RESEARCH NOTE

Transcriptional changes of mitochondrial genes in irradiated cells proficient or deficient in *p53*

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Introduction

The cellular response to ionizing radiation (IR) damage is complex and relies on the simultaneous activation of a number of signalling networks. Recent studies have identified several oxidative stress-induced signalling pathways: ROS metabolism and antioxidant defenses, p53 pathway signalling, nitric oxide (NO) signalling pathway, hypoxia signalling, transforming growth factor (TGF)-beta, bone morphogenetic protein (BMP) signalling, tumour necrosis factor (TNF) ligand-receptor signalling, and mitochondrial function (Sone *et al.* 2010). The IR produces reactive oxygen species (ROS) and induces cell death through the intermediary of mitochondria. ROS are also generated in the mitochondria through normal oxidative metabolism. ROS are responsible for radiation-induced biological effects. Mitochondria are responsible for many cellular processes, including energy production by oxidative phosphorylation (OXPHOS), calcium homeostasis and apoptosis (Borutaite 2010). The mitochondrial genes are transcribed and translated to make several proteins. The mitochondrial genome encodes 13 proteins necessary for the OXPHOS process, involved in adenosine triphosphate (ATP) generation. The remaining proteins needed for the OXPHOS process are encoded by the nuclear genome (Shutt and Shadel 2010). It is shown that radiation induces more damage to mitochondrial DNA as compared to nuclear DNA. The process of transcription continues in the blood cell mitochondria of 10-Gy irradiated mice, whereas the transcription of damaged nuclear DNA is arrested (Evdokimovskii *et al.* 2007). Mitochondria are equipped with major repair pathways to maintain DNA integrity. Mitochondrial DNA has the ability to replicate independently of the nuclear DNA and is capable of repairing DNA damage (Liu and Demple 2010). Due to the increased oxidative stress in the mitochondria, DNA damage is more, and degradation of damaged DNA is tolerated since there are multiple copies (Kang and Hamasaki 2002).

To elucidate the underlying mechanisms involved in the cellular response to radiation-induced oxidative stress, analyses of gene expression are crucially important. The response of cells to ionizing radiation has long been known to result in alterations of nuclear gene expression. Very few studies have investigated the response of mitochondrial genes after IR exposure (Kulkarni *et al.* 2010). Our goal was to examine changes in the mitochondrial gene expression in cells that are proficient or deficient in *p53*. We investigated if *p53* has an impact on the modulation of mitochondrial gene expression in irradiated cells. The p53 protein is a key regulator of gene expression and is essential to maintain genomic stability (Harvey *et al.* 1993). The *p53* is implicated in DNA damage-induced G_1 cell cycle arrest (Kuerbitz *et al.* 1992), apoptosis (Lowe *et al.* 1993), and DNA repair (Bakalkin *et al.* 1994). We took advantage of two well characterized lymphoblastoid cell lines, TK6 and WTK1, that were derived from the same progenitor cell line WIL2, isolated from a single male donor. TK6 exhibits the wildtype *p53* allele while WTK1 is a *p53* negative mutant (Amundson *et al.* 1993). WTK1 has been shown to acquire up to 10 times more mutations at the thymidine kinase (*tk*) locus than TK6 after exposure to IR (Xia *et al.* 1994b). WTK1 cells are more efficient in recombinational repair and have higher resistance to X-irradiation-induced killing than TK6 cells (Xia *et al.* 1994a). Alterations in the expression of many nuclear genes have been suggested to contribute to the differences in the response of these cells to IR (Tsai *et al.* 2006). The changes in the expression of mitochondrial genes in TK6 and WTK1 have not been reported before. We report that mitochondrial genes are modulated in these cells after exposure to IR and these changes are correlated with the *p53* status.

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Keywords. mitochondrial gene expression; TK6 cells; radiation-induced effects.

