Title

Human mammary epithelial cells exhibit a differential p53-mediated response following exposure to ionizing radiation or UV light

Journal

Oncogene, 18(42)

ISSN

0950-9232

Authors

Meyer, Karen M
Hess, Suzanne M
Tlsty, Thea D
et al.

Publication Date

1999-10-14

DOI

10.1038/sj.onc.1202977

Peer reviewed

Oncogene (1999) 18, 5795–5805

http://www.stockton-press.co.uk/onc

Human mammary epithelial cells exhibit a differential p53-mediated response following exposure to ionizing radiation or UV light

Karen M Meyer[1,2], Suzanne M Hess[1], Thea D Tlsty[3] and Steven A Leadon*[,1,2]

[1]*Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, NC 27599, USA;*
[2]*Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, NC 27599, USA;*
[3]*Department of Pathology, University of California at San Francisco, San Francisco, California, CA 94143, USA*

The tumor suppressor protein, p53, plays a critical role as a transcriptional activator of downstream target genes involved in the cellular response to DNA damaging agents. We examined the cell cycle checkpoint response of human mammary epithelial cells (HMEC) and their isogenic fibroblast counterparts to ionizing (IR) and ultraviolet (UV) radiation, two genotoxic agents whose DNA damage response pathways involve p53. Using flow cytometric analysis, we found that both mortal and immortalized HMEC, which contain wild-type p53 sequence, do not exhibit a G1 arrest in response to IR, but show an intact G2 checkpoint. Supportive evidence from Western analyses revealed that there was neither an increase in p53 nor one of its downstream targets, p21^{WAF1}, in HMEC exposed to IR. In contrast, isogenic mammary fibroblasts arrest at the G1 checkpoint and induce the p53 and p21^{WAF1} proteins following IR. By comparison, HMEC exposed to UV displayed an S phase arrest and induced the expression of p53 and p21^{WAF1}. Our results show that the cellular response to DNA damage depends on both the type of damage introduced into the DNA and the specific cell type.

Keywords: p53; p21^{WAF1}; ionizing radiation; ultraviolet radiation; breast

Introduction

Cells are constantly exposed to exogenous and endogenous mutagens that damage DNA and threaten their genomic stability. Because maintenance of the genomic integrity of the cell is crucial to prevent neoplastic transformation, cells have evolved an elaborate defense against DNA damaging agents by utilizing specific proteins involved in cell cycle checkpoint control (Hartwell and Weinert, 1989; Hartwell and Kastan, 1994). These checkpoint proteins ensure delays in cell cycle progression in response to DNA damage so that proper repair of damaged DNA can occur (Hartwell and Kastan, 1994). This prevents the replication of damaged DNA during S phase (G1 checkpoint), as well as the segregation of aberrant chromosomes during M phase (G2 checkpoint). Defects in the cellular response pathways that react to DNA damage may result in the propagation of genetic alterations that lead to genomic instability and contribute to carcinogenesis (Hartwell and Kastan, 1994).

The p53 protein has been implicated in cell cycle checkpoint control in response to a variety of stresses and DNA damaging agents including IR (Maltzman and Czyzyk, 1984; Kastan *et al*., 1991; 1992; Nelson and Kastan, 1994), UV (Maltzman and Czyzyk, 1984; Nelson and Kastan, 1994), hypoxia (Graeber *et al*., 1994), and ribonucleotide depletion (Linke *et al*., 1996). Cells lacking p53, possessing mutant p53, or containing functionally inactive p53, do not arrest at the G1 checkpoint and continue to enter S phase (Kastan *et al*., 1991; Kuerbitz *et al*., 1992). In response to DNA damage, there is a post-transcriptional increase in the level of the p53 protein (Kastan *et al*., 1991), presumably due to increased stabilization of the p53 protein (Reich *et al*., 1983; Rogel *et al*., 1985). However, the regulation of p53 may be influenced by the types of lesions induced. For example, IR generates single- and double-strand DNA breaks and oxidative base damage. Recent studies have suggested that the ataxia telangiectasia gene (ATM) plays a crucial role in detecting DNA damage induced by IR and signaling to activate p53 (McKinnon, 1987; Canman *et al*., 1994; Haines *et al*., 1994; Meyn, 1995). ATM also participates in the UV pathway, since AT cells are unable to induce the G1 checkpoint after UV radiation (Kaufmann and Wilson, 1994). UV radiation produces cyclobutane dimers and (6–4) photoproducts that are repaired via the nucleotide excision repair (NER) pathway. Several lines of evidence have shown that wild-type p53 is necessary for efficient global genomic NER. For example, both Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations (Ford and Hanawalt, 1995) and primary human fibroblasts expressing the human papillomavirus 16 *E6* gene, which enhances degradation of p53 (Ford *et al*., 1998) are deficient in global genomic NER after UV damage. p53 has also been implicated in the NER pathway for UV-induced damage by virtue of its association with the proteins defective in xeroderma pigmentosum groups B and D (Wang *et al*., 1994; 1995).

The signaling pathways activated by damaging agents are also influenced by the specific cell type. The majority of previous investigations have been conducted using transformed cells or fibroblasts to examine the cellular response to damaging agents. However, cells derived from tumors contain several inherent changes at the molecular level, and are therefore, difficult to use as a comparison to the 'normal' response. In the current study, we investigated

*Correspondence: SA Leadon, Department of Radiation Oncology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7512, USA
Received 19 January 1999; revised 5 May 1999; accepted 14 May 1999

the response of HMEC and isogenic mammary fibroblasts to two DNA damaging agents whose damage response pathways both involve the p53 protein. We found that although both normal and immortalized HMEC contain wild-type p53, they do not induce a G1 arrest in response to IR and continue to progress through the cell cycle. In contrast, isogenic mammary fibroblasts arrest at the G1 checkpoint. Western analysis of proteins involved in the IR-induced damage response pathway showed that there was no increase in p53 or $p21^{WAF1}$ in HMEC exposed to IR, while there was an induction of these proteins in irradiated mammary fibroblasts. However, upon exposure of HMEC to UV, there is an induction of both p53 and $p21^{WAF1}$ protein levels. We propose that the damage response pathways that modulate the p53 response in HMEC depend on both the type of DNA damage and the specific cell type.

Results

Mortal and immortal HMEC do not exhibit a G1 checkpoint response following ionizing radiation

We initially examined whether HMEC, immortalized by treatment with benzo[a]pyrene (Stampfer and Bartley, 1985), displayed an intact, DNA damage-inducible G1 checkpoint compared to their normal parental cells following treatment with IR. Normal HMEC184 and the immortal HMEC184A1 were either sham-irradiated or exposed to 4 Gy of γ-irradiation. Flow cytometric analysis was performed to monitor cell cycle progression at various times following exposure to IR (White *et al*., 1994). Dot plots display two dimensional analyses of bromodeoxyuridine (BrdUrd) incorporation, an indication of S phase cells undergoing DNA synthesis, and propidium iodide (PI) staining, an indication of total DNA content (Figure 1a). Figure 1a represents flow analysis at 24 h following IR treatment. We found that both HMEC184 and HMEC184A1 continue to cycle following a dose of 4 Gy as compared to the no treatment control. Time course experiments with the HMEC184 and HMEC184A1 cells have also been conducted and the flow cytometric dot plot data plotted in Figure 1b. HMEC184 and HMEC184A1 continue to enter S phase following IR, suggesting the absence of a G1 arrest. By comparison, both HMEC184 and HMEC184A1 show an accumulation of cells in G2. Similar results were found in HMEC184B5 (data not shown), an immortalized cell strain that is also derived from HMEC184. The absence of a p53-mediated G1 checkpoint response in both normal and immortal HMEC indicates that an alteration in this pathway is not associated with the immortalization of these HMEC.

