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PRINCIPAL INVESTIGATOR: David Gewirtz, Ph.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23298-0568

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David Gewirtz, Ph.D.

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Virginia Commonwealth University
Richmond, VA 23298-0568

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Abstract Studies were designed to understand the role of the p53, Myc, $p21^{waf1/cip1}$, Rb and E2F proteins (as well as the Bax and Bcl-2 proteins) in the pathway leading to growth arrest and apoptotic cell death in the breast tumor cell. We have determined that neither p53 status nor alterations in levels of the Myc protein are critical factors in radiosensitivity. Furthermore, the absence of apoptosis cannot be related to lack of p53/E2F-1 or p53/Myc interactions or to changes in Bax or Bcl-2. Pretreatment of p53 wild type breast tumor cells with Vitamin D3 compounds sensitizes the cells to ionizing radiation - suggesting a role for the combination of Vitamin D3 compounds with radiotherapy in the treatment of breast cancer. Irradiation can also enhance the efficiency of liposomal mediated transgene uptake suggesting that irradiation could be combined with gene therapy in the treatment of breast cancer. Finally, we are attempting to determine how p53 status influences the fidelity of double-strand break repair in apoptosis-proficient 184B5 breast epithelial cells, and the possible relation between apoptosis and tolerance for misrepair. Such studies may suggest additional candidates for transgenic manipulation of the response to radiation.

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INTRODUCTION *Subject and scope of research* This work is directed towards developing an understanding of the molecular and signal-transduction events mediating growth arrest and cell death in breast tumor cells after exposure to ionizing radiation. Our studies have been focused primarily on the p53, $p21^{waf1/cip1}$, Myc and E2F-1 proteins; these proteins have overlapping and possibly mutually exclusive functions in the regulation of cell growth and apoptotic cell death pathways in response to DNA damage. In addition, we are interested in the involvement of the pro-apoptotic and anti-apoptotic proteins of the Bax/Bcl-2 families in the pathway leading to apoptosis in the breast tumor cell. Our findings that the breast tumor cell fails to undergo apoptotic cell death in response to irradiation (as well as in response to adriamycin) have provided the incentive for developing approaches for radiosensitization (and chemosensitization) of the breast tumor cell. In addition, we have discovered that irradiation has the capacity to promote the uptake and expression of exogenous genes, a finding which is the basis for the development of strategies for the delivery of cytotoxic and apoptosis-promoting genes to both p53 wild-type and p53 mutated breast tumor cells. Finally, the possible role of p53 in enforcing the fidelity of double-strand break repair is being investigated in matched p53+ and p53-defective breast epithelial cells. Putative double-strand break misrepair events, induced by bleomycin and detected as *HPRT* mutations, are being analyzed at the DNA sequence level. Chromosomal stability, delayed reproductive death, and radiation-induced apoptosis and cell cycle perturbations are being assessed in the mutant cells in an attempt to determine whether specific types of misrepair events were accompanied by changes in these responses.

Background While ionizing radiation is one of the most effective treatments for breast cancer, the recurrence of disease indicates the limitations of radiotherapy. We believe that breast tumor cells may demonstrate primary resistance to radiotherapy, in part through their refractoriness to the induction of apoptotic cell death. Furthermore, even in breast tumor cells which are initially responsive to radiotherapy, the absence of apoptosis may permit the acquisition of a radioresistant phenotype during the course of treatment.

An extensive literature describes the closely-linked signal transduction pathways which mediate growth arrest and/or cell death in irradiated cells. Irradiation, as well as other modalities which induce DNA damage are known to up-regulate levels of the tumor suppressor protein, p53 (Kuerbitz et al, 1992; Zhan et al, 1993; Dulic et al, 1994; Gudas et al, 1995), which in turn increases levels of the cyclin-dependent kinase inhibitory protein, $p21^{waf1/cip1}$ (Di Leonardo et al, 1994; Dulic et al, 1994; Bae et al, 1995; Gudas et al, 1995). Inhibition of cyclin dependent kinases results in abrogation of the phosphorylation of the tumor suppressor protein, Rb (Nigg et al, 1995; Dimri et al, 1996) - which then binds to and inactivates the transcription factor, E2F (Chellappan et al, 1991; Hiebert et al, 1992; Almasan et al, 1995). E2F is thought to regulate the expression of a spectrum of genes associated with DNA synthesis including c-*myc*, DNA polymerase alpha, thymidine kinase and thymidine synthetase (Almasan et al, 1995; Martin et al 1995). Interference with E2F function is postulated to block DNA synthesis and promote growth arrest (Johnson et al, 1993; Almasan et al, 1995).

While the p53, Myc and E2F proteins are fundamental components of the G1 cell cycle checkpoint, all of these proteins have also been shown to mediate apoptosis or programmed cell death

in a variety of tumor cell models in response to DNA damage (Evan et al, 1992; Almasan et al, 1995; Henneking et al, 1995; Lowe et al, 1995). Conversely, up regulation of $p21^{waf1/cip1}$ in response to DNA damage is though to abrogate the apoptotic pathway (Lin and Benchimol, 1995: Attardi et al, 1996).
).

Although many types of DNA damage can cause an increase in p53 levels and activate the cascade described above, this pathway appears to be particularly sensitive to double strand breaks. Indeed, transfection experiments have suggested that the presence of one double-strand break in a cell nucleus, even on a nonessential plasmid, can activate a p53-dependent checkpoint and arrest the cell in G1 (Huang et al, 1996). In addition, the enhanced apoptotic responses of cell with defective double-strand break repair suggest that double-strand breaks may be the critical triggering lesion for radiation-induced apoptosis as well (Meng et al., 1998; Nussenzweig et al., 1997). Thus, the upstream events in radiation-induced G1 arrest and apoptotic cell death may be intimately linked to the recognition and processing of double-strand breaks.

Purpose The original purpose of these studies was to understand the role of c-*myc* and the p53 protein in the pathway leading to growth arrest in the breast tumor cell. As indicated in this and in the previous report, we have made significant progress relating to this component of the proposal. However, we have concluded that a more clinically relevant aspect of this work involves the relative refractoriness of breast tumor cells to the induction of apoptotic cell death. Consequently, we have developed unique approaches for the promotion of apoptotic cell death in both p53 wild-type and p53 mutated breast tumor cells. An additional component of this work was to investigate the repair of double-strand breaks induced by ionizing radiation (and by the radiomimetic, bleomycin) in breast epithelial cells having wild-type versus mutant p53 genes, and the relationship of double-strand break repair to apoptotic cell death.

BODY

In the first specific aim, we proposed to test the hypothesis that ionizing radiation suppresses the expression of the oncogene, c-*myc* in select breast tumor cell lines and that radiation induced suppression of c-*myc* expression is a downstream event related to the induction of p53 and/or of $p21^{waf1/cip1}$.

In the second specific aim, we proposed to determine whether suppressed c-*myc* expression is required for growth arrest in breast tumor cells.

In the third specific aim, we proposed to assess the influence of ionizing radiation on the level, stability and activity of the Myc protein.

In the fourth specific aim, we proposed to test the hypothesis that suppression of c-*myc* expression and Myc protein activity is, in part, responsible for the relative refractoriness of breast tumor cells to apoptotic cell death.

In the fifth specific aim, we proposed to examine, at the DNA sequence level, errors in double-strand break repair in breast tumor cells treated with radiation and with the radiomimetic drug bleomycin, by analyzing deletions and rearrangements induced by these agents at the *HPRT* locus.

In the sixth specific aim, we proposed to compare double-strand break repair events in breast tumor cells having normal vs. mutant p53 genes, and in possible, in breast tumor cells rendered competent for radiation-induced apoptosis.

A. Involvement of the p53, Myc, $p21^{waf1/cip1}$, Rb and E2F-1 proteins in radiosensitivity, growth arrest and cell death in response to irradiation.

Experimental Methods and Procedures Cell growth and radiation sensitivity were determined by trypan blue exclusion as well as by clonogenic analyses. Standard immunoblotting protocols were utilized to assess the influence of irradiation on select proteins associated with the G1 arrest and apoptotic pathways. Cells were irradiated (10Gy) and proteins was extracted at intervals of 2-4 hours after irradiation as well as after 24 and 48 hours. Apoptotic cell death was evaluated based on cell-cycle analysis, alterations in cell morphology as well as by fluorescent end-labeling (TUNEL assay).

Results and Discussion Utilizing two p53 wild-type (MCF-7 and ZR-75) and two p53 mutated (MDA-MB231 and T-47D) breast tumor cell lines, we have substantiated the absence of apoptotic cell death in response to ionizing radiation and/or the chemotherapeutic agent, adriamycin (Figure 1 and Fornari et al, 1996). Figure 1 presents the results of the TUNEL assay which indicates that 10 Gy of irradiation fails to alter the extent of fluorescent end-labelling of DNA in MCF-7 and ZR-75 breast tumor cells - which indicates that there is no induction of apoptotic DNA damage. Although our previous work suggested that functional p53 might be necessary for suppression of c-*myc* expression and Myc protein levels, our more recent work with ZR-75 appears to argue against this hypothesis. Although p53 was increased to equivalent levels by irradiation in MCF-7 and ZR-75 breast tumor cells (Figure 2), irradiation reduced Myc protein levels in the MCF-7 cells while Myc levels were increased in ZR-75 cells (Figure 2). Furthermore, there was no evident difference in radiosensitivity in these two cell lines (Figure 3).

The induction of $p21^{waf1/cip1}$ in response to DNA damage has been demonstrated to occur in cells exposed to ionizing radiation as well as to other modalities which induce DNA damage (Gudas et al, 1995; Dulic et al, 1994). Exposure of MCF-7 cells to 10 Gy of ionizing radiation resulted in a time-dependent increase in $p21^{waf1/cip1}$ levels (Figure 2). In contrast, Figure 2 indicates that there was a minimal alteration in $p21^{waf1/cip1}$ levels in the ZR-75 breast tumor cell line. These observations suggest that there may be defective regulation of $p21^{waf1/cip1}$ in response to DNA damage in the ZR-75 cells.

The $p21^{waf1/cip1}$ protein acts as a generalized inhibitor of the cyclin dependent kinases (Hiebert et al, 1992). One consequence of this inhibition is conversion of the phosphorylated form of the Rb

tumor suppressor protein to the hypophosphorylated form (Weinberg, 1995), which is thought to activate Rb (Weinberg, 1995), and to facilitate its binding to the transcription factor E2F (Hiebert et al, 1992). The phosphorylation state of Rb in response to ionizing radiation was determined over the same time frame as the levels of the $p21^{waf1/cip1}$ protein. Results of the Western analysis, presented in Figure 2 are similar to what has been reported by other investigators assessing the status of Rb in proliferating tumor cells (Kwon et al, 1996; Gorospe et al, 1996, Wosikowski et al, 1995); that is, in control cells we generally observe the phosphorylated states of the Rb protein. Figure 2 indicates that the dephosphorylated form of Rb was discernible in MCF-7 cells within 2 hours of irradiation, and was further visible throughout the 24 hour interval subsequent to irradiation. ZR-75 cells also demonstrated a conversion of Rb from the phosphorylated to the dephosphorylated forms.

