

1 Risks inherent to mitochondrial replacement

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11 The UK Government has recently been debating whether or not to legislate to
12 allow *mitochondrial replacement* (MR) to be used in the clinic. However, we are
13 concerned that some of the science of MR has been misunderstood, or otherwise
14 given only fleeting consideration. We set out our arguments below and offer a
15 way forward to ensure that MR can safely deliver the health benefits it promises
16 for those suffering from mitochondrial-related diseases

17

18 Recent innovations that enable mitochondrial DNA (mtDNA) mutations to be
19 eliminated from the germline, by replacing mutated mitochondria within an
20 oocyte with mitochondria from a healthy donor female¹⁻³, offer hope for the
21 eradication of several debilitating and lethal mitochondrial diseases. The
22 potential for clinical application of MR has received widespread support^{4,5}, but
23 has also provoked safety and ethical concerns from the public and biomedical
24 practitioners^{4,6}. In addition to currently addressed safety concerns related to
25 technical details of the procedures⁷, a further safety concern exists that cannot
26 be easily addressed by methodological refinements. Embryos produced by all
27 variants of MR (pronuclear transfer; maternal spindle transfer; polar body
28 transfer) will acquire genetic material from three different individuals (nuclear
29 DNA from the prospective parents, and mtDNA from a donor female), and some
30 of these novel combinations of genetic material may not be fully compatible with
31 one another (i.e. may be mismatched). For example, various combinations of
32 donor mtDNA and recipient nuclear genomes have experimentally been shown
33 to negatively affect offspring health and fitness in vertebrate and invertebrate

34 models, even though the donated mitochondria were putatively healthy⁸. This
35 evidence has, however, been suggested to have low relevance to humans^{3,7,9,10} for
36 three proposed reasons (Box 1). Here we address each of those reasons, and
37 explain why none of them refute compellingly the potential for mitochondrial-
38 nuclear (mito-nuclear) mismatches to affect the outcomes of MR in humans.

39

40 **Box 1. *Three proposed reasons why MR should not result in alterations of***
41 ***human phenotypes***

42

43 **Reason 1. MR, like sexual reproduction, randomly shuffles mitochondrial**
44 **and nuclear genomes each generation.**

45 This is based on the argument that sexual reproduction results in the random
46 mixing of two parental genomes. Thus, under sexual reproduction, the father's
47 haploid genome is as evolutionarily 'foreign' to the mother's mtDNA, as will the
48 mother's nuclear genome be to a donor's mtDNA under MR⁹. Under the
49 additional assumption that the mito-nuclear combinations found in the offspring
50 are a random subset of those determined at fertilization (i.e. the absence of
51 selection is assumed), there will be little scope for high performing mito-nuclear
52 allelic combinations to be preserved across generations. MR has therefore been
53 described as being equivalent to sexual reproduction, in terms of generating
54 healthy offspring containing novel combinations of mitochondrial and nuclear
55 alleles. In section 1, we explain why the process of co-transmission of mtDNA
56 and maternal nuclear DNA, coupled with selection, renders this proposed reason
57 unconvincing.

58

59 **Reason 2. Genetic diversity in humans is too low to cause incompatibilities.**

60 It has been suggested that mito-nuclear mismatches are unlikely to occur in
61 humans because the genetic diversity within the human population is so small
62 that any disruptions will be negligible⁷. It has been argued that mito-nuclear
63 compatibility should be widespread, given that humans are "a freely
64 interbreeding species"¹⁰. In section 2, we outline why the potential for mito-
65 nuclear incompatibilities in humans remains a credible possibility.

66

67 **Reason 3. Incompatibilities do not occur in non-human primates.**

68 Empirical data in a primate model³ has been used as evidence that mito-nuclear
69 mismatching will not occur, or will not be important, in humans. This reasoning
70 is based on the production of four healthy male macaques born to three mothers,
71 following MR-assisted IVF attempts on twelve mothers³. The individuals were
72 apparently derived from two distinct, although unspecified¹, sub-species of
73 *Macaca mulatta*⁹. In section 3, we outline why the macaque studies, to date, do
74 not provide a strong base on which to dispel concerns regarding mito-nuclear
75 incompatibilities manifesting in humans.