While the G1 checkpoint is absent in the mortal and immortalized HMEC, our flow cytometric analysis suggested that the G2 checkpoint was intact following IR. To directly assess the G2 checkpoint, we quantitated the fraction of cells undergoing mitosis following irradiation. The mitotic fraction of HMEC184, HMEC184A1, and HMEC184B5 was dramatically reduced by 90–100% within 2 h following a dose of 1 Gy (data not shown). HMEC 184 and 184B5 surpassed sham-irradiated levels by 4 h post-irradiation, with 184A1 showing a 70% recovery. Dose-response analyses were conducted at 2 h post-irradiation, a time when inhibition of mitosis is maximal (Kaufmann *et al*., 1995). A dose of 0.5 Gy yielded an 80–97% decrease in the mitotic fraction in the cell lines examined (data not shown). Taken together, these data show that the G2 checkpoint response in HMEC is both sensitive and rapid.

Matched epithelial and fibroblast cells show a differential response to IR

We examined the effect of IR on cell cycle progression in isogenic sets of epithelial and fibroblast cells to determine whether tissue specificity played a role in the cell cycle checkpoint response to IR. Three sets of cells were obtained as finite life span human mammary epithelial or fibroblast cells from reduction mammoplasty patients (Stampfer, 1985). Flow cytometric analyses reveal that unlike HMEC184, the isogenic fibroblasts 184Fb possess an intact G1 checkpoint in response to IR (Figure 2a). When a second matched set of epithelial and fibroblasts were examined, HMEC161 were found to continue to enter S phase following a dose of 4 Gy, confirming the absence of a G1 arrest (Figure 2b). However, the matched fibroblast counterparts of these epithelial cells possess an intact G1 checkpoint (Figure 2c). Although both matched sets are isogenic, a differential response is detected between the epithelial and fibroblast cells. Similar results have also been observed in a third matched set of mammary epithelial and fibroblast cells (48R and AC350, respectively).

HMEC do not induce the expression of p53 or $p21^{WAF1}$ following IR

The absence of a G1 checkpoint in HMEC treated with IR prompted further investigation into the p53-mediated damage response pathway. Western analyses were conducted to examine the expression of two proteins associated with G1 arrest, p53 and $p21^{WAF1}$, in HMEC184 and their isogenic fibroblast counterparts exposed to IR. These experiments revealed that following a dose of 4 Gy, there is an induction of p53 within 1 h in 184Fb, but there is not an early induction of p53 in irradiated HMEC184 (Figure 3a). Similarly, $p21^{WAF1}$ levels increase within 3 h in 184Fb, but no induction of $p21^{WAF1}$ was detected in HMEC184 upon exposure to IR (Figure 4a). These Western results, along with a more extensive time course analysis, were quantitated and the relative fold induction for p53 and $p21^{WAF}$ were determined after normalization with β-actin (Figures 3b and 4b). There was no increase in either p53 or $p21^{WAF1}$ levels in HMEC184 post-irradiation over the time course examined. In contrast, 184Fb showed a transient p53 increase beginning at 1 h with a maximal induction of 2–2.5-fold by 3 h post-irradiation (Figure 3b). The expression of $p21^{WAF1}$ in 184Fb increased at 3 h after irradiation, with a maximal increase of approximately 4-fold occurring at 6 h post irradiation (Figure 4b). The lack of an induction of both the p53 and $p21^{WAF1}$

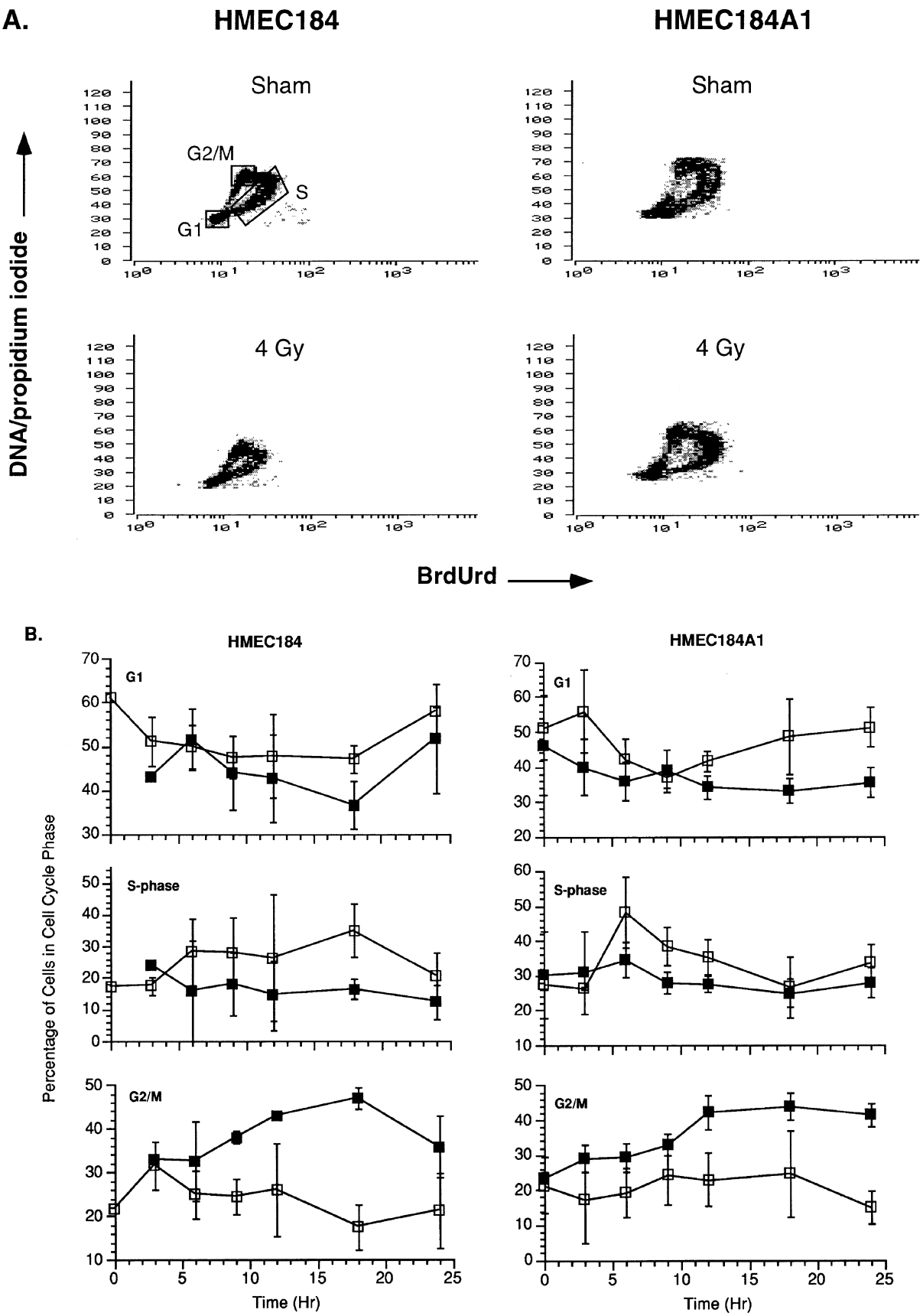

Figure 1 Flow cytometric analysis of mortal HMEC184 and immortalized HMEC184A1 cells following IR. Cells were either sham-irradiated or exposed to 4 Gy of IR. The population of cells present in G1, S, and G2/M was determined using a FACScan flow cytometer (Becton Dickinson). (**a**) Dot plot displays of two dimensional analysis of bromodeoxyuridine (BrdUrd) incorporation, an indication of S phase cells undergoing DNA synthesis, and propidium iodide (PI) staining, an indication of DNA content at 24 h post-irradiation. (**b**) Graphs showing the percentage of HMEC184 and immortalized HMEC184A1 cells in each phase of the cell cycle over a 24 h period following 4 Gy of IR. Bar graphs represent the average of 2–4 experiments. Open symbols: Sham-irradiated. Filled symbols: irradiated with 4 Gy

