Modulating mitochondrial quality in disease transmission: towards enabling mitochondrial DNA disease carriers to have healthy children

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Abstract

1 in 400 people has a maternally-inherited mutation in mitochondrial DNA (mtDNA) potentially causing severe, incurable disease. In so-called heteroplasmic disease, mutant and normal mtDNA co-exist in the cells of carrier women. The severity of the disease in the offspring of carriers depends largely on the proportion of inherited abnormal mtDNA molecules.

Families who have had a child die of severe, maternally-inherited mtDNA disease need reliable information on the risk of recurrence in future pregnancies. However, prenatal diagnosis and even estimates of risk are fraught with uncertainty because of the complex and stochastic dynamics of heteroplasmy. These complications include a mtDNA bottleneck, whereby hard-to-predict fluctuations in the proportions of mutant and normal mtDNA may arise between generations.

In “mitochondrial replacement therapy” (MRT), damaged mitochondria are replaced with healthy ones in early human development, using nuclear transfer. We are testing whether non- invasive alternatives can be used, notably activating autophagy, a cellular quality control mechanism, in which damaged cellular components are engulfed by autophagosomes. Mathematical theory, supported by recent experiments, suggests that this strategy may be fruitful in controlling heteroplasmy.

Using mice that are transgenic for fluorescent LC3 (the hallmark of autophagy) we quantified autophagosomes in cleavage stage embryos. We confirmed that the autophagosome count peaks in 4 cell embryos and this correlates with a drop in the mtDNA content of the whole embryo. This suggests removal by mitophagy (mitochondria-specific autophagy). We suggest that modulating heteroplasmy by activating mitophagy may be a useful complement to mitochondrial replacement therapy.

The problem of heteroplasmic mtDNA disease and preimplantation development

Mitochondrial diseases range from severe to very mild and common, potentially affecting up to one in 400 individuals [1]. Those caused by pathogenic mutations in mitochondrial DNA (mtDNA) are problematic because of the unique biology of maternal inheritance. Prenatal diagnosis [2-4] and even estimates of risk are fraught with uncertainty [5] because of heteroplasmy (co-existing normal and mutant mtDNA). There is also a threshold effect in most mtDNA diseases, with the level of heteroplasmy required for symptoms to become manifest varying from <10% to 100% mutant mtDNA in different tissues. In addition, there is a mtDNA bottleneck whereby dramatic and unpredictable fluctuations in the proportions of mutant and normal mtDNA may arise between generations (illustrated in figure 1 left panel): recent work combining mathematical theory and experiments has helped elucidate the debated mechanism of this process [6]. During transmission of mtDNA from mother to child, significant fluctuations are already apparent in oocytes in both controls [7] and carriers of mtDNA disease [8-10].

The clinical implications of the mitochondrial bottleneck and the strategies for preventing transmission of mtDNA disease are illustrated in figure 1. Oocyte donation (figure 1B) is an appropriate strategy for all maternally transmitted mtDNA disease because it effectively reduces the risk of transmission to the population prevalence. Pre-implantation genetic diagnosis (PGD, figure 1C) [2] is licensed in the UK to reduce transmission of mtDNA diseases from mother to offspring [11]. In PGD the mutant load of embryos produced by in vitro fertilization (IVF) is estimated from either 1-2 cells taken from cleavage stage embryos, or approximately five trophoblast cells from blastocysts cultured *in vitro.* The embryo with the lowest mutant mtDNA load can be selected for transfer to the uterus, greatly reducing the risk of mtDNA disease in any resulting pregnancy [12], [3]. Estimating mtDNA mutant load from a single blastomere of a cleavage stage embryo [13] is accurate, but measurements based on trophoblast cells in a blastocyst biopsy are more controversial [14], [15]. This might be because mtDNA segregation coincides with the increase in oxidative phosphorylation that occurs at implantation.

Mitochondrial replacement therapy (MRT, figure 1D) is now available in the UK as an alternative approach to PGD, apparently successful in monkeys [16] and mice [17], and imminently to be performed in humans. 'Cytoplasmic transfer' of donated, healthy mitochondria has been applied clinically with a view to improving function in aged human oocytes. A low level of injected mtDNA was detectable in the resulting "transmitochondrial" children [18]. One transmitochondrial child born after cytoplasmic transfer was held to be autistic [19], but the numbers were insufficient to determine whether this procedure was overall beneficial or detrimental. Introducing the maternal nucleus into a donor cell with healthy mtDNA immediately before (metaphase spindle transfer[16]) or after (pronuclear transfer, along with the male pronucleus[20]) fertilization is more effective in increasing the proportion of normal mtDNA [10]. However, there difficulties in synchronising menstrual cycles [21], risks from imprinting of nuclear DNA [22] and from compatibility between nuclear and mitochondrial DNA [23] and ethical concerns around having three genetic parents [24].

