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Cytoplasmic transfer of heritable elements other than mtDNA from SAMP1 mice into mouse tumor cells suppresses their ability to form tumors in C57BL6 mice

Akinori Shimizu [a, 1], Haruna Tani [b, 1], Gaku Takibuchi [b, 1], Kaori Ishikawa [b, c], Ryota Sakurazawa [b], Takafumi Inoue [b], Tetsuo Hashimoto [b, c], Kazuto Nakada [b, c], Keizo Takenaga [d], Jun-Ichi Hayashi [e, *]

[a] Department of Microbiology and Immunology, Faculty of Medicine, Fukuoka University, Fukuoka 814-0180, Japan
[b] Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[c] Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[d] Department of Life Science, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan
[e] University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

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ABSTRACT

In a previous study, we generated transmitochondrial P29mtSAMP1 cybrids, which had nuclear DNA from the C57BL6 (referred to as B6) mouse strain-derived P29 tumor cells and mitochondrial DNA (mtDNA) exogenously-transferred from the allogeneic strain SAMP1. Because P29mtSAMP1 cybrids did not form tumors in syngeneic B6 mice, we proposed that allogeneic SAMP1 mtDNA suppressed tumor formation of P29mtSAMP1 cybrids. To test this hypothesis, current study generated P29mt(sp)B6 cybrids carrying all genomes (nuclear DNA and mtDNA) from syngeneic B6 mice by eliminating SAMP1 mtDNA from P29mtSAMP1 cybrids and reintroducing B6 mtDNA. However, the P29mt(sp)B6 cybrids did not form tumors in B6 mice, even though they had no SAMP1 mtDNA, suggesting that SAMP1 mtDNA is not involved in tumor suppression. Then, we examined another possibility of whether SAMP1 mtDNA fragments potentially integrated into the nuclear DNA of P29mtSAMP1 cybrids are responsible for tumor suppression. We generated $P29^{H}$(sp)B6 cybrids by eliminating nuclear DNA from P29mt(sp)B6 cybrids and reintroducing nuclear DNA with no integrated SAMP1 mtDNA fragment from mtDNA-less P29 cells resistant to hygromycin in selection medium containing hygromycin. However, the $P29^{H}$(sp)B6 cybrids did not form tumors in B6 mice, even though they carried neither SAMP1 mtDNA nor nuclear DNA with integrated SAMP1 mtDNA fragments. Moreover, overproduction of reactive oxygen species (ROS) and bacterial infection were not involved in tumor suppression. These observations suggest that tumor suppression was caused not by mtDNA with polymorphic mutations or infection of cytozoic bacteria but by hypothetical heritable cytoplasmic elements other than mtDNA from SAMP1 mice.

1. Introduction

Mammalian mtDNA with pathogenic mutations, which induce mitochondrial respiration defects, could be responsible not only for mitochondrial diseases [1–6], but also for aging and age-associated disorders including tumor formation [1–3,7–9]. However, we have shown that age-associated respiration defects in human fibroblasts occur not because of pathogenic mtDNA mutations or the resultant ROS overproduction [10,11], but because of reversible epigenetic changes of nuclear genes [12,13].

With respect to the tumor formation, mtDNA with pathogenic mutations has been proposed to function as a tumor enhancer because of its induction of the respiration defects and the resultant enhanced glycolysis under normoxic conditions (the Warburg effect) [9]. This concept has been supported by our previous findings that mouse mtDNA with a pathogenic mutation that simultaneously induces respiration defects and ROS overproduction functions as a tumor enhancer [14,15].

* Corresponding author.
E-mail address: jih45@biol.tsukuba.ac.jp (J.-I. Hayashi).
[1] These authors contributed equally to this work.

