MITOCHONDRIAL DNA REPLICATION IN CELLS SUFFERING FROM NUCLEOTIDE POOL IMBALANCES

MAIJA TOPPILA

Master’s Thesis
University of Eastern Finland
Department of Environmental and Biological Sciences
2016
Mitochondria are organelles which have an important role in cell energy metabolism and also in other cellular functions. Mitochondrial malfunctions such as oxidative phosphorylation dysfunction, iron sulfur cluster synthesis defects, redox imbalance or nucleotide pool imbalances can cause mitochondrial disorders.

Mitochondrial neurogastrointestinal encephalomyopathy syndrome (MNGIE) is a severe chronic and progressive disease with symptoms ranging from neurological and intestinal to muscular symptoms. It is caused by a mutation in the nuclear TYMP gene which encodes thymidine phosphorylase (TP). This mutation impairs the enzyme activity of TP and leads to accumulation of its substrates: thymidine and deoxyuridine. This causes imbalances in the nucleotide pools and as a consequence mtDNA copy number is reduced in one or more tissues due to stalled replication.

Currently, there is no cure for MNGIE. Most of the therapeutic strategies focus on removing deoxythymidine and deoxyuridine from the blood stream or reducing their effects. A long-term improvement is so far produced only by allogeneic hematopoietic stem cell transplantation. However, this treatment has many limitations such as finding suitable donors or a risk of graft-versus-host disease. To be able to avoid these risks, the researchers in the Erasmus Medical Center in Rotterdam investigate whether MNGIE could be treated by patient’s own modified bone marrow.

The purpose of this study was to test whether mtDNA replication stalls in MNGIE due to high thymidine levels and whether reintroducing an intact TYMP gene reduces thymidine levels and corrects the stalling. Additionally, the sensitivity of mtDNA replication to the elevated thymidine levels was compared between different tissues.

Different tissues from double knock-out mice and MEF cells were used in this study and analyzed with Southern blotting and 2D gel electrophoresis.

The results indicate that the elevated levels of thymidine lead to stalling of mtDNA replication. However, these results are only considered as approximate due to the large variation in the results. They are inadequate to answer whether the introduction of a functional TYMP gene is sufficient to correct this stalling but it seems that the treatment as such is not efficient enough.

The copy number levels of knock-out samples were elevated or at the same level compared to the wild type samples both in the brain and liver tissues. In the intestine the wild type and the knock-out samples had relatively similar mtDNA copy numbers. There was also a lot of variation in these results and a better data can hopefully be measured with qPCR.

The used methods needs to be remodeled before this study can be repeated to find out whether the reintroducing of TYMP can correct the replication stalling phenotype and how much of this gene needs to be transferred.
## CONTENTS

1 INTRODUCTION .................................................................................................................. 2

2 MITOCHONDRIA .................................................................................................................. 2

2.1 MtDNA ................................................................................................................................. 3

2.2 MtDNA replication ............................................................................................................... 4

3 MITOCHONDRIAL DISEASES ............................................................................................. 7

3.1 Mitochondrial Neurogastrointestinal Encephalomyopathy ............................................... 8

3.1.1 Characteristics of MNGIE .......................................................................................... 8

3.1.2 Therapeutic strategies under investigation .................................................................... 9

4 GOALS AND HYPOTHESES ............................................................................................... 11

5 MATERIALS AND METHODS ............................................................................................... 11

5.1 Used tissues and cells ......................................................................................................... 11

5.2 Cell culture .......................................................................................................................... 12

5.3 Extraction of mitochondria from tissues ............................................................................ 12

5.4 Extraction of mitochondria from MEF’s ............................................................................ 13

5.5 MtDNA extraction .............................................................................................................. 13

5.6 Total DNA extraction ........................................................................................................ 14

5.7 DNA copy number levels by Southern blot ....................................................................... 14

5.8 2D agarose gel electrophoresis ......................................................................................... 15

5.9 Antibody testing with BrdU labelling ............................................................................... 18

6 RESULTS ............................................................................................................................... 18

6.1 Relative replication levels in brain tissue .......................................................................... 18

6.2 Relative replication levels in liver tissue .......................................................................... 21

6.3 Copy numbers .................................................................................................................... 23

6.3.1 MtDNA copynumber in MEF-cells .............................................................................. 25

6.4 BrdU .................................................................................................................................. 26

7 DISCUSSION ......................................................................................................................... 26

8 CONCLUSION AND PERSPECTIVE .................................................................................. 28

9 ACKNOWLEDGEMENTS ....................................................................................................... 29

REFERENCES ............................................................................................................................ 29
1 INTRODUCTION

Mitochondria are organelles where oxidative phosphorylation takes place and most of the cell’s adenosine triphosphate (ATP) is formed. In addition to energy metabolism, mitochondria also have other significant functions in the cell such as synthesis of amino acids and hormones and apoptosis. Mitochondria have multiple copies of their own DNA which encodes components for protein complexes required for oxidative phosphorylation (Nelson & Cox, 2008).

Defects in the oxidative phosphorylation of the respiratory chain lead to disturbances in cell functions and ultimately to apoptosis (Nelson & Cox, 2008). Additionally, mitochondrial nucleotide pool imbalances or problems in mtDNA maintenance, for instance, lead to mitochondrial malfunctions (González-Vioque et al. 2011, Nunnari & Suomalainen 2012). Mitochondrial metabolism defects are also involved in many other human diseases such as cancers and metabolic disorders (Andreux et al. 2013). Mitochondrial diseases are caused by a mutation, either in their own mitochondrial DNA or in the nuclear DNA encoded genes with mitochondrial function, or due to improper regulation of these genes, for example, for epigenetic reasons (Taylor & Turnbull, 2005).