Materials and methods

Cell culture and maintenance

The human lymphoblast cell line TK6 was purchased from American Type Tissue Collection (ATCC) (Manassas, USA) and WTK1 cell line was obtained from Dr Howard Liber, Colorado State University, Fort Collins, USA. These cells were grown exponentially in suspension in a T75 flask using RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, New York, USA), 100 mg/mL streptomycin and 100 U/mL penicillin. The cell cultures were maintained at a density of 2–5 $\times$ 10^5 cells/mL in a 37°C incubator with 5% CO_2 and 100% humidity.

Ionizing radiation treatment

Irradiation of 3 $\times$ 10^6 cells was performed with a RAD Source 2000 X-ray Biological irradiator (Alpharetta, GA, USA) available at the University of Vermont. Exponentially growing cells were seeded at a density of 3 $\times$ 10^6 per mL and 2 Gy dose at 1.7 Gy per minute was administered at room temperature. The control sample was treated in the same way, except for irradiation. The treated cells were incubated at 37°C and harvested at 0, 4, 8, 12, and 24 h for isolating RNA. The experiment was repeated in triplicate.

RNA isolation

The cells for RNA isolation were counted with a hemocytometer and approximately 5 $\times$ 10^6 cells were pelleted by centrifugation at 1500 rpm for 5 min, and washed with 1 mL Dulbecco's phosphate-buffered saline (PBS) without $MgCl_2$ and $CaCl_2$ (Invitrogen, Carlsbad, USA). Total RNA was isolated with RNeasy™ kit (Qiagen, Valencia, USA). The quantity and quality of the total RNA was measured on the NanoDrop 2000 (Thermo Scientific, West Palm Beach, USA) and by analysis on 2% agarose gels stained with ethidium bromide on BioSpectrum® Imaging System (UVP, Upland, USA).

Mitochondrial gene targets

Assays-on-demand for *MT-ND1*, *MT-ND2*, *MT-ND3*, *MT-ND4*, *MT-ND4L*, *MT-ND5*, *MT-ND6*, *MT-CO1*, *MT-CO2*, *MT-CO3*, *MT-CYB*, *MT-ATP6*, and *MT-ATP8* (table 1) were purchased from Applied Biosystems (Foster City, CA, USA). Standard TaqMan assays were designed using Primer-Express software (Applied Biosystems, Carlsbad, USA). RNA samples for gene expression analysis were normalized based on the TaqMan Gene Expression Assays for human endogenous hypoxanthine phosphoribosyltransferase (*HPRT*) gene.

Reverse transcription and cDNA synthesis reactions

Total RNA was treated with DNAase prior to reverse transcription in order to avoid genomic DNA contamination. The cDNA was generated from total RNA with random hexamer primers using cDNA synthesis kit from Applied Biosystems following recommendations of the manufacturer. Reverse transcriptase reactions contained RNA samples, 50 nM hexamer primer, 1$\times$ RT buffer, 0.25 mM each of dNTPs, 3.33 U/μL MultiScribe™ reverse transcriptase and 0.25 U/μL RNase inhibitor. The 15 μL reactions were incubated in Techne TC-312 thermocycler (Burlington, NJ, USA) for 30 min. at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. All reverse transcriptase reactions, including no template controls and RT minus controls, were run in duplicate.

Quantitative real-time polymerase chain reaction (QPCR) and data analysis

QPCR was performed on an Applied Biosystems 7900HT Sequence Detection System by using a standard TaqMan PCR kit protocol. The 10 μL PCR contained 0.67 μL RT product, 1 $\times$ TaqMan Universal PCR Master mix, 0.2 μM TaqMan probe, 1.5 μM forward primer and 0.7 μM reverse primer. The reactions were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s

Table 1. Mitochondrial genes analysed in TK6 and WTK1 cells.

