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Transcriptional Response of Human Umbilical Vein Endothelial Cells to Low Doses of Ionizing Radiation

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Ionizing radiation/Adaptive response/Transcriptional modulation/DNA microarrays/Gene clustering.

We used cDNA microarray hybridization technology to monitor the transcriptional response of Human Umbilical Vein Endothelial (HUVEC) cells to x-rays doses ranging from 2 to 200 cGy. An early time window from irradiation (4h) was selected in order to minimize the effects of the cell cycle blockage eventually induced at high doses of irradiation. Three different gene-clustering algorithms have been used to group the 4134 monitored ORF based on their transcriptional response in function of the irradiation dose. The results show that while few genes exhibit a typical dose-dependent modulation with a variable threshold, most of them have a different modulation pattern, peaking at the two intermediate doses. Strikingly even the lowest dose used (2 cGy) seems to be very effective in transcriptional modulation. These results confirm the physiological relevance of sublethal-dose exposures of endothelial cells and strengthens the hypothesis that alternative dose-specific pathways of radioadaptive response exist in the mammalian cells.

111 genes were found to be modulated at all doses of irradiation. These genes were functionally classified by cellular process or by molecular function. Genes involved in coagulation and peroxidase activity and structural constituent of ribosomes were over-represented among the up-regulated genes as compared with their expected statistical occurrence.

Three genes coding for regulatory kinase activities (CDK6; PRCKB1 and TIE) are found down-regulated at all doses of irradiation.

INTRODUCTION

Molecular responses to various genotoxic stresses, including wounding, nutrient depletion, changes in oxygen tension, oxidative stress and DNA-damaging agents such as ultraviolet and ionizing radiation and chemical mutagens, are complex and are mediated in all eukaryotes by a variety of regulatory pathways. A very important component of these responses is constituted by the transcriptional modulation of several cellular genes, initially identified in simple eukaryotes, such as yeast,[1)] where up to 1% or more of the genome appeared to be involved.[2–4)] It has now rapidly become clear that mammalian cells also respond to genotoxic stress at the transcriptional level.[5–8)] Many target genes have been identified. Some of them are specific to a particular genotoxic stress, while others could simply reflect a general response of the cell to injury. Some of the signaling pathways involved in the regulation of these target genes have been elucidated.[9,10)] In recent years the usefulness of functional genomics approaches to simultaneously analyze the transcriptional modulation of thousands of genes in response to ionizing radiation has been shown in yeast[2–4)] and in mammalian cells.[8,11,12)] The analysis of the great extent of data generated by using DNA microarrays technologies is facilitated by clustering algorithms that are now largely available and that can help the researchers to focus on important aspects of their data.[13,14,15)]

Historically, most published studies on transcriptional response to ionizing radiation in mammalian cells have used extremely high, even supralethal doses to ensure strong gene activation and only few reports focused on physiologically relevant doses.[10,16–18)] In particular, one of these studies[18)] has demonstrated that, in peripheral blood lymphocytes, sev-

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eral genes can consistently respond to ionizing radiation doses as low as 2 cGy. Moreover even the lowest dose (2 cGy) tested in this and in previous studies[18,19] resulted in transient changes in cell-cycle kinetics, suggesting a physiological relevance of the observed changes in gene expression.

One of the aspects of the transcriptional response to ionizing radiation is its defined cell type-specificity. For this reason it is very important to extend the study of the low-dose transcriptional effects to cellular lines of particular medical interest.

Endothelial cells play a pivotal role in modulating the inflammatory response. Modulation of apoptosis by ionizing radiation in endothelial cells is a very important issue for its possible implications for radiotherapy approaches due to the very important role that apoptosis has in the process of angiogenesis.[20] The knowledge of the effects of the exposures of these cells to low doses of ionizing radiation can therefore be crucial for the success of anticancer radiotherapic protocols. We show here that in the HUVEC cells several genes are modulated at all the tested doses, while others show a dose-dependent up-regulation. Strikingly, the lowest dose tested (2cGy) seems to be very effective in transcriptional modulation, suggesting a very high sensitivity of HUVEC cells to ionizing radiation, possibly due to bystander effects.

MATERIALS AND METHODS

Cells and Reagent

HUVEC cells and the medium for culture (Medium 200 with addition of LSGS) were purchased from Cascade Biologics, Inc. (Portland, Oregon, USA). Cells were grown in accord with Cascade protocol (http://www.cascadebiologics.com/Html/cellcult/HUVEC.html) in a humidified, 5% CO_2 atmosphere in a 37°C incubator and used at passage 4.

Irradiation and RNA Extraction

Cells approximately 80% confluent in tissue culture dishes of 15 cm were irradiated with X-rays (Gilardoni MGL 200/8D, 0.2 mm copper filtration, 200 KVp, 6mA) with doses 2, 50, 100 and 200 cGy (0.20 Gy/min). To maximally reduce variations in culture condition the cells were irradiated inside an incubator at 37°C, connected with X-ray generator and the controls were sham-irradiated. After x-rays exposure the cells were incubated at 37°C for 4 h, this early time window was selected in order to minimize the effects of the cell cycle blockage eventually induced at high doses of irradiation. RNA was subsequently isolated by using TRIzol Reagent according to the manufacturer's instructions (Invitrogen™ life technologies, http://www.invitrogen.com). RNA quality was insured by gel visualization and spectrophotometric analysis ($OD_{260/280}$). The quantity of RNA was measured using the OD_{260}.

cDNA microarrays

All the hybridizations were performed using Human 'Named Genes' GeneFilters® Microarrays Release 1 (GF211 Research Genetics) contains 4134 genes, all of which are of known function (See Supplementary Material SM1 on http://www.di.uniroma1.it/ale/fortuneCookie). RNA labelling with ^{33}P dCTP (reverse transcription) and filter hybridization were performed using 5 μg of total RNA for each experiment according to the manufacturer's protocols (See Supplementary Material SM4A on http://www.di.uniroma1.it/ale/fortuneCookie).

Data Analysis

The membranes of hybridized filters were placed in cassettes overlapped to phosphor image screens (Packard) and various exposures were performed for each experiment. Images were scanned by using a Cyclone Phosphor System (Packard). The resulting digital images were analyzed and compared using Pathways™ 4 software (Research Genetics) developed exclusively for use with Gene Filters membranes (See Supplementary Material SM4B on http://www.di.uniroma1.it/ale/fortuneCookie).

