

Ubiquitination and Downregulation of BRCA1 by Ubiquitin-Conjugating Enzyme E2T Overexpression in Human Breast Cancer Cells

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Abstract

Breast cancer is generated through a multistep genetic and epigenetic process including activations of oncogenes and inactivations of tumor suppressor genes. Here, we report a critical role of ubiquitin-conjugating enzyme E2T (UBE2T), an E2 ubiquitin-conjugating enzyme, in mammary carcinogenesis. Immunocytochemical staining and *in vitro* binding assay revealed that UBE2T interacted and colocalized with the BRCA1/BRCA1-associated RING domain protein (BARD1) complex. Knocking down of UBE2T expression with small interfering RNA drastically suppressed the growth of breast cancer cells. Interestingly, *in vivo* ubiquitination assay indicated BRCA1 to be polyubiquitinated by incubation with wild-type UBE2T protein, but not with C86A-UBE2T protein, an E2 activity–dead mutant, in which the 86th residue of cysteine was replaced with alanine. Furthermore, knocking down of UBE2T protein induced upregulation of BRCA1 protein in breast cancer cells, whereas its overexpression caused the decrease of the BRCA1 protein. Our data imply a critical role of UBE2T in development and/or progression of breast cancer through the interaction with and the regulation of the BRCA1/BARD1 complex. [Cancer Res 2009;69(22):8752–60]

Introduction

Breast cancer is the most common cancer among women worldwide; 1.15 million new cases and 410,000 deaths due to breast cancer in 2002 (1). Incidence of breast cancer is increasing in most countries and the increasing rate is much higher in countries where its incidence was previously low (1). Like other types of cancer, a multistep genetic and epigenetic process including activations of oncogenes and inactivations of tumor suppressor genes is known in etiology of this disease (2). Particularly, the link between the *BRCA1/BRCA2* tumor-suppressor genes and hereditary breast cancer is well established (3, 4), but the role of these genes in nonfamilial cancers remains un-

clear. Therefore, further characterization of the pathophysiologic roles of BRCA-related pathways is very critical for better understanding of breast carcinogenesis and for development of better management to breast cancer patients.

Gene expression profiles obtained by microarray analysis have been proven to provide detailed characterization of individual cancers and such information should contribute to choose more appropriate clinical strategies to individual patients (5). Through the genome-wide gene expression profile analysis of 81 breast cancers using a cDNA microarray consisting of 23,040 genes (6), we have been identifying genes that function as oncogenes in the process of development and/or progression of breast cancer (7–11). We here describe identification of ubiquitin-conjugating enzyme E2T (UBE2T) as a novel therapeutic target for breast cancer. *UBE2T* was reported to be one of the genes whose expression was upregulated in human fibroblasts with serum stimulation (12). Moreover, UBE2T protein was shown to bind FANCL, the Fanconi anaemia core complex, and to be necessary for the efficient DNA damage–induced monoubiquitination of FANCD2, one of Fanconi anaemia core complex subunits (13, 14). However, any roles of UBE2T in breast carcinogenesis have not been implicated. In this study, we report characterization of UBE2T that was highly overexpressed in the great majority of breast cancer cells.

Materials and Methods

Cell lines and clinical materials. HCC1937, MCF-7, MDA-MB-231, MDA-MB-435S, SK-BR-3, T47D, YMB-1, BSY-1, BT-20, ZR-75-1, BT549, HeLa, HEK293T, and COS7 cell lines were obtained from American Type Culture Collection (ATCC). No abnormalities were observed on the cellular morphology of these cell lines both at low and high densities of cultures by microscopy according to the guideline from ATCC (15). HBC4 and HBC5 were kind gifts from Dr. Takao Yamori (Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan) with Materials transfer agreement. We monitored the cell morphology of these cell lines by microscopy, and confirmed to maintain their morphologic images in comparison with the original morphologic images from the above affiliation. Human mammary epithelial cells and MCF10A were purchased from Cambrex BioScience, and were used in this study for the first time after purchase. All cells were cultured according to previous (7, 11). No *Mycoplasma* contamination was detected in cultures of all of these cell lines by using Mycoplasma Detection kit (Roche). Tissue samples from surgically resected breast cancers and their corresponding clinical information were obtained from Department of Breast Surgery, Cancer Institute Hospital, Tokyo, Japan and Division of Breast and Endocrine Surgery, Department of Surgery, St Marianna University School of Medicine, Kanagawa, Japan after obtaining

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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written informed consent. This study as well as the use of all clinical materials described above was approved by individual institutional Ethical Committees.

Semiquantitative reverse transcription-PCR analysis. Extraction of total RNA and subsequent cDNA synthesis were performed as described previously (6). The PCR primer sequences were 5'-CAAATATTAGGTG-GAGCCAACAC-3' and 5'-TAGATCACCTTGCCAAAGAACAC-3' for *UBE2T*; 5'-CCTGTGTTACAAAGGCAGA-3' and 5'-CGTGGTATGATCTTGGCTCA-3' for *BRCA1*; 5'-CGACCACTTGTCAAGCTCA-3' and 5'-GGTTGAGCA-CAGGGTACTTTATT-3' for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a quantitative internal control.

Northern blot analysis. Northern blot membrane for breast cancer cell lines was prepared as described previously (7). The breast cancer blot and human multiple tissue blots (Takara Clontech) were hybridized with [α^{32} P]-dCTP-labeled PCR products of *UBE2T* (541 bp) prepared by reverse transcription-PCR (RT-PCR) using a primer set as described in semiquantitative RT-PCR section or with [α^{32} P]-dCTP-labeled β -actin, respectively. Prehybridization, hybridization, and washing were performed as described previously (16). The blots were autoradiographed with intensifying screens at -80°C for 14 d.

Construction of expression vectors. To construct *UBE2T* expression vectors, the entire coding sequence of *UBE2T* cDNA was amplified by PCR using KOD-Plus DNA polymerase (Toyobo) with two primers, *UBE2T* forward (5'-CCGGAATTCATGCAGAGAGCTTCAGTCTG-3') and *UBE2T* reverse (5'-CCGCTCGAGAACATCAGGATGAAATTTCTTTTC-3'; the italics indicate restriction enzyme sites). The PCR products were inserted into the *EcoRI* and *XhoI* sites of pCAGGSnHA expression vector in frame with COOH-terminal HA-tag (pCAGGSn-*UBE2T*-HA). The construct used to express enzyme-dead mutant in which the 86th cysteine was substituted to alanine (C86A) was generated with the Quick-Change II XL Site-Directed Mutagenesis kit (Stratagene) using the following primers: 5'-CTGCTGGAAGGATTGCTCTGGATGTT-3', 5'-AA-CATCCAGAGCAATCTTCCAGCAG-3' [the italics indicate the sequence differ from that of wild-type (WT)]. The expression plasmids for full-length *BRCA1* (4 \times Flag-*BRCA1*) and *BRCA1*/*BRCA1*-associated RING domain protein (*BARD1*; 3 \times Myc-*BARD1*) are kind gifts from Dr. Richard Baer (Institute for Cancer Genetics, Columbia University Medical Center, New York, NY; ref. 17). The HA-tagged ubiquitin expression plasmid is a kind gift from Dr. Kohei Miyazono (Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; ref. 18).

Generation of anti-*UBE2T*-specific polyclonal antibody. Plasmid designed to express a fragment of *UBE2T* (codons 128–197) with His-tag at its COOH terminus was prepared using pET21a (+) vector (Merck). The recombinant *UBE2T* peptide was expressed in *Escherichia coli*, BL21 Codon-Plus (Stratagene), and purified using Ni-NTA resin agarose according to the supplier's protocol (Qiagen). The purified recombinant *UBE2T* peptide was inoculated into rabbits (Medical and Biological Laboratories). The immune sera were subsequently purified on antigen affinity columns using Affigel 10 gel, according to supplier's instructions (Bio-Rad).

