

**CHAPTER 6****Response of Cells to Ionizing Radiation****Wei Han, Kwan Ngok Yu\****Department of Physics and Materials Sciences, City University of Hong Kong, Hong Kong**\*Corresponding author; E-mail: appetery@cityu.edu.hk*

**Abstract:** Ionizing radiation is encountered in our natural environment and is also generated and used by mankind, e.g., for medical uses. A better understanding of the biological effects of ionizing radiation will lead to better use of and better protection from radiation. In this chapter, the response of cells to radiation will be described and discussed. Some basic concepts of ionizing radiation will be briefly given in the beginning. The significant consequences of various types of radiation-induced DNA damages show that DNA is the principle target for biological effects of radiation. The misrepaired or unrepaired DNA damages, in particular DNA double strand breaks, will induce chromosomal aberrations and gene mutations. On the other hand, radiation-induced DNA double strand breaks play an important role in the induction of apoptosis and cell cycle arrest. Radiation-induced bystander response, adaptive response and genomic instability are currently “hot-pots” in the radiobiological research. These three phenomena indicate the complexity of cellular responses to radiation, and will be introduced and discussed in this chapter.

**BASICS OF RADIOBIOLOGY****INTRODUCTION**

In 1895, the German physicist Wilhelm Conrad Röntgen discovered “a new kind of ray” which could blacken photographic films enclosed in a light-tight box. He named these rays as X-rays, which meant unknown rays. The first medical use of X-rays was reported in 1897, when a German surgeon, Wilhelm Alexander Freud, demonstrated the disappearance of a hairy mold after treatments with X-rays. In 1898, radioactivity was discovered by Antoine Henri Becquerel. In the same year, Pierre and Marie Curie discovered and isolated radium successfully.

Just like other new discovers, the potential hazards of radiations or radioactive materials were not adequately acknowledged at the beginning of their discoveries. Along with the applications of radiation in industrial and medical areas, the hazards of radiations were revealed in some unique populations, such as uranium miners who were often exposed to high levels of radon progeny. Most of our understanding of radiation harm comes from several well-known acute exposures. The atomic bombings in Japan in 1945 and the cancer incidence in survivals and their progeny informed us of the potential hazard of nuclear radiations. The leakage of a nuclear

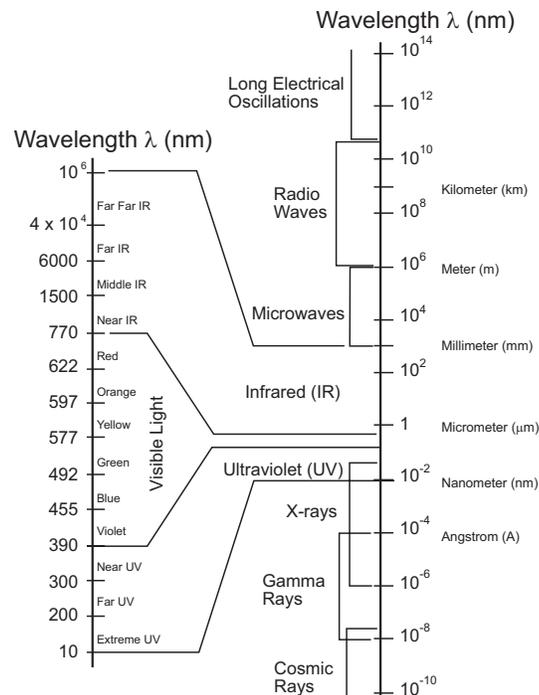
power plant in Chernobyl, Ukraine, in 1986 demonstrated a risk of acute nuclear exposure during the peacetime. The application of radiation in industry, medical diagnostics and cancer therapy shows an indispensable role of radiation in our daily lives. The present chapter will provide basic understanding of radiation biology to enable safer and more effective applications of radiation. In this section, the basic concepts of radiation are introduced [1].

## TYPES OF IONIZING RADIATION

The raising of an electron in an atom or molecule to a higher energy level without actual ejection of the electron is called excitation. If a radiation has sufficient energy to eject one or more orbital electrons from an atom or molecule, the process is called ionization, and the radiation is called an ionizing radiation. Ionizing radiation can be broadly categorized into electromagnetic or particulate radiations.

### Electromagnetic Radiation

X- and  $\gamma$ -rays are two forms of electromagnetic radiation, which are commonly employed in medical and biological applications. X- and  $\gamma$ -rays do not differ in nature or properties; the designations x- or  $\gamma$ -rays merely reflect the way they are produced. X-rays are produced outside the atomic nucleus while  $\gamma$ -rays are produced within the nucleus.



**Fig. 1.** The wavelengths of various electromagnetic radiations.

X-rays are usually produced in an X-ray tube when accelerated electrons hit a metal target like tungsten and are decelerated, thereby emitting a spectrum of bremsstrahlung radiation. Here, part of the kinetic energy of the electrons is converted into X-rays. (Bremsstrahlung photons are generated when a charged particle is accelerated or decelerated.) The emitted spectrum is filtered or modulated to produce a clinically useful X-ray beam. Gamma-rays, in contrast, are produced spontaneously. They are emitted by radioactive isotopes when their nuclei in excited states give off the excess energy and return to the ground state. Radio waves, infrared and visible light are also electromagnetic radiation. All electromagnetic radiations have the same velocity, but with different frequencies and wavelengths (Figure 1). The photon energy is proportional to the frequency and inversely proportional to the wavelength. Usually, electromagnetic radiations are considered ionizing if they have photon energies larger than 124 eV, which corresponds to a wavelength shorter than about  $10^{-8}$  m. In this way, only X-rays and  $\gamma$ -rays are considered to be involved in electromagnetic-radiation induced biological effects. Other electromagnetic radiations, of which the wavelengths are smaller than about  $10^{-8}$  m, are not covered (Figure 1).

### **Particulate Radiations**

Another type of ionizing radiation encountered in our nature, or used experimentally or clinically is particles such as electrons, protons,  $\alpha$  particles, heavy charged ions and neutrons.

### **ABSORPTION OF RADIATION**

The process through which X-ray photons are absorbed depends on the energy of the photons and the chemical composition of the absorbing material. In the high-energy range most widely used in radiotherapy (viz., 100 keV-25 MeV), the Compton effect dominates the energy deposition in tissues. Part of the photon energy is given to the electron as kinetic energy and the photon with the remaining energy continues on its way but is deflected from its original path. In diagnostic radiology, lower photon energies are used, for which both Compton and photoelectric absorption processes occur, with Compton absorption dominating at the higher end of the energy range and photoelectric effect being more important at lower energies. In the photoelectric absorption process, the photon gives up all its energy to a bound electron; a part is used to overcome the binding energy of the electron while the remaining energy is given to the electron as kinetic energy.

Gamma-ray photons with energy  $>1.02$  MeV may interact with a nucleus to form an electron-positron pair. Beyond the energy provided to the rest masses of the electron and positron (0.51 MeV), the excess amount will be carried away equally by these two particles. The positron is eventually captured by an electron, and annihilation of

the two particles releases two photons (each with an energy of 0.51 MeV). These two photons can further lose energy through Compton scattering or photoelectric effect.

## **DIRECT AND INDIRECT ACTIONS OF RADIATION**

The biological effect of radiation can be related to damages of the DNA. Any form of radiation has a potential to directly interact with target structures to cause ionization, thus initiating the chain of events that leads to biological changes. This is called the direct action of radiation, which is the dominant process for radiations with high linear energy transfer (LET), such as neutrons or  $\alpha$  particles.

Radiation can also interact with atoms or molecules in a cell (particularly with water) to produce free radicals, which are able to diffuse far enough to interact with the critical targets and cause damages. This is called indirect action of radiation. Free radicals have unpaired electrons, which result in high chemical reactivity. Most of the energy deposited in cells is absorbed initially in water, which is the main component of cells, leading to a rapid production of oxidizing and reducing reactive hydroxyl radicals. Hydroxyl radicals ( $\cdot\text{OH}$ ) may diffuse over distances to interact with DNA to cause damages. Fortunately, some defensive systems or responses in cells can protect the cells from the damages. Thiol compounds in cells, such as glutathione and cysteine, which contain sulfhydryl groups, can react chemically with the free radicals to reduce their damaging effects. Vitamins C and E and intracellular manganese superoxide dismutase (MnSOD) also have an ability to scavenge free radicals. In general, the contribution of free radical processes for sparsely ionizing radiation exceeds that for direct action of radiation.

## **RADIATION-INDUCED DNA DAMAGES IN CELLS**

Accumulated evidence in radiological studies indicates that DNA is the principle target for the biologic effects of radiation. Radiation-induced DNA lesions through induction of gene mutation and chromosome aberration are fundamental to investigating and understanding radiation-induced cell killing, cell transformation and carcinogenesis. In this section, we focus on radiation-induced DNA lesions, repair, and damage signal transduction, as well as cellular response to DNA damages [3].

## **DNA STRUCTURE AND RADIATION-INDUCED DNA DAMAGES**

### **Introduction to DNA structure**

DNA is a large molecule with a double helix structure. It consists of two long polynucleotide chains, each of them containing four types of nucleotide subunits. The two chains are held together by hydrogen bonds between the bases of the nucleotides. Nucleotides form the “backbone” of the DNA, and are composed of a five-carbon

sugar to which one or more phosphate groups and a nitrogen-containing base are attached. The genetic codes of DNA are represented by groups of four kinds of bases with specific sequences. In DNA, the sugar is deoxyribose (and hence the name deoxyribonucleic acid), which is attached to a single phosphate group and the base can be adenine (A), cytosine (C), guanine (G) or thymine (T). The nucleotides are covalently joined together in a chain through the sugars and phosphates, and form a backbone of alternating sugar-phosphate-sugar-phosphate chain.

