochondrial diseases that show maternal inheritance are considered to be due to mtDNA with pathogenic mutations that induce respiration defects upon their accumulation [1–3]. However, we cannot exclude the possible involvement of nuclear abnormalities in the pathogenesis of these diseases. To confirm that mtDNA mutations are responsible for these diseases, we have to generate transmitochondrial mice (mito-mice) harbouring mtDNA with large-scale deletions (ΔmtDNA) or point mutations that are orthologous to those found in patients with mitochondrial diseases. These mice are effective for precise investigation of disease pathogenesis and for development of effective gene therapies for these diseases.

In previous studies [7, 8], we generated mito-mice∆, which harbor ∆mtDNA and therefore are disease models for CPEO, by transferring ∆mtDNA from a mouse cell line into the germline of female B6 mice. In addition, we recently generated new mito-mice that harbor mtDNA with a pathogenic G7731A mutation in the $tRNA^{Lys}$ gene (mito-mice-$tRNA^{Lys7731}$), which may be useful as models for MERRF [9]. These mito-mice share the B6 nuclear background and express disease phenotypes only when respiration defects are induced by accumulation of mtDNA with pathogenic mutations [7–9], suggesting that the mtDNA mutations are responsible for respiration defects and the resultant expression of abnormalities (Fig. 1).

Mito-mice∆ have also been used for development of gene therapy in embryos [10] and in adult mice [11]. Because inheritance of mammalian mtDNA is strictly maternal (from mother to children) [12], maternal transmission of mitochondrial diseases to progeny has been effectively prevented by the use of nuclear transplantation into enucleated zygotes of normal mice [10]. In adult mice, bone marrow transplantation from normal mice to mito-mice∆ has been shown to be effective for partial prevention of the expression of disease phenotypes [11].

Subsequently, human embryos are used to perform nuclear transplantation from oocytes of one woman into enucleated oocytes from an unrelated woman for future prevention to transmit mitochondrial diseases [13-15]. However, there are few cases for which this technology is applicable. Because most children born from mothers affected by mitochondrial diseases, such as MERRF or MELAS, do not express disease phenotypes [16, 17], most of the oocytes of affected mothers do not have sufficient amounts of the mutated

mtDNA to induce diseases, probably because oocytes with large amounts of mutated mtDNA are not viable.

We recently reported an alternative therapeutic strategy that does not use oocytes from unrelated women and instead uses oocytes from affected mothers [9]. MERRF model mice (mito-mice-tRNA^{Lys7731}) show stochastic segregation of the heteroplasmic mtDNA (mtDNA with and without the mutation), and the resultant marked variation in the proportions of G7731A mtDNA among pups due to bottleneck effects [18–20]. Considering that abnormalities are not observed in mito-mice-tRNA^{Lys7731} with low proportions of G7731A mtDNA [9], simple selection of oocytes with extremely low proportions of the mutated mtDNA from affected mothers likely would be sufficient to yield unaffected children (Fig. 2).

Regulation of metastasis by mutations in mtDNA

Respiration defects and the resultant upregulation of glycolysis under normoxia enable cell growth under hypoxia and thus are thought to be involved in tumor development [21−24]. Because pathogenic mtDNA mutations also induce upregulation of glycolysis under normoxia by inducing mitochondrial respiration defects, pathogenic mtDNA mutations or somatic mutations in mtDNA have been proposed to induce tumor development [1−3].

If this proposal is correct, patients with mitochondrial diseases can be expected to preferentially develop tumors, because patients express upregulation of glycolysis under normoxia due to the pathogenic mutations in mtDNA. However, no statistical evidence for

association between mitochondrial diseases and tumor development in patients has been reported. Moreover, if we assume that some mtDNA mutations, irrespective of whether they are pathogenic or polymorphic, induce tumor development, all family members sharing a mother carrying such mutations should develop tumors as a result of the maternal inheritance of mtDNA, but no bias toward maternal inheritance of tumor development has been reported. Nonetheless, it is still possible that mtDNA mutations are involved in processes other than tumor development (oncogenic transformation of normal cells into tumor cells); for example, mtDNA mutations may be involved in malignant transformation of tumor cells into highly metastatic tumor cells.