Conclusions and Implications We can conclude from these as well as the preceding studies (Watson et al, 1997) that neither p53 status nor alterations in levels of the Myc protein are critical factors in radiosensitivity, at least in those cases where the tumor cell fails to undergo apoptotic cell death. The fact that E2F-1 levels decline in the MCF-7 cell line raises questions as to the extent of formation of the Rb-E2F-1 complex which is thought to be responsible for arrest in G1. Similarly, the low extent of dephosphorylation of Rb in the ZR-75 cells suggests that the Rb-E2F complex which is a transcriptional repressor may not readily form after irradiation. Studies are currently in progress to determine the influence of irradiation on Rb-E2F complex formation in the breast tumor cell since the extent of Rb-E2F complex formation is thought to be a critical component of the G1 cell cycle checkpoint.

B. Involvement of the p53, Myc, $p21^{waf1/cip1}$, E2F-1, Bax and Bcl-2 proteins in the refractoriness of the breast tumor cell to apoptosis in response to irradiation.

Experimental Methods. Standard immunoblotting protocols were utilized to assess the influence of irradiation on select proteins associated with apoptotic pathways. Cells were irradiated (10Gy) and proteins was extracted at intervals of 2-4 hours after irradiation as well as after 24 and 48 hours.

Results and Discussion In the MCF-7 cell line, levels of the Myc and E2F-1 proteins were reduced, with pronounced effects evident after 24 hours. However, neither E2F-1 nor Myc levels were reduced in ZR-75 cells (Figure 2). Levels of E2F-4 were unaltered by radiation in both MCF-7 and ZR-75 cells. Bax and Bcl-2 levels were essentially invariant in MCF-7 cells (Figure 4). In ZR-75 cells, Bax levels were increased markedly in response to irradiation, but Bcl-2 was undetectable.

Conclusions and Implications These studies suggest that the absence of apoptosis in response to irradiation in p53 wild-type cells cannot be readily ascribed to changes in Myc, E2F-1, Bax or Bcl-2. Since p53 is increased in both MCF-7 and ZR-75 cells, but Myc and E2F-1 are decreased only in MCF-7 cells, the absence of apoptosis cannot be related to lack of p53/E2F-1 or p53/Myc interactions. The absence of alterations in Bax levels in MCF-7 cells after irradiation is surprising, and suggests the existence of a defect in this signalling pathway upstream of this protein. It is possible that the profound increase in levels of $p21^{waf1/cip1}$ blocks the apoptotic pathway in MCF-7 cells (Lin and Benchimol, 1995; Attardi et al, 1996). In ZR-75 cells, the minimal increase in $p21^{waf1/cip1}$ may be

permissive for Bax expression in the apoptotic pathway while the absence of detectable Bcl-2 would suggest that apoptotic function should not be compromised. Studies in progress are designed to determine whether regulation at the level of other anti-apoptotic proteins such as bcl-X_l may be critical for prevention of the apoptotic response to radiation in the ZR-75 breast tumor cell.

C. Studies to develop a breast tumor cell line expressing p21 antisense. p21 antisense studies to assess the effects of dysregulation of the cyclin-dependent kinase inhibitor p21 on the response of breast cancer cells to ionizing radiation.

The goal of these studies was to characterize the effect of dysregulation of the cyclin-dependent kinase inhibitor on the radiation response of human breast cancer cells (MCF-7). To this end, attempts were undertaken to stably transfect MCF-7 cells with a construct expressing $p21^{WAF1/CIP1}$ in the antisense configuration as we have previously described in two human myeloid leukemia cell lines (Freemerman, Leukemia 11:504-513, 1997, and Wang, Exp Cell Research, In press). Electroporation was used to transfect MCF-7 cells with either an empty vector control (pREP4) or vector containing the p21 cDNA in the antisense configuration, each containing a hygromycin-resistance marker. Cells were electroporated at 300 V and 500 μF. The cells were then selected in medium containing 400 μg/ml hygromycin, and single-cell clones obtained by limiting dilution. Following expansion, each of the surviving clones was assessed for p21 induction by ionizing radiation (IR) (Figures 5 and 6).

In the uppermost panel (Figure 5), results obtained with 3/10 empty vector control lines are shown. In these studies, cells were exposed to 10 Gy, protein isolated after 6 hr, and p21 expression determined by Western blot analysis. It can be seen that untreated empty-vector controls (V1,2 and 3/0) each displayed a significant increase in p21 expression following irradiation (V1,2,3/IR). Also shown are results obtained with untransfected wild-type cells (WT), which are similar.

Two separate transfections were attempted to obtain p21-antisense expressing clones. Each resulted in the isolation of > 10 individual clones. Representative results of studies involving treatment of these clones are shown in the upper and lower panels of Figure 6. In the upper panel of Figure 6, it can be seen that both empty vector as well as AS clones 1-5 each exhibited significant expression of p21 following IR with 10 Gy. For comparison, the effects of stable p21 antisense expression on the response of human leukemia cells (U937) are shown (upper panel of Fig 6, right). In these cells, empty vector controls displayed a clear increase in p21 expression after a 24-hr exposure to 10 nM PMA. In contrast, this response was essentially abrogated by p21 antisense expression.

Similar results were obtained following a second round of transfections (lower panel). It can be seen that significant expression of p21 was observed in 11 representative MCF-7 clones transfected with the p21 antisense construct.

Based upon these initial results, it is clear that we have not as yet isolated stable MCF-7 transfectants exhibiting dysregulation of p21 in response to IR. It is possible that the p21 AS construct that we are using, while effective in blocking p21 expression in human leukemia cells exposed to PMA, is less effective in blocking p21 expression in breast cancer cells exposed to IR. A more likely possibility is that we have not as yet isolated clones stably expressing a functional p21 antisense construct. Two approaches are now being taken to deal with this problem. First, another

round of transfections is in progress in which we have employed Lipofectamine instead of electroporation to transfect this cell line. Second, collaborative efforts are in progress with Dr. Kris Valerie, Associate Professor of Radiation Oncology, and director of the Adenoviral Core Facility of the Massey Cancer Center, to employ a p21 antisense-containing adenovirus to transfect the MCF-7 line. In view of the high degree of success of adenoviral tranfections in epithelial cell lines such as MCF-7, we are quite confident that these efforts will be fruitful. Once suitable antisense-expressing lines are isolated and characterized, the effect of p21 dysregulation on the IR response of breast cancer cells will be explored in depth.

D. Radiosensitization of the breast tumor cell; promotion of apoptosis by exposure of cells to Vitamin D3 and the hypocalcemic Vitamin D3 analog EB 1089

Experimental Methods Cells were exposed to Vitamin D3 or the Vitamin D3 analog EB 1089 for two days prior to irradiation (10 Gy). Cell number was determined after an additional 24 hours. The induction of apoptosis was assessed based on cell morphology and by terminal transferase labelling.

Results and Discussion We have determined that Vit D3 and EB 1089 induce growth arrest alone and fail to promote apoptotic cell death in the MCF-7 breast tumor cell line (Figure 7). Similarly, ionizing radiation fails to induce cell killing (Watson et al, 1997). However, the combination of either Vit D3 or EB 1089 with estradiol resulted in an enhanced antiproliferative effect (Figure 8) accompanied by the promotion of apoptosis. Figure 9 indicates that the combination increased DNA fragmentation based on terminal transferase labelling (9A) as well as measurement of DNA fragmentation (9B). In addition, Figure 10 indicates that the combination of Vit D3 or EB 1089 with irradiation failed to increase the extent of initial DNA damage, which is consistent with the possibility that a differentiation signal from the Vit D3 compounds may conflict with a growth arrest signal from irradiation to promote apoptosis.

Conclusions and Implications These studies demonstrate that pretreatment of p53 wild type breast tumor cells with Vitamin D3 compounds sensitizes the cells to ionizing radiation. Sensitization appears to occur through the promotion of apoptosis and not through an increase in the extent of initial DNA damage. Preliminary studies suggest that a similar sensitizing effect by Vitamin D3 and EB-1089 may also be evident when used in combination with the chemotherapeutic drug, adriamycin. We propose that the Vitamin D compounds can be used to enhance the effectiveness of radiotherapy and chemotherapy in the clinical treatment of breast cancer.

E. Utilization of ionizing radiation to promote gene uptake and apoptotic cell death in p53 mutated breast tumor cells.

The Vitamin D compounds promote apoptosis in response to irradiation in p53 wild-type breast tumor cells; however a similar effect was not evident in p53 mutated MDA-MB231 cells or T-47 D cells. Consequently, since many breast cancers present with mutated p53 genes, we have been interested in developing approaches for inducing cell death in p53 mutated cells.

Experimental Methods MDA-MB231 and MCF-7 cells were transfected with a liposomal SV40-luciferase complex and irradiated (10 Gy) immediately after, 24 hours after or 24 hours prior to transfection. Luciferase expression was measured using a luminescence assay while the amount of luciferase in the cell was determined after a Hirt extraction procedure (Hirt 1967).

Results and Discussion Ionizing radiation enhanced the liposome-mediated delivery and expression of the SV-40 luciferase transgene in MDA-MB231 breast tumor cells both in the absence and the presence of serum (Figure 11). Improved transgene delivery and expression was observed at a clinically relevant dose of 2 Gy and was dose-dependent over a range of 2-10 Gy in both MCF-7 and MDA-MB231 breast tumor cells (Figure 12).Furthermore, enhancement of gene uptake was observed with irradiation prior to, coincident with and after transfection (Figure 13).

Conclusions and Implications These findings indicate that irradiation can enhance the efficiency of liposomal mediated transgene uptake. We propose that irradiation could be combined with gene therapy in the treatment of breast cancer.Studies are in progress to promote the delivery and expression of apoptosis-inducing (p53, Myc, E2F1) as well as other cytotoxic transgenes to both p53 wild-type and p53 mutated breast tumor cells with the goal of identifying approaches to improve the utility of gene therapeutic approaches in the treatment of this disease.

