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DNA REPAIR BY HDR IN EXPERIMENTAL TUMORIGENESIS

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Stockholm 2008
To my family
ABSTRACT

The aim of this thesis was to further understand how defects in the homology-directed repair (HDR) pathway affect tumor formation and development. To study proteins involved in HDR is challenging since most of its members are required for cell viability. For our studies we have therefore taken a dominant-negative approach to address the importance of the BRCA1 interacting protein BARD1 in HDR (paper I) and investigate the role of HDR in PDGFB-induced gliomagenesis by interfering with a key protein, RAD51 (paper II). In addition we have also investigated how Platelet-derived growth factor A receptor (PDGFA) is transcriptionally regulated by interleukin-1β (IL-1β) (paper III).

The breast cancer susceptibility gene BRCA1 is frequently mutated in hereditary breast and ovarian cancers. BRCA1 has been implicated in many different cellular processes, among them DNA repair by HDR. In Paper I we investigated if BARD1 was involved in HDR through its interaction with BRCA1. We could show that expression of a truncated BARD1 decreased HDR of an induced double-strand break and that this decrease was even more pronounced in Brca1-deficient cells expressing a splice variant of Brca1 that still are able to bind BARD1. We could also show that the role of BARD1 in HDR was dependent on binding to BRCA1 and that the HDR defect resulting from the truncated BARD1 was caused by lack of regulatory elements in the C-terminal end of BARD1 and not caused by improper cellular localization of either the BARD1 construct or the endogenous Brca1 protein. We conclude that BARD1 is important for HDR and that the repair function of BARD1 is dependent on its interaction with BRCA1.

The expression of PDGFRα is strictly regulated during embryogenesis and aberrant PDGFRα expression can lead to malignant transformation, in brain tumors for example. Highly malignant gliomas are the most frequent primary tumor of the central nervous system in adults. To study the effect of different genetic changes in gliomagenesis, glioma-like tumors can be induced by intracerebral injections of oncogene carrying retroviruses. The RCAS/tv-a model system provides the possibility to study combinations of different genetic alterations in a specific cell type. In paper II we have used wild type and Arf-/- nestin tv-a (Ntv-a) transgenic mice, where expression of the RCAS constructs were directed to neural progenitor cells. It has previously been shown that expression of PDGFB can induce glioma-like tumors in these mice. To investigate the role of HDR in tumorigenesis we co-expressed RAD51 or a DNA repair deficient RAD51 (RAD51KR) construct with PDGFB in either wild type or Arf-/- Ntv-a mice. We could show that co-expression of RAD51 or RAD51KR can suppress PDGFB-induced tumorigenesis in wild type mice. However, only RAD51 was able to suppress tumor formation in the Arf-null background. We could also show that all the PDGFB-induced tumors were aneuploid, independent of genotype, tumor grade or tumor size. Interestingly, expression of RAD51 or RAD51KR reduced aneuploidy in the PDGFB-induced tumors.
LIST OF PUBLICATIONS


III. Afink G, Westermark UK, Lammerts E, Nistér M. C/EBP is an essential component of PDGFRA transcription in MG-63 cells. *BBRC 2004;315(2):313-318*
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<tr>
<td>ARF</td>
<td>p14/p19&lt;sup&gt;Arf&lt;/sup&gt;</td>
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<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
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<td>Ataxia Telangiectasia and Rad3 related</td>
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<td>Breast cancer</td>
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<td>C/EBP</td>
<td>CCAT/enhancer-binding element</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>DDR</td>
<td>DNA damage response</td>
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<td>DR-GFP</td>
<td>Direct repeats-green fluorescent protein</td>
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<tr>
<td>DNA-PK</td>
<td>DNA-protein kinase</td>
<td></td>
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<tr>
<td>DSB</td>
<td>Double strand break</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ES cell</td>
<td>Embryonic stem cell</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>γH2AX</td>
<td>Phosphorylated H2AX</td>
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<td>HDR</td>
<td>Homology-directed repair</td>
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<td>IL-1β</td>
<td>Interleukin-1β</td>
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<td>INK4a</td>
<td>Inhibitors of kinase 4a, p16</td>
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<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
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<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<td>MMC</td>
<td>Mitomycin C</td>
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<td>MMLV</td>
<td>Moloney murine leukemia virus</td>
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<tr>
<td>MRN</td>
<td>Mre11/RAD50/Nbs1</td>
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<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
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<tr>
<td>Abbr.</td>
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<tr>
<td>Ntv-a</td>
<td>Nestin tv-a</td>
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<tr>
<td>OIS</td>
<td>Oncogene induced stress</td>
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<tr>
<td>p53</td>
<td>Tp53 gene product</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PIKK</td>
<td>Phosphoinositide 3-kinase-related protein kinase</td>
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<tr>
<td>RCAS</td>
<td>Replication competent ALV splice acceptor</td>
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<tr>
<td>RPA</td>
<td>Replication protein A</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>Tva</td>
<td>Receptor for RCAS</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>WRN</td>
<td>Werner syndrome gene</td>
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1 HOMOLOGY DIRECTED REPAIR

1.1 INTRODUCTION

Tumorigenesis is known to result from multiple genetic changes. To protect the cell against these changes rigorous cellular mechanisms exist to repair DNA damage caused by endogenous and environmental insults, or to eliminate those cells that are irreparably damaged. It is of great importance for the maintenance of genome integrity to repair DNA breaks. Failure to repair these lesions could lead to genomic instability, a common feature of nearly all solid tumors (Lengauer et al., 1998). There are two major DNA repair pathways to repair double strand breaks (DSBs) in mammalian cells; non-homologous end joining (NHEJ) and homology directed repair (HDR) (also called homologous recombination). The NHEJ pathway is potentially error-prone, allowing deletion and insertion of nucleotides at the site of the DSB. HDR, on the other hand, is error-free, when an identical sister chromatid is used as template for the repair. The HDR pathway also play a pivotal role in replication, where it guards and repair replication errors at the replication forks (Pierce et al., 2001). The HDR pathway will be the DNA repair pathway of primary focus in this thesis.

1.2 HDR PATHWAY

In response to DNA damage, checkpoint kinases are activated (e.g. ATM, ATR, DNA-PK). The DSB lesions are recognized by the Mre11/Rad50/ Nbs1 (MRN) complex (Carson et al., 2003). The MRN complex recruits ATM to the site of the break by direct binding to ATM and Nbs1. ATM phosphorylates histone H2AX (γ-H2AX), which in turn works as a docking site for Mdc1. Mdc1 binds both γ-H2AX and Nbs1 and can facilitate spreading of H2AX phosphorylation, thereby promotes remodeling of the chromatin. Other proteins known to be recruited to sites of DSBs at this point are 53BP1 and the BRCA1-BARD1 complex. The accumulation of ATM phosphorylates ATM targets, such as the Chk2 kinase. This initial response at the chromatin is very fast and these proteins are recruited to the site of the break within a minute of the DSB formation (Bartek and Lukas, 2007). Of note, exactly how and in which order these proteins are recruited to DSBs is not completely understood and hence the above description represent one suggested scenario.
ATM and the MRN complex are also important for resection of DNA around the break creating a single stranded DNA (ssDNA) to facilitate ATR-dependent signaling and DNA repair by HDR. Resection of DNA is only possible in the S and G2-phase of the cell cycle and entails activation of cyclin-dependent kinases (CDKs) (Ira et al., 2004). The ssDNA is stabilized and coated by replication protein A (RPA). It has recently been shown that RPA plays an active role in initiating HDR by binding RAD52 and recruiting RAD51 to the break (Sleeth et al., 2007). This process is dependent on the checkpoint kinase Chk1. Chk1 is needed for dissociation of RPA from ssDNA and subsequent loading of RAD51 onto the ssDNA. It was also shown that in cells lacking RPA DSBs persists (Sleeth et al., 2007).

