

—Original—

Selection of Rodent Species Appropriate for mtDNA Transfer to Generate Transmitochondrial Mito-Mice Expressing Mitochondrial Respiration Defects

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Abstract: Previous reports have shown that transmitochondrial mito-mice with nuclear DNA from *Mus musculus* and mtDNA from *M. spretus* do not express respiration defects, whereas those with mtDNA from *Rattus norvegicus* cannot be generated from ES cybrids with mtDNA from *R. norvegicus* due to inducing significant respiration defects and resultant losing multipotency. Here, we isolated transmitochondrial cybrids with mtDNA from various rodent species classified between *M. spretus* and *R. norvegicus*, and compared the O₂ consumption rates. The results showed a strong negative correlation between phylogenetic distance and reduction of O₂ consumption rates, which would be due to the coevolution of nuclear and mitochondrial genomes and the resultant incompatibility between the nuclear genome from *M. musculus* and the mitochondrial genome from the other rodent species. These observations suggested that *M. caroli* was an appropriate mtDNA donor to generate transmitochondrial mito-mice with nuclear DNA from *M. musculus*. Then, we generated ES cybrids with *M. caroli* mtDNA, and found that these ES cybrids expressed respiration defects without losing multipotency and can be used to generate transmitochondrial mito-mice expressing mitochondrial disorders.

Keywords: interspecies mtDNA transfer, multipotency, *Mus caroli* mtDNA, respiration defects, transmitochondrial ES cybrids

Introduction

Accumulation of mitochondrial DNA (mtDNA) with pathogenic mutations that induce respiration defects has been proposed to be responsible for mitochondrial diseases, aging, and age-related disorders [20, 21]. Gen-

eration of transmitochondrial mito-mice expressing respiration defects by the introduction of exogenous mtDNA with pathogenic mutations would provide an ideal system for precise investigation of the pathogenesis of these disorders. However, it is impossible to generate transmitochondrial mito-mice carrying artifi-

(Received 20 May 2013 / Accepted 1 July 2013)

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cially mutagenized mtDNA, because no procedures have yet been established for introducing exogenous mtDNA into mitochondria.

One way of generating transmitochondrial mito-mice expressing respiration defects is to detect mtDNA with a somatic mutation that induces respiration defects in cultivated mouse cell lines. Our previous studies generated transmitochondrial mito-mice carrying mtDNA with pathogenic mutations and expressing various disorders by introduction of mitochondria carrying mtDNA with somatic mutations accumulated in mouse tumor cell lines into fertilized mouse eggs [6] or into mouse ES cells [4, 7, 11, 23].

Another procedure used to generate transmitochondrial mito-mice expressing respiration defects is to introduce mtDNA from different rodent species. Because most mitochondrial respiratory complexes consist of subunits encoded by both nuclear DNA and mtDNA [21], transmitochondrial cybrids with nuclear DNA from mice (*M. musculus*) but with mtDNA from a different rodent species express respiration defects owing to incompatibility between the nuclear and mitochondrial genomes from different rodent species [2, 13, 14, 22]. Similar incompatibility has been reported in transmitochondrial cybrids with human nuclear DNA but with mtDNA from different primate species [1].

However, no reports have succeeded in obtaining transmitochondrial mito-mice expressing respiration defects by introducing mtDNA from different rodent species. For example, transmitochondrial mito-mice with nuclear DNA from mice (*M. musculus*) but with mtDNA from a different mouse species (*M. spretus*) do not express respiration defects and disease phenotypes [11, 15], whereas transmitochondrial mito-mice with mtDNA from rats (*R. norvegicus*) cannot be generated [11]. The latter failure is due to the induction of significant respiration defects and the resultant loss of multipotency in mouse ES cybrids with rat mtDNA [11]. Therefore, we need to find a rodent species of which we can use its mtDNA to induce respiration defects but not induce loss of multipotency in mouse ES cells.

Here, we addressed the issue by isolating transmitochondrial cybrids with nuclear DNA from *M. musculus* and mtDNA from rodent species that are phylogenetically classified between *M. spretus* and *R. norvegicus*. We found that one of these rodent species was an appropriate mtDNA donor for generating mito-mice expressing respiration defects and mitochondrial disorders.

Materials and Methods

Cells and cell culture

Mouse mtDNA-less (ρ^0) B82 cells derived from fibroblasts of *M. musculus* [5], transmitochondrial cybrids B82mtB6, B82mtSpr, B82mtRat [22], B82mtCOI^M [11], B82mtCar, and B82mtAsp isolated in this study were grown in normal medium: RPMI1640 (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal calf serum, 50 ng/ml uridine, and 0.1 mg/ml pyruvate. Mouse ES cells (TT2-F, an XO subline established from XY TT2 cells) [11] and mtDNA-repopulated ES cybrids were cultivated on mitomycin C-inactivated feeder cells derived from mouse embryonic fibroblasts, in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% KNOCKOUTTM Serum Replacement (Invitrogen), 1× non-essential amino acids (MP Biomedicals LLC, OH, USA), leukemia inhibitory factor (10^5 units/ml, Invitrogen), and 100 μ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA).