Western blot analysis. Cells were lysed with lysis buffer [50mmol/L Tris-HCl (pH 8.0), 0.1% SDS, 150 mmol/L NaCl, 0.5% deoxycholate, 1% NP40, 1 mmol/L Na_3VO_4 , and 50 mmol/L NaF] including 0.1% protease inhibitor cocktail III (Calbiochem). The amount of total protein was estimated by a protein assay kit (Bio-Rad), and then proteins were mixed with SDS sample buffer and boiled before loading at 7.5% to 12% polyacrylamide gel. After electrophoresis, the proteins were blotted onto polyvinylidene difluoride membrane (GE Healthcare), and blocked with 4% BlockAce solution (Dainippon Pharmaceutical). The membranes were incubated with anti-*BRCA1* (C-20; Santa Cruz), anti-*BARD1* (H-300; Santa Cruz), or anti-*UBE2T* polyclonal antibodies for detection of endogenous *BRCA1*, *BARD1*, or *UBE2T* proteins. The exogenously expressed proteins were detected using anti-Flag (Sigma Aldrich) or anti-Myc polyclonal antibodies (A-14, Santa Cruz), or an anti-HA monoclonal antibody (3F10, Roche). As a nuclear fraction-specific marker, proliferating cell nuclear antigen (PCNA) protein was detected with

anti-PCNA monoclonal antibody (PC-10, Santa Cruz). Finally, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h and protein bands were visualized by enhanced chemiluminescence detection reagents (GE Healthcare).

Immunohistochemical staining analysis. Slides of paraffin-embedded breast cancer tissues and other normal human tissues (lung, heart, liver, and kidney; Biochain) were processed for antigen retrieval by the antigen retrieval solution pH9 (DAKO Cytomation), and treated with peroxidase blocking reagent (DAKO Cytomation) and protein blocking reagent (DAKO Cytomation). Tissue sections were incubated with anti-*UBE2T* polyclonal antibody as a primary antibody (1:300 dilution) followed by HRP-conjugated secondary antibody (DAKO Cytomation). Specific immunostaining was visualized with peroxidase substrate (3, 3'-diaminobenzidine tetrahydrochloride; DAKO liquid DAB chromogen, Dako Cytomation). Finally, tissue specimens were stained with hematoxylin to discriminate nucleus from cytoplasm.

Immunocytochemical staining and imaging analysis. T47D cells were seeded at the density of 5×10^4 cells in a 35-mm dish with a Col-I-coated glass (Iwaki). To examine expression of endogenous *UBE2T* and *BRCA1* proteins, T47D cells were fixed with PBS (-) containing 4% paraformaldehyde for 15 min, and rendered with a hypotonic lysis solution at 4°C for 15 min, as described previously (19). Subsequently, the cells were covered with 3% BSA in PBS (-) for 1 h at room temperature to block nonspecific hybridization, followed by incubation with affinity-purified rabbit anti-*UBE2T* antibody and mouse anti-*BRCA1* antibody (Ab-1; Calbiochem) at 1:500 and 1:50 dilutions, respectively. After washing with PBS (-), the cells were stained by an Alexa488 and Alexa594-conjugated anti-rabbit and anti-mouse secondary antibodies (Molecular Probe). To detect the HA-tagged *UBE2T* protein, the cells were incubated with anti anti-HA (Roche) antibody at 1:1,500 and Alexa488 conjugated anti-rat secondary antibody (Molecular Probe). Nuclei were counter-stained with 4', 6'-diamidino-2'-phenylindole dihydrochloride (DAPI). Fluorescent images and nuclear intensity of *BRCA1* were obtained under a TCS SP2 AOBs microscope (Leica). The nuclear signal intensities of *BRCA1* were measured from *UBE2T*-expressing cells (WT and C86A) as well as nontransfected cells.

Extraction of nuclear protein. Cells were harvested and washed twice with cold PBS (-) followed by resuspending with a cytoplasmic lysis buffer [20 mmol/L HEPES (pH7.6), 20% glycerol, 10 mmol/L NaCl, 1.5 mmol/L MgCl_2 , 0.2 mmol/L EDTA, 1 mmol/L DTT, 0.1% NP40, 0.1% protease inhibitor cocktail III] on ice for 10 min. After centrifugation at 2,000 rpm for 5 min at 4°C , the nuclei pellets were separated from the supernatant, and incubated with a nuclear lysis buffer (the cytoplasmic lysis buffer including 500 mmol/L of NaCl) on ice for 30 min. The supernatant containing nuclear proteins were collected by centrifugation at 14,000 rpm for 15 min at 4°C .

Gene-silencing effect by small interfering RNA. We had established a vector-based RNA interference expression system using psiU6BX3.0 siRNA expression vector as described previously (20). The target sequence of synthetic oligonucleotides for siRNA (sense) were as follows; si#1, 5'-CCTCCTCAGATCCGATTTC-3'; si#2, 5'-TGCCAGACAGTGGACAGAG-3'; and siEGFP, 5'-GAAGCAGCAGCACTTCTTC-3'. DNA sequences of all constructs were confirmed by DNA sequencing. Human breast cancer cell lines, T47D or BT-20, were plated onto 10-cm dishes (2×10^6 or 1×10^6 cells/dish) and transfected with 8 μg each of psiU6BX3.0-EGFP (siEGFP) and psiU6BX3.0-*UBE2T* (si#1 and si#2) using FuGENE6 reagent (Roche). Forty-eight hours after transfection, cells were reseeded for RT-PCR (1×10^6 or 5×10^5 cells/10-cm dish), Western blot (1×10^6 or 5×10^5 cells/10-cm dish), colony formation assay (1×10^6 or 5×10^5 cells/10-cm dish), and MTT assay (2×10^5 or 1×10^5 cells per well). We selected the psiU6BX3.0-introduced T47D or BT-20 cells, with medium containing 0.7 or 0.6 mg/mL of neomycin (geneticin, Invitrogen). We changed culture medium twice a week. To evaluate an effect of siRNAs by semiquantitative RT-PCR, total RNAs were extracted from the transfected cells after 7-d incubation with geneticin (Invitrogen). The specific primer sets for semiquantitative RT-PCR were used as described in semiquantitative RT-PCR analysis section.

