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The mitochondrial function:

a crossroad for the genetic and epigenetic regulation

of aging and age-related phenomena

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Sommario

La qualità e la velocità dell'invecchiamento umano dipendono dalla complessa interazione tra fattori genetici, epigenetici e ambientali. In questo contesto, numerose evidenze hanno dimostrato come un ruolo centrale sia giocato dai mitocondri e dalla variabilità del DNA mitocondriale (mtDNA). Essi hanno, infatti, un ruolo cruciale nel mantenimento dell'omeostasi energetica e redox, nella regolazione dei processi epigenetici e nell' influenzare i meccanismi di risposta allo stress, elementi chiave per raggiungere la longevità.

Il presente lavoro fornisce una panoramica delle suddette assunzioni riportando, in quattro sezioni i risultati di studi condotti *in vivo* e *in vitro*.

Nelle prime due sezioni sono riportate una serie di evidenze sperimentali che dimostrano la correlazione tra il declino fisiologico tipico dell'età avanzato, le varianti ereditarie del DNA mitocondriale ed i livelli di metilazione globale del DNA. I dati ottenuti hanno dimostrato come i cambiamenti dei livelli di metilazione del DNA, la modificazione epigenetica più ampiamente caratterizzata, sono associati al declino funzionale di individui e correlano alla cambiamenti fisiologici che si verificano nell'uomo nel corso della vita. È emerso inoltre che il rimodellamento nello stato di metilazione del globale DNA durante l'invecchiamento è influenzato dalle varianti ereditarie del DNA mitocondriale, presumibilmente attraverso la regolazione differenziale della funzionalità della OXPHOS. Tali risultati hanno dunque dimostrato come processi epigenetici sono modulati da segnali bidirezionali tra genoma mitocondriale e nucleare, strettamente regolati dalla variabilità dell'mtDNA.

Le ultime due sezioni presentano i dati ottenuti da uno studio volto ad esplorare il ruolo della variabilità ereditaria del DNA mitocondriale nella regolazione dei profili di espressione di due classi di geni nucleari coinvolti nella risposta cellulare allo stress:

sirtuine e Heat Shock Proteins (HSP). Tale studio è stato ispirato da dati di letteratura riportanti come l'efficienza della risposta cellulare allo stress, elemento chiave per il raggiungimento della longevità, è fortemente influenzata da segnali bi-direzionali tra il DNA mitocondriale e nucleo. In tali lavori, ci si è avvalsi della *tecnologia dei cibridi* per dimostrare che tali pathway di comunicazione, in condizioni di stress ossidativo e termico, sono stati in grado di influenzare, rispettivamente, l'espressione dei geni codificanti per le Sirtuine e le HSP, le prime sensori dell'omeostasi energetica e redox, le seconde regolatori dei processi di folding e migrazione proteica nonché dell'apoptosi. Dall'analisi dei profili di espressione delle sette sirtuine è emerso che il gene *SIRT3* ha mostrato downregolazione in condizioni di stress ossidativo correlata alla sequenza del DNA mitocondriale alla compromissione della funzionalità mitocondriale stessa. Per quanto riguarda lo studio di espressione dei geni codificati per le HSP, è emerso come sia HSP60, sia HSP75 sono diversamente modulati in condizioni di shock stress da calore in base alla variabilità del mtDNA. Tali risultati suggeriscono che la correlazione tra la variabilità del DNA mitocondriale e livelli di espressione di geni nucleari coinvolti nella risposta allo stress può essere considerato come un fenomeno generale.

Summary

The rate and quality of human aging depend on a complex interplay among genetic, epigenetic and environmental factors. In this scenario mitochondria and mitochondrial DNA (mtDNA) variability are emerging as major players. This is basically due to their crucial role in maintaining energetic and redox homeostasis, in modulating the intracellular epigenetic program and in influencing cell stress response mechanisms, which are key elements to achieve longevity.

The present work provides an overview of the above assumptions, reporting, in four sections, a series of *in vivo* and *in vitro* investigations. In the first two sections experimental evidences about a correlation among the age-specific functional decline, the mitochondrial DNA inherited variants and the global DNA methylation levels are reported. The data we show demonstrate that the remodeling of DNA methylation levels, that represent the best characterized epigenetic modification, is associated to the functional decline of aged individuals and correlates to their physiological changes occurring over the lifetime. We also demonstrate that the global DNA methylation remodeling during aging is influenced by mitochondrial DNA inherited variants, probably via the different regulation of OXPHOS machinery. So that, we prove as epigenetics processes are modulated in response to mtDNA-specific cross signaling between mitochondrial and nuclear genome.

The last two sections present data obtained in the studies on the role of mtDNA inherited variability in modulating the expression profiles of two classes of stress responder nuclear genes: Sirtuins and HSPs. These sections were inspired by evidences reporting that the efficiency of cellular stress response, a key element for attending longevity, is highly regulated at both nuclear and mitochondrial level through activating bi-directional signaling pathways between mitochondrial and nuclear DNA. In these

works we availed of cybrid technology to demonstrate that the above pathways, in oxidative and heat shock stress conditions, were able to influence the expression of Sirtuin and HSP genes, respectively; the former are regulators of energetic and redox homeostasis, the latter are regulators of protein folding and migration as well as apoptosis in both stressed and non-stressed cells. As for sirtuin genes, we found that only *SIRT3* gene was down-expressed depending on the mtDNA sequence in oxidative stress condition and that this down-expression was correlated to the impairment of mitochondrial function. As for HSPs, either *HSP60* or *HSP75* were differently modulated according to mtDNA variability, in heat shock stress conditions. Thus, the consistency of these results suggest that the correlation between mtDNA variability and expression levels of stress-responder nuclear genes is a general phenomenon.

List of abbreviations

5-MCDG	5-methylcytosine DNA glycosylase
8-OH-dG	8-Hydroxyl-2'-deoxyguanosine
AceCoA	Acetylcoenzyme A
AceCS2	Acetylcoenzyme A synthase 2
AD	Alzheimer's disease
ADL	Activities of daily living
AKAP12	A kinase (PRKA) anchor protein 12
ANT	Adenine nucleotide translocaton
AP-1	Activator Protein-1
AP-2	Activator Protein-2
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
ATP(6-8)	ATP synthetase F0 subunit (6-8)
BER	Base excision repair
BMD	Bone mineral density
BMI	Body mass index
BRCA1	Breast Cancer 1
C/EBPβ	CCAAT/enhancer binding protein beta
CAMKIV	calcium/calmodulin-dependent protein kinase IV
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
CH_3	Methyl group
CHOP	C/EBP Homology Protein

ClpP	ClpP caseinolytic peptidase, ATP-dependent, proteolytic subunit homolog
c-Myc/Myn	myelocytomatosis viral oncogene
CO(I-III)	Cytochrome c Oxidase subunit (I-III)
CoQ	Coenzyme Q
CR	Caloric restriction
CREB	cAMP response element-binding
CRP	C-reactive protein
CVD	Cardiovascular disease
Cytb	Cytochrome b
DAPK	Death-associated protein kinase
DHEA-S	Adrenal androgen dehydroepiandrosterone-sulfate
D-Loop	Displacement-loop
d^mC	Deoxymethylcytosine
DMEM	Dulbecco's modified eagle medium
DNMT1	Maintenance DNA methyltransferase
DNMT3(a-b)	De novo methyltransferase (a-b)
DNMTs	DNA Methyltransferases
DRD2	Dopamine receptor D2
E2F	E2F transcription factor
eNOS	Endothelial nitric oxide synthase
EORS	Epigenetic oxidative redox shift
ERCs	Extrachromosomal ribosomal DNA circles
ETC	Electron transport chain
FBS	Fetal bovine serum

FMR1	Fragile X mental retardation 1
FOXO	Forkhead box-containing protein type O subfamily
GAD1	Glutamate decarboxylase 1
GDH	Glutamate dehydrogenase
GDS	Geriatric depression scale
GH	Growth hormone
GPx	Glutathione peroxidase
GSH	Glutathione
GSTP1	Glutathione S-transferase P1
H	Heavy strand
H_2O_2	Hydrogen peroxide
HDAC1	Histone deacetylase 1
HDL	High-density lipoprotein
HO	Heme oxygenase
HOXB5	omeobox protein Hox-B5
HSP10	Heat shock protein 10
HSP60	Heat shock protein 60
HSPs	Heat Shock Proteins
HVR(I-II)	Hypervariable region (I-II)
ICDH2	Isocitrate dehydrogenase 2
ICR	Imprinted control region
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IL-6	Interleukin-6
IMM	Inner mitochondrial membrane

JNK	c-Jun N-terminal kinase
Kaiso	transcriptional regulator Kaiso
L	Light strand
LHON	Leber's hereditary optic neuropathy
MAPK	Mitogen-activated protein kinase
MAT	Methionine adenosiltransferase
MBD	Methyl binding domain
MBD(1-3)	Methylcytosine binding protein (1-3)
MBD2b	Methyl-CpG-binding domain protein 2
MBD4	Methyl-CpG-binding domain protein 4
MBPs	Methylcytosine binding proteins
MeCP2	methyl CpG binding protein 2
MEF2	myocyte enhancer factor-2
MEFs	Mouse embryonic fibroblasts
MLH1	MutL protein homolog 1
MLSP	Maximum lifespan
MMSE	Mini mental state examination
MPO	Myeloperoxidase
MPT	Mitochondrial permeability transition
mtDNA	Mitochondrial DNA
mtTFA	Mitochondrial transcription factor A
mtTFB	Mitochondrial Transcription Factor B
MZ	Monozygotic
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

ND(1-6; 4L)	NADH dehydrogenase subunit (1-6; 4L)
NFAT	nuclear factor of activated T-cells
NF-KB	Nuclear factor-kappa B
NO	Nitric oxide
NRF1-2	Nuclear Respiratory Factor (1-2)
O2	Molecular oxygen
O2-	Superoxide anion
OH	H-strand replication Origin
OH·	Hydroxyl radical
OL	L-strand replication Origin
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative Phosphorylation
p53	tumor protein p53
PDAC	Protein deacetylase
PGC-1(α-β)	Peroxisome proliferator-activated receptor co-activator 1 (α-β)
PGC-1α	PPARγ coactivator 1α
PH	H-strand transcription Origin
Pi	Inorganic phosphate
PKC	Protein kinase C
PL	L-strand transcription Origin
PLAU	Urokinase type plasminogen activator
PolgA	mtDNA polymerase subunit A
PPARγ	Peroxisome proliferator activated receptor γ
PPIEL	Peptidiylprolyl isomerase E-like
PRC	peroxisome proliferator-activated receptor gamma, coactivator-

	related 1
RASSF1	Ras association domain-containing protein 1
Rb	Retinoblastoma associated protein
RELN	Reelin
RNAi	RNA interference
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
Rtgs	Retrograde proteins
S100A4	S100 calcium binding protein A4
SAM	S-adenosyl-L-methionine
Sir2	Silent informator regulator 2
SIRT1-7	Sirtuin 1-7
SOD	Superoxide dismutase
Sp-1	Sp1 transcription factor
SRHS	Self-reported health status
STK11	Serine/threonine kinase 11
Tfam	Mitochondrial transcription factor A
THO	Tyrosine hydroxylase
THO	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor ?
TRD	Transcriptional repressory domain
tRNA	Transfer RNA
tRNAAsp	Aspartic acid transfer RNA
UCPs	Uncoupling proteins

UPR	Unfolded Protein Response
USF	Upstream stimulatory factor
VADC	Voltage dependent anion conductance
VHL	Von Hippel-Lindau tumor suppressor
XCI	X chromosome inactivation

1. Introduction

Over the past two decades, a growing interest in the research of the biological basis of human longevity has emerged, in order to clarify the biological and the environmental factors affecting the quality and the rate of human aging. In this scenario, many studies have focused on the involvement of mitochondrial function (and dysfunction) in aging. In fact, evidences are accumulating that these organelles are able to modulate numerous intracellular signaling pathways which appear to be critically important for the maintenance of cellular homeostasis.

In the frame of the research on aging and longevity, an emerging field that promises exciting findings is represented by the epigenetic changes affecting DNA during the lifetime. Also in this field of research, a complex interplay between mitochondrial function and epigenetic modifications in aging has been observed, although only a few aspects have been so far elucidated.

1.1 Mitochondria and mitochondrial DNA

Mitochondria are intracellular organelles located in the cytoplasm of eukaryotic cells. As it regards the structure, mitochondria are bounded by a double membrane. The outermost mitochondrial membrane (OMM) is smooth while the inner mitochondrial membrane (IMM) has many folds called cristae, that enhance the "productivity" of cellular respiration by increasing the available surface area.

Mitochondria are emerging to be involved in several cellular processes, including ion homeostasis, cell proliferation and differentiation, but their primary function is the generation of energy in form of ATP, via the electron transport chain (ETC) and the oxidative phosphorylation (OXPHOS) system located within the inner membrane

(IMM) of the organelle. The OXPHOS machinery is composed by five multiprotein complexes each composed by subunits encoded by both nuclear and mitochondrial genome, plus two molecules, ubiquionone (Coenzyme Q) and cytochrome c, that acts as diffusible electron carriers (Fig. 1).

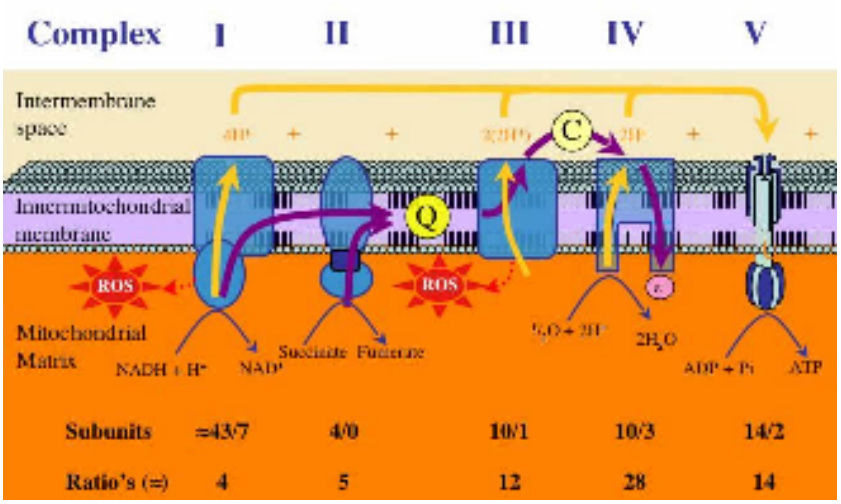

Fig. 1: Electron Transport Chain (ETC). *The reducing equivalents in NADH or $FADH_2$ enter in the electron transport chain through the complex I (NADH dehydrogenase) and Complex II (Succinate dehydrogenase) respectively. While electron are then transferred from NADH to coenzyme Q (CoQ) and to Complex III (Ubiquinine-cytochrome c reduttase), and the cytochrome c to Complex IV (Cytochrome c oxidase), protons are translocated from matrix to the intermembrane space. The electrochemical grandient established across the IMM represents the driving force of ATP synthesis catalized by the Complex V (ATP syntase).*

Every cell contains a variable number of mitochondria, and each mitochondrion harbors 2-10 copies of their own genome, the mitochondrial DNA (mtDNA) (Anderson et al. 1981).

The human mitochondrial DNA is a 16569 bp closed-circular double-stranded molecule, containing 37 genes encoding for 2 ribosomal RNAs (rRNA 12S and 16S), 22 transfer RNAs and 13 subunits of the OXPHOS system (Fig. 2).

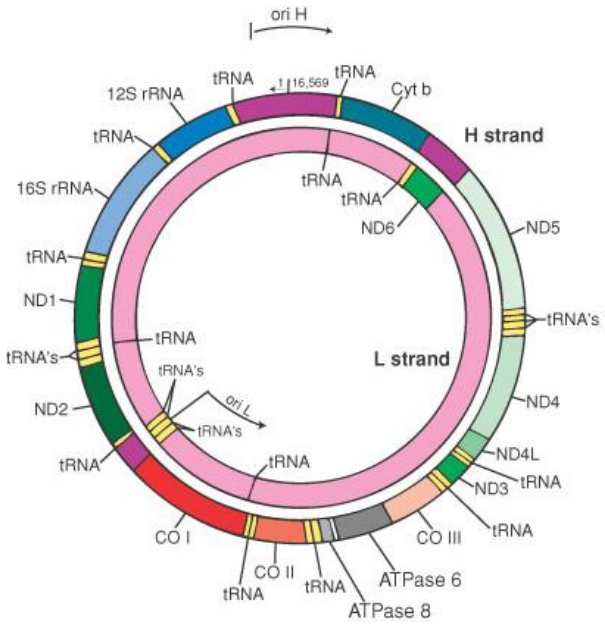

Fig. 2: Human mitochondrial DNA. *mtDNA has two strands, a guanine-rich heavy strand (H) and a cytosine-rich light strand (L) carrying 28 and 9 genes, respectively. 24 of these genes encode for the translational machinery of the mtDNA, including 22 tRNAs and 2 rRNAs. The remaining 13 genes encode for subunits of OXPHOS system, including seven subunits of Complex I (ND1-6 and ND4L), one subunit of Complex III (Cytb), three subunits of Complex IV (COI-III) and two subunits of Complex V (ATP6-8).*

The structure of mtDNA is very compact; in fact, mitochondrial genes have no introns, intergenic sequences are absent or limited to a few bases, some genes overlap and termination codons are generated post-transcriptionally from polycistronic transcripts. The sole non coding region, the Displacement-loop (D-Loop), is a region of 1121 bp containing two hypervariable regions, HVRI (nt 16024-16383) and HVRII (nt 57-372), the origin of replication of H-strand (OH), the promoter region for H (PH) and L-strand (PL) transcription and regulatory elements for both mitochondrial replication and transcription.

MtDNA is inherited maternally, so that the paternal lineage does not contribute mtDNA to the offspring, and it is not subject to significant recombination (Elson et al. 2001).

Therefore, in the course of evolution, inherited mutations have accumulated sequentially along mtDNA independent lineages. This inherited mtDNA variability has been extensively studied in human population genetics, representing an extraordinarily informative tool for the knowledge of human population history (Torroni and Wallace, 1994; Torroni et al. 1994). Indeed, groups of ancestral-associated polymorphisms (haplogroups) and cluster of these groups (haplogroup clusters) have provided additional insights about the origin and relationships of populations and the process of human colonization of continents, and have been used to define branches of the human phylogenetic tree for mtDNA (Torroni et al. 1996). The African haplogroup cluster L is the most ancient of all clusters, from which, approximately 65,000 years ago, have diverged two lineages (M and N) originating Asiatic and European haplogroups (Rose et al. 2002). As to European population, Torroni and coworkers (1996) found that about the 95% of the mtDNAs fall within nine different mtDNA haplogroup; of these, H is the most common once, followed by J, T, U, I, X, K, W and V.

The mtDNA inherited variants are likely to be non-neutral. In particular, several data have demonstrated that the different mtDNA lineages are qualitatively different from each other. In this field it is important to mention the study of Torroni and coworkers (1997) demonstrating that the penetrance of two primary mutations (11778 and 14484), which cause the Leber's Hereditary Optic Neuropathy (LHON), is increased if they occurr on mtDNA molecules belonging to J haplogroup. In addition, a significant association has been found between common mtDNA polymorphisms and age-related pathologies, such as Parkinson and Alzheimer diseases (Ghezzi et al. 2005; Khusnutdinova et al. 2008; Maruszak et al. 2009; Takasaki, 2009).

Moreover, several studies have associated specific inherited variants of mtDNA with human longevity. For example, haplogroup J is over-represented in northern Italians,

Irish and Finnish centenarians (De Benedictis et al. 1999; Ross et al. 2001; Niemi et al. 2003). By contrast, the J haplogroup was underrepresented in southern Italians and in Chinese Uygur long-living people, supporting the idea that the effect of mtDNA inherited variants on longevity is population- and sex-specific, probably according to individual-specific nuclear genetic backgrounds and stochastic events (Dato et al. 2004; Ren et al. 2008).

Besides to this inter-individual variability, also an intra-individual mtDNA variability exists. Due to the multiple copy nature of mtDNA within a cell, somatic mutations in mtDNA molecules may coexist with the wild-type mtDNA, in a condition termed "heteroplasmy".

1.1.1 Nucleus-mitochondria cross-talk

Although mitochondria contain their own genome, the vast majority of the mitochondrial proteins, including the entire complement of proteins involved in mtDNA replication and transcription, structural and transport proteins of mitochondrial membranes, the mitochondrial peptide involved in mitochondrial metabolism and TCA cycle, as well as most of the peptide subunits of the respiratory complexes (the protein-coding capacity of mtDNA is limited to 13 respiratory subunits) are encoded by nuclear DNA. Moreover, mitochondria are seat of multiple metabolic pathways, including β-oxidation of fatty acids and tricarboxylic acid and urea cycles, control intracellular Ca_2^+ metabolism and signaling, regulate thermogenesis and settle the cell fate by integrating numerous death signals. This implies that a sticky coordinated expression of two genomes occur to ensure the biosynthesis and the functional activity of mitochondria, in both physiological and pathological conditions (Garesse and Vallejo, 2001). Thus, a

complex interplay of bidirectional signaling linking nucleus and mitochondria emerges also by the crucial role played by mitochondria in cell physiology.

Signals from nucleus to mitochondrion are essential for maintaining an adequate mitochondrial structure and function. In fact, several nuclear-encoded transcription factors and co-activators have been identified as modulators of the mitochondrial replication and transcription. Two distinct classes of regulatory proteins regulate nucleus-mitochondria interactions at the transcriptional level. The first class comprises transcription factors that bind the promoter regions of both nuclear and mitochondrial genes. In particular, it is well known that the mitochondrial Transcription Factor A (Tfam) and the mitochondrial Transcription Factor B (mtTFB), isoforms TFB1M and TFB2M, work in conjunction with the mitochondrial RNA polymerase to confer promoter specificity and to enhance the rate of transcription initiation of mtDNA genes (Bonawitz et al. 2006; Scarpulla, 2006). Additionally, the second group of transcription factors, including the Nuclear Respiratory Factors NRF-1 and NRF-2 and the PGC-1 family coactivators (PGC-1alpha, PGC-1beta, and PRC), acts predominantly on nuclear genes whose products are required for respiratory chain expression and biological function (Fig. 3).

These findings indicated that nuclear signals are able to modulate the expression of both nuclear and mitochondrial genes in a coordinate way. However, mitochondrial activity also depends on a flow of information from mitochondria to nucleus. In fact, mitochondria are able to signal to the nucleus in response to metabolic disorders or damages that occur in mitochondria.

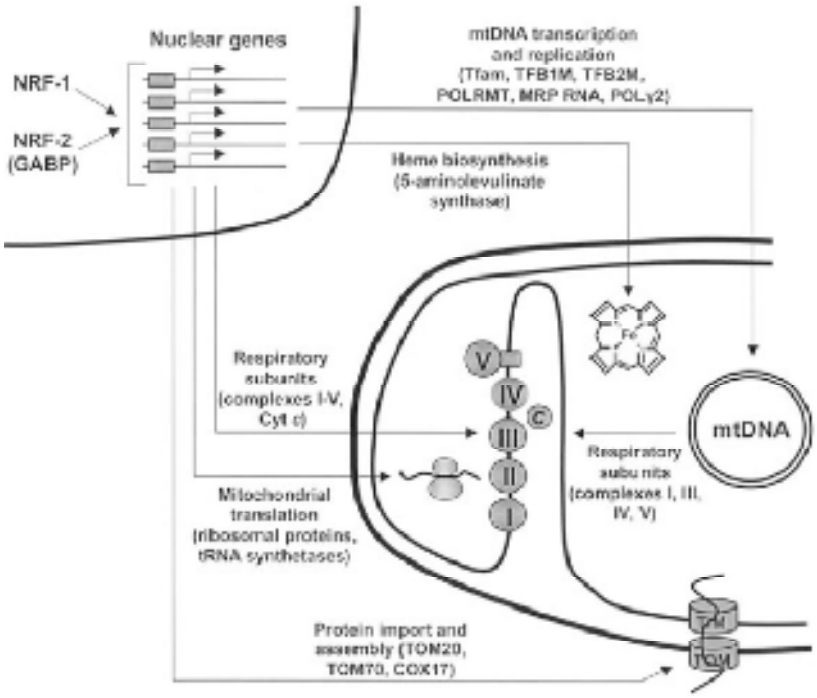

Fig. 3: Nuclear respiratory factors (NRF-1 and NRF-2) in the expression of nuclear genes governing mitochondrial respiratory function. *NRFs act on the majority of nuclear genes encoding for the subunits of the five OXPHOS complexes and on many other genes whose products direct the expression and assembly of the respiratory apparatus.*

This signaling pathway, widely known as retrograde response (also referred to as mitochondrial stress signaling), is broadly defined as cellular response to changes in the functional state of mitochondria and results in wide-ranging changes in nuclear gene expression.

Most of the available data on the retrograde response mechanisms and function have been obtained by analyzing the consequences of mitochondrial dysfunctions in the budding yeast, *Saccharomyces cerevisiae* (Parikh et al. 1987). In particular, the decline of mitochondrial membrane potential, typical of yeast cells lacking of mtDNA (Rho^0 petite), upregulates the Rtg pathway that, in turn, increases the expression level of several genes involved in biogenesis and function of mitochondria, including those of the Tricarboxylic Acid Cycle (TCA), the mitochondrial protein import and the

OXPHOS apparatus (Sekito et al. 2000; Traven et al. 2001). Moreover, it has been demonstrated that retrograde signaling is an important determinant of the yeast life span extension: the age-related accumulation of Extrachromosomal Ribosomal DNA Circles (ERCs) and their deleterious effects are mitigated by the retrograde response, with consequent increase of the lifespan (Jazwinski et al. 2005).

The mitochondrial retrograde response has been also documented in mammalian cells. Altered nuclear gene expression in response to mitochondrial dysfunctions has been extensively observed in different Rho^0 cell lines (Marusich et al. 1997; Wang and Morais, 1997; Biswas et al. 1999).

Literature data have suggested that several forms of mitochondrial stress are able to activate different mitochondrial stress signaling (Fig. 4).

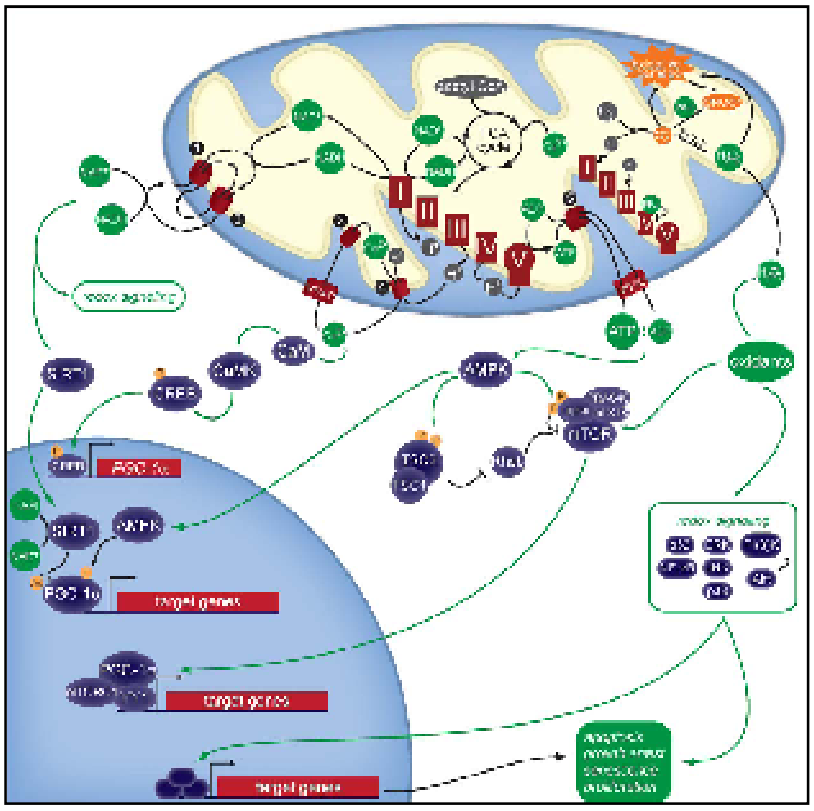

Fig. 4: Mammalian mitochondrial retrograde signaling pathways.

For example, the accumulation of mutant or unfolded protein in the mitochondrial matrix induces the activation of a mitochondrial Unfolded Protein Response (UPR), that results in the activation of mitochondrial chaperone and protease genes, including HSP60, HSP10 and ClpP, by the transcription factors CHOP and C/EBPβ thus reestablishing the normal cellular function (Zhao et al. 2002; Ryan and Hoogenraad, 2007). Experimental data suggest that the mitochondrial UPR is a two-stage regulatory process. First, the sensing of unfolded proteins in mitochondria leads to retrograde signaling to the nucleus and subsequent activation of CHOP gene. Second, CHOP, in conjunction to C/EBPβ, binds to target promoters and activates the transcription of mitochondrial-responsive genes.

Moreover, the mitochondrial release of molecules and/or metabolites, such as Ca^{2+}, nitric oxide (NO), ROS and NAD+/NADH are able to activate several mitochondrial signaling pathways in response to a number of other types of mitochondrial stress such us alteration in energy production or structural damage.

Mitochondrial structural anomalies or disruption of OXPHOS machinery generally induce a drop in mitochondrial membrane potential ($\Delta\Psi m$), that disrupts mitochondrial Ca^{2+} homeostasis and produce a rise in cytosolic free Ca^{2+}. The latter event, in turn, activates calcineurin, several Ca^{2+}-dependent kinases (PKC, JNK, MAPK, CAMKIV) and a wide spectrum of transcription factors, including NFAT, MEF2 and NF-KB, to induce transcriptional up-regulation and thus produce an appropriate cellular stress response (Butow and Avadhani, 2004; Mellström et al. 2008; Finley and Haigis, 2009).

