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# The DNA-Damage Response to Ionizing Radiation in Human Lymphocytes

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## 1. Introduction

The human genome is constantly subjected to DNA damage derived from endogenous and exogenous sources. Normal cellular metabolism can give rise to DNA damage through free radicals production and replication errors, whereas environmental agents, such as ultraviolet (UV) and ionizing radiation (IR), induce specific types of lesions. DNA damage can ultimately lead to genomic instability and carcinogenesis if not properly addressed, thus an elaborate network of proteins has evolved in cells to maintain genome integrity through a pathway termed the DNA-damage response (DDR). DDR allows DNA damage detection, signal propagation and transduction to a multitude of effector proteins, which promote cell survival and activate cell cycle arrest to allow DNA repair. When cells are unable to properly repair DNA, apoptosis or senescence pathways may be triggered, thus eliminating the possibility of passing on damaged or unrepaired genetic material to its progeny. The ultimate goal of DDR is to protect the integrity of genetic information and its faithful transmission, either to DNA by replication or to mRNA by transcription. Therefore, dysregulation of DDR pathway can contribute to carcinogenesis and developmental defects. Ionizing radiation represents a mutagen agent to which human population is exposed due to environmental, professional or accidental reasons. The biological effects of IR depend on the quality and the dose of radiation and on the cell type. Linear energy transfer (LET) represents the energy lost per unit distance as an ionizing particle travels through a material, and it is used to quantify the effects of IR on biological specimens. High-LET radiation (i.e. alpha-particles, neutrons, protons) are densely IR since they lose the energy throughout a small distance, causing dense ionization along their track with high localized multiple DNA damage. Low-LET radiation, such as X and  $\gamma$ -rays, are sparsely IR since they produce ionizations sparsely along their track and, hence, almost homogeneously within a cell. The biological effect of high-LET radiations are in general much higher than those of low-LET radiations with the same energy. This is because high-LET radiation deposits most of its energy within the volume of one cell and the damage to DNA is therefore larger (Anderson et al., 2002; Brenner & Ward, 1992; Prise et al., 2001). Radiation is potentially harmful to humans, because the ionization it produces can significantly alter the structure of molecules within a living cell. The exposure to ionizing radiation elicits a complex cell response to overcome the dangerous effects of DNA-radiation interaction, such as reactive oxygen species (ROS) production, base oxidation and DNA breaks formation (i.e. single-

strand breaks, SSBs and double-strand breaks, DSBs ). In particular, DSBs represent the most severe form of damage, since an inefficient or inaccurate repair may lead to cell death or genomic instability (Wyman & Kanaar, 2006). The presence of DSBs leads to a cascade of post-translational modifications of a wide variety of proteins, including phosphorylation, ubiquitylation, sumoylation, poly(ADP-ribosylation), acetylation and methylation (Huen & Chen, 2010). The early DSB response utilizes phosphorylation-dependent protein-protein interactions to coordinate DNA damage recognition and signal amplification. Following DSB formation the histone H2AX, a histone H2A variant that comprises 10-15% of total cellular H2A in higher eukaryotes, is rapidly phosphorylated on its serine residues 139 ( $\gamma$ -H2AX) (Rogakou et al., 1998) by members of the phosphatidylinositol-3-OH kinase (PI(3)K)-like family, such as ataxia telangiectasia mutated (ATM), DNA-PK and ataxia telangiectasia and Rad3 related (ATR) (Kinner et al., 2008).  $\gamma$ -H2AX formation occurs within minutes after damage, and extends for up to 1-2 megabases from the site of the break in mammalian cells, providing a platform for subsequent DNA repair protein recruitment and amplification at DSBs (Harper & Elledge, 2007). The phosphorylation of H2AX creates a signal recognized by many proteins of the DNA damage response, which are recruited to the sites of DSBs, forming the ionizing radiation-induced foci (IRIF, Lukas et al., 2004). The biological function of IRIF is thought to shelter the broken DNA ends from decay and prevent illegitimate repair processes, to amplify the DNA damage signal and to provide a local concentration of DDR factors relevant for DNA repair and metabolism. Stabilization of DDR factor recruitment to  $\gamma$ -H2AX nucleosomes is achieved through the recruitment of a wide variety of proteins regulating ubiquitylation, sumoylation, acetylation, methylation. The mediator of DNA damage checkpoint 1 (MDC1) is the major protein to localize to the sites of DNA breaks in a  $\gamma$ -H2AX-dependent pathway (Riches et al., 2008; Stucki, 2009) MDC1 has a role in controlling the assembly of multiple repair factors at DNA breaks and in amplifying the DNA damage signal. MDC1 orchestrates the recruitment of IRIF-associated proteins, specifically the MRN complex (MRE11, RAD51, NBS1) and many DNA damage repair proteins, including p53-binding protein 1 (53BP1) and BRCA1 (breast cancer 1). DDR is characterized by the synthesis of ubiquitin conjugates at the sites of damage-induced repair foci (Tanq & Greenberg, 2010). Recently, there has been intense interest regarding the role of ubiquitin and ubiquitin-like molecules in DNA damage repair and signalling, along with its interplay with phosphorylation (Al-Hakim et al., 2010). Protein ubiquitylation has emerged as an important regulatory mechanism that impacts almost every aspect of the DNA damage response, in particular in concentrating DNA repair proteins at the sites of DNA damage. The ubiquitylation cascade involves the activities of at least three enzymes: (i) the ubiquitin-activating enzyme (E1); (ii) the ubiquitin-conjugating enzyme (E2); and (iii) the ubiquitin ligase (E3) (Ciechanover et al., 1982; Hershko et al., 1983). E1 employs ATP to adenylate ubiquitin at its C-terminus, which then forms a thioester bond with the E1 active-site cysteine. The modified ubiquitin is then passed on to the E2 enzyme to form another thioester intermediate (the E2~Ub). Finally, ubiquitin is conjugated to its substrate with the aid of an E3 ubiquitin ligase (Al Hakim et al., 2010). The first E3 ubiquitin ligase that acts in this cascade is RING finger protein 8 (RNF8), which accumulates at DSBs via phospho-dependent interactions between its N-terminal fork head associated (FHA) domain and ATM-phosphorylated TQXF motifs on MDC1 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). At damaged chromatin, RNF8 cooperates with the E2 conjugating enzyme UBC13 to ubiquitylate histones that likely include H2A and H2AX (Huen, et al., 2007; Mailand et al., 2007, Wu et al., 2008). The ubiquitin ligase RNF8 plays an instrumental role in

promoting the maturation of DSB-associated chromatin (Huen et al., 2007; Mailand et al., 2007; Kolas et al., 2007; Wang et al., 2007). Through its direct interaction with MDC1, RNF8 is recruited to DSB sites along with the other factors in the initial wave of protein accumulation at IRIF (Mailand et al., 2007). Here, RNF8 initiates a complex and tightly regulated ubiquitylation cascade of histones H2A and H2AX at the DSB-flanking chromatin, which causes chromatin restructuring (through incompletely understood mechanisms) associated with the generation of binding sites for protein complexes that accumulate downstream of these early factors (Huen et al., 2007; Mailand et al., 2007). The covalent attachment of small ubiquitin-like modifier (SUMO) proteins to specific lysine residues of target proteins, a process termed sumoylation, is a recently discovered protein modification that plays an important role in regulating many diverse cellular processes. Sumoylation is a signalling mechanism which, analogous to and in parallel with ubiquitination, plays an important role in chromatin remodelling at DSB sites. Sumoylation is catalyzed by SUMO-specific E1, E2, E3s and is reversed by a family of Sentrin/SUMO-specific proteases, SENPs. The SUMO E3 ligases PIAS1 and PIAS4 are required for recruitment of proteins BRCA1 and 53BP1 to IRIF, respectively, and both SUMO1 and SUMO2/3 accumulate at IRIF (Galanty et al., 2009; Morris et al., 2009). Moreover, replicating protein A (RPA70) sumoylation facilitates recruitment of RAD51 to the DNA damage foci to initiate DNA repair through homologous recombination (Dou et al., 2010).

## 2. Cellular effects of ionizing radiation in human lymphocytes

### 2.1 Surviving fraction, *HPRT* mutant frequency and molecular characterization of mutations in irradiated human lymphocytes

To contribute to the understanding of the DDR pathway following radiation-induced damage, we studied the effects of IR on human peripheral blood lymphocytes (PBL) irradiated *in vitro* with different doses of  $\gamma$ -rays and low-energy protons (0.88 MeV; LET: 28keV/ $\mu$ m). Irradiated PBL were assayed for cell viability, for mutant frequency at the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene, and for molecular characterization of mutations. The *HPRT* gene, which in humans covers 44 kb and encodes a non-essential protein, allows a wide variety of mutations, from point mutation to total gene deletion, to be detected by using the *HPRT* mutation assay. Deletion of DNA segments is the predominant form of radiation damage in cells that survive irradiation and the mechanisms for producing deletion mutations appear to be very complex and dependent on target cell, gene studied, dose, dose-rate and radiation quality (Schwartz et al., 2000). Large deletions are thought to derive from two DNA double strand breaks close enough to interact each other. Thus, deletion frequency should be dependent on radiation dose and dose-rate. All PBL samples, irradiated either with  $\gamma$ -rays or protons, showed a dose-dependent cell survival decrease and a *HPRT* mutant frequency increase. In Table 1 we report the data of survival and *HPRT* mutant frequency in human PBL irradiated with different doses of  $\gamma$ -rays and low-energy protons.

