Novel treatment strategies for hereditary breast cancer
Evers, B.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 1

*Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects*

Published in *Oncogene* 2006; 25: 5885-5897
Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency

Mouse models of BRCA1 and BRCA2 deficiency: past lessons, current understanding and future prospects

Bastiaan Evers and Jos Jonkers
Division of Molecular Biology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Germline mutations in BRCA1 and BRCA2 are responsible for a large proportion of hereditary breast and ovarian cancers. Soon after the identification of both genes in the mid-1990’s, investigators set out to develop mouse models for the associated disease. Whereas conventional Brca1 and Brca2 mouse mutants did not reveal a strong phenotype in a heterozygous setting, most homozygous mutations caused embryonic lethality. Consequently, development of mouse models for BRCA-associated tumorigenesis required the generation of tissue-specific conditional knockout animals. In this review, we give an overview of the conventional and the conditional mouse models of BRCA1 and BRCA2 deficiency generated over the last decade, as well as the contribution of these models to our understanding of the biological and molecular functions of BRCA1 and BRCA2. The most advanced mouse models for BRCA1- and BRCA2-associated tumorigenesis mimic human disease to the extent that they can be used in studies addressing clinically relevant questions. These models will help to resolve yet unanswered questions and to translate our increasing knowledge of BRCA1 and BRCA2 biology into clinical practice.

Introduction
Breast cancer is the most common malignancy in women of the Western world, affecting up to 10% of the female population1. An estimated 5% of all breast cancers is ascribed to hereditary predisposition. Intensive research in the early 1990’s has led to the identification of two major breast cancer susceptibility genes, BRCA12-3 and BRCA24,5. Individuals carrying mutations in one allele of either BRCA1 or BRCA2 have a life-time risk of up to 80% for developing breast cancer and also display increased risk for ovarian cancer (~40% for BRCA1 mutation carriers and ~20% for BRCA2 carriers, respectively)6. In most breast cancers arising in BRCA1/2 carriers, inactivation of the wild-type allele has occurred by means of loss of heterozygosity (LOH), thus abolishing normal protein expression7,8. BRCA1 tumors show a rather uniform tumor type of high-grade invasive ductal carcinomas, which are usually estrogen receptor (ER) and HER2/neu negative9,10. These properties are reminiscent of "triple-negative" (ER-, progesterone receptor (PR)- and HER2/neu-negative) basal-like sporadic breast cancers11. Indeed, cluster analysis of gene expression data from human breast cancers revealed strong similarity between BRCA1-mutated tumors and basal-like breast cancers12. BRCA2 deficient tumors, on the other hand, show larger variety in histological classification and are not easily distinguished from the overall spectrum of sporadic tumors. While parity in the general population has been associated with decreased breast cancer risk, parity per se was not found to influence risk in BRCA1 mutation carriers, although multiparity did seem to protect as well. For BRCA2, data suggest that increasing parity is associated with increased risk of breast cancer. BRCA2-mutation carriers also have an increased risk of developing pregnancy-associated breast cancer13.
Conventional Brca1 and Brca2 mutants
Following identification of the BRCA1 and BRCA2 breast cancer genes in humans, investigators sought to recapitulate the effects of these genetic lesions in mouse models. Several groups have generated a range of conventional Brca1 and Brca2 knockout mice with mutations in different portions of the genes (see Figures 1 and 2). Somewhat disappointingly, none of these mouse mutants showed a strong tumor predisposing phenotype in a heterozygous setting. When bred to homozygosity, most Brca1 and Brca2 mouse mutants displayed severe embryonic lethal phenotypes. Although these traits have hindered in vivo analysis of tumor suppressor functions of Brca1 and Brca2, conventional mouse mutants have provided valuable insight into their biological roles.

Functional clues
Soon after the generation of Brca1 and Brca2 mouse mutants, it was acknowledged that the embryonic lethality of Brca1-deficient mice closely resembled the phenotype of mouse mutants of Rad51, a homologue of the bacterial RecA protein known to be involved in DNA damage repair\textsuperscript{14}. Subsequently, Rad51, Brca1 and Brca2 proteins were shown to have similar expression patterns during mouse embryogenesis\textsuperscript{15-18} and during cell cycle progression\textsuperscript{19,20}. In addition, Brca1, Brca2 and Rad51 were found to co-localize in nuclear dot patterns\textsuperscript{21,22} and to interact with each other\textsuperscript{15,21,22}, suggesting a role for the BRCA proteins in DNA damage repair. This notion was supported by the observation that Brca-deficiency results in chromosomal instability and increased sensitivity to DNA damaging agents in cultured mouse cells\textsuperscript{15,23-25} and in living animals\textsuperscript{26}. These phenotypes were subsequently attributed to defects in repair of double-strand breaks (DSBs) by homologous recombination (HR)\textsuperscript{27-29}.

Whereas initial studies in Brca1 and Brca2 mouse mutants have highlighted similarities between both proteins, other research efforts have also revealed clear differences\textsuperscript{30}. BRCA1 appears to be more of a signal integrator, linking together sensors and response mechanisms of several types of DNA damage. In contrast, BRCA2 is thought to be more directly involved in homology-directed DSB repair, as it mediates the formation of a RAD51-DNA nucleoprotein filament that catalyzes strand invasion during HR\textsuperscript{31}.

