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Radiation-Induced Changes in Gene-Expression Profiles for the SCC VII Tumor Cells Grown *In Vitro* and *In Vivo*

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ABSTRACT

SCCVII tumor cells that grow *in vitro* or *in vivo* as a solid tumor were used to compare and contrast gene-expression profiles with or without exposure to two doses of ionizing radiation. Exponentially growing SCCVII cell cultures and tumors (1 cm diameter) were treated with 0, 2, or 10 Gy, and RNA was collected 1, 3, 6, 12, and 24 h after treatment. Growth under *in vitro* conditions increased the expression of genes associated with the unfolded protein response (UPR) including ATF4, Ero-1 like, and cystathionase. Growth *in vivo* indicated that the HIF-1a genes were not upregulated, whereas genes such as hemoglobin α and crystallin α B were significantly upregulated. Ninety genes of 16K were found to be significantly modulated under either growth condition by radiation treatment. Gene expression was not dose dependent. Sixty percent of these genes exhibited similar modulation under both *in vitro* and *in vivo* conditions; however, 29% of the genes were modulated by radiation under *in vivo* conditions only. Gene-expression profiles for the same tumor cells can differ, dependent on growth conditions, underscoring the influence that the tumor microenvironment exerts on gene expression for both growth of solid tumors and their response to radiation treatment. *Antioxid. Redox Signal.* 8, 1263–1272.

INTRODUCTION

TECHNIQUES ENABLING THE GROWTH OF TUMOR CELLS in vitro have made a major impact on our understanding of the cellular and molecular biology of cancer. The ability to study tumor cells reproducibly in the laboratory under defined and controlled conditions has allowed the quantification of cell killing after treatment with a variety of anticancer modalities and the study and elucidation of complex biochemical and molecular mechanisms associated with a multitude of cellular functions. However, given these advantages, specific limitations exist to using in vitro models.

Single cells grown *in vitro* are not under the neurologic, metabolic, or physiologic control of the host. Cells grown *in vitro* are typically maintained at ambient oxygen levels (21%); in contrast to most cells in a host that encounter much lower levels of oxygen, normally between 2 and 5%, and even lower for cells in solid tumors. Bringing cells from relatively low oxygen levels encountered *in vivo* to ambient oxygen levels may impose an oxidative stress. For example, human tumor cell lines grown in culture at 21% oxygen had significantly higher glutathione (GSH) levels than the same cells growing in the peritoneal cavity of a mouse, implying that *in vitro* growth conditions imposed an increased oxidative environment (19). Thus, tumor microenvironment and that observed in tissue-culture growth impose very different conditions such as hypoxia, low pH, nutrient deprivation, and osmotic pressure. These differing conditions, as well as altered intracellular redox status, could exert profoundly different effects on tumor cells grown either *in vitro* or *in vivo*.

The introduction of cDNA microarray technologies and genomic analysis has enabled the study of complex gene-expression profiles of various tumor cell types. In addition, several analysis methods allow the comparison of cells after

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the treatment of cells with various exogenous agents that induce intracellular stress including that from exposure to anticancer agents. In most of these studies, the collection of RNA comes directly from either patient or animal biopsies or from collecting cells growing *in vitro*. In the latter case, efforts have been made to simulate a variety of conditions that might be encountered *in vivo*: the tumor microenvironment (7).

However, it can be difficult to simulate accurately and reproducibly *in vivo* conditions by using an *in vitro* model. In the present study, we hypothesize that the gene-expression pattern from tumor cells grown *in vitro* will be significantly different from that in the same cells grown as a solid tumor, because of conditions related to cell–cell interactions and tumor microenvironment. To address this idea, we have taken advantage of a murine tumor cell model, SCCVII, that grows both *in vitro* and *in vivo* as a solid tumor. This system was subsequently used to determine contrasting differences in gene-expression profiles between *in vitro* and *in vivo* tumor cell growth and the subsequent tumor cell response when exposed to two doses of ionizing radiation. The findings suggest a significant impact of the tumor microenvironment on gene-expression profiles alone or after exposure to radiation.

MATERIALS AND METHODS

In vitro *studies*

SCCVII cells (murine squamous cell carcinoma) were grown in MEM medium supplemented with 10% fetal calf serum, penicillin, and streptomycin and were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. Cells from exponentially growing stock cultures were plated into a number of 150-mm dishes (2×10^6 cells/dish) and incubated 3 days before treatment. Cells were treated with 0, 2, or 10 Gy, and total RNAs were extracted from unirradiated or irradiated cells at 1, 3, 6, 12, and 24 h after treatment for cDNA microarrays (two complete replicate experiments were performed). Cytotoxicity was assessed in parallel plates by clonogenic assay for each treatment group immediately after treatment, as previously described (22). Cell and animal irradiation was done by using an Eldorado 8 ^{60}Co teletherapy unit (Theratronics International Ltd., Kanata, Ontario, Canada, formerly Atomic Energy of Canada, Ltd.) at dose rates between 200 and 250 cGy/min. Decay corrections were done monthly, and full electron equilibrium was ensured for all irradiations.

