HYPOTHESIS

BRCA1 functions as a breast stem cell regulator

W D Foulkes

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BRCA1 is an important susceptibility gene for breast cancer, which confers substantial lifetime risks of breast cancer, particularly in the pre-menopausal age group. Typically, carriers of *BRCA1* mutations develop breast tumours that grow rapidly and are high grade and oestrogen receptor negative. They also possess a basal epithelial phenotype, as defined by cytokeratin expression, that is not present in most breast cancers. It has recently been proposed that the adult breast stem cell expresses only basal keratins. Others have indicated a CD44 positive, CD24 negative phenotype for breast cancer stem cells. In this paper, I argue that the biology of human *BRCA1* and its rodent homologues and the clinicopathological features of breast cancer related to *BRCA1* support the notion that one of the key functions of *BRCA1* is to act as a stem cell regulator. This has implications for the management of carriers of mutations of *BRCA1*, in part because support for the role of *BRCA1* as a stem cell regulator would emphasise the distinct nature of breast cancer related to *BRCA1*.

Tissues of complex organisms are comprised of three populations: terminally differentiated static cells that have little or no proliferative capacity, transit amplifying cells that have a short and finite lifespan before becoming terminally differentiated, and stem cells that are capable of self renewal.[1] Such cells can be totipotent or multipotent, depending on the tissue's type and age. Given that cancer and stem cells share a self renewal capacity, the concept that cancer is a stem cell disease is not new.[2] The concept recently has received considerable attention in breast cancer research, however, for several reasons. Firstly, the continued failure of polychemotherapy to cure metastatic breast cancer has led some to reconsider the origin, character, and behaviour of the cell that the treatment is trying to eradicate.[3] Secondly, microarrary studies have indicated that the natural history of breast cancer may be determined by events that occur early in the life of the tumour.[4–6] Thirdly, one group recently claimed to have identified a breast cancer stem cell, because the malignant behaviour of the entire tumour can be reduced to the presence of one rare cell type.[7]

The work of Sun, Möll and others defined subtypes of breast cells and breast cancers based on their expression of cytokeratins.[8 9] This has been recognised to reflect particular topological orientations of breast cells. For example, basal cells of the breast stain with cytokeratin (CK) 5/6, 14, and 17, whereas luminal cells are characterised by expression of CK 7, 8, 18, and 19.[8 9] Myoepithelial cells stain with α smooth muscle actin, S100, and other markers.[10] It is important to note that basal oriented cells and myoepithelial cells are not necessarily the same.[11]

BRCA1 is an important susceptibility gene for human breast cancer. Breast cancer that is occurring in carriers of mutation of *BRCA1* has a distinctive phenotype.[12 13] In addition, mouse models of *BRCA1* function have established an important role for *BRCA1* in mammary gland development, which emphasises the connection between breast development and cancer. Human data are less clear, but they also suggest a role for *BRCA1* in the determination of the structure and function of the adult female breast.[14 15] A large amount of data has accumulated with respect to the molecular genetics of *BRCA1* and its murine homologue,[16] but much less is known from a cellular viewpoint. In this paper, I suggest that the available data support an important role for *BRCA1* in the regulation of breast stem cells.

BRCA1 STEM CELL HYPOTHESIS

I hypothesise that wildtype *BRCA1* functions as a stem cell regulator, in that one of its roles is to promote the orderly transition to the glandular epithelial breast phenotype. Germline mutations or somatic mutations, or both, in *BRCA1* damage this regulatory function, so that failure of transition, a persistence of the "primitive" basal epithelial phenotype, and loss of tight proliferative control over stem cells occur. Many of these cells may die, as programmed cell death is initiated if other genes such as *TP53* are intact. A very small fraction of cells escape this mechanism of death, however, and the resulting clones then grow rapidly and become disseminated, mainly through the bloodstream. I suggest that many of the functional characteristics of wildtype and mutated *BRCA1* genes are consistent with this hypothesis. Moreover, the unusual basal phenotype of breast cancers related to *BRCA1* suggests that *BRCA1* regulates lineage choice in breast development. *BRCA1* also may be implicated in stem cell regulation in other cell types, such as neurons,[17 18] but the influence of mutations of *BRCA1* on cancer predisposition clearly is much more limited than the expression profile of *BRCA1*. A role for *BRCA1* in non-cancerous conditions, such as senescence, has not been excluded, however, and non-cancer phenotypes in carriers of mutations of *BRCA1* deserve further attention.

