The effect of energetic stress and metformin on cells with the 3243A>G mitochondrial mutation

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Introduction

The m.3243A>G mutation is one of the commonest mtDNA mutations, causing oligosymptomatic presbycusis or diabetes rather than mitochondrial myopathy in most cases. Mutant mtDNA co-exists with wild type (heteroplasmy), with the mutant load determining severity. Mitophagy (recycling of damaged mitochondria) is likely to be an important determinant of disease progression in such heteroplasmic mtDNA disease. Documenting mitophagy is technically demanding, however we have validated a technique using high throughput fluorescence microscopy and LC3-II (an autophagy marker) to examine changes in mitophagy. We reasoned that mitophagy might be apparent when heteroplasmic mtDNA mutants are lost during culture conditions requiring oxidative metabolism.

Metformin has been in therapeutic use since 1958 as a hypoglycemic agent, however only recently have the other potential applications of the drug been realised. Through activation of the AMPK signalling pathway, metformin is thought to have widespread uses as a neuro-protective, cardio-protective and tumour suppressive agent (Liu, Tang et al. 2014), (Johnson, Simpson et al. 2005). The novel uses for the drug led us to examine the effect on mitophagy.

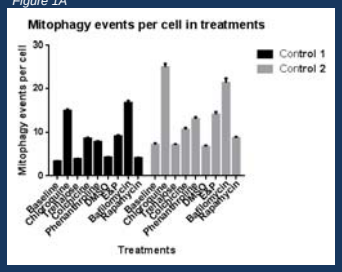
Aims

To determine whether mitophagy underlies the drop in load of mutant mtDNA seen during m.3243A>G cultures under mitochondrial energetic stress.

Validating InCell 1000 for detecting mitophagy

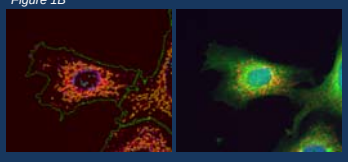
To validate the ability to detect any changes in mitochondrial morphology, autophagy levels and co-localisation between mitochondria and autophagosomes (mitophagy), several known modulators of mitochondrial dynamics and autophagy were used. The results are presented in the accompanying figure and show that known activators (Phenanthroline, Bafilomycin, Trehalose) and inhibitors (E64d/PepA, Colchicine,Chloroquine, Rapamycin) of autophagy induce an increase in the autophagosomes detected as well as of the co-localisation between mitochondria and autophagosomes.

Figure 1A



Detection of mitochondria and autophagosomes using the INCell 1000 system. The images are acquired in 3 different fluorescent channels (DAPI, FITC, TRITC). Computerised binarisation allows for nuclei, cell boundary, autophagosomes, short and long mitochondria segmentation. Co-localisation of mitochondria and autophagosomes (is assessed by combining the binarised images for LC3 and mitochondria. Merge overlay of the images and output of the segmented cell is shown in figure 1B- short mitochondria are in red, long mitochondria in yellow, nuclei in blue, autophagosomes in purple, mitochondria/autophagosome co-localisation in blue and cell boundary in yellow.

Figure 1B



Metformin attenuates the loss of mutant load and reduces mitophagy in m.3243>G cells

We investigated metformin, an agent that is widely used for treating mitochondrial diabetes. We found that the drop in mutant load correlates significantly (p=0.05) with abundance of the autophagy marker, LC3-II on western analysis.

We developed an Assay for quantifying the effect of drugs on mitophagy by using fibroblasts that are heteroplasmic for pathogenic mtDNA mutant m.3243A>G as a marker of dysfunctional mitochondria. The m.3243A>G mutant load is stable in glucose but drops when cells are cultured under energetic stress, that is when they are cultured in media where glucose is replaced with galactose, forcing them to use oxidative phosphorylation

Figure 2B

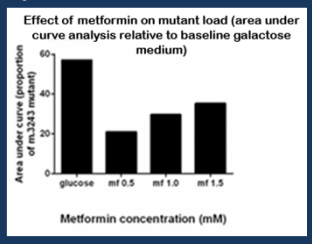


Figure 2A

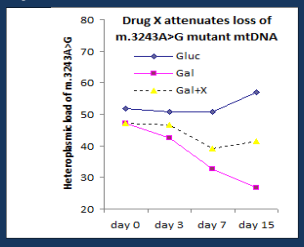
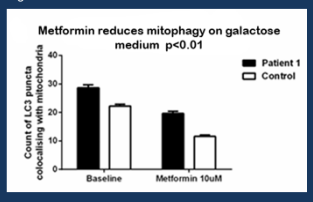