2 MITOCHONDRIA

Mitochondria are organelles with a double membrane where oxidative phosphorylation takes place and most of a cell’s adenosine triphosphate (ATP) is formed. In the oxidative phosphorylation electrons flow through an electron transport chain (complexes I-IV) and the protons are pumped from mitochondrial matrix to the space between the mitochondrial membranes. This creates an electrochemical gradient which causes protons to flow back to the mitochondrial matrix through ATP-synthase, giving energy for ATP production. In addition to energy metabolism, mitochondria also have other significant functions in the cell. These are, for example, synthesis of amino acids and hormones, apoptosis, intracellular signaling and calcium buffering (Nelson & Cox 2008).

According to the endosymbiotic theory the origin of mitochondria are in the α-proteobacteria that were capable of aerobic metabolism. The primitive anaerobic eukaryotes acquired the ability of ATP production through oxidative phosphorylation by establishing a symbiotic relationship with the aerobic bacteria. After a long evolution the most of the aerobic symbiont’s
genes were relocated to the nucleus and its functions were reduced to energy production and a few metabolic pathways. Thus the bacteria became mitochondria of the host cell (Nelson & Cox 2008, Gray et al. 1999).

2.1 MtDNA

The mammalian mitochondrial DNA (mtDNA) molecule is about 17 kb double-stranded circular chromosome (Cantatore & Saccone 1987). Mitochondria have multiple copies of mtDNA which encodes 13 polypeptides that are core components of protein complexes required for oxidative phosphorylation, two ribosomal RNA and 22 transfer-RNA. The nuclear DNA encodes all other genes necessary for the mitochondrial respiratory chain and other functions, such as proteins needed for transcription, translation and replication (Taylor & Turnbull 2005).

MtDNA is packed into nucleoids by proteins and can also form supercoiled monomeric circles, catenanes, oligomers and complex multimeric networks (Picture 1.) (Pohjoismäki & Goffart 2011). MtDNA has only one non-coding region (Picture 2) and thus it is a very tightly organized chromosome. Additionally, the most of the polypeptide and rRNA genes are separated by only one or more tRNA genes and a few non-coding nucleotides. The non-coding region (NCR) contains promoter regions for both strands (LHP, HSP), origins of replication (O_H, Ori-b), stretches of evolutionary conserved sequence blocks (CSB) and a replication-termination-associated sequence (TAS) (Picture 2.) (Holt et al. 2007).

![Picture 1. MtDNA can exist in monomeric open (OC), supercoiled (sc) circles, interconnected catenates (cat) with head-to-tail circular dimers (Pohjoismäki & Goffart 2011).](image)
4

2. The human mtDNA molecule is an about 17 kb double-stranded circular chromosome which is packed into nucleoids by proteins. The non-coding region contains promoters for heavy and light strands (HSP and LSP) and initiation origin for heavy strand replication (OH). The initiation origin for light strand (OL) is outside the non-coding region (Schon et al. 2012).

Mammalian mtDNA is usually inherited maternally i.e. from mother to offspring. During mitosis mitochondria and their genomes are divided randomly into daughter cells (Giles et al., 1980, Zeviani & Di Donato 2004).

2.2 MtDNA replication

Since mtDNA is isolated from the nucleus, it has its own transcription, translation and replication mechanisms. However, all replication proteins of mitochondria are encoded in the nucleus. Some of these replication factors remain still unknown, but polymerase $\gamma$, its accessory subunit POLG2, the DNA helicase Twinkle, the mitochondrial single-stranded DNA-binding protein, the RNA polymerase POLMRT, transcription factor TFAM and mTERFm have already been identified. Three different replication mechanisms have been found in mitochondria: two of them are found in most tissues and cultured cells and one mechanism is only been found so far in the human heart (Pohjoismäki & Goffart 2011).

In the strand-displacement model presented in 1972 the leading (heavy strand) and lagging (light strand) strands replicate at different times so that replication of the lagging strand starts
significantly later than the replication of the leading strand. According to this model the synthesis of the leading strand begins at the initiation origin called $O_H$ and the synthesis of the lagging strand at $O_L$ (Robberson et al 1972, Pohjoismäki & Goffart 2011).

According to the strand-displacement model the heavy strand replicates first, while the parental light strand creates a single-stranded stretch (Clayton 1982). A more recent model suggests that the lagging strand replication starts later than the leading strand replication but the parental lagging strand is covered with RNA molecules (Picture 3.) (Yasukawa et al. 2006). This creates double-stranded RITOLS intermediates. These at least 800 bp long RNA molecules can be synthesized by POLMRT on a single stranded DNA (Pohjoismäki & Goffart 2011). Alternatively long RNA molecules might attach to the single stranded DNA at the replication fork and be later on modified into short RNA pieces (bootlace model) (Reyes et al. 2013).