Each sugar has a phosphate group at the 5' position on one side and a hydroxyl group at the 3' on the other, and each completed chain is formed by linking the 5' phosphate to the 3' hydroxyl so that all of the subunits are lined up in the same orientation. This polarity of the DNA chain is defined by referring to one end as the 3' end and the other as the 5' end. The bases, A, C, T, or G are arranged in specified sequences to form the code that stores the biological messages, and the DNA messages in turn encode proteins to perform biological functions. The complete set of information in the DNA of an organism is called its genome. In other words, the genome carries the information for all the proteins the organism will synthesize.

The three-dimensional structure of DNA, viz., the double helix, arises from the chemical and structural features of the two polynucleotide chains. Since the two chains are held together by hydrogen bonds between the bases (A and T; G and C) on the opposite side, all the bases are on the inside of the double helix while the sugar-phosphate backbones are on the outside. In each case, a bulkier two-ring base (a purine) is paired with a single-ring base (a pyrimidine). The two sugar-phosphate backbones wind around each other to form the double helix, with one complete turn every 10 base pairs. The members of each base pair can fit together only if the two strands are anti-parallel, i.e., if the polarity of one strand is oriented in a direction opposite to that of the other. In other words, each strand contains a sequence of nucleotides which is exactly complementary to that of the opposing strand.

### **Introduction to radiation-induced DNA damages**

In the early days, radiation-induced DNA damages were studied under two different conditions, viz., irradiating the DNA molecule itself (direct effect) or irradiating it in a dilute aqueous solution (indirect effect). The direct effect was a result of direct deposition of energy in the DNA, while the indirect effect was a result from reactions of radicals produced by ionizing the molecules in the solution.

An ionization can occur in any molecule in the cell creating a cation radical and an electron. The produced electron can attach to another molecule or it can become solvated before further reactions. The radical site can be transferred to another nearby molecule. At the same time, the cation radicals can react and become neutralized through losing a proton, such as in the case of the cation produced by ionization of

water.  $\text{H}_2\text{O}^+$  reacts with a neighboring water molecule immediately (10-14 s) to produce the ( $\cdot\text{OH}$ ). As a result of these reactions, cellular DNA can be damaged in different ways, including direct ionization of the DNA, reactions between the DNA and electrons or solvated electrons,  $\cdot\text{OH}$  or  $\text{H}_2\text{O}^+$ , or other radicals.

Radiation damages leading to cell deaths were studied in *Escherichia coli* by Johansen [4]. In this study, *Escherichia coli* was protected against radiation killing through scavengers of free radicals added to the medium. Approximately 65% of the cell killing was in fact found to be caused by the  $\cdot\text{OH}$  radicals, while no radioprotection was found for scavengers of electrons. Subsequently, similar experiments [5,6] were carried out in mammalian cells and the same conclusion was reached, i.e., 65% of the cell killing was caused by  $\cdot\text{OH}$  radicals. These studies were then extended to other systems. For low LET radiation such as X-rays or  $\gamma$ -rays, high concentrations of  $\cdot\text{OH}$  radical scavengers can reduce the yield of DNA breaks, chromosome aberration and mutations besides protection against cell killing. Based on these data, damages can also be classified as scavengable and non-scavengable.

## TYPES OF DNA DAMAGES

It is now well known that radiation produces a wide spectrum of DNA lesions, including damages to nucleotide bases (base damages), DNA single and double-strand breaks (SSBs and DSBs).

### Base damages or modifications

Damages to bases by ionizing radiation have been extensively studied *in vitro* by irradiation of free bases, nucleosides, oligonucleotides or DNA in aqueous solution or in the solid state. The chemistry of lesion formation is already relatively well understood, and detailed accounts can be found in the literature [7-9]. Although certain types of DNA base damages such as 8-hydroxydeoxyguanosine have significant biological significance, available data indicate that such isolated base damages probably have a minor role in radiation mutagenesis [10]. As such, we will not go into detailed discussions on radiation-produced base damages here. In general, the damaged bases can be repaired through the base excision repair pathway.

### DNA single strand break

An SSB is formed by abstraction of any of the deoxyribose hydrogens [10]. The initial radical reactions involve abstraction of a hydrogen atom from the deoxyribose moiety by an  $\cdot\text{OH}$  radical [7]. The radical can then react with an oxygen molecule and form a peroxy radical. Damages to DNA bases will result in destabilization of the N-glycosidic bond and abasic deoxyribose residues are then formed. Other lesions, which are generated by  $\cdot\text{OH}$  radicals, can be converted into strand breaks by

treatment with hot alkalis, so these break sites are referred to as alkali-labile sites. Irradiation in the presence of oxygen will increase the number of alkali-labile sites [11]

Studies on radiation damages to individual sites in DNA suggest that these lesions are not important in mammalian cells. The DNA SSBs generated in mammalian cells through hydrogen peroxide treatment at 0 °C were found not to cause cell killing [12]. On the other hand, production of these breaks is inhibited by an ·OH radical scavenger, implying an ·OH radical as an intermediate reactive species causing the breaks. At a lethal dose, the number of singly damaged sites present in each cell is in the order of  $10^5$ - $10^6$ , while the number of multiply damaged sites (MDS) such as DSB is smaller than 100. It has been established that most of the strand breaks induced by ionizing radiation are repaired by a DNA ligation step [7].

### **DNA double strand break**

A DSB is the most important lesion produced by ionizing radiation where the breaks in the two strands are opposite to each another, or separated by only a few base pairs. Unrepaired or mis-repaired DSBs are the most important lesions in the induction of chromosomal abnormalities and gene mutations [13,14]. Furthermore, the close association of radiation damages, which create “clustered” damages, has recently become recognized as an important feature of radiation damage. Such clustered damages can arise from the combination of direct damages induced by the track of radiation, and indirect damages induced by secondary reactive species which are produced by subsequent ionization events [15,16]. Clustered damages can involve SSBs or DSBs associated with base damages, or more complex associations including multiple closely scattered DSBs. The LET of the radiation determines the frequency and complexity of clustered damages. Modeling studies have shown that about 30% and 70% of DSBs induced by low and high LET radiations, respectively, are of a complex form involving two or more DSBs. If the breaks associated with base damages are included, the proportions will become 60% and 90% for low and high LET radiations, respectively [17-19]. When the damages are more complex, it is more difficult for them to be repaired and they are more likely to lead to biological consequences.

The similarity between the damages induced by exposures to low dose irradiation and those induced endogenously is important for the studies of the damages. It is known that a large amount of oxidative damages can be inflicted in the cells by reactive oxygen species (ROS) generated during normal cellular metabolism. These ROS induced damages appear to be similar to those induced by ionizing radiations (IRs), although there are also important differences. One important aspect is the nature of the termini for ROS- and IR-induced damages, which can affect the repair process. The breaks induced by restriction enzymes have 3'-hydroxyl and 5'-phosphate

moieties at the termini, which is a prerequisite for enzymatic ligation. On the other hand, the majority of breaks generated by ROS and IR have “damaged” termini, most frequently with 3'-phosphate or 3'-phosphoglycolate end groups [10]. Some 5' termini with hydroxyl end groups are also formed. Such termini will require processing before ligation. Excision of a damaged nucleotide will likely lead to base loss at the break. The types of DNA damages induced by ROS and IR are also different. Base damages and SSBs dominate the ROS-induced damages. The frequency of DSBs generated by ROS varies for different reactive species but is typically less than 0.5% of the induced damages, and these DSBs are distributed relatively uniformly throughout the DNA. On the contrary, even low doses of IR can lead to complex lesions with clustered damages due to inhomogeneous energy deposition.

### **DNA-protein cross-links (DPC)**

Cross-linking of DNA to nuclear proteins will affect DNA processes such as replication, transcription and repair, but the role of DPCs in the response to IR is not clear. Twenty-nine proteins were found to have cross-linked to DNA, including structural proteins, regulators of transcription, stress response, and cell cycle regulatory proteins [20]. A linear dose-response relationship was identified for hamster and human cells in the low dose range (0-1.5 Gy), and no difference in the DPC induction was revealed under aerated and hypoxic conditions.

### **DNA DOUBLE-STRAND BREAK REPAIR**

DSBs induced by ionizing radiation and other carcinogenic chemicals are considered the most relevant lesion for mutations and carcinogenesis. DSBs can also be generated in a number of natural processes including replication, meiosis, and production or formation of antibodies.

Unrepaired and misrepaired DSBs are serious threats to the genomic integrity [21]. DSBs lead to chromosomal aberrations which can simultaneously affect many genes and cause malfunction and death in cells [22]. It is noted here that DSBs are continuously induced in normal living organisms as a consequence of oxidative metabolisms [23,24].

Genome protection requires the capability to repair DSBs and to ensure that repair is performed with sufficient fidelity. There are two main pathways of DSB repair, namely, homologous recombination (HR) and nonhomologous end joining (NHEJ), which are error-free and error-prone, respectively. The pathways are conserved from *Saccharomyces cerevisiae* to mammalian cells, despite the different relative importance. It is generally considered that HR and NHEJ dominate DSB repairs in

yeast and in mammalian cells, respectively. DSB repair has been the subject of a number of excellent reviews [25-31].