In vivo studies

Female C3H mice, produced by the National Cancer Institute Animal Production Area (Frederick, MD), were used for this study. The mice were aged 6–8 weeks at the time of experimentation and weighed between 20 and 30 g. All experiments were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the *Guide for the Care and Use of Laboratory Animal Resource,* (1996) National Research Council. Tumor cells were injected subcutaneously (s.c.) as a single-cell suspension of 105 SCCVII cells in the right hind leg. The tumors grew to 1.0 cm diameter (~500 mm^3) in ~8 days, at which time, tumor-bearing mice were placed in customized Plexiglas jigs that allow immobilization of the right leg without the use of anesthetics. Special care was taken to avoid irradiation of other body parts by using lead shields specifically designed for the jigs. Tumors were irradiated with single radiation doses (2 or 10 Gy). Animals were euthanized at 1, 3, 6, 12, and 24 h after irradiation, tumors were removed, and RNA was extracted as described later. For each time point, an entire tumor was processed, and two complete replicate experiments were performed.

p53 Analysis

To isolate genomic DNA, 1×10^6 cells were washed twice with PBS (4°C) and followed by centrifugation (1,000 rpm at 4°C, 5 min), and resuspended in 200 ml PBS. The genomic DNA was further purified by using Qiagen DNeasy Tissue Kit (Valencia, CA) according to the manufacturer's instructions. Polymerase chain reactions (PCRs) were performed in 25-ml reaction volumes containing 12.5 ml HotStarTaq Master Mix Kit (Valencia, CA), 0.3 m*M* primer, and 0.1 mg extracted DNA was used as the template. Cycling conditions included an initial denaturation at 94°C for 10 min followed by 35 cycles of 94°C for 20 sec, 54°C for 30 sec, and 72°C for 30 sec, followed by a final extension cycle for 10 min at 72°C. Primer sequences for all 11 exons of p53 were designed, but only the results for exons 1–5 are shown. PCR products were visualized by standard gel electrophoresis.

Flow cytometry

Cells were isolated from in vitro cultures and fixed with 70% ethanol. Tumors excised from animals were digested with enzymes (collagenase, DNAse) and fixed with 70% ethanol. Fixed cells were washed and incubated with 50 mg/ml propidium iodide for DNA analysis. All samples were analyzed by using a BD FACSscan (BD Biosciences, San Jose, CA).

RNA extraction

For each collection point, cell monolayers were washed once with PBS (4°C), and cells (~15 x 10^6) were scraped in 10 ml PBS (4°C), followed by centrifugation (1,000 rpm at 4°C, 5 min**)**. Total RNA was extracted with the use of Trizol reagent (Invitrogen, Carlsbad, CA) and the Qiagen RNAeasy Mini Kit, according to the manufacturer's instructions (Valencia, CA). The lysate was spun at 10,000 *g* in a microcentrifuge for 10 min. The aqueous phase was collected, and 0.5 volumes of isopropyl alcohol were added. Samples were stored at −80°C.

At appropriate times, postradiation tumors were removed and homogenized in 5 ml Trizol reagent, 1.0 ml of chloroform was added, and the samples were spun at 4,000 *g* for 20 min. The aqueous phase was collected, and 0.5 volumes of isopropyl alcohol were added. Samples were stored at −80°C until ready for further use.

Microarray fabrication

The cDNA microarrays used for this study were developed by the Radiation Oncology Sciences Program, National Can-

cer Institute (NCI), by using a cDNA library from the National Institute on Aging (NIA) containing 15,478 murine cDNA clones. The gene list of this murine microarray can be found at http://nciarray.nci.nih.gov. All 15K cDNAs were spotted onto poly-L-lysine-coated slides (Mm-ROSP-NIA 15K) by using an OmniGrid arrayer (GeneMachines, San Carlos, CA) according to Eisen and Brown (9).

Probe labeling and microarray hybridization

The methods for probe labeling reaction and microarray hybridization were used as described previously (16), with a few modifications. For all experiments, the cDNA probes from untreated and treated SCCVII cells/tumors were compared against a reference probe that was generated from a mouse universal reference RNA (Stratagene, La Jolla, CA), which consisted of total RNA from 11 mouse cell lines derived from different tissues including embryo, embryo (fibroblast), kidney, liver (hepatocyte), lung (alveolar macrophage), B lymphocyte, T lymphocyte (thymus), mammary gland, muscle myoblast, skin, and testis. Then 40 μg of tumor cell RNA or 20 μg universal reference RNA was labeled with Cy5 and Cy3, respectively, by using Superscript II Reverse Transcriptase (Invitrogen). The arrays were prehybridized with buffer [5× saline-sodium citrate (SSC), 0.1% SDS, 1% BSA] at 42°C for 1 h. Slides were washed in deionized water followed by 2-propanol. Cy5- and Cy3-labeled cDNA samples were mixed with 1 μl COT1-DNA (10 μg/μl) (Invitrogen), polyA (8–10 μg/μl) (Amersham Pharmacia Biotech, Piscataway, NJ), and yeast tRNA (4 μg/μl) (Ambion, Austin, TX) for hybridization. The mixed samples were denatured, and after the addition of 20 μl of 2× hybridization buffer (50% formamide, 10× SSC, 0.2% SDS), the entire sample was loaded onto the slides for overnight hybridization at 42°C. After hybridization, the hybridized slides were then washed in 2× SSC, 0.1%, 1× SSC, 0.1% SDS, and 0.2× SSC for 4 min each, followed by a 1-min wash in 0.05× SSC. Slides were then placed in 2-propanol followed by a spin for drying.

