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Accumulation of DNA double strand breaks in normal tissues after fractionated low dose irradiation

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Abbreviation

53BP1	tumor suppressor p53-binding protein 1
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia mutated and Rad3-related
BLM	Bloom syndrome protein
BRCA1	breast cancer 1 susceptibility protein
BRCA2	breast cancer 2 susceptibility protein
BRCT	BRCA1 C-terminal
CHK	checkpoint kinase
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	(DNA) double-strand break
ERCC1	Excision repair cross-complementing protein 1
γ–H2AX	phosphorylated histone H2AX
HAT	histone acetyltransferase
HR	homologous recombination
HRS	hyper-radiosensitivity
hSSB1	human single-strand DNA-binding protein 1
IRR	increasing radioresistance
LNT	linear-no-threshold
LigIV	Ligase IV
MDC1	mediator of DNA damage checkpoint 1

MDM2	murine double minute 2 oncoprotein
MRN	Mre11-Rad50-Nbs1 complex
NHEJ	non-homologous end-joining
PARP-1	Poly [ADP-ribose] polymerase 1
PCNA	proliferating cell nuclear antigen
PIKKs	phosphatidylinositol 3-kinase-related kinases
RIF	radiation-induced foci
ROS	reactive oxygen species
RPA	replication protein A
SCID	severe combined immunodeficiency
SSB	(DNA) single-strand break
STAT1	Signal Transducer and Activators of Transcription factor 1
TBP	TATA-box-binding protein
TEM	transmission electron microscopy
Tip60	Tat interacting protein of molecular weight 60kDa
WRN	Werner syndrome protein
XLF	XRCC4-like factor
XRCC	X-ray repair cross-complementing protein
XPF	Xeroderma pigmentosum group F-complementing protein

1. Abstract

1.1 Purpose

Health effects of low dose radiation continue to be a matter of debate for concerns of radiological estimate and protection. In this present study, we investigated the effects of fractionated low dose radiation exposure on accumulation of DSBs in normal tissues using an *in vivo* murine model. This *in vivo* model may help us to estimate the effects of exposure to environmental and occupational sources of low dose radiation among normal people and help us to assess the long-term effects of normal tissue irradiation in the context of fractionated radiotherapy.

1.2 Materials and methods

After whole body fractionated low dose irradiation of wild-type male C57BL/6 mice, the induction and repair of DSBs were analyzed in kidney and intestine by enumerating 53BP1 foci.

1.3 Results

A single acute low dose irradiation with 10 mGy or 100 mGy induced nearly the identical foci level in the kidney and the small intestine. Persistent foci levels at different time points varied slightly during fractionated irradiation with 10 mGy while slightly elevated, but consistent foci levels were observed during fractionated irradiation with 100 mGy in both kidney

and small intestine. The values of persistent foci in the tubular cells in kidney were always higher than those in the epithelial cells in intestine, independent of the dose per fraction, or analyzed time points.

1.4 Conclusion

A single acute low dose radiation has the same ability to induce DSBs in kidney and small intestine. Persistent foci levels in the tubular cells in kidney were always higher than those in the epithelial cells in the intestine, independent of the dose per fraction, or analyzed time points, which might indicate they have different ability to deal with complex DSBs and chromatin alterations induced by fractionated low dose irradiation.

2. Introduction

Ionizing radiation (IR) is a ubiquitous environmental mutagen and a prototypical DNA-damaging agent. The effects of low-dose and low-dose-rate ionizing radiation continue to be of interest because of the potential dangers posed by exposure to environmental and occupational sources of radiation [1] and because of the potential clinical benefits of radiotherapy [2]. Epidemiological studies indicate that the cancer risk increases with IR exposure even at low doses [3-5], however some experimental evidences support that low-dose radiation increases cellular antioxidant activity [6], facilitates DNA damage repair [7], stimulates immune surveillances [8-10], and suppresses tumor growth, metastasis and carcinogenesis [10, 11]. Large uncertainties remain concerning in the relationship between the ionizing dose in the low dose range and the biological effects.

Ionizing radiation induces a variety of DNA lesions, including single- and double-strand breaks, DNA-protein cross-links, and various base damages. Among all kinds of DNA damages, DNA double-strand breaks (DSBs) are the most closely related to the deleterious consequences. Based on data obtained from high-dose irradiation *in vivo* and *in vitro*, it is assumed that the induction of DSBs is dependent on the irradiation dose. Since direct evidence to prove the dose-response relationship of DSBs in the low dose range irradiation is very limited, the effect of low-dose radiation is usually estimated by an extrapolation from the high-dose range. However, the effect of

radiation-induced DSBs may be different in the high dose versus the low dose levels. Recent findings in radiation and molecular biology strongly challenge this extrapolation and several new conceptions have been proposed on the low dose irradiation.

2.1 DNA double-strand break

2.1.1 DNA damage response (DDR)

A well accepted paradigm in radiation biology is that DNA double-strand break (DSB) is the most deleterious form of DNA damage, making serious threat to the integrity of eukaryotic genomes. DSBs can be induced by exposure to exogenous agents such as ionizing radiation (IR), external mutagens and chemotherapeutics, as well as endogenous agents such as reactive oxygen species (ROS). It can also arise from collapsed replication forks [12, 13] and the processes of V(D)J recombination and class switch recombination (CSR) in the development and maturation of immune system [14-16]. Unrepaired DSBs can lead to cell death while misrepaired DSBs have the potential to produce chromosomal translocations and genome instability, which can contribute to malignant transformations. To protect the integrity of the genome, cells have evolved efficient and rapid repair responses, a sophisticated signal transduction cascade, known as DNA damage response (DDR) which initiates processes such as cell-cycle arrest, DNA repair and apoptosis before DNA replication and cell division [17]. Numerous proteins

engage in this network and can be classified as DNA-damage sensors, mediators, transducers and effectors [18]. Sensors are thought to detect the presence of DNA damage. Although the nature of these sensors and the mechanisms of detection remain unclear, the earliest detectable DNA DSB-induced events involve the MRN [MRE11 (meiotic recombination 11)-RAD50-NBS1 (Nijmegen breakage syndrome 1)] complex [19] and the PIKKs (phosphatidylinositol 3-kinase-related kinases) family such as ATM (ataxia-telangiectasia mutated) and ATR (ATM and RAD3-related) [20, 21]. From these proteins, the DNA damage signal is transmitted to transducer kinases such as CHK1 (checkpoint kinase 1) and CHK2 (checkpoint kinase 2), which target downstream DDR components, as well as amplifying the DDR signal [22]. The signaling between sensors and transducers is thought to be facilitated by mediators or adaptor proteins, such as MDC1 (mediator of DNA damage checkpoint 1), 53BP1 (p53-binding protein 1) and BRCA1 (breast cancer 1 susceptibility protein), which are believed to receive a structural modification by the sensor proteins and this modification is converted to a compatible form for signal amplification by the transducer proteins [23]. The activated sensor and transducer kinases proceed to phosphorylate a number of downstream effectors, resulting in appropriate biological responses depending on the severity of the damage and cell cycle status. Figure 1 shows the current model of the mammalian DNA damage response.

Many proteins known to be involved in DNA damage repair or in signaling

the presence of DNA damage have been shown to localize to the sites of DSBs within seconds to minutes following ionizing radiation, resulting in the formation of microscopically visible nuclear domains referred to as radiation-induced foci (RIF). These proteins include γ-H2AX, ATM, 53BP1, MDC1, RAD51, and the MRN complex (MRE11/RAD50/NBS1). The RIF are dynamic structures and the analysis of these DNA damage related foci allow researchers to gain deeper insights into the effects of ionizing radiation.

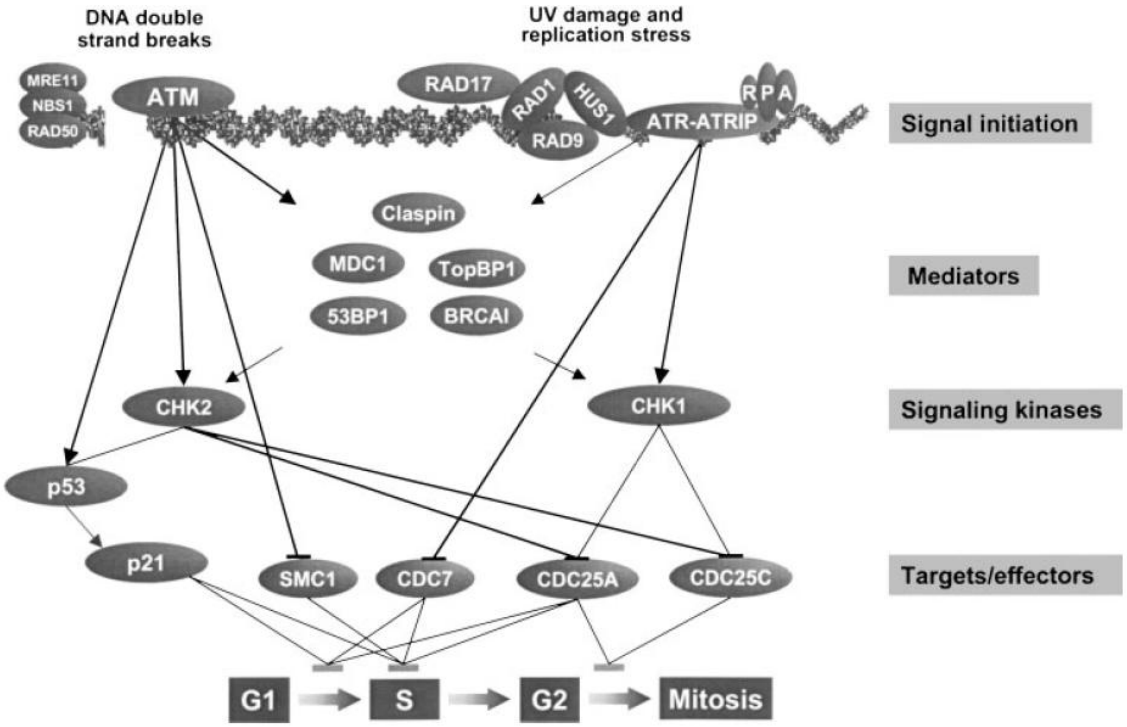

Figure 1: Current model of the mammalian DNA damage and replication checkpoints. A line ending with an arrowhead indicates activation. A line ending with a bar indicates inhibition. Figure was taken from Lei Li and Lee Zou, 2005 [24].

2.1.2 DSB repair pathways

There are two major pathways for DSB repair, namely non-homologous

end-joining (NHEJ) and homologous recombination (HR). In mammalian cells, the majority of radiation-induced DSBs are repaired by NHEJ. NHEJ functions throughout the cell cycle and is the major pathway for the repair of DSBs that arise during G0 and G1 [25]. In addition, NHEJ is responsible for the repair of programmed DSBs generated during V(D)J recombination [14, 16]. In contrast, HR functions preferentially in late S and G2 when an intact sister chromatid is available to act as a template for repair. HR can also use a homologous chromosome as a template and some HR is observed in G0/G1 cells, albeit at a level lower than in S/G2/M phase [26]. One of the main functions of HR is to repair endogenous DSBs that are produced when replication forks collapse [27, 28].

2.1.2.1 Non-homologous end-joining

Non-homologous end-joining (NHEJ) simply pieces together the broken ends and reseals them previously trimmed by various nucleases. The basic steps in NHEJ are (1) detection of the DSB and tethering of the DNA ends; (2) processing the DNA termini to remove non-contactable end groups; (3) religation of the processed DNA ends. Since DNA ends are joined with little or no base pairing at the junction and end joining can be accompanied by insertions or deletions, NHEJ is widely regarded as being error prone. Six core proteins are known to be required for NHEJ in mammalian cells: the Ku70/80 heterodimer (KU), the catalytic subunit of the DNA-dependent protein kinase

(DNA-PKcs), XRCC4, DNA LigIV (Ligase IV), XLF (XRCC4-like factor, also called Cernunnos) and a DNA processing factor, Artemis [29-31]. Deletion or inactivation of any of these core NHEJ proteins induces radiation sensitivity as well as defects in DSB repair and V(D)J recombination [12].

The first step in NHEJ is detection of the DSB by the KU heterodimer. The KU complex is conserved throughout evolution and has a high affinity to the ends of double-stranded DNA, with little or no DNA sequence specificity [30, 32]. It can be rapidly recruited to sites of DNA damage and independently of any other NHEJ or DSB repair proteins [33]. Once bound to the DSB, KU acts as a scaffold to which other NHEJ proteins are recruited. Cells lacking KU are radiosensitive and KU-deficient mice are immunodeficient due to defective V(D)J recombination as well as growth and telomere defects [34, 35]. One of the first proteins shown to interact with KU is DNA-PKcs. DNA-PKcs, the product of the PRKDC gene, is a member of the PIKKs family of serine-threonine protein kinases, and like other PIKKs family members: ATM and ATR, DNA-PKcs phosphorylate its substrate primarily on serines or threonines. DNA-PKcs has very weak protein kinase activity which is stimulated 5-10 fold by its interaction with DNA-bound KU to form the DNA-dependent protein kinase complex, DNA-PK. After assembly of the DNA-PK complex, which tethers the DNA ends together protecting them from inappropriate end processing, DNA-PKcs undergoes autophosphorylation in trans, leading to dissociation of DNA-PKcs from the

DNA ends, which facilitates access of downstream factors to the ends of the DSB [30, 36]. Another role of DNA-PKcs is to recruit the end-processing factor Artemis to DSBs [37]. Artemis has 5'-3'exonuclease activity. In the presence of DNA-PKcs and ATP, Artemis displays endonuclease activity towards DNA hairpins and single stranded DNA flaps [38]. This nuclease activity is required for opening DNA hairpins formed at coding joints during V(D)J recombination. Once the DNA ends have been processed, they are ligated by the XRCC4-DNA LigIV complex. XRCC4 and DNA LigIV are required for both NHEJ and V(D)J recombination and mice lacking either protein die in uterus due to massive neuronal apoptosis [39, 40]. Figure 2 exhibits a schematic illustration of the NHEJ process.