Data point normalization algorithms are used to correct for global intensity shift across multiple experimental images. This technique generates normalized intensities by dividing all sampled intensity by the mean sampled intensity of all clones, except the control points of total genomic DNA spots. These values are used in all calculations performed by the software to compare several conditions. For each dose four independent experiments were analyzed using the Student's test plugged in Pathways™ 4 software, which analyzes each clone at 95% confidence range in a condition quadruple to determinate if the difference in intensities is statistically significant. The test operates determining if the difference between four condition-averaged clones, at the specified confidence, is significant compared to the standard deviation of sampled intensities for each of the condition-averaged clones. This analysis offers both a Gaussian distributed and a distribution free form. The data presented in the table indicate the ratio between the normalized intensities of the filters hybridized with the RNA from the control filters for unirradiated cells and those from filters for irradiated cells. Values >1 represent up-regulation and values <1 represent down-regulation To group the genes significantly modulated we applied two different gene-clustering methods based on the intensities ratio between irradiated cells and controls in function of the irradiation dose.

In the first one we used an average-linkage hierarchical clustering system. In this technique the distance between clusters is calculated using average values calculated with the unweighted pair-group average (UPGMA) method.[13,14] The average distance is calculated from the distance between each point in a cluster and all other points in another cluster. The two clusters with the lowest average distance are joined

together to form a new cluster.

In the second a KMean Clustering algorithm is used to generate associations between clones within a given analysis set. This algorithm find points which are close together in a multi-dimensional clustering data space, using the intensity as cluster variable and an Euclidean distance between points in data space.[14)]

Validation by real-time PCR

Normal human umbilical vein endothelial cells (HUVEC) pooled from multiple donors, were irradiated 2, 50 100 or 200 cGy in the same conditions used for the microarrays analysis. RNA was extracted as described above. Real-time PCR was performed using the Applied Biosystem 7300 system and pre-developped TaqMan Gene Expression Assays (Applied Biosystem). cDNA was obtained as detailed in the TaqMan Gene Expression Assays accompanying protocol. Reactions were performed in duplex, amplifying the selected genes with the TaqMan Fam-labelled probe and primers included in the Gene Expression Assays together with the TaqMan Vik-labelled probe and primers for the endogenous housekeeping control which in our case was GADPH. This gene appeared not modulated by irradiation in the microarray analysis. 10–50 ng of cDNA were used for each assay.

Two independent experiments (each one loaded in duplicate) were performed for each of the six selected genes. Relative quantitation was performed using the 7300 software (Applied Biosystem) and the unirradiated control as calibrator.

The following Gene Expression Assays were used:

GADPH: 4326317E; CDK6: HS00608037; EDN1: HS00174961; LMAN: HS00194366; SERPINE1: HS001677155; SOD1: HS00166575; VWF: HS00168575. All the informations about primers sequences and probes are available at: http://marketing.appliedbiosystems.com/mk/get/0905_ALLGENE_LANDING?isource=From_www.allgenes.com

RESULTS

We used a system of high-density filter-based cDNA microarrays constituted by single membranes containing approximately 4,000 human ORFs of known function (see Materials and Methods). This system has been used before for transcriptional profiling of human cells in response to different physiological and environmental stimuli[21–23)] and proved to be very sensitive and accurate. HUVEC cells were grown and irradiated as described in Materials and Methods. Total RNA was isolated from irradiated or unirradiated control cells 4 h after irradiation. The Venn diagram reported in Fig. 1 shows the numerical distribution of the genes significantly up-regulated at least two fold in function of the irradiation dose. It is evident that even the lowest dose (2 cGy) is effective in eliciting a transcriptional response. A limited number of genes are up-regulated at one dose only, confirming that the use of four replicas and statistical filtering eliminated most of the false positives from our analysis.

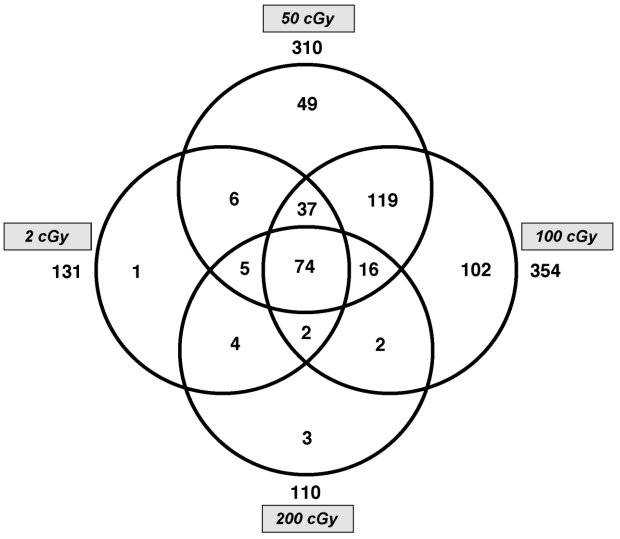

Fig. 1. Venn diagram showing the overlaps of genes up-regulated at different doses. The genes up-regulated at 2 and 100 cGy only (2 genes) and those up-regulated at 50 and 200 cGy only (4 genes) are not shown for graphical reasons.

The two intermediate doses (50 and 100 cGy) show a consistently higher number of up-regulated genes, compared with the other two doses. Moreover the genes up-regulated at the two intermediate doses exhibit the highest degree of overlapping (92%) among genes up-regulated at least at two doses. Overlaps among all the other doses is much lower: 45% between 2 and 50 cGy; 43% between 2 and 100 cGy; 37% between 50 and 200 cGy; 35% between 100 and 200 cGy and 56% between 2 and 200 cGy. When we look at three doses overlaps, we find the highest score for 2, 50 and 100 (41%) and the lowest for 2, 100 and 200 (28%).

Finally we find 74 genes up-regulated at least two fold at all doses.

From this quantitative analysis we can conclude that:

a) There is a very high degree of correlation among the genes significantly up-regulated at the various doses (the number of genes ranging from 15 to 27 σ).

b) A relatively high number of genes is up-regulated at the lowest dose (2 cGy).

c) There is a consistent increase in the number of up-regulated genes at the two intermediate doses (50 and 100 cGy) that show the highest degree of overlapping.

These data do not consider the differences in fold induction of the genes in function of irradiation dose. We therefore selected all the genes significantly modulated at least three doses and applied two different clustering methods to group them based on the intensities ratio between irradiated cells and controls in function of the irradiation dose.

Various clustering algorithms can be applied to the identification of pattern in gene-expression data.[13,14)] The most

used clustering algorithms are hierarchical; the resultant classification has an increasing number of nested classes and the result resembles a phylogenetic classification. The various hierarchical clustering algorithms differ in the manner in which distances are calculated between the growing clusters and the remaining data set, including other clusters. We used an average-linkage hierarchical clustering system (see materials and methods).

The results of this analysis are shown in Fig. 2 A. We can clearly identify two clusters of up-regulated genes (shown in red). The first one (cluster 1) includes genes similarly up-regulated at three or four doses.

The second cluster (cluster 2) groups a large number of genes that show the highest level of up-regulation at the two intermediate doses (50 and 100 cGy).

A limited number of genes are significantly down-regulated (shown in green) at at least three doses. They do not show a clear dose dependent modulation.

