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## Involvement of Non-Homologous End-Joining in Radiation-Induced Genomic Instability

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### 1. Introduction

DNA double-strand breaks are the most detrimental form of DNA damage induced by either endogenous and exogenous sources. DNA double-strand breaks are generated in response to ionizing radiation, radiomimic drugs, and topoisomerase inhibitors. They also created during V(D)J and class switch recombination in lymphocytes, meiotic recombination in germ cells, and by retroviral integrations (Downs et al., 2007, Polo and Jackson, 2011). Since DNA double-strand breaks discontinue chromosome structure, they may result in cell death that are associated with radiosensitivity, immunodeficiency, neurodegeneration, and developmental defects (Jackson and Bartek, 2009, Mahaney et al., 2009, O'Driscoll and Jeggo, 2006, Weterings and Chen, 2007, Wyman and Kanaar, 2006). Thus, cells have evolved sophisticated mechanisms, by which DNA double-strand breaks are repaired. Two major pathways to repair DNA double-strand breaks are non-homologous end-joining and homologous and homologous recombination (Hartlerode and Scully, 2009, Mahaney et al., 2009, Weterings and Chen, 2007, Wyman and Kanaar, 2006). While rejoining of DNA breaks are indispensable for the survival of cells, DNA repair, by itself, may threaten the stability of the genome. (Burma et al., 2006, Pastink et al., 2001, Sonoda et al., 2006, van Gent et al., 2001). In particular, non-homologous end-joining, which is the primary DNA repair pathway functions in G1 phase, is error-prone (Hartlerode and Scully, 2009, Lieber, 2010). It causes loss or rearrangement of the genetic information through mis-rejoining of DNA double strand breaks. Processing of DNA broken ends by exonucleases and endonucleases also provide another chance to alter DNA sequences. Consequently, surviving cells can avoid lethal effects of DNA double-strand breaks but it results in a loss of heterozygosity as well as gross genome rearrangements that are associated with cancer predisposition. Although most genome rearrangements have been thought to be generated directly by the initial radiation exposure (Leonhardt et al., 1999), recent findings have demonstrated that the integrity of the genome is also endangered eventually, if the cells were survived exposure to DNA damaging agents. In this chapter, the results showing that delayed DNA double-strand breaks are induced several generations after the initial insult in the progenies of surviving cells are presented, and a role of non-homologous end-joining on delayed

manifestation of radiation effects and the integrity of the genome in the cells surviving radiation exposure will be discussed.

## 2. Radiation-induced genomic instability

It has been well established that ionizing radiation induces delayed effects in the progeny of surviving cells (Little, 2003, Lorimore et al., 2003, Morgan et al., 1996, Suzuki et al., 2003). This phenomenon is now called radiation-induced genomic instability, which is manifested as the delayed expression of various radiation effects, such as delayed reproductive death, delayed chromosomal instability, and delayed mutagenesis (Figure 1). Radiation-induced genomic instability has been commonly observed in many cell culture systems as well as in various animals (Lorimore et al., 2003, Morgan, 2003). In addition, there are a series of studies showing that radiation-induced genomic instability is attributed to transgenerational effects in mice using hypervariable minisatellite sequences, which have been renamed as expanded simple tandem repeats (Niwa, 2006). Radiation-induced genomic instability results in accumulating gene mutations and chromosomal rearrangements in addition to the direct genome damage caused by the primary radiation exposure. Therefore, it has been thought to play a pivotal role in accelerating the process of radiation-induced carcinogenesis (Huang et al., 2003, Kadhim et al., 1992, Niwa, 2003, Suzuki, 1997).

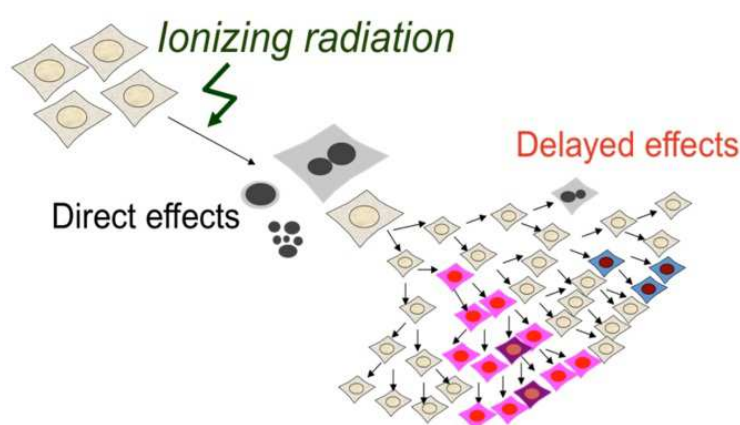


Fig. 1. Radiation-induced genomic instability.