One potential complication arising from these therapies is the risk of introducing non-compatible mtDNA [23], so that mtDNA segregation favours the pathogenic mutant mtDNA. To analyse this issue, Burgstaller et al. produced four heteroplasmic mouse models by ooplasm transfer [23] , placing various naturally-occurring mtDNA haplotypes from mice captured from the wild in Europe onto a common laboratory mouse mtDNA and nuclear background (C57BL/6N). The wild-derived haplotypes used differ considerably from each other and from C57BL/6N, leading to variable genetic

distances between haplotypes in the four crosses. A mathematical framework facilitated the direct comparison of many of these mice, revealing that tissue-specific segregation was very common (including within post-mitotic tissue types), the magnitude of segregation increasing with the genetic distance between the mtDNA haplotypes [23]. These data suggest that unpredictable segregation of mutant mtDNA could impair the effectiveness of mitochondrial replacement therapy unless donor and recipient mtDNA haplotype are closely matched [25]. This would be of particular concern if heteroplasmy *per se* were in some way detrimental [26].

Mitophagy improves mitochondrial quality

Mitophagy is a mitochondria-specific type of autophagy (self-degradation by cells) with the potential to remove mtDNA mutants, illustrated in figure 2. Mitophagy is regulated by both mitochondrial membrane potential and dynamics. In the best-known description, mitochondrial depolarisation activates PINK1 to recruit ubiquitin ligase, Parkin, to mitochondria, leading to clearance. On the other hand excessive mitochondrial fission leads to depolarisation-independent mitophagy [27]. This may be exacerbated by reduced expression or ubiquitinylation of pro-fusion proteins OPA1 and the mitofusins (Mfn1 and Mfn2) or activation of pro-fission proteins such as DRP1. Damaged mitochondria are recruited to the autophagosome via the mitochondrial proteins Nix and BNIP3 and the adapters P62 and LC3-II. Autophagy proteins ATG5 and ATG7 regulate formation of the autophagosome. The autophagosome then fuses with a lysosome and its contents degraded at low pH. Small regions of the mitochondrial network serviced only by detrimental mutant mtDNA may have a decreased membrane potential and may be isolated by fission [28]. The clearance of these non-functional, isolated mitochondria could filter out damaged mtDNA preventing transmission to the next generation [29-33].

Although widely discussed, this type of mitophagy may be quantitatively less important than mitophagy that is activated by nutrient deprivation. These processes have been designated type II and type I mitophagy respectively [34]. Type 1 mitophagy declines with age [35] and may remove the bulk of reactive oxygen species (ROS) that result from oxidative phosphorylation. It could underlie the beneficial effect of low nutrient intake on longevity [36]. Type 2 mitophagy involves PINK1 and Parkin and appears to be important in preventing neurodegeneration. While dysfunctional mitochondria often fragment, other types of mitochondrial energetic stress, including nutrient deprivation and exposure to the mitochondrial poison doxorubicin[37], induce fusion of the network, variously described as mitochondrial "elongation"[38] and "stress-induced mitochondrial hyper-fusion" (SIMH) [39]. This pro-survival mitochondrial response to stress prevents mitophagy [38] and increases cellular ATP, likely enabling cells to tolerate high levels of mutant mtDNA [40]. While an adaptive response in the short term, SIMH prevents mitochondrial fragmentation and may impair mitochondrial quality control by mitophagy, as well as playing other physical and chemical roles in cells [41]

Autophagy is essential for normal pre-implantation development in mice [42]. Embryos lacking oocyte-specific expression of Atg5 and hence normal autophagosomes failed to develop beyond the 4- to 8-cell stage [43]. While mitophagy could be critically important for controlling mtDNA segregation at this stage, little or nothing is known about mitophagy in the germline.

