CHAPTER SEVEN:

DISCUSSION
AND FUTURE PLANS

7.1 INTRODUCTION

The study of *BRCA1* and its functions has now spanned over a decade. An enormous amount of work has been done to describe the functions of this gene and the consequences of *BRCA1* mutations, both in humans and in model organisms. Despite this, a number of questions still remain: what is the mechanism by which loss of BRCA1 causes breast cancer? Which of the many interactions are biologically relevant, and how do they contribute to tumourigenesis and the normal functions of BRCA1? What are the normal roles of BRCA1 in the cell, and which of these functions are conserved between species?

In the preceding chapters, the generation and characterization of several novel mutant alleles of murine *Brca1* have been described. The *Brca1*$^{-}$ allele generated in this study (*Brca1*^{Brdm1}) has the hallmarks of previously-defined null knockout alleles: double-targeted ES cells could not be generated, homozygous mutant embryos were not viable, and heterozygous mutant mice did not have an increased tendency to tumourigenesis, even on an *Blm*$^{-/-}$ background. This allele was generated for two purposes: as part of a conditional ES cell line, and to be tested in conjunction with a *Blm* mutation in tumourigenesis studies. However, most of the work described in previous chapters has focused on the novel allele *Brca1*^{Brdm2} or *gol*, which generates an N-terminal truncated Brca1 protein predicted to be missing the majority of the highly-conserved RING domain. The viability of this allele in homozygous ES cells was unexpected, based on the similarity between it and the *Brca1*$^{-}$ allele, but, as described in Chapter 6, it seems likely that these two alleles are regulated differently. This chapter includes some general discussion points which follow on from the discussions included in previous chapters, and describes several future studies which might be performed using this novel allele.

7.2 THE *gol* ALLELE AND HUMAN *BRCA1* MUTATIONS

The *gol* allele is unique among mouse mutant *Brca1* alleles in that it generates an N-terminal truncation of the Brca1 protein, resulting in a deletion of the highly-conserved Zn-finger RING domain of BRCA1. This domain has been conserved in BRCA1 homologues from plants to *C. elegans* to mammals (see Figure 1.11), and is very highly conserved between mouse and human (see Figure 1.3). To date, no report of a functional N-terminal truncated human BRCA1 protein has been published, although one group has reported a mutation in the "initiation" codon of pseudo-exon 2 of *BRCA1* (in the 5' UTR of *BRCA1*), and *in vitro* studies have indicated that if this mutation occurred in the *BRCA1* gene itself, transcription would re-initiate at a downstream AUG (Signori, 2001). Four different mutations of the AUG initiation codon in exon 2 have been reported in the Breast Cancer Information Core (BIC) database, and it is possible that re-initiation may occur in these alleles (BIC, 2003). The human *BRCA1* transcript possesses fewer alternative start codons than the mouse, but there is a second AUG in exon 2 which occurs in a good consensus Kozak setting (the second, in exon 5, is not so well-placed). According to the BIC, mutations in the RING domain/exon 2 account for ~24%/18% of reported mutations in *BRCA1*. Whether any of these mutations result in re-initiation is unknown (BIC, 2003).

7.3 ALTERNATIVE ALLELES OF *Brca1*

Brca1^{-} and *gol* join a growing list of mutant alleles of murine *Brca1*. This list has expanded to include several conditional mutations, a C-terminal truncation allele, and alleles with partial function. The latter group includes alleles such as the *Brca1* $\Delta X.11$ allele which mimics a natural splice isoform (found in both humans and the mouse) lacking the whole of exon 11. One group has generated one ΔX.11 double-targeted ES cell line and a small number of *Brca1*$^{\Delta X.11/\Delta X.11}$*, p53*$^{-/-}$ mice (Table 1.3 #5) (Gowen, 1996; Cressman, 1999a; Cressman, 1999b); another has shown that their *Brca1* $\Delta X.11$ allele is viable on a *p53*$^{+/-}$ or *p53*$^{-/-}$ background, although the mice tend

to develop cancers, including thymic lymphomas, especially on the *p53*$^{-/-}$ background (Table 1.3 #7) (Xu, 2001b; Bachelier, 2003).

