Dear Sir/Madam,

Call for evidence: Scientific review of the methods to avoid mitochondrial transfer

At a previous Scientific and Clinical Advances Advisory Committee (SCAAC) meeting on the 13th of May 2010, the production of (1) pronuclear transfer-derived human embryos reaching the blastocyst stage containing on average <2 % mitochondrial DNA (mtDNA) carry-over from the original zygote and (2) spindle transfer-derived monkeys exhibiting normal development, was reported. The publication relating to the latter, reported no mtDNA carry-over1.

1. I would like to draw the core panel’s attention to the numerous somatic and embryonic cell nuclear transfer animal studies which show that mtDNA carry-over from the original cells to embryos, foetuses and offspring is a regular phenomenon2-12. Somatic and embryonic cell nuclear transfer involves transferring a nucleus from a somatic or an embryonic cell, respectively, and placing into an oocyte that has had its nucleus removed, making the techniques analogous to that of pronuclear and spindle transfer. Indeed, mtDNA carry-over has been detected in 165 out of 204 (~ 54 %) cases, with up to 59 % mtDNA carry-over reported in one offspring3. As this amount was far in excess that present immediately after the somatic cell nuclear transfer, the authors suggested the mtDNA of the original somatic cell had been preferentially amplified during development3. While these studies involved animals (not humans), transferring nuclei from somatic/embryonic cells with healthy mitochondria (not pronuclei or nuclear DNA from embryos or oocytes, respectively, with unhealthy mitochondria), they raise the strong possibility, that despite best efforts, the unhealthy mitochondria will be carried over and preferentially amplified to levels that could cause mitochondrial disease in “mitochondrial-replacement” babies. Not least because deleted mtDNA in unhealthy mitochondria has been shown to be preferentially amplified over non-deleted (‘wild-type’) mtDNA in healthy mitochondria, in at least one cybrid study13. Cybrids are somatic cell lines produced by transferring a nucleus from one cell into another that has had its nucleus removed.

2. Mitochondria are the powerhouses of cells and their correct assembly is vital for power to be generated, a process that relies on numerous interactions between nuclear DNA and mtDNA. These interactions are a result of millions of years of co-evolution between the two genomes; transplanting a nucleus from one mitochondrial background into that of another (e.g. during pronuclear or spindle transfer) may, therefore, result in nuclear-mitochondrial incompatibility, unhealthy mitochondria and symptoms reminiscent of mitochondrial disease, in "mitochondrial replacement" babies produced. Although the subject of several reviews14-16, the consequences of nuclear-mitochondrial incompatibility in embryo and adult development and beyond, when nuclei are transferred between the cells of the same species are not fully understood. This said, numerous studies involving the transfer of nuclei between the cells of different species, where development rarely reaches beyond the preimplantation development
stage (in the case of somatic cell nuclear transfer\textsuperscript{17-20}) or where mitochondrial health is compromised (in the case of cybrid studies\textsuperscript{21-26}), demonstrate nuclear-mitochondrial compatibility is important.

Ninety-five percent of people in Europe belong to one of 10 mtDNA haplogroups (i.e. share the same specific single nucleotide polymorphisms in their mtDNA\textsuperscript{27}). Different phenotypic traits are associated with these different mtDNA haplogroups\textsuperscript{9,9,27-29}, bringing their compatibility, or rather the compatibility of their nuclei with alternative haplogroups, into question. Recently, I have developed a model which predicts, using selected nuclear DNA and mtDNA sequences; whether or not nuclear-mitochondrial (in) compatibility can be expected following nuclear transfer. Although not yet validated, the model predicts nuclear-mitochondrial incompatibility is likely if pronuclei from an embryo (or nuclear DNA from an oocyte) belonging to haplogroups V, K, H and M (the only haplogroups run through the model so far) are placed into an embryo (or oocyte) with an alternative haplogroup, with incompatibility being more likely in certain combinations (e.g. M vs. H, K or V) than others (e.g. V vs. K, V vs. H and K vs. H), raising the possibility that the mtDNA haplogroup(s) of embryos/oocytes should be determined prior to pronuclear/spindle transfer to prevent potential nuclear-mitochondrial incompatibility during subsequent development.


Yours sincerely,

Rhiannon E. Lloyd, PhD

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15 March 2011

Dear Ms Darby,

**Review of methods to avoid mitochondrial disease**

Thank you for the opportunity to contribute to this HFEA review. We would like to make the following points:

1. **Safety of nuclear transfer techniques**

   There is already abundant evidence from animal studies that in vitro techniques for culturing and manipulating human embryos can cause major risks for offspring born as a result of these techniques. In general, the greater the amount of manipulation, the higher the frequency and severity of the problems in the offspring. The source of many of these problems is incorrect epigenetic modification of the zygote genome, especially in imprinted genes.

   In addition to this body of evidence, there is also experience with the manipulation of human embryos to avoid mitochondrial disease. Early experiments involving the less invasive technique of cytoplasm transfer were prohibited by the US Food and Drug Administration, due to the health problems in children. There is other evidence in the literature on the use of various techniques to avoid mitochondrial genetic disease showing that these techniques can lead to abnormalities which are detectable at the embryo stage.

   Thus, it is clear that these techniques carry significant potential risks to the offspring. Our current state of understanding of how embryo manipulation leads to abnormalities in gene expression is very rudimentary. As a result, there is still an extremely long way to go before it will be possible to reduce these risks.

2. **The risks cannot be eliminated**

   However much epigenetic risks can be reduced, they cannot be eliminated, since embryos can only be grown to the blastocyst stage, whereas many of the results of epigenetic and other gene expression abnormalities do not manifest themselves until much later in gestation or after birth.

   As a result, the first attempts to create children using these techniques will be experiments. This is, of course, the normal situation in the field of reproductive technology: in contrast to the situation with the testing of new pharmaceuticals, testing of the safety of new reproductive technologies can only be done by actually creating new individuals.
3. There is a safe alternative

There already exists a perfectly safe and well-established technique for avoiding the transmission of mitochondrial genetic diseases, i.e. egg donation. It is not true, as has been suggested by advocates of the nuclear transfer techniques, that there is no other way in which women carrying mitochondrial genetic conditions can have children without passing on the condition. Thus, the only advantage in employing nuclear transfer techniques is that the woman with the mitochondrial genetic condition becomes the nuclear genetic parent of her child. However, even those parents who feel very strongly that they wish to be the genetic parents of their child will have to accept that their child’s mitochondria come from a third, unrelated person.