For BRCA1, a wealth of data describes many interactions with other proteins, as well as many functions besides the involvement in the maintenance of genetic stability. Thus, apart from DNA repair, functions have been described for BRCA1 in all phases of the cell cycle\textsuperscript{32}, transcription\textsuperscript{33} and DNA decatenation\textsuperscript{34}. Studies with the Brca1 mouse mutants – or primary cells derived thereof – have revealed several distinct activities of Brca1, including its potential roles in the G2-M cell cycle and spindle assembly checkpoints\textsuperscript{35,36}, maintenance of telomere integrity\textsuperscript{37} and transcriptional repression of unsynapsed chromosomal regions during meiosis\textsuperscript{38}. Interesting with respect to the gender specificity observed in BRCA-associated cancers is the reported role for BRCA1 in maintenance of X-chromosome inactivation\textsuperscript{39}. Recent studies have suggested that also BRCA2 may, in addition to its role in HR, have other functions in for example transcriptional regulation\textsuperscript{40}. Notably, primary mouse embryonic fibroblasts derived from hypomorphic Brca2 mutants have provided important clues regarding possible roles of Brca2 in stabilization of stalled DNA replication forks\textsuperscript{41} and cytokinesis\textsuperscript{42}. 
The BRCA paradox
The developmental failure of both Brca1- and Brca2-deficient animals was mainly ascribed to a proliferation defect\textsuperscript{43-46}. This striking observation introduced a paradox in the biology of BRCA1 and BRCA2. Whereas BRCA-deficient tumor cells proliferate rapidly \textit{in situ}, cells in developing Brca-deficient embryos suffer from a proliferation defect. Although this paradox is still unresolved, part of it might be explained by the genetic interactions between BRCA1/2 and the p53 pathway. Growth-arrested Brca1- and Brca2-deficient embryos showed activation of the \textit{Cdkn1a} gene encoding the cyclin-dependent kinase (cdk) inhibitor p21, a p53 downstream target known to function in the G1-S cell cycle checkpoint\textsuperscript{44,46}. To investigate the role of p53 and p21 in Brca-associated proliferation arrest, compound mutant animals lacking both Brca1/2 and p53 or p21 were produced by cross-breed-
Whereas embryonic survival of Brca1 mutant embryos was partially rescued in a p53- or p21-deficient background, rescue of Brca2 knockout animals by disruption of p53 was less clear. Together, these results indicate that in addition to p53 other factors play a role in the proliferation defect of Brca1- or Brca2-deficient embryos.

### Genotype-phenotype correlations

As shown in Figures 1 and 2, the various Brca1 and Brca2 mouse mutants display large phenotypic variation. Viability and life span differ considerably between models, as did the rescue of embryonic lethality on a p53-null background. Indeed, p53-nullizygosity results in extensive phenotypic rescue of hypomorphic Brca1 mutants, but only delays embryonic lethality of Brca1-null mutants. Apart from differences in targeted mutations in Brca1 or Brca2, genetic background differences between the various Brca1 and Brca2 mutant mouse strains are likely to play an important role in the phenotypic differences between the published models.

### Truncated proteins

Both Brca1 mouse mutants that are predicted to fail to produce Brca1 protein (i.e. Brca1ex2, Brca1s1) die around E7.5-9.5 when bred to homozygosity (see Figure 1). Embryonic lethality in these Brca1null models is characterized by reduced cell prolifera-
tion without signs of increased apoptosis\(^4\). Embryonic lethality is also observed in the \(Brca1^{1700T}\) model, carrying a C-terminal truncating mutation that removes the second BRCT repeat\(^5\). Compared to the \(Brca1^{null}\) models, however, homozygous \(Brca1^{1700T/1700T}\) embryos show a delayed embryonic lethality marked by continued cell proliferation and differentiation until an apoptotic response is activated around E9.5-10.5. These data suggest that C-terminal truncating \(Brca1\) mutations may have different effects on normal cell function than \(Brca1^{null}\) mutations.

Phenotypic differences are also found for the different \(Brca2\) mutations generated to date (see Figure 2). Based on these differences, it was previously suggested that embryos carrying \(Brca2\) mutations that did not encode any BRC repeat could not survive, whereas embryos carrying \(Brca2\) mutations that encoded at least three BRC repeats were partially viable\(^5\). This, however, appears not to be the case for the embryonic lethal \(Brca2^{tm1Mhun}\) mutation, which encodes a truncated protein with four BRC repeats\(^5\).

Although several \(Brca1\) and \(Brca2\) mutations have been described as resulting in truncating alleles (Figures 1 and 2), in most cases the presence of a truncated protein has not been demonstrated. Some of these mutations could be effectively null alleles if the mRNA or protein is unstable. Nevertheless, the large phenotypic variation of the various mouse mutants predicts that also different human \(BRCA1\) or \(BRCA2\) germline mutations will have different effects on normal cell function and tumor predisposition. Additional mouse models mimicking defined germline mutations are needed to address this important issue. The first "humanized" mouse models have already been generated via introduction of a BAC transgene containing the human \(BRCA1\) gene into homozygous \(Brca1\) knockouts. Whereas embryonic lethality was completely rescued by wild-type \(BRCA1\), no rescue was observed for a human \(BRCA1^{C64G}\) allele with a missense mutation in the RING finger domain\(^5\).

**Alternative splice products**

An important and often underappreciated aspect that has complicated proper interpretation of the \(Brca1\) mutant models, is the fact that \(Brca1\) transcripts are subject to alternative splicing. As a result of this, interpretations of targeted mutations in \(Brca1\) should not only take into account the effects on the full-length transcript, but also on all possible splice variants. For humans, several alternative splice products have been described\(^5\). Of all splice variants, only the \(BRCA1-\Delta11\) and \(BRCA1-IRIS\) variants have been functionally analyzed to date\(^6\). The \(Brca1-\Delta11\) splice variant is conserved in mice\(^6\), whereas existence of a rodent \(Brca1-IRIS\) splice variant is still undetermined.

