Constitutive activation of JAK–STAT3 signaling by BRCA1 in human prostate cancer cells

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Abstract Germ-line mutations of the breast cancer susceptibility gene 1 (*BRCA1*) confer a high risk for breast and ovarian cancer in women and prostate cancer in men. The BRCA1 protein contributes to cell proliferation, cell cycle regulation, DNA repair and apoptosis; however, the mechanisms underlying these functions of BRCA1 remain largely unknown. Here, we showed that, in Du-145 human prostate cancer cells, enhanced expression of BRCA1 resulted in constitutive activation of signal transducer and activator transcription factor 3 (STAT3) tyrosine and serine phosphorylation. Moreover, Janus kinase 1 (JAK1) and JAK2, the upstream activators of STAT3, were also activated by BRCA1. Immunoprecipitation assay showed that BRCA1 interacted with JAK1 and JAK2. Blocking STAT3 activation using antisense oligonucleotides significantly inhibited cell proliferation and triggered apoptosis in Du-145 cells with enhanced expression of BRCA1. These findings indicate that BRCA1 interacts with the components of the JAK–STAT signaling cascade and modulates its activation, which may provide a new critical survival signal for the growth of breast, ovarian and prostate cancers in the presence of normal BRCA1.

Key words: Breast cancer susceptibility gene 1; Janus kinase; Signal transducer and activator transcription factor 3; Breast cancer; Prostate cancer

1. Introduction

Germ-line mutations of the breast cancer susceptibility gene 1 (*BRCA1*) on human chromosome 17q21 confer a high risk for breast and ovarian cancers in women and prostate cancer in men [1–5]. BRCA1 encodes a 1863 amino acid, 220 kDa nuclear phosphoprotein containing a conserved amino-terminal RING finger domain and an acidic carboxyl-terminal transcriptional activation domain [6,7]. The BRCA1 protein acts as tumor suppressor and plays important roles in cell proliferation, cycle regulation, DNA damage and repair, genetic instability and various transcriptional pathways, although its precise functions have not been elucidated (reviewed in [8–11]). Brca1 (−/−) mice with targeted deletion of Brca1 developed normally and were fertile, but died by embryo day 7.5, suggesting that Brca1 is essential for embryonic cell proliferation in mice [12]. Similar findings were reported by other researchers, confirming the importance of Brca1 in development [13,14]. Overexpression of BRCA1 inhibited cell growth and conferred increased susceptibility to apoptosis caused by cytotoxic agents [15–17]. Our recent studies indicated that BRCA1 suppressed the transcriptional activity of the estrogen receptor (ER-α), suggesting a role for BRCA1 in regulating estrogen-stimulated mammary cell growth and a specific mechanism of hormone organ cancer suppression [18]. Although it has been known that some BRCA1 functions may be associated with its interaction with cell regulatory genes and factors, such as cyclins, cyclin-dependent kinases, Rad51, c-Myc, RB, p53 and p53-targeted genes and others, the mechanisms of these activities are not well understood (reviewed in [8–11]).

The signal transducer and activator transcription factor (STAT) signaling pathway can be activated by more than 35 cytokines and growth factors, and plays critical roles in a wide variety of cellular functions in the hematopoietic, immunologic, neuronal and hepatic systems (reviewed in [19–21]). It is well established that the binding of a cytokine or growth factor to its receptor activates the receptor-associated tyrosine kinases, known as JAK1–3 and Tyk2, followed by activation of STATs (reviewed in [19–21]). Tyrosine and serine phosphorylation of STATs are required for their transactivation [22,23], nuclear translocation [24], DNA binding [25,26] and dimerization [27]. In addition, many viral oncoproteins, including v-Src, v-Abl, HTLV-1, v-Ros and v-Eyk, have been shown to activate STATs [28–32]. Constitutively activated STATs have been identified in many tumor cell lines and primary tumors, including breast and prostate cancer [33–36]. The essential role of constitutive activation of STAT3 in tumorigenesis is clearly demonstrated by the fact that overexpression of constitutively activated STAT3 in 3Y1 cells caused cell transformation and tumor formation [37]. Recent studies have also shown that BRCA1 can interact with STAT1 and regulate the transcription of a subset of interferon-gamma (IFN-γ) target genes [38]. In this study, we inves-

