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A somatic T15091C mutation in the *Cytb* gene of mouse mitochondrial DNA dominantly induces respiration defects

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ABSTRACT

Our previous studies provided evidence that mammalian mitochondrial DNA (mtDNA) mutations that cause mitochondrial respiration defects behave in a recessive manner, because the induction of respiration defects could be prevented with the help of a small proportion (10%–20%) of mtDNA without the mutations. However, subsequent studies found the induction of respiration defects by the accelerated accumulation of a small proportion of mtDNA with various somatic mutations, indicating the presence of mtDNA mutations that behave in a dominant manner. Here, to provide the evidence for the presence of dominant mutations in mtDNA, we used mouse lung carcinoma P29 cells and examined whether some mtDNA molecules possess somatic mutations that dominantly induce respiration defects. Cloning and sequence analysis of 40–48 mtDNA molecules from P29 cells was carried out to screen for somatic mutations in protein-coding genes, because mutations in these genes could dominantly regulate respiration defects by formation of abnormal polypeptides. We found 108 missense mutations existing in one or more of 40–48 mtDNA molecules. Of these missense mutations, a T15091C mutation in the *Cytb* gene was expected to be pathogenic due to the presence of its orthologous mutation in mtDNA from a patient with cardiomyopathy. After isolation of many subclones from parental P29 cells, we obtained subclones with various proportions of T15091C mtDNA, and showed that the respiration defects were induced in a subclone with only 49% T15091C mtDNA. Because the induction of respiration defects could not be prevented with the help of the remaining 51% mtDNA without the T15091C mutation, the results indicate that the T15091C mutation in mtDNA dominantly induced the respiration defects.

1. Introduction

The mitochondrial theory of aging proposes that age-associated accumulation of somatic mutations in mitochondrial DNA (mtDNA) and the resultant mitochondrial respiration defects are involved in mammalian aging [1–7]. However, there is as yet no convincing evidence of whether accumulation of somatic mutations in mtDNA with aging is responsible for the age-associated respiration defects and the resultant aging phenotypes. Moreover, our previous studies revealed that mammalian mitochondria exchange genetic products as a consequence of intermitochondrial interaction [8–10]. Such exchanges could prevent induction of mitochondrial respiration defects, even when somatic mutations have accumulated in mtDNA with aging.

Recently, the mitochondria theory of aging is supported, in part, by the finding that homozygous mtDNA mutator mice with a proofreading-deficient mtDNA polymerase show accelerated accumulation of somatic mutations in mtDNA, resulting in the expression of mitochondrial respiration defects and premature aging phenotypes [11,12]. More recently, Mito et al. [13] revealed that mtDNA abnormalities in homozygous mtDNA mutator mice are responsible for respiration defects by demonstrating the co-transfer of mtDNA and the respiration defects from the mtDNA

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mutator mice into mtDNA-less (ρ^0) mouse cells. A question that then arises is how mtDNA mutator mice express respiration defects in the presence of intermitochondrial interaction.

This controversial issue can be resolved by assuming either the clonal expansion of mtDNA with pathogenic mutations that induce respiration defects on their predominant accumulation [14], or accumulation of small proportions of mtDNA with various kinds of dominant mutations [15]. In the latter case, point mutations in protein-coding genes are likely to function in the dominant manner by producing abnormal polypeptides, resulting in the failure of proper assembly of mitochondrial respiratory complexes. Probably, the creation of various kinds of dominant mtDNA mutations could reduce mitochondrial respiratory function by their additive or synergic effect, even when each dominant mutation has not accumulated predominantly.

In our current study, we focused on somatic missense mutations that are present on small proportions of mtDNA molecules in mouse lung carcinoma P29 cells to determine whether some of the mutations dominantly induce respiration defects before their predominant accumulation. To screen for missense mutations that are likely pathogenic, we carried out cloning and sequence analysis of mtDNA from P29 cells, and showed that a missense T15091C mutation in the *Cytb* gene could dominantly regulate respiration defects.

2. Materials and methods

2.1. Mouse cell lines and cell culture

Lewis lung carcinoma P29 cells [16,17], their subclones, mtDNA-less (ρ^0) B82 cells [17], and transmitochondrial cybrids (B82mtP29 and B82mt15019) were grown in DMEM (Sigma–Aldrich, St. Louis, Missouri, USA) containing 10% fetal calf serum (FCS), uridine (50 ng/ml), and pyruvate (100 ng/ml).

