Novel BRCA2-interacting protein BJ-HCC-20A inhibits the induction of apoptosis in response to DNA damage

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The major hereditary breast cancer susceptibility gene *BRCA2* is associated with familial breast and ovarian cancer. BRCA2 plays a role in DNA repair, transcription, cell cycle regulation, maintenance of genomic stability in response to DNA damage, centrosome regulation, and cytokinesis. To further understand the function of BRCA2, we used a yeast two-hybrid method and identified a novel BRCA2-interacting protein, BJ-HCC-20A, which is reported to be a potential cancer–testis antigen. We confirmed the interaction between endogenous BJ-HCC-20A and BRCA2 in mammalian cells, and showed that BJ-HCC-20A interacts with a portion of the highly conserved region of BRCA2 in various mammals, and M phase-specific phosphorylation of the binding region of BRCA2 modulates BJ-HCC-20A binding. Overexpression of BJ-HCC-20A increases cell growth, and downregulation of endogenous BJ-HCC-20A expression using small interfering RNA suppresses cell growth and leads to the induction of apoptosis. Importantly, the BJ-HCC-20A mRNA level is downregulated by adriamycin (ADR)-induced DNA damage and depletion of BJ-HCC-20A expression by small interfering RNA promotes the reduction of BRCA2 expression and enhances cell apoptosis in response to DNA damage. Additionally, the recovery of BJ-HCC-20A expression in ADR-induced DNA damage inhibits ADR-induced apoptosis. The data suggest that BJ-HCC-20A promotes cell growth and may regulate the induction of cell apoptosis in response to DNA damage in cooperation with BRCA2 in an M phase-dependent manner. Therefore, we speculate that targeting BJ-HCC-20A may aid in the treatment of breast tumors. (*Cancer Sci* 2008; 99: 747–754)

Genetic factors contribute to an ill-defined proportion of breast cancer incidence, estimated to be approximately 5% of all breast cancer cases.[1] The two major hereditary breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are associated with familial breast and ovarian cancer. Germline mutations in the *BRCA1* gene increase the risk of development of early onset breast cancer and ovarian cancer.[2] Alterations in the *BRCA2* gene result in an increased risk of development of breast cancer in both women and men,[3,4] and a moderately increased risk of a variety of other cancers, including carcinomas of the ovary, pancreas, prostate, and colon.[5] Because inactivation of both alleles of either *BRCA1* or *BRCA2* is a key feature in neoplastic development in hereditary cancers, these genes are believed to act as tumor-suppressor genes required for cell growth. BRCA2 plays an important role in DNA repair in response to DNA damage.[6–10] In addition, BRCA2 is also involved in the regulation of transcription,[11–13] cell cycle checkpoints,[14–16] inhibition of cell growth,[17,18] maintenance of genomic stability,[19–22] centrosome regulation,[23] and cytokenesis.[24] BRCA2 is specifically phosphorylated in M phase.[10,25,26] Phosphorylation of BRCA2 by the cyclin-dependent kinase cdk1 in M phase blocks C-terminal interaction between BRCA2 and RAD51, and reduces the efficiency of recombinational repair.[10]

BRCA2 has several regions that are highly conserved in various mammals. Several proteins interact with these conserved regions of BRCA2 and are reported to play important roles, especially in DNA damage repair.[10,13,15,27] Therefore, functional analysis of the conserved regions has important significance in understanding the roles of BRCA2 in breast carcinogenesis. To further understand the function of BRCA2, we used the yeast two-hybrid system to identify novel proteins that interact with the conserved regions of BRCA2. We identified BJ-HCC-20A as a novel BRCA2-interacting protein. Human BJ-HCC-20 is reported to be a potential CT antigen.[28] Because the function of BJ-HCC-20 is largely unknown whereas a number of CT antigens are involved in cell growth,[18,29] we investigated the effects of BJ-HCC-20A overexpression on cell growth and apoptosis, and the effects of knocking down BJ-HCC-20 expression using siRNA. We further determined the role of BJ-HCC-20A in response to DNA damage because it forms *in vivo* complexes with BRCA2. The data suggest that BJ-HCC-20A may serve as an effecter or regulator of cell growth in cooperation with BRCA2 in the DNA damage/cell apoptosis pathway.

Materials and Methods

Yeast two-hybrid screening. A yeast two-hybrid screening assay using the ProQuest two-hybrid system (Invitrogen, Carlsbad, CA, USA) was carried out according to the manufacturer's instructions. In brief, the cDNA coding for the conserved region (amino acids 121–465) of *BRCA2* was subcloned into pDBLeu. This was used as the bait in the yeast two-hybrid screening of the mouse embryo (10.5-day) cDNA library and the human fetal brain cDNA library.

Cell cultures, synchronization, and transfection. MCF-7 and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. U2OS cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine. Cells were treated with 30 ng/mL nocodazole (Sigma-Aldrich, St Louis, MO, USA) for 24 h or 0.5 μg/mL ADR (Sigma-Aldrich) for 24 h. Cells were transfected with expression plasmids using FuGENE 6 (Roche

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Abbreviations: ADR, adriamycin; *BRCA*, breast-cancer susceptibility gene; BrdU, 5-bromo-2′-deoxy-uridine; CT antigen, cancer–testis antigen; DAPI, 4′,6-diamidino-2-phenylindole; G418, geneticin; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemaglutinin; IB, immunoblot; IP, immunopracipitation; Plk, Polo-like kinase; RT-PCR, reverse transcription–polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrlamide gel electrophoresis; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling.

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Diagnostics, Tokyo, Japan) and Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Plasmids. BJ-HCC-20A cDNA was isolated by RT-PCR from human testis total RNA (BD Clontech, Palo Alto, CA, USA) and cloned into pcDNA3.1HA and pEGFP-C1 (BD Clontech). Flag-tagged BRCA2 was prepared as described previously.[23] GST-tagged BJ-HCC-20A, BJ-HCC-20A mutants, and BRCA2 mutants were cloned into pGEX4T1 (Amersham Biosciences, Piscataway, NJ, USA).