Isolation of transmitochondrial cybrids

Platelets of *M. caroli*(RBRC00123) were provided from RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Platelets of *Apodemus speciosus* were provided from Dr. Hitoshi Suzuki (Hokkaido University, Japan). Platelet mtDNA was introduced into ρ^0 B82 cells by the fusion of the platelets and ρ^0 B82 cells in the presence of 50% (w/v) polyethylene glycol (PEG) as described previously [9]. The fusion mixture was cultivated in selection medium RPMI1640 without pyruvate and uridine, in which unfused ρ^0 B82 cells without mitochondrial respiratory function were unable to grow [8].

Isolation of transmitochondrial ES cybrids

Mouse ES cybrids with mtDNA from *M. caroli* were isolated based on the procedure as reported previously [11]. Briefly, the host ES cells were pretreated with rhodamine 6G (R6G; 0.38–1.5 μ g/ml in 3% ethanol) for 48 h in medium supplemented with uridine (50 ng/ml) and pyruvate (0.1 μ g/ml) to eliminate endogenous mitochondria and mtDNA [17]. Then, they were washed with phosphate-buffered saline (PBS) and suspended in R6G-free medium for 2 h to allow recovery. The mtDNA donor B82mtCar cybrids pretreated with cytochalasin B (10 μ g/ml) for 10 min were centrifuged at 15,000×g for

30 min at 37°C for enucleation. The resultant cytoplasts were fused with R6G-pretreated ES cells using polyethylene glycol, and the fusion mixture was cultivated in selective medium with HAT (hypoxanthine aminopterin thymidine). Due to the absence of thymidine kinase activity of nuclear donor B82 cells, B82mtCar cybrids carrying nuclear genome from B82 cells could not survive in the presence of HAT. Seven days after fusion, growing colonies were picked up for further examination.

Construction of phylogenetic trees

Sequences of the cytochrome b (*mt-Cytb*) gene, a *mt-Dcr* region, and the *mt-Rnr1* gene in mtDNA (Supplementary Table 1) were manually aligned using SEAVIEW program (<http://pbil.univ-lyon1.fr/software/seaview.html>). For each locus pairwise distances were inferred on the basis of Kimura's two-parameter model [12], with among-site rate heterogeneity taken into consideration by assuming discrete distribution with 4 categories. Using the distance matrix obtained phylogenetic tree was constructed by NEIGHBOR program implemented in PHYLIP software (<http://www.phylip.com/>) under the assumption of evolutionary rate constancy among lineages. On the basis of the phylogenetic tree distances between *M. musculus* and other organisms were calculated.

Genotyping of mtDNA

Total cellular DNA (0.2 µg) extracted from cultivated cells was used as a template. Restriction fragment length polymorphism (RFLP) analysis was carried out for detection of *M. caroli* mtDNA. A 306-bp fragment was amplified by PCR with the following primers 5'-CTCTG-GTCTTGTAACC-3' and 5'-GACTGTATGGTG-TATATCAG-3', which corresponded to mouse mtDNA sequences (GenBank Accession No. AY172335) from positions 15306 to 15322 and from 15807 to 15787, respectively. The cycle times were 30 s for denaturation at 94°C, 30 s for annealing at 46°C and 30 s for extension at 72°C for 30 cycles. The PCR amplicon contains a region of the mtDNA *mt-Dcr* with a *Dra* I (Takara) restriction site (control mouse mtDNA was not cleaved), and generates 267-bp and 39-bp fragments on *Dra* I digestion. Similarly, detection of *A. speciosus* mtDNA was achieved by RFLP analysis. A 250 bp fragment was amplified by PCR with the following primers 5'-GGT-GTCCTAGCCTTAATC-3' and 5'-CGATAATTCCT-

GAGAGATTGGT-3', which corresponded to mouse mtDNA sequences (GenBank Accession No. AY172335) from positions 15012 to 15029 and from 15261 to 15242, respectively. The cycle times were 30 s for denaturation at 94°C, 30 s for annealing at 54°C and 30 s for extension at 72°C for 30 cycles. The PCR amplicon contains a region of the *mt-Cytb* gene with an *Mbo* II (NEB) restriction site (control mouse mtDNA was not cleaved), and generates 219-bp and 31-bp fragments on *Mbo* II digestion. These restriction fragments were separated by electrophoresis in a 3% agarose gel. For quantification of the mtDNA from *M. caroli* and mtDNA from *M. musculus*, we used the NIH IMAGE program.

