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Mitochondrial Gene Expression in Human Mononuclear Cells

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MITOCHONDRIAL GENE EXPRESSION IN HUMAN MONONUCLEAR CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Biophysics at the Medical College of Virginia,
Virginia Commonwealth University

by

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I would like to dedicate this thesis to my fiancé Nicholas, who has been so supportive and encouraging during the completion of this thesis, as well as my Mother, Father and my sisters, Kinga and Iwona.

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Abstract

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Adult neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), have been intensively studied in recent years in pursuit of mechanisms responsible for origin and progression. One emerging theme is mitochondrial energetic deficiency as a mechanism of neuronal death. Recent descriptions of protocols to generate induced pluripotent stems cells (iPSCs) from living patients offer the potential to create unique disease models. This model can potentially lead to crucial advances in developing treatment options for a wide variety of neurodegenerative diseases. In this thesis, we attempt to induce iPSCs from mononuclear cells (MNC) in peripheral blood acquired from patients with ALS and healthy control (CTL) subjects, and analyze their mitochondrial genomes. The reprogramming of MNC to yield iPSC was done by nucleofection of an episomal plasmid pEB- C5, expressing OriP sequences of the Epstein-Barr and five reprogramming transgenes Oct4, Sox2, Klf4, c-Myc and Lin28. We investigated the expression of mitochondrial DNA genes, ND2, ND4, COXIII and 12s rRNA in the ALS and CTL MNC before and after their culturing. The results implicate deregulated mitochondrial bioenergetics as a characteristic of ALS. Future work will

establish whether these abnormalities in mitochondrial bioenergetics persist in iPSC's
and iPSC-derived neurons from ALS subject

Chapter 1:

1.1 Amyotrophic Lateral Sclerosis (ALS)

Neurodegeneration is a debilitating process in which nerve cells slowly lose their functionality and/or die over many years, producing clinical manifestations of neurodegenerative diseases. These widespread diseases of the nervous system affect humans in different ways and produce symptoms both within the brain and in different somatic tissues, impeding their proper functioning. Common neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis (ALS). The origins of these diseases are thought to be multi-factorial, genetic, as well as environmental.

Jean Martin Charcot first described "Lou Gehrig's Disease," the eponym created in the US in honor of a Yankee baseball player diagnosed with ALS at age 39, in 1869 as a motor neuron disease (Charcot and Joffroy, 1869). He observed an abnormal appearance of white matter and reactive astrocytes and microglia in lateral corticospinal tracks. This myelin pallor (pallere -be pale in Latin, lack of color and sickliness) and gliosis (reactive response to damage) indicated degeneration of axons of upper motor neurons descending from the brain's precentral gyrus (primary motor cortex) to the spinal cord. Originally named Charcot's sclerosis, the disease was eventually named amyotrophic lateral sclerosis as the axonopathy of the upper motor neurons was associated with degeneration of lower motor neurons in the spinal cord and manifested in the atrophy of muscles (Boillee et al., 2006; Nelson, 2003). In Europe, ALS is called motor neuron disease,

which unambiguously conveys an explanation of the source of muscle weakness that is motor neuron degeneration (Nelson, 2003).

The lifetime risk for ALS has been estimated as 1 in 1000, with 1-2 per 100000 of new cases per year and 4-6 per 100000 of affected individuals (Boillee et al., 2006). That translates, according to the ALS Association, to approximately 5,600 people in the U.S. diagnosed with ALS each year, and 30,000 Americans having the disease at any given time. Typically the disease affects middle-aged people (40-60 years old) but it can also strike at the younger age. Currently, there is no cure for ALS. It has been reported that 50% of the deaths occur within three years from the disease onset (Saris et al., 2009; Dupuis et al., 2011; Boillee et al., 2006). The most common form of ALS is sporadic, which have been thought to account for 90% of all ALS cases (Saris et al., 2009; Dupuis et al., 2011; Boillee et al., 2006). However, a recent Medline literature review and meta analysis of previously reported data, indicated that the familial form of ALS accounts for only 5.1 % (not 10 %) of all ALS cases, with even lower rate in Southern Europe. This discrepancy have been attributed to the fact there was a preferential recruitment of familial ALS patients, and that there is no clear definition of relatedness with only two members of extended family to be considered related (Byrne et al., 2014). Most ALS cases thus occur sporadically ~95% of the time.

The first manifestation of ALS differs between individuals. Some patients experience muscle weakness and cramping in hands, arms or legs. Others have difficulty speaking and projecting their voice as a result of facial muscle weakness. The disease progresses to

speech impairment and spasticity, and at more advanced stages to muscle atrophy, shortness of breath and consequently to paralysis of respiratory muscles and death. ALS not only affects the nervous system but also other body tissues as shown by genome wide studies of sporadic ALS (Dupuis et al., 2011).

As a consequence of the multivariate onset and presentation of ALS, it is sometimes called a syndrome rather than the nosological disease entity. The multifaceted and versatile presentation of ALS challenges its diagnosis. Clinicians perform meticulous neurological examinations and tests to exclude other neurological diseases, rather than identify ALS by looking solely at its symptoms. Some of these tests include electromyography (EMG), nerve conduction velocity (NCV), high-resolution serum protein electrophoresis, lumbar puncture, myelograms (now MRI scans) of cervical spine and muscle and/or nerve biopsy.

Mutations in many different genes are associated with Familial ALS (FALS)

Studies of familial forms of ALS (FALS) indicate that this autosomally dominant disease is associated with the mutations of genes located on at least six different chromosomes. Mutations leading to typical ALS, with a late-onset, progressive deterioration of upper and lower motor neurons are located on chromosomes 21, 18, 16, and 20 at the loci called ALS1, ALS3, ALS6 and ALS7 respectively.

The only gene identified for the clinically typical ALS is the one that encodes Copper/Zinc Super Oxide Dismutase (Cu/Zn SOD1), which converts (“dismutates”) superoxide to hydrogen peroxide and is localized mainly to cytosol. It is located on

chromosome 21 at the locus ALS1 (Dupuis et al. 2011; Boillee et al. 2006; Nelson, 2003).

Out of two forms of ALS with dementia, frontal temporal dementia (FTD) and FTD with Parkinson's disease (FTDPD), only the locus called ALS-FTD has been successfully related to a gene (MAPT), which is a microtubule-associated protein. This locus is located on chromosome 17 and ALS-FTD locus is located on chromosome 9. An atypical, heterogeneous form of ALS, manifested by unusual tremor, is caused by a mutation in vesicle associated membrane protein B (VAMB) gene that is located on chromosome 20 at the locus called ALS8. The gene product is involved in a vesicular transport from endoplasmic reticulum to Golgi apparatus and transport of cargo to membrane in an axon. Another atypical form of ALS related to a mutation on chromosome 2 at the locus associated with progressive lower motor neuron disease. The gene found at this locus, DCTN1, codes for a dydactin subunit, which is a part of axonal transport. The onset of this form of ALS is observed in adults and leads to vocal-cord paralysis.

The names of the loci ALS2, ALS4, and ALS5 located on chromosomes 2, 9 and 15 respectively, are in some way misnomers because those mutations are associated with juvenile forms of motor neuron diseases, not ALS (Rosen et al., 1993; Hand et al., 2002; Chen et al., 2004; Hadano et al., 2001; Nishimura et al., 2004; Boillee et al., 2006).

Numbers of studies have also identified genes that promote ALS progression. According to gene analysis of human postmortem sensory and motor cortexes, ALS subjects showed

differential expressions of 275 genes (Wang et al., 2006). Ten of those genes were upregulated; six associated with surface activity and two with glutamate receptors (consistent with excitotoxicity of glutamate). The remaining, down-regulated more than four fold genes were: zinc finger protein 36, C3H type-like 1, inhibitor of DNA binding 4, and heterogeneous nuclear ribonucleoprotein H1 (group of genes were involved in transcription), ribosomal protein S11, (protein/amino acid synthesis and turnover group) nuclear receptor subfamily 4, group A, member 3, and inhibin, beta C (neurotransmission, hormones group), vascular endothelial growth factor (survival and growth), small protein effector 1 of Cdc42 (signaling), dynein 2 light intermediate chain (cytoskeletal tubulin and cell adhesion molecule), transducer of ERBB2, 1 (differentiation or proliferation), protein regulated in glioma and forming binding protein 3 (growth factor receptor-bound protein) (Wang et al., 2006).

A decrease in vascular endothelial growth factor (VEGF) as a consequence of a mutation in a promoter region of VEGF gene has been linked to increased risk of ALS in Belgium, Sweden, and Britain (Lambrechts et al., 2003; Gros-Louis et al., 2003). This growth factor is essential in angiogenesis not only the developmental, but also hypoxia-induced (Wang, 2006.) Angiogenesis can be also negatively affected by a mutation in angenin gene ANG, mainly in the core subunit (Boillee et al., 2006; Greenway et al., 2006).

Neurofilament's mutations may be also a risk factor for ALS although there is no conclusive linkage between the disease and the NF aggregates observed in familial and sporadic ALS patients (Garcia et al., 2006). Other down-regulated genes were involved in

ubiquitin- proteasome function, apoptosis, lipid metabolism, inflammation, oxidative stress and energy metabolism. Specifically, down regulated genes related to mitochondrial energy metabolism included: methionine adenosyltransferase II alpha, myotubularin related protein, thioredoxin, and CD24 antigen (small cell lung carcinoma cluster 4 antigen) (Wang et al., 2006).

