Journal of Bioenergetics and Biomembranes, Vol. 36, No. 4, August 2004 (© 2004)

Development and Initial Characterization of Xenomitochondrial Mice

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Received April 2, 2004; accepted May 7, 2004

Xenomitochondrial mice harboring trans-species mitochondria on a *Mus musculus domesticus* (MD) nuclear background were produced. We created xenomitochondrial ES cell cybrids by fusing *Mus spretus* (MS), *Mus caroli* (MC), *Mus dunni* (Mdu), or *Mus pahari*(MP) mitochondrial donor cytoplasts and rhodamine 6-G treated CC9.3.1 or PC4 ES cells. The selected donor backgrounds reflected increasing evolutionary divergence from MD mice and the resultant mitochondrial–nuclear mismatch targeted a graded respiratory chain defect. Homoplasmic (MS, MC, Mdu, and MP) and heteroplasmic (MC) cell lines were injected into MD ova, and liveborn chimeric mice were obtained (MS/MD 18 of 87, MC/MD 6 of 46, Mdu/MD 31 of 140, and MP/MD 1 of 9 founder chimeras, respectively). Seven MS/MD, 1 MC/MD, and 11 Mdu/MD chimeric founder females were mated with wild-type MD males, and 18 of 19 (95%) were fertile. Of fertile females, only one chimeric MS/MD (1% coat color chimerism) and four chimeric Mdu/MD females (80–90% coat color chimerism) produced homoplasmic offspring with low efficiency (7 of 135; 5%). Four male and three female offspring were homoplasmic for the introduced mitochondrial backgrounds. Three male and one female offspring proved viable. Generation of mouse lines using additional female ES cell lineages is underway. We hypothesize that these mice, when crossbred with neurodegenerative-disease mouse models, will show accelerated age-related neuronal loss, because of their suboptimal capacity for oxidative phosphorylation and putatively increased oxidative stress.

KEY WORDS: mtDNA; cybrid; xenomitochondrial; ES cells; mouse models; neurodegeneration.

INTRODUCTION

Mitochondrial DNA (mtDNA) diseases in humans are pleiotropic, with neurological pathology common. All mtDNA gene products comprise subunits of the multimeric oxidative phosphorylation (OXPHOS) complexes. The reasons some mtDNA mutations affect certain neuronal populations, while others lead to muscle or endocrine disease, for example, remain mysterious (see DiMauro and Schon, 2001). The potential contribution of OXPHOS defects to the expression of common sporadic neurodegenerative diseases is also often discussed (see Manfredi and Beal, 2000), yet there is no mouse model with mtDNA-based OXPHOS impairment available to directly test this hypothesis.

We have recently demonstrated the feasibility of replacing the endogenous mtDNA of the laboratory mouse, *Mus musculus domesticus*, with that of different mouse species of increasing evolutionary divergence (McKenzie *et al.*, 2004). Our objective in creating such xenomitochondrial mice is to enable a model system where decremental impairments of mtDNA-based

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OXPHOS exist in viable mice. Such mice will then allow direct investigation of different degrees of OXPHOS impairment in tissue pathogenesis, and furthermore allow superimposition of such impairment on nuclear gene models of neurodegenerative disease in crossbreeding experiments.

Since female embryonic stem (ES) cells have not been widely developed for mouse transgenesis, we have been limited by the relatively poor germline success rate of existing lines, having obtained only a few live male animals to date. We have now succeeded in obtaining a homoplasmic female founder, bringing us an important step closer to the creation of mouse lines. Here we describe the genetic basis for the models and their potential utility for studying subtle to mild metabolic disturbances based on mtDNA defects, and for exploring hypothetical OXPHOS contributions to pathogenesis in neurodegenerative diseases.