F. Molecular analysis of mutations induced in plateau-phase 184B5 (p53+) and 184B5-E6c6 (p53-defective) breast epithelial cells by the radiomimetic antibiotic bleomycin

Revisions to Specific Aims Results obtained thus far have led to a revision of Specific Aims 5 and 6, as follows:

1. Since a p53-defective derivative of the 184B5 cell line chosen for mutagenesis studies had already been constructed (Gudas et al., 1995), we are using this cell line, rather than constructing our own lines. This has allowed the mutagenesis experiments with p53+ and p53- cells to be performed concurrently and by the same personnel, thus making the results more strictly comparable.

2. Although we plan to assess the radiation response of mutant cells harboring bleomycin-induced gene rearrangements, we have decided to concentrate on mutations induced by a single agent, bleomycin, in order to be able to analyze a sufficient number of mutants.

3. Due to the recent acquisition of equipment for Spectral Karyotyping (a newly developed chromosome painting technology) by the Department of Human Genetics, we plan to determine karyotypes of any mutants which show rearrangements at the molecular level, in order to assess whether each rearrangement is inter- or intrachromosomal and whether it was accompanied by a global loss of chromosome stability.

Hence, the revised aims are as follows:

Specific Aim 5: To compare the frequency and molecular nature of both small deletions and gene

rearrangements induced by bleomycin in 184B5 (p53+) and 184B5-E6c6 (p53-) cells.

Specific Aim 6: To determine whether gene rearrangements in the two cell lines are accompanied by (1) translocations specifically involving the X chromosome, (2) global chromosomal instability, (3) changes in radiation-induced cell cycle perturbations, (4) apoptosis and (5) delayed reproductive death.

Experimental Methods Medium used for routine culture of 184B5 cells (hereafter referred to as serum-containing medium) was a 1:1 mixture of Ham's F12 and Dulbecco's media, reconstituted from powder (Gibco), sterile-filtered, and supplemented with 0.5 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 25 μg/ml insulin, 5% horse serum, and antibiotics. Procedures requiring more stringently controlled growth, or cloning of individual cells, were performed using commercially prepared Mammary Epithelial Cell Growth Medium (MEGM) from Clonetics Corp. This medium contains, in addition to the usual low-molecular-weight species, bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone and antibiotics, but no serum.

Cells were grown in 100-mm plastic Petri dishes, and were routinely subcultured when they reached 80% confluence. For subculturing, cells were washed with PBS and detached with 0.25% trypsin in PBS containing 1 mM EDTA. An equal volume of serum-containing medium was added, and the cells were pelleted (100 × *G*, 10 min) in order to remove trypsin. The cells were resuspended and seeded at a density of 10^5 per dish in the serum-containing medium.

For mutagenesis experiments, each 100-mm plate was seeded with 8×10^5 cells in serum-containing medium. The next day, the medium was replaced with identical medium but containing hypoxanthine, amethopterin and thymidine (HAT), in order to eliminate any preexisting *HPRT* mutants. When the cells reached 80% confluence, the medium was replaced with MEGM medium without HAT and lacking epidermal growth factor. Beginning 48 hr later, these G_0 cells were treated with bleomycin (0.5 - 5 μg/ml) for two days, with both medium and drug being replaced after one day. Following the 2-day treatment, the drug was removed, the cells washed, and the medium again replaced with drug-free, growth factor-free MEGM. After 4 hr of recovery in this medium, the cells were trypsinized and an aliquot was plated at 800 cells per plate in complete MEGM medium to determine survival. The bulk of the cells were seeded at 10^5 cells per plate in the serum-containing medium, with 10 plates for each treated culture. The cells usually reached confluence about 12 days later, and at that time they were again trypsinized and plated in MEGM medium containing 10 μM 6-thioguanine (5×10^4 cells per plate, two plates from each subculture, 20 plates from each initial treated culture) in order to select mutants. After 12 days, colonies were counted and two mutant colonies from each initial culture were trypsinized inside a cloning ring. The trypsinized cells were seeded into 100-mm plates containing serum-containing medium. The cells were subcultured once and then trypsinized, washed, and frozen in medium containing 10% DMSO prior to storage in liquid nitrogen.

For molecular analysis of *HPRT* mutations, each mutant clone was expanded, and whole-cell RNA was isolated from ~5×10^6 cells using an RNA-STAT/60 kit (Tel-Test). Following cDNA synthesis with Moloney murine leukemia virus reverse transcriptase, the *HPRT* message was amplified by two-step PCR using nested *HPRT* primers (McGregor et al., 1991), and then sequenced using several primers from within the coding sequence. A RETROscript kit (Ambion) was used for cDNA

synthesis and amplification, and an Epicentre Cycle sequencing kit was used for DNA sequencing.

For cytogenetic analysis, 184B5 cells (Walen and Stampfer, 1989), its E6-transfected derivative 184B5-E6c6 (Gudas et al., 1995), and various bleomycin-induced *HPRT* mutants of these lines, were grown to 80% confluence and incubated in the presence of colcemid to accumulate mitotic cells. The cells were swollen by a 10-min exposure to 0.75 M KCl, fixed with 3:1 methanol/acetic acid, and dropped onto cold, wet slides. The slides were hybridized for 2 days at 37°C in a humidified atmosphere with the SkyPaint mixture of fluorescent probes (Applied Spectral Imaging), which stains each human chromosome a different color. Karyotype analysis was performed using a Leica DMRBE microscope with CCD camera, and SpCube 2.0 software.

Results and Discussion

Bleomycin-induced cytotoxicity and mutagenesis in G_0-phase 184B5 cells As described in the previous annual report, we had already shown that bleomycin was significantly mutagenic in 184B5 cells, but that the bleomycin-induced increase in mutation frequency was sometimes marginal, as little as twofold. In an attempt to increase this ratio, cells were grown in medium containing hypoxanthine, aminopterin and thymidine (HAT). Aminopterin blocks *de novo* nucleotide synthesis pathways, so that only cells with intact *HPRT* genes (and thus able to make purine nucleotides from hypoxanthine through the salvage pathway) can survive (Peterson et al., 1975). Thus, HAT medium can decrease the spontaneous mutation frequency by eliminating any *HPRT* mutants that were already present prior to bleomycin treatment. As shown in Table I, this strategy did in fact reduce the frequency of spontaneous mutants, from about 2×10^{-5} in the absence of HAT, to approximately 5×10^{-6}, thus allowing an increase in mutation frequency of at least fivefold to be achieved consistently in the bleomycin-treated samples.

A striking feature of the mutagenesis data is that for the bleomycin-treated samples, the mutants from any single treated culture dish tended to be concentrated in just a few (1 to 3) of the 10 progeny subcultures grown from that dish. Given the known number of surviving clonogenic cells seeded for each subculture, this overdispersion in the distribution of the mutants could not be explained on the basis of chance (i.e., a Poisson distribution of mutants among the subcultures). One possibility is that the mutant cells grow faster than the bulk of nonmutant cells during the expression period, resulting in a higher mutant frequency in the final selection of mutant cells than in the initially seeded culture. This hypothesis is currently being tested.

Molecular analysis of mutant clones Within the past few months, conditions have been established for reverse transcription and nested amplification (RT-PCR) of the *HPRT* mRNA (Fig. 14), and the first few mutants from the 184B5 parental (p53+) line have been sequenced. Results obtained thus far indicate that most but not all of the mutants have an approximately normal-length *HPRT* mRNA, implying that, at most, only a minority of the bleomycin-induced mutations are large-scale deletions or rearrangements. However, three mutants show deletions or rearrangements within the *HPRT* cDNA, in the range of 50-100 bp. One of these has been fully sequenced and contains a deletion in the cDNA encompassing all of exon 5 and parts of exons 4 and 6. The upstream and downstream breakpoints are 92 bp apart in the cDNA but are predicted to be 7 kb apart in genomic DNA. In the

cDNA, a 32-bp non-*HPRT* sequence was inserted in place of the 92-bp deletion.

Point mutants included three with base substitutions, and one with a single-base deletion. The deletion occurred at bp 42, which is a GTT sequence (underlined T deleted), and thus is a potential site of bleomycin-induced double-strand cleavage (Povirk et al, 1989). This type of mutation rarely occurs spontaneously and is a hallmark of bleomycin-induced mutagenesis; it probably results from repair of a double-strand break, with loss of only the single base pair destroyed in formation of the break (Povirk et al, 1994). Although the data are still very preliminary, initial indications are that human cells are more susceptible to bleomycin-induced intrachromosomal deletions/rearrangements, but less susceptible to bleomycin-induced interchromosomal exchanges, than are hamster cells.

Cytogenetic analysis GTG banding of the parental 184B5 cell line confirmed the chromosome rearrangements reported previously (Walen and Stampfer, 1989). The karyotype, while altered, was pseudodiploid and appeared to be stable. The karyotype of the p53-defective derivative 184B5-E6c6 likewise appeared to be stable, and identical to that of 184B5. The apparent karyotypic stability of both cell lines implies that it should be possible to easily determine whether individual bleomycin-induced *HPRT* gene rearrangements are accompanied by chromosomal exchanges specifically involving the X-chromosome, as well as whether the mutant cell line had acquired a global chromosomal instability.

In a pilot study (Figure 15), SKY analysis (24-color chromosome painting with chromosome-specific fluorescent probes) of one of the mutant cell lines confirmed the chromosomal translocations previously reported in the parent line, and permitted more precise assignment of some of the breakpoints. This analysis also suggested that at least one of the rearrangements was more complex than reported previously; the rearranged chromosome 19, originally designated as a translocation of part of chromosome 5, now appears to contain small segments of chromosomes 11 (light blue) and 17 (dark blue) as well. However, it remains to be determined whether this complex rearrangement was present in the parent line or is peculiar to this mutant.

Progress summary for mutagenesis studies Results obtained thus far confirm that the 184B5 and 184B5-E6c6 cell lines are suitable for comparing bleomycin-induced gene rearrangements in p53+ and p53-defective backgrounds. Nearly all of the methodology has been established and data collection is well underway. Generation of additional bleomycin-induced mutants will continue for about half of the coming year. All mutants will be first analyzed by RT-PCR, and the alterations in the *HPRT* sequence will be determined. Mutants that yield no RT-PCR products will be subjected to Southern blotting with the *HPRT* cDNA probe, and those showing rearrangements will be subjected to (1) sequence analysis of the rearrangement, (2) SKY chromosomal analysis, (3) assessment of radiation response in terms of apoptosis and cell cycle arrest and (4) assessment for the "delayed reproductive death" phenotype (Chang and Little, 1991). Again, the basic question to be addressed is whether loss of p53 makes the cells tolerant of specific types of rearrangements, and whether rearrangements generated in p53+ cells are associated with acquisition of other phenotypic markers involving radiation response and chromosomal stability.