The mitochondrial loss of redox homeostasis and the resulting oxidative stress can also trigger signaling pathways. In this context, nitric oxide (NO) plays multiple effects. NO (or NO donors) have been found to increase the concentration of mitochondrial proteins and mtDNA in some cells in culture (Nisoli, 2003). NO stimulation of mitochondrial

biogenesis was apparently due to the cGMP upregulation of several transcription factors, including PGC-1 alpha (peroxisome proliferator-activated receptor gamma coactivator-1 alpha), NRF-1 (nuclear respiratory factor-1) and mtTFA (mitochondrial transcription factor A) (Nisoli et al. 2003). The role of NO in mitochondrial biogenesis has been also supported by several studies on model organisms. In particular, endothelial NO synthase (eNOS) knockout mice present lower mitochondrial levels in many tissues indicating that NO from eNOS physiologically regulates mitochondrial density in tissues (Nisoli et al. 2003). Moreover, calorie-restricted mice have increased eNOS expression and increased mitochondrial density and oxidative phosphorylation, and this increase was prevented in eNOS knockout mice (Nisoli et al. 2005). At moderate levels NO can also increase O_2^- and H_2O_2 production by inhibiting mitochondrial respiration, while at higher levels it inhibits H_2O_2 production by scavenging the precursor superoxide, resulting in peroxynitrite production (Brown et al. 2007). In this context it has been also reported that NO exerts a protective role against ROS and Reactive Nitrogen Species (RNS) damage by increasing the expression and the activity of the antioxidant protein Heme Oxygenase (HO) in several cell lines (Motterlini et al. 2002). It is important to note that the HO enzyme belongs to the family of Heat Shock Proteins (HSPs), which exerts a protective role in a wide variety of unfavorable conditions, including stress response (Calabrese et al. 2000).

Moreover, several transcription factors (AP-1, Sp-1, NF-KB) are redox sensitive, and the oxidation of conserved residues of these proteins by oxidants is able to induce changes in their transcriptional activity (Finley and Haigis, 2009).

Besides, based on the magnitude of the oxidative stress, pro-survival or pro-death pathways can be triggered directly by mitochondria (Finkel and Holbrook, 2000). In fact, when the stress response in not enough to counteract the intracellular impairment

principally due to a severe state of oxidative stress, mitochondria may directly influence cell viability and trigger both apoptotic and necrotic cell death (Gogvadze and Orrenius, 2006). In particular, a combination of increased mitochondrial Ca_2^+, intracellular oxidative stress, ATP depletion, high inorganic phosphate (P_i) and mitochondrial depolarization act synergistically to induce irreversibly the Mitochondrial Permeability Transition (MPT), a large conductance channel formed through a conformational change of several constituent proteins of mitochondrial membrane, including the Adenine Nucleotide Translocaton (ANT) in the IMM, the voltage dependent anion conductance (VADC) in the OMM and cyclophilin D in the mitochondrial matrix (Crompton, 2000). This process on the one hand could causes mitochondrial swelling, cytocrome c release, caspase activation and apoptotic cell death or, on the other hand, induces the collapse of the mitochondrial potential, ATP consumption and depletion and energetic collapse followed by necrotic cell death (Caroppi et al. 2009; Kitsis and Molkentin, 2010).

Additionally, the NAD^+/NADH ratio, strictly connected to mitochondrial metabolism, can change the redox status and plays a critical role in various cellular functions, including regulation of calcium homeostasis and gene expression as well as in cell death by regulating numerous stress responders NAD^+/NADH-dependent enzymes, such as dehydrogenases, poly(ADP-ribose) polymerases, Sir2 family proteins (sirtuins), mono(ADP-ribosyl)transferases, and ADP-ribosyl cyclases (Ying, 2006).

On the basis of the above consideration, the signaling network implemented by mitochondria brought out the idea that they can be considered as receiver/integrator organelles that play a pivotal role in cellular stress response and in determining the cell fate (Goldenthal and Garcia, 2004).

The mitochondria-nucleus signaling has been strictly correlated with mitochondrial and nuclear DNA variability in several age-related complex phenotypes (Gaweda-Walerych et al. 2008; Maruszak et al. 2009).

Several data have demonstrated that epistatic interactions with nuclear genetic background are a significant component of the mitochondrial genetics of aging. This epistasis may explain why some mtDNA mutations have very different phenotypic effects in different individuals, possibly obscuring the mtDNA effects in human aging and disease. A few examples of this epistatic interaction in humans are reported. In particular, De Benedictis and coworkers (2000), by analyzing the distribution of the mtDNA inherited variants by Tyrosine Hydroxylase (THO) genotypes in sample groups of increasing ages, observed a non-random association between the mtDNA and nuclear DNA variability in centenarians, and an over-representation of the U haplogroup in centenarians carrying a THO genotype unfavorable to longevity. Moreover, the penetrance of the maternally inherited deafness, associated with the A1555G mutation in the mitochondrial 12S ribosomal RNA (rRNA) gene, and of the Leber Hereditary Optic Neuropathy (LHON), a disease caused by missense mutations in the mitochondrial DNA (mtDNA), requires additional environmental or genetic changes for phenotypic expression so that the mitochondrial mutation appears to depend on additive effects of several nuclear genes (Bykhovskaya et al. 2000; Shankar et al. 2008). In the last case, linkage analysis in a large family harboring a homoplasmic G11778A mtDNA mutation on a haplogroup J background identified a novel LHON susceptibility locus on chromosome Xq25-27.2 (Shankar et al. 2008). These findings support the hypothesis that some human aging traits and diseases imply particular interactions between mtDNA and nuclear DNA.

Besides to the population data, *in vitro* models have been developed to understand how the inherited mtDNA variation can modulate cellular functionality. The best known of these models is represented by cytoplasmic hybrids also known as cybrids. Cybrid cell lines, first described by King and Attardi (1989), are engineered cells that share the same nuclear genome but have different mitochondrial genome. The preparation of cybrids starts from the creation of mtDNA-null cells (Rho^0 cells) obtained by completely depleting cells of their own mitochondria though a long-term exposure to low concentration of Ethidium Bromide (EtBr). This compound inhibits mtDNA replication and transcription, without inducing any detectable effect on nuclear DNA division (King and Attardi, 1996). Rho^0 cells are then repopulated with exogenous mitochondria derived from enucleated cells (often platelets) harboring particular type of mtDNA molecules. In this way it is possible to obtain different strain of cybrids with the same nuclear genome, that comes from the parental Rho^0 cell and mtDNA of different sequence. By using this methodological approach it is possible clarify the influences that mtDNA variability has on the important cellular processes involving the mitochondrion-nucleus cross-talk. In particular, in cybrids with different mitochondrial genomes, the cell viability, the intracellular calcium dynamics, the mtDNA copy number, the mitochondrial reactive oxygen species (ROS) production, and the expression levels of several nuclear-encoded genes, including some cytokines, HSP60 and HSP75, have been demonstrated to be dependent by the interaction between nuclear and mitochondrial variability (Vives-Bauza 2006; Bellizzi et al. 2006, 2009; Kazuno et al. 2008; Suissa et al. 2009; Smits et al. 2010).

1.1.2 Mitochondrial role in aging

The role of mitochondria in the aging process has been a topic of intense interest for many years; in fact age-related changes in mitochondrial content, structure and function, as well as in mitochondrial DNA have been extensively documented.

A series of studies have demonstrated a decline of the mitochondrial respiration efficiency with age in human and primate and that this decline has been attributed either to a progressive down regulation of genes encoding for mitochondrial proteins such as several subunits of cytochrome-c oxidase, NADH dehydrogenase and ATP synthase, either to the decline of mitochondrial biogenesis with aging (Fernandez-Silva et al. 1991; Calleja et al. 1993; Barrientos et al. 1997; Welle et al. 2000; Short et al. 2005; Reznick et al. 2007).

It has been also well documented that the decline in OXPHOS activity correlates with a wide spectrum of mtDNA mutations, including point mutations, large scale deletions and duplications which progressively accumulate in post-mitotic tissues during human aging (Lee et al. 1994; Michikawa et al. 1999; Bua et al. 2006). For instance, an accumulation of mtDNA deletions has been found in skeletal muscle fibers deficient in electron transport activity in rodents and humans (Bua et al. 2006; Herbst et al. 2007). Studies on the substantia nigra neurons have shown high levels of somatic mtDNA deletions in both elderly control subjects and patients with Parkinson (Bender et al. 2006; Kraytsberg et al. 2006; Reeve et al. 2008). Moreover, an age-related decline in mtDNA content in skeletal muscle from mice and humans has been related to decreases in both mitochondrial ATP production rate and oxidative phosphorylation coupling (Short et al. 2005; Li et al. 2010).

Among the variety of mtDNA alteration, the most prevalent age-associated point mutations of mtDNA are A3243G and A8344G transition, while the most common

mtDNA deletion in aging human tissues is the 4977 bp deletion (Majamaa-Voltti et al. 2006; Pavicic and Richard 2009).

The relation between mtDNA mutation and aging phenotypes has been provided by several investigations using mtDNA mutator mice, that are knock-in mutant mice expressing a proofreading-deficient version of the mitochondrial DNA polymerase γ gene, that is the nuclear-encoded catalytic subunit of mtDNA polymerase (PolgA). Authors demonstrated that the accumulation of point mutations in mtDNA leads to the synthesis of respiratory chain subunits with amino acid substitutions that impair complex mitochondrial stability and cause progressive respiratory chain deficiency which, in turn, leads to premature aging (Trifunovic et al. 2004; Edgar et al. 2009).

The large increase in somatic mtDNA mutations and the deficit in mitochondrial respiratory function have been extensively attributed to the progressive and irreversible accumulation of oxidative damage by Reactive Oxygen Species (ROS), a critical aspect of the aging process, as emerged in the mitochondrial theory of aging (Harman, 1972; 1973; Wei et al. 2001).

ROS are by-product of normal cellular metabolism; the mitochondrial respiratory chain is the major source of several ROS, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), generated as by-products of cellular energy. Moreover, ROS are produced by cytochrome P450 and peroxisomes metabolism, during the immune-inflammatory response, in the detoxification of xenobiotics and in response to several environmental agents, including γ-ray, ultraviolet light irradiation and non-genotoxic carcinogens (Franceschi et al. 2000; Inoue et al. 2003; Valko et al. 2006).

Mammalian cells possess a multi-level ROS defense network of enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) that

cooperate to convert ROS into more stable molecules, such as water and O_2. Besides antioxidant enzymes, also non-enzymatic antioxidants, for instance ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH) and carotenoids function as direct scavengers of ROS (Valko et al. 2007).

A growing body of evidences have shown that ROS play a dual role within cells (Valko et al. 2006). At low/moderate concentrations ROS have beneficial effects regulating the cell cycle, the processes of cellular defense against infectious agents and a number of cellular signaling pathways involved in oxidative stress response, in immune response and in apoptosis (Wojcik et al. 2010). In contrast, an excessive ROS production and/or a decline in the capacity of intracellular antioxidant defense result in the establishment of a state of oxidative stress that damages various cellular constituents, including proteins, lipids, and DNA. Generally, the ROS-induced protein modifications may change the structure and the catalytic activity of key enzymes whereas the lipid and DNA oxidation may alter the fluidity of membranes and the transcriptional processes respectively.

Mitochondrial DNA is especially susceptible to attack by ROS, for several reasons: i) its close proximity to the electron transport chain, the major site of ROS production; ii) the size and the compactness of the genome; iii) the lack of protective histones; iv) the absence of adequate mitochondrial DNA repair systems. At the DNA level, ROS react with both purine and pyrimidine DNA bases, as well as the deoxyrybose backbone.

The most studied mtDNA base lesion induced by oxidative stress in aging is the formation of 8-Hydroxyl-2'-deoxyguanosine (8-OH-dG) and an inverse correlation between the steady-state concentration of 8-OH-dG in mitochondrial DNA and the maximum lifespan (MLSP) of a wide range of mammalian species has been found (Barja and Herrero, 2000; Hamilton et al. 2001; Stevnsner et al. 2002).

As previously reported, mtDNA mutations accumulate progressively during lifetime and influence directly the cellular oxidative phosphorylation activity, thus leading to an enhanced ROS production. In turn, increased ROS production results in an increased rate of mtDNA damage and mutagenesis, thus causing a "vicious cycle" which ultimately culminates in cell death (Fig. 5).

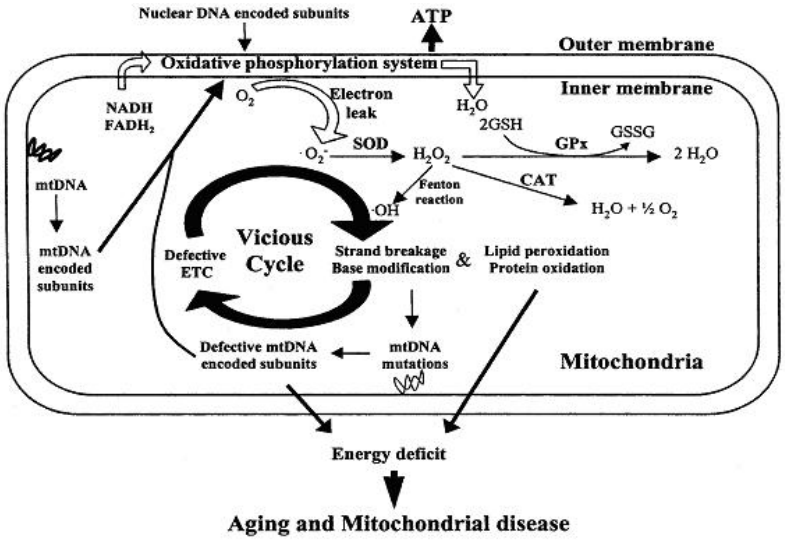

Fig. 5: "Vicious Cycle" of mtDNA damage. *ROS produced from the OXPHOS activity damage mtDNA, thus altering mtDNA transcription and ETC activity, resulting in even higher levels of ROS production.*

The link between cellular ROS and lifespan has been suggested by *in vitro* and *in vivo* studies in which the oxidant scavenging systems were enhanced. Indeed, Serra and coworkers (2003) found that the superoxide dismutase overexpression in human fibroblasts with low antioxidant capacity induce a decrease of the intracellular peroxide content, a slowdown of the telomere shortening rate, and an elongation of the life span of these cells, confirming also the causal role of oxidative stress for age-related telomere shortening. Similarly, Schriner and coworkers (2005), by using transgenic mice over-expressing human catalase observed a significant increase in mice lifespan,

associated to a lower susceptibility for mtDNA to ROS damage. Conversely, knockdown of SOD by using RNAi was demonstrated to induce cellular senescence through p53 (Blander et al. 2003). These results have provided strong support of the "Mitochondrial theory of aging", reinforcing the role that oxidative stress has in mammals lifespan determination (Harman, 1972; 1973).

The oxidative stress hypothesis to explain senescence proposes that reducing the production of reactive oxygen species within the mitochondria concomitantly decreases their deleterious effects on survival. It has been shown that the magnitude of the proton gradient across the inner mitochondrial membrane is directly correlated with superoxide production by the electron transport chain (Korshunov et al. 1997); therefore, a possible method to altering free radical production may be manipulating the proton gradient across the mitochondrial inner membrane. Accordingly, in the last few years several evidences have demonstrated the emerging role of uncoupling proteins (UCPs) in this field of search. UCPs belong to a family of anion transporters located in the inner mitochondrial membrane and are involved in the uncoupling of respiration from energy production generating a leakage of protons into the mitochondrial matrix with heat production. This activity decreases the mitochondrial membrane potential and inhibits the generation of ROS. The role of UCP in modulating the mitochondrial ROS production has been suggested by a number of data confirming the "uncoupling-to-survive" hypothesis, proposed by Martin Brand (2000). As results, new items have emerged shedding light on a possible mechanisms implicated in the buffering of ROS and consequently in the process of aging (Feng et al. 2001; Holzenberger et al. 2003; Fridell et al. 2005; Andrews and Horvath, 2009). Interestingly, recent observations have suggested that the mitochondrial uncoupling process has similar effects of the caloric restriction, the sole known non-genetic enhancer of lifespan (Caldeira da Silva et al.

2008). Indeed, also the limitation in dietary calories uptake extends lifespan in several model organisms stimulating the respiratory rate, that, in turn, decreases the coupling between oxygen consumption and oxidative phosphorylation, supporting the central role for mitochondrial metabolism in the aging process (Xiao et al. 2004).

Aging is often associated with a sedentary lifestyle and it is known that if there are no demands for the extra energy that can be produced by aerobic oxidative phosphorylation, cells may down regulate the ETC components and survive adequately on glycolysis. Also the increased in sugar consumption may enforce the reliance on glycolysis (Johnson et al. 2009). As consequence, an epigenetic oxidative redox shift (EORS) has been recently proposed by Brewer (2010) to ensure ample supplies of NAD^+ for glucose oxidation and maintain redox balance with impaired mitochondrial NADH oxidoreductase by upregulating other oxidoreductases. The activity of these other oxidoreductases has the 100% efficiency in generating oxyradicals (DeGrey, 2005). Thus, to avoid this catastrophic cycle, lactate dehydrogenase is upregulated at the expense of lactic acid acidosis. Overall, the oxidative redox shift is able to change the activity of numerous redox-sensitive transcription factors, including NF-KB, SP1, HOXB5 and USF and enzymes, including oxidoreductases and lactate dehydrogenase as previously reported. Also enzyme involved in the control of the epigenetic mark are reprogrammed. These includes histone acetylases, deacetylases (with their substrate requirement for NAD^+) and methyltransferases. Together, these mediators impose the metabolic shift away from use of mitochondrial energy toward reliance on glycolysis. Thus, the EORS in aging results in a spiral of inability to respond to energy demands or stress which leads to stress-induced initiation of cellular death pathways and organ failure (Brewer, 2010).

1.2 Frailty as aging phenotype

As people age, they progressively accumulate impairment in multiple physiological systems, and become more prone to adverse health outcome. Thus, by analyzing the heterogeneity of health status amongst elderly people, a different degree of successful aging and pathological aging phenotypes can be observed, depending on the different capability that everyone has in counteracting extrinsic and intrinsic factors and restoring the physiological balance. Moreover, as a reflection of multisystem deficits, a wide range of semi-pathological (or intermediate) phenotypes, largely recognized as frailty, is also observed in population.

Frailty belongs to the family of geriatric syndromes, and therefore it can be considered as the resultant of a multidimensional interplay of genetic, biological, psycosocial and environment factors (Rockwood and Mitnitski, 2007). In fact, it has been defined as *"a state of increased vulnerability to stressors that results from decreased physiological reserves and multi-system dysregulation, limited capacity to maintain homeostasis and to respond to internal and external stresses. Frailty is an aggregate expression of risk resulting from age- or disease-associated physiological accumulation of subtreshold decrements affecting multiple physiologic systems resulting in adverse outcomes"* (Fried et al. 2004).

Current data has identified a wide range of geriatric phenotypes that are common in frailty syndrome, including weight loss and sarcopenia, muscle weakness with low grip strength, low activity level, exhaustion, low body mass index, blood pressure instability and balance and gait abnormalities (Fried el al. 2004; Bergman et al. 2007; Topinková 2008; Davis et al. 2010). Although this syndrome is generally due to the reduced levels of physical activity, it is also influenced by multisystem impairments including

immune/inflammatory, multiple hormonal and neuromuscular dysregulations, as well as metabolic and vascular alterations and oxidative stress that commonly occur with aging (Fig. 6) (Ferrucci et al. 1999; Leng et al. 2004; Schalk et al. 2004; Walston et al. 2006; Barzilay et al. 2007; Landi et al. 2008; Blaum et al. 2009; Hubbard et al. 2009; Desai et al. 2010; Evans et al. 2010; Hyde et al. 2010; Lustosa et al. 2010; Maggio et al. 2010).

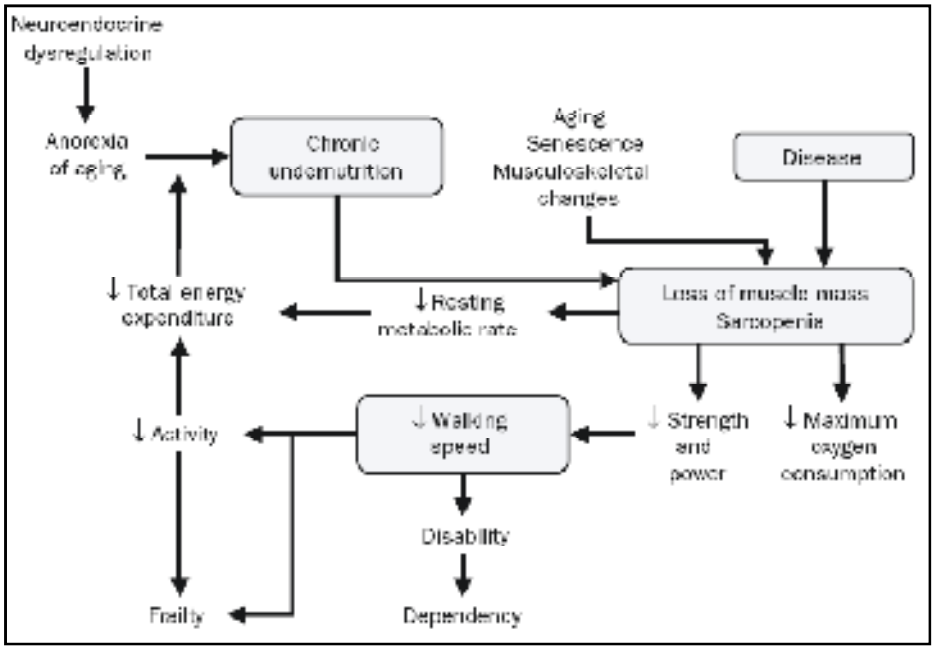

Fig. 6: Frailty Cycle. *This cycle combines elements such age-related musculoskeletal changes, neuroendocrine dysregulations, diseases and nutrition into a patho-physiologic pathway (Singh et al. 2008).*

Inflammation is one of the most recognized factors influencing frailty (Ershler and Keller, 2000). Hallmarks of inflammation, such as C-reactive protein (CRP), inflammatory cytokines and leukocytosis have been widely associated with aging and chronic age-related disease, including cardiovascular diseases (CVD), Alzheimer's disease and diabetes (Lombardi et al. 1999; Alexandraki et al. 2006; Tuomisto et al. 2006). It has been also observed that the increase of the inflammatory markers could at

least partially explain the sarcopenia, the unintentional weight loss and the reduction of physical activity, that represents the most physiological characteristics of frailty (Pel-Littel et al. 2009). In particular, high serum levels of IL-6, TNF-α, and CRP are associated with mobility limitation, disability and mortality in older persons (Ferrucci et al. 1999; Penninx et al. 2004; Gallucci et al. 2007). What is more, stress-responsive genes in monocyte-mediated inflammatory pathway results significantly upregulated in the frailty syndrome (Qu et al. 2008).

Furthermore, several data associate the dysregulation of lipoprotein metabolism to inflammation and frailty of elderly individuals. Recently, elevated systemic levels of myeloperoxidase (MPO), a pro-oxidant enzyme that catalyzes the initiation of lipid peroxidation and affects nitric oxide levels, have been associated with unfavourable clinical outcomes in frail people (Giovannini et al. 2010). In addition, Landi and coworkers (2008) have demonstrated that high levels of high-density lipoprotein (HDL) cholesterol, a powerful anti-inflammatory agent, are associated with better survival, reporting that it can be considered as a reliable marker of frailty and poor prognosis among the oldest elderly. Moreover an interaction between the inflammatory system and the coagulation pathways has been suggested occurring in aging. In fact, procoagulant markers, including D-dimer, fibrinogen and factor VIII, increase in population with age and the incidence of venous thrombosis and pulmonary emboli increase sharply in geriatric population, and represent a major cause of morbidity and mortality in the elderly (Walston et al. 2002; Folsom et al. 2007; Tita-Nwa et al. 2010). This is noteworthy, because it could be explain: i) why comorbidities, like heart failure, myocardial infarction, peripheral vascular diseases, and hypertension increase the risk of frailty (Klein et al. 2005); ii) why the presence of a chronic inflammatory status links frailty to the development of several age-related diseases, such as atherosclerosis,

cardiovascular diseases, predementia syndromes, and diabetes (Libby, 2002; Panza et al. 2006; Fulop et al. 2006).

Like the inflammatory and the cardiovascular system, endocrine and neuroendocrine systems seem to influence both aging and frailty. In fact, the age-related decline of the adrenal androgen dehydroepiandrosterone-sulfate (DHEA-S), growth hormone (GH) and insulin-like growth factor (IGF-1) serum levels as well as the resulting reduction of their intracellular signaling efficiency has been broadly associated with the progressive loss of muscle mass, bone mineral density (BMD) and body mass index (BMI) (Perrini et al. 2010). Furthermore, signs of neurological dysfunction, often associated with reduced physical activity, falls and depression, are frequently observed in older people free of any form of neurological disease (Perrin et al. 1997; Panza et al. 2005).

Several genetic variants have been associated with the development of frailty. A significant association between frailty syndrome and polymorphisms of genes involved in vitamin B12 transport and metabolism, APOE gene, inflammation-related genes and mitochondrial DNA control region has been observed (Kulminski et al. 2008; Moore et al. 2010; Marioni et al. 2010; Matteini et al. 2010).

Recent investigations on the understanding the telomere biology have given rise the hypothesis that telomere shortening, that commonly occurs with aging, may form the biological basis for frailty. Although Cawthon and coworkers (2003) have demonstrated that telomere shortening in human beings contributes to mortality in many age-related diseases, no association between telomere length and i) frailty index (a parameter summarizing the physical, psychological, and functional deficits), ii) overall survival, iii) death from several cause (osteoporosis or fractures, infectious diseases, cancer, or cardiac and cerebrovascular diseases), has been observed (Woo et al. 2008; Sanders et al. 2009; Njajou et al. 2009). Thus, these findings suggest that although telomere length

could be considered a biomarker of cellular senescence, this relationship may not be extrapolated to the functional level represented by the frailty phenotype.

Lastly, lifestyle, including nutritional deficiencies or excesses, contributes to frailty (Khaw et al. 2008). Low serum levels of vitamins A, D, E, B(6), B(12), 25-hydroxyvitamin D and other micronutrients such as carotenoids, folate and zinc are associated with frailty amongst older adults (Semba et al. 2006; Wilhelm-Leen et al. 2010). The high incidence of type 2 diabetes in older people is usually due to excess of nutrients (Morley, 2000). In addition, a sedentary lifestyle and malnutrition inexorably reduces the mobility of aged people (Lee and Tanaka, 1997).

It is noteworthy that these physiological pathways are modulated in a complex manner with aging in response to genetic predispositions, diseases, reactive oxygen species production and mitochondrial dysfunctions. Indeed, as well as in aging, mitochondria influences the functional decline in later life, thus contributing to frailty.

As previously mentioned, sarcopenia is a prime characteristic of the frailty phenotype. This progressive atrophy in skeletal muscle is complex and has not been yet clearly defined, although recent evidences have indicated that in aging it occurs mainly through enhanced activation of apoptosis (Whitman et al. 2005; Dupont-Versteegden, 2005). Given the central role of mitochondria in oxidative stress and in regulating apoptosis, their integrity and functionality give a fundamental contribution to sarcopenia. In particular, Wanagat and coworkers, (2001) reported that reduction in the activity of Complex I and IV of the ETC, due progressive accumulation of mtDNA mutations, correlates with sarcopenia. More recently, Herbst and coworkers (2007) found that a progressive accumulation of mtDNA mutations occurs in muscle fibers with aging, inducing their dysfunction and breakage. Lastly, Moore and coworkers (2010)

demonstrated that some mtDNA polymorphisms in the DNA control region significantly correlate with frailty.

Also mitochondrial biogenesis decreases with aging, and the expression of the mitochondrial encoded ETC subunits decreases as well (Zahn et al. 2006; Reznick et al. 2007); these processes result in an overall loss of mitochondrial function determining a progressive loss of muscle mass (Dirks et al. 2006).

Besides the role played by mtDNA, mitochondrial ROS can results in more direct deleterious effects for cells; for example, an immediate consequence of oxidative damage is the oxidation of cardiolipine, a lipide of the inner mitochondrial membrane, that can directly promote the release of apoptogenic factors from mitochondria (Petrosillo et al. 2003; Gonzalvez and Gottlieb, 2007). Reactive oxygen species are also involved in the direct activation of inflammation via NFKB pathways (Kunsch and Medford, 1999).

Over the years, different multidimensional methodological approaches have been developed for establishing the presence of frailty and for regrouping subjects with homogenous phenotype. In general, these methods incorporates items of physical, cognitive and psycosocial signs, as well as diseases and disabilities, to classify the subject analyzed in a well defined phenotypic class of frailty. However, the wide heterogeneity in the quality of aging population that occur in different population as consequence of genetic variations and sociocultural differences, has revealed the need to develop population-specific models for monitoring aging (Jeune et al. 2006).

In recent years, many operational definitions of frailty have been proposed. Some of them consider frailty as a continuum accumulation of self-reported deficits, reflecting the proportion of potential disabilities present in a person (Mitnitski et al. 2002; Rockwood 2005, 2006); others, consider frailty as a distinct clinical syndrome non

synonymous of co-morbidity or disability and are based on direct measurement of functional parameters as grip strength, walking speed, unintentional weight loss and low physical activity (Fried et al. 2001). In this context, a novel methodology in frailty definition has been developed by Montesanto and coworkers (2010). In particular, well established geriatric parameters, including Mini Mental State Examination (MMSE), Hand Grip strength and geriatric Depression Scale (GDS), Activities of Daily Living (ADL) and Self-reported health status (SRHS) have been used as parameters in a hierarchical cluster analysis (CA), in order to define different aging phenotypes. The diagnostic and predictive soundness of this classification were confirmed by a detailed survival analysis showed higher survival chance for subjects characterized by lower frailty.

1.3 Aging epigenetics: DNA methylation

In the frame of the research on aging and longevity, an emerging field that promises exciting revelations about the determinants of cell senescence and organism aging is represented by the "Aging epigenetics".

Epigenetics refers to the study of mitotically and, in some cases meiotically, hereditable changes of a phenotype that are unrelated to alterations in the DNA sequence. In vertebrates these changes are crucial for all the biological processes, as they regulate the expression of genetic information.

During the early stages of the life, the epigenetics status undergoes several changes to ensure an appropriate process of cell development and differentiation. However, non-random mechanisms such as environmental stimuli or stochastic errors in maintaining fixed the epigenetics patterns are able to induce changes in epigenetics profiles at both early and later in the life. Indeed, epigenetics anomalies have been found to be cause of

congenital disorders and multifactorial pediatric syndromes and adult-onset diseases as well (Schumacher and Petronis 2006; Vidal et al. 2007). Although epigenetics role in development, differentiation and pathological states has been extensively investigated, little is yet known about the relationship between epigenetics and aging.

The best known epigenetic modifications refer to DNA methylation and histone modifications, including methylation, acetylation, ubiquitylation and phosporylation. Both these processes are interdependent and cooperating in chromatin remodeling thus leading a dynamic regulation of gene expression in higher eukaryotic cells.