Correspondence to: Professor W D Foulkes, Program in Cancer Genetics, Departments of Oncology and Human Genetics, McGill University, Room L10-116 Montreal General Hospital, 1650 Cedar Ave, Montreal, QC, Canada H3G 1A4; william.foulkes@mcgill.ca

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SUPPORT FOR THE HYPOTHESIS

Breast cancer stem cells and *BRCA1*

Previous work suggested that in rodents and humans, breast stem cells are situated in the terminal end buds and are basal, or suprabasal, in position.[11 19 20] The precise nature of breast stem cells is not known, but putative rat mammary stem cells have been shown to stain with antibodies raised against CK6.[19] Recent experiments have suggested that these cells are sialomucin (MUC) negative, but epithelial specific antigen (ESA) positive and CK19 positive,[21] whereas others have postulated a CK19 negative phenotype for breast stem cells.[22] Whether or not breast stem cells and breast cancer stem cells necessarily are always the same cell is not clear,[3] and it seems likely that early genetic ''hits'' to a pluripotent breast stem cell could result in the formation of a proliferatively competent breast cancer stem cell. As stated previously, one group recently claimed to have identified definitively a breast cancer stem cell by using a combination of ESA, CD44, and CD24 cell surface markers common to about 2% of all breast cancers.[7] Of relevance to the argument presented here, Böcker and colleagues put forward a new model for the origin of breast cancer.[23] With a combination of morphological analysis and double label immunohistochemistry, they propose that adult breast epithelial stem cells are a suprabasally situated CK5/6 positive cells. Previous studies by this group had suggested that some normal breast luminal cells, which have lost contact with the myoepithelial layer, were CK5/6 positive (that is, they expressed a basal phenotype) but were negative for almost all differentiation antigens.[24] In the skin, a small fraction of basal CK5/6 positive cells are thought to be stem cells or transit amplifying cells.[25]

Wildtype *BRCA1* has a key role in DNA repair,[16] is expressed in basal and luminal layers of the breast,[26] and is also expressed in embryonic stem cells.[27] Loss of *BRCA1*, therefore, could result in persistent errors in DNA replication in immortal cells, with potentially serious downstream effects. Immortal cells need few mutational ''hits'' to become frank cancers, and this might explain the early age of onset of breast cancer related to *BRCA1* compared with non-hereditary breast cancers or breast cancers related to *BRCA2*.[28] Notably, the capacity to propagate after relatively minor mutational derangement may be a specific property of stem cells.[29] In many childhood cancers, fewer mutational events seem to be needed to cause cancer, and this has been attributed to the likely stem cell origin of the cancers.[30] To consider breast cancers related to *BRCA1* as being in the same class as some of these childhood cancers might be useful.

Breast stem cells have been argued to be oestrogen receptor negative cells that are surrounded by oestrogen receptor positive cells that can exert a paracrine effect on the stem cells.[31 32] Notably, grade 3 ductal breast cancers related to *BRCA1* are highly significantly more likely to be oestrogen receptor negative than grade 3 ductal breast cancers not related to *BRCA1* in women of the same age (Foulkes *et al*, manuscript submitted), which suggests that the oestrogen receptor negative status of these cancers is not simply a reflection of their high grade, but rather is determined very early in the life of a cancer and could reflect their stem cell origin.