2.2. Cloning and sequencing of mtDNA

Total DNAs extracted from cells were used as templates for PCR reactions. The PCR reactions amplified seven mtDNA fragments (F1–F7) using primer pairs, which were designed to generate 2.5–3.0 kbp overlapping fragments. The sequences of primers were based on the standard mtDNA sequences of B6 strain mice (GenBank accession no. AY172335) as follows: F1 forward primer: n.p. 4058–4077; F1 reverse primer: n.p. 6568–6549; F2 forward primer: n.p. 5931–5950; F2 reverse primer: n.p. 8626–8606; F3 forward primer: n.p. 8050–8069; F3 reverse primer: n.p. 11,008–10,989; F4 forward primer: n.p. 10,370–10,390; F4 reverse primer: n.p.12,972–12,953; F5 forward primer: n.p. 12,394–12,413; F5 reverse primer: n.p. 15,087–15,068; F6 forward primer: n.p. 14,426 to 14,445; F6 reverse primer: n.p. 654–635; F7 forward primer: n.p. 72–91; F7 reverse primer: n.p. 2735–2716. All PCR amplifications were performed in 50 μl of solution consisting of 1 × PCR buffer, 0.2 mM dNTPs, 0.6 mM primers, 1U Ampli Taq Gold DNA polymerase (Perkin–Elmer Applied Biosystems, Lincoln, California, USA), and 1 μg of cellular DNA as template. Reaction conditions were 95 °C for 10 min with cycle times of 60 s for denaturation at 95 °C, 60 s for annealing at 50–56 °C, and 150 s for extension at 72 °C for 35 cycles. The PCR products were ligated with pUC118 (Takara Bio, Shiga, Japan), and then introduced into DH5α (Takara Bio). Sequence templates were prepared with a TempliPhi DNA Sequencing Template Amplification Kit (Amersham Pharmacia Biosciences, Little Chalfont, Buckinghamshire, England) following the manufacturer's protocol. Sequence reactions were performed by Dye Termination Methods (Takara PCR Thermal Cycler GP). Samples were then sequenced on MegaBACE1000 (Amersham Pharmacia Biosciences).

2.3. Genotyping of mtDNA

To detect the T15091C mutation, a 324-bp fragment containing the nucleotide position 15091 was PCR-amplified by using the nucleotide sequences 15047–15066 and 15351–15370 as primers. The PCR amplicon derived from mtDNA without the T15091C mutation contained an SspI restriction site and generated 279- and 45-bp fragments upon SspI digestion; in contrast, the amplicon from T15091C mtDNA was not cleaved. To detect the G9976A mutation, a 140-bp fragment containing nucleotide position 9976 was PCR-amplified by using the nucleotide sequences 9942–9962 and 10061 to 10081 as primers. The PCR amplicon derived from mtDNA without the G9976A mutation contained a BstNI restriction site and generated 108- and 32-bp fragments upon BstNI digestion, whereas the amplicon derived from G9976A mtDNA was not cleaved. Restriction fragments were separated by electrophoresis in a 3% agarose gel. For quantification of T15091C mtDNA, we used ImageJ (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014) software.

2.4. Estimation of oxygen consumption rates

Cells were washed once in phosphate buffered saline (PBS) and then resuspended in PBS at a density of 8×10^7 cells/ml. The cell suspension (2 ml) was transferred to a polarographic cell, and basal respiration (i.e., oxygen [O_2] consumption) was measured immediately under constant stirring by using an oxygraph equipped with a Clark-type electrode (YSI model 5300; Yellow Springs Instruments Inc., Yellow Spring, Ohio, USA) at 37 °C.

2.5. Blue native polyacrylamide gel electrophoresis

Blue native polyacrylamide gel electrophoresis (BN–PAGE) and mitochondrial complex I in-gel activity assays were performed with digitonin-isolated mitochondria from each P29 subclones based on the procedures described previously [18].