Microarray data analysis

Data were obtained from two separate time experiments using two doses of radiation (2 and 10 Gy) and replicated twice. The times after radiation at which mRNA was collected were 0, 1, 3, 6, 12, and 24 h. Analyses were performed by using BRB ArrayTools, developed by Dr. Richard Simon and Amy Peng Lam. The data collated by the BRB ArrayTools were filtered to include only wells with ≥50% of the data available for ratio calculations. All filtered wells were then median log-ratio normalized. Initially, the control, unirradiated samples were compared with each time point after radiation, and only clones greater or less than 1.5-fold were selected. From the five time points, two doses (2 and 10 Gy), and two conditions used (in vitro versus in vivo), these 20 different conditions resulted in 343 genes passing the 1.5-fold criterion. These 343 genes were then reanalyzed by first comparing each individual experimental time series with the average time series (obtained from both experiments) by using a Pearson's correlation function. Clones with Pearson coefficients >0.7, for both experiments, were deemed reliable for inclusion in the final expression map. Clones were selected only if the Pearson coefficients were acceptable for all doses (2 and 10 Gy) and conditions (in vitro and in vivo) used. Additional data were collected to compare untreated SCC cells grown under either in vivo or in vitro conditions. Here a total of four mRNA samples were collected from in vitro SCC cultures and five mRNA samples from the SCC tumors grown in vivo. Clones either up or down twofold and having a t test <0.05 were selected for comparison. The 90 final clones selected were analyzed by the hierarchical cluster program developed by Eisen et al. (10). The linear ratio data were log transformed and unsupervised average linked clustering was done by using uncentered Pearson correlation coefficients. The final cluster map was displayed by using the TreeView program also written by Eisen et al. (10). The percentage of normalized clone expression in mouse tissues was determined by using Stanford's S.O.U.R.C.E unification tool with the unigene cluster information for each clone.

RESULTS

Cell-cycle analysis of SCCVII cells/tumor

To define growth and cell-cycle differences between *in vivo* and *in vitro* growth, DNA flow-cytometry analysis was carried out on SCCVII cells. SCCVII cells grow rapidly both *in vitro* (population doubling times, ~14–16 h) and *in vivo* (tumor volume doubling times, ~24–48 h). DNA analysis of SCCVII cells grown *in vitro* had a $G_1/S/G_2$-M percentage of 51%/40%/9%, respectively; whereas cells isolated from tumors had $G_1/S/G_2$-M percentages of 65%/30%/5%, respectively (Table 1). The high *S*-phase fraction of SCCVII cells isolated from tumors is indicative of cells in a rapidly growing tumor cell environment. Cells were subsequently exposed to a single fraction of 10 Gy of ionizing radiation, and the changes in cell-cycle profile were determined (Table 1). Radiation treatment of cells *in vitro* induced a G_2-M block with no G_1 block, as cells continued to exit G_1 for *S* phase and ac-

TABLE 1. CELL CYCLE AFTER 10 GY IN SCC *IN VITRO* AND *IN VIVO*

	Time after radiation (h)					
% Cell cycle	*0*	*3*	*6*	*12*	*24*	
G1	51	38	34	62	56	
S	40	44	37	27	34	*In vitro – 2 Gy*
G2/M	9	18	29	11	10	
G1	51	32	30	15	38	
S	40	53	27	25	40	*In vitro – 10 GY*
G2/M	9	15	43	60	22	
	Time after radiation (h)					
% Cell cycle	*0*	*6*	*16*	*24*	*30*	
G1	65	56	46	47	47	
S	30	18	24	23	33	*In vivo – 10 Gy*
G2/M	5	22	30	30	20	

cumulate in G_2/M over a 12-h period. Tumor cells isolated from solid tumor were also analyzed, and in contrast to cultured cells, exhibited a G_2-M block for 10-Gy irradiation, with only a small decrease in the number of cells in the G_1 phase of the cell cycle (Table 1).

p53 Status of SCCVII

The lack of a pronounced block in G_1 after radiation treatment of SCCVII cells suggested a loss of wild-type p53 function in these cell lines. This is consistent with the findings of Saito *et al*., who found that p21 was abnormally regulated in the SCCVII tumor, suggesting an abnormal p53 function; however, no mutations in exons 5–8 of p53 were previously reported (31). Analysis of p53 exons 2 and 3–4 was conducted that revealed mutations that could account for the lack of p53 radiation response seen in these cells. Figure 1 shows that the PCR products from exons 2, 3–4 in SCCVII cells were missing. In contrast, the control wild-type p53 NIH 3T3 cells had products in all the exons examined, consistent with a normal p53 gene in these cells.

Cell survival after radiation

Although the primary purpose of this study was to examine the transcriptional response of SCCVII cells to ionizing radiation, survival information was obtained for cells under both *in vivo* and *in vitro* growth so that appropriate comparisons could be made for cells receiving similar radiation damage. Clonogenic cell survival analysis was carried out for SCCVII cells grown both *in vitro* and *in vivo* exposed to either 2 or 10 Gy of ionizing radiation. The mean surviving fraction for *in vitro* radiation treatment was 0.72 (n = 2) after 2-Gy irradiation and 0.01 (n = 2) after 10 Gy. Relative surviving fraction after an *in vivo* short-term 10-Gy dose (with subsequent isolation of cells from the tumor and plating) was 0.42. The plating efficiency was 0.41 for the *in vivo* tumors, which is consistent with the proportions of ~50% host cells in the SCCVII tumors, which would not be expected to form colonies (18, 26). From these survival results, a comparison between the 10-Gy *in vivo* dose (0.42 survival) and 2-Gy *in vitro* dose (0.72 survival) should provide the most appropriate analysis to observe any changes caused by environmental differences for cells with similar initial radiation damage.

Comparison of gene-expression profiles of SCCVII cells grown in vitro *or* in vivo

The genomic data from mRNA from all the SCCVII cells grown *in vitro* or *in vivo* were compared against a universal mouse RNA standard from Stratagene, which uses RNA from 11 different mouse cell lines representing many of the normal tissues in a mouse. The purpose of this analysis was to isolate genes responsible for the growth and transformation of the SCCVII tumor cells. Genes were selected only if they had ratios >5 under *in vitro* conditions and showed no more than a twofold reduction when the cells were grown *in vivo*. A twofold reduction *in vivo* would suggest that the resident host cells did not express these genes, and hence these genes may be of particular importance for SCCVII tumor growth and survival. Reductions much greater than twofold, when going from *in vitro* growth to *in vivo* growth, would indicate that these genes may be modulated by other physiologic conditions, and these genes are considered later. This analysis showed that six genes were significantly overexpressed (more than fivefold) under *in vitro* conditions with acceptable standard deviations (Table 2). These included BAI1-associated protein 2–like 1, adipose differentiation-related protein, and cyclin D1. Cyclin D1 protein has been reported to be significantly overexpressed in SCCVII cells grown *in vivo* (21), and thus these data suggest that the increase in cyclin D1 is due to transcriptional regulation rather than to posttranslational control.

