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Authors

Ma, Hong
Gutierrez, Nuria Marti
Morey, Robert
et al.

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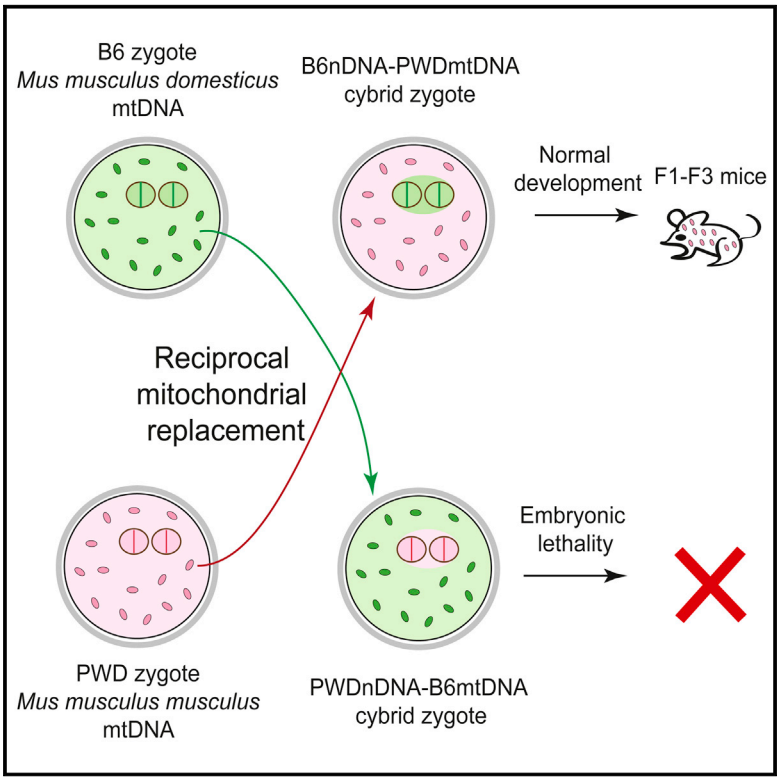
Graphical Abstract



Highlights

- mtDNA replacement (MR) between B6 and PWD mice supports preimplantation development
- MR in PWD zygotes, but not in B6, caused post-implantation embryonic lethality
- Divergence of mtDNA contributes to interspecies reproductive isolation in mammals

Authors

Hong Ma, Nuria Marti Gutierrez, Robert Morey, ..., Don P. Wolf, Louise C. Laurent, Shoukhrat Mitalipov

Correspondence

mitalipo@ohsu.edu

In Brief

Compatibility between nuclear and mitochondrial genomes is important for normal reproductive fitness. Mitalipov and colleagues show that reciprocal mtDNA replacement in zygotes between two mouse strains (B6 and PWD) results in post-implantation embryonic lethality, suggesting that mtDNA sequence divergence between mammalian species contributes to a reproductive barrier.

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Article

Incompatibility between Nuclear and Mitochondrial Genomes Contributes to an Interspecies Reproductive Barrier

Hong Ma,[1,2,5] Nuria Marti Gutierrez,[1,2,5] Robert Morey,[3] Crystal Van Dyken,[1,2] Eunju Kang,[1,2] Tomonari Hayama,[1,2] Yeonmi Lee,[1,2] Ying Li,[1,2] Rebecca Tippner-Hedges,[1,2] Don P. Wolf,[1,2] Louise C. Laurent,[3] and Shoukhrat Mitalipov[1,2,4,*]
[1]Center for Embryonic Cell and Gene Therapy, Oregon Health & Science University, 3303 SW Bond Avenue, Portland, OR 97239, USA
[2]Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Oregon Health & Science University, 505 NW 185th Avenue, Beaverton, OR 97006, USA
[3]Department of Reproductive Medicine, University of California, San Diego, Sanford Consortium for Regenerative Medicine, 2880 Torrey Pines Scenic Drive, La Jolla, CA 92037, USA
[4]Knight Cardiovascular Institute and Departments of Obstetrics and Gynecology, Molecular and Medical Genetics, and Biomedical Engineering, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA
[5]Co-first author
*Correspondence: mitalipo@ohsu.edu
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SUMMARY

Vertebrate cells carry two different genomes, nuclear (nDNA) and mitochondrial (mtDNA), both encoding proteins involved in oxidative phosphorylation. Because of the extensive interactions, adaptive coevolution of the two genomes must occur to ensure normal mitochondrial function. To investigate whether incompatibilities between these two genomes could contribute to interspecies reproductive barriers, we performed reciprocal mtDNA replacement (MR) in zygotes between widely divergent *Mus m. domesticus* (B6) and conplastic *Mus m. musculus* (PWD) mice. Transfer of MR1 cybrid embryos (B6nDNA-PWDmtDNA) supported normal development of F1 offspring with reduced male fertility but unaffected reproductive fitness in females. Furthermore, donor PWD mtDNA was faithfully transmitted through the germline into F2 and F3 generations. In contrast, reciprocal MR2 (PWDnDNA-B6mtDNA) produced high embryonic loss and stillborn rates, suggesting an association between mitochondrial function and infertility. These results strongly suggest that functional incompatibility between nuclear and mitochondrial genomes contributes to interspecies reproductive isolation in mammals.

INTRODUCTION

Species divergence can occur when reproductive barriers between populations are established through breeding failure secondary to mating discrimination or failed fertilization because of gamete incompatibility (prezygotic isolation) (Lee et al., 2008; Rieseberg and Willis, 2007). Reproductive barriers can also involve detrimental hybrid postzygotic development because of chromosome number differences, chromosomal rearrangements, and other nDNA divergencies (Rieseberg and Willis, 2007). Moreover, nDNA to mtDNA incompatibility caused by sequence divergence may also play a major role in interspecies hybrid survival (Burton et al., 2013; Gershoni et al., 2009).

Maternally inherited mammalian mtDNA is typically present at high copy numbers (from hundreds to thousands) compared to only two copies of nuclear genes (Wallace, 2007). House mouse mtDNA is a 16,300 bp, double-stranded circular molecule encoding 13 mitochondrial proteins of OXPHOS, 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) (Koopman et al., 2012; McBride et al., 2006). The remaining OXPHOS proteins are encoded by the nuclear genome (Staubach et al., 2012). These protein subunits encoded by different genomes must be highly compatible to maintain the structural and biochemical properties required for uncompromised enzymatic function. Moreover, mtDNA replication, transcription, and translation are exclusively governed by factors encoded by nDNA (Woodson and Chory, 2008). Nuclear-encoded proteins must recognize and bind to regulatory motifs in mtDNA for proper function. Because of these close interactions, mitochondrial and nuclear genomes undergo adaptive co-evolution to maintain fitness in aerobic metabolism (Bayona-Bafaluy et al., 2005; Burton et al., 2013; Camus et al., 2015; Dowling et al., 2008; Pichaud et al., 2012; Wolff et al., 2014). Interspecies nDNA-mtDNA incompatibility is clearly demonstrated in experimentally induced, interspecies cytoplasmic hybrid (cybrid) cells when human mtDNA is maintained in the presence of great ape nuclear backgrounds, albeit with severe OXPHOS abnormalities (Bayona-Bafaluy et al., 2005). Moreover, mouse cybrid cells carrying rat mtDNA display a slower growth rate, reduced O_2 consumption, and reduced OXPHOS complex I and IV activities in the presence of normal mitochondrial protein synthesis. This combination could represent problems in the assembly of the OXPHOS system (Dey et al., 2000).