Gene symbol	Gene name	Assay ID	Amplicon length
MT-ND1	NADH dehydrogenase 1	Hs02596873_s1	143
MT-ND2	NADH dehydrogenase 2	Hs02596874_g1	148
MT-ND3	NADH dehydrogenase 3	Hs02596875_s1	150
MT-ND4	NADH dehydrogenase 4	Hs02596876_g1	150
MT-ND4L	NADH dehydrogenase 4L	Hs02596877_g1	130
MT-ND5	NADH dehydrogenase 5	Hs02596878_g1	142
MT-ND6	NADH dehydrogenase 6	Hs02596879_g1	151
MT-CO1	Cytochrome c oxidase I	Hs02596864_g1	94
MT-CO2	Cytochrome c oxidase II	Hs02596865_g1	135
MT-CO3	Cytochrome c oxidase III	Hs02596866_g1	143
MT-CYB	Cytochrome b	Hs02596867_s1	151
MT-ATP6	ATP synthase 6	Hs02596862_g1	150
MT-ATP8	ATP synthase 8	Hs02596863_g1	120

and 60°C for 1 min. The relative expression values of cycle thresholds were calculated using the comparative delta delta cycle threshold; $\Delta\Delta C_t$ method (Livak and Schmittgen 2001) by normalization to the endogenous control *HPRT* and to the control nonirradiated sample. There was no significant difference in the quantity of total RNA from irradiated versus unirradiated, and between the two cell lines. No significant differences in the *HPRT* internal control were seen among the two cell lines at different postirradiation time points. The threshold cycle (C_t) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Statistical significance was determined using analysis of variance (ANOVA). The statistics and data analyses were performed with ABI prism (Applied Biosystems, Carlsbad, USA) and GraphPad Prism 5 software (GraphPad Software, Ja Jolla, USA), both licensed to the University of Vermont. C_t values of the 13 mitochondrial genes in irradiated and bystander cells were statistically evaluated using a one-way *t*-test ($P < 0.05$). The experiments were repeated thrice and the expression differences between the irradiated and bystander cells were statistically determined.

Results

We were interested in examining the response of mitochondrial genes in a TK6 and related WTK1 cell lines. After irradiation with 2 Gy dose of X-rays, the cells were sampled at 0 h, 4 h, 8 h, 12 h and 24 h time points for investigating the modulation of mitochondrial genes. The relative gene expression was determined by the QPCR method. The results obtained with the analysis of mitochondrial NADH dehydrogenase genes *MT-ND1*, *MT-ND2*, *MT-ND3*, *MT-ND4*, *MT-ND4L*, *MT-ND5*, and *MT-ND6* expressions are shown in figure 1. Most of these mitochondrial genes were upregulated in irradiated TK6 cells. The *MT-ND5* was induced until 8 h in irradiated TK6 cells and then its expression level declined (figure 1f).

In contrast, most of the mitochondrial NADH dehydrogenases were not induced in irradiated WTK1 cells. The *MT-ND4* was induced in irradiated WTK1 cells (figure 1d). The overall expression of *MT-ND4* was higher in WTK1 cells as compared to the TK6 cells.

The differences in the expression of *MT-ND1* ($P = 0.01$), *MT-ND2* ($P = 0.0006$), *MT-ND3* ($P = 0.001$), *MT-ND4* ($P = 0.003$), *MT-ND4L* ($P = 0.002$), and *MT-ND6* ($P = 0.006$) were statistically significant between the irradiated TK6 and WTK1 cells.

The modulation of cytochrome oxidases *MT-CO1*, *MT-CO2*, *MT-CO3*, *MT-CYB*, and ATP synthases *MT-ATP6*, *MT-ATP8* in irradiated TK6 and WTK1 cells is shown in figure 2. In irradiated TK6 cells *MT-CO1*, *MT-CO2*, *MT-CYB* and *MT-ATP6* were upregulated following IR exposure (figure 2, a, b, d&e respectively). The maximum level of *MT-ATP6* induction was seen at 8 h after irradiation in TK6 cells followed by a decline in its expression that continued until 24 h time point (figure 2e). The expression of *MT-ATP8* was first repressed at 8 h time point and this mRNA was later induced at 12 h and 24 h time points. The expression pattern of *MT-CO3* and *MT-ATP8* in TK6 cells was similar (figure 2, c&f). The mRNAs of these genes were sharply declined at 8 h after radiation exposure followed by an induction at 12 h time point.

