

# Loss of B-Cell Translocation Gene-2 in Estrogen Receptor–Positive Breast Carcinoma Is Associated with Tumor Grade and Overexpression of Cyclin D1 Protein

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## Abstract

**The B-cell translocation gene-2 (BTG2) is present in the nuclei of epithelial cells in many tissues, including the mammary gland where its expression is regulated during glandular proliferation and differentiation in pregnancy. In immortalized mammary epithelial cells and breast cancer cells, BTG2 protein localized predominantly to the nucleus and cytoplasm, respectively. The highly conserved domains (BTG boxes A, B, and C) were required for regulating localization, suppression of cyclin D1 and growth inhibitory function of BTG2. Expression analysis of BTG2 protein in human breast carcinoma ( $n = 148$ ) revealed the loss of nuclear expression in 46% of tumors, whereas it was readily detectable in the nuclei of adjacent normal glands. Loss of nuclear BTG2 expression in estrogen receptor- $\alpha$  (ER $\alpha$ )-positive breast tumors correlated significantly with increased histologic grade and tumor size. Consistent with its ability to suppress cyclin D1 transcription, loss of nuclear BTG2 expression in ER-positive breast carcinomas showed a significant correlation with cyclin D1 protein overexpression, suggesting that loss of BTG2 may be a factor involved in deregulating cyclin D1 expression in human breast cancer. (Cancer Res 2006; 66(14): 7075-82)**

## Introduction

The B-cell translocation gene-2 (BTG2) belongs to a class of proteins known as the Tob and BTG antiproliferative protein family, which is defined by the presence of two highly conserved domains known as BTG boxes A and B separated by a spacer sequence of 20 to 25 nonconserved amino acids. A short stretch of amino acids designated box C is present only in BTG1 and BTG2 and is located between positions 118 and 128 (1–5). BTG2 can be induced by p53 and is a key effector of p53-dependent growth arrest of mouse and human fibroblasts transduced with oncogenic ras (6, 7). BTG2 expression is also regulated by activators of nuclear factor- $\kappa$ B, such as tumor necrosis factor- $\alpha$  and the Mullerian inhibiting substance, a member of the transforming growth factor- $\beta$  superfamily (7, 8).

BTG2 is expressed in the epithelium of many tissues (9), including the mammary gland where its expression declines sharply during gestation. Estrogen and progesterin, which regulate proliferation and

differentiation in the mammary gland during pregnancy, suppress BTG2 mRNA. BTG2 mRNA and protein were not detectable in the mammary gland during lactation, but expression recovered rapidly when lactation ceased (8). Analysis of human breast carcinoma showed loss of nuclear BTG2 expression and faint cytoplasmic staining in tumors (8). Similarly, in high-grade prostatic intra-epithelial neoplasia, BTG2 was limited to weak cytoplasmic staining, suggesting that aberrant expression and localization of BTG2 protein may contribute to disease progression (10). These results prompted us to examine the relevance of the conserved domains of BTG2 in localization and function.

BTG2 expression strongly inhibits growth by impairing G<sub>1</sub> to S progression in retinoblastoma (Rb)-positive and Rb-negative cells (11, 12). In Rb-negative cells, growth inhibition by BTG2 involves suppression of cyclin E and cyclin-dependent kinase 4 (CDK4) expression and a decrease in cyclin E-associated CDK activity, as measured by histone H1 phosphorylation assay (11). In Rb-positive cells, hypophosphorylation of Rb by BTG2 is achieved by lowering the kinase activity of the CDK4/complexes, which occurs predominantly by repression of cyclin D1 expression by BTG2 (12).

Consistent with its ability to suppress cyclin D1 (6, 8, 12), decline in BTG2 expression in the mammary epithelium during gestation was associated with an increase in cyclin D1. Similarly, suppression of BTG2 mRNA in estrogen- and progesterone-treated breast cancer cells correlated with increased cyclin D1 levels (8). Cyclin D1 protein is overexpressed in 50% of mammary carcinomas (13, 14), and overexpression of cyclin D1 in mammary glands results in abnormal mammary cell proliferation, including the development of mammary adenocarcinomas (15), suggesting that increased levels of cyclin D1 may contribute to neoplastic transformation of the breast. Amplification of 11q13, the locus on which the *cyclin D1* gene is localized, is seen in 13% to 15% of breast carcinomas. However, the mechanisms that govern excessive cyclin D1 mRNA and protein expression observed in ~45% to 50% of primary breast cancers (13, 14, 16–19) remain unknown. Although experimental data support that BTG2 is a negative regulator of cyclin D1 expression (6, 8, 12), clinical evidence to support this concept is yet to be established.

In this study, we have characterized the intracellular localization of wild-type and mutant BTG2 proteins in immortalized human mammary epithelial and breast cancer cells and studied their growth inhibitory properties and regulation of cyclin D1. We evaluated the expression of BTG2 in a large cohort of human breast carcinomas of various histologic types and its relationship to clinicopathologic variables and cyclin D1 overexpression. Our results show that loss of nuclear BTG2 expression in estrogen receptor- $\alpha$  (ER $\alpha$ )-positive tumors correlates with increasing tumor

**Note:** H. Kawakubo and E. Brachtel contributed equally to this work.

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doi:10.1158/0008-5472.CAN-06-0379

grade, tumor size, and overexpression of cyclin D1, suggesting that loss of BTG2 may be a factor involved in deregulating cyclin D1 expression in human breast cancer.

## Materials and Methods

**Cell lines.** MCF7 cells were grown in DMEM supplemented with 10% female fetal bovine serum, glutamine, and penicillin/streptomycin. MCF10A cells (American Type Culture Collection, Rockville, MD) were grown in mammary epithelial growth medium (Clonetics, San Diego, CA) supplemented with 100 ng/mL cholera toxin (Calbiochem, San Diego, CA).