Comparison of in vitro *versus* in vivo *gene expression*

As mentioned earlier, a second class of genes elevated *in vitro* and that had *in vivo* ratios <0.8 were considered as genes that may be more environmentally responsive. The top 10 genes upregulated by growth under *in vitro* conditions are shown (Table 3). A significant number of genes, identified in our analysis, involved the regulation of the unfolded protein response (UPR) were increased by *in vitro* growth, including activating transcription factor 4 (ATF4), asparagine synthetase (Asns), ERO1-like (Ero1l), Tribbles homolog 3 (Skip3), and Solute carrier family 3, member 2 (Slc3a2) (13). In addition, two genes involved in cellular redox reactions,

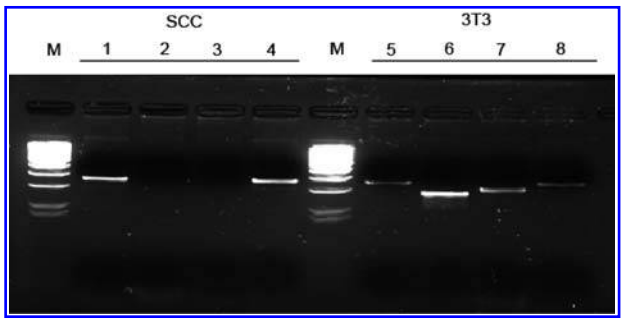

FIG. 1. PCR analysis of p53 exons 1–5 for both SCCVII and 3T3 cells (normal p53). Lanes 1 and 5: exon 1; lanes 2 and 6, exon 2; lanes 3 and 7, exons 3–4; and lanes 4 and 8, exon 5 from SCCVII and 3T3 cell, respectively.

TABLE 2. GENES OVER-EXPRESSED IN SCC (COMPARED TO MOUSE REFERENCE)

Name	*In vitro*	*In vivo*
BAI1-associated protein 2-like 1	25.9 (14.5)	13.8 (3.1)
Uridine phosphorylase 1	20.5 (10.5)	7.0 (2.4)
Calreticulin	10.3 (0.12)	6.1 (1.1)
Cyclin D1	6.3 (1.3)	3.4 (0.4)
Adipose differentiation related protein	7.0 (1.3)	3.4 (1.4)
Transmembrane 7 superfamily member 1	5.1 (1.1)	2.3 (1.3)

*n = 4 *in vitro* samples, n = 5 *in vivo* samples.
**Numbers in parenthesis are standard deviations.

TABLE 3. SCC TUMORS—TOP 10 *IN VITRO*

Description	*CloneID*	In vivo	In vitro	*Ratio*
Cystathionase	H3103D05	0.6 (0.3)	17.5 (4.7)	0.04
ERO1-like (S. cerevisiae)	H3019D08	0.2 (0.1)	1.7 (0.2)	0.13
Glutamic pyruvate transaminase 2	H3095C02	0.7 (0.1)	5.1 (1.1)	0.14
Immediate early reponse 3	H3057B07	0.2 (0.1)	1.5 (0.9)	0.15
Asparagine synthetase	H3154F02	0.6 (0.1)	3.7 (0.7)	0.16
Pleomorphic adenoma gene-like 1	H3118H02	1.9 (2.2)	11.8 (1.9)	0.17
Arginyl-tRNA synthetase	H3016E12	1.1 (0.5)	5.5 (0.7)	0.17
Phosphoserine phosphatase	H3139A09	0.8 (1.1)	4.8 (1.4)	0.18
Phosphoserine aminotransferase 1	H3095G01	0.6 (1.4)	3.6 (1.3)	0.18
Tribbles homolog 3 (Drosophila)	H3035B01	0.6 (0.1)	3.5 (2.1)	0.18

*Numbers listed are mean ± standard deviation.

cystathionase (Cth) and ornithine decarboxylase (Odc), were also significantly upregulated *in vitro*.

The top genes upregulated by *in vivo* growth are shown in Table 4. These results were separated into two sections; the first represents genes that had *in vitro* ratios between 0.9 and 1.4, and the other section includes genes in which the *in vitro* ratios decreased to >0.8. This segmentation was done in an attempt to identify genes more closely associated with the hematopoietic cells (CD45+), which are present in the SCCVII tumors *in vivo* (18, 26). Genes with ratios <0.8 under *in vitro* growth were considered less likely to be associated with SCCVII cells grown *in vivo*. In addition, clones were identified if their top three normalized RNA expressions in tissue were either from bone marrow, lymph node, or blood. Significantly, eight of 11 genes were expressed in the listed tissues when the ratios were <0.8. Conversely, no genes were found to be primarily expressed in either bone marrow, lymph node, or blood when the ratio was >0.9. One gene of interest was hemoglobin α, adult chain 1 (Hba-1α), in which nine separate clones had *in vivo*/*in vitro* ratios >3.0 (data not shown).

A number of hypoxia-related genes (Table 5), identified from the literature, were observed to be elevated after short-term hypoxic exposures (*in vitro* cell lines) (4, 12, 15, 17, 30). The majority of these genes showed no significant differences under either *in vitro* or *in vivo* growth in SCCVII cells. One gene listed in Table 5, crystallin α B, has been associated with moderate hypoxia (5% pO_2) and was elevated threefold *in vivo* compared with *in vitro* growth (23). Thus, it appears that genes normally associated with hypoxia (*i.e.*, HIF-1α–related genes) are not operating efficiently in the SCCVII model.