The expression of *MT-CO1*, *MT-CO3*, *MT-CYB* and *MT-ATP8* in WTK1 cells remained repressed throughout the 24 h time period after exposure to IR (figure 2). For *MT-CYB* the lowest level of gene repression was at 24 h time point (figure 2d). The *MT-CO2* gene was induced in WTK1 cells until 12 h postirradiation and then its expression declined (figure 2b).

The differences in the expression of *MT-CO1* ($P = 0.0001$), *MT-CO2* ($P = 0.04$), *MT-CYB* ($P = 0.02$), and *MT-ATP6* ($P = 0.0003$) were statistically significant between the irradiated TK6 and WTK1 cells.

Discussion

Alteration of p53 affects cellular responses to DNA damage after treatment with IR. Various cells exhibit a wide range of sensitivities to radiation-induced killing. The p53 negative mutant cell line WTK1 has higher resistance to the toxicity of IR than wildtype TK6 (Xia *et al.* 1994b). Apart from the involvement of p53, the mechanism(s) behind the differential radiosensitivity of TK6 and WTK1 remains unknown. We postulated that a differential mitochondrial gene expression could be responsible for responses to IR in TK6 and WTK1 cells. We previously reported the ability of IR to modulate mitochondrial genes in human cells (Chaudhry *et al.* 2003). In the current study, we asked if functional p53 controls the mitochondrial gene expression. We found that most of mitochondrial NADH dehydrogenases genes were upregulated in irradiated p53 wildtype TK6 cells. In contrast, majority of mitochondrial NADH dehydrogenases were not induced in irradiated p53 mutant WTK1 cells. *MT-ND4* was the only mitochondrial NADH dehydrogenase that was induced in both irradiated TK6 and WTK1 cells (figure 1). An opposite gene expression profile in p53 proficient and deficient cells indicates that p53 is responsible for controlling the mitochondrial NADH dehydrogenase expression. Other studies have shown that the *MT-ND1* and *MT-ND4* subunits were overexpressed in H_2O_2 conditioned cells resistant to gamma-irradiation. It was suggested that the alteration in the expression of NADH dehydrogenase could be involved in the recovery of gamma-irradiated cells through inhibition of apoptosis (Ghosh and Girigoswami 2008). The expression of NADH dehydrogenase *MT-ND1*, *MT-ND4* was decreased in gamma-irradiated cells (Ghosh and Girigoswami 2008). Other studies have reported that the levels of *MT-ND2* and *MT-ND4* transcripts were decreased after irradiation in mouse tissues (Evdokimovsky *et al.* 2011). The NADH dehydrogenase

