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13. ABSTRACT *(Maximum 200*

Our long term goal of this proposal is to study the molecular events leading to the progression of breast cancer with emphasis on the role of elk-1 gene. Previously we observed increase in the expression of elk-1 RNA in the presence of estrogen in breast cancer cells. Recently, we have studied and have found no significant effect of estrogen on the transcriptional activation and expression of elk-1 protein, suggesting that the estrogen regulation of elk-1 could be at the level of transcription. Previously, we found BRCA1 gene product to function as a regulator of elk-1 gene. We have developed human breast cancer cell lines expressing BRCA1. Our results demonstrate BRCA1 to function as an inducer of apoptosis. These results suggest that BRCA1 and elk-1 genes may play a critical role in the regulation of apoptosis. Thus a wide variety of human malignancies like breast cancers have a decreased ability to undergo apoptosis. This could be due to lack/decreased levels of functional BRCA1 proteins. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

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Veena N Rao 12/10/96

PI - Signature Date

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INTRODUCTION

Majority of the ets-oncogene superfamily members, which we and others have previously isolated, cloned and characterized are involved in leukemias, lymphomas and solid tumors (1-8). One of the members elk-1 (4) was shown to form a SRF (serum response factor) dependent ternary complex with the SRE (serum response element) similar to P62TCF (ternary complex factor) (9). Elk-1 was previously shown to be involved in the growth factor mediated signal transduction pathway involving activation of MAP kinase (mitogen activated protein kinase), leading to the transcriptional activation of c-fos proto oncogene both of which are activated in tumor cells. Since the fos regulatory function of elk-1 is dependent on the presence of a growth factor regulated transcriptional activation domain whose activity is dependent on phosphorylation by MAP kinase (10-13) and JNK kinases (Jun amino terminal kinase) (14) in vivo both of which are activated in tumor cells, we speculated that elk-1 proteins may be obligatory intermediates in the estrogen and growth factor mediated signal transduction pathway leading to the progression of breast cancer.

We, therefore, tested this hypothesis (which forms the basis of this proposal) by studying the levels of expression of elk-1 protein in breast tumor samples as well as in several breast cancer derived tumor cell lines and compared expression with histologically normal breast samples by western blot analysis using the elk-1 polyclonal or peptide

antibody available with us and correlate expression with stages of the disease. If we find high levels of expression in tumor samples, it would indicate involvement of elk-1 in the progression of breast cancer. We observed elevated levels of expression of elk-1 protein ~ 3-8 fold higher in samples derived from invasive breast cancer than in normal breast. These results which were reported previously have strengthened the objectives of this proposal. These experiments pertain to task 1 of the grant proposal, which have been completed.

Breast cancer has been described in patients over time to change from an estrogen dependent in initial stages of the disease to a hormonally independent tumor. We have speculated that elk-1 proteins may be obligatory intermediates in the estrogen (E2) and growth factor mediated signal transduction pathway. To test this hypothesis we studied the expression of elk-1 in MCF-7 cells that have been made quiescent by depletion of steroids and growth factors and stimulated to enter the cell cycle by the addition of E2 by RT-PCR analysis. We found a rapid induction of elk-1 and Δ elk-1 RNA following growth stimulation by E2 in MCF-7 cells. These results indicated the involvement of elk-1 in the E2 induced signal transduction pathway in human breast cancer cells. These experiments were proposed in Task 2 (a) of the grant proposal have been completed. Since the elk-1 mRNA levels doesn't necessarily reflect differences in the level of elk-1 protein, we plan to study the expression of elk-1 protein by immunoprecipitation analysis. These experiments also comprise Task 2 (a) of the grant proposal.

Since elk-1 proteins show both autonomous and SRF dependent transcriptional activation, we plan to check whether any of these functions are modulated in MCF-7 cells that have been stimulated with E2 and growth factors. These experiments form part of Task 2 (b) of the grant proposal.

We have previously shown the purified recombinant-elk-1 proteins to be substrates for c-src kinase using an in vitro kinase assay indicating that elk-1 could be a physiological target for c-src kinase. With this we have completed Task 2 (c) of the grant proposal.

In an attempt to identify proteins that might interact with elk-1 proteins, we have isolated several putative elk-1 interacting proteins (EIP). We are presently characterizing these cDNA clones. These experiments form part of Task 2 (g) of the grant proposal.

Previously, v-ras H oncogene has been shown to convert the MCF-7 cell line from an absolute hormone dependence for in vivo tumor formation to an estrogen-independent fully tumorigenic phenotype. These studies indicate that an exogenous activated oncogene can bypass the hormonal requirement for tumor formation and convert a previously estrogen-dependent cell line into an independent tumorigenic line. On this basis, we wanted to investigate whether high level expression of elk-1 or ∆elk-1 protein could lead to cellular transformation. Our results suggest that high level expression of elk-1 and ∆elk-1 proteins can transform mouse fibroblasts in vitro and induce tumors in

nude mice indicating that elk-1 and Δelk-1 proteins are oncogenic. These results correlate well with the high expression seen in breast tumor samples. These experiments pertain to Task 2 (h) of the grant proposal have been completed successfully.

Recently a familial breast and ovarian cancer susceptibility gene BRCA1 was identified (15) and shown to be either lost or mutated in families with breast and ovarian cancer (16-20). Recent results on the expression of BRCA1 mRNA in sporadic breast cancer indicated decrease in the level of BRCA1 mRNA levels during the transition from carcinoma in situ to invasive cancer (21). But our results (Rao, unpublished results) indicated increase in the expression of BRCA1 in tumor compared to normal samples, similar to elk-1 protein. At this juncture, we were tempted to look for any correlation between BRCA1 expression and elk-1 expression or vice versa. Previously, we performed the SRE TK CAT (Serum Response Element, Thymidine Kinase, Chloramphenicol acetyl transference reporter vector) functional assay for checking the levels of elk-1 protein in cells that have been transfected with antisense RNA to BRCA1 ($BRCA^{AS}$) that have been developed by us (Ref. 25 Reprint enclosed). We observed high levels of CAT activity which made us to speculate that BRCA1 could be a regulator of elk-1 gene. But we know that there are many elk-1 related proteins which would also respond to this reporter like SAP-1, SAP-2, etc. These experiments form part of Task 2 (I) and have been completed previously.

In order to confirm the results whether BRCA1 regulates elk-1, we plan to develop mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1. The expression of BRCA1 protein in the BRCA1 transfectants will be analyzed by immunofluorescence and immunohistochemistry. We plan to study the expression of elk-1 protein in these cells by western blot analysis and correlate expression with those in parental cells. If we find high levels of expression in $BRCA1^{S}$ cells than parental cells, it would indicate BRCA1 to be a regulator of elk-1 gene. Since our recent results (Rao unpublished results) indicate BRCA1 to be a nuclear phosphoprotein which functions as a suppressor of growth and tumor suppressor. We also plan to investigate the role of BRCA1 in the apoptosis of human breast cancer cell. Recent evidence suggests that hormone dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. It may be possible that inability to undergo apoptosis in these cancers may be due to the decrease levels of functional BRCA1 proteins and BRCA1 downstream signals like elk-1 proteins. These experiments will give us a clue as to the possible regulation of elk-1 function in breast cancer. Future experiments will be directed toward studying the role of elk-1 in apoptosis.

BODY

Task 1 **COMPLETED PREVIOUSLY**

Task 2 **IN PROGRESS**

Task 2 (a)

To test the hypothesis whether elk-1 proteins are intermediates in the E2 mediated proliferation of human breast cancer cells. We have previously studied the expression of elk-1 RNA in MCF-7 cells that have been made quiescent by depletion of steroids and growth factors and stimulated to enter the cell cycle by the addition of E2 for 60 min., we observed a rapid induction of elk-1 and ∆elk-1 RNA following growth stimulation by E2 in MCF-7 cells. These results indicated involvement of the elk-1 protein in the E2 induced signal transduction pathway in human breast cancer cells. **RNA EXPRESSION BY RT-PCR ANALYSIS COMPLETED**.

Since the elk-1 mRNA levels doesn't necessarily reflect differences in the level of elk-1 proteins, we studied the expression of elk-1 at the protein level by ^{35}S-methionine labeling of cells after exposure to E2 for 0, 30 min., 60 min. and 120 min. and subjecting the cell lysate to immunoprecipation using elk-1 polyclonal antibody available with us. We observed no significant difference in the levels of expression of elk-1 protein in uninduced compared to E2 stimulated samples. (Data not given.) These results suggests that E2 could regulate elk-1 expression at the transcriptional level. **WITH THESE RESULTS WE HAVE COMPLETED TASK 2 (a).**

Task 2 (b)

In an attempt to investigate the mechanism by which E2 and peptide growth factors stimulate breast cancer cell proliferation using MCF-7 as a model system, we have studied the autonomous and SRF dependent transcriptional activation of elk-1 and ΔElk-1 proteins in MCF-7 cells that have been stimulated with E2 and EGF as described in the grant proposal. We observed no significant difference in the autonomous and SRF dependent transcriptional activation of elk-1 and ΔElk-1 proteins in MCF-7 cells that have been growth arrested for 24 hours and stimulated with E2 or EGF for 24 hours. These results suggests that E2 or EGF have no effect on the autonomous and SRF dependent transcriptional activation of full length elk-1 and ΔElk-1 proteins. **WITH THESE RESULTS, TASK 2 (b) HAS BEEN COMPLETED.**

Task 2 (c)

Previously we have shown purified recombinant elk-1 protein to be an in vitro substrate for c-src kinase - **TASK 2 (c) HAS BEEN COMPLETED PREVIOUSLY.**

Task 2 (g)

We have isolated elk-1 interacting proteins (EIP) and we are presently characterizing these cDNA clones. **TASK 2 (g) IS IN PROGRESS**.