Energy deregulation plays a central role in ALS progression. In a healthy person intake of energy in form of food and production of energy normally equilibrates energy expenditure. An overeating, static life style leads to insulin resistance, which results in a decreased energy delivered to cells as in diabetes mellitus type 2. On the other hand raised oxygen consumption, decreased food intake or mitochondrial dysfunction lead to weight loss and malnutrition. In ALS patients, for reasons currently unknown, the energy balance is interrupted by hypermetabolism, which precedes denervation. Studies of sporadic ALS of genes responsible for metabolism led to no conclusive results. What is also baffling is that ALS patients experience hyper lipidemia with increased LDL/HDL ratio (Dupouis et al., 2011).

Does study of motorneuron groups help us understand their vulnerability in ALS?

To understand selective vulnerability to degeneration of motor neurons in different areas of the nervous system in different neurodegenerative diseases, neurons of rats were isolated by laser capture microdissection and following areas were analyzed: oculomotor/trochlear (cranial nerve 3/4, unaffected in ALS), hypoglossal (cranial nerve 12, affected in ALS) and lateral motor column of the cervical spinal cord (highly affected in ALS). Analyzed gene expression of these regions revealed that all regions exhibited

unique gene expression profiles; however, there was some similarity in CN12 and lateral spinal column gene expression. All of the regions had differential expression of Hox genes, mitochondrial functions, ubiquitination, apoptosis regulation, nitrogen metabolism, calcium regulation, transport, and RNA processing genes (Hedlung et al, 2010). This is consistent with observations of aggregates caused by mutations in the genes of ubiquitin, TAR DNA binding protein of 43-kDa (TDP-43), and fused sarcoma protein (FUS) in ALS mouse models (Kwiatkowski et al., 2009; Dupuis et al., 2011). These studies did not reveal any selective properties of ALS-vulnerable neuron populations compared to those that typically do not degenerate in ALS.

SOD1 mutations can cause Familial ALS (FALS)

Out of all genetically inherited forms of ALS, it has been previously reported that mutation in SOD1 accounts for 10-20% of FALS cases (Boillee et al., 2006; Wang et al., 2006). Specifically, mutation of alanine to valine-SOD^{A4V} elicits the most aggressive form of ALS and is the most prevalent one in the US (50%) (Boillee et al., 2006). However, according to the only population based study that was conveyed in Italy, the SOD1 mutation accounted for only 13.6% of FALS (Chio et al., 2008; Byrne et al., 2014).

The gene product of SOD1 gene is 153 amino acids long and it is mainly cytosolic, but some appears to be mitochondrial. Its function is to detoxify superoxide anion by converting it to hydrogen peroxide that is ultimately detoxified to water. It had been therefore postulated that the loss of this dismutase function might be associated with ALS phenotype. However this did not seem to be the case because in a course of different research undertakings, it was shown that active and inactive enzyme result in similar

disease presentation (Fischer et al., 2004; Frey et al., 2000; Pun et al., 2006) Moreover, the deletion of the enzyme in mice does not produce an ALS phenotype (Reaume et al., 1996). SOD1 requires Cu and Zn for proper functioning. Copper, toxic if free in the cell, loaded into SOD1 aids the oxidative process. A study of mice with eliminated copper showed however no difference in ALS phenotype (Subramaniam et al., 2002; Boillee et al.2006; Wang et al., 2006). On the other hand it has been shown in SOD1 mutant mice that zinc deficiency can accelerate the disease manifestation and in that situation mitochondrial SOD1 generates more free radicals (Wang et al., 2006).

In a search for an explanation of unknown source of toxicity of SOD1 mutation it has been proposed that it might be a result of a defective protein folding. This could explain aggregations of SOD1, which are one of the trademarks of ALS (Wang et al., 2003). These SOD1 aggregates bind to many cellular components negatively affecting their function. Among some of them are proteasomes, in which SOD1 aggregate binding leads to abnormal protein degradation, and chaperones, which lead to impaired protein folding. Moreover, mutant SOD1 that is imported to mitochondria interrupts calcium buffering, electron transport chain, and protein import by blocking import machinery, and might be a cause of initiating apoptotic pathways by binding to and inactivating Bcl-2 (Boillee et al., 2006). ALS also affects mitochondria structurally, based on observations of enlarged, disorganized mitochondrial membranes in motor neurons (Dupuis et al., 2003).

Global gene expression profiling study of SOD1mutant overexpression in mice showed that the instigation of the disease relates to motor neurons, while advances of the disease are promoted by astrocytes and microglia (Hedlung et al., 2010). A healthy motor

neuron consists of up to ~one-meter-long axon, through which transport of cargo takes place. Healthy motor neurons receive excitatory signals from a glutaminergic neuron that releases glutamate, which binds to glutamate receptors. A motor neuron is also surrounded by microglia and astrocytes, which participate in the removal of glutamate from the synaptic cleft by transporting it through EAAT2 transporter into their cell bodies. It has been observed in SOD1 mutant mouse model that the first event in the ALS development is in a muscle that loses the synaptic connection between the motor neuron and a muscle, seen as motor neuron retraction (Pun et al., 2006). This motor neuron terminal retraction in the early stage of the disease might be due to accumulation of SOD1 aggregates or neurofilament mis-accumulation, which affects the transport within the axon, and mitochondrial dysfunction derived from defective mitochondrial transport into and out of nerve endings.

Activated microglia at the symptomatic stage of the disease, driven by SOD1 released from the motor neuron, produce toxic factors such as nitric oxide (NO) and tumor necrosis factor (TNF) alpha that target the same neuron. At the same time, high levels of glutamate in the synaptic cleft cause constant firing of the motor neuron and calcium entry through AMPA receptors (Boillee et al., 2006). This glutamate driven toxicity has been a target of some drugs like riluzole. Toxic factors like nerve growth factor (NGF) are also released from the astrocytes, which at this point of a disease progress, lose the expression of glutamate transporter EAAT2. All of those events lead to caspase activation and motor neuron apoptosis and muscle atrophy at the end stage.

Motor neuron damage in ALS therefore appears to be caused not only

autonomously but also by neighboring cells. Microglia activation, and cd16 + monocytes increased in peripheral blood have been observed in both sporadic and familial ALS. The abnormally activated monocyte/macrophages correlated to the lipopolysaccharide (LPS) levels in the peripheral blood (Zhang et al., 2011). Also studies of SOD1 mutant mouse have shown that expressing SOD1 mutation exclusively in the motor neuron and not in neighboring cells did not induce a disease in some mice.

The fact that neuro-protection can be achieved by just targeting neighboring cells of a neuron has motivated trials for potential treatment options. Microglia treatment with minocycline inhibits their activation. TNFalpha antagonist injections or cyclooxygenase 2 (COX2) inhibitor injections showed some promise when it comes to slowing disease onset (Zhu et al., 2002; Drachmanetal, 2002; West et al., 2004). Another nonneuronal neighbor target for the disease treatment is the astrocyte. Since expression of EAAT2 glutamate transporter decreases during disease progression, beta-lactam antibiotics that act as transcriptional inducers of EAAT2 produce improved survival in transgenic SOD1 mice (Rothstein et al., 2005). Also blocking release of glutamate could decrease injury, which appears to be a major action of riluzole. Although its mechanisms of action are not fully understood, they also include inactivation of voltage dependent sodium channels or prevention of glutamate binding to its receptors. In a study of SOD1 ALS mouse model, the drug extended their survival (Doble, 1996), and riluzole is currently the only FDA-approved drug for ALS.

Muscles as therapeutic targets in ALS

Muscles have been also used as therapy targets because muscles die without

neuronal stimulation. Increasing mass of muscle by decreasing expression of myostatin, which slows muscle development, didn't improve ALS in SOD1 mutant mice (Holzbaur et al., 2006). Injecting insulin like growth factors IGF-1 caused revival of muscle fibers and life prolongation of SOD1 mice, therefore opened a possibility of growth factor therapies (Dobrowolny et al., 2005). Exercising, especially concomitant with IGF1, showed improvement in ALS transgenic mice (Kaspar et al., 2005).

Growth factors show some benefit in ALS

Infusion of growth factor BDNF through into the brain by ICV or spinal cord intrathecal injection showed small benefits in patients (Nagano et al., 2005b). In mice VEGF encoding retrovirus delivery infusion to muscle or ICV injection of VEGF increased survival (Zheng et al., 2004).

1.2 Mitochondrial genome

Both genetic and sporadic forms of amyotrophic lateral sclerosis present clinically in very similar ways (Saris et al., 2009, Dupuis et al., 2011, Boillee et al., 2006). This relative homogeneity of the clinical phenotype of ALS led to a conclusion that there might be few pathways that are significant in the disease's evolution. Since some of those pathways include signaling pathways in peripheral blood cells, it became evident that the peripheral blood could be used for the gene profiling and become a biomarker for diagnosis of ALS (Saris et al., 2009; Maes et al., 2007). Another study showed that throughout the course of ALS damage occurs not only to motor neurons but also to non-neural tissue, including peripheral lymphocytes (Curti et al., 1996). This damage affects bioenergetics of cells with mitochondria, being the energy factory, centrally involved in that damage (Dupuis et

al., 2011; Heath et al., 2013).