**Generation of BTG2 mutants.** BTG2 constructs lacking boxes A, B, and C were generated by PCR amplification of fragments flanking the NH<sub>2</sub> terminus and COOH terminus of the deletions such that ligation of the two fragments would fuse in frame to generate proteins lacking the intended amino acid sequences with no other change in sequence.

The primers used to generate the NH<sub>2</sub>-terminal fragment flanking box A were forward, CCCC(GAATTC)CGCGACATGAGCCACGGGAAG and reverse, CCCC(AGTACT)GAAGACCTTAAGCCTCTGCTC. The sequences within parentheses in the forward and reverse primers represent *EcoRI* and *ScaI* restriction sites, respectively. The primers used to generate the COOH-terminal fragment flanking box A were forward, CCCC(AATATT)CGCATCAACCACAAGATGGAC and reverse, CCCC(GTCGAC)GCTGGAGACTGCCATCAC. The sequences within parentheses in the forward and reverse primers represent *SspI* and *SalI* restriction sites, respectively.

The forward and reverse primers used to amplify the NH<sub>2</sub> and COOH termini of BTG2 box A were used to amplify the NH<sub>2</sub>-terminal and COOH-terminal fragments flanking the deletion of boxes B and C. The other primers used to generate BTG2 boxes B and C are described below.

The reverse primer used to generate the NH<sub>2</sub>-terminal fragment flanking box B was CCCC(AGTACT)GGGCAGCAGCTGGTGCAGCTG. The sequence within parenthesis represents a *ScaI* restriction site. The forward primer used to generate the COOH-terminal fragment flanking box B was CCCC(AATATT)GGGGAGGACGGCTCCATCTGC. The sequence within parenthesis represents a *SspI* restriction site.

The reverse primer used to generate the NH<sub>2</sub>-terminal fragment flanking box C was CCCC(GATATC)CTCCCAATGCGGTAGGACAC. The sequence within parenthesis represents an *EcoRV* restriction site. The forward primer used to generate the COOH-terminal fragment flanking box C was CCCC(AGGCCT)CTGGCCGCTCTGTGGG. The sequence within parenthesis represents a *StuI* restriction site.

The amplified fragments were gel purified and ligated in frame into a cytomegalovirus (CMV) expression vector containing a hemagglutinin (HA) Tag. The constructs were sequenced to ensure that they did not contain additional deletions or mutations.

**Antibodies and Western blot analysis.** MCF7 cells were transfected with wild-type or mutant BTG2 using Fugene-6. Proteins were extracted with radioimmunoprecipitation assay buffer and analyzed by Western blot as described (20). The E-cadherin, mouse monoclonal anti-HA, and anti-human cyclin D1 antibodies were from Zymed (South San Francisco, CA), Covance (Berkeley, CA), and Novocastra (Newcastle, United Kingdom), respectively. The mouse anti-c-myc and the rabbit anti-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

To analyze nuclear localization, wild-type and mutant BTG2 expression constructs were transfected into MCF7 cells. After 48 hours, cells were harvested in cold PBS, resuspended in 200  $\mu$ L TKM [10:10:1; 10 mmol/L Tris (pH 8), 10 mmol/L KCl, and 1 mmol/L MgCl<sub>2</sub>], and lysed with 0.1% Triton X-100. Nuclei were pelleted by centrifugation at 5,000 rpm at 4°C, and proteins were extracted in buffer containing 10 mmol/L HEPES (pH 7), 420 mmol/L NaCl, and 1 mmol/L EDTA. Nuclear and cytoplasmic fractions representing an equal number of cells were analyzed by Western blot to assess nuclear localization of BTG2 proteins.

**Immunofluorescence technique.** The cells were plated on coverslips 24 hours before transfection, and the pCMV vector and wild-type or mutant BTG2 expression constructs were transfected using Fugene-6 or Cellfectin. After 48 hours, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 3 minutes. The

permeabilized cells were treated with 3% bovine serum albumin (fraction V from Fisher Scientific, Fairlawn, NJ) in PBS for 30 minutes and incubated with mouse anti-HA antibodies at a dilution of 1:1,000 for 1 hour at room temperature. After extensively washing with PBS, cells were incubated with FITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch Lab, Inc., West Grove, PA) followed by staining with 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR).

**Growth inhibition assays.** To determine the ability of wild-type and mutant BTG2 to inhibit cellular proliferation, MCF7 cells were transfected with empty pCDNA vector and wild-type or mutant BTG2 expression constructs along with a hygromycin resistance plasmid. Cells were grown in hygromycin-containing medium for 2 to 3 weeks and stained with crystal violet, and drug-resistant colonies were counted.

**Patients and tissue samples.** One hundred forty-eight cases of human breast carcinoma were selected from the files of the Massachusetts General Hospital (MGH) Pathology Department according to protocols approved by the Human Research Committee at MGH. The samples consisted of formalin-fixed, paraffin-embedded tissue from 135 invasive ductal carcinomas and 13 invasive lobular or mixed ductal-lobular carcinomas from 110 excisions and 38 mastectomies. Tissues were obtained after all diagnostic tests were completed and included tumor and uninvolved breast parenchyma. Histologic diagnosis and grading of tumors were made in accordance with the WHO classification criteria (21). Tumor size, lymph node involvement, and distant metastases were recorded following the American Joint Committee on Cancer staging system (22). Case-matched H&E stains were reviewed for each patient. Clinical history and follow-up relevant to mammary pathology were obtained by review of the clinical notes.

