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Clues to the Function of the Tumour Susceptibility Gene BRCA2

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ABSTRACT: The breast cancer susceptibility gene BRCA2 was isolated in 1995. BRCA2 is a large gene comprising 10,254 nucleotides and 26 coding exons. Neither the nucleotide nor the predicted protein sequences (comprising 3,418 amino acids) have provided substantial clues about its function. As a result, researchers have been trying to elucidate the function using a combination of cell biological and biochemical methods and the construction of animal models using gene targeting in mice. Recent data suggest that BRCA2 may participate in pathways associated with recombination or double-strand DNA break repair and may act by either sensing or responding to DNA damage. In addition, there is evidence to suggest that BRCA2 functions in a manner similar to the previously isolated breast cancer susceptibility gene BRCA1.

INTRODUCTION

Germline mutations of *BRCA2* confer a high lifetime risk of female breast cancer and are associated with an increased risk of male breast cancer, ovarian cancer, prostate cancer and pancreatic cancer [1–7]. The gene is thought to behave as a tumour suppressor; tumours from families linked to BRCA2 show consistent loss of the wild-type allele. In addition, loss of heterozygosity studies indicate that the BRCA2 locus (on chromosome 13q12–13) is frequently deleted in sporadic breast and ovarian cancers [8–10]. However, few somatic BRCA2 mutations have been found in these tumours [10–14].

The nucleotide sequence and predicted BRCA2 protein shows very little homology to previously isolated genes; no domains have been identified which provide obvious clues to the function of the protein [2,3]. One potentially important functional domain located in a central portion of the gene is an amino acid motif which is repeated eight times (termed the BRC repeats) [15]. The majority of these repeats are well conserved between human, mouse, monkey, pig, dog and hamster even though the overall conservation of BRCA2 is poor [16]. Yeast two-hybrid screens have recently shown that six out of eight BRC repeats specifically bind to the RAD51 protein which has been implicated in recombination or double-strand DNA break repair [17]. At the amino terminus, another region of the BRCA2 gene which is highly conserved between human and mouse is homologous to the activation domain of c-Jun, a known transcription factor [18]. This region, which includes exon 3, has been shown to activate transcription *in vitro*. The transcriptional activation ability is significantly reduced when a naturally occurring missense mutation is introduced into the sequence.

The BRCA2 gene is widely transcribed at relatively low levels [2,3]. Two studies show that BRCA2 mRNA expression is cell cycle dependent and is induced at the G1/S boundary [19,20]. Expression appears to be greatest in rapidly proliferating cells and is down regulated in response to growth factor deprivation. In addition, BRCA2 is coordinately expressed with BRCA1 in mammary epithelial cells suggesting that these genes may be induced by, and function in, overlapping regulatory pathways. There is also evidence to suggest that BRCA2 transcription and translation are coregulated with

each other. Antibodies recognising different epitopes of the BRCA2 protein have been used to study its cell-cycle regulation as well as its cellular localisation. Cell fractionation followed by western blotting indicates that BRCA2 is predominantly nuclear [21]. Western blot analyses also suggest that BRCA2 expression levels vary during the cell cycle; BRCA2 expression is induced in MCF7 cells in late G1/early S-phase following release from serum starvation [21].

The murine homologue of BRCA2 has been cloned. Mouse BRCA2 is poorly conserved; it shows an overall 59% identity and 72% similarity with human BRCA2 [22,23]. This is similar to the mouse:human homology observed for the BRCA1 gene but is significantly less than across species conservation of other tumour suppressor genes. Analysis of the expression pattern of mouse BRCA2 reveals that it is widely transcribed, like human BRCA2. Quantitative reverse transcription PCR and northern analysis suggest that expression is greatest in the thymus, testis and ovary [22,23]. In addition, BRCA2 appears to be highly expressed in rapidly proliferating cells in the developing mouse embryo, similar to the expression pattern of BRCA1 [23].