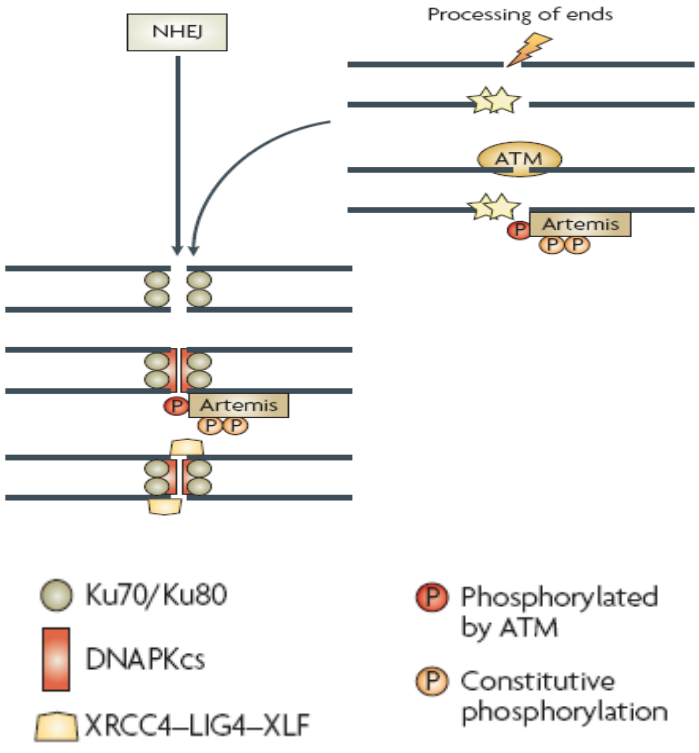

Figure 2: Schematic illustration of the NHEJ process. Figure was taken from Markus Loebrich and Penny A. Jeggo, 2007 [41].

2.1.2.2 Homologous recombination

The second major pathway for the repair of DSBs is homologous recombination (HR). It is a slow process requiring homologous sequences in the form of sister chromatids, homologous chromosomes or DNA repeats as template. Various proteins are involved in this process including RAD51, RAD52, and RAD54 as well as RAD51 paralogues including XRCC2, XRCC3, RAD52B, RAD51C and RAD51D [42]. HR is activated primarily during late S and G2 phase of the cell cycle and considered to be a relatively error free repair pathway [28, 43-45].

As shown in Figure 3, the initial step in HR is detection of the DSB by the MRE11-RAD50-NBS1 complex (MRN), which is followed by 5'-3' resection to produce a long ssDNA 3' extension [28, 43-45]. The ssDNA binding protein RPA binds to the long 3'-ssDNA extension, which prevents premature strand invasion and then the RAD51 forms nucleoprotein complex on ssDNA tails coated by RPA to form a presynaptic filament. Multiple proteins, including RAD52, BRCA2 and the RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) are subsequently involved in the replacement of RPA by RAD51, and stabilization of the filament [44, 45]. After that, the 3'-ssDNA strand acquires the ability to invade the homologous sequence to form an intermediate called a D loop, which named "strand invasion". Then the 3'-DNA end is extended by a DNA polymerase, and a second DSB end is captured by annealing to the extended D loop. This leads to the formation of

one or more crossed strand structure termed Holliday junctions. Finally, the Holliday junctions are resolved to produce either crossover or non-crossover products, depending on which HR subpathway is utilized [28, 43-45]. Other proteins that have been implicated in these steps include RAD54, WRN, BLM, p53, XPF, ERCC1, DNA polymerases delta and epsilon, PCNA and DNA ligaseI [43].

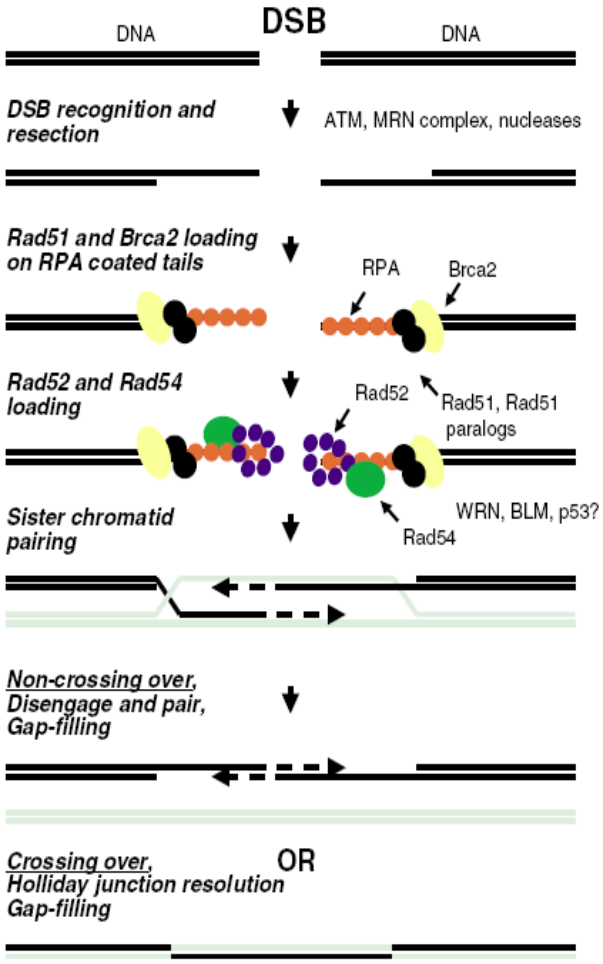

Figure 3: Schematic illustration of homologous recombination. Figure was taken from Valerie *et al.*, 2003 [46].

2.1.2.3 DSB repair pathway choice

The utilization of either NHEJ or HR can be regulated by the nature of the DSB and by cell type and cell-cycle phase. NHEJ is efficient at rejoining

DNA ends with cohesive overhangs, but is less efficient for blunt ends or ends without cohesive overhangs, which can be left to HR [47, 48]. An elegant study analyzed the selective requirement for HR and NHEJ during nervous system development [49] by using mice carrying a germ line disruption of XRCC2 (HR defective) and ligaseIV (NHEJ defective), the two pathways of recombination were found to be spatiotemporally distinct: HR inactivation was crucial from the early steps of embryogenesis leading to abundant apoptosis, whereas defective NHEJ had deleterious consequences only at late developmental stages. Since the late stages of embryogenesis are characterized by massive differentiation, these results may imply that the HR pathway has an essential protective role against DSB-induced cytotoxicity in proliferating cells and become dispensable in post-mitotic cells where NHEJ is the pathway of election. On the other hand, there are multiple pathways that can detect and repair radiation-induced DSBs, how the various DSB repair pathways are coordinated are still poorly understood [50, 51]. What determines whether a given DSB is detected by KU (to initiate NHEJ) or MRN (to activate ATM and subsequently ATR) is unknown. Laser microbeam irradiation experiments reveal that KU and MRN are recruited to the same sites of DNA damage independently throughout the cell cycle but whether they compete for binding to the same DSB or bind to different DSBs is not known [33]. The chromatin structure in the vicinity of the break may have a major effect on DSB repair and pathway choice [52].

2.1.3 Proteins involved in DSB repair

2.1.3.1 ATM

ATM is the product of the gene mutated in Ataxia-Telangiectasia (A-T), a debilitating genetic disorder characterized by progressive neurodegeneration, immune system dysfunction, hypersensitivity to ionizing radiation and a marked predisposition to cancer. Cells derived from A-T patients were found to exhibit chromosomal instability and extreme sensitivity to DSB-inducing agents including ionizing radiation, but no or little sensitivity to other forms of DNA damage. ATM is a large protein (3056 amino acid residuals) largely composed of HEAT repeats [53], which are predicted to form a superhelical structure with concave and convex faces. The C-terminal end of ATM is composed of the PIKK-family kinase domain and a region named the “FATC” motif. Another domain, termed FAT, is juxtaposed N-terminal to the kinase domain. Taken as a protein serine-threonine kinase, ATM is included in the PIKK family and sharing several features with other family members such as DNA-PKcs and ATR.

In undamaged cells, ATM is in a dimeric (or multimeric) inactive form with kinase domain bound to an internal domain of a neighboring ATM molecule containing serine 1981 [54], present throughout the nucleus. Following radiation, the kinase domain of one ATM molecule phosphorylates serine 1981 of an interacting ATM molecule. This phosphorylation event does not directly regulate the activity of the kinase, but disrupts ATM oligomers

and allows accessibility of substrates to the ATM kinase domain. The phosphorylated ATM dissociates from the complex and localizes to the chromatin that surrounds the DSB [55, 56]. Actually, ATM is considered as a “hierarchical kinase”, capable of initiating many pathways simultaneously [57]. After recruitment to sites of DNA damage, ATM phosphorylates a number of substrates, including c-Abl, CHK-1, CHK-2, RPA, BRCA1, BRCA2, NF-kB/IkB alpha, β-adaptin, hSSB1 and autophosphorylation of ATM itself [57], which in turn targets other proteins to induce cell cycle arrest or apoptosis and facilitate DNA repair. A complex web of interplay among these proteins enhances their retention at the DSB. The interaction of ATM with proteins such as BRCA1, NBS1 and CHK-2 suggests its key role in both NHEJ and HR.

ATM kinase activation seems to be an initiating event in cellular response to ionizing radiation and is linked to other factors; principal among these is the MRN complex. Studies show that the MRN complex acts as a double-strand break sensor for ATM and recruits ATM to broken DNA molecules, which occurs via its interaction with the C-terminus of NBS1 [58], The ability of the MRN complex to tether broken DNA ends provides a scaffold for recruitment of critical signaling kinases such as ATM. Cells in which the MRN complex is compromised can affect ATM localization to DNA damage-induced foci or show decreased ATM activity and reduced phosphorylation of ATM substrates [59-61]. Changes in chromatin structure

can also activate ATM, even in the absence of DSBs, suggesting a model whereby altered chromatin structures lead to phosphorylation of Ser1981 of ATM in trans and dissociation of inactive ATM dimers into active monomers [54], however, defined ATM-dependent foci only arise at DSBs [54].

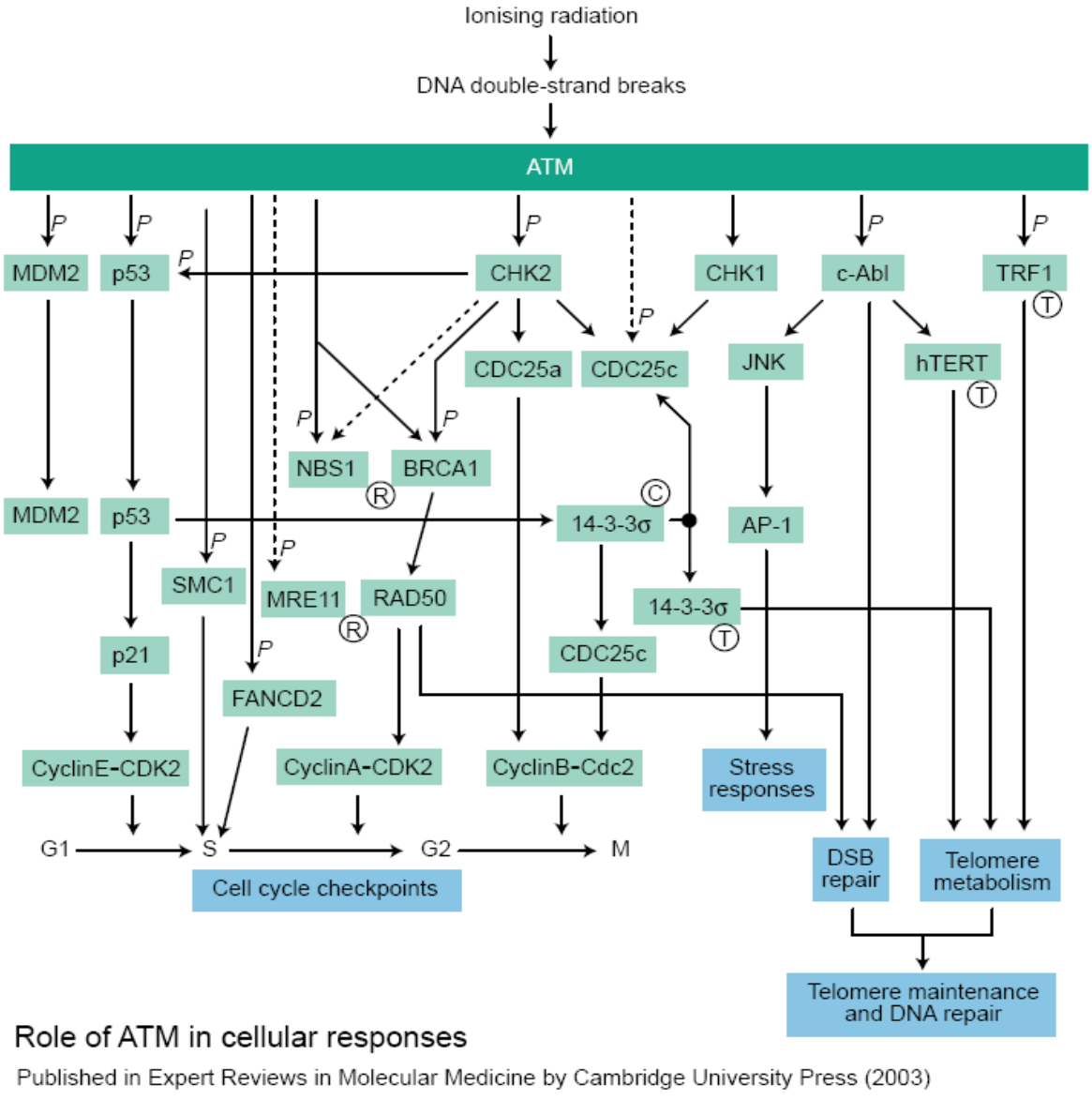

Figure 4: Role of ATM in cellular responses. ATM (ataxia-telangiectasia mutated) kinase activity increases immediately after the induction of DSBs following exposure to ionizing radiation. ATM mediates the early stages of the rapid induction of several signaling

pathways, which include regulation of the cell-cycle checkpoint controls, activation of the DSB repair pathways, activation of stress responses and maintenance of telomeres. Figure was taken from Tej K. Pandita, 2003 [57].