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Abbreviations: BRCA1, breast cancer susceptibility gene 1; JAK–STAT, Janus kinase–signal transducer and activator transcription factor; IP, immunoprecipitation; IL-6, interleukin-6; TCN, tetracycline

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tigated whether BRCA1 affects activation of STAT3, JAK1 and JAK2. We found that BRCA1 physically interacted with JAKs followed by activation of STAT3 in Du-145 human prostate cancer cells. Moreover, inactivation of STAT3 inhibited cell proliferation and induced apoptosis in BRCA1-overexpressing Du-145 cells.

2. Materials and methods

2.1. Cell culture and transfection

The Du-145 human prostate cell line was obtained from ATCC (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin G. The establishment and characterization of stably transfected Du-145 cell clones with BRCA1 were described previously [17]. An expression vector encoding a wild-type (wt) BRCA1 cDNA was generated by inserting the corresponding polymerase chain reaction (PCR)-generated full-length human BRCA1 cDNA into pcDNA3 plasmid (*Hin*dIII–*Not*I sites) containing a *neomycin*-resistant gene (kindly provided by M. Erdos, NIH, Bethesda, MD, USA). Du-145 cells were transfected with wt BRCA1-pcDNA3 plasmid or control pcDNA3 plasmid using lipofectamine (Life Technologies) and selected in medium containing 0.5 μg/ml G-418. G-418-resistant clones were isolated, expanded and screened by immunoblot assay and semi-quantitative reverse transcription-PCR assay. Clones expressing the highest expression of BRCA1 protein were chosen for further studies. A tetracycline (TCN)-regulated BRCA1 expression system was developed using a Tet-Off system (Clontech), which has two components: (1) a regulator plasmid pTet-Off, which expresses a fusion of the Tet repressor and VP16 activation domain of herpes simplex virus under the control of the strong immediate early CMV promoter and (2) a response plasmid pTRE, which contains the Tet-responsive element (TRE) upstream of the minimal immediate early CMV promoter and a multicloning site. Du-145 cell clones with transfection of TCN-inducible BRCA1 plasmid were established by two separate stable transfections, first plasmid pTet-Off and secondly plasmid pTRE with full-length BRCA1 cDNA cloned into the pTRE vector (pTRE-BRCA1). Tet-Off/BRCA1 Du-145 cell clones with double transfections were screened for BRCA1 expression induced by removal of TCN and maintained in medium containing 2 ng/ml TCN.

2.2. Immunoprecipitation (IP) and immunoblot analysis

Total cell extracts were prepared in JAK lysis buffer (25 mM HEPES, pH 7.5, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Nonidet P-40, 0.1% (v/v) 2-mercaptoethanol, 10 nM okadaic acid, 5 mM benzamidine, 0.5 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml antipain, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The concentration of protein was determined with the Bradford reagent (Bio-Rad). The extracts were subjected to IP for 16 h at 4°C with antibodies to BRCA1 (Ab1+Ab2+Ab3, Oncogene), JAK1 or JAK2 (Upstate Biotechnology, Lake Placid, NY, USA). After the addition of protein A/G agarose (Life Technologies, NY, USA), the reaction mixtures were incubated for an additional 4 h. The immunoprecipitates were extensively washed with JAK lysis buffer and subjected to electrophoresis through sodium dodecyl sulfate (SDS)–polyacrylamide gel. The separated proteins were then transferred to a nitrocellulose membrane followed by immunoblot analysis.

Immunoblot analysis was described previously [39]. Cells were resuspended in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, 1% Nonidet P-40, 10% glycerol) and then centrifuged for 10 min at 4°C. Protein concentration was quantitated using the Bradford reagent (Bio-Rad). An aliquot of 40 μg of protein was mixed with an equivalent volume of 2× protein loading buffer containing β-mercaptoethanol and boiled for 5 min before loading onto an SDS/8% polyacrylamide gel. STAT3, JAK1, JAK2 and anti-phosphotyrosine antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phospho-STAT3 antibodies (Tyr^{705} and Ser^{727}) were obtained from New England Bio-Lab. Protein bands were revealed by using an enhanced chemiluminescence detection system (Amersham Life Science) as instructed by the manufacturer.