1.3.1 DNA methylation basic mechanisms

DNA methylation is a covalent biochemical modification that consists of the addition of a methyl group to the aromatic ring of a single DNA base. The methyl group protrudes into the major groove of the DNA double helix, preventing the binding of transcription factors that otherwise bind locally DNA sequences (Bell and Felsenfeld, 2000; Hark et al. 2000) and facilitating the binding of methyl-binding proteins (Jorgensen and Bird, 2002).

In eukaryotes, methylation occurs at 5-carbon position of deoxycytosine thus forming deoxymethylcytosine (d^mC). In particular, in humans, this process accounts for 3-6% of the total cytosine (Callian and Feinberg, 2006).

In mammalian genome DNA methylation takes place predominantly when cytosine base is located 5' to a guanosine, a so-called CpG dinucleotide (CpG) (Clark et al. 1995).

CpG dinucleotides are vastly under-represented as compared with what would be expected, probably because they act as a hotspot mutation. In fact, a depletion of about

20% of the expected frequency of CpG dinucleotides results from the process of deamination of cytosines in uracils that, in turn, are replaced by thymine after DNA replication, or from the direct hydrolytic deamination of 5-methylcitosine to thymine (Singal and Ginder, 1999) (Fig. 7).

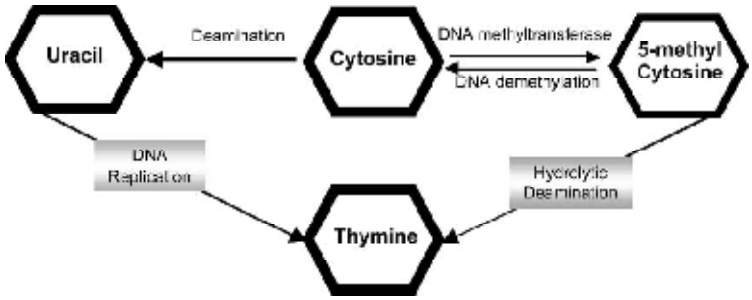

Fig. 7: Molecular processes contributing to the under-representation of cytosine in the vertebrate genome. *Both methylated and non methylated cytosines can be converted to thymine by deamination processes (Liu et al. 2003)*

Within mammalian genome about 70% of CpG dinucleotides are methylated. This process regards prevalently CpGs located into CpG-poor regions, localized in intergenic and intron region of genome. Furthermore, CpG methylated are localized in repetitive sequences, most of which derived from transposable elements, thus hindering the event of amplification and new insertion in the genome (Callian and Feinberg, 2006). On the other hand, most unmethylated CpG pairs are found in CpG-rich regions, termed **CpG islands**, sequences of about 1 kb in length and having a CG content greater than 55%, with an observed/expected CpG ratio of 0.65 (Jones, 1999; Takai and Jones, 2002).

In the humans about 30,000 CpG islands were estimated. They are not distributed throughout the genome, but appear most often associated to the promoter regions and to the first exons of almost 60% of genes, including most housekeeping genes and half of all tissue-specific genes (Ioshikhes and Zhang, 2000).

In mammals, the process of DNA methylation takes places after DNA replication and is mediated by a family of DNA Methyltransferases (DNMTs) that includes *DNMT1*, *DNMT3a*, *DNMT3b* and *DNMT3L* (Klose et al. 2006; Cheng and Blumenthal, 2008). These enzymes catalyze the transfer of the methyl group from S-adenosyl-L-methionine (SAM) to deoxycytosine, producing 5-methylcytosine and S-adenosylhomocysteine (Bestor et al. 1988).

In particular, DNA methylation patterns are established during development by the *de novo methyltransferases* DNMT3a and DNMT3b and their regulator DNMT3L, which have high affinity for previously unmethylated DNA (Okano et al. 1999). Conversely, these patterns are replicated in somatic cells during mitosis by a semiconservative *maintenance DNA methyltransferase*, DNMT1 (Fig. 8) (Bestor et al. 1988).

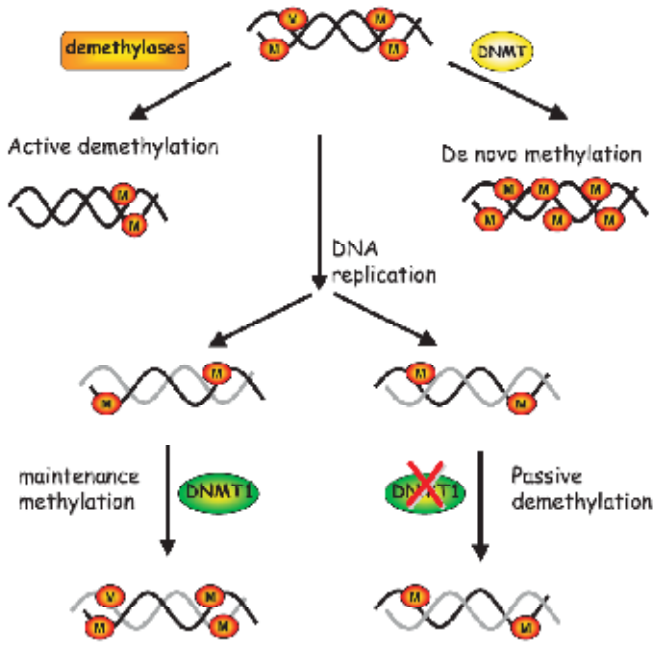

Fig. 8: DNA methylation reactions. *De novo methyltransferases inserts methyl group (CH3) in CpG sites that were not previously methylated. Once DNA methylation pattern are set by de novo methyltransferases and demethylases, they are maintained by DNMT1 during DNA replication. In absence of DNMT1, cells undergo to a passive DNA methylation (D'Alessio and Szyf, 2006).*

DNMT2 is an highly evolutionary conserved member of methyltransferases. In particular this enzyme in Drosophila (dDNMT2), mouse (mDNMT2) and human (hDNMT2) contains the conserved methyltransferase motifs, and maintains the methyltransferase activity (Hung et al. 1999; Okano et al. 1999). However, recent studies have revealed that the primary target of DNMT2 proteins are non-CpG sites (Liu et al. 2003; Kunert et al. 2003); In fact, in human, DNMT2 does not methylate DNA but instead methylates a small RNA. In fact, mass spectrometry showed that DNMT2 specifically methylates cytosine 38 in the anticodon loop of the aspartic acid transfer RNA (tRNAAsp) (Goll et al. 2006).

The activity of DNMTs is crucial in establishing and maintaining methylation patterns; homozygous loss of DNMT1, DNMT3a or DNMT3b in mice results lethal (Okano et al. 1999; Li et al. 1992).

DNA methylation patterns are also regulated by DNA demethylation proteins, that operate by at least two different mechanisms:

i) **Passive DNA demethylation**

   During DNA replication, symmetrically methylated strands of DNA in the parental chromosome segregate to daughter chromatids, thus containing hemimethylated DNA. Normally, the symmetric methylation is restored by the maintenance methyltransferase activity of DNMT1. However it is possible that regulatory nucleoprotein complexes are recruited in the sites of DNA methylation, preventing the access of DNMT1 to the methylation sites and thus leading to the progressive demethylation of DNA (Fig. 8) (Matsuo et al. 1998; Hsieh, 1999);

ii) **Active DNA demethylation**

5-methylcytosine is replaced during demethylation reactions through the action of **i)** 5-methylcytosine DNA glycosylase enzymes (5-MCDG or MBD4) which recognize methylated CpGs and cleave the bond between the methylated cytosine and the deoxyribose. The apyrimidine deoxyribophosphate is then removed and DNA repair systems adds back the cytosine in nucleotide form (Fig. 9A) (Jost et al. 1997, Gehring et al. 2008); **ii)** demethylase enzymes, as MBD2b that hydrolyzes 5-methylcytosine to cytosine and methanol (Fig. 9B) (Ramchandani et al. 1999).

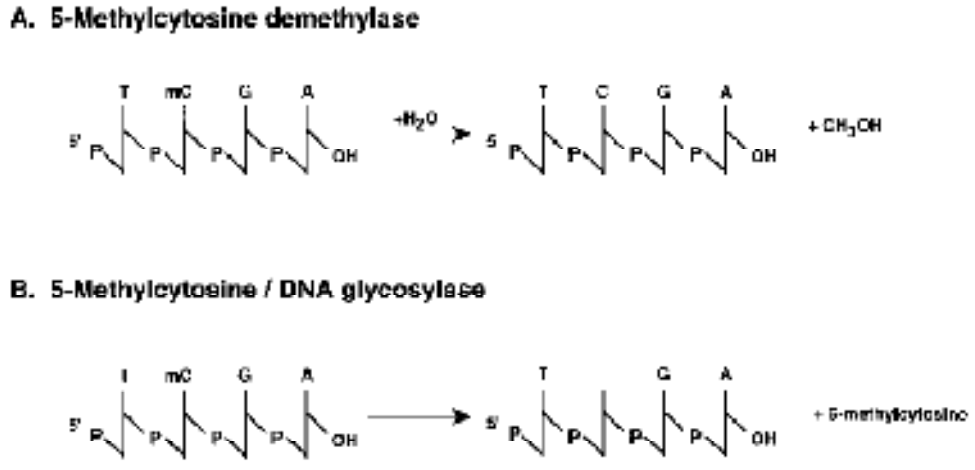

Fig. 9: Active DNA demethylation mechanisms. A. *The 5-methylcytosine demethylase hydrolyzes 5-methylcytosine to cytosine and water.* ***B.*** *The 5-methylcytosine DNA glycosylase removes 5-methylcytosine from the phosphodiester backbone, which then is repaired by using endonuclease (Wolffe et al. 1999)*

1.3.2 DNA methylation functions

Experimental data have demonstrated the role of DNA methylation in modulating different biological functions as:

- Development and differentiation; the homozygous loss of DNMT1, DNMT3a and DNMTb alleles in the early stage of embryonal development results in embryonic death (Okano et al. 1999; Li et al. 1992). Furthermore, somatic DNA methylation contributes to differentiation by repressing key genes in the germline and irreversibly forging the cell to differentiate (Weber et al. 2007; Zilberman, 2007). Moreover, centromeric instability and several forms of mental retardation and cancer have been associated with mutations in the DNA methylation machinery (Amir et al. 1999; Hansen at al. 1999; Gaudet et al. 2003).
- Genomic imprinting; DNA methylation of the imprinted control region (ICR) is the key mechanism by which one copy of a gene is preferentially silenced and retain the same mono-allelic expression as their parental origin: in particular, *DNMT3* family is implicated in maternal imprinting, and the disruption of DNMT3a and DNMT3b in germ cells, by conditional knockout technology, results in methylation lacking and allele-specific expression at all maternally imprinted loci examined (Ferguson-Smith and Surani, 2001; Kaneda et al. 2004).
- X chromosome inactivation; During the embryogenesis of females, one of the X chromosomes is inactivated by DNA methylation in order to preclude the expression of genes located in this chromosome and allow an equal male and female gene dosing for X-linked genes (Park and Kuroda, 2001).
- Parasitic DNA suppression; Mobile DNA elements, such as retrotrasposons or retroviral sequences, acquired in the human genome over time, are usually neutralized by DNA methylation, then suggesting that DNA methylation

processes serve also as defense mechanism (Yoder el al. 1997; Matzke et al. 1999; Bourc'his and Bestor, 2004).

- Transcriptional noise suppression; Methylation-dependent chromatin condensation may silence genes inappropriately activated by transcription factors (Bird, 1995).

The main function of the DNA methylation of CpG dinucleotides is the transcriptional silencing of the associated genes, thus explaining the inverse correlation between density of DNA methylation within the regulatory sequences of a gene and its transcription levels. This correlation has been extensively investigated and validated in the last 20 years by several lines of evidence. In particular, *in vitro* transfection experiments on methylated DNA demonstrated that DNA methylation inhibits gene expression (Kass et al. 1997; Steele et al. 2009). Moreover, silent genes in cultured cell lines are activated upon treatment with 5-azacytidine, a potent methyltransferases inhibitor (Meng et al. 2007; Brueckner et al. 2010).

DNA methylation is able to suppress gene expression through several mechanisms. It was well established that DNA methylation can directly impede the binding of transcriptional factors to their recognition sequences localized in the regulatory elements of genes. Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F, and NFkB, recognize sequences that contain CpG residues, and their binding is inhibited by methylation (Comb and Goodman, 1990).

In addition, a family of methylcytosine binding proteins (MBPs), including MBD1, MBD2, MBD3, MeCP2 and Kaiso have been shown to play a role in methylation-dependent gene silencing (Sansom et al. 2007). The mechanism by which these proteins inhibit the transcriptional processes is not yet well clarified. However, it has been

reported that MBDs can interact with chromatin inactivation complexes containing histones deacetylases thus promoting chromatin condensation into an inactive configuration (Nan et al. 1998; Harikrishnan et al. 2005). Furthermore, MeCP2 has been shown to localize to methyl-CpG reach DNA sequences, and contains methyl binding domain (MBD) and transcriptional repressory domain (TRD) (Nan et al. 1997). Indeed this protein has been defined as an anchor for binding to the DNA a methyl-repressory complex that causes histones deacetylation and chromatin remodeling.

Lastly, it has also been reported that all three DNA methyltransferases are able to suppress gene transcription being component of histone deacetylase repressory complexes (Satoh et al. 2002). DNMT3a binds deacetylases and recognizes the sequence specific repressory DNA binding protein PR58 (Fuks et al. 2001). Moreover, DNMT1 complexes with Rb, E2F1 and HDAC1, repressing transcription from E2F-responsive promoters (Robertson et al. 2000).

1.3.3 DNA methylation role in aging and age-related diseases

DNA methylation patterns are not fixed; during various stages of mammalian development they are reprogrammed to ensure the normal mammalian embryogenesis and cell differentiation. In particular, during mammalian development in germ cells and in preimplantation embryos two waves of genome-wide epigenetic reprogramming occur, generating cells with a broad developmental potential (Reik et al. 2001).

Therefore, DNA methylation patterns can also change during the lifetimes in response to several cues from the external and internal environments. Alteration in DNA methylation status has been documented to begin before bird; for example, nutritional deficiencies can alter the fetal DNA methylation, impairing the normal epigenetic programming for the lifetime (Okano et al. 1999; Obican et al. 2010). Also other

environmental stimuli, such as metals and aromatic hydrocarbons found in water, cigarette smoke and fossil fuel emission may potentially function as epigenetic modifiers (Hillemacher et al. 2008).

These stimuli are able to induce loss or gain of DNA methylation that can be propagated during cell division and sometimes transmitted across generations, resulting in permanent maintenance of the acquired phenotype. As a result, epigenetic modifications of DNA and histones might be crucial for understanding the molecular basis of numerous complex phenotypes (Johannes et al. 2009). Indeed, several evidences have reported the influence of single-locus DNA methylation variants (epialleles) in influencing a wide range of phenotype, such as flower shape or fruit pigmentation in plants and tail shape or coat pigmentation in mice (Cubas et al. 1999; Morgan et al. 1999; Rakyan et al. 2003; Manning et al. 2006).

Recently, epigenomics alterations have been correlated to many pathological situations, such mental retardation syndromes, cardiovascular diseases, cancer and other complex disorders (Calvanese et al. 2008; Tost, 2010). Various neurological diseases are associated with alterated DNA methylation patterns. Examples includes: i) the hypermethylation of the dopamine receptor D2 (*DRD2*) and of the reelin gene (*RELN*) and demethylation of *GAD1* promoter in schizophrenia (Zhang et al. 2007; Huang and Akbarian, 2007; Tochigi et al. 2008); ii) the demethylation in the peptidiylprolyl isomerase E-like promoter (*PPIEL*) in bipolar disease (Kuratomi et al. 2008; Rosa et al. 2008); iii) the hypermethylation of CGG repeats 5' of the *FMR1* gene in Fragile X disease (Tassone et al. 2000). Moreover, epigenetics changes may constitute a basic molecular mechanism in the pathophysiology of Alzheimer's disease (AD). Recent data from studies on set of monozygotic twins discordant for AD reports a significant reduction in DNA methylation levels of the temporal neocortex neuronal nuclei of the

AD twin. These results supports the hypothesis that epigenetic mechanisms may mediate at the molecular level the effects of life events on AD risk, and provide a potential explanation for AD discordance despite genetic similarities (Mastroeni et al. 2009).

Global DNA methylation alterated patterns were also observed in cardiovascular disease (CVD) or in its predisposing conditions such as hypertension, atherosclerosis, diabetes and obesity, occurring either directly, or as consequence of insufficient uptake of nutritional factors in diet (Castro et al. 2003; Plagemann et al. 2009; Bell et al. 2010; Kim et al. 2010; Lai et al. 2010).

Lastly, DNA methylation plays a pivotal role in tumorigenesis. In cancer, a global process of promoter hypomethylation of the genome occurs, in association with specific patterns of hypermethylation of tumor-suppressing genes.

A wide spread process of hypomethylation is observed in the promoter region of numerous proto-oncogenes, growth factors and genes involved in cancer cell proliferation, invasion, and metastasis (Szyf et al. 2004). It was well established that the loss of imprinting of the second allele and increased biallelic expression of *IGF-2* efficiently stimulates malignant cells proliferation (Vu et al. 2003). Moreover, the hyperexpression of genes such as urokinase type plasminogen activator (*PLAU*), heparanase and calcium binding protein (*S100A4)* by demethylation promotes malignant cell motility through the extracellular matrix and metastasis (Pakneshan et al. 2005; Senolt et al. 2006).

Hypomethylation of retrotrasposons also induces chromosomal aberrations common in several cancers, destabilizing genome by insertional mutagenesis and recombination between non-allelic repeats (Eden et al. 2003; Gaudet et al. 2003; Ehrlich et al. 2006).

Moreover, it has been shown that *DNMT1* deficiency or the overexpression of inactive variants of *DNMT3b* also results in a reduction of its methyltransferase activity and in a constitutive chromosomal instability (Weisenberger et al. 2004; Karpf and Matsui, 2005).

At the same time specific patterns of hypermethylation at the promoter regions of tumor-suppression genes were observed. The number of genes inactivated by hypermethylation during carcinogenesis is rapidly increasing. These include genes involved in signal transduction (*AKAP12*, *STK11*), DNA damage repair (*BRCA1*, *MLH1*), detoxification (*GSTP1*), cell cycle regulation (*TP53*, *p15*, *p16*, *RASSF1*), angiogenesis (*VHL*) and apoptosis (*caspases*, *DAPK*) (Sanchez-Cespedes et al. 2002; Choi et al. 2004; Amara et al. 2008; Almeida et al. 2009; Kamimatsuse et al. 2009; Braggio et al. 2010; Greco et al. 2010; Leite et al. 2010; Matuschek et al. 2010; Cho et al. 2010; Shima et al. 2010).

Overall, the role of epigenetics in complex traits is still in its infancy, but it will shed light on the possible link between the epigenotype and the susceptibility to complex phenotypes.

Epigenomics alterations are now increasingly recognized as part of aging and aging-related disease. A recent study on the dynamics of DNA methylation during the course of development, maturation and aging of human brain has evidenced a robust and progressive rise in DNA methylation levels across lifespan for several loci, in association with a process of hypomethylation of ALU and other repetitive elements with aging (Siegmund et al. 2007). Moreover, a complex relation between epigenetic control and X-linked and imprinted genes occurs in aging. In particular, several lines of evidences have demonstrated an age-reduction in DNA methylation of the inactive X chromosome, particularly in the myeloid cell lineage of peripheral blood cells (Busque

et al. 1996; 2009). As a result, beside some cancers, autoimmune disorders and other diseases, a positive correlation between age and degree of somatic X chromosome inactivation (XCI) skewing was observed (Hatakeyama et al. 2004; Yin et al. 2007; Lose et al. 2008; Uz et al. 2009).

In the frame of the investigation of the epigenetic role in human aging, a significant contribution has been provided by the study of discordant monozygotic (MZ) twins. Fraga and coworkers (2005) reported that global and gene-specific epigenetic differences in a large cohort of monozygotic twins increase overtime within different tissue and cells. Specifically it was observed that the epigenetics marks of MZ twins, indistinguishable early in life, exhibit remarkable differences as they become older, mainly in 5-methylcytosine levels and histone modifications. These differences in genetically identical individuals could result both from stochastic (due to random epimutations) or systematic events, in response to external factor and environment changes. The relationship between the epigenetic changes and aging was also confirmed by observation of time-dependent changes in global DNA methylation levels within the same individuals in Iceland and Utah population (Bjornsson et al. 2008). In addition, the evidence that DNA methylation changes exhibits familiar clustering in individuals that do not share house-holds suggests that the DNA methylation stability is genetically determined (Bjornsson et al. 2008).

Overall, a gradual loss of DNA methylation with age occurs in most vertebrate tissues and in humans as well (Vanyushin et al. 1973; Romanov and Vanyushin 1980; Bjornsson et al. 2008). For instance, Bollati and coworkers (2009) found a gradual decrease through aging in repetitive element DNA methylation, particularly in Alu sequences. Beside this process of global DNA hypomethylation, a number of specific loci have been described to becoming hypermethylated with aging. Examples of genes

with increased promoter methylation during aging include those encoding for ribosomal DNA clusters in liver and germ cells of senescent rats and for E-caderin, collagen α1(I) and for the tumor suppressive genes Lysyl Oxidase (LOX), p16INK4a, runt-related transcription factor 3 (RUNX3), TPA-inducible gene 1 (TIG1) in various human tissues (Takatsu et al. 1999; Bornman et al. 2001; Oakes et al. 2003; So et al. 2006). For instance, an increase of methylation in ribosomal DNA cluster has been observed in livers of senescent rats, and in several tumor suppressing genes in humans (Oakes et al. 2003; Fraga and Esteller, 2007). These studies demonstrate a loss of the epigenetic control in aging, suggesting its contribution in the age-related phenotypes, such as psycophysical decline and/or development of age-related diseases. However, the functional relevance of these abnormalities in the epigenetic machinery are still unclear.

1.4 Mitochondria as modulators of aging epigenetics

Also in this field of research, mitochondria, given their involvement in the homeostasis redox and bioenergetics, are emerging as major players.

As above described, ROS, that play a relevant role in carcinogenesis, neurodegenerative disorders and aging as well, induce DNA strand breaks and adducts a number of hydroxyl radical attack to DNA bases, and one of the principal consequence of this process is the production of 8-Hydroxyl-2'-deoxyguanosine (8-OH-dG) (Olinski et al. 1992). Turk and coworkers (1995a; 1995b) reported that the presence of 8-OH-dG in CpG nucleotide sequence diminishes the ability of DNA methyltransferase to methylated the adjacent cytosine, and of restriction nucleases to cleave the DNA. In addition, Valinluck and coworkers (2004) demonstrated that the oxidation of guanosine in 8-OH-dG and/or 5-methylcytosine in 5-hydroxymethylcytosine in a CpG dinucleotide significantly reduces the binding affinity of MBDs to their recognition

sequences, thus leading alteration in transcription. Furthermore, the intracellular redox state is also able to modulate the activity of Methionine adenosiltransferase (MAT), enzyme responsible for the synthesis of S-adenosylmethyonine (SAM) from L-methionine and ATP. In particular, oxidative/nitrosative stress, associated to a low ratio of GSH/GSSH reduces MATs activity (Pajares et al. 1992; Avila et al. 1998).

These evidences have demonstrated the direct role for oxidative damages in the determination of aberrant DNA methylation patterns and, in particular, in the process of global hypomethylation, associated to transcription activation, commonly observed in several cancers and in aging.

Epigenetic processes also depends by the cellular bioenergetic systems, regulated by the mitochondrial electron transport chain activity and, in turn, by mitochondrial DNA. ATP, Acetyl CoA, NADH and NAD^+ are the main substrate of chromatin phosphorylation, acetylation and deacetylation reactions, thus contributing to the epigenetic process of chromatin remodeling (Wallace and Fan, 2010). Lastly, mitochondria contributes to epigenetics modification through the folate metabolism and the production of S-adenosyl-methyonine, the methyl donor of the methylation reactions (Fig. 10) (Naviaux, 2008).

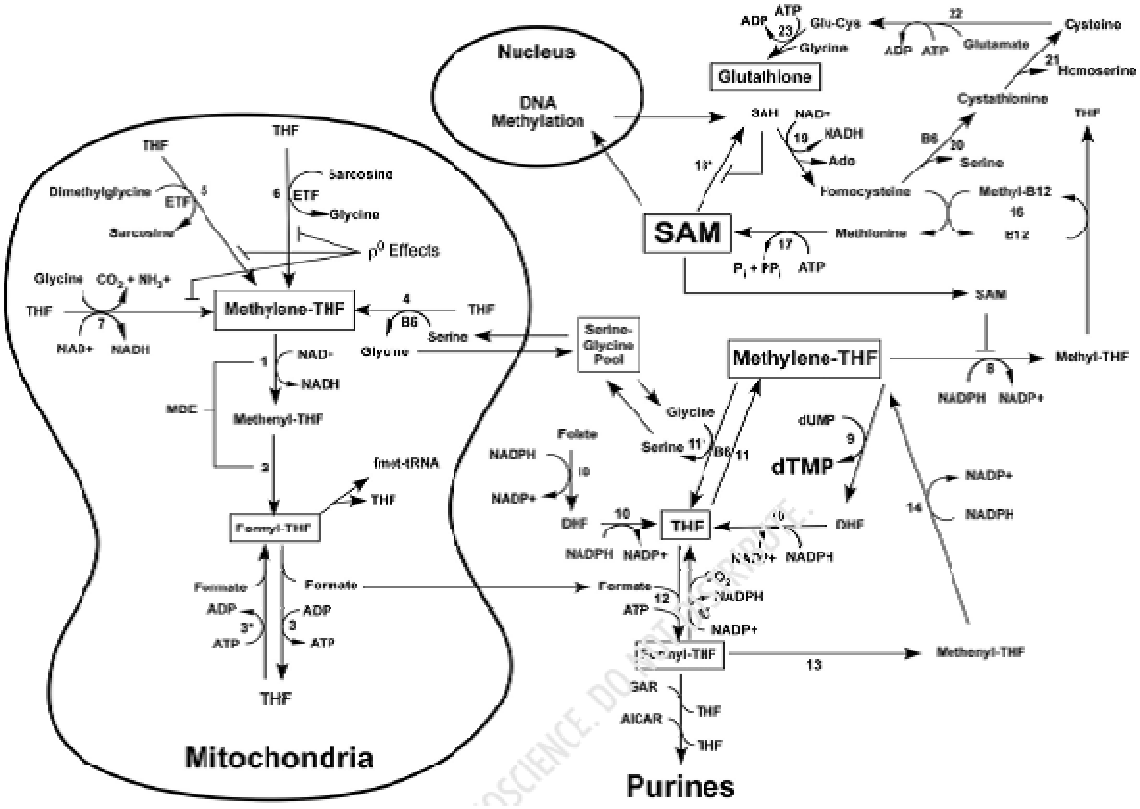

Fig. 10: The One-carbon Cycle: The molecular interplay between mitochondrial folate metabolism and DNA methylation. *(Naviaux, 2008)*

When OXPHOS is inhibited or insufficient, the mitochondrial NADH/NAD^+ ratio increases, inhibiting the mitochondrial production of methylene-tetrahydrofolate and serine. This reduces methionine and, consequently, SAM production.

These data demonstrated a role played by mitochondria in epigenetics mechanisms and regulation the pattern of gene expression. Moreover, to evaluate a direct involvement of mitochondrial DNA in epigenetic DNA modifications, Smiraglia and coworkers (2008) found that in response to the depletion and repletion of mtDNA, DNA methylation patterns of several nuclear genes were significantly influenced, providing the first direct evidence that mtDNA modulates the epigenetic modifications in the nucleus, probably through the well known cross talk between mitochondrial and nuclear genome.

1.5 Plan of the thesis

The present work is organized in 4 sections. The first two sections are represented by two manuscripts still unpublished aimed at exploring a possible correlation among the age-specific functional decline, the mitochondrial DNA inherited variants and the global DNA methylation levels. In particular, in the manuscript *"Frailty and global DNA methylation in elderly people: an intriguing correlation"* we investigated whether the frailty status of elderly people was associated to global DNA methylation levels, thus searching a correlation between the age-specific functional decline and epigenetic modifications. On the other hand, in the manuscript "*Mitochondrial DNA variation modulates global DNA methylation levels"* we searched for a possible influence of mitochondrial DNA inherited variants in age-related epigenetic changes.

The last two sections present two manuscripts exploring the role of mtDNA variability in modulating the expression profiles of two classes of stress responders nuclear genes: Sirtuins, considered as regulators of energetic and redox homeostasis, and HSPs, key regulators of protein folding and migration and apoptotic pathways.

The manuscript *"SIRT3 gene expression: A link between mitochondrial DNA inherited variants and oxidative stress response*" is still unpublished while the other "*Mitochondrial DNA variability modulates mRNA and intra-mitochondrial protein levels of HSP60 and HSP75: experimental evidence from cybrid lines*" was published in 2009.

Global DNA methylation in old subjects is correlated with frailty

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SUMMARY

Epigenetic variations occurring during aging can be considered as a drawbridge across genetic and environmental factors. To verify if this is correlated with the inter-individual phenotypic variability in frailty status of elderly people, we searched for a correlation between global DNA methylation levels and frailty. The results obtained demonstrated that the global DNA methylation levels were correlated to the frailty status in middle/advanced-aged subjects, suggesting that the relaxation of the epigenetic control in aging is more specifically associated to the functional decline that to the chronological age of individuals. A longitudinal study revealed also that a the worsening in the frailty status was associated to a significant decrease in the global DNA methylation levels, thus suggesting that the DNA methylation modifications may play a roles in determining physiological changes over the lifetime. Overall, this work demonstrated that the progressive functional decline during aging is not only under the control of the genetic system but is regulated also by the individual epigenetic background.

KEYWORDS: Frailty; global DNA methylation; aging.