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In contrast, mouse mtDNA with some of polymorphic mutations, which do not induce respiration defects, has been shown to function as a tumor suppressor rather than a tumor enhancer [16–18]. For example, mtDNA from allogeneic mouse strains has many polymorphic mutations, and transmitochondrial P29mtNZB cybrids (generated by replacing mtDNA in P29 tumor cells derived from the C57BL6 mouse strain [hereafter referred to as B6] with mtDNA from the allogeneic strain NZB) are unable to form tumors in B6 mice because of the recognition of these cybrids by B6 innate immune cells [18]. To generalize this idea, our subsequent studies [19,20] generated transmitochondrial P29mtC3H cybrids [19] and P29mtSAMP1 cybrids [20] with mtDNA derived from the allogeneic strains C3H and SAMP1, respectively. P29mtC3H cybrids formed tumors in B6 mice [19], whereas the ability of P29mtSAMP1 cybrids to form tumors in B6 mice was completely suppressed because of their recognition by B6 innate immune cells [20]. On the basis of these observations, we have proposed that the innate immune system recognizes polymorphic mutations in mtDNA from NZB and SAMP1 mice but not from C3H mice [18,20].

However, it remains unclear which polypeptides encoded by mtDNA are recognized by the innate immune cells of B6 mice, if mtDNA from allogeneic strains is responsible for tumor suppression. It is also possible that some nuclear-encoded polypeptides denatured by SAMP1 mtDNA–induced ROS are recognized by innate immune cells. To address these issues, we replaced allogeneic SAMP1 mtDNA in P29mtSAMP1 cybrids with syngeneic B6 mtDNA, and showed that neither SAMP1 mtDNA–encoded polypeptides nor SAMP1 mtDNA–induced ROS overproduction is responsible for tumor suppression of P29mtSAMP1 cybrids in B6 mice.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of Tsukuba, Japan (Permit Number: 15314).

2.2. Cell lines and cell culture

The P29 cells are B6 mouse–derived Lewis lung carcinoma cells [21]. Transmitochondrial cybrids (P29mtB6 and P29mtSAMP1) were established in our previous study [20; Fig. S1A] by isolation of mtDNA-less (ρ^0) P29 cells. Both P29mtB6 and P29mtSAMP1cybrids had nuclear DNA from ρ^0P29 cells and mtDNA from B6 mice (P29mtB6) or SAMP1 mice (P29mtSAMP1). All cells were grown in DMEM (Sigma, St. Louis, MO, USA) containing 10% fetal calf serum (Sanko Junyaku, Tokyo, Japan), 50 mg/ml uridine (Sigma, St. Louis, MO, USA), and 0.1 mg/ml sodium pyruvate (Wako Pure Chemical Industries, Osaka, Japan).

2.3. Isolation of ρ^0 P29mt(b6) cells, ρ^0 P29mt(sp) cells, and ρ^0 P29^H cells

To obtain ρ^0 P29mt(b6) cells and ρ^0 P29mt(sp) cells, P29mtB6 and P29mtSAMP1cybrids [20], respectively, were treated with 1.5 mg/ml ditercalinium, an antitumor bis-intercalating agent [22], for 1 week, and mtDNA elimination was confirmed by PCR analysis. To obtain hygromycin-resistant (Hyg-R) ρ^0 P29 cells (ρ^0 P29^H cells), ρ^0 P29 cells [20] were transfected with a Hyg-R plasmid (pTK-Hyg Vector; Clontech Laboratories, Mountain View CA, USA) and cultivated in the presence of hygromycin (200 μg/ml) for 2 weeks.

2.4. Isolation of new transmitochondrial cybrids

To obtain P29mt(*b*6)B6 and P29mt(sp)B6 cybrids, nuclear donors (ρ^0 P29mt(b6) or ρ^0 P29mt(sp) cells) and mtDNA donors (platelets from B6 mice) were fused the presence of polyethylene glycol (polyethylene glycol 1500; Roche, Basel, Switzerland); unfused nuclear donor cells were eliminated in selection medium without uridine and sodium pyruvate (UP-)(Table 1, Fig. S1A).

