DISSERTATION

DEPLETION OF RAD54, DNA-PKcs AND TANKYRASE 1 BY SMALL INTERFERING RNA AND THE EFFECTS ON RADIATION-INDUCED MUTAGENESIS, TOXICITY AND TELOMERE FUNCTION IN HUMAN CELLS

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JUNQING ZHOU ENTITLED DEPLETION OF RAD54, DNA-PKcs AND TANKYRASE 1 BY SMALL INTERFERING RNA AND THE EFFECTS ON RADIATION-INDUCED MUTAGENSIS, TOXICITY AND TELOMERE FUNCTION IN HUMAN CELLS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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The double-strand break (DSB) is one of the most severe types of DNA damage. Incorrect repair of such lesions results in chromosomal rearrangements and mutations that can lead to cancer and heritable defects in the progeny. There also is evidence that DNA DSBs are induced in bystander cells, which have not been directly irradiated but nevertheless respond to their exposed neighbors. Studies have suggested that the bystander response is potentially tumorigenic.

Small interfering RNA (siRNA) was used to silence expression of two genes, Rad54 and DNA-PKcs, that are known to be involved in DSB repair pathways and then the phenotypic consequences were examined in cells with different p53 status and also in directly irradiated and bystander cells.

In directly irradiated cells, knockdown of Rad54 resulted in increased

radiosensitivity in WTK1 (mutant p53), but this did not occur in TK6 (wild type p53) or NH32 (null p53) cells. Importantly, the radiosensitivity of Rad54-deficiency in WTK1 cells was evident in the S/G2 phases of the cell cycle but not in G1 phase. The immunoprecipitation studies showed that mutant p53, as well as wild type p53, associates with Rad54.

Mutagenesis caused by deficiency of Rad54 and DNA-PKcs in directly irradiated and bystander cells was studied. For directly irradiated mutagenesis, knockdown of Rad54 led to increased ionizing radiation (IR) -induced mutation at the autosomal heterozygous thymidine kinase (tk) locus in WTK1 and NH32 cells, but the knockdown did not affect mutagenesis in p53 wild-type TK6; knockdown of DNA-PKcs led to increased IR-induced mutation in WTK1 cells but mutagenesis was decreased in TK6 and NH32 cells. For bystander mutagenesis, the incubation of WTK1 bystander cells with unirradiated WTK1, TK6 or NH32 donor cells did not alter the background mutation fraction in bystander cells. The same was true for TK6 and NH32 bystander cells cultured with unirradiated WTK1, TK6 and NH32 donor cells. Co-culture with directly irradiated donor cells led to increases in mutation fraction in all three bystander cell lines regardless of p53 status. Knockdown of DNA-PKcs and Rad54 in the three irradiated donor cell lines resulted in no effect on bystander mutagenesis, suggesting that DNA repair status in donor cells is not important. However, the DNA DSB repair status in bystander cells

did turn out to be important. Knockdown of DNA-PKcs resulted in increased bystander mutagenesis in all three cell lines, but knockdown of Rad54 did not result in increased bystander mutagenesis.

Increasing evidence has accumulated to show that some DSB repair proteins are required for normal function of mammalian telomeres and that some telomere maintenance proteins also serve as modulators of the cellular responses to DSB damage. siRNA was used to knock down tankyrase1, a protein known to be involved in telomere elongation and maintenance, and phenotypic consequences on the processes of DNA damage response/repair were examined. Knockdown of tankyrase 1 resulted in an increased frequency of telomere sister-chromatid exchanges. Knockdown of tankyrase 1 also resulted in an increase in radiosensitivity and IR-induced mutagenesis. Surprisingly, knockdown of tankyrase 1 decreased levels of DNA-PKcs, and this may account for the observed phenotypic changes.

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CHAPTER 1

Introduction

1. DNA double-strand breaks: sensing, signaling and repair

a. DNA double-strand breaks (DSBs) and genomic instability

Of the various forms of DNA damages, the DSB is the most dangerous lesion with respect to cell survival and preservation of genomic integrity. DSBs arise when the two DNA complementary strands are broken simultaneously within a few base pairs; such a DSB may result in the chromatin structure collapsing and snapping into two pieces. DSBs can be induced by exogenous agents such as ionizing radiation (IR) or chemotherapeutic agents, or they also can arise as a consequence of natural processes, such as V (D) J recombination (a lymphoid specific process required for gene rearrangement and maturation of T and B cells), DNA replication and meiosis (Jackson, 2002; Khanna and Jackson, 2001; Karagiannis and El-Osta, 2004).

Though DSBs are rare in a normal environment - about 8 spontaneous DSBs per day in a mammalian cell (Bernstein and Bernstein, 1991) - they are difficult to repair by their natures and can lead to cell death and mutations. One non-repaired DSB can lead to cell death by inactivating an essential gene or triggering apoptosis (Rich, et al., 2000). Erroneous repair of DSBs may lead to translocations, inversions, deletions and/or amplification of genomic materials which can lead to the inactivation of tumor suppressor genes and the activation of proto-oncogenes, and may further lead to genetic instability

and eventually cancer. (Hoeijmakers, 2001; Jackson, 2002; Lengauer, et al., 1998; Vamvakas, et al., 1997; van Gent, et al., 2001). Moreover, increasing evidence has shown that defects in the factors involved in DSB signaling and repair lead to an increased level of carcinogenesis (Ferguson and Alt, 2001; Scott and Pandita, 2006; van Gent, et al., 2001).

In response to the threats posed by DSBs, mammalian cells have evolved efficient systems to rapidly detect these lesions, transduce signals and execute various biological responses to damage which include transcriptional regulation, cell-cycle control, apoptosis and DSB repair.

b. Sensing damage and signaling responses

The phosphatidyl inositol-like kinases (PIK) ATM (mutated in ataxia telangiectasia), ATR (ATM and Rad3-related kinase), and DNA-dependent protein kinase (DNA-PK), phosphorylate a variety of substrates and activate a number of important DNA damage response effectors (Khanna and Jackson, 2001).

At or close to the 'start' of the response to a DSB, the inactive ATM dimer auto-phosphorylates at serine 1981, dissociating the dimer and releasing the monomers, now free to phosphorylate their multiple substrates. It also has been suggested that ATM might be activated by monitoring chromatin structure changes that result from DSBs (Bakkenist and Kastan, 2003). Evidence has shown that activation of ATM by autophosphorylation also occurs in response to changes in chromatin structure in the apparent absence of DNA breaks (Bakkenist and Kastan, 2003). In addition, it has been suggested that the highly conserved MRN complex, which contains the Mre11, Rad50 and Nbs1 proteins, is required for ATM activation (Uziel, et al., 2003).

ATM phosphorylates histone H2AX on serine residue 139 and phosphorylated H2AX (γ-H2AX) forms en masse at the sites of DNA damage. γ-H2AX formation may lead to alteration of chromatin structure at the site of DSBs, and thus it may serve as a platform to recruit repair factors that are involved in DNA damage (Rogakou, et al., 1999; Rogakou, et al., 2000). Studies have shown that γ-H2AX is colocalized with repair factors, such as MRN complex, RAD51 and BRCA1 (Kobayashi, et al., 2002; Paull, et al., 2000) and H2AX-deficient mice were highly radiation sensitive (Bassing, et al., 2002; Celeste, et al., 2002). These results indicate that H2AX function is essential for mammalian DNA repair and genomic stability. Even though it has been suggested that γ-H2AX forms only at DSB, a recent study indicated that γ-H2AX foci were detectable 96 h after X irradiation when virtually all DNA breaks had been rejoined; the study has suggested that the remaining γ-H2AX foci were caused by an aberrant chromatin structure resulting from illegitimate rejoining but not a DNA double-strand break itself (Suzuki, et al., 2006).

In addition, activated ATM exerts control over several signaling pathways by phosphorylating key factors in an intricate network of proteins including Chk1, Chk2, Rad17, p53, NBS1, BRCA1, BLM, SMC1, 53BP1, and MDC1 (Kurz and Lees-Miller, 2004; Lavin, et al., 2005; Lou, et al., 2003; Mirzoeva and Petrini, 2001).

ATR is another DSB sensor protein and may serve as a partial backup system for ATM since they phosphorylate common substrates. Unlike ATM which responds mainly to IR, ATR is an important protein involved in responses to UV and bulky lesions and plays a particularly important role in signaling DNA damage during S-phase (Abraham,

2001; Bomgarden, et al., 2004; Brown and Baltimore, 2000; Cliby, et al., 1998; Karagiannis and El-Osta, 2004; Shiloh, 2001).

c. DSB repair pathways

Eukaryotes have developed two mechanisms for DSB repair, including non-homologous end joining (NHEJ) and homologous recombination (HR). In lower eukaryotes as well as prokaryotes, HR is the primary pathway for DNA DSB repair; however, in higher eukaryotes, NHEJ plays the major role in DSB repair (Dudas and Chovanec, 2004; Pastwa and Blasiak, 2003).

(1) Repair of DSBs by NHEJ. It is generally agreed that NHEJ is active throughout the cell cycle and the majority of DSBs are rejoined by NHEJ in mammalian cells. NHEJ, an error-prone process, rejoins the broken DNA ends together without a requirement for the presence of homologous sequences in the genome and usually causes the loss of nucleotides at the site of the DNA break. However, in a complex genome where only a small percentage of the DNA codes for protein, the risks of using an error-prone repair pathway may pose a less serious threat than the risk of an unrepaired DSB (Jackson, 2002; Smith and Jackson, 1999; Takata et al,. 1998).

Core proteins required for NHEJ include the Ku 70/80 heterodimer, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, DNA ligase IV, and Artemis (Burma, et al., 2006; Lieber, et al., 2003). Various studies have led to the following model for NHEJ: First, Ku heterodimer forms a ring-like structure and binds the DNA broken ends. It has been suggested that the Ku complex is a primary damage detector and can prevent damaged ends from degradation (Walker, et al., 2001). Ku then recruits DNA-PKcs to the site of DSBs to form the DNA-PK complex (Dynan and Yoo, 1998). Artemis,

a 5′–3′ exonuclease, forms a complex with DNA-PKcs and cleaves both 5′ and 3′ overhangs for NHEJ repair (Ma, et al., 2002). Finally the XRCC4–Ligase IV complex is recruited to the DNA end and completes the repair by re-ligation of the broken DNA ends (Grawunder, et al., 1997; Pastwa and Blasiak, 2003).

DNA-PKcs has been proposed to function as an activator due to its serine/threonine kinase activity (Kim, et al., 1999; Meek, et al., 2004). Studies have shown that DNA-PKcs is a member of the related PI3-K like kinase (PIKK) family which have a clear amino acid similarity to the phosphatidylinositol-3-kinase (PI3-K) family of kinases (Salles, et al., 2006) and its kinase activity requires binding to DNA broken ends (Hartley, et al., 1995). Ku heterodimer (Ku70 and Ku86) binds to free ends and recruits DNA-PKcs to DSBs (Cary et al., 1997; Pang et al., 1999; Gottlieb and Jackson, 1993). Once bound to a DNA end, DNA-PKcs phosphorylates itself (probably in trans) and a large number of substrates including Ku70, Ku86, Artemis, XRCC4, TP53 and RPA, thus activating or altering their functions (Baumann and West, 1998; Calsou, et al., 1999; Chan, et al., 2002; Chen, et al., 2005; Drouet, et al., 2005; Lee, et al., 2000). However, to date only *in vitro* data are available and there have been no links to function *in vivo*. The autophosphorylation of DNA-PKcs is required for its dissociation from Ku to make the DNA-ends accessible to subsequent processing and ligation steps (Calsou, et al., 1999; Chan, et al., 2002; Ding, et al., 2003). DNA-PKcs with mutations at the auto-phosphorylation sites are able to express the protein but are deficient in DSB repair, and therefore DNA-PKcs phosphorylation appears to be important for DSB repair (Burma and Chen, 2004; Chen, et al., 2005). Since DNA-PKcs is a large protein of about 470 KDa (Gottlieb and Jackson, 1993) and has a cage-like structure with channels and

cavities within the interior of the structure (Leuther, et al., 1999), it has been suggested to function as a scaffold protein for other repair factors to dock with and/or it may align DNA termini and promote their ligation (DeFazio, et al., 2002; Jeggo, et al., 1995; Meek, et al., 2004; Yaneva, et al., 1997).

Many studies have examined phenotype alterations that are associated with DNA-PKcs deficiency. Reintroduction of DNA-PKcs complements the DSB repair defects in M059J and V3 cell lines known to be deficient in DNA-PKcs, suggesting that the absence of DNA-PKcs is responsible for the phenotypes in these cells (Hoppe, et al., 2000; Kurimasa, et al., 1999; Lees-Miller, et al., 1995). Cells derived from highly radiosensitive SCID (severe combined immune deficiency) mice showed impaired DSB repair, neoplastic transformation susceptibility and genomic instability, and mutated *DNA-PKcs* gene has been suggested to be responsible for the malignant phenotypic changes (Blunt, et al., 1996; Danska, et al., 1996; Lun, et al., 1999). Cells from radiosensitive, cancer-prone BALB/c mice showed inefficient end joining of γray-induced DSBs which was accompanied by a significantly reduced expression level of DNA-PKcs protein as well as a lowered DNA-PK activity level (Okayasu, et al., 2000). Furthermore, female BALB/c mice have been shown to be more susceptible to ionizing radiation (IR)-induced breast cancer (Yu, et al., 2001).Thus, DNA-PKcs, a damage response and repair factor, may associate with breast cancer risk. Studies of siRNA knockdown of DNA-PKcs have shown that suppression of DNA-PKcs led to an increase in radiation- induced cell killing, chromosomal aberrations and mutation frequency phenotype and defective in DSB repair (Peng, et al., 2002).

DNA-PKcs-defective mice have been shown to have a shorter life span, an earlier onset of ageing-related pathologies, and higher incidences of T cell lymphomas and infections than the corresponding wild-type littermates (Espejel, et al., 2004).

Interestingly, a recent study has suggested a role of DNA-PK in Alzheimer's disease (AD), a neurodegenerative disease identified by progressive memory loss and cognitive impairment, as affected cells exhibited significantly deficient end joining activity and this was correlated with DNA-PKcs (Shackelford, 2006).

In addition, DNA-PK is found at the end of the chromosomes and defects or deficiencies in DNA-PKcs result in telomere-telomere fusions, on fusions between telomeres and DSB. This suggests a role for DNA-PKcs in telomere maintenance and the prevention of chromosome end-to-end fusion (Bailey et al., 1999 and 2004c).

(2) Repair of DSBs by HR. In contrast to NHEJ, HR utilizes a homologous sequence in the form of sister chromatids, homologous chromosomes or DNA repeats to template repair of a DSB, and, as a result, it is usually an error-free pathway (Sonoda et al., 2006). Generally, HR is most active in late S/G2 when sister chromatids are available. A large number of proteins in the RAD52 epistasis group are known to be involved in HR, including RAD51 and its paralogs, RAD52, RAD54, Rad55, Rad57, Rad59, BRCA1, BRCA2, XRCC2, XRCC3, and the MRN complex (Symington, 2002; Takata et al., 1998; Essers et al., 2000).

A processing model of HR initially involves strand resection of the DSB: both of the 5′ ends of the DSB are resected by the action of a specific nuclease to form 3′ single-strand overhangs. Subsequently, the 3′ overhangs of DNA associate with Rad52 and subsequently with polymerized Rad51, and the Rad51 nucleoprotein filaments invade an

intact homologous duplex and generate a D-loop structure with the help of Rad54. The 3' end of the noninvading strand is also extended by DNA synthesis. This process, followed by ligation, leads to the formation of two Holliday junctions which are four-stranded branched structures. During the last step of HR, resolution of the two Holliday junctions by randomly cutting the crossed or non-crossed strands is expected to yield equal numbers of crossover and noncrossover products (Dudas and Chovanec, 2004).

The highly conserved Rad54 protein plays an essential role in repairing DNA damage through homologous recombination. The human and mouse RAD54 genes encode 83.4 kDa proteins of 747 amino acids. Rad54 is a member of the SWIZ/SNF2 family, which contain seven evolutionarily conserved motifs, including a DNA-dependent ATPase motif (Eisen, et al., 1995). Unlike typical DNA helicases which display ssDNA-dependent/enhanced ATPase activity and use the energy of ATP hydrolysis to translocate on ssDNA (Wolner and Peterson, 2005), Rad54 protein uses the energy of ATP hydrolysis to translocate on dsDNA at an early step in the repair process, thus inducing topological changes in DNA structure and opening of DNA duplex strands, which is indispensable for D-loop formation during HR (Kiianitsa, et al., 2002; Sigurdsson, et al., 2002; Swagemakers, et al., 1998). X-ray structure and biochemical data provide evidence for a model in which Rad54 translocates along duplex DNA (Durr, et al., 2005; Thoma, et al., 2005). However, a recent report proposed that Rad54 can also translocate on single molecules of dsDNA (Amitani, et al., 2006). ATPase and strand opening activity of Rad54 are significantly increased in the presence of the Rad51-ssDNA/dsDNA nucleoprotein filaments. Moreover binding of Rad54 to the Rad51–ssDNA nucleoprotein filament substantially increases its stability (Mazin, et al., 2003), suggesting that *in vivo*

Rad54 functions in concert with Rad51, possibly acting at multiple stages during HR (Heyer, et al., 2006; Kiianitsa, et al., 2002; Solinger, et al., 2001). A recent study also proposed that Rad54 has DNA branch-migration activity at later stages of homologous recombination (Bugreev, et al., 2006).

RAD54-deficient mouse embryonic stem (ES) cells are two-to-four fold more sensitive to IR, and HR activity is five-to-ten fold reduced compared to wild-type cells. Homozygous *RAD54*$^{-/-}$mutant clones in the chicken B cell line DT40 were highly X-ray sensitive compared to wild-type cells and reexpression of the *RAD54* cDNA restored radiation resistance and targeted integration activity (Bezzubova, et al., 1997). However, on the other hand, some somatic cells deleted for Rad54 show only a slight increase in radiosensitivity (Essers, et al., 1997; Essers, et al., 2000; Takata, et al., 1998). Evidence showed that Rad54 alterations may be involved in carcinogenesis. Out of 132 primary tumors examined, three mutations in rad54 have been identified, with one each in a breast tumor, a colon cancer, and a lymphoma (Matsuda, et al., 1999). Another study has shown that loss of heterozygosity of *RAD54* is observed in breast cancer. In addition, mutations in genes with consensus helicase homology have been found in cancer-prone syndromes such as xeroderma pigmentosum, Bloom syndrome, Werner's syndrome, and the X-linked mental retardation with alpha-thalassemia syndrome (ATR-X), which suggest hRAD54, containing all seven of the consensus segments of DNA helicase superfamily, may have a role in those carcinogenic processes (Rasio, et al., 1997; Gibbons et al., 1995; Ellis et al., 1995; Yu et al., 1996).

Homozygous mutations at highly conserved positions of RAD54B, an analogue of Rad54 in human cells, were observed in human primary lymphoma and colon cancer,

which suggest the some cancer arise may through alteration of hRAD54B (Smirnova, et al., 2004).

Moreover, Rad54-deficient mice exhibited significantly shorter telomeres, even though telomerase activity is normal, thus Rad54 activity has been suggested to play an essential role in telomere length maintenance. Rad54 deficiency also causes an increased frequency of end-to-end chromosome fusions, suggesting a role of Rad54 in telomere capping (Jaco, et al., 2003; Tarsounas and West, 2005).

d. p53 is involved in DSB response and repair.