Because radiation-induced genomic instability is induced in a certain fraction of the progenies originated from a single survived cell, not a single gene mutation but some epigenetic changes could be involved in the initiation of radiation-induced genomic instability (Wright, 2010). Persistently elevated levels of oxidative stress was found is association with radiation-induced genomic instability (Azzam et al., 2003, Kim et al., 2006a, Limoli et al., 2003). Further association was confirmed in studies where chronic hydrogen peroxide treatment initiated instability. Administration of free-radical scavengers, antioxidant treatment, and reducing oxygen tension each reduced delayed chromosomal instability, suggesting a role of oxidative stress in perpetuating genomic instability (Roy et al., 2000). Moreover, unstable clones showed mitochondrial dysfunction, which might explain elevated levels of oxidative stress (Kim et al., 2006b). An inflammatory response has also been proposed to be involved in radiation-induced genomic instability particularly in bone marrow (Wright, 2010). Furthermore, possible involvement of bystander effects has also been discussed. Bystander effects are responses observed in unirradiated cells as a

result of receiving signals from irradiated cells (Mothersill and Seymour, 2004, Prise and O'Sullivan, 2009). A variety of responses have been described including DNA damage induction, chromosomal instability, and cell death. As bystander effects have been observed in coculture of irradiated and unirradiated cells, and after the transfer of medium from irradiated cells to unirradiated cells, secreted factor(s) may be involved in transducing the bystander signals (Sowa and Morgan, 2004). It has been hypothesized that increased secretion of transforming growth factor beta results in stimulation of production of reactive oxygen species through a membrane NADPH oxidase. In fact, previous study demonstrated that transforming growth factor beta increased oxidative stress through decreased activity of mitochondrial complex IV (Kim et al., 2006b).

Although oxidative stress is surely involved in perpetuation of radiation-induced genomic instability (Azzam et al., 2003, Coates et al., 2008, Miller et al., 2008, Kim et al., 2006a, Limoli et al., 2003, Wright, 2007), alternative mechanisms could be associated with manifestation of radiation-induced genomic instability in non-hematopoietic cells. We have shown that delayed unscheduled induction of DNA double strand breaks is involved in the manifestation of delayed phenotypes (Suzuki et al., 2003). In fact, our study indicated that increased phosphorylated histone H2AX foci, which correspond to DNA double-strand breaks, were frequently detected in the progeny of normal human diploid cells surviving X-rays. Moreover, delayed reactivation of p53 in response to DNA damage was manifested in the surviving clones. Delayed induction of DNA double strand breaks was also confirmed by delayed induction of chromosomal aberrations (Toyokuni et al., 2009). Thus, it is evidenced that induction of DNA double strand breaks is induced indirectly in surviving cells from exposure to radiation, indicating that DNA repair pathways could play roles in amending delayed DNA double-strand breaks in surviving cells.

Previously, Chang and Little reported that radiation-induced genomic instability was absent in *xrs5* cells, which are NHEJ-deficient Chinese hamster cells defective in Ku80 protein (Chang and Little, 1992a). Interestingly, delayed reproductive death was not observed in these cells, even though they harbor sufficient amount of DNA double-strand breaks. Since the mechanism of delayed reproductive death has not been fully described yet, we have hypothesized that defective NHEJ in *xrs5* cells decreases the chance of mis-rejoining of the broken ends, which result in the formation of dicentric chromosomes involved in division halt. To test this possibility we examined delayed chromosomal instability in two NHEJ-defective cells, *xrs-5* and *xrs-6* cells, and compared the frequency with the wild-type CHO cells. Furthermore, delayed induction of dicentric chromosomes was examined in cells defective in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs).

### 3. Non-homologous end-joining and its mutants

Non-homologous end-joining is one of the two major pathways involved in amending DNA double-strand breaks in multicellular eukaryotes. It primarily plays a critical role during G0-, G1- and early S-phase of cell cycle (Lieber 2010, Polo and Jackson, 2011). Non-homologous end-joining is initiated by binding a heterodimeric protein complex consists of Ku80 and Ku70 to both ends of DNA double-strand breaks (Jackson and Bartek, 2009, Mahaney et al., 2009, O'Driscoll and Jeggo, 2006, Weterings and Chen, 2007, Wyman and Kanaar, 2006). Then, DNA-PKcs, a catalytic subunit of DNA-dependent protein kinase, is recruited to Ku-DNA complex, which results in activation of the protein kinase activity of DNA-PKcs and tethering two broken DNA ends. DNA-PKcs phosphorylates a number of proteins,

including Ku80, Ku70, XRCC4, Artemis, DNA ligase IV and DNA-PKcs itself (Mahaney et al., 2009, Weterings and Chen, 2007). Autophosphorylation alters the conformation of DNA-PKcs, allowing the recruitment of DNA end-processing enzymes, such as MRE11 and Artemis (Huertas, 2010). MRE11 exhibits a 3' to 5' exonuclease activity and plays a critical role in homologous recombination, but its function in non-homologous end-joining is still to be determined. On the other hand, Artemis shows a 5' to 3' exonuclease activity, and it is suggested to be involved in a subset of DNA double-strand breaks. In fact, cells defective in both nucleases do not show significant radiation sensitivity. Processing of broken ends might create DNA single-strand breaks, which are amended by the DNA polymerase X family, such as polymerase mu and polymerase lambda, and terminal deoxyribonucleotidyltransferase. DNA double-strand breaks are finally rejoined by a complex composed of DNA ligase IV, XRCC4, and XLF.