MATERIALS AND METHODS

Cell Lines

Primary fibroblast lines were created from a 2-day-old laboratory mouse (*Mus musculus domesticus*, CBA × C57BL/6 Fl cross), a 5-week-old *Mus spretus* mouse (*SPRET/Ei*, a gift from Dr. Simon Foote, WEHI, Melbourne, Australia), and newborn pups from *Mus pahari* (*Mus pahari/EiJ*) and *Mus caroli* (*Mus caroli/EiJ*) bred from animals purchased from the Jackson Laboratory (McKenzie *et al*., 2003). A *Mus dunni* primary fibroblast cell line, designated III8C, was obtained from the American Type Culture Collection (Manassas, VA). The mouse ρ^0 cell clone LMEB3 was derived from the parental line LMTK$^-$ by exposure to ethidium bromide (Trounce *et al*., 2000; see also Pinkert and Trounce, 2002).

Production of Xenomitochondrial L-Cell Cybrids

Mouse cybrids were produced by enucleation of mitochondrial donor cells and fusion of the cytoplasts with mouse ρ^0 cells followed by selection for respiratory competent transformants. Procedures were described in detail previously (McKenzie and Trounce, 2000). Cells used as mitochondrial donors included the MD, MS, Mdu, MC, and MP primary fibroblast lines described above.

Production of Xenomitochondrial ES Cell Cybrids

The mouse female ES cell line CC9.3.1 was derived from a 129SvEv-Gpil^c (129S6-Gpil^c) embryo (A. Bradley, Baylor College of Medicine, Houston, TX) and was previously shown to have the potential to contribute to the female germ line and produce normal fertile females (Sligh *et al*., 2000). ES cells were treated with rhodamine 6-G for 3 days prior to fusion with cytoplasts made by enucleation of L-cell xenocybrids, and cybrids selected as previously described (McKenzie *et al*., 2004).

mtDNA Genotyping by Restriction Enzyme Analysis and DNA Sequencing

A 461-bp mtDNA fragment containing part of the D-loop and the tRNA-Phe gene was amplified as previously described (McKenzie *et al*., 2003), and restricted with either StyI or Bsu36I to generate species-specific restriction fragments. The complete cytochrome *b* gene was also amplified from each species' DNA and sequenced (McKenzie *et al*., 2003).

Production of Transmitochondrial Mice

Procedures used for the collection and manipulation of blastocysts have been described in detail elsewhere (Polites and Pinkert, 2002). Recently, xenomitochondrial ES cell injections into mouse blastocysts were also described (Pinkert and Trounce, 2002, in press; McKenzie *et al*., 2004). Briefly, progeny resulting from ES cell injections were initially identified by coat color chimerism (chimeras exhibited varying percentages of agouti fur) and all founders were genotyped using species-specific PCR analyses. Tail DNA from non-coat-color chimeras (black coat color) were also analyzed by PCR for chimerism; Clone S2 male #22 was identified as carrying *Mus spretus* mtDNA in this fashion. Dead or euthanatized animals were dissected and the DNA was extracted from various tissues as described above. PCR and restriction digests were performed to confirm mitochondrial genotypes and tissue distribution.

Founder chimeric (heteroplasmic for MS or Mdu mitochondria) females (G_0) were mated to control C57BL/6NTac males (homoplasmic for MD mitochondria). Offspring (G_1) were homoplasmic as expected, either for the host blastocyst or ES-cell-derived mitochondria. Tail biopsies were taken and DNA extraction, PCR, and restriction digests were performed for confirmation of genotype.

All mice were maintained in a specific pathogen-free barrier facility and were anesthetized by Avertin (2,2,2-tribromoethanol) injection during any potentially painful procedure. Mice were monitored carefully after

Table I. Amino Acid Differences in Species-Specific mtDNA Translation Products (Extrapolated from Cytochrome *b* Sequences)