2.3. DNA mobility gel shift assay (DMSA)

Activation of STAT3 DNA binding was determined by DMSA, as described previously [40], using oligo m67 (a high affinity serum induce element m67) (5′GTG CAT TTC CCG TAA ATC TTG TCT ACA3′).

2.4. JAK autophosphorylation assay

Exponentially growing cells were harvested, washed twice with phosphate buffered saline containing 1 mM Na_3VO_4, and lyzed in 0.5 ml of lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1% Nonidet P-40, 10% glycerol). Immunoprecipitated complexes were washed twice with lysis buffer and once with kinase buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1 mM Na_3VO_4). Pellets were resuspended in 50 μl of kinase buffer containing 5 μCi [γ-^{32}P]ATP (Dupont, NEN) and incubated at 30°C for 10 min. Beads were washed twice with 500 μl of stop buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA), and then boiled in SDS sample buffer containing 2.5% 2-mercaptoethanol for 5 min. The solubilized proteins were resolved by SDS–PAGE, followed by autoradiography.

2.5. [^{3}H]Thymidine uptake

Following transfection of cells with STAT3 sense or antisense oligonucleotides for 48 h, [^{3}H]thymidine (2–5 μCi/ml) was added to the culture for 2 h. Cells were then dissolved by the addition of 1.0 ml of 0.5 N NaOH, collected, mixed with 1.5 ml of H_2O, and precipitated with 0.5 ml of 50% trichloroacetic acid (TCA). The precipitate was collected on glass filters, washed twice with 5% TCA, and the retained radioactivity determined by liquid scintillation spectrometry. Each time point for each set of experiment was determined in triplicate.

2.6. Apoptosis assay

Cell apoptosis was measured by 3′-end labeling DNA with terminal deoxynucleotidyl transferase (Life Technologies) as described previously [41]. Briefly, genomic DNA was isolated and subjected to labeling reaction, which was performed in a final volume of 50 μl, containing 31 μl DNA, 10 μl 5×reaction buffer (1 M potassium cacodylate, 0.125 M Tris–HCl, 1.25 mg/ml bovine serum albumin; pH 6.6), 5 μl $CoCl_2$, 5 μl [α^{32}P]ddATP (17 pmol; 50 μCi), and 1 μl (25 U) terminal transferase enzyme. The reaction is allowed to proceed for 60 min at 37°C, and terminated by addition of 5 μl 0.25 M EDTA (pH 8.0). Labeled DNA is separated from unincorporated radionucleotide by using Chroma spin column (Clontech, Palo Alto, CA, USA). The labeled sample is loaded onto a 2% agarose gel and separated by electrophoresis. The gel is then dried and exposed to X-ray film. The amount of radiolabeled ddATP incorporated into the high (>20 kb) and low (<20 kb) molecular weight DNA fractions can be quantitated by cutting the respective fraction of DNA from the dried gel and counting for 1 min in a β-counter.

3. Results and discussion

To investigate whether BRCA1 interacts with STAT3, we first compared the expression of BRCA1 protein in Du-145 human prostate cancer cells: parental, control pcDNA3 vector transfected (Neo) clones and wt BRCA1 transfected (wtBRCA1) clones. Parental Du-145 cells contain a low basal level of endogenous BRCA1 and stable transfection of full-length BRCA1 cDNA significantly increased the BRCA1 protein expression (Fig. 1a). Establishment and characterization of the transfection clones have been previously described in detail [17]. Du-145 clones transfected with wtBRCA1 exhibited only a slight decrease in growth rate (average doubling time = 22 h), as compared with Du-145 parental cells and the control Neo vector transfected clones (average doubling time = 25 h). As a set of representative results shown in Fig. 1a, enhanced expression of BRCA1 resulted in the constitutive activation of STAT3 binding in all wtBRCA1 clones compared to Neo clones. To further define the molecular mechanism by which enhanced expression of BRCA1 activates

STAT3, wtBRCA1 clone 2 (BRCA1–2) was chosen for further experiment. As shown in Fig. 1b, significant tyrosine (Tyr^{705}) and serine (Ser^{727}) phosphorylation of STAT3 were observed in BRCA1–2 clone but not in parental or Neo clones, while the levels of STAT3 protein expression were unaffected in all three group cells.