The tumor suppressor protein p53 is referred to as the 'guardian of the genome' and mutations of p53 are found in over 50% of all human tumors. In an unstressed cell, the p53 protein is kept at a low concentration by its relatively short half-life (Levine, 1997; May and May, 1999). However, in response to damaged DNA, nucleotide depletion, hypoxia, and other genotoxic stresses, p53 is activated and becomes stabilized and accumulates in the nucleus. P53 can be activated through post-translational modification such as phosphorylation, acetylation or even dephosphorylation (Appella and Anderson, 2001). For example, in response to DNA DSB damage, sensor proteins such as members of PIKK family become activated and further phosphorylate various downstream substrates including p53. ATM can directly phosphorylate p53 or indirectly phosphorylate, through ATM-mediated regulation of other protein kinases, such as Chk1, Chk2. Other members of the PIKK family, such as ATR and DNA-PKcs can also phosphorylate p53 in response to different DSB inducers and may be able to compensate for loss or dysfunction of ATM (Kurz, et al., 2004; Kurz and Lees-Miller, 2004). Once activated, p53 exerts multifactorial effects on cell cycle control and apoptosis through its

transcriptional regulation of downstream genes, including p21, WAF1, Bax, etc. Phenotypic studies (Chao, et al., 2000; Jimenez, et al., 2000) have shown that p53 effects in cell-cycle, apoptosis and its tumour-suppressor function requires its transcriptional activity: a mouse model with deficient transcriptional activity of p53 showed defects in cell-cycle regulation and apoptosis even though the level of mutant p53 was stable and DNA-binding activity was apparently normal, but p53 did not accumulate after DNA damage and the expression of downstream genes was not induced. Moreover, both mutant and p53-null mouse embryonic fibroblasts (MEFs) were readily transformed by oncogenes, and the corresponding mice were prone to tumors (Jimenez, et al., 2000).

Although much of the literature has the view that p53 effects on genome stability are through its regulatory roles in cell cycle and apoptosis, p53 also may help maintain genome stability by modulating the DSB response and repair pathways: p53 has been recently proposed to be involved in the initial steps of DNA damage signaling and in fact it colocalizes with γ-H2AX foci (Al Rashid, et al., 2005). Moreover, p53 can positively mediate NHEJ, at least the rejoining of breaks with short complementary ends of single-stranded DNA (Tang, et al., 1999). Bill et al postulated that the presence of mutant p53 or lacking p53 protein can promote end-joining activity compared to wild type p53 TK6 lymphoblastoids (Bill, et al., 1997). Studies also showed that p53 negatively regulates NHEJ since DNA end joining was more active in p53-null MEFs than in MEFs of wild-type p53 (Okorokov, et al., 2002). Moreover, p53 may prevent error prone NHEJ by inhibiting mismatched DNA annealing (Dahm-Daphi, et al., 2005). Other studies have shown that p53 has regulatory roles in HR. Many studies have shown that deficiency of p53 led to increased HR (Bertrand et al., 1997; Livingstone, et al., 1992; Mekeel et al.,

1997). Moreover, introduction of mutants of p53 in mouse cells with wild-type p53 status resulted in an increase in spontaneous and radiation-induced HR (Saintigny et al., 1999) P53 is suggested to prevent inappropriate recombination by directly or indirectly interacting with essential HR proteins, including BLM, BRCA1, BRCA2, Rad52, Rad51 and Rasd54 (Helton and Chen, 2007; Sturzbecher, et al., 1996). However, there is evidence showing that p53 can also regulate HR by transcriptional repression of Rad51 (Arias-Lopez, et al., 2006) and promoting the clearance of Rad51 foci (Orre, et al., 2006).

Moreover, there is evidence showing that p53 effects on DSB response and repair are responsible for its effects on mutagenesis: Patients with the Li-Fraumeni syndrome (LFS), having heterozygous TP53 germline mutations, are susceptible to spontaneous and radiogenic cancers and approximately 60% of LFS families have germline mutations of the TP53 gene (Varley, 2003). Human lymphoblastoid WTK1 cells (mutant p53) show delayed onset of X-ray-induced apoptosis, an increased ability to catalyze recombination and a remarkably increased mutability at the thymidine kinase locus (tk) compared to TK6 (wild-type p53). Moreover, after transfection and subsequent overexpression of the known dominant negative mutant p53 in TK6, there were significantly enhanced spontaneous and X-ray-induced mutant frequencies at the tk locus. These results indicated that p53 dysfunction can lead to increased mutagenicity in human lymphoblastoidoid cell lines either through delayed apoptosis in response to DNA damage or by mediating increased recombination (Xia and Liber, 1997; Xia, et al., 1995).

2 . Radiation-induced bystander effects (BSE): evidence, mechanism and significance

a. Radiation-induced bystander effects

Even though it is well accepted that many of the deleterious effects of IR may result from direct damage to cellular DNA, there is also much evidence showing that cells which have not been directly irradiated respond to their exposed neighbors. This phenomenon has been termed the bystander effect (BSE) (Nagasawa and Little, 1992; Prise, et al., 2006). BSE can be observed after a variety of different exposure strategies. These include co-culture of irradiated and non-irradiated cells (Gerashchenko and Howell, 2003), the use of very low fluences of alpha particles where the majority of cells have not been irradiated (Azzam, et al., 1998), irradiation of targeted cells within a population of cells using charged-particle microbeams (Folkard, et al., 1997; Zhou, et al., 2000), and the transfer of medium from irradiated cells to non-irradiated cells (Mothersill and Seymour, 1997). BSE has been observed using end points including sister chromatid exchange (SCE) (Nagasawa, et al., 2002; Nagasawa, et al., 2005), chromosomal aberrations (Nagasawa and Little, 2002), cell killing (Mothersill and Seymour, 1997; Mothersill, et al., 2006; Zhu, et al., 2005), gene mutation (Nagasawa, et al., 2003; Persaud, et al., 2005; Zhou, et al., 2000), neoplastic transformation (Sawant, et al., 2001), and changes in gene expression (Azzam, et al., 1998; Azzam, et al., 2001). All of these endpoints are consistent with the presence of DNA damage in bystander cells. Since such damage in bystander cells is likely to be potentially tumorigenic (Sawant, et al., 2001), understanding the bystander phenomenon is of extreme importance to risk estimation, radiation protection and cancer therapy.

b. Mechanisms of BSE

The basic mechanisms by which damage signals are transmitted from irradiated to

unirradiated cells, as well as what factors (both in irradiated and unirradiated cells) influence the BSE, are beginning to emerge. That individual cells in a multi-cellular organism communicate is not unexpected or unusual. They do this in many well defined, and some less well defined ways. Understanding BSE demands identification of the transmitted signal and how this signal provokes a response in a non-irradiated cell. On one hand, the transfer of medium from irradiated to non-irradiated cells implicates a soluble secreted factor. On the other, the use of low fluences of alpha particles and appropriate cell lines demonstrates a role for intercellular gap junction communication. The cell-to-cell gap junction mediated transfer of information appears to require connexin 43 (Azzam, et al., 2001; Zhou, et al., 2000), indicating that the size of the signaling factor is relatively small. Membrane signaling has also been implicated in the induction of SCE, micronuclei formation, and gene mutation in bystander cells (Nagasawa, et al., 2002). These mechanisms are not mutually exclusive, and there is likely a role for multiple processes in communicating the bystander response. BSE mechanisms appear to involve enhanced oxidative metabolism, with a role for reactive oxygen and nitrogen species as well as proteins associated with cellular stress responses being the major factors (Azzam, et al., 2003; Azzam, et al., 2002; Bishayee, et al., 2001; Lorimore, et al., 2001; Lyng, et al., 2000).

c. Role of DSB repair in BSE

The majority of the bystander responses described are also observed after targeted exposure to IR, i.e., in cells where radiation-induced energy deposition events occurred. These responses include cytogenetic damage (chromosomal aberrations, SCEs and micronuclei), mutagenesis, transformation, changes in gene expression and cell killing.

For many years the DSB has been considered the primary genotoxic lesion induced by IR, suggesting that this lesion might also be involved in BSE. Formation of DSBs induces phosphorylation of histone H2AX, and this phosphorylated form, γ-H2AX, forms foci at the sites of DNA cleavage. Irradiation of target cells induces the formation of γ-H2AX foci in bystander cell populations (Sokolov, et al., 2005). After 18 h co-culture with cells irradiated with 20 alpha-particles, the fraction of bystander cells showing multiple γ-H2AX foci increased 3.7-fold. Similar changes occurred in bystander populations mixed and cultured with cells irradiated with γ-rays, and in cultures containing media conditioned by γ-irradiated cells. This study indicates that H2AX phosphorylation may well be an early step in the BSE and that DNA DSBs may be responsible for the observed BSE. A similar result was also reported (Yang, et al., 2005). Since DNA DSBs have been found in bystander cells, recent studies have addressed the involvement of DSB repair factors in the bystander response (Nagasawa, et al., 2003; Nagasawa and Little, 2002; Nagasawa, et al., 2005; Yang, et al., 2005).

Conditioned medium harvested from irradiated DNA repair deficient cells has been demonstrated to be more toxic than the medium harvested from repair proficient cells, as measured by IR induced cell killing in recipient cells (Mothersill, et al., 2006). Mouse embryonic cells with a deficiency in the radioresistance gene Rad9 were more sensitive to IR-induced bystander apoptosis and micronucleus formation, but not cell killing (Zhu, et al., 2005); this suggested that DNA repair factors may have different effects on individual bystander response end points. The NHEJ pathway has been suggested to play a role in low fluence α-particle and targeted soft x-rays induced bystander effects in Chinese hamster cells, as measured by cell killing, micronuclei, chromosome aberrations

and SCE (Kashino, et al., 2004; Little, et al., 2003; Nagasawa, et al., 2005). Mutational spectra analyses at the hypoxanthine-guanine phosphoribosyltransferase *(hprt)* locus have shown that mutations induced in DNA repair proficient bystander cells were mainly point mutation; this is in contrast to directly irradiated cells, in which total and partial gene deletions predominate (Huo, et al., 2001). In addition, the mutational spectra in DSB repair deficient bystander cells (XRS-5, Chinese hamster ovary cells with a Ku80 deficiency, NHEJ deficient) were predominantly partial or complete deletions (Huo, et al., 2001; Nagasawa, et al., 2003). Since gene mutations frequently result from processing by DSB repair mechanisms, these results suggest that DSB repair status affects mutagenesis differently in irradiated and bystander cells.

d. Role of p53 in BSE

The p53 tumor suppressor protein functions in cell cycle checkpoint control and the induction of apoptosis (Helton and Chen, 2006). In addition, p53 is involved in spontaneous and DSB-triggered HR, and it contributes as well to nucleotide excision, base excision and mismatch repair (Ford, 2005; Gatz and Wiesmuller, 2006). Moreover, p53 has also been implicated in modulating mutagenesis; p53 mutations lead to a hypermutable phenotype in human cells (Wiese, et al., 2001; Xia, et al., 1994; Xia and Liber, 1997). The role of p53 in BSE is not clear. p53 has been suggested to play a role by inducing export of growth suppressive stimuli from damaged cells to neighboring cells (Komarova, et al., 1998). An interesting report described a p53-mediated abscopal antitumor effect; the observation was made in mice that localized irradiation resulted in a slower growth rate of tumors distant to the irradiated site (Camphausen, et al., 2003). However, the effect of p53 status on IR-induced bystander mutagenesis has not been

reported.

3. Interplay between DSB repair and telomere stability

a. Telomere structure and function

Telomeres are specialized chromatin structures present at the natural ends of eukaryotic chromosomes. Telomeres are composed of telomeric DNA and specific proteins. In human, the telomeric DNA consists of 5-10 kb of tandem arrays of short, repetitive G-rich sequence (TTAGGG) that are extended several thousand base pairs and oriented 5'to 3' towards the end of the chromosome. There is a 3' single-stranded overhang (Makarov, et al., 1997), which invades the double-stranded telomeric DNA, forming circular structures called t-loops (Griffith, et al., 1999). Studies have shown specific proteins that are associated with telomeric DNA; these include telomerase, telomeric repeat binding factors 1 and 2 (TRF1, TRF2), Pot1 and DNA damage repair factors such as DNA-PKcs, Ku. These proteins form large complexes and regulate telomere length and structure.

b. Link between telomere maintenance and DSB response/ repair

About 70 years ago, classic experiments done by Herman Joseph Muller and Barbara McClintock revealed that telomeres were not subject to fusion with other telomeres or to broken chromosomal fragments caused by IR. This is an important aspect of telomeres, the so-called "capping" function, providing protection for natural chromosomal ends against various possible reactions. Many studies have shown that the "capping" function of telomeres can prevent the recognition of chromosomal ends as double-stranded DNA breaks, and thus the subsequent degradation, recombination, or

fusions that otherwise might result; capping also aids in avoiding a DNA damage response and thus can have an important role in preventing mammalian cells from premature aging and carcinogenesis (Blasco, 2003). In contrast to telomeres, broken chromosome ends produced by DNA damage are highly recombinogenic. In order to maintain genomic and chromosomal stability, how do cells distinguish these two types of DNA ends and then process them appropriately? There is evidence suggesting that telomere maintenance and DNA damage response mechanisms are closely linked.

Studies have demonstrated that proteins involved in DSB response and repair pathways also have important roles in telomere maintenance. Cells from AT (ataxia telangiectasia) patients not only show increased chromosomal instability, acute sensitivity to IR and severely impaired G_1, S, and G_2 phase checkpoint functions after IR (Paules, et al., 1995), but also showed accelerated telomere shortening and elevated frequencies of end-to-end chromosome fusions (Metcalfe, et al., 1996). DSB repair factors from the NHEJ and HR pathways are found at telomeres and are required for normal mammalian telomeric end-capping function (Slijepcevic and Al-Wahiby, 2005). Cells from severe combined immunodeficiency (SCID) mice, which are natural DNA-PKcs mutants, show telomere fusions (Bailey, et al., 1999; Goytisolo, et al., 2001). Cells from Ku deficient mice showed elevated frequencies of end-to-end chromosomal fusions with telomeric sequences clearly detectable at the fusion points (Bailey, et al., 1999; Hsu, et al., 2000; Samper, et al., 2000). By using RNAi, DNA-PKcs or Ku-deficient mammalian cells have been shown to have increased levels of telomeric dysfunction (Jaco, et al., 2004; Myung, et al., 2004; Zhang, et al., 2005a). Cells from mice defective in Rad54 show shortened telomeres compared to wild-type mice, this telomere shortening caused an increased

incidence of telomeric fusion (Al-Wahiby, et al., 2005). There also is evidence that RAD51D, a RAD51 paralog, is located at telomeres and plays an important role in telomere maintenance (Tarsounas, et al., 2004). Furthermore, a telomere dysfunction phenotype was observed in primary mouse embryonic fibroblasts defective in RAD51D and p53. These cells showed both significant telomere shortening and increased frequencies of end-to-end chromosome fusions in comparison with appropriate control cells (Slijepcevic and Al-Wahiby, 2005).

Telomere binding proteins have been suggested to have direct roles in DSB damage response. Deficiency of the telomeric binding protein Pot1 promotes chromosomal instability and tumorigenesis (Wu et al., 2006). A recent study has shown a direct role of TRF2 (telomeric repeat binding factor 2), in that it associates with DSBs within 2 s of laser microbeam irradiation; this occurs even before the association of ATM with DSBs and activation of the ATM-dependent DSB response network and suggest that TRF2 is involved in early response to DSB response within a few seconds following exposure to a very high energy UV laser microbeam (Bradshaw, et al., 2005). More recent studies have shown that the co-localization of γ-H2AX foci and TRF2 does not occur following damage induced by ionizing radiation (Williams et al, 2007). Conversely, Interplay between telomere maintenance and DSB response/ repair have been studied intensively for the past few years. However, there are still questions that remain unanswered. How do the same DNA damage/response factors bind both DSB and telomere ends, distinguish between them and determine whether to facilitate end joining or end protection? What are the direct roles of classical telomeric proteins at DSB sites? How do telomeric proteins influence the damage response to DSBs, and thus modulate the genetic consequences of

DNA damage? Much more research is needed on the interplay between telomere maintenance and DNA damage response.

c. Telomere elongation by telomerase or alternative lengthening of telomeres

Conventional semiconservative DNA replication machinery does not allow DNA polymerase to fully replicate chromosome ends, so telomere length is predicted to decrease with each round of replication and eventually shortening becomes critical and causes cellular senescenc (Meeker and De Marzo, 2004; Shay and Wright, 2005). Telomerase elongates telomeric DNA at chromosome ends to maintain telomere length (Greider and Blackburn, 1985). Mammalian telomerase consists of an RNA component (telomerase RNA component [TERC]) that serves as a template for the synthesis of new telomeric TTAGGG repeats by the telomerase reverse transcriptase component (TERT) (Greider and Blackburn, 1987). In primary human cells, where telomerase activity is not detected, telomere length decreases with increasing number of cell divisions and cells eventually senesce. However, in the germ line, and in some cancer and immortalized cell lines, telomere length is maintained through active telomerase which can compensate for telomere loss as cells proliferate (Greider, 1999; Kim, et al., 1994).

There is evidence showing that some mammalian cells with no telomerase activity are able to maintain telomere lengths, and this suggested that non-telomerase mechanisms for elongation also exist (Bryan, et al., 1997; Bryan, et al., 1995). This mechanism is called Alternative Lengthening of Telomeres (ALT). It is now clear that ALT activity has only been found in abnormal situations, including human tumors, immortalized human cell lines, and in telomerase-null mouse cell lines (Bryan, et al., 1997; Henson, et al., 2002). Studies have shown that ALT cells have heterogeneous telomere length and ALT-

associated PML bodies (APBs), which are nuclear structures that include telomeric DNA and the telomere binding proteins, TRF1 and TRF2 (Kim, et al., 1994; Murnane, et al., 1994; Yeager, et al., 1999). It has been suggested that APBs may be involved in ALT related protein modification or removal of by-products. Many of the proteins that are involved in DNA damage response and recombination have been identified in APBs. These proteins include RAD51, RAD52, RPA, MRE11, RAD50, NBS1, BLM, WRN and BRCA1 (Henson, et al., 2002; Johnson, et al., 2001; Le, et al., 1999; Lombard and Guarente, 2000; Wu, et al., 2003; Wu, et al., 2000).

d. Tankyrase 1

Tankyrase 1 (TRF1-interacting ankyrin-related ADP-ribose polymerase 1) is a telomere specific PARP. In general, poly(ADP-ribosyl)ation is involved in various physiological events, including DNA replication, DNA repair, gene expression, chromatin decondensation, malignant transformation, cellular differentiation. Tankyrase 1 was originally identified as a TRF1-binding protein. TRF1 forms part of the TRF1-TIN2-TPP1-POT1 telomeric protein complex and limits telomerase access, while tankyrase 1 polyADP-ribosylates and releases it from telomeres, allowing access of telomerase to telomeres (Smith, et al., 1998). By enhancing access to telomerase, tankyrase 1 thus works as a positive regulator for telomere elongation by telomerase. Studies have shown that tankyrase 1-mediated telomere elongation is not observed in the absence of telomerase activity (Chang, et al., 2003; Cook, et al., 2002; Seimiya, et al., 2005).

Tankyrase 1 protein consists of four characteristic domains which determine its multiple functions. The one striking feature of tankyrase 1 is that it has an ANK domain which is composed of a long stretch of 24 ANK repeats, providing a platform for protein-

protein interactions. The ANK domain of tankyrase 1 is further divided into five well-conserved subdomains and each subdomain, designated as ARC (ANK repeat cluster) I-V, works as an independent binding site. This protein structure of tankyrase 1 suggests it can act as a scaffolding molecule (De Rycker, et al., 2003; Seimiya and Smith, 2002). Another striking feature of tankyrase 1 is the C-terminal PARP domain, which catalyses poly(ADP-ribosyl)ation of acceptor proteins using NAD as a substrate. This post-translational modification provides significant negative charges to the acceptor proteins and often disrupts interactions between the acceptor proteins and the DNA.

PARP family members have been shown to play important roles in single-strand break and DSB repair as well as in base excision repair (Ariumi, et al., 1999; Masson, et al., 1998; Ruscetti, et al., 1998). PARP-1 and PARP-2 have been suggested to have important roles in the cellular response to IR (Chalmers, et al., 2004; Fernet, et al., 2000; Shall and de Murcia, 2000), and this could be via a role in DSB response/repair (Audebert, et al., 2006; Rudat, et al., 2001; Wang, et al., 2006). Studies have suggested an interaction between DNA-PK and PARP-1 (D'Silva, et al., 1999; Morrison, et al., 1997). It was shown that purified DNA-PK can phosphorylate PARP and protein kinase activity of DNA-PK can be stimulated by ADP-ribosylation activity of PARP. Moreover, the ADP-ribosylation of DNA-PKcs was independent of the Ku70/86 complex; the study suggested that DNA-PKcs and PARP may function coordinately in vivo in response to DNA damage (Ruscetti, et al., 1998). In addition, PARP members have important roles in maintenance of genomic stability. One group of scientists reported telomere shortening and telomere fusion phenotype in cells from PARP-1-defective mice (d'Adda di Fagagna, et al., 1999). Moreover, Lindahl *et al.* suggested that PARP-1 has a protective role against

recombination (Lindahl, et al., 1995). Cells with non-functional PARP-1 show increased levels of sister chromatid exchange (SCE), suggesting a hyper recombination phenotype in these cells (Bailey, et al., 2004a; Meyer, et al., 2000; Schwartz and Weichselbaum, 1984).