Gene expression after radiation treatment: 2 versus 10 Gy

SCCVII cells were exposed to both 2- and 10-Gy radiation, and RNA was collected at 1, 3, 6, 12, and 24 h after

TABLE 4. SCC TUMORS—TOP CLONES *IN VIVO*

Description	*CloneID*	In vivo	In vitro	*Ratio*
Crystallin, alpha B	H3143B04	4.4	1.4	3.0
Endomucin	H3034H08	5.6	1.3	4.2
SPARC-like 1	H3134F06	7.4	1.1	6.6
Transmembrane, prostate androgen induced RNA	H3152F10	4.2	1.1	3.8
Myosin light chain	H3104G01	12.1	1.1	11.2
Hemoglobin alpha, adult chain 1	H3045A12	9.9	1.1	9.2
Putative homeodomain transcription factor 1	H3083F09	3.4	0.9	3.6
Lysozyme	H3054F05	8.6	0.9	9.5
Cathepsin C	H3055G02	20.1	0.3	58.1
Lymphocyte cytosolic protein 1	H3011D10	2.7	0.2	15.0
Fc receptor, IgG, low affinity III	H3135G11	8.0	0.6	14.0
Chemokine (C-C motif) receptor 2	IMAGE:635981	6.9	0.7	9.6
Glycoprotein 49 A	H3159A08	2.0	0.3	7.2
Complement component factor h	H3146G02	0.8	0.1	7.0
H19 fetal liver mRNA	H3005A04	1.3	0.2	6.4
Ring finger protein 130	H3101F04	1.6	0.3	5.8
Guanine deaminase	H3085D10	1.6	0.3	5.5
Lipoprotein lipase	H3007A07	1.1	0.2	5.4
Aspartate-beta-hydroxylase	H3045B10	1.9	0.3	5.3

TABLE 5. HYPOXIA RELATED GENES

Description	In vivo	In vitro	*Ratio*	*Reference*
Eroll–ERO1-like (S. cerevisiae)	0.21	1.63	0.13	(16)
ATF4–activating transcription factor	0.94	3.78	0.25	(15)
Glyceraldehyde-3-phosphate dehydrogenase	0.60	1.06	0.57	(13)
Pyruvate kinase, muscle	0.75	1.71	0.44	(13)
Urokinase plasminogen activator receptor	1.67	1.91	0.88	(13)
Heme oxygenase (decycling) 1	1.29	1.64	0.78	(13)
Transferrin receptor	1.17	0.83	1.41	(13)
Transferrin receptor	0.80	0.65	1.22	(13)
N-myc downstream regulated gene 1	0.37	1.19	0.31	(12)
Prolyl 4-hydroxylase, beta polypeptide	0.86	0.76	0.89	(12)
Hypoxia inducible factor 1, alpha subunit	0.99	1.81	0.55	(23)
Cbp/p300-interacting transactivator	0.58	1.47	0.39	(14)
Lactate dehydrogenase 1, A chain	0.60	1.11	0.54	(13)
Vascular endothelial growth factor C	1.27	1.12	1.14	(13)
Adipose differentiation related protein	3.32	6.98	0.48	(39)
Mdm2	2.10	1.23	0.59	(19)
Crystallin, alpha B	4.4	1.40	3.00	(17)

treatment. Genes significantly altered after exposure to irradiation were determined by three criteria: (a) at least a 1.5-fold up or down induction compared with untreated controls, (b) having Pearson correlation coefficients ≥ 0.7 between the average time response and each individual experiment, and (c) the gene response was statistically valid in all four of the experimental conditions. In addition, only genes with known annotations were included in the final list. A hierarchical cluster map of the time response after radiation treatment for both *in vivo* and *in vitro* conditions was generated (Fig. 2), and in total, 90 genes were altered according to the criteria listed earlier.

Comparison of gene-expression profiles between the two radiation doses used showed minimal differences. The hierarchical cluster map was further subdivided into five groups to highlight similarities and differences between *in vivo* and *in vitro* conditions. Group I consisted of 18 genes upregulated only under *in vivo* conditions after either 2 or 10 Gy radiation. Members of this group included Hba-1a, prenylcysteine oxidase 1, and glutamate oxaloacetate transaminase 2 (Got2). Group II, represented genes upregulated by radiation (2 or 10 Gy) only for in vitro conditions. Group III included 14 unique genes, which were significantly upregulated under both the *in vivo* and *in vitro* growth conditions. These included cyclin G1, glutathione *S*-transferase, mu-1 and 2, pituitary tumor protein 1, and sorbitol dehydrogenase. Group IV included genes that were downregulated by radiation under both growth conditions. These included two clones of ornithine decarboxylase, ATF4, cyclin D1, and sterol C4-methyl oxidase. In this group of genes, 32 of 40 clones had *in vitro* ratios (untreated controls) >1.9, and 17 of the 32 had *in vitro* ratios >3.

One gene predominantly affected by radiation under *in vitro* conditions was cyclin D1, which declined continuously after either 2 or 10 Gy (~50% reduction). In contrast, cyclin D1 was not significantly affected under *in vivo* growth conditions with radiation (<10% reduction). Finally, group V genes had a similar response to radiation as group I genes, in that an opposite response to radiation was found in the *in vivo* cells as compared with the *in vitro* cells. One member in this group was the Abcc3, a multidrug-resistant protein, which increased to 1.7-fold at 1 h after radiation but quickly returned to normal by 6 h when irradiated *in vivo*.

