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Expression profiles of stress-responder nuclear genes in relationship to common mitochondrial DNA variability

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Sommario

Tutti gli organismi e le cellule viventi sono continuamente esposti, nel corso della vita, ad agenti stressanti che possono compromettere l'omeostasi cellulare. Tali agenti possono essere di natura esogena oppure endogena, come le Specie Reattive dell'Ossigeno (ROS) che sono i principali induttori dello stress ossidativo. Le cellule viventi hanno sviluppato una serie di meccanismi di difesa nei confronti dei differenti agenti stressanti, noti come meccanismi di risposta allo stress, che agiscono a livello molecolare, cellulare e sistemico allo scopo di evitare o risolvere i danni connessi alle diverse condizioni di stress. L'efficienza della risposta allo stress è garantita da meccanismi biologici altamente regolati sia a livello nucleare che a livello mitocondriale. In questo contesto, diverse evidenze sperimentali hanno mostrato che mutazioni e polimorfismi del DNA mitocondriale (mtDNA) agiscono come fattori di suscettibilità nei tratti complessi modulando la risposta allo stress attraverso una comunicazione bi-direzionale tra nucleo e mitocondrio.

Scopo del presente lavoro di tesi è stato di verificare se la variabilità comune del DNA mitocondriale influenza la risposta allo stress modulando l'espressione di geni nucleari stress-responder attraverso un cross-talk tra il genoma nucleare e quello mitocondriale. A tale scopo, abbiamo preparato cinque linee cellulari cibride in cui è stata analizzata l'espressione dei geni codificanti citochine e loro recettori, sia in condizioni basali che in condizioni di stress ossidativo, e quella dei geni codificanti heat shock proteins (HSP), sia in condizioni basali che in condizioni di heat shock. Le linee cibride sono state ottenute ripopolando cellule di osteosarcoma 143B.TK- precedentemente depletate del proprio mtDNA (cellule rho^0) con mitocondri caratterizzati da aplogruppo mitocondriale H, J, U, X e T.

I principali risultati ottenuti nel presente lavoro sono riassunti di seguito.

1) In condizioni basali, l'espressione dei geni codificanti citochine e HSP è modulata in maniera specifica dalla variabilità del mtDNA. In particolare, abbiamo dimostrato l'esistenza di effetti mtDNA-specifici sull'espressione dei geni codificanti interleuchina-1β (*IL-1*b); interleuchina 6 (*IL-6*) e il recettore 2 del fattore di necrosi tumorale (*TNFR2*), nonché dei geni codificanti heat shock protein 60 (*HSP60*) e heat shock protein 75 (*HSP75*). Tale risultato fornisce una

prima evidenza sperimentale che la variabilità del mtDNA è in grado di influenzare l'espressione di due diverse classi di geni nucleari stress-responder.

2) Le differenze nei pattern di espressione genica osservate tra le linee cibride in condizioni basali scompaiono in condizioni di stress. Infatti, sia lo stress ossidativo che lo stress termico equalizzano i pattern di espressione genica in modo tale che essi risultano indipendenti dalla variabilità comune del DNA mitocondriale.

3) Confrontando la linea parentale con la linea rho^0, abbiamo riconfermato l'esistenza di una risposta retrograda che induce l'espressione del gene *IL6* in risposta allo stress.

4) Infine, il trattamento termico ha indotto un incremento nei livelli proteici di HSP60 soltanto nel cibrido H. Questo risultato mostra, per la prima volta, una correlazione tra la variabilità comune del DNA mitocondriale e l'accumulo nei mitocondri di una proteina che ha un ruolo cruciale nella risposta ai danni indotti da stress.

Nel complesso, i risultati ottenuti forniscono una importante evidenza sperimentale che i polimorfismi comuni del mtDNA sono in grado di influenzare la risposta cellulare a condizioni stressanti modulando i diversi componenti coinvolti in tale risposta.

Summary

All organisms and living cells are continuously exposed, in the course of life, to several stressors, that can compromise cellular homeostasis. These stressors can be exogenous or endogenous, such as Reactive Oxigen Species (ROS) that are main inducers of oxidative stress. Living cells have developed a complex repertoire of physiologic and behavioural responses forming the adaptive stress response. Stress response mechanisms act at molecular, cellular, and systemic level in order to escape or solve damages occurring during stressful conditions. The efficiency of stress response is guaranteed by biological mechanisms highly regulated at nuclear and mitochondrial level. In this frame, experimental evidences have revealed that mitochondrial DNA (mtDNA) mutations and polymorphisms act as susceptibility factors in complex traits by modulating the stress response through a bi-directional communication between nucleus and mitochondria.

Aim of this PhD thesis was to investigate whether the common mtDNA variability influences the stress response by modulating the expression of stress-responder nuclear genes through a cross-talk between mitochondrial and nuclear DNA. To this purpose, we prepared five cybrid cell lines in which we analyzed the expression of cytokine and cytokine receptor genes at both basal and oxidative stress condition, and of heat shock protein genes at both basal and heat shock stress condition. We obtained the cybrid lines by repopulating 143B.TK$^-$ osteosarcoma cells previously depleted of their own mtDNA (rho^0 cells) with foreign mitochondria having mtDNA classified within H, J, U, X and T haplogroups.

The main results we obtained are summarized as follows.

1) At basal conditions, the expression of genes encoding cytokines and HSPs is specifically modulated by mtDNA variability. In particular, we demonstrated the existence of mitochondrial-specific effects on the expression of interleukin-1β (*IL-1*b)•,interleukin 6 (*IL-6*) and tumor necrosis factor receptor 2 (*TNFR2*) genes, and on the expression of heat shock protein 60 (*HSP60*) and heat shock protein 75 (*HSP75*) genes. This finding provides a first experimental evidence

that mtDNA variability is able to affect the expression of two distinct classes of stress-responder nuclear genes.

2) The differences in gene expression patterns observed among the cybrid lines disappear under stress conditions. In fact, both oxidative and heat shock stress equalize the gene expression patterns so that they become independent of mtDNA common variability.

3) By comparing native and rho^0 cells, we confirmed for the second time the existence of a retrograde response which enhances *IL6* gene expression in response to stress.

4) Finally, the heat shock treatment induced an increase of HSP60 in the H cybrid only (Western blotting). This result shows, for the first time, a correlation between mtDNA common variability and accumulation within mitochondria of a protein which has a crucial role in coping with stress damage.

On the whole, the results provide an important experimental evidence that mtDNA common polymorphisms are able to influence the cellular response to stressful conditions by modulating different components involved in this response.

List of Abbreviations

AD	Alzheimer's disease
ANOVA	Analysis of variance
ATF	Activating transcription factor
ATP	Adenosine Triphosphate
ATP (6-8)	ATP synthase F0 subunit (6-8)
CAT	Catalase
CO (I-III)	Cytochrome *c* Oxidase subunit (I-III)
Cyt*b*	Cytochrome *b*
D-loop	Displacement loop
DMEM	Dulbecco's Modified Eagle Medium
d-Rib	2-deoxy-D-ribose
DTT	Dithiothreitol
$\Delta\psi$	Mitochondrial membrane potential
EDTA	Etilen diamin tretacetic acid
EtBr	Ethidium Bromide
ETC	Electron Transport Chain
FOXO3a	Forkhead transcription factor 3a
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GM-CSFR	Granulocyte-Macrophage Colony-Stimulating Factor Receptor
GPx	Glutathione peroxidase
GSH	Glutathione
H	Heavy strand
HD	Huntington's disease
4-HNE	4-hydroxy-2-nonenol
H_2O_2	Hydrogen peroxide
$HO^{\cdot}$	Hydroxyl radical
HSP	Heat shock protein
HSP10	Heat shock protein 10

HSP27	Heat shock protein 27
HSP40	Heat shock protein 40
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
HSP72	Heat shock protein 72
HSP75	Heat shock protein 75
HSP90	Heat shock protein 90
HSP110	Heat shock protein 110
HVRI	Hyper Variable Region I
HVRII	Hyper Variable Region II
IFN-γ	Interferon-
IL-1	Interleukin-1
IL-1β	Interleukin 1- beta
IL-6	Interleukin-6
IL-6R	Interleukin- 6 Receptor
IL-10	Interleukin-10
L	Light strand
LHON	Leber's Hereditary Optic Neuropathy
MDA	Malondialdehyde
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes
MMP	Mitochondrial Membrane Potential
MRP	Mitochondrial ribonuclease protein
mtDNA	Mitochondrial DNA
MTG	MitoTracker Green
mtTFA	Mitochondrial transcription factor A
NADH	Nicotinamide Adenine Dinucleotide
ND (1-6; 4L)	NADH Dehydrogenase subunit (1-6; 4L)
nDNA	Nuclear DNA
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor *kappa* B
NO	Nitric oxide

NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
8-OH-G	8-hydroxy-2-deoxyguanosine
O_2	Molecular oxygen
O_H	H-strand replication Origin
O_L	L-strand replication Origin
OXPHOS	Oxidative Phosphorylation
$O_2^{\cdot-}$	Superoxide anion
ONO_2H	Peroxynitrite
PCR	Polymerase Chain Reaction
PD	Parkinson’s disease
PGC-1	Peroxisome proliferator-activated receptor coactivator
PKD1	Serine/threonine protein kinase D1
RFLP	Restriction Fragment Length Polymorphisms
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rRNAs	ribosomal RNAs
tRNAs	transfer RNAs
Rtg1-2-3	Retrograde protein 1-2-3
RT-PCR	Reverse Transcription –Polymerase Chain Reaction
SOD	Superoxide dismutase
TK	Thymidine Kinase
TMRM	Tetra Methyl Rhodamine Methylesther
TNF α	Tumor Necrosis Factor α
TNFR(1-2)	Tumor Necrosis Factor Receptor (1-2)

Note that to distinguish genes from the corresponding proteins the first ones are written in italics.

1. Introduction

All organisms are continuously exposed to stressful events that can compromise cellular homeostasis. Because these events are very frequent, living cells have developed different mechanisms in order to cope with their detrimental effects. On the whole these mechanisms are known as “stress response”. The stress response machinery is highly regulated at molecular level and several nuclear genes are involved in this process. In addition, recent data showed that besides the nuclear genome also the mitochondrial genome plays a pivotal role in stress response. In particular, a correct interplay between the two genomes is fundamental to maintain cellular homeostasis.

In this Introduction we will focalize our attention on stress and stress response mechanisms with particular regard to heat shock and oxidative stress. Then, we will analyze the role of mitochondria as key components of the stress response, with reference to the involvement of mitochondrial DNA (mtDNA) genetic variation in the modulation of complex traits and cellular phenotypes. Finally, we will consider some aspects of the well established cross-talk between nuclear and mitochondrial DNA.

1.1 An overview on stress and stress response

Environment affects all living organisms: to survive, organisms interact with the environment and make adjustments within their cells in order to adapt, maintain function, and survive to stressful conditions. Excessive or chronic stress can lead to dysregulation and/or failure of the adaptive mechanisms and subsequent increased morbidity and mortality, such as cardiovascular diseases, metabolic syndromes, immunosuppression and depression (Tsigos and Chrousos, 2006).

Stress is not only induced by exogenous factors (physical and biological agents) but many endogenous stressors are active within the cells. As far as these endogenous stressors, a particular attention has been devoted to Reactive Oxygen Species (ROS). These species include unstable oxygen radicals such as superoxide anions ($O_2^{\cdot -}$) and hydroxyl radicals ($HO^{\cdot}$), and non radical molecules like hydrogen peroxide (H_2O_2), which is freely diffusible, reactively stable and long lived. An additional group of endogenous stressors is represented by reactive nitrogen species (RNS) including nitric oxide (NO) and peroxynitrite (ONO_2H) (Zmijewski, 2005).

ROS are products of normal cellular metabolism and normally generated by tightly regulated enzymes, although they occur mostly within mitochondria during oxygen transport and cell respiration in response to the variation of oxygen levels. ROS are also produced during phagocyte activity, cytochrome P450 metabolism, inflammatory cell activation or detoxification of xenobiotics (Inoue, 2003; Burkle, 2002). Furthermore, they can be produced by several exogenous processes. Environmental agents including γ-ray, ultraviolet light irradiation and non-genotoxic carcinogens can directly generate or indirectly induce ROS (Valko, 2007)

ROS are known to play a dual role in biological systems, since they can be either beneficial or harmful to living cells. This “two-faced” character of ROS, as reported by Valko and col. (2004), is clearly proved. At low/moderate concentrations beneficial effects of ROS occur that involve physiological roles in cellular responses to noxia, in induction of a mitogenic response and in activation of cellular signalling systems (Valko, 2006). In the signalling pathways, ROS activate a series of second messengers, such as the serine/threonine protein kinase D1 (PKD1), that in turn act on several transcription factors, for example FOXO3a and NF-•B (Storz, 2006). Thus, through

these factors, ROS are able to modulate the expression of genes involved in defence processes, such as oxidative stress response, immune response and apoptosis.

In contrast, at high concentrations, ROS can determine an alteration of the intracellular homeostasis that leads to damage of several key structures such as nucleic acids, lipids and proteins (Blumberg, 2004). ROS react with all the DNA components, damaging both purine and pyrimidine bases, as well as the deoxyribose backbone. The most extensively studied DNA lesion is the formation of 8-hydroxy-2-deoxyguanosine (8-OH-G). Permanent modifications of the genetic material resulting from these damages represent the first step of processes whose outcome are mutagenesis, carcinogenesis, and ageing. ROS-induced lipid peroxidation determines an accumulation of products strongly detrimental for the biological systems, for instance 4-hydroxy-2-nonenol (4-HNE), the major toxic product of lipid peroxidation, and malondialdehyde (MDA), which is mutagenic in bacterial and mammalian cells and carcinogenic in rats (Valko, 2007). At protein level ROS-mediated damage can determine the formation of disulfide bonds at level of cysteine residues carbonyl derivatives, and many other oxidized residues, such as methionine sulfoxide. These alterations in protein structure lead to functional changes with a substantial physiological impact (Kregel and Zhang, 2007). The above considerations indicate that a delicate balance between beneficial and detrimental effects played by ROS is fundamental to the maintenance of cellular homeostasis. This balance is achieved by mechanisms called *redox regulation*. A shift of this equilibrium results in a deleterious process, the *oxidative stress*, that can be defined as an excessive bio-availability of ROS deriving from an imbalance between production and destruction of these species (Kregel and Zhang, 2007). The redox regulation protects living organisms from oxidative stress and maintains "redox homeostasis" by controlling the redox status *in vivo* (Dröge, 2002). Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing (Dalle-Donne, 2006; Dhalla, 2000; Jenner, 2003; Sayre, 2001).

In order to escape or overcome pathologic stress conditions, living cells have developed an intricate repertoire of physiologic and behavioral responses that form the adaptive stress response. This response, which aims at re-establishing the challenged cell equilibrium, is obtained through several mechanisms that can act at molecular level, at

cellular level and at systemic level (Franceschi, 2007). These defence mechanisms involve: (i) preventative mechanisms, (ii) repair mechanisms and (iii) antioxidant defences.

Among preventative and/or reparative mechanisms, a primary role is played by Heat Shock Proteins (HSP) which were firstly discovered in *Drosophila melanogaster* after exposure to elevated temperature (Ritossa, 1962). For this reason these proteins were first termed heat shock proteins. Initially, an increased expression of HSPs was associated to the phenomenon of thermotolerance, which includes an abrupt rise of cell capability to withstand the damaging effect of extreme heat exposure (Rikhvanov, 2005). Now, it is well established that the induction of HSPs is a common primary event of a wide range of stressful conditions, that can be either physiological (growth factors and hormonal stimulation), environmental (heavy metals and ultraviolet radiation), or pathological (inflammation, autoimmune reaction and ROS over-production) (Gasch, 2000). Indeed, protein damage, as protein misfolding or unfolding, is prevented by the expression of HSPs. They promote the refolding of partially denatured proteins or irreversibly damaged target proteins that, finally, are eliminated by proteolitic systems, such as lysosomes and proteasome (Gupta & Knowlton, 2005: Soti & Csermely, 2003).

Among antioxidant defences, a pivotal role in the stress response mechanisms is played by a network of compartmentalized antioxidant enzymes that are usually distributed within the cytoplasm and among various cell organelles. These enzymes, for example superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), work in a complex series of integrated reactions to convert ROS into more stable molecules, such as water and O_2. Besides antioxidant enzymes, non-enzymatic antioxidants, for instance ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione, carotenoids, natural flavonoids and melatonin, can also function as direct scavengers of ROS (Valko, 2007). In addition, estrogens are known to exert anti-oxidant effects by free radical scavenging. In particular, this scavenging has been thought to cause interruption of free-radical chain reactions and, thus, to prevent oxidative damage to biological macromolecules (Prokai, 2006).

1.2 Mitochondria and mitochondrial DNA variability in complex traits

Mitochondria are intracellular organelles located in the cytoplasm of eukaryotic cells where they are present in several copies. These organelles are able to perform multiple cellular functions including energy production, cell proliferation and apoptosis (Birch-Machin, 2006). Mitochondrial energy is produced through electron transport and oxidative phosphorylation (OXPHOS) within the inner membrane. The OXPHOS machinery is composed by five complexes which cooperate to transduce the energy of nutrient-derived substrates into energy stored in ATP (Fig 1.1).

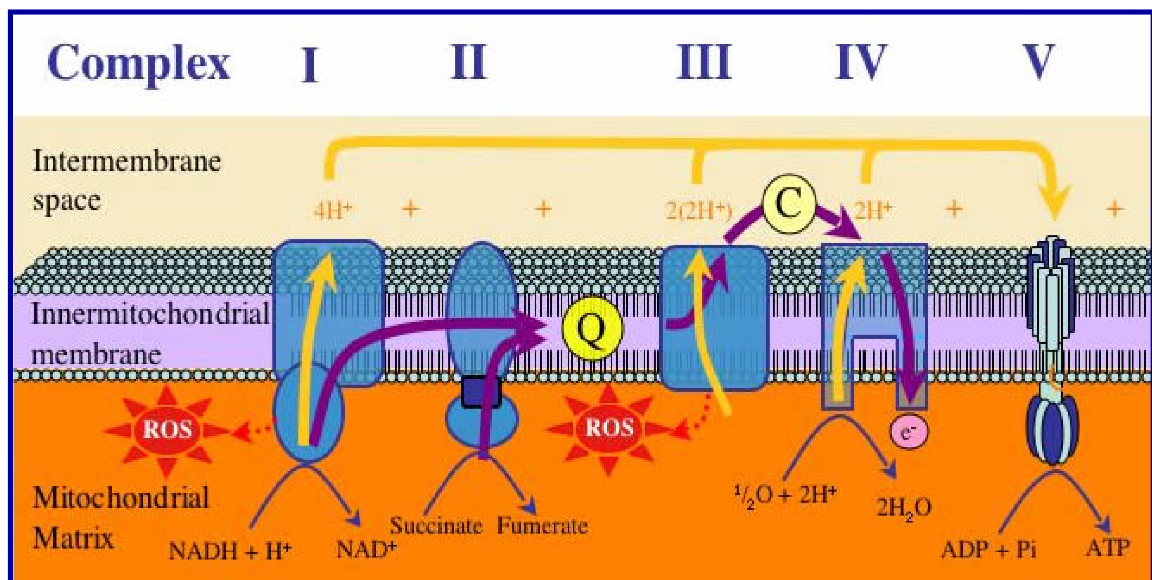


Fig 1.1 Electron Transport Chain. The Electron Transport Chain (ETC) is composed by five complexes: the NADH dehydrogenase complex (complex I), the succinate dehydrogenase complex (complex II), the cytochrome *c* reductase complex (complex III), the cytochrome *c* oxidase complex (complex IV) and the ATP synthase complex (complex V). The mitochondrial respiratory chain also includes two molecules, ubiquinone (coenzyme Q) and cytochrome c, that act as freely-diffusible electron carriers.

Each complex is composed by subunits some of which are encoded by nuclear genes while others by mitochondrial genes. The human mitochondrial DNA (mtDNA) is a

16,569 bp closed-circular molecule present in multiple copies, normally 1000-10000 molecules per cell (Anderson, 1981) (Fig. 1.2).

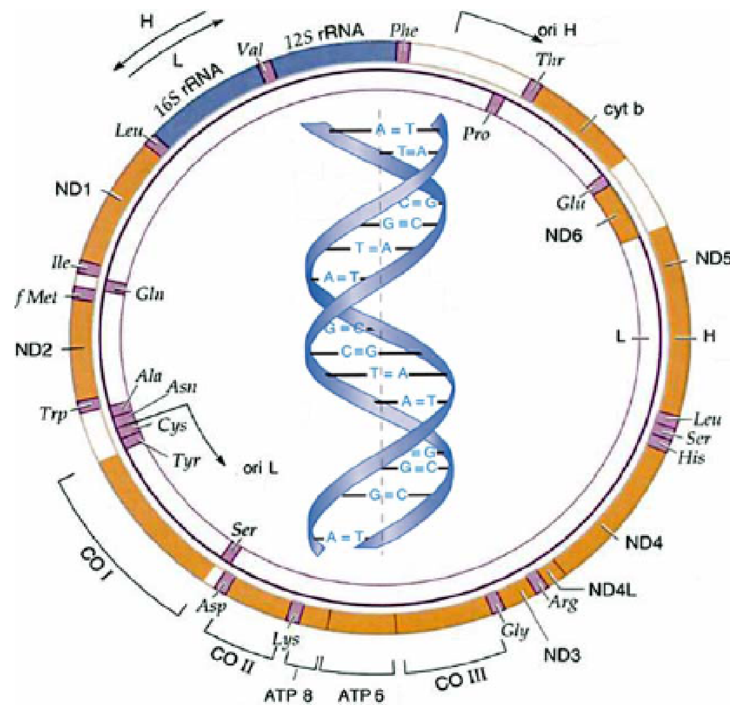


Fig. 1.2 Human mitochondrial DNA. Human mitochondrial DNA (mtDNA) has two strands, a guanine-rich heavy (H) strand and a cytosine-rich light (L) strand. MtDNA contains thirty-seven genes. Twenty-four encode for the translational machinery of the mtDNA (22 tRNAs, in violet and 2 rRNAs in blue). The remaining 13 genes encode subunits of the OXPHOS system: *ND1–6* and *NDL4* for complex I (NADH dehydrogenase); *cyt c* for complex III (bc1 complex); *COI-III* for complex IV(cytochrome c oxidase); *ATPase 6* and *8* for complex V (ATP synthetase). MtDNA is replicated from two origins: the origin of replication of the H-strand (ori H) and the origin of replication of the L-strand (ori L).