		Number of amino acid replacements compared with *Mus musculus*				
mt gene product	# Amino acids	*M. spretus*	*M. dunni*	*M. caroli*	*M. pahari*	*Rattus*
nad1	315	5	9	10	15	32
nad2	345	14	24	27	41	88
coxI	514	2	4	4	7	14
coxII	227	1	1	1	1	3
atp8	67	2	4	4	7	14
atp6	226	2	3	4	6	12
coxIII	261	1	2	3	4	9
nad3	114	2	4	5	7	15
nad4L	97	2	4	4	7	14
nad4	459	9	16	18	27	59
nad5	607	21	36	41	61	133
nad6	172	5	9	11	16	34
cytb	**381**	**4**	**7**	**8**	**12**	**26**
Total	3785	70	123	140	211	453

Note. Values for cytochrome *b*, shown in bold, are from complete sequences. Other values are extrapolations based on the relative divergence from *Rattus norvegicus* (*Rattus*).

anesthesia until full recovery. Mice were euthanatized by CO_2 inhalation or Avertin sedation and cervical dislocation. All procedures followed the AVMA guide per institutional guidelines.

RESULTS

We first produced mouse L-cell cybrids harboring mitochondria from the four species of interest. The species were chosen based on increasing evolutionary divergence so that increasing mismatching of the foreign mtDNA subunits and the *Mus musculus* nuclear-encoded subunits would result in mild, increasing OXPHOS defects (McKenzie *et al.*, 2003). These L-cells are amenable for large-scale cultures needed to isolate sufficient intact mitochondria for full OXPHOS characterization. As discussed in detail in McKenzie *et al.*, (2003), these xenocybrids indeed showed a graded mild respiratory deficiency. Only the MP cybrids showed a significant defect of an OXPHOS enzyme, with a 40% deficiency of cytochrome oxidase activity amid preserved activity of other complexes. Yet, all four cybrids showed elevated lactate production around 2-fold in MS and MC cybrids and 2.5-fold in Mdu and MP cybrids (McKenzie *et al.*, 2003). This indicated that subtle OXPHOS defects existed in these cybrids. Increasing the mismatch level by using further diverged mtDNA donors (e.g., *Rattus norvegicus*) resulted in severe OXPHOS defects (McKenzie *et al.*, 2003). The approximate number of amino acid substitutions in mtDNA genes of the four mouse species used, based on the cytochrome *b* sequences and compared with *Mus musculus*, are shown in Table I.

We then produced ES cell cybrids with all four constructs, by first treating the female ES cell line CC9.3.1 with rhodamine 6-G prior to fusion with cytoplasts from the mitochondrial donor cybrids. These cybrids were genotyped to identify homoplasmic or heteroplasmic clones, and chosen clones injected into mouse blastocysts.

Eleven xenomitochondrial ES cell clones (nine homoplasmic and two heteroplasmic clones) were injected into mouse blastocysts (see Table II). In total, 1684 blastocysts were injected yielding 319 pups. Based on coat-color chimerism and/or confirmatory PCR genotyping, 56 chimeras were obtained, with percentage of chimerism ranging from approximately 1% to over 99%. Nineteen chimeric females were weaned and bred to control C57BL/6NTac males (Table III). Of these breeders, 18 pairs were fertile, producing 583 pups. Five of the female chimeras (all resulting from injection of homoplasmic xenomitochondrial ES cells) that were 1–90% chimeric on the basis of coat color proved germline competent; however, the yield of homoplasmic mutants was low. Of their 135 combined/analyzed offspring, there were 4 males and 3 females produced that were homoplasmic for the introduced mitochondrial genotype. While most germline-competent chimeras delivered a single homoplasmic mutant, one female did deliver 3 *M. dunni* xenomitochondrial offspring; however, all three were dead at birth. At this

Table II. Generation of Chimeric Heteroplasmic Founder Mice

Clone	Ova injected	Founders	Weaned	Chimeras
S2 (*Mus spretus*)	227	66	42	13/61
S3 (*Mus spretus*)	194	28	14	5/26
C8 (*Mus caroli*)	23	4	4	1/4
S15 (*Mus caroli*)	154	30	11	5/26
C23 (*Mus caroli*)	152	22	7	0/16
D6 (*Mus dunni*)	169	43	30	7/38
D7 (*Mus dunni*)	589	116	59	24/102
P5 (*Mus pahari*)	63	5	0	1/5
P12 (*Mus pahari*)	45	0	0	0
P17 (*Mus pahari*)	59	5	1	0/4
P24 (*Mus pahari*)	11	0	0	0

