An evaluation of mitochondrial DNA replication and transcription as well as the transcription of selected nuclear genes in *in vitro* models for OXPHOS deficiencies

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‘n Evaluasie van mitochondriale DNS replisering en transkripsie sowel as die transkripsie van geselekteerde kern-gekodeerde gene in *in vitro* modelle van OKSFOS defekte

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OPSOMMING

Defekte van die oksidatiewe fosforilerings (OKSFOS) sisteem, wat uit vyf ensiemkomplekse (I-V) bestaan, lei tot ‘n verskeidenheid van sellulêre gevolge. Dit sluit veranderde Ca^{2+} homeostase, verminderde adenosientrifosfaat produksie en verhoogde produksie van reaktiewe suurstofspesies (RSS) in. Een van die sekondêre gevolge van sulke defekte is die aanpassende transkripsionele reaksies van etlike mitochondria- en kern-gekodeerde gene wat by OKSFOS biogenese betrokke is. Daarbenewens word ook etlike ander gene, soos metallotioniene, wat by ’n verskeidenheid funksies betrokke is, differensiëel uitgedruk. In hierdie studie is twee hipoteses ondersoek: eerstens dat die verhoogde uitdrukking van metallotioniene (MTs), spesifiek MT1B en MT2A, in selle met ‘n kompleks I defek ‘n beskermende effek teen RSS-verwante gevolge van ‘n kompleks I defek tot gevolg het. Die tweede hipotese was dat gene wat by mitochondriale replisering en transkripsie betrokke is, differensiëel uitgedruk word in sellyne met OKSFOS defekte.

Eerstens is die uitdrukking en rol van MTs in ‘n *in vitro* model met ‘n kompleks I defek ondersoek. Die verhoogde uitdrukking van verskillende MT isovorme in die teenwoordigheid van die kompleks I inhibitor, rotenoon, is in HeLa-selle bevestig. In hierdie model het die ooruitdrukking van MT1B en veral MT2A isovorme teen RSS, opening van die mitochondriale binnemembraan deurlaatbaarheidsporie, apoptose en RSS-geïnduseerde nekrose beskerm. Dié data ondersteun die hipotese dat verhoogde uitdrukking van MT2A ‘n beskermende effek teen die fatale sellulêre gevolge van rotenoon-behandelde HeLa selle het.

Tweedens is die differensiële uitdrukking van selektiewe mitochondria- en kerngekodeerde gene, wat by OKSFOS funksie en regulering betrokke is, ondersoek. Twee eksperimentele *in vitro* modelle is in dié ondersoek ontwikkel en gebruik. Eerstens is ‘n tydelike ribonukleïensuur (RNS) ingreep van die NDUFS3 subeenheid van kompleks I in 143B-selle ontwikkel en gekarakteriseer. Daarna is die effek van die ingreep op verskeie biochemiese parameters (RSS en ATP vlakke), mitochondriale deoksiribonukleïensuur (mtDNS) kopiegetal, totale mitochondriale RNS vlakke, en RNS vlakke van verskeie kern- en mitochondria-gekodeerde transkripte wat vir strukturele- en funksionele proteïene kodeer, bepaal. Addisioneel, om die effek van stabiele OKSFOS defekte te bepaal, is stabiele RNS ingrepe van die NDUFS3 subeenheid van kompleks I asook die Rieske subeenheid van kompleks III ontwikkel en gekarakteriseer.

Die tweede hipotese, wat handel oor die effek van OKSFOS defekte op mtDNS replisering en transkripsie, kon egter nie duidelik deur die data ondersteun of weerspreek word nie. Uit die data is vasgestel dat ‘n OKSFOS defek, wat nie lei tot verhoogde ROS vlakke nie, nié die regulering van mtDNS replisering/transkripsie of kern-gekodeerde OKSFOS geentranskripsie betekenisvol verander het nie. Waar ‘n OKSFOS defek egter met verhoogde RSS vlakke gepaard gegaan het,

was sommige mitochondria-gekodeerde transkripte en regulerende kern-gekodeerde transkripte, naamlik ND6, D-lus, DNApolγ en TFB2M, verhoog. Nietemin, verhoogde RSS produksie in teenwoordigheid van ‘n OKSFOS defek is waarskynlik nie uitsluitlik verantwoordelik vir die reaksies van alle regulatoriese proteïene wat by mtDNS replisering/transkripsie *in vitro* betrokke is nie. Verder mag hierdie kompenserende regulering meer afhanklik wees van mtDNS transkripsie as mtDNS kopiegetal en die data dui aan dat TFB2M ‘n sleutel regulatoriese proteïen mag wees wat vroeg in die meganisme, voor enige ander regulatoriese proteïene geaffekteer word, betrokke is.

Sleutel terme: mitochondria, metallotioniene, OKSFOS defek, geenuitdrukking, mitochondria-kern kommunikasie

ABSTRACT

Deficiencies of the oxidative phosphorylation system (OXPHOS) that consists of five enzyme complexes (I-IV) lead to a diversity of cellular consequences. This includes altered Ca^{2+} homeostasis, reduced ATP production and increased ROS (reactive oxygen species) production. One of the secondary consequences of such deficiencies is the adaptive transcriptional responses of several mitochondrial- and nuclear-encoded genes involved in OXPHOS biogenesis. Additionally, several other genes that are involved in several other functions, such as metallothioneins (MTs), are differentially expressed. In this study we investigated two hypotheses: firstly, that in complex I deficient cells the increased expression of MTs, specifically MT1B and MT2A, has a protective effect against ROS-related consequences of a complex I deficiency. The second hypothesis stated that genes involved in mitochondrial replication and transcription are differentially expressed in OXPHOS deficient cell lines.

Firstly, the expression and role of metallothioneins (MTs) in an *in vitro* complex I deficient model was investigated. The increased expression of different MT isoforms in the presence of the complex I inhibitor rotenone in HeLa cells was confirmed. In this complex I deficient model over-expression of MT1B and especially MT2A isoforms also protected against ROS, mtPTP opening, apoptosis and ROS-induced necrosis. This data supports the hypothesis that increased expression of MT2A has a protective effect against the death-causing cellular consequences of rotenone-treated HeLa cells.

Secondly, we investigated the differential expression of selected mitochondrial- and nuclear genes involved in OXPHOS function and regulation. Two experimental *in vitro* models were developed and utilized in the study. Firstly, a transient siRNA knockdown model of the NDUFS3 subunit of complex I in 143B cells was developed, characterized and introduced. Then the effect of the knockdown on several biochemical parameters (ROS and ATP levels), mtDNA copy number, total mtRNA levels, and RNA levels of several nuclear- and mitochondrial-encoded transcripts encoding structural as well as functional proteins was determined. Additionally, to investigate the effect of stable OXPHOS deficiency, stable shRNA knockdown models of the NDUFS3 subunit of complex I, as well as the Rieske subunit of complex III were introduced and characterized.

The second hypothesis about the effect of OXPHOS deficiencies on mtDNA replication and transcription could not, without a doubt, be supported or contradicted by the data. It was determined from the data that an OXPHOS deficiency, which does not result in increased ROS levels, does not significantly affect the regulation of mtDNA replication/transcription or nuclear OXPHOS gene transcription. However, when OXPHOS deficiency was accompanied by increased ROS levels, some structural mitochondrial-encoded transcripts and regulatory nuclear-encoded

transcripts were up-regulated, specifically ND6, D-loop, DNApolγ and TFB2M. Nonetheless, increased ROS production in the presence of OXPHOS deficiency is probably not exclusively responsible for responses of all regulatory proteins involved in mtDNA replication/transcription *in vitro*. Additionally, this compensatory regulation might be more dependent on mtDNA transcription than mtDNA copy number, and the data showed that TFB2M might be a key regulatory protein involved early in this mechanism before any other regulatory proteins are affected.

Key terms: mitochondria, metallothioneins, OXPHOS deficiency, gene expression, mitochondrial-nuclear communication

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LIST OF ABBREVIATIONS AND SYMBOLS

LIST OF SYMBOLS

α	alpha
β	beta
ρ^o	rho0, mtDNA depleted cells
$\Delta\psi$	electrochemical gradient, membrane potential
I	Complex I, NADH:ubiquinone oxidoreductase
II	Complex II, succinate:ubiquinone oxidoreductase
III	Complex III, ubiquinol:ferricytochrome *c* oxidoreductase, *cytochrome* bc_1 *complex*
IV	Complex IV, ferrycytochrome:oxygen oxidoreductase, *cytochrome c oxidase*, COX
V	Complex V, F_1F_0-ATP synthase
#	number
µ	micro: 10^{-6}
n	nano:10^{-9}
e^-	electron
%	percent

LIST OF ABBREVIATIONS

2-DE/MS	two-dimensional electrophoresis/mass spectrometry
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
ANT	adenine nucleotide translocator
AP-1	activator protein 1
ARE	antioxidant response element
Asp	aspartic acid
ATP	adenosine triphosphate
b	base
BCA	bicinchoninic acid
β-2-MG	β-2-microglobulin
BNIP3	Bcl-2/E1B 19 kDa interacting protein
BN-PAGE	blue-native polyacrylamide gel electrophoresis
bp	base pair
C	cytosine

ºC	degrees centigrade
Ca	calcium
CaMK	Ca^{2+}/calmodulin-dependent kinase
Cd	cadmium
CDKN	cyclin-dependant kinase inhibitor
cDNA	complementary DNA
CFLAR	CASP8 and FADD-like apoptosis regulator
Cl	chloride
CPEO	chronic progressive external ophthalmoplegia
COX	ferricytochrome:oxygen oxidoreductase or *cytochrome c oxidase*
CREB	cAMP response element-binding
CSB	conserved sequence blocks
Ct	cycle threshold value
Cu	copper
cyt b	cytochrome b
Da	Dalton
DCFHDA	2',7'-dichlorofluorescin diacetate
D-loop	displacement loop
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DNApol γ	mitochondrial DNA polymerase γ
ds	double-stranded
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERRA	estrogen-related receptor a
ETC	electron transport chain
FSHMD	facio-scarpulohumeral muscular dystrophy
g	grams
x g	gravitational force of the earth (~$10 m.s^{-1}$)
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCE	hypertrophic cardiomyopathy and encephalomyopathy
H_2O	Water
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia-inducible factors
HMG	high mobility group
HPEM	highly progressive encephalomyopathy
hr	hour
H-strand	heavy strand
IT_L	light stand initiation of transcription site
JNK	c-Jun N-terminal kinases

kb	kilo base pairs (thousand base pairs)
KDa	kilo Dalton
KSS	Kearns–Sayre syndrome
LDD	Leigh-like disease
LHON	Leber's hereditary optic neuropathy
L-strand	light strand
LSP	L-strand promoter region
M	molar (moles/litre)
MAPK	Mitogen-activated protein kinases
MDMD	maternally transmitted diabetes mellitus and deafness
MEF-2	myocyte enhancer factor-2
MELAS	mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF	myoclonus epilepsy with ragged red fibres
min	minutes
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MNGIE	mitochondrial neurogastrointestinal encephalomyopathy
Mn	manganese
MRE	metal responsive element
mRNA	messenger RNA
MT	metallothionein
MT-1B	metallothionein isoform1B
MT-2A	metallothionein isoform 2A
mtDNA	mitochondrial DNA
MTF-1	metal-responsive element-binding transcription factor 1
mtPTP	mitochondrial permeability transition pore
mtRNA	mitochondrial RNA
mtSSB	mitochondrial single-stranded binding protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	number
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NARP	neurogenic muscle weakness, ataxia, and retinitis pigmentosa
ND	NADH:ubiquinone oxidoreductase subunit
nDNA	nuclear DNA
NFAT	nuclearfactor of activated T cell
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf	nuclear factor-erythroid 2 p45 subunit-related factor
NRF	nuclear respiratory factor
O_2	oxygen
$O_2^{\cdot -}$	superoxide
$OH^{\cdot}$	hydroxyl free radical
OXPHOS	oxidative phosphorylation

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	pyruvate dehydrogenase deactivation protein
PEO	progressive external ophthalmoplegia
PGC1	peroxisome proliferator-activated receptor gamma coactivator 1
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
POLRMT	mitochondrial RNA polymerase
PRC	polycomb repressor complexes
Q cycle	ubiquinone cycle
RNA	ribonucleic acid
ROS	reactive oxygen species
RP II	RNA polymerase II
MRP	mitochondrial RNA processing enzyme
RNS	reactive nitrogen species
rRNA	ribosomal RNA
16s rRNA	16 Svedberg units ribosomal RNA
12s rRNA	12 Svedberg units ribosomal RNA
SD	standard deviation
SDHA	succinate dehydrogenase complex subunit A
SDHB	succinate dehydrogenase complex subunit B
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
sec	seconds
Ser	serine
siRNA	small/short interfering RNA
shRNA	small/short hairpin RNA
TCA	tricarboxylic acid cycle
t-BHP	*t*-butylhydroperoxide
TFAM	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B1
TFB2M	mitochondrial transcription factor B2
TMRM	tetramethylrhodamine methylester
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UCS	citrate synthase activity
VDAC	voltage dependant anion channel, porin
v/v	volume per volume
Zn	zinc

CHAPTER ONE

INTRODUCTION

1.1. BACKGROUND

The majority of cellular energy, in the form of ATP (adenosine triphosphate), is produced by the mitochondrial oxidative phosphorylation (OXPHOS) system. This system is controlled on a genetic level by both the nuclear and mitochondrial genomes. The circular mitochondrial genome of ~ 16.6 kb encodes 22 tRNAs, 2 rRNAs and 13 subunits of complexes I, III, IV and V (Anderson *et al.*, 1981), while the rest of the proteins involved in OXPHOS, mtDNA maintenance and replication/transcription, translation, post-translational modification, transport and assembly, are encoded by the nuclear genome. The nuclear-mitochondrial communication, which forms the foundation for coordinate expression of these mitochondrial and nuclear encoded proteins, also relies on complex regulatory mechanisms (Cannino *et al.*, 2007). Recent studies have shown that OXPHOS disorders have an incidence of one in every 5 000 - 8 000, suggesting these deficiencies are one of the most frequent groups of metabolic disorders (Thorburn *et al.*, 2004; Cree *et al.*, 2009). These OXPHOS deficiencies also lead to a spectrum of clinical disease, from exercise intolerance to lethal multi-systemic disorders (Bénit *et al.*, 2009, Distelmaier *et al.*, 2009a).

Some of the consequences often, but not inevitably, associated with OXPHOS deficiencies include altered calcium homeostasis, decreased ATP production and increased reactive oxygen species (ROS) production (Ermak & Davies, 2002; Vives-Bauza *et al.*, 2006, Verkaart *et al.*, 2007a, Koopman *et al.*, 2007, Smeitink *et al.*, 2006, Brookes *et al.*, 2004; Turrens *et al.*, 1980; Dröse & Brandt, 2008). In turn, this could lead to oxidative damage to lipids, proteins and DNA, altered mitochondrial membrane potential and ultimately to apoptosis. Complex I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol cytochrome *c* reductase) are considered to be the main sources of superoxide radical production in the OXPHOS system (Turrens *et al.*, 1980; Dröse & Brandt, 2008), with the exception of a deficiency of the Rieske protein, which is part of the Qo site of complex III, which would result in limited superoxide production (Chen 2003). Deficiencies of

complex I, the first complex in the system, are some of those most frequently encountered (Loeffen *et al.*, 2000). Deficiencies of complex III are the least common (Bénit *et al.*, 2009), although a more significant involvement of complex III, with or without combination association of complex II (possibly through coenzyme Q deficiency), has been reported in South African paediatric mitochondrial disorders (Smuts *et al.*, 2010). Both complexes I and III are multi-subunit enzyme complexes encoded by both mitochondrial- and nuclear genomes (Hunte *et al.*, 2003; Bénit *et al.*, 2009; Carroll *et al.*, 2006; Hirst *et al.*, 2003).

Another consequence associated with OXPHOS deficiency is the differential expression of genes associated with OXPHOS function and regulation (see Chapter Two, as summarised in Reinecke *et al.*, 2009). It has been proposed that this differential expression might be due to increased ROS production leading to oxidative damage of mtDNA and mtRNA (Yakes & van Houten, 1997; Lee & Wei, 2005), and that once a certain threshold of reduced energy production has been reached, a compensatory mechanism that increases transcription of genes involved in OXPHOS is activated by stress-related retrograde effectors, and in particular, increased oxidative stress (Heddi *et al.*, 1999; Lee & Wei, 2005; Seidel-Rogol & Shadel, 2002; Miranda *et al.*, 1999; Davis *et al.*, 1996; Virbasius & Scarpulla, 1994).

In addition to the differential expression of several mtDNA-encoded OXPHOS transcripts and nuclear-encoded genes involved in OXPHOS function and regulation, a study of inherited complex I deficient fibroblasts during carbon source transition from glucose to galactose also showed induced expression of metallothioneins (MTs) (Van der Westhuizen *et al.*, 2003). Metallothioneins (MTs) belong to a super family of intracellular metal-binding proteins, present in virtually all living organisms. MTs are small proteins (6-7 kDa) with high cysteine content that can bind metals, particularly Zn and Cd, and scavenge ROS in a similar way to glutathione (Kägi *et al.*, 1974; Thornalley & Vašák, 1985). In humans, MT1 and MT2 isoforms are thought to be ubiquitously expressed, with MT2A appearing to be the predominantly expressed isoform in human cell lines (Palmiter *et al.*, 1992; Quiafe *et al.*, 1994; Hidalgo *et al.*, 2001; Heguy *et al.*, 1986). MT expression is regulated via *cis*-acting metal responsive elements (MREs) and the antioxidant

response element (ARE) is responsive to a wide range of effectors, including ROS (Andrews, 2000; Haq *et al.*, 2003).

Since the description of the first mitochondrial disorder by Luft *et al.* (1962) and especially since the elucidation of the genes involved in these highly heterogeneous disorders over the past three decades, significant progress has been made in the understanding of mitochondrial function and mitochondria-nuclear communication. This has been facilitated by unprecedented technological advances in the past decade to investigate genes (genomics), as well as transcriptional- (transcriptomics), translational- (proteomics) and metabolic- (metabolomics) profiles. As suggested by Smeitink *et al.*, (2006), such a systems biology approach will be required to understand the complexities of this highly heterogeneous group of disorders.

1.2. PROBLEM STATEMENT AND HYPOTHESIS

This study is a consequence of the advent of a systems biology approach to investigate gene expression in mitochondrial disorders, initiated in 2000 at the Nijmegen Centre for Mitochondrial Disorders, Radboud University Medical Centre, Nijmegen, The Netherlands. As briefly described in the previous section, one of the consequences of OXPHOS deficiencies is the differential expression of mitochondrial- and nuclear-genes involved in, amongst others, mitochondrial biogenesis and defense. In addition to the differential expression of several mtDNA-encoded OXPHOS transcripts and nuclear-encoded genes involved in regulation of mtDNA replication/transcription, a study of inherited complex I deficient fibroblasts during carbon source transition from glucose to galactose also showed induced expression of metallothioneins (MTs) (van der Westhuizen *et al.*, 2003). It is generally believed that MTs play an important role in metal ion homeostasis and prevention of oxidative damage in cells (Thornalley & Vašák, 1985; Andrews, 2000; Ebadi *et al.*, 2005), although a clearly distinctive role for MT isoforms remains unclear. In addition, the functionality of its increased expression in the context of complex I (and possibly other) deficiencies of the OXPHOS system remains to be established (Lindeque, *et al.*, 2010). An investigation into the expression of different MT isoforms in a characterized complex I deficient

model, and the functionality of the most important MT isoforms in such a model, might confirm if such a protective role of metallothioneins to prevent oxidative damage in complex I and other deficiencies does exist, and which isoforms are involved in this protective effect.

Additionally, from the limited data in a variety of disease models of OXPHOS deficiency, it is evident that the differential expression responses associated with OXPHOS deficiency are highly diverse and sometimes inconsistent (reviewed in Chapter Two, Reinecke *et al.*, 2009). The diversity in disease models amongst these reports, including the type of cell lines/tissues, phenotypes, mutations, experimental designs, and genetic background, prevents an accurate assessment among these models. In addition, key information on OXPHOS enzyme activities, which is necessary for making a comparison based on enzyme deficiencies, is mostly not present or is inconclusive. By determining whether increased ROS production and altered transcription of regulatory proteins involved in mtDNA replication/transcription and structural OXPHOS transcripts exist in a characterized OXPHOS deficient model, it might confirm that the differential expression is most probably due to increased ROS production as a mechanism of mitochondrial-nuclear communication to elicit a compensatory mechanism as proposed by Heddi *et al.* (1999). To further confirm this, it might be possible to reduce ROS production in an OXPHOS deficient model to see if the differential expression is still present. This was addressed by the Rieske subunit knockdown model that has been shown to not lead to increased ROS production and no significant differential expression.

In this study two hypotheses were investigated using *in vitro* models of OXPHOS deficiencies. In the first place it was hypothesized that the increased expression of MTs, and in particular MT1B and MT2A, in complex I deficient cells, has a protective effect against ROS-related consequences of complex I deficiency. The second hypothesis stated that genes involved in mitochondrial replication and transcription are differentially expressed in OXPHOS deficient cell lines. Although the investigation into the existing data pertaining to these hypotheses was done using human OXPHOS deficiencies in general, the model used to investigate these hypotheses was complex I deficiency and for the second hypothesis was also complex III.

1.3. RESEARCH AIMS AND METHODOLOGY

The rationale of the study was to evaluate mitochondrial DNA replication and transcription as well as the transcription of selected nuclear genes in *in vitro* models with OXPHOS deficiencies, to confirm and elucidate the hypotheses as described in Section 1.2.

Firstly the putative protective role of metallothionein expression in a characterized *in vitro* complex I deficient model was investigated. This was done by confirming the increased expression of different MT isoforms in the presence of the classic chemical inhibitor rotenone and secondly by determining the effects of over-expression of MT2A and MT1B isoforms on key parameters, including ROS production, ATP production, mitochondrial membrane potential and apoptosis in such a model. This would indicate whether different MT isoforms would lead to different levels of protection in this complex I deficient cell line and also if MT over-expression might not be neutralized by regulatory mechanisms etc. which could eliminate a possible therapeutic role for MTs. It would also have been possible to indicate a possible protective effect of MTs via inhibition of the adaptive response of MTs, possibly through gene knockdown or knockout models, however, it would not prove that over-expression of MTs might have a future therapeutic effect.

Secondly, the differential expression of selected mitochondrial- and nuclear genes involved in OXPHOS function and regulation was investigated in complex I and III deficiency *in vitr*o models. This was done by first introducing a transient siRNA knockdown model of the NUDFS3 subunit of complex I *in vitro* and to determine its effect on several biochemical parameters (ROS and ATP levels), mtDNA copy number, total mtRNA levels and RNA levels of several nuclear- and mitochondrial-encoded transcripts encoding structural as well as functional proteins. Additionally, to investigate the differential expression in the presence of long-term OXPHOS deficiency, stable shRNA knockdown models of complex I (NDUFS3 subunit) and complex III (Rieske protein) were introduced, in either glucose-rich or galactose-rich medium to better challenge mitochondrial energy metabolism.

1.4. STRUCTURE OF THESIS AND DECLARATION OF ORIGINALITY OF WORK

A review of OXPHOS gene expression and control in mitochondrial disorders, based on a published article (Appendix A) is presented in Chapter Two. Chapter Three details the investigation into metallothionein expression and its putative protective function in *in vitro* complex I deficiency by means of rotenone incubations, based on the published article presented in Appendix B. Parts of this chapter describe work published before (Reinecke 2004; Olivier, 2004), although significant additional data were generated for publication of this work (Reinecke *et al.*, 2006). The investigation into the consequences of the transient *in vitro* siRNA knockdown model of the NDUSF3 subunit of complex I on mitochondrial DNA replication/transcription is presented in Chapter Four. In Chapter Five, the consequences of stable *in vitro* shRNA knockdown models of complexes I and III on mitochondrial DNA replication/transcription are described. A significant part of the model development and subsequent experimental work was conducted by Dr. O. Levanets (Centre for Human Metabonomics, North-West University, Potchefstroom). At the same institution, enzyme analyses were done by Prof. F.H. van der Westhuizen. BN-PAGE and in-gel activity assays for the same study were performed by Dr. L. Nijtmans (Nijmegen Center for Mitochondrial Disorders, Radboud University Nijmegen Medical Center, Nijmegen). Data analysis and preparation of the manuscript for publication based on the complex III deficient model in glucose medium (Appendix C) were conducted by Fimmie Reinecke. Some of the information contained in Chapters Four and Five formed the foundation for an article submitted for publication for which the manuscript was prepared by Dr. O. Levanets (Appendix D). Recognition for assistance of other people is given in the Acknowledgements. The concluding remarks are presented in Chapter Six.

CHAPTER TWO

OXPHOS GENE EXPRESSION AND CONTROL IN MITOCHONDRIAL DISORDERS

The information contained in this chapter formed the basis for the published article in Biochimica et Biophysica Acta (2006), which is attached in Appendix A.

2.1. INTRODUCTION

The mitochondrial oxidative phosphorylation (OXPHOS) system, which produces the majority of cellular energy in the form of ATP, is controlled on genetic level by two distinct genomes: the circular mitochondrial genome (mtDNA) and the nuclear genome. The circular mitochondrial genome of ~16.6 kb encodes thirteen structural subunits of complex I, III, IV and V as well as 22 tRNA and two ribosomal RNA genes used for RNA translation (Anderson *et al.*, 1981). The nuclear genome encodes the additional genes required for mtDNA maintenance, replication, transcription, translation, post-translational modification, transport and assembly exclusively. In addition, the nuclear genome controls all other aspects of mitochondrial biosynthesis and function. Nuclear–mitochondrial communication is a highly complex process dominated by the nucleus (Cannino *et al.*, 2007).

A deficiency in mitochondrial function is caused by a dysfunction of one (or more) of hundreds of nuclear- or mitochondrial-encoded proteins. Over the past two decades, it has become clear that the interplay between the mitochondrion and nuclear genome affects mitochondrial disease expression, as evident in diseases that result from mutations in genes involved in the mtDNA replication machinery and in nucleotide metabolism. This impacts qualitatively and/or quantitatively on mtDNA, such as progressive external ophthalmoplegia (PEO), mitochondrial DNA depletion syndrome and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (Spinazzola *et al.*, 2005).

Mitochondrial interplay with the nuclear genome is also evident in the disorders of nuclear and mtDNA encoded subunits of the OXPHOS complexes. Primary deficiencies of the OXPHOS system impact directly on mitochondrial function and result in several disease phenotypes (Smeitink *et al.*, 2001). With recent advances in systems biology for investigating gene expression and function, key aspects of nuclear–mitochondrial communication in these deficiencies have been revealed. As with these deficiencies in which the mtDNA replication machinery has been primarily compromised, differential expression of mtDNA and nuclear OXPHOS genes occurs in cells with mtDNA or nuclear mutations of structural subunits of the OXPHOS system. Differential expression of OXPHOS and related genes has a significant impact on disease expression because of the importance of these genes in energy metabolism, which is compromised in these disorders. This article highlights these observations and investigates the underlying cellular mechanisms that control mitochondrial and nuclear OXPHOS gene expression.

2.2. CELLULAR BIOCHEMICAL CONSEQUENCES OF OXPHOS DEFICIENCIES

Oxidative phosphorylation and deficiencies thereof involve and modulate a great number of cellular functions and metabolic processes upstream and downstream of the five enzyme complexes. Moreover, in considering the effect of OXPHOS deficiencies, it is essential to recognise that deficiencies of the individual enzyme complexes may result in varied biochemical responses. This is evident from existing (but limited) reports of biochemical and gene expression responses to various deficiencies of OXPHOS as discussed in this article.

An initiator of the immediate and downstream consequences of OXPHOS deficiencies is the production of superoxide. Mitochondrial superoxide production can originate from the ineffective transfer of electrons through the various subunits of the electron transport chain (ETC; complexes I–IV) and the ineffective transfer of carriers (ubiquinone, cytochrome c) through the inner mitochondrial membrane. This can lead to the accumulation of electrons and excessive leaking to oxygen to produce ROS, particularly when there is an increased supply of reducing equivalents to the ETC. Complex I (at the bound flavine on the matrix side) and complex III (at the

ubiquinol oxidation side) are generally regarded as the main sources of superoxide radicals originating from the ETC (Turrens & Boveris, 1980; Dröse & Brandt, 2008). The percentage of oxygen converted in this way to ROS under steady state conditions is considered to be much less than the previously estimated 1 to 2% (Smeitink *et al*., 2006) and, in case of respiratory chain deficiencies, would quantitatively be dependent on the amount of electron transfer through the chain and the site of a deficiency within the chain. In fact, a significant part of mitochondrial superoxide production may also originate from the tricarboxylic acid cycle enzyme, α-ketoglutarate dehydrogenase (Starkov *et al*., 2004) and through a deficiency of the complex II subunit SdhB (Guzy *et al*., 2008). Superoxide can result in the generation of other ROS and nitrogen species (RNS), if not dismutated by superoxide dismutases on either side of the mitochondrial inner membrane (Mn and Cu/Zn) or by the radical scavenging effects of antioxidants (vitamins E and C), metallothioneins, or quinone reductase (Koopman *et al*., 2004; Reinecke *et al*., 2006). Hydrogen peroxide, which is formed by SOD, can be converted to water by catalase and glutathione peroxidase but can alternatively be converted to hydroxyl radicals by means of the Fenton reaction.

The damaging effects of ROS, RNS and particularly hydroxyl radicals on macromolecules have been extensively documented (Dröge, 2002; Jones, 2008) and, through oxidation of these molecules, have been shown to have a direct impact on the viability of genetic and functional molecules inside the mitochondrion and elsewhere in the cell. However, ROS and RNS also act as key messengers in signalling mechanisms that lead to the induction of genes often involved in maintenance and restoration of the cellular redox balance (Zhang & Gutterman, 2007; Genestra, 2007). They can also act more directly by altering protein function, such as the activation of uncoupling proteins in brown adipose tissue and the subsequent shift from mitochondrial coupling towards thermogenesis (Echtay *et al*., 2002).

Abundant evidence of increased superoxide production in OXPHOS deficiencies exists. However, in several reports increased superoxide production is not detected, or an increased superoxide level has no detectable effect on parameters associated with oxidative damage or

changes in metabolic homeostasis. Moreover, the origin of superoxide production is not clearly established; for example, ROS production is reported to occur in cell lines harbouring mtDNA mutations (Vives-Bauza *et al.*, 2006), as well as nuclear mutations of complex I (Verkaart *et al.*, 2007b; Koopman *et al.*, 2007). However, ROS production was not detected in a pathogenic mutation of the NDUFS4 subunit of complex I (Iuso *et al.*, 2006) or in HeLa cells containing no mtDNA (ρ^o) (Schauen *et al.*, 2006). ROS production in OXPHOS deficiencies is, therefore, not a generalised occurrence and depends on several factors, including the position and severity of the dysfunction, the source of production and the mechanisms that protect the cell against its possible harmful effects (Koopman *et al.*, 2007; Verkaart *et al.*, 2007a; Dassa *et al.*, 2008; Quinzii *et al.*, 2008).

Deficiencies of OXPHOS also result in other immediate and downstream metabolic, structural and functional effects. These effects are closely associated with mitochondrial dysfunction and are briefly described here. The nicotinamide dinucleotide (NAD) redox balance, which is converted to the reduced state in OXPHOS deficiencies, is a fundamental mediator of several biological processes, such as energy metabolism, calcium homeostasis, cellular redox balance, immunological function and gene expression (Munnich & Rustin, 2001; Ying, 2008). Not surprisingly, ATP production, and subsequently ATP/ADP homeostasis, is disturbed in OXPHOS deficiencies (Smeitink *et al.*, 2006). Cellular calcium handling also becomes disturbed during an increased oxidative state, with an influx of Ca^{2+} into the cytoplasm, nucleus and mitochondria (Ermak & Davies, 2002). This has an effect on cellular signalling events, where Ca^{2+} is often a key messenger, and more specifically mitochondrial Ca^{2+} loading, which is compounded by ROS, opens the mitochondrial transition pore, disrupts the inner membrane potential ($\Delta\Psi$) and increases cell death through apoptosis (Jacobson & Duchen, 2001; Brookes *et al.*, 2004). In complex I deficient fibroblasts, the depolarisation of $\Delta\Psi$ itself and the subsequent reduced supply of ATP to Ca^{2+}-ATPases leads to reduced cellular Ca^{2+} stores (Willems *et al.*, 2008).

The varied biochemical changes that occur in cases of OXPHOS deficiencies have a direct effect on cellular functions. Yet, they are also key underlying mediators of the (retrograde)

communication between the mitochondrion and the nucleus, which results in specific gene expression of both nuclear and mitochondrial genomes.

2.3. DIFFERENTIAL EXPRESSION OF MITOCHONDRIAL AND NUCLEAR GENES IN HUMAN OXPHOS DEFICIENCIES

The biochemical and structural changes that occur because of deficiencies of the OXPHOS system involve the nuclear and mitochondrial genomes. Differential expression of nuclear and mitochondrial genes has been reported for various *in vivo* and *in vitro* OXPHOS deficiency models. Initial reports using targeted investigations of RNA and protein expression have revealed the interaction between the nuclear and mitochondrial genome. However, the development of system biology tools over the past decade has rapidly expanded the number of cellular processes that are affected when a deficiency of the OXPHOS system occurs. In addition, these tools have shown that energy metabolism plays a major role in several related diseases that are not discussed in this article (Shutt & Shadel, 2007). Table 2.1 summarises the main findings of several studies investigating gene expression in the presence of mitochondrial disorders and highlights the expression of nuclear and mitochondrial OXPHOS and related genes. The diversity of the disease models used is evident; thus, except for perhaps the data on muscle in patients harbouring common mtDNA mutations and deletions, these profiles cannot be directly compared with confidence. Several factors that greatly affect gene expression are significantly different among these reports, including the type of cell lines/tissues, phenotypes, mutations, experimental designs and genetic background. In addition, key information on OXPHOS enzyme activities, which is necessary for making a comparison based on enzyme deficiencies, is mostly not present or inconclusive.

Initial investigations of the expression of targeted nuclear and mitochondrial encoded genes were conducted on the tissues of patients with mitochondrial DNA mutations, deletions, or depletion phenotypes (Heddi *et al.*, 1993; Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonod-Bidaud *et al.*, 1999). Expression of nuclear genes involved in mitochondrial (*ATPsynβ* and *ANT1/2*) and

glycolytic bioenergetics was often increased in muscle. However, many exceptions were observed, which included most of the various phenotypes and mutation types where expression of these genes was either decreased or similar to the controls (Heddi *et al.*, 1993; Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonod-Bidaud *et al.*, 1999). Marusich *et al.* (1997) report decreased expression of nuclear genes encoding four OXPHOS subunits—*COXVI*, *COXVa*, *SD30* and *SD70*—in mtDNA depleted fibroblasts. In addition, mitochondrial gene transcripts were generally found to be increased in these patients, although exceptions in a CIII deficiency (Collombet *et al.*, 1997) and a MELAS and KSS patient have been reported (Bonod-Bidaud *et al.*, 1999). Interestingly, among the cases of mtDNA mutations or deletions that mostly had a complex I and complex IV deficiency, a similar expression profile also occurred in a patient with complex II deficiency (Collombet *et al.*, 1997). With the one exception of a KSS patient (Bonod-Bidaud *et al.*, 1999), mtDNA/nDNA ratios were generally found to be decreased (Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonod-Bidaud *et al.*, 1999) and reduced processing (light/heavy strand) of mtDNA transcripts was observed (Heddi *et al.*, 1993).

It was proposed from these early observations that the general increased expression of selected genes involved in ATP synthesis was due to a compensatory mechanism that increases transcription of genes involved in energy production. It was further suggested that this increased transcription only occurs when a certain threshold of reduced energy production has been reached (Heddi *et al.*, 1993; Heddi *et al.*, 1999). This was evident from the study by Heddi *et al.* (1999), in which expression levels in different tissues of a patient identified with a MELAS mutation were measured. They found increased expression of all selected nDNA-encoded genes involved in OXPHOS and the glycolysis pathway in all tissues. All tissues with more than 88% mutant mtDNA showed increased mtDNA transcripts, while kidney tissue with only 73% mutant mtDNA showed decreased transcripts of cyt b, ND5/6, COXI and COXII; increased tRNA-Ser and -Asp; and unchanged 12S rRNA levels.

Table 2.1. Summarised findings of OXPHOS and other gene expression investigations of human mitochondrial disorders

Tissue	Phenotype/deficiency (genotype)	Technique	nDNA expression	mtDNA expression	Reference
Skeletal muscle	MERRF (mtDNA 8344), MELAS (mtDNA 3243), KSS (mtDNA del)	mRNA, Northern blot	• ATPsyn*β*, ANT1 ↑, GAPDH ↓ (MELAS, MERRF) • ATPsyn*β*, ANT, GAPDH ↓ (KSS)	• Transcripts ↑ • Processing (light/heavy strand transcript ratios) ↓	Heddi *et al*., 1993
Skeletal muscle (cultured)	CIII deficiency (mtDNA cytb), MELAS (mtDNA 3243, *CI+IV*), MELAS+CM (mtDNA 3243, *CI+IV*), CPEO+PM (mtDNA del, *CI+IV*), KSS (mtDNA del, *CI+IV*), CII deficiency (nuclear), CIV deficiency (nuclear)	mRNA, Northern blot	• ATPsyn*β*, GAPDH ↑ (MELAS, MELAS + CM, CPEO, CII deficiency) • ATPsyn*β*, GAPDH ↓ (CIII deficiency)	• Transcripts ↑ (excl. CIII deficiency) • Transcripts ↓ (CIII deficiency) • mtDNA/nDNA ↓	Collombet *et al*., 1997
Skeletal muscle, Heart muscle, Liver, Kidney, Brain	LHON (mtDNA 11778), NARP (mtDNA 8993), MELAS (mtDNA 3243, *CI+IV*), MERRF (mtDNA 8344/9344), MDMD (mtDNA del/dup), CPEO (mtDNA del), FSHMD (nuclear)	mRNA, Northern blot	• ATPsyn*β*, ANT1/2 ↑ (excl. MDMD) • Glycolytic/bioenergetic genes generally ↑	• Transcripts ↑ • mtDNA/nDNA ↓ (most tissues for MELAS)	Heddi *et al*., 1999
Skeletal muscle (cultured)	MELAS (mtDNA 3243), KSS (mtDNA del)	mRNA, Northern blot, Competitive RT-PCR	• ATPsyn*β* ↓ (MELAS)	• ND2 ↑ • mtDNA/nDNA ↓ (MELAS) • mtDNA/nDNA ↑ (KSS)	Bonod-Bidaud *et al*., 1999
Fibroblasts	mtDNA depletion (*CII+III, CIV*), RhoO (EtBr induced, *CII+III, CIV*)	Protein, Western blot	• COXVIc absent • COXVI, COXVa, SD30, SD70 ↓	Absent due to defect	Marusich *et al*., 1997
Skeletal muscle	Myopathy (mtDNA del), PEO (mtDNA 3243), MELAS (mtDNA 3243) (for all groups, varying deficiencies of combined CI, CI+III, CII+III and CIV are reported)	mRNA, Microarray (Affymetrix HG U133A, 22 283 oligonucleotide targets)	• OXPHOS structural genes ↑ (mtDNA del) • Genes involved in urea cycle/arginine catabolism ↑ • CDKN1A, -1C (cell cycle G1 arrest, DNA repair mediators) and other cell cycle regulators ↑ • CFLAR (anti-apoptosis) ↑ • PEX6 (peroxisomal biogenesis), MAOA (neurotransmitter catabolism) ↓ • RNA Pol II regulation ↑ (MELAS) ↓ (PEO) • Neurobiological structures, fatty acid oxidation, detoxification of H_2O_2, cell signalling ↓ (PEO)	Not reported	Crimi *et al*., 2005a
Cybrids	LHON (mtDNA 11778 and 3460), mtDNA depletion	mRNA, Microarray (Affymetrix U95Av2, 12 599 oligonucleotide targets)	• Respiratory chain genes, TCA and other aerobic bioenergetic pathways, Pol II promoter transcription and regulation, anti-apoptosis mostly ↑ (mtDNA depletion) • Aldose reductase (aldehyde reduction), integral membrane protein 2B (anti-apoptotic), H2A histone O (chromosome organization/biogenesis) ↑ (LHON cell line shared) • Scaffold protein TUBA (dynamin/actin regulatory), MTHFD (THF/purine metabolism), sialyltransferase 1 (sialic acid transfer/cell surface antigens/determinants), Raf1 (signal transduction/proliferation/differentiation/apoptosis), lipin 1, immunoglobin super family member 3 ↓ (LHON cell line shared)	Not reported	Danielson *et al*., 2005
Lymphoblasts	mtDNA depletion	mRNA, Microarray (Affymetrix HG U133A, 22 283 oligonucleotide targets)	• Lipid, amino acid metabolism, bioenergetics and transport, intracellular homeostasis, DNA/RNA binding, transcription, translation, redox balance, cell cycle control, growth arrest, signalling, apoptosis, DNA damage and oxidative stress protection ↑	Absent due to defect	Behan *et al*., 2005

Table 2.1 ***(Continued)***

Tissue	Phenotype/deficiency (genotype)	Technique	nDNA expression	mtDNA expression	Reference
143B cells (osteosarcoma)	mtDNA depletion	Protein, 2-DE/MS	• Respiratory chain complexes (excl. CII and CV) ↓ (not uniformly) • Mitochondrial translation apparatus ↓ • Mitochondrial transport systems ↓ • Catabolic energy metabolism ↓ • Hax-1 (anti-apoptotic), Smac protein (pro-apoptotic), rhodanese, hydroxysteroid dehydrogenase ↓	Absent due to defect	Chevallet *et al.*, 2006
Fibroblasts	CV deficiency (mtDNA 9205 and nuclear uncharacterised)	mRNA, Microarray (custom-made, 1632 oligonucleotide targets)	• OXPHOS structural genes for complex IV and V, cell growth, differentiation and transduction ↓ (CV nDNA defects) • Cell cycle regulation, Krebs cycle and gluconeogenesis, mitochondrial transcription regulation (TFAM, TFB1M), CytC, NFκB (apoptosis) ↓ (CV mtDNA 9205) • Branched chain amino acid and fatty acid oxidation, complex I structural genes and apoptosis ↑ (CV nDNA defects)	• MTATP6, MTATP8, MTCOX2 ↓ (CV mtDNA 9205) • ND1, ND2, ND4, ND4L ↑ (CV nDNA defects)	Cízková *et al.*, 2008
Fibroblasts (differentially cultured)	CI deficiencies (nuclear): LLD (NDUFS4, NDUFS7, NDUFS8), HCE (NDUFS2), HPEM (NFUFV1)	mRNA, Microarray (custom–made, 618 cDNA targets)	• Metallothioneins (ROS scavenging, heavy metal regulation), ATP1G1, heat shock proteins ↑ • Pro-apoptotic protein (BNIP3), pyruvate dehydrogenase de-activation (PDK1) ↓	• Transcripts ↓ (selected cell lines)	van der Westhuizen *et al.*, 2003

Respiratory chain enzyme deficiencies are shown in italics where reported. The following abbreviations are used: LHON (Leber's hereditary optic neuropathy); NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa); CPEO (chronic progressive external ophthalmoplegia); KSS (Kearns–Sayre syndrome); MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes); MERRF (myoclonic epilepsy and ragged red fibres); MDMD (maternally transmitted diabetes mellitus and deafness); FSHMD (facio-scarpulohumeral muscular dystrophy); LLD (Leigh-like disease); HCE (hypertrophic cardiomyopathy and encephalomyopathy); HPEM (highly progressive encephalomyopathy); 2-DE/MS (Two-dimensional electrophoresis/mass spectrometry).

A more detailed overview of expression profiles in patients with OXPHOS deficiencies was obtained in recent times using micro-arrays (van der Westhuizen *et al.*, 2003; Crimi *et al.*, 2005b; Crimi *et al.*, 2005b; Behan *et al.*, 2005; Cízková *et al.*, 2008; Danielson *et al.*, 2005). For example, the differential expression of several genes in the muscle of patients with common mitochondrial DNA mutations (A3243G MELAS/PEO and 4977 bp deletion) that lead to varied combined deficiencies of OXPHOS enzymes, excluding complex II, are reported (Crimi *et al.*, 2005b). Many genes showed induced expression in all patients in the form of urea cycle/arginine metabolism; anti-apoptotic factor; CFLAR; and selected cell cycle regulators, including cyclin-dependant kinase inhibitor (CDKN), which is involved in G1 arrest and DNA repair. Only a few genes showed

decreased expression in all patients. Significantly, it was shown that some genes were differently expressed in the MELAS and PEO patient subsets, which contained the same mutation but had varied levels of combined enzyme deficiencies, even within phenotype groups. These differently expressed genes include those involved in RNA polymerase II regulation, which were increased in the MELAS subset but decreased in the PEO subset, and genes involved in fatty acid oxidation, hydrogen peroxide detoxification, cell signalling and the development of neurobiological structures. Increased expression of nuclear encoded OXPHOS genes were observed only in the mtDNA macro-deletion subset of patients and it is striking to note that the enzyme deficiencies within this patient group were varied but similar to the other phenotypes. Although this is contrary to initial reports on similar patient tissues (Heddi *et al.*, 1993; Heddi *et al.*, 1999; Collombet *et al.*, 1997) in which general increased expression is reported for one nuclear OXPHOS gene, *ATPsynβ*, differential expression of OXPHOS genes was not associated to mtDNA mutations (LHON 11778 and 3460) in cybrids but rather strongly associated to mtDNA depletion (Danielson *et al.*, 2005). In Danielson *et al.* (2005), the depletion process of mtDNA had a significant effect on genes involved in mitochondrial bioenergetics pathways, which included increased expression of seventeen genes involved in OXPHOS. Supporting observations have been reported in ρ^o lymphoblasts (Behan *et al.*, 2005). In 143B (osteosarcoma) cells, however, conflicting reports indicate either the decreased expression (Chevallet *et al.*, 2006) or unaffected expression (Duborjal *et al.*, 2002) of OXPHOS genes in mtDNA depleted cells.

Differential expression of nuclear encoded structural OXPHOS genes is mostly not reported in micro-array data sets (from which it is assumed they are unaffected) in which deficiencies originate from either mitochondrial or nuclear mutations and appear to be exclusively associated with mtDNA depletion. In fact, Cízková *et al.* (2008) reported a decreased expression of complex IV and V genes in fibroblasts of isolated complex V deficient patients harbouring nuclear mutations. In Chevallet *et al.* (2006), differential levels of decreased respiratory complex subunits, translation apparatus (particularly mitochondria ribosomal proteins) and ion and protein import systems, such as membrane proteins, were found in 143B ρ^o cells when compared to wild-type cells. The

decreased levels of subunits of respiratory complexes were not significant or uniform (CII and CV subunits remained unchanged), indicating that some stable sub-complexes can survive in ρ^o mitochondria. It was suggested that this is because some sub-complexes have other unknown functions or because they are important for mitochondrial stability, or else because of unregulated coordinated nuclear transcription.

Similarities in the differential gene expression of ρ^o lymphoblasts (Behan *et al.*, 2005) and cybrids (Danielson *et al.*, 2005) have been reported. Increased expression of the genes involved in mitochondrial energy metabolism, including TCA cycle and ETC, in addition to transcription regulation occurred in these cell lines. Dissimilarities were observed in the induction of anti-apoptotic factors in cybrids, while several pro-apoptotic factors were increased in lymphoblasts. This again demonstrates the cell-specific regulation of gene expression and indicates that, in the case of apoptosis, the energy pathway predominance of the cell type can direct apoptosis induction (Li *et al.*, 2003).

Comprehensive expression profiles of nuclear encoded OXPHOS deficiencies of the OXPHOS system are limited, including only a comparison of expression under defined energy source changes in isolated complex I deficient fibroblasts (van der Westhuizen *et al.*, 2003) and, recently, in nuclear encoded complex V deficiency (Cízková *et al.*, 2008). In both these cases as well, similarities and marked variations of expression profiles were detected, even in patients that harboured the same mutation. Furthermore, no correlation could be made with the levels of enzyme deficiency. Significant increased expression of the ROS scavenging and metal regulating family of proteins (metallothioneins) and decreased expression of pro-apoptotic protein (BNIP3) and pyruvate dehydrogenase deactivation protein (PDK1) occurred in complex I deficient cells when culture conditions were changed from glucose to galactose, in order to challenge oxidative energy production (van der Westhuizen *et al.*, 2003). In selected patients and notably in the patient with the most severe deficiency, significantly decreased expression of mtDNA transcripts occurred. However, increased expression of mtDNA transcripts was detected in nuclear encoded complex V

deficient fibroblasts (Cízková *et al*., 2008). This was accompanied by increased expression of fatty acid catabolism, complex I structural genes and apoptosis, while decreased expression of nuclear complex IV and V structural genes, cell growth, differentiation and transduction were reported. In the same report, and in contrast to the reports referring to mitochondrial DNA mutations and deletions mentioned previously, mtDNA mutations of complex V resulted in decreased expression of genes of the TCA cycle, cell cycle regulation, mitochondrial transcription and apoptosis.

In addition to the differential expression of several mtDNA-encoded OXPHOS transcripts and nuclear-encoded genes involved in mtDNA replication/transcription, a study of inherited complex I deficient fibroblasts during carbon source transition from glucose to galactose also showed induced expression of metallothioneins (Van der Westhuizen *et al*., 2003). Metallothioneins (MTs) belong to a super family of intracellular metal-binding proteins, present in virtually all living organisms. MTs are small proteins (6-7 kDa) with highly conserved high cysteine content (typically 23-33 %) and lack of aromatic and hydrophobic amino acid residues. They can bind metals, particularly Zn, Cu and Cd through thiolate bonds and scavenge ROS in a similar way to glutathione (Kägi *et al*., 1974; Thornalley & Vašák, 1985). In humans, MT1 and MT2 isoforms are thought to be ubiquitously expressed, with MT2A appearing to be the predominantly expressed isoform in human cell lines (Palmiter *et al*., 1992; Quiafe *et al*., 1994; Hidalgo *et al*., 2001; Heguy *et al*., 1996). MT expression is regulated via *cis*-acting metal responsive elements (MREs) and an antioxidant response element (ARE), is responsive to a wide range of effectors, including ROS (Andrews, 2000; Haq *et al*., 2003). Although a clearly distinctive role for MT isoforms remains unclear, it is generally believed that MTs play an important role in metal ion homeostasis and prevention of oxidative damage in cells (Thornalley & Vašák, 1985; Andrews, 2000; Ebadi *et al*., 2005).

It is thus evident from studies of differential expression in mitochondrial disorders that there is great variation in the expression of both nuclear and mitochondrial genes. For OXPHOS genes in particular, the variation in expression also occurs under steady state levels over a more than two-fold range between various tissues and cells and of different sources (Duborjal *et al*., 2002).

This is an important observation, as the varying levels of steady state expression are similar to what is often regarded as 'differential expression' when pathology is investigated. In the limited published data of a highly varied group of patient cell lines and enzyme deficiencies, induced expression of genes involved in energy metabolism occurs in most of the cases. However, the diversity of expression of these genes and apparent lack of correlation with the type and level of OXPHOS enzyme deficiency strongly underscores the significant influence of genetic make-up in cellular response.

2.4. REGULATION OF NUCLEAR OXPHOS GENE EXPRESSION

Nuclear gene expression of OXPHOS and other genes involved in mitochondrial function and protection is controlled by retrograde (mitochondria-to-nucleus) signalling mechanisms. These signalling pathways are modulated in part by metabolites controlled by the mitochondrion, including Ca^{2+}, ROS and ATP. The interplay between these metabolites in the mitochondrion and their control of the mitochondrial permeability transition pore has previously been reported on (Brookes *et al.*, 2004; Willems *et al.*, 2008; Duborjal *et al.*, 2002; Szabadkai, 2008). Much less is known, however, about the downstream signalling mechanisms of these retrograde effectors in eukaryotes. Calcium-mediated signalling can involve one or more of several pathways, including activation of calcineurin (an activator of NFAT and NFκB), Ca^{2+}-dependant PKC, JNK/MAPK and CaMK IV (and CREB) pathways (Biswas *et al.*, 2005; Newsholme *et al.*, 2007). An extensive number of enzymes and other proteins involved in cell signalling are targets of ROS or are sensitive to redox state changes. These include phospholipases A2, -C and -D; tyrosine phosphatases; guanylyl cyclase; ion and calcium channels; AP-1 and NFκB transcription factors; several protein kinases; HIF-1α; and the JNK/MAPK pathways that activate, amongst other, nuclear factor-erythroid 2 p45 subunit-related factors 1 and 2 (Nrf1 and -2), which have similar but distinct functions in the expression of antioxidant defence and xenobiotic-metabolizing genes containing one or more antioxidant responsive elements (ARE) (Guzy *et al.*, 2008; Genestra, 2007; Dassa *et al.*, 2008; Ohtsuji *et al.*, 2008). Evidence also indicates that increased oxidative stress is involved in the expression of the nuclear respiratory factor-1 (NRF-1, unrelated to Nrf), which is a

key transcription factor in the expression of several genes involved in mitochondrial function (Miranda *et al.*, 1999; Suliman *et al.*, 2003). NRF-1 activation and increased cytochrome c expression have also been associated with the activation of the nuclear-localised, AMP-activated protein kinase (AMPK) as a result of, among others, decreased ATP/AMP ratio (Bergeron *et al.*, 2001).

Gene expression data from several of the studies presented in Table 2.1 support the possibility that regulation of OXPHOS genes may be co-regulated. Recently, Van Waveren and Moraes (2008) have shown that not only OXPHOS genes are co-expressed, but also that subunits within OXPHOS complexes are co-expressed (van Waveren & Moraes, 2008). This co-expression is statistically associated with a selection of *cis*-acting elements in human OXPHOS gene promotors, which include well-known elements found in OXPHOS genes or related genes; NRF-1; NRF-2; and the less specific elements ERRA, SP1, MEF-2, YY1 and CREB (van Waveren & Moraes, 2008; Scarpulla, 2006; Kelly & Scarpulla, 2004; Lee & Wei, 2005). Van Waveren and Moraes (2008) suggest that these factors can act independently or synergistically, in order to allow co-regulation and with other factors can lead to diversity in expression. An example of this diversity and tissue-specific expression has been described for complex IV (COX) expression in muscle (Ramachandran *et al.*, 2008). In mammals, COX may contain ubiquitous liver (L) and heart/muscle-specific (H) isoforms for subunits COX6a and COX7a (Scarpulla, 2006). The promotor regions of these genes lack NRF sites but contain conserved myosyte enhancer factor 2 (MEF2) elements. Expression of MEF2A is induced by NRF-1 over expression and, with PGC1α (a co-activator that also binds PPARα), results in the induction of muscle-specific gene expression of COX (Ramachandran *et al.*, 2008; Gleyzer *et al.*, 2005).

