Journal of Mammary Gland Biology and Neoplasia (2019) 24:257–270
https://doi.org/10.1007/s10911-019-09431-5

BRCA1 Attenuates Progesterone Effects on Proliferation and NFκB Activation in Normal Human Mammary Epithelial Cells

H. N. Hilton[1] · L. J. Patterson McDonald[1] · N. Santucci[1] · F. R. van der Bent[2] · A. Silvestri[1] · J. D. Graham[1] · C. L. Clarke[1]

Received: 15 August 2018 / Accepted: 21 April 2019 / Published online: 18 May 2019

Abstract

Germline mutations in the breast cancer susceptibility gene *BRCA1*, encoding a tumor suppressor protein, greatly enhance the risk of breast and ovarian cancer. This tissue-specificity implicates the role of ovarian hormones. Indeed, BRCA1 has been demonstrated to regulate the signalling axis of the hormone, progesterone, and its receptor, the progesterone receptor (PR), and progesterone action has been implicated in BRCA1-related tumorigenesis. BRCA1 also plays important roles in oxidative stress and activating nuclear factor kappaB (NFκB) signalling pathways. Like wildtype BRCA1 function, PR signalling has also been shown to inhibit NFκB activation. Although PR and BRCA1 networks are known to interact, their interaction at the level of NFκB activation in the human breast is not understood. This study investigates the effect of reduced BRCA1 expression on proliferation and NFκB activation in human breast cells, and the impact of progesterone on these effects. The major findings are that: 1) Reduced BRCA1 levels inhibit cell growth in normal human mammary cells and breast cancer cells; 2) Reduced BRCA1 levels stimulated inflammatory targets and NFκB activity in normal human mammary cells; 3) Wildtype BRCA1 inhibited the pro-proliferative effects of progesterone in normal mammary epithelial cells, and; 4) Progesterone attenuated BRCA1-mediated NFκB activation in normal human mammary cells. These data have important implications for our understanding of progesterone action in BRCA1 mutation carriers, and how inhibition of this action may potentially delay tumorigenesis or impart a more favourable prognosis.

Keywords Progesterone · BRCA1 · Breast cancer · NFκB · Proliferation

Introduction

Breast cancer remains the most common cancer in women worldwide, and germline mutations in the breast cancer susceptibility gene, *BRCA1*, encoding a tumor suppressor protein, significantly enhance the risk of breast and ovarian cancer. Furthermore, BRCA1 mutations have also been identified as drivers in a subset of sporadic breast tumors [1]. BRCA1 function (including roles in DNA repair, cell cycle checkpoint control and transcriptional regulation [2]), displays striking tissue-specificity, with mutation carriers having dramatic increases in susceptibility to both breast and ovarian cancer, thereby implicating the role of the ovarian hormones [3]. One of these hormones is progesterone, which plays a pivotal role in normal female reproduction. Although recognised to be critical in the growth and proliferation of the breast during normal development, signalling through the progesterone receptor (PR) has been implicated in breast cancer, and synthetic progesterone analogues have been associated with increased breast cancer risk [4].

Despite mutations in BRCA1 contributing to tumor formation via the genomic instability associated with impaired DNA damage repair machinery, it is interesting that in women this susceptibility only occurs specifically in two hormone-related cancers, that is, breast and ovarian tumors, suggesting that tissue-specific interactions between BRCA1 and ovarian hormone signalling pathways may be implicated in this effect. Indeed, BRCA1 has previously been demonstrated to regulate signalling of the progesterone pathway, via PR [5].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10911-019-09431-5) contains supplementary material, which is available to authorized users.

✉ J. D. Graham
dinny.graham@sydney.edu.au

[1] Centre for Cancer Research, The Westmead Institute for Medical Research, Sydney Medical School – Westmead, The University of Sydney, Westmead, NSW 2145, Australia

[2] Department of Medicine, Academic Medical Center, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Specifically, wild-type BRCA1, but not mutant BRCA1, inhibited PR activity in human breast cancer cell lines, knockdown of BRCA1 enhanced progesterone-stimulated PR activity, and BRCA1 directly interacted with PR. Importantly, exogenous progesterone stimulated proliferation in the mammary glands of Brca1-deficient mice [5] and RU486, a PR antagonist, prevented mammary tumorigenesis in Brca1/p53-deficient mice [6], providing in vivo evidence that PR signalling is involved in BRCA1-related tumorigenesis. Finally, BRCA1 mutation carriers are reported to have higher levels of serum progesterone [7], and normal epithelium adjacent to mutant BRCA1 tumors, or from prophylactic mastectomies in BRCA1 mutation carriers, display dysregulated PR expression [8–11]. Thus, altered PR signalling may be a factor in the increased risk of cancer in BRCA1-related breast tumorigenesis.

The role of BRCA1 in cell cycle checkpoint control underpins the observations of aberrant growth in cells which harbour a BRCA1 mutation, however these effects can vary and are dependent on the cellular type and context, levels of BRCA1 as well as experimental conditions. While some studies have shown that reduced levels of BRCA1 allowed increased cell proliferation [12, 13], others have shown that conversely, reduced levels or haploinsufficiency of BRCA1 suppressed cell proliferation by inducing premature senescence [14–17]. Progesterone is also critical in breast proliferation during normal development, clearly illustrated by the PR knockout mouse model, which displays severely limited lobuloalveolar development [18]. Progesterone is now recognised as a major proliferative hormone in both the mouse mammary gland and the normal human breast [4], and is required to promote the massive proliferation which occurs during early pregnancy. The influence of progesterone on proliferation is cell- and tissue-specific; for example in the normal human breast, progenitor cells are stimulated to proliferate [19], whereas in human breast cancer cell lines, progesterone can exert proliferative or anti-proliferative effects in a context-dependent manner [20–22]. Whether there is functional overlap in the proliferative roles of BRCA1 and progesterone in human breast remains uncertain.

In addition to roles in DNA damage repair and cell cycle checkpoint control, BRCA1 has also been shown to provide protection against oxidative DNA damage due to increased levels of reactive oxygen species [23], while conversely, inactivation of BRCA1 has been shown to induce high levels of oxidative stress [24, 25]. This particular function of BRCA1 has been suggested to contribute to its role as a tumor suppressor, as oxidative stress can activate a variety of signalling pathways (eg. nuclear factor kappaB [NFκB] activation) leading to up-regulation of chemokines, cell cycle regulatory molecules and inflammatory cytokines, and potentially increased susceptibility to tumorigenesis [26]. The NFκB family of transcription factors, which regulates the growth, differentiation and apoptosis of several tissues and is critical in regulating the immune response, has recently been shown to be activated in the absence of BRCA1 in normal murine mammary cells [27] and a subset of human breast tumors [28]. This suggests a role for wildtype BRCA1 in inhibiting activation of the NFκB pathway, which can underpin many hallmark features of cancer, including survival, invasion and metastasis. Similarly, PR signalling has also been shown to inhibit NFκB activation and to antagonise inflammatory response pathways [29, 30]. Whether BRCA1 inhibits NFκB activation in the human breast, and whether progesterone modulates this effect, has not yet been determined. Moreover, whether the interplay between BRCA1 and progesterone signalling proceeds via the same mechanisms in normal breast and breast cancer is unknown. Using normal human mammary epithelial cells and human breast cancer cells, this study investigates the effect of reduced BRCA1 expression on proliferation and NFκB activation, and the impact of progesterone on these effects.

Materials & Methods

Cell Culture

AB32 cells [31] were maintained in 1 : 1 DMEM : Hams-F12 medium (phenol red-free) supplemented with cholera toxin (0.1 μg/ml), insulin (0.28 units/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (0.02 μg/μl), and 5% horse serum. T47D breast cancer cells were purchased from the American Type Culture Collection (atcc.org, Manassas, VA, USA) and maintained in RPMI1640 medium (phenol red-free) containing 10% fetal calf serum and 0.25 units/ml insulin. Lentivirus was generated in human embryonic kidney cells (HEK-293 T), which were grown in DMEM medium containing 20 mM HEPES, 4 mM L-glutamine and 10% fetal calf serum. For hormone treatments, cells were treated for 6 h (unless otherwise indicated) with 100 nM progesterone (Sigma-Aldrich), 10 nM ORG2058 (Amersham Biosciences; GE Healthcare, Rydalmere, Australia), 10 nM MPA and/or vehicle control.

Virus Generation and Transduction

Two individual BRCA1 shRNA sequences were cloned into pSIH-H1-puro expression lentiviral vectors, which express puromycin resistance (System Biosciences, CA, USA). A nonsilencing negative control shRNA in pSIH-H1-puro was used as a control. Lentiviral particles were generated by co-transfecting each BRCA1shRNA-pSIH-H1-puro vector and lentiviral packaging constructs into HEK-293 T cells, and virus was accumulated in the medium for 48–72 h. AB32 and T47D cells were infected using a multiplicity of infection of 5,

and incubated for at least 24 h to allow expression of the BRCA1 shRNAs.