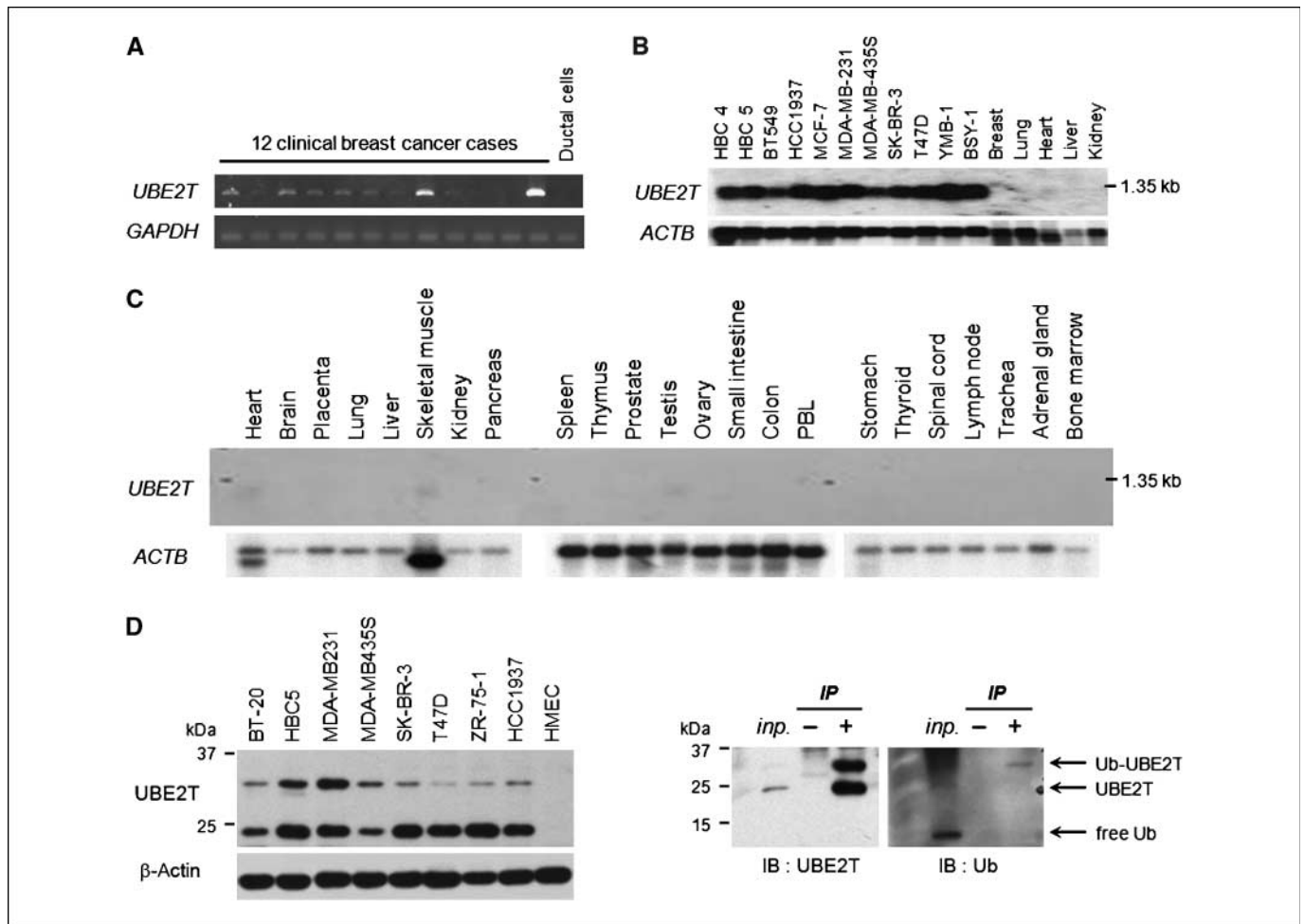


Figure 1. Expression profiles of UBE2T. *A*, semiquantitative RT-PCR results of *UBE2T* in microdissected tumor cells from 12 breast cancer tissues and normal human tissues (Ductal cells, normal breast ductal cells). *B* and *C*, Northern blot analysis of the *UBE2T* in 11 breast cancer cell lines (*B*) and in 23 normal human tissues (*C*). *ACTB* served as a loading control. *D*, expression of endogenous UBE2T protein in eight breast cancer cell lines examined by Western blot analysis (left). From T47D cells, the endogenous UBE2T proteins were immunoprecipitated (IP) and immunoblotted (IB) by anti-ubiquitin (Ub) monoclonal antibody (right).

T47D or BT-20 cells expressing siRNA were grown for 28 d in selective media containing 0.7 or 0.6 mg/mL of neomycin, and then fixed with 100% methanol for 10 min at room temperature before staining with Giemsa's solution (Merck) to assess colony formation. To quantify cell viability, MTT assay was performed using the cell counting kit-8 at 7 d after transfection according to manufacturer's recommendation (Wako). Absorbance at 570 nm wavelength was measured with a Microplate Reader 550 (Bio-Rad). These experiments were performed in triplicate.

We also used siRNA oligonucleotides (Sigma Aldrich Japan) to achieve the high transfection efficiency for further verification of the knockdown effects of *UBE2T*. The sequences targeting each gene were as follows: siUBE2T, 5'-AGAGAUGCUGAUAUAUCUA-3'; siUBE2T-2, 5'-GAAGGCCA-GUCAGCUAGTA-3'; and siEGFP (control), 5'-GCAGCAGACUUCU-CAAG-3'. HEK293T, T47D, and BT-20 cells (2.5×10^5 cells in 6-cm dish for Western blotting analysis) were transfected with either of the siRNAs using Lipofectamin RNAiMAX in OptiMem medium (Invitrogen) according to the instructions of the manufacturer. Forty-eight hours after the transfection, we examined the knockdown effect by Western blot and semiquantitative RT-PCR analyses.

Generation of UBE2T recombinant protein. The entire coding sequence of *UBE2T* was subcloned into the pGEX-6P-1 vector (GE Healthcare). The glutathione *S*-transferase (GST)-UBE2T recombinant protein was expressed in *E. coli* strain BL21 codon-plus RIL competent cells (Stratagene). Purification of the recombinant proteins was performed us-

ing Glutathione Sepharose 4B beads (GE Healthcare) under the native condition according to the supplier's instructions.

In vitro binding assay. HEK293T cells (3×10^6 cells in a 15-cm dish) were transfected with plasmid constructs encoding Flag-tagged BRCA1 and Myc-tagged BARD1. After treatment with 10 μ mol/L of MG132 for 12 h, the cell lysates were immunoprecipitated using anti-Flag monoclonal antibody (M2; Sigma-Aldrich) and rec-Protein G Sepharose 4B (Invitrogen), as described previously (21). Finally, the protein complex of BRCA1/BARD1 was isolated by incubation with 300 μ L of elution buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1 mol/L DTT] including 3% of Flag peptide (Sigma Aldrich) at 4°C for overnight. The eluted BRCA1/BARD1 complex was incubated with 8 nmol of the GST-fused UBE2T proteins ($\sim 400 \mu$ g) at 4°C for 3 h. After washing by 0.5% NP40 buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.05% Triton X-100] thrice, the beads were boiled in 30 μ L of Laemmli sample buffer for 5 min.

In vivo ubiquitination of BRCA1. HEK293T cells were transiently co-transfected with four plasmid constructs, each encoding Flag-tagged BRCA1, Myc-tagged BARD1, HA-tagged UBE2T, or HA-tagged ubiquitin. After treatment with 10 μ mol/L of MG132 for 12 h, the cell lysates were immunoprecipitated using anti-Flag monoclonal antibody to isolate exogenously expressed BRCA1 protein, as described above. After SDS-PAGE, the membrane was incubated with anti-HA (Roche) or anti-ubiquitin (P4D1; Santa Cruz) monoclonal antibodies.

Statistical analysis. Statistical significance was calculated by Student's *t* test, using Statview 5.0 software (SAS Institute). A difference of $P < 0.05$ was considered to be statistically significant.

