Review Article

Translational research in radiation-induced DNA damage signaling and repair

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Abstract: Radiotherapy is an effective tool in the fight against cancer. It is non-invasive and painless, and with advanced tumor imaging and beam control systems, radiation can be delivered to patients safely, generally with minor or no adverse side effects, accounting for its increasing use against a broad range of tumors. Tumors and normal cells respond to radiation-induced DNA damage by activating a complex network of DNA damage signaling and repair pathways that determine cell fate including survival, death, and genome stability. DNA damage response (DDR) proteins represent excellent targets to augment radiotherapy, and many agents that inhibit key response proteins are being combined with radiation and genotoxic chemotherapy in clinical trials. This review focuses on how insights into molecular mechanisms of DDR pathways are translated to small animal preclinical studies, to clinical studies of naturally occurring tumors in companion animals, and finally to human clinical trials. Companion animal studies, under the umbrella of comparative oncology, have played key roles in the development of clinical radiotherapy throughout its >100-year history. There is growing appreciation that rapid translation of basic knowledge of DNA damage and repair systems to improved radiotherapy practice requires a comprehensive approach that embraces the full spectrum of cancer research, with companion animal clinical trials representing a critical bridge between small animal preclinical studies, and human clinical trials.

Keywords: DNA repair; DNA damage checkpoint signaling; radiotherapy; comparative oncology

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Introduction

Cells respond to DNA damage by activating checkpoint signaling and DNA repair pathways, collectively termed the DNA damage response (DDR), which promotes cell survival, and suppresses cancer by promoting genome stability and by triggering programmed cell death pathways. Components of the DDR are often defective in cancer, but because the DDR is a complex network of interacting/cross-talking pathways, a defect in one DDR component can be compensated by alternative pathways. The DDR is a major determinant of cancer cell responses to chemo- and radiotherapy, most of which cause DNA damage directly or indirectly, thus DDR components are enticing targets in the quest to augment cancer therapy (1-6).

Compensatory pathways within the DDR network also represent formidable obstacles to successful cancer treatment. By improving our understanding of DDR pathways, synthetic lethal relationships can be identified among different parts of the network that can be exploited to augment cancer therapy in general, and to develop personalized therapies based on knowledge of specific

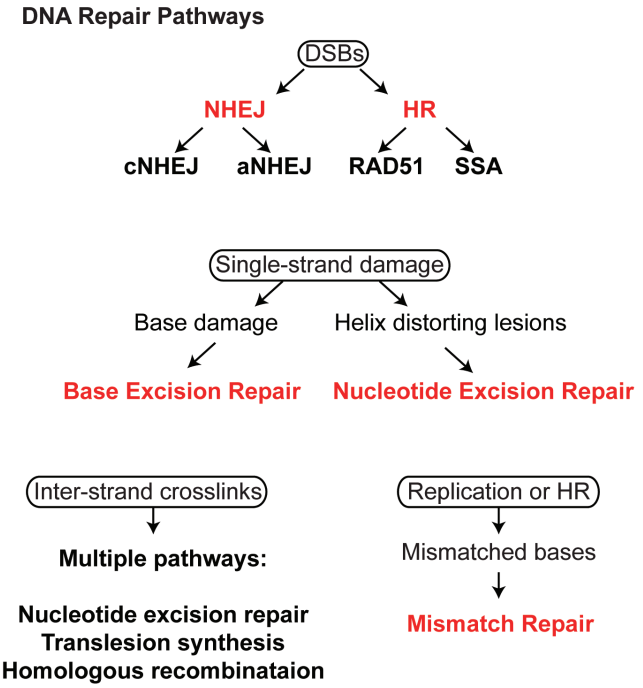

Figure 1 Various types of DNA damage (boxes) are processed by five distinct repair pathways (red font). Interstrand crosslinks require multiple repair pathways.

DDR defects in patient tumors (7-10). This information may also be used to predict how further changes in the DDR (comprising DDR component inactivation, down-regulation, or up-regulation) may compensate for prior defects and confer resistance to general or personalized therapeutics. Such predictions may permit oncologists to monitor tumor response to initial therapy and insert a new line of attack when resistance develops, or perhaps block the compensatory resistance pathway as part of the initial therapeutic strategy. This latter strategy may ultimately prove the most effective given recent insights that contrary to prior models that posited that cancer therapeutics induced mutations that confer resistance, the vast genetic heterogeneity characteristic of most solid tumors indicates that mutations that confer resistance to therapeutics are present subclonally prior to treatment (11). In this view, chemo- or radiotherapy simply selects for pre-existing resistant cancer cells, which can account for the significant rate of failed local tumor control in clinical settings. Translational research in ionizing radiation-induced DNA damage is a very active field, as the complexities of the DDR challenge researchers to define DDR defects in particular tumor types (and in specific patient tumors) and determine how to exploit these defects in personalized treatment to improve therapeutic outcomes. A particular focus is to develop rational strategies to inhibit redundant DDR pathways, so-called synthetic lethal approaches (6,9,10,12,13).

