Neuromuscular junction formation in co-cultures of myotubes and motoneurons with m.3243A>G mutation

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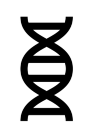
1. Background

Mitochondrial diseases are enormously difficult to both diagnose and treat as they can affect any organ or tissue and most often manifest with an extensive range of symptoms. M.3243A>G mutation is present in **80% of individuals with MELAS Syndrome** (Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes).



m.3243A>G substitution mutation in the MT-TL1 gene affects $tRNA^{Leu\ (UUR)6}$. When the tRNA is altered, incorporation of amino acids into a protein is affected. Consequently, synthesis of many proteins, which are encoded in mitochondrial DNA is impaired. Nervous tissue and muscle tissue are amongst the most highly affected due to their high energy requirements.

Modelling mitochondrial diseases has been challenging due to their complex nature. The physiological model used in my project was **co-cultures of myotubes and motoneurons**. In a living organism, these form synapses between the terminal end of motoneuron and a muscle, called **neuromuscular junctions** (NMJ).

2. Aims

- Generate and characterise hiPSC-derived motoneurons carrying m.3243A>G mutant load of 90% and isogenic control. Myotubes used in this project have previously been characterised in terms of the expression of specific myogenic markers and function.
- Analyse the NMJ formation in co-cultures of wild-type motor neurons and myotubes with m.3243A>G mutation levels 0%, 50%, 90%.

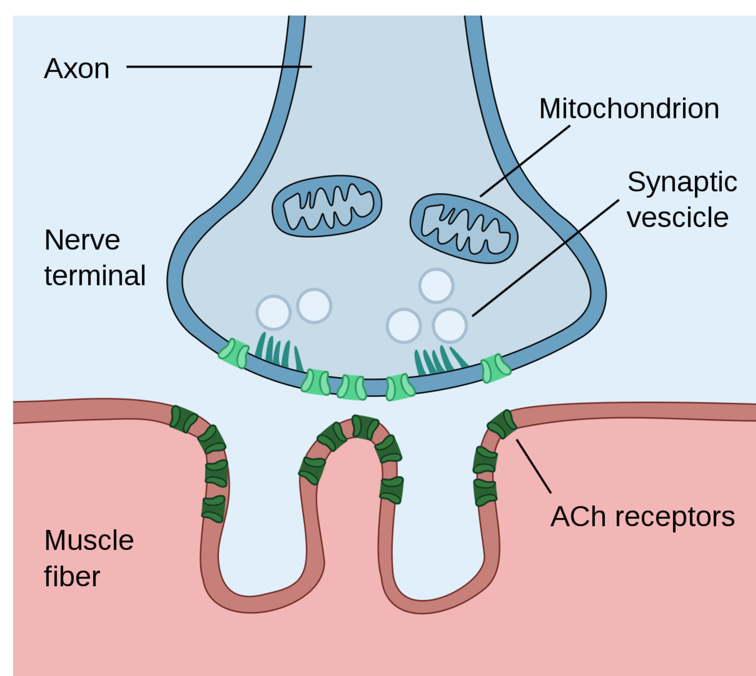


Figure 1. A schematic of a neuromuscular junction.

3. Materials and methods

Cell differentiation and cell culture

Firstly, fibroblasts with patients with m.3243A>G mutations were reprogrammed to hiPSCs. The hiPSC colonies, which formed embryoid bodies, were later differentiated into motoneurons and myotubes with respective mutant loads.

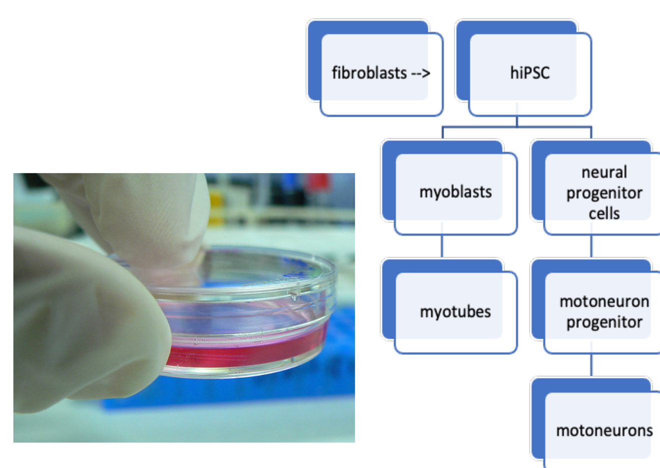


Figure 2. Fibroblasts are reprogrammed to hiPSCs and these can be differentiated into different lineages, in this project into myotubes and motoneurons.