### **Homologous recombination**

HR is a high-fidelity and efficient mechanism to repair DNA DSBs. HR retrieves the information lost at the break site from the undamaged sister chromatid or homologous chromosome. In the course of HR, the damaged DNA physically contacts an undamaged DNA with a homologous sequence, and uses it as a template for repair. From yeast to mammalian cells, HR is mediated through the Rad 52 epistasis group of proteins that includes Rad 50, Rad 51, Rad 52, Rad 54, and meiotic recombination 11 (Mre11). Rad family proteins are Ras-related proteins which lack typical C-terminal amino acid motifs for isoprenylation.

HR starts with a nucleolytic resection of the DSB in the 5'-3' direction by the Mre11-Rad50-Nbs1 (MRN) complex. The 3' single-stranded DNA is bound by a heptameric ring complex formed by Rad 52 proteins, which protects it against exonucleolytic digestion. The competition between Rad 52 and the Ku complex for binding to DNA ends may determine whether the DSB is repaired via the HR or the NHEJ pathway. Single-strand annealing (SSA) is a process for rejoining DSBs by exploiting the homology between the two ends of the joined sequences. The process relies on regions of homology to align the DNA strands to be rejoined. Single stranded regions are created adjacent to the break that extends to the repeated sequences. When this process has proceeded far enough to reveal the complementary sequences, the two DNAs are annealed and then ligated. The genes which define SSA belong to the Rad 52 epistasis group of HR.

### **Nonhomologous end joining (NHEJ)**

DNA repair via the NHEJ pathway is rough and emergent. The process rejoins the two ends of a DSB without the requirement of sequence homology between the two ends. The process can be described as a series of steps:

Initial step: binding of a heterodimeric complex to the damaged DNA. The complex consists of the proteins Ku70 and Ku80 (alias XRCC5). Binding of the complex will protect the DNA from exonuclease digestion. In the binding, Ku80 is distal to while Ku70 is proximal to the DNA break. The Ku70/Ku80 heterodimer can translocate from the DNA end in an ATP-independent manner.

Formation of DNA-PKcs. Following the binding, the Ku heterodimer associates with the catalytic subunit of DNA-PK (XRCC7, DNA-PKcs) to form the active DNA-PK holoenzyme. DNA-PKcs is activated by interaction with a single-strand DNA at the site of DSB and displays Ser/Thr kinase activity.

Linkage of two ends. XRCC4 forms a stable complex with DNA ligase IV, and this complex binds to the ends of DNA molecules and links together duplex DNA molecules with complementary but non-ligatable ends. The XRCC4–ligase IV complex cannot directly re-ligate most DSBs generated by DSB inducing agents. Instead, they have to be processed first. In yeast, DSB processing is performed by the MRN complex which removes excess DNA at 3' flaps. Furthermore, 5' flaps are removed by the flap endonuclease 1 (FEN1). Deficiency of this protein will result in reducing the usage of the NHEJ pathway.

Removal of NHEJ related factors. The NHEJ related factors must be removed from the DNA before the re-ligation of the DSBs. The auto-phosphorylation of DNA-PKcs and/or DNA-PK mediating the phosphorylation of accessory factors are important in the release of DNA-PKcs and Ku from the DSB before end-joining. Finally, the DSB repair is completed, although nucleotides are often lost, which results in an inaccurate repair.

## **SENSING AND SIGNALING TRANSDUCTION OF DNA DOUBLE-STRAND BREAK AFTER RADIATION**

### **Sensing and Signaling: Activation of Related Factors**

The core components of responses to DNA damages include the signal, sensors of the signal, transducers and effectors. Recent studies have also identified another class of molecules, mediators (also called adaptors), which do not possess catalytic activities but facilitate signaling through promoting physical interactions between other proteins. In the next part, the main sensors, mediators and effectors will be briefly introduced.

#### **(I) Role of ATM/ATR**

In response to DSBs, ataxia-telangiectasia mutated (ATM) shows distinct changes that include monomerization of dimers/oligomers and intermolecular auto-phosphorylation on Ser1981. ATM was first discovered in ataxia-telangiectasia (A-T), a multi-system disorder associated with diverse characteristics that include cancer predisposition and clinical radiosensitivity [32]. The cells derived from A-T patients show defects in the cellular response to DSBs in the activation signaling pathways, including the ability to arrest at the G1/S, S and G2/M cell cycle checkpoints [33-35].

ATM can play as a Ser-Thr protein kinase both *in vivo* and *in vitro*, and can phosphorylate the serine 15 residue of p53 specifically. p53 regulates the cell cycle and thus functions as a tumor suppressor which that is involved in preventing cancers [36-38]. In contrast to normal cells, p53 levels in A-T cell lines are not elevated following radiation. This suggests that ATM acts in the upstream of p53 as an early

damage sensor in response to radiation-induced DSBs [39,40]. Further studies showed the complex function of ATM in sensing DSBs. Phosphorylation of serine 15 residue was not a key factor in controlling the p53 stability, and ATM could also phosphorylate other sites on p53. ATM could also phosphorylate other kinases such as Chk1 and Chk2, and kinases could phosphorylate p53 on serine 20 residue to keep the stability of p53. Other kinases including DNA-PK and ATR could phosphorylate the serine 15 residue of p53. Furthermore, ATM could also phosphorylate MDM2, which could affect the stability of p53. Thus, ATM/ATR were important through phosphorylation in regulating p53 in a direct or indirect manner. These findings suggested that ATM was critical in sensing DSBs and in initiating signal transduction pathways by phosphorylation to control cell cycle arrest.

The MRN complex is required in many mammalian cell lines to activate ATM in response to DSBs under normal conditions [41-43]. Recent studies indicated that MRN/MRX complexes activated ATM by independently promoting both monomerization and auto-phosphorylation. First, the MRN complex mediated monomerization of ATM *in vitro* in the occurrence of dense DSBs [44,45]. One role of MRN/X might be to tether DNA and to increase the local concentration of DSBs to allow ATM monomerization, which was revealed based on the findings that MRX proteins in budding yeast could associate with DSBs *in vivo* and purified MRN complexes could tether DNA molecules *in vitro* [46-49]. Another role of MRN was to promote ATM kinase activity via binding of Nbs1 to ATM. Binding to Nbs1 was required in the auto-phosphorylation of ATM on Ser1981 residue *in vitro* regardless of the DSB concentration [44]. Association of MRN/X with DSB sites would help ATM target the proper sites to execute its function.

In contrast to ATM, ATR showed no changes in modification or activity after a genotoxic stress. ATR relocated on DSB sites via the association with ssDNA-RPA complexes. ToBP1 (Topoisomerase II Binding Protein 1) was found to act as a mediator, binding to and stimulating the kinase activity of Xenopus and human ATR *in vitro* [50,51].

## **(II) Role of Nbs1, hMre11 and hRad50**

Nijmegen Breakage Syndrome (NBS) is a phenomenon associated with cancer predisposition and radiosensitivity [52,53]. The cell lines derived from A-T and NBS have similar phenotypes such as radiosensitivity, cell cycle checkpoint deficiency and decreased ability to stabilize p53. The gene defective in NBS was indicated to encode protein Nbs1 or p95 [54,55]. Nbs1 interacts strongly with hMre11 and hRad50 to form an MRN complex in sensing and repairing of DSBs. The finding that mutations in the ATM gene resulted in A-T and Mre11 gene mutations and caused A-T-like disorder (ATLD) further strengthened the link between A-T and NBS [56]. hMre11 and hRad50 null mice showed embryonic lethality. Mutations in hMre11 in ATLD

impaired but did not inactivate hMre11 function, a feature which was consistent with the milder clinical features of this variant class of A-T. NBS cells also had cell-cycle checkpoint defects in response to ionizing radiation, and these defects were notably in the S-phase checkpoint to show the radioresistant DNA synthesis [57]. The complex of hMre11, hRad50 and p95 (MRN complex) co-localized at the DSB sites [58]. In yeast and vertebrates, MRX played an important role in both HR and NHEJ pathways [59]. However, in mammalian cells, it was not an essential component of the NHEJ pathway [60]. There was also evidence that the MRN complex played an important role in either directly activating ATM or in aiding ATM-dependent phosphorylation events [61,43]. As such, MRN is activated with a number of damage response factors including p53 via dependent and independent processes, and acts with ATM as an early sensor complex.

### **(III) Role of H2AX**

Mice lacking H2AX are viable but show genomic instability and radiosensitivity [62]. H2AX is a member of the histone H2A family, and the histone H2A together with the other members of the histone family provide the backbone for wrapping of the DNA. H2AX becomes phosphorylated to  $\gamma$ -H2AX in response to DNA damages and is critical in the recruitment of repair factors at DSB sites [62,63]. H2AX phosphorylation is a rapid response following formation of DSBs. Phosphorylation will extend rapidly to H2AX molecules located up to 3 megabase pairs beyond the DSB site [64].  $\gamma$ -H2AX can be observed as discrete foci under a fluorescent microscope through immunofluorescence with the use of phosphospecific antibodies. All DSBs are marked by the presence of such foci and the number of  $\gamma$ -H2AX foci equals the number of DSBs [65].  $\gamma$ -H2AX is also important in recruiting some repair factors such as Rad 51 and MRN complex to localize at the site of broken DNA [66]. After the DSB repair,  $\gamma$ -H2AX will dephosphorylate to its original form, i.e., H2AX. The analysis of foci number is used as a tool to monitor the formation and repair of DSBs.