CAST clustering algorithms group data points close together in a "greedy" fashion without *a-priori* knowledge of the number of clusters. CAST randomly selects a point and forms a cluster from it finding all points sufficiently close to it, then repeats this process until all points are assigned to one cluster. A final optimisation swaps points between clusters increasing cluster similarity.

In this analysis CAST is used to evaluate number of clusters in the dataset, it shows only four clusters with more than ten data points. To validate this hypothesis, a Figure-Of-Merit was built with KMean algorithm changing the number of clusters and the results show that with four clusters the algorithm error is low.

KMean algorithms group data points into a specified number of clusters by finding the centre of the cluster and assigning data points to the nearest cluster centre in an iterative fashion. KMean is considered to be a partitional clustering algorithm because the clustering process consists of finding the best cluster (partition) for each clone. We used a KMean clustering algorithm to group genes that show a similar modulation in function of irradiation dose. Based on the results of the CAST analysis we fixed the maximum number of clusters to 4 and the number of interactions to 20 with tolerance to 1×10^{10}. In this case up-regulated and down-regulated genes showing the same intensity ratio profile are grouped in the same clusters. The results are shown in Fig. 2 B.

We can classify the four clusters obtained in 2 different categories:

– Cluster 1 corresponds to hierarchical cluster 2, grouping the genes which show the highest level of up-regulation at the two intermediate doses of irradiation but includes also the genes witch show the highest level of down-regulation at the two extreme doses (2 and 200 cGy).

– Clusters 2, 3 and 4 do not show a particular dose dependent modulation pattern.

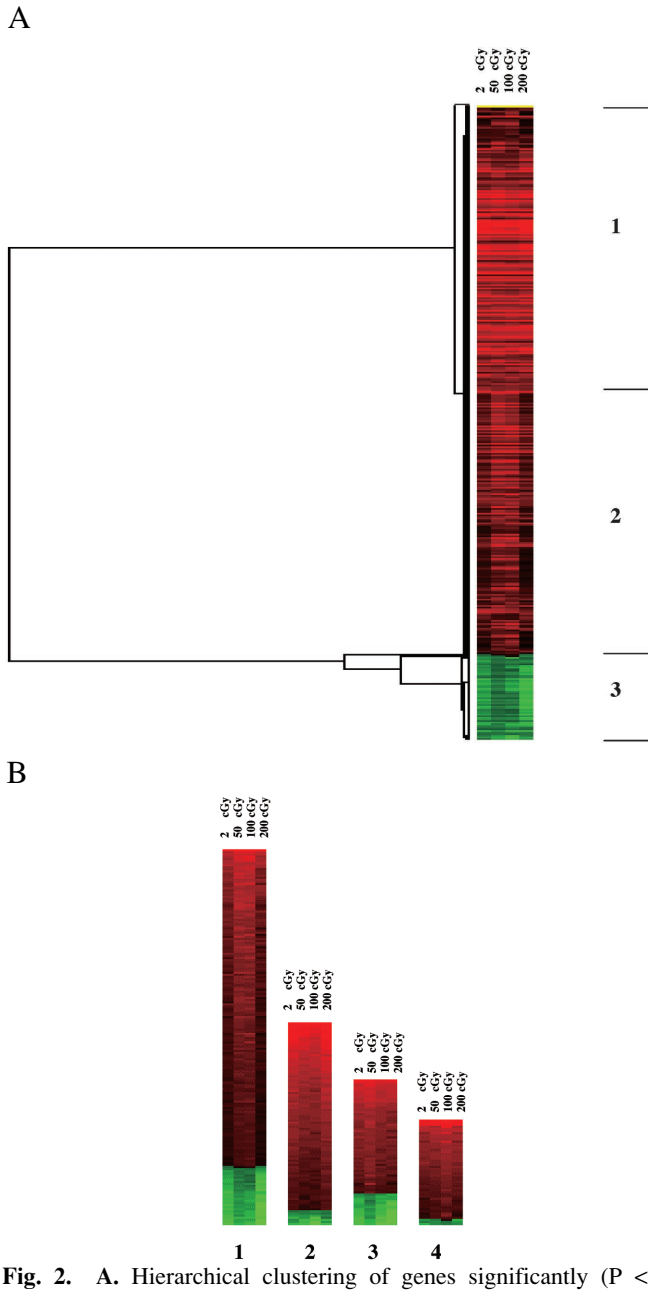

Fig. 2. **A.** Hierarchical clustering of genes significantly ($P < 0.05$) modulated at least two fold at three doses. The four columns represent the indicated doses of irradiation; the rows represent the modulated genes. In red up-regulated genes; in green down-regulated genes. **B.** KMean clustering of genes significantly ($P < 0.05$) modulated at least two fold at three doses. The four clusters are reported separately. Columns, rows and colours as in Fig. 2A.

DISCUSSION

From these analyses it is clear that a high number of genes shows the highest level of up-regulation at the intermediate doses. This observation is in agreement with what has been shown by the analysis of the quantitative distribution of the

up-regulated genes in function of the irradiation dose. The intermediate doses are indeed more effective in eliciting a transcriptional response. This general behavior has interesting implications in the interpretation of the radioadaptive response. The radioadaptive response is a phenomenon whereby the damaging effects of a high dose of ionizing radiation can be mitigated if the cells were previously exposed at a lower dose.[24] In mammalian cells the radioadaptive response has an optimum dose range below 0.1 Gy,[25] it occurs in metabolically active cells but not in dormant G0 cells, reaches is maximum 4–5 hours after irradiation but lasts at least 24 hours.[25] In order to explain the low optimum dose range of the adaptive response it has recently been proposed that two different and alternative response pathways exist in mammalian cells.[26] The first one would be activated by low doses by the protein kinase C through p38 MAP kinase and would activate P53. At higher doses, ERK and JNK kinases and WIP phosphatase would be activated. These proteins act as dominant negative regulators of p38 MAP kinase suppressing and/or competing its action on P53.[26,27] The differences in the transcriptional modulation pattern that we observe could be interpreted according to this model: the first pathway would respond to doses as low as 2 cGy and would activate a first set of genes. It would then be inactivated at doses higher than 100 cGy. A second pathway would be activated at 50 cGy remaining active at higher doses. Based on this scenario we would expect the highest activation at 50 and 100 cGy where the activity of the two pathways overlaps.

From this set of data we can also derive a selection of genes modulated at all doses of irradiation. They are presented in Table 1 and respond to the following criterion: they show modulation of at least 2 fold at all doses in the 95% confidence range according to Student T-test.