Figure 2C



A genetic knockdown (KD) of essential autophagy gene ATG7 reduces mitophagy

Figure 4

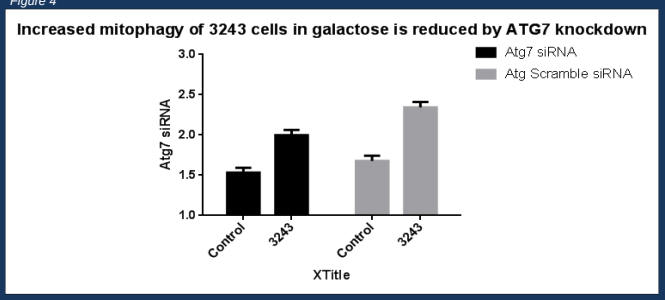


Figure 4A) Atg7 is required for autophagy, our rationale is that knocking out this gene would result in decreased mitophagy in cells with the 3243 mutation. Mitophagy is still seen to some extent in scramble condition because knocking down the gene only partly reduces its activity as opposed to knock outs.

4B) Western showing that the essential autophagy protein, ATG7, was knocked down by siRNA.

4C) SiRNA to the essential autophagy protein, ATG7 attenuates both the baseline and the increase in co-localisation of mitochondrial and LC3 signal resulting from exposure to E+P.

Figure 4B

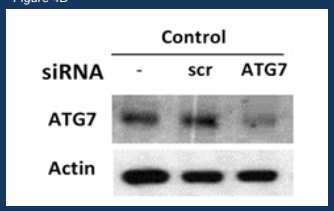


Figure 4C

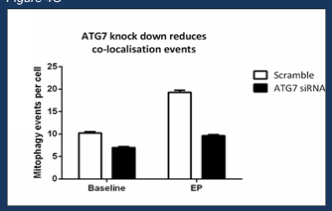
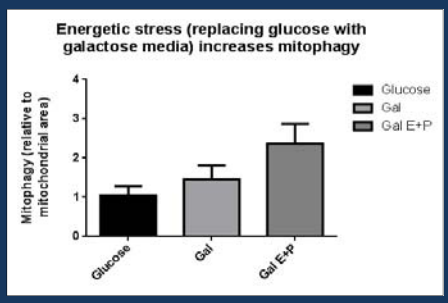


Figure 3. Energetic stress increases mitophagy, measured by InCell 1000 in control and patient fibroblasts

Co-localisation of mitochondrial and LC3 signal is increased by energetic stress (media where glucose is replaced with galactose, forces control cultures to use oxidative phosphorylation) and by exposure to lysosomal inhibitors E64D and pepstatin A (E+P).



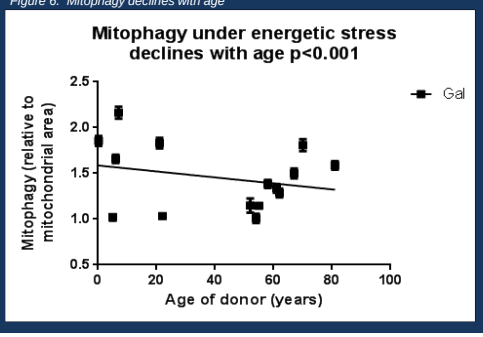
Growth on galactose causes an increase in the expression of LC3-II
Standardised to actin, the level of LC3-II on western analysis increases in galactose relative to glucose, reflecting the increased co-localisation in control cells under energetic stress for 3 days.

Figure 5.

Mitophagy declines with age

Figure 6. Mitophagy declines with age

There is a significant age-related decline in co-localised mitochondrial and LC3 signal in both glucose (not shown) and galactose (p<0.001).



Conclusion

By monitoring load of heteroplasmic mutant mtDNA, we show that mitochondrial energetic stress reduces mutant mtDNA in fibroblasts. Mitophagy is implicated because it is accompanied by increased LC3 puncta containing mitochondria, (the hallmark mitophagy) and the mutant load correlates inversely with the level of LC3-II (p=0.05). Co-localisation of LC3 puncta with mitochondria was significantly increased in heteroplasmic cells compared to wild type under energetic stress caused by galactose but not at baseline. Inhibiting mitophagy either genetically or with drugs reduced co-localisation of LC3 with mitochondria. We conclude that Mitophagy removes pathogenic mutant mtDNA from heteroplasmic patient cultures under mitochondrial energetic stress.

We used this system to screen for the effects of various drugs on mitophagy. We found that a commonly used oral hypoglycaemic agent (metformin) impaired mitophagy. This could have long-term effects on accumulation of mutant mtDNA by heteroplasmic patients. Furthermore it could be particularly damaging under mitochondrial energetic stress such as fasting.