Task 2 (h)

We have previously shown that high level expression of elk-1 and ∆elk-1 proteins transform cells and induce tumors in nude mice. **TASK 2 (h) HAS BEEN COMPLETED PREVIOUSLY.**

Task 2 (i)

We have previously studied the expression of elk-1 in $BRCA1^{AS}$ transfectants using a SRECAT functional assay and observed enhanced CAT activity indicating the possibility that BRCA1 could regulate elk-1 function. **THIS PORTION OF TASK 2 (i) HAS BEEN COMPLETED.**

In an attempt to investigate further whether BRCA1 regulates the expression of elk-1 gene, since both BRCA1 and elk-1 protein levels have been found to be high in invasive breast tumors compared to normal samples, and to investigate the role of BRCA1 in apoptosis, we have developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1 (see Ref. 26, Reprint enclosed). The expression of BRCA1 protein the BRCA1 transfectants were analyzed by immunofluorescence and immunohistochemistry. The BRCA1 transfectants showed a flattened morphology compared to the parental cells (see Reprint 2 enclosed).

One of the most efficient ways of triggering the apoptotic response in fibroblasts is the removal of serum and in thymocytes by calcium ionophore A23187. We found that serum deprivation or calcium inophore treatment of BRCA1 transfectants resulted in programmed cell death (see Reprint 2 enclosed). We also studied the expression of elk-1 protein in these cells by western blot analysis using elk-1 peptide antibody available with us (data not given). Our preliminary results suggest high levels of elk-1 protein in $BRACA1^{S}$ transfected MCF-7 cells when compared to parental MCF-7 cells. These results suggest that BRCA1 gene may regulate the expression of elk-1 gene and BRCA1 genes play a critical role in the regulation of apoptosis. We plan to express high levels of elk-1 protein in MCF-7 cells and then study their effect on the growth, tumor suppression

and apoptosis of breast cancer cells. If elk-1 is found to induce apoptosis, we then plan to identify the death inducing domain of elk-1 protein and identify downstream targets of elk-1 protein.

CONCLUSION

Our studies are designed to investigate the role of elk-1 oncogene in the progression of breast cancer. In summary, our results suggest that BRCA1 gene product which is a nuclear phosphoprotein with tumor suppressor properties functions as an inducer of apoptosis similar to rho-c-myc,p53, E1A and Rel. Our preliminary results suggests BRCA1 to be a regulator of elk-1. Our results also suggest that E2 can regulate the function of elk-1 only at the RNA level and has no significant effect on the transcriptional activity of elk-1.

Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis. Our results suggest that lack or decreased levels of expression of functional BRCA1 gene product in breast and ovarian cancers may be responsible for the increased resistance of these cells to undergo apoptosis (26, Reprint enclosed). Treatments that are aimed at increasing the apoptic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

Alternatively, one can use therapeutic agents that can activate BRCA1 downstream signals involved in apoptosis like elk-1 for the treatment of breast cancers. Results from this work would be utilized in the early detection, diagnosis and also treatment of breast cancer. Primers of elk-1 cDNA can also be used for RT-PCR analysis of breast tumor samples for early diagnosis of this disease. We also plan to make mutant of elk-1 proteins, introduce them into breast tumor cells and look for reversion from the transformed phenotype.

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Induction of apoptosis by the tumor suppressor protein BRCA1

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The breast and ovarian cancer susceptibility gene BRCA1, is a nuclear phosphoprotein which functions as a tumor suppressor. To investigate the role of BRCA1 in apoptosis, we have developed mouse fibroblast cell lines and human breast cancer cell lines expressing BRCA1. The expression of BRCA1 protein in the BRCA1 transfectants were analysed by immunofluorescence and immunohistochemistry. The BRCA1 transfectants showed a flattened morphology compared to the parental cells. We show that serum deprivation or calcium ionophore treatment of BRCA1 transfectants resulted in programmed cell death. These results indicate that BRCA1 genes may play a critical role in the regulation of apoptosis. Thus, since a wide variety of human malignancies like breast and ovarian cancers have a decreased ability to undergo apoptosis, this could be due to lack/decreased levels of functional BRCA1 proteins. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies.

Keywords: BRCA1 proteins; tumor suppressor; breast and ovarian cancers; apoptosis; therapy

Introduction

Breast cancer is one of the most common malignancy affecting women in the United States and ovarian cancer although less frequent than breast cancer is the fourth leading cause of cancer mortality among women. The breast and ovarian cancer susceptibility gene BRCA1, was recently isolated and the conceptual cDNA encodes a 1863 aminoacid protein with an amino-terminal Zinc finger domain and a carboxy terminal acidic region (Miki *et al.*, 1994). We and others have identified multiple BRCA1 proteins approximately 185-220, 160, 145, 100, 52 and 38 kD in both human and mouse cells (Rao *et al.*, 1996; Chen *et al.*, 1995). It remains to be seen whether these proteins represent isoforms of BRCA1 or its related proteins. Recently, we like others have identified alternatively spliced transcripts of the BRCA1 gene (Rao, unpublished results). A recent report indicated that the BRCA1 gene product is localized in the nucleus of several normal cell lines including breast and tumor cells other than breast and ovary (Chen *et al.*, 1995). They have detected BRCA1 mainly in the cytoplasm of almost all breast and ovarian cancer cell lines examined. These results suggested aberrant subcellular localization of BRCA1 in breast cancer (Chen *et al.*, 1995). Interestingly, our results show BRCA1 to be localized mainly in the nucleus (or perinuclear) or cytoplasm or both of several normal or cancer cells (Rao, unpublished results) indicating variable subcellular localization of the BRCA1 proteins. Our results suggest that the subcellular localization of BRCA1 may be determined by the cell cycle status of the cells (Rao, unpublished results). Our results show that BRCA1 interacts with cyclin dependent kinases suggesting a role for BRCA1 in cell cycle regulation (Rao, unpublished results). We have previously reported that the BRCA1 gene product to be a nuclear protein with tumor suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 resulted in neoplastic transformation (Rao *et al.*, 1996). We have recently observed that introduction of variant BRCA1 gene into human cancer cells results in suppression of growth and neoplastic phenotype (Rao *et al.*, unpublished results) implicating a direct role for BRCA1 in growth and tumor suppression.

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Results and discussion

To study the function of BRCA1 genes in the regulation of apoptosis, we have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1. These BRCA1^s cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis (Figure 1a) and immunoperoxidase staining (Figure 1a) using BRCA1 polyclonal antibody as described previously (Rao *et al.*, 1996). The nuclear and cytoplasmic staining was brighter and stronger in BRCA1^s transfectants compared to parental NIH3T3 cells (Figure 1a). The morphology of the BRCA1^s transfectants were different from that of the parental NIH3T3 cells. The BRCA1^s cells are shorter and flatter when compared to the parental NIH3T3 cells (Figure 1b) and with several weeks in continuous culture the BRCA1^s cells become spindle shaped with elongated processes leading to their detachment. These results suggest that constitutive high level expression of the BRCA1 gene product for a prolonged period of time may result in apoptosis.

One of the most efficient ways of triggering the apoptotic response in fibroblasts is the removal of serum (Jimenez *et al.*, 1995). Thus we next investigated the effect of serum withdrawal on the induction of apoptosis in the BRCA1^s transfectants and compared it with that of the NIH3T3 cells. Subconfluent NIH3T3 and BRCA1^s cells were grown in medium containing

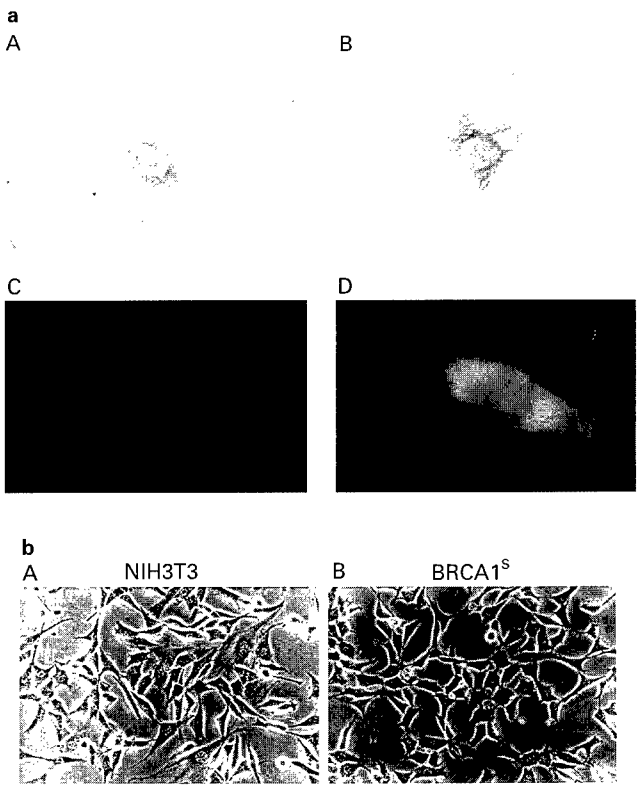


Figure 1 Detection of BRCA1 protein(s) in NIH3T3 and BRCA1^S cells by immunohistochemically and immunofluorescence analysis. (**a**) Immunoperoxidase (A, B) and immunofluorescence (C, D) analysis. A. NIH3T3; B, BRCA1^S; C, NIH3T3; D, BRCA1^S. (**b**), Morphology of the BRCA1^S transfectants. Phase-contrast photomicrographs of NIH3T3 (A) and BRCA1^S cell lines cultured in normal media (10% FBS/DMEM)

either 10% or 0% fetal bovine serum (FBS). After 24 h the cells were collected and subjected to flow cytometric analysis. The BRCA1^S transfected cells showed enhanced rates of apoptosis under serum depleted conditions, as measured by the appearance of an additional 'sub G1' peak on flow cytometry (Figure 2a). This peak is associated with high levels of DNA degradation. High levels of apoptotic cells in the sub G_0/G1 peak (A_p) population were measured in BRCA1^S cells (A_p value 75%) whereas the control cell line NIH3T3 showed lower levels of apoptosis under identical conditions (Figure 2a). BRCA1^S cells did not show any significant apoptosis levels when grown under normal conditions of DMEM supplemented with 10% FBS (Figure 2a).