Mitochondrial DNA has two strands, a guanine-rich heavy (H) strand and a cytosine-rich light (L) strand. The mtDNA encodes for thirteen essential subunits of the OXPHOS system, including seven subunits of complex I (ND1-6 and ND4L), one subunit of complex III (Cyt*b*), three subunits of complex IV (COI-III) and two subunits of complex V (ATP6-8). In addition, mtDNA encodes for the major part of the mitochondrial translation system (two rRNAs and 22 tRNAs). Remaining mitochondrial

OXPHOS proteins, metabolic enzymes, DNA and RNA polymerases, ribosomal proteins and mtDNA regulatory factors are encoded by nuclear genes, synthesized in the cytoplasm and then imported into the organelle (Wallace, 1997).

The structure of mtDNA is very compact, as introns are absent, and all the coding sequences are contiguous. There is only one non-coding region, the displacement loop (D-loop), a region of 1121 bp that contains the origin of replication of the H-strand (O_H) and the promoters for L and H-strand transcription. The D-loop includes also a small fragment containing two hypervariable regions, HVRI (nt 16024-16383) and HVRII (nt 57-372), and regulatory elements for both mitochondrial replication and transcription.

MtDNA replicates in a relaxed fashion independently of the cell cycle which is defined only by the nuclear DNA replication.

A peculiar feature of mtDNA is its strictly maternal inheritance so that the paternal lineage does not contribute mtDNA to the offspring. Thus mtDNA escapes recombination, although the debate whether mtDNA recombination occurs is still open (Schwartz and Vissing, 2003). Because of uniparental inheritance, mtDNA variability is due to mutations that accumulate along independent lineages. Therefore, mtDNA is characterized by a series of inherited sequence point mutations that define specific mtDNA types. According to the presence/absence of restriction sites at evolutionarily conserved positions, mtDNA types are categorized into haplogroups. A mitochondrial haplogroup is a cluster of different mtDNA molecules that share a common origin. The major haplogroups have been identified by searching for population polymorphic variants, initially through the use of RFLP analyses and then through direct sequencing of both mtDNA coding and noncoding regions (Macaulay, 1999). The analysis of mtDNA polymorphisms revealed that mtDNA haplogroups display a continent specific distribution (Torroni, 2006). In particular, in the European population, about 99% of the mtDNAs fall within nine different haplogroups, identified by Torroni et al. (1996) on the basis of information gained from RFLP analysis of the coding region: H is the most common one, representing 41% about of mtDNA types in our continent, followed by J, T, U, I, X, K, W and V. More recently, the complete mtDNA sequencing allowed to subdivide haplogroups into smaller groups, known as sub-haplogroups. For instance, haplogroup H comprehends at least 15 different sub-haplogroups (Achilli, 2004).

By considering that mitochondria, through ATP production, are key organelles in cell physiology, it is not surprising that mitochondrial dysfunctions are involved in several diseases. On this proposal, it must be remarked that in the course of life there is a progressive accumulation of somatic mutations because mtDNA is highly prone to oxidative damage owing to its localization in close proximity to the electron transport chain; in addition, mtDNA is not equipped with an efficient repair system. When a mutation arises in a cell, a new mixed population of mtDNA molecules is generated. Normally, the mitochondrial genotype of an individual is composed of a single mtDNA species, a condition known as homoplasmy. However, the intrinsic propensity of mtDNA to mutate randomly can occasionally determine a transitory condition known as heteroplasmy, where the wild-type and the mutant genomes can co-exist intracellularly (Attardi, 2002). Several studies have associated both heteroplasmic and homoplasmic mtDNA point mutations with diseases, commonly referred as *mitochondrial disorders* (Zeviani and Di Donato, 2004).

Besides somatic mutations, also mtDNA inherited polimorphisms may be involved in pathological phenotypes. In fact for many years mitochondrial polymorphisms were used only for phylogeny analysis and population studies, but recently a series of experimental evidences has suggested that these variants are not neutral, and an association between common mtDNA polymorphisms and complex traits is progressively emerging. In this frame, a particular mention should be reserved to metabolic disorders. In particular, the type 2 diabetes mellitus has been associated with the T16189C polymorphism and also with haplogroup J (Mohlke, 2005). Furthermore, in a recent work Pravenec and coll. (2007) showed that conplastic strains of rats sharing identical nuclear genome but having different mitochondrial genomes exhibit different risk factors for type 2 diabetes. Interestingly, the conplastic strains differ for variants in some genes coding OXPHOS proteins, thus suggesting that oxidative metabolism plays an important role in predisposing to type 2 diabetes.

Besides metabolic disorders, another group of diseases in which mtDNA polymorphisms are involved is represented by neurodegenerative disorders. In particular, several studies have reported association between mtDNA haplogroups and these pathologies. For example, by studying the association between mtDNA haplogroups and Parkinson's disease in a population of European ancestry, a negative

correlation with haplogroups K and J has been observed, that indicated a protective role of the polymorphisms associated to these haplogroups (van der Walt, 2003). In contrast, haplogroup J was found to be a risk factor for Alzheimer's disease (Chagnon, 1999). Interestingly, haplogroup J has been also associated with longevity. In particular, three independent studies, carried out in Italy (De Benedictis, 1999), in Northen Ireland (Ross, 2001) and in Finland (Niemi, 2003) have shown that this haplogroup is over-represented in centenarians, thus suggesting that some mtDNA types included in the J haplogroup are favourable to attain longevity. On the whole, these results reveal that an association between mtDNA common variability and complex traits exist and that it is population specific. The apparently contrasting results showing that haplogroup J is involved in age-related diseases but also in longevity can be explained by considering the role played by mitochondria in ROS production. In fact, certain mtDNA haplotypes included in the J haplogroup may be characterized by a scarce OXPHOS activity that originates a critical energetic situation if other mutations are present in the genetic background. However, if a low OXPHOS efficiency increases ROS production, it may also induce a response by nuclear genes encoding for detoxifying enzymes that in turn prevents ROS damage thus favoring longevity. In any case, the association between mtDNA common variability and complex traits is quite well established; what is more, this association is population specific (Rose, 2002).

MtDNA common variability is involved also in several cellular phenotypes. Recently, experimental evidences of this involvement have been reported in both mouse and humans. In mouse, by analyzing cell lines sharing the same nuclear genome but with different mtDNA types, a significant correlation has been observed between types of mtDNA and cell respiratory efficiency (Moreno-Loshuertos, 2006). Also in humans, some mtDNA variants have been associated with the cellular respiration rate: indeed, cybrid cell lines carrying mtDNA pathologic mutations showed low levels of oxygen consumption that resulted in a reduction of the respiration rate (Floreani, 2005; Chen, 2006).

1.3 Cellular stress response and complex traits

In both stress and stress response mitochondria play a central role. This role is clear by considering that these organelles are the main source of ROS. These species, produced during OXPHOS, have mtDNA as main target because of its proximity to electron transport chain. Thus, increasing damage to mtDNA inevitably leads to compromised mitochondrial function and integrity. Furthermore, damaged mitochondria release more ROS thus generating a vicious cycle of increasing DNA damage leading to increased ROS production that in turn leads to more DNA damage. The decrease of mitochondrial functionality and the consequent excessive production of ROS are most common features in several pathological conditions, where a causative role is played by the failure of stress response mechanisms. In turn, such a failure determines a further over-production of ROS. For example, the excessive production of ROS concurs to the formation of abnormally aggregated proteins which is observed in Alzheimer's, Parkinson's and Huntington's diseases, as well as in amyotrophic lateral sclerosis and Friedreich's ataxia (Calabrese, 2006). Likewise, a redox imbalance due to excessive ROS production has been observed in various cancer cells which might be related to oncogenic stimulation (Valko, 2007). Mitochondrial oxidative stress plays a role also in various cardiovascular diseases such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies and cardiac hypertrophy (Molavi and Mehta, 2004).

Dysfunctional mitochondria are observed not only in pathological conditions but also in aging. The aging process is characterized by a decline of physiological functions, in which the most prominent causal factor is represented by ROS-mediated oxidative stress. The *oxidative stress theory* holds that a progressive and irreversible accumulation of oxidative damage caused by ROS is a critical aspect of the aging process. This theory is supported by strong evidences indicating that aging is characterized by an accumulation of oxidative damage to lipids, proteins and DNA (Valls, 2005; Ward, 2005; Grune, 2005). In addition, aging is associated with an aberrant regulation of redox-sensitive signal pathways (Zhang, 2004; Li and Holbrook, 2003). What is more, aging is characterized by a decline in antioxidant defence systems, leading to the gradual loss of prooxidant/antioxidant balance and accumulation of oxidative damage (Hagen, 2003).

On the whole, the above considerations indicate that the mitochondrial efficiency in stress response plays a pivotal role in a wide variety of cellular pathways involved in pathological conditions and complex traits. This role can be explained by the molecular interplay existing between mitochondria and nucleus.

1.4 Nucleus-mitochondrion cross-talk

The communication between nucleus and mitochondria is important for a variety of cellular functions, including the stress response. Although the two genomes are physically distinct, a close interaction exists between them so that the two genomes should be considered interdependent from a functional point of view. This communication is bi-directional and operates broadly at two levels: signals from nucleus to mitochondria and signals from mitochondria to nucleus (Fig 1.3).

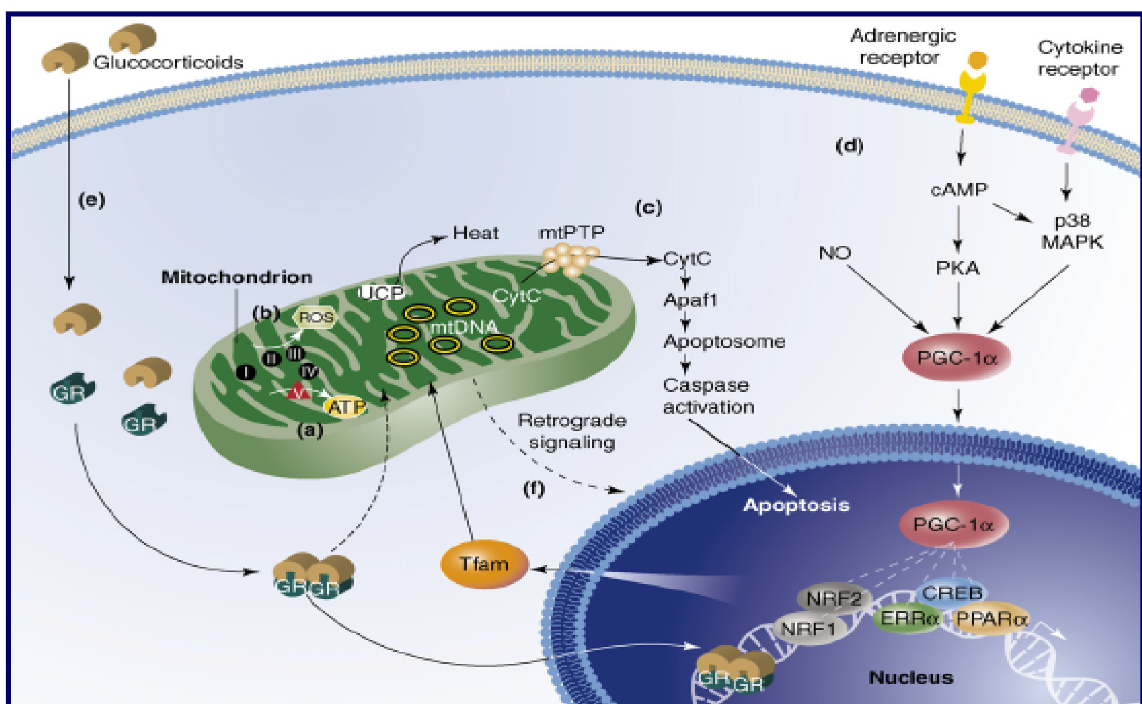


Fig. 1.3 Mitochondrial-nuclear signalling pathways. (a) energy (ATP) production and (b) reactive oxygen species (ROS) generation, (c) induction of apoptosis as well as the main signaling pathways (d)–(f) between hormone or cytokine receptors in the plasma membrane or cytoplasm and the nuclear and the mitochondrial DNA (mtDNA) (mitochondrion) during stress (Manoli, 2007).

Signals from nucleus to mitochondria are fundamental for the co67rrect mitochondrial functionality. The activity of the respiratory chain depends on the expression of thirteen genes encoded by the mitochondrial genome and hundred of genes encoded by the nuclear genome. This implies that a precise coordination of gene expression between the two genomes is fundamental to allow the biosynthesis of functional mitochondria (Traven, 2001). Several literature data, focused on the mechanisms which regulate mitochondrial respiratory genes encoded by the nucleus, have revealed the existence of a set of transcription factors and co-activators. A key transcription factor for the expression of respiratory genes is NRF1 that binds the promoter of several genes encoding OXPHOS proteins, such as cytochrome *c* oxidase (Kelly and Scarpulla, 2004). Furthermore, NRF1 is involved in the regulation of mitochondrial transcription factor A (mtTFA) and mitochondrial ribonuclease protein (MRP), that are the two main factors involved in mtDNA replication/transcription (Garesse and Vallejo, 2001). Moreover, NRF1 seems to be related to the expression of mitochondrial enzymes and components that are involved in the protein import into mitochondria (Scarpulla, 2006).

Besides NRF1, the NRF2 transcription factor is part of the machinery which regulates respiratory genes. In particular, NRF2 regulates the transcription of mtTFA (Kelly and Scarpulla, 2004) and of all the nuclear-encoded cytochrome *c* oxidase (COX) subunits (Ongwijitwat and Wong-Riley, 2005). The activity of these transcription factors is enhanced by several co-activators, that are located within the nucleus and are highly regulated. These co-activators bind to a wide range of nuclear receptors and transcription factors, thus creating a regulatory cascade that results in the transcriptional activation of respiratory genes. In particular, the peroxisome proliferator-activated receptor coactivator 1 (PGC-1) plays a critical role in the regulation of these gene, by interacting with both NRF1 and NRF2 (Wu, 1999).

The above findings are compatible with the idea that nuclear signals are able to modulate the expression of both nuclear and mitochondrial OXPHOS genes. Nuclear signals may also affect the expression of regulatory proteins that coordinate key nuclear-encoded mitochondrial genes with the replication/transcription of the mtDNA.

The regulation of mitochondrial activity depends also on a flow of information from mitochondria to nucleus. Indeed, the regulation of mitochondrial functions requires that the nucleus is constantly informed about the functional status of mitochondria.

Metabolic disorders, or damages that occur within mitochondria, can determine wide changes in nuclear gene expression *via* intracellular signalling from mitochondria to nucleus. This intracellular signalling pathway, known as *retrograde response,* was discovered in the yeast *Saccharomyces cerevisiae* (Parikh, 1987) where it involves three specific transduction proteins: Rtg1, Rtg2 and Rtg3. Rtg1 and Rtg3 are transcription factors translocated during retrograde communication from cytoplasm to nucleus; Rtg2 is a member of the heat shock factor family involved in this translocation (Liao and Butow, 1993; Rothermel, 1997). In yeast the retrograde response is induced by partial or complete deletion of mtDNA. In particular, the decline of mitochondrial membrane potential ($\Delta\psi$), typical of yeast cells lacking of mtDNA, activates Rtg2 factor. The net effect of this activation is the Rtg2-mediated translocation of the Rtg1–Rtg3 complex from cytoplasm to nucleus (Sekito, 2000), and the resulting induction of numerous nuclear genes encoding for cytoplasmic, mitochondrial and peroxisomal proteins (Jazwinski, 2005; Epstein, 2001).

The *retrograde response* is also documented in human cells. Mitochondrial retrograde signalling in mammalian cells was described initially in C2C12 skeletal myoblasts and later confirmed in human lung carcinoma A549 cells (Biswas, 1999; Amuthan, 2002). As in yeast, the early event of the retrograde response is the loss of mitochondrial membrane potential, but in mammalian cells Rtg proteins are not involved in the retrograde signalling pathway. In this case, the loss of $\Delta\psi$ is accompanied by changes of cytosolic levels of Ca^{2+} which in turn are responsible for the activation of transcription factors (NF-κB, NFAT, ATF) that mediate the cellular response (Leo, 2005) (Fig. 1.4).

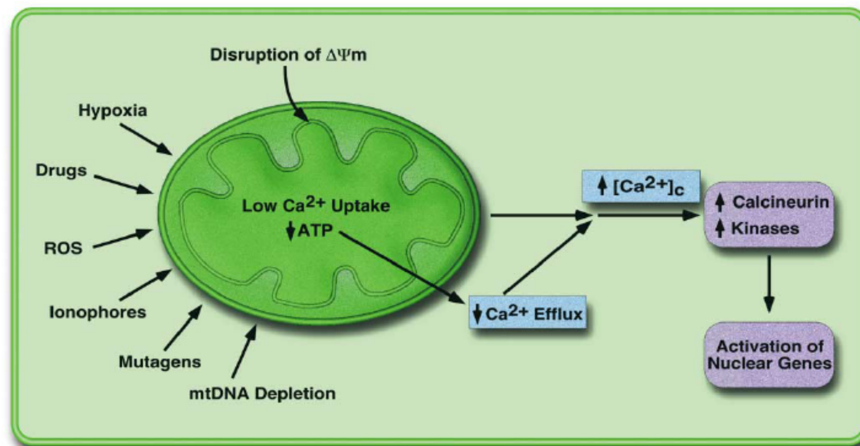


Fig. 1.4 Retrograde Response in mammals. Retrograde signalling in mammalian cells occurs through increased cytosolic Ca^{2+} (Butow and Avadhani, 2004).

The nuclear genes induced by the retrograde response in mammals are less well defined. Furthermore, in some cases they depend on the specific cell type. Miceli and Jazwinski (2005) have analyzed the nuclear genes modulated by mitochondrial dysfunctions: they found that such genes are involved in various cellular pathways, for example those implicated in glucose metabolism and stress response. The most characteristic change is the induction of the transcription factor MYC, which is important for the up-regulation of glycolysis in cell lacking of mtDNA. Another gene involved in retrograde response is WRN, which encodes for a helicase thus suggesting the compensation of loss of nuclear genome stability in mtDNA-less cells (Jazwinski, 2005). Finally, also the genes involved in Ca^{2+} transport and storage (Goffart and Wiesner, 2003), as well as a large number of transcription factors (Wu, 1999), are activated in the mammalian retrograde response.

Since the retrograde response is activated when the functional state of mitochondria is compromised, the general net effect of the activation of this gene network is to facilitate recovery of physiological function. Thus, mitochondria can be considered as receiver/integrator organelles, which receive, integrate and transmit signals which are critically important for determining the fate of the cell (Goldenthal and Garcia, 2004).

2. Expression profiles of stress-responder nuclear genes in relationship to common mitochondrial DNA variability

Work hypothesis

The research faced in this PhD thesis started from two observations: i) common polymorphisms of mtDNA act as susceptibility factors in complex phenotypes; ii) the cell capability to cope with a variety of intrinsic and extrinsic stress factors contributes to some of these phenotypes.

We could hypothesize that the two observations are linked by the role played by mtDNA variability on the expression of nuclear genes involved in stress response through mitochondria-to-nucleus communication. To check the above hypothesis we developed cybrid cell lines in which stress-consequent modifications of gene transcription patterns could be analyzed. In particular, we investigated if oxidative stress or heat shock stress were able to modify the transcription profile of cytokine and cytokine-receptor genes, and heat shock protein genes, respectively.

Cybrids technology.

Cybrid technology represents one of the most valuable tools for studying *in vitro* the mitochondria-to-nucleus communication and for understanding the effects of specific mtDNAs on cellular phenotypes. Cybrid cell lines, first described by King and Attardi (1989), share the same nuclear genome but have different mitochondrial genomes. The preparation of cybrids starts from the creation of mtDNA-null cells (rho^0 cells). Many human cells lines can be rendered rho^0 in culture by growth in the presence of low concentrations of the intercalating agent ethidium bromide (EtBr). This compound inhibits mtDNA replication and transcription, without any detectable effect on nuclear DNA division owing to its packing into chromatin (King and Attardi, 1996). Rho^0 cells are able to survive, are auxotrophic for uridine and pyruvate, and are devoid of the ability to respire meeting their energy needs *via* anaerobic glycolisis, even though cultured at atmospheric oxygen concentrations. Rho^0 cells represent a good model for understanding the role of mtDNA on various cellular phenotypes (Miceli and Jazwinski, 2005) and for studying the impact of mtDNA deficiency on cell biology. The absence of mtDNA could rise to different results regarding gene expression of proteins involved in

mtDNA maintenance and replication: in some cases it induces a decrease of such proteins (for example mtTFA), in other cases the cell is insensitive to the lack of mtDNA (for example in the case of polymerase gamma).

In cybrid technology, rho^0 cells are repopulated with exogenous mitochondria derived from enucleated cells harbouring particular types of mtDNA molecules. The resulting cybrid cell lines are characterized by nuclear DNA that comes from the parental rho^0 cell and mtDNA that comes from the donor cells.

The model of cybrids has been widely used in mitochondrial research to characterize the biochemical phenotype of cells harbouring specific mutations in their mtDNA. Several studies have demonstrated enhanced mitochondrial ROS-production and antioxidant defense in cybrids carrying specific mtDNA point mutations (Zeviani and Di Donato 2004). Some of these mutations were associated with pathogenesis of Leber's hereditary optic neuropathy (LHON), mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and with increasing tumorigenicity (Zeviani and Di Donato, 2004). Cybrids technology has been also utilized to explore the role of altered mitochondrial function in the pathogenesis of neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases (Ghosh, 1999).