Formulations such as, 'there is no other way for parents who insist upon being the nuclear genetic parents to have children', are no better: the simple fact is that egg donation is available. Whilst we understand that parents would like to be the nuclear genetic parents of their children, an absolute insistence upon this is unreasonable.

4. It is not ethical to subject the child to significant risk, simply for the sake of an extra 50% genetic relatedness.

In the normal course of development of reproductive technology, there are, as noted above potential risks to the children. The mainstream ethical view is that it is justifiable to submit the child to such risks, because there is genuinely no other way in which parents can have a child.

Here, the only justification for submitting the child to serious risk is that the mother wishes to be the nuclear genetic parent of the child. In our view, this benefit, which is minor in comparison to being able to have a child at all, does not justify the very significant risks that these technologies hold for children. We would therefore argue that it would be unethical to use this technique in preference to egg donation.

Note that, since in our view the use of such techniques would be fundamentally unethical, there can be no reason to invest scarce research funds in the development of these techniques. We also believe, therefore, that the HFEA was wrong to grant the licence to the Newcastle group to begin this research.

Since this review focuses on safety issues, we have not made arguments concerning the ethical, social, and psychological consequences to the child of having three genetic parents, nor the major social issues raised by the use of this technique, which is arguably a form of germline genetic engineering. There would need to be extensive public consultations upon these issues before the technique could be legalised for treatment. However, we note that the existence of these concerns strengthens our argument that, on the basis of standard risk/benefit analysis, the use of these techniques cannot be justified.

We look forward to the results of your review.

Yours sincerely,

Dr David King
Director, Human Genetics Alert
Dear Colleagues,

Thank you for the invitation to respond to the HFEA document Call for evidence: Scientific review of the methods to avoid mitochondrial disease. Having worked on mitochondrial diseases for two decades it is very reassuring to read such a sensible, balanced document on current and potential methods to avoid mitochondrial disease. I agree with the summary of previous considerations about the potential for pronuclear transfer and spindle transfer to be extremely useful for prevention of mtDNA disease and also with the comments on potential safety issues that need to be resolved. I cannot provide any new evidence on safety issues related to such techniques but will list some points below that may be relevant to your review, and am happy to respond to any questions.

- Prenatal diagnosis for mtDNA mutations by CVS or amniocyte analysis can be extremely useful for some couples at risk of transmitting mtDNA disease but has a number of limitations. Our experience agrees with the consensus view that PND is generally best suited for women with relatively low levels of heteroplasmy and low recurrence risk. My group in Melbourne has been involved in approximately 10 mtDNA prenatal diagnoses, mostly for the m.8993T>G mutation, for which the best data are available on correlating genotype and phenotype.

Preimplantation genetic diagnosis is another useful option for some couples, particularly since it avoids the need to terminate an existing pregnancy and can provide information on heteroplasmic loads in individual oocytes or embryos. This information can guide future approaches even if a successful pregnancy is not achieved. I collaborated with Melbourne IVF, in providing PGD for one couple where the mother carried the m.8993T>G mutation. In the second cycle, an embryo with a low heteroplasmic load (<5% mutant) was implanted and resulted in a successful pregnancy and birth of a healthy child. We collected data on the distribution of heteroplasmy in blastomeres from embryos that were not implanted and confirmed that the level of heteroplasmy in cord blood and multiple regions of placenta corresponded to those found during PGD. These data are summarised in the attached abstract, presented at the Australian Health & Medical Research Congress in Melbourne last November. They were also presented at the ENMC meeting in March 2010 on PGD for mtDNA disease, also attended by Prof. Braude, and summarised by Poulton J & Bredenoord AL (Neuromuscul Disord. 2010 Aug;20(8):559-63). Full details will be submitted shortly in a manuscript in preparation. Our data, and those of the Paris group (Monnot et al., Hum Mutat. 2011 Jan;32(1):116-25), support the use of PGD for prevention of mtDNA disease. As with PND though, PGD is likely to remain most useful for women with relatively low levels of heteroplasmy and low recurrence risk.
• PND and PGD are unsuitable for the substantial numbers of women at risk for transmitting an mtDNA mutation who carry high levels of heteroplasmic or are homoplasmic for the mutation. This provides a strong incentive for investigating methods such as pronuclear transfer and spindle transfer, which show great promise in recent results from the US study on monkeys and the UK study on karyotypically abnormal human embryos. I agree with the comments in Section 4.3 of the Call for Evidence. That is to say, that I strongly support the continuation and extension of these lines of research but do have concerns about potential safety issues that need to be addressed prior to such techniques being offered as assisted reproduction methods.

• The safety concerns are largely theoretical but also prompted by earlier (uncontrolled) studies that detected chromosomal abnormalities in pregnancies following cytoplasmic transfer between embryos. They relate to the potential for epigenetic modifications and the possibility that a heteroplasmic mixture of mtDNA from two maternal ancestors could result in some form of genomic incompatibility or instability. Some data relevant to these concerns are cited in pp. 251-252 of the attached chapter from the book Mitochondrial Medicine (S DiMauro, M Hirano, EA Schon, eds) Parthenon Publishing, Lancaster, UK (2006). Thus, I strongly support the suggestions for further research on safety in Section 4.3, both in the primate model and in studies of human embryos.

Please feel free to contact me regarding any specific issues and thank you for the important role you are playing in oversight of this fascinating and important area.

Sincerely yours,

David R. Thorburn

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Response to call for evidence:

Scientific review of the methods to avoid mitochondrial disease

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Thank you for asking us to submit evidence regarding the current status of research into prevention of human mitochondrial disease.

1. Prenatal diagnosis (PND) and preimplantation genetic diagnosis (PGD)

It is currently estimated that ~80% childhood-onset and ~50% of adult-onset mitochondrial disease is actually caused by mutations in nuclear genes whose products are essential for normal mitochondrial function, and that only a minority of mitochondrial disease is caused by mitochondrial DNA (mtDNA) mutation [1].