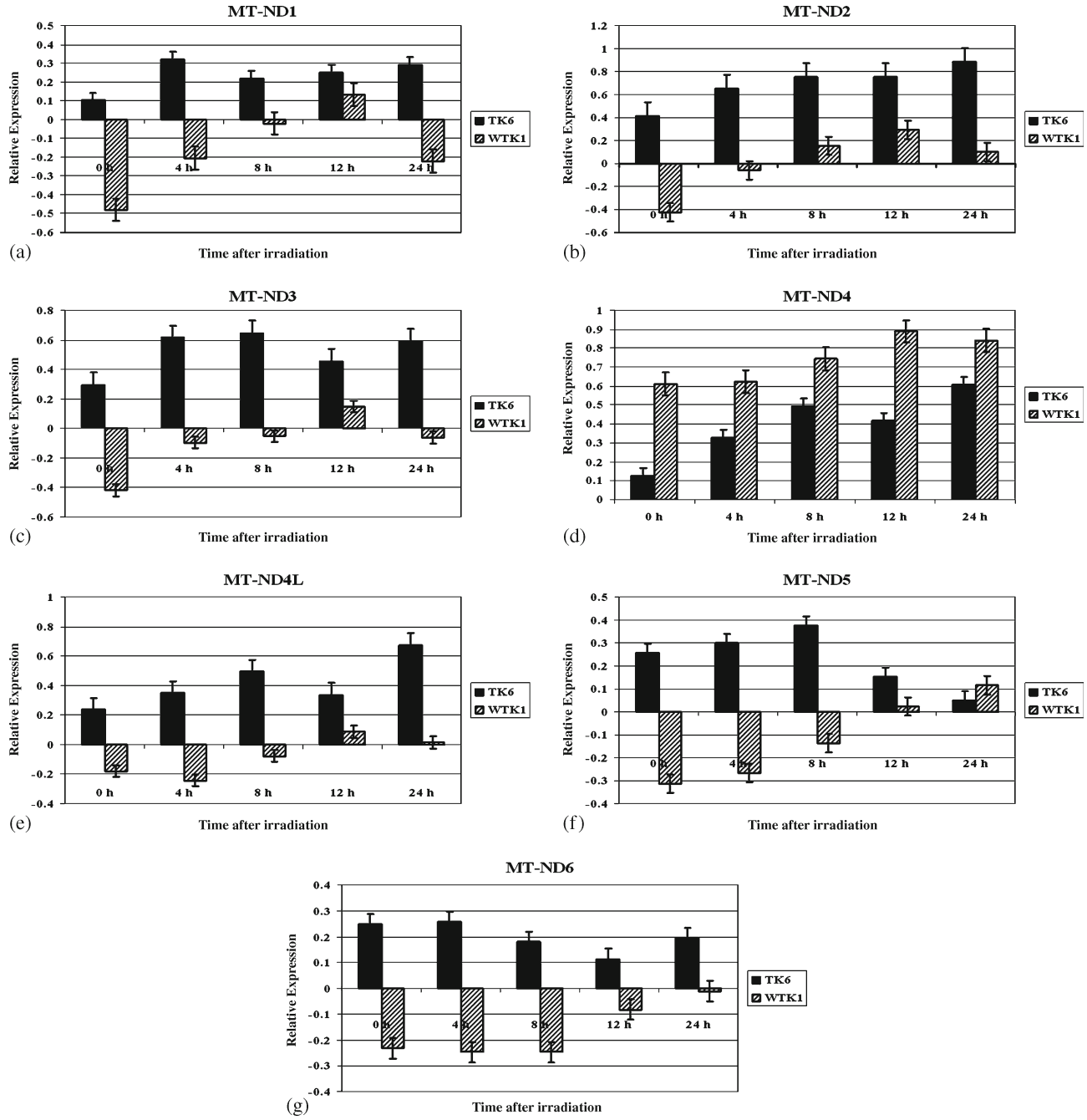

Figure 1. Modulation of various mitochondrial NADH dehydrogenases in irradiated TK6 and WTK1 cells. The relative expression, shown as $\log_2$ values, was computed at 0 h, 4 h, 8 h, 12 h, and 24 h time points. a, *MT-ND1*; b, *MT-ND2*; c, *MT-ND3*; d, *MT-ND4*; e, *MT-ND4L*; f, *MT-ND5*; g, *MT-ND6*. (■) TK6 cells, ▨ WTK1 cells. The error bars indicate the standard error of the mean (SEM) for three independent experiments.

MT-ND4 has shown to be increased after irradiation, with elevated expression persisting for at least 24 h (Gong *et al.* 1998). None of these studies examined the role of p53 influencing the mitochondrial NADH dehydrogenases. Mitochondria could be a target of signalling by IR since it is known to be at the site of cellular oxidative stress. The differences in the modulation of mitochondrial NADH dehydrogenases in p53 positive or negative cells might have a role in defining the responses of these cells after IR treatment. Changes in the mitochondrial gene expression have been correlated with cellular radiosensitivity (Kulkarni *et al.* 2010). Our data suggests that p53 plays a role in this process. The higher resistance to the toxicity of IR seen in WTK1 cells could be due to the inability of these cells to induce mitochondrial NADH dehydrogenase due to lack of functional p53.