Antibodies. Rabbit anti-BJ-HCC-20 antibody was raised against the synthetic peptide BJ-HCC-20 (amino acids 97–112), and affinity-purified by MBL, (Nagoya, Japan). Anti-BRCA2 (Ab1; Calbiochem, San Diego, CA, USA), anti-Flag (Sigma-Aldrich), anti-HA (3F10; Roche Diagnostics), anti-GFP (Nacalai Tesque, Kyoto, Japan), anti-GST (Nacalai Tesque), and anti-α-tubulin (Sigma-Aldrich) antibodies were used.

Immunoprecipitation and immunoblot analysis. Cells were suspended in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_3, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL pepstatin), and then incubated on ice for 30 min. After centrifugation, cell lysates were immunoprecipitated with anti-BJ-HCC-20 or anti-BRCA2 antibodies, protein A (Amersham Biosciences) or G (Zymed Laboratories, South San Francisco, CA, USA) sepharose, or anti-HA affinity matrix (Roche Diagnostics). The immunoprecipitates were washed three times with 0.1% Nonidet P-40 lysis buffer and immunoblot analysis was carried out. Cell lysates or immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters (Bio-Rad, Tokyo, Japan) or polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then incubated with anti-BJ-HCC-20, anti-BRCA2, anti-HA, anti-GFP, or anti-α-tubulin antibodies. Immunoreactive protein bands were visualized using chemiluminescence (PerkinElmer, Wellesley, MA, USA).

***In vitro* binding assays.** GST fusion proteins were expressed in *Escherichia coli* and purified on glutathione sepharose (Amersham Biosciences). Cell lysates were incubated with purified GST fusion proteins. The adsorbates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-Flag or anti-HA antibodies.

Small interfering RNA transfection. Cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The siRNA duplex for BJ-HCC-20 was synthesized and purified by Invitrogen (Stealth RNAi). The Stealth RNAi sequence was 5′-UGGAUGUUGUGCCUCGCCAUCUUUA-3′ for BJ-HCC-20 siRNA1 and 5′-CCAUAGCCAUUGUUUCUGAAGAUUU-3′ for BJ-HCC-20 siRNA2. Scrambled siRNA was used as a negative control (Invitrogen).

Reverse transcription–polymerase chain reaction analysis. Total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). The relative mRNA expression was analyzed by RT-PCR using the SuperScript III One-Step RT-PCR System according to the manufacturer's instructions (Invitrogen). The primer sequences were as follows: BJ-HCC-20 forward, 5′-ATGCTCAGGCTTCTGAGACTTGC-3′; BJ-HCC-20 reverse, 5′-TTAAATGCTTTGGCTCCGACGTTC-3′; BRCA2 forward, 5′-GCACATTTACAGAGTAGTGTAGTTG-3′; BRCA2 reverse, 5′-GTCATTTTTCAACAGGCCAGCAAA-3′; and β-actin using the β-actin RT-PCR Primer Set (Toyobo, Osaka, Japan).

Colony-formation assay. HeLa cells transfected with GFP vector or GFP-BJ-HCC-20A were cultured with an appropriate concentration of G418 for 15 days. The cells were fixed with 70% ethanol and stained using Giemsa solution (Wako, Osaka, Japan).

Cell-proliferation assay. Cell proliferation was detected using BrdU incorporation by immunofluorescence. After transfection, the cells were incubated with BrdU for 1 h and stained with anti-BrdU antibody according to the manufacturer's instructions (Roche Diagnostics).

Apoptosis assay. To visualize the apoptotic nuclear changes, the cells were fixed and stained with DAPI. The percentage of cells with a condensed or fragmented nucleus was determined by counting at least 800 cells (nuclei stained with DAPI). Apoptotic cells were detected using the TUNEL assay with the DeadEnd colorimetric TUNEL system (Promega, Madison, WI, USA).

Results

Identification of BJ-HCC-20A as a BRCA2-interacting protein and confirmation of the interaction *in vivo*. To identify novel proteins that interact with BRCA2, we used the yeast two-hybrid system. After screening the mouse embryo cDNA library using a portion of the highly conserved region of BRCA2 (amino acids 121–465) as bait, several candidate clones were obtained and characterized using DNA sequence analysis. Of the candidate clones identified, five independent clones coded for 4930502E18Rik were located on the mouse chromosome X. Using a BLAST analysis, we determined that the human homolog of 4930502E18Rik was BJ-HCC-20, located on human chromosome X. There is 42.5% identity in amino acids between the mouse clone and human BJ-HCC-20. Human BJ-HCC-20 is reported to be a potential CT antigen that has two splicing isoforms, BJ-HCC-20A and BJ-HCC-20B, differing at the C-terminal region (Fig. 1a).

An interaction between BJ-HCC-20 and BRCA2 was confirmed in mammalian cells. HA-tagged BJ-HCC-20A was overexpressed in HeLa cells, and coimmunoprecipitation and immunoblot analysis were carried out. As shown in Figure 1b, BJ-HCC-20A interacts with BRCA2 in the overexpression system. To further characterize the binding region of BJ-HCC-20A and BRCA2, groups of GST-fusion proteins of BRCA2 and BJ-HCC-20A fragments were purified. Using GST pull-down assays, BRCA2 (amino acids 121–235) interacted with BJ-HCC-20A, and BJ-HCC-20A (amino acids 1–105) interacted with BRCA2 (Fig. 1c). Importantly, the binding region of BRCA2 (amino acids 121–235) with BJ-HCC-20A identified here includes several M phase-specific phosphorylation sites by Plk1 (Ser193, Thr203, Ser205, Ser206, and Thr207).[25] To determine the interaction between endogenous BJ-HCC-20 and BRCA2, we generated rabbit polyclonal anti-BJ-HCC-20 antibody against the synthetic peptide corresponding to BJ-HCC-20 amino acids 97–112. As shown in Figure 2a, this antibody detected endogenous BJ-HCC-20A as a 29-kDa protein in MCF-7 cells. However, this antibody did not detect endogenous BJ-HCC-20B as the 27-kDa protein, indicating that the BJ-HCC-20B protein may be expressed at an extremely low level. A similar result was obtained with HeLa and U2OS cells (data not shown). As shown in Figure 2b, coimmunoprecipitation and immunoblot analysis using anti-BJ-HCC-20 and anti-BRCA2 antibodies showed an interaction between endogenous BJ-HCC-20A and BRCA2 in MCF-7 cells. For this reason, we focused on BJ-HCC-20A in the present study.