Until now, several mutants defective in DNA double-strand break repair have been cloned (Jeggo, 1998). They are highly sensitive to radiation and show chromosome instability in response to DNA damaging agents. As shown in Table 1, at least four independent groups were identified. XR-1 mutant belong to X-ray repair complementing defective repair in Chinese hamster cells 4 (XRCC4) group holds mutations in the XRCC4 gene. Both xrs-5 and xrs-6 cells are the members of the XRCC5 group, and they show profound defect in the Ku80 function (Singleton et al., 1997). In xrs-5 cells, the expression of the Ku80 gene is significantly compromised. In xrs-6 cells, a 13-base pair insertion causes a truncation of KU80 protein, which accelerates degradation of KU80 protein. The responsible gene for the XRCC7 group is the DNA-PKcs (Zdzienicka, 1999). Both scid and V-3 mutants harbor premature termination of the protein in the C-terminus. In irs-20 a mutation that causes substitution of the amino acid located in the C-terminal region.

Mutants	Complementation group	Gene
XR-1	XRCC4	XRCC4
xrs-5, xrs-6	XRCC5	Ku80
-	XRCC6	Ku70
scid, V3	XRCC7	DNA-PKcs

Table 1. Rodent cell mutants with defective non-homologous end-joining repair.

Radiation sensitivity of these mutants was determined by colony formation assay. A single cell is able to form a cluster of progenitor cells, namely, a colony, when it is incubated for 10 days or more. After X-irradiation, numbers of colonies are decreased in a dose dependent manner. Consequently, cell survival can be calculated by dividing the number of colonies after X-irradiation by the number of colonies formed by the control cells. As shown in Figure 2, both xrs-5 (closed diamond), xrs-6 (closed triangle), and scid (closed square) cells show significant reduction of cell survival as compared to CHO cells (open circle). These cells lose the shoulder of the survival curve observed in CHO cells, indicating that they lose DNA repair capacity. To compliment the defect in xrs5 cells, the human Ku86 gene was introduced by electroporation, and as a result, radiation sensitivity was significantly recovered (open diamond), confirming that a single gene mutation causes profound DNA repair defect.



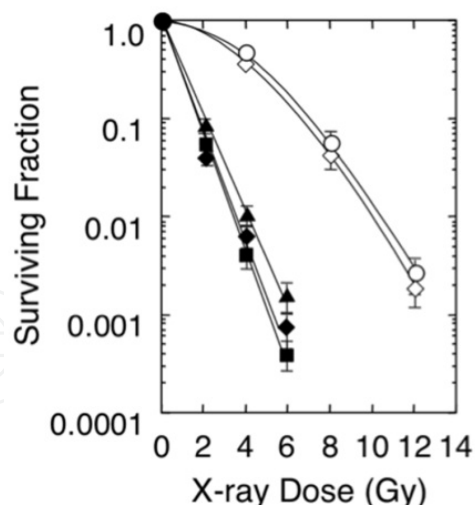


Fig. 2. Survival curves of CHO cells and Ku80-deficient cells.

These cells were irradiated with X-rays from an X-ray generator at 150 kVp and 5 mA with a 0.1-mm copper. The dose rate was 0.44 Gy/min. Dose rates were determined with an ionization chamber.

#### 4. Induction of delayed phenotypes in DNA repair-deficient cells

##### 4.1 Methods for detection of delayed phenotypes

Since radiation-induced genomic instability is manifested as the expression of delayed effects in the progeny of surviving cells, most of the study isolated colonies formed by the cells surviving X-irradiation. The primary colonies were cloned 10 days after irradiation. The cells obtained from each colony have already passed 15 to 20 population doublings. Then, the primary clones were subjected to the secondary colony formation. After 10 day, the secondary clones were isolated, and the cells were at 30 to 35 population doubling levels after irradiation. Delayed reproductive death was examined by colony-forming ability. Colonies derived from the surviving cells often contain giant cells, which occupied an area in the colony several times greater than the rest of the cells. Colonies containing at least one giant cell were judged as giant cell-positive colony. Delayed chromosomal bridges were detected between two dividing daughter nuclei in the anaphase cells in the surviving colonies. Delayed chromosomal aberrations were examined in metaphase cells derived from the primary and the secondary clones. Both chromatid- and chromosome-type aberrations were analyzed, and total 200 metaphases were counted per each sample.