Analysis of mitochondrial respiratory function

Oxygen consumption rates were measured by trypsinizing cells, incubating the suspension in phosphate-buffered saline, and recording oxygen consumption in a polarographic cell (2.0 ml) at 37°C with a Clark-type oxygen electrode (Yellow Springs Instruments, OH). Cytochemical analysis of cytochrome *c* oxidase (COX) activity was carried out by examining the rate of cyanide-sensitive oxidation of reduced cytochrome [16].

Analysis of multipotency of ESmtCar cybrids

To test the multipotency of the ESmtCar cybrids, 1×10^6 cells were inoculated subcutaneously into the backs of 6-week-old nude mice (JCL, BALB/c-nu/nu; CLEA Japan). The resulting teratomas (tumors) were fixed in 10% neutral-buffered formalin, embedded in paraffin, stained with hematoxylin and eosin (HE) and examined histologically.

Animal experiments

All animal experiments were performed in accordance with protocols approved by the Experimental Animal Committee of the University of Tsukuba.

Statistical analysis

We analyzed data with the (unpaired or paired) Student's *t*-test. Values with $P < 0.05$ were considered significant.

Results

Isolation of mouse transmitochondrial cybrids with mtDNA from different rodent species

On the basis of the phylogenetic trees constructed by

comparing the sequences of the *mt-Cytb* gene in the mtDNA (Fig. 1A), we used *M. caroli* and *A. speciosus*, which are phylogenetically classified between *M. spretus* and *R. norvegicus* [18, 19], as candidate mtDNA donor species. As mtDNA recipients, we used ρ^0 B82 cells without mtDNA and with the nuclear genetic background of *M. musculus* [5].

Cytoplasmic transfer of mtDNA from *M. caroli* and *A. speciosus* into ρ^0 B82 cells was performed by the fusion of ρ^0 B82 cells with platelets from *M. caroli* and *A. speciosus*, respectively. Colonies grown in selective medium to exclude unfused ρ^0 B82 cells were isolated clonally as transmitochondrial cybrids and were named B82mtCar cybrids and B82mtAsp cybrids (Table 1). Genotyping of mtDNA showed that B82mtCar cybrids possessed *M. caroli* mtDNA and B82mtAsp cybrids possessed *A. speciosus* mtDNA (Fig. 1B).

For further examination of mitochondrial respiratory function, we used B82mtB6, B82mtSpr, and B82mtRat possessing mtDNA from *M. musculus*, *M. spretus*, and *R. norvegicus*, respectively [11], as control cybrids (Table 1). We furthermore used B82mtCOI^M cybrids possessing *M. musculus* mtDNA with a pathogenic T6589C mutation in the *mt-Co1* gene as control cybrids (Table 1), because we had already successfully generated transmitochondrial mito-miceCOI^M expressing respiration defects and mitochondrial disease phenotypes by introducing the T6589C mtDNA into a mouse female germ line [11].

Effect of phylogenetic distance on respiratory function in transmitochondrial cybrids

We used transmitochondrial cybrids possessing mtDNA from various rodent species or possessing mouse mtDNA with a pathogenic mutation to compare O₂ consumption rates, which reflected overall mitochondrial respiratory function (Fig. 1C). B82mtCar cybrids with mtDNA from *M. caroli*, which belongs to the same genus *Mus*, had a 35% reduction in O₂ consumption rates, indicating that B82mtCar cybrids expressed relatively mild respiration defects. In contrast, B82mtAsp cybrids with mtDNA from *A. speciosus* belonging to a different genus *Apodemus* had a 66% reduction in O₂ consumption rates. These results suggest that mitochondrial respiratory function of B82 cybrids with mtDNA of different rodent species is reduced in accordance with the phylogenetic distance from *M. musculus* (Figs. 1A and C).

To explore this idea further, we estimated the phylo-

genetic distances of the rodent species used here by comparing the sequences of the *mt-Cytb* gene (Fig. 2A), the *mt-Dcr* region (Fig. 2B), and the *mt-Rnr1* gene (Fig. 2C) in the mtDNA. Phylogenetic distance and O₂ consumption rates were well correlated negatively in transmitochondrial cybrids with mtDNA from various rodent species (Fig. 2). Thus, the increase in respiration defects would be due to increased incompatibility between the nuclear genome from *M. musculus* and the mitochondrial genomes from other rodent species.

Selection of rodent species appropriate for generating transmitochondrial mito-mice

Our previous study [11] proposed that failure to obtain transmitochondrial mito-mice with rat mtDNA was due to the induction of significant respiration defects by the introduction of rat mtDNA and the resultant loss of multipotency in mouse ES cybrids with rat mtDNA. Because B82mtAsp cybrids showed a 66% reduction of O₂ consumption rates, which are comparable to the reduction rates observed in B82mtRat cybrids (Figs. 1C and 2), mtDNA from *A. speciosus* would not be appropriate for generating transmitochondrial mito-mice.