Phosphorylation by Plk1 modulates BJ-HCC-20A binding. It has been shown that BRCA2 is phosphorylated (an alteration in electrophoretic mobility of the BRCA2 protein) in M phase by Plk1,[25,26] and the region of BRCA2 (amino acids 121–235) binding with BJ-HCC-20A identified in the present study has several M phase-specific Plk1 phosphorylation sites. To investigate the interaction between BJ-HCC-20A and BRCA2 during M phase, HeLa cells were synchronized in M phase by nocodazole treatment. As shown in Figure 3, an alteration in electrophoretic mobility of the BRCA2 protein was detected after treating the cells with nocodazole (Fig. 3, lanes 2, 4, and 6); however, BJ-HCC-20A didn't interact with the M phase-specific

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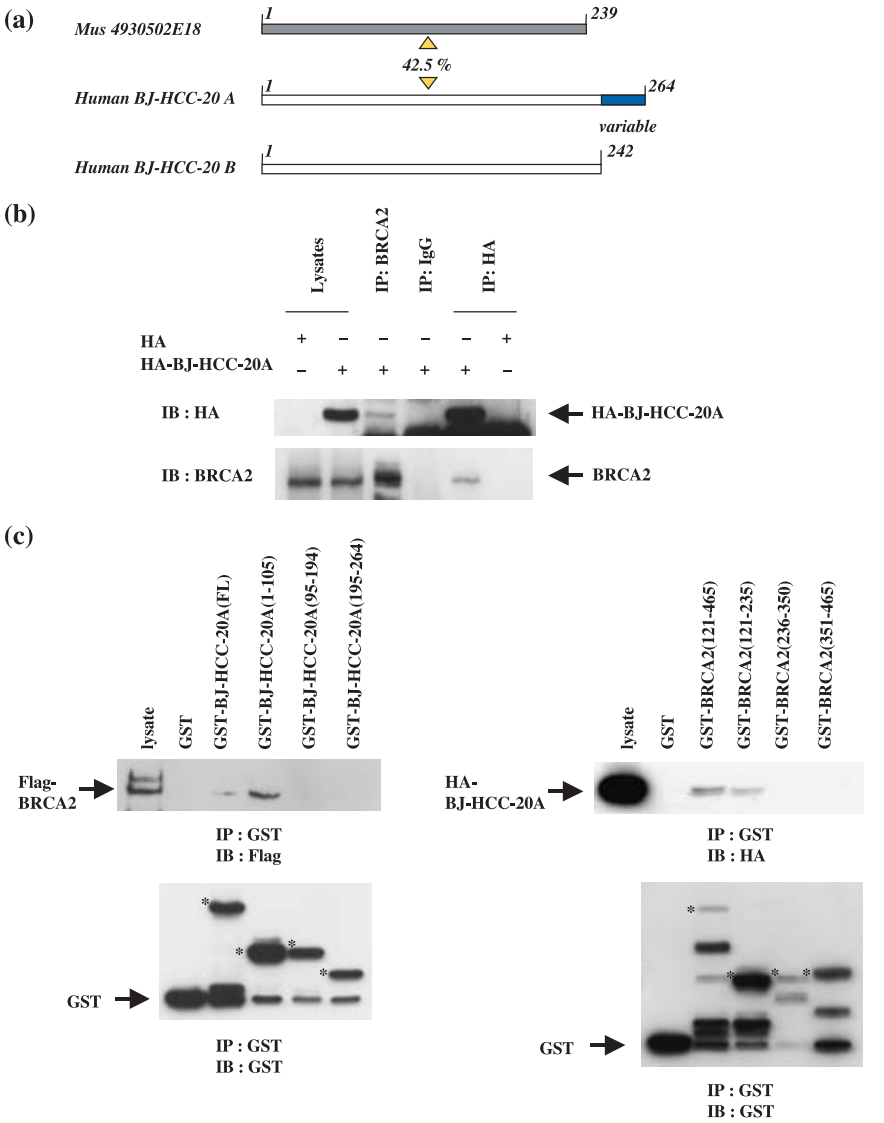

Fig. 1. Interaction of BJ-HCC-20A and BRCA2. (a) Schematic representation of mouse 4930502E18 and human BJ-HCC-20 showing the structures of BJ-HCC20A and BJ-HCC-20B. The blue box indicates the variable region of BJ-HCC-20A and BJ-HCC-20B. (b) Coimmunoprecipitation of endogenous BRCA2 and hemaglutinin (HA)-BJ-HCC-20A. HA-BJ-HCC-20A was expressed in HeLa cells. Cell lysates were immunoprecipitated with anti-HA, normal mouse IgG, or anti-BRCA2 followed by immunoblotting with anti-HA (upper panel) or anti-BRCA2 (lower panel). Cell lysates were also analyzed by immunoblotting with anti-HA (upper panel) or anti-BRCA2 (lower panel). (c) Glutathione S-transferase (GST) pull-down assays of BRCA2 and BJ-HCC-20A. Cell lysates expressing Flag-tagged BRCA2 or HA-tagged BJ-HCC-20A were incubated with each GST fragment as indicated above the panels. The adsorbates were analyzed by immunoblotting with anti-Flag, anti-HA, or anti-GST. Each band specific for the GST-BJ-HCC-20A and BRCA2 fragments is indicated by an asterisk. IB, immunoblot; IP, immunoprecipitation.

phosphorylated BRCA2 with nocodazole treatment (Fig. 3, lane 10). This suggests that the interaction between BJ-HCC-20A and BRCA2 is at least in part regulated by phosphorylation in an M phase-dependent manner.