Results

Upregulation of UBE2T in breast cancers. We verified the elevated *UBE2T* expression in 7 of 12 microdissected breast cancer cases compared with normal ductal cells by semiquantitative RT-PCR analysis (Fig. 1A). Subsequent Northern blot analysis using a *UBE2T* cDNA fragment as a probe detected an ~1.3-kb *UBE2T* transcript at a very high level in all of 11 breast cancer cell lines examined, whereas its expression was hardly detectable in the normal breast, lung, heart, liver, and kidney (Fig. 1B). Additionally, multiple tissue Northern blot analysis revealed the hardly detectable level of *UBE2T* expression in any of normal human tissues examined except skeletal muscle and testis with a very low level of expression (Fig. 1C), indicating a minimum risk of adverse reactions by targeting this molecule. We subsequently developed a polyclonal antibody against residues 128-197 of UBE2T, a segment that bears no homology to other human proteins (see Materials and Methods). Western blot analysis using this antibody detected ~32 kDa and 25 kDa of the endogenous UBE2T proteins in all of eight breast cancer cell lines examined, but its expression was hardly detectable in human mammalian epithelial cells (Fig. 1D, left). To clarify a possible modification of the UBE2T protein, we performed an immunoprecipitation experiment using anti-UBE2T antibody, and subsequent Western blot analyses revealed that the upper band of ~32 kDa was a monoubiquitin conjugated form of UBE2T protein (Fig. 1D, right). Furthermore, immunohistochemical staining with anti-UBE2T antibody detected the protein in the nucleus of three different histologic subtypes of breast cancer, papillotubular, solid-tubular, and scirrhous carcinomas. On the other hand, no

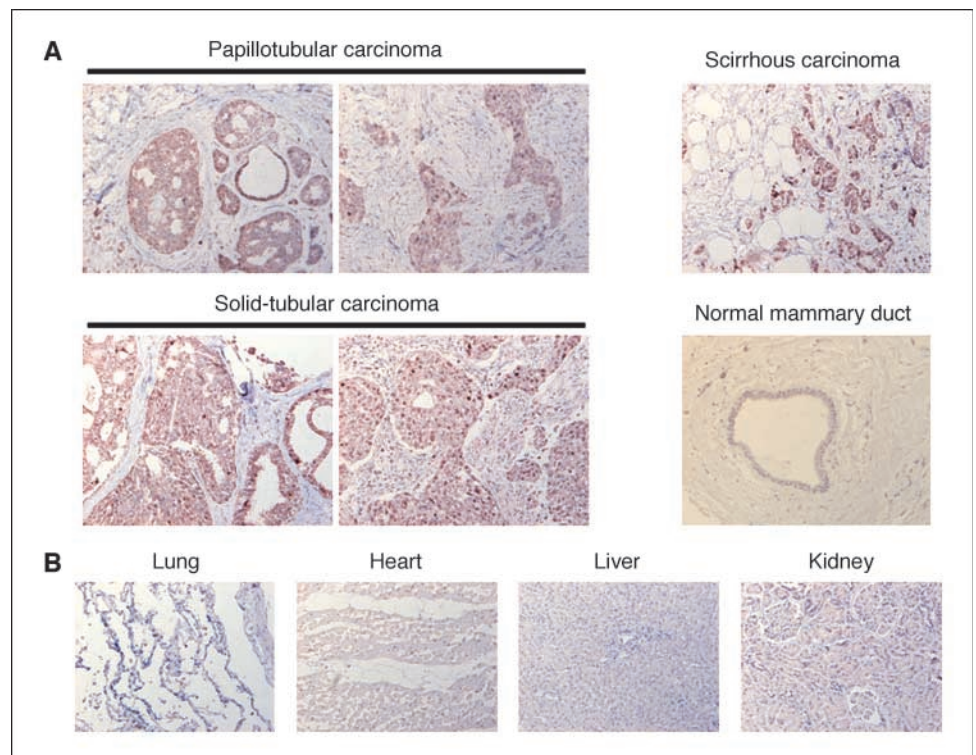
staining of UBE2T was observed in any of normal mammary ductal cells (Fig. 2A) or in any of lung, heart, liver, or kidney tissues in concordance with the results of Northern blot analyses (Fig. 2B).

Knockdown effects of UBE2T on breast cancer cell growth.

To assess whether UBE2T is essential for growth or survival of breast cancer cells, we knocked down the expression of the endogenous UBE2T in breast cancer cell lines, T47D and BT-20 that abundantly expressed UBE2T, by means of the mammalian vector-based RNA interference (RNAi) technique. Semiquantitative RT-PCR and Western blot analyses showed that introduction of UBE2T-specific siRNA (si#2) significantly reduced its expression at both transcriptional and protein levels, compared with psiU6BX-EGFP (siEGFP) construct as a control, whereas another one (si#1) achieved a moderate knockdown effect (Fig. 3A and C). As concordant to the knockdown effects, colony formation and MTT assays (Fig. 3B and D) revealed that treatment with si#2 significantly suppressed growth of T47D and BT-20 cells (MTT assay; $P < 0.0001$, unpaired *t* test); si#1 revealed a moderate effect on the growth suppression. Taken together, these findings suggested that *UBE2T* was likely to play an important role of breast cancer cell growth.

UBE2T interacts with an E3 ubiquitin ligase, BRCA1. To further understand the biological function of UBE2T as an E2-ubiquitin conjugating enzyme, we attempted to find a potential E3 ubiquitin ligase interacting with UBE2T. Because BRCA1, an E3 ubiquitin ligase, is known to attribute to mammary carcinogenesis (21–23), we examined a possible interaction of UBE2T with BRCA1 by *in vitro* binding assay. We cotransfected Flag-tagged full-length BRCA1 and Myc-tagged BARD1 constructs into HEK293T cells, and the eluted protein complex was incubated with GST-tagged recombinant UBE2T protein (GST-UBE2T) *in vitro*. As shown in Fig. 4A, the BRCA1/BARD1 complex was pulled down with GST-UBE2T. Furthermore, we showed that the endogenous

Figure 2. Immunohistochemical staining analyses. A, representative images of immunohistochemical staining of UBE2T in breast cancer and normal tissue sections. B, immunohistochemical staining results of UBE2T in four vital organs: lung, heart, liver, and kidney. Endogenous UBE2T protein was stained by anti-UBE2T polyclonal antibody (original magnification, $\times 100$).



BRCA1 protein was colocalized with endogenous UBE2T protein in the nucleus of T47D cells (Fig. 4B). BRCA1 protein was clearly observed in the nucleus under the treatment with MG132 (Fig. 4B). These findings suggest a possibility that UBE2T might interact with the BRCA1/BARD1 complex in breast cancer cells and cause the degradation of BRCA1 through a proteasome-dependent proteolysis pathway.

It has been reported that the BRCA1/BARD1 complex catalyzes the polyubiquitination of nucleophosmin/B23 protein (24) and monoubiquitination of estrogen receptor (25). However, the degradation of BRCA1 protein by its autopolyubiquitination has not yet been elucidated. Therefore, we hypothesized that BRCA1 in the BRCA1/BARD1 complex might be polyubiquitinated through the interaction with UBE2T protein. To examine whether UBE2T enhances ubiquitination of BRCA1 *in vivo*, we cotransfected plasmids encoding full-length of Flag-tagged BRCA1, Myc-tagged BARD1, HA-tagged UBE2T, and HA-tagged ubiquitin proteins into HEK293T cells. After immunoprecipitation with anti-Flag antibody, the polyubiquitination of BRCA1/BARD1 complex was assessed by immunoblotting with anti-HA or anti-ubiquitin antibodies. Consequently, we found that the BRCA1 protein was ubiquitinated under the presence of UBE2T, but BARD1 was not (Fig. 5A). Additionally, a plasmid encoding UBE2T mutant (UBE2T-C86A), which was reported to lack E2 ubiquitin-conjugating enzyme activity (13), was also transfected and compared with WT of UBE2T (Fig. 5B). The results showed that the UBE2T-C86A abolished ubiquitination of BRCA1 protein, implying the crucial role of UBE2T as an E2 enzyme for polyubiquitination of BRCA1 protein (Fig. 5C).