Measuring mitophagy

Mitophagy is notoriously even more difficult to measure than autophagy. The abundance and flux of autophagosomes engulfing mitochondria are specific measures of mitophagy that are preferred to

quantifying key autophagy proteins [44]. We used pharmacological modulators of autophagy to validate two techniques for quantifying mitophagy [35] . First we used the IN Cell 1000 analyzer to quantify mitochondrial co-localisation with LC3-II positive autophagosomes (figure 2). Unlike conventional fluorescence and electron microscopy, this high-throughput system is sufficiently sensitive to detect transient low frequency autophagosomes. Secondly, because mitophagy preferentially removes pathogenic heteroplasmic mtDNA mutants, we developed a heteroplasmy assay based on loss of m.3243G mtDNA, during culture conditions requiring oxidative metabolism ("energetic stress"). The effects of the pharmacological modulators on these two measures were consistent, confirming that the high throughput imaging output (autophagosomes co-localising with mitochondria) reflects mitochondrial quality control. To further validate these methods, we performed a more detailed study using metformin, the most commonly prescribed antidiabetic drug that is still sometimes used in Maternally Inherited Diabetes and Deafness (MIDD). This confirmed our initial findings and revealed that metformin inhibits mitophagy at clinically relevant concentrations, suggesting that it may have novel therapeutic uses.

MtDNA copy number during pre-implantation development

While relatively few mtDNA genomes (~200 in mouse) are present in primordial germ cells, the earliest stages of germ line differentiation, there is a massive expansion in mtDNA content to 200,000-300,000 [45] as the oocyte grows and matures. While oocytes must contain at least 40,000-50,000 copies of mtDNA in order for an embryo to give rise to a viable foetus [46], unusually high levels of mtDNA at the blastocyst stage of pre-implantation development are associated with failure of implantation [47]. Most authors state that mtDNA content of the oocyte/embryo remains constant between ovulation and morula [48-50], but this is not the case in cows [51] where mtDNA declines by 60% between the 2 and 4/8 cell stage. Immediately after fertilisation, mtDNA barely replicates [48-50] and metabolism is slow [52]. At the blastocyst stage, a proportion of undifferentiated cells become the inner cell mass which eventually gives rise to the body of the embryo, the remainder differentiating into the placenta and membranes. A tiny minority of cells become the precursors of primordial germ cells that transmit mtDNA to future generations.

We studied mtDNA copy number in oocytes and pre-implantation embryos generated *in vitro* using quantitative PCR. The mtDNA of these mice is wild type C57Bl6. Figure 3 shows that the total mtDNA content of the oocyte developing through cleavage stage embryo to blastocyst does not remain constant as described by previous authors [48-50]. Rather, there is a progressive decline in (total per oocyte/embryo) mtDNA to ~50% by the 4/8 cell stage. The initial drop is apparent in other publications but has barely been reported[53]. Unfertilised oocytes had a higher mtDNA content than either those that failed to fertilise or single cell zygotes. This strongly suggests that mtDNA turns over during IVF development. The reason for the drop in mtDNA content is unclear. However, it could involve mitophagy as discussed below.

Mitochondrial dysfunction drives cells to increase mtDNA copy number in both tissue culture models and *in vivo*, a compensatory adjustment that may be ROS dependent [54]. Both mtDNA content and heteroplasmic load increase with developmental stage in oocytes and cleavage embryos carrying the m.3243G mutant mtDNA [55]: potentially mitochondrial dysfunction drives proliferation of mitochondria and mtDNA as it does in muscle and placenta [56].

Selection against detrimental mtDNA mutants contributes to the bottleneck

Purifying selection against detrimental mtDNA mutants in mouse [29, 30, 57] may have evolved to maintain germline homoplasmy. In perhaps the first transmitochondrial mice, we found that mutant mtDNA was rapidly lost [31]. In a second model, maternal transmission of mtDNA rearrangements was attenuated by advancing maternal age [32]. Thirdly, using a proof reading mutation of the mtDNA polymerase to generate multiple mtDNA mutations, Larsson demonstrated selection against transmission of deleterious mtDNA mutations to the offspring [29] that was not apparent in oocytes [58] [30]. A marked difference in distribution has also been shown between different pathogenic mtDNA in heteroplasmic mutant oocytes, such as m.3243A>G, rearrangements [8] and m.8993T>G [3, 12].

Around the onset of biparental gene expression, there is a surge of autophagy [42]. This was neatly demonstrated by Mizushima and colleagues, who visualised autophagosomes by making a mouse in which they tagged the autophagy protein, LC3, with the fluorescent marker, GFP [59]. This could be a critical stage in purifying selection of transmitted mtDNA. We used Mizushima's mouse to investigate whether the drop in mtDNA we had documented soon after ovulation was accompanied by evidence of autophagy. Figure 3 shows data that autophagosome counts are high at the stage when mtDNA content is dropping. The lowest mtDNA copy number is at the morula stage, by which time the preceding surge in autophagosomes has disappeared.