There is also another replication model in mammalian mtDNA. In the COSCOFA (conventional, strand-coupled Okazaki fragment associated) model the replication intermediates are double stranded DNA (Reyes et al. 2013). The initiation origin for COSCOFA replication can be in a large upstream of $O_H$ (Picture 3.). The replication progresses to both directions also using the Okazaki fragments. When one of the replication forks reaches the $O_H$ replication terminus, the fork regression is stopped and only the other replication fork proceeds undirectionally (Pohjoismäki & Goffart 2011).

The quantity of RITOLS and COSCOFA intermediates vary in different tissues. The RITOLS intermediates are usually found in cultured cells, liver and kidney, whereas COSCOFA intermediates are found in skeletal muscle and mouse heart (Pohjoismäki & Goffart 2011).
There are several replication mechanisms found in the mitochondria. A) In the RITOLS mechanism, the replication of the leading-strand initiates at the non-coding region and the replication of the lagging-strand initiates at the OL significantly later. The parental leading strand is covered with RNA molecules. This creates double-stranded RITOLS intermediates which can be synthesized by POLMRT on single stranded DNA. B) In the COSCOFA mechanism the replication strand-coupled bi-directional replication initiates at a wider origin zone (Ori-Z) downstream of the NCR. The replication is bi-directional and both stands are synthesized simultaneously. The replication continues unidirectionally after the first replication fork has reached the terminus in the NCR. C) When the replication forks meet at the NCR, they form four-way junctional molecules, which resemble recombination junctions (Pohjoismäki & Goffart 2011).

RDR (recombination-dependent replication) is also a possible mechanism for replication in the mitochondria. A strand invasion of the linear DNA template at a homologous sequence initiates the RDR (Picture 4). The template starts to elongate from the 3’-end while leading- and lagging-strands are synthesized. By using RDR, the junctional obstacles and torsional stress can be avoided and therefore this replication mechanism is found from complex mitochondrial structures in the adult human heart (Pohjoismäki & Goffart 2011, Pohjoismäki et al. 2009).
3 MITOCHONDRIAL DISEASES

Mitochondrial disorders result most often from oxidative phosphorylation dysfunction. Mitochondrial metabolism dysfunctions are also involved in many other human diseases - often associated with aging. Such diseases include, for example, cancers, metabolic disorders, and neurodegeneration (Zeviani & Donato 2004, Andreux et al 2013). There is also mitochondrial diseases caused by other malfunctions, for example, iron sulfur cluster synthesis defects, redox imbalance or nucleotide pool imbalances (Isaya 2014, González-Vioque et al 2011, Leloup et al. 2011).

Errors in the respiratory chain normally lead to disturbances in cell function and, finally, apoptosis. For this reason, mitochondrial diseases occur mostly in tissues with a high metabolism rate such as nerve, muscle and heart tissue and ocular endocrine organs and tissues (Keogh & Chinnery 2013).

Mutations in either mtDNA or nuclear genes that affect mitochondria can cause mitochondrial dysfunction and therefore mitochondrial disorders may be caused by mutations in either of these genomes. Thus, mitochondrial disorders can be inherited in autosomal dominant, recessive or X-linked way or they can be maternally inherited via mitochondria (Keogh & Chinnery 2013).

MtDNA mutations can be divided into large-scale deletions and inherited point mutations (Zeviani & Di Donato 2004). Point mutations in mtDNA can cause structural changes in the subunits of the respiratory chain proteins. If a point mutation is in a gene encoding for a tRNA, it may cause errors in the protein synthesis. Large-scale deletions may, on the other hand, remove one or more important genes for mitochondrial function (Keogh & Chinnery 2013). Mitochondrial diseases caused by mutations in the mtDNA are for example LHON (Leber...
hereditary optic neuropathy), KSS (Kearns-Sayre syndrome), PEO (progressive external ophthalmoplegia) and Pearson syndrome (Zeviani & Di Donato 2004). Mutations in the nuclear POLG or OPA1 genes cause a variety of mitochondrial diseases which are inherited either autosomal recessive or dominant way (Hudson & Chinnery 2006, Anon. 2009).

3.1 Mitochondrial Neurogastrointestinal Encephalomyopathy

3.1.1 Characteristics of MNGIE

Mitochondrial neurogastrointestinal encephalomyopathy syndrome (MNGIE) is a severe autosomal recessive disorder that is chronic and progressive. The symptoms can be leukoencephalopathy, loss of hearing and vision, peripheral nerve pain and severe gastrointestinal dysmotility that causes nausea, diarrhea, malabsorption and weight loss. MNGIE is caused by mutations in the TYMP gene, which encodes thymidine phosphorylase (TP). This mutation impairs the enzyme activity of TP and leads to accumulation of its substrates thymidine and deoxyuridine. This causes imbalances in the nucleotide pools and as a consequence the mtDNA replication is disturbed. The imbalance doesn’t affect the nuclear replication as much, as in the proliferating cells the nucleotide pools are rather high. Only mtDNA is replicating under low nucleotide pool levels, and there the imbalance has an influence. The defective replication of mtDNA causes reduction of mtDNA molecules and mtDNA damage such as deletions and point mutations (Hirano 2005, updated 2016, Torres-Torronteras et al 2014, Copeland 2012). The level of TP impairment defines the manifestation of the disease (Boschetti et al. 2014).