INTRODUCTION

Frailty represents a clinical syndrome which is prevalent in older population. It is characterized by the decrease of total physiological reserves and a progressive deregulation in the homeostatic network underlying maintenance and repair in the ageing body (Lang et al. 2009; Bandinelli et al. 2010). As a consequence, frailty increases the individual vulnerability to age-related disorders. Indeed, a wide range of geriatric phenotypes are commonly observed to be associated with frailty including weight loss and sarcopenia, muscle weakness with low grip strength, low activity level, exhaustion, low body mass index, blood pressure instability and balance and gait abnormalities (Fried et al. 2004; Bergman et al. 2007; Topinková 2008; Davis et al. 2010). In addition, an association between frailty and the risk of mild cognitive impairment and a rapid rate of cognitive decline was described (Buchman et al. 2007; Boyle et al. 2010). Furthermore, frailty was associated to the development and progression of different age-related diseases, such as myocardial infarction, rheumatoid arthritis, diabetes and hypertension (Hubbard et al. 2010). Finally, in the characterization of the clinical phenotype of frailty, it has been very important the demonstration of the role in this syndrome of multi-system impairments including immune/inflammatory, multiple hormonal and neuromuscular deregulations, as well as metabolic and vascular alterations and oxidative stress that commonly occur with aging (Ferrucci et al. 1999; Leng et al. 2002; Schalk et al. 2004; Walston et al. 2006; Barzilay et al. 2007; Landi et al. 2008; Hubbard et al. 2009; Blaum et al. 2009; Serviddio et al. 2009; Desai et al. 2010; Lustosa et al. 2010; Evans et al. 2010; Hyde et al. 2010; Maggio et al. 2010; Hubbard and Woodhouse 2010). In recent years, many operational definitions of frailty have been proposed. Some of them consider frailty as a continuum accumulation of self-reported deficits, reflecting the proportion of potential disabilities

present in a person (Mitnitski et al. 2002; Rockwood et al. 2006); others, consider frailty as a distinct clinical syndrome non synonymous of co-morbidity or disability and are based on direct measurement of functional parameters as grip strength, walking speed, unintentional weight loss and low physical activity (Fried et al. 2001). Among these last ones, Montesanto et al. (2010) by using a hierarchical cluster analysis that availed of specific geriatric parameters, identified specific aging phenotypes. The diagnostic and predictive soundness of this classification were confirmed by a detailed survival analysis showed higher survival chance for subjects characterized by lower frailty. Commonly, frailty is considered as the resultant of a multidimensional interplay of genetic, biological, psychosocial and environmental factors (Fried et al. 2001; Rockwood et al. 2004; Rockwood and Mitnitski, 2007; Bergman et al. 2007; Lang et al. 2009; Fulop et al. 2010; Landi et al. 2010).

Several lines of evidence suggest that epigenetic reprogramming of gene expression occurs during aging (Fraga 2009; Kawakami et al. 2009; Gravina and Vijg 2010; Rando 2010). A correlation between epigenetic DNA modifications and quality of aging has been shown by Fraga et al. (2005), who found global and locus-specific differences in DNA methylation in identical twins of various ages influenced by different environmental factors and lifestyle. *In vitro* models and studies carried out in tissues and blood DNA samples demonstrated a decrease in global cytosine methylation during aging. This resulted either from demethylation in transposable repetitive elements, or from a hypermethylation in promoter regions of specific genes, with a consequent decrease of correspondent mRNA levels (Castro et al. 2003; Bjornsson et al. 2008; Bollati et al. 2009). In humans, this hypermethylation has been observed for genes involved in cell cycle regulation, tumor-cell invasion, DNA repair, apoptosis, metabolism and cell signaling, implicating their potential role in age-related diseases

(Wilson et al. 1987; Oakes et al. 2003; Richardson 2003; Fuke et al. 2004; Fraga and Esteller 2007; Ling et al. 2008; Arai et al. 2010; Lee et al. 2010). In the present study, we investigated whether the frailty status of elderly people was associated to global DNA methylation levels, thus searching a correlation between the age-specific functional decline and epigenetic modifications. In addition we determined whether the above levels were subject to changes over time in agreement to changes in the frailty status of aging individuals. Global DNA methylation was measured by applying the assay developed by Anisowicz and coll. (2008) to peripheral blood DNAs collected from 318 subjects classified for frailty phenotype according to Montesanto et al. (2010). In a subsample both frailty evaluation and DNA methylation analysis were repeated after 7 years.

RESULTS

We carried out a series of control experiments in order to validate the reproducibility of the CpGglobal assay described in Matherials and Methods (Anisowicz et al. 2008). To this purpose we analyzed two sample of bacteriophage lambda DNAs and three human genomic DNAs as quality control.

Global DNA Methylation levels in control samples

Control Lambda DNA

Two samples of bacteriophage lambda DNA were analyzed: one that was unmethylated and the other that was in vitro methylated by using the bacterial M. HpaII methylase. Firstly, to verify the restriction conditions and the fully methylation we treated the two samples with the methyl-sensitive HpaII and the methyl-unsensitive MspI restriction endonucleases. As shown in Fig.1A, with respect to unmethylated DNA, no HpaII restriction fragments were observed in the methylated lambda DNA. In fact, HpaII

activity was greatly inhibited by the methylation on the internal cytosine residue in its recognition sequence. As expected, the MspI activity was not affected by the absence/presence of methylated cytosines. Then, the *CpG*lobal assay was applied to determine the GDMI values in the two lambda DNA samples. As expected, the GDMI value was significantly lower (about 0.1) in the methylated DNA sample than in the unmethylated DNA (about 0.9) (Fig.1B).

Control human genomic DNAs

Subsequently, *CpG*global assay was applied to three control samples of human genomic DNA: one fully unmethylated, one fully methylated and another obtained by mixing an equal ratio of unmethylated and methylated DNA. We restricted the samples as above described. We observed that when fully methylated human DNA was digested with HpaII, no restriction fragments were observed (Fig. 2A). Conversely, when fully unmethylated human DNA was digested with the same enzyme, several restriction fragments were observed, indicating the complete digestion of the sample. Moreover, the DNA sample obtained by mixing an equal ratio of unmethylated and methylated human DNA showed an intermediate result. In fact, the intensity of the lane of undigested DNA approximates the fifty percent of that untreated with HpaII, as from densitometric tracings (data not shown). As expected, the GDMI values in the three samples were about 0.1, 0.5 and 0.9 for the fully methylated DNA, for the mix of methylated and unmethylated DNA and for the fully unmethylated DNA, respectively (Fig. 2B).

On the whole, the results obtained by the above control experiments demonstrated the accuracy and an overall high reproducibility of the *CpG*lobal assay in measuring the global DNA methylation levels of the different sample analyzed.

Global DNA Methylation levels in the population sample

After the validation of the procedure, the *CpG*lobal assay was applied to measure the GDMI values of the DNA samples extracted from peripheral venous blood collected from 318 subjects (65- to 105- year-old).

The frequency of the GDMI values distribution in the population sample is reported in Figure 3. The distribution of these values in the sample is quite normal with a mean value of about 0.517. In addition, they were quite similar for men and women (p-value=0.424) and were not correlated with age (r=-0.040, p-value=0.474). These results suggested that the global DNA methylation levels do not correlate neither with the age nor with the gender of sample analyzed.

Subsequently, we wondered whether the GDMI values were correlated to the frailty status rather than to chronologic age. To answer this question we availed of the HCA classifications reported in Montesanto and co-workers (2010), that allowed to classify this sample in different aging phenotypes (see Materials and Methods). The mean GDMI values across the S1 and S2 groups were shown in Figure 4. We can observe that frail subjects of S_1 group exhibit GDMI values significantly higher than those prefrail (0.658±0.201 *vs* 0.508±0.223, respectively, p-value=0.006) and nonfrail (0.658±0.201 vs 0.521±0.196, respectively, p-value=0.006). In S_2 group no difference in GDMI values was detected across the frailty phenotypes (0.484±0.191 and 0.509±0.197 for very frail and frail, respectively). Moreover, GDMI values were quite similar for men and women in both groups (in S1 sample 0.534 ± 0.220 vs 0.522 ± 0.210, respectively; p-value=0.668; in S2 sample 0.514±0.217 vs 0.477±0.167, respectively; p-value=0.335). These results indicated that a correlation between the global DNA methylation levels and the frailty phenotype exists in middle-aged subjects. On the contrary, this correlation was not observed in centenarians.

Then, in order to better evaluate the relationship between DNA methylation levels and degree of frailty, 37 prefrail and nonfrail subjects of S_1 sample were revisited after 7 years from the baseline visit. Figure 5 shows GDMI values at baseline (black histogram) and after the follow-up period (grey histogram) with respect to the changes in the frailty status after this period. We can observe that in subjects who after the follow-up period have maintained their nonfrail or prefail frailty status or have changed their frailty status from nonfrail to prefrail, mean GDMI value did not show significant changes over time (about 0.4). On the contrary, in subjects who became frail mean GDMI value was significantly increased (about 0.7) over time compared to the first measurement.

DISCUSSION

Understanding the mechanisms that modulate the quality of aging remains one the most challenging research topics. Several lines of evidence have demonstrated how the characterization of frailty, that represents a state of vulnerability for adverse health outcomes, may contribute to disentangle the molecular mechanisms influencing the functional decline of elderly people and thus to characterize and to better define the aging process (Fried et al. 2004). The impact of genetic variants of both nuclear and mitochondrial DNA on the inter-individual susceptibility to functional decline and vulnerability to diseases in the elderly people has been largely demonstrated (Maggio et al. 2006; Moore et al. 2010; Matteini et al. 2010). Similarly, different reports have shown the influence of environmental and social factors on frailty.

A “drawbridge” across genetic factors and environments may be represented by epigenetic variations which in turn depend on hereditary, environmental and stochastic factors and might explain the inter-individual variability in the frailty status (Sutherland

and Costa 2003; Fraga 2009; Schneider et al. 2010). Indeed, most studies on DNA methylation demonstrated that aging is associated with a relaxation of epigenetic control, thus contributing to some of age-related phenotypes such as functional and cognitive decline and the development of pathologies.

In the present study we looked at epigenetic changes occurring in blood collected from individuals classified by a precise frailty phenotype based on the individual degree of cognitive, functional and psychological status (Montesanto et al. 2010). We availed of a method that, although could not provide precise quantitative measurements of the DNA methylation levels, it is highly effective in comparative analyses of these levels among different samples. It is also worth mentioning that the use of DNA from peripheral blood can be effective in global DNA methylation studies, although this analysis misses the important effects of tissue specific methylation (Thompson et al. 2010).

As it regards 65-85 years old subjects, we found no correlation between global DNA methylation levels and age but frail individuals (with high vulnerability) exhibit lower global methylation levels than those that are in a better condition (prefrail and nonfrail). On the contrary, no difference was observed in these levels among centenarians, despite their differential classification in frail and very frail subjects. To our knowledge, this is the first study applied to an elderly population demonstrating as a correlation between global methylation levels and frailty occurs in aging. Therefore the loss of global DNA methylation in aging seems to be more specifically associated to the functional decline and not to chronological age. This assumption is particularly interesting because it could provide a re-interpretation of some studies in which variations in methylation levels were not observed analyzing middle age-advanced individuals without comparing them to younger control (Tra et al. 2002; Siegmund et al. 2007; Schneider et al. 2010). In fact, in agreement with literature data, the more significant changes in DNA

methylation levels during the lifetime occur predominantly before the middle age. Moreover, epigenetic age-related changes have been mainly observed in studies where locus-specific and not global epigenetic differences were analyzed (Fraga et al. 2005; Poulsen et al. 2007; Christensen et al. 2009). To this regard, the recent article published by Bollati and coworkers showed a decrease at Alu methylation during aging, while the LINE-1 methylation was less consistent. Thus, the association between age and methylation levels could be restricted to specific genomic components.

In addition, we did not observe a link between DNA methylation levels and gender, although several studies found higher global DNA methylation levels in males regardless to age of the subjects analyzed (Fuke et al. 2004; Shimabukuro et al. 2006). However, as in the case of the association between age and global methylation, gender-associated differences in DNA methylation were restricted to specific loci and often to mitotic cells (El-Maarri et al. 2007).

In this paper we reported also the results obtained in a longitudinal study where DNA methylation levels and frailty status were reanalyzed after 7 years of follow-up. Methylation levels were unchanged in subjects who after the follow-up period did not became frail. On the contrary, in subjects who were prefrail at baseline and became frail after the follow-up period, these levels were significantly decreased and were comparable to those observed in frail subjects at baseline.

Since methylation is associated to gene silencing, it is possible that in old frail subjects, a reduction in DNA methylation could activate genes being in a repressed status. Some of these genes might be needed to maintain the reserve capacity and the efficiency in the response to adverse stressors at levels ensuring the survival (Coneyworth et al. 2009). Likely, the hypomethylation observed in frail subjects can be associated to an accentuated deficiency in to achieve the remethylation after DNA replication that, as

reported in Gravina and Vjig (2010), occurs during aging. In addition, the above hypomethylation could derepress silenced retrotransposons, such as L1 or IAP, thus inducing genome instability that might account for the decline typical of frailty status (Barbot et al. 2002; Menendez et al. 2004; Maslov and Vijg 2009). Moreover, longitudinal data provide an important contribute to studies which consider frailty as a dynamic process. In fact, our findings are in line with those reported by Bjornsson et al. (2008) who observed time-dependent changes in global DNA methylation within the same individual in two separate populations (one from Iceland and one from Utah) exhibiting both decrease and increase in methylation greater than 20% over an 11- to 16- year span. A progressive loss DNA methylation over time in repetitive elements disperse through the genome, particularly in Alu elements, was also reported by Bollati et al. (2009).

On the whole, our results contribute to understand how changes in methylation levels are associated with the frailty status of the individuals. We are not able to know whether the changes in frailty status, likely determined by processes under genetic control, induce the changes in methylation levels or, vice versa, whether these levels determine the frailty status. In any case our results suggest, in agreement with a series of previous evidences, that specific age-related phenotypes are modulated by not only genetic factors but also dietary, life style and environment.

EXPERIMENTAL PROCEDURES

Population sample

A total of 318 unrelated individuals (144 men and 174 women) 65-105 years old (median age 79.5 years) participated in the present study. All the subjects lived in Calabria (South of Italy) and their origin in the area had been ascertained up to the

grandparents generation. A more detailed sample description can be found elsewhere (De Rango et al. 2010). Health status was ascertained by medical visit carried out by a geriatrician who also conducted a structured interview including questions on common diseases occurred in the past. At the same time of the visit, peripheral venous blood samples were also obtained. Before the interview each subject consented to her/his phenotypic and genetic data to be used anonymously for genetic studies on aging (informed consent).

In a previous work, by using a hierarchical cluster analysis (HCA), by availing of specific geriatric parameters, this sample was used to identify specific aging phenotypes (Montesanto et al. 2010). In particular, the sample was analyzed considering two different age groups. The first (S_1) included 217 subjects (94 males and 123 females) 65-89 years old (median age 75 years); the second (S_2) included 101 subjects (50 males and 51 females) older than 90 years (median age 99 years). Then, within each group, a HCA which considered physical, cognitive, and psychological parameters as classification variables were used to identify groups of subjects homogeneous for aging phenotypes. By using this approach in the first sample (65-89 years old subjects) three clusters were identified. The analysis of the classification variables within these clusters allowed the authors to define the three clusters as nonfrail (the cluster with subjects showing the best scores for the classification variables), frail (the clusters with subjects showing the worst scores for the classification variables), and prefrail (the cluster with subjects showing intermediate scores for the classification variables). In the second sample (subjects older than 90 years) two clusters were identified. Similarly to the first classification, the two clusters obtained were defined as frail (the cluster with subjects showing the best scores for the classification variables) and very frail (the cluster with subjects showing the worst scores for the same variables).

The diagnostic and predictive soundness of these classifications were confirmed by a 3-year longitudinal study. In fact, a detailed survival analysis showed higher survival chance for subjects characterized by lower frailty in these classifications.

In order to better evaluate the relationship between DNA methylation levels and degree of frailty, a randomly selected sample consisting of 98 prefrail and nonfrail subjects of S_1 group were revisited after 7 years from the baseline visit. During the follow-up period 55 subjects (56%) died. Of the remaining 43 alive subjects, 6 refused the visit, while for the remaining other (37) a new multidimensional geriatric evaluation was performed and a new blood sampling for the evaluation of DNA methylation was provided.

In vitro DNA methylation of control lambda DNA

500 nanograms of lambda DNA (Sigma) were completely methylated by using 2.5 U of M.HpaII methylase (New England Biolabs). The mix was incubated at 37°C for 3 hours and successively at 65°C for 15 minutes to inactivate the enzyme.

Restriction analysis of control lambda DNAs and control human genomic DNAs

100 nanograms of methylated and unmethylated Lambda DNA were separately incubated with 5 U of HpaII and MspII restriction endonucleases (New England Biolabs) at 37°C overnight and successively at 65°C for 20 minutes to inactivate the endonucleases. The samples were loaded on to a 1.4 % agarose gel, electroforesed in TAE (Tris/Acetate/EDTA) buffer and stained with etidium bromide.

100 nanograms of CpGenome^{TM} Universal Methylated DNA (Chemicon), 100 nanograms of CpGenome^{TM} Universal Unmethylated DNA (Chemicon) and 100 nanograms of a mixture obtained combining 50 ng of CpGenome Universal methylated and 50 ng of unmethylated DNA were separately incubated with 5 U HpaII and MspII restriction endonucleases at 37°C overnight and successively at 65°C for 20 minutes to

inactivate the endonucleases. The samples were loaded on to 1.4 % agarose gel, electrophoresed in TAE (Tris/Acetate/EDTA) buffer and stained with ethidium bromide.

Measurement of Global DNA Methylation

DNA was extracted from blood buffy coats following standard procedures. The DNA concentration and 260/280 absorbance ratio were determined using a biophotometer (Eppendorf). The Global DNA methylation levels of control Lambda DNA, control Human DNAs and population sample DNAs were estimated by using the *CpG*lobal method, designed and developed by Anisowicz et al. (2008). Briefly, 2 μM of both Biotin-11-dCTP and Biotin-11-dGTP (Perkin Elmer) were added to digested DNA samples in an end-fill reaction of 20 μl carried out in presence of biotynilation buffer (40mM Tris-HCl pH 7.5, 20 mM Tris-HCl, 50 mM NaCl), and 2 U of Sequenase (USB Corporation). After incubation at 37°C for 30 minutes, 100 μl of Reacti-BindTM DNA Coating Solution (Pierce) were added and the samples were shaken in an orbital platform at room temperature overnight. The solution was removed and the samples were washed 3 times with Dulbecco's Phosphate Buffered Saline (PBS, Sigma). Then, 200 μl of the Detector Block Solution (KPL) were added and the mixtures were incubated at room temperature for 30 minutes. After the removal of the solution, 150 μl of the Detector Block Solution containing 0.5 μg/ml of HRP Streptavidin (KPL) were added and the samples were incubated at room temperature for 30 minutes. After the incubation, the Detector Block Solution was removed and the samples were washed 4 times with Biotin Wash Solution 1X (KPL). Then, 150 μl of LumiGlo Chemiluminescence substrate (KPL) were added and after 2 minutes the chemiluminescence emitted from each sample was quantified in a Lumat LB9507 luminometer (EG&G Bertold). Each sample was analyzed three independent times in

triplicates. In order to determine the possible "background noise" and, thus, to calculate the net luminescence for each sample a control lacking of enzyme was also analyzed. The data were calculated as Global DNA Methylation Index (GDMI) by dividing the mean net luminescence values for the HpaII enzyme to the mean net luminescence values for the MspI enzyme. Thus, the GDMI values inversely correlate to the global DNA methylation levels.

Statistical analysis

Descriptive statistics for continuous and categorical variables were used to describe socio-demographic characteristics, the frailty phenotypes and the GDMI values of the study sample. For continuous variables measures of central tendency and dispersion, including mean, median and standard deviations (SD) were reported. Categorical variables were examined by analyzing the relevant frequency distributions. ANOVA test followed by multiple comparison test (Dunnett's test) was used to compare the values of GDMI among the frailty groups defined by the cluster analysis (CA) approach. Linear regression analysis was performed for testing the dependence between GDMI and age.

After the follow-up period, in order to determine the actual frailty status of the 37 subjects (re-classification) a Classification Tree (CT) model has been used (Breiman et al. 1984). In fact, the main HCA drawback is that classification of new cases is usually not affordable, without repeating the whole HCA analysis. Thus, we used a supervised classification approach in order to obtain easy – to – understand model that can be used for classifying the revisited subjects. In brief, we applied the CT algorithm to the baseline data by considering the classification provided by HCA analysis in S1 group as dependent variable, while the geriatric parameters (HG, MMSE, ADL and SRHS) as independent variables. Adjusted values (i.e. with normalization) were used for all

variables (Montesanto et al. 2010). The performance of the CT model was evaluated in terms of accuracy using a 10-fold cross-validation strategy. The obtained model was then used to re-classify the revisited subjects.

Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, Ill). A significance level of $\alpha = 0.05$ was chosen in all the tests.

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LEGENDS OF FIGURES

Figure 1. Control DNA lambda bacteriophage analysis. **A)** Ethidium bromide-staining agarose gel showing unmethylated and methylated human DNAs digested with HpaII and MspI.

ND: human DNA not digested. **B)** GDMI values of unmethylated and methylated DNAs. The values represent the main of three independent triplicate experiments with standard error mean.

Figure 2. Control human DNA analysis. **A)** Ethidium bromide-staining agarose gel showing unmethylated and methylated human DNAs and of a mixture of equal amount of unmethylated and methylated human DNAs digested with HpaII and MspI.

ND: human DNA not digested. **B)** GDMI values of unmethylated, methylated and of a mixture of unmethylated and methylated human DNAs. The values represent the main of three independent triplicate experiments with standard error mean.

Figure 3. Frequency distribution of GDMI values in the total population sample

Figure 4. Mean GDMI values across the groups defined by Cluster Analysis in S_1 sample

Figure 5. Mean GDMI values across the groups defined by Cluster Analysis in S_2 sample

Figure 6. Variations of GDMI values with respect to the variations of the frailty status after the follow-up period

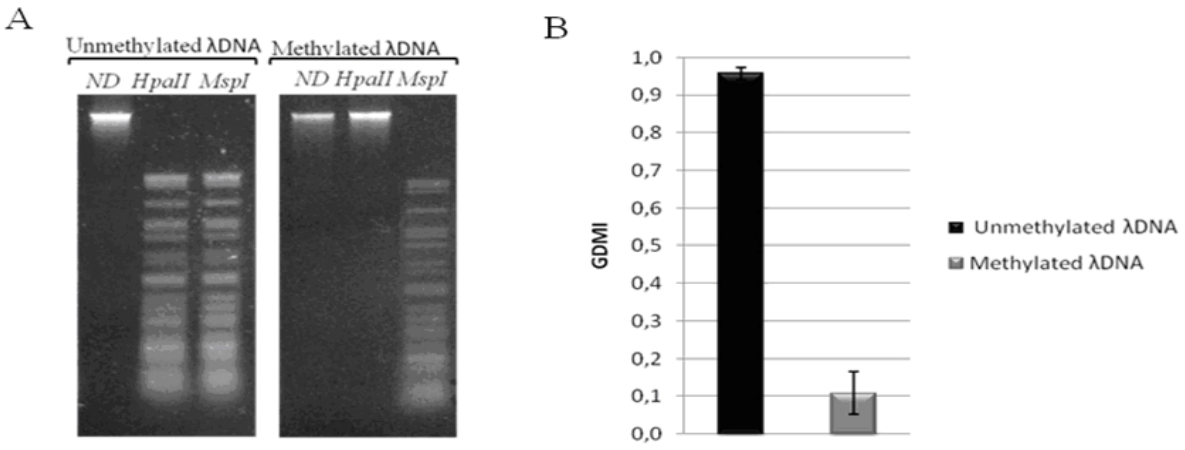

A
Unmethylated λDNA
Methylated λDNA
ND HpaII MspI
ND HpaII MspI
B
GDMI
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
Unmethylated λDNA
Methylated λDNA

Figure 1.

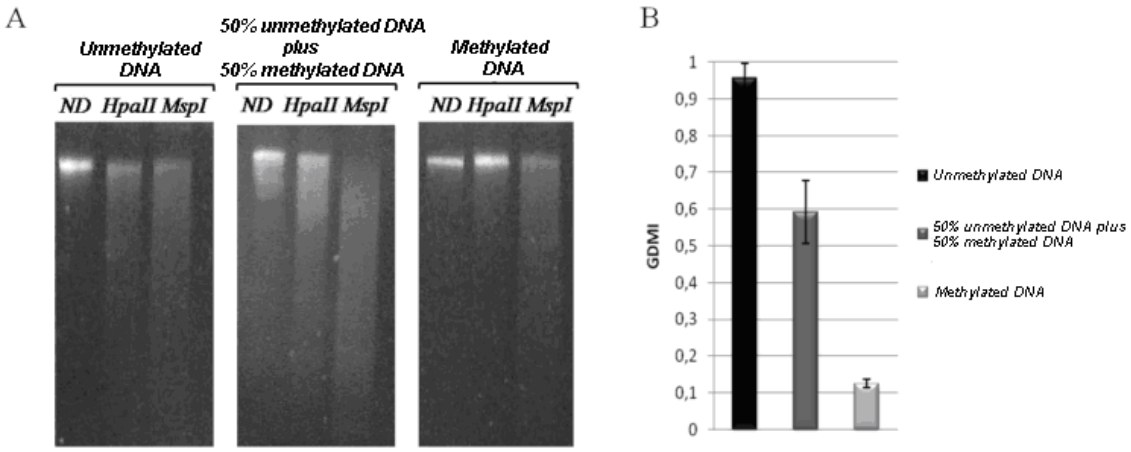

A
Unmethylated DNA
50% unmethylated DNA plus 50% methylated DNA
Methylated DNA
ND HpaII MspI
ND HpaII MspI
ND HpaII MspI
B
GDMI
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Unmethylated DNA
50% unmethylated DNA plus 50% methylated DNA
Methylated DNA

Figure 2.

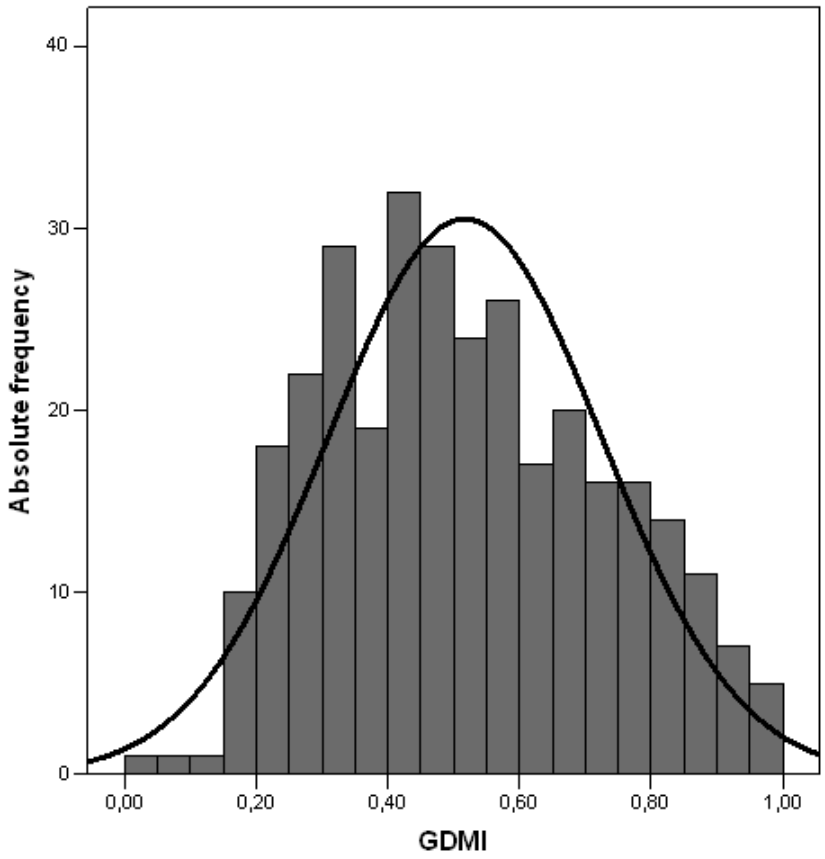


Figure 3.

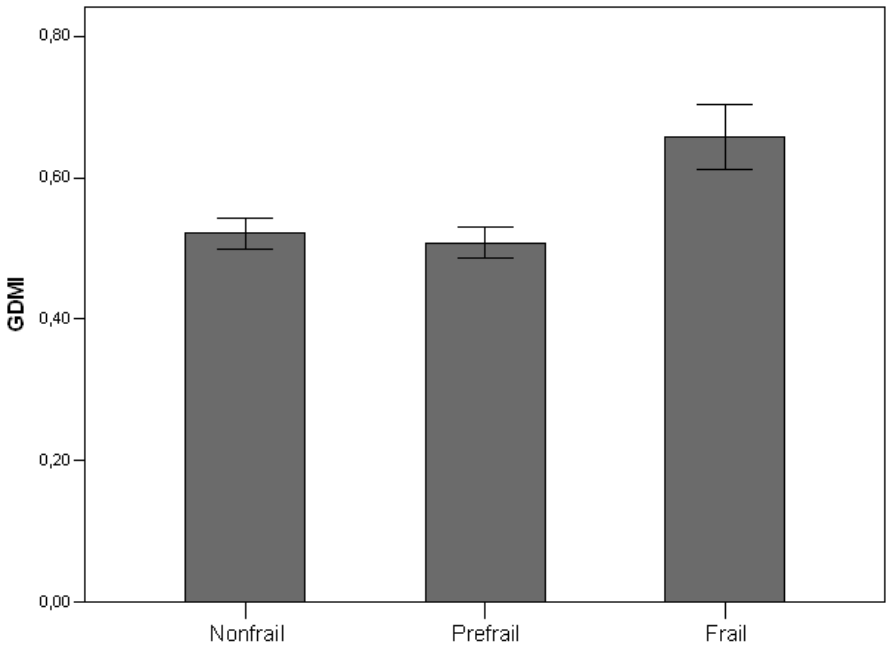


Figure 4.