To obtain P29^{H}mt(b6)B6 and P29^{H}mt(sp)B6 cybrids, nuclear donors (ρ^0 P29^H cells) and mtDNA donors (enucleated P29mt(b6) B6 or P29mt(sp)B6 cybrids) were fused; unfused nuclear donor cells and unenucleated mtDNA donor cells were eliminated in UP-selection medium with hygromycin (Table 2, Fig. S1B).

2.5. Genotyping of B6 mtDNA and Hyg-R nuclear DNA in the cybrids

To distinguish between B6 mtDNA and SAMP1 mtDNA, we used a polymorphic mutation (A11181G) in the *ND4* gene (encoding NADH dehydrogenase subunit 4) of SAMP1 mtDNA [19]. Briefly, a 194-bp fragment containing the 11181 site was amplified by PCR with the primers AAC AAT ACT AAT AAT CGC ACA TGG (nucleotide positions: 11,102–11,125) and CTA TTA GAT TGA TTG AAG GGG GTA (11,295–11,272). SAMP1 mtDNA has an EagI restriction site (EagI cleavage produces 115-bp and 79-bp fragments), whereas B6 mtDNA has no EagI site and generates a 194-bp fragment. The size of the fragments was analysed in a 3% agarose gel [19].

To detect the Hyg-R plasmid in ρ^0 P29^H cells, P29^{H}mt(b6)B6 cybrids, and P29^{H}mt(sp)B6 cybrids, we used primers GAT GTA GGA GGG CGT GGA TA (nucleotide positions 118–137 in the coding sequence of the hygromycin B phosphotransferase gene) and ATA GGT CAG GCT CTC GCT GA (267–248).

2.6. Measurement of mitochondrial ROS

Generation of mitochondrial ROS (superoxide) was quantified by using the mitochondrial superoxide indicator MitoSOX-Red (Invitrogen, Carlsbad, CA, USA) as reported previously [14]. Briefly, cells were mildly trypsinized and suspended in PBS (1×10^5 cells/ml). The cells were then incubated with 5 μM MitoSOX-Red for 15 min at 37 °C in PBS, washed twice with PBS, and analyzed with a FACS calibur (Becton Dickinson, Mountain View, CA, USA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.7. Assays of tumor formation

Tumor formation was examined by subcutaneous inoculation of 5×10^6 cells in 100 μl PBS into the back of 6-week–old male B6 mice, which were obtained from CLEA (Tokyo, Japan). The growth of tumors was monitored assuming their spherical shape [20]. When a tumor mass was visually detectable, its maximum (a) and minimum (b) diameters and height (h) were recorded every 5 days. The volume of each tumor was calculated as $V = \pi abh/6$. The recipient mice were euthanized by cervical dislocation when the tumor volume reached 500 mm^3.

2.8. Examination of bacterial infection in transmitochondrial cybrids

To examine whether transmitochondrial cybrids might be infected with bacteria, we used PCR to detect bacterial 16S rRNA genes. Total genomic DNA was extracted from each cybrid line with a Gentra Puregene Cell Kit (Qiagen, Hilden, Germany) and used in PCR with Takara Ex Taq (Takara Bio, Shiga, Japan) and a primer pair designed to

Table 1
Genetic characteristics of cells, fusion combinations, and obtained transmitochondrial cybrids with B6 mtDNA.

Cells	Mouse strain		Fusion combination	Selection
	Nuclear DNA	mtDNA	Nuclear donor × mtDNA donor	
Parent cells				
(nuclear donors)				
ρ^0 P29mt(b6) cells[a]	B6	–		
ρ^0 P29mt(sp) cells[a]	B6	–		
(mtDNA donors)				
B6 platelets	–	B6		
Cybrids				
P29mt(b6)B6	B6	B6	ρ^0 P29(b6) cells × B6 platelets	UP-[b]
P29mt(sp)B6	B6	B6	ρ^0 P29(sp) cells × B6 platelets	UP-[b]

[a] ρ^0 P29mt(b6) cells and ρ^0 P29mt(sp) cells were obtained from P29mtB6 cybrids with B6 mtDNA and P29mtSAMP1 cybrids with SAMP1 mtDNA, respectively, by complete depletion of their own mtDNA.
[b] For selective isolation of P29mt(b6)B6 and P29mt(sp)B6 cybrids, we used the selection medium without uridine and sodium pyruvate (UP-) to exclude unfused ρ^0 P29(b6) and ρ^0 P29(sp) cells, which do not survive without uridine and sodium pyruvate because of mtDNA depletion.