The central protein to repair DSBs by HDR is the RecA homolog RAD51. RAD51 is loaded onto the ssDNA, a process aided by BRCA2 (Yang et al., 2005) and Chk1 (Sleeth et al., 2007), replacing RPA. RAD51 forms nucleoprotein filaments in an ATP-dependent manner around the ssDNA. This is a central event in HDR since it catalyses DNA strand exchange reaction between ssDNA and the homologous double-stranded DNA (dsDNA) in the sister chromatid (Baumann et al., 1996; Benson et al., 1994; Gupta et al., 1997). The accumulated RAD51 can be visualized as so called nuclear foci (Haaf et al., 1995; Tan et al., 1999). In these foci RAD51 co-localizes with the RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) (Masson et al., 2001), other members of the RAD52 epistasis group (e.g. RAD52, RAD54) (Golub et al., 1997; Tan et al., 1999) as well as other proteins implicated in DNA repair, such as the breast cancer susceptibility genes BRCA1 and BRCA2 (Breast cancer 1 and 2) (Chen et al., 1998; Scully et al., 1997; Sharan et al., 1997), BARD1 (Brca1 associated Ring domain 1) (Jin et al., 1997; Wu et al., 1996), γH2AX (Paull et al., 2000), 53BP1 (Schultz et al., 2000) and FANCD2 (Fanconi anaemia D2) (Garcia-Higuera et al., 2001) amongst other known and unknown proteins.

Nearly all proteins involved in the HDR pathway are essential for cell survival, as most of the HDR genes cause embryonic lethality when knocked-out in mouse models (e.g. Rad51, Brca1, Bard1, Brca2, Rad51B, Rad51C, Rad51D and xrcc2) (Deans et al., 2000; Gowen et al., 1996; Lim and Hasty, 1996; Liu et al., 1996; McCarthy et al., 2003; Pittman and Schimenti, 2000; Smiraldo et al., 2005). Some of them can be rescued or embryonic life can be extended by p53 deficiency (Hakem et al., 1997; Ludwig et al., 1997; McCarthy et al., 2003; Smiraldo et al., 2005). Cells with defects in
the HDR pathway show extensive chromosomal instability, sensitivity to cross-linking agents and irradiation (Jeggo, 1998; Moynahan et al., 2001a; Moynahan et al., 2001b; Sonoda et al., 1998; Thompson and Schild, 1999). This implies that disruption in recombination processes has devastating consequences to genome integrity and that this could potentially lead to tumorigenesis. On the other hand, the sensitivity of HDR mutants to DNA damaging agents has also been suggested to be potential targets for cancer therapy.

1.3 RAD51

RAD51 is cell cycle regulated with highest expression in S/G2-phase of the cell cycle and lowest in resting cells. In S-phase RAD51 can be found in discrete nuclear foci located at replication forks (Flygare et al., 1996; Haaf et al., 1995; Scully et al., 1997). Upon DNA damage RAD51 is relocated to sites of DSBs (Haaf et al., 1995; Raderschall et al., 1999).

Exactly how RAD51 is regulated is not completely understood. However, it has been shown that p53 can regulate RAD51 by binding to the oligomerization domain of RAD51 and thereby preventing nucleoprotein filament formation (Linke et al., 2003; Sturzbecher et al., 1996). BRCA2 can also regulate RAD51 either by interaction via its BRC repeats, thereby keeping RAD51 in an inactive monomeric state or stabilizing RAD51 by binding the RAD51 polymer through the C-terminal end of BRCA2 (Davies and Pellegrini, 2007; Esashi et al., 2005; Esashi et al., 2007; Galkin et al., 2005; Petalcorin et al., 2007). It has also been shown that RAD51 can be phosphorylated and upregulated by the STAT5 pathway (Slupianek et al., 2002; Slupianek et al., 2001).

Disruption of Rad51 in mouse embryonic tissue leads to lethality early in gestation (Lim and Hasty, 1996; Tsuzuki et al., 1996), and the inducible loss of the Rad51 protein in chicken DT-40 cells resulted in severe chromosome aberrations and cell death (Sonoda et al., 1998). Overexpression of RAD51, on the other hand, has also been reported to lead to genomic rearrangements (Richardson et al., 2004). It has also been shown that elevated RAD51 levels can increase HDR, which could possibly lead to DNA repair with less fidelity (Lundin et al., 2003; Richardson et al., 2004; Vispe et al., 1998; Xia et al., 1997). This implicates the importance of proper regulation of
RAD51 in maintaining genome integrity, which is believed to be of utmost importance to prevent malignant cell transformation.

1.3.1 Rad51 in tumorigenesis

Mutations in RAD51 are rarely found in human tumors (Kato et al., 2000; Maacke et al., 2000a; Maacke et al., 2000b), but deletions of the chromosomal region where RAD51 is located (15q15.1) and subsequent loss of heterozygosity (LOH) in breast cancer has been reported (Gonzalez et al., 1999; Nowacka-Zawisza et al., 2007). Moreover, there are several reports showing that a polymorphism in the 5’ untranslated region (UTR) of RAD51 increases the risk of breast cancer in carriers of mutations in the breast cancer associated gene BRCA2 (Antoniou et al., 2007; Jara et al., 2007; Kadouri et al., 2004; Levy-Lahad et al., 2001). It has been suggested that this polymorphism leads to a decrease in RAD51 levels (Antoniou et al., 2007). RAD51 also interacts with proteins known to be involved in tumorigenesis, such as p53 (Sturzbecher et al., 1996), BLM (Wu et al., 2001), BRCA1 (Scully et al., 1997) and BRCA2 (Sharan et al., 1997), suggesting a direct or indirect role for RAD51 in tumorigenesis.

Overexpression of RAD51 is found in several different types of tumor tissues as well as in tumor derived cell lines (Han et al., 2002; Maacke et al., 2000a; Maacke et al., 2000b; Raderschall et al., 2002b; Xia et al., 1997; Yanagisawa et al., 1998). This overexpression seems not to be caused by amplification of the RAD51 gene (Raderschall et al., 2002b), suggesting that the overexpression is due to increased transcription or post-translational modifications. Secondary effects of mutations in regulators of RAD51 could also cause the elevated levels of RAD51 seen in tumor tissues. TP53, for example, has been suggested to inhibit RAD51 expression through direct interaction and as a consequence of that RAD51 expression has shown to be elevated in p53 deficient cells (Arias-Lopez et al., 2006; Hannay et al., 2007). Similarly, overexpression of RAD51 could reflect the disruption of its normal cleavage by caspase-3 during apoptosis (Huang et al., 1999), since the apoptotic pathways commonly are bypassed in malignant cells. For example, tumor cells expressing different fusion tyrosine kinases (e.g. BCR/ABL, TEL/PDGFRβ, TEL/ABL, TEL/JAK2) have elevated levels of RAD51 and they show increased resistance to DNA damaging drugs. These fusion tyrosine kinases upregulate STAT5 expression
(Slupianek et al., 2002) and STAT5 has been shown to transactivate the RAD51 promoter and inhibit RAD51 cleavage by caspase-3 (Slupianek et al., 2001). Hence, RAD51 is upregulated in these cells and contributes to drug resistance (Slupianek et al., 2002). The replicative stress caused by upregulation of oncogenes in tumor tissue and the DNA breaks caused by that could potentially also lead to elevated levels of RAD51 in tumor tissue.

Overexpression of RAD51 has also been correlated to histological grade in breast carcinomas (Maacke et al., 2000b). In this study no correlation was found between RAD51 overexpression and BRCA1 deficiency. However, BRCA1 deficiency had an inverse correlation to tumor grade (Maacke et al., 2000b). Other studies have found that RAD51 was upregulated in BRCA1-deficient tumors (Honrado et al., 2005; Martin et al., 2007) at a higher frequency than in sporadic tumors and that this higher RAD51 expression was independent of cell cycle variables (Martin et al., 2007). In the same study by Martin et al. it was further shown that growth and DNA repair defects in BRCA1-deficient chicken DT-40 cells could be rescued by upregulation of the HDR pathway. They suggested that elevated HDR activity in BRCA1-deficient cells could provide a permissive genetic context for tumor development in carriers of BRCA1 mutations (Martin et al., 2007). In normal cells BRCA1 interacts and inhibits c-Abl. Loss of BRCA1 leads to constitutively elevated c-Abl levels (Foray et al., 2002). In turn, RAD51 can be phosphorylated and upregulated by c-Abl (Chen et al., 1999; Yuan et al., 1998) through the STAT5 pathway, as mentioned above. This could be one explanation why RAD51 is upregulated in a BRCA1-deficient background.