2.5. MITOCHONDRIAL DNA TRANSCRIPTION AND REPLICATION, REGULATION, DAMAGE AND REPAIR

The mechanism involved in the transcription and replication of mtDNA is well documented (Shadel, 2008; Asin-Cayuela & Gustafsson, 2007; Falkenberg *et al*., 2007) and, as is evident from the report summary in Table 2.1, clearly involved in expression of OXPHOS disorders. The 1.1 kb displacement-loop (D-loop) region is the only non-coding region where mtDNA replication and transcription is initiated from. The initiation of mtDNA synthesis is dependent on the initiation of transcription of the L-strand. Transcription of the L-strand begins at the transcription initiation site (IT_L), which is part of the L-strand promoter region (LSP) and proceeds until the region of the conserved sequence blocks (CSB I, II, III) are reached (Taanman 1999). Here, cleavage of the newly synthesised RNA sequence with RNase MRP (mitochondrial RNA processing enzyme) yields a primer for mitochondrial DNA polymerase γ (DNApol γ) to catalyse H-strand DNA synthesis and this region is thus where transition of RNA synthesis to DNA synthesis occurs (Taanman, 1999; Falkenberg *et al*., 2002).

In order for successful initiation of transcription and replication of mtDNA to take place, the presence of several nuclear-encoded trans-acting transcription factors are necessary. Firstly, TFAM must bind to and unwind double-stranded mtDNA at the enhancer site upstream from the promoter region. TFAM, which contains two high mobility group (HMG)-box domains, contributes to mtDNA maintenance and regulation of transcription and replication. Due to its abundance and its ability to bind mtDNA without sequence specificity, it can wrap mtDNA completely into a stable nucleoid structure (Fisher, 1988; Takamatsu, 2002). The common high mobility group domain can weakly bind up to 20 bp DNA at specific binding sites (Wolffe, 1994). This unwinding of the double-stranded mtDNA by TFAM enables either TFB1M or TFB2M to bind to the promoter region (Falkenberg *et al*., 2002; Gaspari, 2004), which in turn enables mitochondrial RNA polymerase (POLRMT) to bind and initiate transcription. Both TFB1M and TFB2M are expressed ubiquitously, although as described before, distinct roles for these two transcription factors are now proposed

(Cotney, 2007; Falkenberg *et al.*, 2002; Gaspari, 2004). The stabilization of the D-loop region, where initiation of transcription starts, and maintenance of mtDNA are assisted by mitochondrial single-stranded binding protein (mtSSB) which binds single-stranded mtDNA (Takamatsu, 2002). The expression of these proteins also seems to be directly involved in regulation of mtDNA copy number (Schultz, 1998).

In normal tissue, mitochondrial DNA copy number varies relative to oxidative capacity and energy needs (Lee & Wei, 2005). These variations may be markedly affected in OXPHOS deficiencies and reports show either a decreased (Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonon-Bidaud *et al.*, 1999) or increased (Bonon-Bidaud *et al.*, 1999) mtDNA/nDNA ratio, assuming that no mutations in replication/transcription genes exist in these patients. It has been proposed that mitochondrial gene expression is regulated not only by nuclear encoded transcriptional or post-transcriptional mechanisms, but also by the mtDNA copy number of the cell itself (Williams, 1986; Kaufman *et al.*, 1996). The mechanism for this is unclear, although several of the regulatory proteins involved in mtDNA transcription/replication (TFAM, POLRMT, mtSSB) are co-ordinately expressed with changes in mtDNA copy number (Seidel-Rogol & Shadel, 2002; Virbasius & Scarpulla, 1994; Davis *et al.*, 1996). Miranda *et al.* (1999), however, demonstrate induced expression of NRF-1 and TFAM mRNA in ρ^o HeLa cells that correlated with ROS levels. Increased expression of these factors is also found in OXPHOS deficient human cells, in aged skeletal muscle (Lee & Wei, 2005) and in HeLa cells treated with menadione that leads to increased superoxide formation (Miranda *et al.*, 1999). Another key regulator of mtDNA maintenance and copy number is Twinkle helicase (Tyynismaa *et al.*, 2004), although very little is known about its regulation. Notably, the regulation of mitochondrial DNA polymerase, DNApolγ, appears not to be affected by mtDNA levels or its maintenance and is expressed at levels sufficient to support variation among tissues (Schultz *et al.*, 1998; Davis *et al.*, 1996).

Knowledge of the regulation of nucleus-to-mitochondria (anterograde) signalling that controls these events is still largely lacking. Mitochondrial transcription involves binding of

mitochondrial RNA polymerase on three possible promotor sites, in association with TFAM and one of two transcription factor B paralogues, TFB1M and TFB2M (Shadel, 2008). These transcription factors have distinct roles *in vivo* and it is proposed that TFB2M is primarily involved in transcription and transcription-primed replication, while TFB1M over expression does not affect these processes but still increases mitochondrial biogenesis (Cotney *et al.*, 2007). Furthermore, over expression of TFB2M induces TFB1M expression, which suggests that there is a retrograde signalling pathway that co-ordinately expresses these transcription factors (Cotney *et al.*, 2007). It is interesting to note that the expression of these controlling factors of mtDNA transcription also contains the NRF-recognition sites that are *trans*-activated by the PGC1 family co-activators, PGC1α and PRC (Gleyzer *et al.*, 2005; Virbasius & Scarpulla, 1994). These controlling elements are thus shared between the expression of both nuclear and mitochondrial encoded OXPHOS genes.

It is well known that methylation of DNA plays an important role in epigenetic events: DNA-methyltransferases (DNA-MTase) catalyse the addition of a methyl group to a cytosine ring in CpG dinucleotides leading to 5-methylcytosine, which is generally associated with reduced gene expression. This occurs either by blocking binding of transcription factors, binding transcription repressors, or changing chromatin structure (Singal & Ginder, 1999). For the mitochondrion evidence of DNA methylation is lacking. However, TFB1M and TFB2M are homologues to rRNA methyltransferases and the role of TFB1M in ribosome biogenesis is proposed (Asin-Cayuela & Gustafsson, 2007; Seidel-Rogol & Shadel, 2002; Cotney *et al.*, 2007; Matsushima *et al.*, 2005). Although methylation and other epigenetic events play an important role in nDNA transcription regulation and maintenance, information on the occurrence and role of these factors in mtDNA expression and maintenance needs further study.

Damage to mtDNA is often highlighted as a factor affecting expression of mtDNA genes and thus contributing to secondary consequences of OXPHOS deficiencies. Considering its structure, maintenance and close localisation to sources of oxidative damage, this may well be a significant factor in disease expression. Owing to its close proximity to the site of mitochondrial

ROS production and comparatively less efficient mtDNA damage repair, mtDNA is more sensitive to oxidative damage than nDNA (Yakes & van Houten, 1997). It has also been demonstrated that oxidative damage is more likely to occur in the controlling D-loop region (Lee & Wei, 2005; Mambo *et al*., 2003). The compounding factors for oxidation of mtDNA are the close proximity of metal ions that act as catalysts and ROS damage to OXPHOS complexes that result in secondary ROS (Yakes & van Houten, 1997). The lack of protective histones around the mtDNA is also suggested to contribute to the sensitivity of the mtDNA to oxidative damage, although the presence of regulating proteins, such as TFAM, on mtDNA may have a protective effect against ROS damage (Fisher & Clayton, 1988; Takamatsu *et al*., 2002). It has now been established that mtDNA damage is primarily repaired through the ATP-dependent base excision repair pathway (Mandavilli *et al*., 2002; Grishko *et al*., 2001; Bogenhagen, 1999; Croteau & Bohr, 1997; Bohr, 2002).

The factors regulating mtDNA replication and transcription are thus highly diverse and tightly controlled by mitochondria–nucleus signalling. In addition, more immediate factors such as ROS, metabolic regulation and defence mechanisms that determine levels of oxidative stress and possibly epigenetic factors, control expression and maintenance. These factors are frequently evaluated separately. Considering the diverse expression profiles that were reported for mtDNA in OXPHOS deficiencies, as well as the regulation of the factors controlling its expression, the interplay of all these factors need to be evaluated to obtain a better understanding of how the mitochondrion responds to OXPHOS deficiencies.

2.6. CONCLUSION

The complex and almost unpredictable nature of disease phenotypes associated with OXPHOS deficiency has been a considerable impediment in the characterisation and treatment of OXPHOS deficiencies. The effect of OXPHOS deficiencies on mitochondrial and nuclear DNA expression and regulation has been investigated in several studies in an attempt to clarify the adaptive responses in OXPHOS disease phenotypes. These include genes involved in mtDNA maintenance, uncoupling, biogenesis, defence mechanisms, apoptosis and metabolic regulation. A

striking observation from these investigations is the great variation that exists in the differential expression of both the nuclear and mitochondrial DNA, which prompted this review of expression profiles and the mechanisms involved.

As summarized in Figure 2.1, the immediate (such as ROS and ATP) and secondary (such as Ca^{2+}) consequences of OXPHOS deficiencies are key mediators in retrograde signalling events that induce expression of nuclear OXPHOS and other genes involved in various cellular processes. Many of the signalling elements and promotor binding sites that control expression of these nuclear genes have now been identified and demonstrate that, in the case of OXPHOS and mtDNA maintenance genes, coordinate (ROS and Ca^{2+} sensitive) expression occurs. The origin and role of ROS is diverse, as its function in the oxidation of macromolecules, including mtDNA and RNA, also contributes to the way mtDNA transcription and replication occur. An aspect that needs to be investigated further is the occurrence and possible role of epigenetic events in the mitochondrion, which may, similar to nuclear DNA, have a marked effect on the expression and maintenance of mtDNA.

Although current data of the expression of genes involved in energy metabolism in OXPHOS deficiencies exhibit diverse profiles and (often) inconsistencies, it is clear that the expression of these genes contributes to the disease expression of OXPHOS deficiencies. In order to obtain an improved understanding of the intricate consequences and adaptive responses in OXPHOS disorders, the expression of nuclear and mitochondrial genes needs to be evaluated holistically, in combination with the signalling processes and metabolites involved and in well-defined disease models. Such an investigation poses a significant challenge, but recent developments in systems biology technologies may soon overcome any difficulties posed.

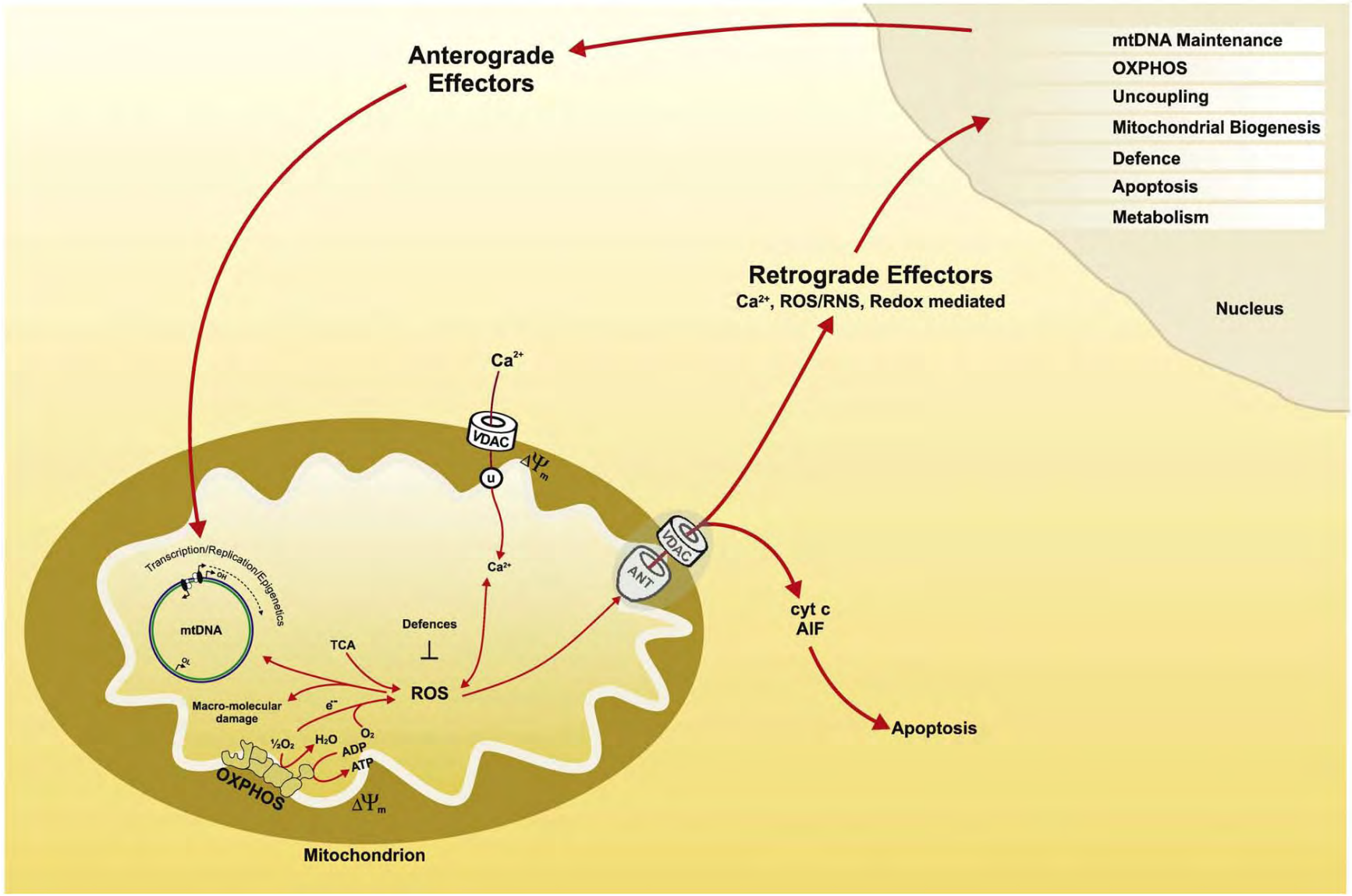

Figure 2.1. Summarized display of mitochondria–nucleus interactions that control the expression of OXPHOS and related genes. Changes in homeostasis of ROS, Ca^{2+} and ATP/ADP control the mitochondrial permeability transition pore (including ANT/VDAC). Such changes can lead to the induction of apoptosis through the intrinsic apoptosis pathway either directly or through secondary signalling pathways (retrograde effectors; see text for details), expression of nuclear genes involved in OXPHOS, and other mitochondria-related functions. Replication and transcription of mtDNA are mainly controlled by the nuclear encoded elements but are also affected indirectly by retrograde signalling and directly by epigenetic factors.

CHAPTER THREE

METALLOTHIONEIN-2A EXPRESSION IS INDUCIBLE AND PROTECTS AGAINST ROS-MEDIATED CELL DEATH IN ROTENONE TREATED HELA CELLS

The information contained in this chapter forms the basis for the published article in the Biochemical Journal (2006), which is attached in Appendix B. It also contains, to a limited extent, information of two former publications (Reinecke, 2004; Olivier, 2004).

3.1. INTRODUCTION

The production of ATP through the process of oxidative phosphorylation (OXPHOS) involves the successive transport of electrons through four mitochondrial enzyme complexes. The first of these complexes, NADH:ubiquinone oxidoreductase (complex I, E.C. 1.6.5.3), is assembled from 45 subunits of bi-genomic origin (Carroll *et al*., 200). Deficiencies of this complex are amongst the most common OXPHOS deficiencies (Loeffen *et al.*, 2000) and may lead to a diversity of disease expression phenotypes (Smeitink *et al*., 2001). The excessive formation of reactive oxygen species (ROS) as a contributing factor to the pathology of this deficiency has been well established along with several other biochemical consequences, including loss of ATP production, loss of mitochondrial membrane potential, calcium regulation and apoptosis (Barrientos & Moraes, 1999; Li *et al*., 2003; Vazquez-Memije, 1996; Visch *et al*., 2004; Moudy *et al*., 1995).

Recent studies using either inherited or rotenone induced complex I deficient cell lines have indicated that several nuclear and mitochondrial genes are differential expressed in this disorder (van der Westhuizen *et al*., 2003; Heddi *et al*., 1999; Collombet *et al*., 1997). Among these, expression of metallothioneins (MTs) was induced in inherited complex I deficient fibroblasts during carbon source transition from glucose to galactose (Van der Westhuizen *et al*., 2003). Although it

was suggested that MT expression may impart a protective effect in complex I deficiency, the functionality of its expression in the context of complex I and possibly other deficiencies of the OXPHOS system remains to be established. MTs are small proteins (6-7 kDa) with high cysteine content that can bind metals, particularly Zn and Cd, and scavenge ROS in a similar way to glutathione (Kägi *et al.*, 1974; Thornalley & Vašák, 1985). MT expression is regulated via *cis*-acting metal responsive elements (MREs) and an antioxidant response element (ARE), both located in the proximal MT promoter and is responsive to a wide range of effectors, including ROS (Andrews, 2000; Haq *et al.*, 2003). In humans, MT1 and MT2 isoforms are thought to be ubiquitously expressed, with MT3 and MT4 only selectively expressed in neurons and squamous epithelial cells, respectively (Palmiter *et al.*, 1992; Quiafe *et al.*, 1994; Hidalgo *et al.*, 2001). MT2A appears to be the predominantly expressed MT isoform in human cell lines *in vitro*, including HeLa cells (Heguy *et al.*, 1986). Although a clearly distinctive role for MT isoforms remains unclear, it is generally believed that MTs play an important role in metal ion homeostasis and prevention of oxidative damage in cells (Thornalley & Vašák, 1985; Andrews, 2000; Ebadi *et al.*, 2005).

In light of the responsiveness of MTs to oxidative stress and generally protective role associated to MTs against reactive oxygen species, we hypothesized that MT expression would be responsive to a deficiency of complex I and, furthermore, that MTs may be involved in the pathology of such a deficiency. Although MT expression may be responsive to disruption of a number of mitochondrial functions that leads to oxidative stress, we focused on its responsiveness to an induced defect of the first component of the OXPHOS system, i.e. complex I, using rotenone. We investigated the expression of the predominant forms of MT, i.e. MT1 (isoforms A and B) and MT2A, in rotenone treated HeLa cells against several control interventions, including metals, ROS producing *tert*-butylhydroperoxide (*t*-BHP) and the cytochrome c reductase inhibitor, myxothiazol. To evaluate the role of MT expression in complex I deficiency, we investigated the effect of over expressed MT2A and MT1B on key parameters, including ROS production, ATP production, mitochondrial membrane potential and apoptosis in rotenone treated HeLa cells.

3.2. EXPERIMENTAL

3.2.1. Materials

HeLa cells were purchased from the National Repository for Biological Materials of the National Cancer Association of South Africa. Tissue culturing reagents were obtained from Gibco, Invitrogen, Co., Auckland, New Zealand. The pIRESneo2 expression vector was obtained from Clontech, BD Biosciences, Mountain View, CA, USA, whilst all restriction endonuleases were purchased from Fermentas, Vilnius, Lithuania. M-MLV reverse transcriptase, random hexamer primers and the Apo-ONE Homogeneous Caspase 3/7 Assay kit were acquired from Promega Madison, WI, USA. The QIAzol lysis reagent was purchased from Qiagen, Hilden, Germany. The XtremeGENE Q2 transfection reagent and Cell Death Detection ELISAPlus from Roche, Penzberg, Germany. The iQ SYBR Green Supermix was acquired from Bio-Rad, Hercules, CA, USA and the probes, 2',7'-dichlorofluorescin diacetate (DCFHDA), tetramethylrhodamine methyl ester (TMRM) and Mitotracker Green were obtained from Molecular Probes, Eugene, OR, USA. All other reagents, including ATP assay reagents were obtained from Sigma Chemical Co., St Louis, MO, USA. MT cDNA clones with accession numbers as indicated in the text were obtained from The Resource Center of the German Human Genome Project (Berlin, Germany).

3.2.2. Cell culture and rotenone treatment

HeLa cells were cultured at 37 ºC and 5% CO_2 in a humidified incubator. The culturing medium, DMEM, was supplemented with 2 mM L-glutamine, 5% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin. For rotenone and myxothiazol treatments the medium of separate culture monolayers that were approximately 90% confluent were supplemented with inhibitors ranging between 0 and 2500 nM. Ethanol or dimethylsulfoxide content, which was used as solvents for inhibitors were kept constant at 0.1%. Incubations were carried out for 24 hr. When required, cells were collected by trypsinization unless otherwise stated and washed twice with PBS before analyses.

3.2.3. Enzyme assays

Rotenone-sensitive NADH:ubiquinone oxidoreductase (complex I) and antimycin A sensitive cytochrome c reductase (complex III) activities were measured in enriched mitochondrial preparations isolated from 2 x 10^8 cells as described before (Rahman *et al.*, 1996). Enzyme activities were normalized to citrate synthase activity (Robinson *et al.*, 1987). Protein content in these and other preparations was determined using the BCA method (Smith *et al.*, 1985).

3.2.4. Metallothionein RNA expression analyses

Rotenone and myxothiazol incubations were performed as described in the previous paragraph. In addition, as controls for MT expression, treatments with $CdCl_2$ (12.5 µM) and $ZnCl_2$ (250 µM) were performed. To increase ROS levels in cells, *t*-BHP at concentrations of 0.5, 0.8 and 1.0 mM were included and incubated for 3 hr before cells were harvested. Total RNA was isolated from 2 x 10^6 cells using QIAzol reagent according to the manufacturer's instructions and the RNA integrity was verified by agarose gel-electrophoresis and ethidium bromide staining. RNA (3 µg) was reverse transcribed with 200U M-MLV reverse transcriptase in a volume of 40 µl using 0.5 µg random hexamer primers. In addition to MT transcripts, several other (housekeeping genes) transcripts were evaluated for suitability as normalization controls. The primers for real-time PCR (in 5'-3' notation) for the various genes (with GenBank accession numbers in brackets) are as follows: GAPDH (NM_002046), β-actin (NM_001101), β-2-microglobulin, (NM_004048) and RNA polymerase II (X63564) were used as reported before (Radonic *et al.*, 2004); 18S rRNA (X03205) forward primer GTGCATGGCCGTTCTTAGTT, reverse primer CGGACATCTAAGGGCATCAC; MT1A (NM_005946) forward primer TCCTGCAAATGCAAAGAGTG, reverse primer TTCCAAGTTTGTGCAGGTCA; MT1B (NM_005947) forward primer GAACTCCAGGCTTGTCTTGG, reverse primer GATGAGCCTTTGCAGACACA; MT2A (NM_005953) forward primer TCCTGCAAATGCAAAGAGTG, reverse primer CAGGTTTGTGGAAGTCGCGT.

Real-time PCR was performed using an iCycler iQ (Bio-Rad) in a final volume of 20 µl using SYBR Green for detection. The PCR reaction consisted of 10 µl iQ SYBR Green Supermix, 500 nM of forward and reverse primers and 75 ng of cDNA (3 ng for 18S rRNA primers). The protocol included an initial denaturation step (3 min at 95 °C) followed by 35 cycles of denaturation at 95 °C for 20 sec, primer annealing at 60 °C for 10 sec, extension at 72 °C for 20 sec and an additional step at 82 °C (84 °C for 18S rRNA primers) with a single fluorescense measurement. A final extension at 72 °C for 5 min followed by a melting curve analysis (55-95 °C with a heating rate of 0.5 °C per 5 sec and fluorescent measurement every 5 sec) concluded the run. All samples were amplified in triplicate and the mean value was used for further calculations. Every assay included a no-template control, five serial dilution points (in steps of five-fold) of a cDNA mixture and each of the test cDNAs. Mean results (Ct values) from the iCycler iQ Real-time Detection System (iCycler iQ Real-time Detection System Software, version 3.0, BioRad, Hercules, CA) were analyzed by Statistica Version 7 software (StatSoft, Tulsa, OK) and BestKeeper software tool (24). PCR efficiency for each primer set was calculated by serial dilutions method using the REST software tool (Pfaffl *et al.*, 2002). The relative expression quantities for each sample were calculated by the comparative Ct method and gene expression stability was analyzed using the GeNorm software tool (Vandesompele *et al.*, 2002).

3.2.5. Metallothionein protein analyses

For MT protein analysis, the same interventions were performed as described for RNA analysis. After collection, cells were sonicated on ice for 3 bursts of 3 sec. MT content was determined in the 13 000 x g supernatants of homogenates by a highly sensitive radioimmunoassay (RIA) as previously described (Gasull *et al.*, 1993). This antibody fully cross-reacts with MT1 and -2 isoforms, but not with MT3, and has been validated for human MT2A (generously provided by Dr. Milan Vasak, Switzerland).

3.2.6. Metallothionein over expressing HeLa cell lines

The cDNA encoding human MT1B (GenBank accession no. NM_005947) was amplified using 5'-CCTAGGAACTCCAGGCTAGC-3' as a forward primer and 5'-AAAGAATGTAGCAAACCGGTC-3' as a reverse primer. Human MT2A (GenBank accession no. NM_005953) cDNA was amplified with 5'-GCGAACCCGCGTGCAACCGGTCCC-3' as forward primer and 5'-CAGGTTTGTGGAAGTCGCGT-3' as reverse primer. After confirming the sequences the MT1B and MT2A cDNAs were cloned into the *Eco*RV / *Pin*AI and *Eco*RV / *Eco*RI sites of the pIRESneo2 mammalian expression vector, respectively. Expression with this vector is driven by the cytomegalovirus (CMV) major immediate early promoter. After verifying the sequence of the constructs, HeLa cells were transfected with the MT expression constructs as well as the base vector, pIRESneo2, using X-tremeGENE Q2 transfection reagent according to the manufacturer's instructions. Cell lines successfully transfected with pIRESneo2-MT1B, -MT2A and pIRESneo2 constructs were denoted as MT1B-, MT2A over expressing cells and control cells, respectively. Selection of transfected cells were performed with 1 mg/ml geneticin in addition to the standard medium supplements. After a three week selection period the standard culturing medium contained 200 µg/ml geneticin throughout all subsequent incubations and analyses. The presence of the MT cDNAs was confirmed by PCR. Briefly, pIRESneo2 specific primers, 5'-TAATACGACTCACTATAGG-3' (forward) and 5'-GCCCTAGATGCATGCTCG-3' (reverse) were used to amplify the cDNAs using isolated DNA from clones. DNA was isolated by phenol/chloroform extraction and ethanol precipitation based on the procedure originally described by Maniatis *et al.* (1982). The presence of the correct length amplicons were used to confirm the presence of the cDNAs and MT RNA and protein expression were evaluated as described in the previous two sections.

3.2.7. ROS production

Cell lines were seeded in microtiter plates at 2 x 10^4 cells per well and treated with rotenone, myxothiazol and metals as described before. Incubations with *t*-BHP (0.5 to 1.0 mM) were used to induce additional ROS production. The fluorescent probe, DCFHDA (10 µM), was

used to measure ROS production essentially as described before (Wang & Joseph, 1999). Fluorescence (excitation at 485 nm and emission at 530 nm) was measured and the mean of eight samples was used in data analysis and expressed relative to protein content.

3.2.8. Cell viability assay

Cells were seeded into microtiter plates at a density of 2 x 10^4 cells. Rotenone and *t*-BHP incubations were performed as describe before. As positive control for loss of cell viability, a 30 min incubation with 6% (v/v) acetic acid was included. Cell viability (MTT assay) was determined by measuring formazan formation as described before (Denizot & Lang, 1986). The mean of three replicates was calculated and expressed relative to protein content.

3.2.9. Membrane potential assessment

The potentiometric fluorescent dye, TMRM, was used in confocal microscopy analyses to visually estimate mitochondrial membrane potential. Cell lines were seeded on sterile glass cover slips in 6-well plates (NUNC) at densities of 2 x 10^5 cells per well in 2 ml culture medium. After overnight incubations to allow attachment, rotenone was added and incubated for 24 hr. TMRM (0.5 µM) and Mitotracker Green (0.5 µM) was added and incubated for 30 min at 37°C after which the cover slips were washed with three changes of media. The cover slips with adherent cells were placed in an applicable flow cell bath in the presence of 2 ml medium. Confocal images were monitored using a Nikon (PCM2000) inverted confocal microscope. Ar and He/Ne Spectra-physics lasers, with excitation at 475 nm and 505 nm and emission at between 505 nm and 568 nm (green) as well as long pass > 565 nm (red) were employed. In order to minimize photo bleaching of the sample and free radical formation in the cells, the smallest available pinhole was used (0.5 µm), together with a neutral density filter of 10%. Magnification was obtained with a Nikon 60x/1.40 Apo Planar oil objective and bars in the micrographs indicate size. Laser power and capturing settings were kept constant in comparative experiments, to enable quantitative analysis. A scan speed of 3 µs/scan was typically used and capturing was averaged to obtain representative micrographs.

3.2.10. ATP and apoptosis analyses

To determine the ATP content in rotenone treated cells, a luminescence based assay reagent was used as instructed and expressed relative to protein content. Cells were seeded in microtiter plates at 2.0 x 10^4 cells per well, allowed to adhere overnight and incubated with rotenone for 8 hr before ATP measurements. Caspase 3/7 activity in treated cells was measured fluorimetrically using a commercial kit as instructed by the manufacturer. Briefly, cells were seeded in microtiter plates at densities of 2.0 x 10^4 cells per well, allowed to adhere overnight and incubated with rotenone as described before. A two hr incubation with 1 µg/ml staurosporine was included as positive control for apoptosis. Assays were carried out kinetically and expressed relative to protein content. As an indicator for DNA degradation during apoptosis, cytosolic nucleosome enrichment in cells was determined using a commercial ELISA (Roche) as instructed by the manufacturer.

3.2.11. Statistical analyses

All results were analyzed with Statistica (version 7) software. Statistical comparisons of MT expression in HeLa cells were made using one-way analysis of variance (ANOVA) with post hoc comparison (Tukey test). For statistical analysis of values obtained from different MT over expressing and control cell lines, two-way ANOVA was performed. For these analyses and interpretation of results the interactions of the concentration (of either rotenone or *t*-BHP) and MT expression were evaluated as indicated by a significant F-value (test statistic). Statistically significance were considered when $p < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1. MT expression and ROS production in rotenone treated cells

The treatment of HeLa cells with varying concentrations of rotenone (0-2500 nM) resulted in a dose-dependent decrease in residual complex I activities as measured in enriched mitochondrial preparations (Table 3.1). Values were similar as reported before for fibroblasts (Koopman *et al*., 2005) and resulted in a useful range of complex I activities, ranging between 0 and 100%, to compare the responsiveness of the parameters that were investigated in this study. Similarly, complex III could be inhibited with myxothiazol treatment over a range of 0-1000 nM that resulted in dose dependent decrease of activity. Treatments with rotenone and myxothiazol were limited to 24 hrs to limit the contribution of media composition changes on cellular function.

Table 3.1. Complex I and III activities in rotenone and myxothiazol treated HeLa cells.

Rotenone (nM)	Complex I (nmol/min/UCS)	Complex III (nmol/min/UCS)
0	108.7 ± 13.4 (100%)	61.7 ± 2.5 (100%)
10	55.9 ± 6.3* (51%)	60.6 ± 1.5 (98%)
100	36.1 ± 3.5* (33%)	64.6 ± 7.7 (105%)
1000	16.8 ± 4.5* (15%)	62.8 ± 5.9 (102%)
2500	0.0 ± 3.8* (0%)	59.8 ± 4.0 (97%)
Myxothiazol (nM)		
10	111.6 ± 3.8 (103%)	42.9 ± 4.2* (70%)
100	106.2 ± 4.5 (98%)	27.9 ± 4.0* (45%)
500	105.1 ± 2.1 (97%)	6.8 ± 1.5* (11%)
1000	106.7 ± 7.2 (98%)	0.0 ± 0* (0%)

Rotenone-sensitive complex I and antimycin A-sensitive complex III activities were determined in mitochondrial enriched preparations that were prepared from rotenone or myxothiazol treated (24 hr) HeLa cells. Values are means ± S.D. (n = 4, *$p < 0.05$) with percentage activities relative to untreated cells given in brackets.

Real-time PCR was performed on total RNA samples obtained from HeLa cells that were treated with rotenone. We included known inducers of MT expression, CdCl and ZnCl as well as the ROS inducer, *t*-BHP, as controls for MT expression. In addition, myxothiazol was included to evaluate the mechanistic possibilities of MT expression. For RNA expression studies northern blotting gave poor, unspecific results which were probably due to the homology that exists between the different MT isoforms. Real-time PCR was used which allowed isoform RNA expression analysis. The stability of several commonly used "housekeeping genes" involved in diverse biological activities, including glycolysis, cytoskeleton structure and kinetics, immune response, gene expression and protein biosynthesis were investigated under the interventions mentioned. To ensure comparability between the analyses of all five housekeeping genes as well as MT isoforms, we determined the reaction efficiency of each individual assay by measuring serial dilutions of 75 ng cDNA in triplicate (Pfaffl *et al.*, 2002). All PCR reactions displayed efficiencies of between 88% and 100%. The variations in the cycle threshold (Ct) values, which represents the cycle where a significant increase in amount of PCR product occurs during the various interventions, are summarized in Figure 3.1. Comparing the median expression values (Ct values) of the housekeeping genes, the variability for housekeeping gene expression was clearly less in the case of the 18S rRNA as compared to the other genes. Ct values were also expressed as relative expression quantities and analyzed using the GeNorm software tool. The results of this analysis are presented as GeNorm expression stability values (or gene stability measures, M), which are defined as average pair wise variations of a particular gene with all other control genes are summarized in Table 3.2 (Vandesompele *et al.*, 2002). Genes with the lowest M values generally have the most stable expression. As a result, the three most stably expressing genes, 18S rRNA, β-2-Microglobulin and GAPDH, were subsequently used for normalization of MT expression.

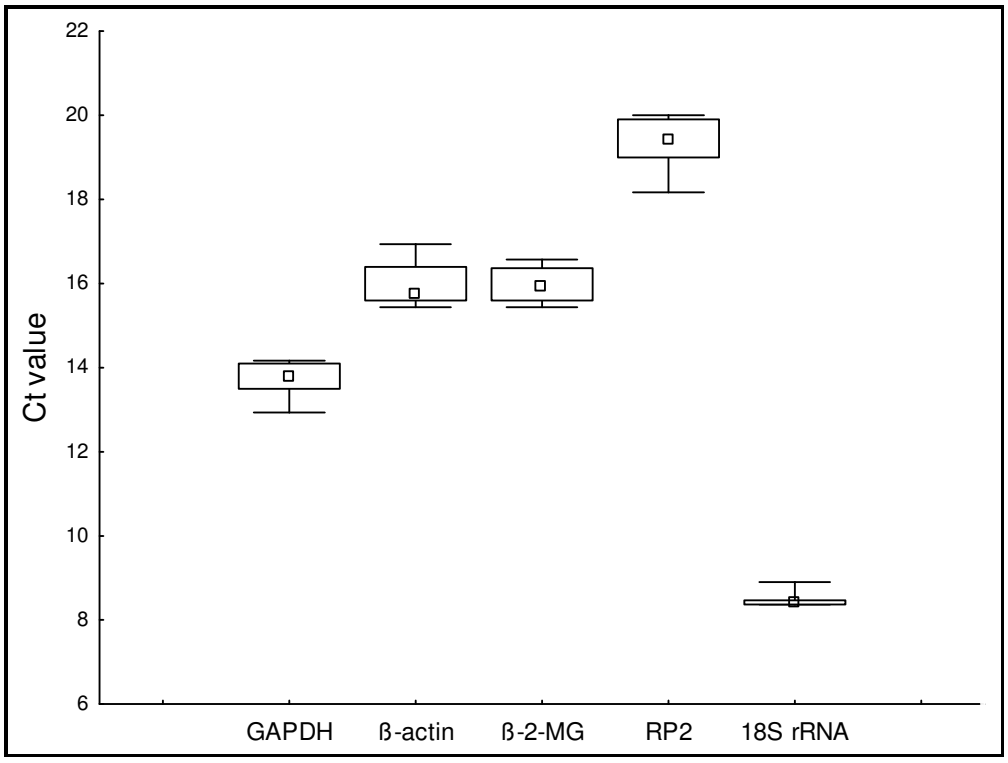

Figure 3.1. Evaluation of RNA transcription level variation of housekeeping genes. Variation in real-time PCR generated Ct values of selected housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, β-2-microglobulin (β-2-MG), RNA polymerase II (RP2) and 18S rRNA) of HeLa cells treated with either rotenone (0-10 μM), $CdCl_2$ (12.5 μM), $ZnCl_2$ (250 μM) and *t*-BHP (0-1 μM) are shown. Incubations were carried out for 24 hr (rotenone, metals) and 3 hr (*t*-BHP). The median values are indicated by small squares, 25-75% percentiles are indicated by the boxes and minimum and maximum values indicated by whiskers..

Table 3.2. Stability of housekeeping genes expression.

Gene	M (24 hr)	M (48 hr)
18S rRNA	0.466	0.402
Beta-2-microglobulin	0.548	0.503
GAPDH	0.575	0.506
Beta-actin	0.682	0.512
RNA polymerase II	0.730	0.532

Results are shown as GeNorm expression stability values or M, the internal control gene-stability measure, defined as average pair wise variation of a particular gene with all other control genes (Vandesompele *et al*., 2002). Genes with the lowest M values have the most stable expression.

Under the experimental *in vitro* conditions and interventions performed in this study we could not detect any expression of MT1A. In addition, an almost undetectable basal expression of MT1B was observed. From the controls for PCR, using cDNA templates for the various isoforms, we concluded that the PCR was efficient and specific to the isoforms. Treatment of cells with any of the other possible inducers mentioned did not result in detectable changes of expression levels of these common MT1 isoforms either. In contrast, MT2A RNA basal levels in untreated HeLa cells were easily detectable and, as presented in Figure 3.2, highly inducible by $CdCl_2$ (± 50-fold) and *t*-BHP (up to fivefold), but not $ZnCl_2$. Myxothiazol treatment up to levels that completely inhibited complex III activity did not result in any significant induction of MT2A expression. Rotenone treatment, however, significantly induced MT2A expression. MT2A levels were slightly elevated with 10 nM rotenone treatment after which expression levels were significantly increased three-fold upon treatment with 100 nM rotenone. Surprisingly, expression levels at 1000 nM were consistently lower than 100 nM. This result was the same in three independent experiments. This phenomenon was also observed when rotenone treatment was extended to 48 hr (results not shown). At rotenone levels higher than 1000 nM, which resulted in almost complete inhibition of rotenone-sensitive complex I activity, MT2A expression was significantly higher and remained constant with higher rotenone levels at approximately seven-fold compared to basal levels. MT protein analysis, which did not enable a distinction between MT1 and MT2 isoforms in treated cell

homogenates, confirmed the increased expression observed with rotenone treatment (Table 3.3). A slight, albeit significant, dose-dependent increase in MT expression was detected at levels up to 2500 nM. As with RNA expression, expression levels at 2500 nM were notably higher than at 1000 nM rotenone. Treatment with *t*-BHP also significantly increased expression of MTs on protein level. However, unlike MT2A RNA expression where only Cd-mediated induction was detected (Figure 3.2), both $CdCl_2$ and $ZnCl_2$ markedly induced MT protein expression.

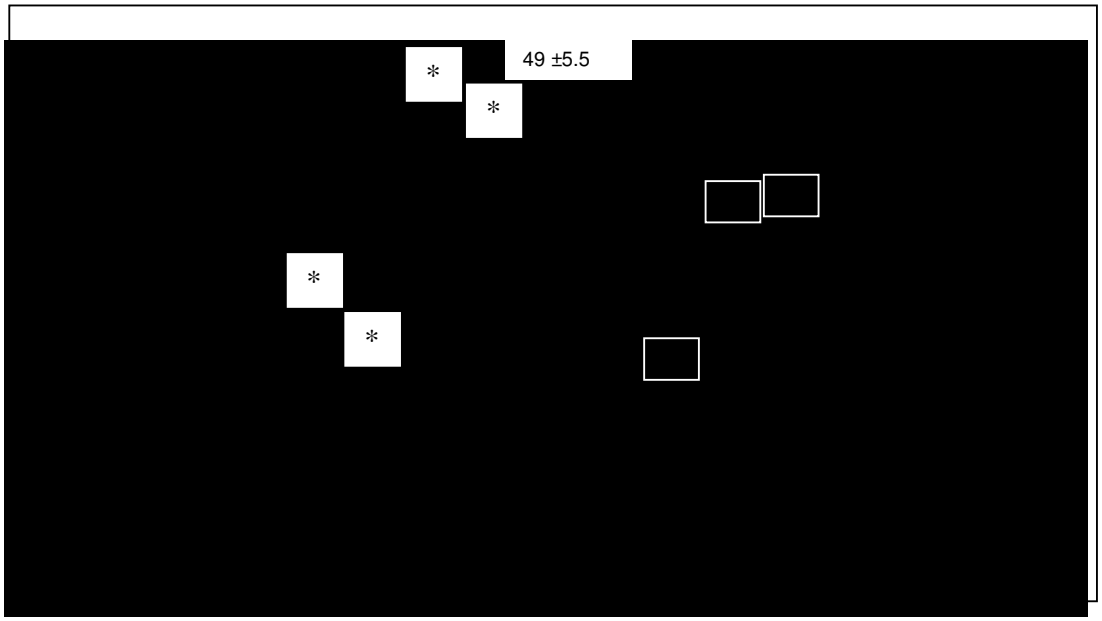

Figure 3.2. MT2A RNA expression in rotenone, metal, t-BHP and Myxothiazol treated HeLa cells. MT2A RNA expression in cells was determined with real-time PCR. Normalized RNA expression levels are expressed as the mean ratio +S.D. (n = 3) relative to untreated cells (open bar). Asterisks indicate statistically significant values ($p < 0.05$) compared to the untreated cells. Expression levels were compared in cells treated with rotenone, metal inducers $CdCl_2$ (12.5 μM) and $ZnCl_2$ (250 μM), *t*-BHP and myxothiazol as indicated. The value for Cd induced expression is indicated above the bar.

Table 3.3. MT protein levels in rotenone, metal and t-BHP treated HeLa cells.

Treatment	MT* (ng/mg)
Untreated	34.7 (32.3 - 37.1)
Rotenone 100 nM	47.0 (44.1 - 49.8)
Rotenone 500 nM	52.1 (51.3 - 52.8)
Rotenone 1000 nM	53.8 (53.1 - 54.4)
Rotenone 2500 nM	71.7 (71.1 - 72.4)
$CdCl_2$ (12.5 µM)	875
$ZnCl_2$ (250 µM)	481
t-BHP (0.5 mM)	56.2 (47.7 - 64.5)
t-BHP (0.8 mM)	61.9 (57.6 - 66.2)
t-BHP (1.0 mM)	91.5 (86.1 - 96.8)

*Total metallothionein (MT1 + MT2) levels are expressed relative to total protein content in cell homogenates (10 mM Tris.Cl, pH 8.0). Mean values are shown with ranges in brackets (n = 2, except metal controls where n = 1).

Treatment of cells with rotenone elevated ROS levels significantly only at levels higher than 100 nM (± 36% residual activity) (Figure 3.3). At complete inhibition of complex I activity (2500 nM), ROS levels increased approximately two-fold compared to untreated cells. As expected *t*-BHP treatment had a marked effect on ROS levels up to almost five-fold at 1 mM. Although a complete inhibition of complex III with myxothiazol was demonstrated (Table 3.1), surprisingly, no significant effect on ROS production could be observed in HeLa cells.

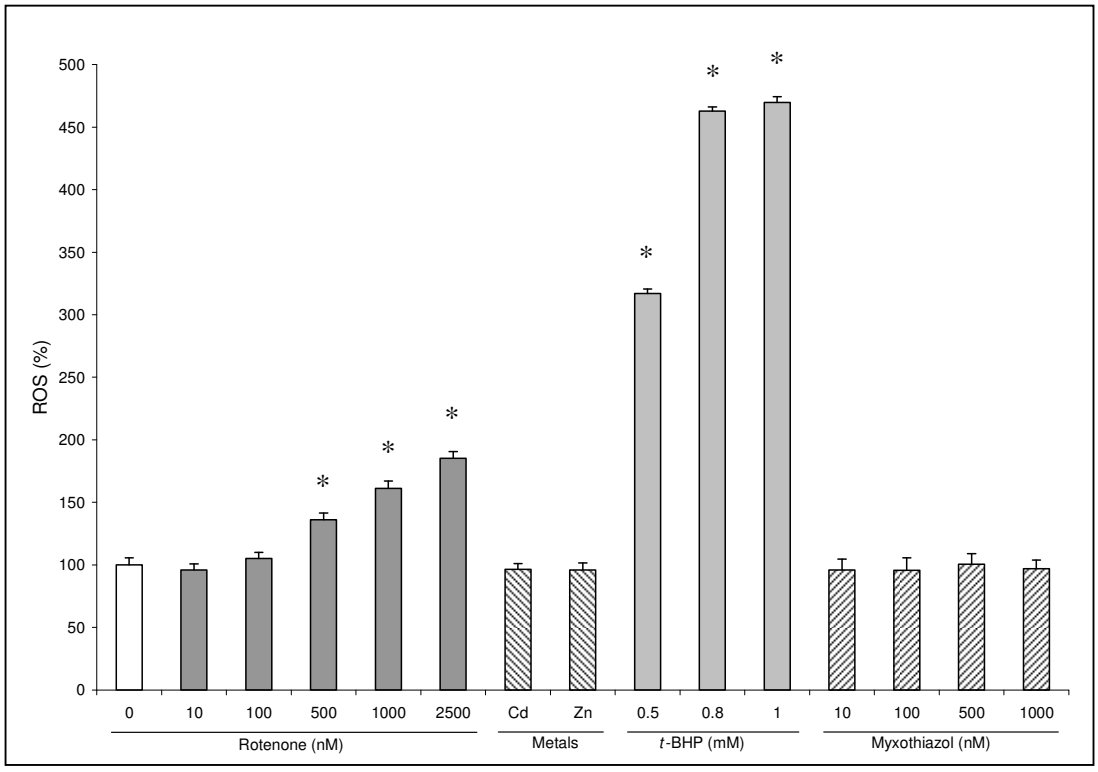

Figure 3.3. ROS production in rotenone metal, t-BHP and Myxothiazol treated HeLa cells. ROS production was measured fluorimetrically in cells treated with rotenone, metal inducers $CdCl_2$ (12.5 μM) and $ZnCl_2$ (250 μM), *t*-BHP and myxothiazol using the ROS-sensitive probe, DCFHDA, and normalized to protein content. Values, which were normalized to protein content, are means ±S.D. (n = 8) and expressed as % relative to untreated cells.

3.3.2. MT over expressing HeLa cells

HeLa cells were transfected with plasmid constructs containing either the pIRESneo2 base vector (control cells), MT1B- or MT2A plasmid constructs. After confirming stable transfer of plasmid DNA, normalized expression ratios of MT1B and MT2A of the various transfected cell lines were determined as summarized in Table 3.4. Basal MT1B expression in control cells was barely detectable (Ct values similar to negative controls), similar to a previous report (Haq *et al.*, 2003). Consequently, a significantly higher MT1B expression ratio in MT1B over expressing cells was calculated. Comparatively, on the background of a relatively high basal expression level of MT2A in control cells (Ct values ± 18), the MT2A over expressing cells resulted in only a two-fold

increase in expression of MT2A. The MT expression levels in transfected cell lines were measured on separate occasions and remained similar to those indicated in Table 3.4 over the time period of the investigation. The total MT protein content, which represents combined MT1 and MT2 levels, however, were similar in both MT over expressing cell lines and approximately 20% higher than control cells. From this we concluded that the additional levels of expression of either MT1B or MT2A in the respective MT over expressing cell lines were similar and were suitable for use in comparative studies.

The differences in total MT protein content in Tables 3.3 and 3.4 can be explained by the fact that the control cell line in Table 3.4 was also transfected with the pIRESneo2 vector whilst ethanol was added to the untreated cell line in Table 3.3 (to eliminate the effect of ethanol in which rotenone was dissolved) . When looking at Tables 3.3 and 3.4 one can draw the conclusion that induction of MT proteins might be more effective than over-expression of MTs when comparing total MT protein content in the cells. However, when looking for a possible therapeutic role for MTs in the presence of OXPHOS deficiencies, it would not be advisable to induce MT expression via further inhibition of the OXPHOS system or increasing ROS production, which could lead to even further oxidative damage. Increasing heavy metals such as cadmium in cells or disrupting homeostatis of physiologically important metals such as Zn in these situations would probably add to the severity of disease models in these patients. It is also true that rotenone and *t*-BHP causes much higher increases in MT protein expression than MT-over-expression. This might be due to post-transcriptional and/or post-translational regulation of MTs and maybe even different rates of degradation of the proteins under different conditions.

Table 3.4. MT RNA and protein expression in recombinant MT over expressing HeLa cell lines.

	RNA expression ratio		Protein (ng/mg)
Cell line	MT1B	MT2A	MT
Control	1.0 ± 1.6	1.0 ± 0.3	151± 6.6
MT1B-HeLa	133 000 ± 38 000*	1.1 ± 0.3	188 ± 8.8*
MT2A-HeLa	1.2 ± 0.8	2.2 ± 0.2*	186 ± 7.6*

RNA expression of MT1B or MT2A in cell lines was analysed by real time PCR and normalized relative to the expression of GAPDH and β-2-microglobulin. RNA expression values represent the expression ratios relative to pIRESneo2-transfected HeLa cells (control). Total MT protein levels were quantified in homogenates (PBS containing 1% Tween20) using a radio immuno-assay and expressed relative to protein content. All values are means ± S.D (n = 3, $*p < 0.05$).

3.3.3. ATP analyses in MT over expressing cells

Rotenone treatment of control and MT over expressing cell lines resulted in a dose-dependent decrease in ATP levels (Figure 3.4). With complete inhibition (1000 nM rotenone), ATP levels decreased to 68% in control cells. Comparatively, ATP levels were 76% and 78% in MT1B- and MT2A over expressing cells, respectively. A clear and significant variance in response to increasing rotenone treatment occurred between the cell lines with ATP levels in both MT over expressing cell lines decreasing notably slower to the levels mentioned before, compared to the control cell line.

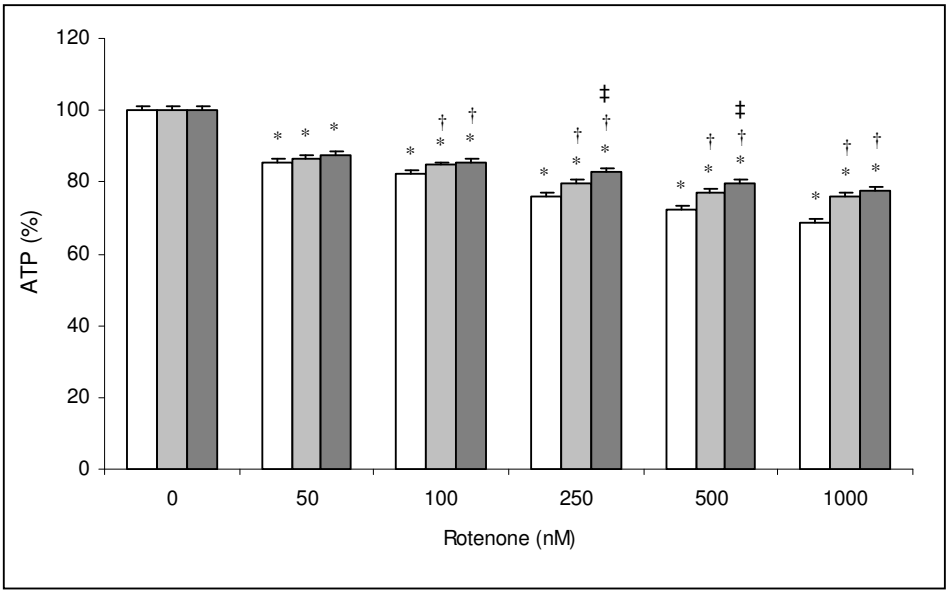

Figure 3.4. ATP levels in rotenone treated MT over expressing HeLa cells. Total cellular ATP content was measured in rotenone treated MT1B- (pIRESneo2-MT1B transfected, light grey bars), MT2A over expressing (pIRESneo2-MT2A transfected, dark grey bars) and control (pIRESneo2- transfected, open bars) HeLa cells. Values were normalized to protein content and expressed as a mean percentage of untreated cells (+S.D., n = 4), $^{*}p < 0.05$ when compared to untreated cells of the same cell line; $^{\dagger}p < 0.05$ when compared to control cell line at same treatment; $^{\ddagger}p < 0.05$ when comparing MT2A to MT1B over expressing cell line at same treatment.

3.3.4. Rotenone- and *t*-BHP induced ROS production

As with HeLa cells that were not genetically modified (Figure 3.3), total cellular ROS production in genetically modified HeLa cells increased significantly only after treatment with rotenone levels higher than 100 nM (Figure 3.5), which represents approximately 33% residual complex I activity. As before, ROS levels further increased dose-dependently only to levels approximately two-fold higher at 2500 nM rotenone. At this concentration, ROS levels in MT2A, which were higher at 500 nM, were significantly, albeit only slightly, lower compared to the other two cell lines. ROS production could be further and significantly induced using *t*-BHP (Figure 3.5). A clear variance in response to this treatment could be observed with MT2A over expressing cells

which had significantly lower ROS levels (± 40%) compared to control and MT1B over expressing cells.

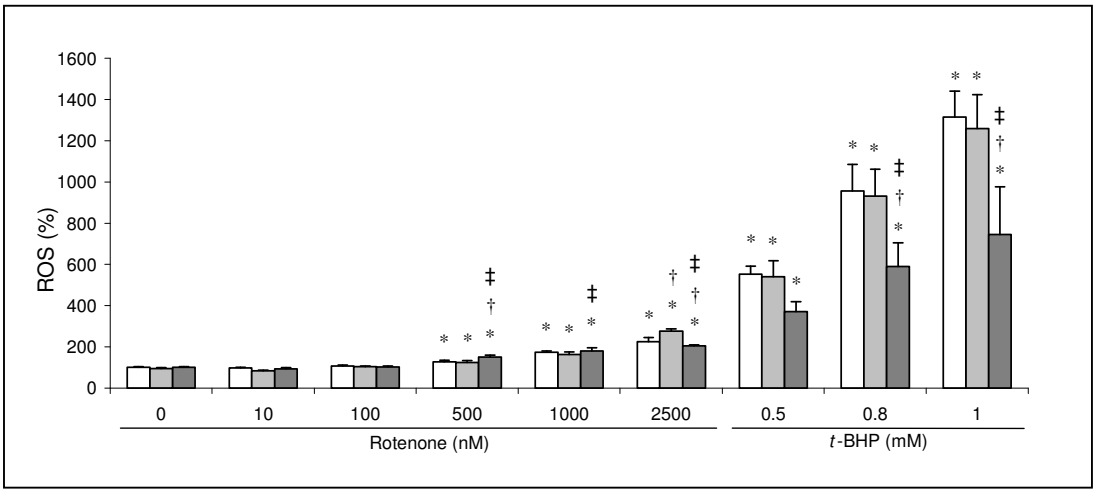

Figure 3.5. ROS production in rotenone and t-BHP treated MT over expressing HeLa cells. ROS production was measured in control (open bars), MT1B- (light grey bars) and MT2A (dark grey bars) over expressing cell lines treated with rotenone or *t*-BHP. Values, which were normalized to protein content, are means +S.D. (n = 8) and expressed as percentage relative to untreated cells, $^{*}p < 0.05$ when compared to untreated cells of the same cell line; $^{\dagger}p < 0.05$ when compared to control cell line at same treatment; $^{\ddagger}p < 0.05$ when comparing MT2A to MT1B over expressing cell line at same treatment.