Our previous studies [25–27] have provided convincing evidence that mtDNA with a pathogenic G13997A mutation in the *ND6* gene (G13997A mtDNA) controls the malignant transformation of B6-derived lung carcinoma cells from poorly metastatic into highly metastatic (Fig. 1), whereas this mutation does not control tumor development (transformation of normal cells into tumor cells). Moreover, because the induction of high metastasis is due not to the respiration defects but rather to overproduction of ROS, expression of high metastatic potential can be reversibly controlled by treatment with antioxidants [25–27], indicating that antioxidant administration effectively prevents development of high metastatic potential in tumor cells.

The effects of G13997A mtDNA on aging and tumor development in living mice can be examined by generating mito-mice-ND6^{13997}, which carry the nuclear genome from B6 mice and G13997A mtDNA from B6-derived lung carcinoma cells [28]. Mito-mice-ND6^{13997} do not express premature aging phenotypes and have a normal lifespan but show

lactic acidosis, hyperglycemia, and lymphoma development, indicating the possible involvement of mtDNA mutations in lymphoma development in B6 mice (Fig. 1). However, considering that the B6 nuclear background is prone to lymphoma development, G13997A mtDNA might not develop lymphoma independently. By generating mito-mice with G13997A mtDNA and a nuclear background from the A/J strain mice, which are not prone to lymphoma development, we show that these mito-mice do not develop lymphoma [29]. These results indicate that G13997A mtDNA simply enhances the frequency of lymphoma development, which is due primarily to abnormalities in the B6 nuclear genome.

Epigenetic regulation of human age-associated respiration defects

The mitochondrial theory of aging also holds that ROS overproduction and the resultant accumulation of somatic mutations in mtDNA are responsible for aging and age-associated respiration defects [1–6]. This contention is supported partially by subsequent studies [30, 31], which have generated mtDNA mutator mice expressing a proofreading-deficient mtDNA polymerase: mtDNA mutator mice show accelerated accumulation of somatic mutations in mtDNA due to the proofreading deficiency and the resultant expression of respiration defects and early-onset aging phenotypes. However, it appears controversial that mtDNA mutator mice do not show ROS overproduction [31], and mito-mice-ND6^{13997} overproducing ROS do not show early-onset aging phenotypes [28]. Moreover, one important question that has to be addressed is whether elderly human subjects really

accumulate such amounts of somatic mtDNA mutations as the mtDNA mutator mice do in their tissues.

Our previous studies have suggested the involvement of nuclear mutations in the age-associated respiration defects found in elderly human fibroblasts, as indicated by experiments involving mtDNA transfer from elderly fibroblasts into mtDNA-less HeLa cells [32] and by experiments involving nuclear DNA transfer from mtDNA-less HeLa cells into elderly fibroblasts [33]. Because transfer of the nuclear genome from mtDNA-less HeLa cells (that is, the nuclear genome uncontaminated by mtDNA) into elderly fibroblasts restores the respiration defects, these defects must be caused not by mtDNA mutations but by nuclear-recessive mutations [33]. However, our findings can also be explained by assuming the involvement of epigenetic regulation of nuclear genes in the absence of nuclear-recessive mutations.