1.3.2 RAD51 overexpression in experimental models

Overexpression of RAD51 in vitro has shown to decrease proliferation, delay cell cycle progression, increase apoptosis and increase resistance to DNA damaging treatments (Flygare et al., 2001; Ohnishi et al., 1998; Raderschall et al., 2002a; Russell et al., 2003; Vispe et al., 1998). High levels of RAD51 can lead to higher order nuclear structures in experimental models (Raderschall et al., 2002a; Westermark et al., 2008). Whether these structures also occur in human tumors overexpressing RAD51 is unclear and the importance of these structures in tumorigenesis is so far unknown.
The role of overexpression of RAD51 in maintaining genome integrity remains elusive. One report shows that overexpression of RAD51 in a variety of different human cell lines led to fewer chromosomal aberrations (Raderschall et al., 2002a). In paper II in this thesis, we show that RAD51 overexpression has a protective effect on genome stability, in a mouse tumor model (Westermark et al., 2008). However, it has also been shown in a mouse embryonic stem (ES) cell model that overexpression of RAD51 can lead to increased chromosomal instability (Richardson et al., 2004). However, it was later shown that overexpression of RAD51 in ES cells generated more translocations after an induced DSB compared to myeloid cells (Francis and Richardson, 2007), suggesting cell specific differences in the response to RAD51 overexpression.

A study by Lundin et al. has provided a possible explanation to the observed decrease in genomic instability by RAD51 overexpression (Lundin et al., 2003). They induced different types of DNA lesions at replication forks in cells overexpressing RAD51, using etoposide (VP16) and hydroxy urea (HU) to induce DSBs at the replication forks and thymidine to slow down replication without inducing DSB. However, thymidine treatment leads to assembly of HDR proteins at replication forks, implying that thymidine induces a DNA damage response before DSBs are formed. They could show that RAD51 was involved in repairing all these different types of lesions. They could also show that cells overexpressing RAD51 more efficiently repaired VP16 induced DSB and that overexpression of RAD51 suppressed long-tract HDR but not global HDR. They propose that RAD51 can rescue stalled replication forks before DSB formation, supported by the involvement of RAD51 in thymidine lesions. This data taken together implies that overexpression of RAD51 could protect against genomic instability by more efficient DSBs repair, rescuing stalled replication forks and thereby preventing formation of DSBs. Suppression of long-tract HDR could also benefit genome stability, since long-tract HDR has been associated with increased risk of genomic instability.

1.3.3 Dominant-negative RAD51 mutants in experimental models

A high frequency of tumors was induced when a dominant-negative RAD51 was expressed in p53-defective Chinese hamster ovary (CHO) cells and subsequently injected into nude mice (Bertrand et al., 2003). The tumors exhibited faster growth,
spontaneous centrosome duplication defects and aneuploidy compared to injection of control CHO cells or CHO cells overexpressing wild type RAD51.

Another dominant-negative RAD51 used in experimental model is the RAD51-K133R (RAD51KR) mutant. This RAD51 mutant has a point mutation at position 133 where a lysine been exchanged for an arganine. The RAD51KR mutant is incapable of ATP hydrolysis (Morrison et al., 1999). The mutant can still bind DNA and catalyze strand exchange between homologous DNAs (Chi et al., 2006). However, ATP hydrolysis seems to be required for dissociation of RAD51 from the nucleoprotein filament. Thereby the RAD51KR mutant can stabilize the presynaptic filament and enhance homologous pairing (Chi et al., 2006; Forget et al., 2007). In mouse ES cells the hRAD51KR mutant has been shown to exhibit typical characteristics of an HDR mutant (Stark et al., 2002). This DNA repair phenotype was less pronounced in a study where hRAD51KR was expressed in chicken DT-40 cells (Morrison et al., 1999). In human cells the K133R mutant was incapable of DNA repair when the cells lacked endogenous RAD51 (Forget et al., 2007). Taken together this data suggests that RAD51KR behaves as a dominant-negative mutant for DNA repair function in mammalian cells possibly by stalling the dissociation of the RAD51 nuclear filament and thereby reducing the free pool of endogenous RAD51 available for DNA repair.

1.4 BRCA1/BARD1 COMPLEX

An inherited germline mutation in either BRCA1 or BRCA2 dramatically increases the risk to develop ovarian and breast cancer. In these tumors the other BRCA allele is typically lost (Collins et al., 1995; Cornelis et al., 1995). The BRCA1 gene encodes a large protein of 1863 amino acids (Miki et al., 1994). The protein has a RING finger domain in its amino-terminal and tandem BRCT repeats in its carboxy-terminal end. More than half of the protein is encoded by exon 11 that includes a nuclear localization signal (NLS). Many different biological functions have been assigned to BRCA1 such as DNA repair, cell cycle control, apoptosis, centrosome duplication and transcriptional regulation (Jasin, 2002; Moynahan et al., 1999; Moynahan et al., 2001a; Scully et al., 2000; Welcsh and King, 2001).

At the RING finger domain BRCA1 interacts with another RING finger protein, BRCA1-Associated Ring Domain (BARD1). Like BRCA1 BARD1 also contains two
BRCT domains in its C-terminal end. In addition BARD1 also have three tandem ankyrin repeats upstream of the BRCT repeats (Wu et al., 1996). BARD1 interacts with BRCA1 and thereby stabilizes the BRCA1 protein (Hashizume et al., 2001; Joukov et al., 2001; Xia et al., 2003) and the interaction has also been reported to enhance the affinity of BRCA1 for DNA (Simons et al., 2006). Overexpression of BARD1 increases BRCA1 stability and thereby inhibits cell cycle dependent degradation of BRCA1 (Choudhury et al., 2004; Hashizume et al., 2001; Joukov et al., 2001; Xia et al., 2003). Mice null for either BRCA1 or BARD1 show similar phenotype including early embryonic lethality and mouse embryonic fibroblasts (MEFs) generated from these mice display severe chromosome aberrations (Gowen et al., 1996; Hakem et al., 1997; Liu et al., 1996; Ludwig et al., 1997; McCarthy et al., 2003; Snouwaert et al., 1998). The close link between BRCA1 and BARD1 function and the fact that the two proteins are typically found in a complex with each other suggests that the BRCA1-BARD1 heterodimer is the physiological relevant form of BRCA1 (Yu and Baer, 2000).

1.4.1 BRCA1/BARD1, ubiquitin ligase activity

Apart from stabilizing BRCA1 the BRCA1-BARD1 heterodimeric RING finger also acquires E3 ubiquitin ligase activity in an E2-dependent manner (Hashizume et al., 2001). This ligase activity is abolished by common tumor associated mutations in the RING finger of BRCA1 (Brzovic et al., 2003; Hashizume et al., 2001; Lorick et al., 1999; Ruffner et al., 2001), strongly suggesting a tumor suppressive function for the BRCA1-BARD1 complex. Cells carrying these BRCA1 mutations are hypersensitive to irradiation (Ruffner et al., 2001), implying a role for the ubiquitin ligase activity in the DNA damage repair function by the complex.

The most common function of polyubiquitylation is targeting proteins for degradation by isopeptide linkage using lysine residues of ubiquitin to form polypeptid chains on the targeted protein. The most common lysine used to build these chains is K48. However, the E3-ligase activity by the BRCA1-BARD1 heterodimer primarily directs polymerization of ubiquitin by the less common K6 lysine (Wu-Baer et al., 2003), which is an indication that the E3-ligase activity by the heterodimer is not primarily degradation. At sites of replication stress or DNA damage the BRCA1-BARD1 heterodimer is auto-ubiquitylated by forming K6-linked ubiquitin structures (Morris
and Solomon, 2004), which in turn increases the ligase activity of the complex 20-fold (Chen et al., 2002; Mallery et al., 2002; Wu-Baer et al., 2003).