3.3.5. Cell viability

Rotenone treatment of the cell lines resulted in a general decrease in cell viability as determined by the MTT test (Figure 3.6). The cell lines, however, showed no consistent variation in cell viability across the range of rotenone concentrations, i.e. compared to MT1B, MT2A over expressing cells had significantly higher viability at 500 nM rotenone with lower viability at 2500 nM rotenone. As with ROS production, a more pronounced response of cell lines was observed when *t*-BHP was used to increase ROS production and consequently lowering cell viability to less than 20%. Also in this case a clear and statistically significant variation in response to *t*-BHP treatment

occurred in the MT2A over expressing cells compared to both control and MT1B over expressing cells. This is strikingly obvious at 0.5 and 0.8 nM *t*-BHP, where MT2A over expressing cells had significantly higher viability. Unlike the responses in ROS levels (compare Figure 3.5), at 1 mM *t*-BHP, viability of all three cell lines was similar at levels lower than 20% of the untreated cells.

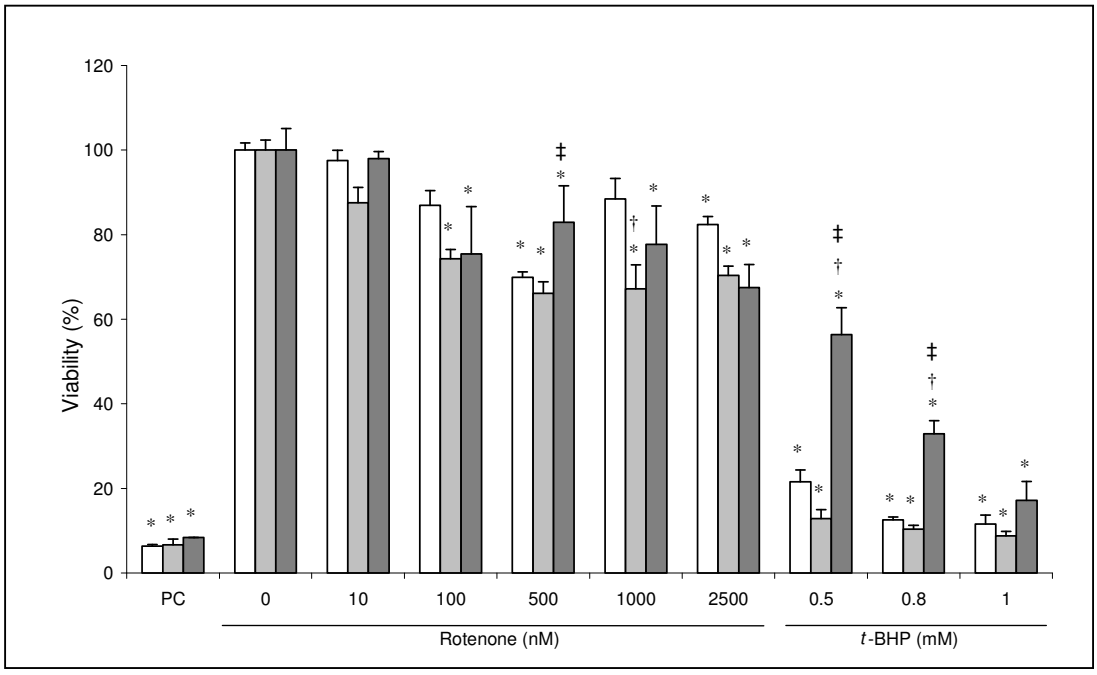

Figure 3.6. Cell viability in rotenone- and t-BHP treated MT over expressing HeLa cells. Cell viability was measured using the MTT test in MT over expressing (MT1B, light grey bars; MT2A dark grey bars) and control cell lines (open bars) treated with rotenone or *t*-BHP. Values, which were normalized to protein content, are means +S.D. ($n = 3$) and expressed as percentage viability relative to untreated cells. Acetic acid (6%) was used as positive control (PC). $^{*}p < 0.05$ when compared to untreated cells of the same cell line; $^{\dagger}p < 0.05$ when compared to control cell line at same treatment; $^{\ddagger}p < 0.05$ when comparing MT2A to MT1B over expressing cell line at same treatment.

3.3.6. Mitochondrial membrane potential

The membrane potential was visualized with confocal microscopy using TMRM staining. Fluorescence from TMRM generally co-localized with green fluorescence from mitochondrial staining (results not shown). The control cell line (Figure 3.7A) showed a clear decrease in membrane potential with increasing rotenone concentration, having almost no visual membrane potential when treated with 1000 nM rotenone. Both MT over expressing cell lines had visibly more membrane potential remaining when treated with rotenone (Figure 3.7B-C). Compared to MT1B, MT2A over expressing cells (Figure 3.7C) had a visibly smaller decrease in membrane potential with increased rotenone treatment.

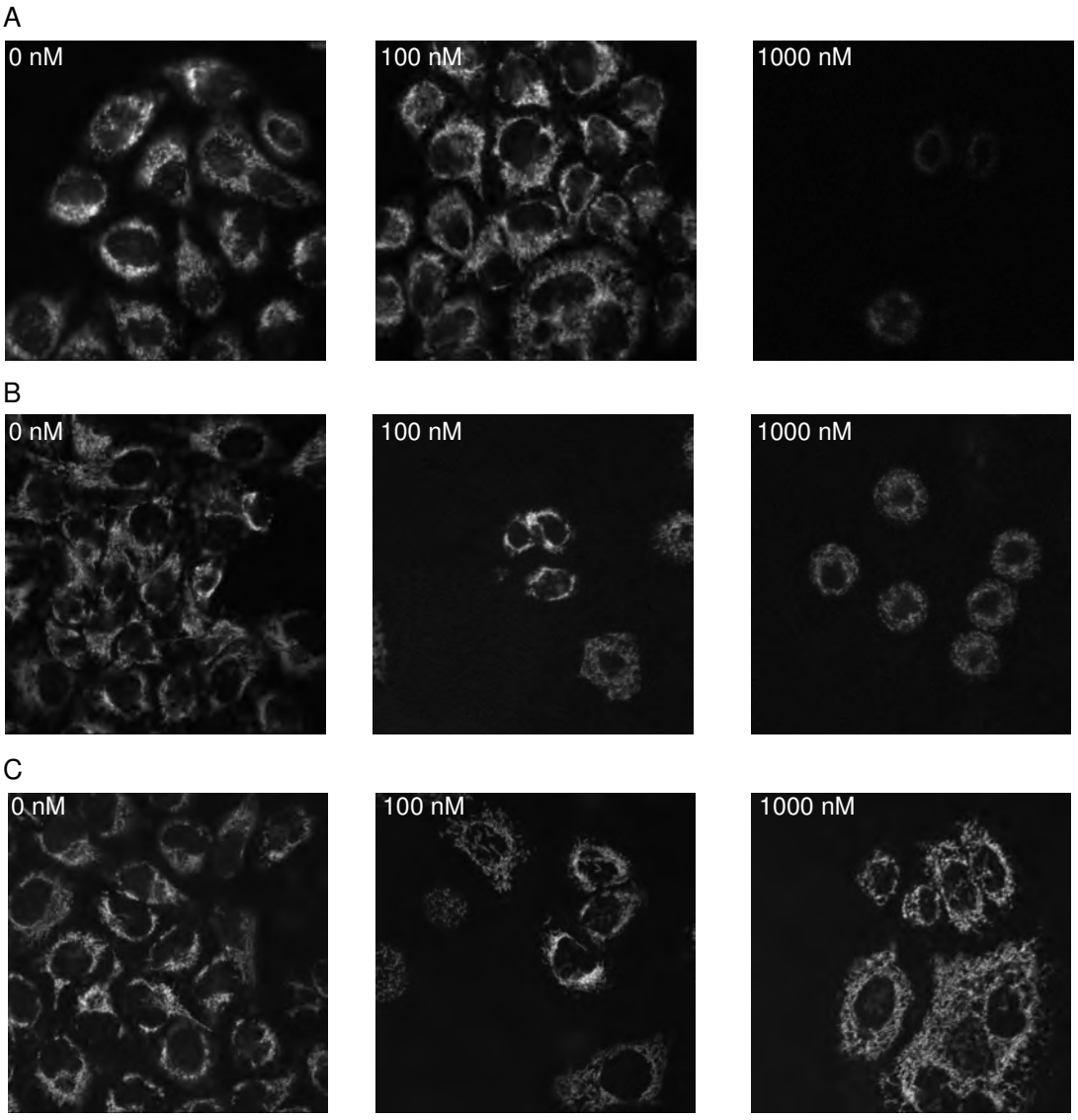

Figure 3.7. Assessment of mitochondrial membrane potential in rotenone treated MT over expressing cells. Membrane potential of control (A), MT1B- (B) and MT2A over expressing (C) HeLa cells treated with 0, 100 and 1000 nM rotenone was visualized by confocal microscopy after TMRM staining.

3.3.7. Apoptosis

Staurosporine treatment (positive control) of all cell lines resulted in the induction of similar caspase 3/7 activities, indicating that MT over expression in these cell lines did not result in changes in protein kinase-mediated caspase activation (Figure 3.8). In all cell lines, caspase 3/7

activity increased approximately in a dose dependent way over the full range of rotenone incubations used. Both MT over expressing cell lines had a significant variation in response to rotenone treatment. In MT1B over expressing cells, caspase activity increased much less compared to similar activities of control cells at 250 nM rotenone. At higher rotenone levels, caspase activity in this cell line was significantly less than control cells and at 2500 nM rotenone were ± 35% lower than control cells. Induction of caspase 3/7 activity in MT2A over expressing cells was significantly lower than both control and MT1B over expressing cells with complete inhibition of complex I. Activities remained relatively constant up to 250 nM rotenone and up to 2500 nM increased only to levels of ± 45% lower than the control cells.

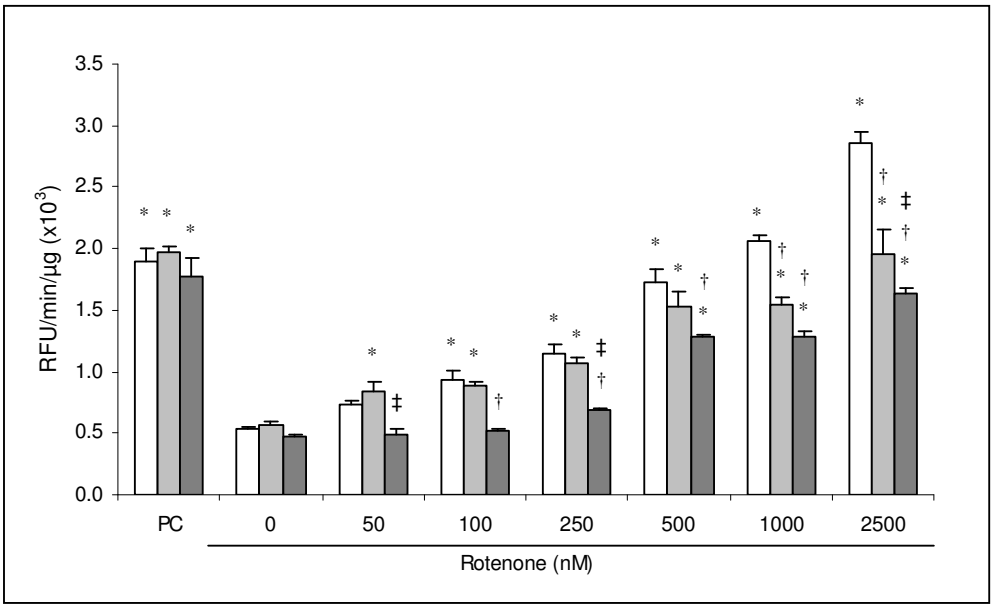

Figure 3.8. Caspase 3/7 activation in rotenone treated MT over expressing HeLa cells. Caspase 3/7 activity was measured in MT over expressing (MT1B, light grey bars; MT2A dark grey bars) and control cell lines (open bars) treated with rotenone. Reaction velocities are indicated as change in relative fluorescence units (RFU) per µg total protein. Staurosporine treatment (1 µg/ml for 2 hr) were the positive control (PC) for caspase activation. Values are means +S.D. (n = 3), *p < 0.05 when compared to untreated cells of the same cell line; $^{\dagger}p$ < 0.05 when compared to control cell line at same treatment; $^{\ddagger}p$ < 0.05 when comparing MT2A to MT1B over expressing cell line at same treatment.

Over the incubation period used in this study (24 hr), visible DNA laddering was not detected using gel electrophoresis analysis (results not shown). However, cytosolic nucleosome enrichment which is a result of DNA degradation could be detected with rotenone treatment in all three cell lines using an immunological assay (Figure 3.9). In view of the caspase 3/7 data the onset of DNA fragmentation in high concentraitons can also be seen in the comparatively less significant increase in nucleosome formation with staurosporine treatment in all three cell lines. In the rotenone treated cells nucleosome formation increased significantly only at levels higher than 100 nM. For control cell lines the nucleosome formation increased up to 14-fold at 2500 nM rotenone. Comparatively, in MT1B and MT2A over expressing cell lines, nucleosome formation increased significantly less at rotenone levels higher than 100 nM (36% of residual complex I activity) to reach levels of approximately six-fold and five-fold, respectively, at 2500 nM compared to baseline activity. Thus, as with caspase 3/7 activation, a significant variance in response to increasing rotenone treatment occurred between the cell lines. In addition, nucleosome formation in rotenone treated MT2A over expressing cells increased less than in MT1B over expressing cells and were significantly lower at the higher levels of rotenone treatment.

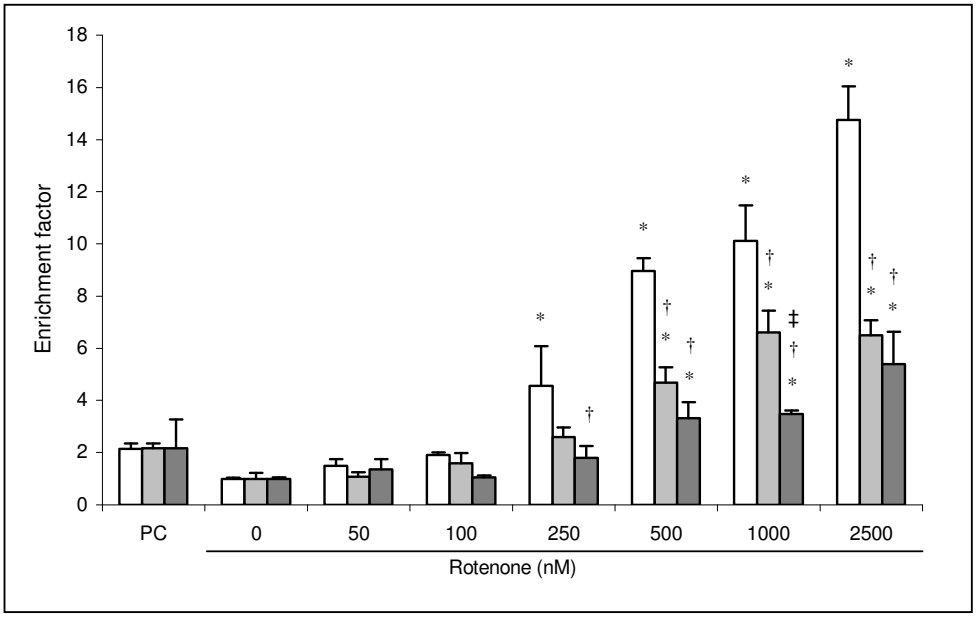

Figure 3.9. Cytosolic nucleosome enrichment in rotenone treated MT over expressing HeLa cells. Cytosolic nucleosome enrichment was determined in MT1B (light grey bars), MT2A (dark grey bars) over expressing and control (open bars) cells treated with rotenone for 24 hr or staurosporine (1 µg/ml for 2 hr) as positive control (PC). Values, normalized relative to protein content, are indicated as a mean fold change (+S.D., n = 3) relative to untreated cells, $^{*}p < 0.05$ when compared to untreated cells of the same cell line; $^{\dagger}p < 0.05$ when compared to control cell line at same treatment; $^{\ddagger}p < 0.05$ when comparing MT2A to MT1B over expressing cell line at same treatment.

3.4. CONCLUSIONS

The contribution of nuclear and mitochondrial gene expression in the pathophysiology of mitochondrial disorders has been recognized and investigated over the past decade as summarized in Chapter Two (Reinecke *et al.*, 2009; Van der Westhuizen *et al.*, 2003; Heddi *et al.*, 1999; Collombet *et al.*, 1997; Miranda *et al.*, 1999). One of the model systems often used to investigate disorders associated with the mitochondrial respiratory chain includes the inhibition of complex I with irreversible inhibitors such as rotenone. Among the diversity of genes differentially expressed in inherited and induced complex I deficient cell lines, the marked over expression of

MTs occurred in complex I deficient fibroblasts when mitochondrial energy metabolism was challenged by changing the carbon source of the medium. It was hypothesized that MT expression points to a possible beneficial adaptive response under such conditions (Van der Westhuizen *et al.*, 2003). It is conceivable that the expression and function of MTs are related to disorders of the respiratory chain as these proteins are not only induced by ROS, which is a common feature in such disorders, but also scavenges hydroxyl radicals (Thornalley & Vašák, 1985; Andrews, 2000; Thomas *et al.*, 1986). Evidence linking MT expression responsiveness to oxidative stress and associated protection against oxidative stress in *in vitro* and *in vivo* models are mounting (Thornalley & Vašák, 1985; Andrews, 2000; Ebadi *et al.*, 2005; Kumari *et al.*, 1998). These include reports of MT1 mediated protection of key mitochondria associated functions, such as apoptosis, coenzyme Q10 synthesis and mitochondrial genome integrity, against neurotoxin treated murine neuronal cells (Sharma & Ebadi, 2003).

We demonstrated that MT2A expression in HeLa cells is highly inducible with rotenone treatment. This is in contrast to a previous report that MT1 RNA expression is decreased in the striatum cells of rats treated with MPTP (Rojas *et al.*, 2000), which also binds and inhibits complex I (Ramsay *et al.*, 1991). Our results show a significant increase in expression of MT2A to occur only after residual complex I activity was inhibited to levels below 50%. Expression of MT2A also did not increase in a coordinate way relative to rotenone concentration and a clear biphasic expression pattern was observed. The reason for this phenomenon is not clear and has to be investigated further. Some clues as to the mechanism of rotenone induced MT expression may, however, be revealed by the lack of both MT expression and ROS production by treatment with the complex III inhibitor, myxothiazol. It was evident that both MT2A expression and elevated ROS production occurred at residual complex I activities lower than 50%, which was not the case with complete inhibition of complex III activity. This result supports a ROS related mechanism of rotenone induced MT2A expression. Lack of myxothiazol induced ROS production confirmed previous findings that showed that inhibition at the Qo site of complex III (as with myxothiazol) does not lead to increased superoxide production (Turrens, 2003; Bénit *et al.*, 2009), although some contradictive reports observed increased production of ROS in the presence of myxothiazol

treatment (Young *et al.*, 2002). It is important to also note that increased ROS production and decrease in ATP levels induced by rotenone treatment was limited (although statistically significantly), possibly because consequences as a result of deficient electron transport chain in the predominantly glycolytic cancerous HeLa cells may, in general, be less pronounced than in cell lines which rely more on oxidative phosphorylation (Warburg, 1956; Reitzer *et al.*, 1979).

MT2A expression was also inducible by Cd but not Zn, both of which modulate MT expression by a different mechanism than oxidative stress-related inducers (Andrews, 2000; Saydam *et al.*, 2002; LaRochelle *et al.*, 2001). However, both Cd and Zn induced combined MT1/2 protein expression. Although Cd and H_2O_2 share a common MTF-1 activation pathway in HeLa cells that is dependent on the release of Zn from MT-bound Zn (Zhang *et al.*, 2003), it is not clear why $ZnCl_2$ treatment had no effect on MT2A expression. Similar observations were also made in human proximal tubule cells (Garrett *et al.*, 1998). In addition, surprisingly neither MT1A nor MT1B RNA expression induction was detected with any of the inducers used. Lack of MT1B expression in HeLa cells was previously reported (Heguy *et al.*, 1986). Other MT1 isoforms in HeLa cells may be expressed that could explain the observed Zn induction of MT protein expression.

The effect of over expressed MT2A on ATP and ROS levels, cell viability and apoptosis in rotenone cells treated where evaluated in parallel to MT1B over expression, which, as mentioned before, were not induced by any of the possible MT inducers used in this study. ATP levels, which were only decreased to ±70% in control cells with rotenone treatment, remained slightly albeit significantly higher in MT over expressing cells. ROS production in MT1B and MT2A over expressing HeLa cells were similar to control cells when treated with rotenone up to levels where almost no rotenone-sensitive complex I activity could be measured (1000 nM). As mentioned before and evident from our data, HeLa cells rely mainly on glycolytic ATP production. ROS production via inhibition of the OXPHOS system in HeLa cells may, therefore, be limited compared to cells that have a greater dependence on ATP produced from OXPHOS (Li *et al.*, 2003; Vrbacky *et al.*, 2003). With the induction of more ROS, i.e. by treatment with *t*-BHP which generates ROS via microsomal cytochrome-P450 activity (Davies, 1989) in addition to opening the mtPTP

(Nieminen *et al.*, 1995), a markedly higher level of ROS could be induced. ROS levels under these conditions were significantly less and cell viability significantly higher in MT2A over expressing HeLa cells and to a lesser extent in MT1B over expressing cells, compared to control cells.

Increased production of ROS has been found to quantitatively relate to apoptosis induction in cells treated with rotenone (Barrientos & Moraes, 1999; Li, 2003). The mechanism of rotenone induced cell death in HeLa cells has previously been investigated to a limited extent only. Apoptotic cell death predominantly occurs in cells such as HeLa cells which are less dependent on pyruvate/malate supported ATP production (Li, 2003; Vrbacky *et al.*, 2003). Apoptosis induction, in addition to caspase activation, also results in the opening of the mtPTP (Isenberg & Klaunig, 2000), which in turn results in a breakdown of mitochondrial membrane potential (Zoratti & Szabo, 1995). The qualitative results obtained with 100 and 1000 nM rotenone treatment of all three cell lines indicated that sustaining of membrane potential was improved in MT over expressing cells, but more so for MT2A, compared to control cells. Similar to previous observations, activation of caspase 3/7 and subsequent nucleosome formation occurred at much lower rotenone levels than the levels needed to induce cellular toxicity (Barrientos & Moraes, 1999). Rotenone induced caspase 3/7 activation increased dose dependently after 24 hr period in control cells to levels similar to that induced by staurosporine in 2 hr. Over the rotenone concentration range, caspase 3/7 activity was clearly significantly lower in MT2A over expressing cells and, to a lesser extent, also in MT1B over expressing cells. This protective, delaying effect of MT expression on apoptosis was strongly supported by the significantly lower nucleosome formation in MT2A - and, to a lesser extent, MT1B over expressing cells, compared to control cells.

Studies on the function of MT are not conclusive as to its role in the prevention of oxidative stress. It was recently reported that MTs present in the inter membrane space of liver mitochondria could inhibit mitochondrial respiratory chain complexes I and III, through transfer of Zn to the complexes (Ye *et al.*, 2001; Simpkins *et al.*, 1994; Simpkins *et al.*, 1998). However, this phenomenon did not occur in heart muscle mitochondria (Ye *et al.*, 2001; Zhou & Kang, 2000). In general, studies show that MT expression is associated with a protective effect against

interventions leading to oxidative stress. Our results have shown that in rotenone induced complex I deficient HeLa cells over expression of MT2A indeed had a lowering effect on oxidative stress and increased cell viability, which were especially clear when further challenged with *t*-BHP treatment. Furthermore, MT2A over expression had a preventative or delaying effect on rotenone induced apoptosis in HeLa cells. MT1B, at similar over expressed levels, in general did not show the same responsiveness as MT2A.

To conclude, recent interest in the downstream adaptive responses to deficiencies of the OXPHOS system has revealed that, via differential gene expression, several genes may be involved in novel responses apart from those already associated with the deficiency, such as induction of apoptosis and changes in redox status (Van der Westhuizen *et al.*, 2003; Heddi *et al.*, 1999; Collombet *et al.*, 1997). Of these responses, little, if any, have been further investigated or reported. We have investigated the expression and role of MTs in an *in vitro* complex I deficiency model and concluded that the induced expression of MTs, specifically MT2A, has a protective effect against death-causing cellular consequences of rotenone treated HeLa cells. Although our data supports a ROS-related mechanism, since induction of MT expression was absent in myxothiazol treated cells that showed complex III inhibition but no increase in ROS, it remains to be determined what the mechanistic properties of this expression are and if MT expression is functionally relevant to complex I and other inherited OXPHOS deficiencies *in vivo*. Our data are comparable to current literature reports on the functional properties associated with MT expression but specifically reveals MT2A expression to be a beneficial downstream adaptive response in complex I deficient cells.

CHAPTER FOUR

INVESTIGATION OF THE CONSEQUENCES OF NADH:UBIQUINONE OXIDOREDUCTASE DEFICIENCY ON MITOCHONDRIAL DNA REPLICATION/TRANSCRIPTION BY MEANS OF INDUCING A TRANSIENT NDUFS3 KNOCKDOWN IN 143B CELLS

Some of the information contained in this chapter was included in the paper in preparation to be submitted for publication in Analytical Biochemistry (2010 - Appendix D).

4.1. INTRODUCTION

The mitochondrial OXPHOS (oxidative phosphorylation) system, which is responsible for most of the cellular ATP production, consists of five complexes. The first of these enzymes, NADH:ubiquinone oxidoreductase (complex I) is assembled from 45 subunits (Carroll *et al.*, 2006) and as for three of the other complexes, these subunits are encoded by both mitochondrial- and nuclear genomes (Anderson *et al.*, 1981, Hirst *et al.*, 2003). Most often, deficiencies of the OXPHOS system are associated with complex I (Loeffen *et al.*, 2000) and biochemical consequences of such deficiencies often, but not inevitably, include increased formation of reactive oxygen species (ROS), loss of ATP production as well as mitochondrial membrane potential, calcium regulation and apoptosis (Vives-Bauza *et al.*, 2006, Verkaart *et al.*, 2007a, Koopman *et al.*, 2007, Smeitink *et al.*, 2006, Brookes *et al.*, 2004).

Another consequence is the differential expression of several nuclear and mitochondrial genes involved in mitochondrial bioenergetics that have been detected in inherited complex I deficient or rotenone-treated cell lines (van der Westhuizen *et al.*, 2003; Heddi *et al.*, 1999, as summarised in Reinecke *et al.* 2009). It has been proposed that this differential expression might either be due to increased ROS production leading to oxidative damage of mtDNA and mtRNA

(Yakes & van Houten, 1997; Lee & Wei, 2005), or because of differential regulation of mtDNA replication/transcription (Heddi *et al*., 1999; van der Westhuizen *et al*., 2003).

To evaluate the status of mtDNA replication and transcription processes in the presence of complex I deficiency *in vitro*, a transient knockdown of the NDUFS3 subunit of complex I in 143B cells was introduced. In Chapter 3, the chemical inhibitor, rotenone, that binds specifically and irreversibly to complex I, was utilised. However, secondary toxic effects (also leading to apoptosis) unrelated to complex I activity has been documented with use of rotenone above concentrations of 100 nM or more than 100% complex I inhibition as utilised in Chapter 3 (Barrientos & Moraes, 1999). To simulate a complex I deficiency more precisely *in vitro*, synthetic short interfering RNA molecules (siRNA) were transfected into 143B cells to directly interfere with NDUFS3 target mRNA (summarized by Levanets *et al.* 2010). NDUFS3 is one of the key subunits involved in electron transfer in complex I and is known to be incorporated early on in complex I assembly (Ugalde *et al*., 2004a; Vogel *et al*., 2007). Deficiency of this subunit has also been observed to result in complex I deficiency with associated clinical presentations (Bénit *et al*., 2004). This model for transient complex I deficiency was initially used in this study to investigate the hypothesis as stated in Chapter 2. Several parameters were selected to first evaluate and characterise the induced deficiency (such as RNA, protein and functionality) as well as key biochemical parameters (ROS and ATP levels) and secondly to evaluate the transcription and replication of mtDNA in this transient complex I deficient model. The latter included an evaluation of mtDNA copy number, total mtRNA levels and RNA levels of several nuclear- and mitochondrial-encoded transcripts encoding structural as well as functional proteins.

4.2. EXPERIMENTAL

4.2.1. Cell culture and siRNA knockdown

143B206 ρ^0 and 143B control cybrids (Ugalde *et al.* 2003) were a kind gift from Prof. J.A.M. Smeitink (NCMD, Radboud University Nijmegen Medical Centre). Cells were cultured at 37 ℃ with 5% CO_2 in DMEM, supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin (all reagents obtained from Gibco™). 143B206 ρ^0 cells were additionally supplemented with 50 µg/ml uridine and 100 µg/ml pyruvate (both from Sigma-Aldrich) as suggested by King & Attardi (1996). One day before transfections, cells were plated with a cell density of 2 x 10^5 cells per well in a 6-well plate. Cells were transfected with any one of three target NDUFS3 siRNA duplex sequences (Genbank® accession number NM_004551) synthesised by IDT® and designed using the si*DESIGN*® Center tool (Dharmacon, see Table 4.1). This was done in the presence of Oligofectamine™ (Invitrogen) to a final concentration of 10 nM or 100 nM siRNA. Cells were harvested after 72 hours of incubation without any change of medium.

Table 4.1. Duplex NDUFS3 siRNA sequences

	Antisense strand (5′-3′)	Sense strand (5′-3′)
Target 1	UAGGUCUUCACACGGAUCCdTdT	GGAUCCGUGUGAAGACCUAdTdT
Target 2	AUCAUCAUAACGUAACUCAdTdT	UGAGUUACGUUAUGAUGAUdTdT
Target 3	UUCAUCAUCAUAACGUAACdTdT	GUUACGUUAUGAUGAUGAAdTdT

4.2.2. RNA transcript quantification (Real-time PCR)

Total RNA was isolated from all cell cultures using QIAzol™ reagent according to the manufacturer's instructions (Qiagen®). Reverse transcription of 3 µg RNA with 200 U M-MLV reverse transcriptase (Promega) was carried out in a volume of 40 µl using 0.5 µg random hexamer primers (Inqaba Biotech™). Real-time-PCR was performed using an iCycler iQ™ (Bio-Rad) instrument in a final volume of 20 µl that contained the iQ™ SYBR Green® Supermix and 500

nM forward and reverse primers. NDUFS3 transcripts (see Table 4.2), as well as other transcripts, were amplified using 75 ng cDNA in addition to the forward and reverse primers. The amplifications were normalised to β2-microglobulin transcripts (Table 4.2; Radonic *et al.*, 2004) which were shown to remain stable in an induced complex I deficiency (Reinecke *et al.*, 2006).

Relative quantification was also determined for other mitochondrial- and nuclear encoded transcripts (Table 4.2) and normalised to β2-microglobulin transcripts. The mean Ct values (n = 3), from the iCycler iQ™ Real-time Detection System Sortware, version 3.0 (Bio-Rad) of all amplifications were analyzed with BestKeeper software tool (Pfaffl *et al.* 2002). The GeNorm software tool (Vandesompele *et al.*, 2002) was utilised to calculate the relative expression quantities for each sample via the comparative Ct method.

Table 4.2. Primer sequences for mitochondrial- and nuclear-encoded transcripts.

Transcript	Forward primer 5′-3′	Reverse primer 5′-3′	Genbank® accession number
Reference transcript			
β2-microglobulin	AGCGTACTCCAAAGATTCAGGTT	ATGATGCTGCTTACATGTCTCGAT	NM_004048
Mitochondrial transcripts			
ND1	GTCTCAGGCTTCAACATCG	CGAATTCATAAGAACAGGGAG	NC_012920
ND6	CTCCTCAATAGCCATCGCTG	GATTGTTAGCGGTGTGGTCG	NC_012920
COXII	CATCCTAGTCCTCATCGCC	GATTAGTCCGCCGTAGTCG	NC_012920
D-loop	CCTAACACCAGCCTAACCAG	TGATGAGATTAGTAGTATGGGAG	NC_012920

Transcript	Forward primer 5′-3′	Reverse primer 5′-3′	Genbank® accession number
Nuclear-encoded structural transcripts			
NDUFS3	GCTGACGCCCATTGAGTCTG	GGAACTCTTGGGCCAACTCC	NM_004551
NDUFA9	GGAGCAACAGGATTCCTGGG	CTGTGTTGTACTACTCGTCGG	NM_005002
CII-70kD FP	GACGTCACGAAGGAGCCG	CGGTTGGCACCATGTACCG	NM_004168
CII-30 kD IP	CTTATGCAGGCCTATCGCTG	CTTGTGCAGTTCATGATGGTG	NM_003000
CIII-core 2	GTCGTTGGGAAGTAGCTGAC	GGATTAGCCAAGGCATTCCG	NM_003366
CV-α subunit	GGTCGTGTAGTTGATGCCC	CAGTCTGCATTGGTTCCCG	NM_004046
Nuclear-encoded functional transcripts			
TFAM	CCGGCTGTGGAAGTCGAC	GTTCCCTCCAACGCTGGGC	NM_003201
mtSSB	GAGATGTGGCGATCAGGG	GATTGTTGTTGCTTGTCGCC	NM_003143
TFB2M	CGCGAAAGCATTTGCCGGC	TCACTTTCGAGCGCAACCAC	NM_022366
POLRMT	CAAGCCGCATCGTTCCTCTC	GCCTCGAAGGTCAGCGTGG	NM_005035
DNApolγ	GGTTGCTGAACGGGCATGG	GAGGTTGGTGATCTGCAAGG	NM_002693

4.2.3. Total mitochondrial RNA levels (Northern blot analysis)

Total RNA (3 µg per lane) was separated by agarose gel electrophoresis using a 1.5% (w/v) agarose gel containing formaldehyde. The separated RNA was transferred to Hybond™-N+ nylon membranes (Amersham Biosciences), hybridised and washed as described before (Brown *et al.*, 1997). The mitochondrial probe utilised in hybridisation was generated with a Random Primed labelling kit (Roche) labelled with Redivue (α-^{32}P)dTTP (Amersham Biosciences). The template for labelling, which contained the full length mtDNA sequence, was generated as described by Reynier *et al.* (1998) using the TripleMaster™ PCR System (Eppendorf®). Unincorporated nucleotides were removed by using the Wizard® SV Gel and PCR Clean-up System (Promega). Membranes were exposed overnight at -70ºC to BioMax® light film (Kodak™) and the ECL signals were quantified (densitometrically) with GeneTools 3.06 Imaging software (SynGene).

4.2.4. Protein analyses and complex I activity

Cells were re-suspended in PBS (Gibco™) after harvesting and treated as described before (Nijtmans *et al.*, 2002) for SDS-PAGE analysis. Protein content was determined with the BCA method (Smith *et al.*, 1985) and 10 µg protein loaded onto a 10% (v/v) SDS-PAGE gel. BN-PAGE gradient gels (5-15%) were prepared and loaded with 40 µg mitochondrial fraction as described before (Nijtmans *et al.*, 2002) after which an in-gel activity assay for complex I was carried out. Western blotting was performed with primary antibodies against NDUFS3 and Cyclophilin D (Mitosciences®) and CII-70 for normalisation of in-gel activity, together with a secondary goat anti-(mouse Ig) Ig peroxidase antibody (Zymed). Signal detection was performed with ECL Western blotting substrate (Pierce) and the blots exposed to BioMax® light film (Kodak™). The signals were quantified with GeneTools 3.06 Imaging software (SynGene).

4.2.5. ADP/ATP ratio

ADP/ATP ratios were quantified using a modification of a method described previously (Au, Su & Weintjes, 1989). Whole cell pellets, collected after scraping cells in PBS and centrifugation at 600 x g, were resuspended in 100 µl double de-ionized water (MilliQ, Millipore), sonicated on ice for 5 seconds and 300 µl acetonitrile added to precipitate proteins. After addition of 100 µl double de-ionized water, samples were incubated on ice for 10 minutes and centrifuged for 10 minutes at 16 000 x g. The supernatants were dried under nitrogen at 40 °C and dissolved in 1 M KH_2PO_4 (pH 6). The chromatographic system consisted of a HPLC pump (Dionex P580A HPG), equipped with an online degasser (DG-2410), an automated sample injector (ASI-100) and a Dionex UV/VIS detector (UVD 170S/340S). A Phenomenex C18 column (2 mm x 250 mm) was used, protected by a guard column (4 mm x 2mm) containing a silica-based C18 sorbent packing. Buffer A consisted of 0.1 M KH_2PO_4 with 8 mM tetrabutylammoniumbromide (TBA) (pH 6.0) and Buffer B consisted of 0.1 M KH_2PO_4, 8 mM TBA and 30% (v/v) acetonitrile (pH 6.0). Of the sample, 50 µl was injected and the following program used for separation: the flow rate was kept constant at 0.2 ml/minute over the entire run. Initially, 100% (v/v) Buffer A was used for 2.5 minutes. The concentration of

Buffer B was gradually increased to 10% (v/v) over the next 1.5 minutes and kept constant for 1 minute. Buffer B was increased to 20% (v/v) over 1 minute and then increased to 40% (v/v) over the next 10 minutes. For the next 15 minutes, Buffer B was increased to 60% (v/v) and then to 100% over the next 1 minute. The concentration of Buffer B was kept constant at 100% for 10 minutes before it was returned to the initial concentration over 1 minute. The initial concentration was maintained for 15 minutes to recondition the C18-column for the next sample. ADP and ATP was monitored at 254 nm and quantified using a calibration curve, obtained by analyzing five standards in duplicate ($R^2 > 0.99$).

4.2.6. ROS production

To measure ROS levels, cells were incubated for 30 minutes with 10 µM of the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA, Molecular probes®). Cells were trypsinated and re-suspended in PBS before detection on a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson). Cells were analyzed at a flow rate of 1000 events per second and 10 000 events were collected for each sample. Cells were excited with an argon laser emitting at 488 nm. Forward and side scatter was collected on a linear scale and DCF fluorescence was detected by FL1 PMT using log amplification. Unstained 143B cells were used as negative control and as a positive control for ROS production, control cells were treated for 24 hours with 100nM rotenone (results not shown). Data were acquired using CELLQuest PRO (Becton Dickinson) and analysed by FCS Express (version 3, De Novo Software). Results were expressed as ratios between mean fluorescence intensities, normalised to events.

4.2.7. Relative mitochondrial DNA copy number

Total DNA was isolated as described previously (Driggers *et al.*, 1997). Relative mitochondrial DNA (mtDNA) copy number was determined using relative quantification of the mtDNA *ND1* gene in 100 ng total DNA with real-time PCR as described in Section 4.2.2. Copy number was quantified relative to *GAPDH* (GenBank AY340484; forward primer 5′-

CTCACGTATTCCCCCAGG- 3′ and reverse primer 5′-GAAGATGGTGATGGGATTTC-3′). As negative control, 143B ρ0 cybrid cells (mtDNA less) were included (results not shown).

4.2.8. Statistical analyses

All results were analyzed with GraphPad Prism (version 5) software as mean ± standard deviation. Statistical comparisons were made using one-way analysis of variance (ANOVA) with post hoc comparison (Tukey test). Statistically, significance was considered when $p < 0.05$.

4.3. RESULTS AND DISCUSSION

4.3.1. Establishment of NDUFS3 knockdown in 143B cells and evaluation of relevant biochemical parameters

To investigate the consequences of complex I deficiency *in vitro*, the initial objective of this study was to develop a transient (siRNA) knockdown of the NDUFS3 subunit of complex I in 143B cybrid cells. Initially three different siRNA sequences were selected (Table 4.1) and their knockdown efficiencies evaluated based on protein expression level as well as enzyme activity of Targets 2 and 3 (Target 1 was eliminated after the protein evaluation). Initial experiments over 0-96 hours with 100 nM siRNA showed decreased NDUFS3 protein levels after 72 hours (results not shown), with Target 3 showing significant knockdown on protein level (Figure 4.1.A), and significantly reduced enzyme activity as measured with BN-PAGE in-gel activity (Figure 4.1.B). Based on these results it was decided to use Target 3 in all subsequent experiments due to higher knockdown and better cell viability (as observed visibly, but not measured) compared to Targets 1 and 2 with repeated transfections.

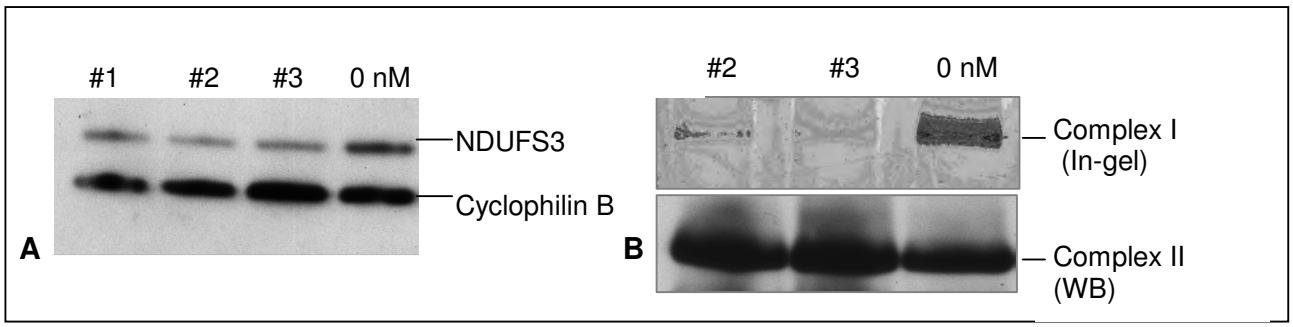

Figure 4.1. Protein expression and complex I in-gel activity analysis after siRNA knockdown with different targets. (A) Western blotting of NDUFS3 protein expression relative to cyclophilin B expression. (B) BN-PAGE in-gel activity of complex I relative to complex II protein expression after transfection (72 hrs) with Targets 1, 2 and 3 (100 nM) as well as transfections with only oligofectamine (0 mM). Western blotting was used to quantify protein expression of NDUFS3, relative to cyclophilin B expression.

To investigate the effect of different concentrations of siRNA transfection of Target 3 into the cells, two different concentrations of siRNA were used in all experiments (10 nM and 100 nM final concentrations). Analyses with real-time PCR showed an 85% and 82% decrease in NDUFS3 transcript ratios respectively for 10 nM and 100 nM siRNA knockdown relative to control 0 nM siRNA knockdown (Figure 4.2.A) when normalised to β2-microglobulin expression. Protein expression levels, as evaluated by western blotting, confirmed the mRNA expression results, with 10 and 100 nM of Target 3 both leading to similar levels of significant knockdown in NDUFS3 protein level relative to control, when normalised to cyclophilin B protein levels (Figure 4.2.B). BN-PAGE in-gel activity for complex I (Figure 4.2.C) also showed significantly reduced activity for 10 nM and 100 nM knockdown when compared to 0 nM and normalised to complex II expression. Thus, 10 nM and 100 nM siRNA transfections of Target 3 siRNA lead to successful complex I deficiency profile, with 10 nM leading to slightly more reduced NDUFS3 expression (on RNA and protein level) than 100 nM.

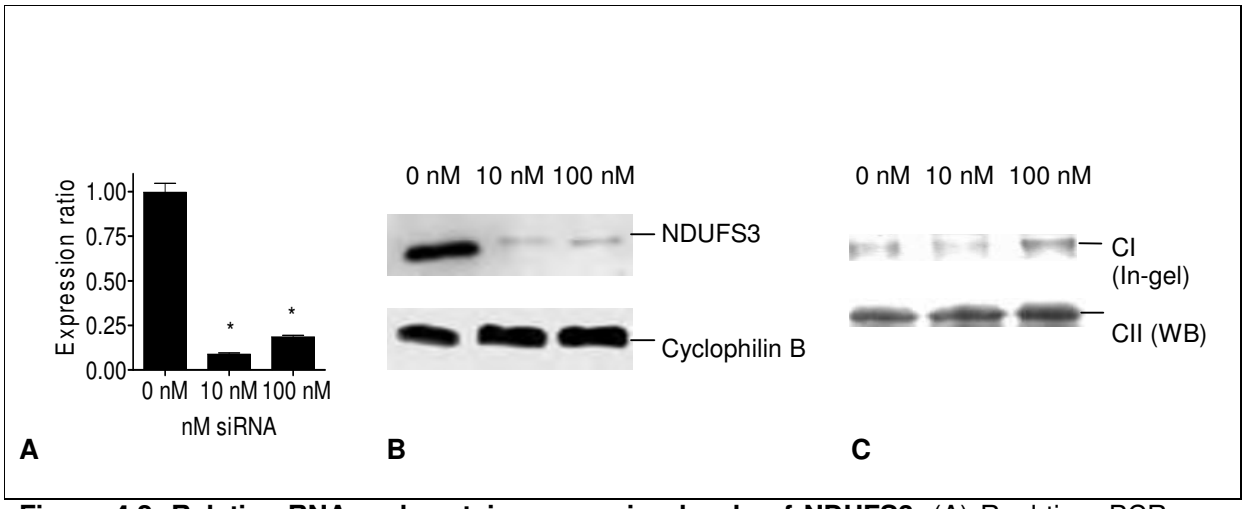

Figure 4.2. Relative RNA and protein expression levels of NDUFS3. (A) Real-time PCR was used to quantify NDUFS3 mRNA in Target 3 transfected (10 and 100 nM) and untransfected (0 nM) 143B cells as a ratio normalized to β2-microglobulin expression (n = 6), *p<0.05 (ANOVA, Tukey test). (B) Western blotting was used to quantify protein expression of NDUFS3 in Target 3 transfected (10 and 100 nM) and untransfected (0 nM) 143B cells, relative to cyclophilin D expression. (C) BN-PAGE in-gel activity of complex I, relative to complex II protein expression was used to quantify complex I enzyme activity in Target 3 transfected (10 and 100 nM) and untransfected (0 nM) 143B cells.

Two biochemical parameters often associated with deficient respiratory chain function, ADP/ATP ratio (Figure 4.3) and ROS production (Figure 4.4), were measured and revealed additional information on the effect of the complex I intervention. ADP/ATP ratios (Figure 4.3) increased slightly and not statistically significantly, with NDUFS3 knockdown, with 15% and 17% increases for 10 and 100 nM siRNA respectively, relative to the control (0 nM). Previous studies found a strong correlation between decreased ATP levels and decreased complex I activity (Heidari *et al.*, 2009, Distelmaier *et al.*, 2009b). In the present study, the ATP levels only decreased slightly with significant complex I enzyme inhibition due to NDUFS3 siRNA knockdown which might correlate with a previous study by Visch et al. (2004) where unstimulated ATP levels were unchanged in complex I deficient fibroblasts. This finding may be attributed to the observation that cancerous cells such as 143B cells rely more on glycolysis, rather than OXPHOS, for ATP production and this is known as the Warburg effect (Warburg, 1956; Reitzer *et al.*, 1979). To better

challenge mitochondrial energy metabolism *in vitro,* a medium in which galactose is the carbon energy source can be utilised. This induces increased glutaminolysis by the TCA cycle, which provides reducing equivalents to the OXPHOS system and, therefore, forces the cells to rely on OXPHOS rather than glycolysis for energy (Reitzer *et al.*, 1979), as described in Section 5.2.1. However, due to the observation that poor cell division occurs when using galactose (results not shown), this was not done for the transient model described here.

Although only a slight increase (9%) in ROS levels was detected with 10 nM siRNA knockdown (Figure 4.4), a 100 nM siRNA knockdown resulted in an 83% increase in ROS levels relative to the 0 nM control. Non-specific or off-target effects are changes in mRNA expression due to interaction between the siRNA guide strand and partially complementary but unrelated sites (Alemán *et al.*, 2007). It has been proposed that these off-target effects increase with increased siRNA concentration, especially above 10 nM (Semizarov *et al.*, 2003; Tschuch *et al.*, 2008; Persengiev *et al.*, 2004). It is therefore important to consider these nonspecific effects when interpreting experiments with 100 nM siRNA transfection. Since 10 nM and 100 nM knockdown both showed similar significant inhibition in complex I enzyme activity, but showed significant differences in ROS levels (9 and 83% increase respectively), this increase in ROS levels at 100 nM was interpreted to be due to nonspecific effects that resulted in cellular stress and subsequently increased oxidative stress.

As discussed also in Chapters 2 and 3, increased ROS production has been reported with several studies involving complex I deficiency (Vives-Bauza, 2006) and it has been proposed that increased ROS production could correlate with the severity of the deficiency, or decreased complex I activity (Koopman *et al.*, 2007; Verkaart *et al.*, 2007a; Quinzii *et al.*, 2008). However, there are exceptions to this, such as has been reported for a mutation in NDUFS4 (Iuso *et al.*, 2006). Therefore, when considering the 10 nM siRNA knockdown with only 9% increase in ROS, it is striking that the significantly reduced complex I activity in the 10 nM siRNA knockdown did not lead to significantly increased ROS levels. As was discussed before, this may also have been affected by the energy metabolism in these cells being mainly directed to glycolysis, thus leading to

relatively less electrons flowing through the respiratory chain with less leakage by a deficient complex I.

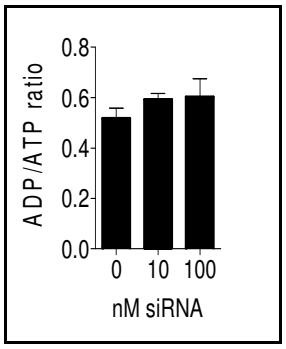

Figure 4.3. Relative ADP/ATP ratio evaluation of NDUFS3 transient knockdown. ADP/ATP ratios ratio (n = 7) were measured with reverse-phase (ion-pairing) HPLC in Target 3 transfected (10 and 100 nM) and untransfected (0 nM) 143B cells, *p<0.05 (ANOVA, Tukey test).

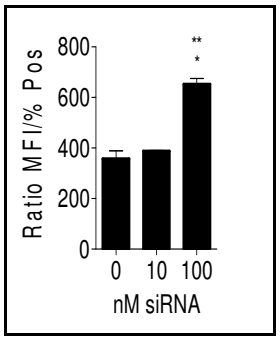

Figure 4.4. Relative ROS production evaluation of NDUFS3 transient knockdown. Flow cytometry with the DCFH-DA probe as used to measure ROS production in Target 3 transfected (10 and 100 nM) and untransfected (0 nM) 143B cells (n = 3), *p<0.05 (ANOVA, Tukey test) compared to 0 nM, ** p<0.05 compared to 10 nM.

4.3.2. Evaluation of relative mtDNA and mtRNA levels

Real-time-PCR analyses of the mitochondrially encoded ND1 gene normalised to the nuclear encoded GAPDH gene showed 92% and 102% of control copy number for the 10 nM and 100 nM siRNA knockdown, respectively (Figure 4.5). This indicates no significant changes in the

mtDNA copy number with NDUFS3 knockdown. Several previous studies showed mostly decreased copy numbers in skeletal muscle, liver and brain tissue (Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonod-Bidaud, 1999) and unchanged or increased mtDNA copy number ratios in heart, kidney and skeletal muscle (Bonod-Bidaud, 1999), depending on the OXPHOS deficiency. It has been suggested that the tissues with the highest energy demand (like brain, heart and muscle) are most affected by OXPHOS deficiencies (Scheffler, 2001) and this may lead to the differentially affected mtDNA copy number ratio in different tissues. In this study, the osteosarcoma-derived 143B cells were used, which does not have such a high OXPHOS energy demand such as muscle, brain and heart tissue and this might, at least in part, explain the lack of differential mtDNA copy number ratios in the presence of the siRNA knockdown of complex I. It is interesting to note that the 100 nM siRNA transfections, which had increased ROS levels (albeit unspecific to complex I deficiency) did not result in changed mtDNA copy number ratio. This would suggest that cellular ROS increase alone in these cells does not result in changes in the mitochondrial replication machinery functions.

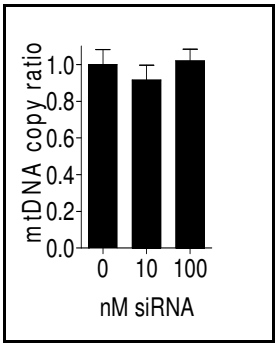

Figure 4.5. Relative mtDNA copy number. ND1 mitochondrial copy number was normalized to GAPDH nuclear copy number ($n = 9$), *$p<0.05$ (ANOVA, Tukey test). 143B ρ0 cells showed relative mtDNA copy number of 0.05±0.01.

Relative quantification of mitochondrial RNA (Figure 4.6) showed almost no change (4%decrease) in total mtRNA levels with knockdown using the 10 nM siRNA concentration. However, when using 100 nM, a significant decrease of 19% was detected. One of the first possibilities may be that this could be due to increased oxidative damage to mtRNA or differential

regulation of genes involved in mtDNA replication/transcription at 100 nM siRNA transfections. However, in these transient knockdown experiments, complex I activity did not correlate with increased ROS levels when compared to results from Figure 4.2 and 4.4. Therefore, it can be concluded that off-target effects at 100 nM siRNA knockdown might have been responsible for, not only the increased ROS levels, but also the observed decreased total mtRNA levels. It is unlikely that the decreased mtRNA levels at 100 nM siRNA knockdown resulted from regulatory responses due to OXPHOS deficiency, including those evaluated further in this study, since the mtRNA levels at 10 nM are not similar to those of 100 nM, although they both have similar complex I enzyme inhibition.

To further investigate and better quantify the mitochondrial transcriptional responses, levels of four mitochondrial encoded transcripts were evaluated and, as was the trend when evaluating total mtRNA, a decreased expression with NDUFS3 knockdown was observed for all four transcripts (Figure 4.7). Both H-strand transcripts (of ND1 and COXII genes) showed approximately 17% (10 nM) and 32% (100 nM) decrease, whilst the L-strand transcript (for ND6 gene) showed 0% (10 nM) and 22% (100 nM) decrease. The displacement loop (D-loop), the only non-coding region where replication/transcription was initiated from, showed 9% (10 nM) and 32% (100 nM) decrease in transcript levels, respectively for the two transfections. It appeared from these results that the complex I deficiency due to NDUFS3 knockdown (at 10 nM siRNA) reduced H-strand transcript levels more than L-strand levels, which were transcribed by two different promoters. Differences between H- and L-strand transcript levels were also detected in earlier studies and it was proposed that these were probably due to different rates of processing or transcription and/or different half lives for the two strands (Heddi *et al.*, 1993, Duborjal *et al.*, 2002, Gelfand & Attardi, 1981). These differences in H- and L-transcript levels were also observed for the stable knockdown cell lines described Chapter 5.3.2. The expression pattern of the four mitochondrial transcripts with increased siRNA concentration was similar to that of the general observation for total reduced mtRNA levels (Figure 4.6), probably due to the reasons mentioned above.