The above results of apoptosis in BRCA1^S transfectants was further comfirmed by DNA fragmentation assay. Here NIH3T3 and BRCA1^S transfectant cells were cultured in serum free media for 24, 48 and 72 h and then analysed for DNA fragmentation in agarose gels. The BRCA1^S transfected cell line showed the production of a typical oligonucleosomal DNA ladder (Figure 2b; B, lanes 1–3) indicating activation of apoptosis. This effect was readily seen for BRCA1^S cell lines cultured in serum free media but not for the parental NIH3T3 cells grown under identical conditions (Figure 2b; A, lanes 1 to 3). These results indicate that over expression of BRCA1 accelerates apoptosis in serum depleted NIH3T3 cells.

Apoptosis can be induced by calcium ionophore, A23187, in thymocytes (McConkey *et al*., 1989). This led us to examine apoptosis in BRCA1^S cells during A23187 treatment. NIH3T3 and BRCA1^S cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by flow cytometry with propidium iodide staining method (Bendall *et al*., 1994). Histogram of the DNA content and the percentage of cells in G1, S and G2 plus M of the cell cycle were evaluated by computer analysis using EPICS profile analyzer. The BRCA1^S transfected cells showed accelerated rates of apoptosis (A_p value 82%) in presence of calcium ionophore (Figure 3a). Measurement of apoptosis through the sub G1 peak in the DNA histogram has the advantage of simplicity. But it has the disadvantage that since all the cells are fixed there is no distribution between viable and dead cells. Therefore the viability of both NIH3T3 cells and BRCA1^S cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that majority of the BRCA1^S cells treated with calcium ionophore A23187 were dead whereas most of the control NIH3T3 cells survived (Figure 3). These results suggest that BRCA1 induces death in NIH3T3 cells.

Apoptosis in the BRCA1^S transfectants was further confirmed in two ways. First, NIH3T3 and BRCA1^S cells were cultured in the presence of calcium ionophore to induce apoptosis and the incidence of cell death was determined by phase contrast microscopy after staining the cultures with Hochest 33258 (Oberhammer *et al*., 1994). Majority of the nuclei of BRCA1^S showed strong chromatin condensation and nuclear degradation into small, spherial nuclear particles of condensed chromatin characteristic of apoptosis (Figure 3c), whereas the parental NIH3T3 cells did not show any significant change in the staining pattern (Figure 3). Second, the analysis of DNA degradation upon treatment of BRCA1^S transfectants with calcium ionophore confirmed induction of apoptosis. Figure 3d shows that the DNA of BRCA1^S cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder whereas the parental NIH3T3 cells had no significant DNA degradation (Figure 3d). These results suggested that calcium ionophore induces apoptosis more readily in BRCA1^S cells than NIH3T3 cells. All the above results shown for one clone of NIH3T3–BRCA1^S cells, have been reproducibly obtained with several other independent clonal isolates of NIH3T3–BRCA1^S cell lines (data not shown).

In an attempt to understand the role of BRCA1 genes in the regulation of apoptosis of human breast cancer cells, we have transfected MCF7 cells with pcDNA expression vector or pcDNA expression vector containing human BRCA1 cDNA and obtained stable G418 resistant cell lines expressing BRCA1 (Rao *et al*., unpublished results). These BRCA1^S cell lines were analysed for BRCA1 protein expression by indirect immunofluorescence analysis, immunoperoxidase staining and Western blot analysis (Rao *et al*., unpublished results). Apoptosis in the MCF-7 BRCA1^S transfectants were analysed after treatment with calcium ionophore A23187. MCF-7 and BRCA1^S cells were treated with calcium ionophore A23187 for 24 h and the cell cycle distribution was determined by Flow cytometry with propidium iodide staining. The BRCA1^S transfected MCF-7 cells showed accelerated

rates of apoptosis (A_p value 75%) in the presence of calcium ionophore (Figure 4a). The viability of both MCF-7 cells and BRCA1^S cells cultured in the presence of calcium ionophore was tested by crystal violet staining. Cell viability staining showed that a vast majority of the MCF-7 BRCA1^S cells treated with calcium ionophore A23187 were dead whereas most of the control MCF-7 cells survived (Figure 4b). These results suggest that BRCA1 induces death in MCF-7 cells. The induction of apoptosis in the BRCA1^S transfectants was further confirmed by analysis of DNA fragmentation upon treatment of BRCA1^S transfectants with calcium ionophore. The DNA of BRCA1^S cells treated with calcium ionophore was broken into oligonucleosomal DNA ladder unlike the parental MCF-7 cells (Figure 4c). These results suggest that over expression of BRCA1 in breast cancer cells results in apoptosis.

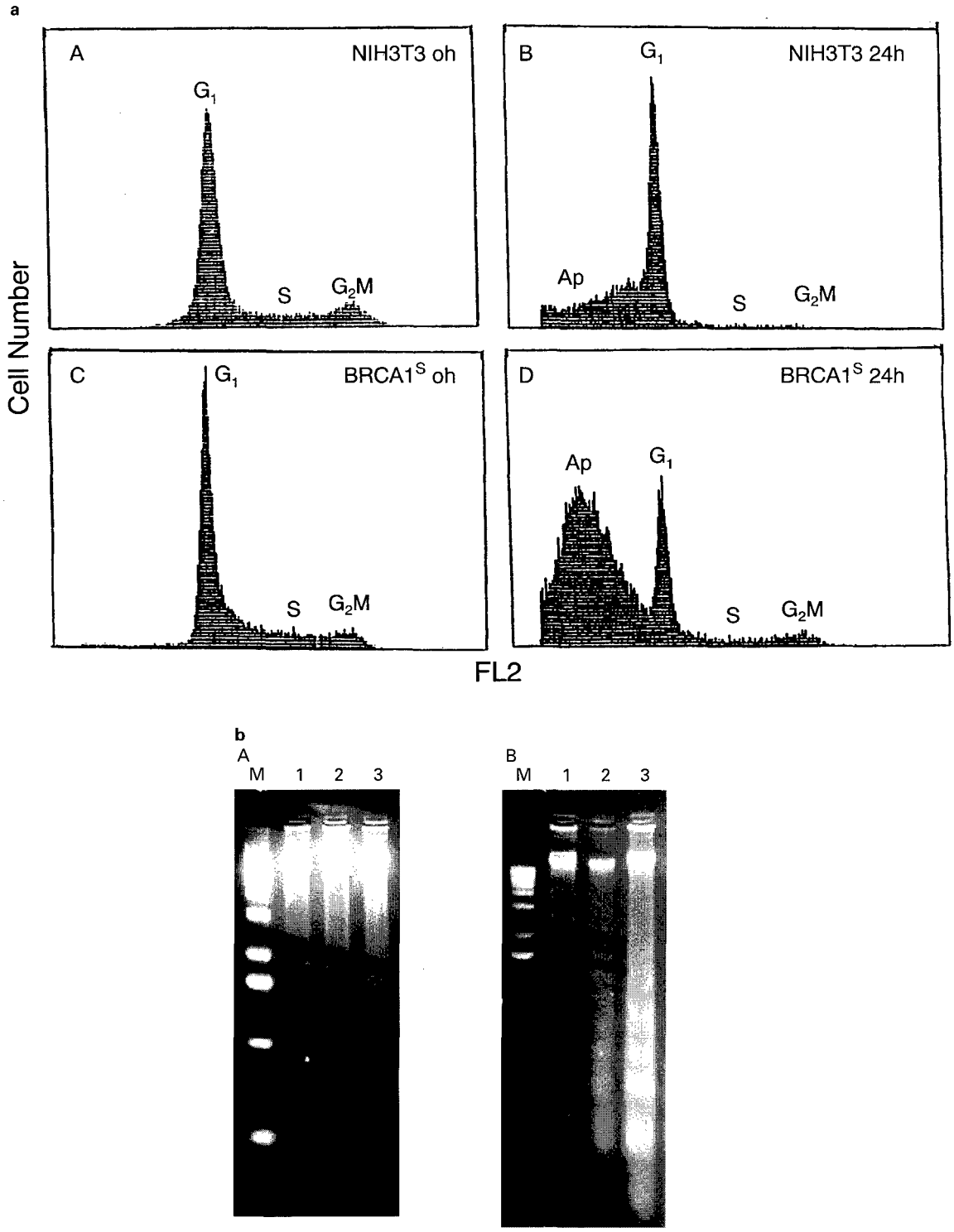
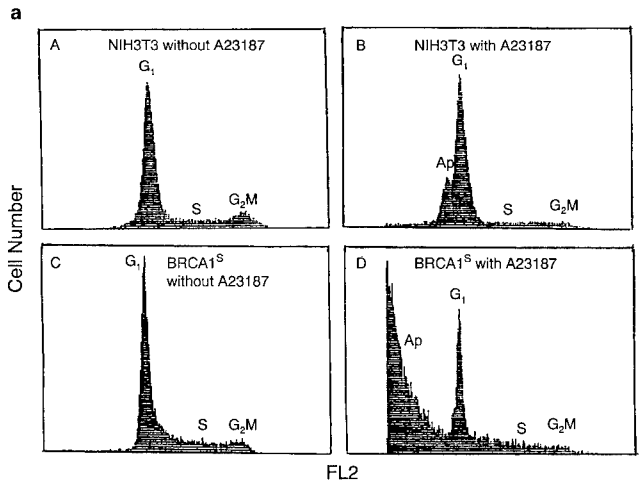
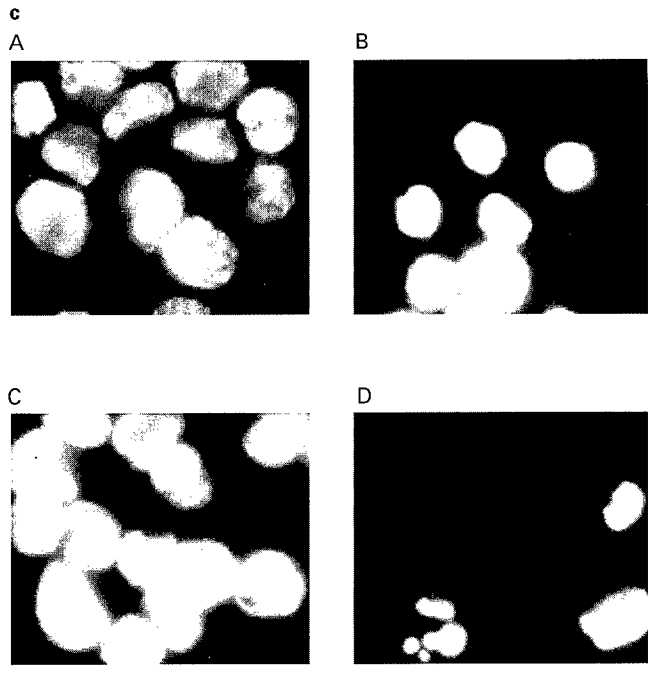