2.1.3.2 γ-H2AX

H2AX is one of a set of histone H2A variant proteins which constitutes 2-25% of the mammalian histone H2A depending on the organism and cell type [62, 63]. In response to DSB induction, H2AX is rapidly phosphorylated at serine 129 in mouse (serine 139 in human) on its C–terminal tail at a conserved SQE motif by members of the PIKKs, including ATM, ATR and DNA-PKcs [20, 62]. ATM is the major kinase to phosphorylate histone H2AX under physiological conditions [20, 64]. Numerous H2AX are phosphorylated in chromatin regions flanking the lesion [65, 66], which can be easily detected by antibodies against γ-H2AX, leading to the formation of discrete nuclear foci visible in immunofluorescence microscopy. In our latest study, we have shown by transmission electron microscopic approach that γ-H2AX, as well as other repair factors forming RIF visualized by fluorescence microscopy, appeared exclusively in heterochromatin, which support the idea that these components may promote localized chromatin decondensation necessary for repair in more complex heterochromatin [67]. Discrete γ-H2AX foci appear within 3 minutes after irradiation [65], then grow in size and number and reach a plateau within 10 to 30 minutes after irradiation [66]. The level of the plateau is proportional to the radiation dose [62]. Once DSBs are repaired, the

γ-H2AX will be removed by two non-exclusive mechanisms: dephosphorylation of γ-H2AX by protein phosphatases [18] and removal of γ-H2AX from the chromatin through histone exchange with unphosphorylated H2AX from the nucleoplasm (and dephosphorylation after its displacement from chromatin) [68]. These processes persist until 2 to 24 hours following irradiation. It has been reported that the kinetics of γ-H2AX foci loss is related to the DSB repair capacity in somatic cells [69] and germ cells [70]. Persistent γ-H2AX foci after the initial induction of DNA damage indicate that some of the damage remains unrepaired. Because of its sensitivity and simplicity, detection of γ-H2AX has been widely used to monitor DNA damage, especially induced by IR in the low dose range, and subtle changes caused by radiation-induced bystander effects [71] or by genomic instability [72]. Co-localization of γ-H2AX foci with other proteins involved in DNA damage repair allows spatial and temporal dissection of these processes. However it should be noted that there is a variable background level of γ-H2AX signals primarily associated with DNA replication and expressed mostly in S-phase cells [73].

It is believed that H2AX phosphorylation stabilizes the interaction of DSB response proteins, such as 53BP-1 [74], BRCA1 and NBS1 at the repair site [75-77] and acts as an assembly platform to facilitate the accumulation of DNA damage response proteins onto damaged chromatin. These DNA repair-related proteins subsequently congregate at the γ-H2AX foci during the

repair process. Although their recruitment to DSBs is not completely dependent on H2AX phosphorylation, H2AX is an important element in proper damage response foci formation by enhancing the retention of repair factors after their initial recruitment [75]. The γ-H2AX binds directly to the BRCT repeat of MDC1 [78]. This complex formation regulates H2AX phosphorylation and is required accordingly for recruitment of DSB response proteins to flanking chromatin [18, 79, 80] and for normal radioresistance [78]. Cells lacking H2AX are able to undergo checkpoint activation and cell cycle arrest [76], but are unable to maintain arrest in the presence of persistent damage [81]. This defective G2/M checkpoint response is likely to be caused by the impaired accumulation of checkpoint factors such as MDC1, 53BP1 at DSBs, which serve as an amplification step at low levels of DNA damage. H2AX-/- mice have moderate defects including radiation sensitivity, growth retardation, male specific infertility and immunodeficiency which are consistent with deficiencies in DNA repair [76, 82].

2.1.3.3 53BP1

The p53 binding protein 1 (53BP1), a protein identified through its ability to bind to p53, is initially proposed to function as a transcriptional coactivator of the tumor suppressor p53. It has two tandem C-terminal BRCT domains, which are present in a number of proteins involved in DNA damage signaling and DNA repair. BRCT domains are protein-protein interaction domains and

in 53BP1 they mediate its interaction with p53 [83, 84]. Unlike other p53 binding proteins such as MDM2 and TBP [85, 86], which bind to the N-terminal transcriptional activation domain of p53, 53BP1 binds to the central domain of p53, a region responsible for sequence-specific DNA binding that is normally mutated in human tumors [87].

53BP1 is found to be a nuclear protein that localizes rapidly to discrete foci following treatment with agents that cause DSBs, such as IR [88-90]. 53BP1 foci are formed within five minutes after irradiation with doses as low as 0.5 Gy. The number of 53BP1 foci increases over time, reaching a maximum at about 15-30 minutes after irradiation [88] and then decreases rapidly in the first few hours after irradiation followed by a slower dispersing process at later time. The persistent foci exhibiting after 24 hours or later may be correlated with unrepaired or misrepaired DSB or alteration in the chromatin structure [91]. The maximum value of 53BP1 foci, about 20 foci per cell per Gy, closely parallels the number of DSBs. Furthermore, the kinetics of 53BP1 foci resolution appears to be very similar to the kinetics of DSB repair following IR. Therefore, 53BP1 foci may represent “sites of DSBs”, a hypothesis that is supported by the colocalization of 53BP1 foci with other foci known to mark sites of DSBs, such as γ-H2AX [62, 65, 88-90, 92].

Radiation-induced 53BP1 foci occur in almost all cell cycle phases except those in mitosis [90]. In terms of the early response after IR, the specificity for

agents inducing DSB, and the involvement of the entire population of irradiated cells, 53BP1 gains an advantage as a biomarker over lots of other proteins, such as MRE11, NBS1, RAD50, RAD51, RAD54, BLM and BRCA1 [92-94]. The mechanism of initial recruitment of 53BP1 is still unclear. Two observations suggest that 53BP1 might be a sensor of DSBs: first, 53BP1 recruitment to sites of DSBs does not require ATM or other DNA damage checkpoint proteins; second, depletion of 53BP1 by siRNA leads to reduced ATM phosphorylation [88, 95]. A proposed hypothesis is that DSB-associated changes in chromatin structure increase the exposition of methylated histone tails facilitating initial recruitment of 53BP1. Subsequent phosphorylation of histone H2AX stabilizes the binding of 53BP1 to chromatin. Further recruitment of 53BP1 into focal structure is also a highly complicated process involving many regulatory steps and multiple posttranslational modifications of various proteins including phosphorylation of H2AX, recruitment of MDC1 and the E3 ubiquitin ligase-protein RNF8, methylation of histone H3 and H4, and Tip60 HAT activation [96]. Lack of interactions between these factors impairs the formation of 53BP1 foci.

Knockout or knockdown of 53BP1 results in cell cycle checkpoint defects and genome instability which is typified by increased levels of chromatid gaps, breaks and exchanges. 53BP1-/- mice and cell lines exhibits an increased sensitivity to exogenous DNA damage, which indicates that 53BP1 is involved in DSB repair [74]. Evidence shows that 53BP1 Tudor domains are

able to stimulate the ligase activity of the DNA ligase IV/XRCC4 complex *in vitro* [97, 98], suggesting that there may exist a 53BP1-dependent pathway distinct from the core NHEJ pathway. In addition, 53BP1 was reported to contribute to the NHEJ repair by an ATM-Artemis-LigIV/XRCC4-dependent pathway, which is required in late repair kinetics in G1 phase [23, 52].

2.2 Low-dose radiation

First of all, we should clarify what the definition of low-dose radiation as well as low dose-rate radiation means. The ICRP (International Commission on Radiological Protection) applied absorbed doses below 200 mGy and from higher absorbed doses when the dose rate is less than 100 mGy per hour as low-dose radiation and low dose-rate radiation. However, the NRPB (National Radiological Protection Board) conservatively adopted lower criteria than ICRP and the values may be more reasonable for practical purpose: low acute doses are those below 100 mGy and low dose-rates are those below 5 mGy per hour [99]. Although low-dose radiation has no immediately noticeable effects on organisms, it attracts great interest since it may have long-time biological effects on cancer induction in exposed individuals and genetic defects in their progeny. Various models have been developed to account for the features of radiation dose-response relationships. The no-threshold model means that there is no safe level, because all dose levels (regardless how small the radiation doses might be) are potentially harmful. The available biological

and biophysical data provide support for this linear no-threshold (LNT) risk model which postulates that the risk of cancer induction from radiation exposure is assumed to be proportional to the absorbed dose. Therefore, any exposure to radiation (above zero-dose), linearly increases the risk of radiation sickness, cancer or cell death, suggesting that no level of radiation is safe. Because of absence of direct data, the effects of low-dose radiation are currently estimated by extrapolating from the effects of high-dose radiation [100] which is by using the LNT model. However, recent findings in radiation and molecular biology strongly challenge this LNT concept in the low dose range. The biological mechanisms and responses are considerably more complex than predicted by that LNT model. Several new conceptions have been proposed, including hyper-radiosensitivity, untargeted effects and adaptive-response mechanisms.

2.2.1 Hyper-radiosensitivity and increasing radioresistance

Studies by Joiner *et al*. [101, 102] and Wouters *et al*. [103-105] revealed that many human tumor cell lines exhibit a low-dose hypersensitivity to radiation (termed HRS for hyper-radiosensitivity), which describes a phenomenon that cells die from excessive sensitivity to small single doses of ionizing radiation. This effect usually manifested at doses $<$0.5 Gy as a clear deviation from the standard linear-quadratic cell survival response, extrapolated from higher doses back to 0 Gy. It is accompanied by an increase in radioresistance at dose in the range of 0.5 Gy- 1 Gy (termed IRR for

increased radioresistance). By fitting cell survival data with Joiner's Induced-Repair-model [101, 106], Brian *et al*. propose a more detailed description of the HRS/IRR phenomena: the mammalian cells exhibit hyper-radiosensitivity (HRS) to radiation doses of less than ~0.3 Gy when given at acute dose-rate, over the ~0.3 - 0.6 Gy dose range, a more radioresistant response per unit dose was evident, as illustrated by the shallower slope of the radiation dose–response curve [107]. At values greater than 1 Gy, a downward-bending survival curve was observed, that is well described by a linear quadratic expression [107]. The exact molecular mechanisms responsible for the HRS/IRR phenomena still remain unclear. Using the γ-H2AX assay, Wykes *et al*. [108] found no relationship between the initial or residual levels of DSBs and the prevalence of HRS, indicating that HRS does not reflect a failure of DNA break recognition. However, Simonsson *et al*. [107] reported a hypersensitive dose response pattern for the persistence of γ-H2AX foci in epidermal skin cells in patients biopsy samples 30 minutes after a 0.3 Gy therapeutic dose, indicating a tentative relationship with DSB repair and HRS. The most credence so far is that cellular repair processes elicited in response to radiation-induced damage overcome HRS and trigger IRR. The dose-dependent activation pattern for ATM activity would produce a changeover point in the low-dose range radiation, exactly as what has been demonstrated with the transition from HRS to IRR [54, 107, 109, 110], which indicates that the notable role of ATM in the differential effectiveness in

low-dose radiation. However, more data showed that ATM activation does not play a primary role in initiating radioresistance. Furthermore, a relationship was discovered between the function of the downstream ATM-dependent early G2-phase checkpoint and the prevalence and overcoming of HRS [111]. Consequently, the early G2-phase checkpoint was proposed as critical event controlling the transitional low-dose radiation exposure [112]. In the meanwhile, MRN complex, DNA-PK [113, 114] and PARP-1 [115, 116] have all been demonstrated for overcoming HRS and instigating IRR response, which are all involved in the major repair pathways of radiation-induced DSB. Although ATM activation alone is not the key determinant for overcoming HRS, apoptotic response mediated through the p53-dependent activation of Caspase 3, which is also a part of the signal cascade downstream of ATM activation, has been identified as important for HRS [117]. Therefore it appears that HRS might be a default mechanism to prevent potentially mutagenic G2 phase cells from entering mitosis [118]. Accordingly, it may be that HRS is not important for slowly proliferating normal tissues. As HRS has been strongly linked with the rapid progression of radiation-damaged G2-phase cells, therefore HRS is more likely to affect early-responding proliferating tissues rather than late-responding tissues with regard to radiation injury.

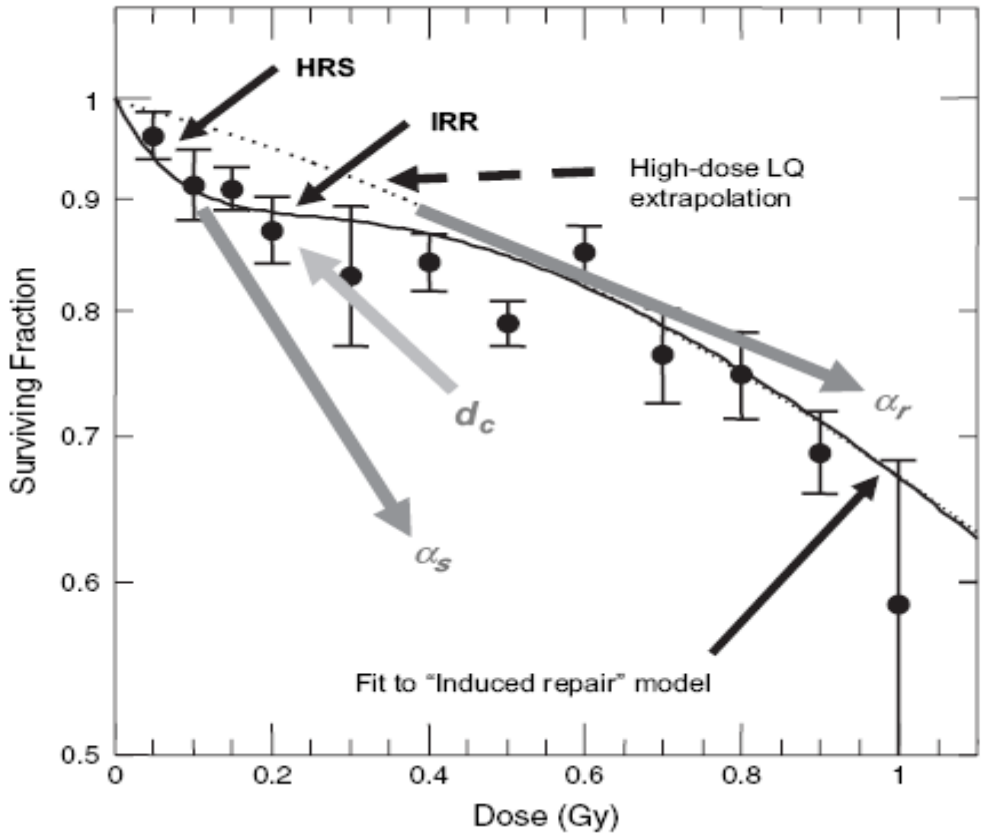

Figure 5: Typical cell survival curve with evidence of hyper-radiosensitivity (HRS). Figure was taken from Brian Marples, 2008 [107].