Note. Chimeric heteroplasmic founder mice were generated from different ES xenomitochondrial cybrid clones. *Mus spretus* and *Mus dunni* data were previously reported (McKenzie *et al*., 2004). Using restriction digest analysis, injected clones were homoplasmic for the respective species mtDNA with the exception of two *Mus caroli* cell lines (C15 and C23) that were purposefully heteroplasmic (approx. 50% *Mus caroli* and 50% *Mus musculus domesticus* mitochondria) toward development of mitochondrial dynamics/competition model systems.

Table III. Breeding of Chimeric Founder Females

Clone	Female (% chimerism)	Offspring	Germline transmission	Germline offspring, status
S2 (*Mus spretus* homoplasmic clone; 13 chimeras in total)				
	13 ♀(20)	19	0/19	
	26 ♀(15)	54	0/47	
	33 ♀(5)	10	0/5	
	37 ♀(1)	20	1/15	1 ♂, viable
S3 (*Mus spretus* homoplasmic clone; 5 chimeras in total)				
	02 ♀(10)	46	0/35	
	16 ♀(20)	29	0/27	
	17 ♀(5)	9	0/9	
C15 (*Mus caroli* heteroplasmic clone; 5 chimeras in total)				
	06 ♀(40)	32	0/17	
D6 (*Mus dunni* homoplasmic clone; 7 chimeras in total)				
	01 ♀(20)	48	0/48	
	27 ♀(20)	33	0/33	
D7 (*Mus dunni* homoplasmic clone; 24 chimeras in total)				
	01 ♀(90)	31	0/26	
	02 ♀(10)	32	0/29	
	34 ♀(80)	34	1/33	1 ♂, viable
	35 ♀(80)	No offspring		
	55 ♀(90)	31	3/23	1 ♂ + 2 ♀ dead
	62 ♀(95)	68	0/38	
	63 ♀(85)	64	1/51	1 ♂, viable
	87 ♀(85)	42	0/33	
	89 ♀(80)	21	1/13	1 ♀, viable

Note. Weaned chimeric founder females (heteroplasmic for Ms, Mc, or Mdu mitochondria) were bred with C57BL/6NTac (B6) males to generate heteroplasmic or homoplasmic offspring. Viable xenomitochondrial offspring were all derived from homoplasmic cell lines and exhibited agouti fur pigmentation. Homoplasmy was confirmed by PCR and Southern blot analysis. All other offspring from female lineages bred to control B6 males had black fur. Chimera S3 #16 died at 5 weeks of age. Ovaries were harvested and transferred to nude female recipients to attempt lineage rescue. Pups from chimera D7 #55 died or were killed within 24 h of birth; gender and homoplasmy were confirmed by PCR and/or Southern blot analysis. Portions of these data were previously reported (McKenzie *et al*., 2004).

time, *M. dunni* female #89 and her homoplasmic daughter represent the first mutant lineage that has the capacity to go beyond the first generation.

It should be noted that male chimeras and male first-generation homoplasmic mutants were all bred to control C57BL/6NTac females. Some of the chimeric males were infertile; others sired only black offspring—none of which harbored introduced mitochondria. In contrast, the homoplasmic males were fertile, and while both black and agouti offspring were obtained, as expected, none harbored the introduced (*M. spretus* or *M. dunni*) mitochondria, on the basis of PCR analyses.

While survival of pups to weaning was lower than anticipated, survival rates of control litters on unrelated projects were correspondingly low and likely reflected transition into a new animal facility. Over the last year, survival rates across all projects have increased and now reflect a seasonally correlated pattern (but under a 15% loss across the last year).