Patient age ranged from 31 to 94 years with a median of 57 years. The status of hormone receptor proteins (ER $\alpha$  and progesterone receptor) was determined by immunohistochemistry. Tumor size ranged from 0.5 to 10 cm with a median of 2.3 cm. There were 79 patients who had no lymph node metastasis (N<sub>0</sub>); 37 showed metastatic carcinoma in one to three ipsilateral lymph nodes (N<sub>1</sub>); 10 patients showed metastatic carcinoma in four to nine lymph nodes (N<sub>2</sub>); 6 patients showed metastatic carcinoma in  $\geq 10$  lymph nodes (N<sub>3</sub>); and nodal status was unknown (N<sub>x</sub>) in 16 patients. There was no metastatic disease at presentation in 118 patients (M<sub>0</sub>); 11 patients showed metastatic disease, and metastatic status was unknown for 19 patients (M<sub>x</sub>). Follow-up was available for up to 93 months with a median of 53 months. On follow-up, five patients had died (three of metastatic disease and two of other causes); 24 were alive with metastatic disease; 2 were alive with metastatic other tumors; and 111 were alive with no evident disease.

**Immunohistochemical analyses.** Immunohistochemistry was done on formalin-fixed, paraffin-embedded 5- $\mu$ m tissue sections according to standard procedures as described (8). Briefly, deparaffinized tissue sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase. After antigen retrieval, sections were incubated with 2.5% normal goat serum to block nonspecific protein binding. For BTG2 detection, sections were incubated with rabbit antibodies against human BTG2 at a final antibody concentration of 1  $\mu$ g/mL diluted in blocking serum solution. For cyclin D1 staining, the antibody was used at a dilution of 5  $\mu$ L/mL. After incubation with biotinylated link antibody and peroxidase-labeled streptavidin, staining was developed by reaction with diaminobenzidine or Nova Red. Detection chemistries were purchased from Vector Laboratories (Burlingame, CA) and used according to manufacturer's instructions. The sections were lightly counterstained with hematoxylin and mounted.

The stains for ER $\alpha$  protein had been done during the initial pathologic evaluation for diagnostic purposes. For quantification of immunohistochemistry, slides were evaluated semiquantitatively for staining intensity and extent as described in ref. (23), and the predominant staining intensity of BTG2 and cyclin D1 was compared with adjacent uninvolved breast tissue on the same slide. Membranous Her2/c-erbB protein expression was assessed during the initial pathologic evaluation for diagnostic purposes as previously described (24).

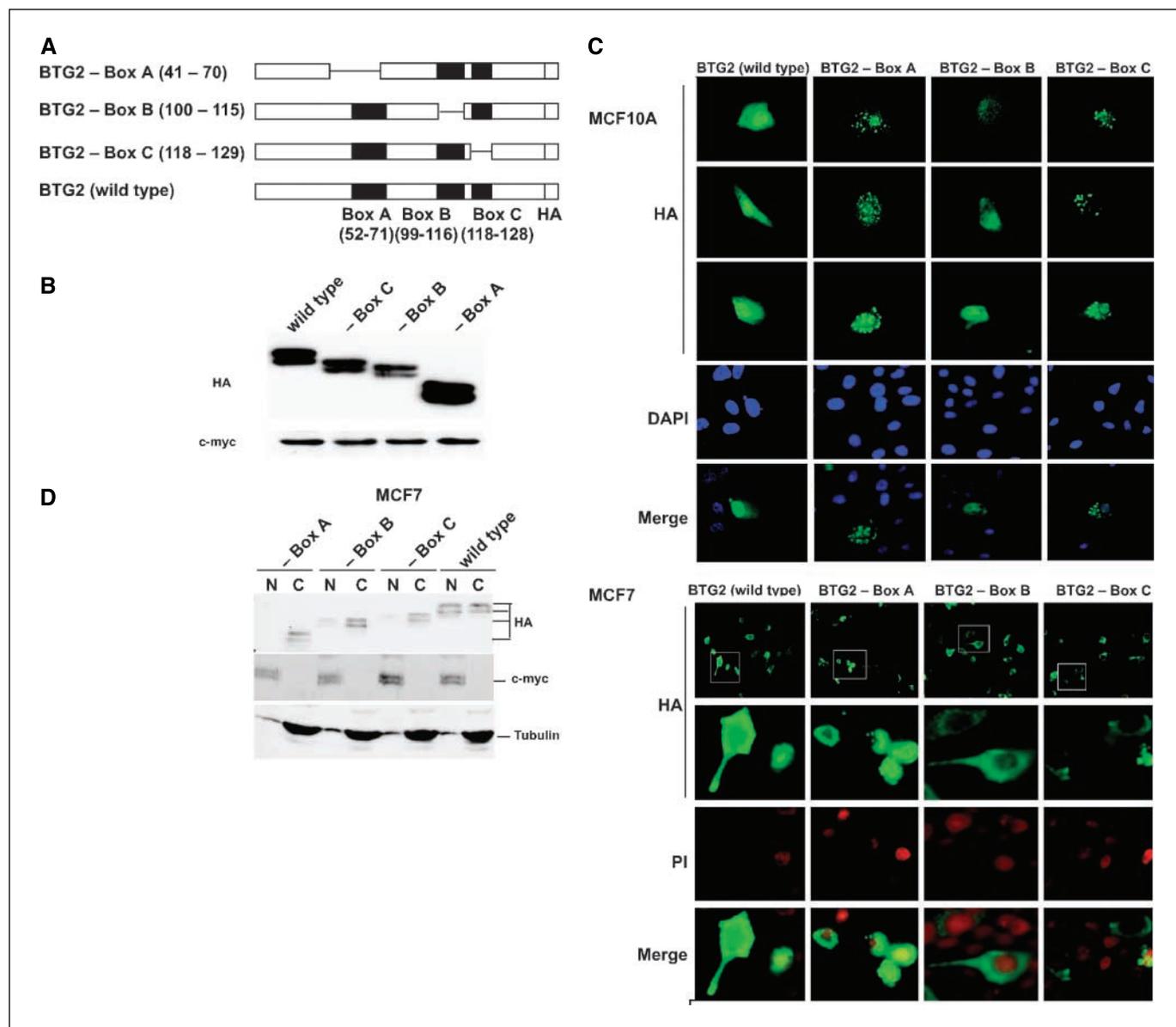
**Statistical analysis.**  $\chi^2$  test was done to assess the significance of the association between BTG2 expression and other tumor characteristics

(grade, tumor size of <2 or >2 cm, Her2 and cyclin D1 expression, nodal status, and metastasis). The analysis was repeated in the subgroups classified by ER $\alpha$  status. *P*s < 0.05 were considered statistically significant.