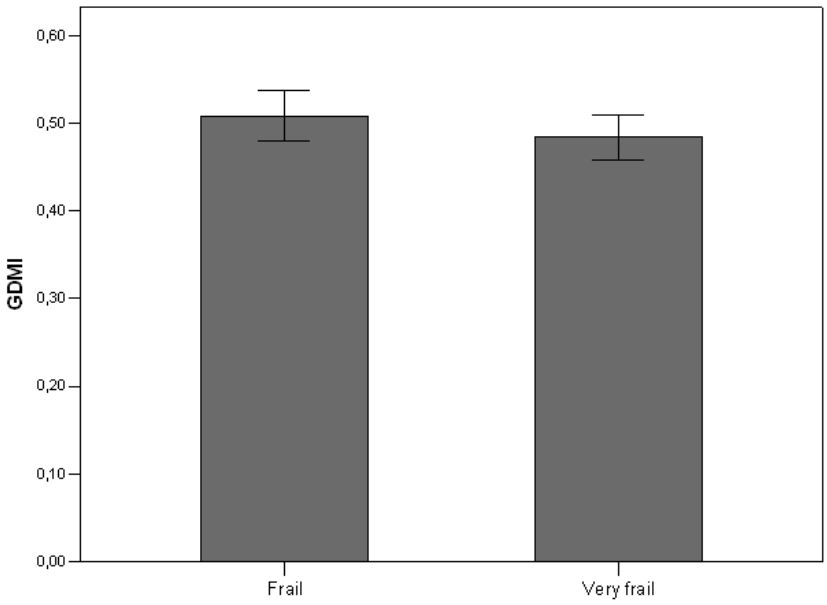
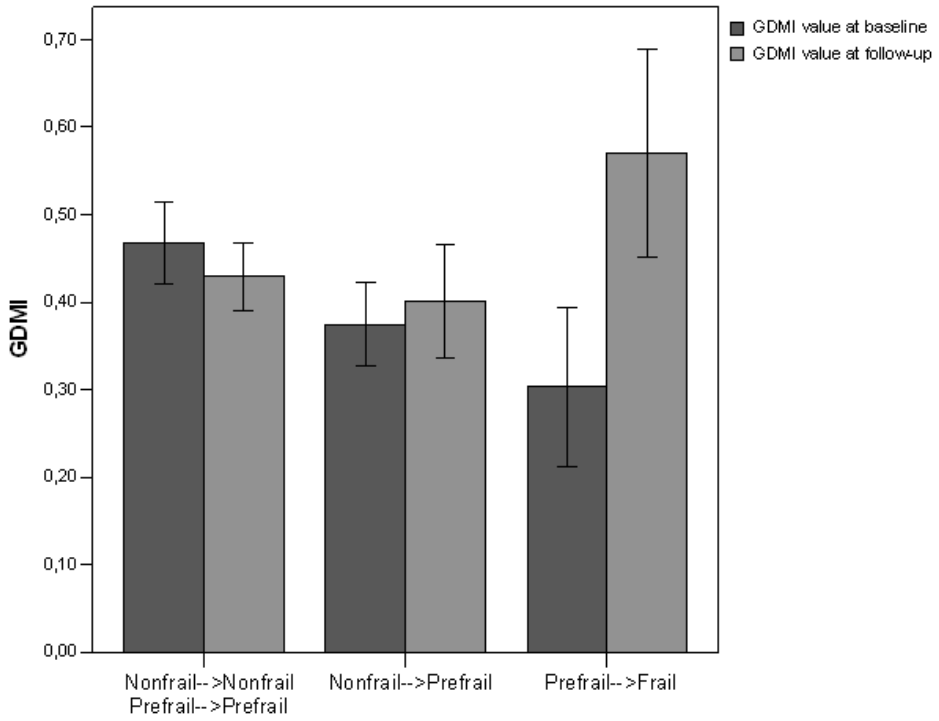


Figure 5.



NF-NF or PF-PF P-value=0.486
NF-PF P-value=0.684
PF-F P-value=0.132

Figure 6.

The variability of mitochondrial DNA modulates global DNA methylation levels

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ABSTRACT

Epigenetic changes are modulated by environmental and genetic factors. We investigated whether these changes are affected by mitochondrial DNA (mtDNA) inherited variants. In a population of elderly subjects, we found that global DNA methylation levels were higher in J haplogroup carriers than in non J carriers. This observation was consistent with results obtained by *in vitro* studies on cybrid lines harbouring different mtDNA, where DNA hypermethylation in J cybrid was observed, correlated with an overexpression of the *MAT1A* gene. Our findings indicated that mtDNA-specific interactions between mitochondria and nucleus regulate epigenetic changes.

KEYWORDS: cybrid cells; mtDNA variability; global DNA methylation; *MAT1A* gene; ATP; ROS.

1. INTRODUCTION

The increasing interest of researchers in disentangling the relationships between mitochondrial efficiency and complex phenotypes has highlighted the central role played by mitochondria in a wide variety of cellular pathways involved in aging and age-related diseases (Wallace 2005; Swerdlow et al. 2010; Winklhofer and Haass 2010). This is due to the central position of mitochondria between energy uptake and energy production. As a consequence, they are involved in a number of crucial cellular processes such as heat production, apoptosis regulation, cellular differentiation, but especially in the production and the regulation of the Reactive Oxygen Species (ROS), toxic by-products of the cellular metabolism (Kowaltowski et al. 2009; Circu and Aw 2010; Koopman et al. 2010). In particular, a ROS-related decrease of mitochondrial DNA (mtDNA) copies and an accumulation of somatic mtDNA deletions or point mutations, with consequent general decay of the mitochondrial function, have been extensively described to occur during aging (Michikawa et al. 1999; Barazzoni et al. 2000; Terzioglu and Larsson 2007; Clay Montier et al. 2009; Wallace 2010; Larsson 2010). Several lines of evidences also demonstrated that, besides somatic mutations, inherited variants of mtDNA are associated with aging (De Benedictis et al. 1999; De Benedictis et al. 2000a; Ross et al. 2001; Niemi et al. 2005; Santoro et al. 2006; Nishigaki et al. 2010; Moore et al. 2010). In fact, these variants, determining mtDNA haplotypes and groups of haplotypes named haplogroups, contribute to the interindividual susceptibility to aging and late-onset pathologies, such as Alzheimer's and Parkinson's diseases, cardiovascular disease, diabetes and cancer (van der Walt et al. 2003; van der Walt et al. 2004; Takagi et al. 2004; Khusnutdinova et al. 2008; Fang et al. 2010).

The molecular mechanisms by which mtDNA variability influences these traits are not yet completely clarified. However, it seems to be likely that mtDNA haplotypes can modulate nuclear genome, possibly by modulating the efficiency of oxidative phosphorylation (Tranah 2010; Gomez-Duran et al. 2010; Gil Borlado et al. 2010).

In mice and humans, a few examples of these interactions between mitochondrial and nuclear DNA have been reported, supporting the hypothesis that some age related traits and diseases require particular interactions between the two genomes (De Benedictis et al. 2000b; Bykhovskaya et al. 2000; Johnson et al. 2001; Roubertoux et al. 2003; Pravenec et al. 2007; Shankar et al. 2008; Finley and Haigis 2009). Evidences of this communication have also emerged by studies carried out in cybrid cell lines, engineered cells that share the same nuclear genome but harbor different mitochondrial genome (King and Attardi, 1989). In particular, in these cells, the cell viability, the intracellular calcium dynamics, the mtDNA copy number, the mitochondrial ROS production, and the expression levels of several nuclear-encoded genes, including those encoding some cytokines, HSP60 and HSP75, were demonstrated to be influenced by the mtDNA variability (Vives-Bauza 2006; Bellizzi et al. 2006; 2009; Kazuno et al. 2008; Suissa et al. 2009; Smits et al. 2010).

The mitochondria correlation with ageing is more recently emerging also from a different perspective that is the analysis of epigenetic changes affecting DNA. Among these changes, the methylation of cytosines in DNA plays an important role in regulating the expression of genes (Goldberg et al. 2007; Lennartsson and Ekwall 2009). In fact, the efficiency of mitochondrial energy producing machinery can modulate the activity of Methionine Adenosyl Transferase (MAT), enzyme responsible for the synthesis of S-Adenosyl Methyonine (SAM) from L-methionine and ATP (Pajares et al. 1992; Avila et al. 1998; Wallace and Fan 2010). It follows that

dysfunctions in mitochondrial activity may have direct effects on epigenetic markers and, consequently, lead to a disarrangement of the pattern of gene expression and of cellular and organism functioning. Indeed, the presence of 8-Hydroxyl-2'-deoxyguanosine (8-OH-dG), the most common ROS-induced DNA base lesion, in CpG nucleotide sequence diminishes the ability of DNA methyltransferases to methylate cytosines (Turk et al. 1995a; 1995b; Valinluck et al. 2004). Smiraglia and coworkers (2008), reported that the depletion of mtDNA (Rho^0 cells) induced changes in DNA methylation of different genes and that these changes were partially reversed by the re-introduction of mtDNA into the Rho^0 cells. On the other hand, as mitochondrial functions strongly rely on proteins that are coded by the nuclear genome, epigenetic changes in the methylation status of nuclear genes, and thus in their expression, affect the above mitochondrial function with the onset of a vicious cycle. This very complex interplay of mitochondrial function and epigenetic modifications has important consequences on the ageing process, although only a few aspects have so far been elucidated.

Starting from these observations, we carried out a population study and *in vitro* studies in order to investigate the relationship between age-related epigenetic modifications and mtDNA-specific interactions between mitochondria and nucleus. Global DNA methylation was measured by applying the assay developed by Anisowicz and coworkers (2008) to peripheral blood DNAs collected from elderly subjects and to DNA samples extracted from cybrid cell lines harbouring mtDNA molecules of different haplogroups.

2. MATERIAL AND METHODS

2.1 Population sample

A total of 354 (163 men and 191 women) unrelated individuals 65-107 years old (median age 81 years) participated in the present study. All the subjects lived in Calabria (South of Italy) and their origin in the area has been ascertained up to the grandparents generation. A more detailed sample description can be found elsewhere (De Rango et al. 2010). In brief, health status was ascertained by medical visit carried out by a geriatrician, who also conducted a structured interview including questions on common diseases occurred in the past. At the same time of the visit, peripheral blood samples were also obtained. Before the interview each subject consented to her/his phenotypic and genetic data to be used anonymously for genetic studies on aging (informed consent).

2.2 Cell lines and culture conditions

143B.TK$^-$ osteosarcoma cells and cybrid cell lines (H, J, U, X, and T) were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 4.5 g/l glucose and 110 μg/ml pyruvate, supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 50 μg/ml gentamycin (Invitrogen) (Bellizzi et al. 2009). Rho^0 cells were grown in the above medium supplemented with 50 μg/ml uridine (Sigma). 143B.TK$^-$, cybrid cell lines and Rho^0 cells were cultured in a water-humidified incubator at 37°C in 5% CO_2/ 95% air.

2.3 DNA samples

Six milliliters of venous blood was drawn from each subject. Plasma/sera were used for routine laboratory analyses, while DNAs were extracted from blood buffy coats following standard procedures.

DNA samples from 143B.TK$^-$ osteosarcoma cells, Rho^0 cells and cybrids were obtained by phenol/chloroform purification. The DNA concentration and 260/280 absorbance ratio were determined spectrophotometrically.

2.4 ***In vitro DNA methylation of control lambda DNA***

500 nanograms of lambda DNA (Sigma) were completely methylated by using 2.5 U of M.HpaII methylase (New England Biolabs). The mix was incubated at 37°C for 3 hours and successively at 65°C for 15 minutes to inactivate the enzyme.

2.5 Restriction analysis of DNA samples

100 nanograms of methylated and unmethylated Lambda DNA were incubated separately with 5 U of HpaII and MspII restriction endonucleases (New England Biolabs) at 37°C overnight and successively at 65°C for 20 minutes to inactivate the endonucleases. The samples were loaded on to a 1.4 % agarose gel, electrophoresed in TAE (Tris/Acetate/EDTA) buffer and stained with ethidium bromide.

100 nanograms of CpGenome^{TM} Universal Methylated DNA (Chemicon), 100 nanograms of CpGenome^{TM} Universal Unmethylated DNA (Chemicon) and 100 nanograms of a mixture obtained combining 50 ng of CpGenome Universal methylated and 50 ng of unmethylated DNA were incubated separately with 5 U HpaII and MspII restriction endonucleases at 37°C overnight and successively at 65°C for 20 minutes to inactivate the endonucleases. The samples were loaded on to 1.4 % agarose gel, electrophoresed in TAE (Tris/Acetate/EDTA) buffer and stained with ethidium bromide.

100 nanograms of each of population sample DNAs and DNAs extracted by the cell lines were incubated separately with 5 U of HpaII and 5 U of MspII restriction endonucleases in a total volume of 30 µl at 37°C overnight and successively at 65°C for 20 minutes to inactivate the two endonucleases.

2.6 Measurement of Global DNA Methylation levels

The Global DNA methylation levels of control DNA samples, population sample DNAs and DNAs extracted by the cell lines were estimated by using the *CpG*lobal method,

designed and developed by Anisowicz et al. (2008). Briefly, 2 μM of both Biotin-11-dCTP and Biotin-11-dGTP (Perkin Elmer) were added to digested DNA samples in an end-fill reaction of 20 μl carried out in presence of biotynilation buffer (40 mM Tris-HCl pH 7.5, 20 mM Tris-HCl, 50 mM NaCl), and 2 U of Sequenase (USB Corporation). After incubation at 37°C for 30 minutes, 100 μl of Reacti-BindTM DNA Coating Solution (Pierce) were added and the samples were shaken in an orbital platform at room temperature overnight. The solution was removed and the samples were washed 3 times with Dulbecco's Phosphate Buffered Saline (PBS, Sigma). Then, 200 μl of the Detector Block Solution (KPL) were added and the mixtures were incubated at room temperature for 30 minutes. After the removal of the solution, 150 μl of the Detector Block Solution containing 0.5 μg/ml of HRP Streptavidin (KPL) were added and the samples were incubated at room temperature for 30 minutes. After the incubation, the Detector Block Solution was removed and the samples were washed 4 times with Biotin Wash Solution 1X (KPL). Then, 150 μl of LumiGlo Chemiluminescence substrate (KPL) were added and after 2 minutes the chemiluminescence emitted from each sample was quantified in a Lumat LB9507 luminometer (EG&G Bertold). Each sample was analyzed three independent times in triplicates. In order to determine the possible "background effect" and, thus, to calculate the net luminescence for each sample, a control lacking of enzyme was also analyzed. The data were calculated as Global DNA Methylation Index (GDMI) by dividing the mean net luminescence values for the HpaII enzyme to the mean net luminescence values for the MspI enzyme. Thus, the GDMI values inversely correlate to the global DNA methylation levels.

2.7 MtDNA analysis

Haplogroups typing was carried out by restriction analyses of mtDNA according to Torroni et al. (1996). The DNA analyses to ascertain the haplogroup classification are described elsewhere (De Benedictis et al. 1999).

2.8 Gene Expression Analysis

Total RNA was extracted from cells by using RNeasy mini Kit (Qiagen). The RNA concentration was measured for each sample by using a spectrophotometer at an absorbance ratio of 260/280 nm. RNA samples were treated with DNA-free DNase to remove any residual genomic DNA contamination. Reverse transcription was carried out by using the ImPromII Kit (Promega). An RT mix including 500 ng of total RNA and 0.5 μg of oligo-dT primers was preheated at 70°C for 5 min. The reaction was carried out in a 40 μl final volume containing 1X RT buffer, 0.5 mM of each dNTP, 3 mM $MgCl_2$, 20 U RNase inhibitor, and 5 U reverse transcriptase. The mix was incubated at 25°C for 5 min, then 37°C for 1 h and, successively, at 95°C for 10 min to inactivate the reverse transcriptase. The cDNAs obtained were then used as a template for Real-time PCRs was carried out by using the SYBR Green qPCR Master Mix (Promega) in a StepOne Plus machine (Applied Biosystems). Forward primers and reverse primers were as follows: DNMT1For 5'-AGAACGGTGCTCATGCTTACA-3'; DNMT1Rev 5'-GGGGCTAGGTGAAGGTTCAG-3'; DNMT3AFor 5'-CCGATGCTGGGGACAAGAAT-3'; DNMT3ARev 5'-CCCGTCATCCACCAAGACAC-3'; DNMT3BFor 5'-CCAATCCTGGAGGCTA TCCG-3'; DNMT3BRev 5'-ACTGGGGTGTCAGAGCCAT-3'; MBD2For 5'-CCCACAACGAAT GAATGAACAGC-3'; MBD2Rev 5'-TGAAGACCTTTGGGTAGTTCCA-3'; MBD4For 5'-CCCCACCGTCACCTCTAGT-3'; MBD4Rev 5'-GTAGCACCAAACTGAGCAGAA-3'; MAT1AFor 5'-

CAGTGTGCAAGACCGGCAT-3'; MAT1ARev 5'-TAGCCGATGTGCTTGAT GGTG-3'; MAT2BFor 5'-TCTGGGAATTTAGCAAAGGAAGC-3'; MAT2BRev 5'-GCTCCTAGATTGTTCTCCAGGAC-3'; GAPDHFor 5'-ATGGGGAAGGTGAAGGTCG-3'; GAPDHRev 5'-GGGGTCATTGATGGCAACAATA-3'. The PCR mixture (10 µl) contained 1 µl of cDNA, 1X GoTaq® qPCR Master Mix, 0.2 µM of each primer, and 1X CXR Reference Dye. The thermal profile used for the reaction included a 2-minute heat activation of the enzyme at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds, followed by melt analysis ramping at 60°C to 95°C. All measurements were taken in the log phase of amplification. Negative controls (in which water instead of cDNA was added) were also run in each plate. Furthermore, contamination of the RNA samples by genomic DNA was excluded by an analysis without prior cDNA conversion. StepOne Software V 2.0 was used to analyze data, in which the results obtained in the 143B.TK$^-$ Osteosarcoma cell line were used as reference sample and the relative expression levels were determined comparing with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression.

2.9 ATP levels measurement

The ATP measurement was performed by using ENLITEN ATP assay system bioluminescence detection kit for ATP measurement (Promega), according to the manufacturer's protocol. Briefly, 1x10^6 143B.TK$^-$, Rho^0, and cybrid cells were seeded in 6 well/plates. In the exponential growth phase, cells were washed with PBS, trypsinized and counted on a hemocytometer with an inverted light microscope. 5 x10^5 cells were collected and centrifuged at 1500 rpm for 5 min at 4°C. Then, the intracellular ATP was extracted by resuspending pellets 100 µl in 0.5% trichloroacetic acid (TCA). After ATP extraction, the TCA in the samples was neutralized and diluted by additing Tris-Acetate

buffer pH 7.75. Lastly, the bioluminescence was assessed by using a Lumat LB9507 luminometer (EG&G Berthold). The ATP content was expressed as Relative Luminescence Units (RLU). Three independent experiments were carried out in triplicate, and negative controls were also analyzed to determine the amount of background that has been subtracted from each sample.

2.10 Total ROS/Superoxide detection

Intracellular reactive oxygen species were quantified by using two fluorescent dye reagents: Oxidative Stress Detection Reagent (green), that reacts directly with a wide range of ROS/RNS species and Superoxide Detection Reagent (orange), that reacts specifically with superoxide (O^{2-}) (Total ROS/Superoxide Detection Kit, Enzo Life Science).

5x105 143B.TK^{-}, Rho^{0}, and cybrid cells were seeded in 6 well/plates. In the exponential growth phase, cells were washed with 1X Wash buffer (Enzo Life Science), collected and centrifuged at 1500 rpm for 5 min. Pellets were washed with 1X wash buffer (Enzo Life Science), and the cell suspensions were centrifuged at 1500 rpm for 5 min. Then, the supernatant was discarded and the cell pellet was resuspended in 500 µl of ROS/Superoxide Detection mix (Enzo Life Science). The cell suspensions was incubated for 30 minutes at 37°C in the dark and then analyzed by flow cytometry.

2.11 Statistical analysis

Descriptive statistics for continuous and categorical variables were used to describe socio-demographic characteristics, mtDNA haplogroup classification and the Global DNA Methylation Index (GDMI) values. For continuous variables measures of central tendency and dispersion, including mean, median and standard deviations (SD), were reported. Categorical variables were examined by analyzing the relevant frequency

distributions. Student's t-test was used to compare the values of GDMI among the mtDNA haplogroup classification.

Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, Ill). A significance level of $\alpha = 0.05$ was chosen in all the tests.

For gene expression levels and ATP and ROS/Superoxide quantification we adopted one-way analysis of variance for multiple comparisons and student's t-test for pair-wise comparisons. Significance level was defined as $\alpha=0.05$.

3. RESULTS

A series of control experiments was carried out in order to validate the reproducibility of the *CpG*global assay above described. To this purpose we analyzed two sample of bacteriophage lambda DNAs and three human genomic DNAs as quality control.

3.1 Global DNA Methylation levels in control samples

Control Lambda DNA

Two samples of bacteriophage lambda DNA were analyzed: one was unmethylated; the other was *in vitro* methylated by using the bacterial M.HpaII methylase. Firstly, to verify the restriction conditions and the fully methylation we treated the two samples with the methyl-sensitive HpaII and the methyl-unsensitive MspI restriction endonucleases. Fig.1A shown that the unmethylated DNA was digested by HpaII while no HpaII restriction fragments were obtained in the methylated lambda DNA. By contrast, the MspI activity was not affected by the absence/presence of methylated cytosines. Then, the *CpG*lobal assay was applied to determine the GDMI values in the two lambda DNA samples. As expected, the GDMI value was significantly lower (about 0.1) in the methylated DNA sample than in the unmethylated DNA (about 0.9) (Fig.1B).

Control human genomic DNAs

Subsequently, *CpG*global assay was applied to three control samples of human genomic DNA: one fully unmethylated, one fully methylated and another obtained by mixing an equal ratio of unmethylated and methylated DNA.

We restricted the samples as above described. We observed that when fully methylated human DNA was digested with HpaII no restriction fragments were observed (Fig. 2A). Conversely, when fully unmethylated human DNA was digested with the same enzyme, several restriction fragments were observed, indicating the complete digestion of the sample. Moreover, the DNA sample obtained by mixing an equal ratio of unmethylated and methylated human DNA showed an intermediate result. In fact, the intensity of the lane of undigested DNA, measured by densitometric tracings, approximates the fifty percent of that untreated with HpaII (data not shown).

As expected, the GDMI values in the three samples were about 0.1, 0.5 and 0.9 for the fully methylated DNA, for the mix of methylated and unmethylated DNA and for the fully unmethylated DNA, respectively (Fig. 2B).

On the whole, the results obtained by the above control experiments demonstrated the accuracy and the reproducibility of the *CpG*lobal assay in measuring the global DNA methylation levels of the different sample analyzed.

3.2 Global DNA methylation level analysis in population DNA samples

After the validation of the procedure, the *CpG*lobal assay was applied to measure the GDMI values of DNA samples extracted from peripheral venous blood collected from 354 subjects (65- to 107- years-old).

As shown in Figure 3, the distribution of the GDMI values in our sample is quite normal with a mean value of about 0.513 (SD: 0.203). In Table 1 is reported the frequency distribution of mtDNA haplogroups of the analyzed sample. The frequencies observed

in the present study are in agreement with those previously reported for the Calabrian population (De Benedictis et al. 1999).

Table 2 reports the mean GDMI values according to the mtDNA haplogroups classification. We observed that subjects carrying mtDNA belonging to J haplogroup exhibit higher methylation levels then those carrying mtDNA belonging to non J haplogroup (0.372±0.210 vs 0.523±0.199, respectively; p-value<0.001).

3.3 Global DNA methylation level analysis in cybrid cell lines

Then, in order to better clarify the influence of the mtDNA variability on DNA methylation, we carried out an *in vitro* study by applying the *CpG*lobal assay to cybrid cell lines harbouring mtDNA of H, J, U, X and T haplogroups, and to the 143B.TK$^-$ native cell line and its derivative Rho^0 line, as control (Bellizzi et al. 2006; 2009). The results indicated that global DNA methylation levels were different among cibrids (Fig. 4) ($p<0.001$) with the J cybrid showing higher methylation levels than the other lines (Fig. 5). Since mtDNA is the sole variant among the cybrid lines, these results demonstrate a correlation between mtDNA variability and global DNA methylation levels, thus confirming the evidence emerged from the population study. Moreover, the comparison between the global methylation levels of the 143B.TK$^-$ and Rho^0 cell lines demonstrated that these levels are also associated with the depletion of mtDNA (Fig. 5) as Rho^0 cells exhibited higher levels with respect to the native line ($p=0.009$).

3.4 Gene expression analyses

Once the global DNA methylation status was found to be increased in the J cybrid line, quantitative real-time PCR assays were carried out in order to identify which genes, involved in DNA methylation processes, were responsible of the above hypermethylation. To this purpose we analyzed the expression levels of: *DNMT1* (DNA (cytosine-5-)-methyltransferase 1), *DNMT3A* (DNA (cytosine-5-)-methyltransferase 3

alpha), *DNMT3B* (DNA (cytosine-5-)-methyltransferase 3 beta), *MAT1A* (methionine adenosyltransferase I, alpha), *MAT2B* (methionine adenosyltransferase II, beta), *MBD2* (methyl-CpG binding domain protein 2) and *MBD4* (methyl-CpG binding domain protein 4).

By comparing the mRNA levels of the seven genes among cybrids, a significant difference was observed only for *MAT1A* gene (Fig. 6). More specifically, as shown in Fig. 7, the J cybrid showed higher mRNA levels of *MAT1A* gene than the non J cybrids ($p<0.001$). Therefore, this result suggests that the mtDNA variability is able to modulate the expression of *MAT1A* gene and that the hyperexpression of this gene might be involved in the hypermethylation status of J cybrids.

The role of mtDNA in modulating the expression profiles of the above genes has emerged also by comparing the Rho^0 cell to the parental cell line. Indeed, as J cybrid, the Rho^0 cells showed higher *MAT1A* mRNA levels with respect to the parental cell line (Fig. 6 and 7).

3.5 ATP and ROS level measurements

In order to investigate molecular mechanisms underlying the differences in the *MAT1A* expression profiles previously reported between J and non J cybrids, we measured the intracellular ATP levels, that is MAT1A substrate, and ROS levels, that are negative regulators of the MAT1A activity. The J cybrid shows lower intracellular ATP (Fig. 8A) and lower ROS (Fig. 8B) levels with respect to the non J cybrids ($p=0.047$ and $p=0.037$, respectively). Interestingly, Rho^0 cells exhibit lower ATP levels but higher ROS levels with respect to the parental cell line ($p<0.001$).

4. DISCUSSION

The aim of the present study was to investigate whether inherited mitochondrial DNA variants can trigger changes in the global DNA methylation profiles. Indeed, while it is quite clear that the these variants are able to regulate several intracellular functions as well as to induce changes in the nuclear gene-expression through a cross signaling between mitochondria and nuclear genome, nothing is documented about their influence on epigenetic processes. From our population association study and *in vitro* analyses, it has emerged that elderly individuals and cybrid cells harboring mtDNA molecules belonging to J haplogroup have higher global DNA methylation levels than non J. These data indicate a clear correlation between mtDNA variability and DNA methylation. In particular, since the above correlation has been observed in subjects of middle/advanced age and in centenarians, our findings may highlight a novel effect of the haplogroup J in aging. In fact, besides our previously findings about the role of mtDNA haplogroup J in increasing the individual chance to attain longevity, here we demonstrate that the J haplogroup is also able to regulate epigenetic processes (De Benedictis et al. 1999; Bellizzi et al. 2006; 2009).

As to the analyses of cell lines, it is important to note that the difference in DNA methylation levels between native and Rho^0 cells, in line with the data reported by Smiraglia and coworkers, provides a further evidence for the existence in human cells of a *retrograde response* (Smiraglia et al. 2008). Gene expression studies reported in this paper are also relevant to demonstrate that the hypermethylation observed in J cybrid might be due to *MAT1A* gene that is hyperexpressed in this cells with respect to the other cybrids. Considering the role of the MAT1A in the synthesis of S-AdenosylMethionine, the major methyl group donor in cell metabolism as well as in DNA methylation, our results suggest that mtDNA variability influences the DNA methylation by regulating the expression of a gene that plays a crucial role in the

methylation process. We hypothesize that this influence is exerted through the activation of mediators of the cross-talk between mitochondria and nucleus. ATP, key substrate of the activity of MAT1A, and ROS could be among these mediators. Low ATP and ROS levels occurring in J cybrid might induce the activation of transcriptional activators of the *MAT1A* gene, leading to its over-expression and thus to DNA hypermethylation. Previous reports proposed that the mutations defining J haplogroup occur in complex I protein subunits thus impairing the OXPHOS performance (De Benedictis et al. 1999). Data about the low ATP levels here reported represent the first experimental evidence of the above suggestion. In addition, the low ROS levels we observed are in line with literature data reporting that a decrease in OXPHOS efficiency, with consequent low production of ATP, induce a decrease in intracellular ROS due to an hyperexpression of nuclear genes encoding for detoxifying enzymes (Valko et al. 2007). On the other hand it seems inconsistent that low ATP levels correlated with high expression of *MAT1A*, that uses ATP as substrate. It is that cells hyperexpress *MAT1A* gene in order to maximize the utilization of the low ATP availability; an alternative possibility is that the hyperexpression of *MAT1A* gene is more strictly associated to the low ROS levels. The second hypothesis is confirmed by literature data demonstrating as ROS may lead to epigenetic modifications and regulate MAT1A activity (Avila et al. 1998; Lim et al. 2008). It is possible that low ROS levels we observed in J cybrids are not able to induce DNA lesions, which have been shown to interfere with the ability of DNA to function as a substrate for methylase enzymes, thus resulting in the global hypermethylation (Franco et al. 2008). This correlation ROS-global hypermethylation of DNA can lead to review the role of ROS in aging, generally considered harmful in this process.

On the whole our data provide the first evidence that mtDNA variability modulates global DNA methylation levels, possibly via the regulation of OXPHOS efficiency. This can represent an alternative to explain the remodeling in gene expression that commonly take place during the lifetime and especially during aging. We also believe that our results contribute to add another piece to the puzzle of the signaling pathways that are activated between mitochondria and nucleus and that play a key role in different complex traits.

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LEGENDS OF FIGURES

Figure 1. Control DNA lambda bacteriophage analysis. **A)** Ethidium bromide-staining agarose gel showing unmethylated and methylated human DNAs digested with HpaII and MspI.

ND: human DNA not digested; **B)** GDMI values of unmethylated and methylated DNAs. The values represent the mean of three independent triplicate experiments with standard error mean.

Figure 2. Control human DNA analysis. **A)** Ethidium bromide-staining agarose gel showing unmethylated and methylated human DNAs and of a mixture of equal amount of unmethylated and methylated human DNAs digested with HpaII and MspI.

ND: human DNA not digested; **B)** GDMI values of unmethylated, methylated and of a mixture of unmethylated and methylated human DNAs. The values represent the mean of three independent triplicate experiments with standard error mean.