It has also been shown, in vitro, that the BRCA1-BARD1 heterodimer can ubiquitylate components of the RNA Pol II complex after DNA damage and thereby controlling cell-cycle progression by degrading these components (Kleiman et al., 2005; Starita et al., 2005). It has also been reported that the E3 ligase activity of the BRCA1-BARD1 complex can monoubiquitylate histone H2AX, which implies a role in chromatin remodeling by the complex (Chen et al., 2002; Mallery et al., 2002).

Another study proposes that the E3 ligase activity of the BRCA1-BARD1 complex can ubiquitylate centrosome components, including γ-tubulin (Starita et al., 2004). Inability to do so led to centrosome amplification. This taken together strongly supports the importance of the ubiquitin ligase activity of the heterodimer in DNA repair.

1.4.2 BARD1 in DNA repair

Paper I in this thesis is the first article showing experimental evidence that BARD1 is important in BRCA1-mediated DSB repair by the HDR pathway (Westermark et al., 2003). As described above, the acquired E3-ubiquitin ligase activity of the BRCA1-BARD1 heterodimeric RING finger appears to play an important role in DNA repair function. Furthermore, it has recently been shown that all motifs in BARD1 are essential for repair of an induced DSB by HDR (Laufer et al., 2007). Deletion of either the RING domain, the ankyrin repeats or the BRCT repeats of BARD1 leads to a decrease in DNA repair efficiency (Laufer et al., 2007). In addition, deletion of the ankyrin or the BRCT repeats led to chromosomal instability when expressed in a Bard-null mammary carcinoma cell line (Laufer et al., 2007). Mutations of putative phosphorylation sites within the BRCT repeats in BARD1 did not ablate the DNA repair ability of BARD1 neither did cancer-associated missense mutations in this area (Laufer et al., 2007; Westermark et al., 2003).

It has been shown that BARD1 can be phosphorylated after DNA damage on serine and threonine residues located within the BRCT repeats (Kim et al., 2006). The phosphorylation of BARD1 was suggested to be dependent on the phosphoinositide 3-kinase-related protein kinase (PIKK) family and also on BRCA1 interaction (Kim et
al., 2006). Mutations of these phosphorylation sites abrogated the inhibition of polyadenylation and degradation of RNA polymerase II after DNA damage (Kim et al., 2006). This indicates that phosphorylation of BARD1 is of importance in response to DNA damage. Since different phosphorylation sites were mutated in the work of Laufer et al. (Laufer et al., 2007) compared to the study by Kim et al. (Kim et al., 2006) these results does not necessarily contradict each other.

It has recently been shown that the BRCT domains of BRCA1 can interact with the ubiquitin-binding protein RAP80 (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). RAP80 recruits the BRCA1-BARD1 complex to ubiquitin structures at sites of DSBs. In addition, interaction with BARD1 enhances the DNA binding ability of BRCA1 (Simons et al., 2006).

1.4.3 BARD1 in tumorigenesis

The close association between BRCA1 and BARD1 has rendered the interest to study possible BARD1 mutations in breast and ovarian cancer patients. So far no deleterious mutations of BARD1 have been found. However, several point mutations have been identified, two of them was also found in the germ line of cancer patients (Ishitobi et al., 2003; Thai et al., 1998). One of the germ line mutation, Gln564His, was observed in a patient diagnosed with sporadic endometrial, breast and ovarian cancer. In at least one of these tumors the wild type allele was lost. This mutation was not found in any of the normal tissues tested (Thai et al., 1998). Cells with the Gln564His mutation have been shown to have defect in polyadenylation in response to DNA damage (Kleiman and Manley, 2001) and it is also defective in apoptotic activity (Irminger-Finger et al., 2001).

In several studies an elevated frequency of a Cys557Ser mutation in patients with breast and ovarian cancer was reported (Ghimenti et al., 2002; Karppinen et al., 2006; Karppinen et al., 2004; Sauer and Andrulis, 2005; Stacey et al., 2006). The highest prevalence of this mutation was found in breast cancer patients without a family history of ovarian cancer (Karppinen et al., 2004). The Cys557Ser mutation were also observed in two other studies, but in these studies were conceived as a normal polymorphism (Ghimenti et al., 2002; Thai et al., 1998). Interestingly, Gln564His and Cys557Ser mutations are found in close proximity to each other in a region of the
BARD1 protein proposed to be crucial for apoptosis (Feki et al., 2005; Feki et al., 2004; Jefford et al., 2004). Thus, the apoptotic function of BARD1 might be of importance in preventing tumor formation.

BARD1 has also been shown to be overexpressed in the cytoplasm of cells in breast and ovarian cancers (Wu et al., 2006). A majority of these tumors expressed a truncated BARD1 protein lacking the 5’ RING domain. This overexpression was associated with high malignancy and poor prognosis (Wu et al., 2006). Different BARD1 isoforms, with possible oncogenic potential, has also been reported (Li et al., 2007). Suggesting a role for aberrant BARD1 expression in tumor progression.

1.4.4 BRCA1 independent role of BARD1

There are several reports suggesting a BRCA1 independent role for BARD1 in apoptosis (Feki et al., 2005; Feki et al., 2004; Irminger-Finger and Leung, 2002; Irminger-Finger et al., 2001; Jefford et al., 2004; Rodriguez et al., 2004). Overexpression of BARD1, both in vivo and in vitro, shows an increase in cell death, with typical characteristics of apoptosis. Moreover, BARD1-repressed cells have been shown to be deficient in apoptosis after genotoxic stress (Irminger-Finger et al., 2001). BARD1 expression levels are increased in apoptotic cells and a significant shift from the nucleus to the cytoplasm has been noted (Jefford et al., 2004; Rodriguez et al., 2004). It has been proposed that BARD1 induces apoptosis by binding, stabilizing and phosphorylating p53 on serine-15 in the cytoplasm (Feki et al., 2005; Irminger-Finger et al., 2001). The apoptotic function of BARD1 appears to be dependent on functional p53, but independent of BRCA1 (Feki et al., 2005; Irminger-Finger et al., 2001). BRCA1 expression seems even to inhibit the apoptotic function of BARD1 (Irminger-Finger et al., 2001; Rodriguez et al., 2004). It has recently been shown that cytoplasmic BARD1 can localize to the mitochondria and contribute to oligomerization of the pro-apoptotic factor Bax, thereby inducing apoptosis. BRCA1 was not required for the mitochondrial localization of BARD1 (Tembe and Henderson, 2007). On the other hand, it has also been suggested that BARD1 can have an inhibitory effect on apoptosis by inhibiting BRCA1 induced apoptosis by nuclear retention of BRCA1 (Fabbro et al., 2004).
A plausible model based on these data could be that BARD1 can direct the cells fate after genotoxic stress either to DNA-repair by binding and stabilizing BRCA1 in the nucleus, or induce apoptosis by binding and stabilizing p53 in the cytoplasm.

1.5 DNA DAMAGE RESPONSE IN TUMORIGENESIS

Activation of DNA damage response (DDR) proteins is rarely found in normal tissues. The only tissues, which scored positive for these proteins, are tissues that undergo genome rearrangements and meiotic recombination, i.e. lymphocytic cells in the bone marrow and spermatocytes in adult testes (Bartkova et al., 2005a). However, upregulation of DDR genes have been found in pre-malignant lesions of colon, urinary bladder, skin, prostate and lung (Bartkova et al., 2005b; Bartkova et al., 2007; Bartkova et al., 2006; Di Micco et al., 2006; DiTullio et al., 2002; Fan et al., 2006; Gorgoulis et al., 2005; Nuciforo et al., 2007; Tort et al., 2006), while corresponding malignant lesions showed significantly lower frequency in expression of these proteins. This suggests that DDR could serve as an inducible barrier to prevent tumor formation by inducing cell death or cellular senescence. Defects in genes involved in DDR, for example p53, ATM, 53BP1 and BRCA1 and 2, could lead to escape from checkpoint control and subsequently lead to chromosomal rearrangements and genetic instability (Bartek et al., 2007a; Bartek et al., 2007b), typical hallmarks of cancer (Hanahan and Weinberg, 2000).