RNA Preparation and Quantitative PCR (qPCR)

RNA was harvested with the innuPREP RNA Mini Kit (Analytik Jena, Germany) or RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific), according to the manufacturer's instructions, and reverse transcribed with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) or High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA was amplified by Platinum SYBR Green qPCR Supermix (Thermo Fisher Scientific) on a Rotor-gene 6000 real-time cycler (Corbett Research, Australia) using the TATA-binding protein (TBP) as an internal control gene. Primer sequences used are shown in Supp Table 1.

RT^2 Profiler PCR Arrays

cDNA was prepared from 0.5 μg of RNA using the RT^2 HT First Strand Kit (Qiagen), as per the manufacturer's instructions. cDNA was added to 2x RT^2 SYBR Green Mastermix and RNAse-free water and 25 μl of the final mixture was transferred to each well of the RT^2 PCR array (Human NFκB Signaling Pathway Plus PCR Array) and assayed using a BioRad CFX96 real-time PCR detection system. All conditions were done in triplicate and normalised to the vehicle control shRNA sample, and results were analysed using web-based analysis software. Changes were considered significant when $p < 0.05$.

Gene Expression Profiling and Bioinformatic Analysis

Total RNA was amplified and labelled using the Illumina™ TotalPrep™ RNA Amplification kit (Thermo Fisher Scientific). Human HT-12 v4 Expression beadchips (Illumina, San Diego, CA, USA) were processed using Illumina reagents and scanned on an Illumina BeadStation scanner. Data was analysed using GenomeStudio version V2009.1 (Illumina, San Diego, CA, USA). Average signal intensities were normalized using the cubic spline function in GenomeStudio (without background subtraction) and the Illumina Custom function was used to obtain a differential expression score and *p* value for each gene. The Database for Annotation, Visualization, and Integrated Discovery online tool, version 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) was used to identify gene ontology categories which were enriched (using high stringency) within differentially expressed gene lists. Unsupervised hierarchical clustering analysis of sets of differentially expressed transcripts, and heatmap visualization were performed using the Morpheus online tool (https://software.broadinstitute.org/morpheus).

Cell Proliferation Assays

Cells which had been transduced with BRCA1shRNA or control shRNA were plated at a density of 1×10^4 (AB32) or 5×10^3 (T47D) cells per well in 96-well plates. The next day cells were treated with progestins or vehicle, as indicated. Cell growth was monitored by IncuCyte (Essen BioScience, Michigan, USA) live-cell imaging system. Images were captured of each well every 4 h continuously for 5–6 days. An in-built confluency algorithm assessed the percentage of confluency, and proliferation was determined as an increase in percentage of confluency.

Immunoblotting

Protein samples were prepared by lysis of whole cells in RIPA buffer (10 mM $NaPO_4$ (pH 7.0), 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 0.1% β-mercaptoethanol) containing 10 mM $NaMoO_4$, 1% aprotinin, 0.5 mM phenylmethylsulfonylfluoride, and rotated for 30 min at 4 °C. Insoluble debris was removed by centrifugation at 14,000×g, 15 min at 4 °C. Protein concentration was estimated using Bradford dye reagent (Bio-Rad, Regents Park, Australia). Lysates were prepared in NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent (Thermo Fisher Scientific) and fractionated on NuPAGE 4–12% Bis-Tris acrylamide gels run in NuPAGE MOPS buffer (Thermo Fisher Scientific), followed by transferring to nitrocellulose membrane. PR was detected using hPRa6 and hPRa7 in-house mouse monoclonal antibodies (1 : 100 each) and goat anti-mouse horseradish peroxidase conjugated secondary antibody (Dakocytomation, Glostrup, Denmark). Protein bands were visualized by chemiluminescent reaction using ECL reagents (Quantum Scientific, Murrarie, Australia) and imaged on a ChemiDoc MP Imaging System (Bio-Rad) followed by analysis on Image Lab software, version 5.2.1 (Bio-Rad).

Immunohistochemistry

For immunofluorescence (IF) staining, the antigens were revealed by incubation of BRCA1 (clone D-9; Santa Cruz Biotechnology) and Ki67 (clone MIB1; Dako) primary antibodies, followed by detection by an appropriate biotinylated secondary antibody (goat anti-mouse; Dako) and a streptavidin-conjugated fluorescent label (Alexa Fluor 594; Thermo Fisher Scientific). Sections were mounted in ProLong Gold antifade reagent containing the nuclear counterstain DAPI (Thermo Fisher Scientific). To ensure antibody specificity, adjacent sections were stained without the primary antibody, using the biotinylated secondary antibody and streptavidin-conjugated fluorescent label only.

Results

Reduced BRCA1 Levels Inhibit Cell Growth in Normal Human Mammary Cells and Breast Cancer Cells

BRCA1 knockdown was demonstrated in T47D breast cancer cells (Supp Fig. 1A-C) and AB32 cells, a stable PR expressing clone of the MCF-10A immortalized normal breast cell line [31] (Fig. 1a, Supp Fig. 1D). To determine the effect that reduced BRCA1 levels had on the growth of the AB32 cells, we monitored cell proliferation in real-time following transduction with a pool of two individual BRCA1 shRNA constructs using the IncuCyte live-cell imaging system. Compared to controls, a reduction in BRCA1 levels significantly decreased cell proliferation ($p = 0.0001$ at 92 h; two-tailed *t*-test), with BRCA1 knockdown cells showing virtually no increase in cell confluency over 5 days (Fig. 1b and c). This was also supported by decreased immunofluorescent staining of the proliferation marker, Ki67, in AB32 cells after BRCA1 knockdown, compared with control AB32 cells (data not shown). This is consistent with the critical requirement for BRCA1 in regulation of cell growth and cell cycle checkpoint control. To determine whether BRCA1 deficiency had a similar effect in human breast cancer cells, T47D cells were transduced with BRCA1 shRNA constructs (Supp Fig. 1A-C), and real-time cell growth curves determined using the IncuCyte live-cell imaging system showed that reduced BRCA1 also decreased cell proliferation in T47D cells (Fig. 2). These data showed that BRCA1 was required for cell proliferation in both normal and cancer cells.

To identify the processes regulated when BRCA1 levels were manipulated in AB32 cells, we performed whole genome expression profiling. This revealed that 6603 gene identifiers (IDs) were significantly differentially detected ($p < 0.01$) upon reduction of BRCA1 expression. When looking specifically at targets that were regulated by greater than 1.5-fold, 1680 of these were up-regulated upon reduced BRCA1 expression, and 1982 were down-regulated. Using the DAVID gene functional classification tool [32, 33], the most highly up-regulated genes were enriched in processes related to negative regulation of apoptosis (Table 1). The most down-regulated genes were enriched in cell cycle processes (Table 1), with a number of cyclins showing decreased levels (Supp Fig. 2), consistent with an inhibition of cell proliferation.

Whole genome expression profiling of T47D breast cancer cells with reduced BRCA1 expression identified that there were 3231 gene IDs that were significantly differentially expressed ($p < 0.01$) upon BRCA1 knockdown. When looking specifically at targets that were regulated by greater