### **(IV) BRCA1 and BRCA2**

BRCA1 and BRCA2 are genes deficient in familial breast cancer patients. Germline mutations in BRCA1 and BRCA2 induced a high risk of breast and ovarian tumors [67,68]. There were evidences that both gene products were involved in damage response mechanisms and that cells deficient in either protein showed pronounced genomic instability [69]. BRCA1 deficient cells showed marked genomic instability, impaired ability in the HR pathway [70] and impaired checkpoint function, including impaired S and G2/M checkpoint arrest [71]. BRCA1 was phosphorylated after DSB induction and localized to H2AX foci after DNA damage and thus co-localizes with MRN complex, 53BP1 and the mediator of DNA damage checkpoint 1 (MDC1) [63]. BRCA1 was also found necessary to facilitate some ATM-dependent phosphorylation events to help other repair related factors localize to the H2AX foci [72,73]. All these

results suggested that BRCA1 was critical in controlling checkpoint signaling and in triggering the HR pathway. On the other hand, cells deficient in BRCA2 did not appear to show cell cycle checkpoint defects but showed an impaired ability in the HR pathway [74]. BRCA2 was proposed to be required for the localization of Rad 51 to the sites of single stranded DNA since Rad 51 foci did not form in BRCA2 defective cells [75,76].

#### **(V) MDC1 and 53BP1**

53BP1 was originally identified through its ability to bind to p53 via C-terminal BRCT repeats present in 53BP1 [77]. MDC1 was identified as a binding partner of the Mre11 complex simultaneously by several laboratories. The encoded protein is required to activate the intra-S phase and G2/M phase cell cycle checkpoints in response to DNA damages [78,79]. Evidence showed that both proteins form foci and co-localize with H2AX and MRN foci at the sites of DSB after irradiation [80-82] and these proteins were required for the normal function of checkpoint responses. Lack of these proteins would lead to at least some radiosensitivity.

#### **(VI) Role of p53**

An increase in the p53 levels was found as an early response of mammalian cells (within minutes) to DNA damages [83]. p53, known as protein 53 or tumor protein 53, is a transcription factor which in humans is encoded by the TP53 gene. Changes in p53 expression result in the transcription of some key proteins involved in a number of distinct damage response pathways, since the capability of p53 functioning as a transcriptional activator may also be increased [84,85] after the formation of damages. The role of p53 in the response to radiation damages is complex since it affects some aspects of DNA repair, controls checkpoint cell cycle arrest and initiates apoptosis, etc. [86]. Studies on patients deficient in p53 (Li-Fraumeni syndrome patients) and p53 knock-out mice [87-89] demonstrated the importance of p53 in damage response mechanisms. In addition, mutations in p53 are found in around 40% of tumors covering all the cancer types.

Regulation of p53 is strict and the network of p53 regulation in mammalian cells is complex [84,85]. Mdm2 was found as a key protein in controlling p53 [90], and binding of Mdm2 to the amino-terminus of p53 would target it for ubiquitination and subsequent degradation by ubiquitin controlled proteasomes ([91]. Knock-out mice for Mdm2 were found to be embryonic lethal due to the high endogenous levels of p53, but double mutant p53/Mdm2 knock out mice were found to be viable. Mutations in Mdm2 were commonly found in tumors, in particular those with normal p53 function. In normal undamaged cells, p53 is maintained at low levels *via* Mdm2 binding and ubiquitin-dependent degradation. In response to radiation damages, Mdm2 negatively regulates both stabilization of p53 and its function. Changes to p53

and/or Mdm2 decrease their binding potential with subsequent increase in the lifetime of p53, and Mdm2 binding suppresses p53 as a transcription activator [92].

### Cell Cycle Arrest after Radiation

Mammalian cell cycle contains G1, S, G2 and M phases, and variations in radiosensitivity are shown in the different phases. The following views concerning the different radiosensitivities associated with different phases of the cell cycle are widely accepted in cellular radiobiology [93,94]:

- mitotic cells are the most radiosensitive;
- if G1 has an considerable length, there is normally a period with a resistance, which declines towards the S phase;
- the resistance increases in the S phase, with a maximum increase in the latter part of the phase;
- the G2 phase is almost as sensitive as the mitotic phase.

Progression from one phase to the next occurs by phosphorylation or dephosphorylation of cyclin dependent kinases (Cdks). DNA damages will often lead to arrest at cell cycle checkpoints, which are also called DNA integrity checkpoints. Besides checkpoints at the boundaries between adjacent phases, there is also an S phase checkpoint that recognizes a stalled replication fork. These checkpoint responses have been extensively studied in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, but the operation of checkpoints is also evident in mammalian cells. In the following, the processes in mammalian cells will be briefly reviewed.

The checkpoint responses are conserved between organisms, although the multiple steps and mechanisms are less well understood in mammalian cells [95,96]. In mammalian cells, PI3-K related protein kinases (PIKK kinases), ATM (ataxia telangiectasia mutated protein)/ATR (ataxia telangiectasia and Rad3-related protein) are important in controlling checkpoint responses. These pathways target p53 which then mediate permanent cell cycle arrest or apoptosis besides induction of transient delays at cell cycle transitions.

#### (I) G1/S arrest

Two types of G1/S arrest were observed in mammalian cells, namely, prolonged arrest and a more transient response [97,98]. The former is a p53-dependent response and the latter is similar to the G1/S response observed in yeast. Protein 21 (p21) is a major p53 response protein required for G1/S arrest [99], and p53 regulates strictly p21 as a transcription factor. Neither p53 nor ATM null cells showed a prolonged radiation induced G1/S arrest. p21 is an inhibitor of cyclin dependent kinases. It plays an important role in the G1/S arrest by binding to the cyclin D- and cyclin E-associated kinases, and by inhibiting the ability to phosphorylate the retinoblastoma

gene product (pRb) [100]. p21 has another role in controlling growth arrest by preventing proliferating cell nuclear antigen (PCNA) from activating DNA polymerase, which is essential in DNA replication. G1/S arrest after radiation does not help in the survival of cells, based on the observation that p53 null cell lines or transformed fibroblasts (which normally lack this arrest due to p53 inactivation) showed elevated radioresistance compared to p53 wild cells [101].

### **(II) S phase arrest**

Cell cycle arrest in the S phase enables repairs of the radiation-induced damages before these are permanently fixed by DNA replication into irreparable chromosomal breaks. Among all cellular checkpoints, the S-phase checkpoint is the most complex. Evidence showed that early S phase arrest after irradiation was ATM-dependent but later S phase arrest was associated with ATR [102]. Cells deficient in genes of ATM and NBS displayed a phenotype called radioresistant DNA synthesis, and S phase arrest was not observed before the damages were repaired [103]. S phase arrest included inhibition of ongoing replication forks, stabilization of replication forks and inhibition of late firing replicons [104,105]. Chk2 and Chk1 were involved in mediating S phase arrest via Cdc25A degradation [106,107]. Chk1 and Chk2 are two serine/threonine kinases involved in the induction of cell cycle checkpoints. Phosphorylation of Chk1 by ATM was required for effective radiation-induced degradation of Cdc25A, and stabilization of p53 was required in this process. The irreversible slower response to DNA damages required p53 stabilization [108].

### **(III) G2/M arrest**

The contribution of G2/M arrest to cell survival after induction of damages by radiation remains unclear, although it is generally agreed that the arrest enhances cell survival and reduces the probability of genomic alterations. The G2/M checkpoint is regulated by protein kinases Chk1 and Chk2. Phosphorylation of Chk and its activation as a result of the induced damages are both ATM-dependent. Evidence suggests that ATM phosphorylates Cds1 and/or Chk1, which in turn phosphorylates and inactivates Cdc25. Some differences in G2/M arrest were identified between the normal cells and ATM deficient cells after  $\gamma$ -irradiation. After irradiation, the normal cells demonstrated a delay in entering the M phase from the G2 phase, and A-T cells showed a reduced delay compared to normal cells. This indicated that the G2/M arrest was at least partially ATM dependent [109]. After a high-dose irradiation, asynchronous A-T and normal cells could have a permanent arrest at G2/M, which was showed to be ATR-dependent [110].

## **RADIATION-INDUCED CHROMOSOME ABERRATION**

Radiation-induced chromosome aberrations can be classified as chromatid or chromosome-type aberrations. When cells in the early interphase (i.e., in the G1

phase) are exposed to radiation, the first mitoses exclusively give chromosome-type aberrations, which are results from damages to the G1 chromosome. When this chromosome is replicated during the S phase, the lesion is replicated and both sister chromatids at the same site are affected. In contrast, if the cells are irradiated in the S or G2 phase, i.e., after the DNA is replicated, aberrations with defects in only one chromatid are produced, and are thus referred to as chromatid-type aberrations [1,2].