Figure 3. Frequency distribution of GDMI values in the total population sample

Figure 4. GDMI values determined in 143B.TK$^-$, Rho^0 and cybrid cell lines. The values represent the mean of three independent triplicate experiments with standard error mean.

Figure 5. GDMI values determined in 143B.TK$^-$, Rho^0, J and non J cybrids. The values represent the mean of three independent triplicate experiments with standard error mean. The GDMI values of non J cybrids represent the mean of the GDMI values of H, U, X and T cybrids.

Figure 6. Expression profiles of *DNMT1*, *DNMT3*, *DNMT3B*, *MAT1A*, *MAT2B*, *MBD2* and *MBD4* genes measured in J and non J cybrids. These levels are reported as the mean of Relative Quantification values (RQ), measured in three independent triplicate experiments, with standard error mean.

The RQ values of non J cybrids represent the mean of the RQ values of H, U, X and T cybrids.

Figure 7. mRNA levels of *MAT1A* gene measured in 143B.TK$^-$, Rho^0, J and non J cybrids. These levels are reported as the mean of Relative Quantification values (RQ), measured in three independent triplicate experiments, with standard error mean. The RQ values of non J cybrids represent the mean of the RQ values of H, U, X and T cybrids.

Figure 8. (A) Intracellular ATP levels measured in 143B.TK$^-$, Rho^0, J and non J cybrid cell lines. The levels are reported as the mean of Relative Luminescence Units (RLU) determined in three independent triplicate experiments with standard error mean. The RLU values of non J cybrids represent the mean of the RLU values of H, U, X and T cybrids. **(B)** Intracellular ROS levels measured in 143B.TK$^-$, Rho^0, J and non J cybrid cell lines. These levels are represented as percentage of ROS-staining positive cells. Data represent the mean of three independent experiments with standard error mean.

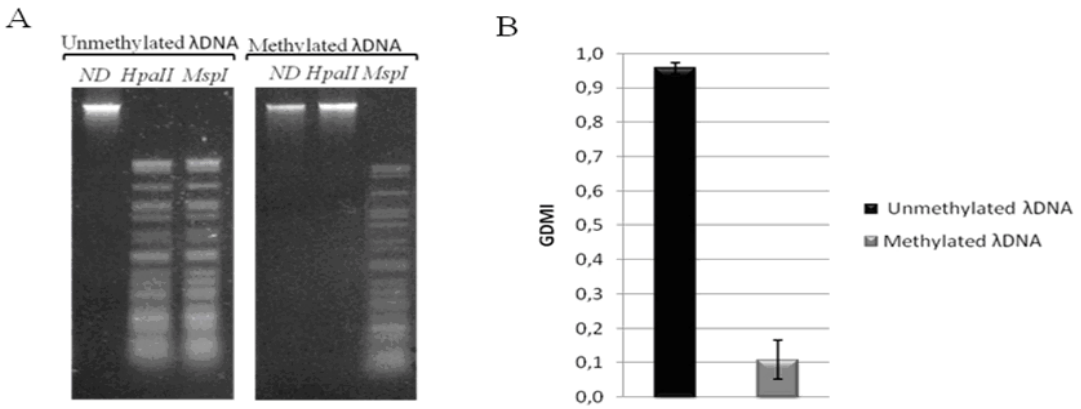

A
Unmethylated λDNA
Methylated λDNA
ND HpaII MspI
ND HpaII MspI
B
GDMI
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
Unmethylated λDNA
Methylated λDNA

Figure 1.

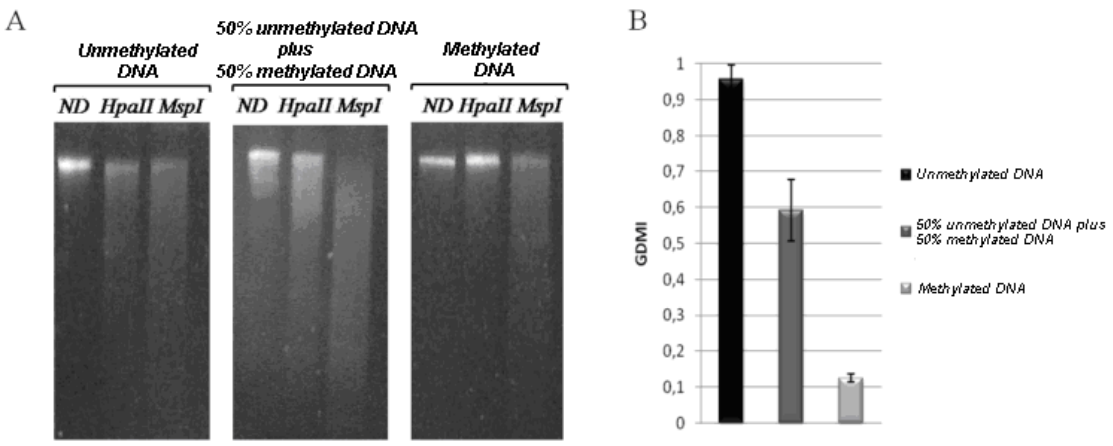

A
Unmethylated DNA
50% unmethylated DNA plus 50% methylated DNA
Methylated DNA
ND HpaII MspI
ND HpaII MspI
ND HpaII MspI
B
GDMI
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Unmethylated DNA
50% unmethylated DNA plus 50% methylated DNA
Methylated DNA

Figure 2.

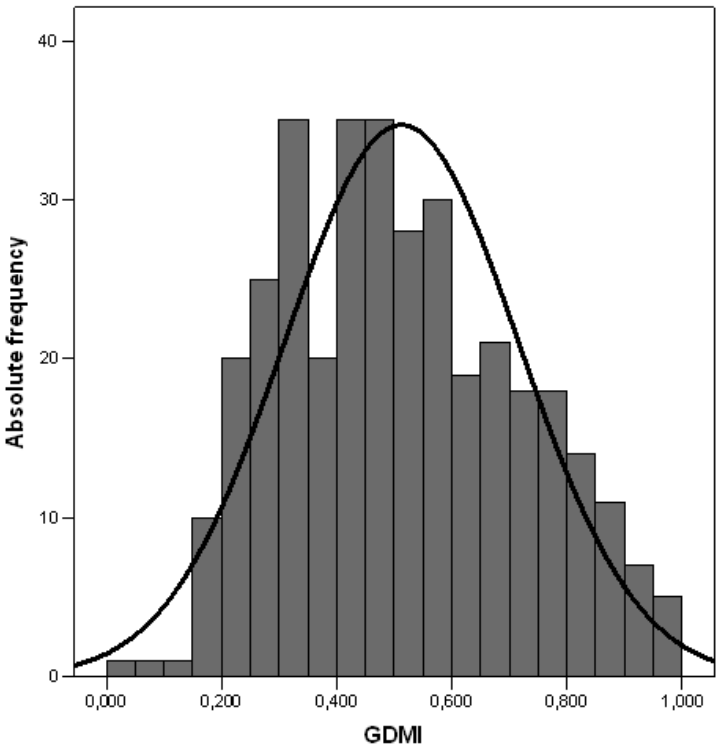

Absolute frequency
40
30
20
10
0
0,000
0,200
0,400
0,600
0,800
1,000
GDMI

Figure 3.

Table 1: Frequency distribution of mtDNA haplogroup in the analyzed sample.

mtDNA haplogroup	N	Rel. Freq. ± SE
H	120	0.339±0.025
I	8	0.023±0.008
J	25	0.071±0.014
K	36	0.102±0.016
T	38	0.107±0.016
U	40	0.113±0.017
V	5	0.014±0.006
W	22	0.062±0.013
X	21	0.059±0.013
OTHERS	39	0.110±0.017

Table 2: Mean GDMI values with respect to the mtDNA haplogroups classification in the analyzed sample.

mtDNA haplogroup	N	Mean ± SE
H	120	0.514±0.017
I	8	0.465±0.082
J	25	0.372±0.042
K	36	0.542±0.035
T	38	0.523±0.037
U	40	0.513±0.030
V	5	0.644±0.135
W	22	0.514±0.049
X	21	0.562±0.036
OTHERS	39	0.527±0.028

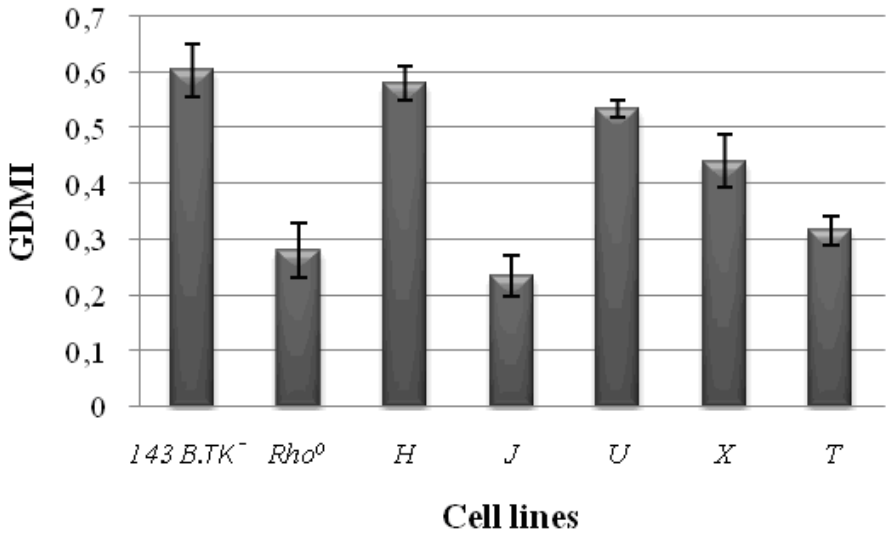


Figure 4.

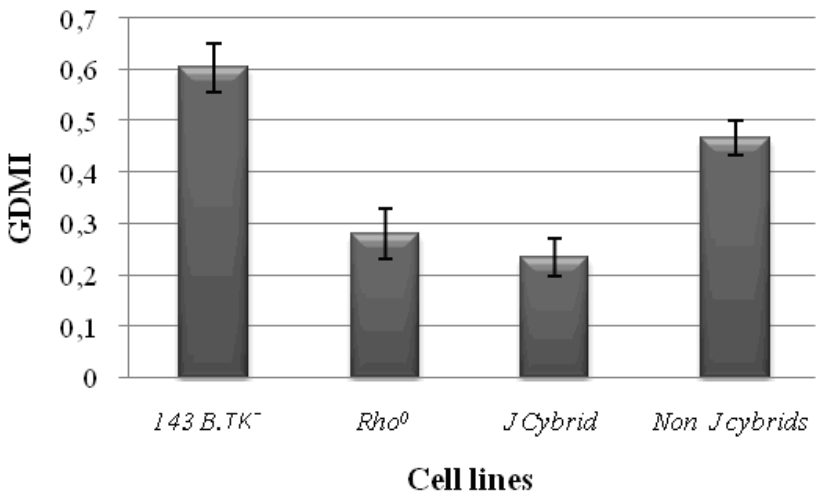

0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
GDMI
143 B.TK⁻
Rho⁰
J Cybrid
Non J cybrids
Cell lines

Figure 5.

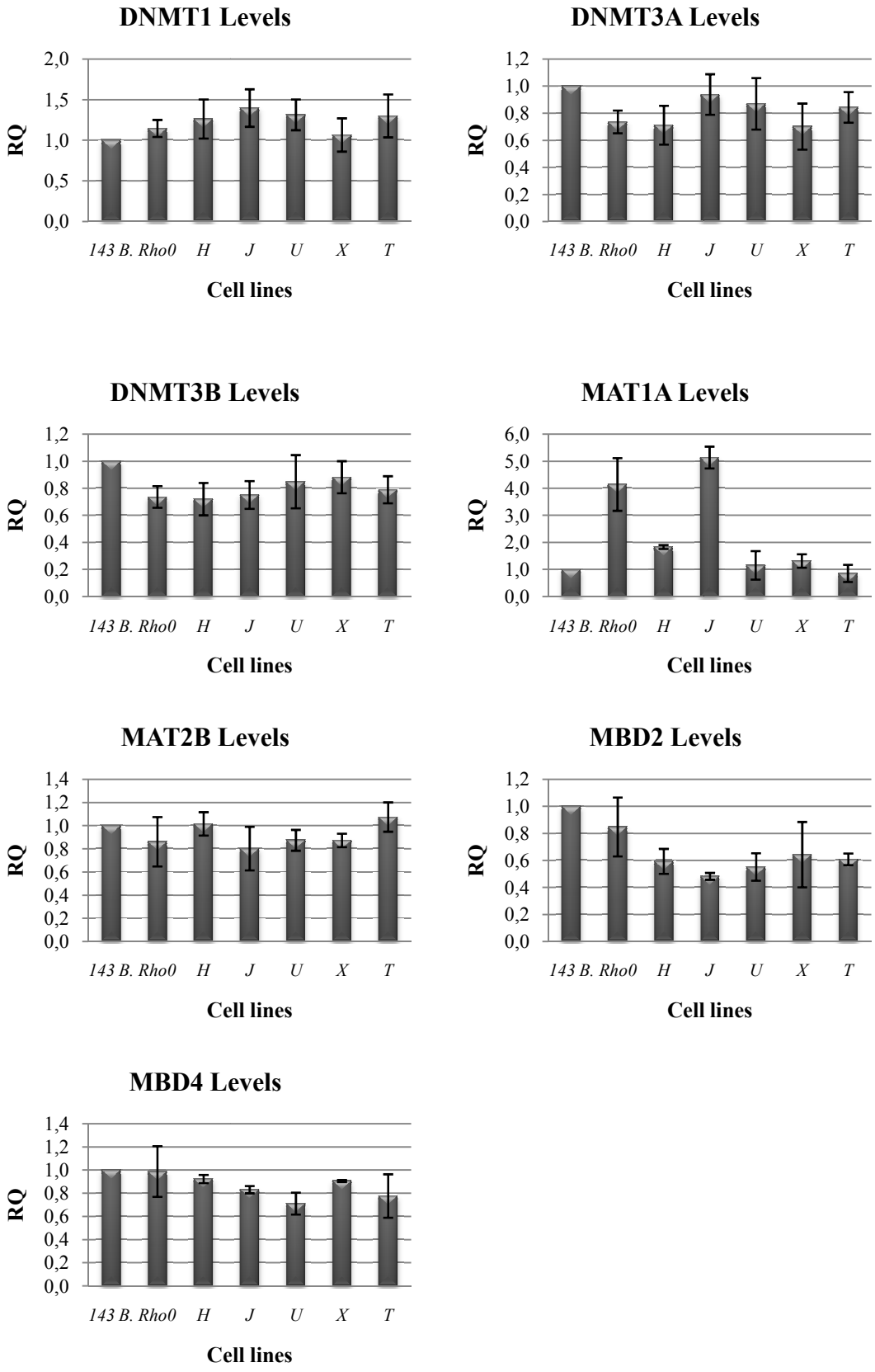

DNMT1 Levels
RQ
2,0
1,5
1,0
0,5
0,0
143 B. Rho0 H J U X T
Cell lines
DNMT3A Levels
RQ
1,2
1,0
0,8
0,6
0,4
0,2
0,0
143 B. Rho0 H J U X T
Cell lines
DNMT3B Levels
RQ
1,2
1,0
0,8
0,6
0,4
0,2
0,0
143 B. Rho0 H J U X T
Cell lines
MAT1A Levels
RQ
6,0
5,0
4,0
3,0
2,0
1,0
0,0
143 B. Rho0 H J U X T
Cell lines
MAT2B Levels
RQ
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
143 B. Rho0 H J U X T
Cell lines
MBD2 Levels
RQ
1,2
1,0
0,8
0,6
0,4
0,2
0,0
143 B. Rho0 H J U X T
Cell lines
MBD4 Levels
RQ
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
143 B. Rho0 H J U X T
Cell lines

Figure 6.

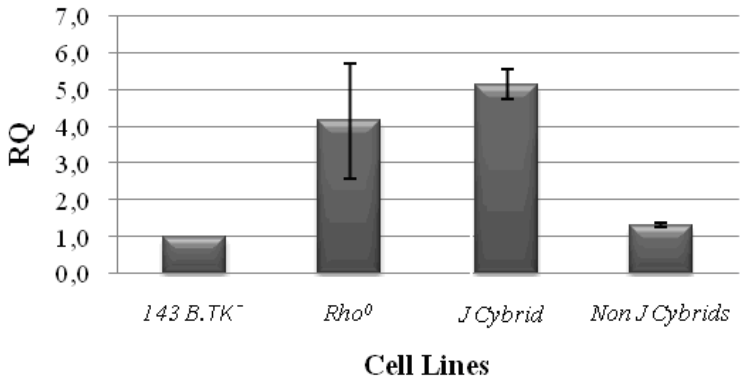
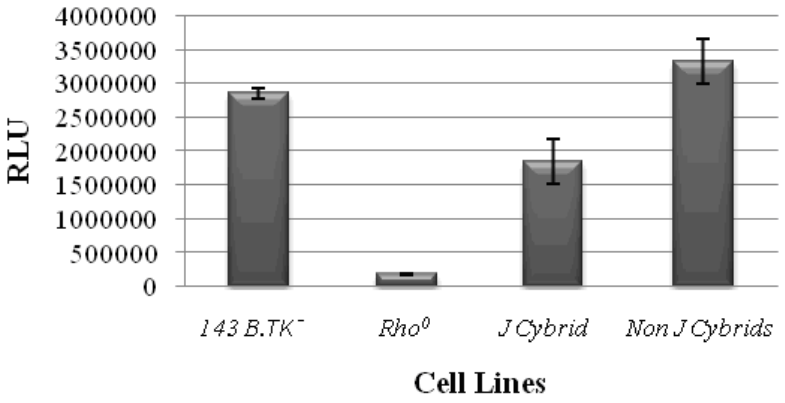

7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
RQ
143 B.TK-
Rho0
J Cybrid
Non J Cybrids
Cell Lines

Figure 7.

A


4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
RLU
143 B.TK-
Rho0
J Cybrid
Non J Cybrids
Cell Lines

B

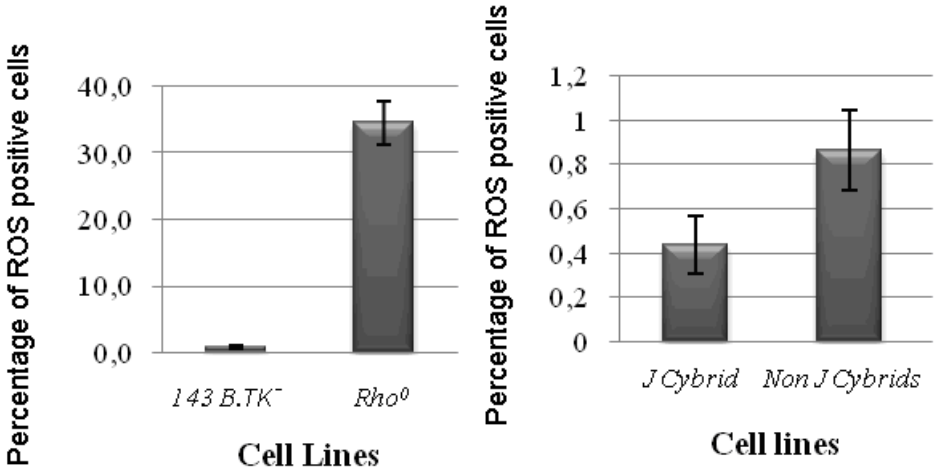

Percentage of ROS positive cells
40,0
30,0
20,0
10,0
0,0
143 B.TK-
Rho0
Cell Lines
Percentage of ROS positive cells
1,2
1
0,8
0,6
0,4
0,2
0
J Cybrid
Non J Cybrids
Cell lines

Figure 8.

***SIRT3* gene expression: a link between mitochondrial DNA inherited variants and oxidative stress**

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ABSTRACT

Cross-signalling between mitochondria and nucleus is activated in order to preserve cellular homeostasis, especially in the event of oxidative stress. We investigated in cybrids whether mitochondrial DNA (mtDNA) variability modulates the expression profiles of the seven mammalian sirtuins in oxidative stress. We found that the expression of *SIRT3* gene was down-regulated, depending on the mtDNA sequence, and this was correlated to the impairment of mitochondrial function, resulting in the decline of ATP and NAD^+/NADH levels. Data here reported link SIRT3 (a mitochondrial deacetylase involved in cell metabolism), mitochondrial DNA variability, and mitochondrial functionality, three fundamental components of the cellular stress response.

KEYWORDS: Sirtuins; SIRT3; cybrid cells; mtDNA variability; ATP; NAD^+/NADH.

1. INTRODUCTION

Reactive Oxygen Species (ROS), physiological products of the cell metabolism, have a central role in regulating the cell cycle, the cellular defence against infectious agents and a number of intracellular signalling pathways (Valko et al. 2006; Valko et al. 2007; Groeger et al. 2009; Hamanaka and Chandel 2010). On the other hand, high ROS concentrations can damage key biological molecules, alter cellular signalling pathways, activate apoptotic or necrotic cell death, thus contributing to the pathogenesis of degenerative and chronic diseases (Patten et al. 2010; Kondo et al. 2010) Redox regulation mechanisms maintain a fine balance of ROS. In case of an imbalance between ROS production and neutralization, a number of oxidative stress responsive genes are activated (Aon et al. 2010; Kregel and Zhang 2007). In this context a central role is played by mitochondria, which, besides their role in aerobic ATP generation and in different metabolic pathways, are the major intracellular source of ROS (Kamata and Hirata 1999; Inoue et al. 2003; Koopman et al. 2010). As widely recognized, an accumulation of ROS and a consequent decline in mitochondrial function is one of the main factors contributing to rate and quality of aging and age-related pathologies (Loeb et al. 2005; Wallace and Fan 2009; Seo et al. 2010). It has been demonstrated that the cells have developed a complex repertoire of stress response mechanisms highly regulated by a network of nuclear and mitochondrial signalling pathways (Srinivasan et al., 2010; Mammucari and Rizzuto 2010; Cataldi 2010). The genes encoding components involved in sensing of oxidative stress and/or in redox signalling play a key role in this network (Motterlini et al., 2002; Gogvadze and Orrenius, 2006; Brown, 2007; Finley and Haigis 2009). From this perspective, sirtuin genes require a close attention. Sirtuins are highly conserved proteins for which a basic role in the regulation of lifespan has often been reported (Dali-Youcef et al. 2007; Guarente 2007; Kaeberlein

2008; Donmez and Guarente 2010). Mammals sirtuins (Sir-2-related enzymes) are a class of seven NAD^+-dependent deacetylases with a mono-ADP-rybosylation activity, that play a crucial role in a broad spectrum of cellular functions (Michan and Sinclair 2007; Greiss and Gartner 2009; Verdin et al. 2010; Haigis and Sinclair 2010; Lomb et al. 2010; Qiu et al. 2010) Indeed, a sirtuin-mediated transcriptional silencing due the deacetylation of histones as well as of various transcription factors has been widely reported (Yeung et al., 2004; Kobayashi et al., 2005; Rodgers et al., 2005; Nemoto et al., 2005; Ford et al., 2006). In addition, sirtuins are involved in regulating cell proliferation, stress resistance and apoptosis (Imai and Guarente, 2010). Functional studies of human SIRT2 indicated that this is over-expressed during mitosis and it is associated with chromatin, thus affecting mitotic exit (Dryden et al. 2003; Vaquero et al., 2009). Likewise, SIRT6 is involved in maintaining genome integrity by regulating the base excision repair (BER) of single-stranded DNA breaks (Mostoslavsky et al., 2006). Moreover, several studies reported that SIRT1 is able to modulate the activity of several regulatory factors in response to different intracellular stress (van der Horst et al., 2004; Brunet et al., 2004; Kobayashi et al., 2005). In particular, it inhibits p53 transactivation activity and suppresses apoptosis in response to oxidative stress and DNA damage, thus increasing cell survival (Cheng et al., 2003; Luo et al., 2001). In addition, the DNA repair capacity of cells and survival are improved by the deacetylation of Ku70 by SIRT1 and SIRT3 (Jeong et al., 2007; Sundaresan et al., 2008). Finally, SIRT1, SIRT3, SIRT4 and SIRT5, the last three located in mitochondria, play an important role in regulating the cellular metabolism. In particular, recent studies demonstrated that SIRT3 controls reversible lysine acetylation in this organelle, by modulating the acetylation level of a large number of mitochondrial proteins, such as acetylcoenzyme A synthase 2, glutamate dehydrogenase and isocitrate dehydrogenase 2

(Shi et al., 2005; Hallows et al., 2005; Schwer et al., 2006; Lombard et al., 2007; Schlicker et al., 2008). Moreover, SIRT3 is involved in the maintenance of basal ATP levels through the modulation of the acetylation level of multiple components of mitochondria electron transport Complex I (Ahn et al., 2008).

The involvement of NAD^+ as a substrate for sirtuin-mediated deacetylation has encouraged the hypothesis that perturbations in NAD^+ metabolism provide a link to connect energy metabolism to signaling in the nucleus (Ying 2008; Imai 2010). For this reasons, the idea that sirtuins could act as transducers of metabolic information in cells is progressively emerging, alongside a new interpretation of the role of sirtuins, namely as sensors of intracellular energy and redox state (Denu, 2003; Hipkiss et al., 2008). Moreover, as genetic studies on a range of organisms have indicated that sirtuins strictly control aging and life-span, it has also been reported that the link between sirtuins activity and cell senescence is represented by the intracellular NAD^+/NADH ratio. In particular, the progressive aging delay under caloric restriction (CR) has been observed to occur in several organisms as a consequence of the decrease of glycolytic NAD^+ demand associate to an increase in NADH oxidation, that regenerate NAD^+ (Hipkiss et al., 2008).

Starting from these considerations, in the present work we availed of cybrid technology to reveal if pathways of communication between mitochondrial and nuclear genomes are able to modulate in oxidative stress conditions the expression profiles of sirtuin genes depending on mitochondrial DNA inherited variants.

2. MATERIAL AND METHODS

2.1 Cell lines and culture conditions

143B.TK$^-$ osteosarcoma cells and cybrid cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 25 mM glucose and 1 mM sodium pyruvate, supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 0.1 mM gentamycin (Invitrogen). The Rho^0 cell line, obtained by culturing 143B.TK$^-$ in the routine growth medium containing 50 ng/mL ethidium bromide with regular replenishment of medium for about 2 months (King and Attardi, 1996), were maintained in DMEM supplemented with 10% FBS and 0.2 mM uridine (Sigma).

143B.TK$^-$, Rho^0 cells and cybrid cell lines were cultured in a water-humidified incubator at 37 °C in 5% CO_2/95% air. Experiments for quality control of mtDNA depletion in Rho^0 cells and mtDNA haplogroups analyses in cybrid cell lines were described in Bellizzi et al. (2006).

2.2 Oxidative stress treatment conditions

1×10^6 143B.TK$^-$, Rho^0 and cybrid cells were seeded in regular growth medium in 100-cm^2 plates. In the exponential growth phase, the growth medium was discarded and replaced with DMEM containing different concentrations of H_2O_2 (0.1, 0.5, 1, 1.5 and 2 mM). The cells were incubated to 37 °C for 8 hours. In both conditions, untreated cells were also analyzed as control.

2.3 Experiments for quality control of stress treatment

2.3.1 Viability/proliferation assay

The viability of treated and untreated cell lines were assayed by Trypan Blue exclusion assay.

Floating and adherent cells were collected and 200 μl of cellular suspension were added to an equal volume of 0.4% Trypan Blue solution (Sigma). The, viable and non-viable cells were counted on a hemocytometer with an inverted light microscope using a 20 X magnification.

For each cell lines, three independent experiments were carried out, and the percentage of cell viability was calculated as the ratio between the number of viable cells and the total cell number.

2.3.2 DNA fragmentation analysis

Treated and untreated cells were trypsinized and centrifuged at 3000 g for 5 min. The pellet was incubated on ice for 20 min in 400 µl of lysis buffer (10 mM Tris-HCl pH 8, 20 mM EDTA, 0.2% Triton-X100). After a 12000 g centrifugation for 20 min, an equal volume of phenol/chloroform was added to the supernatant. Then, after a new 12000 g centrifugation for 5 min, an equal volume of chloroform was added to supernatant and centrifuged again at the same conditions. The supernatant was collected and stored at −20 °C overnight after adding 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volumes of ethanol to precipitate DNA. DNA was pelleted by centrifugation at 12,000 g per 20 min, rinsed with 70% ethanol and then resuspended in TE buffer containing 100 mg/mL RNase A. After 2 h of incubation at 37 °C, the DNA samples were loaded onto a 1.5% agarose gel, electrophoresed in 1 X TAE (Tris/Acetate/EDTA) buffer and stained with ethidium bromide.

2.4 Expression profile analyses of sirtuin genes

Total RNA was extracted from untreated and treated cells by using RNeasy Mini Kit (Qiagen) and samples were treated with DNA-free DNAse to remove any residual genomic DNA contamination. The RNA concentration was measured for each sample by using a spectrophotometer at an absorbance ratio of 260/280 nm.

The RT-PCR (Reverse Transcriptase-PCR) reactions were carried out by using the ImPromII Reverse Transcription System Kit (Promega). Firstly, an RT mix including 0.5 µg of total RNA and 500 ng of Oligo(dT)15 Primers was preheated at 70 °C for 5 min. Then, the reaction was carried out in a 40 µl final volume containing 1 X RT

Buffer, 3 mM of $MgCl_2$, 0.5 mM of each dNTP, 20 U of RNase inhibitor and 5 U of MMLV (moloney murine leukemia virus) reverse transcriptase. The mix was incubated at 25°C for 5 minutes, at 37 °C for 60 minutes and, successively, at 95 °C for 10 minutes to inactivate the reverse transcriptase.

Forward primers and reverse primers were as follows: SIRT1For 5'-GAAGACGACGACGACGAG-3'; SIRT1Rev 5'-GCCACAGTGTCATATCATCC-3'; SIRT2For 5'-AAGGAGCGTCTGCTGGAC-3'; SIRT2Rev 5'-AGATGGTTGGCTTGAACTGC-3'; SIRT3For 5'- ATGGAACCTTTGCCTCTG-3'; SIRT3Rev 5'-CAAGTCCCGGTTGATGAG-3'; SIRT4For 5'-ATGTGGATGCTTTGCACACC-3'; SIRT4Rev 5'-TGGCCTCCACATTGAACGCA-3'; SIRT5For 5'- AGGAGCTGGAGGTTATTG-3'; SIRT5Rev 5'-CAGCCACAACTCCACAAG-3'; SIRT6For 5'- ACTTCAGGGGTCCCCACG-3'; SIRT6Rev 5'-TCGGCGTTCCTGCTGGCC-3'; SIRT7For 5'-GAGCAGACACCATCCTGTGT-3'; SIRT7Rev 5'-ACAGCGACTTCCGACTGTGG-3'; GAPDHFor 5'-GACAACTTTGGTATCGTGGA-3'; GAPDHRev 5'-TACCAGGAAATGAGCTTGAC-3'.

