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Abstract

Women who have familial breast cancer often have a germline mutation of the breast cancer susceptibility gene known as BRCA1. The function of BRCA1 is not totally understood. Previously we observed a correlation between the expression of BRCA1 and $p27^{Kip1}$ in a series of breast cancer cell lines. The $p27^{kip1}$ is a member of the universal cyclin-dependent kinase inhibitor family. We have shown that BRCA1 can transcriptionally activate the $p27^{Kip}$ promoter. The BRCA1-responsive element was defined as a 35 bp region from position -545 to -511. We determined that within this region is also a potential binding site for the transcription factor FOXA1. FOXA1 could activate the $p27^{Kip1}$ promoter. Co-transfection of BRCA1 and FOXA1 resulted in a synergistic activation of the $p27^{Kip1}$ promoter. Mutation of the FOXA1 DNA binding site in the $p27^{Kip1}$ promoter significantly diminished the activity of FOXA1 alone or in combination with BRCA1. EMSA analysis demonstrated that FOXA1 could bind to the $p27^{kip1}$ promoter. Using siRNA to suppress BRCA1 protein levels, we observed decreased $p27^{Kip1}$ promoter activity and reduced FOXA1 protein expression. Co-immunoprecipitation experiments indicated that FOXA1 and BRCA1 proteins interacted *in vivo*. In summary we discovered a role for BRCA1 in the regulation of $p27^{Kip1}$ transcription and a possible interaction with FOXA1, and we identified high expression of FOXA1 in breast cancer cell lines and tissues.

Table of Contents

Introduction

The breast cancer susceptibility gene BRCA1 is a tumor suppressor gene which is mutated frequently in hereditary breast cancers. The BRCA1 protein has been assigned a number of different functions, including DNA repair, cell cycle regulation and as a transcription factor. Cell cycle progression is governed by a family of cyclin-dependent kinases, whose activity is regulated by phosphorylation, activated by cyclin binding and inhibited by various inhibitors, such as $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. A number of studies have also examined $p27^{Kip1}$ expression in a series of tumors to determine if there is any diagnostic or prognostic significance. It has been shown that $p27^{Kip1}$ protein decreases during tumor development and progression in breast, colon, prostate and ovarian cancers. Previous studies in this lab observed a correlation between the expression of BRCA1 and $p27^{Kip1}$ in a series of breast cancer cell lines (Elstner et al, 2002). We have shown that BRCA1 could transcriptionally regulate the cyclin-dependent kinase inhibitor $p27^{Kip1}$, suggesting that this might be a mechanism for the regulation of the cell cycle by BRCA1. We determined that the BRCA1-responsive element of the $p27^{Kip1}$ promoter was localized to a 35 bp region at positions -545 to -511. Computer analysis using a number of transcription factor data bases suggested that the BRCA1-reponsive element contained a potential binding site for the transcription factor FOXA1. The Forkhead box (FOX) family of transcription factors contains more than 50 members which all share homology in their winged-helix DNA binding domain (Kaestner *et al*, 2000). Members of this family have been shown to play roles in cell proliferation, differentiation and metabolic homeostasis (Costa *et al*, 2001, Kaestner *et al*, 2000, Kaufmann and Knochel, 1996, Zaret 1999). Since the BRCA1-responsive element identified in the $p27^{Kip1}$ promoter contained a potential FOXA1 DNA binding site, we examined the expression of FOXA1 in breast cancer cell lines, and further examined the role of FOXA1 in conjunction with BRCA1 on the regulation of the cyclin dependent kinase inhibitor $p27^{Kip1}$.

Body

Research findings:

1: BRCA1 can transactivate expression of p27 ^{Kip1}: Using transient transfections we examined the effect of BRCA1 on both mouse and human $p27^{Kip1}$ promoter reporter gene expression in Cos, MCF-7 breast cancer and HCT116 colon cancer cells (Figure 1a, b). pCR3-BRCA1 activated the mouse $p27^{Kip1}$ promoter by 10-fold in Cos cells and 5-fold in MCF-7 and HCT116 cells (Figure 1b), as compared to the pCR3 vector.

2: Determined the specificity of p27Kip1 induction by BRCA1 using various synthetic and tumor associated BRCA1 mutants: Four different tumor-associated BRCA1 Mutants and BRCA1 mutant (del-500-1863) were unable to significantly transactivate the p27Kip1 promoter reporter compared to wild-type BRCA1 (Figure 2a, b). However, a second mutant lacking only the RAD51-interacting domain but with a functional nuclear localization signal and C-terminal transactivation domain, BRCA1 (del 515-1091), was able to transactivate the p27 promoter nearly as efficiently as wild type BRCA1 (Figure 2b).

3: Investigated the mechanism for regulation of $p27^{Kip1}$ by BRCA1. Western immunoblot analysis of HCT116 cells demonstrated that endogenous $p27^{Kip1}$ protein expression was up-regulated by wild-type BRCA1 but not by either the control vector pCR3 or a mutated BRCA1 (Gln1756insC) (Figure 3a).Next, we performed PCR on RNA from cells transiently transfected with control vector, wild-type BRCA1 or mutated BRCA1. At 30 cycles a PCR product for $p27^{Kip1}$ was detected only in the cells transfected with wild-type BRCA1 (Figure 3b). Thus these results suggest that the regulation of $p27^{Kip1}$ by BRCA1 is transcriptional.

4: Identification of a putative BRCA1-responsive element in the $p27^{Kip1}$ promoter. Initial experiments were carried out using deletion mutants of the mouse $p27^{Kip1}$ promoter. Deletion up to position 7774 increased the activity of BRCA1 on the $p27^{Kip1}$ promoter reporter constructs (Figure 4a), which is consistent with a previous report showing that the mouse $p27^{Kip1}$ promoter contains negative regulatory elements in the region 7609 to 7925 (Kwon et al., 1996). Further deletion to 7615 did not significantly decrease the response of the $p27^{Kip1}$ promoter to BRCA1 (Figure 4a). However, BRCA1 responsiveness was lost by deletions up to position 7511. These results suggested that a putative BRCA1-responsive element was located between positions 7615 and 7511 of the