The constitutive activation of STAT3 in Du-145 clones over-expressed with wtBRCA1 may have occurred during

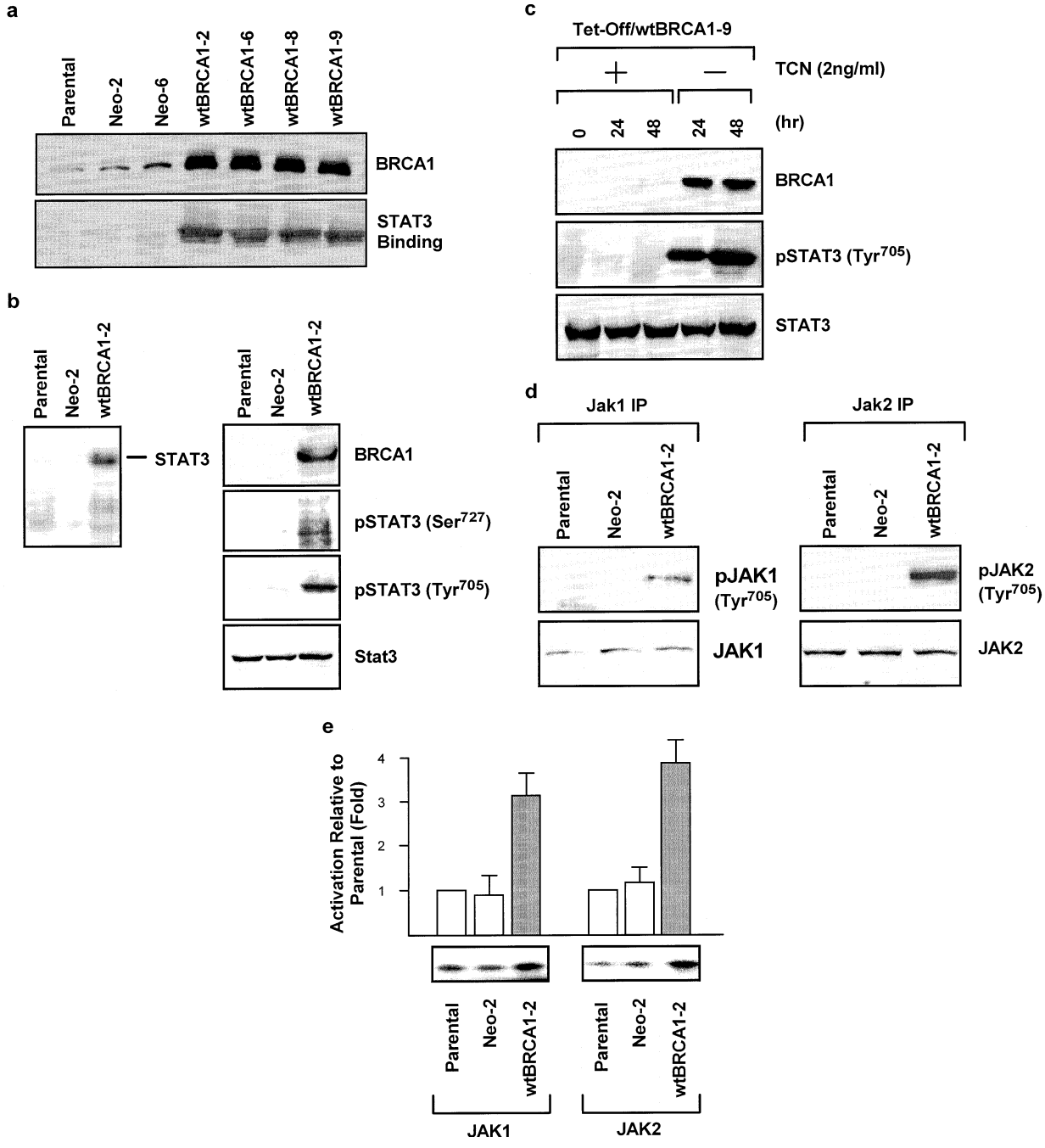

Fig. 1. Constitutively active JAK–STAT3 in Du-145 human prostate cancer cells containing overexpression of BRCA1. Clones were established and characterized in Du-145 cells stably transfected with control pcDNA3 vector containing *neomycin* resistance gene (Neo) or full-length wtBRCA1 cDNA (BRCA1) as described previously [17]. a: Exponentially growing Du-145 parental cells, two pcDNA3 vector transfected clones and four wtBRCA1 clones were harvested for immunoblot analysis using BRCA1 antibody (C-20, Santa Cruz Biotechnology, CA, USA) or DMSA using m67 oligo as a probe. b: Exponentially growing parental Du-145 cells, Neo-2 clone and BRCA1–2 clone were harvested for Western blotting. c: Du-145 cells were stably transfected with an inducible BRCA1 in TCN-off vector as described in Section 2. BRCA1 transfected Du-145 cells were grown in the growth media in the presence (+Tet) or the absence (−Tet) of TCN (2 ng/ml) for the indicated time. Total cell extracts were prepared and subjected to immunoblot analysis with anti-BRCA1, anti-STAT3, and anti-phospho-STAT3 (Tyr^{705}). d and e: Total cell extracts were prepared from parental Du-145 cells, Neo-2 clone and BRCA1–2 clone, respectively, and subjected to IP with antibodies to JAK1 or JAK2. The resulting immunoprecipitates were then subjected to immunoblot analysis with anti-phosphotyrosine antibodies (d), or subjected to in vitro autophosphorylation assay (e). The radioactivities on the blots were quantified by phosphorimaging. Values shown are means ± S.E.M. from three independent experiments, expressed as fold changes over parental control.