A Gene Ontology classification by "Cellular Process" (Table 2), performed with the "FatiGO" software (http://fatigo.bioinfo.cnio.es/), identifies only one category, "Coagulation", which is over-represented in the genes up-regulated at all doses (6.7%) as compared with the total population of the genes present on the array (1.4%). This category is also enriched in the genes up-regulated at any dose with 10 up-regulated genes (3.3%) (see Supplementary Materials Sm1, Sm2, Sm3A-B-C on http://www.di.uniroma1.it/ale/fortuneCookie). These genes are two membrane regulators of complement (CD59 and CD9); the plasminogen inhibitor Serpine 1, whose up-regulation correlates with thrombosis risk;[28] the thrombin receptor F2R; the gene coding for fibrinogen gamma polypeptide FGB; the ITGB3 gene coding for Beta 3 Integrin, involved in platelet signaling; the interleukin 10 receptor beta (IL10RB); the A5 annexin; the endothelial protein C receptor and VWF, coding for von Willebrand factor, a protein whose secretion in the blood is correlated with the signalling of endothelial damage.[29]

A classification of the genes up-regulated at all doses by "Molecular Function" identifies two over represented categories. The first one is "Structural Constituent of Ribosome" with 10.9% as compared with 0.59% in the complete list of the genes in the microarray. Stimulation of ribosomal proteins genes at doses unable to block the cell cycle and early times from irradiation has been previously observed,[3] while higher doses and later times are generally down-regulating the genes coding for protein synthesis apparatus components.[4]

Among the 74 genes up-regulated at all doses we also find 2 genes, PRDX1 and GPX1 (2.7%) out of 18 genes in the category of "Peroxidase Activity" present on the microarray (0.48%). Other 3 genes of the same category (PRDX2, PTGS1 and CAT) are up-regulated at some of the irradiation doses. Moreover, SOD1, coding for superoxide dismutase 1, another important antioxidant activity, is up-regulated at all doses. Induction of these enzymatic activities by ionizing radiation has been previously observed. Generally the inductions have been observed at doses higher than 2 cGy. In fact, the up-regulation of glutathione peroxidase at relatively low doses (50 cGy of gamma-ray) of ionizing radiation has been observed before[30] and is justified by the prominent role that this enzymatic activity has in protecting the cells from oxidative damage. PRDX1 has also been shown to have an essential role in antioxidant defense[31] but clear evidence of its inducibility at low dose of irradiation was still lacking. Although we do not observe enrichment in any other molecular functional category it is clear that some other genes previously shown to be involved in defense from irradiation in different cellular types are up-regulated at all doses.

We infact observe a net up-regulation at all doses also of two heat shock proteins mRNAs (HSPA8 and HSPCB) that could have a protective role against radiation damage[32] and of two translation initiation factor subunits (EIF3S2 and EIF2S2) whose induction by radiation has been previously shown in another cellular system.[33]

Another gene whose up-regulation by ionizing radiation could be expected is XRCC5 (Ku80), known as an important member of the non-homologous end-joining (NHEJ) pathway of DNA double strand break (DSB) repair, and previously shown to be responsive to ionizing radiation in other cellular systems.[33] Once again, its inducibility at low doses is novel.

Among the genes most strongly up-regulated at all doses (>10 fold) we find BRCA1, a key gene in DNA damage sensing and repair.[34]

Finally, we also find up-regulated at all doses a gene that has very important functions in the endothelial cells: EDN1, coding for endothelin 1 a protein secreted by endothelial cells that has crucial effects in a series of human pathologies.[35] When we look at the 37 genes down-regulated at all doses we find that the only Molecular Function category which is enriched (20% versus 9%) is "Kinase activity". 5 down-regulated genes are classified in this category: CDK6,

Table 1. ($p < 0.05$; $0.5 < ratio > 2$).

Genes up-regulated					
acc	gene	Radiation dose (cGy)			
		2	50	100	200
R40850	ACTR1A	5,33 ± 2,48	9,25 ± 5,22	10,48 ± 5,00	5,00 ± 2,33
H14808	ATP1B2	4,35 ± 2,32	3,45 ± 1,70	3,66 ± 1,81	4,71 ± 2,80
AA779480	BMP8	5,44 ± 3,34	4,87 ± 2,75	4,92 ± 2,73	6,87 ± 4,43
H90415	BRCA1	18,71 ± 12,50	14,06 ± 9,26	11,16 ± 6,70	23,82 ± 17,12
R83000	BTF3	4,29 ± 2,28	4,41 ± 1,68	4,73 ± 2,14	4,07 ± 2,05
R44288	CALM2	3,93 ± 1,93	6,98 ± 2,79	5,98 ± 3,81	4,48 ± 2,58
R37953	CAP	2,41 ± 1,07	6,94 ± 2,25	5,24 ± 2,41	2,11 ± 0,91
N57964	CCR6	3,84 ± 1,77	2,95 ± 1,31	2,95 ± 1,49	4,93 ± 2,58
H07880	CCT6A	2,73 ± 1,19	4,45 ± 1,37	3,53 ± 1,93	2,24 ± 0,85
H60549	CD59	2,85 ± 1,73	4,99 ± 2,71	3,89 ± 1,84	2,68 ± 1,65
AA421296	CD68	4,33 ± 2,55	3,61 ± 2,11	2,88 ± 1,27	3,82 ± 2,14
AA113872	CLTA	2,55 ± 1,03	3,94 ± 1,74	3,22 ± 1,62	2,25 ± 0,88
AA284856	CNN2	3,71 ± 1,88	3,43 ± 1,25	3,68 ± 0,96	3,61 ± 1,88
AA425664	COMT	2,92 ± 1,50	7,60 ± 3,95	5,12 ± 2,62	2,13 ± 1,21
AA485427	CRIP2	2,58 ± 1,27	3,84 ± 1,73	3,14 ± 1,75	2,05 ± 1,04
T65118	CTNNA1	2,86 ± 1,04	4,25 ± 1,28	3,82 ± 1,67	2,62 ± 1,01
AA487452	DFFA	7,35 ± 3,58	6,60 ± 3,02	6,86 ± 3,05	9,95 ± 5,15
H11003	EDN1	3,36 ± 1,32	3,41 ± 1,00	3,40 ± 1,35	2,62 ± 1,08
R93621	EIF2S2	7,14 ± 4,11	7,78 ± 3,48	6,06 ± 3,72	6,79 ± 3,54
AA936783	EIF3S2	4,66 ± 2,45	5,43 ± 2,64	5,65 ± 2,23	5,74 ± 3,23
AA669674	EIF3S6	6,43 ± 4,23	5,68 ± 2,73	6,23 ± 3,56	4,96 ± 3,04
H09590	EIF4A1	3,86 ± 1,82	7,88 ± 3,49	7,29 ± 4,66	5,21 ± 2,43
H05919	EIF4A2	2,98 ± 0,51	3,67 ± 0,63	2,90 ± 0,99	3,12 ± 0,83
T98887	G6PC	4,14 ± 2,21	4,84 ± 2,49	4,08 ± 2,02	3,91 ± 2,20
AA629909	GARS	4,11 ± 2,39	3,94 ± 1,43	2,62 ± 1,20	2,94 ± 1,51
AY251759	GNAS1	3,71 ± 2,69	5,02 ± 2,71	3,40 ± 1,91	2,92 ± 1,82
R96220	GNB2L1	8,96 ± 5,46	6,28 ± 2,57	6,26 ± 3,87	9,05 ± 5,25
AA485362	GPX1	3,37 ± 1,55	3,81 ± 1,57	3,48 ± 1,50	2,91 ± 1,13
R20554	GRP58	2,15 ± 0,35	3,32 ± 0,52	3,53 ± 1,15	2,13 ± 0,52
H62527	GW128	5,73 ± 3,15	6,52 ± 3,55	6,38 ± 3,50	7,31 ± 4,51
AA448261	HMGIY	3,57 ± 1,35	2,97 ± 0,86	3,18 ± 1,71	2,86 ± 1,02
R37286	HNRPA1	9,26 ± 4,03	11,93 ± 4,32	16,09 ± 9,08	10,25 ± 5,06
AL391241	HSPA8	5,52 ± 2,97	6,60 ± 3,18	7,09 ± 3,58	3,36 ± 1,66
R44334	HSPCB	3,83 ± 1,69	6,50 ± 2,97	6,59 ± 3,13	3,76 ± 1,89
AA504656	LTBP1	3,06 ± 1,69	2,85 ± 1,04	2,08 ± 0,98	2,95 ± 1,50
N32199	MLANA	3,44 ± 1,82	4,58 ± 2,07	4,97 ± 1,82	4,05 ± 2,32
AL356259	MRPL3	3,32 ± 1,66	6,41 ± 3,52	5,58 ± 2,96	2,61 ± 1,23