To determine whether the mtDNA content at this stage could be modulated pharmacologically we exposed two-cell embryos to activators of mitophagy, either rapamycin or phenanthroline for 4 hours. Figure 3 shows preliminary evidence that both of these are able to activate mitophagy, rapamycin significantly increasing the number of autophagosomes and phenanthroline decreasing the mtDNA content (both $p<0.01$).

A theoretical framework with which to analyse and predict the effects of these experimental interventions has recently been developed. Johnston et al. combined mathematical modelling with a range of existing and new experimental measurements of heteroplasmy during development to quantitatively describe the processes altering developing mtDNA populations in mice [6]. The theory, supported by existing and new experiments, predicts that interventions increasing mitophagy will both exacerbate any selection against detrimental mutants and increase the power of the developmental bottleneck to increase heteroplasmy variance between oocytes. These arise because increased mitophagy must either decrease the size of cellular mtDNA populations or provoke a compensatory increase in mtDNA replication to stabilise copy number. Both of these outcomes strengthen the effects of selection and random drift, either due to a smaller population size or due to an increase in the rates of the underlying cellular processes. The relative importance of random drift and selection can be accounted for by this theory. This mathematical treatment reinforces preliminary experimental findings suggesting that mitophagy activators like rapamycin or phenanthroline may constitute new axes of intervention to increase the power of mtDNA bottlenecking and selection to decrease mutant load.

Conclusions

We have developed methods for investigating mitophagy based on high throughput imaging in order to understand mtDNA segregation in heteroplasmic mitochondrial disease. We showed that mitophagy can be increased in cultures from patients with m3243G by energetic stress and by drug modulators of mitophagy. We studied mitophagy during preimplantation development in the mouse, obtaining data that is consistent with autophagic activity that results in a drop in mtDNA

copy number soon after ovulation. Preliminary data suggest that mtDNA copy number may be driven both by mitochondrial dysfunction and by drug modulators of mitophagy. Theoretical treatments based on mathematical modelling and data-drive statistical analysis support the idea that increasing mitophagy may help to robustly remove mutant mtDNA. More data is needed to inform regulators of mitochondrial replacement therapy about these important processes that may determine its success or failure.

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Figure legends

Figure 1. Transmission of mtDNA disease and strategies to prevent transmission of mtDNA mutations. Three available ways to reduce the risk of transmitting mitochondrial DNA disease: oocyte donation, preimplantation genetic diagnosis, and mitochondrial replacement therapy. Red represents mutant mitochondrial DNA, pink and white represent successively higher proportions of normal mitochondrial DNA. Blue represents genetic material from an unrelated donor. A) No intervention: offspring's mutant mitochondrial DNA load will vary greatly. B) Oocyte donation: current availability in the United Kingdom is limited by the availability of oocyte donors. C) Preimplantation genetic diagnosis: is available in the UK for most mitochondrial DNA diseases. D) Mitochondrial replacement therapy (MRT) Nuclear transfer: being developed in the UK, first cases likely this year, not yet available in USA.

Figure 2. The mitophagy pathway. The mitochondrial network is dynamic with events of membrane fusion and fission within the network. The morphology of the mitochondrial network observed reflects the balance between these events. An increase in fission or a decrease of fusion is favourable to mitophagy when an increase of fusion or decreased fission inhibits the mitophagy. A simplified view of mitophagy is represented in the cartoon; briefly a dysfunctional mitochondria is targeted, potentially by Parkin and Pink1 proteins, to a forming autophagosome, the phagophore. The phagophore formation is under the control of Atg5/7 proteins notably. The autophagosome matures and engulfs the mitochondria before fusing with a lyososome for degradation. Our IN Cell system is able to measure the co-localisation between mitochondria and autophagosome.

Figure 3 Evidence of autophagy in mouse oocytes and pre-implantation embryos. A) Autophagosomes of oocytes and preimplantation embryos of mice with GFP-tagged LC3 were visualised by fluorescence microscopy and autophagosomes per embryo counted. Relative mtDNA content (AU for arbitrary units) was assessed by single embryo qPCR. The results show that mtDNA declines during active autophagy. B) 2-cell embryos have been treated with rapamicin (red bars), phenanthroline (green bars) or not (blue bars) for 4h to activate mitophagy; rapamycin 100nM increased autophagosomes number per embryo and mtDNA dropped after exposure to 10uM phenanthroline
Key *P<0.05, **P<0.01, ***P<0.001 T-test. T tests where distribution was normal, Mann-Whitney elsewhere.

