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 strengthen 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 yeast²⁻⁴ 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 studies¹⁸ has demonstrated that, in peripheral blood lymphocytes, sev-
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 radiotherapeutic 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\(^{\circ}\)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\(^{\circ}\)C, connected with X-ray generator and the controls were sham-irradiated. After x-rays exposure the cells were incubated at 37\(^{\circ}\)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\(^{\text{TM}}\) 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\(^{\text{TM}}\) 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 \(^{3}P\) dCTP (reverse transcription) and filter hybridization were performed using 5 \(\mu\)g 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\(^{\text{TM}}\) 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\(^{\text{TM}}\) 4 software, which analyzes each clone at 95\% confidence range in a condition quadruple to determine 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.\textsuperscript{14}

\textbf{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: HS001174961
- LMAN: HS00194366
- SERPINE1: HS001677155
- SOD1: HS00166575
- VWF: HS00168575

All the informations about primers sequences and probes are available at: http://mark	eting.appliedbiosystems.com/mk/get/0905_ALL

\textbf{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\textsuperscript{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 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.

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:

\begin{enumerate}
\item 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\(\sigma\)).
\item A relatively high number of genes is up-regulated at the lowest dose (2 cGy).
\item 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.
\end{enumerate}

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 patterns in gene-expression data\textsuperscript{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 $1 \times 10^{10}$. 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 which 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.

**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

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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.
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.\(^{25,26,27}\) 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://fati-go.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/fortune-Cookie). 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 inhibitor 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,\(^{30}\) while higher doses and later times are generally down-regulating the genes coding for protein synthesis apparatus components.\(^{31}\)

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 2cGy. In fact, the up-regulation of glutathione peroxidase at relatively low doses (50 cGy of gamma-ray) of ionizing radiation has been observed before\(^{32}\) 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).

| 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 |
| AA79480 | 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 | CTT6A    | 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 |
| AAS04656 | 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 |

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## Transcriptome of Irradiated HUVEC

| 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| 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 |
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| 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| 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 |

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| 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 | PPI      | 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 |

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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 | Genes up and down-regulated reported in Table 1. Software by Bioinformatics Unit - CNIO (http://fatigo.bioinfo.cnio.es/). Datamining with Gene Ontology (GO). |
|-------------------|--------------------------------------------------------------------------------------------------|
| **cell communication** | VDR, CTNNA1, PTGES, TIE, EFNB1, EDN1, PPI, GRP58, SLC6A1, DFFA, COMT, MAPK8, PRKCB1, VWF, CCR6, RAB6A, NR4A2, CD59 |
| **mesoderm development** | BMP4, TIE |
| **metabolism** | VDR, BRCA1, MRPL3, PTGES, PTPRZ1, SSX3, TIE, NME2, CDC34, PFKP, EIF4A2, RPL35, RPL21, CCT6A, LMAN1, HSPA8, PPI, AMPD3, ZNF74, GRP58, DDB1, CDK6, CTSE, XRCC5, RPL35A, HSPCB, POR, PCK1, GPX1, DFFA, EIF2S2, COMT, MAPK8, CASP4, S1P, PRKCB1, GARS, HNRP1, RPL5, G6PC, SOD1, TCEB2, NR4A2, EIF3S2, OAZ1, BTF3, PURA, LAF4, NACA |
| **pathogenesis** | EDN1 |
| **morphogenesis** | BMP4, SPTA1, TIE, EFNB1, SOD1, PRDX1 |
| **growth** | BMP4 |
| **regulation of cellular process** | BRCA1, NME2, EDN1, CASP4 |
| **coagulation** | SERPINE1, LMAN1, VWF, CD59 |
| **organismal physiological process** | PTGES, EDN1, SERPINE1, LMAN1, CTSE, SLC6A1, COMT, VWF, CCR6, NR4A2, CD59 |
| **cellular physiological process** | SLC25A6, BMP4, SPTA1, BRCA1, NME2, EDN1, CDC34, N2N, LMAN1, CLTA, GRP58, CDK6, CTSE, SLC6A1, SC65, DFFA, MAPK8, CASP4, PFN1, HNRP1, VWF, CCR6, RAB6A, MSN, ATP1B2, PSMD2, PURA, PRDX1 |
| **response to stimulus** | BRCA1, PTGES, HSPA8, DDB1, CTSE, XRCC5, HSPCB, GPX1, MAPK8, VWF, SOD1, CCR6, NR4A2, CD59 |
| **regulation of physiological process** | 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, HMG1Y, KIAA1029, KIAA1247, LILRB2, NT5B, PIG11, PIG8, POLR2L, RAC1, RPL19, RPL24, RPL32, RPS15A, RPS27A, RPS4X, RPS4Y, RPS8, SCYA14, SR-BP1, SST, TMSB4X, UBA52, VSNL1, ZP3A

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MAPK8, PCK1, PRKCB1 and TIE.

CDK6 is a very important regulator of response to damage and its transcriptional down-regulation has been correlated with radiosensitivity.36 The beta subunit of protein kinase C could also be involved in regenerative 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 estimate 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.

Variability indicated in parenthesis.

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### 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) |

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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™ 4 software user’s guide