Several \(Brca1\) mutations that encode truncated versions of the full-length protein could, at least in theory, still lead to the production of \(Brca1-\Delta11\) splice variants (see Figure 1), and the increased viability of some of the homozygous mouse mutants strongly suggests that these alternative splice products have a biological function. Indeed, similar to full-length \(Brca1\), \(Brca1-\Delta11\) is expressed in a cell-cycle regulated manner and localizes to discrete nuclear foci, despite the fact that this splice product lacks the nuclear localization signals of full-length \(Brca1\). DNA damage-induced \(Brca1\) phosphorylation and Rad51 focus formation, on the other hand, were severely impaired in cells that only express \(Brca1-\Delta11\).
Also Brca1-IRIS may have distinct biological activities, as it is capable of stimulating DNA synthesis, presumably through its interaction with pre-replication complexes. The fact that full-length BRCA1 and BRCA1-IRIS are expressed from different promoters raises the interesting possibility that functions of Brca1 towards tumor suppression and embryonic development might reside on different locations in the gene. Indeed, the only Brca1 deletion mutant with an intact hypothetical Brca1-IRIS coding unit does not suffer from a proliferation defect, yet dies at E10.5 due to apoptosis. Further investigation of these exciting new aspects of BRCA1 biology will help to dissect the different functions exerted by distinct BRCA1 splice variants.

**Genetic background effects**
Several lines of evidence point towards a strong influence of modifier genes on survival of Brca1- and Brca2-deficient animals. Embryonic survival of a lethal Brca2 mutation increased significantly from embryonic day E8.5 on a 129 background to E10.5 on a BALBc background, and postnatal viability of two truncating Brca2 mutations was significantly increased for homozygous mutant mice generated in mixed 129/B6/DBA or 129/MF1 backgrounds compared to 129/B10 or 129/129 genetic backgrounds. Likewise, embryonic lethality of a hypomorphic Brca1 mutation (which still encodes for Brca1-Δ11) was completely rescued when the mixed 129/B6 mice were backcrossed onto 129/Sv or outcrossed using MF1. Obviously, it would be interesting to perform backcross experiments in combination with whole-genome scanning studies in the mouse, in order to map and eventually identify modifier genes that affect embryonic lethality of Brca1 and Brca2 mutants. Provided that similar mechanisms mediate viability of Brca-deficient embryos and survival of BRCA-deficient tumor cells, such genes might form interesting therapeutic targets.

**Tumor predisposition**
Whereas most Brca1 and Brca2 mouse mutants were embryonic lethal when bred to homozygosity, some gave rise to sub-Mendelian ratios of viable offspring. Viable Brca1<sup>h/tr</sup> mutants, encoding the Brca1-Δ11 splice variant, were found to be tumor-prone, although only 12 out of 92 neoplasms were mammary tumors. A bias towards lymphoid and sarcomatoid tumors became even more pronounced when these animals were crossed onto a p53<sup>-/-</sup> background; nevertheless, Brca1<sup>h/tr</sup>;p53<sup>-/-</sup> mice developed tumors significantly faster than the p53 single knockouts. Similarly, Cressman et al. described three Brca1Δ223-763/Δ223-763;p53<sup>-/-</sup> animals that succumbed to lymphomas. Finally, heterozygous p53 mutations introduced in mice harboring two Brca1<sup>Δ11</sup> alleles gave rise to mammary tumors in most of the animals produced, although also here lymphomas were observed. For Brca2, all three models that enabled survival of animals showed increased tumorigenesis in the absence of p53 mutations. However, the Brca2 models displayed a strong bias towards developing thymic lymphomas. In conclusion, while these data clearly demonstrate tumor suppressor activities for Brca1 and Brca2, the utility of these models to investigate properties of human breast cancer is limited because of the low incidence of mammary carcinomas.

**Conditional Brca1 and Brca2 mutants**
Besides embryonic lethality and development of non-epithelial tumors, another important limitation of conventional Brca1 and Brca2 homozygous mutants is that they cannot model development of sporadic cancer, which arises amidst a genetically "normal" background. To overcome these limitations, conditional mutagenesis strat-
Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency

Strategies have been developed, which rely on Cre recombinase that catalyzes specific genetic deletion of genomic regions flanked by loxP recombination sites. These recombination sites are inserted in non-coding sequences to ensure that expression of the gene is not disrupted prior to Cre-mediated recombination. Introduction of Cre recombinase into these conditional mouse mutants – via intercrosses with Cre transgenic mice or via somatic delivery – results in tissue-specific and/or time-controlled inactivation of conditional tumor suppressor genes.

Targeting Cre expression to mammary epithelium

Several promoters have been used to achieve mammary specific Cre expression, all with their own advantages and disadvantages. Wagner and co-workers published the creation of transgenic mice expressing Cre under the control of the whey acidic protein (WAP) promoter as well as the mouse mammary tumor virus long terminal repeat (MMTV) promoter, whereas Selbert et al. chose the ovine β-lactoglobulin (BLG) promoter to drive Cre expression. An important limitation of these three mammary gland-specific promoters is that their activity is strongly influenced by steroid hormones. Furthermore, models employing the WAP-Cre and MMTV-Cre promoters often require one or even multiple rounds of pregnancy and lactation for effective induction of mammary tumor formation. To overcome these limitations, we created a transgenic line expressing Cre under the control of the cytokeratin-14 (K14) promoter. Besides K14-Cre expression in salivary glands and skin, stochastic Cre-recombinase activity was found both in luminal epithelial cells and in myoepithelial cells of virgin mammary glands, suggesting that K14-Cre is expressed in both cell types or in a common progenitor. The latter hypothesis is supported by the observation that K14 is expressed in Lin CD29+CD24- mammary progenitor cells or CD24+CD49fhigh mammary repopulating unit (MRU) cells. The stochastic activity of K14-Cre results in Cre-mediated gene switching in only a small fraction of mammary epithelial cells. While this trait is advantageous for sporadic tumor development, it precludes assessment of direct consequences of Brca loss-of-function.