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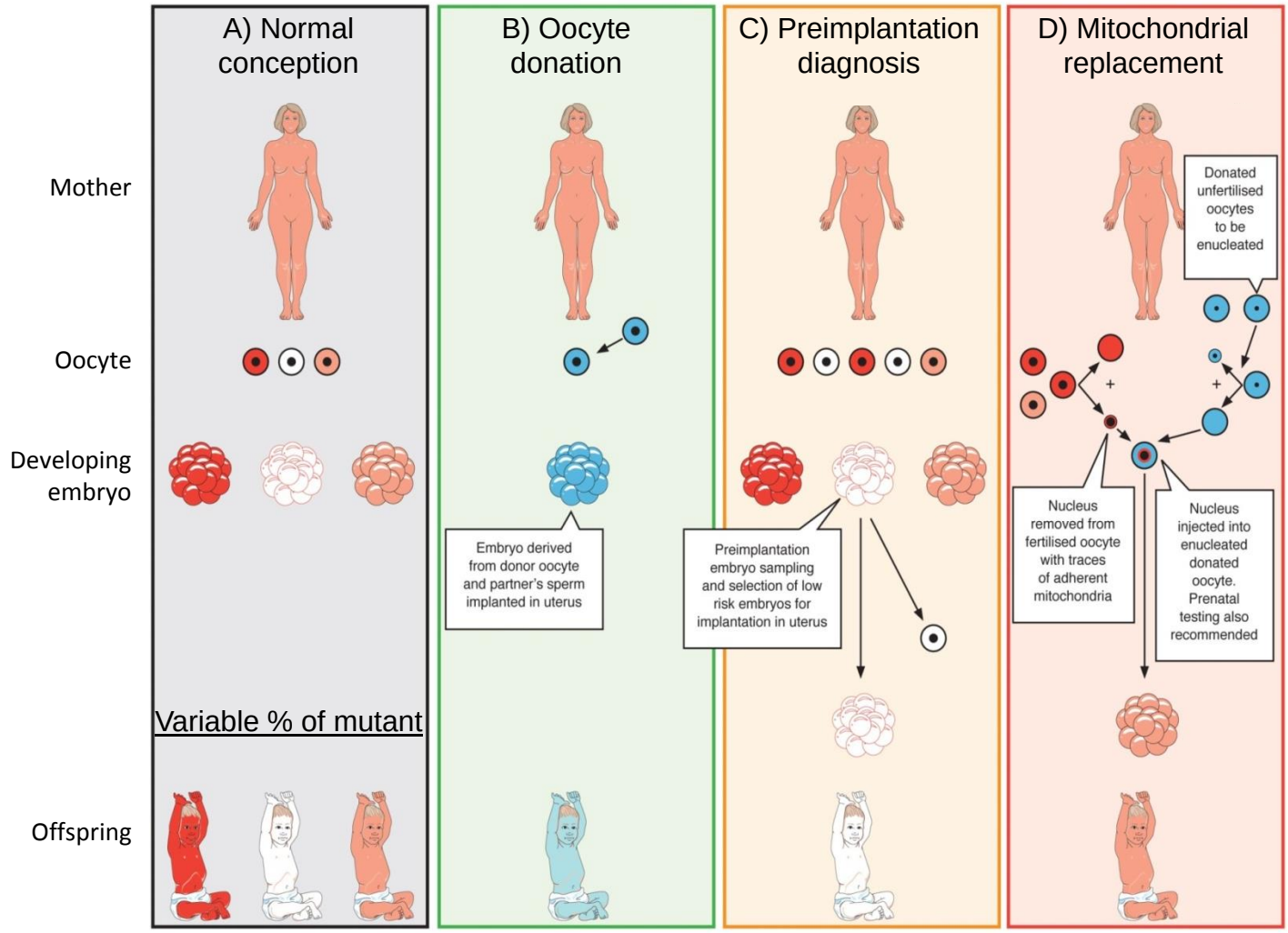


Figure 1. Transmission of mtDNA disease and strategies to prevent transmission of mtDNA mutations. Three available ways to reduce the risk of transmitting mitochondrial DNA disease: oocyte donation, preimplantation genetic diagnosis, and mitochondrial replacement therapy. Red represents mutant mitochondrial DNA, pink and white represent successively higher proportions of normal mitochondrial DNA. Blue represents genetic material from an unrelated donor. A) No intervention: offspring's mutant mitochondrial DNA load will vary greatly. B) Oocyte donation: current availability in the United Kingdom is limited by the availability of oocyte donors. C) Preimplantation genetic diagnosis: is available in the UK for most mitochondrial DNA diseases. D) Mitochondrial replacement therapy (MRT) Nuclear transfer: being developed in the UK, first cases likely this year, not yet available in USA.

