In search of the tumour-suppressor functions of BRCA1 and BRCA2

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Abstract

Hereditary breast and ovarian cancer syndromes can be caused by loss-of-function germline mutations in one of two tumour-suppressor genes, *BRCA1* and *BRCA2* (ref. [1]). Each gene product interacts with recombination/DNA repair proteins in pathways that participate in preserving intact chromosome structure. However, it is unclear to what extent such functions specifically suppress breast and ovarian cancer. Here we analyse what is known of *BRCA* gene function and highlight some unanswered questions in the field.

The amino acid sequences of BRCA1 and BRCA2 originally provided few clues to their specific functions (Fig. 1a). BRCA1 was found to localize to sub-nuclear foci during the S and G2 phases of the cell cycle. These foci also contain Rad51, a protein central to homologous recombination[2]. Endogenous BRCA1-Rad51 complexes were detected in cell extracts. BRCA2 also interacts and co-localizes with Rad51 and BRCA1 (refs [3] and [4]). The three genes are co-expressed in development[1].

Human Rad51, like its homologues in other eukaryotes (RAD51) and prokaryotes (RecA), operates in the repair of double-strand DNA breaks (DSB) by binding single-stranded (ss) DNA to form a nucleoprotein filament that can invade a homologous duplex DNA molecule. *Saccharomyces cerevisiae rad51* mutants are defective in double-strand break repair (DSBR) and meiosis, two processes dependent upon homologous recombination.

Rad51 also exhibits characteristic localization patterns during meiosis. The synaptonemal complex (SC) is a linear protein/DNA-bearing structure that assembles at the onset of meiotic prophase I, a tetraploid stage during which homologous chromosomes become paired before meiotic recombination. The SC forms by the apposition ('synapsis') of two homologous axial elements, each containing two identical sister chromatids and associated proteins. Rad51 binds axial elements. Strikingly, BRCA1 and BRCA2 act similarly[2,4] (Fig. 1b), suggesting that BRCA proteins function in meiotic recombination, and hinting at a role in sister chromatid interactions.

Relocation of BRCA1 to sites of DNA synthesis

After hydroxyurea (HU)-mediated replication arrest or exposure of S-phase cells to ultraviolet (UV) light, BRCA1 rapidly relocalizes from its 'native' nuclear foci to sites of DNA synthesis and becomes hyperphosphorylated[5]. This suggests that BRCA1 is involved in the repair of abnormal DNA structures generated at sites of DNA synthesis after DNA damage. Among the

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proteins that interact and co-localize with BRCA1 in nuclear foci are BRCA2 and BARD1. Like BRCA1, BARD1 contains an amino-terminal RING domain and carboxy-terminal BRCT motifs[6]. BARD1, BRCA2 and Rad51 all accompany BRCA1 in its re-localization after DNA damage[4,5]. This implies that these multiprotein complexes are dedicated, at least in part, to repair of replication-associated DNA damage.

In yeast, cell cycle control responses to HU or to certain DNA damaging agents delivered in the S phase are controlled by S-phase checkpoint gene products, which include *S. cerevisiae* MEC1 and *Schizosaccharomyces pombe* rad3, two large nuclear kinases with C-terminal PI3 kinase-like domains. Atr is a mammalian homologue of these proteins and, like BRCA proteins, it also localizes to axial elements during meiotic prophase I[7]. Recently, Atr was found to colocalize with BRCA1 in somatic cells, both before and after replication arrest[41]. Atr, in part, controls BRCA1 phosphorylation following HU treatment[41]. BRCA1 is also phosphorylated following gamma irradiation[5], under the control of a related kinase, Atm[8].

Death by checkpoint

Mouse gene targeting experiments provided insights into the relationship between *BRCA* mutation and cancer (reviewed in refs [1] and [9]). Nullizygous *BRCA1* or *BRCA2* embryos die around the time of gastrulation. Unexpectedly, these embryos reveal a proliferative defect and induction of the *p53*-dependent cell cycle inhibitor, *p21* (ref. [10]). It was argued that, if BRCA1 regulates DNA repair, its inactivation might lead to spontaneous abnormalities in DNA structure. If so, induction of *p21* in *BRCA1*^{-/-} embryos might reflect the activation of a DNA damage-dependent checkpoint[2]. The resulting cell cycle delay could have catastrophic effects on a gastrulating embryo, leading to 'death by checkpoint'. Consistent with this hypothesis, *p53* or *p21* nullizygosity delays the death of *BRCA1*^{-/-} or *BRCA2*^{-/-} embryos (reviewed in refs [1] and [9]).

