Mitochondrial Myopathies and Muscle Stem Cells

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This thesis is submitted for the degree of Doctor of Philosophy at Newcastle University

Mitochondrial Research Group

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Author's Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Newcastle University. The research was conducted in the Mitochondrial Research Group under the supervision of Professor D.M. Turnbull and Professor H. Lochmuller and unless otherwise stated, all work is my own.

I certify that none of the material offered in this thesis has been previously submitted by me for a degree or qualification at this or any other university.

Abstract

Mitochondrial Myopathies and Muscle Stem Cells

Mitochondrial myopathies are a group of progressive muscle disorders caused by mutations in the mitochondrial genome (mtDNA) for which there is no effective treatment. Culturing of myoblasts from patients with sporadically occurring mitochondrial diseases has suggested that mtDNA mutations may be lower or absent in muscle stem cells (satellite cells). The activation of muscle satellite cells and subsequent repair of muscle fibres may favourably shift the balance of delete to wild-type (WT) mtDNA, thereby decreasing mtDNA mutation load in affected muscle. This research has investigated muscle precursor cells from patients with mitochondrial myopathy due to sporadically occurring mtDNA deletions. This was to determine if they will benefit from attempts to "gene shift" the balance of WT and mutated mtDNA in their muscles using high intensity resistance training.

Fluorescently Activated Cell Sorting (FACS) on the basis of CD56 (NCam) was used to isolate satellite cells and real time PCR to analyse them. In all eight patients investigated mtDNA deletions were detected in satellite cells at levels similar to mature muscle. In most of these patients the mtDNA deletions were lost during the culturing of their myoblasts. In some patients, however, the mutation was maintained, although there was a gradual decline in mutation load as the myoblasts headed towards differentiation. It was hypothesised that this difference between patients in the maintenance or loss of mutations in their myoblasts was attributable to an mtDNA bottleneck effect at the point of satellite cell activation. A second selection point occurred during the process of myoblast proliferation, possibly mediated by segregation of WT and delete mtDNA after cell division. Daughter cells that inherit large amounts of delete mtDNA will be unable to continue to proliferate.

If efforts to "gene shift" in these patients will involve the activation of satellite cells to repair damaged muscle, it is paramount that this process does not exhaust the muscle stem cell pool. Satellite cell numbers have been determined in patients harbouring sporadically occurring mtDNA deletions, who will be considered potential beneficiaries

of exercise based interventions. No significant difference was observed in satellite cell numbers when patients were compared to controls. In addition, a single patient was examined for satellite cell numbers over eleven years and no reduction in numbers was found.

Given that the large majority of single deletion patients will lose their mtDNA mutation during the process of muscle regeneration and that they will not suffer from an exhaustion of the satellite cell pool, “gene shifting” remains a viable therapy in these patients. However, the mechanisms behind the process are somewhat different to what was originally hypothesised.

Acknowledgements

First and foremost I would like to offer my gratitude to the patients involved in this research. The study they undertook required considerable commitment over a long period of time, and the willingness to undergo invasive tests. Secondly I would like to thank my supervisors Doug Turnbull and Hanns Lochmuller, not only for giving me the opportunity to undertake a PhD, but for their support, advice, and patience.

The samples used in this research have been obtained from participants in an exercise trial at Newcastle University which has required the collaboration of many different specialists. I would like to thank Grainne Gorman for performing many of the biopsies, Jane Newman for overseeing the exercise, and the Newcastle Mitochondrial Diagnostic Group for preparing many of the samples. In addition, all of the myoblast cultures were set up by Mojgan Reza at the Newcastle MRC Biobank and FACS sorting was performed with the help of Ian Dimmick, to both I am extremely grateful.

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When undertaking a PhD there is a tendency to become consumed with the task, but I have been fortunate enough to be surrounded by a great group of people who have reminded me that there may possibly be more to life than science. Three people have been there from the very beginning; Elaine, Helen, and Alison. You will probably never know how important your presence has been, but I will be eternally grateful. It's been a fantastic thirty years, and I'm excited to see what the next thirty bring! To many other friends in and out of the lab, on and off court, thank you for keeping me laughing, running, climbing, playing, and sane…ish.

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List of abbreviations

A	adenine
A-band	anisotrophic band
ABC	avidin-biotin peroxidase complex
ADOA	autosomal dominant optic atrophy
ADP	adenosine diphosphate
AGE	agarose gel electrophoresis
ANT	nucleotide translocator
ATP	adenosine 5'- triphosphate
ATPase	ATP synthase
BER	base excision repair
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pair
BSA	bovine serum albumin
C	Celsius
Ca^{2+}	calcium ions
CD56	cluster of differentiation molecule 56 (neural cell adhesion molecule)
CO_2	carbon dioxide
CoA	coenzyme A
COX	cytochrome *c* oxidase
CPEO	chronic progressive external ophthalmoplegia
CRS	Cambridge Reference Sequence

Cu	copper
Cys	cysteine
Da	Dalton
DAB	3.3’diaminobenzidine
DAPI	4’-6-diamidino-2-pheylindole
dATP	2’-deoxyadenosine 5’-triphosphate
dCTP	2’-deoxycytosine 5’-triphosphate
dGTP	2’-deoxyguanosine 5’-triphosphate
dH_2O	deionised water
DMD	Duchene Muscular Dystrophy
DMEM	Dulbeccos’s modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	2’-deoxynucleotide 5’triphosphate
E	embryo
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
ETC	electron transport chain
FACS	fluorescently activated cell sorting
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin dinucleotide
FeS	iron sulphur

FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
FSC	forward scatter
G	guanine
g	grams
Glu	glutamate
GTP	guanosine triphosphate
H^+	proton
HDAC	histone deacetylases
H & E	haematoxylin and eosin
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HRP	horseradish peroxidase
H strand	heavy strand
HSP	heavy strand promoter
HSP-2	second heavy strand promoter
HVR	hypervariable region
I-band	isotrophic band
IM	inner mitochondrial membrane
IT_{H1}	H strand transcription initiation site
IT_{H2}	second H strand transcription initiation site

IT_{L1}	L strand transcription initiation site
Kbp	kilobase pair
KDa	kilo Dalton
KSS	Kearns Sayre Syndrome
L strand	light strand
Leu	leucine
LHON	Leber's hereditary optic neuropathy
LSP	light strand promoter
LSP-2	second light strand promoter
M	molar
MCU	calcium uniporter
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke like episodes
MERRF	myoclonic epilepsy with ragged-red fibres
Mfn	mitofusin
Mg	magnesium
$MgCl_2$	magnesium chloride
MHC	myosin heavy chain
ml	millilitre
MRF	myogenic regulatory factors
MRI	magnetic resonance imaging
mRNA	messenger RNA

mtDNA	mitochondrial DNA
mt-tRNA	mitochondrial tRNA
Na^+	sodium ions
NAD+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium
NCam	neural cell adhesion molecule
NER	nucleotide excision repair
ND	NADH dehydrogenase
nDNA	nuclear DNA
NDUFS	NADH dehydrogenase ubiquinone iron-sulphur
NDUFV	NADH dehydrogenase ubiquinone flavoprotein
NGS	normal goat serum
nt	nucleotide position
$\cdot O_2^-$	superoxide anion
$\cdot OH^-$	hydroxyl radical
O_2	oxygen
O_H	origin of heavy strand replication
O_L	origin of light strand replication
OM	outer mitochondrial membrane
OPA1	optic atrophy 1

OXPHOS	oxidative phosphorylation
Pax 7	paired box protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDL	poly-d lysine
PE	phycoerythrin
PFA	paraformaldehyde
PMS	phenazine methosulphate
POLG	polymerase γ
Q	ubiquinone
QH_2	ubiquinol
RITOLS	ribonucleotide incorporation throughout the lagging strand
RRF	ragged red fibres
RNA	ribonucleic acid
ROS	reactive oxygen species
Rpm	revolutions per minute
rRNA	ribosomal ribonucleic acids
S^{2-}	sulphide ions
SDH	succinate dehydrogenase
SKCM	skeletal muscle cell media
SSC	saline sodium citrate
SSC	side scatter

T	thymidine
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline triton
TCA	tricarboxylic acid
TFAM	mitochondrial transcription factor
TIM	translocator inner membrane
TNF	tumour necrosis factor
TOM	translocator outer membrane
tRNA	transfer RNA
T_m	melting temperature
Val	valine
VDAC	voltage dependent anion channel
w/v	weight per volume
Zn	zinc
µm	micrometre
µl	microlitre

Chapter 1 Introduction

1.1 Mitochondria

The more that is learned about the mitochondrion and its DNA (mtDNA), the more its importance is realised. Whether it is the vital role this organelle plays in generating energy and buffering cells or the devastating effects of mutations within its DNA. This review aims to give general background information on mitochondrial biology and DNA, and then focus in on the problems that can be caused when it goes wrong. In addition, a possible source of therapy for some conditions, muscle stem cells, will be introduced.

1.1.1 Mitochondrial Origins

"Cells without nuclei were the first to evolve. Cells with nuclei, however, are not merely mutant descendants of the older kind of cell. They are the product of a different evolutionary process: a symbiotic union of several cells without nuclei"

(Margulis, 1971).

Mitochondria evolved through one such relationship, they descended from eubacteria which underwent an endosymbiosis with a nucleus containing host. When Lynn Margulis proposed the endosymbiotic theory of mitochondrial origins, she highlighted the fact they contain their own DNA, messenger RNA, ribosomes, and are capable of protein synthesis, as evidence they were once able to live independently.

This endosymbiotic event is believed to have occurred between 1.5-2 billion years ago. It is still debated whether it happened in a nucleus containing, amitochondrial cell, or the bacterial endosymbiont entered a prokaryotic host which later developed eukaryotic characteristics (Embley and Martin, 2006).

Sequencing the genome of the intracellular parasite *Rickettsia prowazekii*, an α-proteobacteia, revealed many similarities to mitochondria, including genes encoding components of the tricarboxylic acid (TCA) cycle and the respiratory chain complexes (Andersson et al., 1998). Coupled with phylogenetic reconstructions (Gray et al., 1999), the evidence would point towards this early mitochondrion being a member of the α division of the proteobacteria. Other members of this group include *Ehrichia* which

cause the tick borne disease Ehrlichiosis, and *Rickettsi* which have been implicated in the spread of typhus.

Prior to the symbiosis, early mitochondria contained the entire DNA required for independence. As the symbiosis developed there was a gradual loss of mtDNA to the host genome leaving behind a compact molecule consisting of 16,569 base pairs (bp).

1.1.2 Structure

Mitochondria are present in the cytoplasm of eukaryotes; their numbers, size, and shape fluctuate depending on the energy requirements of the cell. Investigations into the structure of mitochondria have relied a great deal upon electron microscopy (EM), and have progressed as the technology and tissue preparation methods have improved.

While sizes vary, mitochondria are usually between 1-4μm long and 0.3-0.7μm in diameter. Early investigations reported cylindrical or rod shaped organelles, with smooth outlines, a mitochondrial matrix, and an outer membrane. Images also revealed an internal system of lamellae lying perpendicular to the long axis of the mitochondria. These lamellae were described as “ridges” or “folds” protruding from the inside surface of the mitochondrial membrane that failed to reach the other side (Palade, 1952). Palade named these lamellae *cristae mitochondriales*. Later EM studies identified a double plasma membrane, consisting of an inner membrane (IM) and an outer membrane (OM). The lamellae were hypothesised to be cristae continuous with the IM (Palade, 1953), which has since been shown to occur through dynamic tubules termed cristae junctions (Frey and Mannella, 2000, Frey et al., 2002).

These early EM studies resulted in the “Baffles” model of mitochondrial structure (Figure 1.1A). An alternative model has been suggested in which the lamellae, termed septa, crossed the matrix and divided the mitochondria into compartments (Figure 1.1B) (Sjostrand, 1956).

The OM contains large amounts of the transmembrane channel porin, also known as voltage dependant anion channel (VDAC), which is freely permeable to ions less than 10KDa in size. In order for proteins to enter the organelle the OM contains

translocases of the outer membrane (TOMS) and the IM has translocases of the inner membrane (TIMS).

The IM is folded into many cristae and houses the protein components responsible for energy generation; the electron transport chain (ETC) and ATP synthase. In addition it provides a barrier across which the respiratory chain complexes can generate a chemiosmotic gradient in order to generate energy. The folding of the IM increases its surface area approximately five times, allowing for a greater area across which oxidative phosphorylation (OXPHOS) can occur.

1.1.2.1 Mitochondrial Structure in Muscle

Mitochondrial morphology and numbers differ according to the energy demand of their host tissue. In skeletal muscle they sit underneath the sarcolemma and penetrate the myofibrils. The greater energy demand of muscle is satisfied by an increase in cristae packing and organisation. This gives around a threefold increase in cristae surface area, thus allowing for greater amounts of energy to be generated.

In his original EM investigations Palade (1953) observed tightly packed cristae and a smaller matrix in skeletal muscle mitochondria when compared to liver cells. But even within muscle, human studies have revealed differences in mitochondrial morphology and positioning (Ogata and Yamasaki, 1997). Using scanning EM, Ogata and colleagues investigated three types of skeletal muscle fibres from the *triceps brachii* and *vastus lateralis*, that they termed red, white, and intermediate. The diameter of the mitochondria differed, with white fibres containing the smallest at 0.3µm, the red fibres the largest 0.9µm, and the intermediate fibres in the middle at 0.6µm.

Striated muscle contains a reticulum of mitochondria penetrating the myofibrils and tethered to the cytoskeleton. All three fibre types contain paired mitochondria at the I-band; in the white fibres these mitochondria circle the myofibril at this position. While the red and intermediate fibres contain a stalk of mitochondria crossing the A-band (Figure 1.18B) to the next row of mitochondria (Ogata and Yamasaki, 1997).

In terms of mitochondrial numbers, the greatest densities are observed in the highly oxidative muscle types; for example cardiac muscle has 30-50% muscle volume, while

animal studies have shown skeletal muscle to have between 1-10% (Moyes and Hood, 2003).

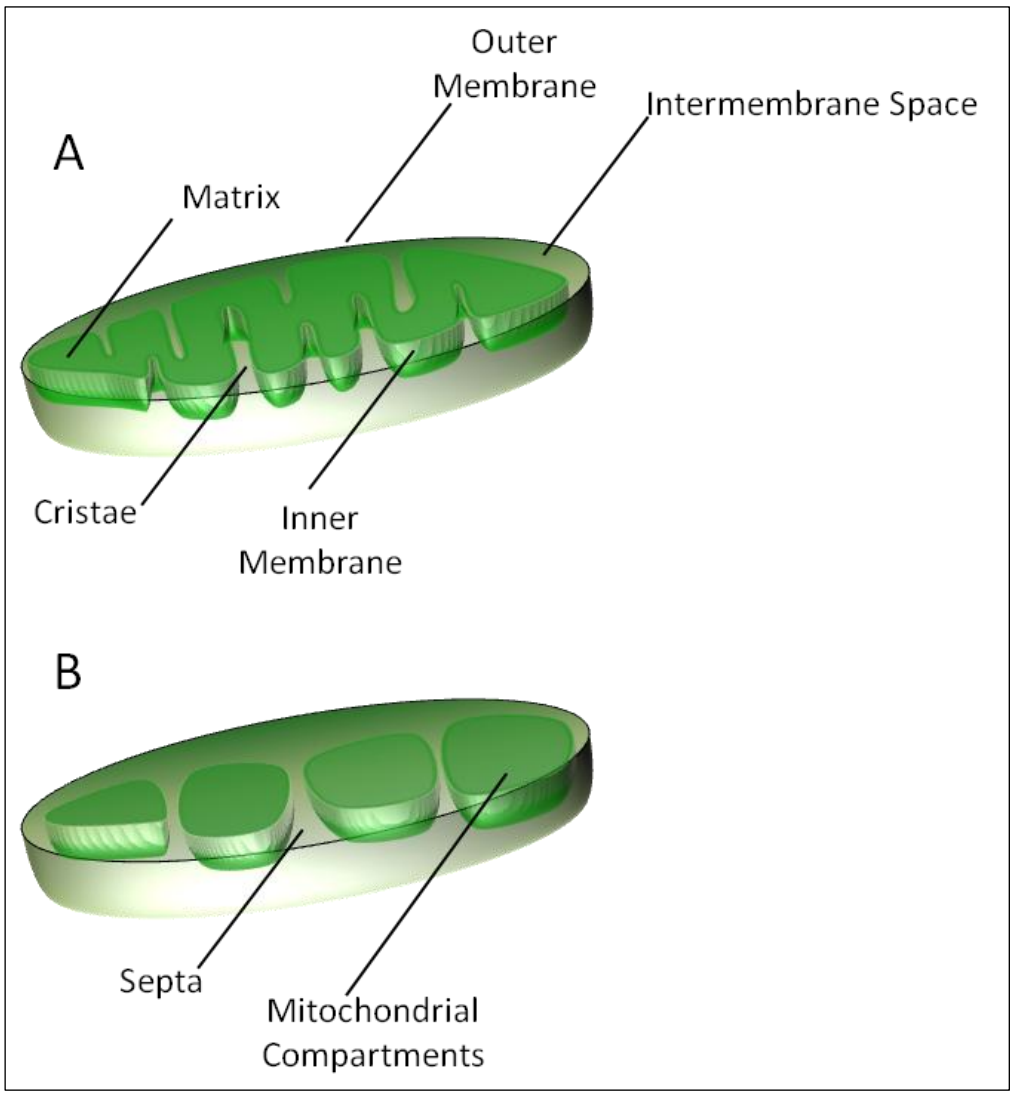

Figure 1.1. Mitochondrial structure. A. The "Baffles" model of mitochondrial structure. EM investigations revealed mitochondria to consist of a double membrane, with the inner membrane folding inwards to create cristae. Within the confines of the inner membrane is the matrix, which houses the mtDNA and RNA, along with various enzymes required for the TCA cycle. **B**. An alternative interpretation of the early EM images was the "Septa" model, whereby the cristae or septa crossed the width of the mitochondria dividing it into compartments.

1.1.2.2 Dynamic Organelles

While the singular oval shaped "Baffles" model of mitochondria are still presented in many images, these organelles are in reality, extremely lively structures. The dynamic nature of mitochondria was hinted at by Palade (1952), when he reported their "true branching". This "branching" is the process of fission and fusion. Fission results in the division of mitochondria into separate structures, while fusion is their unification via inner and outer membranes (Figure 1.2). This process results in changes to morphology, size, and numbers of mitochondria, and also allows for the exchanging of contents. The trading of mitochondrial contents is important for the health of the mitochondria. Many of the proteins involved in the process of fission and fusion were first identified in yeast, though their mammalian homologues are now being elucidated.

1.1.2.2.1 Mitochondrial Fission

In order for fission to occur in yeast, a dynamin-related protein (Dnm1) is recruited from the cytosol, it assembles itself into punctate spots around the mitochondria. This forms a ring like structure around the mitochondrial tubule, encircling and constricting it (Bleazard et al., 1999). In human cells this function is performed by the mammalian homologue Drp1, which localises to evenly spaced areas on the mitochondria which coincide with sites of constriction and division (Smirnova et al., 2001).

In yeast, the process of fission also requires an outer mitochondrial membrane protein termed Fis1, this protein binds to Dnm1 through an adaptor protein, Mdv1, and allows for the proper distribution and function of Dnm1 (Mozdy et al., 2000). The human protein hFis1 has been suggested as a possible homologue to Fis1, and its binding to Drp1 has been demonstrated (Yoon et al., 2003). However, discussions are ongoing as the loss of hFIS1 does not decrease Drp1 binding to mitochondria (Lee et al., 2004). The exact mechanism of IM fission is still being debated, although this could occur through the force generated by the OM components (Scott et al., 2003). Alternatively it could be via an IM protein for example transmembrane protein 11 (TMEM11), although the exact mechanism of action of TMEM11 is unknown (Rival et al., 2011).

1.1.2.2.2 Mitochondrial Fusion

Mitochondrial fusion requires the joining of both the IM and OM, with the resultant mixing of the matrix contents. One of the first components identified in the process of fusion was the fuzzy onion protein (Fzo1) found in *Drosophila*.

The fusion of the OM in mammalian mitochondria requires two large GTPases of the dynamin family, mitofusins 1 and 2 (Mfn1, Mfn2), which are both encoded by the nuclear genome (Santel and Fuller, 2001). These are required to act on adjacent mitochondria. Both Mf1 and Mf2 contain two hydrophobic heptad repeats, termed HR1 and HR2 which sit on either side of two hydrophobic transmembrane (TM) segments. In addition they contain a GTPase domain. HR2 is involved in the tethering of adjacent mitochondria through coiled-coil interactions, while the GTPase domain is required for the full fusion of the membranes (Koshiba et al., 2004).

IM fusion also requires a GTPase, in the form of optic atrophy 1 (Opa1), the deletion of which does not affect fusion of the OM. Opa1 is a member of the dynamin related protein family and is localised to the intermembrane space. Unlike the mitofusins, Opa1 is only required in one of the two mitochondria that will undergo fusion. As well as requiring the homologue of Opa1, which is Mgm1, fusion in yeast also needs an adaptor protein, Ugo1. This binds to both Fzo1 and Mgm1 and coordinates the fusion of the two membranes. As yet, no mammalian homologue of Ugo1 has been determined (Song et al., 2009).

The importance of the proteins involved in mitochondrial fusion can be seen through the disorders witnessed when their genes are mutated. In 50-60% of cases of autosomal dominant optic atrophy (ADOA), the disease is caused by a mutation in the Opa1 gene. This results in the destruction of the optic nerve, via degeneration of the retinal ganglion cells that constitute the nerve. Consequently, there is a loss of the central field and a sparing of the peripheral vision. Many pathogenic mutations causing ADOA have been identified in the GTPase domain and the central dynamin regions (Detmer and Chan, 2007, Yu-Wai-Man et al., 2011).

Mutations in Mfn2 have been implicated in Charcot-Marie-Tooth 2A disease, one of the commonest hereditary neuropathies. Symptoms include distal limb weakness and sensory loss, atrophy, and compromised mobility. It is caused by the degeneration of the long peripheral nerves, and as a result symptoms often start in the hands and feet (Detmer and Chan, 2007). The exact reasons why Mfn2 mutations disrupt the peripheral nerves are not known. One theory suggests that the disrupted fusion affects the transport and distribution of mitochondria in the long nerves, where energy is often required a long way from the soma (Cartoni and Martinou, 2009).

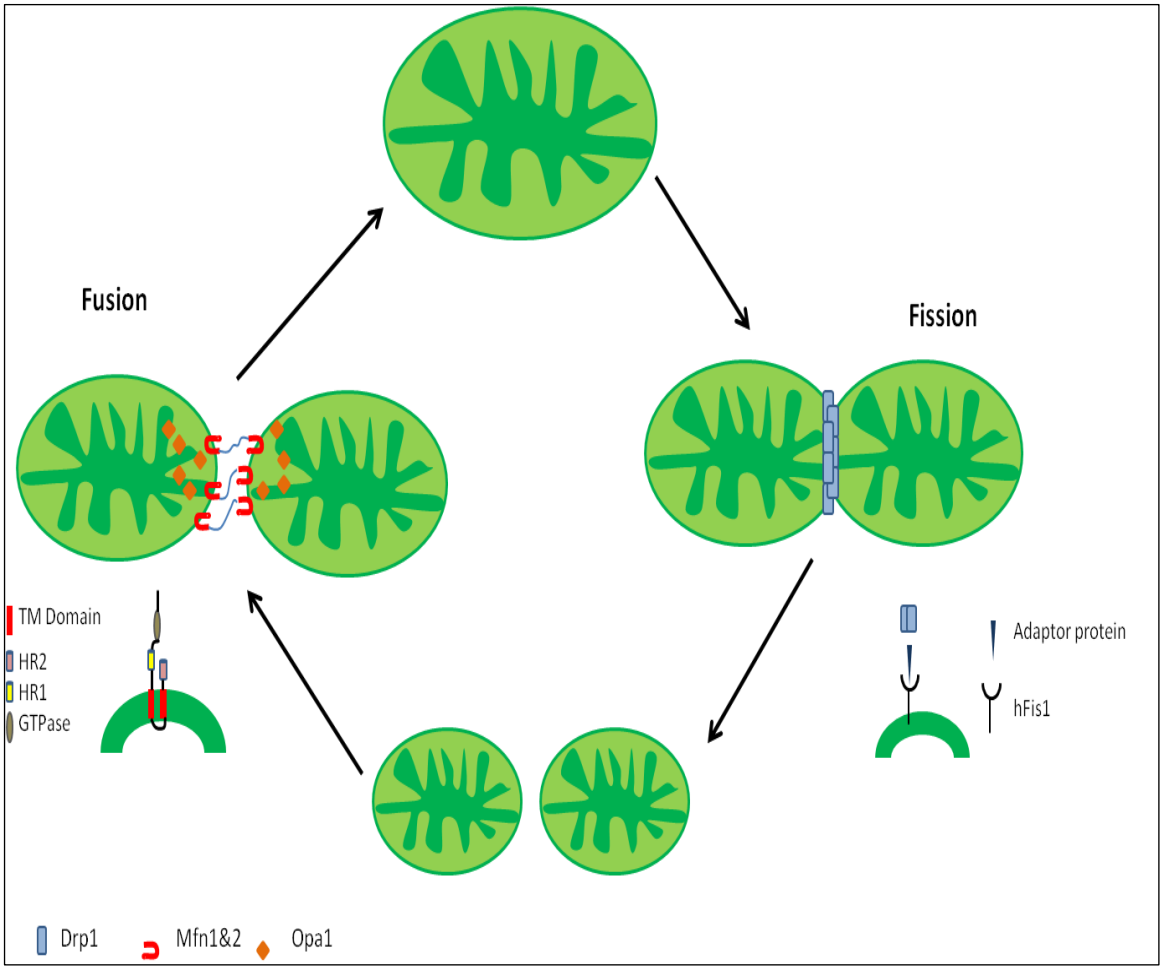

Figure 1.2 Fission and fusion. Mitochondria are continuously undergoing the process of fission and fusion, allowing them to regulate their size, morphology, number, and matrix content. Fusion requires the joining of both inner and outer membranes, involving the mitofusins (Mfn) and optic atrophy 1 (Opa1) respectively. Fission is the budding of mitochondria from the mitochondrial network. It requires Drp1 being recruited to the membrane, where it surrounds and constricts the organelle.

1.1.3 Mitochondrial Functions

Mitochondria perform a number of functions in the cell. While they are popularly referred to as “batteries of the cell”, a reference to their energy generating capabilities, they also have roles in calcium regulation, reactive oxygen species generation, apoptosis, and the production of iron-sulphur clusters.

1.1.3.1 Oxidative Phosphorylation

A major function of mitochondria is to generate energy in the form of ATP via the process of oxidative phosphorylation (OXPHOS). The ninety or so protein components of OXPHOS, are embedded in the mitochondrial inner membrane. It consists of five protein complexes; I-IV comprising the electron transport chain (ETC), and complex V the ATP synthase. In addition there are a further two electron carriers (Figure 1.3). In the OXPHOS system electrons are transported through the ETC; this flow of electrons gives a free energy change which is used to create a proton (H^+) gradient. This gradient is generated by complexes I, III, and IV, pumping H^+ across the inner membrane into the intermembrane space. The gradient is then used by the last complex, the ATP synthase to generate ATP from ADP. The use of an electrochemical gradient across the inner membrane to generate energy is termed the chemi-osmotic hypothesis (Mitchell, 1961).

While some of the ATP is utilised by the mitochondria themselves for their own needs, the majority of it is transported out of the organelles by the adenine nucleotide translocator (ANT) for use by the cell.

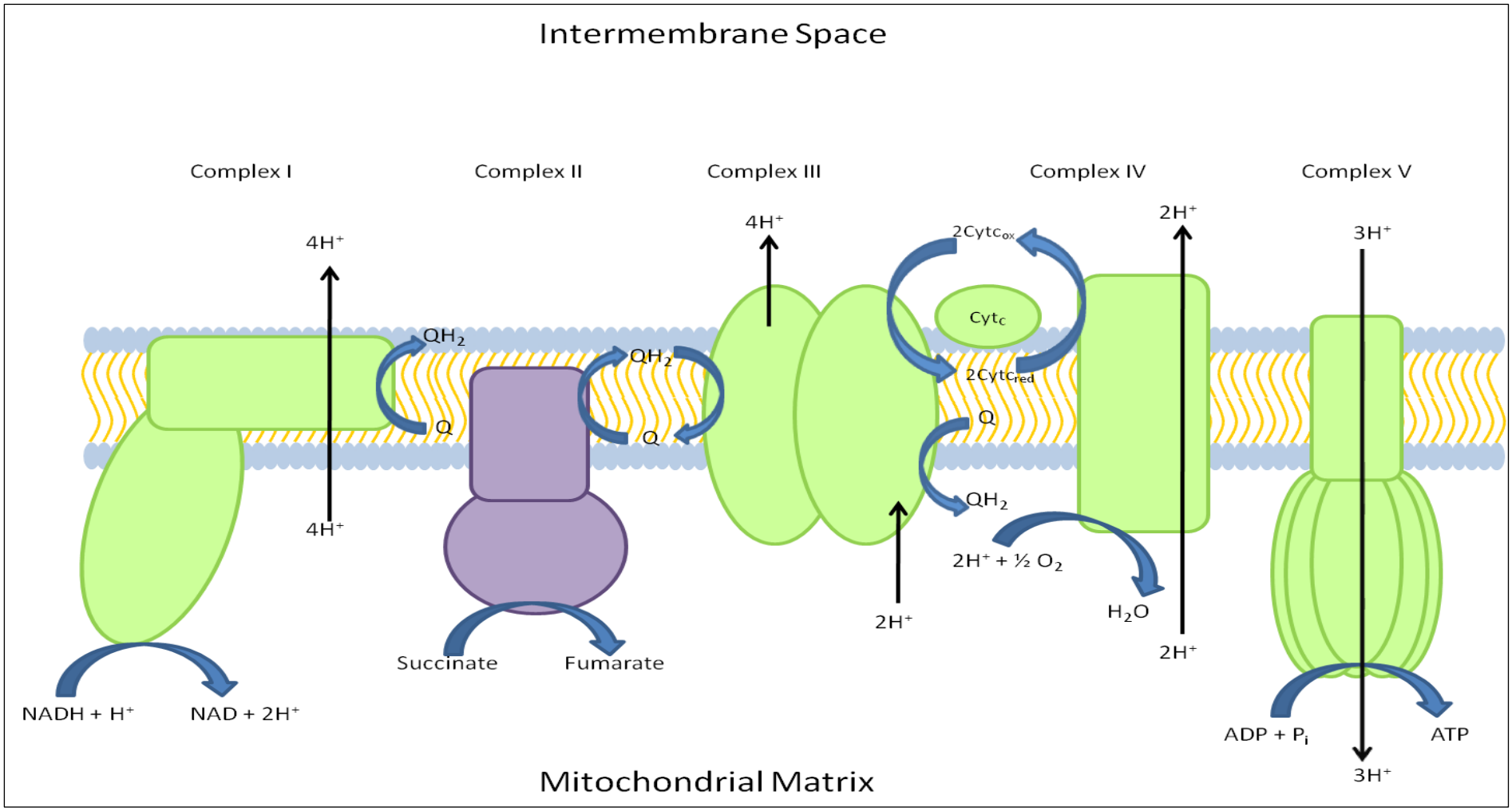

Figure 1.3 Oxidative phosphorylation. Energy is generated for the cell by mitochondria via the process of oxidative phosphorylation. This involves the transport of electrons through a series of protein complexes by redox groups. As the electrons pass through the redox groups free energy is generated; this is used to pump protons across the membrane. The resultant proton gradient is used by complex V to generate ATP from ADP.

1.1.3.1.1 Complex I (NADH Ubiquinone Oxidoreductase)

Complex I catalyses the first step in the ETC, utilising NADH, the products of the TCA cycle, and the β–oxidation of fatty acids. It transfers two electrons from NADH to ubiquinone and couples this with the translocation of four H^+ across the inner mitochondrial membrane.

Many of the experiments performed to determine the structure of complex I have been carried out using bovine enzyme as a model for the human enzyme. Complex I is the largest of the respiratory chain enzymes at almost 1000kDa. It is often depicted in an L shape and consists of a hydrophobic membrane domain, containing a number of transmembrane helices, and a hydrophilic peripheral arm lying perpendicular to the membrane component. In total there are forty five subunits (Carroll et al., 2006), of which seven are encoded by the mtDNA, and the rest by the nuclear genome (nDNA).

Fourteen of these subunits are termed “core” subunits as they are conserved between eukaryotes and prokaryotes. Seven of these “core” subunits are hydrophilic, in the peripheral arm, and encoded by the nDNA; NDUFS1, NDUFS2, NDUFS3, NDUFS7, NDUFS8, NDUFV1 and NDUFV2. The remaining seven “core” subunits are encoded by the mtDNA, ND1-6, and ND4L and sit in the hydrophobic membrane arm (Lazarou et al., 2009, Zickermann et al., 2009).

The exact mechanism of complex I assembly has caused many discussions, mainly due to its large nature and bigenomic control. But its importance is paramount, as many defects in complex I that cause diseases occur as a result of impaired assembly. The subunits encoded by the nDNA must be imported into the mitochondria and then assembled alongside mtDNA encoded subunits. Rather than a linear assembly, it has been suggested that nDNA encoded subunits are continuously imported into the mitochondria where they assemble into existing subunits in the complex (Lazarou et al., 2007). In contrast, mtDNA encoded subunits need to assemble into intermediate complexes before being integrated. A recent study, using mouse L929 cells (adipose fibroblasts) with mutations in ND4, ND5, and ND6, concluded that there are five entry points for the mtDNA encoded subunits into complex I. ND1 joins the peripheral arm subunit complexes to form a ~400kDa intermediate that then anchors itself to the inner mitochondrial membrane. This is followed by a second sub complex (~460kDa) containing ND2, ND3, and ND4L that joins

the ND1 assembly. A third entry point is for ND4, followed by incorporation of ND6, and finally ND5 (Perales-Clemente et al., 2010).

Complex I catalyses the oxidation of NADH to NAD^+, this releases two electrons which are passed to a flavin mononucleotide (FMN) prosthetic group. This reduces FMN to $FMNH_2$, the electrons are then passed through a series of eight iron-sulphur (Fe-S) clusters. This takes place in the peripheral arm of the complex. The two electrons are then passed to a ubiquinone (Q) molecule reducing it to ubiquinol (QH_2). This reaction is coupled with the pumping of $4H^+$ from the matrix into the intermembrane space (Figure 1.4). The proton translocation takes place in the membrane arm of the enzyme and utilises the energy generated in the peripheral arm via the redox reactions.

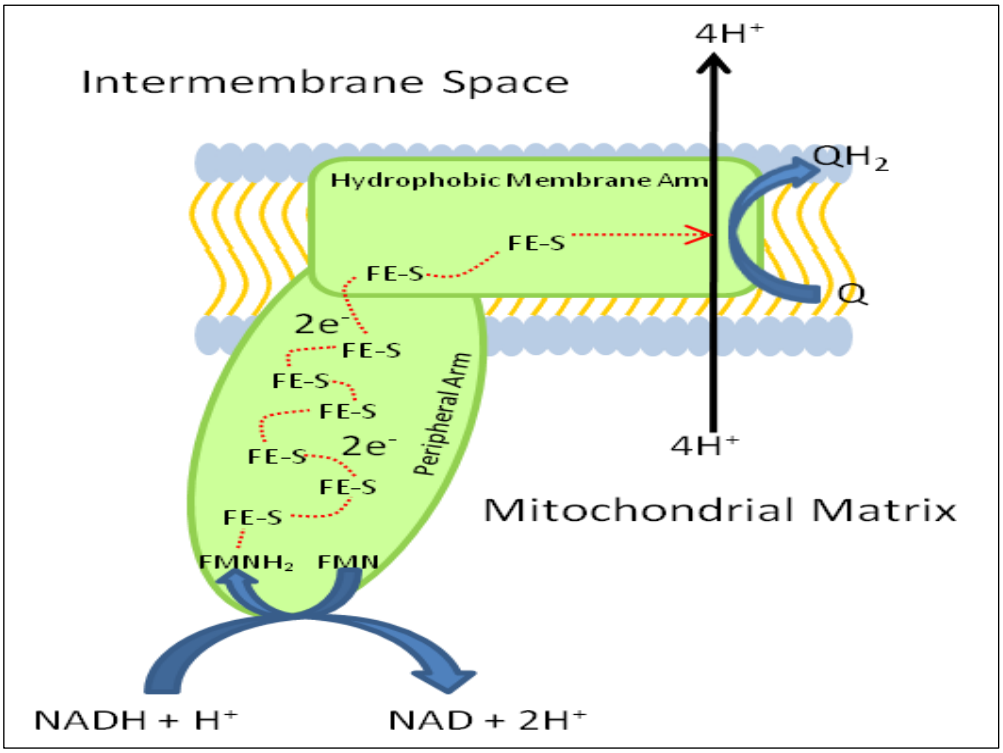

Figure 1.4 Complex I. Complex I is also known as NADH-ubiquinone oxidoreductase or NADH dehydrogenase. It consists of a hydrophobic and hydrophilic domain, often depicted in an L shape. It catalyses the reaction: NADH +Q+$5H^+$>NAD^++QH_2+$4H^+$. The complex contains a number of redox groups that carry electrons, the first of which is the flavin mononucleotide (FMN). The electrons then pass through eight iron-sulphur clusters to reduce ubiquinone (Q) to ubiquinol (QH_2). This reaction results in four protons being translocated.

1.1.3.1.2 Complex II (Succinate Dehydrogenase)

Complex II is the smallest component of the ETC, comprising four polypeptides (Capaldi et al., 1977), termed A (73kDa), B (27kDa), C (15kDa), and D (12.5kDa). In humans these four subunits are encoded by the nDNA. A and B are hydrophilic and present on the matrix side of the membrane, while C and D are hydrophobic and integral membrane proteins. Within the hydrophilic component of the complex, subunit A contains a flavin adenine dinucleotide (FAD) prosthetic group, and B houses three Fe-S clusters (2Fe-2S, 4Fe-4S, 3Fe-4S). The hydrophobic subunits C and D contain a haem group sandwiched between α-helices (Figure 1.5).

Complex II catalyses the oxidation of succinate to fumarate, a step in the TCA cycle. This allows a second entry point of two electrons to enter the ETC. These two electrons reduce FAD to FADH2 and are then passed through three Fe-S clusters before reducing ubiquinone to ubiquinol. Complex II is thought to contain two ubiquinone binding sites, one on the matrix side of the complex, and the other close to the intermembrane space (Sun et al., 2005). The enzyme is unusual in that it is the only component of the ETC where the transfer of electrons is not coupled to the translocation of H^+, probably because the free energy change from succinate to ubiquinone is too small.

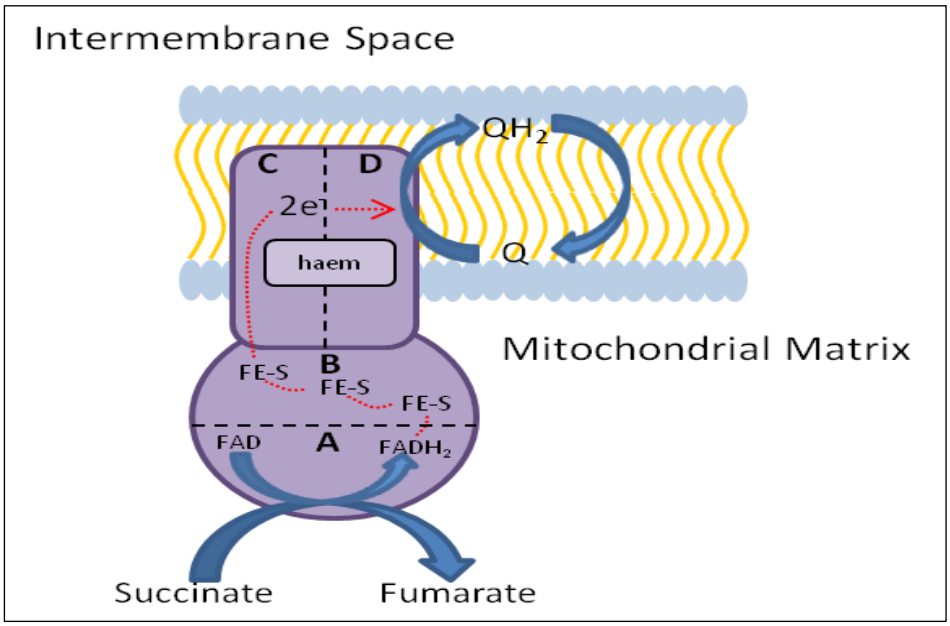

Figure 1.5 Complex II. Complex II is also known as succinate: ubiquinone oxidoreductase. It consists of two hydrophilic polypeptides, A and B, and two membrane hydrophobic polypeptides, C and D. It catalyses the reaction: succinate+Q> fumarate+QH_2. The movement of electrons through the redox groups does not result in enough free energy for the movement of protons across the membrane, but the complex is a source of further electrons for the ETC.

1.1.3.1.3 Complex III (Cytochrome *bc*1, Ubiquinol-cytochrome c oxidoreductase)

Complex III (248kDa) is an integral membrane protein catalysing the electron transfer from ubiquinol to cytochrome *c*. It is a dimer, with each monomer consisting of eleven subunits, three of which contain prosthetic groups (Figure 1.6). Subunit III has two haem containing groups: cytochrome b_L and b_H. Subunit IV contains one haem group: cytochrome c_1, and subunit V contains a 2Fe-2S containing Rieske protein (Xia et al., 1997). These three subunits constitute the essential subunits, with the other eight termed supernumerary subunits.

The two monomers of complex III form cavities through which substrate pockets can be accessed. The complex can be divided into three regions: intermembrane space, transmembrane helix, and matrix. The intermembrane space domain contains the functional part of cytochrome c_1 and Fe-S, along with subunit VIII. Each transmembrane monomer contains eight helices from cytochrome *b*, and five from cytochrome c_1, Fe-S, and subunits VII, X, XI. More than half the mass of the enzyme is contained in the matrix region and consists of subunits I, II, VI, and IX, as well as parts of subunit VII, Fe-S, and cytochrome c_1 (Yu et al., 1999). Except for cytochrome *b* which is encoded by the mtDNA, complex III is nuclear encoded.

Complex III catalyses the transfer of electrons from ubiquinol to cytochrome *c* and couples this transfer with the translocation of four H^+. This electron transfer is thought to occur via the Q cycle, involving a bifurcation of electron flow and two active sites (Mitchell, 1976). The first step in this cycle is the oxidation of ubiquinol at Q_o which generates two electrons. One of the electrons is then passed via the Rieske Fe-S centre to cytochrome c_1 and is used to reduce the soluble electron acceptor cytochrome *c*. The second electron is recycled through cytochrome b_L and then b_H, and is used to reduce ubiquinone to ubisemiquinone at the Q_i site. A second Q cycle further reduces ubisemiquinone to ubiquinol. Two protons are translocated for every quinol oxidised and in a complete cycle two ubiquinols are oxidised with the resultant two electrons being transferred to the Q_i site and one ubiquinone being reduced, resulting in a total of four H+ being translocated (Gao et al., 2003, Yu et al., 1999).

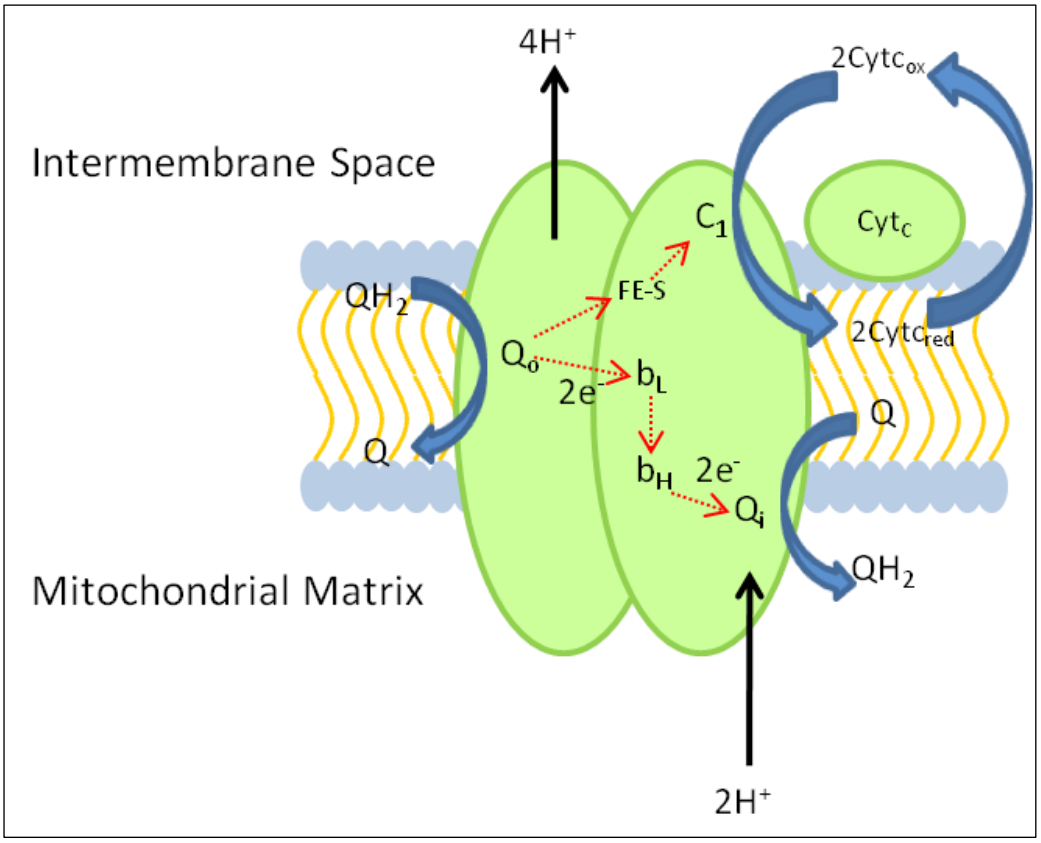

Figure 1.6 Complex III. Complex III may also be named ubiquinone-cytochrome *c* oxidoreductase and bc*1* complex. It is a dimer composed of two monomers, each consisting of eleven subunits. The complex catalyses the reaction: QH_2+2cytc+$2H^+$>Q+2cytc+$4H^+$, which results in four protons being pumped into the intermembrane space.

1.1.3.1.4 Cytochrome *c*

Cytochrome *c* is a 13kDa, water soluble, haem containing protein, consisting of one hundred and four amino acids. It is mobile within the plane of the mitochondrial inner membrane and is reduced by complex III. It then donates these electrons to complex IV. It is entirely nuclear encoded and evolutionary conserved (Huttemann et al., 2011).

1.1.3.1.5 Complex IV (Cytochrome *c* Oxidase)

Complex IV is the last component of the ETC and houses the terminal electron acceptor. It catalyses the transfer of two electrons to molecular oxygen (O_2) to generate water (H_2O) and results in two H^+ being pumped into the intermembrane space.

Complex IV (204kDa) is composed of two monomers each containing thirteen subunits. The largest subunits, I-III, are encoded by the mtDNA and form the functional core of the protein, while IV-XIII are encoded by the nDNA. The active site for the enzyme is located in the transmembrane region on subunit I. The complex contains two cytochrome containing haems (haem a, haem a_3) and two copper centres (Cu_A, Cu_B), as well as additional metal centres of Zn and Mg. The haem a and haem a_3 prosthetic groups along with the Cu_B group are bound to subunit I in the transmembrane region, which also contains twelve transmembrane helices. The Cu_A centre is located in subunit II on the cytosolic side of the complex. The Zn centre is in the nuclear encoded subunit V on the matrix side of the complex, while the Mg centre sits between subunits I and II (Tsukihara et al., 1995, Tsukihara et al., 1996).

Cytochrome *c* binds to complex IV and donates electrons to the primary electron acceptor, Cu_A. The electron is then transferred to haem a and then on to the catalytic site which contains haem a_3 and Cu_B. The reduction of molecular O_2 to H_2O requires four electrons, and cytochrome *c* can only donate one at a time. As a result cytochrome *c* cycles through four states, with each state being coupled to H^+ uptake and translocation across the membrane (Faxen et al., 2005).

1.1.3.1.6 Complex V (ATP synthase)

Complex V is the last component of the OXPHOS system. It is a large protein complex of approximately 500kDa and is responsible for the synthesis of ATP from ADP and inorganic phosphate (Pi) on the matrix side of the membrane. It utilises the flow of protons across the membrane and down the electrochemical gradient to do this.

The complex contains sixteen subunits, two of which are encoded by the mtDNA, ATPase 6 and 8. It consists of two domains, the transmembrane F_o part and the water soluble F_1 segment which is on the matrix side of the membrane (Figure 1.7). F_o contains a proton channel and is composed of a subunit c ring and an ab complex. F_1 is composed of four subunits α_3, β_3, δ, and ε in a cylindrical shape surrounding a fifth, γ component. Three catalytic sites are present on the β subunit in F_1 (Yoshida et al., 2001). In addition the complex contains two stalks (b), one connecting the F_o and F_1 regions and another extending from the bottom of F_o to the top of F_1 (Bottcher et al., 1998).

H^+ translocation through the F_o proton channel drives the rotation of the γ subunit within the F_1 segment, with each 120^0 turn driving the release of one ATP molecule. The side stalks prevent unwanted rotation of the stationary parts of the protein. The catalytic sites for ATP hydrolysis are on the β subunit, and exists in one of three states, open (O), loose (L), and tight (T). When ADP and Pi are bound to a site it is “loose”, ATP is “trapped/tight” in the second site, and the third site will be empty (O). A rotation of F_1 allows the ATP to be released, and ADP + Pi becomes ATP.

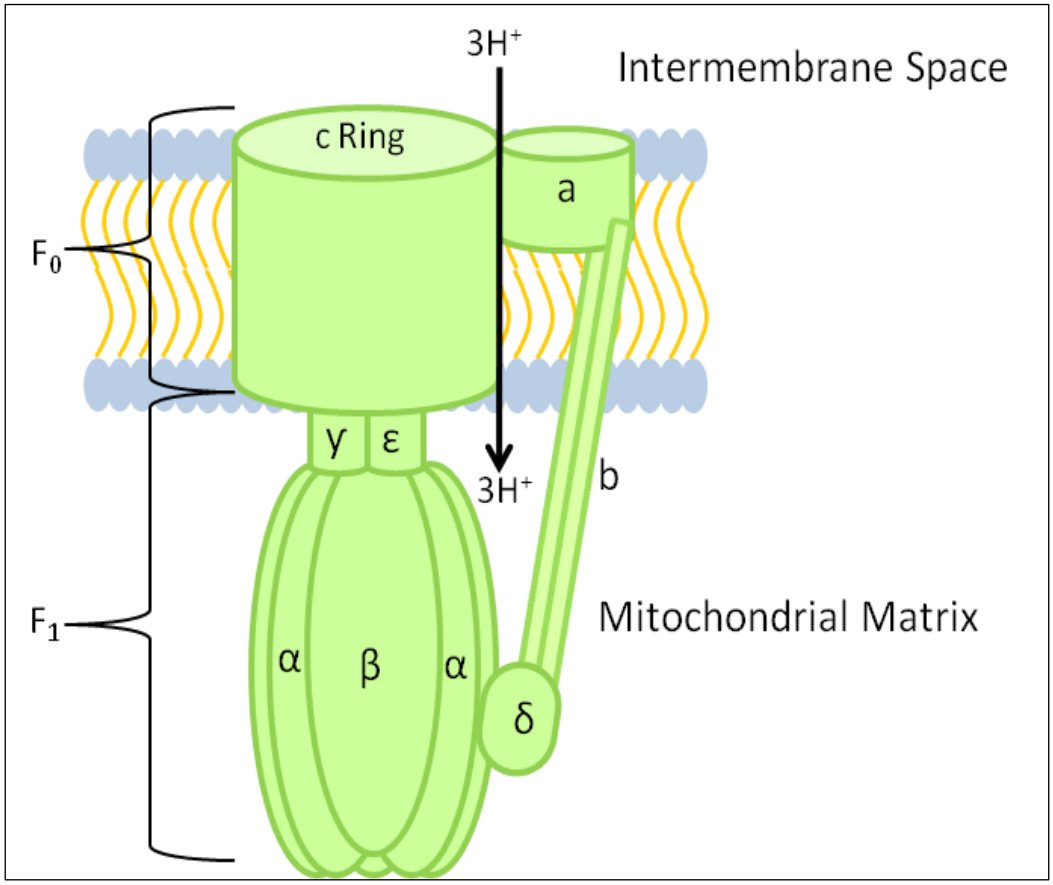

Figure 1.7 ATP synthase. The ATP synthase consists of a membrane component (F_0), and a hydrophilic catalytic portion (F_1). F_0 is composed of a c ring, an ab complex and a proton channel. The c ring along with the γ and ε domains make up the rotating segment of the enzyme. The b stalk connects the F_0 and F_1 regions. The catalytic domain consists of α_3, β_3, δ, ε, and γ.

1.1.3.1.7 Adenine Nucleotide Transporter (ANT)

ANT catalyses the exchange of ATP for ADP across the inner membrane in a 1:1 stoichiometry. It is an integral membrane protein of approximately 32kDa and is encoded by the nDNA. Its role in OXPHOS is to exchange ATP out of the mitochondria for ADP in, but is able to work in reverse during times of stress (Chinopoulos et al., 2009).

1.1.3.1.8 Mitochondrial Supercomplexes

The OXPHOS system is often depicted as a sequence of proteins embedded in the membrane where they are able to move freely by lateral diffusion with electrons flowing between them connected by the mobile carriers ubiquinone and cytochrome *c*. This is referred to as the "Random Diffusion Model". However, recent research has shown that the individual complexes form aggregates with each other which are termed supercomplexes. Investigations into supercomplex structures have been greatly helped with the development of Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE). This has revealed that the exact compositions of these supercomplexes appears to vary depending on the organism being investigated and the detergents used to solubilise the mitochondrial membrane (Lenaz and Genova, 2007).

A recent paper demonstrated that supercomplexes can also contain ubiquinone and cytochrome *c*. In addition they were shown to respire, thus demonstrating their functional capabilities and earning them the name "respirasomes" (Acin-Perez et al., 2008). Complex I has been found to associate with complex III in a I_1III_2 ratio, as well as in supercomplexes containing both III and IV. Only small amounts of complex I have been found in the free form. While initially not thought to form supercomplexes, complex II has now been demonstrated in these associations (Acin-Perez et al., 2008, Lenaz et al., 2010).

It is thought that this organisation allows the close proximity of enzymes catalysing consecutive reactions, thus preventing OXPHOS intermediates being lost from the chain into the surrounding area. It can also aid the structural stabilisation of the complexes (Lenaz and Genova, 2007).

1.1.3.2 Apoptosis

Apoptosis is the programmed cell death that occurs in many eukaryotes. It confers an advantage to the organism as it allows for control of cell numbers, tissue size, and the disposal of old or rogue cells. It differs from necrosis, which occurs in response to external factors outside the cell, for example injury, infection, or toxins.

There are two main pathways of apoptosis, both of which result in the activation of caspases. Caspase activation leads to cell death through the modification of regulatory and structural proteins. The extracellular route is initiated by cell surface receptors and produces caspase-8 activation, while the intracellular route is mediated by mitochondria (Figure 1.8).

The extracellular pathway is initiated by the ligation of "death-receptors", which are members of the tumour necrosis factor (TNF) receptor family, for example FAS, or TNF receptor-1 (TNFR1). These receptors contain an intracellular domain which is able to activate caspase-8. This causes downstream activation of effector caspases, for example 3 or 6 (Youle and Strasser, 2008).

The intracellular route is mediated by mitochondrial release of cytochrome *c* from the cristae. This is controlled upstream by the Bcl-2 family of proteins, which in turn is controlled by BH3-only proteins. Upon the initiation of apoptosis, BH3-only proteins inhibit the anti-apoptotic Bcl-2 proteins, which allow the pro-apoptotic BAK and BAX proteins to permeabilise the outer mitochondrial membrane (Suen et al., 2008). This permeabilisation causes the release of cytochrome *c*, which leads to caspase activation. Cytochrome *c* binds to APAF1, which induces the assembly of an apoptosome which binds to caspase-9, causing caspase-3 initiation and cell death (Youle and Strasser, 2008).

The release of cytochrome *c* happens close in time to BAX activation, which can then be observed to localise to the outer mitochondrial membrane at small focused areas; these foci become sites of mitochondrial splitting. This release occurs just after or simultaneously to mitochondrial fission, and is thought to be mediated by the protein Drp1, the binding of which to mitochondria increases during apoptosis (Landes and Martinou, 2011). Using short hairpin RNAs for RNA inhibition it has been shown that down regulation of Drp1 reduces cytochrome *c* release and eventual cell death. Inhibition

of hFIS1 decreases apoptosis to an even greater extent, possibly because it is required for BAX recruitment to the Drp-1 foci (Lee et al., 2004).

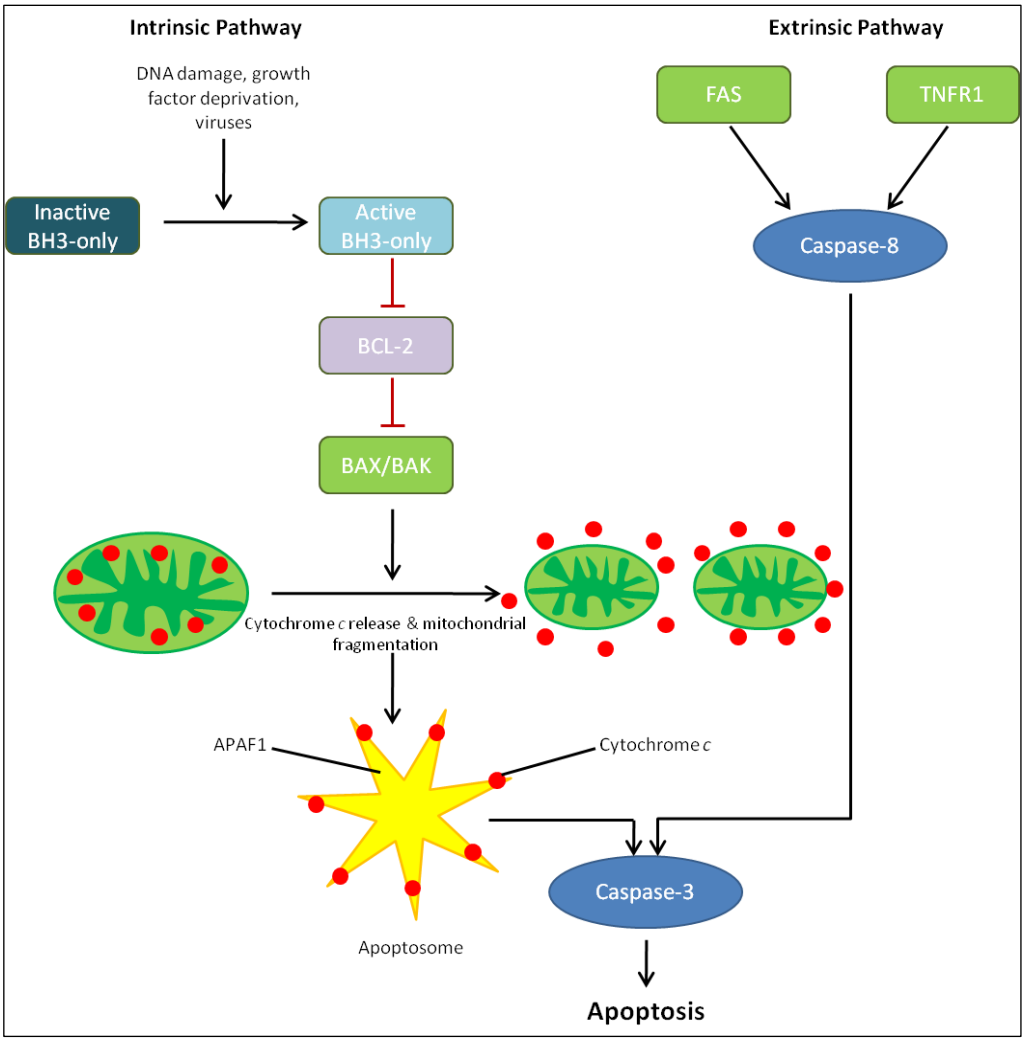

Figure 1.8 Apoptosis. Apoptosis may be initiated via an extrinsic or intrinsic route. The extrinsic pathway is activated by cell surface receptors FAS and TNFR1, and then through the activation of caspase-8 the caspase cascade is initiated. The intrinsic pathway relies on mitochondria. In response to a stress on the cell, BH3 is activated, it then stops the inhibition of BAX/BAK proteins, which causes the fission of mitochondria and cytochrome *c* release from the intermembrane space. This activates the apoptosome which again induces caspase activation.

1.1.3.3 Mitochondria and Calcium

Calcium (Ca^{2+}) serves many functions in the cell, including signalling, differentiation, apoptosis, and muscle fibre contraction. Its regulation is therefore very important. Mitochondria play a role in this regulation through the storage and buffering of Ca^{2+} (Contreras et al., 2010, Duchen, 2000). High cytoplasmic levels of Ca^{2+} are responded to by an uptake and storage of the ion by the mitochondria, thus protecting the cell from overload. The reverse is also witnessed when levels in the cell drop (Collins and Meyer, 2010). Within mitochondria Ca^{2+} is able to stimulate ATP production through its activation of dehydrogenases coupled to the TCA cycle, and possibly via its interactions with the ETC (Hoppe, 2010).

Mitochondria accumulate Ca^{2+} via the Ca^{2+} uniporter (MCU), in the inner mitochondrial membrane. This occurs against a concentration gradient and is driven by a negative potential rather than ATP-dependent pumping. Movement out of the mitochondria occurs via a Na^{+}/Ca^{2+} exchanger. The outer membrane is permeable to Ca^{2+} through VDAC channels that allow the passage of molecules up to 10kDa. There is debate if VDAC is freely permeable to Ca^{2+} or provides some regulation (Hoppe, 2010).

A recent study identified a regulator of the MCU through a series of focused genetic screens and knockdown experiments (Perocchi et al., 2010). The authors used a bank of mitochondrial proteins that they narrowed down to IM proteins that were present in the majority of mammalian tissues. They then ruled out all proteins that did not have homologues in vertebrates and kinetoplastids, but were present in yeast, as yeast does not demonstrate uniport activity. Eventually they were left with thirteen genes with which to perform RNA interference experiments. They identified one protein, the mitochondrial Ca^{2+} uptake 1 (MICU1), as a regulator of Ca^{2+} uptake. This 54kDa protein contains two EF-hands that are separated by a long helix, which are important for the Ca^{2+} uptake, as it is these sites the ion binds to. The channel is also sensitive to Ca^{2+} concentrations, suggesting it may additionally function as a Ca^{2+} regulator (Perocchi et al., 2010).

1.1.3.4 Reactive Oxygen Species (ROS) Generation

Reactive oxygen species (ROS) are termed reactive as they are able to react with reducible agents. ROS include hydrogen peroxide (H_2O_2), superoxide (O_2^-) and the hydroxyl radical (·OH). The ETC produces O_2^-, which is converted into H_2O_2 by superoxide dismutase (SOD), this can be further converted into ·OH (Camello-Almaraz et al., 2006). ROS is mainly generated at complexes I and III through the incomplete reduction of oxygen. Complexes I and II release ROS into the mitochondrial matrix while complex III produces it on both sides of the inner mitochondrial membrane (Muller et al., 2004).

ROS generation may be considered a double-edged sword. While it has important roles to play in cellular signalling, especially pathways related to autophagy, it has also been implicated in the ageing process.

When cells are exposed to hypoxia, adaptive signalling pathways are activated in order to decrease energy consumption and oxygen usage. The cellular responses to hypoxia require the activation of hypoxia inducible transcription factors (HIFs), an upstream regulator of which is thought to be H_2O_2 released from the mitochondria (Hamanaka and Chandel, 2009). In addition, mitochondrial ROS is thought to regulate tumour necrosis factor alpha (TNFα) mediated cell death, modulate inflammation, and play a role in Ca^{2+} signalling (Hamanaka and Chandel, 2010, Camello-Almaraz et al., 2006). However, mitochondrial ROS has also been suggested as one of the factors limiting lifespan (Balaban et al., 2005).

1.1.3.5 Iron – Sulphur (Fe-S) Cluster Biogenesis

Iron enters the mitochondrial IM via mitoferrin, however it is not yet known how it crosses the OM. Once in the mitochondria it is used for haem and Fe-S cluster synthesis, or stored in mitochondrial ferritin.

Mitochondria are a major site of Fe-S biogenesis formation, consisting of iron and sulphide anions (S^{2-}), which assemble into clusters of 2Fe-2S, or 4Fe-4S. These clusters become protein cofactors, which can proceed to perform functions in electron transport, DNA repair, enzyme structural stability, ROS sensing, and iron homeostasis (Rouault and Tong, 2005, Richardson et al., 2010).

The importance of Fe-S clusters can be observed in patients with Friedreich's ataxia. This is a neurodegenerative disorder caused by a decrease in the frataxin gene due to a GAA repeat in its first intron. Frataxin is synthesised in the cytoplasm, targeted to the mitochondria and thought to participate in Fe-S biogenesis. Patients with Friedreich's ataxia demonstrate a decrease in the activities of Fe-S proteins and have iron accumulation in their mitochondria, leading to oxidative damage and failure (Rouault and Tong, 2005, Stemmler et al., 2010).

1.1.4 The Mitochondrial Genome

1.1.4.1 Structure and Organisation

Mitochondria contain their own genome separate from the nDNA. It is present in multiple copies in each cell and is a covalently closed double stranded loop. The two strands are named heavy (H strand,) abundant in guanine, and light (L strand) which is rich in cytosine (Figure 1.9). The two strands were given their name because of their separate levels in a cesium chloride (cs-cl) separation gradient. In addition, the genome contains a short three stranded DNA region, termed the displacement loop (D-loop). The possibility of this structure being an intermediate in a repair mechanism, or a precursor of replication has been suggested, but its true function remains a mystery (Kasamatsu et al., 1971).

While often depicted as a circular structure, mtDNA is in fact a supercoiled molecule. In addition, punctate staining of mtDNA in the cell suggests that it is packaged up with various proteins into nucleoids. These are thought to regulate the genetic content and intracellular distribution of mtDNA. Each nucleoid contains between two to ten copies of mtDNA and is located at discrete sites uniformly along the length of the organelle in the matrix. The mtDNA is tethered through the inner membrane to cytoskeleton proteins (Gilkerson and Schon, 2008, Iborra et al., 2004).

An experiment solubilising mitochondria with detergent to release mitochondrial transcription factor A (TFAM) showed it was associated with mtDNA. In addition, it was not until the mtDNA was digested with DNAse I that the TFAM was released. It was further suggested that the ratio of TFAM to mtDNA was 900:1, enough to completely encapsulate the mtDNA (Alam et al., 2003). There is also evidence that TFAM is directly involved with regulating mtDNA copy number (Ekstrand et al., 2004). The above findings suggest TFAM is a nucleoid associated protein.

Other proteins associated with the nucleoids include prohibitin, polymerase γ (POLG), mitochondrial single stranded binding protein (mtSSB) and TWINKLE (Gilkerson and Schon, 2008).