However, the mechanisms underlying tankyrase 1 involvement in DNA damage response and repair are still unknown. Our work should expand our understanding of the network of tankyrase-mediated biological processes.

4. Study goals

The overall goal of this study is to produce deficiencies of critical DSB repair components and examine the subsequent phenotypic consequences, both in directly irradiated and bystander cells. I have used small interfering RNA (siRNA) to knockdown gene products known to be involved in the two major pathways for DSB repair, namely homologous recombination (HR) or nonhomologous DNA end-joining (NHEJ), and then examined the consequences of the resulting perturbations of DSB repair on radiosensitivity and radiation-induced mutagenesis both in directly irradiated and bystander cells. I have extended this examination to include different genetic backgrounds, specifically different status of p53 due to the fact that p53 is involved in both DSB repair and mediates cell killing and growth in bystander cells.

I also have broadened my investigation to examine the effects of suppressing expression of critical telomere maintenance proteins on the processes of DNA damage repair. The studies of others have shown that telomere-associated proteins are involved in DNA damage response/repair. My results show that tankyrase 1, a telomere-associated

protein, may influence DSB repair by mediating levels of DNA-PKcs protein.

CHAPTER 2

Rad54 knockdown by small interfering RNA increases radiosensitivity in human lymphoblastoid cells with mutated p53 but does not affect cells with wild-type p53 and null p53

Introduction

Ionizing radiation (IR) introduces DNA double strand breaks (DSB) to irradiated cells that are likely to cause cell death if not properly repaired (Jackson, 2002). Therefore, radiotherapy is widely used for the treatment of many malignant cancers. However, cancers are often resistant to radiotherapy, and that is usually the cause of treatment failure. In response to IR-induced DSB, mammalian cells have evolved efficient repair systems, namely non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ, an error-prone process, rejoins the broken DNA ends together without a requirement for the presence of homologous sequences in the genome and is usually active through the cell cycle (Jackson, 2002). Unlike NHEJ, which simply rejoins two broken ends, HR utilizes a homologous sequence as a template to repair DNA damage. HR is most active in late S/G2 when sister chromatids are available (Weinstock, et al., 2006b).

The highly conserved Rad54 is a member of the SWIZ/SNF2 family of helicase-like proteins. It contains an evolutionarily conserved DNA-dependent ATPase motif (Eisen, et al., 1995) and plays an essential role in repairing DNA damage through HR. Rad54

protein uses the energy of ATP hydrolysis to translocate on dsDNA at an early step in the repair process, thus inducing topological changes in DNA structure and an opening of DNA duplex strands, which is indispensable for D-loop formation during HR (Kiianitsa, et al., 2002; Petukhova, et al., 1998; Sigurdsson, et al., 2002; Swagemakers, et al., 1998). Moreover, binding of Rad54 to the Rad51–ssDNA nucleoprotein filament substantially increases its stability (Heyer, et al., 2006; Mazin, et al., 2000a; Mazin, et al., 2000b; Solinger, et al., 2002; Solinger, et al., 2001). At later stages of HR, DNA branch-migration activity of Rad54 helps to resolve Holliday junctions (Bugreev, et al., 2006; Solinger, et al., 2002). Phenotypic studies have also shown the important role of Rad54 in repairing DSB. RAD54-deficient mouse embryonic stem (ES) cells are two-to-four fold more sensitive to IR, and HR activity is five-to-ten fold reduced compared to wild-type cells. Homozygous *RAD54*$^{-/-}$mutant clones in the chicken B cell line DT40 were highly X-ray sensitive compared to wild-type cells and reexpression of the *RAD54* cDNA restored radiation resistance and targeted integration activity (Bezzubova, et al., 1997). On the other hand, some somatic cells deleted for Rad54 show only a slight increase in radiosensitivity (Essers, et al., 1997; Essers, et al., 2000; Takata, et al., 1998), so perhaps HR varies in its importance among different cell types. Evidence showed that Rad54 alterations sometimes may be involved in carcinogenesis. Out of 132 primary tumors examined, three mutations in rad54 have been identified, with one each in a breast tumor, a colon cancer, and a lymphoma (Matsuda, et al., 1999). Another study has shown that loss of heterozygosity of *RAD54* is observed in breast cancer (Rasio, et al., 1997).

The p53 tumor suppressor gene has a variety of biological activities, including critical roles in cell cycle regulation, apoptosis, maintenance of genetic integrity of cells, control

of angiogenesis, and senescence. Mutations in the p53 gene occur in more than 50% of human cancers (Greenblatt, et al., 1994), and are often responsible for cancer resistance to radiotherapy and other DNA-damaging therapeutic agents. It has been suggested that p53 may play a role in DNA repair and the mutation of p53 may alter cellular resistance to γ-radiation induced DNA damage (Lee and Bernstein, 1993).

Reducing the capacity of cancer cells to repair DSBs could sensitize tumors to radiotherapy. Many investigators have used gene therapy strategies to down-regulate or inactivate proteins involved in the repair of DSBs in order to reduce the survival of cancer cells. Even though RAD54 has the potential of being a target for gene therapy aiming to increase the radiosensitivity of cancer cells, no direct experimental evidence is available.

Silencing of gene expression by RNA interference (RNAi) has become a powerful tool for modulating the functions of human genes (Sledz and Williams, 2005; Zhang, et al., 2005b; Zhang, et al., 2006). In brief, introduction of short (19-25 nucleotides) double-stranded RNA induces sequence-specific degradation of the homologous mRNA, and subsequent cessation of protein production. Thus, RNAi has the potential of being used as a promising gene therapy strategy. In this study, by using RNA interference techniques, we knocked down Rad54 in three closely related human lymphoblastoid cells with different p53 status. Our results indicated that Rad54 knockdown preferentially increased IR-induced cell killing in p53 mutated cells but does not affect the cells with wild type p53 and null p53. Furthermore, radiosensitivity of Rad54-deficiency in the p53 mutated cells was evident in the S/G2 phases of the cell cycle but not in G1 phase. The immunoprecipitation result found that mutant p53, as well as wild type p53, associates

with Rad54.

Materials and methods

Cell culture and γ-irradiation. The human B-lymphoblastoid cell lines, TK6, WTK1 and NH32 were derived from the same progenitor, WIL2 (Liber and Thilly, 1982; Xia, et al., 1995). TK6 cells have a wild-type p53, WTK1 cells have a mutant form of p53 (Ile237) and NH32 have null p53. All cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated horse serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cultures were incubated at 37°C in 5% CO2 and 100% humidity and maintained at densities of 1-10x10^5 cells/mL by subculture at regular intervals. γ-irradiations were done at room temperature in a calibrated Mark I ^{137}Cs γ-irradiator (J.L. Shepherd and Associates, Glendale, CA). Log-phase cells were irradiated at 1 x 10^6 per mL in 10 mL of growth medium in T-25 flasks with doses of 1 to 3 Gy. Dose rates ranged from 0.20 to 0.25 Gy/min.

Small interfering RNA transfection. The siRNA sequence (leading strand) used for *Rad54* gene silencing was: r(GCCGUAGCAGUGACAAAGU)d(TT). Searches of the human genome database (BLAST) were carried out to ensure that the sequences would not target other gene transcripts. Transfections were done according to the instructions of the supplier (Qiagen, Valencia, CA), in serum-free RPMI 1640 at a density of 8 x 10^5 cells/mL. The concentration of siRNAs were 20 nmol/L in transfections, which was prepared in the form of siRNA/LipofectAMINE2000 (Invitrogen, Carlsbad, CA) complexes (1:1.5). Horse serum was added to a final concentration of 10% 6 h later. We carried out tandem transfections; cells were given two transfections on successive days to

produce maximal knockdown, as described previously (Zhang, et al., 2005b; Zhang, et al., 2006).Transfected cells were collected daily to determine levels of Rad54 by both Western Blot and immunocytochemistry.

Western blots and immunocytochemistry assay. Cells (6×10^6) were harvested, washed with cold PBS and lysed in 200 μL ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA (pH 8.0)] containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.1% leupeptin, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride) on ice for 15 min. Following centrifugation, supernatants containing protein were collected. Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Cell lysates were loaded and electrophoresed on 10% SDS polyacrylamide gels. After wet-blotting to nitrocellulose, protein levels were analyzed using the corresponding primary rabbit polyclonal antibodies Rad54 (Santa cruz biotechnology), p53 (Calbiochem), ß-actin (Abcam), then horseradish peroxidase–conjugated goat anti-rabbit IgG (Promega) or goat anti-mouse IgG (Promega) were used as secondary antibodies. The immunoreactive bands were visualized by chemiluminescence (ECL kit; Amersham Pharmacia) on X-ray films. For the immunofluorescence assay, cells were centrifuged onto slides and then fixed with 4% paraformaldehyde at 4°C for 10 minutes. After permeabilization with 0.2% Triton X-100 in PBS for 5 minutes, cells were incubated with Rad54 antibody (Scbt) for 2 hours at romm temperature, rince with PBS for three times, and then incubated with FITC-conjugated goat anti-rabbit IgG (Oncogene) for 1 hour at room temperature. Slides were examined by fluorescence microscopy.

Cell survival. Cells were irradiated with a single dose of 0 to 3 Gy as described above. Immediately after irradiation, cells were seeded in 96-well plates at densities of 1-50 cells/well, depending on the radiation doses, and incubated at 37°C for 11 days to allow colony formation. Plating efficiencies were determined as previously described (Furth, et al., 1981) and surviving fractions calculated by normalizing the plating efficiencies of treated cells to those of untreated cells.

DNA labeling and cell sorting. Appropriate numbers of cells were inoculated into T25 flasks 22-26 hrs before DNA labeling so that exponentially growing cells were present at the time of labeling. Cells were labeled with Hoechst 33342, an A-T rich region specific DNA dye (Sigma Chemical Co., St. Louis, MO). The stock solution of 1 mM Hoechst 33342 in deionized water was stored at 4°C until use. Hoechst 33342 was added to the exponentially growing cells in the flask at a final concentration of 10 μM in the medium. After 1.5 hr of incubation at 37 °C, cells were collected, centrifuged and resuspended with ice-cold PBS to minimize the leaking of the dye. All cell samples were analyzed and sorted with a Coulter EPICS V cell sorter (Coulter, Miami, FL) interfaced to a Cicero data acquisition and display system DakoCytomation, Inc., Fort Collins, CO) using 200 mW UV line. The blue fluorescence was measured between 457 and 497 nm. Fluorescence histograms were gated on forward angle light scattering to exclude debris and clumped cells. Gating on peak versus integral fluorescence of the Hoechst 33342 signal was set to eliminate clumped cells. G1 and S/G2 cells in the cell cycle histogram were sorted into the collection tube and used immediately.

Immunoprecipitation. Cells were lysed in 200 μL ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-

100, 5 mmol/L EDTA (pH 8.0)] containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.1% leupeptin, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride) and protein concentrations were determined. Samples were incubated overnight at 4°C with the indicated primary Rad54 antibody (Scbt) or an equivalent amount of the appropriate control (mouse IgG) (Ams Biotechnology). Protein complexes were then precipitated with agarose- or Sepharose-conjugated protein G, and resolved using SDS-PAGE. Transfer of proteins to nitrocellulose filters and western blotting were performed using the ECL detection systerm (Amersham) according to the protocol of the manufacturer. Anti-p53 antibody and protein A/protein G agarose were obtained from Ams Biotechnology. Experiments were repeated at least twice, and representative blots are shown.

Results

Suppression of Rad54 protein expression by small interfering RNA in TK6, WTK1 and NH32 cells. In TK6, WTK1 and NH32 cells, three days after initial siRNA transfection, western blots showed that the levels of Rad54 were reduced obviously compared with mock-transfected cells (Fig. 1A). An immunofluorescence assay also showed a similar result; Rad54 protein was barely detectable in siRNA-transfected TK6, WTK1 and NH32 cells compared with mock-transfected cells (Fig. 1B). Therefore, we collected cells at 3 days after initial transfection to perform the cytotoxicity assays described below.

Rad54-targeted siRNA transfection increased radiosensitivity in p53 mutated WTK1 cells but not in p53 wild type TK6 or p53 null NH32 cells. TK6, WTK1 and

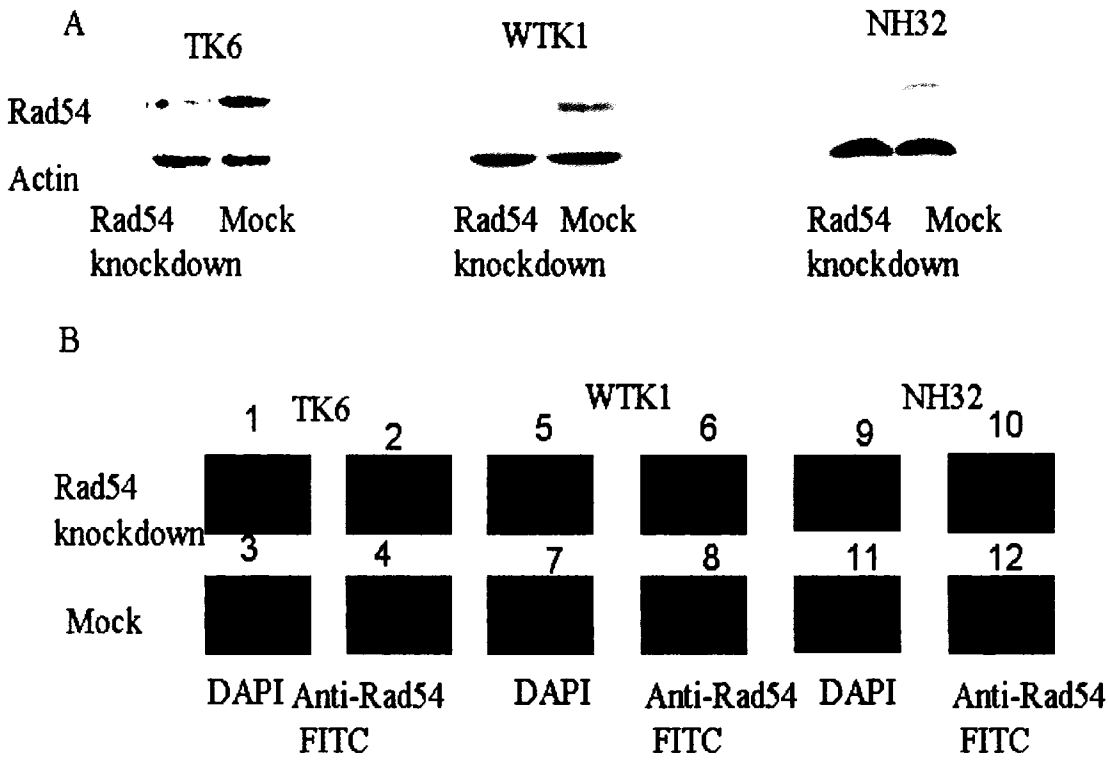

Figure 1. Expression of Rad54 in TK6, WTK1 and NH32 cells after Rad54 siRNA knockdown on day 3 after initial transfection. A, western blots from TK6 (left panel), WTK1 (middle panel) and NH32 cells (right panel). Upper bands were probed with anti-Rad54 antibody and lower bands are actin loading controls. B. Immunocytochemistry images from TK6 (left panel), WTK1 (middle panel) and NH32 cells (right panel) after Rad54 siRNA knockdown on day 3 after initial transfection. Slides were stained with DAPI, probed with anti-Rad54 antibody, and a secondary FITC-conjugated antibody. Panels 3, 4, 7, 8, 11 and 12 are mock-transfected cells; 1, 2, 5, 6, 9 and 10 are siRNA-transfected cells; 1, 3 5, 7, 9 and 11 are the slides under DAPI filter to identify nuclei; 2, 4, 6, 8, 10 and 12 are the same fields under FITC filter to detect Rad54 protein level.

NH32 cells were γ-irradiated with 0-3 Gy on day 3 after the initial Rad54 siRNA transfection. The surviving fractions were determined by colony forming assay (Fig. 2). By using a regression model in S-plus software, we compared the slopes of the survival curves of mock-transfected cells and Rad54 siRNA-transfected cells. The log-transformed surviving fraction showed a reasonable linear fit with the radiation dose. As shown in Fig. 2A, the slopes for mock-transfected sets and Rad54 siRNA-transfected sets were not different for TK6; The calculated D_0's (dose that reduces survival to 37% in the linear portion of the curve) for mock- and siRNA-transfected TK6 cells were 0.56 and 0.54 Gy, respectively. This experiment showed that there was no effect of Rad54 knockdown on cell killing in TK6. Similarly, as shown in Fig. 2C, the slopes for mock-transfected and Rad54 siRNA-transfected sets were not different for NH32; The calculated D_0's for mock- and siRNA-transfected NH32 cells were 0.62 and 0.59 Gy and again showed that there was no effect of Rad54 knockdown on cell killing in NH32. However, Rad54 knockdown did increase the radiosensitivity of WTK1 cells compared with those in the mock-transfected cells. As shown in Fig. 2B, in WTK1 the slopes for mock-transfected sets and Rad54 siRNA-transfected sets were significantly different. The D_0 values for mock- and Rad54 siRNA-transfected cells were 1.13 and 0.67Gy, respectively ($p=0.03$). Overall, our results suggest that Rad54 knockdown preferentially increased radiosensitivity in p53 mutated but not in p53 wild type and p53 null human lymphoblastoid cells.

The increased radiosensitivity of Rad54-deficiency in WTK1 cells was evident in S/G2 phase, but not in G1 phase. It is well accepted that cell cycle position has a substantial influence on radiosensitivity, that is, cells in G1 and S phases are more

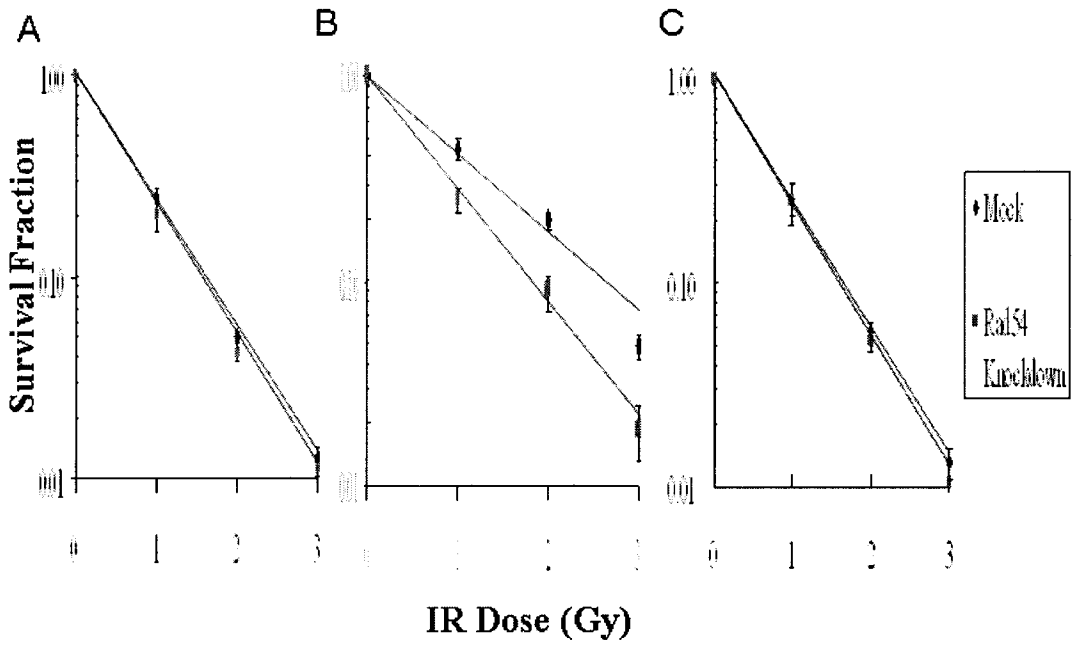

Figure 2. Radiation-induced cell killing in TK6 (A), WTK1 (B) and NH32 (C) cells after Rad54 siRNA knockdown. On day 3 after initial transfection, cells were seeded in 96-well plates, immediately after γ-radiation over a range of 0-3 Gy. Points, average of three experiments; bars, SD. Mock transfection (♦), Rad54 siRNA transfection (■).

resistant to IR-induced cell killing than G2 phase. To further examine how Rad54 siRNA knockdown radiosensitized WTK1 cells varied with respect to cell cycle, mock-transfected and Rad54 siRNA-transfected WTK1 cells were sorted into G1 and S/G2 phases by flow cytometry immediately after γ-irradiation on day 3 after initial transfection and subjected to the colony forming assay (Fig. 3). As shown in Fig. 3A, the slopes of survival curves for mock-transfected and Rad54 siRNA-transfected sets were not different for G1 phase; the calculated D_0's for mock- and siRNA-transfected G1 phase WTK1 cells were 1.1 and 1.09 Gy, indicating that there was no effect of Rad54 knockdown on cell killing in G1 phase. However, in S/G2 phase (Fig. 3B), the slopes for mock-transfected sets and Rad54 siRNA-transfected sets were markedly different; the calculated D_0's for mock- and siRNA-transfected were 1.19 and 0.72 Gy, respectively, with $p=0.02$. These results suggest that the radiosensitivity of Rad54-deficiency in WTK1 cells was evident in S/G2 phases of the cell cycle but not in G1 phase.