DDR pathways

The DDR includes five major DNA repair pathways (some with sub-pathways; *Figure 1*) that process different types of DNA lesions. DNA damage can arise spontaneously as a result of chemical lability of DNA, or is induced by reactive oxygen species produced by normal cellular metabolism, nucleases such as RAG1/2 (14) or exogenous genotoxins including most cancer chemotherapeutics and ionizing radiation. The DDR also includes two DNA damage checkpoint signaling pathways (*Figure 2*), one centered on ATM that responds to double-strand breaks (DSBs), and one centered on ATR that is triggered by single-stranded DNA (ssDNA) that forms when replication forks are blocked by DNA lesions (replication stress), and when broken ends of DSBs are resected (15,16). ATM and ATR are members of the PI3-kinase-like kinase (PIKK) family that also includes DNA-PK. Together, PIKKs are "early responders" to DSBs and replication stress caused by single-strand damage and intra- and inter-strand crosslinks. Once activated, PIKKs phosphorylate large networks of proteins (17-19) including the downstream effector kinases Chk1 and Chk2 that phosphorylate p53 and other targets to arrest the cell cycle in response to damage, promote DNA repair, and promote programmed cell death pathways when damage is too extensive (16,20-22). The DDR thus presents two general targets that can be manipulated for therapeutic gain: inhibiting DNA repair sensitizes cells to damage, and inhibiting checkpoint signaling prevents cell cycle arrest in response to damage, which increases replication stress (23-27). Great strides have been made in recent years to improve our understanding of DDR proteins and processes and this has been exploited to translate basic knowledge from the lab bench to preclinical models and in several notable cases to human clinical trials and clinical practice. Because the DDR is a complex network of cross-talking and redundant pathways, inhibiting a single pathway may have limited utility. For cancers with a specific defect, targeting a redundant pathway can cause synthetic lethality (9,10). This strategy is exemplified by the successful treatment of HR-defective (BRCA1/2-mutant) breast and ovarian cancers with PARP1 inhibitors (7,28). However, the highly

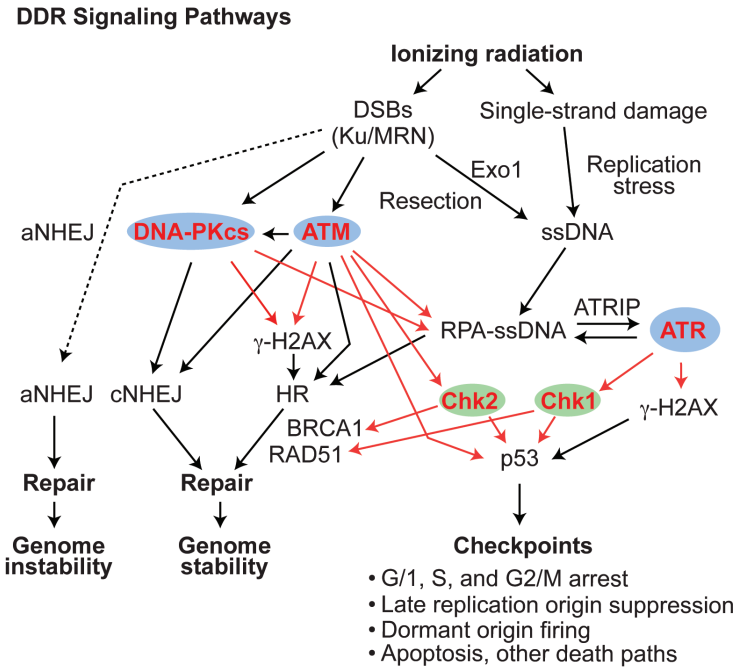

Figure 2 DDR signaling pathways. Ionizing radiation creates DSBs and single-strand damage that triggers PIKK activation (blue), which activates downstream effector kinases (green). Black arrows indicate functional pathways, red arrows indicate target phosphorylation by protein kinases. DDR, DNA damage response; PIKK, PI3-kinase-like kinase.

networked DDR also provides means for tumor cells to develop resistance to targeted therapies, and strategies are being developed to identify and block resistance pathways (29-32).

Repair of IR-induced DNA damage

IR creates DNA DSBs and many types of single-strand damage (33). DSBs have gained the most attention due to their greater cytotoxicity and risk of triggering genome instability (22,34). In mammalian cells, DSBs are primarily repaired by classical non-homologous end joining (cNHEJ). cNHEJ is fast and error-prone, but it tends to produce small deletions and insertions at junctions so it prevents large-scale genome instability and suppresses cancer (35,36). DSBs are also repaired by homologous recombination (HR), which is slow and generally accurate, but it is largely limited to S/G2 phases (37). Alternative NHEJ (aNHEJ; also called microhomology-mediated end joining) can serve as a back-up to cNHEJ (38). aNHEJ is more error-prone than cNHEJ and more likely to create large-scale genome rearrangements such as translocations (38-40). Since cNHEJ is faster and more efficient than aNHEJ, the risk of genome destabilization by aNHEJ is low unless cNHEJ is defective (40-43). Single-strand damage (single-strand breaks, base damage, abasic sites) are repaired by a set of base excision repair (BER) pathways regulated by PARP1 (44), and by nucleotide excision repair (NER).