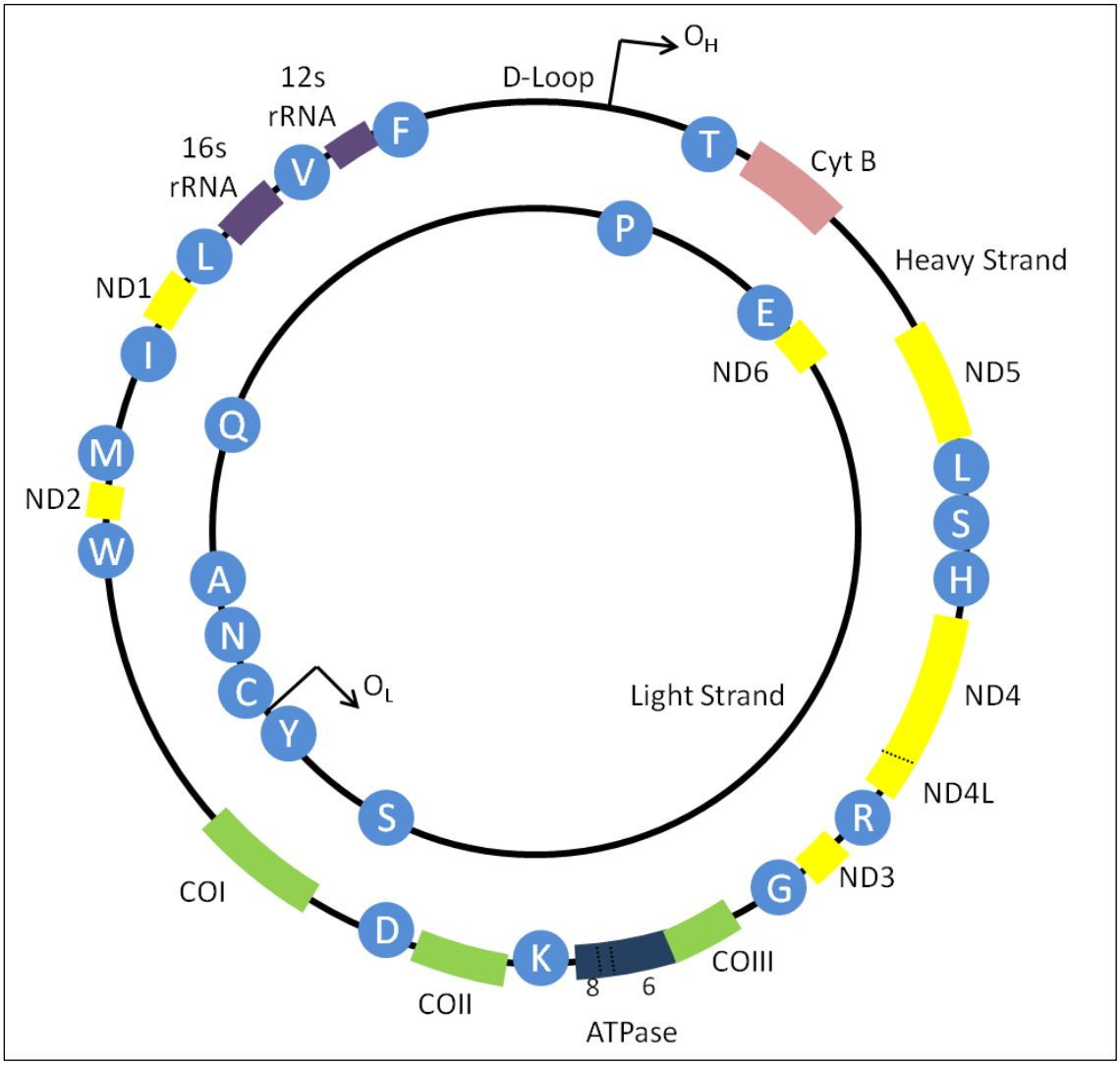

Figure 1.9 MtDNA structure. MtDNA is a double stranded genome, consisting of a heavy and light component. It encodes thirty seven genes which are unevenly distributed between the two strands. It encodes seven genes of complex I (yellow), one of complex III (pink), three of complex IV (green) and two of complex V (dark blue). In addition it encodes twenty two tRNAs (blue), and two rRNAs (purple). The origins of light and heavy strand replication are also indicated.

1.1.4.2 Genetic Code

Sequenced in its entirety back in 1981, mtDNA is extremely tightly packed, in that it contains very few non coding regions with no introns, and is highly conserved among mammals. It is 16569bp long and encodes thirty seven genes (Figure 1.9); these are thirteen structural proteins, twenty two tRNAs, and the 12S and 16S rRNAs (Anderson et al., 1981). The thirteen structural proteins are core subunits of the OXPHOS system, with the tRNAs and rRNAs that are required for their translation in the cell. These genes are distributed unevenly between the H and L strands of the DNA, with eight tRNAs and one structural protein on the L strand and everything else on the H strand. The remaining components required by the mitochondria are encoded by the nuclear genome and imported into the organelle. The genome is confined within the inner membrane (Tuppen et al., 2010, Smeitink et al., 2001).

The genome sequenced in 1981 formed the Cambridge Reference Sequence, however the mtDNA was sourced from a single human, HeLa cells, and bovine cells. This has resulted in some discrepancies in the sequence. A more recent paper (Andrews et al., 1999) sought to correct these discrepancies and produced the Revised Cambridge Reference Sequence.

1.1.4.3 MtDNA Replication

Work in mouse L cells (fibroblasts) has demonstrated that mtDNA replication occurs independently of the cell cycle (Bogenhagen and Clayton, 1977). The genome also continually replicates in post mitotic tissues, for example muscle and brain. However, the exact mechanism of mtDNA replication is still under debate. Two models have emerged in this discussion, the strand asynchronous or displacement model (Robberson and Clayton, 1972, Clayton, 1982), and the strand synchronous or coupled model (Holt et al., 2000).

1.1.4.3.1 Strand Asynchronous Model

In this model, replication initiates at the origin of heavy strand replication (O_H), which causes displacement of the L strand from the H strand. Replication then proceeds in a clockwise direction until the origin of light strand replication (O_L) is exposed, which occurs when about 67% of the H strand has been synthesised. At this point, L strand replication is initiated in the opposite direction, using the exposed L strand as a template (Figure 1.10A). This order of replication has resulted in the heavy and light strands being termed "leading" and "lagging" respectively. When replication of the strands is complete they are covalently closed. When both daughter strands have been synthesised and dissociated from their templates super-helical turns are introduced. It has been suggested that this whole process is complete in two hours (Clayton, 1982).

1.1.4.3.2 Strand Synchronous Model

While earlier work investigating mtDNA replication involved the utilisation of Cs-Cl density gradients, later work involved the use of 2D-PAGE techniques, allowing for the separation of DNA molecules on the basis of both size and shape. Using this approach the strand synchronous model was proposed (Holt et al., 2000).

As in the asynchronous model, H strand synthesis is thought to be instigated at O_H and proceeds in a clockwise direction, again being termed the leading strand. Shortly afterwards this initiates replication of the "lagging" L strand in the opposite direction (Figure 1.10B). However, the L strand is generated in short segments (replication intermediates) that have been named Okazaki Fragments (Holt et al., 2000). The researchers did leave open the possibility that both mechanisms of replication could occur within the mitochondria. With the strand asynchronous model occurring when cells require large amounts of mtDNA for example during growth, and the synchronous model being used for cell maintenance.

In a development of the strand synchronous model it has been proposed that the lagging L strand is initially composed of RNA, before being converted to DNA in maturation steps. This "RNA incorporation throughout the lagging strand" (RITOLS) model would allow for the stabilization of the displaced DNA strand during replication (Yasukawa et al., 2006).

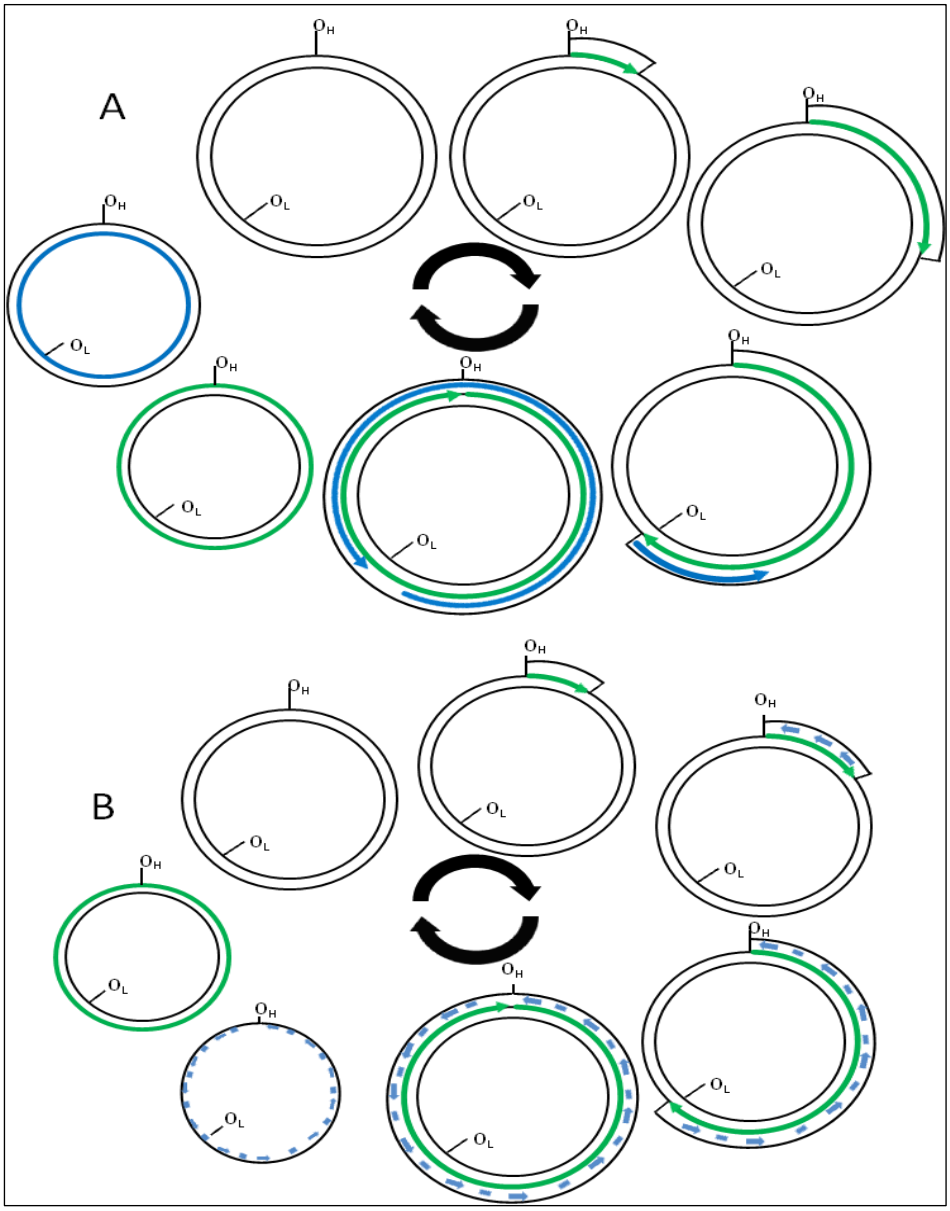

Figure 1.10 MtDNA replication. A. Strand asynchronous model of mtDNA replication. Replication begins at the origin of heavy strand replication (O_H), it then proceeds until the origin of light strand replication (O_L) is exposed. Replication then commences in the opposite direction until both strands are complete. **B**. Strand synchronous model. Replication is also initiated from O_H, but very quickly replication of the light strand begins. This occurs through the production of Okazaki Fragments. Both strands continue to be replicated until two genomes have been generated. Green arrow = heavy strand replication, blue arrow = light strand replication.

1.1.4.3.3 Protein Machinery for MtDNA Replication

The proteins required for mtDNA replication are encoded by the nDNA, translated in the cytosol and then imported into the mitochondria. These enzymes include POLG, a replicative DNA helicase TWINKLE, the mitochondrial single stranded DNA-binding protein (mtSSB), and mitochondrial RNA polymerase (POLRMT) (Figure 1.11).

The TWINKLE helicase moves in a 5' to 3' direction unwinding the double stranded mtDNA. MtSSB instigates TWINKLE activity, in addition to stabilising the single strands created and stimulating POLG activity. POLG has both 5'-3' and 3'-5' exonuclease activity, and is a heterotrimer consisting of one catalytic subunit (POLGα) with two accessory subunits (POLGβ). POLRMT is the O_L specific primase and synthesises the RNA primer needed for lagging strand synthesis (Wanrooij and Falkenberg, 2010).

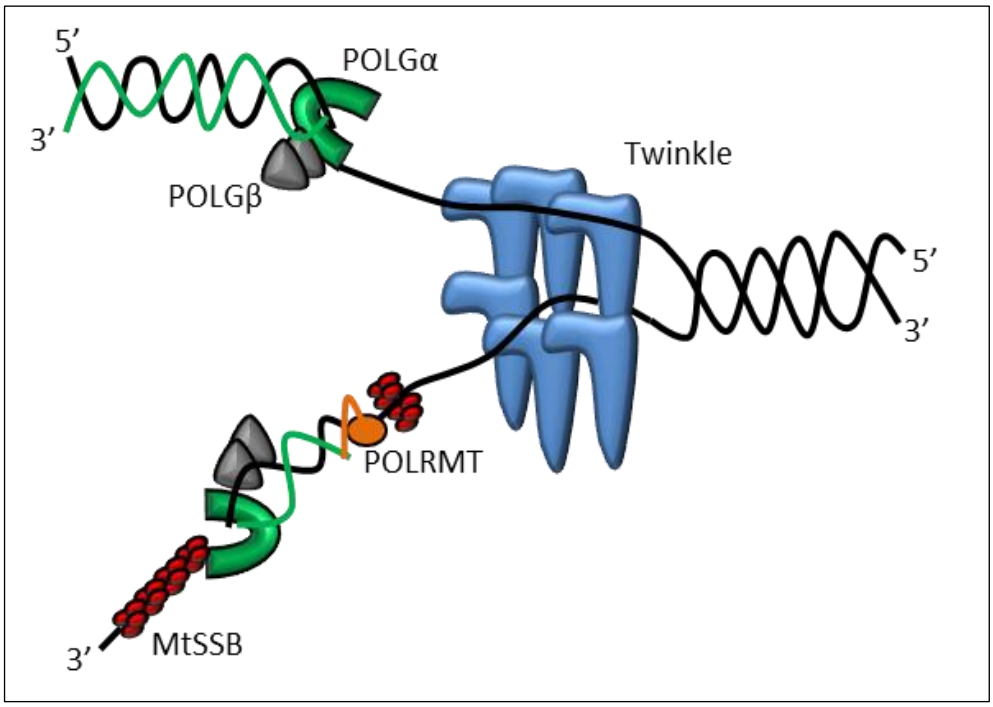

Figure 1.11 Proteins involved in mtDNA replication. TWINKLE helicase (blue) unwinds the double stranded DNA into single strands which are then stabilized by mitochondrial single stranded binding protein (mtSSB) (red). The new strands are then synthesised by POLG (α&β) (grey & green), while POLRMT (orange) synthesises the RNA that is required for the laggings strand synthesis that is initiated at O_L.

1.1.4.4 MtDNA Transcription

MtDNA transcription is initiated in the D-loop, from one promoter on the light strand (LSP) and two promoters on the heavy strand (HSP1 & 2) (Figure 1.12). Transcription from LSP is initiated from the transcription initiation site L (IT_L) at nucleotide 407 in the D-loop and proceeds to generate a polycsistronic molecule almost the entire length of the L strand. Transcription from HSP2 begins at transcription initiation site H (IT_{H2}) and also results in long molecules corresponding to the entire length of the genome. Another transcription initiation site H (IT_{H1}) is present in HSP1 and transcribes the two rRNAs (12S & 16S), mt- $tRNA^{Phe}$ and mt-$tRNA^{Val}$ (Chang and Clayton, 1984, Taanman, 1999, Ojala et al., 1981, Montoya et al., 1982).

Transcription activator A (TFAM) is a 25kDa protein composed of two high mobility group (HMG) domains, separated by a twenty five amino acid residue. It binds upstream of HSPs and LSB, allowing for the protein to initiate structural alterations in the promoter region and is essential for transcriptional activation. While it strongly promotes L strand transcription, its effect on H strand transcription is somewhat weaker. Mitochondria have their own polymerase, POLRMT, initially identified in yeast and then in humans. This is a 1230 amino acid protein that cannot initiate transcription on its own, but works in combination with TFAM and TFB2M. TFB2M forms a heterodimeric complex with POLRMT that is also required for transcription initiation (Falkenberg et al., 2007).

The termination of transcription is mediated by mTERF, a 39kDa protein. It is thought to attenuate both L and H strand transcription through binding to the 3' end of the mt-$tRNA^{Leu(UUR)}$ gene and providing a physical barrier (Taanman, 1999). The importance of this protein is seen with the m.3243A>G mutation, often causing the disease mitochondrial encephalopathy lactic-acidosis and stroke-like episodes (MELAS). The mutation reduces the binding affinity for mTERF and results in impaired transcription termination (Taanman, 1999).

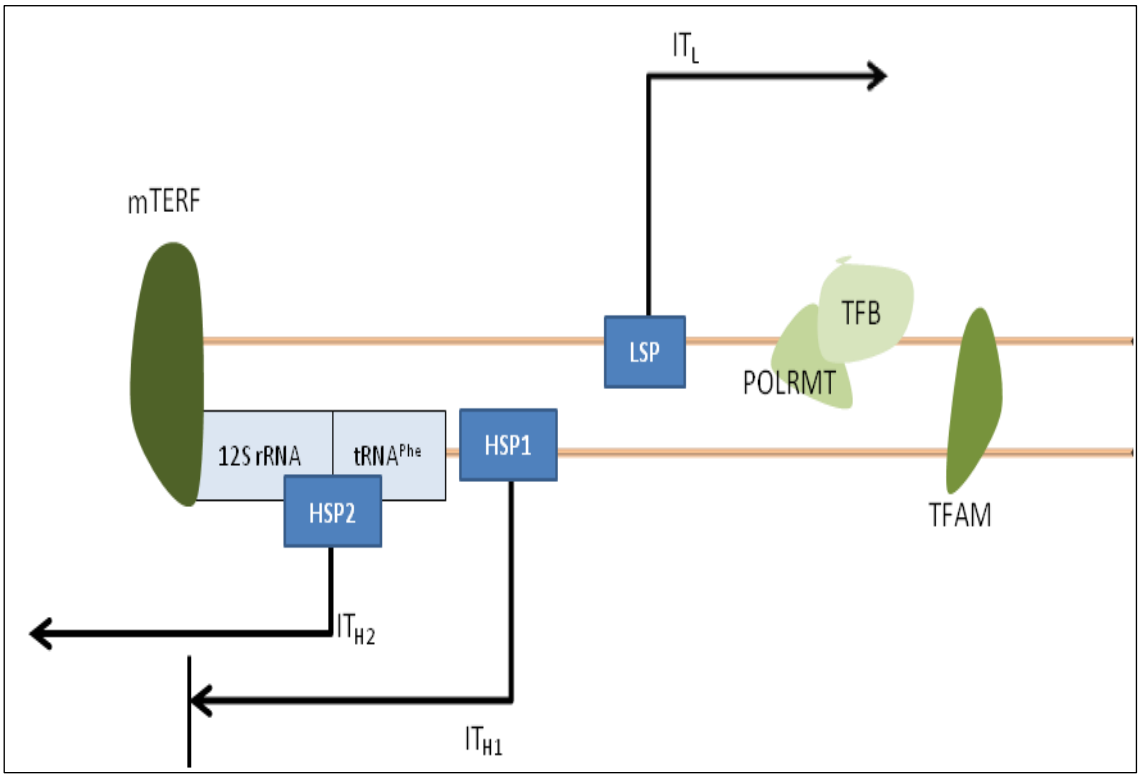

Figure 1.12 MtDNA transcription. MtDNA transcription is initiated from the D-loop. It commences from one promoter on the light strand (LSP) and from two promoters on the heavy strand (HSP1 & 2). Transcription from LSP and HSP2 creates long transcripts almost the length of the entire genome, while that initiated from HSP1 only transcribes two rRNAs and two tRNAs. TFAM works in combination with POLRMT and TFB to initiate transcription, while mTERF is required for its termination.

1.1.4.5 MtDNA Translation

Many of the proteins required by the mitochondria are encoded by the nDNA and imported into the organelle through channels, for example through the outer membrane via TOMs (translocases outer membrane) or the inner membrane via TIMs (translocases inner membrane). However, the mtDNA still encodes a few of the proteins it requires using its own translation system, although this system is somewhat different to that of the cytoplasm.

Mitochondria only use the codons UAA, and UAG for termination. While AGG and AGA were originally thought to be stop codons it has recently been shown that they simply cause a -1 frameshift allowing for the recognition by the termination factors (Temperley et al., 2009). In mitochondria, UGA codes for tryptophan rather than a stop codon, and AUA is a methionine rather than an isoleucine. In addition mRNAs have no or few 5' un-translated nucleotides, they are uncapped and have a poly (A) tail added after the last stop codon.

MtDNA translation consists of three stages, initiation, elongation, and termination. During the initiation phase, initiation factor 3 (IF3) catalyses the separation of the mitoribosome into its components, the small (12S) and the large (16S) subunits. This allows for the binding of the mRNA start codon (AUG) in the peptidyl site (P-site) on 28S subunit. The fMet-tRNA^{Met} also binds to the 12S subunit which requires initiation factor 2 (IF2). The 12S and 16S subunits then recombine dissociating IF3 and IF2 in the process.

The elongation phase requires elongation factors EFG1 and 2, EFTs and EFTu. ETFu forms a structure with the tRNA and carries it to the acceptor site (A-site), where the codon-anticodon interaction can occur. Peptide bond formation occurs on the 16S subunit and an amino acid is added to the growing peptide (Smits et al., 2010).

The termination phase requires a stop codon (UAA, UAG,) to pass through the acceptor site (A-site). This is recognised by a termination release factor mtRF1a causing the protein to be released (Soleimanpour-Lichaei et al., 2007).

1.1.4.6 MtDNA Inheritance

MtDNA is inherited in a non-Mendelian manner. In many animals and plants mtDNA is inherited from one parent, in humans this is the mother. This maternal inheritance was first demonstrated by Giles *et al.* (1980). Through the isolation of mtDNA from blood platelets and examination of *Hae II* restriction enzyme patterns it was demonstrated that mtDNA is maternally inherited. An atypical *Hae II* cleavage pattern was observed for all three children as well as in the mother, while the father contained a typical set of cleaved fragments that was not detectable in the children (Giles et al., 1980).

It is currently thought that in humans maternal inheritance occurs through the destruction of the male mitochondria in the sperm cell. This is performed by proteosomes and lysosomes in the egg cell. At the point of fertilisation, sperm mitochondria are able to enter the egg, but ubiquitination, which has occurred in both the male reproductive tract and inside the egg, marks these mitochondria out for destruction (Sutovsky et al., 1999).

There has however been a finding of paternal mtDNA inheritance identified in humans. Schwartz and Vissing (2002) reported the case of a twenty eight year old male, who presented with a mitochondrial myopathy that included exercise intolerance. Histochemical investigations into a muscle biopsy revealed accumulation of abnormal mitochondria within the fibres, termed Ragged Red Fibres (RRFs). Genetic investigations revealed a 2bp mtDNA deletion present in 90% of his muscle DNA. The mutation occurred in the *ND2* gene, causing a defect in complex I. In addition, the patient was shown to have two different mtDNA haplotypes. His blood haplotype matched his mother's, while his muscle haplotype was that of his father. It was hypothesised that a breakdown had occurred in the male mtDNA elimination mechanisms (Schwartz and Vissing, 2002).

While paternal leakage has currently only been reported in one human case, it would appear to occur more frequently in animals, including birds and lizards. This is hypothesised to occur in hybrid zones, where closely related species meet and breed. Due to the reproductive barriers not being fully developed, the mechanisms in place to eliminate male mitochondria may not be functioning, resulting in paternal leakage (Kvist et al., 2003, Ujvari et al., 2007).

Despite discussions regarding the clonality of mtDNA (Eyre-Walker et al., 1999, Elson et al., 2001a, Macaulay et al., 1999), its sequence and polymorphisms therein have been used extensively charting evolutionary history and population genetics.

1.1.5 Mitochondrial DNA in Disease

1.1.5.1 Mitochondrial Mutations

It was not until 1988 that mutations of mtDNA were linked with mitochondrial diseases. Holt *et al.* (1988) described patients with mitochondrial myopathies caused by large scale single mtDNA deletions (Holt et al., 1988). Then later that year Wallace *et al.* (1988) reported a point mutation that was necessary, though not sufficient, to cause Leber's Hereditary Optic Neuropathy (LHON) (Wallace et al., 1988). Since then, advances in molecular biology and in particular genetics, have resulted in mtDNA mutations being recognised as a major cause of genetic diseases.

MtDNA mutations can be grouped into point mutations, which are single base pair substitutions, and rearrangements, which include deletions and duplications. In addition mtDNA can become depleted. Mutations can affect mitochondrial protein synthesis genes for example tRNA genes, and protein coding genes, for example complex I mutations.

However, linking mtDNA mutations with particular diseases is not straight forward, as a particular genetic mutation may cause many different phenotypes and a single phenotype may be caused by many different mutations. MtDNA behaviour demonstrates threshold effects, heteroplasmy, and segregation, with resulting difficulties trying to define a genotype-phenotype relationship.

1.1.5.2 Mutation Rate and Repair Mechanisms

Due to its close proximity to the respiratory chain complexes, its exposure to reactive oxygen species, and a lack of protective histones, mtDNA has a very high mutation rate. In addition, the mtDNA POLG is substantially less accurate than the nDNA polymerase α (Kunkel and Loeb, 1981). All these factors result in a mtDNA error rate in higher primates of around ten times that of the nDNA (Brown et al., 1979). Adding to this mutation rate is the fact mtDNA is very efficient, which means any mutations are more likely to be present in coding regions and therefore pathogenic.

While it was originally thought that mammalian mitochondria lacked a DNA repair mechanism (Clayton et al., 1974) it has now been shown that this is not the case. Mitochondria undergo base excision repair (BER). The first step of which, is the cleavage of the glycosydic bond between the base to be removed and the sugar. Lyase activity then breaks the phosphodiester bond 3' to the site, and an endonuclease cleaves the 5' bond. The space created is then filled by DNA POLG, and the repair site sealed by DNA ligase III (Mandavilli et al., 2002). In addition, human mitochondria have recently been shown to have mismatch-repair (MMR) activity, that is independent from the nuclear MMR system and involves the protein YB-1 (de Souza-Pinto et al., 2009).

1.1.5.3 Point Mutations

MtDNA point mutations involve the substitution of a single base pair, are often maternally inherited, and frequently affect tRNA genes. They may be transversions, when a purine (A & G) is substituted for a pyrimidine (C & T), or vice versa. Alternatively they may be transitions which involve the substitution of a purine for another purine, or a pyrimidine for a pyrimidine. Because they can drastically alter the shape of a molecule, transversions are often more severe than transitions. If the resultant amino acid remains the same then it is also known as synonymous, if the amino acid is changed then it is a nonsynonymous mutation. The first point mutation to be implicated in a mitochondrial disease, was the m.11778G>A transition, which results in a nonsynonymous change of an arginine amino acid for a histidine and is needed for the LHON phenotype (Wallace et al., 1988).

1.1.5.4 Rearrangements

MtDNA rearrangements can be duplications of the genome or deletions of portions of the molecule.

1.1.5.4.1 Duplications

MtDNA duplications can consist of two delete mitochondrial genomes joined together (deletion dimers), or a delete molecule inserted into a WT one. They have been linked with sporadically occurring myopathies (Poulton and Holt, 1994), Pearson's syndrome (Rotig et al., 1995), and maternally inherited diabetes and deafness (MIDD) (Martin Negrier et al., 1998).

MtDNA duplications were first identified as being causative agents in mitochondrial disease in the 1980's, when restriction digest of blood mtDNA revealed linearised bands of 24.5kbp long, suggesting part of the mtDNA had been duplicated (Poulton et al., 1989). One study suggested they were primarily associated with Kearns-Sayre Syndrome (KSS). Investigations into eighteen patients with chronic progressive external ophthalmoplegia (CPEO) and KSS revealed all patients with KSS harboured both mtDNA deletions and duplications, while the CPEO patients contained only deletions. It was suggested that the relative proportion of these rearrangements present in the tissue accounts for the differing phenotypes of these two conditions (Poulton et al., 1994b).

It has been hypothesised that duplications are recombination intermediate forms that will eventually become deletions. This requires the delete molecule to be excised from the enlarged genome. This hypothesis has been put forward after results showing that the amounts of duplicated genomes reduce over time, coinciding with an increase in delete molecules (Poulton et al., 1993).

1.1.5.4.2 Single Deletions

MtDNA deletions have long been accepted as a major cause of mitochondrial disease (Holt et al., 1988), and are thought to represent around 18% of adults presenting with mitochondrial conditions (Schaefer et al., 2008). While original investigations often relied on restriction enzymes and Southern blots, there are now a whole host of methods to determine both the presence and levels of mtDNA deletions, including long range and real time PCRs.

While mtDNA deletions could potentially arise anywhere in the molecule, they tend to occur in the major arc region of the genome, between the two origins of replication O_H and O_L (Figure 4.13). They vary in size, but can remove up to the majority of the genome. The "common deletion" is a 4977bp deletion that removes mtDNA between nt.8469 and nt.13447. It is the most frequently reported mtDNA deletion and has been associated with both KSS and CPEO (Zeviani et al., 1998, Moraes et al., 1989a, Samuels et al., 2004).

The majority of deletions are flanked by short repeats, between 4-16bp long, one of which will be removed by the deletion. About 60% of deletions are flanked by perfect repeats and are termed class I deletions. Class II deletions are flanked by imperfect repeats and make up around 30% of deletions, while the remaining 10% are not flanked by repeats and are class III deletions (Samuels et al., 2004).

While mtDNA deletions have long been linked with mitochondrial disease, they have more recently been associated with aging phenotypes. MtDNA deletions have been shown to accumulate in ageing muscle, potentially leading to fibre breakage and eventual fibre loss. This process could be instrumental in the age related muscle loss observed in humans termed sarcopenia (Bua et al., 2006). Other studies have demonstrated mtDNA deletions in the substantia nigra of older control subjects (Bender et al., 2006) and patients suffering from neurodegeneration (Reeve et al., 2008).

1.1.5.4.3 Causes of Deletions

There is some debate regarding the exact cause of single mtDNA deletions. The finding that the majority of single deletions occur in the major arc and are flanked by repeats has led to the development of a "slip-strand" mechanism of formation. This suggests that the deletion occurs during the process of mtDNA replication via the asynchronous (or strand displacement) model. If during mtDNA replication the heavy strand is displaced, and its 3' direct repeat then anneals to the 5' direct repeat on the light strand, the excess DNA would loop out and eventually be degraded (Shoffner et al., 1989).

Problems with this model have been raised by Krishnan *et al.* (2008). The model's reliance on a strand displacement mode of replication does not fit with more recent evidence that mtDNA replication involves a strand asynchronous mechanism, with the incorporation of protective RNAs (Yasukawa et al., 2006, Holt et al., 2000). This prevents the regions of single stranded DNA that the slip-strand mechanism requires. In addition, not all mtDNA deletions are flanked by repeats, and some have been found which remove the 3' repeat, which is required to bind with the 5' repeat on the light strand (Reeve et al., 2008).

Krishnan *et al*. propose a model based on double stranded breaks (DSBs). A DSB allows 3' to 5' exonuclease activity on the DNA, which results in single strands. The 3' and 5' repeats then ligate, with the degradation of the unbound single strands (Krishnan et al., 2008).

1.1.5.5 Nuclear DNA Mutations

MtDNA encodes thirteen polypeptides, two rRNAs, and twenty two tRNAs needed by the mitochondria. The remainder of the machinery is encoded by the nuclear genome. Nuclear DNA mutations are also an important cause of mitochondrial disease. They may induce secondary mutations in the mtDNA for example causing multiple deletions, or they may affect mitochondrial function directly. As a result many mitochondrial disorders have been linked to defects in the nDNA.

TWINKLE is the 5' to 3' helicase which unwinds double stranded DNA during the process of mtDNA replication. Mutations in this gene have been linked to autosomal dominant progressive ophthalmoplegia (adPEO). These patients harbour multiple mtDNA deletions (Spelbrink et al., 2001). Another protein important for mtDNA replication is POLG, again with mutations causing multiple mtDNA deletions. Early reports of *POLG* mutations came from Van Goethem and colleagues, who mapped mutations to the *POLG* gene in patients with adPEO (Van Goethem et al., 2001). Since then numerous other mutations of *POLG* resulting in a wide variety of clinical conditions have been described (Horvath et al., 2006).

The adenine nucleotide translocator (ANT) is a homodimer gate responsible for the exchange of ATP for cytosolic ADP across the inner mitochondrial membrane, thus providing energy to the cytosol. Two mutations in the ANT1 isoform have been linked with adPEO in both a sporadic case, and in several families (Kaukonen et al., 2000). In the first report of a recessive case, a patient presented with myopathy, hypertrophic cardiac myopathy, lactic acidosis, and exercise intolerance, but no opthalmoplegia (Palmieri et al., 2005).

1.1.5.6 MtDNA Depletion

Again caused by nuclear defects, genetic mutations have been linked with mtDNA depletion, which is a severe reduction in the amount of mtDNA. Many sufferers present in infancy with muscle weakness and liver problems, possibly due to the high amounts of mitochondrial biogenesis that occur in these tissues during the initial years of life.

In 1991, Carlos Moraes reported the first case of mtDNA depletion syndrome in patients with autosomal recessive disorders. Southern blot analysis clearly showed the depletion

was segregated to the liver and muscle, and while they could not identify the responsible mutation, they did hypothesise it was nuclear in origin (Moraes et al., 1991).

Since then many genes have been associated with depletion syndromes, including *POLG* (Naviaux et al., 1999), thymidine kinase 2 (Saada et al., 2001), h-mtTFA (Poulton et al., 1994a), and deoxyguanosine kinase (*DGUOK*) (Freisinger et al., 2006). Interestingly, the depletion observed in myotubes with *DGUOK* mutations can be rescued with dAMP/dGMP supplementation (Bulst et al., 2009).

1.1.5.7 MtDNA Behaviour in Disease

The number of identified mutations in both the nDNA and mtDNA is growing, as is the list of diseases associated with these mutations. However, there is no clear link between a particular genotype and phenotype. While a single mutation may present in a variety of clinical conditions, a single disease may be caused by a range of mutations. There are a number of different factors that will affect the severity, tissue distribution, and age of onset of any mitochondrial disease.

1.1.5.7.1 Heteroplasmy and Homoplasmy

Unlike nuclear DNA, the mitochondrial genome exists in multiple copies within a single mitochondrion, and therefore in thousands of copies within a single cell. This can lead to a phenomenon termed heteroplasmy. This was noted when mtDNA mutations were first linked with mitochondrial disease. Holt and colleagues noted a patient harbouring two populations of mtDNA, one WT and one that was “partially deleted” (Holt et al., 1988).

Normally all copies of mtDNA within a cell are the same, a situation termed homoplasmy. However the mitochondrial genome is prone to mutations and if the genomes within a cell are different it is said to be heteroplasmic (Figure 1.13). A homoplasmic cell could consist of either 0% or 100% mutant genomes, a heteroplasmic cell will contain anything in between. Heteroplasmy is normally defined as the percentage of mtDNA with a mutation. A disease causing mtDNA mutation can be either homoplasmic or heteroplasmic. While heteroplasmy and homoplasmy are usually referred to in relation to disease causing mutations, a recent study has demonstrated that many people are heteroplasmic for mitochondrial polymorphisms. Furthermore, these changes increase with age (Sondheimer et al., 2011).

When cells divide during mitosis, the mutant genomes can segregate in any ratio to the two daughter cells. This can result in marked variations between different tissues in terms of how much mutation they contain, and therefore result in varying phenotypes and age of onsets in patients with the same genetic mutation. The heteroplasmy levels are very important for patients as they have been linked to the frequency of clinical features (Chinnery et al., 1997).

It is difficult to obtain a general consensus on how heteroplasmy levels change in a patient over time. It is well known that mutations may be lost, and heteroplasmy levels changed over time in rapidly dividing tissues, for example blood. However, it is harder to examine changes in post mitotic tissue, such as in the brain or muscle. One group has reported an increase from 70% to 90% in a tRNA mutation in the muscle of a mitochondrial myopathy patient over twelve years (Weber et al., 1997). However, a patient in our lab with the "common deletion" has remained at approximately 70% heteroplasmy over thirteen years (unpublished observations).

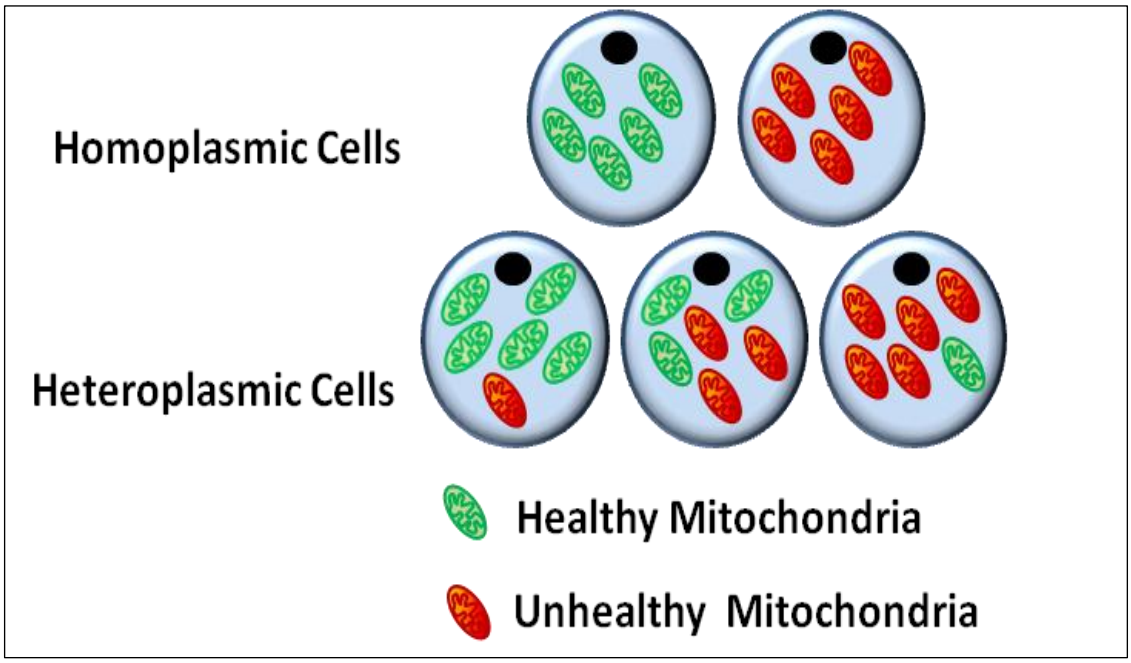

Figure 1.13 Heteroplasmy and homoplasmy. The multi copy nature of mtDNA can result in cells containing mixtures of WT and mutated mtDNA. If all the copies of mtDNA in a cell are the same then the cell is said to be homoplasmic. If the copies differ then the cells are heteroplasmic. Heteroplasmy is quite common in mitochondrial disease, where patients often harbour a mixture of WT and mutated mtDNA.

1.1.5.7.2 Threshold

The presence of heteroplasmy in a cell leads to another interesting phenomenon in mitochondrial genetics, that of threshold. A decrease in mitochondrial function caused by mutant mtDNA can initially be compensated for by the WT mtDNA in the mitochondria or even through an increase in glycolysis. This is partly attributable to the excess of enzymes, mRNAs, tRNAs, and other factors required by the mitochondria for normal function. Eventually the healthy mitochondria are unable to compensate further and at this point a biochemical defect is expressed in the cells (Figure 1.14) (Rossignol et al., 2003). The amount of delete DNA required for the biochemical defect to be observed is the threshold level and is given as a percentage of total mtDNA.

The threshold varies depending on the organ affected, which is partly attributable to the differing energy requirements of the tissue. In addition, different complexes of the respiratory chain appear to have different thresholds in different tissues, so depending on the complex affected by the mtDNA mutation, the tissue may be more or less affected (Rossignol et al., 1999).

The tissue specificity effect was nicely demonstrated by Greaves and colleagues, who examined threshold levels in extra-ocular muscles (EOM) and the *vastus lateralis*, in patients with CPEO. The EOMs demonstrated greater numbers of COX deficient fibres, which was caused by a lower level of heteroplasmy than needed in the *vastus lateralis* muscle fibres to elicit the same COX deficiency. They also noted that a greater number of WT mtDNA molecules were needed in the EOM to maintain COX activity, suggesting that the percentages of WT and delete molecules may not be as important as the absolute WT mtDNA copy number. The greater metabolic demand of the EOM was hypothesised to account for the different threshold values (Greaves et al., 2010b).

The threshold level also varies with mutation type. With the m.8344A>G mutation linked to myoclonic epilepsy with ragged red fibres (MERRF), it is around 90% (Shoffner et al., 1990), while a report examining the m.3243A>G mutation suggested it is between 50-65% (Jeppesen et al., 2006a). Threshold values between 50-85% have been reported for mtDNA deletions (Porteous et al., 1998, Sciacco et al., 1994, Rossignol et al., 2003).

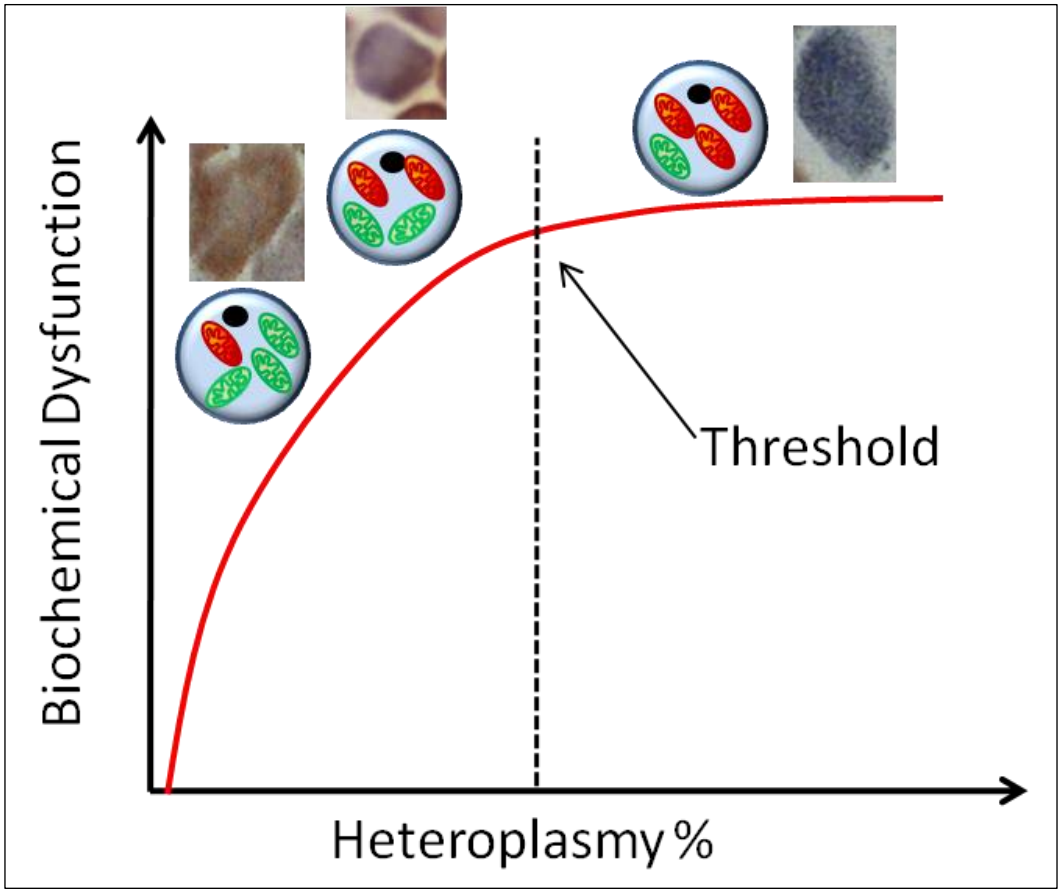

Figure 1.14 Threshold. Heteroplasmy within a cell leads to a threshold effect. While a small amount of mutant mtDNA can be tolerated by the cell due to a functional redundancy, as the levels increase the cell is unable to compensate further and a biochemical defect is displayed. This can be shown with complex IV histochemistry (see section 2.2.5.2); a small amount of mtDNA mutation still yields a brown product in a muscle fibre, demonstrating no biochemical defect. Once the threshold level is passed the cell can no longer cope and the muscle fibre appears blue, suggesting an absence of complex IV activity.

1.1.5.7.3 The MtDNA Bottleneck

The first experimental evidence of an mtDNA bottleneck was provided by Hauswirth and Laipis (1982). They examined segregation of an mtDNA polymorphism that introduced an extra *HaeIII* restriction enzyme site in the mitochondrial genome. Two mitochondrial genotypes, on the basis of this polymorphism, were shown to exist in the progeny of a single female cow. While they considered the possibility of mutations, and paternal leakage, their conclusions were that an mtDNA bottleneck had caused the variations in genotype observed in the female cow's descendants. If the original cow had been heteroplasmic for the polymorphism then amplification of a few of her mtDNA copies could create differing genotypes (Hauswirth and Laipis, 1982).

The bottleneck serves an important evolutionary function, as it reduces the number of mutations that can be inherited from a mother to child. The mtDNA is prone to mutations and without a bottleneck these would be passed on to the child. In terms of mitochondrial disease the bottleneck is important for patients as it can result in marked differences between mothers and children in their heteroplasmy levels (Figure 1.15) (Larsson et al., 1992, Blok et al., 1997). This makes giving advice about pregnancy to women with mitochondrial mutations somewhat difficult.

There is ongoing debate regarding the exact mechanism of the mtDNA bottleneck. Cao *et al.* (2007) have suggested it occurs through the aggregation of mtDNA into segregating units which would be inherited. A year later Cree *et al.* (2008) proposed it was attributable to a decrease in mtDNA copy number followed by segregation of the remaining genomes. Finally, Wai *et al.* (2008) have suggested it is attributable to selective amplification of a subpopulation of mtDNA. These different theories are discussed further in chapter 6.

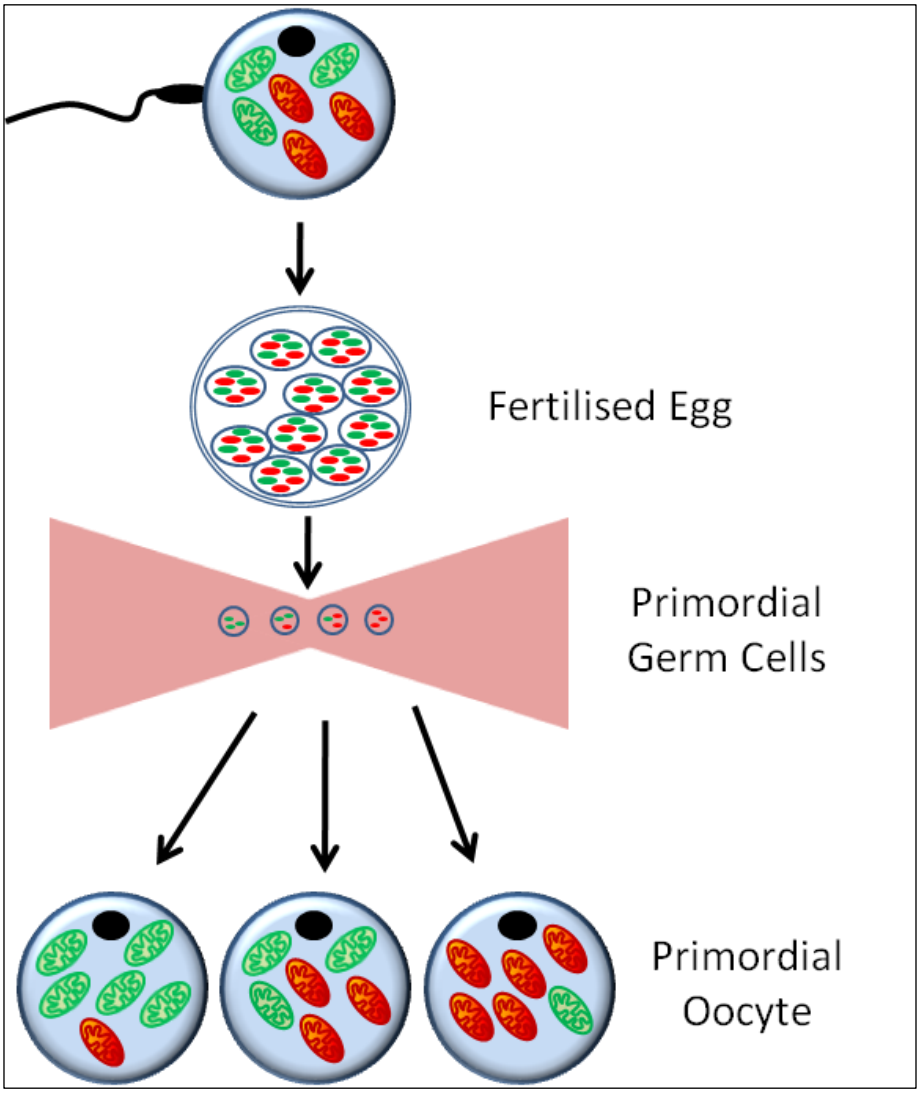

Figure 1.15 The mtDNA bottleneck. Fertilization of a heteroplasmic oocyte can result in a bottleneck effect. Amplification of a small population of genomes can result in extremely varied heteroplasmy levels in terms of mtDNA mutations in resultant cells, and consequently in progeny.

1.1.5.7.4 Clonal Expansion

Clonal expansion is the amplification of mtDNA mutations within tissues. Through this mechanism a mutation in a single mtDNA can increase within either a mitotic or post mitotic cell. If the level of the mutation supersedes the threshold value then the cell will demonstrate a biochemical defect. If a mutation has segregated at birth to many cells then it may eventually expand to cause problems in the tissue.

An early hypothesis for the mechanism of clonal expansion suggested that mtDNA containing a deletion was able to replicate faster than a full size WT mtDNA (Wallace, 1992). This hypothesis gained some backing when it was shown that mtDNA containing a deletion was able to repopulate cells faster that WT mtDNA following a depletion assay similar to that described in chapter 6. This pattern, however, was not observed for mtDNA harbouring a point mutation (Diaz et al., 2002). Evidence against this smaller replicating molecule hypothesis comes from findings that the whole replication process is complete in under two hours, which is shorter than the time between replications (Clayton, 1982).

A mechanism that would explain both the clonal expansion of mtDNA deletions and point mutations is the “sick mitochondria” theory. This involves mitochondria with a respiratory chain defect proliferating in order to compensate for their impairment. This was suggested after experiments revealed the preferential replication of mtDNA with the m.3243A>G mutation over WT mtDNA (Yoneda et al., 1992).

Another possible theory is random genetic drift. MtDNA is subject to genetic drift, with molecules being chosen randomly to replicate (Birky, 1994). Using a computer simulation to model random drift in post mitotic cells, Elson *et al.* (2001) demonstrated that relaxed replication was sufficient to result in clonal expansion (Elson et al., 2001b).

An interesting field of research is currently examining the role of clonal expansion in ageing. As people age they accumulate mtDNA mutations in different tissues, including the colon (Greaves et al., 2010a), substantia nigra (Bender et al., 2006), and muscle (Bua et al., 2006). While these mutations may be at low levels in the tissue, within individual cells a mutation may clonally expand until a COX deficiency is observed.

1.1.6 Mitochondrial DNA Diseases

1.1.6.1 Epidemiology

Mitochondrial disease has historically been under diagnosed. The wide variety of clinical presentations, numerous causative genetic mutations, and non-Mendelian mode of inheritance, has resulted in mitochondrial disease not receiving adequate focus. Improvements in diagnostic tests, greater public awareness, and improved inter centre collaborations, have all helped provide a clearer picture regarding both the scale of mitochondrial disease and the requirements of its sufferers.

A recent paper found that one in every two hundred people harbour a potentially pathogenic mtDNA mutation (Elliott et al., 2008). Thankfully, the number of people in which these mutations cause disease is considerably less.

An early study examining the prevalence of the m.3243A>G mutation in a population of Finnish people found a one in six thousand occurrence of this particular point mutation (Majamaa et al., 1998). A later investigation examining the prevalence of mitochondrial disease in the North East of England suggested that one in ten thousand people have a symptomatic mitochondrial disease. Additionally, it was estimated a further one in six thousand children and adults are at risk of developing a condition (Schaefer et al., 2008).

While the contribution of mitochondrial disease to morbidity is being increasingly recognised, the rate at which new mutations are being found suggests these numbers will increase further.

1.1.6.2 Investigating Mitochondrial DNA Disease

The diagnosis of mitochondrial disease can be somewhat problematic due to the multisystem nature of the condition. Initial investigations into suspected cases require extensive clinical assessment, with symptoms and family history being important considerations.

Following this, blood tests can be performed to examine creatine kinase (CK), lactate, glucose levels, and blood counts. While some conditions can be diagnosed through such tests, a negative result does not preclude a mitochondrial disease.

The gold standard for diagnosing mitochondrial disease is a muscle biopsy. This allows for histochemical, molecular, and biochemical analysis of the muscle. Histological analysis

often includes the cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) COX/SDH, analysis, which is explained further in chapter 2. When this staining is performed all fibres with a low or absent amount of mutated mtDNA will stain positive for COX (brown precipitate). If a fibre has a COX deficiency it will not pick up the stain. Further incubation with SDH will stain all the COX deficient fibres blue. This gives the muscle section the characteristic mosaic pattern of brown and blue fibres (Figure 1.16A). Another stain occasionally used is Gomori trichrome; this picks up subsarcolemmal accumulations of mitochondria, and stains them red (Figure 1.16B). These fibres demonstrate a characteristic red colour and are termed ragged red fibres (RRFs) (Taylor et al., 2004).

Once suspected conditions have been narrowed down, molecular investigations can be performed to determine the exact genetic defect. The genetic mutation sought is very much determined by the clinical and muscle biopsy findings. Tests performed may include Southern blot and long range PCR to look for mtDNA rearrangements, or genetic sequencing to look for point mutations (Chinnery and Turnbull, 1997).

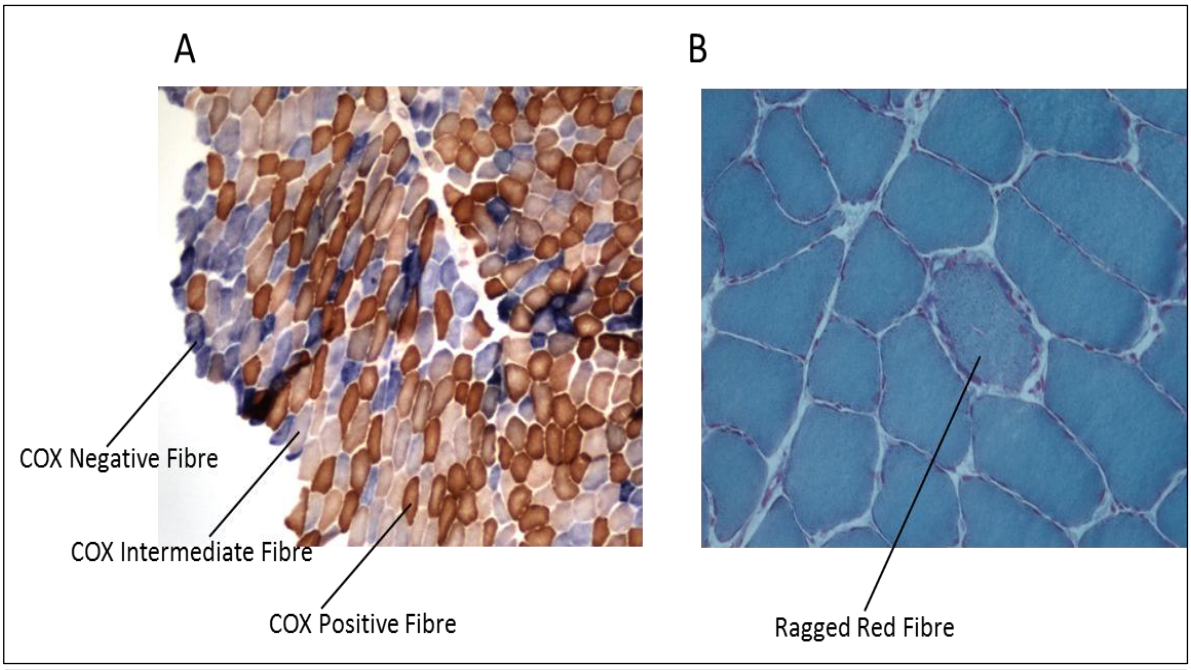

Figure 1.16 Diagnostic muscle biopsies. **A**. Performing the COX/SDH histochemistry protocol on a biopsy from a patient with a mitochondrial disease may reveal a mosaic pattern of staining. With brown fibres indicating normal mitochondrial function (COX positive fibre), and blue fibres revealing impaired mitochondrial function (COX negative fibre). COX intermediate fibres are often visible, this corresponds to intermediate levels of mitochondrial function. **B**. A Gomori trichrome stain can reveal muscle fibres that have an abnormal subsarcolemmal accumulation of mitochondria, this appears red, and earns them the name ragged red fibres (RRF). Gomori trichrome image supplied by Gavin Falkous.

1.1.6.3 MtDNA Diseases

Due to the ubiquitous nature of mitochondria, a disease can present in a plethora of different ways, with just about any system being vulnerable (Figure 1.17). Neurological presentations are quite common, but patients may also present with cardiomyopathy, liver failure, short stature, and a whole host of other symptoms. The next few pages will describe some of the common conditions associated with sporadically occurring single mtDNA deletions, which is the main mitochondrial mutation investigated in this research. In addition, a few other conditions will be described, demonstrating the wide clinical and genetic heterogeneity associated with mitochondrial disease.

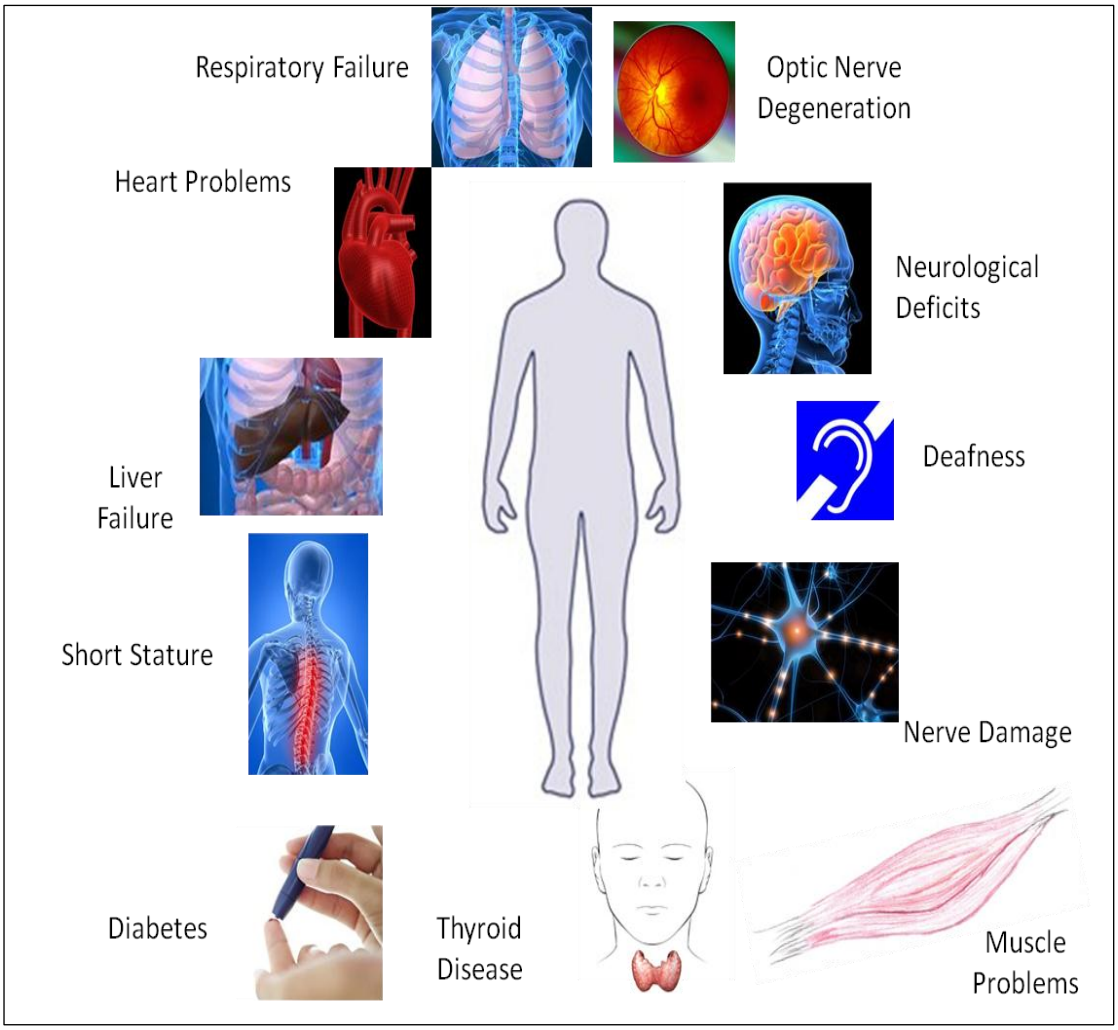

Figure 1.17 Mitochondrial disease phenotypes. While neurological symptoms of mitochondrial disease may predominate, just about any system in the body can be affected. This can result in delays in diagnosing mitochondrial disease, as patients are referred to various specialists for investigations.

1.1.6.3.1 Chronic Progressive External Ophthalmoplegia (CPEO)

A common presentation of mitochondrial disease, and the condition the patients in this study suffer from, is CPEO. Many CPEO patients harbour sporadically occurring single mtDNA deletions (Laforet et al., 1995), although it has also been associated with nuclear mutations resulting in secondary mtDNA mutations, and with mtDNA point mutations. While it can occur in combination with other symptoms, it may sometimes be given as a diagnosis in itself.

The condition usually presents in the third or fourth decade of life and is caused by a progressive weakening of the extra-ocular muscles, resulting in a restriction of the eye movements. Patients will also experience ptosis, a drooping of the eye-lids (Laforet et al., 1995). As the condition progresses patients may start to experience a more general myopathy, with exercise intolerance, muscle wasting, and weakness (Zeviani and Donato, 2004).

1.1.6.3.2 Kearns-Sayre Syndrome (KSS)

KSS is a multisystem disorder that has traditionally been characterised by the "triad" of CPEO, pigmentary retinopathy, and onset before the age of twenty. Although proximal myopathy, cerebella ataxia, cardiomyopathy, and heart block may also occur. Muscle biopsies may demonstrate RRF and COX deficiency. While often linked to a sporadically occurring large scale single mtDNA deletion or duplication, other mutations have also been implicated (Pistilli et al., 2003, Zeviani and Donato, 2004).

1.1.6.3.3 Pearson Marrow-Pancreas Syndrome

This disease is a multisystem condition, presenting very early in life. Symptoms include vacuolization of marrow precursor cells, reduction of blood cells, sideroblastic anemia, and pancreatic dysfunction. It has been associated with both deletions and duplications of the mtDNA. Interestingly, if children are able to survive infancy they may proceed to develop a KSS phenotype (Rotig et al., 1995, Simon and Johns, 1999).

1.1.6.3.4 Mitochondrial Encephalopathy Lactic-Acidosis and Stroke-Like Episodes (MELAS)

MELAS is one of the commonest mitochondrial disorders (Chinnery and Turnbull, 2001). It is characterised by encephalopathy, stroke-like episodes caused by brain lesions, and lactic acidosis. Other neurological symptoms may be present including dementia and hearing problems.

It is most commonly associated with the maternally inherited m.3243A>G point mutation in the $tRNA^{Leu(UUR)}$ gene. It was first identified by Goto *et al.* (1990) when they found the mutation in twenty six out of thirty one patients with MELAS (Goto et al., 1990). However, other mutations have also been implicated in this phenotype (Morten et al., 1993, Manfredi et al., 1995, Horvath et al., 2008b). All of these mutations were heteroplasmic and present in highly conserved nucleotide positions.

1.1.6.3.5 Myoclonic Epilepsy with Ragged Red Fibres (MERRF)

MERRF sufferers have a progressive myoclonus, epilepsy, cerebellar ataxia, and myopathy. Occasionally associated with MERRF are lipomas. These are benign tumours of adipose tissues that have a tendency to accumulate in the trunk and cause severe discomfort to sufferers. Muscle biopsies commonly demonstrate RRFs, attributable to the accumulation of mitochondria. It is frequently associated with the maternally inherited, heteroplasmic m.8344A>G mutation in the tRNA for lysine (Shoffner et al., 1990, Zeviani and Donato, 2004).

1.1.6.3.6 Leber's Hereditary Optic Neuropathy (LHON)

One of the first diseases to be linked to an mtDNA point mutation, and indeed the commonest mitochondrial disease, is LHON (Chinnery and Turnbull, 2001). Affected individuals suffer a loss of central vision due to the degeneration of the retinal ganglions cells.

The condition has been linked to three mitochondrial mutations, m.3460G>A, m.11778G>A, and m.14484T>C. The first of these mutations was found by Wallace *et al.* (1988). They reported the m.11778G>A point mutation and noted that it was necessary but not sufficient to cause the disease (Wallace et al., 1988). Since then investigations into this condition have focused on finding the disease modifier that is required for full phenotypic expression.

The condition is maternally inherited but there is a propensity for males to be affected. This has resulted in suggestions that a modifier may be present on the X chromosome. Other factors investigated as possible co-conspirators are mtDNA haplogroup, nuclear genes, and hormones, in addition to a number of potential environmental factors including smoking, alcohol, head trauma, and chemical toxins (Yu-Wai-Man et al., 2011). However, the culprit still remains elusive.

1.1.6.4 Treatments and Therapies for Mitochondrial Disease

Strides have been taken in recent years in both the diagnosis of mitochondrial disease and in understanding the pathological mechanisms underlying the conditions. However, treatments and therapies are still somewhat limited and geared toward symptomatic relief.

1.1.6.4.1 Symptomatic Relief

Surgery can help with some of the symptoms patients suffer from. CPEO patients with ptosis may have their eye lids lifted, lipomas may be excised from patients with MERRF, and pacemakers can be implanted in KSS patients with heart block. As a last resort organ transplantation can be performed. The diabetes that many MELAS and KSS patients suffer from can be managed, as can the seizures that are also common (Schon and DiMauro, 2003, Horvath et al., 2008a).

1.1.6.4.2 Genetic Counselling

Genetic counselling is particularly helpful for patients wishing to have children. However, it can be very difficult to give definite answers on the probability of a condition being passed on. For example large scale single mtDNA deletions are often sporadically occurring and will not be transmitted, and since mitochondria are maternally inherited, defects of mtDNA in men will not be passed on. Knowledge about the type of mutation, heteroplasmy level, and probability of the mutation being transmitted will help clinicians' counsel women and their partners. This highlights the necessity of further research identifying and examining the behaviour of the genetic mutations that cause mitochondrial disease.

1.1.6.4.3 Preventing Transmission

One area of therapy that is making rapid developments is the prevention of transmission of mitochondrial diseases. Pre-implantation genetic diagnosis involves the removal of a cell from the embryo and testing for the genetic defect, before selecting which embryos to put back. However, this decision can become difficult when embryos with low levels of mutation are generated. While the heteroplasmy is low, segregation of the mutation may still result in a symptomatic child. Prenatal diagnosis can also be performed using chorionic villus sampling or amniotic fluid. However, there are still questions regarding how representative these tissues are in the foetus (Poulton et al., 2009).

One avenue currently being explored by Craven *et al.* (2010) is pronuclear transfer. This involves the removal of the pronucleus from the fertilised egg and its transfer into another fertilised egg that has had its nucleus removed. This leaves behind the mitochondria that contain the mutation. Current investigations into this treatment involve determining how much "carry-over" of mitochondria there is from the original egg cell, and ensuring the technique is compatible with onward embryo development.

1.1.6.4.4 Genetic Manipulation

This approach is centred on changing the amounts or balance of WT and mutant mtDNA. Many attempts have been made to get WT mtDNA into the mitochondria, but the ability to scale up to the cellular and tissue level is still problematic. An alternative is to inhibit the replication of mutated mtDNA, thus allowing WT mtDNA to dominate within the cell, hopefully bringing the cell below threshold. However, these approaches are still to come to fruition (Chinnery and Turnbull, 2001).

Another strategy to redress the imbalance of WT and mutated mitochondrial in patients with myopathy is resistance training. The theory behind this is discussed further in chapter 4, but briefly, it involves the activation of muscle stem cells through high intensity exercise that damages the muscle. Through the incorporation of new muscle cells that have lower or no mtDNA mutations the heteroplasmy level in the muscle will be favourably changed (Gardner et al., 2007).

1.2 Muscle

Skeletal muscle is responsible for locomotion, postural stability, and assists with breathing. It's remarkable structure and contractile ability allows it to perform these functions. When through injury, disease, disuse, or atrophy it ceases to work as efficiently, the consequences are disabling.

1.2.1 Muscle Structure

Muscle is organised into compartments by connective tissue. The muscle belly is surrounded by the epimysium, which separates it from other muscles. Within the muscle are many fascicles; these are bundles of muscle fibres that are enclosed by a perimysium. This allows the major blood vessels and nerves to penetrate the muscle between these fascicles. Surrounding the individual muscle fibres is the endomysium, and running between the fibres are capillaries (Figure 1.18A).

Muscle fibres are composed of cylindrical structures called myofibrils and in mammals, are around 50-100μm in diameter. Surrounding the myofibrils are mitochondria, sarcoplasmic reticulum, and the t-tubule system. The myofibrils appear striated due to being comprised of sarcomeres, the contractile unit of the fibre. It is the number of sarcomeres that determines the length of the fibre, with each sarcomere being between 2-2.5μm long at rest.