Ludwig *et al.* have generated a very interesting alternative allele of *Brca1*; this allele mimics the results of a human cancer-related nonsense mutation within exon 11. This allele generates a C-terminal truncation product lacking nearly half the protein, yet homozygous mutant mice are viable (depending in part on strain background). Male homozygotes were infertile, but homozygotes were generally healthy, although prone to tumours (86% of animals developed tumours of some type after a mean latency of 1.4 years (Ludwig, 2001)). Mammary tumours were observed in some mice, but the overall tumour spectrum encompassed lymphomas, sarcomas, and adenomas in various tissues. The long tumour latency suggested that the mutated Brca1 protein was not the only factor responsible for tumourigenesis, and further study of some tumours indicated that the expression of several other gene products was altered (interestingly, the amount of *p53* gene product was altered in only 3 of 10 mammary tumours tested). They also observed, like Bachelier *et al*., that animals on a *p53*$^{-/-}$ background developed thymic lymphomas more rapidly than did *p53*$^{-/-}$ control animals (Ludwig, 2001; Bachelier, 2003).

These models indicated that mice homozygous for a *Brca1* mutation can shorten the tumour latency of *p53*$^{-/-}$ mice, supporting the role of Brca1 as a caretaker tumour- suppressor in mice (Ludwig, 2001; Bachelier, 2003; Jonkers and Berns, 2003). *Brca1* may have similar roles in the cell as does human *BRCA1*, but the fact remains that heterozygous murine carriers of a *Brca1* mutation do not have an increased predisposition to tumourigenesis compared to wildtype mice, in contrast to human mutation carriers. In numerous studies, *Brca1*$^{+/-}$ mice on a *p53* mutant background, an *Apc*$^{+/min}$ background, and (in this work), on a *Blm*$^{-/-}$ background, do not speed the time-to-tumour compared to mice without a *Brca1* mutation (Cressman, 1999b; Hohenstein, 2001). This was also the experience of Jonkers *et al.* who showed, using mice co-conditional for both *Brca1* and *p53*, that tumours formed more rapidly in *Brca1*$^{-/-}$, *p53*$^{-/-}$ mice than in *Brca1*$^{+/-}$, *p53*$^{-/-}$ mice, but that all tumours which did form had lost both copies of *p53* (Jonkers and

Berns, 2003). These experiments suggest that the *Brca1*-related tumour profile in mice may differ from the spectrum developing in human heterozygous *BRCA1* mutation carriers (Lane, 1995; Marquis, 1995; Scully, 1997c; Chen, 1998).

7.4 DIFFERING TUMOUR SPECTRA AND A RANGE OF PHENOTYPES: TUMOUR SUPPRESSOR MODEL SYSTEMS

Neither the range of phenotypes observed in mice carrying different alleles nor the difference between mouse and human tumour spectra nor the are rare events amongst models of human cancer-related genes. As was mentioned in Chapter 4, mouse *Brca2* mutant alleles have a similar range of viability, including null alleles which are embryonic lethal (Ludwig, 1997; Sharan, 1997) and a range of alternative alleles. Mice homozygous for some of these alleles succumb early in life to tumours, generally thymic lymphomas (Connor, 1997; Friedman, 1998). An allele which deletes exon 27 results in viable homozygous mice and cells which are hypersensitive to DNA damaging agents (mice are prone to tumourigenesis at an earlier age than heterozygotes or wildtype animals (Morimatsu, 1998; Donoho, 2003)). The three published mutant alleles of *Blm* also differ from one another; the one used in this study generates viable homozygous mutant mice with an increased tumour predisposition that will accelerate tumourigenesis in $Apc^{+/min}$ mice (Luo, 2000). However, mice homozygous for the other two alleles are not viable, and these alleles appear to be haploinsufficient, as a heterozygous mutant background will accelerate tumourigenesis in $Apc^{+/min}$ mice (Chester, 1998; Goss, 2002).