Conditioned Brca1 and Brca2 alleles

Three different conditional Brca1 alleles have been generated to date, i.e. Brca1F5-6, Brca1Co and Brca1F5-13 (Liu et al., manuscript in preparation; see Figure 1). Cre-mediated deletion of exons 5-6 or exons 5-13 from the Brca1F5-6 or Brca1F5-13 allele, respectively, induces a frameshift mutation that abrogates production of all three splice products (full-length Brca1, Brca1-Δ11 and the putative Brca1-IRES). In contrast, deletion of exon 11 of the Brca1Co allele results in a hypomorphic mutation that still allows for expression of Brca1-Δ11. For Brca2, four different conditional alleles have been published to date (Figure 2). Of these, the Brca2F9-10 and Brca2F3-4 alleles encode – upon Cre-mediated switching – truncated transcripts that are susceptible to nonsense-mediated mRNA decay. Although the Brca2F11 allele results in an in-frame deletion after Cre expression, early embryonic lethality of animals homozygous for the Brca2Δ11 allele suggests that also this allele is a functional null. In contrast, Cre-mediated deletion of the Brca2F27 allele did not result in embryonic lethality of homozygous mutants, despite the fact that the BRCA2 C-terminal domain encoded by exon 27 contains a nuclear localization signal, interacts with RAD51 in a CDK-dependent manner, and is required for maintaining genomic stability after DNA damage.
Mammary development in conditional Brca1/2 mutants

To achieve mammary gland-specific inactivation of Brca1, Xu and colleagues crossed both MMTV-Cre and WAP-Cre transgenic lines with mice carrying one conventional Brca111- allele and one conditional Brca1Co allele. Female mice from both models showed abnormal development of mammary glands. Defects included incomplete ductal outgrowth, alveolar differentiation and involution, suggesting that Brca1 function is indispensable for normal mammary gland development. Similar analyses for Brca2 showed reduced ductal side branching in mammary glands from Brca2Δ27/Δ27 female mice. These data contrast to results obtained with MMTV-Cre;Brca2F9-10/F9-10 and with WAP-Cre;Brca2F9-10/F9-10 animals, in which mammary gland development or involution appeared to be unaffected. However, no firm conclusions can be drawn from the latter study, since Cheung et al. did not demonstrate the existence of Brca2-deficient mammary epithelial cells with both Brca2F9-10/F9-10 alleles recombined.

Mammary tumorigenesis in conditional Brca1 mutants

With the aim of creating a mouse model for breast cancer, MMTV-Cre;Brca1Co/11- or WAP-Cre;Brca1Co/11- female mice were continuously mated to induce sustained high-level expression of Cre. Some of the females indeed developed mammary tumors of diverse types, albeit with long latency. This model was subsequently improved by introduction of a single p53 null allele, yielding MMTV-Cre;Brca1Co/Co;p53−/− mice which developed mammary tumors with reduced latency. Follow-up studies showed that this model mimicked several aspects of human BRCA1-associated carcinogenesis. Tumors were ERα-negative and displayed gross genomic instability as shown by comparative genomic hybridization (CGH) and spectral karyotyping (SKY). Tumorigenesis in the MMTV-Cre;Brca1Co/Co;p53−/− model appeared to be critically dependent on abrogation of p53 function, since most tumors showed loss of the wildtype p53 allele. This is similar to the human situation where aberrant p53 signaling was more commonly found in BRCA1-associated breast cancers, compared to sporadic tumors. Cooperation between p53 and Brca1 in mammary tumorigenesis was also demonstrated in a K14-Cre;Brca1F5-13/F5-13;p53F2-10/F2-10 mouse model with tissue-specific inactivation of both Brca1 and p53. Female mice of this strain developed, besides skin tumors, mammary tumors that closely resembled their human counterparts. Most mammary tumors of K14-Cre;Brca1F5-13/F5-13;p53F2-10/F2-10 females were highly aneuploid solid carcinomas with basal-like characteristics, including high proliferation index, poor differentiation, lack of ERα expression and continuous pushing margins.

Mammary tumorigenesis in conditional Brca2 mutants

To achieve mammary gland-specific recombination of a Brca2F3-4 conditional allele, Ludwig et al. used a knock-in targeting strategy to place the Cre gene under the transcriptional control of the endogenous mouse Wap gene promoter. Crossing of the resulting Wapcre/+ mice with Brca2ex11/F3-4 animals resulted in Wapcre/+;Brca2ex11/F3-4 female mice, which, upon continuous mating to induce Cre expression, succumbed to non-metastatic mammary carcinomas or adenosquamous carcinomas after a relatively long latency of ~1.4 years. The tumors displayed aneuploidy and chromosomal aberrations, were ErbB2/neu-negative and usually ERα- and cyclin D1-positive. Also MMTV-Cre;Brca2F9-10/F9-10 animals developed mammary carcinomas after long latency periods of ~1.6 years. This latency was significantly reduced in a p53
heterozygous background. Although there was a difference in tumor latency between MMTV-Cre;Brca2<sup>F9-10/F9-10</sup>;p53<sup>+/−</sup> mice and MMTV-Cre;Brca2<sup>F9-10/F9-10</sup>;p53<sup>+/−</sup> or p53<sup>−/−</sup> animals, the shift in tumor spectrum was the most striking effect of mammary gland-specific Brca2 inactivation in p53 heterozygous mice. Combined tissue-specific inactivation of both Brca2 and p53, on the other hand, resulted in highly efficient mammary tumor formation in K14-Cre;Brca2<sup>F11/F11</sup>;p53<sup>F2-10/F2-10</sup> female mice. A control group of K14-Cre; Brca2<sup>F11/F11</sup>;p53<sup>F2-10/F2-10</sup> females showed a much longer median tumor latency (10 instead of 6 months), demonstrating that Brca2 and p53 loss-of-function have a synergistic effect on mammary tumorigenesis.