2.6. Isolation of transmitochondrial cybrids

We used ρ^0 B82 cells as recipients for mtDNA with or without the T15091C mutation. ρ^0 B82 cells are resistant to bromodeoxyuridine (BrdU) but sensitive to hypoxanthine-aminopterin-thymidine (HAT) owing to their deficiency of thymidine kinase activity [17]. Furthermore, ρ^0 B82 cells are unable to grow in the absence of uridine and pyruvate owing to their lack of mtDNA. P29 cells or P29-95 cells (cells with >95% T15091C mtDNA) were used as mtDNA donors. They were pretreated with cytochalasin B (10 μg/ml) for 10 min and centrifuged at 7500 × *g* for 10 min at 37 °C for enucleation. The resultant enucleated cells (cytoplasts) were fused with ρ^0 B82 cells by using polyethylene glycol [17]. The fusion mixture was cultured in selection medium containing BrdU (30 μg/ml) and lacking uridine and pyruvate.

2.7. Statistical analysis

Data are presented as mean ± SD and were analysed by using Student's *t* test or ANOVA followed by Tukey's HSD test; *P* values less than 0.05 were considered significant. Excel software was used for all statistical analysis.

3. Results

3.1. Screening of somatic missense mutations in protein-coding regions of mtDNA

To screen for somatic missense mutations that might induce respiration defects on their accumulation, we sequenced the protein-coding regions of mouse mtDNA molecules from Lewis lung carcinoma P29 cells [16,17]. Seven primer sets (F1–F7) were used so that the resultant PCR products covered all protein-coding genes in mtDNA (Fig. S1). Because the proportion of somatic mutations at each nucleotide position of the mtDNA population was expected to be very small, direct sequence analysis of mtDNA could not be used to detect somatic mutations. Therefore, we cloned and sequenced the PCR products corresponding to 40–48 mtDNA molecules. The resultant sequences (submitted to GenBank) were compared with the reference mtDNA sequence of P29 cells (Table S1; GenBank, accession no. EU312160).

We found 173 mutations in the protein-coding regions (Table S1 and Fig. S1), 108 of which were missense mutations. Of the missense mutations, only two mutations, the T10660C mutation in the *ND4* gene and the T15091C mutation in the *Cytb* gene (Fig. S1), corresponded to human orthologous mutations, which have been reported in patients with Leber's hereditary optic neuropathy [19,20] and in a patient with cardiomyopathy [21], respectively. Therefore, we considered that these two mutations are likely pathogenic mutations that induce respiration defects on their accumulation.

The T10660C mutation and the T15091C mutation were present in one of 45 clones and in one of 40 clones, respectively (Table S1), suggesting that they were present in approximately 2–2.5% of the mtDNA population in P29 cells (Fig. S1). Such low proportions of the mutated mtDNAs (T10660C mtDNA and T15091C mtDNA) would be difficult to be detected by conventional restriction fragment length polymorphism (RFLP) analysis of the PCR products, and would likely be insufficient to induce respiration defects, even if they were dominant mutations. We therefore considered that isolation of cells possessing more than a proportion of T10660C or T15091C mtDNA detectable by RFLP analysis (i.e., >5%) would be required to determine whether these mutations are pathogenic and whether their pathogenicity is expressed in a dominant or a recessive way.

3.2. Isolating P29 subclones with high proportions of T15091C mtDNA

Given that two different mtDNA haplotypes present within single cells segregate stochastically during cell division [22,23], individual cells in the P29 cell population eventually either lose or accumulate detectable proportions of the mutated mtDNA by RFLP analysis. Therefore, we isolated 127 subclones from P29 cells and performed RFLP analysis to detect the T10660C and T15091C mtDNA in the subclones. RFLP analysis, based on HhaI (for T10660C mtDNA) and SspI (for T15091C mtDNA) digestions of the PCR products of mtDNA (see Materials and Methods), showed that 5 of 127 subclones possessed 16%–22% T15091C mtDNA, while none of 127 subclones possessed detectable amounts of T10660C mtDNA. Therefore, we focused on T15091C mtDNA in subsequent experiments.

To isolate P29 subclones with higher proportions of T15091C mtDNA, we selected the subclone P29-22 possessing the largest proportion (22%) of T15091C mtDNA (Fig. 1A) in the above RFLP analysis. We cultured this subclone for an additional 2 months expecting that stochastic segregation would produce variation of the T15091C mtDNA level within the P29-22 cell population. We then isolated 34 subclones from P29-22 cells, and obtained a subclone P29-34 possessing 34% T15091C mtDNA (Fig. 1B). After twice repeating the recloning process, we obtained a subclone P29-69 with 69% T15091C mtDNA. Because the RFLP analysis provides only indirect evidence for the presence of the T15091C mutation, we carried out deep sequence analysis of the whole mtDNA from P29-69. The results showed that P29-69 cells indeed possessed the T15091C mutation in mtDNA, and that its proportion was 58% (Table 1), comparable to the proportion estimated by RFLP. The sequence analysis also showed that a small proportion of T15091C mtDNA molecules in P29-69 cells had an additional G9976A missense mutation in the *ND4L* gene (Table 1).