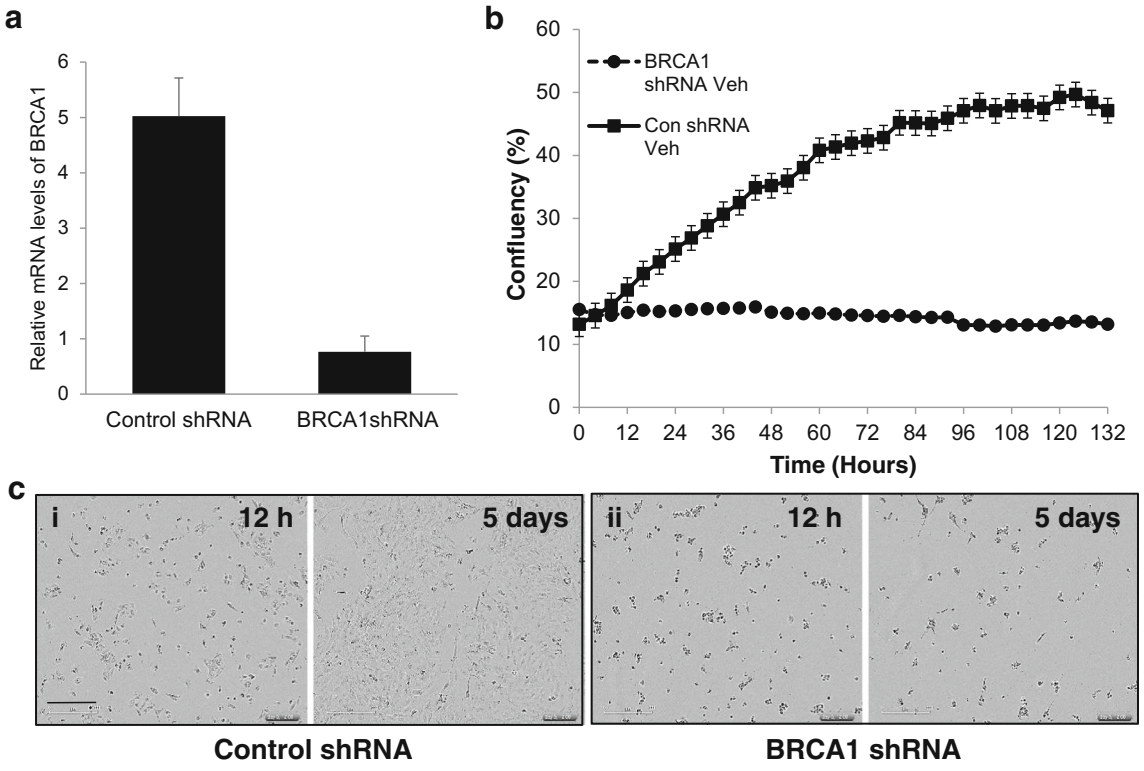

Fig. 1 **a** Relative reduction in BRCA1 transcripts in AB32 cells in IncuCyte live-cell imaging experiments ($n = 2$). Error bars indicate standard error. **b** BRCA1 shRNA inhibits cell proliferation in AB32 cells. Cell growth is expressed as an increase in percentage of confluence. Chart represents mean ± standard error ($n = 4$) **c** Representative images of AB32 cells transduced with (i) control shRNA or (ii) BRCA1 shRNA taken 12 h after plating (left panels) or 5 days after plating (right panels). Scale bar = 300um

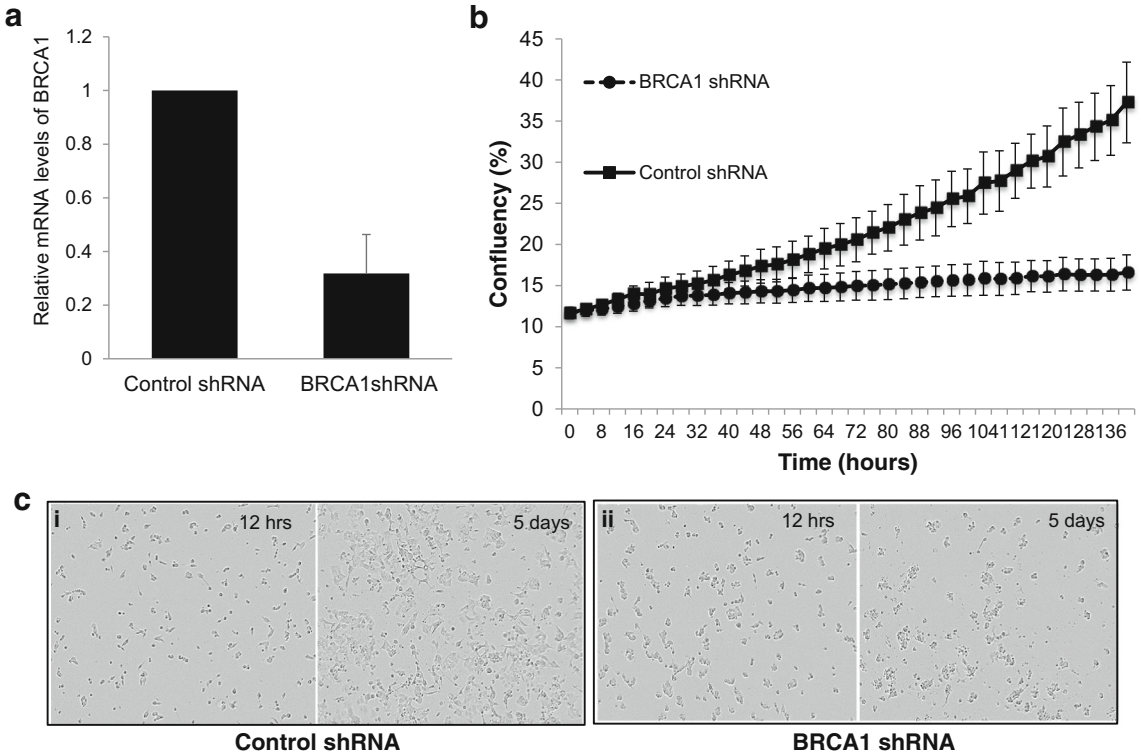

Fig. 2 **a** Relative reduction in BRCA1 transcripts in T47D cells in IncuCyte live-cell imaging experiments ($n = 3$). Error bars indicate standard error. **b** BRCA1 shRNA inhibits cell proliferation in T47D cells. Cell growth is expressed as an increase in percentage of confluence. Chart represents mean ± standard error ($n = 4$). **c** Representative images of T47D cells transduced with (i) control shRNA or (ii) BRCA1 shRNA taken 12 h after plating (left panels) or 5 days after plating (right panels). Scale bar = 300um

than 1.5-fold, 938 of these were up-regulated, and 1291 were down-regulated upon reduced BRCA1 expression. Similar to AB32 cells, the genes that were most down-regulated (>2-fold) were highly enriched in processes involved in cell cycle

Table 1 Functional clustering of genes enriched when BRCA1 is knocked down in AB32 and T47D cells

Category	Enrichment score	*p*-value
Up-regulated genes (>2.5-fold) when BRCA1 is knocked down in AB32 cells		
Negative regulation of apoptosis	6.04	6.3×10^{-8}
Cytokine activity	5.93	8.6×10^{-9}
Cell death	4.54	1.2×10^{-5}
Chemokine activity	3.14	1.6×10^{-4}
Chemotaxis	3.08	4.7×10^{-4}
Down-regulated genes (>2.5-fold) when BRCA1 is knocked down in AB32 cells		
Positive regulation of cellular protein metabolic process	3.86	3.4×10^{-6}
Cell division	3.30	1.6×10^{-4}
Mitotic cell cycle	2.90	6.9×10^{-4}
Negative regulation of apoptosis	2.58	2.5×10^{-3}
Cell cycle checkpoint	2.33	1.3×10^{-3}
Down-regulated genes (>2-fold) when BRCA1 is knocked down in T47D breast cancer cells		
M phase; cell cycle	39.35	3.8×10^{-43}
Mitosis; cell division	33.04	3.5×10^{-38}
Condensed chromosome kinetochore	10.84	1.2×10^{-13}
Establishment of mitotic spindle localization	4.34	2.3×10^{-5}
Meiosis	3.84	1.4×10^{-4}

processes (Table 1) with the same cyclins as in AB32 cells showing decreased levels in T47D cells (Supp Fig. 3). This was further supported by the mitosis and cell cycle categories that were most highly enriched in the gene IDs which were commonly differentially expressed between both AB32 and T47D cells upon BRCA1 knockdown (Supp Table 2). It is interesting to note the concordance of BRCA1 reduction inhibiting cell growth in both normal AB32 cells and T47D breast cancer cells, via similar impacts on cell cycle processes and reduction in the levels of the same cyclins. However, the overall mechanisms resulting in decreased confluency in the normal and cancer cells are likely to be distinct, given that the profiles of genes regulated by BRCA1 were overall distinct between each cell line, particularly when looking at genes which were regulated in the same direction (Fig. 3a–c). While 1359 IDs were commonly regulated in both cell lines, 476 (or 35%) of these IDs were regulated in opposite directions between the two cell lines. The different impact on gene expression of BRCA1 manipulation in AB32 and T47D cells is illustrated by the heatmap in Fig. 3d. Despite the probable differences suggested by these data in specific components of cell cycle-related pathways regulated by BRCA1 in each cell line, BRCA1 deficiency inhibited cell proliferation in both normal mammary AB32 cells and T47D breast cancer cells.