Molecular analyses of *HPRT* mutants were carried out in clones derived from PBL exposed to  $\gamma$ -rays (1-4 Gy) and to low-energy protons (0.5-2Gy), and in non-irradiated clones of the same donors. Among the mutant clones obtained from  $\gamma$ -irradiated PBL, point mutations were the only kind of mutation in 1Gy irradiated clones, whereas deletions were the prevalent mutations among clones irradiated at 4Gy. In contrast, no partial or total deletions of the *HPRT* gene were detected in mutant clones isolated after proton irradiation. Figure 1

shows the percentages of mutation types calculated over the total number of mutations derived from human PBL irradiated with both radiation qualities. The difference of the mutational spectrum between  $\gamma$ -rays and protons probably depends on the nature of IR. Complex gene rearrangements and deletions are assumed to be a specific signature of exposure to high-LET radiation in mammalian cells. Nevertheless, the absence of these kind of mutations in PBL irradiated with protons could be due to their lower survival in comparison with  $\gamma$ -irradiated PBL, as a consequence of the more cytotoxic than mutagenic lesions induced.

	SF (%) $\pm$ S.E.	HPRT MF ( $\times 10^{-6}$ ) $\pm$ S.E.
$\gamma$ -rays		
0.5 Gy	99.5 $\pm$ 0	2.8 $\pm$ 0
1.0 Gy	83.85 $\pm$ 11.36	10.5 $\pm$ 3.8
2.0 Gy	44.7 $\pm$ 7.36	29.2 $\pm$ 3.7
3.0 Gy	13.7 $\pm$ 4.56	50.6 $\pm$ 17.9
4.0 Gy	6.2 $\pm$ 2.4	24.7 $\pm$ 11.1
Protons		
0.5 Gy	60.84 $\pm$ 7.82	5.33 $\pm$ 2.18
1.0 Gy	39.87 $\pm$ 6.28	9.25 $\pm$ 2.8
1.5 Gy	38.42 $\pm$ 11.35	16.72 $\pm$ 4.86
2.0 Gy	35.5 $\pm$ 0	11.7 $\pm$ 1
2.5 Gy	29.9 $\pm$ 11.35	5.06 $\pm$ 0

Table 1. Surviving fraction (SF) and *HPRT* mutant frequency ( $\pm$  standard error, S.E.) in human PBL irradiated with  $\gamma$ -rays and low-energy protons.

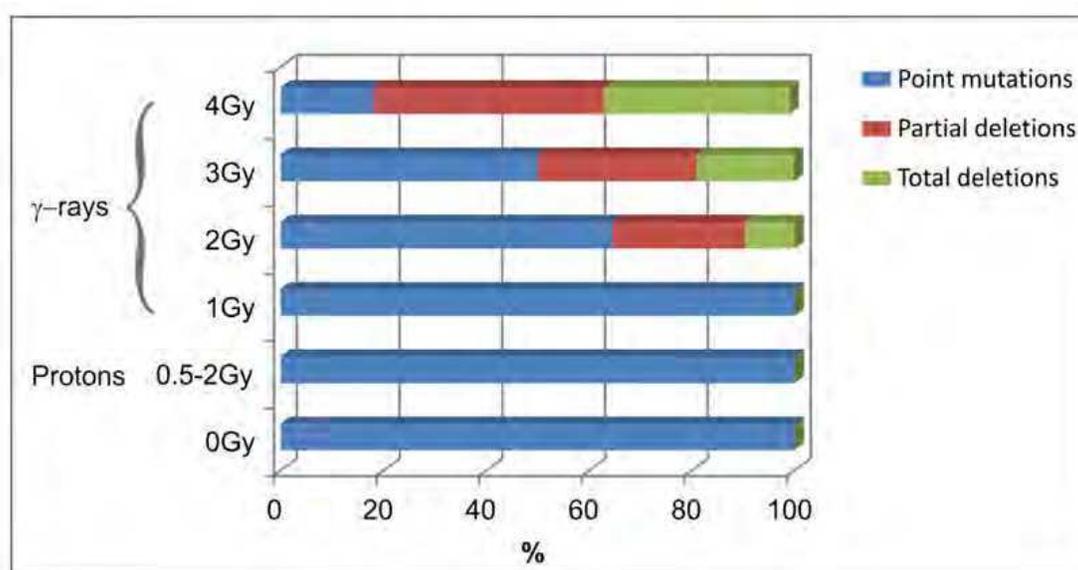


Fig. 1. Characterization of *HPRT* mutant clones derived from PBL irradiated with  $\gamma$ -rays and protons or non-irradiated (0Gy).

## 2.2 Double strand break repair in irradiated human lymphocytes

To evaluate the repair of DSBs in PBL irradiated with  $\gamma$ -rays or low-energy protons, we analyzed  $\gamma$ -H2AX kinetics through foci formation and disappearance. The presence of nuclear foci was monitored by *in situ* immunofluorescence at different time points after IR. Figure 2 shows the different  $\gamma$ -H2AX foci pattern at 2h after IR with high- and low-LET radiation, reflecting the sparsely and densely nature of IR.

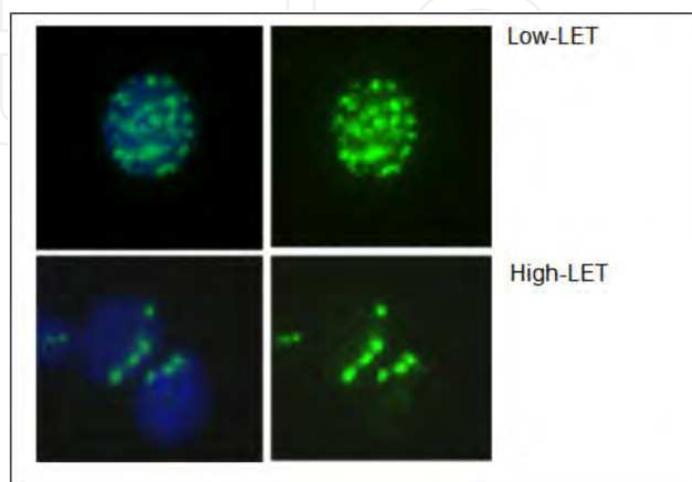


Fig. 2. Visualization by *in situ* immunofluorescence of  $\gamma$ -H2AX foci in human PBL irradiated with  $\gamma$ -rays or low-energy protons. The pattern of  $\gamma$ -H2AX localization within the nucleus is strictly dependent on the quality of radiation. Low-LET radiation, such as  $\gamma$ -rays, hit the cells throughout all directions, and DSBs are sparsely distributed; on the contrary, high-LET radiation such as protons, give raise to clustered DNA damage along tracks.

In irradiated PBL the kinetics of DSB repair was different according to the quality of radiation. In particular, the fraction of foci-positive cells was higher in  $\gamma$ -irradiated than in proton-irradiated lymphocytes at all times, except at 24h after IR. Early after irradiation (30 min and 2h)  $\gamma$ -H2AX foci were present in 80% and 43% of PBL, irradiated respectively with  $\gamma$ -rays and protons (Fig. 3A). This difference is mainly due to the quality of radiation: while sparsely IR as  $\gamma$ -rays lose their energy throughout all directions thus hitting all nuclei, densely IR as protons, hits the fraction of cells along their track. The preferential production of complex aberrations is related to the unique energy deposition patterns produced by densely ionizing radiation, causing highly localized multiple DNA damage. At 6h after IR the percentage of foci-positive cells decreased, revealing the repair capacity of DSBs in both kind of irradiated lymphocytes, although the repair kinetics was faster in  $\gamma$ -irradiated PBL. At 24h after IR the percentage of  $\gamma$ -H2AX foci positive cells tended to reach the value of non-irradiated PBL, either in  $\gamma$ - and in proton-irradiated PBL.

The mean number of  $\gamma$ -H2AX foci per nucleus was higher in PBL irradiated with  $\gamma$ -rays than with protons, at all times after IR (Fig. 3B). In our experiments, most of PBL displayed 10–20 or more  $\gamma$ -H2AX foci/nucleus 30 min after irradiation, giving a maximum yield of 4 foci/Gy, a number similar to that reported for human PBL irradiated with X-rays (about 10 foci/Gy) (Sak et al., 2007; Schertan et al., 2008), but much lower than that determined in human fibroblasts (32.2 foci/Gy) (Hamada et al., 2006). It has been reported that the number of  $\gamma$ -H2AX foci is well consistent with the number of theoretically calculated DSB/Gy of sparsely ionizing radiation (i.e. about 40) (Ward, 1991), if one DSB is contained per focus.

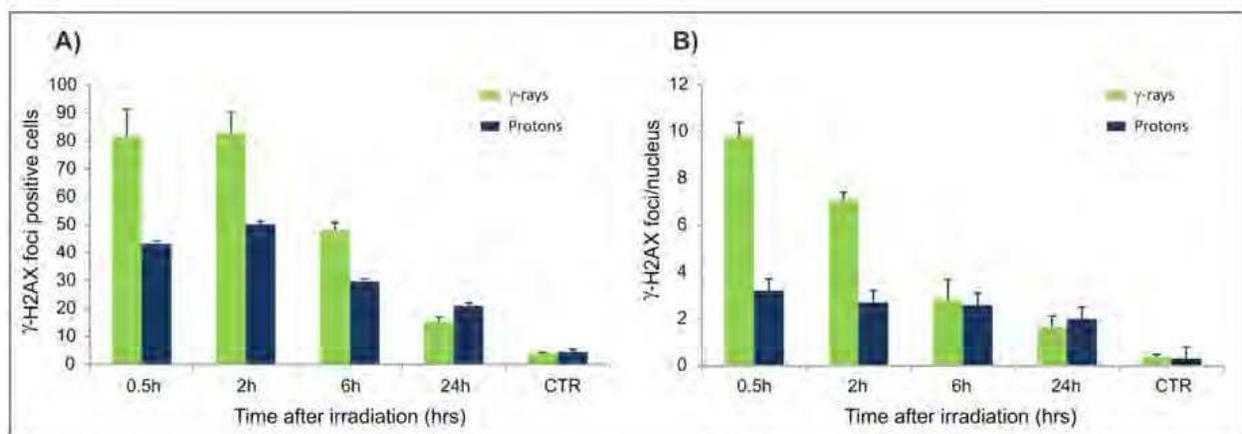


Fig. 3. Kinetics of  $\gamma$ -H2AX foci in PBL irradiated with  $\gamma$ -rays and low energy protons during the time after irradiation. A) Fraction of cells positive for  $\gamma$ -H2AX foci and B) mean number of  $\gamma$ -H2AX foci per nucleus.