In contrast, the 35% reduction in O₂ consumption rates in B82mtCar cybrids appears comparable to the 31% reduction in B82mtCOI^M cybrids with mouse T6589C mtDNA (Figs. 1C and 2). Considering that mouse T6589C mtDNA can be effectively introduced into the female germ line and induces disease phenotypes in transmitochondrial mito-miceCOI^M in the absence of embryonic lethality [11], *M. caroli* is a suitable mtDNA donor species for generating transmitochondrial mito-mice expressing phenotypes related to mitochondrial diseases.

Isolation of transmitochondrial mouse ES cybrids with mtDNA from M. caroli

Next, we isolated transmitochondrial mouse ES cybrids with mtDNA from *M. caroli*. For exclusive isolation of the ES cybrids with mtDNA from *M. caroli*, we used B82mtCar cybrids as mtDNA donors, because unenucleated B82mtCar cybrids can be excluded from the fusion mixture by using HAT selection medium owing to their deficiency in thymidine kinase (Table 1; Materials and Methods). As nuclear DNA donors and mtDNA recipients, we used female-type mouse ES cells, because mtDNA is exclusively inherited via the female germ line [10, 17].

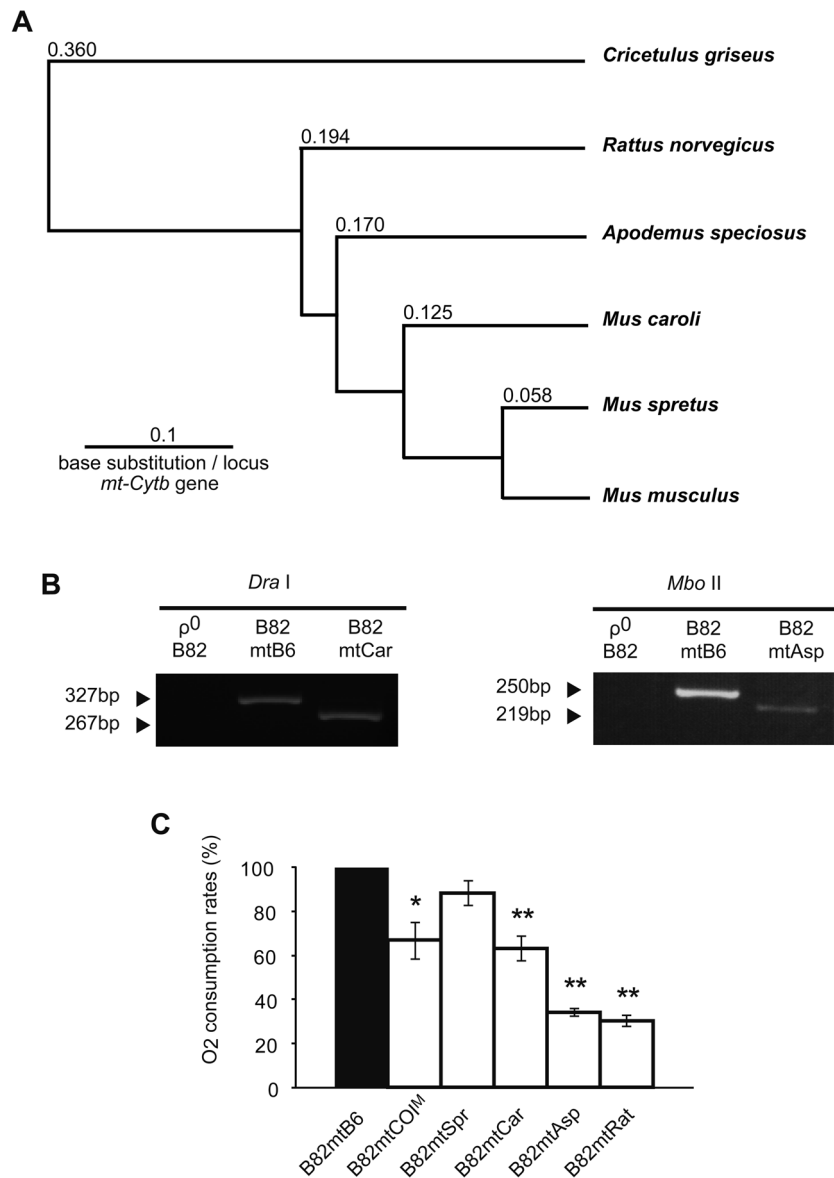


Fig. 1. Characterization of transmitochondrial cybrids with mtDNA from various rodent species. Transmitochondrial cybrids B82mtB6, B82mtSpr, B82mtCar, B82mtAsp, and B82mtRat possessed nuclear DNA from *M. musculus* and mtDNA from *M. musculus*, *M. spretus*, *M. caroli*, *A. speciosus* and *R. norvegicus*, respectively. B82mtCOI^M cybrids possessed *M. musculus* mtDNA with a pathogenic T6589C mutation in the *mt-Co1* gene that induces respiration defects [11]. (A) Phylogenetic trees constructed by comparison of the sequence of the *mt-Cytb* gene encoded by mtDNA. On the basis of Kimura's two-parameter model [24], we used *mt-Cytb* gene sequence data (positions 14139 to 15266) to create phylogenetic trees with PHYLIP software (<http://www.phylip.com/>). Branch lengths show evolutionary distance from *M. musculus*. The tree is rooted using *Cricetulus griseus*(Chinese hamster) sequence data. Values on each branch indicate