Table 2
Genetic characteristics of cells, fusion combinations, and obtained transmitochondrial cybrids with nuclear DNA from ρ^0 P29^H cells.

Cells	Mouse strains		Fusion combination	Selection
	Nuclear DNA	mtDNA	Nuclear donor × mtDNA donor	
Parent cells				
(nuclear donors)				
ρ^0 P29^H cells[a]	B6, Hyg-R	–		
(mtDNA donors)				
enP29mt(b6)B6[b]	–	B6		
enP29mt(sp)B6[b]	–	B6		
Cybrids				
P29^{H}mt(b6)B6	B6, Hyg-R	B6	ρ^0 P29^H cells[a] × enP29mt(b6)B6[b]	UP-, Hyg
P29^{H}mt(sp)B6	B6, Hyg-R	B6	ρ^0 P29^H cells[a] × enP29mt(sp)B6[b]	UP-, Hyg

[a] ρ^0 P29^H cells were obtained from ρ^0 P29 cells by transfection with the Hyg-R plasmid. ρ^0 P29 cells were obtained in our previous study [11] by complete depletion of mtDNA from P29 cells.
[b] enP29mt(b6)B6 and enP29mt(sp)B6 represent enucleated P29mt(b6)B6 and P29mt(sp)B6 cybrids, respectively. To obtain P29^{H}mt(b6)B6 and P29^{H}mt(sp)B6 cybrids, we used selection medium with hygromycin and without uridine and sodium pyruvate (UP-): unenucleated cybrids were eliminated in the presence of hygromycin, whereas unfused ρ^0 P29^H cells were eliminated in the absence of uridine and sodium pyruvate (UP-).

amplify 16S rRNA gene fragments from diverse bacterial phyla. The sequences of the primers were based on the highly conserved 16S rRNA gene sequence of *Rickettsia prowazekii* (GenBank accession no. AJ235272): forward AGAGTTTGATCCTGGCTCAG (nucleotides 17–36) and reverse TAAGGAGGTGATCCAGCC (1507–1490). The PCR products were separated by 1% agarose gel electrophoresis, and DNA bands of the expected length (~1500 bp) were extracted with a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The purified DNA fragments were cloned into the pGEM vector using the TA cloning reaction in a LigaFas Rapid DNA Ligation System (Promega, Fitchburg, WI, USA). The resultant plasmids were transformed into *E. coli*, and we performed screening on IPTG/Xgal plates. White *E. coli* colonies were selected, and their DNA was sequenced on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Each obtained sequence was subjected to BLASTN search to identify the PCR product.

2.9. Statistical analysis

All experiments were conducted at least three times. Differences between groups of values were assessed by Dunnett's test. All values are means ± S.D. P values < 0.05 were considered to be statistically significant.

3. Results

3.1. SAMP1 mtDNA is not involved in tumor suppression in B6 mice

Previously we proposed that polypeptides encoded by SAMP1 mtDNA in P29mtSAMP1 cybrids are responsible for the suppression of the ability of these cybrids to form tumors in B6 mice [20]. To test this hypothesis, in the current study we replaced the mtDNA of P29mtSAMP1 cybrids with B6 mtDNA (Fig. S1A). First, we completely excluded SAMP1 mtDNA from P29mtSAMP1 cybrids and obtained mtDNA-less P29mtSAMP1 cybrids, named ρ^0 P29mt(sp) cells. To introduce B6 mtDNA, we fused ρ^0 P29mt(sp) cells with platelets from B6 mice and isolated new transmitochondrial P29mt(sp)B6 cybrids (Table 1).