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Dose (μg/ml)	Survival (%)	Mutation Frequency	Individual Plate Counts
0	(100)	2×10^{-6}	0,0,0,0,0,0,0,0,0,2
0	(100)	6×10^{-6}	0,0,0,0,0,0,0,1,1,4
3	13	9.6×10^{-5}	0,0,0,0,0,0,0,1,14,81
3	33	4.2×10^{-5}	0,0,0,0,0,1,1,7,8,26,
6	17	8.7×10^{-5}	0,0,0,0,1,1,3,10,35,37
6	14	6.3×10^{-5}	0,0,0,0,0,0,0,3,12,48

Table I. Representative bleomycin mutagenesis experiment in 184B5 cells. Confluence-arrested cells were treated with bleomycin and each treated or control culture was split into 10 subcultures of 10^5 cells each and grown for 8 days. Then two plates of 5×10^4 cells were seeded from each subculture and incubated in the presence of thioguanine to select *HPRT* mutants. Individual plate counts are the sum of colonies in the two plates from each of the 10 subcultures.

Legends to Figures

Figure 1 TUNEL assay for the induction of DNA damage by ionizing radiation in MCF-7 and ZR-75 breast tumor cells. MCF-7 and ZR-75 breast tumor cells (both adherent and non-adherent) were isolated on microscope slides at the indicated times after irradiation, and DNA fragmentation was assessed by fluorescent end-labeling.
Left Panel: Control ZR-75 cells and ZR-75 cells at 24, 48 and 72 hours after 10 Gy of irradiation.
Right Panel: Control MCF-7 cells and MCF-7 cells at 24, 48 and 72 hours after 10 Gy of irradiation.

Figure 2 Influence of ionizing radiation (10 Gy) on levels of p53, $p21^{waf1/cip1}$, Rb, E2F-1, E2F-4 and Myc proteins in MCF-7 and ZR-75 breast tumor cells. Right panels: Quantitative representation of data. Cells were exposed to 10 Gy of radiation, and protein was isolated at the indicated times. Phosphorylation state of Rb:The upper band is indicative of the multiple phosphorylated states of the Rb protein. The lower band is the hypophosphorylated form of Rb.

Figure 3. Influence of ionizing radiation on proliferation of MCF-7 and ZR-75 breast tumor cells. The two breast tumor cell lines were irradiated with various doses of irradiation (0.5 to 10 Gy) and growth inhibition was calculated based on the relative growth rates of control and irradiated cells after 24 hours - where growth of control cells is taken as 100%.

Figure 4. Influence of ionizing radiation on the levels of the Bcl-2 and Bax proteins in MCF-7 and ZR-75 breast tumor cells. Cells were isolated for determination of protein levels at the indicated times after exposure to 10 Gy of ionizing radiation.

Figure 5. Influence of ionizing radiation on the induction of p21 in cells transfected with p21 antisense. Cells were irradiated with 10Gy and lysed 6 hours later to monitor p21 expression by Western blot analysis. Empty vector controls; 3 representative clones are shown.

Figure 6. Conditions are the same as described for Figure 5. Upper panel. Five (of nine) clones were assayed for p21 induction. On the right is shown induction of p21 by PMA in U937 controls (leukemic cells) and in antisense expressing cells. Lower panel. Following a second transfection, 11 U937 clones were isolated and monitored for p21 induction 6 hours after exposure to 10 Gy of irradiation.

Figure 7. Influence of Vit D3 and EB 1089 on the proliferation of MCF-7 breast tumor cells. Cells were treated with Vit D3 or EB 1089 (100nM) for 24, 48 and 96 hours. Final cell count was performed by trypan blue exclusion.

Figure 8. Enhanced antiproliferative activity of the combination of Vit D3 or EB 1089 with ionizing radiation in MCF-7 and ZR-75 breast tumor cells. Cells were treated with 100nM Vit D3 or EB1089 for 48 hours prior to irradiation. Cell numbers were determined after an additional incubation time of 24 hours.

Figure 9. Promotion of apoptotic cell death in MCF-7 breast tumor cells by the combination of EB 1089 with ionizing radiation. Cells were treated as above. DNA fragmentation was assessed by the TUNEL assay as well as by the Hoechst dye essay (Jarvis et al 1994).

Figure 10. The absence of an increase in initial DNA damage by Vit D3 or EB 1089 in combination with ionizing radiation. DNA damage was determined immediately after irradiation using the alkaline unwinding assay as described previously (Watson et al, 1997).

Figure 11. Enhancement in liposomal-mediated uptake and expression of SV-40 luciferase by ionizing radiation in MDA-MB231 breast tumor cells in the presence of serum. Studies were performed in the presence of various concentrations of serum and with a radiation dose of 710 Gy.

Figure 12. Dose-dependent increase in liposomal mediated uptake and expression of SV-40 luciferase in MCF-7 and MDA-MB231 breast tumor cells. Cells were irradiated with 2-10 Gy within 10 minutes of the initiation of transfection. Luciferase activity was determined after an additional 72 hours.

Figure 13. Capacity of ionizing radiation to increase the liposome mediated uptake of SV-40 luciferase with irradiation prior to, coincident with and after transfection. The abscissa indicates the time in hours either prior to or post-initiation of transfection when cells were irradiated. The uptake phase is considered to be the 5 hours after initiation of transfection.

Figure 14. RT-PCR of *HPRT* cDNA in two bleomycin-induced mutant lines. The *HPRT* PCR product is 721 bp. A 361-bp fragment of the *rig/S15* cDNA was amplified as an positive control.

Figure 15. Spectral Karyotyping (SKY) analysis of spontaneous mutant A1C1. Top panels show the metaphase spread with white light and with fluorescent labeling. Bottom panels show the karyotype of the same metaphase spread. Each chromosome is painted a different color, so that rearrangements can be detected as multicolor chromosomes. Note the complex rearranged chromosome 19, containing segments of chromosomes 5, 11 and 17.

Figure 1

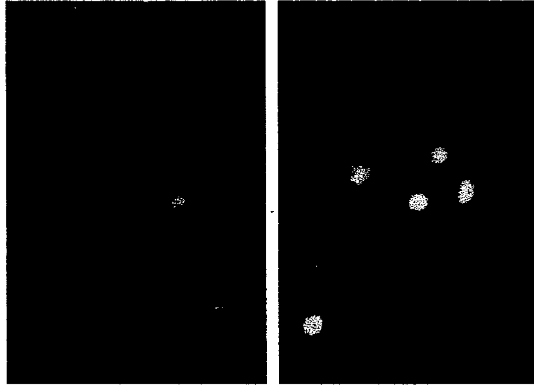
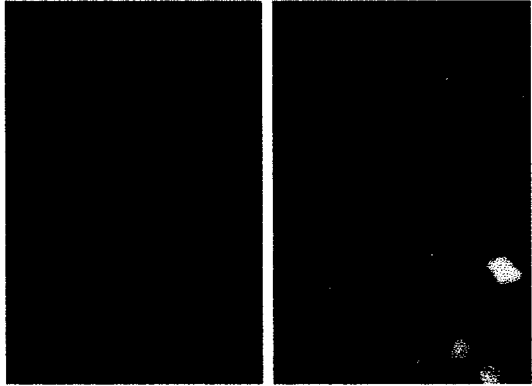
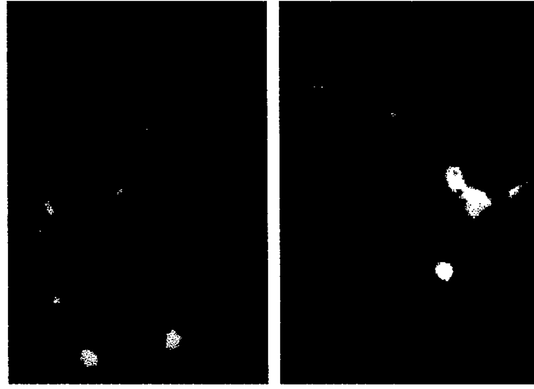
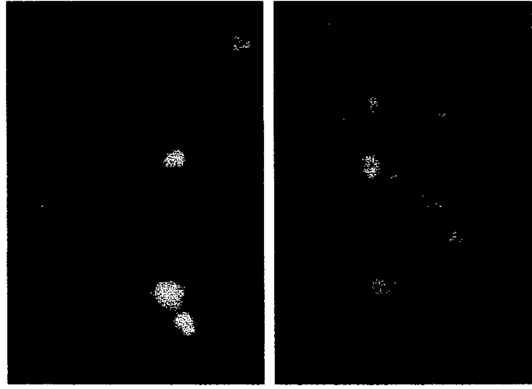
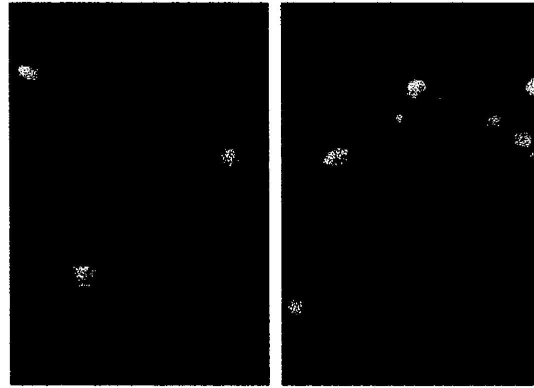
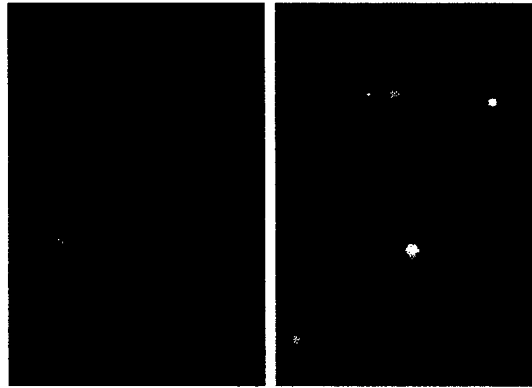
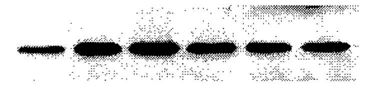
	ZR75 (control, 10 Gy IR)	MCF7 (control, 10 Gy IR)
24 h		
48 h		
72 h		