Nuclear-encoded mitochondrial diseases can easily be prevented by conventional PND and PGD techniques, providing the responsible mutation(s) have been identified in the index case. Patients in our service have received PND to prevent further cases affected by the following diseases:

i. Leigh syndrome (subacute necrotising encephalomyelopathy) caused by mutations in the SURF1 gene, which encodes an assembly factor for the cytochrome oxidase enzyme (the fourth enzyme in the mitochondrial respiratory chain).

ii. Alpers disease and other mitochondrial DNA depletion syndromes caused by mutations in the POLG gene, which encodes the catalytic subunit of the polymerase gamma enzyme responsible for replicating mtDNA.

However, identification and characterisation of the nuclear genes responsible for mitochondrial disease are still at relatively early stages. The challenge at present is that the underlying genetic defect cannot be identified using routine diagnostic methods in most patients who are suspected to have mitochondrial disease caused by nuclear gene mutations. The most efficient method for preventing human mitochondrial disease would therefore be to identify more of the nuclear genes causing these disorders.

Evidence for PND and PGD for mtDNA-related disease is limited to women who have low levels in blood of the m.8993T>G mutation in the MT-ATP6 gene [reviewed in 2]. We currently offer PND for
m.8993T>G to women with low levels of this mutation in blood presenting to the NCG mitochondrial diagnostic service in London, if they request this test after appropriate genetic counselling.

2. **Pronuclear transfer**

Pronuclear transfer and spindle transfer are exciting techniques which show some promise for prevention of mtDNA-related disease [3-5]. However we note that cytoskeletal inhibitors were used in this research. It is clearly important that the long term safety of cytoskeletal inhibitors on a developing embryo is fully assessed. For this reason, we would be in favour of a licence to extend research in this area, but do not consider that these techniques have reached the stage of being clinically applicable yet.


London, 15th March 2011
The Muscular Dystrophy Campaign welcomes the Human Fertilisation and Embryology Authority’s (HFEA) scientific review of the technology described as “pronuclear transfer” to avoid inheritance of mitochondrial disease from mother to child.

In 2010, promising results from research funded by the Muscular Dystrophy Campaign at Newcastle University showed that pronuclear transfer may be an effective method to prevent mitochondrial diseases being passed from mother to child. To date, the Muscular Dystrophy Campaign has invested over £500,000 into this research.

The researchers, led by Prof. Doug Turnbull, used embryos and eggs donated to research by couples undergoing in vitro fertilisation (IVF). These eggs had fertilised abnormally and could not be used for IVF so would have been discarded. The DNA contained within the nucleus of the embryos was transferred into eggs that had previously had their own nucleus removed. The embryos were allowed to develop in the laboratory for 6-8 days. Of the manipulated embryos 8.3% successfully developed to the blastocyst stage. Pronuclear transfer results in an embryo that carries 99.9% of the genes from its parents and less than 0.1% of the genes from the donor egg.

The researchers then measured if any of the mitochondria from the embryos were transferred over to the donor eggs. On average less than 2% was carried over and often no transfer of mitochondria could be detected. Previous research has shown that levels of abnormal mitochondria this low would not be expected to cause mitochondrial disease. The full results were published in *Nature*. A copy of the article is submitted alongside this document as evidence.

An estimated 6,000 people in the UK have a mitochondrial disease, 3,500 of these have a mitochondrial myopathy. There is currently no treatment for mitochondrial myopathies available and no prospect of an efficient treatment being developed in the near future. Moreover, because the defect sits on mitochondrial DNA and not chromosomal DNA, it is extremely difficult to predict how severely the child will be affected. This makes genetic counselling for prospective parents almost impossible. Pronuclear transfer has the potential to become the treatment or even cure for future generations that families have waited so long for, as this will give them the opportunity to have healthy children.
The researchers have carried out a number of further pre-clinical tests and have now exhausted all of the possible laboratory tests. They are now preparing to move this promising technology forward into clinical trial and we would strongly welcome the introduction of regulations which would allow research into the potential benefits and risks of pronuclear transfer to be carried out in the UK. The UK has been at the forefront of this research and our concern is to ensure that this technology will get tested for the first time in a controlled and regulated way.

It is unfortunate, however, that the proposed panel selected to review this issue comprised of scientists alone. There is no patient representative, despite patients ultimately bearing the related risks and given the complexity of the moral and ethical issues involved. In order to conduct a fair and relevant review, we believe dialogue must be opened up between the scientific and patient communities and would urge the inclusion of a patient representative on the panel that will decide whether this treatment is allowed to move forward into clinical trial. We would also urge the HFEA to co-opt a patient representative on to the Ethics and Law Advisory Committee to further consider how to move this research forward.

Dr Marita Pohlschmidt, Research Director
Dear Hannah,

I'm replying to your request for evidence for treatments to avoid mitochondrial disease. I am replying on behalf of Prof Robert W Taylor and myself, both of us working for the NCG Rare Mitochondrial Diseases Service and are based in Newcastle upon Tyne. Prof Taylor heads the mitochondrial diagnostic service, while I lead the children's clinical service for mitochondrial disease. We are both actively engaged in research programmes regarding mitochondrial disease. Prof Douglass Turnbull, lead clinician for the adult mitochondrial disease service, will be submitting his own evidence independently.

Mitochondrial disease is unique in that it can result from mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA). There are some unusual aspects of mtDNA biology that are worthy of mention as they are highly relevant to interpreting the outcome of some of the tests performed.

1. mtDNA is present in multiple copies per cell  
2. Two populations (wild-type and mutant) of mtDNA may co-exist within the same cell - a situation known as heteroplasmy  
3. One major factor in determining the pathogenicity of an mtDNA mutation is the proportion of mutated mtDNA present - once a tissue-specific 'threshold' is exceeded then disease will ensue.