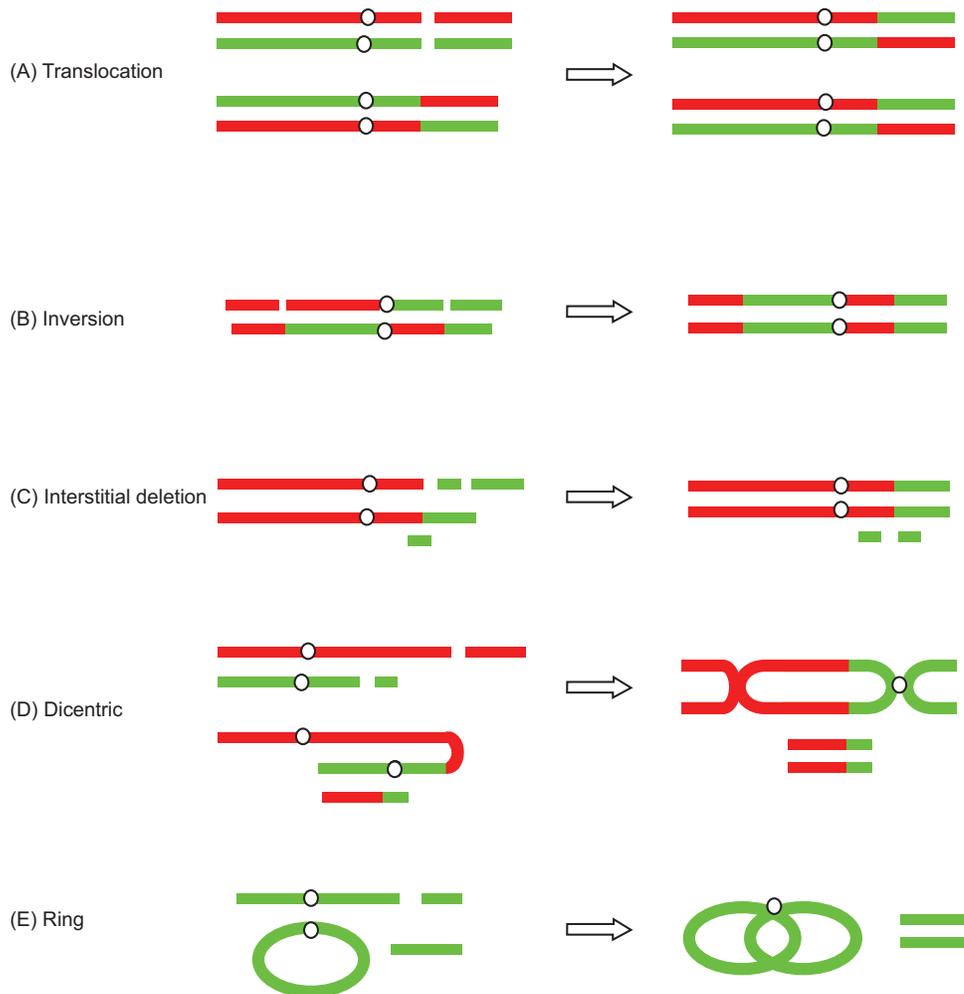
## **CHROMOSOME AND CHROMATID ABERRATIONS**

### **Chromosome aberrations produced by IR**

Radiation-induced G1 or early S phase (pre- or early DNA replication) chromosome aberrations can be categorized as symmetrical or asymmetrical. Symmetrical rearrangements involve reciprocal translocations (Figure 2A) and inversions. Reciprocal translocations are generated by the exchange of the broken fragments of two pre-replication chromosomes. On the other hand, inversions (Figure 2B) are formed when two breaks occur within the same chromosome. The inversion is either paracentric if both breaks occur in the same chromosome arm, or pericentric (encompassing the centromere) if the breaks occur in both chromosome arms. Symmetrical aberrations do not produce gross distortions in the chromosome structure and are thus likely compatible with cell survival.

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In contrast, asymmetrical chromosome aberrations are more likely lethal due to incorrect distribution of genetic materials between the daughter cells at mitosis. An arm of a pre-replicated chromosome with two breaks may rejoin in a way that an interstitial fragment is lost, leading to an interstitial deletion (Figure 2C) and thus loss

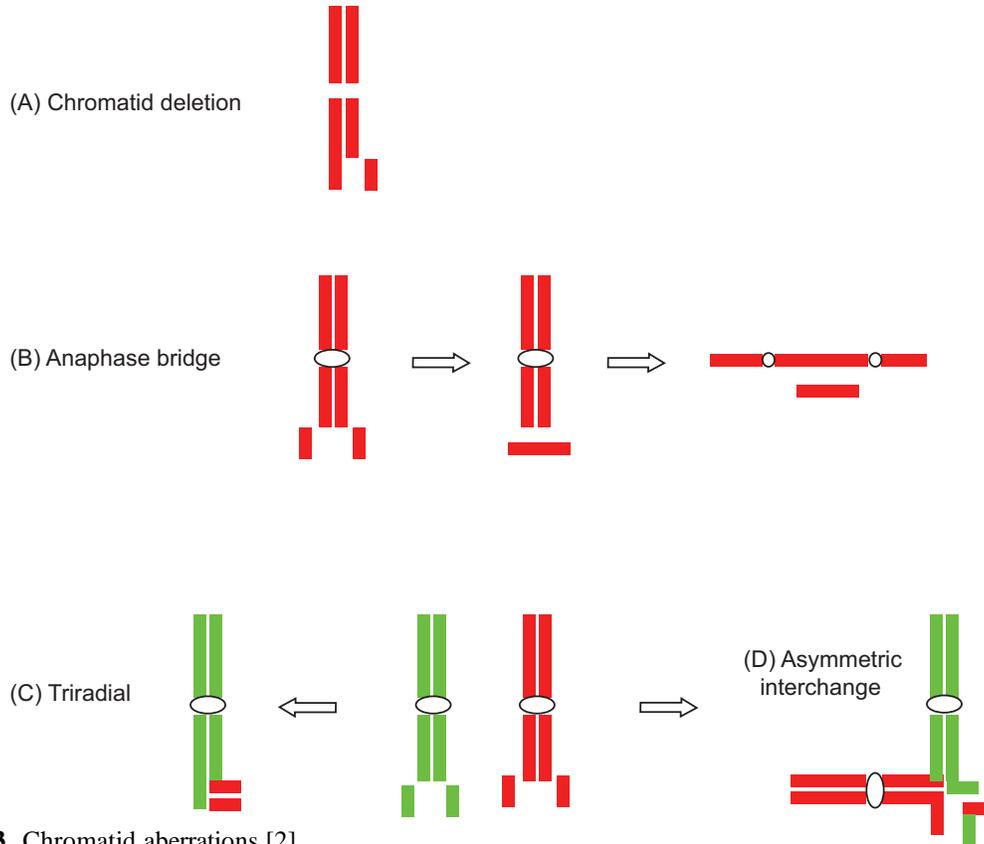


**Fig. 2.** Chromosome aberrations [2].

of genetic materials. On the other hand, if two breaks occur in two separate chromosomes in the early interphase, and if the ends are close to each other, they can rejoin as shown in Figure 2D. The entire structure can be replicated during the S phase to form a distorted chromosome with two centromeres, i.e., a dicentric, together with a fragment without centromeres (acentric fragment). Yet there is another possible structure if the breaks occur in both arms of one chromosome and the ends rejoin to form a ring and a fragment (Figure 2E). Here, when the chromosome replicates, two overlapping rings with one centromere are formed together with two acentric fragments.

### Chromatid Aberrations produced by IR

After DNA replication, each chromosome will consist of a pair of chromatids which are joined at the centromere. Chromatid aberrations can be produced at this time, which can be induced by interactions between two chromatid arms of different chromosomes or the same chromosome. Figure 3 summarizes the different possibilities. Figure 3A depicts a chromatid deletion where a terminal fragment of one chromatid arm is deleted. Figure 3B illustrates the formation of an anaphase bridge, which occurs when breaks occur in each of the two chromatid arms of one chromosome and the pairs of broken ends join to form a sister union and an acentric fragment. Figure 3C is a triradial which involves deletions and rejoining of terminal fragments from separate chromosomes. Figure 3D describes an asymmetrical



**Fig. 3.** Chromatid aberrations [2].

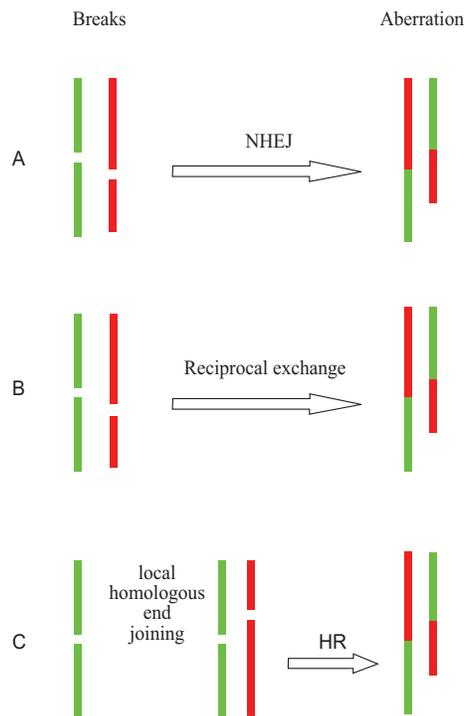
interchange where two chromosomes are formed through deletion of a fragment from one chromatid arm of each, which leads to the formation of a dicentric structure and an acentric fragment. Finally, Figure 3E describes a symmetrical interchange between terminal fragments of chromatid arms from each of the two chromosomes. At the anaphase, when the sister chromatids try to separate from each other, the two centromeres of the dicentric will go towards the two opposite poles of the cell, but separation of the daughter cells cannot be completed. At the same time, the acentric fragment is lost because of the absence of a centromere.

