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13. ABSTRACT (Maximum 200 Words) Penicillamine (PEN) causes apoptosis in tumor-derived cells but not in normal cells, suggesting that it may have potential as a therapeutic agent. This proposal focused on whether p53 and/or BRCA1 phosphorylation are required for PEN-induced apoptosis. Replacement of serine with alanine at BRCA1 1423 protected HCC1937 cells from apoptosis as did wild-type BRCA1. Replacement of serines 1387 and 1524 with alanines sensitized cells to PEN. Truncated BRCA1 retains these three serines, but lacks the second BRCT domain which binds p53 and regulates its transcriptional activity. Therefore both phosphorylation of serines 1387 and 1524, and the second BRCT domain are required for protection of HCC1937 cells from PEN-mediated apoptosis. To determine whether p53 expression had an effect on PEN-induced apoptosis in Saos-2 cells (p53-null), they were transfected with an inducible wild-type p53 construct, then treated with PEN. The sensitivity of these cells was not affected by p53 expression, preventing evaluation of a role for phosphorylation. However, in HCC1937 cells expressing truncated BRCA1, PEN-induced apoptosis was higher when p53 was present. Mutation of p53 serines 15 and 46 had no effect, indicating that phosphorylation of p53 is not required for PEN-induced apoptosis in HCC1937 cells.				
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INTRODUCTION

Mutations in BRCA1 (breast cancer gene 1) are linked to inherited, early-onset breast cancers. Because carriers of BRCA1 mutations are sensitive to acquiring additional mutations resulting from radio- or chemotherapies, the presence of defective BRCA1 could impair reparation of DNA damage in normal cells. Therefore it is important to fully understand the role BRCA1 plays in DNA repair, cell cycle arrest, and apoptosis. Its role in these processes has not been clearly defined and with regard to apoptosis its expression has been purported to induce it (1) or to prevent it (2). It has recently been discovered that penicillamine (PEN) causes apoptosis in tumor-derived cells but not in normal cells (3). Because of PEN's selectivity, it has potential as a therapeutic agent. The mechanism utilized in PEN-induced apoptosis is unknown, but appears to involve p53 and ATM. Both BRCA1 and p53 are phosphorylated by ATM; phosphorylation of BRCA1 is required for survival following DNA damage (4) and phosphorylation of p53 serine 15 is required for its transcriptional activation (5). The presence of BRCA1, ATM and p53 in a large multi-protein complex involved in DNA repair (BASC), suggests that perturbations, possibly phosphorylation of either p53 or BRCA1, could switch the cell's focus between DNA repair, cell cycle arrest, or apoptosis. Therefore, the objectives of this proposal are to determine whether ATM-mediated p53 and/or BRCA1 phosphorylation is required for PEN-induced apoptosis and if so, whether any of these phosphorylation events disrupt BASC.

BODY

Task 1. Determine whether ATM-mediated BRCA1 phosphorylation is required for PEN-induced apoptosis and if so, which of the four ATM-phosphorylatable sites are involved.

a. Determine which BRCA sites are phosphorylated in response to PEN treatment.

There were problems with this subtask. Preliminary experiments were carried out with Saos-2 and A549 cells. As a positive control these cells were exposed to ionizing radiation, which has been shown to induce phosphorylation of BRCA1 serines 1387, 1423, 1457 and 1524 (4, 6). As early as one hour following exposure to 2 Gy, BRCA1 mobility was slowed in both cell types, which is indicative of phosphorylation and consistent with published results in the literature (Figures 1A and 1B). In Saos-2 cells there was one band running at a slightly lower mobility following exposure, while for A549 cells there was a doublet, with some unphosphorylated or less phosphorylated species always present. However, when extracts from A549 cells were probed with site-specific antibodies (phospho-BRCA1 1387, 1423, 1457 or 1497), there was either no binding of the site-specific antibody following exposure to radiation or there was non-specific binding to other proteins in the cell extracts. Blots were probed using BSA or non-fat milk as blocking agents and either incubated 1 hour or overnight. I eventually concluded that these antibodies were not going to work and proceeded to the site-directed mutagenesis.