At present, preventing transmission of mitochondrial disease is reliant on accurate genetic diagnosis, counselling and in the case of affected women, (or the mothers of affected individuals), the availability of prenatal chorionic villus biopsy in early pregnancy. In the case of mtDNA mutations, amniocentesis is less satisfactory for this purpose, as the cells sampled (highly differentiated skin cells) may not accurately reflect the mtDNA heteroplasmy level found in other fetal tissues. Interpretation of CVB for nDNA mutations causing mitochondrial disease is straightforward - the mutation is either present or not. Heteroplasmy complicates the interpretation of CVB for mtDNA mutations. In general, we have counselled prospective parents that <30% mutation heteroplasmy level carries an extremely low level of clinical disease; 30 - 70% is an intermediate level and carries a definite lifetime risk of disease, but that this is unlikely to manifest in early childhood; >70% mutation is regarded as high level and correspondingly high risk of disease. These interpretations of low/intermediate/high levels of heteroplasmy are in line with our reporting of heteroplasmy levels in other diagnostic tissues such as skeletal muscle. Rare exceptions to these guidelines are recognized and dealt with on a case-by-case basis. A number of the mutations for which we have been asked to perform CVB analysis have been novel, with little clinical data available outside of the immediate family.

In very rare circumstances, CVB cells can be cultured and a biochemical analysis of complex IV of the mitochondrial respiratory chain can be performed. The outcome of such an analysis is reliable if positive i.e complex IV deficiency is detected, but a negative result can be due to multiple confounding factors and the family should be counselled accordingly. This technique is only applicable to presumed nDNA mutations, where complex IV has been demonstrated to be deficient in muscle and no genetic cause has been identified. To our knowledge, this technique is only offered at one centre in the UK, (Oxford, Dr Garry Brown).
We have recently undertaken a brief review of prenatal diagnoses performed within the NCG Rare Mitochondrial Disease Service (3 centres - Newcastle, London and Oxford) between Jan 2007 and Oct 2010. During that time period 33 chorionic villus biopsies were analysed and 2 amniocenteses. Of these, 33 were performed because the mother was known to harbour a pathogenic mutation or a previous child had been affected. MtDNA mutations accounted for 8 cases. In 15 cases the mutation being screened for was positively identified (11 nDNA and 4 mtDNA). In 2 cases the heteroplasmy level for mtDNA mutation exceeded 70%. CVB analyses were offered for the following mutations:

- mtDNA: m.3243A>G; m.8344A>G; m.8993T>C; m.14453G>A; m.13513A>G; m.11777C>A
- nDNA: RARS2; PDHA1; MPV17; DGUOK; NDUFS2; TK2; SURF1; POLG1

An alternative method of avoiding mitochondrial disease, in families where it has been identified, is the use of Pre-implantation Genetic Diagnosis (PGD). This IVF technique involves biopsy (removal of a single cell) from an 8-cell blastocyst and analysis (sequencing) for the nDNA or mtDNA mutation, before returning the selected blastocyst(s) to the uterus. Again, for nDNA mutations this simply involves the detection or absence of the mutation. For mtDNA disease we have elected to use the same low/intermediate/high risk categories based on the level of mutant heteroplasmy detected. We (in Newcastle) have not yet completed a PGD cycle, but have 3 couples who will be going through this procedure within the next few months.

I hope this information is of value to you in making your deliberations regarding the strategies which should be adopted to prevent these dreadful diseases. If you require any further information, then Prof Taylor or I will be happy to oblige.

Regards,

Dr Robert McFarland MA MBBS PhD MRCP MRCPCH DoH/HEFCE Clinical Senior Lecturer in Paediatric Neurology Mitochondrial Research Group Newcastle University
www.mitochondrialncg.nhs.uk
We have previously submitted evidence to the Scientific and Clinical Advances Advisory Committee on 13\textsuperscript{th} May 2010 (Appendix 1)

**Pronuclear Transfer and Spindle Transfer**

Our recent work on pronuclear transfer (PNT) between abnormally fertilised human embryos revealed that PNT is compatible with onward development to the blastocyst stage (at a rate \(~50\%\) of that for unmanipulated abnormal embryos). We also showed that the carry-over of donor zygote mtDNA is minimal (<2\%), with many embryos containing no detectable levels of donor mtDNA (Craven et al, 2010; Appendix 2). We believe these results demonstrate that PNT has the potential to prevent the transmission of mtDNA disease in humans.

Our studies on metaphase II spindle transfer between human oocytes have revealed very similar findings. Our results show that metaphase II spindle transfer between human oocytes is compatible with onward development to the blastocyst stage following artificial activation. We have also shown that the carry-over of donor oocyte mtDNA is minimal (<2\%) (unpublished research; Appendix 3), which again suggests that spindle transfer has the potential to prevent the transmission of mtDNA disease in humans.

A recent study performed spindle transfer between metaphase II oocytes from non-human primates and resulted in the birth of live offspring with undetectable levels of spindle donor mtDNA (Tachibana et al, Nature 2009;461;367-72). The infants were healthy and their birth weights and gestational lengths within the normal range for the primate used. In addition, blastocysts derived from reconstituted oocytes gave rise to embryonic stem cell lines in which there were no detectable chromosomal abnormalities.
In their previous considerations, the Scientific and Clinical Advances Committee (SCAAC) stated that follow-up studies of primates created using spindle transfer would be pertinent. The growth of these offspring has been monitored monthly and to date, no difference has been noted between experimental monkeys born following metaphase II spindle transfer and controls. Their development is also within the normal range (Appendix 3). We have also been able to look at multiple tissues from 3 primates (at approximately 12 months old) who underwent metaphase II spindle transfer and very low, or undetectable levels, of carry over mtDNA was detectable (Appendix 3).

**Vitrification**

If pronuclear transfer is to be offered as a treatment to prevent the transmission of mtDNA disease, it will be necessary to have embryos from the mitochondrial patient (containing unhealthy mitochondria) and embryos from a donor (containing healthy mitochondria). It may be difficult to synchronize IVF cycles for both the patient and the donor, or there could be surplus embryos. We have therefore performed experiments to determine whether PNT could be performed successfully on frozen/thawed zygotes. Following optimisation of vitrification procedures, 91.6% of abnormally fertilised human zygotes survived thawing. Embryos were subsequently cultured to assess developmental potential following vitrification or used for pronuclear transfer. Manipulated embryos showed onward development to the cleavage stage at similar levels to those which had not been manipulated (unpublished research; Appendix 4).