Genes up-regulated					
acc	gene	Radiation dose (cGy)			
		2	50	100	200
R22977	MSN	2,50 ± 0,99	2,88 ± 0,98	3,55 ± 0,90	2,72 ± 1,13
AA664241	NACA	4,32 ± 2,40	4,28 ± 1,78	4,15 ± 2,21	3,89 ± 2,33
AA496628	NME2	4,75 ± 2,77	5,30 ± 2,79	3,97 ± 2,44	4,66 ± 2,97
AA404619	NT5B	4,67 ± 2,52	3,46 ± 1,74	3,31 ± 1,59	4,16 ± 2,12
AA487466	OAZ1	2,30 ± 1,03	4,22 ± 1,74	3,02 ± 1,48	2,44 ± 0,98
R38433	PFKP	2,90 ± 1,32	3,31 ± 1,35	2,66 ± 1,04	2,00 ± 1,12
AY890164	POLR2L	2,52 ± 1,43	3,81 ± 1,47	2,61 ± 1,48	2,88 ± 1,52
AA775803	PRDX1	3,33 ± 1,70	7,89 ± 4,51	6,12 ± 3,04	2,79 ± 1,46
H05893	PSMD2	2,75 ± 1,31	6,43 ± 3,77	5,44 ± 2,98	2,05 ± 0,86
AY888255	RAB6A	35,36 ± 25,20	26,27 ± 17,68	21,08 ± 14,09	39,72 ± 28,80
AA626787	RAC1	5,04 ± 3,22	5,17 ± 2,52	4,73 ± 2,59	3,56 ± 1,92
AA083485	RPL19	3,40 ± 1,73	4,46 ± 1,89	3,61 ± 1,71	4,19 ± 2,18
AA464743	RPL21	10,47 ± 6,07	10,53 ± 5,20	8,23 ± 5,03	11,50 ± 6,59
AA633768	RPL24	15,11 ± 9,15	11,37 ± 4,25	9,48 ± 5,55	17,38 ± 10,43
R43544	RPL32	3,03 ± 1,11	2,70 ± 0,54	2,85 ± 0,54	3,11 ± 1,16
AA625634	RPL35	5,77 ± 3,33	10,89 ± 5,68	10,47 ± 6,07	6,57 ± 3,44
AA873351	RPL35A	7,73 ± 5,26	6,61 ± 3,53	5,65 ± 3,01	9,53 ± 6,42
AA496880	RPL5	9,38 ± 6,85	7,42 ± 4,44	6,46 ± 3,72	10,16 ± 7,04
AA872341	RPS15A	12,09 ± 8,45	10,19 ± 5,40	9,34 ± 6,09	11,90 ± 7,67
AA625632	RPS27A	9,71 ± 5,27	10,00 ± 4,29	6,98 ± 3,74	11,47 ± 6,54
AA888182	RPS4X	12,60 ± 9,18	10,47 ± 6,93	8,54 ± 5,41	12,98 ± 9,18
T69468	RPS4Y	7,49 ± 4,19	11,64 ± 7,32	8,28 ± 3,47	7,72 ± 4,68
AA683050	RPS8	19,26 ± 10,55	27,04 ± 12,32	27,18 ± 15,01	18,16 ± 8,50
AA011215	SAT	2,93 ± 1,25	2,40 ± 0,95	3,06 ± 1,60	2,38 ± 1,15
W81191	SC65	16,54 ± 10,28	10,89 ± 6,12	9,24 ± 5,13	16,13 ± 10,78
AY410859	SERPINB6	4,99 ± 3,25	4,69 ± 2,49	4,07 ± 2,46	6,79 ± 4,53
AY81655	SERPINE1	2,52 ± 1,20	5,54 ± 2,33	4,64 ± 2,20	2,22 ± 1,03
N26026	SIP1	20,82 ± 14,56	15,87 ± 10,79	12,51 ± 8,26	19,67 ± 14,42
R61295	SLC25A6	2,82 ± 1,25	3,68 ± 1,19	3,88 ± 1,38	2,98 ± 1,41
AA599127	SOD1	3,35 ± 1,02	5,21 ± 1,30	3,51 ± 1,08	3,19 ± 1,08
AA630017	TCEB2	2,50 ± 0,49	2,57 ± 0,76	2,15 ± 0,72	2,41 ± 0,77
AA634103	TMSB4X	4,74 ± 2,98	5,26 ± 2,95	4,81 ± 2,74	4,88 ± 2,97
AA878561	UBA52	4,76 ± 1,96	5,31 ± 2,92	5,89 ± 2,49	4,88 ± 1,87
AA485226	VDR	5,56 ± 3,48	5,15 ± 3,18	4,01 ± 2,31	4,81 ± 2,77
AA485883	VWF	3,48 ± 1,58	2,81 ± 1,17	5,04 ± 2,11	4,31 ± 2,20
AA775355	XRCC5	2,36 ± 1,19	3,13 ± 1,17	3,87 ± 1,94	2,27 ± 1,09
AA629838	ZNF74	4,06 ± 2,23	3,91 ± 1,28	3,76 ± 1,33	4,55 ± 2,20