The impact of interspecies nDNA-mtDNA incompatibility on the organismal level is less obvious since reproductive barriers prevent the birth of live offspring in most hybrids. However,

Table 1. mtDNA Heteroplasmy in Cybrid Zygotes after Reciprocal MR

Treatment	Nuclear Genotype	Donor mtDNA Genotype	No. of Zygotes	Donor mtDNA (%)	Carryover mtDNA (%)
MR1	B6	PWD	10	84 ± 1.2	16 ± 1.2
MR2	PWD	B6	10	90 ± 0.7	10 ± 0.7

genetic mechanisms responsible for interspecies, postzygotic reproductive isolation have been studied in hybrid house mouse subspecies including *Mus musculus domesticus* and *Mus musculus musculus*. Divergence of these subspecies, from a common ancestor, occurred in the last 0.5 million years, corresponding, in numbers of generations and relative molecular divergence, to chimpanzees and humans (Gregorová et al., 2008). Incompatibilities between genes located on the X chromosome and several autosomal regions have been identified as critical for the phenomenon known as hybrid male sterility (Bhattacharyya et al., 2014; Janoušek et al., 2012). However, while the role of mtDNA compatibility has been considered, it has not been tested experimentally (Dzur-Gejdosova et al., 2012; Lane, 2011).

One limitation in studies of nDNA-mtDNA interactions in hybrid mice is that while mtDNA in F1 hybrid zygotes is exclusively contributed by the female species, the nuclear genome is composed of chromosomes contributed from both populations. The presence of nuclear alleles from the mtDNA donor likely provides an adequate level of nDNA-mtDNA interactions and mitochondrial function for F1 hybrid survival, thus masking potential interspecies incompatibility between the two genomes. Even in conplastic strains derived by multigenerational backcrossing of F1 hybrid females against parental males, positive selection and introgression of chromosomes from the mtDNA donor species allows offspring survival, thereby maintaining intra-species nuclear-to-mitochondrial interaction (Gregorová et al., 2008).

We set out to examine mtDNA-nDNA interactions challenging the concept that incompatibility could be a postzygotic genetic isolation factor that contributes to reproductive barriers and species divergence. We reasoned that nuclear-mitochondrial interactions should be suboptimal in distantly related mouse subspecies. Further, the limitations of classical hybrids mentioned above could be overcome with the generation of cybrid mice by reciprocal mtDNA replacement (MR) through pronuclear transfer (PNT) between C57BL/6N (B6, *Mus m. domesticus*) and conplastic C57BL/6J-mt$^{PWD/Ph}$/ForeJ (PWD) zygotes that carry exclusively *Mus m. musculus* mtDNA and few PWD chromosomal regions on a largely B6 nuclear background (Gregorová et al., 2008). This approach allowed the maintainance of a homozygous nuclear genetic background from one subspecies and an mtDNA genotype from another population. Cybrid mice with PWD nDNA and B6 mtDNA were less fit and showed profoundly impaired fetal development in utero (stillborn) or infertility compared to mice containing the opposite combination (B6 nDNA and PWD mtDNA). Functional rescue of incompatibility occurred in hybrid mice containing mixed nuclear genomes from both subspecies.

RESULTS

Intersubspecies MR Did Not Affect Preimplantation Development

To generate transmitochondrial mice carrying divergent mtDNA genotypes, we selected inbred C57BL/6N (B6) carrying *Mus m. domesticus* mtDNA and the conplastic strain C57BL/6J-mt$^{PWD/Ph}$/ForeJ (PWD) carrying *Mus m. musculus* mtDNA. We analyzed the genetic distance between these two subspecies based on the total number of SNP differences in mtDNA, and the number of SNPs affecting amino acid coding and RNA genes. PWD and B6 mtDNA sequences differed at 391 SNPs as confirmed by whole mtDNA sequencing, corresponding to 2.4% of the genome. These included 26 SNPs in rRNAs, 18 in tRNAs, 34 in noncoding region, and the remaining 313 with 32 non-synonymous substitutions in protein-coding genes (Table S1). In contrast, intraspecies mtDNA differences between NZB and B6 strains (both *Mus m. domesticus)* accounted for a total of 90 SNPs corresponding to about 0.6% of the genome (Sharpley et al., 2012).

Next, we collected in vivo fertilized zygotes and performed reciprocal MR between B6 and PWD mice by PNT (Figure S1A, MR1 and MR2). Comparisons were made to two different controls: autologous MRs performed reciprocally between zygotes from the same strain (Figure S1B, autologous1 and autologous2) and intact zygotes without MR (intact1 and intact2). Survival rates after the PNT procedure were comparable between MR1 (91% ± 2%) and MR2 (93% ± 1%) and to autologous controls (97% ± 1.3% and 95% ± 3% in autologous1 and 2, respectively). Since PNT is associated with carryover of residual cytoplasm surrounding the pronuclei, and hence host mtDNA, we measured mean heteroplasmy levels of both donor and carryover mtDNA in reconstructed cybrid zygotes by amplification refractory mutation system quantitative PCR (ARMS-qPCR). The donor mtDNA level was 84% ± 1.2% in MR1 and 90% ± 0.7% in MR2 (Table 1), consistent with a previous study (Wang et al., 2014).

A total of 495 cybrid zygotes in MR1 and 490 in MR2 was studied (Table S2). Similar to controls, 85% of zygotes in both MR1 and MR2 cleaved, and the percentages of embryos developing to 8-cell in MR1 (73%) and MR2 (71%) were comparable to the autologous groups (86% to 90%) and intact controls (84% to 91%) (Table S2). Significantly reduced rates of development to the blastocyst stage were observed in both MR1 and MR2 (68%) when compared to intact controls (83% to 90%) (Kruskal-Wallis test, $p < 0.05$) (Table S2). However, similar reduced blastocyst development rates in MR1 and MR2 were not significantly different than those observed in autologous MR groups (81% in autologous1 and 82% in autologous2; Table S2), indicating that PNT microsurgery rather than mtDNA incompatibility was the cause.

Since failed fertilization due to gamete incompatibility is recognized as a mechanism of prezygotic isolation (Rieseberg and Willis, 2007), we asked if intersubspecies MR affects fertilization. To this end, spindle-chromosomal structures (spindles) were isolated from unfertilized mature metaphase II (MII) oocytes from B6 mice and transferred (spindle transfer, ST) into enucleated recipient MII oocytes from PWD mice (Figure S2A; ST1). Conversely, spindles from PWD mice were transferred into enucleated MII oocytes from B6 mice (Figure S2A, ST2). The

survival rates after MR were high and comparable between ST1 (91% ± 2.6%) and ST2 (93% ± 3.6%). Reconstructed oocytes after ST were then fertilized with sperm collected from the nuclear donor strain using standard in vitro fertilization (IVF). As control for procedural effects, IVF was also conducted on intact oocytes without MR (Figure S2B). Similar fertilizaton levels were observed in both ST1 (73%) and ST2 (69%) and in controls (72% in IVF1; 78% in IVF2, respectively (Figure S2C), indicating that intersubspecies MR did not affect fertilization.