DISCUSSION

SCCVII Model

The SCCVII *in vitro/in vivo* model afforded the opportunity to compare and contrast potential differences in gene-expression profiles for cells maintained under strict control (*in vitro*) with that of cells influenced by the complexities of the tumor microenvironment. SCCVII tumor cells were chosen because of their robust growth under both *in vivo* and *in vitro* conditions and because of the pronounced hypoxia reported for this tumor (median pO_2 levels, ~1–2 mm Hg) (34). Additionally, SCCVII tumors are reported to be heavily infiltrated with host cell interactions (50%), including infiltrating macrophages, cytotoxic T cells, and other host cells involved in growth and nutrient maintenance (18, 26). Tumor-infiltrating host cells have also been shown to be hypoxic, similar to that observed for tumor cells (26). The SCCVII model is nonmetastatic (38) and has a low apoptotic (31) and necrotic fraction as compared with RIF (radiation-induced fibrosarcoma) or KHT (mouse sarcoma) tumors (34). Thus, it is a tumor that appears highly adapted to surviving in a hypoxic environment.

Realizing that host/tumor cell interactions may potentially complicate the interpretation of *in vivo* gene-expression profiles, we attempted to separate cell suspensions isolated from solid tumors into tumor and normal hematopoietic cell fractions by using CD45 antibody/magnetic bead separation (data not shown). Although we were successful in separating tumor and hematopoietic cell fractions (as judged by DNA content/flow cytometry), this procedure did not provide mRNA of sufficient quality for the array studies to be carried out. The reason for this probably resides in the lengthy

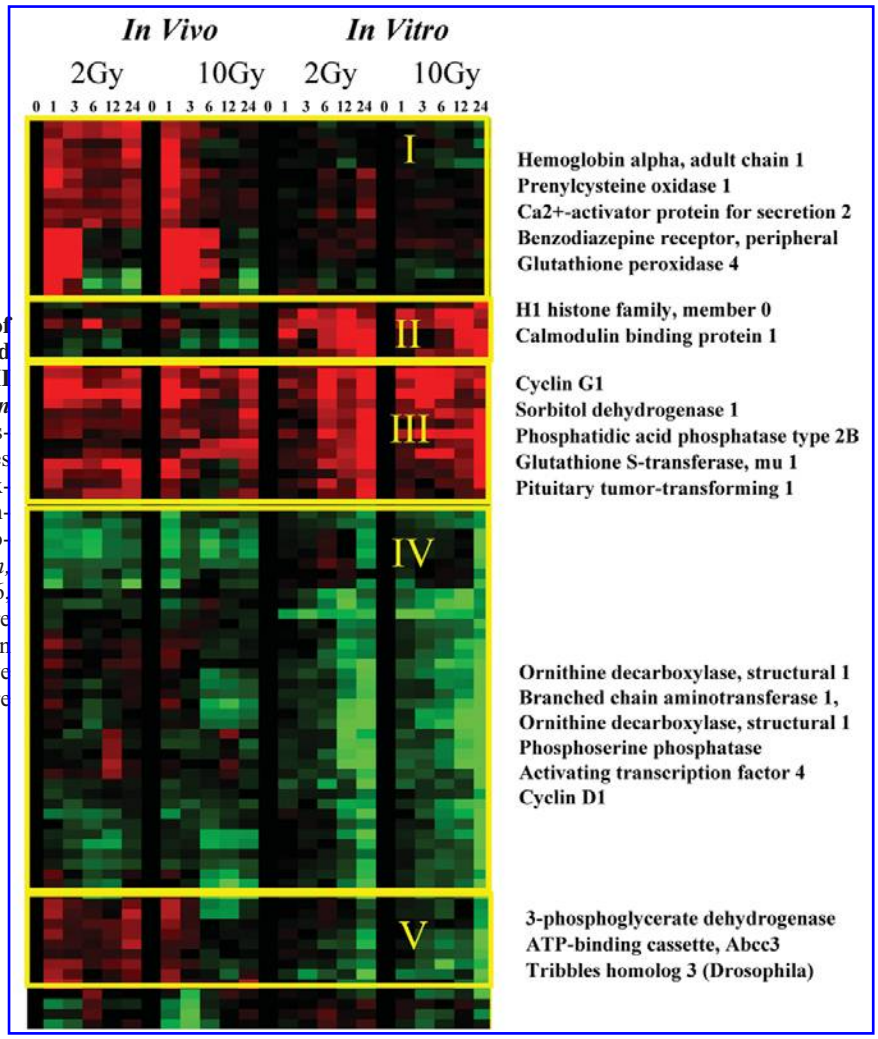

FIG. 2. Hierarchical clustering of RNA expression observed for 2- and 10-Gy radiation doses for SCCVII cells treated under *in vitro* and *in vivo* conditions. Average expressions of the two replicates of 90 genes are shown in logarithmic scale. All expressions shown are relative to untreated cells. Gene-expression profiles (*red*, upregulated; *green*, downregulated) are shown for 1, 3, 6, 12, and 24 h after radiation exposure (left to right). The gene-expression profiles were subdivided into five groups, and selected gene names are shown to the right of each group.

isolation/separation period required, which may have led to degradation of the mRNA.

Cells with abnormal p53, in general, do not respond to radiation-induced DNA damage with gene induction in a dose-dependent manner (14). Saito *et al*. (31) evaluated the p53 status in SCCVII cells and did not find any mutations in exons 5–8, the most frequently mutated sections of p53. They did, however, note that p21 (CDKN1A) was aberrantly regulated in SCCVII tumors, suggesting a compromised p53 induction (31). The present study shows that both the biologic response [lack of G_1 block after radiation (Table 1)] and analysis of the p53 gene structure (mutations in exon 2–4) indicate that SCCVII cells have an abnormal p53 protein.