DSB repair pathway choice is primarily determined by end resection (37). cNHEJ operates on unresected ends, catalyzed by the MRE11/RAD50/NBS1 (MRN) complex which trims radiation-damaged bases at broken ends prior to joining by Ku70/Ku80, DNA-PKcs, Lig IV, and XRCC4 (45). Broken ends at IR-induced DSBs can have a variety of chemical structures, some of which require processing by the Artemis nuclease (46). End resection is initially catalyzed by CtIP; this limited 5' to 3' resection can reveal microhomologies that serve to align ends for aNHEJ (47). HR requires extensive 5' to 3' resection (hundreds to thousands of nt), catalyzed by Exo1-BLM or Dna2-BLM (48-50). The long 3' ssDNA tails are initially bound by RPA, then RPA is exchanged for RAD51, and the RAD51-ssDNA filament searches for and invades a homologous sequence elsewhere in the genome. HR typically uses the sister chromatid as a repair template, as this reduces the chance for HR-mediated genome

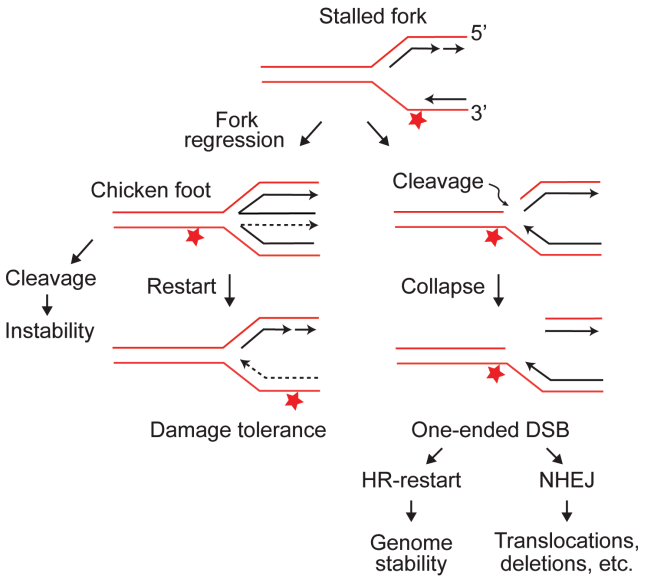

Figure 3 Sample fates of stalled replication forks. Stalled forks may regress to chicken foot structures that can serve as intermediates in an HR-dependent, lesion tolerance pathway; such structures may be cleaved, causing instability. Alternatively, stalled forks may be cleaved, causing fork collapse to one-ended DSBs. Collapsed forks may be restarted by HR, which preserves genome stability, or broken ends may be joined by NHEJ with ends from other collapsed forks, causing genome instability. HR, homologous recombination; NHEJ, non-homologous end joining.

rearrangements and large-scale loss of heterozygosity (6,51). Many proteins function with RAD51 in mediating HR, including BRCA1, BRCA2, PALB2, BCCIP, five RAD51 paralogs (XRCC2/3, RAD51B/C/D), RAD54/B, and a growing number of Fanconi anemia proteins involved in HR repair of certain types of lesions (e.g., interstrand crosslinks) (52).

IR creates DSBs by two distinct mechanisms. IR induces frank DSBs by direct energy absorption or more commonly through production of reactive oxygen species, and evidence of these DSBs (i.e., γ-H2AX foci) is apparent almost immediately after IR. IR also induces secondary DSBs several hours after IR—these appear several hours after irradiation, when replication forks encounter unrepaired single strand breaks or single-strand lesions, causing replication stress (53,54). Note that for every DSB produced by IR, approximately 30-fold more single-strand breaks are produced, along with numerous single-strand lesions such as broken rings, abasic sites, and interstrand crosslinks (55,56). Stalled forks are initially stabilized by checkpoint and repair factors including ATR, BLM, and BRCA2 (27,57-60), but if not restarted in timely manner blocked forks can be cleaved by structure-specific nucleases including the MUS81-EME2 complex and EEPD1, and possibly Metnase, causing fork collapse to DSBs (61-65). Importantly, the replication-associated, secondary DSBs induced by IR contribute to cell killing (53).

Stalled and collapsed forks are preferentially restarted via HR which maintains genome stability and suppresses cancer (66,67). However, HR is a double-edged sword: when forks are not restarted in timely manner, HR factors can mediate formation of branched structures including "chicken feet" (68) (*Figure 3*), a recombination intermediate in a damage tolerance pathway. Some structures are toxic recombination intermediates that can induce genome instability or cause cell death if not resolved properly (69,70). The alternative to HR to repair DSBs at collapsed forks is NHEJ (40), but because DSBs at collapsed replication forks are one-ended (*Figure 3*), joining of DSBs at different collapsed forks causes large-scale genome rearrangements including deletions and chromosome translocations (71-73), or cell death if essential genes are inactivated or dicentric chromosomes are produced. From the discussion above it is clear that the tumor-killing effects of IR-induced DNA damage can be augmented in several ways. Repair of frank DSBs can be suppressed by inhibiting cNHEJ; repair of replication-associated, secondary DSBs can be suppressed by inhibiting HR; and replication stress can be increased by inhibiting repair of single-strand damage, e.g., with PARP1 inhibitors (74) or by blocking replication arrest with checkpoint inhibitors, which increases encounters of replication forks with unrepaired lesions. Given that the highly networked DDR provides tumor cells with ample "escape routes", the most effective approaches may rely on multiple layers of targeting to achieve synthetic lethality, for example, by exploiting specific tumor weaknesses such as HR defects in some breast, ovarian, and other cancers with PARP1 inhibitors, or inducing "artificial synthetic lethality" by targeting multiple repair or checkpoint pathways in combination with radiotherapy (75).