Intersubspecies MR Caused Embryonic Lethality

To determine whether intersubspecies MR affects post-implantation development, cybrid embryos at the blastocyst stage were transferred into pseudopregnant ICR (CD-1) female mice (Kang et al., 2014). After transfer of 341 embryos from MR1 into 37 synchronized recipients, 11 (30%) females became pregnant and 8 of them (73%) delivered 14 live pups (5 males and 9 females) (4% per ET) (Table 2). This live pup rate was comparable to autologous (4% per ET) and intact (3% per ET) controls (Table 2), suggesting that postimplantation development of B6 mice carrying donor PWD mtDNA was unaffected. However, in the reciprocal MR2, although the initial pregnancy rate (32%, 10/31) as determined by weight gain was similar to autologous2 (29%) and intact2 (31%) control, fewer pregnant recipients (40%, 4/10) delivered pups compared to controls (75%) (Table 3). Total number of full-term pups in MR2 (1.7%) was also low compared to autologous2 (7%) and intact2 (6%) controls (Table 3). Only one live pup (0.3% per ET) out of 359 transferred blastocysts was obtained (Table 3), while the remaining five full-term offspring (83%) in MR2 were stillborn (Table S3). These outcomes were significantly different when compared to autologous2 (Kruskal-Wallis test, $p < 0.05$) (Table 3; Table S3). This impedance in post-implantation development in MR2 most likely reflects incompatibility between nuclear and mtDNA genomes.

We speculated that if incompatability between nDNA and mtDNA exists, it might be reversed in hybrid embryos carrying nuclear chromosomes from both subspecies. Therefore, we generated PWD-DBA zygotes by crossing PWD females and DBA/2 males (*Mus m. domesticus*) to produce hybrid zygotes carrying both PWD and DBA nuclear genomes and PWD mtDNA. Parenthetically, DBA/2 males consistently produce larger litters than other inbred strains, and DBA/2 mtDNA only differs from B6 mtDNA at 1 SNP. We also generated B6-DBA zygotes (designated BDF1; Charles River) by crossing C57BL/6 females and DBA/2 males (both *Mus m. domesticus*) carrying both B6 and DBA nuclear genomes and B6 mtDNA. Reciprocal MRs between PWD-DBA and B6-DBA were performed (Figure S1C, MR3 and MR4) in conjunction with autologous MRs (Figure S1D) and intact controls. The post-implantation developmental potential of reconstructed embryos was then examined. As anticipated, the phenomena of embryonic lethality in MR2 was rescued in MR4 hybrid mice when the DBA allele was present in the nuclear genome. After transfer of 276 MR4 blastocysts into 23 recipients, 33 live pups (17 males and 16 females) were obtained at term (11% per ET) (Table 3) comparable to the autologous group and intact controls (Table 3). 14 live pups (4 males and 10 females) were also obtained at term (5% per ET) in the MR3 (Table 2).

Table 2. Full-Term Development of Cybrid B6 and B6-DBA Blastocysts

Treatment	Nuclear Genotype[a]	mtDNA Donor Genotype	Embryos Transferred (ET)	Total Recipients (R)	Pregnant R (%/R)[b]	R Delivered Pups (%/Pregnant)	Total Full-Term Pups (%/ET)		Live Pups (%/ET)	
MR1	B6 (d)	PWD (m)	341	37	11	30%	8	73%	20	6% ± 2%	14	4% ± 2%
Autologous1	B6 (d)	B6 (d)	130	12	4	33%	2	50%	5	4% ± 2%	5	4% ± 2%
Intact1	B6 (d)	B6 (d)	158	13	4	36%	3	75%	6	4% ± 3%	5	3% ± 3%
MR3	B6-DBA (d-d)	PWD (m)	260	21	14	67%	10	71%	27	10% ± 3%	14	5% ± 3%
Autologous3	B6-DBA (d-d)	B6 (d)	161	14	9	64%	4	44%	9	6% ± 3%	9	6% ± 3%
Intact3	B6-DBA (d-d)	B6 (d)	177	13	8	62%	5	63%	19	11% ± 4%	18	10% ± 4%

[a](d) or (m) indicate *Mus m. domesticus* or *Mus m. musculus* mice, respectively

[b]Pregnancy was estimated based on recipient weight gain 1 week after ET (>2 g over initial value)

Table 3. Full-term Development of Cybrid PWD and PWD-DBA Embryos

Treatment	Nuclear Genotype[a]	Donor mtDNA Genotype	Embryos Transferred (ET)	Total Recipients (R)	Pregnant R (%/R)	R Delivered Pups (%/Pregnant)[b]	Total Full-Term Pups (%/ET)		Live Pups (%/ET)[c]	
MR2	PWD (m)	B6 (d)	359	31	10	32%	4	40%	6	1.7% ± 1%	1	0.3% ± 0.3%[a]
Autologous2	PWD (m)	PWD (m)	104	14	4	29%	3	75%	7	7% ± 3%	7	7% ± 3%[b]
Intact2	PWD (m)	PWD (m)	227	11	4	31%	3	75%	14	6% ± 3%	9	4% ± 2%[a]
MR4	PWD-DBA (m-d)	B6 (d)	276	23	15	65%	12	80%	41	15% ± 3%	33	11% ± 3%[b]
Autologous4	PWD-DBA (m-d)	PWD (m)	132	9	5	56%	5	100%	15	11% ± 4%	15	11% ± 4%[b]
Intact4	PWD-DBA (m-d)	PWD (m)	103	7	3	43%	3	100%	16	16% ± 8%	16	16% ± 8%[b]

[a](d) or (m) indicate *Mus m. domesticus* or *Mus m. musculus* mice, respectively.
[b]Based on weight gain measurements, four recipients in MR2 lost pregnancies between days E9.5 and E14.5, while another two recipients lost pregnancies between days E14.5 and E20.5. Only 4 recipients in this group (40%) delivered pups.
[c]Different superscripts within a column are significantly different ($p < 0.05$).

In summary, normal post-implantation development was documented in cybrid MR1 embryos with the B6nDNA-PWDmtDNA combination (Table 2). In contrast, only one live pup out of 359 transferred blastocysts was born from MR2 cybrid embryos with the PWDnDNA-B6mtDNA combination in conjuction with a significantly higher embryonic lethality and stillborn rate (Table 3; Table S3). These results imply that suboptimal PWDnDNA-B6mtDNA interactions are a main barrier to cybrid survival.

Intersubspecies MR Caused Reduced Reproductive Fitness in F1 Progeny

All F1 offspring were weaned at 3 weeks and grew to adulthood without any obvious abnormalities. After achieving sexual maturity, we investigated whether intersubspecies MR affected reproductive ability. For this purpose, we crossed all F1 offspring in MR1 (except for one male sacrified for heteroplasmy assays) and MR2 to control mice with the same nuclear backgound. Breeding pairs were housed two per cage for a maximum of 60 days unless noted, and a minimum of two breedings were attempted per experimental mouse. We also included two females from MR3 and three females from MR4 as controls in the reproductive fitness study.

Two F1 males from the MR1 cross sired one or two litters of normal size, while the other two males, when paired with at least two different control females, did not establish a pregnancy, indicating male infertility. To dissect the causes underlining reduced male fertility, we examined several male reproductive parameters including testis weight, sperm count, and sperm motility. All males were similar in overall body weight, testes weight, sperm count, and sperm motility (Table S4). Further, histological analyses revealed no significant findings in testis and other major organs that might explain male sterility.

In contrast to the reduced reproductive outcome in some males, all nine F1 females in the MR1 cybrids became pregnant; three gave birth to one or two litters of normal size and viability, while six females showed low rates of pup survival with significant stillborn rates varying from 60% to 100% during the 6–7 month trial, reflecting reduced fecundity (Table S5). We also observed significantly higher stillborn rates in these animals when compared to others in the group or to females of hybrid mice in the MR3 group (Tables S5).