## Results

**Conserved domains of BTG2 regulate nuclear localization, repression of cyclin D1, and growth inhibitory function.** We had previously shown that BTG2 nuclear localization is impaired in breast carcinoma (8). BTG2 mutants lacking the conserved boxes A, B, and C were generated to characterize their role in directing

nuclear localization and function of the protein. A schematic diagram representing the three mutants, which lack amino acids 41 to 70 (BTG2 box A), amino acids 100 to 115 (BTG2 box B), and 118 to 129 (BTG2 Box C), is shown (Fig. 1A). The constructs were transfected into MCF7 cells, and protein expression was analyzed by Western blot using an anti-HA antibody (Fig. 1B). The synthesis of two proteins occurs due to the presence of an internal translation initiation site located nine codons downstream of the initiator codon. Both ATG codons are flanked by an identical 5' sequence (CGACATG). The expression of wild-type BTG2, BTG2



**Figure 1.** Conserved domains of BTG2 regulate nuclear localization. *A*, schematic representation of BTG2 deletion mutants. *B*, the constructs shown above were transfected into MCF7 cells. Protein expression was analyzed by Western blot using the anti-HA antibody. The blot was probed with an anti-myc antibody to control for loading in cells. *C*, *top*, MCF10A cells were transfected with 2  $\mu$ g of wild-type BTG2, BTG2 box A, BTG2 box B, and BTG2 box C expression constructs. BTG2 protein expression in cells was analyzed by indirect immunofluorescence using an anti-HA antibody. Three fields, each containing a single cell expressing the wild-type or mutant BTG2 protein (*top three*). 4',6-Diamidino-2-phenylindole (DAPI) staining of nuclei and super imposition of the images in a single field (*bottom*). *Bottom*, MCF7 cells were transfected with 2  $\mu$ g of wild-type BTG2, BTG2 box A, BTG2 box B, and BTG2 box C expression plasmids. BTG2 protein expression in cells was analyzed by indirect immunofluorescence using an anti-HA antibody. Higher magnification of insets, propidium iodide (PI) staining of nuclei, and super imposition of the two images (*bottom three*). *D*, MCF7 cells were transfected with wild-type and mutant BTG2 expression constructs. After 48 hours, nuclear and cytoplasmic protein fractions were analyzed by Western blot using the anti-HA antibody. The purity of the fractions was examined by probing the blots with anti-c-myc and anti-tubulin antibodies. *C*, cytoplasmic fraction; *N*, nuclear fraction.

box B, and BTG2 box C proteins was comparable, whereas in repeated experiments, the expression of BTG2 box A was slightly higher compared with wild-type BTG2 (Fig. 1B) due to increased stability (data not shown).

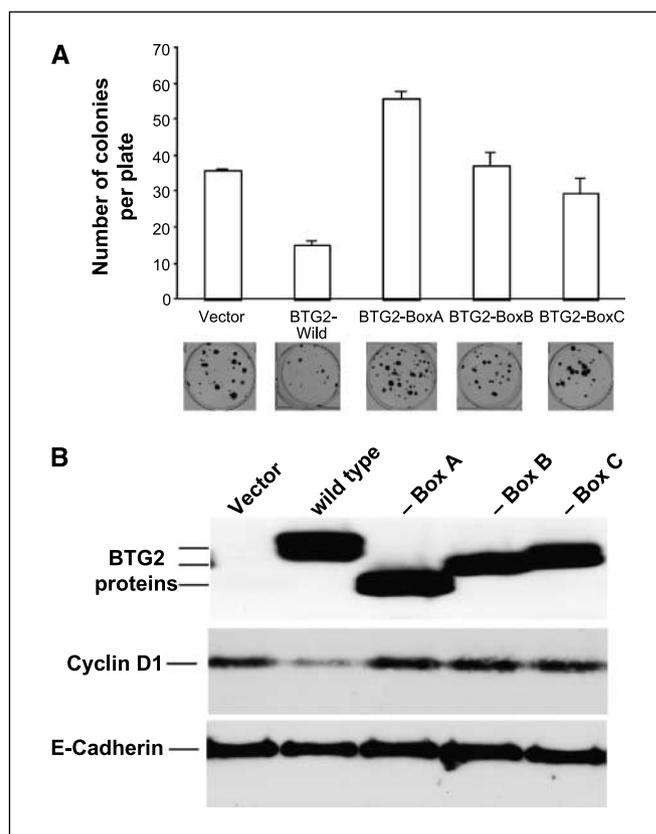
To characterize subcellular localization, the wild-type and mutant BTG2 constructs were transfected into immortalized human mammary epithelial cell line MCF10A and the human breast cancer cell line MCF7, and cells were immunostained with the anti-HA antibody. Although in both cell types, the wild-type protein partitioned between the nucleus and cytoplasm, expression was predominantly nuclear in MCF10A cells and cytoplasmic in MCF7 cells. Deletion of the conserved domains A, B, and C led to increased retention of the protein in the cytoplasm with the absence of boxes A and C, resulting in aggregation in the cytoplasm (Fig. 1C). These observations were confirmed by Western blot of nuclear and cytoplasmic protein fractions derived from wild-type and mutant BTG2-transfected MCF7 cells; removal of the conserved boxes A, B, and C impaired nuclear localization of BTG2 (Fig. 1D). Similar results were obtained upon expression of BTG2 proteins in the Er-negative MDA-MB-468 cells (data not shown).