Expression of BJ-HCC-20A promotes cell growth. A number of CT antigens are involved in cell growth.[18,30] To determine the effect of BJ-HCC-20A on cell growth, BJ-HCC-20A was overexpressed, or endogenous BJ-HCC-20A was downregulated in cells. As shown in Figure 4a, BJ-HCC-20A overexpression significantly increased cell-growth activity compared to the control in HeLa cells using the colony-formation assay. To further confirm whether BJ-HCC-20A is involved in cell growth, expression of endogenous BJ-HCC-20 was knocked down by transfection using BJ-HCC-20 siRNA. In Figure 4b, immunoblot analysis shows that cells transfected with BJ-HCC-20 siRNA1 reduced the level of endogenous BJ-HCC-20 expression. The BrdU-incorporation assay showed that downregulation of endogenous BJ-HCC-20 expression results in the suppression of cell growth compared to the controls (untransfected and transfected with scrambled siRNA) in U2OS and HeLa cells (Fig. 4c). A similar result was obtained with MCF-7 cells. These data suggest that BJ-HCC-20A may be involved in the promotion of cell growth.

Downregulation of BJ-HCC-20A causes the induction of apoptosis. In the execution phase of apoptosis, characteristic morphological changes of cells are induced, including dynamic membrane blebbing and nuclear changes. In the previous BrdU-incorporation assay, endogenous BJ-HCC-20 expression was downregulated in U2OS cells using siRNA. In order to visualize the apoptotic nuclear changes (condensation and fragmentation), these cells were fixed and stained with DAPI. As shown in Figure 5a, some U2OS cells exhibited apoptotic nuclear changes, and downregulation of BJ-HCC-20 increased the number of cells with apoptotic nuclear changes compared to the control (transfected with scrambled siRNA) ($8.4 \pm 0.4\%$ vs $0.9 \pm 0.3\%$, $P < 0.0005$). A similar result was obtained with HeLa cells (data not shown). Importantly, these results indicate that downregulation of BJ-HCC-20A expression using siRNA suppresses cell growth and induces apoptotic nuclear changes simultaneously. To confirm that the cellular morphological changes induced by downregulation of BJ-HCC-20A are caused by the induction of apoptosis, the TUNEL assay was carried out. As shown in Figure 5b, downregulation of endogenous BJ-HCC-20A increased the induction of apoptosis compared to the controls (untransfected and transfected with scrambled siRNA) in U2OS cells. A similar result was obtained with HeLa cells

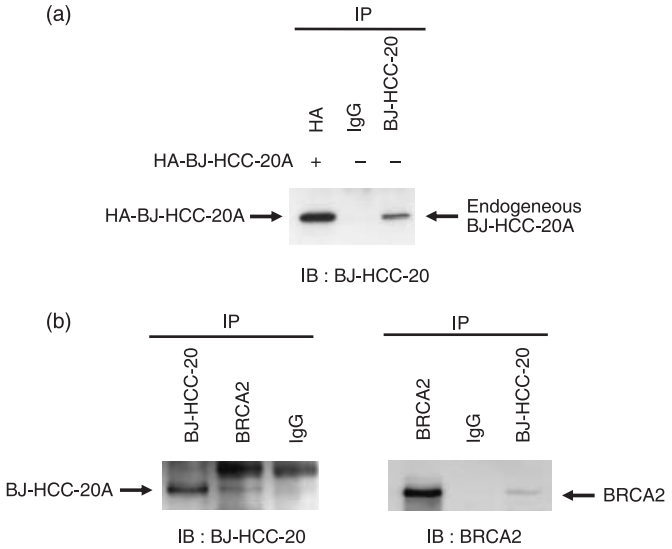

Fig. 2. Interaction of endogenous BJ-HCC-20A and BRCA2. (a) Characterization of the anti-BJ-HCC-20 antibody. Cell lysates from MCF-7 cells were immunoprecipitated and immunoblotted with affinity-purified anti-BJ-HCC-20 polyclonal antibody. Cell lysates from MCF-7 cells transfected with hemaglutinin (HA)-BJ-HCC-20A were used as a control. (b) Cell lysates from MCF-7 cells were immunoprecipitated with anti-BJ-HCC-20 or anti-BRCA2 antibodies followed by immunoblotting with anti-BJ-HCC-20 (left panel) or anti-BRCA2 (right panel) antibodies. Normal rabbit or mouse IgG were used as negative controls for immunoprecipitation. IB, immunoblot; IP, immunoprecipitation.