Rad54 associates with wild-type p53 in TK6 cells as well as mutated p53 in WTK1 cells. It has been reported that wild type p53 associates with Rad54 (Linke et al., 2003). We examined whether wild type p53 in TK6 cells and mutant p53 in WTK1 cells could interact with Rad54. In TK6 cells, the level of wild-type p53 increased 2 h after treatment with 10 Gy IR, and remained elevated for at least 18 h after irradiation. With Rad54 IP, we found that more p53 was associated with Rad54 at both 12 h and 18 h after irradiation than after no treatment (Fig. 4A). The basal level of mutant p53 is high in WTK1 cells, and there was no sign of IR induction, reflecting the mutant status of p53 (no wild type p53 exists in WTK1 cells, and therefore the western blot for WTK1 cells represents only mutant p53). We found that mutant p53 in WTK1 cells also associated with Rad54. In

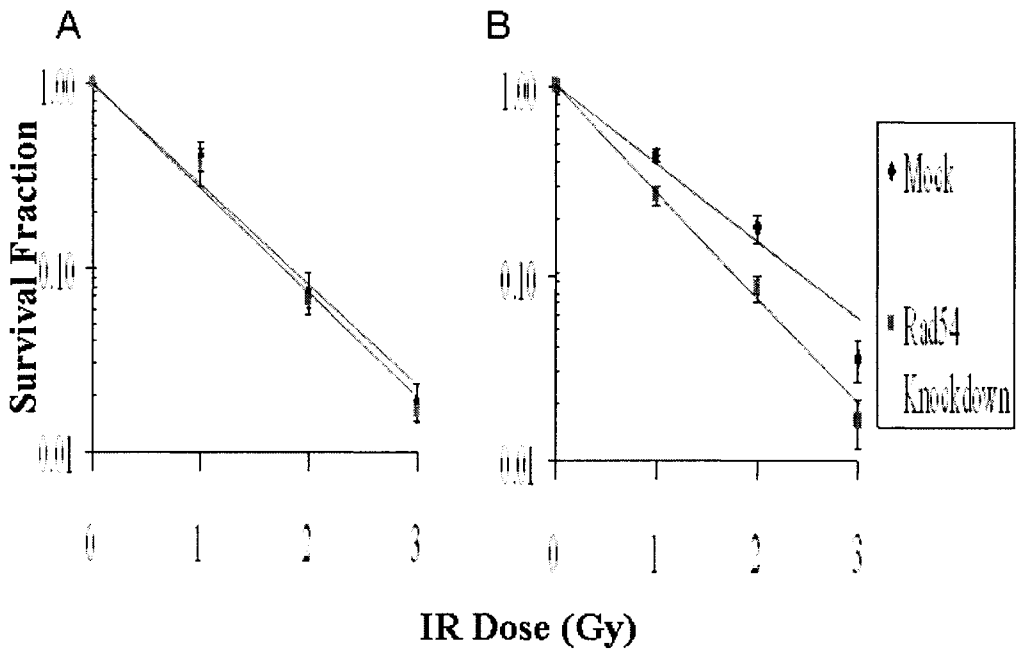

Figure 3. Radiation-induced cell killing in G1 (A) and S/G2 (B) phases of WTK1 cells after Rad54 knockdown. On day 3 after initial transfection, cells were sorted into G1 and S/G2 phases by flow cytometry right after γ-radiation over a range of 0-3 Gy. Points, average of three experiments; bars, SD. Mock transfection (♦), Rad54 siRNA transfection (■).

contrast to TK6 cells, the amount of Rad54 –associated p53 did not change after IR (Fig. 4B). Our results indicate that mutant p53 could modulate HR repair through interacting with Rad54 and the different outcome suggest that mutant p53 may cause dysfunctional HR through the interaction.

Discussion

Disruption of factors involved in the detection, signaling, and repair of DNA damage after exposure to IR are attractive means to increase cellular sensitivity to improve radiotherapy. The pivotal role of Rad54 in repairing DSBs through the HR pathway points to it as a promising candidate for targeted disruption. However, cancer cells could demonstrate less radiosensitivity than normal cells due to their different genetic background. Mutations in the tumor suppressor p53 have been demonstrated in more than 50% of human malignancies, most commonly in lung cancer, head and neck cancer, and colorectal cancer, but in other cancers as well (Weller, 1998). p53 functions in cell cycle checkpoint control and the induction of apoptosis (Helton and Chen, 2006). p53 is also involved in spontaneous and DSB-triggered HR, and it contributes as well to nucleotide excision, base excision and mismatch repair (Ford, 2005; Gatz and Wiesmuller, 2006). In addition, p53 has been implicated in mediating cell killing (Camphausen, et al., 2003; Matsumoto, et al., 2001; Matsumoto, et al., 2000), cell growth (Komarova, et al., 1998), and p21 activation. In most cancer cells, p53 mutations have been linked to a more aggressive tumor phenotype and a poorer prognosis due to increased resistance to chemotherapy and radiation therapy (Gallagher and Brown, 1999). Specific examples include Burkitt's lymphoma (Fan, et al., 1994), astrocytoma (Iwadate, et al., 1998),

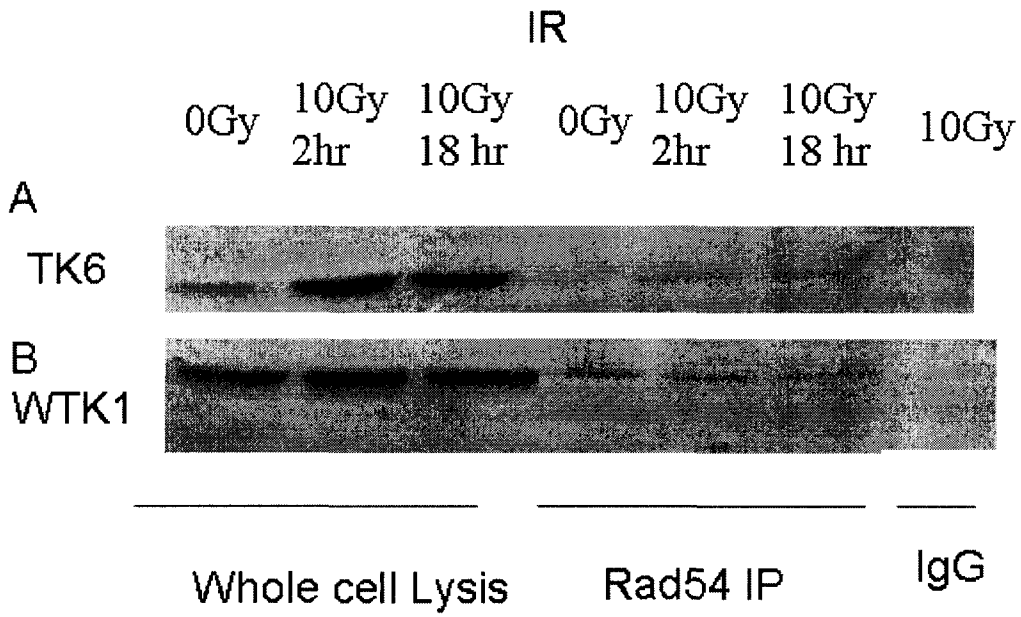

Figure 4. Rad54 associates with wild-type p53 in TK6 cells and mutant p53 in WTK1 cells. Both TK6 (A) and WTK1 (B) cells were treated with γ-radiation at 10 Gy. The lysates (200 μg) were immunoprecipitated with anti-Rad54 or rabbit IgG and then run on a gel along with 40 μg of immunoprecipitation supernants. The blots were probed with anti-p53.

human glioma (Hirose, et al., 2001), and melanoma (Li, et al., 1998a). It has been reported that human ovarian cancer cells transfected with a dominant negative mutant p53 gene became resistant to IR, cisplatin, doxorubicin and 1-beta-D-arabinofuranosylcytosine (Vasey, et al., 1996). The sensitivity of colorectal cancer cells to agents such as 5-fluorouracil is also decreased by a loss of p53-mediated apoptosis (Bunz, et al., 1999). p53 mutation was also associated with early metastasis and decreased response to hormone therapy in patients with prostate cancer (Grignon, et al., 1997). Taken together, strategies to increase the sensitivity of cancer cells with p53 mutation will be important for improving the efficacy of cancer therapy.

Previous studies showed that two human lymphoblastoid cell lines- TK6 and WTK1 derived from a single donor differ in p53 status and have remarkably different phenotypes. WTK1 was much more sensitive to spontaneously arising and IR-induced mutagenesis. Compared to TK6, WTK1 had a 25-fold higher spontaneous mutation rate, and an approximately 20-fold increased sensitivity to 1.5 Gy X-ray-induced mutation at the TK locus (Amundson, et al., 1993). In addition, WTK1 had a 7-fold greater capacity to catalyze intermolecular recombination (Xia, et al., 1994). There were also significant differences in the mutational spectra observed. WTK1 had a higher proportion of large-scale genetic changes than TK6, and the sizes of these changes were more extensive (Xia, et al., 1994), which is consistent with the high level of HR capacity observed in WTK1 cells. Moreover, after transfection and subsequent overexpression of the known dominant negative mutant p53 ($p53Ile^{237}$), which is the same mutation harbored in WTK1 line, we observed significantly enhanced spontaneous and X-ray-induced mutant frequencies at the TK locus (Xia and Liber, 1997). The different HR activity in TK6 and WTK1 cells is

consistent with the role of p53 in DSB repair; wild type p53 suppresses HR (Romanova, et al., 2004) while mutant p53 promotes HR (Xia, et al., 1994). Consequently, HR mediated DSB repair could be a more important pathway in WTK1 than in TK6.

In this study, we used siRNA transfection to silence expression of Rad54 protein in three related human lymphoblastoids with different p53 status and examined IR–induced cell killing. After Rad54 knockdown, we found that TK6 cells with a wild-type p53 and NH32 with null p53 showed no increased sensitivity to IR–induced killing, whereas WTK1 cells with a mutant p53 showed an increased sensitivity (Fig. 2). We believe that the different response to IR-induced cell killing is related to p53 related different DSB repair status in these cell lines.

Rad54 is an important factor in the HR pathway and influence of deficiency of HR by knocking down Rad54 could be more efficient in killing WTK1 cells than TK6 (Fig. 2B). NH32 is a p53 knockout cell line derived from TK6. NH32 did not show much increased levels of IR-induced mutagenesis and cell killing compared to TK6 (Chuang, et al., 1999) and it is reasonable to speculate that the DSB repair status in two lines could also be similar. Similarly as in TK6 cells, our results showed that deficiency of Rad54 in NH32 cells did not increase the radiosensitivity (Fig. 2C), which is consistent with the gain-of-function, but not loss-of-function of p53 in IR-induced cell killing.

Results in Fig. 3 support the view that Rad54 knock down leads to increased cell killing via a HR deficiency. We sorted WTK1 cells into G1 and S/G2 phases by flow cytometry and measured their radiosensitivity. The cell cycle distribution in mock transfected cells was the same as in Rad54 siRNA knockdown cells, which suggested that Rad54 knock down did not radiosensitize cells via cell cycle redistribution (data not

shown). Our results showed that the radiosensitivity of Rad54-deficiency in WTK1 cells was evident in S/G2 but not in G1 phase (Fig. 3), which was consistent with the role of Rad54 in DSB repair. HR usually utilizes a homologous sequence in the form of sister chromatids as a template to repair DSB. Therefore HR should be most active in late S/G2 phase when sister chromatids are available (Jackson, 2002). Suppression of Rad54 induced radiosensitivity in S/G2 cells but not G1; this is consistent with sister-chromatid involvement in DNA repair. It has been suggested that wild type p53 suppresses HR (Linke, et al., 2003; Romanova, et al., 2004; Xia, et al., 1994). Although the exact biochemical process by which mutant p53 is involved in HR repair is largely unknown, several reports have indicated that expression of the tumor-derived p53 mutants leads to elevated HR (Bertrand, et al., 1997; Saintigny, et al., 2005; Saintigny and Lopez, 2002; Saintigny, et al., 1999).

Wild-type p53 has been shown to interact with Rad54 (Linke, et al., 2003) and we observed similar results in TK6 cells (Fig. 4). For the first time we found that mutant p53 in WTK1 cells also interacts with Rad54 *in situ* (Fig. 4), and this may be responsible for the elevated HR level in WTK1 cells. Thus, disruption of HR factors such as Rad54 that are downstream of mutant p53 could be a promising means to overcome p53 mutation mediated radioresistance.

In summary, for the first time, we demonstrated that Rad54 knockdown preferentially radiosensitized p53 mutated cells but did not affect cells with wild-type p53 and null p53. Importantly, the radiosensitivity of Rad54-deficiency in WTK1 cells was evident in the S/G2 phases of the cell cycle but not in G1 phase. Based on the IP results showing that mutant p53 in WTK1 cells interacts with the HR factor Rad54, it is tempting to speculate

that the mutant p53 may modulate IR-induced cell killing via a gain-of-function but not a loss-of-function model. Since many cancers harbor p53 mutations and demonstrate decreased radiosensitivity, the present results provide a potential direction for clinical cancer radiotherapy aimed to improve the radiosensitivity of cancer cells.

CHAPTER 3

Deficiencies of double strand break repair factors lead to different mutagenesis outcomes in γ-irradiated and bystander human lymphoblastoid cells

Introduction

DNA is an important target of ionizing radiation (IR) induced damage, and genotoxicity can result from either direct energy deposition, or the attack of reactive oxygen species (ROS) generated from radiolysis of water (see review (Slupphaug, et al., 2003)). DNA double strand breaks (DSB) are considered to be the most important DNA lesion for causing cell death, if they remained unrejoined (see review (Jackson, 2002)). Probably more detrimental to organisms are misrepaired DSBs, which can lead to chromosome aberrations, gene mutations and presumably carcinogenesis (Shiloh, 2003). DSBs trigger a DNA damage response, a set of cellular events including DNA repair, cell cycle checkpoints and apoptosis, the latter two either to allow time for the repair of DNA lesions or to eliminate cells with potentially mutagenic lesions (Jackson, 2002). Strict coordination among the components of the DNA damage response is required to decrease the risk of mutagenesis.

Even though it is well accepted that many of the deleterious effects of IR may result from direct damage to cellular DNA, there is also much evidence showing that cells which have not been directly irradiated respond to their exposed neighbors. This phenomenon is termed the bystander response (Nagasawa and Little, 1992; Prise, et al.,

2006). It has been observed using end points including sister chromosomal exchange (SCE) (Nagasawa, et al., 2002; Nagasawa, et al., 2005), chromosomal aberrations (Nagasawa and Little, 2002), cell killing (Mothersill and Seymour, 1997; Mothersill, et al., 2006; Zhu, et al., 2005), gene mutation (Nagasawa, et al., 2003; Persaud, et al., 2005; Zhou, et al., 2000), neoplastic transformation (Sawant, et al., 2001), and changes in gene expression (Azzam, et al., 1998; Azzam, et al., 2001). All of these endpoints are consistent with the presence of DNA damage in bystander cells. Since such damage in bystander cells is likely to be potentially tumorigenic (Sawant, et al., 2001), understanding the bystander phenomenon is of extreme importance to risk estimation, radiation protection and cancer therapy. The basic mechanisms by which damage signals are transmitted from irradiated to unirradiated cells, as well as what factors (both in irradiated and unirradiated cells) influence the bystander response, are beginning to emerge. Increasing experimental evidence indicates that ROS and nitric oxide may be involved in the DNA damage observed in bystander cells (Azzam, et al., 2003; Azzam, et al., 2002; Bishayee, et al., 2001; Lorimore, et al., 2001; Lyng, et al., 2000), and also that gap junctions between irradiated and unirradiated cells can be important (Azzam, et al., 2001; Zhou, et al., 2000). On the other hand, membrane signaling has been implicated in the induction of SCE, micronuclei formation, and gene mutation in bystander cells (Nagasawa, et al., 2002).

The p53 tumor suppressor protein functions in cell cycle checkpoint control and the induction of apoptosis (see review (Helton and Chen, 2006)). p53 is also involved in spontaneous and DSB-triggered HR, and it contributes as well to nucleotide excision, base excision and mismatch repair (Ford, 2005; Gatz and Wiesmuller, 2006). Three

closely related human lymphoblastoid lines with different p53 status have been used extensively to examine the role of p53 in radiation mutagenesis; either the mutated p53 in WTK1 cells, or the null mutation in NH32 cells, results in an increased radiation-induced mutant fraction at the *tk* locus, compared with p53 wild type TK6 cells (Peng, et al., 2002; Zhang, et al., 2005a). In addition, p53 has been implicated in mediating cell killing (Camphausen, et al., 2003; Matsumoto, et al., 2001; Matsumoto, et al., 2000), cell growth (Komarova, et al., 1998), and p21 activation. However, the effect of p53 status on IR-induced bystander mutagenesis has not been reported. p53 has also been suggested to play a role by inducing export of growth suppressive stimuli from damaged cells to neighboring cells (Komarova, et al., 1998). An interesting report described a p53-mediated abscopal antitumor effect; the observation was made in mice that localized irradiation resulted in a slower growth rate of tumors distant to the irradiated site (Camphausen, et al., 2003).

Since DNA DSBs have been found in bystander cells, recent studies have addressed the involvement of DSB repair factors in the bystander response (Nagasawa, et al., 2003; Nagasawa and Little, 2002; Nagasawa, et al., 2005; Yang, et al., 2005). Conditioned medium harvested from irradiated DNA repair deficient cells has been demonstrated to be more toxic than the medium harvested from repair proficient cells, as measured by IR induced cell killing in recipient cells (Mothersill, et al., 2006). Mouse embryonic cells with a deficiency in the radioresistance gene Rad9 were more sensitive to IR-induced bystander apoptosis and micronucleus formation, but not cell killing (Zhu, et al., 2005); this suggested that DNA repair factors may have different effects on individual bystander response end points. The NHEJ pathway has been suggested to play a role in low fluence

α-particle and targeted soft x-rays induced bystander effects in Chinese hamster cells, as measured by cell killing, micronuclei, chromosome aberrations and SCE (Kashino, et al., 2004; Little, et al., 2003; Nagasawa, et al., 2005).