THE PHENOTYPES OF BRCA2 INACTIVATION IN THE MOUSE

Analysis of the phenotypic consequences of mutations in whole organisms may provide useful clues to the function of specific genes. The phenotype in mice which have been engineered to be heterozygous for mutations analogous to those identified in humans sometimes closely resembles the phenotype observed in humans (e.g. inactivation of the APC gene in the colorectal cancer susceptibility syndrome familial adenomatous polyposis [24]). In other instances the phenotype is considerably different between mouse and humans (e.g. inactivation of the WT1 gene in Wilms tumour [25]). Despite the different phenotypes observed between mice and humans in some syndromes, it remains likely that the interactions between specific gene products and the biochemical pathways in which they are involved will be conserved across species.

To date, five independent mouse strains have been engineered in which the BRCA2 gene has been targeted and completely or partly knocked out by homologous recombination [26–30]. The locations of the mutations are different but each is predicted to produce a truncated BRCA2 protein, although this has not been formally demonstrated due to the lack of antibodies specific to BRCA2. In all cases, mice which are heterozygous for the BRCA2 mutant allele (BRCA2+/−) have so far failed to show any sign of susceptibility to mammary tumours or indeed any other phenotype. In three of these mouse strains, intercrosses of BRCA2+/− animals produced only wild type and heterozygous progeny suggesting that homozygous mutant progeny (BRCA2−/−) were not viable [26–28]. Analysis of the development of these mice during embryogenesis shows that BRCA2−/− embryos fail at a very early stage, after approximately 6.5 to 7.5 days of gestation. The remaining two mice strains, however, have a different phenotype. In both strains, some homozygotes survived birth. Surviving BRCA2−/− mice were growth retarded compared with BRCA2+/− and BRCA2+/+ littermates and were infertile [29,30]. In addition, the majority of neonates and adult mice had a distinctively kinked tail suggestive of developmental defects in skeletal structures. In both of these strains, BRCA2 null mice died at a relatively early age (between 11 and 22 weeks) concomitant with the development of metastatic thymic lymphomas.

GENOTYPE–PHENOTYPE CORRELATION IN BRCA2 NULL MICE

Why do mice homozygous for different BRCA2 mutations have differing phenotypes? One possible explanation is that the penetrance of the BRCA2 mutation differs depending on the genetic background of the mouse strain. There is

evidence to suggest that this may partly be true in the two strains of BRCA2−/− mice which are viable; in both cases the proportion of homozygotes which survive varies depending on background, suggesting that other genetic factors are modifying penetrance. However, modifying effects of different genetic backgrounds are unlikely to account for all of the phenotypic variation observed amongst these mouse strains.

Another possible explanation for this phenotypic variation is that different mutations have different functional effects, possibly through the loss or retention of functionally important domains in the different predicted truncated proteins. The most significant regions of functional homology identified in BRCA2 to date are the BRC repeats most of which specifically bind RAD51 *in vitro* [15–17]. Three of the five BRCA2 mutations which have been used to generate BRCA2−/− mice are predicted to truncate the BRCA2 protein before the first of these BRC repeats [26–28]; these strains of mice all have an embryonic lethal phenotype. In the two strains in which some BRCA2−/− mice survive, the truncated protein is predicted to retain three and seven of the eight repeats respectively [29–30]. These data suggest that the BRC repeats are functionally significant and that their loss or retention directly affects the phenotype of BRCA2−/− mice.

It is intriguing that the region which contains the BRC repeats is also associated with phenotypic variation in humans [6]. Germline truncating mutations in this region (termed the ovarian cancer cluster region, OCCR, and encompassing nucleotides 3035 to 6629) are associated with an increased risk of ovarian cancer and/or a decreased risk of breast cancer compared with mutations elsewhere in the gene [6]. These data provide further support for a significant functional role of the BRC repeats and their association with RAD51. Figure 1 highlights the similarities between mouse and human BRCA2, summarises current knowledge