Mitochondria were first described as “chlorophyll bodies” and “cell granules” at the end of the nineteenth century. Since then, mitochondria were more fully characterized in the 1950’s. Lynn Margulis, who proposed the endosymbiotic theory, formulated their origins in the early 1970’s. According to this theory, oxidative proteobacteria were symbiotically incorporated into a prokaryotic or early eukaryotic cell and evolved to become modern mitochondria.

The endosymbiotic theory is supported by molecular genetics and sequence homology comparisons between DNA of mitochondria and prokaryotes. The organelle’s ribosome’s are like prokaryotes (70S) and differ from cytoplasm of eukaryotes (80S). Other supportive arguments for the endosymbiotic theory include circular mitochondrion genome and its replication, initiation of RNA translation, and the double membrane of mitochondria (with the inner mitochondrial membrane being high in cardiolipin content, which is like bacterial membranes but different from eukaryotic plasma membranes). Mitochondria within the course of evolution adapted to being inside of the cell by transferring some of its genome into nucleus, reducing in this way their “supplementary” genome and energy expenditures (Wallace, 1999).

Mitochondria control many tasks in a cell. They include but are not limited to: initiation of cell death cascades, regulation of energy production, feedback regulation of Krebs cycle, maintaining calcium homeostasis, and formation of iron sulfur clusters.

Deregulated of calcium homeostasis, as in the peripheral blood lymphocytes of ALS

patients, leads to uncoupling of oxidative phosphorylation (Curti et al., 1996).

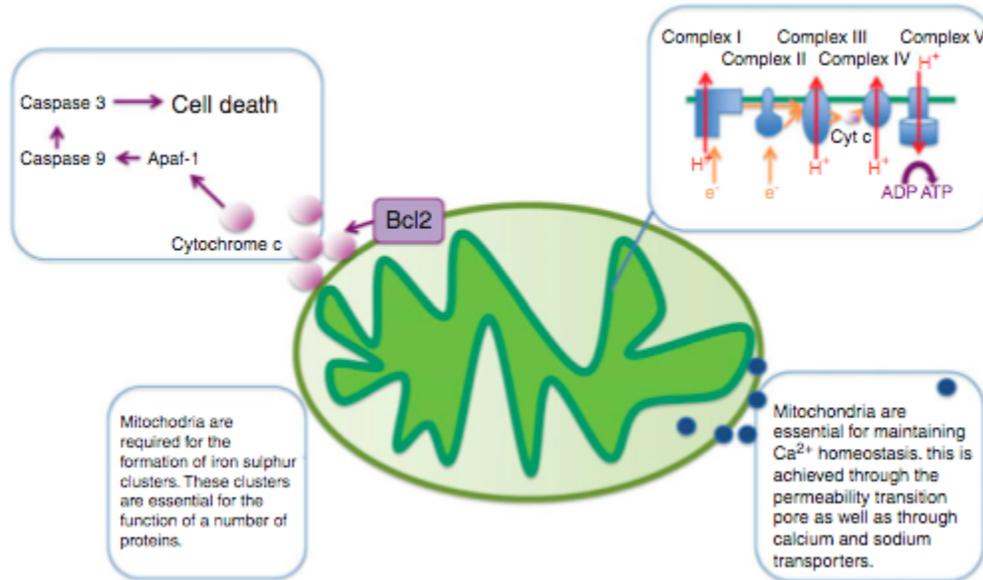


Figure 1.1. Many functions of mitochondrion. Reeve et al., 2012.

Human mitochondrial genome consists of 16568 base pairs contained on heavy and light circular DNA strands. The lighter (based on G-C content), inner strand encodes ND6 and eight tRNA's; the heavier outer strand encodes 12s and 16s rRNAs, 14 tRNAs, six subunits of complex I (ND1-5, ND4L), one subunit of complex III (cytochrome b), three subunits of complex IV (COX I-III), as well as two subunits of complex V (ATP synthase; ATP6, ATP8). These 13 genes encode proteins of the electron transport chain (ETC) that are necessary for the capturing of energy in the form of a proton gradient, and converting it to ATP in a process of oxidative phosphorylation (OXPHOS).

The semiautonomous system of mitochondria relies also on the nuclear genome. The rest of the proteins, including 74 ETC proteins, enzymes, transcription factors, ribosomal

proteins, and polymerases are made outside of the mitochondria then imported and assembled there (Wallace, 1999; Bellance et al., 2009).

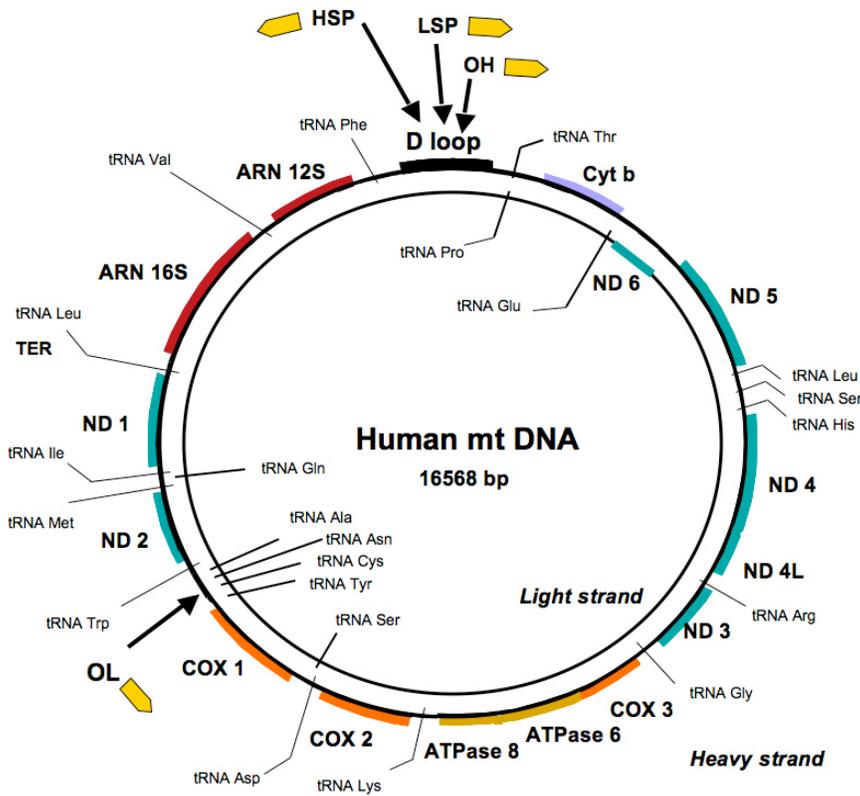


Figure 1.2. Mitochondrial genome. Bellance et al., 2009.

The D-loop is a regulatory, non-coding region, with promoters of heavy and light strands and the origin of replication Heavy strand (OH). According to the ‘strand-asymmetric’ explanation of mtDNA replication, the origin of replication of the light strand (OL) is located approximately 2/3 of the circumference from OH. The replication cycle is about two hours long and starts with the replication of the heavy chain proceeding counterclockwise.

A single mitochondrial polycistronic mRNA encodes several different polypeptide chains (Bellance et al., 2009). The mitochondrial genome is unique in many

aspects. It not only has a distinctive code for translating DNA and replicates independently from the nuclear genome, but also its RNA is capable of enzymatic activity and of joining two transcripts together with so-called trans-splicing (Williams, 2002). Human mitochondrial (mt) genome was thought to be mainly maternally inherited. This was attributed to the fact that paternal mitochondria present in the tail of sperm, do not enter an oocyte, and if they do, they are overcome by abundant maternal mtDNA and damaged by reactive oxygen species (ROS) (Luo et al., 2013; Williams, 2002). Despite this general belief, it has been observed in rare situations that mitochondria from the father can be passed to the offspring in humans. A patient of Schwartz and Vissing with trademarks of mitochondrial myopathy, ragged-red muscle fibers and exercise exertion, is one example of mitochondrial paternal inheritance. The detailed sequencing analysis of the mitochondrial genome in the quadriceps muscle of this patient has shown, that the mutation in the ND2 gene was derived from the father. Interestingly, the mutation was not present in circulating lymphocytes and that is consistent with the fact that mitochondrial mutations are often localized to a specific tissue (Schwartz and Vissing, 2002; Williams, 2002).

Mitochondrial DNA has a larger mutation rate than does nuclear DNA (Wallace, 1999). The mutation frequency increases with age even in a healthy person, and is accelerated in degenerative diseases (Williams, 2002). The mutated state (heteroplasmy) can be transferred to the somatic cell progeny and be a cause of diminishing bioenergetic capacity of a cell by a process known as “Muller’s ratchet” that describes increasing mutational burden associated with non-sexual reproduction of DNA. It follows that the

tissues affected the most by mitochondrial mutations, are the ones with high-energy demands like neurons, cardiac muscle and skeletal muscle. This energy and disease correlation was observed in many studies. For example, the mitochondrial DNA study by Keeney et al. showed increased mtDNA point mutations in spinal cord neurons of patients with ALS as well as decreased cytochrome oxidase activity, which is an important part of ETC. Also, a Parkinson's disease study showed increased abundances of mtDNA deletions that included cytochrome oxidase and complex I of ETC (Bender et al. 2006).