It appears that chimeric founders and homoplasmic male offspring have similar lifespans when compared to other mice within the same vivarium suite (but unrelated to this project). The one observation that has not been fully explained or characterized relates to three chimeric females (two germline and one nongermline) that were moribund or found dead following the birth of their third or fourth litters. While necropsy and histopathological reports point to a periparturient dystocia as a likely cause of death (C. A. Pinkert, R. B. Baggs, and I. A. Trounce, unpublished data), it is unclear if this phenomenon is directly related to their mitochondrial genetics and a stress susceptibility triggered by the periparturient cascade of obviously energy-dependent events.

DISCUSSION

Despite much interest in the potential role of mtDNA variants as contributors to neurodegenerative disease, there remains no technology for creating targeted mutations in the mtDNA. The number of mouse cell mtDNA mutants described in the literature is also quite limited, and of those characterized for OXPHOS enzymology most appear to result in severe defects (see Trounce and Pinkert, in press). Faced with these barriers, we undertook the approach of introducing foreign mouse species mtDNAs directly into zygotes and ES cells. Initially, methods for mitochondrial isolation and inter-species transfer were devised using mitochondria injection into mouse zygotes (Pinkert *et al.*, 1997). Transmitochondrial founders were obtained and germline transmission of the heteroplasmic state was observed in maternal lineages (Irwin *et al.*, 1999; Pinkert and Trounce, 2002). To pursue homoplasmic models, we then used the cybrid route, creating cybrid ES cells followed by blastocyst injection, chimera production, and breeding to identify homoplasmic transmitochondrial offspring (McKenzie *et al.*, 2004).

The CAP^R mutant used by Sligh *et al.* (2000)—to produce the first transmitochondrial homoplasmic mice—had moderate OXPHOS defects consequent to the translation defect associated with the mtDNA 16S rRNA mutation (Levy *et al.*, 1999). Yet, the homoplasmic pups produced by Sligh *et al.* (2000), died shortly after birth with a metabolic acidosis and cardiomyopathy. In view of these important findings, we have aimed for the generation of viable mouse lines with mild OXPHOS defects. Our most divergent *Mus musculus* xenocybrid, harboring *Mus pahari* mtDNA, appears to result in a less severe defect compared to the multiple partial OXPHOS complex defects seen in the CAP^R mutant. Our other xenocybrids should show only very mild defects. How then may these be relevant or informative in modelling mtDNA and OXPHOS contributions to neurodegenerative diseases?

The link between inhibition of OXPHOS complex I and Parkinson's disease (PD) is now firmly established by important toxin models of this disease (see Dawson and Dawson, 2003), first using MPTP in mice (Heikkila *et al.*, 1984) and later rotenone in rats (Betarbet *et al.*, 2000). An emerging view is that the increase in reactive oxygen species (ROS) consequent to the complex I inhibition is more important to dopaminergic cell loss in these models than the decreased ATP production per se (see Sherer *et al.*, 2003). A link between mtDNA polymorphisms in humans and increased or decreased risk of PD can therefore be hypothesized, and our models with increased polymorphisms in the mtDNA-encoded complex I genes may be useful in further probing such links. Claims have indeed been made for association of mtDNA variants with both sporadic PD and Alzheimer's disease (Trimmer *et al.*, 2004a,b), but to date no specific variants have been identified.

The xenomitochondrial approach introduces an "uncontrolled" range of amino acid variation, in the sense that we do not choose the changes. Table I shows the approximate degree of this variation expected for the four species used in this work, compared to *Mus musculus*. Sequencing of the complete mtDNAs of the species used is underway (W. K. Pogozelski, C. A. Cassar, I. A. Trounce, and C. A. Pinkert, unpublished data), so the table represents extrapolation from the limited comparative sequence data we have to date. The most divergent construct, using *mus pahari* mtDNA, has around 1 in 20 amino acids substituted. The most conserved construct, with *Mus spretus* mtDNA, has around 1 in 50 amino acids substituted. It

can be appreciated from the table that these replacements are not distributed evenly throughout the mtDNA, with the complex I genes ND5, ND2, and ND4 accounting for more than half, while genes such as COI, COII, COIII, and ATP6 show a much lower level of replacements. The *Mus pahari* cybrids show a mild cytochrome oxidase deficiency with apparently normal function of the other respiratory chain complexes, while the other three constructs show OXPHOS enzyme function that is not significantly different to control cybrids in vitro (McKenzie *et al*., 2003). Using cell lactate production as a more sensitive indirect measure of oxidative ATP production, all constructs showed significantly elevated lactate compared with the wild-type cybrid (McKenzie *et al*., 2003).