Figure 2 BRCA1 over expression induces apoptosis in serum depleted NIH3T3 cells. (**a**) Flow cytometric analysis of cells induced to undergo apoptosis by serum deprivation. A, NIH3T3, 0 h; B, NIH3T3, 24 h; C, BRCA1^S 0 h; D, BRCA1^S 24 h. (**b**) Over expression of BRCA1 gene induces DNA fragmentation typical of apoptosis in serum deprived cells. A, control NIH3T3 cells serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3) and B, BRCA1^S cell serum starved for 24 h (lane 1); 48 h (lane 2); 72 h (lane 3)

In summary, our results demonstrate that the BRCA1 gene product which is a nuclear phosphoprotein (Rao *et al.*, 1996; Chen *et al.*, 1995) with tumor suppressor properties (Rao *et al.*, 1996; Rao *et al.*, unpublished results) functions as an inducer of apoptosis similar to rho, c-myc, p53, E1A and rel (Jimenez *et al.*, 1995; Fisher, 1994; Hoffman and Libermann, 1994; Yonish-Rouach *et al.*, 1991). The BRCA1 cDNA used in this study lacks majority of exon 11, suggesting that this region of exon 11 is dispensable for the apoptotic function of BRCA1. The precise mechanism by which BRCA1 triggers cell death remains to be investigated. It may be possible that BRCA1 gene products function as transcriptional regulators that may either activate death inducing genes or repress death inhibiting genes leading to apoptosis. Alternatively, BRCA1 may activate apoptosis inducing





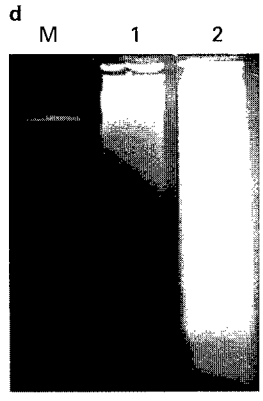
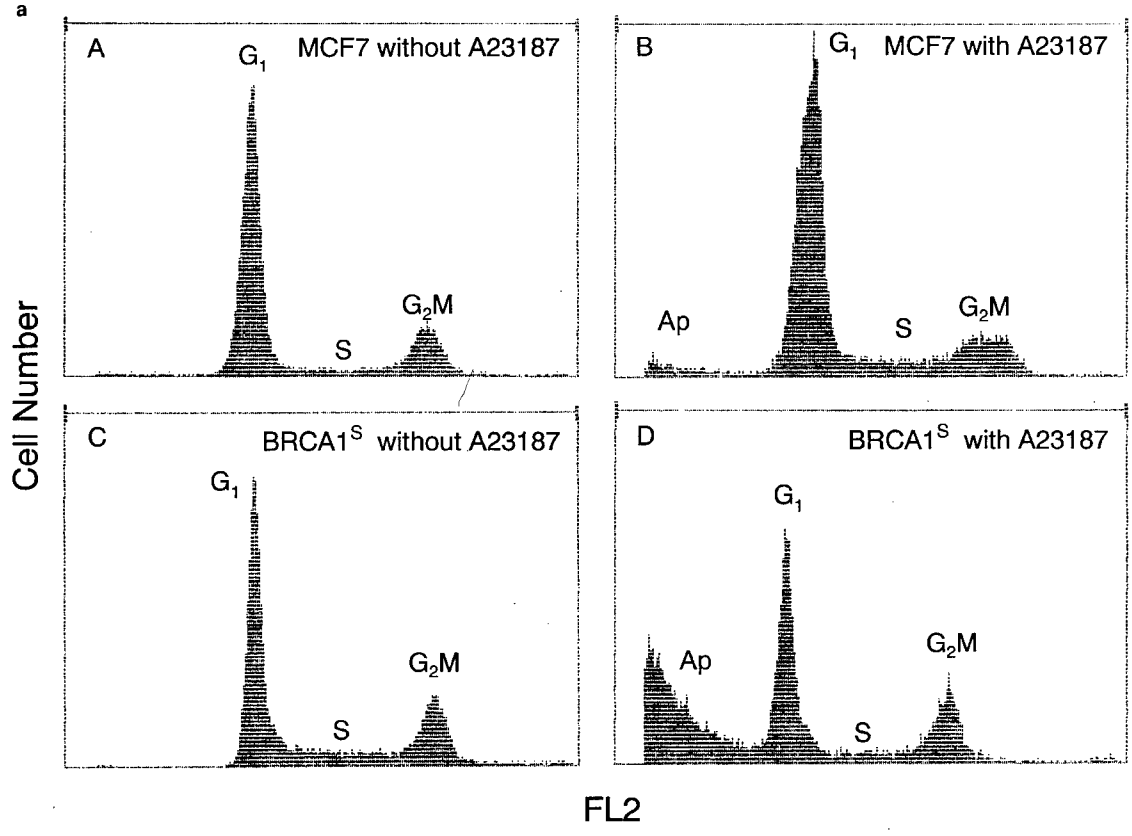
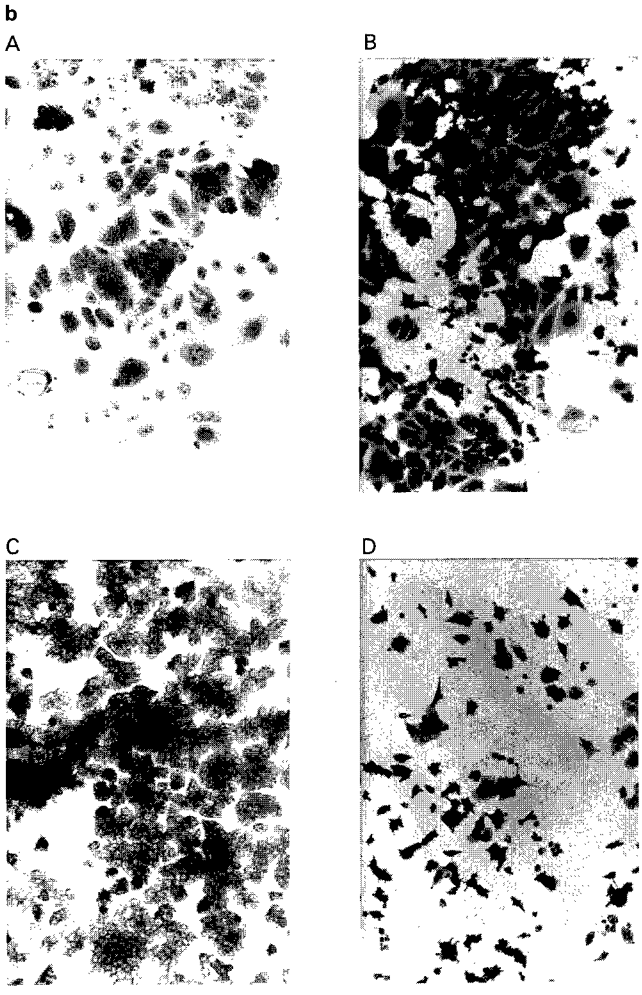