We then tested the effect of these proteins on growth. MCF7 cells were transfected with BTG2, BTG2 box A, BTG2 box B, and BTG2 box C expression constructs along with a hygromycin resistance plasmid, and the number of drug-resistant colonies on the plates was compared. As reported previously, wild-type BTG2 suppressed colony growth, whereas the lack of box A not only abrogated the growth inhibitory effect of BTG2 but slightly enhanced growth compared with that observed in vector-transfected cultures. Moreover, boxes B and C were also required for the growth inhibitory effect of BTG2 (Fig. 2A).

To determine whether the effects of wild-type and mutant BTG2 on growth corresponded with their ability to inhibit cyclin D1, the expression of which has been shown to be suppressed by BTG2 (6, 12), proteins extracted from MCF7 cells transfected with BTG2 constructs were analyzed by Western blot. Wild-type BTG2 expression correlated with suppression of cyclin D1 protein compared with that observed in untransfected cells. The expression of mutant BTG2 proteins did not result in decreased cyclin D1 levels. These results suggest that the ability of BTG2 to block growth corresponds to its ability to suppress cyclin D1 (Fig. 2B).

**Loss of nuclear BTG2 expression correlates with increasing tumor grade.** Characterization of BTG2 expression in breast carcinoma samples showed that BTG2 protein was present in the nuclei of uninvolved glands and absent in the tumor in 15 of 23 cases examined (8). To characterize whether aberrant BTG2 expression in breast tumors correlated with tumor characteristics, we examined 148 breast carcinomas for which clinicopathologic data and follow-up were available (Table 1).

In agreement with previously reported results (8), BTG2 protein localized to the nuclei of epithelial cells lining the normal ducts and lobules but was absent in 46% of the tumors. BTG2 immunostaining of tumor and adjacent normal tissue and the corresponding H&E stains (Fig. 3A) of a representative case of grade 3 breast carcinoma are shown. Although 44% of ER $\alpha$ -positive breast tumors and 50% of ER $\alpha$ -negative breast tumors showed loss of nuclear BTG2 expression, loss of BTG2 did not correlate significantly with ER $\alpha$  status of the tumor. The percentage of tumors showing loss of BTG2 protein significantly increased with the tumor grade; 19% of grade 1, 45% of grade 2, and 54% of grade 3 tumors showed the loss of nuclear BTG2 protein ( $P = 0.033$ ). In ER $\alpha$ -positive patients, the correlation between loss of nuclear



**Figure 2.** Conserved domains of BTG2 are required for suppression of cyclin D1 and growth. **A**, MCF7 cells grown in six-well plates were transfected with 2  $\mu$ g of wild-type or mutant BTG2 expression constructs along with 0.2  $\mu$ g of hygromycin resistance plasmid. Cells were grown in medium containing 100  $\mu$ g/mL of hygromycin, and colonies were stained with crystal violet. Number of colonies on each plate was counted ( $n = 3$ ). Representative culture plate for each sample (bottom). **B**, wild-type and mutant BTG2 constructs shown were transfected into MCF7 cells. Protein expression was analyzed by Western blot using anti-HA and cyclin D1 antibodies. The blot was probed with an E-cadherin antibody to control for loading.

BTG2 expression and the histologic grade of the tumor was more pronounced; loss of expression was observed in 19% of grade 1, 39% of grade 2, and 69% of grade 3 tumors ( $P = 0.0033$ ). Moreover, decreased BTG2 expression in tumors associated with increasing tumor size ( $P = 0.020$ ). No correlation existed between the absence of nuclear BTG2 expression and tumor grade in ER $\alpha$ -negative tumors.

Loss of nuclear BTG2 expression in breast carcinoma did not show a statistically significant correlation with nodal status, metastasis to other organs, or survival ( $P > 0.05$ ). However, the association between loss of BTG2 and overexpression of Her2 in ER $\alpha$ -positive tumors almost approached statistical significance ( $P = 0.051$ ). The loss of nuclear BTG2 expression in a tumor overexpressing Her2, nuclear BTG2 expression in adjacent normal glands, and H&E stains of the tumor and uninvolved tissue are shown (Fig. 3B).

**Loss of BTG2 expression in ER-positive breast tumors correlates with increase in cyclin D1 protein.** Because BTG2 suppresses cyclin D1 expression (12), and because its growth inhibitory function seems to be associated with its ability to suppress cyclin D1 (Fig. 2), we analyzed whether the loss of nuclear BTG2 expression in breast tumors would be related to overexpression of cyclin D1. Cyclin D1 immunostaining in breast tumors was nuclear with faint cytoplasmic staining. Staining

usually was homogenous, and specimens were considered as overexpressing cyclin D1 if they showed moderate to strong nuclear staining compared with that observed in the adjacent matched normal glands. Cyclin D1 protein was overexpressed in 46% of breast tumors compared with that seen in uninvolved matched normal breast tissue. A representative case in which cyclin D1 is overexpressed in the tumor compared with adjacent normal glands along with H&E stains (Fig. 4A) is shown. As reported previously (25–29), overexpression of cyclin D1 was associated with hormone receptor positivity: 48% of ER $\alpha$ -positive tumors and 26% of ER $\alpha$ -negative tumors overexpressed of cyclin D1.

Characterization of BTG2 and cyclin D1 protein expression in breast carcinoma showed an inverse correlation (Fig. 4A and B). In a tumor overexpressing cyclin D1, nuclear expression of BTG2 was not detected, whereas in the matched normal glands, BTG2 expression was nuclear, and cyclin D1 protein expression was low (Fig. 4A). In another tumor where BTG2 expression was nuclear, cyclin D1 expression was almost undetectable, whereas the normal glands showed nuclear BTG2 expression (Fig. 4B).