The cell energy production relies on the interaction of three processes that take place in mitochondria: oxidative phosphorylation, production of the radical oxygen species and apoptosis (Wallace, 1999). The interruption of this state of equilibrium by, for example, cellular mutations, leads to a disease. The energy currency of the cell, ATP is produced in mitochondrial ETC in the process of oxidative phosphorylation. The ETC consists of five complexes; NADH dehydrogenase (complex I; 7mt DNA {ND1-6, ND4L}, 40 nDNA), succinate dehydrogenase (complex II; 0mtDNA, 4nDNA), ubiquinol cytochrome C oxidoreductase (complex III; 1mtDNA {cytochrome b}, 10 nDNA), cytochrome C oxidase (complex IV; 3mt DNA {COX I-III}, 10 nDNA) and ATP synthase (complex V; 2mt DNA {ATP6, ATP8}, 15 nDNA).

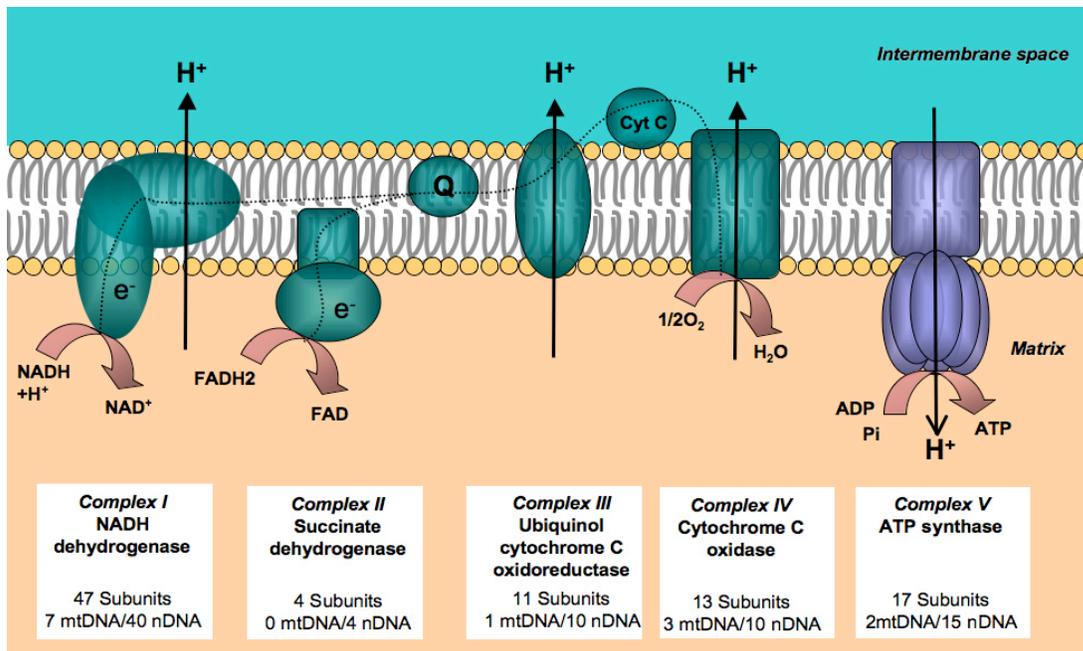


Figure 1.3. Inner membrane Electron Transport Chain of mitochondria. Bellance et al., 2009.

This chain of proteins oxidizes NADH and FADH₂ that were produced in the Krebs cycle from the pyruvate from glycolysis. NADH and FADH₂ enter the ETC at the complexes I and II respectively and electrons released in this process of oxidation are transferred to coenzyme Q. Complex III passes these electrons to cytochrome c, and cytochrome c to complex IV. Oxygen consumption takes place in complex IV where the O₂ is “sucked up” by heme₃ and reduced to H₂O. Complex V of ETC of mitochondria uses electrochemical proton gradient created by ETC (H⁺ moved to inter-membrane space) to generate ATP from ADP and phosphate. ETC although often described as consisting of single complexes is in reality an assembly of supercomplexes so called “respirasomes” that is few subunits of a specific complex bound to each other. The ratio of these complexes varies for different tissues. Respirasomes minimize diffusion distances of substrates and electron leakage (Schägger and Pfeiffer, 2001) and the leakage in ETC can cause oxidative damage to many cellular components including

cellular and mitochondrial membranes as well as it can inactivate the energy production through oxidative phosphorylation.

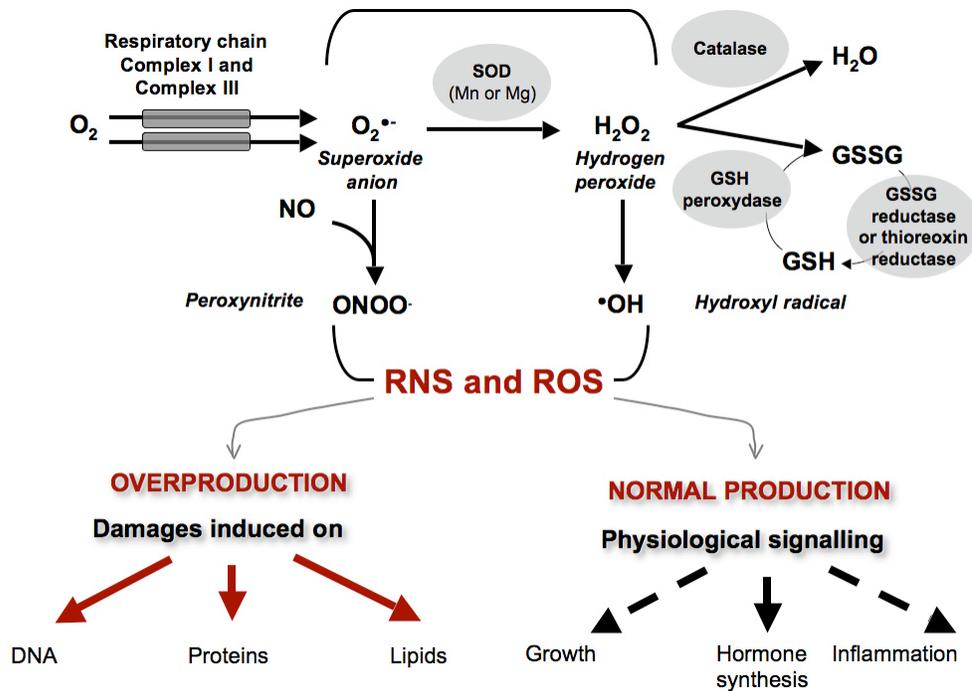


Figure 1.4. Reactive Oxygen and Nitrogen Species. Bellance et al., 2009.

Oxygen molecules that are abundant in mitochondrial matrix can accept free electrons that leave complex I and complex III of the ETC, yielding superoxide anion, that under normal conditions is converted to hydrogen peroxide by Mn/Mg superoxide dismutase (SOD-2). Mitochondria have high levels of reduced glutathione (GSH) that is a component of glutathione peroxidase and catalase can convert hydrogen peroxide to water. If that process of superoxide removal is deregulated it leads to generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and causes oxidative and nitrosative damage of DNA, proteins and lipids.

Increased ROS can also cause opening of mitochondrial permeability transition pore (mtPTP), which leads to the initiation of apoptosis. The cascades of events follows; discharge of the electrochemical gradient of mitochondrial membrane, inflation of inner membrane, release of cytochrome c, activation of caspases and apoptosis. This interplay between oxidative phosphorylation, reactive oxygen species, apoptosis taking place in mitochondria is fundamental in bioenergetics of the cell.

1.3 Induced Pluripotent Stem Cells (iPSCs)

One of the main concerns of neurodegenerative studies is the amount of tissue available for research. Many studies utilize postmortem samples from autopsies (Payne et al., 2014; Gage et al. 2013; Wang, 2006) with limited amount of nucleic acid for analysis from samples that are also at the final stage of the diseases. Also, the animal models created for studies are genetic, which do not necessarily reflect the most commonly occurring (~95%) sporadic forms of human ALS. This pursuit of plausible solutions to overcome these limitations has directed attention to induced pluripotent stems cells (iPSCs), induced neuronal phenotypes (iN), and somatic cell neural transfer (NT) (Wernig et al., 2013; Mertens et al. 2013; Staerk et al. 2010; Wang et al. 2013), and has inspired the development of disease models which could potentially lead to the advances in crucial treatment options for neurodegenerative diseases including ALS. The reprogrammed cells from genetically unique patients would allow studies of specific pathways in motor neurons even ahead of a disease manifestation, and would make for a plausible design of personalized pharmacological treatment (Gage et al., 2013). For therapy, iPSC's could be differentiated to motor neurons, which can be delivered back to

the patient (Oakely et al., 2013). The reports of successful autologous transplantation of stem cells from bone marrow to ALS patients have been seen in studies (Feng et al., 2012).