$p27^{Kip1}$ promoter. However these results did not determine whether $p27^{Kip1}$ activation by BRCA1 was a direct or indirect effect by BRCA1. To investigate the effect of BRCA1 on the $p27^{Kip1}$ promoter we generated oligonucleotides spanning the region of the mouse promoter containing the putative BRCA1-responsive element as identified by the promoter deletion analysis. These oligonucleotides (oligo A 7609 to 7575, oligo B 7577 to 7543, oligo C 7545 to 7511) were used in EMSA with nuclear lysates from two breast cancer cell lines MCF7 and HCC1937, a breast cancer cell line which is unizygous for the BRCA1 5382insC mutation, resulting in termination of BRCA1 protein translation at codon 1829 (Tomlinson et al., 1998). Nuclear extract from MCF7 produced a slowly migrating band with oligo C but did not show any significant binding to oligo A or oligo B (Figure 4b). In contrast, nuclear extract from HCC1937 did not produce a band shift with any of the oligonucleotides (Figure 4b and data not shown). Subsequently we determined by immunohistochemistry that HCC1937 did express BRCA1 protein (Figure 5b). The slowly migrating complex observed with MCF7 nuclear extract and oligo C could be competed 90% by a 10-fold excess of cold oligo C (Figure 4b). Some competition was observed with cold oligo A but there was no competition observed with an excess of an unrelated oligo (oligo U; STAT site from MUC1 promoter). This suggests that the DNA-protein complex is specific. Since nuclear extract from the mutant BRCA1 cell line HCC1937 did not bind the DNA sequence from the $p27^{Kip1}$ promoter, we re-introduced wild-type BRCA1 into this cell line (HCC-BRCA1) and repeated the EMSA analysis. In this experiment a slowly migrating complex was observed with oligo C and nuclear extract from HCC-BRCA1 (Figure 4b). This slowly migrating complex with HCC-BRCA1 was less than that observed for MCF7 but the level of expression of wild-type BRCA1 in HCC-BRCA1 was lower than that observed for MCF7 (data not shown). Thus introduction of wild-type BRCA1 did restore binding to the p27Kip1 promoter sequence. Since it appeared that the BRCA1-responsive element could be localized to a 35 bp region of the p27Kip1 promoter, we generated a construct in which this region was deleted. Transient transfection assays using the deletion construct 7774 and the construct without the putative BRCA1-responsive element 7774 (del 545 ± 511) demonstrated that the removal of this 35 bp region decreased BRCA1 responsiveness of the p27Kip1 promoter by 80% (Figure 4c). Therefore it does appear that the BRCA1-responsive element is located at position 7545 to 7511 of the mouse $p27^{Kip1}$ promoter, which corresponds to 7714 to 7680 of the human p27Kip1 promoter. This region is 100% identical between the mouse and human promoter binding sites (Minami et al., 1997).

5: Decreased $p27^{Kip1}$ protein expression is found in mutant BRCA1-expressing breast cancer cell lines. We compared the endogenous $p27^{Kip1}$ protein levels in cells expressing wild-type BRCA1 (MCF7) versus mutated BRCA1 (HCC1937). Immunohistochemistry demonstrated reactivity of both MCF7 and HCC1937 with an N-terminal BRCA1 antibody (Figure 5a,b). As expected from our transfection assay results, immunoreactivity for $p27^{Kip1}$ was weak for the HCC1937 cell line, as compared to MCF7 which showed strong expression of $p27^{Kip1}$ protein (Figure 5c,d).

6: FOXA1 activates the $p27^{Kip1}$ promoter: Analysis using a number of transcription factor data bases suggested that the region -544 to -536 corresponded to a binding site for the transcription factor FOXA1, a member of the forkhead family of transcription factors. BRCA1 alone activated the $p27^{Kip1}$ promoter 12-14-fold (Figure 6A), FOXA1 alone activated the $p27^{Kip1}$ promoter up to 75-fold and a combination of FOXA1 and BRCA1 appeared to be synergistic in activating the $p27^{Kip1}$ promoter (Figure 6A). Mutation of the potential FOXA1 DNA-binding site in the $p27^{Kip1}$ promoter decreased the activation by FOXA1 either alone or in combination with BRCA1 (Figure 6B). Therefore, these results suggest that FOXA1 can strongly activate the $p27^{Kip1}$ promoter, and this activity is increased dramatically in the presence of BRCA1. Only wild-type BRCA1 and the transcriptionally active mutant, BRCA1del515-1091, were able to synergize with FOXA1 on the $p27^{Kip1}$ promoter (Figure 6C).

7: **Expression of FOXA1 in breast cancer.** RNA was isolated from eight breast cancer cell lines and real-time quantitative PCR was used to determine the expression of FOXA1 mRNA. A comparison of FOXA1 expression between ER+ versus ER- breast cancer cell lines indicated that FOXA1 mRNA expression was 4 times greater in the ER+ breast cancer cell lines compared to the ER- breast cancer cell lines (Figure 7A). However, when we analyzed a series of breast tumors for the expression of FOXA1, the difference between ER+ and ER- was not as dramatic (data not shown), possibly due to the presence of contaminating normal breast tissue. Expression of FOXA1 RNA was similar between normal breast tissue and breast tumor samples (Figure 7A). Next, we investigated the expression of FOXA1 protein in breast cancer cell lines. Nuclear lysates were immunoblotted for FOXA1 and demonstrated high expression of this protein in the ER+ cell lines MCF7, T-47D, ZR-75-1 and BT-474 and very low expression in ER- breast cancer cell lines MDA-MB-231 and BT-20 (Figure 7B, data not shown). Thus, FOXA1 protein expression also correlates with ER expression in these

breast cancer cell lines. Furthermore, we analyzed a number of breast tumor patient samples for the expression of FOXA1 by immunohistochemistry (Figure 7C). This demonstrated that FOXA1 protein is indeed a nuclear protein in breast tissue. Of the 27 ER+ tumors, 74% (20/27) showed very strong expression of FOXA1 protein (Figure 7C, ii). Of the 6 ER- tumors, 83% (5/6) showed weak expression of FOXA1 protein (Figure 7C, iii). Therefore, this confirms the association observed by other studies that FOXA1 expression correlates with ER positivity. FOXA1 is expressed in normal breast tissue, and the expression appears to increase in the ER+ breast tumor tissue (Figure 7C, i vs. ii).

8: FOXA1 binds to the $p27^{Kip1}$ promoter: In order to show that FOXA1 can bind the $p27^{Kip1}$ promoter, we carried out EMSA analysis . We observed a protein-DNA complex in MCF-7 extracts that we had previously determined expressed FOXA1 protein (Figure 8) Confirmation that FOXA1 was present in this complex came when a FOXA1 antibody was included in the binding reaction, causing a supershift of the protein-DNA complex (Figure 8, lane 11). Therefore, these results show that FOXA1 is capable of binding an element in the $p27^{Kip1}$ promoter and directly activating this promoter.

9: FOXA1 protein expression is enhanced by co-expression with BRCA1: Analysis of the nuclear extracts from transiently transfected cells showed that when FOXA1 was co-transfected with BRCA1, greater expression of FOXA1 protein occurred compared to cells transfected with FOXA1 alone (Figure 9a, b). We determined that in cells transiently transfected with FOXA1 alone, the half-life of the FOXA1 protein was approximately 8 hours (Figure 10a). By comparison, the half-life of the FOXA1 protein co-transfected with BRCA1 was greater than 24 hours (Figure 10b). Therefore this result suggests that expression of BRCA1 may indeed stabilize FOXA1 protein.

10: Silencing of BRCA1 by siRNA. MCF-7 cells were transfected with a BRCA1-directed siRNA in combination with the p27Kip1 promoter-luciferase construct. As can be seen, MCF-7 cells transfected with increasing amounts of the BRCA1-directed siRNA showed decreasing activity of the p27Kip1 promoter-luciferase construct (Figure 11A). Thus, BRCA1 does indeed regulate p27Kip1. We also analyzed protein extracts from these siRNA-transfected MCF-7 cells and demonstrate that the reduction of BRCA1 protein expression is accompanied by a 13 decrease in

FOXA1 protein expression (Figure 11B, C). Therefore, this further suggests that the protein levels of BRCA1 can affect the protein levels of FOXA1.