Conditional Brca1/2 mutation in other tissues

Since cancer predisposition of human BRCA1/2-mutation carriers primarily concerns breast cancer, research efforts have primarily focused on modeling this cancer type in Brca1/2 mutant mice. While the reason for this biased tumor spectrum is not understood, it is important to know whether the BRCA gene products exhibit tumor suppression functions in other tissues as well.

T cell-specific deletion of Brca1 in Lck-Cre;Brca1<sup>F5-6/F5-6</sup> mice showed that thymocyte development, but not T cell receptor recombination, is dependent on Brca1 function. Concomitant p53 abrogation or overexpression of the apoptosis inhibitor Bcl-2 rescued thymocyte survival and development. Similar experiments using Lck-Cre;Brca2<sup>F9-10/F9-10</sup> mice did not show any changes in thymocyte cellularity or composition, although in vitro experiments showed increased apoptosis and genomic instability in both Brca1- and Brca2-deficient thymocytes. In line with the lymphoma predisposition of hypomorphic Brca2 mutants, a small acceleration of T-cell lymphomagenesis was reported for Lck-Cre;Brca2<sup>F9-10/F9-10</sup>;p53<sup>−/−</sup> mice, compared to Lck-Cre;p53<sup>−/−</sup> control animals.

Brca1 and Brca2 also have tumor suppressor activity in skin epithelium. K5-Cre;Brca1<sup>Co11/Co11</sup> mice – in which Cre expression from a bovine cytokeratin-5 promoter induces recombination in e.g. the basal cell layer of the epidermis, mammary myoepithelium and oral cavity – develop squamous cell carcinomas. Thus, Brca1 functions as a tumor suppressor in other epithelial tissues besides mammary gland. Interestingly, E2F1 overexpression in these animals dramatically accelerated skin tumor development, suggesting cooperation between the Rb-E2F1 pathway and Brca1 in tumorigenesis. Similar cooperation in skin tumorigenesis was found between Brca1 and p53 and between Brca2 and p53. The latter observation indicates that also Brca2 has tumor suppressor activity in skin epithelium. Tumor suppressor activity of Brca1 and Brca2 in yet other tissues has not been convincingly documented to date. However, broad tumor suppressor activity of Brca1 might be suggested by the fact that Brca1<sup>Tr/Tr</sup> mice developed, in addition to mammary tumors and lymphomas, tumors in many other organs such as lung, liver, uterus and colon. From a clinical perspective, it would be especially relevant to know whether ovarian-specific Brca inactivation would lead to tumors that resemble human BRCA-associated ovarian cancer. In conclusion, despite the tissue bias of human BRCA-associated cancers, Brca1 and Brca2 may have tumor suppressor activity in multiple mouse tissues, suggesting that the tumor spectrum seen in human BRCA-mutation carriers is not due to cell-type specific tumor suppression.
Genetic interactions in development and tumorigenesis

BRCA1 and BRCA2 are unusual tumor suppressors in that they are essential for normal cell survival, yet their inactivation seems pivotal for cancer development in BRCA-mutation carriers. These paradoxical observations suggest that BRCA-associated tumorigenesis might be fostered by (tissue-specific) environmental and/or genetic factors that somehow alleviate BRCA-associated growth suppression.

Genetic interactions with the p53 pathway

It has long been hypothesized that genetic interaction between BRCA1/2 and the p53 pathway results from the DNA damage that accumulates in repair-deficient BRCA-mutated cells. DSBs in these cells would trigger a p53-mediated cell cycle checkpoint that could be alleviated by e.g. mutation of p53 or its downstream target p21. This concept is supported by the observation that inactivation of p53 or p21 results in a prolonged survival of Brca-deficient embryos45,47. Another interesting hypothesis for how p53 mutations might cooperate with BRCA deficiency relates to the recent appreciation of the potential role of tetraploid cells in tumorigenesis85. Tetraploid cells that were obtained through induction of endoreduplication via a transient block in cytokinesis, were found to be dependent on p53 dysfunction for survival. These cells were genetically unstable and tumorigenic, properties that were not displayed by isogenic diploid cells. Interestingly, it was recently shown that Brca2 deficient cells undergo abnormal cytokinesis86, and indeed knockdown of BRCA1 caused accumulation of multinucleated cells86. It is thus conceivable that BRCA-deficiency may foster the generation of endoreduplicated cells that require dysfunctional p53 for survival.

Genetic interaction between BRCA1/2 and the p53 pathway is supported by a large body of data. For example, p53 mutation is more often observed in BRCA-associated tumors than in sporadic cancers87, and BRCA1/2 tumors showed common changes at p53 codons that are not mutation hotspots88,89. As mentioned before, studies in mouse models have documented cooperation between loss of p53 and inactivation of Brca1 or Brca2 in tumorigenesis, although the exact nature of this cooperation remains unclear. However, p53 mutation is not sufficient for complete reversion of Brca-associated growth arrest, given the incomplete rescue of embryonic lethality and proliferation defects of cultured embryonic fibroblasts24. Taken together, these data suggest that abrogation of the p53 pathway is necessary but not sufficient for survival of BRCA-deficient cells.

Other genetic interactions

Besides p53, also other cell-cycle checkpoint and DNA damage response factors have been found to modulate phenotypes in Brca-deficient animals. Lee and colleagues provided evidence that inactivation of the spindle assembly checkpoint may be involved in Brca2-associated tumorigenesis90. Following the observation that lymphomas arising from Brca2Tr/Tr mice contain mutations in the spindle checkpoint genes Bub1 and Mad3L, it was shown that dominant negative Bub1 rescues the proliferation defect of Brca2Tr/Tr embryonic fibroblasts. However, direct cooperation between mutated spindle checkpoint genes and BRCA2 inactivation in tumorigenesis remains to be established.

