Cell Cycle Control of BRCA2

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Abstract

Identifying the conditions and kinetics of the induction of BRCA2 gene expression may implicate roles for the function of the tumor suppressor gene. In this study, expression of BRCA2 mRNA is shown to be regulated by the cell cycle and associated with proliferation in normal and tumor-derived breast epithelial cells. Cells arrested in G0 or early G1 contained low levels of BRCA2 mRNA. After release into a proliferating state, cells produced maximum levels of BRCA2 mRNA in late G1 and the S-phase. Similar cell cycle control of BRCA2 was observed in fractions of exponentially growing cells isolated by centrifugal elutriation. Expression of BRCA2 was shown to be independent of bulk DNA synthesis. In addition, the kinetics of BRCA2 mRNA up-regulation appeared to be similar to those of BRCA1, suggesting that the two genes could be commonly controlled. These results imply that these two tumor suppressor genes are utilized during growth and may have a protective role in cellular proliferation.

Introduction

Approximately 5% of women with breast cancer display a pattern of autosomal dominant transmission for early onset breast carcinomas, indicating an inherited predisposition. Recently, two breast cancer susceptibility genes, BRCA1 and BRCA2, have been identified that appear to be responsible for approximately 45% (1, 2) and 17% (3, 4) of total hereditary breast cancers, respectively. Mutations in BRCA1 and BRCA2 most commonly induce frameshifts that cause protein truncation and presumably loss of protein function (1, 3–6). In addition, tumors from individuals with BRCA1 or BRCA2 mutations display loss of the wild-type allele, suggesting that loss of functional BRCA1 or BRCA2 protein is a causal event for these early onset breast cancers (7, 8). Surprisingly, mutations of BRCA1 or BRCA2 rarely appear in sporadic breast cancer, suggesting that sporadic tumors have different etiologies of mutagenesis than those most frequently found in familial tumors (9, 10). However, it is possible that BRCA1 or BRCA2 are inactivated by means other than mutation in sporadic tumors. This has been suggested in the case of BRCA1 by two studies: in one study, BRCA1 mRNA levels in tumors appeared to be down-regulated relative to normal breast epithelial cells (11), and, in another study, the BRCA1 protein displayed aberrant subcellular localization in tumor cells (12).

Our laboratory has been investigating conditions that control BRCA1 and BRCA2 gene expression in HMECs and tumor cell lines. Identification of the conditions and kinetics for the induction of BRCA1 and BRCA2 gene expression should implicate roles for the function of the genes as well as identify the window of time when potential collaborating proteins may associate with the BRCA1 or BRCA2 gene products. In addition, a fundamental understanding of normal patterns of BRCA1 and BRCA2 expression is crucial to identify potential aberrant patterns of expression in tumors.

While investigating the control of BRCA1 mRNA expression, Guudas et al. (13) as well as investigators in our laboratory found that estrogen induced a dramatic elevation in BRCA1 mRNA levels in certain tumor cell lines. However, the kinetics of induction appeared much slower than the estrogen-responsive gene pS2. Elevated expression of BRCA1 was suggested to be linked to estrogen-mediated proliferation (13). A role for cell proliferation in controlling BRCA1 expression was also suggested by the studies of the developing mouse embryo reported by Marquis et al. (14), which showed that BRCA1 was expressed in a wide array of organ systems undergoing proliferation. Through a variety of cell synchrony regimens, we showed that in both the normal and tumor breast epithelial cells, BRCA1 is induced in a cell cycle-dependent pattern expressing peak mRNA levels prior to entry into the S-phase and maintaining these levels throughout the S-phase (15). Little is known about BRCA2 gene expression except that BRCA2 mRNA has a tissue-specific expression pattern similar to that of the BRCA1 message (6). In this current work, we investigated the control of BRCA2 gene expression and demonstrate cell cycle control of expression similar to BRCA1, with highest levels being induced just prior to entry into the S-phase. We also discuss the potential for coordinate control of the BRCA1 and BRCA2 genes.