Uncontrolled growth caused by upregulation of onogenes and downregulation of tumor suppressor genes is another hallmark of cancer (Hanahan and Weinberg, 2000). Studies in cell culture and mouse models have shown that overexpression of a plethora of oncogenes can cause DNA damage checkpoint activation, e.g. H-ras, mos, cyclin E, Cdc25A, E2F1, cdc6, STAT5 and cMyc (Bartkova et al., 2005b; Bartkova et al., 2006; Di Micco et al., 2006; Frame et al., 2006; Mallette et al., 2007; Powers et al., 2004; Pusapati et al., 2006; Reimann et al., 2007; Tort et al., 2006). Inactivation of the tumor suppressor pRb has also been shown to activate DDR (Pickering and Kowalik, 2006; Tort et al., 2006), while inactivation/activation of other proteins in the pRB pathway, p16ink4a and cyclinD1, did not (Tort et al., 2006).

Even a combination of growth factors (basic fibroblast growth factor, stem cell factor and endothelin-3) injected into human skin xenografts in a mouse model for skin
hyperplasia could induce DDR (Gorgoulis et al., 2005). In this model it was shown that expression of growth factors could induce genomic instability at fragile sites a few weeks after induction of hyperplasia, suggesting that the observed instability was an early event in this tumor model (Gorgoulis et al., 2005). This data corresponds well with results in paper II in this thesis. In paper II we show that by overexpressing the growth factor PDGFB in the brain of newborn mice we can induce hyperplastic lesions within three weeks and that these lesions are aneuploid (Westermark et al., 2008). This implies that the genomic instability seen in these models might primarily be caused by replication stress caused by overexpression of oncogenes, independent of inactivation of care-taker genes. This is further supported by our data showing that inactivation of p19^{Arf} had no apparent effect on genomic instability (Westermark et al., 2008). It also implies that genomic instability can precede the malignant transformation rather than being a consequence of tumor progression.

The proposed model for this phenomenon is that overexpression of an oncogene will cause replicative stress, including stalled replication forks, collapse of the forks and subsequent DNA damage that will activate DDR and check point control. In cells with functional cell cycle regulation this can either lead to cell death or cell cycle arrest. If the cell cycle block becomes permanent it can be manifested as oncogene-induced senescence (OIS) (Bartkova et al., 2006; Di Micco et al., 2006). Thus, activation of DDR will prevent tumorigenesis. However, this oncogenic stress can also induce genomic instability (Gorgoulis et al., 2005; Westermark et al., 2008), which subsequently could lead to tumor progression.

1.6 DNA DAMAGE PATHWAYS AS A POSSIBLE TARGETS FOR CANCER THERAPY

Conventional cancer therapy typically involves different types of DNA damaging agents, such as irradiation and chemotherapy. Even though these types of treatments are rather efficient in many types of cancers it also have serious side effects, since they target all proliferating cells and not only the tumor cells. Moreover, it has become evident that tumor cells can evade this treatment by deregulation of DNA repair pathways. Increased activation of these pathways can potentially overcome DNA damage activated cell cycle checkpoints and allow cells that should have been eliminated to continue proliferation. This could in turn lead to increased genomic instability and tumor progression. Apart from overexpression of DNA repair proteins in
tumors, persons with hereditary mutations in DNA repair genes are predisposed to
tumor development, e.g. BRCA1/2, FANC genes, ATM, BLM (Bloom’s syndrom) and
WRN (Werner syndrome). Pre-clinical models has shown that inhibitors of DNA repair
can enhance the efficacy of DNA damaging anti-cancer drugs. Furthermore, recent
findings indicate that inhibitors of DNA repair also could be used as single agents to
treat patients with known defects in DNA repair pathways. Inhibitors of different DNA
damage pathways are currently being tested in clinical trials (Helleday et al., 2008).

To study cells sensitivity to DNA damaging treatment, RAD51 has been downregulated
in a multitude of ways and in a variety of cell types. Downregulation of RAD51 by
expressing a RAD51 ribozyme mini gene led to increased sensitivity to γ-irradiation in
a prostate tumor cell line (Collis et al., 2001). Sensitivity to γ-irradiation was also
increased in mouse cells when Rad51 was knocked down by antisense nucleotides
(ODNs) (Taki et al., 1996). A combination of anti-sense RAD51 and low dose
irradiation increased survival in a mouse glioma model (Ohnishi et al., 1998). RAD51
siRNA has been shown to enhance the effect of radiation and cisplatin in a variety of
cancer cells both in vitro and in vivo (Ito et al., 2005; Saydam et al., 2007). It has also
been shown that the tyrosine kinase inhibitor, Gleevec/Imatinib, can downregulate
RAD51 in glioma cell lines and thereby sensitize the cells to radiation (Russell et al.,
2003), implying a link between growth factor signaling and RAD51 regulation.

Other members of the HDR network has also been investigated as potential targets for
cancer therapy. Peptide-based inhibition of the RAD51 paralog RAD51C in CHO cells
reduced DNA-damage induced foci and increased the cells sensitivity to cisplatin
(Connell et al., 2004). In another study, the ATM inhibitor caffeine sensitized glioma
cells to irradiation (Short et al., 2007).

Inhibitors of DNA repair have also been shown to work as single agents. Inhibition of
poly (ADP-ribose) polymerase (PARP) can specifically kill cells with deficiency in
proteins involved in the HDR pathway (e.g. BRCA1/2, RAD51, ATM, ATR, CHK1,
CHK2, RPA1, NBS1, FANCA/C/D2) (Bryant et al., 2005; Farmer et al., 2005;
McCabe et al., 2006). However, cells deficient for RAD52 were not sensitive to PARP
inhibition, implying that the PARP inhibition primarily effected repair by gene
conversion and not single strand annealing (SSA) (McCabe et al., 2006). These results
imply the possible benefit of PARP inhibitors for treating tumors with defects in proteins involved in HDR.
2 GLIOMAGENESIS

2.1 PDGF AND THE BRAIN

2.1.1 PDGF ligands and receptors

Platelet-derived growth factor (PDGF) was first identified as a serum component important for proliferation of arterial smooth muscle cells (Ross et al., 1974). Later it was found that PDGF had specific receptors, primarily on cells derived from connective tissue and from normal and malignant glia (Heldin et al., 1981). At present four family members of PDGF is known: PDGF-A, -B, -C and D. They can form homo- and heterodimers (AA, AB, BB, CC and DD) and they bind to the tyrosine kinase receptors PDGFRα and PDGFRβ. The PDGF receptors can also form homo- and heterodimers (αα, αβ and ββ). The different PDGF ligands have different affinity for the PDGF receptors: AA binds αα, AB and CC binds αα and αβ, BB binds all three receptors and DD binds αβ and ββ. Upon binding the PDGF receptors dimerize and are activated by cross phosphorylation of their intracellular tyrosine kinase domains. The phosphorylated tyrosine residues serves as docking sites for several different proteins involved in mitogenic signaling cascades. These signaling pathways includes the Ras-Mitogen Activated Protein Kinase (MAPK), STAT transcription factor, phospholipase Cγ (PLCγ), Src family kinase and phosphatidylinositol 3-kinase (PI3K) pathways, which in turn activate downstream proteins such as Akt, Ras, Rho and Jak kinases. This suggests diverse functions of the PDGF family and also that the different binding combinations of the PDGF family members serve different cellular functions. (Shih and Holland, 2006; Yu et al., 2003).

2.1.2 PDGF in brain development

The central nervous system (CNS) stem cell gives rise to neuronal and glial progenitors, which subsequently give rise to the mature cell types of the CNS: neurons, oligodendrocytes, astrocytes and ependymal cells. This maturation process is tightly regulated by the expression of different growth factors and the different maturation stages have been defined by cell morphology and expression of specific markers (Shih and Holland, 2006).