Incorrectly repaired DSBs result in gene mutation, and therefore it is important to understand the influence of DNA repair factors on mutagenesis. In mammalian cells, DSBs can be repaired by two recombinational pathways, NHEJ and HR. NHEJ is facilitated by DNA-dependent protein kinase (DNA-PK), which is composed of a catalytic subunit, DNA-PKcs, and a hetrodimeric DNA binding regulatory complex Ku (Collis, et al., 2005). DNA-PKcs is recruited to the broken ends of DSBs by Ku and functions in phosphorylating a set of molecules in the NHEJ pathway. HR restores the continuity of DSB by using an intact and homologous DNA as a template. During HR, the damaged DNA ends are processed into single-stranded DNA tails with 3' extensions and enter into synapsis with an undamaged DNA molecule with which the damaged DNA shares extensive sequence homology. After DNA synthesis using the homologous DNA as a template, followed by branch migration, Holliday junction resolution, and DNA ligation, the damaged DNA is restored to exactly the same nucleotide sequence as the homologous template (Iliakis, et al., 2004). Rad54, a DNA-dependent ATPase with nucleosome remodeling activity (Swagemakers, et al., 1998), stabilizes the binding of Rad51 (another key factor in HR) to single-stranded DNA, thus facilitating homologous sequence searching (Tan, et al., 2003). Rad54 also functions in DNA duplex opening, and heteroduplex extension in HR process (Tan, et al., 2003). Disruption of either DNA-PKcs or Rad54 results in DNA repair deficiency in mice (Collis, et al., 2005; Essers, et al., 1997), and both DNA-PKcs and Rad54 could play important roles in mediating

mutagenesis in human cells. Direct evidence of DNA-PKcs involvement in mutagenesis has been presented in our previous work (Peng, et al., 2002). On the other hand, no experimental evidence is available to elucidate the role of Rad54 in mutagenesis in human cells. p53 has also been implicated in modulating mutagenesis; p53 mutations lead to a hypermutable phenotype in human cells (Wiese, et al., 2001; Xia, et al., 1994; Xia and Liber, 1997).

Although the influence of DSB repair factors and p53 in IR-induced mutagenesis are beginning to be understood, little is known about their effect in bystander cells. Mutational spectra analyses at the hypoxanthine-guanine phosphoribosyltransferase *(hprt)* locus have shown that mutations induced in DNA repair proficient bystander cells were mainly point mutations; this is in contrast to directly irradiated cells, in which total and partial gene deletions predominate (Huo, et al., 2001). In addition, the mutational spectra in DSB repair deficient bystander cells (XRS-5, Chinese hamster ovary cells with a Ku80 deficiency, presumably NHEJ deficient) were predominantly partial or complete deletions (Huo, et al., 2001; Nagasawa, et al., 2003). Since gene mutations frequently result from processing by DSB repair mechanism, these results suggest a different role of DSB repair factors in irradiated and bystander cells.

I hypothesized that the status of DSB repair could have different effects on mutagenesis in irradiated and unirradiated bystander cells. In this study, I used three human lymphoblastoidoid cell lines from the same donor that differ in p53 status (wild-type, null, and mutant p53), in combination with RNA interference techniques to knockdown key factors in the DSB repair pathways (DNA-PKcs for NHEJ and Rad54 for HR), to investigate the influence of DNA DSB repair on mutagenesis both in irradiated

and bystander cells.

Materials and methods

Cell culture and γ-rays irradiation. The human B-lymphoblastoid cell lines TK6, NH32, and WTK1 were derived from the same progenitor, WIL2. They differ in p53 status; TK6 has wild type p53, WTK1 has mutant p53 (ile237), and NH32 has null p53 (Chuang, et al., 1999; Liber and Thilly, 1982; Xia, et al., 1995). All cell lines were maintained in RPMI 1640 medium supplemented with 10% horse serum, 100 U/ml penicillin, and 100 g/ml streptomycin. γ-irradiations were performed at room temperature in a calibrated Mark I 137Cs γ-irradiator (J. L. Shepherd and Associates, Glendale, CA). Log-phase cells were irradiated in 6-well dishes (Becton Dickinson Labware, Franklin Lakes, NJ) with 2 Gy at a dose rate of 3.9 Gy/min. As reported previously (Yang, et al., 2005), we used cell culture transwell inserts (Becton Dickinson Labware, Franklin Lakes, NJ) to study the bystander effect of mutagenesis; the bottom of the insert is a membrane with 1.0 μm pores at a density of $1.6\times10^6/cm^2$ to allow the passage of molecules (Figure 1). Immediately after irradiation, cell culture insert dishes with 5 mL unirradiated cells at a density of 4×10^5/mL were placed into the 6-well dishes for 1 day. The cells then were harvested into T-75 flasks and allowed another 2 days culture in normal media before mutant fractions (MF) were determined.

Small interfering RNA transfection. The siRNA sequences used for gene silencing were (leading strand): r(GGCGUGUCAGUUGAUGAAA)d(TT) for *DNA-PKcs* and r(GCCGUAGCAGUGACAAAGU)d(TT) for *Rad54*. BLAST searches of the human genome database were done to ensure that the sequences would not target other gene

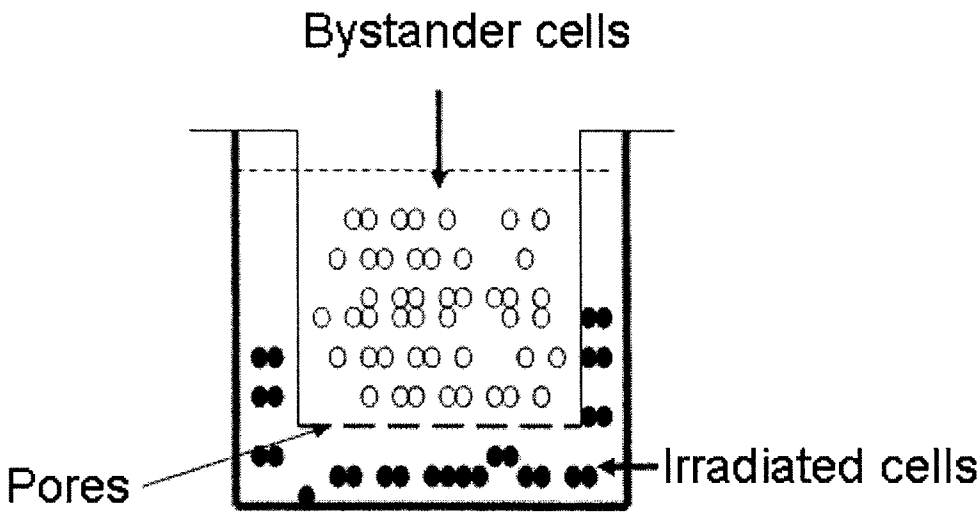

Figure 1. The transwell insert culture system to study the bystander effect on mutagenesis. Cells can be cultured in two compartments without mixing to facilitate further independent analysis. The bystander and irradiated cells are separated by a membrane with 1.0 μm pores which prevents the mixture of cells but allows the free passage of molecules between the two cell compartments.

transcripts. Transfections were done according to the instructions of the supplier (Qiagen, Valencia, CA), in serum-free RPMI 1640 at a density of 8 x 10^5 cells/mL. The concentrations of siRNAs in transfections were 20 nmol/L, and they were prepared in the form of siRNA/LipofectAMINE2000 (Invitrogen, Carlsbad, CA) complexes (1:1.5). Horse serum was added to a final concentration of 10% 6 hours later. We carried out tandem transfections; cells were given two transfections on successive days to produce maximal knockdown, as described previously (Zhang, et al., 2005b; Zhang, et al., 2006). Cell samples were collected and analyzed by Western blotting to measure the efficiencies of the knockdowns.

Western blots. Cells (6 x 10^6) were lysed in 200 μL ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA (pH 8.0)] containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.1% leupeptin, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride). Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Cell lysates were loaded and electrophoresed on SDS polyacrylamide gels. After wet-blotting to nitrocellulose, protein levels were analyzed using the corresponding primary antibodies to DNA-PKcs(Neomarkers, Fremont, CA), Rad54(Scbt, Santa Cruz, CA), and ß-actin (Abcam, Cambridge, MA), followed by horseradish peroxidase–conjugated goat anti-mouse IgG (Abcam, Cambridge, MA). The immunoreactive bands were visualized by chemiluminescence on X-ray films. Protein levels were quantified using ImageQuant V5.1 software (Molecular Dynamics, Piscataway, NJ) and normalized to the loading controls.

Mutagenesis studies at the *tk* locus. We used standard procedures to determine the MF at the *tk* locus (Liber and Thilly, 1982). Briefly, cells were treated with CHAT (deoxycytidine, hypoxanthine, aminopterin and thymidine) medium for 2 days to reduce $tk^{-/-}$ backgrounds, and then allowed to recover for 1 day in THC (deoxycytidine, hypoxanthine, and thymidine) medium. For both irradiated and bystander samples, the cells were incubated for 3 days in normal medium to allow for phenotypic expression of mutants. Cells then were plated in the presence of 2 μg/mL trifluorothymidine for $tk^{-/-}$ mutant selection; mutants survive in the presence of this selective agent and form colonies. These colonies were scored after 20 days incubation. Plating efficiencies were calculated using the Poisson distribution; specifically, $Po = e^{-x}$, where x is the average number of colony-forming cells per well and Po is the observed fraction of negative wells. The MF is the ratio of plating efficiency in the presence of selective agent to that in its absence.

Statistical Analysis. Statistical analysis was done on the means of the data obtained from three independent experiments. All results were presented as means ± SD. Significance was assessed by using student's t test at $p < 0.05$.

Results

siRNA transfection reduces levels of DNA-PKcs and Rad54. WTK1, TK6, and NH32 cells were transfected with siRNAs for either DNA-PKcs, or Rad54 by the tandem protocol described in Materials and Methods. Western blotting confirmed that these treatments were quite effective at reducing the levels of protein. At either 5 days after initial transfection for DNA-PKcs, or 3 days for Rad54, the protein levels were barely

detectable (Figure 2). Therefore, on either the fifth day after DNA-PKcs siRNA transfection, or the third day after Rad54 siRNA transfection, we initiated the mutagenesis experiments described below.

p53, both in irradiated and bystander cells, is not required for the induction of bystander mutagenesis. We studied bystander mutagenesis in three human lymphoblastoidoid cell lines with different p53 status. Each of the cell lines was used as the irradiated cells receiving 2 Gy of γ-rays, or as the bystander cells. The data are presented in Figure 3.

The incubation of WTK1 cells with unirradiated WTK1, TK6, or NH32 did not alter the background mutant fractions (BMF) in bystander cells. The observed BMFs in WTK1 cells were within the normal range and no statistical differences were found among those co-cultured with unirradiated WTK1, TK6, and NH32 cells ($p>0.05$). The same was true for TK6 and NH32 cells when co-cultured with unirradiated WTK1, TK6, and NH32 cells ($p>0.05$). However, co-culture with directly irradiated cells led to increases in MFs of 1.5-3-fold in all three cell lines, but p53 did not appear to be a significant factor. For WTK1 as the bystander cells (Figure 3a), the MFs after co-culture with irradiated WTK1, TK6, and NH32, showed significant increases comparing to those co-cultured with unirradiated cells; all of these values were significantly different from their respective controls ($p<0.01$), but none were different from one another ($p>0.05$). The same patterns were observed in TK6 (Figure 3b) and NH32 cells (Figure 3c). Co-culture with either irradiated WTK1, or TK6, or NH32 significantly increased the MFs in TK6 bystander cells ($p<0.01$). No statistical differences were found among TK6 bystander cells co-cultured with either irradiated WTK1, or TK6, or NH32 cells. Similar

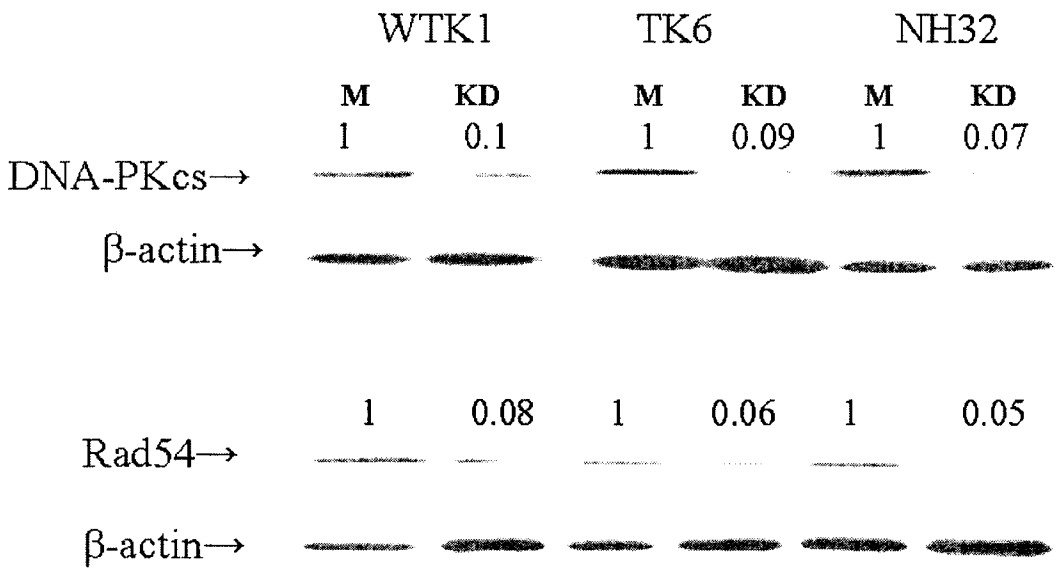

Figure 2. DNA-PKcs and Rad54 knockdown mediated by siRNA transfection.Cells were transfected with siRNA targeted to DNA-PKcs or Rad54 mRNA by the tandem procedures described in Materials and Methods. Cells were harvested five days after the initial transfection with DNA-PKcs siRNA, or at 3 days after initial transfection with Rad54 siRNA. Western blotting assays were performed. β -actin was included as loading controls. The numbers above the bands indicate the ratio of protein levels to those in mock transfected cells, as determined by the ImageQuant system. M: mock tranfected cells, KD: siRNA transfected cells.

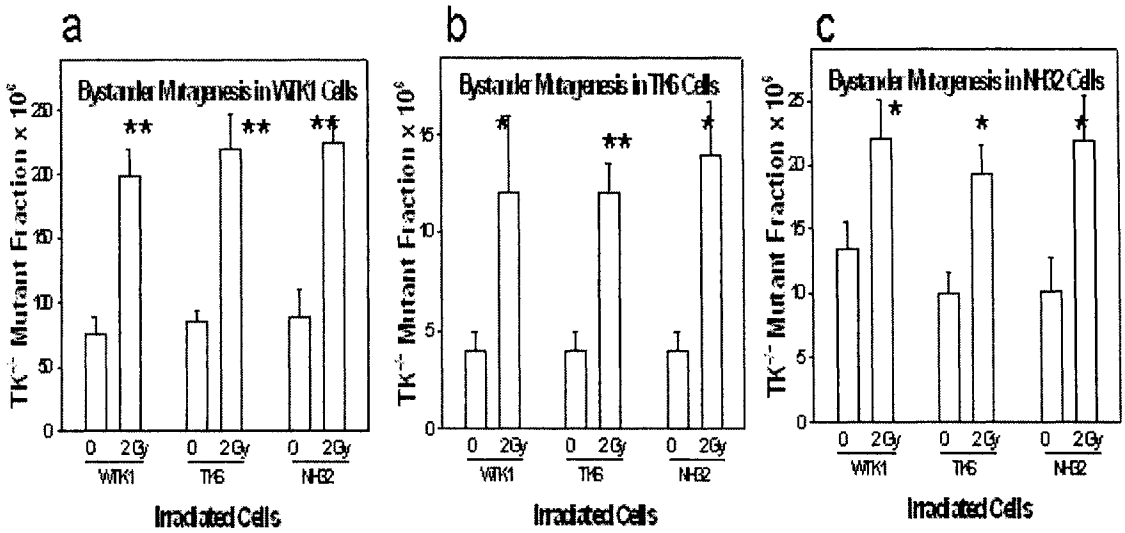

Figure 3. Effects of p53 status on bystander mutagenesis. The bystander mutagenesis was studied by the transwell insert system illustrated in Figure 1. A total of 2 × 106 cells in inserts were placed into 6-well dishes containing either unirradiated or 2 Gy γ-irradiated cells. After 1 day culture in the transwell insert system, the cultures were separated and allowed an additional 2 days culture in T-75 flasks; at this time mutant fractions were determined. WTK1 (p53 mutant), TK6 (p53 wild-type), and NH32 (p53 null) were used either as the irradiated cells or as the bystander target. All 9 combinations are shown. Each panel shows bystander mutagenesis for a particular line, after co-culture with unirradiated or 2 Gy γ-irradiated cells. *Columns* are the average of three experiments, *bars* are SD. (* $p<0.05$, ** $p<0.01$ compared to bystander cells co-cultured with unirradiated cells).

to the observation for WTK1 and TK6 bystander mutagenesis, co-culture with either WTK1, or TK6, or NH32 significantly increased the MFs in NH32 bystander cells ($p<0.01$). No statistical differences were found among NH32 bystander cells co-cultured with either irradiated WTK1, or TK6, or NH32 ($p>0.05$).

The results suggest that IR can induce an obvious bystander mutagenesis effect in co-cultured unirradiated human lymphoblastoidoid cells regardless of the p53 status of either the irradiated or bystander cells. The magnitudes of the MFs in bystander cells were ordered WTK1>NH32>TK6, which is similar to both spontaneous and IR-induced mutant fractions of those cells, and thus reflects the intrinsic characteristics of those cells in their response to mutagenic stresses. Therefore, at least for the endpoint of mutagenesis, p53 is not required to initiate or respond to the bystander signal.

Effect of DNA-PKcs and Rad54 knockdown on mutagenesis in irradiated cells. As can be seen in Figure 4, knockdown of DNA-PKcs or Rad54 did not increase the BMF at the *tk* locus in WTK1, TK6, or NH32 cells ($p>0.05$). However, an approximate 2-fold statistically significant increase of IR-induced MF was observed in WTK1 cells (Figure 4a) after DNA-PKcs knockdown ($p<0.05$). In contrast, the IR-induced MFs in TK6 (Figure 4b) and NH32 cells (Figure 4c) dropped significantly to 66% and 53% of the mock transfected control cells, respectively ($p<0.05$). Rad54 knockdown resulted in significant 1.4-fold increases of IR-induced MF in both WTK1 and NH32 cells ($p<0.05$). However, the MF in TK6 cells was not influenced by Rad54 knockdown ($p>0.05$). The mechanism underlying the differential response to IR-induced mutagenesis among these cell lines has not been proven, but we believe that since all three cell lines are derived from the same donor, the most straightforward explanation is that the difference in p53

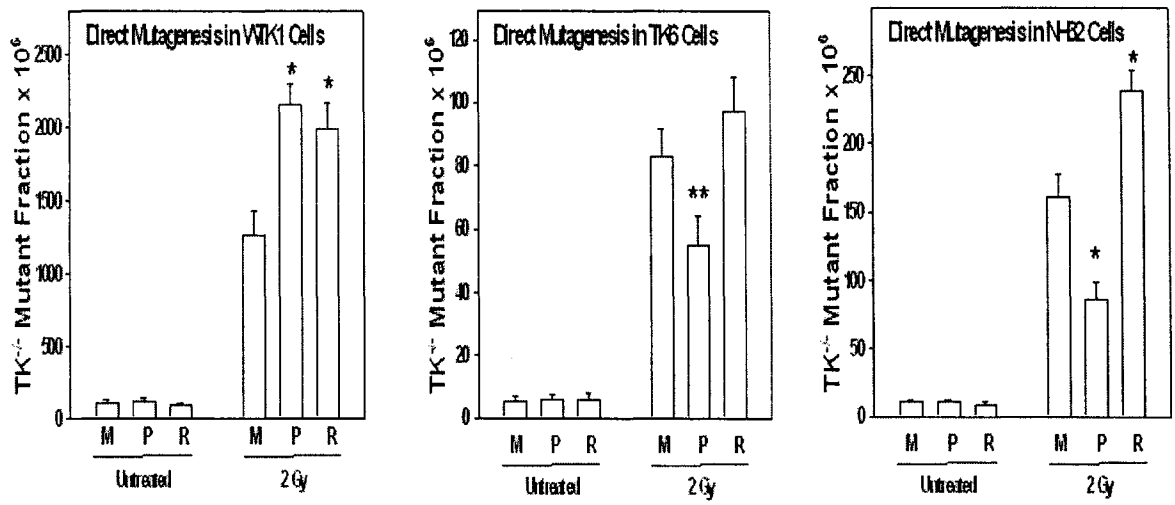

Figure 4. Effects of DNA-PKcs and Rad54 knockdowns on the mutagenesis of directly irradiated cells. WTK1, TK6, and NH32 cells were either mock transfected (M), knocked down for DNA-PKcs (P), or for RAD54 (R). They were untreated or irradiated with 2 Gy γ-rays. MFs were determined three days later. *Columns* are the average of three experiments, *bars* are SD. (* $p<0.05$, ** $p<0.01$ compared to mock transfected cells).

status is responsible.