Among the somatic cell types that have been effectively reprogrammed into iPSC are mononuclear cells (MNC) (Dupuis et al., 2013). These blood cells, isolated from either the umbilical cord (CB) or adult peripheral blood (PMNC), offer several advantages among which is their low number of somatic mutations as a result of their short life span, compared to skin fibroblasts (Oakely et al., 2013; Feng et al., 2012) which are also used for iPSC generation. The other advantage is their short culture time as well as accessibility (Dupuis et al., 2013). Originally human iPSC were derived using a retroviral vectors, however, the questionable safety of injecting a retro or lentivirus into humans and the resulting mutations they created (Yu et al., 2009) redirected attention of researchers to recombinant proteins usage. This approach, though plausible, was very inefficient (Dupuis et al., 2011) therefore a method of using non-integrating or excisable vectors and transduction of the reprogramming factors was developed (Dupuis et al., 2013; Jaenish et al., 2013; Yu et al., 2009).

The other experimental parameter that affects the quality of iPSC is the number of reprogramming factors and donor cells (Jaenish et al. 2013). Dupuis et al. reported a successful single plasmid transfection of pEB-C5 plasmid containing EBNA1/OriP OriP sequences of the Epstein-Barr with five reprogramming factors Oct4, Sox2, Klf4, c-Myc and Lin28. The plasmid was not integrated into the genome so it is much safer than using a retrovirus, and preferentially expanded erythroblasts over T lymphocytes before

reprogramming, since T lymphocytes have high number of somatic VDJ rearrangements (Dupuis et al., 2011; Yu et al., 2009). To improve reprogramming efficiency of PMNC sodium butyrate can be added to the media (Chou et al., 2011).

Chapter 2:

2.1 Materials and Methods

Tissue Culture

Peripheral blood of two ALS and one non-ALS healthy control (CTL) patients was collected at the Parkinson's and Movement Disorder's Center Medical College of Virginia according to VCU IRB-approved protocol. Within four hours after blood collection, mononuclear cells (MNC) were separated from other blood cells using Ficoll-Paque Premium density-based centrifugation. In this method MNC, being of lower density, stay in the top layer above Ficoll which is a hydrophilic polysaccharide and the separation agent for this method. QIAzol Lysis Reagent (Qiagen) was used to remove any remaining red blood cells. The peripheral blood MNC (PMNC) was stored at -80 degrees Celsius.

The generation of human induced pluripotent stem cells from PMNC was attempted by following the protocol of Dowey et al., 2012.

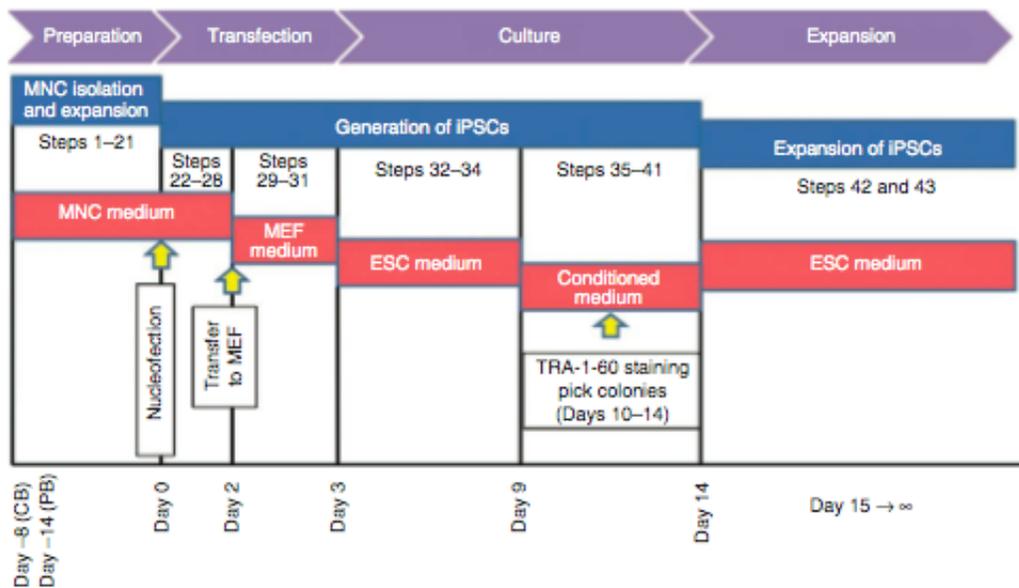


Figure 2.1. Generation of integration free human iPSC protocol. Dowey et al., 2012.

MNC were isolated and expanded in MNC medium for 14 days. This medium was serum free with cytokines that would favor erythroblasts over lymphocytes development. On day 14 MNC were transfected with episomal plasmid pEB- C5, expressing OriP sequences of the Epstein-Barr and five reprogramming transgenes Oct4, Sox2, Klf4, c-Myc and Lin28. The 4D-Nucleofector Core Unit and X Unit (Lonza) were used for reprogramming. This event started the generation of iPSCs. The cells were then transferred to feeder-coated plates containing mouse embryonic fibroblasts (MEF) and the medium was switched from MNC to MEF medium with antibiotic antimycotic and l-glutamine. On the third day after transfection, MNC medium was replaced with embryonic stem cell (ESC) medium. The iPSCs colonies start multiplying on day 14.

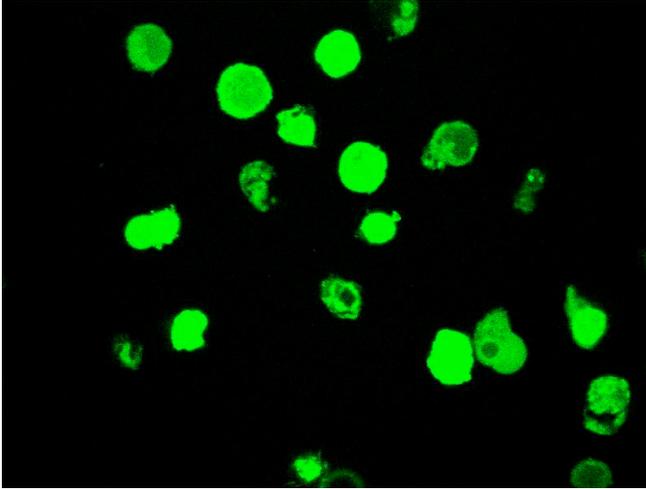


Figure 2.2. An image of a commercial MNC (All Cells) that were transfected in previous experiments by another research group member. Image is of MNC two days after transfection stained with a GFP green fluorescent protein (GFP) plasmid. It was a positive control for the transfection process.

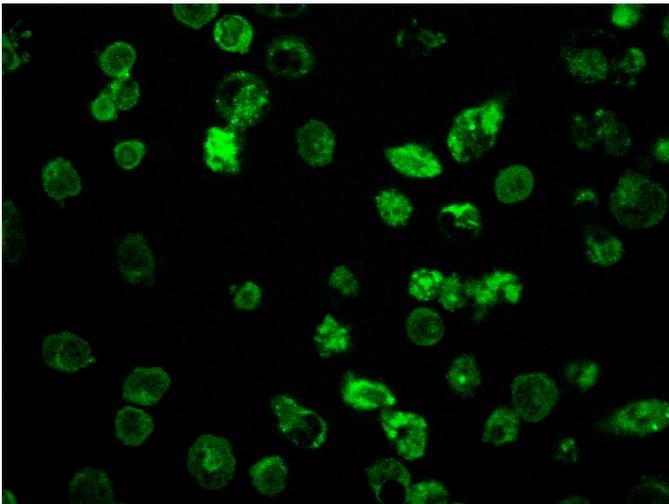


Figure 2.3. An image of a commercial MNC (All Cells) that were transfected in previous experiments by another research group member. Image is of MNC two days after transfection stained with just PBS as a negative control.

Two weeks after the transfection, cells can be stained with TRA-1-60 antibody to confirm the identity of successfully reprogrammed iPSCs colonies. Unfortunately, in my

experiment after morphological assessment of the cell culture, no iPSC were identified, therefore TRA-1-60 staining was not performed.

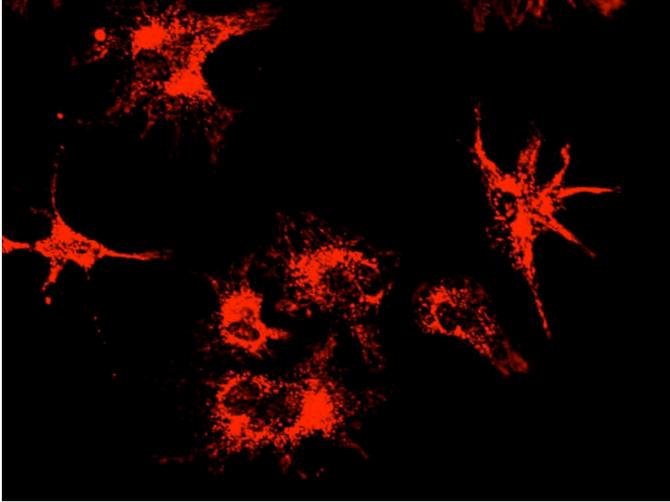


Figure 2.4. An image of a commercial MNC (All Cells) that were transfected in previous experiments by another research group member. Image is of the same MNC cell line as in Figure 2.2 and Figure 2.3 fourteen days after transfection with plasmids. It has been stained with an antibody to TRA-1-60, which is an early marker of pluripotency.