The reduction of mtDNA molecules is usually caused by mutations in genes that are somehow involved in the deoxyribonucleoside triphosphate (dNTP) metabolism. MtDNA is continuously replicated and therefore it requires a constant supply of nucleotides. The excess of dNTPs doesn’t affect the replication rate of mtDNA whereas the replication rate is limited by the amount of the dNTP which is available the least. The delay of mitochondrial DNA replication rate is likely not caused by excess of deoxythymidine triphosphate (dTTP) itself, but instead, the overload of dTTP produces a secondary deoxycytidine triphosphate (dCTP) depletion that stalls the mtDNA replication (Picture 5.) (González-Vioque et al 2011).
3.1.2 Therapeutic strategies under investigation

Currently, there is no cure for MNGIE (Boschetti et al. 2014). Most of the therapeutic strategies focus on removing deoxythymidine and deoxyuridine or reducing their effects. Since the thymidine circulates in the body removing it in one organ should affect the levels also in the rest of the body. There are attempts to reduce the excess thymidine levels by reducing renal reabsorption of thymidine by blocking the Na+/thymidine transporter, by dialysis and by enzyme replacement therapy (Hirano 2005, updated 2016). Some treatments are efficient but only for a limited time. For example, the peritoneal dialysis is used to lower thymidine levels.
but the effect lasts only for a few hours (Boschetti et al. 2014). Enzyme replacement therapies include allogeneic stem cell transplantation, carrier erythrocyte entrapped thymidine phosphorylase and platelet transfusion (Hirano 2005, updated 2016). One option for treating MNGIE patients may be an organ transplantation. Some studies suggest that a liver transplantation in MNGIE patients could provide an efficient treatment (Boschetti et al. 2014).

A permanent reduction in toxic levels of these nucleosides and a long-term improvement is so far produced by allogeneic hematopoietic stem cell transplantation (Hirano et al. 2006, Torres-Torronteras et al 2014). However, this treatment has many problems such as finding suitable donors, the procedure can be unsuccessful or the transplantation can cause a graft-versus-host disease. In addition, delayed diagnosis often leads to poor condition in MNGIE patients and therefore the mortality is high (Torres-Torronteras et al 2014).

As stated before, one limitation of hematopoietic stem cell transplantation is the difficulty of finding suitable donors. Researchers in the Erasmus Medical Center in Rotterdam have studied whether the patient’s own bone marrow could be modified in such manner that it would eventually cure or at least halt the disease. A similar therapy with donor bone marrow is currently used in treating MNGIE patients with quite efficient results (Hirano et al. 2006, Halter et al. 2011, Peedikayil et al. 2015). Therefore it is likely that this treatment with modified bone marrow could be at least as efficient as the current treatment. In addition, it would solve the difficulty of finding suitable donors as well as remove the possibility of the body’s rejection to the transplantation.

Murine models are used as models for MNGIE. In humans the deficient TP gene causes MNGIE. However, unlike human uridine phosphorylase (UP), murine UP cleaves also thymidine in addition to uridine. Therefore, a TP-UP double-knockout (TP(-/-) UP(-/-)) mice were generated to gain a proper clinical picture (Haraguchi et al. 2002).
4 GOALS AND HYPOTHESES

The purpose of this study was to test whether mtDNA replication stalls in MNGIE due to high thymidine levels and reintroducing an intact TP gene reduces thymidine levels and corrects the stalling. Also the sensitivity of mtDNA replication to the elevated thymidine levels was compared between different tissues. This was done since the symptoms of MNGIE are pronounced in the brain and the intestine but not in some other organs like liver or skin, for example. Different tissues from the TP-UP double knock-out mice and MEF cells were used in this study and analyzed with Southern blotting and 2D gel electrophoresis.

Hypothesis 1: MtDNA replication stalls in MNGIE due to high thymidine levels (or indirectly due to low dCTP levels).

Hypothesis 2: Reintroducing an intact TP gene somewhere in the body reduces thymidine levels and alleviates/corrects the stalling.

5 MATERIALS AND METHODS

5.1 Used tissues and cells

In this study the double-knockout mice were used as models for MNGIE. These mice had no functional genes for thymidine phosphorylase (TP) and uridine phosphorylase (UP). Bone marrow samples from these mice were modified with lentivirus vectors to reintroduce an intact thymidine phosphorylase gene. This therapeutic gene was a functional TP gene driven either by the phosphoglycerate kinase (PGK) promoter or the spleen focus forming virus (SFFV) promoter. The bone marrow cells of mice of the same strain were killed and the modified bone marrow was transplanted into these mice. Some mice, so called mock-treated mice, were treated similarly but they received bone marrow cells treated with empty vectors. All of these procedures were done by the group of Prof. de Coo in Rotterdam. Frozen liver, intestine, brain and muscle samples from healthy (wild type), double-knockout, treated and mock-treated mice were sent from Erasmus Medical Center (Rotterdam, Netherlands).
I analyzed four samples of each untreated wildtype mice, double knock-out mice deficient for TP and UP and mice transplanted with genetically modified bone marrow. Additionally, I analyzed two mice transplanted with mock-treated bone marrow. To analyze the effect of thymidine overload in a more simplified system also cultured MEF cells (mouse embryonic fibroblast) were used in this study.

5.2  Cell culture

MEF cells were cultured in DMEM with 4,5% glucose, 1 mM Na-Pyruvate, 2mM L-Glutamine with 10% fetal bovine serum (FBS) and 1x Penicillin/Streptomycin. They were cultured at 8,5 % level of CO₂, 100 % humidity and 37 ℃.