As controls, we used P29mtB6 cybrids harboring both nuclear DNA and mtDNA from the B6 strain, and isolated corresponding mtDNA-less P29mtB6 cybrids, named ρ^0 P29mt(b6) cells. We reintroduced B6 mtDNA from platelets of B6 mice into ρ^0 P29mt(b6) cells, and obtained new transmitochondrial P29mt(b6)B6 cybrids (Table 1). Genotyping showed that P29mt(sp)B6 and P29mt(b6)B6 cybrids had B6 mtDNA but not SAMP1 mtDNA (Fig. 1A).

Then, we examined tumor formation in B6 mice inoculated with P29mt(sp)B6 or P29mt(b6)B6 cybrids. Even though both cybrids had nuclear DNA and mtDNA from the B6 strain (Fig. S1A), P29mt(b6)B6 but not P29mt(sp)B6 cybrids formed tumors (Fig. 1B). These observations suggest that the suppression of tumor formation by P29mt(sp)B6 cybrids in B6 mice was not caused by SAMP1 mtDNA.

3.2. Nuclear-integrated SAMP1 mtDNA fragments are not involved in tumor suppression in B6 mice

Another possibility is that nuclear DNA in P29mtSAMP1 cybrids

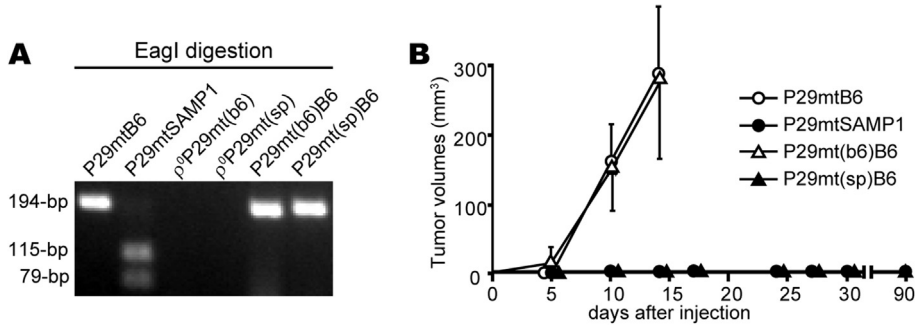

Fig. 1. Effects of an mtDNA replacement of P29mtSAMP1 cybrids from SAMP1 to B6 on tumor formation. (A) mtDNA genotyping. The absence of mtDNA in ρ^0 P29mt(b6) and ρ^0 P29mt(sp) cells was confirmed by the absence of its PCR products. To genotype mtDNA in the cybrids (see Fig. S1A), the PCR products of their mtDNA were digested with EagI. The SAMP1 mtDNA, which has the A11181G mutation, produces 115-bp and 79-bp fragments due to the gain of an EagI site. B6 mtDNA has no such mutation and produces a 194-bp fragment resistant to EagI digestion. All cybrids except P29mtSAMP1 had only B6 mtDNA. (B) P29mt(sp)B6 cybrids do not form tumors. Cells (5×10^6) were inoculated under the skin of B6 mice. No tumors were formed by P29mt(sp)B6 cybrids for up to 3 months, whereas P29mt(b6)B6 cybrids formed tumors within 10 days after inoculation.

acquired integrated SAMP1 mtDNA fragments (nuclear mtDNA sequences, NUMTs) [23,24] during cultivation, and that the products of NUMTs are translocated onto the cell surface, where they are recognized by B6 innate immune cells to suppress tumor formation. In this case, SAMP1 mtDNA–derived NUMTs could be transferred via ρ^0 P29mt(sp) cells into P29mt(sp)B6 cybrids (Fig. S1A), resulting in tumor suppression (Fig. 1B).