## Mechanisms of Aberration

Possible mechanisms of aberration formation were reviewed by Bryant [111,112]. Briefly, some debates focused on the three pathways as shown in Figure 4. These pathways are not necessarily exclusive and can exist in parallel.

1. The breakage and reunion mechanism (Figure 4 A) corresponded at the molecular level to a process of double-strand breakage followed by joining of the broken ends through a process of NHEJ.

2. The one-hit model (Figure 4 C) depicted that a single radiation-induced DSB would be enough to initiate an exchange with an otherwise undamaged portion of the genome [113]. This could involve an HR process with interaction between limited sequence homologies at different sites. This process is also known as recombinational misrepair.



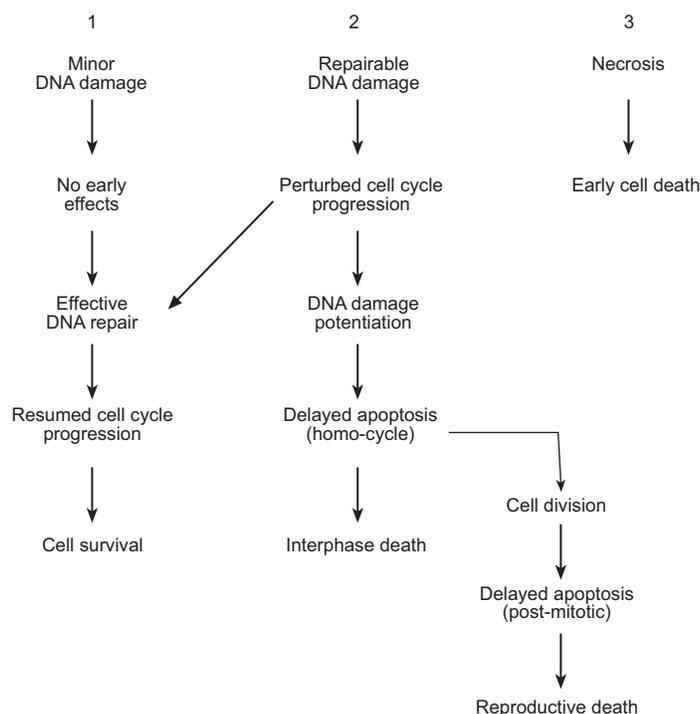
**Fig. 4.** Models for formation of exchanges: (A) breakage-and-reunion, (B) exchange theory, and (C) recombinational misrepair (one hit)[2].

3. The third pathway (Figure 4B) [114] viewed primary lesions destined for pairwise interaction as damages that did not immediately compromise the integrity of the chromosome. After the primary lesions had attempted the exchange process, the chromosomal structure was disrupted in such a way that the aberrations were visible

at mitosis. Failures in the exchange process, instead of unrejoined breaks, led to most terminal deletions.

## RADIATION-INDUCED CELL KILLING: NECROSIS AND APOPTOSIS

Radiation-induced cell killing has been known for nearly one century since radiation was discovered. In fact, this is the basis for radiotherapy, in which radiation is used to kill tumor cells. Following irradiation, mammalian cells can die in a number of ways, including necrosis and apoptosis. The fate of mammalian cells following irradiation is illustrated in Figure 5.



**Fig. 5.** Fate of irradiated cells [117].

## Apoptosis

Apoptosis is a form of programmed cell death, which is a physiological “cell-suicide” program essential for embryonic development, immune-system function, and the maintenance of tissue homeostasis in multicellular organisms. Dysregulation of apoptosis has been involved in numerous pathological conditions, including neurodegenerative diseases, autoimmunity and cancer. Apoptosis in mammalian cells is mediated by a family of cysteine proteases, which are known as the caspases. To control the apoptotic program, caspases are initially expressed in cells as inactive procaspase precursors that are activated by oligomerization, and they cleave the precursor forms of effector caspases. Activated effector caspases then cleave a specific set of cellular substrates, leading to a group of biochemical and

morphological changes associated with the apoptotic phenotype. Caspase activation can be triggered by extrinsic and intrinsic apoptotic pathways.

### **Necrosis**

Necrosis is a disorganized and unregulated process of traumatic cell destruction. The process completes by the release of intracellular components. A distinctive set of morphological features is observed, including membrane distortion, organelle degradation and cellular swelling. Necrosis is usually a consequence of a pathophysiological condition, including infection, inflammation or ischaemia.

### **Radiation-Induced Apoptosis**

The pathways and possible mechanisms of radiation-induced apoptosis were reviewed in the past decades [115,116]. Here, some of them will be briefly introduced.

### **Pre- vs Post- mitotic Apoptosis**

After irradiation, the cells can undergo apoptosis at different cell cycle stages. Considering the cell cycle stage in which apoptosis takes place, apoptosis can have two categories [117], namely, the pre-mitotic type and the post-mitotic type. Nevertheless, the different mechanisms for the pre-mitotic and post-mitotic apoptosis remained to be investigated. For example, irradiation of U937 cells at different X-ray doses led to apoptosis in both categories, and decision on the category would have been made according to the extent of cell damage [118].

Pre-mitotic apoptosis refers to apoptosis which occurs before cell division. This type of apoptosis is associated with an immediate (within several hours) activation of caspase-3, a decrease in the mitochondrial transmembrane potential and DNA strand breaks. The apoptotic cell death occurs mainly in the S phase fraction. In general, a high-dose irradiation will likely lead to pre-mitotic apoptosis. For example, a 20 Gy X-ray irradiation can induce a rapid and strong apoptosis in a pre-mitotic manner [118].

Post-mitotic apoptosis refers to apoptosis which occurs after at least one cell division. Here, the caspase activation and DNA strand breaks do not take place until the cells complete the mitosis. There is a cell cycle arrest at G2/M, but the cell does not die during or immediately after the cell-cycle block. There are no apparent variations in the susceptibility to cell death within different cell-cycle phases. On the other hand, low-dose irradiation is likely to lead to post-mitotic apoptosis. For example, 5 Gy X-ray can induce post-mitotic apoptosis in U937 cells [118].

The mechanism at the molecular level of pre-mitotic apoptosis is quite different from that of post-mitotic apoptosis. Since pre-mitotic apoptosis is a rapid mode of cell

death, a prompt activation of pre-existing cytoplasmic caspase-3 may be involved. Shinomiya *et al.* verified this hypothesis. In the study, caspase-3 inhibitor was added to the culture media at different time points, and apoptosis-related events such as PARP cleavage and DNA fragmentation were examined [118]. Regarding the 20 Gy-irradiated U937 cells, suppression on PARP cleavage was effective when the caspase-3 inhibitor was added before irradiation, but not effective if it was added after irradiation (even within 1 h). This clearly indicated that activation of caspase-3 was a very early event in pre-mitotic apoptosis and that pre-existing caspases were important here. Furthermore, for the 20 Gy-irradiated cells, DNA fragmentation was significantly decreased when the caspase-3 activity was suppressed by its inhibitor. In contrast, for the 5 Gy-irradiated cells (i.e., the case of post-mitotic apoptosis), this inhibitor did not reduce the apoptotic rate. This proposed an alternative pathway for the post-mitotic apoptosis. It is known that post-mitotic apoptosis requires a transient G2/M blockade. Furthermore, post-mitotic apoptosis took a longer incubation period (more than 24 h) to execute when compared to pre-mitotic apoptosis. As such, the post-mitotic apoptosis was suggested not only due to primary damages, but also due to accumulation of secondary changes that occurred during the cytostatic phase. In this way, down-regulation of anti-apoptosis genes and up-regulation of apoptosis-related genes were considered likely to be involved in post-mitotic apoptosis.

## **DNA damage-dependent or independent apoptosis**

### **(I) DNA damage-dependent apoptosis**

One type of apoptosis is initiated *via* intrinsic signaling pathways and is often DNA damage-dependent [119]. The intrinsic signaling pathways initiate apoptosis by producing mitochondrial-initiated intracellular signals and act directly on targets within the cell. Here, DNA damages are regarded to trigger the apoptosis. The stimuli, including radiation, toxins, hypoxia and free radicals etc., that initiate the intrinsic pathways produce intracellular signals that may act in either a positive or negative way. All these stimuli can lead to changes in the inner mitochondrial membrane, then an opening of the mitochondrial permeability transition pore, loss of the mitochondrial trans-membrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the inter-membrane space into the cytosol [120].

The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi [121], and the first group proteins function to activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9 to form an “apoptosome” [122,123] and the clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi can inhibit the activity of apoptosis inhibitor proteins to promote apoptosis [124,125]. The second group of pro-apoptotic proteins, apoptosis-inducing factor (AIF),

endonuclease G and endonuclease CAD, are released from the mitochondria during apoptosis. The release of these proteins occurs in the late phase of apoptosis and they cause DNA fragmentation. The released AIF, CAD and endonuclease G translocate to the nucleus and initiate DNA fragmentation in two steps. In the first step, AIF causes DNA fragmentation into ~50-300 kb pieces and condensation of peripheral nuclear chromatin [126], and Endonuclease G cleaves nuclear chromatin to produce oligonucleosomal DNA fragments [127]. In the second step, CAD is cleaved by caspase-3, and then it leads to oligonucleosomal DNA fragmentation and a more pronounced and advanced chromatin condensation [128,129].