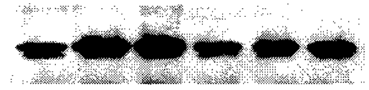
Figure 2

ZR75 | MCF7

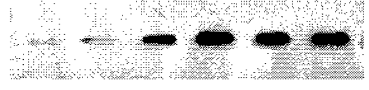
0 2 4 6 8 24 | 0 2 4 6 8 24

p53

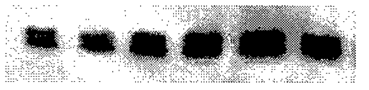


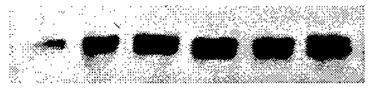


p21

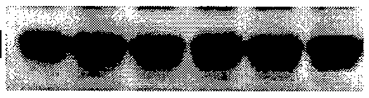


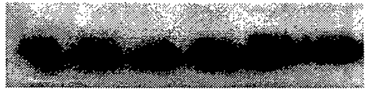
Rb





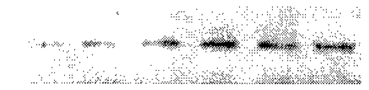
E2F-1

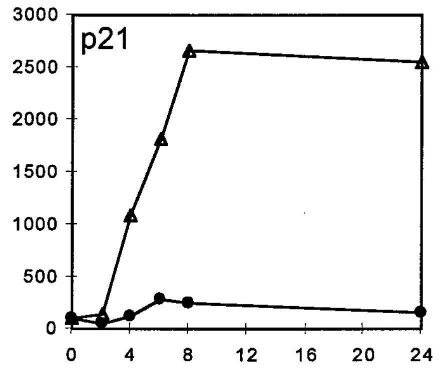


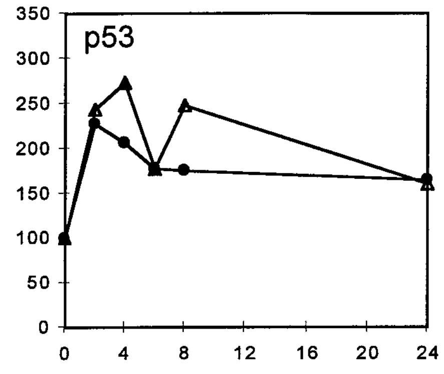


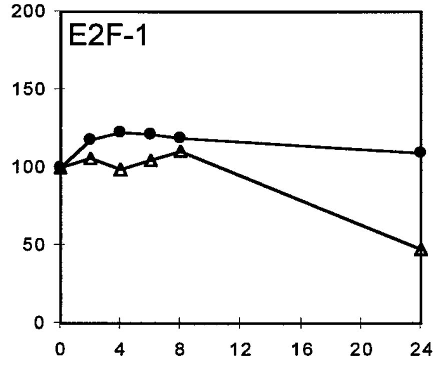
E2F-4

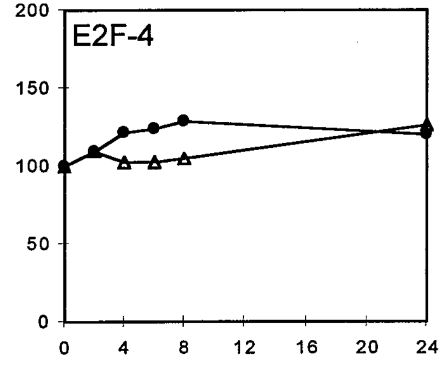
Myc

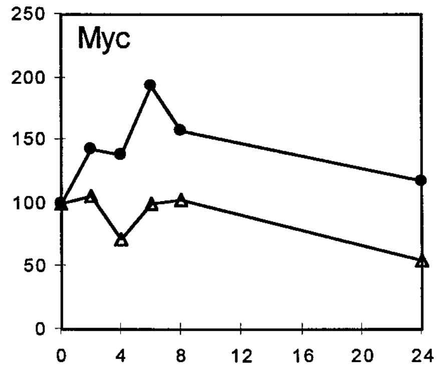












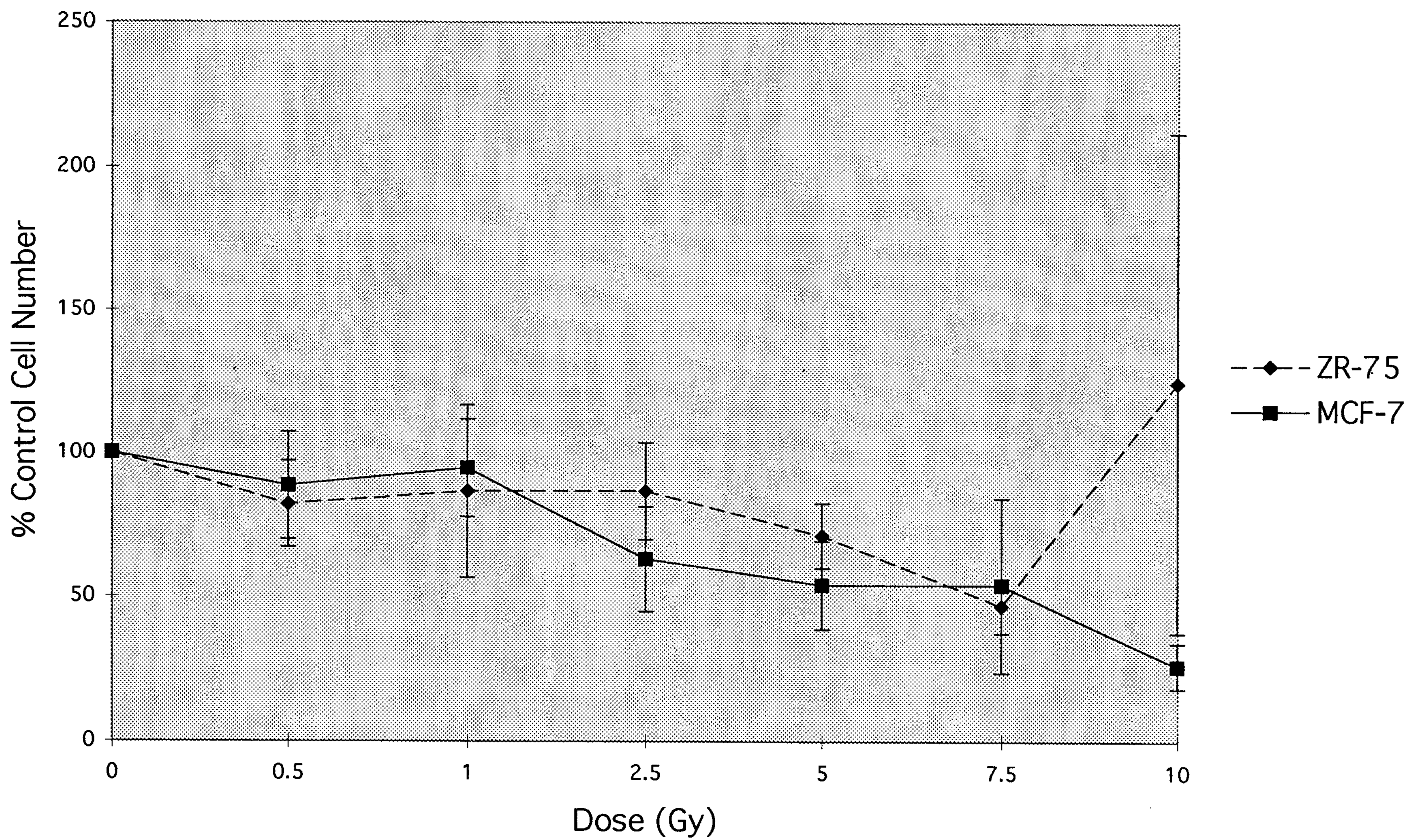
ZR75

MCF7

x-axis: Time (hours)

y-axis: Relative Protein Levels (%T=0)

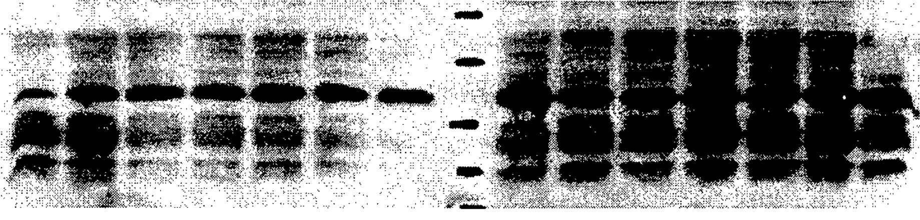
Figure 3

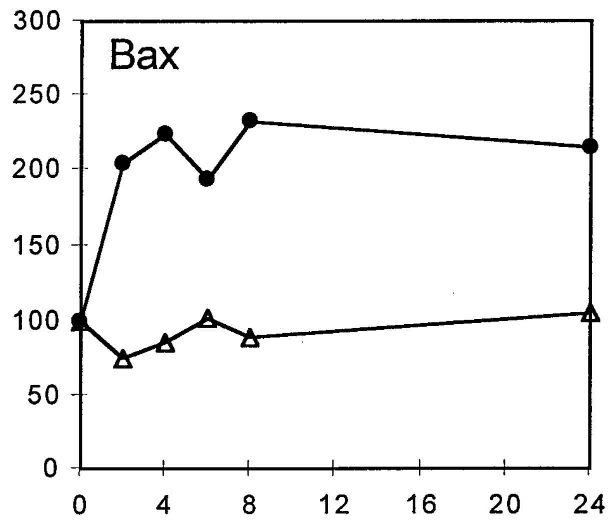


ZR75 MCF7

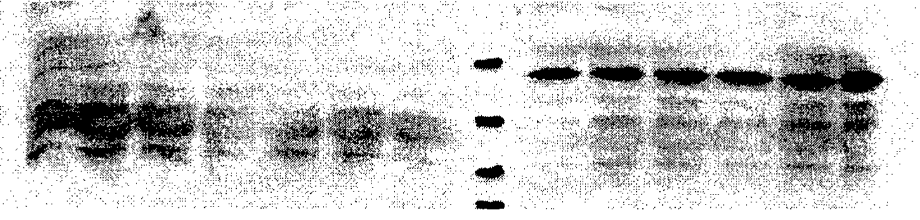
0 2 4 6 8 24 48 0 2 4 6 8 24 48

Bax





Bcl-2



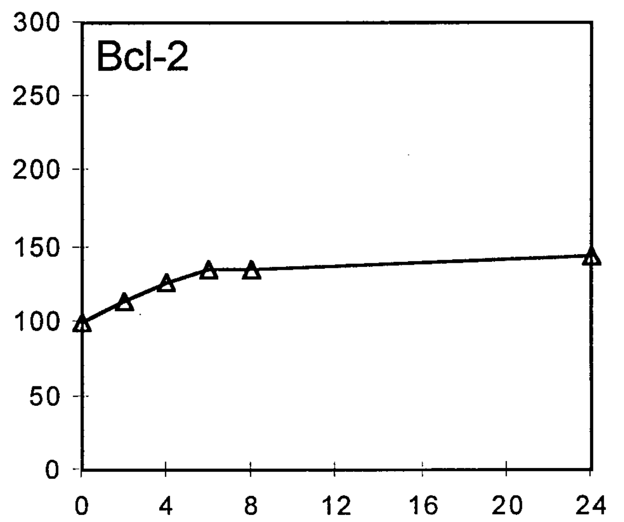


Figure 4

Figure 5

Mcf-7/Prep4

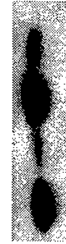
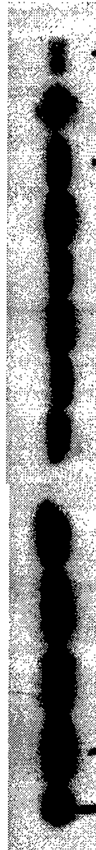