The lower number of foci detected in peripheral lymphocytes could depend on the large amount of heterochromatin of resting cells, from which  $\gamma$ -H2AX foci are mostly excluded (Cowell et al., 2007) as well as on the small nuclear volume, where overlapping foci are difficult to detect separately. Thus, in accordance with the observations of Scherthan et al., (2008) we hypothesize that  $\gamma$ -H2AX foci detected very early after irradiation contained more than one DSB; later on, the number of foci decreased and probably each foci contained only one DSB. Furthermore, we found a size increase of  $\gamma$ -H2AX foci in cells irradiated with protons, as compared with gamma irradiations, probably as a consequence of DSBs clusters induced by high-LET radiation. Our results are in accordance with those in melanoma cells exposed to low- and high-LET radiation (Ibañez et al., 2009).

### 3. Cellular effects of ionizing radiation in human lymphocytes cultured in microgravity condition

The cellular response to ionizing radiation besides on genetic and physiological features of the biological systems, depends also on environmental conditions occurring during DNA repair. Space missions expose humans to an exogenous environment not encountered within our biosphere, in particular the contemporary presence of radiation and a condition of weightlessness called microgravity ( $10^{-4}$ – $10^{-6}g$ ). One of the important aspects of risk estimation during space flights, is whether the effects of radiation on astronauts are influenced by microgravity. The combination of microgravity and ionizing radiation has been demonstrated to have a synergistic action on human cells, both *in vivo* and *in vitro*. The effects of space environment experienced by astronauts include loss of calcium and minerals from bone, decreased skeletal muscle mass and depressed immune function (Longnecker et al., 2004). *Ex vivo* astronaut studies, in-flight cell cultures, and ground models of microgravity studies, have consistently demonstrated inhibition of lymphocyte proliferation and suppressed or altered cytokine secretion (Lewis et al., 1998; Grimm et al., 2002). Among the biological effects of the reduced gravity in human cell cultures, were described apoptosis induction, cytoskeletal alteration, cell growth inhibition and increased frequency of chromosome aberrations (Lewis et al., 1998; Grimm et al., 2002; Cubano et al., 2000; Sytkowski et al., 2001; Mosesso et al., 2001; Durante et al., 2003). Gene expression analyses

on human cells grown in microgravity during space flights or in modeled microgravity (MMG) on Earth, report changes among genes involved in apoptosis induction, cell adhesion, cytoskeletal features and cell differentiation, even if large differences in culture conditions, cell types and methods to simulate microgravity were adopted in those experiments (Hammond et al., 2000; Lewis et al., 2001, Torigoe et al., 2001, Infanger et al., 2007). While the genotoxic effects of ionizing radiation have been intensely studied, the consequence of the reduced gravity together with radiation is still unclear. Therefore, it is of special importance to verify whether DDR is affected by the combined effects of IR and microgravity, in view of the prolonged permanence of man in future space missions. To analyze the possibility that a reduced gravitational force impairs the DDR pathway, increasing the risk of the exposure to conditions occurring during spaceflight, we studied the DDR to ionizing radiation in human PBL incubated in MMG and in parallel static conditions. Microgravity was simulated by culturing PBL in the Rotating Wall Vessel bioreactor (Synthecon, Cellon, Fig. 4) placed inside a humidified incubator, vertically rotating at 23 rpm.

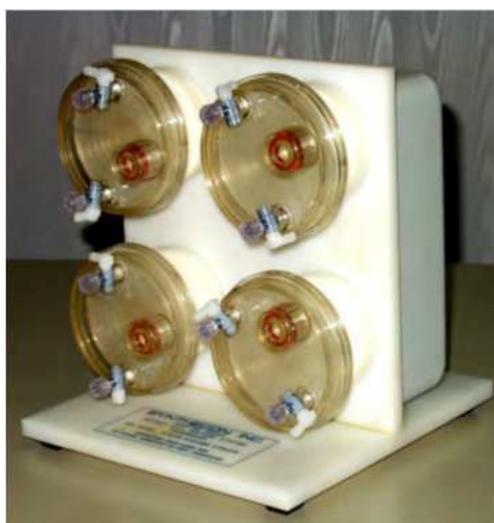


Fig. 4. Rotating Wall Vessel Bioreactor (Synthecon).

The Rotating Wall Vessel was developed at the NASA Johnson Space Center (Houston, TX) to simulate, as accurately as possible, culture conditions predicted to occur during experiments in space. In the rotating system, the gravity is balanced by equal and opposite mechanical forces (centrifugal, Coriolis and shear components), and the gravitational vector is reduced to about  $10^{-2}$  g. In these conditions, single cells are nearly always in suspension, rotating quasi-stationary with the fluid, in a low-shear culture environment (Unsworth 1998, Maccarone et al., 2003). Ground based (1 g) PBL cultures, both irradiated and non-irradiated, were kept at the same cell density in flasks inside a humidified incubator for 24 h.

### 3.1 The DNA-damage response of human peripheral lymphocytes cultured in microgravity after $\gamma$ -irradiation

The DNA-damage response was investigated in human PBL irradiated *in vitro* with different doses of gamma rays and incubated for 24 h in 1 g or in modeled microgravity (MMG). While cell survival was only slightly affected by MMG, the *HPRT* mutant frequency significantly increased in PBL incubated in MMG after irradiation compared with those

maintained in 1 g. Given the increase of *HPRT* mutants in MMG, we investigated whether the reduced gravity affected the progression of the rejoining of double strand breaks (DSBs) in human PBL irradiated with  $\gamma$ -rays and incubated in MMG or in 1g. The kinetics of  $\gamma$ -H2AX foci was monitored during the repair incubation, showing that DSBs rejoining was slower in MMG than in 1g at 6 and 24 h after irradiation. In addition, the mean number of  $\gamma$ -H2AX foci per nucleus was significantly higher in MMG than in 1g at the same time-points (Fig. 5).

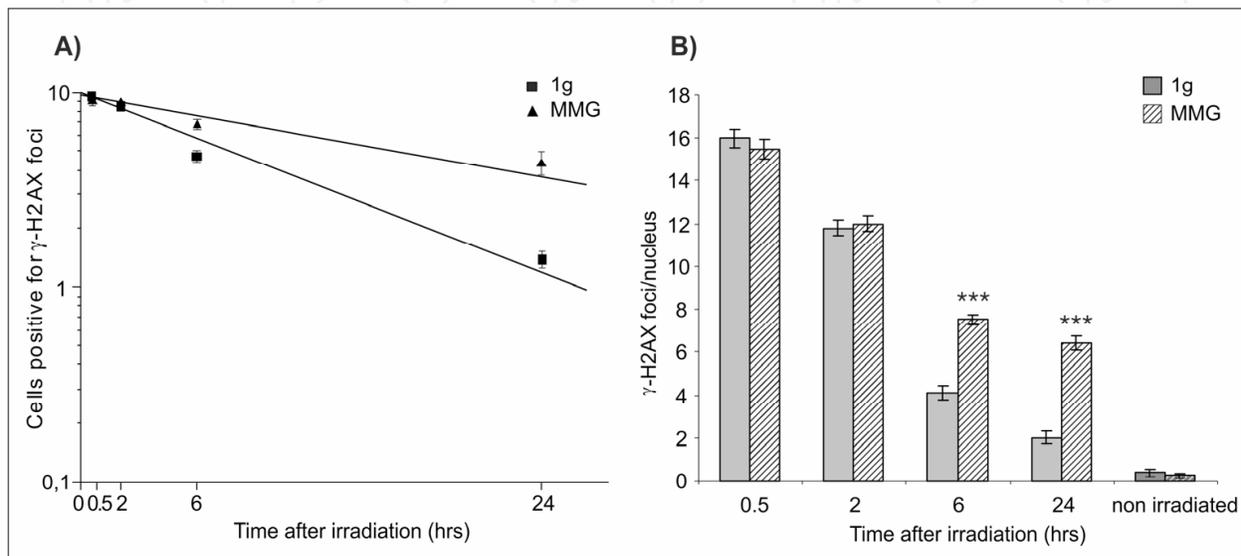


Fig. 5. Kinetics of  $\gamma$ -H2AX foci in PBL irradiated with 5Gy of  $\gamma$ -rays and incubated in 1g or MMG during the repair time. A) Fraction of PBL positive for  $\gamma$ -H2AX foci. B) Mean number of  $\gamma$ -H2AX foci/nucleus (\*\*\*) $P < 0.001$ , *t*-test).

To verify whether the disappearance of  $\gamma$ -H2AX foci correlated with the rejoining of double strand breaks, we subjected irradiated lymphocytes to a non-radioactive PFGE assay (Gradzka et al., 2005). The fraction of DNA released (FR) from the plug after PFGE was considered a measure of DSB level. The kinetics of DSB removal in lymphocytes irradiated and incubated in 1g exhibits a typical fast initial component and a decreasing rate at longer repair intervals, in accordance with data from other authors (Stenerlow et al., 2000; Gradzka et al., 2005). Both the methods we used to quantify DNA fragmentation, reported a lower rate of DSB rejoining in lymphocytes incubated in MMG compared to those in 1g, in agreement with the kinetics of  $\gamma$ -H2AX foci. Our results provide evidences that MMG incubation during DNA repair delayed the rate of radiation-induced DSB rejoining, and increased, as a consequence, the genotoxic effects of ionizing radiation.