BRCA1 dysfunction might have similar effects in adult cells; it may precipitate spontaneous abnormalities of DNA structure and, thereby, provoke 'death by checkpoint'. If, however, loss of *BRCA1* function were to occur in a pre-malignant cell that had already suffered inactivation of key checkpoints, the aberrant DNA structures resulting from *BRCA1* inactivation might be tolerated without cell cycle arrest[2] (Fig. 2). This might promote neoplastic development. *BRCA1* or *BRCA2* inactivation, if it were to lead to cancer, would then not be the first 'hit' during tumorigenesis. Alternatively, *BRCA* gene inactivation in an otherwise wild-type cell would have to be closely followed by the inactivation of key checkpoints.

Support for the notion that *BRCA* genes suppress genome instability comes from several sources. Embryonic tissue lacking *wt* (wild-type) *BRCA1* or *BRCA2* reveals ionizing radiation (IR) hypersensitivity, consistent with a defect in DSBR[3,9]. *BRCA1* or *BRCA2* homozygous mutant mouse embryo fibroblasts (MEFs) undergo spontaneous chromosome breakage accompanied by checkpoint-mediated growth arrest[9,11]. Moreover, severe aneuploidy and centrosome amplification are observed in these cells[1,9]. The full identity of the checkpoints responsible for cell cycle arrest of *BRCA1* or *BRCA2* mutated cells is unclear. Among the proposed candidates is the spindle pole checkpoint[1,9]. *Rad51*^{-/-} mouse embryos also suffer early death that is partially rescued by *p53* germline inactivation, and cultured cell lines deprived of Rad51 undergo spontaneous chromosome breakage.

Interestingly, *BRCA* gene and checkpoint-mediated mechanisms may also suppress genome instability in breast tissue. Recently, breast-specific murine *BRCA1* gene inactivation was achieved[9]. These mice developed late-onset breast cancer with frequent, tumour-associated *p53* mutations. On a *p53*^{+/-} background, earlier-onset and higher-frequency disease was detected. *p53* hemizygosity was also found to promote breast tumour development in *BRCA1*^{+/-} mice exposed to ionizing radiation[1,9]. Thus, *p53*-mediated checkpoint functions may

contribute to proliferation control of *BRCA1*-deficient breast ductal cells. Loss of these p53 functions could increase tolerance of genome instability and promote tumorigenesis.

BRCA-mediated recombination and tumour suppression

Human genetics has defined numerous mutant *BRCA1* and *BRCA2* alleles defective in tumour suppression. This has provided a tool for testing the relationship between BRCA1 tumour suppressor function and its role in genome integrity maintenance. Recently, a breast cancer cell line, HCC1937, that lacks *wtBRCA1* was found to be hypersensitive to IR and to exhibit delayed kinetics of DSBR. Stable expression of *wtBRCA1* in these cells reversed IR sensitivity and restored efficient DSBR[13]. In contrast, several clinically important mutant alleles, each bearing a missense mutation in one of three distinct BRCA1 protein domains—the N-terminal RING domain, the C-terminal BRCT motifs, or a centrally located region—failed to rescue the phenotype. The DSBR function of BRCA1, therefore, arises from the participation of diverse structural elements of the protein. As each of these domains also serves as an interaction centre for associated proteins, a scaffolding role for BRCA1 in DSBR seems possible. Moreover, given the inactivation of this function by a diverse set of disease-producing, missense mutations, DSBR may be a key BRCA1 tumour-suppressor function.