base substitution in the *mt-Cytb* gene. (B) Genotyping of mtDNA. On *Dra* I digestion of the PCR products, B82mtB6 cells with *M. musculus* mtDNA gave a 327-bp fragment, whereas B82mtCar cells with *M. caroli* mtDNA gave a 267-bp fragment and a 39-bp fragment (not detectable) by the gain of a *Dra* I site and a 21-bp deletion in the *mt-Dcr* region. On *Mbo* II digestion of the PCR products, B82mtB6 cybrids with *M. musculus* mtDNA gave a 250-bp fragment, whereas B82mtAsp cybrids with *A. speciosus* mtDNA gave a 219-bp fragment and a 31-bp fragment (not detectable) by the gain of an *Mbo* II site in the *mt-Cytb* gene. (C) Estimation of O₂ consumption rates. B82mtB6 cells with nuclear and mitochondrial genomes from *M. musculus* were used as standards expressing normal respiratory function. Asterisks indicate a *P*-value less than 0.05 and double asterisks indicate a *P*-value less than 0.01.

Table 1. Genome composition of transmitochondrial cybrids with imported mtDNA from various rodent species

Transmitochondrial cybrids	Nuclear genetic marker	Rodent species	
		Nuclear genome	Mitochondrial genome
B82mtB6*	HAT sensitive	<i>M. m. domesticus</i>	<i>M. m. domesticus</i>
B82mtCOI ^{M**}	HAT sensitive	<i>M. m. domesticus</i>	<i>M. m. domesticus</i>
B82mtSpr*	HAT sensitive	<i>M. m. domesticus</i>	<i>M. spretus</i>
B82mtCar	HAT sensitive	<i>M. m. domesticus</i>	<i>M. caroli</i>
B82mtAsp	HAT sensitive	<i>M. m. domesticus</i>	<i>A. speciosus</i>
B82mtRat*	HAT sensitive	<i>M. m. domesticus</i>	<i>R. norvegicus</i>

*, Established in our previous report study [10]. **, Possessing *M. m. domesticus* mtDNA with a T6589C mutation in the *mt-Co1* gene; established in our previous report study [5].

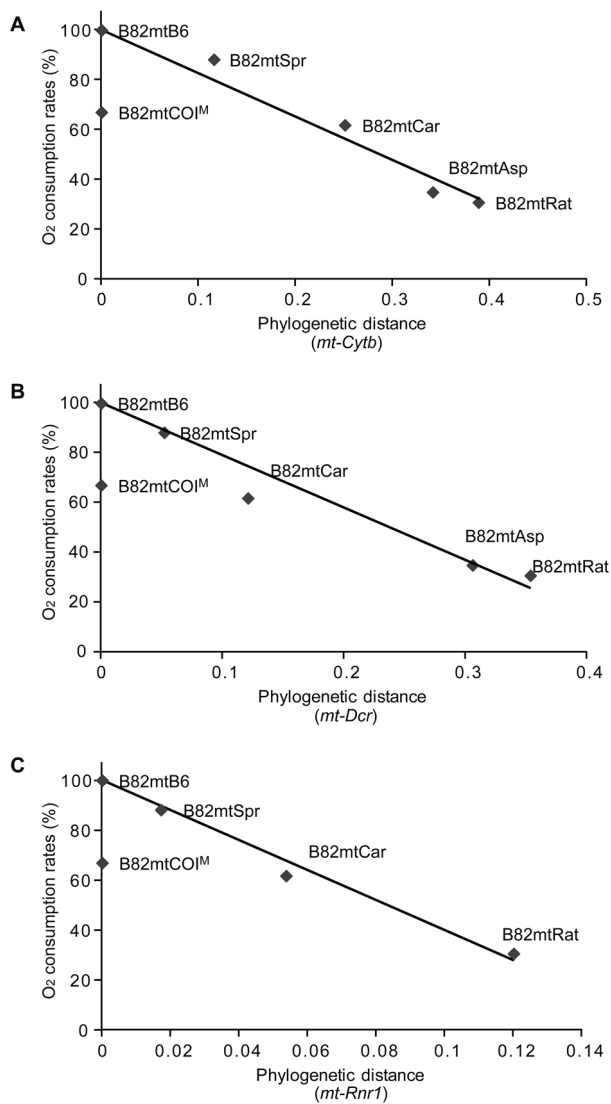


Fig. 2. Correlation between phylogenetic distance and reduction rates of O₂ consumption in transmitochondrial cybrids. Phylogenetic distance was estimated from base substitution in (A) the *mt-Cytb* gene, (B) the *mt-Dcr* region, and (C) the *mt-Rnr1* gene in mtDNA. Correlation coefficients were 0.964, 0.951 and 0.984 in (A), (B) and (C), respectively.