Cyclin D1 was overexpressed in 39% of tumors in which BTG2 expression was detected and in 53% of tumors in which nuclear BTG2 expression was absent ( $P = 0.097$ ). However, in ER $\alpha$ -positive tumors, a statistically significant inverse correlation was observed by between overexpression of cyclin D1 and loss of nuclear BTG2 expression; 68% of samples, which had no nuclear BTG2 protein, overexpressed cyclin D1, whereas only 43% of those that retained BTG2 expression overexpressed cyclin D1 ( $P = 0.018$ ). No significant correlation between loss of BTG2 and increased cyclin D1 expression was observed in ER $\alpha$ -negative breast cancer.

## Discussion

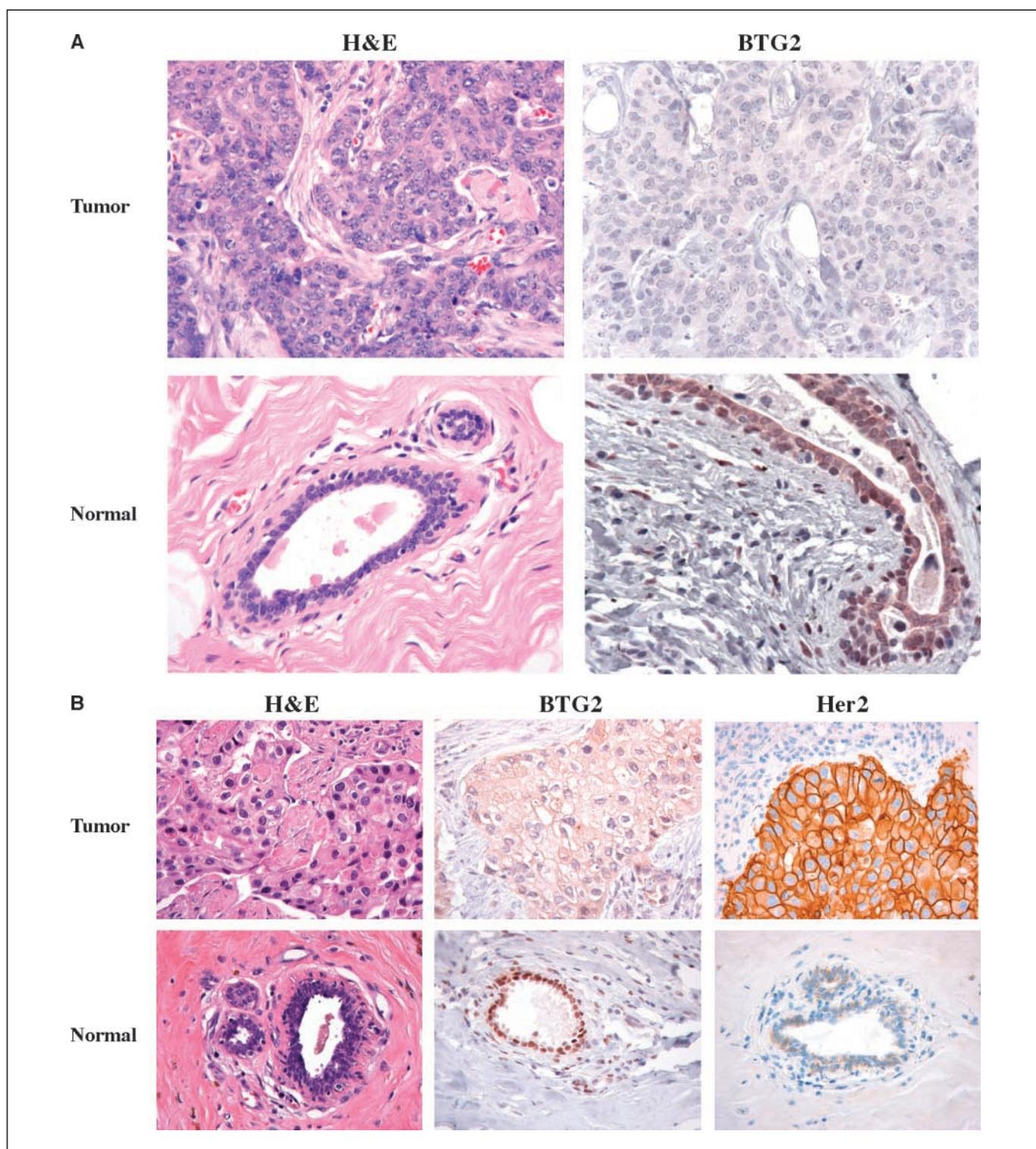
The expression patterns of BTG2 in the normal breast and breast cancer suggest that BTG2 may be a regulator of growth and differentiation in the mammary gland. Immunolocalization studies revealed that although BTG2 protein partitions between the nucleus and cytoplasm in cells, nuclear expression predominates in immortalized human mammary epithelial cells, whereas cytoplasmic expression is more prominent in breast cancer cells, suggesting that breast cancers may be expressing components that facilitate cytoplasmic retention and/or inhibit nuclear localization of BTG2 protein. Deletion of the conserved domains (boxes A, B, and C) of BTG2, which mediate the interaction of BTG2 with other cellular proteins (3, 5, 30), decreased nuclear localization and increased cytoplasmic retention.