2.2.2 Untargeted effects

Untargeted effects are responses observed in cells that were not subject to energy deposition events induced by ionizing radiation. These cells have received damaging signals produced by irradiated cells (radiation-induced bystander effect) or are the descendants of irradiated cells (radiation-induced genomic instability). Radiation-induced genomic instability is characterized by a number of delayed adverse responses including chromosomal abnormalities, gene mutations, and loss of reproductive potential and cell death, which is accepted as one of the most important aspects of carcinogenesis. Bystander effect can be exhibited in many ways, such as

damage-inducible stress response [119-122], sister chromatid exchange [123-125], micronucleus formation [126, 127], apoptosis [126], gene mutation [128-130] and chromosomal instability [131]. It can also be demonstrated as increased cell proliferation [132, 133], release of growth inhibitory factors [134] and radioresistance to subsequently challenge radiation dose [135, 136]. It seems that there are both damaging and protective cell signals that are encompassed within the general field of bystander effect. In the low-dose radiation range, bystander effect postulates that the effect may be even more complex than that predicted by the LNT model, since irradiated cells may signal their distress to the neighbors and initiate response in them. Such signals may be from direct cell-to-cell interaction via cell gap junctions or the molecules secreted by the irradiated cells to the medium. Some possible candidates for the signal are reactive oxygen, nitrogen species and various cytokines such as IL-8 [100, 137, 138]. Bystander effect is not a universal phenomenon, not all cells are able to produce bystander signals or respond to them, which depend on the genetic background of the cell, cell type, cell cycle phase and the property and dose of the radiation. In general, bystander effect appears to be a low-dose phenomenon [139]. Some *in vitro* studies have reported the effects could exhibit at gamma rays radiation dose as low as 2 mGy and disappear as the radiation dose increased up to around 1 Gy [140].

2.2.3 Adaptive response

When cells were exposed to a very low dose of radiation, a priming dose, followed with a larger dose after a short time, the challenge dose, the frequency of chromosomal aberrations induced by the challenge dose was substantially reduced comparing with that from the challenge dose given alone [141, 142]. This has been termed the adaptive response. Adaptive response can be divided into three successive biological phenomena, the intracellular response, the extracellular signal and the maintenance. The intercellular response leading to adaptation of a single cell is a complex biological process including induction or suppression of gene groups. An extracellular signal, the nature of which is still unknown, may be sent by the affected cell to neighbouring cells causing them to adapt as well. Adaptive response can be maintained for periods ranging from of a few hours to several months, even the entire life span, which may be related with the constantly increased levels of ROS (reactive oxygen species) or NO (nitric oxide) in adaptive cells [143, 144]. Radioadaptive responses have been observed *in vitro* and *in vivo* using indicators of cellular damage, such as cell lethality, chromosomal aberrations, mutation induction, radiosensitivity, and DNA repair [141, 145-149]. Adaptation has been shown in response to both low LET (X-rays, γ-rays, β-particles) [150-152] and high LET (neutrons, α−particles) [149, 153] radiation and is known to show a high degree of inter- and intra-individual variability. The variability may depend on factors such as dose-rate [151],

time interval between the doses [154, 155], genetic variation among individuals [156, 157] and experimental conditions [158]. In general, in experimental set-ups resulting in adaptive behavior the values have been found to range from 0.01 to 0.5 Gy and from 0.01 to 1.0 Gy/min for priming dose and priming dose rate, respectively [159]. An interval of at least 4 hours is necessary between the priming dose and the challenge dose for the maximum adaptive radioresistance to be seen [150].

The adaptive response phenotype has been associated with an increase of cellular functions such as DNA repair and stress response. The activation of PARP (poly ADP-ribose polymerase), known to be involved in DNA repair and cellular ageing, is required for adaptation [155, 160]. Khodarev *et al*. found that STAT1, a component of the cytokine IFN signaling pathway, was significantly up-regulated during adaptation [161]. A study by Coleman *et al*. compared transcript profiles of human lymphoblastoid cell lines showing or not showing adaptive response and found that many of the genes up- or down-regulated in adapting cell lines could be associated with p53 functions [162]. In general, cell lines showing adaptive response showed induction of groups of genes associated with DNA repair and stress response while down-regulated genes that could be associated with cell cycle control and apoptosis [162].

2.3 Early- and late-responding tissues in radiation

Radiotherapy is an important curative and palliative modality in the treatment of cancer, but associated toxicities of neighbouring normal tissues remain the most important obstacle in delivering dose intensity. Radiotherapy toxicity is generally separated into acute toxicity and late toxicity. Acute toxicities (early responses) occurs during or shortly after the radiotherapy and usually arise in rapidly renewing tissues where a small number of stem cells divide slowly and regularly to provide a steady supply of proliferative progenitor cells which differentiate into mature, nonproliferative, functioning cells. The proliferative damage of radiation-vulnerable stem cells and progenitor cells causes a transient decrease in the number of specific functioning cells, but this acute toxicity heals by proliferation of surviving stem cells. Late toxicities (late responses) can manifest itself months to years after the completion of the treatment and usually arise in slowly renewing tissues composed of highly differentiated cell populations performing specialized functions, for example, the tubule epithelium in kidney, the cardiomyocytes in heart, the alveolar epithelium in lung and the oligodendroglia in brain. Only a low percentage of these functional cells maintain the capacity of proliferation on demand. In contrast to transient, clinically manageable acute responses, late normal tissue injury is often progressive with few therapeutic options and substantial long-term morbidity and mortality.

Kidneys, which are made up of millions of nephrons that act as individual filtering units, are the main and the most radiosensitive part of the urinary system which helps maintain homeostasis by removing metabolic waste from the blood and regulating fluid and electrolyte balance in the body. As dose-limiting organs for local radiotherapy involving upper abdomen and total body irradiation, radiation-induced kidney injury can manifest as benign or malignant hypertension, elevated creatinine levels, anemia, and renal failure, which can be lethal. The adult kidney is a slow-turnover tissue. Studies with tritiated thymidine have shown that both tubular and endothelial cells have low labeling indices of 0.4% and 0.1% [163], indicating that the normal turnover of these cells is slow. However, the kidney is capable of responding to injury, such as surgery, chemicals and irradiation, by transient increased proliferation [164, 165]. Irradiation can induce an early, dose related increase in proliferation in tubules [164] and this stimulated proliferation after irradiation can precede the onset of functional damage and persist for 6 to 12 months [164]. But this proliferation is not associated with increased cell number, indicating that cell proliferation is matched by cell loss [166, 167].

Intestine is an important normal tissue at risk during the radiotherapy in the abdomen or pelvis. The absorptive epithelium of the small intestine, which is ordered into crypts and villi, constantly undergoes shedding and replenishment. These epithelial cells proliferate in the crypts, migrate along the villi, and then are eventually shed into the intestinal lumen. The cell cycle

time for the majority of proliferating cells in the mouse intestine crypt is in the order of 12-13 hours, whereas the cell cycle time for crypt stem cells is considerably longer at approximately 24 hours. The total transit time for cells from the crypt base to the villus tip is about 6-8 days and it takes 48-72 hours from when a cell enters the villus base until it is shed from its tip [168]. Acute radiation injury to the intestine manifests when cells in the differentiated cellular compartment in the villus are no longer adequately replaced by cells from the progenitor compartment in the crypt. Accelerated compensatory proliferation is initiated on this damage, when crypt cell cycle times may be as short as 6 hours [169-171].

2.4 Aim of this project

Low dose irradiation is ubiquitous in our environment. The biological effect is more complicated than high dose irradiation which is estimated by the linear-quadratic (LQ) model (a model in which the effect is a linear-quadratic function of dose). Hyper-radiosensitivity, increasing radioresistance, untargeted effects, and adaptive effects are all involved to complicate the ultimate response to ionizing radiation. In the present study, we investigated the effects of fractionated low-dose radiation exposure on the accumulation of DSBs in normal tissues using an *in vivo* model with repair-proficient mice. This *in vivo* model may help us to estimate the effects of exposure to environmental and occupational sources of low-dose radiation

among the normal population; it may also help us to assess the long-term effects of normal tissue irradiation in the context of fractionated radiotherapy.

3. Material and methods

3.1 Animals

8 week-old male C57BL/6 (wild-type, C57BL/6NCrl) mice of comparable weight were obtained from Charles River Laboratories (Sulzfeld, Germany). All mice were housed 6-7 per cage in laminar flow hoods under identically standard laboratory conditions (temperature 22±2 ^{0}C, 55 ±10 % humidity, and light-dark cycle 12:12), and had free access to sterilized laboratory food and water during the whole course of experiments. Before use, the mice were allowed to acclimatize from shipping for 1 week. All animal studies were performed according to the Institutional Animal Care and Use Guidelines, and the experimental protocol was approved by the Animal Care and Use committee of the Saarland University.

3.2 Irradiation

Whole body irradiation was performed in a special plastic cylinder with a 6-MV linear accelerator at a dose rate of 2 Gy/min. The isodose distributions were evaluated by ADAC Pinnacle three-dimensional treatment planning system, revealing that the 95% isodose enclosed the whole body of each individual mouse.

For the DSB induction, three mice were sacrificed at 30 min after a single dose radiation of 10 mGy or 100 mGy, respectively. For the fractionated low-dose radiation experiments, the mice were exposed to 10 mGy or 100

mGy, respectively, once every day from Monday to Friday, with time intervals of 24 hours between radiation exposures. After 2, 4, 6, 8, and 10 weeks of daily low-dose radiation, three mice were sacrificed at 24 or 72 hours after their last fraction.

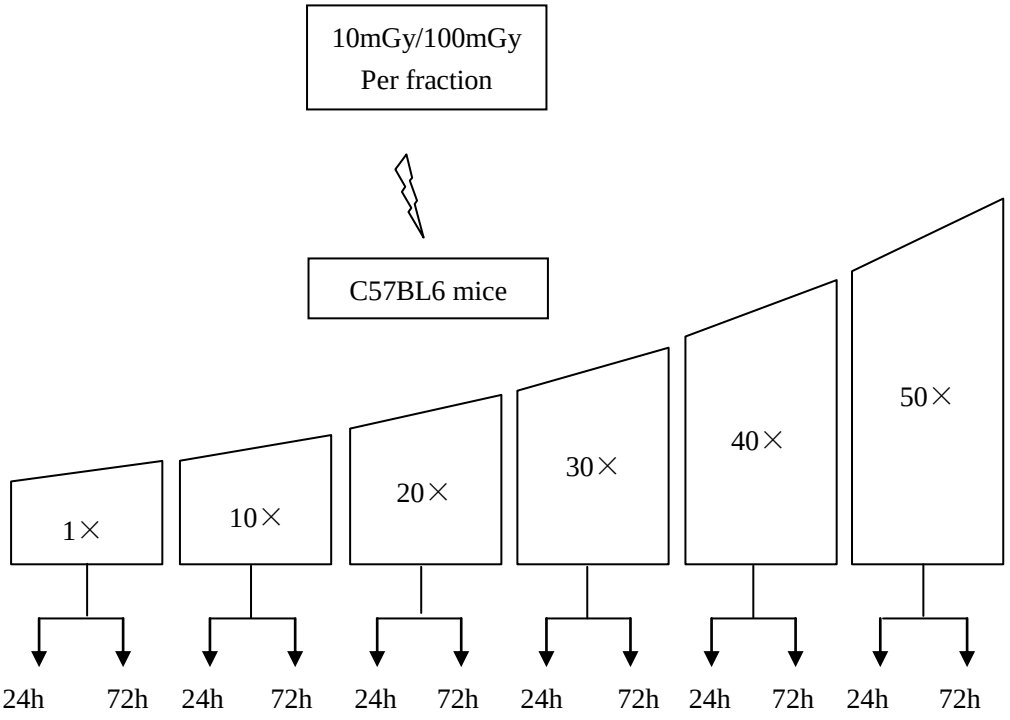

Figure 6: Fractionated irradiation schedule.

3.3 Tissue sampling

After anesthesia by an intraperitoneal injection of Xylazine (Rompun) and Ketamine (Rompun 1 ml and Ketamine 0.75 ml, diluted in 8.25 ml 0.9% natrium chloride solution, 0.1 ml/ 10 g), different organs (skin, heart, lung, liver, intestine, kidney, testis and brain) were quickly harvested. Each tissue was fixed in 4% neutral buffered formaldehyde 16h at room temperature. After dehydration with a graded series of ethanol and xyline by an automatic

tissue dehydration apparatus, the tissues were imbedded in paraffin and then cut into sections at an average thickness of 4 μm.

3.4 Immunofluorescence staining

To analyze the residual DSBs in differentiated cells of kidney and intestine, we performed the immunofluorescence staining with antibodies against 53BP1 according to the following protocols:

Paraffin sections were dewaxed three times in 100% xyline (7 min each), and hydrated by graded dilute ethanol series: 100% ethanol, 96% ethanol, 90% ethanol, 80% ethanol, 70% ethanol and distilled water (5 min each), followed by boiling in the citrate buffer (Dako REAL Target Retrieval Solution, #S-2031, pH 6.0) for 60 min at 96^0C to unmask the antigenic sites. After 10 min natural cooling, sections were incubated at room temperature for 1 hour with Roti-ImmunoBlock (T144.1, ROTH) to impede the non-specific interaction whilst preserve specific binding signals, reducing background and false-positive signals. Afterwards, the tissue sections were incubated with the primary rabbit polyclonal antibody against 53BP1 (Bethyl Laboratories, Cat.-No.IHC-00001, USA) at a dilution of 1:200 in a humidified chamber overnight at 4^0C, then incubated with Alexa Fluor 488-conjugated goat-anti rabbit LgG (Invitrogen, Cat.-No.A11034, Germany) as secondary antibody at 1:400 dilution in a humidified chamber for 60 min in dark at room temperature. Finally the sections were mounted by using DAPI-containing

mounting medium (VECTASHIELD Hard-set mounting medium, Vector Laboratories, Cat.-No.H-1400, USA) and stored overnight at 4^0C before use. Between each step of incubation and mounting, the sections were gently washed with PBS three times (10 min each) on a shaker.

3.5 Quantification of radiation-induced foci

Sections were examined by using Nikon E600 epifluorescent microscope equipped with charge-coupled-device camera and acquisition software (Nikon, Düsseldorf, Germany). For quantitative analysis, the foci were scored in a blinded manner to avoid bias and counted by eye under ×600 or ×1000 microscope magnification. For each data point, the kidney and intestine sections from three different mice were analyzed and the mean value was calculated. Counting was performed until at least 40 foci and 40 cells were registered for each sample. The error bars represented the SEM from the number of cells analyzed.

3.6 Statistical analysis

To evaluate the potential differences in residual DSB of normal tissue cells at different time point, the Mann-Whitney U test was performed. The criterion for the statistical signification was $p \leq 0.05$.

4. Results

4.1 DSB induction at low dose irradiation

To investigate the induction of DSB in kidney and intestine after fractionated low dose irradiation, we performed 53BP1 immunofluorescence staining in the kidney and intestine sections. As described previously, well-defined regions encompassing the characteristic cell populations of the tissues were chosen for the quantitative 53BP1 foci analysis. In the kidney, the renal tubular epithelial cells in the renal cortex with the glomeruli and proximal and convoluted tubules were analyzed. In the small intestine, the enumerating of 53BP1 foci was confined to the epithelial cells of the mucosal surface forming the middle part of the villi, in order to exclude the disturbance of proliferating precursor cells in the crypts and the senescent cells at the top of the villi.