**Preimplantation Genetic Diagnosis (PGD)**

PGD is a technique that determines the level of mutant mitochondrial DNA in a single blastomere to estimate whether or not the level of mutation in the embryo will give rise to mitochondrial DNA disease. The Scientific and Clinical Advances Committee (SCAAC) previously considered this technique to avoid mitochondrial
DNA disease and suggested that there should be further research to investigate the effects of the mitochondrial bottleneck and the implications of the reliability of a diagnosis based on preimplantation stages.

As noted by the Committee, a small number of studies have looked at levels of mtDNA mutation in blastomeres from disaggregated embryos and reported less variation between cells than might be expected (Steffan et al, J Med Genet. 2006;43:244-7; Thorburn et al, Molecular Genetics and Metabolism 2009; 98; 6; Monnot et al, Hum Mutat. 2011;32:116-25). We have also examined the level of mtDNA mutation in blastomeres from two patients carrying different mtDNA mutations. The results revealed very little variation in the mutation load between blastomeres disaggregated from a single embryo (unpublished research; Appendix 5). We also show close correlation between mutation load detected in the blastomere and that observed in the trophectoderm from the same embryo cultured to the blastocyst stage (Appendix 5), again supporting that PGD is likely to be a helpful technique for mothers carrying heteroplasmic mtDNA mutations.
Pronuclear/metaphase II spindle transfer

• Why do we need this technique?
• What have we done so far?
• What are the risks?
• What do we need to do in the future?
"I’ve lost six newborn babies’

Sharon Bernardi tells new! about the curse that has robbed her of six children

When she first fell pregnant 25 years ago, Sharon Bernardi had no idea of the nightmare ahead.

After a normal pregnancy, she gave birth to a healthy girl. But only 28 hours later, her baby was dead. Doctors could give no explanation.

And so, she was bafﬂed and devastated. This tragic tale repeated itself ﬁve more times, as each of Sharon’s babies lived for just a few hours. Only one, Edward, survived.

Sharon had just celebrated Edward’s 18th birthday, full-time mother Sharon, who lives with partner Neil Smith, 36, in Sunderland, tells new! how she’s coped with the loss of six babies.

Aside from some terrible morning sickness, my ﬁrst pregnancy was uneventful. It was only after the delivery that things began to go wrong. My daughter, Gemma Louise, was born a perfect pink, chubby baby. But half an hour later, she developed breathing problems, and was whisked to intensive care. After 24 hours, she died.

This was meant to be the happiest day of my life. Instead, I was mourning my baby’s death and preparing myself for her burial. It was heartbreaking. The doctors could give no explanation, but I knew that I wanted to be a mother and didn’t hesitate to try again.

A couple of years later, in 1984, I gave birth to another baby – a boy, Ian. This time I was more aware. I knew that while my baby was still inside me, he was safe. I was almost scared of giving birth.

I had a Caesarian section because he was breech. I was under general anaesthetic and woke up two hours after, to be told my son had died.

Again, there was a postmortem and, again, I was given no explanation why Ian died. The babies were perfectly healthy on delivery but just didn’t seem able to survive on their own. As there seemed to be no medical reason, I was never warned against trying for more children.

Three years later, in 1987, I had Geoffrey, again born by Caesarian. As the hours went by and nurses came in and out, doing checks, I started to feel hopeful. Each minute I didn’t hear anything, I took as a positive sign – it meant my baby was still alive. But 30 hours after the birth, the doctor’s footsteps came and so did the news. I was crying. Geoffrey, like the two babies before him, had died.

Family curse

I began asking myself if it was my fault. That’s when my mum admitted that she had been through a similar history. She had had four babies, the ﬁrst three of whom died a few hours after birth. I was the fourth – the lucky one. It made me wonder if it was something I had inherited from her, but it also gave me hope. I’d survived – maybe one day I’d have a baby that would live.

"Edward, my fourth child, born two years later in 1989, would be that survivor. He was terribly poorly and I was warned of his chances of survival were slim, but the hours turned into days, then weeks. The doctors didn’t know why he was different to the other babies, but after a blood transfusion and ﬂu shot, a ventilator, he made it. Five weeks after he was born, I took him home.

Finally, I was the mother I’d longed to be. Edward was a bouncy baby, reaching all his milestones – walking by 14 months and talking as normal. Then, when he was four, he began falling over a lot. Experts ran tests and scored our medical history. It was then that Leigh’s disease was mentioned. I learned it was a rare, inherited disorder that caused degeneration of the central nervous system. Symptoms could include: a lack of muscle control and breathing, and heart problems. It was likely that my mum had the abnormal gene on to me and as a carrier, I had passed it on to my three babies – and now Edward."

Only child

Doctors didn’t think Edward would make it to his ﬁfth birthday. But he was a fighter. I learned that although there was no cure, Leigh’s disease could be managed by diet and medication. The disease has limited Edward’s quality of life – he is conﬁned to a wheelchair and suffers with epilepsy. But he was a happy child with lots of friends and a great sense of humour.

"I learned to accept what I had in Edward. But I didn’t want him to be an only child and I know that there was a chance I could have another baby that would survive. I was aware of the risks, but I wanted to try for more children. I had Holly in 1991 and Olivia in 1990, but neither survived.

In 1996, I split from my husband. I met Neil in a club two years later. We had one baby together, Caroline, in 2000 but she died after 29 hours. It never got easier. I’d leave hospital exhausted, with no baby to show for it – instead, I’d have a funeral to arrange."

I feel sad that Edward never had a brother or sister to play with, but he’s aware of the brothers and sisters he never knew. We’ve got photos of my lost babies round the house and if anyone asks, I tell them I have one child – but I’ve had seven.

"Neil has been supportive but as a mother, losing your children is something you never ever get over. I think about them every day, and have kept their photographs and ﬁngerprints. They’re a part of my family history. I can’t afford to break down because of Edward. I need to be strong for him, and it’s important that he enjoys a good quality of life. If it gets too much or I feel upset, I’ll deal with it privately.