Effects of DNA-PKcs and Rad54 knockdown on mutagenesis in bystander cells. Figure 5a shows the results of using knockdowns as the irradiated cells (i.e., the bystander signal donors). Therefore in this set of experiments, the cells receiving direct DNA damage were perturbed for either NHEJ or HR, but the recipient bystander cells were not treated with siRNA. As can be seen, there were no differences in the resulting bystander mutagenesis among any of the knockdown conditions in irradiated cells ($p>0.05$). Therefore the repair status of the irradiated cells was not important for their ability to generate bystander signals.

However, the repair status of the bystander cell did turn out to be important. This is shown in Figure 5b. In this set of experiments, the irradiated cells (bystander signal donors) had not been siRNA treated, and therefore they maintained their respective normal repair capabilities. Since we have shown that p53 status in irradiated cells is not important for generating bystander signals (Figure 3), we used irradiated TK6 cells in all experiments as the donor of the bystander signals. Recipient bystander cells were knocked down for either DNA-PKcs or Rad54, and at the time of maximal knockdown, co-cultured with irradiated TK6 cells. Figure 5b shows that depletion of DNA-PKcs in the bystander cells led to increased mutagenesis. The MFs for DNA-PKcs knockdown WTK1, TK6, and NH32 cells were all significantly different from mock transfected control cells ($p<0.05$). However, knockdown of Rad54 had no effect. The MFs for Rad54 knockdown cells all showed no statistical difference from mock transfected control cells ($p>0.05$). From these data we can conclude that NHEJ is involved in preventing bystander mutagenesis. These results also indicate that the perturbation of

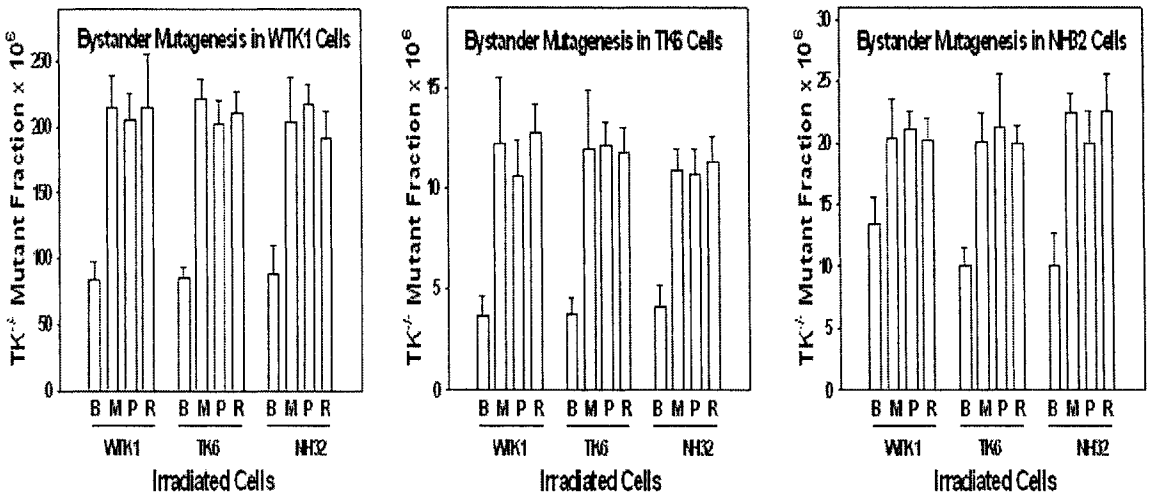

Figure 5. Effect of DNA-PKcs and Rad54 knockdown on mutagenesis in bystander cells. Mutagenesis was studied using the transwell insert system illustrated in Figure 1. A total of 2 × 106 cells in inserts were placed into 6-well dishes containing either unirradiated or 2 Gy γ -irradiated cells. After 1 day culture in the transwell insert system, the cultures were separated and allowed an additional 2 days culture in T-75 flasks; at this time mutant fractions were determined. a, Effects of siRNA knockdown of DNA DSB repair proteins in irradiated cells on bystander mutagenesis. Either DNA-PKcs (P) or Rad54 (R) was knocked down in WTK1, TK6, and NH32 cells. These repair-compromised cells (P or R), as well as mock-transfected cells (M), were irradiated with 2 Gy γ -rays at the time of maximal knockdown and then co-cultured with various bystander target cells (none of which had been subjected to siRNA). Each panel shows bystander mutagenesis for a particular line. Cells co-cultured with unirradiatedcells represent the background MF (B). *Columns* are the average of three experiments, *bars* are SD.

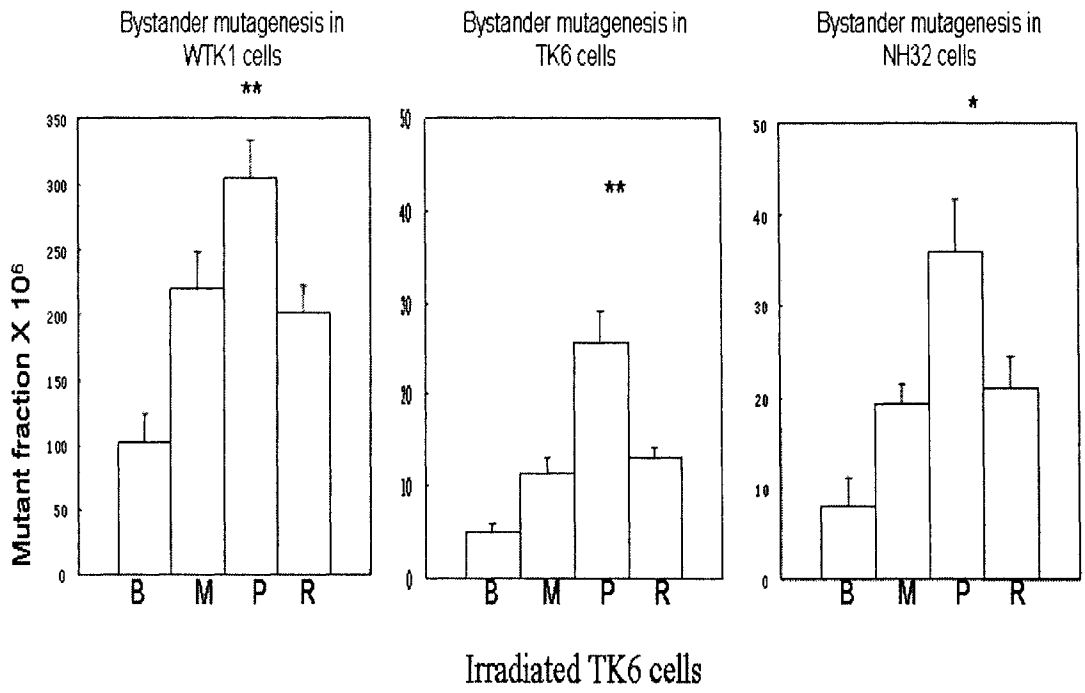

Figure 5b, Effects of siRNA knockdown of DNA DSB repair proteins in bystander cells on bystander mutagenesis. Either DNA-PKcs (P) or Rad54 (R) was knocked down in WTK1, TK6, and NH32 cells. These repair-compromised cells (P or R), as well as mock-transfected cells, were used as bystander cells. They were co-cultured with irradiated TK6 cells that not been subjected to siRNA knockdown. Each panel shows bystander mutagenesis for a particular line. Cells co-cultured with unirradiated cells represent the background MF (B). *Columns* are the average of three experiments, *bars* are SD. (* $p<0.05$, ** $p<0.01$ compared to mock transfected cells).

DNA DSB repair factors differentially influence direct and bystander mutagenesis. This further suggests that these two mutagenic processes have different mechanisms.

Discussion

It previously has been found that gene mutation can be induced in cells neighboring those that are "hit" by radiation; and DSBs have been found in bystander cells. However, little is known about the impact of DSB repair factors on mutagenesis in bystander human cells. Previous studies have indicated that the deficiency of some molecules involved in DSB repair, as well as p53 status, alters IR-induced mutagenesis in human cells (Peng, et al., 2002; Zhang, et al., 2005a; Zhang, et al., 2005b). In this study, siRNA transfection was used to transiently and selectively decrease the levels of either DNA-PKcs, which mediates NHEJ, or RAD54, which is involved in HR. By utilizing a transwell cell culture system where irradiated cells and bystander cells are not in direct contact, but share a common media through a permeable membrane (Figure 1), the impact of DSB repair factors on bystander mutagenesis was studied.

The present results showed that p53 is not required for the transmission and reception of mutagenic bystander signals in human lymphoblastoid cells (Figure 3). Previous studies have shown that p53 is involved in both spontaneous and IR-induced mutagenesis in human lymphoblastoid cells, and that sensitivity is ordered as WTK1>NH32>TK6 (Peng, et al., 2002). In this study, I found that irradiated cells with either wild-type, null, or mutant p53 can all generate signals that increase mutant fractions in bystander cells. In addition, bystander cells with either wild-type, null, or mutant p53 respond to mutagenic bystander signals. The magnitudes of mutant fractions in the bystander cells

are proportional to their intrinsic mutability, that is WTK1>NH32>TK6. The above mentioned results indicate that p53 is not absolutely required for either the generation or receipt of mutagenic bystander signals. In contrast, p53 status in irradiated mouse fibroblast cells has been implicated as important for sending proliferation suppressive factors to unirradiated neighboring cells (Komarova, et al., 1998), as well as for the production of nitric oxide in irradiated human glioblastoma cells to induce micronuclei and cell killing in bystander cells (Matsumoto, et al., 2000; Shao, et al., 2004). Comparing those results with our results for mutagenesis shown in Figure 3, suggests either that (i) p53 involvement in bystander signal generation/receipt is cell type dependent, or (ii) the mutagenic bystander signals may be different from those that induce micronuclei or cell killing.

After DNA-PKcs or Rad54 knockdown, WTK1, TK6, and NH32 showed either increased, unchanged, or decreased IR-induced MFs. After DNA-PKcs knockdown, IR-induced MFs increased in WTK1 cells but decreased in TK6 and NH32 cells; this is consistent with our previous reports (Peng, et al., 2002; Zhang, et al., 2005a). After Rad54 knockdown, IR-induced MFs increased in WTK1 and NH32 cells but were unchanged in TK6 cells; this is the first report on the effect of HR on mutagenesis in these human cells. Considering the complexity of the mechanisms of mutagenesis and the different p53 status of these three cell lines, it is not yet straightforward to account for these differences in mutagenesis in cells with either NHEJ or HR deficiency.

It is well accepted that IR-induced mutations could be a consequence of the incorrect processing of DNA damage by DNA repair machinery. Upon the perturbation of DSB repair status in cells with DNA damage, there are at least two general potential causes for

increase in the resulting MF. First is the increased probability of converting a premutagenic lesion into a mutation. In this case, cells may make use of alternative pathways which increase the likelihood of sequence alteration upon rejoining (i.e., error-prone or mutagenic repair), instead of utilizing an error-free repair process, thus, increasing the mutant fraction (Ma, et al., 2003; Stewart, et al., 1999; Udayakumar, et al., 2003; Zhuang, et al., 2006). For example, it is possible that the disruption of DNA-PKcs-dependent NHEJ could force cells to use a different, more "error-prone" alternative end joining mechanism(s), and this could be responsible for the increased mutant fractions in WTK1. Second, an increase in the tract size of the DNA lesions, as a result of improper DNA repair, including large deletions or HR events that mediate LOH, can effectively increase the target size for mutagenesis. Little is known about how DNA repair factors influence the tract size of DNA lesions; however, our previous data showed that mutant p53 facilitates an increased recombinational DNA repair in WTK1 cells which leads to larger tract sizes of the DNA lesions, thus, these cells are highly mutable (Xia, et al., 1994). In addition, the mutant p53 mediated recombination could be even more active in the case of DNA-PKcs mediated NHEJ deficiency due to the competition of DNA repair factors between NHEJ and HR (Allen, et al., 2003; Allen, et al., 2002).

The decreased radiation mutagenesis in NHEJ deficient TK6 and NH32 cells is intriguing. It should be mentioned that only those cells with genetic damage influencing the exons of the target gene will display a mutant phenotype and be recorded as mutants in all existing models used in mutagenesis research, including our *tk* gene system. For those cells in which genetic damage has been either properly repaired, or those that harbor unreparable damage that leads to cell death, the mutant fraction of a specific gene

marker does not necessarily display an increase. Therefore, unchanged or even decreased mutant fractions are possible alternative outcomes. Considering that all three lines have been derived from the same donor, it is tempting to speculate that the different p53 status in the three lines is responsible, at least in part, for the differential responses to mutagenic stress under NHEJ deficient conditions. Nevertheless, we can not exclude the possibility that other preexisting differences related to the DNA damage response reside in these three lines. In the case of HR deficiency, due to the competition of NHEJ and HR pathways in DSB repair (Allen, et al., 2003; Allen, et al., 2002), cells may make use of more error-prone NHEJ pathways to repair IR-induced DSBs, thus, resulting in the increased mutant fractions observed in Rad54 knockdown WTK1 and NH32 cells. However, Rad54 knockdown in TK6 cells did not show changes in mutant fraction, which again suggest the role of p53 in mediating the cellular response to mutagenic stresses.

Interestingly, mutagenesis in bystander cells displayed a different spectrum of response to DNA DSB repair deficiency, compared to what was observed in directly irradiated cells. In DNA-PKcs deficient cells, in contrast to the increased MF in irradiated WTK1 cells and the decreased MF in irradiated TK6 and NH32 cells, all three cell lines showed an increased mutant fraction in bystander cells. Rad54 knockdown resulted in increased IR-induced MFs in WTK1 and NH32 cells, and no change in TK6 cells; however, no changes at all were observed for bystander mutagenesis in all three lines. These results suggest different mechanisms of mutagenesis exist in irradiated and bystander cells.

It is well accepted that severe biological consequences induced by IR, including gene

mutation, are predominantly from DSBs. Moreover, DSBs have been reported in human fibroblast cells co-cultured with medium from x-irradiated cells by utilizing the same transwell system used in the present study (Yang, et al., 2005). Therefore, I propose that either different molecular natures of the DNA lesions or different repair dynamics between irradiated and bystander cells would be responsible for the different mutagenic consequences. It has been reported that the biological effects of radiation could be mediated by the interaction of radicals such as ROS and nitric oxide with proteins, lipids, and nucleic acids; these reactive species may be generated from primary ionization events or through secondary amplification systems (Huo, et al., 2001). Some reactive species are membrane-permeable and are sufficiently stable to allow diffusion over significant distances between cells (Huo, et al., 2001). Generation of ROS and nitric oxide have been considered to be a cause of the bystander effect, and treatment of cells with antioxidants or nitric oxide scavengers reduce the bystander effect (Azzam, et al., 2002; Balajee, et al., 2004; Shao, et al., 2004; Yang, et al., 2005). It has been hypothesized that the mutagenic damage in bystander cells could result from clustered DNA damage comprised of closely opposed base damage and single strand breaks, whereas DSBs in direct irradiated cells are mainly responsible for the mutagenesis (Little, et al., 2003). The HPRT mutants in low fluence α-particle induced bystander cells are mainly composed of point mutations, in contrast to partial or total deletions observed in irradiated cells (Nagasawa, et al., 2003); this is also true for $CD59^-$ mutants in bystander cells neighboring microbeam α-particle irradiated A_L cells (Zhou, et al., 2000). It is tempting to speculate that the molecular nature of DNA damage is different between irradiated and bystander cells; as a result, DSB repair factor deficiency may differentially

influence mutant induction. On the other hand, the magnitude of DNA damage is different between irradiated and bystander cells, since the MFs in cells irradiated with 2 Gy are more than 15 times those of unirradiated controls, whereas bystander cells exhibit increases of only 1.5-3 fold over background levels. In a study where primary human fibroblast cells received a high or a low dose of IR and then were assayed for the dynamics of the formation and removal of γ-H2AX foci, the low dose-induced γ-H2AX foci remained for weeks whereas the high dose IR-induced γ-H2AX foci were removed quickly. This was interpreted to mean that DSB repair was not activated when the damage did not reach a threshold, and that therefore efficient repair was not available (Rothkamm and Lobrich, 2003). It is also possible that a relatively smaller amount of DNA damage in bystander cells renders the dynamics of repair different from that in irradiated cells, especially when cells are in a DSB repair deficient status.

In summary, the status of DNA DSB repair factors in irradiated cells does not significantly influence bystander mutagenesis, whereas the DSB repair status in bystander cells is important. p53 is not required, either in irradiated or in bystander cells, for bystander mutagenesis to occur. The present results demonstrate that DSB repair factors have differing roles in mediating mutagenesis in irradiated and bystander cells, therefore, providing us with a better understanding of the mutagenic effects of exposure to IR.

CHAPTER 4

siRNA silencing of tankyrase 1 increases telomeric recombination, radiosensitivity and mutagenesis in human cells

Introduction

Mammalian telomeres are composed of telomeric DNA stabilized by telomeric proteins (de Lange, 2005). The telomeric DNA duplex consists of TTAGGG/AATCCC repeats ending in a 3'single-stranded overhang thought to form a higher order structure called a t-loop (Griffith, et al., 1999). The telomeric complex protects natural chromosome ends from degradation, recombination or fusion, and telomeric instability is associated with early phases of carcinogenesis (de Lange, 2004; Greenberg, 2005; Sharma, et al., 1996).

In normal human cells, conventional semi-conservative DNA replication machinery does not allow DNA polymerase to fully replicate chromosome ends, so telomere length is predicted to decrease with each round of replication and eventually shortening becomes critical and causes cellular senescence (Meeker and De Marzo, 2004; Shay and Wright, 2005). However in most cancer cells, telomere length is maintained through active telomerase, which can compensate for telomere loss as cells proliferate (Greider, 1999; Kim, et al., 1994). Some mammalian cells are able to maintain telomere lengths with no telomerase activity suggesting that non-telomerase dependent mechanisms of elongation also exist (Bryan, et al., 1997; Bryan, et al., 1995). This mechanism has been termed

Alternative Lengthening of Telomeres (ALT), and is characterized by heterogeneous telomere length and ALT-associated PML bodies (APBs) (Kim, et al., 1994; Murnane, et al., 1994; Yeager, et al., 1999). The mechanism of telomere lengthening in ALT is currently unknown, but it has been proposed to be a recombination-based process based on the fact that recombination proteins have been identified in APBs (Henson, et al., 2002).

PARP-1((poly(ADP-ribose)polymerase) has been shown to play important roles in suppressing recombination (Tong, et al., 2001). The influence of PARP-1 on genomic sister chromatid exchange (G-SCE) frequency, which can be used to assess stability and recombination rates occurring within the mitotic cell cycle, has been studied extensively by abrogating PARP-1 activity (Bailey, et al., 2004a; Meyer, et al., 2000; Schwartz and Weichselbaum, 1984). However, reduced levels of PARP did not influence telomeric SCE (T-SCE) levles. Tankyrase 1 (TRF1-interacting ankyrin-related ADP-ribose polymerase 1), a telomere specific poly(ADP-ribose)polymerase (PARP) enzyme, is proposed to serve as suppressor of T-SCE (Bailey, et al., 2004a). Tankyrase 1 was originally identified as a TRF1-binding protein (Smith, et al., 1998). TRF1 forms part of the TRF1-TIN2-TPP1-POT1 telomeric protein complex and serves to regulate telomerase access; tankyrase 1 poly(ADP-ribosyl)ates and releases TRF1 from telomeres, faciliting access of telomerase (Smith, et al., 1998). By enhancing to telomerase accissiblity, tankyrase 1 functions as a positive regulator of telomere elongation by telomerase. Studies have shown that tankyrase 1 has multiple functions in accordance with its presence in a variety of compartments (De Rycker and Price, 2004; Seimiya, 2006). Recent studies have shown that tankyrase 1 is required for the dissociation of cohesins in sister chromatid

resolution (Dynek and Smith, 2004) and also has an important role in the assembly of bipolar spindles (Chang, et al., 2005) during mitosis. By using PARP inhibitors to induce telomere shortening, it has been proposed that tankyrase 1 may be a suitable target for telomere-directed cancer therapy (Seimiya, et al., 2005) that may be especially effective when used combined with modalities such as IR.