UBE2T downregulates BRCA1 in breast cancer cells. Although downregulation of BRCA1 protein in sporadic breast cancers

was known to be caused by its genetic mutation, epigenetic silencing, or haploinsufficiency causes (26, 27), other mechanisms to cause it are also suspected. Therefore, we assumed that BRCA1 protein might be downregulated through its polyubiquitination under the overexpression of UBE2T in breast cancer cells. Hence, we first examined the expression levels of endogenous BRCA1 and UBE2T proteins in various kinds of cell line, and found that a human mammary epithelial cell line (MCF10A) had high expression of the BRCA1 protein in the absence of UBE2T (Fig. 6A). On the other hand, the BRCA1 protein was hardly detectable in the breast cancer cells, BT20 and T47D, which had high expression of UBE2T protein (Fig. 6A). Then, we examined the expression level of BRCA1 protein when the UBE2T expression was knocked down by siRNA. Upregulation of BRCA1 protein, but not the BRCA1 transcript (Supplementary Fig. S1), was clearly observed by knocking down of UBE2T protein in breast cancer cells, T47D and BT-20, as well as HEK293T cells (Fig. 6B). These results were reproducible when we used another siRNA duplex against UBE2T transcript (Supplementary Fig. S2). Additionally, we transfected HA-tagged WT-UBE2T or C86A-UBE2T constructs into MCF10A cells to assess their effect on the amount of the endogenous BRCA1 protein. Forty-eight hours after the transfection with either of the HA-UBE2T plasmids, we analyzed by immunocytochemistry using anti-HA and anti-BRCA1 antibodies, and found that introduction of HA-WT-UBE2T protein diminished the nuclear staining of BRCA1 (*yellow arrows*), whereas HA-UBE2T-C86A mutant did not (*white arrows*; Fig. 6C). These findings suggested that overexpression of UBE2T protein resulted in the downregulation of BRCA1 protein. Additional imaging analysis revealed that the nuclear intensities of BRCA1 protein was significantly decreased in the

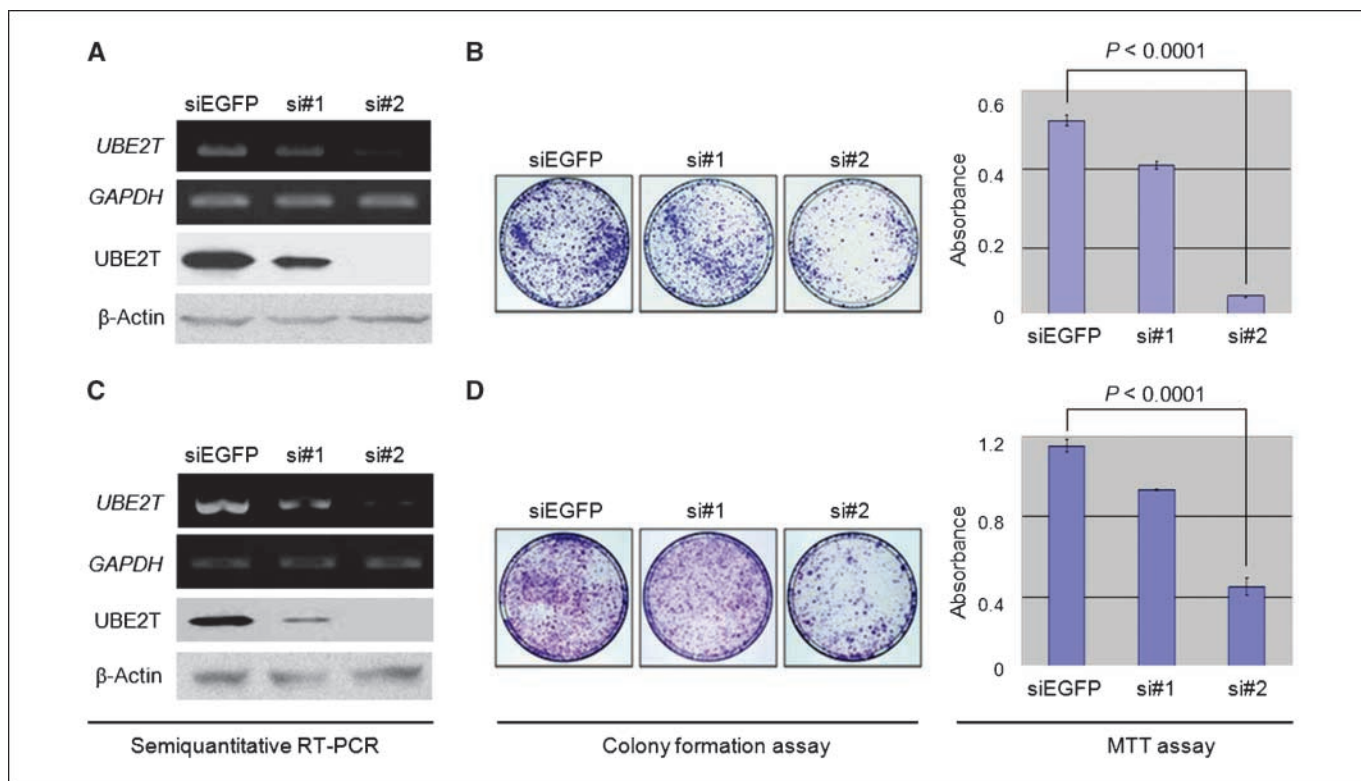
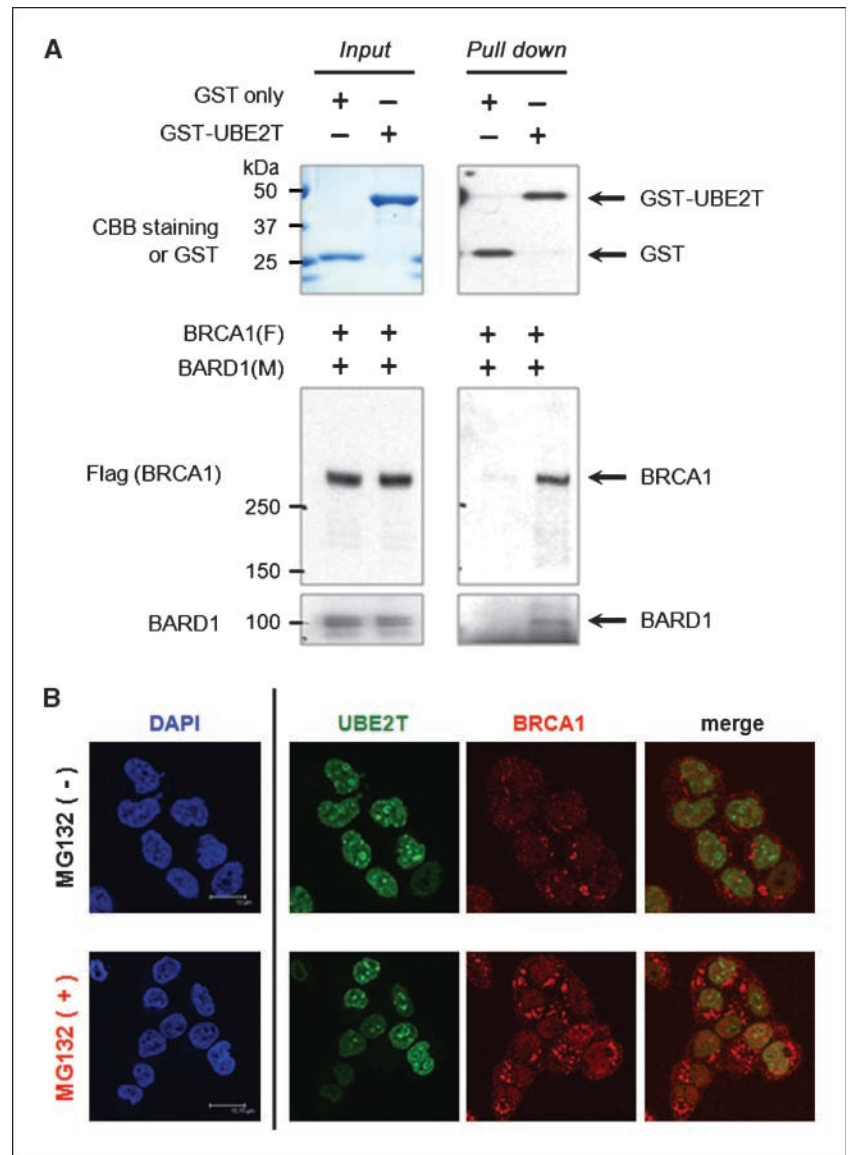


Figure 3. Growth-inhibitory effects of siRNA-targeting UBE2T in breast cancer cells, T47D (A and B) and BT-20 (C and D). A and C, knockdown of UBE2T by siRNAs was validated by semiquantitative RT-PCR (*top*) and Western blot (*bottom*) analysis. B and D, colony formation assay (*left*) demonstrating a decrease in the number of colonies by knockdown of UBE2T. MTT assay (*right*) demonstrating a decrease in the number of cells by knockdown of UBE2T (si#2; $P < 0.0001$, unpaired *t* test).