"Of course, sometimes I wish I’d had another child or that I could make Edward perfectly healthy – but I know now that it just wasn’t meant to be. So, although I’ll always remember my six other children, writing cards and lighting candles on each of their birthdays, I also appreciate what I’ve got."

Edward has just celebrated his 18th birthday. Most kids with Leigh’s disease die between the ages of two and eight, so he’s done so well to get this far. I didn’t know what the future holds, but we take each day as it comes.

"Maybe one day, he could have his own family. If so, as a male he won’t pass on the condition and we will ﬁnally have broken the family curse."

If you want to share your amazing true story with new! call 0871 520 7780 or email truelife@express.co.uk

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March 24 2004
Pregnant Lesley Purvis has more reasons than most to be nervous about the birth of her third child. A dark cloud has cast a shadow over the young mum’s joy in the form of a cruel familial curse.

The news of an ultimate family condition has already taken most of her family, killing many and leaving her heartbroken. Her 28-year-old only son that her two nieces —8-year-old Beth Purvis and her twin, Beth Pritchard — will escape its clutches.

Lesley suffers from MELAS Syndrome which can cause life-threatening problems for her family. The term MELAS stands for mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes.

Her 32-year-old sister Marilyn was the first in the line, but she managed to escape the illness. However, her sister-in-law, 30-year-old Michelle Purvis, and her niece, 8-year-old Beth Purvis, are also suffering from the same condition.

Lesley told the Daily Mail that the disease has only slightly affected her, and she is looking forward to being a grandmother. She said, “It’s a little scary to think about, but I’m just hoping for the best.”

“By the time my children are grown up, there might not be much left of me,” she added.

“BAPSY”

MELAS is a rare genetic disorder that can affect any part of the body, including the brain, muscles, and heart. It causes symptoms that can range from mild to severe and can vary from person to person.

There is no single cure or treatment for MELAS, as it can affect each person differently. However, managing symptoms and lifestyle changes can help improve quality of life.

LESLEY PURVIS

“Just don’t know what the future holds for my children.”

— Lesley Purvis

GENERATIONS OF SUFFERING

MELAS FACTFILE:

MELAS — Mitochondrial Encephalopathy Lactic Acidosis and Stroke-like episodes — is a rare killer disease. It is caused by mutations in the DNA and means the body can’t break down food to make energy.

Symptoms include loss of balance, tingling, deafness, diabetes, and other allergies, headaches, and brain disorders. It is caused by a build-up of toxic cells in the brain, temporary local problems and dementia.

MELAS affects each person differently, and the symptoms can vary from person to person. It can affect the age of 20 years old, although most affect symptoms before the age of 3 years of age. There is no cure for MELAS, which is progressive and fatal.
Equitable access to care

National Commissioning
Pronuclear transfer

- Chromosomes enclosed by a clearly visible nuclear envelope
- Proven to be compatible with development in mice (Meirelles & Smith, 1997; Brown et al, 2006)
- Proven to prevent transmission of a mtDNA deletion in mice (Sato et al, Proc Natl Acad Sci, 2005)
Recipient zygote

Donor zygote

Enucleation

Fusion

Reconstituted zygote

Culture *in vitro*

Embryo disaggregated for mtDNA analysis

Embryo cultured to assess development
ALL sequences 263 and 16519 unless marked otherwise
m.16519T>C

HaeIII

PNT embryo

U  B1  B2  B3  B4  B5  B6  D

(bp)

223
132
91
60
31
Mitochondrial gene replacement in primate offspring and embryonic stem cells

Masahito Tachibana¹, Michelle Sparman¹, Hathaitip Sritanaudomchai¹, Hong Ma¹, Lisa Clepper¹, Joy Woodward¹, Ying Li¹, Cathy Ramsey¹, Olena Kolotushkina¹ & Shoukhrat Mitalipov¹,²,³

doi:10.1038/nature08368
Human metaphase II oocyte viewed using Oosight

The spindle is marked with an arrow
Pronuclear/metaphase II spindle transfer

• Why do we need this technique?
• What have we done so far?
• What are the risks?
• What do we need to do in the future?
Heteroplasmic mitochondrial DNA mutations in normal and tumour cells

Yiping He¹, Jian Wu¹, Devin C. Dressman¹, Christine Iacobuzio-Donahue², Sanford D. Markowitz³, Victor E. Velculescu¹, Luis A. Diaz Jr¹, Kenneth W. Kinzler¹, Bert Vogelstein¹ & Nickolas Papadopoulos¹

In particular, they demonstrate that individual humans are characterized by a complex mixture of related mitochondrial genotypes rather than a single genotype.
ALL sequences 263 and 16519 unless marked otherwise
Mitochondrial Genetics: maternal inheritance

- Egg
- Sperm
- Fertilisation
- Nuclear DNA
- Female pronucleus
- Male pronucleus
- Paternal mitochondria destroyed after fertilisation
- Maternal inheritance
Next steps (1)

- Optimise the pronuclear transfer and metaphase II transfer procedures
  - Increase development to the blastocyst stage
  - Minimise mtDNA carryover
Next steps (2)

• Effects of pronuclear transfer and metaphase II spindle transfer on embryo development
  – Can reconstituted eggs/zygotes undergo normal cell division?
  – Do they develop to the blastocyst stage?
  – Are these blastocysts normal?

• Studies on offspring of non-human primates
  – In collaboration with Dr Shoukhrat Mitalipov, National Primate Centre, Oregon
The Newcastle System: Environmental control during manipulations

Fully enclosed system protects against temperature and pH fluctuations, and VOCs

40% of embryos donated to research develop to the blastocyst stage
Are blastocysts from reconstituted oocytes/zygotes normal?

- Total Cell number
- Number of cells in the Inner Cell Mass (ICM)
Lineage restriction in the ICM

We will determine whether the events associated with lineage specification in the ICM can be used as an additional Marker to assess the effect of Pronuclear / spindle transfer in Human embryos.