We examined this possibility by replacing the nuclear DNA of P29mt(sp)B6 cybrids with nuclear DNA that does not contain SAMP1 mtDNA–derived NUMTs (Table 2, Fig. S1B). As nuclear donors without SAMP1 mtDNA–derived NUMTs, we used mtDNA-less P29 cells (ρ^0 P29 cells), which we isolated previously [11]. For selection and identification of the cells carrying nuclear DNA from ρ^0 P29 cells, we generated Hyg-R ρ^0 P29 cells (ρ^0 P29^H cells) by transfecting ρ^0 P29 cells with the Hyg-R plasmid. Then, we fused ρ^0 P29^H cells with enucleated P29mt(sp)B6 cybrids and used selection medium with hygromycin and without uridine and pyruvate to eliminate unenucleated P29mt(sp)B6 cybrids and unfused ρ^0 P29^H cells, respectively. A colony that grew in the selection medium was clonally isolated and named P29^{H}mt(sp)B6 cybrids (Table 2, Fig. S1B). As a control, we also fused enucleated P29mt(b6)B6 cybrids with ρ^0 P29^H cells in the same selection medium. A colony was clonally isolated and named P29^{H}mt(b6)B6 cybrids (Table 2, Fig. S1B).

Genotyping of mtDNA and nuclear DNA showed that both P29^{H}mt(sp)B6 and P29^{H}mt(b6)B6 cybrids had B6 mtDNA (Fig. 2A) and the Hyg-R plasmid (Fig. 2B), whereas P29mt(b6)B6 and P29mt(sp)B6 cybrids had no Hyg-R plasmid (Fig. 2B). Thus, the nuclear DNA of P29^{H}mt(sp)B6 and P29^{H}mt(b6)B6 cybrids had no NUMTs derived from SAMP1 mtDNA (Fig. S1B).

To examine tumor formation by P29^{H}mt(b6)B6 and P29^{H}mt(sp)B6 cybrids, we inoculated them under the skin of B6 mice. P29^{H}mt(sp)B6 cybrids did not form tumors, whereas P29^{H}mt(b6)B6 cybrids developed into tumors (Fig. 2C), even though both cybrids harbored B6-derived nuclear DNA and B6-derived mtDNA (Fig. S1B). Because nuclear donors (ρ^0 P29^H cells) had nuclear DNA without NUMTs derived from SAMP1 mtDNA, we concluded that tumor suppression was not caused by such NUMTs.

3.3. ROS overproduction or bacterial infection is not involved in tumor suppression

Previously we found a slight increase in the amounts of ROS (superoxide) in P29mtSAMP1 cybrids compared to P29mtB6 cybrids [20]. In this study, we found no statistically significant difference in the amounts of ROS between P29^{H}mt(b6)B6 and P29^{H}mt(sp)B6 cybrids (Fig. 3), even though tumor formation only by P29^{H}mt(sp)B6 cybrids was suppressed (Fig. 2C). These observations, together with the finding that replacement of SAMP1 mtDNA in P29mtSAMP1 cybrids with B6 mtDNA did not restore tumor formation in B6 mice (Fig. 1), suggest that ROS overproduction induced by SAMP1 mtDNA is not involved in suppression of tumor formation.

We also examined a possible involvement of cytozoic bacteria that might be transferred from SAMP1 mice to P29^{H}mt(sp)B6 cybrids; to detect such bacteria, we cloned and sequenced PCR products potentially corresponding to the bacterial 16S rRNA gene. BLAST searches showed that all seven sequenced PCR products were derived from the mouse nuclear genome, not from bacterial genomes (Table 3). These data indicate that tumor suppression is not due to cytozoic bacteria.

Overall, the results of our current study suggest that heritable cytoplasmic elements other than mtDNA and bacteria from SAMP1 mice are responsible for tumor suppression in B6 mice (Fig. S2).