The ES cells were pretreated with rhodamine 6G (R6G) to eliminate endogenous mitochondria and mtDNA, and were then fused with enucleated B82mtCar cybrids. Unfused R6G-pretreated ES cells were unable to grow owing to the absence of mitochondria and mtDNA, and unenucleated B82mtCar cybrids failed to grow in the HAT selection medium. Three colonies growing in the selection medium were isolated clonally. They were named ESmtCar-1, -2, and -3, and mtDNA genotyping showed that they possessed 39%, 42% and 42% mtDNA, respectively, from *M. caroli* (Fig. 3A). The ESmtCar cybrids were therefore transmitochondrial ES cybrids with heteroplasmic mtDNA consisting of *M. caroli* mtDNA from the B82mtCar cybrids and *M. musculus* mtDNA in the host ES cells, probably due to incomplete elimination of their own mtDNA by the R6G pretreatment.

Considering that the ESmtCar cybrids possessed nuclear DNA exclusively from *M. musculus*, it is possible that the *M. caroli* mtDNA would eventually be excluded from the ESmtCar cybrids during cultivation by preferential replication of *M. musculus* mtDNA. We examined this possibility by mtDNA genotyping after prolonged cultivation of the ESmtCar-2 cybrids for 6 weeks after cloning. The results showed that the proportion of *M. caroli* mtDNA did not change substantially (Fig. 3B). Thus, *M. caroli* mtDNA can replicate and propagate stably into subsequent generations, even in the presence of host *M. musculus* mtDNA in ESmtCar cybrids.

Effects of M. caroli mtDNA on respiratory function and multipotency of ESmtCar cybrids

A question was whether ESmtCar cybrids expressed respiration defects. Because it was difficult to obtain a sufficient number of ESmtCar cybrids (5×10^6 cells) for estimation of O₂ consumption rates without feeder cell