b. Generate constructs for expression of wild-type and mutant BRCA1. BRCA1 cDNA will be cloned into pcDNA3.1D/V5-His-TOPO, then serines replaced by alanines by site-directed mutagenesis at positions 1387, 1423, 1457, and 1524. I was unable to generate BRCA1 cDNA from RNA isolated from Saos-2 cells using the following primers: forward (5' ATGGATTTATCTGCTCTTCGCGTTGAAGAAG-3') and reverse (5'-CTAGGGGGTGTCCGGTGATGACT-3'). I then obtained a pcDNA3 vector containing BRCA1 cDNA from the Livingston lab (7). This vector was used to transform TOP10 *E. coli* and minipreps were carried out to isolate DNA for restriction digests and partial sequencing to confirm that the vector contained BRCA1 sequence. PvuI digestion linearized the vector which was 11.1 kb-5.4 kb for the pcDNA3 vector and 5.7 kb for the BRCA1 insert. For site-directed mutagenesis, serines 1387, 1423 and 1524 were changed to alanines using the following primers: 5'-CTGCTCAGGGCTATCCGCTCAGAGTGAC-3' and 5'-GTCACTCTGAGCGGATAGCCCTGAGCAG-3'; 5'-GTGTTAGAACAGCATGGGGCCCAGCCTTCTAACAGC-3' and 5'-GCTGTTAGAAGGCTGGGCCCCATGCTGTTCTAACAC-3'; 5'-GAATAGAACTACCCAGCTCAAGAGGAGCTCATTAAAG-3' and 5'-CCTTAATGAGCTCCTCTTGAGCTGGGTAGTTTCTATTC-3', respectively. The mutated BRCA1 constructs were then used to transform TOP10 *E. coli* and following isolation of DNA, sequenced to confirm the mutations. The fourth site, 1457, was not a good site for mutagenesis, so there was no mutant construct made for this site.

c. Make stable transfectants of HCC1937, a cell line expressing a non-functional BRCA1, using the BRCA1 constructs listed above. HCC1937 cells (8) synthesize a truncated BRCA1 protein encoded by a mutant allele (5382insC) causing a frame shift in codon 1756. This cell line has lost the wild-type allele and no wild-type protein is expressed (9). When these cells are transfected with wild-type BRCA1 they exhibit decreased sensitivity to ionizing radiation and a reduced number of double-strand DNA breaks (10). However, transfection of HCC1937 cells with a mutated BRCA1 (serines 1423 and 1524 replaced with alanines) fail to rescue the sensitivity of HCC1937 cells to ionizing radiation (4). With this in mind, HCC1937 cells were transfected with wild-type BRCA1, BRCA1 (S1387A), BRCA1 (S1423A), or BRCA1 (S1524A). Each of the constructs was linearized using Pvu I, cleaned up with a Qiagen kit, and used to transfect cells at a 6:1 ratio of Fugene 6 (Invitrogen):DNA. Transfectants were selected 48 hours later using G418 at a concentration of 200 µg/ml. Clones were picked using cloning rings 14-21 days after transfection and transferred to 12 well plates. Following expansion of cells into T25 flasks, cells were harvested, assayed for protein and run on 7.5% gels to check for BRCA1 expression. Each transfection generated between 12-20 stable transfectants but only 1-3 clones from each transfection expressed BRCA1, see Figure 2. To verify that the clone expressing wild-type BRCA1 was protected from ionizing radiation, cells were exposed to 0-8 Gy then allowed to recover for 14 days and stained with crystal violet. Survival of cells expressing wild-type BRCA1 was significantly higher than for the HCC1937 parent cell line (data not shown) confirming that cells expressing wild-type BRCA1 behaved as reported by others (10).

d. Analyze transfectants expressing wild-type and mutant BRCA1 to determine which mutations affect PEN-induced apoptosis. The contribution of BRCA1 to DNA-damage induced apoptosis is unresolved with some reports indicating that overexpression of BRCA1 induces apoptosis (1), while others suggest the opposite effect (2). BRCA1 expression has also been reported to enhance p53's transcriptional activity (11), and this is effected by p53 binding to the second BRCT domain (12). A recent study of mouse fibroblasts indicates that BRCA1 expression enhances p53-mediated growth suppression but not apoptosis (2). To determine the sensitivity of HCC1937 cells expressing truncated BRCA1, these cells were treated with 15 mM PEN for 24 and 48 hr. A549 cells which are sensitive to PEN were used as a positive control. The disappearance of intact PARP was followed as an indicator of apoptosis. Comparison of HCC1937 cells with A549 cells revealed a small decrease in PARP at 24 hr following PEN treatment of HCC1937 cells compared to A549 cells which contained no intact PARP at this time point. If PEN treatment was allowed to progress for 48 hr, there was less intact PARP in HCC1937 cells than at 24 hr, though the 85 kd cleavage product was still absent, Figure 3.