the process of clonal selection. To distinguish changes that occur within days from those occurring more slowly, we established clones with TCN-regulated wtBRCA1 expression as described in Section 2. As a set of representative results shown in Fig. 1c, in Tet-Off/wtBRCA1 clone 9 (Tet-Off/wtBRCA1-9), removal of TCN (−TCN) from the culture medium markedly increased expression of BRCA1 protein and activated the tyrosine (Tyr^{705}) phosphorylation of STAT3, while it did not affect STAT3 protein expression. Similar results were obtained in other Tet-Off/wtBRCA1 clones (data not shown). These observations, consistent with the assays of stably transfected Du-145 clones shown in Fig. 1a,b, suggest that BRCA1 does constitutively activate STAT3. Interestingly, other STATs, such as STAT1 and STAT5 were not activated in either transiently or stably transfected Du-145 cells with wtBRCA1 (data not shown).

We next determined whether the constitutive activation of STAT3 in wtBRCA1-overexpressing Du-145 cells is triggered by activation of STAT3 upstream activators, JAK1 and JAK2, by BRCA1. The activation of JAK1 and JAK2 was determined using either IP-immunoblot assay (Fig. 1d) or kinase assay (Fig. 1e). As shown in Fig. 1d, significant tyrosine phosphorylation of JAK1 or JAK2 protein was observed in the immunoprecipitates from wtBRCA1 clones compared to Du-145 parental cells and control Neo clones. JAK1 and JAK2 activities were also markedly increased in BRCA1 clones as demonstrated by an in vitro kinase assay (Fig. 1e). These findings indicate that the constitutive activation of STAT3 may result from the BRCA1 activation of JAK1 and JAK2.