acc	gene	Radiation dose (cGy)			
		2	50	100	200
Genes down-regulated					
R01733	AMPD3	0,25 ± 0,16	0,31 ± 0,18	0,30 ± 0,19	0,24 ± 0,13
AA459663	AOE372	0,19 ± 0,10	0,23 ± 0,11	0,24 ± 0,12	0,21 ± 0,10
N51018	BGN	0,26 ± 0,22	0,36 ± 0,23	0,25 ± 0,14	0,17 ± 0,11
AA463225	BMP4	0,36 ± 0,18	0,44 ± 0,19	0,38 ± 0,14	0,38 ± 0,15
N53512	CACNA2D2	0,28 ± 0,16	0,40 ± 0,20	0,38 ± 0,20	0,27 ± 0,13
H44953	CASP4	0,23 ± 0,18	0,34 ± 0,22	0,29 ± 0,19	0,24 ± 0,15
H20743	CDC34	0,26 ± 0,12	0,28 ± 0,09	0,30 ± 0,10	0,37 ± 0,20
H73724	CDK6	0,22 ± 0,19	0,32 ± 0,21	0,34 ± 0,24	0,18 ± 0,13
H94487	CTSE	0,24 ± 0,19	0,42 ± 0,28	0,39 ± 0,25	0,21 ± 0,11
AA608557	DDB1	0,27 ± 0,19	0,41 ± 0,24	0,31 ± 0,18	0,21 ± 0,12
AA428778	EFNB1	0,23 ± 0,17	0,32 ± 0,20	0,30 ± 0,19	0,19 ± 0,14
H49443	KIAA1029	0,29 ± 0,18	0,23 ± 0,13	0,15 ± 0,06	0,32 ± 0,15
AA156793	KIAA1247	0,27 ± 0,18	0,42 ± 0,23	0,44 ± 0,22	0,27 ± 0,15
H99588	LAF4	0,25 ± 0,21	0,36 ± 0,22	0,30 ± 0,18	0,17 ± 0,11
H54023	LILRB2	0,25 ± 0,20	0,34 ± 0,21	0,32 ± 0,19	0,16 ± 0,12
AA446103	LMAN1	0,18 ± 0,14	0,30 ± 0,18	0,22 ± 0,14	0,16 ± 0,11
T94169	MAPK8	0,20 ± 0,21	0,35 ± 0,26	0,18 ± 0,11	0,19 ± 0,15
AA598611	NR4A2	0,27 ± 0,18	0,37 ± 0,19	0,46 ± 0,26	0,37 ± 0,21
AA405731	PCK1	0,19 ± 0,15	0,28 ± 0,19	0,21 ± 0,17	0,16 ± 0,12
AA521431	PFN1	0,49 ± 0,32	0,33 ± 0,18	0,33 ± 0,20	0,24 ± 0,19
H11660	PIG11	0,34 ± 0,18	0,37 ± 0,15	0,39 ± 0,16	0,46 ± 0,23
AA702548	PIG8	0,19 ± 0,17	0,25 ± 0,17	0,16 ± 0,09	0,29 ± 0,20
T73294	POR	0,19 ± 0,15	0,30 ± 0,21	0,20 ± 0,14	0,18 ± 0,12
AA676404	PPIC	0,26 ± 0,16	0,42 ± 0,20	0,28 ± 0,14	0,21 ± 0,12
AA479102	PRKCB1	0,23 ± 0,20	0,38 ± 0,27	0,31 ± 0,24	0,14 ± 0,10
AA436163	PTGES	0,25 ± 0,18	0,36 ± 0,23	0,25 ± 0,15	0,19 ± 0,14
AA476461	PTPRZ1	0,16 ± 0,04	0,24 ± 0,11	0,38 ± 0,16	0,47 ± 0,28
H46425	PURA	0,31 ± 0,21	0,45 ± 0,24	0,42 ± 0,20	0,35 ± 0,20
R96626	SCYA14	0,37 ± 0,28	0,24 ± 0,13	0,22 ± 0,10	0,28 ± 0,15
H46254	SLC6A1	0,25 ± 0,17	0,21 ± 0,13	0,12 ± 0,05	0,23 ± 0,11
AA001897	SPTA1	0,23 ± 0,19	0,33 ± 0,21	0,26 ± 0,15	0,16 ± 0,11
W47485	SR-BP1	0,19 ± 0,13	0,31 ± 0,17	0,30 ± 0,16	0,15 ± 0,06
R51912	SST	0,21 ± 0,19	0,35 ± 0,25	0,30 ± 0,22	0,14 ± 0,12
AA609599	SSX3	0,22 ± 0,14	0,33 ± 0,18	0,34 ± 0,26	0,21 ± 0,12
AA432062	TIE	0,28 ± 0,18	0,29 ± 0,14	0,22 ± 0,10	0,24 ± 0,11
H65066	VSNL1	0,24 ± 0,18	0,37 ± 0,23	0,29 ± 0,17	0,20 ± 0,14
AA425602	ZP3A	0,31 ± 0,13	0,42 ± 0,20	0,24 ± 0,09	0,42 ± 0,14

Table 2. Genes up and down-regulated reported in Table 1. Software by Bioinformatics Unit - CNIO (http://fatigo.bioinfo.cnio.es /). Datamining with Gene Ontology (GO).

GO process Genes	
cell communication	**regulation of cellular process**
VDR CTNNA1 PTGES TIE EFNB1 EDN1 PPIC GRP58 SLC6A1 DFFA COMT MAPK8 PRKCB1 VWF CCR6 RAB6A NR4A2 CD59	BRCA1 NME2 EDN1 CASP4
mesoderm development	coagulation
BMP4 TIE	SERPINE1 LMAN1 VWF CD59
metabolism	**organismal physiological process**
VDR BRCA1 MRPL3 PTGES PTPRZ1 SSX3 TIE NME2 CDC34 PFKP EIF4A2 RPL35 RPL21 CCT6A LMAN1 HSPA8 PPIC AMPD3 ZNF74 GRP58 DDB1 CDK6 CTSE XRCC5 RPL35A HSPCB POR PCK1 GPX1 DFFA EIF2S2 COMT MAPK8 CASP4 SIP1 PRKCB1 GARS HNRPA1 RPL5 G6PC SOD1 TCEB2 NR4A2 EIF3S2 OAZ1 BTF3 PURA LAF4 NACA	PTGES EDN1 SERPINE1 LMAN1 CTSE SLC6A1 COMT VWF CCR6 NR4A2 CD59
pathogenesis	**cellular physiological process**
EDN1	SLC25A6 BMP4 SPTA1 BRCA1 NME2 EDN1 CDC34 CNN2 LMAN1 CLTA GRP58 CDK6 CTSE SLC6A1 SC65 DFFA MAPK8 CASP4 PFN1 HNRPA1 VWF CCR6 RAB6A MSN ATP1B2 PSMD2 PURA PRDX1
morphogenesis	**response to stimulus**
BMP4 SPTA1 TIE EFNB1 SOD1 PRDX1	BRCA1 PTGES HSPA8 DDB1 CTSE XRCC5 HSPCB GPX1 MAPK8 VWF SOD1 CCR6 NR4A2 CD59
growth	**regulation of physiological process**
BMP4	VDR BRCA1 SSX3 NME2 EDN1 EIF4A2 ZNF74 HSPCB TCEB2 NR4A2 EIF3S2 BTF3 PURA LAF4
	death
	BRCA1 DFFA CASP4
Genes with GO but NOT in this ontology process	
BGN CACNA2D2 CD68 CRIP2 LTBP1 MLANA SAT SERPINB6	
Genes without GO annotated	
ACTR1A AOE372 BMP8 CALM2 CAP EIF3S6 EIF4A1 GNAS1 GNB2L1 GW128 HMGIY KIAA1029 KIAA1247 LILRB2 NT5B PIG11 PIG8 POLR2L RAC1 RPL19 RPL24 RPL32 RPS15A RPS27A RPS4X RPS4Y RPS8 SCYA14 SR-BP1 SST TMSB4X UBA52 VSNL1 ZP3A	