Materials and Methods

Cell Culture and Synchrony. Cell culture and synchrony methods were performed as described previously (15). Briefly, normal HMECs, derived from the breast reduction surgery of a 29-year-old woman, were grown on 60-mm Falcon plates in DFCI-1 media seeded at 3 × 10⁵ cells/plate. MCF-7 cells were grown on 60-mm Falcon plates in RPMI 1640 supplemented with 10% FBS seeded at 5 × 10⁵ cells/plate. Normal ovarian epithelial cells, NOSE1, were derived from spontaneously immortalized ovarian epithelial cells from the ovary of a 46-year-old woman. Cells were grown at subconfluent levels in RPMI 1640 and 10% FBS. To produce G0 synchrony, cells were allowed to grow 2 days after plating, after which cells were washed and grown in serum-depleted medium for 24 h. For G0 synchronization, DFCI-3 medium was used (16) for HMECs, and RPMI 1640 was used for MCF-7. G0 cells were stimulated back into the growth cycle by changing cultures into complete medium.

The inactive lactone form of Lovastatin was supplied by Alfred Alberts of Merck, Inc. and activated according to Keyomarsi et al. (17). MCF-7 cell were cultured in complete medium containing 60 μM lovastatin for 36 h and then released into growth phase by washing plates once with complete medium and then culturing cells in complete medium with 500 μM mevalonic acid. In experiments synchronizing cells at the G1-S-phase boundary with mimosine (Sigma), MCF-7 cells were cultured in RPMI 1640 lacking serum for 24 h. Cells were then cultured in media consisting of 200 μM mimosine in RPMI

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2 To whom requests for reprints should be addressed, at Box 3873, Duke University Medical Center, Durham, NC 27710.
3 The abbreviations used are: HMEC, human mammary epithelial cell; BrUrd, 5-bromodeoxyuridine; FBS, fetal bovine serum; NOSE1, normal ovarian surface epithelial cell; PI, propidium iodide.
1640 plus 10% FBS for 16 h. Cells were then washed once in RPMI 1640 and grown in RPMI 1640 plus 10% FBS.

**Flow Cytometry.** Cell cycle profiles were generated to measure cell synchrony by using either PI staining or a combination of PI staining and BrdUrd detection. Cells were fixed in 70% ethanol overnight and then pelleted and washed twice with cold PBS. For PI staining, cells were then resuspended in 5 ml of 50 μg/ml of PI and 0.5 mg/ml of RNase and incubated at 4°C overnight before being read by a Becton Dickinson FACS Star Plus. DNA synthesis measured by BrdUrd detection was performed as described by Lowe et al. (18).

**Centrifugal Elutriation.** NOSE1 cells (1 × 10⁸) were removed from plates by trypsin digestion, washed once in PBS, resuspended in 5 ml of PBS, and incubated at 5 ml/min into an elutriation chamber housed within a Beckman J-6B refrigerated centrifuge retrofitted with a Beckman 3569405-TY-A elutriation rotor with a 5-m1 collection chamber. Countercurrent flow rate was increased at 2-ml/min increments from 5 to 25 ml/min, and 100-ml fractions were collected at each flow rate. Fractions were pelleted, washed in PBS, and divided into two samples for both cell cycle analysis and RNA isolation.

**RNA Isolation and Analysis.** At each time point, cells from a 60-mm plate were lysed in 1 ml of Trizol (Life Technologies, Inc.) for 5 min. Lysates were stored at −100°C. RNA was isolated using methods recommended for the Trizol reagent by Life Technologies, Inc. Seven to 10 μg of total RNA from each sample were loaded onto a 1% formaldehyde-agarose gel, electrophoresed, electroblotted, and probed as described previously (19). Blots were hybridized with cDNA 32P probes of BRCA2, BRCA1, histone H2A, pS2, and c-myc.