We reasoned that compromised mitochondrial respiratory function secondary to suboptimal nDNA-mtDNA interactions could explain reduced reproductive fitness. A total of 32 non-synonymous substitutions exists between B6 and PWD mtDNA with 26/32 (81%) located in genes encoding protein subunits for OXPHOS complex I and 2 non-synonymous substitutions (6%) located in genes encoding subunits for complex IV. Therefore, we performed complex I and IV activity measurements in muscle tissues recovered from all males and females in MR1 and two females in MR3. Males 3 and 4 with no opffsring showed levels of mitochondrial complex I and IV activities similar to those of males with normal fertility from the same group ($p > 0.05$, Table S5). However, one female with high stillborn rates (female 6) showed significantly reduced mitochondrial complex I and IV activity while the other three (females 7–9) showed activities similar to MR3 controls or to the females with low stillborn rates from the same group (Table S5). Thus, reduced male fertility and female fecundity and increased stillborn rates in some MR1 F1 pups

Table 4. Fertility and Mitochondrial Activity in MR2 and MR4 F1 Females

Group	Female	Nuclear Genotype	Donor mtDNA Genotype	Pregnant	No. Term/ No. Pregnant	Live F2 Pups (%/Term)		Complex I Activity (%)	Complex IV Activity (%)
MR2	Female1	PWD	B6	–	–	–	–	65 ± 1.4[a]	81 ± 3.1
MR4	Female1	PWD-DBA	B6	+	13/2	13	100	72 ± 2.2[b]	78 ± 9.0
	Female2	PWD-DBA	B6	+	24/2	24	100	79 ± 5.1[b]	82 ± 5.9
	Female3	PWD-DBA	B6	+	20/2	20	100	90 ± 0.4[b]	84 ± 4.5
Mean								80 ± 2.6	81 ± 6.5

[a]Different superscripts within a column indicate significant difference ($p < 0.05$).
[b]Different superscripts within a column indicate significant difference ($p < 0.05$).

did not correlate with decreased mitochondrial OXPHOS activity. This outcome could reflect compromised regulation of transcription or translation of mtDNA genes secondary to suboptimal nDNA-mtDNA interactions.

In contrast to the 100% fertility rate observed in F1 females from MR1, the only surviving F1 female in MR2, when paired for 5 months with males, did not become pregnant before she expired, suggesting infertility (Table 4). Pathological examinations of the ovary and other major organs for abnormalities that might cause infertility revealed no significant findings. Over the same time period, three hybrid F1 females in the control MR4 group had two gestations and produced 100% living F2 pups (Table 4). The complex I activity in the MR2 female was 65% ± 1.4%, significantly lower than the mean activities in the MR4 females at 80% ± 2.6% (Student's t test, $p < 0.05$) (Table 4). We also measured complex IV activity in muscle tissues from the same animals, and its activity in the MR2 female was 81% ± 3.1%, indistinguishable from the mean activity of 81% ± 6.5% in MR4 control females (Table 4). These findings suggest that infertility in the MR2 female is associated with impared mitochondrial OXPHOS activities secondary to detrimental nDNA-mtDNA interactions, particularly in complex I function.

Candidate Nuclear Genes Responsible for mtDNA Incompatibility

Since an estimated 99.87% of nDNA sequences in the C57BL/6J-mt$^{PWD/Ph}$/ForeJ conplastic mouse are from B6, with only 0.13% from the wild-type PWD mouse (Gregorová et al., 2008), we hypothesized that the regions of introgressed PWD sequence in the nDNA of the conplastic strain might be responsible for compatibility with PWD but not with B6 mtDNA. To identify these regions, we performed whole genome sequencing (WGS) on nDNA from the conplastic strain and called SNPs that were discordant compared to B6 ($p < 0.0001$; n = 15,439; Table S6). For initial confirmation of the wild-type PWD sequences, we compared this list of discordant SNPs to a database of 3,432 SNPs known to distinguish between the B6 and PWD mice (dbSNP Build 137; http://www.informatics.jax.org/snp) and found 28 PWD-specific SNPs in conplastic C57BL/6J-mt$^{PWD/Ph}$/ForeJ (Table S7). To find additional genomic regions containing sequences retained from the PWD genome, we filtered for discordant SNPs that were located in regions of the genome that coded for mRNAs. These discordant SNPs were located in 2,139 genes (Table S8), 79 of which were found to encode gene products with a high confidence score for mitochondrial localization (Calvo et al., 2016). Furthermore, we discovered SNPs in: *Immp2l*, which is involved in mitochondrial protein processing; *Gfm2*, which is important for mitochondrial protein translation; *Bcl2l1*, a gene involved in mitochondrial organization; and *P2rx7*, which contributes to regulation of mitochondrial depolarization. These data support the hypothesis that the nDNA of the conplastic PWD mice used in the current study underwent positive selection and contains PWD nuclear DNA sequences that promote offspring survival and reproductive fitness in the conplastic mice by maintaining intra-species (PWD-to-PWD) nuclear-to-mitochondrial genome interactions. Conversely, in the MR2 cybrids, disruption of these intra-species mechanisms leads to the observed incompatibility with the B6 mtDNA.

Segregation of mtDNA Heteroplasmy in F1 Progeny

Recognizing the presence of carryover mtDNA after the PNT process, and hence mtDNA heteroplasmy in cybrid zygotes, we next investigated heteroplasmy dynamics and associations with postnatal development of F1 pups and adults. We collected skin tissues from stillborn fetuses in the MR1 and MR2 groups and performed tail biopsies from each F1 live pup at the age of 30 days. Additionally, mtDNA heteroplasmy was measured in six organs and tissues from each female involved in the reproductive fitness assessments after sacrifice (180–820 days). The percentage of donor PWD mtDNA genotype in three stillborn MR1 pups (88%–99%) was higher than the mean proportion (84%) measured initially in cybrid zygotes (Table 1). Thus, carryover mtDNA levels were reduced during embryonic and fetal development. In only one stillborn was the percentage of donor mtDNA heteroplasmy (62%) lower than the initial zygotic value (Figure 1A). A similar segregation bias, trending toward homoplasmy of donor mtDNA, was seen in live females from the MR1 group (Figure 1A) and in their organs at the time of sacrifice as adults (Table 5). Carryover values in tissues from MR3 mice with the B6-DBA nDNA and PWD mtDNA genotype were also lower than the initial value except for the heart and liver in MR3 female 1 (Table 5).

In skin tissue of two of the three stillborns in the MR2 group, expected high levels of donor B6 mtDNA were noted (Figure 1B). Similarly high levels of donor B6 mtDNA in liver, heart, kidney, and spleen were detected in one stillborn animal (stillborn-3) (Figure 1C). In the only live MR2 F1 pup, the initial level of donor B6 mtDNA (90% at zygote stage) declined to 50% in tail (Figure 1B; Table 5) with low but variable values in the other six tested tissues at death, ranging from 15% in liver to 75% in spleen (Figure 1C; Table 5). We reasoned that higher levels of carryover (host) PWD mtDNA in high energy demanding tissues