In the adult brain the largest germinal center and source of neural stem cells (B-cells) is in the subventricular zone (SVZ) in the lateral ventricle, which give rise to mature
neurons and oligodendrocytes (Menn et al., 2006). B-cells express PDGFRα and it has been shown that PDGFRα is important for oligodendrogenesis from these cells (Jackson et al., 2006). PDGFA is also expressed in the SVZ, while PDGFB is primarily expressed in the cortex. The importance of PDGF in regulation of B-cells in the SVZ was further emphasized by a study showing that forced expression of PDGFA in mouse SVZ increased PDGFRα phosphorylation and proliferation of B-cells, leading to hyperplasia. These hyperplasias were dependent on PDGFA stimulation (Jackson et al., 2006). The same study also showed phosphorylation of PDGFRα in the SVZ in human adult brain, suggesting that endogenous PDGF signaling occur not only during development but also in the adult brain (Jackson et al., 2006).

PDGFB and PDGFRβ are important for vascular development in the mouse. Endothelial cells produce PDGFB and PDGFRβ is expressed by vascular smooth muscle cells, also called pericytes (Holmgren et al., 1991; Lindahl et al., 1997). Deletion of either of these genes causes embryonic lethality in mice due to microvascular hemorrhage and edema, caused by lack of pericytes (Hellstrom et al., 2001; Kaminski et al., 2001; Leveen et al., 1994; Lindahl et al., 1997). The pericytes surrounds and protects the endothelial cells in the blood vessel (Sims, 1986). This strongly suggests a paracrine loop between PDGFB ligand on endothelial cells in blood vessels and PDGFRβ on pericytes during angiogenesis. It has also been shown that PDGF ligands and receptors often are co-expressed on tumor cells suggesting an important role for autocrine/paracrine stimulation by PDGFs in the development of tumors (Hermanson et al., 1992; Uhrbom et al., 2000; Westermark et al., 1995).

2.2 BRAIN TUMORS
Similar to normal brain, primary brain tumors are composed of multiple cell types: neurons, glia, smooth muscle and endothelial cells based on the similarity to their normal counterparts in morphology and expression of cell-type specific markers. Brain tumors are also composed of cells of various differentiation stages of each particular cell type (Collins, 2004; Ohgaki and Kleihues, 2005).

Gliomas are classified into different grades (I-IV) based on malignancy according to the World Health Organization (WHO), where glioblastoma multiforme (GBM) is classified as the most malignant form (grade IV). Gliomas grow diffusely and infiltrate
the surrounding tissue and are therefore very challenging to surgically remove. The prognosis for glioma patients is poor and only a minority of patients reaches long-term survival. Low-grade gliomas are divided into two groups: astrocytomas and oligodendrogliomas. Diffuse lower grade tumors have a strong tendency for malignant progression and commonly recur as a GBM in the proximity of the primary tumor location within a few years after surgery. The majority of patients diagnosed with GBM die within a year of diagnosis (Holland, 2001; Ohgaki and Kleihues, 2005; Rich and Bigner, 2004).

It has previously been believed that gliomas arise from glial or neural progenitors, since cells in glioma mostly resemble immature astrocytes and/or immature oligodendrocytes. Another possible explanation could be that mature astrocytes and oligodendrocytes dedifferentiate due to the genetic changes they encounter during tumor development. However, in recent years the cancer stem cell hypothesis has evolved suggesting that only a minority of tumor cells have the capacity to self-renew and give rise to new tumors (Fomchenko and Holland, 2005).

Brain tumor stem cells have been isolated from human brain tumors based on their expression of the surface antigen CD133. These cells could form neurospheres in culture, were able to self-renew indefinitely under the right conditions and could initiate glioma-like tumors in serial-transplantations in mice (Galli et al., 2004; Singh et al., 2003). Only CD133 positive cells from human tumors had the capacity to generate tumors in mice that closely resembled the original tumor. CD133 negative cells from the same tumor were not able to establish tumors. This supports the idea that the CD133 positive brain tumor stem cell might be the initiating cell in gliomagenesis (Singh et al., 2004).

It has also been shown that injection of a retroviral PDGFB-GFP construct into the brain of adult rats could induce brain tumors in all animals and with short latency (Assanah et al., 2006). Only a fraction of the tumor cells expressed the GFP-tag used in this study, implying that uninfected cells were recruited to the tumor by paracrine stimulation by PDGFB. This was further supported by the recruitment of perivascular smooth muscle cells to the tumor, possibly by activation of the PDGFRβ. However, PDGFRβ expression in the vasculature was not investigated in this study. Both GFP positive and negative cells in the tumors expressed glial progenitor cell markers such as
nestin, olig2, NG2 and PDGFRα, but not the astrocytic marker GFAP suggesting that these tumors had arisen from white matter progenitors (Assanah et al., 2006). It has also been shown using the RCAS/tv-a model system that PDGFB can induce tumors from both nestin and GFAP expressing cells (Dai et al., 2001; Dai et al., 2005; Shih et al., 2004; Tchougounova et al., 2007). It is noteworthy that brain tumors in humans can occur throughout the whole brain and not only in the vicinity of the SVZ where the majority of normal neural stem cells reside. Thus, the cell-of-origin in gliomas remain elusive. However, one hypothesis does not necessarily override the other.

2.2.1 Genetic changes in glioma

Gliomas are markedly heterogeneous tumors, even within the same tumor. Despite the heterogenous appearance of gliomas, the typical genetic targets for alteration are within specific cellular processes, primarily in two main functional groups: activation of receptor tyrosine kinase (RTK) signaling pathways and loss of cell cycle regulation. It appears that modifications in both pathways are required for gliomas to occur. Common alterations in the RTK signaling pathways are overexpression of growth factors (e.g. PDGF, FGF) and overexpression/amplification of tyrosine kinase receptors (e.g. PDGFR, EGFR). An excess of growth factors and their receptor counterpart is frequently seen in the same cell, which can lead to autocrine stimulation and increased activity of down-stream effectors in the same pathway. Activation of the RTK pathway is typically accompanied by inactivation of the p53 pathway (e.g. p53, p14ARF) and/or the RB pathway (e.g. RB, p16INK4a). Not surprisingly, the number of genetic alterations often correlates with tumor grade, where an increase in gene amplifications and deletions give rise to tumors of higher grade (Collins, 2004; Ohgaki and Kleihues, 2005; Rich and Bigner, 2004; Sanson et al., 2004).

2.2.2 Glioma therapy

Gliomas are often resistant to radiation therapy. It would therefore be of great benefit for treatment of gliomas to sensitize the tumor cells to radiotherapy. Downregulation of proteins involved in DNA repair has proven to be quite efficient in radiosensitizing tumor cells and tumors in animal models (Bao et al., 2006; Golding et al., 2007; Ohnishi et al., 1998; Russell et al., 2003; Saydam et al., 2007; Short et al., 2007). It has been suggested that the CD133 positive brain cancer stem cells promotes radioresistance in glioma by activating DDR. In a study by Bao et al. they showed that
CD133 positive cells more efficiently activated DDR in response to radiation compared to the CD133 negative cells and that the pool of CD133 positive cells increased after the radiation treatment (Bao et al., 2006). Targeting DNA repair pathways, especially the HDR pathway, could be of special gain in brain tumors since in the majority of normal post-mitotic neuronal cells DNA repair is diminished and the treatment would thereby specifically target the tumor cells. However, the benefits of such combined therapy in the clinic remain to be shown.

The most efficient therapy to treat gliomas, at present, is treatment with the alkylating agent, Temozolomide. However, many gliomas are resistant to Temozolomide by expressing the DNA repair enzyme, O\textsuperscript{6}-Methylguanine-DNA-Methyltransferase (MGMT). Several different strategies have been developed to silence MGMT and thereby improve treatment by Temozolomide (Esteller et al., 2000; Gerson, 2004; Hegi et al., 2005; Stupp et al., 2005).