Typical exposure times for Kodak AR film with a single intensifying screen were 24 h for BRCA2 and BRCA1, 4 h for c-myc, 4 h for pS2, and 1 h for histone H2A. The following probes were utilized: for BRCA2, a 731-bp fragment from exon 11 spanning nucleotides 4140–4870 (Genbank accession no. U43746); for BRCA1, a 2520-bp fragment spanning nucleotides 1026–3545 (Genbank accession no. U14680) from exon 11; for human histone H2A, a 430-bp HindIII/BamH1 fragment derived from a cDNA clone, a kind gift from W. Bonner; for pS2, an excised 600-bp PstI fragment was derived from a cDNA clone, a kind gift from D. McDonnell; and for c-myc, a 1.8-kb EcoRI fragment was derived from a cDNA clone, a kind gift from J. Nevins.

**Results**

To examine regulation of BRCA2 in normal cells transiting a quiescent state and entering the cell cycle, HMECs derived from a breast reduction mammoplasty were cultured in RPMI 1640 for 36 h. Upon adding growth medium, cultures were harvested between 0 and 24 h for RNA extraction. Sequential probing of the Northern blot shows the kinetics of cell cycle control for mRNA levels of BRCA2, BRCA1, histone H2A, and c-myc (Fig. 1). An ethidium bromide stain of the blot shows the ribosomal RNA levels as loading control.

In contrast, expression of histone H2A mRNA was elevated after release from growth factor deprivation (17). MCF-7 cells were blocked in G₁ by Lovastatin for 36 h and then released from the Lovastatin block by adding fresh medium containing mevalonic acid. To measure the population of cells active in the S-phase after drug treatment, duplicate plates of cells were labeled with BrdUrd and analyzed with flow cytometry after staining with anti-BrdUrd antibody and PI. Cell cycle profiles show that after 5 h of release from drug, 5% of the cells were in the S-phase, and BRCA2 and BRCA1 mRNA expression was low (Fig. 2). At 23 h after release, 21% of the population was in the S-phase and by this time both BRCA2 and BRCA1 mRNA levels were elevated. In addition, throughout the later time points, high levels of BRCA2 and BRCA1 messages were maintained as was a high proportion of the S-phase cells. Levels of mRNA from the estrogen-inducible gene pS2 were at their highest levels during the Lovastatin block and at 5 and 23 h but declined at later time points. Therefore, the estrogen-inducible pS2 gene can be uncoupled from BRCA2 and BRCA1 expression by cell cycle arrest with Lovastatin.

![BRCA2 mRNA expression is up-regulated after release from starvation in normal HMECs producing a pattern of expression similar to that of BRCA1. Cells were cultured in minimal media (DFCI-3) for 36 h. Complete growth medium (DFCI-1) was added at time 0. Total RNA was isolated between 0 and 24 h and electrophoresed on a 1% formaldehyde gel. The gel was electroblotted and probed sequentially with cDNAs for BRCA2, BRCA1, c-myc, and histone H2A. Ethidium bromide stain of the blot shows the ribosomal RNA levels as loading control.](cancerres.aacrjournals.org)
Centrifugal elutriation is a velocity sedimentation method which separates cells into subpopulations based on size. Since cells increase in volume between G1 and G2-M, this technique can allow enrichment of different cell cycle subpopulations (for review, see Ref. 20). Centrifugal elutriation was utilized to demonstrate that a normally cycling population of cells, little BRCA2 and BRCA1 mRNA is present in cells within early G1, and the two genes appear to be up-regulated in cell populations committed to proliferation.

To demonstrate that BRCA2 expression could be uncoupled from active DNA replication, MCF-7 cells were synchronized with the replication inhibitor mimosine. Cells were released from 24 h of serum starvation into complete medium containing 200 μM mimosine. After 16 h in mimosine, a cohort of cells becomes synchronized at the G1-S boundary (21). Cell cycle analysis using PI staining showed that mimosine excluded cells from entering the S-phase (Fig. 4). Both the BRCA2 and BRCA1 mRNAs were highly elevated when replication was effectively blocked by mimosine (Fig. 4). The S-phase content increased from 2% at the mimosine block to 22% in the S-phase at the 5-h time point, yet little increase in BRCA2 or BRCA1 expression was evident in that time interval. However, histone H2A synthesis dramatically increased at the 5-h time point. These results suggest that upon reaching the G1-S boundary, BRCA2 expression is highly elevated and similar to BRCA1 expression but distinct from histone H2A expression, which requires high levels of active DNA replication.