Delayed induction of DNA double strand breaks was determined by 53BP1 foci by immunofluorescence. 53BP1 is a protein, which was originally discovered as a p53-binding protein. Lately, it turns to be clear that 53BP1 is a critical component of DNA damage signalling pathway (Polo and Jackson, 2011). ATM-dependent DNA damage checkpoint plays a central role in protecting integrity of the genome (Kastan and Bartek, 2004, Lavin, 2008, Shiloh and Kastan, 2001, Shiloh, 2003). ATM, which forms dimer or oligomer in the control cells, dissociates into monomer through autophosphorylation at serine 1981 in response to ionizing radiation (Bakkenist and Kastan, 2003). Activated ATM transduces DNA damage signal through phosphorylation of down-stream factors, including histone H2AX, MDC1 and 53BP1 (Ciccio and Elledge, 2010, Kastan and Lim, 2000)(Figure 3), and DNA damage signal is amplified through the formation of ionizing radiation-induced foci

(Bonner et al., 2008). Activated ATM also phosphorylates p53 and CHK2/Cds1, which result in an induction of apoptosis as well as cell cycle arrest. Furthermore, ATM phosphorylates MRE11, NBS1, and Artemis, by which DNA repair ability may be regulated. Previously, it was demonstrated that the number of DNA double-strand breaks and the number of foci are well correlated, and since then, ionizing radiation-induced foci have been treated as a trustable marker for DNA double-strand breaks (Bonner et al., 2008). Foci of phosphorylated histone H2AX at serine 139 were the first ones to be used as DNA damage marker (Paull et al., 2000). Subsequently, foci of phosphorylated ATM at serine 1981 were demonstrated to illustrate low level of DNA double-strand breaks (Suzuki et al., 2006). Compared to these foci, 53BP1 foci create larger discrete foci, which can be detected even in tissue specimens (Nakashima et al., 2008). Thus, 53BP1 foci have been treated as DNA damage marker.

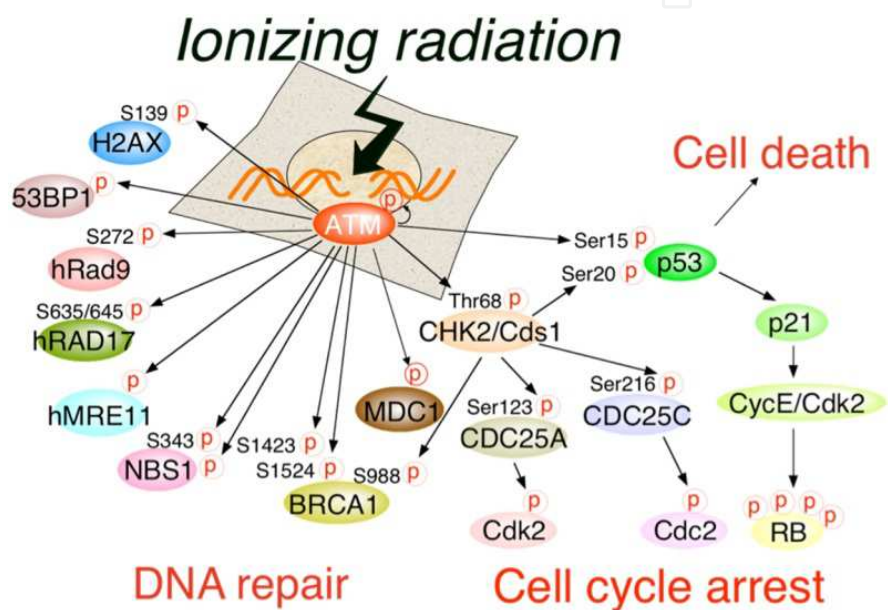


Fig. 3. ATM-dependent DNA damage checkpoint signalling.

53BP1 foci were detected by a specific antibody against the human 53BP1 protein. The primary antibody was visualized by Alexa594-labelled anti-rabbit IgG antibody. The nuclei were counterstained with DAPI. The samples were examined with an Olympus fluorescence microscope. Digital images were captured by a cooled CCD camera, and the images were analyzed by image analysis software.