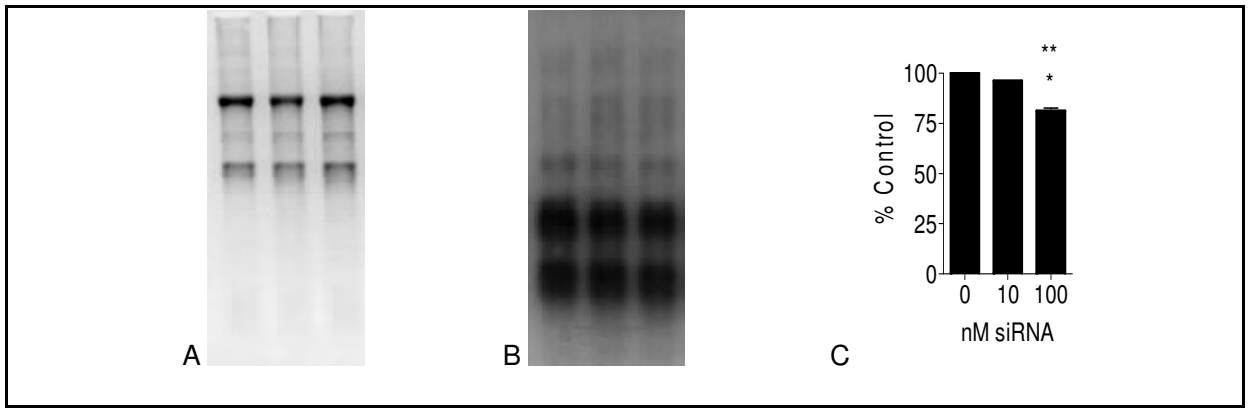

Figure 4.6. Northern blot of total mtRNA. A) 3 µg total RNA loaded on 1.5% denaturing agarose gel to confirm equal loading. B) Northern blot hybridised with mtDNA probe. C) Ratio of total RNA relative to 0 nM siRNA (n = 2), on Northern blot, *p<0.05 (ANOVA, Tukey test) compared to 0 nM, ** p<0.05 compared to 10 nM.

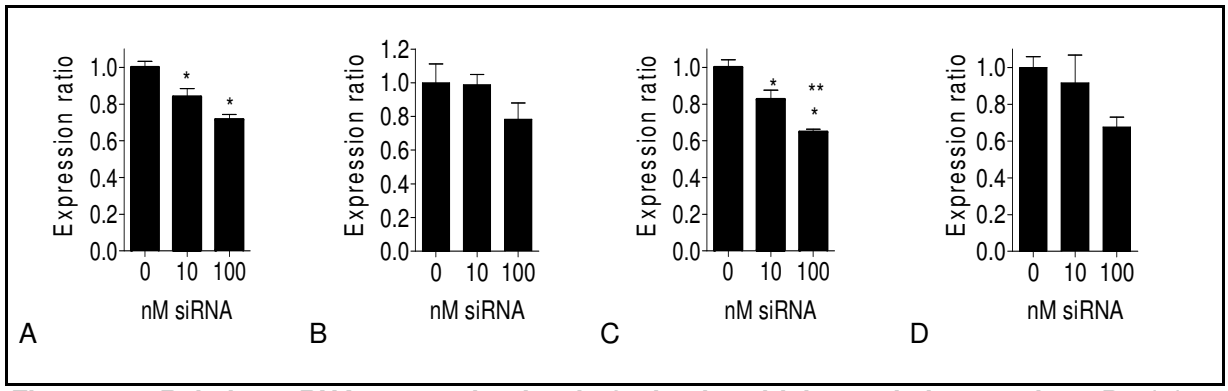

Figure 4.7. Relative mRNA expression level of mitochondrial encoded transcripts. Real-time PCR was used to quantify A) ND1 (n = 9), B) ND6 (n = 3), C) COXII (n = 3) and D) D-loop (n = 9) mRNA as a ratio normalized to β2-microglobulin expression, *p<0.05 (ANOVA, Tukey test) compared to 0 nM, ** p<0.05 compared to 10 nM.

4.3.3. Evaluation of selected nuclear transcript levels

Of the five nuclear encoded structural transcripts, there were no general statistically significant changes in RNA levels with transfections at 10 nM or 100 nM siRNA (Figure 4.8). Expression of CII-70kD was more reduced than CII-30kD expression with 10 nM siRNA knockdown and CV-α expression was also slightly reduced, whilst the rest of the transcripts were unchanged. In a previous study by Chevallet and coworkers (2006) it was found that in 143Bρ0

cells (depleted of mtDNA) CII and CV subunits did not show any changes in protein expression, whilst subunits from CI, CIII and CIV showed variable levels of reduction in protein levels and expression of some subunits of CI and CIV was not altered at all. Several other studies also showed different ratios of increased or decreased expression of OXPHOS subunits in the presence of OXPHOS deficiencies (Collombet *et al.*, 1997, Bonond-Bidaud *et al.*, 1999, Heddi *et al.*, 1999, as discussed in Reinecke *et al.*, 2009; Chapter 2). It was proposed that this might be due to some sub-complexes having additional functions, or because they have different stabilities, or thirdly, owing to a lack of coordinated regulation of nuclear transcription (Chevallet *et al.*, 2006, Marusich *et al.*, 1997).

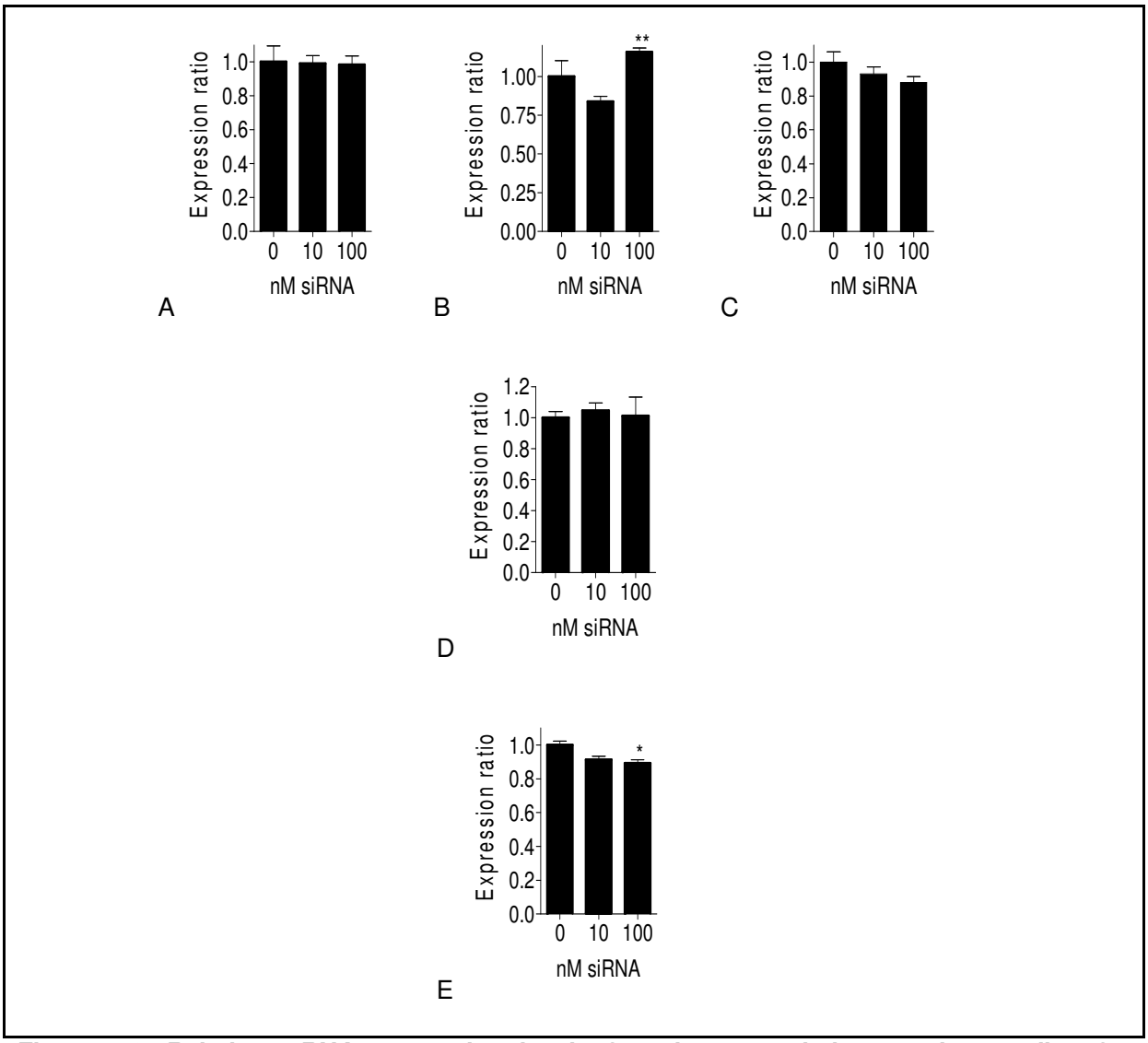

Figure 4.8. Relative mRNA expression level of nuclear encoded transcripts coding for structural proteins. Real-time PCR was used to quantify A) NDUFA9 (*n* = 6), B) CII-70kD FP (*n* = 9), C) CII-30 kD IP (*n* = 3), D) CIII-core 2 (*n* = 3) and E) CV-α subunit (*n* = 3) mRNA as a ratio normalized to β2-microglobulin expression, **p*<0.05 (ANOVA, Tukey test) compared to 0 nM, ** *p*<0.05 compared to 10 nM.

4.3.4. Evaluation of selected transcripts involved in mtDNA regulation

Investigations into the expression levels of five nuclear-encoded transcripts for proteins involved in mtDNA replication/transcription (Figure 4.9) showed, with the exception of TFB2M and DNApolγ, a slight decrease in expression, though none statistically significant. Compared to control

cells, expression of TFAM mRNA was 91% (10 nM) and 89% (100 nM) with siRNA transfections, whilst expression of mtSSB mRNA was comparable with 86% (10 nM) and 89% (100 nM) and POLRMT expression was 90% (10 nM) and 80% (100 nM). Previous studies showed that expression of TFAM, mtSSB and POLRMT tightly correlates with mtDNA copy number (Schultz *et al.*, 1998, Seidel-Rogol *et al.*, 2002, Davis *et al.*, 1996; Ekstrand *et al.*, 2004). This observation is similar to the results of the 10 nM knockdown showing 92% mtDNA copy number when compared to the control sample and very slight reduction in TFAM, mtSSB and POLRMT expression (85-91%). TFAM expression is regulated by the two nuclear transcription factors, NRF-1and NRF-2, which have also been shown to be redox sensitive (Piantadosi & Suliman, 2006; Miranda *et al.*, 1999; Leung *et al.*, 2003) and increased TFAM expression was also linked to increased ROS levels in mtDNA-depleted cells (Miranda *et al.*, 1999). In the current study, however, TFAM expression was only slightly reduced and ROS levels slightly were increased (9%), though both statistically insignificant.

The DNApolγ expression level was even more reduced (to 74% (10 nM) and 63% (100 nM, statistically significant) compared to controls) than with TFAM, mtSSB and POLRMT. According to various studies, DNApolγ expression is not affected by mtDNA copy number (Schultz *et al.*, 1998, Davis *et al.*, 1996), although a study by Hance *et al.* (2005) showed that DNApolγ expression was increased as a result of increased mtDNA copy number due to TFAM over-expression and that a partially DNApolγ-deficient animal model had slightly reduced mtDNA copy number. In the current study DNApolγ expression levels were reduced (74%) in 10 nM siRNA knockdown cells, with mtDNA copy number, TFAM, mtSSB and POLRMT only slightly reduced. The reduced expression pattern of DNApolγ was also similar to the expression pattern of the H-strand transcripts with siRNA transfection.

Interestingly, the only transcript encoding a regulatory protein that showed a different expression pattern was that of TFB2M (Figure 4.9), with a 33% (10 nM) and 61% (100 nM) increase in expression. The two transcription factors, TFB1M and TFB2M, showed close resemblance to a family of rRNA methyltransferases. The presence of either one in addition to

TFAM is necessary in order for POLRMT to initiate mtDNA transcription (Falkenberg *et al.*, 2002; Asin-Cayuela & Gustafsson, 2007). It has been suggested that the primary function of TFB2M is to act as transcription factor for mtDNA replication/transcription, whilst TFB1M is primarily involved with rRNA methylation (Asin-Cayuela & Gustafsson, 2007). The increased expression of TFB2M transcripts in this study might indicate that TFB2M is a key player in the proposed compensatory mechanism for OXPHOS deficiency as described by Heddi and coworkers (1999).

The two nuclear transcription factors NRF-1 and NRF-2, which are involved in TFB2M and TFAM transcription (Gleyzer *et al.*, 2005; Miranda *et al.*, 1999), have also been shown to be redox sensitive (Miranda *et al.*, 1999; Leung *et al.*, 2003). The transcriptional control of NRF-1 and NRF-2 could explain the slightly increased expression of TFB2M with slightly increased ROS levels in 10 nM siRNA knockdown cells and even more so at 100 nM transfections, but not the slightly reduced expression of TFAM with slightly increased ROS production. Also, in the past it was shown that over expression of either TFB2M or TFAM led to increased mtDNA copy number (Scarpulla, 2008), which is in contrast to the slightly reduced expression of TFAM and mtDNA copy number, and increased TFB2M expression, found with 10 nM siRNA knockdown in this study. In a previous study by Seidel-Rogol *et al.* (2002), increased TFAM and POLRMT expression was delayed relative to mtDNA copy number when partially mtDNA-depleted cells were grown without ethidium bromide. The mitochondrial transcript levels were also increased early after recovery of the cells from ethidium bromide, whilst mtDNA copy number, TFAM and POLRMT levels were still low and the authors concluded that one reason for this might be that another transcription factor, such as TFB2M, might be responsible for this early increase in transcription/replication. It is therefore possible that TFB2M might be the first regulatory transcript to be increased as part of the compensatory mechanism in OXPHOS deficient cells and in particular, considering this model to be a transient one, before responses (increases) in mtDNA copy number, TFAM and POLRMT may occur.

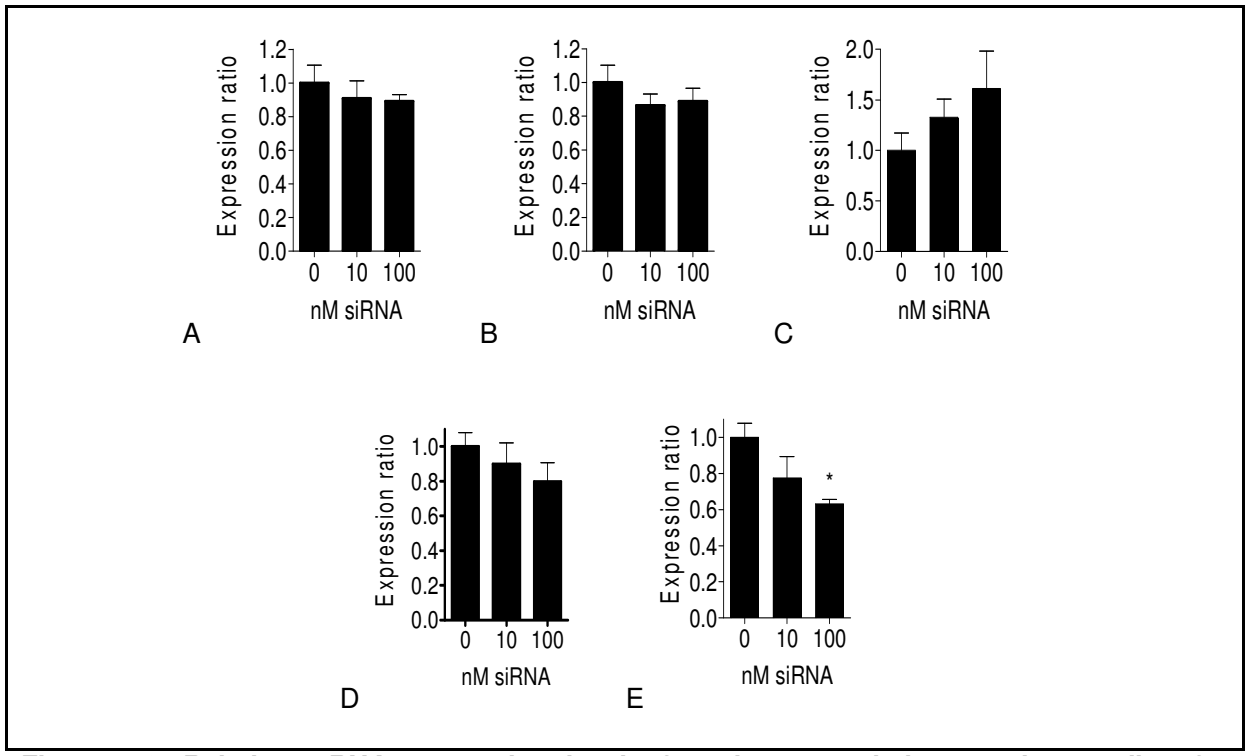

Figure 4.9. Relative mRNA expression level of nuclear encoded transcripts coding for proteins involved in mtDNA transcription/replication. Real-time PCR was used to quantify A) TFAM (*n* = 3), B) mtSSB (*n* = 3), C) TFB2M (*n* = 6), D) POLRMT (*n* = 3) and E) DNApolγ (*n* = 6) mRNA as a ratio normalized to β2-microglobulin expression, **p*<0.05 (ANOVA, Tukey test).

4.4. SUMMARY

The status of mitochondrial DNA (mtDNA) and mtRNA as well as transcriptional expression of genes involved in mtDNA replication and transcription was investigated after establishing transient NADH:ubiquinone oxidoreductase (complex I) deficiency *in vitro* via siRNA knockdown of the subunit, NDUFS3. Significant knockdown of NDUFS3 on both RNA and protein levels in 143B cells was observed and this was supported by the significant (levels associated with a deficiency) inhibition of complex I activity. Decreased ATP production was measured (although not statistically significant), but ROS levels in the cell lines transfected with 10 nM siRNA were not significantly increased. In addition, mtDNA copy number and total mtRNA levels were not significantly decreased with 10 nM knockdown. When evaluating this knockdown model and the subsequent evaluation of the effect on mtDNA replication and transcription the use and limitations of siRNA

have to be considered. These limitations will be discussed in more detail in Chapter 6. Concerning the use of siRNA, one limitation that has to be recognised is the influence of the oligonucleotide transfections used for RNA interference, which was evident from the use of two siRNA concentrations (10 nM and 100 nM) in this study. Both 10 nM and 100 nM transfections showed similar significant inhibition in complex I enzyme activity, but showed significant differences in ROS levels (9 and 83% increase respectively) and also significant decrease in total mtDNA and mtRNA transcripts levels with 100 nM transfection. The changes in the 100 nM siRNA knockdown cells are, therefore, not related to the level of complex I knockdown or deficiency, but rather to a secondary off-target effect of the siRNA transfection.

In the current study the successful inhibition of complex I activity with 10 nM siRNA knockdown was not accompanied by increased ROS production. This could support the finding by Iuso *et al.* (2006) that not all complex I deficiencies lead to increased ROS production. It also implies that any changes in mitochondrial or nuclear transcript levels, or the levels of transcripts involved in mtDNA regulation, are not due to increased oxidative damage. This includes the significant decrease in mitochondrial H-strand transcripts (which is probably due to differential regulation or half-lives), the decrease in DNApolγ expression (although not significant) and the increased expression of TFB2M (also not significant). As also discussed in the previous sections, another factor that has to be recognized is the limited dependence on OXPHOS by cancerous cell lines which limit the effect of a stressor on the OXPHOS system on the parameters measured in this study (ADP/ATP and ROS). With the use of transiently induced deficiencies and the methodology used for it (i.e. using glucose as carbon source for optimal cell growth) it is also not feasible to redirect energy metabolism.

The reduced expression of DNApolγ (although not significant) with siRNA transfection contradicts the general perception that its expression is not differentially affected even when mtDNA copy number or other regulatory transcripts are affected. Although mtDNA copy number was relatively unchanged, the DNApolγ expression pattern was similar to that of the H-strand transcripts. Because of the lack of significant ROS production with the 10 nM siRNA transfection,

we must conclude that this differential expression is not due to redox-sensitive regulation of DNApolγ, but it might be due to the coordinate regulation of factors involved with mtDNA regulation/transcription due to retrograde signalling not involving ROS production.

Because TFB2M expression was increased (although not significantly) with siRNA transfection, whilst TFAM, mtSSB and POLRMT transcripts encoding was only slightly decreased, it is possible that TFB2M might be the first regulatory transcript to be increased as part of the compensatory mechanism in OXPHOS deficient cells, before responses (increases) in mtDNA copy number, TFAM and POLRMT may occur. To further investigate this, it will be necessary to evaluate the differential expression of these genes in a stable knockdown model, where long-term effects can be evaluated, and where the secondary effects of siRNA transfections can be eliminated from the investigation (see Chapter 5).

CHAPTER FIVE

INVESTIGATION OF THE CONSEQUENCES OF COMPLEXES I AND III DEFICIENCY ON MITOCHONDRIAL DNA REPLICATION/TRANSCRIPTION BY MEANS OF INDUCING STABLE KNOCKDOWN IN 143B CELLS

The information contained in this chapter in part forms the basis for the paper entitled "Mitochondrial DNA replication and OXPHOS gene transcription show weak responsiveness to Rieske protein knockdown in 143B cells" submitted for publication in Biochimie (2010), which is attached in Appendix C and the paper in preparation to be submitted for publication in Analytical Biochemistry (2010), which is attached in Appendix D.

5.1. INTRODUCTION

Complex I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol cytochrome *c* reductase) are considered to be the main sources of superoxide radical production in the OXPHOS system (Turrens *et al.*, 1980; Dröse & Brandt, 2008). Both are multi-subunit enzyme complexes encoded by mitochondrial- and nuclear DNA (Hunte *et al.*, 2003; Bénit *et al.*, 2009; Carroll *et al.*, 2006; Hirst *et al.*, 2003) and both lead to a spectrum of clinical diseases, from exercise intolerance to lethal multi-systemic disorders (Bénit *et al.*, 2009, Distelmaier *et al.*, 2009a), although deficiencies of complex I are much more common than those of complex III (Loeffen *et al.*, 2000). Biochemical consequences of such deficiencies often, but not inevitably, include increased formation of ROS (which could damage lipids, proteins and DNA), loss of ATP production as well as mitochondrial membrane potential, calcium regulation and apoptosis (Vives-Bauza *et al.*, 2006, Verkaart *et al.*, 2007a, Koopman *et al.*, 2007, Smeitink *et al.*, 2006, Brookes *et al.*, 2004).

Another consequence of OXPHOS deficiencies is the differential expression of several nuclear and mitochondrial genes involved in mitochondrial bioenergetics (as summarised in

Reinecke *et al*., 2009). For mtDNA, which is reported to respond to oxidative capacity and energy demand (Lee & Wei, 2005), an up-regulation of replication and transcription appears to be a fitting response considering that several of its nuclear encoded control elements also respond to stress-related retrograde effectors and in particular increased oxidative stress originating from mitochondria (Lee & Wei, 2005; Seidel-Rogol & Shadel, 2003; Miranda *et al*., 1999; Davis *et al*., 1996; Virbasius & Scarpulla, 1994). However, increased oxidative stress is not always observed where even severe deficiencies of the OXPHOS system occur (Iuso *et al*., 2006; Schauen *et al*., 2006). This raises the question if mtDNA is indeed differentially expressed and replicated in OXPHOS deficiencies that do not result in significantly elevated superoxide production. Also, from the *in vitro* transient NDUFS3 knockdown model of complex I deficiency (discussed in Chapter 4), several questions arose as to the suitability of a transient siRNA knockdown model to study the effects of OXPHOS deficiency on differential gene expression.

We investigated these questions using complex I and III deficient *in vitro* models, using stable knockdown of the NDUFS3 subunit (complex I) as well as the Rieske protein (complex III) in 143B cells. Complex III is assembled from 11 subunits, of which three (Rieske Fe-S protein, cytochrome *b* and cytochrome *c*1) transport electrons from ubiquinol to cytochrome *c* through the ubiquinone (Q) cycle. Although complex III is one of the major sites in the respiratory chain responsible for the production of superoxide radicals, which occurs at both the ubiquinol oxidizing (Qo) and the ubiquinone reducing (Qi) sites (Hunte *et al*., 2003; Zhang *et al.* 1998), a deficiency of the Rieske protein (forming part of the Qo site) would result in limited superoxide production but would still prevent electron transport to cytochrome *c* (Chen, 2003; Muller *et al*., 2004).

From Chapter 4, where a transient NDUFS3 siRNA knockdown model of *in vitro* complex I deficiency was studied, no significant changes in ADP/ATP ratios, ROS levels or differential gene expression were found (with the exception of mitochondrial H-strand transcripts) with 10 nM siRNA transfection. Cancerous cells such as 143B cells rely more on glycolysis rather than OXPHOS for ATP production (Warburg, 1956; Reitzer *et al*., 1979), which might explain the lack of response in the transient complex I knockdown model. To better challenge mitochondrial energy metabolism in

the stable *in vitro* knockdown models, a medium in which galactose is the carbon energy source was also utilised. This induces increased glutaminolysis by the TCA cycle, which provides reducing equivalents to the OXPHOS system and, therefore, forces the cells to rely on OXPHOS rather than glycolysis for energy (Reitzer *et al.*, 1979). As in Chapter 4, several parameters were selected to first evaluate and characterise the induced deficiency (such as RNA, protein, functionality and ROS levels) and then to evaluate the transcription and replication of mtDNA in this transient complex I deficient model. This included an evaluation of mtDNA copy number RNA levels of several nuclear- and mitochondrial-encoded transcripts encoding structural as well as functional proteins.

5.2. EXPERIMENTAL

5.2.1. Cell culture and siRNA knockdown

The anti-Rieske shRNA-expressing construct in pSIREN-RetroQ plasmid was a kind gift from Prof. N.S. Chandel (Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago). Except where indicated, all tissue culture reagents were obtained from Gibco. 143B control cybrid cells (Ugalde *et al.* 2003), were cultured at 37 ºC with 5 % CO_2 in medium, supplemented with 10% fetal bovine serum dialysed with phosphate buffered saline, 100 units/ml penicillin and 100 µg/ml streptomycin (all reagents obtained from Gibco). For the galactose-rich medium, DMEM with no glucose, 1 mM sodium pyruvate and 10 mM galactose was used, whereas for the glucose-rich medium DMEM with no galactose, 1 mM sodium pyruvate and 4.5 g/L glucose was used. Transfected cells were also supplemented with 3µg/ml puromycin (Sigma-Aldrich). Cells were transfected with either the empty pSIREN-RetroQ-TetP vector (Clontech), the anti-Rieske shRNA-expressing construct (Brunelle *et al.*, 2005), the unrelated anti-eGFP shRNA (Clontech, Du *et al.*, 2006) with target sequence 5´-GCACGACTTCTTCAAGTCC-3´, or the anti-NDUFS3 shRNA with target sequence 5´-GTTACGTTATGATGATGAA-3´ in the presence of ExGen 500 *in vitro* Transfection reagent (Fermentas). Cells were selected for 10-14 days and isolated colonies selected by the dilution method (Puck & Marcus, 1955). Prior to analysis, cells were cultured for at least 72 hours without antibiotics in DMEM containing 1 mM

sodium pyruvate, 4.5 g/L glucose and 10 % (v/v) fetal bovine serum dialysed with phosphate buffered saline. During this time, cells were seeded at a density of 4,000 cells per cm^2 and harvested when a confluency of ~90% was reached.

5.2.2. Relative RNA quantification

Total RNA was isolated from all cell cultures using QIAzol reagent according to the manufacturer's instructions (Qiagen). Reverse transcription of 4 µg RNA with 200 U M-MLV reverse transcriptase (Promega) was carried out in a volume of 40 µl using 0.5 µg random hexamer primers (Inqaba Biotech). Realtime-PCR was performed using an ABI7300^{TM} (Applied Biosystems) in a final volume of 20 µl with the KAPA SYBR® FAST ABI Prism® 2X qPCR Master Mix (Kapa Biosystems) and 250 nM forward and reverse primers. Relative quantification was determined for selected mitochondrial- and nuclear encoded transcripts (Table 5.1). Transcripts were amplified from 20 ng cDNA with forward and reverse primers and normalised to β2-microglobulin transcripts (Table 5.1; Radonic *et al*., 2004, Reinecke *et al*., 2006). This transcript has been shown to remain unaffected when a complex I deficiency was induced with rotenone and was thus considered to be a suitable normalising gene (24). The C_T-values obtained from the 7300 System Sequence Detection Software (version 1.4), from Applied Biosystems, were exported to REST-384 Version 2 software (Relative Expression Software Tool) for analysis and PCR efficiency for each primer set was calculated by serial dilutions method using the REST software tool (Pfaffl, *et al*., 2002).

Table 5.1. Primer sequences for mitochondrial- and nuclear-encoded transcripts.

Transcript	Forward primer (5′-3′)	Reverse primer (5′-3′)	Genbank® accession number
Reference transcript			
β2-microglobulin	AGCGTACTCCAAAGATTCAGGTT	ATGATGCTGCTTACATGTCTCGAT	NM_004048
Mitochondrial transcripts			
ND1	GTCTCAGGCTTCAACATCG	CGAATTCATAAGAACAGGGAG	NC_012920
ND6	CTCCTCAATAGCCATCGCTG	GATTGTTAGCGGTGTGGTCG	NC_012920
COXII	CATCCTAGTCCTCATCGCC	GATTAGTCCGCCGTAGTCG	NC_012920
D-loop	CCTAACACCAGCCTAACCAG	TGATGAGATTAGTAGTATGGGAG	NC_012920
Nuclear-encoded structural transcripts			
NDUFS3	GCTGACGCCCATTGAGTCTG	GGAACTCTTGGGCCAACTCC	NM_004551
NDUFA9	GGAGCAACAGGATTCCTGGG	CTGTGTTGTACTACTCGTCGG	NM_005002
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	NM_004168
CIII-core 2	GTCGTTGGGAAGTAGCTGAC	GGATTAGCCAAGGCATTCCG	NM_003366
CIII-Rieske	GGAAATTGAGCAGGAAGCTG	CCACCAAAATCTCCTGCATT	NM_006003
COX4I1	CTAGTTGGCAAGCGAGCAAT	TCACGCCGATCCATATAAGC	NM_001861
CV-α subunit	GGTCGTGTAGTTGATGCCC	CAGTCTGCATTGGTTCCCG	NM_004046
Nuclear-encoded functional transcripts			
TFAM	CCGGCTGTGGAAGTCGAC	GTTCCCTCCAACGCTGGGC	NM_003201
mtSSB	GAGATGTGGCGATCAGGG	GATTGTTGTTGCTTGTCGCC	NM_003143
TFB2M	CGCGAAAGCATTTGCCGGC	TCACTTTCGAGCGCAACCAC	NM_022366
POLRMT	CAAGCCGCATCGTTCCTCTC	GCCTCGAAGGTCAGCGTGG	NM_005035
DNApolγ	GGTTGCTGAACGGGCATGG	GAGGTTGGTGATCTGCAAGG	NM_002693

5.2.3. Relative mitochondrial DNA copy number

The total DNA was extracted from the cultured cells using the NucleoSpin kit (Macherey-Nagel). The relative mtDNA copy number was measured by real-time PCR and calculated using a nuclear gene as a normaliser. For the real-time PCR, the primers and probe for the nuclear β-globin gene were: 5'-GTGCACCTGACTCCTGAGGAGA-3' (forward), 5'-CTTGATACCAACCTGCCCAG-3' (reverse) and 5'-FAM-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA-3' (probe) respectively (Metabion International). For mtDNA amplification, MT-ND2 TaqMan Gene Expression Assay from Applied Biosystems was used (assay Hs02596874_g1, Applied Biosystems). The PCR was performed in the ABI 7300 Real Time PCR System in 25 µL reaction volumes. Each reaction mixture contained 12.5 µL TaqMan Universal PCR Master Mix, No AmpErase UNG (2X) (Applied Biosystems), 0.5 µM of forward and reverse primers and 0.2 µM of probe for β-globin gene. For the MT-ND2 gene, a 1x dilution of primers/probe mixture and 10 ng of DNA was used. The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C and annealing/extension at 60°C for 1 minute with fluorescence measurement during this step. All reactions were performed in triplicate and every assay included a no-template control and three serial dilution points (in 5-fold steps) of a DNA mixture. Relative mtDNA copy number was calculated as described for the relative RNA quantification.

5.2.4. Enzyme activities

All reagents used for enzyme and protein analyses were purchased from Sigma-Aldrich. Analyses were performed using 600 x *g* supernatants that were prepared from homogenizing cells suspended in an isotonic buffer (mannitol, 210 mM; sucrose, 70 mM; HEPES, 5 mM; EGTA, 0.1 mM; pH 7.2). Protein content was determined using the BCA method (Smith *et al.*, 1985). Mitochondrial respiratory chain (RC) enzymes complex II (succinate:ubiquinone oxidoreductase, EC 1.3.5.1), complex III (ubiquinol:ferricytochrome-c oxidoreductase, EC 1.10.2.2) and complex IV (ferrocytochrome-c:oxygen oxidoreductase, EC 1.9.3.1), were measured essentially as described previously (Rahman *et al.* 1996). Citrate synthase (CS; EC 2.3.3.1) activity was determined by the

method of Shepherd and Garland (1969). The RC enzyme activities were expressed as a ratio to CS (as mitochondrial marker enzyme) to compensate for mitochondrial enrichment in the sample.

5.2.5. Denaturing and non-denaturing PAGE analyses

Freshly prepared whole cells were re-suspended in PBS after harvesting. An enriched mitochondrial pellet was prepared by homogenisation and differential centrifugation up to 10 000 x *g* in the isotonic buffer used for enzyme analyses. The pellets were further prepared for SDS-PAGE as described before (Ugalde *et al.*, 2004). After total protein quantification (Smith *et al.*, 1985), 10 µg of mitochondria-enriched extract was loaded onto a 12 % (w/v) SDS-PAGE gel. Blue-native PAGE gradient gels (5-15%) were prepared and loaded with 40 µg mitochondrial fraction as described before (Nijtmans *et al.*, 2002). In-gel activity assay for complex I was carried out as described by Nijtmans *et al.* (2002). Western blotting was performed with primary antibodies as indicated (Mitosciences), together with a secondary HRP-Goat Anti-Mouse IgG (H+L) antibody (Zymed) at the suggested dilutions and conditions according to the suppliers. Signal detection was performed with ECL Western blotting substrate (Pierce) and the blots exposed to BioMax light film (Kodak). The chemiluminescent signals on the exposed film were quantified using GeneTools 3.06 Imaging software (SynGene).

5.2.6. Measurement of ROS levels

To measure ROS levels, cells were incubated for 30 min with 10 µM of the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA, Molecular probes®). Cells were trypsinized and re-suspended in PBS. All treated cell preparations were then analysed for ROS-induced fluorescence using a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson). Cells were analyzed at a flow rate of 1,000 events per second and 10,000 events were collected for each sample. Cells were excited with an argon laser emitting at 488 nm. Forward and side scatter was collected on a linear scale and DCF fluorescence was detected by FL1 PMT using log

amplification. For every assay unstained cells of each cell line were used as controls (results not shown). As a positive control for ROS production, a set of control cells in glucose-medium was treated for 3 hours with tert-butyl hydroperoxide (*t*-BHP) at 1mM. Data were acquired using CELLQuest PRO (Becton Dickinson) and analysed by FCS Express (version 3, De Novo Software). Results were expressed as ratios between mean fluorescence intensities per percentage positively stained cells.

5.2.7. Statistical evaluation

All results were analyzed with GraphPad Prism (version 5) software as mean ± standard deviation. Statistical comparisons were made using two-way analysis of variance (ANOVA) with Bonferroni post tests. Statistical significance was considered when $p < 0.05$.

5.3. RESULTS AND DISCUSSION

5.3.1. Characterization of stable NDUFS3 and Rieske knockdown models

To investigate the consequences of complex I and III deficiency *in vitro,* stable (shRNA) NDUFS3 and Rieske protein knockdown models were introduced in 143B cybrid cells. Individual clones were analysed for relative RNA expression levels of Rieske protein and one clone with most significant reduction in RNA levels was selected for further experiments (results not shown). The stable knockdown models were evaluated based on relative expression levels of RNA and protein, as well as enzyme activity of complexes I and III (Figure 5.1). Real-time PCR analysis of the relative expression level of NDUFS3 transcripts (Figure 5.1.A) in the NDUFS3 knockdown model (complex I) showed a 69% and 54% reduction in the glucose- and galactose medium respectively compared to 143B control cells. More revealing, a 71% and 65% reduction in NDUFS3 transcripts in the NDUFS3 knockdown model was observed compared to the cells transfected with the empty vector. However, western blot analysis of the NDUFS3 knockdown showed only slightly reduced NDUFS3 protein level compared to both 143B cells and cells transfected with the empty vector

(Figure 5.1.C) when normalised to cyclophilin D. Expression is relative compared to cyclophilin D expression to compensate for any differences in protein loading to each well. Furthermore, fully assembled native complex I, as seen in the western blotting of the BN-PAGE (Figure 5.1.D) did not show significant decrease in the NDUFS3 knockdown model and the in-gel activity enzyme assay (Table 5.2) confirmed that there was no significant reduction in complex I enzyme activity in the stable NDUFS3 knockdown model. The stable NDUFS3 knockdown model utilised the same target sequence as the transient siRNA knockdown model in Chapter 4 that showed significant reduction in NDUFS3 RNA and protein levels and also showed significant reduction in complex I enzyme activity (Figure 4.2). This might be due to the fact that, unlike the high concentration of siRNA oligonucleotides present with transient siRNA transfections, shRNA is present at physiological concentrations and additional intracellular processing of shRNA is required before it can act as siRNA sequences (summarized by Levanets *et al.*, 2010; Appendix C).

Real-time PCR analysis of the relative expression level of Rieske transcripts (Figure 5.1.B) showed an 88% reduction in the Rieske-shRNA clone compared to both 143B cells and cells transfected with the empty vector in both the glucose- and galactose-rich medium. Western blot also showed significant reduction of the Rieske protein level in the Rieske-shRNA transfected clone compared to both 143B cells and cells transfected with the empty vector (Figure 5.1.C), confirming the successful Rieske knockdown model as originally shown by Brunelle *et al.* (2005). A significant reduction in the level of fully assembled native complex III, as seen in the western blotting of the BN-PAGE (Figure 5.1.D) and longer exposure revealed the presence of the lower molecular weight sub-complex (Figure 5.1.E). In the Rieske-shRNA clone, the enzyme activity of complex III was reduced to approximately 23% in both the 143B cells and the empty vector cells. This confirmed that the Rieske-shRNA clone showed significant knockdown of Rieske protein, on both RNA and protein level, and this led to a significant decrease in complex III enzyme activity.

Interestingly, in both glucose- and galactose medium, the NDUFS3 knockdown model also showed significant changes (27% reduction and 26% increase in glucose and galactose medium respectively) of complex I (NDUFS3) subunit transcripts (Figure 5.1.B). Furthermore the Rieske

knockdown model showed significant reduction (42% and 45% in glucose and galactose medium respectively) of complex III (Rieske) subunit transcripts (Figure 5.1.A). The Rieske knockdown model also showed reduced protein levels of NDUFS3 subunit (Figure 5.1.C) and reduced fully assembled complex I (Figure 5.1.D). A strong correlation between deficiency of complex I subunits and reduced fully-assembled complex III was also found previously (Ugalde *et al*., 2004a) and it is known that complexes I, III and IV form supercomplexes or respirasomes, which could explain the correlation between reduced expression of NDUFS3 (complex I) and Rieske (complex IIII) transcripts with the complex I and III deficiency models.

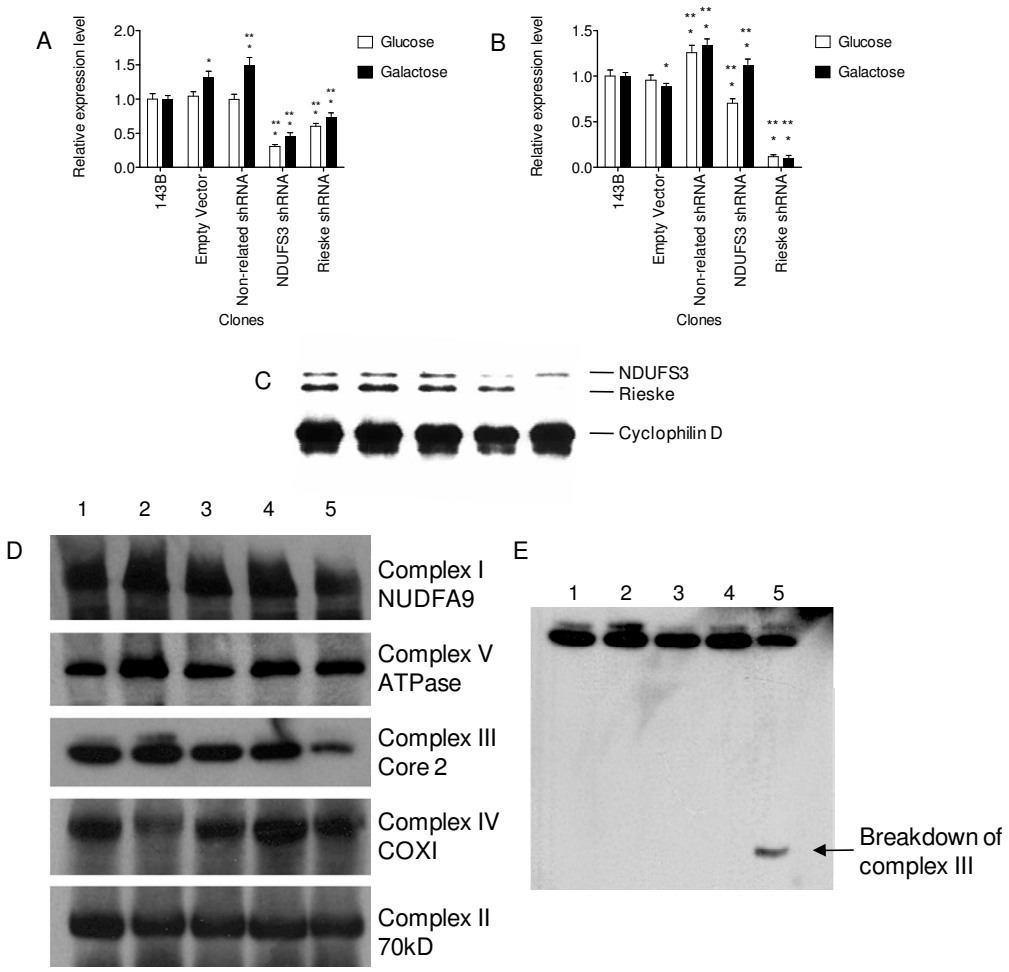

Figure 5.1. Confirmation of Rieske protein stable knockdown. Relative expression level of (A) NDUFS3 and (B) Rieske transcripts with real-time PCR in galactose- and glucose-rich medium with 1) 143B cells, 2) empty vector, 3) unrelated shRNA, 4) NDUFS3 shRNA and 5) Rieske shRNA clones (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, two-way ANOVA). (C) Protein expression with SDS-PAGE and (D) BN-PAGE with antibodies for western blotting as indicated. (E) Long term exposure of BN-PAGE western blot with antibody against complex III-core 2 protein showing lower molecular weight subcomplex as indicated by black arrow.

Table 5.2. Respiratory chain enzyme activities in cell lines

	Complex I[#]	Complex II	Complex III	Complex IV
		(nmol/min/UCS)	(nmol/min/UCS)	(nmol/min/UCS)
143B cells	1.00	235 ± 5.3	140 ± 19.8	7.9 ± 2.6
143B cells + empty vector	0.86	252 ± 8.7	150 ± 2.8	7.5 ± 1.0
143B cells + non-related shRNA	1.19	213 ± 1.1	156 ± 13.4	9.4 ± 1.3
143B cells + NDUFS3 shRNA	1.05	289 ± 2.0	239 ± 19.8	9.2 ± 0.7
143B cells + Rieske shRNA	0.86	203 ± 1.8	34 ± 12.8	9.6 ± 1.4

[#]Complex I activities (arbitrary value) were measured using BN-PAGE in-gel activity, normalised to complex IV activity by densitometry. All other activities (average ± SD, n = 3) were measured kinetically as described in the experimental section and normalised to citrate synthase activity (UCS).

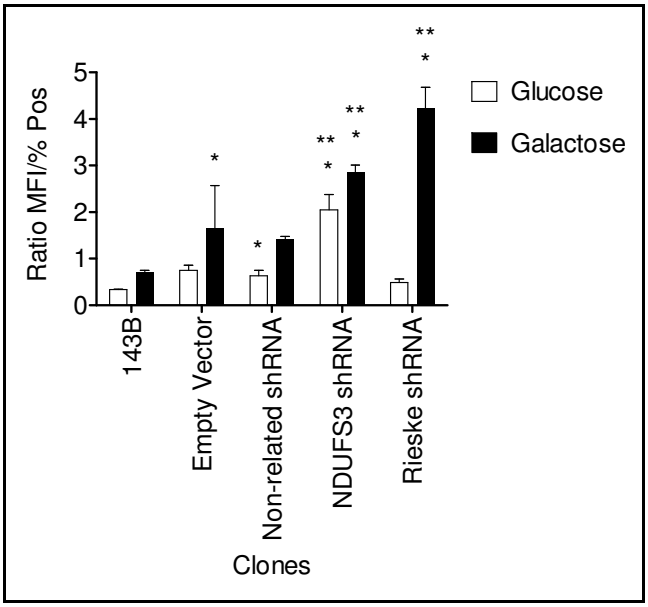

Figure 5.2. Relative ROS production with flow cytometry in galactose- and glucose-rich medium. The positive control (1 mM *t*-BHP) had a ratio of 37 ± 5.39 (not shown). Levels expressed as mean fluorescence intensity per percentage positively stained cells (MFI/%Pos, n=3, **p<0.05* compared to 143B, ** *p<0.05* compared to empty vector, two-way ANOVA).

An important biochemical parameter often associated with deficient complex I or III activity is the elevated levels of ROS. In the NDUFS3 knockdown model, ROS production was significantly increased: 174% and 74% in the glucose and galactose medium respectively compared to the cells transfected with the empty vector and 600% and 400% respectively compared to the 143B cells. In the stable NDUFS3 knockdown model protein levels and enzyme activity of complex I was not significantly altered and, therefore, the increased ROS levels is likely due to secondary consequences of the shRNA transfection. Due to the stable nature of the shRNA, it is unlikely that these secondary consequences are due to the transfection reagent or toxicity of the shRNA as can occur with transient siRNA transfections (summarized by Levanets *et al.*, 2010). Instead, the secondary consequences might be related to if/where the pSIREN-RetroQ-TetP vector with inserted NDUFS3 shRNA sequence has been incorporated into the 143B genome, or it might be due to off-target effects of the shRNA sequence as documented previously (Bridge *et al.*, 2003; Persengiev *et al.*, 2004) and seen with the transient siRNA knockdown model (Section 4.3.1). In these cell lines we observed significantly increased ROS production in the 100 nM siRNA concentration compared to 10 nM siRNA concentration, even though both siRNA concentrations showed similar NDUFS3 protein levels and complex I enzyme activity.

In the current study ROS production in the Rieske-shRNA clone was not significantly altered in the glucose-rich medium (48% increased compared to 143B cells and 44% reduced compared to cells transfected with the empty vector), but in the galactose rich medium a significant increase of 600% was seen compared to 143B cells, or 260% compared to cells transfected with the empty vector (Figure 5.2). The ROS production in the galactose-rich medium was generally much higher in all cell lines compared to the glucose-rich medium, which correlates with previous studies (Quinzii *et al.*, 2008, Benard *et al.*, 2007). It was suggested that this might be due to increased reverse ATP synthase activity to maintain mitochondrial potential in glutaminolytic cells with increased OXPHOS and, therefore, increased ROS production and increase in apoptosis (Wu *et al.*, 2007). In previous studies (Bell *et al.*, 2007; Brunelle *et al.*, 2005) it was found that the Rieske protein is necessary for production of ROS at the Qo site of the ubiquinone (Q) cycle and that knockdown of Rieske protein leads to a lack of increased ROS production at this site in the

presence of hypoxia. In the present study the ROS production seen in the galactose-rich medium would, therefore, be attributed to ROS production at another site than Qo, such as the Qi site or maybe at complex I.

5.3.2. Evaluation of mtDNA and mtRNA levels

Real-time-PCR analyses of the mtDNA in the stable NDUFS3 knockdown model showed small changes in relative mtDNA copy number compared to 143B cells (10% increase and 11% decrease in glucose and galactose medium respectively). Compared to the cells transfected with the empty vector, the NDUFS3-shRNA cells showed no change (1% increase) in mtDNA copy number ratio (Figure 5.3). Since there was no significant reduction in complex I activity with the stable NDUFS3 knockdown model, it was expected that the mtDNA copy number would stay unchanged. It is also evident that the significantly increase in ROS levels (albeit unspecific to OXPHOS deficiency) in this stable knockdown model did not alter the relative mtDNA copy number (similar to the effect seen in the transient knockdown model in Section 4.3.2). Again, this would suggest that cellular ROS increase alone in these cells does not result in changes in the mitochondrial replication machinery functions.

In the Rieske-shRNA knockdown model, real-time-PCR analyses of the relative mtDNA copy number showed a 4% increase and an 8% reduction (significant) in the glucose and galactose medium respectively, compared to 143B cells. Compared to the cells transfected with the empty vector, the Rieske-shRNA cells showed slightly reduced (4%) mtDNA copy number ratio in the glucose-treated cells and slightly increased (5%) mtDNA copy number ratio in the galactose-treated cells. It is, therefore, clearly evident that despite a significantly deficient complex III function via Rieske protein knockdown, and including all the consequences associated with it, mtDNA replication was not markedly affected. This corresponds to the unchanged mtDNA copy number ratio in the transient NDUFS3 knockdown of complex I in Chapter 4. Several previous studies showed mostly decreased copy number in skeletal muscle, liver and brain tissue (Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonod-Bidaud, 1999) and unchanged or increased mtDNA copy

number ratios in heart, kidney and skeletal muscle (Bonod-Bidaud, 1999), depending on the OXPHOS deficiency. It is thought that the tissues with the highest energy demand (like brain, heart and muscle) are affected most by OXPHOS deficiencies (Scheffler, 2001) and this might lead to the differentially affected mtDNA copy number ratio in different tissues. In this study, the osteosarcoma-derived 143B cells were used, which does not have such a high OXPHOS energy demand such as primary muscle, brain and heart tissue and this might explain the lack of differential mtDNA copy number ratios in the presence of the stable Rieske knockdown of complex III, even in the presence of the galactose medium which enhances energy production via OXPHOS.

In the 143B cells, the relative mtDNA copy number ratio in the galactose-rich medium was higher than in the glucose-rich medium. This is in contrast to a study by Rossignol *et al.* (2004) where the mtDNA copy number ratio in HeLa cells was similar in galactose-rich and glucose-rich medium, but it supports the compensatory mechanism as described by Heddi *et al.* (1999) whereby OXPHOS transcript expression was up-regulated to compensate for increased OXPHOS energy demand.

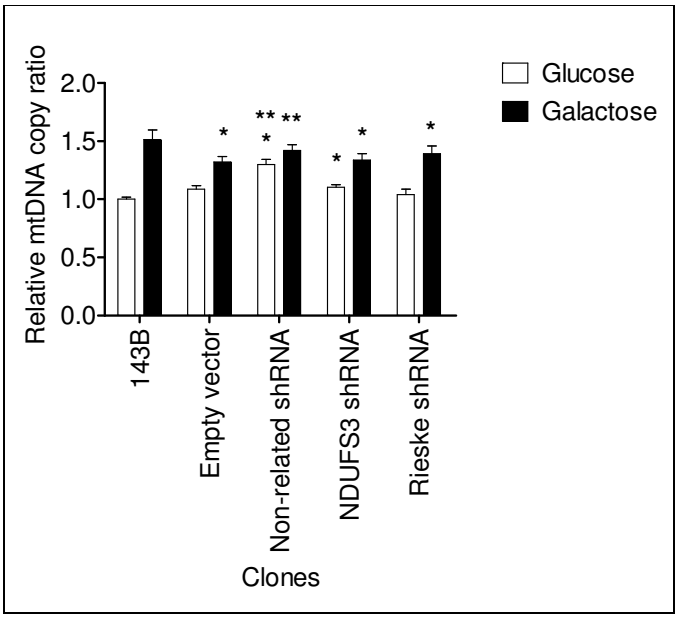

Figure 5.3. Relative mtDNA copy ratio. ND2 (mtDNA) expression relative to β-globin (nDNA) expression (n=3, *$p<0.05$* compared to 143B, ** $p<0.05$ compared to empty vector, two-way ANOVA).

To further evaluate mtDNA transcriptional responses to the stable knockdown models, levels of three mitochondrial encoded transcripts were quantified (Figure 5.4). In the NDUFS3-shRNA clone, the H-strand transcript (COXII) was 44% (significant) and 9% reduced in the glucose and galactose medium, respectively, compared to the cells transfected with the empty vector (Figure 5.4.A). The L-strand transcript (ND6) was 5% reduced and 15% increased in the glucose and galactose medium, respectively, compared to the cells transfected with the empty vector (Figure 5.4.B). The displacement loop (D-loop), the only non-coding region where replication/transcription was initiated from, was 72% increased and 27% decreased in the glucose and galactose medium respectively, compared to cells transfected with the empty vector (Figure 5.4.C). Since no significant changes in complex I activity were detected in the stable NDUFS3 knockdown model, it is concluded that any changes detected were due to the secondary effects described in Section 5.3.1.

In the Rieske-shRNA clone, the H-strand transcript (COXII) showed approximately 45% (glucose-rich) and 18% (galactose-rich) decrease compared to the cells transfected with the empty vector (Figure 5.4.A). The L-strand transcript (ND6) showed 0% and 82% increase in the glucose and galactose medium, respectively (Figure 5.4.B), compared to the cells transfected with the empty vector. The D-loop, from where mtDNA replication and transcription is initiated, showed 12% and 134% increase in glucose and galactose medium respectively (Figure 5.4.C). It appears from these results that the complex III deficiency due to Rieske-shRNA knockdown may reduce H-strand transcription, which encodes the majority of mtDNA genes. Differences between H- and L-strand transcript levels were also detected in earlier studies and it was proposed that this was probably due to different rates of processing or transcription and/or different half lives for the two strands (Heddi *et al.*, 1993; Duborjal *et al.*, 2002; Gelfand & Attardi, 1981). The significantly increased expression of the ND6 and D-loop mitochondrial transcripts in the Rieske-shRNA clone in galactose medium supports the hypothesis that once the threshold of deficiency has been reached (as in the galactose-rich medium where cells are more dependent on OXPHOS energy production), expression of most of the genes encoding for these OXPHOS proteins increase, probably *via* redox-related transcription factors, to compensate for the decrease in energy production (Heddi *et al.*, 1993; Heddi *et al.*, 1999).