Prior to carrying out experiments with HCC1937 cells expressing exogenous mutant BRCA1, HCC1937 cells were compared to HCC1937 cells expressing wild-type BRCA1 (HCC1937/BRCA1wt). HCC1937, cells which express a truncated BRCA1 (1-1756) lack one BRCT domain, but retain phosphorylatable serines 1387, 1423 and 1524. The missing BRCT domain would be expected to have a negative effect on transcriptional activation of p53 since one of the p53 binding sites resides in this region. Comparison of HCC1937 versus HCC1937/BRCA1wt cells in response to PEN treatment for 24 hr indicates that both cell types are resistant to apoptosis. However, in cells transfected with wild-type p53 prior to PEN treatment, there is a decrease in the amount of PARP in cells expressing truncated BRCA1, Figure 4A. This is consistent with the study showing that BRCA1 enhances p53 growth arrest, since expression of wild-type BRCA1 protected cells from PARP cleavage. Moreover, when HCC1937 cells expressing truncated BRCA1 are transfected with either wild-type or mutant p53 (m=S15A, S46A), and then treated with PEN, these cells become more sensitive to PEN as shown in Figure 4B. In contrast, HCC1937 cells expressing wild-type BRCA1 are insensitive to PEN regardless of whether they are transfected with p53 prior to treatment, Figure 4C. Since the replacement of serines with alanines at residues 15 and 46 had no effect in either HCC1937 or HCC1937/BRCA1wt cells, phosphorylation was not required either to protect or to enhance apoptosis induced by PEN treatment in these cells.

In contrast, phosphorylation of BRCA1 did have an effect. HCC1937/BRCA1¹³⁸⁷, HCC1937/BRCA1¹⁵²⁴, and two different HCC1937/BRCA1¹⁴²³ mutants exhibited different sensitivities. HCC1937 cells which expressed alanine instead of serine at 1423 were resistant to PEN, behaving like the wild-type cells, whereas the other two mutants, 1387 and 1524 were somewhat sensitive, with a decrease in the amount of intact PARP as was the case for HCC1937 cells expressing truncated BRCA1, Figures 5A and 5B.

Task 2. Determine whether ATM-mediated p53 phosphorylation is required for PEN-induced apoptosis, and which of the two ATM-phosphorylatable sites are involved.

a. Generate stable transfectants of a p53-null cell line (Saos-2) using constructs encoding wild-type and mutant p53 (site-directed mutagenesis at positions 15 and 46). Stable transfectants expressing either an empty vector, wild-type p53, mutant p53 (S15A), or the double mutant (S15A, S46A) were generated in Saos-2 cells, but p53 expression was extremely low compared to the amount of p53 induced in A549 cells treated with PEN. For this reason I decided to use an inducible system. Saos-2 cells were first transfected with pcDNA6/TR encoding the tetracycline repressor, TetR (Invitrogen). Prior to transfection, the vector was linearized with Sap I. Stable transfectants expressing TetR were selected using 3 µg/ml blasticidin. The 1.85 kb insert encoding p53 was excised from pRc/CMV/p53 using Xba I (13) and cloned into pcDNA4/TO, which contains two copies of the tetracycline operator for high-level regulated expression (Invitrogen). Cells from five of the twelve clones selected with blasticidin were then transfected with pcDNA4/TO/wtp53. To test inducibility of p53, 4×10^5 cells were seeded into wells of a 6-well plate and 24 hr later were transfected with pcDNA4/TO/p53. At 48 hours, 1 µg/ml tetracycline was added and at 72 hours cells were harvested, lysed and run on gels. Following transfer to nitrocellulose, blots were probed with an antibody to p53. All of the clones expressed high levels of p53 following addition of tetracycline, but of the clones tested, B3 and C3 expressed the least in the absence of tetracycline, so these were chosen for further study. To determine whether p53 expression affected PEN-induced apoptosis, cells (B3 clone) were set up in a similar way; one set of three were treated with PEN, while the second set were untreated (no PEN), Figures 6A and 6B. There was a small amount of p53 expression even in the absence of tetracycline, but in its presence (1 µg/ml), there was a 5-10-fold increase in p53, Figure 6A. Following PEN treatment 24 hr later, the amount of PARP was lower in all three treated wells, and there was also a moderate amount of caspase 3 cleavage, Figure 6B, indicating that p53 expression did not enhance apoptosis in PEN-treated Saos-2 cells.

b. Determine whether phosphorylation at each site is required for PEN-induced apoptosis. Since p53 expression did not make a difference in PEN-induced apoptosis in Saos-2 cells, this sub task was not pursued further.