Cytokine and cytokine-receptor genes

Cytokines are evolutionarily conserved molecules which play a pivotal role in the homeostasis of the organism by mediating and regulating immunity, inflammation and hematopoiesis (Sawada, 1995; Hirano, 1999; Quelle, 1998; Matarese and La Cava, 2004). The proteins act by binding to specific receptors which modulate the expression of a series of downstream genes. In the inflammation process cytokines act not only as pro-inflammatory but also as anti-inflammatory factors. The scientific literature on the role played by cytokines, and their receptors, in cell and organism biology is impressive, and a summary of such literature is out of the aims of this PhD thesis. The point to be stressed is that all literature confirms the key role played by these proteins in stress response. This is well documented within the OMIM keys reported in Table 1.1 which lists the cytokine and cytokine receptor genes we analyzed.

Cytokine and cytokine receptors genes			
Gene symbol	**Description**	**Chromosome localization**	**OMIM**
TNF-α	tumor necrosis factor alpha	6p21.3	191160
GM-CSFR	granulocyte-macrophage colony stimulating factor receptor	Xp22.32 -Yp11.3	306250-425000
IL-1β	interleukin 1 beta	2q14	147720
TNFR1	tumor necrosis factor receptor superfamily, member 1A	12p13.2	191190
GM-CSF	granulocyte-macrophage colony stimulating factor	5q31.1	138960
IL-6	interleukin 6	7p21	147620
IL-6R	interleukin 6 receptor	1q22	147880
TNFR2	tumor necrosis factor receptor superfamily, member 1B	1p36.3-p36.2	191191

Tab 1.1 Cytokine and cytokine receptor genes. Gene symbol, description, chromosome localization and OMIM reference identification number are shown.

Tumor necrosis factor α (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) are classical pro-inflammatory cytokines. They contribute to the activation of the inflammatory cells, and together with chemokines, induce the expression of adhesion molecules thus causing their local recruitment. IL-6 is a multifunctional cytokine produced by immune and non-immune cells. Inappropriate expression and production of IL-6 is thought to be involved in the pathogenesis of numerous diseases, including osteoporosis, atherosclerosis and Alzheimer's disease (Hak, 2003).

Colony-stimulating factors (CSFs) are proteins necessary for survival, proliferation, and differentiation of hematopoietic progenitor cells. In particular, the *GM-CSF* gene encodes granulocyte/macrophage colony-stimulating factor. The gene encoding for its receptor (*GM-CSFR*) is located in the pseudoautosomal region (PAR) at the end of the short arm of the X and Y chromosomes. The reaction between the GM-CSF protein and its receptor activates a series of differentiation processes whose outcome is the re-

direction of common lymphoid progenitors (that give rise exclusively to T, B, and natural killer lymphocytes) to the myeloid lineage (Kondo, 2000).

Since their crucial role in immune response and inflammation, cytokines are involved also in age-related phenotypes (Licastro, 2005). During aging, cytokine levels undergo a progressive increment which is due to the continuous attrition caused by life-long exposition to stressors. This increment is coupled with a pro-inflammatory status, which represents a risk factor for many age-associated diseases (Franceschi, 2000), such as cardiovascular dysfunctions (Licastro, 2005), Alzheimer's disease (Akiyama, 2000), cancer (Caruso, 2004; Schwartsburd, 2004). By considering that both iper- and hypo-responsiveness to inflammatory stimuli can be detrimental, an appropriate expression of cytokine and cytokine-receptor genes in stress response is important for escaping diseases and reaching the extreme limits of human lifespan (Franceschi, 2000). In agreement, association studies have shown that cytokine gene polymorphisms, which are correlated to different rates of gene transcription, are significantly associated with longevity (Terry, 2000; Bonafè, 2001; Lio, 2002). Therefore, the understanding of mechanisms and factors involved in the regulation of cytokine and cytokine-receptor genes during stress response constitutes a challenge of primary importance in the research on age-related diseases.

Heat shock proteins.

Heat Shock Proteins (HSPs) are ubiquitously expressed, highly conserved proteins present in virtually every species. HSPs are found in the cytosol, nucleus, endoplasmic reticulum, mitochondria and chloroplasts (Siu, 2005). HSPs have been classified into four major families according to their molecular weight: HSP40, HSP60, HSP70, HSP90, HSP110 and small HSPs (Jolly & Morimoto, 2000). Each family is composed of members which are expressed in either constitutive or inducible way and targeted to different sub-cellular compartments (Garrido, 2001). Under normal conditions, HSPs act as ATP-dependent molecular chaperones by assisting the folding of newly synthesized polypeptides in the cytosol, endoplasmic reticulum and mitochondria, the assembly of multiprotein complexes, the transport of proteins across cellular membranes, the degradation of unstable proteins, and the refolding of misfolded proteins (Parcellier, 2003).

A characteristic of HSPs is that they are located preferentially in the cytosol of eukaryotic cells but, if necessary, are redistributed in each cellular compartment. For instance, HSP60 carries out its chaperone activity in the cytoplasm but, when this function is required within mitochondria, it is rapidly imported. Here, in association with HSP10, HSP60 assists the folding of proteins imported from the cytosol or synthesized within mitochondria (Itoh, 2002). Furthermore, the reallocation of cytosolic proteins into mitochondria requires HSP75, the mitochondrial form of HSP70, in an ATP-dependent process (Mokranjac, 2005).

At stress conditions, an increased HSP gene expression has been observed both *in vivo* and *in vitro,* that limits the intracellular accumulation of denatured or misfolded proteins (Morimoto, 1998). Another protective effect of HSPs is related to their ability to interact with various components of the programmed cell death machinery. In this context HSPs play a central role in both extrinsic and intrinsic apoptotic pathways, by acting as anti- or pro-apoptotic factors. HSP70, HSP27, HSP90, and HSP60 have been implicated in protection against apoptosis induced by numerous signals, such as heat shock, nutrient withdrawal, ROS, endoplasmic reticulum stress, proteasome inhibition, UV radiation and chemotherapy-induced DNA damage. The role of HSP60 in apoptosis is limited to the intrinsic pathway, in which this protein, in association with HSP10, acts as pro-apoptotic factor. In particular, under stress conditions, the HSP60/HSP10 complex is released by mitochondria so that it carries out its pro-apoptotic function in the cytosol, by activating procaspase-3 (Samali, 1999).

The HSP genes we analyzed in the present work are reported in Table 1.2

Heat Shock Protein Genes			
Gene symbol	**Description**	**Chromosome localization**	**OMIM**
HSP72	Heat Shock Protein 72	6p21.3	140550
HSP60	Heat Shock Protein 60	2q33.1	118190
HSP75	Heat shock Protein 75	16p13.3	606219

Tab 1.2 Heat shock protein genes. Gene symbol, description, chromosome localization and OMIM reference identification number are shown.

HSP72 was used to test the efficacy of the heat shock treatment, since it is the heat-inducible form of *HSP70*. The other genes (*HSP60* and *HSP75*) have been chosen because they encode proteins acting into mitochondria where they play a pivotal role in the functions above mentioned.

2.1 Results and Discussion

2.1.1 Cybrid cell lines

In the present study we re-cloned H and J cybrid cell lines previously produced (Bellizzi, 2006) and, in addition, we produced *ex-novo* three cybrid cell lines by fusing 143B.TK$^-$ 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. Therefore, we named the new cybrid cell lines according to the name of the respective mtDNA haplogroup. After fusion, the cell lines were cultured in regular growth medium without uridine, thus confirming the effective repopulation of the rho^0 cells. Then, all the cell lines (143B.TK$^-$ and rho^0 cells, H, J, U, X, T cybrids) were cultured for about two months for their stabilization before use.

Although the mtDNA molecules of the X cybrid line and those of the 143B.TK- parental line were both classified within the X haplogroup, a further RFLP analysis showed sequence differences between them. This result is in line with literature data demonstrating that, within each haplogroup, a large mtDNA variability can be observed, especially in very ancient haplogroups such as the X haplogroup (Torroni, 2006). Thus, possible differences in the expression profiles of different cybrid lines should to be referred to the entire mtDNA sequence and not to the specific haplogroup to which the mtDNA molecule belongs.

2.1.2 Experiments for quality control of the cybrid cell lines

Before starting with gene expression analyses, we carried out a series of control experiments aimed at verifying the cellular state of the cybrid cell lines.

Proliferation assay. In order to assess the growth characteristics of the cybrid cell lines, we carried out a proliferation assay, in which 143B.TK$^-$ parental line and its derivative rho^0 cells were also analysed as control. Growth curves were obtained by counting cells at regular intervals of 24, 48 and 72 hours. Fig. 2.1 shows the results of three

independent experiments carried out for each time point. It must be noted that we added uridine to the rho^0 cell culture.

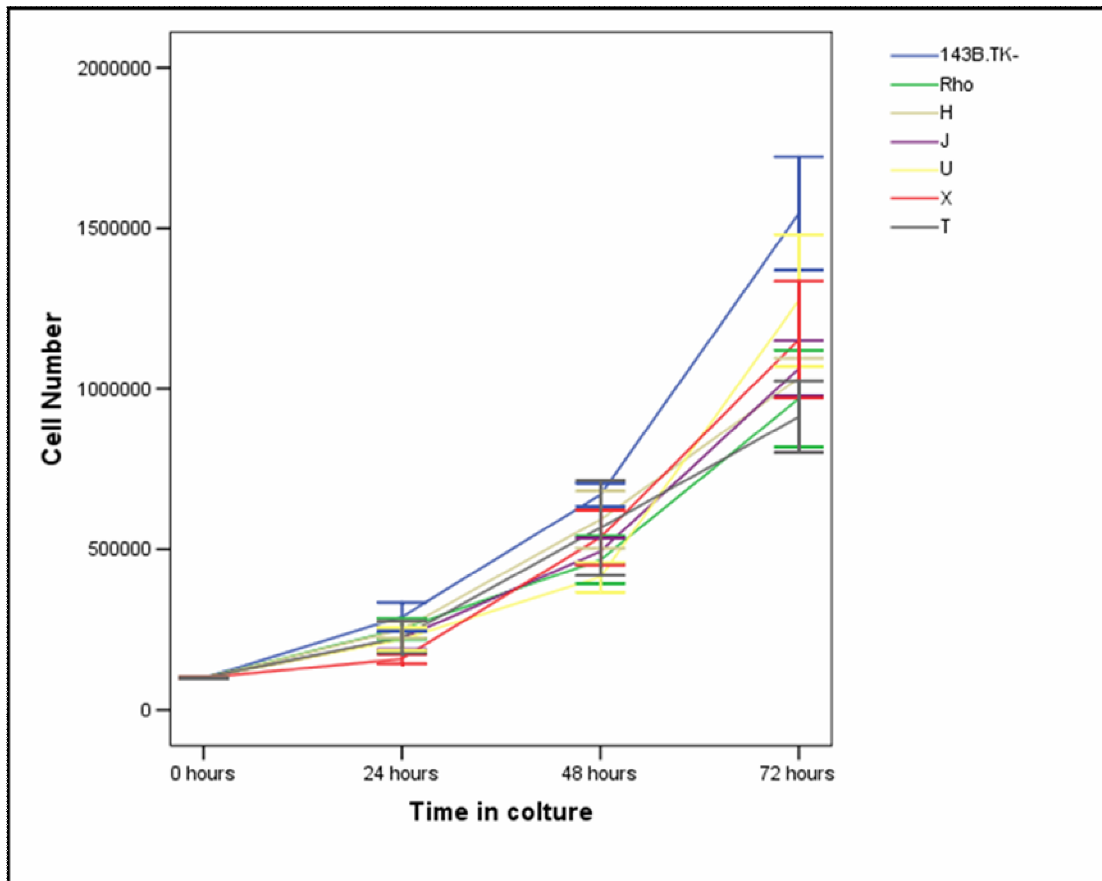


Fig 2.1 Growth curves of 143B.TK$^-$, rho^0, H, J, U, X and T cell lines. Results are expressed for each time point as mean of three independent experiments with standard deviation.

We did not observe significant differences among the seven cell lines, thus indicating that the cybrid cell lines maintained the proliferation capability of the parental cells.

Quantification of mtDNA. In order to exclude significant differences in the amount of mtDNA among 143B.TK$^-$ and cybrid cell lines, we estimated the number of copies of

mtDNA per cell by using Quantitative Real Time PCR. In Fig. 2.2 we show the mtDNA amount estimated in each cell lines.

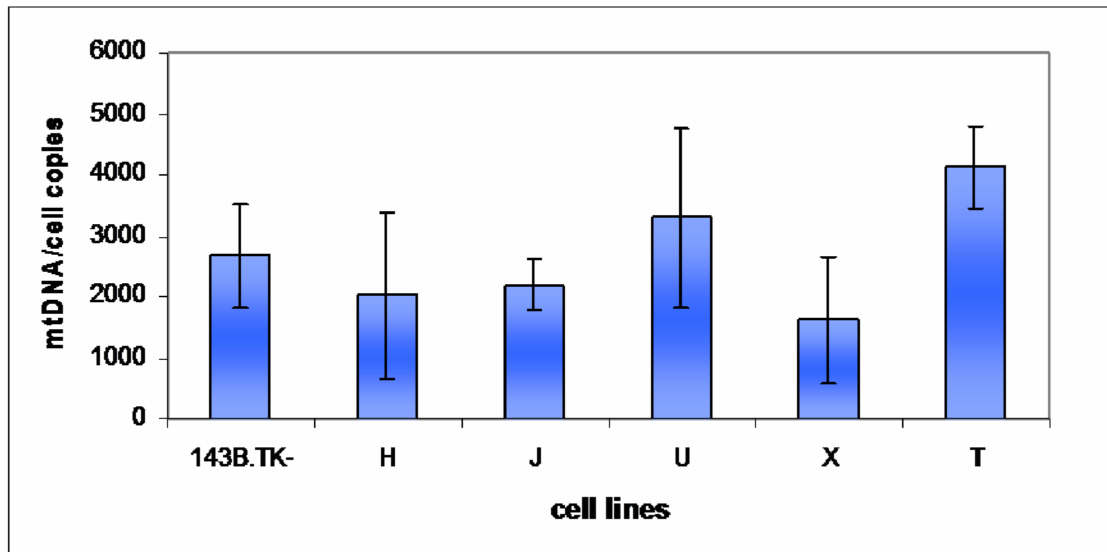


Fig 2.2 Quantitative analysis of mitochondrial DNA amount in 143B.TK$^-$ and cybrid cell lines. The values represent the mean of three independent experiments with standard deviation**.**

By applying ANOVA test no significant difference was observed among the six cell lines (p = 0,237), thus showing that the experimental manipulation did not affect significantly the amount of mtDNA.

Mitochondrial membrane potential (MMP) assay. We verified the mitochondrial functionality of the cybrid cell lines by measuring the Mitochondrial Membrane Potential (MMP). We carried out a double staining assay by using the probes MTG and TMRM, specific for mitochondrial mass and for MMP respectively. For each cell line (143B.TK$^-$, rho^0 and cybrid cell lines) we measured MMP as ratio between TMRM and MTG fluorescence values to normalise the mitochondrial polarisation level with respect to the mitochondria cell content. Fig. 2.3 shows the quantitative analysis of MMP in each cell lines.

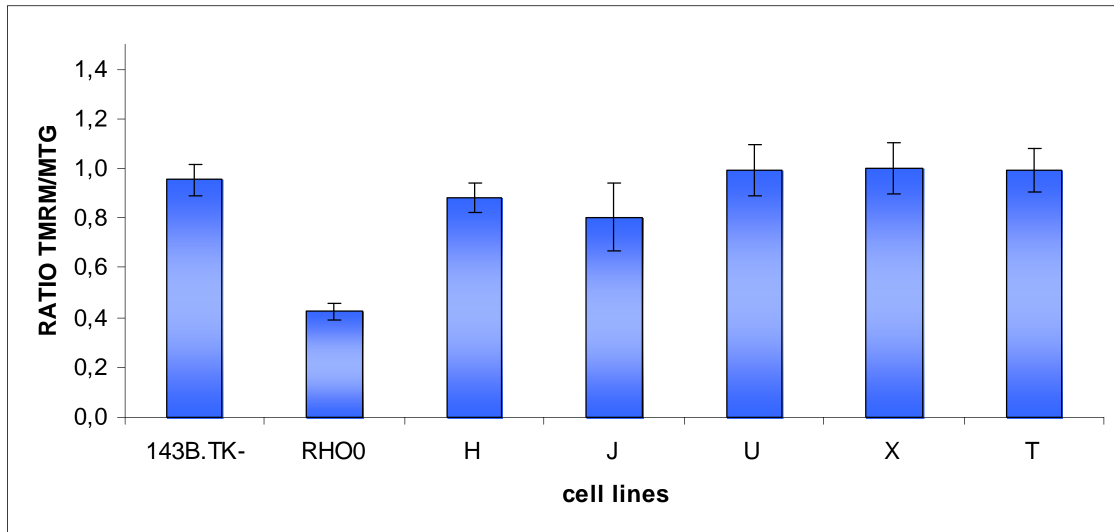


Fig 2.3 Quantitative analysis of Mitochondrial Membrane Potential in 143B.TK$^-$, rho^0 and cybrid cell lines. The values represent the mean of three independent experiments with standard deviation**.**

By applying ANOVA test no significant difference was observed among the six cell lines having active mitochondria (p = 0,123), thus showing that cybrid lines were metabolically active. On the contrary, when the MMP value of rho^0 cells was included in the ANOVA test, a significant difference was observed among the seven cell lines (p = 0,000).

From Fig. 2.3 it is evident that MMP, although lower in rho^0 cells than in the others, is still maintained in cells lacking of active mitochondria. This result is in agreement with literature data showing that rho^0 cells can still maintain a mitochondrial membrane potential (Appleby, 1999). Studies on yeast and mammalian rho^0 cells suggest that the membrane potential is generated by electrogenic exchange of ATP^{4-} for ADP^{3-} through the adenine nucleotide carrier (Appleby, 1999; Buchet and Godinot, 1998). However, considerable uncertainties persist about the magnitude of the membrane potential in mammalian rho^0 cells. Likely, these uncertainties depend on experimental conditions, that is the method used to evaluate MMP (Salvioli, 1997).

On the whole, the above control experiments showed that the cybrid cell lines functioned normally and did not differ significantly from the parental line. This result is important taking into account the debate on the reliability of the results pursued by cybrid techonology (Danielson, 2005). Indeed, a genetic instability or cellular stress

could occur during the cybrid experimental manipulation, that is the long-term cell treatment with ethidium bromide, known carcinogen and powerful mutagen, and the reintroduction of exogenous mtDNA in rho^0 cells (cybridization process). In addition, the parental cell line (143B.TK-) is an osteosarcoma line and, like other tumoral cells, could be prone to genetic mutations, probably because of the routine maintenance of the cell cultures. The results obtained in the control experiments led us to conclude that the manipulation we carried out did not interfere with the physiological state of the cell lines. For this reason, we were confident that the following gene expression analysis did not feel the effect of technical manipulations.

2.1.3 Oxidative stress: cytokine and cytokine receptors genes

After assessing the functionality of the seven cell lines, we induced oxidative stress by treating cells with 2-deoxy-D-Ribose (dRib). Literature data show that this compound induces depletion of the intracellular reduced glutathione (GSH), thus determining an increased cellular level of ROS, which in turn are responsible for cell death (Barbieri, 1994; Kletsas, 1998).

Control experiments of the oxidative stress status

We carried out two control experiments to check the oxidative stress conditions (dosage and treatment time): cell viability and DNA fragmentation assays. In other words, by these control experiments we used apoptosis as a tool to verify the occurrence of oxidative stress.

Cell viability assay. In order to calculate the percentage of living cells we performed a Trypan blue exclusion assay after treatment with dRib 20 mM for 24, 48 and 72 hours. As control, we also analysed the viability of the cells at basal condition. In Fig 2.4 the results of this assay are reported.

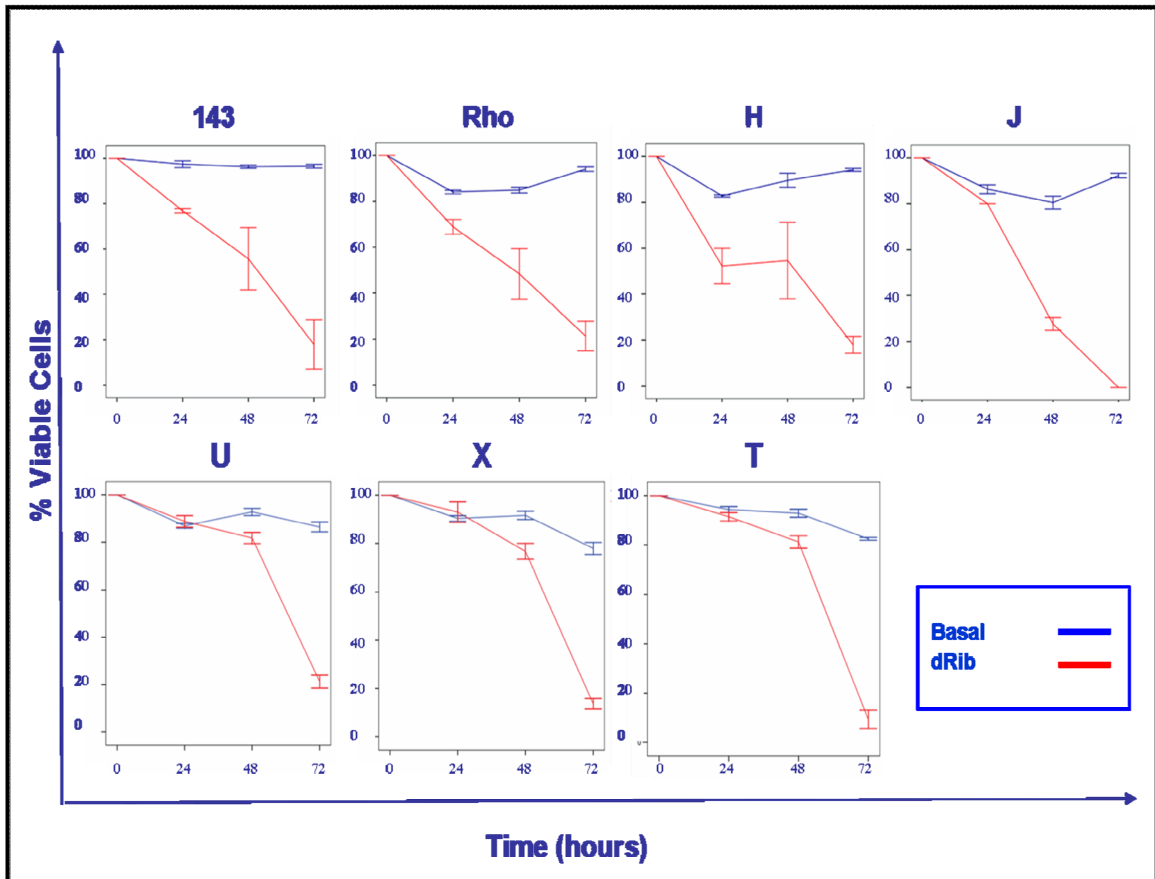


Fig 2.4 Viability curves of 143B.TK$^-$, rho^0, H, J, U, X and T cell lines. In each graph, untreated cells (blue line) and cell treated with dRib 20mM for 24, 48 and 72 hours (red line) are shown.