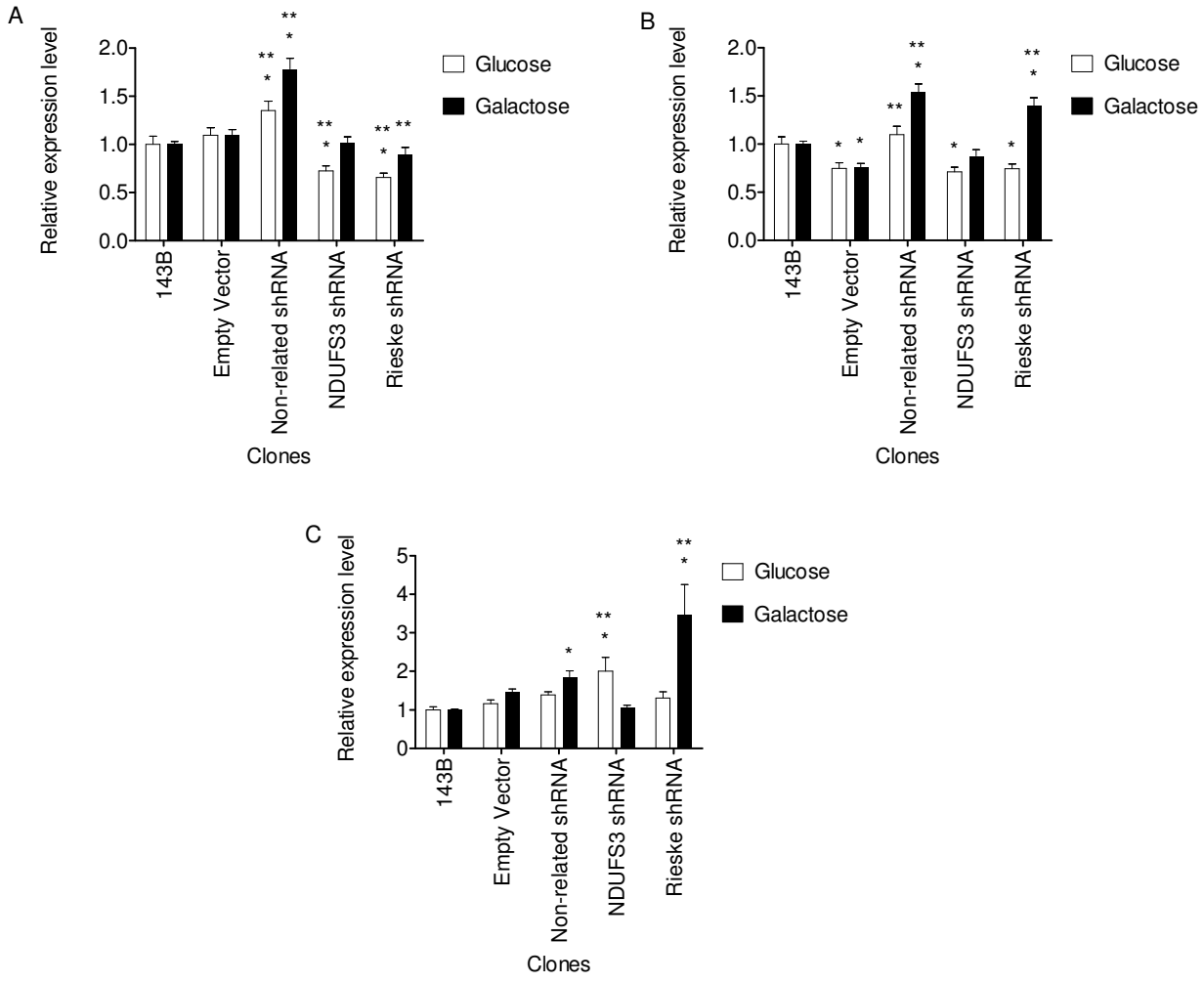

Figure 5.4. Relative expression level of mitochondrial transcripts in galactose- and glucose-rich medium. Relative expression level of (A) COXII, (B) ND6 and (C) D-loop in a variety of stable knockdown cell lines (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, two-way ANOVA).

5.3.3. Evaluation of selected nuclear transcript levels

The mRNA expression of five nuclear genes encoding structural subunits of the various OXPHOS complexes were relatively quantified (Figure 5.5), in addition to the two nuclear encoded genes quantified in Section 5.3.1 (NDUFS3 and Rieske transcripts). In the stable NDUFS3 knockdown model (with no significant reduction in complex I enzyme activity), the NDUFA9 (Figure 5.5.A), CIII-core2 (Figure 5.5C) and Rieske (Figure 5.1.B) transcript levels were

significantly reduced in the glucose medium compared to the cells transfected with the empty vector, while the other transcripts were slightly (but insignificantly) reduced. In the galactose medium, the transcripts were slightly reduced, except for the Rieske transcript which was significantly increased and SDHA transcript which was significantly reduced.

When compared to empty-vector control cells, the stable Rieske knockdown model showed statistically significant reduced expression for the complex I subunit, NDUFS3 (Figure 5.1.A) in both glucose and galactose medium as discussed in Section 5.3.1. Although the transcripts of NDUFA9, SDHA, CIII-core 2, COX4I1 and CV-α were not statistically different, the transcript levels of these subunits appears to be generally lower in the knockdown cells in both glucose and galactose medium compared to the cells transfected with the empty vector. The exceptions were that of NDUFA9, CIII-core2 and CV-α transcripts which were slightly, but insignificantly, increased in galactose medium and the COX4I1 transcript which was significantly decreased in the glucose medium compared to cells transfected with the empty vector. It thus appears from this data that these selected OXPHOS genes are indeed differentially expressed as is often reported for OXPHOS deficiencies, possibly due to alternate functions or stability (Chevallet *et al.*, 2006, Marusich *et al.*, 1997), but that their expression is to a limited extent also coordinately reduced (Reinecke *et al.*, 2009; van Waveren & Moraes, 2008). The differential expression in the two complex I subunits, NDUFS3 and NDUFA9, in the current study highlights this limited coordinate expression, possibly because NDUFS3 is part of the catalytic core of complex I, whilst NDUFA9 is not (Hirst *et al.*, 2003; Ugalde *et al.*, 2004b).

From the results we can also see that (although mostly statistically not significant) expression of nuclear subunits of complexes I-V was reduced more in glucose medium, but in the galactose medium, where cells were forced to rely on OXPHOS energy production, transcripts were less reduced or even increased (complex I, III and V). This again supports the hypothesis of compensatory regulation of genes involved with OXPHOS in the presence of OXPHOS deficiency.

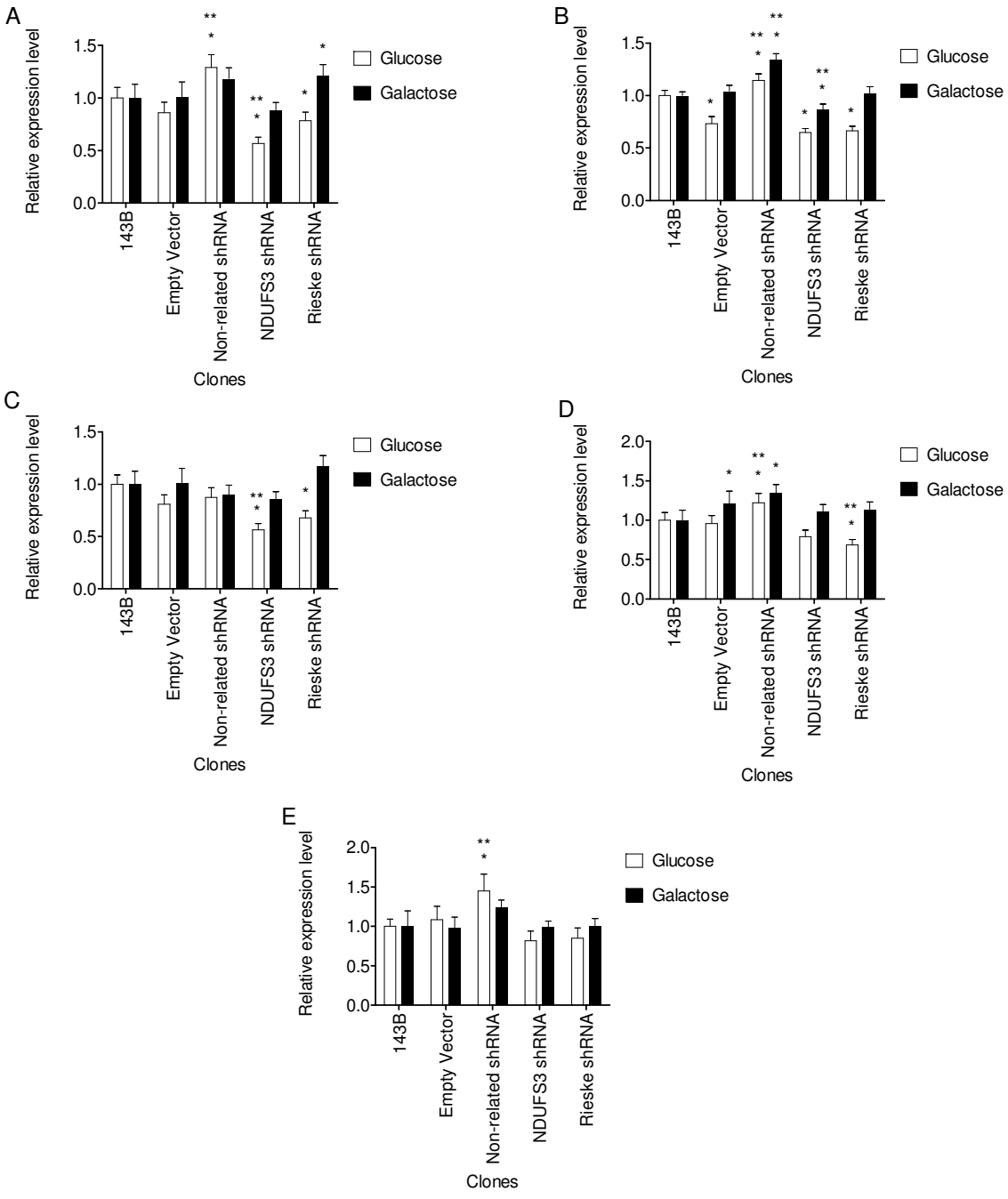

Figure 5.5. Relative expression level of nuclear transcripts in galactose- and glucose-rich medium. Relative expression of (A) NDUFA9, (B) SDHA, (C) CIII-core 2, (D) COX4I1 and (E) CV-α in a variety of stable knockdown cell lines (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, two-way ANOVA).

5.3.4. Evaluation of selected transcripts involved in mtDNA regulation

As the transcription/replication machinery of mtDNA in mammals is regulated by several nuclear encoded genes (Asin-Cayuela & Gustafsson, 2007; Falkenberg *et al.*, 2007; Shadel, 2008), the expression of several of these key regulators was evaluated in the NDUFS3 and Rieske knockdown models. In the NDUFS3 stable knockdown model, with no significant reduction in complex I enzyme activity, two of these regulators, POLRMT (Figure 5.6.A) and TFAM (Figure 5.6.B), showed no significant changes in both glucose and galactose medium. The mtSSB transcript levels were 35% (significant) reduced and 3 % increased in the glucose and galactose medium respectively (Figure 5.6.C). In the same model, DNApolγ was 5% reduced and 21% (significantly) increased in the glucose and galactose medium respectively, compared to the cells transfected with the empty vector (Figure 5.6D). Finally, TFB2M expression was 3% reduced and 550% increased (significantly) in glucose and galactose medium respectively (Figure 5.6.E).

With the Rieske knockdown model, expression levels of POLRMT (Figure 5.6.A) and TFAM (Figure 5.6.B) mRNA levels showed no significant changes when compared to empty vector transfected cells in either glucose or galactose medium. Previous studies reported that expression of TFAM, mtSSB and POLRMT tightly correlates with mtDNA copy number (Schultz *et al.*, 1998, Seidel-Rogol *et al.*, 2002, Davis *et al.*, 1996; Ekstrand *et al.*, 2004). Considering these reports, the relatively unchanged TFAM and POLRMT expression observed in this study is in accordance with a relatively stable mtDNA copy number ratio in the Rieske protein knockdown cell line in both glucose and galactose medium. TFAM expression is also associated with increased ROS levels in mtDNA depleted cells (Miranda *et al.*, 1999). The expression of these regulatory proteins (TFAM, TFB2M, POLRMT, mtSSB and DNApolγ) is regulated by the two nuclear transcription factors, NRF-1and NRF-2, which have also been shown to be redox sensitive (Piantadosi & Suliman, 2006; Miranda *et al.*, 1999; Bruni *et al.*, 2010). In contrast, with the current study in the stable Rieske knockdown model, TFAM and POLRMT mRNA levels as well as mtDNA copy number were relatively unchanged in both the glucose and galactose medium, whilst ROS levels were significantly increased in the galactose medium.

In the Rieske knockdown model, expression of another protein that is reported to correlate with mtDNA copy number, mtSSB, was statistically significantly reduced by 26% and 17% in glucose and galactose medium respectively (Figure 5.6.C). However, this result is in contrast to previous studies (and the transient knockdown model of complex I in Section 4.3.3) where mtSSB expression changes were comparable to changes of mtDNA copy number ratio (Seidel-Rogol &. Shadel, 2002; Schultz *et al.*, 1998). It is known that mtSSB binds and stabilizes single-stranded DNA, including D-loops (Takamatsu *et al.*, 2002), but in the current study the mtSSB expression was reduced in both media, whereas D-loop expression was significantly increased in the galactose medium. Another study by Yin *et al.* (2004) found that mtSSB expression closely compared with mtDNA copy number in non-tumerous liver tissue, but in hepatocellular carcinoma tissue mtSSB expression was increased whilst mtDNA copy number was unchanged or reduced.

DNApolγ expression in the Rieske-shRNA knockdown cells was reduced with 6% and increased with 16% (statistically significant) in glucose and galactose rich medium, respectively, compared to the cells transfected with the empty vector (Figure 5.6.D). According to various studies, DNApolγ expression is not affected by mtDNA copy number (Schultz *et al.*, 1998, Davis *et al.*, 1996), although a study by Hance *et al.* (2005) showed that changes in DNApolγ expression corresponded with changes in mtDNA copy number and TFAM expression. In the current study the DNApolγ expression level in the cells, grown in glucose medium, were comparable with the mtDNA copy number ratio and TFAM expression in the Rieske-shRNA knockdown cells, but although the mtDNA copy number and TFAM expression were relatively unchanged in the galactose-rich medium, the DNApolγ expression level was statistically significantly increased. It is known that, in addition to being involved in mtDNA replication, DNApolγ is also involved in the ATP-dependent base excision repair pathway to repair DNA damage in mitochondria. Glycosylases without lyase activity employ the deoxyribophosphodiesterase activity of DNApolγ to remove the 5'-sugar-phosphate residue after which DNApolγ then catalyzes a one-nucleotide incorporation whilst mtDNA ligase repairs the nick (Mandavilli *et al.*, 2002; Croteau & Bohr, 1997; Graziewicz *et al.*, 2006). One possible explanation for the increased DNApolγ mRNA levels in the galactose medium might be that, due to the increased ROS production in the galactose-treated cells, the impending

oxidative damage to the mitochondrial DNA might stimulate the increased expression of DNApolγ to aid in the repair of the mtDNA.

Another regulatory protein that showed increased mRNA levels in the Rieske-shRNA knockdown cells cultured with galactose-rich medium was that of TFB2M (Figure 5.6.E), with a 9% and 561% (statistically significant) increase in expression in the glucose and galactose medium respectively. The two transcription factors, TFB1M and TFB2M, closely resemble a family of rRNA methyltransferases. The presence of either one in addition to TFAM is needed in order for POLRMT to initiate mtDNA transcription (Falkenberg *et al*., 2002; Asin-Cayuela & Gustafsson, 2007). It has been suggested that the primary function of TFB2M is to act as transcription factor for mtDNA replication/transcription, whilst TFB1M is primarily involved with rRNA methylation (Asin-Cayuela & Gustafsson, 2007). The increased expression of TFB2M transcripts in this study (and also in the transient NDUFS3 knockdown model in Section 4.3.3) might indicate that TFB2M is a key player in the proposed compensatory mechanism for OXPHOS deficiency as described by Heddi and co-workers (1999). However, it is important to note that the TFB2M mRNA level in the empty vector control cells in galactose-rich medium was significantly lower than in the glucose-rich medium. This is in contrast to the generally higher mRNA levels of the various transcripts in the galactose-rich medium than the glucose-rich medium for the empty vector control cells and further studies should, therefore, be conducted to confirm the mRNA levels in the empty vector control cells.

As mentioned earlier, the expression of these regulatory proteins (TFAM, TFB2M, POLRMT, mtSSB and DNApolγ) is regulated by the two nuclear transcription factors, NRF-1and NRF-2, which have also been shown to be redox sensitive (Piantadosi & Suliman, 2006; Miranda *et al*., 1999; Bruni *et al*., 2010). This could explain the large increases in ROS levels and TFB2M expression levels, and also to a lesser extent the increase in DNApolγ expression, in the Rieske-shRNA knockdown cells in the galactose-rich medium. However, in the past it was shown that over-expression of either TFB2M or TFAM leads to increased mtDNA copy number (Scarpulla, 2008), which is in contrast to the very slightly increased expression of TFAM and mtDNA copy

number found in the Rieske-shRNA knockdown cells in the galactose-rich medium in this study. In a previous study by Seidel-Rogol *et al.* (2002), increased TFAM and POLRMT expression was delayed relative to mtDNA copy number when partially mtDNA-depleted cells were grown without ethidium bromide. The mitochondrial transcript levels were also increased early after recovery of the cells from ethidium bromide, whilst mtDNA copy number, TFAM and POLRMT levels were still low and the authors concluded that one reason for this might be that another transcription factor(such as TFB2M) might be responsible for this early increase in transcription/replication. In the current study significant increases were seen, not only in TFB2M and DNApolγmRNA levels and ROS levels, but also in the mitochondrial ND6 and D-loop mRNA levels in the Rieske-shRNA knockdown cells in the galactose-rich medium. It is, therefore, possible that TFB2M might be the first regulatory transcript to be increased as part of the compensatory mechanism in OXPHOS deficient cells to increase mtDNA transcription, before increases in mtDNA copy number, TFAM and POLRMT can be seen.

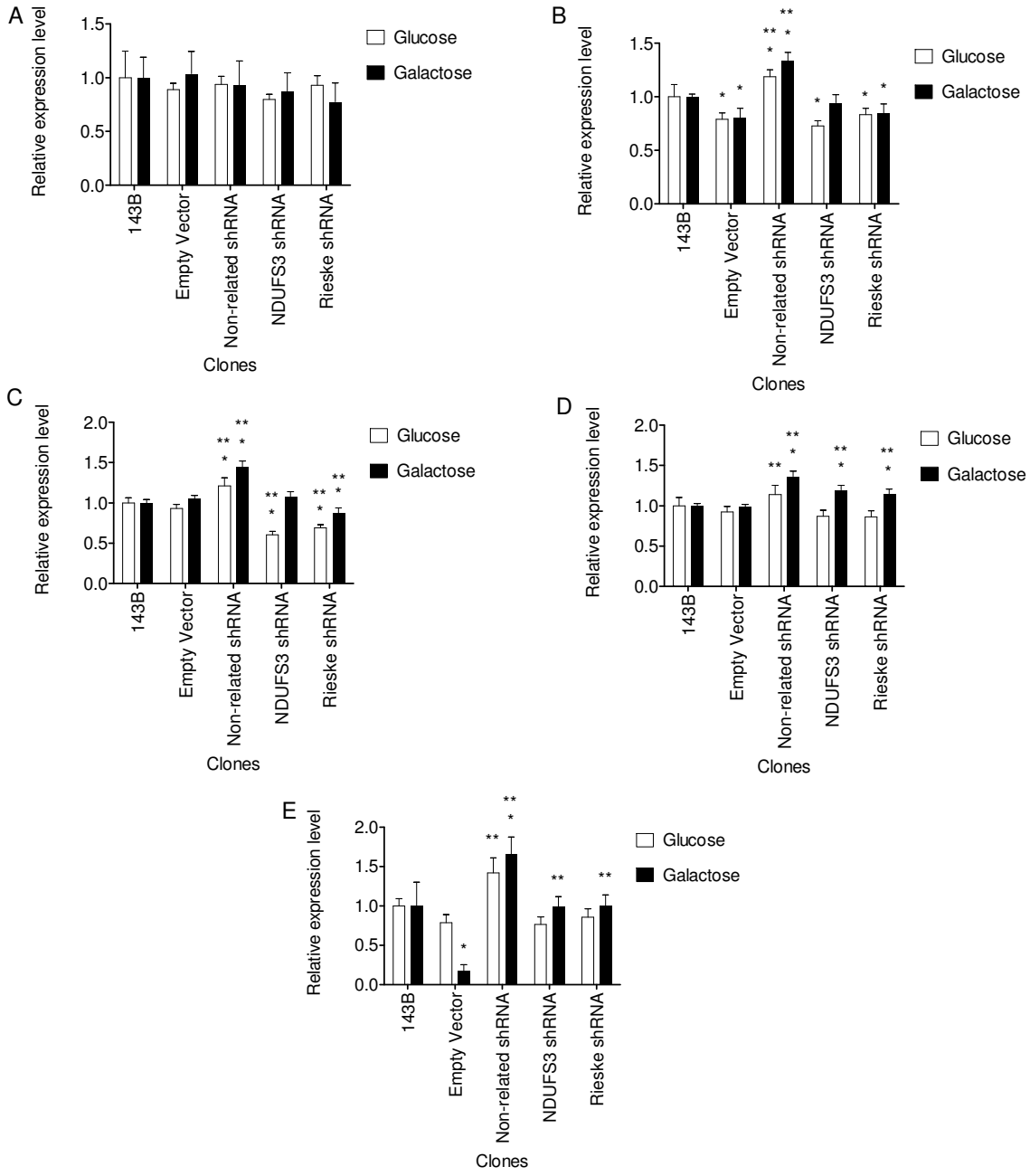

Figure 5.6. Relative expression level of regulatory nuclear transcripts in galactose- and glucose-rich medium. Relative expression of (A) POLRMT, (B) TFAM, (C) mtSSB, (D) DNApolγ and (E) TFB2M in a variety of stable knockdown cell lines (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, two-way ANOVA).

5.4. SUMMARY

Increased ROS production and differential expression of genes involved in mitochondrial biogenesis are thought to be some of the primary consequences of OXPHOS deficiencies, especially when complexes I and III are involved (as summarized in Reinecke *et al*., 2009; Dröse & Brandt, 2008). In Chapter 4, where a transient NDUFS3 siRNA knockdown model of *in vitro* complex I deficiency was studied, no significant changes in ADP/ATP ratios, ROS levels or differential gene expression were found, with the exception of reduced mitochondrial H-strand transcripts with 10 nM siRNA transfection. To exclude the effects of siRNA toxicity and evaluate long-term effects of an RNA knockdown model *in vitro*, a stable NDUFS3 knockdown model was introduced into the 143B cells. Also, to better challenge energy production in these cancerous 143B cells, that rely more on glycolysis than on OXPHOS for ATP production (Warburg, 1956; Reitzer *et al*., 1979), a medium in which galactose is the carbon energy source was included. This induces increased glutaminolysis by the TCA cycle and, therefore. forces the cells to rely on OXPHOS rather than glycolysis for energy (Reitzer *et al*., 1979).

Even though the stable NDFUS3 knockdown model showed significant reduction in NDUFS3 RNA levels, surprisingly no significant reduction in protein levels or fully assembled complex I was observed. There were also no significant changes in complex I enzyme activity. This might be due to the fact that, unlike the high concentration of siRNA oligonucleotides present with transient siRNA transfections, shRNA is present in physiological concentrations and additional intracellular processing of shRNA is required before it can act as siRNA sequences (summarized by Levanets *et al*., 2010; Appendix C). In spite of this, increased ROS production and some differential gene expression (COXIII, D-loop, NDUFA9, CIII-core2, Rieske, SDHA, mtSSB, DNApolγ and TFB2M transcripts) were observed in this knockdown model. Therefore, the increased ROS levels and differential gene expression might be due to secondary consequences of the shRNA transfection, rather than a complex I deficiency. Due to the stable nature of the shRNA, it is unlikely that these secondary consequences are due to the transfection reagent or toxicity of the shRNA as can occur with transient siRNA transfections (as summarized by Levanets *et al*., 2010). Instead, the secondary consequences might be related to if/where the pSIREN-

RetroQ-TetP vector, with inserted NDUFS3 shRNA sequence, has been incorporated into the 143B genome, or it might be due to off-target effects of the shRNA sequence, such as secondary interferon response (Bridge *et al.*, 2003; Persengiev *et al.*, 2004). This was seen with the transient siRNA knockdown model (Section 4.3.1) where we observed significantly increased ROS production in the 100 nM siRNA concentration compared to 10 nM siRNA concentration, even though both siRNA concentrations showed similar NDUFS3 protein levels and complex I enzyme activity. Furthermore, these changes in ROS production and differential gene expression did not significantly alter mtDNA copy number, which would suggest that cellular ROS increase alone in these cells does not result in changes in the mitochondrial DNA copy number.

Increased oxidative stress is not always observed with OXPHOS deficiencies (Schauen *et al.*, 2006; Verkaart *et al.*, 2007b). Specifically in complex III, the production of superoxide radicals occurs at both the ubiquinol oxidizing (Qo) and the ubiquinone reducing (Qi) sites (Hunte *et al.*, 2003). In previous studies deficiency of the Rieske protein (forming part of the Qo site) resulted in limited superoxide production whilst still preventing electron transport to cytochrome *c* (Chen, 2003; Muller *et al.*, 2004; Brunelle *et al.*, 2005; Bell *et al.*, 2007). We investigated the effect of such a complex III deficiency *in vitro*, by means of stable Rieske protein knockdown, on mtDNA replication/transcription processes. Successful stable knockdown of the Rieske subunit was confirmed on RNA, protein and enzymatic levels. Although a significant change in ROS levels was not observed in the glucose medium (as expected), the knockdown model showed significantly increased ROS levels in the galactose medium. In view of the fact that the Rieske protein is necessary for production of ROS at the Qo site of the Q cycle (Bell *et al.*, 2007; Brunelle *et al.*, 2005), the ROS production seen in the galactose-rich medium in the stable Rieske knockdown model would, therefore, be attributed to ROS production at another site than Qo, such as the Qi site or maybe at complex I.

Even though the coordinate changes in mtDNA copy number with OXPHOS deficiency has been proven before (Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonod-Bidaud, 1999), there was no change in mtDNA copy number in the Rieske knockdown model in either glucose or galactose

medium. From these results we can conclude that a stable Rieske knockdown model, with all the associated consequences, did not alter mtDNA copy number, even when the cell line was forced to rely on OXPHOS energy production and ROS production was significantly increased. At the same time, the significantly increased expression of the ND6 and D-loop mitochondrial transcripts in the Rieske-shRNA clone in galactose medium supports the hypothesis that once the threshold of deficiency had been reached (as in the galactose-rich medium where cells are dependent on OXPHOS energy production and showed significantly increased ROS production), expression of most of the genes encoding for these OXPHOS proteins increase, probably via redox-related transcription factors, to compensate for the decrease in energy production (Heddi *et al.*, 1993; Heddi *et al.*, 1999). Additionally, from the results, it also appeared that the complex III deficiency due to Rieske-shRNA knockdown may reduce H-strand transcription, which encodes the majority of mtDNA genes. Differences between H- and L-strand transcript levels were also detected in earlier studies and it was proposed that these were probably due to different rates of processing or transcription and/or different half lives for the two strands (Heddi *et al.*, 1993; Duborjal *et al.*, 2002; Gelfand & Attardi, 1981).

The nuclear encoded transcript levels of NDUFA9, SDHA, CIII-core 2, COX4I1 and CV-α were generally (mostly statistically insignificant) lower in the Rieske knockdown cells in both glucose and galactose medium, or only slightly increased in the galactose medium. It thus appears that these selected OXPHOS genes are indeed differentially expressed as is often reported for OXPHOS deficiencies, possibly due to alternate functions or stability (Chevallet *et al.*, 2006, Marusich *et al.*, 1997). The differential expression in the two complex I subunits, NDUFS3 and NDUFA9, highlights this limited coordinate expression, possibly because NDUFS3 is part of the core catalytic core of complex I, whilst NDUFA9 is not (Hirst *et al.*, 2003; Ugalde *et al.*, 2004). However, to a certain extent, the expression of these selected OXPHOS genes are also coordinately reduced (Reinecke *et al.*, 2009; van Waveren & Moraes, 2008).

From the results it can also be observed that (although mostly statistically not significant) expression of nuclear, mitochondrial and even regulatory transcripts were mostly reduced in

glucose medium, but in the galactose medium where cells are forced to rely on OXPHOS energy production and increased ROS levels were observed, transcripts were less reduced or even increased (as was seen with mtDNA copy number). This again supports the hypothesis of compensatory regulation of genes involved with OXPHOS in the presence of an OXPHOS deficiency. However, in view of the fact that poor cell division occurred when using galactose medium (results not shown), it is important that any changes in the galactose medium compared to glucose medium be taken into consideration with caution, in view of the fact that there might be secondary consequences to the use of galactose (and glutaminolysis) as primary energy source in the 143B osteosarcoma cells.

Previous studies reported that expression of the regulatory proteins, TFAM, mtSSB and POLRMT, tightly correlates with mtDNA copy number (Schultz *et al*., 1998, Seidel-Rogol *et al*., 2002, Davis *et al*., 1996; Ekstrand *et al*., 2004) and expression of these regulatory proteins (TFAM, TFB2M, POLRMT, mtSSB and DNApolγ) are in turn regulated by the two nuclear transcription factors, NRF-1and NRF-2, which have also been shown to be redox sensitive (Piantadosi & Suliman, 2006; Miranda *et al*., 1999; Bruni *et al*., 2010). Considering these reports, the relatively unchanged TFAM and POLRMT expression observed in this study is in accordance with the relatively stable mtDNA copy number ratio in the Rieske protein knockdown cell line in both glucose and galactose medium, but in contrast with the increased ROS levels in the galactose medium. Also, mtSSB expression was statistically significantly reduced in the Rieske protein knockdown cells even though mtDNA copy number remained unchanged. This can be explained by the observation by Yin *et al.* (2004) that found a strong comparison between mtSSB expression and mtDNA copy number in non-tumorous tissues, but was absent in tumorous tissues. It is, therefore, also possible that the reduction in mtSSB transcripts is specific to the 143B cell line investigated here.

The expression of DNApolγ was significantly increased in the Rieske-shRNA knockdown cells grown in galactose medium, as expected, since, as mentioned earlier, the transcription factor involved to a certain extent in DNApolγ transcription, NRF-2, is also redox sensitive. It is known

that, in addition to being involved in mtDNA replication, DNApolγ is also involved in the ATP-dependent base excision repair pathway to repair DNA damage in mitochondria. One possible explanation for the increased DNApolγ mRNA levels in the galactose medium might be that, due to the increased ROS production in the galactose-treated cells, the impending oxidative damage to the mitochondrial DNA might stimulate the increased expression of DNApolγ to aid in the repair of the mtDNA.

Another regulatory protein that showed increased mRNA levels in the galactose medium was that of TFB2M. The increased expression of TFB2M transcripts in this study (and also in the transient NDUFS3 knockdown model in Section 4.3.3) might indicate that TFB2M is a key player in the proposed compensatory mechanism for OXPHOS deficiency as described by Heddi and co-workers (1999). However, it is important to note that the TFB2M mRNA level in the empty vector control cells in galactose-rich medium was significantly lower than in the glucose-rich medium. This is in contrast to the generally higher mRNA levels of the various transcripts in the galactose-rich medium than the glucose-rich medium for the empty vector control cells and further studies should, therefore, be conducted to confirm the mRNA levels in the empty vector control cells.

In a previous study by Seidel-Rogol *et al.* (2002), increased TFAM and POLRMT expression was delayed relative to mtDNA copy number when partially mtDNA-depleted cells were grown without ethidium bromide, although mitochondrial transcript levels were increased early after recovery. The authors concluded that one reason for this might be that another transcription factor might be responsible for this early increase in transcription/replication. In the current study significant increases were seen, not only in TFB2M mRNA levels, DNApolγ mRNA levels and ROS levels, but also in the mitochondrial ND6 and D-loop mRNA levels in the Rieske-shRNA knockdown cells in the galactose-rich medium. It is, therefore, possible that TFB2M might be the first regulatory transcript to be increased as part of the compensatory mechanism in OXPHOS deficient cells to increase mtDNA transcription, before increases in mtDNA copy number, TFAM and POLRMT can be seen.

Therefore, we conclude that a deficient complex III function via Rieske subunit knockdown, which does not result in increased ROS levels, do not significantly affect the regulation of mtDNA replication or transcription as well as nuclear OXPHOS gene transcription. However, when this deficiency was accompanied by increased ROS levels, such as with the galactose medium where cells rely more strongly on OXPHOS energy production, these mitochondrial, nuclear and regulatory transcripts were slightly less decreased, whilst a few, such as N6, D-loop, DNApolγ and TFB2M were increased. This means that even though mtDNA copy number might be unaffected, OXPHOS genes could be up-regulated to compensate for increased OXPHOS energy demand and that certain regulatory proteins (TFB2M and DNApolγ) might be up-regulated earlier than other regulatory proteins. Furthermore, considering the regulation elements involved in these processes, increased ROS production in OXPHOS deficiency is probably not enough for significant responses of all regulatory proteins involved in mtDNA replication/transcription *in vitro*.

CHAPTER SIX

CONCLUSIONS

6.1. INTRODUCTION

Disorders of OXPHOS are regarded as one of the most frequent groups of metabolic disorders and they lead to a spectrum of clinical disease, from exercise intolerance to lethal multi-systemic disorders (Bénit *et al.*, 2009; Distelmaier *et al.*, 2009; Thorburn *et al.*, 2004; Cree *et al.*, 2009). On a genetic level, this system is controlled by both the mitochondria and the nucleus and the nuclear-mitochondrial communication that forms the foundation for coordinate expression of proteins involved in OXPHOS relies on complex regulatory mechanisms (Cannino *et al.*, 2007). Deficiencies of OXPHOS often, but not inevitably, lead to altered calcium homeostasis, decreased ATP production and increased production of ROS (Ermak & Davies, 2002; Vives-Bauza *et al.*, 2006, Verkaart *et al.*, 2007, Koopman *et al.*, 2007, Smeitink *et al.*, 2006, Brookes *et al.*, 2004; Turrens *et al.*, 1980; Dröse & Brandt, 2008). In turn, this could lead to oxidative damage to lipids, proteins and DNA, altered mitochondrial membrane potential and apoptosis.

Along with other aspects that include gene expression responses, cellular threshold to tolerate a dysfunction and genetic composition, various tissues and ultimately the organism as a whole, may present a disorder that clinically manifests itself. Clinically and biochemically, these disorders are highly heterogeneous and complex. Although significant progress has been made to elucidate this complex nature of primary mitochondrial energy deficiencies in the past two decades, the cellular responses that form the basis of disease expression remains uncharted territory to a great extent. As discussed in Chapter 1 and expressed before (Smeitink *et al.*, 2006), a better understanding of the processes involved in these disorders requires insight on a global/holistic/untargeted cellular level. In addition, as expressed by Reinecke *et al.* (2009), well defined disease models are another requirement. In this study, which resulted from observations that combined these two aspects, i.e. a transcriptomic investigation of well defined cell lines with complex I deficiency (van der Westhuizen *et al.*, 2003), specific (targeted) areas of cellular

responses to a primary OXPHOS deficiency were investigated. This, therefore, represents a hypotheses-driven, empirical study.

6.2. THEORETICAL BACKGROUND, RATIONALE AND HYPOTHESES

Differential expression of genes that encode proteins involved in mitochondrial biogenesis is a consequence associated with OXPHOS deficiencies, as summarized in Chapter Two (Reinecke *et al*., 2009). It has been proposed that this differential expression might be due to increased ROS production leading to oxidative damage of mtDNA and mtRNA (Yakes & van Houten, 1997; Lee & Wei, 2005) and furthermore, that once a certain threshold of reduced energy production has been reached, a compensatory mechanism that increased transcription of genes involved in mitochondrial biogenesis will be activated by stress-related retrograde effectors, and in particular, by increased oxidative stress (Heddi *et al*., 1999; Lee & Wei, 2005; Seidel-Rogol & Shadel, 2003; Miranda *et al*., 1999; Davis *et al*., 1996; Virbasius & Scarpulla, 1994). Amongst others, these proteins include structural OXPHOS proteins, proteins involved with regulation of mtDNA replication/transcription and metallothioneins (van der Westhuizen *et al*., 2003). Metallothioneins have been identified as one of the scavengers of ROS in a manner similar to that of glutathione, which is due to its high cysteine content (Kägi *et al*., 1974; Thornalley & Vašák, 1985; Ebadi *et al*., 2005). In humans, MT1 and MT2 isoforms are ubiquitously expressed, with MT2A appearing to be the predominantly expressed isoform (Palmiter *et al*., 1992; Quiafe *et al*., 1994; Hidalgo *et al*., 2001; Heguy *et al*., 1996). In a study by van der Westhuizen *et al.* (2003), increased expression of metallothioneins in inherited complex I deficient fibroblasts during carbon source transition from glucose to galactose was observed. Although a clearly distinctive role for MT isoforms remains unclear, a structural and functional association with mitochondria has recently been documented in the review by Lindeque *et al.* (2010). The rationale behind the first part of this study was that the observed expression of MTs in complex I deficient cells may be an indication of their involvement in mitochondrial function as a protective adaptive response. The hypothesis was that the increased expression of MTs, and in particular MT1B and MT2A, in complex I deficient cells has a protective effect against ROS-related consequences of complex I deficiency. For this

investigation MT expression was characterized in an *in vitro* complex I deficient model, by evaluating expression of different MT isoforms in the presence of the complex I inhibitor rotenone and by determining the effects of over-expression of MT2A and MT1B isoforms on key parameters, including ROS production, ATP production, mitochondrial membrane potential and apoptosis (Chapter Three).

From the limited data in a variety of disease models of OXPHOS deficiency, the differential expression of genes associated with OXPHOS was found to be highly diverse and sometimes inconsistent (reviewed in Chapter Two; Reinecke *et al.*, 2009). However, accurate assessments between the various reports are prevented by the diversity of the disease models, including the types of cell lines/tissues, mutations, experimental designs and genetic background and insufficient data relating to OXPHOS enzyme activities. In addition, the measurement of especially ROS production, in addition to altered transcription of regulatory proteins involved in mtDNA replication/transcription and structural OXPHOS transcripts, is often lacking in these reports. However, to consider ROS production as the main modulator of differential expression of genes and to assume that this leads to compensatory adaptive responses as proposed by Heddi *et al.* (1999), is a generalization that is unlikely to be true in all cases of OXPHOS deficiencies.

The rationale behind the second part of the study was that the observed differential expression of mtDNA encoded genes and increased ROS production in complex I and other OXPHOS deficient cells may be an indication of the aberrant mtDNA replication/transcription as an adaptive response. The second hypothesis stated that genes involved in mitochondrial replication and transcription are differentially expressed in OXPHOS deficient cell lines. This hypothesis was investigated by evaluating mitochondrial DNA replication and transcription as well as the transcription of selected nuclear genes in *in vitro* models with OXPHOS deficiencies (Chapters Four and Five).

6.3. MT EXPRESSION IN ROTENONE INDUCED COMPLEX I DEFICIENT HELA CELLS

In the first part of this thesis it was demonstrated that MT2A expression in HeLa cells is highly inducible with rotenone treatment, with a significant increase in expression of MT2A and ROS production only after residual complex I activity was inhibited to levels below 50%. Treatment with myxothiazol, an inhibitor of complex III at the Qo site, that has also previously been shown to not lead to increased ROS production (Turrens, 2003; Bénit *et al*., 2009) showed no significant increases in ROS production or MT expression. It, therefore, supports a ROS related mechanism of rotenone induced MT2A expression. In addition, surprisingly, neither MT1A nor MT1B RNA expression induction was detected with any of the inducers used.

In general, increased rotenone concentrations in HeLa cells lead to reduced ATP levels, cell viability and membrane potential, although these reductions were less severe in the MT2A over expressing cell lines. Simultaneously, in control cells rotenone treatments lead to increasing caspase 3/7 activity, cytosolic nucleosome enrichment and to a lesser extent ROS production, while in the MT2A-, and to a lesser extent the MT1B over expressing cells, these increases were less severe. In the MT1B over expressing cell line, ATP levels and membrane potential were also less reduced in the rotenone treated cells, although investigations into cell viability showed no protective effect of MT1B over expression against rotenone treatment. The MTT assay (which was utilised as measurement of cell viability) is much more selective to measure necrosis than apoptosis. This assay indicated that MT1B over expression resulted in protection against apoptosis, whilst MT2A over expression resulted in protection against both apoptosis and ROS-induced necrosis. This could also indicate why rotenone treated cells did not show more than 30% decrease in cell viability, contrary to what was expected. In a state of reduced ATP generation as observed following rotenone treatment, apoptosis may proceed until cellular ATP stores are depleted or reduced to a point where cell death becomes necrotic rather than apoptotic (Isenberg & Klaunig, 2000). It is possible that this state of necrosis has not been reached in these cells under the set conditions, as cell viability decreased only to approximately 70%.

It is important to also note that increased ROS production as well as decrease in ATP levels induced by rotenone treatment was limited, possibly because consequences as a result of deficient electron transport chain in the predominantly glycolytic HeLa cells may, in general, be less pronounced than in cell lines which rely more on oxidative phosphorylation (Warburg, 1956; Reitzer *et al.*, 1979; Li *et al.*, 2003; Vrbacky *et al.*, 2003). With the induction of more ROS, i.e. by treatment with *t*-BHP, a markedly higher level of ROS could be induced. ROS levels under these conditions were significantly less and cell viability significantly higher in MT2A over expressing HeLa cells, and to a lesser extent in MT1B over expressing cells, compared to control cells. Our results have shown that in rotenone induced complex I deficient HeLa cells over expression of MT2A indeed had a lowering effect on oxidative stress and increased cell viability (specifically ROS-induced necrosis), both of which were especially clear when further challenged with *t*-BHP treatment. Furthermore, MT2A over expression had a preventative or delaying effect on rotenone induced apoptosis in HeLa cells. MT1B, at similar over expressed levels, in general did not show the same responsiveness as MT2A.

The protective property of MTs is probably due to ROS scavenging through the formation of disulfide bonds between the free thiol groups of the MTs and ROS ($O_2^{\cdot-}$ and H_2O_2, but particularly $^{\cdot}OH$), although this study did not conclusively prove direct ROS protection. It does appear, however, that MT2A seems to be more effective in protection against ROS, mtPTP opening, apoptosis and ROS-induced necrosis than MT1B. This could suggest why MT1B are expressed in very low, barely detectable, levels in HeLa cells compared to relatively high expression of MT2A in these cells. This MT1B expression also showed to be un-inducible by rotenone, cadmium and zinc. Heguy *et al.* (1986) stated that methylation of the MT1B gene could be the reason for this observation. Therefore, in normal cells, except in liver and kidney cells where MT1B expression levels are higher, MT2A could be the main protector against the deleterious biochemical effects found with complex I deficiency. A possible explanation for this difference in efficiency to bind and scavenge ROS may be found in the difference in structure between the two isoforms. They differ by nine amino acids. Most of these differences occur directly next to one, or in between two, cysteine residues. MT1B also has one more cysteine residue than MT2A. This would affect the

structural environment surrounding the cysteine residues to which ROS is thought to bind, possibly causing 'knobs' obstructing this binding of ROS. Based on this preliminary investigation, the involvement of MTs with mitochondrial structure and function warrants further investigations. It was subsequently demonstrated by Pretorius (2006) that expression of MTs in rotenone-treated rat tissues is induced *in vivo*. Furthermore, a recent review by Lindeque *et al.* (2010) has highlighted empiric scientific data that support MT involvement in mitochondrial function.

6.4. MTDNA REPLICATION, TRANSCRIPTION AND SELECTED NUCLEAR GENE EXPRESSION IN *IN VITRO* MODELS OF COMPLEX I AND III DEFICIENCY

In the second part of this thesis the differential expression of selected mitochondrial- and nuclear genes involved in OXPHOS function and regulation, including mtDNA, was investigated. This was done firstly by developing a transient siRNA knockdown model of the NUDFS3 subunit of complex I *in vitro* and to determine its effect on several biochemical parameters (ROS and ATP levels), mtDNA copy number, total mtRNA levels and RNA levels of several nuclear- and mitochondrial-encoded transcripts encoding structural as well as functional proteins (Chapter Four). Additionally, to investigate the differential expression in the presence of long-term OXPHOS deficiency, a stable shRNA knockdown models of complex I (NDUFS3 subunit) and complex III (Rieske protein) were developed, in either glucose-rich or galactose-rich medium to better challenge mitochondrial energy metabolism (Chapter Five).

Significant transient knockdown of NDUFS3 lead to decreased ATP production (although not statistically significant), but ROS levels in the cell lines transfected with 10 nM siRNA was not significantly increased. In addition, mtDNA copy number and total mtRNA levels were not significantly changed with 10 nM knockdown. This supports the finding by Iuso *et al.* (2006) that not all complex I deficiencies lead to increased ROS production. It also implies that any changes in mitochondrial or nuclear transcript levels, or the levels of transcripts involved in mtDNA regulation, are not due to increased oxidative damage or redox-related regulation in this model. This includes

the significant decrease in mitochondrial H-strand transcripts (which is probably due to differential regulation or half-lives), the decrease in DNApolγ expression (although not significant) and the increased expression of TFB2M (also not significant).

The reduced expression of DNApolγ (although not significant) with transient siRNA transfection contradicts the general perception that its expression is not differentially affected even when mtDNA copy number or other regulatory transcripts were affected. Although mtDNA copy number was relatively unchanged, the DNApolγ expression pattern was similar to that of the H-strand transcripts. Due to the lack of significant ROS production with the 10 nM siRNA transfection, we must conclude that this differential expression is not due to redox-sensitive regulation of these two transcripts, but it might be due to the coordinate regulation of factors involved with mtDNA regulation/transcription due to retrograde signalling not involving ROS production. The coordinate regulation of all the transcripts investigated is confirmed by the generally slightly reduced expression, with the exception of TFB2M. Because TFB2M expression was increased (although not significantly) with transient siRNA transfection, whilst TFAM, mtSSB and POLRMT transcripts were only slightly decreased, it is possible that TFB2M might be the first regulatory transcript to be increased as part of the compensatory mechanism in OXPHOS deficient cells, before responses (increases) in mtDNA copy number, TFAM and POLRMT may occur.

When evaluating this transient knockdown model and the subsequent evaluation of the effect on mtDNA replication and transcription the use and limitations of siRNA have to be considered. One limitation that has to be recognised is the influence of the oligonucleotide transfections used for RNA interference. In addition to the toxicity of the transfections on the cells, non-specific or off-target effects have also been associated with the use of siRNA. These are changes in mRNA expression due to interaction between the siRNA guide strand and partially complementary, but unrelated sites (Alemán *et al.*, 2007). It has been proposed that these off-target effects increase with increased siRNA concentration, especially above 10 nM (Semizarov *et al.*, 2003; Tschuch *et al.*, 2008; Persengiev *et al.*, 2004). It is, therefore, important to consider these non-specific effects when interpreting experiments with 100 nM siRNA transfection. Since 10 nM

and 100 nM knockdown both showed similar significant inhibition in complex I enzyme activity, but showed significant differences in ROS levels and expression levels of the transcripts evaluated at 100 nM, it is most probable that these changes are not related to the level of complex I knockdown or deficiency, but rather to a secondary off-target effect of the siRNA transfection. Additionally, the lack of significant changes in ADP/ATP ratio, ROS production or general transcript expression might be due to the transient nature of the siRNA knockdown model. Alternatively, it might be explained by the limited dependence on OXPHOS by cancerous cell lines which limits the effect of a stressor on the OXPHOS system on the parameters measured in this study and possibly the threshold of deficiency, that would lead to a compensatory increase in expression of proteins involved in OXPHOS, was not yet reached.

To exclude the toxicity and off-target effects of siRNA knockdown and evaluate long-term effects of an RNA knockdown model *in vitro*, a stable NDUFS3 knockdown model was introduced into the 143B cells. Also, to better challenge energy production in these cancerous 143B cells, that rely more on glycolysis rather than OXPHOS for ATP production (Warburg, 1956; Reitzer *et al.*, 1979), a medium in which galactose is the carbon energy source was included. This induces increased glutaminolysis by the TCA cycle and, therefore, forces the cells to rely on OXPHOS rather than glycolysis for energy (Reitzer *et al.*, 1979). Unfortunately, even though the stable NDFUS3 knockdown model showed significant reduction in NDUFS3 RNA levels, surprisingly no significant reduction in protein levels, fully assembled complex I, or complex I enzyme activity was observed. This might be due to the fact that, unlike the high concentration of siRNA oligonucleotides present with transient siRNA transfections, shRNA are present at physiological concentrations and additional intracellular processing of shRNA is required before it can act as siRNA sequences (summarized by Levanets *et al.*, 2010; Appendix C). Additionally, the coordinate expression of proteins involved in OXPHOS and post-translational regulation of proteins could be responsible for this phenomenon. In spite of this, increased ROS production and some differential gene expression (COXIII, D-loop, NDUFA9, CIII-core2, Rieske, SDHA, mtSSB, DNApolγ and TFB2M transcripts) were observed in this knockdown model. Therefore, the increased ROS levels and differential gene expression might be due to secondary consequences of the shRNA

transfection, rather than a complex I deficiency. Due to the stable nature of the shRNA, it is unlikely that these secondary consequences are due to the transfection reagent or toxicity of the shRNA as can occur with transient siRNA transfections (as summarized by Levanets *et al.*, 2010). Instead, the secondary consequences might be related to if/where the pSIREN-RetroQ-TetP vector, with inserted NDUFS3 shRNA sequence, has been incorporated into the 143B genome, or it might be due to off-target effects of the shRNA sequence (as seen with the 100 nM NDUFS3 siRNA model), or a secondary interferon response (Bridge *et al.*, 2003; Persengiev *et al.*, 2004). Furthermore, these changes in ROS production and differential gene expression did not significantly alter mtDNA copy number, which would suggest that cellular ROS increase alone in these cells does not result in changes in the mitochondrial DNA copy number.

To further investigate the role of ROS production in the differential expression of genes in the presence of OXPHOS deficiency, we introduced a successful stable complex III deficiency *in vitro*, by means of stable Rieske protein knockdown. Previous studies have shown that deficiency of the Rieske protein (forming part of the Qo site) resulted in limited superoxide production whilst still preventing electron transport to cytochrome *c* (Turrens, 2003; Chen, 2003; Muller *et al.*, 2004; Brunelle *et al.*, 2005; Bell *et al.*, 2007). Although a significant change in ROS levels was not observed in the glucose medium (as expected), the knockdown model showed significantly increased ROS levels in the galactose medium. In view of the fact that the Rieske protein is necessary for production of ROS at the Qo site of the Q cycle (Bell *et al.*, 2007; Brunelle *et al.*, 2005), the ROS production seen in the galactose-rich medium in the stable Rieske knockdown model would, therefore, be attributed to ROS production at another site than Qo, such as the Qi site or maybe at complex I.

From the results it can also be observed that (although mostly statistically not significant) expression of nuclear, mitochondrial and even regulatory transcripts were mostly reduced in glucose medium, but in the galactose medium where cells were forced to rely on OXPHOS energy production and increased ROS levels were observed, transcripts were less reduced or even increased (as was seen with mtDNA copy number). This again supports the hypothesis of

compensatory and coordinate regulation of genes involved with OXPHOS in the presence of an OXPHOS deficiency. Even so, the coordinate expression between transcripts was limited, possibly due to alternate functions or stability (Chevallet *et al.*, 2006, Marusich *et al.*, 1997). Two specific examples are the differential expression of the two complex I subunits, NDUFS3 and NDUFA9, and the L-strand and H-strand mitochondrial transcripts. However, in view of the fact that poor cell division occurred when using galactose medium (results not shown), it is important that any changes in the galactose medium compared to glucose medium must be evaluated with caution, in view of the fact that there might be secondary consequences, such as slow growth rates and thus difference in cell cycle, to the use of galactose (and glutaminolysis) as primary energy source in the 143B osteosarcoma cells. Additionally and significantly, in a study by Kok (2009) it was shown that global methylation was significantly increased in these transfected 143B cells and that the energy resource and culture time also greatly affected the methylation status of the cells. It is well known the methylation plays an important role in epigenetic events and it is possible that changes in methylation status might account for some of the differential expression seen in the current OXPHOS deficient models.

Even though the coordinate changes in mtDNA copy number with OXPHOS deficiency have been proven before (Heddi *et al.*, 1999; Collombet *et al.*, 1997; Bonod-Bidaud, 1999), there was no change in mtDNA copy number in the Rieske knockdown model in either glucose or galactose medium, even though significant complex III deficiency was confirmed and a significant increase in ROS levels was measured in the galactose medium. From these results we can conclude that a stable Rieske knockdown model, with all the associated consequences, does not alter mtDNA copy number, even in the presence of increased ROS production and that any changes in the expression of transcripts are not due to regulation of mtDNA copy number, but rather to a regulation of mtDNA transcription.

The unchanged expression of TFAM and POLRMT and unchanged mtDNA copy number in the stable Rieske knockdown model in both glucose and galactose medium is in accordance with previous observations (Schultz *et al.*, 1998, Seidel-Rogol *et al.*, 2002, Davis *et al.*, 1996; Ekstrand

et al., 2004). However, it is in contrast with the increased ROS levels in the galactose medium, since expression of TFAM and POLRMT is regulated by the two nuclear transcription factors, NRF-1and NRF-2, which have also been shown to be redox sensitive (Piantadosi & Suliman, 2006; Miranda *et al.*, 1999; Bruni *et al.*, 2010). The same studies also indicated that mtSSB expression tightly correlates with mtDNA copy number, which is in contrast to the reduced mtSSB expression in this study. This can be explained by the observation by Yin *et al.* (2004) that found a strong comparison between mtSSB expression and mtDNA copy number in non-tumorous tissues, but was absent in tumorous tissues. It is, therefore, possible that the reduction in mtSSB transcripts is specific to the 143B cell line investigated here. The expression of DNApolγ was significantly increased in the Rieske-shRNA knockdown cells grown in galactose medium, as expected, since the redox-sensitive transcription factor NRF-2 is also involved in DNApolγ transcription (Piantadosi & Suliman, 2006; Miranda *et al.*, 1999; Bruni *et al.*, 2010). It is known that, in addition to being involved in mtDNA replication, DNApolγ is also involved in the ATP-dependent base excision repair pathway to repair DNA damage in mitochondria. One possible explanation for the increased DNApolγ mRNA levels in the galactose medium might be that, due to the increased ROS production in the galactose-treated cells, the impending oxidative damage to the mitochondrial DNA might stimulate the increased expression of DNApolγ to aid in the repair of the mtDNA.

The increased expression of TFB2M transcripts in the galactose medium (and also in the transient NDUFS3 knockdown model in Section 4.3.3) might indicate that TFB2M is a key player in the proposed compensatory mechanism for OXPHOS deficiency as described by Heddi and co-workers (1999), although further investigations will be necessary to confirm this. Seidel-Rogol and co-workers (2002) suggested that before TFAM and POLRMT expression might regulate mtDNA copy number and transcription, another transcription factor might be responsible for early changes in transcription/replication. In the current study significant increases were seen, not only in TFB2M mRNA levels, DNApolγ mRNA levels and ROS levels, but also in the mitochondrial ND6 and D-loop mRNA levels in the Rieske-shRNA knockdown cells in the galactose-rich medium. It is, therefore, possible that TFB2M might be the first regulatory transcript to be increased as part of the

compensatory mechanism in OXPHOS deficient cells to increase mtDNA transcription, before increases in mtDNA copy number or TFAM and POLRMT expression can be seen.