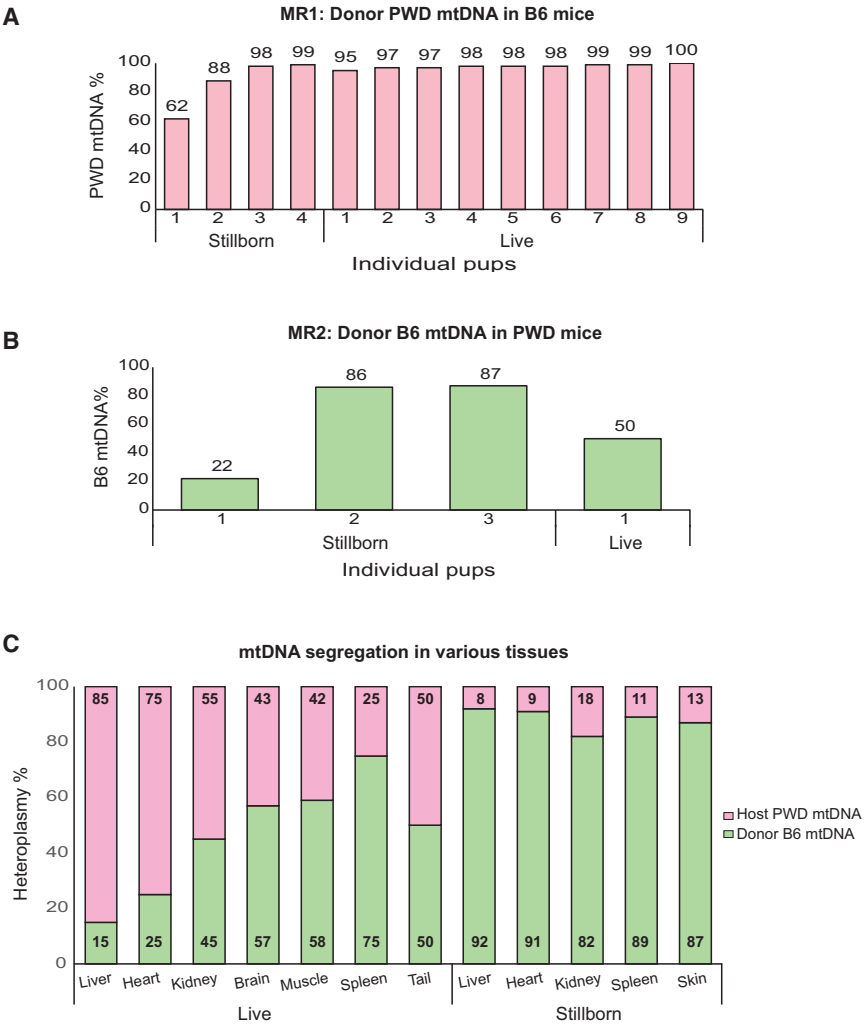

Figure 1. mtDNA Heteroplasmy Segregation in Individual Pups of MR1 and MR2

(A) Percentages of donor PWD mtDNA allele in individual stillborn and live MR1 pups with B6 nuclear genome.

(B) Percentages of donor B6 mtDNA allele in individual stillborn and live MR2 pups with PWD nuclear genome.

(C) Heteroplasmy percentages of host PWD mtDNA (pink) and donor B6 mtDNA allele (green) in various tissues collected from one live and one stillborn pup (3 in B) in MR2.

and organs contributed to survival of this female. Divergent segregation of the donor B6 mtDNA genotype in different tissues was also observed in the presence of the PWD-DBA hybrid nuclear background in MR4 (Table 5), suggesting random segregation of donor B6 mtDNA in the PWD or PWD-DBA nuclear background. A previous mouse study showed a preferential segregation of the donor mtDNA and the correlation between genetic distance and segregation strength (Burgstaller et al., 2014). Our results were in agreement with MR1 segregation bias toward homoplasmy of donor PWD mtDNA in every tissue studied, but MR2 displayed random divergent segregation of donor B6 mtDNA. The difference could be due to either different donor mtDNA heteroplasmy levels at the initial starting MR or replicative advantage of different mtDNA molecules (Lane, 2012).

In summary, these results indicate a strong bias favoring progression to homoplasmy for donor PWD mtDNA in B6 or B6-DBA cybrid mice within one generation. In contrast, donor B6 mtDNA levels in surviving PWD cybrids were lower than initial levels, suggesting that high heteroplasmy or homoplasmy for donor B6 mtDNA is detrimetal to MR2 offspring survival.

Germline Transmission of Donor PWD mtDNA in F2 and F3 B6 Females

To futher investigate reproductive outcome and donor mtDNA transmission in subsequent MR1 progeny, two F2 females produced from F1-female 1 with no history of stillborn pups and two F2 females derived from F1-female 5 with high stillborn rates were crossed with B6 males (Table S5). All four F2 females became pregnant; three gave birth to one or two litters of normal size (Table S9) and the fourth died in labor. F2-female 3, derived from F1-female 5 with a high frequency of stillborns, produced 29 live pups from 4 pregnancies with no stillborns (Table S9). Therefore, the tendency to produce stillborn pups is not transmitted to the subsequent generation. We futher crossed three F3 females with B6 males resulting in normal fecundity with the production of 37 live offspring (Table S10).

To track germline transmission of donor mtDNA, we performed tail biopsies on all 40 F2 live pups and on 20 F3 pups (14 live and 6 dead) in the MR1 group at the age of 30 days. The level of donor PWD mtDNA genotype in seven F2 pups was 98%–99%, while the remaining F2 pups (82.5%) were homoplasmic. Similar outcomes were found in the F3 generation where 17 F3 pups were homoplasmic (85%) and three were heteroplasmic at 96%–97%. These results indicate that donor PWD mtDNA was stably transmitted through the germline into F2 and F3 generations in B6 cybrids.

DISCUSSION

Hybrid sterility and other forms of intrinsic postzygotic isolation are likely caused by genetic incompatibility factors (termed Dobzhansky-Muller (DM) incompatibilities) (Dobzhansky, 1936; Johnson, 2002). Although DM incompatibilities were originally linked to interactions between nuclear genes, miscommunication between nuclear and mitochondrial genomes has been implicated in yeast (Lee et al., 2008), *Drosophila* and other insects (Dickman and Moehring, 2013; Foley et al., 2013), fish (Bolnick et al., 2008; Niehuis et al., 2008), and primate

Table 5. Donor mtDNA Levels in Various Tissues

Treatment	F1 Females	Donor mtDNA Genotype	Donor mtDNA Levels (%)[a] Tail Biopsy at 30 Days	Tissues Collected at Days	Brain	Heart	Kidney	Liver	Muscle	Spleen
MR1	Female 1	PWD	98	395	97	99	99	99	100	99
	Female 2	PWD	95	365	98	98	97	96	97	99
	Female 3	PWD	99	365	91	99	99	100	100	95
	Female 4	PWD	98	515	100	99	100	98	100	100
	Female 5	PWD	93	820	100	100	97	100	100	100
	Female 6	PWD	98	365	100	100	100	100	100	100
	Female 7	PWD	100	395	98	99	99	99	100	98
	Female 8	PWD	99	240	99	99	99	99	100	85
	Female 9	PWD	100	515	100	99	100	99	100	100
MR2	Female 1	B6	50	180	57	25	45	15	59	75
MR3	Female 1	PWD	98	450	82	73	94	61	99	99
	Female 2	PWD	98	450	99	97	100	98	100	100
MR4	Female 1	B6	91	700	53	64	40	51	100	37
	Female 2	B6	90	515	74	87	100	99	90	73
	Female 3	B6	52	700	27	35	21	66	24	25

[a]Percentage of donor mtDNA levels in each tissue was measured at the time that tissue was collected. For female 4, for example, the level of donor PWD mtDNA in tail was 98% or 100% (homoplasmic) in brain, respectively.