11: FOXA1 and BRCA1 proteins interact *in vivo*. Since the data suggested that BRCA1 could stabilize FOXA1 protein, we hypothesized that this was due to a physical interaction between these two proteins *in vivo*. Immunoprecipitation of nuclear lysates with a BRCA1 antibody and subsequent probing with an antibody against FOXA1 indicated that *in vivo* BRCA1 and FOXA1 were in a protein complex together in these two breast cancer cell lines (Figure 12).

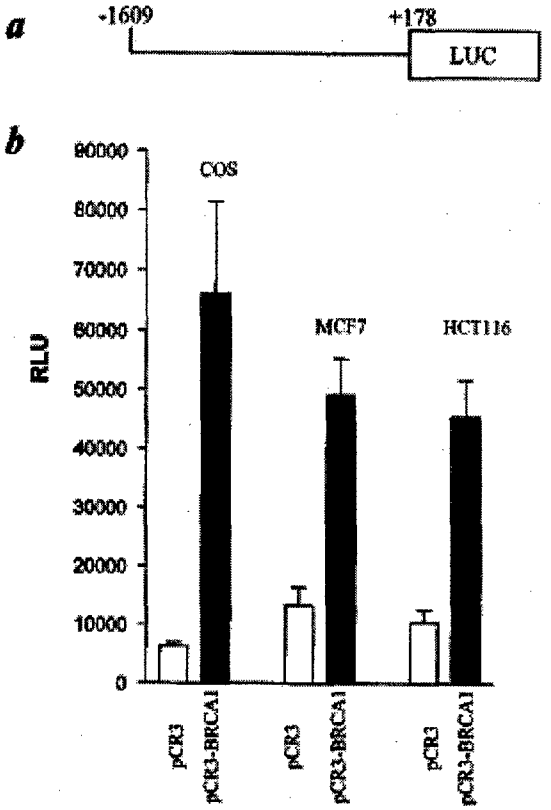

Figure 1 **BRCA1 transactivates the mouse p27Kip1 promoter.** (a) Schematic representation of the p27Kip1 promoter luciferase promoter. The 3' boundary is 178 bp downstream of the translation start site. (b) COS, MCF7 and HCT116 were co-transfected with p27ptr-luc and either pCR3 or pCR3-BRCA1 and luciferase activity was measured 48 h later. These results represent three independent experiments. Results are shown as RLU which is the ratio of the luciferase units observed for the promoter construct compared to that seen for the pRL-SV40.

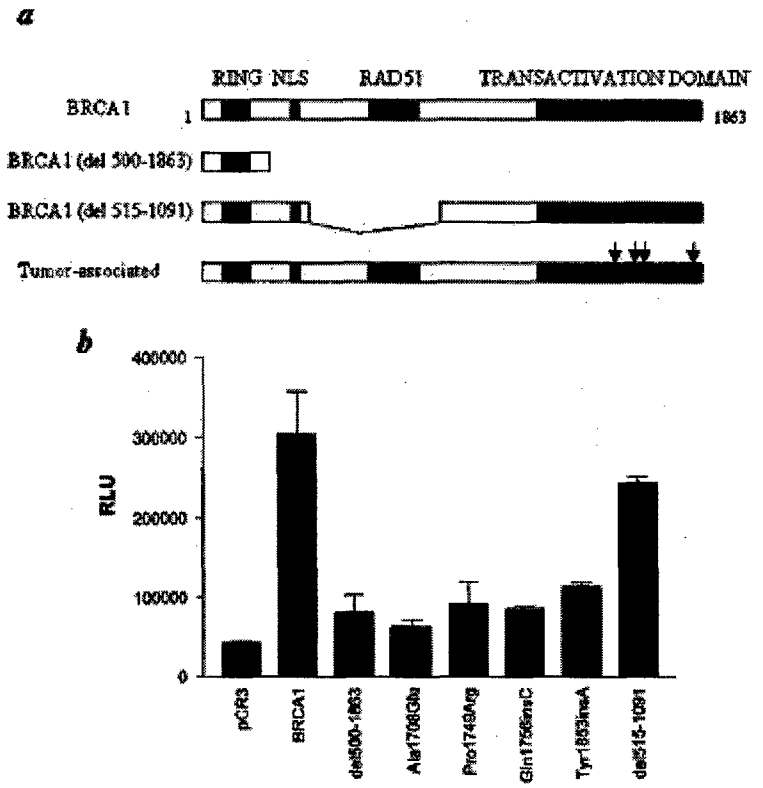

Figure 2 **BRCA1 mutants lacking the C-terminal transactivation domain are defective for activation of p27Kip1.** (a) Schematic representation of the BRCA1 mutants indicating the important domains for BRCA1 function. The arrows indicate the position of the C-terminal mutations of the BRCA1 gene. (b) COS cells were co-transfected with p27ptr-luc and pCR3 or either wild-type or mutant pCR3-BRCA1 expression plasmids as indicated. Luciferase activity was measured 48 h post-transfection as in Figure 1. These results represent two independent experiments

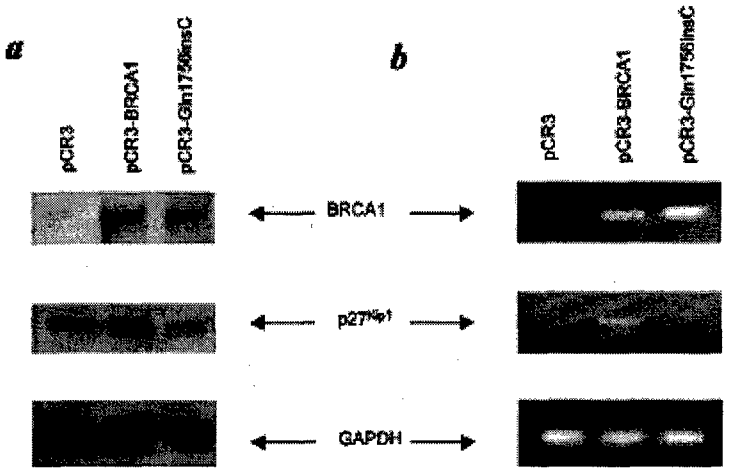

Figure 3 **BRCA1 transcriptionally regulates p27Kip1 expression.** (a) HCT116 colon cancer cells were transfected with pCR3, pCR3-BRCA1 or pCR3-BRCA1(Gln1756insC). Proteins were harvested 48 h post-transfection and analysed by SDS±PAGE and Western blot. Expression of endogenous p27Kip1 protein in up-regulated only in the presence of wild-type BRCA1. Equal protein loading is demonstrated by re-probing the blot with an antibody against GAPDH. (b) HCT116 cells were transfected as in (a) and RNA harvested by Trizol 48 h post-transfection. Reverse transcription-PCR demonstrated that both wild-type and mutant BRCA1 were being expressed. Expression of p27Kip1 was observed only in the cells transfected with wild-type BRCA1. Equality between samples was shown by using primers for GAPDH