Figure 4. UBE2T interacts with BRCA1. *A*, *in vitro* binding assay. After incubation with the GST-fused proteins (8 nmol), the protein complex pulled down with GST-UBE2T was examined by Western blot analysis. *B*, colocalization of UBE2T (green) and BRCA1 proteins (red). The nuclei were counter-stained with DAPI (blue). To block the proteasomal protein degradation, the cells were treated with 10 $\mu\text{mol/L}$ of MG132 for 12 h before staining (bottom).



cells transfected with WT-UBE2T, compared with those with UBE2T-C86A or nontransfected parental cells (Supplementary Fig. S3; Fig. 6D). Taken together, these results suggest a possibility that overexpression of UBE2T might enhance polyubiquitination and degradation of BRCA1 protein in breast cancer cells.

Discussion

Cumulative evidences have suggested that the ubiquitin-proteasome system, which consists of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-ligase (E3), and the 26S proteasome, play critical roles in controlling the function of various cellular proteins including critical regulators of signal transduction, cell proliferation, and apoptosis for tumorigenesis (28–32). Therefore, several cancer-therapeutic inhibitors targeting the ubiquitin-proteasome system have been developed: PYR-41 (a cell permeable inhibitor of the ubiquitin E1, UBE1), HLI98 (a small compound inhibitor which block Hdm2-mediated p53 ubiquitination), and bortezomib (a dipeptide boronate inhibitor of 26S proteasome; ref. 33). However, because PYR-41 or bortezomib are

distinct from target-specific inhibition, these inhibitors are thought to block whole cellular system mediated by ubiquitin pathways (33, 34). In addition, the development of E3 ligase inhibitors such as HLI98 has been challenged by structural similarities among E3 enzymes that are present in >600 proteins in human cells, consequently resulting in off target effect (33).

In mammalian cells, >40 putative E2 enzymes are estimated to work with many different E3 ligases to promote ubiquitination of target proteins (33). It has been reported that some E2 ubiquitin-conjugating enzymes are also associated with the tumorigenesis (35–37). Moreover, the cycline-selective ubiquitin conjugase murine E2-C was reported to be upregulated in the NIH3T3 cells transformed by EWS/FLI1 fusion gene, which acts as an oncogene in Ewing's sarcoma (32). These facts imply that E2 ubiquitin-conjugating enzymes are alternative targets for cancer therapy. In this study, our data also provide significant evidences that an E2 ubiquitin-conjugating enzyme, UBE2T is exclusively upregulated in breast cancers (Figs. 1 and 2), and plays indispensable roles in breast cancer cell proliferation (Fig. 3). In addition, we have reported upregulation of UBE2T

in other types of cancer including bladder, lung, and prostate cancers (38–40). Hence, these findings suggest UBE2T as an ideal therapeutic target and may enable us to develop an anticancer agent with fewer adverse effects.

Accumulating reports indicated that the inactivation of BRCA1, breast cancer susceptibility gene, in sporadic breast cancers is due to its genetic mutation, epigenetic silencing, or haploinsufficiency (25, 26). This protein it encodes has been implicated in a number of biological processes to prevent tumor progression (41). Consequently, downregulation of BRCA1 by other mechanisms is thought to cause sporadic breast cancers (26). Our findings in this study have shown for the first time that UBE2T overexpression possibly cause degradation of BRCA1 via autoubiquitination and proteasomal degradation such as other E3 ubiquitin ligases, Siah2, and Mdm2 (42) through its interaction with BRCA1/BARD1 (Figs. 4 and 5). This assumption was further supported by the results of UBE2T depletion in breast cancer cell lines (Fig. 6B) as well as UBE2T overexpression in normal breast epithelial cells (Fig. 6C and D). Furthermore, we found that BRCA1 expression in MCF10A cells is much higher than those in breast cancer cells, suggesting the inverted correlation between UBE2T and BRCA1 expressions (Fig. 6A). These results is well correlated with the previous findings that downregulation of BRCA1

impairs differentiation but enhances proliferation of MCF10A cells (43). This fact suggests that overexpression of UBE2T may contribute to breast carcinogenesis throughout downregulation of BRCA1. In addition, knocking down of UBE2T protein induced upregulation of BRCA1 protein in breast cancer cells, whereas its overexpression caused the decrease of the BRCA1 protein (Fig. 6C and D), suggesting that the E2 enzymatic activity of UBE2T protein is important for BRCA1 downregulation in breast cancer cells although there is a possibility that BRCA1 might be served as a substrate rather than E3 ligase. Moreover, considering the number of E3 than E2 enzymes, each E2 is likely to interact with multiple E3 ligases (33). Therefore, we are unable to exclude a possibility that knocking down of UBE2T might influence simultaneously on the other Ub-pathway, for example, the FANC pathway constituting the DNA damage repair network (13), resulting in growth suppression of breast cancer cells synergistically with BRCA1 downregulation. Collectively, we assume that the screening of compounds to inhibit the ubiquitin-conjugating enzyme activity of UBE2T may be able to develop an anticancer agent that suppresses breast cancer cell growth by prevention of BRCA1 degradation.

Although several mechanisms of BRCA1 downregulation have been reported (26, 27), the mechanism that BRCA1 can be