These initial findings suggest that polymorphism in the complex I genes is better tolerated than that in the more highly conserved complex IV and ATP synthase genes. Our homoplasmic MS male mouse (70 amino acid replacements) and two Mdu males (120 replacements), have shown outwardly normal development and aging in our studies to date. We anticipate that the MC and MP mice will begin to exhibit signs of age-related disease, but full evaluation of these different models awaits the production of females and mouse lines.

Production of transmitochondrial mouse models has proven to be a daunting challenge. In addition to early microinjection studies, and the ES-cell-based work, we have also attempted nuclear transfer studies to try to expedite development of xenomitochondrial models (K. Takeda *et al*., unpublished data).

Breeding of founder females allowed production of germline mice, albeit at low rates, most likely related to the low germline competency of the ES cells. Alternatively, it is possible that homoplasmic offspring may have to surmount a metabolic crisis based on a mitochondrial–nuclear mismatch that is developmentally critical as noted in children. Boles and coworkers (2003) have described a severe but reversible cardiomyopathy in a number of infants, that was associated with an mtDNA D-loop heteroplasmy. Reversible cytochrome oxidase deficiencies, in particular, have also been previously described (see DiMauro and Schon, 2001). As such, it is possible that a comparable situation exists in our mice. If this hypothesis holds, then the homoplasmic first-generation offspring represent those animals that survive beyond the early postnatal period surmounting a prenatal or perinatal metabolic crisis. Following this time point, they then develop on through adulthood, where both *Mus spretus* and *Mus dunni* “xenomitomice” appear quite normal.

It is most exciting that a live female has been produced indicating that if an animal must surmount a putative metabolic crisis as we have hypothesized, then females may very likely have a comparable viability to the males that we preliminarily characterized. While this female may represent a developmental anomaly, she does illustrate that the model is valid. Further studies with new murine ES cell lineages may also heighten our overall experimental efficiencies (C. A. Cassar, E. M. Vollmers, I. A. Trounce, and C. A. Pinkert, unpublished data). In addition, from preliminary analysis of chimeric founders, it appears that the animals on hand have preferentially maintained wild-type mitochondrial genotypes at the expense of the introduced (mutant) mitochondrial genomes. This observation would also account for the limited germline transmission results obtained to date.

Because of the unique features of mitochondrial genetics, nuclear gene knockout mice will not supersede such models, and the creation of mitochondrial mutants will shed additional light upon mitochondrial function and dynamics of heteroplasmy. Such mice will also be of great interest to researchers of Mendelian and sporadic neurodegenerative diseases where oxidative stress or secondary mitochondrial impairment is implicated. Lastly, by crossbreeding mutant models with nuclear gene knockout mice, a new realm of experimental genetics will be available for investigating nuclear–mitochondrial interactions.

ACKNOWLEDGMENTS

We gratefully acknowledge the scientific discussion and insights from D. Cotton, R. B. Baggs, M.P. Massett, J. P. Corsetti, M. H. Irwin, and L. W. Johnson. We appreciate the assistance of Maria Chiotis, Jamie Littleton, Kendra A. Bussey, Ellen M. Vollmers, C. Owen Smith, Vishwala Kasbekar, Dong Woong Kim, and Christopher Deigel. The CC9.3.1 cell line was a gift of A. Bradley. This work was supported in part by grant 145719 from the NH and MRC of Australia (IAT), NIH grants RR16286 and DE12634, and institutional funds (CAP).

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