We then assessed whether MMG incubation affected IR-induced apoptosis. Human lymphocytes, irradiated and non-irradiated, were scored for the presence of fragmented nuclei and apoptotic bodies. Apoptotic index (A.I.) increased with time after irradiation and at 24 h it was significantly higher in PBL incubated in MMG compared to those in 1g (19.3% vs. 13.7% respectively,  $P < 0.001$ ). Since DSBs can be induced, besides radiation, also by DNA fragmentation during early apoptosis, we measured caspase-3 activation at the same time-points by the cleavage of the peptide substrate DEVD-AFC. Caspase-3 activation was only slightly higher in PBL maintained in MMG than in 1g, in contrast to the high persistence of foci-positive cells ( $P < 0.01$ ), and foci number/nucleus ( $P < 0.001$ ), suggesting

that the level of H2AX phosphorylation was principally correlated to a delayed DSB resolution rather than apoptosis induction.

We then tested for the possibility that MMG incubation affects DNA damage response by altering the recruitment of the signaling proteins, 53BP1, NBS1-p343 and ATM-p1981, which co-localize with  $\gamma$ -H2AX foci to DSB sites (Fig. 6A). After irradiation ~90% of cells became foci-positive for the three proteins in both gravity conditions (not shown). In contrast to  $\gamma$ -H2AX, the fraction of foci-positive cells persisted high up to 24 h after irradiation in 1g and no differences between the two culture conditions were detected. The number of foci/nucleus significantly decreased during post-irradiation incubation from 14–16 foci/nucleus at 30 min to 4–5 foci/nucleus at 24 h (Fig. 6B), without differences between samples in 1g and MMG. The discrepancies with the kinetics of  $\gamma$ -H2AX foci suggest that these proteins could represent the remaining scaffold structure used for DSB repair that persisted after the repair has been completed (Markova et al., 2007, van Veelen et al., 2005).

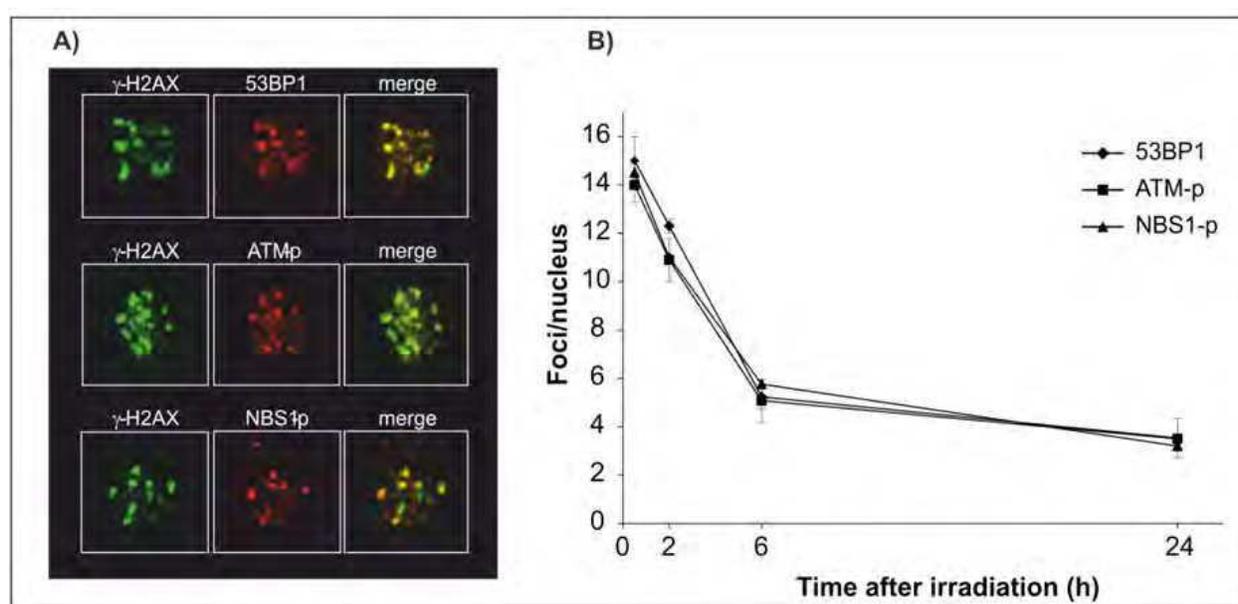


Fig. 6. Kinetics of 53BP1, ATM-p1981, NBS1-p343 foci in PBL irradiated with  $\gamma$ -rays and incubated in 1g. A) Co-localization with  $\gamma$ -H2AX foci to form the ionizing radiation-induced foci (IRIF). B) Mean number of foci per nucleus.

### 3.2 The DNA-damage response of human tumoral lymphocytes cultured in microgravity after $\gamma$ -irradiation

We analyzed the DNA damage response to radiation also in human tumoral lymphocytes (TK6 cells, lymphoblastoid B cells) irradiated with  $\gamma$ -rays (1, 2, 4 Gy) and incubated in 1g or in MMG during the repair time. In irradiated TK6 cells, we observed a higher survival in MMG than in 1g, and the difference was significant at 4Gy. In addition, in cells maintained in MMG rather than in 1g after  $\gamma$ -irradiation, higher frequency of HPRT mutants was observed at all irradiation doses, particularly at 4Gy (Figure 7A). Remarkably, at this dose, mutant frequency may often be underestimated, since cells with many and severe mutations are unable to repair DNA damage and die. Instead, in TK6 cells cultured in MMG after irradiation, mutant frequency increased with doses up to 4Gy (Figure 7A). The frequency of micronucleated cells was measured in both gravity conditions after irradiation. At the end of

post-irradiation incubation (24 h time-point), the percentage of micronuclei (MN) was significantly higher in both non-irradiated and in irradiated cells incubated in MMG compared with 1g (Fig.7B). Eighteen hours later (42 h from irradiation), the percentage of MN in cultures incubated in MMG was higher than in 1g only at 2Gy  $\gamma$ -ray dose. At 48 h time-point, MN frequencies observed in 1g or MMG were comparable. As expected, MN significantly increased after irradiation in both gravity conditions with respect to non-irradiated cells; a significant difference was still observed at 48 h after irradiation at both 1 and 2Gy. The significant increase of micronucleated cells in MMG suggested that MMG itself was able to induce chromosome damage.

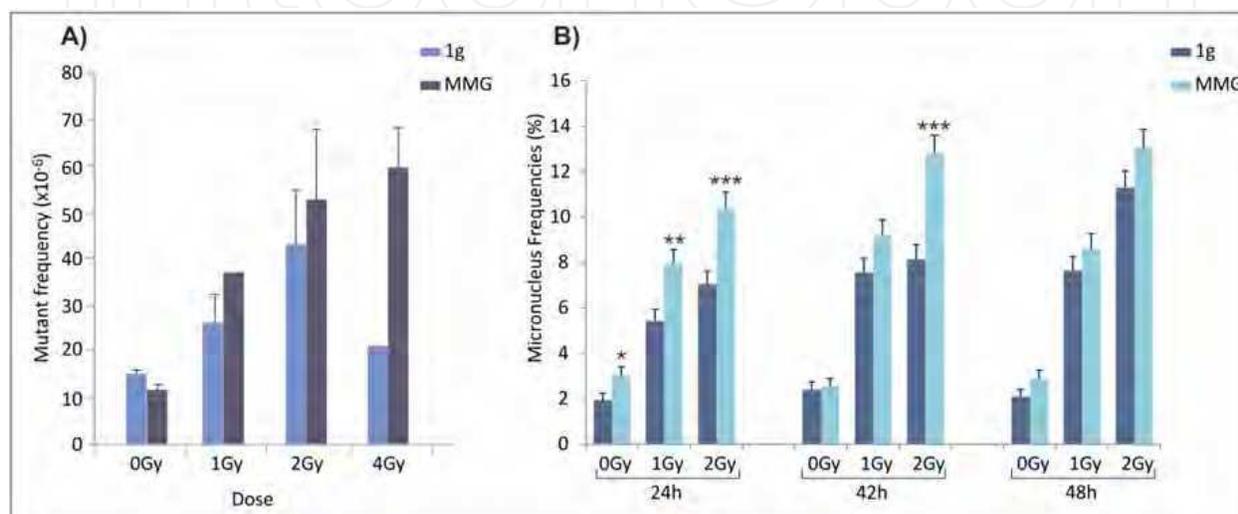


Fig. 7. A) Mutant frequency at the HPRT locus of irradiated and non-irradiated TK6 cells incubated for 24h in 1g or in modeled microgravity. B) Micronucleus frequencies (%) in irradiated and non-irradiated TK6 cells incubated in 1g or MMG for the first 24h after irradiation and then cultured in 1g or MMG up to 48 h. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (G test).

The effect of MMG incubation on cell cycle alteration induced by  $\gamma$ -ray exposure was assessed by flow cytometry analysis. Figure 8 shows the cell cycle distribution of TK6 cells at various time-points from irradiation and incubation in MMG or 1g by representative DNA histograms.  $\gamma$ -ray irradiation induced an increase in G2/M-phase cells and a reduction in S-phase cells, both in TK6 maintained in 1g and MMG after irradiation. At the end of MMG or 1g incubation (24 h time-point), the percentages of cells in G1-phase were higher in cultures irradiated with 2-4 Gy and incubated in MMG compared with cells maintained in 1g. Moreover, the G2/M block after irradiation was less evident in MMG than in 1g condition. Also radiation-induced apoptosis was affected in TK6 cells by MMG incubation. Induction of apoptosis was significantly lower in irradiated TK6 cells incubated in MMG compared with cells irradiated with the same dose and incubated in 1g. The differences were more pronounced in cells analyzed at long post-incubation times (72 h time-point).