More clear are the genetic interactions between Brca1 and Chk2, which have been documented in several Brca1 models. Chk2 deficiency was found to restore T-lymphocyte cellularity and development
Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency

in Lck-Cre;Brca1F5-6/F5-6;Chk2-/- mice and to promote mammary tumor formation in WAP-Cre;Brca1F5-6/F5-6;Chk2-/- mice91. Furthermore, embryonic lethality of homozygous Brca1Δ11/Δ11 mutants was rescued in a Chk2-deficient or in a Atm-haploinsufficient background92. Together, these data suggest that loss-of-function of either Atm or Chk2 can substitute for p53 inactivation in Brca1-associated embryonic lethality and tumorigenesis, thus providing strong evidence for the activation of the Atm–Chk2–p53 DNA damage response pathway in Brca1-deficient cells. Activation of this pathway by DSBs leads to p53-induced cell cycle arrest, senescence and apoptosis93. Chk2 activates p53 through phosphorylation, and may therefore not only function as an "amplifier" of DNA damage response but also as a tumor suppressor. Indeed, heterozygous germline CHK2 mutations were found in families with non-p53 Li-Fraumeni syndrome94. In addition, the CHK2*1100delC variant, encoding an unstable truncated protein lacking the kinase domain, was identified as a low-penetrance breast cancer susceptibility gene in noncarriers of BRCA1 or BRCA2 mutations95.

GADD45a is a growth arrest- and DNA damage-inducible gene, and a transcriptional target of BRCA196. Since also Gadd45A-deficient mouse embryonic fibroblasts exhibited centrosome amplification97, genetic interactions between Brca1 and Gadd45 were studied in Brca1Δ11/Δ11;Gadd45a-/- mice. Compound mutant embryos died at E9.5-10.5, exhibiting exencephaly and high rates of apoptosis, probably due to activation of p53 as a result from increased genetic instability98. The relevance of this interaction for BRCA1-associated tumor formation is still unclear.

Applications of current Brca1 and Brca2 mouse models

Validation studies

The development of mice with mammary gland-specific deletion of Brca1 or Brca2 has not only improved our understanding of BRCA-associated breast cancer in humans, but also provided tools that can be used to test novel therapeutic intervention and tumor prevention strategies. To this end, it is important to validate the different mouse models with respect to the human disease. Ideally, validation of the mouse models should not only include comparative histopathology and cross-species comparison of gene expression profiles and molecular genetic alterations, but also therapeutic benchmarking by testing responses of mouse mammary tumors to conventional chemotherapy and radiotherapy. Although not necessarily all human aspects need to be recapitulated in one mouse model for it to be useful, careful assessment of all differences and similarities is important for correct interpretation of results obtained with the mouse model.

Most Brca1 and Brca2 mouse mammary tumor models generated to date have been validated to only a limited extent (Table 1). In general, they show a relatively high incidence of mammary carcinomas, have deregulated p53 pathways and show genetic instability in the resulting tumor cells. Several mouse models, especially the Brca2 mammary tumor models, developed mammary tumors with histopathological features that were rather different from their human counterparts. Whether this is reflective of a difference in cell-of-origin or a more general difference between both species remains unclear. Cross-species comparison of gene expression data from mammary tumors of the K14-Cre;Brca1Δ13/Δ13;p53F2-10/F2-10 model revealed gene expression signatures with fea-
Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency

Table 1 - Human vs mouse comparison of BRCA-associated mammary tumors.

<table>
<thead>
<tr>
<th>Property</th>
<th>Human BRCA1 breast cancers (compared to sporadic tumors)</th>
<th>B6C3F1/1−/+ [Ref. 67]</th>
<th>B6C3F1/1−/+; p53−/− [Ref. 69]</th>
<th>MMTV-Cre; Brca1Co/Δ11 [Ref. 75]</th>
<th>MMTV-Cre; Brca1Co/Δ11; p53−/− [Ref. 75]</th>
<th>WAP-Cre; Brca1Co/Δ11 [Ref. 77]</th>
<th>K14-Cre; Brca1Co/Δ11 [Ref. 80]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Invasive ductal carcinoma</td>
<td>+/−</td>
<td>ND</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>ER expression</td>
<td>Less common</td>
<td>+/−</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>PR expression</td>
<td>Less common</td>
<td>+/−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HER2/neu overexpression</td>
<td>Less common</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>p53 mutations</td>
<td>More common</td>
<td>+</td>
<td>ND</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Aneuploidy</td>
<td>More common</td>
<td>ND</td>
<td>ND</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tumor incidence</td>
<td>High</td>
<td>-</td>
<td>+/−</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Property</th>
<th>Human BRCA2 breast cancers (compared to sporadic tumors)</th>
<th>Wap-Cre; Brca2Co/Δ11/F5-13/F5-13 [Ref. 76]</th>
<th>MMTV-Cre; Brca2Co/Δ11/F9-10/F9-10 [Ref. 79]</th>
<th>MMTV-Cre; Brca2Co/Δ11/F9-10/F9-10; p53−/− [Ref. 79]</th>
<th>K14-Cre; Brca2Co/Δ11/F11/F11 [Ref. 71]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Invasive ductal carcinoma</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>+/−</td>
</tr>
<tr>
<td>ER expression</td>
<td>No difference</td>
<td>+/−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PR expression</td>
<td>No difference</td>
<td>+/−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HER2/neu overexpression</td>
<td>No difference</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>p53 mutations*</td>
<td>No difference</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aneuploidy</td>
<td>More common</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tumor incidence</td>
<td>High</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*It is unclear whether BRCA2 tumors show more p53 mutations, compared to sporadic tumors. However, since p53 mutations are found in 40-50% of BRCA2 tumors, we have scored p53 mutations in the mouse Brca2 models as being in concordance with the human condition. For each property, a ‘+’ indicates similarity to the human situation, whereas a ‘−’ indicates lack of similarity. ‘+/−’ symbols indicate heterogeneity of the factor involved, and ‘ND’ indicates no information has been reported.