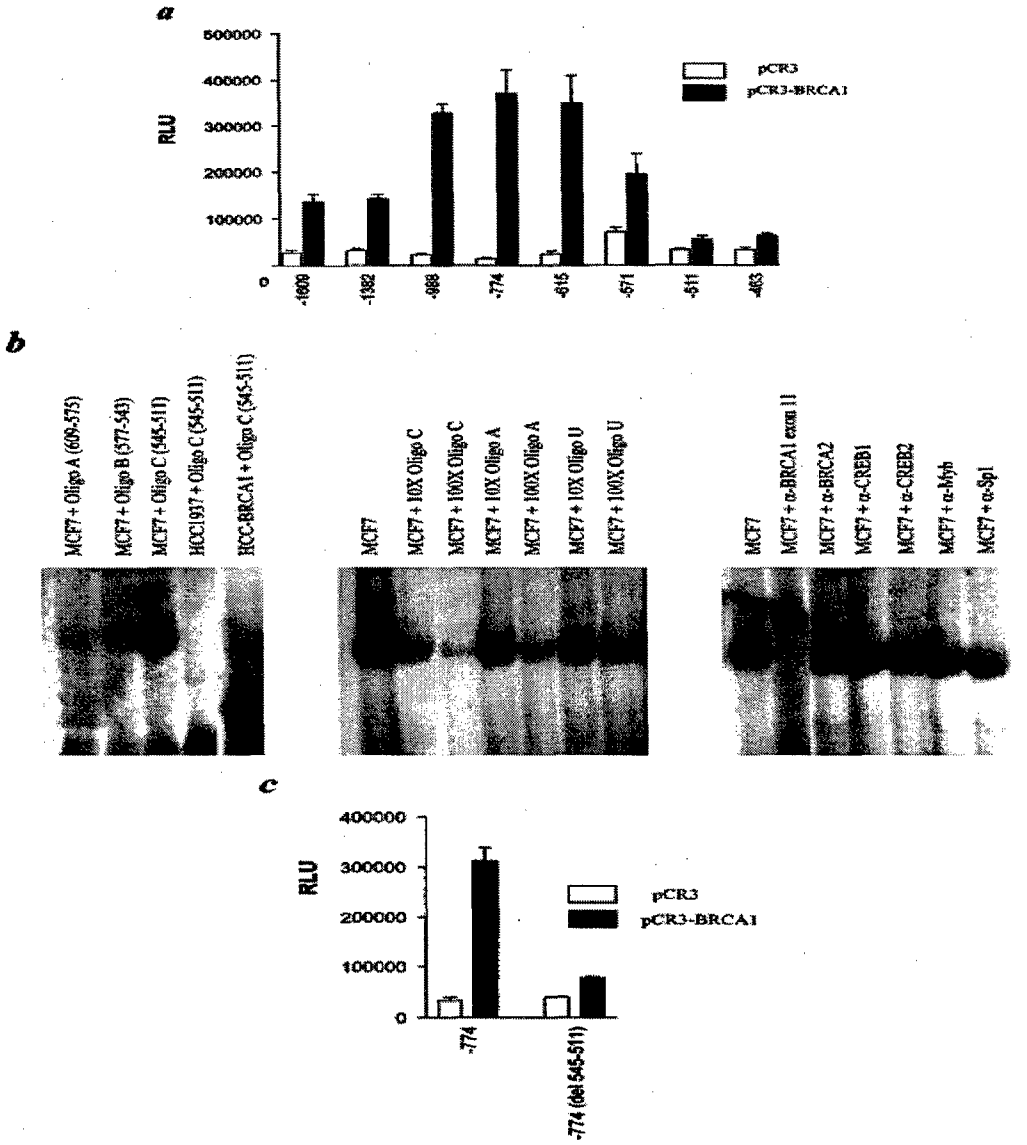

Figure 4 **Region of mouse p27Kip1 promoter containing the putative BRCA1-responsive element identified by 5' deletion mapping.**(a) COS cells were co-transfected with the 5'-deletion p27Kip1 promoter reporter constructs and either pCR3 or pCR3-BRCA1, and luciferase activity was measured 48 h later. These results represent three independent experiments. (b) Nuclear lysate from MCF7 was incubated with oligo A (7609 to 7575), oligo B (7577 to 7543) and oligo C (7545 to 7511) of the p27Kip1 promoter. Nuclear lysate from HCC1937 and HCC-BRCA1 was incubated with oligo C. Cold competition was carried out with an excess of oligo C, oligo A or oligo U (an unrelated 35 bp sequence). The complex is supershifted by an antibody against exon 11 of BRCA1. (c) MCF7 cells were transfected with a 5'-deletion p27Kip1 promoter construct (7774) or with the construct minus the putative BRCA1-responsive element (7774 (del 545-511)). Luciferase activity was measured as in Figure 1. These results represent three independent experiments.

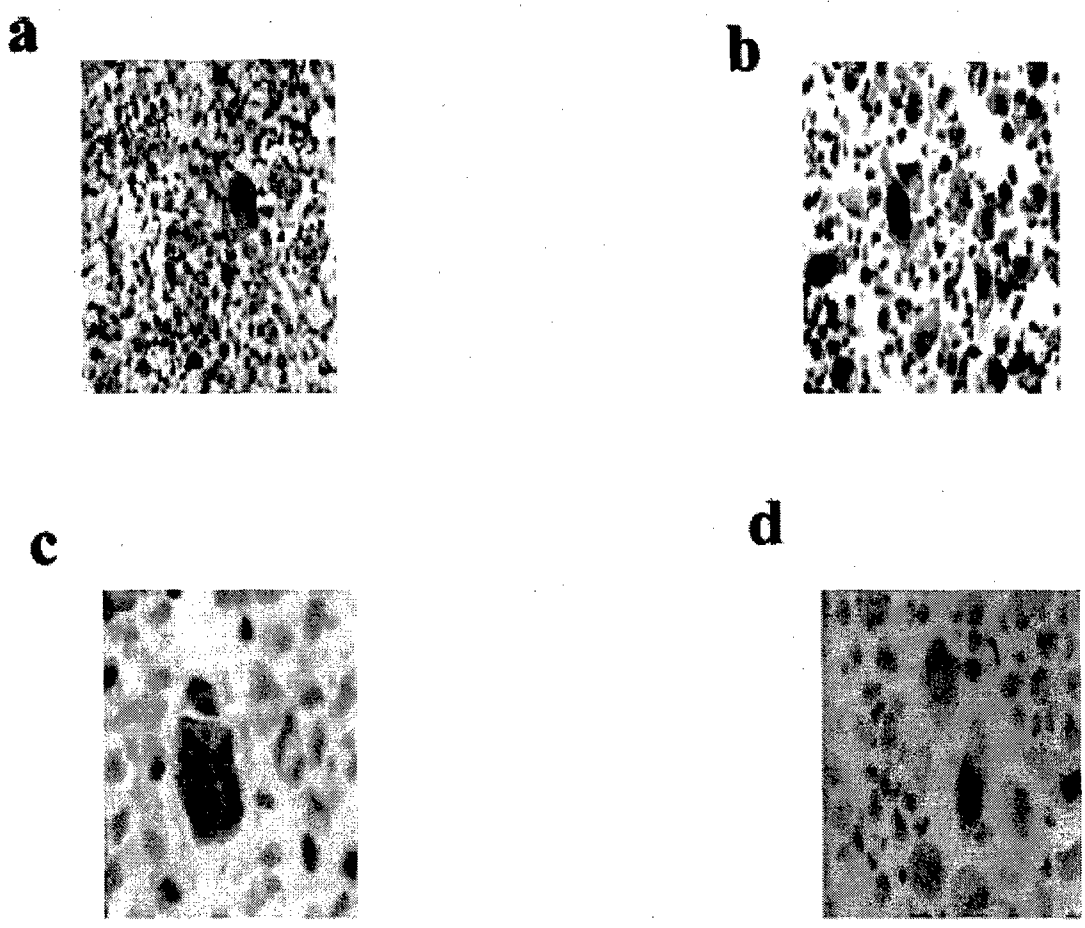

Figure 5 **Expression of p27Kip1 is decreased in cells expressing mutated BRCA1.** (a) (c) Formalin-fixed paraffin-embedded MCF7. (b) (d) Formalin-fixed paraffin-embedded HCC1937. (a) (b) N-terminal anti-BRCA1. (c) (d) Anti-p27Kip1. (a) (b) are 46 magnification. (c) (d) are 106 magnification