Discussion

In this study, BRCA2 gene expression is shown to be cell cycle controlled and associated with cell proliferation. Both normal HMECs and the breast tumor cell line MCF-7 displayed low levels of BRCA2 mRNA in early G1 and elevated expression as cells approached the G1-S boundary. Release from Lovastatin G1 arrest also induced high BRCA2 mRNA levels in MCF-7 cells but failed to up-regulate mRNA levels for the estrogen-responsive gene pS2. Cell cycle control of BRCA2 message levels in normally growing cells was demonstrated by centrifugal elutriation of NOSE1 cells. These results showed that

Fig. 2. Up-regulation of BRCA2 mRNA after release from Lovastatin is uncoupled from pS2 expression but coupled to proliferation. MCF-7 cells were cultured in 60 μM for 36 h and then released in fresh media containing 500 μM mevalonic acid at time 0. Cells were cultured for the times indicated, and total RNA was prepared. Blots were probed with BRCA1, BRCA2, and pS2 cDNAs. Cell cycle analysis of parallel cells released from Lovastatin gave the following values: 5 h release, G1, 84%; S-phase, 5%; and G2-M, 11%; 23 h release, G1, 71%; S-phase 21%; and G2-M, 8%; 27 h release, G1, 65%; S-phase 24%; and G2-M, 11%; 31 h release, G1, 62%; S-phase, 23%; G2-M, 16%; 35 h release, G1, 70%; S-phase 17%; G2-M, 13%. Percentages were calculated using flow cytometry of BrdUrd incorporation and PI staining of a minimum of 10,000 events.

Centrifugal elutriation is a velocity sedimentation method which separates cells into subpopulations based on size. Since cells increase in volume between G1 and G2-M, this technique can allow enrichment of different cell cycle subpopulations (for review, see Ref. 20). Centrifugal elutriation was utilized to demonstrate that a normally cycling cell population controls expression of BRCA2 in a cell cycle-dependent manner (shown in Fig. 3). Because HMECs are heterogeneous and contain variable cell sizes, these cells could not be reliably subjected to elutriation. Instead, a spontaneously immortalized normal ovarian epithelial cell line (NOSE1) was utilized. Elutriation was performed under conditions that maximize G1 separation. An aliquot of each sample was stained with PI and analyzed for cell cycle content based on DNA content using flow cytometry. Cell cycle profiles demonstrated a modest but definite enrichment of the S-phase cells with increasing fraction numbers (Fig. 3). Northern blot analysis of RNA from NOSE1 fractions showed a very low level of BRCA2 transcript in early G1 (fractions 1 and 2). In fractions 3 and 4, which consisted primarily of larger G1 cells and a greater proportion of the S-phase cells, BRCA2 mRNA levels were elevated. In each of the remaining fractions (fractions 5–8), there was a greater proportion of S-phase and G2 cells, confirming that increasingly larger cells were being isolated by elutriation and that there was a trend toward further elevation in BRCA2 mRNA expression. These fractions were also probed for BRCA1 mRNA, yielding a similar pattern of mRNA expression as was seen for BRCA2 mRNA. These results showed that within a normal cycling population of cells, little BRCA2 and BRCA1 mRNA is present in cells within early G1, and the two genes appear to be up-regulated in cell populations committed to proliferation.