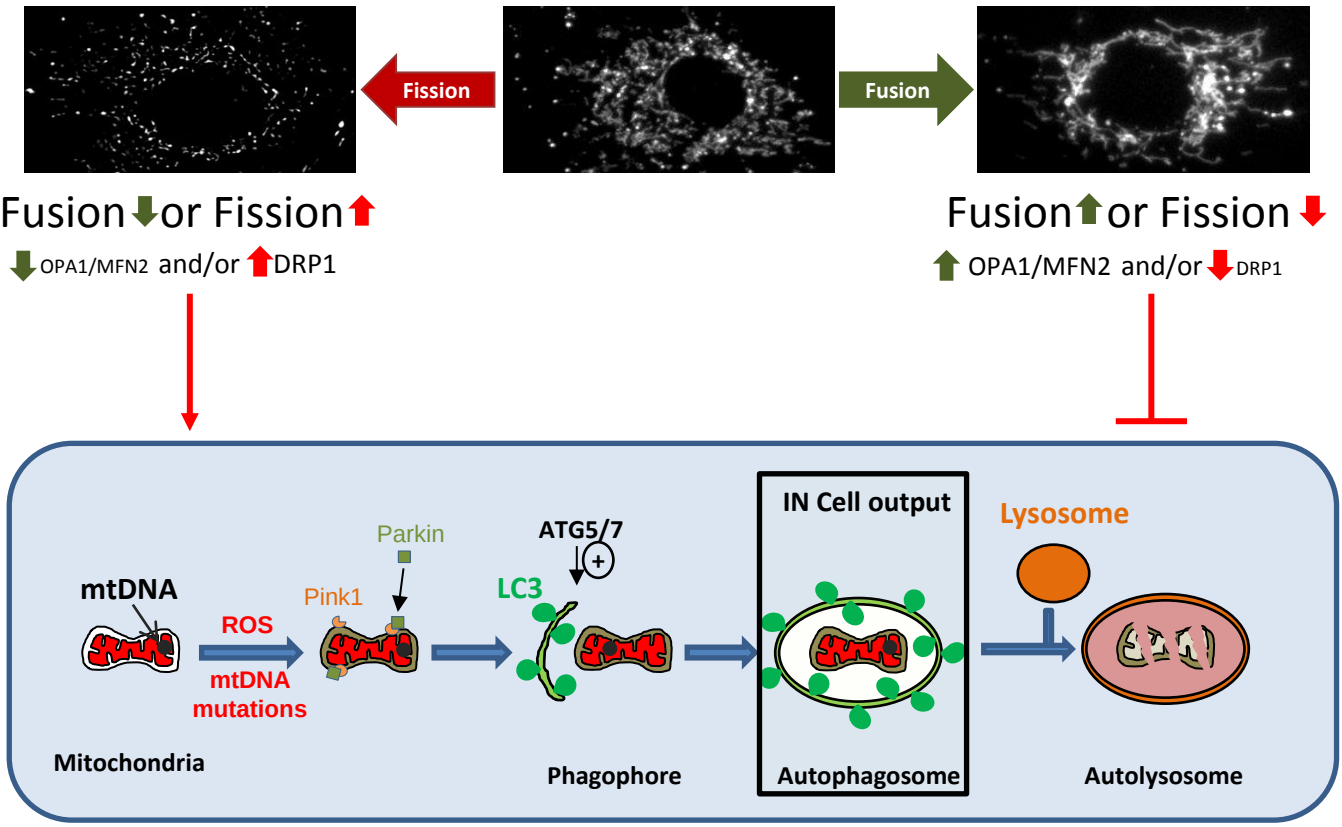
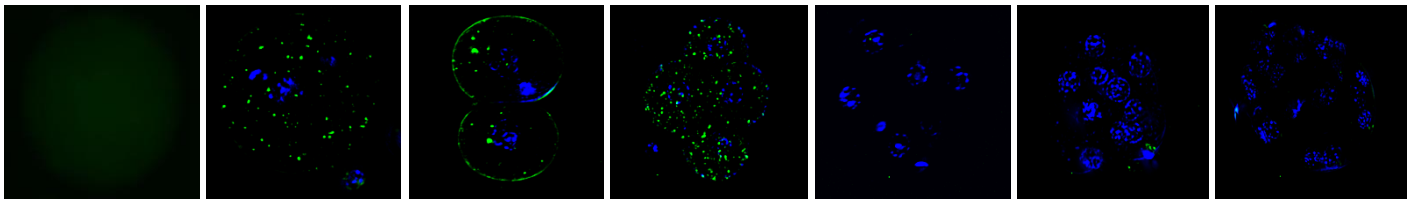
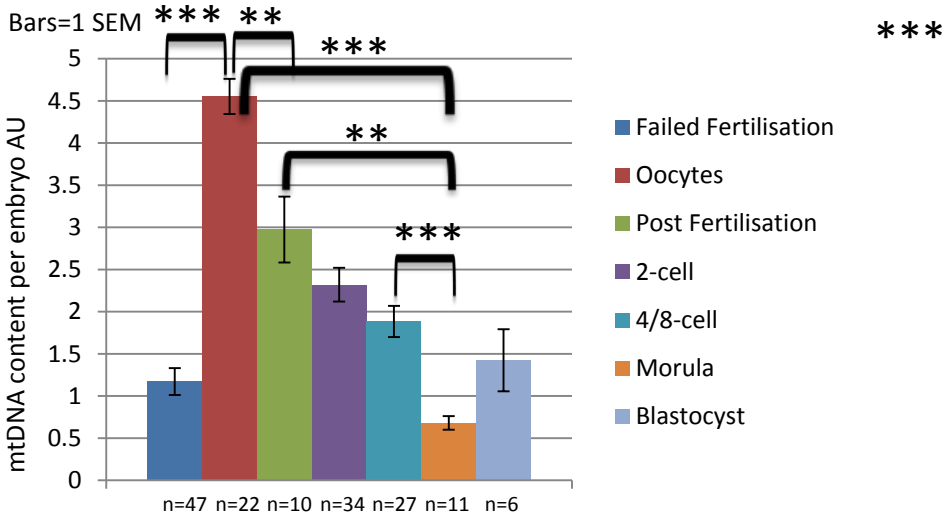