The PCR mixture was carried out in 25 µl volume containing 1 µl of cDNA, 1 X Reaction Buffer, 2 mM $MgCl_2$, 0.2 mM for each dNTP and 1.5 U Taq Dinazyme (Finnenzymes).

In order to appropriately measure the expression level of a gene by RT-PCR analysis it is important that both the PCR product of the target gene and its corresponding internal control (*GAPDH*) are analyzed in the frame of the exponential phase of the saturation curve, that is before the amplification products reach the saturation phase. To this purpose, for each sirtuin gene and its corresponding GAPDH we carried out a series saturation curves to set-up the best RT-PCR protocol. When the internal control PCR

product reached the saturation phase before the PCR product of the corresponding target gene, the *GAPDH* primers were added to the PCR reaction after an appropriate number of cycles. Thus, the PCR conditions were different for each gene analyzed as indicated in Table1. On the whole, after an initial denaturation step at 94°C for 1 minute, 30 cycles were carried out at 95°C for 1 minute, 60-61°C for 1 minute and 72°C for 1 minute. The final step was an incubation at 72°C for 10 minutes.The PCR products were analyzed on 2% agarose gel electrophoresed in 1 X TAE (Tris/Acetate/EDTA) buffer and stained with ethidium bromide. Fluorescence intensity of each band was calculated using densitometer analysis (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290), and then normalized with respect to GAPDH band intensity (Relative Band Intensity, RBI). Three independent RT-PCR experiments were carried out in duplicate for each cell line, then, the average values with the standard error mean were calculated.

2.5 Transfection experiments

The *SIRT3* promoter activity was determined in 143B.TK$^-$, Rho^0, H and J cybrids by transfection experiments of the luciferase reporter construct containing the *SIRT3* promoter sequence (Bellizzi et al., 2006). Transfections were performed by using the Fugene6 Reagent, as recommended by the manufacturer (Roche Molecular Biochemicals).

$6x10^4$ 143B.TK$^-$, Rho^0 and cybrid cells were seeded in 24-well plates with 1 ml of regular growth medium/well. In the exponential growth phase, the growth medium was discarded and replaced with DMEM containing a transfection mixture of Fugene6, 1 μg of reporter plasmid and 1 ng of pRL-CMV (Promega), a plasmid that contains the Renilla luciferase gene under the cytomegalovirus promoter and is utilized as an internal control to normalize the effects of transfection efficiencies. After 10 hours, the

medium was discarded and incubated for 8 hours in DMEM containing 0.1 mM H_2O_2. Then, untreated and treated cells were lysed by 100 µl of Passive Lysis Buffer (Promega). 20 µl of cell lysate were used for luciferase reporter assay, by using the Dual Luciferase reporter Assay Kit (Promega), according to the manufacturer's protocol. Light intensity was quantified in a Lumat LB507 luminometer (EG&G Berthold). The Luciferase activity of the SIRT3 reporter construct was normalized the Renilla luciferase activity.

Three independent transfection experiments were carried out in duplicate for each cell line, then, the average values with the standard error mean were calculated.

2.6 ATP levels measurement

The ATP measurement was performed using ENLITEN ATP assay system bioluminescence detection kit for ATP measurement (Promega), according to the manufacturer's protocol. Briefly, $1x10^6$ 143B.TK$^-$, Rho^0, and cybrid cells were seeded in 6 well/plates. In the exponential growth phase, each well washed with PBS, trypsinized and counted on a hemocytometer with an inverted light microscope. $5x10^5$ cells were collected and centrifuged at 1500 rpm for 5 min at 4°C. Then, the intracellular ATP was extracted by resuspending pellets 100 µl in 0.5 % trichloroacetic acid (TCA). After ATP extraction, the TCA in the samples was neutralized and diluted by additing pH 7.75 Tris-Acetate buffer. Lastly, the bioluminescence was assessed using a Lumat LB9507 luminometer (EG&G Berthold). The ATP content was expressed as luminescence values (RLU). In each experiment, samples were done duplicate and negative controls were run to determine the amount of background that has been subtracted from each sample RLU.

2.7 NAD/NADH ratio measurements

The NAD^+/NADH ratio measurement was performed using NAD^+/NADH Quantification Kit (BioVision) according to the manufacturer's protocol. Briefly, $5x10^5$ cells were seeded in 6 well/plates. In the exponential growth phase, treated and untreated cells were washed with cold PBS, collected and centrifuged at 1500 rpm for 5 minutes. Then, cells were lysed by two cycles of freeze/thaw in NADH/NAD Extraction Buffer and subsequently vortexed for 10 seconds. The cellular extracts were transferred into labeled 96-well plate in duplicates, incubated with NAD Cycling Mix for 5 min into each well. NADt will be quantified by adding NADH developer to each sample and reading the plate at OD450 nm for 30 min. To detect NADH the extracted samples were incubated at 60°C for 30 minutes in order to decompose the NAD^+. Then, samples were incubated with NAD Cycling Mix for 5 min, and, after the addition of NADH developer, were read as previously reported. The NAD^+/NADH ratio was calculated as: (NADt – NADH)/NADH.

2.8 Statistical Analyses

Results are expressed as means ± standard error mean.

Statistical analyses were performed using SPSS 15.0 statistical software for windows (SPSS Inc., Chicago, Illinois). One-way analysis of variance (ANOVA) and Student's t-test were adopted, with a significance level defined as α=0,05.

3. RESULTS

3.1 Control experiments of the oxidative stress status

A series of control experiments were carried out in order to set-up the oxidative stress conditions following treatment of the cells with hydrogen peroxide (H_2O_2).

Cell viability assay. In order to calculate the percentage of living cells we performed a Trypan Blue Exclusion Assay after treatment with hydrogen peroxide at 0.1, 0.5, 1, 1.5, 2 mM for 8 hours.

As control, we also analyzed the viability of the cells at basal conditions.

As shown in Fig. 1, an H_2O_2 dosage-dependent decrease in the percentage of living cell was observed in the four cell lines.

In particular, after treatment with 0.1 mM H_2O_2, the percentage of living cells was around 95%. Treatment with 1 mM H_2O_2 was able to reduce the cell viability at around 50% while treatment with higher concentrations of H_2O_2 resulted in the complete death of the cells.

DNA fragmentation assay. In order to check the apoptotic status we carried out a DNA fragmentation assay by which was possible to visualize the inter-nucleosomal DNA fragmentation typical of apoptotic cell death.

As shown in Fig. 2, an H_2O_2 dosage-dependent increase in inter-nucleosomal DNA fragmentation was observed. In particular, the four cells lines did not show DNA fragmentation after treatment with 0.1 mM H_2O_2 while this fragmentation can be observed at higher concentrations of H_2O_2.

Therefore, considering the results obtained in the above control experiments, we chose 0.1 mM and 1 mM H_2O_2, the former as an inducer of mild oxidative stress, the latter as an inducer of a more severe oxidation stress.

3.2 Sirtuin gene expression profiles in cybrids

SIRT1-7 gene expression levels were analyzed in H and J cybrid cells and in 143B.TK$^-$ and Rho^0, as control, at both basal and oxidative stress conditions (0.1 and 1 mM H_2O_2) by semi-quantitative RT-PCR reactions. Representative RT-PCR electrophoresis patterns of the seven sirtuin genes are shown in Fig. 3. By comparing the mRNA levels of the seven genes between basal and stress condition, we found significant difference only for *SIRT3* gene in all cells (Fig. 4). In particular, *SIRT3* gene was under-expressed in the J cybrid with respect to the H cybrid at 0.1 mM H_2O_2 ($p=0.023$). This difference

disappeared at 1 mM H_2O_2 where the under-expression of the gene is observed in both cybrids. These results show that *SIRT3* is the sole sirtuin gene the expression of which is influenced by the oxidative stress. In addition, the difference in the expression levels of the *SIRT3* gene between H and J cybrids give an interesting evidence about a correlation between the expression of this gene and mtDNA inherited variants under mild oxidative stress conditions.

3.3 Promoter activity of the SIRT3 gene in cybrids

In order to investigate whether the down-regulation of the *SIRT3* gene in response to oxidative stress was carried out at transcription level, the cell lines were transiently transfected at both basal and stress conditions with the luciferase reporter construct containing the *SIRT3* promoter sequence (Bellizzi et al., 2007). The transfections revealed a lower *SIRT3* promoter activity in J cybrid with respect to H cybrid in mild oxidative stress (p=0,011). Under severe oxidative stress, the above activity was decreased with respect to basal condition in both cybrids (p=0,004, p=0,002, respectively) (Fig. 5).

Therefore, the transfection results displayed the same trend of the gene expression profiles, demonstrating that the response of the *SIRT3* gene to oxidative stress is regulated by molecular mechanisms carried out at transcription levels dependent on the mtDNA variability.

3.4 ATP levels measurement

Since SIRT3 is a NAD^+ dependent deacetylase, its activity is strictly dependent on the efficiency of the mitochondrial energy producing machinery. Thus, in order to investigate if the differences in the *SIRT3* expression profiles between H and J cybrids previously reported were due to differences in OXPHOS efficiency, we measured the intracellular ATP levels in the four cell lines. We observed that the J cybrid showed

lower intracellular ATP levels (Fig. 6) than the H cybrid at both basal and mild oxidative stress conditions (p=0,039, p=0,017, respectively). On the contrary, under severe oxidative stress the ATP levels in H cybrid decreased up to the levels of the J cybrid. On the basis of the data obtained we infer that ATP levels might act as mediators of the cross-talk between mitochondria and nucleus and thus regulate the differential *SIRT3* gene expression in mild oxidative stress depending on mitochondrial DNA variability.

3.5 NAD^+/NADH ratio measurement

Considering that NADH is the substrate of the inner mitochondrial membrane dehydrogenases that catalyze the transfer of electrons from NADH to coenzyme Q during oxidative phosphorylation and that NAD^+ is a substrate of sirtuins, we measured the intracellular NAD^+/NADH ratio in the four cell lines at both basal and stress conditions. By comparing the NAD^+/NADH ratio between H and J cybrids, we observed a significant difference after treatment with 0.1 mM H_2O_2 (Fig. 7). In particular, the J cybrid has a lower NAD^+/NADH ratio than the H cybrid (p=0,032). This difference disappear at more severe oxidative stress conditions, where it is possible to observe a low NAD^+/NADH ratio in both cybrids with respect to the basal conditions (p=0,038, p=0,031, respectively).

4. DISCUSSION

The present study aims to investigate whether the mitochondrial DNA inherited variants influence the cellular stress response by modulating the expression profile of sirtuin genes. Previous results we obtained in cybrid cells demonstrated that the above variants

are able to modulate the expression of some stress responder nuclear genes, such as those encoding for cytokines and cytokine receptors, HSP60 and HSP70 proteins, in oxidative and heat shock stress conditions (Bellizzi et al., 2006; 2009). In this work we extended our study to a further class of stress responder genes, that is those encoding for sirtuins, which are emerging as sensors of the energetic and redox state of the cells. Here we demonstrated that the *SIRT3* gene is negatively regulated by a state of intracellular oxidation. As SIRT3 has a deacetylase activity, its under-expression might result in a global increase in gene expression. Indeed, it has been reported that genes encoding for detoxifying enzymes significantly increase their expression after an oxidative stress (Valko e al. 2006; 2007). Furthermore, the differences observed in the expression levels of the *SIRT3* gene between the H and J cybrid revealed a correlation between the expression of this gene and mtDNA inherited variants under mild oxidative stress conditions. This result is in agreement with those previously reported for genes encoding for cytokines and cytokine receptors, HSP60 and HSP75 proteins, thus suggesting that the correlation between mtDNA variability and expression levels of stress-responder nuclear genes could be a general phenomenon (Bellizzi et al., 2006; 2009). Interestingly, the differences observed between the cybrid lines disappeared under more severe oxidative stress, thus suggesting that, in this condition, the cells tend to maximize gene expression patterns regardless of the mtDNA variability. Data here reported demonstrated that the response of the *SIRT3* gene to oxidative stress is regulated by acting at transcription levels. Indeed, numerous evidence indicate that the mitochondrial genome is able to regulate a series of nuclear target genes by transcription factors acting as mediators of the well known cross-talk nucleus-mitochondrion (Biswas et al. 2005). It is important to note that some of these factors, such as SP1, Ap2 and NFkB, have several motif binding sites located within the *SIRT3*

promoter region and have been demonstrated to be involved in the regulation of the expression of different stress responsive genes (Lavrovsky et al. 2000; Bellizzi et al., 2006; Ak and Levine 2010).

As it regards the results observed in Rho^0 cells, the under-expression of *SIRT3* with respect to the native cells observed after treatment with 1 mM H_2O_2 might demonstrate the existence of a *retrograde response* also in mammals (Butow and Avadhani 2004; Biswas et al., 2005). On the other hand, since these cells are in very stressful conditions due to mitochondrial dysfunctions, it seems inconsistent that the above under-expression is detectable only at severe oxidative stress condition. One possible explanation is that Rho^0 cells can adopt a series of mechanisms to compensate their intrinsic oxidative stress status.

The different ATP levels between J and H cybrids under mild oxidative stress conditions demonstrated that, besides to the above transcription factors, also ATP can be considered a mediator of the cross signaling between mitochondria and nucleus. Indeed, the intracellular ATP levels is strictly linked to the regulation of a number of molecules and/or metabolites, such as Ca^{2+}, nitric oxide (NO) and NAD^+/NADH that, in turn modulate several stress responder signaling and, on the basis of the magnitude of the stress status, trigger directly pro-death pathways (Motterlini et al., 2002; Brown et al., 2007; Mellström et al., 2008; Finley and Haigis, 2009; Caroppi et al., 2009; Kitsis and Molkentin, 2010). In addition, the decline in ATP levels, observed in J cybrid under mild oxidative stress and in both cybrids at severe stress conditions, is in line with literature data demonstrating this decline in different cellular systems following oxidative stress inducers, and, particularly, after treatment with H_2O_2 (Miyoshi et al., 2006; Teepker et al., 2007; Zmijewski et al., 2010). Interestingly, this decline is not

observed in H cybrid under mild oxidative stress, thus highlighting as the role played by mtDNA could be also correlated to the oxidative stress conditions.

It is important to note that the lower ATP levels of the J cybrid with respect to the H cybrid at basal condition are in line with previous reports indicating that the mutations defining J haplogroup occur in complex I protein subunits impair the OXPHOS performance. On the other hand, despite the low ATP levels, in J cybrids we did not observe low *SIRT3* expression, suggesting that at basal conditions, these low levels can still activate the normal gene expression processes.

The decline in NAD^+/NADH ratio observed in J cybrid at mild cellular oxidation and in both cybrid under severe oxidative stress is in line with several data reporting that both NAD^+ and NADH levels in cells exposed to H_2O_2 decrease significantly from normal levels. Here we demonstrate that this decline is influenced by mtDNA variability (Gilad et al., 1997; Hwang et al., 1999; Xie et al., 2009). On the whole, data reported here link, in a global overview, SIRT3, a mitochondrial deacetylase involved in cell metabolism, mitochondrial DNA inherited variants and mitochondrial functionality, three main components that have been considered to play a fundamental role in stress response.

5. References

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LEGENDS OF FIGURES

Figure 1 Viability curves of 143B.TK$^-$, Rho^0, H and J cybrid cell lines at both basal and stress conditions. Stress was carried out with 0.1, 0.5, 1, 1.5, 2 mM H_2O_2 for 8 hours.

Figure 2 DNA fragmentation electrophoresis pattern of 143B.TK$^-$, Rho^0, H and J cybrid cell lines at both basal and stress conditions. Stress was carried out with 0.1, 0.5, 1, 1.5, 2 mM H_2O_2 for 8 hours.

Figure 3 RT-PCR electrophoresis patterns of the seven sirtuin genes (*SIRT1-SIRT7*) in 143B.TK$^-$, Rho^0, H and J cybrid cell lines at both basal and stress conditions (0.1 mM and 1 mM H_2O_2 for 8 hours). *GAPDH*: Glyceraldehyde Phosphate Dehydrogenase.

Figure 4 Densitometric analysis of the *SIRT3* gene expression in 143B.TK$^-$, Rho^0, H and J cybrid cell lines at both basal and stress conditions. Data are reported as Relative Band Intensity obtained by normalizing the band intensity of the *SIRT3* PCR product to that of the *GAPDH* PCR product used as internal control. The values represent the mean of three independent triplicate experiments with standard error mean

Figure 5 Luciferase activity of the reporter construct containing the *SIRT3* promoter transiently transfected in 143B.TK$^-$, Rho^0, H and J cybrid cell lines at both basal and stress conditions. This activity is reported as Fold Induction (FI) with respect to the pGL_2-Basic vector. The values are the mean of three independent triplicate experiments with standard error mean.

Figure 6 Intracellular ATP levels measured in 143B.TK$^-$, Rho^0, H and J cybrid cell lines at both basal and stress conditions. These levels are reported as the main of

Relative Luminescence Units (RLU), determined in three independent triplicate experiments, with standard error mean.

Figure 7 Intracellular NAD^+/NADH ratio measured in 143B.TK^-, Rho^0, H and J cybrid cell lines at both basal and stress conditions. This ratio is reported as the main of absorbance values (ABS, OD_{450}), determined in three independent triplicate experiments, with standard error mean.

Table 1: RT-PCR conditions conditions for each SIRT gene analyzed.

Gene	Annealing Temperature	GAPDH Number of cycles	Sirtuin Primer concentrations	GAPDH Primer concentration
SIRT1	60°C	30	0.25 µM	0.16 µM
SIRT2	60°C	30	0.30 µM	0.16 µM
SIRT3	60°C	23	0.25 µM	0.25 µM
SIRT4	61°C	25	0.25 µM	0.25 µM
SIRT5	60°C	23	0.25 µM	0.25 µM
SIRT6	61°C	26	0.30 µM	0.30 µM
SIRT7	61°C	25	0.50 µM	0.30 µM

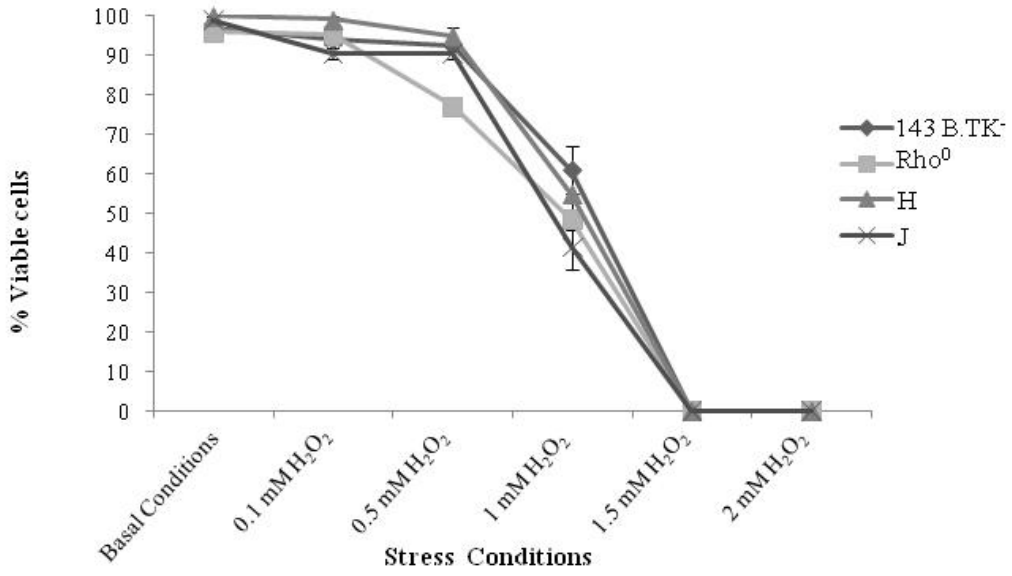
100
90
80
70
60
50
40
30
20
10
0
% Viable cells
Basal Conditions
0.1 mM H_2O_2
0.5 mM H_2O_2
1 mM H_2O_2
1.5 mM H_2O_2
2 mM H_2O_2
Stress Conditions
143 B.TK-
Rho^0
H
J

Figure 1

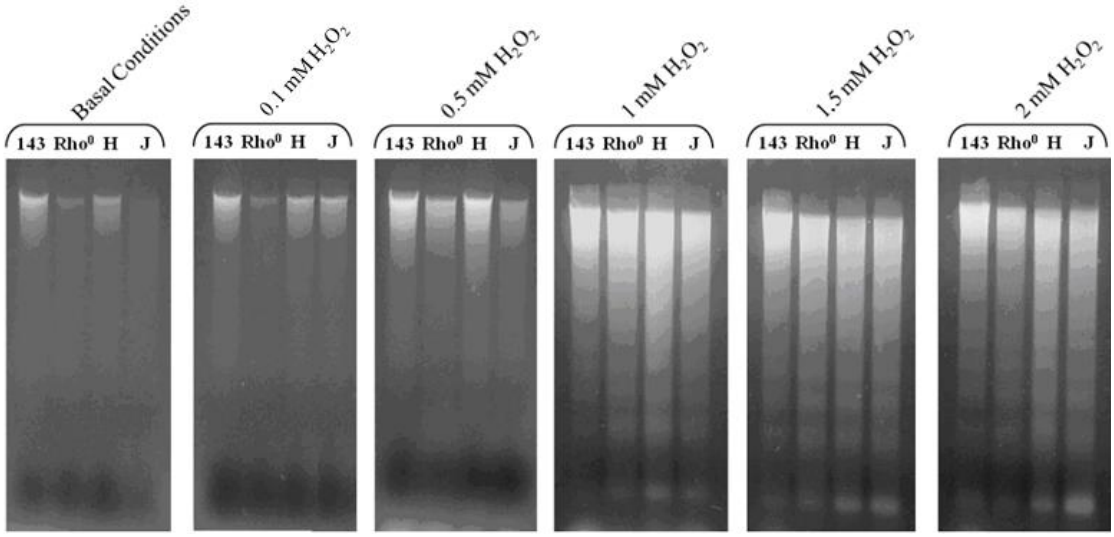
Basal Conditions
0.1 mM H_2O_2
0.5 mM H_2O_2
1 mM H_2O_2
1.5 mM H_2O_2
2 mM H_2O_2
143 Rho^0 H J

Figure 2

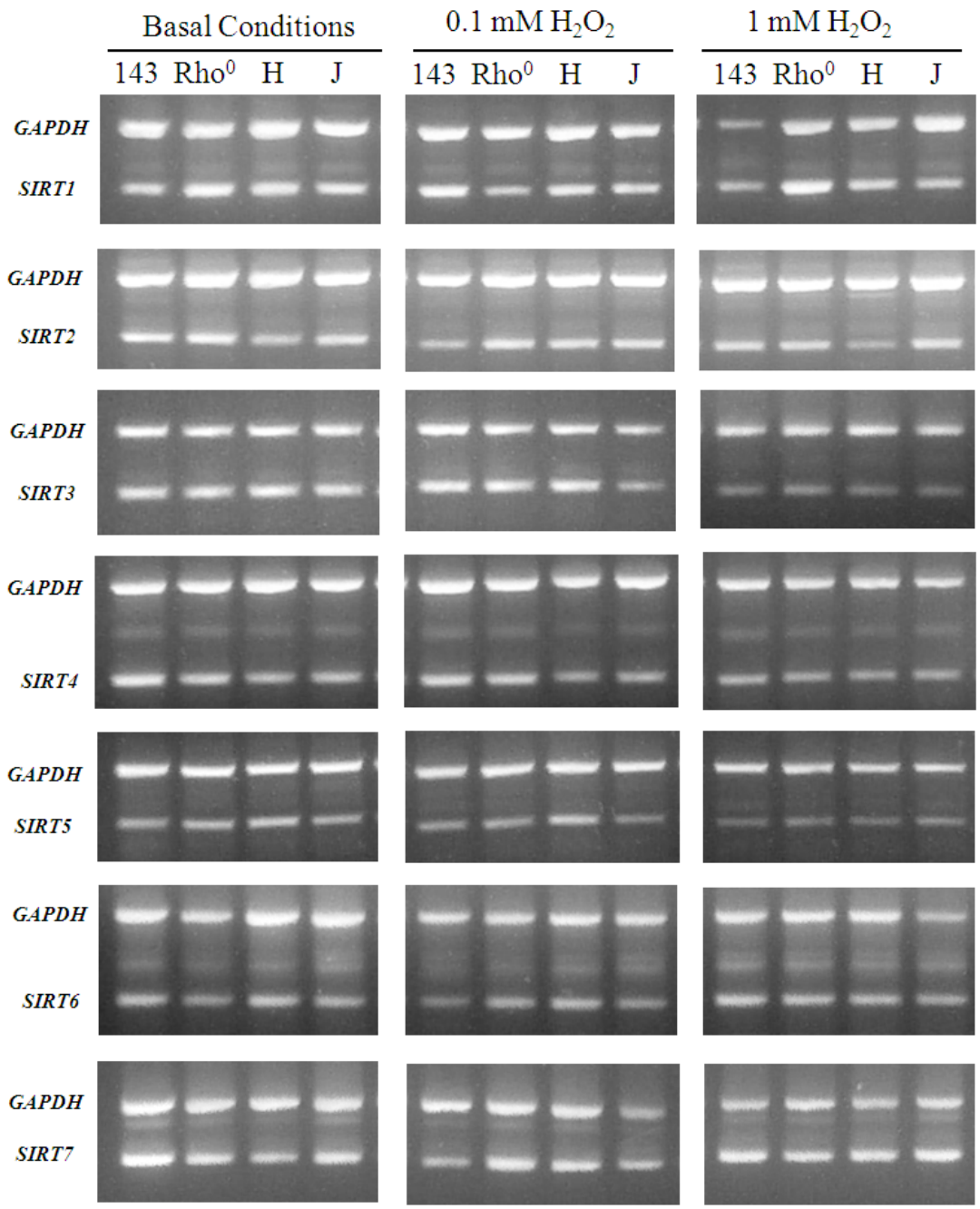
Basal Conditions
0.1 mM H_2O_2
1 mM H_2O_2
143 Rho^0 H J
143 Rho^0 H J
143 Rho^0 H J
GAPDH
SIRT1
GAPDH
SIRT2
GAPDH
SIRT3
GAPDH
SIRT4
GAPDH
SIRT5
GAPDH
SIRT6
GAPDH
SIRT7

Figure 3

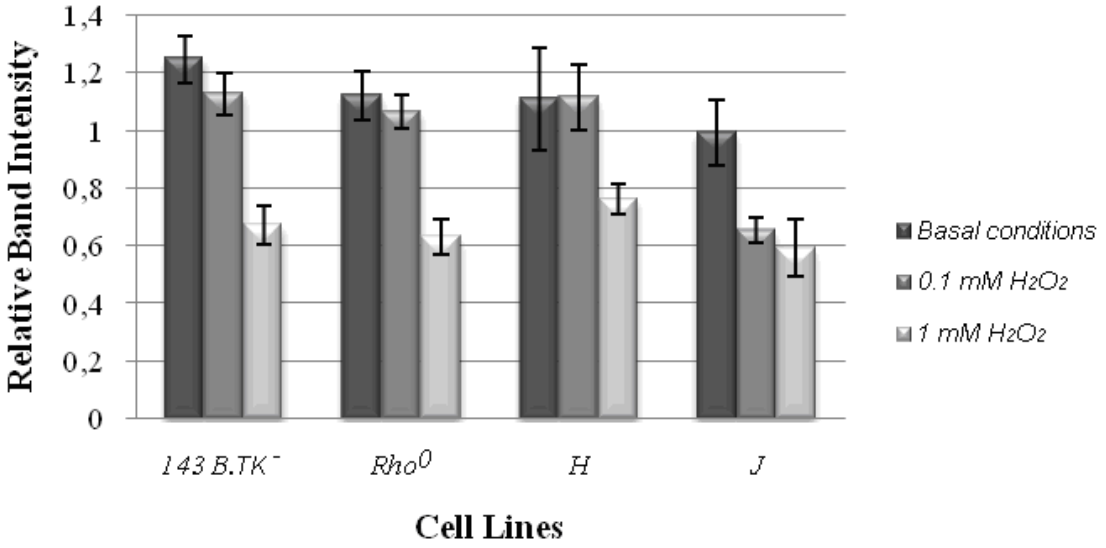
Relative Band Intensity
1,4
1,2
1
0,8
0,6
0,4
0,2
0
143 B.TK⁻
Rho⁰
H
J
Cell Lines
Basal conditions
0.1 mM H2O2
1 mM H2O2

Figure 4

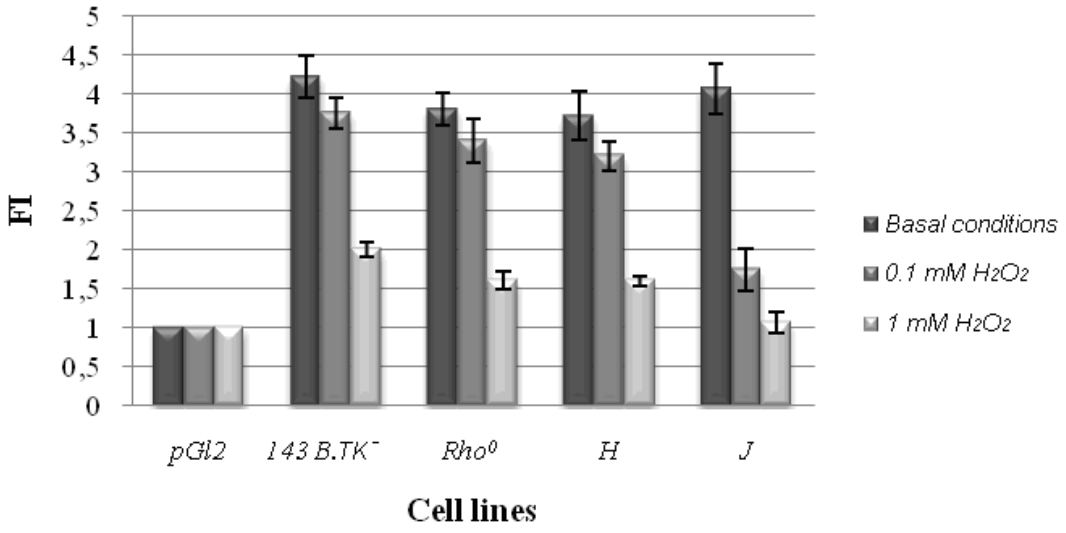
FI
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
pGl2
143 B.TK-
Rho0
H
J
Cell lines
Basal conditions
0.1 mM H2O2
1 mM H2O2