2.3 ANIMAL MODELS OF GLIOMAGENESIS

To further understand the molecular mechanisms behind gliomagenesis several mouse models have been developed. Models where brain tumors are induced by somatic-cell gene-transfer have proven to be very informative. The genes are delivered to the brain cells by intracerebral injection of oncogene-carrying retroviruses. These models allow the tumors to develop from somatic cells in their normal cellular environment and they show striking histopathological similarities with their human counterparts. By injecting different combinations of oncogene-expressing retroviruses in mice with deletions of different tumor suppressor genes it is possible to delineate the impact of various genetic alterations as well as combination of alterations in brain tumor formation (Uhrbom and Holland, 2001).

2.3.1 PDGFB/MMLV

The PDGFB/MMLV (Moloney murine leukemia virus) model system was developed to investigate the role of PDGFB in brain tumorigenesis. PDGFB expressing retrovirus was intracerebrally injected in newborn mice and highly malignant brain tumors of varying histological appearance developed in 40% of the animals. A majority of the tumors showed characteristics of human gliomas and all of them co-expressed PDGFB and PDGFRA mRNA, suggesting a critical role for PDGF in tumor initiation. It was
also shown that a cell line established from these tumors was dependent on autocrine PDGF stimulation for its proliferation (Uhrbom et al., 1998). However, it is known that PDGF overexpression and autocrine stimulation alone is not sufficient to cause glioma formation in humans. It was therefore hypothesized that additional mutations were caused by retroviral insertional mutagenesis to induce these highly malignant tumors. The PDGFB-induced tumors were investigated for proviral tagging of cellular genes. Several known and novel genes of tumor progression were tagged multiple times in the analyzed tumors. Of special interest for this thesis is that two genes associated with HDR were identified, the Rad51 paralog Rad51b and Fancc, which were tagged in two different tumors respectively. Rad51b displaying elevated expression similar to the Pdgfrα expression in these tumors (Johansson et al., 2004; Johansson et al., 2005). This offers the possibility that abnormalities in the HDR pathway could contribute to progression of PDGFB induced gliomagenesis.

2.3.2 RCAS/t-va

The receptor for MMLV is expressed in many different cell types in the mouse and produced virus can thereby infect a variety of cell types when injected into the brain of newborn mice. To address the question of cell-of-origin in gliomagenesis as well as the impact of combinations of different genetic alterations, the RCAS/tv-a model system was developed (Holland et al., 1998a; Holland et al., 1998b). The model is based on replication competent ALV splice acceptor (RCAS) and transgenic mice expressing the receptor for RCAS, tv-a. The tv-a receptor is normally only expressed in avian cells. This makes it possible to engineer transgenic mice to express the tv-a receptor from cell specific promoters and the expression of the RCAS virus will thereby only be directed to cells that express this promoter. The RCAS virus is replication incompetent in mammalian cells (Holland et al., 1998a; Holland et al., 1998b; Uhrbom and Holland, 2001).

Two transgenic tv-a mouse lines have been established to study gliomagenesis, one expressing tv-a from the nestin promoter (Ntv-a) and the other expressing tv-a from the GFAP promoter (Gtv-a). In the Ntv-a transgenic mouse the RCAS vector will deliver genes by infecting neural progenitors and in Gtv-a mice the genes carried by the RCAS vector will be transferred to astrocytes (Uhrbom and Holland, 2001). It is worth noting that neural stem cells in the SVZ (B-cells) also express GFAP (Jackson et al., 2006). Hence, the RCAS virus can also target these cells in the Gtv-a mice. The RCAS/tv-a
mice can be crossed with mice with targeted deletions of different tumor suppressor
genes (Holland et al., 1998a; Holland et al., 1998b; Tchougounova et al., 2007; Uhrbom et al., 2002; Uhrbom et al., 2005; Uhrbom et al., 2004). The impact of
different gene alterations on brain tumorigenesis can be studied by intercerebral
injection in newborn mice of RCAS producing cells that express a gene of interest, by
itself or in combination with other RCAS producing cells. This model offers a
possibility to study a variety of combination of genetic alterations in specified cell types
(Uhrbom and Holland, 2001).

The RCAS/tv-a mouse model has been extensively used to elucidate the role of
different oncogenes and tumor suppressor genes in brain tumor formation, by
themselves or in various combinations. In the first study using the RCAS/tv-a model
system the EGFR was overexpressed. Overexpression of EGFR alone in wild type or
p53-deficient Ntv-a mice did not induce tumors, but when expressed in an Ink4a-Arf
null background a high frequency of tumors developed (Holland et al., 1998a).

Overexpression of PDGFB in Ntv-a as well as in Gtv-a mice induces a large number of
tumors, similar to what has previously been seen in the PDGFB/MMLV gene transfer
model, however, the tumors where primarily oligodendroglomas (Ntv-a, Gtv-a) and
mixed oligoastrocytomas (Gtv-a) (Dai et al., 2001). Thus, when PDGFB is directed to
specific cell types it gives a more defined tumor population. PDGFB was also
overexpressed in an Ink4a-Arf null background, the incidence in Ntv-a mice was similar
to wild-type mice but the in the Gtv-a mice tumor incidence was elevated almost 2-
fold. The tumors were more malignant and had shorter latency in both Ntv-a and Gtv-a
mice. Of note, this study demonstrated that overexpression of PDGFB in p53-null
animals produced similar result to wild type animals (Dai et al., 2005). This is
consistent with the fact that TP53 mutations rarely occur in oligodendroglomas, while
silencing of the Ink4a-Arf locus by hypermethylation is quite frequent (Collins, 2004).
By elevating the PDGFB levels, tumors of higher grade and with increased
vascularization was induced. Tumor maintenance was dependent on PDGFB (Shih et
al., 2004).

The respective roles of the two members of the Ink4a-Arf locus, p16\textsuperscript{INK4a} and
p14/p19\textsuperscript{ARF}, in PDGFB-induced gliomagenesis have also been investigated
(Tchougounova et al., 2007). From this study it was found that the two tumor
suppressors had differential roles in a cell type specific manner in this tumor model system. Both tumor suppressors induced a significant increase in tumor incidence compared to wild type in the \textit{Gtv-a} mice and the \textit{Ink4a}-null background increased tumor initiation compared to the other genotypes. Tumor incidence in \textit{Ntv-a} mice was only slightly increased by the tumor suppressor loss. However, \textit{Arf}-loss had a pronounced effect on malignancy compared to \textit{Ink4a}-loss, in both \textit{Gtv-a} and \textit{Ntv-a} mice. Based on \textit{in vitro} data in primary brain cultures it was suggested that the increase in tumor malignancy in the \textit{Arf}-null background was through deregulation of MAPK pathway (Tchougounova et al., 2007).

In paper II we could confirm that \textit{Arf}-loss led to increased malignancy in PDGFB-induced gliomagenesis, using \textit{Ntv-a} mice. The most malignant tumors in this study (grade III) showed significant vascularization and this vasculature clearly expressed PDGFR\(\beta\), while the tumor cells expressed high levels of PDGFR\(\alpha\), suggesting autocrine/paracrine PDGFB stimulation in the formation of these tumors. No grade III tumors or extensive vascularization was seen in tumors in the wild type background (Westermark et al., 2008). It has been shown that \textit{Arf} play an important role in the regulation of perivascular cells, such as pericytes, in the developing eye. Loss of \textit{Arf} led to excessive proliferation of these cells. It was further shown that this hyperproliferation was dependent on PDGFR\(\beta\) and that \textit{Arf} negatively regulated PDGFR\(\beta\) expression independently of p53 and Mdm2 (Silva et al., 2005; Thornton et al., 2005). These results offer a rather persuasive explanation why \textit{Arf}-loss contributes to malignant progression in PDGFB-induced tumorigenesis, but not in \textit{Ink4a}- and \textit{p53}-null animals. However, it has also been shown that \textit{Arf} is the major tumor suppressor in \textit{Gtv-a} and \textit{Ntv-a} mice in \textit{K-ras} induced gliomagenesis (Uhrbom et al., 2005), suggesting an additional and more general tumor suppressor function for \textit{Arf} in the brain.
3 AIM OF THESIS