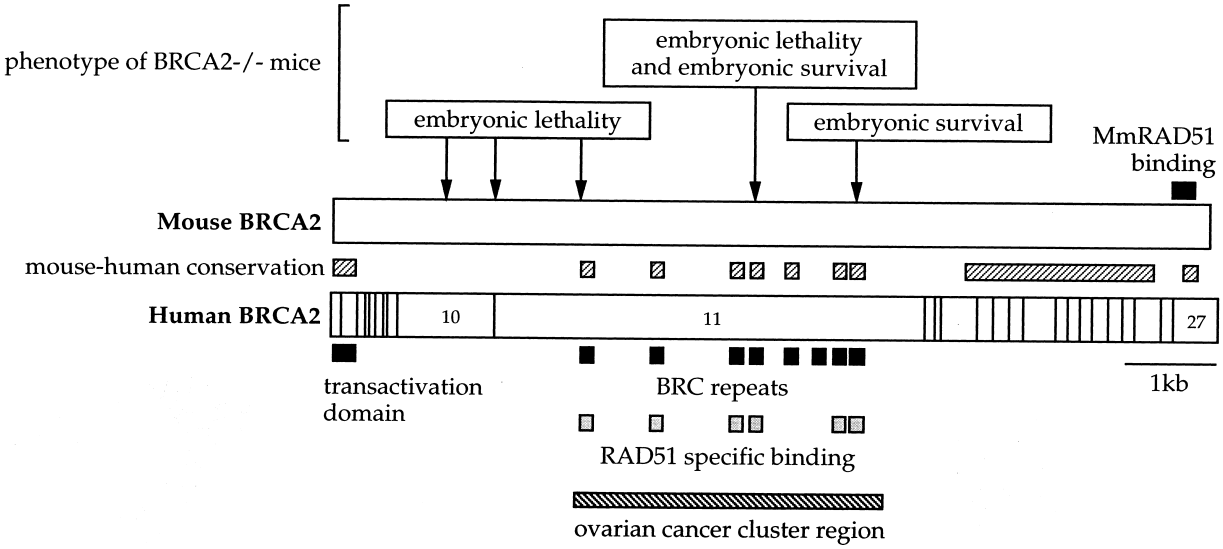

Fig. 1. Comparison between human and mouse BRCA2 genes. The genomic structure of human BRCA2 is known [3]; vertical lines along the length of the gene represent the approximate location of coding exons. The genomic structure of mouse BRCA2 is unknown [16–18,26]. Black bars represent regions of putative functional significance [16–18,26]. Hatched bars represent regions of high identity between mouse and human genes. Shaded bars represent regions of RAD51 specific binding. The location of truncating mutations in mice are illustrated with respect to the phenotypes of BRCA2−/− mice. A region of phenotypic variation in humans, the ovarian cancer cluster region [6] is also illustrated.