Neurofibromatosis type 2 (NF2) is a familial dominant disorder characterized in humans by schwannomas and meningiomas caused by a germline mutation in one copy of *NF2*. *NF2* mutations are also found in sporadic schwannomas. While $Nf2^{+/-}$ mice are cancer-prone, they exhibit osteosarcomas and hepatocellular carcinomas at an advanced age, and null mutants die in early embryogenesis, which is not an accurate model of the human condition (McClatchey, 1997; McClatchey, 1998). Mice carrying

mutant *Rb1* alleles are cancer-prone, but they do not accurately mimic the human phenotype (including retinoblastoma, a childhood malignancy of the retina, as well as osteosarcomas, prostate, and breast cancers (reviewed in Zheng and Lee, 2001). *Rb1*$^{+/-}$ mice develop pituitary gland tumours between 6 and 8 months of age, and *Rb1*$^{-/-}$ mice die in late embryogenesis, although the developing retina appears normal (Clarke, 1992; Jacks, 1992; Lee, 1992). In both cases, better models of the human condition were generated by using either conditional alleles driven by tissue-specific Cre transgenes, or secondary mutations (Lee, 1996; Robanus-Maandag, 1998; Giovannini, 2000). This is not unlike the use of *WAP*-, MMTV-, or *K14*- Cre transgenes in conjunction with *Brca1* conditional mice to try and restrict expression to the mammary gland (Xu, 1999b; Jonkers and Berns, 2003).

The embryonic lethality of homozygous mutant *Brca1* mice and the predisposition to cancers in heterozygous *BRCA1* carriers has often been described as paradoxical, as these two outcomes describe cell death and uncontrolled cell growth, respectively. However, these two outcomes are likely to be consistent with *BRCA1* being a tumour-suppressor gene expressed in growing and differentiating cells. In the cells of an adult human carrier of a *BRCA1* mutation, loss or mutation of the second allele is likely to lead to genomic instability, which eventually leads to cancer via additional mutations acquired in the cells. This is exactly what is likely to occur in embryonic tissues of *Brca1*-mutant homozygotes, too: loss of *Brca1* leads to additional mutations, which eventually leads to enough genetic disorder that the cell can no longer function. However, unlike the hypothetical adult cell, the homozygous mutant cells of the embryo are rapidly undergoing a huge amount of growth and differentiation, and the burden of mutations is likely to become overwhelming more quickly. Additionally, *Brca1* is normally expressed in the developing embryo, which suggests that any effects of its loss would likely be observed more quickly than in an adult cell where it may not be expressed (Marquis, 1995). An increased burden of mutations in *Brca1*-mutant embryos is supported by the studies of Shen *et al.* who showed that chromosomal instability is increased in *Brca1*$^{\Delta X.11/\Delta X.11}$ embryos compared to wildtype embryos (Table 1.3 #6). The addition of a second mutation (*p53*

deficiency) resulted in a greater amount of genomic rearrangement (Shen, 1998).

Based on these previous studies, the *gol* allele may serve as a useful model for *BRCA1*-related tumourigenesis, but may be more advantageous when used in studies of the functions of *Brca1* in the cell (which indirectly apply to tumourigenesis). The original aim of this project was to investigate the functions of *Brca1* in the cell, with a view to understanding the molecular causes behind its role in tumourigenesis. The *gol/gol* cells provide a tool for investigating the functions of Brca1, both in well-studied areas such as the response to γ-irradiation, nuclear focus formation, and phosphorylation, and in others such as nuclear import and export, RING-domain interactions and their consequences, degradation of the Brca1 protein, and possible targets of the Brca1-Bard1 E3 ligase.

7.5 THE FUNCTIONS OF BARD1

Bard1 was isolated on the basis of its interaction with Brca1, and the majority of subsequent studies involving this protein have focused on its functions in tandem with Brca1. A few studies investigating Bard1 as a possible tumour-suppressor gene have unanimously concluded that Bard1 is rarely, if at all, involved in mammary tumourigenesis (Thai, 1998; Yoshikawa, 2000; Ishitobi, 2003). However, there have been some glimpses into the functions of Bard1, including possible roles in cell cycle control and the response to DNA damage.