Some of the cells were treated with thymidine to create an artificial overload of thymidine in cells. For this purpose the MEF cells were arrested by changing the medium to low serum conditions (1% serum) for two weeks. While the control cells were kept in this medium without addition of thymidine, the cells with increased thymidine levels were created by addition of 40 μM thymidine to the culture medium for these two weeks.

5.3  Extraction of mitochondria from tissues

Frozen tissues were first washed in H-buffer (225 mM Mannitol, 75 mM Sucrose, 20 mM HEPES pH 7.4, 10 mM EDTA). All tubes, other equipment and used solutions were precooled on ice. Next, a small piece was cut from the tissue for total DNA extraction. The rest of the tissue was cut in small pieces and washed again with H-buffer to remove most of the blood. The pieces in H-buffer were then homogenized using a Dounce homogenizer using 10 strokes. Next, the samples were centrifuged two times for five minutes at 800 g at 4 ℃ to pellet unbroken cells and nuclei. The supernatant was centrifuged 5 minutes at 12 000 g at 4 ℃ to pellet mitochondria. The pellet was resuspended in H-buffer and centrifuged again.

The crude mitochondrial pellet was resuspended in H-buffer and overlayed over a two-step sucrose gradient (1,5 M/1 M sucrose in 10 mM HEPES, 10 mM EDTA pH 7,4) and centrifuged at 50 000g in a swing-out rotor (Beckman Avanti) for an hour at 4 ℃. After that the middle layer containing the mitochondria was removed, diluted in H-buffer and centrifuged at 12 000 g for 5 minutes. The resulting mitochondrial pellet was used for mtDNA extraction.
5.4 Extraction of mitochondria from MEF’s

The cells were detached from culture dishes by trypsinisation and then suspended in 10 ml medium for harvesting. Then cells were pelleted by centrifuging for 3 minutes at 300 g at 4 °C. Then the pellet was washed with cold PBS and recentrifuged, washed again with 0,1 x homogenization buffer and centrifuged. Cells were suspended in 0,1 x homogenization buffer and homogenized using a dounce homogenizer. 1/10 Vol of 10x homogenization buffer was added. Further preparation was similar to the extraction of mitochondria from tissues.

5.5 MtDNA extraction

The sucrose-purified mitochondria were resuspended in 1 ml H-buffer and 10 µl proteinase K (10 mg/ml) was added to degrade extra-mitochondrial enzyme contaminations that break down DNA. The solution was left to incubate for 10 minutes in room temperature and then the mitochondria were pelleted by centrifugation at 12 000 g for 5 minutes. Next, the mitochondria were lysed in lysis buffer (75 mM NaCl, 50 mM EDTA, 0,5 % SDS) for 20 min at room temperature and again 20 µl proteinase K was added to degrade proteins.

After that, mtDNA was extracted by phenol-chloroform extraction. 1 Vol. Phenol-chloroform mix (1:1) was added on top of the mitochondria solution. The samples were mixed carefully by manual shaking and then centrifuged for 5 minutes at 15 000 g. The upper water phase was transferred into a fresh tube. This was repeated approximately three times until no white interphase was formed. Finally, chloroform was added to the water phase, mixed, centrifuged and the upper phase was again transferred into a fresh tube.

Depending on the amount of mtDNA, either two sample volumes of 100 % ethanol (1 ml) or one volume of isopropanol (500 µl) and 1/10 volume of 3 M Na-Acetate (50 µl) was added into the solution. Then the samples were frozen at -80°C for 15 minutes or overnight in a regular freezer (-20°C). After freezing, the samples were centrifuged for 15 minutes at 16 000 g, washed with 70 % ethanol and recentrifuged. MtDNA was left to dry before dissolving the pellet in 50-200 µl 20 mM HEPES (pH 7,2).
5.6 Total DNA extraction

Pieces of sample tissues were cut in small pieces and homogenized with a small homogenizer (suitable for Eppendorf tubes). Next, the cells were lysed in 500 µl lysis buffer (recipe see above) and 20 µl proteinase K (10 mg/ml) was added. The cells were left to incubate at 37 °C in a turning wheel overnight. After incubation DNA was extracted with phenol-chloroform extraction and alcohol precipitation in similar way that was used when extracting mtDNA and then dissolved in 40-200 µl 20 mM HEPES (pH 7.2). The harvesting and washing of MEF cells for total DNA extraction was done like in the extraction of mitochondria from MEF’s. After that the protocol was similar to the total DNA extraction.

5.7 DNA copy number levels by Southern blot

Southern blot was used to determine the DNA copy number levels in tissues. The DNA concentrations from the total DNA samples were measured by using a Nanodrop ND-1000 spectrophotometer. 1-5 µg DNA per sample was digested with Hind III and digestion buffer that contained loading dye. The samples were loaded onto a 0,8% agarose gel containing 0,3 µg/ml ethidium bromide in TAE-buffer. The gel was run overnight at about 35 V.

After the electrophoresis the gel was washed two times in 0,2 M HCl (depurination solution) for 15 minutes and two times in denaturation solution (0,5 M NaOH and 1,5 M NaCl) for 20 minutes. The gel was transferred for blotting and DNA from the gel was blotted to Hybond XL nylon membrane by capillary blotting. Two wet filter papers, tissue papers and a weight were placed on top of the membrane. When the blotting was completed the membrane was neutralized by using 6xSSC and then crosslinked at 80 °C for two hours.