To determine whether BRCA1 activation of JAK1 and JAK2 is due to direct physical interaction of BRCA1 with JAKs, an IP assay was performed. Cell extracts from control Neo clones or BRCA1 transfected clones were immunoprecipitated by using BRCA1, JAK1 or JAK2 antibody and followed by immunoblot analysis with JAK1, JAK2 or BRCA1. As a set of representative results shown in Fig. 2, both JAK1 and JAK2 proteins were detected in BRCA1 immunoprecipitation of BRCA1 transfected clone (BRCA1–2), but not of the control Neo clone (Neo-2). Under the same condition, STAT3 and gp130 were not co-precipitated with BRCA1 (data not shown). BRCA1 protein was also detectable in JAK1 and JAK2 immunoprecipitation. These findings indicate that BRCA1 is physically associated with JAK1 and JAK2.

Interleukin-6 (IL-6) has been found to activate STAT3 in prostate cancer cells [42,43], we thus determined whether the constitutive activation of STAT3 in wtBRCA1 clones is affected by IL-6 stimulation. Control Neo clones and BRCA1 transfected clones were exposed to IL-6 in the presence of or in the absence of IL-6 antibody and then subjected to immunoblot analysis. As a set of representative results are shown in Fig. 3a, IL-6 significantly activated tyrosine (Tyr^{705}) phosphorylation of STAT3 in parental Du-145 cells and Neo clones, and slightly increased tyrosine phosphorylation of STAT3 in wtBRCA1 Du-145 clones as well. Neutralizing antibody to IL-6 abrogated the IL-6-activated tyrosine phosphorylation of STAT3 in parental Du-145 cells or Neo clones, but did not cause any influence on the constitutive activation of STAT3 in wtBRCA1 clones (Fig. 3b). The level of the *IL-6* gene expression was similar in Neo clones and in BRCA1 transfected clones (data not shown). These findings suggest

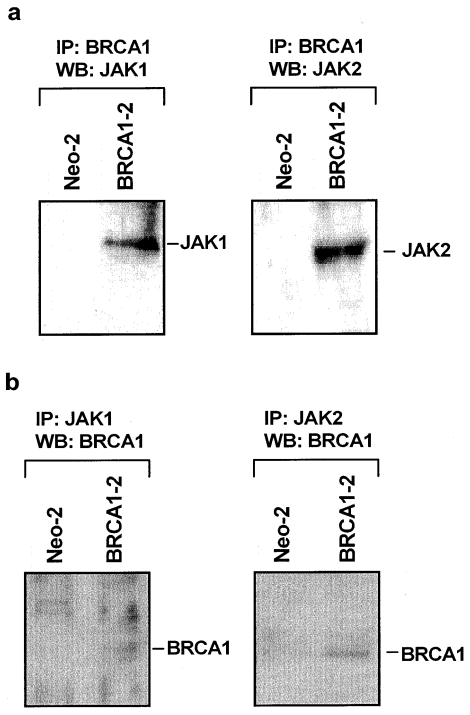

Fig. 2. Co-precipitation of BRCA1 with JAK1 and JAK2. Total cell extracts were prepared from control Neo-2 clone and BRCA1–2 clone in JAK lysis buffer and subjected to IP with antibodies to BRCA1. The resulting immunoprecipitates were subjected to immunoblot analysis with antibodies to JAK1 or JAK2 as indicated.

that the constitutive STAT3 activation in wtBRCA1 Du-145 clones is not due to IL-6 stimulation.

It has been shown that BRCA1 inhibited or delayed growth of breast and ovarian cancer cells and enhanced apoptosis induction in response to cytotoxic agents, including anti-cancer agents [8–11]. As described previously [17], overexpression of BRCA1 only caused a slightly decrease in the proliferation rate of Du-145 cells (average doubling time = 25 h for Neo clones and 22 h for BRCA1 clones). However, BRCA1 significantly increased apoptosis induction after DNA damage caused by anti-cancer agents. Since constitutively active STAT3 has been shown to play a critical role in cell transformation and tumor formation [37], we, therefore, determined whether constitutively active STAT3 exerts any effects on the proliferation and apoptosis of Du-145 cells in the presence of wtBRCA1. As shown in Fig. 4, blocking STAT3 expression using STAT3 antisense oligonucleotides significantly inhibited cell proliferation and induced apoptosis in BRCA1 clones but not in Neo clones, while sense STAT3 oligonucleotides did not significantly affect the proliferation or apoptosis in both Neo- and BRCA1 clones. These findings indicate that the constitutive activation of STAT3 by BRCA1 is a survival signal for escaping the tumor suppressing activity of BRCA1. Although all the results described above are obtained from one clone (Neo-2, wtBRCA1–2, Tet-Off/wtBRCA1-9), similar findings were also observed in several other transfected clones.