At least two distinct processes, homologous recombination (HR) and non-homologous end joining (NHEJ), contribute to DSBR in mammalian cells. The interaction of BRCA1 with the NBS1–MRE11–Rad50 complex (reviewed in ref. [1]) potentially implicates it in either of these processes. Current data suggest a specific contribution of *BRCA* genes to HR. For example, *BRCA1* mutant mice reveal arrested spermatogenesis in meiotic prophase I, the stage at which BRCA1 is normally localized to the axial elements of developing synaptonemal complexes (reviewed in refs [1] and [9]). A unique, viable *BRCA1* homozygous mutant murine embryonic stem (ES) cell clone revealed reduced rates of HR or single-strand annealing in response to a site-specific DSB[14]. Gene targeting, an HR-dependent process, was enhanced by expression of *wtBRCA1* in the same *BRCA1*-mutated ES cell clone[15]. By contrast, where measured, NHEJ appeared normal in *BRCA1* or *BRCA2* mutant cells[11,14].

DNA polymerase stalling and S-phase checkpoint activation

If the *BRCA* genes act as tumour suppressors by supporting HR, which HR-dependent process is the key disease-relevant target? One candidate is sister chromatid recombination (SCR), a potentially error-free process which operates, in part, in the repair of recombinogenic DNA lesions generated during S phase[16]. SCR may be provoked by attempted replication across lesions that induce DNA polymerase stalling, such as those caused by UV irradiation or DNA adduction[17]. Indeed, work in prokaryotes indicates that replication arrest is a common event during DNA synthesis and that recombination functions are required for replication restart, genome integrity maintenance, and cell viability (reviewed in ref. [18]).

Hydroxyurea exposure may mimic such DNA polymerase-stalling events by causing genome-wide replication arrest. HU or UV treatment of S phase cells may each give rise to persistent ssDNA tracts—'daughter strand gaps' (DSG)—in close proximity to a replication fork[19] (Fig. 3). We propose that such DSGs contribute to the rapid relocalization of BRCA1 and associated proteins to sites of replication following HU or UV treatment[5]. DSGs, or their derivatives, might activate S phase checkpoint signalling and also serve as substrates of recombinational responses, such as SCR (Fig. 3). Atr now appears to be a regulator of these events[41]. Interestingly, *ATR*^{-/-}embryos suffer early embryonic lethality with spontaneous chromosome breakage—a phenotype reminiscent of *BRCA*^{-/-} and *Rad51*^{-/-} embryos[20].

Whereas S-phase 'checkpoint' kinases signal to BRCA1, little is known of how these phosphorylations alter BRCA1 function. We do know that, unlike Atr, intact BRCA proteins

are not required for S-phase checkpoint function in itself (ref. [11]; R.S. and D.M.L., unpublished work). This implies that BRCA1 plays a checkpoint-associated not a checkpoint-intrinsic function, possibly a direct role in SCR (Fig. 3). This is supported by the frequent "chromatid-type" errors that arise in *BRCA*-mutant MEFs[11,16].

Homozygous germline mutations in *ATM* produce genome instability and a cancer-prone syndrome in humans, and Atm participates in certain checkpoint responses to IR[21]. Indeed, Atm and a second checkpoint kinase, Chk2, signal to BRCA1 after IR[8,22], but Atm does not appear to contribute to BRCA1 phosphorylation after HU exposure[5,8]. Moreover, unlike *ATR*^{-/-} embryos, *ATM*^{-/-} mice are viable. Taken together, the available data imply that the pathways that trigger BRCA1 phosphorylation by these two proteins and the biological outcomes of these sets of events are, at least in part, distinct. Perhaps Atr and Atm activation are triggered by distinct categories of DNA structure.

Other genome integrity functions of the BRCA complex

Defective transcription-coupled repair of oxidative DNA damage was demonstrated in *BRCA1* and *BRCA2* mutated cells[23,42]. Cells homozygous for a *BRCA1* allele lacking exon 11 revealed anomalous G2/M checkpoint responses to gamma irradiation, implying a role for BRCA1 in this process[9]. Recently, a partial purification of BRCA1 revealed its association with mismatch repair proteins, the Bloom's syndrome helicase, and certain replication factors[24]. The functional significance of these particular associations is unclear. However, it is worth noting that the Bloom's Syndrome gene and certain mismatch repair genes have established tumour-suppressor function.

Unlike the homologues of the *RAD52* epistasis group of *S. cerevisiae*, which are represented in all eukaryotic genomes, there are no clear homologues of *BRCA* genes in yeast, flies or worms. Therefore, the biochemical contributions of BRCA1 and BRCA2 to DNA repair may be as regulators of more conserved repair functions.