Distinctions between low and high linear energy transfer (LET) IR-induced DNA damage and repair

DNA repair systems evolved to deal with the constant threat of DNA damage from exogenous sources (UV light, low-level background ionizing radiation, genotoxic

chemicals) and from endogenous ROS produced during oxidative metabolism. For the most part, DNA damage from these sources is widely dispersed, and repair pathways are highly efficient repairing such damage (76). LET is a measure of ionization density along a radiation track, and low LET IR largely produces dispersed DNA lesions. High LET IR, such as carbon and iron ions, create densely ionizing tracks that create complex, clustered DNA lesions including clustered DSBs, and clustered single-strand damage (77-82). Clustered DNA damage is repaired slowly or not at all, and this is a key reason why high LET carbon ions have 2–3-fold higher relative biological effect than low LET IR (83,84). It has been argued that the cytotoxic effects of low LET IR are largely due to occasional clustered lesions (78,83). In this view, the increased cytotoxicity of high LET IR simply exploits the intrinsic weakness of DNA repair systems to process clustered lesions. This can be understood as a consequence of the lack of natural selective pressure to develop systems capable of efficiently repairing clustered DNA damage. Clinical success with high LET carbon ions probably also reflects the fact that high LET IR has lower dependence on oxygen (83,84), thus hypoxic tumors resistant to traditional radiotherapy (e.g., head/neck, melanoma, and pancreatic cancers) are more readily controlled by carbon ion radiotherapy (85,86). The natural resistance of hypoxic tumors to low LET IR is a growing concern given recent evidence that most solid tumors have hypoxic regions (87).

It is well established that clustered damage is repaired less efficiently than dispersed damage (78,79,81,83). Several studies indicate that HR plays a more important role in repair of damage created by high LET IR than low LET IR (82,88,89), at least in part due to increased resection of complex lesions induced by high LET IR (90), which promotes HR and suppresses cNHEJ. The difficulty in achieving proper repair of clustered DSBs does not depend on associated (clustered) single-strand damage, as clustered DSBs at engineered I-SceI nuclease sites greatly increases cell killing and causes massive genome instability (chromothripsis, chromosome translocations) that increases with increasing DSB density (91). The chromosome translocations were formed by aNHEJ, indicating that clustered DSBs are refractory to repair by cNHEJ (91). These results also indicate that high LET IR-induced chemical modifications at broken ends does not account for inhibition of cNHEJ, as I-SceI-induced DSBs have "clean" ligatable ends. It appears instead that DSB clustering *per se* suppresses cNHEJ.

Targeting the DDR to sensitize tumor cells to IR

Because of the cytotoxicity of DSBs, DSB repair proteins are attractive targets to sensitize tumors to radiotherapy. As cNHEJ is a primary determinant of resistance to low LET IR (X-rays, γ-rays, protons) throughout the cell cycle (45,92,93), blocking cNHEJ has long been pursued as a general strategy to radiosensitize tumors to IR, principally with inhibitors of DNA-PKcs (94,95). However, early DNA-PKcs inhibitors were relatively non-specific (96), and later versions with high specificity suffered from other problems, such as low bioavailability and rapid clearance or inactivation *in vivo* (97). The Turchi lab is currently investigating Ku inhibitors (3). LigIV inhibitors have also been investigated, but to date they have shown off-target effects or activities are too low to be therapeutically useful (98,99). In general, the promise of cNHEJ inhibition revealed in biochemical and cellular studies has not translated well to preclinical or clinical studies (3).

With the recognition that certain cancers harbor HR defects, the success in treating such cancers with PARP1 inhibitors, and the importance of HR for managing replication stress, including IR-induced replication stress, there is increasing interest in targeting HR, and RAD51 in particular, for therapeutic gain. RAD51 is frequently overexpressed in cancers (100), which is associated with therapeutic resistance, poor prognosis, and increased metastasis (101). Overexpression of RAD51 is likely both an adaptation to oncogenic replication stress (27,102) and a driver of tumor progression via genome destabilization (103,104). RAD51 may be targeted by genetic means (e.g., siRNA knockdown or expression of micro-RNAs that inhibit RAD51), or by using small molecules to inhibit RAD51 biochemical functions (101). Downregulation of RAD51 preferentially sensitized tumor *vs.* normal cells to cisplatin-induced DNA damage (105). Although this suggests similar benefits may be seen with radiotherapy, one study failed to show such benefits with glioma cells (106). This might reflect the fact that HR plays a lesser role than cNHEJ in conferring resistance to IR. The effects of RAD51 inhibition might be more pronounced if cell survival was more dependent on managing IR-induced replication stress, an idea supported by improved outcomes when RAD51 inhibition is combined with PARP1 inhibition (106).