Immunostaining

For immunofluorescence staining of co-cultures of myotubes and motoneurons markers MyHC (myotube marker), ChaT (neuronal late marker), alpha-bungarotoxin (acetylcholine receptor marker) and Hoechst (nuclear stain) were chosen.

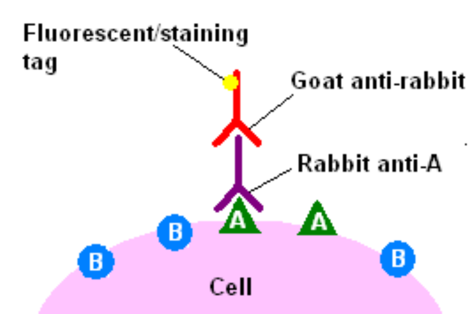


Figure 3. A schematic showing the mechanism of immunofluorescence.

Confocal microscopy

Stained samples were analysed with Zeiss imaging system and with a laser confocal scanning microscope at 63× oil magnification. ImageJ software (Fiji) was used for qualitative analysis.

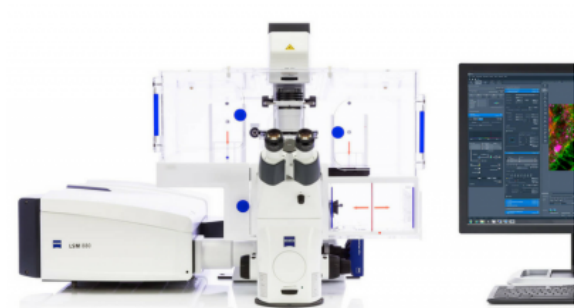


Figure 4. Zeiss LSM 880 Airyscan Confocal Laser Scanning Microscope used in the project.

4. Results

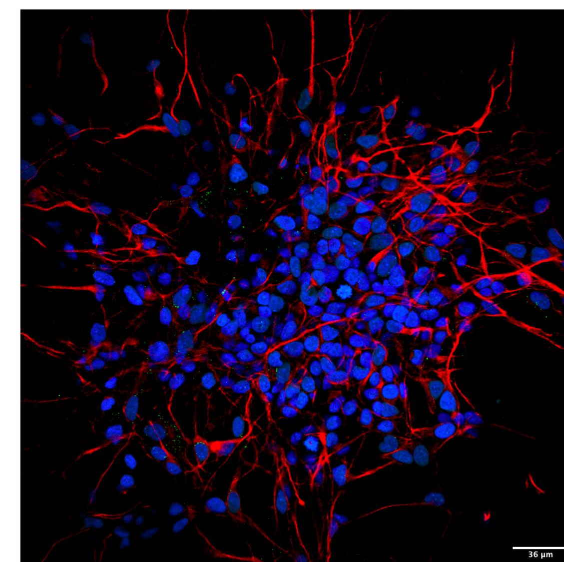


Figure 5. Motoneuron progenitors.

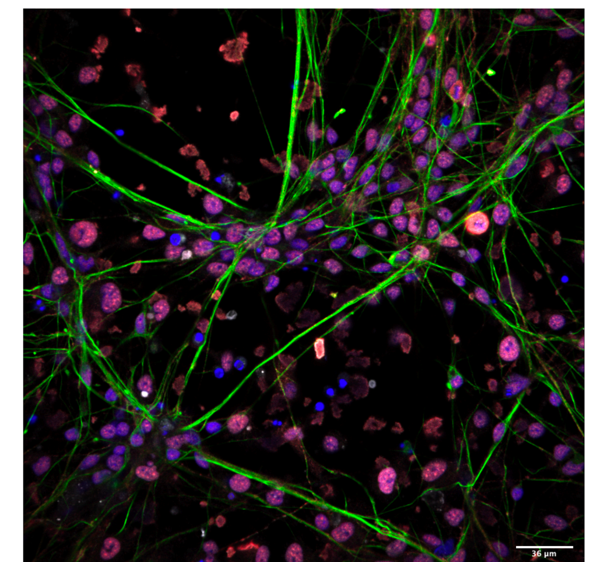


Figure 6. Motoneurons, isogenic control.

Motoneuron progenitor (Figure 5) identity was confirmed with use of Tuj1, marker of neuronal identity, in red; Hoechst marker for cell nuclei, in blue; HB9, marker for a transcription factor essential in motor neuronal development, in green.

Identity of motoneurons with 0 and 90% mutant was confirmed through markers: ChAT, MN late marker, in red; N200, neuronal maturity, in grey; Tuj1,neuronal identity, in green; Hoechst, nuclear stain, in blue. ChaT was present in nuclei in Figure 6 but not in Figure 7 and stained neurons more strongly in Figure 6.

Figures 5, 6, 7 were acquired from my project supervisor Gabriel Valdebenito.