76

77 **1. MR is more likely than sexual reproduction to disrupt coevolved mito-**
78 **nuclear genetic combinations**

79 *1.1 Co-transmission.* During sexual reproduction, but not during MR, offspring
80 invariably receive an entire haploid copy of the nuclear genome from their
81 mother, alongside their maternally-inherited mtDNA. In other words,
82 mitochondrial alleles co-transmit with 50% of the autosomal nuclear alleles in
83 100% of the cases (and with two-thirds of the X-chromosome linked alleles,
84 since females carry two copies of the X-chromosome and males carry only one).
85 In contrast to sexual reproduction, MR can create entirely novel allelic
86 combinations of mito-nuclear genotypes, because the mtDNA has been donated
87 from a third-party (the donor female) – thus the co-transmission rate between
88 the patient's nuclear DNA and the donated mtDNA is 0%.

89

90 *1.2 Selection.* The co-transmission of mtDNA and nuclear alleles facilitates the
91 preservation of high performing (coevolved) combinations, across generations.
92 The greater the percentage of co-transmission between mtDNA and nuclear DNA,
93 the higher the potential for mito-nuclear co-adaptation. In natural conceptions,
94 embryos carrying better-performing mito-nuclear allelic combinations may be
95 more likely to survive through development, to reach reproductive age, and
96 ultimately to successfully reproduce. Because the best-performing combinations
97 may be more likely to be passed on, coadapted mito-nuclear allelic pairings are
98 likely to be preserved across generations within any particular maternal lineage.
99 Similarly, germline selection against incompatible mito-nuclear combinations

100 might occur at the oocyte stage, with poorly-performing oocytes potentially re-
101 adsorbed. By contrast, MR-assisted IVF creates combinations of mito-nuclear
102 alleles that are potentially novel (i.e. never before placed together), and not
103 previously screened by natural selection (or previously screened and selected
104 against). This lack of prior screening means that the sample of oocytes and
105 embryos created under MR will contain individuals that may be inherently more
106 likely to exhibit incompatibilities between the mitochondrial and nuclear
107 genomes⁸. The mito-nuclear allelic combinations carried by the offspring will be
108 under selection across life-stages, from before fertilization through to the
109 sexually mature adult (with this selection manifested as differential patterns of
110 survival or fertility among offspring carrying different mito-nuclear allelic
111 combinations). Reduced fertility, especially of males, as a result of epistatic
112 interactions during hybridization between alleles at different loci, including
113 those spanning different genomes, is expected theoretically¹¹ and supported
114 empirically, including for mito-nuclear complexes in *Drosophila melanogaster*
115 ^{12,13}.

116

117 **2. Genetic diversity in humans**

118 The human population is generally thought to show lower mean levels of genetic
119 divergence at nuclear loci than other species^{e.g.} ¹⁴. While the probability of MR
120 resulting in mito-nuclear incompatibilities would presumably be low if there was
121 complete genetic admixture within the nuclear genome, it is clear that genetic
122 population stratification does exist¹⁵⁻¹⁷. This stratification has its origins in
123 historical and demographic patterns of selection and migration¹⁸, and positive
124 assortative mating between individuals of similar phenotypes may contribute to
125 its maintenance^{19,20}.

126

127 However, the level of divergence across mtDNA sequences is also relevant when
128 it comes to the question of whether or not MR may result in mismatched mito-
129 nuclear genotypes. In humans, the percentage divergence in mtDNA between
130 major human haplogroups is around 0.5% (Fig 1, Table S1), essentially
131 equivalent to the divergence exhibited across mtDNA haplotypes within the fruit
132 fly, *D. melanogaster* (0.4%; Fig 1, Table S2), which exhibit clear signatures of

133 mito-nuclear incompatibilities, particularly in males^{12,13}. Haplogroup matching,
134 proposed as a way of circumventing this issue^{21,22}, might not always be
135 successful in preventing mito-nuclear incompatibilities. By definition, when
136 probing variation within human macro-haplogroups, divergence across mtDNA
137 haplotypes will persist (~0.1%, see Table S3 for estimates within haplogroup H,
138 the most common European macro-haplogroup, or 0.2% if the non-coding region
139 is included in the analysis [Fig 1]; similar patterns are found within H1 [Table S4,
140 Fig 1]). The mechanisms of the incompatibilities are largely unknown and the
141 identity of the causative interacting loci is undetermined²³. However, it seems
142 that several loci of small effect are involved in *Drosophila*²⁴. While this suggests
143 that less distantly-related genomes may result in smaller incompatibility
144 effects²⁴, it will be difficult to make predictions about the likelihood of
145 incompatibilities based on the specific alleles that delineate haplotypes, given
146 that it has been previously shown that single nucleotide differences in the
147 mtDNA can cause male sterility when interacting with particular nuclear
148 genotypes^{13,25}. Further research into the degree of mismatch manifested with
149 increasing mitochondrial genetic divergence between putative donor and
150 patients should be a priority.