Personalizing cancer therapy based on known defects in DDR factors through synthetic lethal approaches is a rapidly expanding research area, both in the lab and in clinical practice (2,4,6-10,12,13,107-111). However,

there are additional opportunities for targeting cancer by inducing "artificial synthetic lethality", that is by combining radiotherapy with drugs that inhibit redundant DNA repair pathways. Studies of inhibitors of heat shock protein 90 (Hsp90) illustrate the value of this approach. Hsp90 is a molecular chaperone responsible for the conformational maintenance of a number of client proteins that play key roles in cell cycle arrest, DNA damage repair, and apoptosis following radiation (112). Inhibition of Hsp90 by different substances increases radiosensitivity of various cancer cell lines; however, although Hsp90 is the target for inhibition, radiosensitization reflects effects on various downstream proteins (113). Early Hsp90 inhibitors were based on the natural compound geldanamycin and its derivatives, 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-DMAG (114,115), and next evolved to the orally bioavailable BIIB021 (116), all of which sensitize cells to IR. Studies of 17-AAG demonstrated radiosensitization of tumor cells resulted from downregulation of HR via BRCA2 degradation, and decreased RAD51 function (117). Subsequent studies revealed that second and third generation Hsp90 inhibitors PU-H71 and TAS-116 downregulated both HR and cNHEJ factors (118-120), thus these agents inhibit both major DSB repair pathways, and therefore induce a type of artificial synthetic lethality in combination with IR. Importantly, Hsp90 adopts different conformations in tumor *vs.* normal cells (121), thus Hsp90 inhibitors radiosensitize tumor cells, but not normal cells (117-120). In addition, these effects are seen in both p53 WT and p53 mutant tumor cells (122), suggesting the potential for broad use in cancer therapy. The advanced Hsp90 inhibitors that block both HR and NHEJ may prove especially useful in combination with high LET IR, given the more balanced reliance on both cNHEJ and HR in repairing clustered DNA damage (123).

ATM and ATR are master regulators of DNA damage checkpoints (cell cycle arrest) through Chk2 and Chk1, respectively (15,16), and they also promote HR (124,125). Given that these kinases phosphorylate many hundreds of targets (19), it is difficult to pinpoint radiosensitization mechanisms. Nonetheless, it is well-established that ATR suppresses origin firing, protects stressed replication forks by stabilizing the replisome, and promotes fork restart through RPA phosphorylation (15), and ATM is an important regulator of the G1/S checkpoint. Thus, inhibiting checkpoint proteins can enhance IR-induced cell killing by at least two mechanisms: inhibiting DNA repair, and increasing replication stress. ATM inhibitors such as KU55933 and KU60019 sensitize cells to IR (124,126-129) and the latter has been shown to sensitize cancer (glioma) cells but not normal fibroblasts (129). VE-821 and AZD6738 are specific ATR inhibitors currently in clinical trials as mono-therapy and in combination with radiotherapy for solid tumors (110). Chk1 inhibition by siRNA increases DNA damage-induced apoptosis and radiosensitizes p53-deficient cancer cells (130), and selective Chk1 inhibitors, CEP-3891, Chir-124, and UCN-01 also enhanced cellular radiosensitivity (131). Small molecule inhibitors of Chk1 (SAR-020106), Chk2 (PV1019), and Chk1/Chk2 (AZD7762 and XL-844) have also been tested in preclinical studies in combination with IR (110). PV1019 is particularly interesting as it selectively kills or suppresses growth of cancer cells that overexpress Chk2 while protecting normal thymocytes from IR-induced apoptosis (132).

Translational research in IR-induced DNA damage: rodent models

The ability to study IR-induced DSBs in an animal model system have allowed a comprehensive evaluation of biological effects, as well as the development of novel agents for radiotherapy, radiation protection, and treatment of radiation injury. There are numerous spontaneous and experimentally induced mutation syndromes caused by the dysfunction of various components of the DSB repair pathways that manifest as certain radiation hypersensitivity and chromosomal instability phenotypes. For example, mutation of the NHEJ process critical for T- and B-lymphocyte receptor development was used to create scid mice (133,134). When scid mice were irradiated, it was discovered that myeloid cells and fibroblasts were markedly more radiosensitive than those from control mice (133). Below we highlight examples of translational studies utilizing preclinical rodent models to understand how manipulation of IR-induced DNA damage repair and signaling can lead to clinical advances in radiation oncology. As described, pathways involved in signaling and repair of IR-induced DSBs are critical targets for cytotoxicity and the outcome of radiation therapy. Translational studies using rodent models to test the efficacy of combined DDR-modulating compounds and IR have been performed with inhibitors of ATM, DNA-PKcs, Chk1/Chk2, PARP1, and Hsp90.

Mutations in ATM cause radiation hypersensitivity in patients with the autosomal recessive disorder, ataxia-

telangiectasia; therefore, inhibition of ATM within tumors is a therapeutic approach designed to disrupt DSB repair and cause tumor radiosensitization. KU55933 is a relatively specific ATM inhibitor, but its analog KU60019 is more effective at blocking ATM phosphorylation (129). Using orthotopic xenograft models of glioblastoma multiforme in mice, it was demonstrated that combined IR and KU60019 significantly increased the survival of mice by 2- to 3-fold when compared with controls (135); importantly, tumor radiosensitization was more pronounced in mice harboring p53-mutant glioblastoma xenografts than mice with genetically matched p53-wild-type tumors (135). The ability of ATM inhibitors to sensitize p53-deficient cancer cells to IR is critical in light of the tumor resistance to therapy associated with p53 mutations, and the high frequency of p53 mutations in malignant tumors (136).