As shown in Figure 7, the nuclei were uniformly stained with DAPI. In the background of diffuse 53BP1 staining, obvious discrete foci could be seen and the number of foci slightly increased in both kidney and intestine sections after irradiation, while the sham irradiated controls were predominantly negative for 53BP1 staining. The foci exhibited different characteristics; some were big and bright while some were small and dim.

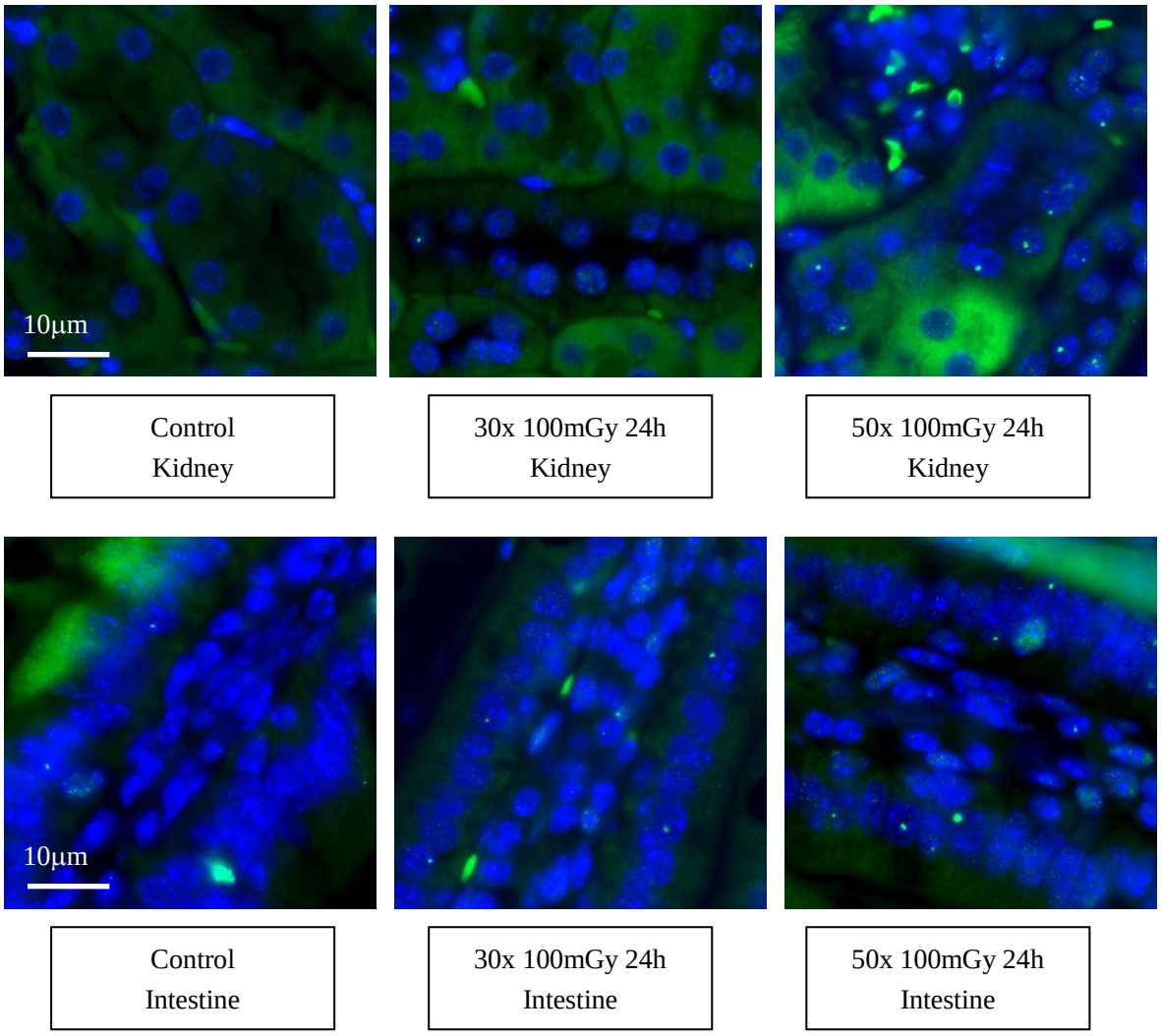

Figure 7: Immunofluorescence staining of 53BP1 (green foci) in kidney and intestine at 24h after 30 and 50 fractions of 100 mGy, respectively, compared to unirradiated control tissues.

In unirradiated normal tissue, we observed a low background level of foci with 0.07-0.11 foci per cell. At 30 min after a single dose irradiation with 10 mGy or 100 mGy, both kinds of tissues exhibited nearly the same value of foci, with 0.2 foci per cell after 10 mGy and 1.3 foci per cell after 100 mGy. After subtraction of background level, 10 mGy induced 0.11 foci per cell and 100 mGy induced 1.21 foci per cell. In our previous study, we have used

γ-H2AX foci to analyze the induction and repair of DSBs in mouse tissues after relative high doses and suggested a linear dose correlation for the induction of γ-H2AX foci from 0.1 Gy - 1 Gy in all analyzed mouse tissues. The induction of DSBs was 8 foci /cell /Gy measured 10 min after irradiation, with the background level of 0.04 foci per cell. As 53BP1 foci were supposed to colocalize with γ-H2AX foci in these tissues, present data were slightly higher than previous results, partly due to differences in sample staining and the criterion of foci scoring.

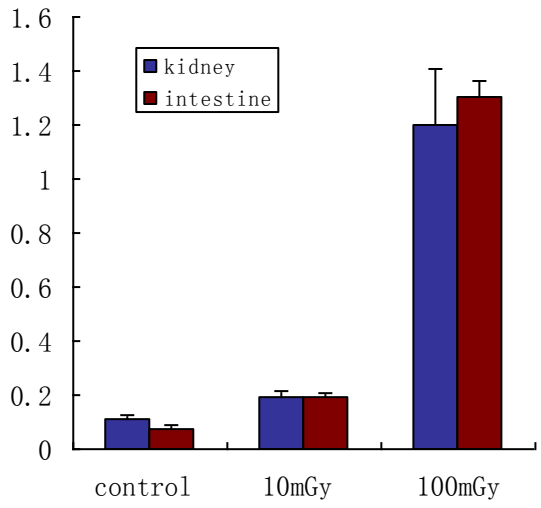

Figure 8: DSB induction quantified by enumerating 53BP1 foci in kidney and intestine at 30 min after 10 mGy or 100 mGy irradiation. Error bars represent standard error of mean from three experiments.

4.2 Residual foci in kidney

In our previous study, we have assessed the formation and rejoining of DSBs in various normal tissues after fractionated irradiation in clinic relevant scheme [172]. Here, we evaluated the effects of fractionated low-dose irradiation in these normal tissues. First, we analyzed kidney tissues obtained

at defined time points. As shown in Figure 8, the foci per cell did not vary obviously as dose increased in kidney. After fractionated low-dose radiation with 10 mGy, the average number of foci per cell in kidney fluctuated between 0.15 and 0.19 foci/cell at 24 hours and between 0.11 to 0.18 foci/cell at 72 hours after the last fraction. In the 100 mGy per fraction group, the foci per cell in kidney ranged between 0.27 and 0.38 foci/cell at 24hours after the last fraction, and decreased to 0.23 to 0.31 foci/cell at 72 hours after the last fraction.

In the 10 mGy per fraction group, nearly identical foci levels at the different time points during irradiation, although the residual foci at 72 hours after the last fraction varied obviously compared to the values at 24 hours. In the 100 mGy per fraction group, slightly elevated, but consistent foci levels could be observed during irradiation. The residual foci at 72 hours peaked at 50 fractions (cumulative dose 5 Gy) and the value at 24 hours peaked at 20 fractions (cumulative dose 2 Gy) in this group.

The values of foci per cell in the group of 100 mGy per fraction were constantly higher than that in the corresponding 10 mGy per fraction group. The residual foci levels at 24 hours after last fraction were higher than the corresponding levels at 72 hours, revealing some sort of recovery from the damage.

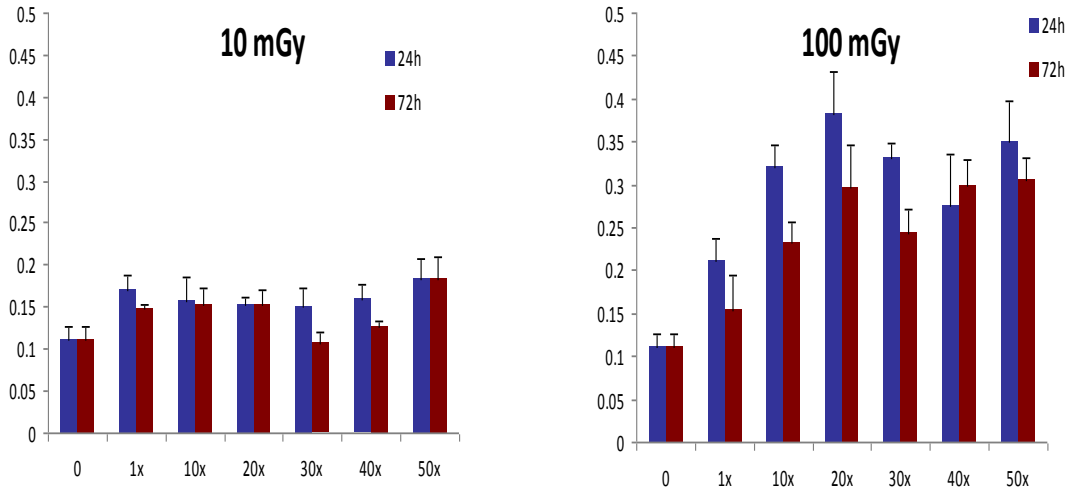

Figure 9: Residual 53BP1 foci per cell in tubular epithelial cells in kidney at 24 and 72 hours after the last exposure to fractionated irradiation (10x, 20x, 30x, 40x, 50x) with 10 mGy and 100 mGy, respectively, compared to single dose irradiation (10 mGy, 100 mGy) and unirradiated control tissues. Error bars represent standard error of mean values from three samples.

4.3 Residual foci in intestine

To investigate the effect in the typical early-responding tissue during fractionated low-dose irradiation, we next analyzed small intestine tissues at defined time points. As Figure 10 showed, in the 10 mGy per fraction group, the average number of foci per cell fluctuated between 0.07 and 0.11 foci/cell at 24 hours and between 0.07 and 0.11 foci/cell at 72 hours after irradiation. When 100 mGy was given per fraction, the foci per cell ranged between 0.09 and 0.16 foci/cell at 24 hours post irradiation and decreased between 0.06 and 0.16 foci/cell at 72 hours post irradiation. In the 10 mGy per fraction group, residual foci varied very slightly, with no obvious minimum and maximum. In the 100 mGy per fraction group, by contrast, we observed low levels of residual foci after 10 fractions (cumulative dose 1 Gy) but clearly higher foci

levels after 40 fractions (cumulative dose 4 Gy) and 50 fractions (cumulative dose 5 Gy), respectively.

In contrast to kidney, the values of foci per cell in the group of 100 mGy per fraction were not always higher than those of the corresponding 10 mGy per fraction group.

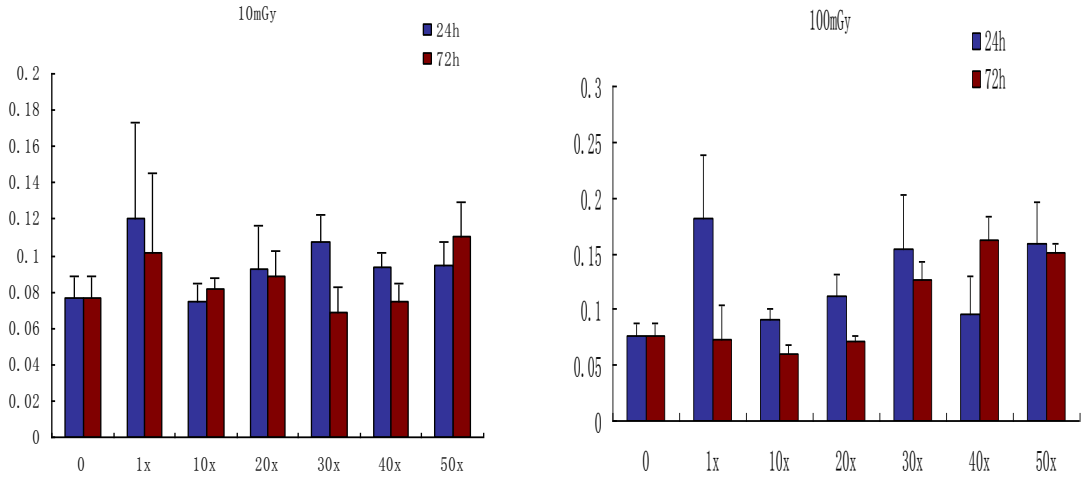

Figure 10: Residual 53BP1 foci per cell in the epithelial cells in intestine at 24 and 72 hours after the last exposure to fractionated irradiation (10x, 20x, 30x, 40x, 50x) with 10 mGy and 100 mGy, respectively, compared to single dose irradiation (10 mGy, 100 mGy) and unirradiated control tissues. Error bars represent standard error of mean values from three samples.

4.4 DSB repair in different organs

The values of persistent foci in the tubular cells in kidney were always higher than those in the small intestine, independent of the dose per fraction, or analyzed time points, which reflect a lower efficiency in dealing with persistent foci. In previous studies our study group observed nearly identical DSB rejoining kinetics in differentiated somatic tissue cells after single-dose

irradiation with 2 Gy [172]. However, in these fractionated low dose irradiation experiments, we observed some differences regarding persistent foci levels between early- and late-responding tissues, which might be related to the ability to deal with complex DSBs or chromatin disruptions induced by low dose radiation.