Chazaud et al. (2008) Dev Cell (10)
Epigenetic Analysis

Expression of lineage-specific transcription factors e.g. Nanog, Gata6

DNA methylation in human blastocysts

Santos et al, 2010 Hum. Reprod. 25(9)

Nanog-expressing cells are hypermethylated
Genetic analysis of blastocysts

- Nuclear genome
  - Karyotypes
  - Array-based CGH

- Mitochondrial genome
  - mtDNA copy number
  - mtDNA mutations
Summary (1) Analysis of human blastocysts

- Development to the blastocyst stage

- Analysis of blastocysts
  - Morphology
  - Cell number (Total cell number + ICM number)

- Nanog expression in the ICM

- Epigenetics (Direct assessments)
  - DNA methylation
    - *other parameters subject to the development of satisfactory assays*

- Genetic analysis
  - Nuclear genome
  - Mitochondrial genome
Summary (2) Analysis of offspring (rhesus monkey)

- Growth characteristics – body weight
- Epigenetic analysis
Why not just use PGD? It has already been used successfully to avoid the disease (1-4). Simultaneous selection of male embryos could also prevent 3rd generation inheritance altogether (4).


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UMDNJ-Graduate School of Biomedical Sciences at NJMS, Biomedical Science Program
I would worry about longer term toxicity from CCD, nocodazole and Sendai virus fusion. How to monitor in humans? Are primate studies sufficient and how old are the youngsters born? Are they being monitored for disease/behaviour? Why is use of nocodazole needed? What studies are in hand to check chromosomal/epigenetic variations in embryos reaching blastocyst stage? Has short term exposure to CCD, Nocodazole or sendai virus in non-manipulated or ICSI generated human embryos been undertaken?

Use of Noc and even CCd questionably necessary in these?

Nuclear transfer and electrofusion in bovine in vitro-matured/in vitro-fertilized embryos: effect of media and electrical fusion parameters.

Van Stekelenburg-Hamers AE, Van Inzen WG, Van Achterberg TA, Kruip TA, de Laat SW, Weima SM

Somatic cell nuclear transfer in the pig: control of pronuclear formation and integration with improved methods for activation and maintenance of pregnancy.


Biol Reprod. 2002 Mar;66(3):642-50..

and see
Chromosome malsegregation and embryonic lethality induced by treatment of normally ovulated mouse oocytes with nocodazole.

Generoso WM, Katoh M, Cain KT, Hughes LA, Foxworth LB, Mitchell TJ, Bishop JB.

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http://www.pdn.cam.ac.uk/johnson/
&
http://www.christs.cam.ac.uk/college-life/people/fellows-list/display.php?id=73
Dear Ms Darby,

Re: Cytochalasin B. Our ref: ICE 11/0407

Thank you for your recent enquiry to the Medicines and Healthcare products Regulatory Agency (MHRA).

According to our licensing records cytochalasin B is not currently licensed for use in any medicinal products.

N.B. Information is not released on Marketing Authorisations that are currently under assessment.

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Yours sincerely

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Dear Helen

In response to your questions

What are the reasons for using cytochalasin and nocodazole in your pronuclear transfer experiments?
Cytochalasin and nocodazole are reversible inhibitors of actin and microtubule polymerisation. Actin and microtubules form a dynamic fibre-like network which acts a cytoskeleton in all cell types. In preliminary experiments we found that removal of pronuclei in the absence of cytoskeletal inhibitors was not compatible with survival of human zygotes. This was because we had to penetrate the plasma membrane with a large bore pipette, which resulted in persistent leakage of cytoplasm and degeneration of the zygote. We therefore adapted the technique described by McGrath and Solter (1983), who successfully performed pronuclear transfer between mouse zygotes by incubating them in cytochalasin and nocodazole. This approach enabled us to "pinch off" the karyoplast consisting of the pronuclei surrounded by a small amount of cytoplasm enclosed in plasma membrane. During the course of our research we have optimised the exposure to these agents to reduce the size of the karyoplast. It has been long established that the cytoskeleton reassembles upon removal of cytochalasin and nocodazole from the incubation medium.

What agents have you used in your spindle transfer experiments and what are the reasons for using these?
Cytochalasin B, which inhibits polymerisation of actin is used during the spindle transfer experiments to enable us to remove the spindle in a membrane-bound karyoplast. It is neither desirable nor necessary to depolymerise the microtubule network by nocodazole treatment of oocytes. This is because the microtubule network becomes reorganised in eggs and other dividing cells to form the spindle.

Why are you activating the eggs following spindle transfer and not fertilising them?
These experiments were initially performed as part of a somatic cell nuclear transfer project which involved artificial activation rather than fertilisation. By measuring mtDNA content of spindle karyoplasts and in reconstituted embryos, we were able to maximise the data obtained from these experiments. In so doing we made progress in understanding whether spindle transfer would provide a useful option for preventing transmission of mtDNA disease. Since then we have obtained permission from the HFEA to fertilise human oocytes specifically for this purpose.

In view of the very interesting discussion at the workshop on the review of the effectiveness and safety of methods to avoid mitochondrial disease about the possibility of segregation of transmitted mutations to different tissues we are also enclosing details of tissue levels of two common mitochondrial DNA mutations. These results are from several post mortem samples and show remarkably little variation between tissues.

Hope this all you need - if not please get in touch Doug

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**Cytoplasmic transfer**

Cytoplasmic transfer can be performed by injection of a small amount of cytoplasm (e.g., 5-15%) into a mature or a fertilized oocyte. This can be accomplished, respectively, by direct injection of cytoplasm into oocyte’s cytoplasm using a very fine pipette (e.g., an ICSI pipette [1]) or by electrical fusion of a cytoplast (previously introduced in the perivitelline space) with a zygote [2].