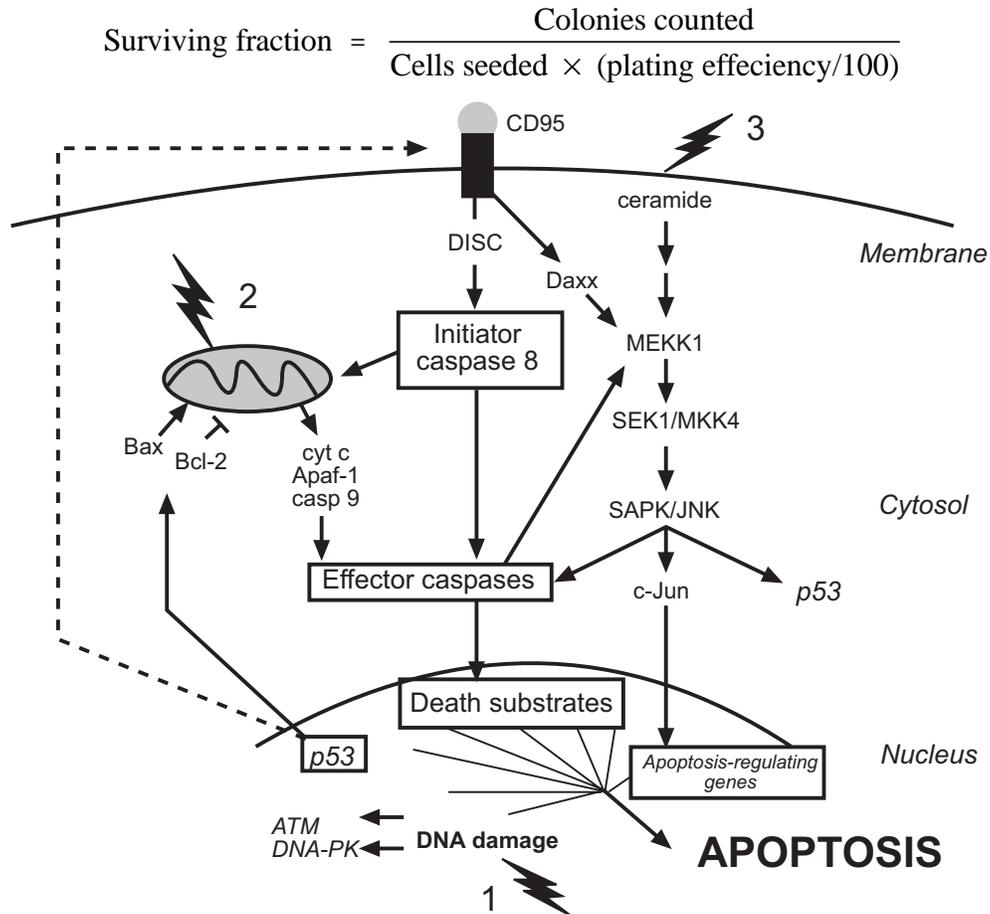
Members of the Bcl-2 family of proteins control and regulate the apoptotic mitochondrial events [130], and the tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins [131]. The exact mechanisms have not yet been completely understood. The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. These proteins are very important in that they can determine whether a cell commits apoptosis or aborts the process. A possible action of the Bcl-2 family of proteins is to regulate cytochrome c release from the mitochondria *via* alteration of mitochondrial membrane permeability.

## **(II) DNA damage-independent apoptosis**

Another type of apoptosis is not initiated by DNA damages, but instead by reception of apoptosis related factors on the cellular membrane. This type of apoptosis is also referred to as mediated by extrinsic signaling pathways. The involved factors are members of the tumor necrosis factor (TNF) receptor gene superfamily [132]. To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF- $\alpha$ /TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 [133]. The members of the TNF receptor family have similar cyteine-rich extracellular domains and a cytoplasmic domain, called the "death domain". This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways after interacting with the membrane receptors.

The extrinsic apoptosis signaling pathway consists of two steps, namely, binding with receptor and activation of caspase 8. Clustering of receptors is found on the cell membrane. Upon ligand binding, cytoplasmic adapter proteins are recruited, which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor leads to the binding of the Fas-associated death domain (FADD) protein, and the binding of TNF ligand to TNF receptor leads to the binding of the TNF receptor-associated death domain (TRADD) protein, with recruitment of FADD and RIP [134]. FADD then associates with procaspase-8 via dimerization of the death effector domain, resulting in the auto-catalytic activation of procaspase-8 [135]. Once caspase-8 is activated, the execution of apoptosis is triggered.

A simplified model of radiation-induced apoptotic pathways is shown in Figure 6.

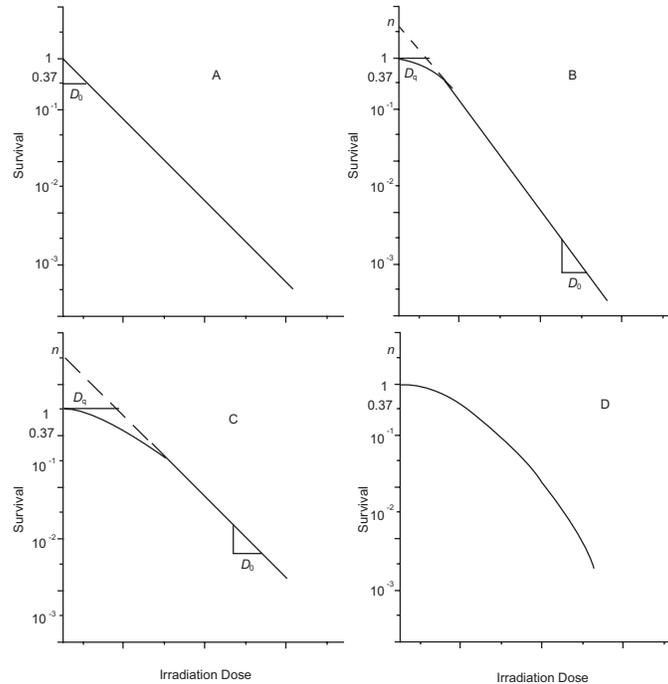


**Fig. 6.** A simplified model of radiation-induced apoptotic pathways. 1: Radiation-induced DNA damage initiates apoptosis *via* p53-dependent mechanisms, e.g. by regulating the expression of Bcl-2 family members. 2: Mitochondria are triggered to release caspase-activating factors, such as cytochrome c, by various stimuli, including Bax, reactive oxygen intermediates. 3: Activation of the pro-apoptotic SAPK/JNK pathway may occur downstream of membrane-derived signals, including ceramide and Daxx, a CD95-binding protein [116].

## CELL-SURVIVAL CURVES

Experimental radiation survival curves are based on the clonogenic assay, which does not differentiate among different modes of cells killed in an irradiated cell population [1,2]. From the viewpoint of radiotherapy, loss of clonogenicity represents the most significant consequence of exposure to ionizing radiation. Generally speaking, the surviving fraction is given by

**Target Theory:** The target theory of cell survival is based on the model in which a number of critical targets have to be inactivated for a cell to be killed. For example, the single-target survival curve is a straight line on a semi-logarithmic plot (Figure 7 A). This kind of survival curves has been identified for inactivation of viruses and bacteria, and can be applicable to the response of certain very radiosensitive mammalian cells (normal and malignant) to very low-dose rates, as well as to the response to high LET radiation.



**Fig. 7.** Illustration of survival curves. (A) Single-hit, single-target survival curve; (B) Multi-target survival curve; (C) Two-component survival curve; (D) Linear quadratic model of cell killing [2].

The target theory and derivation of simple cell-survival relationships in terms of targets and hits have been in use for a long time. A drawback, however, is that specific or idiographic radiation targets have not been identified in mammalian cells. Furthermore, although the two-component model (Figure 7 C) predicts cell killing in the low-dose region, the nearly linear change in cell survival in the dose range from 0 to D<sub>q</sub> implies no sparing when the fraction size is below 2 Gy, which does not agree with experimental or clinical data.

## **RADIATION-INDUCED CELL MUTATIONS**

Although the mutagenic capability of radiation was first described by Muller in 1927, it is only within the past two decades that information has been obtained regarding the molecular changes involved in mutations of mammalian cells. Radiation can induce a wide spectrum of mutations, from point mutations in single genes to

deletions of several genes, based on the earlier studies with the hemizygous X-linked HPRT gene [136]. The mutation spectrum induced by radiation is different from spontaneous mutations induced by ultraviolet light and chemical mutagens, the majority of the latter being consequences of point mutations. Most evidence at the molecular level indicated that the DNA deletions resulting in gene loss were the primary events leading to mutagenic effects of ionizing radiation. Since mutations or deletions of some essential genes are closely related to survival of cells, this limits the ability to detect large deletions in certain genes, especially some lethal genes.