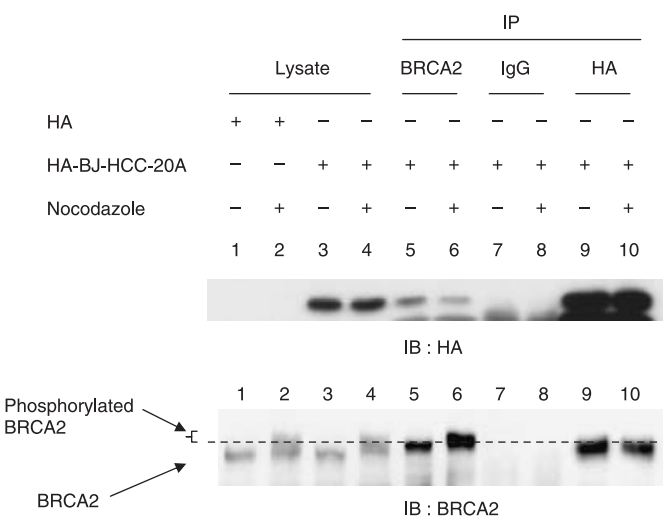

Fig. 3. Modulation of BJ-HCC-20A binding with an M phase-specific phosphorylated BRCA2. HeLa cells were transfected with hemaglutinin (HA) vector or HA-BJ-HCC-20A. At 24 h post-transfection, the cells were treated with dimethyl sulfoxide or 30 ng/mL nocodazole for 24 h. Cell lysates were immunoprecipitated with anti-HA or anti-BRCA2 antibodies followed by immunoblotting with anti-HA or anti-BRCA2 antibodies. IB, immunoblot; IP, immunoprecipitation.

(data not shown). These suggest that BJ-HCC-20A may regulate the induction of apoptosis.

BJ-HCC-20A is involved in the inhibition of apoptosis in response to DNA damage. Reports show that BRCA2 is involved in DNA damage-induced apoptosis.[6,22,31] Several DNA-damaging agents are shown to downregulate BRCA2 expression in cancer cells.[30,32–35] To determine the effect of BJ-HCC-20A in response to DNA damage, U2OS cells were treated with the DNA-damaging agent ADR. As shown in Figure 6a, ADR caused decreased expression of BJ-HCC-20A mRNA. Similar results were obtained with HeLa and MCF-7 cells (data not shown). This suggests that BJ-HCC-20A may be involved in response to DNA damage. Next, U2OS cells were transfected with HA-BJ-HCC-20A, and then treated with ADR for 24 h. As shown in Figure 6b, expression of BJ-HCC-20A inhibited ADR-induced apoptosis significantly. To further investigate the involvement of BJ-HCC-20A in response to DNA damage, U2OS cells were transfected with BJ-HCC-20 siRNA, and then treated with ADR for 24 h. As shown in Figure 6c, the combination of downregulation of BJ-HCC-20 expression and DNA damage increased ADR-induced apoptosis compared to a combination of scrambled siRNA and DNA damage, or downregulation of BJ-HCC-20A expression alone. This suggests that downregulation of BJ-HCC-20A expression enhanced the sensitivity of cells to DNA damage-induced apoptosis. Because BRCA2 is involved in DNA repair and forms a complex with BJ-HCC-20A, we determined whether downregulation of BJ-HCC-20A affects BRCA2 expression in response to DNA damage. U2OS cells were transfected with BJ-HCC-20 siRNA, and then treated with ADR for 24 h. As shown in Figure 6d, downregulation of BJ-HCC-20A promoted the reduction of BRCA2 expression in response to DNA damage compared to the controls (untransfected and transfected with scrambled siRNA). This suggests that BJ-HCC-20A expression may be involved in the inhibition of apoptosis in response to DNA damage through the interaction with BRCA2.

Discussion

Here, we identified that the protein BJ-HCC-20A interacts with a portion of a highly conserved region of BRCA2 using a yeast two-hybrid system, and showed an interaction between endogenous BJ-HCC-20A and BRCA2 *in vivo*. Human BJ-HCC-20 is reported to be a potential CT antigen. Because CT antigens are expressed in a variety of cancers and in the normal testis, some of the CT antigens are being studied as potential therapeutic cancer vaccines. In addition, a number of CT antigens are reported to be involved in cell growth and contribute to some features of neoplastic phenotypes including immortality, invasiveness, and metastatic capacity.[36,37] Here, we show that overexpressed BJ-HCC-20A results in the promotion of cell growth, and downregulation of endogenous BJ-HCC-20 expression using siRNA results in the suppression of cell growth. This suggests that BJ-HCC-20A may have an oncogenic role. Downregulation of endogenous BJ-HCC-20 expression by siRNA leads to the induction of apoptosis, suggesting that BJ-HCC-20 has an antiapoptotic role. Taken together, BJ-HCC-20 may promote cell growth by inhibiting the induction of apoptosis.

DNA damage disrupts the balance between DNA repair/cell survival and DNA damage/cell apoptosis signaling pathways in cells. Other studies have shown BRCC (BRCA1/2-containing complex) is involved in the imbalance between the DNA damage/cell apoptosis and DNA repair/cell survival signaling pathways.[38] Severe damage using high-dose ADR, as in the present study, may lead cells to DNA damage and cell apoptosis. BRCA2 is involved in DNA damage repair and apoptosis,[6–8,22,31] and several DNA-damaging agents, including ADR, camptothecin, and ultraviolet, have been shown to down-regulate BRCA2 expression in cancer cells.[30,32–35] Here, we show that similar to BRCA2, the BJ-HCC-20 mRNA level is also decreased by ADR-induced DNA damage, and the depletion of BJ-HCC-20 expression by siRNA enhances cell apoptosis and promotes the reduction of BRCA2 expression in response to DNA damage. In addition, the recovery of BJ-HCC-20A expression