RNA and DNA extractions

The extraction process is used to separate DNA and RNA from biological samples using chemical and physical methods. The intricacy of the RNA extraction lies in the realization of omnipresence of RNAases that degrade RNA and making a great effort to eliminate them.

In this experiment RNA and DNA were extracted from PBMC samples using an AllPrep DNA/RNA Mini Kit (Qiagen), which allowed simultaneous purification of genomic DNA and total RNA. The first step of DNA/RNA extraction was disruption of plasma membrane using RLT buffer containing guanidine thiocyanate followed by samples' homogenization to reduce the viscosity of the lysate. This would allow for better binding of DNA and RNA to mini spin columns. The DNA was separated from RNA using

AllPrepDNA mini spin column to which DNA bound. Consequently, ethanol was added to the eluted RNA to precipitate it. The next steps for RNA and DNA extraction consisted of adding RW1 buffer with ethanol to the RNA column and AW1 buffer with Guanidinium hydrochloride to DNA column, then washing them.

Following the DNA and RNA extraction, both nucleic acids were subjected to quality control (QC) using Nanodrop 2000c spectrophotometer (Thermo Scientific) for DNA and Experion Automated Electrophoresis System (Bio-Rad) for RNA.

Experion RNA StdSens analysis kit was used to determine total RNA concentration and establish its integrity using RNA quality indicator (RQI) number with a scale 1-10, 10 being the highest. After the chip preparation, which included adding gel stain containing fluorescent dye and sieving matrix, the chip was inserted into the electrophoresis station with voltage sensitive electrodes. Charged RNA fragments were separated on their size basis (virtual gel matrix), and the fluorescent dye penetrated the fragments. Following the detection by a photodiode of fluorescence of the laser-exciting sample fragments, the software created fluorescence intensity versus time plots (electropherogram). The total RNA sample was separated into two peaks that represented 18S and 28S ribosomal RNA (rRNA) and the software compared the area under the electropherogram to calculate total concentration of the sample. RNA was then reversely transcribed to cDNA using iScript (Bio-Rad) using GeneAmp PCR system 9700 (Applied Biosystems).

Nanodrop 2000c spectrophotometer (Thermo Scientific) was used for quality control (QC) of DNA, which measured the intensity of light transmitted through the sample. The

absorbance at a given wavelength was then calculated according to the equation:

$$\text{Absorbance} = -\log \left[\frac{\text{Intensity of sample}}{\text{Intensity of blank}} \right]$$

The Beer-Lambert equation was used to correlate the calculated absorbance with concentration:

$$A = \epsilon * b * c$$

Where **A** is the absorbance represented in absorbance units (A), ϵ is the extinction coefficient liter/mol*cm, **b** is the length of solution the light passes through in cm, and **c** is the analyte concentration in moles/liter or molarity (M).

The reference buffer used in this experiment was Tris (hydroxymethyl) aminomethane - Ethylenediaminetetraacetic acid (Tris- EDTA that is TE) buffer in which DNA was dissolved. TE solubilizes DNA while protecting it from degradation. To assess the purity of DNA the ratio of absorbances at 260nm/280nm was calculated. DNA absorbs UV light at 260 and 280 nanometers and pure DNA has a ratio of 1.8. A lower ratio may indicate presence of protein, phenol or other contaminants because aromatic proteins absorb UV light at 280 nm. The secondary measurement of 260nm/230nm additionally assesses nucleic acid purity. A ratio of 260/230 lower than 1.8-2.2 indicates the presence of copurified contaminants.

Selection of Genes of interests and Quantitative PCR

Real time polymerase chain reaction (qPCR) is a technique that amplifies and simultaneously quantifies a targeted molecule utilizing the physiochemical properties of nucleic acids and DNA polymerase. It provides an amplification plot based on the changes in fluorescence, which is proportional to the amount of the target. QPCR is also a common technique for studying gene expression of neurological diseases. The advantage of this method is the sensitivity of the measurement to small amounts of mRNA. However, this technique entails the necessity of normalization to housekeeping genes that are transcribed continuously at a constant rate, which guarantees a stable level of mRNA regardless of the experimental condition. Geometric means need to be used because the absolute expression levels of reference genes can vary widely, and geometric means (as opposed to typical arithmetic means) distribute this difference. GeNorm is used to find the least variable reference genes in the cells of interest, independently of their absolute expression levels. Also, cell types and disease progression, and their amount, would be comparable to the analyzed genes. According to Penna et al. (2011) and their GeNorm and NormFinder analysis of gene stability, the most stable reference gene in postmortem brain was CYC1, however; they recommended a combination of reference genes. Following the experience from our research group (Keeney et. al., 2010) the housekeeping genes chosen for normalization were TOP1 (encodes DNA topoisomerase type I which enzymatically breaks and rejoins a single strand of DNA), GAPDH (encodes glyceraldehyde-3-phosphate dehydrogenase, an enzyme that catalyzes the sixth step of glycolysis, which has been extensively used in different studies as a reference gene (Penna et al. 2011), YWHAH (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, which encodes a 14-3-3 family of proteins that

mediate signal transduction in cells by binding to phosphoserine containing proteins) and CYC1 (encodes cytochrome C1, mitochondrial heme protein). The normalization factor for mtDNA was a geometric mean of GAPDH and YWHAH for mtDNA and TOP1 and CYC1 for mt cDNA.

It was crucial to establish suitable candidates for the gene analysis. According to Keeney et al. (2011), the mitochondrial genes encoding respiratory proteins ND4 and COX III are frequently deleted in cervical spinal cord major arc ALS patients and ND2 is also deleted but less frequently. The 12s rRNA gene product is involved in the assembly of proteins within cells present only in mitochondria.

QPCR was performed with human mtDNA copy number standards (for mtDNA qPCR) or human fetal brain cDNA (for mt cDNA qPCR) using iQ Multiplex Power Mix in a CFX-96 Real-Time PCR instrument iCycler with aniQ5 detection system (BioRad, Hercules, CA). MtDNA genes ND2, ND4, COX III, and 12s rRNA were measured using dyes (FAM- ND2, Texas Red- ND4, TET- COX III, Quasar- 12s rRNA) as well as sense and antisense primers. Each sample and standard was quadrupled. The cycling protocol consisted of activation at 95°C for 5 min followed by 50 cycles of: 95°C melting for 10 seconds (allowing for the separation of the double chain), and 50°C for 1 minute (allowing for the binding of the primers with the DNA template). Data was analyzed using the iQ5 software. QPCRs for the reference genes GAPDH, YWHAH, TOP1, CYC1 were performed individually using EvaGreen® Supermix (BioRad) and primers designed for PCR. The standard curves generated showed similar efficiencies of the qPCR runs.

2.2 Results

Tissue culture

In this study another research group member cultured MNC from ALS patients. I cultured MNC from a CTL subject. My two additional MNC cultures from ALS patients were contaminated during the course of the study, and the efforts of reprogramming MNC to iPSCs from the CTL subjects were unsuccessful for the reasons addressed in discussion. After morphological assessment of my cell culture, no iPSC were identified; therefore TRA-1-60 staining was not performed.

RNA and DNA Quality Control

For ALS and control PBMC samples the quality indicator (RQI) numbers were between 9.8 -10. For the CTL sample before culturing, however (sample number 9), there was a critical anomaly error, which could have been due to some contamination. It could not have been a result of RNA concentration, which was not the lowest one (62 ng/ul). The RNA concentrations ranged between 38.25 ng/ul for the ALS sample I before culturing, and 257.39 ng/ul for the ALS sample II after culturing (See **Table 2.1**).

Well ID	Sample Name	RNA Area	RNA Concentration (ng/μl)	Ratio [28S/18S]	RQI	RQI Classification	RQI Alert
L	Ladder	836.14	160				
1	ALS46 I	199.89	38.25	0.02	10	Green	
2	ALS 46#1	862.8	165.1	1.88	9.8	Green	
3	ALS 46#2	774.77	148.26	1.73	9.9	Green	
4	ALS 46#3	831.14	159.04	1.96	10	Green	
5	ALS 48 II	217.87	41.69	2.05	10	Green	
6	ALS 48#1	801.03	153.28	1.64	9	Green	
7	ALS 48#2	687.32	131.52	2.07	10	Green	
8	ALS 48#3	1345.07	257.39	1.85	9.9	Green	
9	CTL 47 I	324.12	62.02	2.25	N/A		Critical Anomaly
10	CTL 47#1	303.05	57.99	0.06	10	Green	

Table 2.1. Quality Control results of RNA using Experion Automated Electrophoresis System (Bio-Rad).

The measured 260/280 ratios for the DNA samples were within 1.86-1.89 range, which confirmed their purity from aromatic proteins. However, there might have been some other contaminant in the CTL sample before culturing (sample number 9), because the ratio 260/230 was much lower than the purity range that is 0.63.