Taken together, we demonstrate for the first time that BRCA1 associates with JAK1 and JAK2 in vivo, and constitutively activated the JAK–STAT3 signaling pathway in Du-145 cells. Abrogation of STAT3 activation markedly inhibited cell proliferation and induced apoptosis in wtBRCA1 cells compared to control Neo cells. Therefore, these novel findings

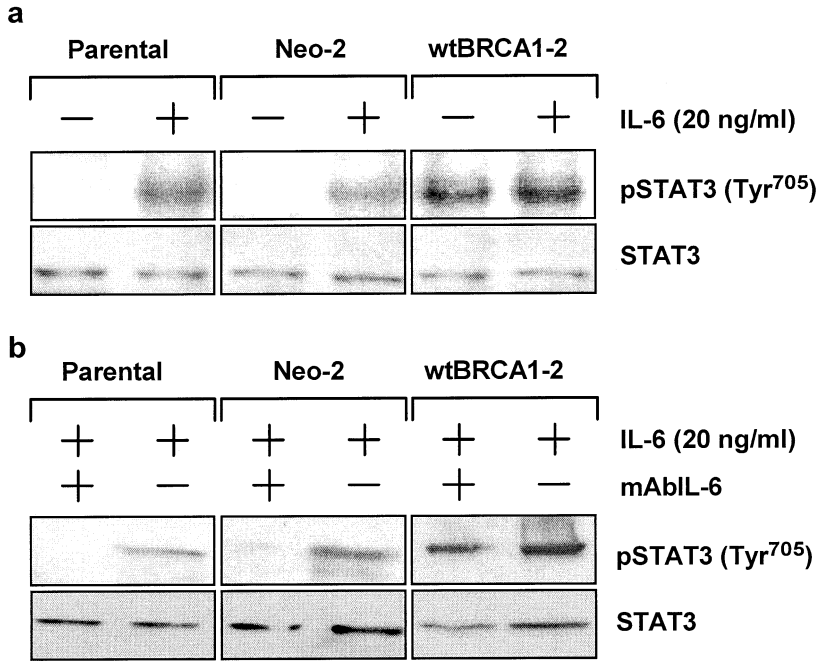

Fig. 3. Constitutively active STAT3 is not due to IL-6 stimulation. a: Parental Du-145 cells, control Neo-2 clone, or BRCA1–2 clone were exposed to IL-6 (20 ng/ml) for 30 min, total cell extracts were then subjected to immunoblot analysis with antibodies to STAT3 tyrosine phosphorylation (Tyr^{705}) or STAT3. b: Parental Du-145 cells and control Neo-2 clone were exposed to IL-6 (20 ng/ml) for 8 h in the presence or in the absence of anti-IL-6 antibody. BRCA1 transfected cells were incubated directly with anti-IL-6 antibody for 24 h. Total cell extracts were then subjected to immunoblot analysis with antibodies to STAT3 tyrosine phosphorylation (Tyr^{705}) or STAT3.

suggest that BRCA1 promotes cell survival signal via activating the JAK–STAT3 signal pathway, which may provide a new mechanism for the growth of breast, ovarian and prostate cancers in the presence of normal BRCA1, since 90% sporadic breast cancers express normal BRCA1 [8–11]. Indeed, several breast cancer cell lines and primary breast tumors express constitutively active STAT3 [33–36], which has been proposed as a target for tumor therapy [33]. Our unpublished data showed that several prostate cancer cell lines also expressed constitutively active STAT3. Whether endogenous BRCA1 is responsible for the constitutive activation of STAT3 in these cells remains unknown. We have tried to examine whether suppression of *BRCA1* gene expression attenuated constitutive STAT3 activation in these cells. However, unfortunately, we failed to knock out *BRCA1* gene expression by either using antisense oligonucleotides or over-expressing BRCA1 antisense mRNA in prostate cancer cells, this is probably due to the fact that BRCA1 mRNA is very large and may contain