SCCVII (in vitro, in vivo) versus normal mouse cell lines

Initial comparisons of *in vitro* and *in vivo* gene-expression patterns were made against a mouse normal-tissue reference. Because host cells make up ~50% in SCCVII tumors, we attempted to identify tumor-specific genes by first finding genes highly upregulated *in vitro* (compared with the normal mouse reference). Next, we considered only genes reduced by twofold after *in vivo* growth because infiltrating host cells dilute the ratio by 50%. This was accomplished by choosing only genes that were elevated *in vitro* but were reduced by twofold after *in vivo* growth (Table 2). One of the genes identified was cyclin D1, which was sixfold higher in the SCCVII cells (*in vitro*) compared with the mouse reference library, and indeed has been shown to be elevated in SCCVII tumors by protein analysis (21).

In vivo, cyclin D1 expression was approximately half that observed for *in vitro* growth, consistent with a ~50% "dilution" of tumor cells *in vivo* because of host cell infiltration. This is based on the assumption that infiltrating host cells are noncycling and hence their cyclin D1 levels would be expected to be low. Uridine phosphoralase 1 has been shown to be elevated in breast, lung, and colon tumors versus normal tissue (28). These genes, along with the others shown in Table 2, were not modified by *in vivo* growth (after correction for host cell infiltration) and hence might represent genes that are important molecular targets for defining the inherent growth of the SCCVII cells.

Comparison of in vitro *versus* in vivo *gene expression*

A number of genes were found to be upregulated *in vitro* when compared to *in vivo*. Because *in vitro* cells were main-

tained at 21% oxygen levels (compared with much lower oxygen levels *in vivo*), we anticipated that specific genes involved in buffering oxidative stress should be upregulated for *in vitro* conditions. In this regard, cystathionase, which produces cysteine, and glutamic pyruvate transaminase 2, which produces glutamate, were elevated significantly, suggesting that these gene products would increase amino acids necessary for GSH synthesis. In this context, GSH is an important cellular redox molecule that provides protection against oxidative stress by detoxification of hydrogen peroxide in conjunction with GSH peroxidase. In addition, that the genes involved in GSH synthesis were upregulated *in vitro* compared with *in vivo* is consistent with the concept that cells growing under higher oxygen levels experience higher levels of oxidative stress and consequently higher GSH levels (19).

Another pathway strongly upregulated in *in vitro* SCCVII cells involved the endoplasmic reticulum (ER) stress or the unfolded protein response (UPR). Several mechanisms are known to regulate UPR, including amino acid starvation, oxidative stress, and hypoxia (20). UPR leads to the production of unfolded proteins, which accumulate in the ER and, if not corrected, can result in cell death (20). Although the UPR is clearly upregulated by anoxic conditions (30), none of the genes identified with the UPR response observed in the present study were upregulated by *in vivo* SCCVII growth (ATF4, Aspargine synthase, Slc3a2, Nars, and ERO1-like) (Table 3).

Although contradicting other ATF4 hypoxic studies, the ATF4 response in the SCCVII cells was consistent with the lack of the HIF response observed in the SCCVII cells under hypoxic *in vivo* conditions (Table 5). Collectively these results would indicate that SCCVII cells are highly adapted to hypoxic growth.

Analysis of genes increased in the *in vivo* growth model is more complicated than that in cells grown *in vitro*. First, SCCVII cells grown in mice clearly contain significant numbers of host cells (26). Second, hypoxia and other environmental factors can clearly influence gene regulation. An unexpected finding was that few (if any) of the known hypoxia-response genes were upregulated under *in vivo* growth (Table 5). Surprisingly, HIF-1α itself was actually increased under *in vitro* growth. Although strongly associated with hypoxia, HIF-1α can be modulated by other stresses such as iron chelation, cobalt chloride, or proteosome inhibition (32). In addition, Sobhanifar *et al*. (33) have shown that anoxia along with nutrient deprivation fails to upregulate HIF-1α (33). Another possibility may lie with the aberrant p53 status of the SCCVII cells, because recent studies have indicated antagonistic interactions between p53 and HIF-1α (2). These studies showed that elevated levels of p53 or certain mutations in p53 can inhibit the upregulation of HIF-1α by hypoxia (3).

Only one gene, crystallin α B (CryAB) was upregulated significantly *in vivo*. CryAB has been shown to be regulated by reduced oxygen levels (23). It is also interesting that CryAB levels were determined in a series of 62 head and neck cancer patients before treatment (8). Patients who lacked positive CryAB staining had no recurrences, even for patients who were initially node positive. In contrast, patients whose tumor was positive CryAB had a 37% risk of recurrence at 5 years after treatment (even the node-negative patients) (8). Overexpression of CryAB has been found in other malignancies such as glioblastomas, astrocytomas, and renal cancers (27).

Table 4 is separated into two parts: the top part is thought to represent genes that may be tumor associated, as the *in vitro* ratios were between 0.9 and 1.4. As mentioned earlier, CryAB has been shown to be elevated in some tumors (27). Sparc-like 1 (also called Hevin) production has been demonstrated primarily in tumor cell lines (6). Hemoglobin (Hba-a1) obviously may be related to the extensive hypoxia found in SCCVII tumors. Myosin light chain 6 overexpression has been found in pancreas tumors with wild-type K-ras (11). The bottom portion of Table 4 is thought to represent genes associated more with the normal host cell infiltration. Eight of 11 of the top clones in this category were seen to be predominantly expressed in either bone marrow, lymph nodes, or blood, as reported in the Stanford's SOURCE database.

It is of interest to note that another microarray study of SCCVII tumors was published by Yang *et al*. (37); the peripheral portions of SCCVII tumors were compared with the central portions of SCCVII tumors by using MRI and the Affymetrix arrays (37). In stage 2 tumors (2–4 weeks after implantation), a significant difference in the hemoglobin gene expression (threefold to fourfold) was found between the peripheral and central portions of the SCCVII tumors. These data coupled with the results of the present study indicate that hemoglobin genes appear to be regulated by SCCVII cells.