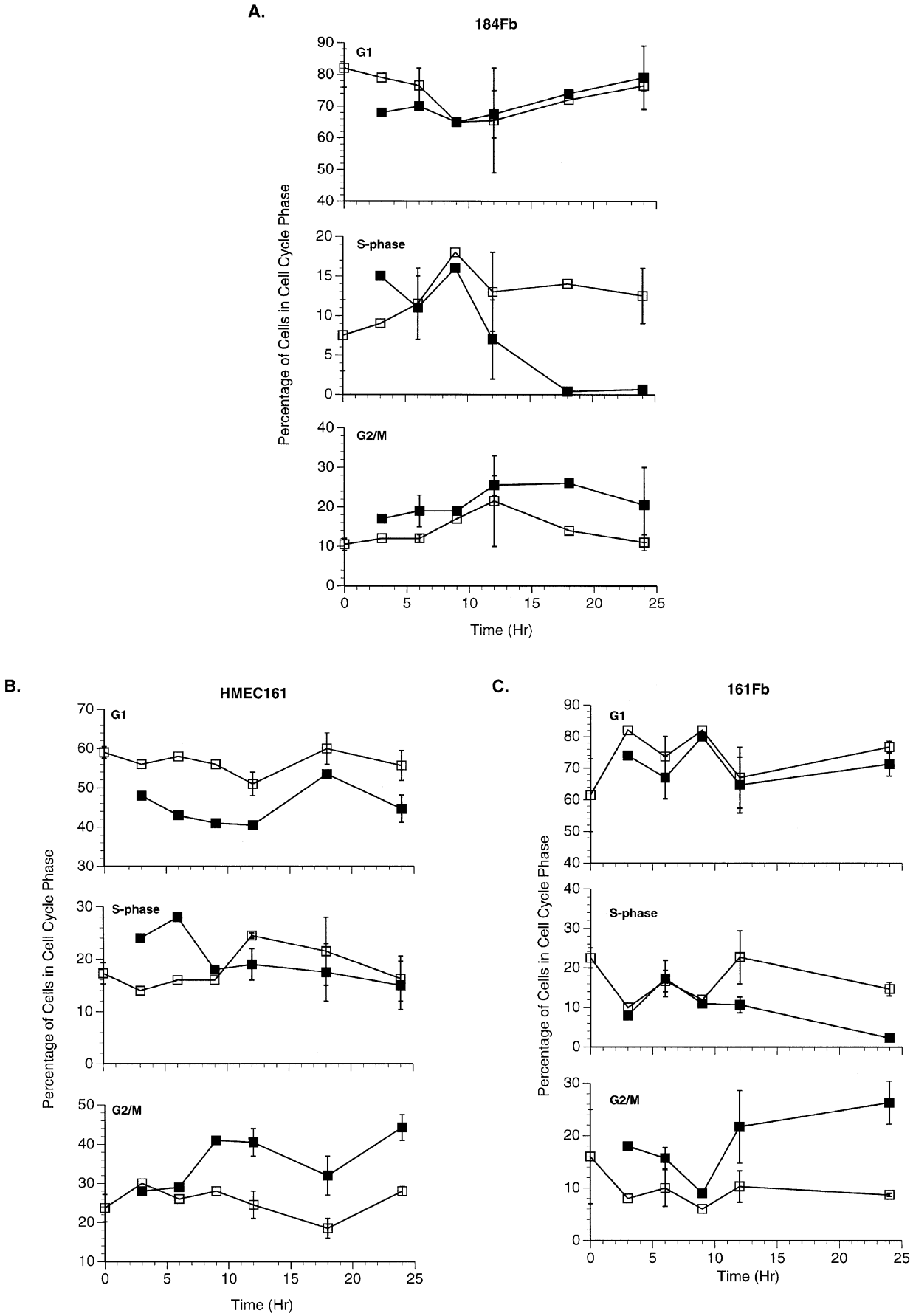

Figure 2 Flow cytometric analysis of isogenic 184 and 161 pairs following IR. Cells were either sham-irradiated or exposed to 4 Gy of IR. The population of cells present in various stages of the cell cycle was determined using a FACScan flow cytometer (Becton Dickinson). Time course of the percentage of (**a**) 184Fb, (**b**) HMEC161, and (**c**) 161Fb cells in G1, S phase and G2/M. Results are the average of 2–4 experiments. Open symbols: sham-irradiated. Filled symbols: irradiated with 4 Gy

proteins in HMEC184 supports our flow cytometry data showing an absence of a G1 checkpoint following IR. Similar Western results for p53 and $p21^{WAF1}$ were also obtained with the HMEC161 and 161Fb pair (data not shown).

HMEC184 contain wild-type p53

Because earlier studies had demonstrated that HMEC184 had a finite lifespan and displayed a normal, diploid karyotype (Stampfer, 1985), the absence of a p53-mediated response to IR in HMEC184 was unexpected, suggesting that p53 may be mutated in HMEC184. However, Lehman *et al*., (1993) had previously sequenced the highly conserved region of p53 known as the mutational hot-spot region (exons 4–9) in HMEC184, HMEC184A1 and HMEC184B5 and found a wild-type p53 sequence. However, p53 mutations have been found outside of the hot-spot region in some breast cancers (Hartmann *et al*., 1995). Therefore, to determine whether HMEC184 contained mutations in p53 outside of the hot-spot region, four primer sets were used to clone the complete p53 coding sequence in HMEC184. At least four independent sequences were analysed from each of the p53-specific PCR products generated. No mutations in p53 were found in all of the sequences examined (data not shown). However, the p53 sequences analysed from HMEC 184 displayed a homozygous arginine residue at codon 72. This previously characterized germline polymorphism at codon 72, which contains either an arginine (CGC) or a proline residue (CCC), has been observed at this position in the p53 sequence of several human p53 clones derived from normal and transformed cells (Matlashewski *et al*., 1987; Buchman *et al*., 1988). Therefore, these data confirm that HMEC184 contain a wild-type p53 protein.

Normal HMEC and fibroblast cells have similar radiation sensitivities to IR

We next examined whether the absence of a p53-dependent G1 checkpoint in HMEC affected their sensitivity to IR relative to that of fibroblasts. Radiation survival experiments were conducted with normal mammary epithelial cells (HMEC184 and 161), immortalized mammary epithelial cells (HMEC184A1 and HMEC184B5), and normal human skin fibroblasts (GM38). All five strains showed similar levels of radiation sensitivity (data not shown). The D_o value (inverse of the slope of the exponential portion of the survival curve) for the HMEC161 and HMEC184 cells was 2.1 and 2.0, respectively, 1.6 for the HMEC184A1 cells, and 2.2 for the HMEC184B5 cells. Although flow cytometric experiments and Western analyses were conducted with the 184Fb, these mammary fibroblast cells did not produce colonies under conditions used for the clonogenic survival experiment. Therefore, GM38 cells, which had been previously used in clonogenic survival experiments (Leadon and Cooper, 1993), were used for a comparison. The D_o value for the GM38 was 2.1 Gy.