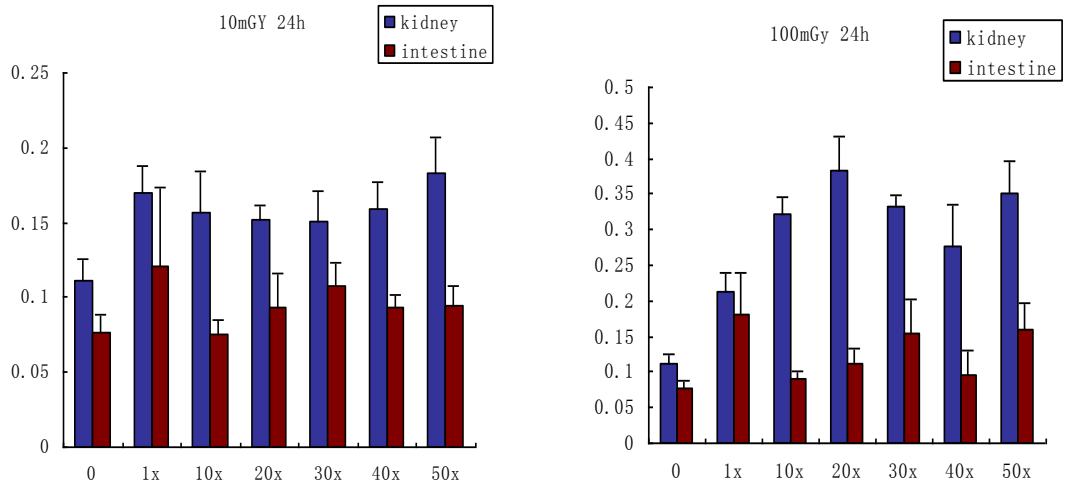

Figure 11: Residual 53BP1 foci per cell in tubular epithelial cells in kidney and in the epithelial cells in intestine at 24 hours after the last exposure to fractionated irradiation (10x, 20x, 30x, 40x, 50x) with 10 mGy and 100 mGy, respectively, compared to single dose irradiation (10 mGy, 100 mGy) and unirradiated control tissues. Error bars represent standard error of mean values from three samples.

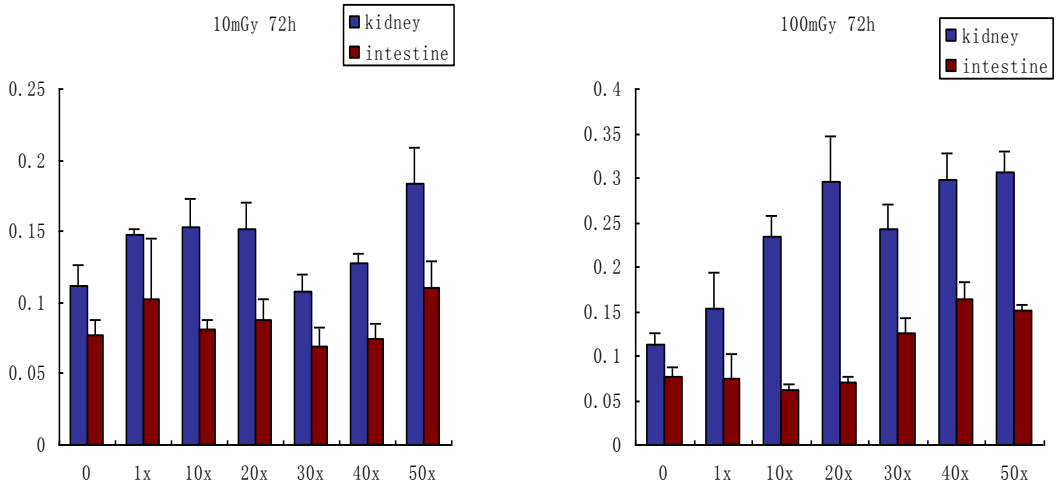

Figure 12: Residual 53BP1 foci per cell in tubular epithelial cells in kidney and in the epithelial cells in intestine at 72 hours after the last exposure to fractionated irradiation (10x, 20x, 30x, 40x, 50x) with 10 mGy and 100 mGy, respectively, compared to single dose irradiation (10 mGy, 100 mGy) and unirradiated control tissues. Error bars represent standard error of mean values from three samples.

5. Discussion

Low dose radiation continues to be of great interests because of its potential dangers posed by exposure to environmental and occupational sources of radiation and because of the potential clinical benefits of radiotherapy. But the estimate of its biological effects has always been controversial due to no enough available evidences. In our present study, we established an *in vivo* model with repair –proficient mice to investigate the effects of fractionated low-dose radiation exposure on the accumulation of DSBs in normal tissues with different radiation characteristics. We found that a single acute low dose irradiation with 10 mGy or 100 mGy induced nearly the identical foci level in kidney and small intestine, which are the typical late- and early-responding tissues in radiation. In both kidney and small intestine, persistent foci levels at different time points varied slightly during fractionated irradiation with 10 mGy while slightly elevated, but consistent foci levels could be observed during fractionated irradiation with 100 mGy. However, the values of persistent foci in the tubular cells in kidney were always higher than those in the small intestine, independent of the dose per fraction, or analyzed time points, which may reflect a lower efficiency in dealing with persistent foci.

5.1 RIF approach analysis *in vivo*

In the present study, we used 53BP1 immunofluorescence assay to assess

the persistent DSBs in typical early- and late-responding tissues with repair-proficient murine model after fractionated low dose irradiation. Traditional methods for quantification of DSBs in cells were based on the separation of DNA molecules according to their size by gel electrophoresis techniques, including pulse-field gel electrophoresis (PFGE) [173, 174], which is considered as the gold standard to detect DSBs. These physical methods of DSB quantification are not especially sensitive, typically requiring the use of doses above 5 Gy for a reliable assessment for the rejoining kinetics, which exclude their utilization in evaluating the effects of low dose radiation and detecting persistent and subtle DSBs after radiation. Enumerating foci with microscopy has been identified to assess the induction and repair of DSBs based on the close correlation between foci level and DSBs numbers and between the rate of foci loss and DSBs repair, providing a sensitive assay to monitor DSB in individual cells using physiological doses [91, 175, 176]. Despite of all the advantages of this RIF approach, recent studies have suggested that some foci do not represent unrepaired DSBs and foci formation and dispersion may not follow the actual fate of the physical breaks but rather registers accompanying chromatin modifications [177, 178]. Furthermore, in our latest findings gained by transmission electron microscopy (TEM) analysis [67], we have revealed this RIF approach does not possess the sensitivity to detect all unrepaired DSBs. We have confirmed that the repair factors forming RIF are not evenly distributed throughout the entire nucleus

but exclusively allocated in the heterochromatic domains. Additionally, the RIF were in a great dimension (up to a diameter of 1 μm), clustering over time and overlapping in the space. All these factors above may lead to the underestimation of unrepaired DSBs. However, this foci-approach is still widely used as a valuable indirect monitor of DSBs because of its sensitivity and simplicity, with more careful consideration in analyzing the obtained data.

5.2 Residual foci in tissues with different radiation characteristics

Pogribny *et al.* have confirmed that fractioned whole body low-dose radiation exposure resulted in a significant decrease in global DNA methylation and led to the accumulation and/or persistence of DNA damage as monitored by γ-H2AX foci in the murine thymus [179]. But no more data are available about its biological effect in complex normal tissues during the course of clinically relevant fractionation schedules. In the present study, we analyzed the residual DSBs measured by enumerating 53BP1 foci with different fractionated dose size and overall radiation dose in a clinical schedule in two other tissues: kidney and intestine, which are the two most involved organs when abdomen is irradiated and often become dose-limiting organs for radiotherapy of the upper abdomen, which may help us predict the injury of them in a new model other than LQ model.

As we quantified the foci microscopically by eye, we didn’t get more exact information about the shape, size or intensity of the foci. Belyaev *et al*.

have previously reported that residual foci induced by low LET were significantly larger in size than the initial RIF [180, 181] and proposed that initial RIF are formed at the sites of DSB induction and cover megabase-size of chromatin domains at places of actual DSB localizations. Fast repair occurs in the initial foci usually 1-30 min after irradiation. Complex foci may require longer time periods to be repaired, some complex DSBs move along with corresponding chromatin domains/foci to produce clustering of the initial foci, which result into secondary larger foci representing sites for formation of chromosomal exchanges. In the present study, comparing the foci visible at 30min after acute low dose irradiation, the sizes of foci in the tissue of kidney and intestine revealed no visible differences, respectively. However the majority of foci in the epithelia cells in intestine and tubular cells in kidney are smaller than those induced by relative high doses.

The induction of foci measured 30 min after low dose irradiation were a little bit higher than our previous result, but they still demonstrate that low dose irradiation induce identical foci numbers in different normal tissues, regardless of their clinical characteristics. This result was in consistence with what Grudzenski and his colleagues have reported [182].

The precise role of residual RIF that persist 24 hours and longer in cells is presently unknown, its possible links to chromatin alterations, delayed repair and misrepaired of DSB, apoptosis, activity of several kinases and phosphatases, and checkpoint signaling have been suggested [180, 183-185].

Rothkamm *et al*. have previously reported that some residual 53BP1 and γ-H2AX foci remain in cells for a relative long time (≥ 24h) after irradiation and suggested possible correlation between cellular radiosensitivity and the numbers of residual foci [176]. However, no clear correlation has been established until now. Kidney and small intestine typically exhibit different radiosensitivity in clinic radiotherapy. In our present study, we estimate the persistent foci level in these two tissues. As the figures showed previously, the values of persistent foci in the tubular cells in kidney were always higher than those in the small intestine, independent of the dose per fraction, or analyzed time points. This might be related to their ability to deal with complex DSBs and the alteration in chromatin induced by low dose irradiation. However, we should also consider that kidney is a slowly turn-over organ; the majority of the tubular cells are quiescent. Despite the radiation has the potential to stimulate proliferation in tubules, considering the efficiency of this stimulation and the small dose given, the cells still retain and have enough time to accumulate all the damages. On the contrast, the small intestine is a rapidly proliferating tissue. The cells lining the villi would turn over nearly every 6-8 days. Accordingly, at a single cell level, the dose it has received is much lower than the accumulative dose we have given.

As Grudzenski *et al*. demonstrated [182], foci were lost much less efficiently following a single 10 mGy dose comparing to the 100 mGy dose, with 5% unrepaired foci after 24 hours after a dose of 100 mGy but more than

50% of the initial induced foci persisted for up to 24 hours after 10 mGy, indicating that cells exhibited different repair efficiency after 10 mGy and 100 mGy irradiation. Additionally, unrepaired DSBs accumulated in the fractionated irradiation course. As previously stated, increasing radioresistance, untargeted effects and adaptive response all had their thresholds and transition points, respectively, that means they would only trigger when the cumulative dose or damage reach some level, and these level may vary in different cell types or tissues. Hyper-radiosensitivity was exhibited in the range less than ~0.3 Gy, and the intervals between fractions which allowed recovery was another factor. Marples and Joiner [186] have shown that 6-24 hours were necessary between fractions to allow for recovery of HRS in V79 cells. Short *et al*. also suggested that when consecutive low doses were given, a lower than predicted cell survival, consistent with repeated HRS, only occurred when the doses were spaced by certain intervals [187]. At the molecular level, the trigger dose and recovery time for a particular molecule, such as ATM, early G2-phase checkpoint, and so on, remain unknown in complex physical environment, which have been revealed to be important in what way the cells decide to response to low dose irradiation. All of these different effects, depending on the fractionated dose size, accumulative dose, time left for repair, tissue characteristics etc, may explain that the foci levels reach their minimum and maximum value at different time points in kidney and intestine.

6. References

[1]. Upton, A.C., Carcinogenic effects of low-level ionizing radiation: problems and prospects. In Vivo, 2002. 16(6): p. 527-33.

[2].Murtha, A.D., Review of low-dose-rate radiobiology for clinicians. Semin Radiat Oncol, 2000. 10(2): p. 133-8.

[3]. Wakeford, R., The cancer epidemiology of radiation. Oncogene, 2004. 23(38): p. 6404-28.

[4]. Feinendegen, L.E., A.L. Brooks and W.F. Morgan, Epidemiology of radiation-induced cancer. Health Phys, 2011. 100(3): p. 303.

[5]. Brenner, D.J., et al., Cancer risks attributable to low doses of ionizing radiation: assessing what we really know. Proc Natl Acad Sci U S A, 2003. 100(24): p. 13761-6.

[6]. Yamaoka, K., R. Edamatsu and A. Mori, Increased SOD activities and decreased lipid peroxide levels induced by low dose X irradiation in rat organs. Free Radic Biol Med, 1991. 11(3): p. 299-306.

[7]. Feinendegen, L.E., et al., Cellular mechanisms of protection and repair induced by radiation exposure and their consequences for cell system responses. Stem Cells, 1995. 13 Suppl 1: p. 7-20.

[8]. Boice, J.J., Study of health effects of low-level radiation in USA nuclear shipyard workers. J Radiol Prot, 2001. 21(4): p. 400-3.

[9]. Cuttler, J.M., Health effects of low level radiation: when will we acknowledge the reality? Dose Response, 2007. 5(4): p. 292-8.

[10]. Yu, H.S., et al., Effects of low-dose radiation on tumor growth, erythrocyte immune function and SOD activity in tumor-bearing mice. Chin Med J (Engl), 2004. 117(7): p. 1036-9.

[11]. Kojima, S., K. Nakayama and H. Ishida, Low dose gamma-rays activate immune functions via induction of glutathione and delay tumor growth. J Radiat Res (Tokyo), 2004. 45(1): p. 33-9.

[12]. O'Driscoll, M. and P.A. Jeggo, The role of double-strand break repair - insights from human genetics. Nat Rev Genet, 2006. 7(1): p. 45-54.

[13]. Povirk, L.F., Biochemical mechanisms of chromosomal translocations resulting from DNA double-strand breaks. DNA Repair (Amst), 2006. 5(9-10): p. 1199-212.

[14]. Jankovic, M., A. Nussenzweig and M.C. Nussenzweig, Antigen receptor diversification and chromosome translocations. Nat Immunol, 2007. 8(8): p. 801-8.

[15]. Stavnezer, J., J.E. Guikema and C.E. Schrader, Mechanism and regulation of class switch recombination. Annu Rev Immunol, 2008. 26: p. 261-92.

[16]. Bassing, C.H., W. Swat and F.W. Alt, The mechanism and regulation of chromosomal V(D)J recombination. Cell, 2002. 109 Suppl: p. S45-55.

[17]. Kastan, M.B. and J. Bartek, Cell-cycle checkpoints and cancer. Nature, 2004. 432(7015): p. 316-23.

[18]. Bekker-Jensen, S., et al., Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. J Cell Biol, 2006. 173(2): p. 195-206.

[19]. Rupnik, A., M. Grenon and N. Lowndes, The MRN complex. Curr Biol, 2008. 18(11): p. R455-7.

[20]. Stiff, T., et al., ATM and DNA-PK Function Redundantly to Phosphorylate H2AX after Exposure to Ionizing Radiation. CANCER RESEARCH, 2004. 64: p. 2390-2396.