151

152 **3. Proof-of-principle studies do not allow epidemiological predictions of** 153 **incompatibilities**

154 Several studies demonstrated the technical feasibility of surgical MR^{1-3,26}, using
155 macaques, human cell lines and mice. The number of mitochondrial × nuclear
156 genotype combinations covered by all these studies together appears to be 15, or
157 less. In addition, with the exception of two studies^{3,27}, no maternal replicates
158 were used per mito-nuclear combination, preventing an examination of whether
159 any effect is due to a particular maternal effect associated with the study subject
160 or inherent to a particular mitochondrial × nuclear genotype combination. In
161 other words, these studies^{1-3,26,27} were not designed to test for mito-nuclear
162 incompatibilities, and cannot be used to predict the population-wide likelihood
163 of mito-nuclear incompatibilities manifesting post-MR. Doing so will likely lead
164 to a high rate of Type II errors – the failure to detect effects that are present.

165

166 There are also additional issues with some of these studies. For example,
167 Tachibana et al.³ used 98 human oocytes (from 7 donors) for MR, and concluded,
168 there was no difference in zygote survival to normal IVF controls. However, this
169 conclusion might warrant reappraisal. In their study, the authors derived six
170 embryonic stem cells (ESCs), from 19 blastocysts, from a starting stock of 64
171 oocytes that underwent MR treatment (ESC success rate: $6/64 = 9\%$, blastocyst
172 rate: $19/64 = 30\%$). This compares to nine ESCs, from 16 blastocysts, from a
173 starting stock of 33 oocytes in the control group (ESC rate: $9/33 = 27\%$,
174 blastocyst rate: $16/33 = 48\%$). The differences in ESC isolation rate between
175 treatment and control groups are in fact statistically significant (ESC: Fisher's
176 exact test, 1df, two-tailed, $p = 0.035$; Blastocysts: $p = 0.078$). This suggests
177 further evaluation of developmental success post-MR should be a priority.

178

179 Paull et al.²⁶ obtained 7 blastocysts out of 18 MR oocytes. The cell lines derived
180 from these blastocysts showed lower activity in all four respiratory chain
181 enzyme complexes than control cells. While differences were not statistically
182 significant, they represent reductions of between 2 to 19 % (average across four
183 enzymes: 11%) compared to parthenogenetically-induced controls, and given the
184 low sample sizes involved, again suggest that further scrutiny into possible
185 effects of MR is warranted.

186

187 Finally, Craven et al.² report that development to blastocyst stage was
188 approximately 50% lower for zygotes receiving MR treatment ($18/80 = 22.5\%$)
189 than for controls; a difference that is likely to be statistically significant, although
190 the controls were unmanipulated and therefore do not represent a true control
191 for the manipulation.

192

193 In the light of these three examples, it is noteworthy, that MR affected
194 development and respiration in many other studies on non-primate vertebrates
195 and invertebrates⁸.

196

197 **Conclusions**

198 MR-assisted IVF could place novel allelic combinations of interacting mtDNA and
199 nuclear genes alongside each other in the offspring, and these combinations
200 might not have been previously screened by selection (or in the worst case may
201 have already been removed from the population by selection). Therefore, mito-
202 nuclear allelic combinations created following MR (which are characterized by
203 0% co-transmission from parents to offspring) are theoretically not equivalent to
204 those found in individuals produced under sexual reproduction. This insight is of
205 fundamental importance, but apparently underappreciated in the literature
206 pertaining to MR. Given that mito-nuclear allelic combinations contribute to
207 encoding life's critical function of energy conversion, natural selection must be
208 assumed to be particularly intense on these combinations. We suggest that it is a
209 real possibility that novel combinations created under MR could result in mito-
210 nuclear mismatches. This possibility has also been predicted by evolutionary
211 theory²⁸ and experimentally supported in several taxa^{8,29}, including several with
212 comparable levels of mitochondrial genetic diversity to the human
213 population^{12,13}.