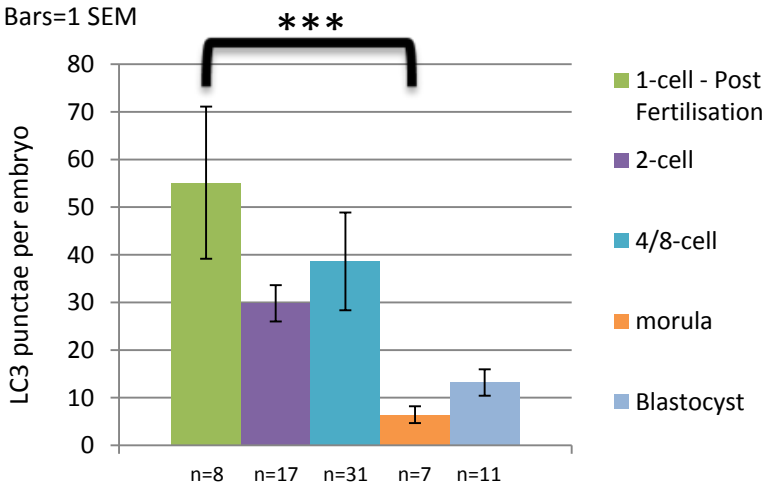
Figure 2. The mitophagy pathway. The mitochondrial network is dynamic with events of membrane fusion and fission within the network. The morphology of the mitochondrial network observed reflects the balance between these events. An increase in fission or a decrease of fusion is favourable to mitophagy when an increase of fusion or decreased fission inhibits the mitophagy. A simplified view of mitophagy is represented in the cartoon; briefly a dysfunctional mitochondria is targeted, potentially by Parkin and Pink1 proteins, to a forming autophagosome, the phagophore. The phagophore formation is under the control of Atg5/7 proteins notably. The autophagosome matures and engulfs the mitochondria before fusing with a lyososome for degradation. Our IN Cell system is able to measure the co-localisation between mitochondria and autophagosome.