6.5. FINAL CONCLUSIONS

To summarize, recent interest in the downstream adaptive responses to deficiencies of the OXPHOS system has revealed that, via differential gene expression, several genes may be involved in novel responses apart from those already associated with the deficiency, such as induction of apoptosis and changes in redox status (van der Westhuizen *et al.*, 2003; Heddi *et al.*, 1999; Collombet *et al.*, 1997). Of these responses, few, if any, have been investigated further or reported. We have investigated the expression and role of MTs in an *in vitro* complex I deficiency model and concluded that the induced expression of MTs, specifically MT2A, has a protective effect against death-causing cellular consequences of rotenone treated HeLa cells through a redox-related mechanism. Our data are comparable to current literature reports on the functional properties associated with MT expression but specifically reveals MT2A expression to be a beneficial downstream adaptive response in complex I deficient cells. In addition, we have established that an OXPHOS deficiency, which does not result in increased ROS levels, does not significantly affect the regulation of mtDNA replication/transcription or nuclear OXPHOS gene transcription. However, when OXPHOS deficiency was accompanied by increased ROS levels, some structural mitochondrial-encoded transcripts and regulatory nuclear-encoded transcripts were up-regulated, specifically ND6, D-loop, DNApolγ and TFB2M. This supports current literature reports that propose a compensatory mechanism in OXPHOS deficiencies once a threshold of deficiency has been reached. Additionally, this compensatory regulation might be more dependent on mtDNA transcription than mtDNA copy number and TFB2M might be a key regulatory protein involved early in this mechanism. Furthermore, DNApolγ might be up regulated in response to increased ROS production in OXPHOS deficiency as a possible protective mechanism against oxidative damage to mtDNA. Moreover, increased ROS production in OXPHOS deficiency is probably not exclusively responsible for responses of all regulatory proteins involved in mtDNA replication/transcription *in vitro*. However, as certain limitations of *in vitro* models have become

apparent in this study, it remains to be determined what the mechanistic properties of altered mtDNA copy number and this differential expression of MTs and selected genes involved in OXPHOS biogenesis are and whether the expression is functionally relevant to complex I and other inherited OXPHOS deficiencies *in vivo.*

Even though various other analyses could be conducted to expand on or substantiate the results generated in this study, it would be feasible to strongly consider extending the study to *in vivo* models or fully characterized OXPHOS-deficient patient cell lines/tissues. This would eliminate the secondary effects of chemical inhibition, transient siRNA models and its off-target effects, or cancerous cells relying mostly on glycolysis for energy production. Most importantly, it might also disprove or support the lack of differential expression in an *in vivo* model for all regulatory proteins that did not show differential expression in the current study. The possible induction of MTs as a biomarker of increased ROS production and as a therapeutic agent in mitochondrial disorders *in vivo* should also be investigated in future. Confirmation of TFB2M as a key player in the early regulation of mtDNA replication/transcription, and the mechanism behind it, could prove valuable for future investigations into the possible therapeutic effect of up-regulation of mtDNA replication/transcription to compensate for OXPHOS deficiencies. Further investigations into the functionality of DNApolγ expression and its putative protective role to prevent oxidative damage to mtDNA in the presence of OXPHOS deficiency might also prove valuable for future therapeutic investigations.

In conclusion, this study has investigated the expression of genes involved in human OXPHOS deficiencies. One of the contributions of the study was an extensive literature review that revealed the striking diversity and complexity of transcriptional responses that were reported for these disorders. As an empirical scientific study, two specific hypotheses were derived from these observations and investigated using *in vitro* models for complex I and III deficiencies. This has resulted in conclusive evidence that supported the first hypothesis, and under certain conditions, also the second hypothesis. Although certain results were considered inconclusive owing to the limitations of the *in vitro* systems used, valuable new knowledge about the consequences of

OXPHOS disorders was generated and published, or submitted for publication, during the course of this study. Considering that the consequences of energy metabolism deficiencies contribute to cellular health and death, which is relevant to many diseases and not only to inherited metabolic diseases, the hope is expressed that such investigations will be supported, that it may continue and contribute to better and extended human health.

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APPENDIX A

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Review

OXPHOS gene expression and control in mitochondrial disorders

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ABSTRACT

The cellular consequences of deficiencies of the mitochondrial OXPHOS system include a variety of direct and secondary changes in metabolite homeostasis, such as ROS, Ca^{2+}, ADP/ATP, and NAD/NADH. The adaptive responses to these changes include the transcriptional responses of nuclear and mitochondrial genes that are mediated by these metabolites, control of the mitochondria permeability transition pore, and a great variety of secondary signalling elements. Among the transcriptional responses reported over more than a decade using material harboring mtDNA mutations, deletions, or depletions, nuclear and mitochondrial DNA OXPHOS genes have mostly been up-regulated. However, it is evident from the limited data in a variety of disease models that expression responses are highly diverse and inconsistent. In this article, the mechanisms and controlling elements of these transcriptional responses are reviewed. In addition, the elements that need to be evaluated, in order to gain an improved perspective of the manner in which OXPHOS genes respond and impact on mitochondrial disease expression, are highlighted.

1. Introduction

The mitochondrial oxidative phosphorylation (OXPHOS) system, which produces the majority of cellular energy in the form of ATP, is controlled on the genetic level by two distinct genomes: the circular mitochondrial genome (mtDNA) and the nuclear genome. The circular mitochondrial genome of ~16.6 kb encodes thirteen structural subunits of complex I, III, IV, and V 22 tRNA and two ribosomal RNA genes used for RNA translation [1]. The nuclear genome encodes the additional genes required for mtDNA maintenance, replication, transcription, translation, post-translational modification, transport, and assembly exclusively. In addition, the nuclear genome controls all other aspects of mitochondrial biosynthesis and function. Nuclear–mitochondrial communication is a highly complex process dominated by the nucleus [2].

A deficiency in mitochondrial function is caused by a dysfunction of one (or more) of hundreds of nuclear- or mitochondrial-encoded proteins. Over the past two decades, it has become clear that the interplay between the mitochondrion and nuclear genome affects mitochondrial disease expression, as evident in diseases that result from mutations in genes involved in the mtDNA replication machinery and in nucleotide metabolism. This impacts qualitatively and/or quantitatively on mtDNA, such as progressive external ophthalmoplegia (PEO), mitochondrial DNA depletion syndrome, and mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) [3].

Mitochondrial interplay with the nuclear genome is also evident in the disorders of nuclear- and mtDNA-encoded subunits of the OXPHOS complexes. Primary deficiencies of the OXPHOS system impact directly on mitochondrial function and result in several disease phenotypes [4]. With recent advances in systems biology for investigating gene expression and function, key aspects of nuclear–mitochondrial communication in these deficiencies have been revealed. As with these deficiencies in which the mtDNA replication machinery has been primarily compromised, differential expression of mtDNA and nuclear OXPHOS genes occurs in cells with mtDNA or nuclear mutations of structural subunits of the OXPHOS system. Differential expression of OXPHOS and related genes has a significant impact on disease expression because of the importance of these genes in energy metabolism, which is compromised in these disorders. This article highlights these observations and investigates the underlying cellular mechanisms that control mitochondrial and nuclear OXPHOS gene expression.

2. Cellular biochemical consequences of OXPHOS deficiencies

Oxidative phosphorylation and deficiencies thereof involve and modulate a great number of cellular functions and metabolic processes upstream and downstream of the five enzyme complexes. Moreover, in considering the effect of OXPHOS deficiencies, it is essential to recognize that deficiencies of the individual enzyme complexes may result in varied biochemical responses. This is evident from existing (but limited) reports of biochemical and gene expression responses to various deficiencies of OXPHOS as discussed in this article.

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Table 1
Summarized findings of OXPHOS and other gene expression investigations of human mitochondrial disorders.

Tissue	Phenotype/deficiency (genotype)	Technique	nDNA expression	mtDNA expression	Reference
Skeletal muscle	MERRF (mtDNA 8344), MELAS (mtDNA 3243), KSS (mtDNA del)	mRNA, Northern blot	•ATPsynβ, ANT1 ↑, GAPDH ↓ (MELAS, MERRF) •ATPsynβ, ANT, GAPDH ↓ (KSS)	•Transcripts ↑ •Processing (light/heavy strand transcript ratio) ↓	[32]
Skeletal muscle (cultured)	CIII deficiency (mtDNA cytb), MELAS (mtDNA 3243, *CI + IV*), MELAS + CM (mtDNA 3243, *CI + IV*), CPEO + PM (mtDNA del, *CI + IV*), KSS (mtDNA del, *CI + IV*), CI deficiency (nuclear), CIV deficiency (nuclear)	mRNA, Northern blot	•ATPsynβ, GAPDH ↑ (MELAS, MELAS + CM, CPEO, CII deficiency) •ATPsynβ, GAPDH ↓ (CIII deficiency)	•Transcripts ↑ (excl. CIII deficiency) •Transcripts ↓ (CIII deficiency) •mtDNA/nDNA ↓	[34]
Skeletal muscle, heart muscle, liver, kidney, brain	LHON (mtDNA 11,778), NARP (mtDNA 8993), MELAS (mtDNA 3243, *CI + IV*), MERRF (mtDNA 8344/8344), MDMD (mtDNA del/dup), CPEO (mtDNA del), FSHMD (nuclear)	mRNA, Northern blot	•↓ ATPsynβ, ANT1/2 ↑ (excl. MDMD) •Glycolytic/bioenergetic genes generally ↑	•Transcripts ↑ •mtDNA/nDNA ↓ (most tissues for MELAS)	[33]
Skeletal muscle (cultured)	MELAS (mtDNA 3243), KSS (mtDNA del)	mRNA, Northern blot, competitive RT-PCR	•ATPsynβ ↓ (MELAS)	•ND2 ↑ •mtDNA/nDNA ↓ (MELAS) •mtDNA/nDNA ↑ (KSS)	[35]
Fibroblasts	mtDNA depletion (*CI + III, CIV*), RhoO (EtBr induced, *CI + III, CIV*)	Protein, Western blot	•COXVic absent •COXVI, COXVa, SDH0, SDH70 ↓	Absent due to defect	[36]
Skeletal muscle	Myopathy (mtDNA del), PEO (mtDNA 3243), MELAS (mtDNA 3243) (for all groups, varying deficiencies of combined CI, CI + III, CII + III, and CIV are reported)	mRNA, microarray (Affymetrix HG U133A, 22 283 oligonucleotide targets)	•OXPHOS structural genes ↑ (mtDNA del) •Genes involved in urea cycle/arginine catabolism ↑ •CDKN1A, -1C (cell cycle G1 arrest, DNA repair mediators), and other cell cycle regulators ↑ •CFLAR (anti-apoptosis) ↑ •PEX5 (peroxisomal biogenesis), MAOA (neurotransmitter catabolism) ↓ •RNA Pol II regulation ↑ (MELAS) ↓ (PEO) •Neurobiological structures, fatty acid oxidation, detoxification of H_2O_2, cell signalling ↓ (PEO)	Not reported	[38]
Cybrids	LHON (mtDNA 11778 and 3460), mtDNA depletion	mRNA, microarray (Affymetrix U95Av2, 12 599 oligonucleotide targets)	•Respiratory chain genes, TCA and other aerobic bioenergetic pathways, Pol II promoter transcription and regulation, anti-apoptosis mostly ↑ (mtDNA depletion) •Aldose reductase (aldehyde reduction), integral membrane protein 28 (anti-apoptotic), H2A histone O (chromosome organization/biogenesis) ↑ (LHON cell line shared) •Scaffold protein TUBA (dynamin/actin regulatory), MTHFD (THF/purine metabolism), sialyltransferase 1 (sialic acid transfer/cell surface antigens/determinants), Raft1 (signal transduction/proliferation/differentiation/apoptosis), lipin 1, immunoglobin superfamily member 3 ↓ (LHON cell line shared)	Not reported	[42]
Lymphoblasts	mtDNA depletion	mRNA, microarray (Affymetrix HG U133A, 22 283 oligonucleotide targets)	•Lipid, amino acid metabolism, bioenergetics and transport, intracellular homeostasis, DNA/RNA binding, transcription, translation, redox balance, cell cycle control, growth arrest, signalling, apoptosis, DNA damage and oxidative stress protection ↑	Absent due to defect	[40]

Table 1 (continued)

Tissue	Phenotype/deficiency (genotype)	Technique	nDNA expression	mtDNA expression	Reference
143B cells (osteosarcoma)	mtDNA depletion	Protein, 2-DE/MS	•Respiratory chain complexes (excl. CII and CV) ↓ (not uniformly) •Mitochondrial translation apparatus ↓ •Mitochondrial transport systems ↓ •Catabolic energy metabolism ↓ •Hax-1 (anti-apoptotic), Smac protein (pro-apoptotic), rhodanese, hydroxysteroid dehydrogenase ↓	Absent due to defect	[43]
Fibroblasts	CV deficiency (mtDNA 9205 and nuclear uncharacterized)	mRNA, microarray (custom-made, 1632 oligonucleotide targets)	•OXPHOS structural genes for complex IV and V, cell growth, differentiation and transduction ↓ (CV nDNA defects) •Cell cycle regulation, Krebs cycle and gluconeogenesis, mitochondrial transcription regulation (TFAM, TFB1M), CytC, NFκB (apoptosis) ↓ (CV mtDNA 9205) •Branched chain amino acid and fatty acid oxidation, complex I structural genes and apoptosis ↑ (CV nDNA defects)	•MTATP6, MTATP8, MTCOX2 ↓ (CV mtDNA 9205) •ND1, ND2, ND4, ND4L ↑ (CV nDNA defects)	[41]
Fibroblasts (differentially cultured)	CI deficiencies (nuclear): LLD (NDUFS4, NDUFS7, NDUFS8), HCE (NDUFS2), HPEM (NRUFV1)	mRNA, microarray (custom-made, 618 cDNA targets)	•Metallothioneins (ROS scavenging, heavy metal regulation), ATP1G1, heat shock proteins ↑ •Pro-apoptotic protein (BNIP3), pyruvate dehydrogenase de-activation (PDK1) ↓	•Transcripts ↓ (selected cell lines)	[37]

Respiratory chain enzyme deficiencies are shown in italics where reported. The following abbreviations are used: LHON (Leber's hereditary optic neuropathy); NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa); CPEO (chronic progressive external ophthalmoplegia); KSS (Kearns–Sayre syndrome); MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes); MERRF (myoclonic epilepsy and ragged red fibers); MDMD (maternally transmitted diabetes mellitus and deafness); FSHMD (facio-scapulohumeral muscular dystrophy); LLD (Leigh-like disease); HCE (hypertrophic cardiomyopathy and encephalomyopathy); HPEM (highly progressive encephalomyopathy); 2-DE/MS (Two-dimensional electrophoresis/mass spectrometry).

An initiator of the immediate and downstream consequences of OXPHOS deficiencies is the production of superoxide. Mitochondrial superoxide production can originate from the ineffective transfer of electrons through the various subunits of the electron transport chain (ETC; complexes I–IV) and the ineffective transfer of carriers (ubiquinone, cytochrome *c*) through the inner mitochondrial membrane. This can lead to the accumulation of electrons and excessive leaking to oxygen to produce reactive oxygen species (ROS), particularly when there is an increased supply of reducing equivalents to the ETC. Complex I (at the bound flavine on the matrix side) and complex III (at the ubiquinol oxidation side) are generally regarded as the main sources of superoxide radicals originating from the ETC [5,6]. The percentage of oxygen converted in this way to ROS under steady state conditions is considered to be much less than the previously estimated 1 to 2% [7] and, in case of respiratory chain deficiencies, would quantitatively be dependent on the amount of electron transfer through the chain and the site of a deficiency within the chain. In fact, a significant part of mitochondrial superoxide production may also originate from the tricarboxylic acid cycle enzyme, α-ketoglutarate dehydrogenase [8], and through a deficiency of the complex II subunit SdhB [9]. Superoxide can result in the generation of other ROS and nitrogen species (RNS), if not dismutated by superoxide dismutases on either side of the mitochondrial inner membrane (Mn and Cu/Zn) or by the radical scavenging effects of antioxidants (vitamins E and C), metallothioneins, or quinone reductase [10,11]. Hydrogen peroxide, which is formed by SOD, can be converted to water by catalase and glutathione peroxidase but can alternatively be converted to hydroxyl radicals by means of the Fenton reaction. The damaging effects of ROS, RNS, and particularly hydroxyl radicals on macromolecules have been extensively documented [12,13] and, through oxidation of these molecules, have been shown to have a direct impact on the viability of genetic and functional molecules inside the mitochondrion and elsewhere in the cell. However, ROS and RNS also act as key messengers in signalling mechanisms that lead to the induction of genes often involved in maintenance and restoration of the cellular redox balance [14,15]. They can also act more directly by altering protein function, such as the activation of uncoupling proteins in brown adipose tissue and the subsequent shift from mitochondrial coupling towards thermogenesis [16].

Abundant evidence of increased superoxide production in OXPHOS deficiencies exists. However, in several reports increased superoxide production is not detected, or an increased superoxide level has no detectable effect on parameters associated with oxidative damage or changes in metabolic homeostasis. Moreover, the origin of superoxide production is not clearly established; for example, ROS production is reported to occur in cell lines harboring mtDNA mutations [17], as well as nuclear mutations of complex I [18,19]. However, ROS production was not detected in a pathogenic mutation of the NDUFS4 subunit of complex I [20] or in HeLa cells containing no mtDNA (ρ^0) [21]. ROS production in OXPHOS deficiencies is therefore not a generalized occurrence and depends on several factors, including the position and severity of the dysfunction, the source of production, and the mechanisms that protect the cell against its possible harmful effects [19,22–24].

Deficiencies of OXPHOS also result in other immediate and downstream metabolic, structural, and functional effects. These effects are closely associated with mitochondrial dysfunction, and are briefly described here. The nicotinamide dinucleotide (NAD) redox balance, which is converted to the reduced state in OXPHOS deficiencies, is a fundamental mediator of several biological processes, such as energy metabolism, calcium homeostasis, cellular redox balance, immunological function, and gene expression [25,26]. Not surprisingly, ATP production, and subsequently ATP/ADP homeostasis, is disturbed in OXPHOS deficiencies [7]. Cellular calcium handling also becomes disturbed during an increased oxidative state, with an influx of Ca^{2+} into the cytoplasm, nucleus, and mitochondria [27]. This has an effect on cellular signalling events, where Ca^{2+} is often a key messenger, and more specifically mitochondrial Ca^{2+} loading, which is compounded by ROS, opens the mitochondrial transition pore, disrupts the inner membrane potential ($\Delta\Psi$), and increases cell death through apoptosis

[28,29]. In complex I deficient fibroblasts, the depolarization of $\Delta\Psi$ itself and the subsequent reduced supply of ATP to Ca^{2+}-ATPases leads to reduced cellular Ca^{2+} stores [30].

The varied biochemical changes that occur in cases of OXPHOS deficiencies have a direct effect on cellular functions. Yet, they are also key underlying mediators of the (retrograde) communication between the mitochondrion and the nucleus, which results in specific gene expression of both nuclear and mitochondrial genomes.

3. Differential expression of mitochondrial and nuclear genes in human OXPHOS deficiencies

The biochemical and structural changes that occur because of deficiencies of the OXPHOS system involve the nuclear and mitochondrial genomes. Differential expression of nuclear and mitochondrial genes has been reported for various in vivo and in vitro OXPHOS deficiency models. Initial reports using targeted investigations of RNA and protein expression have revealed the interaction between the nuclear and mitochondrial genome. However, the development of system biology tools over the past decade has rapidly expanded the number of cellular processes that are affected when a deficiency of the OXPHOS system occurs. In addition, these tools have shown that energy metabolism plays a major role in several related diseases that are not discussed in this article [31]. Table 1 summarizes the main findings of several studies investigating gene expression in the presence of mitochondrial disorders and highlights the expression of nuclear and mitochondrial OXPHOS and related genes. The diversity of the disease models used is evident; thus, except for perhaps the data on muscle in patients harboring common mtDNA mutations and deletions, these profiles cannot be directly compared with confidence. Several factors that greatly affect gene expression are significantly different among these reports, including the type of cell lines/tissues, phenotypes, mutations, experimental designs, and genetic background. In addition, key information on OXPHOS enzyme activities, which is necessary for making a comparison based on enzyme deficiencies, is mostly not present or inconclusive.

Initial investigations of the expression of targeted nuclear- and mitochondrial-encoded genes were conducted on the tissues of patients with mitochondrial DNA mutations, deletions, or depletion phenotypes [32–35]. Expression of nuclear genes involved in mitochondrial (*ATPsynβ* and *ANT1/2*) and glycolytic bioenergetics was often increased in muscle. However, many exceptions were observed, which included most of the various phenotypes and mutation types where expression of these genes was either decreased or similar to the controls [32–35]. Marusich et al. report decreased expression of nuclear genes encoding four OXPHOS subunits—*COXVI*, *COXVa*, *SD30*, and *SD70*—in mtDNA depleted fibroblasts [36]. In addition, mitochondrial gene transcripts were generally found to be increased in these patients, although exceptions in a CIII deficiency [34] and a MELAS and KSS patient have been reported [35]. Interestingly, among the cases of mtDNA mutations or deletions that mostly had a complex I and complex IV deficiency, a similar expression profile also occurred in a patient with complex II deficiency [34]. With the one exception of a KSS patient [35], mtDNA/nDNA ratios were generally found to be decreased [33–35], and reduced processing (light/heavy strand) of mtDNA transcripts was observed [32].

It was proposed from these early observations that the general increased expression of selected genes involved in ATP synthesis was due to a compensatory mechanism that increases transcription of genes involved in energy production. It was further suggested that this increased transcription only occurs when a certain threshold of reduced energy production has been reached [32,33]. This was evident from the study by Heddi et al., in which expression levels in different tissues of a patient identified with a MELAS mutation were measured [33]. They found increased expression of all selected nDNA-encoded genes involved in OXPHOS and the glycolysis pathway in all tissues. All tissues with more than 88% mutant mtDNA showed increased mtDNA transcripts, while kidney tissue with only 73% mutant mtDNA showed decreased transcripts of cyt *b*, ND5/6, COI, and COII; increased tRNA-Ser and -Asp; and unchanged 12S rRNA levels.

A more detailed overview of expression profiles in patients with OXPHOS deficiencies was obtained in recent times using micro-arrays [37–42]. For example, the differential expression of several genes in the muscle of patients with common mitochondrial DNA mutations (A3243G MELAS/PEO and 4977 bp deletion) that lead to varied combined deficiencies of OXPHOS enzymes, excluding complex II, are reported [38]. Many genes showed induced expression in all patients in the form of urea cycle/arginine metabolism; anti-apoptotic factor; CFLAR; and selected cell cycle regulators, including cyclin-dependent kinase inhibitor (CDKN), which is involved in G1 arrest and DNA repair. Only a few genes showed decreased expression in all patients. Significantly, it was shown that some genes were differently expressed in the MELAS and PEO patient subsets, which contained the same mutation but had varied levels of combined enzyme deficiencies, even within phenotype groups. These differently expressed genes include those involved in RNA polymerase II regulation, which were increased in the MELAS subset but decreased in the PEO subset, and genes involved in fatty acid oxidation, hydrogen peroxide detoxification, cell signalling, and the development of neurobiological structures. Increased expression of nuclear encoded OXPHOS genes were observed only in the mtDNA macro-deletion subset of patients, and it is striking to note that the enzyme deficiencies within this patient group were varied but similar to the other phenotypes. Although this is contrary to initial reports on similar patient tissues [32–34] in which general increased expression is reported for one nuclear OXPHOS gene, *ATPsynβ*, differential expression of OXPHOS genes was not associated to mtDNA mutations (LHON 11778 and 3460) in cybrids but rather strongly associated to mtDNA depletion [42]. In Danielson et al., the depletion process of mtDNA had a significant effect on genes involved in mitochondrial bioenergetics pathways, which included increased expression of seventeen genes involved in OXPHOS [42]. Supporting observations have been reported in mtDNA depleted (ρ^0) lymphoblasts [40]. In 143B (osteosarcoma) cells, however, conflicting reports indicate either the decreased expression [43] or unaffected expression [44] of OXPHOS genes in mtDNA depleted cells. Differential expression of nuclear encoded structural OXPHOS genes is mostly not reported in micro-array data sets (from which it is assumed they are unaffected) in which deficiencies originate from either mitochondrial or nuclear mutations and appear to be exclusively associated with mtDNA depletion. In fact, Cizkova et al. report a decreased expression of complex IV and V genes in fibroblasts of isolated complex V deficient patients harboring nuclear mutations [41]. In Chevallet et al., differential levels of decreased respiratory complex subunits, translation apparatus (particularly mitochondria ribosomal proteins), and ion and protein import systems, such as membrane proteins, were found in 143B ρ^0 cells when compared to wild-type cells [43]. The decreased levels of subunits of respiratory complexes were not significant or uniform (CII and CV subunits remained unchanged), indicating that some stable sub-complexes can survive in ρ^0 mitochondria. It was suggested that this is because some sub-complexes have other unknown functions or because they are important for mitochondrial stability, or else because of unregulated coordinated nuclear transcription.

Similarities in the differential gene expression of mtDNA depleted (ρ^0) lymphoblasts [40] and cybrids [42] have been reported. Increased expression of the genes involved in mitochondrial energy metabolism, including TCA cycle and ETC, in addition to transcription regulation occurred in these cell lines. Dissimilarities were observed in the induction of anti-apoptotic factors in cybrids, while several pro-apoptotic factors were increased in lymphoblasts. This again demonstrates the cell-specific regulation of gene expression, and indicates

that, in the case of apoptosis, the energy pathway predominance of the cell type can direct apoptosis induction [45].

Comprehensive expression profiles of nuclear encoded OXPHOS deficiencies of the OXPHOS system are limited, including only a comparison of expression under defined energy source changes in isolated complex I deficient fibroblasts [37] and, recently, in nuclear encoded complex V deficiency [41]. In both these cases as well, similarities and marked variations of expression profiles were detected, even in patients that harbored the same mutation. Furthermore, no correlation could be made with the levels of enzyme deficiency. Significant increased expression of the ROS scavenging and metal regulating family of proteins (metallothioneins) and decreased expression of pro-apoptotic protein (BNIP3) and pyruvate dehydrogenase de-activation protein (PDK1) occurred in complex I deficient cells when culture conditions were changed from glucose to galactose, in order to challenge oxidative energy production [37]. In selected patients, and notably in the patient with the most severe deficiency, significantly decreased expression of mtDNA transcripts occurred. However, increased expression of mtDNA transcripts was detected in nuclear encoded complex V deficient fibroblasts [41]. This was accompanied by increased expression of fatty acid catabolism, complex I structural genes, and apoptosis, while decreased expression of nuclear complex IV and V structural genes, cell growth, differentiation, and transduction were reported. In the same report, and in contrast to the reports referring to mitochondrial DNA mutations and deletions mentioned previously, mtDNA mutations of complex V resulted in decreased expression of genes of the TCA cycle, cell cycle regulation, mitochondrial transcription, and apoptosis.

It is thus evident from studies of differential expression in mitochondrial disorders that there is great variation in the expression of both nuclear and mitochondrial genes. For OXPHOS genes in particular, the variation in expression also occurs under steady state levels over a more than two-fold range between various tissues and cells and of different sources [44]. This is an important observation, as the varying levels of steady state expression are similar to what is often regarded as 'differential expression' when pathology is investigated. In the limited published data of a highly varied group of patient cell lines and enzyme deficiencies, induced expression of genes involved in energy metabolism occurs in most of the cases. However, the diversity of expression of these genes and apparent lack of correlation with the type and level of OXPHOS enzyme deficiency strongly underscores the significant influence of genetic make-up in cellular response.

4. Regulation of nuclear OXPHOS gene expression

Nuclear gene expression of OXPHOS and other genes involved in mitochondrial function and protection is controlled by retrograde (mitochondria-to-nucleus) signalling mechanisms. These signalling pathways are modulated in part by metabolites controlled by the mitochondrion, including Ca^{2+}, ROS, and ATP. The interplay between these metabolites in the mitochondrion and their control of the mitochondrial permeability transition pore has previously been reported on [29; 30; 44; 47]. Much less is known, however, about the downstream signalling mechanisms of these retrograde effectors in eukaryotes. Calcium-mediated signalling can involve one or more of several pathways, including activation of calcineurin (an activator of NFAT and NFκB), Ca^{2+}-dependent PKC, JNK/MAPK, and CaMK IV (and CREB) pathways [46,48]. An extensive number of enzymes and other proteins involved in cell signalling are targets of ROS or are sensitive to redox state changes. These include phospholipases A2, -C and -D; tyrosine phosphatases; guanylyl cyclase; ion and calcium channels; AP-1 and NFκB transcription factors; several protein kinases; HIF-1α; and the JNK/MAPK pathways that activate, amongst other, nuclear factor-erythroid 2 p45 subunit-related factors 1 and 2 (Nrf1 and -2), which have similar but distinct functions in the expression of antioxidant defense and xenobiotic-metabolizing genes containing one or more antioxidant responsive elements (ARE) [9,15,23,49]. Evidence also indicates that increased oxidative stress is involved in the expression of the nuclear respiratory factor-1 (NRF-1, unrelated to Nrf), which is a key transcription factor in the expression of several genes involved in mitochondrial function [50,51]. NRF-1 activation and increased cytochrome *c* expression have also been associated with the activation of the nuclear-localized, AMP-activated protein kinase (AMPK) as a result of, among others, decreased ATP/AMP ratio [52].

Gene expression data from several of the studies presented in Table 1 support the possibility that regulation of OXPHOS genes may be co-regulated. Recently, Van Waveren and Moraes have shown that not only OXPHOS genes are co-expressed, but also that subunits within OXPHOS complexes are co-expressed [53]. This co-expression is statistically associated with a selection of *cis*-acting elements in human OXPHOS gene promotors, which include well-known elements found in OXPHOS genes or related genes; NRF-1; NRF-2; and the less specific elements ERRA, SP1, MEF-2, YY1, and CREB [53–56]. Van Waveren and Moraes suggest that these factors can act independently or synergistically, in order to allow co-regulation, and with other factors can lead to diversity in expression [53]. An example of this diversity and tissue-specific expression has been described for complex IV (COX) expression in muscle [57]. In mammals, COX may contain ubiquitous liver (L) and heart/muscle-specific (H) isoforms for subunits COX6a and COX7a [54]. The promotor regions of these genes lack NRF sites but contain conserved myocyte enhancer factor 2 (MEF2) elements. Expression of MEF2A is induced by NRF-1 over expression and, with PGC1α (a co-activator that also binds PPARα), results in the induction of muscle-specific gene expression of COX [57,58].

5. Mitochondrial DNA transcription and replication, regulation, damage, and repair

The mechanism involved in the transcription and replication of mtDNA is well documented [59–61] and, as is evident from the report summary in Table 1, clearly involved in expression of OXPHOS disorders. In normal tissue, mitochondrial DNA copy number varies relative to oxidative capacity and energy needs [56]. These variations may be markedly affected in OXPHOS deficiencies, and reports show either a decreased [33–35] or increased [35] mtDNA/nDNA ratio, assuming that no mutations in replication/transcription genes exist in these patients. It has been proposed that mitochondrial gene expression is regulated not only by nuclear encoded transcriptional or post-transcriptional mechanisms, but also by the mtDNA copy number of the cell itself [62,63]. The mechanism for this is unclear, although several of the regulatory proteins involved in mtDNA transcription/replication (TFAM, POLRMT, mtSSB) are co-ordinately expressed with changes in mtDNA copy number [64,69,66]. Miranda et al., however, demonstrate induced expression of NRF-1 and TFAM mRNA in ρ° HeLa cells that correlated with ROS levels [50]. Increased expression of these factors is also found in OXPHOS deficient human cells, in aged skeletal muscle [56], and in HeLa cells treated with menadione that leads to increased superoxide formation [50]. Another key regulator of mtDNA maintenance and copy number is Twinkle helicase [67], although very little is know about its regulation. Notably, the regulation of mitochondrial DNA polymerase, DNA poly, appears not to be affected by mtDNA levels or its maintenance and is expressed at levels sufficient to support variation among tissues [65,66].

Knowledge of the regulation of nucleus-to-mitochondria (anterograde) signalling that controls these events is still largely lacking. Mitochondrial transcription involves binding of mitochondrial RNA polymerase on three possible promotor sites, in association with TFAM and one of two transcription factor B paralogs, TFB1M and TFB2M [60]. These transcription factors have distinct roles in vivo, and

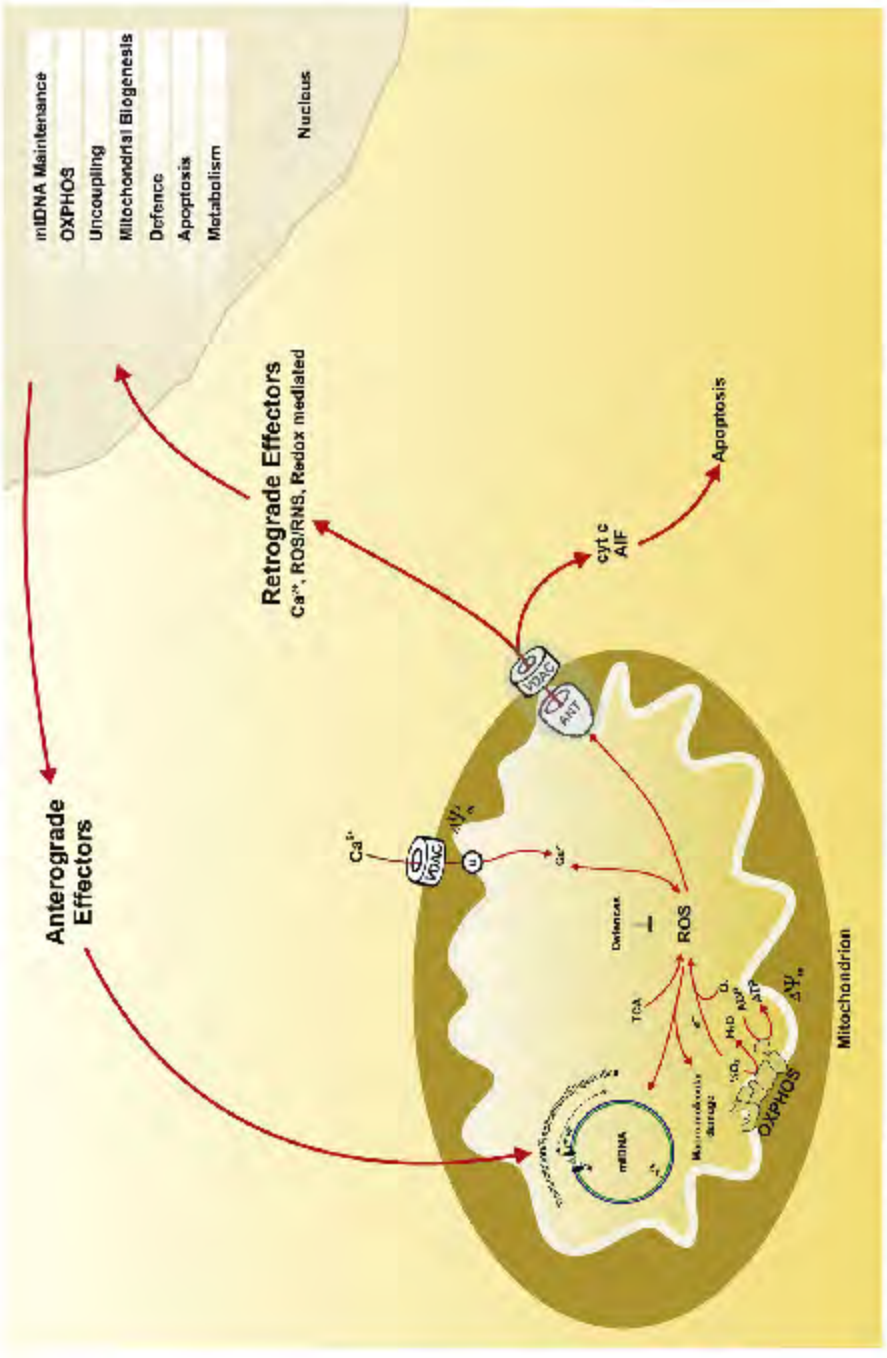

Fig 1. Summarized display of mitochondria–nucleus interactions that control the expression of OXPHOS and related genes. Changes in homeostasis of ROS, Ca^{2+}, and ATP/ADP control the mitochondrial permeability transition pore (including ANT/VDAC). Such changes can lead to the induction of apoptosis through the intrinsic apoptosis pathway either directly or through secondary signalling pathways (retrograde effectors; see section for details), expression of nuclear genes involved in OXPHOS, and other mitochondria-related functions. Replication and transcription of mtDNA are mainly controlled by the nuclear encoded elements but are also affected indirectly by retrograde signalling and directly by epigenetic factors.

it is proposed that TFB2M is primarily involved in transcription and transcription-primed replication, while TFB1M over expression does not affect these processes but still increases mitochondrial biogenesis [68]. Furthermore, over expression of TFB2M induces TFB1M expression, which suggests that there is a retrograde signalling pathway that co-ordinately expresses these transcription factors [68]. It is interesting to note that the expression of these controlling factors of mtDNA transcription also contains the NRF-recognition sites that are *trans*-activated by the PGC1 family co-activators, PGC1α and PRC [58,69]. These controlling elements are thus shared between the expression of both nuclear- and mitochondrial-encoded OXPHOS genes.

It is well-known that methylation of DNA plays an important role in epigenetic events: DNA-methyltransferases (DNA-MTase) catalyse the addition of a methyl group to a cytosine ring in CpG dinucleotides leading to 5-methylcytosine, which is generally associated with reduced gene expression. This occurs either by blocking binding of transcription factors, binding transcription repressors, or changing chromatin structure [70]. For the mitochondrion evidence of DNA methylation is lacking. However, TFB1M and TFB2M are homologues to rRNA methyltransferases and the role of TFB1M in ribosome biogenesis is proposed [60,64,68,71]. Although methylation and other epigenetic events play an important role in nDNA transcription regulation and maintenance, information on the occurrence and role of these factors in mtDNA expression and maintenance needs further study.

Damage to mtDNA is often highlighted as a factor affecting expression of mtDNA genes and thus contributing to secondary consequences of OXPHOS deficiencies. Considering its structure, maintenance, and close localization to sources of oxidative damage, this may well be a significant factor in disease expression. Owing to its close proximity to the site of mitochondrial ROS production and comparatively less efficient mtDNA damage repair, mtDNA is more sensitive to oxidative damage than nDNA [72]. It has also been demonstrated that oxidative damage is more likely to occur in the controlling D-loop region [56,73]. The compounding factors for oxidation of mtDNA are the close proximity of metal ions that act as catalysts and ROS damage to OXPHOS complexes that result in secondary ROS [72]. The lack of protective histones around the mtDNA is also suggested to contribute to the sensitivity of the mtDNA to oxidative damage, although the presence of regulating proteins, such as TFAM, on mtDNA may have a protective effect against ROS damage [74,75]. It has now been established that mtDNA damage is primarily repaired through the ATP-dependent base excision repair pathway [76–80].

The factors regulating mtDNA replication and transcription are thus highly diverse and tightly controlled by mitochondria–nucleus signalling. In addition, more immediate factors such as ROS, metabolic regulation and defense mechanisms that determine levels of oxidative stress and possibly epigenetic factors, control expression and maintenance. These factors are frequently evaluated separately. Considering the diverse expression profiles that were reported for mtDNA in OXPHOS deficiencies, as well as the regulation of the factors controlling its expression, the interplay of all these factors need to be evaluated to obtain a better understanding of how the mitochondrion responds to OXPHOS deficiencies.

6. Conclusion

The complex and almost unpredictable nature of disease phenotypes associated with OXPHOS deficiency has been a considerable impediment in the characterization and treatment of OXPHOS deficiencies. The effect of OXPHOS deficiencies on mitochondrial and nuclear DNA expression and regulation has been investigated in several studies in an attempt to clarify the adaptive responses in OXPHOS disease phenotypes. These include genes involved in mtDNA maintenance, uncoupling, biogenesis, defense mechanisms, apoptosis, and metabolic regulation. A striking observation from these investigations is the great variation that exists in the differential expression of both the nuclear and mitochondrial DNA, which prompted this review of expression profiles and the mechanisms involved.

As summarized in Fig. 1, the immediate (such as ROS and ATP) and secondary (such as Ca^{2+}) consequences of OXPHOS deficiencies are key mediators in retrograde signalling events that induce expression of nuclear OXPHOS and other genes involved in various cellular processes. Many of the signalling elements and promotor binding sites that control expression of these nuclear genes have now been identified and demonstrate that, in the case of OXPHOS and mtDNA maintenance genes, coordinate (ROS and Ca^{2+} sensitive) expression occurs. The origin and role of ROS is diverse, as its function in the oxidation of macromolecules, including mtDNA and RNA, also contributes to the way mtDNA transcription and replication occur. An aspect that needs to be investigated further is the occurrence and possible role of epigenetic events in the mitochondrion, which may, similar to nuclear DNA, have a marked effect on the expression and maintenance of mtDNA.

Although current data of the expression of genes involved in energy metabolism in OXPHOS deficiencies exhibit diverse profiles and (often) inconsistencies, it is clear that the expression of these genes contributes to the disease expression of OXPHOS deficiencies. In order to obtain an improved understanding of the intricate consequences and adaptive responses in OXPHOS disorders, the expression of nuclear and mitochondrial genes needs to be evaluated holistically, in combination with the signalling processes and metabolites involved and in well-defined disease models. Such an investigation poses a significant challenge, but recent developments in systems biology technologies may soon overcome any difficulties posed.

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APPENDIX B

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Metallothionein isoform 2A expression is inducible and protects against ROS-mediated cell death in rotenone-treated HeLa cells

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The role of MT (metallothionein) gene expression was investigated in rotenone-treated HeLa cells to induce a deficiency of NADH:ubiquinone oxidoreductase (complex I). Complex I deficiency leads to a diversity of cellular consequences, including production of ROS (reactive oxygen species) and apoptosis. HeLa cells were titrated with rotenone, resulting in dose-dependent decrease in complex I activity and elevated ROS production at activities lower than 33%. Expression of MT2A (MT isoform 2A), but not MT1A or MT1B RNA, was significantly inducible by rotenone (up to 7-fold), t-BHP (t-butyl hydroperoxide; 5-fold) and $CdCl_2$ (50-fold), but not $ZnCl_2$. Myxothiazol treatment did not elevate either ROS or MT2A levels, which supports a ROS-related mechanism for rotenone-induced MT2A expression. To evaluate the role of MT2A expression, MT2A and MT1B were overexpressed in HeLa cells and treated with rotenone. Compared with control and MT1B-overexpressing cells, ROS production was significantly lower and cell viability higher in MT2A-overexpressing HeLa cells when ROS production was enhanced by treatment with t-BHP. Mitochondrial membrane potential was noticeably less reduced in both MT-overexpressing cell lines. MT2A overexpression in rotenone-treated cells also significantly reduced or delayed apoptosis induction, as measured by caspase 3/7 activity and cytosolic nucleosome enrichment. We conclude that MT2A offers significant protection against the main death-causing consequences of rotenone-induced complex I deficiency in HeLa cells. Our results are in support of the protective role against oxidative stress ascribed to MTs and provide evidence that MT2A expression may be a beneficial downstream adaptive response in complex I-deficient cells.

Key words: apoptosis, metallothionein, mitochondria, NADH: ubiquinone oxidoreductase, reactive oxygen species (ROS), rotenone.

INTRODUCTION

The production of ATP through the process of OXPHOS (oxidative phosphorylation) involves the successive transport of electrons through four mitochondrial enzyme complexes. The first of these complexes, NADH:ubiquinone oxidoreductase (complex I; EC 1.6.5.3), is assembled from 46 subunits of bigenomic origin [1]. Deficiencies of this complex are among the most common OXPHOS deficiencies [2] and may lead to a diversity of disease expression phenotypes [3]. The excessive formation of ROS (reactive oxygen species) as a contributing factor to the pathology of this deficiency has been well established, along with several other biochemical consequences, including loss of ATP production, loss of mitochondrial membrane potential, calcium regulation and apoptosis [4–8].

Recent studies using either inherited or rotenone-induced complex I-deficient cell lines have indicated that several nuclear and mitochondrial genes are differentially expressed in this disorder [9–11]. Among these, expression of MTs (metallothioneins) was induced in inherited complex I-deficient fibroblasts during carbon-source transition from glucose to galactose [9]. Although it was suggested that MT expression may impart a protective effect in complex I deficiency, the functionality of its expression in the context of complex I and possibly other deficiencies of the OXPHOS system remains to be established. MTs are small proteins (6–7 kDa) with a high cysteine content that can bind metals, particularly Zn and Cd, and scavenge ROS in a similar way to glutathione [12,13]. MT expression is regulated via *cis*-acting metal responsive elements and an antioxidant response element, both located in the proximal MT promoter, and is responsive to a wide range of effectors, including ROS [14,15]. In humans, MT1 and MT2 isoforms are thought to be ubiquitously expressed, with MT3 and MT4 only selectively expressed in neurons and squamous epithelial cells respectively [16–18]. MT2A (MT isoform 2A) appears to be the predominantly expressed MT isoform in human cell lines *in vitro*, including HeLa cells [19]. Although a clearly distinctive role for MT isoforms remains unclear, it is generally believed that MTs play an important role in metal ion homoeostasis and prevention of oxidative damage in cells [13,14,20].

In light of the responsiveness of MTs to oxidative stress and the generally protective role associated with MTs against ROS, we hypothesized that MT expression would be responsive to a deficiency of complex I and, furthermore, that MTs may be involved in the pathology of such a deficiency. Although MT expression may be responsive to disruption of a number of mitochondrial functions that leads to oxidative stress, we focused on its responsiveness to an induced defect of the first component

Abbreviations used: Ct, cycle threshold; DCFDA, 2′,7′-dichlorofluorescin diacetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMLV, Moloney-murine-leukaemia virus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MT, metallothionein; MT2A, MT isoform 2A; mtPTP, mitochondrial permeability transition pore; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; t-BHP, t-butyl hydroperoxide; TMRM, tetramethylrhodamine methyl ester.

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of the OXPHOS system, i.e. complex I, using rotenone. We investigated the expression of the predominant forms of MT, i.e. MT1 (isoforms A and B) and MT2A, in rotenone-treated HeLa cells against several control interventions, including metals, ROS-producing t-BHP (tert-butyl hydroperoxide) and the cytochrome *c* reductase inhibitor, myxothiazol. To evaluate the role of MT expression in complex I deficiency, we investigated the effect of overexpressed MT2A and MT1B on key parameters, including ROS production, ATP production, mitochondrial membrane potential and apoptosis in rotenone-treated HeLa cells.

EXPERIMENTAL

Materials

HeLa cells were purchased from the National Repository for Biological Materials of the National Cancer Association of South Africa. Tissue culturing reagents were obtained from Gibco, Invitrogen (Auckland, New Zealand). The pIRESneo2 expression vector was obtained from ClonTech, BD Biosciences (Mountain View, CA, U.S.A.), whilst all restriction endonucleases were purchased from Fermentas (Vilnius, Lithuania). MMLV (Moloney-murine-leukaemia virus) reverse transcriptase, random hexamer primers and the Apo-ONE Homogeneous Caspase 3/7 Assay kit were acquired from Promega (Madison, WI, U.S.A.). The QIAzol lysis reagent was purchased from Qiagen (Hilden, Germany). The X-tremeGENE Q2 transfection reagent and Cell Death Detection ELISA^{PLUS} were from Roche (Penzberg, Germany). The iQ SYBR Green Supermix was acquired from Bio-Rad (Hercules, CA, U.S.A.), and the probes DCFDA (2′,7′-dichlorofluorescein diacetate), TMRM (tetramethylrhodamine methyl ester) and Mitotracker Green were obtained from Molecular Probes (Eugene, OR, U.S.A.). All other reagents, including ATP assay reagents, were obtained from Sigma (St. Louis, MO, U.S.A.). MT cDNA clones with accession numbers as indicated in the text were obtained from The Resource Center of the German Human Genome Project (Berlin, Germany).

Cell culture and rotenone treatment

HeLa cells were cultured at 37 °C and 5 % CO_2 in a humidified incubator. The culturing medium, Dulbecco's modified Eagle's medium, was supplemented with 2 mM L-glutamine, 5 % (v/v) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. For rotenone and myxothiazol treatments, the medium of separate culture monolayers that were approx. 90 % confluent was supplemented with inhibitors ranging from 0 to 2500 nM. Ethanol or DMSO content, which was used as solvents for inhibitors, was kept constant at 0.1 %. Incubations were carried out for 24 h. When required, cells were collected by trypsinization unless otherwise stated and washed twice with PBS before analyses.

Enzyme assays

Rotenone-sensitive NADH:ubiquinone oxidoreductase (complex I) and antimycin A-sensitive cytochrome *c* reductase (complex III) activities were measured in enriched mitochondrial preparations isolated from 2×10^8 cells as described previously [21]. Enzyme activities were normalized to citrate synthase activity [22]. Protein content in these and other preparations was determined using the bicinchoninic acid method [23].

MT RNA expression analysis

Rotenone and myxothiazol incubations were performed as described in the previous paragraph. In addition, as controls for MT expression, treatments with $CdCl_2$ (12.5 μM) and $ZnCl_2$ (250 μM) were performed. To increase ROS levels in cells, t-BHP at concentrations of 0.5, 0.8 and 1.0 mM was included and incubated for 3 h before cells were harvested. Total RNA was isolated from 2×10^6 cells using QIAzol reagent according to the manufacturer's instructions, and the RNA integrity was verified by agarose-gel electrophoresis and ethidium bromide staining. RNA (3 μg) was reverse-transcribed with 200 units of MMLV reverse transcriptase in a volume of 40 μl using 0.5 μg of random hexamer primers. In addition to MT transcripts, several other (housekeeping gene) transcripts were evaluated for suitability as normalization controls. The primers for real-time PCR (in 5′–3′ notation) for the various genes (with GenBank® accession numbers in parentheses) are as follows: GAPDH (glyceraldehyde-3-phosphate dehydrogenase; NM_002046), β-actin (NM_001101), β_2-microglobulin, (NM_004048) and RNA polymerase II (X63564) were used as reported previously [24]; 18 S rRNA (X03205) forward primer GTGCATGGCCGTTCTTAGTT and reverse primer CGGACATCTAAGGGCATCAC; MT1A (NM_005946) forward primer TCCTGCAAATGCAAAGAGTG and reverse primer TTCCAAGTTTGTGCAGGTCA; MT1B (NM_005947) forward primer GAACTCCAGGCTTGTCTTGG and reverse primer GATGAGCCTTTGCAGACACA; MT2A (NM_005953) forward primer TCCTGCAAATGCAAAGAGTG and reverse primer CAGGTTTGTGGAAGTCGCGT. Real-time PCR was performed using an iCycler iQ (Bio-Rad) in a final volume of 20 μl using SYBR Green for detection. The PCR reaction consisted of 10 μl of iQ SYBR Green Supermix, 500 nM of forward and reverse primers and 75 ng of cDNA (3 ng for 18 S rRNA primers). The method included an initial denaturation step (3 min at 95 °C) followed by 35 cycles of denaturation at 95 °C for 20 s, primer annealing at 60 °C for 10 s, extension at 72 °C for 20 s and an additional step at 82 °C (84 °C for 18 S rRNA primers) with a single fluorescence measurement. A final extension at 72 °C for 5 min followed by a melting curve analysis (55–95 °C with a heating rate of 0.5 °C per 5 s and fluorescent measurement every 5 s) concluded the run. All samples were amplified in triplicate and the mean value was used for further calculations. Every assay included a no-template control, five serial dilution points (in steps of 5-fold) of a cDNA mixture, and each of the test cDNAs. Mean results [Ct (cycle threshold) values] from the iCycler iQ Real-time Detection system (iCycler iQ Real-time Detection System Software, version 3.0; Bio-Rad) were analysed by Statistica Version 7 software (StatSoft, Tulsa, OK, U.S.A.) and the BestKeeper software tool [24]. PCR efficiency for each primer set was calculated by serial dilutions method using the REST software tool [25]. The relative expression quantities for each sample were calculated by the comparative Ct method, and gene expression stability was analysed using the GeNorm software tool [26].

MT analysis

For MT protein analysis, the same interventions were performed as described for RNA analysis. After collection, cells were sonicated on ice for three bursts of 3 s. MT content was determined in the 13000 *g* supernatants of homogenates by a highly sensitive RIA as previously described [27]. This antibody fully cross-reacts with MT1 and MT2 isoforms, but not with MT3, and has been validated for human MT2A (generously provided by Dr Milan Vasak, Institute of Biochemistry, University of Zurich, Zurich, Switzerland).

MT-overexpressing HeLa cell lines

The cDNA encoding human MT1B (GenBank® accession no. NM_005947) was amplified using 5′-CCTAGGAACTCCAGGCTAGC-3′ as a forward primer and 5′-AAAGAATGTAGCAAACCGGTC-3′ as a reverse primer. Human MT2A (GenBank®

accession no. NM_005953) cDNA was amplified with 5′-GCGAACCCGCGTGCAACCGGTCCC-3′ as forward primer and 5′-CAGGTTTGTGGAAGTCGCGT-3′ as reverse primer. After confirming the sequences, the MT1B and MT2A cDNAs were cloned into the EcoRV/PinA1 and EcoRV/EcoRI sites of the pIRESneo2 mammalian expression vector respectively. Expression with this vector is driven by the CMV (cytomegalovirus) major immediate early promoter. After verifying the sequence of the constructs, HeLa cells were transfected with the MT expression constructs as well as the base vector, pIRESneo2, using X-tremeGENE Q2 transfection reagent according to the manufacturer's instructions. Cell lines successfully transfected with pIRESneo2-MT1B, -MT2A and pIRESneo2 constructs were denoted as MT1B-, MT2A-overexpressing cells and control cells respectively. Selection of transfected cells was performed with 1 mg/ml Geneticin in addition to the standard medium supplements. After a 3-week selection period, the standard culturing medium contained 200 μg/ml Geneticin throughout all subsequent incubations and analyses. The presence of the MT cDNAs was confirmed by PCR. Briefly, pIRESneo2-specific primers, 5′-TAATACGACTCACTATAGG-3′ (forward) and 5′-GCCCTAGATGCATGCTCG-3′ (reverse), were used to amplify the cDNAs using isolated DNA from clones. DNA was isolated by phenol/chloroform extraction and ethanol precipitation based on the procedure originally described by Maniatis et al. [28]. The presence of the correct-length amplicons was used to confirm the presence of the cDNAs, and MT RNA and protein expression were evaluated as described in the previous two sections.

ROS production

Cell lines were seeded in microtitre plates at 2×10^4 cells per well and treated with rotenone, myxothiazol and metals as described in the 'Cell culture and rotenone treatment' section. Incubations with t-BHP (0.5–1.0 mM) were used to induce elevated ROS production. The fluorescent probe, DCFDA (10 μM), was used to measure ROS production essentially as described previously [29]. Fluorescence (excitation at 485 nm and emission at 530 nm) was measured and the mean for eight samples was used in data analysis and expressed relative to protein content.

Cell viability assay

Cells were seeded into microtitre plates at a density of 2×10^4 cells. Rotenone and t-BHP incubations were performed as described in the 'Cell culture and rotenone treatment' section. As a positive control for loss of cell viability, a 30 min incubation with 6% (v/v) acetic acid was included. Cell viability [MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay] was determined by measuring formazan formation as described previously [30]. The mean for three replicates was calculated and expressed relative to protein content.