For each line, we observe a decrease in the cell viability after the oxidative treatment with d-Rib. In particular, after treatment at 48 hours, the percentage of living cells was intermediate between those shown at 24 hours and 72 hours.

DNA fragmentation assay. In order to determine the apoptotic state of our cells we carried out a DNA fragmentation assay, which is able to visualize the inter-nucleosomal DNA fragmentation typical of apoptotic cell death. In Fig 2.5 we show fragmentation patterns for each cell line both at basal condition and after stress treatment.

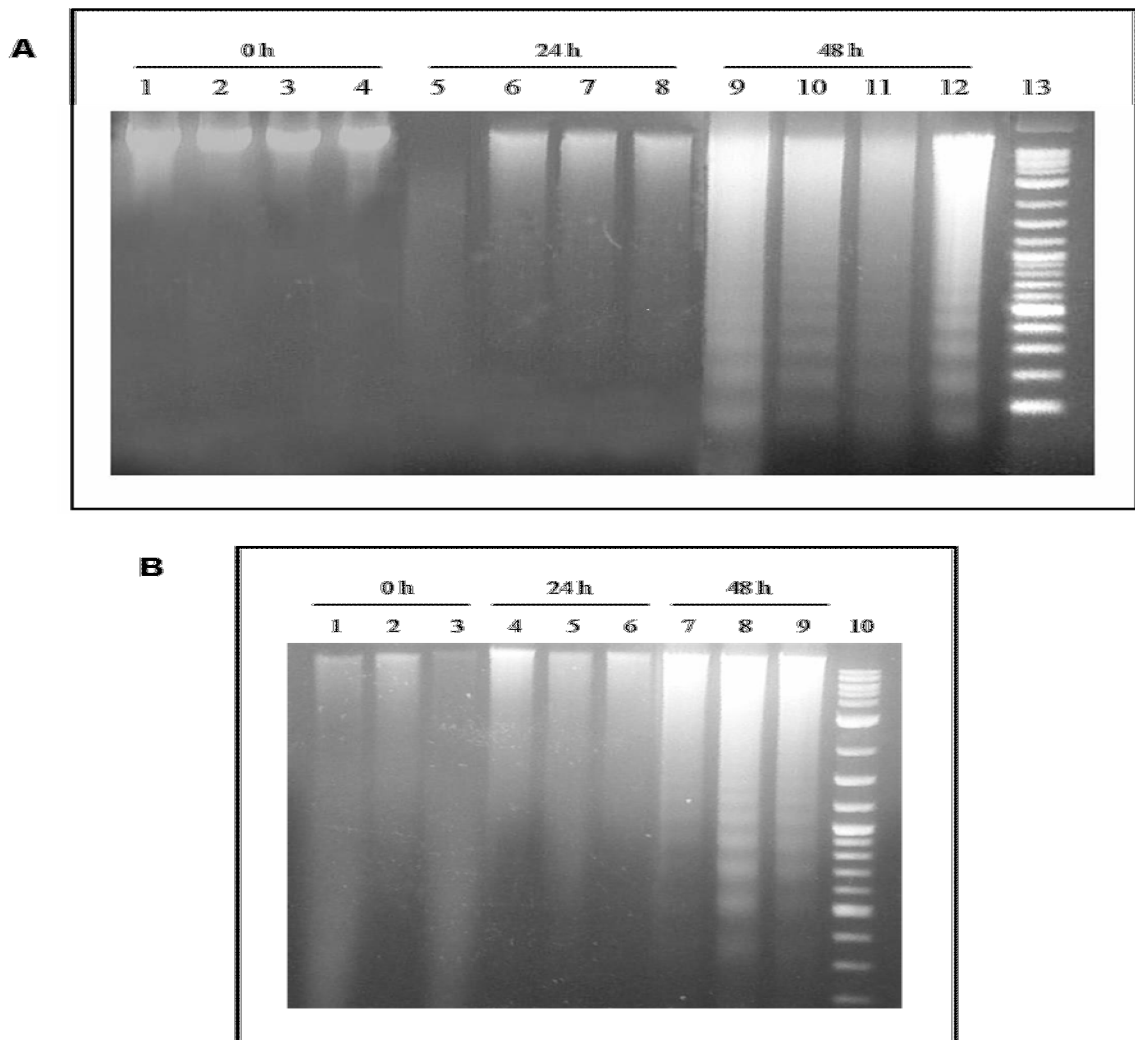


Fig 2.5 DNA fragmentation electrophoresis pattern of 143B.TK-, rho^0, H and J cell lines (A) and of U, X and T cell lines (B). (A) Lanes 1-4 contain, in the order, DNA isolated from 143B.TK, rho^0, H and J cells at basal condition; lanes 5-8 and 9-12 contain, in the same order, DNA isolated from cells treated with 20mM dRib for 24 and 48 hours, respectively; lane 13 contains molecular weight 100 bp ladder. **(B)** Lanes 1-3 contain, in the order, DNA isolated from U, X and T cells at basal condition; lanes 4-6 and 7-9 contain, in the same order, DNA isolated from cells treated with 20mM dRib for 24 and 48 hours, respectively; lane 10 contains molecular weight 100 bp ladder.

All the cell lines clearly show DNA fragmentation after treatment with d-Rib at 48 hours. Therefore, both the control experiments showed that the treatment with dRib 20 mM for 48 hours was able to induce a severe stress condition.

Gene expression analysis

Cytokine and cytokine-receptor gene expression was analyzed in 143B.TK-, rho^0 and cybrid cell lines at basal and stress condition (d-Rib 20 mM for 48 hours) by a semiquantitative RT-PCR.

We analyzed a panel of eight genes coding for the cytokines and cytokine-receptors reported in Tab.1.1: TNFα (tumor necrosis factor alpha), GM-CSFR (granulocyte-macrophage colony-stimulating factor receptor), IL-1β (interleukin 1- beta), TNFR1 (tumor necrosis factor receptor 1), GM-CSF (granulocyte-macrophage colony-stimulating), IL-6 (interleukin 6), IL-6R (interleukin 6 receptor), TNFR2 (tumor necrosis factor receptor 2).

In order to escape false positive results due to the cybridization process, for each cybrid line we replicated the analysis of gene expression profiles not only in triplicate starting from the same clone, but also on independent clones when positive results were obtained. We found consistent results in all the cases (data not shown).

What is more, we compared cybrid lines among each other: therefore possible differences in gene expression patterns were only due to the different mtDNAs we used to repopulate rho^0 cells. In Fig 2.6 a representative RT-PCR electrophoresis pattern is shown.

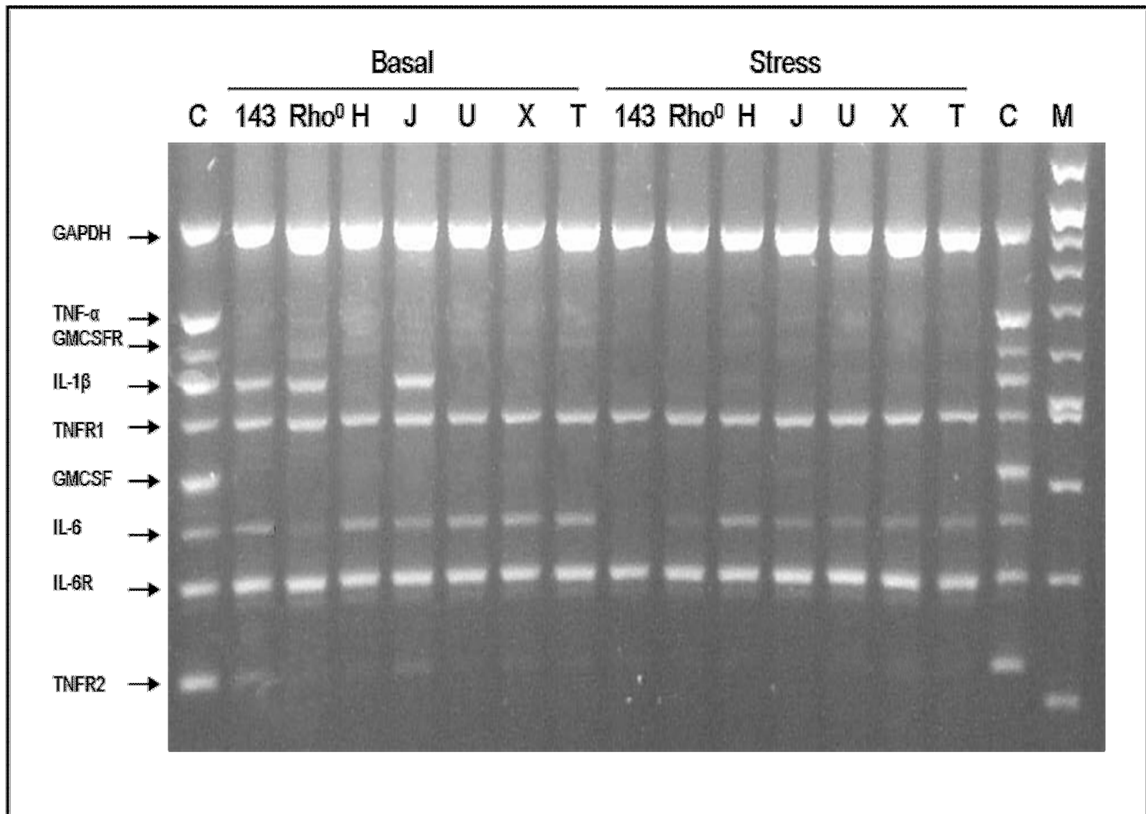


Fig 2.6 RT-PCR electrophoresis pattern of cytokine and cytokine receptor genes in 143B.TK$^-$, rho^0, H, J, U, X and T cell lines at basal and stress condition (dRib 20mM for 48 hours). Ctr: control cDNA supplied by the MPCR (Multiplex PCR) Amplification kit; GAPDH: Glyceraldeide phosphate deydrogenase. MW: molecular weight 100 bp ladder.

The results of quantitative analyses of gene expression are summarized in Tables 2.1 and 2.2, where the average densitometer measures of three independent experiments are reported.

Basal condition.

Table 2.1 reports the results obtained at basal conditions

Basal Condition

	143B.TK- M (SD)	Rho^0 M (SD)
TNF-α	0,000 (0,000)	0,000 (0,000)
GMCSFR	0,000 (0,000)	0,000 (0,000)
IL-1β	0,493 (0,168)	0,606 (0,226)
TNFR1	0,733 (0,309)	0,804 (0,211)
GMCSF	0,000 (0,000)	0,000 (0,000)
IL-6	0,765 (0,109)	0,000 (0,000)
IL-6R	1,553 (0,324)	1,693 (0,397)
TNFR2	0,341 (0,059)	0,000 (0,000)

	H M (SD)	J M (SD)	U M (SD)	X M (SD)	T M (SD)
TNF-α	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
GMCSFR	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
IL-1β	0,355 (0,131)	0,828 (0,364)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
TNFR1	0,857 (0,216)	0,785 (0,124)	0,720 (0,290)	0,720 (0,190)	0,704 (0,187)
GMCSF	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
IL-6	0,845 (0,107)	0,268 (0,054)	0,894 (0,082)	0,516 (0,158)	0,593 (0,266)
IL-6R	1,520 (0,286)	1,491 (0,141)	1,460 (0,229)	1,462 (0,111)	1,393 (0,023)
TNFR2	0,000 (0,000)	0,170 (0,024)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)

Tab 2.1 Densitometer analysis of gene expression of cytokines and cytokine receptors in 143B.TK$^-$, rho^0 and cybrid cell lines at basal condition. The values represent the mean of three independent experiments with standard deviation**.**

A) Comparison between 143B.TK- and rho^0 cells

By comparing 143B.TK- and rho^0 cells, we obtained the following results (Table 2.1): i) *IL-6* and *TNFR-2* genes were not expressed in the rho^0 cells; ii) the expression of *IL-1*b, *TNFR1* and *IL-6R* genes was not substantially different between parental and rho^0 cells; iii) *TNF*a, *GM-CSF* and *GM-CSFR* genes were not expressed in any cell line.

Gene expression profiles of cytokines and their receptors observed in 143B.TK– cell line were consistent with those observed in other osteosarcoma cells, as well as with those observed in primary human osteoblast-like cells (Bilbe, 1996). Since the culture medium constituents may exert both stimulatory and inhibitory effects on gene transcription, the agreement of our findings with those reported in literature is very important: in fact it demonstrates that our experimental conditions did not affect the gene expression profiles we observed.

Interestingly, the differences observed between rho^0 and 143B.TK- cells showed that the expression patterns of both *IL-6* and *TNFR-2* genes were dependent on the presence of active mitochondria.

B) Comparison among cybrid lines.

By comparing cybrid cell lines among each other we observed the following results (Table 2.1). First, the expression of *IL-1*b, *IL-6* and *TNFR-2* genes was different among cybrids. In particular, *IL-1*b was only expressed in H and J cell lines while not the other ones; *IL-6* was expressed in all the lines but at different levels ($p = 0{,}044$ by ANOVA test); *TNFR2* was expressed in the J line only. Second, the expression of *TNFR1* and *IL-6R* genes was not substantially different among the lines ($p > 0{,}05$ by ANOVA test). Third, *TNF*a, *GM-CSF* and *GM-CSFR* genes were not expressed in any cybrid line.

In particular, *IL-1*b and *TNFR-2* genes were up-regulated, while *IL-6* was down-regulated, in the J cybrid with respect to the other cybrid lines. Thus, the J cybrid showed an unique expression pattern not shared with the other cybrid lines. This result is interesting by considering, as discussed in the Introduction, that the J haplogroup has been often considered as a particular haplogroup which is associated with many complex traits.

Oxidative stress condition.

Table 2.2 reports the results obtained at stress conditions.

Stress Condition

	143B.TK- M (SD)	Rho^0 M (SD)
TNF-α	0,000 (0,000)	0,000 (0,000)
GMCSFR	0,000 (0,000)	0,000 (0,000)
IL-1β	0,000 (0,000)	0,000 (0,000)
TNFR1	0,730 (0,197)	0,664 (0,412)
GMCSF	0,000 (0,000)	0,000 (0,000)
IL-6	0,000 (0,000)	0,544 (0,172)
IL-6R	1,346 (0,085)	1,408 (0,182)
TNFR2	0,000 (0,000)	0,000 (0,000)

	H M (SD)	J M (SD)	U M (SD)	X M (SD)	T M (SD)
TNF-α	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
GMCSFR	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
IL-1β	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
TNFR1	0,646 (0,079)	0,680 (0,155)	0,676 (0,082)	0,634 (228)	0,641 (0,110)
GMCSF	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)
IL-6	0,574 (0,069)	0,373 (0,075)	0,489 (0,111)	0,549 (0,166)	0,467 (0,158)
IL-6R	1,443 (0,006)	1,595 (0,239)	1,547 (0,052)	1,627 (0,332)	1,666 (0,289)
TNFR2	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)	0,000 (0,000)

Tab 2.2 Densitometer analysis of gene expression of cytokines and cytokine receptors in 143B.TK$^-$, rho^0 and cybrid cell lines at stress condition. The values represent the mean of three independent experiments with standard deviation**.**

A) Comparison between 143B.TK- and rho^0 cells

By comparing 143B.TK- with rho^0 cells, we observed that *IL-6* gene was up-regulated in rho^0 cells. In fact, the gene is not expressed at all in the 143B.TK- native line. This result provides the first experimental evidence that *IL6* gene expression is activated in a retrograde response elicited by rho^0 cells. In short, the expression of this gene requires the presence of active mitochondria at basal condition (Table 2.1) and is elicited by a retrograde response at stress condition (Table 2.2). Taking into account that IL6 protein is a key component of the stress response at organismal level, and that mtDNA damages accumulate with aging, this result may provide a clue to interpret the quality of aging in terms of efficacy of the IL6-related retrograde response.

B) Comparison among cybrid lines.

By comparing cybrid cell lines among each other no significant difference was observed in contrast with the results obtained at basal conditions (Table 2.1). In fact the transcriptional profiles resulted to be more variable among the cell lines at basal than at stress condition, because the differences observed among the cybrids at basal condition disappear under stress (see Fig. 2.7). We can speculate that each cell line tends to maximize stress response independently of mtDNA variability.

Finally, in order to have a synthetic picture of the stress effect, we compared gene expression profiles, *within a single cell line before and after stress*. By using the data of Tables 2.1 and 2.2, we assembled the histograms shown in Fig. 2.7 where the genes that were expressed in one cell line at least are reported.

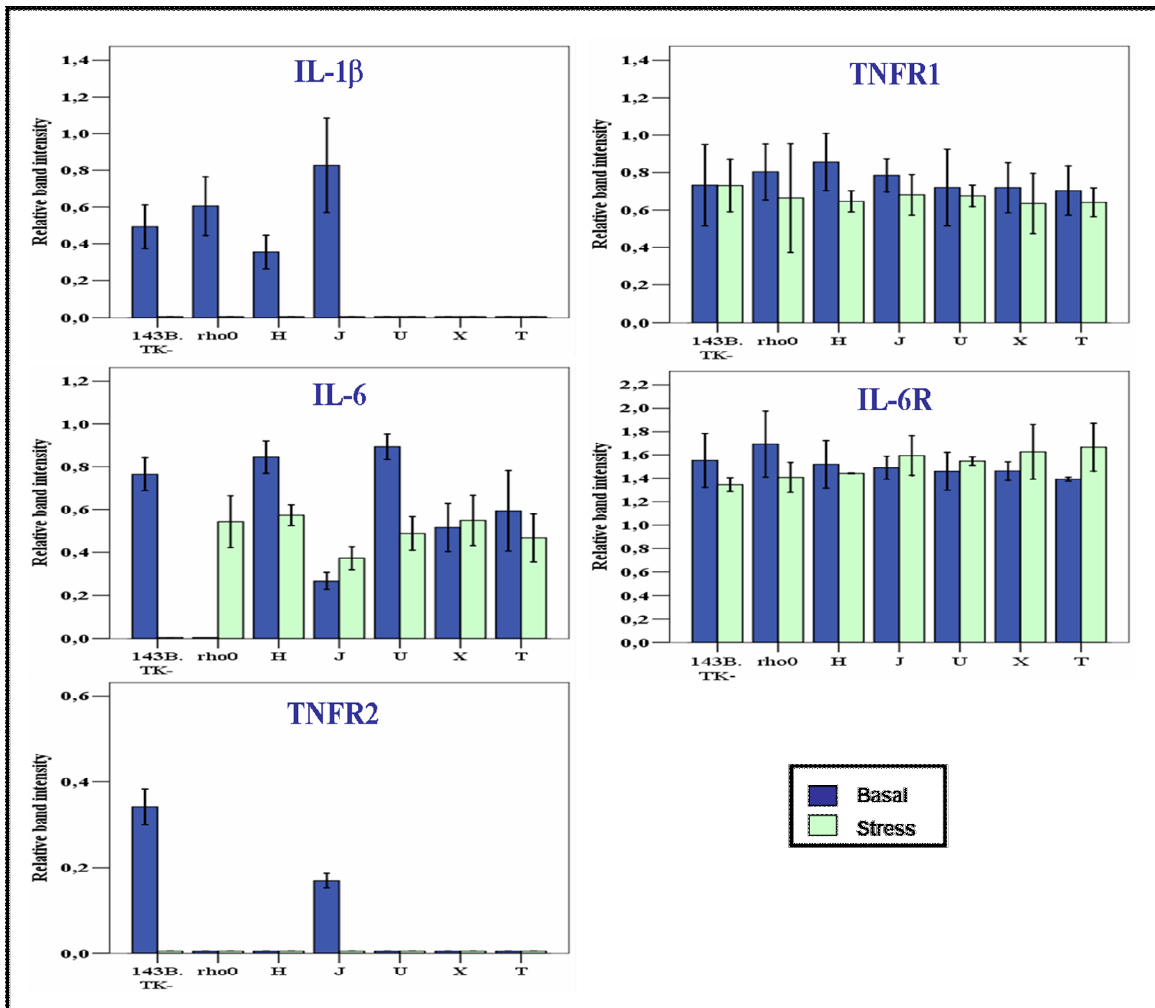


Fig 2.7 Stress effect on IL-1•, TNFR1, IL-6, IL-6R and TNFR2 gene expression in 143B.TK^{-}, rho^{0} and cybrid cell lines. The histogram is assembled by the densitometric data reported in Tables 2.1 and 2.2.