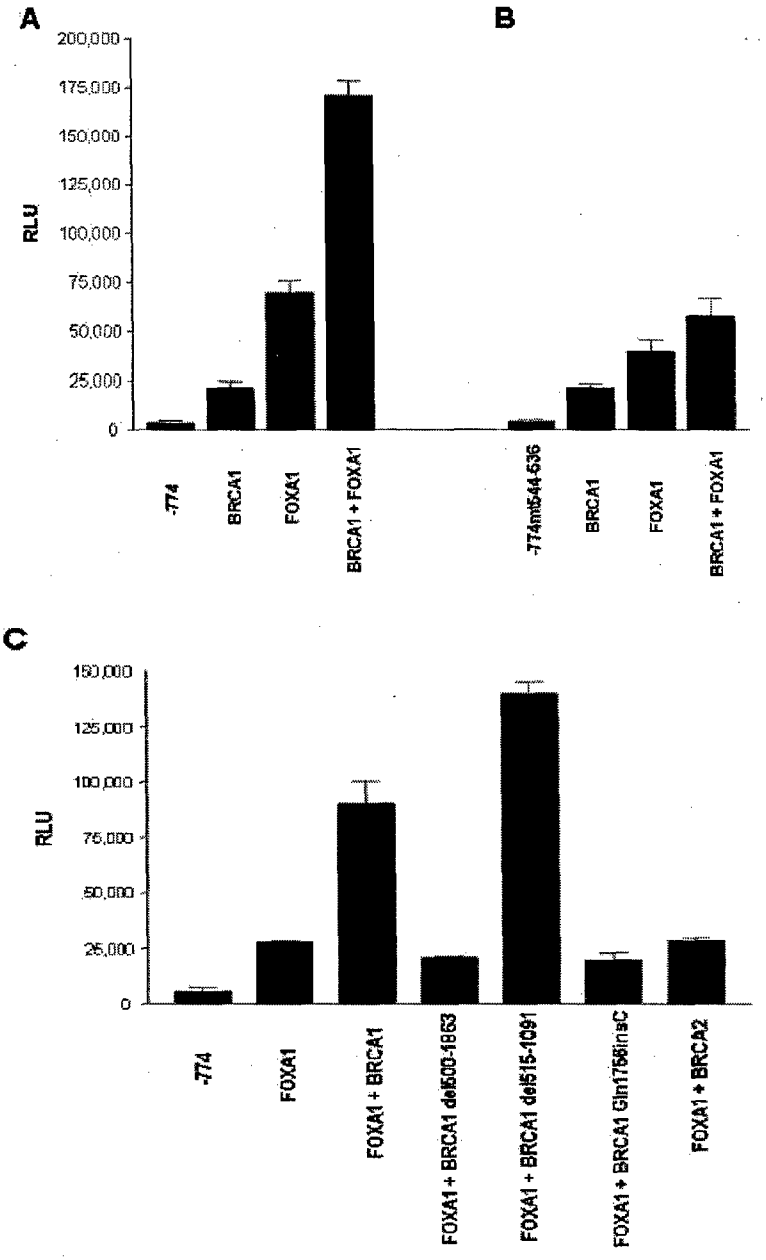

Figure 6. **FOXA1 and BRCA1 together activate the p27^{Kip1} promoter-luciferase reporter construct.** (A) HCT116 cells were transiently transfected with either FOXA1 and/or BRCA1. The p27^{Kip1}-774 promoter-luciferase construct was used in these assays since this construct was previously shown to have the greatest induction of activity by BRCA1. Lysates were harvested 48 hours post-transfection. The results shown here represent the results from 5 independent experiments. (B) The potential FOXA1 binding site in the p27^{Kip1} promoter was mutated and the transient transfection reporter assays were repeated as described above. These results represent 5 independent experiments. (C) Transient transfection reporter assays were repeated using FOXA1 in combination with wild type and mutant BRCA1 and BRCA2 for activation of the p27^{Kip1} promoter.