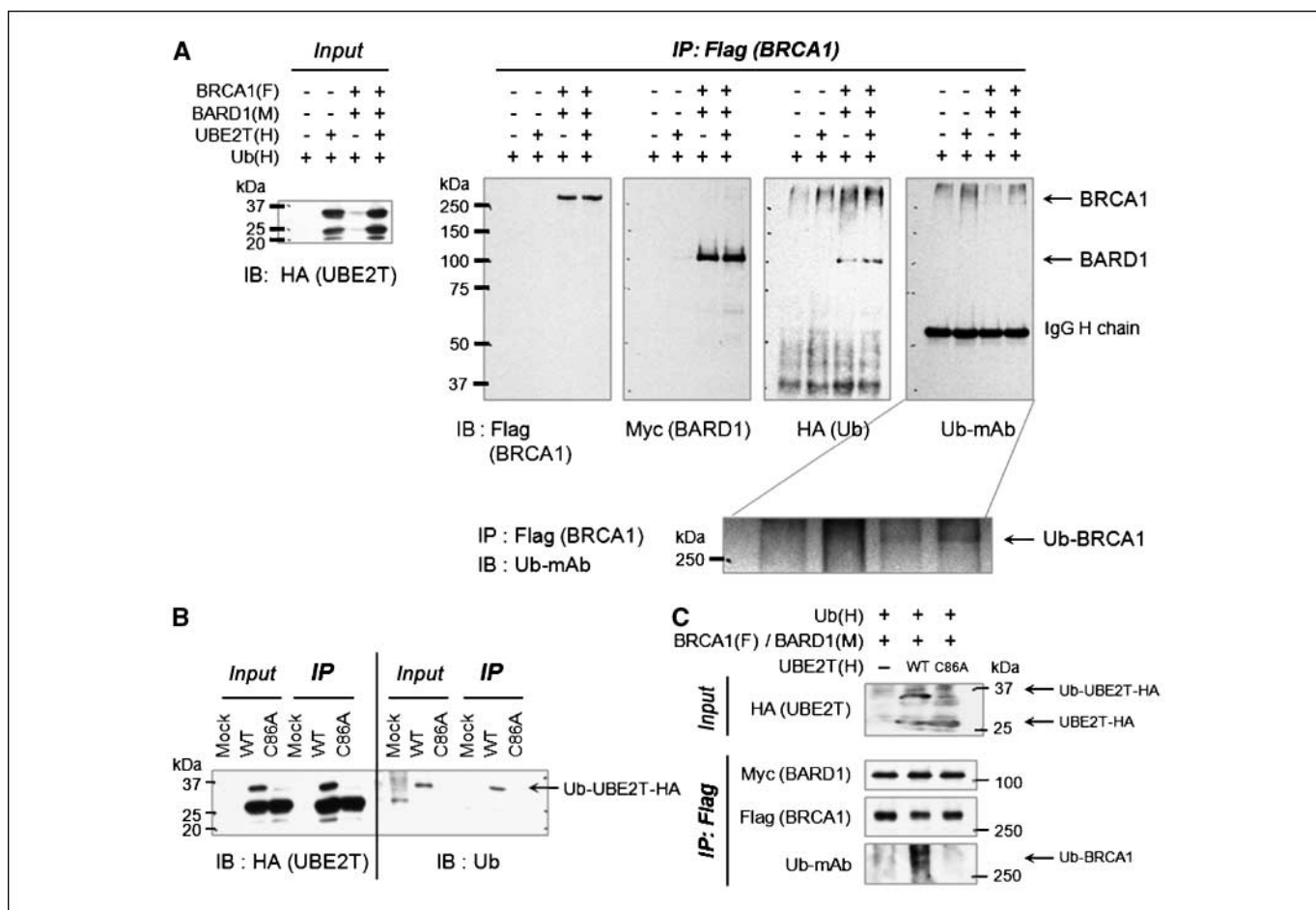


Figure 5. UBE2T contributes to ubiquitination of BRCA1. *A*, ubiquitination of BRCA1 by UBE2T *in vivo*. In HEK293T cells, exogenous proteins of Flag-BRCA1, Myc-BARD1, and HA-UBE2T, and HA-ubiquitin (*Ub*) were expressed in condition with MG132. The BRCA1/BARD1 complex was immunoprecipitated (*IP*) by anti-Flag antibody, and immunoblotted (*IB*) by anti-Flag, anti-Myc, anti-HA, and anti-Ub antibodies. *B*, generation of an enzyme-dead mutant (C86A) of UBE2T. In HEK293T cells, the WT or C86A were expressed and immunoprecipitated by anti-HA antibody. *C*, validation assay of ubiquitinated BRCA1 using C86A that is a mutant lacking E2 enzymatic activity.

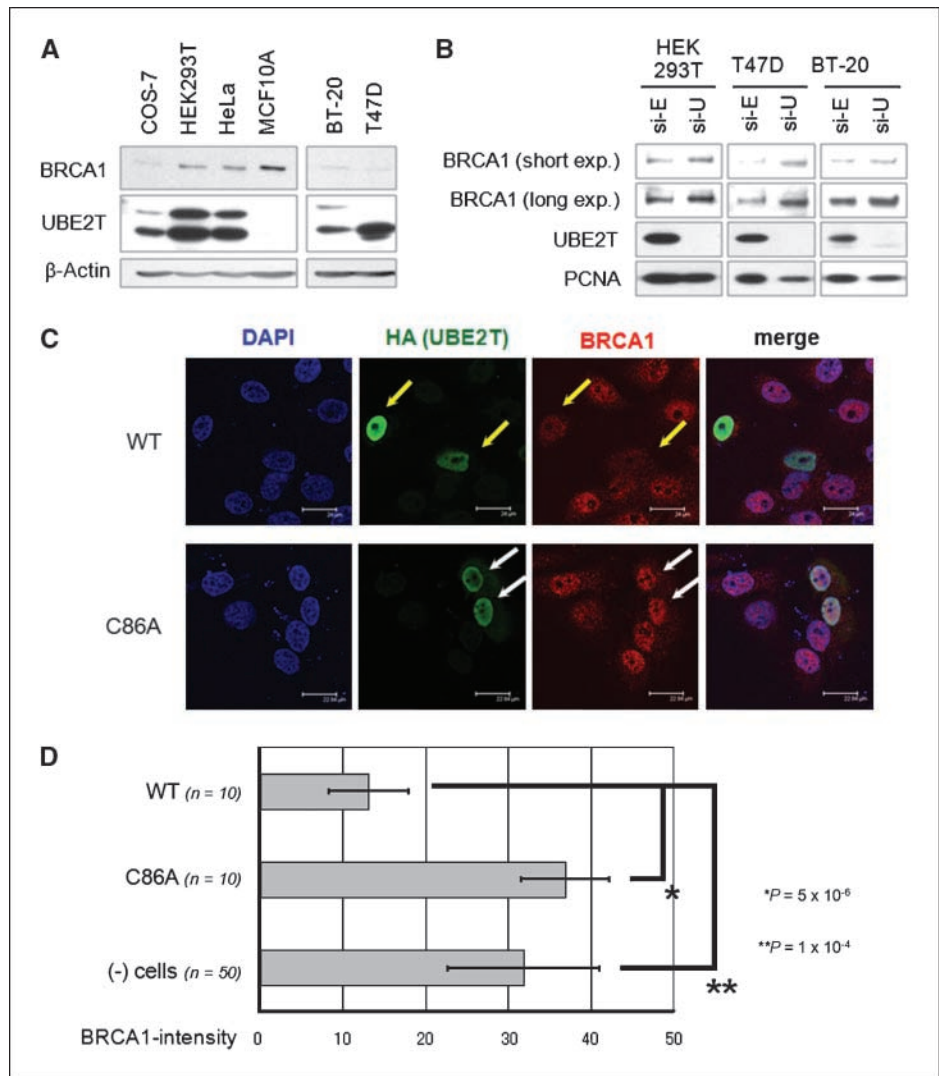


Figure 6. Downregulation of BRCA1 by UBE2T. *A*, expression of BRCA1 and UBE2T proteins by Western blot analysis. *B*, restoration of BRCA1 protein by knocking down of the UBE2T expression. Forty-eight hours after transfection with si-EGFP (control, *si-E*) or si-UBE2T (*si-U*), the endogenous proteins were investigated by immunoblotting using nuclear proteins. *C*, exogenous expression of UBE2T in MCF10A cells. Forty-eight hours after transfection with HA-tagged UBE2Ts, MCF10A cells were immunostained with anti-HA (UBE2T, *green*) and anti-BRCA1 (*red*) monoclonal antibodies. *Arrows*, the cells expressing UBE2T of WT (*yellow arrows*) or C86A mutant (*white arrows*). *D*, WT of UBE2T (WT) caused significant reduction of BRCA1 protein, compared with C86A-UBE2T transfected cells ($P = 5 \times 10^{-6}$, unpaired *t* test) or nontransfected cells ($P = 1 \times 10^{-4}$, unpaired *t* test).

inactivated by proteasomal degradation pathway shown above was not described previously. Our data imply a critical role of UBE2T in the degradation of BRCA1 protein in breast cancer cells, and that the inhibitors for enzymatic activity of UBE2T or their UBE2T-BRCA1/BARD1 interaction would be valuable targets to develop therapeutic modalities against breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;51:74–108.
2. Nathanson KL, Wooster R, Weber BL. Breast cancer genetics: what we know and what we need. *Nat Med* 2001;7:552–6.
3. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66–71.