The stress condition induced down-regulation of *IL-1*b in the cell lines where the gene was expressed at basal conditions (143B.TK-, rho^{0}, H and J cybrids) so that the global outcome was the absence of gene expression in all the lines. The same result is observed as for *TNFR2* that was expressed at basal condition in 143B.TK- and J cell lines, but not expressed in any line under stress condition. These findings are in agreement with literature data reporting a repression of gene expression by oxidative stress (Morel & Barouki, 1999). In fact, a growing number of studies shows that oxidative stress can specifically down-regulate the expression of various genes (Boetkjaer, 2007; Kayurma, 2007; Kumar, 2007). Our findings add an important piece of information to these literature data, as they show for the first time that mtDNA common variability

contributes by a down-regulation of specific genes to the global outcome in oxidative stress response.

The result observed as for *IL-6* is of particular interest: under stress condition this gene was down regulated in 143B.TK- cells, as well as in H and U cybrids but up-regulated in the rho^0 cells. This result confirms that mtDNA variability contributes to the down-regulation of a stress-responder gene, as oxidative stress induces a different response within the cybrid lines. In addition, the up-regulation observed in cells lacking of active mitochondria provides a further evidence of the existence in human cells of a retrograde response activated in response to oxidative stress. Therefore, oxidative stress modulates the expression of *IL-6* and this effect is related both to the presence of active mitochondria and to mtDNA variability.

From the whole set of experiments, two conclusions can be drawn: a) mtDNA variability affects the expression of cytokine and cytokine receptor genes independently of the oxidative stress stimulus (Table 2.1); b) mtDNA variability affects the global outcome of the oxidative stress response by modulating the expression of cytokine and cytokine receptor genes (Fig.2.7).

The results we obtained in H and J cybrids confirm those previously reported (Bellizzi, 2006, see reprint in the End Section). This is particularly important because the soundness of the results obtained in cybrid cell lines is largely debated due to the genomic instability of tumoral cells repopulated with foreign mitochondria (Danielson, 2005). In the present case the expression profiles obtained in H and J cybrids are identical to those previously observed, despite the use of different H and J clones prepared across an interval of three years about.

Therefore, we can conclude that cybrid technology provides an useful tool to explore the role of mtDNA common variability on cell phenotypes provided that high quality control experiments are carried out.

2.1.4 Heat shock stress: heat shock protein genes

We induced heat shock stress by treating cell lines at 42°C for 2, 4 and 6 hours. Literature data show that heat shock is induced in living cells by treating them with a temperature that exceeds the normal life temperature (37°C) by five degrees (Wang, 1999).

Control experiments of the heat shock stress status

We verified the heat shock stress status by two different approaches: i) cell viability and DNA fragmentation assays to assess the apoptotic condition; ii) expression analysis of the heat inducible gene *HSP72* (Heat Shock Protein 72).

Cell viability assay. We calculated the percentage of living cells by a Trypan blue exclusion assay after heat shock at 42°C for 2, 4 and 6 hours. As a control, we also analyzed the viability of the cells at basal condition. The percentage of cell viability at the different times of stress treatment is reported in Fig. 2.8.

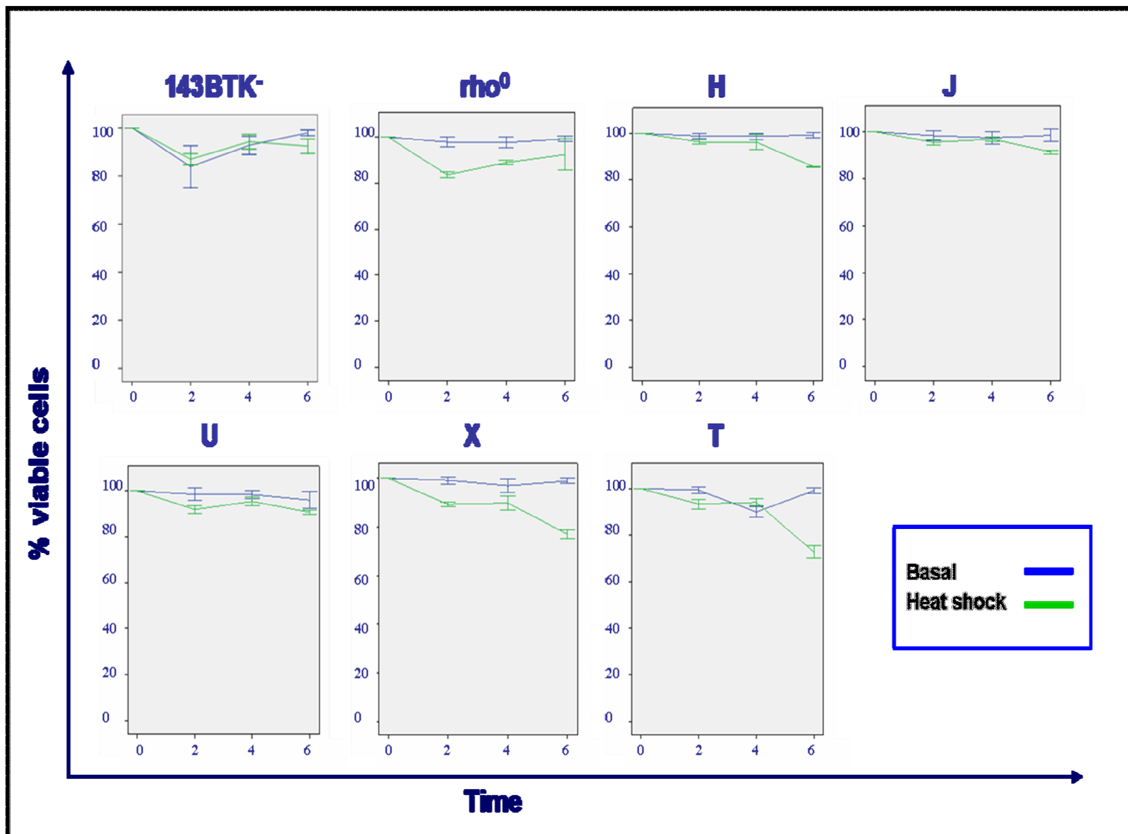


Fig 2.8 Viability curves of 143B.TK$^-$, rho^0, H, J, U, X and T cell lines. In each graph, untreated cells (blue line) and cell treated at 42°C for 2, 4 and 6 hours (green line) are shown.

It is interesting to remark the difference which is observed by comparing Fig. 2.4 (oxidative stress) with Fig. 2.8 (heat shock stress). In the first case, we adopted rather extreme conditions in order to be sure of the stress effectiveness. In fact we see in Fig. 2.4 that, at 48 hours of treatment, the percentage of living cells varied within the range 20% (J line) - 80% (U, X and T lines). In the second case, as we could check the

effectiveness of the heat shock stress by looking at *HSP72* gene expression, we adopted less extreme conditions. In fact we see in Fig. 2.8 that, at 4 hours of treatment, the percentage of living cells was 90% about in all the lines.

DNA fragmentation assay. In order to check the apoptotic status induced by heat treatment, we carried out a DNA fragmentation assay. The results are shown in Fig 2.9.

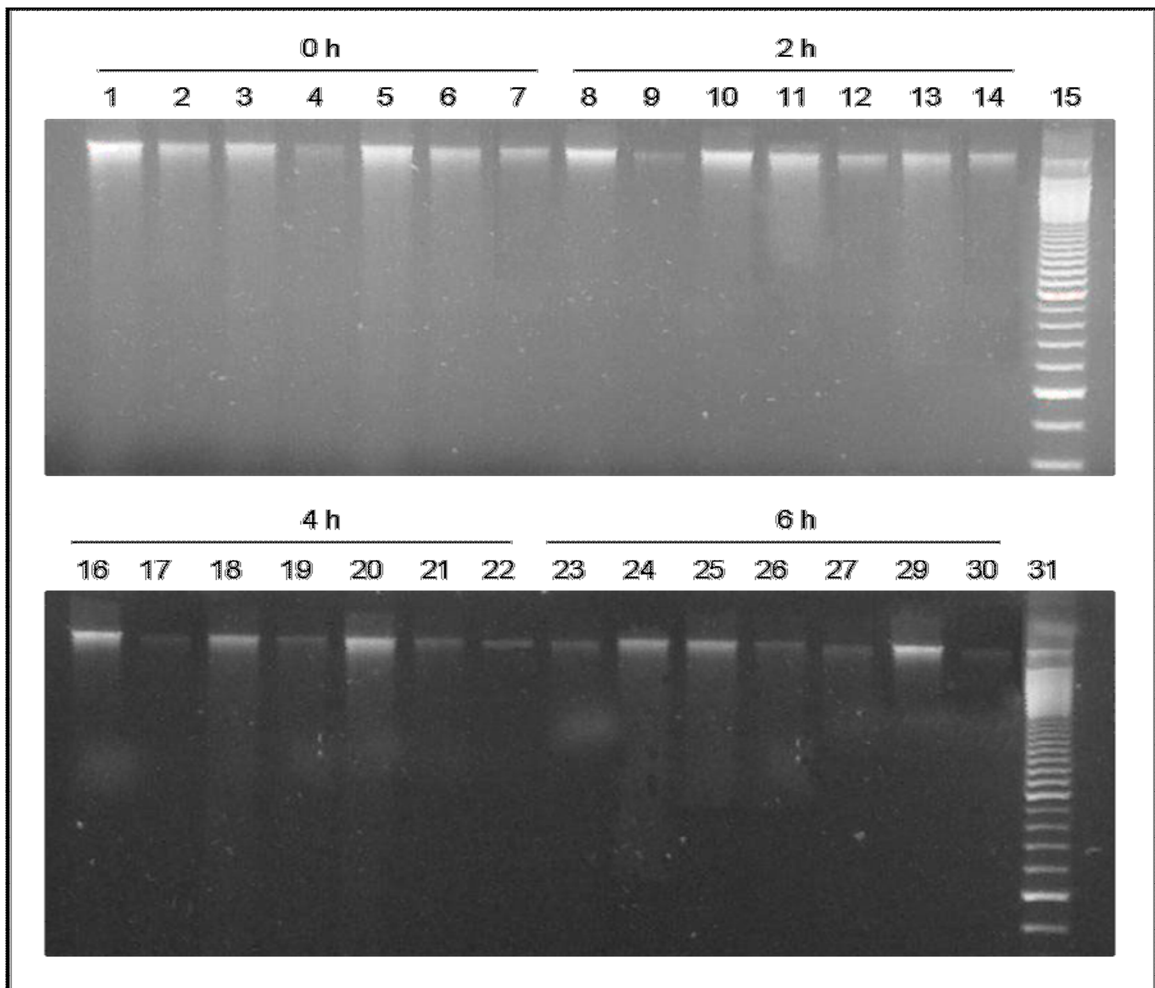
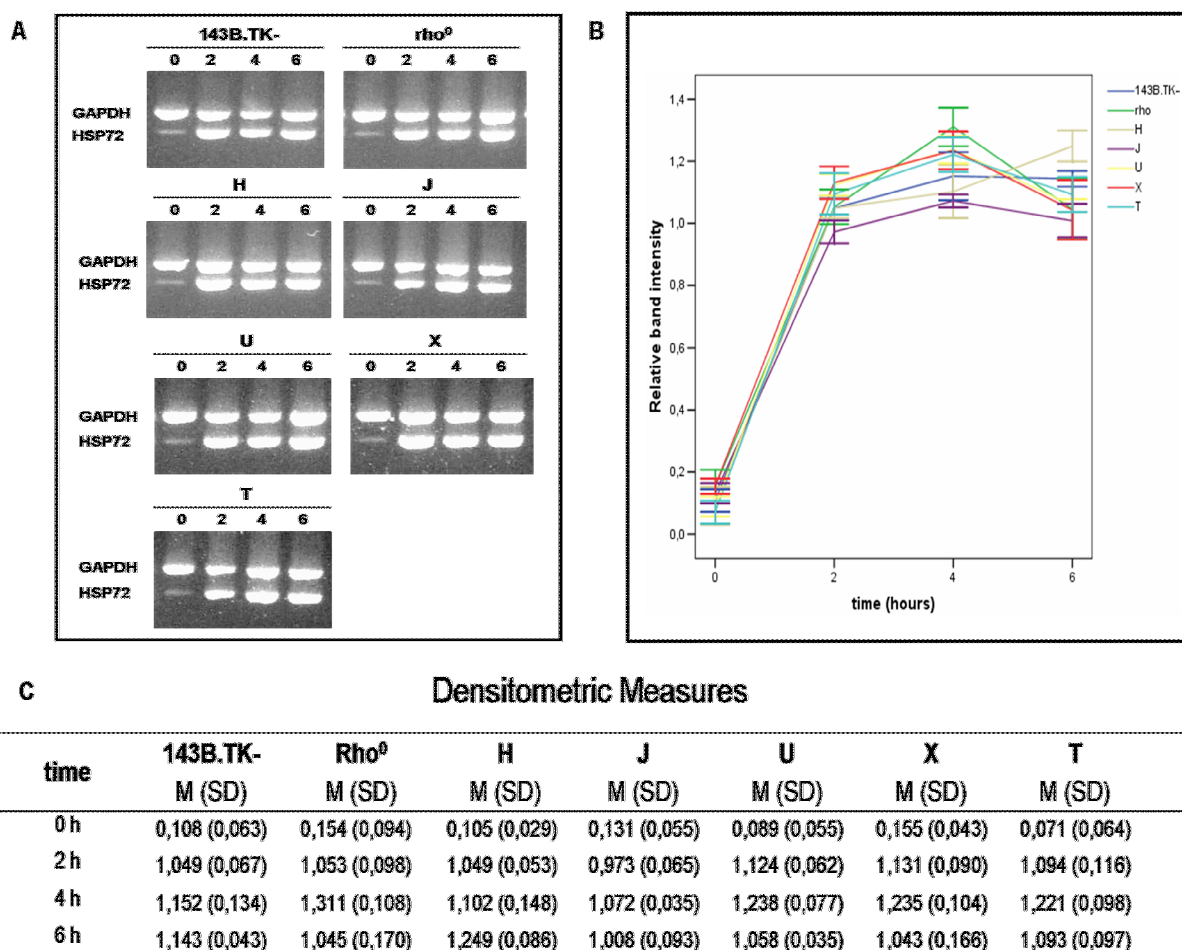


Fig 2.9 DNA fragmentation electrophoresis pattern of 143B.TK-, rho^0, H and J cell lines (A) and of U, X and T cell lines (B). Lanes 1-7 contain, in the order, DNA isolated from 143B.TK, rho^0, H, J, U, X and T cells at basal condition; lanes 8-14, 16-22, 23-30 contain, in the same order, DNA isolated from cells treated at 42°C for 2, 4 and 6 hours, respectively; lanes 15 and 31 contain molecular weight 100 bp ladder.

In line with the results obtained by cell viability assay, no cell line did show inter-nucleosomal DNA fragmentation at any time point.

***HSP72* gene expression analysis.** The above control experiments showed that the treatment at 42°C for 4 hours does not induce cell death significantly. Therefore, in order to be sure of the effectiveness of the stress protocol we checked the expression profile of the heat inducible gene *HSP72*. The results are reported in Fig 2.10 where a representative electrophoresis pattern (A) and the relevant graph (B) assembled by densitometric measures in three independent experiments (C) are reported.



Densitometric Measures

time	143B.TK- M (SD)	Rho^0 M (SD)	H M (SD)	J M (SD)	U M (SD)	X M (SD)	T M (SD)
0 h	0,108 (0,063)	0,154 (0,094)	0,105 (0,029)	0,131 (0,055)	0,089 (0,055)	0,155 (0,043)	0,071 (0,064)
2 h	1,049 (0,067)	1,053 (0,098)	1,049 (0,053)	0,973 (0,065)	1,124 (0,062)	1,131 (0,090)	1,094 (0,116)
4 h	1,152 (0,134)	1,311 (0,108)	1,102 (0,148)	1,072 (0,035)	1,238 (0,077)	1,235 (0,104)	1,221 (0,098)
6 h	1,143 (0,043)	1,045 (0,170)	1,249 (0,086)	1,008 (0,093)	1,058 (0,035)	1,043 (0,166)	1,093 (0,097)

Fig. 2.10 HSP72 gene expression analysis. (**A**) Representative RT-PCR electrophoresis pattern of *HSP72* gene after 2, 4 and 6 hours of heat shock treatment (42°C); (**B**) Relevant graph assembled by densitometric measures in three independent experiments (**C**)

At 4 hours of treatment the *HSP72* gene was induced significantly (p = 0,000 by pair-wise comparisons between basal and stress condition in each cell line). In fact, mRNA levels increased about 10-fold in all the seven cell lines after exposure to heat shock.

The results reported in Fig. 2.10 confirmed that the 4 hours stress treatment was appropriate to induce heat shock in our cells.

Gene expression analysis

HSP gene expression was analyzed in 143B.TK-, rho^0 and cybrid cell lines at basal and stress conditions by a semiquantitative RT-PCR. Three genes were analyzed: *HSP60* (Heat Shock Protein 60); *HSP75* (Heat Shock Protein 75) and *HSP72* as a control.

In Fig 2.11, a representative RT-PCR electrophoresis pattern is shown.

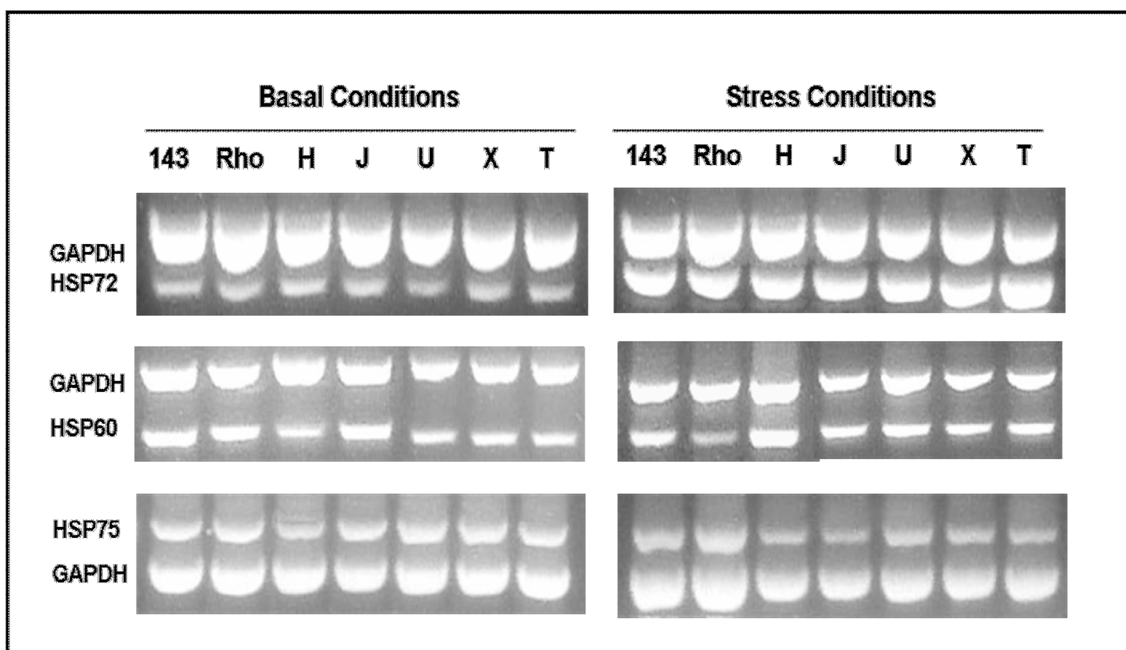


Fig 2.11 RT-PCR electrophoresis pattern of heat shock protein genes in 143B.TK$^-$, rho^0, H, J, U, X and T cell lines at basal and stress condition (42°C for 4 hours). GAPDH: Glyceraldeide phosphate deydrogenase.

Quantitative analyses of gene expression profiles are reported in Table 2.3 (basal condition) and Table 2.4 (stress condition) where the average densitometer measures of three independent experiments are reported.

Basal condition.

Table 2.3 reports the results obtained at basal conditions.

Basal Condition

	143B.TK- M (SD)	**Rho^0** M (SD)
HSP72	0,108 (0,063)	0,154 (0,094)
HSP60	0,573 (0,160)	0,550 (0,219)
HSP75	0,465 (0,090)	0,416 (0,112)

	H M (SD)	**J** M (SD)	**U** M (SD)	**X** M (SD)	**T** M (SD)
HSP72	0,105 (0,029)	0,131 (0,055)	0,089 (0,055)	0,155 (0,043)	0,071 (0,064)
HSP60	0,245 (0,178)	0,675 (0,172)	0,532 (0,112)	0,640 (0,131)	0,555 (0,101)
HSP75	0,212 (0,085)	0,342 (0,049)	0,365 (0,034)	0,516 (0,096)	0,461 (0,124)

Tab 2.3 Densitometer analysis of gene expression of HSPs in 143B.TK$^-$, rho^0 and cybrid cell lines at basal condition. The values represent the mean of three independent experiments with standard deviation**.**

A) Comparison between 143B.TK- and rho^0 cells.

By comparing 143B.TK- and rho^0 cell lines no significant difference was observed either for *HSP60* or for *HSP75*, thus indicating that the expression of the two genes is independent of the presence of active mitochondria.

B) Comparison among cybrid lines.

By comparing cybrid cell lines, we found that the expression levels of both *HSP60* and *HSP75* differed among the lines (p=0.005 and p=0.001, respectively, by ANOVA test). In particular, both genes were under-expressed in the H cybrid with respect to the other cybrid lines. Since mtDNA is the sole variant among the cybrid lines, we can conclude that a correlation exists between the expression of *HSP60* and *HSP75* and mtDNA variability. This result is in agreement with that found in the analysis of cytokines and cytokine receptors described above. Also in the case of *HSP60* and *HSP75* the gene expression is affected by mtDNA variability independently of the stress stimulus. By considering the role of the two HSPs in the processes of protein import into mitochondria and subsequent protein folding, the expression pattern shown by the H cybrid is very interesting. In fact, this result suggests that the efficiency of the above processes is in some way dependent on mtDNA variability. What is more, taking into account the concerted action of HSP60 and HSP75 in protein import and folding (Mokranjac, 2005; Itoh, 2002), we could hypothesize that mtDNA variability modulates the expression of *HSP60* and *HSP75* genes through a shared signaling pathway which acts on the two genes in a coordinate way.