Figure 6

MCF-7/P21AS
Test 1

Test 2



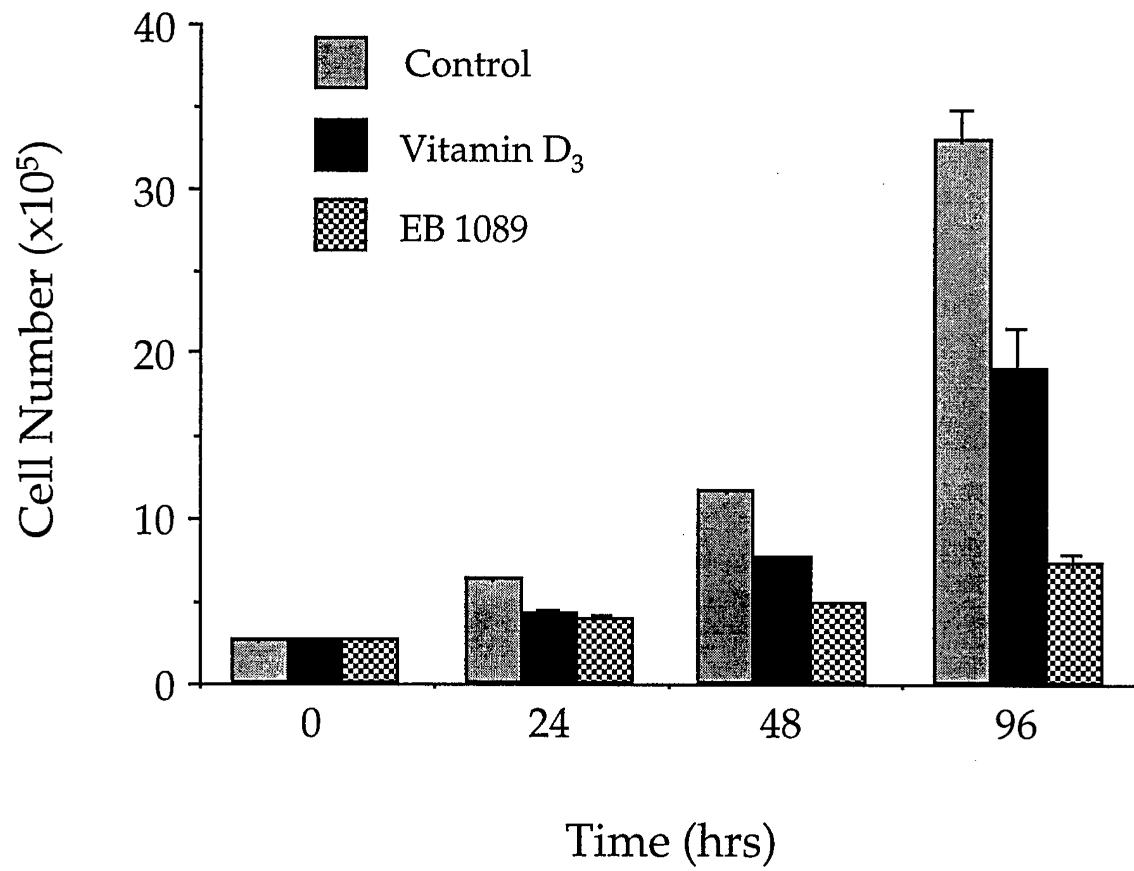
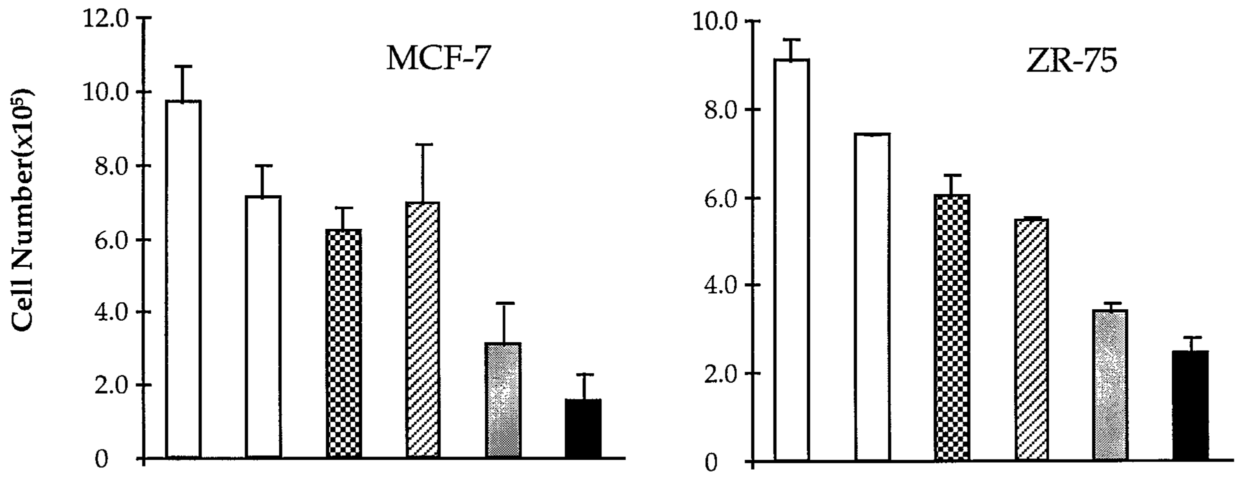