#### 4.2 Manifestation of delayed phenotypes

Radiation-induced genomic instability needs to be examined at the same survival levels, so that 8 Gy and 10 Gy of X-rays were irradiated to CHO, while 2 and 4 Gy of X-rays were exposed to xrs-5 and xrs-6 cells, respectively. The primary clones were isolated, and they were subjected to the secondary colony formation. As shown in Figure 4, colony-forming ability is significantly reduced in CHO cells derived from surviving clones compared with those derived from the control clones. In contrast, no such difference is observed in both xrs-5 and xrs-6 cells, indicating that delayed reproductive death was completely absent in both xrs-5 and xrs-6 cells. In CHO cells, the frequency of giant cells and chromosomal bridges is also increased only in surviving clones, whereas, delayed induction of these phenotypes was completely abrogated in xrs-5 and xrs-6 cells (Figures 5 and 6).

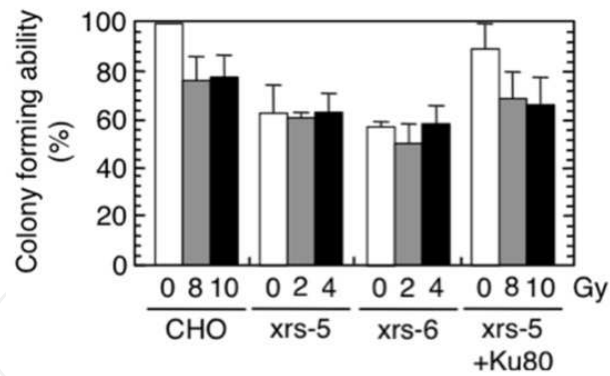


Fig. 4. Delayed reproductive death at 30-35 population doublings post-irradiation.

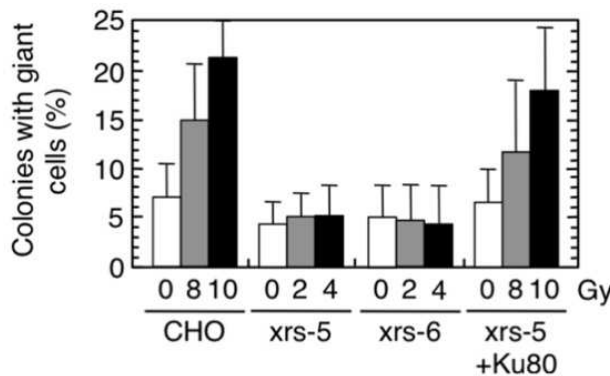


Fig. 5. Delayed giant cell formation at 30-35 population doublings post-irradiation.

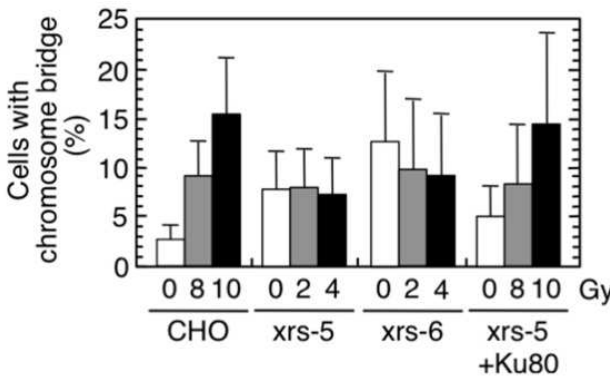


Fig. 6. Delayed chromosome bridge (right) at 30-35 population doublings post-irradiation.

In order to confirm that defective induction of these delayed phenotypes are caused by the simple Ku80-deficiency, the human Ku86 gene was introduced into xrs-5 cells. Expression of human KU80 protein was confirmed by western blotting, and sufficient amount of KU80 protein was detected in the complimented cells. Accordingly, it was confirmed that complementation of the defective Ku80 function in xrs-5 cells simultaneously restored delayed reproductive death, giant cell formation and delayed chromosomal bridge formation to the levels observed in CHO cells. Thus, the results clearly indicated that Ku80-dependent rejoining is involved in the manifestation of delayed phenotypes in the progenies of X-ray-surviving cells.



### 4.3 Delayed induction of DNA double-strand breaks

It is possible that delayed phenotypes are caused by delayed DNA double strand breaks and Ku80-dependent mis-rejoining. Delayed induction of DNA double strand breaks were examined by 53BP1 foci in cells at 30-35 PDL post-irradiation (Figure 7). Since the primary antibody against 53BP1 is visualized by the secondary antibody labeled with Alexa594, 53BP1 foci are detected as discrete large red dots within the blue nuclei. Upon X-irradiation, the foci of DNA damage checkpoint factors become detectable within a few minutes after exposure. At 1 to 2 hours after X-irradiation, the initial foci are detectable in all exposed cells. Many small foci, most of which diameter distributed between 0.4 and 1.0 micrometer, were observed. The number of foci is decreased thereafter, indicating DNA repair. While the number of the initial foci reduced significantly during the first 6 to 10 hours, some fraction of the initial foci is remained as residual foci. Diameter of these residual foci increased time-dependently, and they are quite large in size after 24 hours (Yamauchi et al., 2008). The number of foci shows no significant change thereafter, however, the cells with large residual foci are diluted out, when the foci-negative cells dominate the population. Thus, the background foci is rarely detectable even in the cells surviving 10 Gy of X-rays. While the frequency of 53BP1 foci per cell in the control CHO cells was approximately  $0.14 \pm 0.07$ , it was increased to  $0.41 \pm 0.19$  in 10 Gy-surviving cells. The frequency of 53BP1 foci in the unirradiated xrs5 cells were relatively higher ( $0.18 \pm 0.08$ ) compared to the CHO cells, and it was about  $0.51 \pm 0.27$  in 4 Gy-surviving cells.