The eukaryotic linear motif resource for predicting functional sites in proteins and “prediction and analysis of nuclear localization signals” did not identify any sequence within BTG2 that shared homology with previously described nuclear localization signals. However, Rodier et al., in a study to identify domains involved in cellular trafficking of BTG1, a BTG family member highly homologous to BTG2, reported several BTG1 domains to be involved in nuclear localization. The highly conserved B box of BTG1 induced significant nuclear localization of  $\beta$ -galactosidase; it was enhanced by the presence of the COOH-terminal moiety. Moreover, the NH<sub>2</sub>-terminal LxxLL motif of BTG1, which mediates interaction with members of the nuclear receptor superfamily, also favored nuclear localization (31). The domains of BTG2 required for directing nuclear localization are consistent with these observations; the conserved boxes B and C of BTG1 and

**Table 1.** BTG2 and cyclin D1 expression in relation to clinicopathologic variables

	Total cases, no. cases (%)	BTG2 T < U, no. cases (%), <i>n</i> = 64 of 139 (46)	BTG2 T = U, no. cases (%), <i>n</i> = 75 of 139 (54)	Cyclin D1 T > U, no. cases (%) <i>n</i> = 60 of 131 (46)
Patient age, y ( <i>n</i> = 148)				
≥50	96 (65)	40 (63)	44 (59)	37 (62)
<50	52 (35)	24 (37)	31 (41)	23 (38)
ER ( <i>n</i> = 146)				
ER positive	99 (68)	40 (63)	51 (69)	48 (80)
ER negative	47 (32)	23 (37)	23 (31)	12 (20)
Her2 staining ( <i>n</i> = 77)				
Strong overexpression	9 (12)	6 (17)	3 (8)	5 (16)
Histologic grade ( <i>n</i> = 148)				
G <sub>1</sub>	18 (12)	3 (5)	13 (18)	4 (7)
G <sub>2</sub>	59 (40)	25 (39)	31 (41)	27 (45)
G <sub>3</sub>	71 (48)	36 (56)	31 (41)	29 (48)
Tumor size, cm ( <i>n</i> = 145)				
≤2 (T <sub>1</sub> )	84 (58)	34 (53)	47 (64)	37 (62)
>2 (T <sub>2</sub> , T <sub>3</sub> , T <sub>4</sub> )	61 (42)	30 (47)	26 (36)	23 (38)
Lymph nodes ( <i>n</i> = 132)				
N <sub>0</sub> /N <sub>1</sub>	116 (88)	46 (82)	64 (93)	46 (88)
N <sub>2</sub> /N <sub>3</sub>	16 (12)	10 (18)	5 (7)	6 (12)
Follow-up findings ( <i>n</i> = 142)				
No disease	111 (78)	48 (79)	58 (79)	28 (76)
Alive with disease	24 (17)	12 (20)	10 (14)	8 (22)
Deceased of disease	3 (2)	1 (1)	1 (1)	0 (0)
Other conditions	4 (3)	0 (0)	4 (6)	1 (2)

Abbreviations: T, tumor; U, uninvolved glandular tissue.



**Figure 3.** Nuclear expression of BTG2 is absent in breast carcinoma (images in original magnification,  $\times 400$ ). *A*, tumor cells lack nuclear BTG2 protein, whereas the adjacent normal glands strongly express BTG2 in the nucleus. *B*, tumor cells lack nuclear BTG2 and show strong membranous Her2/C-erbB staining. BTG2 expression is nuclear in matched normal glands negative for Her2/C-erbB staining.

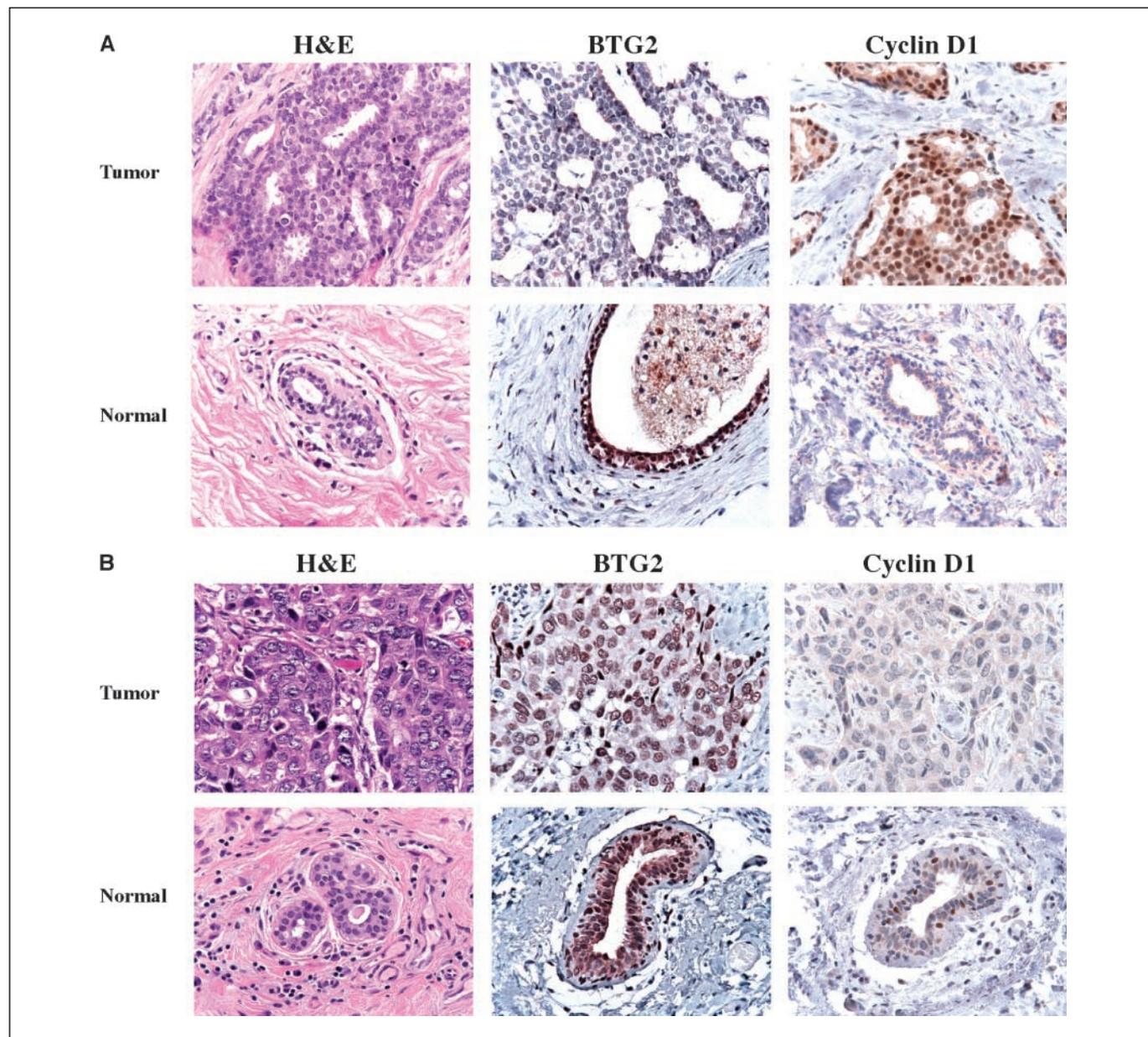
BTG2 share 100% and 80% homology, respectively. Moreover, BTG2 also contains an L(SS)LL motif between positions 19 and 23.