Figure 7

p53 wild type

p53 mutant



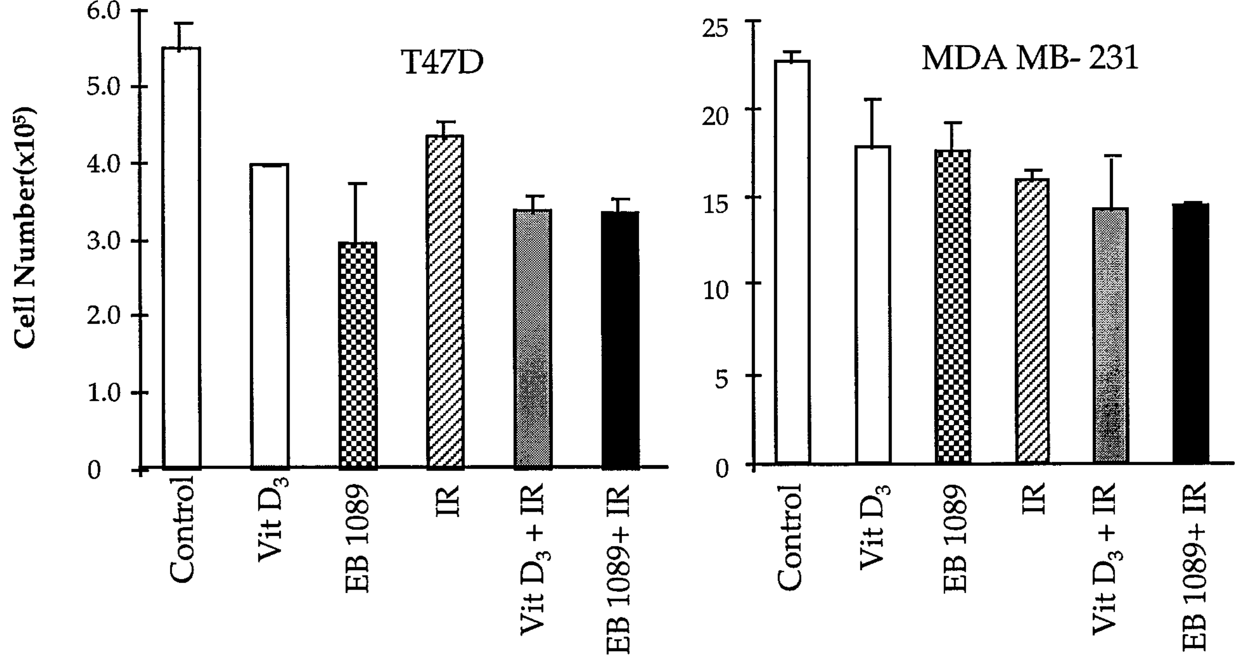


Figure 8

Figure 9A

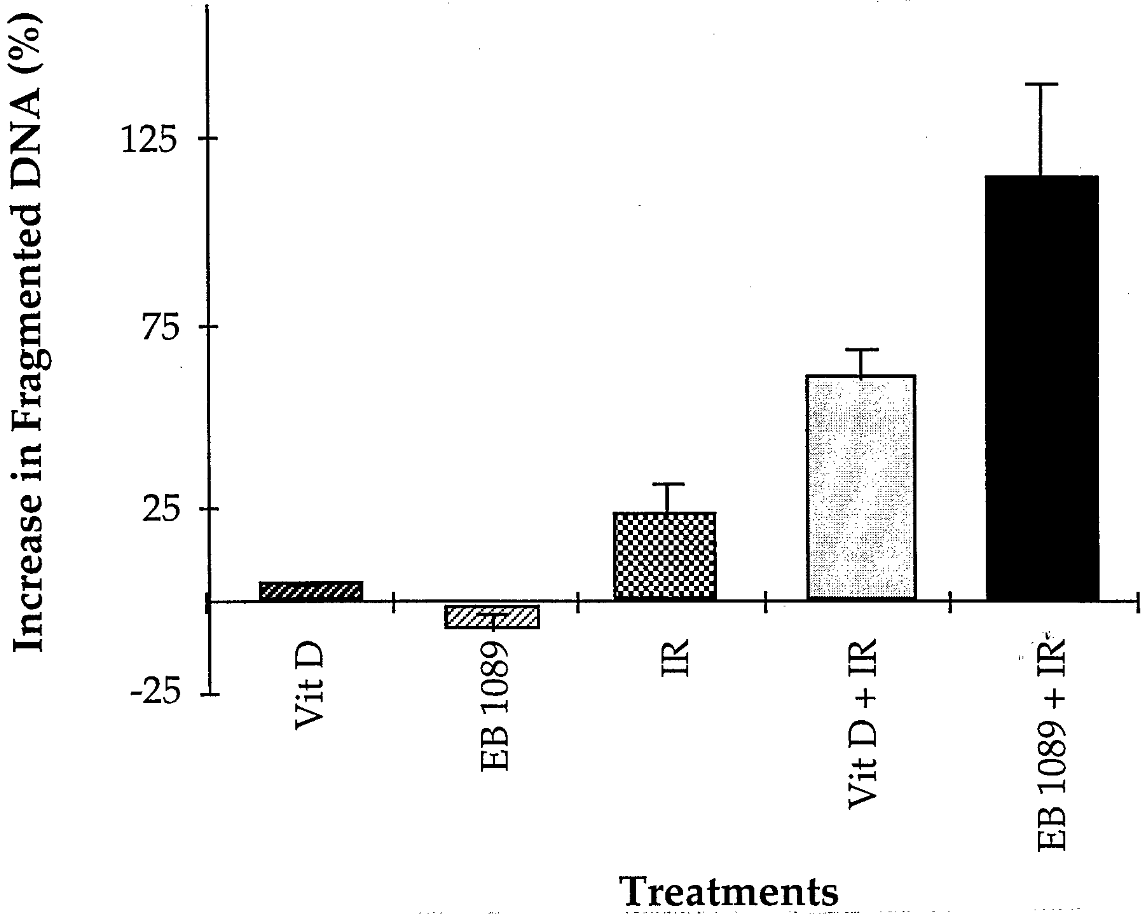
Control

IR

Vitamin D_3 + IR

EB 1089 + IR

Figure 9B



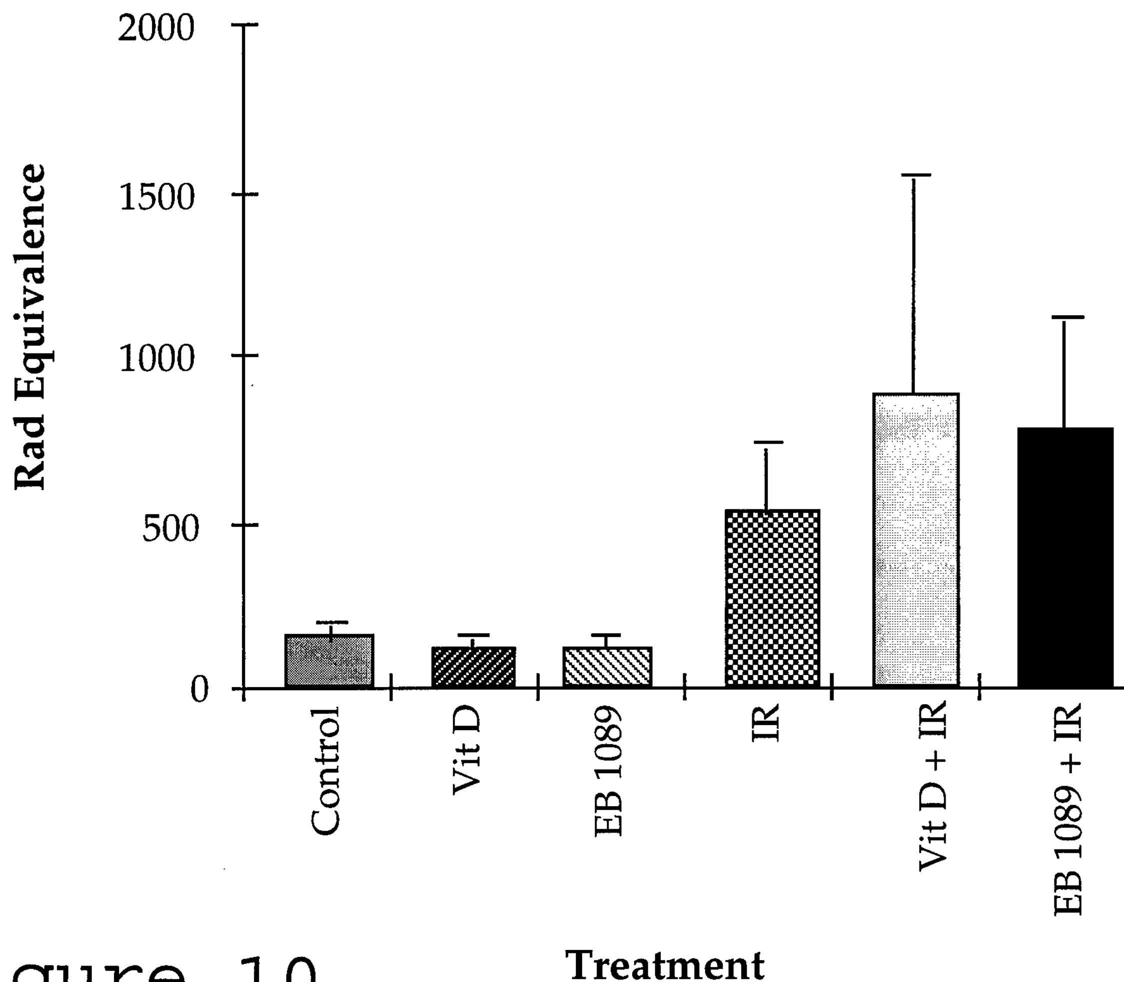


Figure 10

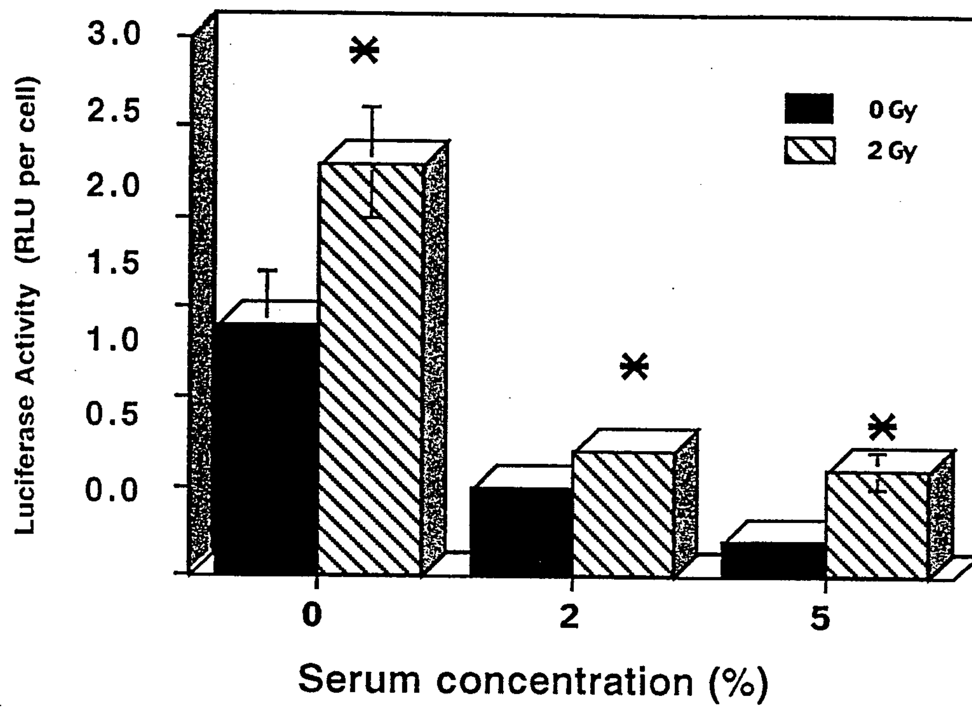
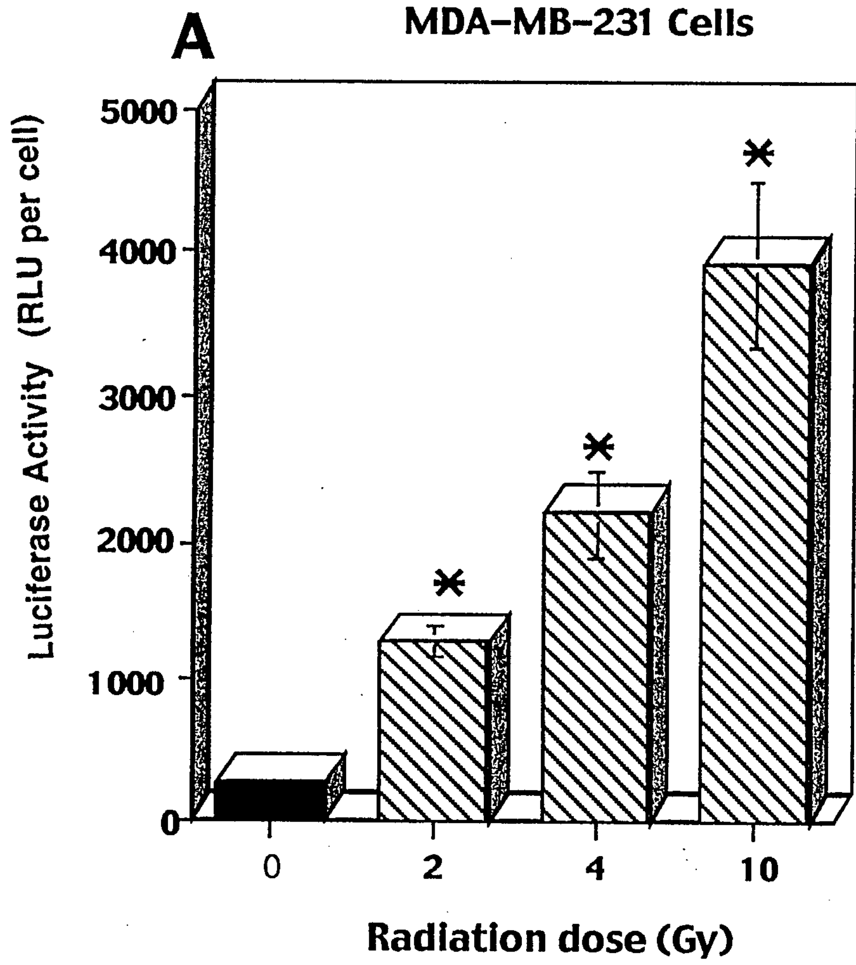