Antisense-mediated depletion of *Bard1* in cultured cells has indicated that cells with decreased expression of *Bard1* tend to have a prolonged cell cycle and a higher amount of aneuploidy or polyploidy (Irminger-Finger, 1998). *Bard1* also appears to be transcriptionally upregulated following genotoxic stress (UV treatment), and may be upregulated or induced during apoptosis (Irminger-Finger, 2001). A second group has shown that UV exposure or a DNA replication block induced by HU treatment results in a temporary block in 3’ cleavage of pre-mRNAs. BARD1 is likely to be necessary for this damage-

induced block, as it does not occur if BARD1 is mutated (Kleiman and Manley, 2001). Overexpression of BARD1 also appears to induce apoptosis and increase the amount of p53 in the cell – similar to what has been observed in cells overexpressing BRCA1 (Irminger-Finger, 2001).

BARD1 may also play a role in homologous recombination repair (HRR); when a dominant-negative truncated version of BARD1 (BARD1 can interact with BRCA1 but is missing its C-terminus), is transfected into *Brca1*$^{+/+}$ or *Brca1*$^{\Delta X.11/\Delta X.11}$ ES cells (Table 1.3 #5), an I-SceI repair assay indicates that both cell lines have a lower efficiency of HRR than the parental cell line (Westermark, 2003). *gol/gol* cells could be used to determine if the interaction of Bard1 and Brca1 was necessary for this result (the Δ*X.11* isoform of *Brca1* used in the study described above retains the RING domain). If Bard1 does not depend on its interaction with Brca1 to affect HRR efficiency, then transfection of the dominant-negative Bard1 protein into *gol/gol* cells should result in a decrease in the efficiency of HRR compared to that of *gol/gol* cells alone.

Normally, Brca1 appears to be escorted to (and retained in) the nucleus by Bard1; Brca1 appears to be similarly involved in Bard1 import and retention (Rodriguez, 2004). However, in *gol/gol* cells, lack of both the RING domain and (likely) the NES of Brca1 appears to result in normal nuclear import of the protein, as Brca1^{gol} is observed by immunofluorescence in both the nucleus and cytoplasm. This does not mean that the Brca1^{gol} protein is able to shuttle back and forth between the nucleus and cytoplasm, though, as the ΔX.11 form of the Brca1^{gol} protein would not be expected to be able to enter the nucleus (lacking, as it does, both the RING domain and the NLSs from exon 11) and should be observed in the cytoplasm. It might be worthwhile to investigate whether the Brca1^{gol} protein does shuttle in and out of the nucleus, and whether or not the ΔX.11 form of Brca1^{gol} is able to enter the nucleus.

7.6 THE E3 UBIQUITIN LIGASE ACTIVITY OF BRCA1-BARD1: A POSSIBLE FEEDBACK MECHANISM

Many RING-containing proteins are E3 ubiquitin ligases, involved in the 26S proteasome-mediated protein degradation pathway. Both BRCA1 and BARD1 have been shown to have E3 ligase activity, although it is increased when the two heterodimerize (Hashizume, 2001; Kentsis, 2002), and increased further by the formation of autopolyubiquitin chains on the heterodimer (Chen, 2002; Mallery, 2002). Much speculation has surrounded the E3 ubiquitin ligase capabilities of BRCA1 and BRCA1-BARD1 and whether substrates other than itself exist. The mouse protein Mouse double-minute 2 (Mdm2), a regulator of p53, is an E3 ligase which not only ubiquitinates p53, but also undergoes autoubiquitination to regulate its own stability (Fang, 2000). This suggests that BRCA1-BARD1 might have protein targets in addition to itself.