The issue of whether age-associated respiration defects observed in elderly human subjects are due to mutations or to epigenetic changes is resolved by reprogramming of human fibroblasts [34]. First, we show that age-associated mitochondrial respiration defects are expressed in the absence of either ROS overproduction in the mitochondria or the accumulation of somatic mutations in mtDNA indicating that these aging phenotypes expressed in human fibroblasts are not caused by mtDNA mutations [34]. Moreover, when elderly fibroblasts are reprogrammed by the creation of induced pluripotent stem cells (iPSCs), reduced respiratory function is restored, suggesting that epigenetic—not genetic—regulation confers these human aging phenotypes [34]. It is therefore likely that these age-associated respiration defects are controlled neither by ROS overproduction nor by

mutations but rather by reversible epigenetic regulation of nuclear genes (Figs. 1 and 3). A recent study [35] proposes that reprogramming also recovers other aging phenotypes of human fibroblasts, such as abnormal mitochondrial metabolism, shortened telomeres, and limited proliferation capacities. Therefore, most age-related phenotypes in human fibroblasts are under epigenetic regulation.

The question then arises as to which nuclear genes cause age-associated mitochondrial respiration defects by means of epigenetic regulation? A microarray analysis has revealed that epigenetic downregulation of nuclear-coded genes, including *GCAT* and *SHMT2*, which regulate glycine production in mitochondria, results in respiration defects [34]. Given that the age-associated respiration defects in elderly fibroblasts are likely due in part to reduced translation activity in the mitochondria [32, 33], defects in glycine metabolism in the mitochondria as a result of a reduction in *SHMT2* and *GCAT* expression would be partly responsible for the reduction in mitochondrial translation, resulting in the expression of age-associated respiration defects. For generalization of the concept that age-related phenotypes in human fibroblasts are under epigenetic regulation, further works are necessary to show whether epigenetic regulation of aging phenotypes are common to other human tissues.

Our major arguments are summarized in Fig. 1.

References

[1] Larsson N-G, Clayton DA (1995) Molecular genetic aspects of human mitochondrial disorders. *Annu Rev Genetics* 29:151-178.

[2] Wallace DC (1999) Mitochondrial diseases in man and mouse. *Science* 283:1482-1488.

[3] Taylor RW, Turnbull, DM (2005) Mitochondrial DNA mutations in human disease. *Nature Rev Genet* 6, 389-402.

[4] Jacobs, HT (2003) The mitochondrial theory of aging: dead or alive? *Aging Cell* 2:11-17.

[5] Khrapko, K, Vija, J (2008) Mitochondrial DNA mutations and aging: devils in the details? *Trends Genet* 25: 91-98.

[6] Bratic A, Larsson NG (2013) The role of mitochondria in aging. *J Clin Invest* 123: 951-957.

* [7] Inoue K, et al. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nat Genet* 26:176-181.

Transmitochondrial mice (mito-mice) harbouring mtDNA with a deletion mutation that is an orthologous mtDNA mutation found in patients with CPEO are generated and provide evidence that respiration defects caused by the pathogenic mtDNA mutation are responsible for disorders.

[8] Nakada K, et al. (2001) Inter-mitochondrial complementation: Mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. *Nat Med* 7:934-940.

[9] Shimizu A, et al. (2014) Transmitochondrial mice as models for primary prevention of diseases caused by mutation in the $tRNA^{Lys}$ gene. *Proc. Natl. Acad. Sci. USA* 111: 3104-3109.

[10] Sato A, et al. (2005) Gene therapy for progeny of mito-mice carrying pathogenic mtDNA by nuclear transplantation. *Proc Natl Acad Sci USA* 102: 16765-16770.

[11] Inoue S, et al. (2006) Suppression of disease phenotypes of adult mito-mice carrying pathogenic mtDNA by bone marrow transplantation *Hum Mol Genet* 15: 1801-1807.

[12] Kaneda H, et al. (1995) Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proc Natl Acad Sci USA* 92: 4542-4546.

* [13] Craven L, et al. (2010) Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA diseases. *Nature* 465: 82-85.

This report succeeds in pronuclear transplantation without contamination of mtDNA from oocytes into enucleated oocytes from unrelated women for future prevention to transmit mitochondrial diseases.

[14] Tachibana M, et al. (2013) Towards germline gene therapy of inherited mitochondrial diseases. *Nature* 493: 627-631.