Figure 3 BRCA1 induces apoptosis in NIH3T3 cells after calcium ionophore treatment. (**a**) Flow cytometric analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. (**b**) Viability of NIH3T3 and BRCA1^{s} cells treated with and without calcium ionophore for 24 h. A, NIH3T3; B, NIH3T3 treated with calcium ionophore; C, BRCA1^{s}; D, BRCA1^{s} treated with calcium ionophore. (**c**) Chromatin condensation shown morphologically by Hoeschst staining of the BRCA1^{s} cell line induced to undergo apoptosis by calcium ionophore treatment for 24 h. A, NIH3T3 in the absence of calcium ionophore; B, NIH3T3 in the presence of calcium ionophore; C, BRCA1^{s} in the absence of calcium ionophore; D, BRCA^{s} in the presence of calcium ionophore. (**d**) DNA fragmentation induced by BRCA1 overexpression. 1, NIH3T3 treated with calcium ionophore; 2, BRCA1^{s} treated with calcium ionophore





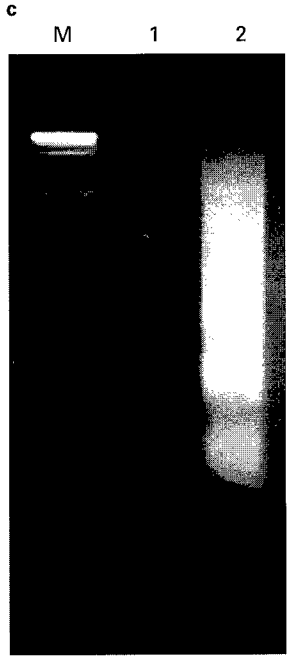


Figure 4 BRCA1 over expression induces apoptosis in MCF-7 cells after calcium ionophore treatment. (**a**) Flow cytometry analysis of cells induced to undergo apoptosis by calcium ionophore A23187 treatment for 24 h. (**b**) Cell viability of MCF-7 and BRCA1^{s} cells treated with and without calcium ionophore for 24 h. A, MCF-7; B, MCF-7 cells treated with calcium ionophore; C, BRCA1^{s}; D, BRCA1^{s} treated with calcium ionophore. (**c**) DNA fragmentation induced by BRCA1 over-expression. Lane 1, MCF-7 cells treated with calcium ionophore; 2, BRCA1^{s} cells treated with calcium ionophore

proteins or target apoptosis inhibiting proteins through direct protein-protein interactions. In the mouse mammary gland BRCA1 expression was found to be elevated during pregnancy following treatment with ovarian hormones (Lane *et al.*, 1995; Marquis *et al.*, 1995) and in human breast cancer cells BRCA1 mRNA levels were found to be regulated by steroid hormone estrogen and progesterone (Gudas *et al.*, 1995). Recent evidence suggests that hormone-dependent tumors like breast and ovarian cancers have a decreased ability to undergo apoptosis (Thompson, 1995). Our results suggest that lack or decreased levels of expression of functional BRCA1 gene product in breast and ovarian cancers may be responsible for the increased resistance of these cells to undergo apoptosis. Treatments that are aimed at increasing the apoptotic threshold by BRCA1 gene therapy may have the potential to prevent the progression of these malignancies. Alternatively, one can use therapeutic agents that can activate BRCA1 downstream signals involved in apoptosis for the treatment of breast and ovarian cancers.

Materials and methods

Cell lines

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FCS), 1% Penicillin-streptomycin and 200 μg/ml of G418 under 5% CO_2 atmosphere. MCF-7 cells were transfected with BRCA1 cDNA as described previsouly (Rao *et al.*, 1996; Rao *et al.*, unpublished results).

Immunohistochemistry

NIH3T3 cells stably overexpressing the BRCA1 gene product has been previously described (Rao *et al.*, 1996). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-streptomycin and 200 μg/ml of G418 under 5% CO_2 atmosphere. NIH3T3 and BRCA1^s transfectant cells were cultured in chamber slides and processed for immunohistochemistry using the BRCA1 peptide antibody as described previously (Rao *et al.*, 1996).

Immunofluorescence

Immunofluorescence analysis was done as described previously (Dyck *et al.*, 1994). In brief, NIH3T3 and BRCA^s cells cultured in chamber slides were fixed in 3.7% formaldehyde at room temperature, following by washing with PBS and blocking in blocking solution (3% bovine serum albumin-0.5% Triton X-100 in PBS) for 30 min at room temperature. The cells were incubated with primary BRCA1 peptide antibody diluted 1:100 for 2 h at room temperature, then washed three times with PBS and were incubated with the secondary antibody (FITC conjugated goat anti rabbit IgG (Cappel). After rinsing in PBS, the slides were mounted with fluoresecence mounting media (Vector) and photographed on an immunofluorescence microscope.

Flow cytometry analysis

Subconfluent to confluent NIH3T3, BRCA1^s cells were incubated in 10% FBS or 0% FBS media. After 24 h both adherent and nonadherent cells were pooled, washed in PBS and fixed in 80% cold ethanol at −18°C overnight. Cells were pooled, washed in PBS and stained with propidium iodide (20 μg/ml) and incubate with 20 μg/ml of RNAse A at 4°C in the dark overnight. Samples were analysed using a EPICs profile analyzer. Histograms showing the total DNA content at FL2 vs cell number are shown.

Flow cytometry analysis of NIH3T3, MCF7 and BRCA1^s cells treated with calcium ionophore A23187 was done similar to that described in Figure 2 legend except for the treatment with 20 μM calcium ionophore A 23187 (Sigma) for 24 h.

DNA fragmentation

DNA fragmentation assay was done as described (Kondo *et al.*, 1995). In brief, confluent NIH3T3 and BRCA1^s cells grown in DMEM supplemented with 10% FBS and penicillin streptomycin were changed into media containing 0% FBS for 24, 48 and 72 h. After the indicated incubation periods, both adherent and detached cells were collected ($2-5\times10^6$ cells), washed once in TBS buffer and lysed in 1 ml of 100 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS and 20 μg/ml RNAse A (pH 8.0) and incubated at 37°C for 30 min. Proteinase K at a final concentration of 100 μg/ml was added and further incubated for 3 h at 55°C. After extraction with an equal volume of phenol:chloroform:isoamyl alcohol, followed by re-extraction with phenol:chloroform: isoamyl alcohol. The DNA was precipitated from the aqueous phase with sodium acetate and two volumes of ethanol, the DNA pellet was dissolved in TE buffer and analysed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining.

DNA fragmentation analysis of NIH3T3, MCF7 and BRCA1^s cells treated with calcium ionophore A23187 was done as described in Figure 2 legend except for the treatment of A23187 (20 μM) for 24 h before analysis on a 2% agarose gel.

Cell viability

Subconfluent NIH3T3, MCF-7-BRCA1^s cells seeded into six well plates and grown in DMEM 10% FBS were treated with 20 μM calcium ionophore A23187 for 24 h. After which cells were washed with PBS and fixed in methanol:glacial acetic (3:1). Cells were stained with crystal violet. After washing in water, the cells were visualized and photographed under an Olympus microscope at 200× magnification.

Chromatin condensation

NIH3T3 and BRCA1 cells treated with or without 20 μM calcium ionophore for 24 h were cultured on glass cover slips and fixed in methanol:glacial acetic acid (3:1) at −18°C for 30 min. The cells were washed in PBS and stained with 8 μg/ml Hochest 33258 for 5 min in dark. The coverslips were rinsed in water and mounted with fluorescence mounting media. The cells were visualized and photographed under the fluorescence microscope.

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Wet thank G Chipitsyna for excellent technical help. We thank other colleagues of Rao and Reddy laboratories for their cooperation. This work is supported in part by NIH grants CA57322 to VNR and NIH grant CA 58642 and ACS grant VM#69 to ESPR and DAMD17-94-4280 by US Army medical research (VNR). The contents of the information does not necessarily reflect the position or the policy of the government and no official endorsement should be inferred.

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Antisense RNA to the putative tumor suppressor gene BRCA1 transforms mouse fibroblasts

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Recently, BRCA1, a familial breast and ovarian cancer susceptible gene has been cloned and shown to be either lost or mutated in families with breast and ovarian cancers. BRCA1 has been postulated to encode a tumor suppressor, a protein that acts as a negative regulator of tumor growth. We have characterized the BRCA1 gene products by Western blot and immunoprecipitation analysis in mouse and tumor cells. Multiple BRCA1 polypeptides of approximately 225, 185, 160, 145, 100, 52 and 38 kD were identified in these cells. BRCA1 proteins were found to be localized mainly in the nucleus of normal Rat1 cells and human breast cancer cells. In order to understand the role of BRCA1 in cell transformation, we have established a stable NIH3T3 cell line expressing BRCA1 antisense RNA. The inhibition of expression of endogenous BRCA1 protein was detected in NIH3T3 transfectants by Western blot analysis. The antisense BRCA1 expressing NIH3T3 cells showed accelerated growth rate, anchorage independent growth and tumorigenicity in nude mice unlike the parental and sense transfectants. These results provide the first direct biological evidence for the possible function of BRCA1 as a tumor suppressor gene.