[21]. Ward, I.M. and J. Chen, Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J Biol Chem, 2001. 276(51): p. 47759-62.

[22]. Zhou, B.B. and S.J. Elledge, The DNA damage response: putting checkpoints in perspective. Nature, 2000. 408(6811): p. 433-9.

[23]. Kobayashi, J., et al., Current topics in DNA double-strand break repair. J Radiat Res (Tokyo), 2008.

49(2): p. 93-103.

[24]. Li, L. and L. Zou, Sensing, signaling, and responding to DNA damage: organization of the checkpoint pathways in mammalian cells. J Cell Biochem, 2005. 94(2): p. 298-306.

[25]. Rothkamm, K., et al., Pathways of DNA Double-Strand Break Repair during the Mammalian Cell Cycle. MOLECULAR AND CELLULAR BIOLOGY, 2003. 23(16): p. 5706-5715.

[26]. Saleh-Gohari, N. and T. Helleday, Conservative homologous recombination preferentially repairs DNA double-strand breaks in the S phase of the cell cycle in human cells. Nucleic Acids Res, 2004. 32(12): p. 3683-8.

[27]. Essers, J., et al., Homologous and non-homologous recombination differentially affect DNA damage repair in mice. EMBO J, 2000. 19(7): p. 1703-10.

[28]. Li, X. and W.D. Heyer, Homologous recombination in DNA repair and DNA damage tolerance. Cell Res, 2008. 18(1): p. 99-113.

[29]. Lieber, M.R., The mechanism of human nonhomologous DNA end joining. J Biol Chem, 2008. 283(1): p. 1-5.

[30]. Mahaney, B.L., K. Meek and S.P. Lees-Miller, Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. Biochem J, 2009. 417(3): p. 639-50.

[31].Weterings, E. and D.J. Chen, The endless tale of non-homologous end-joining. Cell Res, 2008. 18(1): p. 114-24.

[32]. Downs, J.A. and S.P. Jackson, A means to a DNA end: the many roles of Ku. Nat Rev Mol Cell Biol, 2004. 5(5): p. 367-78.

[33]. Kim, J.S., et al., Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells. J Cell Biol, 2005. 170(3): p. 341-7.

[34]. Gu, Y., et al., Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. Proc Natl Acad Sci U S A, 1997. 94(15): p. 8076-81.

[35]. Gu, Y., et al., Growth retardation and leaky SCID phenotype of Ku70-deficient mice. Immunity, 1997. 7(5): p. 653-65.

[36]. Meek, K., V. Dang and S.P. Lees-Miller, DNA-PK: the means to justify the ends? Adv Immunol, 2008. 99: p. 33-58.

[37]. Ma, Y., et al., Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. Cell, 2002. 108(6): p. 781-94.

[38].Ma, Y., K. Schwarz and M.R. Lieber, The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps. DNA Repair (Amst), 2005. 4(7): p. 845-51.

[39]. Barnes, D.E., et al., Targeted disruption of the gene encoding DNA ligase IV leads to lethality in embryonic mice. Curr Biol, 1998. 8(25): p. 1395-8.

[40]. Frank, K.M., et al., DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. Mol Cell, 2000. 5(6): p. 993-1002.

[41]. Lobrich, M. and P.A. Jeggo, The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer, 2007. 7(11): p. 861-9.

[42]. Karran, P., DNA double strand break repair in mammalian cells. Curr Opin Genet Dev, 2000. 10(2): p. 144-50.

[43]. Helleday, T., et al., DNA double-strand break repair: From mechanistic understanding to cancer treatment. dna repair, 2007. 6: p. 923-935.

[44]. San, F.J., P. Sung and H. Klein, Mechanism of eukaryotic homologous recombination. Annu Rev

Biochem, 2008. 77: p. 229-57.

[45]. Sung, P. and H. Klein, Mechanism of homologous recombination: mediators and helicases take on regulatory functions. Nat Rev Mol Cell Biol, 2006. 7(10): p. 739-50.

[46]. Valerie, K. and L.F. Povirk, Regulation and mechanisms of mammalian double-strand break repair. Oncogene, 2003. 22(37): p. 5792-812.

[47]. Frank-Vaillant, M. and S. Marcand, NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the ligase IV pathway. Genes Dev, 2001. 15(22): p. 3005-12.

[48]. Lee, S.E., et al., Role of yeast SIR genes and mating type in directing DNA double-strand breaks to homologous and non-homologous repair paths. Curr Biol, 1999. 9(14): p. 767-70.

[49]. Orii, K.E., et al., Selective utilization of nonhomologous end-joining and homologous recombination DNA repair pathways during nervous system development. Proc Natl Acad Sci U S A, 2006. 103(26): p. 10017-22.

[50]. Shrivastav, M., L.P. De Haro and J.A. Nickoloff, Regulation of DNA double-strand break repair pathway choice. Cell Research, 2008. 18(1): p. 134-147.

[51]. Branzei, D. and M. Foiani, Regulation of DNA repair throughout the cell cycle. nature reviews | molecular cell biology, 2008. 9: p. 297-308.

[52]. Goodarzi, A.A., et al., ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. Mol Cell, 2008. 31(2): p. 167-77.

[53]. Perry, J. and N. Kleckner, The ATRs, ATMs, and TORs are giant HEAT repeat proteins. Cell, 2003. 112(2): p. 151-5.

[54]. Bakkenist, C.J. and M.B. Kastan, DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature, 2003. 421(6922): p. 499-506.

[55]. Andegeko, Y., et al., Nuclear retention of ATM at sites of DNA double strand breaks. J Biol Chem, 2001. 276(41): p. 38224-30.

[56]. Berkovich, E., R.J. Monnat and M.B. Kastan, Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. Nat Cell Biol, 2007. 9(6): p. 683-90.

[57].Pandita, T.K., A multifaceted role for ATM in genome maintenance. Expert Rev Mol Med, 2003. 5(16): p. 1-21.

[58]. Paull, T.T. and J.H. Lee, The Mre11/Rad50/Nbs1 complex and its role as a DNA double-strand break sensor for ATM. Cell Cycle, 2005. 4(6): p. 737-40.

[59]. Lee, J.H. and T.T. Paull, ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science, 2005. 308(5721): p. 551-4.

[60]. Carson, C.T., et al., The Mre11 complex is required for ATM activation and the G2/M checkpoint. EMBO J, 2003. 22(24): p. 6610-20.

[61]. Difilippantonio, S., et al., Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. Nat Cell Biol, 2005. 7(7): p. 675-85.

[62]. Rogakou, E.P., et al., DNA Double-stranded Breaks Induce Histone H2AX Phosphorylation on Serine 139. THE JOURNAL OF BIOLOGICAL CHEMISTRY, 1998. 273(10): p. 5858-5868.

[63]. Redon, C., et al., Histone H2A variants H2AX and H2AZ. Curr Opin Genet Dev, 2002. 12(2): p. 162-9.

[64]. Burma, S., et al., ATM Phosphorylates Histone H2AX in Response to DNA Double-strand Breaks. THE JOURNAL OF BIOLOGICAL CHEMISTRY, 2001. 276(45): p. 42462-42467.

[65]. Rogakou, E.P., et al., Megabase Chromatin Domains Involved in DNA Double-Strand Breaks In Vivo. The Journal of Cell Biology, 1999. 146(5): p. 905-915.

[66]. Pilch, D.R., et al., Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. Biochem Cell Biol, 2003. 81(3): p. 123-9.

[67]. Rube, C.E., et al., DNA repair in the context of chromatin: new molecular insights by the nanoscale detection of DNA repair complexes using transmission electron microscopy. DNA Repair (Amst), 2011. 10(4): p. 427-37.

[68]. Svetlova, M.P., L.V. Solovjeva and N.V. Tomilin, mechanism of elimination of gamma-H2ax from chromatin after repair of DSBs. Mutation Research, 2010. 685: p. 54-60.

[69]. Nazarov, I.B., et al., Dephosphorylation of histone gamma-H2AX during repair of DNA double-strand breaks in mammalian cells and its inhibition by calyculin A. Radiation research, 2003. 160(3): p. 309--317.

[70]. Chicheportiche, A., et al., Characterization of Spo11-dependent and independent phospho-H2AX foci during meiotic prophase I in the male mouse. Journal of cell science, 2007. 120(Pt 10): p. 1733.

[71]. Sedelnikova, O.A., et al., DNA double-strand breaks form in bystander cells after microbeam irradiation of three-dimensional human tissue models. Cancer Res, 2007. 67(9): p. 4295-302.

[72]. Sedelnikova, O.A., et al., Delayed kinetics of DNA double-strand break processing in normal and pathological aging. Aging Cell, 2008. 7(1): p. 89-100.

[73]. MacPhail, S.H., et al., Cell cycle-dependent expression of phosphorylated histone H2AX: reduced expression in unirradiated but not X-irradiated G1-phase cells. Radiat Res, 2003. 159(6): p. 759-67.

[74]. Ward, I.M., et al., Accumulation of checkpoint protein 53BP1 at DNA breaks involves its binding to phosphorylated histone H2AX. J Biol Chem, 2003. 278(22): p. 19579-82.

[75]. Celeste, A., et al., Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol, 2003. 5(7): p. 675-9.

[76]. Celeste, A., et al., Genomic instability in mice lacking histone H2AX. Science, 2002. 296(5569): p. 922-7.

[77]. Paull, T.T., et al., A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol, 2000. 10(15): p. 886-95.

[78]. Stucki, M., et al., MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell, 2005. 123(7): p. 1213-26.

[79]. Lou, Z., et al., MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals. Mol Cell, 2006. 21(2): p. 187-200.

[80]. Lukas, C., et al., Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. EMBO J, 2004. 23(13): p. 2674-83.

[81]. Fernandez-Capetillo, O., et al., DNA damage-induced G2 – M checkpoint activation by histone H2AX and 53BP1. nature cell Biology, 2002. 4: p. 993-997.

[82]. Celeste, A., et al., H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. Cell, 2003. 114(3): p. 371-83.

[83]. Iwabuchi, K., et al., Stimulation of p53-mediated transcriptional activation by the p53-binding proteins, 53BP1 and 53BP2. J Biol Chem, 1998. 273(40): p. 26061-8.

[84]. Joo, W.S., et al., Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure. Genes Dev, 2002. 16(5): p. 583-93.

[85]. Chen, J., V. Marechal and A.J. Levine, Mapping of the p53 and mdm-2 interaction domains. Mol Cell Biol, 1993. 13(7): p. 4107-14.

[86]. Seto, E., et al., Wild-type p53 binds to the TATA-binding protein and represses transcription. Proc Natl Acad Sci U S A, 1992. 89(24): p. 12028-32.

[87]. Vogelstein, B., D. Lane and A.J. Levine, Surfing the p53 network. Nature, 2000. 408(6810): p. 307-10.

[88]. Schultz, L.B., et al., p53 Binding Protein 1 (53BP1) Is an Early Participant in the Cellular Response to DNA Double-Strand Breaks. The Journal of Cell Biology, 2000. 151(7): p. 1381-1390.

[89]. Rappold, I., et al., Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J Cell Biol, 2001. 153(3): p. 613-20.

[90]. ANDERSON, L., C. HENDERSON and Y. ADACHI, Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. MOLECULAR AND CELLULAR BIOLOGY, 2001. 21(5): p. 1719-1729.

[91]. Costes, S.V., et al., Spatiotemporal characterization of ionizing radiation induced DNA damage foci and their relation to chromatin organization. Mutation Research, 2010. 704: p. 78-87.

[92]. Maser, R.S., et al., hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks. Mol Cell Biol, 1997. 17(10): p. 6087-96.

[93]. Haaf, T., et al., Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes. Proc Natl Acad Sci U S A, 1995. 92(6): p. 2298-302.

[94]. Scully, R., et al., Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell, 1997. 90(3): p. 425-35.

[95]. Mochan, T.A., et al., 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. Cancer Res, 2003. 63(24): p. 8586-91.

[96]. FitzGerald, J.E., M. Grenon and N.F. Lowndes, 53BP1: function and mechanisms of focal recruitment. Biochem Soc Trans, 2009. 37(Pt 4): p. 897-904.

[97]. Iwabuchi, K., et al., Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. J Biol Chem, 2003. 278(38): p. 36487-95.

[98]. Iwabuchi, K., et al., 53BP1 contributes to survival of cells irradiated with X-ray during G1 without Ku70 or Artemis. Genes Cells, 2006. 11(8): p. 935-48.

[99]. Wakeford, R. and E.J. Tawn, The meaning of low dose and low dose-rate. J. Radiol. Prot., 2010. 30: p. 1-3.

[100].Bonner, W.M., Low-dose radiation: Thresholds, bystander effects and adaptive responses. PNAS, 2003. 100(9): p. 4973-4975.

[101]. Marples, B. and M.C. Joiner, The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. Radiat Res, 1993. 133(1): p. 41-51.

[102]. Joiner, M.C., et al., Hypersensitivity to very-low single radiation doses: its relationship to the adaptive response and induced radioresistance. Mutat Res, 1996. 358(2): p. 171-83.

[103]. Wouters, B.G. and L.D. Skarsgard, The response of a human tumor cell line to low radiation doses: evidence of enhanced sensitivity. Radiat Res, 1994. 138(1 Suppl): p. S76-80.

[104]. Wouters, B.G., A.M. Sy and L.D. Skarsgard, Low-dose hypersensitivity and increased radioresistance in a panel of human tumor cell lines with different radiosensitivity. Radiat Res, 1996. 146(4): p. 399-413.

[105]. Wouters, B.G. and L.D. Skarsgard, Low-dose radiation sensitivity and induced radioresistance to cell killing in HT-29 cells is distinct from the "adaptive response" and cannot be explained by a subpopulation of sensitive cells. Radiat Res, 1997. 148(5): p. 435-42.

[106]. Joiner, M.C. and H. Johns, Renal Damage in the Mouse: The Response to Very Small Doses per Fraction. Radiation Research, 1988. 114(2): p. 385-398.

[107]. Brian Marples, P.D. and P.D. Spencer J. Collis, Low-dose hyper-radiosensitivity: past, present and future. Int. J. Radiation Oncology Biol. Phys., 2008. 70(5): p. 1310-1318.