A



Oocyte Fertilised 2-cell 4-cell 8-cell Morula Blastocyst





B

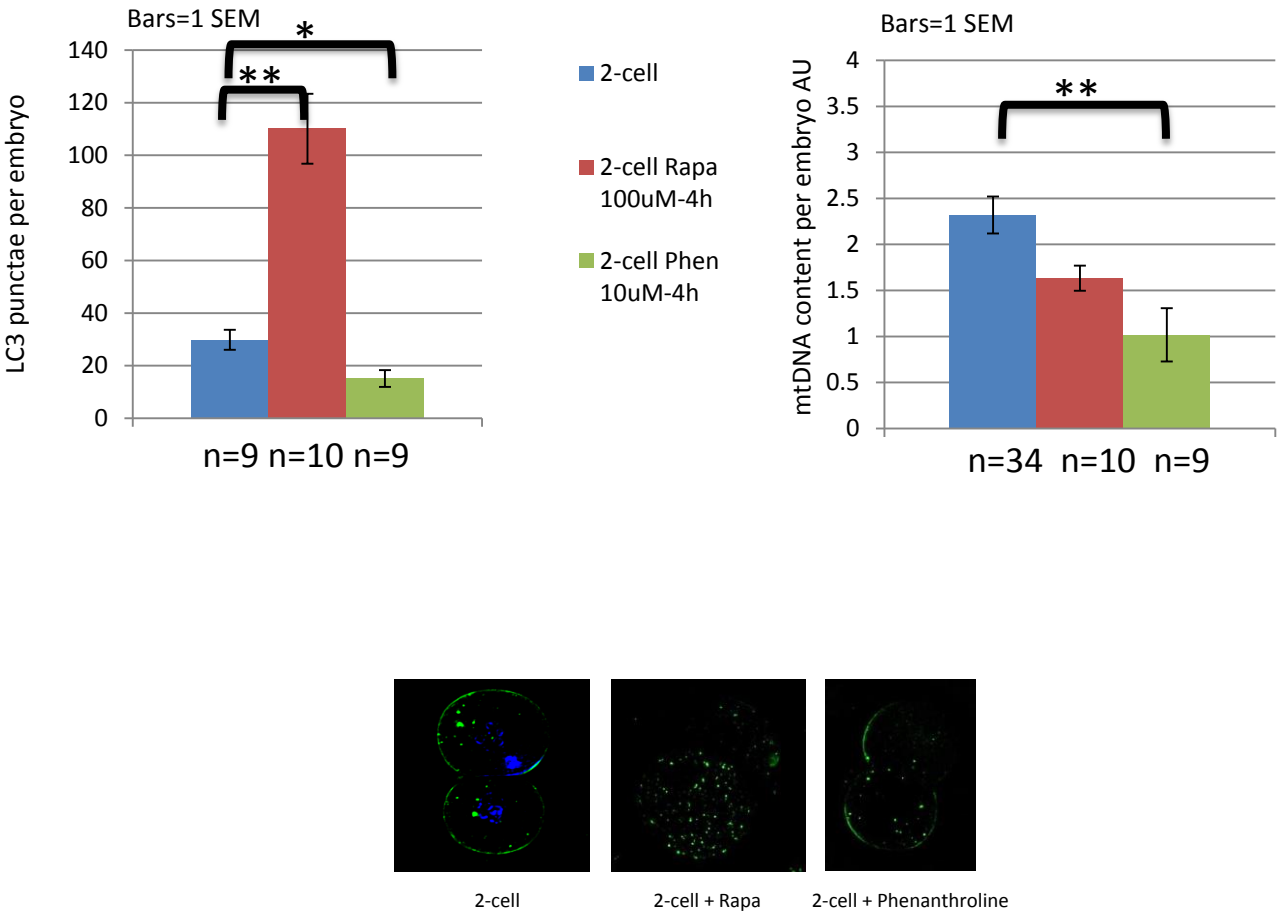


Figure 3 Evidence of autophagy in mouse oocytes and pre-implantation embryos.

A) Autophagosomes of oocytes and preimplantation embryos of mice with GFP-tagged LC3 were visualised by fluorescence microscopy and autophagosomes per embryo counted. Relative mtDNA content (AU for arbitrary units) was assessed by single embryo qPCR. The results show that mtDNA declines during active autophagy .

B) B) 2-cell embryos have been treated with rapamicin (red bars), phenanthroline (green bars) or not (blue bars) for 4h to activate mitophagy; rapamycin 100nM increased autophagosomes number per embryo and mtDNA dropped after exposure to 10uM phenanthroline

Key *P<0.05, **P<0.01, ***P<0.001 T tests where distribution was normal, Mann-Whitney elsewhere