Membrane potential assessment

The potentiometric fluorescent dye, TMRM, was used in confocal microscopy analyses to estimate visually mitochondrial membrane potential. Cell lines were seeded on sterile glass coverslips in 6-well plates (Nunc) at densities of 2×10^5 cells per well in 2 ml of culture medium. After overnight incubations to allow attachment, rotenone was added and incubated for 24 h. TMRM (0.5 μM) and Mitotracker Green (0.5 μM) were added and incubated for 30 min at 37 °C, after which the coverslips were washed with three changes of media. The coverslips with adherent cells were placed in an applicable flow cell bath in the presence of 2 ml of medium. Confocal images were monitored using a Nikon (PCM2000) inverted confocal microscope. Ar and He/Ne Spectra-Physics lasers, with excitation at 475 and 505 nm and emission at between 505 and 568 nm (green) as well as long pass > 565 nm (red), were employed. In order to minimize photobleaching of the sample and free radical formation in the cells, the smallest available pinhole was used (0.5 μm), together with a neutral density filter of 10%. Magnification was obtained with a Nikon × 60/1.40 Apo Planar oil objective, and bars in the micrographs indicate size. Laser power and capturing settings were kept constant in comparative experiments, to enable quantitative analysis. A scan speed of 3 μs/scan was typically used and capturing was averaged to obtain representative micrographs.

ATP and apoptosis analyses

To determine the ATP content in rotenone-treated cells, a luminescence-based assay reagent was used as instructed by the manufacturer and expressed relative to protein content. Cells were seeded in microtitre plates at 2.0×10^4 cells per well, allowed to adhere overnight and incubated with rotenone for 8 h before ATP measurements. Caspase 3/7 activity in treated cells was measured fluorimetrically using a commercial kit as instructed by the manufacturer. Briefly, cells were seeded in microtitre plates at densities of 2.0×10^4 cells per well, allowed to adhere overnight and incubated with rotenone as described in the 'Cell culture and rotenone treatment' section. A 2 h incubation with 1 μg/ml staurosporine was included as positive control for apoptosis. Assays were carried out kinetically and expressed relative to protein content. As an indicator for DNA degradation during apoptosis, cytosolic nucleosome enrichment in cells was determined using a commercial ELISA as instructed by the manufacturer.

Statistical analyses

All results were analysed with Statistica (version 7) software. Statistical comparisons of MT expression in HeLa cells were made using ANOVA with *post hoc* comparison (Tukey test). For statistical analysis of values obtained from different MT-overexpressing and control cell lines, two-way ANOVA was performed. For these analyses and interpretation of results, the interactions of the concentration (of either rotenone or t-BHP) and MT expression were evaluated as indicated by a significant F-value (test statistic). Statistical significance was considered when $P < 0.05$.

RESULTS

MT expression and ROS production in rotenone-treated cells

The treatment of HeLa cells with varying concentrations of rotenone (0–2500 nM) resulted in a dose-dependent decrease in residual complex I activities as measured in enriched mitochondrial preparations (Table 1). Values were similar as reported previously for fibroblasts [31] and resulted in a useful range of complex I activities, ranging between 0 and 100%, to compare the responsiveness of the parameters that were investigated in the present study. Similarly, complex III could be inhibited with myxothiazol treatment over a range of 0–1000 nM that resulted in a dose-dependent decrease of activity. Treatments with rotenone and myxothiazol were limited to 24 h to limit the contribution of media composition changes on cellular function.

Real-time PCR was performed on total RNA samples obtained from HeLa cells that were treated with rotenone. We included known inducers of MT expression, $CdCl_2$ and $ZnCl_2$ as well as the ROS inducer, t-BHP, as controls for MT expression. In addition, myxothiazol was included to evaluate the mechanistic possibilities of MT expression. For RNA expression studies, Northern blotting

Table 1 Complex I and III activities in rotenone- and myxothiazol-treated HeLa cells

Rotenone-sensitive complex I and antimycin A-sensitive complex III activities were determined in mitochondrial-enriched preparations that were prepared from rotenone- or myxothiazol-treated (24 h) HeLa cells. Values are means ± S.D. ($n = 4$, *$P < 0.05$) with percentage activities relative to untreated cells given in parentheses. UCS, units of citrate synthase.

	Complex I (nmol · min^{-1} · UCS^{-1})	Complex III (nmol · min^{-1} · UCS^{-1})
Rotenone (nM)		
0	108.7 ± 13.4 (100 %)	61.7 ± 2.5 (100 %)
10	55.9 ± 6.2* (51 %)	60.6 ± 1.5 (98 %)
100	35.1 ± 3.5* (33 %)	64.6 ± 7.7 (105 %)
1000	16.8 ± 4.5* (15 %)	62.8 ± 5.9 (102 %)
2500	0.0 ± 3.8* (0 %)	59.8 ± 4.0 (97 %)
Myxothiazol (nM)		
10	111.6 ± 3.8 (103 %)	42.9 ± 4.2* (70 %)
100	106.2 ± 4.5 (98 %)	27.9 ± 4.0* (45 %)
500	105.1 ± 2.1 (97 %)	6.8 ± 1.5* (11 %)
1000	106.7 ± 7.2 (98 %)	0.0 ± 0* (0 %)

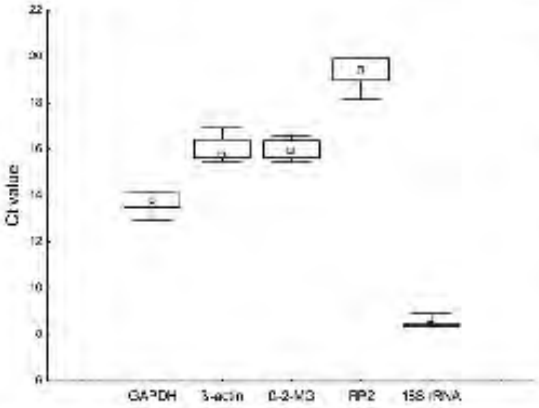

Figure 1 Evaluation of RNA transcription level variation of housekeeping genes

Variation in real-time PCR-generated Ct values of selected housekeeping genes of HeLa cells treated with rotenone (0–10 μM), $CdCl_2$ (12.5 μM), $ZnCl_2$ (250 μM) and t-BHP (0–1 μM) are shown. Incubations were carried out for 24 h (rotenone, metals) and 3 h (t-BHP). The median values are indicated by small squares, 25–75 % percentiles are indicated by the boxes and minimum and maximum values indicated by whiskers. Genes are GAPDH, β-actin, β_2-microglobulin (β-2-MG), RNA polymerase II (RP2) and 18 S rRNA.

gave poor, unspecific results, which were probably due to the similarity that exists between the different MT isoforms. Real-time PCR was used, which allowed isoform RNA expression analysis. The stability of several commonly used 'housekeeping genes' involved in diverse biological activities, including glycolysis, cytoskeleton structure and kinetics, immune response, gene expression and protein biosynthesis, were investigated under the interventions mentioned. To ensure comparability between the analyses of all five housekeeping genes as well as MT isoforms, we determined the reaction efficiency of each individual assay by measuring serial dilutions of 75 ng of cDNA in triplicate [25]. All PCR reactions displayed efficiencies of between 88 and 100 %. The variations in the Ct values, which represent the cycle where a significant increase in amount of PCR product occurs during the various interventions, are summarized in Figure 1. Comparing the median expression values (Ct values) of the

Table 2 Stability of housekeeping genes expression

Results are shown as GeNorm expression stability values or *M*, the internal control gene-stability measure, defined as average pairwise variation of a particular gene with all other control genes [26]. Genes with the lowest *M* values have the most stable expression.

Gene	*M* (24 h)	*M* (48 h)
18 S rRNA	0.466	0.462
β_2-Microglobulin	0.548	0.503
GAPDH	0.575	0.506
β-Actin	0.682	0.512
RNA polymerase II	0.730	0.532

housekeeping genes, the variability for housekeeping gene expression was clearly less in the case of the 18 S rRNA as compared with the other genes. Ct values were also expressed as relative expression quantities and analysed using the GeNorm software tool. The results of this analysis are presented as GeNorm expression stability values (or gene-stability measures, *M*), which are defined as average pairwise variations of a particular gene with all other control genes, and are summarized in Table 2 [26]. Genes with the lowest *M* values generally have the most stable expression. As a result, the three most stably expressing genes, 18 S rRNA, β_2-microglobulin and GAPDH, were subsequently used for normalization of MT expression.

Under the experimental *in vitro* conditions and interventions performed in the present study, we could not detect any expression of MT1A. In addition, an almost undetectable basal expression of MT1B was observed. From the controls for PCR, using cDNA templates for the various isoforms, we concluded that the PCR was efficient and specific to the isoforms. Treatment of cells with any of the other possible inducers mentioned did not result in detectable changes of expression levels of these common MT1 isoforms either. In contrast, MT2A RNA basal levels in untreated HeLa cells were easily detectable and, as presented in Figure 2, highly inducible by $CdCl_2$ (±50-fold) and t-BHP (up to 5-fold), but not $ZnCl_2$. Myxothiazol treatment up to levels that completely inhibited complex III activity did not result in any significant induction of MT2A expression. Rotenone treatment, however, significantly induced MT2A expression. MT2A levels were slightly elevated with 10 nM rotenone treatment after which expression levels were significantly increased 3-fold upon treatment with 100 nM rotenone. Surprisingly, expression levels at 1000 nM were consistently lower than 100 nM. This result was the same in three independent experiments. This phenomenon was also observed when rotenone treatment was extended to 48 h (results not shown). At rotenone levels higher than 1000 nM, which resulted in almost complete inhibition of rotenone-sensitive complex I activity, MT2A expression was significantly higher and remained constant with higher rotenone levels at approx. 7-fold compared with basal levels. MT protein analysis, which did not enable a distinction between MT1 and MT2 isoforms in cell homogenates, confirmed the increased expression observed with rotenone treatment (Table 3). A slight, albeit significant, dose-dependent increase in MT expression was detected at levels up to 2500 nM. As with RNA expression, expression levels at 2500 nM were notably higher than at 1000 nM rotenone. Treatment with t-BHP also significantly increased expression of MTs on protein level. However, unlike MT2A RNA expression where only Cd-mediated induction was detected (Figure 2), both $CdCl_2$ and $ZnCl_2$ markedly induced MT protein expression.

Treatment of cells with rotenone elevated ROS levels only significantly at levels higher than 100 nM (±36 % residual

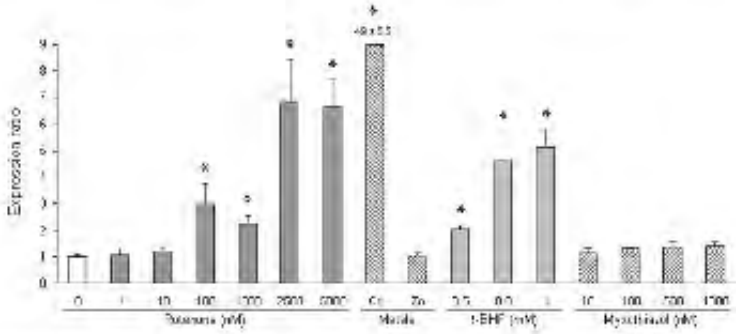

Figure 2 MT2A RNA expression in rotenone-treated HeLa cells

MT2A RNA expression and total MT protein levels in cells were determined as described in the Experimental section. Normalized RNA expression levels are expressed as the mean ratio + S.D. ($n = 3$) relative to untreated cells (open bar). Asterisks indicate statistically significant values ($P < 0.05$) compared with the untreated cells. Expression levels were compared in cells treated with rotenone, metal inducers $CdCl_2$ (12.5 μM) and $ZnCl_2$ (250 μM), t-BHP and myxothiazol as indicated. The value for Cd-induced expression is indicated above the bar.

Table 3 MT protein levels in rotenone-, metal- and t-BHP-treated HeLa cells

Total metallothionein (MT1 + MT2) levels are expressed relative to total protein content in cell homogenates (10 mM Tris/HCl, pH 8.0). Mean values are shown with ranges in parentheses ($n = 2$, except metal controls where $n = 1$).

Treatment	MT (ng/mg)
Untreated	34.7 (32.3–37.1)
Rotenone	
100 nM	47.0 (44.1–49.8)
500 nM	52.1 (51.3–52.8)
1000 nM	53.8 (53.1–54.4)
2500 nM	71.7 (71.1–72.4)
Metal controls	
$CdCl_2$ (12.5 μM)	875
$ZnCl_2$ (250 μM)	481
t-BHP	
0.5 mM	56.2 (47.7–64.5)
0.8 mM	61.9 (57.6–66.2)
1.0 mM	91.5 (86.1–96.8)

activity) (Figure 3). At complete inhibition of complex I activity (2500 nM), ROS levels increased approx. 2-fold compared with untreated cells. As expected, t-BHP treatment had a marked effect on ROS levels up to almost 5-fold at 1 mM. Although a complete inhibition of complex III with myxothiazol was demonstrated (Table 1), surprisingly, no significant effect on ROS production could be observed in HeLa cells.

MT-overexpressing HeLa cells

HeLa cells were transfected with plasmid constructs containing either the pIRESneo2 base vector (control cells), MT1B or MT2A plasmid constructs. After confirming stable transfer of plasmid DNA, normalized expression ratios of MT1B and MT2A of the various transfected cell lines were determined as summarized in Table 4. Basal MT1B expression in control cells was barely detectable (Ct values similar to negative controls), similar to a previous report [19]. Consequently, a significantly higher MT1B expression ratio in MT1B-overexpressing cells was calculated. Comparatively, on the background of a relatively high basal expression level of MT2A in control cells (Ct values ± 18), the MT2A-overexpressing cells resulted in only a 2-fold increase in expression of MT2A. The MT expression levels in transfected cell lines were measured on separate occasions and remained similar to those indicated in Table 4 over the time period of the investigation. The total MT protein content, which represents combined MT1 and MT2 levels, however, was similar in both MT-overexpressing cell lines and was approx. 20 % higher than control cells. From this we concluded that the additional levels of expression of either MT1B or MT2A in the respective MT-overexpressing cell lines were similar and were suitable for use in comparative studies.

ATP analyses in MT-overexpressing cells

Rotenone treatment of control and MT-overexpressing cell lines resulted in a dose-dependent decrease in ATP levels (Figure 4). With complete inhibition (1000 nM rotenone), ATP levels decreased to 68 % in control cells. Comparatively, ATP levels were 76 and 78 % in MT1B- and MT2A-overexpressing cells respectively. A clear and significant variance in response to increasing rotenone treatment occurred between the cell lines, with ATP levels in both MT-overexpressing cell lines decreasing notably more slowly to the levels mentioned above, compared with the control cell line.

Rotenone- and t-BHP-induced ROS production

As with HeLa cells that were not genetically modified (Figure 3), total cellular ROS production in genetically modified HeLa cells increased significantly only after treatment with rotenone levels higher than 100 nM (Figure 5), which represents approx. 33 % residual complex I activity. As before, ROS levels further increased dose-dependently only to levels approx. 2-fold higher at 2500 nM rotenone. At this concentration, ROS levels in MT2A, which were higher at 500 nM, were significantly, albeit only slightly, lower compared with the other two cell lines. ROS production could be further and significantly induced using t-BHP (Figure 5). A clear variance in response to this treatment could be observed with MT2A-overexpressing cells, which had significantly lower ROS levels (±40 %) compared with control and MT1B-overexpressing cells.

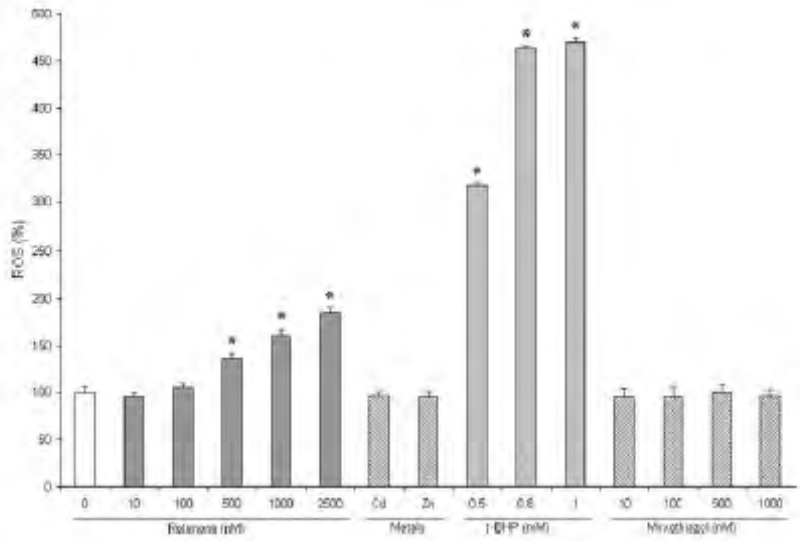

Figure 3 ROS production in rotenone-treated HeLa cells

ROS production was measured fluorimetrically in cells treated with rotenone, metal inducers $CdCl_2$ (12.5 μM) and $ZnCl_2$ (250 μM), t-BHP and myxothiazol using the ROS-sensitive probe, DCFDA, and normalized to protein content. Values are means ± S.D. ($n = 8$) and expressed as percentage relative to untreated cells.

Table 4 MT RNA and protein expression in recombinant MT-overexpressing HeLa cell lines

RNA expression of MT1B or MT2A in cell lines was analysed by real-time PCR and normalized relative to the expression of GAPDH and β_2-microglobulin. RNA expression values represent the expression ratios relative to pIRESneo2-transfected HeLa cells (control). Total MT protein levels were quantified in homogenates (PBS containing 1 % Tween 20) using an RIA and expressed relative to protein content. All values are means ± S.D. ($n = 3$, $^*P < 0.05$).

	RNA expression ratio		Protein (ng/mg)
Cell line	MT1B	MT2A	MT
Control	1.0 ± 1.6	1.0 ± 0.3	151 ± 6.6
MT1B-HeLa	133000 ± 38000*	1.1 ± 0.3	188 ± 8.8*
MT2A-HeLa	1.2 ± 0.8	2.2 ± 0.2*	186 ± 7.6*

Cell viability

Rotenone treatment of the cell lines resulted in a general decrease in cell viability as determined by the MTT test (Figure 6). The cell lines, however, showed no consistent variation in cell viability across the range of rotenone concentrations, i.e. compared with MT1B, MT2A-overexpressing cells had significantly higher viability at 500 nM rotenone with lower viability at 2500 nM rotenone. As with ROS production, a more pronounced response of cell lines was observed when t-BHP was used to increase ROS production and consequently lowering cell viability to less than 20 %. Also, in this case, a clear and statistically significant variation in response to t-BHP treatment occurred in the MT2A-overexpressing cells compared with both control and MT1B-overexpressing cells. This is strikingly obvious at 0.5 and 0.8 nM t-BHP, where MT2A-overexpressing cells had significantly higher viability. Unlike the responses in ROS levels (compare Figure 5), at 1 mM t-BHP, viability of all three cell lines was similar at levels lower than 20 % of the untreated cells.

Mitochondrial membrane potential

The membrane potential was visualized with confocal microscopy using TMRM staining. Fluorescence from TMRM generally co-localized with green fluorescence from mitochondrial staining (results not shown). The control cell line (Figure 7A) showed a clear decrease in membrane potential with increasing rotenone concentration, having almost no visual membrane potential when treated with 1000 nM rotenone. Both MT-overexpressing cell lines had visibly more membrane potential remaining when treated with rotenone (Figures 7B and 7C). Compared with MT1B, MT2A-overexpressing cells (Figure 7C) had a visibly smaller decrease in membrane potential with increased rotenone treatment.

Apoptosis

Staurosporine treatment of all cell lines resulted in the induction of similar caspase 3/7 activities, indicating that MT overexpression in these cell lines did not result in changes in protein kinase-mediated caspase activation (Figure 8). In all cell lines, caspase 3/7 activity increased approximately in a dose-dependent way over the full range of rotenone incubations used. Both MT-overexpressing cell lines had a significant variation in response to rotenone treatment. In MT1B-overexpressing cells, caspase activity increased much slower to similar activities of control cells at 250 nM rotenone. At higher rotenone levels, caspase activity in this cell line was significantly lower than control cells and, at 2500 nM rotenone, was ± 35 % lower than control cells. Induction of caspase 3/7 activity in MT2A-overexpressing cells was significantly lower than both control and MT1B-overexpressing cells with complete inhibition of complex I. Activities remained relatively constant up to 250 nM rotenone and, up to 2500 nM, increased only to levels of ± 45 % lower than the control cells.

Over the incubation period used in the present study (24 h), visible DNA laddering was not detected using gel-electrophoresis analysis (results not shown). However, cytosolic nucleosome enrichment which is a result of DNA degradation could be detected

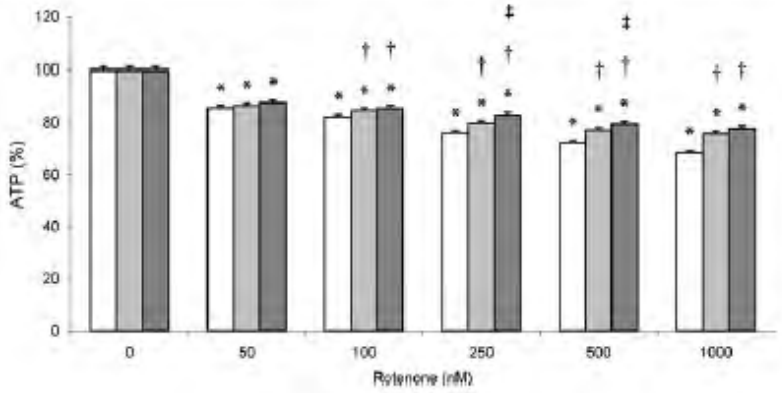

Figure 4 ATP levels in rotenone-treated MT-overexpressing HeLa cells

Total cellular ATP content was measured in rotenone-treated MT1B- (pIRESneo2-MT1B-transfected; light grey bars), MT2A-overexpressing (pIRESneo2-MT2A-transfected; dark grey bars) and control (pIRESneo2-transfected; open bars) HeLa cells as described in the Experimental section. Values were normalized to protein content and expressed as a mean percentage of untreated cells (±S.D., $n = 4$). *$P < 0.05$ when compared with untreated cells of the same cell line; †$P < 0.05$ when compared with control cell line at the same treatment; ‡$P < 0.05$ when comparing MT2A- with MT1B-overexpressing cell line at the same treatment.

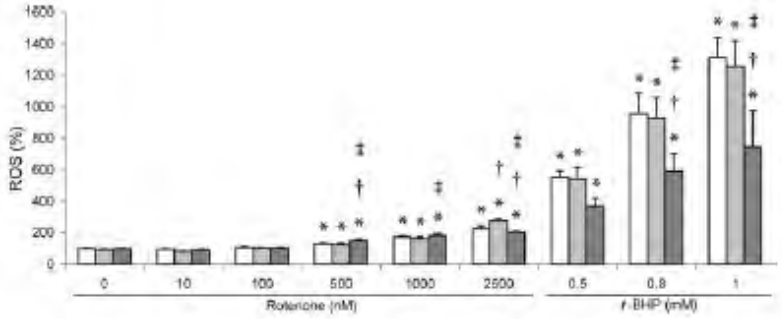

Figure 5 ROS production in rotenone- and t-BHP-treated MT-overexpressing HeLa cells

ROS production was measured in control (open bars), MT1B- (light grey bars) and MT2A- (dark grey bars) overexpressing cell lines treated with rotenone or t-BHP. Values, which were normalized to protein content, are means ± S.D. ($n = 8$) and expressed as percentage relative to untreated cells. *$P < 0.05$ when compared with untreated cells of the same cell line; †$P < 0.05$ when compared with control cell line at the same treatment; ‡$P < 0.05$ when comparing MT2A- with MT1B-overexpressing cell line at the same treatment.

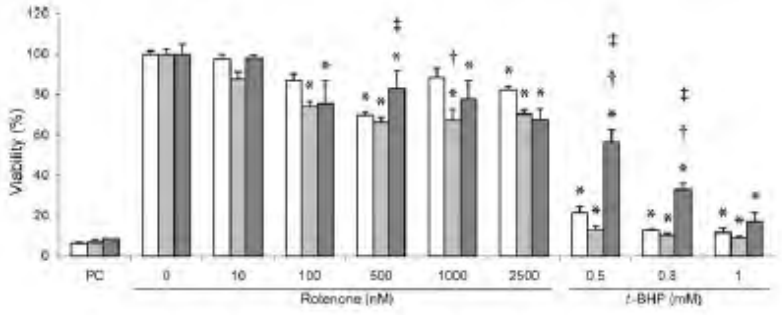

Figure 6 Cell viability in rotenone- and t-BHP-treated MT-overexpressing HeLa cells

Cell viability was measured using the MTT test in MT-overexpressing (MT1B, light grey bars; MT2A, dark grey bars) and control cell lines (open bars) treated with rotenone or t-BHP as described in the Experimental section. Values, which were normalized to protein content, are means ± S.D. ($n = 3$) and expressed as percentage viability relative to untreated cells. Acetic acid (6%) was used as positive control (PC). *$P < 0.05$ when compared with untreated cells of the same cell line; †$P < 0.05$ when compared with control cell line at the same treatment; ‡$P < 0.05$ when comparing MT2A- with MT1B-overexpressing cell line at the same treatment.

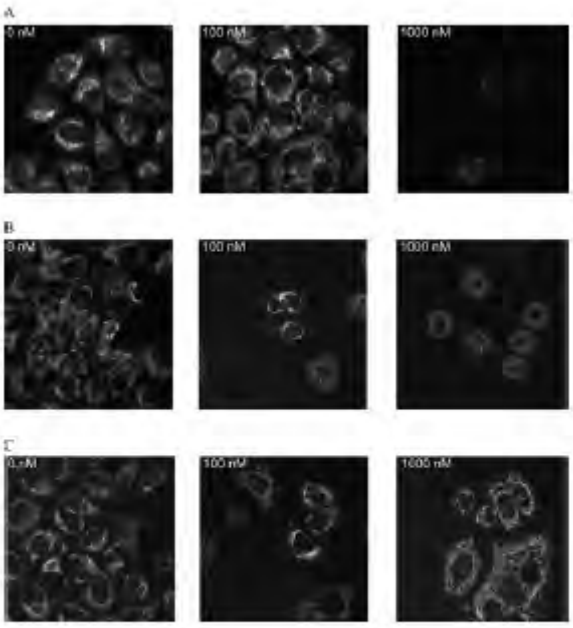

Figure 7 Assessment of mitochondrial membrane potential in rotenone-treated MT-overexpressing cells

Membrane potential of control (**A**), MT1B- (**B**) and MT2A-overexpressing (**C**) HeLa cells treated with 0, 100 and 1000 nM rotenone was visualized by confocal microscopy after TMRM staining.

with rotenone treatment in all three cell lines using an immunological assay (Figure 9). In view of the caspase data, the generally slower onset of DNA fragmentation can also be seen in the comparatively lower increase in nucleosome formation with staurosporine treatment. In the rotenone-treated cells, nucleosome formation increased significantly only at levels higher than 100 nM. For control cell lines, the nucleosome formation increased up to 14-fold at 2500 nM rotenone. Comparatively, in MT1B- and MT2A-overexpressing cell lines, nucleosome formation increased significantly more slowly at rotenone levels higher than 100 nM (36 % of residual complex I activity) to reach levels of approx. 6- and 5-fold respectively at 2500 nM compared with baseline activity. Thus, as with caspase activation, a significant variance in response to increasing rotenone treatment occurred between the cell lines. In addition, nucleosome formation in rotenone-treated MT2A-overexpressing cells increased more slowly than in MT1B-overexpressing cells and was significantly lower at the higher levels of rotenone treatment.

DISCUSSION

The contribution of nuclear and mitochondrial gene expression in the pathophysiology of mitochondrial disorders has been recognized and investigated over the past decade [9–11,32]. One of the model systems often used to investigate disorders associated with the mitochondrial respiratory chain includes the inhibition of complex I with irreversible inhibitors such as rotenone. Among the diversity of genes differentially expressed in inherited and induced complex I-deficient cell lines, the marked overexpression of MTs occurred in complex I-deficient fibroblasts when mitochondrial energy metabolism was challenged by changing the carbon source of the medium. It was hypothesized that MT expression points to a possible beneficial adaptive response under such conditions [9]. It is conceivable that the expression and function of MTs are related to disorders of the respiratory chain as these proteins are not only induced by ROS, which is a common feature in such disorders, but scavenges hydroxyl radicals [13,14,33]. Evidence linking MT expression responsiveness to oxidative stress and associated protection against oxidative stress in *in vitro* and *in vivo* models is mounting [13,14,20,34]. These include reports of MT1-mediated protection of key mitochondria-associated functions, such as apoptosis, coenzyme Q_{10} synthesis and mitochondrial genome integrity, against neurotoxin-treated murine neuronal cells [35].

We demonstrated that MT2A expression in HeLa cells is highly inducible with rotenone treatment. This is in contrast with a previous report that MT1 RNA expression is decreased in the striatum cells of rats treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [36], which also binds and inhibits

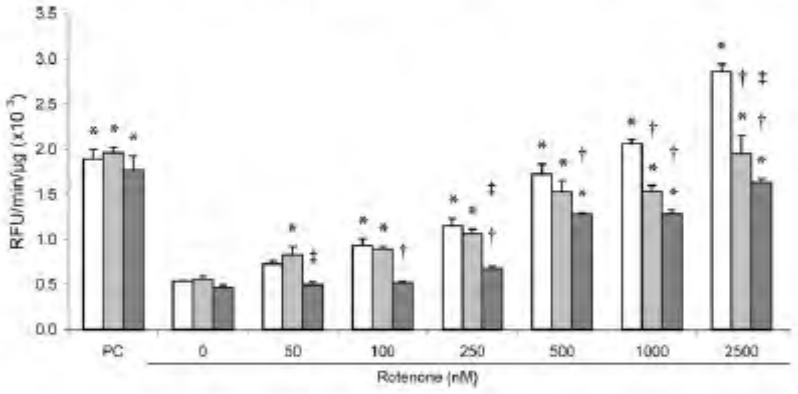

Figure 8 Caspase 3/7 activation in rotenone-treated MT-overexpressing HeLa cells

Caspase 3/7 activity was measured in MT-overexpressing (MT1B, light grey bars; MT2A, dark grey bars) and control cell lines (open bars) treated with rotenone as described in the Experimental section. Reaction velocities are indicated as change in relative fluorescence units (RFU) per μg of total protein. Staurosporine treatment (1 μg/ml for 2 h) was the positive control (PC) for caspase activation. Values are means ± S.D. ($n = 3$). *$P < 0.05$ when compared with untreated cells of the same cell line; †$P < 0.05$ when compared with control cell line at the same treatment; ‡$P < 0.05$ when comparing MT2A- with MT1B-overexpressing cell line at the same treatment.

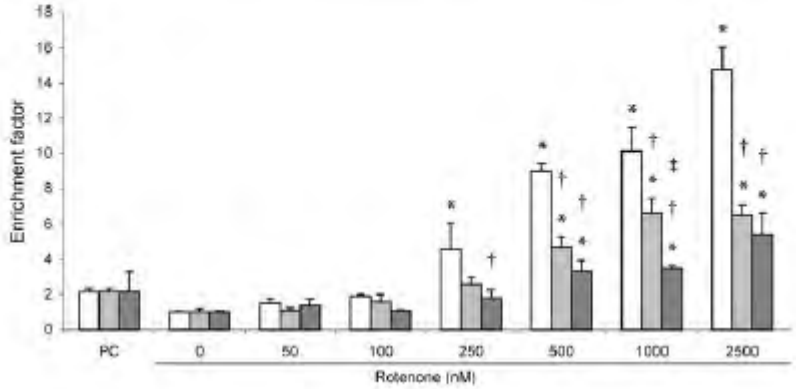

Figure 9 Cytosolic nucleosome enrichment in rotenone-treated MT-overexpressing HeLa cells

Cytosolic nucleosome enrichment was determined in MT1B- (light grey bars), MT2A- (dark grey bars) overexpressing and control (open bars) cells treated with rotenone for 24 h or staurosporine (1 μg/ml for 2 h) as positive control (PC). Values, normalized relative to protein content, are indicated as a mean fold change ($\pm$S.D., $n = 3$) relative to untreated cells. *$P < 0.05$ when compared with untreated cells of the same cell line; †$P < 0.05$ when compared with control cell line at the same treatment; ‡$P < 0.05$ when comparing MT2A with MT1B-overexpressing cell line at the same treatment.

complex I [37]. Our results show a significant increase in expression of MT2A to occur only after residual complex I activity was inhibited to levels below 50%. Expression of MT2A also did not increase in a co-ordinate way relative to rotenone concentration, and a clear biphasic expression pattern was observed. The reason for this phenomenon is not clear and has to be investigated further. Some clues as to the mechanism of rotenone-induced MT expression may, however, be revealed by the lack of both MT expression and ROS production by treatment with the complex III inhibitor, myxothiazol. It was evident that both MT2A expression and elevated ROS production occurred at residual complex I activities lower than 50%, which was not the case with complete inhibition of complex III activity. This result supports a ROS-related mechanism of rotenone-induced MT2A expression. Lack of myxothiazol-induced ROS production was an unexpected result, as the associated electron-transfer-inhibitory effect of myxothiazol has been well documented [38]. The reason for this lack of detectable ROS production is not clear, although ROS production as a result of deficient electron transport chain in the predominantly glycolytic HeLa cells may, however, in general be less pronounced than in cell lines that rely more on OXPHOS. This is evident from the limited ROS production in addition to the limited decrease in ATP levels induced by rotenone treatment.

MT2A expression was also inducible by Cd, but not Zn, both of which modulate MT expression by a different mechanism from oxidative-stress-related inducers [14,39,40]. Both Cd and Zn induced combined MT1/MT2 protein expression. Although Cd and H_2O_2 share a common MTF-1 activation pathway in HeLa cells that is dependent on the release of Zn from MT-bound Zn [41], it is not clear why $ZnCl_2$ treatment had no effect on MT2A expression. Similar observations were also made in human proximal tubule cells [42]. In addition, surprisingly, neither MT1A nor MT1B RNA expression induction was detected with any of the inducers used. Lack of MT1B expression in HeLa cells was previously reported [19]. Other MT1 isoforms in HeLa cells may be expressed that could explain the observed Zn induction of MT protein expression.

The effects of overexpressed MT2A on ATP and ROS levels, cell viability and apoptosis in rotenone-treated cells were evaluated in parallel to MT1B overexpression, which, as mentioned above, were not induced by any of the possible MT inducers used in the present study. ATP levels, which were only decreased to $\pm$70% in control cells with rotenone treatment, remained slightly, albeit significantly, higher in MT-overexpressing cells. ROS production in MT1B- and MT2A-overexpressing HeLa cells was similar to control cells when treated with rotenone up to levels where almost no rotenone-sensitive complex I activity could be measured (1000 nM). As mentioned above and evident from our results, HeLa cells rely mainly on glycolytic ATP production. ROS production via inhibition of the OXPHOS system in HeLa cells may therefore be limited compared with cells that have a greater dependence on ATP produced from OXPHOS [5,43]. With the induction of more ROS, i.e. by treatment with t-BHP, which generates ROS via microsomal cytochrome P450 activity [44] in addition to opening the mtPTP (mitochondrial permeability transition pore) [45], a markedly higher level of ROS could be induced. ROS levels under these conditions were significantly less and cell viability was significantly higher in MT2A-overexpressing HeLa cells compared with control and MT1B-overexpressing cells.

Increased production of ROS has been found to relate quantitatively to apoptosis induction in cells treated with rotenone [4,5]. The mechanism of rotenone-induced cell death in HeLa cells has previously been investigated to a limited extent only. Apoptotic cell death predominantly occurs in cells such as HeLa cells, which are less dependent on pyruvate/malate-supported ATP production [5,43]. Apoptosis induction, in addition to caspase activation, also results in the opening of the mtPTP [46], which results in a breakdown of mitochondrial membrane potential [47]. The qualitative results obtained with 100 and 1000 nM rotenone treatment of all three cell lines indicated that sustaining of membrane potential was improved in MT-overexpressing cells, but more so for MT2A, compared with control cells. Similar to previous observations, activation of caspase 3/7 and subsequent nucleosome formation occurred at much lower rotenone levels than the levels needed to induce cellular toxicity [4]. Rotenone-induced caspase 3/7 activation increased dose-dependently after a 24 h period in control cells to levels similar to that induced by staurosporine in 2 h. Over the rotenone concentration range, caspase 3/7 activity was clearly significantly lower in MT2A-overexpressing cells and, to a lesser extent, also in MT1B-overexpressing cells. This protective delaying effect

of MT expression on apoptosis was strongly supported by the significantly lower nucleosome formation in MT2A- and, to a lesser extent, MT1B-overexpressing cells, compared with control cells.

Studies on the function of MT are not conclusive as to its role in the prevention of oxidative stress. It was recently reported that MTs present in the intermembrane space of liver mitochondria could inhibit mitochondrial respiratory-chain complexes I and III, through transfer of Zn to the complexes [48–50]. However, this phenomenon did not occur in heart muscle mitochondria [48,51]. In general, studies show that MT expression is associated with a protective effect against interventions leading to oxidative stress. Our results have shown that in rotenone-induced complex I-deficient HeLa cells, overexpressing MT2A indeed had a lowering effect on oxidative stress and increased cell viability, which were especially clear when further challenged with t-BHP treatment. Furthermore, MT2A overexpression had a preventative or delaying effect on rotenone-induced apoptosis in HeLa cells. MT1B, at similar overexpressed levels, in general did not show the same responsiveness as MT2A.

To conclude, recent interest in the downstream adaptive responses to deficiencies of the OXPHOS system has revealed that, via differential gene expression, several genes may be involved in novel responses apart from those already associated with the deficiency, such as induction of apoptosis and changes in redox status [9–11]. Of these responses, little, if any, have been further investigated or reported. We have investigated the expression and role of MTs in an *in vitro* complex I deficiency model and concluded that the induced expression of MTs, and specifically MT2A, has a protective effect against death-causing cellular consequences of rotenone-treated HeLa cells. Although our results support a ROS-related mechanism, it remains to be determined what the mechanistic properties of this expression are, and if MT expression is functionally relevant to complex I and other inherited OXPHOS deficiencies *in vivo*. Our results are comparable with current literature reports on the functional properties associated with MT expression, but specifically reveal MT2A expression to be a beneficial downstream adaptive response in complex I-deficient cells.

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APPENDIX C

Mitochondrial DNA replication and OXPHOS gene transcription show weak responsiveness to Rieske protein knockdown in 143B cells

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Keywords:

OXPHOS deficiency, gene expression, Rieske knockdown

Abbreviations:

C_T, Cycle threshold; DCFH-DA, 2',7'-dichlorofluorescin diacetate ; D-loop, displacement loop; DNApolγ, DNA polymerase γ; H-strand, heavy strand; L-strand, Light strand; mtDNA, mitochondrial DNA; mtSSB, mitochondrial single-stranded binding protein; NRF, nuclear transcription factor; OXPHOS, oxidative phosphorylation; POLRMT, mitochondrial RNA polymerase; RC, respiratory chain; ROS, reactive oxygen species; shRNA, short hairpin RNA; TFAM, mitochondrial transcription factor A; TFB2M, mitochondrial transcription factor B2;

ABSTRACT

Complexes I and III are considered to be the main sources of superoxide radical production in the oxidative phosphorylation (OXPHOS) system, which could damage lipids, proteins and DNA, and also lead to differential expression of several genes involved in mitochondrial biogenesis. However, increased oxidative stress is not always observed with such deficiencies. We investigated the effect of a complex III deficiency, by means of Rieske protein knockdown in 143B cells, on mitochondrial DNA (mtDNA) replication/transcription processes. Despite the efficient knock down of Rieske protein and subsequent complex III decrease, no significant ROS increase was observed but strikingly the knockdown showed weak but significant effects on mitochondrial DNA (mtDNA) replication and OXPHOS gene transcription. Mitochondrial L-strand and D-loop transcript levels remained unchanged, while the H-strand transcript for COXII was reduced. Nuclear-encoded OXPHOS gene transcripts were slightly reduced (statistically insignificant), except for NDUFS3 and COX4I1 transcripts. The deficiency of complex III also did not result in a change in mtDNA copy number or expression of several key regulatory proteins involved in mtDNA replication and transcription (DNA polymerase γ, mitochondrial RNA polymerase, mitochondrial transcription factor A and mitochondrial transcription factor B2). However, mitochondrial single-stranded binding protein (mtSSB) expression was reduced in the Rieske protein knockdown cells. We conclude that a deficient complex III function via Rieske subunit knockdown in 143B cells, which does not result in increased ROS levels, results in a weak response in mtDNA replication and both nuclear and mitochondrial OXPHOS gene transcription. These responses may prove to be more significantly affected by OXPHOS deficiencies that do result in increased oxidative stress.

1. Introduction

The immediate intracellular consequences resulting from deficiencies of the mitochondrial OXPHOS system are greatly diverse with regard to the range, potency and final outcome. These consequences have been widely documented and include a great diversity of primary and secondary changes in metabolites, structural modifications, and signalling events that controls transcriptional and replication responses [1-7]. For OXPHOS enzyme deficiencies, existing reports on the transcription and replication of mitochondrial DNA (mtDNA), which have a significant impact on mitochondrial disease expression, reveals an inconsistent and incomparable profile, as recently reviewed by Reinecke et al. [8]. For mtDNA, which responds to oxidative capacity and energy demand [9], an up-regulation of replication and transcription appears to be a fitting response considering that several of its nuclear encoded control elements also respond to stress-related retrograde effectors, and in particular increased oxidative stress, which mainly originate from mitochondria [9-13]. However, increased oxidative stress is not always observed in even severe deficiencies of the OXPHOS system [14, 15], which raises the question of whether mtDNA is indeed differentially expressed and replicated in OXPHOS deficiencies that do not result in significantly elevated superoxide production.

We investigated this question using a cytochrome *c* reductase (complex III) deficiency *in vitro* model, using a stable knockdown of the Rieske protein in 143B cells. Complex III is assembled from 11 subunits, of which three (Rieske Fe-S protein, cytochrome *b*, and cytochrome *c*1) transport electrons from ubiquinol to cytochrome *c* through the ubiquinone (Q) cycle. Although complex III is one of the major sites in the respiratory chain responsible for the production of superoxide radicals, which occurs at both the ubiquinol oxidizing (Qo) and the ubiquinone reducing (Qi) sites [16], a deficiency of the Rieske protein (part of the Qo site) would result in limited superoxide production but still prevent electron transport to cytochrome *c* [17, 18]. Here we report the general lack of response of mtDNA replication and transcription in Rieske protein deficient 143B cells, where a severe complex III deficiency do not result in elevated superoxide production.

2. Materials and methods

2.1 Cell culture and shRNA knockdown

The anti-Rieske shRNA-expressing construct in pSIREN-RetroQ plasmid was a kind gift from Prof. N.S. Chandel (Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL). Except where indicated, all tissue culture reagents were obtained from Gibco (Invitrogen, Carlsbad, CA)unless indicated otherwise Generally, 143B control cybrid cells [19] were cultured at 37 ºC with 5% CO_2 in DMEM, supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Transfected cells also supplemented with 3µg/ml puromycin (Sigma-Aldrich St. Louis, MO). Cells were transfected with either the empty pSIREN-RetroQ-TetP vector (Clontech, Biosciences, Heidelberg, Germany), the anti-Rieske shRNA-expressing construct [20], or the unrelated anti-eGFP shRNA-expressing construct (Clontech, BD Biosciences, Heidelberg, Germany) [21] in the presence of ExGen 500 *in vitro* Transfection Reagent (Fermentas, Vilnius, Lithuania). Cell selection continued for 10-14 days and isolated clones selected by the dilution method [22]. Prior to analysis cells were cultured for at least 72 hours without antibiotics in DMEM containing 1 mM sodium pyruvate, 4.5 g/L glucose, and 10 % (v/v) fetal bovine serum dialysed with phosphate-buffered saline. During this time, cells were seeded at a density of 4,000 cells per cm^2 and harvested when a confluency of ~90% was reached.

2.2 Relative RNA quantification

Total RNA was isolated from cultured cells using QIAzol reagent according to the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription of 4 µg RNA with 200 U M-MLV reverse transcriptase (Promega, Madison, WI) was carried out in a volume of 40 µl using 0.5 µg random hexamer primers (Inqaba Biotech, South Africa). Realtime-PCR was performed using an ABI7300 (Applied Biosystems, Foster City, CA) in a final volume of 20 µl with the KAPA SYBR FAST ABI Prism 2X qPCR Master Mix (Kapa Biosystems, Woburn, MA) and 250 nM forward and reverse primers. Relative quantification was determined for other mitochondrial- and nuclear encoded transcripts (Table 1). Transcripts were amplified from 20 ng cDNA with forward and reverse primers and normalised to β2-microglobulin transcripts [23]. This transcript

has been shown to remain unaffected when a complex I deficiency was induced with rotenone and were thus considered to be a suitable normalising gene [24]. The C_T-values obtained from the 7300 System Sequence Detection Software (version 1.4), from Applied Biosystems (Foster City, CA), were exported to REST-384 Version 2 software (Relative Expression Software Tool) [25]. PCR efficiency for each primer set was calculated by serial dilutions method using the REST software tool [25].

2.3 Relative mitochondrial DNA copy number

Total DNA was extracted from cultured cells using the NucleoSpin kit (Macherey-Nagel, Duren, Germany). The relative mtDNA copy number was measured by real-time PCR and calculated using a nuclear gene as a normaliser. For PCR, the primers and probe for the nuclear β-globin gene were: 5'-GTGCACCTGACTCCTGAGGAGA-3' (forward), 5'-CTTGATACCAACCTGCCCAG-3' (reverse) and 5'-FAM-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA-3' (probe), respectively (Metabion International, Martinsried, Germany). For mtDNA amplification, MT-ND2 TaqMan Gene Expression Assay from Applied Biosystems was used (assay Hs02596874_g1, Applied Biosystems, Foster City, CA). The PCR reactions, in 25 µL volumes, were performed using an ABI 7300 Real Time PCR System. Each reaction mixture contained 12.5 µL TaqMan Universal PCR Master Mix, No AmpErase UNG (2X) (Applied Biosystems, Foster City, CA), 0.5 µM of the forward and reverse primers and 0.2 µM of the probe for β-globin gene. For the MT-ND2 gene, a 1x dilution of primers/probe mixture and 10 ng of DNA was used. The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C and annealing/extension at 60°C for 1 min with fluorescence measurement during this step. All reactions were performed in triplicate and every assay included a no-template control and three serial dilution points (in 5-fold steps) of the DNA sample. Relative mtDNA copy number was calculated as described for the relative RNA quantification.

2.4 Enzyme analyses

All reagents used for enzyme and protein analyses were purchased from Sigma-Aldrich (St. Louis, MO). Analyses were performed using 600 x *g* supernatants that were prepared from homogenizing cells suspended in an isotonic buffer (mannitol, 210 mM; sucrose, 70 mM; HEPES, 5 mM; EGTA, 0.1 mM; pH 7.2). Protein content was determined using the Bicinchoninic acid (BCA) method [26]. Mitochondrial respiratory chain (RC) enzymes complex II (succinate:ubiquinone oxidoreductase, EC 1.3.5.1), complex III (ubiquinol:ferricytochrome-c oxidoreductase, EC 1.10.2.2), and complex IV (ferrocytochrome-c:oxygen oxidoreductase, EC 1.9.3.1), were measured essentially as described previously [27]. Citrate synthase (CS; EC 2.3.3.1) activity was determined by the method of Shepherd and Garland [28]. The RC enzyme activities were expressed as a ratio to CS (as mitochondrial marker enzyme) to compensate for mitochondrial enrichment in the sample.

2.5 Denaturing and non-denaturing PAGE analyses

Freshly prepared whole cells were re-suspended in phosphate buffered saline after harvesting. An enriched mitochondrial pellet was prepared by homogenisation and differential centrifugation up to 10 000 x g in the isotonic buffer used for enzyme analyses. The pellets were further prepared for SDS-polyacrylamide gel-electrophoresis (PAGE) as described before [29]. After total protein quantification [26], 10 μg of mitochondria-enriched extract was loaded onto a 12 % (w/v) SDS-PAGE gel. Blue-native (BN) PAGE gradient gels (5-15%) were prepared and loaded with 40 μg mitochondrial fraction as described before [30]. In-gel activity assay for complex I was carried out as described by Nijtmans et al. [30]. Western blotting was performed with primary antibodies against Rieske protein, complex III-core 2 protein and cyclophilin D (Mitosciences, Eugene, OR), together with a secondary HRP-Goat Anti-Mouse IgG (H+L) antibody (Zymed, San Francisco, CA) at the suggested dilutions and conditions according to the suppliers. Signal detection was performed with ECL Western blotting substrate (Pierce, Rockford, IL) and the blots exposed to BioMax light film (Kodak, Rochester, NY). The chemiluminescent signals on the exposed film were quantified using GeneTools 3.06 Imaging software (SynGene, Frederick, MD).

2.6 ROS production

To measure reactive oxygen (ROS) levels, cells were incubated for 30 min with 10 µM of the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA, Molecular probes, Eugene, OR). Cells were trypsinized and re-suspended in phosphate buffered saline. All treated cell preparations were then analysed for ROS-induced fluorescence using a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA). Cells were analyzed at a flow rate of 1,000 events per second and 10,000 events were collected for each sample. Cells were excited with an argon laser emitting at 488 nm. Forward and side scatter were collected on a linear scale and DCF fluorescence were detected by FL1 PMT using log amplification. For every assay unstained cells, both treated and untreated were used as controls. Data was acquired using CELLQuest PRO (Becton Dickinson, San Jose, CA) and analysed by FCS Express (version 3, De Novo Software). Results were expressed as ratios between mean fluorescence intensities per percentage positively stained cells.

2.7 Statistical evaluation

All results were analyzed with GraphPad Prism (version 5) software as mean ± standard deviation. Statistical comparisons were made using one-way analysis of variance (ANOVA) with post hoc comparison (Tukey test). Statistically significance were considered when $p < 0.05$.

3. Results

3.1 Characterization of stable Rieske protein knockdown of 143B cells

To characterize the success of inducing a stable (shRNA-mediated) Rieske protein knockdown in 143B cells, individual clones were analysed for relative mRNA expression of the Rieske gene (results not shown). A clone with the most significant reduction in mRNA expression was selected for further investigations. The Rieske protein knockdown cell line was evaluated based on relative expression levels of mRNA and protein, as well as enzyme activity of complex III (Table 2). Real-time PCR analysis of the relative expression level of Rieske transcripts (Figure 1A) showed an 88% reduction in the Rieske-shRNA clone compared to both 143B cells and cells

transfected with the empty vector. Western blot analysis also showed the significant reduction of the Rieske protein level in the Rieske-shRNA transfected clone compared to both 143B cells and cells transfected with the empty vector (Figure 1B), confirming the successful Rieske protein knockdown, as originally demonstrated by Brunelle et al. [20]. A significant reduction in the level of fully assembled native complex III could be observed, as seen in the western blot of the BN-PAGE with other enzyme complexes remaining relatively unchanged (Figure 1C). Longer exposure revealed the presence of a lower molecular weight sub-complex (Figure 1D). In addition, the complex III (cytochrome *c* reductase) activity of this clone was reduced to approximately 23% compared to the values of the native 143B and empty vector-transfected cell lines, but with comparable complex I, II and IV activities (Table 2). This result confirmed that the Rieske protein knockdown indeed resulted in a significant decrease in complex III enzyme activity. Although a slight drift towards lower knockdown effectiveness was observed, these results were generally reproducible over several months of culture, which indicates a stable knockdown of the protein was obtained.

The production of ROS in the Rieske knockdown cell line were approximately 40% (statistically insignificant) higher than native 143B cells, but significantly lower than the vector- and nonrelated-shRNA transfected clones (Figure 2). This indicates that although higher ROS levels are formed in the Rieske protein knockdown, its formation is more a result of the genetic modification as it is due to complex III knockdown. This observation is in accordance with previous studies that reported that the Rieske protein knockdown limits ROS production at the Qo site [31, 20].

3.2 mtDNA and mtRNA expression

Real-time-PCR analyses of the mtDNA showed unchanged relative mtDNA copy number in the Rieske-shRNA knockdown, compared to cells transfected with the empty vector. Compared to 143B cells, the Rieske-shRNA cells showed a small, but insignificant increase (5%) in mtDNA copy number ratio (Figure 3). It is therefore clearly evident that despite a significantly deficient complex

III function via Rieske protein knockdown, and including all the consequences associated with it, mtDNA replication were not measurably affected by it.

To evaluate mtDNA transcriptional responses to the knockdown, levels of three mitochondrial encoded transcripts were quantified (Figure 4). Compared to the empty-vector control, the H-strand transcript of COXII showed a reduction of approximately 45% in Rieske knockdown cells, whereas the L-strand transcript of the ND6 gene showed no change. The non-coding displacement loop (D-loop) transcript showed a small but insignificant (12%) increase. It appears from these results that the complex III deficiency due to Rieske-shRNA knockdown may reduce H-strand transcription, which encodes the majority of mtDNA genes. Differences between H- and L-strand transcript levels were also detected in earlier studies and it was proposed that this is probably due to different rates of processing or transcription and/or different half lives for the two strands [32, 33].

3.2 Nuclear OXPHOS gene mRNA expression

The mRNA expression of six nuclear genes encoding structural subunits of the various OXPHOS complexes were quantified (Figure 5). When compared to empty-vector control cells, statistically significant reduced expression for the complex I subunit, NDUFS3 (42%, Figure 5A), and the complex IV subunit, COX4I1 (28%, Figure 5E) transcripts were detected. The significantly reduced expression of NDUFS3 is also evident from the SDS-PAGE data shown in Figure 1B. Although the transcripts of NDUFA9, SDHA, CIII-core 2 and CV-α were not statistically different, the transcription of these subunits appears to be generally lower in the knockdown cells. It thus appears from this data that these selected OXPHOS genes are indeed differentially expressed as is often reported for OXPHOS deficiencies, but that their expression are to a limited extent also coordinately reduced [8, 34].

3.3 mRNA expression of mtDNA replication and transcription regulators

As the transcription/replication machinery of mtDNA in mammalians is regulated by several nuclear encoded genes [35-37], the expression of several of these key regulators was evaluated in the Rieske protein knockdown cell line. As is clearly evident in the results shown in Figure 6, the expression levels of DNA polymerase γ (DNApolγ), mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B2 (TFB2M) mRNA levels showed no significant changes when compared to empty vector transfected cells. Previous studies reported that expression of TFAM, mtSSB and POLRMT tightly correlates with mtDNA copy number [11, 12, 38, 39]. Considering these reports, the relatively unchanged TFAM and POLRMT expression observed in this study is in accordance with a relatively stable mtDNA copy number ratio in the Rieske protein knockdown cell line. TFAM expression is also associated with increased ROS levels in mtDNA depleted cells [10]. The expression of these regulatory proteins (TFAM, TFB2M, POLRMT, mtSSB and DNApolγ are regulated by the two nuclear transcription factors, NRF-1and NRF-2, which have also been shown to be redox sensitive [10, 40-41]. The expression of another protein that is reported to correlate with mtDNA copy number, mtSSB, was statistically significantly reduced by 26% in the Rieske protein knockdown cells (Figure 6C). However, this result is in contrast to previous studies where mtSSB expression changes were comparable to changes of mtDNA copy number ratio [11, 38].