The hybridization buffer was preheated to 65°C as well as the hybridization oven. The membrane containing the sample DNA was prehybridized in Church’s hybridization buffer (7 % SDS, 0,25 M NaPO₄, pH 7,6) for 30 minutes.

Next, a mouse cytochrome B-probe (CytB, nts. 14783-15333 of mouse mtDNA) and a mouse 18S probe (nts. 23-527 of mouse 18s rDNA) were used in the radioactive labeling. 0,71 µl 18S-probe and 44,4 µl TE-buffer (10mM Tris pH 8,0 and 1 mM EDTA) or 2 µl CytB-probe and 43 µl TE-buffer were used for one labeling. First the probe was denatured at 95 °C and then snap cooled on ice. Then the probe was pipetted to a reaction tube (Amersham Rediprime II
DNA Labeling system, GE Healthcare) and 5 µl of [α-38P]dCTP was added to the mixture. The radioactive probe was incubated for 20 minutes at 37 ºC and then pipetted on G50-purification column (Mini Quick Spin Column, Roche). The column was centrifuged at 1600 g for four minutes, then the probe was incubated at 95 ºC for 5-10 minutes and finally added into the hybridization solution. The membrane was left to hybridize overnight. Both CytB and 18S probes were used separately to copy number membranes but only the CytB probe was used on 2D membranes.

When the hybridization was completed the membrane was washed for 5 minutes with washing solution (1xSSC, 0.1 % SDS) and then again three times for 20 minutes. Next, the membrane was transferred into a cassette (Kodax BioMax) with an autoradiography film (Carestream, BioMax MS Film, Maximum Sensitivity-Radioisotope) on top. If a cassette didn’t contain an intensifying screen itself, a separate screen was placed on top of the film. The cassette was placed in -80ºC for several days (depending on how strong the signal was) before developing the film.

5.8 2D agarose gel electrophoresis

2D agarose gel electrophoresis was used to determine relative replication level of mtDNA. About 5 µg of mtDNA was digested with 2 µl ClaI-enzyme, 5 µl 10 x digestion buffer that contained loading dye and water so that the final volume was 50 µl. The samples were left to digest at 37 ºC for at least 2 hours. Then 50 µl of phenol-chloroform mix was added on top, mixed briefly and centrifuged at 16 000g for 3 minutes. The yellow water phase was pipetted to a fresh tube and 5 µl of loading dye added. The first dimension was run in a 0,4 % ultrapure agarose gel in TBE-buffer at about 35 V overnight. The next morning the gel was stained with ethidium bromide. The gel was cut into slices so that there was one sample in each slice. The slices were placed sideways on a tray and a new gel was poured on a tray and left to solidify (Picture 6). The second dimension was run in a 0,95 % ultrapure agarose gel in TBE-buffer that was constantly recirculated. This time both gel and buffer contained ethidium bromide at 1ug/ml. The gel was run at high voltage in a cold room so that the gel didn’t warm up and the samples didn’t run too fast. After the electrophoresis the gel was blotted and hybridized as described above. Quantitative data was collected using a molecular imaginer and QuantityOne Software.
2D agarose gel electrophoresis. The first dimension is run in lower agarose concentration (0.4%) to separate different sized strands. These strands are cut into slices and are then run sideways in higher agarose concentration (Reyes et al 2007).

The fragments created by the restriction digest enabled the visualization of the replication intermediates separated by the 2D agarose gel electrophoresis (Picture 7. and 8.). The linear arc consist of linear mtDNA fragments. Fragments that are not replicating are in the 1n spot. If a molecule contained an intact replication bubble it migrates on the so-called bubble-arc. If an intermediate is cut in a way that it contains only part of the replication bubble it forms a fork structure and moves on the Y-arc. Molecules with two replication forks moving towards each other are named double-y-molecules and move within a zone next to the descending y-arc. An X-spike is formed by molecules containing crossover sites, e.g. recombinating molecules. When a replication intermediate is cut from the mtDNA molecule at the end of the replication, in a way that replication bubble is cut from both ends, it creates an X. Different mouse tissues have differences in their 2Ds: in liver tissue the Y-arc is prominent, while in brain tissue the X-spike is strong (Picture 8.).
Picture 7. The second dimension of 2D agarose gel electrophoresis separates different replication intermediates. They form the bubble-arc, the X-arc, the Y-arc and the linear arc (Picture from Anu Hangas)

Picture 8. Different replication intermediates are visualized in the second dimension of the 2D agarose gel electrophoresis. The X-spike is clearly visible in the brain samples.
5.9 Antibody testing with BrdU labelling

The MEF cells were cultured as mentioned before in 10 % FBS medium and later in 1% medium. 10 µl BrdU solution (10 mM dissolved in PBS) was pipetted on top of one petri dish. The cells were left to incubate with BrdU for 4 hours. Then the medium was sucked off and the cells were washed once with PBS. The cells were harvested in 1 ml PBS and spinned down. Then PBS was pipetted off and replaced with 500 µl of lysis buffer and 20 µ proteinase K (10 mg/ml). Next total DNA was extracted from the cells as described before.