[15] Paull D, et al. (2013) Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants. *Nature* 493: 632-637.

[16] Hammans SR, et al. (1993) The mitochondrial DNA transfer RNA^{Lys} A → $G^{(8344)}$ mutation and the syndrome of myoclonic epilepsy with ragged red fibres (MERRF). *Brain* 116 (Pt 3): 617-632.

[17] Chinnery P, Howell N, Lightowlers RN, and Turnbull DM (1998) MELAS and MEFFR: The relationship between maternal mutation load and the frequency of clinically affected offspring. *Brain* 121 (Pt 10): 1889-1894.

[18] Cao L, et al. (2007) The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. *Nat Genet* 39: 386-390.

[19] Cree LM, et al. (2008) A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. *Nat Genet* 40: 249-254.

[20] Wai T, Teoli D and Shoubridge EA (2008) The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. *Nat Genet* 40: 1484-1488.

[21] Baysal BE, et al. (2000) Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* 287: 848-851

[22] Niemann S, Muller U (2000) Mutations in SDHC cause autosomal dominant paraganglioma, type3. *Nat Genet* 26: 268-270

[23] Gottlieb E, Tomlinson IP (2005) Mitochondrial tumor suppressors: a genetic and biochemical update. *Nat Rev Cancer* 5: 857-866

[24] Koppenol WH, Bounds PL, Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* 11: 325-337

* [25] Ishikawa K, et al. (2008) ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science* 320: 661-664

By complete exchange of mtDNA between poorly and highly metastatic mouse lung carcinoma cell lines, this study provides direct evidence for the involvement of specific mtDNA mutations in malignant transformation of tumor cells from poorly to highly metastatic but not in transformation of normal cells to be tumor cells.

[26] Ishikawa K, et al. (2008) Enhanced glycolysis induced by mtDNA mutations does not regulate metastasis. *FEBS Lett* 582: 3525-3530

[27] Koshikawa N, et al. (2009) Reactive oxygen species-generating mitochondrial DNA mutation up-regulates hypoxia-inducible factor-1α gene transcription via phosphatidylinositol 3-kinase-Akt/protein kinase C/histone deacetylase pathway. *J Biol Chem* 284: 33185-33194.

[28] Hashizume O, et al. (2012) Specific mitochondrial DNA mutation in mice regulates diabetes and lymphoma development. *Proc Natl Acad Sci USA* 109: 10528–10533

[29] Hashizume O, et al. (2015) A specific nuclear DNA background is required for high frequency lymphoma development in transmitochondrial mice with G13997A mtDNA. *PLOS ONE* 10: e0118561.

[30] Trifunovic A, et al. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 429: 417-423.

[31] Kujoth GC, et al. (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* 309: 481-484.

[32] Hayashi J-I, et al. (1994) Nuclear but not mitochondrial genome involvement in human age-related mitochondrial dysfunction. *J Biol Chem* 269: 6878-6883.

[33] Isobe K, et al. (1998) Nuclear-recessive mutations of factors involved in mitochondrial translation are responsible for age-related respiration deficiency of human skin fibroblasts. *J Biol Chem* 273: 4601-4606.

* [34] Hashizume O, et al. (2015) Epigenetic regulation of the nuclear-coded GCAT and SHMT2 genes confers human age-associated mitochondrial respiration defects. *Sci Rep* 5: 10434.

Deep sequence analysis of mtDNA and reprogramming of human elderly fibroblasts show that age-associated respiration defects in elderly human fibroblasts are due not to mtDNA mutations but to epigenetic regulation of nuclear-coded genes.

[35] Lapasset L, et al. (2011) Rejuvenating senescent and centenarian human cells by reprogramming through the pluripotent state. *Genes Dev* 25: 2248-2253.