After two more rounds of recloning, we finally obtained a subclone P29-95 with >95% T15091C mtDNA (Fig. 1A and B). A question that then arises is whether some of the T15091C mtDNA molecules in P29-95 cells also harbor the G9976A mutation (Table 1). Therefore, we estimated the proportion of mtDNA with the G9976A mutation (G9976A mtDNA) in P29-05 cells (cells with no detectable T15091C mtDNA) and P29-95 cells (cells with only T15091C mtDNA), respectively, by a BstNI digestion of the PCR products. The results showed that 55% of mtDNA in P29-95 cells possessed the G9976A mutation, whereas P29-05 cells did not contain detectable levels of the G9976A mutation (Fig. 1C); this indicates that ~55% of mtDNA with the T15091C mutation in the *Cytb* gene simultaneously included the G9976A mutation in the *ND4L* gene, which might affect the activity of mitochondrial respiratory complex I.

3.3. Determining pathogenicity of the T15091C and G9976A mutations

First, we examined the pathogenicity of the T15091C mutation by using three P29 subclones, P29-05, P29-49, and P29-95, possessing <5%, 49%, and >95% T15091C mtDNA, respectively. If the T15091C mutation is pathogenic and induces the respiration defects in a dominant way, respiration defects must be expressed in P29-49 cells. In contrast, in the case of recessive mutation, P29-49 would not express the respiration defects due to compensation by the mtDNA without the T15091C mutation via intermitochondrial interactions [8–10]. To determine the pathogenicity of T15091C mtDNA, we estimated O_2 consumption rates, which reflect overall mitochondrial respiration. The results showed that respiration defects were observed in both P29-49 and P29-95 cells (Fig. 2A). These observations suggest that the T15091C mutation is pathogenic and induces the respiration defects dominantly. Then, we examined the pathogenicity of the additional G9976A mutation by estimating the mitochondrial respiratory complex I activity. Neither P29-49 nor P29-95 cells showed reduction of complex I activity, which excludes the involvement of the G9976A mutation in the respiration defects (Fig. 2B).

Next, we performed cytoplasmic transfer of the T15091C mtDNA from P29-95 cells into ρ^0 B82 cells (see Materials and Method) to exclude the possibility that the repeated recloning processes used to isolate P29-95 cells (Fig. 1B) preferentially selected cells with nuclear abnormalities that induce respiration defects. In the case of nuclear abnormalities, respiration defects would not be transferred to ρ^0 B82 cells along with the transfer of the T15091C mtDNA from P29-95 cells. Thus, we performed intercellular transfer of T15091C mtDNA by fusion of enucleated P29-95 cells with ρ^0 B82 cells. Fusion mixtures were cultivated in the selection medium to exclude un-enucleated P29-95 cells and unfused ρ^0 B82 cells, respectively, and to allow exclusive growth of transmitochondrial cybrids (ρ^0 B82 cells with T15091C mtDNA).

One colony grown in the selection medium was isolated clonally and its cells were named B82mt15091 cybrids. Genotyping of mtDNA showed the presence of 88% T15091C mtDNA in the