Total DNAs from both BrdU treated and regular MEF cells were each dissolved in 200 µl of 20mM HEPES. 2µg BrdU labelled DNA and 2 µg not-labelled DNA were dropped onto a southern blot membrane and crosslinked in UV-light for 5 minutes. Then DNA was blocked in 4 % milk in TBST at 4°C. 2 ml of the 1st antibody solution (1:200 in 4% milk, Anti-BrdU mouse antibody Sigma B8434) was added onto the membrane and left to incubate for 1 hour. Then the membrane was washed 3 times for 5 minutes with TBST before adding 2 ml of the 2nd antibody solution (1:10 000 in TBST) and left to incubate for another hour. Then the membrane was washed 6 times for 10 minutes with TBST. 990 µl luminol and 10 µl enhancer were added on top and left to affect for a couple of minutes. Finally, the membrane was exposed to a film.

6 RESULTS

The relative replication levels of brain and liver tissues were determined using 2D electrophoresis Southern blot and phosphorimager quantification.

6.1 Relative replication levels in brain tissue

The relative replication levels in brain tissues were measured by comparing X-spikes to 1n-spots from 2D electrophoresis data.

The relative level of mtDNA replication in the knock-out mice was elevated compared to the levels in the wild type mice (Picture 9.). The treatment with either the viral promoter or the TP-promoter did not decrease the replication levels to normal values in brain tissues, although there was some lowering effect. On average the replication level in the brain tissue in TP-treated mice was lower than in SFFV-treated mice. However, the variation within sample groups was
very big, especially in the knock-out and the viral promoter samples, allowing no statistical analysis after quantification of four samples.

Picture 9. Relative level of replication in brain samples. The relative level of replication in knock-out mice was elevated compared to the levels in wild type mice. The treatment with either promoter did not decrease the replication levels to normal values.

Accurate replication differences were impossible to interpret with bare eyes and thus molecular imager and QuantityOne Software were used to get the results (Picture 10.).
Picture 10. 2D electrophoresis picture of wild type, knock-out and treated brain samples.
The transplantation without a therapeutic vector did not have a significant influence onto the replication level, indicating that the procedure itself does not significantly alter replication levels (Picture 11.). Hereby the alteration of results from treated mice can be considered to be caused by the different expression of TP in these mice and not by the transplantation procedure.

![Relative replication level in brain tissue](image)

Picture 11. The mock-treatment i.e. a transplantation without a therapeutic vector did not significantly change replication levels.

6.2 Relative replication levels in liver tissue

The relative replication levels in liver tissues were measured by comparing Y-arcs to 1n-spots (Picture 12.).

The relative replication levels in liver samples were quite equal in the wild type and the knock-out mice indicating that the disease does not change the level of replication in liver samples.

The mock-treated wild type mice had quite low levels of replication and thus the procedure itself did not alter replication levels either in the liver tissue. In contrast, the mock-treated knock-out mice had almost twice the amount of replication than the mock-treated wild type or the wild type mice. This could indicate that the mock-treatment in the knock-out mice caused some effect in the replication or, more likely, this level of replication should have been the result of the regular knock-out mice and the treatment didn’t cause any difference.
The replication levels were increased in treated mice and especially in the mice treated with the vector with the TP-promoter. The replication in the SFFV-treated mice wasn’t as high as it was in the TP-treated mice, but still higher than in the wild type mice. The results indicate that the treatment had not only been ineffective but also made things worse in liver tissue. Even though the variation within the sample groups was quite big, the pattern is still clear.

Picture 12. The relative level of replication in the liver samples. The relative replication levels were quite equal in wild type and knock-out mice and were increased in the treated mice.

Again accurate replication levels were measured with molecular imaginer and QuantityOne Software (Picture 13.).
6.3 Copy numbers

In the brain samples mtDNA copy numbers had increased in the knock-out and in the treated mice compared to the wild type mice (Picture 14.). The copy numbers were relatively at the same level in both treatments and also in the knock-out samples even though there is some dispersion in the results. In both cases of the mock-treated mice the copy number levels were even higher than in the knock-out or treated mice.
The copy number levels were increased in all cases compared to the wild type mice.

In the liver tissue the knock-out mice, the mock-treated and the treated mice had more mtDNA molecules than the wild type samples (Picture 15.).

In the intestine it appeared that the wild type and the knock-out mice had relatively similar mtDNA copy numbers (Picture 16.). On the other hand, the copy number levels were reduced in the other cases in the intestine.
25

Picture 16. Copy number levels in the intestine samples. The wild type and the knock-out mice had relatively similar mtDNA copy number levels whereas the levels were reduced in the other cases.

6.3.1 MtDNA copy number in MEF-cells

MtDNA copy numbers were elevated in case of the MEF-cells with extra thymidine (Picture 17.).

Picture 17. Copy number levels in the MEF-cells. The copy numbers were elevated in the MEF-cells with extra thymidine.
6.4 BrdU

Antibodies reacted only with BrdU-labelled DNA indicating that the antibodies were working as wanted and are effective antibodies to use in studies that require BrdU-labelling (Picture 18.). The results were visible already after 1 minute exposure to the film. After 5 minutes exposure, it was clear that the antibody was working properly. Due to time constraint this method was not used any further.

![Image](image.png)

**After 1 min.**  **After 5 min.**

Picture 18. Antibodies reacted only with BrdU-labelled DNA (marked with +). The result was visible already after 1 minute exposure to the film.