The sarcomeres are separated from each other by a Z disk. These provide anchors for the thin filaments, which are composed of pairs of polymerised actin monomers. In addition, the thin filaments contain tropomyosin, a long filament protein lying in the groove created by the actin, which in turn contains troponin molecules attached to the filament. Between the thin filaments sit the thick myosin filaments. These are composed of myosin molecules, containing two globular heads attached to a long tail formed by two coiled α-helices. The interaction between these two sets of filaments causes muscle contraction (Figure 1.18B) (Jones et al., 2009).

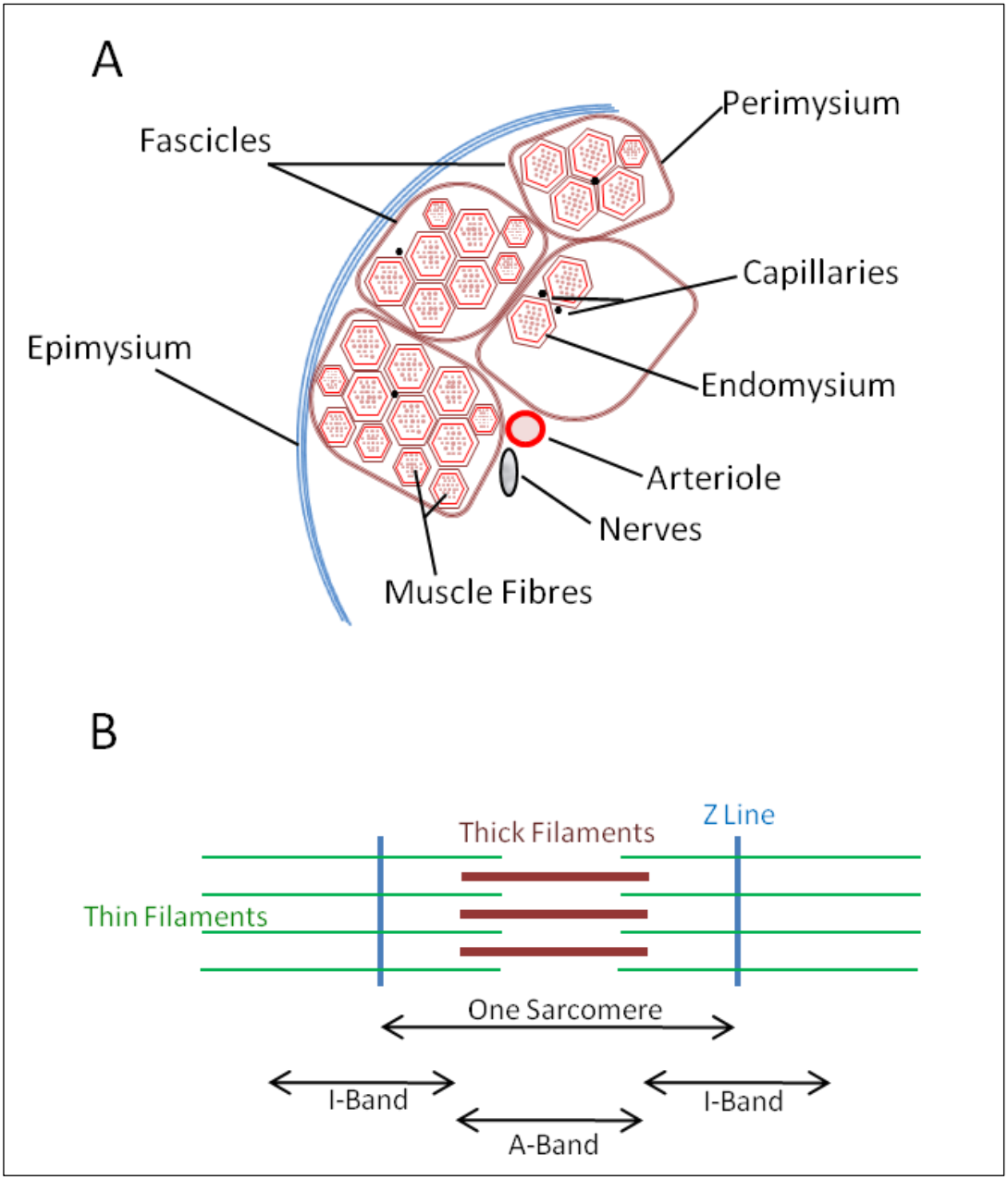

Figure 1.18 Muscle structure. **A**. Muscle is bound by connective tissue, mostly made up of collagen. The epimysium surrounds the whole muscle, then the perimysium divides the fibres up into fascicles and finally each fibre is surrounded by an endomysium. **B**. The contractile unit of the muscle fibre is the sarcomere. Z-lines delineate the boundaries of the sarcomere, and provide anchor points for the filaments. The "sliding filament" hypothesis of muscle contraction states that the thin filaments, composed of actin, slide over the thick myosin filaments and the sarcomere reduces in size.

1.2.2 Contraction

Contraction of muscle occurs by a sliding filament mechanism. This hypothesis was first proposed by Huxley and colleagues back in the 1950s when experiments using the frog revealed a shortening of the observed striations (Huxley and Hanson, 1954, Huxley and Niedergerke, 1954).

When an action potential arrives at the neuromuscular junction it stimulates the release of acetylcholine (Ach) from the endplate. When Ach binds to the receptors on the post synaptic membrane it stimulates the opening of voltage gated sodium channels and an action potential is generated in the muscle fibre. This passes through the sarcolemma which encloses the muscle fibres, and moves into the t-tubule system. The t-tubule system penetrates the muscle fibre and conducts the impulse from the surface of the fibre to its interior. The depolarisation of the t-tubules causes Ca^{2+} release from the sarcoplasmic reticulum which surrounds the myofibrils.

The Ca^{2+} binds to the troponin on the thin filaments which causes a conformational change in the filament and exposes the myosin binding sites on the actin. The myosin heads bind to these sites and then pivot, which draws the thin filaments in. The dissociation of myosin from the actin requires the hydrolysis of a molecule of ATP. In order to terminate the contraction, the muscle fibre pumps Ca^{2+} back into the sarcoplasmic reticulum (MacIntosh et al., 2006).

1.2.3 Embryological Origin

Skeletal muscle originates from the somites that have divided off from the mesoderm. The somites then segment further into the sclerotome, which forms the vertebrae and cartilage, and the dermomyotome which will give rise to the dermis, and skeletal muscles. The epaxial myotome forms the muscles of the back, while the hypaxial myotome forms the muscles of the rest of the body. Muscle progenitor cells split from the dermomyotome to form the myotome (Figure 1.19). Progenitors migrate from both the dorsomedial and ventromedial lip of the somite, as well as the caudal and rostral borders (Gros et al., 2004).

The formation of the myotome is dependent on Pax3, and the myogenic regulatory factors Myf4 and 5. The cells then proliferate and are expressing MyoD and Myf5. With

the up-regulation of myogenin they differentiate into myotubes (Buckingham et al., 2003, Buckingham, 2007).

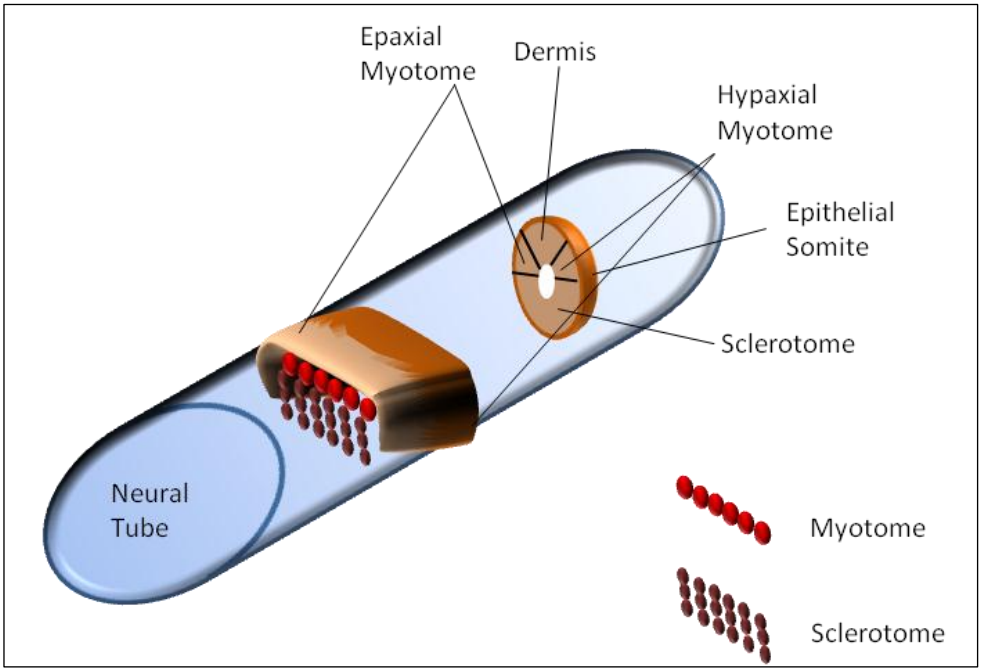

Figure 1.19 Muscle origins. Skeletal muscle originates from the mesoderm which segments into somites. These then give rise to muscle progenitor cells that split from the dermomyotome to form the muscle of the trunk and limbs.

1.2.4 Muscle Damage and Repair

Muscle is a post mitotic tissue, and as such there is minimal turnover of nuclei. However, it does have the remarkable ability to repair itself in response to damage caused by injury or disease.

The initial stage of repair is necrosis of the muscle fibre, which is helped by the disruption of the fibre sarcolemma. Increased calcium entry into the cell activates proteases that break down the structural proteins and lipid membranes (Jones et al., 1984). To clear up debris caused by the injury, inflammatory cells are mobilised. Neutrophils are first to respond to chemotactic signals released by the damaged fibre. Following neutrophil invasion of the area, macrophages arrive to clear up the debris using phagocytosis. One of the indicators of muscle damage is CK; this is released from the fibres after disruption and can often be detected in the blood (Charge and Rudnicki, 2004).

After the degeneration of the muscle the next stage is regeneration. This is mediated by muscle stem cells (satellite cells).

1.2.5 Satellite cells

Satellite cells were first reported by Mauro (1961), after using EM to study muscle from frog. Satellite cells are mononucleated cells that sit under the basal lamina and on top of the sarcolemma. They protrude inwards into the muscle fibre and have been named based upon their location (Mauro, 1961). They have very little cytoplasm, and while they do have mitochondria, these are few in numbers, generally rounder, paler, and harbour fewer cristae than those in muscle (Roth et al., 2000).

1.2.5.1 Satellite Cell Origins

Satellite cells have the same embryological origins as skeletal muscle, in that they both derive from the somites. Gros *et al.* (2005) used electroporation to insert green fluorescent protein (GFP) into chick somites along with quail-chick grafting techniques to map the development of muscle precursor cells. They demonstrated that a population of cells translocated from the central dermomyotome into the myotome. The movement of the cells was preceded by a cell division, with one cell moving into the myotome and the other remaining in the dermomyotome (Gros et al., 2005).

It is difficult to distinguish satellite cells from other myogenic cells during development. It is only after they have moved into position and have a basal lamina formed over them,

that they can be truly identified by their location. In mice the satellite cells move into position by E10.5, and the basal lamina forms between E16.5 -18.5 (Relaix et al., 2005). This is quite late considering the gestational time for mice is around 19-21 days.

1.2.5.2 Satellite Cells and Muscle Repair

Satellite cells are responsible for post natal muscle growth and regeneration (Figure 1.20). Within adult muscle, satellite cells account for between 1% to 7% of muscle nuclei (Kadi et al., 2005), although the levels may be much higher in postnatal muscle while it is still developing.

Before activation, satellite cells are quiescent and have exited from the cell cycle. While quiescent they are expressing the transcription factor paired box 7 (Pax7), and in some muscles Pax3. In addition many quiescent cells also express the basic helix-loop-helix myogenic regulatory factor 5 (Myf5), the calcium-dependent cell adhesion molecule (M-cadherin), the hepatocyte growth factor receptor (c-met), and the neural cell adhesion molecule (NCam) also known as CD56/Leu19 (Negroni et al., 2006).

After muscle injury, satellite cells are activated through signals received from the damaged muscle fibre and possibly from the inflammatory cells that have been tasked with cleaning up the remnants of the damage. They start to proliferate and are then referred to as muscle precursor cells or myoblasts. At this stage they are expressing Pax7, Myf5, and myogenic differentiation factor 1 (MyoD). Their remarkable proliferative ability was demonstrated by single myofibre grafts into radiation-ablated mouse muscle. A single fibre containing as few as seven satellite cells was able to generate over one hundred muscle fibres, in addition to repopulating the host muscle with new satellite cells (Collins et al., 2005).

The myoblasts then head towards differentiation, which involves their fusion into long cells referred to as myotubes. At this stage myogenin becomes important, and Pax7 is now down regulated (Olguin and Olwin, 2004).

Satellite cells are the muscle stem cell and as such need to maintain an adequate supply for the muscle. After activation the cells divide asymmetrically and one of the resultant daughter cells returns to quiescence under the basal lamina after exiting from the cell cycle. This cell will continue to express Pax7, but will down regulate MyoD (Zammit et al., 2004).

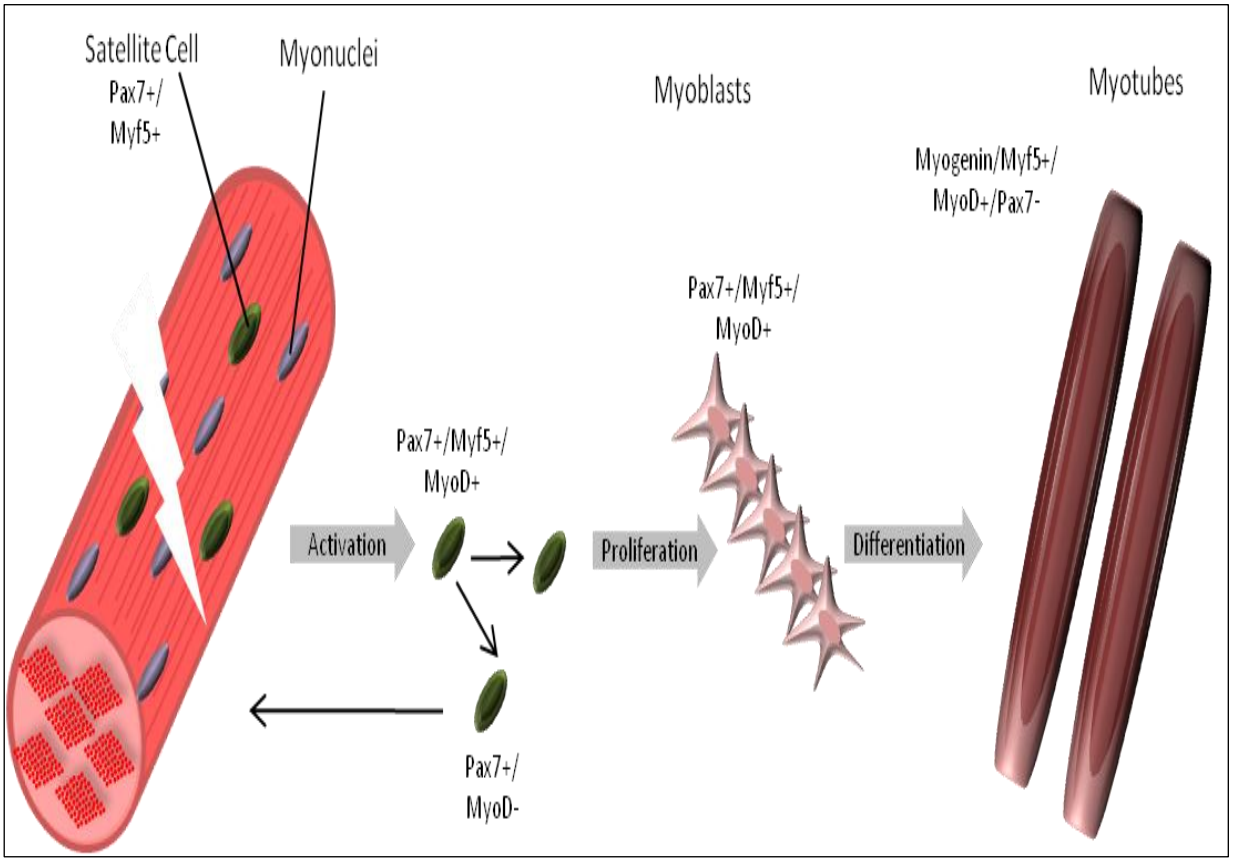

Figure 1.20 Muscle regeneration. In response to injury satellite cells are activated. They express Pax7, Myf 5, and MyoD. After division one daughter cell will down regulate MyoD and return to quiescence. The other will continue to express MyoD and proliferate. With the induction of myogenin, the myoblasts commence differentiation into myotubes, which are able to fuse into the damaged muscle fibre to repair it.

1.2.5.3 Satellite Cell Heterogeneity

There have been suggestions that other cells may contribute to the satellite cell pool. Satellite cells cultured from mouse muscle have been shown to possess characteristics of bone-marrow derived hematopoietic stem cells, and are thought to arise from the hematopoietic system (Jackson et al., 1999, McKinney-Freeman et al., 2002). While other stem cells have been shown to have myogenic potential, including mesoangioblasts (Sampaolesi et al., 2003), and adult bone marrow derived cells (LaBarge and Blau, 2002). However, while these cells may localise to the satellite cell position and display some characteristics of muscle progenitor cells, they are not innately myogenic and may require modification in order to contribute to regeneration (Sherwood et al., 2004, Meng et al., 2010).

While many experiments examining satellite cells have used mice, some caution must be observed. Studies have suggested that adult muscle fibres from both mice and humans have similar numbers of satellite cells, but the markers used to identify them may be species dependent. For example, CD34 marks satellite cells in mice but not in humans, and NCam is expressed in quiescent satellite cells in humans but is only present in activated cells in mice (Tedesco et al., 2010, Boldrin et al., 2010).

1.2.5.4 Satellite Cells as Therapy

One particular muscle disease where the potential therapeutic use of satellite cells has been extensively investigated is Duchenne's Muscular Dystrophy (DMD). This is a severe X-linked recessive muscular dystrophy that affects about one in three and a half thousand male births. It is caused by a defect in the gene for dystrophin (Hoffman et al., 1987), which is an important protein for plasma membrane integrity. The protein is important in linking the muscle fibre cytoskeleton to the extracellular matrix. Disruption of dystrophin results in tears in the sarcolemma which allow Ca^{2+} into the cells, with the damaged fibre undergoing necrosis. The injured fibre then requires satellite cells to repair it. However, the repaired fibre will still lack the dystrophin protein and there will be subsequent cycles of degeneration and regeneration.

For this reason, the possibility of using donor myogenic cells has been extensively researched. This involves injecting expanded myoblasts from non-infected individuals into the muscle of sufferers. Problems associated with this technique are rejection by the host, lack of migration of the introduced cells, fusion of the cells into host muscle, and

replenishment of the satellite cell pool. Although it has been shown that immunocompromising the host muscle improves migration, rejection, and integration of the introduced myoblasts (Smythe et al., 2000). In addition it has been shown that transplanting human foetal muscle precursor cells into mice can result in functional satellite cells that repopulate the stem cell compartment and can form functional myoblasts (Ehrhardt et al., 2007). More recently, irradiating the host muscle aided the restoration of dystrophin in both young and old mdx mice by donor satellite cells (Boldrin et al., 2009). However, the problem of delivering myoblasts throughout the body still remains.

Another dystrophy that may benefit from satellite cell derived progeny is fascio-scapulo-humeral muscular dystrophy. This condition commonly affects the face, shoulder, and upper arm muscles, with sparing of the lower body. This has opened up the possibility of injecting myoblasts derived from the patients themselves into the affected muscles to restore function. The small areas affected and use of patient tissue would overcome problems of rejection and migration faced by attempts to transplant muscle precursor cells in other dystrophies (Cooper et al., 2006).

The research described in this project is also investigating the use of muscle satellite cells to ameliorate a muscle condition. Mitochondrial myopathies are a common feature of mitochondrial disease, with patients often presenting with proximal weakness and exercise intolerance. Patients may progress to display other organ involvement or the condition may be confined to muscle, with more distal muscles becoming involved. Other symptoms may include muscle pain, cramps, and stiffness. These symptoms are caused by the inability of the mitochondria to generate the energy required by the muscles. The satellite cell mediated treatment for mitochondrial myopathies involves the replacement of dysfunctional mitochondria with WT ones through the incorporation of myoblasts with healthy mitochondria into the muscle. This approach involves using the patient's own satellite cells to repair the muscle and as a result there will be no problems with host rejection or the generation of new muscle stem cells.

1.3 Aims of this Research

Mitochondrial DNA mutations have been shown to be a significant cause of disease. Conditions can range from the quite mild to devastating. While the list of causative mutations is growing exponentially, the possible sources of therapy are not as extensive. Developing treatments and therapies for patients with mitochondrial disease is therefore paramount.

Resistance training has been hypothesised to help patients with sporadically occurring mtDNA mutations. This is based on findings of low or absent levels of mtDNA mutations in the proliferating myoblasts of patients with sporadically occurring conditions (Fu et al., 1996, Weber et al., 1997). On the basis of these findings it has been hypothesised that muscle stem cells (satellite cells) contain little or no mutation. The rationale behind resistance training is that through the activation and incorporation of developing muscle cells with low levels or absence of mutation, the heteroplasmy levels in muscle of affected patients will be changed. This should help ameliorate some of the muscle symptoms patients experience.

The aim of this research is to test the theory behind the rationale for using resistance training as a therapy for patients with sporadically occurring mtDNA mutations. It aims to examine myoblast precursors and determine if mtDNA mutations are absent from the satellite cells. In addition it is interested in establishing the reasons why mutations may be absent from either the satellite cells or myoblasts. It is hoped by revealing the mechanisms of any loss it will be possible in the future to predict which patients will benefit from performing resistance training to adjust their heteroplasmy levels.

A further aim of this research is to investigate the possibility that patients with mitochondrial myopathies may exhaust their satellite cell pool. This could occur through excessive turnover of muscle fibres due to the myopathy, with a resultant increase in the cycles of degeneration and regeneration this causes. If resistance training is to be offered as a treatment for these patients it is important that this has no detrimental effect in terms of stem cell numbers.

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Chapter 2 Materials and Methods

2.1 Equipment, Consumables, and Reagents

2.1.1 Equipment

ABI 3130xI Genetic Analyzer	Applied Biosystems
ABI Step One Plus Real-Time PCR System	Applied Biosystems
Autoclave	Astell
Balance: Sartorius Basic	Sartorius
Bench-top microcentrifuge PCV-2400	Grant-Bio
Bench-top centrifuge 1-14	Sigma
Bench-top centrifuge 5417 R	Eppendorf
Binder general purpose incubator	Philip Harris
Cerenkov counter, type 6-20	Mini Instruments
Cryostat (Cryo-star HM 560M)	Microm International
Dry heat block	Techne
Electrophoresis power supply model 250EX	Life Technologies
Horizontal agarose gel electrophoresis systems	Amersham Biosciences
Hot water bath	Techne
Laminar flow hood	Jencons-PLS
Luminometer TD-20e	Turner
Microflow biological safety cabinet	Bioquell
Nanodrop ND-1000 spectrophotometer	Labtech International
3510 pH meter	Jenway
Olympus microscope BHX54	Olympus

PhosphorImager, Storm 860	Molecular Dynamics
Roche Lightcycler	Roche
Rotating hybridisation oven	Hybaid
Thermal cycler (9700)	Applied Biosystems
UV gel documentation system and	
AlphaImage series 2200 Software	Flowgen
Axioplan 2iE Light microscope	Zeiss
AxioCam HRc digital camera	Zeiss
AxioVision imagecapture software	Zeiss

2.1.2 Consumables

0.2ml PCR tubes	Biogene
1.5ml microcentrifuge tubes	Biogene
2.0ml microcentrifuge tubes	Biogene
Graduated filter tips	Starlabs
Coverslips (22x22mm, 22x40mm, 22x50mm)	Merck
Cellstar® Disposable Pipettes	Greiner
Eppendorf Biopipette (P1000, P200, P20, P10)	Eppendorf
Falcon tubes (15ml and 50ml)	Costar
Gel extraction kit	Qiagen
Lightcycler capillaries	Roche
Microscope slides (76x26x1.0-1.2mm)	Merck
Tissue extraction kit	Qiagen

Cellstar® tissue culture flasks (25cm^3, 75cm^3)	Greiner
Whatman Grade IV filter paper	Merck
10ml universal tubes	Greiner
96-Well Fast Optical Reaction Plates	Applied Biosystems
Cap strips, domed	Eppendorf
96-well PCR plate	Starlabs

2.1.3 Chemicals and Reagents

2.1.3.1 Tissue Preparation

Iso-pentane	Merck
Liquid nitrogen	BOC
OCT cryoembedding matrix	Raymond Lamb

2.1.3.2 Histological and Histochemical Reagents

Avidin/ Biotin Blocking Kit	Vector Laboratories
Catalase	Sigma
Cytochrome *c*	Sigma
3. 3'diaminobenzidine tetrahydrochloride	Sigma
DPX™ Mountant	Merck
Ethanol Analar	Merck
Histoclear™	National Diagnostics
Hydrogen peroxide	BDH
Nitro blue tetrazolium	Sigma
Rabbit anti-mouse HRP conjugated antibody	DakoCytomation
Methanol Analar	Merck

Paraformaldehyde	Sigma
Pax7 (IgG1) primary antibody	Developmental Studies Hybridoma Bank
Phosphate buffered saline tablets	Oxoid
Sodium azide	Sigma
Sodium chloride	Sigma
Sodium dihydrogen phosphate	Sigma
Sodium succinate	Sigma
Triton X-100	Sigma
X-Cell-Plus HRP detection with DAB	A. Menarini diagnostics

2.1.3.3 Polymerase Chain Reaction and Sequencing Reagents

Amplitaq Gold DNA polymerase	Applied Biosystems
BigDye Terminator v3.1 cycle sequencing kit	Applied Biosystems
Bovine serum albumin	New England Biolabs
Deoxynucleotide triphosphates	Rovalab
DMSO (molecular biology grade)	Sigma
ExoSAP-IT	GE Healthcare
Expand Long Template PCR system	Roche
HiDi	Applied Biosystems
SYBR Green	Invitrogen
Tamra labelled probe	Applied Biosystems
TaqMan® mastermix	Roche

2.1.3.4 Gel Electrophoresis Reagents

Agarose MP	Roche
Bromophenol blue	Sigma
1Kb DNA ladder	Gibco BRL
24Kb DNA ladder	Fermentas
Ethidium bromide	Merck
GelRed Nucleic Acid Stain	Biotium
Hyperladder IV	Bioline
Sodium dodecyl sulphate (SDS)	BDH
Tris acetate EDTA (TAE:x10)	Sigma

2.1.3.5 Southern Blot Reagents

[α-^{32}P]-dCTP (3,000 Ci/mmol)	Perkin Elmer
BamHI restriction enzyme	New England Biolabs
Dextran sulphate sodium salt	Pharmacia
Dithiothreitol (DTT)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Hybond XL nylon membrane	Amersham Pharmacia
Hydrochloric acid (HCl)	BDH
Klenow enzyme	New England Biolabs
Multiprime kit	Amersham Pharmacia
Random hexamers Pd $(N)_6$ 5'PO_4 Na^+salt	Pharmacia
Salmon testes DNA (10mg/ml)	BDH
Sephadex G-5- Nick Column	Amersham Pharmacia
SnaBI restriction enzyme	New England Biolabs

Sodium chloride (NaCl)	BDH
Sodium hydroxide (NaOH)	BDH

2.1.3.6 Tissue Culture Reagents

Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Foetal calf serum	Gibco
Gentamicin	Gibco
L-Glutamine	Gibco
MycoAlert mycoplasma detection kit	Lonza
Penicillin and streptomycin solution (Pen-Strep)	Gibco
Pyruvate	Sigma
Skeletal muscle cell growth medium	PromoCell
Trypsin	Gibco
Uridine	Sigma

2.2 Methods

2.2.1 Ethics

Approval for this research was granted by the Newcastle and North Tyneside Local Research Ethics Committees 2202/205 for Doug Turnbull from 2002 to the end of 2012.

2.2.2 Biopsies

Muscle biopsies were performed by Dr Grainne Gorman of the Biomedical Research Centre using the needle biopsy technique. To obtain muscle samples, biopsies were taken from the mid-portion of the *vastus lateralis*. Samples for satellite cell isolation were place in solution A (30mM HEPES,130mM NaCl, 3mM KCl, 11mM Glucose, 0.567mg phenol red dissolved in 500ml of dH_2O) for storage until Fluorescently Activate Cell Sorting (FACS) could be performed. Samples for molecular biology studies were snap frozen in pre-cooled isopentane and stored at -80^{o}C. Samples for histochemistry were mounted on Whatman grade IV filter paper with OCT adhesive and also snap frozen in isopentane.

A small portion of the sample was transferred to the Newcastle MRC Centre for Neuromuscular Diseases Biobank for myoblast culture.

2.2.3 Cryostat Sections

Muscle cryostat sections were cut from frozen tissue using a Cryo-star HM 560 cryostat. Tissue was orientated transversely and sections of 12µm were cut and mounted onto slides. Sections were left to air dry for one hour and then stored at -20^{o}C until required.

2.2.4 Isolation of Satellite Cells

2.2.4.1 Cell Dissociation

To dissociate satellite cells from the muscle samples FACS sorting was performed. The muscle sample was placed in a Medicon (BD Biosciences) with approximately 500µl of sterile phosphate buffered saline (PBS), and placed into a Medimachine (BD Biosciences) for approximately fifteen seconds. The Medicon, consisting of a blade and grid, was placed in the Medimachine which propels the blade. The resultant cell solution passes through the grid, leaving any connective tissue on top. This allows for a sterile, automated cell dissociation system. The cell solution was aspirated with a 5ml syringe, and to remove muscle fibres it was passed through a 30µm filter (CellTrics®, Partec) into a FACS sorting tube. Cells were then counted using a Beckman Coulter Cell Viability Analyser. Phycoerythrin (PE) labelled Mouse Anti-Human CD56 IgG_1 (BD Pharmingen^{TM}), recognising the Neural Cell Adhesion Molecule (NCam), was added to the cells (10µl antibody per million cells) to incubate in the dark for thirty minutes. The sample was then washed using the BD Lyse Wash Assistant (BD Biosciences). This centrifuges the cells to the wall of the tube and washes them using a buffer, removing residual PBS or solution A. The cells were then immediately FACS sorted.

2.2.4.2 Fluorescently Activated Cell Sorting (FACS)

CD56 positive (CD56+) cells were sorted using a FACS Aria (BD Biosciences). The FACS Aria takes up the sample and uses laminar flow with sheath fluid to create a stream of cells. The cells then pass through the optimal point of light source and are interrogated with a laser. This allows data on light scatter (forward and side) and fluorescence to be collected.

The PE labelled Mouse Anti-Human CD56 IgG_1 was excited at 488nm by the FACS Aria and a signal was emitted by the fluorescently labelled cells at 585nm. This signal was detected by the machine which then sorted the cells into CD56+ and CD56-. The CD56+ cells were collected in a microcentrifuge tube, centrifuged at 1500rpm (166g) for five minutes, the supernatant was pipetted off and the cells stored at -20^{o}C until needed.

2.2.5 Histochemistry

2.2.5.1 Haematoxylin and Eosin (H & E) Staining

H & E staining was routinely used to visualise tissue morphology. In order to stain muscle nuclei, haematoxylin staining was performed. The oxidised form of haematoxylin; hemalum, gives a blue colour when added to sections after becoming bound to arginine rich nucleoproteins such as histones. While eosin, an acidic dye, stains eosinophilic structures pink. In the case of muscles sections, this will mostly be the cytoplasm of the fibre which is basic.

In order to perform the staining, sections were defrosted in slide mailers for thirty minutes, and then air dried for one hour. The sections were then fixed in formal calcium for fifteen minutes, and rinsed in tap water. The slides were totally immersed in haematoxylin for one minute, and then rinsed in tap water to blue the nuclei. Once the water ran clear the sections were placed in eosin (1% eosin yellowish, 0.4% erythrosine B, 0.2% phloxin B in dH_2O) for thirty seconds, and then washed again in tap water.

To dehydrate the sections, the slides were passed through a graded ethanol series (75%, 95%, 2 x 100%), being left in the final 100% solution for ten minutes. They were then cleared through two stages of histoclear, mounted using DPX mountant and a cover slip was applied.

2.2.5.2 Dual Cytochrome c Oxidase (COX) and Succinate Dehydrogenase (SDH) Histochemistry

COX/SDH histochemistry (Old and Johnson, 1989) was performed on frozen muscle sections to assess the activity of complexes II and IV. Through combining these two stains it was possible to identify COX deficient fibres. COX deficient fibres will not demonstrate the brown reaction product (oxidised DAB) during the first incubation, but will stain blue following the second incubation with SDH. This is because SDH is encoded by the nuclear genome so any mtDNA mutations will not affect its activity, while mtDNA mutations will affect the activity of complex IV and prevent DAB oxidation.

Muscle sections were defrosted in slide mailers for thirty minutes, and then air dried for one hour. All staining and incubations were carried out in a humidified chamber. The COX incubation medium was prepared by adding 200µl of 500µM cytochrome *c* to 800µl of 5mM 3.3'diaminobenzidine (DAB) with approximately 20µg of catalase enzyme. The solution was then vortexed and 200µl per section was added to the slides. The sections

were incubated for forty five minutes at 37^{o}C and then immersed in PBS three times to wash.

The SDH incubation medium was prepared by adding 100µl of 1.3M sodium succinate, 100µl of 2mM phenazine methosulphate (PMS) and 10µl of 100mM sodium azide to 800µl of 1.875mM NitroBlue tetrazolium (NBT). The mixture was vortexed and 200µl was added to the sections. Again, the sections were incubated for forty five minutes at 37^{o}C, before being immersed three times in PBS to wash.

The sections were dehydrated by passing the slides through a graded ethanol series (75%, 95%, 2 x 100%), being left in the final 100% solution for ten minutes. They were then cleared through two stages of histoclear, mounted using DPX mountant and a cover slip was applied.

2.2.6 Immunohisochemistry

All immunohistochemistry protocols, unless otherwise stated, were performed on the bench at room temperature. A control muscle section, without any primary antibody was included with the staining to check for any cross reactivity. All brightfield images were taken on a Zeiss Axioplan 2 microscope using AxioVision 4.8.1 software.

2.2.6.1 Pax7 Staining

Pax7 is a nuclear marker expressed in quiescent satellite cells, and is down regulated as cells head towards differentiation (Buckingham, 2006). Pax7 stained satellite cells within a muscle section are visible as brown (DAB) nuclei around the periphery of the fibres. Pax7 staining was performed on muscle sections to enable satellite cell counting in patients with mitochondrial myopathies and control subjects.

Muscle sections were defrosted in slide mailers for thirty minutes and then air dried for one hour. A hydrophobic pen was used to draw around the sections to stop solutions from dispersing around the slide. The sections were then fixed in 4% paraformaldehyde (PFA) for thirty minutes, before rinsing in distilled water to remove excess PFA.

Antigen retrieval was performed on the sections by continuously submersing the slides in and out of boiling EDTA (pH 8) for one minute. This helps break protein bonds and brings the antigens to the surface. They were placed in distilled water and then into 3% H_2O_2 for thirty minutes to block any endogenous peroxidase. After washing in distilled water they were given a further three washes. This consisted of tris buffered saline (TBS)(pH 7.4) being pipetted onto the sections for five minutes twice, and then tris buffered saline with tween (TBST) (pH 7.4) being applied once for five minutes. Tween is a detergent that helps the solution spread evenly around the slide.

Then a blocking solution of 1% normal goat serum (NGS) in TBS was made, of which 100µl was applied to the sections for thirty minutes. This was to block non-specific goat antibody binding sites, as the secondary antibody was raised in goat. The primary antibody, Pax7 (IgG1) (Developmental Studies Hybridoma Bank), was made up at a 1:50 dilution in TBS, and 100µl per section was applied to the slides. The slides were then either left at 4^{o}C overnight or at room temperature for ninety minutes.

The primary antibody was washed off with two five minute washes in TBS and one five minute wash in TBST, before a secondary antibody was applied. 100µl of anti-mouse IgG–

biotinylated antibody (raised in goat; Vector Laboratories) was applied at a 1:200 dilution in 1% NGS, for thirty minutes. While the sections were incubating a streptavidin/biotinylated (ABC) solution (Vector Laboratories) was made by adding one drop of solution A and one drop of solution B to 5ml of TBS. After the sections had been washed in TBS and TBST, 100µl of the ABC solution was added for thirty minutes. The next two washes consisted of TBS being pipetted onto the sections for five minutes, and then the slides were immersed in distilled water. To visualise the staining, 3,3-diaminobezidine (DAB) was used. This was made up by adding one DAB and one urea hydrogen peroxidase tablet to 5ml of nanopure water and vortexing until they dissolved. 100µl of this solution was applied for six minutes.

The slides were washed in distilled water and counter stained with haematoxylin for one minute to visualise nuclei. They were then washed in tap water until it ran clear, and put through a graded ethanol series to dehydrate, (75%, 95%, 2 x 100%), being left in the final 100% solution for ten minutes. They were cleared through two stages of Histoclear^{TM}, mounted using DPX mountant and a cover slip was applied.

2.2.6.2 Neural Cell Adhesion Molecule (NCam) Staining

NCam is a transmembrane protein expressed in satellite cells that serves as a cell surface marker. It is important for cell adhesion and is expressed in satellite cells, as well as regenerating muscle fibres. Any satellite cells in a muscle section are visible by the presence of a stained halo around the outside of the nuclei. NCam staining was performed in order to determine which fibres in a muscle section were regenerating.

Methods for defrosting, fixing, antigen retrieval, blocking endogenous peroxidases, and blocking with NGS, were as they were for Pax7 (section 2.2.6.1). The primary antibody, NCam (IgG1) (gift from Eric Shoubridge) was made up at a 1:50 dilution in TBS, and 100µl per section was applied to the muscle sections. The slides were then left at 4^{o}C overnight or alternatively at room temperature for ninety minutes.

Methods for visualising the staining with a secondary antibody, ABC solution, and DAB were the same as they were for Pax7 (section 2.2.6.1). The slides were then washed in distilled water and counter stained with haematoxylin for one minute to visualise nuclei. They were then washed in tap water until it ran clear, and put through a graded ethanol series to dehydrate, (75%, 95%, 2 x 100%), being left in the final 100% solution for ten

minutes. They were then cleared through two stages of histoclear, mounted using DPX mountant and a cover slip was applied.

2.2.6.3 Complex I Subunit 20kDa Staining

Complex I subunit 20kDa (complex I:20) reacts against NDUFB8, which will have an impaired entry into the complex I assembly pathway if the mtDNA encoded ND4 is deficient. This is because a problem with ND4 impairs complex I assembly at an early stage and prevents incorporation of NDUFB8 at a later time point (Perales-Clemente et al., 2010). Complex I:20 staining was performed on muscle biopsy sections to determine which muscle fibres were complex I deficient.

Methods for defrosting, fixing, antigen retrieval, and blocking of endogenous peroxidase, were as they were for Pax7 (section 2.2.6.1). The method of visualisation used for this antibody does not require the addition of a secondary antibody to the sections, which for previous protocols has been raised in goat. As a result the use of this antibody does not require a blocking step of 1% NGS.

The primary antibody, complex I subunit NDUFB8 monoclonal antibody (IgG1) (MitoSciences) was made up in TBS at a 1:600 dilution, and 100µl was added to the muscle sections. The slides were then either left at 4^{o}C overnight or at room temperature for ninety minutes. After antibody incubation the slides were washed twice for five minutes in TBS, and once for five minutes in TBST. A Universal Probe, recognising the mouse epitope of the primary antibody, was added to the sections for thirty minutes. After a further wash, a horseradish-peroxidise (HRP)-polymer solution was added to the section for thirty minutes. This binds to the Universal Probe and amplifies the signal, thus increasing the sensitivity of the protocol. The slides were then washed twice in TBS and immersed in distilled water. To visualise the staining 3,3-diaminobezidine (DAB) was used, made up as previously stated (section 2.2.6.1).

The slides were washed in distilled water and counter stained with haematoxylin for one minute to visualise nuclei. They were washed in tap water until it ran clear, and put through a graded ethanol series to dehydrate, (75%, 95%, 2 x 100%), being left in the final 100% solution for ten minutes. They were then cleared through two stages of Histoclear^{TM}, mounted using DPX mountant and a cover slip applied.

2.2.7 Cell Culture

2.2.7.1 Myoblasts

In order to determine what happens to mtDNA deletions as muscle cells develop into myotubes, myoblast cultures were performed. At the time of biopsy a small sample of muscle was transferred into the MRC Centre for Neuromuscular Diseases Biobank, where myoblast cultures were set up by the biobank technician Mojgan Reza.

Briefly, the sample was transferred to a Petri dish containing ATE (solution A 49.7ml, trypsin 0.5ml, EDTA 0.2ml), and cut up using a scalpel. The sample, along with 10ml of ATE was transferred to a Wheaton flask with a magnetic flea which was placed in a beaker containing water warmed to 37^{o}C. The beaker and Wheaton flask went on a magnetic stirrer for fifteen minutes. Once the fragments had settled the liquid was poured off into a Falcon tube. This process was repeated a further two times. The Falcon tubes were centrifuged at 160g for ten minutes and the supernatants discarded. Each pellet was suspended in 2ml of skeletal muscle cell medium (SKCM) (PromoCell), combined, and transferred to a Petri dish and placed in the incubator at 37^{o}C, 5% CO_2. The medium was changed every two to three days. After around two weeks the cells were trypsinised and placed in a T25 flask and transferred from the biobank to the Mitochondrial Research Group. Once received, the myoblasts were cultured further and eventually differentiated into myotubes. Cells were grown in SKCM supplemented with 10% foetal calf serum (FCS), 1.5% L-glutamine, and 0.06% of gentamicin.

2.2.7.2 Passaging and Harvesting Cells

When cells were about 85% confluent the media was aspirated off, and the cells washed with 5ml of PBS to remove all traces of FCS. The PBS was removed and 2ml of trypsin was added to the flask. This was placed back in a humidified incubator at 37^{o}C, 5% CO_2, for approximately five minutes until the cells had detached from the growing surface and were free floating. Following this, 5ml of serum containing media was added to the flask to quench the trypsin and the cells were transferred to a 10ml universal tube. The cells were then centrifuged at 160g for five minutes. The supernatant was discarded and the cell pellet re-suspended in 10ml of SKCM. Some of the cells were then added to a T75 flask and the media made up to 20ml. Cells for harvesting were placed in a microcentrifuge tube and re-centrifuged at 160g, the supernatant discarded and the resultant pellet frozen at -20^{o}C until DNA extraction could be performed.

2.2.7.3 Freezing Cells

The cells were harvested as stated in the previous section, and those for freezing were placed in a microcentrifuge tube, spun at 166g for a further five minutes and the supernatant discarded. 2ml of freezing medium (DMEM 70%, FCS 20%, dimethyl sulfoxide (DMSO) 10%, all sterilised through a 0.22μm filter), was added to the pellet, the cells were re-suspended and then transferred to a cryotube, which was quickly placed in the -20^{o}C freezer. The cells were then placed at -80^{o}C for twenty four hours before being transferred to liquid nitrogen for long term storage.

2.2.7.4 Differentiation of Myoblasts to Myotubes

Differentiation of myoblasts was achieved through the changing of SKCM for a reduced serum medium. When myoblasts were about 80% confluent the medium was removed and the cells washed with PBS. 15ml of differentiating medium (98% DMEM, 2% horse serum) was added to the flask which was then returned to the incubator. After about five days myotubes were visible in the flask. These are elongated multinuclear cells that appear to line up alongside each other (Figure 2.1). The myotubes were then harvested and pelleted as described previously (section 2.2.7.2) and frozen until DNA could be extracted.

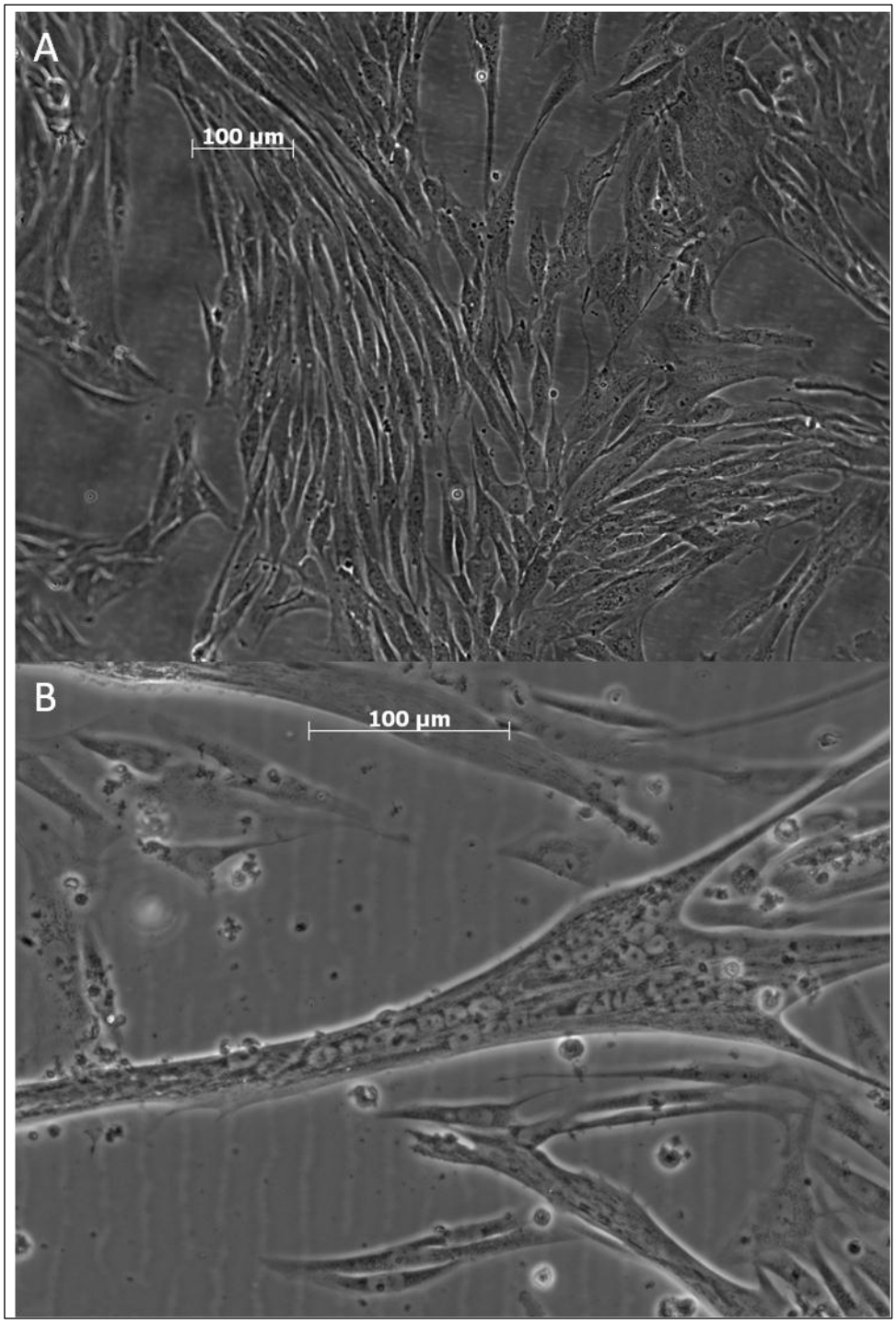


Figure 2.1 Images of myoblasts and myotubes. **A**. Myoblasts before the addition of differentiation medium. All cells have a single nucleus and are still dividing. **B**. After the addition of differentiation medium cells start to line up and fuse together, myotubes with multiple nuclei can be observed.

2.2.7.5 Counting Cells

When cells were harvested and re-suspended in 10ml of fresh SKCM they were also counted. A cover slip was applied to a haemocytometer and 15µl of cell sample was pipetted on the edge of the cover slip and drawn up via capillary action. Using x10 magnification, the microscope was focused onto the grid lines to view the cells. The four corners of the haemocytometer contain a grid of sixteen squares, in which all cells were counted. Any cell positioned on the left hand or bottom boundary was included in the counts, while those on the right hand and upper boundaries were not included. The counts for the four corners were then averaged. The average number of cells for the four corners of the haemocytometer was multiplied by 10^4 to give the number of cells per ml.

2.2.7.6 DNA Extraction of Cultured Cells

DNA was extracted from myoblasts and myotubes using a Qiagen DNeasy Blood & Tissue Kit according to manufacturer's instructions for the 'Purification of Total DNA from Animal Blood or Cells' (Spin-column Protocol). DNA was eluted from the column in 40µl of dH_2O after two minutes incubation, and stored at -20^oC. In order to determine the DNA concentration of the samples 1µl was loaded onto a NanoDrop® ND-1000 Spectrophotometer to give a value of ng/µl. Samples were stored at -20^oC until needed.

2.2.7.7 Mycoplasma Testing

At the first passage after receiving myoblasts from the biobank all cells were tested for mycoplasma using the MycoAlert® Mycoplasma Detection Kit (Lonza). The cells being cultured were primary human cell lines, in which mycoplasma is a common problem as it is present on the skin of most people. When the medium was aspirated off 5mls were transferred to a 10ml universal tube, sealed with Parafilm, and stored at 4^oC until testing. In order to perform the mycoplasma test, 2ml of media were transferred into a microcentrifuge tube and spun at 166g for five minutes to pellet any contaminating cells. Whilst being careful not to pipette any air bubbles, 25µl of clear supernatant was transferred to a luminometer cuvette. Then 25µl of MycoAlert® Reagent was added to the cuvette and the sample left for five minutes. The tube was then placed in the luminometer and three readings were taken to give an average. After this, 25µl of MycoAlert® Substrate was added to the sample and left for ten minutes, before a final three readings were taken on the luminometer. The ratio between the readings was taken by dividing the substrate result by the reagent results. Providing this ratio was below one, the cells could be considered to be mycoplasma free.

2.2.7.8 Seeding Cover slips

In order to perform immunofluorescence and COX/SDH on the myoblasts, cells were grown on cover slips to aid staining. Autoclaved circular cover slips were placed into each well of a six well plate. After harvesting, cells were re-suspended in 3ml of medium. To the centre of each cover slip 500µl of cell containing medium was pipetted, before placing the plate back into the incubator to allow the cells to adhere to the cover slip. After one hour a further 1.5ml of medium was added to the wells and the plate returned to the incubator.

2.2.7.9 Immunofluorescent Staining of Myoblasts

In order to confirm the purity of the myoblast culture, cells were labelled for desmin. Desmin is an intermediate filament found in the sarcomeres of striated muscle cells.

Medium was removed from the wells and the cells washed in 2ml of PBS. The cells were then fixed in 4% PFA for ten minutes in the laminar flow hood. PFA was removed from the wells and the cover slip washed three times in PBS to remove any excess PFA. The cover slips were then removed from the wells and adhered to a slide using nail varnish to aid staining, which was performed at the bench. Cells were blocked in 5% NGS in PBST (to permeabilise the membrane) for one hour. Monoclonal mouse anti-human desmin IgG_1 (Dako) was diluted in blocking reagent 1:100 and added to the cells for one hour at room temperature. Following this, the cells were washed three times in PBST, each time for five minutes, and Alexa Fluor® -488 goat anti-mouse (FITC) (1:100 in blocking reagent) was applied for one hour, again at room temperature. Cells were then washed once in PBST and twice in PBS. Nuclei were visualised using VECTASHIELD® Mounting Medium with DAPI.

2.2.7.10 Immunofluorescent Labelling of Fibroblasts

Fibronectin staining was performed to determine if cultured cells were fibroblasts. Fibronectin localizes to the matrix of cultured human fibroblasts.

Medium was removed from the wells and the cells washed in 2ml of PBS. The cells were then fixed in 4% PFA for ten minutes in the tissue culture hood. PFA was removed from the wells and the cells washed three times in PBS to remove any excess PFA. The cover slips were then removed from the wells, attached to slides using nail varnish, and transferred to the bench. Cells were blocked in 5% NGS in PBST (to permeabilise the membrane) for one hour. Monoclonal anti-fibronectin antibody IGg_1 (Sigma-Aldrich)

made in mouse, diluted 1:200 in blocking reagent was added to the cells for one hour at room temperature. Following this, the cells were washed three times in PBST, each time for five minutes, and Alexa Fluor® -488 goat anti-mouse (FITC) (1:100 in blocking reagent) was applied for one hour, again at room temperature. Cells were then washed once in PBST and twice in PBS. Nuclei were then visualised using VECTASHIELD® Mounting Medium with DAPI. Images were taken on a Zeiss Axioplan 2 microscope using AxioVision 4.8.1 software.

2.2.7.11 COX Staining on Myoblasts

COX staining was performed in order to determine the enzymatic activity of the cultured myoblasts from patients.

Cells were grown on cover slips in six well plates. The medium was removed from the wells and the cells washed in PBS. The cover slips were removed from the wells and mounted on slides; all subsequent steps were performed at the bench. The COX incubation medium was prepared as previously described (section 2.2.5.2) and approximately 250µl were added to each cover slip in order to ensure all the cells were covered. The slides were then incubated for ninety minutes at 37^{o}C, before being washed three times in PBS.

The cover slips were then dehydrated in an ethanol series, placed in Histoclear™, and then removed from their slides before being re-mounted using DPX mountant on fresh slides.

2.2.8 Molecular Biology Techniques

2.2.8.1 DNA Extraction

Muscle homogenate DNA was extracted using the EZ1® Advanced Workstation and the Qiagen EZ1® DNA Tissue Kit by the Newcastle Mitochondrial Diagnostic Service.

2.2.8.1.1 Qiagen Kit

DNA was extracted from CD56+ and myoblasts cells using a Qiagen DNeasy Blood & Tissue Kit according to manufacturer's instructions for the 'Purification of Total DNA from Animal Blood or Cells' (Spin-column Protocol). DNA was eluted from the column in 40µl of water after two minutes incubation, and stored at -20^{o}C. DNA concentrations were determined using the NanoDrop® ND-1000 Spectrophotometer.

2.2.8.1.2 Lysis Method

Single cell DNA extraction was carried out using a lysis method in a ninety six well plate. The lysis medium consisted of 50µl of 500nM Tris-HCL, 250µl of 1% Tween 20, 190µl of autoclaved nanopure water and 5µl of proteinase K (20mg/ml). To each cell 15µl was added, and the plate was lightly shaken, the cells were then incubated at 55^{o}C for two hours before a final proteinase K inactivation step of heating to 95^{o}C for ten minutes. Samples were stored at 4^{o}C until required.

2.2.8.2 Standard Polymerase Chain Reaction (PCR)

2.2.8.2.1 Standard PCR

Standard PCR reactions were performed in order to amplify fragments of mtDNA for DNA sequencing and to generate template DNA for SYBR Green assay optimisation (see section 4.3.1).

Standard PCR's were set up on the bench and performed in 25µl volumes. The reaction mixture consisted of 16.87µl of autoclaved water, 2.5µl of 10 x dNTP's (ROVALAB), 2.5µl of 10 x PCR buffer (10mM Tris-HCl pH8.3 15mM $MgCl_2$, 50mM KCL, 0.001% w/v gelatine), 0.13µl of AmpliTaq Gold® DNA polymerase (Applied Biosystems), and 1µl of both forward and reverse primer (20µM) (Table 2.1). 1µl of wild type (WT) blood DNA was used as a positive control, while 1µl of dH_2O was used as a negative control. Reactions were carried out in a GeneAmp® PCR System (Applied Biosystems) thermal cycler under the following cycling conditions: 95°C for ten minutes, thirty cycles of: 94°C for forty five seconds (denaturing), 58°C for forty five seconds (annealing), and 72°C for one minute (extension), with a final extension of 72°C for eight minutes.

2.2.8.2.2 Gel Electrophoresis

Results of the PCR were then visualised using gel electrophoresis and a 1% agarose gel. To make the gel, 1g of powdered agarose was added to 100ml of 1 x TAE buffer with 2µl of ethidium bromide added to stain the DNA. The mixture was then heated in a microwave to dissolve the agarose and once cooled the gel was poured. To each well 5µl of PCR product, combined with 0.5µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in dH_2O), was added. An appropriately sized ladder was also loaded onto the gel to allow for an estimation of the product size. The gel was run for thirty minutes at 70V in a tank containing 1xTAE buffer, and bands were visualised on the UV gel documentation system.

2.2.8.3 Long Range PCR

In order to amplify large sections of DNA and enable detection of mtDNA deletions, a two-step long range PCR method was employed (Expand Long Range Template PCR System®, Roche). A first round PCR was carried out using primers (nt.5855-nt.5875, nt.129-nt.110) (Table 2.1) to amplify a ~10.8kbp fragment. The master mix consisted of 1µl of DNA template, 5µl reaction buffer 3 (Roche), 8.75µl 0.35mM dNTPs, 10µl 1mg/ml bovine serum albumin (BSA), 0.7µl Expand Taq (Roche), 1.5µl of both forward and reverse primers 10pmol/µl, and 21.55µl dH_2O, to give a 50µl reaction mixture. Reactions were performed on a thermal cycler under the following conditions: three minutes at 93^{o}C; ten cycles of 93^{o}C for thirty seconds, 58^{o}C for thirty seconds, and 68^{o}C for twelve minutes; twenty cycles of 93^{o}C for thirty seconds, 58^{o}C for thirty seconds, and 68^{o}C for twelve minutes with an additional five seconds for every cycle. Then there was a final extension of eleven minutes at 68^{o}C.

The samples were then diluted in dH_2O to give a DNA template, from which 1µl was taken for the second round of PCR. This time primers were used to generate a fragment of ~10.2kbp (nt.6358-nt.6377, nt.019-nt.001), all other reaction reagents were as for the first round. Cycling conditions were also kept the same. The amplified products were separated on a 0.7% agarose gel, with 10µl of product being combined with 0.5µl of loading dye, and run for two hours at 50V.

PCR Primers			
Primer Pair	Forward Primers (5'-3') Nucleotide Number	Reverse Primers (5'-3') Nucleotide Number	Fragment Size (Including Primers)
1	721-740	1268-1248	548
2	1157-1177	1709-1689	553
3	1650-1671	2193-2175	544
4	2091-2111	2644-2625	536
5	2549-2569	3087-3068	539
6	3017-3036	3574-3556	558
7	3505-3524	4057-4037	553
8	3965-3984	4577-4556	613
9	4518-4537	5003-4983	486
10	4932-4952	5481-5462	550
11	5367-5386	5924-5906	558
12	5855-5875	6430-6410	576
13	6358-6377	6944-6924	587
14	6863-6882	7396-7376	534
15	7272-7293	7791-7773	520
16	7713-7723	8301-8283	589
17	8196-8215	8740-8720	545
18	8656-8676	9201-9183	546
19	9127-9146	9661-9641	535
20	9607-9627	10147-10128	541
21	10085-10104	10649-10629	565
22	10534-10553	11109-11089	576
23	1101-11030	11605-11586	596
24	11541-11561	12054-12034	514
25	11977-11997	12545-12527	569
26	12478-12498	13009-12991	532
27	12940-12959	13453-13435	514
28	13365-13383	13859-13839	495
29	13790-13809	14374-14356	585
30	14317-14341	14857-14838	540
31	14797-14815	15368-15349	572
32	15295-15315	15896-15877	602
D1	15758-15777	019-001	812
D2	16223-16244	129-110	476
D3	16548-16569	389-370	412
D4	323-343	771-752	449

Table 2.1 Primer pairs. Primers used to amplify the mitochondrial genome. The primers amplify the genome in thirty six overlapping fragments, with each primer pair generating a product of around 600bp.

2.2.8.4 DNA Sequencing

This was performed on the mtDNA D-loop in order to ascertain sample identity and also to determine mtDNA break points for patients involved in this study in cases where they were not already known.

2.2.8.4.1 D-Loop

The D-loop region of sample mtDNA was amplified using standard PCR methods and primers D1 forward (nt.15758-nt.15777) reverse (nt.019-nt.001), D2 forward (nt.16223-nt.16244) reverse (nt.129-nt.110), D3 forward (nt.16548-nt.16569) reverse (nt.389-nt.370), and D4 forward (nt.323-nt.343) reverse (nt.771-nt.752) (Table 2.1). After running the samples on a thermal cycler under standard PCR conditions (see section 2.2.8.2.1), the products were visualised on an agarose gel and any portions unamplified were repeated. The PCR products were then sequenced.

2.2.8.4.2 MtDNA Deletion Break Points

50µl of long range PCR product were run on a 0.7% agarose gel. These DNA bands were then excised from the gel using a scalpel and UV box, before having the DNA extracted using the Qiagen Gel Extraction Kit, as per manufacturer's instructions.

2.2.8.4.3 Purification of PCR Products

5µl of each product was pipetted into a ninety six well plate over ice and 2µl of ExoSap-IT (VWR) was added to each sample. A sealing mat was applied to the plate, which was then vortexed and pulse spun down. The mixture was incubated in a thermal cycler at 37^{o}C for fifteen minutes, followed by a fifteen minute inactivation step at 80^{o}C. The ExoSap-IT uses exonuclease I and shrimp alkaline phosphatase to remove any unreacted dNTPs and primers in the PCR mixture that could otherwise interfere with the DNA sequencing analysis.

2.2.8.4.4 Cycle Sequencing

A master mix comprising 7µl of dH_2O, 3µl of 5 x sequencing buffer, 1µl of Universal forward primer, and 1µl of BigDyesv3.1, was created, 12µl of which was added to each well of the plate containing a sample. The plate was sealed with strip caps and placed in a thermal cycler under the following conditions: 96^{o}C for one minute, then twenty five cycles of 96^{o}C for ten seconds, 50^{o}C for five seconds, and 60^{o}C for four minutes.

2.2.8.4.5 Precipitation and Sequencing

Precipitation was performed to concentrate and de-salt the DNA. EDTA was diluted to a working concentration of 125mM and 2µl was added to each sample, and tapped down into the well. 2µl of 3M sodium acetate was then added to the well and the plate pulse spun down. The salt nature of the sodium acetate neutralises the negative charges on the DNA making them less hydrophilic and thus less soluble in water.

Following this, 50µl of 100% EtOH was added to the wells to facilitate the precipitation, and the plate inverted four times and left at room temperature for fifteen minutes. The plate was then centrifuged at 2090g for thirty minutes, before the supernatant was discarded through a brief upside down spin on white paper. Then 70µl of 70% EtOH was added to the samples to wash any residual salt from the pelleted DNA, and the plate centrifuged at 1650g for fifteen minutes. Again the supernatant was discarded through an upside down spin and the plate was left to dry in the dark for fifteen minutes.

10µl of HiDi^{TM} formamide was added to each well and the samples were incubated at 95^{o}C for two minutes to separate the DNA strands, the plate was then placed on ice. The HiDi helps with this denaturation by lowering the melting point of the DNA. The plate was then placed in an ABI 3130xl Automated DNA Sequencer to run. Using SeqScape software, the fragments were aligned and compared to the Cambridge Reference Sequence (Anderson et al., 1981).

2.2.8.5 Real Time PCR (qPCR)

2.2.8.5.1 MTND1/MTND4 qPCR TaqMan® Assay

The MTND1/MTND4 qPCR multiplex assay (Krishnan et al., 2007) was used to determine mtDNA deletion levels in patients. MtDNA contains a region that is seldom deleted in patients with sporadically occurring large scale deletions, this is the ND1 region. The ND4 region is however, absent in the majority of these patients (Figure 4.13). This assay uses fluorescent probes (VIC and FAM) along with primers for these specific regions, which are two complex I genes. By comparing the levels of fluorescence created by the probes once they have been freed of their quencher, it is possible to determine heteroplasmy levels.

All qPCR reactions were set up in the UV hood in ninety six well plates. ND1 forward (L3485-3504, 5'-CCC TAA AAC CCG CCA CAT CT-3'), reverse (H3532-3553, 5'-GAG CGA TGG TGA GAG CTA AGG T-3') and ND4 forward (L12087-12109, 5'-CCA TTC TCC TCC TAT CCC TCA AC-3') and reverse (H12140-12170 5' – CAC AAT CTG ATG TTT TGG TTA AAC TAT ATT T-3') primers were reconstituted to 200pmol/µl for stock solutions, from which 1:20 aliquots were made for a working stock of 10µM and kept at -20^{o}C. The ND1 probe (nt.3506-nt.3529, 5'-CCA TCA CCC TCT ACA TCA CCG CCC-3') contained a VIC reporter tag at the 5' end and a TAMRA non fluorescent quencher molecule at the 3' end. While the ND4 probe (nt.12111-nt.12138, 5'-CCG ACA TCA TTA CCG GGT TTT CCT CTT G) contained a reporter FAM tag at the 5' end and a TAMRA quencher at the 3' end. Probes were diluted to 5µM stock concentration.

While the probe is intact no fluorescent signal is generated. Once the probe and primers have annealed the 5'nuclease of the DNA polymerase cleaves the quencher from the fluorescent tag and a signal is emitted (Figure 4.12).

The master mix consisted of 12.5µl of TaqMan® Universal MasterMix, 0.5µl 5µM ND1 probe, 0.5µl 5µM ND4 probe, 0.75µl 10µM of ND1 forward and reverse primer, 0.75µl 10µM of ND4 forward and reverse primer, and 7.5µl of dH_2O. This was added to 1µl of sample DNA, to give a 25µl reaction volume. The plate was sealed using a MicroAmp™ Optical Adhesive Film and pulse spun down. The plate was then transferred to an ABI Step One Plus Real Time PCR System. Cycle conditions were two minutes at 50^{o}C, ten minutes at 95^{o}C, followed by forty cycles of fifteen seconds at 95^{o}C and sixty seconds at 60^{o}C.

The results were exported from the machine and analysed using Microsoft Excel. The proportion of wild type (WT) mtDNA to delete mtDNA was determined using the ΔCt method ($2^{-\Delta Ct}$), where Ct is calculated as ($Ct_{ND1-ND4}$) (Krishnan et al., 2007, He et al., 2002). Ct is the cycle threshold, defined by the number of cycles required for the level of fluorescence to cross the threshold which is set in the exponential phase of the amplification (Figure 4.3). A blood sample was also run, the ΔCt of which was subtracted from the sample results.

2.2.8.5.2 18S rRNA Assay to Determine Relative Copy Number

The 18S assay is able to determine relative amounts of mtDNA to a nuclear marker. The 18S rRNA gene is present in multiple copies in the nuclear genome and since the mtDNA copy number is determined relative to this unknown amount, it is not possible to quantify absolute mtDNA copy number using this method. Primers were used to amplify up the ND1 region (see section 2.2.8.5.1), and the 18S region (Forward 5'-GCC GCT AGA GGT GAA ATT CTT G-3', Reverse 5'-CAT TCT TGG CAA ATG CTT TCG-3'), working concentration 10μM. The ND1 probe along with a 5' tagged FAM 18S probe (5'-CCG GCG CAA GAC GGA CCA GA-3') were used at 5μM concentrations.

The ND1 master mix consisted of 12.5μl of TaqMan® Universal MasterMix, 0.75μl of ND1 forward and reverse primer, 0.5μl of ND1 Vic tagged probe, and 7.5μl of dH_2O. This was added to 1μl of sample DNA, to give a 25μl reaction volume. The 18S master mix consisted of 12.5μl of TaqMan® Universal MasterMix, 0.75μl of reverse primer (10μM), 0.13μl of forward primer (10μM), 0.5μl of probe (5μM), and 10.12μl of dH_2O. The ND1 and 18S reactions were set up in parallel, in triplicate. The plate was sealed using a MicroAmp™ Optical Adhesive Film and centrifuged down. The plate was then placed in an ABI Step One Plus Real Time PCR system. Cycle conditions were two minutes at 50°C, ten minutes at 95°C, followed by forty cycles of fifteen seconds at 95°C and sixty seconds at 60°C. MtDNA copy number was calculated using the equation $R=2^{-\Delta Ct}$ (He et al., 2002).

2.2.8.5.3 SYBR Green qPCR

The MTND1/MTND4 qPCR TaqMan® assay has a lower threshold of detection sensitivity of around 30% heteroplasmy, so any cells with less than 30% delete mtDNA will not give an accurate result. In order to determine levels of delete mtDNA below this threshold, deletion specific SYBR Green qPCR assays were utilised. SYBR Green is a fluorescent dye that binds to all double stranded DNA in a PCR reaction and will fluoresce once bound. As the DNA polymerase amplifies the target sequence, the dye will bind to all new double stranded DNA formed, and an increase in fluorescence will be registered.

Three deletion specific SYBR Green assays were optimised for this research; the details of specific assays are described in chapter 4. Here, the general method for optimising and performing the assay is described.

Depending on the size and position of the mtDNA deletion, primers were chosen to generate an approximately 600bp fragment of the patient's mtDNA that incorporated their deletion break points. This template DNA was then serially diluted down to 1 in 10^{12}. Primers were designed using Primer 3 (v.0.4.0) (Rozen and Skaletsky, 2000), that would amplify up an approximately 100bp fragment of either wild type (WT) mtDNA or mtDNA containing a deletion. Primers were reconstituted in dH_2O to a 200pmol/µl concentration for a stock solution; 1:20 aliquots were then taken to give a 10µM working concentration.