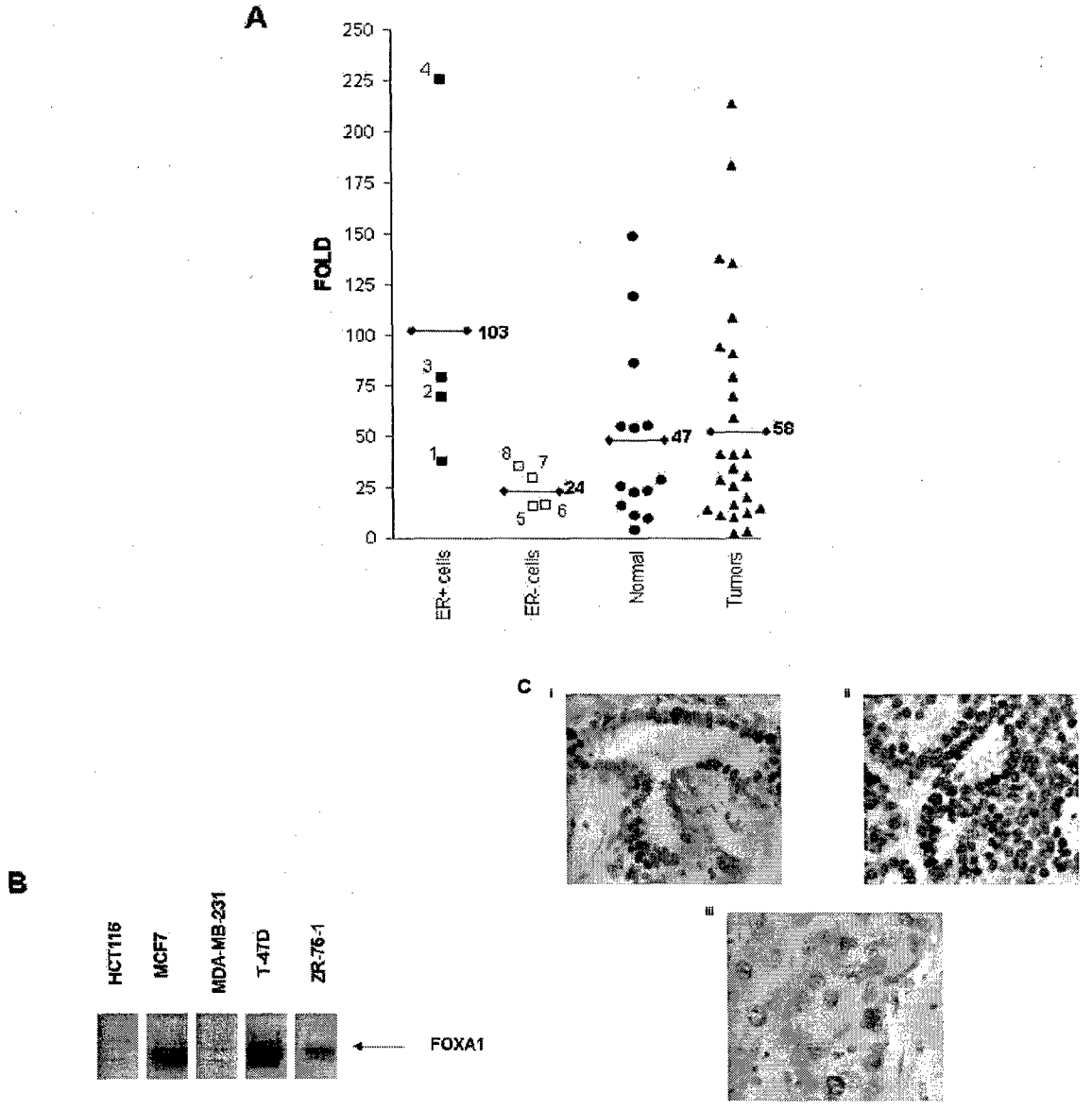

Figure 7. **Expression of FOXA1 correlates with estrogen receptor expression in breast cancer cell lines.** (A) RNA was isolated from breast cancer cell lines, and FOXA1 expression was determined by quantitative real time-PCR. The results were grouped according to estrogen receptor positivity of the breast cancer cell lines. Mean expression of FOXA1 mRNA was 4 times greater in the ER+ breast cancer cell lines compared to the ER- breast cancer cell lines. represents the mean value of FOXA1 RNA expression in the groups tested. Little difference was observed for the comparison of normal breast tissue to breast tumor tissue. Breast cancer cell lines are as follows: 1, MCF7; 2, BT474; 3, T-47D; 4, ZR-75-1; 5, MDA-MB-468; 6, SKBr3; 7, MDA-MB-231; and 8, BT-20. (B) Nuclear proteins were isolated from breast cancer cell lines and the HCT116 colon cancer cell line, and analyzed by Western immunoblotting with an antibody directed at the C-terminus of FOXA1. FOXA1 protein expression correlated with ER positivity of the cell line (MCF-7, T-47D, ZR-75-1). Equal loading of the nuclear extracts was visualized by Ponceau S staining of the membrane prior to immunoblotting (data not shown). (C) Paraffin sections of breast tumor tissue analyzed by immunohistochemistry for expression of FOXA1 using the antibody described above. The tissue samples are as follows: (i) normal breast tissue adjacent to invasive cancer; (ii) ER+ breast tumor; (iii) ER- breast tumor. Magnification X100.

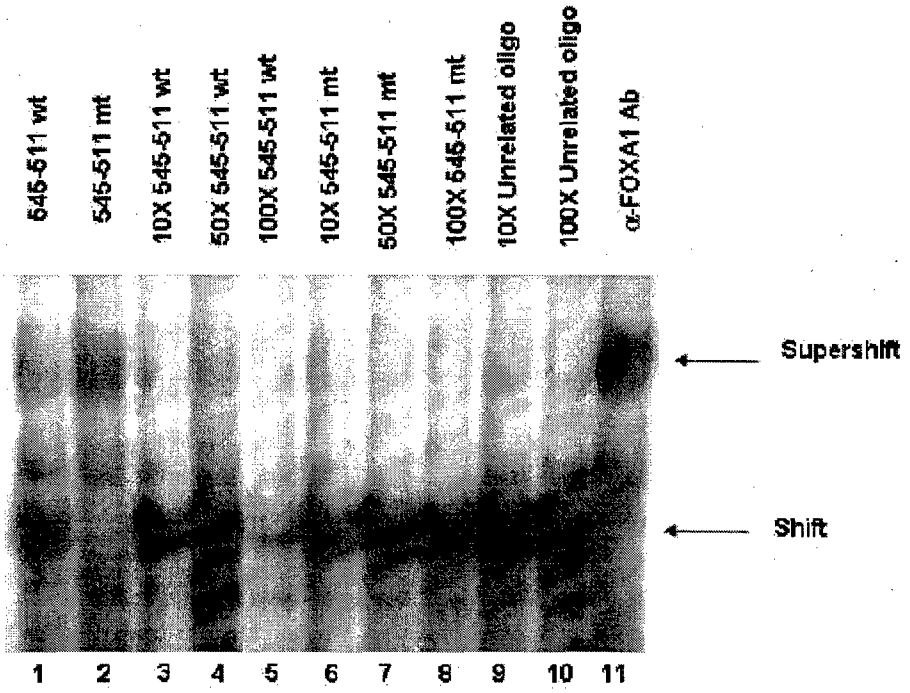

Figure 8. **FOXA1 protein binds to the potential DNA binding site in the $p27^{Kip1}$ promoter.** EMSA analysis using nuclear protein extracts from the MCF7 breast cancer cell line and the following oligonucleotides: $p27^{Kip1}$ promoter 545-511 wild-type, $p27^{Kip1}$ promoter 545-511 mutant (544-536), and an unrelated DNA sequence. The mutation of the FOXA1 site in the oligonucleotide was identical to the mutation made of this site in the $p27^{Kip1}$ promoter-luciferase reporter construct. The protein-DNA complex observed with the MCF7 nuclear protein extracts could be supershifted by the inclusion of an antibody directed against FOXA1 in the binding reaction.