The overall aim of this thesis was to investigate the role of HDR in tumor initiation, tumor progression and genetic instability. The specific aims were:

- To investigate whether BARD1 was involved in DNA repair by HDR of DSBs and whether the interaction between BARD1 and BRCA1 was essential for the two proteins role in HDR. (Paper I)

- To elucidate whether a potential cancer causing germline mutation in BARD1 gave rise to repair defects in the HDR pathway. (Paper I)

- To study the effect of overexpression of either wild type RAD51 or a dominant negative RAD51 on PDGFB-induced brain tumor formation in wild type and Arf-/- mice. (Paper II)

- To identify the responsive element/elements for IL-1β downregulation of PDGFRA in the promoter region of PDGFRA. (Paper III)
4 RESULTS

4.1 PAPER I

To investigate whether the BRCA1 interacting protein BARD1 is important in HDR of a DSB we took a dominant negative approach. For this study we used wild type mouse embryonic stem (ES) cells and Brca1-deficient ES cells with an integrated repair substrate, DR-GFP. The Brca1-deficient cells express an alternative spliced Brca1 that skips exon 11, Brca1Δ11. This cell line has previous been shown to have defects in HDR of an induced DSB and is hypersensitive to DNA-damaging agents such as mitomycin C (MMC) (Moynahan et al., 2001a). To elucidate the role of BARD1 in HDR we expressed either full length BARD1 or a truncated BARD1 with an intact N-terminal RING domain but with deleted C-terminal structural motifs in wild type and Brca1-deficient ES cells. We confirmed by two-hybrid analysis and immunoprecipitation that this truncated BARD1 could interact with the endogenous Brca1 and Brca1Δ11.

We could show that transient expression of the truncated BARD1 construct decreased HDR of an induced DSB in wild type ES cells. The effect of the dominant negative BARD1 was even more pronounced in Brca1-deficient ES cells. Expression of full length BARD1 had no effect on repair in either wild type ES cells or Brca1-deficient ES cells.

To examine if the observed repair defect was dependent on the BARD1 constructs interaction with the endogenous Brca1, two different point mutations were introduced in the RING domain of the truncated BARD1, C83G and L107P. Missense mutations in key cysteine residues in BRCA1 are associated with human tumors and mutations in these residues have also been shown to interfere with RING interacting proteins (Castilla et al., 1994; Friedman et al., 1994). The C83G point mutation in BARD1, however, did not interfere with the heterodimerization between BRCA1 and BARD1. The expression levels of this mutant were significant lower compared to the truncated BARD1 without the point mutation and the effect on HDR were intermediate, probably due to the low expression level of the C83G mutant. The L107P mutant was constructed to completely abolish the interaction between the BARD1 construct and endogenous Brca1. This was confirmed by two-hybrid analysis. No significant decrease
in HDR was observed when this construct was expressed in wild type and Brca1-deficient cells.

To confirm that the repair defects observed were not due to improper cellular localization of either the transfected BARD1 constructs or the endogenous Brca1, immunoprecipitation and cellular fractionation was performed on transfected ES cells. We could show that the transfected BARD1 construct was predominantly nuclear and that Brca1 and Brca1Δ11 were exclusively nuclear in the BARD1 transfected ES cells. These data support the interpretation that the HDR defect is a direct dominant negative effect due to heterodimer formation between endogenous Brca1 and truncated BARD1 and not due to mislocalization of either of the proteins.

At the time of this study only one potential cancer causing germline mutation in BARD1 had been reported, Gln564His (Q564H) (Thai et al., 1998). We wanted to test whether expression of a full length BARD1 with the Q564H mutation would cause a HDR defect. Our data show that expression of BARD1 with the Q564H mutation had no effect on HDR in our model system.

From this study we conclude that BARD1 is involved in DNA repair by HDR of a DSB. Expression of a dominant negative BARD1 decreases HDR and this decrease is dependent on the heterodimer formation between BARD1 and Brca1. Our result suggests the BRCA1-BARD1 heterodimer as the functional unit for HDR. We could also show that a reported germline mutation in BARD1 had no effect on HDR in this model system.

### 4.2 PAPER II

The aim of this study was to investigate the effect of deficient DNA repair in PDGFB-induced gliomagenesis in wild type and Arf-null Ntv-a mice. For this study we used the RCAS/tv-a mouse model system. The model is based on the RCAS retroviral vector and transgenic mice expressing the receptor for RCAS, tv-a, from a cell type specific promoter. For our study we used the nestin promoter tv-a (Ntv-a) mouse. These mice only express the tv-a receptor in nestin positive cells, i.e. neural progenitor cells. Tumors can be induced by injection of cells expressing various RCAS virus constructs intracerebrally in newborn tv-a expressing mice. It has previously been shown that
injection of PDGFB virus causes glioma like brain tumors in Ntv-a mice and that Arf-loss increase tumor malignancy in these mice.

To investigate the effect of alterations in DNA repair in PDGFB-induced gliomagenesis, we injected cells expressing the central HDR protein RAD51 or a RAD51 mutant, RAD51-K133R, which has a dominant-negative effect on DNA repair function. These RAD51 constructs were expressed by themselves or in combination with PDGFB virus in wild type and Arf-/- Ntv-a mice.

Expression of the RAD51 constructs by themselves did not induce brain tumors in the Ntv-a mice. However, when co-expressed with PDGFB they where able to suppress PDGFB-induced gliomagenesis. We could show that overexpression of wild type RAD51 suppressed tumor formation in wild type animals and the suppressive effect was even more pronounced in the Arf-null background. The RAD51KR mutant strongly suppressed PDGFB-induced gliomagenesis in wild type animals but not in the Arf-/- mice.

Another intriguing finding in this study was that all the PDGFB-induced tumors were aneuploid, independent of genotype, grade or size. Interestingly, co-expression of PDGFB and the two RAD51 constructs could decrease the rate of aneuploidy and could even render diploid tumors.

We conclude that aneuploidy was an early event in PDGF-induced gliomagenesis independent of genotype, tumor grade or tumor size. Co-expression of RAD51 constructs decreased the aneuploidy caused by PDGFB and suppressed tumor development.

4.3 PAPER III

The aim of this paper was to investigate transcriptional regulation of PDGFRA by interleukin-1β (IL-1β). In this paper we used the osteosarcoma cell line MG-63. This cell line was previously shown to downregulate PDGFRA after stimulation by IL-1β and this regulation was primarily at the transcriptional level.

To find the responsive element/elements for this regulation we used different length of the PDGFRA 5’promoter region coupled to the luciferase gene. This enabled us to
measure PDGFRA activity by luciferase assay. We also performed bandshift assays to study protein binding to putative responsive DNA sequences in the PDGFRA promoter region. We also introduced mutations in the regions of interest in the PDGFRA promoter to try to abolish the inhibitory effect by IL-1β on PDGFRA promoter activity.

We were able to map an IL-1β responsive area within the PDGFRA promoter region. Although we found a strong correlation between the IL-1β effect and binding of CCAT/enhancer binding proteins (C/EBPs) to the promoter region, we could not abolish the inhibitory effect of IL-1β on PDGFRA expression by mutation of these regions. However, our data clearly showed that C/EBP is essential for basal PDGFRA transcription in MG-63 cells.
5 CONCLUDING REMARKS

Regulation of DNA repair appears to be a double-edged sword in the evolution and treatment of cancer. On one hand upregulation of DNA repair proteins protects cells from malignant transformation, but on the other hand increased DNA repair activity decreases cells sensitivity to DNA damaging drugs and thereby complicate the treatment of cancer. Moreover, deficiency in DNA repair is associated with chromosomal instability and increased risk of tumorigenesis. Taken together, this indicates that proper function of DNA repair pathways is of utmost importance for prevention and in treatment of cancer. Thus, further understanding of how the DNA repair pathways are regulated in normal and malignant cells have the potential to be of great use in the development of new and more efficient treatments of cancer in the future.
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7 REFERENCES