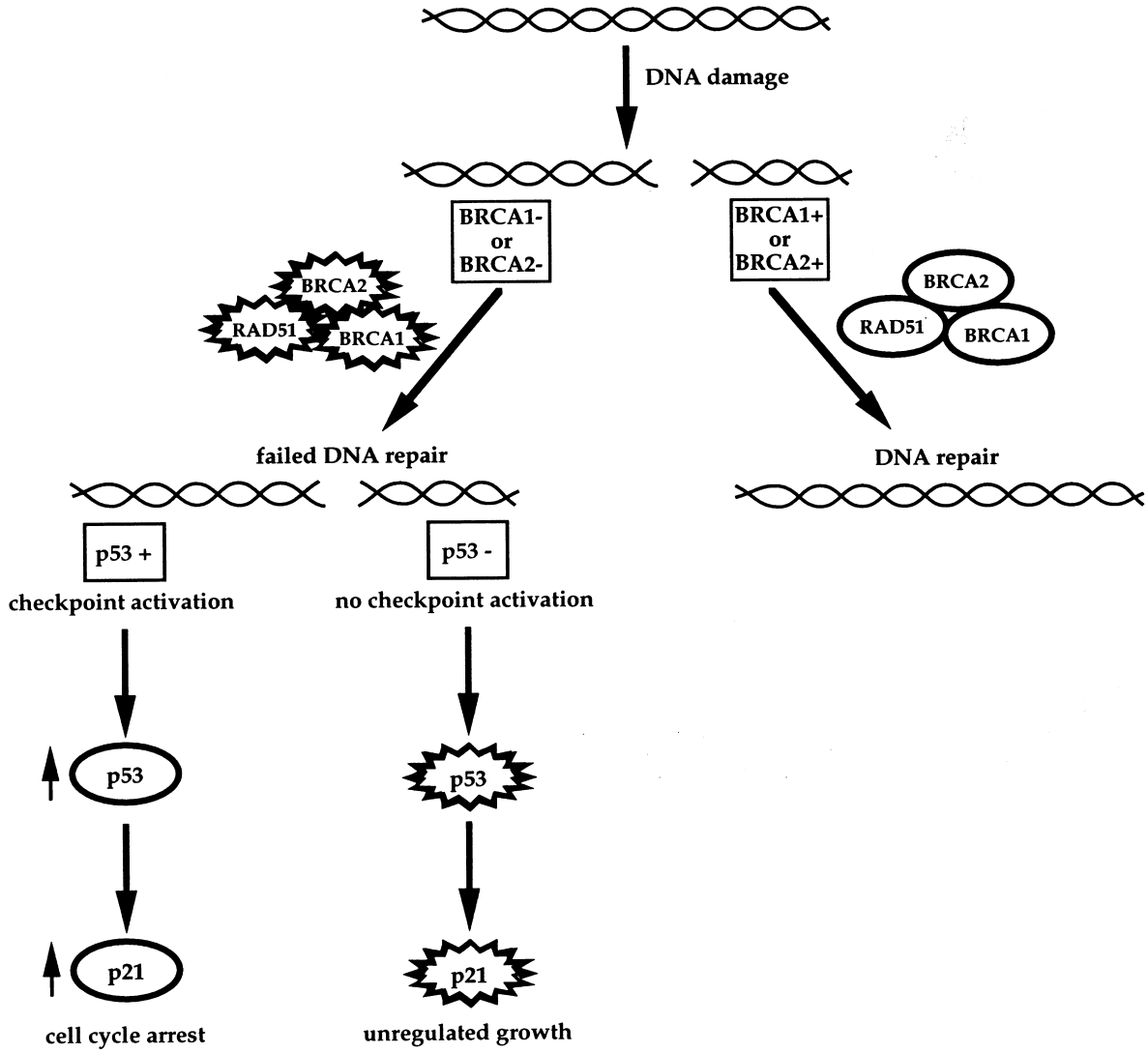

Fig. 2. Hypothetical explanation for the putative interaction between BRCA2, BRCA1 and RAD51, and their postulated role in repairing DNA damage (adapted from [39]). Mutations in these genes (represented by "rough" ovals) lead to the accumulation of unrepaired DNA damage. p53, p21 and other cell cycle checkpoint genes are upregulated in response to this damage leading to cell cycle arrest. However, when these genes also become the victim of unrepaired DNA damage cells lose the ability to initiate cell cycle arrest leading to unregulated growth and tumourigenesis.

with respect to regions of the gene which appear to be functionally significant and correlates the phenotypic variation observed in mice and humans with genotype.

REDUCED CELLULAR PROLIFERATION IN BRCA2−/− MICE

Mice which are nullizygous for BRCA1 exhibit growth retardation and embryonic lethality similar to that of three strains of BRCA2−/− mice [27,31,33]. Growth arrest in BRCA1−/− mice was shown to be caused by a decrease in the rate of cellular proliferation rather than an increase in the rate of apoptosis [31]. In BRCA2−/− mice the rate of cellular proliferation has been investigated at various stages of embryogenesis by measuring the incorporation of 5-bromo-2'-deoxyuridine (BRDU) into DNA in the S phase of the cell cycle and by direct measurement of the rates of proliferation of mouse embryonic fibroblasts (MEF) [26,29,30]. Homozygous mutant embryos do not exhibit any significant proliferative differences from heterozygous or wild-type embryos up to 6.5 days. However, at E7.5 the proliferation ability of BRCA2−/− embryos is severely impaired [28]. Likewise, homozygous mutant MEFs initially divide at rates similar to heterozygous and wild type MEFs before undergoing a striking proliferative impairment with successive passages [29,30].