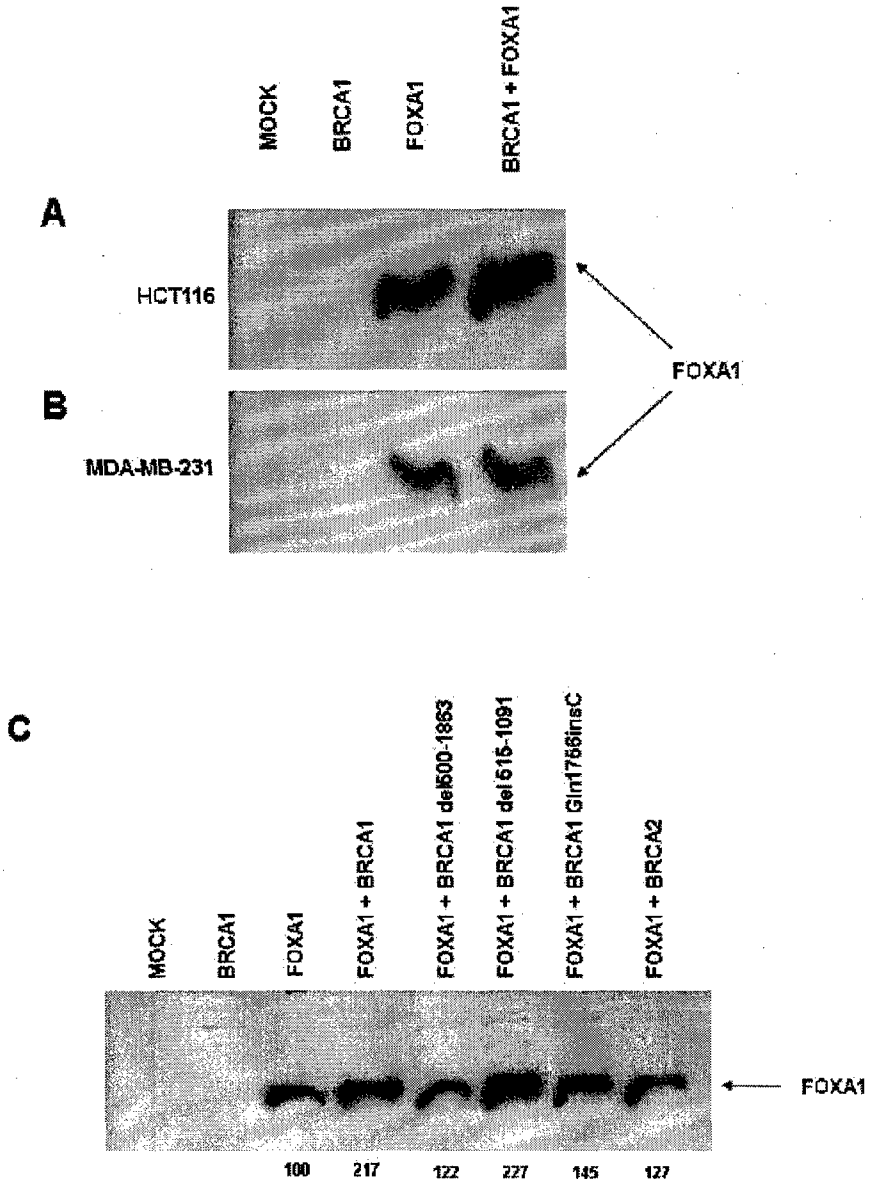

Figure 9. **Co-transfection of wild type BRCA1 increases the expression of FOXA1 protein.** Nuclear extracts were prepared from (A) HCT116 and (B) MDA-MB-231 transiently transfected with either FOXA1 and/or BRCA1. The extracts were immunoblotted for FOXA1 protein. (C) Nuclear extracts from HCT116 transiently co-transfected with FOXA1 and either wild-type or mutant BRCA1. Equal loading of the nuclear extracts was visualized by Ponceau S staining of the membrane prior to immunoblotting.

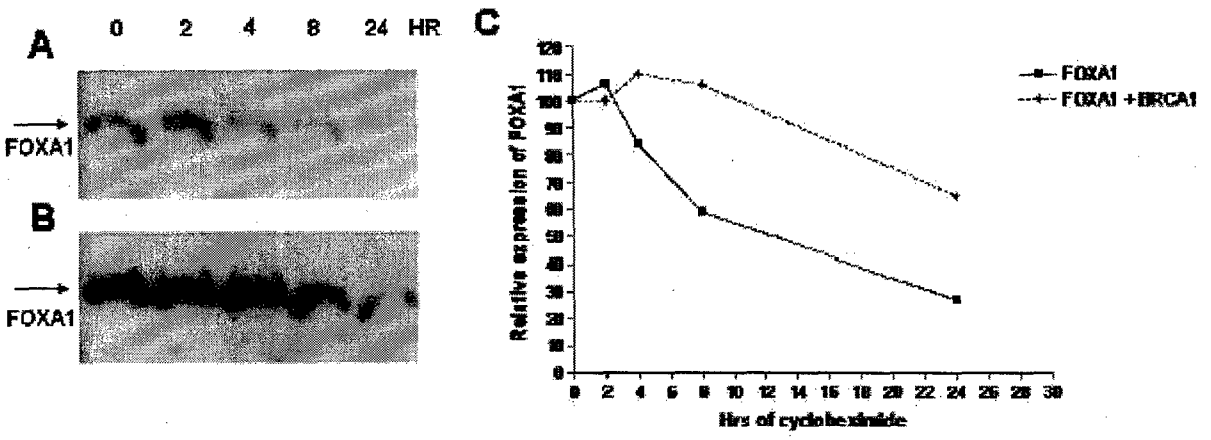

Figure 10. **Co-transfection of BRCA1 with FOXA1 increases the half-life of FOXA1 protein.** HCT116 cells were transiently transfected with FOXA1 in the absence (A) and presence (B) of BRCA1. These cells were treated with cycloheximide (10 μg/ml) and cells harvested for nuclear protein extraction at time points up to 24 hours. Nuclear extracts were separated by SDS-PAGE and immunoblotted for FOXA1 protein. (C) Ratio of protein concentration and densitometry from the Western blot determined the relative amounts of FOXA1 protein in the samples during the treatment with cycloheximide.