4. Discussion

In this study, we replaced SAMP1 mtDNA in P29mtSAMP1 cybrids with B6 mtDNA (Fig. S1A), and showed that the resultant P29mt(sp)B6 cybrids did not regain tumor formation in B6 mice (Fig. S2). Therefore, SAMP1 mtDNA does not suppress tumor formation by P29mt(sp)B6 cybrids in B6 mice. Then, we replaced nuclear DNA in P29mt(sp)B6 cybrids with that from ρ^0 P29^H cells, which did not contain NUMTs derived from SAMP1 mtDNA (Fig. S1B), and showed that the resultant P29^{H}mt(sp)B6 cybrids still did not regain tumor formation in B6 mice (Fig. S2). Therefore, neither SAMP1 mtDNA nor SAMP1 mtDNA–derived fragments in nuclear DNA suppresses tumor formation by P29mt(sp)B6 or P29^{H}mt(sp)B6 cybrids.

It has been proposed that ROS overproduction promotes tumor growth [25]. Our previous study [14] supported this idea by showing that P29mtA11 cybrids with mtDNA from highly metastatic lung carcinoma A11 cells had a high metastatic potential because of A11 mtDNA–mediated ROS overproduction. In contrast, our recent study [20] indicated possible involvement of ROS overproduction caused by SAMP1 mtDNA in the suppression of tumor formation of P29mtSAMP1 cybrids. However, current study showed that tumor suppression was still observed in P29mt(sp)B6 cybrids even in the absence of SAMP1 mtDNA (Fig. 1B). Moreover, P29^{H}mt(b6)B6 but not P29^{H}mt(sp)B6 cybrids developed into tumors (Fig. 2C), even though no substantial difference in the amounts of ROS was observed between them (Fig. 3). Therefore,