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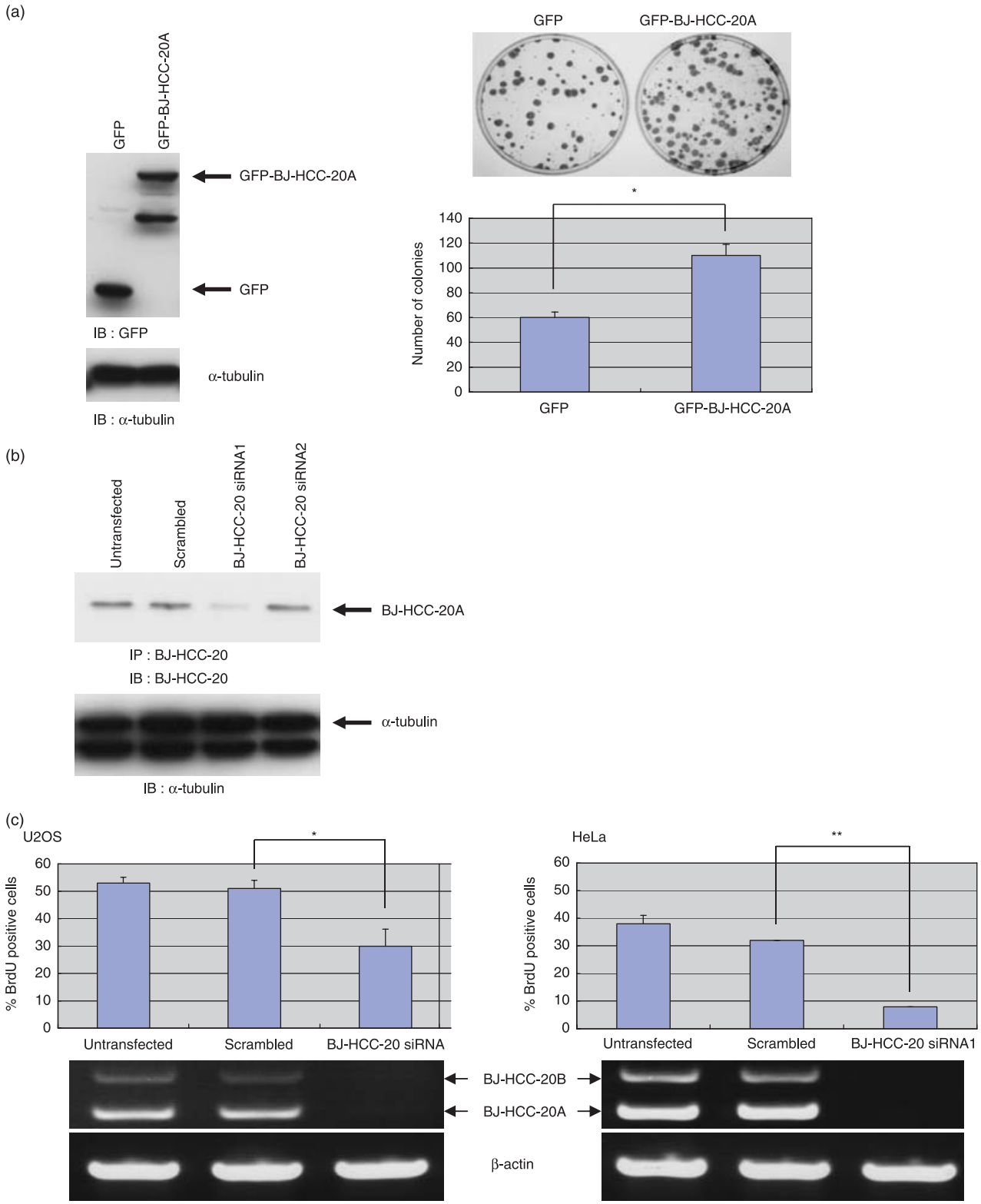

Fig. 4. Expression of BJ-HCC-20A promotes cell growth. (a) Expression of green fluorescent protein (GFP) vector or GFP-BJ-HCC-20A in HeLa cells was analyzed by immunoblotting with anti-GFP (left panels). The colony-formation assays showed that BJ-HCC-20A promotes cell growth in HeLa cells (right panels) (carried out in triplicate). Statistical analysis was carried out using Student's *t*-test. **P* < 0.005. (b) Reduction of endogenous BJ-HCC-20A expression by BJ-HCC-20 small interfering RNA (siRNA). MCF-7 cells were transfected with the indicated siRNA for 48 h. BJ-HCC-20A expression was determined by immunoprecipitation and immunoblotting with anti-BJ-HCC-20 (upper panel). Immunoblot of cell lysates with anti-α-tubulin was used as a control (lower panel). (c) Suppression of cell growth by BJ-HCC-20 siRNA. U2OS and HeLa cells were transfected with the indicated siRNA for 48 h, and the effects on cell growth were determined using 5-bromo-2′-deoxy-uridine (BrdU)-incorporation assays. At least 700 cells (4′,6-diamidino-2-phenylindole) from five fields were counted and the percentage of BrdU-positive cells was calculated. The results are shown as the mean ± SD of three independent experiments (upper panel). The lower panel shows the knockdown effects of BJ-HCC-20 mRNA expression by BJ-HCC-20 siRNA in U2OS and HeLa cells. Statistical analysis was carried out using Student's *t*-test. **P* < 0.05, ***P* < 0.005. IB, immunoblot; IP, immunoprecipitation.