A novel and potent mTOR inhibitor, NVP-BEZ235, was shown to also inhibit ATM and DNA-PKcs (137), which have catalytic domains highly homologous to phosphoinositide 3-kinases (138,139). mTOR is also a PIKK family member, thus NVP-BEZ235 is a general PIKK inhibitor. NVP-BEZ235 blocks both cNHEJ and HR repair pathways and significantly attenuates DSB repair (137). In addition to reducing phosphorylation of ATM targets and G2/M cell cycle checkpoint activation, NVP-BEZ235 was shown to radiosensitize a panel of glioblastoma multiforme cell lines. Although the effects of IR and NVP-BEZ235 on tumor growth delay were not investigated, mice bearing subcutaneous glioblastoma multiforme xenografts were used to demonstrate that NVP-BEZ235 significantly impairs IR-induced DSB repair, validating the efficacy of this drug as a DNA repair inhibitor *in vivo* (137). However, as discussed by Goldstein and Kastan (136), a potential drawback of NVP-BEZ235 is that it also inhibits ATR, which is crucial in resolving stalled replication forks, and more generally for cell survival (140). Disruption of ATR is lethal at both the cellular and organism levels (141,142). As such, NVP-BEZ235 was predicted to induce unacceptable systemic toxicities in patients (136). Indeed, a phase II study of NVP-BEZ235 in patients with advanced pancreatic neuroendocrine tumors revealed that the drug was poorly tolerated and, because of this, it failed to reach the second stage of the study despite observed disease stability (143).

As noted above, tumor radiosensitization can be achieved via inhibition of Chk1- and/or Chk2-mediated checkpoint signaling. Rodent models have been used to demonstrate the efficacy of Chk1 inhibitors as cancer therapeutics. UCN-01 proved to be efficacious as a single agent in delaying tumor growth of orthotopic and subcutaneous xenograft glioblastoma multiforme tumor models in mice (144), and the Chk1 inhibitor MK8776 sensitized subcutaneous pancreatic cancer xenografts to combination therapy with gemcitabine and IR (145). In an alternative approach, a genetically modified mouse model revealed that $Chk2^{-/-}$ mice were more resistant to normal tissue radiation damage than WT mice, as characterized in splenic lymphocytes, thymocytes, and neurons of developing brains (146). As noted above, the Chk2 inhibitor PV1019 protects normal thymocytes from IR-induced apoptosis while simultaneously showing antiproliferative effects in cancer cells that express Chk2 at high levels (132), but this compound has not yet been tested in animal models.

Because IR-induced base modifications and single-strand breaks can indirectly induce toxic DSBs by blocking replication forks, inhibition of BER provides another means of radiosensitization. PARP1 promotes repair of single-strand lesions and it has long been known that PARP1 inhibition or genetic deletion enhances radiosensitivity (147-149). Genetically-modified $PARP1^{-/-}$ mice are hypersensitive to whole body irradiation compared to WT controls (150), and PARP1 inhibitors have been demonstrated to radiosensitize rodent xenograft models of human cervical carcinoma (151), colorectal cancer (152), lung cancer (153,154), head and neck squamous cell carcinoma (155), glioblastoma (156), and colon cancer (157). These preclinical studies were critical to the translation of PARP1 inhibitors to human clinical trials.

Hsp90 inhibitors suppress HR by downregulating RAD51 and/or BRCA2 (112,117-120). Preclinical studies of Hsp90 inhibitors have been promising. *In vivo* radiosensitization with various Hsp90 inhibitors has been shown in human tumor xenograft models of cervical (158), prostate (159), and head and neck squamous cell carcinoma (116). A synthetic alternative, NVP-AUY922, was also developed and shown *in vivo* to delay tumor growth and increase end-point survival in a head and neck squamous cell carcinoma xenograft model (112). Visual impairment has been a serious toxicity effect associated with early-generation Hsp90 inhibitors, and this has been largely overcome by the development of TAS-116 (160). Lee *et al.* (119) investigated the radiosensitizing effects of TAS-116 in low LET X-ray and high LET carbon ion-irradiated human cancer cells and mouse tumor xenografts. TAS-116 decreased cell survival of both X-ray and carbon ion-irradiated human cancer cell lines (HeLa and H1299 cells), and similar to other Hsp90 inhibitors, it did not affect radiosensitivity of

noncancerous human fibroblasts. The combined treatment of mouse subcutaneous cervical tumor xenografts with carbon ions and TAS-116 significantly delayed tumor growth compared to controls (119). In another HR targeting approach, growth of HeLa cell tumor xenografts in mice was significantly reduced by cisplatin combined with RAD51 knockdown (105), suggesting similar effects might be obtained by combining RAD51 inhibition with radiotherapy.