Fig. 1. Mutations in mtDNA regulate mitochondrial diseases and tumor phenotypes but do not regulate age-associated respiration defects

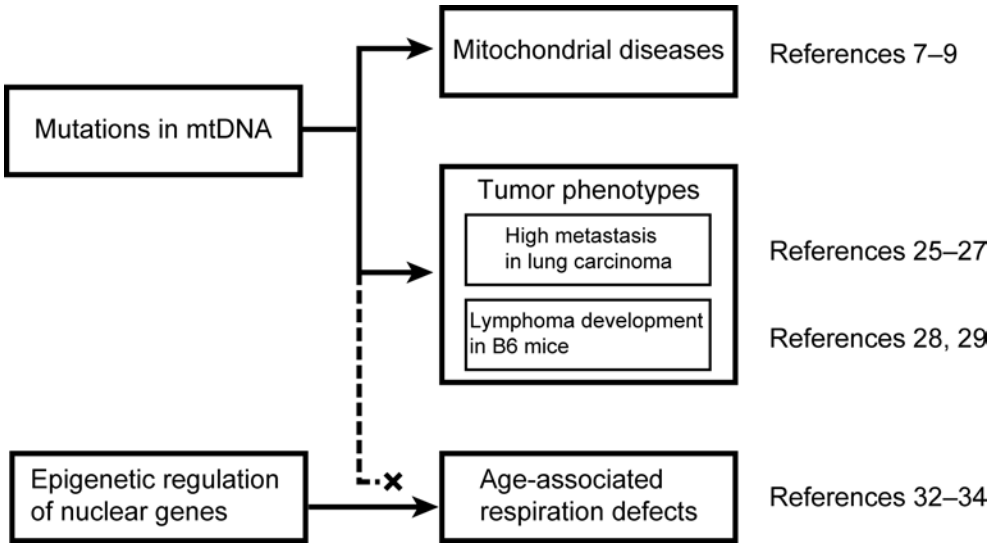

Fig. 2. Two strategies, nuclear transplantation and oocyte selection, for primary prevention of maternal inheritance of mitochondrial diseases

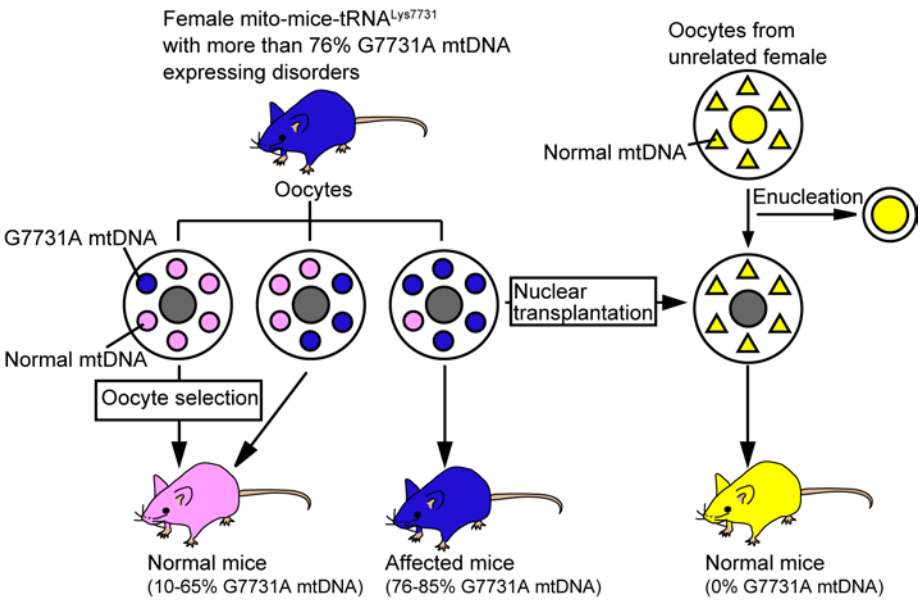

Fig. 3. Reversible regulation of age-associated respiration defects observed in fibroblasts from elderly human subjects