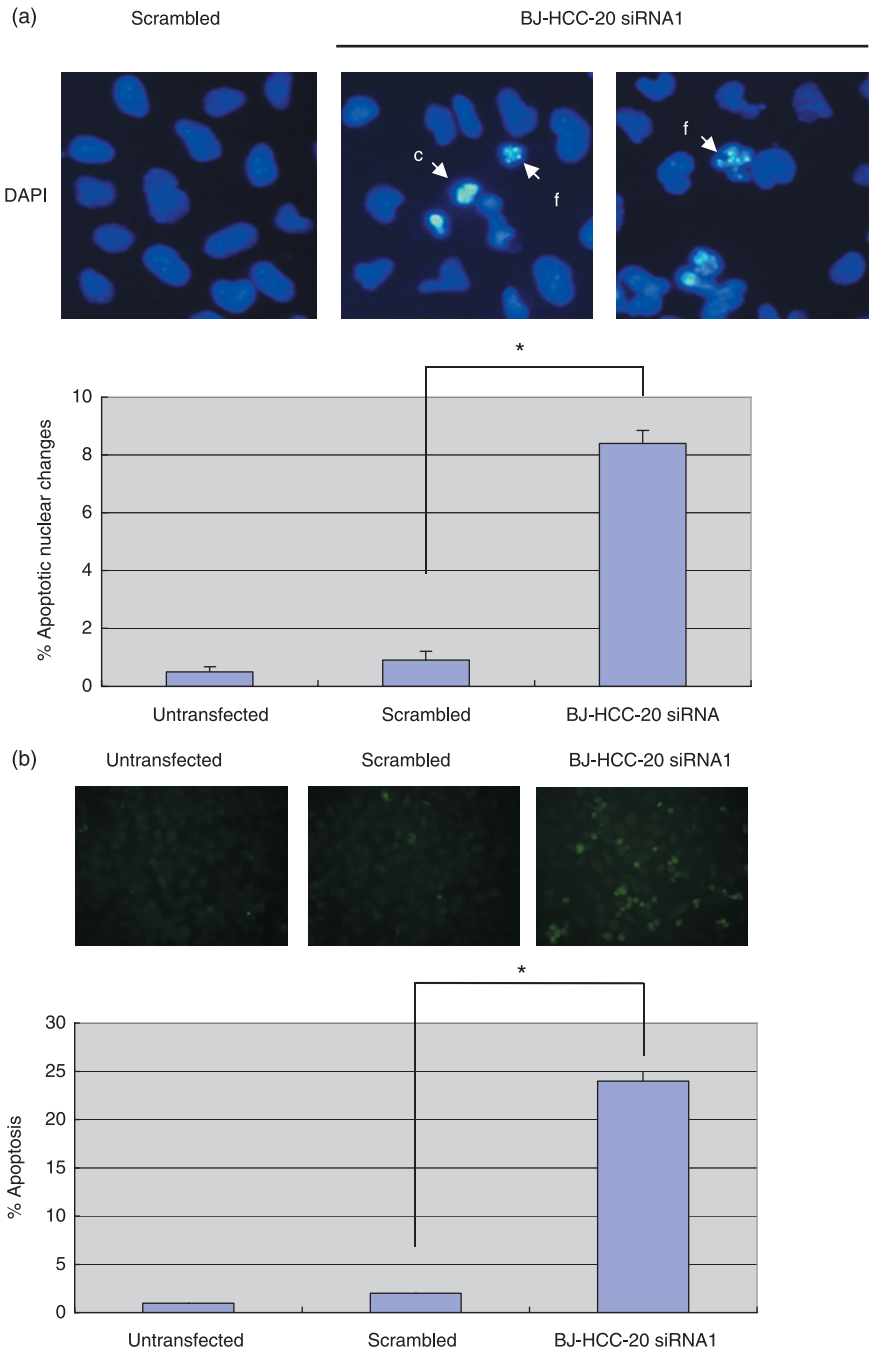

Fig. 5. Downregulation of BJ-HCC-20 causes the induction of apoptosis. (a) Cell morphological changes in the nucleus caused by BJ-HCC-20 small interfering RNA (siRNA). U2OS cells that were transfected with the indicated siRNA for 48 h in the BrdU-incorporation assays were stained with 4′,6-diamidino-2-phenylindole (DAPI). Arrowheads indicate the apoptotic nuclear changes (c, condensation; f, fragmentation). At least 800 cells (nuclei stained with DAPI) from five fields were observed and the percentage of cells exhibiting apoptotic nuclear changes was calculated. The results are shown as the mean ± SD of three independent experiments. Statistical analysis was carried out using Student's *t*-test. $*P < 0.0005$. (b) Induction of cell apoptosis by BJ-HCC-20 siRNA. U2OS cells were transfected with the indicated siRNA for 72 h, and the effects on apoptosis were determined using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay. At least 600 cells (DAPI) from five fields were counted and the percentage of TUNEL-positive cells was calculated. The results are shown as the mean ± SD of three independent experiments. Statistical analysis was carried out using Student's *t*-test. $*P < 0.005$.

in ADR-induced DNA damage inhibits DNA damage-induced apoptosis. Based on these findings, we propose that in the DNA damage/cell apoptosis signaling pathway, downregulation of BJ-HCC-20 promotes the induction of apoptosis and simultaneously interferes with DNA repair by promoting the downregulation of BRCA2, resulting in the acceleration of cell apoptosis. Considering that the enhancement of ADR-induced apoptosis occurs by depletion of BJ-HCC-20 expression using siRNA, in addition to the fact that BJ-HCC-20A is a potential CT antigen, BJ-HCC-20A may serve as a potential target for the treatment of human tumors using DNA-damaging agents coupled with small molecules inhibiting BJ-HCC-20A or siRNA drugs to induce tumor death.

The mechanism by which BJ-HCC-20 may prevent the reduction of BRCA2 expression following DNA damage is not clear. p53 may be involved in the induction of apoptosis and sensitivity to the reduction of BRCA2 expression in response to DNA damage by downregulation of BJ-HCC-20 expression. Interestingly, previous studies have shown that p53 interacts with BRCA2,[12] and is involved in downregulation of BRCA2 mRNA and protein levels in response to DNA damage.[35] This hypothesis requires further investigation.