Keywords: BRCA1 proteins; antisense RNA; tumor suppressor; breast and ovarian cancers

Introduction

Breast cancer is one of the most frequent cancers affecting women. Although majority of cases are thought to be sporadic, about 5% of cases are estimated to be familial (Claus *et al.*, 1991). The clinical progression of human breast cancer reflects accumulated molecular defects in specific genes that are important in regulating the growth of normal breast tissue. The breast cancer susceptible gene BRCA1 gene was shown to be lost or mutated in families with hereditary breast and ovarian cancers (Miki *et al.*, 1994; Takanashi *et al.*, 1995; Easton *et al.*, 1993). Some recent reports have also implicated a role for BRCA1 directly in sporadic cancers (Merajver *et al.*, 1995; Hosking *et al.*, 1995; Futreal *et al.*, 1994). Studies of allele loss in tumors from breast and ovarian cancer affected families suggesting that BRCA1 is a tumor suppressor gene (Smith *et al.*, 1992). Thus the inherited mutation results in inactivation of one copy of the gene by mutation and the loss of the second wild type allele (Smith *et al.*, 1993; Kelsell *et al.*, 1993). These results implicate a key role for tumor suppressor genes like BRCA1 in the genesis and progression of breast cancers. The BRCA1 gene is composed of 22 coding exons stretching roughly 100 Kb of genomic DNA (Miki *et al.*, 1994). The gene codes for a 1863 amino-acid protein with an amino terminal zinc finger domain and a carboxy terminal acidic region typical of several transcriptional factors (Miki *et al.*, 1994). The first insight into the potential role of BRCA1 in breast tumor progression came from the work described by Thompson *et al.* (1995) where they show inhibition of BRCA1 expression with antisense oligonucleotides resulted in accelerated growth of normal and malignant mammary cells but not non-mammary epithelial cells. These results suggested that BRCA1 negatively regulates the proliferation of mammary epithelial cells. Taking all these results into consideration, we reasoned that if BRCA1 functions as a tumor suppressor regulating cell growth and division, allelic loss or damage by mutation of BRCA1 as seen in patients with breast cancer could result in loss of function of BRCA1 protein and uncontrolled cell growth leading to cancers. To test the hypothesis whether BRCA1 is a tumor suppressor gene, we used antisense RNA methodolgy (Iszat and Weintraub, 1985). We reasoned that if BRCA1 functions as a growth regulator in normal cells, inhibiting its expression should result in transformation. Our results demonstrate that antisense RNA to BRCA1 transforms mouse fibroblasts, providing the first direct biological evidence for the possible function of BRCA1, as a tumor suppressor gene.

Results and discussion

The experimental strategy that we have used to test the hypothesis whether BRCA1 is a tumor suppressor gene is shown in Figure 1. NIH3T3 cells express significant levels of a major $\approx$100 kD and a minor $\approx$145 kD BRCA1 protein as analysed by Western blot analysis (Figure 2a, lane 1). Similar sized BRCA1 proteins were also observed in several human cell lines (Figure 2c). In addition to these bands, we have also observed both higher $\approx$225 kD (using nuclear extracts, data not shown), 185 kD (Figure 2c, lane 3), $\approx$160 kD (data not given) and lower ($\approx$52 and $\approx$38 kD) molecular weight BRCA1 polypeptides (Figure 2c). Interestingly, we have detected high level of expression of $\approx$185–200 kD and $\approx$38kD BRCA1 proteins in HL 60 cells by immunoprecipitation analysis (Figure 2e). These results suggest that Western blot and immunoprecipitation analysis detect different size and level of expression of BRCA1 proteins. Detection of

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different size BRCA1 proteins is consistent with the presence of alternatively spliced transcripts of BRCA1 in different cells (Miki *et al.*, 1994; Rao, unpublished results). Our results suggest that these BRCA1 proteins undergo phosphorylation (Rao, unpublished results). We have isolated and characterized a BRCA1 cDNA corresponding to $\approx$100 kD BRCA1 protein in human cells (Rao, unpublished results). It remains to be seen whether these multiple protein bands represent other isoforms of BRCA1 or its related proteins. We have studied the subcellular distribution of BRCA1 proteins in normal (Rat 1 cells, Figure 2d) and in transformed cells (MCF7 cells, Figure 2d) using immunohistochemical methods (Figure 2d) and indirect immunofluorescense staining (data not given). Our results suggest that BRCA1 proteins are localized mainly in the nucleus of Rat 1 and MCF7 cells. However we have also observed weak cytoplasmic staining in the case of MCF7 cells.

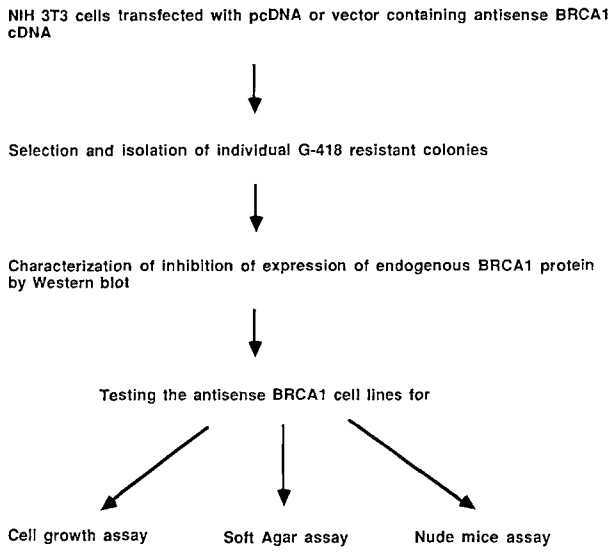
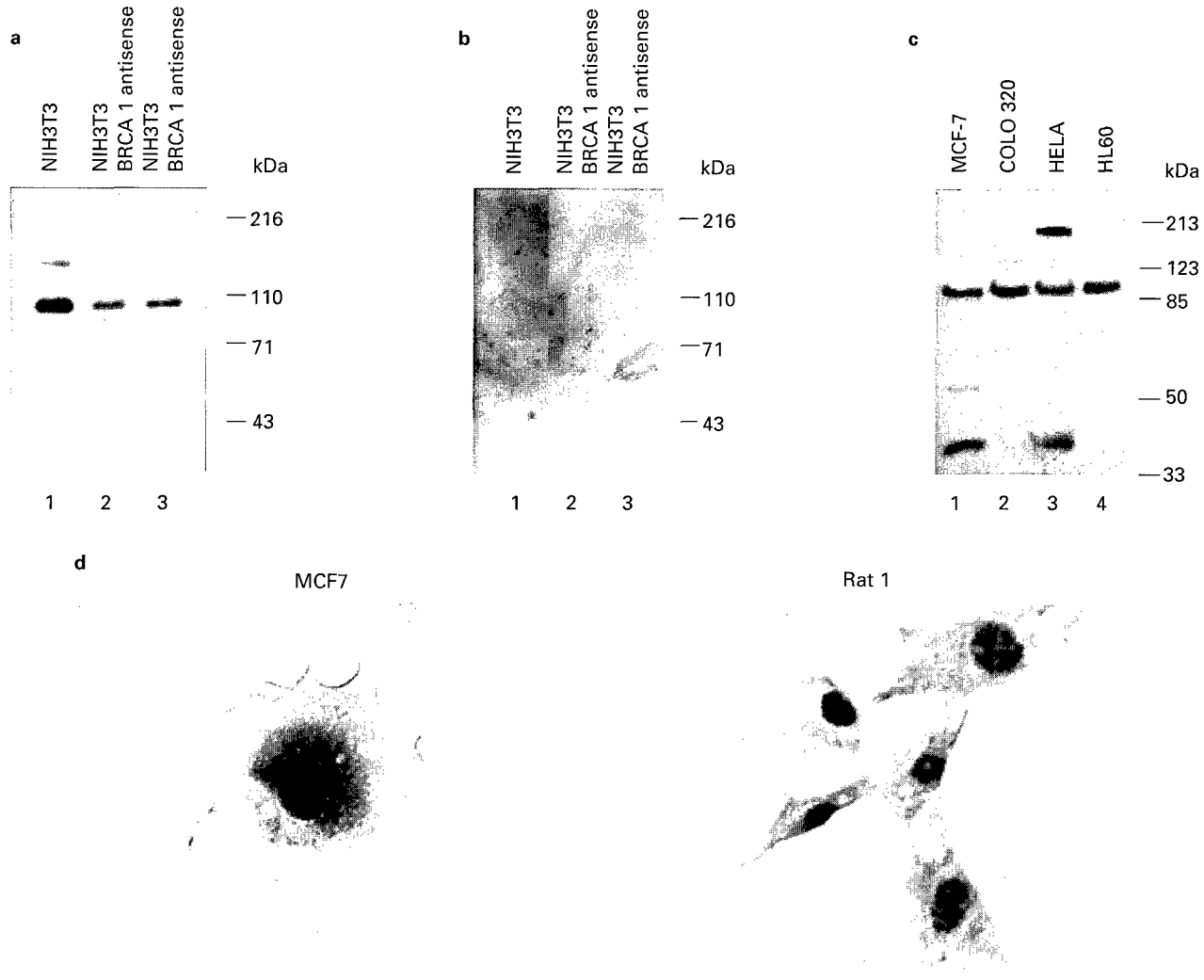


Figure 1 Experimental strategy used for obtaining the antisense BRCA1 transfectants

We have transfected NIH3T3 cells with pcDNA expression vector or pcDNA expression vectors containing antisense BRCA1 cDNA and obtained stable G418 resistant cell lines expressing antisense RNA to BRCA1. Initially we have screened several $BRCA^{AS}$ cell lines for BRCA1 protein expression by Western blot analysis using BRCA1 polyclonal peptide antibody. We selected two cell lines which showed a significant decrease in the expression of BRCA1 protein (Figure 2a, lanes 2 and 3). These lines showed roughly 3–5-fold decrease in the expression of BRCA1



protein when compared to the parental NIH3T3 cells. We have performed BRCA1 peptide competition to show that the protein band(s) seen on the immunoblot are specific for the BRCA1 protein(s) (Figure 2b). We did not find total inhibition of expression with BRCA^{AS} transfectants because in theory, a high concentration of antisense RNA is necessary to completely inhibit any target gene, however, our results (discussed below) suggests that total inhibition may not be necessary to observed a biological change, since mRNA molecule can synthesize several copies of protein.