All reactions were set up in a UV hood after UV treatment of all water and assay consumables. Pairs of primers were designed that would selectively amplify up either WT mtDNA or deleted mtDNA. The master mix consisted of 12.5µl of Platinum®SYBR®Green qPCR SuperMix-UDG, 0.5µl of both forward and reverse primers, and 10.5µl of dH_2O. The $MgCl_2$ concentration was optimised for different assays, 0.5µl of $MgCl_2$ was added to increase the $MgCl_2$ concentration to 4mM, without this addition the reaction was performed at 3mM. Master mix was added to 1µl of sample DNA. Assays were optimised for primer and $MgCl_2$ concentrations, as well as annealing temperatures.

Reactions were initially set up in LightCycler Capillaries and performed on the Roche LightCycler. Later assays were carried out in a ninety six well plate and performed on the ABI Step One Plus Real Time PCR system. Cycling conditions were optimised for each assay but generally consisted of a ten minute hold at 95°C followed by forty cycles of: 95°C for fifteen seconds and 60°C for one minute (primer dependent). This was followed

by a melt curve analysis of 95°C fifteen seconds, 60°C one minute followed by a 0.3°C per fifteen second increase up to 95°C.

Once conditions had been optimised for both the WT and delete mtDNA reactions, a percentage deletion curve was generated through diluting template delete mtDNA with WT mtDNA. This provided a set of standards which were run in duplicate or triplicate with every assay. The results were exported and analysed using Microsoft Excel. The percentage deletion (log) was plotted against ΔCT(Ct_{Del-WT}) and the equation of the line was generated from a line of best fit. The ΔCT value of the samples could be inserted into the equation to determine the percentage of delete mtDNA.

2.2.8.6 Southern Blot

In order to determine if one of our patients harboured an mtDNA duplication a Southern blot was performed.

2.2.8.6.1 Linearization of DNA and Gel Electrophoresis

This was performed using a restriction digest. 2-3µg of whole DNA were digested using either *SnaBI* (nt10736) or *BamHI* (nt14258) (New England Biolabs) at 37°C for ninety minutes. The digest contained 17µl of DNA/H_2O mix, 2µl of 10x buffer, and 1µl of enzyme. Following digestion, the samples and a 1kb ladder were run on a 0.7% agarose gel, containing ethidium bromide, for sixteen to twenty four hours at 30V. The gel was photographed using the gel documentation system along with a fluorescent ruler to allow an estimation of the size of the bands. The size of the gel was measured and a corner of the gel was nicked to correspond with the membrane.

2.2.8.6.2 Southern Blotting

The DNA was denatured by soaking the gel in 0.25M HCL for ten minutes and the excess HCL removed by rinsing in dH_2O. To neutralise the gel it was agitated in 0.4M NaOH/0.6M NaCl for thirty minutes, and then in 1.5M NaCl/0.5M Tris-HCL pH 7.5 for thirty minutes. GeneScreen membrane was cut to the same size as the gel and pre-wet in dH_2O for a few seconds. It was then equilibrated in 10x sodium citrate, sodium chloride solution (SSC) for fifteen minutes. This consisted of 3M NaCl and 0.3M sodium citrate dissolved in 800mls of dH_2O, adjusted to pH 7 and then made up to 1000ml. The DNA was transferred to the positively charged nylon membrane from the gel via capillary action, using an overnight Southern blot with 10x SSC as the transfer solution.

After the transfer was complete, the membrane was removed from the blot and then agitated in 0.4M NaOH for one minute in order to denature the DNA. The membrane was then neutralised in 0.2M Tris-HCL pH 7.5/1x SSC for one minute. DNA was cross linked to the membrane through exposure to UV light for four and a half minutes.

2.2.8.6.3 Probe Generation

The probe was provided by Emma Blakely of the Newcastle Mitochondrial Diagnostic Service. A PCR fragment of about 2000bp was generated to correspond to the mtDNA D-loop region. This was run on a 1% agarose gel and the bands extracted using the Qiagen Gel Extraction Kit. The DNA was then denatured and re-natured in the presence of random hexamer sequences. These hexamers are incorporated into the DNA and act as primers for a reaction incorporating ^{32}P into the probe. The probe was purified by running it through a NICK^{TM} column (GE Healthcare); these are designed for the purification of radioactively labelled DNA fragments from unincorporated nucleotides.

2.2.8.6.4 Hybridisation

The membrane was placed into an autoclaved hybridisation tube and had 10ml of pre-hybridisation solution added (10% dextran sulphate, 1% SDS, 1mM NaCl); it was then rotated for one hour at 65^{o}C. Sheared salmon sperm DNA (100µg/ml) was added to the probe and the mixture incubated at 95^{o}C for three minutes to denature the DNA. The DNA present in the sheared salmon sperm binds to the membrane to prevent none specific binding of the probe. The probe was then added to the centre of the hybridisation tube and hybridisation was performed overnight at 65^{o}C in a rotating oven.

2.2.8.6.5 Removal of Non-Specific Radioactivity and Imaging of bands

Following incubation the membrane was subjected to six washes; 2xSSC at room temperature for five minutes twice, 2xSSC + 1% SDS 65^{o}C for thirty minutes twice, and 0.1x SSC at room temperature for three minutes twice. The membrane was then wrapped in saran wrap and exposed to a phosphoimage screen and left for at least six hours. The bands were imaged on a phosphoimager machine.

Chapter 3 Sample Source, Isolation, and Analysis

3.1 Introduction

In patients with mitochondrial disorders muscle (cardiac, smooth and skeletal) is a commonly affected tissue. It has been suggested that this could be attributable to the high energy demand of the tissue. In addition, muscle is a post mitotic tissue which could allow for the accumulation of mtDNA mutations over time that will not be lost through selection after cell division.

Clinical manifestations of myopathy can include exercise intolerance, fatigue, muscle weakness, ptosis, and CPEO. In addition, patients may experience muscle cramps, pain, and stiffness. Histological investigation of muscle from these patients often demonstrates RRF and COX negative blue fibres (Larsson and Oldfors, 2001). Mitochondrial myopathies have been associated with mtDNA rearrangements, and specifically with sporadically occurring large scale single mtDNA deletions (Holt et al., 1988, Zeviani et al., 1988).

Therapies for patients with mitochondrial myopathies are limited, and are mainly geared towards managing symptoms. Advice regarding stretching, posture, and help obtaining aids to assist with everyday tasks may be provided. In addition, patients have been given antioxidants, for example vitamin C, and respiratory chain co-factors, for example succinate and co-enzyme Q10. However, the benefits of these treatments are still debateable (Chinnery et al., 2006).

One approach that has been suggested for patients harbouring sporadically occurring mutations is resistance training (Taivassalo and Haller, 2004). The theory behind this suggestion is discussed in detail in chapter 4.

In order to determine the effects of exercise training on patients with mitochondrial myopathies, our lab is currently performing an exercise intervention in a group of patients harbouring a sporadically occurring large scale single mtDNA deletion. The biopsies being performed for this trial not only provided a source of muscle, but also allowed for current research investigating the theory behind the use of resistance training in patients with sporadically occurring mtDNA mutations. In order to test this theory, satellite cells needed to be isolated and characterised from these patients.

3.2 Aims

This aims of this chapter are to describe the methods used to obtain satellite cells from exercise trial patients, and optimisation steps performed to ascertain how myogenic these cells were. In addition it will also describe some of the initial molecular techniques performed on the samples to characterise the mutation and ensure sample identity.

3.3 Methods

3.3.1 Exercise Trial

Muscle samples for this study have come from eight patients enrolled on an exercise trial at Newcastle University. This trial was designed to assess the effects of both resistance and aerobic training on patients harbouring a sporadically occurring large scale single mtDNA deletion. Patients underwent an initial assessment involving strength testing, needle muscle biopsy, and an MRI scan. They undertook six months of resistance training, a follow up assessment, and were then given a six month aerobic exercise program before a final assessment (Figure 3.1).

Resistance training was carried out three times per week and patients performed three sets of eight repetitions of their 80-85% one repetition maximal. The exercise was performed on a standard leg extension weight-stack piece of equipment designed to primarily exercise the quadriceps muscle group. Patients were asked to perform the exercise in order to maximise the eccentric contraction phase of the movement. This is muscle contraction that involves the muscle lengthening as it is active, and is thought to induce micro damage.

Aerobic exercise was performed on a stationary bike three times a week at an intensity allowing patients to work at 70-80% of their maximal heart rate.

3.3.2 Patients

Eight patients (two female: mean age 46 ±15 years; six male: mean age 42.5 ± 10 years) were enrolled on the trial. All patients suffered myopathy with CPEO, and in most cases exercise intolerance and ptosis. The mean age of onset was 23.1 ± 11.3 years old (Table 3.1).

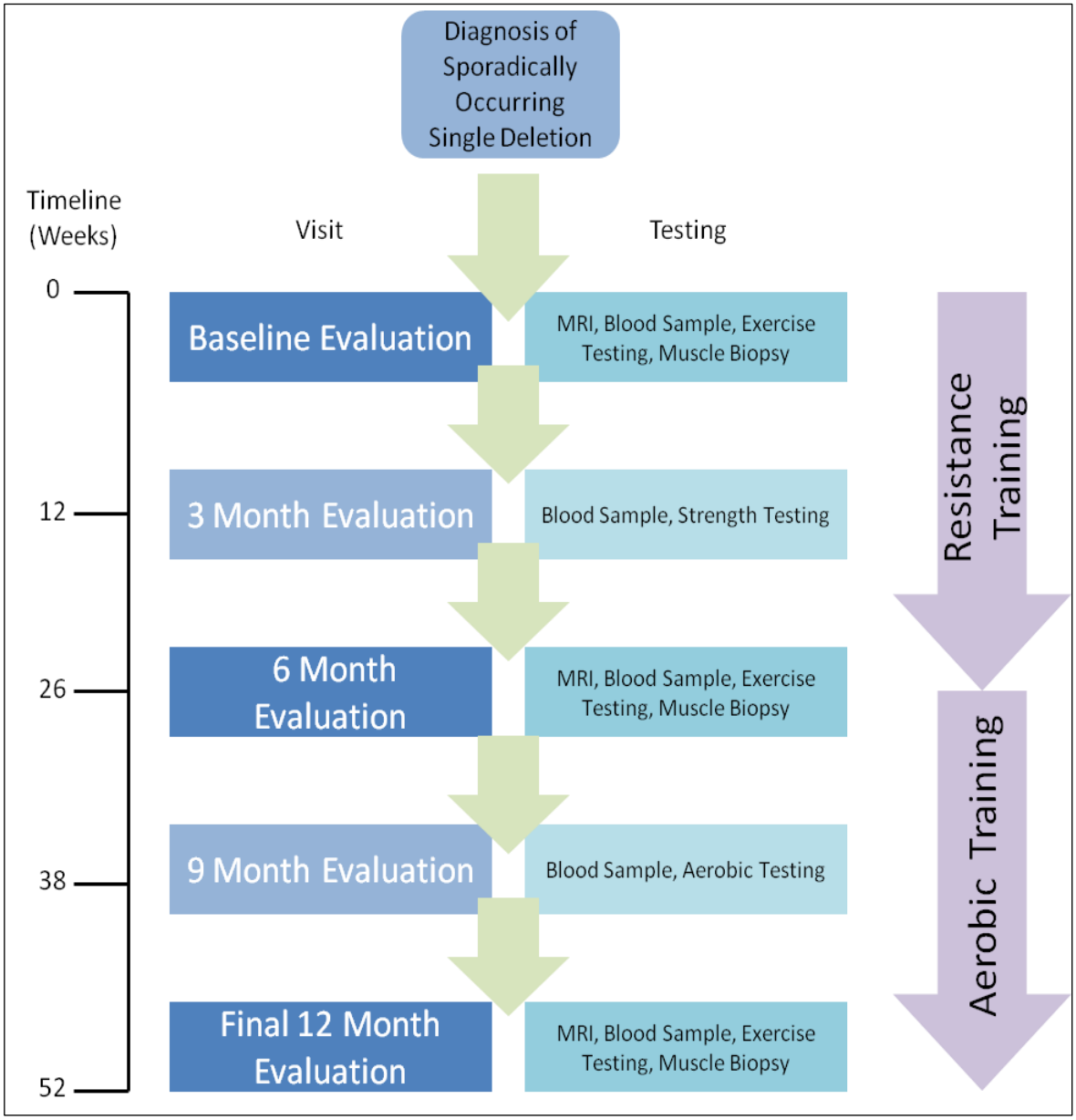


Figure 3.1 Flow diagram of exercise interventions. Eight patients with a single mtDNA deletion were given baseline evaluations and a fifty two week exercise program. At regular intervals samples were taken for molecular and histochemical analyses. Strength testing was also performed.

Patient	Sex	Age	Age at onset	Symptoms
1	F	58	37	CPEO, exercise intolerance, fatigue
2	M	46	28	CPEO, exercise intolerance, fatigue, gastrointestinal tract problems
3	M	39	16	CPEO, exercise intolerance, fatigue, gastrointestinal tract problems
4	M	59	10	CPEO, exercise intolerance, fatigue, ptosis
5	M	45	29	CPEO, exercise intolerance, fatigue, ptosis
6	M	35	33	CPEO, exercise intolerance, fatigue, gastrointestinal tract problems
7	M	31	16	CPEO, exercise intolerance, fatigue, SVT
8	F	34	9	CPEO, ptosis, mild dysphagia

Table 3.1 Clinical symptoms of patients enrolled on the exercise trial. All patients displayed symptoms of myopathy, with chronic progressive external opthalmoplegia (CPEO) and either ptosis or exercise intolerance. The age range at the time of intervention was 31-58, and age of onset varied from 9 to 37. SVT = supraventricular tachycardia

3.4 Methods Optimisation and Results

3.4.1 Sample Isolation

Many studies examining sporadically occurring mtDNA mutations in patients have investigated cultured myoblasts from muscle biopsies and found no mutation in these cells. They have suggested this may be because the mutations are not present in the satellite cells (Fu et al., 1996, Weber et al., 1997). However, sporadically occurring mtDNA mutations are known to be lost or lower in rapidly dividing cells (Moraes et al., 1989a, Bonod-Bidaud et al., 1999) and it was necessary to ensure that there would be no selection occurring in the cells used for this study. Any period of selection acting on the myoblasts prevents inferences about the mutational status of the satellite cells. It was decided to obtain satellite cells through FACS sorting on the basis of CD56, a marker of these stem cells.

Patients underwent a biopsy of the *vastus lateralis* to obtain a muscle sample. This is one of the muscles in the quadriceps group, and is being exercised during the trial. The sample was then placed in a Medicon and a Medimachine to dissociate the cells, as described in chapter 2. The cell solution was passed through a filter to remove any residual muscle fibres and the cells counted on an automated cell counter (Figure 3.2).

Depending on the number of cells obtained, between 10-20µl of human PE labelled CD56 IgG1 antibody was added to the solution. This antibody recognises the neural cell adhesion molecule (NCam) on the surface of satellite cells. The sample was then sorted into CD56 positive (CD56+) and CD56 negative (CD56-) fractions using the FACS Aria machine. The sorting of the sample with the FACS Aria was performed by Ian Dimmick of the North East Stem Cell Institute (NESCI). Two samples were passed through the machine, one with the antibody added, and one without; it was then possible to determine the fluorescent cell population we wanted to isolate (Figure 3.3).

First a gate was drawn to discard any particularly large or small cells as these were thought to be debris (Figure 3.3A&G). Then the samples were gated allowing only single cells to flow through the machine. This reduces contamination as it stops the collection of non-fluorescent cells that are stuck to fluorescent cells, and is carried out on the basis of side scatter (SSC) which is a measurement of granularity (Figure 3.3 B&H). From the singlets it was possible to gate out any DAPI positive cells (Figure 3.3 C&I), which

correspond to dead cells. Dead cells will have permeabilised membranes which will take up the DAPI. This was performed to aid culturing of the cells after the sort. Then from the remaining cells the highly fluorescing ones (Figure 3.3D) were selected as our CD56+ fraction.

As can be seen from the gate areas of the collected CD56+ and CD56- cells (Figure 3.3 F&L), even without the antibody a few auto-fluorescing cells are visible. This will mean that a few CD56- cells will have been collected in the CD56+ fraction. However, the vast majority of the cells collected were CD56+.

While all patients were originally due to undergo three biopsies, this has not been possible in all cases. Patients 1 and 4 dropped out of the trial before the final biopsy. The sample obtained from patient 8's first biopsy was not sufficient to undergo FACS sorting, and patients 6, 7, and 8 will undergo further biopsies in the coming year. In addition there was not always enough of the sample to provide muscle for the Newcastle MRC Centre for Neuromuscular Disease Biobank, and consequently myoblasts have not always been available. The muscle sample sizes varied between patients, as did the number of cells isolated (range $0.025x10^6$ to $27.98x10^6$), and the numbers of CD56+ cells obtained (range 1251 to 36136) (Table 3.2).

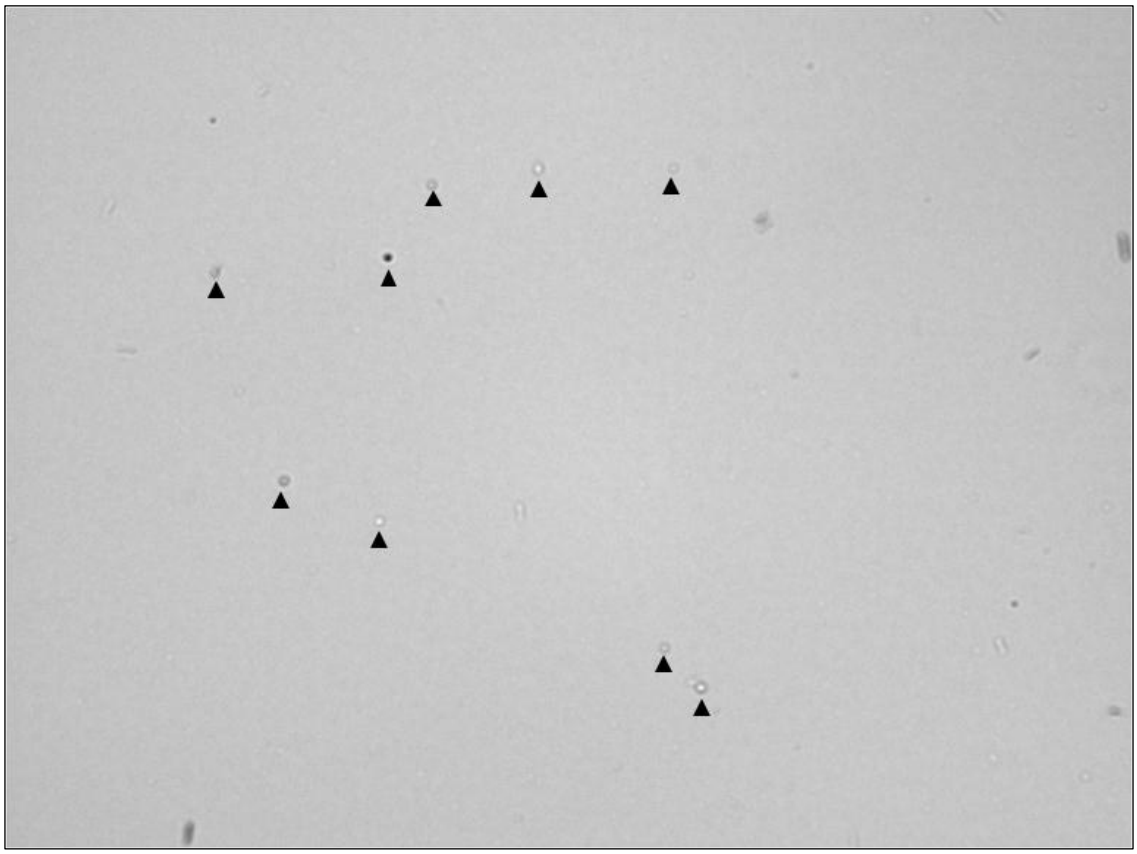


Figure 3.2 Image of cells dissociated using a Medicon and Medimachine. After dissociation the cells were counted on a Beckman Coulter Cell Viability Analyser, which captures up to fifty images of the cells. This image shows isolated cells (arrow heads), demonstrating the ability of the Medicon to give a well dissociated sample. The average diameter of the cells ranged from 3.4 to 7.99 microns with an average of 5.8 ± 1.05 microns.

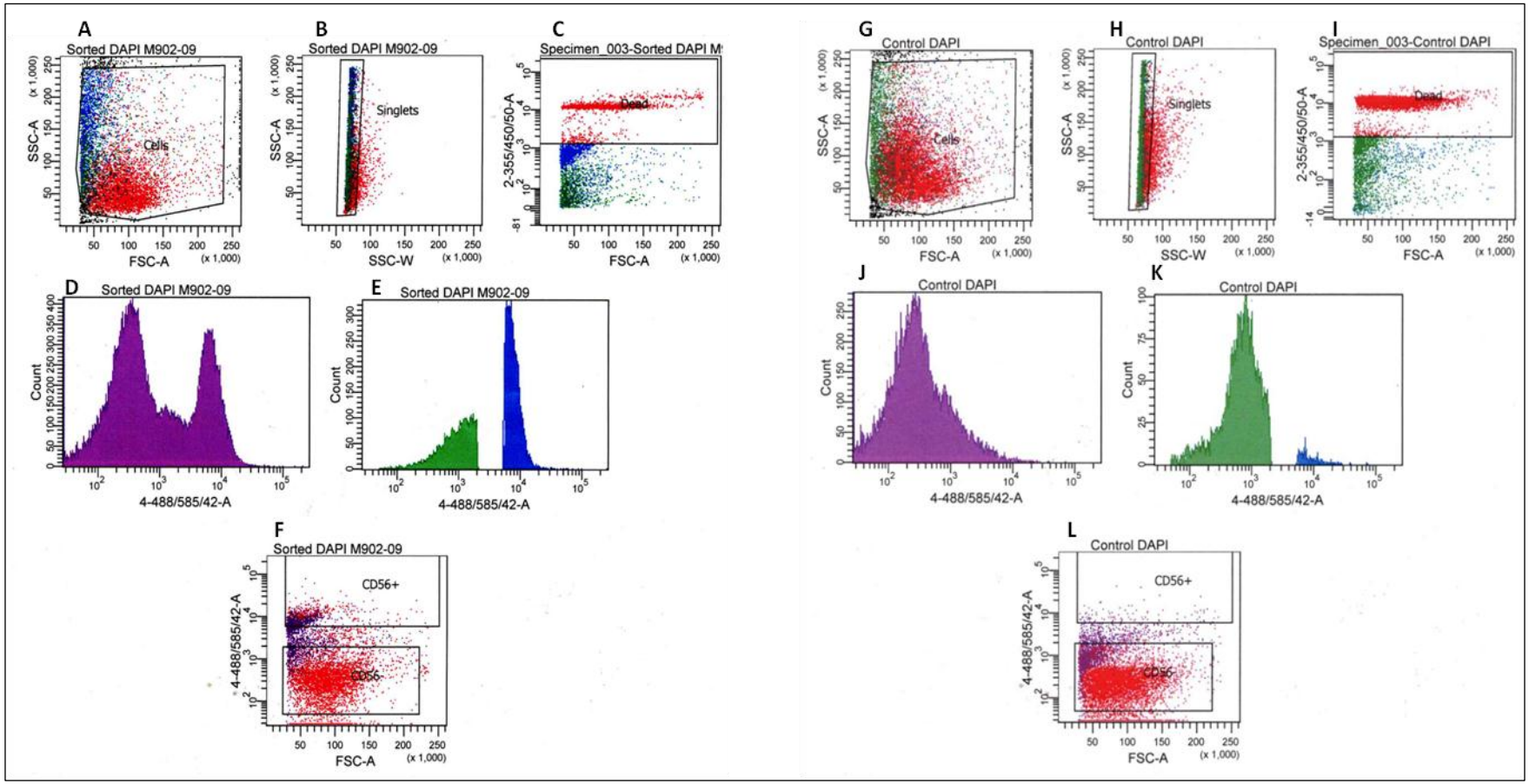


Figure 3.3 FACS sorting of CD56+ and CD56- cells. **A-F** with CD56, G-L with no CD56. **A &G** Whole cell populations with cells at the extreme end of the scales gated out. **B & H** Only cells passing through the detector as singlets were collected. **C & I** Dead cells were excluded on the basis of DAPI uptake. **D & J** Fluorescence levels (horizontal axis) of remaining cells, with **D** showing a clear highly fluorescing population of cells. **E & K** Separation of cells collected in terms of fluorescence level, the blue fraction is what was collected as CD56+. **F & L** Positioning of the gates to collect CD56+ and CD56- cells. While a clear distinction between the stained and unstained populations can be observed, some auto fluorescing cells will have been collected in the CD56+ sample. **SSC** Side scatter (an indicator of granularity), **FSC** Forward scatter (an indicator of size)**, 4-488/585/42A** Laser excitation and antibody fluorescing wavelengths, gives an indication of level of fluorescence, the further along the axis the higher the fluorescent signal.

Patient	Biopsy	Number of dissociated cells	CD56+ cells collected
1	1st	0.025x10⁶	1783
	2nd	3.09x10⁶	7919
2	1st	Not available	1810
	2nd	27.98x10⁶	3135
	3rd	0.66x10⁶	7337
3	1st	12x10⁶	4506
	2nd	1.6x10⁶	7741
	3rd	0.42x10⁶	5990
4	1st	4x10⁶	2386
	2nd	1.38x10⁶	36136
5	1st	3x10⁶	1251
	2nd	3.29x10⁶	6049
	3rd	0.61x10⁶	15710
6	1st	0.32x10⁶	2535
7	1st	0.28x10⁶	14411
	2nd	0.48x10⁶	2247
8	2nd	2.59x10⁶	33340

Table 3.2 Numbers of cells isolated and numbers of CD56+ cells acquired. Cells were dissociated and sorted on the FACS Aria at the NESCI Core FACS Facility at the Newcastle Centre for Life. Numbers of cells dissociated and collected varied from patient to patient and biopsy to biopsy.

3.4.2 Characterising CD56+ Cells

While antibodies recognising the NCam antigen have been used many times in immunohistochemistry (Mackey et al., 2007, Charifi et al., 2003, Crameri et al., 2004, Murphy et al., 2008) and in FACS sorting to isolate satellite cells (Webster et al., 1988, McKay et al., 2010), it was decided to try and characterise the CD56+ cells further.

Historically, satellite cells have been defined by their anatomical position on the periphery of the muscle fibre, however, there is ongoing debate regarding which of these cells are the "true" muscle precursor cells (Biressi and Rando, 2010). It has been demonstrated that fibre type renewal is mediated by heterogeneous fast or slow/fast type satellite cells through the expression of myosin heavy chain (MHC) (Feldman and Stockdale, 1991). Quiescent satellite cells are heterogeneous on the basis of m-cadherin (Cornelison and Wold, 1997), CD34, and Myf5 (Beauchamp et al., 2000), and when activated they display different patterns of Myf5, MyoD, and Pax7 (Cooper et al., 1999, Zammit et al., 2004). A recent study by Kuang *et al.* (2007) demonstrated that quiescent satellite cells were heterogeneous on the basis of Pax7/Myf5, and that the pattern of expression determined the expression of these factors in later stages of development. The eventual fate of these cells (myogenesis or self-renewal) was dependent on the cell's orientation under the basal laminar (Kuang et al., 2007).

3.4.2.1 Growing CD56+ Cells

One way to show these cells are myogenic is to grow them in cell culture. During the FACS sort some of the cells were sorted directly into a twenty four well plate containing skeletal muscle cell media (SKCM). Dead cells were gated out of the CD56+ fraction using DAPI (Figure 3.3C). After sorting, the plates were placed in the incubator at 37^{o}C, with 5% CO_2, and the media changed every two days. After the first attempt no myoblasts were visible on the bottom of the wells after a period of ten days.

It was thought this may be attributable to a lack of growth factors in the medium, so it was decided to use conditioned medium. In order to obtain conditioned medium control myoblasts were grown in SKCM for two days, after which the medium was removed, filtered through a 0.2µm filter and stored at 4^{o}C until required. Again, CD56+ cells were FACS sorted into conditioned medium in a twenty four well plate. This approach was combined with attempts varying the numbers of cells sorted into each well, using ninety six well plates, and coating the bottom of the wells with BD BioCoat^{TM}Matrigel^{TM} (Becton

Dickinson), to both adhere the cells to the bottom of the well, and provide extra growth factors. However, to date it has not been possible to culture CD56+ cells.

3.4.2.2 Immunohistochemistry

It was decided to try and stain CD56+ cells for Pax7, a marker of satellite cells, to give a better indication of the characteristics of the cells collected. Pax7 marks a population of activated and proliferating cells, although again with heterogeneous expression (Olguin and Olwin, 2004). It is an early marker of proliferating cells, but becomes down regulated as the cells develop (Zammit et al., 2006).

When muscle biopsies were FACS sorted, the CD56+ cells were collected in 1.5ml microcentrifuge tubes in a small amount of FACS Flow solution. The first step in the immunohistochemistry protocol was to adhere enough cells to a slide in order to perform staining. This was done using a cytocentrifuge. A coated cytoslide was placed on a filter card containing a single hole and placed in a cytoclip. The cell sample in approximately 200µl of FACS flow liquid was pipetted into a cytofunnel, which was attached to the cytoclip with the opening of the funnel placed in the hole of the filter card next to the slide (Figure 3.4). The cytoclip was then loaded into the cytorotor, and placed in the cytocentrifuge. The samples were spun at 800rpm for ten minutes. The slides were left to air dry for at least one hour, to allow any excess liquid to evaporate. When the cells were put through an immunohistochemistry protocol and mounted with DAPI Vecta-shield, many cells were visible. However, when this was repeated with a CD56+ fraction no Pax7 nuclei could be observed (Figure 3.5A).

Attempts to permeabilise the cell membranes using 1% sodium citrate, with 0.1% triton for two minutes over ice, and fixing the cells in ice cold 100% methanol instead of PFA also did not yield any Pax7 positive cells (Figure 3.5B).

In order to check if there were problems with the antibody, a muscle section was stained for Pax7 using both chromagens and fluorescence. While Pax7 worked well with a secondary antibody, ABC, and DAB (Figure 3.5C), it was not possible to see anything on the section that had been stained fluorescently (Figure 3.5D), suggesting it was something specific about the fluorescent protocol that was not working.

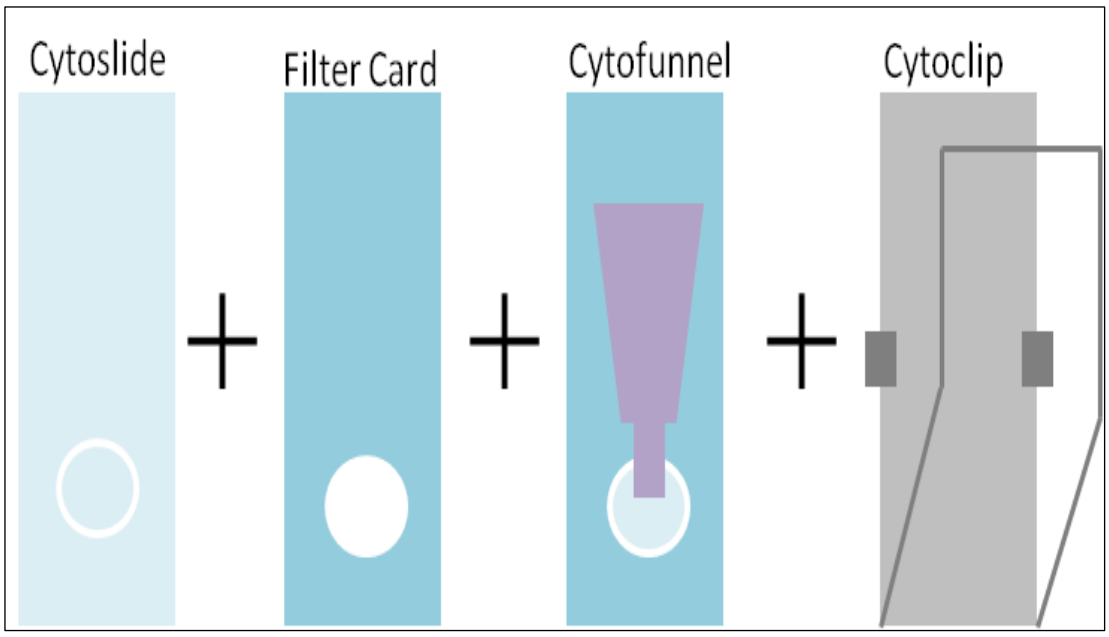


Figure 3.4 Set up of a cytospin slide. FACS sorted CD56+ cells in a small amount of liquid were placed in a cytofunnel in close proximity to a cytoslide, and then loaded onto a cytoclip and cytorotor. They were then placed in the cytocentrifuge and spun for ten minutes. The cells come out of solution and adhere to the slide, with excess liquid being absorbed by the filter card.

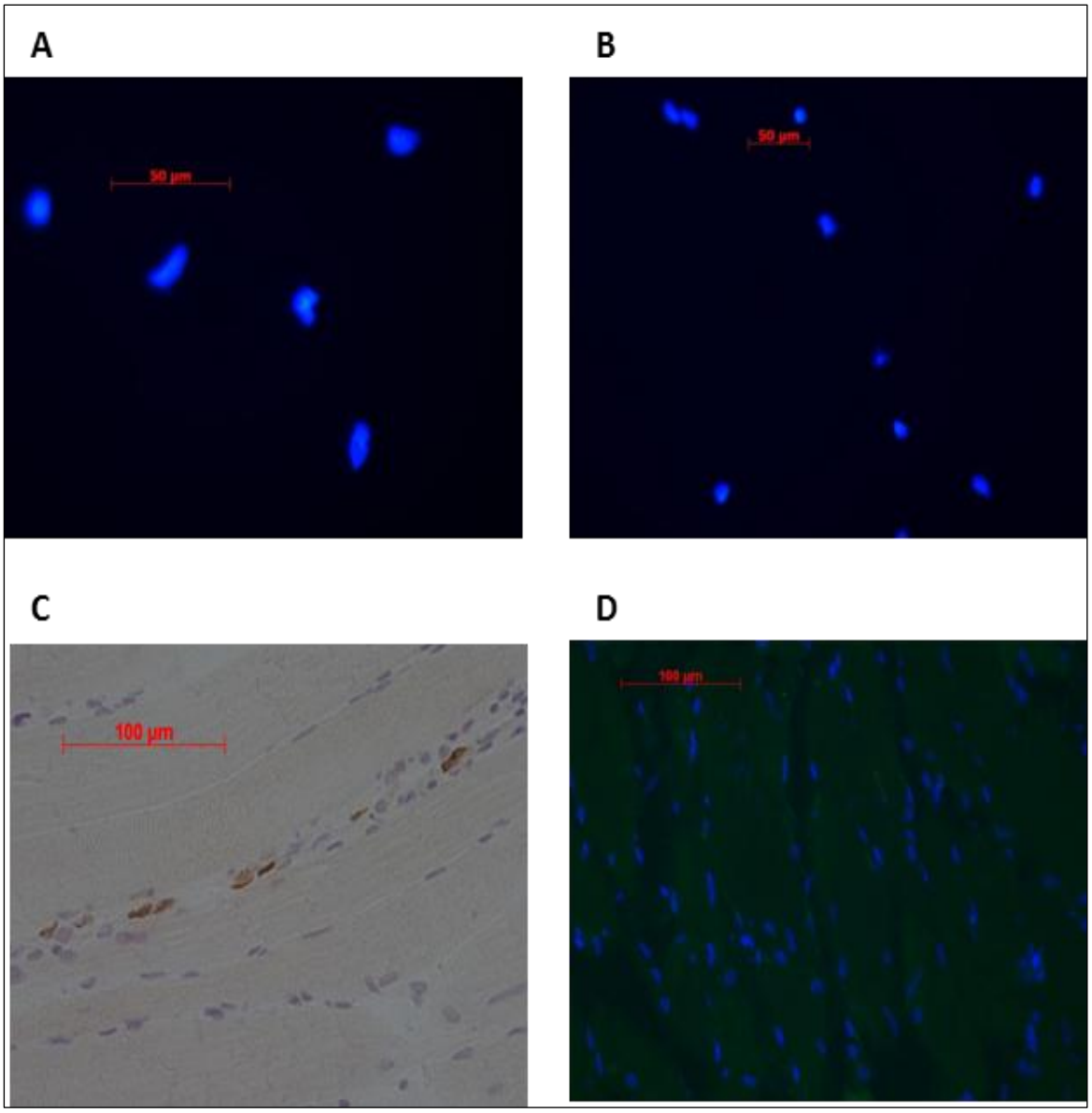


Figure 3.5 Images of cytospun FACS sorted cells. **A**. Cytospin of CD56+ onto slides resulted in many cells adhering; however, these cells did not stain for Pax7. **B**. Permeabilising the cells and fixing in methanol did not result in any Pax7 positive cells. **C**. When used with a secondary antibody and DAB on a muscle section Pax7 positive cells could be seen. **D**. But not when used on a muscle section with a fluorescence protocol and FITC. Blue = DAPI, Green = FITC.

3.4.2.3 Western Blot

It was decided to see if any Pax7 protein could be detected by Western blot. It was assumed that large amounts of cells would be required to attain enough protein, so muscle samples were obtained from orthopaedic surgery patients undergoing operations to repair their anterior cruciate ligament (ACL). This resulted in 28324 CD56+ cells and 69836 CD56- cells. These were then lysed and a Bradford assay used to determine protein concentrations. Despite the large numbers of cells used it was still not possible to obtain enough protein from either the positive or negative populations of cells to progress with the protocol.

3.4.2.4 FACS Analysis of CD56+ Cells

Recent attempts to characterise these CD56+ cells have involved fluorescently activated cell analysis. It could be possible to show that the CD56+ cells are also positive for Pax7. Due to the nuclear expression of Pax7 a permeabilisation step is required in addition to conjugating the antibody to a flurophor. In order to permeabilise the cells the Fix & Perm kit® (Invitrogen) was used, with Pax7, and Donkey IgG (BD Systems) as a secondary antibody. A control sample with no Pax7 and just secondary antibody was also analysed. With the Pax7 antibody many highly fluorescing cells were visible (Figure 3.6A). However there were also many fluorescing cells in the same region (Figure 3.6B) which would make it difficult to distinguish a true Pax7 population. Due to lack of muscle samples it has not been possible to optimise this method further.

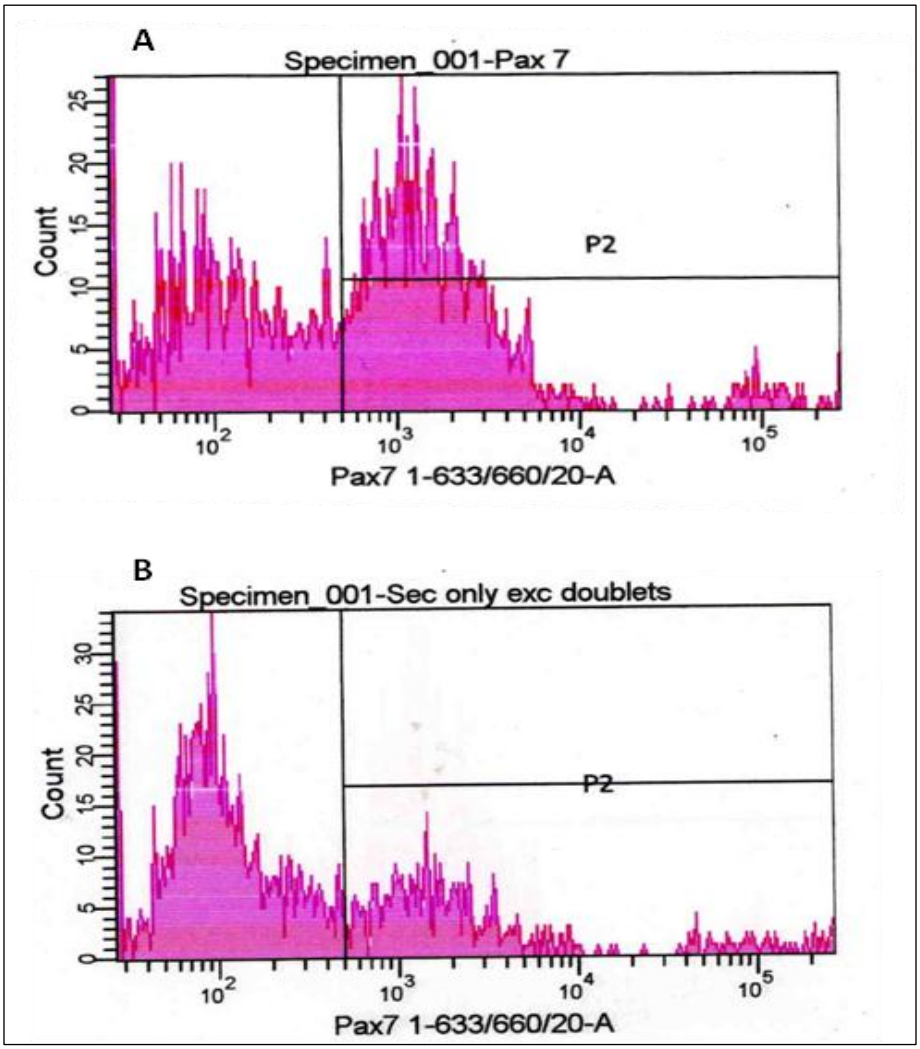


Figure 3.6 FACS analysis of Pax7 expression. **A.** A muscle sample was dissociated, fixed, permeabilised, stained with Pax7, and had a fluorescing secondary antibody applied, many cells stained with Pax7. These are visible in the top right corner marked P2. **B**. When a sample of cells with only the secondary antibody was analysed there appeared to be fluorescing cells. These are visible in the bottom right corner. While there are not as many as the sample with the primary antibody added, they are still a considerable number. These will be fluorescing because of auto-fluorescence or non-specific binding of the secondary antibody. The vertical axis denotes numbers of cells and the horizontal axis is fluorescence.

3.4.3 Single Muscle Fibre Culture: Another Method for Isolating Satellite Cells

Another way of collecting human satellite cells has been described which involves the isolation of single muscle fibres (Bonavaud et al., 2002). This paper described the use of samples obtained from the *gluteus maximus*, intercostal, and abdominal muscles after orthopaedic surgeries and post-mortems. A few days after isolating single fibres from these muscle samples, and plating in cell culture conditions, cells could be observed to migrate away from the fibre to proliferate. While this method strictly describes a method for collecting myoblasts and not satellite cells it was thought it may be useful to collect myoblasts very early on during the developmental process.

A method adapted from D. Rosenblatt (Rosenblatt et al., 1995) was used for the isolation. Briefly, a needle muscle biopsy was obtained from a patient who had been referred to the Newcastle Mitochondrial Diagnostic Service. The sample was digested using collagenase for thirty minutes; single fibres were then detached from the muscle by gentle titration with wide-mouthed pipettes. The individual fibres were serially transferred through three Petri dishes containing DMEM for washing. They were then plated on a drop of Matrigel^{TM} in a twenty four well plate with SKCM, and incubated at 37^{o}C, 5% CO_2.

After a few days, while the fibres had adhered to the Matrigel^{TM} many appeared dark and truncated, and no migrating cells were visible. It is probable that the biopsy method will have cut many of the fibres. When performing this technique it is important to get the whole fibre to avoid them hyper-contracting, it is possible this is what happened. In order to get the technique to work, larger samples should probably be used, possibly sourced from open biopsies.

This method is advantageous in that it allows for the isolation of truly myogenic cells, the separation of myoblasts derived from specific muscle fibre types, and the ability to get cultures free from fibroblasts. However, the need for large amounts of muscle and thus open biopsies is a disadvantage.

3.4.4 D-Loop Sequencing

After the biopsies were taken the muscle was divided up into three samples: for myoblast culture to be performed in the MRC Neuromuscular Biobank, molecular and histological analyses in the Newcastle Mitochondrial Diagnostic Service, and for FACS sorting at the North East Stem Cell Institute Core Facility. It was decided to sequence the D-loop of the samples once they had been brought together for this research project in order to ensure the identity of the different samples from each patient.

The D-loop, part of the control region, is a section of mtDNA that contains an extra replica of the H strand. It is formed when extension of the H strand is arrested (Doda et al., 1981). It is non-coding and contains the origin of heavy chain replication (O_H), and promoter elements for both the light and heavy strands. While the D-loop itself is conserved between mammals, the nucleotide sequence and size of the region is not.

The D-loop contains hypervariable regions and many polymorphisms. These are probably created due to the structure of the region, and persist due to the non-coding nature of the area. The variable nature of this region has led to the D-loop sequence being utilised in many fields of research, from authenticating remains of Tsar Nicholas of Russia, his wife and children (Ivanov et al., 1996), to determining the variability and origins of Sada goats in the Mediterranean area (Vacca et al., 2010).

The D-loop was therefore thought a good region to use in order to confirm the identity of samples. The D-loop region of skeletal muscle homogenate DNA, FACS sorted CD56+ cells, and cultured myoblasts, was amplified using primers corresponding to regions D1 (Forward nt.15758-nt.15777, Reverse nt.019-nt.001), D2 (Forward nt.16223-nt.16244, Reverse nt.129-nt.110), D3 (Forward nt.16548-nt.16569, Reverse nt.389-nt.370), and D4 (Forward nt.323-nt.343, Reverse nt.771-nt.752) (Table 2.1). The products were then run on the ABI sequencer and analysed with SeqScape software. They were compared to the Cambridge Reference Sequence (CRS) (Anderson et al., 1981), and any differences recorded. Sequences are shown in the following tables, patients 1, 2, and 3 (Figure 3.7), 4 and 5 (Figure 3.8), 6 (Figure 3.9), 7 and 8 (Figure 3.10).

All of the patients contained polymorphisms in their D-loop, mainly in the D1, D2, and D3 regions. Patients 2, 4, and 6 contained many differences, while 1, 3, 5, 7, and 8 contained fewer.

Patient 1

Region	D2	D3				D4
Position & CRS	16520T	152T	199T	263A	315	250A
Sample						
SKM Homogenate	C	C	C	G	C In	G
CD56+ 1st Biopsy	C	C	C	G	C In	G
CD56+ 2nd Biopsy	C	C	C	G	C In	G

Patient 2

Region	D1		D3							D4			
Position & CRS	16069C	16127T	73A	228G	263A	295C	310T	315	325	462C	250A	489T	750A
Sample													
SKM Homogenate	T	C	G	A	G	T	C	C In	X	T	G	X	X
CD56+ 1st Biopsy	T	C	G	A	G	T	C	CIn	T	T	G	X	X
Myob 1st Biopsy	T	C	G	A	G	T	C	C In	T	T	G	X	X
Myob 2nd Biopsy	T	C	G	A	G	T	C	CIn	X	T	G	C	G

Patient 3

Region	D2	D3		D4
Position & CRS	16519T	263A	315	750A
Sample				
SKM Homogenate	C	G	C In	G
CD56+ 1st Biopsy	C	G	C In	X
CD56+ 2nd Biopsy	C	G	C In	G
Myob 2nd Biopsy	C	G	C In	G
CD56+ 3rd Biopsy	X	G	C In	G
Myob 3rd Biopsy	X	G	C In	G

Figure 3.7 Differences from the Cambridge Reference Sequence (CRS). Patients 1, 2, and 3, had their D-loop sequenced and compared to the CRS. D1-4 refers to the region of the D-loop. Position & CRS refers to the nucleotide position in the mtDNA and the base pair that is present in the CRS. X denotes areas that did not sequence and the base pair is not known, C In = C insertion.

Patient 4

Region	D1			D3						D4		
Position & CRS	15836A	16069C	16126T	73A	185G	228G	252T	263A	295C	462C	489T	750A
Sample												
SKM Homogenate	X	X	X	G	A	A	C	C	T	T	C	G
CD56+ 2nd Biopsy	G	T	C	G	A	A	C	C	T	X	X	X
Myob 2nd Biopsy	G	T	C	G	A	A	C	C	T	T	C	G

Patient 5

Region	D1		D3				D4	
Position & CRS	15883C	16304T	146T	204T	263A	315	456C	750A
Sample								
SKM Homogenate	T	C	C	C	G	CIn	T	G
CD56+ 1st Biopsy	T	C	C	C	G	CIn	T	G
Myob 1st Biopsy	T	C	C	C	G	CIn	T	X
CD56+ 2nd Biopsy	X	X	C	C	G	CIn	T	X
CD56+ 3rd Biopsy	T	C	C	C	G	CIn	T	G

Figure 3.8 Differences from the Cambridge Reference Sequence (CRS). Patients 4 and 5 had their D-loop sequenced and compared to the CRS. D1-4 refers to the region of the D-loop. Position & CRS refers to the nucleotide position in the mtDNA and the base pair that is present in the CRS. X denotes areas that did not sequence and the base pair is not known, C In = C insertion.

Patient 6

Region	D1									D3							D4		
Position & CRS	15928G	15929G	16111C	16126T	16153G	16163A	16186C	16294C	16304T	73A	146T	152T	195T	204T	263A	315	456C	709G	750A
Sample																			
SKM Homogenate	A	A	C	C	G	G	T	T	T	G	C	C	C	T	G	CIn	C	A	G
CD56+ 1st Biopsy	A	A	C	C	G	X	X	T	X	G	C	C	C	T	G	CIn	C	X	X
Myob 1st Biopsy	X	A	X	C	X	G	T	T	T	G	C	C	C	X	G	CIn	X	A	G

Figure 3.9 Differences from the Cambridge Reference Sequence (CRS). Patient 6 had his D-loop sequenced and compared to the CRS. D1-4 refers to the region of the D-loop. Position & CRS refers to the nucleotide position in the mtDNA and the base pair that is present in the CRS. X denotes areas that did not sequence and the base pair is not known. C In = C insertion.

Patient 7

Region	D1				D3		D4	
Position & CRS	15884G	16224T	16245C	16311T	146T	315	497C	750A
Sample								
SKM Homogenate	A	C	T	C	C	Cln	T	G
CD56+ 1st Biopsy	A	C	T	C	C	Cln	T	G
Myob 1st Biopsy	A	C	T	C	C	Cln	T	G
CD56+2nd Biopsy	A	C	T	C	C	Cln	T	G
Myob 2nd Biopsy	A	C	T	C	C	Cln	T	G

Patient 8

Region	D1			D3					D4
Position & CRS	16042G	16096	16189T	150C	152T	263A	315	345C	751A
Sample									
SKM Homogenate	A	T	C	T	C	G	Cln	T	G
CD56+2nd Biopsy	A	T	C	T	C	G	Cln	T	G
Myob 2nd Biopsy	X	X	X	T	C	G	Cln	T	G

Figure 3.10 Differences from the Cambridge Reference Sequence (CRS). Patients 7 and 8 had their D-loop sequenced and compared to the CRS. D1-4 refers to the region of the D-loop. Position & CRS refers to the nucleotide position in the mtDNA and the base pair that is present in the CRS. X denotes areas that did not sequence and the base pair is not known, C In = C insertion.

3.4.5 MtDNA Deletion Break Point Analysis

As will be discussed in chapter 4, it was necessary to have the break points for the patient's mtDNA deletions in order to create qPCR protocols to determine mtDNA heteroplasmy levels. While many of the mtDNA deletion break points had been determined by other members of our lab, patient 1 had not been characterised.

In order to do this, a sample of the patient's muscle homogenate DNA was amplified with two sets of primer pairs 15F and 29R (Forward nt.7272-nt.7293, Reverse nt.14374-nt.14356), 16F and 30R (Forward nt.7713-nt.7723, Reverse nt.14857-nt.14838) (Table 2.1) using a long range PCR. These primer pairs would normally result in fragment sizes of 7063bp and 7115bp respectively. However, if a deletion is present in the mtDNA of these regions it would remove some of the base pairs, and smaller fragments would be generated. After running the PCR product on a 0.7% agarose gel at 50V for two hours along with a 1Kb ladder (Invitrogen) bands corresponding to fragment sizes of 3900bp (15F, 29R) and 3950bp (16F, 30R) were observed (Figure 3.11A). Given the fragment sizes that would have been generated with WT DNA (7063bp and 7115bp); this suggested the patient harboured an mtDNA deletion of approximately 3160bp.

The entire PCR sample was then run on an agarose gel. The mtDNA bands excised, purified using the Qiagen Gel Extraction kit, and then run on the ABI 3130xI Sequencer.

However, this fragment did not sequence, probably due to its large size, so primer pairs generating a smaller fragment were used. Once the primer binding site is inside the deletion area it will not bind and thus not elongate, and no PCR product will be generated. Using primers 20F and 27R (Forward nt.9607-nt.9627, Reverse nt.13453-nt.13435) generating a 3808bp fragment, a product was observed of approximately 600bp (Figure 3.11B). This band was gel extracted and run on the sequencer to determine the brake point. A 3693bp mtDNA deletion was observed between nt.9756-nt.13449, with a 13bp indirect repeat (5' repeat CTCCCTTCACCAT, 3' repeat CTCCCTCACCAT). The 5' repeat was removed by the deletion.

This breakpoint was added to that of the other patients (Table 3.3) and used to create mtDNA deletion specific qPCR.

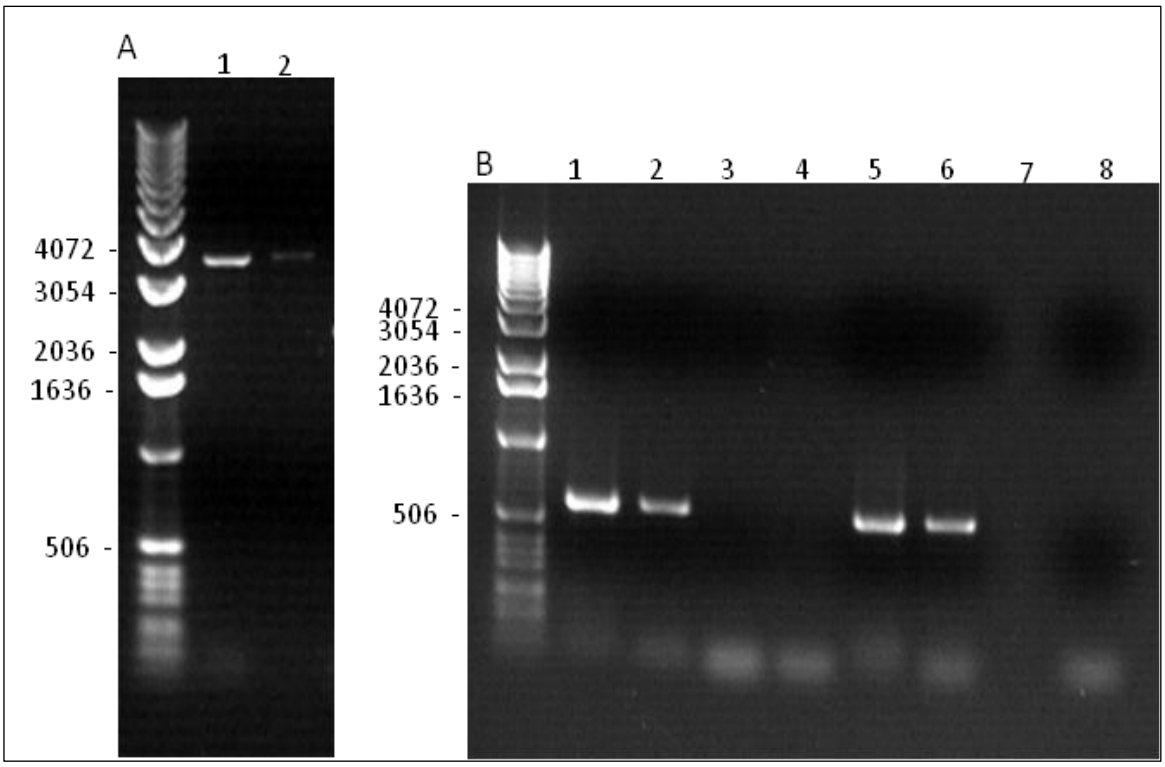


Figure 3.11 Generating a PCR fragment for sequencing analysis. A. A sample of patient muscle homogenate mtDNA was amplified using primers 15F & 29R (Lane 1) and 16F & 30R (Lane 2). In WT mtDNA these primers would generate fragments of 7063bp and 7115bp respectively. The bands observed here correspond to fragments of around 3900bp, which suggests the patient harbours an mtDNA deletion of approximately 3160bp. **B**. A second set of primers was used to amplify smaller fragments of around 4000bp. Bands corresponding to fragment sizes of ~600bp were observed with primer pairs 20F & 28R (lane 1), and 20F & 27R (Lane 2), again suggesting an mtDNA deletion of around 3200bp. However when the primers were stepped in to 20F & 26R (lane 3) and 20F & 25R (lane 4) no products were visible as the reverse primer was inside the deletion break point. +Lanes 5 & 6 = WT DNA with primers 17F&R and 20F & R respectively (giving a 600bp fragment). Lane 7 = Empty, Lane 8 negative control (20F & 20R).

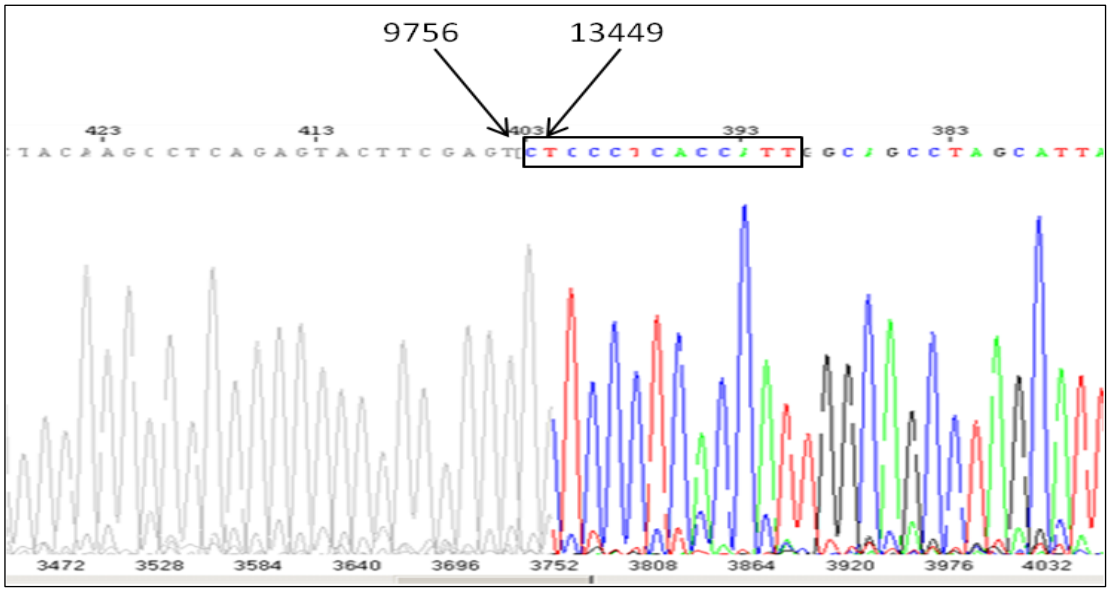


Figure 3.12 Electropherogram of patient 1's break point. When run on the sequencer, a breakpoint at nucleotide 9756 to 13449 was observed. The coloured section defines where the software was able to match up the sequence to the CRS, but then the deletion resulted in the next sequence being unaligned and therefore in grey. The sequence in grey corresponds to that leading up to nucleotide 9756. The break contained an indirect 13bp repeat (Boxed).

Patient	Deletion Size	Deletion Break Point
1	3693	9756-13449
2	4978	8469-13447 (CD)
3	4113	11262-15375
4	4978	8469-13447 (CD)
5	7959	7845-15440
6	8039	7637-15676
7	4978	8469-13447 (CD)
8	5340	6714-12054

Table 3.3 Molecular information on mtDNA single deletion patients. All patients harboured a large scale single deletion of the mtDNA in the major arc region. Three of the patients had the common deletion (CD). Patients 3, 5, and 6 had break points determined by the Newcastle Mitochondrial Diagnostic Service, patients 2 and 4 by Dr. J. Murphy and patients 7 and 8 by Georgia Campbell.

3.5 Discussion and Future Work

The use of exercise therapy in patients with sporadically occurring mitochondrial myopathies is currently being investigated in many labs (Murphy et al., 2008, Jeppesen et al., 2009, Jeppesen et al., 2006b, Cejudo et al., 2005, Taivassalo et al., 2001). The theory behind the use of resistance training in myopathy patients with sporadically occurring mtDNA mutations relies somewhat on the characteristics of muscle satellite cells. It is therefore very important that we are able to examine these cells before any changes could occur in the population through selection.

FACS sorting on the basis of CD56 allowed isolation of a cell population that has not been examined in these patients before, as previous experiments have focused on myoblasts, the cultured progeny of these cells. Further analysis of these cells should provide an indication of their mtDNA deletion heteroplasmy levels. In addition FACS sorting allowed isolation of cells in sufficient numbers to perform molecular studies characterising mtDNA deletion break points and D-loop sequencing.

While CD56 has been shown to be a proximal marker of commitment to myogenic differentiation (Capkovic et al., 2008), the inability to stain the cells with Pax7 was disappointing. Though the lack of activity seen in muscle sections using the same antibody, suggests this is more of a protocol related issue rather than cell related. Characterisation of the cells using FACS analysis shows promise but needs further optimisation.

While FACS sorting has been used to characterise satellite cells in humans (McKay et al., 2010), many of the studies that have gone on to culture these FACS sorted cells have been performed in mice (Montarras et al., 2005, Pallafacchina et al., 2010). Satellite cells are extremely reliant on their stem cell niche. FACS sorting cells into medium in order to obtain a myoblast culture not only takes them away from growth factors needed to maintain the cells, but also away from any factors that may be necessary to activate the cells from their quiescent state. In addition, the passage through the FACS machine may damage surface markers on the cells making it difficult for them to become established in the wells. Further attempts to culture CD56+ cells could use a fibroblast feeder layer.

Once CD56+ cells were isolated, the DNA was extracted and a number of experiments performed on the cells to characterise them. The sequencing of the D-loop of all the

samples from the patients allowed their identity to be confirmed when all the samples were brought together for this project.

While all patients exhibited a large scale single deletion of the mtDNA, the size and breakpoints of these deletions varied. Three of the patients harboured the common deletion while others had deletions ranging from around 3500bp to 8000bp. It was therefore highly important to characterise the deletions before any molecular analysis could be performed. The breakpoint characterisation allowed for the design of deletion specific SYBR Green qPCR assays which are described in chapter 4. In addition it showed that all the deletions occurred in the major arc region of the mtDNA, allowing for the use of the MTND1/MTND4 qPCR assay (see section 4.3.5).

Chapter 4 MtDNA Deletions in Satellite Cells

4.1 Introduction

4.1.1 Satellite Cells and Regeneration

Treatments for patients with mitochondrial myopathies are limited. Resistance exercise has long been suggested as a possible intervention for patients harbouring sporadically occurring mtDNA mutations (Taivassalo and Haller, 2004). It is proposed that resistance training can activate satellite cells which will go on to regenerate muscle, and in the presence of heteroplasmy, change the balance of WT to mutated mtDNA (Shoubridge et al., 1997). This process has been referred to as "gene shifting".

Postnatal muscle growth (hypertrophy) and regeneration is achieved through satellite cell activation. In response to stress caused by injury to the fibre, the satellite cells activate and re-enter the cell cycle, and in a pathway that demonstrates similarities to muscle embryogenesis, repair the damage.

Quiescent satellite cells sit underneath the basal laminar and on top of the sarcolemma on the muscle fibre (Figure 4.1). In their quiescent state they have up-regulated genes that encode negative regulators of the cell cycle, including Rgs2 (regulator of G-protein signalling2), and Cdkn1c (cyclin-dependent kinase inhibitor 1C). They have up regulated myogenic inhibitory molecules, for example Bmp4 (bone morphogenic protein 4), and Notch molecules which are negative regulators of MyoD expression. In addition, they exclusively express the calcitonin receptor which further inhibits their activation (Fukada et al., 2007).

The activation of satellite cells is probably initiated by a number of factors including hepatocyte growth factor/scatter factor (HGF/SF) (Tatsumi et al., 1998), and nitric oxide (Anderson et al., 2002). Mechanical stretch has been shown to stimulate both these factors (Tatsumi et al., 2006), suggesting a possible mechanism for contraction induced injury and satellite cell activation. In addition there may be a role for inflammatory cells that flock to the site of injury in order to phagocytose the debris (Ten Broek et al., 2010).

Following activation, the cells become proliferating myoblasts under the control of Myf5 and MyoD. During this process some cells become Pax7+MyoD- and return to quiescence in order to replenish the stem cell pool. While Pax7+MyoD+ cells become committed to a

muscle cell fate through differentiation (Tedesco et al., 2010). This is followed by down regulation of Pax7 in the myoblasts and up regulation of the myogenic regulatory factors, myogenin and Myf4. These myoblasts then fuse to each other to form myotubes which are able to fuse into the damaged fibre to repair it (Figure 1.20).

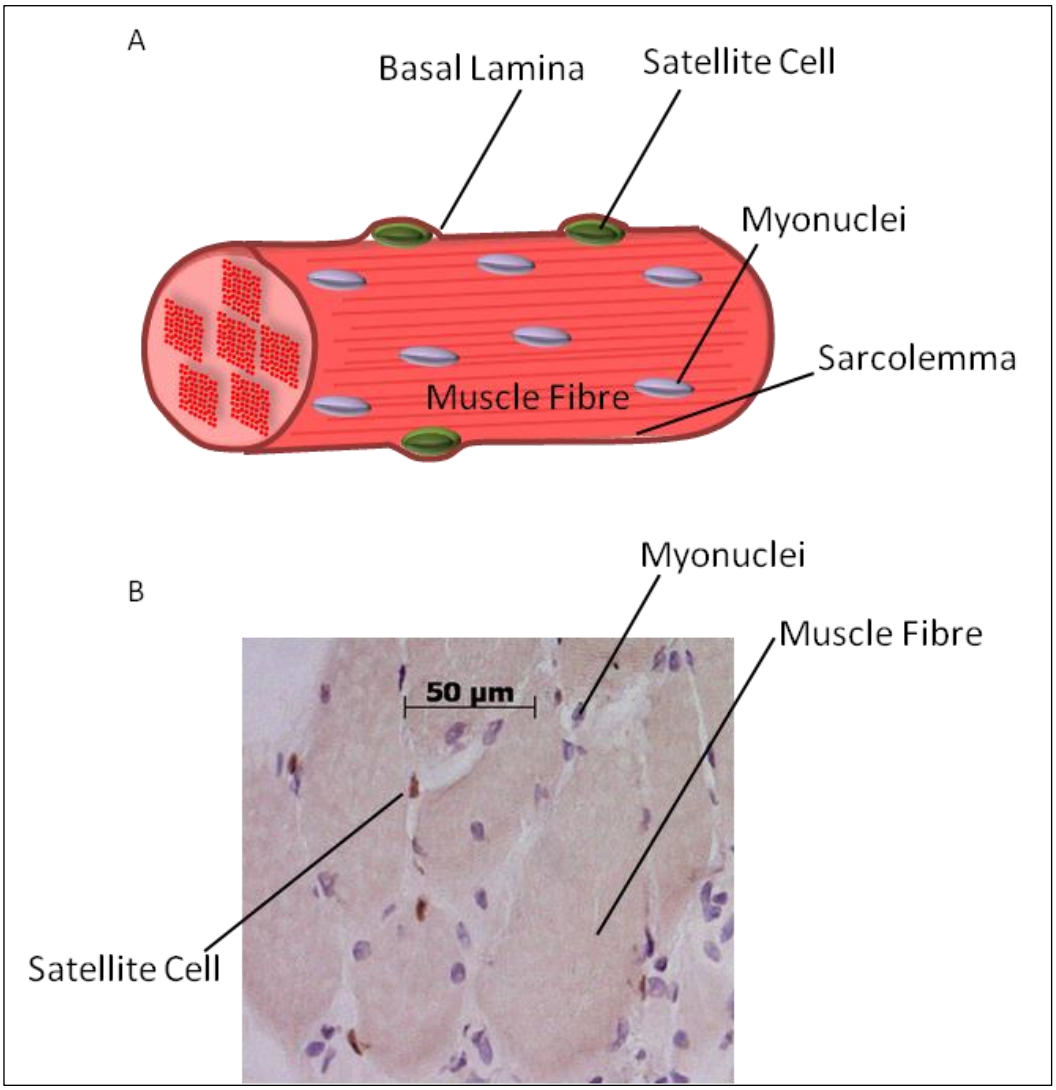


Figure 4.1 Satellite cells. Satellite cells sit on the periphery of the fibre, in a "satellite" position. **A**. They lie under the basal laminar and on top of the sarcolemma. **B**. In transverse sections they can be seen in between the muscle fibres with Pax 7 staining. Brown= satellite cells (DAB), purple = myonuclei (haematoxylin).