214

215 Lack of evidence from small-scale proof-of-principle experiments for MR effects
216 should not be used to conclude mito-nuclear incompatibilities are unlikely to
217 manifest post-MR, because these experiments cover few mito-nuclear
218 combinations and their statistical inferences, in some cases, appear open to
219 question. In fact, there is actually an extensive, but largely overlooked, body of
220 experimental evidence that indicates mito-nuclear interactions are important in
221 determining health outcomes in humans³⁰⁻⁴², as well evidence for mito-nuclear
222 incompatibilities following the similar procedure of somatic cell nuclear transfer
223 in cattle^{43,44}. Furthermore, the only previous attempt of using pronuclear
224 transfer in humans was not successful⁴⁵. Future work should, therefore, address
225 to what extent the risk of mismatching can be reduced by matching the donor
226 and maternal mitochondrial haplotypes, since genetic variation across many
227 interacting loci are likely to be involved²⁴, and given the genetic variation
228 between and within human mtDNA haplogroups that we have outlined here. As a
229 suggested design, two oocytes should be used for every donor; each enucleated.
230 One of these is assigned to a control, and re-populated with the donor's own

231 nuclear genetic material, and the other to the MR treatment. By then comparing
232 the success of MR-treated to control eggs, and provided sufficient replication
233 across donors, this design would provide an explicit test for mito-nuclear
234 incompatibilities post-MR.

235

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241

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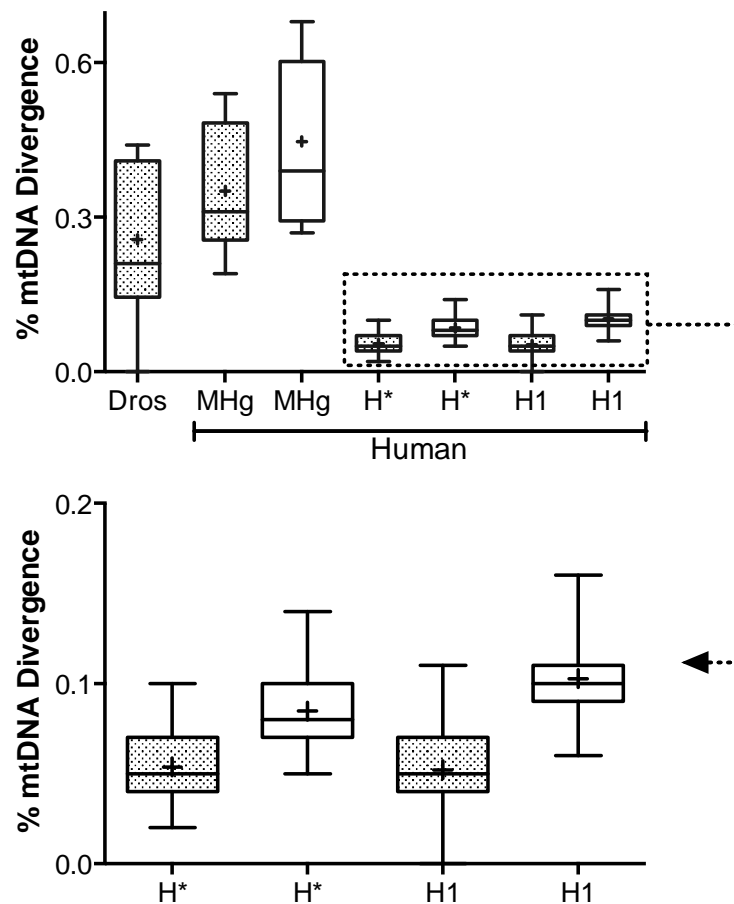
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364

365 **Figure 1:**

366



367

368 **Figure 1:** Boxplots depicting variation in mtDNA divergence (%) across naturally-
369 occurring human mtDNA sequences, with comparison to mtDNA divergence across
370 global fruit fly (*Drosophila melanogaster*) populations. The *Drosophila* (Dros) plot is
371 based on protein coding regions of 13 *Drosophila melanogaster* populations that were
372 previously used in published studies showing effects of mitochondrial
373 replacement^{12,13,24}. Human data are first presented using sequence polymorphisms
374 found only in the protein coding region (denoted by hashed boxes; to enable direct
375 comparison to the *Drosophila* plots, in which non-protein coding sequences were
376 unavailable), and secondly using the full sequence data (protein and non-coding
377 regions; denoted by open boxes). Human data are presented at three scales; first at the
378 scale of human mitochondrial macro haplogroups M, N, R, L0, and L3 (MHg), second
379 at the scale of human mitochondrial haplogroup H (sub-clades H1 to H10 [H*]) – the
380 most common haplogroup among Europeans, and third at the scale of haplotype,
381 specifically 20 mitochondrial haplotypes sampled from haplogroup H1 (H1). Box
382 plots show median values (line within box), 2nd, and 3rd quartile (box outline),
383 maximum data range (whiskers), and mean (+). At each scale, plots are generated
384 using pairwise divergence estimates for all combinations of mtDNA sequence.

385