...tures of basal-like breast cancers and strong resemblance to human BRCA1-mutated breast cancers. Analysis of DNA copy number alterations in mammary tumors from the MMTV-Cre;Brca1Co/Δ11, WAP-Cre;Brca1Co/Δ11 and WAP-Cre;Brca1Co/Δ11;p53−/− models by comparative genomic hybridization (CGH) and spectral karyotyping (SKY) revealed a pattern of chromosomal gains and losses that resembled the pattern in human breast cancers, although a cross-species comparison with data from human BRCA1-associated tumors was not performed.

With the most advanced models currently mimicking several aspects of human BRCA-associated tumorigenesis, the time seems ripe to start using these mouse models for addressing clinically relevant questions. In fact, the first translational studies have already been reported.
Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency

Hormone dependency of BRCA1-associated tumorigenesis
Disruption of ubiquitously expressed BRCA genes leads to a tumor spectrum that shows specificity towards steroid hormone-responsive target organs, such as the mammary glands, ovaries and prostate. Both BRCA1 and BRCA2 expression are, albeit indirectly, responsive to estrogen levels\textsuperscript{99}, and conversely, BRCA1 was found to inhibit ERα mediated signaling\textsuperscript{100,101}. Therefore, it was suggested that estrogen signaling might be important in BRCA-associated tumorigenesis, thus providing a possible explanation for the observed tissue specificity. In support of this, it has been shown that premenopausal oophorectomy exerts a \textasciitilde 50\% reduction of the risk of breast cancer in BRCA1 mutation carriers\textsuperscript{102}. Also, adjuvant anti-estrogenic tamoxifen therapy resulted in a decreased risk of second primary carcinomas and an improved overall survival of BRCA mutation carriers\textsuperscript{103,104}. The mechanism by which oophorectomy exerts a protective effect remains to be elucidated. Paradoxically, BRCA1-associated tumors express ERα less often than sporadic tumors\textsuperscript{9}. Since certain effects of estrogen on normal mammary epithelial proliferation and morphogenesis are mediated through paracrine mechanisms\textsuperscript{105}, it is conceivable that similar paracrine signals from surrounding ERα-positive cells might stimulate proliferation of ERα-negative BRCA1-deficient tumor cells. Alternatively, tumors in which BRCA1 loss-of-function resulted in loss of negative control on estrogen signaling, may require less ERα expression (beneath the detection limit in histochemical assays) for stimulation by estrogen. A third possibility is that ERα expression is expressed during initial stages of BRCA1-associated tumorigenesis but is lost in the process of tumor progression.

To gain more insight in the role of estrogen signaling in BRCA-associated tumorigenesis, the MMTV-Cre;Brca1\textsuperscript{Co/Co;p53+/−} mouse model developed by the group of Deng, has been used to study the effects of interventions in this pathway\textsuperscript{106}. Concurrent with the human situation, there was a significant protective effect of oophorectomy on the occurrence of mammary cancer in older animals. Although the mechanism underlying this effect remains unclear, it was shown that mammary glands of oophorectomized mice underwent regression. Of note, oophorectomy studies in the MMTV-Cre;Brca1\textsuperscript{Co/Co;p53+/−} model are compromised by the fact that extent of MMTV-Cre mediated Brca1 mutation – and thus the incidence and multiplicity of mammary tumors – may be influenced by levels of steroid hormones. Hence, reduced mammary tumor formation in oophorectomized MMTV-Cre;Brca1\textsuperscript{Co/Co;p53+/−} females might be an artifact, caused by decreased Cre activity. Hormone dependency of MMTV-Cre might also explain the unexpected results from tamoxifen treatment studies in the MMTV-Cre;Brca1\textsuperscript{Co/Co;p53+/−} model, showing accelerated development of mammary tumors in tamoxifen-treated female mice\textsuperscript{107}. While the experiments performed in this study suggest that loss of full-length Brca1 expression alters the agonist/antagonist activity of tamoxifen to the extent that the agonistic function becomes more pronounced, firm conclusions can only be drawn when these results are confirmed in a different model.

Despite the technical limitations associated with the MMTV-Cre;Brca1\textsuperscript{Co/Co;p53+/−} model, the studies of Bachelier et al. and Jones et al. provide a proof-of-concept for the utility of mouse Brca1/2 mammary tumor models to investigate clinically relevant questions. This type of research will no doubt help us to address clinically relevant questions and provide knowledge that can subsequently be translated into clinical practice.
**Targeting BRCA-deficient cells and tumors**

Another example that highlights the value of conditional Brca1/2 mammary tumor models for preclinical studies concerns the research on antitumoral and tumor preventive activities of chemical inhibitors of poly-(ADP-ribose)-polymerase-1 (PARP1). PARP1 deficiency results in increased Rad51 foci formation and sister chromatid exchanges, but does not affect DSB repair via HR. Based on these observations, it was hypothesized that inhibition of PARP1 activity in BRCA-deficient cells with defective HR would result in cytotoxicity due to rapid accumulation of DNA damage. Since DNA lesions in BRCA-proficient cells can be repaired by homologous recombination, PARP1 inhibition may have a wide therapeutic window. Indeed, several groups showed that chemical inhibitors of PARP1 activity increased DNA damage and caused specific killing of cells with impaired BRCA1 or BRCA2 function. In both studies, treatment with PARP1 inhibitors induced regression of Brca2-deficient tumors in transplantation models.