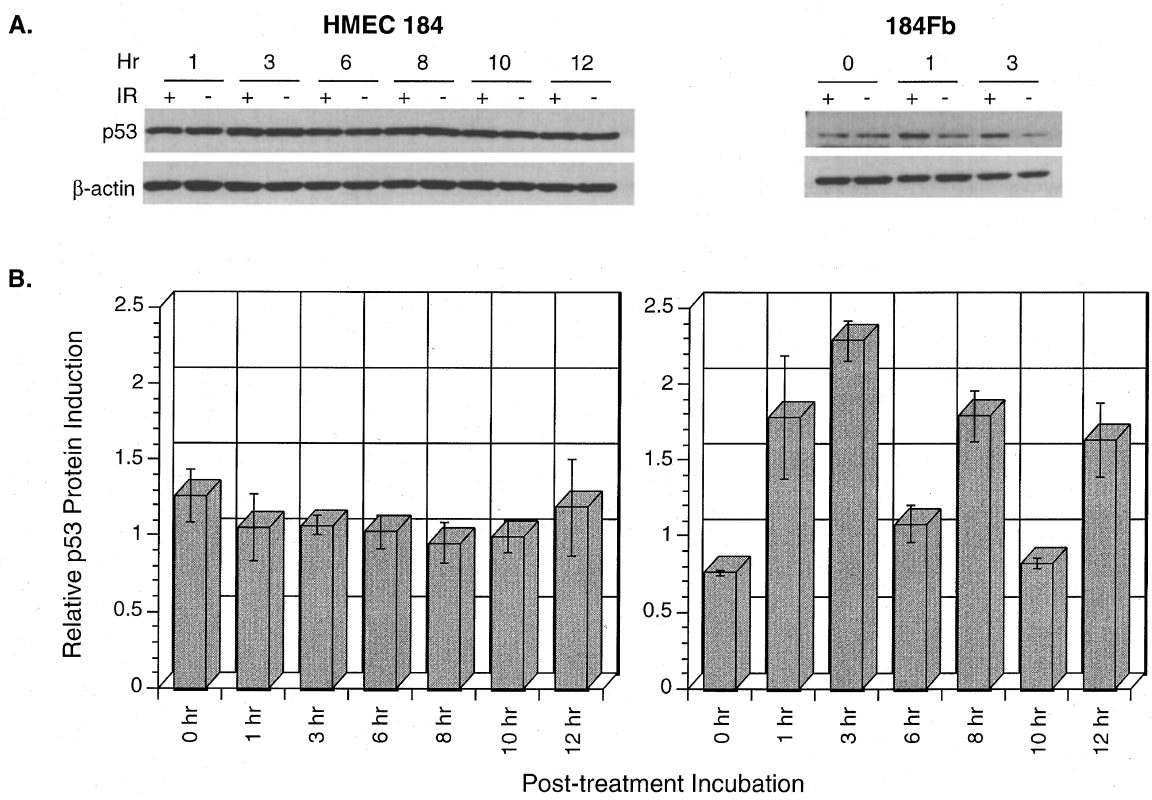

Figure 3 Expression of p53 in HMEC184 and 184Fb cells following IR. Cells were either sham-irradiated or irradiated with 4 Gy of IR and lysates collected at various times post-irradiation (0–12 h). (**a**) Autoradiograms from representative Western analyses. (**b**) Quantitation and relative fold induction for each protein after normalizing with the β-actin protein. Points indicate the means of 2–3 experiments; bars indicate standard error

This data indicates that the differential response to IR between the HMEC and fibroblasts does not result in an increased resistance of the mammary epithelial cells to IR.

HMEC arrest following UV treatment

Since the cellular response involving p53 can depend on the type of damage introduced into the DNA, we next examined cell cycle progression of HMEC184 and 184Fb exposed to UV radiation using flow cytometry. HMEC184 and 184Fb were either sham-irradiated or exposed to 5 J/m^2 of UV radiation. Clonogenic survival curves indicate that there is an 80% survival of the HMEC at a dose of 5 J/m^2 (data not shown). At 6 h following UV treatment, HMEC184 showed a decrease in the fluorescence intensity of BrdUrd incorporation. The nuclei shifted to the left in the S phase population, which suggests an inhibition of replication initiation (Figure 5). HMEC184 recover by 15 h after irradiation (data not shown). The response of 184Fb to UV also shows an inhibition of BrdUrd incorporation at 6 h. By 12 h following UV treatment, the 184Fb recover and continue to progress through the cell cycle (Figure 5). These results indicate that both the mammary epithelial cells and fibroblasts undergo an S phase arrest in response to UV irradiation.

p53 and p21^{WAF1} protein levels increase in HMEC after UV radiation treatment

Increased levels of p53 protein and activation of downstream targets of p53 involved in repair and arrest have been shown to occur in cells irradiated with UV light (Maltzman and Czyzyk, 1984; Ljungman and Zhang, 1996; Cistulli and Kaufmann, 1998). To determine whether the absence of a p53-mediated response following exposure to IR was specific to this DNA damaging agent, Western analyses were performed to examine the protein levels of p53 and p21^{WAF1} following UV radiation. Lysates were collected from either sham- or UV-irradiated (5 J/m^2) HMEC184 and 184Fb over a 24 h time course. In response to UV radiation, there was an increase in the level of the p53 protein compared to sham-irradiated controls in both HMEC184 and 184Fb (Figure 6a). However, temporal differences were observed. HMEC184 showed an increase in p53 protein levels beginning at 14 h and then a gradual decrease by 24 h after treatment. Irradiated 184Fb displayed an earlier induction of p53, which occurred by 3 h, and this response was maintained through 16 h. The quantitation of these data is shown in Figure 6b. There was a maximal threefold increase in the level of p53 protein present in 184Fb at 3 h, while the induction of p53 (2.0–2.5-fold) in HMEC184 peaked at 14 h. Since an increase in p53 protein was observed, its downstream target p21^{WAF1} was subsequently examined to determine whether there was a corresponding induction after exposure to UV. p21^{WAF1} was induced in HMEC184 beginning at 15 h and peaked at 20 h after UV treatment (Figure 7a, 7b). The p21^{WAF1} protein levels in 184Fb were induced at an earlier time, beginning at 6 h post-irradiation and with a maximal induction of approximately threefold occurring at 16 h (Figure 7b). The increase in both p53 and p21^{WAF1} proteins in