Reduced BRCA1 Levels Stimulated Inflammatory Targets and NFκB Activity in Normal Human Mammary Cells

In addition to apoptosis, the genes that were up-regulated following BRCA1 knockdown in AB32 cells were also enriched in cytokine and chemokine activity (Table 1). Further analysis identified that a number of these proteins were involved in inflammation and NFκB signalling. To confirm this, we chose a number of specific targets involved in these processes (IL6, IL8, CXCL1, CCL20, IL1B, BIRC3) and qPCR confirmed their elevated transcript numbers following knockdown of BRCA1 (Fig. 4). To confirm that these observations were not particular to the AB32 cell line that we generated to stably express PR, we transduced the parental cell line, MCF-10A (which is PR-negative), with BRCA1 shRNA. While each target was indeed also up-regulated in MCF-10A cells, the fold change in increase was much less (on average around 3-fold) compared with the AB32 cells (on average around 50-

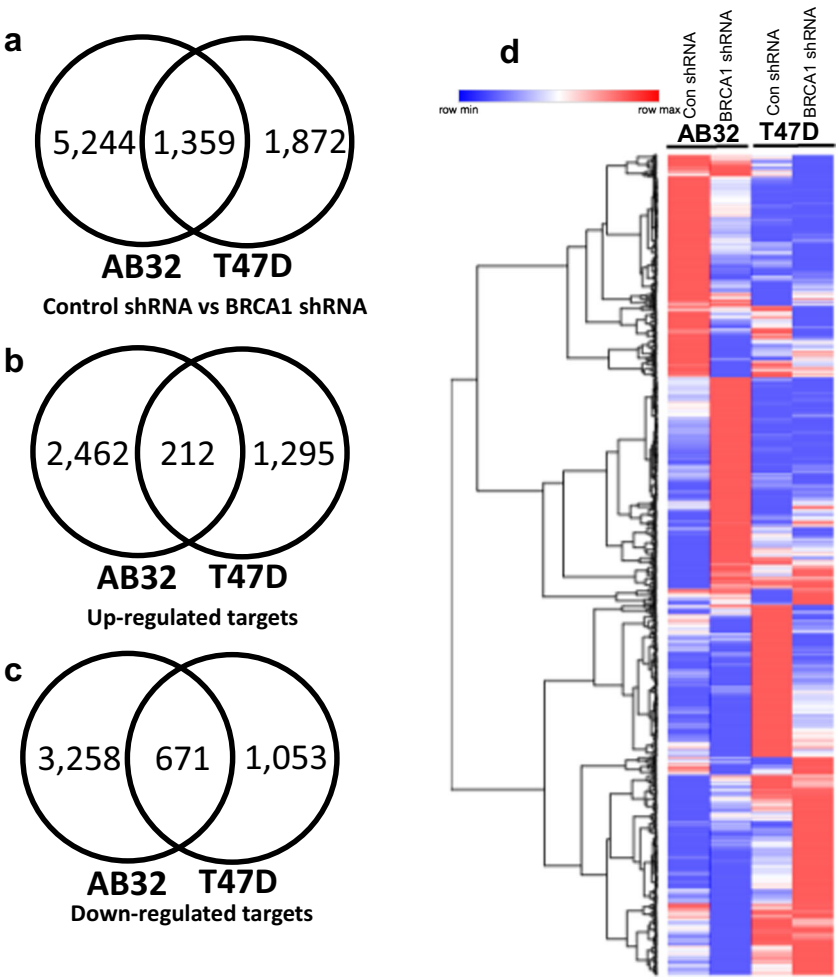

Fig. 3 Comparison of gene IDs which were significantly differentially expressed ($p < 0.01$) upon BRCA1 knockdown in AB32 and T47D cells. Venn diagrams depict gene IDs which were **a** commonly regulated overall, **b** commonly up-regulated only, or **c** commonly down-regulated only. **d** Unsupervised hierarchical cluster analysis of differentially expressed transcripts between control shRNA and BRCA1 shRNA transduced AB32 and T47D cells. Red represents higher expression and blue represents lower expression

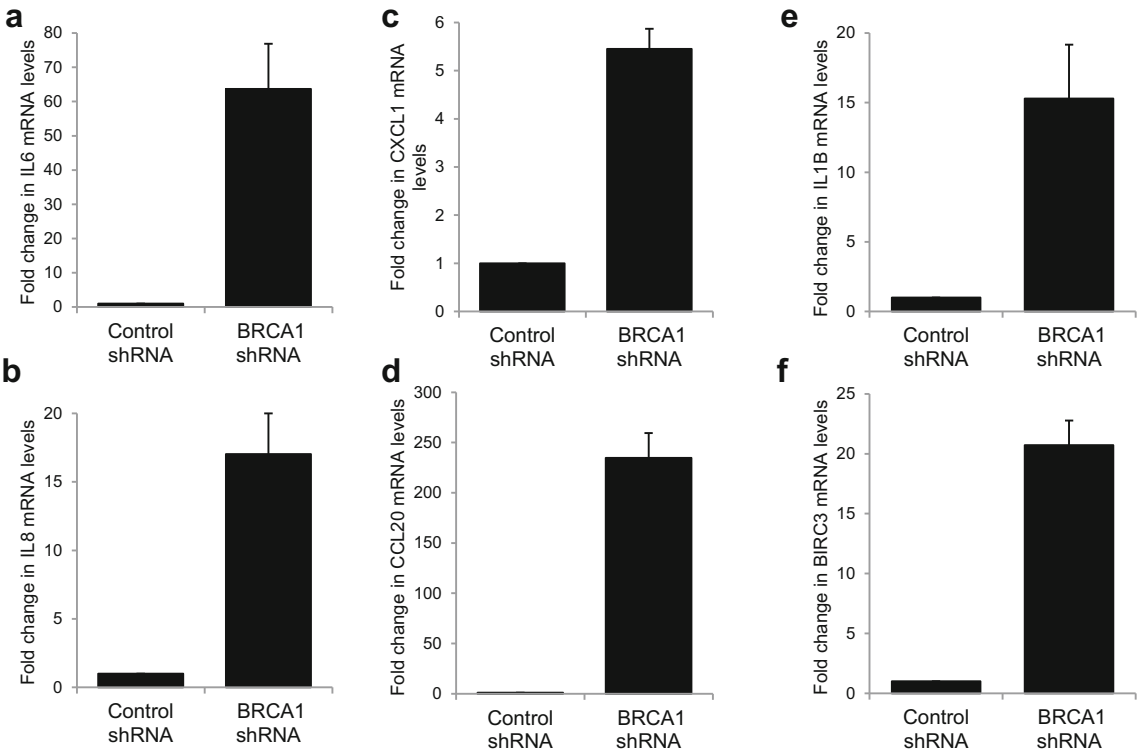

Fig. 4 Average fold change in mRNA levels relative to control shRNA of **a** IL6, **b** IL8, **c** CXCL1, **d** CCL20, **e** IL1B and **f** BIRC3, as determined by qPCR in AB32 cells ($n = 3$). Error bars indicate standard error

fold) (Supp Fig. 4A and B). This could possibly be due to the fact that AB32 cells expressed higher basal levels (approximately 2.7-fold; Supp Fig. 4C) of BRCA1, and were therefore potentially more sensitive to a significant reduction in BRCA1 expression.

In contrast to AB32 cells, 5 of the 6 inflammation and NFκB signalling targets quantitated in Fig. 4 were not detectable in T47D breast cancer cells by microarray, or were at the limit of detection by qPCR, both in the presence or absence of BRCA1 (data not shown). As others have previously shown, T47D cells exhibit very little basal NFκB activity [34], and we did not observe any significant stimulation of NFκB activity in T47D cells upon reduced BRCA1 expression. However, BIRC3, which is also an anti-apoptotic target, was up-regulated in BRCA1-deficient T47D cells (data not shown), as has also been shown previously in MCF7 breast cancer cells [35].

To further explore the effect of BRCA1 knockdown on NFκB activity in AB32 cells, we used a qRT-PCR array containing 84 key genes related to NFκB-mediated signalling. Of these genes, 46 were significantly ($p < 0.05$) regulated following BRCA1 knockdown (Table 2). While 30 of these targets were down-regulated, the ones that were most down-regulated included many that are important in mitogenic pathways (eg. EGFR, AKT1, FOS) and pro-apoptotic signalling (eg. TLR3, NOD1, EGR1), consistent with BRCA1 deficiency inhibiting cell growth. Therefore, BRCA1 deficiency stimulated NFκB activity and targets of inflammation in normal human mammary cells.

Wildtype BRCA1 Inhibited the pro-Proliferative Effects of Progesterone in Normal Mammary Epithelial Cells

Given the interaction between the BRCA1 and PR signalling networks, we were interested in the effect of progesterone treatment on these specific effects of BRCA1 deficiency. While AB32 cells express PR [31], it was interesting to note that the levels of PR were greatly induced in BRCA1-deficient AB32 cells by approximately 16-fold, compared with controls (Supp Fig. 5A), and this induction was confirmed at the protein level (Supp Fig. 5B-C), confirming a role for BRCA1 in depressing PR levels in these cells. Indeed, increased PR expression has previously been shown to correlate with reduced BRCA1 expression in murine mammary epithelial cells [6] and in T47D breast cancer cells [36]. In both control shRNA- and BRCA1 shRNA-treated cells, progesterone and the synthetic progestins, ORG2058 and MPA, reduced PR expression (Supp Fig. 5A and B), consistent with the known down-regulation of PR by progesterone [37]. Thus, this normal human breast cell line responds as expected to progesterone treatment.