In contrast to telomeres, which serve to maintain chromosome end stability, broken chromosome ends produced by DNA double-strand breaks (DSBs) are highly recombinogenic. Erroneous repair of DSBs may result in translocation, inversion, deletion and/or amplification of genomic sequences, all of which can lead to genetic instability and eventually cancer (Mills, et al., 2003). In order to maintain genomic and chromosomal stability, it is important for cells to correctly distinguish between these two types of DNA ends, telomeres and DSBs, and then process them appropriately, with the ideal result being the protection of telomere ends and the repair of DSBs. Increasing evidence suggests that telomere maintenance and DSB damage response/repair mechanisms are closely linked, and indeed, common factors bind both telomeric and DSB ends. Deficiencies of DSB repair factors from the NHEJ and HR pathways such as DNA-PKcs, Ku, Rad54 and Rad51 lead to telomere dysfunction phenotypes (Al-Wahiby, et al., 2005; Jaco, et al., 2004 & 2003; Myung, et al., 2004; Tarsounas, et al., 2004; Zhang, et al., 2005a;). Deficiency of the telomeric binding protein Pot1 (protection of telomeres) promotes chromosomal instability and tumorigenesis (Wu, et al., 2006). A recent study demonstrated an apparently direct role of TRF2 (telomeric repeat binding factor 2) in DSB repair; TRF2 was found to associate with γ -H2AX foci formed within a few seconds following exposure to a very high energy UV laser microbeam (Bradshaw, et al.,

2005). Although this led to the suggestion that TRF2 is involved in the early response to DSB response, more recent studies have shown that the co-localization of γ -H2AX foci and TRF2 does not occur following damage induced by ionizing radiation (Williams et al, 2007). Concurrently, the functions, phenotypes and mechanisms of telomere proteins in the DSB response/repair are not well understood.

In this study, in order to examine the roles of the telomere specific PARP, tankyrase 1, in the T-SCE process and in the DSB response/ repair, we utilized siRNA knockdown of tankyrase 1. We observed that tankyrase 1 deficiency led to increased levels of T-SCE, radiosensitivity and mutagenesis. Most surprisingly, we found that knockdown of tankyrase 1 also led to a rapid decrease in protein levels of DNA-PKcs, perhaps explaining its apparent role in repair.

Materials and methods

Cell culture and γ-irradiation. Li-Fraumeni cells (MDAH 087) were derived from cancer-affected individuals with Li-Fraumeni syndrome (LFS), who inherit a germ-line defect in one p53 allele (single–base mutations in p53 at codon 248) (Malkin, et al., 1990; Varley, et al., 1997). Li-Fraumeni (087) cells are high passage cells without detectable telomerase activity. Normal human fibroblast 5C cells are primary low passage telomerase negative. Both Li-Fraumeni and 5C cells were maintained in culture using α-MEM (Hyclone, Logan, Utah) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 100 units/mL penicillin, and 100 μg/mL streptomycin. The human B-lymphoblastoidoid cell line, WTK1 was derived from WIL2 and carries a mutant form of p53 (Liber and Thilly, 1982; Xia, et al., 1995). WTK1 cells were maintained in RPMI

1640 supplemented with 10% heat-inactivated horse serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cultures were incubated at 37°C in 5% CO2 and 100% humidity. 3-Aminobenzamide (3-AB, from Sigma, St. Louis, MO) was dissolved in DMSO at a final concentration of 0.1 mM and was used to inhibit PARP poly(ADP-ribosyl)ation. 3AB was added alone or 30 minutes before irradiation and removed 24 hours later. γ-irradiations were done at room temperature in a calibrated Mark I ^{137}Cs γ-irradiator (J.L. Shepherd and Associates, Glendale, CA). The fibroblasts were seeded at various concentrations in 60 mm dishes 3 hours before the cells were irradiated (doses ranged from 0 to 8 Gy). Log-phase WTK1 cells were irradiated at a density of 1 x 10^6 per mL in 10 mL of growth medium in T-25 flasks with doses of 0 and 1.5 Gy. Dose rates ranged from 0.20 to 0.25 Gy/min.

Small interfering RNA transfection. Two different siRNA sequences (leading strand) were used for *tankyrase 1* gene silencing: r(AGGAAGGAGACACAGAUAU)d(TT) and r(CCUGGAAGUAGCUGAAUAU)d(TT). Searches of the human genome database (BLAST) were carried out to ensure that these sequences would not target other gene transcripts, thus avoiding off-target effects. Transfections were done according to manufactures instructions (Qiagen, Valencia, CA), in serum-free MEM or RPMI media. The concentration of siRNAs used was 20 nmol/L and were prepared in the form of siRNA/LipofectAMINE2000 (Invitrogen, Carlsbad, CA) complexes (1:1.5). Fetal bovine or horse serum was added to a final concentration of 10% 6 h later. Transfected cells were collected daily to determine protein levels of tankyrase 1 by Western blot analyses.

Western blots. Cells (6 x 10^6) were harvested, washed with cold PBS and lysed in 200 μL ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% sodium

deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA (pH 8.0)] containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.1% leupeptin, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride) on ice for 15 min. Following centrifugation, supernatants containing protein were collected. Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Cell lysates were loaded and electrophoresed on 10% SDS polyacrylamide gels. After wet-blotting to nitrocellulose, protein levels were analyzed using the corresponding primary antibodies tankyrase 1 (Scbt, Santa Cruz, CA) or DNA-PKcs (AB-4) (Neomarkers, Fremont, CA), then horseradish peroxidase–conjugated goat anti-rabbit IgG (Promega, WI) or goat anti-mouse IgG (Promega, WI) were used as secondary antibodies. The immunoreactive bands were visualized by chemiluminescence (ECL kit; Amersham Pharmacia) on X-ray films.

Cell cycle analysis. Cells were trypsinized, fixed in cold 70% ethanol, then stained with 50 ug/ml propidium iodide for at least 20 minutes before analysis by flow cytometry. 500 mW at 488 nm was used for excitation and fluorenscence was measure at wavelength > 610 nm.

Cell survival. Two hours before exposure, exponentially growing cells were seeded in 60 mm dishes at various densities depending on the radiation dose (0 to 8Gy of γ-rays). After IR exposure, plates were incubated at 37°C for 14-20 days in growth medium to allow for colony formation. Plates were rinsed, fixed with methanol and stained with methylene blue. Colonies with >50 cells were counted; plating efficiency and surviving fractions for each dose were calculated.

Mutagenesis assays. We used standard procedures to determine mutant fractions (MF) at the TK locus (Liber and Thilly, 1982). Briefly, cells were pretreated with CHAT (deoxycytidine, hypoxanthine, aminopterin and thymidine) for 2 days to reduce $TK^{-/-}$ backgrounds, then allowed to recover in THC (deoxycytidine, hypoxanthine, and thymidine) for 1 day. To determine MFs, cells were grown in normal medium for 3 days to allow phenotypic expression of newly induced mutants at TK. Then cells were seeded at 2000 cells/well in 96-well dishes in the presence of 2 μg/ml trifluorothymidine to measure mutation. Cells were also seeded at 1 cell/well to determine plating efficiency. Plates were scored 20 days later, and total MF (mutants per surviving cell, including both normal and slowly growing varieties) were calculated using the Poisson distribution (Furth, et al., 1981).

Chromosome-Orientation Fluorescence In Situ Hybridization (CO-FISH). CO-FISH is a strand-specific modification of FISH, developed by Dr. Susan Bailey and colleagues (Bailey, et al., 2004b). Its purpose is to allow detection of only one of the telomeric sequences. Briefly, 18 hours after siRNA transfection, 100 μ*M* 5′-bromo-2′-deoxyuridine (BrdU; Sigma) was added to cultures, to allow incorporation into DNA. Twelve hours later, 0.1 μg/ ml colcemid (GiBCO, Grand Island, NY,) was added to halt cells in mitosis. Therefore, a total of 16 hr, or about one cell cycle, elapsed after the addition of the BrdU; consequently, cells are only substituted in one of the DNA strands. Dishes were trypsinized; the cells were fixed in methanol:acetic acid (3:1) and dropped onto cold, wet microscope slides. The slides were treated with 0.5 mg/ml RNase A for 10 min at 37°C , and stained for 15 min at ambient temperature with Hoechst 33258 (Sigma; 0.5 μg/ml Hoescht in 2× SSC, which is 0.3 M NaCl, 0.03 M sodium citrate). Then, the slides were

irradiated for about 30 min with UV light at a wavelength of 365 nm (Stratalinker 1800 UV irradiator); this step produces breaks in the BrdU-substituted strand, but not the other. Next, the slides were subjected to digestion of the newly synthesized strands, i.e., the BrdU -substituted strands, using 3 U/μl of Exonuclease III (Promega) in a buffer consisting of 50 mM Tris–HCl, 5 mM $MgCl_2$, and 5 mM dithiothreitol, pH 8.0. This reaction was for 10 min at room temperature. The exonuclease only works on the strand with the breaks induced by the UV acting on thre BrdU. An additional denaturation step was performed in 70% formamide, 2× SSC at 70°C for 1 min, to complete the removal of the newly replicated BrdU-substituted strands; this was followed by dehydration in a cold ethanol series (70, 85, 100%). Telomeric DNA oligomeric probes contained either the sequence $(TTAGGG)_7$ or $(CCCTAA)_7$. These were labeled by terminal deoxynucleotidal transferase tailing with Cy3-dCTP, as described by the manufacturer (Boehringer Mannheim). The hybridization mixture contained 0.4 μg/ml of probe in 30% formamide and 2× SSC, and was added to the slides. After an overnight hybridization at 37°C in a moist chamber, the slides were washed 5 times for 15 min each in 2× SSC (42°C) and placed in a glycerol solution containing 1 mg/ml of *p*-phenylenediamine HCl, which is an antifade compound, and 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), which is a counterstain for chromosomal DNA. By utilizing CO-FISH, sister chromatid recombination within telomeres are able to distinguished which can be seen as the telomere signal splits between the sister chromatids.

Results

Suppression of tankyrase 1 protein expression by small interfering RNA in Li-Fraumeni (087) and primary normal 5C human fibroblasts. Figure 1 reveals significant suppression of tankyrase 1 protein expression at 18 hours after transfection with two different siRNAs in both Li-Fraumeni (087) (Fig. 1 A) and 5C fibroblasts (Fig. 1B). Similar results were obtained in WTK1 lymphoblastoids (data not shown). Therefore, I collected cells at 18 hours post transfection and performed the cytotoxicity and mutagenesis assays described below.

Tankyrase 1 knockdown resulted in a significant increase in telomere sister chromatid exchange. CO-FISH facilitates scoring of sister chromatid exchange (SCE)-like recombination within the telomere itself; these have been termed telomere sister chromatid exchange (T-SCE) (Bailey, et al., 2004b). Fig. 2A shows CO-FISH images of T-SCE for mock and tankyrase1 siRNA knockdowns. When a T-SCE occurs, the telomere signal splits between the sister chromatids, and the incidence of these are a measure of telomeric recombination activity. Fig. 2B shows that significantly increased frequencies of T-SCEs were observed after using either of two different tankyrase 1 siRNAs to produce the knockdown ($p=0.001$ when compared to mock transfection in Li-Fraumeni (087) cells); frequencies of G-SCE, quantified by the standard 'fluorescence-plus-Giemsa' technique (Perry and Wolff, 1974), were not altered after tankyrase 1 knockdown (Fig. 2B). Similar results were observed in 5C cells (data not shown). Consistent with the role of PARP 1 in suppression of recombination in the genome (Bailey, et al., 2004a; Meyer, et al., 2000), Results here suggest that tankyrase 1, a telomeric PARP, can function to limit recombination within telomeric regions.

siRNA knockdown of tankyrase 1 in Li-Fraumeni fibroblasts results in reduced

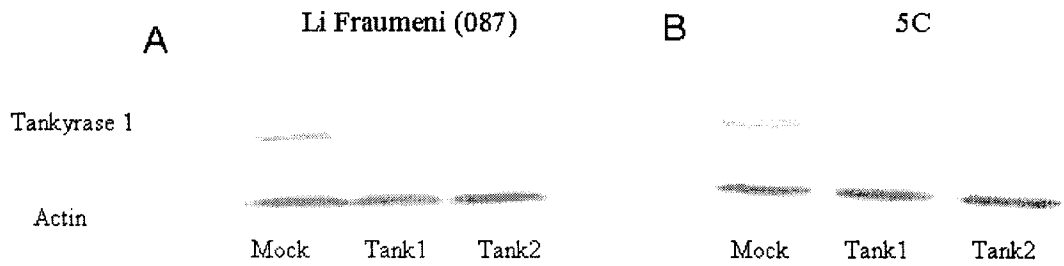

Figure 1. Expression of tankyrase 1 in Li-Fraumeni and 5C cells 18 hours after tankyrase 1 siRNA knockdown . (A). Western blot from Li-Fraumeni cells. (B). western blot from 5C control cells. Upper bands were probed with anti-tankyrase 1 antibody and lower bands with β-actin, used as loading controls.

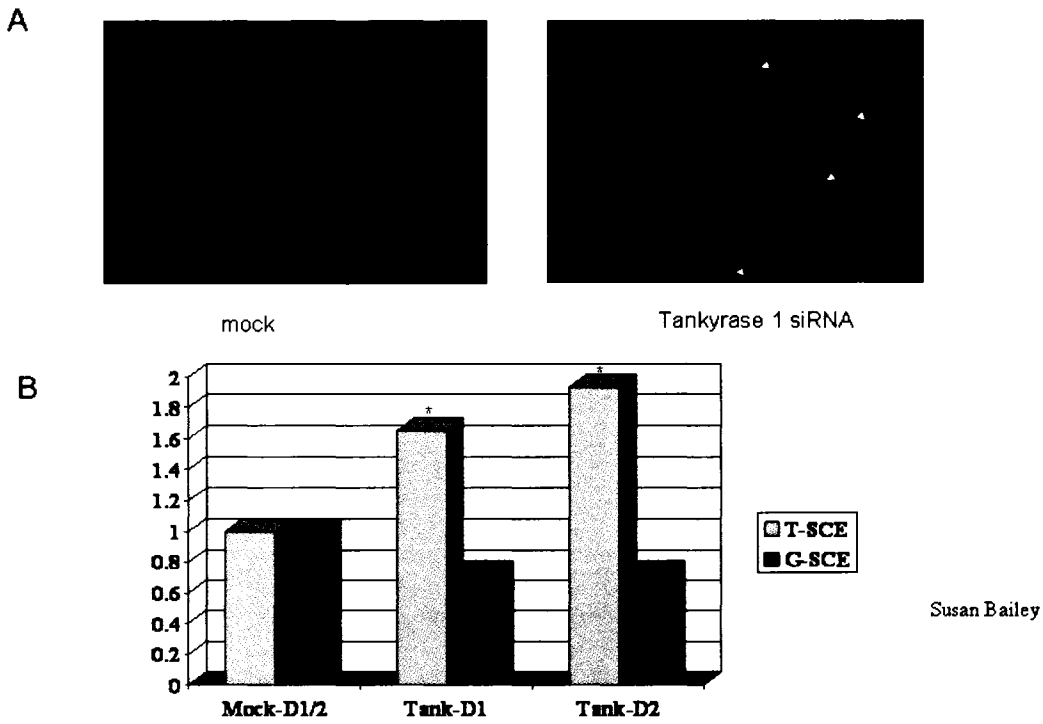

Figure 2. Telomeric SCE (T-SCE) and genomic SCE (G-SCE) after tankyrase 1 knockdown. (A) CO-FISH images of T-SCE (arrow) for mock and tankyrase 1 siRNA. (B) Frequency per chromosome of telomeric (T-SCE) and genomic SCE (G-SCE) in Li-Fraumeni (087) (left panel) cells after two different tankyrase 1 siRNA transfections (ratios relative to control).

protein levels of DNA-PKcs. As shown in Figure 3A, a rapid decrease in the level of DNA-PKcs protein with tankyrase 1 knockdown was observed. Samples were collected at 12 hr, 1 and 2 days after transfection, and the time course of reduction of DNA-PKcs was similar to that of tankyrase 1 depletion. Figure 3B shows that the reduction of DNA-PKcs levels after siRNA knockdown of tankyrase 1 in Li-Fraumeni cells was not affected by a 5 Gy IR exposure, when monitored 12 hours post-transfection. Levels of Ku86 and β-actin remained unaltered over these time and IR points. Because it is known that it takes 4-5 days for RNAi to deplete DNA-PKcs protein levels (Peng, et al., 2002), My results suggest that tankyrase 1 may be required for DNA-PKcs protein stability, i.e., to prevent it from undergoing rapid degradation.

Tankyrase 1-targeted siRNA transfection increased radiation-induced cell killing in Li-Fraumeni (087) and 5C fibroblasts. Li-Fraumeni and 5C fibroblasts were γ-irradiated with 0-8 Gy at 18 hours after tankyrase 1 siRNA transfection. Cell cycle analysis showed that tankyrase 1 knockdown did not change the cell cycle distribution at this time point (Fig. 4). The surviving fractions were determined by the colony forming assay. As shown in Figure 4, the log-transformed surviving fractions showed a reasonable linear-quadratic fit with the radiation dose and there were marked radiosensitization of tankyrase 1-targeted siRNA-transfected cells relative to the mock transfection in both Li-Fraumeni and 5C fibroblasts (the calculated α and βvalues are shown). The results here demonstrate that reduced levels of tankyrase 1 significantly reduces survival in Li-Fraumeni (087) and 5C fibroblasts following IR exposure.

3AB treatment results in an increased spontaneous mutant fraction (MF) and IR-induced MF; however, tankyrase 1 knockdown only results in an increased IR-

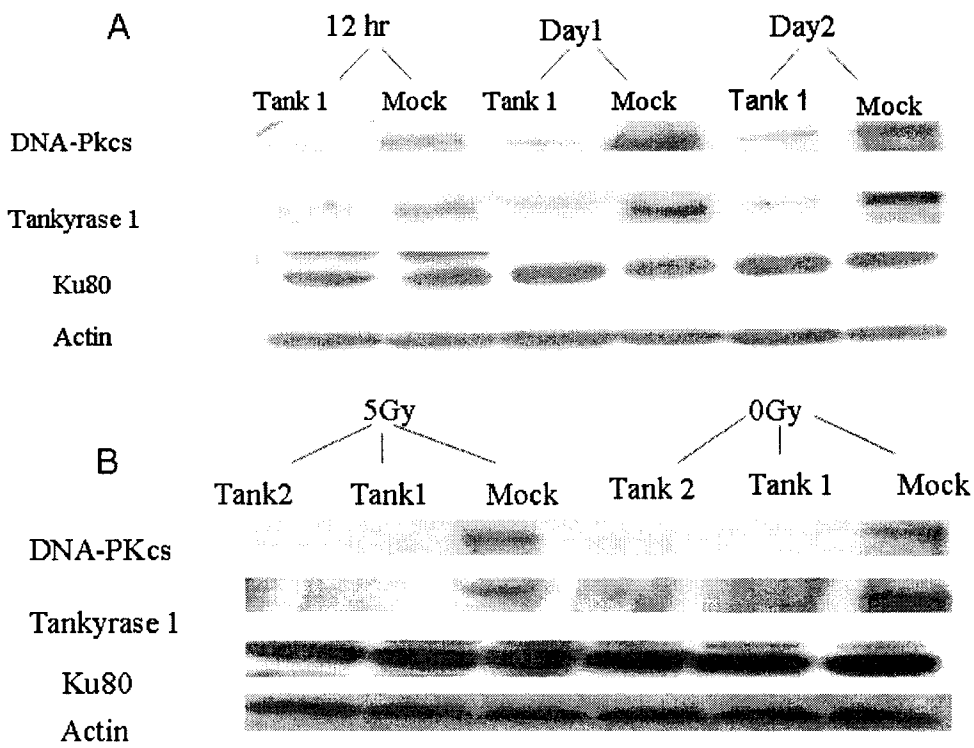

Figure 3. Reduction of DNA-PKcs protein levels after tankyrase 1 siRNA transfection in Li-Fraumeni fibroblasts. (A) Levels of DNA-PKcs protein in Li-Fraumeni cells after tankyrase 1 or mock siRNA transfection, at12 hours, 1 or 2 days after knockdown . (B) Levels of DNA-PKcs protein in Li-Fraumeni cells after tankyrase 1 or mock siRNA transfection, which were done 12 hours after irradiation with 0 or 5 Gy of γ-rays. Levels of Ku86 and β-actin did not change over these time and IR points.