xeno-mitochondrial cybrid cell lines (Bayona-Bafaluy et al., 2005). Previous studies in *Drosophila* found male-specific infertility associated with mitochondrial polymorphism (Clancy et al., 2011; Innocenti et al., 2011). Other studies in the marine copepod *Tigriopus californicus* reported that low fitness of F3 hybrids is rescued in the offspring of maternal but not paternal backcrosses, suggesting that fitness loss is attributable to nuclear-mitochondrial genomic interactions (Burton et al., 2013; Ellison and Burton, 2008). Somatic cell nuclear transfer (SCNT), as another method of cybrid generation, has been successfully carried out only by MR within the same species or between species that are closely related to each other, such as sheep/mouflon and cattle/gaur, but not in species with large phylogenetic seperations. Incompatibility between nuclear and mitochondrial genomes in distant species was considered to be one of the hurdles preventing full-term development of interspecies SCNT (Loi et al., 2011). Previous studies in mice, based on either backcrossing of heteroplasmic mice for 129S6 and NZB mtDNAs onto a C57BL/6J nuclear background (Sharpley et al., 2012) or generating chimeric mice with xenomitochondrial ESC cybrids with cytoplasts from closely related *Mus spretus* (Ms) or the more divergent *Mus dunni* (Md) cells (McKenzie et al., 2004), emphasized the deleterious effects of nuclear-mitochondrial mismatch on the physiological fitness of offspring (Cannon et al., 2011; McKenzie et al., 2004; Roubertoux et al., 2003; Sharpley et al., 2012). However, the impact of nDNA-mtDNA incompatibility in these studies was concealed by the presence of nuclear alleles from mtDNA donors or by host embryo complementation in chimeras.

The present study, based on zygotic mtDNA replacement provides the first direct evidence at the organismal level in mammals that improper nDNA to mtDNA interactions secondary to intersubspecies mtDNA sequence divergence play a major role in reproductive isolation. Thus, we demonstrated that reciprocal MR between *Mus m. musculus* and *Mus m. domesticus* zygotes resulted in cybrid B6nDNA-PWDmtDNA embryos with normal post-implantation development that was comparable to controls. However, PWDnDNA-B6mtDNA embryos with greater than 85% of donor B6mtDNA were profoundly inferior as evidenced by embryo demise and/or stillborn birth post-transfer to recipients. This deficit was rescued when a hybrid PWD-DBA nDNA was present (Figure 2). We believe that this asymmetric embryonic lethality reflects incompatibility between PWDnDNA and B6mtDNA and suggests that the PWD nuclear genome, after being separated evolutionarily from the B6 genome for more than 500,000 years, has largely, if not totally, lost its ability to interact with B6 mtDNA. This functional incompatibility may therefore represent a mechanism underpining postzygotic reproductive isolation and speciation (Burton et al., 2013; Ellison and Burton, 2008).

The reproductive rescue observed in the presence of hybrid PWD-DBA nDNA indicates that the presence of nuclear alleles from the mtDNA donor plays a key role in the cross-talk between nDNA and mtDNA. Furthermore, we speculate that the underlying genetic incompatibilities are recessive. As a result of repeated backcrossing of hybrid females with C57BL/6 males, the nuclear genome of the conplastic C57BL/6J-mt$^{PWD/Ph}$/ForeJ mice, used in the current study, consists largely of C57BL/6 sequences with a small percentage of PWD sequence introgression (Gregorová et al., 2008). Using whole genome sequencing, 79 genes coding for proteins localized to the mitochondria were identified residing in these introgressed regions (Calvo et al., 2016). Among them, *Immp2l*, *Gfm2*, *Bcl2l1*, and *P2rx7* are known to be involved in mitochondrial protein processing/translation or regulation of mitochondrial organization/depolarization. Further functional studies will be necessary to elucidate how these and potentially other genes in the introgressed regions contribute to mtDNA-nDNA compatibility.

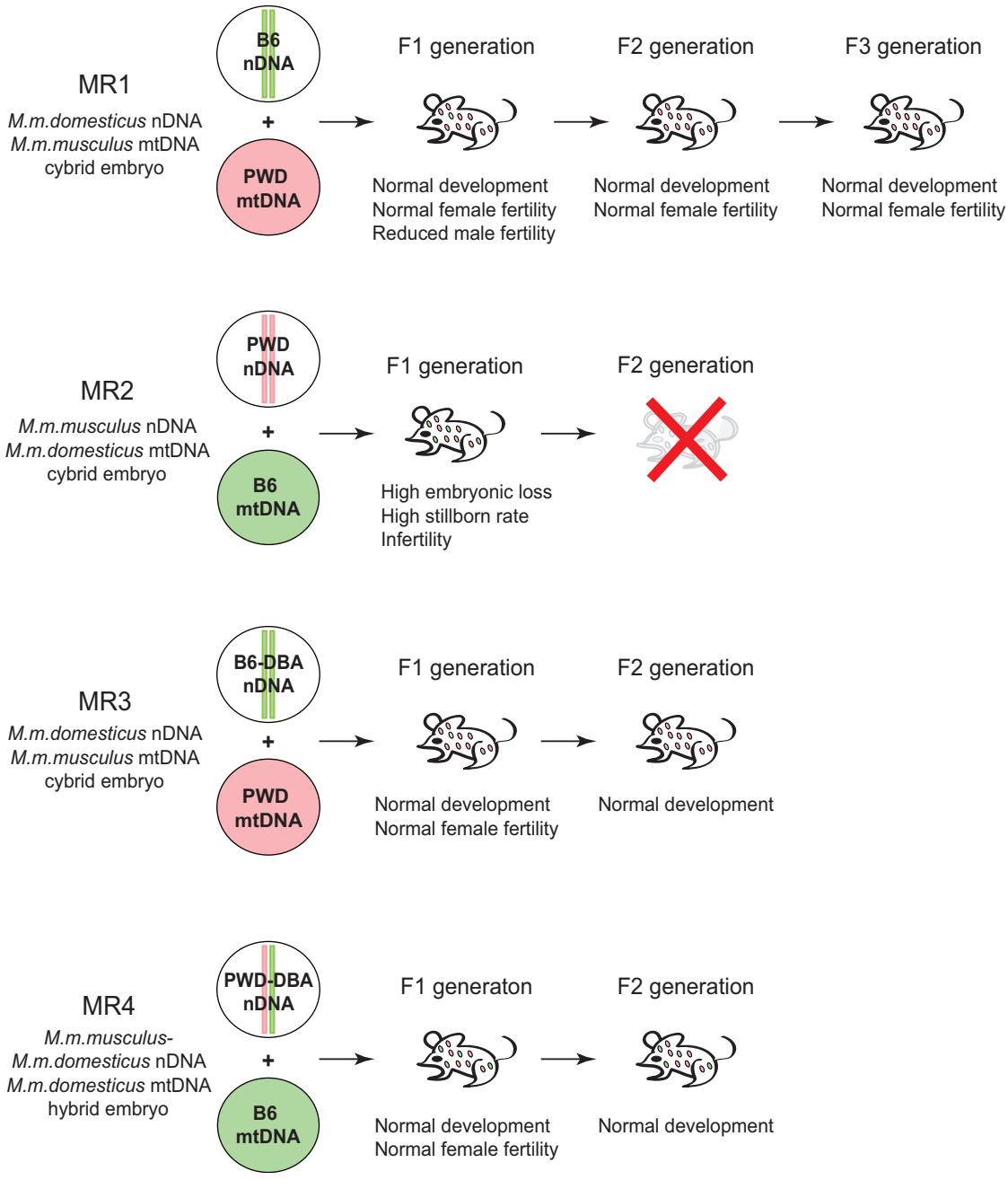

Figure 2. Diagrammatic Representation of Reciprocal Intersubspecies MR between B6, PWD, B6-DBA, and PWD-DBA Zygotes

Reconstructed embryos were tested for their post-implantation developmental potential after transfer to recipients. MR1 embryos supported normal development of F1 offspring with reduced male fertility but unaffected reproductive fitness in females. Embryos in MR2 mostly resulted in stillborns, reflecting incompatibility of the PWD nDNA and B6 mtDNA genome. The only surviving F1 female was infertile. Development of cybrid MR3 embryos with heterozygous B6-DBA nDNA and PWD mtDNA was similar to controls. Inclusion of DBA chromosomes along with PWD nDNA rescued development of hybrid embryos in MR4.