Clinicopathological features of breast cancers related to *BRCA1*

A distinctive phenotype for breast cancers related to *BRCA1* has emerged. These cancers are usually high grade, infiltrating, ductal carcinomas that possess a pushing margin; do not express oestrogen receptors, progesterone receptors, erbB-2 (HER2), $p27^{Kip1}$, or cyclin D1 but do express p53; and frequently exhibit mutations of *TP53*.[12 13 33–35] These features are not due to the early age at diagnosis of breast cancers related to *BRCA1*. Recent microarray studies have shown that the gene expression profile of oestrogen receptor positive and oestrogen receptor negative breast cancers are very different.[4] Among the latter group, there appears to be a further division between those that are positive for erbB-2 and those that are negative for erbB-2. Breast cancers related to *BRCA1* might be expected to be overrepresented in the ER/erbB-2 negative group. Interestingly, tumours that lacked expression of oestrogen receptors and erbB-2 often had a basal phenotype rather than the more common luminal epithelial phenotype.[4] The basal phenotype has been associated with a poor outcome.[36 37] As stated above, CK 5/6, 14, and 17 usually are present in basal cells, whereas luminal cells are characterised by CK 7, 8, 18, and 19.[8 9] We[38] and others[39] have shown that breast cancers related to *BRCA1* are characterised by a basal epithelial phenotype, in that they commonly overexpress CK5/6 (in our study, nine times more frequently than ER/erbB-2 oestrogen receptor negative breast cancers not related to *BRCA1* or *BRCA2*).[38] Interestingly, combined immunohistochemical, molecular genetic, and loss of heterozygosity studies have suggested that CK5/6-positive, CK8/18-negative breast cancer cells have low levels of bcl-2, p21, p27, oestrogen and progesterone receptors and erbB-2, as well as high levels of Ki-67, epidermal growth factor receptor and p53.[40] These features are remarkably similar to those of breast cancers related to *BRCA1*, as shown above. A role for *BRCA1* in determining the cytokeratin profile of breast cancer thus seems probable. Furthermore, this may reflect a regulatory role for *BRCA1* on cell fate.

Breast cancers related to *BRCA1* grow quickly and are often axillary lymph node negative but may have poor outcomes: breast cancer stem cell phenotype

A recent study showed that breast cancers related to *BRCA1* often present as interval cancers[41] (that is, they occur between mammography screens for breast cancer and are fast growing tumours). We recently found that even large (20–30 mm) breast cancers related to *BRCA1* are much more likely to be axillary node negative than are other types of breast cancer.[42] The processes by which cancer cells spread through the blood and lymphatic routes might differ. Microarray studies of breast cancer suggest that bloodborne spread (and concomitantly, long term survival) is determined by events early in the life of a tumour, whereas lymphatic spread may more closely reflect the time that a tumour has been present.[43] The factors that allow breast cancers related to *BRCA1* to metastasise rapidly and independently of local lymphatic spread also may contribute to the surprisingly poor prognosis associated with node negative breast cancers related to *BRCA1*.[44–46] At the same time, this fast growth may limit the time available for nodal metastases to develop. By contrast, breast cancers related to *BRCA2* are usually oestrogen receptor positive, and most survival studies performed for breast cancers related to *BRCA2* suggest that the prognosis is not substantially different from that seen in non-hereditary breast cancer.[47] Interestingly, overexpression of proteins such as cyclin E, which promotes unregulated cell cycling, predicts a poor prognosis after a diagnosis of breast cancer, which is independent of the grade of tumour.[48] Notably, cyclin E is upregulated in cells that have a basal phenotype.[49] Stem cells are exceptionally controlled with respect to proliferation; loss of this control is a key feature of cancer and breast cancers related to *BRCA1* bear all the hallmarks of a cancer where proliferative control is lost early in the genesis of the cancer. Moreover, tumours in which *BRCA1* is inactivated somatically tend to resemble tumours with germline *BRCA1* mutations,[50 51] which suggests a more general role for *BRCA1* in regulation of breast stem cells.

BRCA1, *TP53*, breast development, and breast cancer

Unlike in women without mutations of *BRCA1*, early pregnancy does not protect carriers of mutations of *BRCA1* from breast cancer before age 40 years; indeed, parity may increase their risk of breast cancer. For example, in one study, parous carriers of mutations of *BRCA1* were at significantly higher risk of breast cancer than matched non-parous carriers of mutations of *BRCA1*.[52] Carriers of mutations of *BRCA1* also are prone to develop breast cancer during pregnancy. In the general population, proliferation that occurs in pregnancy temporarily is thought to increase the risk,[53] but later, the risk of breast cancer diminishes because of differentiation of the terminal end buds. Interestingly, in lactating mammary glands, the lobules contain only terminally differentiated lactating end cells that stain for CK8/18 and not CK5/6 (indicating a luminal, glandular phenotype).[23] The breast epithelium of *Brca1*$^{Co/Co}$ *WAP-Cre* conditional knockout mice fails to differentiate properly.[54] Russo and colleagues suggested in humans that the mammary gland architecture of parous carriers of mutations of *BRCA1* resembles that of nulliparous non-carriers.[14] Moreover, carriers of mutations of *BRCA1* had a shorter total duration of breastfeeding than non-carriers.[15] A slight deficiency of BRCA1 protein (that is, heterozygosity)[55] could result in abnormalities in the self renewing breast stem cells, which go on to form the terminal end buds and cause a failure of terminal differentiation after pregnancy and hence a persisting risk of breast cancer.[52] In rodents, these terminal end buds are clonal and derived from a single cell.[56] Lack of BRCA1 protein in puberty could not only cause defective initial ductal formation but also a failure to differentiate the terminal end buds (that normally occur during pregnancy) such that loss of the second *BRCA1* allele becomes more likely.