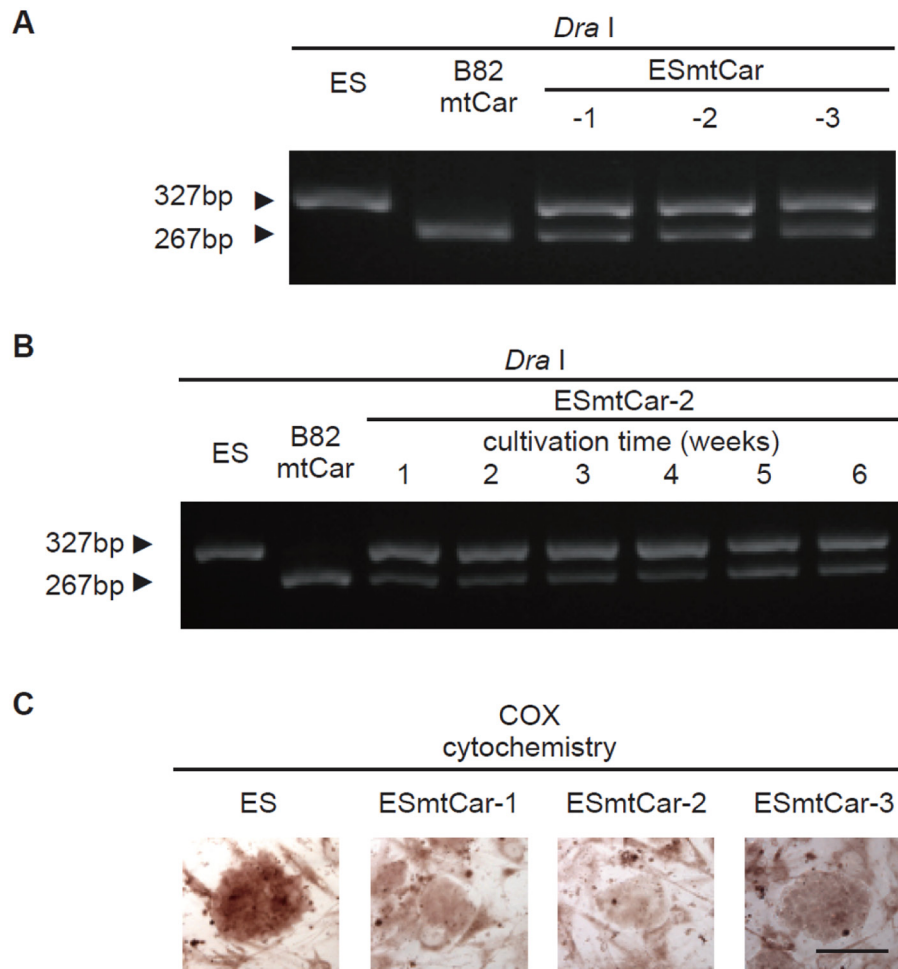


Fig. 3. Characterization of transmitochondrial ESmtCar cybrids. (A) Genotyping of mtDNA from ESmtCar cybrids. On *Dra* I digestion of the PCR products, B82mtB6 cells with *M. musculus* mtDNA gave a 327-bp fragment, whereas B82mtCar cybrids with *M. caroli* mtDNA gave a 267-bp fragment and a 39-bp fragment (not detectable) by the gain of a *Dra* I site and a 21-bp deletion in the *mt-Dcr* region. ES cells with homoplasmic *M. musculus* mtDNA and B82mtCar cybrids with homoplasmic *M. caroli* mtDNA were used as negative and positive controls, respectively. Three ESmtCar cybrid clones showed heteroplasmy of *M. caroli* mtDNA and *M. musculus* mtDNA. ESmtCar-1, -2, and -3 possessed 39%, 42%, and 42% *M. caroli* mtDNA, respectively. (B) Stability of *M. caroli* mtDNA in ESmtCar-2 cybrids cultivated for 1 to 6 weeks after cloning. (C) Analysis of mitochondrial respiratory function of ESmtCar cybrids by COX cytochemistry. Bar, 100 μ m.

contamination, we used cytochemical analysis of COX activity. ESmtCar cybrids with *M. caroli* mtDNA showed respiration defects, whereas parental ES cells did not (Fig. 3C). These observations indicated that mtDNA genotypes and respiration phenotypes were transferred simultaneously from mtDNA donor B82mtCar cybrids to ESmtCar cybrids.

The next question was whether ESmtCar cybrids expressing respiration defects retained their multiple differentiation potential. Our previous report [11] showed

that mouse ES cybrids with rat mtDNA (ESmtRat cybrids) lost their multipotency phenotypes upon subcutaneous inoculation under the back skin of nude mice, resulting in failure to generate transmitochondrial mice with rat mtDNA. Therefore, we tested whether the ESmtCar cybrids were able to differentiate into various tissues under the back skin of nude mice. Both the ESmtCar cybrids and the parental ES cells formed primary tumor masses within 4 weeks after their inoculation (Fig. 4). Histological analysis of their primary tumors showed