The observed decrease of apoptotic response in MMG incubated cultures could allow severely damaged cells, which in 1g condition should be eliminated by selection, to survive, with negative consequences on genomic integrity. Alterations in cell response to ionizing radiation due to MMG incubation during the DNA repair period may be caused by the reduced activity of some proteins, which play a crucial role in damage signaling. Previous data have shown that absence or reduction of gravity can alter gene expression (Walther et

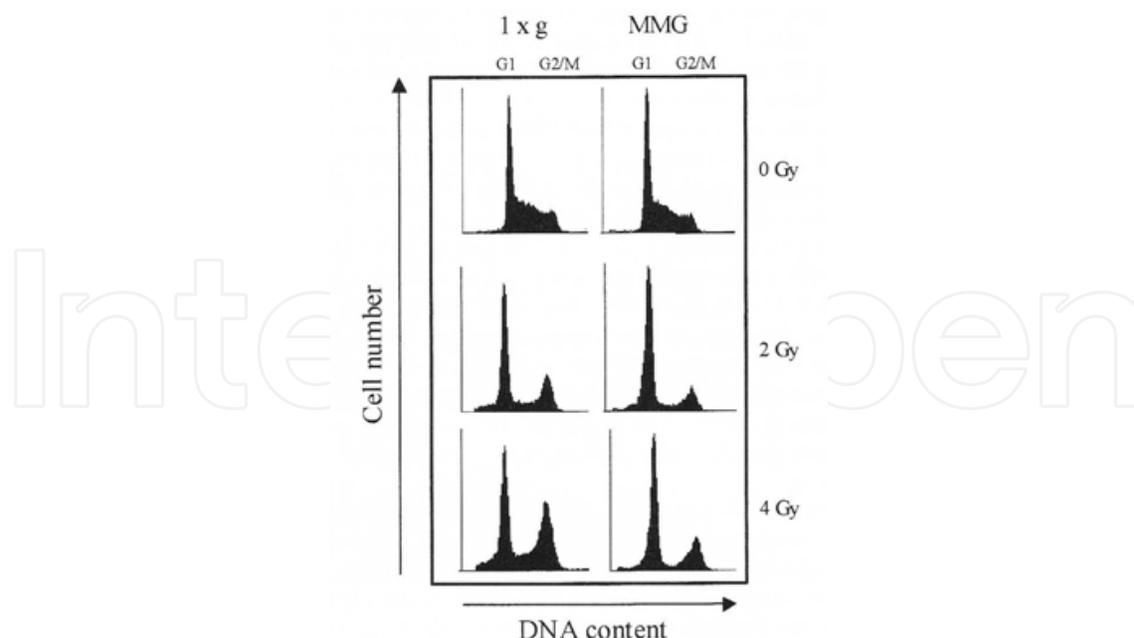


Fig. 8. Cell cycle distribution of irradiated and non-irradiated TK6 cells at the end of 24h incubation in 1g or in MMG.

al., 1998; Hughes-Fulford 2001; Kita et al., 2000), which in turn may explain the results reported here. It remains to be determined if one upstream or several downstream genes belonging to the pathway of the radiation response are involved in the effects induced by MMG incubation.

### 3.3 Gene expression changes in human lymphocytes cultured in microgravity during the DNA-damage response to radiation

Gene expression changes represent an early bio-indicator of radiation exposure. Given the increase of *HPRT* mutants observed in human lymphocytes incubated in modeled microgravity, we investigated whether this gravity condition can alter the transcription of 14 genes representative of the main DNA repair pathways. The genes analyzed are representative of the major DNA repair pathways: four genes (*Ku70*, *Ku80*, *DNA-ligase IV*, *XRCC4*) are involved in non-homologous end joining processes (NHEJ), three genes (*BRCA1*, *BRCA2*, *RAD51*) in homologous recombination (HR), four genes (*XRCC1*, *PCNA*, *GADD45A*, *p21Cip1/Waf1*) in base excision repair (BER) and two genes (*DDB2*, *XPC*) in nucleotide excision repair (NER). *DNA-ligase I*, involved in both BER and NER repair pathways, was analyzed too. Analyses were carried out in three pools of three donor, each by quantitative real time PCR. Results show that almost all BER and NER genes were up-regulated in irradiated PBL, whereas the expression of HR and NHEJ genes was only slightly or not affected by radiation (Fig.9). Incubation in modeled microgravity after irradiation did not significantly change the expression of genes involved in DNA repair, suggesting that transcriptional impairment was not responsible for the increase of mutant frequency observed in irradiated cells incubated in microgravity in comparison to the static 1 g condition. These findings in agreement with previous studies on gene expression of non-irradiated space flown and RWV cultured cells, showing that DNA repair genes were unaffected by low-gravity whereas intracellular signaling, growth regulatory, cytoskeletal and tumor suppressor genes were altered (Lewis et al., 2001; Hammond et al., 2000; Pardo et al., 2005).

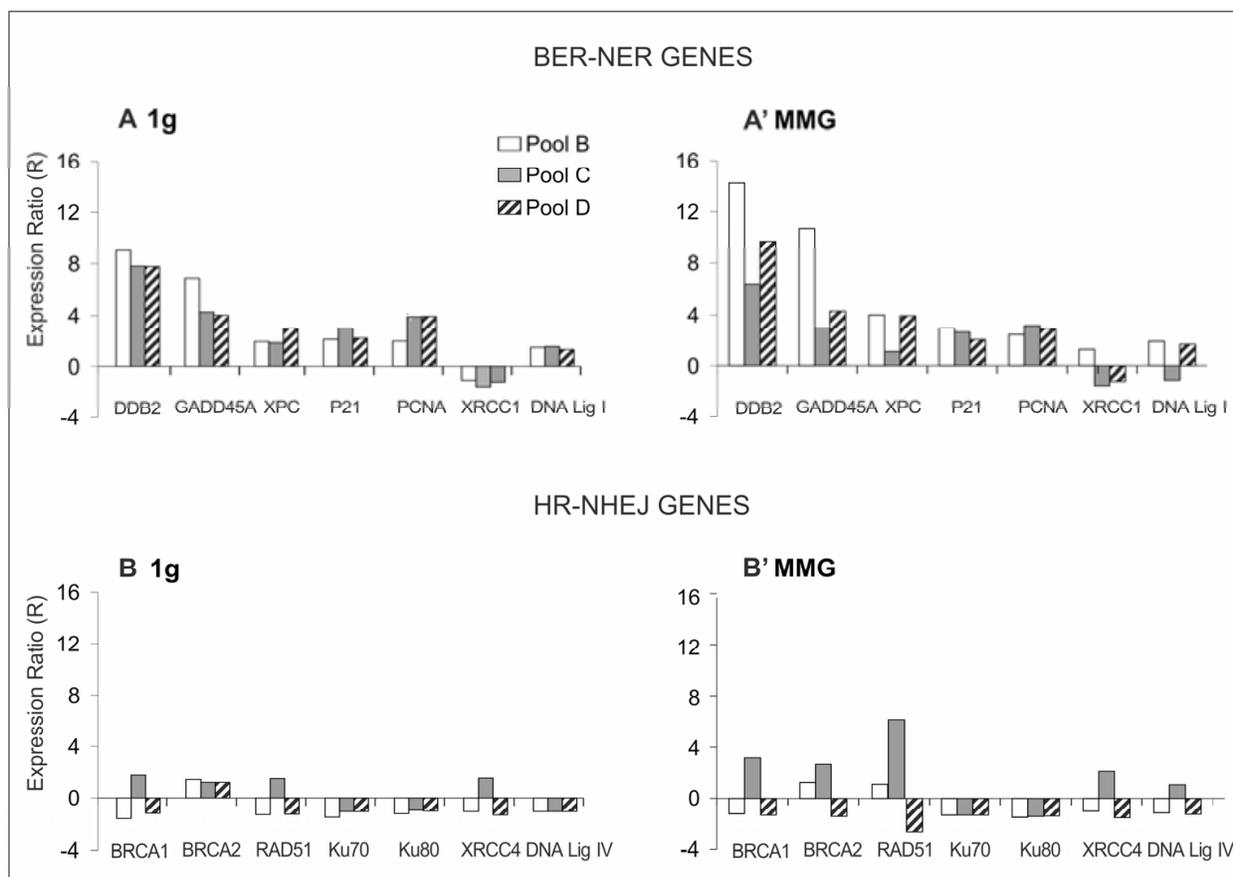


Fig. 9. Expression ratios in PBL of pools B-D incubated in 1 g and modeled microgravity after X-irradiation. (A) *R* values of BER and NER genes in 1g; (A) *R* values of BER and NER in MMG; (B) *R* values of HR and NHEJ genes in 1g; (B) *R* values of HR and NHEJ genes in MMG.

Recently, a new class of important gene modulators has been discovered: microRNAs. They are a large family of small non-coding RNAs of 18-24 nucleotides that negatively regulate gene expression levels by binding to microRNA-binding elements in the 3' untranslated-region (3'UTR) of target mRNAs thereby triggering decreased protein translation mainly through mRNA degradation (Guo et al., 2010). A single miRNA may have broad effects on gene expression networks, such as regulating cell lineage specificity, cellular functions or stress response. By considering the complexity of the DNA-damage response (DDR), addressed to maintain genome integrity through cell cycle arrest, DNA repair and/or apoptosis, it is expected that miRNAs have an important role in this cellular process. Whilst miRNA-mediated DDR has been studied after UV radiation and hypoxic stress (Pothof et al., 2009; Crosby et al., 2009) that of radiation combined with microgravity has not been studied yet and should give important information about risk assessment in space environment. MicroRNAs profiling were carried out by using the platform "Human miRNA Microarray kit (V2)" (Agilent), according to the Agilent miRNA protocol. For mRNA expression profile we used the "Whole Human Genome Oligo Microarray" (Agilent), consisting of ~41.000 (60-mer) oligonucleotide probes, which span conserved exons across the transcripts of the targeted full-length genes. Identification of differentially expressed genes and miRNAs was performed with one and two class Significance Analysis of Microarray (SAM) program (Tusher et al., 2001) with default settings. Figure 10A shows a

dendrogram relative to some miRNAs differentially expressed following ionizing radiation in human PBL.