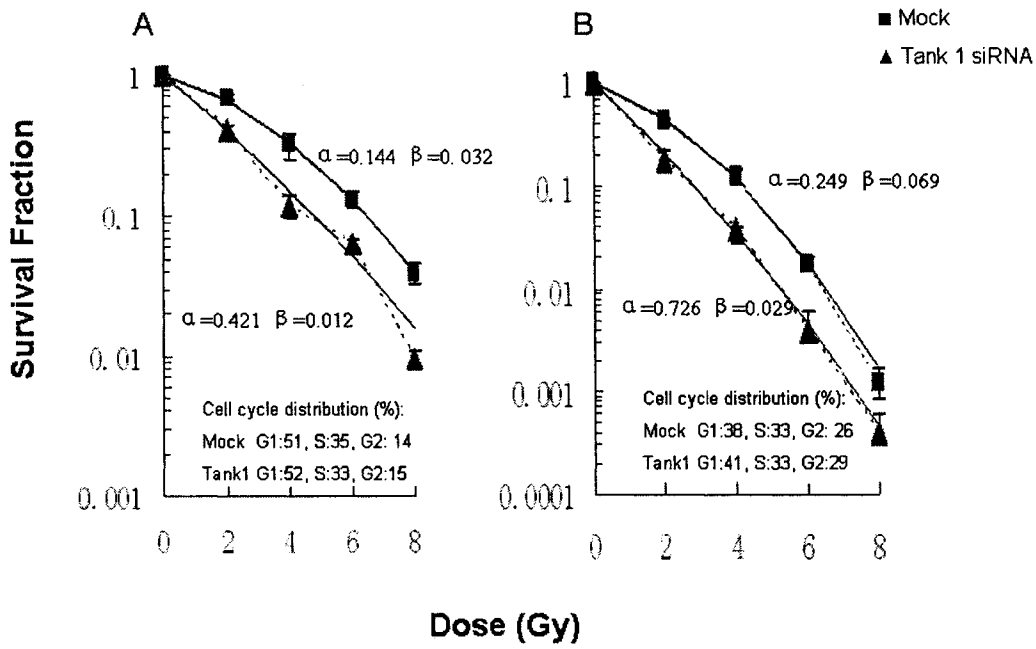

Figure 4. Radiation-induced cell killing in Li-Fraumeni (A) and 5C (B) fibroblasts following tankyrase 1 siRNA knockdown. At 18 hours after siRNA transfection, cells were seeded in 60 mm dishes, then γ-irradiated with 0-8 Gy 2 hours later. Points are averages of three experiments; bars are standard deviations of the mean. Mock transfection (■), tankyrase 1 siRNA transfection (▲).Note that flow cytometry analyses confirmation no change in cell cycle distribution as a result of tankyrase 1 knockdown.

induced MF. Figure 5 shows MFs at the TK locus in WTK1 lymphoblastoid cells under four conditions: (i) controls (mock transfection), (ii) after tankyrase 1 knockdown, (iii) after treatment with the PARP inhibitor (3-aminobenzamide, 3AB) alone and (iv) after 3AB treatment combined with tankyrase 1 knockdown. Spontaneous MF (no IR) revealed no quantitative differences between tankyrase 1 knockdown and mock transfection. However, there was an approximate two-fold increase in spontaneous MF between 3AB treatment alone or 3AB treatment and tankyrase 1 knockdown when compared to mock ($p<0.05$ for both) and tankyrase 1 siRNA transfection ($p<0.05$ for both). After treatment with 1.5 Gy γ-rays, tankyrase 1 knockdown resulted in an increased IR-induced MF at TK locus in WTK1 cells, compared to mock ($p<0.05$); 3AB treatment alone and 3AB treatment combined with tankyrase 1 knockdown resulted in an even higher increased IR-induced MFs compared to mock transfection ($p<0.001$) and tankyrase 1 knockdown alone ($p<0.01$); however, there was no significant difference for IR-induced MF between 3AB treatment alone and 3AB treatment combined with tankyrase 1 knockdown. Our mutagenesis results suggest ionizing radiation–induced MF at the TK locus was increased after tankyrase 1 knockdown; the PARP inhibitor (3AB) treatment enhanced both spontaneous and IR-induced MF at TK locus. However, tankyrase 1 knockdown did not further increase spontaneous or IR-induced MF at TK locus when combined with 3AB treatment.

Discussion

In this study, we used siRNA transfection of tankyrase 1 to suppress protein levels of this telomeric specific PARP, then examined relevant phenotypic consequences including

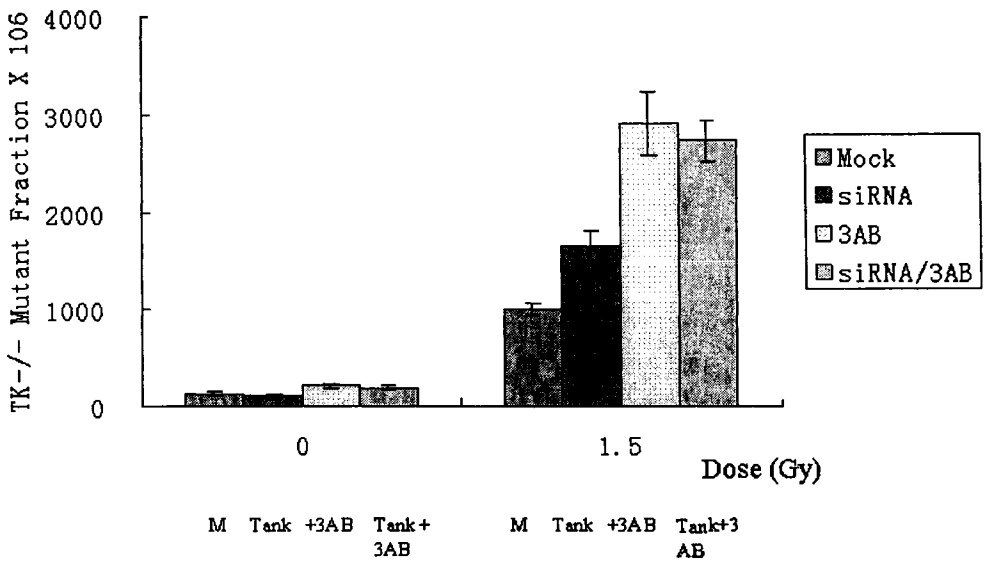

Figure 5. Radiation-induced mutagenesis at the TK locus in WTK1 cells, after tankyrase 1 knockdown and/or 3-AB treatment. WTK1 lymphoblasts were -irradiated 18 hours after tankyrase1 siRNA transfection. They were maintained in normal medium for 3 days to allow phenotypic expression of TK–/– mutants, then plated in the selective medium trifluorothymidine. Colonies were scored 20 days later. Data are the average of three independent determinations; error bars are standard deviations.

telomeric SCE, ionizing radiation–induced cell killing and mutagenesis. Surprisingly, we observed a rapid reduction in the level of DNA-PKcs protein after tankyrase 1 knockdown.

Telomerase has been studied extensively as a potential therapeutic target for cancer treatment, based on the fact that the majority of tumor cells exhibit telomerase activity in order to bypass the telomere checkpoint and to obtain unlimited growth potential (Zimmermann and Martens, 2007). However, how telomerase-negative cancer cells escape the telomere-length checkpoint by the ALT process is still poorly understood (Bryan, et al., 1997). One possibility is that in ALT cells, increased recombination within telomeres could facilitate telomere elongation and therefore long-term survival (Neumann and Reddel, 2002).

We hypothesize that tankyrase 1 is a telomere specific PARP that normally plays an important role in regulating telomere recombination. As we observed, knocking down tankyrase 1 by siRNA transfection increased telomere SCE (Fig. 2B), perhaps by increasing stalled replication forks owing to difficulty in the separation of telomere sister chromatids (Dynek and Smith, 2004). PARP-1 deficiency resulted in an increase in G-SCE but not in T-SCE (Bailey, et al., 2004a). In contrast, we found that knockdown of tankyrase 1 affected only T-SCE, not G-SCE, supporting a model where differential regulation of SCE frequency is dependent on genomic location.

PARPs have been shown to have important roles in DSB response/repair (Audebert, et al., 2006; Rudat, et al., 2001; Wang, et al., 2006). Studies have suggested the interaction between DNA-PK and PARP-1(D'Silva, et al., 1999; Morrison, et al., 1997). A study showed that purified DNA-PK can phosphorylate PARP and protein kinase

activity of DNA-PK can be stimulated by ADP-ribosylation activity of PARP. Moreover, the ADP-ribosylation of DNA-PKcs is independent of the Ku70/80 complex. The study suggested that DNA-Pkcs and PARP may function coordinately in vivo in response to DNA damage (Ruscetti, et al., 1998). In the present study, we found that siRNA knockdown of tankyrase 1 in Li-Fraumeni cells reduced protein levels of DNA-PKcs (Fig. 3). We observed that DNA-PKcs level was significantly decreased as early as 12 hours after tankyrase 1 siRNA transfection, and this was true whether or not cells had been treated with 5 Gy IR after tankyrase 1 siRNA transfection. The level of Ku 86 protein remained unaltered with tankyrase 1 knockdown at all time points and IR treatments. Our result are consistent with a previous study that showed that Ku localizes to the telomere via a high-affinity interaction with TRF1, but which was not depend on DNA-PKcs (Hsu, et al. 2000). That knocking down tankyrase 1 results in a decreased level of DNA-PKcs but an unaltered level of Ku in our study, supports the idea that Ku functions in a different way at the telomere than during the NHEJ pathway of DSB repair. From other studies using siRNAs to knockdown DNA-PKcs, we know that at least 4 days is required to deplete DNA-PKcs protein levels via an RNAi mechanism (Peng, et al., 2002). Therefore, our results suggest that tankyrase 1 may be required for DNA-PKcs protein stability, perhaps preventing its rapid degradation. However, our preliminary immunoprecipitation experiments failed to demonstrate an association between tankyrase 1 and DNA-PKcs (data not shown). Two possibilities could account for this; a direct association between the proteins could be present, but not be strong enough to withstand the rigors of immunoprecipitation, or there may be other mechanisms responsible for tankyrase 1 regulation of DNA-PKcs stability besides direct interaction.

Depletion of the telomeric protein, tankyrase 1 resulted in increased radiosensitivity (Fig. 4) in both Li-Fraumeni and normal human primary 5C fibroblasts. Flow cytometry analyses confirm that tankyrase 1 knockdown did not change the cell cycle distribution at 18 hours after tankyrase 1 knockdown in these two cell lines; this is consistent with a previous study showing no cell cycle changes within 24 hours in HeLa cells after tankyrase 1 knockdown (Dynek and Smith, 2004). It is tempting to speculate that the rapid decrease in the level of DNA-PKcs protein after tankyrase 1 knockdown could be responsible for the increased radiosensitivity. Many previous studies have shown that inhibition of DNA-PKcs results in deficient DNA double-strand break repair and the radiosensitization of cells. Similar results were observed in hyper-radiosensitive DNA-PK knockout cells (Allalunis-Turner, et al., 1995; Collis, et al., 2005; Hoppe, et al., 2000; Jhappan, et al., 1997; Peng, et al., 2002). Dysfunctional telomeres caused by tankyrase 1 knockdown may be another possibility for the increased radiosensitivity we observed. Studies have shown that mechanisms of telomere length maintenance may be linked with mechanisms that confer sensitivity to radiation (Slijepcevic, 2006). Studies have shown that tankyrase 1 and DNA-PKcs have important roles in maintaining functional telomeres. Therefore, tankyrase 1 knockdown and reduced levels of DNA-PKcs protein after tankyrase 1 knockdown may be responsible for the radiosensitivity we observed in this study.

As shown in Figures 5, tankyrase 1 knockdown does not increase spontaneous MF, but does significantly increase in IR-induced MF at the TK locus in WTK1 cells. How tankyrase 1 is involved in mutagenesis also is not clear, but reduced DNA-PKcs levels with tankyrase 1 siRNA transfection could be responsible for the increased IR-induced

MF in WTK1 cells. Previous studies in WTK1 have shown that deficiency of DNA-PKcs resulted in an increased IR-induced MF of the same order as was observed here (Peng, et al., 2002; Zhang, et al., 2006). Moreover, treatment with the PARP inhibitor 3AB resulted in increased spontaneous and IR-induced MF at the TK locus in WTK1 cells. This is consistent with the multiple roles of PARP family members in many processes such as DNA repair, maintenance of genomic stability, cell differentiation and ploidy control. Studies have shown that PARP family members play important roles in single-strand break and DSB repair as well as in base excision repair, and interacts with various proteins engaged in these processes (Ariumi, et al., 1999; Masson, et al., 1998; Ruscetti, et al., 1998). In addition, PARP members have important roles in maintenance of genomic stability. A variety of chromosomal instability endpoints such as SCE (Bailey, et al., 2004a; Dominguez, et al., 2000; Oikawa, et al., 1980), chromosomal ploidy (Simbulan-Rosenthal, et al., 2000), deletion mutations (d'Adda di Fagagna, et al., 1999) and gene amplifications (Burkle, et al., 1987) have been observed in PARP deficient cells. Direct phenotypic studies have also suggested that dysfunction of PARP-1 is unequivocally involved in carcinogenesis in various organs, caused either by an exogenous or endogenous carcinogenic insult (Masutani, et al., 2003). 3AB, which inhibits general PARP activity, could be responsible for the increased spontaneous and IR-induced MF in WTK1 cells in the present study. Since 3AB is a general PARP inhibitor, it presumably also inhibits tankyrase 1 activity, perhaps explaining why we did not observe any further increase in spontaneous or IR-induced MF at TK locus when tankyrase 1 knockdown was combined with 3AB treatment.

In summary, we have shown that reduced levels of the telomeric specific PARP

protein tankyrase 1, results in increased T-SCE, radiosensitivity and mutagenesis in human cells. Importantly, tankyrase 1 knockdown also results in reduced levels of the NHEJ protein DNA-PKcs, which may exert an important role in the repair phenotypes we observed. Our work expands our understanding and appreciation of the association between telomeres and DSB repair and may contribute to improving future approaches in telomere-associated anti-cancer therapy.

CHAPTER 5

Summary and future directions

Summary

The double-strand break (DSB) is one of the most dangerous DNA lesions, because erroneous repair may lead to genetic instability and eventually cancer. In this study, I used small interfering RNA (siRNA) to produce deficiencies of critical DSB repair components both in directly irradiated and bystander cells and examined the subsequent phenotypic consequences. I extended this examination to study the effects of different genetic backgrounds, specifically differential status of p53. I also broadened my investigation to study the effects of suppressing expression of critical telomere maintenance proteins on the processes of DNA damage repair.

Rad54 knockdown by small interfering RNA increased radiosensitivity in human lymphoblastoid cells with mutated p53 but spared cells with wild-type p53 and null p53

The highly conserved Rad54 protein plays an essential role in repairing DNA damage through homologous recombination. Studies have shown that the human Rad54 protein exhibits many biochemical activities, including ATPase, super-coiling, and DNA duplex separation. I used small interfering RNA (siRNA) to silence expression of Rad54 protein and analyzed relevant phenotypes in three strains of related human lymphoblastoids with different p53 status, namely wild type TK6, mutated WTK1 and

null NH32 cells. Knockdown of Rad54 resulted in an increased radiosensitivity in WTK1, but not in TK6 or NH32 cells. Importantly, the radiosensitivity of Rad54-deficiency in WTK1 cells was evident in the S/G2 phases of the cell cycle but not in G1 phase. My experiments with immunoprecipitation showed that mutant p53, as well as wild type p53, associates with Rad54.

Deficiencies of double strand break repair factors lead to different mutagenesis outcomes in γ-irradiated and bystander human lymphoblastoidoid cells

By utilizing RNA interference techniques to knock down key proteins in two major double strand break (DSB) repair pathways [DNA-PKcs for non homologous end joining, (NHEJ) and Rad54 for homologous recombination (HR)] , I investigated the influence of DSB repair factors on radiation mutagenesis at the autosomal thymidine kinase (*tk*) locus, both in directly irradiated cells and in unirradiated bystander cells. I also examined the role of p53 in these processes by using three human lymphoblastoidoid cell lines from the same donor but differing in p53 status (TK6 is p53 wild-type, NH32 is p53 null, and WTK1 is p53 mutant). My results indicated that p53 status did not affect either the production of radiation bystander mutagenic signals or their receipt. In directly irradiated cells, knockdown of DNA-PKcs led to an increased mutant fraction (MF) in WTK1 and decreased MFs in TK6 and NH32. In contrast, knockdown of DNA-PKcs led to increased mutagenesis in bystander cells regardless of p53 status. In directly irradiated cells, knockdown of Rad54 led to increased induced MFs in WTK1 and NH32, but the knockdown did not affect mutagenesis in p53 wild-type TK6. In all cell lines, Rad54 knockdown had no effect on the magnitude of bystander mutagenesis.

siRNA silencing of expression of tankyrase 1 increases telomere sister chromatid exchange, radiosensitivity and mutagenesis in human cells

PARP-1((poly(ADP-ribose)polymerase) has been shown to play important roles to suppress recombination and the deficiency of PARP-1 increased genome SCE frequency, but not telomere SCE. Tankyrase 1, a telomere specific poly(ADP-ribose)polymerase (PARP) enzyme, is proposed to serve as a suppressor of T-SCE. Studies from other laboratories have shown that telomere proteins have important roles in DSB response/repair. In order to examine the roles of telomere specific PARP in the T-SCE process and in the DSB response/ repair, I utilized siRNA to knock down tankyrase 1, and observed that tankyrase 1 deficiency led to increased levels of T-SCE, radiosensitivity and mutagenesis. Surprisingly, knockdown of tankyrase 1 led to a rapid decrease in protein levels of DNA-PKcs.

Future directions

There are questions that remain unanswered.

I have investigated the effects of transient silencing of DNA repair and telomere associated proteins on mutagenesis, cell killing, and telomere dysfunctions in our human lymphoblastoidoid cell systems. However, what the effects will be of permanent gene silencing or over-expression of these proteins are still unknown. In addition, the role of telomere-associated proteins in mediating DNA damage response, such as DNA repair, apoptosis, and gene expression profile is still unknown. Finally, the molecular nature of mutations induced by radiation under conditions of protein deficiency, which can tell us about the mechanism of mutagenesis, is also unknown. Therefore, the future directions

could be the following:

1 We could establish permanent gene silencing models of these proteins by short hairpin RNA or gene knock out approaches, as well as over-expression models by transfecting vectors with these genes into our human lymphoblastoidoid cell systems. We could then study the effects of the different conditions on DNA damage response, using the same or similar endpoints.

2 We could study the role of other telomere-associated proteins on gene expression profiles after DNA damage. At the same time, additional end points related to the DNA damage response, such as cell death, apoptosis, and DNA double strand break repair could be investigated. Thus, we could associate the gene expression profile with the endpoints to better understand the molecular mechanism of telomere-associated proteins in DNA damage response.

3 We could study which mutational classes (e.g., point mutations, large deletions, recombination-generated loss of heterozygosity events) are affected by the deficiencies generated by siRNA. Radiation-induced mutational spectra generated under conditions of Rad54, DNA-PKcs and tankyrase1 deficiencies would indicate which classes are inhibited or enhanced, and this would tell us about mechanism of mutagenesis.

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