4.1.2 Rationale for Exercise Training

4.1.2.1 Early Investigations

The rationale for resistance training as a potential therapeutic intervention comes from early experiments examining patients harbouring sporadically occurring mutations. In 1996 Fu *et al.* described a male patient who presented with CPEO, ptosis, pigmentary retinopathy, and sensorineural hearing loss. When a muscle biopsy was performed, RRFs were present. Molecular analysis showed a sporadically occurring novel point mutation in the mt-tRNA$^{leu(CUN)}$ gene, causing a heteroplasmic G to A transition at conserved position nt.12315. The mutation was present in the muscle at 94%, but was undetectable in blood samples and cultured fibroblasts, suggesting that the mutation had been lost from dividing tissue. Interestingly, when the group cultured myoblasts from this patient no mtDNA point mutation was detected. It was suggested that the mutation may not be present in the patient's satellite cells.

They concluded that the mutation had accumulated in the muscle due to its post mitotic nature, but had been lost by random genetic drift in mitotic cells, fibroblasts and blood. They further suggested that this pattern may be a feature of sporadically occurring conditions. It was hypothesised that by activating satellite cells and incorporating them into mature muscle, the heteroplasmy level could be favourably changed, therefore improving symptoms (Fu et al., 1996).

This study was followed a year later by another group also reporting a patient with a sporadically occurring point mutation (Weber et al., 1997). This patient presented in her mid-thirties with joint pains, progressive proximal weakness, and mild ptosis. Biopsy analysis demonstrated RRFs, and COX negative fibres. Molecular investigations demonstrated an A to G transition at a conserved position (nt.12320), in the mt-tRNA$^{Leu(CUN)}$ gene. The patient had undergone a number of muscle biopsies and harboured the mutation at high levels in this tissue. Over a twelve year period her heteroplasmy level had risen from 69% to 90%. In agreement with Fu *et al.* (1996), they also could not detect the mutation in fibroblasts or blood. In addition, myoblasts grown from one of the biopsies did not harbour the mutation, again raising the possibility that satellite cells contain little or no mutation.

The previous two papers described no mutation in satellite cells, fibroblasts and blood from patients harbouring sporadically occurring mtDNA point mutations. However, other papers have reported lower levels of deletions in fibroblasts, myoblasts and blood cells from patients with KSS (Moraes et al., 1989a, Moraes et al., 1989b). While deletions often occur sporadically (Chinnery et al., 2004) these papers do not state if that was the case here.

These findings opened up the possibility of changing the amount of WT to mutated mtDNA in the muscles of patients, in a process termed "gene shifting". It was suggested that this could be performed through the stimulation and incorporation of satellite cells that contain none or very little of the disease causing defect. This theory was tested by two groups in 1997.

4.1.2.2 Testing the Hypothesis

The first group (Clark et al., 1997) described the use of bupivicaine hydrochloride to induce muscle necrosis in the left *vastus lateralis* of their previously described patient (Weber et al., 1997), in the hope of "gene shifting". Bupivicaine disrupts calcium homeostasis and perturbs the plasmalemma membrane, eventually leading to fibre necrosis. But muscle satellite cells are resistant to this effect. They waited three weeks to allow for satellite cell regeneration of the damaged area, and then removed a sample of the previously injected muscle. While the pre-intervention muscle biopsy contained approximately 75% COX negative fibres, the follow up biopsy not only showed evidence of muscle regeneration, but many of these regenerating fibres were COX positive. Single fibre PCR analysis showed these regenerating COX positive fibres to have little or no mutation.

This study was followed a few months later (Shoubridge et al., 1997) again by a group investigating the effects of instigating muscle regeneration on a previously reported patient (Fu et al., 1996). This time muscle necrosis was initiated by a muscle biopsy of the right biceps. Three weeks later the same site was biopsied again. In regenerating fibres, determined by NCam reactivity, mutant mtDNA was undetectable. It was highlighted however, that the assay used to determine mtDNA mutation load had a sensitivity of greater than one percent, leaving open the possibility that the mutations persisted at levels lower than this.

4.1.3 Possibilities for the Absence of Sporadic Mutations

There are a number of reasons why sporadically occurring mtDNA mutations may not be present in satellite cells of affected patients.

These are sporadically occurring mutations and it is possible that they developed after the satellite cells formed. However, the finding of a sporadically occurring mtDNA deletion in both brothers of a set of identical twins (Blakely et al., 2004) suggests deletions are probably present in the zygote. In addition research has suggested that around 50% of oocytes contain mtDNA with the common deletion, with 0.01% to 0.1% of total mtDNA examined harbouring this 4977bp deletion (Chen et al., 1995). This suggests the mutations did not arise at a later stage of development.

While the mutations were probably present at the beginning of embryogenesis, it is possible that the mutations segregated to the muscle but not the satellite cells. However, the common dermomyotome origin of limb skeletal muscle and satellite cells (Gros et al., 2005), coupled with reports demonstrating that single deletions segregate to many tissues during development (Hurko et al., 1990, Marzuki et al., 1997, Brockington et al., 1995, Pistilli et al., 2003) suggest this is probably not the case.

The above papers did however report lower amounts of mtDNA deletions in mitotic tissue, suggesting that the mutations had originally been present in all tissue but then lost from mitotic cells. While satellite cells do have the ability to divide, they are mitotically quiescent until activated. It is doubtful that they would have undergone enough divisions in the process of repairing muscle and renewing the satellite cell pool to have lost the mutation. Although, it is possible that the myopathic nature of mitochondrial disease could have resulted in more muscle regeneration in these patients and thus more satellite cell divisions. In addition, while we know satellite cells themselves are quiescent, the status of mtDNA replication in the cells is not known, so it is possible that some selection may be acting at the mtDNA or mitochondrial level.

An alternative explanation for the absence of mtDNA mutations in myoblasts from patients with sporadically occurring mitochondrial diseases could be that the mutations were present in the satellite cells but were lost through selection before the harvesting of the myoblasts.

4.1.4 Inducing Muscle Regeneration

While experiments demonstrating the feasibility of "gene shifting" were very successful, the methods employed to initiate muscle necrosis are not workable in a clinical setting due to their invasive nature. For this reason, resistance exercise performed in a way to induce muscle hypertrophy has been suggested as an alternative (Shoubridge et al., 1997).

Muscle can contract in a number of ways. Concentric muscle contractions are when the muscle shortens while active and eccentric contractions involve the muscle lengthening while being active (Figure 4.2). For example, during a bicep curl the bicep muscle would be contracting concentrically when being raised or brought closer to the shoulder, and when being lowered the bicep would be contracting eccentrically

It has been shown that a single bout of eccentric exercise is sufficient to elicit a significant satellite cell response in males (Dreyer et al., 2006, Crameri et al., 2004), although the exact mechanism by which this happens is not fully known. It is thought that satellite cells may be activated by exercise alone, through ultrastructural damage, the release of inflammatory substances or via exercise-induced release of growth factors (Kadi et al., 2005).

In healthy subjects, eccentric muscle contractions induce more pain, soreness, morphological abnormalities, and sarcomere disruption than concentric contractions (Newham et al., 1983). The appearance of serum CK twenty four hours after eccentric exercise suggests that there has been a breakdown in the cell membrane surrounding the muscle fibre (Friden and Lieber, 2001). It is though this damage will activate satellite cells to repair the muscle and hopefully incorporate mitochondria containing WT mtDNA.

It is possible that damage may not be essential to stimulate a satellite cell response. A recent study demonstrated that muscle injury (determined by serum CK) was not required to induce hypertrophy in subjects undergoing an eccentric exercise program. However, since the study was not conducted alongside muscle biopsies it is difficult to ascertain if any micro-damaged occurred in the muscle that would have activated satellite cells but not resulted in the release of CK (Flann et al., 2011). The myonuclear domain hypothesis suggests that after repeated bouts of resistance training the muscle fibre area increases. The up-regulation of myonuclei transcriptional activity can initially compensate

for this, but further hypertrophy requires the activation and incorporation of satellite cells, which occurs independently of muscle damage (Kadi et al., 2005).

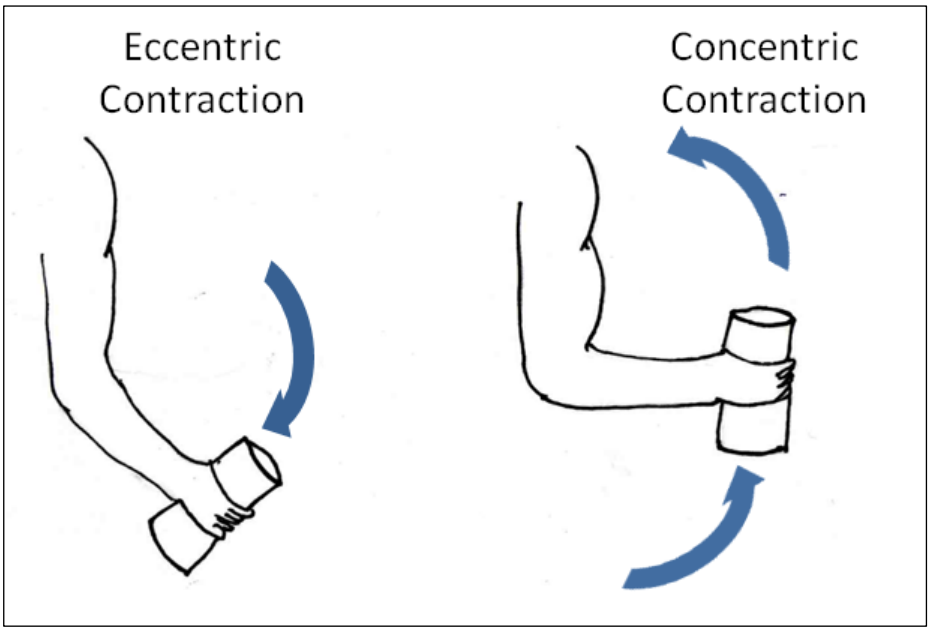


Figure 4.2 Muscle contraction. When the bicep muscle lengthens while holding a weight it is contracting eccentrically. This type of contraction is more likely to damage the muscle fibres. When the bicep shortens while lifting a weight, for example during a bicep curl, it is contracting concentrically.

4.1.4.1 Resistance Training in Mitochondrial Myopathies

One of the earliest studies examining the effects of resistance training in a patient with a sporadically occurring mutation, showed a decrease in COX negative muscle fibres and an increase in WT mtDNA, in muscle exercised both concentrically and eccentrically (Taivassalo et al., 1999). A more recent study involving patients with sporadically occurring single mtDNA deletions also found a reduction in COX negative fibres coinciding with an increase in COX intermediate fibres. However, there was no significant change in muscle heteroplasmy level (Murphy et al., 2008). It may be possible that the protocol performed was not of sufficient intensity to elicit enough damage and satellite cell incorporation to "gene shift".

4.2 Aims

Studies suggesting absent or low levels of mutant mtDNA in the muscle stem cells of patients harbouring sporadically occurring mtDNA mutations have involved culturing satellite cells and then examining their progeny for the genetic defect. This lapse in time could allow for a period of selection to act on the cells and for mutated mtDNA to disappear completely or reduce to low levels. Studies showing the feasibility of gene shifting (Shoubridge et al., 1997, Clark et al., 1997) have also looked at muscle characteristics after a period of regeneration, again allowing for selection to act on the developing cells.

The aims of this chapter were to look for mtDNA deletions in the satellite cells of patients before any selection could occur. In order to do this a number of mtDNA deletion specific SYBR Green qPCR assays were designed and optimised. This chapter describes in detail the optimisation of one of these assays and explains the development of two other SYBR Green assays. It was hypothesised before starting this work that there would be very little or no mutation in the satellite cells of these patients.

4.3 Methods Optimisation and Results

While it would be possible to detect mtDNA deletions using a traditional PCR assay, followed by running the products on an agarose gel, this method has limitations. Traditional PCR can determine if a product is present or not, it is an end point assay, and cannot give any indication of how much product is present. The use of a real time PCR (qPCR) method allows for DNA detection during the exponential phase of the PCR reaction. The amount of DNA present in this phase of the reaction is proportional to the amount of starting DNA. The value obtained for the assay is a cycle threshold (Ct) value, which is the point when the fluorescence level crosses a threshold value during the exponential amplification of DNA (Figure 4.3).

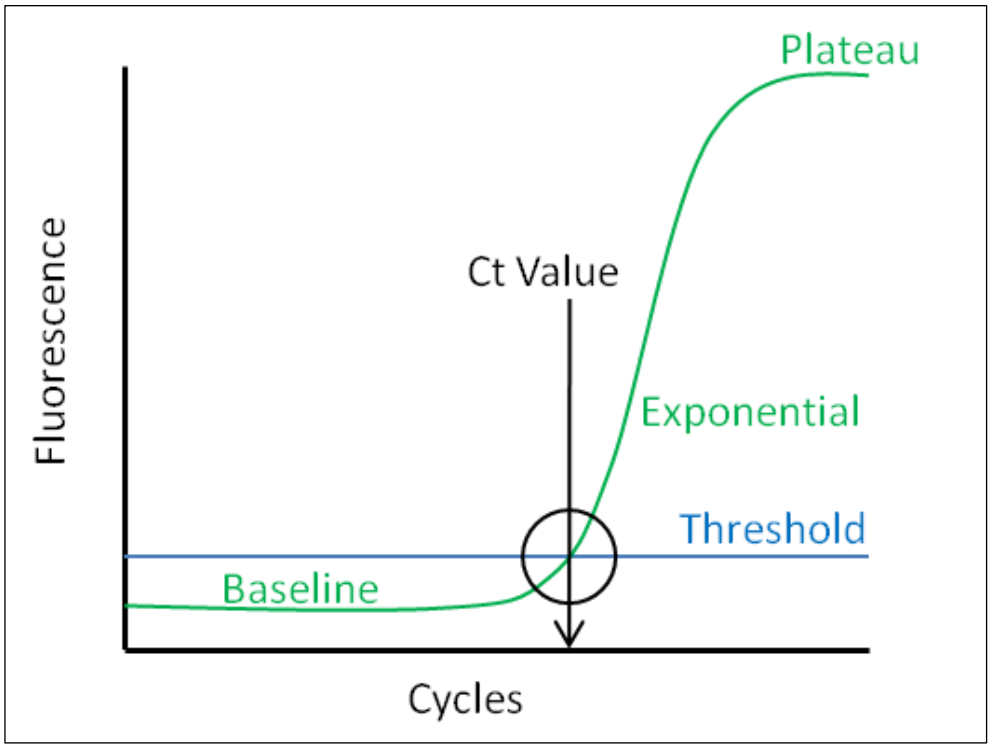


Figure 4.3 Real time PCR (qPCR). Traditional PCR visualizes DNA at an endpoint, or at the plateau phase of amplification, it cannot give an indication as to how much DNA is present. Real Time PCR measures fluorescence generated by a fluorescent dye during the exponential phase of a PCR reaction. It results in a cycle threshold (Ct) value, which is the number of PCR cycles needed before the fluorescence crosses a threshold value.

4.3.1 SYBR Green Assay

Due to previous reports suggesting little or no sporadically occurring mtDNA mutation in satellite cells (Fu et al., 1996, Weber et al., 1997), it was decided to use an extremely sensitive qPCR method. SYBR Green is a fluorescent dye that binds to the minor groove of all double-stranded DNA formed during a PCR reaction, for this reason it is extremely sensitive. When bound to double stranded DNA the intensity of its fluorescence increases (Figure 4.4). As more double stranded products are created the SYBR Green signal will increase. Due to its sensitivity, it is possible to detect single mtDNA deletions below 1%. In addition it allows for the design of assays specific to each mtDNA deletion. Its sensitivity and ability to work at levels below 1% made it an advantageous method to use. However, because of its sensitivity the assay requires a well optimised reaction. Two mtDNA deletion specific qPCR assays have been designed and optimised for this study and a further assay for patient 3 was re-optimised.

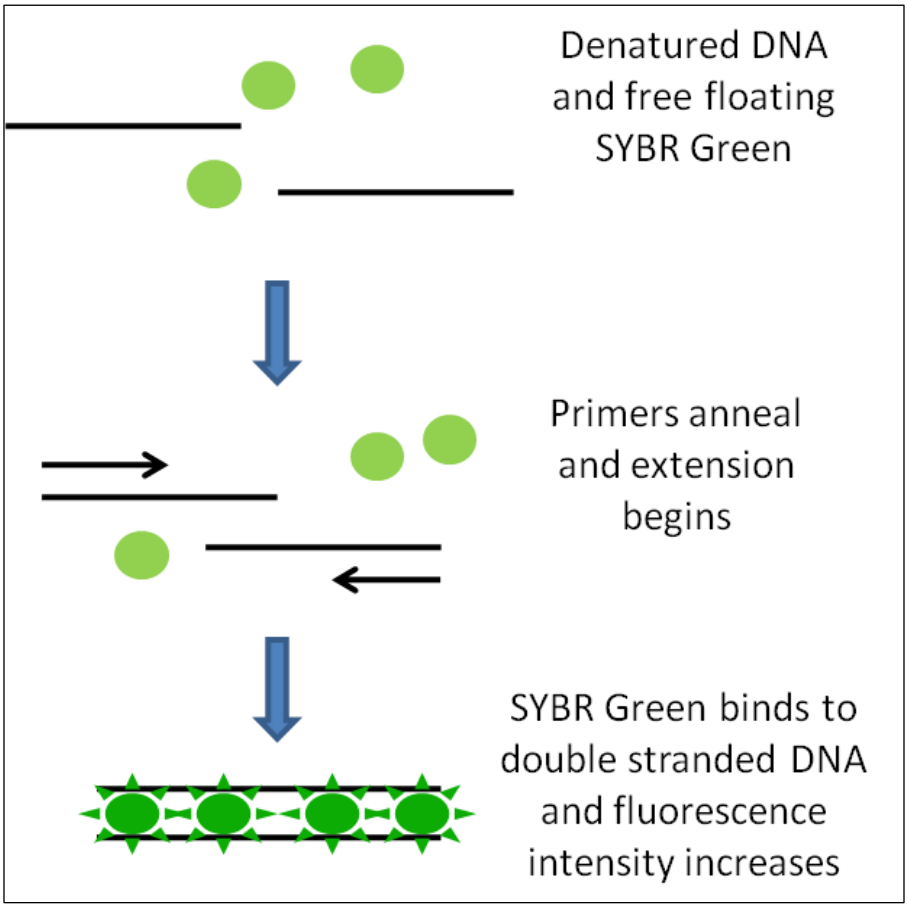


Figure 4.4 SYBR Green. SYBR Green is a fluorescent dye that binds to double stranded DNA. The signal generated is proportional to the amount of DNA present in the reaction and can be quantified using qPCR instruments.

4.3.2 Patient 3

Patient 3 had previously been reported in our lab (Blakely et al., 2004). He presented with bilateral ptosis, and a mild proximal myopathy. A Southern blot analysis of his muscle showed a large scale single deletion of 4115bp between nt.11262 and nt.15375. A SYBR Green assay was optimised and revealed the deletion was present at 66% in his muscle homogenate. Interestingly his identical twin also had the same deletion but at 0.016%. In order to investigate the CD56+cells isolated in chapter 3 from this patient it was decided to use the previously optimised SYBR Green assay (Blakely et al., 2004).

4.3.2.1 Generating Template DNA

SYBR Green binds to all double stranded DNA, so it was necessary to set up two reactions, one to amplify WT mtDNA, and another to amplify delete mtDNA. In order to optimise these two reactions template DNA was generated. Two pairs of primers were chosen to amplify a 600bp fragment of either WT mtDNA (Forward nt.10958-nt.11004, Reverse nt.11605-nt.11586) or mtDNA containing a deletion (Forward nt.10985-nt.11004, Reverse nt.15681-nt.15661) (Figure 4.5A & B). The entire PCR sample was run on a 1% agarose gel (Figure 4.5C), the bands excised and the PCR products purified using the Qiagen Gel Extraction Kit. The amount of DNA in the sample was then determined using the NanoDrop® Spectrophotometer. The WT mtDNA was 25.4ng/µl, and the delete mtDNA was 10.1ng/µl.

The template DNA was then serially diluted in dH_2O to give dilutions of 1:10, 1:100, up to 1:10,000. This was done in order to create a standard curve of DNA which could be used to optimise the WT and delete mtDNA reactions.

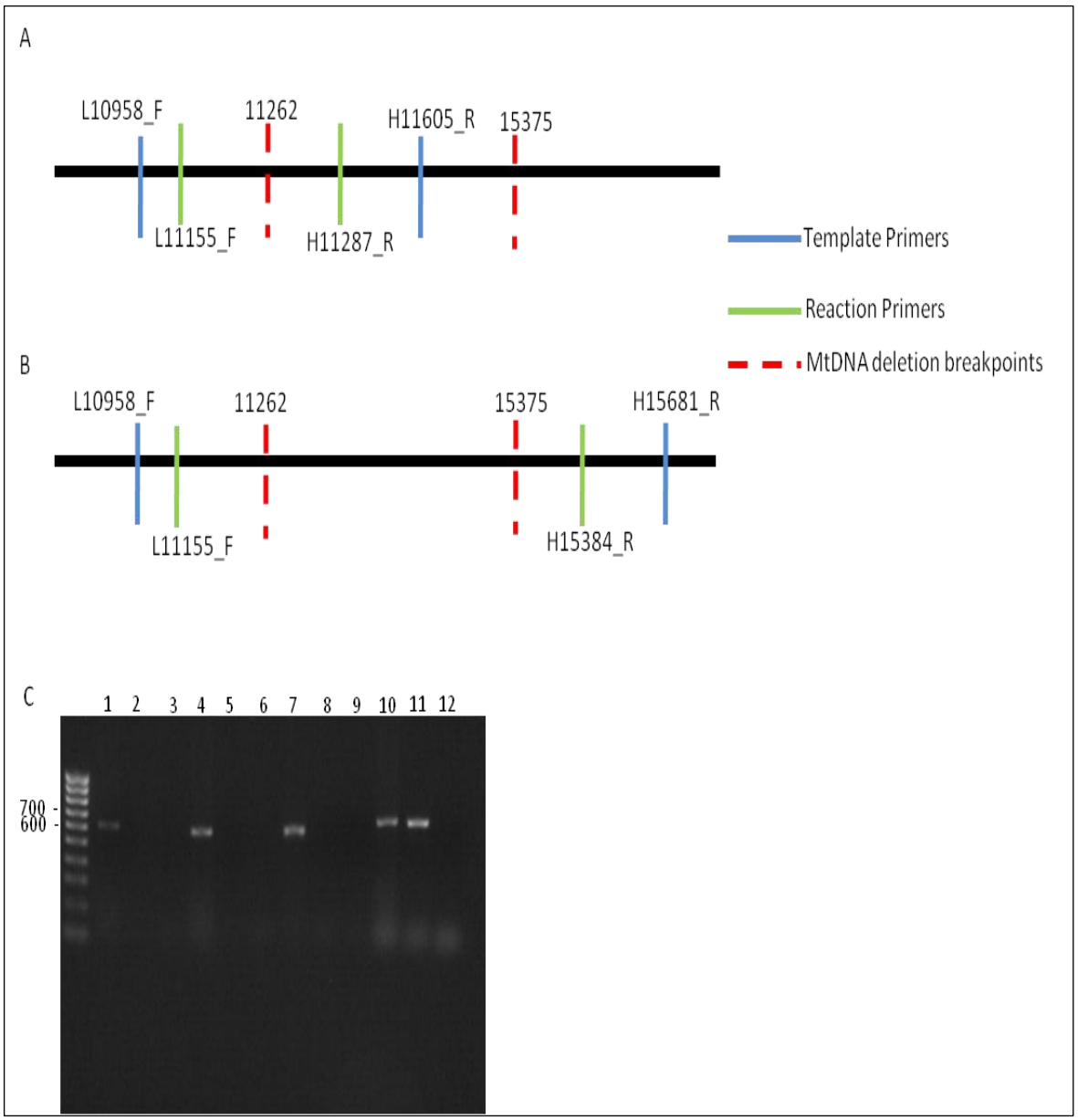


Figure 4.5 Generating template DNA. Primer pairs (blue) were chosen to amplify an approximately 600bp region of either WT **(A)** or delete **(B)** mtDNA. This was to generate template mtDNA that could be used to optimise the SYBR Green assay. Primers chosen to anneal to regions within the breakpoints (red) of the deletion will not amplify delete mtDNA and will therefore give a 600bp fragment of WT mtDNA, while primers chosen to anneal to either side of the breakpoint will only amplify deleted mtDNA due to the extension time and PCR conditions, resulting in a 600bp fragment that incorporates the mtDNA deletion. Green indicates the primers used for the final SYBR Green assay when analysing the patient's samples. These reactions were set up in parallel to amplify WT **(A)** and delete **(B)** mtDNA. **C**. Reaction products were run on a 1% agarose gel showing the generated fragments were approximately 600bp. The products were then gel extracted and used as template mtDNA on which to optimise the final reactions. Lanes 1, 4, and 7, contained primers to amplify delete mtDNA and a sample of patient 3's muscle homogenate. Lane 10 contained primers to amplify WT mtDNA and patient 3's muscle homogenate. Lanes 2, 5, 8 and 11 contained WT control DNA, and lanes 3, 6, and 9 contained water.

4.3.2.2 Optimising the Template DNA Reactions

WT and delete reactions were set up as previously described (Blakely et al., 2004). Two master mixes were created, one with primers to amplify delete mtDNA (Forward nt.11155-nt.11173, Reverse nt.15384-nt.15366) and one with primers to amplify WT mtDNA (Forward nt.11155-nt.11173, Reverse nt.11287-nt.11267) (Figure 4.5A & B), all primers were at 125pmol. In addition, the master mix contained $MgCl_2$ optimised to 3mM for the WT reaction, and to 4mM for the delete reaction. All experiments were set up in 25µl volumes in LightCycler® Capillaries (Roche), to which 1µl of sample DNA was added. They were performed on a Lightcycler® (Roche) using LightCycler® DNA Master SYBR Green I (Roche). The cycling conditions were an initial denaturation step (95°C for 10 minutes), then forty five cycles of amplification consisting of a denaturation step (95°C for 10 seconds), annealing phase (64°C for 5 seconds) and extension phase (72°C for 10 seconds). A fluorescence acquisition step was included at the end of each extension phase. The reaction ended with a melt curve analysis of a rise to 95°C, followed by an annealing step of 65°C for fifteen seconds, and then a slow increase to 95°C (0.1°C/second) with a continuous acquisition of fluorescence levels.

When performed under these conditions with template DNA the WT reaction produced products that were all of very similar Ct values (Table 4.1). The melt curve analysis suggested this was because the reaction had resulted in all the Ct values being generated because of primer-dimer (Figure 4.6A). The results for the delete mtDNA reaction showed a similar pattern. While the first three concentrations of template DNA (1:100, 1:1000, and 1:10,000) produced values attributable to products (Table 4.1), the remaining dilutions all resulted in primer-dimer (Figure 4.6B). With every ten fold dilution an approximately 3.3 Ct value increase would normally be expected.

It was decided to focus solely on optimising the WT reaction. Further attempts involved adjusting the annealing temperature, varying primer and $MgCl_2$ concentrations, and adding more sample DNA, but primer-dimer persisted.

It was decided to move completely away from the cycling conditions used by Blakeley *et al.* (2004), and adjust the conditions to those given by the company supplying the LightCycler® DNA Master SYBR Green I (Roche). These were an initial denaturation step (95°C for 30 seconds), then forty five cycles of amplification consisting of a denaturation step (an increase to 95°C), annealing phase (58°C for 5 seconds) and extension phase

(72^{o}C for 5 seconds). But again no products were visible and further attempts varying reagent and sample concentrations, and performing temperature gradient checks for primer annealing temperatures were unsuccessful.

At this point it was decided to change the SYBR Green to Platinum®SYBR®Green qPCR SuperMix-UDG (Invitrogen). The master mix consisted of Platinum®SYBR®Green qPCR SuperMix-UDG (Invitrogen), $MgCl_2$ at 3mM, and primers at 125pmol. The cycling conditions were changed back to those of Blakeley *et al.* (2004), except with the annealing temperature set at 58^{o}C.

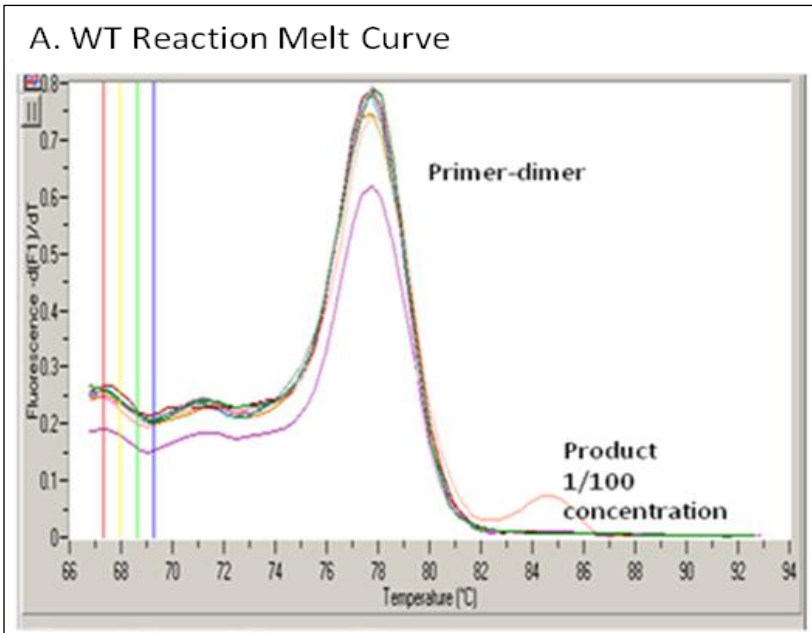
An initial attempt looked promising with peaks being observed for both dilutions run, and the melt curve showed no primer-dimer (Figure 4.7A). However, both negative samples showed small product peaks, suggesting contamination. This run was repeated with fresh reagents and fresh primers from stock solutions. This time there was no primer-dimer and no contamination (Figure 4.7B). In addition, as can be seen from the Ct values, the 1:10 and 1:100 dilutions contained large quantities of DNA. From this stage onwards, all reagents, dH_2O and primers were aliquoted and used fresh each time.

It was then possible to perform a run with the WT mtDNA dilution series in order to construct a concentration graph for the WT reaction. This would give an indication as to how efficient the reaction was (Figure 4.8A). The Ct values stretched to above thirty which meant the assay was able to detect very low amounts of DNA, and the x value for the line was close to 3.3 (3.43) demonstrating a good efficiency. The efficiency of the reaction can be determined by the slope of the line of DNA concentration plotted against Ct values. It was decided to progress to optimising the reaction for the delete mtDNA.

The delete mtDNA template DNA was re-diluted down to 1 in 10^{11}. The concentration of $MgCl_2$ and primers were as they were for the WT reaction, as were the cycle conditions with the annealing temperature at 58^{o}C. These conditions produced a standard curve that extended above a Ct of thirty and had an x value (3.46) close to that of the WT (3.43) reaction (Figure 4.8B).

Delete mtDNA dilution	Ct Value	WT mtDNA dilution	Ct Value
1/100	17.47	1/100	16.27
1/1000	20.55	1/1000	26.53
1/10,000	23.50	1/10,000	16.80
$1/10^5$	24.29	$1/10^5$	16.87
$1/10^6$	24.52	$1/10^6$	16.87
$1/10^7$	24.04	$1/10^7$	16.94
$1/10^8$	24.55	$1/10^8$	16.9
Negative	24.47	Negative	16.91

Table 4.1 Ct values for template DNA optimisation. An initial run attempting to optimise WT and delete mtDNA reactions resulted in many of the dilution series having similar Ct values, including the negative (NTC) reactions.



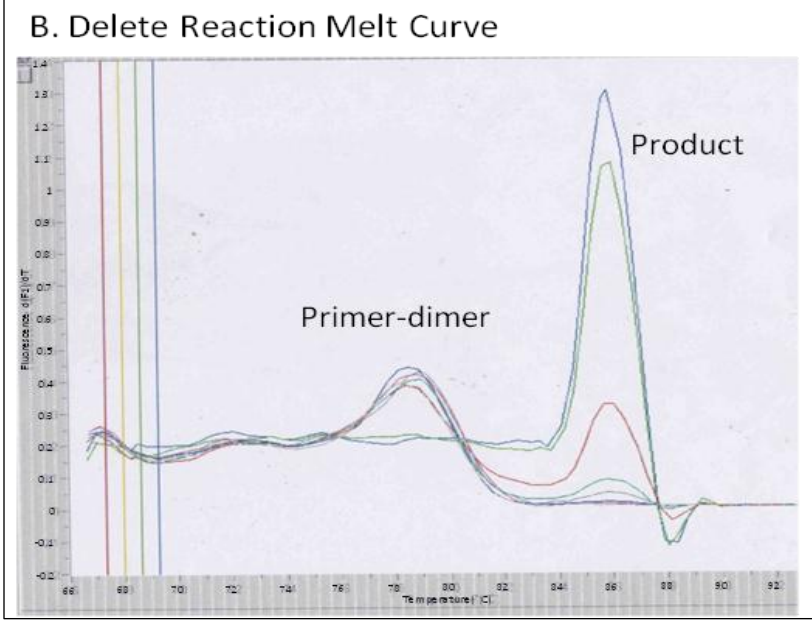


Figure 4.6 Melt curve analysis. A. The WT reaction resulted in only one product peak at around 95°C for the 1:100 dilution, although this sample contained a large primer-dimer peak at 78°C. This suggests that the Ct values obtained for the WT reaction were created by the primers annealing to each other and not to the sample DNA in the reaction mixture. **B**. A few more product peaks were visible for the delete reaction, but again there were primer-dimer peaks. Horizontal axis denotes temperature, and shows at what temperature the products melted. The horizontal axis denotes fluorescence.

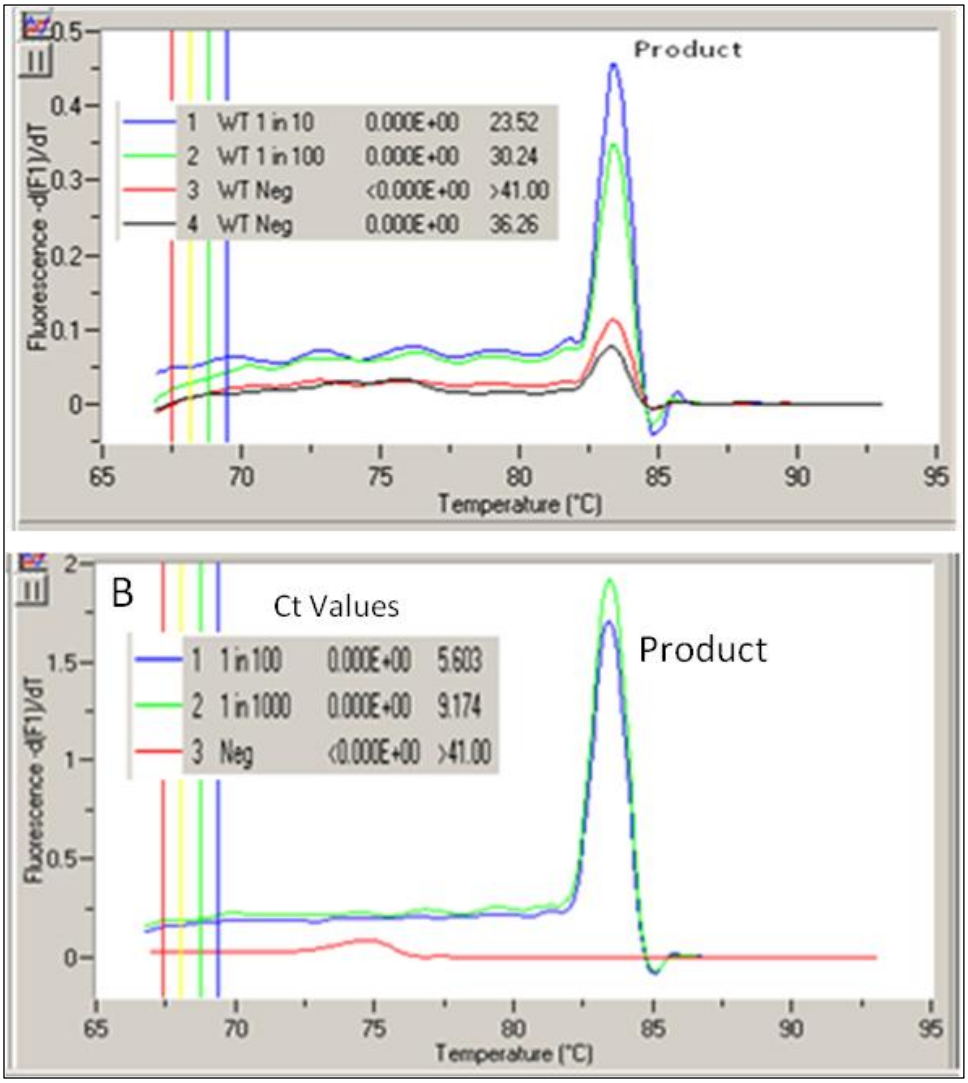


Figure 4.7 Melt curves. A change in SYBR Green supplier appeared to have reduced problems with primer-dimer. **A**. A first run showed no primer-dimer for the two dilutions tested, but a small amount of contamination in the NTC. **B**. Fresh reagents removed the contaminated NTC and primer-dimer.

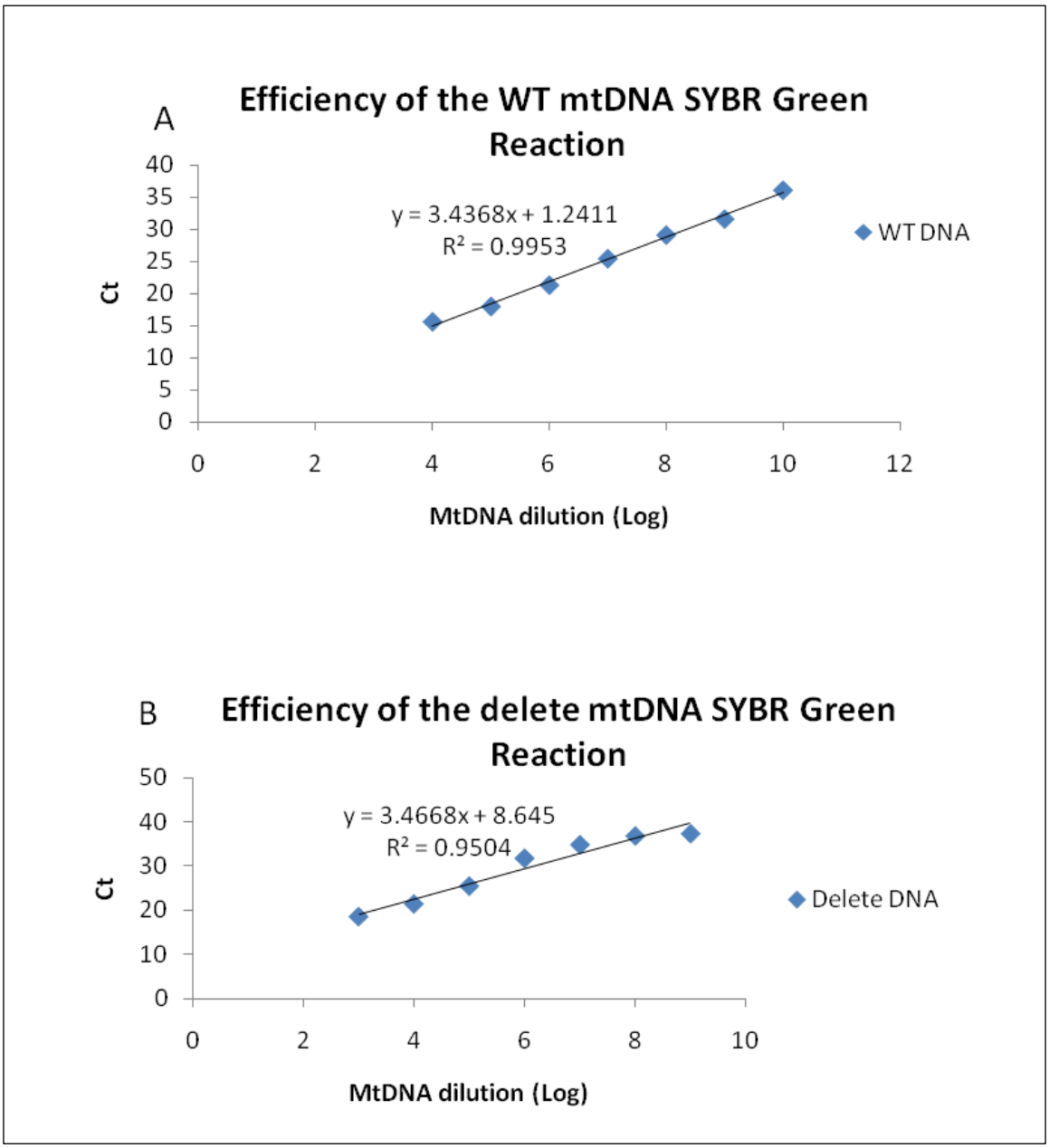


Figure 4.8 Efficiency curves for WT and delete mtDNA reactions. A. A dilution series of WT mtDNA plotted against Ct values gave a good efficiency of 3.4. **B**. A dilution series of delete mtDNA plotted against Ct values also gave an efficiency of 3.4.

4.3.2.3 Optimising a Percentage Deletion Curve

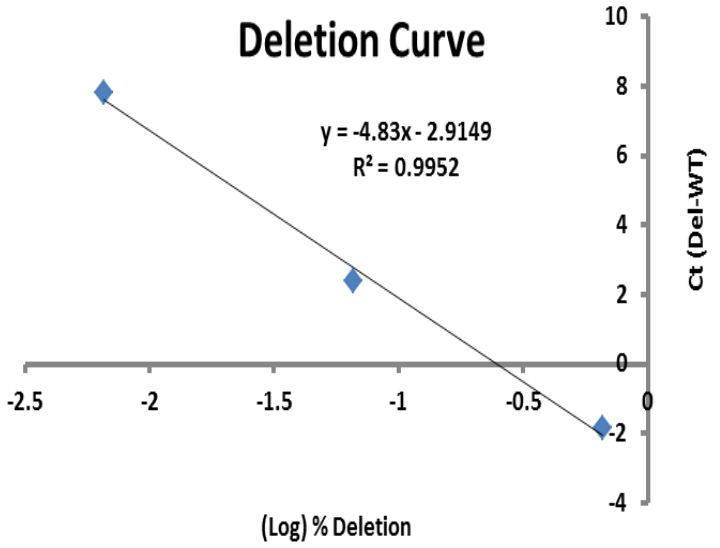
Once the individual reactions had been optimised for WT and delete mtDNA it was necessary to create a deletion curve for this patient. By plotting the amount of delete mtDNA against Ct values it would be possible to create a graph which could be used to determine the heteroplasmy level of a sample by using the Ct value that sample generated.

This patient had previously been shown to harbour the deletion at 66% in his muscle homogenate. A sample of his homogenate mtDNA was obtained from the Newcastle Mitochondrial Diagnostic Service and serially diluted down from 66% to 0.0066% with WT mtDNA to create a percentage deletion curve. These standards were then diluted to 1:100,000 and WT and delete mtDNA reactions run in parallel. The percentage of delete mtDNA in the sample was then plotted against the difference between Ct values for WT and delete mtDNA. An equation of a line of best fit for the data was generated. By re-arranging this equation it was possible to insert Ct values obtained from an unknown sample and determine the heteroplasmy level.

4.3.2.4 Sample Runs

A sample of CD56+ cells and muscle homogenate DNA from this patient's first biopsy were run with primers and reaction conditions to amplify up WT and delete mtDNA in separate reactions. These were set up in duplicate along with deletion standards in order to create a percentage deletion curve. Controls for the assay were a sample of this patient's blood (which should not contain a deletion), and a sample of muscle homogenate DNA from a patient harbouring the m.3243 A>G mutation. This reaction gave a 60% deletion in the CD56+ cells (Figure 4.9). The muscle homogenate sample was 35%, which is perhaps a bit low given previous work published on this patient (Blakely et al., 2004). However, the Ct values for the muscle homogenate sample lay far from the range of the standard curve which could account for this discrepancy. Both the blood and the WT DNA sample came out as 0%. Repeats of this assay showed some variation but the CD56+ cells were averaged out at 51%, and the muscle homogenate at 69%. This assay was later performed on the second biopsy samples and the CD56+ cells had a deletion level of 69% (Table 4.3).

Percentage Deletion	Percentage Deletion (Fraction)	Ct Wt	Ct Del	Percentage Deletion (Log)	Ct (del-wt)		
66	0.66	26.88	25.03	-0.18046	-1.85		
6.6	0.066	26.83	29.23	-1.18046	2.4		
0.66	0.0066	27.28	35.09	-2.18046	7.81		
0.066	0.00066	26.35	no product				
0.0066	0.000066	26.96	no product				
NTC		32.81	>41				
Samples						**X**	**10^X %**
Blood 1/1000		28.38	>41				0%
SKM 1/1000		23.15	22.44		-0.71	-0.4565	35%
CD56+ 1st		26.67	24.62		-2.05	-0.17907	
CD56+ 1st		26.71	25.06		-1.65	-0.26188	
CD56+ 1st Average		26.69	24.84		-1.85	-0.22048	60%
3243A>G (WT)		19.37	41		21.63	-5.08176	0%



X=(ct(del-wt)+2.9149)/(-4.83)

Figure 4.9 Deletion curve and sample runs. Samples of Patient 3's CD56+, blood, and muscle homogenate (SKM) were run alongside deletion standards, WT mtDNA and a NTC. The Ct values for the percentage deletion standards were plotted against percentage deletion to construct a curve. The equation of the line was generated from the line of best fit. This was re-arranged (x=[Ct(Del-WT)+2.9149]/(-4.83)) to provide an equation into which the Ct values for the samples could be inserted. The CD56+ cells were run in duplicate and averaged out. NTC=no template control. 1st refers to first biopsy.

4.3.3 Patients 2 & 4

As previously described in chapter 3, patients 2 and 4 harboured the "common deletion" (nt.8469-nt.13447).Therefore it was necessary to develop an mtDNA common deletion specific qPCR assay.

4.3.3.1 Generating Template DNA

Primers were chosen to amplify both WT (Forward nt.8196-nt.8215, Reverse nt.8740-nt.8720) and delete (Forward nt.8196-nt.8215, Reverse nt.13859-nt.13839) mtDNA giving an approximately 600bp fragment (Figure 4.10). The samples were run on a 1% agarose gel, the bands excised and the products purified using the Qiagen Gel Extraction Kit. The amount of DNA in the sample was then determined using the NanoDrop® Spectrophotometer. The WT mtDNA concentration was 11.7ng/µl, and the delete mtDNA concentration was 13.2ng/µl.

4.3.3.2 Optimising Template DNA

Primers were designed using Primer3 (v.0.4.0) (Rozen and Skaletsky, 2000), that would amplify up an approximately 95bp region, with either WT or delete mtDNA (Figure 4.10). WT and delete mtDNA reactions were then optimised for primer and $MgCl_2$ concentration, and annealing temperature, until the reactions showed similar efficiency. Two master mixes were created, one with primers to amplify WT mtDNA (Forward nt.8430-nt.8449, Reverse nt.8524-nt.8505), and the other to amplify delete mtDNA (Forward nt.8419-nt.8438, Reverse nt.13511-nt.13493). Primers for all reactions were at 125pmol and the $MgCl_2$ concentration for the WT reaction was 3mM and for the delete reaction 4mM. All experiments were set up in 25µl volumes in LightCycler® Capillaries (Roche), with 1µl of sample DNA added. They were performed on a Lightcycler® (Roche) using Platinum®SYBR®Green qPCR SuperMix-UDG (Invitrogen). The cycling conditions were an initial denaturation step (95°C for 10 minutes), forty five cycles of amplification (95°C for 10 seconds, 52.3°C for 5 seconds, 72°C for 10 seconds) with an acquisition step at the end of each cycle, followed by a melt curve analysis (increase to 95°C, 65°C 15 seconds, increase to 95°C (0.1°C/second)), with continuous acquisition.

4.3.3.3 Percentage Deletion Curve

MtDNA containing the common deletion at a heteroplasmy level of 75% was obtained from the Newcastle Mitochondrial Diagnostic Service, along with a sample of WT DNA from a patient harbouring the m.3243A>G mutation. Both samples were diluted in dH_2O so they were at similar concentrations (WT 33.6ng/μl, Delete 36.6ng/μl). The delete mtDNA was then serially diluted down with the WT mtDNA to give a set of deletion standards (75%, to 0.0075%). This was diluted to 1:1000 and run at the same time as the samples from patients 2 and 4.

4.3.3.4 Sample Runs

Samples of CD56+ cells from two biopsies from Patient 2 were run alongside the deletion standard curve, along with a sample of WT mtDNA. The two samples of CD56+ cells from this patient contained mtDNA deletion levels very similar to each other, with the first biopsy CD56+ cells containing 59% heteroplasmy and the second biopsy 57% heteroplasmy, the WT DNA was 0% (Table 4.2). A later run showed the muscle homogenate sample to be considerably higher at 89%.

When samples of CD56+ cells from patient 4 were run with this assay the cells from the first biopsy contained 48% delete mtDNA and the cells from his second biopsy had a 46% heteroplasmy level. The muscle homogenate sample harboured the common deletion at 37% (Table 4.3). Again the samples of CD56+ cells from the two biopsies were fairly similar, with a slight difference between these samples and the muscle homogenate DNA.

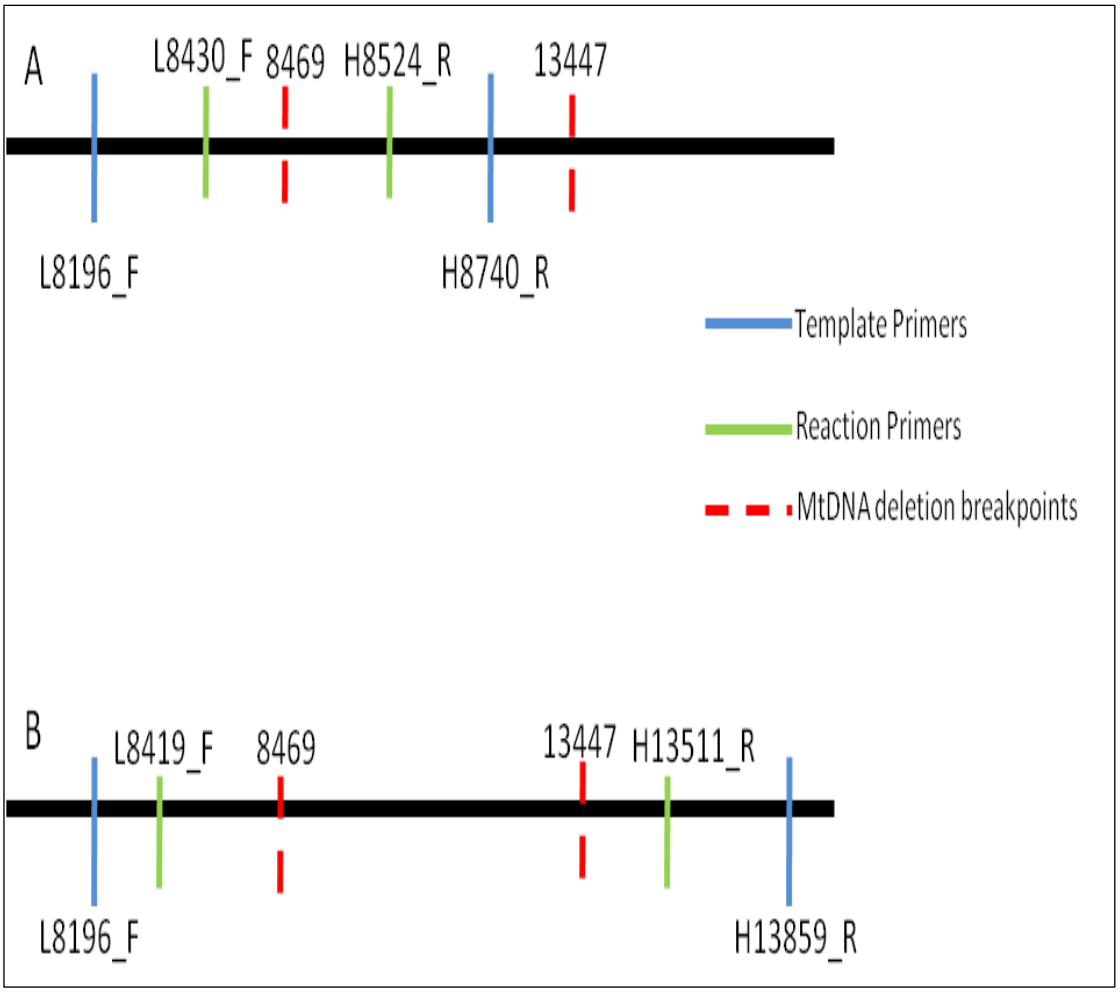


Figure 4.10 Generating template DNA. Primer pairs (blue) were chosen to amplify an approximately 600bp region of either WT mtDNA **(A)** or delete **(B)** mtDNA. This was to generate template mtDNA which could then be used to optimise the SYBR Green assay involving the reaction primers (green). Primers chosen to anneal to regions within the breakpoints (red) of the deletion will only amplify WT mtDNA and not mtDNA harbouring a deletion, because the reverse primer will have nothing to bind to. Primers chosen to anneal to either side of the breakpoint will only amplify deleted mtDNA. This is because the size of fragment between the two primers in WT mtDNA will not amplify due to the extension time and PCR conditions not being capable of amplifying such a large fragment.

Percentage Deletions	Percentage Deletion (Fraction)	Ct WT	Ct Del	Percentage Deletion (Log)	Ct (Del-Wt)		
75	0.75	25.94	24.52	-0.12494	-1.42		
7.5	0.075	22.73	27.92	-1.12494	5.19		
0.75	0.0075	23.95	32.1	-2.12494	8.15		
0.075	0.00075	23.1	34.29	-3.12494	11.19		
0.0075	0.000075	21.93	no product	-4.12494			
NTC		37.32	37.42		0.1		
Samples						**X**	**10^X %**
CD56 + 1st		25.28	25.39				
CD56+ 1st		25.36	25.43				
CD56+ 1st mean		25.32	25.41		0.09	-0.2306	59%
CD56+2nd		25.57	26.09				
CD56+2nd		25.65	25.42				
CD56+2nd mean		25.61	25.755		0.145	-0.24408	57%
3243A>G WT		13.58	32.05				
3243A>G WT		13.12	30.09				
3243A>G WT mean		13.35	31.07		17.72	-4.55273	0%

Table 4.2 Sample runs. CD56+ cells from patient 2's first and second biopsy were run alongside a set of delete mtDNA standards which were used to generate a curve with an equation for the line of best fit. This equation was then re-arranged (x= [Ct (Del-WT) +0.8506]/ (-4.029)), to make X the subject. The values for Ct(Del-WT) for the samples were then inserted into the equation in order to determine the heteroplasmy level for the cells. WT DNA is from a m.3243A>G patient that does not contain a deletion. NTC = No template control. 1st and 2nd refers to first and second biopsies.

4.3.4 Patient 5

This patient had previously been investigated by the Newcastle Mitochondrial Diagnostic Service. A Southern blot revealed that he harboured a single deletion at 9% in his muscle homogenate. Break point analysis showed it was a 7959bp (nt.7845-nt.15440) deletion. Again a SYBR Green assay needed to be designed and optimised to investigate this patient. Previous to the start of this optimisation an ABI StepOnePlus Real Time Detection System became available for use. It was decided to optimise the assay on this machine as it allowed for the use of ninety six well plates, thus enabling the samples to be run in triplicate. In addition it contained settings for a VeriFlex™ step which allowed different annealing temperatures to be used on the same run, which became important for this particular assay.

4.3.4.1 Generating Template DNA

Primers were chosen to amplify both WT (Forward nt.7713-nt.7723, Reverse nt.8301-nt.8283) and delete (Forward nt.7713-nt.7723, Reverse nt.15896-nt.15877) mtDNA giving approximately 580bp fragments (Figure 4.11). The samples were run on a 1% agarose gel, the bands excised and the products purified using a Qiagen Gel Extraction Kit. The amount of DNA in the sample was then determined using the NanoDrop® Spectrophotometer. The delete mtDNA concentration was 8.7ng/µl, and the WT mtDNA concentration was 14.6ng/µl. The WT and delete mtDNA templates were then diluted in dH_2O from 1:10 to $1:10^{12}$.

4.3.4.2 Optimising Template DNA

Primers were designed using Primer3 (v.0.4.0) that would amplify an approximately 60bp region of either WT (Forward nt.7816-nt.7834, Reverse nt.7881-nt.7863) or delete mtDNA (Forward nt.7816-nt.7834, Reverse nt.15445-nt.15463) (Figure 4.11). Reactions for WT and delete mtDNA were then optimised for primer and $MgCl_2$ concentrations and annealing temperature, until the reactions showed similar efficiency.

The WT master mix contained $MgCl_2$ at 4mM, the forward primer (nt.7816-nt.7834) at 75pmol and the reverse primer (nt.7881-nt.7863) at 100pmol; the annealing temperature was 61°C. The delete mtDNA master mix contained $MgCl_2$ at 3mM, the forward primer (nt.7816-nt.7843) at 100pmol, and the reverse primer (nt.15445-nt.15463) at 75pmol; the annealing temperature was 56°C. All experiments were carried out in a 25µl volume on an ABI StepOnePlus Real Time Detection System using Platinum®SYBR®Green qPCR

SuperMix-UDG (Invitrogen). The use of this Real Time machine allowed a VeriFlex™ step whereby different regions of the plate could be taken to different temperatures during the run. The cycling conditions were an initial denaturation step (95°C for 10 minutes), forty cycles of amplification (95°C for 15 seconds, annealing temperature for 1 minute) with an acquisition step at the end of each cycle, followed by a melt curve analysis (95°C for 15 seconds, 60°C for 1 minute, increase to 95°C 0.3°C/15 seconds), with continuous acquisition.

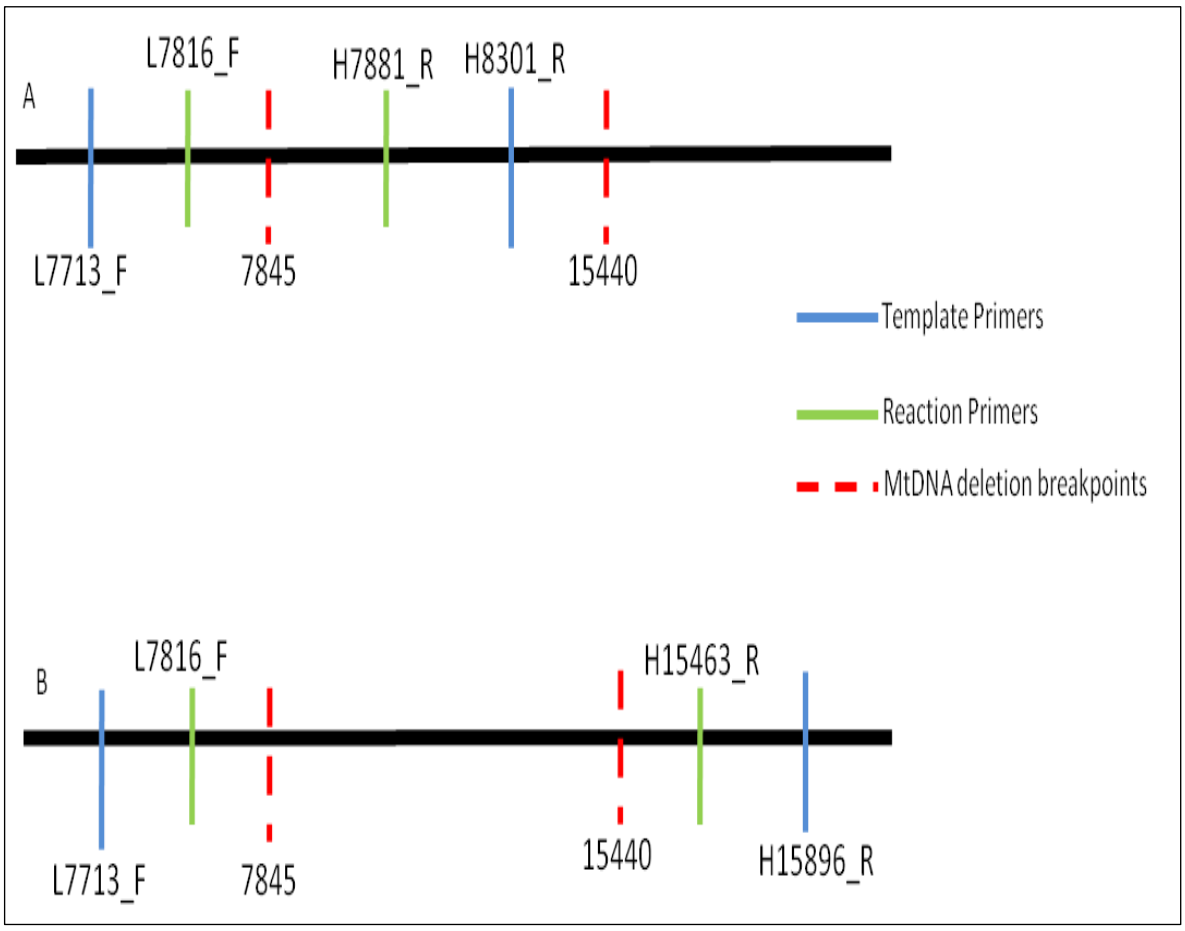


Figure 4.11 Generating template mtDNA. Template primer pairs (blue) were chosen to amplify an approximately 580bp region of either WT **(A)** or delete **(B)** mtDNA. Primers chosen to anneal to regions within the breakpoints (red) of the deletion will not amplify delete mtDNA, while primers chosen to anneal to either side of the breakpoint will only amplify deleted mtDNA due to the extension time and PCR conditions. For the WT and Delete qPCR reactions primers (green) were used to amplify up a ~60bp product.

4.3.4.3 Percentage Deletion Curve

This patient harboured the deletion at 9%, so rather than diluting down a sample of his homogenate DNA, the WT and delete mtDNA templates were mixed to provide a deletion standard curve. Delete template mtDNA of 100% was diluted down using WT mtDNA to give standards at 75%, 50%, 25%, 12.5%, 6%, 3%, and 1.5%. These standards were then diluted to 1/1000 and run alongside samples from this patient.

4.3.4.4 Sample Runs

When CD56+ cells from this patient were run alongside the deletion curve his cells from his first biopsy contained a 0.6% deletion level, and a sample of his muscle homogenate had 10%. Later runs of this assay showed his second biopsy cells to have a 1.3% deletion level and his third biopsy cells to have 3.6% delete mtDNA (Table 4.3).

Patient	Biopsy	CD56+ Cells	SKM
2	1st	59%	89%
	2nd	57%	
3	1st	51%	69%
	2nd	69%	
4	1st	48%	37%
	2nd	46%	
5	1st	0.6%	10%
	2nd	1.3%	
	3rd	3.6%	

Table 4.3 SYBR Green results. Three SYBR Green assays were optimised to examine mtDNA deletion levels in samples of CD56+ cells and muscle homogenate samples from four patients. Patient 5 had all three biopsy samples analyzed using his specific SYBR Green assay while the other three patients had two of their biopsies analyzed. SKM = skeletal muscle homogenate DNA.

4.3.5 MTND1/MTND4 TaqMan® qPCR

Initially it was hypothesised that the CD56+ cells would contain little or no mtDNA deletion. Therefore it was decided to develop SYBR Green qPCRs in order to have complete confidence that any deletion, no matter how low the heteroplasmy level, would be observed. SYBR Green qPCR results suggested that not only were deletions present (Table 4.3), but that they were present at levels that could be determined using a TaqMan® qPCR method.

TaqMan® qPCR assays rely on two principles, Fluorescent Resonance Energy Transfer (FRET) and the 5'nuclease activity of the Taq polymerase. FRET occurs when a high energy dye (reporter) is in close proximity to a low energy dye (quencher).There is a resultant transfer of energy from the high energy molecule to the low energy molecule. Once the probe has bound to the DNA, the 5'nuclease activity of the Taq polymerase allows the reporter to be cleaved from the quencher during primer extension. Once the reporter has separated from the quencher its fluorescence increases (Figure 4.12).

The MTND1/MTDN4 qPCR assay compares the ratio of the MTND1 and MTND4 regions of the mitochondrial genome. Single deletions often occur in the major arc of the mtDNA and take out the *ND4* gene, but not the *ND1* gene. By designing probes that bind to these regions it is possible to detect levels of mtDNA deletions, using a ΔCt calculation (He et al., 2002). By assigning different fluorescent dyes to the probes it is possible to multiplex the assay and improve reliability (Krishnan et al., 2007) (Figure 4.13). The use of probes designed for particular regions of the genome means this assay is a great deal more specific and consistent than SYBR Green qPCR. However, it is not as sensitive as SYBR Green, and cannot accurately detect mtDNA deletions levels below about 30%.

It was decided to test all the CD56+ samples from our patients using this assay. In addition, a sample of the patient's muscle homogenate DNA from the first biopsy was analysed. The samples were run with standards of known deletion levels and a no template control (NTC). This assay was not used on patient 5 as his levels were known to be below 30%, the threshold for detection. Again, all patients examined had single deletions at high levels in their CD56+ cells (Table 4.4). The universal nature of this qPCR method allows it to be used for all patients who harbour single mtDNA deletions in the major arc, which removes the need for patient specific assays.

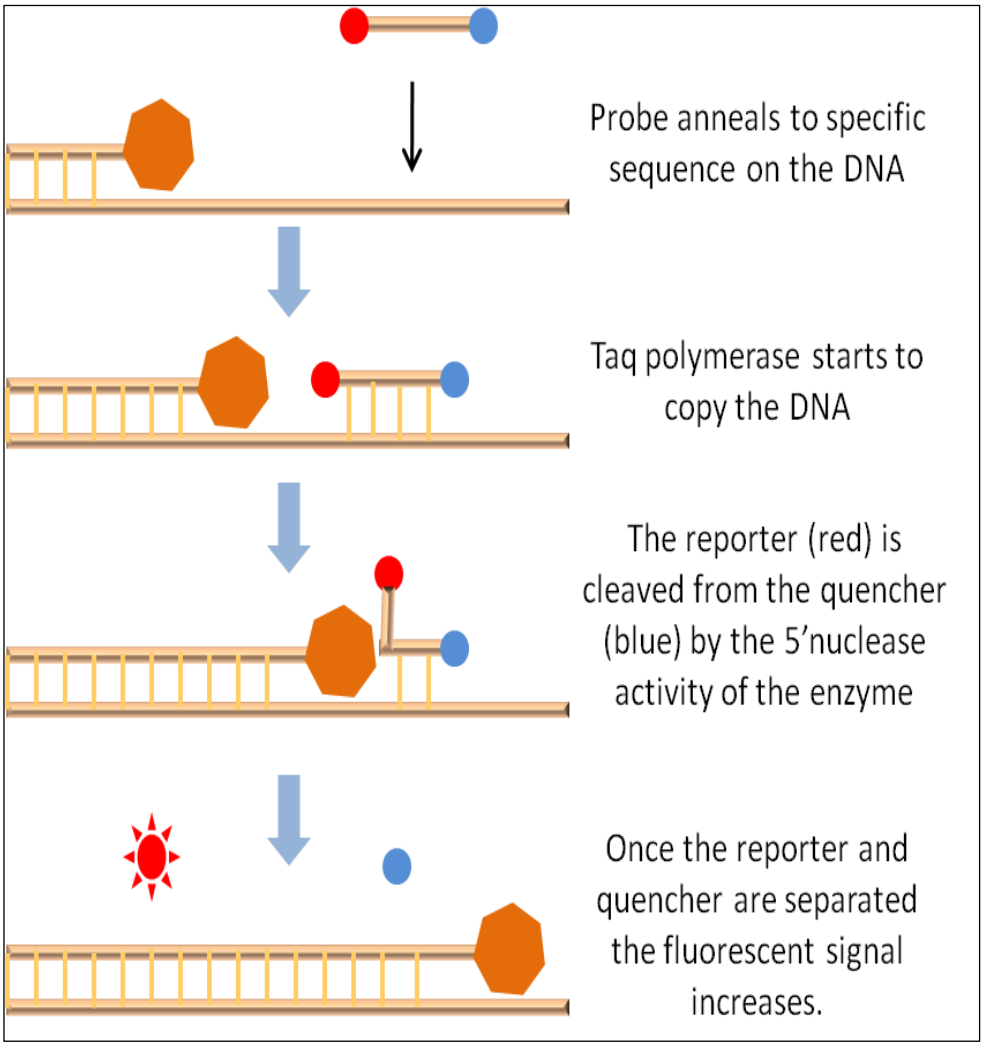


Figure 4.12 Principles of the TaqMan® probe based assay. This assay uses the 5'nuclease activity of the Taq polymerase (orange) to cleave the reporter (red) fluorescent tag from the quencher (blue) molecule.