The BRCA^{AS} transfectants showed no major morphological alterations except for a slightly more flattened phenotype when compared to the parental NIH3T3 cells (compare Figure 3a and d–f). Transformed cells unlike normal cells can proliferate faster and grow in serum-free or low serum culture medium as they become independent of growth factors present in the serum. Thus we investigated the growth of BRCA1^{AS} transfectants in different serum culture conditions (10%, 0.1% and serum free). The BRCA1^{AS} transfectants proliferated at a much faster rate than NIH3T3 cells and also the BRCA1^{AS} cells proliferated in low serum media (Figure 3e and 4a and b) and serum free media (Figure 3f), whereas the parental NIH3T3 cells were unable to proliferate under these conditions (Figures 3b, c and 4b). These data indicate that BRCA1^{AS} transfectants behave like transformed cells in that they become independent of growth factors present in fetal bovine serum.

The accelerated growth rate and growth in serum free media of the BRCA1^{AS} cells raised the possibility that they might have become transformed, hence we tested their ability to grow in soft agar. Interestingly the BRCA1^{AS} cells were anchorage independent (Figure 5a) unlike the parental NIH3T3 cells and cells transfected with the BRCA1 sense constructs (Figure 5a). BRCA1^{AS} transfectant cell line #6 showed high clonogenic affinity (average 150 colonies, Figure 5b) in soft agar assay whereas NIH3T3 cells, NIH3T3/pcDNA and NIH3T3/BRCA1^{S} transfectants cells showed no colonies (Figure 5b). Another BRCA1^{AS} cell line number 3 also showed accelerated growth rate and growth in soft agar but was less tumorigenic than BRCA1^{AS} cell line no. 6 (data not given). Our results clearly suggests that a certain threshold level of BRCA1 protein is required for the regulation of cell growth in both mammary epithelial cells (Thompson *et al.*, 1995) and mouse fibroblasts (this study). Mere down regulation of expression of BRCA1 protein may result in deregulation of BRCA1 function leading to the progression from a normal to a transformed state. These results demonstrate that inhibition of expression of BRCA1 protein in BRCA1^{AS} cells might be sufficient to achieve transformation.

We next tested the tumorigenicity *in vivo* of NIH3T3 cells transfected with either pcDNA vector to BRCA1 antisense cDNA in nude mice. Our results show that subcutaneous injection of BRCA1^{AS} cells into nude mice consistently resulted in the development of tumors at the site of injection with a latency of 3 to 4 weeks (six out of six animals). None of the mice injected with the vector transfectants induced tumors at least up to 6 weeks.

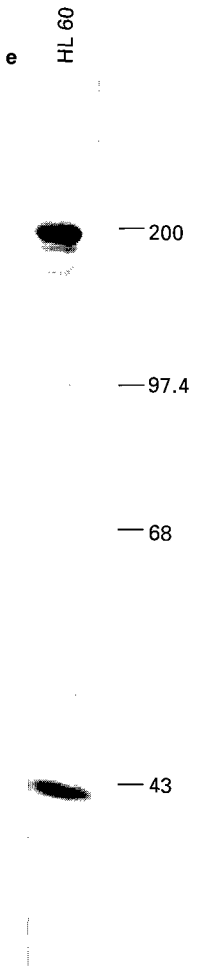


Figure 2 Characterization and subcellular localization of BRCA1 proteins. (**a**) Western blot analysis of total cellular BRCA1 protein levels in NIH3T3 and BRCA^{AS} transfectants. Total cellular extracts from NIH3T3 cells (lane 1), BRCA1 AS cell line no 3 (lane 2) and BRCA^{AS} cell line no 6 (lane 3) were electrophoretically separated, transferred to PVDF membrane and subjected to immunoblot analysis using a BRCA1 antipeptide antibody (Santacruz Biotechnology, Inc.). The apparent molecular weight of the prestained protein standards is shown. The molecular weight of the major BRCA1 protein band corresponds to $\approx$100 kD. (**b**) The same blot as in (**a**) was stripped and reprobed with the BRCA1 peptide antibody but in the presence of excess BRCA1 antigen. (**c**) Total cellular extract ($\approx$70 μg) from MCF-7 cells (human breast cancer cells) (lane 1); Colo 320 (human colon adenocarcinoma) (lane 2); HeLa cell (human epitheloid carcinoma) (lane 3); HL-60 (human promyelocytic leukemia) ($\approx$35 μg) (lane 4) were subjected to Western blot analysis as described in (**a**). (**d**) Immunohistochemical detection of BRCA1 proteins in normal and breast cancer cells. In Rat1 cells BRCA1 is localized only to nucleus and in MCF7 cells BRCA1 is localized mostly in nucleus. Original magnification $\times$400. (**e**) Immunoprecipitation of BRCA1 proteins in HL60 cells. HL60 cells were labelled with [^{35}S] trans label and subjected to immunoprecipitation using a carboxy terminal BRCA1 peptide antibody

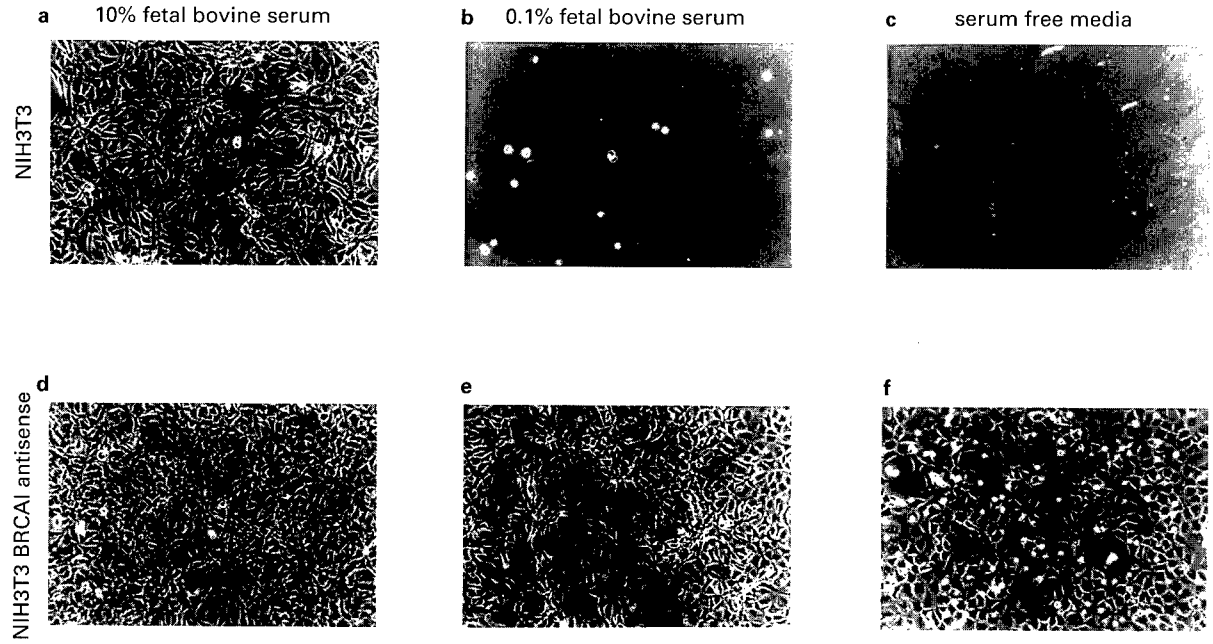


Figure 3 Growth properties and morphology of $BRCA^{AS}$ transfectants. Phase-contrast photomicrographs of NIH3T3 (**a-c**) and $BRCA^{AS}$ cell lines (**d-f**). Cells were cultured in normal media (10% FBS/DMEM) (**a** and **d**); low serum (0.1% FBS/DMEM) (**b** and **e**); serum free media (DMEM alone) (**c** and **f**)