[108]. Wykes, S.M., et al., Low-Dose Hyper-radiosensitivity is not Caused by a Failure to Recognize DNA

Double-Strand Breaks. RADIATION RESEARCH, 2006. 165: p. 516-524.
[109]. B, X., S. Kim and D. Lim, Two Molecularly Distinct G2 M Checkpoints Are Induced by Ionizing Irradiation. Mol cell Biol, 2002. 22: p. 1049-1059.
[110]. Krempler, A., et al., An imperfect G2M checkpoint contributes to chromosome instability following irradiation of S and G2 phase cells. Cell Cycle, 2007. 6(14): p. 1682-6.
[111]. Krueger, S.A., et al., Transition in survival from low-dose hyper-radiosensitivity to increased radioresistance is independent of activation of ATM Ser1981 activity. Int J Radiat Oncol Biol Phys, 2007. 69(4): p. 1262-71.
[112]. Marples, B., et al., Low-dose hyper-radiosensitivity: a consequence of ineffective cell cycle arrest of radiation-damaged G2-phase cells. Radiat Res, 2004. 161(3): p. 247-55.
[113]. Vaganay-Juery, S., et al., Decreased DNA-PK activity in human cancer cells exhibiting hypersensitivity to low-dose irradiation. Br J Cancer, 2000. 83(4): p. 514-8.
[114]. Marples, B., et al., Evidence for the involvement of DNA-dependent protein kinase in the phenomena of low dose hyper-radiosensitivity and increased radioresistance. Int J Radiat Biol, 2002. 78(12): p. 1139-47.
[115]. Chalmers, A., et al., PARP-1, PARP-2, and the cellular response to low doses of ionizing radiation. Int J Radiat Oncol Biol Phys, 2004. 58(2): p. 410-9.
[116]. Marples, B. and M.C. Joiner, Modification of survival by DNA repair modifiers: a probable explanation for the phenomenon of increased radioresistance. Int J Radiat Biol, 2000. 76(3): p. 305-12.
[117]. Enns, L., et al., Low-dose radiation hypersensitivity is associated with p53-dependent apoptosis. Mol Cancer Res, 2004. 2(10): p. 557-66.
[118]. Krueger, S.A., et al., Role of apoptosis in low-dose hyper-radiosensitivity. Radiat Res, 2007. 167(3): p. 260-7.
[119]. Hickman, A.W., et al., Alpha-particle-induced p53 protein expression in a rat lung epithelial cell strain. Cancer Res, 1994. 54(22): p. 5797-800.
[120]. Azzam, E.I., S.M. de Toledo and J.B. Little, Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. Proc Natl Acad Sci U S A, 2001. 98(2): p. 473-8.
[121]. Azzam, E.I., et al., Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. Radiat Res, 1998. 150(5): p. 497-504.
[122]. Azzam, E.I., S.M. de Toledo and J.B. Little, Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. Oncogene, 2003. 22(45): p. 7050-7.
[123]. Lehnert, B.E., E.H. Goodwin and A. Deshpande, Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells. Cancer Res, 1997. 57(11): p. 2164-71.
[124]. Nagasawa, H. and J.B. Little, Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res, 1992. 52(22): p. 6394-6.
[125]. Deshpande, A., et al., Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. Radiat Res, 1996. 145(3): p. 260-7.
[126]. Belyakov, O.V., et al., Delayed lethality, apoptosis and micronucleus formation in human fibroblasts irradiated with X-rays or alpha-particles. Int J Radiat Biol, 1999. 75(8): p. 985-93.
[127]. Belyakov, O.V., et al., Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. Br J Cancer, 2001. 84(5): p. 674-9.
[128]. Nagasawa, H. and J.B. Little, Unexpected sensitivity to the induction of mutations by very low doses of alpha-particle radiation: evidence for a bystander effect. Radiat Res, 1999. 152(5): p. 552-7.

[129]. Zhou, H., et al., Induction of a bystander mutagenic effect of alpha particles in mammalian cells. Proc Natl Acad Sci U S A, 2000. 97(5): p. 2099-104.
[130]. Zhou, H., et al., Radiation risk to low fluences of alpha particles may be greater than we thought. Proc Natl Acad Sci U S A, 2001. 98(25): p. 14410-5.
[131]. Lorimore, S.A., et al., Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. Proc Natl Acad Sci U S A, 1998. 95(10): p. 5730-3.
[132]. Shao, C., M. Aoki and Y. Furusawa, Bystander effect on cell growth stimulation in neoplastic HSGc cells induced by heavy-ion irradiation. Radiat Environ Biophys, 2003. 42(3): p. 183-7.
[133]. Iyer, R., B.E. Lehnert and R. Svensson, Factors underlying the cell growth-related bystander responses to alpha particles. Cancer Res, 2000. 60(5): p. 1290-8.
[134]. Komarova, E.A., et al., Stress-induced secretion of growth inhibitors: a novel tumor suppressor function of p53. Oncogene, 1998. 17(9): p. 1089-96.
[135]. Iyer, R. and B.E. Lehnert, Low dose, low-LET ionizing radiation-induced radioadaptation and associated early responses in unirradiated cells. Mutat Res, 2002. 503(1-2): p. 1-9.
[136]. Iyer, R. and B.E. Lehnert, Alpha-particle-induced increases in the radioresistance of normal human bystander cells. Radiat Res, 2002. 157(1): p. 3-7.
[137]. Ballarini, F., et al., Cellular communication and bystander effects: a critical review for modelling low-dose radiation action. Mutat Res, 2002. 501(1-2): p. 1-12.
[138]. Wright, E.G. and P.J. Coates, Untargeted effects of ionizing radiation: implications for radiation pathology. Mutat Res, 2006. 597(1-2): p. 119-32.
[139]. Seymour, C.B. and C. Mothersill, Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. Radiat Res, 2000. 153(5 Pt 1): p. 508-11.
[140]. Liu, Z., et al., A dose threshold for a medium transfer bystander effect for a human skin cell line. Radiat Res, 2006. 166(1 Pt 1): p. 19-23.
[141]. Wolff, S., The adaptive response in radiobiology: evolving insights and implications. Environ Health Perspect, 1998. 106 Suppl 1: p. 277-83.
[142]. Wolff, S., Aspects of the adaptive response to very low doses of radiation and other agents. Mutat Res, 1996. 358(2): p. 135-42.
[143]. Matsumoto, H. and T. Ohnishi, Contribution of radiation-induced, nitric oxide-mediated bystander effect to radiation-induced adaptive response. Biol Sci Space, 2004. 18(3): p. 108-9.
[144]. Coates, P.J., S.A. Lorimore and E.G. Wright, Damaging and protective cell signalling in the untargeted effects of ionizing radiation. Mutat Res, 2004. 568(1): p. 5-20.
[145]. Cai, L. and S.Z. Liu, Induction of cytogenetic adaptive response of somatic and germ cells in vivo and in vitro by low-dose X-irradiation. Int J Radiat Biol, 1990. 58(1): p. 187-94.
[146]. Cai, L., Research of the adaptive response induced by low-dose radiation: where have we been and where should we go? Hum Exp Toxicol, 1999. 18(7): p. 419-25.
[147]. Yonezawa, M., J. Misonoh and Y. Hosokawa, Two types of X-ray-induced radioresistance in mice: presence of 4 dose ranges with distinct biological effects. Mutat Res, 1996. 358(2): p. 237-43.
[148]. Cramers, P., et al., Pre-exposure to low doses: modulation of X-ray-induced dna damage and repair? Radiat Res, 2005. 164(4 Pt 1): p. 383-90.
[149]. Gajendiran, N., et al., Neutron-induced adaptive response studied in Go human lymphocytes using the comet assay. Journal of radiation research, 2001. 42(1): p. 91--101.
[150]. Olivieri, G., J. Bodycote and S. Wolff, Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. Science, 1984. 223(4636): p. 594-7.

[151]. Shadley, J.D. and J.K. Wiencke, Induction of the adaptive response by X-rays is dependent on radiation intensity. Int J Radiat Biol, 1989. 56(1): p. 107-18.
[152]. Azzam, E.I., G.P. Raaphorst and R.E. Mitchel, Radiation-induced adaptive response for protection against micronucleus formation and neoplastic transformation in C3H 10T1/2 mouse embryo cells. Radiat Res, 1994. 138(1 Suppl): p. S28-31.
[153]. Sawant, S.G., et al., Adaptive response and the bystander effect induced by radiation in C3H 10T(1/2) cells in culture. Radiat Res, 2001. 156(2): p. 177-80.
[154]. Sasaki, M.S., On the reaction kinetics of the radioadaptive response in cultured mouse cells. Int J Radiat Biol, 1995. 68(3): p. 281-91.
[155]. Shadley, J.D., V. Afzal and S. Wolff, Characterization of the adaptive response to ionizing radiation induced by low doses of X rays to human lymphocytes. Radiat Res, 1987. 111(3): p. 511-7.
[156]. Vijayalaxmi, et al., Variability in adaptive response to low dose radiation in human blood lymphocytes: consistent results from chromosome aberrations and micronuclei. Mutat Res, 1995. 348(1): p. 45-50.
[157]. Kalina, I. and G. Nemethova, Variability of the adaptive response to low dose radiation in peripheral blood lymphocytes of twins and unrelated donors. Folia Biol (Praha), 1997. 43(2): p. 91-5.
[158]. Bosi, A. and G. Olivieri, Variability of the adaptive response to ionizing radiations in humans. Mutat Res, 1989. 211(1): p. 13-7.
[159]. Tapio, S. and V. Jacob, Radioadaptive response revisited. Radiat Environ Biophys, 2007. 46(1): p. 1-12.
[160]. Wiencke, J.K., et al., Evidence that the [3H]thymidine-induced adaptive response of human lymphocytes to subsequent doses of X-rays involves the induction of a chromosomal repair mechanism. Mutagenesis, 1986. 1(5): p. 375-80.
[161]. Khodarev, N.N., et al., STAT1 is overexpressed in tumors selected for radioresistance and confers protection from radiation in transduced sensitive cells. Proc Natl Acad Sci U S A, 2004. 101(6): p. 1714-9.
[162]. Coleman, M.A., et al., Low-dose irradiation alters the transcript profiles of human lymphoblastoid cells including genes associated with cytogenetic radioadaptive response. Radiat Res, 2005. 164(4 Pt 1): p. 369-82.
[163]. Glatstein, E., et al., The uptake of rubidium-86 in mouse kidneys irradiated with fractionated doses of x rays. Radiat Res, 1975. 61(3): p. 417-26.
[164]. Otsuka, M. and M.L. Meistrich, Cell Proliferation and Abnormal Nuclei Induced by Radiation in Renal Tubule Epithelium as anEarly Manifestation of Late Damage. Radiation Research, 1990. 123(3): p. 285-291.
[165]. Robbins, M.E., et al., Radiation-induced changes in the kinetics of glomerular and tubular cells in the pig kidney. Radiat Res, 1994. 138(1): p. 107-13.
[166]. Moulder, J.E., et al., Angiotensin II blockade reduces radiation-induced proliferation in experimental radiation nephropathy. Radiat Res, 2002. 157(4): p. 393-401.
[167]. van Kleef, E., et al., In vitro and in vivo expression of endothelial von Willebrand factor and leukocyte accumulation after fractionated irradiation. Radiat Res, 2000. 154(4): p. 375-81.
[168]. Potten, C.S., Structure, function and proliferative organisation of mammalian gut. Radiation and gut, 1995: p. 1--31.
[169]. Lesher, S. and J. Bauman, Cell kinetic studies of the intestinal epithelium: maintenance of the intestinal epithelium in normal and irradiated animals. National Cancer Institute monograph, 1969. 30: p. 185.
[170]. Hagemann, R.F., Compensatory proliferative response of the colonic epithelium to multi-fraction irradiation. International Journal of Radiation Oncology* Biology* Physics, 1979. 5(1): p. 69--71.
[171]. Hagemann, R.F., Intestinal cell proliferation during fractionated abdominal irradiation. British Journal of Radiology, 1976. 49(577): p. 56.

[172].CLAUDIA E. RUEBE, M.D.P.D., et al., DNA double-strand break rejoining in complex normal tissues. Int. J. Radiation Oncology Biol. Phys., 2008. 72(4): p. 1180-1187.

[173]. Stenerlow, B. and E. Hoglund, Rejoining of double-stranded DNA-fragments studied in different size-intervals. Int J Radiat Biol, 2002. 78(1): p. 1-7.

[174].Prise, K.M., et al., A review of dsb induction data for varying quality radiations. Int J Radiat Biol, 1998. 74(2): p. 173-84.

[175]. Löbrich, M., et al., ɣ H2AX foci analysis for monitoring DNA double-strand break repair. Cell Cycle, 2010. 9(4): p. 662-669.

[176]. Rothkamm, K. and M. Loebrich, Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. PNAS, 2003. 100(9): p. 5057-5062.

[177]. Kinner, A., et al., Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res, 2008. 36(17): p. 5678-94.

[178]. Belyaev, I.Y., Radiation-induced DNA repair foci: Spatio-temporal aspects of formation, application for assessment of radiosensitivity and biological dosimetry. Mutation Research, 2010. 704: p. 132-141.

[179]. Pogribny, I., et al., Fractionated Low-Dose Radiation Exposure Leads to Accumulation of DNA Damage and Profound Alterations in DNA and Histone Methylation in the Murine Thymus. Mol Cancer Res, 2005. 3(10): p. 553-561.

[180]. Torudd, J., et al., Dose-response for radiation-induced apoptosis, residual 53BP1 foci and DNA-loop relaxation in human lymphocytes. Int J Radiat Biol, 2005. 81(2): p. 125-38.

[181]. van Veelen, L.R., et al., Analysis of ionizing radiation-induced foci of DNA damage repair proteins. Mutat Res, 2005. 574(1-2): p. 22-33.

[182]. Grudzenski, S., et al., Inducible response required for repair of low-dose radiation damage in human fibroblasts. Proc Natl Acad Sci U S A, 2010. 107(32): p. 14205-10.

[183]. Markova, E., N. Schultz and I.Y. Belyaev, Kinetics and dose-response of residual 53BP1/gamma-H2AX foci: co-localization, relationship with DSB repair and clonogenic survival. Int J Radiat Biol, 2007. 83(5): p. 319-29.

[184]. Yamauchi, M., et al., Growth of persistent foci of DNA damage checkpoint factors is essential for amplification of G1 checkpoint signaling. DNA Repair (Amst), 2008. 7(3): p. 405-17.

[185]. Suzuki, M., et al., Phosphorylated histone H2AX foci persist on rejoined mitotic chromosomes in normal human diploid cells exposed to ionizing radiation. Radiat Res, 2006. 165(3): p. 269-76.

[186]. Marples, B. and M.C. Joiner, The elimination of low-dose hypersensitivity in Chinese hamster V79-379A cells by pretreatment with X rays or hydrogen peroxide. Radiat Res, 1995. 141(2): p. 160-9.

[187]. Short, S.C., et al., Low-dose hypersensitivity after fractionated low-dose irradiation in vitro. Int J Radiat Biol, 2001. 77(6): p. 655-64.

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