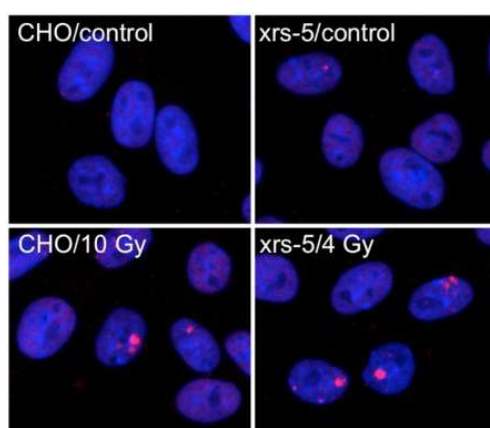


Fig. 7. Delayed induction of 53BP1 foci in CHO and xrs-5 cells at 30-35 population doublings post-irradiation.

Previously, a few studies including ours have suggested that delayed DNA double strand breaks are induced several generations after the initial insult (Barber et al., 2006, Huang et al., 2007, Suzuki et al., 2003), which has been proven by examining the delayed induction of foci of DNA damage checkpoint factors, such as phosphorylated histone H2AX. While phosphorylated histone H2AX foci are frequently used as biochemical markers for DNA double strand breaks, the foci of other DNA damage checkpoint factors, such as phosphorylated ATM foci and 53BP1 foci, are colocalized with phosphorylated histone H2AX foci, and they could also be used as alternative markers for DNA damage. In the present study, we demonstrated that the frequency of 53BP1 foci was higher in the progenies of surviving cells compared to unirradiated cells. Thus, it is confirmed that delayed induction of DNA double strand breaks in the progeny of surviving cells associated with pleiotropic manifestation of radiation-induced genomic instability.

4.4 Mis-rejoining of delayed DNA double-strand breaks

Although the number of spontaneous foci per cell in the foci-positive cells is 1, we found multiple numbers of foci in not a small numbers of surviving cells. Therefore, it is possible that mis-rejoining of delayed DNA double-strand breaks results in delayed chromosomal instability. We tested this possibility by examining delayed induction of dicentric chromosomes at 30-35 PDL post irradiation (Figure 8).

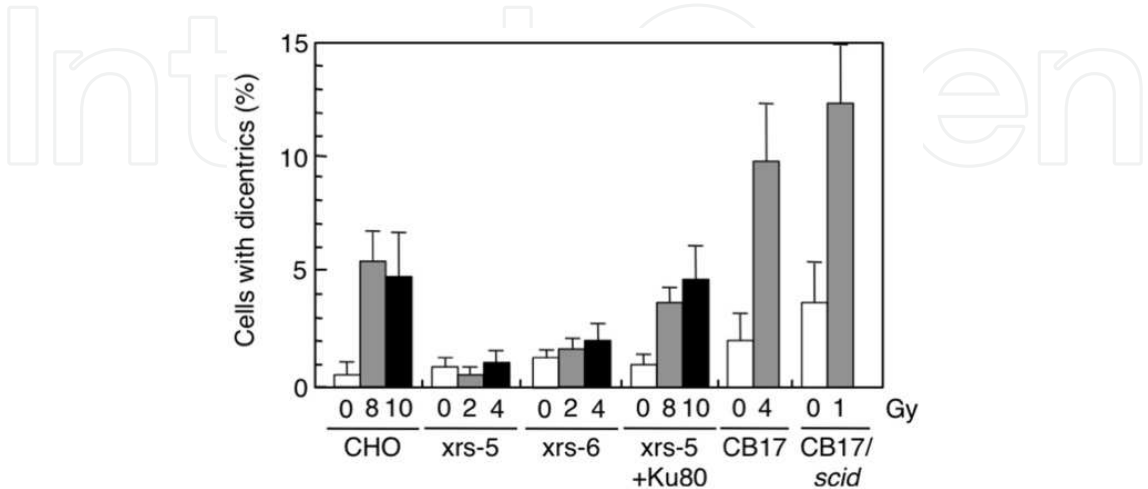


Fig. 8. Delayed chromosomal instability at 30-35 population doublings post-irradiation.