4. Narod SA, Foulkes WD. BRCA1 and BRCA2: 1994 and beyond. *Nat Rev Cancer* 2004;4:665–76.
5. Petricoin EF III, Hackett JL, Lesko LJ, et al. Medical applications of microarray technologies: a regulatory science perspective. *Nat Genet* 2002;32 Suppl:474–9.
6. Nishidate T, Katagiri T, Lin ML, et al. Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: identification of genes associated with progression and metastasis. *Int J Oncol* 2004;25:797–819.

7. Park JH, Lin ML, Nishidate T, Nakamura Y, Katagiri T. PDZ-binding kinase/T-LAK cell-originated protein kinase, a putative cancer/testis antigen having an oncogenic activity in breast cancer. *Cancer Res* 2006;66:9186–95.
8. Shimo A, Nishidate T, Ohta T, Fukuda M, Nakamura Y, Katagiri T. Elevated expression of PRC1, protein regulator of cytokinesis 1, involved in the growth of breast cancer cells. *Cancer Sci* 2007;98:174–81.
9. Lin ML, Park JH, Nishidate T, Nakamura Y, Katagiri T. Involvement of maternal embryonic leucine zipper

- kinase (MELK) in mammary carcinogenesis through interaction with Bel-G, a pro-apoptotic member of Bel-2 family. *Breast Cancer Res* 2007;9:R17.
10. Shimo A, Tanikawa C, Nishidate T, et al. Involvement of kinesin family member 2C/mitotic centromere-associated kinesin overexpression in mammary carcinogenesis. *Cancer Sci* 2008;99:62–70.
 11. Ueki T, Nishidate T, Park JH, et al. Involvement of elevated expression of multiple cell-cycle regulator, DTL/RAMP (denticleless/RA-regulated nuclear matrix associated protein), in the growth of breast cancer cells. *Oncogene* 2008;27:5672–83.
 12. Iyer VR, Eisen MB, Ross DT, et al. The transcriptional program in the response of human fibroblasts to serum. *Science* 1999;283:83–7.
 13. Machida YJ, Machida Y, Chen Y, et al. UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. *Mol Cell* 2006;23:589–96.
 14. Alpi A, Langevin F, Mosedale G, Machida YJ, Dutta A, Patel KJ. UBE2T, the Fanconi anemia core complex, and FANCD2 are recruited independently to chromatin: a basis for the regulation of FANCD2 monoubiquitination. *Mol Cell Biol* 2007;27:8421–30.
 15. American Type Culture Collection (ATCC). Cell line verification test recommendations; ATCC recommends cell line verification tests and guidelines for publishing. *ATCC Technical Bulletin* 2007;no. 8.
 16. Katagiri T, Ozaki K, Fujiwara T, et al. Cloning, expression and chromosome mapping of adducin-like 70 (ADDL), a human cDNA highly homologous to human erythrocyte adducin. *Cytogenet Cell Genet* 1996;74:90–5.
 17. Choudhury AD, Xu H, Baer R. Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression. *J Biol Chem* 2004;279:33909–18.
 18. Suzuki C, Murakami G, Fukuchi M, et al. Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *J Biol Chem* 2002;277:39919–25.
 19. Sengupta S, Robles AI, Linke SP, et al. Functional interaction between BLM helicase and 53BP1 in a Chk1-mediated pathway during S-phase arrest. *J Cell Biol* 2004;166:801–13.
 20. Shimokawa T, Furukawa Y, Sakai M, et al. Involvement of the FGF18 gene in colorectal carcinogenesis, as a novel downstream target of the β -catenin/T-cell factor complex. *Cancer Res* 2003;63:6116–20.
 21. Hashizume R, Fukuda M, Maeda I, et al. The RING heterodimer BRCA1-1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem* 2001;276:14537–40.
 22. Baer R, Ludwig T. The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity. *Curr Opin Genet Dev* 2002;12:86–91.
 23. Brzovic PS. Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc Natl Acad Sci U S A* 2003;100:5646–51.
 24. Sato K, Hayami R, Wu W, et al. Nucleophosmin/B23 is a candidate substrate for the BRCA1-1 ubiquitin ligase. *J Biol Chem* 2004;279:30919–22.
 25. Eakin CM, Maccoss MJ, Finney GL, Kleit RE. Estrogen receptor α is a putative substrate for the BRCA1 ubiquitin ligase. *Proc Natl Acad Sci U S A* 2007;104:5794–9.
 26. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer* 2004;4:814–9.
 27. Elstrodt F, Hollestelle A, Nagel JH, et al. BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res* 2006;66:41–5.
 28. Chen A, Kleiman FE, Manley JL, Ouchi T, Pan ZQ. Autoubiquitination of the BRCA1-1 RING ubiquitin ligase. *J Biol Chem* 2002;277:22085–92.
 29. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997;387:296–9.
 30. Urano T, Saito T, Tsukui T, et al. Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth. *Nature* 2002;417:871–5.
 31. Pagano M, Tam SW, Theodoras AM, et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 1995;269:682–5.
 32. Arvand A, Bastians H, Welford SM, Thompson AD, Ruderman JV, Denny CT. EWS/FLI1 up regulates mE2-C, a cyclin-selective ubiquitin conjugating enzyme involved in cyclin B destruction. *Oncogene* 1998;17:2039–45.
 33. Yang Y, Kitagaki J, Wang H, Hou DX, Perantoni AO. Targeting the ubiquitin-proteasome system for cancer therapy. *Cancer Sci* 2009;100:24–8.
 34. Yang Y, Kitagaki J, Dai RM, et al. Inhibitors of ubiquitin-activating enzyme (E1), a new class of potential cancer therapeutics. *Cancer Res* 2007;67:9472–81.
 35. Okamoto Y, Ozaki T, Miyazaki K, Aoyama M, Miyazaki M, Nakagawara A. UbcH10 is the cancer-related E2 ubiquitin-conjugating enzyme. *Cancer Res* 2003;63:4167–73.
 36. Berlingieri MT, Pallante P, Guida M, et al. UbcH10 expression may be a useful tool in the prognosis of ovarian carcinomas. *Oncogene* 2007;26:2136–40.
 37. Berlingieri MT, Pallante P, Sboner A, et al. UbcH10 is overexpressed in malignant breast carcinomas. *Eur J Cancer* 2007;43:2729–35.
 38. Takata R, Katagiri T, Kanehira M, et al. Predicting response to methotrexate, vinblastine, doxorubicin, and cisplatin neoadjuvant chemotherapy for bladder cancers through genome-wide gene expression profiling. *Clin Cancer Res* 2005;11:2625–36.
 39. Kikuchi T, Daigo Y, Katagiri T, et al. Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph node metastasis and sensitivity to anti-cancer drugs. *Oncogene* 2003;22:2192–205.
 40. Ashida S, Nakagawa H, Katagiri T, et al. Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene expression profiles of prostate cancers and PINs. *Cancer Res* 2004;64:5963–72.
 41. Mullan PB, Quinn JE, Harkin DP. The role of BRCA1 in transcriptional regulation and cell cycle control. *Oncogene* 2006;25:5854–63.
 42. Fang S, Weissman AM. A field guide to ubiquitylation. *Cell Mol Life Sci* 2004;61:1546–61.
 43. Furuta S, Jiang X, Gu B, Cheng E, Chen PL, Lee WH. Depletion of BRCA1 impairs differentiation but enhances proliferation of mammary epithelial cells. *Proc Natl Acad Sci U S A* 2005;102:9176–81.