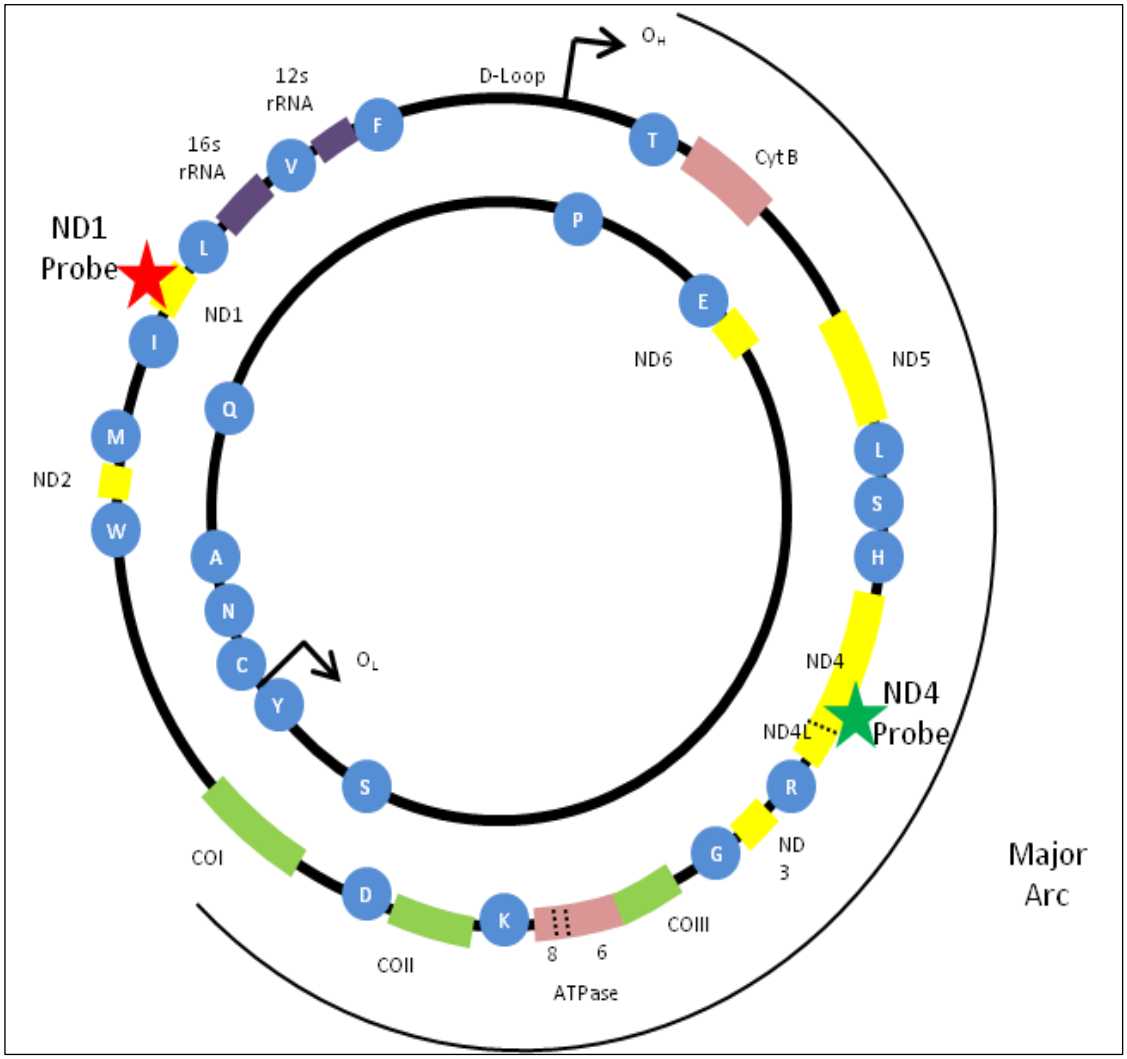


Figure 4.13 Basis of MTND1/MTND4 assay. Single mtDNA deletions mainly occur in the major arc (black line) and often take out the ND4 region of the genome. By designing probes that anneal to this region (green star) and the ND1 region (red star) and comparing the amounts of ND1 and ND4 probe generated fluorescence, it is possible to determine the mtDNA deletion level.

Patient	Biopsy	CD56+ Cells	SKM
1	1st	47%	53%
	2nd	47%	
2	1st	55%	58%
	2nd	43%	
	3rd	60%	
3	1st	83%	67%
	2nd	79%	
	3rd	56%	
4	1st	50%	45%
	2nd	47%	
6	1st	44%	48%
7	1st	50%	62%
	2nd	53%	
8	2nd	47%	43%

Table 4.4 MTND1/MTDN4 results table. CD56+cells and skeletal muscle homogenate (SKM) samples were analysed on the MTND1/MTND4 qPCR assay. The SKM samples were from the first biopsy the patients underwent, before any exercise intervention.

4.3.6 Heteroplasmy Levels in CD56+ Cells and Muscle Homogenate Samples

The MTND1/MTND4 qPCR results were combined with the SYBR Green qPCR results for patient 5. All patients examined harboured deletions in their CD56+ cells and in their muscle homogenate (Table 4.5). The mean heteroplasmy level was 45.1% ±23.4% for the CD56+ cells (n=17), and 48.3% ± 17.6% for the first biopsy muscle homogenate samples (n=8) (Figure 4.14A). Both cell types showed a wide standard deviation, partly attributable to the results from patient 5. A test of normality in Minitab (Anderson-Darling Test) showed the data to be normally distributed. While there was a slightly lower amount of delete mtDNA in the CD56+ cells, a 2 sample t-test revealed this was not a significant difference (P=0.712).

4.3.7 Effects of Six Months Resistance Training

While it was not possible to obtain results for all three biopsies from all eight patients enrolled on the exercise trial, the first two biopsies were available from six of the patients. These two biopsies were taken before and after six months of resistance training. The mean heteroplasmy level for the CD56+ cells before resistance training was 47.6% ± 26.6%, and after resistance training it was 45% ± 25.1% (n=6) (Figure 4.14B). While there was a slight decrease after resistance training a paired t-test showed this was not significant (P=0.290).

4.3.8 SYBR Green and MTND1/MTND4 qPCR

Three of the patients had their heteroplasmy levels determined using both a SYBR Green qPCR and a TaqMan® qPCR. The mean heteroplasmy level with SYBR Green was 58.6% ± 15.4% and with TaqMan® 58.5% ± 14.7% (n=9) (Figure 4.14C). The levels of heteroplasmy obtained from the SYBR Green assay were not significantly different to those obtained with the TaqMan® based assay (paired t-test P=0.984).

Patient	Biopsy	CD56+ Cells	SKM
1	1st	47%	53%
	2nd	47%	
2	1st	55%	58%
	2nd	43%	
	3rd	60%	
3	1st	83%	67%
	2nd	79%	
	3rd	56%	
4	1st	50%	45%
	2nd	47%	
5	1st	0.6%	10%
	2nd	1.3%	
	3rd	3.6%	
6	1st	44%	48%
7	1st	50%	62%
	2nd	53%	
8	2nd	47%	43%

Table 4.5 QPCR results. MTND1/MTND4 qPCR results for all patients were combined with the SYBR Green qPCR results from patient 5. When compared to the heteroplasmy level in the skeletal muscle homogenate there was no significant difference between the amounts of deletion in the CD56+ cells and the skeletal muscle.

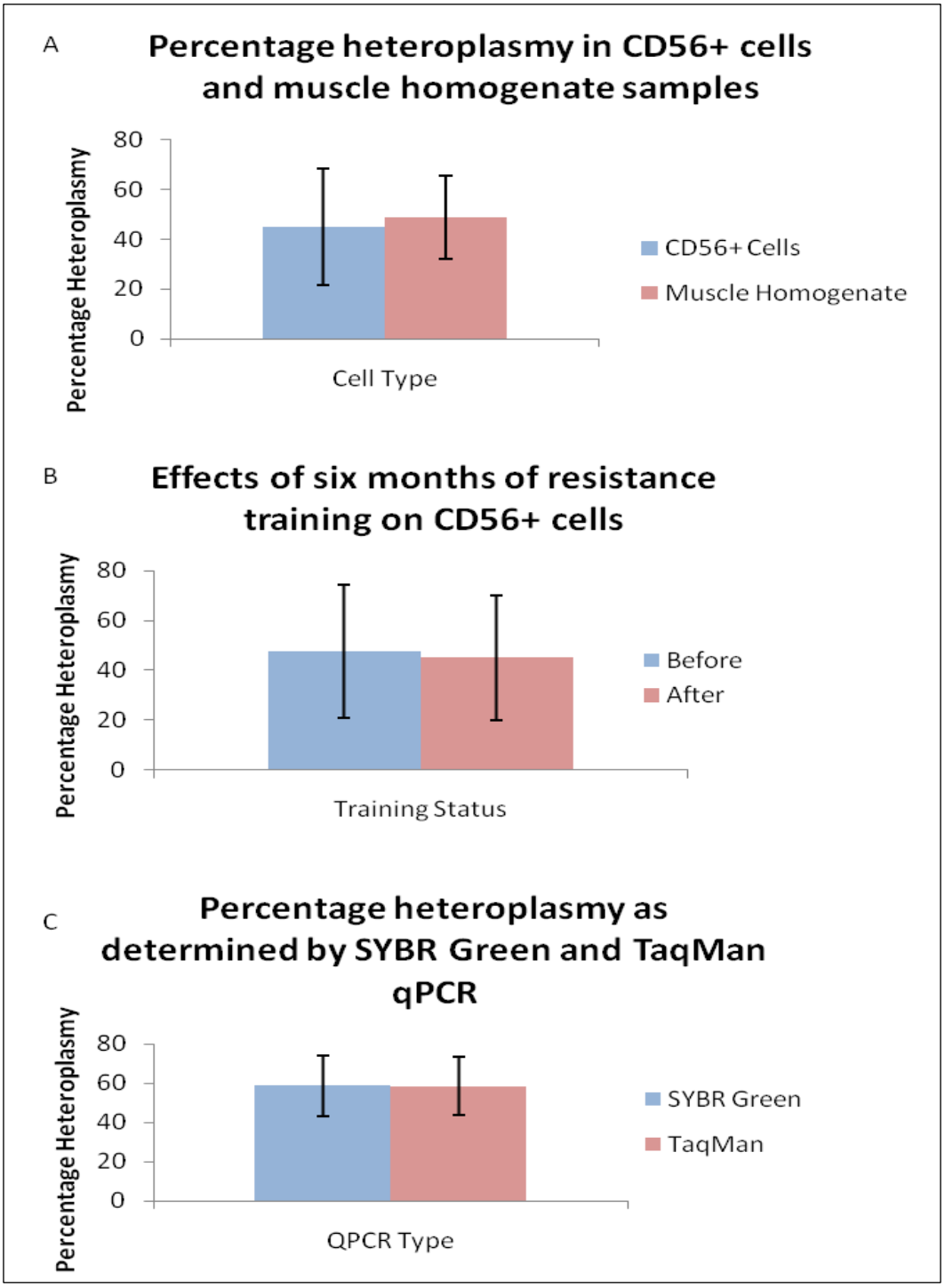


Figure 4.14 Heteroplasmy levels. A. There was a slightly higher level of mtDNA deletion in the muscle homogenate samples when compared to the CD56+ cells, but this was not significant. **B**. A slight decrease in delete mtDNA was observed after six months of resistance training in the CD56+ cells, again this was not significant. **C**. There was no difference observed in heteroplasmy levels obtained with SYBR Green qPCR and TaqMan® qPCR. Lines indicate standard deviation.

4.3.9 Long Range PCR

MtDNA deletions have been shown to be present in the CD56+ cells of patients harbouring sporadically occurring mutations using two different qPCR methods. In order to confirm this finding it was decided to use a non-qPCR method to demonstrate the mtDNA deletion. Consequently, a long range PCR was performed. A traditional PCR is able to generate a fragment of up to 1kbp, while long range PCR is able to amplify a much larger region. If a band is visible on the agarose gel that is shorter than the fragment that would be generated with the primers and Wt DNA, then it suggests that some of the DNA is missing and the mtDNA harbours a deletion.

CD56+ cells from patient 1 were amplified with primers (Forward nt.6863-nt.6882, Reverse nt.14857-nt.14838) designed to generate a 7956bp fragment. PCR products were diluted 1:50 and 1:100, before undergoing a second round of amplification with primers (Forward nt.7272-nt.7293 and Reverse nt.14374-nt.14356) to give a 7063bp fragment (Figure 4.15A). Bands could be observed corresponding to a fragment of ~3500bp indicating a deletion size of 3563bp, which closely matches the deletion size for this patient of 3693bp.

CD56+ cells from patient 6 were amplified with primers (Forward nt.5855-nt.5875, Reverse nt.129-nt.110) designed to generate a 10774bp fragment. PCR products were diluted to 1:50, 1:100, and 1:150, and underwent a second round of long range PCR with primers (Forward nt.6863-nt.6882, Reverse nt.1589-nt.15877) designed to amplify an 8995bp fragment. Bands could be observed at 1000bp (Figure 4.15), which corresponds to an approximately 7995bp deletion which closely matches this patient's deletion size of 8039bp.

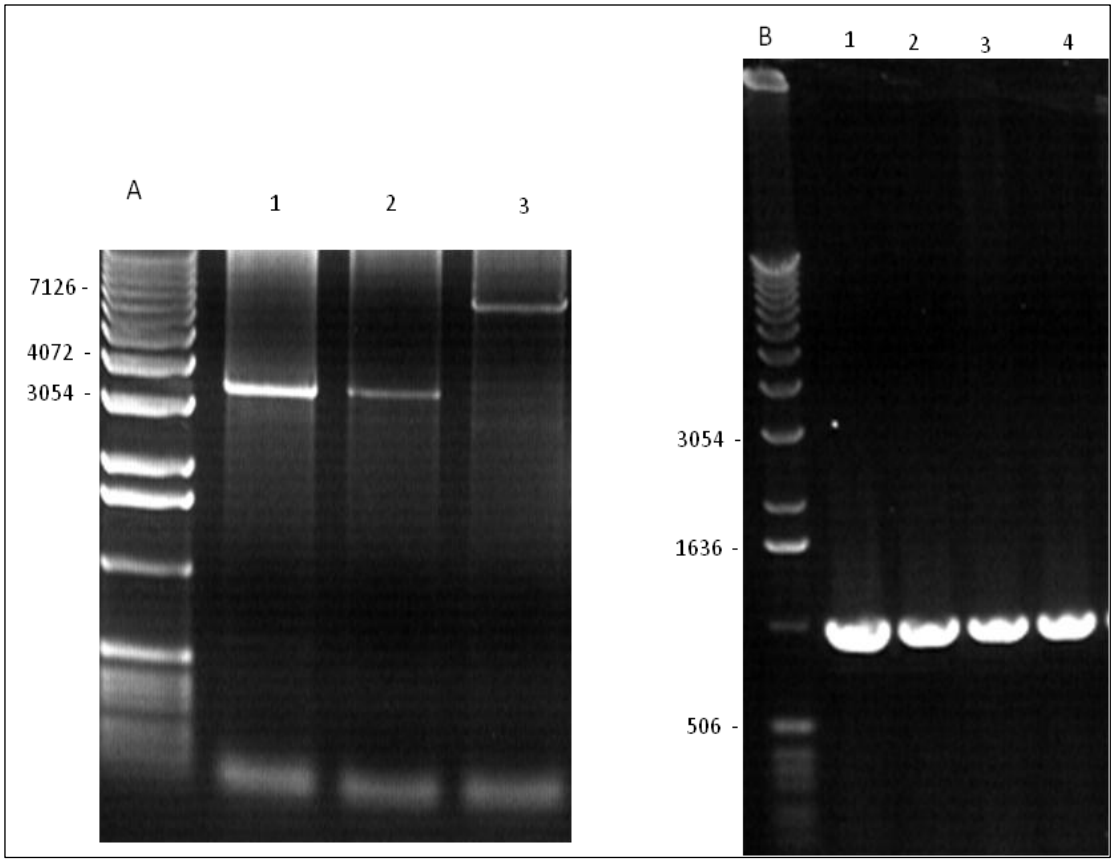


Figure 4.15 Second round long range gels showing mtDNA deletions in CD56+ cells. A. Patient 1's CD56+ cells underwent two rounds of long range PCR. Lanes 1 and 2 are CD56+ cells with primers designed to generate a 7063bp fragment. Bands can be observed at 3500bp suggesting a ~3563bp deletion. Lane 3 is WT mtDNA and therefore a band can be observed at ~7000bp. Lane 1 was diluted 1:50 and lane 2 was diluted 1:100 before this second round of long range PCR. **B**. Patient 6's CD56+ cells underwent two rounds of long range PCR. Lanes 1-4 have primers designed to generate a 8995bp fragment, bands have been generated corresponding to a 1000bp fragment, suggesting this patient harbours a mtDNA deletion of ~7995bp. Lane 1 is a sample of this patient's muscle homogenate DNA diluted 1:200, while lanes 2-4 are CD56+ cells diluted 1:50, 1:100, and 1:150 respectively, before undergoing the second round of amplification.

4.4 Discussion

The aim of this chapter was to develop mutation specific SYBR Green qPCR assays to look for mtDNA deletions in CD56+ cells from patients harbouring a sporadically occurring single mtDNA deletion. It was hypothesised before this research that there would be little or no mutation in these cells and an extremely sensitive qPCR method was therefore employed to investigate this theory. Two mtDNA deletion specific SYBR Green qPCR assays were designed and optimised, and a further assay was re-optimised for this research.

MtDNA deletions have been demonstrated in the satellite cells of patients harbouring sporadically occurring single mtDNA mutations. This has been carried out using two qPCR methods; mtDNA deletion specific SYBR Green assays and a TaqMan® based assay. In addition, for patients 1 and 6, long range PCR gels revealed bands corresponding to the presence of a single deletion of the size previously determined (Table 3.3). The levels of mtDNA deletions in the CD56+ cells did not differ from those present in the skeletal muscle homogenate samples. The similar levels in CD56+ cells and muscle homogenate samples further suggests that there is not a sub population of cells in the CD56+ fraction that contains only WT mtDNA. If there was a more “stem cell like” sub population in the CD56+ fraction that had no mutation, then the heteroplasmy level of the CD56+ cells would be much lower than the homogenate sample.

These findings were somewhat surprising given previous investigations demonstrating the lack of mutations in myoblasts of patients with sporadically occurring mutations (Fu et al., 1996, Weber et al., 1997). In addition, mtDNA deletions have been reported to be absent from patients with CPEO in dividing cells, for example blood, fibroblasts (Moraes et al., 1989a), oral mucosa (Bau and Zierz, 2005), and bone marrow (Marzuki et al., 1997). While CPEO can be multi-systemic, this is not the norm and it may be that the segregation observed in CPEO is responsible for its milder phenotype (Bau and Zierz, 2005).

However, this is not the case in patients with KSS caused by sporadically occurring single mtDNA deletions. KSS is a multi-system disorder where clinical features often present before the age of twenty and can include pigmentary retinopathy, cerebella ataxia, proximal myopathy and cardiomyopathy (Pistilli et al., 2003). In contrast to CPEO, mtDNA single deletions have been reported to be present in dividing cells for example myoblasts (Collombert et al., 1997, Bonod-Bidaud et al., 1999), fibroblasts (Larsson et al., 1990) and

leukocytes (Moraes et al., 1989a). However, these levels are lower than those observed in skeletal muscle. While none of the patients examined in this study demonstrated a KSS phenotype, it could be hypothesised that KSS patients would also be expected to harbour mtDNA deletions in their satellite cells if they were examined.

4.4.1 Sporadically Occurring MtDNA Deletions in Satellite Cells

While these findings were unanticipated, a closer examination of the nature of satellite cells suggests they may not be all that unexpected.

The presence of a mtDNA deletion in both brothers of a set of identical twins (Blakely et al., 2004), and the presence of mtDNA deletions in oocytes suggests that the sporadic mutation arises early in development (Chen et al., 1995). Possibly in the oocytes of the grandmother, followed by segregation into the oocytes of the mother of the affected individual (Chinnery et al., 2004). Interestingly, a recent report suggests that the occurrence of sporadic mtDNA deletions in the grandmothers is not attributable to age (Elson et al., 2010).

Given that the mutation will have been present during embryogenesis and that satellite cells and skeletal muscle have the same embryological origins (Gros et al., 2005), it is probable that the mutation will have segregated to the satellite cells in the same way that it segregated to the muscle.

The presence of mutations at levels similar to the muscle homogenate are intriguing given the assumption that clonal expansion has occurred in the post mitotic muscle fibres. Satellite cells are quiescent, so at what point has clonal expansion acted upon these cells? The mutation has either been laid down at that level during embryogenesis, or has clonally expanded during a very short period of time within the cells while they have been repairing the muscle. While it would initially seem unlikely that the cells have undergone enough divisions for clonal expansion to occur, the low mtDNA copy number in these cells (see section 6.4.1) could mean that clonal expansion requires fewer divisions in which to take place. It is also possible that the mutation was laid down at similar levels in both the satellite cells and muscle cells. A small increase in the mutation level in muscle fibres could have resulted in the muscle cells passing the threshold level.

These experiments have been performed on muscle homogenate samples; they do not take into account differences that could exist between individual cells. For example, at

birth the mutation may be present at 50% in the muscle homogenate, clonal expansion could result in some muscle cells increasing above this level and some cells decreasing, but a heteroplasmy level of 50% would still be maintained.

Satellite cells are quiescent until they activate, and while it is currently not known if there is any mtDNA turnover in quiescent satellite cells there has obviously not been enough to allow an opportunity for negative selection. While it is probable that these satellite cells will have been activated at some point in order to repair muscle before returning to quiescence, again this will not have occurred enough times to allow for selection.

While previous reports suggesting sporadic mutations are not present in the satellite cells of patients harbouring mtDNA mutations, may have been somewhat premature (Fu et al., 1996, Weber et al., 1997, Clark et al., 1997, Shoubridge et al., 1997), the experimental results they obtained can still be explained in light of the current findings.

4.4.2 Explanation of Previous Findings

The original papers (Weber et al., 1997, Fu et al., 1996) based their hypothesis on what they found in cultured myoblasts; this would have allowed for a period of selection to act on the cells. Papers testing the theory of "gene shifting" (Shoubridge et al., 1997, Clark et al., 1997) examined regenerated muscle fibres, again allowing for selection to occur during muscle regeneration.

During the process of myoblast culture or muscle regeneration the mtDNA deletions could have been lost. An mtDNA deletion would be deleterious at both the mitochondrial and the cellular level and could therefore affect the ability of the myoblasts to proliferate. After cell division if there was segregation of WT and delete mtDNA into daughter cells, any cells that inherited large amounts of delete mtDNA may die.

In addition it has been shown that clonal cultures of both fibroblasts and myoblasts containing mtDNA deletions may grow slower (Moraes et al., 1989b). It is possible that this occurred in these instances and resulted in very diluted samples of delete mtDNA.

Furthermore, it is possible that satellite cells containing mutations may not activate. After activation there is a period of intense up-regulation of transcription and replication within satellite cells. Cells with impaired OXPHOS may not be able to survive this process.

It will be necessary to investigate the myoblasts of these patients to determine what happened to these mutations as the satellite cells started to proliferate and differentiate into myotubes.

4.4.3 Stem Cells and MtDNA Mutations

Other investigations examining mtDNA mutations in stem cells have mainly focused on colon tissue and blood stem cells.

Howell *et al.* (2000) examined levels of the m.3460A>G LHON mutation in blood samples from a family with the disease and saw a decrease of approximately 1% per year (over 5-6 years) in the mutation. The levels of a silent polymorphism did not change. They hypothesised this decrease was attributable to the presence of homoplasmic mutant and homoplasmic WT blood stem cells. They suggested the homoplasmic mutant stem cells may produce fewer white blood cells and platelets over time, or that the stem cells may be heteroplasmic and selection acted to favour proliferation of mitochondria with lower levels of mutations (Howell et al., 2000). Computer simulations have demonstrated the loss of the m.3243A>G mutation in blood may also be caused by selection at the stem cell level (Rajasimha et al., 2008).

MtDNA point mutations have been reported in the stem cells of colonic crypts, (Taylor et al., 2003, Greaves et al., 2010a), although not from patients presenting with a mitochondrial disease. In contrast to blood, these mutations were maintained in the stem cell population, and the developing cells. This could be attributable to the position of the colonic crypt stem cell in a tight niche not allowing for selection.

4.4.4 Effects of Six Months Resistance Training

Six months of resistance training had no effect on the levels of mtDNA mutations in the satellite cells of these patients. Due to the high intensity of the resistance training being performed it is reasonable to expect that there will have been some regeneration occurring in the muscle. This will have involved activation of satellite cells and the renewal of the stem cell pool. The absence of any change in heteroplasmy level suggests that the degree of damage induced was not sufficient to induce enough satellite cell divisions and renewal to allow for the loss of the mutation.

4.4.5 SYBR Green and TaqMan® qPCR

While initial attempts to optimise a SYBR Green assay for patient 3 were frustrating, changing the supplier of the reagent was an advantageous step. Both reagents contained Taq DNA Polymerase, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, $MgCl_2$ and a reaction buffer (contents not stated). It can only be assumed these two reagents differed in their reaction buffers or the concentration of contents. This would account for the highly varied results with the two reagents.

Two qPCR methods have been used to quantify mtDNA deletions in muscle stem cells and muscle homogenate samples from patients. Once it was realised that deletions were present in enough quantities to quantify with the MTND1/MTND4 assay, the samples were re-analysed using this technique.

SYBR Green has been used to quantify both mtDNA point mutations (Bai et al., 2004) and deletions (Blakely et al., 2004, Fuke et al., 2008). The results obtained using the SYBR Green and TaqMan® assays were not significantly different from each other. This is in agreement with other studies examining mtDNA deletions in brain tissue, where results obtained with the two techniques showed minimal differences (Sabunciyan et al., 2007).

While the TaqMan® based assays are more consistent and specific for deletions, the ability to use SYBR Green on small DNA concentrations and to quantify low level mtDNA deletions does make it valuable in these situations. If the levels of mtDNA deletions are above 30%, the ability to multiplex the MTND1/MTND4 qPCR allows for more samples and runs in triplicate. Coupled with the ability to compare results between runs, this does make the MTND1/MTND4 qPCR technique superior.

4.5 Conclusions

MtDNA deletions are present in the satellite cells of patients harbouring sporadically occurring large scale single mtDNA deletions, at levels comparable to muscle homogenate samples.

This is probably due to the mutation being present in the oocyte and then not being lost as the tissue develops prenatally and regenerates postnatally. The mutation was either present at current levels at birth or has clonally expanded during short periods of division that have occurred during muscle regeneration.

These results do not contradict earlier findings of little or no mtDNA mutations in the myoblasts of patients harbouring sporadically occurring mutations.

Findings of mutation in the satellite cells of patients with sporadically occurring conditions may have initially been worrying considering current attempts at “gene shifting”. However, the probable loss of the mutation as the cells develop into myoblasts to eventually repair the muscle means that “gene shifting” is still a viable option for these patients.

It will be necessary to grow myoblasts from these biopsies in order to determine what happens to the mtDNA deletions as new muscle cells develop.

Chapter 5 MtDNA Deletions and Myoblasts

5.1 Introduction

Sporadically occurring single mtDNA deletions have been demonstrated in the satellite cells of patients with mitochondrial myopathies. While this was initially surprising given previous reports, it did not contradict those findings or rule out the possibility of "gene shifting" in patients with mitochondrial myopathies caused by sporadic mutations.

The initial experiments reporting no mutations in myoblasts from patients with sporadic mutations examined cultured myoblasts, and experiments demonstrating the feasibility of "gene shifting" examined muscle after a period of intense regeneration (Weber et al., 1997, Fu et al., 1996, Clark et al., 1997, Shoubridge et al., 1997). It is therefore possible and probable that the mutation was lost from myoblasts *in vitro* during cell culture or *in vivo* during muscle regeneration.

5.1.1 Selection

There are a number of time points and a number of different mechanisms that could account for loss of these mutations. This could have occurred in the satellite cells and/or in the myoblasts.

Satellite cell activation could have been impaired, with cells harbouring mutations being unable to activate. Alternatively if satellite cells are heteroplasmic on the basis of mtDNA deletions, then the resultant myoblasts may maintain or lose the mutation depending on which particular stem cells were activated.

Selection could have happened during myoblast proliferation. This could occur at the mitochondrial level, with mitochondria containing high levels of mutation being signalled out for degradation by mitophagy (Twig et al., 2008, Alemi et al., 2007). Alternatively it could happen at the cellular level, where myoblasts with large amounts of deletions and thus impaired OXPHOS die, or are unable to replicate as quickly as WT myoblasts. The differentiation of myoblasts to myotubes may also be impaired in respiratory chain deficient cells.

5.1.1.1 Myoblasts, Myotubes, and Mitochondrial Biogenesis

Quiescent satellite cells have low, if any, transcriptional activity, with most cells in the G0 phase of the cell cycle, a stage characterised by resting. After activation, the myoblasts are proliferating rapidly, and these early stages coincide with an increase in the contribution of glycolysis for energy production (Barani et al., 2003). As myogenesis proceeds it is accompanied by both an increase in mtDNA copy number and mitochondrial protein synthesis, with increases in PGC1α and TFAM (a mitochondrial transcription factor) being observed (Barbieri et al., 2011).

Early experiments have demonstrated the importance of mitochondrial function in myogenesis *in vitro* through the use of a DNA intercalating dye, ethidium bromide (EtBr). EtBr inhibits mtDNA synthesis and reduces copy number. Adding EtBr to myoblasts reversibly inhibits their differentiation into myotubes, but does not prevent their proliferation. Not only is differentiation impaired by a decrease in mtDNA but a successful differentiation is accompanied by an increase in mtDNA and mitochondrial biogenesis (Brunk and Yaffe, 1976, Herzberg et al., 1993). This increase in biogenesis coincides with a shift in energy metabolism from primarily glycolytic to mainly oxidative (Leary et al., 1998). Although other research suggests this shift to OXPHOS occurs later, as the myotubes are maturing (Webster et al., 1990).

The prevention of myotube formation by inhibition of mitochondrial protein synthesis is both morphological and biochemical and is thought to be attributable to a reduction in mitochondrial translation rather that transcription (Hamai et al., 1997). Inhibition of mitochondrial translation by chloramphenicol down-regulates protein and mRNA levels of myogenin, which has an important role in myoblast differentiation into myotubes. This also suggests a role for mitochondrial cell signalling during myogenesis (Rochard et al., 2000).

5.1.1.2 Muscle Regeneration and Mitochondrial Biogenesis

Much of the early work highlighting the importance of mitochondrial biogenesis and function during the proliferation of myoblasts and their differentiation into myotubes involved cell culture. More recent experiments have used *in vivo* models of regeneration to determine the importance of mitochondrial biogenesis.

By injecting rats with 0.75% bupivicaine to necrose the *tibialis anterior* (TA) muscle, Duguez *et al.* (2001) demonstrated that muscle regeneration could be split into two overlapping time periods. Days three to fourteen were characterised by satellite cell activation and myoblast proliferation, followed by a period of myoblast differentiation into myotubes from days five to thirty five. The initiation of skeletal muscle regeneration was accompanied by an increase in mitochondrial biogenesis. At the point of myoblast differentiation (days 5-10) there was a large increase in biogenesis, possibly mediated by PGC-1 and TFAM (Duguez et al., 2001).

These findings not only highlight the importance of mitochondrial function during the process of muscle regeneration, but also suggest a method whereby mutations could be lost from myoblasts in culture, or during muscle regeneration. In the period leading up to differentiation of myoblasts any cells with high levels of mutation may not be able to cope with the switch from a glycolytic state to a one in which OXPHOS is required to generate energy. This increase in demand on the OXPHOS pathway may result in impaired cells either dying or being outnumbered by WT cells.

There are therefore many time points when selection could have occurred in these cells. In order to determine when the mutation is lost it will be necessary to grow and differentiate myoblasts and examine the levels of any deletions present as the cells develop.

5.2 Aims

The aims of this chapter were to culture myoblasts from patients harbouring sporadically occurring single mtDNA deletions in both their muscle homogenate samples and their satellite cells. This was to determine what happens to any mutations present as the cells head towards, and differentiate into, myotubes. It was hoped this would identify the step of muscle regeneration where selection is acting on the developing muscle cells enabling mutations to be lost. Given the likelihood there would be very little or no mutations in these proliferating myoblasts it was necessary to use sensitive SYBR Green assays to have confidence in any results which suggested there was less than 30% delete mtDNA.

5.3 Methods

5.3.1 Myoblast Culture

At the time of biopsy, a small sample of muscle was transferred to the MRC Centre for Neuromuscular Diseases Biobank, where myoblast cultures were set up by the biobank technician Mojgan Reza. After around two weeks the cells were trypsinised and placed in a T25 flask and transferred from the biobank to the Mitochondrial Research Group.

Once the cells were confluent they were transferred to a T75 flask. At this point some medium was taken for mycoplasma testing. Cells were cultured in SKCM, with the medium changed every two days. When cells were at 85% confluence they were passaged. Additionally, some myoblasts were frozen for storage and a pellet was also harvested for mtDNA analysis, the remainder were transferred into a fresh T75 flask.

Myoblasts were grown for around seven passages and then differentiated into myotubes as described in section 2.2.7.4. It was, however, difficult to culture some cell lines up to passage seven, as the cells started to develop a strange morphology and die.

Unfortunately it was not possible to obtain myoblasts for all of the biopsies performed. The patients undergoing this trial have a myopathy, and in some cases this is accompanied by severe muscle wasting, so it was occasionally difficult for a large enough sample to be collected for all the studies that required tissue.

5.3.1.1 Desmin Staining

Samples of patient myoblasts were grown on cover slips and then stained for desmin to ensure they were myoblasts and not fibroblasts. Cells were labelled with monoclonal mouse anti-human desmin (DAKO) and then with Alexa Fluor® -488 goat anti-mouse (FITC) secondary antibody, and mounted with VECTASHIELD® Mounting Medium with DAPI. Counts were performed at x20 magnification on an Axiovert 200M (Zeis), using AxioVision Release 4.6.3 software (Zeiss). The bottom middle edge of the cover slip was assigned as 0,0 and the same set of twelve coordinates were visited on each cover slip. All nuclei (DAPI) and desmin positive cells (FITC) in the field of view were counted.

5.3.2 Molecular Studies

Myoblast pellets had the supernatant discarded and were stored at -20°C until DNA could be extracted. This was carried out using the Qiagen DNeasy Blood & Tissue Kit, with the final elution step being performed in water. The samples were stored at -20°C until qPCR analysis.

5.3.2.1 QPCR Assays

The MTND1/MTND4 assay was performed on all samples of myoblasts to determine heteroplasmy levels. Where these levels were below 30% the relevant SYBR Green assay was used to determine the amount of delete mtDNA. In addition a sample of blood DNA was available for patients 2 and 3, and a urine sample was available from patient 2.

5.3.2.2 Patient 6

In order to determine the heteroplasmy level, if any, in the myoblasts from patient 6, an mtDNA deletion specific SYBR Green assay needed to be optimised. This patient was first biopsied after it had been shown that deletions were present in satellite cells at levels that could be determined using the MTND1/MTND4 qPCR method. It was therefore not necessary to develop a SYBR Green assay for his satellite cells, but use of the MTND1/MTND4 qPCR on his myoblasts showed them to be below 30%, so it became necessary to develop an mtDNA deletion specific SYBR Green qPCR.

In order to generate template DNA, primers were chosen to amplify either WT (Forward nt.7349-nt.7369, Reverse nt.8009-nt.7990), or delete (Forward nt.7349-nt.7369, Reverse nt.16067-nt.16048) mtDNA, giving an approximately 600bp fragment (Figure 5.1). These were used to optimise the WT and delete mtDNA reactions. Reaction primers were designed using Primer3 (v.0.4.0) that would amplify an approximately 100bp reaction product of WT (Forward nt.7592-nt7610, Reverse nt.7680-nt7660) or delete (Forward nt.7592-nt7610, Reverse nt.15717-n.t15735) mtDNA.

Primers for the WT reaction were at 125pmol, and for the delete reaction the forward primer was at 125pmol with the reverse primer at 100pmol. $MgCl_2$ was at 3mM and the annealing temperature for the WT reaction was 56°C and for the delete reaction was 55°C. All reactions were carried out in 25µl volumes on an ABI StepOnePlus Real Time Detection System using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), with 1µl of sample DNA added. The cycling conditions were an initial denaturation step (95°C for 10 minutes), forty cycles of amplification (95°C for 15 seconds, annealing temperature

for 1 minute) with an acquisition step at the end of each cycle, followed by a melt curve analysis (95°C for 15 seconds, 60°C for 1 minute, increase to 95°C (0.3°C/15 seconds)), with continuous acquisition.

Delete template mtDNA from this patient was diluted using WT mtDNA to give standards of 75%, 50%, 25%, 12.5%, 6%, 3% and 1.5%. These were diluted to 1/5000 and analysed with samples of this patient's myoblasts.

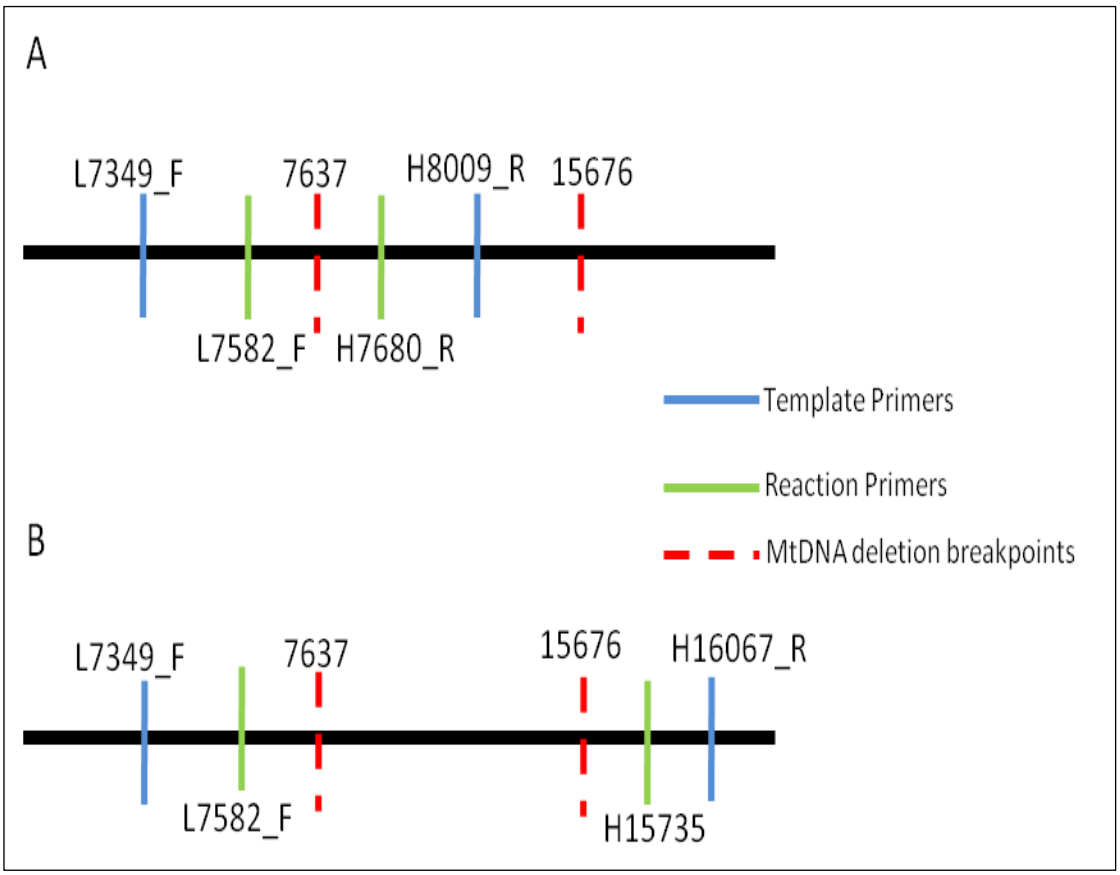


Figure 5.1 Patient 6 SYBR Green assay. Template primer pairs (blue) were chosen to amplify an approximately 600bp region of either WT **(A)** or delete **(B)** mtDNA. Primers chosen to anneal to regions within the breakpoints (red) of the deletion will not amplify delete mtDNA, while primers chosen to anneal to either side of the breakpoint will only amplify deleted mtDNA due to the extension time and PCR conditions. For the WT and delete qPCR reactions primers (green) were used to amplify up an approximately 100bp product.

5.4 Results

5.4.1 Desmin Staining

Desmin staining was performed on seven out of the nine myoblast cell lines available (Figure 5.2). The percentage of desmin positive cells ranged from 69% to 98% (mean 86% ±11%) (Table 5.1). A few of the cell lines with lower desmin staining may have contained some fibroblast contamination. Given that fibroblasts from patients with sporadically occurring mutations do not normally harbour the mutation, it is possible that fibroblasts present in the cultures could have reduced any heteroplasmy levels observed.

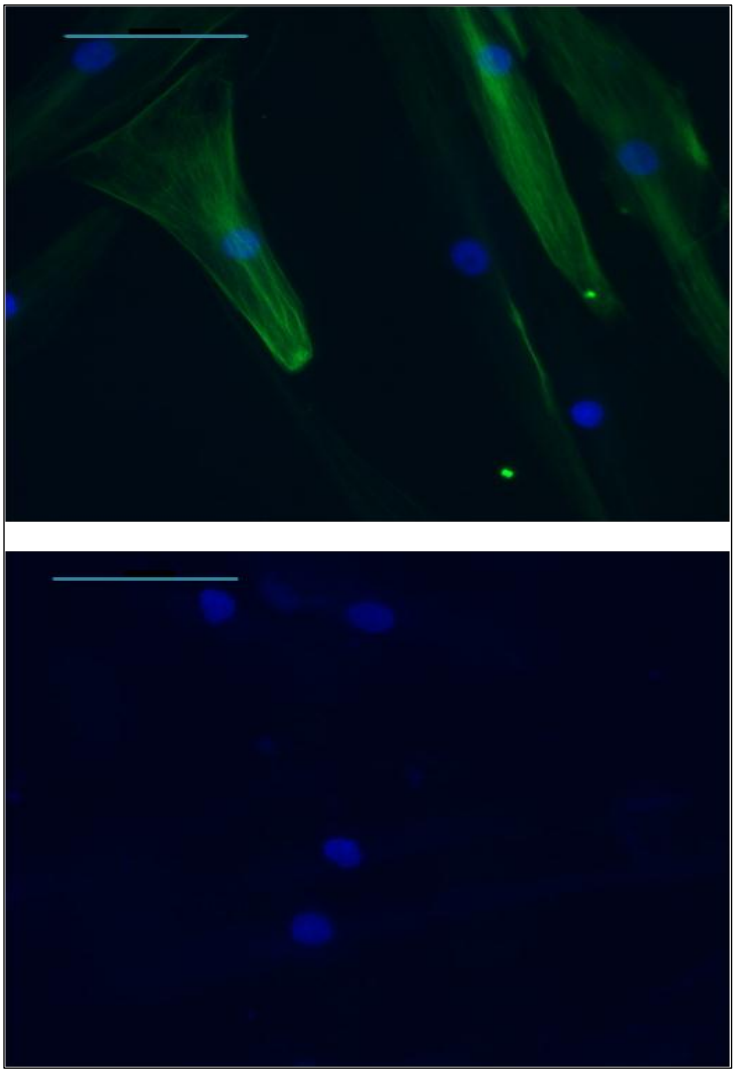


Figure 5.2 Desmin staining. Desmin staining was performed on the myoblast cell lines to determine the percentage of myoblasts and fibroblasts in the cultures. The top image shows desmin visualized with FITC (green), while the bottom image is without the primary antibody. Nuclei were visualized with DAPI. Scale bar is 100µm.

Patient	Biopsy	Percentage Myoblasts
3	2nd	98%
	3rd	76%
4	2nd	98%
5	1st	69%
6	1st	82%
7	1st	95%
8	2nd	86%

Table 5.1 Table of myoblast purity. Myoblasts were grown on cover slips and then stained for desmin to determine the amount of myoblasts present in the culture. The desmin positive cells and nuclei were then counted in order to provide a percentage.

5.4.2 MtDNA Heteroplasmy Levels

The MTND1/MTND4 qPCR was used to determine heteroplasmy levels within myoblasts. Samples of myoblasts were available for seven out of eight patients enrolled on the exercise trial, and for three of these patients myoblasts were available from more than one of their biopsies. There was considerable variation between patients regarding results, as well as one patient displaying marked differences between biopsies.

In all MTND1/MTND4 qPCRs four controls were run; three samples of mtDNA harbouring known deletion levels (37%, 67%, 87%), and a blood sample. In order for a run to be classed as successful the blood sample needed to be 0%, and the three known deletion samples had to be within 10% of their true value. In addition, patient samples were run in triplicate with Ct values only being used if they were within one Ct of each other.

5.4.2.1 Patient 2

Myoblasts from patient 2's first and second biopsies were available for analysis. This included first biopsy myoblasts from passages four to seven, with a sample of myotubes differentiated at passage eight. From his second biopsy, myoblasts from passages three to six were available along with a sample of myotubes differentiated at passage seven.

When the MTND1/MTND4 assay was performed on samples from patient 2's first biopsy, mtDNA deletions were present in his myoblasts at 38%, 30%, 33%, 26% (passages 4-7), which reduced to 24% in his myotubes (analysed with SYBR Green) (Figure 5.3A). Due to the surprising nature of these findings the MTND1/MTND4 assay was performed a further two times (Table 5.2A). These results were all within 10% of the first run, and therefore they were averaged (Table 5.3).

His second biopsy harboured a deletion of around 80% (Table 5.3) in the myoblasts; this level was maintained in the myotubes (Figure 5.3A). These samples were run four times, and each set of results were within 6% of all other runs (Table 5.2A), and have also been given as averages (Table 5.3).

When samples of this patient's urine and blood were run on the same assay the results suggested they were less than 30%. When they were analysed on the "common deletion" SYBR Green assay developed in chapter 4 they were shown to harbour no mtDNA deletion (Table 5.3).

5.4.2.2 Patient 3

Myoblasts from patient 3's second and third biopsies were available for analysis. Second biopsy cells from passages one to five, with myotubes differentiated at passage five were examined, along with myoblasts from his third biopsy passages one to six, and myotubes differentiated at passage seven.

Patient 3 was shown using the SYBR Green assay developed in chapter 4 to harbour an mtDNA deletion at 1% in this first set of myoblasts at passage one, but this was undetectable in all remaining passages. The myoblasts from his third biopsy, however, were shown using the MTND1/MTND4 qPCR to have a deletion of around 70%, this gradually decreased until it was 0% in his myotubes (Figure 5.3B). These samples were run a second time, and apart from one value at passage two, were all within 10% of the first run (Table 5.2B) so heteroplasmy levels were averaged out (Table 5.3).

It was decided to re-grow this patient's myoblasts from passage two and repeat the analysis to determine if the same pattern would be observed. Again the deletion was present, although it appeared to be at a slightly lower level this time (around 55%) and reduced to 13% on differentiation to myotubes (SYBR Green qPCR) (Figure 5.3B). When a sample of blood DNA from this patient was analysed using his deletion specific SYBR Green assay it was shown that there was no mtDNA deletion present (Table 5.3).

A

Biopsy	Repeat	Myob P3	Myob P4	Myob P5	Myob P6	Myob P7	MyoT
1st	1st		29%	30%	33%	26%	23%
	2nd		38%	32%	35%	26%	24%
	3rd		38%	36%	36%	36%	
2nd	1st	77%	77%	79%	77%		
	2nd	82%	82%	83%	83%		
	3rd	78%	79%	81%	77%		80%
	4th	80%	80%	82%	79%		80%

B

Biopsy	Repeat	Myob P1	Myob P2	Myob P3	Myob P4	Myob P5	Myob P6	MyoT
3rd	1st	65%	75%	77%	75%	57%	31%	0%
	2nd	68%	96%	78%	75%	52%	23%	0%

Table 5.2 Heteroplasmy levels of myoblasts from patients 2 and 3. Because of the surprising nature of the results demonstrating single mtDNA deletions in myoblasts of these patients, the samples were re-analysed. **A**. Patient 2 had his 1st biopsy samples run three times and his second biopsy samples run four times. **B**. Patient 3 also demonstrated a deletion in his myoblasts from his third biopsy, so again this assay was repeated. There was a good general agreement between runs.

5.4.2.3 Patient 4

Patient 4 had myoblasts available from passages one to three, obtained from his second biopsy. Myotubes differentiated at passage three were also available. An initial run using the MTND1/MTND4 qPCR revealed levels of mtDNA deletions, if present, were less than 30%. So these samples were analysed using the "common deletion" SYBR Green assay developed in chapter 4. Using this assay, it was shown that there was no mtDNA deletion present in any of his myoblasts or myotubes (Table 5.3).

5.4.2.4 Patient 5

Myoblasts from this patient's first biopsy, passages one to seven, and myotubes differentiated at passage eight were available for analysis. An initial run using the MTND1/MTND4 qPCR showed levels of mtDNA deletions, if present, were less than 30%. So these samples were analysed using his deletion specific SYBR Green assay developed in chapter 4. This assay suggested there were tiny amounts of delete mtDNA in his myoblasts (0.01%), although in some of the passages this was undetectable (Table 5.3).

5.4.2.5 Patient 6

Myoblasts from patient 6's first biopsy were available for analysis, these were from passages three to seven, and myotubes differentiated at passage eight. An initial run using the MTND1/MTND4 qPCR showed levels of mtDNA deletions, if present, were less than 30%. A SYBR Green assay was developed for him in order to determine if there was any delete mtDNA in these samples. Use of his deletion specific qPCR assay showed there was no mtDNA deletion in either his myoblasts or myotubes (Table 5.3).

5.4.2.6 Patients 7 & 8

Patients 7 and 8 began the exercise trial late on during this research. While it was possible to grow the myoblasts from these patients it was not possible to analyse their myoblasts using a SYBR Green assay. The MTND1/MTND4 assay suggests they both have less than 30% delete mtDNA in their myoblasts (Table 5.3), though this could be any amount between 0-30%.

Patient	Biopsy	Satellite Cells	Myob P1	Myob P2	Myob P3	Myob P4	Myob P5	Myob P6	Myob P7	MyoT	SKM	Other
2	1st	55%				35%	33%	35%	29%	24%	58%	0% (Urine) 0% (Blood)
	2nd	43%			79%	80%	81%	79%		80%		
	3rd	60%										
3	1st	83%									67%	0% (Blood)
	2nd	79%	1%	0%	0%	0%	0%			0%		
	3rd	56%	67%	86%	78%	75%	55%	27%		0%		
4	1st	50%									45%	
	2nd	47%	0%	0%	0%					0%		
5	1st	0.6%	0%	0.01%	0%	0%	0%	0%	0.001%	0%	10%	
	2nd	1.3%										
	3rd	3.6%										
6	1st	44%			0%	0%	0%	0%	0%	0%	48%	
7	1st	50%			<30%	<30%	<30%	<30%	<30%	<30%	62%	
	2nd	53%			<30%	<30%	<30%	<30%	<30%	<30%		
8	2nd	47%		<30%	<30%	<30%	<30%	<30%	<30%	<30%	43%	

Table 5.3 Heteroplasmy levels for myoblasts and myotubes. Heteroplasmy levels were determined using either the MTND1/MTND4 qPCR or a deletion specific SYBR Green assay. Myoblasts were not available for patient 1, and only available for some of the biopsies of other patients. Where levels are given as <30%, the MTND1/MTND4 assay has shown these cells have less than 30% delete mtDNA but it has not been possible to run these samples on a SYBR Green assay, so it is not possible to say if they have any mutation or not. Myob = myoblasts, P = passage number, MyoT = Myotubes, SKM = skeletal muscle homogenate levels (as determined in chapter 4).

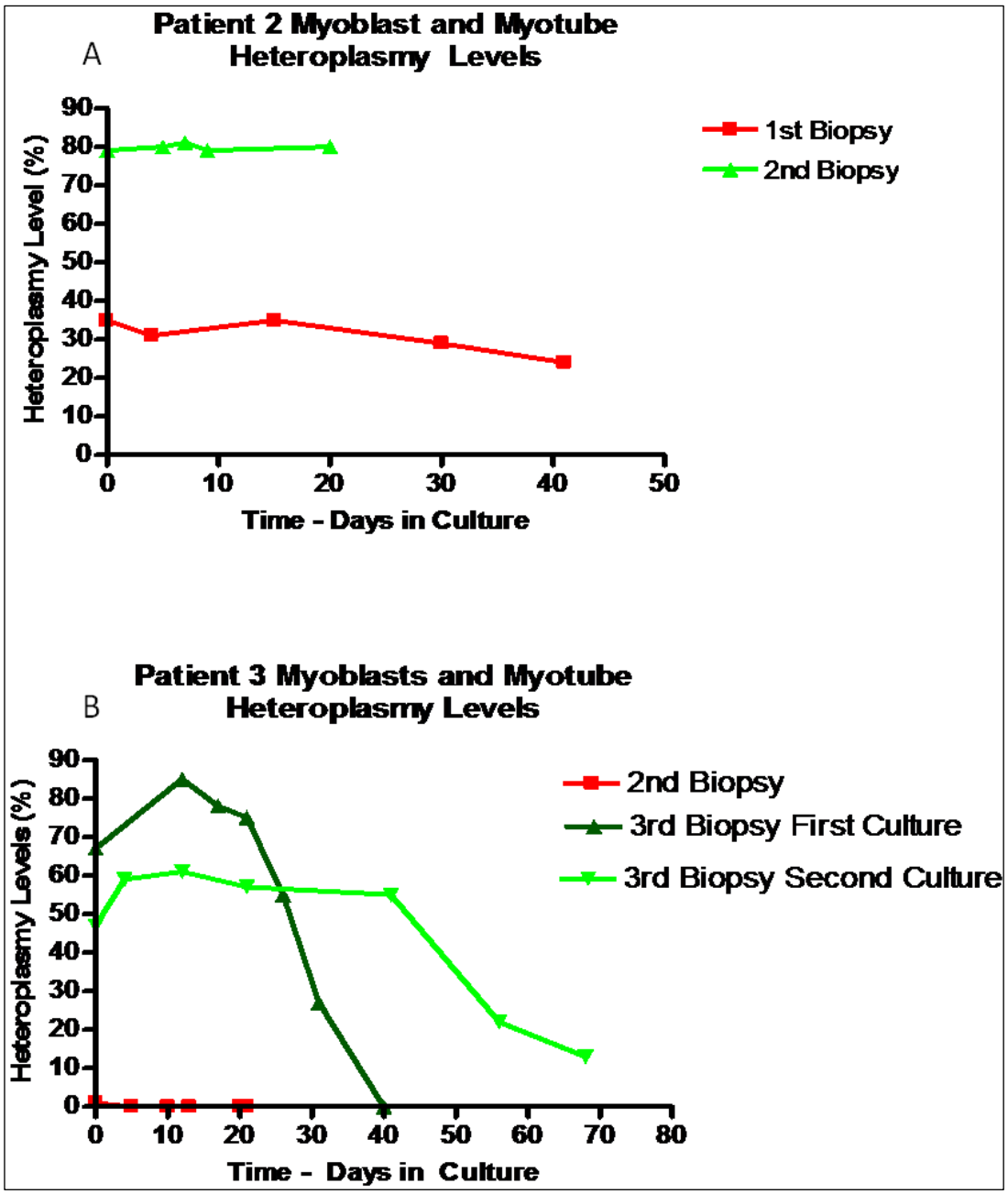


Figure 5.3 Changes in heteroplasmy over time. A. Patient 2 maintained the mutation at around 30% in his myoblasts from his first biopsy, with a slight decrease as the cells differentiated. In his second biopsy the mutation was maintained at around 80%, this did not change as the cells differentiated. **B**. Patient 3 lost the mutation in his second biopsy, but maintained it during his third biopsy, although a decrease was again observed as the cells headed towards differentiation. The same pattern was observed when this cell line was grown again. The last data points on the line correspond to levels in the differentiated myotubes.

5.4.3 Pyruvate and Uridine Supplementation

The cell medium myoblasts are grown in contains FCS, L-glutamine, and after the addition of the supplemental mix provided by the manufacturer, also contains fetuin, basic fibroblast growth factor, epidermal growth factor, insulin and dexamethasone. However, it has been shown that cells with an OXPHOS impairment may divide slower or not at all in culture, and are often dependent on pyruvate and uridine for growth because of the absence of a functional respiratory chain (King and Attardi, 1989). Supplementing the medium with pyruvate and uridine avoids selection occurring against respiratory deficient cells.

To determine if the lack of pyruvate and uridine in the medium could have had a negative selection pressure on the myoblasts some of the cells were re-grown with pyruvate and uridine added to the medium.

Patients 2 and 3 had originally maintained the mutation in their myoblasts. After growing in supplemented medium, patient 2 still harboured the mutation (Table 5.4). This was at a similar level to when the cells were grown without supplementation (Figure 5.4A). Again, patient 3 maintained the mutation in his myoblasts when grown in supplemented medium (Table 5.4). It was shown that when the cells were grown without supplementation there was a decrease in the heteroplasmy levels. When they were grown with supplementation there was a slight increase in the heteroplasmy levels (Figure 5.4B).

All patients who had lost the mutation first time around had MTND1/MTND4 results suggesting the levels were still below 30% (Table 5.4). While we can hypothesise that this will have been 0%, this would need to be demonstrated with the relevant SYBR Green assay to confirm.

Cells grown with supplementation were grown at a later time point than those grown without. While they originated from the same flask of cells they will have undergone freezing and thawing. It is possible that this accounts for the differences in starting heteroplasmy levels at passage four for patient 3. In addition, this was a single experiment and in order to draw any firm conclusions about the effects of supplementation on heteroplasmy levels it should be repeated.

Patient	Biopsy	Satellite cells	Myob P4	Myob P5	Myob P6	Myob P7
2	2nd	43%	79%	81%	76%	71%
3	3rd	56%	37%	38%	49%	48%
5	1st	0.6%	<30%	<30%	<30%	<30%
6	1st	44%	<30%	<30%	<30%	<30%

Table 5.4 Myoblast heteroplasmy levels after supplementation. Some of the myoblasts were re-grown in medium supplemented with pyruvate and uridine, allowing for proliferation of respiratory chain impaired cells. The two patients who had previously maintained the mutation continued to do so, while the two patients who lost the mutation still contained levels below 30%. Heteroplasmy levels were determined using the MTND1/MTND4 qPCR on a single run.

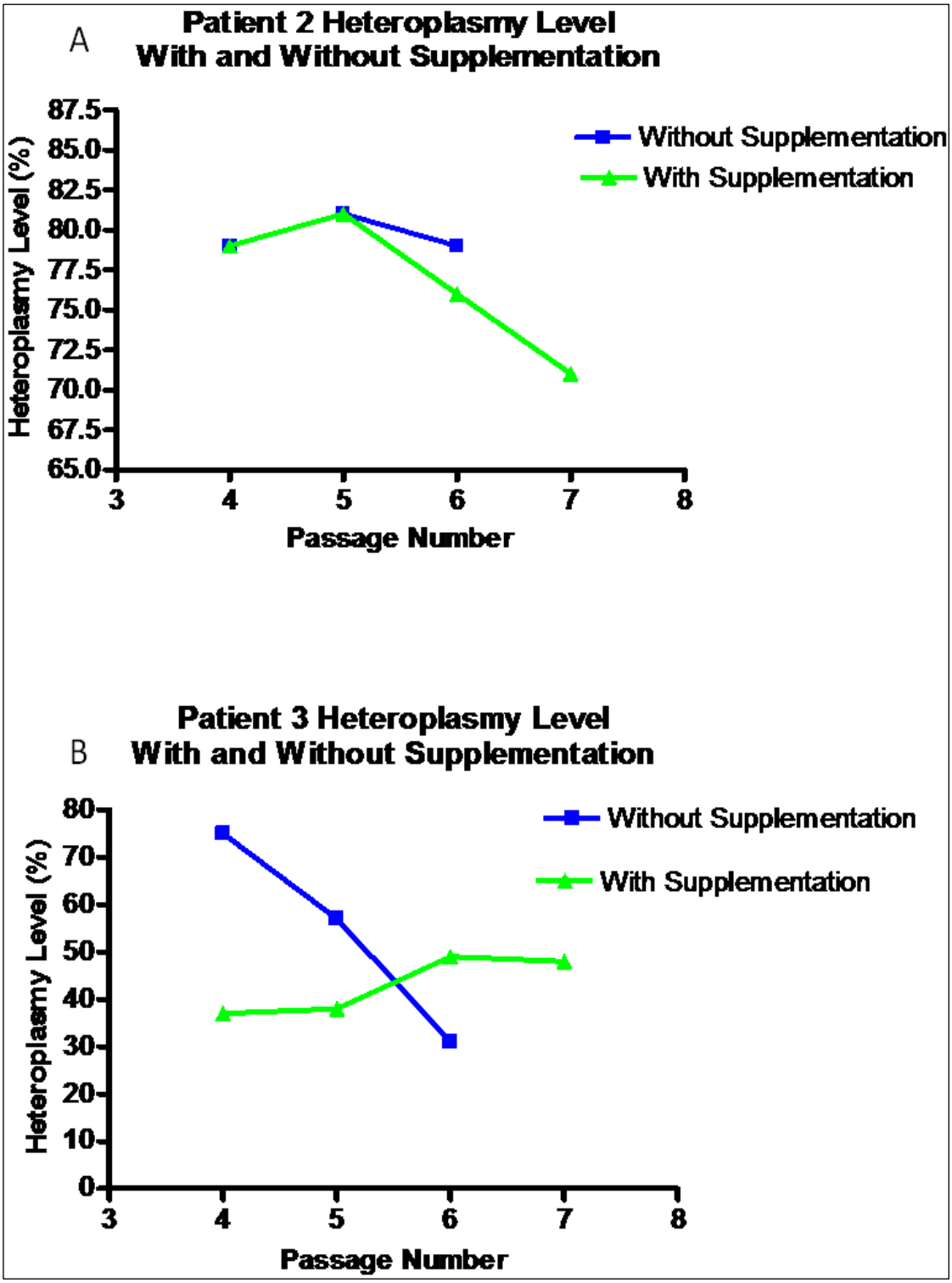


Figure 5.4 Graphs showing changes in heteroplasmy. A Patient 2 maintained the levels of heteroplasmy in his myoblasts both with and without supplementation at similar levels. **B**. Patient 3 maintained the mutation in myoblasts grown with and without supplementation. Although, the cell line that was grown with supplementation had a slightly lower starting level and rose slightly, while the myoblasts that were grown without supplementation started higher, but this was followed by a reduction.

5.4.4 Functional Consequences

Having demonstrated that some sporadically occurring mtDNA deletions could be maintained in myoblasts it was decided to see if these mutations had a functional consequence on the cells. In order to do this COX histochemistry was performed.

A series of optimisations ranging from forty five minutes to three hours were performed to determine incubation times for the COX reagent. Optimal staining was achieved with a two and a quarter hour incubation time, at 37^{o}C.

Myoblasts from one patient who maintained his mtDNA deletion (patient 3) and from two patients who lost their mutations (patients 5 and 6), together with a control cell line, were grown on cover slips and then incubated in COX medium for two and a quarter hours.

Images were taken on a Zeiss Axioplan 2 microscope, and converted to greyscale (Figure 5.5); densometric analysis was performed using AxioVision 4.8.1 software. This gave a value between 0-250 which was an indication of density of staining per unit area, with 1 being the most intense and 250 being the least. Three cover slips were counted for each patient and on each cover slip three areas were counted. Analysis was performed at x20 magnification and all myoblasts visible on the screen were analysed. Values were subtracted from 250 in order to give a scale in which the most densely stained cells had the highest numerical values.

Patient 3 had a mean intensity of staining of 70.96 ± 4.7, patient 5 was 76.10 ± 12.2, and patient 6 was 74.62 ± 6.4, while the control myoblasts had a mean of 90.08 ± 14.1. Patient 3 who harboured the mtDNA deletion in his myoblasts appeared to have weaker staining than both the other two patients and the control cell line. However, it would be necessary to repeat this experiment with more controls before any firm conclusions can be drawn.

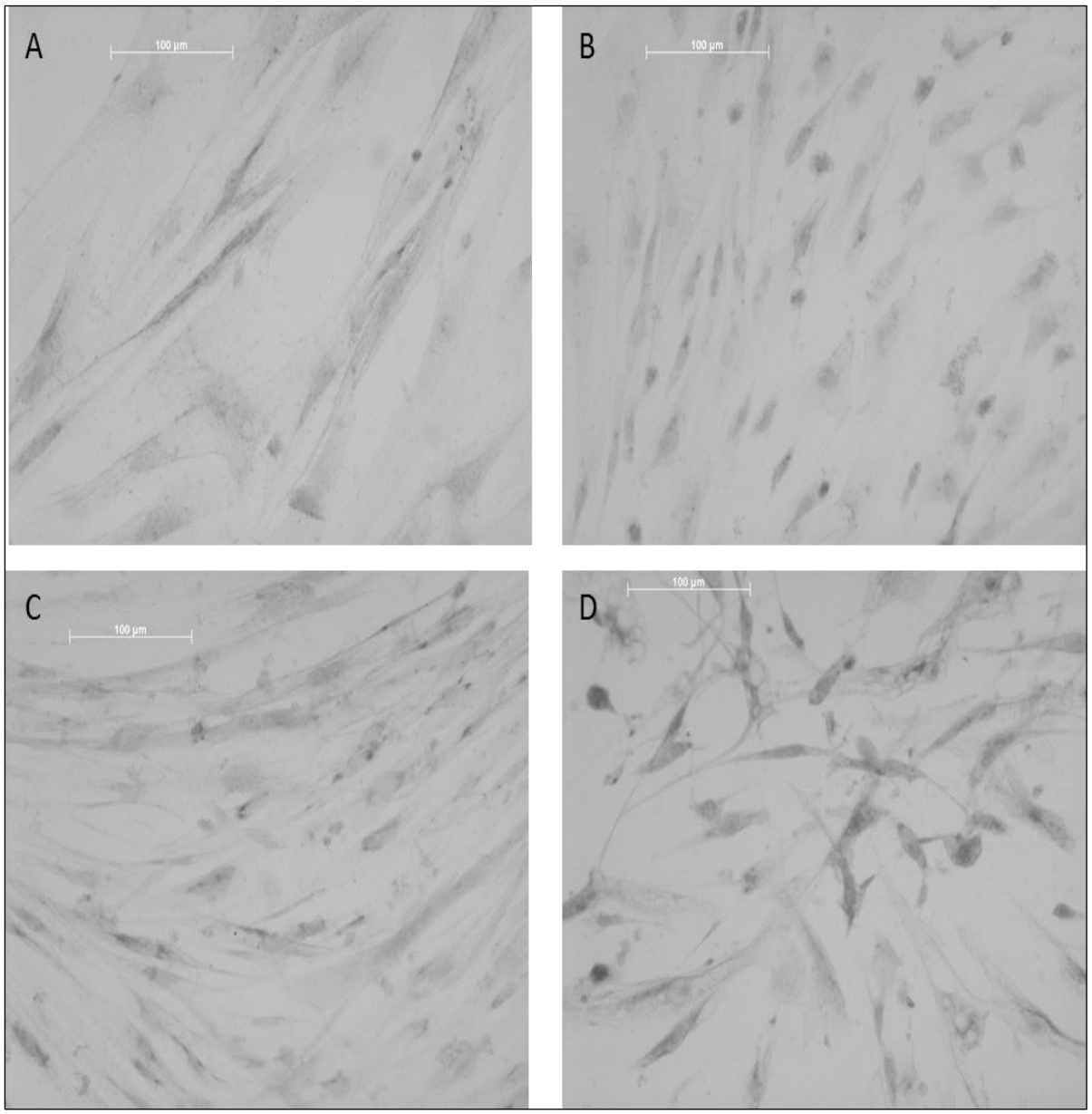


Figure 5.5 COX staining on cultured myoblasts. Myoblasts from patients 3 **(A)**, 5 **(B)** and 6 **(C)** were grown on cover slips along with a sample of control myoblasts **(D)**. Images were taken and converted to grey scale in order for densitometric analysis to determine how intensely the COX had stained.

5.4.5 Testing for an MtDNA Duplication

Given the surprising nature of the results demonstrating the presence of mtDNA deletions in myoblasts of some of our patients, it was decided to examine the possibility that the mutations could in fact be mtDNA duplications. It is thought that mtDNA duplications allow the presence of mtDNA rearrangements in dividing tissues (e.g. blood), which would otherwise eliminate deletions (Poulton and Holt, 1994).