Figure 11



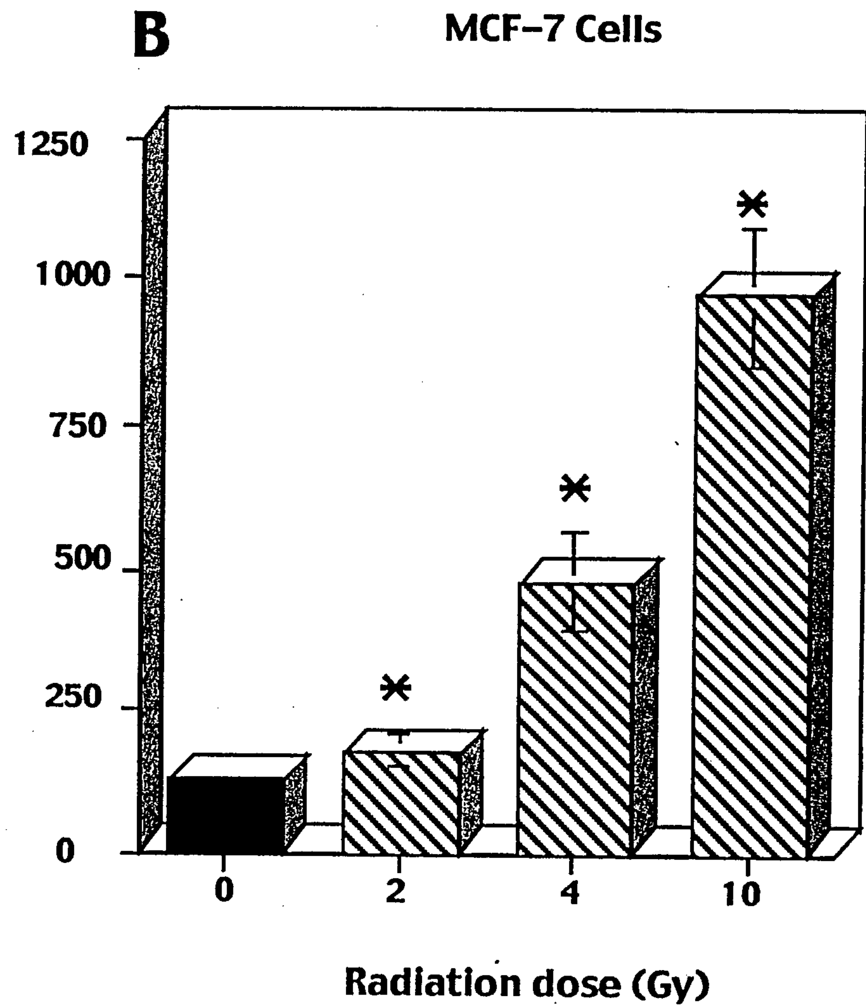


Figure 12

Jain et al

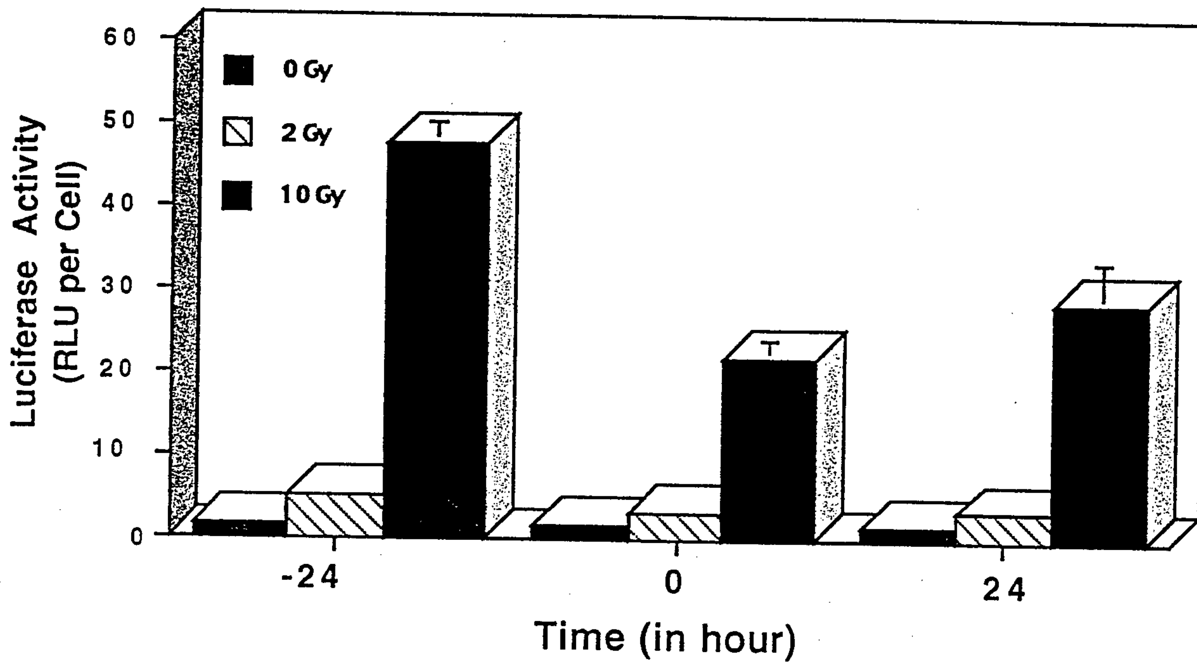


Figure 13

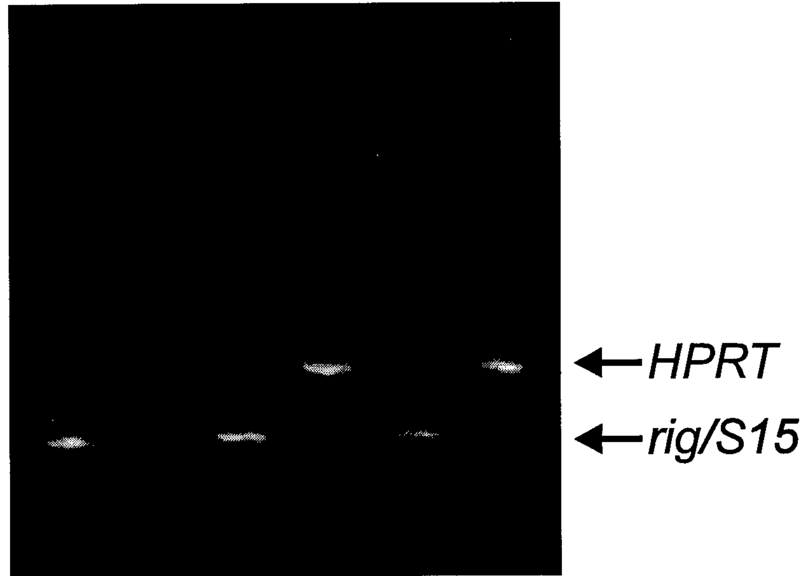
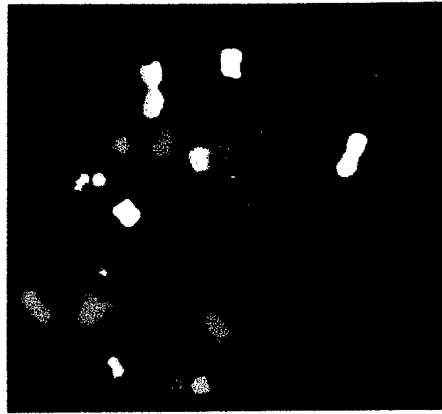
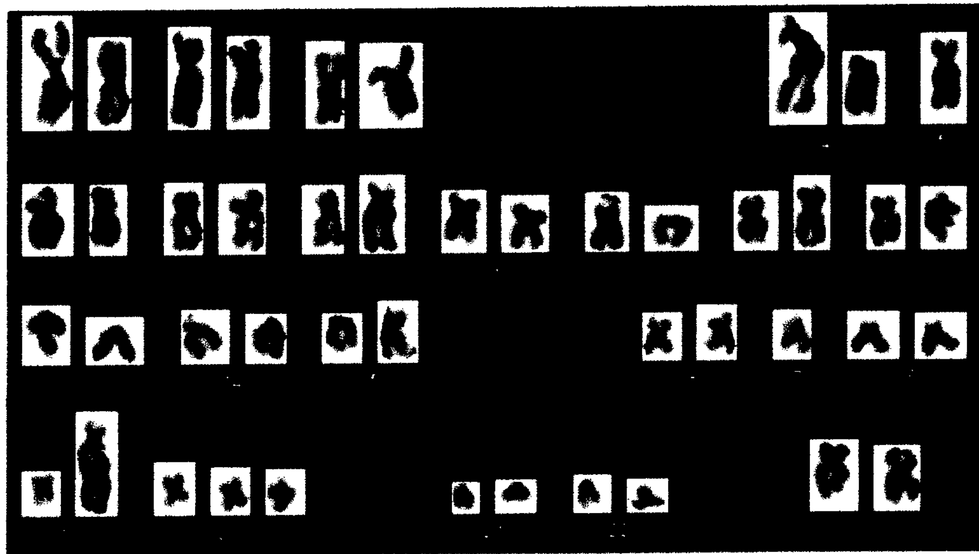


Figure 14





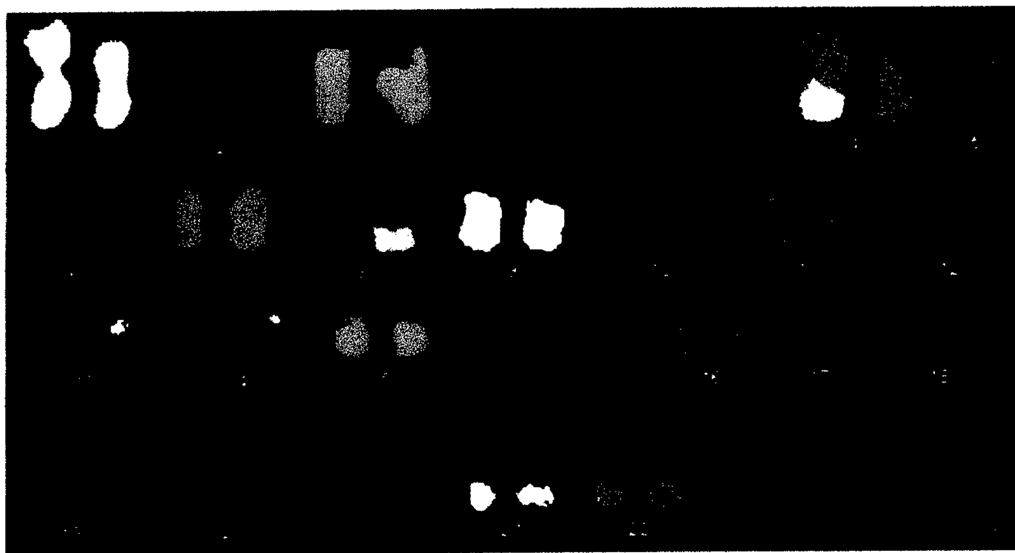


Figure 15

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