Although nuclear localization of BTG2 is impaired in 46% of breast tumors, and although BTG2 mRNA is very low or undetectable in almost all breast cancer cell lines tested (8), analysis of 12 breast

cancer cell lines and 18 primary breast tumors revealed the lack of mutations or polymorphisms in BTG2 (7). Whether loss of important protein(s) that regulate BTG2 nuclear localization and altered posttranslational modification of BTG2 are involved in aberrant localization of BTG2 in breast cancer cells needs to be determined.

Moreover, epigenetic mechanisms may also play a critical role in regulating the levels and localization of BTG2 in breast cancer cells. BTG2 is a potential target of cellular microRNAs (miRNAs), a class of 17- to 24-base single-stranded RNA molecules, which associate with a cellular complex that participates in RNA interference to regulate the stability and translational efficiency of the target mRNA (32–34). Of the several members of the miRNA family that are likely regulators of BTG2 expression in cells (32–34), miR-21 is suppressed in normal mammary epithelial cells but overexpressed in breast cancer cells (35). Whether overexpression of miRNAs that target posttranscriptional processing of BTG2 and/or protein-protein interaction with cellular partners leads to aberrant regulation of BTG2 expression in breast carcinoma remains to be elucidated.

Consistent with the reports that BTG2 suppresses cyclin D1 (12), and down-regulation of BTG2 in human cells being linked to up-regulation of cyclin D1 (6), loss of BTG2 expression in ER $\alpha$ -positive breast tumors correlated with overexpression of cyclin D1 protein. Estrogen- and progesterone-mediated suppression of BTG2 in breast cancer cells correlated with increased cyclin D1 levels (8) and could provide a possible explanation for the statistically significant association between loss of BTG2 and overexpression of cyclin D1 in ER $\alpha$ -positive breast tumors. Moreover, the spatial pattern of BTG2 and cyclin D1 expression (BTG2 is predominantly expressed in the mammary epithelium, and overexpression of cyclin D1 is observed in a majority of breast tumors of luminal epithelial origin) also suggests that the loss of BTG2 and gain of



**Figure 4.** BTG2 and cyclin D1 protein expression shows an inverse correlation in breast carcinoma (images in original magnification,  $\times 400$ ). *A*, a representative case of breast carcinoma shows loss of nuclear BTG2 expression, whereas strong nuclear BTG2 expression is seen in the adjacent normal glands. The tumor overexpresses the cyclin D1 protein with adjacent normal glands showing weak cyclin D1 staining. *B*, another representative case of breast carcinoma in which BTG2 expression is nuclear in the tumor cells, which are negative for cyclin D1. Nuclear BTG2 expression and scattered nuclear staining with the cyclin D1 antibody is observed in the adjacent, uninvolved glands.

cyclin D1 in breast tumors are likely to be related events. This is further supported by our data, which show that BTG2 expression in MCF7 cells suppresses cyclin D1 protein levels.

Many factors, including estrogens (36) and the peptidyl-prolyl *cis/trans*-isomerase-1 (Pin-1) and AIB-1, both of which are reported to be amplified and overexpressed in breast cancer, positively regulate cyclin D1 expression (37–41) and may contribute to the overexpression of cyclin D1 mRNA and protein in breast cancer. In fact, Pin-1 has been shown to interact with Tis21, the mouse homologue of BTG2 (42). Whether BTG2, due to its ability to suppress cyclin D1, can functionally interact with AIB1 and Pin-1 proteins needs further investigation.

In addition to their inverse correlation in tissue expression, BTG2 and cyclin D1 are functionally antagonistic. Cyclin D1 is a positive regulator of cell cycle progression, whereas BTG2 induces a G<sub>1</sub> arrest in cells. In breast cancer cells, estrogen suppresses BTG2 mRNA, and BTG2 in turn mitigates estrogen-induced transactivation of a reporter construct driven by an estrogen response

element (8, 30). Cyclin D1 expression is induced by estrogen and coactivates ER-mediated transcription (39). Whether coordinated inhibition of BTG2 and enhancement of cyclin D1 could constitute a mechanism to increase estrogen responsiveness and proliferation in breast cancer cells and thus serve as a molecular signature to identify patients who would develop resistance to endocrine therapy is an important question that needs to be addressed in the future.

## Acknowledgments

Received 1/31/2006; revised 5/9/2006; accepted 5/17/2006.

**Grant support:** NIH grant CA84441 (P.D. Walden), Department of Defense grant PC030351 (P.D. Walden), and NIH/National Cancer Institute grant CA89138 (S. Maheswaran).

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We thank Drs. Emmett Schmidt, Toshi Shioda, and Dennis Sgroi for critically reading this article.

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