As reduced levels of BRCA1 inhibited cell growth in AB32 cells (Fig. 1) and T47D cells (Fig. 2), we determined the effect of progestin treatment on this in AB32 cells using

Table 2 NFκB targets that are up-regulated (Bold) or down-regulated (Italic) upon BRCA1 knockdown in AB32 cells

Gene symbol	Gene description	Fold change in expression upon BRCA1 knockdown	*p* value
CCL20	Chemokine (C-C motif) ligand 20	**49.18**	0.008754
BIRC3	Baculoviral IAP repeat containing 3	**8.86**	0.017991
IL1B	Interleukin 1, beta	**8.82**	0.020075
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	**7.11**	0.045053
IL1A	Interleukin 1, alpha	**5.31**	0.000071
IRAK2	Interleukin-1 receptor-associated kinase 2	**5.31**	0.042147
IL8	Interleukin 8	**4.26**	0.000297
RELB	V-rel reticuloendotheliosis viral oncogene homolog B	**4.11**	0.005015
CXCL3	Chemokine (C-X-C motif) ligand 3	**3.91**	0.001788
BCL2A1	BCL2-related protein A1	**3.4**	0.00248
CSF3	Colony stimulating factor 3 (granulocyte)	**3.32**	0.000074
IRF1	Interferon regulatory factor 1	**3.26**	0.003903
ICAM1	Intercellular adhesion molecule 1	**2.38**	0.000057
HMOX1	Heme oxygenase (decycling) 1	**2.12**	0.031193
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B cells 2 (p49/p100)	**1.91**	0.012073
TICAM1	Toll-like receptor adaptor molecule 1	**1.68**	0.028193
TBK1	TANK-binding kinase 1	*−1.27*	0.007768
BCL3	B cell CLL/lymphoma 3	*−1.34*	0.040518
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	*−1.39*	0.000931
STAT1	Signal transducer and activator of transcription 1, 91 kDa	*−1.44*	0.034816
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	*−1.48*	0.025084
CFLAR	CASP8 and FADD-like apoptosis regulator	*−1.49*	0.041415
CHUK	Conserved helix-loop-helix ubiquitous kinase	*−1.54*	0.001555
LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	*−1.61*	0.003098
BIRC2	Baculoviral IAP repeat containing 2	*−1.71*	0.019676
CASP8	Caspase 8, apoptosis-related cysteine peptidase	*−1.71*	0.025315
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	*−1.77*	0.010009
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	*−1.87*	0.007168
ATF1	Activating transcription factor 1	*−1.91*	0.000268
FADD	Fas (TNFRSF6)-associated via death domain	*−1.93*	0.006097
RHOA	Ras homolog gene family, member A	*−2.03*	0.007604
PSIP1	PC4 and SFRS1 interacting protein 1	*−2.06*	0.028739
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	*−2.06*	0.007484
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	*−2.13*	0.001729
TRADD	TNFRSF1A-associated via death domain	*−2.15*	0.046246
TLR4	Toll-like receptor 4	*−2.41*	0.033273
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	*−2.5*	0.003439
BCL2L1	BCL2-like 1	*−2.58*	0.003416
CSF1	Colony stimulating factor 1 (macrophage)	*−2.72*	0.004866
EGFR	Epidermal growth factor receptor	*−3.03*	0.000728
EGR1	Early growth response 1	*−3.05*	0.004768
MAP 3 K1	Mitogen-activated protein kinase kinase kinase 1	*−3.07*	0.004229
NOD1	Nucleotide-binding oligomerization domain containing 1	*−3.07*	0.018135
FOS	FBJ murine osteosarcoma viral oncogene homolog	*−3.28*	0.001727
AKT1	V-akt murine thymoma viral oncogene homolog 1	*−3.33*	0.000187
TLR3	Toll-like receptor 3	*−4.49*	0.007614

the IncuCyte live-cell imaging system. Levels of BRCA1 transcripts upon treatment with the progestins, ORG2058 and MPA, are shown in Fig. 5a. Interestingly, progestin treatment increased proliferation, and this was particularly striking when BRCA1 was knocked down (Fig. 5b). This is particularly evident when looking at a single time point, and the fold

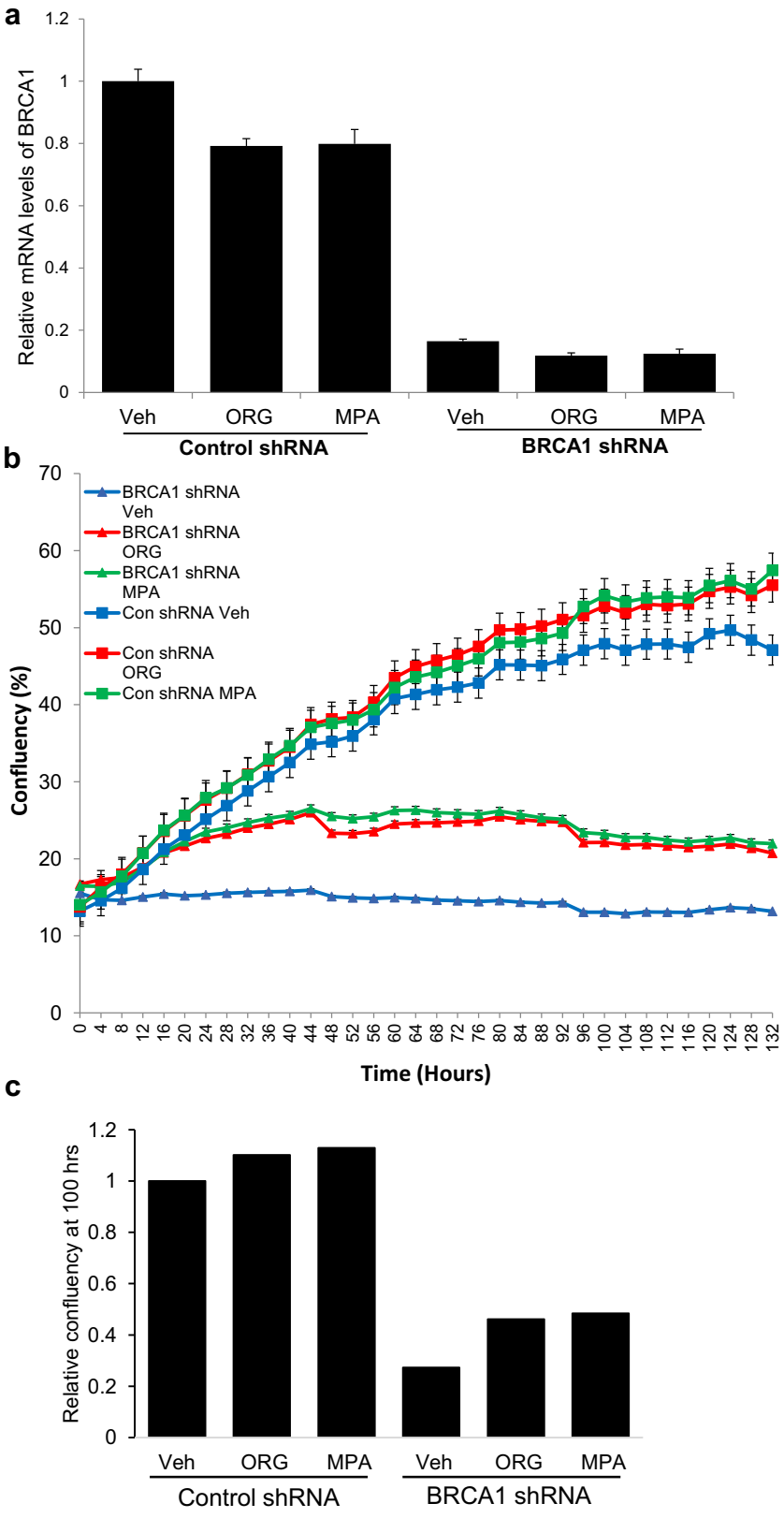

Fig. 5 **a** BRCA1 transcript levels in AB32 cells (± BRCA1 shRNA ± progestin treatment). **b** Cell growth of control shRNA (■) and BRCA1 shRNA (▲) is expressed as an increase in percentage of confluence. Chart represents mean ± standard error ($n = 4$). **c** Fold change in confluency of cells (± BRCA1 shRNA ± progestin treatment), relative to vehicle control shRNA cells at 100 h

increase in confluency in progestin-treated cells, relative to vehicle-treated cells (Fig. 5c). This suggests that wildtype levels of BRCA1 markedly attenuated the pro-proliferative effects of progesterone.

In contrast to the AB32 cells, treatment with progestins in T47D breast cancer cells decreased proliferation in control cells (Supp Fig. 6A) as has previously been demonstrated in T47D cells [20, 21]. This is again evident when looking at the fold increase in confluency at a single specific time point (Supp Fig. 6B). However, unlike in AB32 cells, progestins neither inhibited nor stimulated cell growth in T47D cells treated with BRCA1 shRNA (Supp Fig. 6C). Together these findings demonstrate that while BRCA1 reduction inhibited cell growth in both normal AB32 cells and T47D cells, the capacity of progestin treatment to modulate this inhibitory effect of BRCA1 on growth was dependent on cell type, with progestins having a marked effect in normal AB32 cells, but not in T47D cells.