In contrast to the situation for *brca1*, absence of *Tp53* results in an indefinite lifespan of transplanted normal mouse mammary tissue *in vivo*,[57] and loss or inactivation of one allele of Tp53 in Brca1$^{\Delta 11/\Delta 11}$ knockout mice rescued the ductal development deficiency noted in these mice.[58] Smith suggested that, in this context, p53 functions to suppress the negative effect of deletion of *brca1* on normal allometric mammary ductal growth (G. Smith, personal communication, 2003). More recent studies of these cells indicate that haploid loss of *Tp53* resulted in some escape from senescence caused by the *brca1*$^{\Delta 11/\Delta 11}$ status, but *brca1*$^{\Delta 11/\Delta 11}$/Tp53$^{+/-}$ mutant cells that escaped senescence underwent clonal selection for faster proliferation and had extensive genetic and molecular alterations.[59] Poor development during embryogenesis and an increased risk of cancer later in life thus could be ''two sides of the same coin''. In the gut, loss of p53 leads to decreased radiation sensitivity of stem cell progeny to radiation,[60] which results in less apoptosis and a higher risk of persisting DNA mismatches. If the same process is present in the breast, then survival of abnormal stem cells could be a rare but catastrophic event for the organism. Mutations in *TP53* have been shown to be common in breast cancers related to *BRCA1*, and this could reflect deficiencies in nucleotide excision repair[35]–a viewpoint supported by recent experimental data.[61] Early inactivation of *TP53* in breast stem cells that lack functional *BRCA1* may be a prerequisite for the subsequent apparently aggressive ''basal'' phenotype that studies at our centre and those of others have identified. Notably, *TP53* mutations are much common in breast cancers with a basal phenotype than in cancers with luminal phenotypes.[36]

Stem cells probably are situated in a physical niche,[62] and, as such, the sensitivity of stem cells to mutational damage may be altered by the hormonal milieu. In non-carriers, pregnancy has been argued to force ''cell differentiation out of the stem cell compartment–thereby reducing the number of cells at risk.''[30] For carriers of mutations of *BRCA1*, the lower levels of *BRCA1* may result in failure to complete this transition. Only after menopause, when a significant fall in circulating oestrogen occurs does risk for breast cancers related to *BRCA1* start to decline. According to the model presented here, fewer susceptible stem cells are present after menopause, and hence the risk per decade of life falls.

Somatic mutations in *BRCA1* in breast cancer

Somatic mutations are rare in breast cancers related to *BRCA1*. Various explanations have been proposed for this phenomenon, including the ''mutation window'' of puberty, after which the breasts are not susceptible to mutations of *BRCA1*[63 64] (but they do seem to remain susceptible to mutations in other genes). The lack of somatic mutations in *BRCA1* also has been suggested to reflect a low intrinsic rate of mutations of *BRCA1*.[65] The reason that somatic mutations in *BRCA1* are rare in non-hereditary breast cancer could be related to the size of the target: breasts enlargen greatly during puberty. Before puberty, the breasts are not susceptible to damage after exposure to ionising radiation, so mutations that occur when the ''target'' is small do not have a phenotype. For women who carry mutations of *BRCA1*, each breast stem cell has one mutation in *BRCA1*, as do all the progeny. Only the proliferation competent cells are susceptible to further mutations. For women who do not carry a mutation of *BRCA1*, both alleles have to be inactivated in the same proliferation competent cell for a cancer to develop from this source. As these first and second events will only have a phenotypic effect when far more susceptible cells are present in the breast than are present before puberty, statistically it is much less probable that a single cell will accumulate both mutations than when the first ''hit'' is already present in all cells.