Task 3. Determine whether any of these phosphorylation events disrupt BASC, a complex of BRCA1-associated proteins involved in DNA repair.

a. Stable transfectants expressing mutant and wild-type BRCA1 and p53 proteins will be treated with 15 mM PEN. This subtask was described in 1d.

b. The presence of proteins in BASC will be monitored at different times following treatment (BRCA1, ATM, p53, Rad51, MSH2, MSH6, MLH1, BLM, RAD50, MRE11, and NBS1). Antibodies to all of these proteins are commercially available. I had problems with immunoprecipitating BRCA1, so was unable to carry out this subtask. Several different antibodies were used in an attempt to immunoprecipitate BRCA1. Previous to this grant award, an antibody from Santa Cruz (C-20) worked, but the new antibody from this company did not. Two antibodies from Oncogene (Ab-1 and Ab-3) were also tried; Ab-3 worked to some extent but the amount of BRCA1 immunoprecipitated was small. It did not work at all when conjugated to Protein A Sepharose, which is an important step for detection of p53, which migrates close to the immunoglobulin heavy chain on SDS-gels. The antibody from Pharmingen (556443), which was used for Western blots had not been tested for immunoprecipitation by the company and I did not have the time or funds to explore this. It would have been preferable to have used a tagged BRCA1 construct, such as HA-BRCA1, so that antibodies to the tag could be used and if further studies are carried out this will be done instead.

KEY RESEARCH ACCOMPLISHMENTS

1. HCC1937 cells expressing truncated BRCA1 are somewhat sensitive to PEN
2. HCC1937 cells expressing wild-type BRCA1 are resistant to PEN
3. HCC1937 cells expressing truncated BRCA1 and wild-type or mutant p53(S15A, S46A) are sensitive to PEN
4. HCC1937 cells expressing wild-type BRCA1 and wild-type or mutant p53(S15A, S46A) are resistant to PEN
5. Expression of p53 does not sensitize Saos-2 cells to PEN-induced apoptosis

REPORTABLE OUTCOMES

1. Stable transfectants

Saos-2 cells expressing the TetR protein	(12)
HCC1937 cells expressing wild-type BRCA1-HCC1937/BRCA1wt	(1)
HCC1937 cells expressing BRCA1(S1387A)-HCC1937/BRCA1 ¹³⁸⁷	(2)
HCC1937 cells expressing BRCA1(S1423A)-HCC1937/BRCA1 ¹⁴²³	(2)
HCC1937 cells expressing BRCA1(S1524A)-HCC1937/BRCA1 ¹⁵²⁴	(1)

2. Manuscripts

Manuscripts: Some of the data may be added to a manuscript that is in progress, but this is not a certainty.

CONCLUSIONS

Replacement of serine with alanine at residue 1423 resulted in protection from PEN-induced apoptosis, as was the case for wild-type BRCA1. In contrast, replacement of serines 1387 and 1524 with alanines increased sensitivity to PEN. Truncated BRCA1 retains serines 1387, 1423, and 1524, but lacks the carboxyl terminus containing the second BRCT domain which binds p53 and regulates its transcriptional activity. Therefore both phosphorylation of serines 1387 and 1524, and the presence of the second BRCT domain are required for the protection of HCC1937 cells from PEN-mediated apoptosis. This means that if PEN were used therapeutically, it would only be effective in patients with truncated BRCA1 (lacking the second BRCT domain) or in those expressing BRCA1 containing mutations at serines 1387 and 1524.

p53 is phosphorylated by ATM at serines 15 and 46 in response to ionizing radiation (14); phosphorylation at these sites has been shown to enhance DNA-damage induced apoptosis (5, 15). In the study described here, Saos-2 cells were treated with PEN to induce apoptosis. However, the sensitivity of these cells was not enhanced by p53 expression. Therefore whether the phosphorylation of serines 15 and 46 play a role in PEN-induced apoptosis could not be evaluated in these cells. In future studies, a different cell type for studying the role of these two serines should be used to explore the importance of ATM-mediated p53 phosphorylation in PEN-induced apoptosis. Saos-2 cells may behave differently than other cell types. Treatment was stopped at 24 hr and it is possible that if these cells were treated longer there might be an increase in apoptosis in cells expressing p53. However, since overexpression of p53 in the absence of other treatments can cause apoptosis by 72 hr (16), longer time points were avoided. One possibility for the anomalous behavior of Saos-2 cells is that unlike most tumor-derived cell lines, they do not express telomerase, so if PEN-induced apoptosis works by interfering with telomere function this could account for their resistance to PEN. In contrast to Saos-2 cells, HCC1937 cells (which express a truncated BRCA1 protein and no p53), were sensitized when wild-type p53 was induced. Replacement of serines 15 and 46 with alanines did not diminish apoptosis, indicating that phosphorylation of p53 is not required for PEN-induced apoptosis in HCC1937 cells. The enhanced sensitivity of HCC1937 cells in the presence of p53 indicates that the use of PEN as a therapeutic agent would be most effective in p53-expressing cells that also express a truncated BRCA1 missing the second BRCT domain.