The *gol/gol* cells or mice should be excellent tools for investigating the targets of the Brca1-Bard1 E3 ubiquitin ligase, as the mutation in the RING domain means that neither Brca1 nor Brca1-Bard1 should be a functional E3 ligase. The human cancer cell line HCC1937 has been used to investigate BRCA1-related ubiquitin ligase activity in the past, but not only do these cells carry mutations besides the one in BRCA1, the RING domain is still intact in the mutated version of BRCA1 found in these cells, meaning that BRCA1-related E3 ligase activity may be partially functional in these cells. It would be especially interesting to investigate the involvement, if any, of Brca1 in the ubiquitination of RNA Pol II, an oft-suggested potential target of the Brca1-Bard1 E3 enzyme. Such studies may also reveal if Bard1 or Brca1 alone act as a ubiquitin ligase for other substrates.

Several groups have suggested that autoubiquitination may be a method of stabilizing the components of the BRCA1-BARD1 heterodimer; both mouse and *Xenopus* experiments have demonstrated that loss of one protein appears to result in a downregulation of the other (Joukov, 2001b; McCarthy, 2003). Data generated in this study suggests that the $Brca1^{gol}$ mutation is

upregulated or stabilized compared to wildtype Brca1, perhaps as a result of its inability to interact with Bard1. This result does not necessarily contradict the findings of previous studies, as the *gol* allele produces a mutant protein, while the other studies involved complete loss (or functionally relevant downregulation) of protein.

That autoubiquitination provides stability for the heterodimer is supported by *in vitro* data indicating that ubiquitin monomers are attached to BRCA1/BARD1 via a novel, Lys-6 linkage (different from the more common Lys-48 linkage observed on polyubiquitin chains of proteins destined for 26S proteasome-mediated degradation) (Wu-Baer, 2003). When ubiquitinated BRCA1/BARD1 is presented to the 26S proteasome, it is de-ubiquitinated, but not degraded (Nishikawa, 2004). Other groups have shown both that BRCA1 may not be degraded by the 26S proteasome, but by acid calpains or cathepsins instead, and that degradation of BRCA1 may occur in the nucleus (Blagosklonny, 1999; Choi, 2001). This could suggest that the Lys-6 ubiquitin chain targets the heterodimer to a different protease, or it may be a signal to target the heterodimer to the nucleus or cytoplasm, and not involved in stability or degradation.

The increased amount or increased stability of Brca1^{gol} may stem directly from the perturbation of a Brca1-Bard1 feedback loop. Before embarking on experiments to determine if Brca1 and Bard1 participate in a feedback loop, it would be worthwhile to first determine if Brca1 is more abundant or more stable in *gol/gol* cells. A time-course experiment following cyclohexamide treatment to block protein synthesis should help determine if the half-life of Brca1^{gol} protein is extended compared to wildtype Brca1. Further experiments might include blocking acidic protease and/or 26S proteasome activity, monitoring ubiquitin chain formation on Brca1^{gol}, and investigating the stability, ubiquitination status, and amount of Bard1 protein in *gol/gol* cells.

7.7 POTENTIAL FUTURE EXPERIMENTS WITH THE *gol* ALLELE

One of the most difficult problems when faced with the enormous volume of scientific literature published about BRCA1 biology is trying to link together the many interactions that the BRCA1 protein appears to be involved in. One advantage of having a viable mutant allele lacking a specific domain is that it affords a chance to investigate what that domain of the protein does or does not interact with, without using transgene overexpression or yeast 2-hybrid assays. Such investigations should include additional immunolocalization experiments, involving proteins such as Bard1 and Rad51, and perhaps proteins such as PCNA (following DNA damage). A microarray assessment of *gol/gol* cellular mRNA before and after DNA damage to look at gene induction following damage might also yield interesting results.

Experiments which extend the functional studies described in previous chapters are easily envisioned. It would be worthwhile to determine if a transcriptional repressor element is present in intron 2 of the mouse *Brca1* gene. This could be done via a novel BAC system constructed by a colleague, Haydn Prosser. His system is designed to allow BACs to be introduced into ES cells in a defined location. BACs can now be modified fairly easily through recombineering, and by recombineering a series of BACs carrying mouse *Brca1* with a series of intron 2 deletions, the location of this putative repressor could be located. The efficacy of the repressor could then be confirmed using a reporter gene assay. It would also be worthwhile to determine where the initiation site of the *gol* transcript is – and if more than one is utilized.