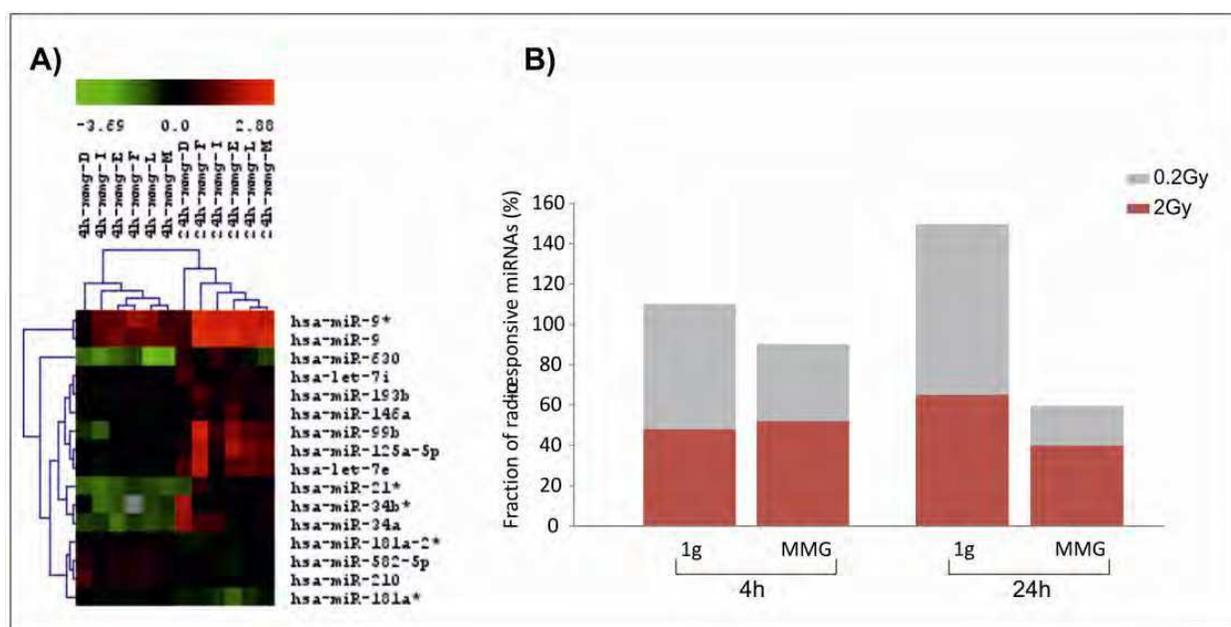


Fig. 10. A) Dendrogram showing several miRNAs differentially expressed in human PBL at 4 and 24h after irradiation with 0.2Gy. Range of expression value is determined as the log<sub>2</sub> ratio of irradiated/non-irradiated sample. Down-regulated and up-regulated miRNAs correspond to green and red boxes, respectively. B) Fraction of radio-responsive miRNAs (%) in human PBL irradiated with 0.2 and 2Gy and incubated for 4 and 24h- in 1g or in modeled microgravity (MMG).

MiRNA expression profile was carried out at 4h and 24h after irradiation with 0.2Gy and 2Gy and incubation in 1g and MMG and compared to that of non-irradiated PBL maintained in parallel conditions. Results showed that in both gravity conditions the miRNA expression profile was dose-specific, as indicated by the low percentage of common miRNA responsive to both doses; moreover, the effects of the higher dose predominated at the late time point. Interestingly, MMG tended to decrease the number of radio-responsive miRNAs respect to 1g condition, in particular at 24h after irradiation (Figure 10B).

To predict the target genes of differentially expressed miRNAs we first performed a computational analyses using PITA algorithm available on line (Kertesz et al., 2007). However, all available software for target prediction are characterized by a large fraction of false positives, thus to identify the most likely targets, we have integrated mRNA and miRNA expression data, obtained on the same lymphocyte samples, using MAGIA (MiRNA And Genes Integrated Analysis) web tool (Sales et al., 2010). We used a non-parametric index (Spearman correlation coefficient), the most indicated statistical coefficient for a small number of measures, to estimate the degree of anti-correlation (e.g. up-regulated miRNA and corresponding down-regulated mRNA target) between any putative pairs of miRNA and mRNA (Xin et al., 2009; Wang and Li 2009). The anti-correlated transcripts were then classified according to DAVID (Database for Annotation, Visualization and Integrated Discovery) web tool (Huang et al. 2009), to determine which Gene Ontology (GO) terms were significantly enriched in our set of genes. Results of GO analysis of anti-correlated

genes showed that in MMG-incubated PBL were not enriched the categories of response to stress, to DNA damage and to apoptosis. miRNA-mRNA anti-correlations of DDR pathway were visualized by using Cytoscape software package (Shannon et al., 2003; Cline et al., 2007) (Figure 11). The results showed that, in most cases, the same mRNA was targeted by different miRNA species according to the different condition of gravity.

Future research is addressed to validate several of the anti-correlations highlighted with our analyses as important in DDR pathway. In particular, we will perform a functional assay to demonstrate the regulatory effect of a particular miRNA on its putative target mRNA. The luciferase assay represents the most efficient approach to evaluate the activity of a miRNA on its anti-correlated mRNA. This assay allows to demonstrate the activity of a miRNA on its anti-correlated mRNA by the quantification of the luminescent signal derived from the luciferase reporter enzyme. Cells are co-transfected with a reporter vector containing the firefly luciferase gene together with the 3'UTR target sequence predicted for that miRNA and the miRNA precursor (pre-miRNA) or inhibitor (anti-miRNA), which respectively mimics and inhibits the endogenous miRNA. The binding of pre-miRNA to the complementary target sequence will cause the repression of luciferase gene expression, whereas the binding of anti-miRNA to the endogenous miRNA will induce the expression of luciferase gene. The quantification of the luminescent signal derived from the luciferase reporter enzyme thus allows to demonstrate the activity of a miRNA on its putative target mRNA. In addition to the luciferase assay, it would be interesting to study the role of selected miRNAs in DDR pathway by a biological approach. Usually, several end points such as cell survival, DNA repair, cell cycle progression and apoptosis induction are analyzed in cells over-or under-expressing the miRNA of interest.

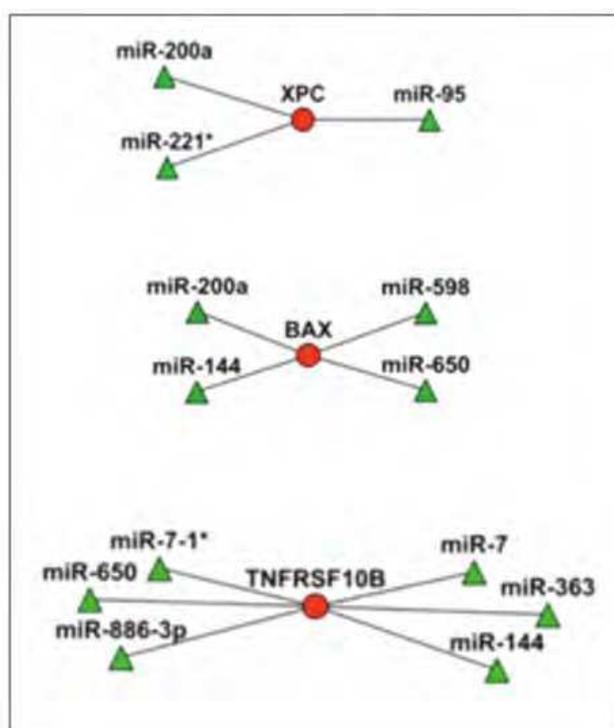


Fig. 11. Example of visualization of inversely correlated miRNA-mRNA relationships in irradiated human PBL. Circles represent transcripts and triangles miRNAs, shown with the color corresponding to the expression value.

#### 4. The DNA-damage response of human lymphocytes to indirect effect of ionizing radiation

In addition the cellular effects arising as a direct response to ionizing radiation, in the last decade it has been suggested that extranuclear or extracellular targets can contribute to the genetic damage in non-irradiated (bystander) cells. The bystander effect (BE) is the biological response of non-irradiated cells induced by contact with irradiated cells. The contact with bystander factors may occur by direct cell-cell interaction or be mediated by the fluid surrounding the cells. It has been reported that the BE causes cell death, cell cycle arrest, apoptosis, changes in gene expression, and increases micronucleus induction, chromosomal aberrations, mutation frequency, and DNA damage in cells neighboring hit cells. In contrast to DNA damage induced by direct irradiation, bystander cell DNA damage is still poorly understood. Many data showed that early events of the radiation induced bystander effect are rapid calcium fluxes and generation of reactive oxygen species in bystander cells. Mitochondria seem to play a central role in bystander signaling: irradiated cell conditioned media can cause changes of mitochondrial distribution, loss of mitochondrial membrane potential, increases in ROS, and increase in apoptosis among the medium receptor cells, which can be blocked by treatments with antioxidants (Chen et al., 2008). Experiments carried out in hepatoma cell lines provide evidence that the BE can be modulated by the p53 status of irradiated cells and that a p53-dependent release of cytochrome-c from mitochondria may be involved in producing BE (He et al., 2011).