The DNA concentrations varied between 128.5 ng/ul for the sample 10 (CLT after culturing) and 311.4 ng/ul for the sample 8 (ALS 48 after culturing) (See **Table 2.2**)

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	1 ALS 46 I	Administrator	5/21/2014 11:35:56 AM	270	ng/ul	5.4	2.9	1.86	2.03	DNA	50
2	2 ALS 46 #1	Administrator	5/21/2014 11:37:36 AM	250	ng/ul	5	2.674	1.87	2.08	DNA	50
3	3 ALS 46 #2	Administrator	5/21/2014 11:38:34 AM	231.4	ng/ul	4.627	2.453	1.89	1.83	DNA	50
4	4 ALS 46 #3	Administrator	5/21/2014 11:39:36 AM	223.5	ng/ul	4.471	2.385	1.87	2.04	DNA	50
5	5 ALS 48 I	Administrator	5/21/2014 11:40:39 AM	197.8	ng/ul	3.955	2.121	1.86	1.4	DNA	50
6	6 ALS 48 #1	Administrator	5/21/2014 11:41:45 AM	295	ng/ul	5.899	3.155	1.87	2.09	DNA	50
7	7 ALS 48 #2	Administrator	5/21/2014 11:42:36 AM	254.9	ng/ul	5.098	2.73	1.87	1.84	DNA	50
8	8 ALS 48 #3	Administrator	5/21/2014 11:43:37 AM	311.4	ng/ul	6.228	3.325	1.87	1.96	DNA	50
9	9 CTL 47 II	Administrator	5/21/2014 11:44:45 AM	242.5	ng/ul	4.85	2.601	1.86	0.63	DNA	50
10	10 CTL 47 #1	Administrator	5/21/2014 11:46:01 AM	128.5	ng/ul	2.57	1.385	1.86	1.54	DNA	50

Table 2.2. Quality Control results of DNA using Nanodrop 2000c spectrophotometer (Thermo Scientific).

Mitochondrial cDNA/ RNA quantification

The results of qPCR were normalized to two housekeeping genes CYC1 and TOP1 by dividing SQ means by the geometric mean of two housekeeping genes. Mt cDNA of

analyzed genes ND2, ND4, COX III and 12s rRNA showed higher gene expression in ALS MNC before culturing compared to CTL before culturing for all genes. Specifically, ND2 expression was increased by 2.4 for ALS comparing to CTL which was increased by 1.6 and COX III expression increased by 2.6 for ALS versus CTL increase of 2. There was no apparent increase of gene expression for 12s rRNA (1.1 for ALS and 0.8 for CTL) and ND4 (1.05 for ALS .95 for CTL)(**Figures 2.5-2.8**).

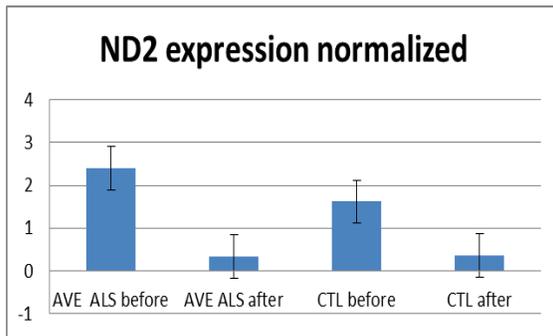


Figure 2.5. ND2 gene expression in ALS versus CTL MNC before and after culturing.

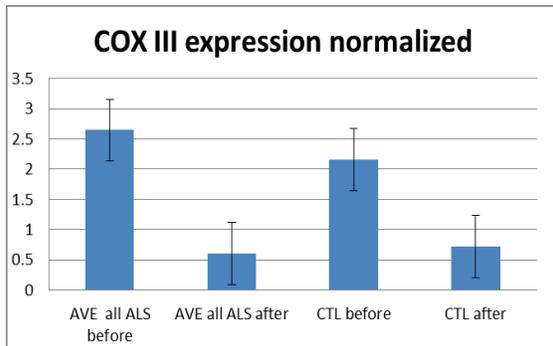


Figure 2.6. COX III gene expression in ALS versus CTL MNC before and after culturing.

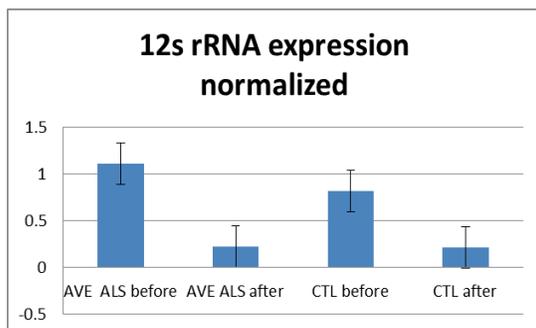


Figure 2.7. 12s rRNA gene expression in ALS versus CTL MNC before and after culturing.

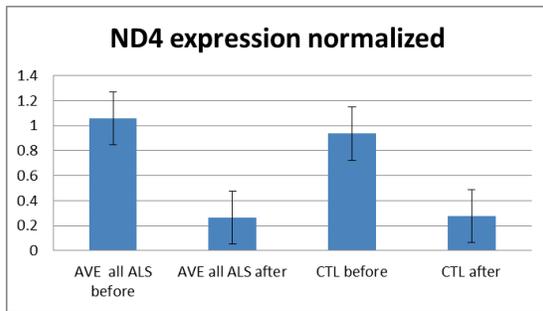


Figure 2.8. ND4 gene expression in ALS versus CTL MNC before and after culturing.

After culturing, ALS and CTL gene expression was decreased for all genes in comparison to ALS and CTL gene expression before culturing. ALS gene expression was comparable to CTL gene expression for all genes.

mtDNA copy numbers

The results of qPCR were normalized to two housekeeping genes GAPDH and YAHWZ by dividing SQ means by the geometric mean of two housekeeping genes. Mitochondrial DNA copy numbers of analyzed genes ND2, ND4, COXIII and 12s rRNA were increased in MNC after culturing compared to copy numbers before culturing for all ALS and CTL samples. There was an increase in copy numbers for the respiratory genes ND2, ND4, COX III which was ~1200000 for ALS compared to ~1000000 copy numbers of CTL. There was no apparent difference in copy numbers of 12s rRNA expression of ALS compared to CTL. Copy numbers for CTL were higher for all genes after culturing comparing to before culturing (**Figures 2.9 – 2.12**).

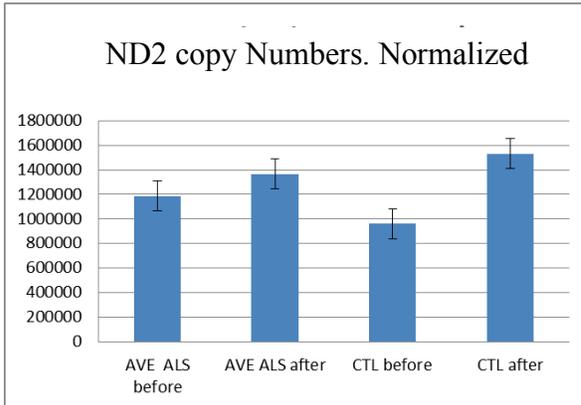


Figure 2.9. ND2 mtDNA copy numbers in ALS versus CTL MNC before and after culturing.

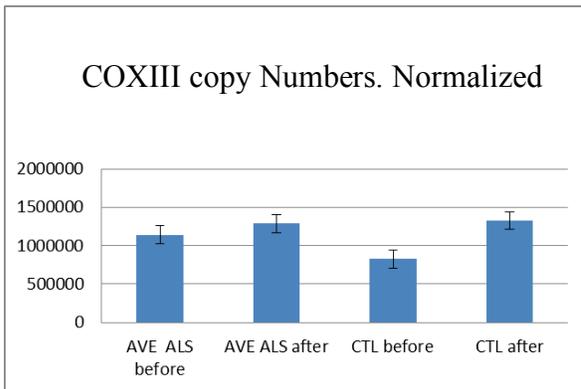


Figure 2.10. COX III mtDNA copy numbers in ALS versus CTL MNC before and after culturing.

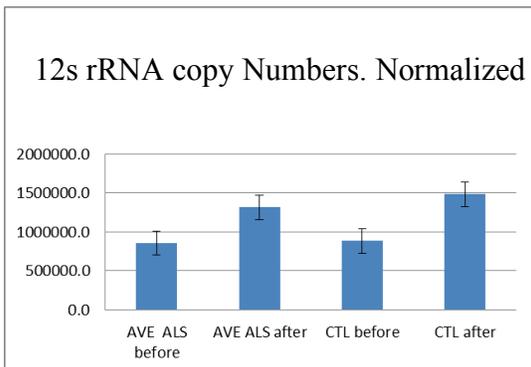


Figure 2.11. 12s rRNA mtDNA copy numbers in ALS versus CTL MNC before and after culturing.

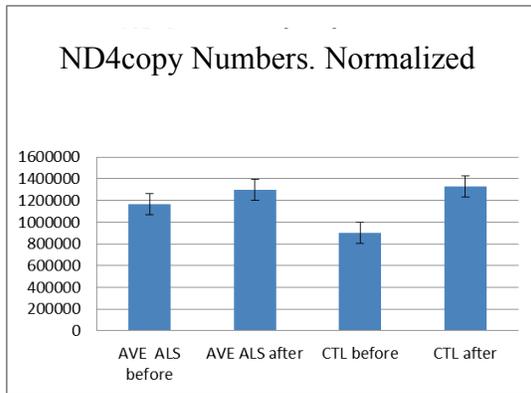


Figure 2.12. ND4 mtDNA copy numbers in ALS versus CTL MNC before and after culturing.