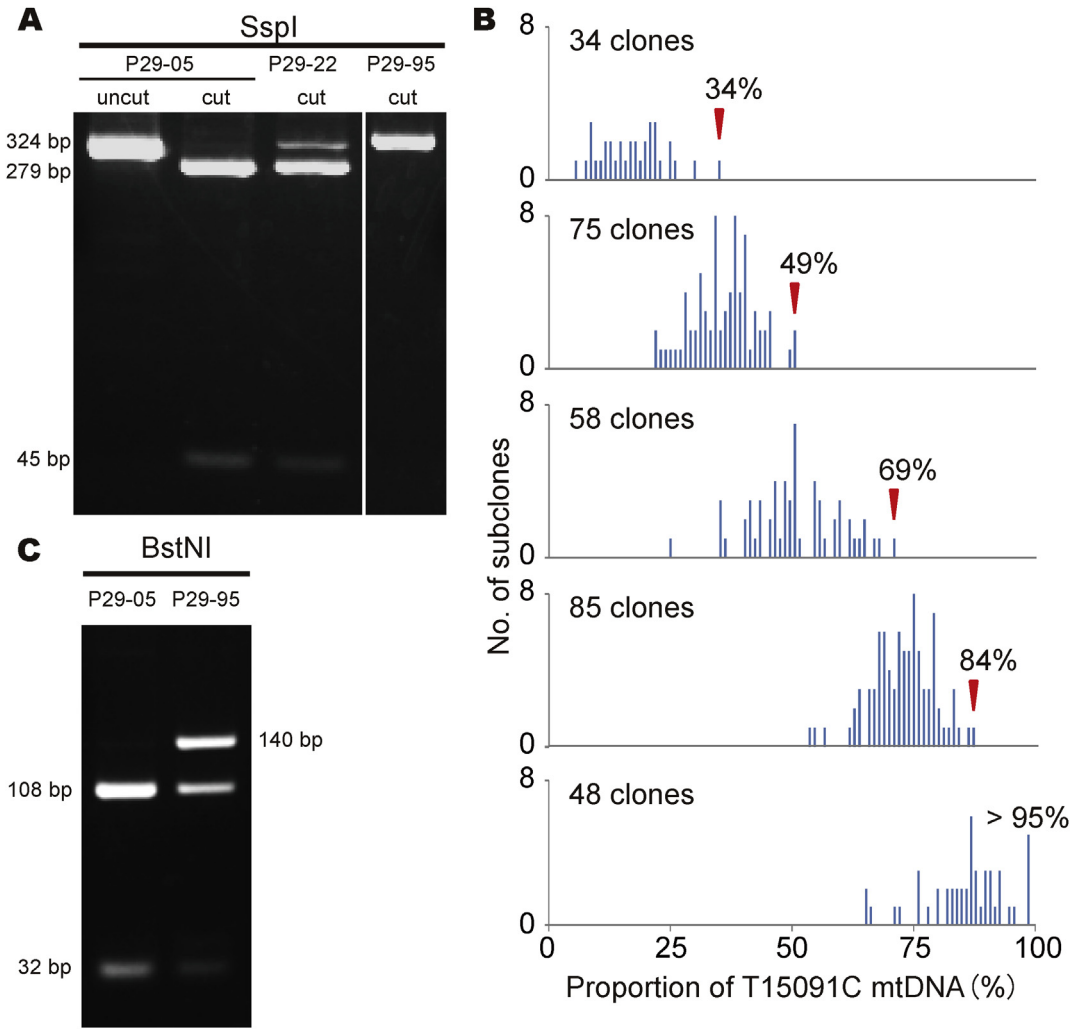

Fig. 1. Isolation of P29 subclones with various proportions of T15091C mtDNA. (A) Identification and quantification of T15091C mtDNA in P29 subclones by an SspI digestion of the PCR products. The T15091C mtDNA produced a 324-bp fragment owing to the loss of an SspI site by mutation, whereas mtDNA without the mutation produced 279-bp and 45-bp fragments owing to the presence of the SspI site. (B) Repeated recloning of P29 subclones to select the subclone with the highest proportion of T15091C mtDNA. A subclone P29-22 with 22% T15091C mtDNA was cultivated for 2 months to allow its stochastic segregation. From this clone, we obtained 34 subclones; the one with the most prevalent T15091C mtDNA possessed 34% T15091C mtDNA. After four more rounds of subcloning, we isolated five P29 subclones with more than 95% T15091C mtDNA, and one of them was used as P29-95. (C) Identification and quantification of T15091C mtDNA with an additional G9976A mutation in the *ND4L* gene in subclone P29-95 by BstNI digestion of the PCR products. The mtDNA without the G9976A mutation produced 108-bp and 32-bp fragments owing to the presence of the BstNI site, whereas mtDNA with the G99976A mutation produced a 140-bp fragment owing to the loss of the BstNI site by mutation. About 55% of T15091C mtDNA possessed the additional G9976A mutation, whereas mtDNA without the T15091C mutation did not possess the G9976A mutation.

Table 1
Deep-sequence analysis of whole mtDNA from a P29 subclone with 69% T15091C mtDNA.[a]

Locus	*ND4L*	*ND6*	*Cytb*	D-loop		
Nucleotide position	9976	13672	15091	15532	16099	16105
P29 reference sequence[b]	G	A	T	T	A	T
Variant sequence	A	T	C	C	C	C
Amino acid alteration	E34K	silent	M316T	–	–	–
A P29 subclone with 69% T15091C mtDNA	18.60%	4.40%	58.30%	95.18%	31.97%	40.73%

[a] P29-69 mtDNA sequence, GenBank accession number AP014886.
[b] P29 mtDNA sequence, GenBank accession number EU312160.