The binding domain of BRCA2 with BJ-HCC-20A includes five M phase-specific phosphorylation sites by Plk1, and M phase-specific phosphorylation of this domain is shown to modulate the interaction between BJ-HCC-20A and BRCA2. This suggests that the interaction is at least in part regulated by phosphorylation in an M phase-dependent manner, and may provide new insights into the involvement of BRCA2 in the DNA damage/cell apoptosis pathway. Importantly, the phosphorylation status of the binding domain by Plk1 may be crucial in regulating

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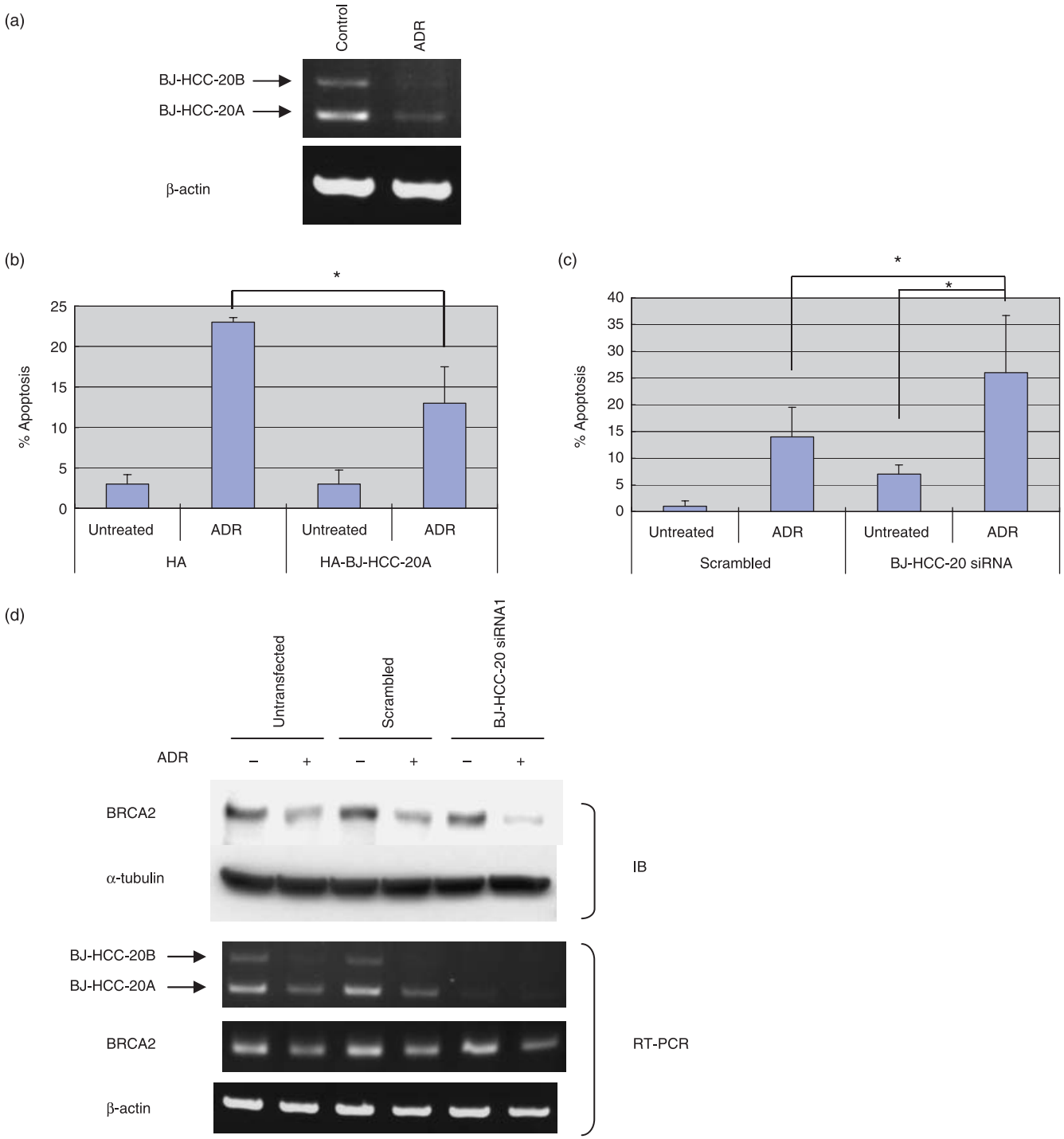

Fig. 6. Involvement of BJ-HCC-20 in apoptosis in response to DNA damage. (a) BJ-HCC-20 expression in response to DNA damage. U2OS cells were untreated or treated with 0.5 μg/mL adriamycin (ADR) for 24 h. A reverse transcription–polymerase chain reaction (RT-PCR) assay of the total RNA was carried out using primer sets for BJ-HCC-20 or β-actin. (b) U2OS cells were transfected with the indicated plasmids for 24 h and then untreated or treated with 0.5 μg/mL ADR for 24 h. Cell apoptosis was then determined using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay. At least 300 cells (4′,6-diamidino-2-phenylindole) from three fields were counted and the percentage of TUNEL-positive cells was calculated. The results are shown as the mean ± SD of three independent experiments. Statistical analysis was carried out using Student's *t*-test. **P* < 0.05. (d) U2OS cells were transfected with the indicated small interfering RNA (siRNA) for 24 h and then untreated or treated with 0.5 μg/mL ADR for 24 h. Cell lysates were analyzed by immunoblotting with anti-BRCA2 or anti-α-tubulin antibodies. A RT-PCR assay of the total RNA was carried out using primer sets for BJ-HCC-20, BRCA2 or β-actin. IB, immunoblot.

the interaction of BJ-HCC-20A with the binding region of BRCA2. We found that ADR treatment resulted in reduced expression of BRCA2 and BJ-HCC-20A, which induced cell apoptosis in response to DNA damage. The complex of BRCA2 and BJ-HCC-20A may regulate the induction of cell apoptosis after DNA-damaging treatment in S phase. None or low levels of phosphorylation in S phase are likely to be important for regulating cell apoptosis by this complex. Conversely, phosphorylation of the binding domain during mitosis may be responsible for inactivation of the DNA damage/cell apoptosis pathway.

In conclusion, we found that the BJ-HCC-20A protein promotes cell growth and is involved in the inhibition of apoptosis induced by DNA damage, and may regulate the induction of cell apoptosis in response to DNA damage in cooperation with BRCA2 in an M phase-dependent manner. Therefore, we speculate that targeting BJ-HCC-20A may aid in the treatment of breast tumors.

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