A considerable amount of time was wasted in trying to find the best antibody for probing Western blots to confirm BRCA1 expression in clones and for immunoprecipitation of BRCA1. A tagged BRCA1 construct would have simplified tracking its expression. It would also have aided in immunoprecipitation which is necessary to follow its association with other proteins. I had originally planned to follow the effect of site-specific phosphorylation of BRCA1 on its association with other proteins, but was unable to do so because of these technical problems.

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Figure 1A. Saos-2 cells were irradiated with 0-8 Gy using a ¹³⁷Cs irradiator (Mark I model, J.L. Shepherd & Associates). Cells were harvested 1 hour following irradiation, run on gels and probed with an antibody to BRCA1.

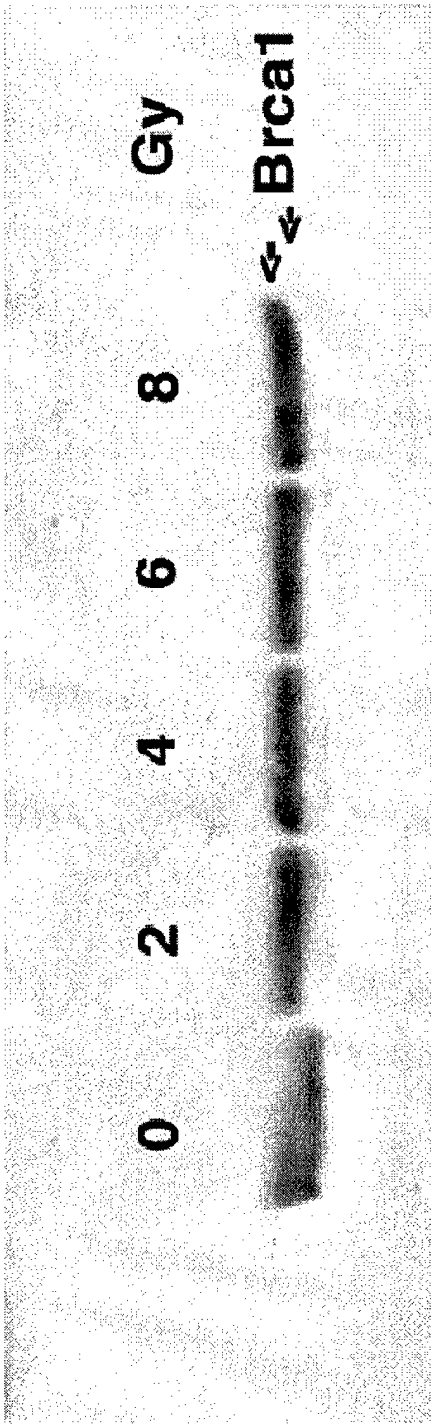


Figure 1B. A549 cells were irradiated with 0-8 Gy using a ¹³⁷Cs irradiator. Cells were harvested 1 hour following irradiation, run on gels and probed with an antibody to BRCA1.



Figure 2. BRCA1 expression in HCC1937 clones. Whole cells extracts (25 ug) were run on gels and probed with an antibody to BRCA1.