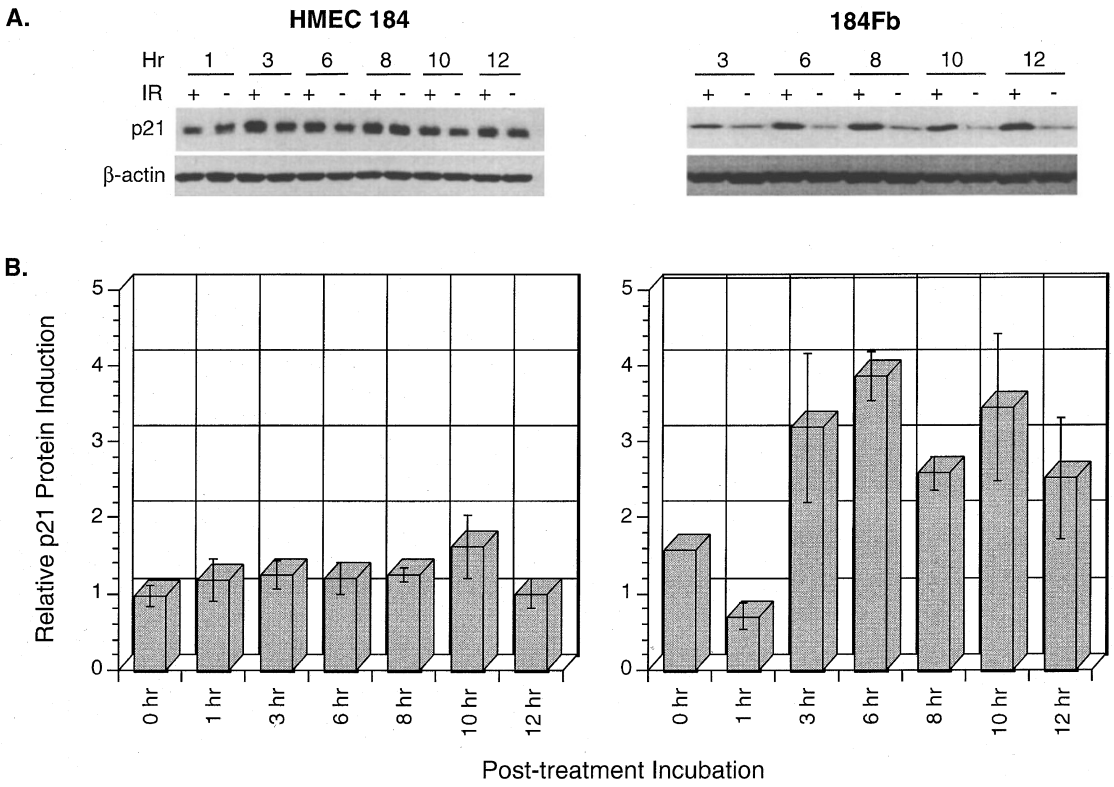

Figure 4 Levels of p21^{WAF1} in HMEC184 and 184Fb cells following IR. Cells were either sham-irradiated or irradiated with 4 Gy of IR and lysates collected at various times post-irradiation (0–12 h) (**a**) Autoradiograms from representative Western analyses. (**b**) Quantitation and relative fold induction of p21^{WAF1} after normalizing with the β-actin protein. Points indicate the means of 2–3 experiments; bars indicate standard error

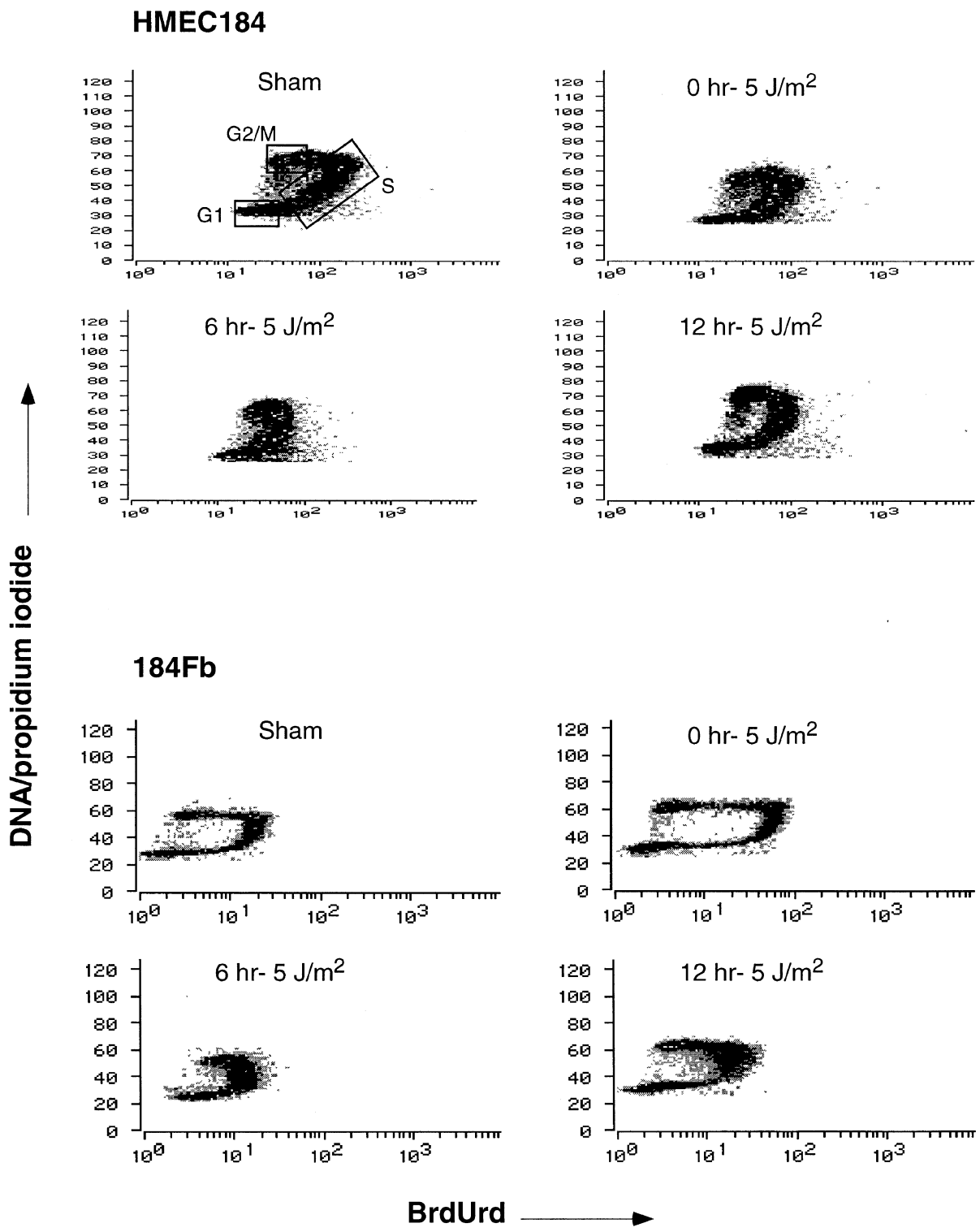

Figure 5 Flow cytometric analysis of isogenic HMEC184 and 184Fb cells following UV radiation. Cells were either sham-irradiated or exposed to 5 J/m^2 UV radiation. Dot plot displays of two-dimensional analysis of BrdUrd incorporation and PI staining

HMEC following UV radiation is in contrast to the lack of p53 and p21^{WAF1} induction observed in HMEC following IR.

Discussion

In this study, we investigated the p53-mediated response of HMEC containing wild-type p53 to two DNA damaging agents, IR and UV. Our results demonstrate that mammary epithelial cells respond differently not only relative to isogenic mammary fibroblasts, but also to the type of DNA damaging agent used. While the G1 checkpoint appears to be intact in mammary fibroblasts, this response is absent in both normal and immortal HMEC following treatment with IR. However, HMEC do exhibit a dramatic G2 checkpoint upon exposure to IR.