While there is great value in these preclinical rodent models for studying radiation-induced DNA damage repair and therapeutic applications of DDR inhibitors, it is important to recognize the limitations of this model for translational science. First, in order to study human cancers using the standard subcutaneous xenograft model *in vivo*, the mouse immune system must be significantly altered, such as with athymic nude or severe combined immunodeficiency (SCID) mice. However, the immune system strongly influences overall therapeutic responses of cancer patients. Further, while murine orthotopic tumors are grown within their host tissue and mimic local tumor growth and metastasis, xenograft models usually rely on highly passaged cell lines and clonal selection may not mimic human tumors arising spontaneously in patients. Alternatives to xenograft models are genetically engineered animal models (GEM), where oncogenes are activated and/or tumor suppressor genes are inactivated to induce tumors *in situ*, generally via the temporally controlled and tissue-specific expression of CRE recombinase (161-163). Tumors initiated within GEM rodent models develop in a more natural microenvironment, with supporting vasculature, stromal cells, and an intact immune system; however, inducible tumors tend to be artificially homogenous, lacking the vast heterogeneity seen in natural, spontaneous cancer (164,165). Finally, murine tumor models frequently error towards false positive therapeutic results, with cancer cures in mice frequently failing to translate to humans (166). Despite these limitations, preclinical research in rodent models is a crucial and necessary step in taking positive therapeutic results from cell lines to the clinic. In the next section we discuss advantages of translational research that involves clinical trials to treat spontaneous tumors in companion animals.

Past, present and future translational research opportunities with clinical trials to treat spontaneous tumors in companion animals

The value of spontaneously occurring tumors as a tool for translational research has recently received increased attention by cancer scientists. In June 2015, the US National Academy of Science Institute of Medicine's National Cancer Policy forum hosted a workshop on comparative oncology. Comparative oncology was defined as the study of naturally occurring cancers in animals as models for human disease. This workshop titled "*The Role of Clinical Studies for Pets with Naturally Occurring Tumors in Translational Cancer Research*" explored a number of topics including the rationale for clinical trials, canine tumor biology, and lessons learned from comparative oncology (167). Although the focus of the workshop was primarily on the role of comparative oncology in drug development, the take-home messages are broadly applicable to cancer therapy in general including radiotherapy.

Current interest not-withstanding, comparative oncology in the field of radiation oncology has been ongoing since the discovery of X-rays. Understanding normal tissue tolerance and regulatory factors is the cornerstone of radiation oncology. The effects of the radiation therapy should not be worse than the disease, so radiation dose is constrained by normal tissue tolerance. While speciation resulted in gradual changes over the course of evolutionary time, DNA repair is critical for the maintenance of genome integrity, and DNA repair mechanisms are highly conserved (168). Thus, the radiation sensitivity, and the radiation tolerance of normal tissues, is similar for most mammalian species. This means that radiation therapy data from humans is valuable for the treatment of veterinary patients, and vice versa. While naturally occurring tumors are used in a wide array of studies, including evaluation of chemotherapy and cancer imaging, here we focus on early studies that evaluated tumor control and radiation effects to demonstrate important principles in radiation oncology, and on more recent molecular advances that highlight the importance of comparative oncology in radiotherapy research.

Shortly after Roentgen discovered the X-ray in 1895, both human and animal skin tumors were treated empirically. With minimal scientific methodology, encouraging responses to treatment were reported in human and veterinary patients, including dogs and horses (169-171). The fields of human and veterinary radiation oncology followed parallel paths and information was frequently shared regarding treatment outcome and normal tissue effects at national and international meetings (172). Despite early excitement, radiation therapy did not provide an effective or easy treatment for cancer. Tumor control

was not durable, and patients were often plagued with severe radiation effects (173). Dr. Henri Coutard was a radiation oncologist at the Curie Institute in Paris whose keen observations changed radiation oncology (174). By the 1920s he was studying fractionation schemes using a more scientific method. He evaluated the impact of dose per fraction, total dose, tumor size, field size, and overall treatment time on tumor control and adverse radiation effects. While these studies did not elucidate an understanding of underlying biology, they did provide a protocol that could be safely delivered and resulted in more durable tumor control. During the same period, Dr. Alois Pommer, a veterinarian at what was then called the Vienna Veterinary High School, received funding from the Rockefeller Institute to start a radiation therapy program for animal patients. Pommer, like Coutard, published extensively on fractionation schedules, tumor control and radiation effects (173,175). These two pioneering radiation oncologists, both of whom used orthovoltage radiation equipment, provided a template for safe treatment for future decades.

By the late 1960s scientists had a rudimentary understanding of radiation biology, based on cell culture experiments and work with rodent tumor models. The Elkind lab reported that mammalian cells in culture could repair radiation damage, explaining one of the benefits of delivering dose in fractions instead of in large single doses (176). This led to other hypotheses about mechanisms underlying the efficacy of fractionated radiation therapy such as Wither's description of the four Rs: repair, redistribution (in the cell cycle), repopulation, and reoxygenation of hypoxic regions (177). But cells in culture are isolated systems, and induced tumors in rodents lacked the complexity of human tumors. Naturally occurring tumors in companion animals (dogs and cats) share many commonalties with human tumors (178-180). Histological appearance and behavior of some animal tumors are markedly similar. The relative size of the tumor to the body is more comparable to that in rodent tumors, yet the gross tumor size is large enough for serial sampling. The size of the patients makes sophisticated imaging, treatment and monitoring possible, using the same technology used for humans. Most importantly, the tumors share similar microenvironments. In both species tumors can be acutely or chronically hypoxic, and intra- and extracellular pH may be altered. Genetic changes observed in human tumors are seen in canine tumors, and unlike many rodent models, the immune system is intact (180).