Table 3. Quantitation of mRNA for the 6 indicated genes in irradiated cells relative to non irradiated control cells by real-time PCR.

Acc	Gene	Radiation dose (cGy)			
		2	50	100	200
	CDK6	0.04 (0.015)	0.06 (0.015)	0.04 (0.02)	0.04 (0.005)
	EDN1	30.1 (0.5)	12.3 (5.2)	2.8 (0.4)	2.7 (0.5)
	SERPINE1	0.73 (0.1)	2.0 (0.3)	1.8 (0.5)	3.5 (0.5)
	LMAN1	0.025 (0.015)	0.015 (0.005)	0.06 (0.01)	0.03 (0.02)
	SOD1	1.91 (0.02)	1.08 (0.06)	2.02 (0.9)	0.83 (0.17)
	VWF	0.21 (0.08)	0.38 (0.15)	0.24 (0.15)	0.33 (0.11)

Variability indicated in parenthesis.

MAPK8, PCK1, PRKCB1 and TIE.

CDK6 is a very important regulator of response to damage and its transcriptional down-regulation has been correlated with radioresistence.[36] The beta subunit of protein kinase C could also be involved in regulative response to irradiation.[26] TIE is a tyrosine kinase with immunoglobulin and epidermal growth factor homology domains which is accurately regulated in endothelial cells.[37] Transcriptional down-regulation by irradiation of these three kinase activities in endothelial cells is a very interesting observation whose implications deserve to be more deeply analyzed .

Genes down-regulated at all doses are not significantly enriched in any "Cellular Function" category.

These genes could constitute sensitive biomarkers for endothelial cells irradiation but to be really useful in biodosimetry should be carefully validated by independent techniques, something that goes beyond the goal of this work. Nevertheless, to get an extimate of the reliability of these data and eventual clone-dependent effects, we decided to test the modulation of six of these genes at all doses of irradiation in a pool of HUVEC clones by Real-Time PCR. We choose six of the genes discussed above: CDK6; EDN1; LMAN1; SERPINE1; SOD1 and VWF. Reactions were performed in duplex, using GADPH as endogenous control. This gene appeared not modulated by irradiation in the microarray analysis.

Two independent experiments (each one loaded in duplicate) were performed for each of the six selected genes. Results, presented in Table 3, show a good qualitative correlation between microarrays and real-time PCR data. Infact five out of six genes show modulation by irradiation with 17/24 experimental points exhibiting the same kind of modulation observed in the microarrays analysis. CDK6, EDN1, and LMAN1 are modulated at all doses, as expected. SERPINE1 is induced at 50, 100 and 200 cGy, while is slightly repressed at 2 cGy. SOD1 appears induced at 2 and 100 cGy and not modulated at 50 and 200 cGy. Only for VWF real-time PCR data contradict microarrays analysis, showing repression instead of induction at all doses. This discrepancy could be explained by a false positive in the microarrays analysis, alternative splicing forms selectively detected by real-time PCR or clonal variability.

From a quantitative point of view, the modulations observed in the real-time experiments appear much stronger than in the microarrays analysis for EDN1, CDK6 and LMAN1 and comparable for SERPINE1 and SOD1.

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REFERENCES

1. Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C. and Elledge, S. J. (1994) The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. **8**: 2401–2415.
2. Jelinski, S. A. and Samson, L. D. (1999) Global response of S. cerevisiae to an alkylating agent. Proc. Natl. Acad. Sci. USA **96**: 1486–1491.
3. De Sanctis, V., Bertozzi, C., Costanzo, G., Di Mauro, E., Negri, R. (2001) Cell cycle arrest determines the intensity of the global transcriptional response of Saccharomyces cerevisiae to ionizing radiation. Radiat Res. **156**: 379–387.
4. Gasch, A. P., Huang, M., Metzner, S., Botstein, D., Elledge, S. J., Brown, P. O. (2001) Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog Mec1p. Mol Biol Cell **12**: 2987–3003.
5. Weichselbaum, R. R., Hallahan, D. E., Sukhatme, V., Dritschilo, A., Sherman, M. L. and Kufe, D. W. (1991) Biological consequences of gene regulation after ionizing radiation exposure. J. Natl. Cancer Inst. **83**: 480–484.
6. Fornace Jr, A. J. (1992) Mammalian genes induced by radiation: activation of genes associated with growht control. Annu. Rev. Genet. **26**: 507–562.