Progesterone Attenuated BRCA1-Mediated NFκB Activation in Normal Human Mammary Cells

Upon treatment with progesterone and progestins, the targets which were induced by BRCA1 deficiency (Fig. 4), were markedly inhibited (Fig. 6). This suggested that the suppressed expression of NFκB activity and inflammatory targets with wild-type levels of BRCA1, which was relieved by BRCA1 shRNA, was reversed by progesterone treatment. Furthermore, using the qRT-PCR array containing 84 key genes related to NFκB-mediated signalling, progesterone significantly regulated 19 targets in control shRNA cells (Supp Table 3), and 14 targets in BRCA1 shRNA cells (Supp Table 4). Pro-apoptotic targets were significantly reduced with BRCA1 knock-down (Table 2), suggesting there was reduced apoptosis in these cells. In contrast, several of these targets (for example, TRADD, TNFRSF10B, MALT1, FADD, FOS, RIPK1 and TNFRSF1A) were up-regulated by progesterone treatment in control shRNA cells (Supp Table 3), but not in BRCA1 shRNA cells. This suggested that progesterone treatment had an opposing effect on apoptosis to that of reduced BRCA1 expression. Moreover, using the "activity score" generated by 16 experimentally derived signature biomarker genes, we showed that progesterone significantly inhibited NFκB activity, both in the presence and absence of BRCA1 (Fig. 7a), attenuating the stimulation of NFκB activity by BRCA1 deficiency.

When comparing targets that were significantly regulated greater than 2-fold by progesterone in the presence or absence of BRCA1, there were 7 targets that were down-regulated by progesterone when BRCA1 was knocked down (CCL20, IL1B, BCL2A1, CSF3, BCL3, IRF1, TNFAIP2; Fig. 7b), but not in control cells (Supp Fig. 7). This suggests that wildtype BRCA1 represses progesterone down-regulation of these targets, which is consistent with previous demonstrations that BRCA1 inhibits progesterone-inducible gene expression [38]. Together these findings demonstrate that in normal human mammary cells, progesterone inhibited NFκB activation in BRCA1-deficient cells.

Discussion

This study has demonstrated that in both immortalised normal human mammary epithelial cells (AB32) and in breast cancer cells (T47D), reduced levels of BRCA1 expression inhibited cell proliferation, highlighting the reliance of breast cell proliferation on wild-type BRCA1 levels. In addition, the finding that BRCA1 deficiency resulted in increased NFκB activity and targets of inflammation in normal human mammary epithelial cells suggests that these pathways are normally suppressed by wildtype BRCA1 levels. Interestingly, treatment with progesterone attenuated these effects of BRCA1 deficiency, suggesting that wildtype BRCA1 may inhibit progesterone action in these cells.

Despite the well-defined role of progesterone in counteracting the proliferative effects of estrogen in the endometrium [39, 40], progesterone is critical in driving the proliferation and development of the normal breast, but also can exert anti-proliferative effects in certain cellular contexts [4]. Ablation of BRCA1 can also exert proliferative or anti-proliferative effects in a context-dependent manner. For example, depletion of BRCA1 in 3D cultures of normal mammary epithelial cells enhanced proliferation in acini [12], and reduced BRCA1 expression in primary human breast epithelial cells stimulated the self-renewal capacity of stem cells [41], while healthy breast tissue from BRCA1 mutation carriers displayed an increase in progenitor cell numbers [10, 11]. Other studies have shown that normal breast tissue from BRCA1 mutation carriers displayed decreased proliferation and premature senescence [16]. This is potentially explained by the demonstration that BRCA1-haploinsufficient mammary epithelial cells have a diminished ability to cope with replication stress [42], which may successively generate double strand DNA breaks, a DNA damage response, activation of DNA damage checkpoints, and thereby lead to apoptosis or cell cycle arrest/senescence [27, 43]. Consistent with this notion, our data has shown that reduced BRCA1 expression inhibited cell growth, in both normal mammary AB32 cells and T47D breast cancer cells. While we observed a significant decrease in BRCA1 transcripts, and reduced BRCA1 protein expression as shown by immunofluorescence upon transduction with BRCA1 shRNA, it will now be important to determine quantitatively the precise levels of BRCA1 protein, and whether there is any effect on BRCA1 stability, in these cell lines.

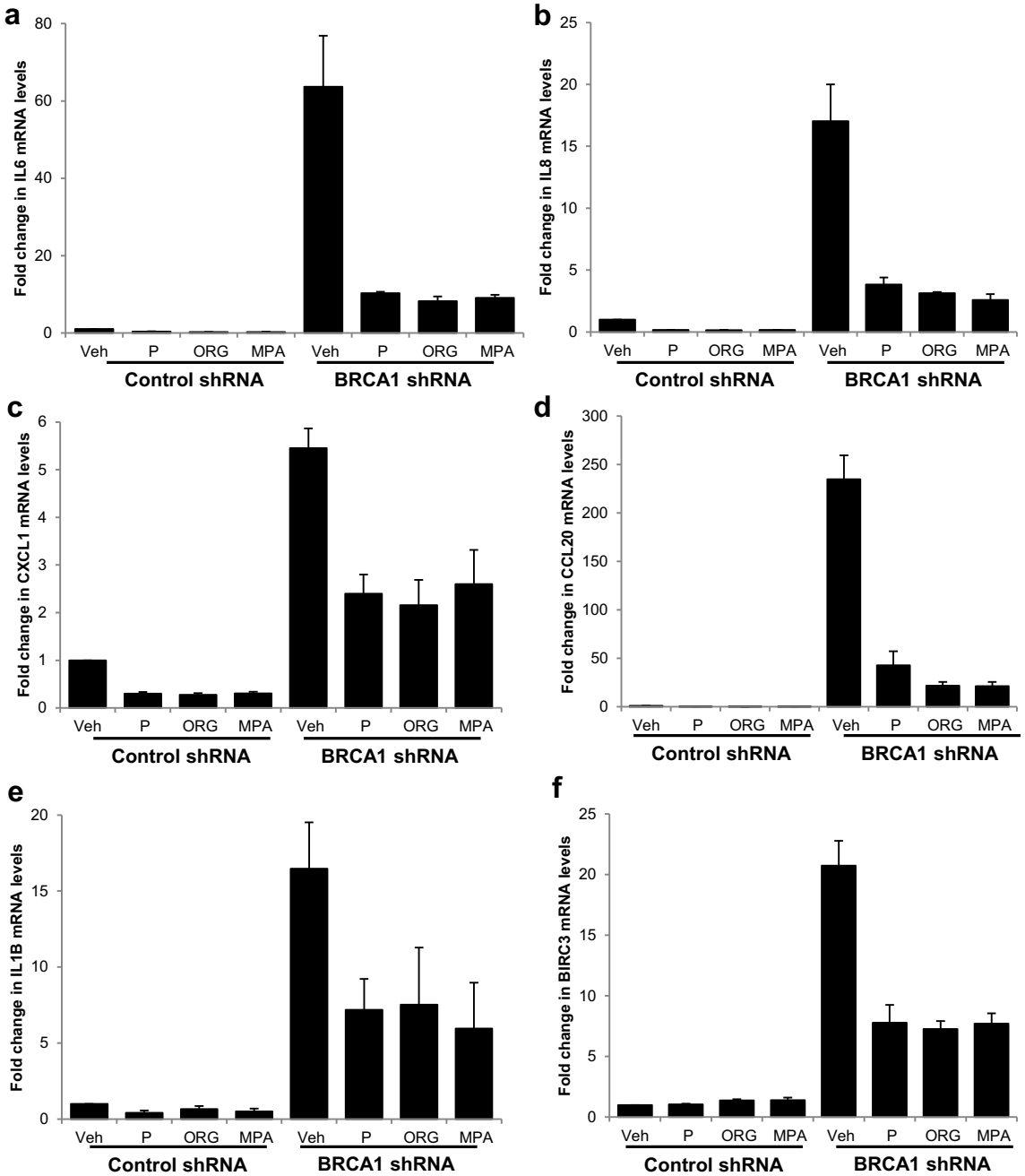

Fig. 6 Average fold change in mRNA levels in AB32 cells ± BRCA1 shRNA following treatment with P (100 nM; $n = 3$) or progestins (10 nM; $n = 2$) of **a** IL6, **b** IL8, **c** CXCL1, **d** CCL20, **e** IL1B and **f** BIRC3. Data is depicted relative to control shRNA and chart represents mean + SE

Our data has also shown that progesterone and progestins stimulated proliferation of normal AB32 cells, and that this was more striking in the absence of BRCA1. However, in contrast to AB32 cells, treatment with progesterone and progestins inhibited cell growth in T47D breast cancer cells expressing wildtype BRCA1, but not in cells with reduced BRCA1 expression. Taken together, deficient BRCA1 expression enhanced the proliferative effects of progesterone in normal mammary epithelial cells, and counteracted the anti-proliferative effects of progesterone in breast cancer cells.