Normal fetal development to full term in vivo and normal fertility performance in adult females were evidenced in the MR1 group. Although some F1 females produced stillborns, perhaps due to subnormal nDNA-mtDNA interactions, the tendency to produce stillborn pups was not transmitted to the next generation. This implies that B6 nuclear genes have largely preserved their ability to interact functionally with PWD mtDNA in the subsequent generation. The germline transmission of donor PWD mtDNA in B6 mice was efficient, with homoplasmy seen in the majority of F2 and F3 progeny. Mice with B6nDNA-PWDmtDNA are genetically stable and can be normally maintained in the laboratory. This new strain of mouse established by intersubspecies MR will represent an addition to the mouse genetic resource. Interestingly, although reproductive fitness in

F1 females was normal in the MR1, infertility was observed in some males from this group. Previous studies linked hybrid male sterility between these mice to loci on the X chromosome (Good et al., 2008) and the autosomes (Dzur-Gejdosova et al., 2012). Additional studies could elucidate the molecular mechanism underlining partial male sterility in some cybrid mice.

Loss of reproductive fitness due to nDNA-mtDNA mismatch is often attributed to either disruption of oxidative phosphorylation or compromised regulation of transcription, or translation of mtDNA genes. The regulation of mitochondrial gene expression mostly occurs through post-transcriptional mechanisms, and all known organelle transcription factors are nuclear encoded proteins such as mtTFB in yeast (TFB1M or TFB2M in mammals) (Asin-Cayuela and Gustafsson, 2007). Since mitochondrial OXPHOS proteins are encoded from both mtDNA and nDNA, sequence divergence and changes in protein structure could cause improper subunit interactions, thereby affecting metabolism and ATP production that is, of course, fundamental to early oocyte and embryo development. We suggest that compromised mitochondrial function caused by incompatibility between nDNA-mtDNA is associated with breakdown of reproductive fitness in F1 females in the MR2 group. A significant decline of mitochondrial metabolic function, mainly in complex I, was observed in the only surviving F1 female from this group who was infertile. Infertility or high rates of intrauterine demise could reflect reduced mitochondrial OXPHOS as ATP levels are required for normal sperm-triggered calcium oscillations at fertilization (Dumollard et al., 2004) and for normal embryonic and fetal development.

Our findings support the concept that compatibility between nuclear and mitochondrial genomes is critical for normal reproductive fitness. It is known that human germline mutations in mtDNA can cause defects in respiratory chain function, resulting in serious disorders including neurological and neuromuscular diseases, diabetes, cancer, and infertility at a frequency of 1 in 10,000 adults (Schaefer et al., 2008). At present, there are no curative treatments for mtDNA disease, and interventions are largely limited to managing symptoms. We have described a strategy to prevent transmission of germline mtDNA mutations to children involving mitochondrial replacement therapy (MRT) using wild-type mitochondria and mtDNA from healthy, donor oocytes (Tachibana et al., 2013). However, a major safety issue currently under debate relates to mtDNA-nDNA incompatibility after MRT (Chinnery et al., 2014). Clearly, if the present results can be extrapolated to humans, such incompatibility within human populations is unlikely. Relevant evidence for this conclusion also comes from studies in other mice where intraspecies mitochondrial replacement between BALB/cByJ (BALB) and NZB/BinJ (NZB) or between BDF1 and NZW/LacJ mice produced viable fertile offspring that were indistinguishable from controls (Jenuth et al., 1996; Wang et al., 2014). Moreover, adult transmitochondrial nonhuman primates have been produced carrying wild-type mtDNA haplotypes from different rhesus macaque subpopulations and in which longitudinal studies demonstrate normal growth and development to adults with unaffected ATP levels and membrane potential (Tachibana et al., 2013). In pre-clinical MRT studies, human embryos and embryonic stem cells carrying "unmatched" donor mtDNA were generated with no apparent abnormalities attributed to mtDNA mismatch (Ma et al., 2015; Paull et al., 2013; Tachibana et al., 2013).

Natural human mtDNA sequence differences, generally denoted as mtDNA haplotypes, are often associated with regional migration and adaptation to climate and are measured by SNP numbers (Wallace et al., 1999). The sequence difference between distant human mtDNA haplotypes, estimated among 7,390 individuals, followed a bimodal distribution with a main peak at 45 SNPs and a secondary peak at 100 SNPs (Blanco et al., 2011). These distances are markedly closer than those in the intersubspecies used in this study with 391 SNP differences. Nevertheless, matching haplotypes between mtDNA donor and recipient would be judicious in any clinical applications of MRT.

EXPERIMENTAL PROCEDURES

Animals

All mice were housed under controlled lighting conditions (12 hr light/12 hr dark) under specific pathogen-free conditions in the Small Lab Animal Unit at the Oregon National Primate Research Center. All animal procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University.

MR by PNT and ST

2- to 3-month-old B6 and PWD or BDF and BDF-PWD females were superovulated with 5 international units pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). MII oocytes or zygotes were collected from excised oviducts 14 hr or 20 hr post hCG injection, respectively. All manipulations were conducted on an inverted microscope (Olympus IX71) equipped with a stage warmer (http://www.tokaihit.com) and Narishige micromanipulators (http://www.hamiltonthorne.com). To perform ST, MII oocytes were placed in a 50 μl manipulation droplet of FHM medium (Lawitts and Biggers, 1993) containing 5 μg/ml cytochalasin B and covered with tissue culture oil. An optical birefringence system (Oosight; http://www.hamiltonthorne.com/index.php/products/oosight-imaging-system) was used for spindle-chromosome observation. To perform PNT, zygotes were placed in a droplet of FHM medium containing 5 μg/ml cytochalasin B and 10 μg/ml nocodazole. MII oocytes and zygotes were positioned using a holding pipette, and zonae pellucidae were drilled with laser pulses before an enucleation pipette was inserted through the opening. The spindle-choromosome complex or pronuclei were aspirated into the pipette with a minimal amount of cytoplasm and surrounding plasma membrane. The resulting karyoplast containing either a metaphase spindle or pronuclei was briefly exposed to HVJ-E extract (Ishihara Sangyo Kaisha) and transferred into the perivitelline space of the recipient enucleated MII oocyte or zygote, respectively. Reconstructed oocytes or zygotes were incubated in KSOM medium (Millipore) until fusion occurred. After fusion, the reconstructed oocytes were subjected to in vitro fertilization. Reconstructed embryos from ST or PNT were then placed in droplets of KSOM medium covered with cell culture oil and cultured at 37°C in 5% CO_2, 5% O_2, and 90% N_2 until the blastocyst stage.