Figure 5

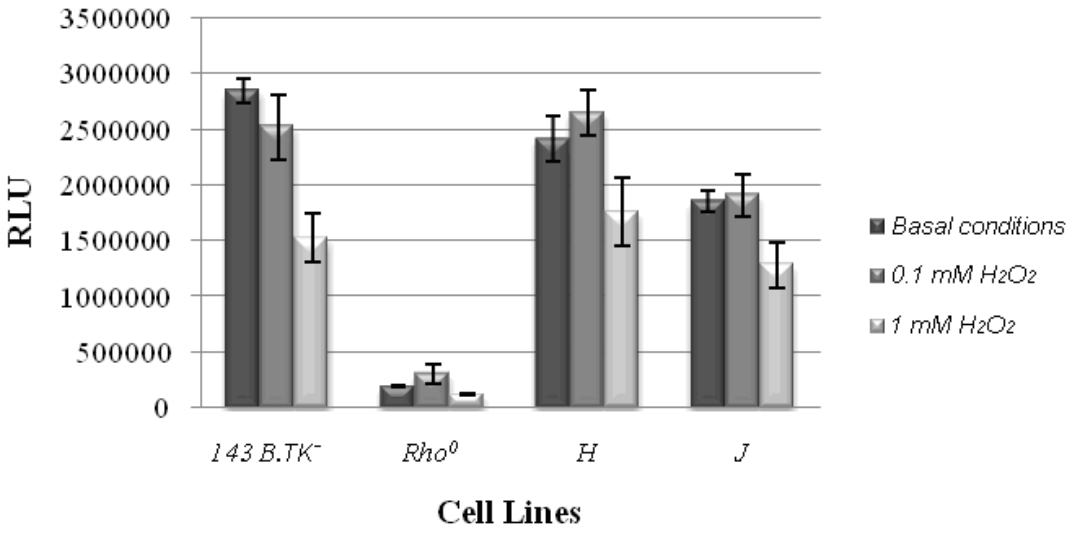
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
RLU
143 B.TK⁻
Rho⁰
H
J
Cell Lines
Basal conditions
0.1 mM H2O2
1 mM H2O2

Figure 6

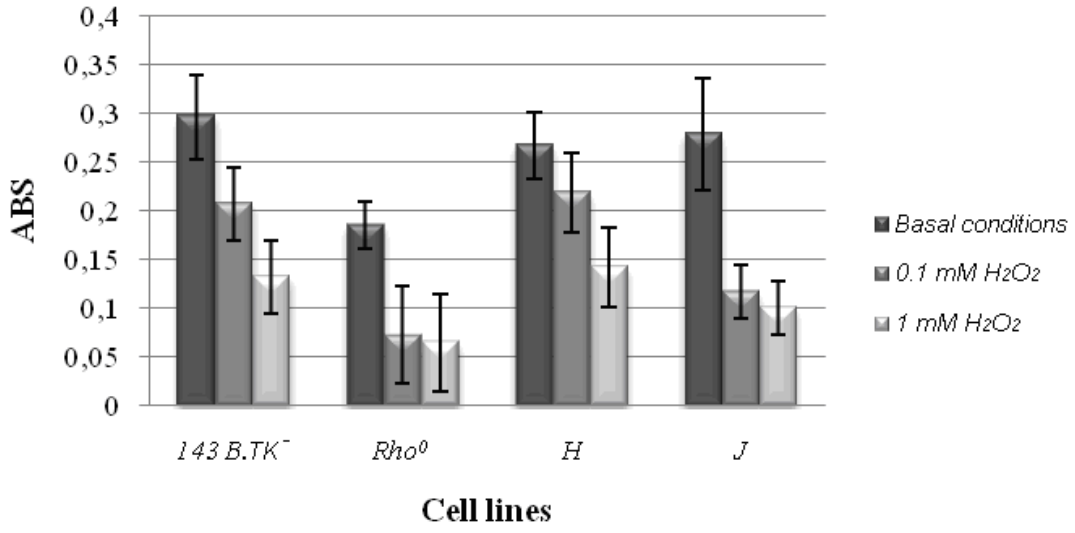
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
ABS
143 B.TK⁻
Rho⁰
H
J
Cell lines
Basal conditions
0.1 mM H2O2
1 mM H2O2

Figure 7

Cell Stress and Chaperones (2009) 14:265–271
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ORIGINAL PAPER

Mitochondrial DNA variability modulates mRNA and intra-mitochondrial protein levels of HSP60 and HSP75: experimental evidence from cybrid lines

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Abstract To explore possible relationships between mitochondrial DNA (mtDNA) polymorphism and the expression levels of stress-responder nuclear genes we assembled five cybrid cell lines by repopulating 143B.TK$^-$ cells, depleted of their own mtDNA (Rho^0 cells), with foreign mitochondria with different mtDNA sequences (lines H, J, T, U, X). We evaluated, at both basal and under heat stress conditions, gene expression (mRNA) and intra-mitochondrial protein levels of HSP60 and HSP75, two key components in cellular stress response. At basal conditions, the levels of HSP60 and HSP75 mRNA were lower in one cybrid (H) than in the others (p=0.005 and p=0.001, respectively). Under stress conditions, the H line over-expressed both genes, so that the inter-cybrid difference was abolished. Moreover, the HSP60 intra-mitochondrial protein levels differed among the cybrid lines (p=0.001), with levels higher in H than in the other cybrid lines. On the whole, our results provide further experimental evidence that mtDNA variability influences the cell response to stressful conditions by modulating components involved in this response. Sentence summary of the article: the results reported in the present study provide important experimental evidence that in human cells mtDNA variability is able to influence the cellular response to heat stress by modulating both the transcription of genes involved in this response and their intra-mitochondrial protein levels.

D. Bellizzi and D. Taverna equally contributed to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s12192-008-0081-x) contains supplementary material, which is available to authorized users.

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Keywords Cybrid cell lines · HSP60 · HSP75 · mtDNA variability · Stress response

Abbreviations

ROS	reactive oxygen species
RFLP	restriction fragment length polymorphism
RT-PCR	reverse-transcriptase-polymerase chain reaction

Introduction

Recent data indicate that polymorphisms of the mitochondrial genome (mtDNA) are not neutral and evidence of an association between mtDNA variability and complex traits is increasing (Wallace 2005; Crispim et al. 2006; Bai et al. 2007). Furthermore, numerous evidences suggest that the different mtDNA lineages are qualitatively different from each other, bearing mutations that can modulate mitochondrial function and consequently influence complex phenotypes (Mishmar et al. 2003). This modulation is carried out either directly by influencing energy production efficiency (Baudouin et al. 2005), or indirectly by interaction with nuclear genes (Ryan and Hoogenraad 2007). In humans, the involvement of nuclear–mitochondrial interactions in modulating complex phenotypes is supported by the observation of non-random associations between mtDNA and nuclear variability (De Benedictis et al. 2000; Carrieri et al. 2001; Maruszak et al. 2008). In mice, by means of conplastic strains expressing different combinations of mitochondrial/nuclear genomes, it has been unequivocally demonstrated that mtDNA variability affects complex phenotypes, such as hearing loss (Johnson et al. 2001), cognitive function (Robertoux et al. 2003) and risk of type 2 diabetes (Pravenec et al. 2007). Finally, in vitro transmitochondrial hybrids have shown that nuclear–mitochondrial interactions

may modulate nuclear gene expression (Jahangir Tafrechi et al. 2005), mitochondrial reactive oxygen species (ROS) production (Vives-Bauza 2006) and intracellular calcium dynamics (Kazuno et al. 2008). Recently, we have provided experimental evidence of such interactions by analyzing human cybrid cell lines that share the same nuclear genome but have different mtDNA (Bellizzi et al. 2006). In these cells, mtDNA variability is associated with expression levels of genes encoding cytokines and cytokine-receptors. In particular, the existence of mitochondrial-specific effects on the expression of interleukin-1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor receptor 2 (TNFR2) genes has been observed at both basal and oxidative stress conditions.

Considering these observations, the aim of the present study was to investigate whether the modulation of stress-responder nuclear genes by mtDNA variability is a general phenomenon concerning not only cytokines and oxidative stress, but also other stress-responder systems, such as heat shock proteins (HSPs) and heat stress.

To this purpose, we developed cybrid cell lines by repopulating osteosarcoma Rho^0 cells with foreign mitochondria having different mtDNA sequences. In these cells we analyzed the expression of two heat shock protein genes, HSP60 and HSP75, at both basal and heat stress conditions. HSP60 (Cpn60) and HSP75 (TRAP1) are mitochondrial chaperones that assist, in both stressed and non-stressed cells, in the folding, unfolding, or disaggregating of proteins either imported from the cytosol or synthesized within mitochondria (HSP60) (Cheng et al. 1989; Frydman 2001; Itoh et al. 2002; Saibil 2008) and in the reallocation of cytosolic protein into mitochondria (HSP75) (Felts et al. 2000; Mokranjac and Neupert 2005). In addition to its chaperone activity, HSP60 has well-documented anti- or pro-apoptotic roles (Arya et al. 2007; Chandra et al. 2007) as well as immunoregulatory properties (Habich and Burkart 2007; Pockley et al. 2008). HSP75 acts as an antagonist of ROS and exhibits anti-apoptotic activity (Hua et al. 2007; Pridgeon et al. 2007).

The results reported here show that the levels of HSP60 and HSP75 mRNAs, and the intra-mitochondrial protein level of HSP60, are correlated to mtDNA variability thereby providing additional evidence for the role played by such variability in the stress response.

Materials and methods

Cell lines and culture conditions

143B.TK$^-$ osteosarcoma cells and cybrid cell lines were grown in Dulbecco's modified eagle medium (DMEM, Invitrogen) containing 25 mM glucose and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 0.1 mM gentamycin (Invitrogen). Rho^0 cells were grown in the above medium supplemented with 0.2 mM uridine (Sigma). 143B.TK$^-$, cybrid cell lines, and Rho^0 cells were cultured in a water-humidified incubator at 37°C in 5% CO_2/95% air.

Heat stress treatment

143B.TK$^-$, Rho^0, and cybrid cells, 1×10^5, were seeded in 24 well/plates. In the exponential growth phase, the cells were incubated at 42°C for 2, 4 and 6 h. Untreated cells were also used as control.

Cell viability assay

Treated and untreated cell lines were assayed for viability by Trypan blue exclusion assay. Floating and adherent cells were collected and 200 μl of cellular suspension were added to an equal volume of 0.4% Trypan Blue solution (Sigma). Then, viable and non-viable cells were counted on a hemocytometer with an inverted light microscope using a ×20 magnification.

RT-PCR of HSP genes

Total RNA was extracted from control and heat-treated cells with RNeasy kit (Qiagen). The reverse-transcriptase-polymerase chain reactions (RT-PCR) were carried out by using the ImPromII Kit (Promega). An RT mix including 500 ng of total RNA and 0.5 μg of oligo-dT primers was pre-heated at 70°C for 5 min. The reaction was carried out in a 40 μl final volume containing 1X RT buffer, 0.5 mM of each dNTP, 3 mM $MgCl_2$, 20 U RNase inhibitor, and 5 U reverse transcriptase. The mix was incubated at 25°C for 5 min, then 37°C for 1 h and, successively, at 95°C for 10 min to inactivate the reverse transcriptase.

The primers used in gene expression analyses were the following:

HSP72	Forward primer	5' AAGTTGCAATGAACCCCACC 3'
	Reverse primer	5' TTGCGCTTAAACTCAGCAA 3'
HSP60	Forward primer	5' ATTCCAGCAATGACCATTGC 3'
	Reverse primer	5' GAGTTAGAACATGCCACCTC 3'
HSP75	Forward primer	5' TGGCAGTTATGGAAGGTAAA 3'
	Reverse primer	5' AGCAATGACTTTGTCTTCTG 3'
GAPDH	Forward primer	5' GACAACTTTGGTATCGTGGA 3'
	Reverse primer	5' TACCAGGAAATGAGCTTGAC 3'

The PCR mixture (30 μl) contained 1.5 μl of cDNA, 1X buffer RB, 0.5 mM of each dNTP, 3.5 mM $MgCl_2$, 0.6 μM of each primer, and 10 U DNA polymerase (EuroTaq). After an initial denaturation step at 94°C for 1 min, the PCR was carried out for 25 cycles at 92°C for 1 min, followed by 56°C for 1 min and 72°C for 1 min. The final step was an incubation at 72°C for 10 min. Then, PCR products were analyzed on 2.5% agarose gel containing 0.5 mg/ml ethidium bromide. Fluorescence intensity of each band was calculated using densitometric analyses (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized to glyceraldehyde phosphate dehydrogenase gene (GAPDH) band intensity.

Isolation of mitochondrial protein fractions

Mitochondrial extracts were prepared by using Mitochondrial Fractionation Kit (Active Motif). 2×10^7 heat-treated and untreated cells were scraped on ice after the addition of 10 ml of ice-cold 1X phosphate-buffered saline (PBS) and then centrifuged at 600×*g* for 5 min at 4°C. Cell pellets were re-suspended in 5 ml of ice-cold PBS and centrifuged at 600×*g* for 5 min at 4°C. Then, cell pellets were resuspended in 250 μl of 1X cytosolic buffer included in the kit and then incubated on ice for 15 min. Successively, cell pellets were homogenized with a homogenizer and the resulting supernatant was centrifuged at 800×*g* for 20 min at 4°C. Then, the supernatant, containing the cytosol and the mitochondria, was removed and centrifuged a second time at 800×*g* for 10 min at 4°C. Then, the supernatant was newly removed and centrifuged at 10,000×g for 20 min at 4°C to pellet the mitochondria. Mitochondrial pellets were washed with 100 μl of 1X cytosolic buffer and then centrifuged at 10,000×*g* for 10 min at 4°C. Finally, mitochondrial pellets were lysed by adding 35 μl of complete mitochondria buffer, prepared by adding mitochondria buffer, protease inhibitor cocktail, and dithiothreitol included in the kit, and incubating on ice for 15 min.

Western blot analyses

Fifteen microgram of mitochondrial extracts were separated on 10% SDS-PAGE and transferred into Hibond-P membranes at 60 V for 1 h at 4°C. Membranes were washed with TBST 1X (0.3 mM Tris–HCl, pH 7.5, 2.5 mM NaCl, 0.05% Tween 20) for 10 min and then incubated overnight at room temperature with 5% non-fat dried milk in TBST 1X. Then blots were washed three times with TBST 1X for 10 min and incubated in TBST containing 1% milk with anti-HSP60 polyclonal mouse antibodies (1:1,000) (Stressgen) and anti-HSP75 polyclonal rabbit antibodies (1:50) (SantaCruz). Then, anti-mouse (1:5,000) or anti-rabbit (1:2,000) antibodies conjugated with horseradish peroxidase (HRP, Amersham) were used as secondary antibodies. Immunoreactivity was determined by means of the ECL chemiluminescence reaction (Amersham). Anti-CoxIV antibody (1:500, Molecular Probes) was used as an internal control of the mitochondrial fraction. Quantitative evaluation of the blots was carried out by using densitometric analyses of immunoreactive band intensities (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized to the internal control.

Statistical analyses

Statistical analyses were performed using SPSS 15.0 statistical software. We adopted one-way analysis of variance for multiple comparisons and student *t*-test for pair-wise comparisons. Significance level was defined as $\alpha=0.05$.

Results

Cell lines

We analyzed the 143B.TK$^-$ native cell line, its derivative Rho^0 line, and the H and J cybrid cell lines which we have previously described (Bellizzi et al. 2006). In addition, we produced ex-novo three cybrid cell lines by fusing Rho^0 cells with platelets isolated from young donors. According to the variability at diagnostic positions (RFLP analyses), mtDNAs of the donor platelets were classified as belonging to U, X, and T haplogroups (Torroni et al. 1996). Therefore, we named the newly produced cybrid cell lines according to the name of the respective mtDNA haplogroup. By analyzing the AvaII8249 polymorphic site, we found that the mtDNA of the X cybrid line and that of the 143B.TK$^-$ native line, although both belonging to the X haplogroup, were of different haplotype (Table S1 in Supplementary Material).

Then, we assessed the cellular state of the newly produced U, X, and T cybrid lines by carrying out control experiments (proliferation assays, quantification of mtDNA, and mitochondrial membrane potential—MMP—assay) as previously described (Bellizzi et al. 2006). Proliferation rate, number of copies of mtDNA per cell and MMP values of U, X, and T cybrid lines did not differ significantly from those of the native cell line (data not shown).

Heat stress

In order to determine the optimal conditions necessary to induce heat stress in our cell lines, we treated the seven cell

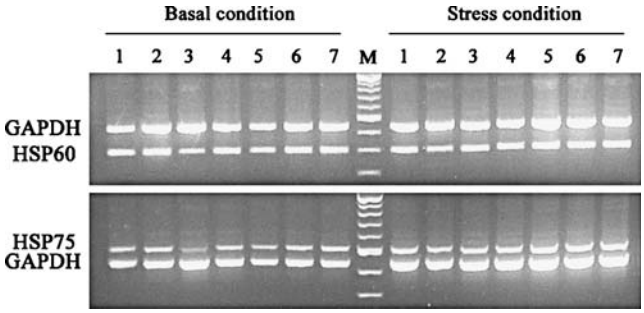

Fig. 1 Representative RT-PCR electrophoresis patterns of HSP60 and HSP75 mRNAs at basal and under stress conditions (42°C for 4 h) in the following cell lines: 143B.TK$^-$ (*1*), Rho^0 (*2*), H (*3*), J (*4*), U (*5*), X (*6*) and T (*7*). HSP60 heat shock protein 60, HSP75 heat shock protein 75, GAPDH glyceraldehyde phosphate dehydrogenase, *M* molecular weight 100 bp ladder

lines at 42°C for 2, 4 and 6 h, and then we checked the cellular state of the seven lines by cell viability assay. In all the lines, the treatment did not induce excessive cell death, as the percentage of living cells was 90% about in all the cases. Slight differences in the percentage of living cells between basal and stress condition were observed at 2 h (Fig. S1 in Supplementary Material), probably because of casual cell fluctuations or experimental manipulation. These differences were minimum at 4 h. Then, since HSP60 and HSP75 are reported to be constitutively expressed genes we measured the cell response to heat stress by looking at the expression pattern of the major heat shock-inducible gene, Heat shock protein 72 (HSP72). In Fig. S2 (Supplementary Material) a representative HSP72 gene expression pattern is shown, while Table S2 (Supplementary Material) reports the densitometric analyses of three independent experiments. We observed that the HSP72 gene is significantly up-regulated by heat, and such up-regulation is maximum at 4 h. Therefore, considering cell viability assays and HSP72 gene expression analysis we choose 4 h of heat treatment as the ideal experimental condition.

HSP60 and HSP75 mRNA level analysis

The levels of HSP60 and HSP75 mRNAs were evaluated in the seven cell lines at basal and stress conditions by semi-quantitative RT-PCRs. We ruled out the possibility that PCRs reached the stationary level by assembling saturation curves for each gene including the glyceraldehyde phosphate dehydrogenase gene, used as an internal control (data not shown).

In Fig. 1, a representative RT-PCR electrophoresis pattern of HSP60 and HSP75 gene expression is shown, while Table 1 summarizes the densitometric analyses for three experiments. Considering the fluctuating values of mRNA levels of HSP60 and HSP75 genes in our cell lines, we pointed our attention only to differences of gene expressions where one cell line consistently had a gene expression level at least twofold higher with respect to another cell line.

At basal conditions, comparing 143B.TK$^-$ and Rho^0 cell lines no significant difference was observed for either HSP60 or HSP75 (p=0.871 and p=0.523, respectively), thus indicating that the expression of the two genes is independent of the presence of active mitochondria. In contrast, under stress conditions HSP60 mRNA levels were significantly lower in Rho^0 cells than in the native line (p=0.003).

As for the cybrid lines, at basal conditions the mRNA levels of both HSP60 and HSP75 differed among the lines (p=0.005 and p=0.001, respectively), with the H cybrid showing mRNA levels lower than those of the other lines (Table 1). This result was confirmed by RT-PCRs carried out on an independent H clone (data not shown). Since mtDNA is the sole variant among the cybrid lines, we can conclude that a correlation exists between mtDNA variability and mRNA levels of HSP60 or HSP75. Interestingly, under heat stress the mRNA levels of both HSP60 and HSP75 in H cybrid increase up to the levels of the other cybrid lines (p=0.924 and p=0.744, respectively). This

Table 1 Densitometric analysis of HSP60 and HSP75 mRNA levels, normalized to GAPDH mRNA levels, in 143B.TK$^-$, Rho^0, H, J, U, X and T cell lines at basal and under stress conditions (42°C for 4 h)

Densitometric analysis							
Basal condition							
	143B.TK$^-$	Rho^0	H	J	U	X	T
HSP60	0.573 (0.080)	0.550 (0.110)	0.245 (0.089)	0.675 (0.086)	0.532 (0.056)	0.640 (0.065)	0.555 (0.051)
HSP75	0.465 (0.045)	0.416 (0.056)	0.211 (0.042)	0.342 (0.024)	0.365 (0.017)	0.416 (0.048)	0.461 (0.062)
Stress condition							
	143B.TK$^-$	Rho^0	H	J	U	X	T
HSP60	0.847 (0.040)	0.489 (0.062)	0.734 (0.065)	0.680 (0.058)	0.764 (0.039)	0.751 (0.065)	0.742 (0.104)
HSP75	0.369 (0.041)	0.272 (0.050)	0.400 (0.032)	0.328 (0.057)	0.413 (0.050)	0.386 (0.050)	0.352 (0.057)

Average values over three experiments are reported with standard error mean in parentheses

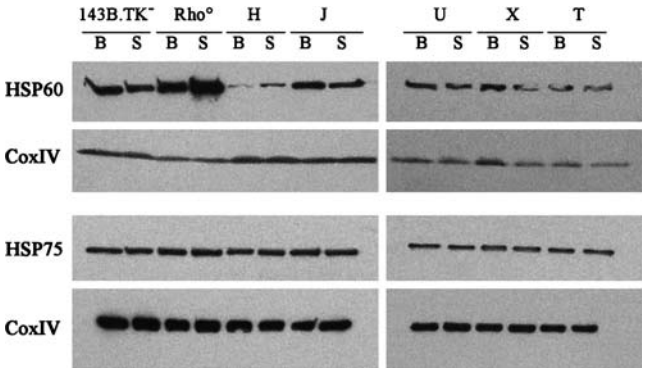

Fig. 2 Representative Western blot electrophoresis patterns of HSP60 and HSP75 intra-mitochondrial proteins in 143B.TK$^-$, Rho^0, H, J, U, X and T cell lines at basal (*B*) and stress conditions (*S* 42°C for 4 h). CoxIV cytochrome c oxidase subunit IV

observation suggests that the H line reacted to heat stress by up-regulating the expression of the two genes.

Western blot analysis

Considering that both HSP60 and HSP75 are mitochondrial proteins, we evaluated whether mitochondrial protein levels were correlated with mtDNA variability. By Western blot we analyzed the amount of HSP60 and HSP75 present in mitochondria in all the cell lines. In Fig. 2, a representative Western blot pattern of the HSP60 and HSP75 intra-mitochondrial protein levels is shown, while Table 2 summarizes the densitometric analyses of three experiments. As referred in the previous paragraph, we pointed our attention only to differences of protein levels where one cell line consistently had levels at least twofold higher with respect to another cell line.

At basal conditions, the HSP60 and HSP75 intra-mitochondrial protein levels were higher in Rho^0 cells than in the native line (p=0.046 and p=0.036, respectively). Under stress conditions, only the HSP60 protein level increased in Rho^0 cells compared to the native line (p=0.015). As for HSP75, although the same trend was observed, the difference between basal and stress conditions was not statistically significant (p=0.258).

By comparing cybrid cell lines at basal conditions, no difference was found either in HSP60 or in HSP75 intra-mitochondrial protein levels (p=0.419 and p=0.064, respectively) thus indicating that these levels are independent of mtDNA variability. On the contrary, under stress conditions the HSP60 protein level differed among the lines (p=0.001), with the H cybrid showing higher levels compared to the other cybrid lines (Table 2). These results were also confirmed by Western blot carried out on an independent H clone (results not shown).

Discussion

The aim of the present study was to investigate whether mtDNA variability affects gene expression levels and/or intra-mitochondrial protein concentration, of HSP60 and HSP75, two key components of the mitochondrial stress response machinery. The question is of general interest due to the well-documented role of HSPs in maintaining cellular homeostasis in response to stress.

The cybrid technology used in our model to answer the question is largely debated in regards to the effect of cybridization on transcription patterns (Danielson et al. 2005). In the present case, however, we are confident of the reliability of our results for two reasons. First, observing Table 1, we see that under the heat stress condition, the cybridization process does not affect either HSP60 or HSP75 mRNA levels with respect to the native line, while these levels are lower in the cells depleted of active mitochondria (Rho^0 cells). Second, the down-regulation of HSP60 and HSP75 genes observed in the H line in basal conditions, as well as the up-regulation observed in this line under stress conditions (Table 1), were confirmed in an independent clone.

To our knowledge, the present study identifies a correlation between mtDNA variability and mRNA levels of HSP genes for the first time. We note that as for the H

Table 2 Densitometric analysis of intra-mitochondrial protein levels of HSP60 and HSP75, normalized to CoxIV protein levels, in 143B.TK$^-$, Rho^0, H, J, U, X and T cell lines at basal and under stress conditions (42°C for 4 h)

Densitometric analysis							
Basal condition							
	143B.TK$^-$	Rho^0	H	J	U	X	T
HSP60	2.078 (0.444)	4.303 (0.880)	2.071 (0.439)	2.023 (0.559)	1.761 (0.277)	1.832 (0.329)	1.524 (0.314)
HSP75	0.171 (0.029)	0.363 (0.060)	0.152 (0.012)	0.151 (0.035)	0.178 (0.010)	0.164 (0.029)	0.159 (0.020)
Stress condition							
	143B.TK$^-$	Rho^0	H	J	U	X	T
HSP60	2.748 (0.445)	5.259 (0.425)	3.939 (0.222)	1.688 (0.185)	1.108 (0.090)	1.264 (0.208)	1.620 (0.473)
HSP75	0.254 (0.092)	0.502 (0.244)	0.132 (0.005)	0.130 (0.019)	0.145 (0.016)	0.115 (0.016)	0.146 (0.018)

Average values over three experiments are reported with standard error mean in parentheses

cybrid, the heat induction of HSP60 gene is in line with literature data, while the up-regulation of the HSP75 gene is unexpected, as it had been reported to be up-regulated by stresses different from heat (Hadari et al. 1997; Ryan et al. 1997; Carette et al. 2002; Zhao et al. 2002; Tokalov et al. 2003; Murray et al. 2004; Volobouéva et al. 2008). Considering the role of the two HSPs in the processes of protein import into mitochondria and subsequent protein folding (Frydman 2001; Mokranjac and Neupert 2005; Saibil 2008), we could hypothesize that mtDNA variability modulates mRNA levels of HSP60 and HSP75 through transcription factors that coordinate the activity of the two genes. In fact, numerous evidence indicate that the mitochondrial genome is able to regulate a series of nuclear target genes by transcription factors, such as NFkB and CEBP, that acts as mediators of the well known cross-talk nucleus-mitochondrion (Biswas et al. 2005). The above hypothesis is also in line with literature data showing that promoters of HSP genes contain common and highly conserved binding sites for transcription factors some of which are specifically required for the heat shock response (Amin et al. 1988; Trinklein et al. 2004). We are currently investigating whether the above transcription factors are able to regulate also genes encoding for heat shock proteins localized in the cytoplasm and not only for those localized in the mitochondria.

The cybrid-specific response to stress (line H in Table 1) is in agreement with the results for cytokines and cytokine-receptors we described previously (Bellizzi et al. 2006). Therefore, the correlation between mtDNA variability and expression levels of stress-responder nuclear genes could be a general phenomenon, even if further studies are needed to generalize this assumption.

We obtained a confirmation of the cybrid-specificity in the response to stress by Western blot data (Table 2): the H line is the sole cybrid line that accumulated HSP60 within mitochondria. This result is very interesting because it suggests a correlation between mtDNA variability and accumulation within mitochondria of a protein which has a crucial role in coping with stress damage. In line with this role, the Rho^0 cells, which are in very stressful conditions due to mitochondrial dysfunction, show an increase in intra-mitochondrial protein levels of both HSP60 and HSP75. The cell mechanisms through which the accumulation of both proteins within mitochondria occurs independently of nuclear gene expression is not known, and further studies are needed to clarify this point.

On the whole, the results reported in the present study provide important experimental evidence that in human cells mtDNA variability is able to influence the cellular response to heat stress by modulating both the transcription of genes involved in this response and their intra-mitochondrial protein levels.

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Conclusive remarks

Years of researches about the biological basis of human longevity have outlined a complex scenario in which both genetic and epigenetic factors have emerged as key determinants. In this context, the articles presented here contribute in disentangling the manuscripts presented here contribute to shed a light on some aspects of these complex mechanisms.

In fact, our works demonstrate a correlation between the age-specific functional decline and DNA methylation levels, suggesting that the relaxation of the epigenetic control in aging is more specifically associated to the functional decline than to the chronological age of individuals. Moreover, these levels might be involved in determining physiological changes over the lifetime. Here we provide the first evidence that the DNA methylation levels are modulated by mtDNA variability through mtDNA-specific cross signaling between mitochondrial and nuclear genome specifically influenced by OXPHOS efficiency. These evidence highlight the importance of the mitochondrial function in age-related processes of gene expression remodeling and in the decrease of the physiological and cellular homeostasis during aging.

The manuscripts about the role of mitochondrial DNA genetic variants in stress response mechanisms, which are crucial for achieving longevity, demonstrate the existence of mtDNA-dependent pathways of communications able to modulate, in stress conditions, the expression of the nuclear genes encoding for SIRT3, HSP60 and HSP75 proteins. These results provide a link among stress responder nuclear genes, mitochondrial DNA inherited variants and mitochondrial functionality, three main components that have been considered to play a fundamental role in stress response.

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