NFκB is a transcription factor which regulates a broad spectrum of genes involved in many physiological processes, including proliferation, angiogenesis, metastasis, immune responses, inflammation and cell survival [44]. NFκB is also a central component in the response to DNA damage and cellular stress [45], and can be constitutively active in breast cancer, among other solid tumors and hematological malignancies [46]. Furthermore, genetic inactivation of NFκB can reduce or attenuate mammary tumors in mouse models [47]. Our data has demonstrated that BRCA1 deficiency stimulated

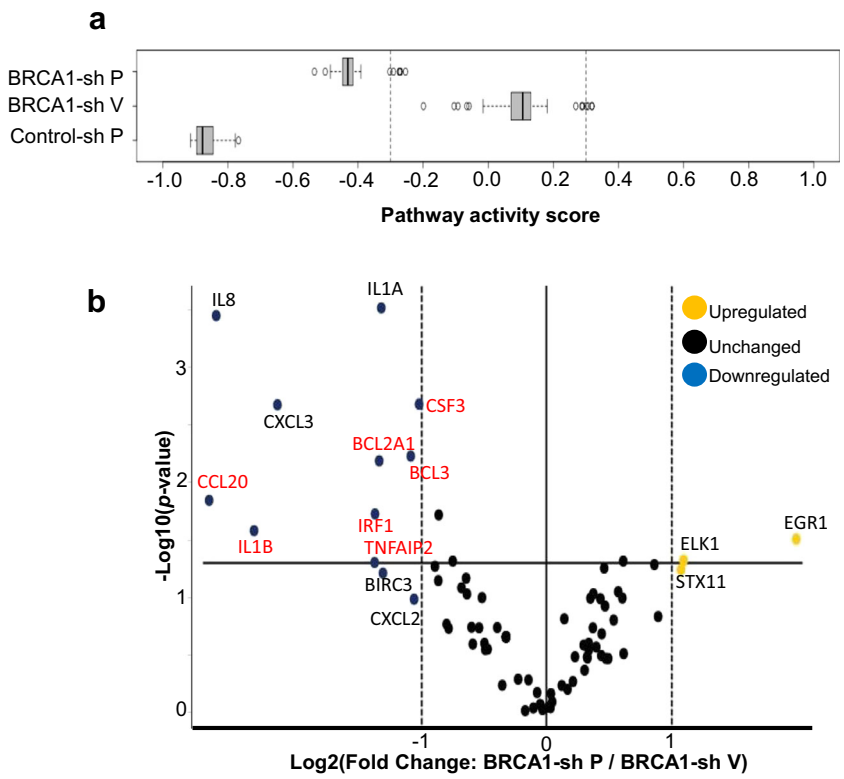

Fig. 7 **a** Inhibition of NFκB pathway activation by progesterone. Pathway activity score of >0.3 or < −0.3 indicates significant pathway activity change. **b** Volcano plot analysis (integrating *p* values and log2 fold changes) for progesterone-regulated targets in AB32 cells treated with BRCA1-shRNA. Blue and yellow dots indicate probes that showed a fold-change ≤2 or ≥ 2, respectively. Gene names in red indicate targets that are significantly down-regulated by progesterone when BRCA1 is knocked down, but not in control (see Supp Fig. 7)

NFκB activity and targets of inflammation in normal human mammary cells. To further elucidate the mechanism by which this happens, it will now be necessary to examine the levels of protein expression and phosphorylation of these NFκB targets, as well as their subcellular localisation. In support of our study however, it has previously been shown that NFκB is persistently activated in BRCA1-deficient progenitor cells in the mouse mammary gland, and also in BRCA1-deficient MCF-10A cells, as measured by phosphorylation of the ser536 residue on the p65 sub-unit [27]. Also concordant with our data, NFκB activation was demonstrated in the normal breast cell line, 184A1, as shown through increases in NFκB target genes, including IL-8, IL-6 and CXCL1, as well as in a number of breast cancer cell lines and a subset of BRCA1-deficient triple negative breast tumors [28]. However, in contrast to this, others have reported that depletion of BRCA1 in MCF-10A cells actually reduced levels of NFκB targets, such as IL-8 and CXCL1 [48]. This discrepancy is potentially due to different experimental conditions, for example, the latter study maintained MCF-10A cells in media supplemented with bovine pituitary extract, rather than horse serum. Taken together however, experimental and clinical studies to date largely support the notion that reduction in wild-type levels of BRCA1 is associated with augmented NFκB activity.

It is interesting to note that the active NFκB signature observed in a subset of BRCA1-deficient triple negative breast tumors was also associated with a better prognosis due to an anti-tumor microenvironment [28], and similarly, estrogen receptor-negative tumors with BRCA1 mutations were associated with over-expression of immune response genes, as well as a good prognosis [49]. We have demonstrated that progesterone treatment inhibited NFκB activation and levels of immune targets in BRCA1-deficient AB32 cells, and that progestin treatment increased proliferation in BRCA1-deficient AB32 cells. While a potential limitation of this study is that these experiments were conducted in in vitro cellular assays employing PR positive cell lines, our observations still raise the question as to whether progesterone signalling, either direct or through paracrine mediators, may contribute to breast tumorigenesis in BRCA1 mutation carriers in vivo. The persistent hormonal influence throughout a woman's reproductive life, and exposure to exogenous hormones, such as to progestins in hormone replacement therapy and oral contraception, are associated with increased breast cancer risk [50–52]. These fluctuating progesterone levels may be particularly relevant to the risk of breast tumorigenesis in BRCA1 mutation carriers. Therefore, it would be interesting to determine whether inhibition of progesterone action in tumors with BRCA1 mutations, or BRCA1 mutation carriers, would delay breast tumorigenesis or impart a better prognosis. Indeed, there has been recent interest using denosumab, an inhibitor of a key paracrine mediator of progesterone signalling (RANKL), as a preventive therapeutic strategy in BRCA1 mutation carriers [53]. Treatment with denosumab inhibited progesterone-mediated proliferation in 3D cultures of pre-neoplastic tissue from BRCA1 mutation carriers, as well as

reducing proliferation in breast biopsies from BRCA1 mutation carriers taking denosumab [49]. Because there have been adverse side effects associated with higher dose denosumab use in the longer term, alternative antagonists of progesterone action may also provide similar benefit to BRCA1 mutation carriers. It should be noted that most synthetic PR antagonists that have been explored in cancer treatment to date have been limited by adverse side effects and thus have not been suitable for long-term applications. However, they remain a promising therapeutic tool, and the development of novel safe and effective PR antagonists for the inhibition of tumor growth continues to be investigated. Moreover, combined PR and RANK targeting could lower the required dose of denosumab, thus diminishing the risk of ongoing side effects. In summary, the results of this study have provided data to support continued exploration of the use of PR antagonists in a clinical setting, particularly in BRCA1 mutation carriers.

Acknowledgments This work was made possible by funding from the National Breast Cancer Foundation (HNH; Career Development Fellowship; Grant ID: ECF-16-007), Cure Cancer Australia Foundation/ Cancer Australia (HNH; Grant ID: APP1098796). CLC is a National Health and Medical Research Council Principal Research Fellow (Grant ID: APP1081334). HNH, CLC and JDG are supported by a project grant from The Cancer Council NSW (Grant ID: RG-15-17).