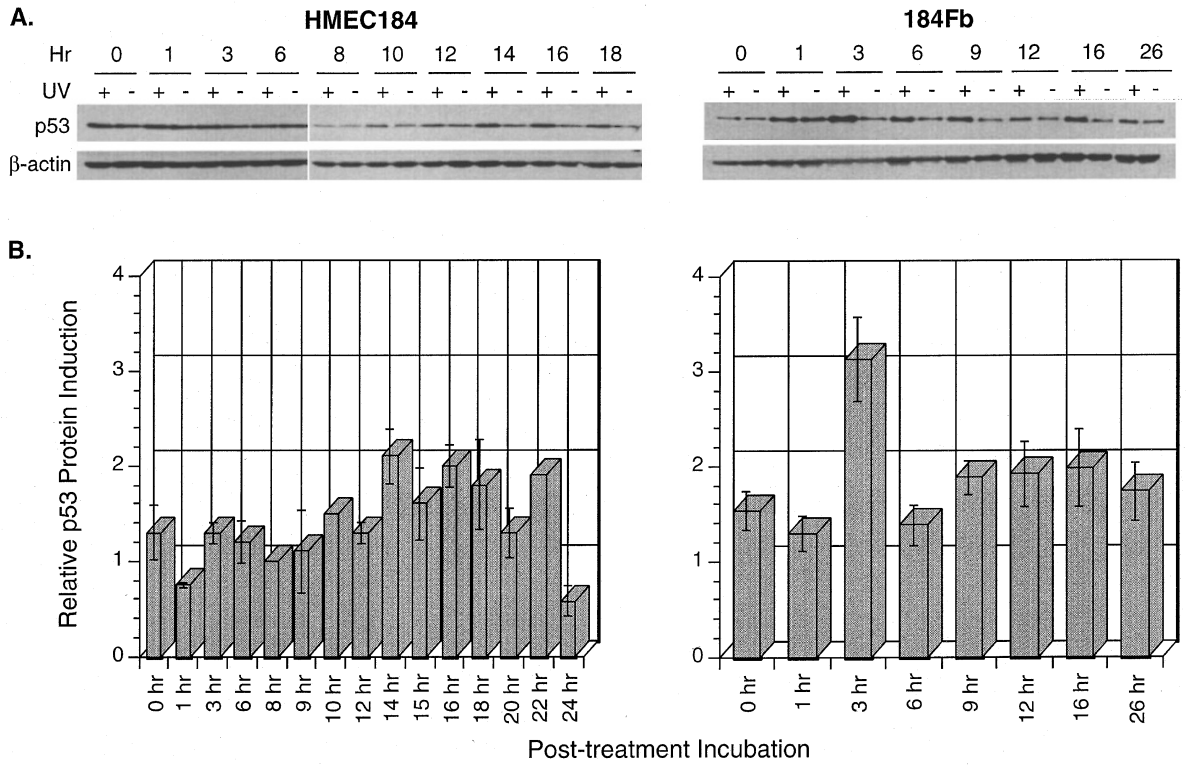

Figure 6 Protein levels of p53 in HMEC184 and 184Fb cells following UV radiation. Cells were either sham-irradiated or irradiated with 5 J/m^2 UV radiation and lysates collected at various times post-irradiation (0–24 h). (**a**) Autoradiograms from representative Western analyses. (**b**) Quantitation and relative fold induction for each protein after normalizing with the β-actin protein. Points indicate the means of 2–3 experiments; bars indicate standard error

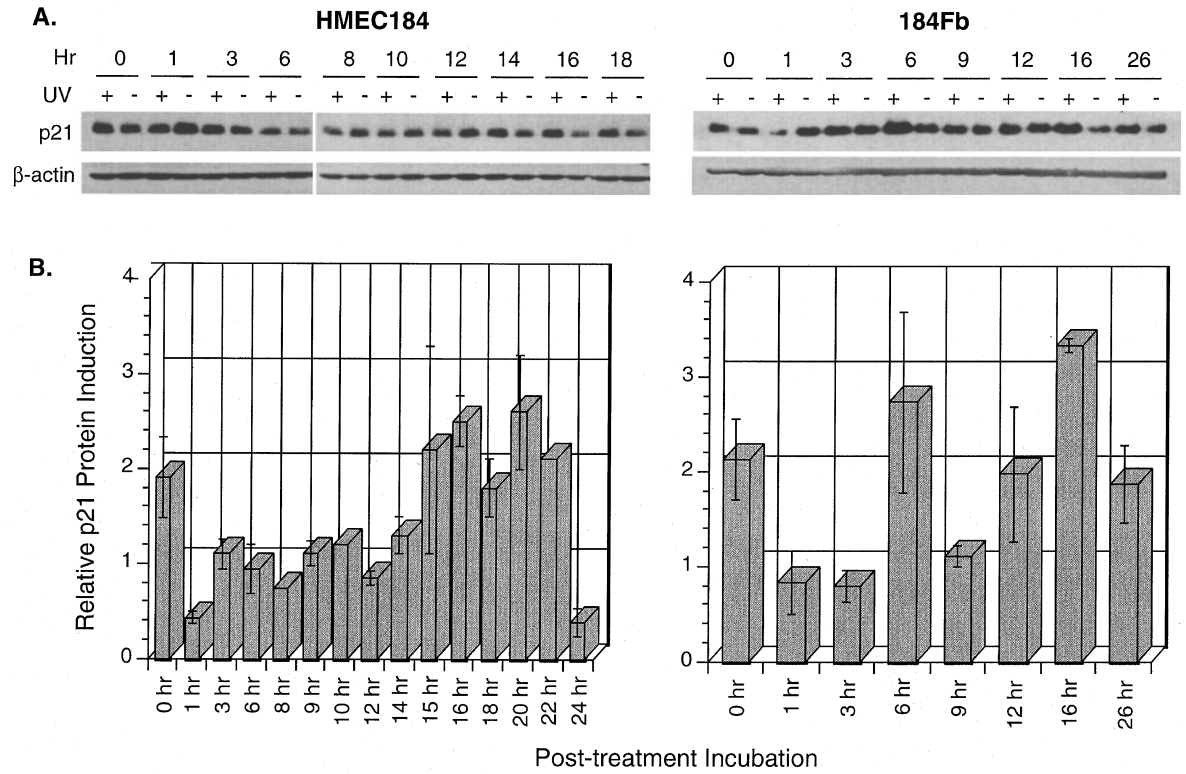

Figure 7 Protein levels of p21^{WAF1} in HMEC184 and 184Fb cells following UV radiation. Cells were either sham-irradiated or irradiated with 5 J/m^2 UV radiation and lysates collected at various times post-irradiation (0–24 h). (**a**) Autoradiograms from representative Western analyses. (**b**) Quantitation and relative fold induction for each protein after normalizing with the β-actin protein. Points indicate the means of 2–3 experiments; bars indicate standard error

Furthermore, in contrast to the mammary fibroblasts, HMEC184 do not show an induction of p53 or $p21^{WAF1}$ proteins upon exposure to IR. The absence of a p53-mediated G1 checkpoint in HMEC is not due to the presence of mutated p53, since our results and those reported by Lehman *et al*. (1993) show that these cells contain a wild-type p53 sequence. In addition, immunohistochemical data suggests that lack of a G1 response is not due to sequestration of the nuclear p53 protein in the cytoplasm following IR (data not shown).

Previous studies have also shown the absence or attenuation of a G1 checkpoint response in prostate epithelial cells, keratinocytes, and human bronchial epithelial cells exposed to IR (Girinsky *et al*., 1995; Gadbois and Lehnert, 1997; Nigro *et al*., 1997). In contrast, Wazer *et al*. (1995) and Namba *et al*. (1995) have reported that mammary epithelial cells and primary thyroid cells, respectively, both show a G1 arrest in response to IR. These studies, however, used media supplemented with serum, while our studies, as well as those by Girinsky *et al*. (1995), Nigro *et al*. (1997) and Gadbois and Lehnert (1997), have all used serum-free media. Thus, the addition of serum to culture media may select for cells with characteristics more similar to fibroblasts.

The absence of an IR-induced G1 checkpoint in HMEC relative to their fibroblast counterparts reflects differences in the regulation of p53 and its downstream targets between the two cell types. One significant difference between these cell types is the half-life of the p53 protein. In human and rodent fibroblasts, the half-life of p53 is approximately 30 min (Reich *et al*., 1983; Rogel *et al*., 1985), while it is greater than 3 h in HMEC (Delmolino *et al*., 1993). Because p53 is targeted for degradation by a ubiquitin-dependent proteolytic pathway (Chowdary *et al*., 1994), this suggests that there are also differences in this pathway between the two tissue types. The extended half-life of p53 in HMEC most likely accounts for the elevated endogenous levels of p53 found in HMEC relative to fibroblasts (Meyer and Leadon, unpublished results; Lehman *et al*., 1993). This could point to important distinctions in how breast epithelial cells modulate the activity of p53 compared to their fibroblast counterparts.

In contrast to our results with IR, exposure of HMEC to UV led to an S phase arrest and an accumulation of both p53 and $p21^{WAF1}$ proteins. This observation is important because it indicates that the p53 present in the HMEC can indeed activate downstream targets. The p53-mediated response to DNA damaging agents is influenced by the type of damage introduced into the DNA and by the processing of these lesions. IR generates single- and double-strand DNA breaks and oxidative base damage. While damage induced by IR has been linked with the signal that leads to the activation of p53, the repair of IR-induced DNA damage via base excision repair has not generally been associated with p53. UV radiation induces the formation of cyclobutane pyrimidine dimers and (6–4) photoproducts in DNA, which are repaired through the NER pathway. Indeed, strand breaks arising from excision repair of UV-induced dimers have been shown to induce p53 (Nelson and Kastan, 1994). Thus, the induction of p53 in the HMEC following treatment with UV may be related to the repair of the lesions induced by this agent.