7 DISCUSSION

According to previous research, imbalanced mitochondrial nucleotide pools disturb the mtDNA replication or repair mechanisms or both (González-Vioque et al. 2011, Lara et al. 2007). In the case of MNGIE the excess thymidine causes secondary depletion of cytidine which might cause the stalling of mtDNA replication (González-Vioque et al. 2011). In this study mice with altered nucleotide levels were used as models for MNGIE. Due to previous studies it was expected that mtDNA replication was disturbed in the knock-out samples. It was also investigated whether a gene therapy reintroducing the defective TP gene had any effect. The replication levels were studied by using 2D-gel electrophoresis and Southern blot from liver and brain tissues of wild type, knock-out, treated and mock-treated mice.

The replication intermediate levels were elevated in the knock-out brain samples indicating that altered nucleotide levels in their mitochondria causes stalling in their mtDNA replication. Due to the slow or stopped replication processes, replication intermediates are accumulated in
the cell. It is also possible that cells even try to compensate the stalling by increasing mtDNA replication. In the knock-out liver samples the replication intermediate levels were not elevated. However, there was more replication in the mock-treated knock-out liver samples suggesting that extra thymidine might cause stalling also in the liver. In other tissues the mock-treated and the wild type or the knock-out samples didn’t vary a lot and therefore they can be considered as same type of samples. These results indicate that the first hypothesis was correct and mtDNA replication stalls due to altered nucleotide levels in mitochondria, but only in some tissues like brain, while others like liver seem unaffected.

According to the second hypothesis reintroducing an intact TP gene would reduce thymidine levels and correct the stalling. However, the replication levels were not decreased enough or at all in any treated samples. Thus, the second hypothesis is not correct and the treatment was insufficient. This could be because the nucleotide levels were altered wrongly or this therapy didn’t alter them at all. It can also be that a double knockout model cannot be fixed by TP reintroduction alone. This questions whether the mouse model is a good model for human MNGIE after all.

Generally there was large variation in the results. This could be due to unequal infection in tested mice or tissue samples were not uniform. In this study it would have been ideal if the results could have been collected from healthy wild type mice, see how its replication levels were altered due to knock-out of TP- and UP-genes and whether reintroducing these genes via bone transplantation corrected possible stalling. However, this was not possible for obvious reasons. The TP levels in the tissues of analyzed mice should have been measured in order to get more reliable results. These measurements were supposed to be made by the group in Rotterdam, but unfortunately, the TP measurements were not successful.

It is also possible that the used methods were not precise enough to get reliable data. Since the variation of the results were so big, they can be considered only as approximate.

It has been studied previously, that copy number levels are reduced at least in muscle tissues of several patients suffering from MNGIE (Hirano et al. 1994, Nishigaki et al. 2004). Thus, it was estimated that this would be the case also in this study. The copy number levels were studied by Southern blotting and radioactive labelling.

However, the copy number levels of the knock-out samples were elevated or at the same level compared to the wild type samples both in the brain and the liver tissues. The same outcome was observed in the MEF-cells and the cells with extra thymidine. In the intestine the wild type and the knock-out samples had relatively similar mtDNA copy numbers. The mock-treated and treated mice had more mtDNA molecules than the wild type samples in brain and
liver tissues, whereas in the intestine samples these levels were lower than in the wild type samples.

The copy number data is, however, also unreliable for similar reasons as were the replication levels. The used methods were probably not precise enough. Better data can hopefully be measured with qPCR. This was not done at this point because I had no access for functional qPCR machine. It should also be considered that maybe copy number levels are not reduced in the liver or brain tissue or at least not as much as in the muscle tissue.

The study could be continued by quantification of the replication speed by BrdU incorporation and South-Western blot. Due to time constraint, it was decided only to set up the South-Western blotting method. One of the difficulties in South-western blotting was to find a suitable membrane where both DNA and antibodies would bind properly. First a Nitrocellulose membrane (Protran) was tried but it didn’t bind the DNA efficiently. Therefore the Nylon membrane (Hybond XL) was used later on. Both tested antibodies worked fine on this membrane, so that the method is ready to be used.

8 CONCLUSION AND PERSPECTIVE

The results indicate that the elevated levels of thymidine lead to stalling of mtDNA replication. However, these results are inadequate to answer whether the introduction of a functional TYMP gene is sufficient to correct this stalling. The huge variability between samples prevents getting reliable data. It is necessary to get rid of the variation to be able to judge the efficacy of the method.

The method to transfer the TYMP gene needs to be sufficiently established that a predictable TYMP level can be reached. Then this study can be repeated to find out whether TYMP can correct the replication stalling phenotype and how much of this gene needs to be transferred.

MNGIE is a rare disorder which affects several systems and is often lethal. Since the diagnosis for MNGIE is ambiguous it often stays unrecognized or is misdiagnosed. Therefore it is difficult to determine its frequency in the population. While methods for diagnosis are improving it also puts pressure for researchers to create more efficient treatments for MNGIE. Hopefully, the introduction of a functional TYMP via modified bone marrow is the key to curing MNGIE, and a working solution can be established.
9 ACKNOWLEDGEMENTS

Special thanks for my instructor Steffi Goffart for patient and excellent guidance. I also want to thank my other instructor Jaakko Pohjoismäki, and Anu Hangas and Rubén Torregrosa for assistance in the laboratory.

REFERENCES

DNA replication proceeds via a ‘bootlace’ mechanism involving the incorporation of processed transcripts. – Nucleic Acids Research 41:5837-5850.


Keogh M., Chinnery P. 2013: How to spot mitochondrial disease in adults. – Clinical medicine vol 13, 1:87-92.