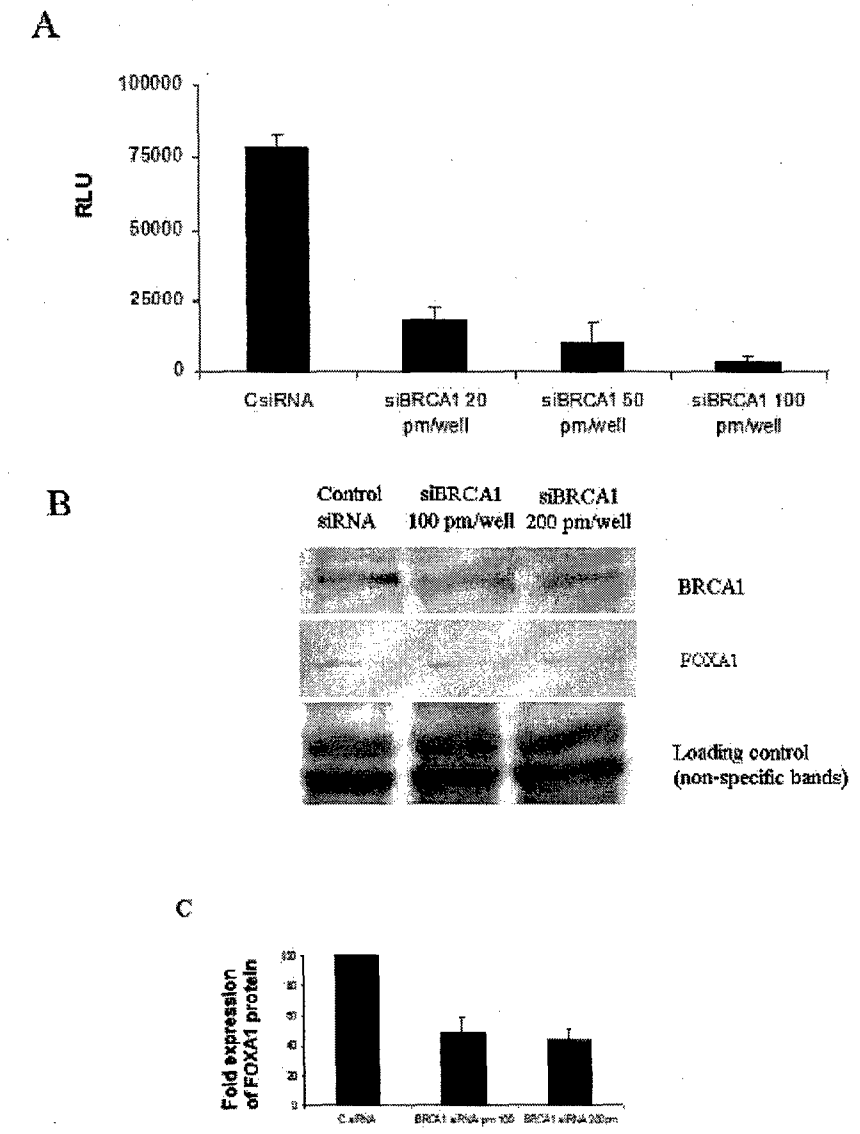

Figure 11. **Silencing of BRCA1 reduces p27Kip1 promoter activity and FOXA1 protein expression.** (A) p27Kip1 promoter activity is reduced in MCF-7 cells transfected with BRCA1-directed siRNA (20, 50, and 100 pmol/well). Luciferase assays were carried out as previously described. Data are shown as mean +/- SD of 3 independent experiments. (B) MCF-7 cells transfected with BRCA1 siRNA (100 and 200 pmol/well), were analyzed by Western immunoblotting to confirm the reduction in BRCA1 and FOXA1 protein expression. (C) Densitometry results confirm a 40-50% decrease in FOXA1 protein expression. Results are for 3 experiments. P>0.001 for each BRCA1 siRNA transfected well compared to the control well.

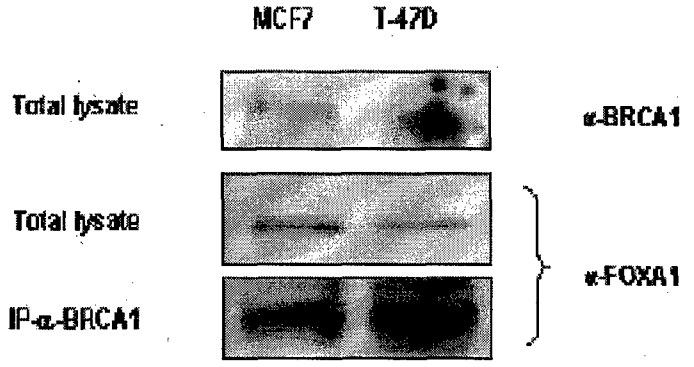

Figure 12. **BRCA1 and FOXA1 occur together in a protein complex *in vivo*.** Nuclear extracts were prepared from MCF7 and T-47D breast cancer cell lines, immunoprecipitated with an antibody against BRCA1, and the resulting Western blot probed with an antibody against FOXA1. Total nuclear lysate was also probed for the expression of these two proteins. FOXA1 protein is observed in the BRCA1-immunoprecipitated samples.

Key Research Accomplishments

- Demonstration that BRCA1 transactivates expression of $p27^{Kip1}$.
- Identification of the BRCA1-responsive element in the $p27^{Kip1}$ promoter
- Identification of FOXA1 binding site within the BRCA1-responsive element in the $p27^{Kip1}$ promoter.
- Showed that expression of FOXA1 correlates with estrogen receptor expression in a panel of breast cancer cell lines and tissues
- Discovered that FOXA1 interacts with BRCA1 to regulate expression of $p27^{Kip1}$.

Reportable Outcomes

BRCA1 Transactivates the cyclin dependent kinase inhibitor p27 . *Oncogene (2002) 21, 3199-3206.*

BRCA1 and FOXA1 proteins co-regulate the expression of the cycle dependent kinase inhibitor p27Kip1. *Oncogene, in press 2005.*

BRCA1 and FOX proteins interact and regulate the expression of the cyle dependent kinase inhibitor $p27^{kip1}$. *Era of Hope Department of Defense Breast Cancer Research Program Meeting. Pennsylvania, 2005.*

Conclusions

We have demonstrated that $p27^{Kip1}$ is a target for BRCA1 transcriptional activation. Wild-type BRCA1 transactivated the $p27^{Kip1}$ promoter in a number of cell lines. However, tumor-associated BRCA1 mutants were defective in transcriptionally regulating $p27^{Kip1}$ indicating that a functional C-terminal transactivation domain of BRCA1 is required for modulating $p27^{Kip1}$. This study suggests that another mechanism for growth inhibition by BRCA1 may be mediated via the upregulation of $p27^{Kip1}$ promoter.

The $p27^{Kip1}$ is decreased in various human cancers but specific mutations have only rarely been reported (Spirin et al., 1996). Transcriptional, translational and post-translational mechanisms contribute to p27Kip1 regulation. It had been considered that the major mechanism for regulating $p27^{Kip1}$ at the protein level was post-translational. However, transcriptional upregulation of $p27^{Kip1}$

by the Forkhead transcription factors has been reported recently (Medema et al.,2000). For breast tumors, this decrease in $p27^{Kip1}$ expression might be a reflection of a loss of functional BRCA1, resulting either from a mutation in the BRCA1 gene or by methylation of the BRCA1 promoter. Recent studies have shown that decreased expression of $p27^{Kip1}$ does indeed correlate either with both the presence of a BRCA1 mutation in breast tumor tissue as well as with BRCA1 promoter methylation (Chappuis et al., 2000; Niwa et al., 2000). Thus, loss of functional BRCA1 might be expected to result in impaired growth inhibition due to inefective regulation of $p27^{Kip1}$. Therefore, understanding the mechanisms controlling $p27^{Kip1}$ expression in breast tumors may provide new strategies to inhibit tumor growth.

BRCA1 has been shown previously to interact with a number of different proteins. We determined that the 35 bp region of the $p27^{Kip1}$ promoter that was responsible for BRCA1 regulation contained a sequence known to bind a liver-specific transcription factor FOXA1. Although FOXA1 has been shown to be important for liver and lung development, very little is known about its expression in breast tissue. Microarray analysis of breast tumor samples suggested that FOXA1 is expressed only in ER+ breast tumors and is reduced significantly in ER- breast tumors (van't Veer *et al*, 2002, West *et al*, 2001). We determined that FOXA1 expression did indeed correlate with ER positivity in breast cancer cell lines. Interestingly, FOXA1 could up-regulate $p27^{Kip1}$ alone to a greater extent than BRCA1 alone, and these two proteins could act in concert upon the $p27^{Kip1}$ promoter and on endogenous p27Kip1 transcription. This appeared to be due to BRCA1 stabilizing the FOXA1 protein, increasing the half-life of this protein. Thus, we hypothesized that BRCA1 and FOXA1 proteins might interact *in vivo* to regulate the expression of $p27^{Kip1}$. Since the effect on protein stability is observed only with a BRCA1 protein having an intact C-terminal BRCT repeat domain, we suggest that the interaction occurs at or near the C-terminus of BRCA1. As a comparison, BARD1 and BRCA1 interact via their N-terminal RING domains, and this interaction and the effect on protein stability is unaffected by C-terminal mutations of BRCA1. Indeed, a study has demonstrated that FOXA1 is decreased in BRCA1-mutated breast cancers (van't Veer *et al*, 2002).

In summary, we have demonstrated that $p27^{Kip1}$ is a target for BRCA1 transcriptional activation and identified FOXA1 as a potential interacting partner for BRCA1. Both can regulate the expression of $p27^{Kip1}$ alone or in concert with BRCA1, and BRCA1 stabilizes FOXA1 protein.

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