To further validate this approach to targeting BRCA-deficient cells, a small intestine-specific inducible Cre line (Ah-Cre) was crossed to mice carrying conditional Brca2 alleles as well as a reporter for Cre-mediated recombination, ROSA26R. Subsequent treatment of mice with PARP1 inhibitors resulted in depletion of reporter-positive cells specifically in Ah-Cre;Brca2 intestines but not in Ah-Cre;Brca2 intestines, suggesting specific depletion of Brca2-deficient cells. These findings pave the way for extending the application of PARP1 inhibitors from therapeutic towards prophylactic settings. If loss of BRCA function is an early event in mammary tumor formation in human mutation carriers, such cells might be specifically depleted by PARP1 inhibition, even before turning malignant.

The potential use of PARP1 inhibitors for targeting BRCA-deficient or “BRCA-like” tumors with defective HR is exciting and, clearly, the current Brca1/2 mammary tumor models are valuable tools for studying both therapeutic and prophylactic applications of PARP1 inhibitors in more detail. Also, evaluation of combination therapies in these mouse models can provide important information for the clinic, where the first clinical trials using PARP1 inhibitors have already started.

**Conclusions and future perspectives**

Since the discovery of BRCA1 and BRCA2, mouse models have proven of paramount importance for obtaining information on the function of these genes, both in development and tumorigenesis. Studies in conventional and conditional Brca1/2 mouse mutants have provided insight into the role of Brca1 and Brca2 in various cellular processes, and established genetic interactions with several other proteins, most notably components of the DNA damage response pathway. Tumor suppressor activity of both BRCA proteins was confirmed in several mouse models, not only in the mammary gland but also in skin and lymphoid compartments. Nevertheless, the tumor spectrum of the mouse models parallels the human tumor spectrum at least to some extent, since a clear bias towards mammary tumorigenesis is reported in conditional Brca1/2 models employing MMTV-Cre and K14-Cre, which express Cre in multiple tissues besides mammary gland. Other features that are recapitulated by at least some of the mouse models include tumor histology and aneuploidy.

Despite our increased understanding of the function of BRCA1 and BRCA2, several important questions remain yet to be...
resolved. The intriguing observation that BRCA inactivation induces growth arrest in normal cells, yet promotes tumor formation in mutation carriers, strongly suggests the presence of secondary suppressor mutations that may overcome such an arrest during BRCA-associated tumorigenesis. Also stromal factors might foster in situ survival of BRCA-deficient mammary tumor cells. Cancer-associated fibroblasts (CAFs) contribute to various stages of tumor growth, and also immune cells can support cancer development. Clearly, mouse models are key to investigating these complex tumor-host interactions in the context of BRCA-associated tumor formation.

Another unresolved aspect of BRCA-associated tumorigenesis concerns gender bias and tissue-specificity of the disease. This tissue specificity might be caused by redundant pathways, specifically absent in mammary epithelium, that could mask BRCA-associated phenotypes in other tissues. However, the embryonic lethality of Brca1/2 homozygous mouse mutants argues against the existence of pathways that are functionally redundant with BRCA1 or BRCA2 in cell proliferation. It has therefore been proposed that the observed bias may be explained by cell type-specific and/or environment-dependent survival of BRCA-deficient mammary and ovarian epithelial cells. Since the spectrum of BRCA-related cancers is biased towards organs that are targets of estrogen, it is conceivable that survival factors in the form of hormones may have a protective effect on BRCA-deficient cells. These and other possibilities may be effectively explored in Brca1 and Brca2 mouse models.

Future research is also expected to provide more insight into the genotype-phenotype correlations that have emerged from the analysis of the various Brca1 and Brca2 mutants. It is conceivable that at least some of these correlations may also be of relevance for pathogenesis and clinical responses of BRCA-associated tumors in human mutation carriers. Phenotypic analysis of mice engineered to express one or more of the observed Brca1 splice variants may shed light on the specific functions of the different Brca1 protein products. Similarly, mouse Brca1 and Brca2 mutants with specific amino acid changes may uncover functions of specific protein domains or phosphorylation sites. One such mutant, Brca15097A, encoding a Brca1 protein with an inactivated Chk2 phosphorylation site, has already been described, showing predisposition to carcinogen-induced tumorigenesis. Clinically more relevant would be the generation of mouse mutants with truncation mutations mimicking human BRCA1/2 germline mutations. These models may be used to assess tumor predisposition and clinical responses of known disease-associated mutations. Recently developed "humanized" mouse models carrying human BRCA1 BAC transgenes or oligo targeting techniques that allow targeted insertion of subtle point mutations and small insertions into mouse embryonic stem cells are particularly suitable for such allelic series of Brca1/2 mutations.

Existing as well as new mouse models for BRCA-associated breast cancer – and perhaps other “BRCA-like” tumors with impaired homologous recombination – are expected to play an increasing role in preclinical and translational research. Robust validation of the utility of mouse models for preclinical applications is important and should include cross-species comparisons of tumor characteristics and responses to chemotherapeutics. Nevertheless, the first studies with targeted therapeutics in some of the existing models have already been reported, thus sparking the hope that breast cancer patients will soon benefit from the...
collective research on mouse models of BRCA1 and BRCA2 deficiency.

Acknowledgements
We thank Peter Bouwman, Rinske Drost, Xiaoling Liu and Karin de Visser for critically reading the manuscript and providing helpful suggestions. B.E. is supported by a grant from the Netherlands Organization for Scientific Research (NWO-VIDI 917.36.347).

References


Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency


Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency


Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency


Chapter 1: Mouse models of BRCA1 and BRCA2 deficiency


122. Morimatsu, M., Donoho, G. and Hasty, P. Cells deleted for Brca2 COOH terminus exhibit hypersensitivity to gamma-radia