Heat shock stress condition.

Table 2.4 reports the results obtained at stress conditions.

Stress Condition

	143B.TK- M (SD)	Rho^0 M (SD)
HSP72	1,152 (0,134)	1,311 (0,108)
HSP60	0,847 (0,081)	0,489 (0,124)
HSP75	0,369 (0,081)	0,272 (0,099)

	H M (SD)	J M (SD)	U M (SD)	X M (SD)	T M (SD)
HSP72	1,102 (0,148)	1,072 (0,035)	1,238 (0,077)	1,235 (0,104)	1,221 (0,098)
HSP60	0,734 (0,130)	0,680 (0,115)	0,764 (0,077)	0,752 (0,130)	0,742 (0,209)
HSP75	0,400 (0,063)	0,328 (0,115)	0,413 (0,101)	0,386 (0,099)	0,352 (0,114)

Tab 2.4 Densitometer analysis of gene expression of HSPs in 143B.TK$^-$, rho^0 and cybrid cell lines at stress condition. The values represent the mean of three independent experiments with standard deviation**.**

The expression level of *HSP75* did not differ either between 143B.TK- and rho^0 cells or among the cybrid lines. On the contrary, the expression level of *HSP60* was significantly lower in the rho^0 cells than in the parental line 143B.TK- (p=0.003 by pair-wise t-Student's test). Interestingly, the expression levels of *HSP60* in the cybrid lines did not differ significantly from that of the parental cell line (p > 0,05 by ANOVA test performed on the six lines having active mitochondria). This result showed that, in the case of *HSP60,* at difference what we found as for *IL6*, not only in rho^0 cells a

retrograde response is not activated, but the gene is down-regulated under heat shock stress.

As before, we visualized the heat shock stress effects by comparing the expression profiles of each gene *within a single cell line* before and after stress. By using the data reported in Table 2.3 and 2.4, we assembled the histograms shown in Fig. 2.12.

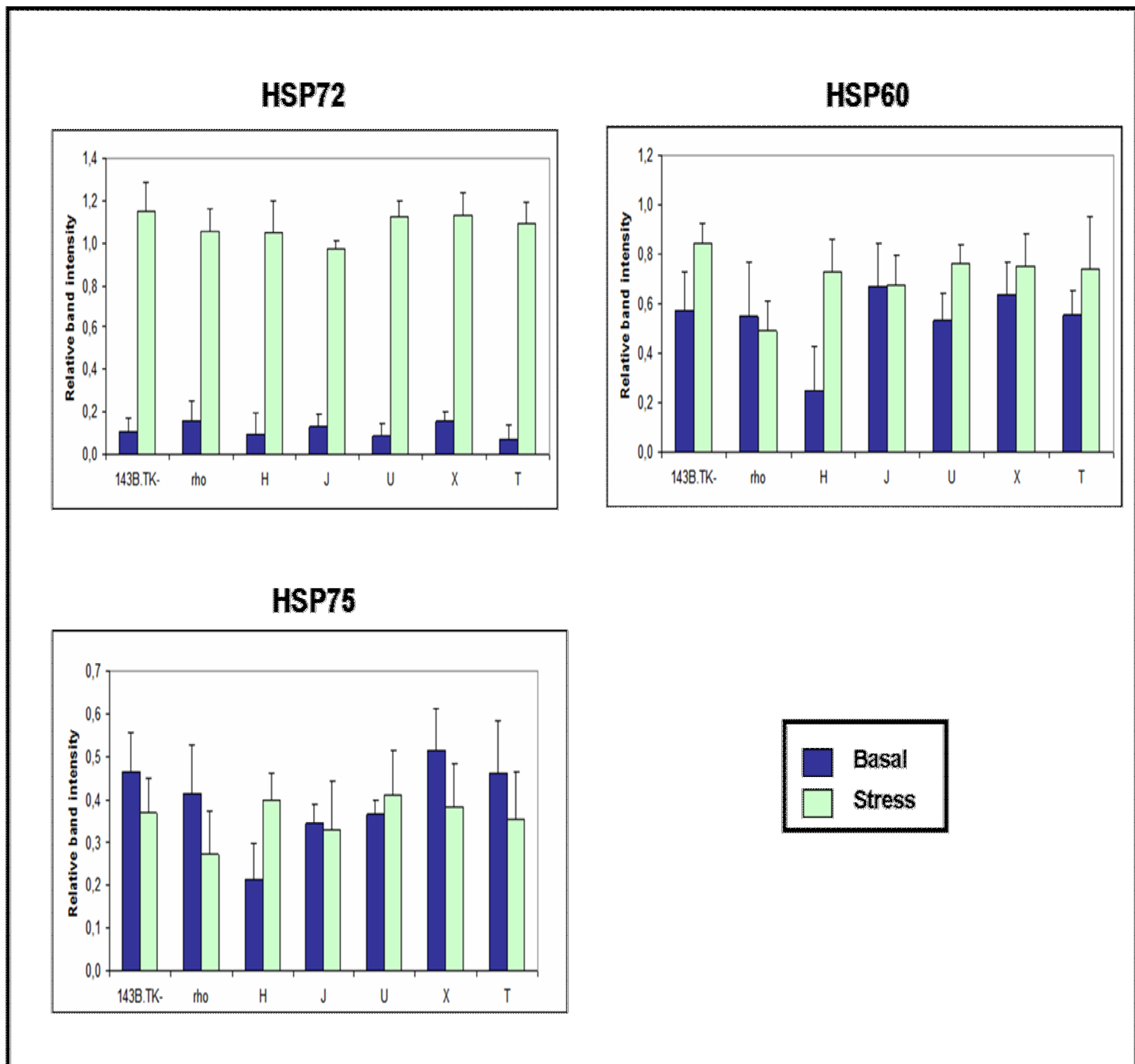


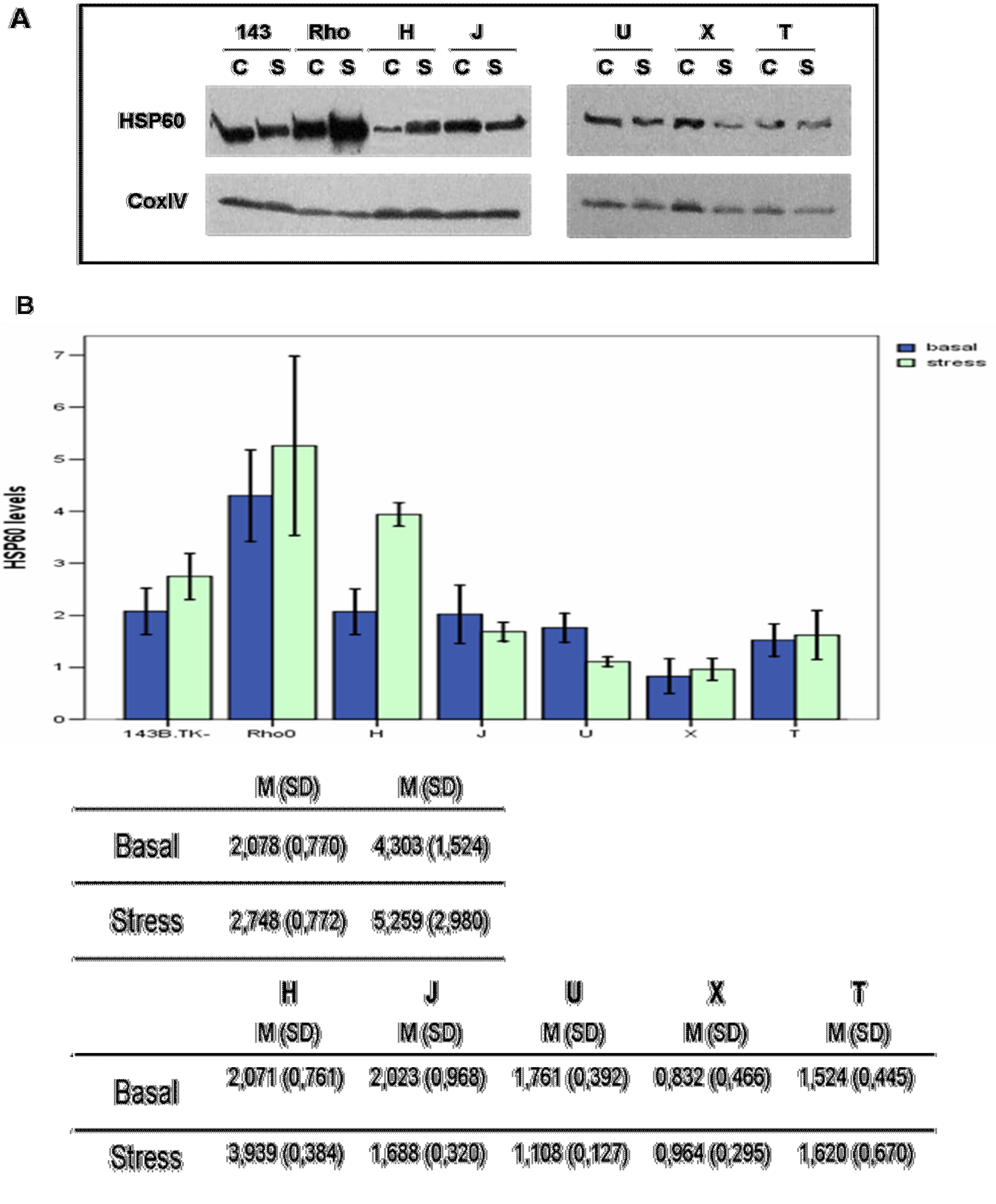
Fig 2.12 Stress effect on HSP72, HSP60 and HSP75 gene expression in 143B.TK^{-}, rho^{0} and cybrid cell lines. The histogram is assembled by the densitometric data reported in Tables 2.3 and 2.4.

Excepted for the H cell line, the expression of *HSP60* and *HSP75* genes was not substantially modified by the heat treatment. This result was rather unexpected because it is well documented that heat shock induces increase of HSPs at both transcription and

translation level (Tang, 2002; Moraitis and Curran, 2007). However, in the H cell line the result was different: at basal condition, *HSP60* and *HSP75* gene expression was lower than in the other cybrids; upon stress, both genes were over-expressed thus reaching levels comparable to those of the other cybrid lines. This result is in line with the results we found in the analysis of *IL-1*b and *TNFR2*: in both cases the stress condition eliminates the differences observed at basal condition (see Fig. 2.7 and Fig.2.12). In the case of *HSP60* and *HSP75*, the effect of the H mitochondrial genetic background, which at basal condition implied a gene expression level lower than that of the other cell lines, was vanished under stress condition.

Western blotting analysis.

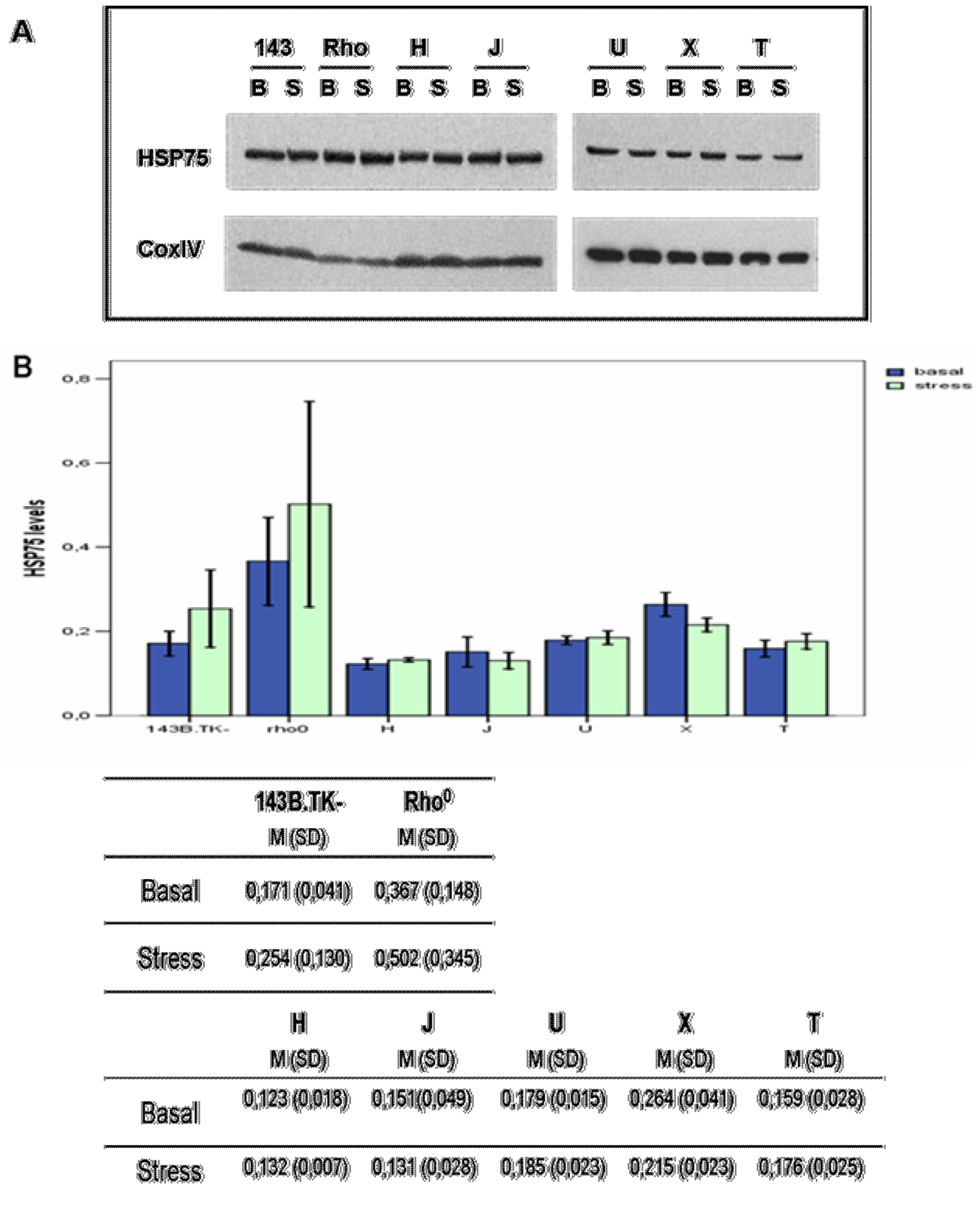
By considering that both HSP60 and HSP75 are mitochondrial proteins, we wished to verify if the amount of protein within mitochondria was different according to mtDNA variability. To this purpose, we measured by Western blotting the amount of HSP60 and HSP75 proteins within mitochondria in all the cell lines. Figures 2.13 (HSP60) and 2.14 (HSP75) show a representative Western blotting pattern (A) and the protein levels quantified by densitometer analysis (B).



	M (SD)	M (SD)
Basal	2,078 (0,770)	4,303 (1,524)
Stress	2,748 (0,772)	5,259 (2,980)

	H	J	U	X	T
	M (SD)	M (SD)	M (SD)	M (SD)	M (SD)
Basal	2,071 (0,761)	2,023 (0,968)	1,761 (0,392)	0,832 (0,466)	1,524 (0,445)
Stress	3,939 (0,384)	1,688 (0,320)	1,108 (0,127)	0,964 (0,295)	1,620 (0,670)

Fig 2.13 HSP60 Western blotting analysis. **(A)** Western blot electrophoresis pattern of HSP60 (mitochondrial fraction) in 143B.TK$^-$, rho^0 and cybrid cell lines at basal (B) and stress (S) condition (42°C for 4 hours). CoxIV: Cytochrome c oxidase subunit IV. (**B**) HSP60 protein levels (mitochondrial fraction) estimated by densitometric measure in 143B.TK$^-$, rho^0 and cybrid cell lines. The values represent the mean of three independent experiments with standard deviation**.**



	143B.TK- M (SD)	Rho[0] M (SD)
Basal	0,171 (0,041)	0,367 (0,148)
Stress	0,254 (0,130)	0,502 (0,345)

	H M (SD)	J M (SD)	U M (SD)	X M (SD)	T M (SD)
Basal	0,123 (0,018)	0,151(0,049)	0,179 (0,015)	0,264 (0,041)	0,159 (0,028)
Stress	0,132 (0,007)	0,131 (0,028)	0,185 (0,023)	0,215 (0,023)	0,176 (0,025)

Fig 2.14 HSP75 Western blotting analysis. (A) Western blot electrophoresis pattern of HSP75 (mitochondrial fraction) in 143B.TK$^-$, rho^0 and cybrid cell lines at basal (B) and stress (S) condition (42°C for 4 hours). CoxIV: Cytochrome c oxidase subunit IV. (**B)** HSP75 protein levels (mitochondrial fraction) estimated by densitometric measure in 143B.TK$^-$, rho^0 and cybrid cell lines. The values represent the mean of three independent experiments with standard deviation**.**

At basal conditions, by comparing 143B.TK- with rho^0 cells, we observed that HSP60 and HSP75 protein levels were higher in rho^0 cells (Figs. 2.13 and 2.14, B; p= 0.046 and p=0.036, respectively, by t-Student test). The increase of HSP60 and HSP75 observed in rho^0 cells with respect to the native cell line is well explained by considering that such cells are in a very stressful conditions due to mitochondrial dysfunctions caused by mtDNA depletion. However, it is worth noting that rho^0 cells do not activate a retrograde response able to increase *HSP60* and *HSP75* gene expression either at basal condition (Table 2.3) or after heat shock (Table 2.4). What are the cell mechanisms by which the accumulation of HSP60 and HSP75 within mitochondria is realized independently of the level of nuclear gene expression is not known and further studies are needed to clarify this point.

By comparing cybrid cell lines, no difference was found either in HSP60 or in HSP75 protein levels (in both cases, p>0,05 by ANOVA test) thus indicating that, at basal conditions, mtDNA common variability does not affect the levels of these proteins within mitochondria.

At stress conditions, by comparing 143B.TK- with rho^0 cells, we observed that the amount of HSP60 was higher in rho^0 cells (p= 0.036 by t-Student test). No difference was observed in HSP75 protein level (p= 0.442 by t-Student test).

By comparing cybrid cell lines, we found that HSP60 protein level differed among the lines (p=0.000 by ANOVA test). In particular, the H cybrid showed increased levels of HSP60 with respect to other cell lines. On the contrary, the difference observed as for HSP75 was not significant (p=0.064 by ANOVA test).

Finally, if we compare heat shock stress effects on the protein levels of HSPs *within a single cell line*, we observe that the heat shock treatment induced an increase of HSP60 in H cybrid only (Fig.2.13B). This result is very interesting, because it shows, for the first time, a correlation between mtDNA common variability and accumulation within mitochondria of a protein which has a crucial role in coping with stress damage. On the contrary, as for HSP75, no correlation was observed between mtDNA variability and accumulation of this protein after stress (Fig. 2.14B). The different results obtained for HSP60 and HSP75 may be explained at the light of the different functions exerted by these proteins. In fact, while HSP75 is implicated in an ATP-dependent process of

import of cytosolic proteins within mitochondria, HSP60 is specifically implicated in mitochondrial protein refolding.

2.2 Matherials and Method

2.2.1 Cell lines and culture conditions

143B.TK$^-$ osteosarcoma cells and cybrid cell lines were grown in DMEM (Gibco) containing 4.5 g/l glucose and 110 µg/ml pyruvate, supplemented with 10% Foetal Bovine Serum (Gibco) and 50 µg/ml gentamicin (Gibco). Rho^0 cells were grown in DMEM (Gibco) containing 4.5 g/l glucose and 110 µg/ml pyruvate, supplemented with 10% Fetal Bovine Serum (Gibco) and 50 µg/ml gentamicin (Gibco) and 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.

2.2.2 Experiments for quality control of cellular state

Proliferation assay. 143B.TK$^-$, rho^0 and cybrid cell lines were seeded in 6-well plates (1 x 10^5 cells/well) in regular growth medium. Cells were trypsinized after 24, 48 and 72 hours culture and counted on a haemocytometer with an inverted light microscope using a 10x magnification. Three experiments were carried out for each cell line, then average values with the relevant standard deviations were calculated.

Quantification of mtDNA. Total DNA was extracted from 143B.TK$^-$ and cybrid cell lines according to standard methods. The cell content of mtDNA was measured by RT-PCR analysis based on target-specific fluorigenic chemistries (TaqMan probes). the TaqMan probe specific for mtDNA product was 5'-FAM-CCTCACGCAAGCAACCGCATCC- BlackHole Quencher1-3' while the TaqMan probe specific for nuclear product was Genprobe (5'-Texasred-CTGTTCCGTTTCCTGCCGGTGCBlackHole Quencher2-3'). The sequences of promers specific for mtDNA were the following:

- mtDir (5'- CACAGAAGCTGCCATCAAGTA-3')
- mtRev (5'-CCGGAGAGTATATTGTTGAAGAG-3')

The sequences of primers specific for nuclear genome, that amplify the FasL gene were the following:

- GenDir (5' GGCTCTGTGAGGGATATAAAGACA-3')

§ GenRev (5' AAACCACCCGAGCAACTAATCT- 3')

Both the two RT-PCR were carried out in a final volume of 20 •l containing a mix of 1X PCR buffer (Promega, Madison, Wisconsin, USA), 3.0 mmol/l magnesium chloride, 400 pmol primers for mtDNA, 0.2 mmol/l dNTP, 2U Taq polymerase (Promega) and the TaqMan probe. The PCR was performed with an initial denaturation step at 95°C for 6 min followed by 45 cycles of amplification (94°C for 30 s, 60°C for 60 s) in an iCycler Thermal cycler (BioRad, Hercules, California, USA). All samples were analysed in triplicate. The relative copies number of mitochondrial and nuclear DNA was detected by using a standard curve for each PCR. The regions used respectively for mtDNA and nuclear DNA amplifications were cloned tail to tail in a vector (pGEM-11Z; Promega) to obtain a 1.1 ratio of the reference molecules. Serial known diluitions of this vector were included in each PCR and amplified in triplicate to obtain the standard curve. The measured values for mtDNA and nuclear DNA were distributed in the range of the standard curve and the correlation coefficient was always> 0.995. Absolute values for the copies of mtDNA per cell were then simply calculated as ratio between the relative values of mtDNA and nuclear DNA (obtained versus the same vector), multiplied by 2 (as two copies of the nuclear gene are present in a cell).