Generation of F1 Offspring

Blastocysts were transferred into the uteri of pseudo pregnant (embryonic day 2.5 [E2.5]) ICR females, and pregnancy was monitored by recipient weight 1 week after embryo transfer. Pregnancy was presumed if recipients gained 2 or more grams. Pregnant recipients were maintained alone in a single cage. All offspring were born naturally and weaned at 21 days or after reaching a body weight of 10 g.

Full-term birth was defined as the day of birth. Stillborn was assumed when pups were either dead or cannibalized at full term.

Generation of F2 and F3 Offspring

After achieving sexual maturity, all F1 offspring in MR1 and MR2 and selected F1 females in MR3 and MR4 were crossed with control mice from the corresponding subspecies to generate F2 animals. Selected F2 females in MR1 were crossed to male mice of B6 to generate F3 animals.

DNA Extraction from Zygotes and Tissues

Reconstructed zygotes were individually transferred into a 0.2 ml PCR tube, and DNA was extracted using an Arcturus Pico Pure DNA extraction kit (Applied Biosystems). Tail samples were collected from offspring after weaning. Tissues (brain, heart, kidney, liver, skin, spleen, and tail) were collected from expired animals, and DNA was extracted using a Purogen DNA purification kit (QIAGEN) according to the manufacturer's instructions. NanoDrop 2000 spectrophotometry (NanoDrop Technologies) was used for DNA mass determinations.

Heteroplasmy Quantification by Amplification Refractory Mutation System-qPCR

ARMs-qPCR assays were performed to measure mtDNA heteroplasmy levels in PNT embryos and offspring as previously described (Lee et al., 2012). For the discriminative assay (D), primers and TaqMan probes (FAM) were designed to target the unique mtDNA region in the ND5 gene for B6 mtDNA (5′F-CCTA CTAATTACACTAATCGCCACT, 5′R-GAGGTCTGGGTCATTTTCGTTA, FAM-AACCGCGTTTTCCCCCCCTAA-BHQ1) and the unique mtDNA region in the ND2 gene for PWD mtDNA (5′F-ACTGCACATAGGACTTATTCTTGTC, 5′R-TTGAGTAGCGGGTAGATTAGG, FAM-TGACAAAAAATCGCCCCCCTAT CAA-BHQ1), respectively. For the nondiscriminative assay (ND), primers and TaqMan probes (TET) were designed to target the common region of the COX3 gene (5′F-TCTAGCCTCGTACCAACACATGAT, 5′R- TGAAACA CCTGATGCTAGAAGTACTGA, TET-TCCTGTTGGAGGTCAGCAGCCTCCT-BHQ1). The ND and D assays were mixed in 15 μl reaction mixtures containing 1× PCR MasterMix from Rotor-Gene Multiplex PCR Kit (QIAGEN), 250 nM of each set of primers,150 nM of each TaqMan probe, and sample DNAs and were run on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). All reactions were run in duplicate with two different amounts of input DNA: 8 ng and 1 ng. The percentage of donor mtDNA in relation to the total mtDNA content was calculated by the equation: heteroplasmy = 100 × (quantity D/quantity ND).

Mitochondrial Respiratory Chain Enzyme Assays

Respiratory chain complex I and IV activities were assayed in skeletal muscle homogenates by spectrophotometric methods as described previously (Spinazzi et al., 2012). A VersaMax microplate reader system (Molecular Devices) in combination with SoftMax Pro software was used for activity measurements and calculations. Enzyme activities were expressed as a percentage relative to citrate synthase. Results were presented as mean ± SEM. One-way ANOVA was used for three-group comparisons, and Student's t test was used for two-group comparisons. A p value less than 0.05 was considered significant.

Quantification of Sperm Number and Motility

Sperm samples were collected from excised epididymides by applying gentle pressure, and sperm suspensions were incubated in 200 μl of HTF medium for 45 min at 37°C and 5% CO_2. Sperm number, presented in million/ml, was based on three counts using a neubauer chamber, while sperm motility was calculated from the number of progressively swimming cells in a population of 50.

Histological Analyses

Mice were euthanized with CO_2 followed by cervical dislocation. Tissues were extracted and fixed in 10% neutral buffered formalin overnight, embedded in paraffin, and processed by the pathology service unit of the Oregon National Primate Research Center.

DNA Library Preparation and Whole Genome Sequencing

DNA was prepared from C57BL/6J (B6) and C57BL/6J-mtPWD/Ph/ForeJ conplastic (PWD) mice obtained from Jackson Laboratories. Sequencing libraries were prepared using an Illumina TruSeq Nano DNA library preparation kit (Illumina) following the manufacturer's instructions. Briefly, 1 μg of genomic DNA from each sample was fragmented by Adaptive Focused Acoustics (E220 Focused Ultrasonicator, Covaris) to produce an average fragment size of ~350 base pairs. Fragmented DNA was purified using the Agencourt AMPure XP beads (Beckman Coulter). The quality of the fragmentation and purification was assessed with the Agilent 2100 Bioanalyzer (Agilent Santa Clara). End repair was performed and adaptors were ligated to the fragments. The resulting DNA library was amplified by six cycles of PCR. Sequencing was performed using the Illumina HiSeq 4000 system, generating an average of 600 million 100 bp paired-end reads per sample. The two samples were sequenced to an average depth of 30×.

Whole Genome Sequencing Analysis

Sequencing data was adaptor and quality trimmed with default parameters using Trim_galore v.0.3.3 (Babraham Bioinformatics). The trimmed data were then mapped to GRCm38/mm10 (UCSC) using Bowtie2 v.2.2.3 (Langmead and Salzberg, 2012) with the following parameters -I 60 -X 800. PCR duplicates were removed using Samtools v.0.1.19. SNPs were called using Samtools v.1.3 mpileup and Varscan v.2.3.6 (Koboldt et al., 2012). SNPs with a p value of less than 0.0001 (Fisher's exact test performed by Varscan) were used. This p value was chosen based on a marked decrease in the ratio of the number of SNPs to the number of mRNAs seen with SNPs with higher p values. To verify regions of PWD sequence in the conpastic PWD sample, we searched for SNPs known to differentiate the B6 and PWD strains (n = 3,432; dbSNP Build 137) (http://www.informatics.jax.org/snp) in our list of called SNPs. These SNPs were then visually verified using the Integrative Genomics Viewer (http://www.broadinstitute.org/igv/home).

Statistical Analysis

For Tables 1, 2, 3, 4, and S2, data are presented as mean ± SEM. Kruskal-Wallis test in conjunction with Mann-Whitney U test was used for multiple group comparisons, and Student's t test was used for two-group comparisons. Contingency tables were used for the comparison of percentages of live and stillborn pups of F1 female animals in Table S5, and one-way ANOVA was used for comparison of complex I activities in the same Table S5. In all analyses, a p value less than 0.05 was considered significant.

ACCESSION NUMBERS

The accession numbers for the whole genome sequencing data reported in this paper are NCBI Short Read Archive: SRP072373, SRP072374.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, and 10 tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.06.012.

AUTHOR CONTRIBUTIONS

S.M., H.M., and N.M.G. conceived and planned the study. N.M.G. carried out PNT experiments. N.M.G., C.V.D., E.K., T.H., and Y.L. performed oocyte and embryo collections, embryo transfers, and colony management. N.M.G., H.M., C.V.D., Y.L., and R.T.-H., performed DNA isolations and detections of mtDNA heteroplasmy and mitochondrial complex activities. R.M. and L.C.L. performed whole genome sequencing. H.M., N.M.G., R.M., L.C.L., D.P.W., and S.M. analyzed data and wrote the manuscript.

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