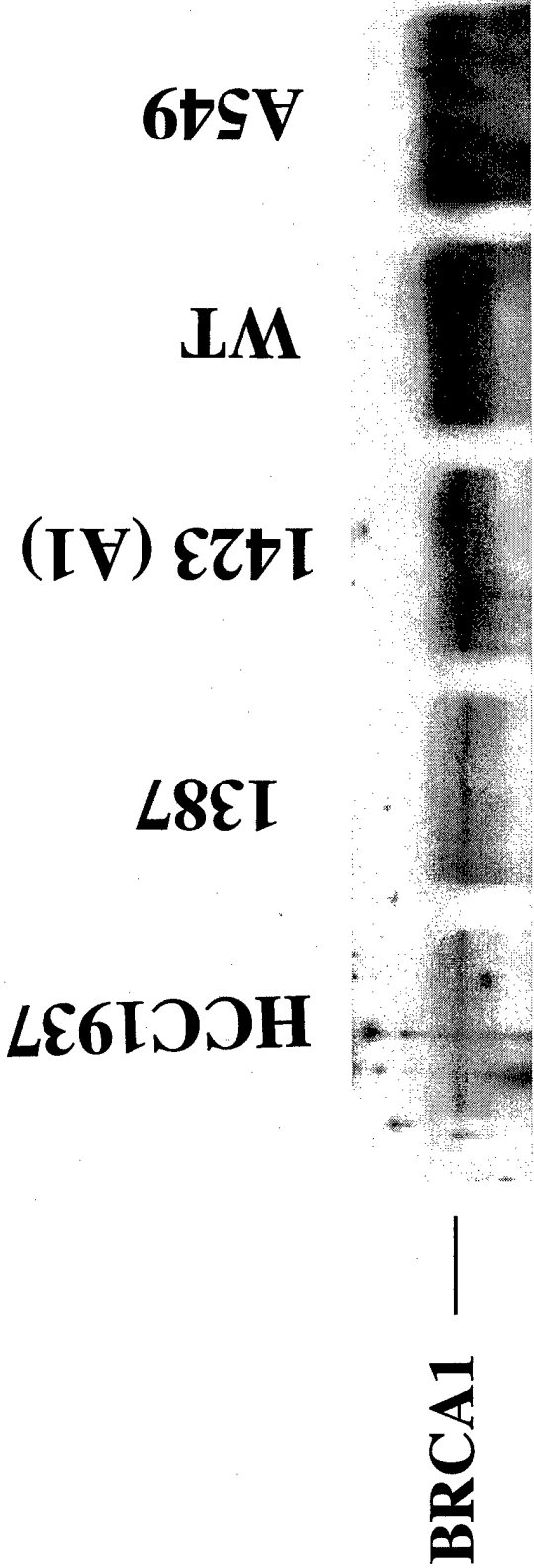


Figure 3. Treatment of HCC1937 and A549 cells with PEN. Whole cell extracts (25 ug) from each cell type were run on gels and probed with an antibody to PARP.

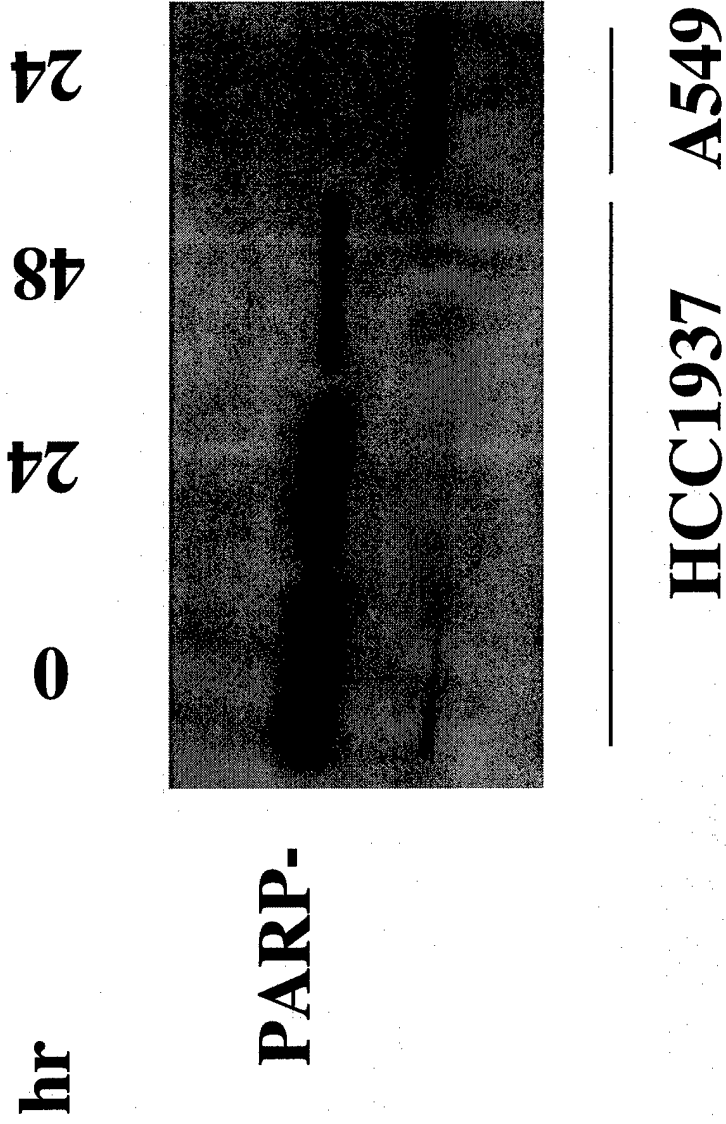
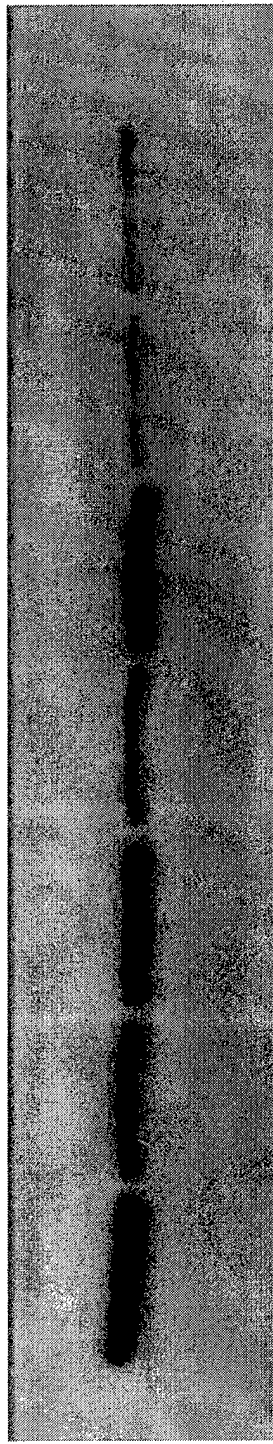
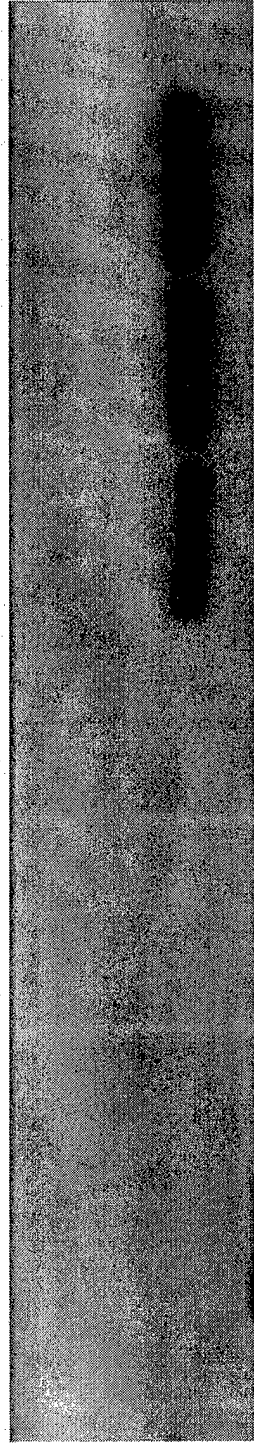