5.4.5.1 Southern Blot

To test for an mtDNA duplication, a sample of patient 2's myoblasts, and a sample of WT mtDNA were cut using the restriction endonucleases *BamHI* and *SnaBI*. The samples were electrophoresed on a 0.7% agarose gel for twenty one hours at 30V and then transferred to a hybridisation membrane by Southern blot. An area corresponding to the D-loop was probed using ^{32}P. Images of the DNA bands were taken using a phosphoimager.

BamH1 cuts once in the mtDNA at position nt.14257 to give a linear 16.5Kb molecule (Figure 5.6A). This band can be clearly seen in the WT sample. In the patient sample, a delete linear band can be seen along with a very faint higher WT band, attributable to the small amount of WT mtDNA this patient has (Figure 5.6B).

The uncut samples will have super coiled mtDNA which will run much higher than the linearised samples. This can be seen in the WT uncut lane, and also a delete molecule super coiled band in the patient 2 uncut lane. Both the WT and the patient lane also contain a smaller band corresponding to some naturally occurring linear mtDNA in the sample.

SnaBI cuts once at position nt.10735, which is within the break points of the "common deletion" (Figure 5.6A). The gel would appear to show that this enzyme has not cut very efficiently. In addition to a 16.5Kb linear band in the WT sample, there would appear to be some closed super coiled DNA that has not been linearised. Furthermore, there is a third band below this one that cannot be explained. Because the restriction site for this enzyme is within the breakpoints for the common deletion, the delete mtDNA from the patient will not have been cut and consequently there is a band corresponding to delete super coiled mtDNA.

If this sample harboured any mtDNA duplications then bands above the WT super coiled fragment would be observed for the uncut samples.

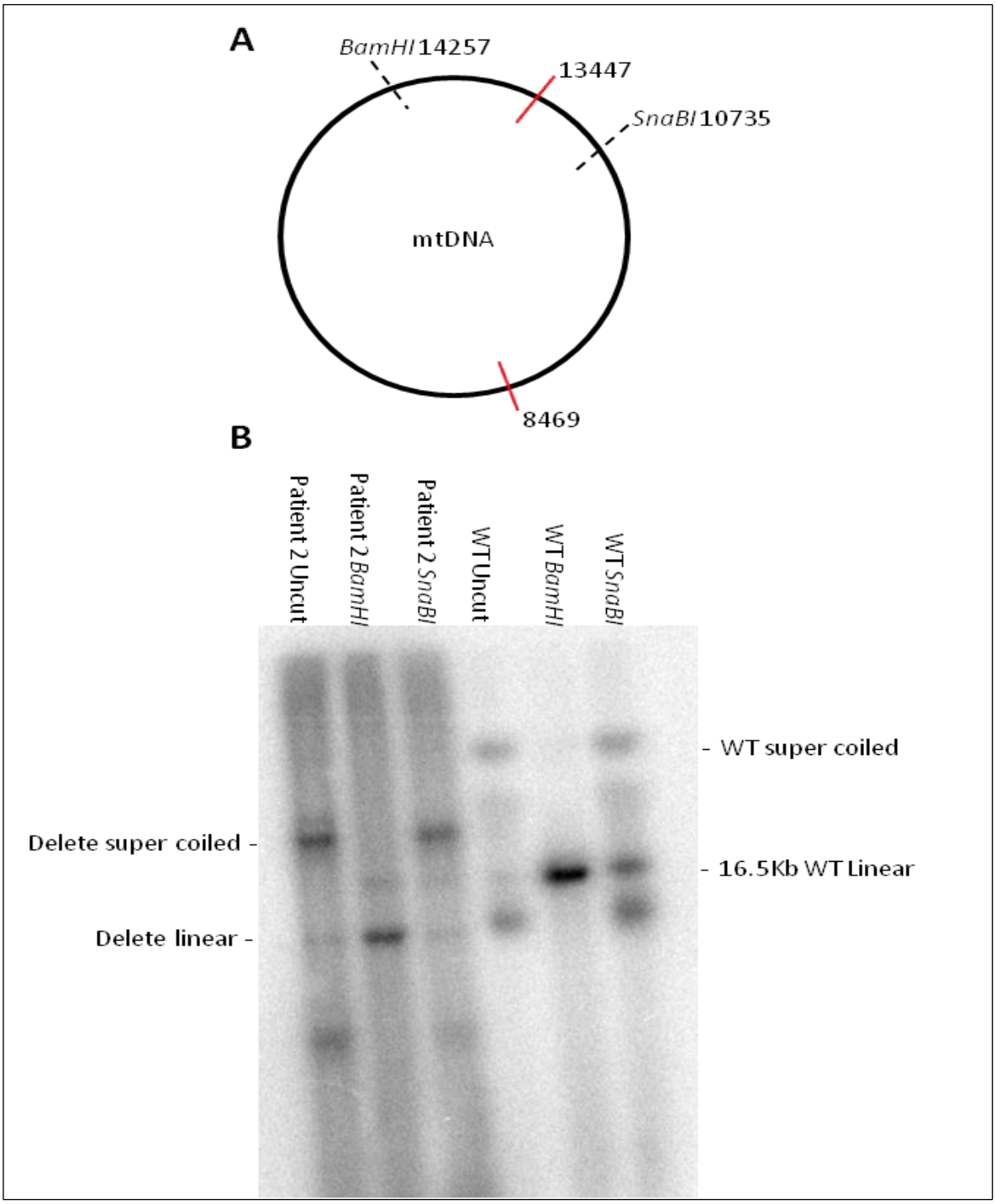


Figure 5.6 Southern blot. A. Myoblast mtDNA from patient 2 was digested using restriction endonucleases that cut at nt.14257 and nt.10735. **B**. From the WT sample a super coiled mtDNA band and a WT linear band can be identified. The *SnaBI* enzyme appears to not have fully digested the mtDNA as there is still a super coiled fragment visible. Using *BamHI* a linear fragment of delete mtDNA can be observed that sits below the linear WT mtDNA band.

5.5 Discussion

The aims of this chapter were to culture myoblasts from patients who had previously been shown to harbour single mtDNA deletions in their satellite cells. This was to determine what happens to any mutations present as the cells progress towards and differentiate into, myotubes. It was hoped this would identify the step of muscle regeneration where selection is acting on these cells enabling mutations to be lost.

5.5.1 MtDNA Deletions in Myoblasts

When these experiments were started it was expected that no mtDNA deletions would be present in the myoblasts from patients harbouring sporadically occurring mutations. Finding single mtDNA deletions in myoblasts from some of the patients is therefore surprising. In addition, the fact that in some patients the mutation is maintained and in others it is lost, adds to the intrigue. Patient 3 is particularly interesting. In his second biopsy he lost the mutation after the second passaging of his cells, but in his third biopsy he maintained the mutation.

When the mutation was lost from his myoblasts the starting level in his satellite cells was higher than it was for his third biopsy which is when it was maintained. This pattern is somewhat repeated in patient 2. Patient 2 maintained the deletion in both his first and second biopsy; however, it was much higher in his second biopsy which is when the level of mtDNA deletion in his satellite cells was the lowest.

This would suggest that whether a patient loses the mutation in their myoblasts is not mutation or patient specific and not dependent on the starting level of deletion in their satellite cells. In addition, patients 2 and 3, who maintained the mutation in at least one of their biopsies, had further samples examined. Blood samples from both patients had no mtDNA deletions and a urine sample from patient 2 had no mutation. These findings were not surprising, given suggestions that sporadically occurring mutations are lost from mitotic cells, for example blood, urinary and buccal cells (Brockington et al., 1995, Marzuki et al., 1997, Aure et al., 2007). This demonstrates that the mutation can be lost from dividing cells in these two patients, but was not always lost from their dividing myoblasts.

5.5.2 An MtDNA Duplication?

It was initially thought that a more complex mtDNA rearrangement may be responsible for the maintenance of the single mtDNA deletion in the dividing myoblasts. MtDNA rearrangements include partial deletions or duplications of the genome. While patients 2 and 3 have been diagnosed as having large scale single mtDNA deletions, it was possible that they also harboured an mtDNA duplication.

Duplications can consist of a WT genome with a delete genome inserted into it, or as deletion dimers (two delete molecules joined together) (Poulton et al., 1993, Grossman and Shoubridge, 1996). The mixture of recombinations observed, and the fact that the proportion of duplicated molecules varies with both time and tissues, has led to suggestions that duplications are in fact an intermediate species. Observed reductions in duplications coinciding with increases in deletion dimers over time, may be caused by the delete molecule being ejected from the enlarged duplicated one (Poulton et al., 1993, Poulton et al., 1995).

MtDNA duplications have been reported in KSS (Poulton et al., 1994b), and in Pearson's syndrome (Rotig et al., 1995). It has been suggested that the balance of duplicated and delete species is partly responsible for the differing phenotypes of these diseases (Poulton and Holt, 1994).

The presence within a duplicated molecule of all the necessary genes that are required for OXPHOS could result in an absence of selection acting on the myoblasts of patients 2 and 3. Indeed, this is part of the reason duplications can be inherited.

However, the results of the Southern blot show that the maintenance of a single mtDNA deletion in the myoblasts of patient 2 is not because the patient harbours an mtDNA duplication. In addition mtDNA duplications are often detectable in blood (Poulton et al., 1994b, Poulton et al., 1993) and analysis of both blood and urine samples from patient 2 and a blood sample from patient 3 found no mtDNA deletion present (Table 5.3).

Additional support for an mtDNA duplication not being involved in the maintenance of mtDNA deletions in the myoblasts of these patients comes from findings by Poulton *et al.* (1994). This paper examined eighteen patients with KSS or CPEO and found duplications associated with 10 out of 10 KSS patients but not in any of the patients presenting with CPEO. This suggests that this particular type of mtDNA rearrangement may be primarily

associated with KSS and not CPEO (Poulton et al., 1994b). All the patients examined in the present study have CPEO, including patients 2 and 3 who maintain the mtDNA deletion in their myoblasts (Table 3.1).

5.5.3 Losing the Mutation

There were marked differences both between patients and in one case between biopsies in the loss and maintenance of mutations. There would appear to be two periods of potential loss. The first occurs at the point of satellite cell activation and is extremely fast and almost absolute; the second is a more gradual decline in mtDNA deletion levels in the myoblasts.

As previously stated the rapid loss at the point of satellite cell activation could be attributable to cells with mutation not being able to activate, although findings in this chapter suggest they can activate. In addition even cells with a high level of mtDNA deletion (patient 2 second biopsy, patient 3 third biopsy) are able to do so. In addition, there is a considerable time period between the investigation of the satellite cells and the myoblasts. This is when the myoblast cultures are being established, and can take up to two weeks. It is possible that mutations are lost during this time frame. However, the maintenance of deletions in three out of ten of the myoblast cell lines suggests this may not be the case.

Alternatively there could be a bottleneck occurring. A bottleneck occurs when there is a reduced amount of DNA followed by replication from a smaller set of genomes. In mitochondrial genetics the bottleneck accounts for the rapid shifts in heteroplasmy that can occur between generations.

There are a few potential ways this could happen during satellite cell activation. If, after activation, satellite cells are dividing asymmetrically to both form myoblasts, and repopulate the stem cell pool, the myoblasts may either maintain or lose the mutation depending on the way the cell divides. Alternatively, if muscle contains satellite cells that have varying amounts of single mtDNA deletions, depending on the exact cells activated, the resultant myoblasts could experience a rapid shift in heteroplasmy levels. These theories are discussed more in the next chapter.

The second phase of selection occurs in the myoblasts. On two out of the three occasions the mutation was maintained, there was a slight drop in levels as the cells progressed

towards differentiation into myotubes (Figure 5.3). Previous reports have suggested that there is an increase in mitochondrial biogenesis as myoblasts proceed towards differentiation as well as a switch from primarily glycolytic energy generation to OXPHOS (Leary et al., 1998, Brunk and Yaffe, 1976, Herzberg et al., 1993, Duguez et al., 2001). This increased reliance on OXPHOS and/or an up regulation of mitochondrial biogenesis could mark a point at which negative selection occurs on the cells. Any cells that have a respiratory chain deficiency may die when these processes begin.

Alternatively, after myoblast division, there may be uneven segregation of WT and delete mtDNA, with cells harbouring large amounts of delete mtDNA dying, or being unable to proliferate as quickly as WT myoblasts. Again, these possibilities are discussed and examined further in the next chapter.

The differences observed between proliferating myoblasts and dividing blood and urinary epithelial cells in patients 2 and 3, may be attributable to the nature of these tissues. Blood stem cells and urinary epithelia are constantly dividing and thus there is a longer period of time over which negative selection could occur. In contrast, selection can only happen in myoblast after injury to the muscle.

5.5.4 Pyruvate and Uridine Supplementation

When cells from patients who had originally lost the mutation in their myoblasts (patients 5 and 6) were grown a second time, but with the addition of pyruvate and uridine, the MTND1/MTND4 qPCR results suggested the levels were still below 30%. While it is possible to make the assumption that the mutation had been lost again, only the relevant SYBR Green assay would confirm this. In addition it was only possible to perform this experiment on myoblasts from passage four onwards, this would allow for a possible period of selection on the myoblasts when they were originally being grown without supplementation up to passage four.

The mutation was maintained in myoblasts from patients 2 and 3 when the cells were grown both with and without supplementation. Patient 2 maintained the mtDNA deletion in his myoblasts at a similar level to the growth without supplementation. Patient 3 harboured the deletion at a lower level when his myoblasts were grown with supplementation. However, this decrease was observable at the first passage, which suggests that when the cells were thawed there may have been an initial cull of cells with high levels of mtDNA deletion. It was intriguing that these cells actually increased their heteroplasmy levels slightly. This did not happen when the cells were grown without supplementation. This suggests that the medium was acting somewhat selectively for these cells when they were grown without supplementation, and that the addition of these factors may have allowed myoblasts with mutations to persist for a short while longer. However, this is based on a single observation in patient 3, and did not occur in patient 2.

It is interesting to note that it was possible to grow the cells for an extra passage for both patients 2 and 3 when the medium was supplemented. It is possible that when the cell cultures were originally set up in the MRC Centre for Neuromuscular Diseases Biobank that the lack of supplementation caused selection to act on some of the myoblasts.

5.5.5 Previous Findings

While the presence of sporadically occurring mtDNA deletions in the myoblasts of these patients was unexpected given previous reports (Fu et al., 1996, Weber et al., 1997), they still do not contradict them. In the current study two patients had no detectable mutation in their myoblasts, and a further patient harboured amounts below the sensitivity of the assays used in the original papers. In addition there have been previous reports of mtDNA deletions in myoblasts, just at lower levels than in muscle homogenate (Moraes et al., 1989a, Moraes et al., 1989b, Bonod-Bidaud et al., 1999).

The papers using cell culture to examine mutations (Fu et al., 1996, Weber et al., 1997) supplemented the media with pyruvate and uridine, at the start of culture. It is probable then that the selection point for these cells occurred at the satellite cell activation stage.

The methods used to induce regeneration when testing the theory of "gene shifting" were bupivicaine (Clark et al., 1997) and biopsying (Shoubridge et al., 1997). These were very severe techniques and muscle necrosis will have been severe. In order to regenerate the muscle a large amount of cell division will have occurred thus allowing for selection. The change from glycolysis to OXPHOS has been reported to occur around day twelve (Leary et al., 1998) and while this was an *in vitro* experiment, a similar switch could have occurred in the three weeks these papers allowed for muscle regeneration. In addition, the mutations could also have been lost at the satellite cell activation stage.

5.6 Conclusions

Sporadically occurring single mtDNA deletions can be maintained in proliferating myoblasts. However, there would appear to be considerable variation both between patients and even between biopsies. Whether the mutation is lost or maintained, is not dependent on the mutation type, heteroplasmy level, or size. It would appear to be a more stochastic process.

There are probably two periods of selection acting on the cells responsible for muscle regeneration. In some cases the mutation is lost at the point of satellite cell activation; and in others it is lost as the myoblasts progress towards differentiation.

While the maintenance or loss of mutation from myoblasts would appear to be a random process, analysis of the myoblasts from patients 7 and 8 using a SYBR Green assay may give a better indication as to the likelihood of maintenance or loss in future patients.

The findings of mtDNA mutations in myoblasts may be initially worrying for the possibilities of “gene shifting” in patients undergoing the exercise trial. However, the fact that in the majority of biopsies performed the mutation was lost and in the others there was a downward trend in deletion levels suggests that “gene shifting” is still a viable option for these patients.

Chapter 6 MtDNA Deletions and Selection Points

6.1 Introduction

The previous two chapters have demonstrated the presence of large scale single mtDNA deletions in the satellite cells of all patients examined. The myoblasts of two of these patients (patients 2 and 3) also contained mtDNA deletions. In patient 2's first biopsy there was a gradual decline as the cells headed towards differentiation, while in his second biopsy there was no such decline. Patient 3 maintained the mtDNA deletion in his third biopsy but by the time the cells had differentiated into myotubes the mutation had been lost.

It has been hypothesised in chapter 5, that the loss of mutation in many of the patients at the point of satellite cell activation is attributable to a bottleneck effect, or possibly selection. Whether the bottleneck occurs through an actual reduction in mtDNA copy number caused by cellular division immediately after activation, or via activation of certain satellite cells and thus replication of subgroups of mitochondrial genomes is unclear.

A second period of selection occurs in the myoblasts that maintain the mtDNA deletion as they head towards differentiation. This suggests that myoblasts with impaired respiratory chain deficiency are dying. This may be attributable to an increase in mitochondrial biogenesis and a switch from a primarily glycolytic nature to one in which OXPHOS is the main supplier of energy (Leary et al., 1998). Although the fact this gradual decline only occurred in two out of three myoblast cell lines that maintained the mutation, opens up the possibility that a different mechanism could be responsible for the cells dying. After myoblast division, if there is uneven segregation of the mtDNA on the basis of deletions, then the cells that have inherited high levels of mtDNA deletions may die.

6.1.1 Bottlenecks and Satellite Cells

Ever since investigations into Holstein cows suggested that "amplification of one or a few mtDNA molecules will yield one predominant genotype"(Hauswirth and Laipis, 1982), research into mtDNA bottlenecks has focused on the shift in genotype that is sometimes observed in embryogenesis. This shift involves a stage in primordial germ cell (PGC) development where a reduction in reproducible mtDNA occurs, amplification of mtDNA then proceeds from this small population (Figure 1.15). However, there is still ongoing debate about the exact mechanism of this particular mtDNA bottleneck (Khrapko, 2008). The differing opinions about the mechanism of the bottleneck fall into three main groups.

Cao *et al.* (2007) proposed the bottleneck occurs via the aggregation of mtDNA into (homoplasmic) segregating units (or nucleoids). They isolated PGCs by staining with alkaline phosphatase and then used qPCR to measure mtDNA copy number. No decrease in copy number was observed and they concluded that the bottleneck was created without an actual reduction in mtDNA. The amplification of a small number of the segregated units could account for the rapid shifts in heteroplasmy (Cao et al., 2007).

However, Cree *et al.* (2008) argued that staining the PGCs with alkaline phosphatase disrupts the extremely sensitive qPCR reaction that is used to determine mtDNA copy number. They used *stella*-GFP transgenic mice and isolated PGCs via FACS sorting. Using a qPCR method they demonstrated an actual reduction in mtDNA copy number in PGCs and hypothesised that the segregation of these much reduced genomes could account for the bottleneck (Cree et al., 2008).

Having found a similar reduction in mtDNA copy number Wai *et al.* (2008) came to a different conclusion. They failed to show a wide variation in heteroplasmy immediately after the initial reduction, leading them to suggest that the variation does not occur until a later stage. Again, contrasting results were attributed to different isolation techniques. Rather than using FACS sorting, this group opted for manual isolation of PGCs, as they found FACS sorting resulted in an unacceptable amount of "debris and doublets". Their proposed mechanism involves the selective replication of a subpopulation of mtDNA copies at a later stage of development, but without a reduction in copy number (Wai et al., 2008).

Given that satellite cells are known to be mitotically quiescent, and that even the very first EM images suggested these cells consisted mostly of nucleus with a “paucity” of cytoplasm (Mauro, 1961), it is reasonable to assume they have a low mtDNA copy number. Replication of a sub-population of these molecules could result in dramatic shifts in the heteroplasmy level. However, like the bottleneck that occurs during embryogenesis, there may be a few possible mechanisms accounting for the effect that occurs here.

While results in chapter 4 suggest that satellite cells are heteroplasmic on the basis of mtDNA deletions, this may not be the case for individual cells. A homogenate sample of satellite cells with a heteroplasmy level of 50% may be the result of all cells harbouring 50% delete mtDNA, the cells being homoplasmic with either 0% or 100% delete mtDNA. Alternatively cells may contain a mixture of different heteroplasmy levels which all average out to 50%.

6.1.2 Replication of a Subgroup of Mitochondrial Genomes

After injury satellite cells are activated, the size of their response is proportional to the extent of the injury. If the basal lamina has been damaged then satellite cells may migrate to adjacent fibres. A smaller injury which has left the basal lamina intact, will allow for satellite cells to migrate underneath it to the site of injury (Hawke and Garry, 2001). However, it is very difficult to give a precise number on how many satellite cells are activated in order to repair an injury. One experiment suggested that a single bout of eccentric training was enough to activate between 5% to 11% of satellite cells in three month old mice (Darr and Schultz, 1987). While in humans, a single bout of exercise resulted in an increase in satellite cells at day two of over 100% (Crameri et al., 2004). But the numbers of these cells that will go on to form myoblasts is unknown, and it is difficult to say how many satellite cells are activated in response to resistance training in our patients.

Nevertheless, there is a possibility that the dramatic shifts in heteroplasmy observed in our patients is caused by an activation of a random subgroup of satellite cells which have either very high or very low amounts of single mtDNA deletion (Figure 6.1B). Alternatively, the satellite cell population may consist of homoplasmic WT and delete cells (Figure 6.1C). Through the activation of primarily WT or delete mtDNA satellite cells, the mutation may be either lost or maintained.

6.1.3 Reduction in MtDNA Copies via Asymmetric Division

Satellite cells are able to proliferate and commit to a myoblast fate, and in addition, are able to self-renew to repopulate the stem cell pool. This divergent fate of satellite cells involves an asymmetric division after activation, which has been demonstrated on the basis of many factors.

The cytoplasmic protein Numb is involved in determining the fate of muscle precursor cells and is thought to exert its influence through attenuating the effects of Notch-1, an inhibitor of these cells. Asymmetric localization of Numb, to one pole of dividing cells, perpendicular to the plane of mitosis, precedes a heterogeneous expression of Numb in daughter cells. Numb positive cells are more committed to a muscle phenotype, as demonstrated by their high levels of Myf5 expression (Conboy and Rando, 2002).

The asymmetric division of satellite cells has also been shown to occur on the basis of template DNA strands, which co-segregate to one daughter cell (Shinin et al., 2006). This provides support for the Cairn's hypothesis, that stem cells retain immortal template DNA during divisions, thus protecting them from replication induced errors (Cairns, 1975).

More recently Kuang and colleagues demonstrated both symmetric and asymmetric satellite cell divisions. Symmetric divisions occur in a planar orientation generating identical daughter cells that either renew the stem cell pool or become muscle progenitor cells. Asymmetric divisions occur in an apical-basal orientation and result in a stem cell at the basal side and a committed muscle precursor cell on the apical surface. This asymmetric division has segregated localisation of Myf5, with the negative cells being self-renewing, and the positive cells becoming committed to a muscle cell fate. In support of Shinin and colleagues (2006) they demonstrated that 41% of Myf5 positive cells labelled for BrdU, while only 14% of negative cells did, suggesting these cells were synthesising DNA and proceeding through the cell cycle (Kuang et al., 2007).

While the above examples of asymmetric satellite cell division suggests the localisation of various signalling molecules and DNA is very targeted, in the case of mtDNA, the localisation would appear to be more stochastic. If the mtDNA copy number is low in satellite cells then a single division would reduce this number further. In addition it would segregate the mtDNA copies into two daughter cells. Depending on the cell that

proceeded to form muscle precursor cells, the resultant myoblasts may either lose or maintain the mutation (Figure 6.1A).

It should be noted that the above mechanisms for a bottleneck are not mutually exclusive. Activation of a subgroup of satellite cells followed by asymmetric division would provide an even stronger bottleneck and given the drastic reduction observed when some of the patients lose their mutation; this may be the causative factor.

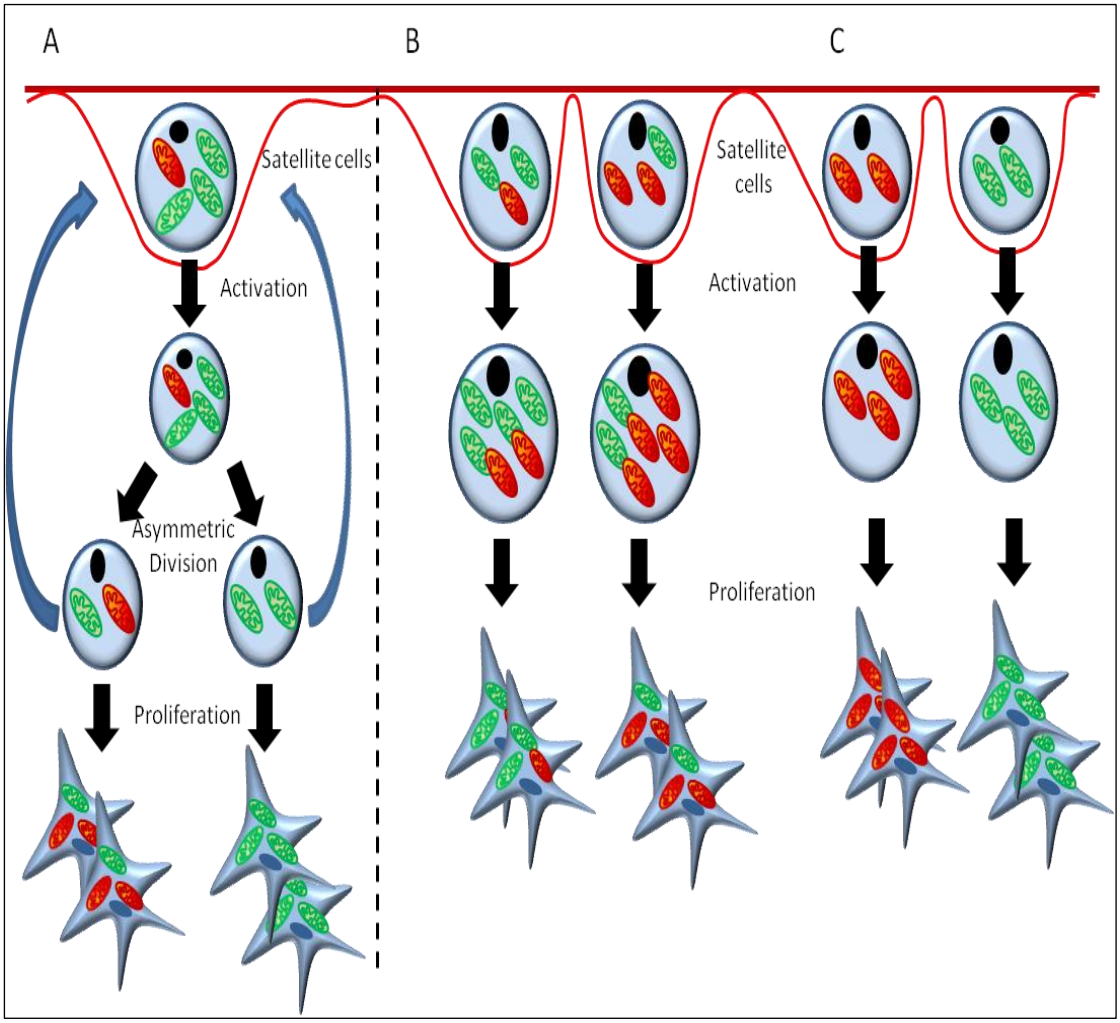


Figure 6.1 Satellite cell activation selection point. At the point of satellite cell activation a large number of patients lose their mtDNA deletion from their satellite cells. This has been hypothesised to occur via a bottleneck. There are a few possible mechanisms by which this could have occurred. **A**. Asymmetric division of the activated satellite cell results in two daughter cells, one which has a mutation and one which does not. Depending on which daughter cell proceeds to form myoblasts, the patient will either lose or maintain the mutation. This mechanism involves an actual reduction in the mtDNA copy number. **B & C** Alternatively it could involve replication of a sub group of mtDNA copies through the activation of satellite cells that have either very large or very small amounts of delete mtDNA **(B)**, or cells that are homoplasmic for the presence or absence of the mutation **(C)**. Red mitochondria indicate high levels of mtDNA deletions, green mitochondria are WT.

6.2 Aims

Results so far suggest that selection is acting on regenerating muscle cells at two points; satellite cell activation, and the proliferation or differentiation of myoblasts into myotubes. The aims of this chapter are to investigate possible mechanisms of selection at these two time points.

It aims to show that a bottleneck is occurring at the first stage, mediated by activation of a sub-population of satellite cells and/or asymmetric division of activated cells.

In addition it is hypothesised that the switch from a glycolytic nature to OXPHOS and an increase in mitochondrial biogenesis is involved in the second selection point

If “gene shifting” is to occur, then muscle fibres with high levels of mutation will need to be able to regenerate in order for the mutation to be lost. This chapter also aims to present some preliminary work showing that respiratory deficient cells are able to regenerate.

6.3 Methods

6.3.1 MtDNA Copy Number

An mtDNA bottleneck involves amplification of DNA from a small population of genomes. In order to determine if that is the case here, a TaqMan® based qPCR method was used to establish the amount of mtDNA present in the satellite cells, proliferating myoblasts and differentiated myotubes of patients. The 18S/MTND1 qPCR compares the ratio of the mitochondrial *ND1* gene to a nuclear marker, *18S*. *ND1* is not in the major arc region and therefore tends to be preserved in patients with large scale single mtDNA deletions. All of the patients in this study harboured mtDNA deletions in their major arc (Table 3.3) and their ND1 region was consequently preserved. The nuclear gene, *18S*, is an rRNA gene present in multiple copies in the cell. This assay produces a number that is a ratio between nDNA and mtDNA.

Samples of the patient's CD56+ cells, myoblasts, myotubes, and muscle homogenate were analysed together on a single qPCR assay. A blood sample and a muscle homogenate sample were analysed as controls. The blood sample would always be expected to have a copy number value less than 1, while the muscle sample should always be above 10. Samples were analysed in triplicate and the mtDNA copy number calculated using the equation $r=2^{-\Delta Ct}$ (He et al., 2002), with ΔCT being the difference in Ct values generated by the ND1 and the 18S probes.

6.3.2 Creating a Bottleneck

To investigate if large shifts in heteroplasmy in muscle cells can result from amplification of a small population of mtDNA, it was decided to create a bottleneck by reducing the amount of mtDNA in myoblasts and allowing it to recover. If a corresponding shift in heteroplasmy occurred, it would suggest that amplification of a small number of genomes could account for the differences observed between patients with regards to their maintenance or loss of mtDNA deletions.

Myoblasts from patient 2's second biopsy (passage five) and from patient 3's third biopsy (passage four), along with control myoblasts, were seeded with SKCM containing pyruvate, uridine and glucose. After five days, half of the plates had their medium changed for that containing 50ng/ml ethidium bromide (EtBr). Due to fears that myoblasts grown with EtBr would not reattach to the flasks after harvesting, cells were seeded in six well plates (~8300 cells per well) and a different well harvested for each time point.

The cells were grown with or without EtBr for fifteen days, after which all cells were grown in medium without EtBr for a further thirty five days to allow the mtDNA copy number to recover. Cells were harvested at days 0, 6, 11, 15, 20, 25, 30, 37, 44, and 50 so that a sample of myoblasts grown both with and without EtBr was available for each time point. The cell harvesting days were chosen to enable many data points early on in the experiment close to the addition and removal of EtBr, as it was assumed most changes would occur around this switch.

Myoblasts had their mtDNA extracted using the Qiagen DNeasy Blood & Tissue Kit. All cells were then analysed for heteroplasmy levels using the MTND1/MTND4 qPCR and copy number using the MTND1/18S qPCR.

6.3.3 Single Cell QPCR

If there is a bottleneck occurring in the satellite cells it could be via a few mechanisms (Figure 6.1). The satellite cells may contain a mixture of WT and delete mtDNA or they could be homoplasmic, containing either WT or delete copies.

In order to determine which of these two situations is correct, single CD56+ cells from patient 6 were FACS sorted with one cell going into each well of a ninety six well plate. The plate was then sealed, centrifuged and stored at -20^{o}C until required.

Thirty two cells from this plate were lysed with single cell lysis reagents (see section 2.2.8.1.2) and then run on the MTND1/MTND4 qPCR in order to determine heteroplasmy levels. It was anticipated that many Ct values would be very high due to the lack of DNA present in the sample, so to ensure that results generated were attributable to mtDNA deletions, only samples that resulted in ND1 Ct values more than three cycles away from the ND1 no template controls were used.

6.3.4 Inducing Differentiation

While the stage of satellite cell activation served as one selection point, a second selection occurred as the myoblasts were proliferating and differentiating. In order to determine if it was during myoblast proliferation or differentiation that the mutation was lost, it was decided to differentiate an early passage of myoblasts into myotubes. Myoblasts at passage five from patient 2's first and second biopsy and patient 3's second and third biopsy were all differentiated into myotubes (as per section 2.2.7.4).

The myotubes were then harvested and run on the MTND1/MTND4 qPCR assay to determine heteroplasmy levels.

6.3.5 Muscle Staining

In order for "gene shifting" to occur muscle fibres containing mtDNA deletions will need to regenerate in order to incorporate developing myoblasts with little or no mtDNA mutation.

To determine the regeneration status of respiratory chain deficient muscle cells, two sets of serial biopsy sections from a female patient harbouring a large scale single mtDNA deletion were stained for complex I subunit 20kDa (complex 1:20), and NCam (see section 2.2.6). Complex I:20 staining was performed to provide an indication of the respiratory chain function, while NCam was used as a marker of muscle regeneration. Images were taken of the sections and fibre status was recorded on the basis of complex I:20 and NCam. The counts for the two sets of serial sections were combined.

6.4 Results

6.4.1 MtDNA Copy Number

In order for a bottleneck to occur there must be amplification of mtDNA from a small population of molecules. To determine if satellite cells have a small population of mtDNA, a qPCR copy number assay was performed.

The MTND1/18S qPCR was used to give a relative indicator of mtDNA copy number of CD56+ cells, myoblasts, and myotubes. Each patient's samples were all analysed on a single qPCR assay in triplicate. This was done to avoid any variation between runs.

In all patients the mtDNA copy number of CD56+ cells was below six. In the first set of myoblasts examined, the copy number had risen to above that of the satellite cells in six out of ten cell lines and by the second set of myoblasts it was eight out of ten. While there was some fluctuation, the general trend was for an increase in mtDNA copy number after satellite cell activation and during myoblast proliferation (Table 6.1). In order to determine how copy number changed with passage number a Pearson correlation analysis was performed on the results. The R values demonstrated the relationships were positive, so as the passage number increased so did the mtDNA copy number (Table 6.1). Two cell lines did not give a significant result, patient 4's second biopsy and patient 2's first biopsy.

Patient	Biopsy	CD56+	Myob P1	Myob P2	Myob P3	Myob P4	Myob P5	Myob P6	Myob P7	MyoT	SKM	Pearson Correlation	R Value
1	1st	2.1									8.3		
	2nd	0.5											
2	1st	2.5				2.1	2.7	3	4.5	3.3		(P=0.082)	0.755
	2nd	1.5			7.6	11.8	12.4	38.9		107	10.6	(P=0.011)	0.912
	3rd	5											
3	1st	2.1											
	2nd	1.8	3.3	5.3	6.6	5.5	9.7			5.5	7.1	(P=0.025)	0.818
	3rd	1.5	18.5	23.4	45.7	59.7	69.4	42.7		53.9		(P=0.013)	0.818
4	1st	2.3									2.6		
	2nd	5	2	2	2.8					5		(P=0.809)	0.151
5	1st	4.5	48.6	45.9	59.1	74.4	76.9	78	73.8	88.4		(P=0.001)	0.896
	2nd	3.8									92.8		
	3rd	4.2											
6	1st	2			8.9	7.1	26.3	17.0	20.5	55.4	12.5	(P=0.026)	0.812
7	1st	5.7			6.5	8.5	9.4	7.6	19.2	35.2	27.2	(P=0.001)	0.954
	2nd	5.9			3.7	6.4	8.3	8.2	12	27.9		(P=0.003)	0.925
8	2nd	5		3.1	4.2	9.2	13	16.8	17.3	23.9	115	(P=0.001)	0.940

Table 6.1 Copy number of cells. CD56+ cells, myoblasts, myotubes, and muscle homogenate samples were run on the MTND1/18S qPCR in order to determine relative copy number. All CD56+ cells had lower levels of mtDNA than the corresponding muscle homogenate sample, apart from patient 4. A gradual increase in mtDNA copy number was observed as the cells were proliferating and heading towards differentiation. The values given are relative to an nDNA marker, so increases in copy numbers also suggest mtDNA is being replicated faster than nDNA. Myob = myoblasts, MyoT = myotubes, SKM = skeletal muscle homogenate.

6.4.2 Creating a Bottleneck

To determine if an mtDNA bottleneck could account for the rapid change in heteroplasmy between satellite cells and myoblasts the effect was recreated. This was performed on myoblasts from patients 2 and 3, which maintained the mutation, and on a control cell line. Myoblasts were grown with and without EtBr for fifteen days before all cells had medium changed for that without EtBr and were grown for a further thirty five days.

The results from the control cell line showed that myoblasts with and without EtBr had a reduction in mtDNA copy number that never recovered (Figure 6.2E).

The myoblasts from patient 2 that contained EtBr had a reduction in copy number, but apart from a high copy number at day twenty, this never recovered. The heteroplasmy level remained between 70-80%, apart from day thirty seven which was 91% (Figure 6.2A). When myoblasts from this patient were grown without EtBr, again the heteroplasmy levels remained steady. While the copy number increased initially, this was followed by a dramatic reduction which never recovered (Figure 6.2B).

The cells from patient 3 that had EtBr added saw an initial reduction in copy number, followed by a slight recovery after the medium was changed to that without EtBr. There was an initial increase in heteroplasmy levels followed by a decrease to around 25% (Figure 6.2C). The cells from this patient that did not contain EtBr saw an initial increase in copy number, but this preceded a general trend downwards, apart from the cells harvested at day thirty seven. In line with this downward trend was a reduction in heteroplasmy level. It was not possible to harvest a set of cells from day fifty for this patient (Figure 6.2D).

When myoblasts were grown without EtBr there was an initial increase in copy number, but this was followed by a decrease around days fifteen to twenty. While the addition of EtBr did cause an initial reduction in mtDNA copies, there was never any recovery and thus no amplification of mtDNA from a small population of genomes. Cell counts were performed on the myoblasts, but were not deemed accurate. While cells were still visible in the wells, low numbers resulted in a value of zero using the haemocytometer. While these counts were disappointing, it suggests the myoblasts were dying, which could be the reason for the reduction in copy number. Therefore no conclusions may be drawn from this experiment about the hypothesised bottleneck mechanism.

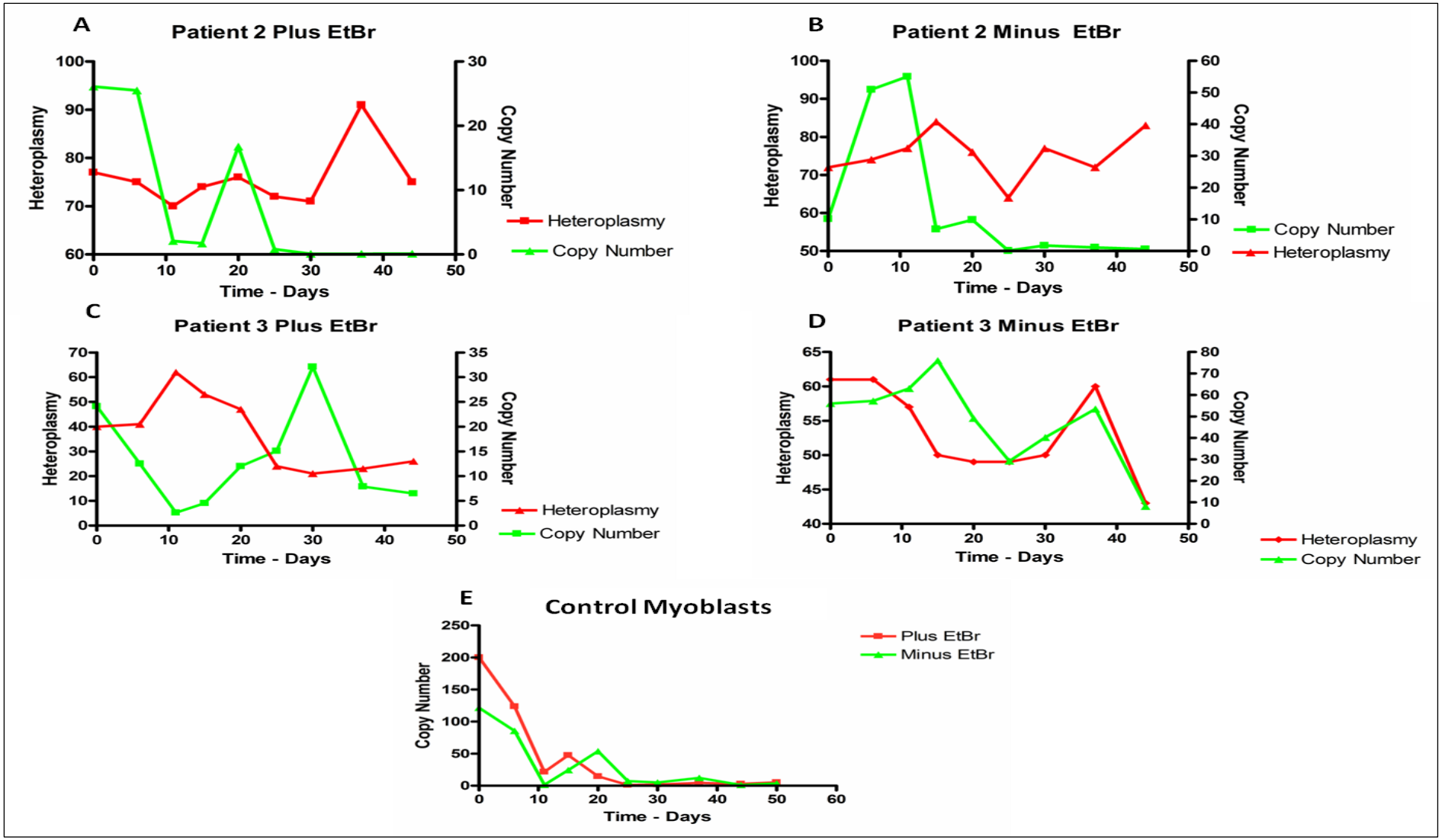


Figure 6.2 Effects of ethidium bromide on myoblasts. Myoblasts from patients 2 and 3 were grown with and without EtBr in order to induce an mtDNA bottleneck. **E**. The control cell line declined in copy number when grown both with and without EtBr. **B & D**. The two patient cells lines both saw an initial increase in copy number when grown without EtBr, but this reduced around day twenty. **A & C**. When grown with EtBr both patient cell lines had a decrease in mtDNA, that never recovered to levels observed before the addition of EtBr. While there were variations in heteroplasmy levels for patients, this did not appear to be related to mtDNA copy number.

6.4.3 Single Cell QPCR

Having hypothesised that the first selection point was attributable to a bottleneck, it was decided to perform single cell qPCR on some of the satellite cells in order to determine if they were heteroplasmic or homoplasmic on the basis of mtDNA deletions. Single CD56+ cells were FACS sorted into wells of a ninety six well plate, lysed and run on a MTND1/MTND4 qPCR to determine deletion levels.

Out of the thirty two cells that were originally lysed and run on this assay, ten resulted in ND1 Ct values that were far enough away from the no template controls to be analysed. Five of these samples were run a second time to confirm the findings.

The cells were heteroplasmic on the basis of mtDNA deletions. Two of the cells had quite high levels of deletions, two had levels around 50% and the rest had levels below the detection sensitivity of this assay, 30%. The level set for sensitivity of this assay (30%) is justified by the results of the repeats. While the cell with a high heteroplasmy level (88%) was consistent in the second run (90%), the cells that had levels below 30% were not consistent between the two runs (Table 6.2).

This patient has a muscle heteroplasmy level of 48% and a satellite cell heteroplasmy level of 44%. The heteroplasmy level for his homogenate sample of satellite cells has been obtained through a mixture of different heteroplasmy values for individual cells.

Position on Plate	E9	H9	B10	C10	G10	A12	B12	D11	G12	H12
1st Run	71%	55%	0%	0%	46%	1%	0%	88%	3%	9%
2nd Run						0%	0%	90%	24%	0%

Table 6.2 Single cell qPCR. Single CD56+ cells from patient 6 were FACS sorted into wells of a ninety six well plate. An MTND1/MTND4 qPCR assay was performed on the lysed cells. The cells were heteroplasmic, with variable levels of mtDNA deletions. Five of the samples were run a second time and confirmed the presence of heteroplasmy.

6.4.4 Inducing Differentiation

In order to determine if myoblast proliferation or the process of differentiation causes the mtDNA deletion to be lost, a number of the myoblast cell lines were differentiated at passage five. This was earlier than they had previously been differentiated. Their heteroplasmy levels were then determined using the MTND1/MTND4 qPCR.

After differentiation all the myotubes still harboured mtDNA deletions and when compared to myoblasts of the same passage there appeared to be no difference. Deletion levels in patient 2's first biopsy passage five myotubes appeared to be a little higher than myoblasts of similar passage number and were much higher than the myotubes differentiated at a later passage. His second biopsy passage five myotubes, were again a little higher than similar passage myoblasts, but generally were no different to cells around the same passage (Table 6.3).

Patient 3 had originally lost the mtDNA deletion after his myoblasts were differentiated into myotubes at passage six, however, that did not occur on this occasion. His passage five myotubes were of a similar level to passage five myoblasts (Table 6.3).

These data would suggest that differentiating myoblasts into myotubes did not induce the mutation to be lost. On the two out of three cases the mutations were lost, it occurred during the process of myoblast proliferation.

Patient	Biopsy	Myob P4	Myob P5	MyoT P5	Myob P6
2	1st	38%	32%	**43%**	35%
	2nd	79%	81%	**87%**	79%
3	3rd	75%	57%	**51%**	31%

Table 6.3 Heteroplasmy levels in myotubes. Differentiating myoblasts did not appear to change their heteroplasmy levels. Levels of mtDNA deletions were maintained as if the cells had carried on to their next passage. Myob = myoblasts, MyoT P5 = myotubes differentiated at passage 5.

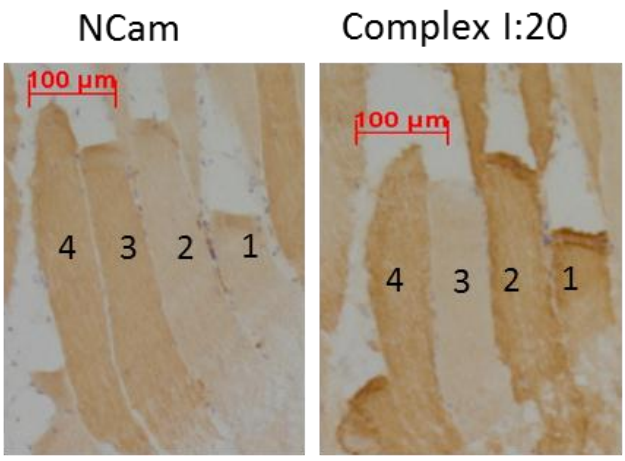
6.4.5 Muscle Staining

If “gene shifting” is to succeed, then muscle fibres with a respiratory chain deficiency will need to be capable of regeneration. Then through the incorporation of cells with lower or absent levels of mtDNA mutation, the phenotype of the fibre may be changed.

Complex I:20 reacts against the NDUFB8 subunit of complex I. If a cell is lacking the ND4 gene because it has been removed by a deletion in the major arc, the assembly pathway of complex I will be inhibited, resulting in a loss of NDUFB8 incorporation into the complex. NCam was used to determine if the fibres were regenerating. Through staining serial muscle sections with these antibodies it was possible to determine the NCam and complex I:20 status for muscle fibres visible on both sections (Figure 6.3A).

In total 206 muscle fibres were counted, of which the majority, 62%, were NCam negative. The complex I:20 staining showed that most of the fibres were complex I:20 positive, 61% (Figure 6.3B). In total there were 80 fibres that were complex I:20 negative. Out of these 80 complex I:20 negative fibres 58% were NCam positive, compared to 25% of complex I:20 positive fibres. This suggests that respiratory deficient muscle fibres were trying to regenerate.

A



B

	NCam Positive	NCam Negative	Totals
Complex 1:20 Positive	32	94	126
Complex 1:20 Negative	46	34	80
Totals	78	128	206

Figure 6.3 Muscle regeneration and respiratory chain function. A. Serial sections were stained for complex I:20 and NCam. Fibres were then identified on both sections and staining for both antibodies recorded. In the above image, fibres 1 and 2 both have a normal respiratory chain and are not regenerating. Fibre 3 however, is complex I:20 deficient and NCam positive suggesting it is trying to regenerate. While fibre 4 is also trying to regenerate, it is complex I:20 positive. **B**. In total 206 fibres were counted and recorded.

6.5 Discussion

The aims of this chapter were to identify selection points and mechanisms of selection acting on the cells responsible for muscle regeneration. It had been hypothesised that selection was acting after satellite cell activation via a bottleneck effect. Then as the myoblasts proliferated and headed for differentiation a second selection point occurred, possibly mediated by a switch in energy generation from mainly glycolytic to primarily oxidative. In addition this chapter sought to determine if respiratory deficient cells could regenerate.

6.5.1 Bottleneck Theory

It was hypothesised that one of the selection points was caused by a bottleneck taking place at the point of satellite cell activation. One of the possible mechanisms by which this could occur is through homoplasmic satellite cells being selectively activated.

While only a few single satellite cells resulted in Ct values that could be used; the qPCR studies revealed that satellite cells are heteroplasmic on the basis of mtDNA deletions. The levels of heteroplasmy were spread out (range of 0-88%), consisting of a couple of cells harbouring high levels of mtDNA deletions, two with intermediate levels and the rest contained low levels. When the homogenate sample of CD56+ cells from this patient was examined, it contained a heteroplasmy level of 44% (Table 4.5). This is a result of individual cells having different levels rather than all cells harbouring 44%. This suggests that the bottleneck is acting on satellite cells that are heteroplasmic for mtDNA deletions.

This experiment was performed after many of the patients had undergone all of their biopsies, so it has only been possible to perform it in one subject. It will be necessary to repeat this experiment in future patients to confirm the findings. In addition, to determine the exact levels of mtDNA deletions in the CD56+ cells with less than 30% heteroplasmy, it will be necessary to use the relevant mtDNA deletion specific SYBR Green assay.

The segregation of mutant mtDNA within the CD56+ cells is intriguing. The mutation is present in the satellite cells but not in all of them. This would be in agreement with the *status quo* in muscle fibres, where a mosaic pattern of COX staining is observed between muscle fibres, which corresponds to a variable mtDNA deletion level (He et al., 2002).

This raises the question of how the CD56+ cells have such a wide variety of heteroplasmy levels. It is possible, given the low mtDNA copy number they contain, that clonal expansion has occurred in some cells, but not in others. This selective clonal expansion could be dependent on which satellite cells have been activated in the past and therefore have undergone mtDNA replication.

Alternatively, the mtDNA deletions are present in all the satellite cells and after the first division there will be a random distribution of delete molecules between the two daughter cells. This could result in very different heteroplasmy levels in these two cells. One of the daughter cells will return to the satellite cell pool until further activation and division. Over time and multiple activations, the levels of mtDNA deletions in the stem cell will head towards one of two possible fates, homoplasmic WT or homoplasmic mutant. Given the low mtDNA copy number in satellite cells, this could occur very rapidly. Computer simulations have suggested this could happen in around 70 divisions in a stem cell with 250 copies of the mtDNA (Coller et al., 2002).

In order for a bottleneck to occur there needs to be amplification from a small population of mtDNA copies. To demonstrate that satellite cells contain relatively few copies of mtDNA when compared to their descendents, a qPCR assay was performed that compared the ratio of mtDNA with nDNA. These data demonstrate that there are very low levels of mtDNA relative to the amounts seen in myoblasts, in some cases in very early stage myoblasts.

As well as providing support for the bottleneck theory of selection, the copy number data also shows the large increase in mitochondrial biogenesis that occurs as the myoblasts proliferate and differentiate.

While the satellite cells and myoblasts come from the same biopsies, the myoblasts were cultured from different satellite cells in the biobank; this could account for the slight delay in myoblast mtDNA copy number exceeding that of the satellite cells.

One of the aims of this chapter was to demonstrate that a bottleneck could create large shifts in heteroplasmy in muscle cells. In order to create a bottleneck effect, the mtDNA content was reduced in myoblasts containing a single mtDNA deletion, through the use of EtBr for fifteen days.

While EtBr succeeded in reducing mtDNA copy number there was never any recovery in levels. In addition, after fifteen days, even the myoblasts that did not contain EtBr started to lose mtDNA. This was probably attributable to myoblasts dying. Similar depletion experiments using EtBr have shown that cell lines containing heteroplasmic mtDNA deletions are repopulated with delete mtDNA two to three times faster than that with WT mtDNA (Diaz et al., 2002). It would have been intriguing to examine such an effect in myoblasts.

However, myoblasts are difficult to culture after a number of passages and length of time. It may be that starting this experiment with cells at passages three and four was too late, especially considering that the whole experiment lasted fifty days. Performing this experiment at an earlier passage, reducing the EtBr concentration, or applying it for less time may help. In addition there are other methods of reducing mtDNA. While drugs such as chloramphenicol and doxycycline have been used to simulate an mtDNA depletion phenotype, they affect mtDNA translation and would not have reduced mtDNA copy number (Olgun and Akman, 2007). An actual reduction in mtDNA copy number can be obtained with nucleoside reverse transcriptase inhibitors (NRTIs), which inhibit the mitochondrial polymerase POLG (Koczor and Lewis, 2010). Treatment of myoblasts with one of these drugs may have resulted in a less harsh response.

While a bottleneck could explain the rapid shift in heteroplasmy observed in some of our patients at the stage of satellite cell activation, it does not result in complete separation of WT and delete mtDNA. Most of the patients who lost the mutation from their cells, lost it completely, for example patients 4 and 6. However, with patient 3's second biopsy and patient 5's first biopsy there was some evidence of a small amount of delete mtDNA persisting in the myoblasts. In addition, the myoblasts that maintained the mtDNA deletion also contained WT mtDNA; this suggests that the segregation is not absolute. This is probably because of small amounts of delete mtDNA present in mitochondria that are otherwise WT, or WT mtDNA in mitochondria which primarily consist of delete mtDNA.

However, taken together the above experiments suggest a bottleneck is responsible for the rapid loss of mtDNA deletions in some patients and its maintenance in others at the point of satellite cell activation. However, it does not distinguish between the two

remaining hypotheses for how it occurs (Figure 6.1A&B). It could be attributable to a physical reduction in mtDNA copy number caused by a cellular division followed by segregation of the genomes. Alternatively it could be caused by random activation of satellite cells that consist primarily of WT or delete mtDNA. A third possibility is that both mechanisms are occurring.

6.5.2 MtDNA Deletion Loss from Myoblasts

A second selection point was hypothesised to occur during myoblast differentiation to myotubes, coinciding with an increase in OXPHOS. In order to test this, cells from patients who maintained the mutation in their myoblasts were differentiated at passage five, this would hopefully push the cells into using OXPHOS for energy. If the switch from glycolysis to OXPHOS was causing selection against cells with impaired respiratory chain activity, then a parallel decrease in heteroplasmy levels as the cells were differentiated should have been observed.

The results show that the process of myoblast differentiation into myotubes did not cause the loss of mtDNA deletions from the cells. This would suggest some other mechanism is responsible for the reduction in heteroplasmy as the cells are proliferating.

Given that mtDNA deletions were lost in two out of three sets of myoblasts, it is possible that a more random mechanism is acting on the cells. As the myoblasts are dividing there could be asymmetric segregation of mtDNA on the basis of mtDNA deletions. Any myoblasts that inherit a large amount of mtDNA with a deletion may be unable to survive or proliferate as rapidly as myoblasts that have inherited very little or no delete mtDNA.

6.5.3 Muscle Regeneration

If gene shifting is to be successful then muscle fibres with a respiratory chain defect will need to be able to regenerate. In order to test their capability to do so, serial sections were stained for complex I:20 and NCam. NCam is expressed in regenerating myofibres and has previously been used in studies examining muscle regeneration (Walsh and Moore, 1985, Shoubridge et al., 1997).

The finding that 58% of complex I:20 negative cells were NCam positive, suggests these fibres were attempting to regenerate. Although it is not possible to determine how advanced this regeneration is, or if it will ultimately be successful. It was interesting to note that 25% of complex I:20 positive cells were also NCam positive. The source of this

biopsy was a female patient with a large scale single mtDNA deletion. While she is not involved in the current exercise trial taking place in Newcastle, she had been undertaking an exercise trial at a centre in Texas. It is possible that there was some degree of regeneration occurring in her muscle caused by this trial.

6.6 Conclusions and Future Work

Muscle regeneration in these patients potentially involves two selection points; the first at the time of satellite cell activation, and a second during myoblast proliferation (Figure 6.4).

While it is very difficult to determine exactly what happens at the point of satellite cell activation, the results of these experiments suggest that a bottleneck is occurring. Satellite cells are heteroplasmic for mtDNA deletions. The mutations are being dramatically lost or maintained through either, asymmetric division and a reduction in mtDNA copy number, or activation of satellite cells that consist primarily of WT or delete mtDNA.

If single fibre culture could be performed with samples from these patients it would be possible to examine copy number and heteroplasmy levels in cells as they were leaving the fibre. This would give a much better indication of what was going on at the point of satellite cell activation and possibly help determine if the bottleneck is occurring via selected satellite cell activation or asymmetric division. In addition, obtaining more single satellite cells from future patients and analysing them with the relevant SYBR Green qPCR would provide a much more accurate picture of what is occurring in these cells.

It would be interesting to attempt to examine absolute copy number in satellite cells. This may be difficult to perform on homogenate samples, as cells will have been lost during the process of centrifugation to remove supernatants, and also DNA extraction. However, if a small number of cells, for example less than fifty, were FACS sorted into a ninety six well plate, they could be lysed in the plate and would hopefully contain enough DNA to run on a qPCR assay. This could be performed using a probe for *MTND1* and a standard curve of mtDNA concentrations.

The second selection point did not coincide with differentiation from myoblasts to myotubes, but probably with myoblast proliferation. While it is possible that the cells with mtDNA deletions were dying, this did not happen in all cases, which suggests it may be a more stochastic mechanism. This could be the asymmetric localisation of predominantly WT and delete mitochondria, with myoblasts inheriting large amounts of delete mtDNA dying.

While both selection points seem somewhat random, out of the nine biopsies that myoblasts were available for and where the mutation levels could be mapped, eight resulted in myotubes with no mutation. This continues to suggest that gene shifting is theoretically possible; especially considering respiratory chain deficient cells are capable of regenerating.

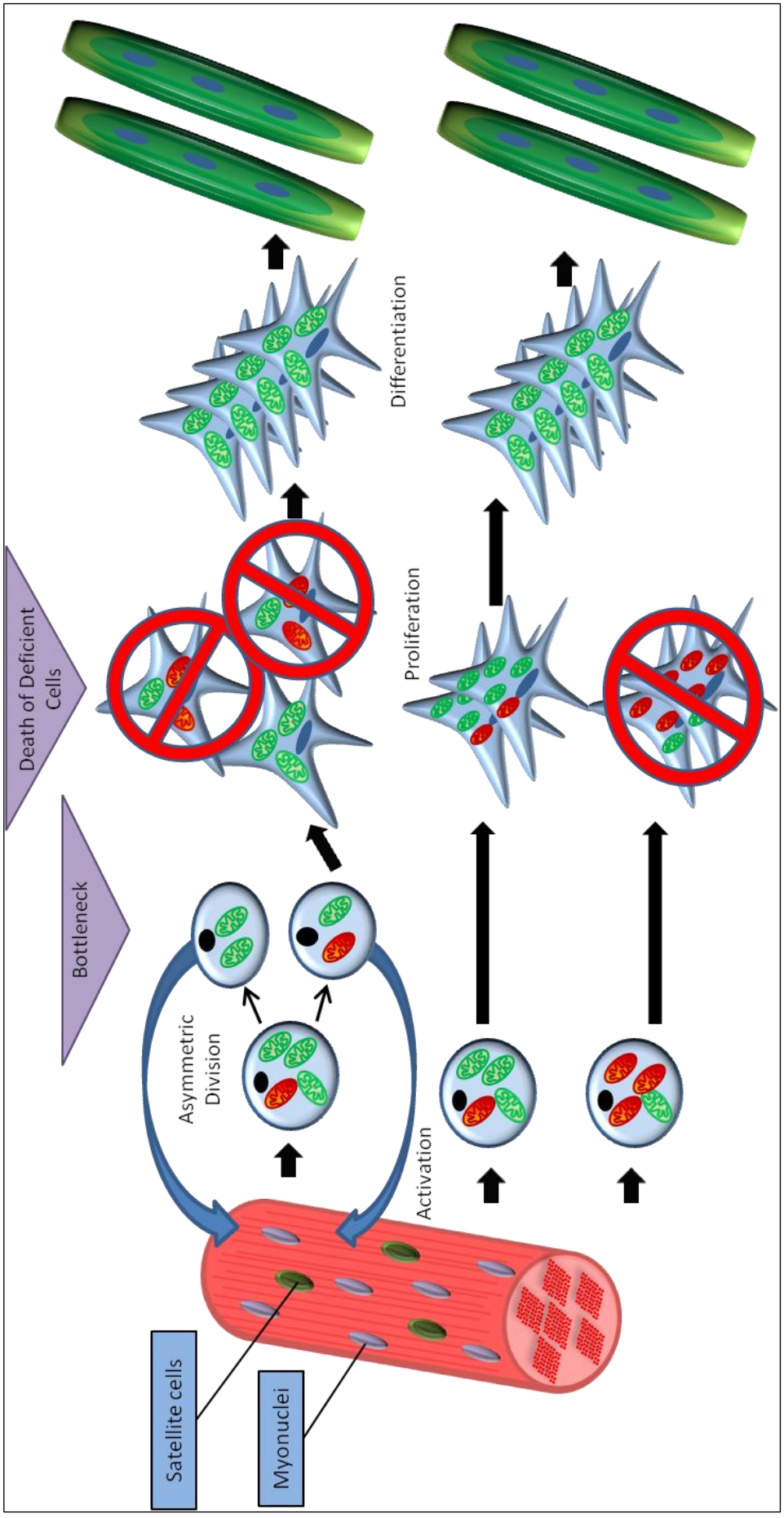


Figure 6.4 Selection points. It has been hypothesised in this chapter that selection acts at two points during the process of muscle regeneration. The first selection point is after satellite cell activation, and occurs through a bottleneck mechanism. This could occur via an asymmetric division with one daughter cell going back to repopulate the satellite cell pool and the other proceeding to form myoblasts, and/or through activation of satellite cells with either a high or low amount of mtDNA mutation. If the mutation is maintained then a second period of selection occurs when the cells divide and any resultant myoblasts with high levels of deletions die.

Chapter 7 Satellite Cell Numbers in Mitochondrial Myopathy Patients

7.1 Introduction

The previous chapters have concluded that the stimulation of muscle satellite cells and loss of mtDNA mutation as the resultant myoblasts head towards differentiation could benefit mitochondrial myopathy patients through the process of "gene shifting". To stimulate satellite cell activation, our lab is using resistance training to induce damage within the muscle. However, for this approach to be successful satellite cells must be present in sufficient quantities to repeatedly activate and repair muscle.

Mutations of mtDNA have often been described as causing an ageing phenotype (Trifunovic et al., 2004). This coupled with the myopathic nature of the conditions the patients in the current study have, give cause for concern regarding the ability of their muscles to regenerate. As well as any muscle wasting caused by their condition, there is the added issue of inactivity in these patients. This will lead to a further decline in the health of their muscles. If these patients are performing resistance training in order to "gene shift" it will be necessary to ensure they have adequate numbers of satellite cells to repair their muscles, and do not experience an exhaustion of the stem cell pool.

7.2 Aims

The aims of this chapter are to examine satellite cell numbers in patients with mitochondrial myopathies caused by sporadically occurring single mtDNA deletions. This is to ensure that efforts to stimulate satellite cells will not be detrimental to patients in terms of stem cell numbers.

To investigate this, satellite cell numbers will be determined in mitochondrial myopathy patients harbouring sporadically occurring single mtDNA deletions under three conditions. The first will be examining satellite cell numbers in patients compared to control samples to determine if the disease itself causes any significant increase or decrease in numbers. Secondly it will investigate how satellite cell numbers change in a patient over eleven years, in order to ascertain if there is likely to be any detrimental increase or decrease as patients' age. Finally, it will examine the effects of six months of resistance training in patients that has been designed to stimulate satellite cells and encourage their incorporation into muscle.

7.2.1 Satellite Cell Numbers in Mitochondrial Myopathy Patients Compared to Control Subjects

7.2.1.1 Satellite Cells in Myopathies

Patients with muscle degenerative conditions will naturally undergo repeated bouts of degeneration and regeneration, with each cycle requiring satellite cells in order to repair the muscle. There is some concern as to if this damages or exhausts the satellite cells.

An early study using electron microscopy (EM) examined satellite cell numbers in patients suffering from a range of neuromuscular disorders. It was found that they contained higher percentages of satellite cells per nuclei (satellite cell frequency) when compared to control subjects. Patients with Duchenne's Muscular Dystrophy (DMD) had a satellite cell frequency of 28.10 ±7.3%, polymyositis (PM) patients had 14.88 ±6.31%, and myotonic dystrophy (MD) patients had 11.6±4.4%. The control subjects had a much lower frequency of 4.32±1.29%. While this data suggests that patients with a neuromuscular disorder have a higher satellite cell frequency, there were marked differences in the age of patients, which was a reflection of typical age of onset. The mean age of the control subjects was thirty years. The DMD patients were around seven years, the PM patients fifty two years, and the MD patients were an average of thirty six years (Ishimoto et al., 1983).

The later age of onset of PM may mean that despite the increase in satellite cell numbers their stem cell pool never becomes exhausted, however this may not be the case in DMD patients. Their greatly increased satellite cell numbers at such a young age may result in an exhausted stem cell pool later on. This could be a problem with the increasing survival age of DMD patients, in no small part helped by the use of non-invasive ventilation (Simonds, 2006). This increased life span results in their muscles undergoing many more rounds of degeneration and regeneration.

The possibility that patients with dystrophic muscle may reduce the regenerative ability of, or exhaust their satellite cells has been partly suggested because of findings in the mdx mouse, an animal model of DMD. These mice have been shown to lack a population of highly proliferative radiation resistant cells, when injected with the strong myotoxin notexin (Heslop et al., 2000). This has been attributed to the

exhaustion of this particular cell lineage through repeated bouts of degeneration and regeneration (Luz et al., 2002).

The repeated bouts of regeneration may not only exhaust the satellite cell pool. Muscle biopsies from dystrophic muscle, caused by DMD and Limb Girdle Muscular Dystrophy 2C (LGMD 2C), have shown significant reductions in telomere length when compared to controls. This signals an increased senescence of the satellite cells (Decary et al., 2000). The shortness of telomeres indicates that the satellite cell have undergone repeated divisions in order to repair the muscle. The participation of satellite cells in this regeneration has been shown to reduce their proliferation potential (Schultz and Jaryszak, 1985).

While the cycles of degeneration and regeneration may not be as numerous in patients with mitochondrial disease compared to those with DMD, it is still necessary to examine the situation in mitochondrial patients, especially if they will be partaking in activities to cause muscle damage.

7.2.1.2 Methods

In order to compare the satellite cell frequencies of patients and controls, Pax 7 staining was performed (see section 2.2.6.1). Muscle biopsies from the *vastus lateralis* of ten patients with a mitochondrial myopathy caused by a sporadically occurring single mtDNA deletion were cut at 12μm by Dr. J. Murphy. These patients included the eight described in chapter 3, from whom sections were obtained from their baseline biopsy. This single mtDNA deletion group consisted of ten subjects, six male and four female, with an average age of forty two. Except for patients 8 and 9, two muscle sections were cut and stained for Pax7. Patients 8 and 9 only had small biopsy samples and tissue was required for other experiments, as a result counts were performed on one muscle section.

Control muscle samples were received from the Freeman Hospital, Newcastle, from patients undergoing operations to repair their anterior cruciate ligament (ACL). When patients undergo this operation part of their hamstring is removed to repair the ligament. When the operation is performed, part of the muscle attached to the ligament is removed along with it, and usually discarded. This is the muscle sample that was received to be used as control tissue.