It could be that the differences in the expression of mitochondrial genes observed in the present study were due to alterations in the copy number of mtDNA after irradiation.

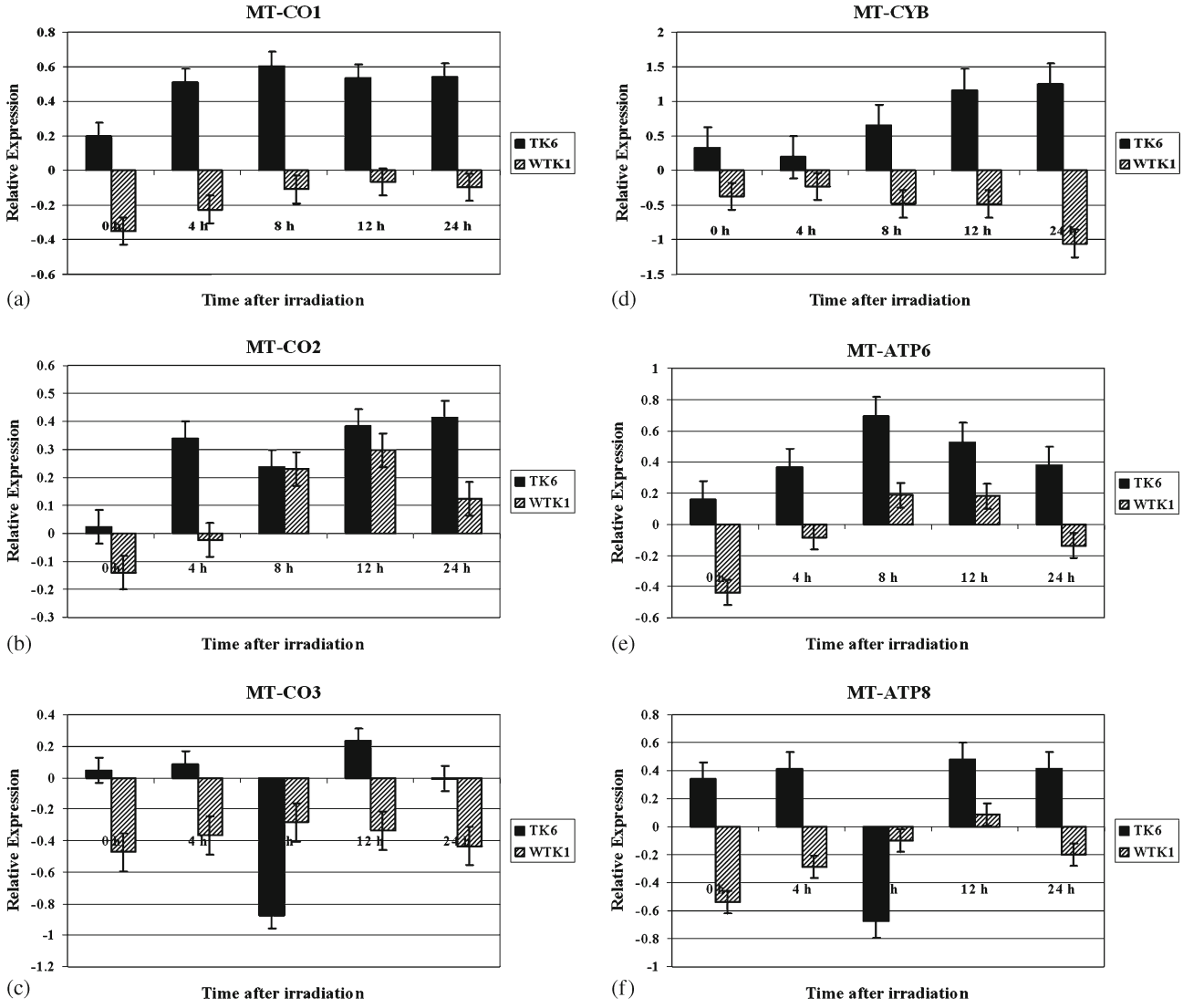

Figure 2. Expression analysis of mitochondrial cytochrome c oxidase subunits, cytochrome b, and ATP synthases genes in 2-Gy irradiated TK6 and WTK1 cells. The data are plotted as $\log_2$ values and indicate relative expression measured at 0 h, 4 h, 8 h, 12 h, and 24 h time points after radiation treatment. a, *MT-CO1*; b, *MT-CO2*; c, *MT-CO3*; d, *MT-CYB*; e, *MT-ATP6*; f, *MT-ATP8*. (■) TK6 cells, ▨ WTK1 cells. The error bars indicate the standard error of the mean (SEM) for three independent experiments.

We examined mtDNA copy numbers in irradiated cells by RT-PCR analysis of the hypervariable region 2 (HVR2) in the mitochondrial D-loop and did not observe any significant differences as compared to unirradiated cells (data not shown). Other studies have reported that after irradiation mtDNA copy number is increased in mouse tissues (Evdokimovsky *et al.* 2011). These differences could be due to a high dose of 10 Gy that was used to irradiate animals or the radiation-induced effects in animal tissues versus human cells grown in culture.

Radiation-induced changes in gene expression and enzyme activity associated with the mitochondrial oxidative phosphorylation process have been reported (O'dowd *et al.* 2009). In the current investigation we observed the upregulation of cytochrome oxidases *MT-CO1*, *MT-CO2*, and *MT-CYB* in irradiated TK6 cells (figure 2). In contrast, the expression of *MT-CO1*, *MT-CO3*, and *MT-CYB* remained repressed in WTK1 cells throughout the 24 h time period after exposure to IR, but *MT-CO2* gene was induced in WTK1 cells (figure 2). Cytochrome oxidases, are enzyme of the mitochondrial electron transport chain that are encoded in the mitochondrial genome. Cytochrome c is a pivotal protein that resides in mitochondria as component of mitochondria respiration and apoptosis initiator. It has been shown that cytochrome oxidase was overexpressed in IR resistant cells (Chaudhuri *et al.