4. Discussion

Increased ROS production is thought to be one of the primary consequences of OXPHOS deficiencies, especially when complexes I and III are involved [42]. However, increased oxidative stress is not always observed with such deficiencies [43, 44]. Specifically in complex III, the production of superoxide radicals occur at both the ubiquinol oxidizing (Qo) and the ubiquinone reducing (Qi) sites [16], and in previous studies deficiency of the Rieske protein (forming part of the Qo site) resulted in limited superoxide production whilst still preventing electron transport to cytochrome *c* [17, 18, 20, 31].

We investigated the effect of such a complex III deficiency *in vitro*, by means of Rieske protein knockdown, on mtDNA replication/transcription processes. From the results we conclude that a deficient complex III function via Rieske subunit knockdown, including all the consequences associated with it, do not affect the regulation of mtDNA replication (i.e mtDNA copy number). This is supported by the unchanged expression of the mitochondrial L-strand - and D-loop transcript levels in the complex III deficient model. The reduced expression of the H-strand transcript in the same model supports previous findings and it was proposed that this is probably due to different rates of processing or transcription and/or different half lives for the two strands [32, 33].

The nuclear-encoded transcripts were slightly reduced (although statistically not significant) in the Rieske protein knockdown cells, except for NDUFS3 and COX4I1 transcripts. It thus appears from this data that these selected OXPHOS genes are indeed differentially expressed as is often reported for OXPHOS deficiencies, but that their expression are to a limited extent also coordinately expressed [8, 34]. The fact that two nuclear transcript levels (NDUFS3 and COX4I1) was significantly reduced, whilst the other nuclear transcript levels were statistically unchanged, points to the possibility of additional functions, different RNA stabilities of the different nuclear subunits, or a lack of coordinated regulation (different promoter elements) of the genes as proposed previously [45, 46]. The differential expression of the two nuclear-encoded complex I subunits (NDUFS3 and NDUFA9) could also be because NDUFS3 is part of the catalytic core of complex I, whilst NDUFA9 is not [47, 48]. A strong correlation between deficiency of complex I subunits and reduced fully-assembled complex III was also found previously [48] and it is known that complexes I, III and IV form supercomplexes or respirasomes, which could explain the correlation between reduced expression of NDUFS3 (complex I) and COX4I1 (complex IV) transcripts with the complex III deficiency.

The deficiency of complex III also did not lead to any up-regulation of the regulatory proteins involved in mtDNA transcription we investigated (DNApolγ, POLRMT, TFAM and TFB2M), as was expected since there were no changes in mtDNA copy number or increased ROS production. Previous studies reported tight correlations between TFAM, mtSSB and POLRMT expression and mtDNA copy number [11, 12, 38, 39]. Expression of these regulatory proteins is

regulated by the two nuclear transcription factors, NRF-1 and NRF-2, which have also been shown to be redox sensitive [40-41], a correlation was evident between ROS levels and TFAM expression in a study by Miranda et al. [10].

The expression of mtSSB, another protein involved in mtDNA replication/transcription, was also found to correlate with mtDNA copy number [11, 38]. In the current study however, mtSSB was statistically significantly reduced by 26% in the Rieske protein knockdown cells even though mtDNA copy number remained unchanged. A study by Yin et al. [49] found that mtSSB expression closely compared with mtDNA copy number in non-tumerous liver-tissues, but in hepatocellular carcinoma tissues mtSSB expression was increased whilst mtDNA copy number was unchanged or reduced.

5. Conclusion

Therefore, we conclude that a deficient complex III function via Rieske subunit knockdown, which does not result in increased ROS levels, does not significantly affect the regulation of mtDNA replication or transcription as well as nuclear OXPHOS gene transcription. Furthermore, considering the regulation elements involved in these processes, increased ROS production, which is often associated with OXPHOS deficiencies, is probably necessary for significant responses of mtDNA replication/transcription *in vitro*.

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Table 1: Primer sequences for mitochondrial- and nuclear-encoded transcripts.

Transcript	Forward primer (5′-3′)	Reverse primer (5′-3′)	Genbank® accession number
Reference transcript			
β2-microglobulin	AGCGTACTCCAAAGATTCAGGTT	ATGATGCTGCTTACATGTCTCGAT	NM_004048
Mitochondrial transcripts			
ND1	GTCTCAGGCTTCAACATCG	CGAATTCATAAGAACAGGGAG	NC_012920
ND6	CTCCTCAATAGCCATCGCTG	GATTGTTAGCGGTGTGGTCG	NC_012920
COXII	CATCCTAGTCCTCATCGCC	GATTAGTCCGCCGTAGTCG	NC_012920
D-loop	CCTAACACCAGCCTAACCAG	TGATGAGATTAGTAGTATGGGAG	NC_012920
Nuclear-encoded structural transcripts			
NDUFS3	GCTGACGCCCATTGAGTCTG	GGAACTCTTGGGCCAACTCC	NM_004551
NDUFA9	GGAGCAACAGGATTCCTGGG	CTGTGTTGTACTACTCGTCGG	NM_005002
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	NM_004168
CIII-core 2	GTCGTTGGGAAGTAGCTGAC	GGATTAGCCAAGGCATTCCG	NM_003366
CIII-Rieske	GGAAATTGAGCAGGAAGCTG	CCACCAAAATCTCCTGCATT	NM_006003
COX4I1	CTAGTTGGCAAGCGAGCAAT	TCACGCCGATCCATATAAGC	NM_001861
CV-α subunit	GGTCGTGTAGTTGATGCCC	CAGTCTGCATTGGTTCCCG	NM_004046
Nuclear-encoded functional transcripts			
TFAM	CCGGCTGTGGAAGTCGAC	GTTCCCTCCAACGCTGGGC	NM_003201
mtSSB	GAGATGTGGCGATCAGGG	GATTGTTGTTGCTTGTCGCC	NM_003143
TFB2M	CGCGAAAGCATTTGCCGGC	TCACTTTCGAGCGCAACCAC	NM_022366
POLRMT	CAAGCCGCATCGTTCCTCTC	GCCTCGAAGGTCAGCGTGG	NM_005035
DNApolγ	GGTTGCTGAACGGGCATGG	GAGGTTGGTGATCTGCAAGG	NM_002693

Table 2. Respiratory chain enzyme activities in cell lines

	Complex I[#]	Complex II (nmol/min/UCS)	Complex III (nmol/min/UCS)	Complex IV (nmol/min/UCS)
143B cells		235 ± 5.3	140 ± 19.8	7.9 ± 2.6
Empty vector		252 ± 8.7	150 ± 2.8	7.5 ± 1.0
Non-related shRNA		213 ± 1.1	156 ± 13.4	9.4 ± 1.3
Rieske shRNA		203 ± 1.8	34 ± 12.8	9.6 ± 1.4

[#]Complex I activities were measured using BN-PAGE in-gel activity, normalised to complex II activity by densitometry. All other activities (average ± SDEV, n = 3) were measured kinetically as described in the Experimental section and normalised to citrate synthase activity (UCS).

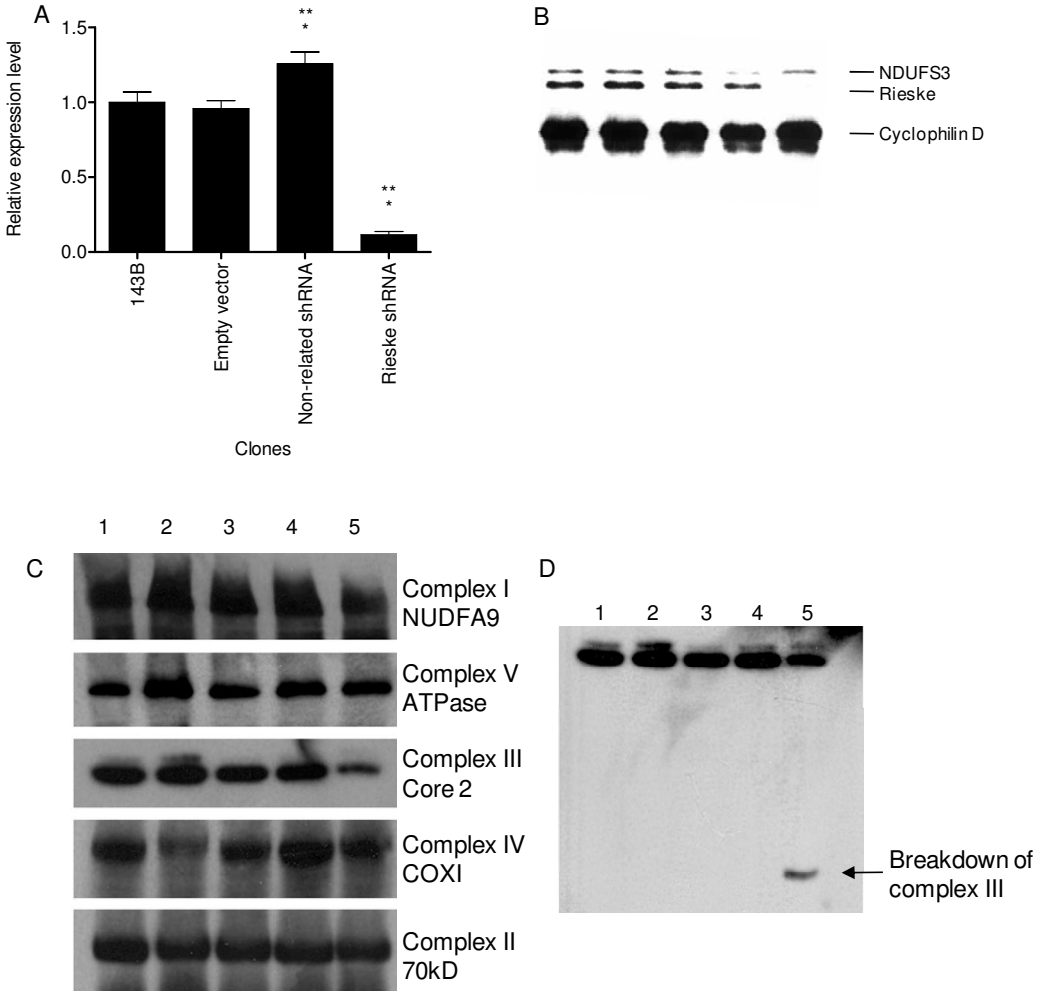


Figure 1: Rieske subunit stable knockdown in 143B cells. (A) Relative expression analysis of Rieske transcripts by means of real-time PCR (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector using a one-way ANOVA, Tukey test). (B) Protein expression with SDS-PAGE and (C) BN-PAGE with western blot analysis of the OXPHOS subunits using antibodies as indicated in 1) 143B cells, 2) empty vector, 3) unrelated shRNA and 4) Rieske shRNA clones. (D) Long term exposure of BN-PAGE western blot with antibody against complex III-core 2 subunit showing a lower molecular weight subcomplex formation as indicated by the black arrow. (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, one-way ANOVA, Tukey test).

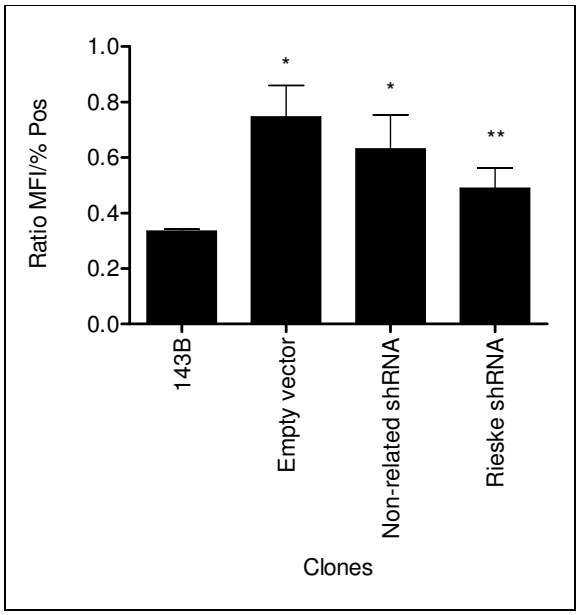


Figure 2: Relative ROS production of Rieske knockdown 143B cells. Levels expressed as mean fluorescence intensity per percentage positively stained cells (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, one-way ANOVA, Tukey test).

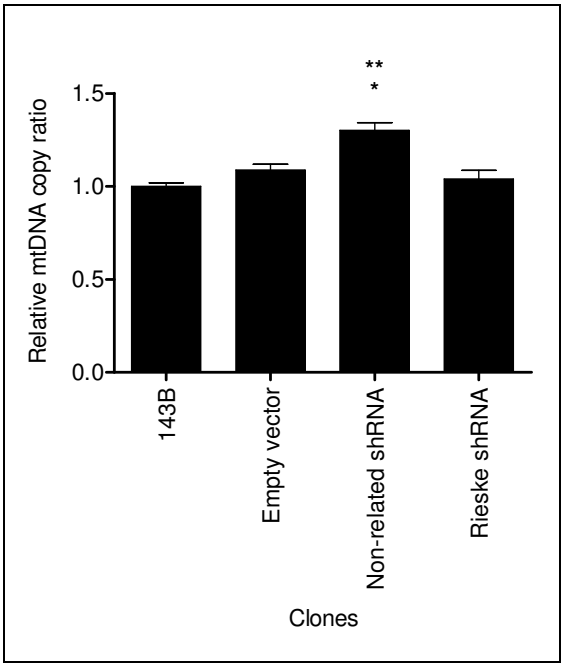


Figure 3: Relative mtDNA copy ratio. ND2 (mtDNA) content relative to β-globin (nDNA) content (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, one-way ANOVA, Tukey test).

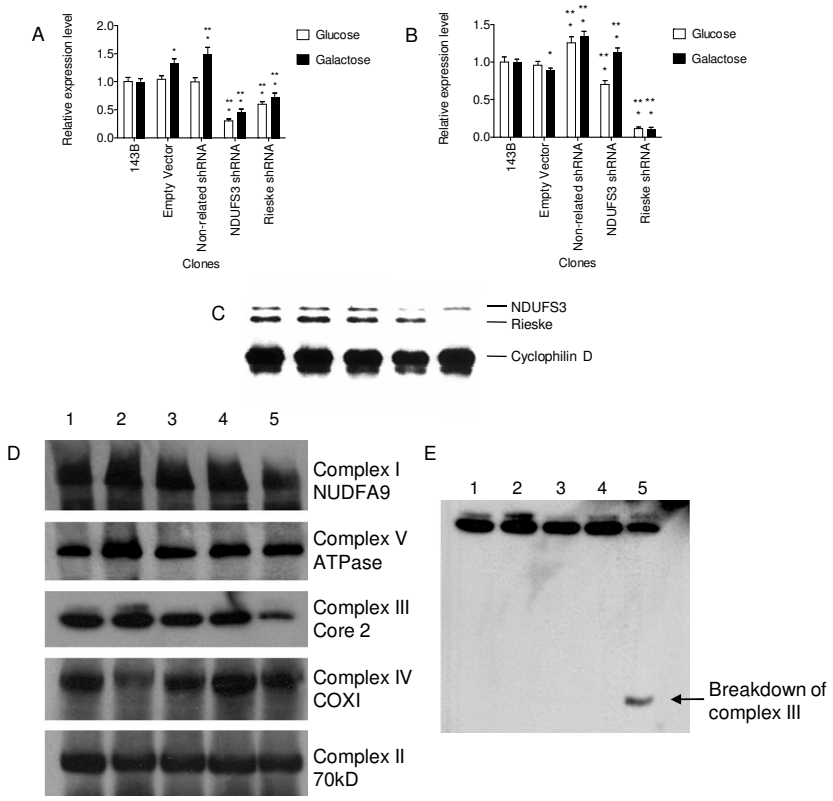
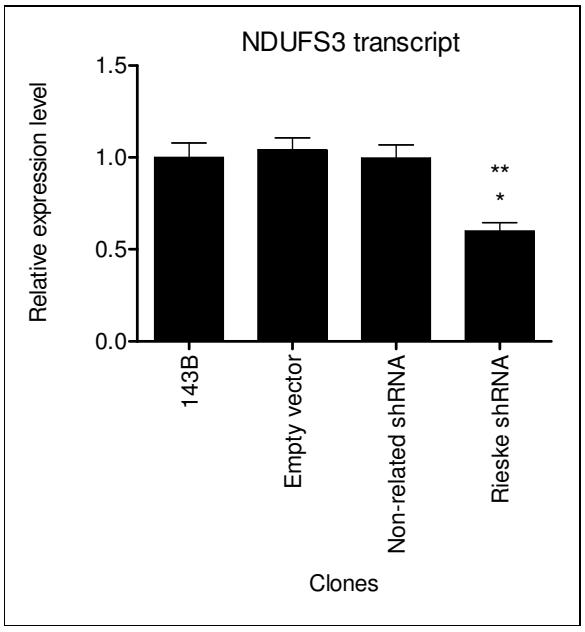
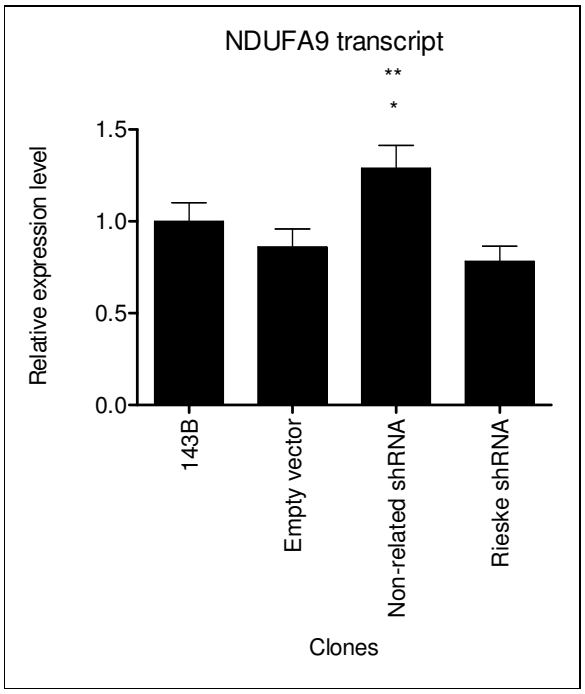


Figure 4: Relative expression level of mitochondrial DNA transcripts in cell lines. Relative transcript levels were quantified using real-time PCR relative to the nuclear gene transcript for β2-microglobulin in the various cell lines grown for (A) ND6, (B) COXII, and (C) D-loop (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, one-way ANOVA, Tukey test).

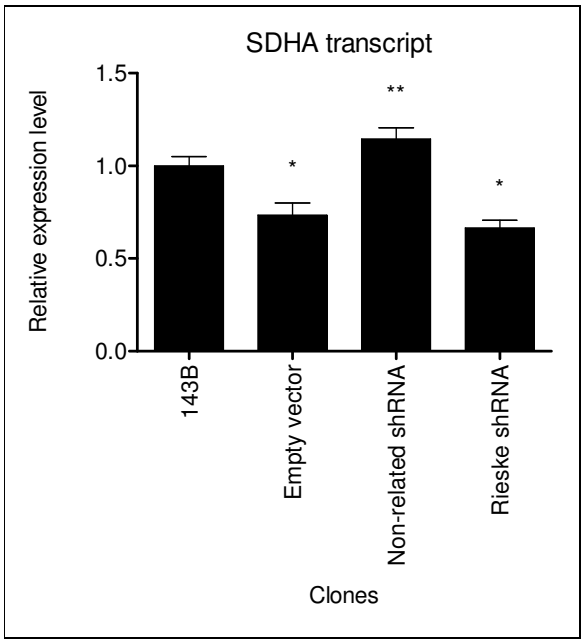
A


NDUFS3 transcript
Relative expression level
1.5
1.0
0.5
0.0
**
*
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

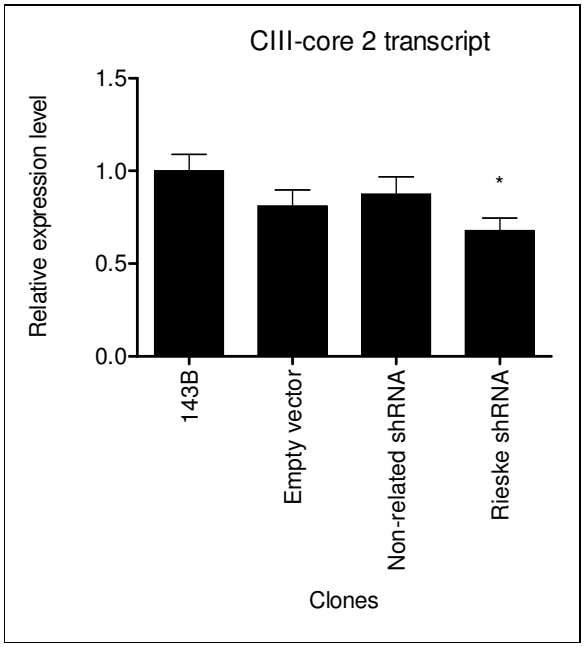
B


NDUFA9 transcript
**
*
Relative expression level
1.5
1.0
0.5
0.0
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

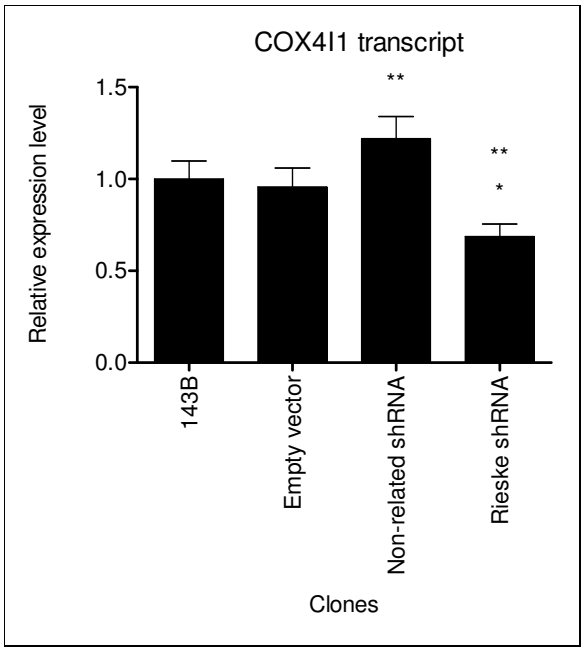
C


SDHA transcript
Relative expression level
1.5
1.0
0.5
0.0
*
**
*
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

D


CIII-core 2 transcript
Relative expression level
1.5
1.0
0.5
0.0
*
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

E



F

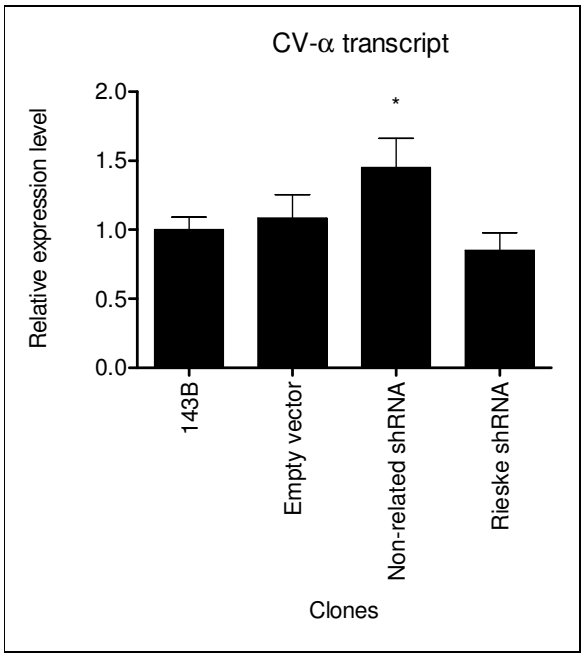
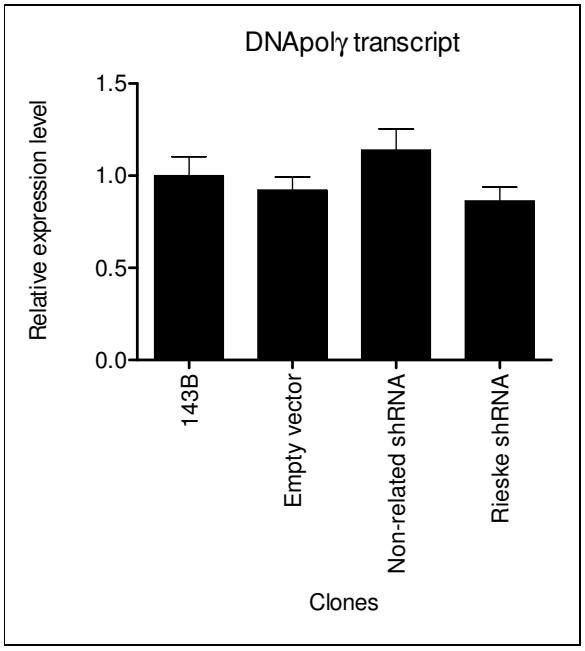


Figure 5: Relative expression level of nuclear OXPHOS gene transcripts. Transcript levels for (A) NDUFS3, (B) NDUFA9, (C) SDHA, (D) CIII-core 2, (E) COX4I1 and (F) CV-α were quantified using real-time PCR relative to the nuclear gene transcript for β2-microglobulin (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, one-way ANOVA, Tukey test).

A


DNApolγ transcript
Relative expression level
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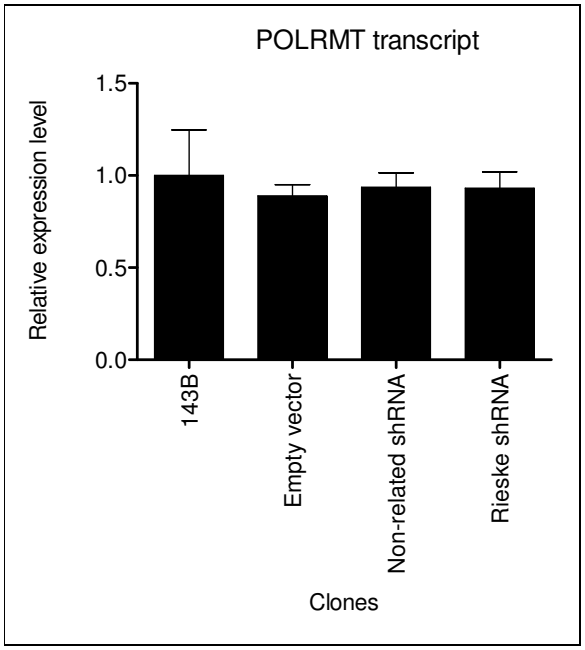
1.0
0.5
0.0
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

B


POLRMT transcript
Relative expression level
1.5
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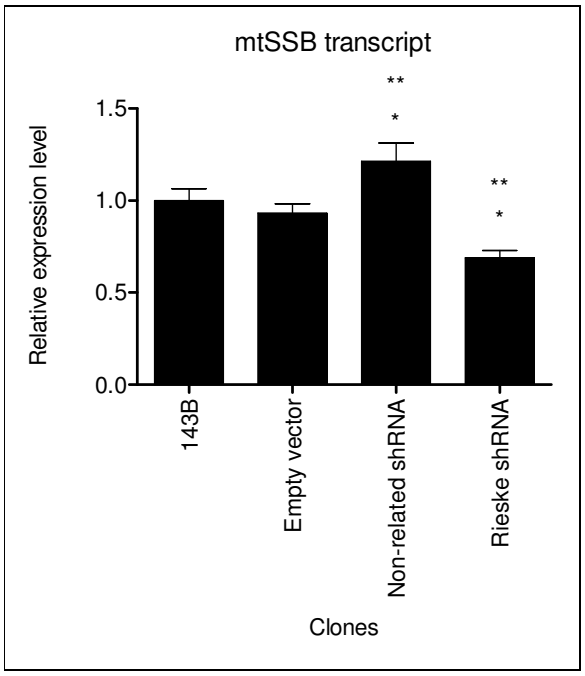
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

C


mtSSB transcript
Relative expression level
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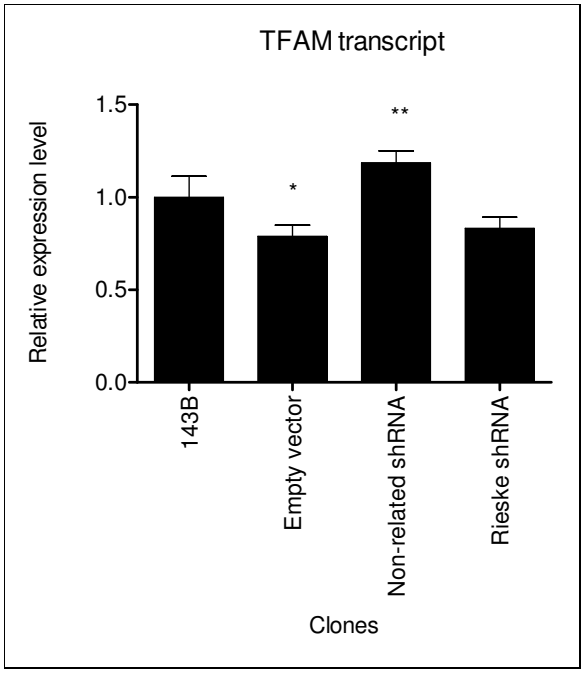
1.0
0.5
0.0
**
*
**
*
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

D


TFAM transcript
Relative expression level
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1.0
0.5
0.0
*
**
143B
Empty vector
Non-related shRNA
Rieske shRNA
Clones

E

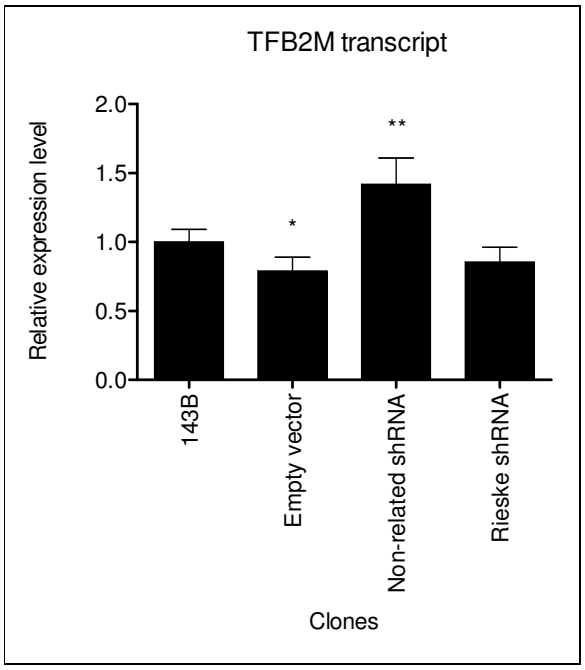


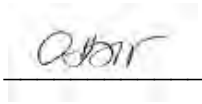
Figure 6: Relative expression level of regulatory nuclear transcript. Real-time PCR quantification relative to the nuclear gene transcript for β2-microglobulin (A) DNApolγ, (B) POLRMT, (C) mtSSB, (D) TFAM, and (E) TFB2M (n=3, *$p<0.05$ compared to 143B, ** $p<0.05$ compared to empty vector, one-way ANOVA, Tukey test).

Permission from co-authors

To whom it may concern,

We, the co-authors of the paper titled "**Mitochondrial DNA replication and OXPHOS gene transcription show weak responsiveness to Rieske protein knockdown in 143b cells**" hereby give permission that the data originating from the experiments conducted on the *in vitro* stable knockdown models of NDUFS3 and Rieske subunits, **as presented in Chapter Five and Appendix C**, may be submitted as part of the thesis submitted for the degree *Philosophiae Doctor* and preparation of a manuscript for publication.

The contributions of the co-authors for this chapter and the resulting manuscript submitted for publication were as follows: Dr. Oksana Levanets and Miss Fimmie Reinecke was responsible for developing the stable knockdown models and conducted all experiments unless otherwise stated; Dr. Lissinda du Plessis conducted the flow cytometry experiments; Dr. Leo Nijtmans was responsible for the BN-PAGE blotting and in-gel activity assays; Prof. Francois van der Westhuizen conducted the enzyme activity assays for complexes II-IV, Fimmie Reinecke was responsible for the preparation of the manuscript and Prof Jan Smeitink and Prof. Francois van der Westhuizen provided scientific supervision.



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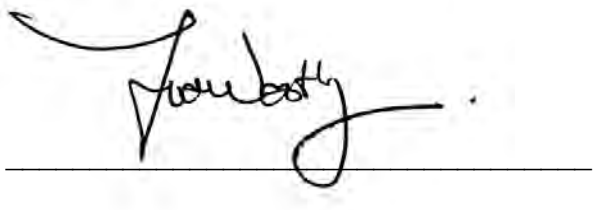

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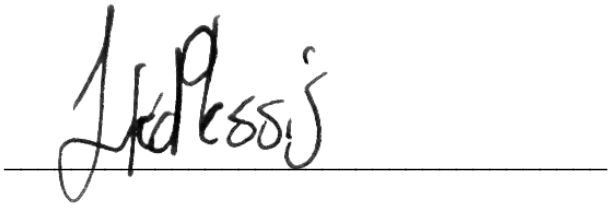

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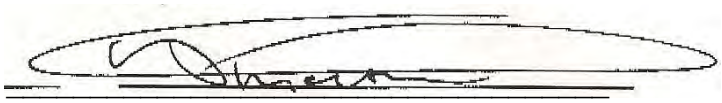

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APPENDIX D

siRNA versus shRNA: lessons learnt from mitochondrial NDUFS3 knockdown

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Short title: siRNA versus shRNA: lessons from NDUFS3 knockdown

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Subject category: Cell Biology, DNA Recombinant Techniques and Nucleic Acids

Abstract

RNA interference is one of the most powerful and commonly used methods to modulate gene expression and to investigate protein function. Here we report on unexpected limitations of this widely used approach. We compared a transient and stable RNA interference of the NDUFS3 subunit in 143B cells in the process of establishing an *in vitro* model of human mitochondrial NADH:ubiquinone oxidoreductase deficiency,. Our results show that small interfering RNA and short hairpin RNA action differs at the mRNA and protein levels in the case of NDUFS3 knockdown. We conclude that results obtained from transient knockdown do not always reflect in stable knockdown on protein level.

Keywords: siRNA, shRNA, mitochondrial diseases, NDUFS3, *in vitro* disease model.

Abbreviations used: RNAi, RNA interference; siRNA, small interfering RNA; shRNA, short hairpin RNA; miRNA, microRNA; OXPHOS, oxidative phosphorylation; PAGE, polyacrylamide gel electrophoresis; BN-PAGE, Blue Native polyacrylamide gel electrophoresis.

Mitochondrial diseases represent a wide group of disorders produced either by genetically transmitted or acquired disruption of the mitochondrial energy production function. Mitochondrial pathologies are relatively common with at least 1 in 5000 births being affected [1, 2]. It has also become apparent that mitochondrial dysfunction is involved in the development of many common cardiovascular and neurological diseases as well as in cancer, diabetes, physiological aging, and other [3]. Understanding the mechanisms of mitochondrial dysfunction is of great significance to the diagnostics and development of therapeutic approaches for this group of diseases. One of the useful tools for the investigation of disease is using *in vitro* models based on human cell lines and in particularly the development of disease models using RNA interference (RNAi) [4]. Small RNA-mediated gene silencing mimics the conditions of normal mitochondrial proteins deficiency and helps to understand the events leading to disease development. There are several different protocols for RNAi-mediated gene knockdown based on the different sources and processing mechanisms of small RNAs. Small interfering RNAs (siRNAs) [4, 5] are chemically synthesized RNA molecules used to transfect the cells. They act directly on target mRNA after minimal processing, but as they come from the exogenous source, they only act as long as they are present in the environment (cell culture medium) insuring therefore the transient target gene knockdown. However, the transient approach has the disadvantage that high concentrations of external small RNAs can be harmful to the cell. The transfection reagent to which the cells are constantly exposed, can also interfere with some analyses. To avoid such problems, short hairpin RNA- (shRNA) [6], or microRNA- (miRNA) based [7] approaches can be used. Unlike siRNAs, both shRNAs and miRNAs are expressed inside the cell. After intracellular processing, shRNAs turn to the same target molecules as siRNAs and act by the same mechanism. Both techniques have

some disadvantages: additional processing needed for shRNA, as well as the above-mentioned off-side effects of the siRNA transfection process, can affect the final results. Another problem associated with shRNA is the vector influence. Mostly retro- or lentivirus-based vectors are used for shRNA expression in the cells. These vectors integrate into cellular genome on a random basis, which can greatly affect shRNA efficiency.

As complex I (*NADH*:ubiquinone oxidoreductase), which is the first and the largest enzymatic complex of the OXPHOS system, is the most frequently encountered deficiency among mitochondrial disorders in humans [8], our primary aim was to develop an *in vitro* model for this enzyme. Human complex I consists of 45 subunits, encoded both by genomic and mitochondrial DNA. Many of its subunits are known to be affected during the development of pathologies, including NDUFS3 [9]. To knockdown the NDUFS3 subunit of complex I (Genbank® accession number NM_004551), we had used both siRNA and shRNA approaches. siRNA (5'-GUUACGUUAUGAUGAUGAAdTdT-3') was designed using siDESIGN® Center (Dharmacon, Inc., Lafayette, CO, *USA*) and synthesized by IDT®, Inc. (Coralville, IA, USA). 143B cells were cultured at 37℃ with 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco®, Carlsbad, CA, USA). Cells were transfected with 10 nM or 100 nM siRNA using Oligofectamine™ (Invitrogen, Carlsbad, CA, *USA) and* harvested for analyses in 72 hours after transfection.

The same target sequence was used to design shRNA (Clontech siRNA Hairpin Oligonucleotide Sequence Designer). Oligonucleotides were synthesized by Bioneer Corporation (Daejeon, South Korea). The Knockout Tet RNAi System P (Clontech Laboratories, Inc., Mountain View, CA, USA) was used for shRNA expression. Synthesized oligonucleotides were annealed and cloned using *Bam*H I and *Eco*R I restriction sites to RNAi-Ready pSIREN-RetroQ-TetP vector according to the manufacturer's recommendations. Resulting plasmids were sequenced and transfected into 143B cells using ExGen transfection reagent (Fermentas, Vilnius, Lithuania). For the development of stable transfected clones, cells were subjected to antibiotic selection (3 mg/ml puromycin) for about 2 weeks, and separate clones were isolated. At least 35 clones were

analysed to select those with the highest gene knockdown rate. Analyses were done using real-time time PCR to estimate gene expression at the mRNA level, western blot with antibodies against mitochondrial proteins (Mitosciences, Inc, Eugene, OR, USA) after SDS polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate the amount of target subunit at the protein level, and in-gel activity analysis after Blue Native PAGE (BN-PAGE) to evaluate the integrity and activity of complex I [10]. Untransfected 143B cells, as well as cells transfected with base pSIREN-RetroQ-TetP vector and vector expressing non-related shRNA against eGFP [11], were used as controls for all analyses.

The results of transient model analyses validated the high efficiency of the designed siRNA (Fig. 1). They demonstrated a ~85% decrease of target transcripts, ~88% reduction of protein expression (Fig. 1A) and ~60% decrease of complex I enzymatic activity (Fig. 1B). For a stable NDUFS3 model, two individual clones were obtained that demonstrated the best gene knockdown results at the mRNA level. As illustrated in Fig. 2A, the target transcript expression was decreased by ~90%, which is similar to transient knockdown results. However, unlike the transient model, at the protein level only ~50% reduction of the NDUFS3 subunit was observed. Moreover, no change of complex I assembly or activity was detected (Fig. 2B).

Despite the fact that shRNA is often concidered as siRNA, expressed intracellular, our results demonstrate that their action was not identical in the case that we investigated, and therefore the common procedure of RNA interference experiments design and target validation was not applicable. The available algorithms for RNA interference target design generate siRNA sequences, whereas shRNA is usually designed on the basis of siRNA by addition of a complementary strand and hairpin structure, as well as sequences for further RNA processing and cloning into the vector. But according to our results, siRNA's and shRNA's actions are not identical *in vitro*.

Our results indicated that, despite the high efficiency in target gene expression knockdown at the mRNA level, the shRNA approach did not translate into disruption of complex I assembly

and function as was the case for the siRNA approach. A number of aspects should be taken into consideration when interpreting these results. One of them is the target's nature. In this case we aimed to knockdown the mitochondrial OXPHOS complex I, which consists of 45 subunits, and which is also involved in the formation of larger super-complexes with other OXPHOS enzymes [12]. Little is known about the coordination of each individual subunit regulation during the synthesis and assembly of the full complex. Apparently, strong expression coordination should be present, especially when taking into account the fact that both nuclear and mitochondrial genomes are involved. According to our data, some regulatory event(s) takes place at the translation level and influences the final result. Recent literature data suggest that additional factors can be involved in OXPHOS complexes expression regulation at the translational level. It can be either direct regulation via proteins binding the corresponding mRNA and regulating its translation like in the case of cytochrome *c* oxidase subunit IV [13]. Or more complex and indirect OXPHOS proteins expression regulation can be also present. For instance, efficient complex I assembly requires the presence of additional proteins – assembly factors and chaperones, in particular, the iron-sulfur cluster assembly proteins (ISCU1/2). Recently, these proteins have been shown to be regulated by miRNA [14], thus implementing an indirect regulation of complex I assembly.

Our data demonstrate the limitations of the widely used shRNA-mediated gene knockdown technique. As shRNA acts at the post-transcriptional level only, the regulation of downstream translational and posttranslational level can significantly influence the final result. The nature of knockdown target also plays an important role in the experiment's success. siRNA is significantly more efficient probably due to high concentrations of siRNA present in the cellular environment (medium) and permanently transfecting the cells, thus implementing gene silencing. However, as mentioned above, the use of siRNA has some disadvantages and limitations. The expression and processing of shRNA, on the other hand, occurs intracellular and at physiological concentrations and its effectiveness is therefore controlled. For practical applications this control can be a disadvantage for target gene silencing, as there are more possibilities for downstream regulation of the corresponding protein expression. One of the possible solutions can be the use of miRNA instead of shRNA in such experiments, as miRNA acts directly at the translational level.

Acknowledgment

We would like to thank the National Research Foundation of South Africa as well as the Department of Science and Technology (BioPAD) for the financial support of this work.

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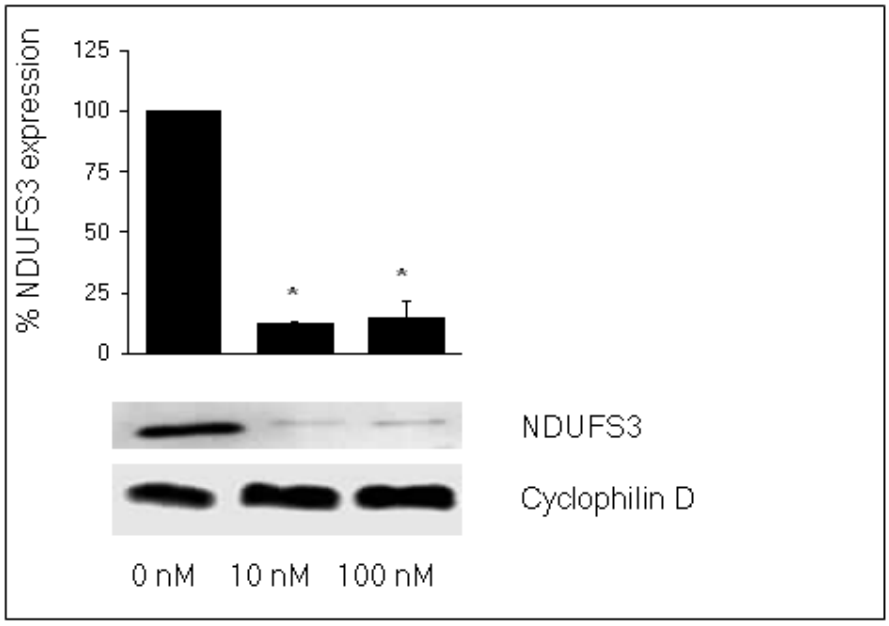

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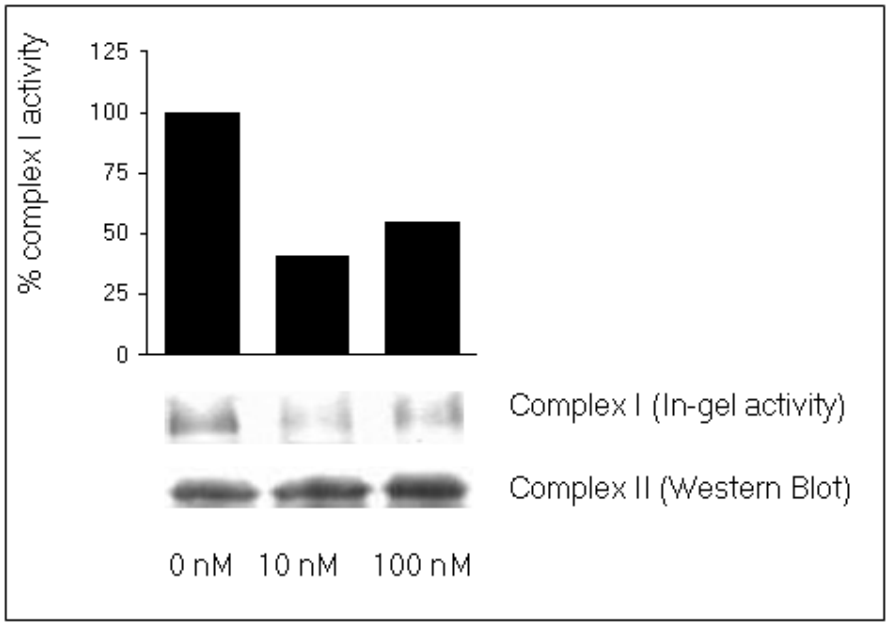
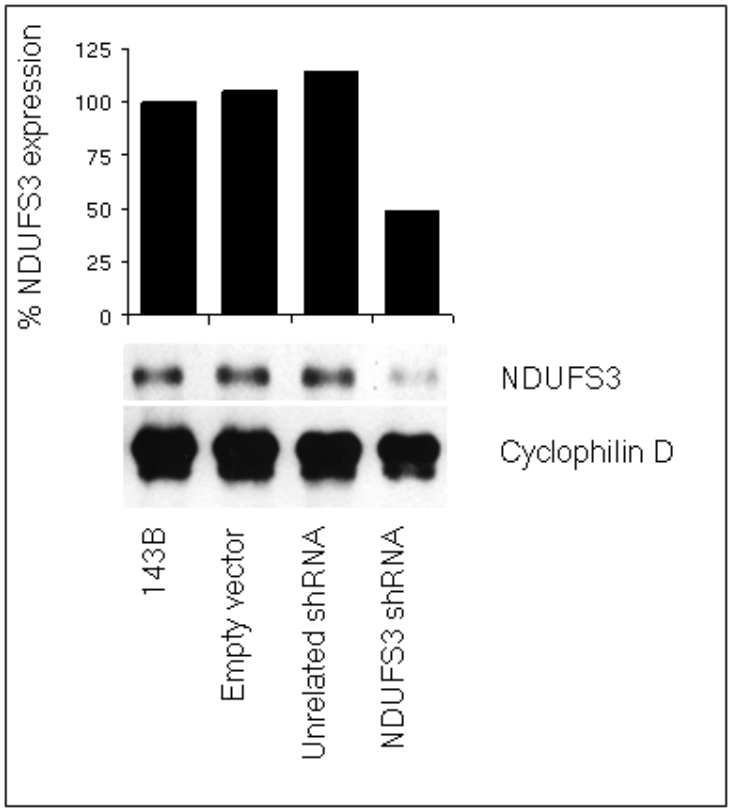


Fig. 1. NDUFS3 subunit expression in siRNA-transfected 143B cells. (A) Protein content of individual NDUFS3 subunit. Western blot of cell lysates separated with SDS-PAGE was used to quantify protein expression of NDUFS3 in 143B cells transfected with 10 and 100 nM of siRNA and untransfected (0 nM) 143B cells, using cyclophilin D as a loading marker (n=3), *p<0.05 (ANOVA, Tukey test). (B) BN-PAGE in-gel activity of complex I. In-gel activity analysis after mitochondria-enriched cell extracts separation by BN-PAGE relative to complex II protein content was used to

quantify complex I enzyme activity in 143B cells transfected with 10 and 100 nM of siRNA and untransfected (0 nM) cells. Densitometric quantification as indicated on the top was performed by GeneTools 3.06 Imaging software (GeneTools software, Syngene, Cambridge, UK).

A



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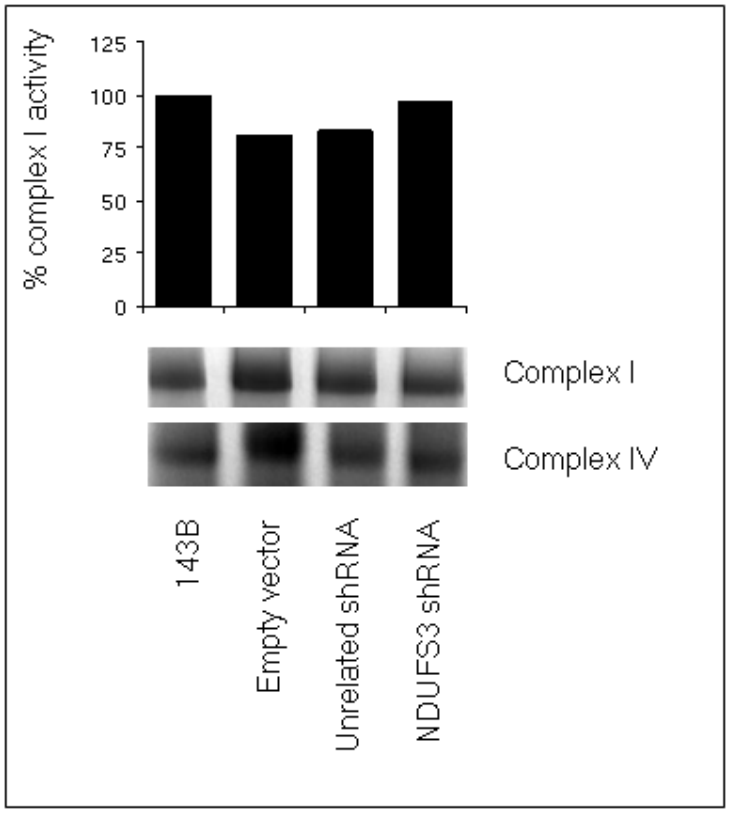


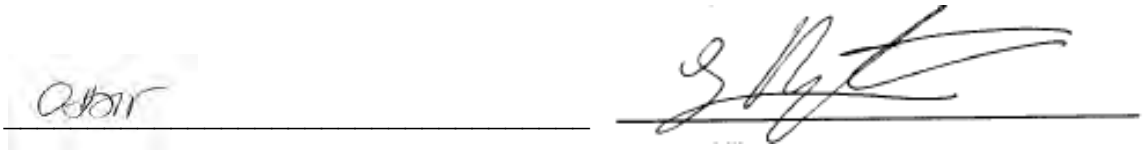
Fig. 2. NDUFS3 subunit in shRNA-transfected 143B cells shows different expression pattern. (A) NDUFS3 protein content in shRNA-expressing 143B cells clones by the means of western blot analysis of SDS-PAGE-separated mitochondria-enriched cellular extracts. Untransfected 143B cells, as well as transfected with base vector and unrelated shRNA were used as negative controls. Cyclophilin D antibody was used as a loading control for quantification. (B) In-gel activity of complex I after mitochondria-enriched extracts separation on 5-15% BN-PAGE relative to complex IV in-gel avtivity. Untransfected 143B cells as well as transfected with base vector and unrelated shRNA were used as negative controls. Densitometric quantification was performed by GeneTools 3.06 Imaging software.

Permission from co-authors

To whom it may concern,

We, the co-authors of the paper titled "**siRNA and shRNA action on mitochondrial complex I subunit NDUFS3 differs on RNA and protein level**" hereby give permission that the data originating from the experiments conducted on the *in vitro* transient and stable knockdown models of NDUFS3 subunits, as presented in Appendix D, may be submitted as part of the thesis submitted for the degree *Philosophiae Doctor* and preparation of a manuscript for publication.

The contributions of the co-authors for this chapter and the resulting manuscript submitted for publication were as follows: Dr. Oksana Levanets and Miss Fimmie Reinecke was responsible for developing the knockdown models and conducted all experiments unless otherwise stated; Dr. Leo Nijtmans was responsible for the BN-PAGE blotting and in-gel activity assays; Prof. Francois van der Westhuizen conducted the enzyme activity assays for complexes II-IV, Dr Levanets was responsible for the preparation of the manuscript and Prof Jan Smeitink and Prof. Francois van der Westhuizen provided scientific supervision.



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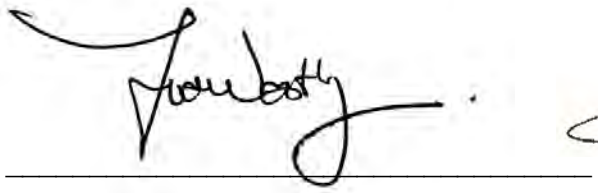

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APPENDIX E

National and International conference proceedings

F.Reinecke, O. Levanets, R. Louw, B. Semete, Y. Olivier, A. Olckers, and F.H. van der Westhuizen. Functional properties of Metallothionein overexpression in rotenoneinduced NADH:ubiquinone oxidoreductase-deficient HeLa cells. 19th South African Society for Biochemisrty and Molecular Biology (SASBMB) Conference, Stellenbosch, 16-20 January, 2005. Poster presentation.

Y Olivier, F Reinecke, O Levanets, T Semete, R Louw, A Olckers and FH van der Westhuizen. Identifying isoform- specific Metallothionein expression in Rotenone induced NADH:ubiquinone oxidoreductase deficiency in HeLa cells. 19th South African Society for Biochemisrty and Molecular Biology (SASBMB) Conference, Stellenbosch, 16-20 January, 2005. Poster presentation.

J Pretorius, M Alessandrini, F Reinecke, R Louw, A Olckers and FH van der Westhuizen. Analysis of Metallothionein expression levels in rotenone-induced mitochondrial NADH:ubiquinone oxidoreductase deficient rat tissue. 9th IUBMB Conference, Budapest, Hungary, 5-9 July 2005. FEBS Journal 272 (s1), N5-022P. Poster.

F Reinecke, O Levanets, R Louw, B Semete, Y Olivier, A Olckers and FH van der Westhuizen. Functional properties of metallothionein overexpression in rotenone-induced NADH:ubiquinone oxidoreductase deficient HeLa cells. 9th IUBMB Conference, Budapest, Hungary, 5-9 July 2005. FEBS Journal 272 (s1), N5-023P. Poster.

F Reinecke, R Louw, LH du Plessis, A Grobler, L Nijtmans, JAM Smeitink. FH van der Westhuizen. Introducing siRNA knockdown of the NDUFS3 subunit of NADH:ubiquinone oxidoreductase: evaluation of mitochondrial DNA replication and transcription. 21st South African Society for Biochemisrty and Molecular Biology (SASBMB) Conference, Grahamstown, 23-26 January, 2008. Oral & poster presentation.