Eleven muscle samples were obtained from these control subjects, eight male and three female with an average age of thirty six. They were cut at 12μm, and stained for Pax7. Counts were only performed on one muscle section from each subject. Given the large size of the muscle sections and therefore the large number of fibres counted this was deemed sufficient to give an accurate impression of the muscle biopsy.

Counts of muscle fibres, satellite cells, and myonuclei were performed on an Olympus BX51, using Stereo Investigator software (MBF Biosciences, USA). To perform the counts, an outline was drawn around the section at a low magnification, ensuring the marked area encompassed a minimum of one hundred fibres. Occasionally staining resulted in a darker band around the outside of the muscle section, so to prevent any ambiguity regarding counting, the boundary was drawn a couple of fibres into the section.

The magnification was then increased, and the computer software scanned the section within the outlined image. On each new image, counts of fibres, myonuclei and satellite cells that lay within the boundary of the area were performed (Figure 7.1). Any fibres that lay across the line were only counted if both the left hand and bottom edge was within the boundary. The numbers of fibres, myonuclei, and satellite cells were recorded every fifty fibres.

The numbers of satellite cells were assessed by determining the satellite cell frequency in each biopsy. This was performed using the equation:

$$Satellite\ cell\ frequency = \frac{Satellite\ cell\ number}{(satellite\ cells + myonuclei)} \text{ X } 100$$

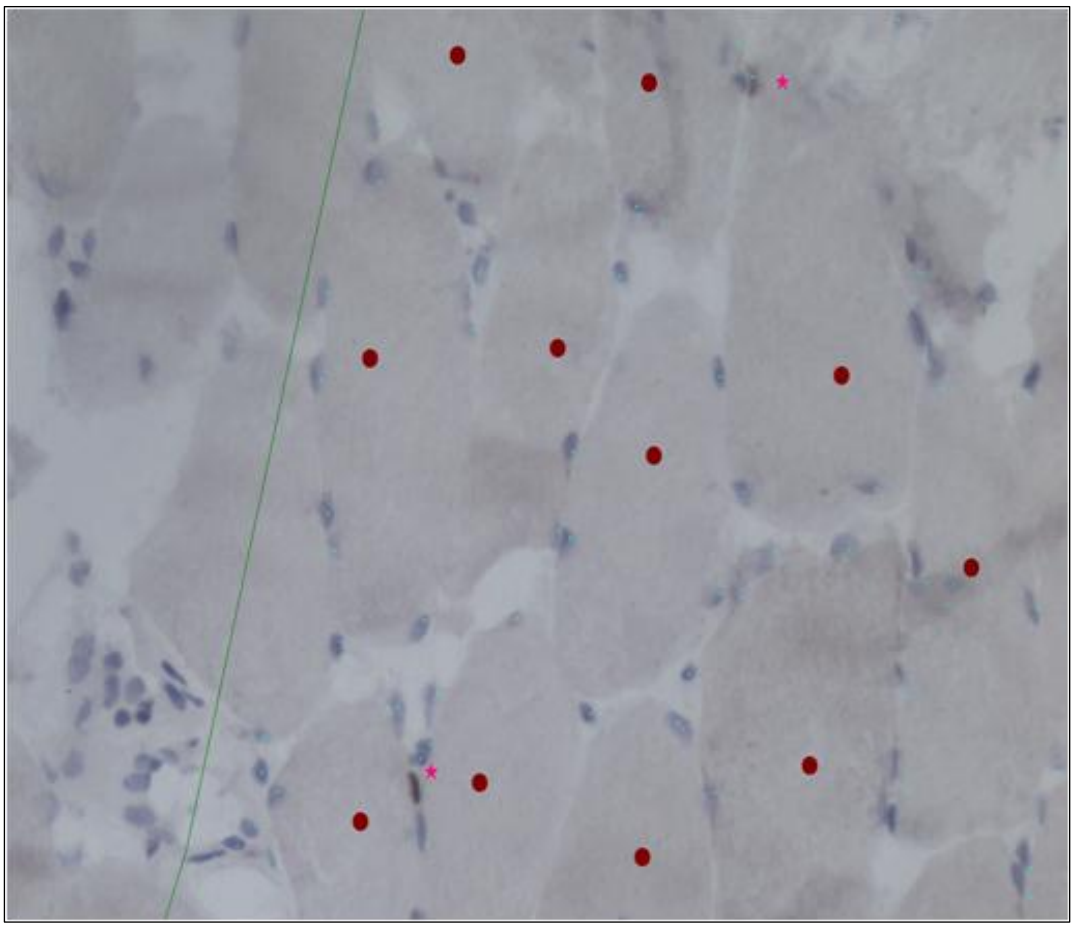


Figure 7.1 Counting cells on Stereo Investigator. On each muscle section a line was drawn around the outside of the muscle section, seen here in green, with fibres to the right of this line being included in the count, and those to the left of the line not being counted. Satellite cells were stained for Pax7 and visualised with DAB. They are therefore visible as brown nuclei. In each area the fibres (red dots), myonuclei (blue dots), and satellite cells (pink stars) were counted.

7.2.1.3 Results

Counts were performed on ten patients (six males: mean age 42.5±9.9, and four females: mean age 42.5±12). There were considerable differences between patients in terms of how many fibres (148 to 1380), myonuclei (3113 to 9694), and satellite cells (51 to 214) that were counted (Table 7.1). This was attributable to the differing sizes of the biopsies.

Control muscle sections were available from the hamstring group of muscles from eleven subjects. While this sample is from a different muscle, its common embryological origin and similar fibre type ratio to the *vastus lateralis,* which was biopsied in the patients, suggested it would provide an appropriate comparison (Garrett et al., 1984). The control muscle samples consisted of eight males (mean age 38 ±10 years) and three females (mean age 32 ±10 years). As previously, there were marked differences between the numbers of fibres (220 to 1792), myonuclei (1348-12030), and satellite cells (57 to 267) counted, which again was attributable to variation in section sizes (Table 7.2). Interestingly this group demonstrated much more variation in numbers of fibres, myonuclei, and satellite cells, as evidenced by their standard deviations. Satellite cell frequencies were determined for both groups using the equation stated previously.

The average satellite cell frequency for the patients was 2.37±0.725, while for the controls it was 2.39± 1.03. As can be seen from the graph and the standard deviations, there was slightly more variation in the satellite cell frequencies of the control subjects (Figure 7.2). An Anderson Darling test showed satellite cell frequencies were not significantly different from a normal distribution. A two sample t-test showed there was no significant difference between the satellite cell frequencies of patients when compared to controls (P=0.958).

Patients	Sex	Age at Biopsy	Fibres	Myonuclei	Satellite Cells	Satellite Cell Frequency
1	F	58	209	2170.5	51.5	2.31
2	M	46	1050.5	4724.5	125	2.57
3	M	39	1038.5	6753.5	169.5	2.44
4	M	59	478	3694.5	72	1.98
5	M	45	651.5	3113	71.5	2.31
6	M	35	1126	4612.5	96.5	2.04
7	M	31	807	3339	58	1.71
8	F	34	235	3373	64	1.86
9	F	32	1380	9694	214	2.15
10	F	46	148	3748	168	4.29
Averages		**42.5**	**712.35**	**4522.25**	**109**	**2.373**
Standard Deviation		**10.1**	**435.23**	**2193.34**	**57**	**0.724**

Table 7.1 Satellite cell numbers in patients. Ten mitochondrial myopathy patients had counts of muscle fibres, myonuclei, and satellite cells performed to determine satellite cell frequency.

Controls	Sex	Age at Biopsy	Fibres	Myonuclei	Satellite Cells	Satellite Cell Frequency
1	M	21	418	3472	109	3.04
2	F	23	1352	10980	266	2.36
3	M	27	550	4451	144	3.13
4	F	31	1060	8376	83	0.98
5	M	35	388	3615	146	3.88
6	M	36	1792	11026	151	1.35
7	M	40	220	1348	57	4.05
8	F	42	1026	12030	267	2.17
9	M	43	431	3940	89	2.20
10	M	47	622	7069	88	1.22
11	M	52	1111	9708	189	1.90
Averages		**36.09**	**815.45**	**6910.45**	**144.45**	**2.39**
Standard Deviation		**9.89**	**488.25**	**3717.58**	**71.24**	**1.03**

Table 7.2. **Satellite cell numbers in control subjects**. Eleven control subjects had counts of muscle fibres, myonuclei, and satellite cells performed. The control subjects appeared to have a wider distribution of fibres, myonuclei, and satellite cells.

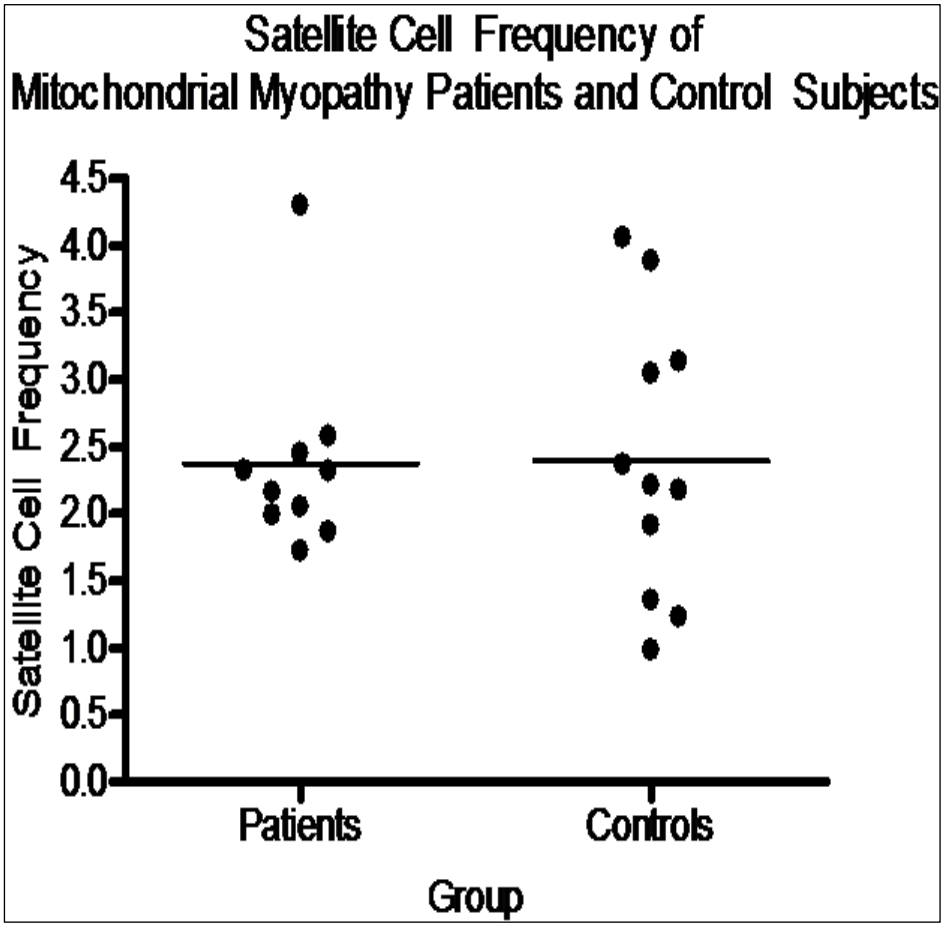


Figure 7.2 Satellite cell frequencies in mitochondrial myopathy patients and control subjects. Satellite cell frequencies were determined for ten patients with a mitochondrial myopathy, and eleven subjects who had undergone an operation to repair their anterior cruciate ligament. The control subjects appeared to have a wider spread of data. The line indicates the average satellite cell frequency for each group.

7.2.2 *Effects of Ageing on Satellite Cell Numbers in Mitochondrial Myopathy Patients*

7.2.2.1 *Satellite Cells in Ageing*

As muscle ages its ability to regenerate diminishes. When this results in a large amount of muscle loss it is termed sarcopenia. Sarcopenia contributes to falls and general weakness, which in turn leads to a greater morbidity in ageing people. While the exact cause is still being debated, many things are thought to contribute to this loss of regeneration potential in aged muscle. Possible influences include malnutrition, injury, hormonal changes, denervation, lack of exercise and general disuse. These factors cause decreased protein synthesis, the accumulation of deleterious effects of reactive oxygen species, altered enzyme activities and impaired glucose metabolism (Carmerli et al., 2002, Fulle et al., 2004).

In addition, a role for mitochondria in sarcopenia has been suggested. Aged muscle fibres from rats were found to contain high levels of mtDNA deletions. When levels of mutation exceeded 90% the fibres displayed impaired COX activity and were prone to splitting (Herbst et al., 2007), a phenomenon often observed in neuromuscular disorders (Swash and Schwartz, 1977). While mtDNA mutations have been found in ageing human tissue, they are at a very low level, less than 1%. However, this heteroplasmy level has been generated through many fibres with no mutation and some containing segments of very high levels. These segments of high mutation load may then be prone to splitting, resulting in muscle loss (Khrapko and Vijg, 2009, Bua et al., 2006).

Satellite cells are also thought to contribute to sarcopenia, although their exact role is still under discussion. A satellite cell's decreased potential to regenerate could be attributable to a systemic factor, problems relating to the muscle itself, changes in the stem cell niche, or something intrinsic to the satellite cell.

The importance of systemic factors in satellite cell mediated muscle regeneration was nicely highlighted by experiments creating parabiotic pairings between old and young mice (Conboy et al., 2005). Exposure of old mice to a young circulatory system restored the proliferation and regenerative capacity of "old" satellite cells. Partly mediated by a restoration of Notch signalling, that had previously been shown to be

impaired in aged muscle (Conboy et al., 2003). Notch is an important regulator of the pathway that leads from activated satellite cells to differentiated myotubes.

Extrinsic factors caused by the stem cell niche could also play a role. As muscle ages it suffers from a reduced blood flow and a consequent decline in factors needed for satellite cell activation. The basal laminar also thickens with age, again making it harder for activation and for other factors to diffuse through to the satellite cells (Brack and Rando, 2007).

Factors intrinsic to the stem cell will also contribute to reduced muscle regeneration. Aging in haemopoetic stem cells has been linked to genetic damage, but whether this holds true for satellite cells that have a lower cellular turnover is somewhat unknown (Brunet and Rando, 2007).

One study found that both intrinsic and extrinsic factors contributed to the decline in satellite cell potential. Experiments isolating aged myofibres and culturing the satellite cell progeny demonstrated that while there was a reduction in myogenic cells, a minority were capable of proliferating, differentiating, and self-renewing. When these aged myofibres were engrafted into young mice, this preserved minority of satellite cells were able to regenerate muscle just as efficiently as “young” satellite cells (Collins et al., 2007).

Other studies examining the contribution of satellite cells to the aging phenotype of muscle, have examined the numbers of these cells available for regeneration as people age. Evidence for a depletion of satellite cells in ageing came from early studies marking a decline in satellite cell frequency in mice from 4.6% to 2.4% as they aged over a period of twenty two months, starting at month eight (Snow, 1977). More recently it was noted that satellite cell numbers as determined by Pax7 staining, in *extensor digitorum longus* (EDL) and *soleus* muscle fibres, significantly decreased towards the end of the life span in mice. It was interesting to note that the decrease in the EDL muscle occurred much quicker than in the *soleus* muscle (Shefer et al., 2006). In rodents the EDL is primarily composed of type II fibres, while the *soleus* is primarily composed of type I fibres (Soukup et al., 2002, Barclay and Weber, 2004), suggesting a susceptibility of type II fibres to satellite cell exhaustion.

However, in humans the debate is somewhat less clear. Roth *et al.* (2000) examined men and women classed as young (20-30 years) and old (65-75 years) and found no difference between any of the groups. Biopsies from the *vastus lateralis* were collected and analysed using EM. Satellite cell frequency as a percentage of total myonuclei was determined. While the older groups had more central nuclei than the younger groups, suggesting more regeneration was occurring in their muscles, it was concluded that there was no reduction in the stem cell pool as people age (Roth et al., 2000).

In contrast to this, Verdijk *et al.* (2007), observed a reduction in satellite cell numbers in old (average 76 years) verses young (average 20 years) males, in the *vastus lateralis*. This reduction was only observed in type II fibres and not in type I fibres and coincided with the atrophy patterns observed in the older subjects, with specific degeneration of the type II fibres (Verdijk et al., 2007). This supported findings of a specific fibre type related atrophy in aging mammals (Shefer et al., 2006).

Other studies have found no age associated differences in the *vastus lateralis* (Dreyer et al., 2006). But declines noted with age when the *tibialis anterior* (Kadi et al., 2004) and the *biceps brachii* and *masseter* (Renault et al., 2002) muscles were studied. Findings that older people did not have significantly shorter telomeres in their satellite cells suggests that any exhaustion of the satellite cell pool may not be attributable to excessive turnover of cells (Renault et al., 2002).

While the previous section suggests that an exhaustion of the satellite cell pool may not initially be an issue in these patients, the combined effects of multiple cycles of regeneration over time, in addition to any changes that occur due to aging, may create a deficiency. This situation may be compounded by the fact mitochondrial disease has often been compared to an aging phenotype. Although it would appear that the reduced ability of satellite cells to repair muscle as people age is multi-dimensional, the opportunity to examine one of these potential factors, satellite cell numbers, must be taken.

7.2.2.2 Methods

In order to determine how the numbers of satellite cells change as a patient ages, a series of biopsies from a patient with a sporadically occurring large scale single mtDNA deletion were examined. This patient had previously been shown to contain COX negative fibres in her muscle. Patient 9 (Table 7.1) has undergone ten muscle biopsies over a period of eleven years (130 months), with her first biopsy being performed at the age of thirty two.

Muscle sections were cut at 12μm, stained for Pax7, and counted as previously described (section 7.2.1.2). Due to the small amount of tissue available from some of the biopsies, it was only possible to obtain two sections from seven out of the ten biopsies performed. The remaining three biopsies only had one section counted. Where two sections were counted the results were averaged.

7.2.2.3 Results

A total of ten biopsies from the *vastus lateralis* of a single patient over eleven years were analysed to determine satellite cell frequencies. The sizes of the biopsies varied, as evidenced by the wide ranging numbers of muscle fibres (332 to 1737), myonuclei (3067 to 9736), and satellite cells (80 to 240). The average number of muscle fibres counted was 886±536; myonuclei 7448±2808, and satellite cells were 190±60. Over the ten biopsies average satellite cell frequency was 2.53±0.45. There appeared to be a slight increase over time (Figure 7.3), which was shown to be significant using a Pearson's Correlation ($P<0.05$).The Pearson's number was 0.644, demonstrating the correlation was positive. In this patient, satellite cell frequency increased with age.

When the satellite cell frequencies for mitochondrial myopathy patients from the previous experiment (section 7.2.1.3) were plotted against age at biopsy, a regression line showed a slight increase had occurred (Figure 7.4A). However, Pearson's Correlation showed there was no relationship between age at biopsy and satellite cell number ($P=0.491$), Pearson's value 0.247. When the same analysis was performed on the control subjects a regression line showed a gradual decrease over time (Figure 7.4B), but again a Pearson's Correlation showed this was not significant. ($P=0.432$), Pearson's number -0.264.

This suggests while an individual patient undergoes some changes in satellite cell numbers, the differences observed between patients are greater. Alternatively, this pattern may be unique to this particular patient. The reason this patient has undergone numerous biopsies over this period of time is due to her participation in a number of different exercise trials. This may have resulted in a gradual accumulation of satellite cells. Importantly however, this patient has not undergone an exhaustion of her satellite cell pool with age.

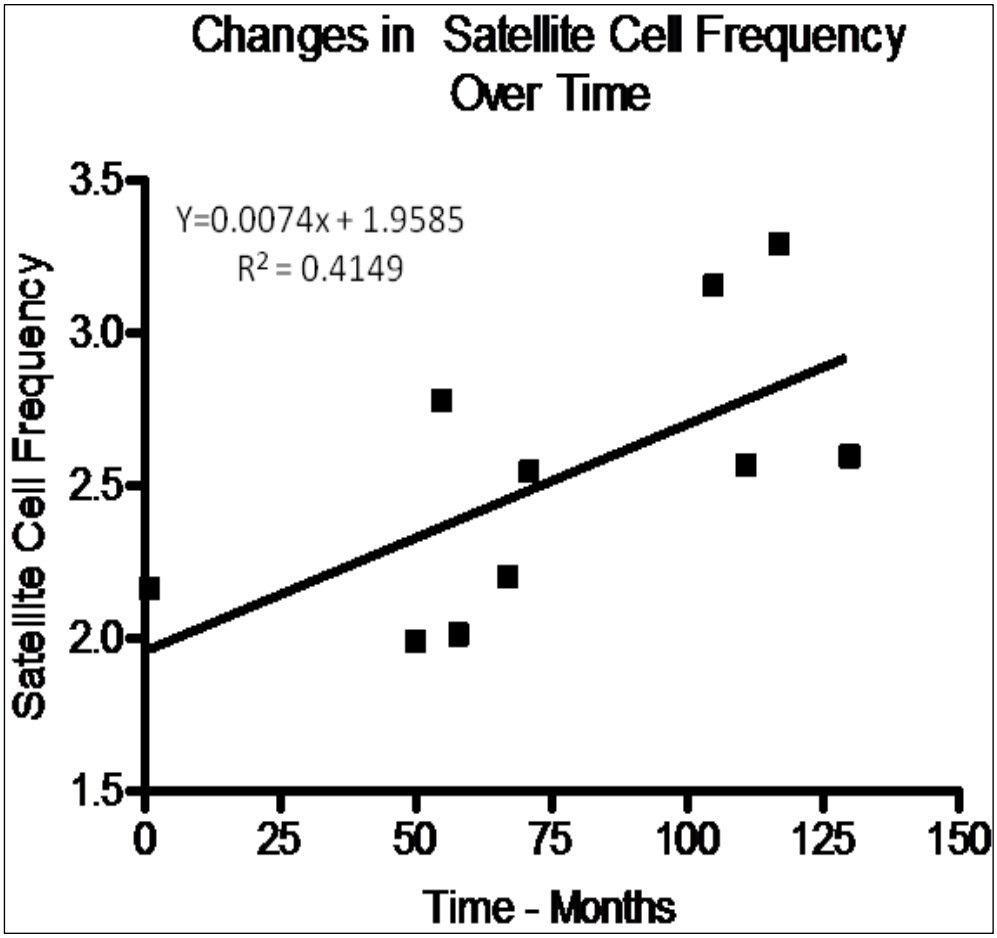


Figure 7.3 Changes in satellite cell frequency over time. A single patient with a mitochondrial myopathy caused by a sporadically occurring single mtDNA deletion underwent ten biopsies over a period of eleven years. There was a gradual increase in her satellite cell frequency over time, although the R^2 value of the line of best fit suggests wide variation in this relationship. This may be attributable to various exercise interventions.

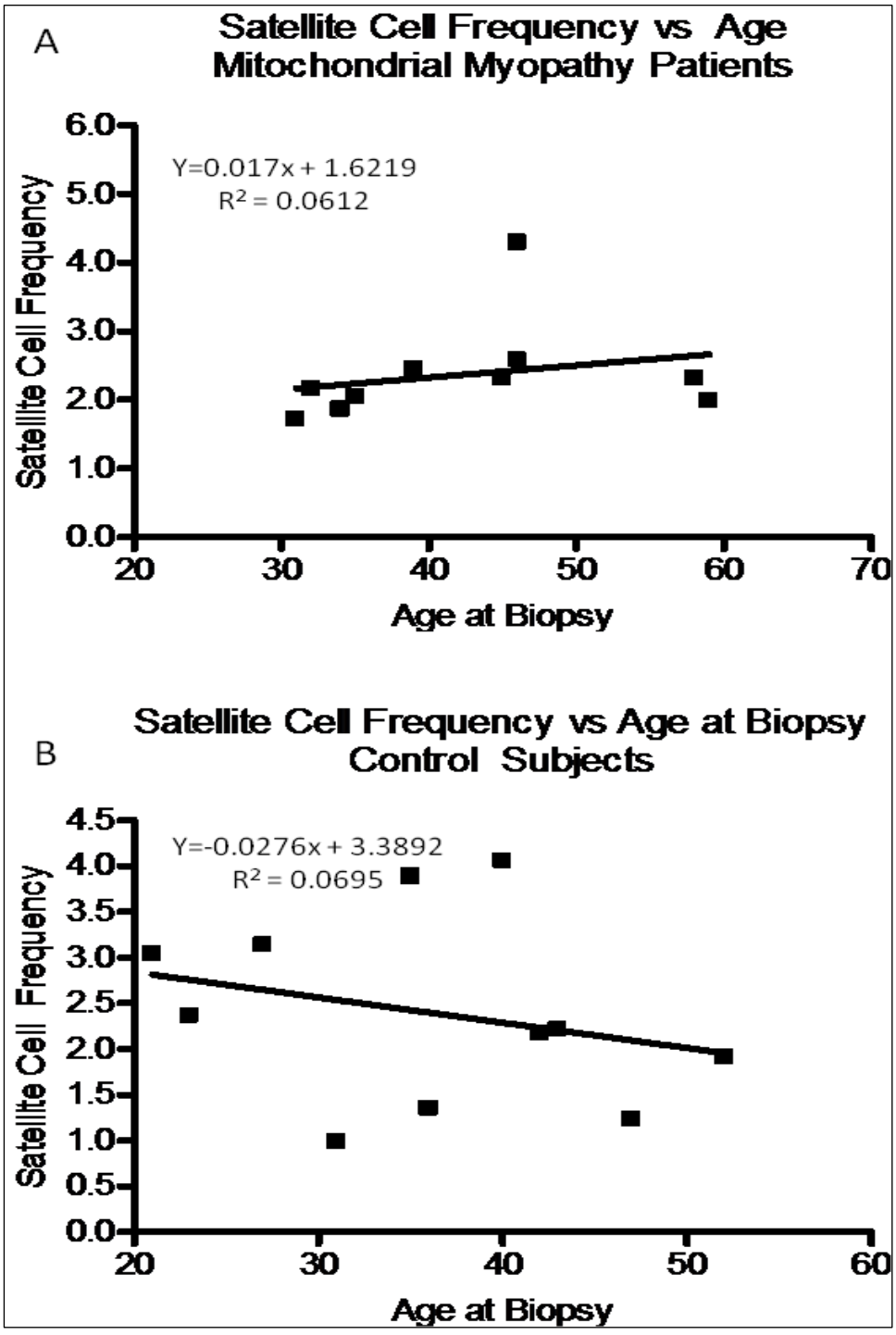


Figure 7.4 Satellite cell frequency with age. A. The satellite cell frequency was plotted against age at biopsy of mitochondrial myopathy patients. A slight increase occurred with age, but this was not significant. **B**. When the satellite cell frequency was plotted against age for the control subjects a decrease with age was observed, again this was not significant.

7.2.3 Satellite Cells in Response to Resistance Training

Eight of the patients being examined for satellite cell numbers are undergoing an exercise trial at Newcastle University (see section 3.3.1). One of the aims of this trial is to attempt "gene shifting" in patients by inducing muscle damage and thus satellite cell activation and eventual incorporation.

While the trial is still ongoing, biopsies are available both before and after the resistance arm of the exercise program for these eight patients. This presented the opportunity of examining the effects of six months of high intensity resistance training on the satellite cell numbers in patients with mitochondrial myopathies.

7.2.3.1 Effects of Resistance Training on Muscle

Many studies have examined the effects of resistance training on muscle. The wide variety of exercise programs undertaken, the muscles examined, and the methods of data collection, makes direct comparisons and obtaining an overall consensus somewhat difficult. An added problem when examining the long term effects of resistance training in healthy people has been that many of the subjects are long term weight lifters, and have often used steroids. In addition it is important to note that increases in strength are only observed when measured using the same type of contraction that the exercise training involved (Higbie et al., 1996). In general, studies examining resistance training have consisted of a five to sixteen week program, two to four sessions a week, with each session exercising a range of muscles with three sets of between eight to fifteen repetitions.

Resistance training is widely known to increase muscle strength and the mechanism of the adaptation has been the focus of many studies. It is thought that some of the increases in strength are attributable to neural adaptations, including increased motor unit recruitment, increased motor unit firing rates, increased activation of synergistic muscles and decreased activation of antagonistic muscles (Higbie et al., 1996, Hakkinen et al., 2003, Duchateau et al., 2006). In addition architectural changes are thought to be responsible for some of this improvement, although there have been conflicting reports regarding this (Blazevich et al., 2007, Aagaard et al., 2001).

Resistance training affects males and females differently, with men tending to gain more muscle size and women gaining more in strength. Initially there were suggestions that both hyperplaesia and hypertrophy could account for the increases in muscle strength and size in response to exercise (Grounds, 1998). It is now thought however, that hyperplaesia only occurs in response to exercise in certain animals, and previous reports in humans were probably attributable to muscle fibre splitting (Toigo and Boutellier, 2006).

Fibre hypertrophy in muscle has been reported in both athletes who perform resistance exercise as part of their training (Tesch et al., 1984, Kadi et al., 1999), and in none athletes undergoing a program of strength training (McCall et al., 1996, Higbie et al., 1996, Aagaard et al., 2001). This increase is particularly strong in the type II fibres, demonstrating their greater adaptive response to exercise (Tesch et al., 1984, McCall et al., 1996, Kadi et al., 1999, Aagaard et al., 2001).

Concurrent with this increase in fibre hypertrophy, an increase in capillarisation also occurs. Although this effect is not visible when determined per fibre, only when determined by area, and has thus occurred relative to hypertrophy (Tesch et al., 1984, McCall et al., 1996).

The increase in fibre hypertrophy is mediated by increases in gene transcription and thus protein synthesis. These have been shown to occur after only a single bout of exercise (Bickel et al., 2005). The early stages of muscle hypertrophy are mediated by an increase in the myonuclei domain of a muscle nucleus. However, there are limits to the area for which a single myonuclei can initiate protein synthesis. Further increases in hypertrophy require the activation and incorporation of satellite cells (Petrella et al., 2008).

7.2.3.2 Effects of Resistance Training on Satellite Cell Numbers

Mechanical stretch is thought to activate satellite cells through the release of hepatocyte growth factor (HGF) from the extracellular matrix in a nitric oxide dependent manner. The HGF binds to the c-met receptor on the satellite cells and initiates a cascade reaction that results in DNA synthesis and eventual satellite cell activation (Toigo and Boutellier, 2006, Tatsumi et al., 1998, Anderson et al., 2002, Tatsumi et al., 2006).

Increases in satellite cell numbers have been reported in weight lifters (Kadi et al., 1999), and in subjects given resistance training programs (Petrella et al., 2006, Roth et al., 2001). This increase in satellite cell number has even been reported to occur after a single bout of exercise (Crameri et al., 2004). The effects of age and sex on the size of the satellite cell response to exercise are still being debated (Petrella et al., 2006, Roth et al., 2001). One report suggested the size of response was dependent on initial starting numbers of satellite cells (Petrella et al., 2008).

In 2000 Kadi and colleagues demonstrated an increase in strength, fibre size, satellite cell numbers and myonuclei per fibre in females undergoing ten weeks of resistance training. This study demonstrated nicely the “myonuclei domain” hypothesis, where satellite cell activation and eventual incorporation leads to an increase in the numbers of myonuclei in muscle fibres and allows continued hypertrophy of the fibre (Kadi and Thornell, 2000).

In a study examining the effects of twelve weeks of resistance training in patients with a mitochondrial myopathy caused by sporadically occurring single mtDNA deletions, Murphy *et al.* (2008) also reported increases in strength and satellite cell frequencies. Although it was not reported if there was an eventual increase in overall myonuclei content of the fibre and there were no significant changes in fibre area, suggesting that hypertrophy may not have occurred (Murphy et al., 2008).

This current study has examined the effects of six months of resistance training in a similar group to Murphy *et al*. It is hoped that the extended duration of the training program will initiate a stronger response.

7.2.3.3 Methods

To determine the effects of six months resistance exercise on patients with a mitochondrial myopathy caused by a sporadically occurring single mtDNA deletion, eight patients underwent a muscle biopsy of the *vastus lateralis* before and after training.

These patients were participating in an exercise trial at Newcastle University. They were biopsied at baseline and then underwent six months of resistance training, which consisted of three sessions per week, with each session comprising three sets of eight repetitions of their 80-85% one repetition maximal. After six months a second biopsy was performed. When performing the experiments in chapters 4 and 5 it was not always possible to get muscle samples from both these biopsies for FACS sorting and myoblast culture. However, the samples for this study were taken from the muscle sample supplied to the Newcastle Mitochondrial Diagnostic Service, which had obtained tissue from all of the biopsies performed.

Muscle biopsies of the eight patients both before and after resistance training were cut at 12μm by Dr. J. Murphy, stained for Pax7, and counted as previously described. In addition, the myonuclei per fibre was determined both before and after training, to see if there had been any satellite cell incorporation into the fibres.

7.2.3.4 Results

Eight patients have completed the resistance arm of the trial and moved on to the endurance section of the program. Biopsy samples are not currently available for immunohistological analysis from the endurance section of the trial. Counts were performed to determine satellite cell frequency before and after resistance training (Table 7.3). There was a slight increase in mean satellite cell frequency after six months of resistance training from 2.15±0.29 to 2.41±0.72. The data appeared slightly more spread after the training period, suggesting there were maybe individual differences in patient's response to exercise (Figure 7.5A). However, a paired t-test (data normally distributed) revealed the increase was not significant (P=0.331).

Interestingly, when myonuclei number per fibre was determined for the eight patients, there was an increase from 7.06±3.66 to 8.37±4.4 (Figure 7.5B), which represented an overall increase of 19%. This increase occurred in six of the eight patients, with three of the six patients having over 100% increase, and the other three patients having an increase of between 8% to 28% (Table 7.3). However, a paired t-test showed this was not a significant difference (P=0.609).

The two patients that did not follow this trend showed a substantial decrease in myonuclei per fibre number of around 60%. For patient 1 this was not surprising as she left the trial after the resistance arm of the exercise program due to a considerable loss of weight. In addition doubts have been raised about the compliance of patient 8 to the exercise program. These patients had mtDNA deletion heteroplasmy levels of 53% and 43% respectively, which was very close to the average level of 48.6% for this patient group. This suggests their response, or lack of it, was not attributable to the amount of delete mtDNA they harboured. When the statistical analysis was performed with these two patients removed, the test showed a significant increase in myonuclei number (P=<0.05). However, these two subjects are part of the trial, and it may be that patients differ in their responses to resistance training. In addition sex may be an influencing factor, as these two patients were the only two females partaking in the study.

An interesting observation was made with patient 2 during this study. His second biopsy, after resistance training, demonstrated a large number of satellite cells as well as many small fibres (Figure 7.6A). This was hypothesised to be an area undergoing a period of strong regeneration, presumably following degeneration. There was a clearly defined border to this area of tissue, and it did not appear indicative of the rest of the biopsy (Figure 7.6B). The normal appearing area did contain some central nuclei, but had fewer satellite cells and no small fibres.

It is possible that this phenomenon occurred because of an injury sustained by the patient in the performance of his previous exercise session, or possibly through the second biopsy hitting the site of the first biopsy. The second reason would be somewhat worrying given that six months have elapsed since the previous biopsy and regeneration would appear not to be complete.

For the purpose of analysis this section was counted twice. Firstly, including the highly regenerative area, resulting in a satellite cell frequency of 7.868, and secondly without the unusual area giving a satellite cell frequency of 3.946. It was the latter value that was used for the statistical analysis.

	Satellite Cell Frequency		Myonuclei per Fibre		
Patients	1st Biopsy	2nd Biopsy	1st Biopsy	2nd Biopsy	Percentage Difference
1	2.31	1.94	10.38	3.28	-68%
2	2.57	3.94	4.49	11.28	151%
3	2.44	2.82	6.50	14.70	126%
4	1.98	1.62	7.72	8.51	10%
5	2.31	1.64	4.77	5.17	8%
6	2.04	2.71	4.09	13.80	237%
7	1.71	2.01	4.13	5.29	28%
8	1.86	2.60	14.35	4.90	-66%
Averages	**2.15**	**2.41**	**7.06**	**8.37**	**19%**
Standard Deviations	**0.29**	**0.77**	**3.66**	**4.40**	**±108%**

Table 7.3 Individual responses to resistance training. Eight patients underwent six months of resistance training intended to injure muscle and activate satellite cells. While five out of eight patients had an increase in satellite cell frequency after training, this was not significant. The variation in the satellite cell frequencies was greater after training. When the myonuclei and fibre counts were used to determine the myonuclei per fibre ratio an increase was observed for six out of the eight patients.

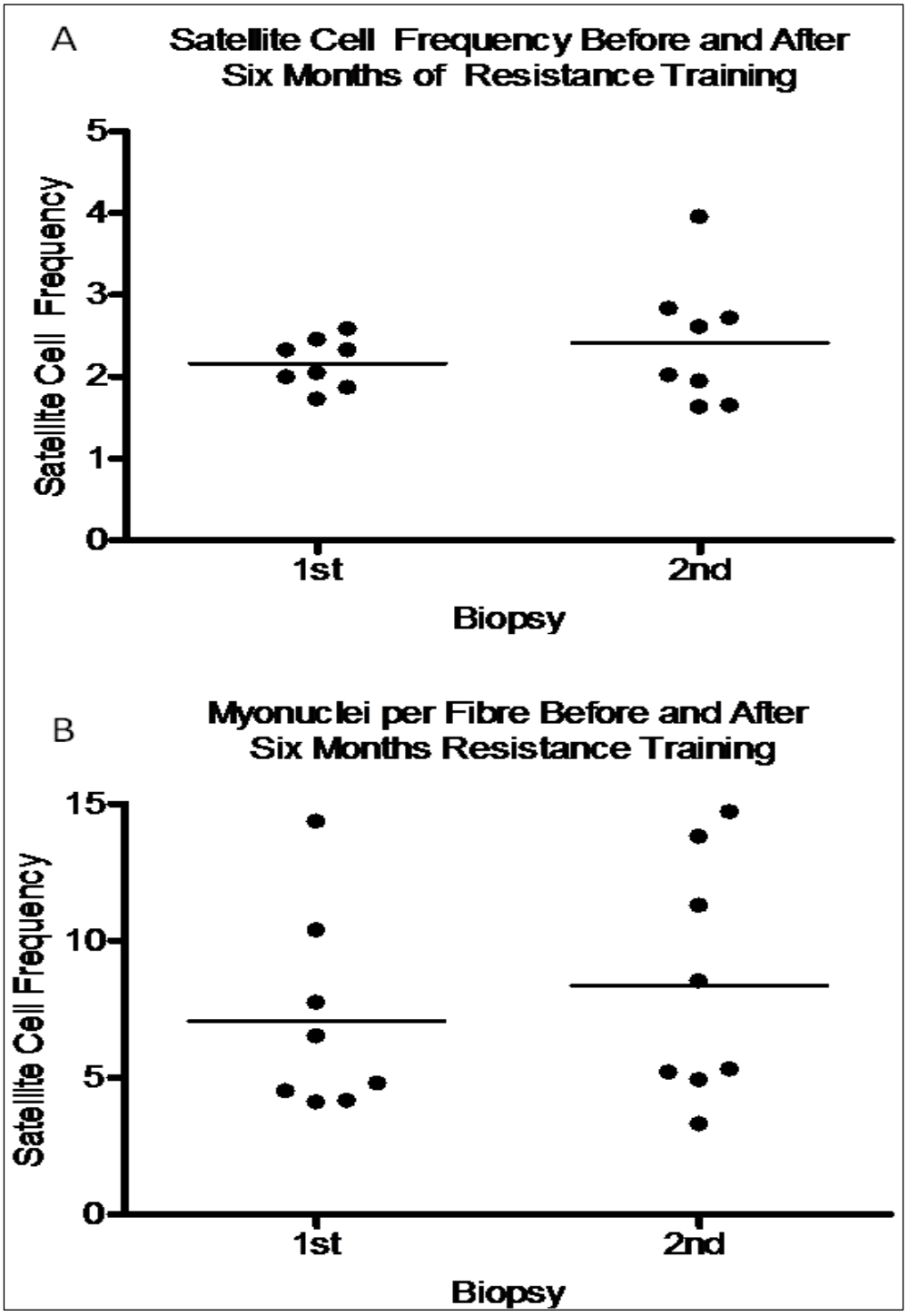


Figure 7.5 Effects of six months of resistance training on satellite cell frequency and myonuclei number. A. Satellite cell frequency before and after six months of resistance training. **B**. Average myonuclei per fibre increased after six months of resistance training; however this increase was not significant. Lines indicate average values for the group.

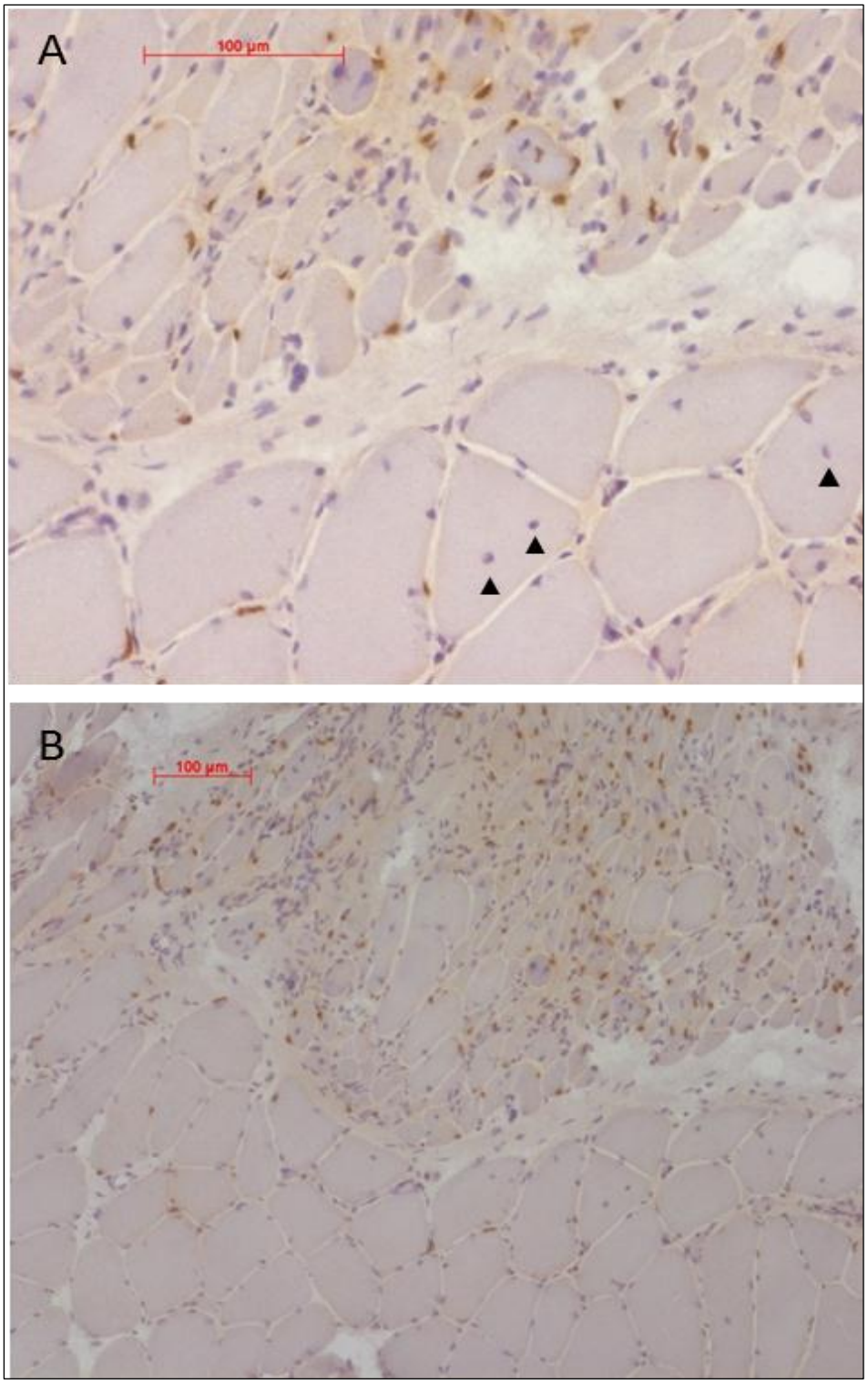


Figure 7.6 Patient 2. After six months of resistance training patient 2 showed a highly unusual area of degeneration/regeneration in his biopsy. **A**. Magnification x20. Many small fibres were visible, with numerous satellite cells. In the "normal" part of the biopsy central nuclei could be observed (arrow heads), suggesting regeneration had also occurred here. **B**. x10 magnification. This unusual response was limited to a defined area in the biopsy.

7.3 Discussion

The aims of this chapter were to investigate the numbers of satellite cells in patients in three scenarios. Firstly this research wanted to determine if patients suffering from a mitochondrial myopathy had fewer or more satellite cells than controls. Secondly it sought to see if there was any decrease in numbers as patients' age. Finally, the effects of six months of resistance training were examined. It was hoped that by investigating satellite cell frequencies under these three conditions it could be determined if efforts to activate satellite cells and "gene shift" would result in detrimental changes in terms of stem cells available for muscle regeneration.

Previous investigations have generally resulted in a satellite cell frequency of between 1.6 ±1.2 to 6.4 ±1.3 in the *vastus lateralis* (Kadi et al., 2005). The values obtained for this research are generally within this range for both mitochondrial myopathy patients and control subjects, suggesting appropriate staining and counting methods have been employed. While this study used Pax7 to stain and count satellite cells, other groups have used NCam (Crameri et al., 2004, Kadi et al., 1999). It would be interesting to use other markers for satellite cells to perform counts.

When assessing the effects of age and exercise on muscle, studies have performed analysis on a wide number of fibres, ranging from 43 fibres per subject (Roth et al., 2000) to 1850 per subject (Crameri et al., 2004). One study suggested a minimum of twenty five type I fibres and twenty five type II fibres should be counted in order to observe any differences (Mackey et al., 2009). The investigations performed here have involved an average of 781 fibres being counted, with a minimum of 148 fibres. This is well within the range previous studies have examined and higher than the recommendation given by Mackey and colleagues.

7.3.1 Satellite Cell Frequencies in Mitochondrial Myopathy Patients

While a very small number of satellite cells are obviously a problem for regenerating muscle, the same may be said for myopathy patients who have very large amounts. For example, findings of large satellite cell frequencies in myopathy patients (Ishimoto et al., 1983), are probably an indicator of large amounts of degeneration and regeneration occurring in the muscle. The danger being, that as the patients age they may experience an exhaustion of the satellite cell pool, as is seen in the mdx mouse (Luz et al., 2002).

Findings in our patients of similar levels of satellite cells when compared to controls is therefore encouraging, especially considering the control subjects were on average younger than the patients. It suggests that the patients have neither exhausted their stem cell reserve, nor are they currently on their way to exhausting it.

Consideration must however be given to the control tissue used in this study. While the fibre types between the hamstring muscle group and *vastus lateralis* are similar, and the embryological origins of the muscle are the same, there may still be differences between these muscles. In addition, these patients were undergoing operations to repair their ACL ligaments, and while the exact cause of the injury is unknown, it is often associated with football. The quick changes of direction required, its stop/starting nature, and the tendency for players to be “side tackled” when their feet are planted, means this injury is quite common in this particular sport. While our control group included both men and women and an age range of 21-52 years, they were still generally younger and more male orientated than our patients. In addition, there may have been a slight tendency for more athletic subjects to be a part of this group. Although finding that our patients had similar satellite cell frequencies to a group of healthy, younger, and possibly fitter subjects is reassuring.

7.3.2 Effects of Ageing

While a "snap shot" of our patients has suggested there is no significant difference between their satellite cell numbers and that of healthy controls, it was still interesting to examine how age and time affected them. Studies examining the effects of age on healthy subjects have reported differing findings. Experiments examining mice have suggested a decrease in satellite cell frequencies with age (Shefer et al., 2006, Snow, 1977), while studies in humans have reported both decreases (Verdijk et al., 2007) and no difference (Roth et al., 2000) in the *vastus lateralis* with age. Although there were slight differences in the ages studies, Roth *et al.* had a "young" group of an average age of twenty five and a half, and an "old" group with an average age of sixty eight. While Verdijk's "young" group were an average of twenty years, and the "old" group were an average of seventy six years. While this may not appear a great difference, the thirteen and a half year gap between the age ranges could have contributed to the conflicting findings.

Our patients will undergo more degeneration and regeneration over their life time due to their myopathy, and the comparisons made between mitochondrial disease and ageing phenotypes does mean the effects of age on our patients should be investigated.

One of our patients has been biopsied repeatedly over eleven years from the age of thirty two to forty three. A correlation analysis showed she had experienced an increase in her satellite cell frequency over time. While this may be an indicator of her progressive myopathy, her satellite cell frequencies were still within the range observed for healthy controls (Table 7.1B). Although, as already highlighted the reason this patient has undergone so many biopsies is her participation in a number of exercise trials. It is possible that this increase is a result of the long term effects of exercise.

It should also be noted that when all satellite cell frequencies for the patients examined in the initial part of this chapter were plotted against age there was no relationship between age and satellite cell frequencies, this was a similar pattern to the control subjects. This suggests that there may be more variation between patients

in terms of satellite cell frequency, than is seen in a single patient over time. Again this is encouraging as it suggests that even with a mitochondrial myopathy this patient group does not experience a detrimental increase or decrease in satellite cell frequency as they age.

Studies examining satellite cell number in aging people have often contained an older group of over sixty years. It may be possible that any differences are only observed when people cross a threshold of around this level. Given that none of the patients examined here are past this age, it may be expected that no differences are observed. It would be interesting to examine our patients as they get older to see if there is any change. In addition it may be intriguing to investigate satellite cell numbers in relation to fibre type and see if these patients suffer from the same type II fibre susceptibility to satellite cell loss that healthy subjects do (Verdijk et al., 2007). While performing multiple biopsies over a period of years is somewhat difficult, it would be interesting to examine other patients or even control subjects longitudinally to investigate any changes.

While this research has suggested that these patients do not experience an exhaustion of their stem cell pool as they age, this does not rule out the possibility that other factors associated with aging may impair the ability of their satellite cells to regenerate their muscle. Other factors that should be examined in these patients include telomere lengths, and the proliferative capabilities of satellite cell progeny in aged muscle.

7.3.3 The Effects of Resistance Training on Patients

The final part of this chapter aimed to examine the effects of six months of resistance training on patients with a mitochondrial myopathy caused by a large scale single mtDNA deletion. A previous exercise trial in a similar group of patients found a significant (P=0.003) increase in satellite cell number after three months of resistance training (Murphy et al., 2008). However, that finding was not repeated here. There was a slight increase in the satellite cell frequency after the resistance training, but this was not significant. While the patients on the previous resistance trial performed a similar exercise protocol, the duration was only twelve weeks, compared to twenty

four weeks on this trial. In addition the methods for staining and counting satellite cells varied between the trials.

Findings of an average 19% increase in myonuclei number per fibre may be important. However, an increase was only observed in six out of the eight patients studied, and was not significant. This may be attributable to differing individual responses to training. When Petrella *et al.* (2008) examined the effects of sixteen weeks of resistance training they found vastly differing responses in subjects. They were able to group their participants into “non-responder”, “moderate responders”, and “extreme responder” on the basis of changes in fibre area. Only the moderate and extreme responders demonstrated an increase in myonuclei number per fibre.

This study did not examine fibre area, but on the basis of myonuclei number per fibre, it is possible to group our patients into similar groups using the percentage change. High responders, patients 2, 3, and 6 (range of 126%-237% increase), moderate responders were patients 4, 5, and 7 (range of 8%-28% increase), and negative responders who were patients 1 and 8 (-66% to -68%). The removal of one of the negative responders from the trial and the questionable compliance of the other may raise doubts about these groupings. Alternatively there could be a sex based difference in the response.

These results may suggest that in at least some of our patients there has been incorporation of satellite cells into muscle fibres. It would be interesting to examine changes in fibre area in these patients, especially the high responders, it would be expected that they would demonstrate greater fibre hypertrophy. If they respond the same way as healthy controls, this may occur in the type II fibres (Tesch et al., 1984) (McCall et al., 1996, Kadi et al., 1999, Aagaard et al., 2001). They may also have responded with greater strength increases.

These results are interesting given that no significant change was observed in satellite cell frequency. Interestingly, all three of the high responders demonstrated a slight increase in satellite cell frequency. Previous research in this patient group demonstrated a significant increase in satellite cell frequency after twelve weeks of resistance training (Murphy et al., 2008), half of what was performed here. In addition,

previous exercise trials in healthy subjects have examined satellite cell frequencies after a maximum of sixteen weeks. It may be that the adaptive response changes in subjects after this length of training program.

The current trial is one of the longest resistance trials in both this particular patient group, and in healthy subjects. It may be that adaptations over the first few months require large increases in satellite cell numbers, but after this a smaller or muted satellite cell response is occurring. This is still enough to result in a steady increase in myonuclei number but small enough not to be significant when analysed. Examination of the response of healthy subjects or high responders from our patient group, to resistance training over a similar or even longer time frame would be interesting.

7.4 Conclusions

These results are very promising for attempts to “gene shift” in patients with a mitochondrial myopathy. This research suggests they will not exhaust their satellite cell pool either due to their myopathy or as they age.

While patients may experience a slight increase in satellite cell number as their myopathy progresses, this increase is still within ranges observed in healthy subjects. It may be necessary to further assess satellite cell numbers in this group of patients as they age further.

Performing resistance training can lead to satellite cell incorporation into the muscle fibres in selected patients. This finding is therefore very important in terms of efforts to “gene shift” in these patients.

However, there may be individual or sex linked differences in the responses to resistance training in these patients, which should be investigated further using greater numbers of participants.

Chapter 8 Final Discussion

8.1 Introduction

Ever since the discovery of mtDNA deletions and point mutations in humans in 1988 (Wallace et al., 1988, Holt et al., 1988) mitochondrial mutations have increasingly been recognised as a major cause of genetic disease. In 2000, the prevalence of people either with or at risk of developing a mitochondrial disease was determined to be 12.48 in 100,000 (Chinnery et al., 2000), while a more recent study put the number at 25.7 in 100,000 (Schaefer et al., 2008). This number may continue to increase through further advances in diagnostic techniques and a greater public awareness of mitochondrial disease.

Patients with mitochondrial disease often require long term health care support, furthermore, the multi-systemic nature of the conditions requires input from many specialities. Developing therapies and treatments for mitochondrial disease is therefore paramount. Pharmacological interventions can help manage some of the neurological symptoms, for example with seizures, and surgery has helped patients suffering from ptosis, and cataracts (Horvath et al., 2008a). In addition, strides are being taken to prevent the transmission of certain mitochondrial diseases (Craven et al., 2010, Poulton et al., 2009).

One avenue being explored is exercise, both endurance and resistance training (Taivassalo et al., 2001, Cejudo et al., 2005, Jeppesen et al., 2009, Murphy et al., 2008). Exercise interventions may be particularly helpful for patients who suffer from a mitochondrial myopathy. In addition to improvements in strength, it may help stall or even reverse the downward spiral that occurs in patients due to a lack of general physical activity.

The rationales behind the two types of exercise are somewhat different. Endurance exercise is thought to increase mitochondrial biogenesis and hopefully increase the amount of mtDNA in the muscle (Taivassalo and Haller, 2004, Taivassalo et al., 2006). In contrast, resistance training aims to activate satellite cells and promote their

incorporation into the muscle. This will serve to increase muscle strength in patients and in the presence of heteroplasmy change the amounts of WT and delete mtDNA.

The aims of this project were to investigate the rationale behind the use of resistance training in patients with mitochondrial myopathies caused by a sporadically occurring single mtDNA deletion. This was to ensure there would be no adverse effects from stimulating satellite cells in terms of variations in the muscle mutation load, and changes in stem cell numbers. It was originally hypothesised that no sporadically occurring mtDNA mutations would be found in the satellite cells of these patients. Accordingly, the original intention of this research was to investigate why there was an absence of mutation in these stem cells. However, early findings during this project in chapter 4 contradicted this hypothesis and, consequently, the research was taken in a different direction.

8.1.1 Rationale for Research

The rationale for using resistance training in mitochondrial myopathy patients with a sporadically occurring mtDNA mutation arose from reports in the 1990s (Weber et al., 1997, Fu et al., 1996). These papers reported finding no mutation in the cultured myoblasts of patients with sporadically occurring conditions. Previous reports in the 1980s had found much reduced levels of single mtDNA deletion in the cultured myoblasts of patients with KSS (Moraes et al., 1989a, Moraes et al., 1989b). It was suggested that the reason for lower or no mutation in cultured myoblasts was that the muscle satellite cells harboured little or no mutation.

These studies then led to successful attempts to change the phenotype of muscle fibres in patients with sporadically occurring mtDNA point mutations by the induction of muscle necrosis and the incorporation of WT cells into the regenerating fibres (Clark et al., 1997, Shoubridge et al., 1997). However, the methods used to induce muscle necrosis by the above groups, bupivicaine (Clark et al., 1997), and biopsying (Shoubridge et al., 1997), were deemed too invasive for clinical application. As a result, resistance training has been suggested as a viable alternative. High intensity resistance training induces sarcomere disruption and the activation of satellite cells to repair the

damage. If the satellite cells harbour no mutation, then the heteroplasmy level of the repaired fibre should change favourably.

However, the above studies were based on molecular findings in either myoblasts or regenerated muscle fibres. This would have allowed for a period of selection to act on the developing cells and it may be that the mutation was lost during the process of cell culture or muscle regeneration. With exercise being increasingly seen as a possible therapeutic intervention in patients with sporadically occurring mitochondrial myopathies, it became increasingly necessary to examine the exact processes that were occurring during muscle regeneration.

The current exercise trial at Newcastle University investigating the effects of both resistance and endurance training on patients with sporadically occurring single mtDNA deletions provided the opportunity to investigate this in a homogeneous group.

8.2 Major Findings

8.2.1 Muscle Satellite Cells

When this research was started it was hypothesised that examination of the satellite cells of these patients would show they were devoid of any mtDNA deletion, or at the very least there were very low levels.

Having isolated satellite cells by FACS sorting on the basis of CD56, it was possible to perform molecular investigations to examine their mutation status. It was shown in chapter 4, using two types of real time PCR and a long range PCR, that single mtDNA deletions were present in the satellite cells of all patients investigated. In addition they were found at similar levels to that found in the patient's muscle samples.

When individual satellite cells were collected and analysed they were shown to be heteroplasmic on the basis of single mtDNA deletions, with some cells containing high levels of mutation and some cells harbouring no mutation or very low levels. This is a very similar pattern to that observed in mature muscle.

This research led to the hypothesis that the mutation was present in the satellite cells during embryogenesis. The variations in heteroplasmy levels could be attributable to one of two processes or indeed a combination of the two. When the satellite cells are activated mtDNA replication will be initiated; given the low copy number in the stem cells, clonal expansion could be rapidly occurring. The other possibility is that the asymmetric division of satellite cells after activation and subsequent return to the stem cell pool of one of the daughter cells could cause a drifting of the genotype within the satellite cell to either homoplasmic WT or delete.

However, in order to get a clearer picture of what is occurring in the satellite cells it will be necessary to perform single cell studies on many more samples and use the relevant SYBR Green assay to determine levels below 30%.

8.2.2 Myoblasts

Myoblast cultures were set up from many of the biopsies performed and grown for a number of passages. When these cell lines were examined for single mtDNA deletions, the results were again surprising and demonstrated marked differences between patients.

It was shown in chapter 5 that while many of the cell lines had lost the disease causing mutation by the first passage, three of them had maintained it. In addition one patient both lost and maintained the mutation in consecutive biopsies. In two out of the three cell lines that maintained the mutation there was a gradual reduction in the heteroplasmy levels as the cells headed towards differentiation.

Using these data it was possible to define two selection points for the loss of the mtDNA mutation. One occurred at the point of satellite cell activation, and the second occurred as the myoblasts proliferated, and not as originally thought, as the myoblasts differentiated.

It has been hypothesised that the two selection points occur through different mechanisms. The loss of mutation at satellite cell activation is hypothesised to occur via a bottleneck mechanism. Two proposals for this bottleneck have been made, although they are not mutually exclusive. Firstly, when muscle damage is initiated satellite cells are activated to repair the damage. If satellite cells that have a primarily WT or delete genotype are activated, the resultant myoblasts could have astoundingly different heteroplasmy levels. Secondly the initial division of the activated satellite cell results in two daughter cells, only one of which will proceed to form myoblasts. If there is uneven segregation of mtDNA in these two cells then the resulting myoblasts may either primarily maintain or lose the mutation. Over time the stem cell that is maintaining the satellite cell pool may tend towards a homoplasmic fate, either WT or mutant.

The second selection point is hypothesised to occur because of the dividing nature of myoblasts. After each cell division there will be segregation of mtDNA. If a myoblast obtains a slightly higher amount of delete mtDNA it may suffer from a respiratory chain defect large enough to result in it dying. The process of myoblast proliferation is very reliant on mitochondrial biogenesis, as evidenced by increasing mtDNA copy number. An increased reliance on OXPHOS will result in any cells with high levels of mtDNA deletions being unable to survive.

These findings still concur with earlier reports examining sporadically occurring mtDNA mutations in proliferating myoblasts and regenerating muscle. Previous experiments

examining the cultured myoblasts from patients with sporadically occurring mutations found no mutation in these cells (Fu et al., 1996, Weber et al., 1997); this research found similar results in seven of the ten cell lines examined. It may be hypothesised that in both these papers and subsequent ones investigating the effect *in vitro* (Shoubridge et al., 1997, Clark et al., 1997) the mutation was lost at the point of satellite cell activation through a bottleneck effect or through negative selection on the dividing myoblasts.

While findings in some patients of mtDNA mutations in their myoblasts were again worrying for attempts to "gene shift", the results presented here suggest it will still be possible. The activation of satellite cells and subsequent loss of the mutation through either a bottleneck or negative selection will result in healthy myotubes being incorporated into the damaged muscle fibre. In addition, it is possible that the exercise being performed by patients will place added stress on the regenerating muscle cells and give a stronger selection pressure on myoblasts with high levels of mtDNA deletions. This should change the amounts of WT and delete mtDNA present in the muscle and will hopefully improve the muscle symptoms of patients.

However, a number of groups have now performed exercise interventions in patients with mitochondrial myopathy and a favourable change in mutation load has not been reported (Taivassalo et al., 2001, Jeppesen et al., 2006b, Murphy et al., 2008, Jeppesen et al., 2009). It may be that any changes will be too subtle to detect or the exercise programs are not sufficient or appropriate for "gene shifting". This highlights the need for further research in this area.

8.2.3 Satellite Cell Numbers

Attempts to "gene shift" involve the activation of satellite cells through inducing muscle damage via high intensity resistance training. Previous suggestions of significantly increased numbers of satellite cells followed by an exhaustion of the satellite cell pool in dystrophic muscle are worrying (Ishimoto et al., 1983, Heslop et al., 2000, Luz et al., 2002). Given that patients with a sporadically occurring single mtDNA deletion often present with myopathic symptoms, including muscle wasting, it was necessary to investigate the possibility the same may occur.

When compared to control subjects our patients had neither an increase nor decrease in satellite cell numbers, which was very encouraging. In addition, we demonstrated a slight increase in satellite cell numbers in a single patient over a period of eleven years. While this may have been caused by an increase in her myopathy, her satellite cell frequencies were still within the ranges observed for all other patients and control subjects.

This research also investigated the effects of six months of resistance training in patients with a mitochondrial myopathy and found no significant increase or decrease in satellite cell numbers. An interesting observation was made regarding the numbers of myonuclei per fibre. While a decrease was observed in two out of the eight patients, the other six showed increases in myonuclei per fibre. The patients were grouped into high, moderate and negative responders on this basis, which coincided with another report of grouped responses to resistance training (Petrella et al., 2008). It suggests that similar to healthy subjects, there may be individual differences in the direction and strength of response to resistance training. However, many more subjects should be examined before any firm conclusions may be drawn.

The increase in myonuclei number in six of our patients was encouraging as it suggests that there has been some satellite cell incorporation into the muscle fibres. This is one of the main requirements for “gene shifting”. In addition, the results suggest hypertrophy maybe occurring in these patients, which should provide strength increases. However, if there are inherent differences between patients in the way they respond to resistance training, it will be necessary to determine how a particular patient will respond before starting any training program.

8.3 Further Work

This research has revealed sporadically occurring mtDNA deletions in satellite cells of all of the patients studied. While the mutation is lost early on in the majority of patients, this is not always the case. In the cell lines that maintain the mutation there is generally a reduction as the myoblasts proliferate. These selection points are caused initially by a bottleneck followed by negative selection, although the exact mechanism of these phenomena is still somewhat ambiguous.

While this research was able to examine satellite cells, and early passage myoblasts, there was still around a two week time gap between these cell types while the cultures were being established in the biobank. In order to determine exactly what is occurring at the point of satellite cell activation, very early stage myoblasts should be examined. If a large piece of muscle could be obtained from these patients, a single muscle fibre culture could be set up. This would allow for the collection of muscle precursor cells just after activation, and as they move away from the fibre in the first couple of days. Individual cells could be picked out for both heteroplasmy and mtDNA copy number analysis, providing a much tighter time frame for the changes observed.

While it is difficult to perform this experiment in human subjects, part of it could be carried out in mice. Single fibre culture and collection of early cells seen migrating away from the fibre could provide information on mtDNA copy number in the early stages of muscle regeneration. Unfortunately, the absence of an appropriate mouse model of single mtDNA deletions precludes the examination of heteroplasmy levels. Mice models have been produced that harbour deletions, but these have been through nuclear mutations, for example the TWINKLE mouse (Tyynismaa et al., 2005), or have modelled an early onset multisystem disease (Inoue et al., 2000).

While the heteroplasmy levels were examined in individual satellite cells, a similar analysis should be performed in individual myoblasts. It can be hypothesised they also contain variable heteroplasmy levels but this should be verified. This could be performed with FACS sorting of individual cells on the basis of desmin or through laser dissection of individual myoblasts from a membrane cell culture plate.

This research has demonstrated that patients will probably not run out of satellite cells as they age. However, it will be important to re-examine satellite cell numbers in patients who are much older than this cohort, probably into their seventies.

While results in this small group of patients suggests that there may be individual differences in the way mitochondrial myopathy patients respond to resistance exercise, this effect should be examined further. The changes observed in myonuclei per fibre need to be considered in relation to any other muscle adaptations the patients undergo. Therefore the extent of hypertrophy of both muscle fibres and overall muscle should be determined. It would be especially interesting to see if patients undergo selected hypertrophy of the type II muscle fibres, as experienced by healthy subjects.

8.4 Strengths, Limitations, and Difficulties

The main strength of this research was also the source of many of its limitations and difficulties. The availability of human tissue has been invaluable during this project. The lack of appropriate mouse models of mitochondrial disease and suggestions that satellite cells are somewhat different between rodents and humans would have meant this research would otherwise have been impossible to perform. The use of human tissue has resulted in findings that are highly relevant to our patients and will enable future exercise studies to proceed with a much greater understanding of the mechanisms involved.

The enrolment of these patients on a particular clinical trial has allowed the examination of a very homogeneous subject group. These patients all have a similar phenotype and harbour a large scale single mtDNA deletion. Consequently, more confidence may be placed in the reported findings, as it is known that differences between patients are not attributable to a difference in genotype or phenotype.

However, the reliance on human samples has also led to gaps in the research. Tissue samples were not always available from all the biopsies performed for FACS sorting and myoblast culture. These patients were primarily being investigated for their response to exercise so it was paramount that sufficient tissue was always acquired for these studies. This particular study required participants to undergo a muscle biopsy every six months and consequently samples from patients were often analysed quite far apart from each other and occasionally on different machines. In addition, as the project developed different experiments were performed on the cells, so in some cases very few samples have been available for the more recent investigations.

There are always issues regarding compliance to exercise studies in humans. When examining the effects of resistance training on muscle adaptations, it is difficult to be confident that a reduced or absent response is a true result or partially attributable to questionable compliance.

Another limitation of this study was its reliance on cell culture as a model of muscle regeneration. Whether the cell culture environment is more or less hostile, when compared to a regenerating muscle, is somewhat unknown. However, the differing

environments will have caused different selection pressures on the myoblasts, which could have influenced heteroplasmy levels.

8.5 Final Conclusions

Attempts to “gene shift” in patients with a sporadically occurring large scale single mtDNA deletion could change the balance of WT to delete mtDNA in their muscles, although via a different mechanism to what was originally envisioned.

Selection acts on developing muscle cells at two points; satellite cell activation through a bottleneck mediated mechanism initially, followed by negative selection during myoblast proliferation.

It is likely that patients will not exhaust their satellite cell pool as a result of their myopathy or as they age. Resistance training will therefore not be detrimental in terms of exhausting their stem cell pool. On the contrary, it should help these patients via the incorporation of WT developing muscle cells and through the increases in strength that are attained through training.

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