Mitochondrial membrane potential (MMP) assay. Mitochondrial membrane potential (MMP) was assessed by double staining with MitoTracker Green^{TM} (MTG, specific for mitochondrial mass) and TetraMethyl Rhodamine Methylesther (TMRM, specific for MMP) (Molecular Probes, Eugene, OR, USA). 143B.TK$^-$, rho^0 and cybrid cells were seeded in 6-wells plates (2 x 10^5 cells/well). After 24 hours, the cells were stained with MTG 100 nM and TMRM 150 nM for 20 minutes at 37°C, then collected and analysed at flow cytometer. Cytofluorimetric analyses were performed by using a Becton Dickinson NJ (FACSscan). The data obtained by cytofluorimetric analyses were analysed by using WinMDI software.

2.2.3 Oxidative stress treatment

2 x 10^5 143B.TK$^-$, rho^0 and cybrid cells were seeded in 100-cm^2 plates. In the exponential growth phase, the medium was discarded and replaced with DMEM

containing 2-deoxy-D-ribose (d-Rib) 20 mM (Sigma). The cells were incubated to 37°C for 24, 48 and 72 hours. Untreated cells were also analysed as control.

2.2.4 Heat shock treatment

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

2.2.5 Experiments for quality control of stress treatment

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). Viable and non viable cells were then counted on a haemocytometer with an inverted light microscope using a 20x magnification.

Three experiments were carried out for each cell line, then average values with the relevant standard deviations were calculated. The percentage of cell viability was calculated from the ratio of the number of viable cells divided by the total number of cells.

DNA fragmentation assay. Treated and untreated cells were trypsinised and centrifuged at 3000 x g for 5 minutes. The pellet was resuspended in 400 µl of lysis buffer (10mM TRIS-HCl pH 8, 20mM EDTA, 0.2% triton-X100) and then incubated on ice for 20 minutes. After a 12,000 x g centrifugation for 20 minutes, an equal volume of phenol/chloroform was added to the surnatant. Then, after a new 12,000 x g centrifugation for 5 minutes, an equal volume of chloroform was added to surnatant and centrifuged again. The surnatant was collected and stored at -20 °C overnight after adding of 0.1 volume of 3M Sodium Acetate pH5.2 and 2 volumes of ethanol to precipitate DNA. DNA was pelleted by centrifugation at 12,000 x g per 20 minutes,

rinsed with 70% ethanol and then resuspended in TE buffer containing 100 mg/ml RNase A. After 1 hours of incubation at 37 °C, the DNA samples were loaded onto a 1.5% agarose gel, electrophoresed in TAE buffer and stained with ethidium bromide.

2.2.6 RT-PCR of cytokine and cytokine-receptor genes

Total RNA was extracted from control and treated 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.

The RT-PCR reactions were carried out by using the RETROscript Kit (Ambion). An RT mix including 10 μg of total RNA and 100 pmoles of random decamers was pre-heated at 70°C for 5 minutes. The reaction was carried out in a 50 μl final volume containing 1X RT Buffer, 0.4 mM of each dNTP, 25U of RNase inhibitor, 275U of MMLV reverse transcriptase. The mix was incubated at 37°C for 1 hour and, successively, at 95°C for 10 minutes to inactivate the reverse transcriptase.

For the cytokine analysis, we used MPCR (Multiplex PCR) Amplification kit (Maxim Bio). This kit have been designed for the simultaneous amplification of specific human inflammatory cytokine genes. 25 μl of the PCR mixture contained 2,5 μl of cDNA, 1X MPCR Buffer, 1X MPCR primers specific for cytokine genes and 1.5U *Taq* DNA polymerase (Eppendorf). As internal control primers specific for GAPDH housekeeping gene were utilized. Two control reactions were carried out: a positive control containing as template the cDNA supplied by the kit and a negative control that did not contain cDNA. After an initial denaturation step at 96°C for 1 minute, the PCR was cycled twice at 96°C for 1 minute and 67°C for 2 minutes, followed by 35 cycles of 96°C for 1 minute and 67°C for 4 minutes. The final step was incubation at 70°C for 10 minutes. Then, PCR products were analysed on 2,2% agarose gel containing ethidium bromide 0.5 mg/ml. Fluorescence intensity of each band was calculated by using a densitometer analysis (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized respect to GAPDH band intensity. Three independent RT-PCR experiments were carried out for each cell line, then the average values with the relevant standard deviations were calculated.

2.2.7 RT-PCR of heat shock protein genes

Total RNA was extracted from control and treated 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.

The RT-PCR reactions 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 minutes. The reaction was carried out in a 40 µl final volume containing 1X RT Buffer, 0.5 mM of each dNTP, 3mM $MgCl_2$, 20U of RNase inhibitor, 5U of reverse transcriptase. The mix was incubated at 25°C for 5 minutes, then 37°C for 1 hour and, successively, at 95°C for 10 minutes to inactivate the reverse transcriptase.

For the analysis of heat shock genes we used primers specific for HSP72, HSP60, HSP75 and, as internal control primers specific for GAPDH housekeeping gene (Tab 2.3).

Primers	Primer forward 5'-3'	Primer reverse 5'-3'
HSP72	AAGTTGCAATGAACCCCACC	TTGCGCTTAAACTCAGCAA
HSP60	ATTCCAGCAATGACCATTGC	GAGTTAGAACATGCCACCTC
HSP75	TGGCAGTTATGGAAGGTAAA	AGCAATGACTTTGTCTTCTG
GAPDH	GACAACTTTGGTATCGTGGA	TACCAGGAAATGAGCTTGAC

Tab 2.3. Primers forward and reverse for HSP72, HSP60, HSP75 and GAPDH.

The PCR mixture was carried out in 30 µl volume contained 1,5 µl of cDNA, 1X Buffer RB, 0,5mM for each dNTP, 3,5mM $MgCl_2$, 0,6 µM for each primer, 10U DNA polymerase (EuroTaq)

After an initial denaturation step at 94°C for 1 minute, the PCR was carried out for 25 cycles at 92°C for 1 minute, 56°C for 1 minute and 72°C for 1 minutes. The final step was incubation at 72°C for 10 minutes. Then, PCR products were analysed on 2,5% agarose gel containing ethidium bromide 0.5 mg/ml. Fluorescence intensity of each band was calculated using a densitometer analysis (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized respect to GAPDH band intensity. Three independent RT-PCR experiments were carried out for

each cell line, then the average values with the relevant standard deviations were calculated.

2.2.8 Western Blot analysis of heat shock proteins

Isolation of mitochondrial protein extracts. Isolation of mitochondrial protein extracts was carried out by using Mitochondrial Fractionation Kit (Active Motif). Treated and untreated cells were scraped on ice after the addition of 5 ml of PBS 1X and then centrifuged at 600 x g for 5 minutes at 4°C. The pellets were resuspended in 350 •l of Cytosolic Buffer 1X and then incubated on ice for 15 minutes. Successively, the pellets were homogenize with a homogenizer and then centrifuged at 800 x g for 20 minutes. The pellets were washed with Cytosolic Buffer 1X and then centrifuged at 10000 x g for 10 minutes at 4°C. Finally, the pellets were resuspended in 35 •l of Complete Mitochondrial Buffer (Mitochondria Buffer, Protease inhibitor Cocktail, DTT) and incubated on ice for 15 minutes.

Immunoblotting. For immunoblotting, 10 •g of total protein and 15 •g of mitochondrial protein extracts were separated by one-dimensional SDS-PAGE 10% polyacrylamide gels at 100 V for 2 hours, and transferred to nitrocellulose membranes at 60 V for 1 hour at 4°C. The blot was washed with TBST 1X for 10 minutes and then incubated overnight with a blocking solution (5% non-fat dried milk/TBST 1X) to block non-specific sites. Then the blot was washed three times with TBST 1X for 10 minutes and incubated with specific antibodies, followed by HRP-conjugated anti-mouse (1:5000) or anti-rabbit (1:2000) IgG (Amersham), and visualized by using the enhanced chemiluminescence kit (ECL) (Amersham). The following antibodies were used: anti-HSP60 (1:1000) (SPA-807, Stressgen), anti-HSP75 (1:50) (H-155, SantaCruz). As internal control of mitochondrial fraction anti-CoxIV antibody (1:500) (A21347, Molecular Probes) was used. Quantitative evaluation of the immunoblots was carried out by using a densitometer analysis (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized respect to internal controls. Three independent western blot experiments were carried out for each cell line, then the average values with the relevant standard deviations were calculated.

2.2.9 Statistical analyses

Results are expressed as means ± standard deviation (SD). Statistical analyses were performed using SPSS 10 statistical software (SPSS Inc., Chicago, Illinois). One-way analysis of variance (ANOVA) and Student's t-test and were adopted. Significance level was defined as $\alpha = 0,05$

3. Conclusive Remarks

Aim of this PhD thesis was to investigate whether the common mtDNA variability influences the stress response by modulating the expression of stress-responder nuclear genes through a cross-talk between mitochondrial and nuclear DNA. We analyzed the expression of cytokine and cytokine receptor genes at both basal and oxidative stress condition, and of heat shock protein genes at both basal and heat shock stress condition.

We applied cybrid technology that provides a useful tool to investigate the influence of mtDNA variation on cellular phenotypes. In fact, by this technology it is possible to keep constant the nuclear background and to change the mitochondrial genome only, thus obtaining cell lines in which the sole variant is the mtDNA. However, some cautions are required to draw conclusions based on cybrids. In fact, mutations occurring in nuclear DNA during the cell maintenance in culture, or possible effects of the experimental manipulation, may introduce artificial results. In our study, not only we carried out appropriate control experiments but also replicated the analyses of gene expression on an independent clone when positive results were observed. The most important evidence of the soundness of our results is provided by the replication of the results we obtained in a previous study (Bellizzi, 2006; see reprint at the End Section). We re-analyzed gene expression of cytokine and cytokine receptors in H and J cybrids cloned at a distance of 3 years about. We obtained the same results in the two preparations of cybrid lines. The reproducibility of the results rules out that stochastic factors affect the expression patterns we observed.

The results here presented show that, at basal condition, mtDNA common variability is correlated to the expression of cytokine and cytokine receptor genes (*IL1*-b, *IL-6* and *TNFR2*), thus confirming our previous results (Bellizzi, 2006). Furthermore, at basal conditions, mtDNA variability is also correlated to the expression of heat shock protein genes (*HSP60* and *HSP75*). In fact, we observed significant differences in the expression profiles of these genes among cybrid cell lines. At our knowledge it is the first time that this correlation is found as for HSP genes. The agreement between the data obtained in cytokines and those obtained in HSPs indicates that the correlation between mtDNA variability and expression levels of stress responder genes may be a general phenomenon. The extension of our approach to further classes of stress

responder genes could verify the above indication. This could be the next step in our research.

Interestingly, the differences observed among the cybrid lines disappear under stress conditions. In fact, both oxidative and heat shock stress equalize the gene expression patterns so that they become independent of mtDNA common variability. A possible biological explanation of this finding could be provided by the crucial biological role of these proteins in stress response. In line with this hypothesis are the results of the cell viability assay carried out to set up the oxidative stress conditions: we see in Fig. 2.4 that the percentage of cells survived to the stress stimulus varies from 20% and 60% in J and H lines, respectively, to 80% in U, X and T lines. Therefore, just in the lines where the percentage of cell survival is lower, the major difference in gene expression were observed. This result indicates that the cell capability to equalize the expression patterns of stress responder genes is correlated with the cell capability to survive to stress.

On the whole, the results we obtained reinforce the idea that mtDNA common polymorphisms are not neutral as previously believed, and that the cross-talk between the two genomes is very important in stress response and, consequently, in complex traits. On this proposal, Roubertoux and coll. (2003) provided direct evidence of mtDNA involvement in cognitive functioning in mice, showing that this genome, by interacting with the nuclear genome, is able to influence learning, exploration, sensory development and the brain anatomy.

We are aware that our findings are obtained *in vitro* and therefore cannot be immediately transferred at organism level. However the results here reported are new and may provide a key to understanding the complex relationship between mtDNA common variability and cellular phenotypes.

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END SECTION

Gene expression of cytokines and cytokine receptors is modulated by the common variability of the mitochondrial DNA in cybrid cell lines

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Some lines of evidence indicate that common polymorphisms of mitochondrial DNA (mtDNA) act as susceptibility factors in complex traits, such as age-related common diseases. There is also evidence that the cell capability to compensate ravages caused by intrinsic or extrinsic stress factors could contribute to some of these diseases. The cross-talk between nuclear and mitochondrial genome may link the above observations if we assume that the transcription of stress-responder nuclear genes is modulated according to the mtDNA common variability. Cytokines and cytokine receptors are key molecules in stress response. We could, therefore, check the above hypothesis by analyzing expression patterns of cytokine and cytokine receptor genes in response to stress in cell lines sharing the same nuclear genome but different mtDNA. By using a cybrid model (143B.TK$^-$ osteosarcoma cells depleted of their own mtDNA and repopulated with foreign mitochondria) we show that the transcription patterns of some of such genes are specifically modulated by the variability of the mitochondrial genome not only under stress conditions (interleukin-6) but also at basal conditions (interleukin-1β and tumor necrosis factor receptor 2). These findings provide a first experimental evidence of a relationship between mtDNA common variability and expression pattern of stress responder nuclear genes in human cells.

Introduction

Mitochondria play a central role in cell biology by converting dietary calories into usable energy. This process generates reactive oxidative species (ROS) as a toxic by-product. As ROS are detrimental to the integrity of the mitochondrial DNA (mtDNA), a 'mitochondrial paradigm' of degenerative diseases, aging and cancer has been proposed, which assumes that the late onset and progressive course of age-related diseases result from the accumulation of somatic mutations in the mtDNA of postmitotic tissues (Linnane *et al.* 1989; Wallace 2005). Two clues suggest that the mtDNA inherited variability also has a role in this performance. First, significant associations have been found between mtDNA common polymorphisms and age-related complex traits, such as Parkinson disease (van der Walt *et al.* 2003; Autere *et al.* 2004; Otaegui *et al.* 2004; Ghezzi *et al.* 2005), Alzheimer disease (Chagnon *et al.* 1999; van der Walt *et al.* 2004), cardiovascular diseases (Takagi *et al.* 2004), longevity (Tanaka *et al.* 1998; De Benedictis *et al.* 1999; Ross *et al.* 2001; Niemi *et al.* 2005); second, significant differences in oxidative phosphorylation performance have been found between sperm having mtDNA of different haplogroups (Ruiz-Pesini *et al.* 2000). Therefore, not only somatic but also inherited mtDNA variability could act as a susceptibility factor in complex phenotypes, according to the cell capability to cope with oxidative stress. In line with this perspective, studies in model organisms showed that signals from mitochondria to nucleus are able to modulate both the expression of stress-responder genes and lifespan (Kirchman *et al.* 1999).

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Cytokines are small secreted proteins which play a pivotal role in the homeostasis of the organism, by mediating and regulating immunity, inflammation and hematopoiesis. They act by binding to specific membrane receptors, which, through secondary messengers, modulate the expression of a series of downstream genes. Therefore, an appropriate expression of cytokine and cytokine receptor genes is crucial for coping with a variety of intrinsic and extrinsic stress factors. If the variability of the mtDNA does play a role in stress-response, cell lines sharing the same nuclear genome but different mtDNAs (cybrid cell lines) should show different expression profiles of these master genes under stress conditions. We checked this hypothesis by analyzing the transcription patterns of cytokine and cytokine receptor genes in cybrid cell lines repopulated with foreign mitochondria having different mtDNA sequences. The results here reported show that, at both basal and stress conditions, some of the above genes are expressed according to mtDNA common variability.

Results

Cell fusion and mtDNA variability in cybrid cell lines

We produced cybrid cell lines by fusing platelets from two young donors with 143B.TK$^-$ osteosarcoma cells depleted of mitochondrial DNA (rho^0 cells). The absence of mtDNA in the rho^0 cells was verified by two approaches. First, the cells were unable to grow without added uridine, and died within 10 days, thus showing loss of functional mitochondria. Second, PCR amplification of the 15996–16401 mtDNA region gave negative results in DNA extracted from the rho^0 cells (results not shown). Positive mtDNA repopulation was confirmed by the growth of clones in selective medium lacking of uridine.

According to the variability at evolutionary conserved positions (RFLP analyses), the mtDNA of the native cell was classified as belonging to the X haplogroup, while those of the donor platelets to H and J haplogroups (Torroni *et al.* 1996). Accordingly, we named the two cybrid cell lines H and J.

MtDNA molecules of both native and cybrid cells were then entirely sequenced to assess their diversity. Results are shown in Table 1. With respect to the revised Cambridge reference sequence (rCRS) (Andrews *et al.* 1999; <www.mitomap.org>), 58 variant sites were found: 24 sites occurred in the-non-coding region, at key points involved in the regulation of mtDNA replication and transcription; 8 sites were in 12S rRNA, 16S rRNA and 1 in the tRNA^{Thr} genes; 25 sites were in the coding region, and 9 of them changed conserved amino acids.

Control experiments

Before starting with the analysis of gene expression profiles, we carried out a series of control experiments on the four lines (parental 143B.TK$^-$, rho^0, H and J), to assess the quality of the experimental conditions.

Cell growth and quantification of mtDNA

The growth curve was not substantially different among the four cell lines, with uridine in the rho^0 culture (Supplementary Fig. S1). In order to verify that the amount of mtDNA did not differ among 143B.TK$^-$, H, and J cell lines, we estimated the number of copies of mtDNA per cell in each line by using Quantitative Competitive-PCR (QC-PCR). No significant difference was observed among the three lines (Supplementary Fig. S2).

Functionality of the cybrid cell lines

We assessed the mitochondrial functionality of the cybrid lines by measuring the Mitochondrial Membrane Potential (MMP). Both H and J cell lines displayed a value of MMP comparable to that of the 143B.TK$^-$ parental line, thus showing that the cybrid lines were metabolically active; as expected, MMP was lower in the rho^0 cells (Supplementary Fig. S3).

Stress conditions

The stress experimental conditions (dosage and treatment time by 2-deoxy-D-ribose) were verified by cell viability assay and DNA fragmentation analysis. By using a viability/proliferation assay, we verified that the viability did not differ among the four cell lines at 48 h, either at basal or stress conditions (Supplementary Fig. S4). Then, we checked the efficacy of the 48 h stress by looking at the internucleosomal DNA fragmentation which is expected in an apoptotic response (Supplementary Fig. S5). After assessing the quality of the cell lines and stress experimental conditions we went on with the analysis of gene expression.

Gene expression profiles

Gene expression was analyzed at both basal and stress conditions by multiplex RT-PCR for a panel of eight genes coding for cytokines and cytokine receptors (tumor necrosis factor-α, *TNF-α*; tumor necrosis factor receptor 1, *TNFR1*; tumor necrosis factor receptor 2, *TNFR2*; Granulocyte Macrophage-Colony Stimulating Factor, *GM-CSF*; Granulocyte Macrophage-Colony Stimulating Factor Receptor, *GM-CSFR*; interleukin-1β, *IL-1β*; interleukin-6, *IL-6*; interleukin-6 receptor, *IL-6R*). We replicated the experiments three times, starting from the

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Table 1 Variant sites found in mtDNA molecules from 143B.TK⁻, H and J cybrid lines

Position (nt)	Locus	Nucleotide change*	143B.TK⁻	Cybrid H	Cybrid J	Amino acid change
73	HV2	A → G	G	—	G	Non coding
114	HV2	C → T	—	T	—	Non coding
146	HV2	T → C	—	C	C	Non coding
152	HV2	T → C	—	C	C	Non coding
153	HV2	A → G	G	—	—	Non coding
195	HV2	T → C	C	C	—	Non coding
225	CSB1	G → A	A	—	—	Non coding
226	CSB1	T → C	C	—	—	Non coding
295	TFY	C → T	—	—	T	Non coding
309	CSB2	Cn → CC ins	—	CC ins	—	Non coding
315	CSB2	Cn → C ins	C ins	C ins	—	Non coding
462	D-loop	C → T	—	—	T	Non coding
489	D-loop	T → C	—	—	C	Non coding
499	D-loop	G → A	—	A	—	Non coding
709	12S	G → A	—	—	A	Non coding
1438	12S	A → G	—	—	A	Non coding
1719	16S	G → A	A	—	—	Non coding
1733	16S	C → T	—	T	—	Non coding
2628	16S	T → C	—	C	—	Non coding
2706	16S	A → G	G	G	—	Non coding
3010	16S	G → A	—	A	—	Non coding
3330	ND1	C → T	—	T	—	Syn
4216	ND1	T → C	—	—	C	Y → H
6221	CO1	T → C	C	—	—	Syn
6267	CO1	G → A	A	—	—	A → T
6371	CO1	C → T	T	—	—	Syn
7028	CO1	C → T	T	—	T	Syn
8269	CO2	G → A	—	—	A	Non coding
9141	ATP6	T → C	—	C	—	Syn
10398	ND3	A → G	—	—	G	T → A
11251	ND4	A → G	A	A	—	Syn
11719	ND4	G → A	A	—	A	Syn
11932	ND4	C → T	—	C	C	Syn
12612	ND5	A → G	—	—	G	Syn
12705	ND5	C → T	T	—	—	Syn
13101	ND5	A → C	—	C	—	Syn
13135	ND5	G → A	A	—	—	A → T
13708	ND5	G → A	—	—	A	A → T
13711	ND5	G → A	—	A	—	A → T
13966	ND5	A → G	G	—	—	T → A
14470	ND6	T → C	C	—	—	Syn
14766	CytB	C → T	T	—	T	I → T
15034	CytB	A → G	G	—	—	Syn
15310	CytB	T → C	C	—	—	Syn
15397	CytB	A → G	G	—	—	Syn
15452	CytB	C → A	—	—	A	L → I
15894	tRNAThr	G → A	A	—	—	Non coding
16069	HV1	C → T	—	—	T	Non coding
16126	HV1-7SDNA	T → C	C	—	—	Non coding
16145	HV1-7SDNA	G → A	—	—	A	Non coding
16189	HV1-7SDNA	T → A	A	—	—	Non coding

Continued overleaf

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Table 1 *Continued*

Position (nt)	Locus	Nucleotide change*	143B.TK⁻	Cybrid H	Cybrid J	Amino acid change
16222	HV1-7SDNA	C → T	—	—	T	Non coding
16261	HV1-7SDNA	C → T	—	—	T	Non coding
16278	HV1-7SDNA	C → T	T	—	—	Non coding
16288	HV1-7SDNA	T → C	—	C	—	Non coding
16302	HV1-7SDNA	A → G	—	G	—	Non coding
16362	HV1-7SDNA	T → C	—	C	—	Non coding
16519	7SDNA	T → C	C	—	—	Non coding

*Nucleotide changes are reported with respect to the revised Cambridge reference sequence (rCRS) (<www.mitomap.org>).
Cn, stretch of numerous (n) cytosines; Cins and Ccins, insertion of 1 (Cins) or 2 (Ccins) cytosines; Syn, synonymous mutation.