We investigated on the mechanisms of the medium-mediated bystander response induced by low doses of  $\gamma$ -rays in human tumoural lymphocytes (TK6 cells), a cell line growing in suspension, in which gap-junction communications are not involved in transferring bystander signals and only medium-mediated molecules may be responsible for BE induction. Cell cultures were irradiated and the culture medium discarded immediately after irradiation and replaced with a fresh one to eliminate ROS originating during irradiation. Irradiated cells were incubated for 6h in fresh medium, which, at the end of incubation time, is referred as conditioned medium (CM) and used to incubate non-irradiated TK6 cells for different times (2-48 h). In bystander cultures, cell mortality at the fixed incubation times ranged between 24 and 19%, very similar values to that of directly irradiated cells (28 and 20%). The mortality percentages for all incubation times were significantly higher with respect to that of the controls (0Gy and 0Gy CM). The survival fraction of directly 1Gy irradiated or CM incubated cells was determined by the clonogenic assay. The data show that both irradiated and bystander TK6 cells had a lower cloning efficiency than their respective controls. Figure 12 reports the results about cell mortality and survival (given as the ratio of the cloning efficiency of treated vs. untreated control cells) in TK6 cells exposed directly to IR or to CM. Apoptosis induction was tested by the presence of fragmented nuclei and apoptotic bodies at 2, 24 and 48h after 1Gy irradiation or CM incubation. The apoptotic index (A.I.) ranged between 7 and 9 % in irradiated cells and between 6 and 7.5 % in bystander cells, and was significantly higher than the relative controls at all times (Figure 13). The induction of apoptosis was also analyzed by the activation of caspase-3, the principal effector caspase, assayed by the cleavage of the peptide substrate DEVD-AFC, at 1, 2, 24 and 48h after irradiation or CM incubation. In bystander cells caspase-3 activation increased from 1.4- to 2.7-fold during the 48h of CM incubation, suggesting that bystander apoptosis increases after 48h. Bystander apoptosis in TK6 cells was sensitive to the inhibitor of caspase-8, the Z-IETD-fmk, added during CM treatment or

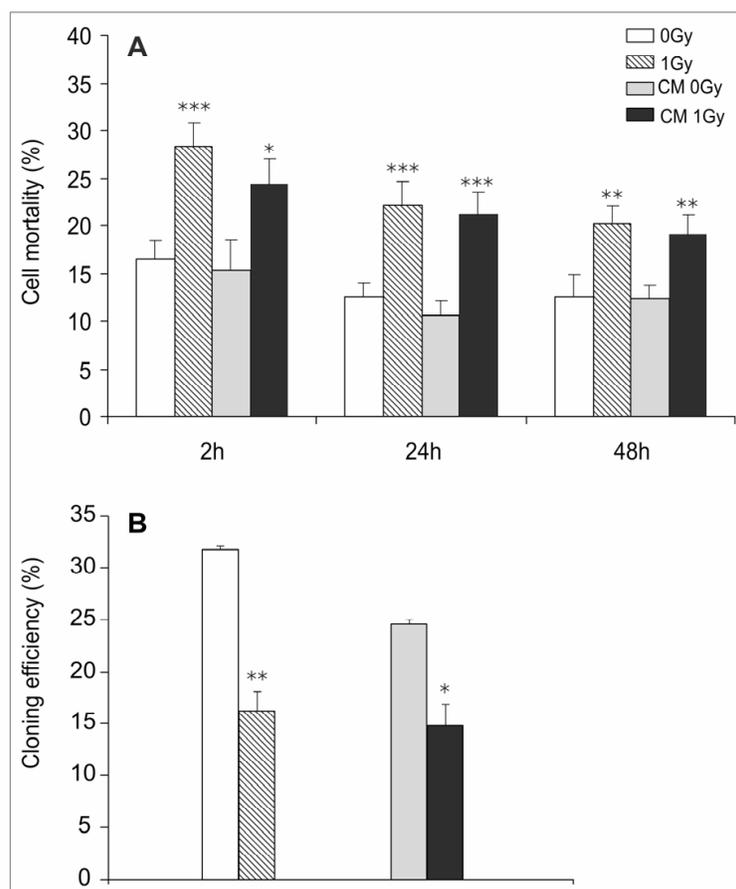


Fig. 12. Cell mortality determined by Trypan blue staining (A) and cloning efficiency (B) in non-irradiated control cells (0Gy), in irradiated cells (1Gy), in cells incubated with conditioned medium from non-irradiated cells (CM 0Gy) and in cells incubated with conditioned medium from irradiated cells (CM 1Gy). Results are the means of 3-5 independent experiments  $\pm$  S.E. (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001,  $t$  test).

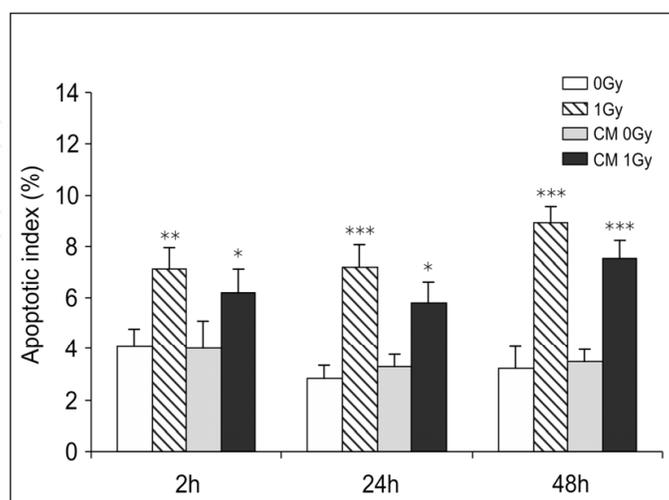


Fig. 13. Apoptotic index (A) in irradiated and bystander cells. Results are the means  $\pm$  S.E. of 5-8 independent experiments. Significant differences were observed in 1Gy and CM 1Gy vs 0Gy and CM 0Gy, respectively (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001,  $t$  test).

post-irradiation incubation. The presence of the inhibitor significantly decreased the induction of apoptosis to the control level, but it did not significantly decrease the level of apoptosis in either irradiated or non-irradiated controls (Figure 14). These results suggest that caspase-8 activation is triggered by signaling molecules present in the conditioned medium. The addition of the ROS scavenger Cu-Zn superoxide dismutase and N-acetylcysteine to the conditioned medium allowed to investigate the involvement of oxidative stress in inducing bystander apoptosis. ROS scavengers did not significantly decrease the apoptotic index in CM cultures; by treating non-irradiated TK6 cells with medium irradiated without cells (IM), we evaluated the contribute of ROS produced by irradiation in inducing bystander apoptosis.

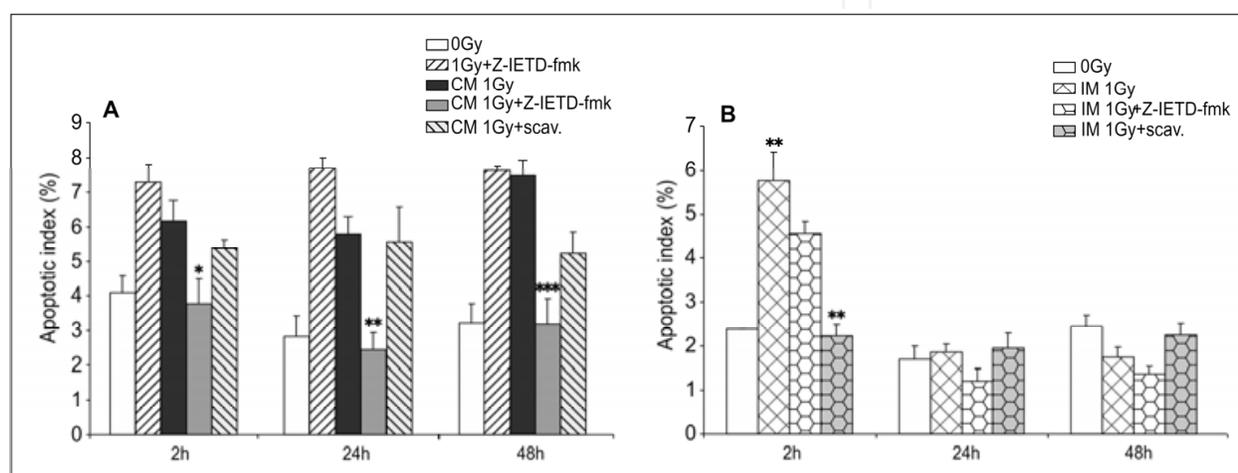


Fig. 14. Apoptotic index in irradiated cells (1Gy), in irradiated cells incubated with caspase-8 inhibitor (1Gy + Z-IETD-fmk), in bystander cells incubated with caspase-8 inhibitor (CM 1Gy + Z-IETD-fmk), and with ROS scavengers SOD and NAC (CM 1Gy + scav.). Results are the means of 3-5 independent experiments  $\pm$  S.E. Significant differences were observed in CM 1Gy vs CM 1Gy + Z-IETD-fmk at all times. **B.** Apoptotic index in cells incubated with irradiated medium (IM 1Gy), with irradiated medium in the presence of caspase-8 inhibitor (IM 1Gy + Z-IETD-fmk), and ROS scavengers (IM 1Gy + scav.). The values are the means of 3 independent experiments  $\pm$  S.E. Significant differences were observed at 2 h of incubation with IM 1 Gy vs IM 1Gy + Z-IETD-fmk (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001,  $t$ -test).

IM incubation for 2h increased the apoptotic index which was totally inhibited by ROS scavengers and little affected by incubation with the caspase-8 inhibitor, whereas at 24 and 48h no significant differences among samples incubated with IM were observed. DSBs induced by ionizing radiation can easily be detected by the extensive H2AX phosphorylation occurring near DNA lesions, forming foci that co-localize with several repair proteins (Fernandez-Capetillo et al., 2003). 85% of TK6 were  $\gamma$ -H2AX foci positive at 2h after irradiation with 1Gy, then this percentage decreased to the level of non-irradiated cells 24h later, fitting DNA repair kinetics. The incubation of cells with CM for 2h significantly increased the percentage of  $\gamma$ -H2AX foci positive cells (9-11%) but, when the CM was kept in contact with bystander cells for 24h the number of positive cells decreased to control levels, suggesting that DNA lesions induced at the beginning of CM incubation are repaired and no new damage accumulates later. Data from other human cells show that  $\gamma$ -H2AX foci induction in bystander cells persists in time, probably as a consequence of the

formation of bystander factors that themselves generate ROS, leading to a self-sustaining system responsible for long-lasting effects (Yang 2005, Sokolov 2005, Kashino 2004, Lyng 2006). In irradiated TK6 cells both 53BP1 and NBS1p343 proteins co-localized with  $\gamma$ -H2AX foci, whereas in bystander cells co-localization was partial or absent (Figure 15).