The phenotype of BRCA2 mutant mice changes when they are crossed with p53 mutant mice to create BRCA2/p53 nullizygotes [27]. Although the embryonic lethal phenotype is maintained, BRCA2/p53 null embryos are partially rescued to the extent that embryonic lethality occurs 1–2 days later in gestation. This may indicate that the early lethality observed in the BRCA2 mutant mice is dependent on the function of p53; cell cycle checkpoint activation and cessation of embryonic development may result from the activation of p53 in response to loss of BRCA2. In cells null for p53 as well as for BRCA2 the same constraints on hyperproliferation may no longer exist and as a result BRCA2−/− embryos are able to survive longer. In support of this, p53 expression is increased in BRCA2 homozygous mutant MEFs and is accompanied by an increase in the expression of p21, a G1 cell cycle inhibitor which is upregulated by p53 as part of the growth arrest response to DNA damage [27–30].

BRCA2 AND RESPONSE TO DNA DAMAGE

The embryonic lethal phenotype which is observed in BRCA2−/− mice resembles the phenotype observed in mice null for BRCA1 and RAD51 [27,31–33]. p53 and p21 are also upregulated in response to the growth arrest which occurs in BRCA1 null mice [27,31]. RAD51 is the homologue of a bacterial recombination protein recA and has been implicated in the repair of double-strand DNA breaks [34,35]. RAD51 associates with BRCA1 and BRCA2 *in vitro* (although a direct interaction has only been shown for BRCA2) and co-localises with BRCA1 on meiotic chromosomes [17,36]. This implies a role for BRCA1 and BRCA2 in sensing and/or responding to DNA damage. The response of BRCA2−/− blastocysts and MEFs to DNA damaging agents provides support for this [26,29,37]. Blastocysts null for BRCA2 were shown to be significantly more senstive to γ-irradiation than wild-type or heterozygous embryos [26]. In another study, BRCA2−/− MEFs were defective in repairing DNA damage induced by exposure to X-irradiation in single cell electrophoresis ("comet") assays [29]. Finally, BRCA2−/− MEFs are significantly more sensitive to UV-light and the monofunctional alkylating agent MMS (methyl-methane sulphonate) than BRCA2 heterozygous and wild-type MEFs [37]. The phenotype of BRCA2−/− MEFs resembles that of mouse cells deficient in excision repair protein ECCR1: in addition to sensitivity to UV light and MMS, ECCR1 deficient cells suffer proliferative impairment with successive passaging and are frequently polyploid [38]. BRCA2−/− cells also become increasingly polyploid and chromosomes

exhibit excessive structural abnormalities with successive passaging [37]. This may imply a role for BRCA2 in DNA excision repair.

How do mutations of BRCA2 cause cancer, and more specifically breast and ovarian cancer?

It has been hypothesised that BRCA2 may form a complex of proteins which includes BRCA1 and RAD51 (Figure 2) [39]. In this model, cells in which BRCA1 or BRCA2 have been inactivated are unable to repair DNA damage. In response, p53 and p21 are upregulated leading to checkpoint activation and cell cycle arrest. However, in the absence of functional BRCA1 or BRCA2 protein, DNA damage which affects p53, p21 and other genes involved in cell cycle regulation would also remain unrepaired. Thus, the brakes on cellular proliferation would be released resulting ultimately in tumour formation.

In summary, evidence is accumulating to suggest that BRCA2 belongs to a class of proteins, which includes the mismatch repair proteins associated with predisposition to colorectal cancer, which behave as 'caretakers' of genome integrity. Furthermore, BRCA2 appears to have many characteristics in common with the previously isolated breast cancer predisposition gene BRCA1, suggesting that these two proteins may be coregulated in overlapping pathways. Considering the universal role which is predicted for BRCA2 and BRCA1, it is unclear why mutations in these genes should specifically predispose individuals to breast and ovarian cancer. Future research is likely to clarify the role of BRCA2 as a protein which either senses or responds to DNA damage, and its putative interaction with RAD51 and BRCA1.

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