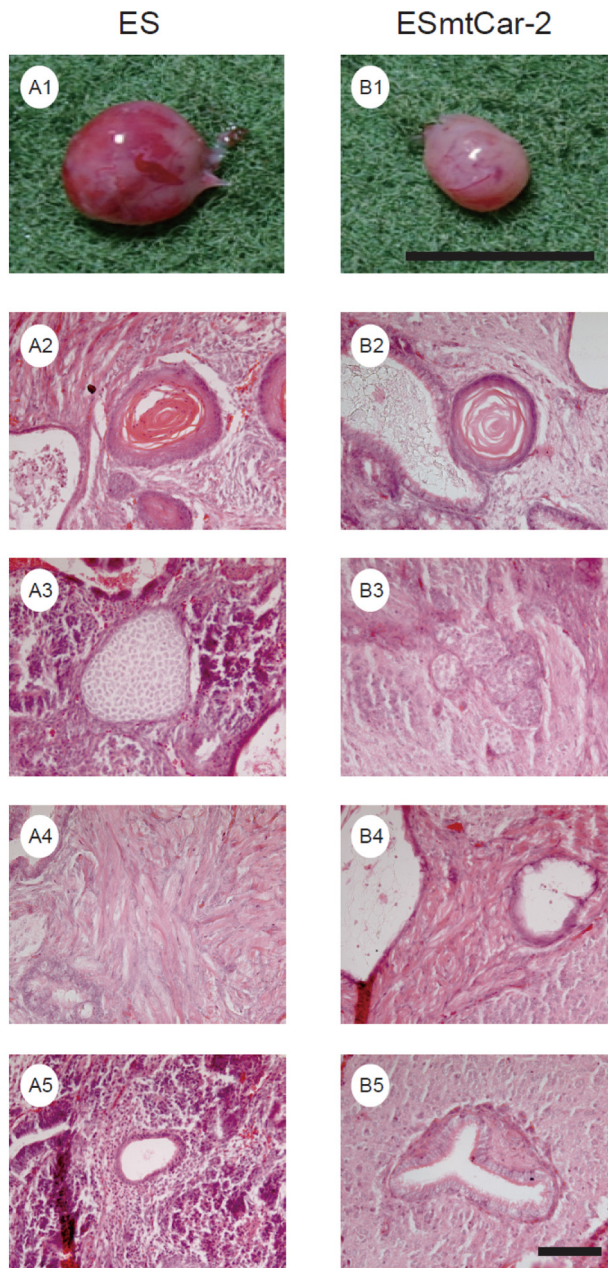


Fig. 4. Effects of respiration defects on multipotency of transmitochondrial ESmtCar cybrids. ES cells and ESmtCar-2 cybrids with 42% *M. caroli* mtDNA were inoculated into nude mice and the resultant primary tumor masses were used for histochemical analysis. (A1–A5), ES cells; (B1–B5), ESmtCar-2 cybrids. A1 and B1 are primary tumor masses formed in nude mice. A2 and B2, A3 and B3, A4 and B4, and A5 and B5 correspond to hair follicles, bone cells, striated muscle and secretory cells, respectively. Bar in B1, 1 cm; bar in B5, 100 μ m.

that both ES cells and ESmtCar-2 cybrids were able to differentiate into multiple tissue types, namely secretory cells, bone cells, striated muscle, and hair follicles

(Fig. 4). These observations suggest that the ESmtCar cybrids still express multipotency, and could therefore be used in future studies to generate transmitochondrial mito-mice with mtDNA from *M. caroli*.

Discussion

This study determined that *M. caroli* was an appropriate mtDNA donor for generating transmitochondrial mito-mice expressing respiration defects by isolating transmitochondrial cybrids with nuclear DNA from *M. musculus* and mtDNA from *M. caroli* or *A. speciosus*, which are phylogenetically classified between *M. spretus* and *R. norvegicus*.

Comparison of the O₂ consumption rates of transmitochondrial cybrids with mtDNA from various rodent species showed a strong negative correlation between phylogenetic distance and reduction of O₂ consumption rates (Fig. 2). Because most mitochondrial respiratory complexes consist of both nuclear genome-coded and mitochondrial genome-coded subunits [21], mitochondrial respiratory function is controlled by both genomes. Therefore, the respiration defects observed in transmitochondrial cybrids with mtDNA from various rodent species (Fig. 2) would have been due to the coevolution of nuclear and mitochondrial genomes and the resultant incompatibility between the nuclear genome from *M. musculus* and the mitochondrial genome from the other rodent species.

Transmitochondrial cybrids with *A. speciosus* mtDNA (B82mtAsp cybrids) showed a 66% reduction in O₂ consumption rates, which were comparable to the reduction rates observed in B82mtRat cybrids (Fig. 1C). Because transmitochondrial mito-mice with rat mtDNA have not been generated owing to the induction of significant respiration defects by the rat mtDNA and the resultant losing multipotency in mouse ES cybrids with rat mtDNA [11], mtDNA from *A. speciosus* or from *R. norvegicus* would not be appropriate for generating transmitochondrial mito-mice. Similar failures to generate transmitochondrial mito-mice were reported, particularly when mouse embryos possessed mouse mtDNA with pathogenic mutations that induce significant respiration defects [3, 7].

In contrast, B82mtCar cybrids showed a 35% reduction in O₂ consumption rates (Fig. 1C). Although a previous report [13] showed that transmitochondrial cybrids with mtDNA from *M. caroli* did not exhibit reduced

activity of each respiration complex, the O₂ consumption rates we obtained here reflect the overall activity of mitochondrial oxidative phosphorylation. Moreover, a good correlation between reduction rates of the O₂ consumption and phylogenetic distance (Fig. 2) suggests that O₂ consumption rates are reliable for estimating overall activity of mitochondrial respiration. Furthermore, the reduction rates of the O₂ consumption induced by *M. caroli* mtDNA were comparable to those induced by T6589C mtDNA of *M. musculus* (Fig. 1C), which is effectively transferred via the mouse female germ line to the following generations and induces disease phenotypes in transmitochondrial mito-miceCOI^M [11]. Therefore, mtDNA from *M. caroli* is a candidate mtDNA that can carry possible pathogenic mutations in the transmitochondrial mito-mice with nuclear DNA from *M. musculus*.

We also succeeded in isolating transmitochondrial ESmtCar cybrids with *M. caroli* mtDNA expressing respiration defects (Fig. 3C) without loss of multipotency (Fig. 4B). Therefore, we next intend to generate transmitochondrial mito-mice with *M. caroli* mtDNA using the ESmtCar cybrids, and to examine whether they can be used as models of mitochondrial disorders.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research S 19100007 (to J.-I.H.), Scientific Research A 23240058 (to K.N.), and Scientific Research on Innovative Areas 24117503 (to J.-I.H.) from Japan Society for the Promotion of Science.

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