* 2003). Other studies have shown that *MT-CO1* and *MT-CO2* were induced after irradiation (Gong *et al.* 1998).

The ATP synthases *MT-ATP6* and *MT-ATP8* were induced in TK6 cells following IR exposure and the expression of *MT-ATP8* in irradiated WTK1 cells was repressed (figure 2). The *MT-ATP6* is a component of Complex V of ATP synthase. ATP levels and the mitochondrial membrane

potential are increased after irradiation. The difference in the modulation of ATP synthases in TK6 and WTK1 cells suggest that the specificity of changes in mitochondrial gene expression after irradiation is dependent on functional p53.

The proteins encoded by mitochondrial mRNAs characterized in this study are part of the mitochondrial respiratory chain, which produces ATP through the process of oxidative phosphorylation. Our observations indicate that the mitochondrial genes regulating energy generation show similar responses in p53 proficient cells. In contrast the mitochondrial gene expression responses are different in p53 deficient cells indicating dissimilar functions in the IR- induced stress response operating in these cells. The role of p53 in regulating cell cycle and apoptosis is well known but the involvement of p53 in other cellular processes such as metabolism is less clear. Recent studies have indicated that p53 affects the mode of energy production (Matoba *et al.* 2006). p53 has been shown to regulate mitochondrial respiration with secondary changes in glycolysis (Ma *et al.* 2007). Our data indicates the involvement of altered expression levels of mitochondrial genes in IR-induced stress response in a p53 dependent manner.

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