Figure 4B. PEN treatment of HCC1937 cells with wild-type or mutant p53. Cells were transfected with empty vector, wild-type p53, or p53 (S15A, S46A). At 24 hr, cells were treated with PEN, and at 48 hr whole cell extracts were prepared and run on gels, then probed with antibodies to PARP and p53.

	0	E	wt	m
PEN	-	+	-	+



PARP

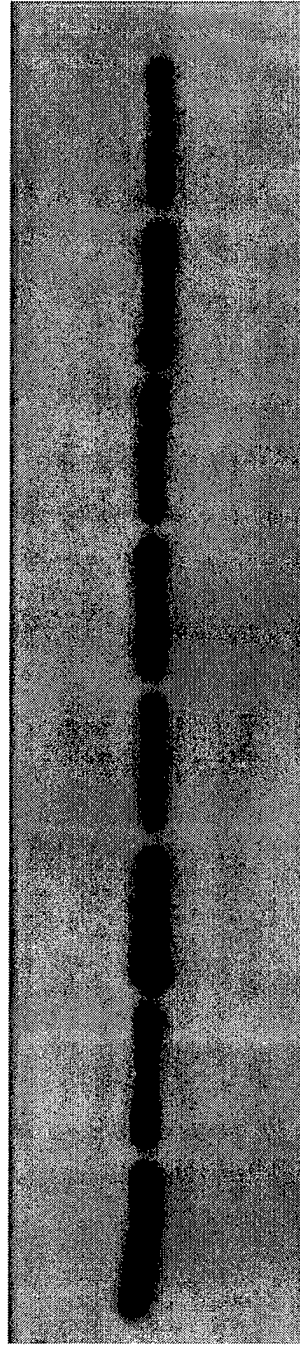


p53

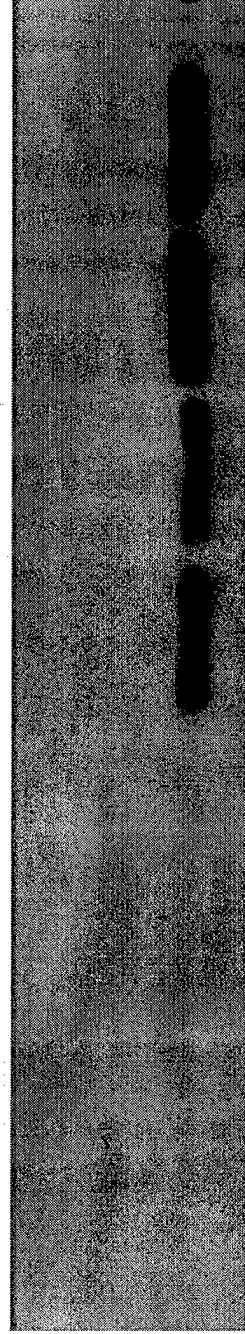
Figure 4C. PEN-treatment of HCC1937/BRCA1wt cells with wild-type or mutant p53. Cells were transfected with empty vector, wild-type p53 or p53(S15A, S46A). At 24 hr, cells were treated with PEN, and at 48 hr whole cell extracts were prepared and run on gels, then probed with antibodies to PARP and p53.