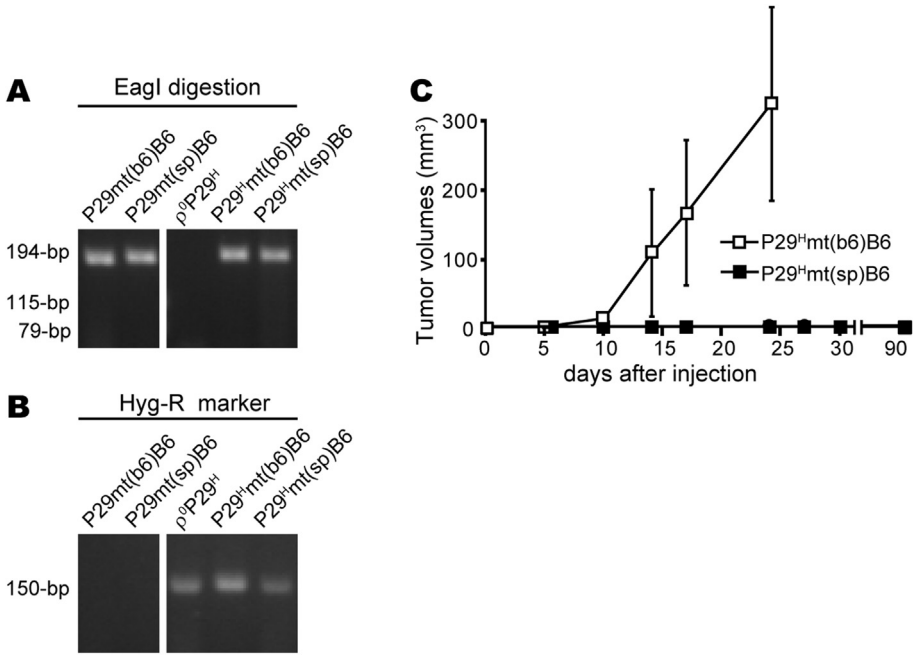

Fig. 2. Effect of a nuclear DNA replacement in P29mt(sp)B6 cybrids with that from ρ^0 P29^H cells on tumor formation. (A) B6 mtDNA but not SAMP1 mtDNA is present in P29^{H}mt(sp)B6 cybrids. The absence of mtDNA in ρ^0 P29^H cells was confirmed by the absence of its PCR products. The presence of a 194-bp fragment resistant to EagI digestion in all cybrids shows that they have B6 mtDNA but not SAMP1 mtDNA. (B) Nuclear DNA with the integrated Hyg-R plasmid is present in P29^{H}mt(b6)B6 and P29^{H}mt(sp)B6 cybrids. Products of PCR with primer sets amplifying the integrated Hyg-R plasmid show that these cybrids and ρ^0 P29^H cells, but not P29mt(b6)B6 or P29mt(sp)B6, have ρ^0 P29^H nuclear DNA. (C) P29^{H}mt(sp)B6 cybrids do not form tumors. Cells (5×10^6) were inoculated under the skin of B6 mice. No tumors were formed by P29^{H}mt(sp)B6 cybrids for up to 3 months, even though they did not have SAMP1 mtDNA or P29 nuclear DNA with potentially integrated SAMP1 mtDNA fragments. In contrast, P29^{H}mt(b6)B6 cybrids formed tumors within 10 days after inoculation.

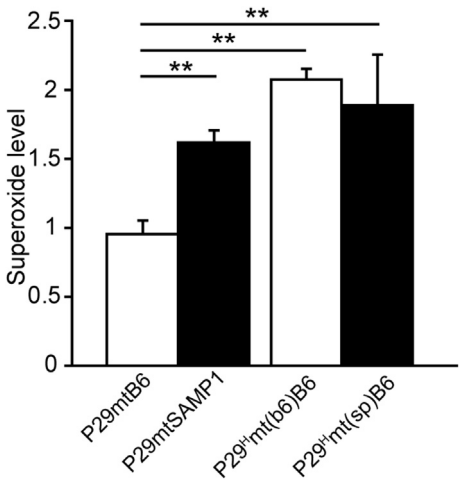

Fig. 3. Levels of mitochondrial ROS (superoxide) in P29^{H}mt(b6)B6 and P29^{H}mt(sp)B6 cybrids. P29mtB6 cybrids were used as a negative control and P29mtSAMP1 as a positive control. Relative superoxide levels are expressed as mean fluorescence intensity of MitoSOX-Red normalized to that in the negative control. Experiments were performed in triplicate; error bars, ± S.D. $^{**}P < 0.01$.

ROS overproduction induced by SAMP1 mtDNA is not responsible for tumor suppression, and some heritable cytoplasmic elements other than mtDNA derived from SAMP1 mice might be present in P29^{H}mt(sp)B6 cybrids and suppress their ability to form tumors (Fig. S2).

We also excluded involvement of cytozoic bacteria derived from SAMP1 mice as heritable cytoplasmic elements other than mtDNA in tumor suppression (Table 3). However, some cytozoic bacteria that could not be detected by our procedure might be present and

Table 3
Mapping of the PCR products of P29^{H}mt(sp)B6 cybrids amplified by using 16S rRNA-primer pair with the sequence universally conserved among prokaryotes.

No. of PCR products	Matched sequence by BLAST		Accession No.
	Species	Chromosome	
1	*M. musculus*	17	LC145625
2	*M. musculus*	17	LC145626

responsible for the tumor suppression. Moreover, endogenous retroviruses might also function as such heritable cytoplasmic factors and be responsible for the recognition of P29mtSAMP1, P29mt(sp)B6, and P29^{H}mt(sp)B6 cybrids by innate immune cells, resulting in suppression of tumor formation in B6 mice. In fact, mouse strains contain various endogenous retroviruses in their nuclear DNA, and some of them are infectious [26]. Although mammalian cells are known to possess no heritable cytoplasmic elements other than mtDNA, our current study warrants further examination of whether some cytozoic bacteria or endogenous retroviruses derived from SAMP1 mice function as such elements.

Author contributions

Conceived and designed the experiments: AS, HT GT J-IH. Performed the experiments: AS HT GT KI RS TI. Helped with the design and coordination of the study: TH KN KT. Wrote the paper: J-IH. All authors reviewed the manuscript.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.09.035.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.09.035.

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