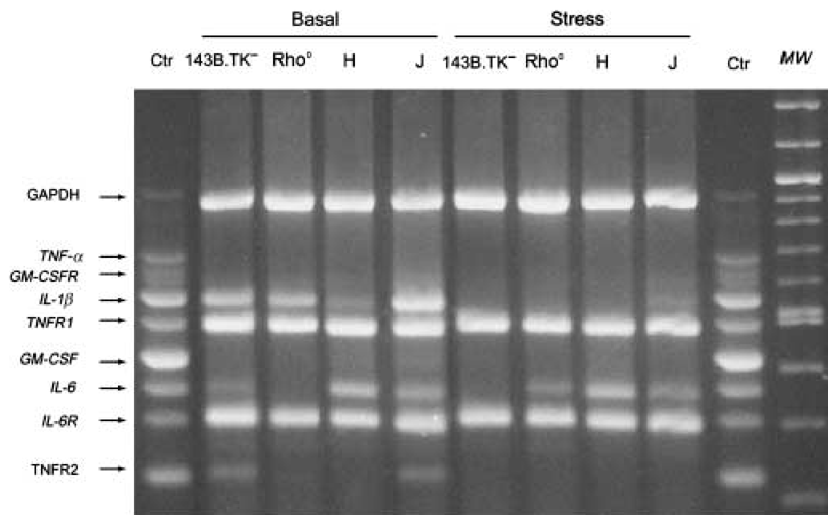

Figure 1 RT-PCR electrophoresis pattern of cytokines and cytokine receptors in 143B.TK⁻, Rho⁰, cybrid H and cybrid J cells at basal and stress conditions. Stress was carried out with 2-deoxy-D-ribose 20 mM for 48 h. Ctr: control cDNA supplied by the kit; *GAPDH*: Glyceraldehyde phosphate dehydrogenase; *TNF-α*: tumor necrosis factor-alpha; *GM-CSFR*: Granulocyte Macrophage-Colony Stimulating Factor receptor; *IL-1β*: interleukin 1-beta; *TNFR1*: tumor necrosis factor receptor 1; *GM-CSF*: Granulocyte Macrophage-Colony Stimulating Factor; *IL-6*: interleukin-6; *IL-6R*: interleukin-6 receptor; *TNFR2*: tumor necrosis factor receptor 2; MW: Molecular Weight 100 bp ladder.

Table 2 Densitometer analysis of gene expression of cytokines and cytokine receptors in 143B.TK⁻, Rho⁰, cybrid H and cybrid J cell lines at basal and stress conditions. Average values over three experiments (Standard Deviation in parentheses) are reported

	Basal conditions				Stress conditions			
	143B.TK⁻ M (SD)	Rho⁰ M (SD)	H M (SD)	J M (SD)	143B.TK⁻ M (SD)	Rho⁰ M (SD)	H M (SD)	J M (SD)
TNF-α	0.000 (0.000)	0.000 (0.000	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
GM-CSFR	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
IL-1β	0.859 (0.160)	0.562 (0.061)	0.312 (0.119)	1.321 (0.163)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
TNFR1	1.248 (0.345)	1.187 (0.207)	1.161 (0.285)	1.252 (0.379)	1.023 (0.245)	1.043 (0.231)	1.284 (0.180)	1.203 (0.185)
GM-CSF	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)
IL-6	0.291 (0.007)	0.000 (0.000)	0.803 (0.108)	0.503 (0.061)	0.000 (0.000)	0.389 (0.104)	0.951 (0.161)	0.327 (0.159)
IL-6R	1.544 (0.411)	1.422 (0.170)	1.506 (0.487)	2.080 (0.163)	1.262 (0.264)	1.238 (0.161)	1.753 (0.400)	1.285 (0.173)
TNFR2	0.202 (0.065)	0.000 (0.000)	0.000 (0.000)	0.144 (0.056)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)

same clone for each cell line: the data were consistent in the three experiments, according to genes, cells, basal/stress conditions. An example of the expression patterns is shown in Fig. 1, while Table 2 summarizes the average densitometric measures over the three experiments. By applying a pair-wise test (*t-Student*) for each gene we found significant differences between H and J lines at both basal (*IL1β*, $P = 0.001$; *TNFR2*, $P = 0.012$) and

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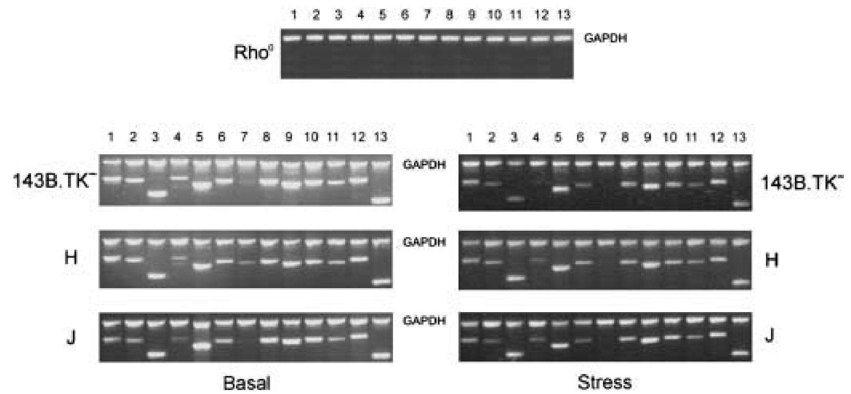

Figure 2 RT-PCR electrophoresis pattern of mitochondrial genes coding for peptides in 143B.TK−, Rho0, cybrid H and cybrid J cells at basal and stress conditions. Stress was carried out with 2-deoxy-D-ribose 20 mM for 48 h. 1: ND1 (NADH Dehydrogenase, subunit 1); 2: ND2; 3: ND3; 4: ND4; 5: ND4L; 6: ND5; 7: ND6; 8: COI (Cytochrome c oxidase I); 9: COII; 10: COIII; 11: CytB (Cytochrome b); 12: ATP6 (ATP Synthase 6); 13: ATP8 (ATP Synthase 8).

stress conditions (*IL-6*, $P = 0.009$). As we used a multiplex RT-PCR (see Experimental procedures) the possibility that PCR did not reach the stationary level for some of the checked genes could not be excluded. However, since the same conditions were applied in each experiment, the differences observed by comparing gene specific mRNAs between the two cybrid lines could be considered reliable.

To exclude the possibility that the observed differences were caused by genetic instability of the nuclear genes (as well as by alteration of the transcription patterns due to experimental manipulation in producing the cybrid lines) we replicated two times the experiment on a further clone from each of the H and J lines, and again obtained consisting results (Supplementary Table S1). Thus, from the whole set of experiments, we concluded that some cytokine and cytokine receptor genes are expressed according to mtDNA variability, at both basal and stress conditions (Table 2: compare H and J lines as it regards *IL1-β* and *TNFR2* at basal conditions; compare H and J lines as it regards *IL6* at stress conditions).

To verify whether the different patterns observed in the above experiments were due to a differential expression of mtDNA genes in cybrid cell lines we analyzed the expression profiles of the 13 genes encoding for the mitochondrial peptides. Results are shown in Fig. 2. We have not observed significant differences between the cell lines at either basal or stress conditions.

Discussion

The cross-talk between nucleus and mitochondria is fundamental in cell biology (Rose *et al.* 2002). Cybrid technology provides a powerful tool to explore if the common variability of the mitochondrial DNA plays a role in this cross-talk. However, cybrid technology requires some caution: first, the effect of a possible genomic instability of the nuclear DNA has to be taken into account; second, gene transcription patterns may be altered by the experimental manipulation (re-introduction of foreign mitochondria into cells depleted of their own mitochondria). For these reasons, before starting with the experiments on gene expression, we carefully checked the quality of native, rho^0, cybrid cell lines (see control experiments).

What is more, to escape artefacts due to technical manipulation, we repeated the experiment three and two times on two independent cybrid clones. As we obtained consistent results in all the cases, we are confident that the differences in gene expression patterns (Fig. 1) are reliable and not due to stochastic factors. In any case, as the H and J cybrid lines were equally manipulated, the different gene expression patterns observed between the cybrid lines should be due to the different mtDNA used to repopulate the rho^0 cell.

In the 143B.TK− cell line the gene expression profiles of cytokines and their receptors were consistent with those observed in other osteosarcoma cells (Bilbe *et al.* 1996). Also the change of the expression patterns observed in the rho^0 cells with respect to the native cells (Fig. 1, Table 2) was in line with recent data showing that the rho^0 cells modulate the expression of specific genes to compensate their status of oxidative stress (Miceli & Jazwinski 2005).

Let us now compare the cybrid lines. We see that, at basal conditions, the *IL-1β* gene is expressed about three times more in the J than in the H line. Likewise, the *TNFR2* gene is expressed in the J but not in the H line. Therefore, the J cell line expresses both the genes more than the H line. Interestingly, the differences observed between the cybrid lines at basal conditions disappear under stress conditions, because neither the H nor the J line expresses *IL-1β* or *TNFR2*. This finding is in agreement with literature data reporting a repression of gene expression by oxidative stress (Morel & Barouki 1999).

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Now, let us look at the expression profile of *IL-6*. For this gene the H and J cell lines do not show different expression patterns at basal conditions; but, under stress conditions, the H line activates *IL-6* transcription more than the J line ($P = 0.009$). Therefore, the connection between mtDNA variability and *IL6* expression pattern seems to be activated under stress conditions only. This hypothesis agrees with the *IL-6* expression patterns shown by the native and the rho^0 cells, which clearly show that the stress condition modifies the expression profile of this gene. Indeed, at basal conditions, *IL-6* is expressed in the native cell while not in the rho^0 cell; *vice versa*, under stress conditions, the gene transcription is suppressed in the native cell, but activated in the rho^0 cell. The comparison of *IL-6* expression between native and rho^0 cells provides a further evidence for the existence in human cells of a stress-dependent *retrograde response* (Miceli & Jazwinski 2005).

Of course, it would be incautious to infer from this data a direct effect of mtDNA diversity on complex phenotypes at organism level. In any case, taking into account the pleiotropic effects that *IL-1β*, *TNFR2* and *IL-6* genes exert on a myriad of cell pathways crucial in age-related common diseases, we believe that the findings described above deserve further functional studies.

In conclusion, the analysis of expression of cytokine and cytokine receptor genes at basal and stress conditions revealed mitochondrial-specific effects for the expression of *IL-1β*, *TNFR2* and *IL-6* genes. Although the findings obtained by means of the cybrid technology cannot be immediately transferred to the situation at organism level, our results provide a first experimental evidence that mtDNA common variability could modulate nuclear gene expression through still unknown mechanisms.

Experimental procedures

Cell lines and culture conditions

143B.TK$^-$ osteosarcoma cells were kindly provided by G. Attardi of the California Institute of Technology, Pasadena, CA, USA. The cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) (Gibco) containing 4.5 g/L glucose and 110 μg/mL pyruvate, supplemented with 10% FBS (Fetal Bovine Serum) (Gibco), 100 μg/mL 5-bromo-2′-deoxy uridine (Sigma) and 50 μg/mL gentamicin (Gibco).

The rho^0 cell line was obtained by culturing 143B.TK$^-$ in the routine growth medium containing 50 ng/mL ethidium bromide (0.22 μm-filtered) with regular replenishment of medium for about 2 months (King & Attardi 1996). After ethidium bromide treatment, the cells were maintained in DMEM supplemented with 10% FBS, 100 μg/mL 5-bromo-2′-deoxy uridine and 50 μg/mL uridine (Sigma). At this stage the cells were plated at low density and individual clones were isolated.

Both 143B.TK$^-$ and rho^0 cells were cultured in a water-humidified incubator at 37 °C in 5% CO_2/95% air.

Experiments for quality control of mtDNA depletion

The complete depletion of mtDNA in rho^0 cells was assayed by both auxotrophic test and PCR amplification of the D-loop mtDNA.

- For auxoptrophic test 6×10^8 rho^0 cells were grown in uridine-free DMEM supplemented with 10% dialyzed FBS (Gibco) and 100 μg/mL 5-bromo-2′-deoxy uridine. Medium was changed at regular intervals (2 days). Cell survival was checked every 48 h.
- D-loop PCR amplification was performed with primers encompassing the mtDNA region comprised between nucleotides 15996 and 16401 (primer forward 5′ CTCCACCATTAG-CACCCAAAGC 3′; primer reverse 5′ TGATTTCACGGAGGATGGTG 3′).

Production of transmitochondrial cybrids

Transmitochondrial cell lines were obtained by the method of Chomyn (1996). Platelets were isolated by differential centrifugation from blood of two 27-years-old donors, and the pellet was utilized to generate cybrids. Platelet donors gave written informed consent, according to the guidelines of the Ethical Committee of the University of Calabria.

rho^0 cells were collected by low-speed centrifugation, re-suspended in DMEM and counted. 10^6 rho^0 cells were mixed with an equal number of platelets and the culture medium was eliminated by centrifugation. Cells were re-suspended for 1 min in 0.1 mL of 42% polyethyleneglycol 1500 (Sigma). The fusion mixture was cultured in standard DMEM for 48 h and then in selective medium uridine-free DMEM supplemented with 10% FBS and 100 μg/mL 5-bromo-2′-deoxy uridine. After 2–3 weeks in the selection medium, several distinct colonies emerged: 20 colonies were isolated by trypsinization in cloning rings and propagated.

To ensure complete stabilization of the mtDNA amount, the functional assessment of selected clones was carried out only after at least 3 months of clone cycling.

MtDNA analyses

For both parental (143B.TK$^-$) and cybrid lines, confluent 100-cm^2 plates were trypsinized and total DNA was isolated by digestion with 1.5 mg/mL proteinase K in 10 mM Tris-HCl, pH 7.4/ 10 mM NaCl/25 mM EDTA/1% SDS at 37 °C for 4 h. Then the DNA was extracted with phenol/chloroform, precipitated by ethanol, dried and re-suspended in TE (Tris/EDTA) buffer.

Haplogroup typing was used to verify whether the mtDNA of the cybrid lines was that of the donor platelets. By RFLP analyses of the coding region (Torroni *et al.* 1996) and D-loop sequencing (15996–16401 PCR fragment) the mtDNA of the native cell

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(143B.TK$^-$) was classified in the X haplogroup, while those of the cybrid lines in the H and J haplogroups. Accordingly, the two cybrid lines were named H and J lines.

To exactly define the whole variability of the mtDNA molecules used in our experiments, sequencing of the entire mtDNA molecule in the three cell lines was performed by the C.R.I.B.I.-BMR, DNA SEQUENCING SERVICE, University of Padua (Italy). The sequences were then aligned by *Genalys 2.0* to find variations with respect to the Cambridge Reference Sequence (MITOMAP: A human mitochondrial genome database; <www.mitomap.org>).

Proliferation assay

143B.TK$^-$, rho^0, cybrid H and cybrid J cells were seeded in 6-well plates (1×10^5/well) in regular growth medium. Adherent cells were trypsinized after 24, 48 and 72 h culture and counted on a hemocytometer with an inverted light microscope using a 10× magnification.

Quantification of mtDNA

The number of mtDNA copies per cell was estimated by a Quantitative Competitive-PCR (QC-PCR) method (Pinti *et al.* 1999; Salvioli *et al.* 2003).

Mitochondrial membrane potential (MMP) assay

Mitochondrial membrane potential (MMP) was assessed by double staining with MitoTracker Green™ (MTG, specific for mitochondrial mass) and TetraMethyl Rhodamine Methylesther (TMRM, specific for MMP) (Molecular Probes, Eugene, OR, USA).

143B.TK$^-$, rho^0 and cybrid cells were collected during the log phase of growth by trypsinization, counted and reseeded in fresh complete medium in 6-well plates (2×10^5 cells/well). After 24 h, the cells were stained with MTG 100 nM and TMRM 150 nM for 20 min at 37 °C, then collected and analyzed at flow cytometer. Parameters for acquisition were set up as follows: Fl1 PMT: 389; Fl2 PMT: 461; Fl1-Fl2 compensation: 0.8%; Fl2-Fl1 compensation: 35.1%. Cells with low MMP were those having low TMRM fluorescence. Cytofluorimetric analyses were performed using a FACScalibur cytometer (BD, San José, CA, USA) equipped with an Argon ion laser tuned at 488 nm. In all analyses, a minimum of 1×10^4 cells per sample were acquired in list mode and analyzed with Cell Quest software.

Oxidative stress treatment

Oxidative stress was induced by treating cells with 2-deoxy-D-ribose (dRib). dRib, a higly reducing sugar, causes depletion of the intracellular reduced glutathione (GSH) and therefore increase of the cell level of ROS, which in turn induce cell death (Barbieri *et al.* 1994; Kletsas *et al.* 1998).

Two $\times 10^5$ 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 with 2-deoxy-D-ribose 20 mM (Sigma). The cells were incubated to 37 °C for 24, 48 and 72 h. Untreated cells were also analyzed as control.

Viability/proliferation 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). Viable and-non-viable cells were then counted on a hemocytometer with an inverted light microscope using a 20× magnification.

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 12 000 ***g*** centrifugation for 20 min, an equal volume of phenol/chloroform was added to the supernatant. Then, after a new 12 000 ***g*** centrifugation for 5 min, an equal volume of chloroform was added to supernatant and centrifuged again. 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 on to a 1.5% agarose gel, electrophoresed in TAE (Tris/Acetate/EDTA) buffer and stained with ethidium bromide.

RT-PCR of cytokine and cytokine receptor genes

Total RNA was extracted from control and treated 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.

The RT-PCR (Reverse Transcriptase-PCR) reactions were carried out by using the RETROscript Kit provided by Ambion.

An RT mix including 10 µg of total RNA and 100 pmoles of random decamers was preheated at 70 °C for 5 min. The reaction was carried out in a 50 µL final volume containing 1× RT Buffer, 0.4 mM of each dNTP, 25 U of RNase inhibitor, 275 U of MMLV (moloney murine leukemia virus) reverse transcriptase. The mix was incubated at 37 °C for 1 h and, successively, at 95 °C for 10 min to inactivate the reverse transcriptase.

For the cytokine analysis, we used MPCR (Multiplex PCR) Amplification kit provided by Maxim Bio. This kit has been designed to direct the simultaneous amplification of specific human inflammatory cytokine genes.

The PCR mixture in 25 µL volume contained 2.5 µL of cDNA, 1× MPCR Buffer, 1× MPCR primers specific for cytokine

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and cytokine receptor genes and 1.5 U *Taq* DNA polymerase (Eppendorf). As internal control, the MPCR primers mixture contained primers specific for human *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene. Two control reactions were carried out: a positive control containing as template the cDNA supplied by the kit and a negative control that did not contain cDNA. After an initial denaturation step at 96 °C for 1 min, the PCR was cycled twice at 96 °C for 1 min and 67 °C for 2 min, followed by 35 cycles of 96 °C for 1 min and 67 °C for 4 min. The final step was incubation at 70 °C for 10 min. Then, PCR products were analyzed on 2.2% agarose gel containing ethidium bromide 0.5 mg/mL. Fluorescence intensity of each band was calculated using a densitometer analysis (Kodak Electrophoresis Documentation and Analysis System 290, EDAS 290) and then normalized with respect to the *GAPDH* band intensity.

RT-PCR of mitochondrial genes

The RT reactions were carried out as described above by using 2 μg of total RNA.

The PCRs were performed with 1 μL of cDNA in a total volume of 25 μL containing 1× amplification Buffer, 200 μM of each dNTP, 0.8 μM of specific primers (Supplementary Table S2), 1.25 U of EuroTaq DNA polymerase. Primers for GADPH gene were used as internal control. Twenty-five cycles of PCR were carried out at 92 °C for 30 s, 58 °C for 30 s and 72 °C for 1 minute. The products were loaded on to a 2% agarose gel, electrophoresed in TAE buffer and visualized with ethidium bromide staining. Fluorescence intensity of each product was calculated by Kodak densitometer and then normalized respect to *GAPDH* band intensity.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1 Growth curves of 143B.TK$^-$, rho^0, cybrid H and cybrid J cell lines.

Figure S2 Electrophoresis pattern of the Quantitative Competitive-PCR (QC-PCR) for the quantification of the mtDNA amount.

Figure S3 Mitochondrial membrane potential (MMP).

Figure S4 Viability/proliferation assay of 143B.TK$^-$, rho^0, cybrid H and cybrid J cell lines.

Figure S5 Internucleosomal DNA fragmentation electrophoresis pattern.

Table S1 Densitometer analysis of gene expression of cytokines and cytokine receptors in cybrid H* and cybrid J* cell lines at basal and stress conditions.

Table S2 Primers utilized in RT-PCR experiments of mitochondrial genes.

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