0 **E** **WT** **M**

PEN - + - + - + - +



PARP —

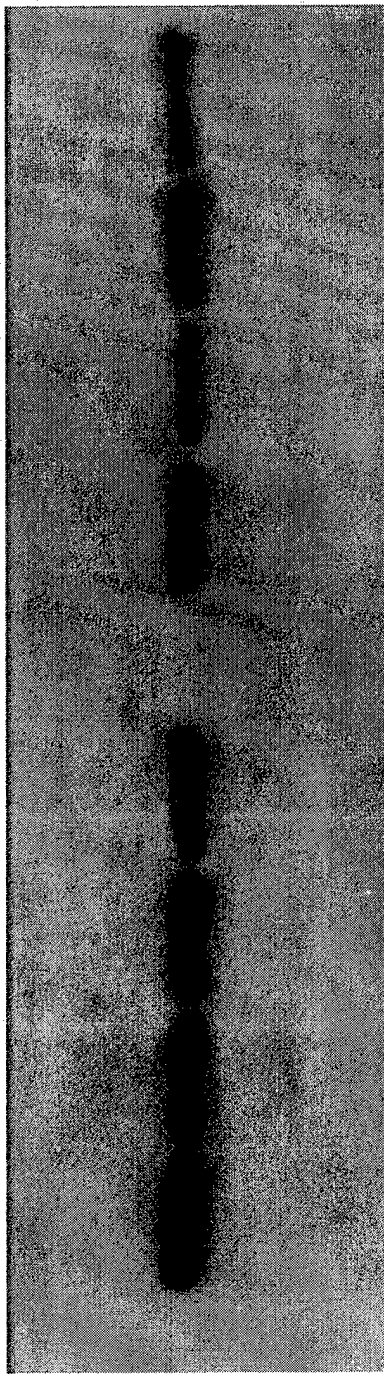


p53 —

Figure 5B. PEN-induced PARP cleavage in HCC1937 clones. Clones were transfected with an empty vector or wild-type p53 as indicated. Following PEN treatment 24 hr later, whole cell extracts were run on gels and probed with antibodies to PARP and p53.

1423 (14B9) 1524

PEN - + - + - + - +



PARP

p53 E wt E wt

Figure 6A. PEN treatment of Saos-2 cells expressing a tetracycline-inducible wild-type p53. p53 was induced with tetracycline as indicated and 24 hr later, cells were treated with PEN. Whole cell extracts were run on gels and probed with a p53 antibody.

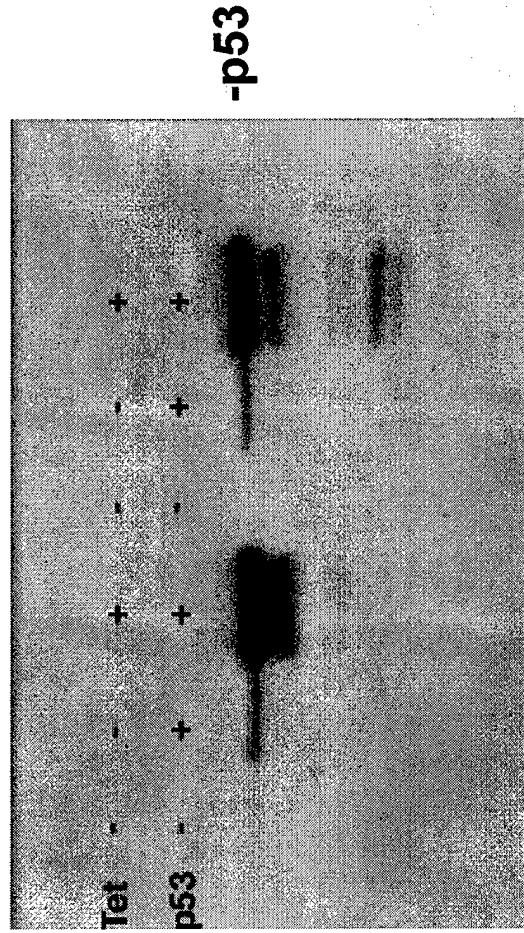


Figure 6B. PEN treatment of Saos-2 cells expressing a tetracycline-inducible wild-type p53. p53 was induced with tetracycline as indicated and 24 hr later, cells were treated with PEN. Whole cell extracts were run on gels and probed with antibodies to PARP and caspase 3.