We found that delayed formation of dicentric chromosomes was significantly increased in CHO cells. Whereas, it is absent in xrs5 and xrs6 cells, while the control levels of dicentric chromosome were comparable among those cells. To confirm whether defective induction of dicentric chromosomes is related to Ku80-deficiency, delayed chromosomal instability was examined in the complimented xrs5 cells. We observed that delayed induction of dicentric chromosomes was increased to the level observed in CHO cells. Delayed chromosomal instability was also examined in cells derived from DNA-PKcs-defective *scid* mouse (Figure 8). Cells were irradiated with equivalent 10% survival doses and delayed induction of dicentric chromosomes was analyzed 30-35 PDL post-irradiation. The frequency of dicentric chromosomes in the unirradiated *scid* was higher than that in the wild-type cells, and increased dicentric frequencies in surviving cells were observed.

5. Role of non-homologous end-joining on radiation-induced genomic instability

Radiation-induced genomic instability has been reported commonly in various cell systems including human and rodent cells (Little, 2003, Lorimore et al., 2003, Morgan et al., 1996, Suzuki et al., 2003). However, Chang and Little demonstrated that delayed reproductive death, one characteristic manifestation of radiation-induced genomic instability, was not observed in Ku80-deficient xrs5 cells (Chang and Little, 1992a). The authors suggested that the cellular processing of DNA double strand breaks during repair must play a role in delayed reproductive death. In fact, our current study confirmed their results and found that not only delayed cell death but also delayed induction of giant cells and chromosome bridge were absent in xrs-5 cells (Figures 5 and 6). Furthermore, other Ku80-deficient cell line, xrs6, also revealed deficiency in the induction of those delayed phenotypes. Thus, it becomes clear that Ku80-dependent non-homologous end-joining is involved in the

manifestation of radiation-induced genomic instability. Then the question should be about the mechanism. One possible explanation of the defective induction of some delayed phenotypes is that error-free DNA repair, such as homologous recombination, reduced the incidence of transmissible damage in the absence of error-prone non-homologous end-joining repair. If so, delayed induction of DNA double strand breaks should be lower in Ku80-defective cells than the control CHO cells. Therefore, we checked whether delayed DNA damage was less frequent in *xrs5* cells. The results clearly indicated that it was not the case. Thus, even without Ku80-dependent repair, genomic instability by itself could be induced in the progenies of surviving cells. The second possibility is that defective DNA repair in *xrs5* cells decreased the chance of mis-rejoining of the broken ends that occurred many generations after the initial insult. In fact, delayed induction of chromosome bridges between two daughter cells was significantly reduced in *xrs-5* and *xrs-6* cells (Figure 6). Furthermore, delayed induction of dicentric chromosomes was completely absent in both *xrs-5* and *xrs-6* cells (Figure 8). Although several studies have reported that chromosome breakages are more frequent in Ku80-deficient cells (Darroudi and Natarajan, 1987, Kemp and Jeggo, 1986), the frequency of dicentric chromosome was relatively low considering the frequency of chromosome breaks. These results support our conclusion that the formation of dicentric chromosome caused by delayed DNA damage was compromised in Ku80-deficient cells. Although a back-up non-homologous end-joining may undertake mis-rejoining of broken ends in the absence of Ku80-dependent DNA repair (Iliakis et al., 2004), it is highly likely that a major pathway of illegitimate rejoining the DNA breaks is Ku80-dependent process (Liang et al., 1996). We also confirmed that radiation-induced genomic instability was manifested in cells derived from DNA-PKcs-defective *Scid* mouse. Moreover, delayed dicentric formation was normally detected in *Scid* cells. Therefore, DNA-PK-independent rejoining, which was suggested previously (Gao et al., 1998), is involved in delayed dicentric formation. Recently, it has been postulated that XRCC4/DNA Ligase IV-dependent but DNA-PKcs-independent rejoining needs Ku80/70 complex. Thus, it is highly possible that Ku80-dependent mis-rejoining is involved in delayed generation of dicentric chromosomes, by which chromosome bridges is generated. Such mis-rejoining inhibits segregation of two daughter cells, which results in delayed induction of giant cells as well as delayed reproductive death.

It has been well described that genomic instability, which is known as gross chromosomal rearrangement, is commonly observed in yeast. Gross chromosomal rearrangements manifest as translocations, chromosomal deletions, and inversions, indicating that they could be means to accelerate multiple genetic alterations associated with carcinogenesis (Kolodner et al., 2002). Although multiple pathways cooperate to suppress gross chromosomal rearrangement, homologous recombination plays a pivotal role in avoiding gross chromosomal rearrangement (Myung et al., 2001). Moreover, the restrained recruitment of homologous recombination proteins has been reported to promote gross chromosomal rearrangement. Thus, non-homologous end-joining has little effect on gross chromosomal rearrangement in yeast.