7. Herrlich, P., Ponta, H. and Rahmsdorf, H. J. (1992) DNA damage induced gene expression: signal transduction and relation to growth factor signalling. Rev. Physiol. Biochem. Pharm. **119**: 187–223.
8. Amudson, A. A., Bittner, M., Chen, Y., Trent, J., Meltzer, P. and Fornace Jr, A. J. (1999) Fluorescent cDNA microarray hybridization reveals complexity and heterogeneity of cellular genotoxic stress responses. Oncogene **18**: 3666–3672.
9. Holbrook, N. J., Liu, Y. and Fornace Jr., A. J. (1996) Signalling events controlling the molecular response to genotoxic stress. In Stress-Inducible Cellular Responses (U. Feige, R. I. Morimoto, I. Yahara and B. S. Polla Eds.), pp. 273–288. Birkhauser Verlag, Basel.
10. Dent, P., Yacoub, A., Contessa, J. *et al*. (2003) Stress and radiation-induced activation of multiple intracellular signalling pathways Rad. Res. **159**: 283–300.
11. Amudson, A. A., Bittner, M., Meltzer, P. Trent, J., and Fornace Jr., A. J. (2001) Induction of gene expression as a monitor of exposure to ionizing radiation. Radiat Res. **156**, 657–661.
12. Tusher, V. G., Tibshirani, R. and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98**: 5116–5121.
13. Eisen, M. B., Spellman, P. T., Brown, P. O. and Bottstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA **95**: 14863–14868.
14. Quackenbush, J. Computational analysis of microarrays data. 2001 Nature Reviews Genetics **2**: 418–427.
15. Yasumoto, J., Imai Y., Takahashi, A., Ohnishi. K., Yuki, K., Kirita, T. and Ohnishi, T. (2003) Analysis of apoptosis-related gene expression after X-ray irradiation in human tongue squamous cell carcinoma cells harboring wild-type or mutated *p53* gene. J. Radiat. Res. **44**: 41–45.
16. Amudson, A. A., Do, K. T., Shahab, S., Bittner, M., Meltzer, P. Trent, J., and Fornace Jr., A. J. (2000) Identification of potential mRNA biomarkers in peripheral blood lymphocytes for human exposure to ionizing radiation. Radiat. Res. **154**: 342–346.
17. Woloshak, G. E., Chang-Liu, C. M., Jones, P. S. and Jones, C. A. (1990) Modulation of gene expression in Syrian hamster embryo cells following ionizing radiation. Cancer Res. **50**: 339–344.
18. Amudson, A. A., Do, K.T. and Fornace Jr., A. J. (1999) Induction of stress genes by low doses of gamma rays. Radiat Res. **152**: 225–231.
19. Salone, B., Grillo, R., Aillaud, M., Bosi, A. and Olivieri, G. (1996) Effects of low-dose (2 cGy) X-ray on cell-cycle kinetics and on induced mitotic delay in human lymphocyte. Mutat. Res. **351**: 193–197.
20. Segura, I., Serrano, A., Gonzalez De Buitrago, G. *et al*. (2002) Inhibition of programmed cell death impairs *in vitro* vascular-like structure formation and reduces *in vivo* angiogenesis. FASEB J. **16**: 833–841.
21. Schaub, F. J., Han, D. K. M., Liles, W. C. *et al*. (2000) Fas/FADD-mediated activation of a specific program of inflammatory gene expression in vascular smooth muscle cells. Nature Medicine, **6**: 790–796.
22. Walker, J. and Rigley, K. (2000) Gene expression profiling in human peripheral blood mononuclear cells using high-density filter-based cDNA microarrays. Journal of Immunological Methods, **239**: 167–179.
23. McCormick, S. M., Eskin, S. G., McIntire, L. V. *et al*. (2001) DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. Proc. Natl. Acad. Sci. USA **98**: 8955–8960.
24. Olivieri, G., Bodycote, J. and Wolf, S. (1984) Adaptive response of human lymphocytes to low concentration of radioactive thymidine Science **223**: 594–597.
25. Shadley, J. D. (1994) Chromosomal adaptive response in human lymphocytes. Radiat. Res. **138**: S9–S12.
26. Sasaki, MS., Ejima, Y., Tachibana, A. *et al*. (2002) DNA demage response in radioaptive response. Mutat. Res. 504, 101–118.
27. Shimizu, T., Kato, T. Jr., Tachibana, A. and Sasaki, M. S. (1999) Coordinated regulation of radioadaptive response by protein kinase C and p38 mitogen-activated protein kinase. Exp. Cell Res. **251**: 424–432.
28. Yamamoto K, Shimokawa T, Yi H, Isobe K, Kojima T, Loskutoff DJ, Saito H. Aging accelerates endotoxin-induced thrombosis : increased responses of plasminogen activator inhibitor-1 and lipopolysaccharide signaling with aging. Am. J. Pathol. 2002 **161**: 1805–1814.
29. Nadar, S. K., Yemeni, E. A., Blann, A. D. and Lip, G. Y. (2004) Thrombomodulin, von Willebrand factor and E-selectin as plasma markers of endothelial damage/dysfunction and activation in pregnancy induced hypertension. Thromb Res. **113**: 123–128.
30. Bravard, A., Luccioni, C., Moustacchi, E. and Rigaud, O. (1999) Contribution of antioxidant enzymes to the adaptive response to ionizing radiation of human lymphoblasts. Int. J. Radiat. Biol. **75**: 639–645.
31. Neumann, C. A. Krause, D. S., Carman, C. V. *et al*. (2003) Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defense and tumor suppression. Nature **424**: 561–565.
32. Kang, C. M., Park, K. P., Cho, C. K. *et al*. (2002) Hspa4 (HSP70) is involved in theradioadaptive response: results from mouse splenocytes. Radiat Res. **157**: 650–655.
33. Otomo, T., Hishii, M., Arai, H., Sato, K. and Sasai, K. (2004) Microarray Analysis of Temporal Gene Responses to Ionizing Radiation in Two Glioblastoma Cell Lines: Up-regulation of DNA Repair Genes. J. Radiat. Res. **45**: 53–60.
34. Stewart, G. and Elledge, S. J. (2002) The two faces of BRCA2, a FANCtastic discovery. Mol. Cell **10**: 2–4.
35. Zsary, A., Szucs, S., Schneider, T. *et al*. (2002) Role of endothelin-1 in the development of a special type of cardiomyopathy. Clinical science **103:** 272–275.
36. Sato,T. N., Tozawa,Y., Deutsch,U., Wolburg-Buchholz,K., Fujiwara,Y., Gendron-Maguire,M., Gridley,T., Wolburg,H., Risau,W. and Qin,Y. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation Nature **376** (6535): 70–74 (1995).
37. Fukuda K, Sakakura C, Miyagawa K, Kuriu Y, Kin S, Nakase Y, Hagiwara A, Mitsufuji S, Okazaki Y, Hayashizaki Y, Yamagishi H. Differential gene expression profiles of

radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation. Br. J. Cancer. 2004 **91**: 1543–1550.

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Supplementary material

SM1 reports the list of all genes in GF211 microarrays used on this analysis.

SM2 includes ratio values and Gene Ontology classification by "Cellular Process" or "Molecular Function" of all genes modulated at least 2 fold at any irradiation dose.

SM3A reports ratio values of all genes significantly up or down-regulated ($p<0.05$) at least two fold at 2 cGy, 50 cGy, 100 cGy, 200 cGy.

SM3B shows Gene Ontology classification of all genes significantly up-regulated reported in SM3A

SM3C shows Gene Ontology classification of all genes significantly down-regulated reported in SM3A

SM4A includes detailed DNA microarray protocols, from isolating RNA to storing GeneFilters (RNA isolation, probe labeling, and hybridisation)

SM4B reports Pathways^{TM} 4 software user's guide