Chemotherapy and breast cancers related to *BRCA1*

Most stem cells exhibit high levels of BCL-2, and this is thought to be one reason why such cells are often resistant to chemotherapy.[3] The presence of low levels of BCL-2 in tumours often is associated with high levels of proliferation, apoptosis, and cell death.[66] Chemotherapy may be more effective in this setting.[67] Breast cancers related to *BRCA1* are more likely than non-hereditary breast cancers or breast cancers related to *BRCA2* to exhibit low levels of BCL-2.[68] We recently suggested that breast cancers related to *BRCA1* may respond surprisingly well to chemotherapy.[45 69] *BRCA1* may influence the fate of breast cancer stem cells through the balance between proapoptogenic and antiapoptogenic factors, and this has an important effect on response to chemotherapy. That compounds that contain platinum seem to be very effective in treating ovarian cancers related to *BRCA1* is interesting.[70] This class of drug has not been used commonly to treat breast cancer, but it may be particularly effective in breast cancers related to *BRCA1*. The effect of platinum based compounds on breast stem cells is not known.

Implications of the hypothesis

Considerable data suggest that breast cancers related to *BRCA1* are distinguishable from breast cancers not related to *BRCA1* on morphological, immunohistochemical, and molecular grounds. Despite this, it is commonly suggested that the natural history of breast cancers related to *BRCA1* can be reduced to the most simple characteristics of these tumours–that is, node negative, oestrogen receptor negative, high grade, p53 positive, and so on. If the hypothesis proposed here were to be supported, it would shift thinking towards the uniqueness of breast cancers related to *BRCA1*. For example, the debate that surrounds the use of tamoxifen to

prevent breast cancers related to *BRCA1* is predicated on the notion that all oestrogen receptor negative breast cancers essentially are alike. The arguments presented here suggest strongly that oestrogen receptor negative breast cancers related to *BRCA1* are not the same disease as oestrogen receptor negative breast cancers in the general population, and, by inference, the rules about prevention and treatment of breast cancers related to *BRCA1* cannot be derived from research conducted in non-carriers of *BRCA1*. This would have important implications for carriers of mutations of *BRCA1*, whether or not they have received a diagnosis of breast cancer.

TESTING THE HYPOTHESIS

As yet, no consensus exists as to the exact nature of breast cancer stem cells, and therefore extensive immunohistochemical studies and analysis of expression array data have not been performed. Existing data, however, suggest that at least one type of breast cancer stem cell is positive for ESA and CD44 but negative for CD24 and lineage markers.[7] I predict that breast cancer stem cells will be more likely than non-stem cells to express basal keratins and to have low levels of BRCA1 protein. Furthermore, the side population in fluorescent activated cell sorter studies of bone marrow and other tissues are believed to be enriched for stem cells.[71] *BRCA1* is predicted to be more commonly expressed in side population cells than other compartments.

One consequence of the maturation arrest theory of carcinogenesis is that cancers do not develop from benign precursors.[2] The precise pathway to breast cancer in carriers of the *BRCA1* mutation is not known, but one prediction would be that breast cancers related to *BRCA1* do not arise from benign precancerous forms but in fact arise *de novo*. The relative lack of ductal carcinoma *in situ* and other more benign lesions in the breasts of women who carry a *BRCA1* mutation[72 73] lends support to the hypothesis advanced here.

Conditional regulated alleles of *brca1* have been created.[53] With the cleared mammary fat pad system,[19] it should be possible to try to create an entire murine breast from a single cell that contains a conditionally regulated allele of *brca1*. One prediction of this hypothesis is that breast development will be possible from a single cell that lacks at least one copy of *brca1*, but that development of the breast will be abnormal, and breast cancer will be more likely, particularly if *Tp53* is also absent.

Whether expression of *BRCA1* in cells that lack *BRCA1* can alter their immunohistochemical phenotype is unknown, but one test might be to establish whether the presence of wildtype *BRCA1* tends to promote the CK8/18 positive luminal phenotype, which is the expected phenotype of most terminally differentiated breast cells, whether normal or otherwise.

CONCLUSION

Much is known about the molecular genetics of *BRCA1*, but its role in shaping cell fate has been studied in less detail. I propose that *BRCA1* is a regulator of breast stem cells and that this is its key function in normal breast tissue. In the schema presented here, *BRCA1* is the nexus between breast development and breast cancer, and perturbations of the gene in humans and other animals significantly alter both processes.

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