2.3 Discussion

ALS, despite tremendous research efforts and palliative therapy advances, remains a lethal motor neuron disease. The etiology of ALS, most of which occurs sporadically, is still unknown and the endeavor dedicated to its characterization and finding consequent treatment options, led scientists to intensive research of cell components. Given the heterogeneity of sporadic ALS clinically, there may be different etiologies and mechanisms responsible for progression. Free radicals' formation, oxidative stress, autophagy, and mitochondrial dysfunction lead to energy deficiencies of cells in animal models (Dupuis et al., 2011; Wallace et al. 1999., Curti et al., 1996; Oakley et al. 2013). Each of these processes, singly or in combination, may underlie the origin(s) and progression of ALS.

Extensive studies have tried to find genes responsible for ALS. In this search, few nuclear genes have been identified that are linked to the disease. Mutation in SOD1 gene

resulting in toxic aggregates causes not only impairment for proteasomes and cellular chaperones but SOD1 transport and accumulation in mitochondria may play a negative affect on calcium channels, electron transport chain and may prematurely trigger apoptosis. Other cellular aggregates like 43-kDa (TDP-43), fused sarcoma protein (FUS) and neurofilaments have been also observed as characteristic of a disease progression (Kwiatkowski et al., 2009; Dupuis et al., 2011; Garcia et al., 2006). Not only the motor neuron itself but also its neighbors are central players in ALS.

Microglia and astrocytes can also release toxic factors that contribute to the disease development. Activation of microglia leads to release of glutamate that resides longer in the synaptic cleft because it can not be removed by glutamate transporters (which are lost from astrocytes during ALS progression). This leads to perpetual activation of the motor neuron and calcium influx.

It has been observed that ALS not only affects the nervous system but also has a holistic impact on the body and all its cells. In particular, peripheral mononuclear cells may be taken into consideration as potential screening candidates for detecting the disease at early stages (Zhang et al., 2011). Looking at bioenergetic deficiency of a cell and mitochondrial gene expression could lead to ALS characterization at different stages of its progress. Also peripheral blood MNC can be used to develop induced pluripotent stem cells (iPSC) leading to potential treatment options of ALS and ~~many~~ other degenerative diseases, such as Parkinson's and Alzheimer's disease.

In this work we tried to induce PSC from mononuclear cells taken from the peripheral blood of patients with ALS and healthy control. The protocol for MNC expansion was used according to Dowey et al., 2012. According to this protocol MNC

were isolated and expanded in serum free MNC medium with cytokines for 14 days. On day 14 MNC were transfected with episomal plasmid containing reprogramming transgenes Oct4, Sox2, Klf4, c-Myc and Lin28, which started the generation of iPSCs. Consequently, transfected MNC expanded in different media favoring development of iPSC.

Starting with three samples, peripheral MNC from two ALS patients and one control CTL, the two ALS samples have been contaminated during the course of MNC expansion. Working with a tissue culture demands a sterile technique. This technique involves special training and different cells have different susceptibility to contamination. I received training on SY5Y cells, which is human neuroblastoma cell line. Working with human MNC samples requires more precise and finer technique comparing to SY5Y because of the amounts of cells and media contained in wells comparing to flasks of SY5Y cells. The factors mentioned above plus multiple steps, elaborate, every day preparation of different media for MNC expansion may have contributed to the contamination of cells. The SY5Y cell line had only one type of media prepared in advance limiting the possibility of contamination. In the course of learning the sterile technique, the CTL cell line was not contaminated. Despite the efforts of expansion and the cell culture, I did not detect any iPSCs. The unsuccessful reprogramming of MNC to iPSCs could be attributed to many factors. Peripheral blood monocytes have lower reprogramming efficiency than cord blood (Chou et al., 2011). The cell counts of my original CTL sample were low at the beginning of the expansion, and the colony was growing poorly, which might be an indicator of sample contamination, especially after considering the quality control index CQ of RNA and DNA for that sample. Additionally,

nucleofection, which transfers substrates (in this case plasmid) into the cell by applying an external electric field, causes excessive cell death and can be responsible for low transfection efficiency. Many of the cells die at this point before colonies can develop.

For future studies to enhance reprogramming efficiency, the starting MNC cell count should be chosen to be higher as compared to Dowey et al. Few adjustments could have been made to my experiment to obtain better results. Being able to ensure the quality of the blood samples before the cell expansion could have been an advantage since the growth of the colonies and quality of DNA and RNA might have been affected by the quality of blood MNC before culturing. No staining experiment was performed to exclude possibility of omitting the iPSC colonies during the transfer to different media. There was also no absolute identification of the colonies after iPSC expansion only the morphological assessment. After the transfection the colonies were picked manually under the microscope, which could have contributed to the excluding iPSC cell lines. The iPSC colonies might have been lost during the course of expansion because some of them did not adhere to the gel.

The significant drawback of my study was the limited sample numbers. Working with more samples could be very beneficial, as it would identify the outliers and minimize the experimental error. Also, having just one control sample with lower quality of DNA could have misled the conclusions.

The qPCR mitochondrial gene expression study showed that mitochondrial cDNA of analyzed genes ND2, ND4, COX III and 12s rRNA was higher in ALS MNC before culturing compared to CTL before culturing for all genes. This increase in cDNA might be an indicator of increased protein numbers in ALS patients as a response to higher energy demands. An increase was observed in mitochondrial DNA copy numbers of analyzed genes ND2, ND4, COX III and 12s rRNA in MNC after culturing compared to copy numbers before culturing for all ALS and CTL samples. This is opposite to what occurred in the cDNA samples. There was a decrease in cDNA gene expression for both ALS and CTL after culturing. Therefore mtDNA copy numbers in genomic DNA increased slightly after culturing, but cDNA consistently decreased after culturing. This discrepancy between mtDNA copy number and mtRNA (as cDNA) expression might be contributed to the fact that we did not determine appropriate reference genes for the MNC after culturing which is a shortcoming of this study. GeNorm analysis would be necessary because monocytes after culturing are in principle different cells then before culturing. Looking at the graphs, the proportional decrease in gene expression after culturing appears greater in ALS compared to CTL, whereas mtDNA gene copy numbers increase more in CTL than in ALS. Therefore ALS samples appear more impaired than CTL in increasing their mtDNA copy numbers and lose more mtDNA gene expression.

There was no apparent difference in copy numbers of 12s rRNA genes of ALS compared to CTL which could be attributed to the fact that 12s rRNA has a separate transcriptional promoter from the genes encoding respiratory proteins ND2, ND4, and COX III which is not affected in the course of ALS. Only COXIII and ND4 had increased expression in ALS patients in comparison to CTL. Although mtDNA copy

numbers were not markedly reduced in ALS MNC blood compared to CTL MNC, it supports results of Keeney et al. where mtDNA gene copy numbers in ALS spinal motor neurons decreased in ALS patients. Overall, these results implicate deregulated mitochondrial bioenergetics at a systems level as a characteristic of ALS.

For future experiments it would be beneficial to see and compare the demographics of patients with ALS to draw accurate conclusions and make connections between age, sex and familial history of ALS. It would be also valuable to carry out a study on patients' family members, and compare their mitochondrial gene expression. Periodical screening of MNC mitochondrial gene expression of the same patient would contribute to understanding of the processes that guide ALS progression and would reveal its correlations to mitochondrial bioenergetics. If that is successful then peripheral mononuclear cells may be considered as potential screening candidates for detecting the disease at early stages. It would be also beneficial to look at potential cross talk between the MNC and other blood components by testing for inflammatory cytokines, growth factors and necrosis factors, which could also change during the disease's course and could be indicators of disease progression and could aid ALS diagnosis.

2.4 Conclusion

The motivation for this thesis has been ~~an examination of~~ was to examine the mitochondrial genome in the mononuclear cells of amyotrophic lateral sclerosis (ALS) patients. The deviations in the gene expression could contribute to the mitochondrial energy deficiency and be an underlying cause of this lethal neurological disorder of

motor neurons. The mitochondrial DNA genes examined were ND2, ND4, COXIII and 12s rRNA. The qPCR mitochondrial gene expression study showed that mitochondrial DNA copy numbers in genomic DNA increased slightly after culturing, but cDNA consistently decreased after culturing. The results implicate deregulated mitochondrial bioenergetics as a characteristic of ALS. Future studies with reevaluated choice of housekeeping genes and potential temporal study of mitochondrial genome could lead to future elucidating the origins of ALS. The development of a disease model from induced pluripotent stems cells (iPSCs) have been explored as a potential treatment option for neurodegenerative diseases including amyotrophic lateral sclerosis. The reprogramming of mononuclear cells (MNC) to iPSCs would allow for a plausible design of personalized pharmacological treatment, as iPSCs differentiated to motor neurons, which could be delivered to the same patient and uniquely target the disease. The technique of transfection of mononuclear cells with non-integrating episomal plasmid has a great potential for successful reprogramming of mononuclear cells into iPSC however for future studies it can be improved by increasing the number of MNC before nucleofection.

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