Transcriptional functions of BRCA1 and BRCA2

Certain fragments of each BRCA protein, when fused to a GAL4 DNA-binding domain, can transactivate a GAL4 reporter, except when they bear clinically relevant mutations[25–27]. This suggested that BRCA1 and/or BRCA2 function as transcriptional regulators of specific target genes, the identification of which might, in turn, shed light on the mechanism of *BRCA* tumour suppression. Interactions have been found between BRCA proteins and sequence-specific transcription factors such as c-myc (BRCA1) and p53 (BRCA1 and BRCA2), as well as with certain transcription regulation proteins that do not recognize a specific, canonical DNA sequence (reviewed in refs [1] and [28]). The search for BRCA1 target genes has also pointed to candidates in the *p53* pathway: *p21* and *GADD45* (reviewed in refs [1] and [28], and references therein). This connection suggests a different kind of link between BRCA1 function and genome integrity control in which its function is tied to the expression of genes that do the work of checkpoint control and/or DNA repair. BRCA1, when overproduced, also suppresses oestrogen receptor (ER) transactivation (cited in ref. [1]). Notably, *p53* mutation and ER negativity are frequent findings in *BRCA1*-linked tumours, so *BRCA1* tumour-suppressor action appears not to be exclusively linked to *p53*- or *ER*-dependent functions.

Chromatin remodelling activity of BRCA multiprotein complexes

Chromatin remodelling functions have been attributed to both BRCA1 and BRCA2[1,28]. A recent biochemical purification of BRCA1 suggests that it interacts with a SWI/SNF-containing complex[29]. The impact of chromatin structure and of its remodelling upon transcriptional events is widely accepted. Equally important is the relationship between

chromatin structure and the repair of recombinogenic DNA lesions. For example, in *S. cerevisiae*, the SIR proteins function in transcriptional silencing of subtelomeric heterochromatin, but are rapidly released from such sites and recruited to the proximity of a DSB where their presence is required for efficient repair[30]. This is an attractive paradigm against which to consider the BRCA proteins. Perhaps a DSB triggers the release of BRCA proteins from their 'civilian' functions or loci and promotes their recruitment to the 'locale' of the break.

If this concept is correct, what defines the 'locale' of a DSB? Recent studies have shown that chromatin is actively modified around a DSB in mammalian cells. Specifically, the mammalian histone H2A subspecies, H2AX, is phosphorylated on a residue in its C-terminal tail within minutes of gamma irradiation. Indeed, phosphorylated H2AX was shown to appear around an experimentally induced 'line' of DSBs in interphase nuclei (cited in ref. [31]). Conceivably, phosphorylation of H2AX participates in defining the 'locale' of a DSB. Some phosphorylated H2AX also exists in undamaged cells, and it forms nuclear foci that co-localize, in part, with the BRCA dots[31]. This suggests that the BRCA dots may contain a specialized form of chromatin structure.

Although there are few clues to the innate biochemical activities of the *BRCA* gene products, it is notable that the BRCA1 RING domain region, which interacts tightly, perhaps stoichiometrically, with the RING domain of BARD1, participates in an *in vitro* ubiquitin ligation reaction, like RING domains of some ubiquitin ligases[32]. Perhaps ubiquitination, sumoylation, or nedd 8 coupling of selected protein targets are mediated by BRCA1 and/or BARD1.

BRCA genes and breast or ovarian cancer predisposition

There is good reason to accept that defective maintenance of genomic integrity, as described in *BRCA*-deficient cells, is an accelerator of cancer progression. If so, how does such a generic defect translate into specifically increased breast or ovarian cancer risk?

The breast epithelium proliferates rapidly during puberty and under the influence of oestrogenic hormones. Unlike the cells of many rapidly proliferating epithelia, such as those of the intestine or of the uterine endometrium, progeny of this proliferative burst are retained within the breast epithelium. This is demonstrated by the finding that breast lobules are clonal[33]. If, before lobular development, a lobular precursor cell had sustained a cancer-predisposing mutation at a relevant locus (such as *p53*), the entire lobule would then carry that mutation. This, in turn, could amplify the risk of subsequent neoplastic progression. Some germline *p53* mutations (such as in Li–Fraumeni syndrome) confer a particularly elevated risk of adult-onset breast cancer. Similarly, high-dose ionizing chest radiation during puberty, or even in early childhood (for example, atom-bomb exposure; mantle zone therapeutic radiation), specifically predisposes women to adult-onset breast cancer[34,35].