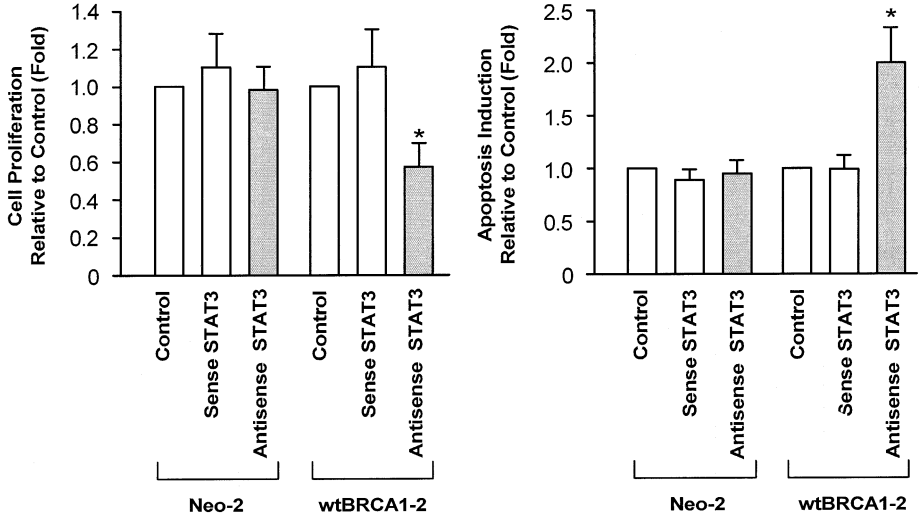

Fig. 4. Constitutively active STAT3 mediates the proliferative and survival signals in BRCA transfected cells. wtBRCA1 or control pcDNA3 vector (neo) transfected Du-145 cells were transfected with sense STAT3 (5′CCATTGGGCCATCCTGTTTCT3′) or antisense STAT3 (5′AGAAACAGGATGGCCCAATGG3′). After 48 h, cells were subjected to proliferation or apoptosis assay. Proliferation assay was conducted by [^{3}H]thymidine incorporation into DNA. Apoptosis was conducted by measuring DNA fragmentation as described Section 2. Values shown in the panel are means ± S.E.M. from three different experiments, expressed as fold changes over corresponding control. Significant difference from corresponding control is indicated by asterisks: $*P < 0.01$.

a secondary structure. Further experiments are required to demonstrate whether endogenous BRCA1 is responsible for STAT3 activation in breast and prostate cancer cells. If endogenous BRCA1 was able to constitutively activate STAT3, the next obvious question is why normal breast and ovarian tissues that express normal BRCA1 do not contain active STAT3. The reason for this is not clear. One possibility is that the tumor cells express an adapter protein(s), which is required for BRCA1-mediated STAT3 activation but does not express in normal tissue cells.

It has been shown that the JAK–STAT signaling pathway can be activated by more than 35 cytokines and growth factors, and this activation is transient [19–21]. Recently, Ouchi et al. [38] reported that BRCA1 is able to bind to the STAT1 transcriptional activation domain and is required for induction of a subset of IFN-γ target genes. Although the possible contribution of other cytokines or growth factors to BRCA1 activation of JAK–STAT3 cannot be ruled out, two lines of evidence suggest that IL-6 stimulation may not be involved. First, we found that IL-6 monoclonal antibody can abrogate IL-6-activated STAT3, but failed to block wtBRCA1-activated STAT3 in Du-145 cells. Second, the *IL-6* gene expression was the same in Neo and BRCA1 clones (unpublished data). In addition to the cytokines and growth factors, many viral oncoproteins have been shown to activate STATs, including v-Src [28], v-Abl [29], HTLV-1 [30], v-Ros [31] and v-Eyk [32]. Whether these oncoproteins are involved in BRCA1-mediated STAT3 activation requires further investigation.

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