B82mt15091 cybrids (Fig. 3A), indicating the success of cytoplasmic transfer of T15091C mtDNA into ρ^0 B82 cells. As control trans-mitochondrial cybrids without T15091C mtDNA, we isolated B82mtP29 cybrids that carried the nuclear genome from B82 cells and mtDNA without T15091C mutation from P29 cells by the fusion of ρ^0 B82 cells with enucleated P29 cells (Fig. 3A).

We found that B82mt15091 cybrids showed decreased O_2 consumption rates compared to those of B82mtP29 cybrids (Fig. 3B).

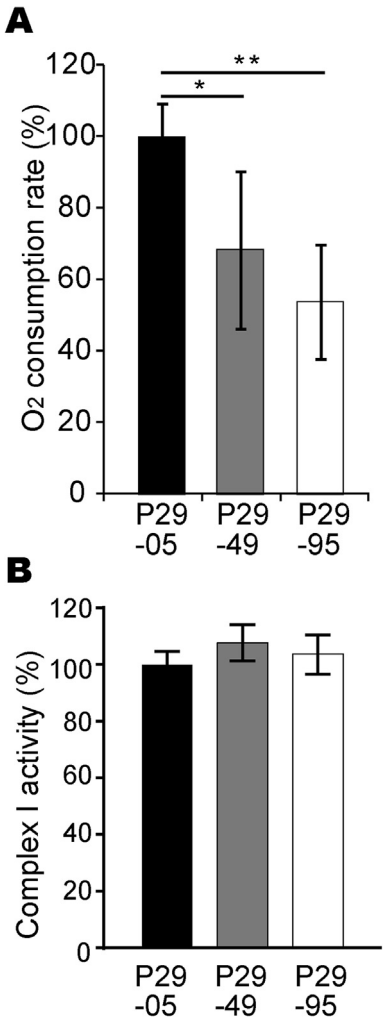

Fig. 2. Estimation of respiratory function to determine the pathogenicities of T15091C and G9976A mutations in mtDNA. (A) Comparison of mitochondrial respiratory function among subclones P29-05, P29-49, and P29-95 by estimation of O_2 consumption rates. The rates were calculated as a percentage of the average level in P29-05 cells. Experiments were performed in triplicate; error bars indicate ±SD. $^{*}P < 0.05$ and $^{**}P < 0.01$; ANOVA followed by Tukey's HSD test. (B) Comparison of mitochondrial respiratory function between subclones P29-05 and P29-95 by estimation of mitochondrial complex I activity using blue native gel electrophoresis. The complex 1 activity was calculated as a percentage of the average level in P29-05 cells. Experiments were performed in triplicate; error bars indicate ± SD.