Dr. Edward L. Gillette helped establish a Veterinary Radiation Oncology program at Colorado State University in the late 1950s, with a vision of using naturally occurring tumors as a model for human disease. In 1968 he worked with Drs. Herman Suit and Rodney Withers at M.D. Anderson Cancer Center (181). These colleagues saw the value of the dog model and they encouraged Gillette to pursue research in comparative oncology. Gillette's work in intraoperative radiation therapy set the standard for normal tissue tolerances in human and veterinary medicine, and his work evaluating the impact of fraction size and field size helped create data on α/β ratios that are still used by both veterinary and human radiation oncologists (181-191). But it was Gillette's work using spontaneous tumors to evaluate radiation biological principles that elevated the value of the naturally occurring tumor model. Therapeutic gain in the context of radiotherapy was based on the concept that to improve treatment outcome (tumor control) while maintaining quality of life (limited late effects), both tumor control and late effects needed to be evaluated and compared between different treatments. Tumor control without complications is the ultimate exploitation of differences in DNA repair characteristics between tumor cells and late responding normal tissues. Gillette was able to prove this principle because of the flexibility for trial design in the naturally occurring tumor model in veterinary patients. He first conducted a trial in which dogs with naturally occurring squamous cell carcinomas of the oral cavity were randomized to receive different total doses of fractionated radiation. The patients were followed through their lifetime for tumor control and late radiation effects. He used this information to determine the dose that provided the best tumor control with least complications, and these radiation dose groups were also tested with hyperthermia treatments (192,193). These studies helped inform the design of clinical trials in human patients.

These early clinical studies of normal tissue and tumor responses to radiation in naturally occurring tumors in dogs led scientists to further utilize this translational model by evaluating DNA repair characteristics in normal dogs and in canine tumors. Sequencing of the canine ATM mRNA demonstrated high homology with the human counterpart, both in the promoter and overall gene structure, facilitating comparative studies of ATM function in dogs and a potential model for ATM deficiency (194). There is significant overlap between deregulated human and canine genes in mammary tumors, as well as from normal mammary tissues (195). ATM mRNA and protein

expression were shown to be downregulated in canine mammary tumors (196), consistent with frequently observed checkpoint and DNA repair defects in human tumors (2,16). Recently the molecular mechanisms of both XLF and Ku-dependent NHEJ in canines was evaluated and proposed as a platform for development of novel chemotherapies for dogs and humans (197,198). Human cancer cell lines have been extensively characterized over the years, but only recently have in-depth studies of radiation sensitivity, including analysis of radiation-induced DSB repair been undertaken. Twenty-seven established canine cell lines were evaluated and radiation-induced DSB repair was related to radiation sensitivity, as previously shown in human tumors (199). Thus, canine cancer models present many translational research opportunities to exploit fundamental knowledge about DNA repair to improve radiotherapy.

Conclusions

There was broad consensus at the Institute of Medicine's National Cancer Policy Workshop that a more detailed characterization of the canine genome, and expansion of comparative oncology research will define similarities and differences in dog and human cancers to benefit both companion animal and human patients (167). There are broad opportunities to apply well-established clinical trials techniques to explore promising leads in radiotherapy research: To augment and personalize radiotherapy using many forms of tumor molecular profiling (transcriptomics, metabolomics, DDR network analysis, etc.) and novel combination chemo-radiotherapies. By developing tools to evaluate DDR proteins in canine cells (197) or in treated tumors, biological responses to therapy can be defined and correlated with treatment outcomes to seek improved therapeutic strategies.

There are many key frontiers in radiation oncology that can be advanced through expansion of comparative oncology research; several are highlighted here. The past decade has produced an explosion of information about DDR "strengths and weaknesses" that are just beginning to be exploited in clinical settings (1,2,5,101,107,110,111,200,201). DDR manipulation can involve radiosensitization of tumors, as well as radioprotection of normal tissue which would allow safe delivery of increased doses to tumors. Companion animals will likely play a key role at the new frontier of radio-immunotherapy with PD-1 and PD-L1 inhibitors, to unleash the power of the immune system to "clean up" micrometastases (and perhaps even well-established metastases) by enhancing the abscopal effect (201,202). Tumor imaging through advanced radiologic methods remains a critical foundation for radiotherapy, and an area with great potential for diagnostic and theranostic progress (again, best studied in large, easily imaged, animals), especially with novel PET isotopes linked to tumor-tropic biologicals or small molecules (203,204). Carbon ion radiotherapy, with >22 years human clinical practice and >20,000 human patients treated, is making great strides (85), but it is clear that to accelerate our understanding of the complexities of tumor and normal tissue responses to heavy and light ion radiation we must attack on all fronts: at the basic cell and molecular level, with preclinical small animal models, and with clinical studies of naturally occurring tumors in companion animals. This integrated approach offers the best chance to rapidly translate life-saving cancer cures to human clinical practice.

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