It has been proposed that UV and IR damage activate p53 through different mechanisms. To support this hypothesis, p53 has been found to be phosphorylated at the casein kinase II site (Ser 389) in RKO cells, rat embryo fibroblasts (Kapoor and Lozano, 1998), and F9 murine testicular carcinoma cells (Lu *et al*., 1998) following UV treatment. This posttranslational modification, which has been associated with specific p53 DNA binding activity *in vitro* (Hupp *et al*., 1992) and growth suppression activity (Milne, 1992), has not been observed following treatment with IR or etoposide-induced DNA damage (Kapoor and Lozano, 1998; Lu *et al*., 1998). In addition, recent studies have shown that ATM phosphorylates p53 at Ser 15 *in vitro* (Banin *et al*., 1998; Canman *et al*., 1998), and that IR but not UV radiation increased the ATM protein kinase activity (Canman *et al*., 1998). Phosphorylation of p53 at Ser 15 in response to DNA damage is associated with increased expression p53 protein and with the ability of p53 to transactivate its downstream target genes (Siliciano *et al*., 1997). Furthermore, mutation of the Ser 15 phosphorylation site has been shown to affect the p53-dependent G1 checkpoint (Fiscella *et al*., 1993). Additionally, recent studies analysing the stability and ubiquitination of p53 following IR and UV radiation have indicated that the p53 protein is differentially modified following treatment with these two agents. Ubiquitination of p53 was detected in unirradiated and γ-irradiated RKO cells, but was absent in UV treated cells (Maki and Howley, 1997), suggesting that the stabilization of p53 after UV results from loss of p53 ubiquitination. Taken together, these data indicate that the posttranslational modifications that lead to the activation of p53 in response to IR may not be occurring in the HMEC. Studies are currently underway to test this possibility.

In conclusion, our results demonstrate that it is important to understand the response of normal cells to DNA damaging agents. This is especially important with respect to epithelial cells since the majority of human cancers are derived from this cells type. Thus, these studies will serve as an essential foundation for providing us with a better understanding of the molecular alterations that can occur during carcinogenesis.

Materials and methods

Cell culture and treatment

The matched sets of mammary epithelial and fibroblast cells, HMEC184 (AC305) and 184Fb (AD014), HMEC161 (AB881) and 161Fb (AB886), HMEC48R (AC170) and 48RFb (AC350), as well as the immortal benzo[a]pyrene transformed HMEC184 cell lines 184A1 and 184B5, were obtained from Dr Martha Stampfer, Lawrence Berkeley National Laboratory (Berkeley, CA, USA). The normal HMEC and isogenic fibroblast cells were obtained as normal mammary tissue from reduction mammoplasty patients (Stampfer, 1985). The immortalized cells were generated from HMEC184 using the chemical carcinogen benzo[a]pyrene (Stampfer and Bartley, 1985). The epithelial cells were grown as previously described (Stampfer, 1985) using serum-free mammary epithethial basal medium

(MEBM) obtained from Clonetics and supplemented with bovine pituitary extract (BPE; 50 μg/ml), epidermal growth factor (EGF; 0.01 μg/ml), isoproterenol (10 μM), transferrin (5 μg/ml), geneticin (0.05 μg/ml), insulin (5 μg/ml), and hydrocortisone (0.5 μg/ml). The human diploid mammary 184, 161, and 48R fibroblasts were maintained in DMEM/F12 containing 10% fetal bovine serum. The epithelial cells were cultured at 37°C in 2% CO_2, and the fibroblast cells were grown at 37°C in 5% CO_2. For both cell cycle progression experiments and Western analyses, epithelial and fibroblast cells were allowed to grow to log phase. Cells were irradiated with either 4 Gy of IR using a ^{60}Co γ-irradiator (Theratron; Atomic Energy of Canada), 5 J/m^2 of UV radiation (wavelength of 254 nm using a germicidal lamp), or sham-irradiated.

Cell cycle analysis

Cell cycle progression was analysed using a flow cytometric assay previously described in (White *et al*., 1994). Briefly, 10 mM bromodeoxyuridine (BrdUrd) was added 3 h prior to a given time point in order to label cells actively synthesizing DNA. Cells were then harvested and fixed with 63% ethanol in PBS. Cells were pelleted, treated with 0.08% pepsin/0.1M HCl, incubated for 20 min at 37°C, and then pelleted again. Nuclei were partially denatured by incubation in 2N HCl for 20 min at 37°C and then neutralized with 3M sodium borate. Nuclei were pelleted, resuspended in IFA/Tween 20 (4% fetal calf serum, 10 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% sodium azide, 0.5% Tween 20), and incubated in the dark on ice for 30 min with an anti-FITC BrdUrd antibody (Becton Dickinson). Samples were then rinsed, counterstained with propidium iodide (PI), and RNase (5 mg/ml) added. The population of cells present in G1, S, and G2 was determined using a FACScan flow cytometer (Becton Dickinson) by two dimensional analysis in which dot plots simultaneously display analysis of S phase DNA synthesis (determined by the BrdUrd incorporation) and DNA content (determined by the PI staining). Analysis of cell cycle distribution was conducted using the CyCLOPS 3.14 Program (Becton Dickinson). A total of 2×10^4 cells were analysed per time point.

Quantitation of G2 delay

G2 delay was analysed by quantitating the mitotic fraction as previously described (Kaufmann *et al*., 1995). Briefly, exponentially growing cells were irradiated with 0–2 Gy of ionizing radiation and returned to the incubator. After 1–4 h incubation, cells were fixed with methanol:acetic acid (3 : 1), rinsed with water, and stained with propidium iodide. Mitotic cells were identified using light microscopy, and minimum of 2000 cells were counted per sample. Mitotic cells were expressed as a fraction of the total number of cells.

Western blot analysis

Cells were rinsed with PBS and then scraped into sample lysis buffer (100 mM Tris-HCl, 8% SDS, 20% glycerol). Lysates were boiled for 5 min and an aliquot was removed for protein determination. Protein concentrations were determined using the modified Bradford assay (Biorad). A volume of $10 \times$ 2-mercatoethanol/bromophenol blue (60% 2-mercatoethanol, 0.02% bromophenol blue) was added to yield a $1 \times$ concentration. 40–50 μg of protein was electrophoresed on 12.5% SDS-polyacrylamide gels and transferred to Immobilon P membranes (Millipore) by electroblotting. The blots were then probed with antibodies against p53 (Oncogene Science; AB-6) or p21^{WAF1} (PharMingen; 6B6). A primary antibody to β-Actin (Sigma; A-5316) was used to normalize for equal protein loading. Blots were washed as previously described. Detection was by enhanced chemiluminescence (Amersham). Quantitation of protein bands was performed using a molecular image analysis program (Biorad).

Sequencing of the coding region of p53 mRNA in HMEC 184

Sequencing was carried out as previously described by Delmolino *et al*. (1993) with brief modifications. 1 μg of total RNA from HMEC 184 was used as a template for reverse transcription PCR using Superscript reverse transcriptase (Stratagene) and Taq DNA polymerase (Promega). Four sets of oligonucleotide primers were used to generate p53-specific PCR products which were cloned into pCR® 2.1-TOPO vectors (Invitrogen). Two separate PCR reactions were performed for each set of primers. Double-stranded plasmid DNA was purified using PEG precipitation and sequenced using M13-specific primers. DNA was sequenced at the UNC-CH automated DNA sequencing facility on a model 377 DNA sequencer (Perkin Elmer, Applied Biosysterms Division) using the ABI PRISM™ Dye Terminate Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division). The primers used for PCR are described below. The lower case letters represent restriction enzyme sites and the numbers in parentheses represent the nucleotide position in the p53 coding sequence (GeneBank accession number x02469).