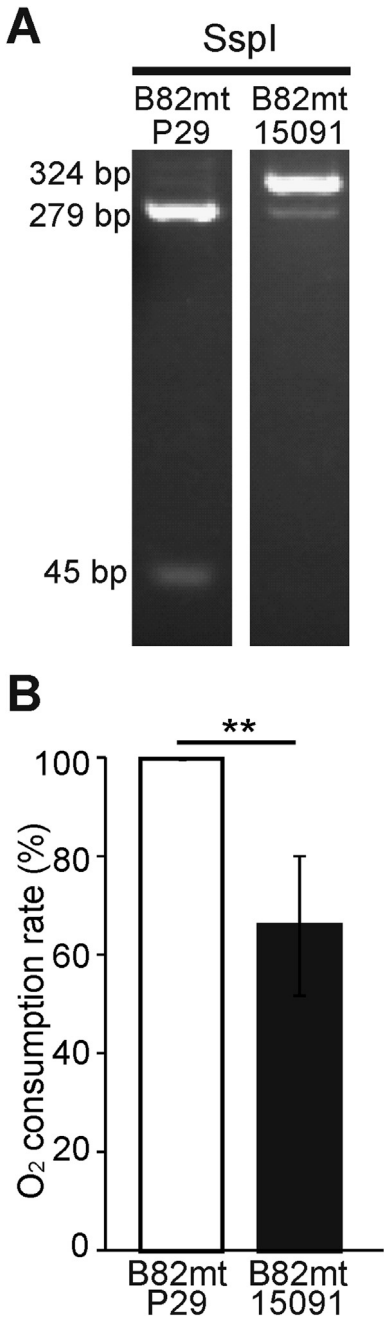

Fig. 3. Characterization of B82mt15091 cybrids to confirm the pathogenicity of T15091C mtDNA. (A) Quantitative estimation of the proportion of T15091C mtDNA in B82mtP29 and B82mt15091 cybrids by SspI digestion of the PCR products. B82mt15091 cybrids had 88% T15091C mtDNA, whereas B82mtP29 cybrids did not contain detectable T15091C mtDNA. (B) Comparison of mitochondrial respiratory function between B82mtP29 and B82mt15091 cybrids. The O_2 consumption rates were calculated as a percentage of the average level in B82mtP29 cybrids. Experiments were performed in triplicate; error bars indicate ±SD. $^{**}P < 0.01$; Student's t test.

Therefore, respiration defects were transferred to B82mt15091 cybrids concurrently with the transfer of T15091C mtDNA. All these observations suggest that the T15091C mutation is a pathogenic mutation that dominantly induces respiration defects.

4. Discussion

Our previous studies provided evidence for the presence of recessive mtDNA mutations that cause respiration defects [8–10]; due to intermitochondrial interactions, the respiration defects caused by these mutations could be prevented by the presence of a small proportion (10%–20%) of mtDNA without the mutations. Here, we discovered an mtDNA T15091C mutation inducing respiration defects that could not be prevented by the presence of a large proportion (51%) of mtDNA without the mutation. Our finding indicates that the T15091C mutation acts as a dominant mutation in mtDNA.

The presence of dominant mutations in mtDNA was predicted from the studies of mtDNA mutator mice, which have a proofleading-deficient mtDNA polymerase [11,12]. These mice simultaneously express respiration defects and premature aging phenotypes because of accelerated accumulation of somatic mutations in mtDNA as a consequence of the proofleading deficiency. A subsequent report [15] proposed an idea that some missense mutations in mtDNA lead to synthesis of subunits with amino acid

substitutions, causing incorrect folding that prevents proper assembly of mitochondrial respiratory complexes and leads to respiration defects. Recently, Mito et al. [13] demonstrated that mtDNA mutations in mtDNA mutator mice are responsible for the respiration defects by showing simultaneous transfer of both mtDNA and the respiration defects from mtDNA mutator mice into mouse ρ^0 B82 cells. Our discovery that the T15091C mtDNA mutation induces respiration defects in a dominant manner in mouse cells suggests that accumulation of various kinds of the somatic missense mutations in mtDNA controls expression of the respiration defects in the mtDNA mutator mice even in the presence of intermitochondrial interactions.

The question that remains is whether respiration defects observed in human elderly subjects are due to the age-associated accumulation of somatic mutations in mtDNA. In a recent study [26], we revealed the absence of age-associated accumulation of somatic mutations in the mtDNA of fibroblasts from the elderly human subjects, even though they express age-associated respiration defects. Moreover, we demonstrated that reprogramming of elderly fibroblasts via isolation of their iPS cells restores the respiration defects [26]. These results indicate that age-associated respiration defects found in elderly human fibroblasts [26–28] are controlled not by mutations in either the nuclear or the mitochondrial genome, but by epigenetic and reversible regulation [26]. Therefore, the mechanisms of expression of age-associated respiration defects found in human fibroblasts are different from those observed in the mtDNA mutator mice that express respiration defects by accumulation of somatic and dominant mutations in mtDNA [11–15].

Because a mutation orthologous to the mouse T15091C mutation has been reported in a patient with cardiomyopathy [21], transmitochondrial mito-mice possessing exogenously introduced T15091C mtDNA could be a disease model for detailed investigation of the pathogenesis of the dominant T15091C mutation. Therefore, we intend to generate the transmitochondrial mito-mice with T15091C mtDNA on the basis of the procedures we described previously [24,25]. Moreover, we cannot rule out any other missense mutations found in this study (Table S1) having a role in the respiration defects, even though the orthologous mutations have not been reported in patients with mitochondrial diseases. Therefore, we also intend to generate transmitochondrial mito-mice with these missense mutations in mtDNA as possible models for mitochondrial diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.052.

Transparency document

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