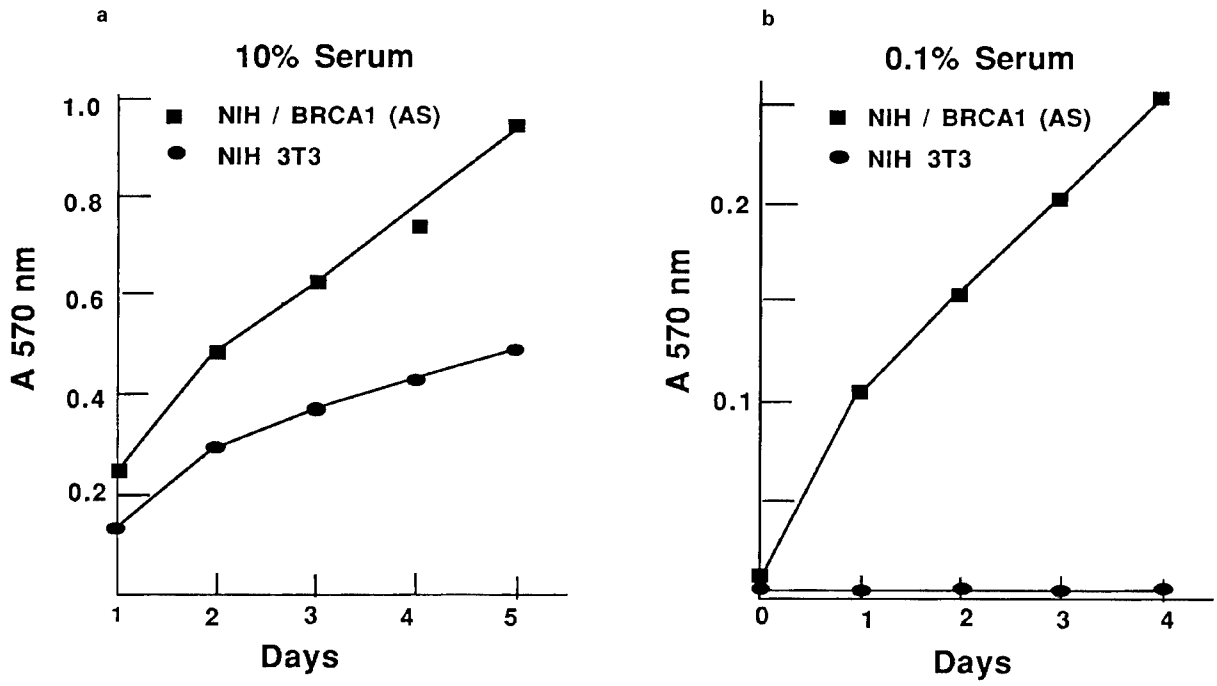


Figure 4 Inhibition of BRCA1 expression by antisense RNA accelerates NIH3T3 cell proliferation. Growth curves represent numbers of viable cells as measured by MTT dye assay (Loveland *et al.*, 1992) after different periods of cultivation in medium containing 10% FBS (**a**) or in 0.1% serum (**b**). The points represent mean of duplicates from a representative experiment

In summary, our results demonstrate that the BRCA1 gene product is a nuclear phosphoprotein which has tumor-suppressor function in mouse fibroblast cells since inhibition of endogenous BRCA1 expression by antisense RNA to BRCA1 results in neoplastic transformation. Our study provides the first direct biological evidence for the role of BRCA1 in transformation. The mechanism by which BRCA1 regulates cell proliferation is not known. The cell lines generated in this study should be useful to study the molecular mechanism involved in the function of BRCA1 gene. Knowledge of the pathways from inhibition of BRCA1 function leading to the progression to cancer will be important for the development of diagnostic kits and for designing targeted therapeutic strategies. Future efforts will be directed towards directly testing the BRCA1 gene product for growth inhibitory function.

Materials and methods

Plasmid construction and transfections

cDNA of BRCA1 (V Rao and ESP Reddy, unpublished results) containing the coding exons were subcloned into pcDNA vector by PCR using the published BRCA1 primers (Miki *et al.*, 1994). Purified DNA (15 μg) of pcDNA expression vector or vector containing the sense/antisense BRCA1 cDNAs were transfected into mouse NIH3T3 cells using the Strategene kit according to the manufacturers instructions. G418 resistant colonies were picked and propagated in the selective medium.

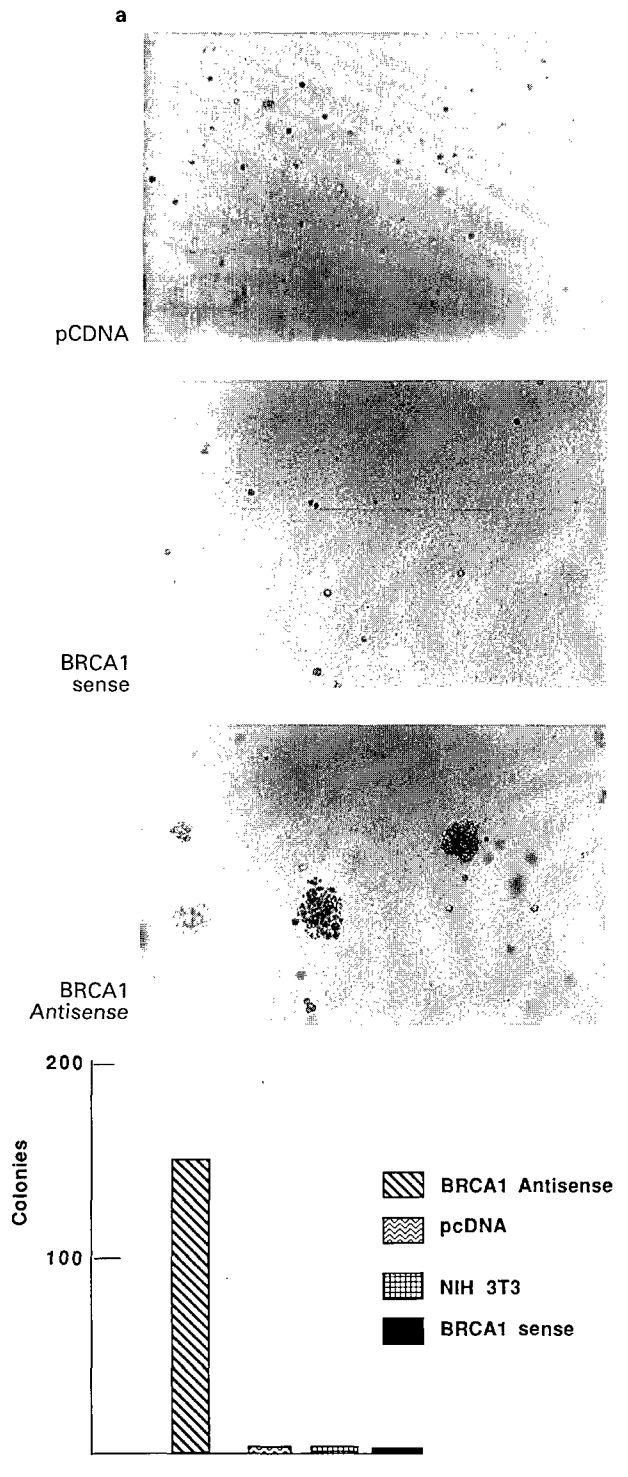


Figure 5 Growth of BRCA1^{AS} cells in soft agar (**a**) 2×10^3 cells per dish of pcDNA vector transfectant, BRCA1^{S} transfectant and BRCA1^{AS} transfectant were analysed for anchorage independent growth. (**b**) clonogenicity of BRCA1^{AS} cells; pcDNA vector cells; parental NIH3T3; and BRCA1^{S} cells

Western blot analysis

Cell extracts were prepared from exponentially growing cells, the protein concentration in the extracts were determined by Bradfords method (Bio Rad) and $\approx 18-87$ μg of protein from each of the extracts were loaded on a 10% SDS–PAGE as described previously (Rao *et al.*, 1989). After electrotransfer onto PVDF membrane, the filter was probed with a polyclonal BRCA1 peptide antibody and visualized using a chemiluminiscent assay as described by the manufacturer (Clontech kit) and exposed to Kodak X-AR film. Duplicate SDS–PAGE gels were run for each experiment and stained with coomassie blue.

MTT dye assay

The MTT metabolic assay was performed as described (Loveland *et al.*, 1992). In brief, cells were seeded at a density of 1×10^4 cells into microtitre plates and incubated in 10% FBS or 0.1% FBS media. At five 24 h intervals viable cells were stained for 4 h with the MTT dye and absorbance was read at 570 nm. The experiments were performed in duplicates or triplicates and reproduced at least three times.

Anchorage independence assay

Soft agar growth assay was done in 0.3% agar/DMEM/10% FBS and plated on a base of 0.5% agar/DMEM/10% FBS. Cells were plated at a concentration of 2×10^3 cells per 35 mm plate in soft agar containing DMEM and 10% FBS. Colonies greater than 100 μm in diameter were scored after 3 weeks. Each soft agar assay was performed in triplicates.

Immunohistochemistry

MCF7 and Rat1 cells cultured in chamber slides were washed in PBS, fixed with 3.7% formaldehyde in PBS at room temperature for 30 min. This was followed by washes in PBS and 30 min block in blocking serum (VECTASTAIN, ABC system from Vector). The specimens were incubated with primary BRCA1 carboxyterminal peptide antibody diluted 1 : 100 at room temperature for 2 h. After washing with PBS, slides were incubated for an hour with diluted biotinylated secondary antibody solution. The slides were washed with PBS and incubated for 30 min with Vectastain ABC reagent. Slides were further washed and incubated for 5 min in 0.01% H_2O_2–0.05% DAB solution. Slides were washed for 5 min in water, mounted in cytoseal 60 (Stephens scientific) and photographed on a immunofluorescence microscope (Olympus).

Immunoprecipitation

Briefly, confluent 100 mm plates of HL60 cells were labelled with [^{35}S] trans label for 3 h. The cells were lysed in radio immunoprecipitation assay buffer. Following sedimentation the supernatants were subjected to immunoprecipitation using rabbit anti-BRCA1 peptide antibody or preimmune serum as described previously (Rao and Reddy, 1993). The samples were subjected to 12% SDS polyacrylamide gel electrophoresis, fluorography and autoradiography.

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