1. sense: 5′-ggggatccATTGGCAGCCAGACTGCC-3′ (#103–120)
   antisense: 5′-gggaattcAGGGACAGAAGATGACAG-3′ (#429–412)
2. sense: 5′-ggggatccGATGAAGCTCCCAGAAT-3′ (#316–332)
   antisense: 5′-ggaattcCTGCTTGTAGATGGCCAT-3′ (#630–613)
3. sense: 5′-ggggatccAAACCACTGGATGGAGAA-3′ (#1096–1113)
   antisense: 5′-ggggaatTCAGTCTGAGTCAGGCC-3′ (#1317–1301)
4. sense: 5′-ggggatccATTGGCAGCCAGACTGCC-3′ (#103–120)
   antisense: 5′-ggggaatTCAGTCTGAGTCAGGCC-3′ (#1317–1301)

Clonogenic survival assays

Human mammary epithelial cells (HMEC184, HMEC184A1, HMEC184B5, and HMEC161) and normal human skin fibroblasts (GM38) were plated in triplicate at various densities and exposed to increasing doses of ionizing radiation or sham-irradiated. After 14 days, cells were fixed with methanol/acetic acid and stained with crystal violet. The percentage of clonogenic survival was derived from the ratio of colony-formation efficiencies of irradiated cells and unirradiated cells (colony defined as >50 cells).

Acknowledgements
We are grateful to Dr William K Kaufmann for enlightening discussions and critical reading of the manuscript. This research was supported by US Public Health Service Grants CA62059 and CA40453 from the National Cancer Institute, and a predoctoral (KMM) and postdoctoral (SMH) traineeship (T32 ES07126) from the National Institute of Environmental Health Sciences.

References

Banin S, Moyal L, Shieh S-Y, Taya T, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y and Ziv Y. (1998). *Science,* **281,** 1674–1677.

Buchman VL, Chumakov PM, Ninkina NN, Samarina OP and Georgiev GP. (1988). *Gene,* **70,** 245–252.

Canman CE, Lim D-S, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB and Siliciano JD. (1998). *Science,* **281,** 1677–1679.

Canman CE, Wolff AC, Chen Jr C-Y, Fornace AJ and Kastan MB. (1994). *Cancer Res.,* **54,** 5054–5058.

Chowdary DR, Dermody JJ, Jha KK and Ozer HL. (1994). *Mol. Cell. Biol.,* **14,** 1997–2003.

Cistulli CA and Kaufmann WK. (1998). *Cancer Res.,* **58,** 1993–2002.

Delmolino L, Band H and Band V. (1993). *Carcinogenesis,* **14,** 827–832.

Fiscella M, Ullrich SJ, Zambrano N, Shields MT, Lin D, Lees-Miller SP, Anderson CW, Mercer WE and Appella E. (1993). *Oncogene,* **8,** 1519–1528.

Ford JM, Baron EL and Hanawalt PC. (1998). *Cancer Res.,* **58,** 599–603.

Ford JM and Hanawalt PC. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 8876–8880.

Gadbois DM and Lehnert BE. (1997). *Cancer Res.,* **57,** 3174–3179.

Girinsky T, Koumenis C, Graeber TG, Peehl DM and Giaccia AJ. (1995). *Cancer Res.,* **55,** 3726–3731.

Graeber TG, Peterson JF, Mitchell T, Monica Jr K, Fornace AJ and Giaccia AJ. (1994). *Mol. Cell. Biol.,* **14,** 6264–6277.

Haines DS, Landers JE, Engle LJ and George DL. (1994). *Mol. Cell. Biol.,* **14,** 1171–1178.

Hartmann A, Blaszyk H, McGovern RM, Schroeder JJ, Cunningham J, DeVries EMG, Kovach JS and Sommer SS. (1995). *Oncogene,* **10,** 681–688.

Hartwell LH and Kastan MB. (1994). *Science,* **266,** 1821–1828.

Hartwell LH and Weinert TA. (1989). *Science,* **246,** 629–634.

Hupp TR, Meek DW, Midgley CA and Lane DP. (1992). *Cell,* **71,** 875–886.

Kapoor M and Lozano G. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 2834–2837.

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW. (1991). *Cancer Res.,* **51,** 6304–6311.

Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Tyler J, Walsh WV, Plunkett BS, Vogelstein Jr B and Fornace AJ. (1992). *Cell,* **71,** 587–595.

Kaufmann WK, Levedakou EN, Grady HL, Paules RS and Stein GH. (1995). *Cancer Res.,* **55,** 7–11.

Kaufmann WK and Wilson SJ. (1994). *Mutat. Res.,* **314,** 67–76.

Kuerbitz SJ, Plunkett BS, Walsh WV and Kastan MB. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 7491–7495.

Leadon SA and Cooper PK. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 10499–10503.

Lehman TA, Modali R, Boukamp P, Stanek J, Bennett WP, Welsh JA, Metcalf RA, Stampfer MR, Fusenig N, Rogan EM and Harris CC. (1993). *Carcinogenesis,* **14,** 833–839.

Linke SP, Clarkin KC, Leonardo AD, Tsou A and Wahl GM. (1996). *Genes Dev.,* **10,** 934–947.

Ljungman M and Zhang F. (1996). *Oncogene,* **13,** 823–831.

Lu H, Taya Y, Ikeda M and Levine AJ. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 6399–6402.

Maki CG and Howley PM. (1997). *Mol. Cell. Biol.,* **17,** 355–363.

Maltzman W and Czyzyk L. (1984). *Mol. Cell. Biol.,* **4,** 1689–1694.

Matlashewski GJ, Tuck S, Pim D, Lamb P, Schneider J and Crawford LV. (1987). *Mol. Cell. Biol.,* **7,** 961–963.

McKinnon PJ. (1987). *Hum. Genet.,* **75,** 197–208.

Meyn MS. (1995). *Cancer Res.,* **55,** 5991–6001.

Milne DM, Palmer RH and Meek DW. (1992). *Nuc. Acid. Res.,* **20,** 5565–5570.

Namba H, Hara T, Tukazaki T, Migita M, Ishikawa N, Ito K, Nagataki S and Yamashita S. (1995). *Cancer Res.,* **55,** 2075–2080.

Nelson WG and Kastan MB. (1994). *Mol. Cell. Biol.,* **14,** 1815–1823.

Nigro JM, Aldape KD, Hess SM and Tlsty TD. (1997). *Cancer Res.,* **57,** 3635–3639.

Reich NC, Oren M and Levine AJ. (1983). *Mol. Cell. Biol.,* **3,** 2143–2150.

Rogel A, Popliker M, Webb CG and Oren M. (1985). *Mol. Cell. Biol.,* **5,** 2851–2855.

Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E and Kastan MB. (1997). *Genes Dev.,* **11,** 3471–3481.

Stampfer MR. (1985). *J. Tissue Cult. Methods,* **9,** 107–116.

Stampfer MR and Bartley JC. (1985). *Proc. Natl. Acad. Sci. USA,* **82,** 2394–2398.

Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR and Harris CC. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 2230–2234.

Wang XW, Yeh H, Schaeffer L, Roy R, Moncollin V, Egly J-M, Wang Z, Friedberg EC, Evans MK, Taffe BG, Bohr VA, Weeda G, Hoeijmakers JHJ, Forrester K and Harris CC. (1995). *Nature Genet.,* **10,** 188–195.

Wazer DE, Liu X-L, Chu Q, Gao Q and Band V. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 3687–3691.

White AE, Livanos EM and Tlsty TD. (1994). *Genes Dev.,* **8,** 666–677.