References

1. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. Nat Rev Cancer. 2004;4(10):814–9.
2. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell. 2002;108(2):171–82.
3. Hu Y. BRCA1, hormone, and tissue-specific tumor suppression. Int J Biol Sci. 2009;5(1):20–7.
4. Hilton HN, Graham JD, Clarke CL. Minireview: progesterone regulation of proliferation in the normal human breast and in breast cancer: a tale of two scenarios? Mol Endocrinol. 2015;29(9):1230–42.
5. Ma Y, Katiyar P, Jones LP, Fan S, Zhang Y, Furth PA, et al. The breast cancer susceptibility gene BRCA1 regulates progesterone receptor signaling in mammary epithelial cells. Mol Endocrinol. 2006;20(1):14–34.
6. Poole AJ, Li Y, Kim Y, Lin SC, Lee WH, Lee EY. Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist. Science. 2006;314(5804):1467–70.
7. Widschwendter M, Rosenthal AN, Philpott S, Rizzuto I, Fraser L, Hayward J, et al. The sex hormone system in carriers of BRCA1/2 mutations: a case-control study. Lancet Oncol. 2013;14(12):1226–32.
8. Mote PA, Leary JA, Avery KA, Sandelin K, Chenevix-Trench G, Kirk JA, et al. Germ-line mutations in BRCA1 or BRCA2 in the normal breast are associated with altered expression of estrogen-responsive proteins and the predominance of progesterone receptor a. Genes Chromosomes Cancer. 2004;39(3):236–48.
9. King TA, Gemignani ML, Li W, Giri DD, Panageas KS, Bogomolniy F, et al. Increased progesterone receptor expression in benign epithelium of BRCA1-related breast cancers. Cancer Res. 2004;64(15):5051–3.
10. Lim E, Vaillant F, Wu D, Forrest N, Pal B, Hart A. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med. 2009;15:907–13.
11. Proia TA, Keller PJ, Gupta PB, Klebba I, Jones AD, Sedic M, et al. Genetic predisposition directs breast cancer phenotype by dictating progenitor cell fate. Cell Stem Cell. 2011;8(2):149–63.
12. Furuta S, Jiang X, Gu B, Cheng E, Chen P-L, Lee W-H. Depletion of BRCA1 impairs differentiation but enhances proliferation of mammary epithelial cells. Proc Natl Acad Sci U S A. 2005;102(26):9176–81.
13. Burga LN, Tung NM, Troyan SL, Bostina M, Konstantinopoulos PA, Fountzilas H, et al. Altered proliferation and differentiation properties of primary mammary epithelial cells from BRCA1 mutation carriers. Cancer Res. 2009;69(4):1273–8.
14. Cao L, Li W, Kim S, Brodie SG, Deng C-X. Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. Genes Dev. 2003;17(2):201–13.
15. Bai F, Smith MD, Chan HL, Pei XH. Germline mutation of Brca1 alters the fate of mammary luminal cells and causes luminal-to-basal mammary tumor transformation. Oncogene. 2012;32:2715–25.
16. Sedic M, Skibinski A, Brown N, Gallardo M, Mulligan P, Martinez P, et al. Haploinsufficiency for BRCA1 leads to cell-type-specific genomic instability and premature senescence. Nat Commun. 2015;6:7505.
17. Scott A, Bai F, Chan HL, Liu S, Slingerland JM, Robbins DJ, et al. p16 loss rescues functional decline of Brca1-deficient mammary stem cells. Cell Cycle. 2017;16(8):759–64.
18. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CAJ, et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 1995;9(18):2266–78.
19. Graham JD, Mote PA, Salagame U, van Dijk JH, Balleine RL, Huschtscha LI, et al. DNA replication licensing and progenitor numbers are increased by progesterone in normal human breast. Endocrinology. 2009;150(7):3318–26.
20. Musgrove EA, Lee CS, Sutherland RL. Progestins both stimulate and inhibit breast cancer cell cycle progression while increasing expression of transforming growth factor alpha, epidermal growth factor receptor, c-fos, and c-myc genes. Mol Cell Biol. 1991;11(10):5032–43.
21. Groshong SD, Owen GI, Grimison B, Schauer IE, Todd MC, Langan TA, et al. Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1). Mol Endocrinol. 1997;11(11):1593–607.
22. Lange CA, Richer JK, Horwitz KB. Hypothesis: progesterone primes breast cancer cells for cross-talk with proliferative or anti-proliferative signals. Mol Endocrinol. 1999;13(6):829–36.
23. Bae I, Fan S, Meng Q, Rih JK, Kim HJ, Kang HJ, et al. BRCA1 induces antioxidant gene expression and resistance to oxidative stress. Cancer Res. 2004;64(21):7893–909.
24. Esteve JM, Armengod ME, Knecht E. BRCA1 negatively regulates formation of autophagic vacuoles in MCF-7 breast cancer cells. Exp Cell Res. 2010;316(16):2618–29.
25. Martinez-Outschoorn UE, Balliet R, Lin Z, Whitaker-Menezes D, Birbe RC, Bombonati A, et al. BRCA1 mutations drive oxidative stress and glycolysis in the tumor microenvironment. Cell Cycle. 2012;11(23):4402–13.
26. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med. 2010;49(11):1603–16.
27. Sau A, Lau R, Cabrita Miguel A, Nolan E, Crooks Peter A, Visvader Jane E, et al. Persistent activation of NF-κB in BRCA1-deficient mammary progenitors drives aberrant proliferation and accumulation of DNA damage. Cell Stem Cell. 2016;19(1):52–65.

28. Buckley NE, Haddock P, Simoes RDM, Parkes E, Irwin G, Emmert-Streib F, et al. A BRCA1 deficient, NFκB driven immune signal predicts good outcome in triple negative breast cancer. Oncotarget. 2016;7(15):19884–96.
29. Hardy DB, Janowski BA, Chen C-C, Mendelson CR. Progesterone receptor inhibits aromatase and inflammatory response pathways in breast cancer cells via ligand-dependent and ligand-independent mechanisms. Mol Endocrinol. 2008;22(8):1812–24.
30. Kobayashi S, Stice JP, Kazmin D, Wittmann BM, Kimbrel EA, Edwards DP, et al. Mechanisms of progesterone receptor inhibition of inflammatory responses in cellular models of breast cancer. Mol Endocrinol. 2010;24(12):2292–302.
31. Clarke CL, Graham JD. Non-overlapping progesterone receptor cistromes contribute to cell-specific transcriptional outcomes. PLoS One. 2012;7(4):e35859.
32. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2008;4(1):44–57.
33. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37(1):1–13.
34. Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin AS. Selective activation of NF-κB subunits in human breast cancer: potential roles for NF-κB2/p52 and for Bcl-3. Oncogene. 2000;19:1123–31.
35. Bae I, Rih JK, Kim HJ, Kang HJ, Hassan B, Kirilyuk A, et al. BRCA1 regulates gene expression for orderly mitotic progression. Cell Cycle. 2005;4(11):1641–66.
36. Calvo V, Beato M. BRCA1 counteracts progesterone action by ubiquitination leading to progesterone receptor degradation and epigenetic silencing of target promoters. Cancer Res. 2011;71(9):3422–31.
37. Mockus MB, Horwitz KB. Progesterone receptors in human breast cancer. Stoichiometric translocation and nuclear receptor processing. J Biol Chem. 1983;258(8):4778–83.
38. Booth BW, Smith GH. Estrogen receptor-α and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. Breast Cancer Res. 2006;8:R49.
39. Fernandez-Valdivia R, Mukherjee A, Mulac-Jericevic B, Conneely OM, DeMayo FJ, Amato P, et al. Revealing progesterone's role in uterine and mammary gland biology: insights from the mouse. Semin Reprod Med. 2005;23(1):22–37.
40. Pan H, Deng Y, Pollard JW. Progesterone blocks estrogen-induced DNA synthesis through the inhibition of replication licensing. Proc Natl Acad Sci U S A. 2006;103(38):14021–6.
41. Liu S, Ginestier C, Charafe-Jauffret E, Foco H, Kleer CG, Merajver SD, et al. BRCA1 regulates human mammary stem/progenitor cell fate. Proc Natl Acad Sci U S A. 2008;105(5):1680–5.
42. Pathania S, Bade S, Le Guillou M, Burke K, Reed R, Bowman-Colin C, et al. BRCA1 haploinsufficiency for replication stress suppression in primary cells. Nat Commun. 2014;5:5496.
43. Sedic M, Kuperwasser C. BRCA1-hapoinsufficiency: unraveling the molecular and cellular basis for tissue-specific cancer. Cell Cycle. 2016;15(5):621–7.
44. Baud V, Karin M. Is NF-κB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov. 2009;8(1):33–40.
45. Hayden MS, Ghosh S. Shared principles in NFκB signaling. Cell. 2008;132(3):344–62.
46. Bassères DS, Baldwin AS. Nuclear factor-κB and inhibitor of κB kinase pathways in oncogenic initiation and progression. Oncogene. 2006;25(51):6817–30.
47. Cao Y. Luo J-l, Karin M. IκB kinase α kinase activity is required for self-renewal of ErbB2/Her2-transformed mammary tumor-initiating cells. Proc Natl Acad Sci U S A. 2007;104(40):15852–7.
48. Gardini A, Baillat D, Cesaroni M, Shiekhattar R. Genome-wide analysis reveals a role for BRCA1 and PALB2 in transcriptional co-activation. EMBO J. 2014;33(8):890–905.
49. Nolan E, Vaillant F, Branstetter D, Pal B, Giner G, Whitehead L, et al. RANK ligand as a potential target for breast cancer prevention in BRCA1-mutation carriers. Nat Med. 2016;22(8):933–9.
50. Hunter DJ, Colditz GA, Hankinson SE, Malspeis S, Spiegelman D, Chen W, et al. Oral contraceptive use and breast cancer: a prospective study of young women. Cancer Epidemiol Biomark Prev. 2010;19(10):2496–502.
51. Beral V, Reeves G, Bull D, Green J. Million women study collaborators. Breast cancer risk in relation to the interval between menopause and starting hormone therapy. J Natl Cancer Inst. 2011;103(4):296–305.
52. Chlebowski RT, Manson JE, Anderson GL, Cauley JA, Aragaki AK, Stefanick ML, et al. Estrogen plus progestin and breast cancer incidence and mortality in the women's health initiative observational study. J Natl Cancer Inst. 2013;105(8):526–35.
53. Nolan E, Lindeman GJ, Visvader JE. Out-RANKing BRCA1 in mutation carriers. Cancer Res. 2017;77(3):595–600.