Fig. 3. mRNA expression of BRCA2 and BRCA1 is associated with commitment to proliferation in elutriated populations of the normal ovarian epithelial cell line (NOSE1). Total RNA from each fraction was isolated and electrophoresed. Blots were probed with BRCA1 and BRCA2 cDNAs. 28S ribosomal RNA levels stained with ethidium bromide are presented as a loading control. Cell cycle analysis of fractions gave the following values: fraction 1. G1, 94%; S-phase, 1%; and G2-M, 5%; fraction 2, G1, 94%; S-phase, 2%; and G2-M, 4%; fraction 3, G1, 90%; S-phase, 4%; and G2-M, 6%; fraction 4, G1, 86%; S-phase, 7%; and G2-M, 7%; fraction 5, G1, 81%; S-phase, 10%; and G2-M, 9%; fraction 6, G1, 72%; S-phase, 13%; and G2-M, 15%; fraction 7, G1, 60%; S-phase, 17%; and G2-M, 23%; and fraction 8, G1, 53%; S-phase, 18%; and G2-M, 29%. Percentages were calculated using flow cytometry of PI staining, and a minimum of 1300 cell cycle events were read.

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changes in *BRCA2* expression levels were not artificially induced by cell synthesis. Mimosine $G_{1}$-S synchrony showed that *BRCA2* message levels could be induced during inhibition of DNA replication and that *BRCA2* levels were maintained throughout the S-phase. These results imply that commitment to proliferation is a fundamental positive control of *BRCA2* gene expression. Since the S-phase fraction of different tumors and cell lines can be quite different, these results suggest that cell proliferation status should be considered when measuring and comparing *BRCA2* levels.

Mutations in *BRCA2* cause a different familial disease than do mutations in *BRCA1*. Families carrying *BRCA2* mutations display a higher incidence of male breast cancer and only a modestly increased incidence of ovarian cancer (3, 4, 22); families carrying *BRCA1* mutations rarely have cases of male breast cancers but do have a higher incidence of ovarian cancer (23). Despite these differences in the tumor risks from mutations in these genes, both genes commonly cause early onset female breast cancer, demonstrate similar tissue-specific expression (6), and as such could be involved in a similar pathway of tumor suppression. This supposition is further supported by our results showing that *BRCA2* and *BRCA1* mRNA expression are controlled similarly in the cell cycle. This similarity of gene expression prompted us to compare the promoter regions of the two genes to identify homologous regions that might suggest that *BRCA2* and *BRCA1* are coordinately controlled by the same transcription factors. Two homologies were found with similar spacing near the start sites of gene transcription. The motif ttcgga for *BRCA2* and ttcggg for *BRCA1* were identified as c-ets binding sites using the TFSEARCH program, and these sites are spaced 44 bp from the homologous motif gcgaggg in each gene. This second motif is repeated again in *BRCA1* as agecggg, sharing a full 8 bp homology with the *BRCA2* motif but is not a known transcription factor binding site. The regions in *BRCA1* homologous with *BRCA2*, the c-ets site, and the two gc boxes are completely conserved among mouse, rat, and human DNA. Recently ETS1, a transcription factor associated with cell differentiation and proliferation, has been shown to have tumor suppressor activity when expressed in colon cancer cells that have lost expression of the gene (24). It is possible that transcription factors of the ets family control expression of a class of tumor suppressor genes including *BRCA1* and *BRCA2*.

Our work on the cell cycle expression of both *BRCA1* and *BRCA2* shows that the highest levels of gene expression are normally attained after commitment to the proliferative state and throughout the S-phase. Although proof that *BRCA2* protein levels are similarly controlled by the cell cycle must await the availability of suitable antibodies, we have previously shown that *BRCA1* protein levels are controlled by the cell cycle in a similar manner to mRNA levels (15). If *BRCA2* protein levels are controlled in a similar manner to mRNA levels, the *BRCA2* gene product may be utilized for cell proliferation. A role for the gene as a protective agent in the proliferation process should be explored. Considering that both *BRCA1* and *BRCA2* are expressed just before and throughout the S-phase, examining whether these genes have a role in protecting the fidelity of DNA replication would be of interest.

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References


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