It should be very interesting to know the consequence of cells harboring mis-rejoined chromosomes. Previously, it was reported that ionizing radiation induced genomic instability in the progeny of surviving CHO cells, which resulted in a heritable mutator phenotypes. For example, mutation frequency at the hypoxanthine-guanine phosphoribosyltransferase locus in surviving clones was persistently higher than the unirradiated progenies (Lim et al., 2000). It was expected that such mis-rejoining caused large deletions at the gene locus, however, multiplex polymerase chain reaction analysis revealed that point mutations are the

predominant type of genetic alterations in the mutants (Chang and Little, 1992b). Because cells with micronuclei were frequently observed among the surviving cells, persistent break induction, which may not be involved in delayed cell death under the p53-dysfunctional condition (Tzang et al., 1999), may be resulted from mis-rejoined chromosomes. In fact, previous study demonstrated that delayed chromosomal instability was mediated by bridge-breakage-fusion cycle, which could also be involved in the perpetuation of radiation-induced genomic instability. It has been proved that ionizing radiation induces delayed genomic instability, which accumulates genetic alterations including gene mutations, loss of heterozygosity, and chromosome rearrangements, concurrently with delayed reproductive death. As Ku80-deficiency compromised delayed cell death through the formation of dicentric chromosomes, it is likely that such cells with defective DNA repair capacity are more susceptible to carcinogenesis induced by DNA damaging agents.

## 6. Molecular nature of radiation signature involved in radiation-induced genomic instability

Radiation-induced genomic instability is transmitted through many generations after the initial X-irradiation, indicating that there should be some mechanism(s), by which the initial radiation insults are memorized (Suzuki, 1997). As delayed DNA double-strand breaks are identified in surviving cells, persistent DNA damage is one candidate for radiation signature. In fact, a recent study has proved that DNA double-strand breaks induced by very low dose of X-rays remain unrepaired for many days in confluent cells (Rothkamm and Lobrich, 2003). Furthermore, if persistent DNA is radiation signature, radiation-induced genomic instability is more prevailing in xrs-5 and xrs-6 cells compared with CHO cells. But, we observed radiation-induced genomic instability at similar level between these cells (Suzuki et al., 2009). Thus, these results exclude the possibility that radiation signature is DNA double-strand breaks *per se*. Previously, we compared delayed aberrations of X chromosome with or without large deletion at the hypoxanthine-guanine-phosphorybosyltransferase gene, which is located at Xq26.1 (Toyokuni et al., 2009, Suzuki et al., 2003). Because cells defective in the gene were able to grow in the presence of 6-thioguanine, we cloned 6-thioguanine resistant clones after 3 Gy of X-rays, and examine gene deletion. We found some of the resistant clones had large deletions expanding over several megabases. Interestingly, these clones with large deletions showed higher probability to induce delayed chromosomal instability. Thus, altered higher-order chromatin structure could be a candidate for radiation signature. It is possible that such altered higher-order chromatin structure results in replication stress, which causes DNA double-strand breaks.

## 7. Conclusion

Ionizing radiation induces delayed destabilization of the genome in the progenies of surviving cells. This phenomenon, which is called radiation-induced genomic instability, is manifested by delayed induction of radiation effects, such as cell death, chromosome aberration, and mutation in the progeny of cells surviving radiation exposure. Previously, it was reported that delayed cell death was absent in Ku80-deficient cells. We have proved that this is because delayed induction of dicentric chromosomes is significantly compromised in those cells. In fact, reintroduction of the human Ku86 gene complimented the defective DNA repair and recovered delayed induction of dicentric chromosomes and



delayed cell death. Thus, our current study demonstrated that DNA repair pathway is an important determinant of cellular response to ionizing radiation not only in the immediate response but also in cells surviving radiation exposure. Survived cells induced DNA double strand breaks many generations after the initial insult. Although the mechanism of delayed DNA damage induction has to be determined, delayed dicentric formation indicated that delayed DNA damage was induced in G1 phase. Such delayed DNA damage could be repaired by NHEJ repair, but it also provided a chance to engender mis-rejoining. These results should bring a new insight into how DNA repair protects the integrity of the genome from the insults of DNA damaging agents.

## 8. Acknowledgements

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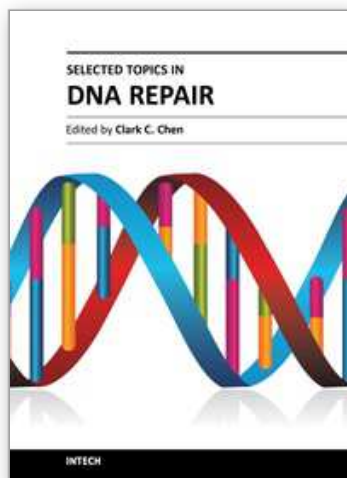
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