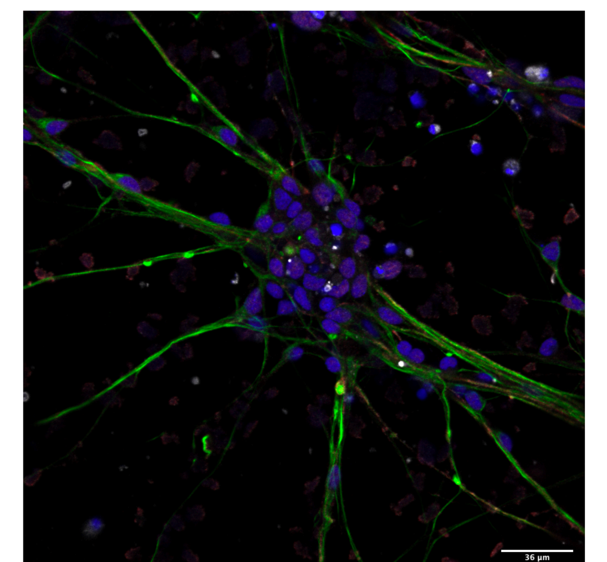


Figure 7. Motoneurons with 90% mutant load.

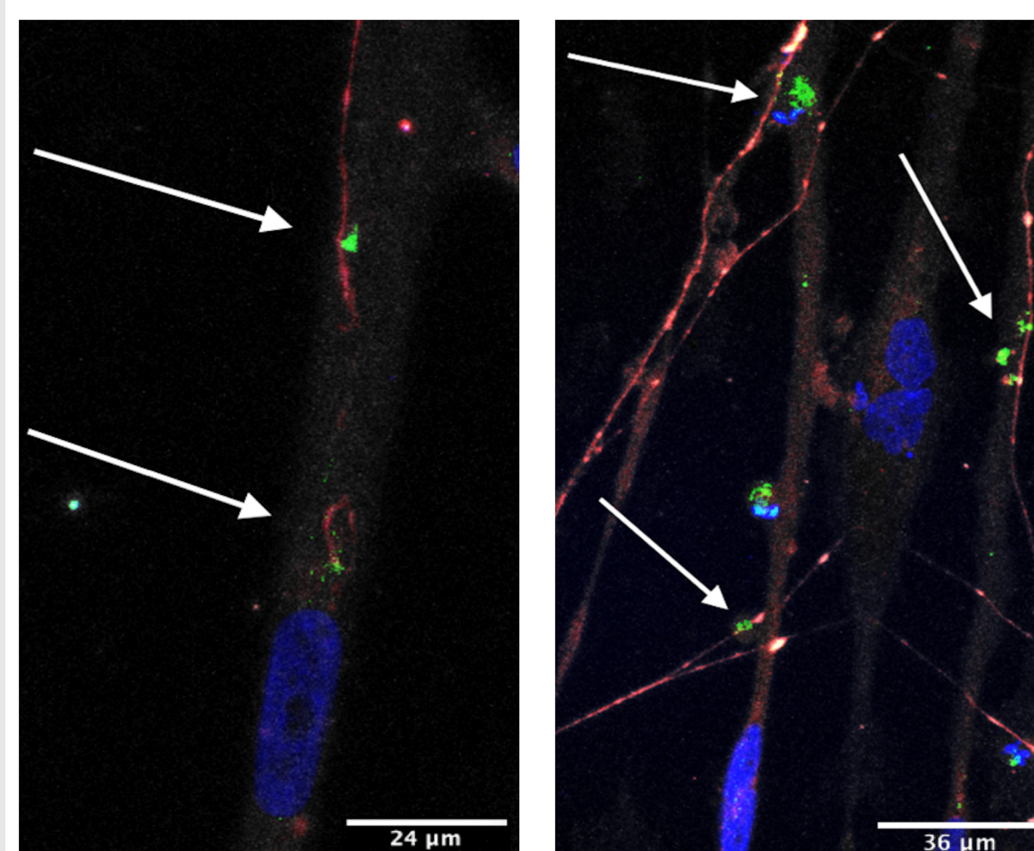


Figure 8. M90 co-culture. NMJs shown with white arrows. Figure 9. M50 co-culture. NMJs shown with white arrows.

In all co-cultures of myoblasts and neuroprogenitors, myoblasts proliferated more rapidly, hence a high disproportion between the number of myotubes and motoneurons.

Co-cultures of myotubes and motoneurons were stained with ChaT (red), Hoechst (blue), MyHC (grey) and alpha-bungarotoxin (green).

It was possible to find NMJs in samples of 90% and 50% mutation level, as shown in Figure 8 and 9. It was not possible to find NMJs in the remaining samples. The highest prevalence of NMJs was found in the 50% mutation level sample.

5. Acknowledgements

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