These observations could be relevant to *BRCA*-linked disease, especially if the *BRCA*$^{+/-}$ genotype were haplo-insufficient in genome integrity maintenance function—an unanswered question at present. *BRCA* gene haplo-insufficiency could, in principle, increase the risk of additional cancer-promoting mutations occurring during breast development, including mutation of the *BRCA* gene, itself. Indeed, if puberty constitutes a limited 'window' during which a *BRCA* mutation carrier is at special risk of developing carcinogenic mutations, this might account for the relative absence of *BRCA* gene inactivation in sporadic breast or ovarian cancer. In sporadic disease, biallelic *BRCA* gene inactivation might occur too late to have an impact on disease risk[36].

That the breast and ovary are oestrogen-responsive tissues could also be relevant to the tissue specificity of *BRCA* disease risk. Some oestrogen metabolites can adduct DNA, and so could act as tissue-specific carcinogens (so-called "remote carcinogenesis")[37]. Conceivably, this effect might be exacerbated by *BRCA* mutation, if the relevant DNA repair pathways were dysfunctional.

BRCA proteins associate with chromosomal pairing events on the synaptanemal complex. In some model organisms, homologous chromosomal pairing is also known to occur in certain somatic cells where it can influence development by affecting transcriptional regulation ("transvection") as well as imprinting[38]. Potentially analogous, homology-dependent transcriptional regulation may also operate in mammals[39] and could represent a link between homologous pairing and tissue-specific gene expression. If homologous chromosomal pairing occurs in the breast or ovarian epithelium, are BRCA proteins involved and does such an involvement contribute to their organ-specific tumour suppression function?

Human cells express alternatively spliced *BRCA1* transcripts, the biological significance of which is unclear[40]. Whether products of such transcripts contribute to the tissue specificity of *BRCA* tumour suppression bears future investigation.

Acknowledgments

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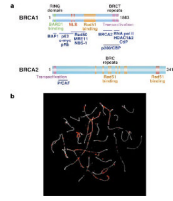
Figure 1.
Structure and localization of BRCA proteins. **a**, Structure of the BRCA1 and BRCA2 polypeptides. BRCA1 shows RING domain, BRCT motifs, implicated in DNA damage response pathways. BRCA2 shows BRC repeats, involved in the Rad51 interaction. Some associated proteins are denoted in blue. **b**, Localization of BRCA1 during meiotic prophase I. Depicted is a single primary, human spermatocyte nucleus, stained with an antibody to the synaptonemal complex protein, SCP3 (white), and with BRCA1 antibody (red). BRCA1 localizes to the unsynapsed regions (axial elements) of developing synaptonemal complexes. Copyright held by Cell Press, reproduced with permission.
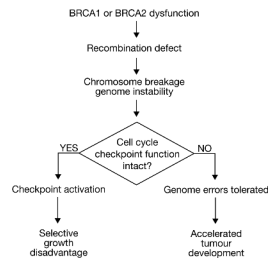
Figure 2.
Checkpoint inactivation and *BRCA* gene-mediated tumorigenesis. Inactivation of DNA damage-responsive cell cycle checkpoints may contribute to the cellular response to *BRCA* gene dysfunction. Loss of *BRCA* function and associated chromosome breakage in a cell trigger checkpoints that deselect it. If such a checkpoint has already been disabled (as in a pre-cancerous cell), the chromosome breakage syndrome may be tolerated and lead to accelerated neoplastic progression.
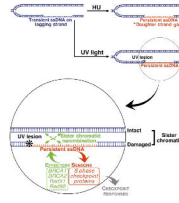
Figure 3.
Proposed role for the BRCA proteins in sister chromatid recombination. DNA polymerase stalling activates S-phase checkpoint signalling and recruits BRCA1, BRCA2, Rad51 and associated proteins to sites of arrested DNA synthesis. We propose that this recruitment arises from the existence of tracts of persistent ssDNA close to a replication fork. Such lesions, or their derivatives, may activate S-phase checkpoint signalling and act as a substrate for BRCA protein-mediated recombinational repair.