The DNA damage phenotypes of *gol/gol* cells could be studied much more extensively; it would be quite interesting to look by SKY or karyotyping analyses to assess the amount of genomic rearrangement in *gol/gol* MEFs or ES cells after a number of passages in culture, or following DNA damage. It should also be possible to determine if the lack of difference in colony-forming ability between *gol/gol* and wildtype ES cells following UV exposure is due to efficient repair or a greater tolerance of damage. Additionally, by

synchronizing either MEFs or ES cells, it could be determined if *gol/gol* cells are susceptible to DNA damage at certain phases of the cell cycle. The results of such an experiment may be helpful in determining whether the slight decrease in HRR efficiency in these cells is functionally relevant, as HRR and NHEJ are generally used at different points in the cell cycle (Takata, 1998; Wang, 2001b).

A mouse *gol* model is in development, and, if viable, is potentially quite interesting with many future applications. For a start, the viability (in terms of expected Mendelian frequency) and/or the fertility of the mice will be investigated. Should the homozygotes be viable, then of course tumorigenic studies will be performed on them. In conjunction with this, might be interesting to see how *gol/gol* mice respond to MMC treatment or γ-irradiation as tumourigenic accelerants. If it is revealed at a later date that expression of mutant *Brca1* in a specific tissue or cell-type would be advantageous, a conditional allele of *gol* exists (*c2*) which could be used. The analysis of any resulting tumours by microarray for changes in expression and possibly for the loss or gain of protein products may also yield useful information.

Generating a *gol/gol, Bard1*$^{-/-}$ model would also be interesting, and at the very least may reveal whether the *Bard1* deficiency is lethal because of the role of Bard1 as nuclear chaperone/anchor to Brca1. If the *gol/gol* mice are not viable, then it might be worth trying to cross the allele onto the *Bard1* knockout background, to see if the two mutations might rescue one another.

7.8 SOME FINAL WORDS

In the mouse, Brca1 appears to contribute to carcinogenesis through its role as a caretaker tumour-suppressor. Although there are several mouse models which provide a variety of models for *BRCA1*-related breast cancer, to date it has been equally worthwhile to investigate the DNA damage response and cell cycle-related functions of Brca1 in cells. The involvement of Brca1 in such processes appears to be well-conserved across species, and in this regard, the mouse makes an excellent model for the human. The original goal of this project was to generate a tool for investigating the function of *Brca1* in cells, and the *gol/gol* cells can indeed be regarded as such a tool. It

is hoped that the *gol* allele will add to the many studies of Brca1 function, leading to a greater understanding of its roles in the cell and how the loss of these actions eventually leads to tumourigenesis.

After nearly ten years of study, involving hundreds of laboratories, it may seem that little progress has been made toward these goals, but the study of *BRCA1*-related breast cancer is not terribly different from that of most cancers. It may be argued that the majority of cancer therapies in use today – indeed, the majority of therapies for any human disorder – do not directly exploit knowledge about the molecular mechanisms involved. This appears disheartening, but taken from another angle, it suggests that huge strides forward will be made in the near future, as the molecular findings from the past and present are translated from understanding into therapies and advanced prevention and diagnostic techniques. Glimpses into this rosier future are given by cancer drugs such as Gleevec, an inhibitor of the kinase product of the BCL-ABL fusion (resulting from translocation) found in many leukaemia patients, or Herceptin, which inhibits growth of breast cancer cells overexpressing the *HER2/neu/ErbB2* gene (reviewed in Shawver, 2002). These therapies were developed using knowledge of the role of specific proteins in specific cancers, and such “smart drugs” may completely transform cancer therapeutics. Added to the rapidly-increasing outpouring of information about molecular interactions and functions of virtually every gene, transcript, and protein in the genome, this makes the next ten years a very exciting prospect.