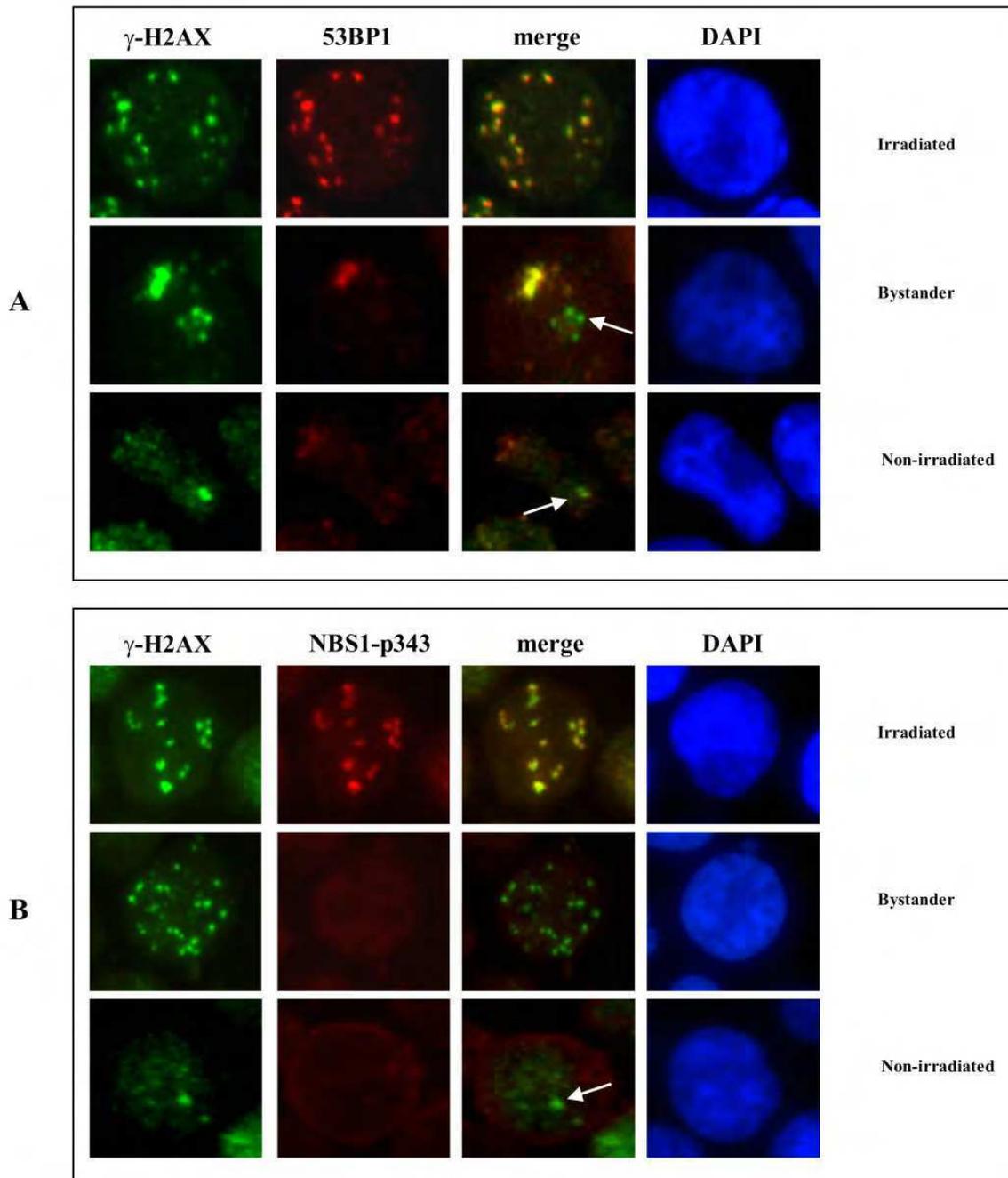


Fig. 15. Non-irradiated, irradiated and bystander TK6 cells were fixed and co-stained with anti-  $\gamma$ H2AX (green), anti-53BP1 and anti-NBS1-p343 (red), at 2 h from irradiation or CM incubation. The red and green images were merged and subjected to co-localization analysis. Arrows indicate  $\gamma$ H2AX foci without co-localization of 53BP1 and NBS1-proteins. Nuclei were counterstained with DAPI.

We suggest that the short-lived ROS released in the medium by irradiated cells are responsible for DNA lesions, unlike double strand breaks, which activate H2AX phosphorylation but do not require the 53BP1 and NBS1p343 proteins to be repaired. It is possible that in our experiments DNA damage induced by CM treatment consisted of a few DSBs, the repair of which requires the recruitment of 53BP1 and NBS1p343 proteins and mainly in other types of DNA lesions, in which repair occurs without these proteins. Recent studies suggest that there are important differences between the DNA damage response in directly irradiated cells and non-targeted cells via bystander signals. The DNA damage in bystander cells seems to persist for a prolonged time (Burdak-Rothkamm et al., 2007), differently from DNA damage induced directly by irradiation which is repaired completely within few hours depending on radiation dose. Studies carried out in p53 wild-type (TK6), p53 null (NH32), and p53 mutant (WTK1) lymphoblastoid cells using siRNA to knockdown DNA PKcs demonstrated the central role of non-homologous end-joining in processing bystander damage, in contrast to the role of homologous recombination which seems to be essential only in inducing sister chromatid exchanges in bystander cells (Zhang et al., 2008). ATM- and Rad3-related (ATR) protein kinases have a central role into DNA damage signaling in bystander cells, with ATM activation occurring downstream of ATR. DNA-PK is not essential in mortality inducing in bystander cells neither for bystander  $\gamma$ H2AX foci induction (Burdak-Rothkamm et al., 2007). These differences between bystander and direct DNA damage response offer new potential targets for repair inhibitors, with the aim to protect bystander normal tissues during cancer radiotherapy.

## 5. Conclusions

The DNA-damage response pathway relies on the recruitment and modification of many different proteins that sense and signal the damage, activate transducer and effector proteins involved in cell cycle arrest, DNA repair and apoptosis. A correct DDR safeguards cells, whereas perturbations/defects in this pathway might contribute to the occurrence or to the acceleration of carcinogenesis. Our results have contributed to highlight cell response of human lymphocytes to DNA damage induced directly or indirectly by ionizing radiation. In particular, novel aspects of low- and high-LET radiation effects on human lymphocytes have been described, such as double strand break repair kinetics, mutational effects, micronuclei induction, apoptosis induction, cell cycle alterations, gene and microRNA expression changes. In addition, we have reported new findings about the cell response of human lymphocytes when ionizing radiation exposure occurred in microgravity, condition which has been experimentally simulated by the Rotating Wall Vessel. The results clearly indicate that modeled microgravity affects the cell response to radiation, thus contributing to increase the risk of radiation exposure during space missions. By considering that the levels of DNA repair genes were not significantly changed in MMG condition, we suppose that perturbations in the cell response to ionizing radiation are due to the altered activity of proteins playing an important role in DDR pathway. Evidences are accumulating on the strict dependence between efficiency in DNA repair and chromatin structural organization (Gontijo et al., 2003, Rube et al., 2011). The elaborate higher-order organization of chromatin appears to be important in assembling the repair machinery, improving the accessibility of DNA lesions to repair complexes. Modifications of cell structure and perturbations of nuclear architecture induced by microgravity may affect the accessibility in chromatin to DNA repair machinery. The preliminary results obtained from miRNA-mRNA profiling

represent new insights about the radio-responsiveness in MMG. They seem promising to clarify the role of miRNAs in DNA-damage response to radiation in microgravity, thus improving the scientific approach towards environmental exposure risk.

The studies on molecular mechanisms of bystander effect could have great implications in evaluating radiation risk of IR exposures, and also have the potential to reassess radiation damage models currently used in radiotherapy. The radiation-induced bystander effect was shown to occur in a number of experimental systems both *in vitro* and *in vivo* and it is supposed to be realized through several pathways of transmission of the stress signal: a direct cellular contact, interaction through gap-junctions and through the culture medium of the irradiated cells. In our experimental system the conditioned medium was the main way by which the irradiated cells communicate their stressed condition to the non-irradiated cells. ROS released by irradiated TK6 cells into the culture medium were short-lived and probably other soluble molecules are necessary to maintain high the level of cell mortality in bystander cells. Recent studies, investigating on the nature of such molecules, suggest that fragments of extracellular genomic DNA, probably released from the apoptotic irradiated cells in the culture medium, are able to induce the bystander effects (Ermakov et al., 2011). Such DNA fragments bind to the Toll-like receptors family, leading to a signaling mechanism whose outcome is the dynamic transformation of the cytoskeleton and alteration in the spatial localization of chromatin portions in the nucleus. Thus, in bystander cells, as for microgravity-incubated lymphocytes, modifications in the nuclear structural organization may affect the assembly of the DNA repair machinery.

## 6. Acknowledgment

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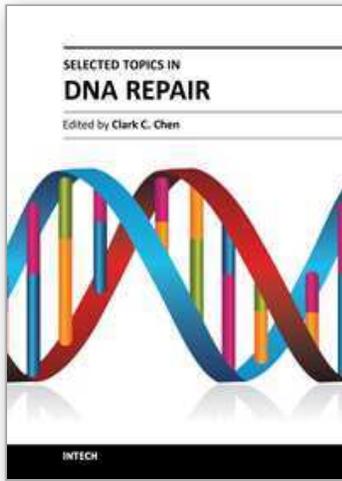
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