Cytoplasmic transfer was introduced in humans in the late 1990s with the aim of treating infertile patients [1]. The procedure did appear to rescue oocyte development leading to the birth of sixteen children [3]. As expected, cytoplasmic transfer results in progeny with mtDNA potentially derived from two sources: mother and cytoplasmic donor [3-5]. Although the contribution of donor mtDNA is small, it far exceeded the expected 5-15% proportion in some cases [5]. Moreover, two human concepts achieved following cytoplasmic transfer were found to be chromosomally abnormal and one child developed a pervasive developmental syndrome at the age of eighteen months [3, 6]. This generated great concern about the use of this technique in humans leading it to be banned in the United States by the Food and Drug Administration [5-8]. However, it is currently known that chromosomal abnormalities occur at a high frequency in human oocytes mainly due to mis-segregation errors during the meiotic division [9]. Thus, the chromosomal abnormalities seen in concepts born following cytoplasmic transfer may not be caused by the manipulation procedure itself, but due to factors intrinsically related to the patients (e.g., mother’s age). This is further supported by the finding that the manipulation procedure needed for ICSI is not far different than that performed in the past for cytoplasmic transfer and has not been associated with a much higher levels of miscarriage and birth defects compared to IVF [10].

The use of cytoplasmic transfer for the purpose of correcting an mtDNA disorder has not yet been attempted, but the potential of this method to provide a cure, or at least an attenuation of symptoms, has generated some interest from researchers within the field [10-14]. Theoretically, cytoplasm donated by oocytes of healthy women could be used to dilute the mutant mtDNA level to below the critical threshold for disease; although a number of technical hurdles and questions of safety would need to be addressed prior to clinical application. To guarantee that offspring will not develop symptoms of the mitochondrial disorder, the mutant mtDNA level should be diluted to a very low level. This is particularly important in diseases where the critical threshold of mutant mtDNA is low [15]. Only 5-15% of cytoplasm was introduced in the early cytoplasmic transfers, and this might be insufficient to lower the mutant load sufficiently. It might be possible to introduce a much larger amount of cytoplasm into the oocyte or to replace a proportion of the oocyte’s cytoplasm. A possible way to do that would be by introducing purified mitochondria instead of cytoplasm. This has been performed using mitochondria isolated from somatic and embryonic cells but it does not seem to be enough to achieve high levels of donor mtDNA in the reconstructed oocyte [16-18]. This might be overcome by partial depletion of mitochondria from the recipient oocyte/zygote following a procedure previously reported [19, 20]. We are currently undertaking several tests to determine whether partial removal of recipient’s mitochondria and supplementation with
purified mitochondria is a viable option to lower the mutant load. Compared to spindle and pronuclear transfer, cytoplasmic transfer has the advantage of not requiring chromosomal manipulation. Moreover, there is no need for generating embryos exclusively to be used as cytoplasmic donors because the cytoplasm can be donated by oocytes [2]. Cytoplasmic transfer can also be accomplished in the absence of drugs potentially toxic such as cytochalasin and nocodazole that are commonly used in spindle and pronuclear transfer. Finally, cytoplasmic transfer does not require a high number of oocytes because it can be efficiently accomplished and has no detrimental effect on developmental rates [2].

Many questions related to the safety of cytoplasmic transfer have also to be addressed. For instance, would the recipient nuclear material be able to regulate cytoplasmic-donor mtDNA? In a recent work we have found a pronounced segregation of donor mitochondria introduced by cytoplasmic transfer [20]. This highlights the need for further investigations aiming to clarify whether this was caused by the procedure itself or, for instance, by a replication advantage of the recipient mtDNA. Moreover, apart from mitochondria, other organelles and molecules are also introduced in the oocyte/zygote during cytoplasmic transfer, which may induce epigenetic alterations [8].

In conclusion, although it may be a promising method, cytoplasmic transfer is not currently a viable option to prevent transmission of mtDNA disorders.

References
Dear Peter,

Here are the answers to your queries.

Cytoplasmic transfer in order to improve implantation was attempted in patients who repeatedly failed implantation after many attempts at IVF. All procedures were performed at The Institute of Reproductive Medicine of Saint Barnabas Medical center in New Jersey between 1997 and 2001. A total of 37 cycles was performed. Patients were not selected based on maternal age. Many of these patients had PGD for chromosomal anomalies previously, but not all. Some patients were deselected when the number of chromosomal anomalies was very high. The findings have been published in a number of papers as well as our mitochondrial heteroplasma findings and preceding mitochondrial mutation work.

The questions pertaining to the health of the offspring are very relevant, but follow-up has been problematic. The clinical team (Richard Scott and co-workers) responsible for all births but one, left late in 1999 to go private. Laboratory staff attended most births and gathered data from the ongoing pregnancies, as well as cord serum and placental tissue when consented. These findings have been published.

The new clinical team at Saint Barnabas was open to follow up, however, this was affected by negative press and the FDA concerns as a result in May of 2001. The FDA did not stop the procedures but instead declared that a so-called IND permit was needed to perform cytoplasmic transfer.

Our team started this application process in 2002. There was a public hearing organized by the FDA in 2002 and we subsequently performed molecular experiments (in mouse and spare human eggs) in order to confirm that there was no evidence of nuclear DNA transfer. We had a successful so-called pre-IND meeting with the FDA to consider our proposal in 2003.

The FDA application was halted when the OBGYN department at Saint Barnabas was asked by the hospital directors to privatize in 2003. We subsequently split into a PGD team (Reprogenetics), a research laboratory (Tyho-Galileo Research Laboratories) and the clinical team (IRMS) which is still located at Saint Barnabas but is private. Funding to continue the FDA application was now gone and the application process was never restarted. As far as I know this was the only effort to obtain an IND permit for Cytoplasmic Transfer.

Follow-up was done by interviews performed by clinicians until 2003. As is not unusual, quite a few patients did not permit us to follow-up.

Part of the negative publicity in 2001 centered on the possibility of chromosomal anomalies after cytoplasmic transfer. One of the miscarriages was successfully screened and showed XO. An ongoing twin pregnancy had amnio and one twin had XO and was reduced. These findings were immediately reported to our Internal Review Board. There was no statistical evidence that this was worrisome and the procedures were continued with disclosure to patients. XO is the most common chromosomal anomaly in pregnancy.
In the follow-up after births (n=17) we had found that one of two male twins showed signs of PDD. Parents did not consent to follow up. I am therefore not sure of the current status of this child. I personally only keep in touch with the mother of one male child who had the largest shift in heteroplasmy tested at birth. Obviously this is a fascinating case. This child is now 11 years old. He is an excellent student and developing normally.

I hope this helps.

Let me know if you need more information.

Jacques