Gene expression after radiation treatment: 2 Gy versus 10 Gy

The effects of the tumor microenvironments on the radiation response of SCCVII cells was evaluated by examining global gene expression for cells irradiated in either *in vitro* or *in vivo* conditions at two different doses. As shown in Fig. 2, 90 of 16,000+ genes were identified as up- or downregulated ≥1.5-fold. First and foremost, the cluster map of gene expression (up- or downregulation) in SCCVII cells showed no radiation dose response under either *in vitro* or *in vivo* growth conditions. This observation is consistent with the aberrant p53 status of SCCVII cells, as p53 regulation dominates the dose-responding genes (1).

The cluster map also provides important information about the relevancy of studying the radiation response of cells grown under *in vitro* conditions (*i.e*., high pO_2, 10% serum, and adequate nutrients) and translating these results to cells growing as solid tumors. Figure 2 [groups III and IV, which included 54 of 90 radioresponsive clones (60%)], showed that the *in vitro* responses were indeed representative of the responses observed *in vivo*. Upregulated genes (group III) were quantitatively similar, whereas the downregulated genes (group IV) were qualitatively similar, with the *in vitro* response showing a stronger downregulation. This may have been related to the stronger cell-cycle blocks occurring under *in vitro* radiation [see Table 1, 2 Gy *in vitro* versus 10 Gy *in vivo* (~ isosurvival)].

The genes in the upregulated group included cyclin G1, GSTmu-1, GSTmu-2, and Pttg-1, which all have some association with DNA damage (25, 29, 35). Other genes, such as sorbital dehydrogenase and laminin α 5, have not been identified as radiation-responsive genes. Laminin α 5 appears to be involved with normal lung development and VEGF production (24). Group IV genes were those primarily downregulated after radiation. One gene of interest, ATF4, was pre-

dominantly downregulated after *in vitro* radiation (Fig. 2). Downregulated along with ATF4 were several genes that have been associated with the UPR ATF4 response, including asparagine synthetase, phosphoserine aminotransferase, phosphoserine phosphatase, and alanyl-tRNA synthetase (13).

As was noted in the results, 32 of 40 clones downregulated by radiation treatment had *in vitro* ratios >1.9. These were genes were already operating at a high level when grown under *in vitro* conditions. Thus, it is possible that a global decrease in mRNA synthesis brought about by radiation may be responsible for the decline in these genes. Whether other DNA-damaging agents would lead to the downregulation of the ATF4 response remains to be studied. Ornithine decarboxylase was downregulated by *in vitro* radiation in SCCVII cells, and this is a response also seen in Syrian hamster cells after x-rays (36). Interestingly, Milas *et al*. (21) found that cyclin D1 was not changed after radiation in tumors irradiated *in vivo*, and we also did not note significant changes after *in vivo* radiation (21). However, cyclin D1 was significantly downregulated by both a 2- and 10-Gy dose under *in vitro* conditions.

Perhaps the most interesting finding of the cluster analysis was that 31 of 90 genes (34%) showed different expression levels depending on whether the cells were irradiated under *in vitro* or *in vivo* conditions. A group of 23 unique genes were upregulated by radiation only under the *in vivo* conditions (Fig, 2, groups I and V). Genes in this category included hemoglobin alpha (Hba-a1), prenylcysteine oxidase, and glutathione peroxidase 4 for group I, and Tribbles homolog 3 and Abcc3 (a multidrug-resistant associated gene) for group V. This cluster of genes may be influenced by either tumor microenvironment differences or because the *in vivo* tumor contained contaminating normal hosts cells.

Hba-a1 showed the strongest response in this category as 21 of 23 different Hba-a1 clones showed increases after radiation (data not shown). Little evidence in the literature suggests that hemoglobin is modulated by radiation exposure. However, a recent study reported that a pre-B cell line treated with various chemotherapy drugs did upregulate Hba-a1 (5). Interestingly, this upregulation was not thought to be due to the oxygen-carrying ability of Hba-a1, but rather that Hba-a1 was acting as a proapoptotic effector in these hematopoietic cells (5). It is possible that the Hba-al response requires both radiation and hypoxia as an inducer of this gene, or it could be marker gene of the radiation response of the normal host cells residing in the tumor. Finally, a small number of genes were identified (Fig. 2, group II) that were only upregulated by radiation treatment *in vitro*. Although the significance of these genes and those shown in groups I and V in the radiation response is unclear, it is apparent that conditions associated with the tumor microenvironment and *in vivo* growth altered their expression.

This study clearly demonstrates that gene-expression profiles for tumor cells are strongly dependent on growth conditions. Highlighted in these studies were differences in gene expression for cells grown *in vitro* and *in vivo* with or without radiation exposure. These differences may serve to advance our understanding of how the tumor microenvironment in conjunction with solid tumor growth modulates gene expression (up or down) and may aid in the identification of target genes that would have been unnoticed by conducting *in vitro* studies exclusively. Further studies will be required to evaluate the significance of these differences, including determination, when possible, of actual protein expression and function.

ABBREVIATIONS

ATF4, activating transcription factor 4; CryAB, crystallin alpha B; ER, endoplasmic reticulum; GSH, glutathione; GST, glutathione *S*-transferase; Hba-a1, hemoglobin alpha; HIF, hypoxia inducible factor; Nars, asparaginyl-tRNA synthetase; PCR, polymerase chain reaction; SSC, saline-sodium citrate; Slc3a2, solute carrier family 3, member 2; UPR, unfolded protein response; VEGF, vascular endothelial growth factor.

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