Radon is a naturally occurring radioactive gas and alpha particles are emitted from radon and its progeny. The US Environmental Protection Agency estimated radon in indoor environments to have caused 21,600 deaths per year through radon-induced lung cancers [137]. To have a better quantitative assessment of the lung cancer risk associated with residential radon exposure, Hei's group investigated the mutation inducing capability of single alpha particles, by using the microbeam facility at the Columbia University to deliver an exact number of alpha particles to the nuclear region of cells [138]. They examined the frequencies and molecular spectrum of S1-mutants induced in human-hamster hybrid ( $A_L$ ) cells by 1, 2, 4, or 8  $\alpha$  particles. Although single-particle traversal was only slightly cytotoxic to  $A_L$  cells (survival fraction  $\sim 0.82$ ), it was very mutagenic, and the average induced mutant fraction was 110 mutants per  $10^5$  survivors. Furthermore, both toxicity and mutant induction were dose-dependent. The presence or absence of marker genes among the mutants was determined by multiplex PCR, and the proportion of mutants with multi-locus deletions was found to increase with the number of particle traversals.

Earlier studies in a number of different biological systems indicated that nuclear irradiation was critical for cytotoxic effects. As such, the nucleus was considered the main target of radiation. In a further study by Hei's group, mutagenesis of cytoplasmic irradiation with low fluences of alpha particles was studied with their microbeam facility [139]. The results showed that cytoplasmic traversals by alpha particles led to more mutations in  $A_L$  cells, but had relatively little effect on cell survival. An approximately doubled spontaneous mutation frequency was observed for a single alpha-particle traversal, and a 2- to 3-fold enhancement in the mutation frequency was observed with up to four particle traversals per cell. No further increase in the mutation frequency was found for larger particle fluences. These results are in contrast to their earlier studies involving nuclear irradiation. For nuclear traversals, mutation frequencies were 2- to 3-fold higher than those for the same number of cytoplasmic traversals. Furthermore, in the case of nuclear radiation, the mutation frequency kept increasing with the fluence up to eight or more particles per cell. It was particularly interesting to note that the spectrum of molecular-structural changes was significantly different between the two types of irradiation. Nuclear irradiation mainly led to large-scale changes such as those described previously for X-rays. On the other hand, cytoplasmic irradiation mainly led to point mutations with

the spectrum resembling that of spontaneously arising mutants. In both cases, an enhanced production of reactive oxygen species was involved [140]. These results concluded that nuclear radiation was not required for the production of important genetic effects.

As regards the mechanism of radiation mutagenesis, the mutagenesis is considered a result of the cell attempting to repair damages based on analyses of the induced mutations. DSBs are regarded as an important initiating lesion in the pathogenesis of large deletions characteristic of ionizing radiation and the involvement of DSB repair pathways in the mutagenic process [141]. DSBs can be repaired by HR in an error-free manner. Nevertheless, most DSBs are repaired by an error-prone process which likely accounts for many of the potentially mutagenic DNA lesions. Multiple DSBs in a cell may thus lead to chromosomal rearrangements and other large-scale changes, which are commonly present in irradiated cells. Repeat sequences in the genome also suggest that NHEJ of DSBs is usually responsible for the mutagenic process when large deletions are involved.

Radiation-induced base damages are also important. It is known that base damages can often lead to base substitutions (point mutations) and that certain repair pathways involved in base damage repair can also be mutagenic.

## **RADIATION-INDUCED BYSTANDER EFFECT**

### **OVERVIEW: OBSERVATION AND SIGNIFICANCE OF BYSTANDER EFFECT**

Biological effects of ionizing radiation have long been considered a consequence of DNA damages in the irradiated cells. Here, unrepaired or misrepaired DNA damages in the irradiated cells are responsible for the genetic effects. At the same time, no effects are expected in cells in the population that have not received radiation exposure. This conventional dogma was, however, challenged by the occurrence of the radiation-induced bystander effect (RIBE).

RIBE was reported back in 1954, when cells exposed to doses of low LET radiation were found to have an indirect effect in producing a plasma-borne factor, which led to chromosome breakage and cytogenetic abnormalities in human bone marrow or lymphocytes and caused tumors in rats [142]. Plasma from high-dose radiotherapy patients were also found to induce various aberrations, including dicentrics and chromatid and chromosome breaks, in normal non-irradiated lymphocytes in short-term culture. The radiation-induced clastogenic factors in the plasma of irradiated patients had low molecular mass, and their production involved lipid peroxidation and oxidative stress pathways. These factors either had long lives or were regenerated

continuously since they persisted in the plasma of atomic bomb survivors even 31 years after exposure.

From the early 1990s, developments in single-cell irradiations either with low  $\alpha$ -particle fluences or with microbeam facility have led to a large amount of experimental data and an immense interest in the bystander effects. In the following, more detailed information or findings for RIBE will be introduced.

Generally, RIBE can be defined as the phenomenon that the irradiated cells (by  $\alpha$  particles, X- or  $\gamma$ -ray, heavy ions etc.) can release some signaling molecule(s), which is transferred via the medium or gap-junctions, so that the same cytotoxicity or genotoxicity can be observed in the non-irradiated cells, which are either close to the irradiated cells or shared the conditioned medium harvested from the irradiated cells. RIBE has challenged the conventional dogma of radiation protection, the guidelines for which are based on prediction of biological effects of low doses of radiation by extrapolating from known epidemiological datasets. These datasets are mainly in the high-dose regions and the main source of information came from Japanese atomic-bomb survivors. The simplest way to perform the extrapolation is to assume a linear no-threshold relationship between the dose and the biological effect even at very low doses. In other words, a dose, however small, always has a finite probability of causing a biological effect. Environmental radon has been suggested to cause about 21,600 lung-cancer deaths in USA each year. The presence of bystander effects implies no direct correlation between the number of cells exposed to radiation and the number of cells at risk of mutation, chromosomal damage or apoptosis. Instead, biological effects depend on complex interactions between the irradiated cells and the bystander cells. The risk is no longer that of a single cell resulted from its radiation damages; instead the risk is “amplified” by the bystander effect and a simple dose–effect relationship is no longer valid.

### **RIBE in a system of low fluence $\alpha$ LPHA-particle irradiation**

One way to study RIBE is to use a broad beam of  $\alpha$  particles with a low fluence, in which case a small proportion of cells are traversed by  $\alpha$  particles. J.B. Little’s lab was the first to report a bystander effect resulting from high LET irradiation. In this study, the frequency of sister chromatid exchanges (SCEs) reached 30% in a cultured population of Chinese hamster ovary (CHO) cells when less than 1% of the cell nuclei were actually traversed by an  $\alpha$  particle (a dose corresponding to 0.31 mGy) [143]. Similar results were later reported for normal human lung fibroblasts. An enhanced frequency of HPRT locus mutations was also found in bystander CHO cells in cultures following an exposure to very low fluences of  $\alpha$  particles. Changes in gene expression in non-irradiated cells were also observed after low fluence of  $\alpha$  particles. It was noted that up-regulation of the p53 damage response pathway occurred in bystander cells in monolayer cultures exposed to very low fluences of  $\alpha$  particles

[144]. Phosphorylation of p53 in bystander cells suggested that p53 up-regulation in bystander cells was a consequence of DNA damages. Activation of the p53 damage response pathways in bystander cells was confirmed by *in situ* immunofluorescence studies which showed up-regulation of p21<sup>Waf1</sup> in clusters of cells in the monolayer population, of which only 12% of the cell nuclei had been traversed by an  $\alpha$  particle [145].

Direct measurements of DNA damages in bystander cells after exposure to low fluence  $\alpha$  particles have been made possible through advancement of the associated experimental techniques. Phosphorylated H2AX, a member of the histone family, is a marker of DSBs. With *in situ* immunofluorescence of  $\gamma$ -H2AX, excessive DSBs were measured in the bystander cells after even 1 cGy  $\alpha$ -particle irradiation, for which less than 1/10 nuclei were hit by the particles [146].

### **Studies of RIBE with Charged Particle Microbeam Facility**

Microbeam techniques can deliver exactly one particle (or more) to irradiate precisely the cell nucleus or cytoplasm [147]. This technique has been used to investigate the bystander effect for  $\alpha$ -particle irradiation for a variety of biological end points, including cell lethality, DNA damage, mutagenesis and oncogenic transformation [148]. One endpoint used in the RIBE study with the microbeam facility at the Gray Cancer Institute, UK, was micronuclei assay. In their study, human glioblastoma T98G cell nuclei were individually irradiated with an exact number of helium ions. It was found that when only 1 cell in a population of  $\sim 1200$  cells was targeted, with one or five ions, the cellular damage measured as induced micronuclei was increased by 20%. When a fraction from 1% to 20% of cells was individually targeted, the micronuclei yield in the population greatly exceeded the yield predicted with no bystander effect. Another end point investigated with the Columbia microbeam facility was mutagenesis. When a near-lethal dose of 20 alpha particles were directed to each nucleus of 20% of human–hamster hybrid ( $A_L$ ) cells, the surviving fraction was found to be less than 1%. On the other hand, by employing mutations in human chromosome 11 as the endpoint, the mutation yield was found to be four times that of the background. Since the irradiated cells were exposed to a lethal dose, the mutations should come from the non-irradiated bystander cells. The mutation spectrum evaluated for the bystander cells was significantly different from the spontaneous spectrum, which hinted different mutagenic mechanisms [149].

In another study of bystander-mediated oncogenesis mouse fibroblast (C3H10T12), cells were plated in a monolayer and either every cell or every tenth cell was irradiated with 1 to 8 alpha particles directed to the cell nucleus. The cells were subsequently replated at low density and the transformed foci, based on the morphology, were counted 6 weeks later. More cells were found inactivated than the number actually traversed by alpha particles. Furthermore, when 10% of the cells on

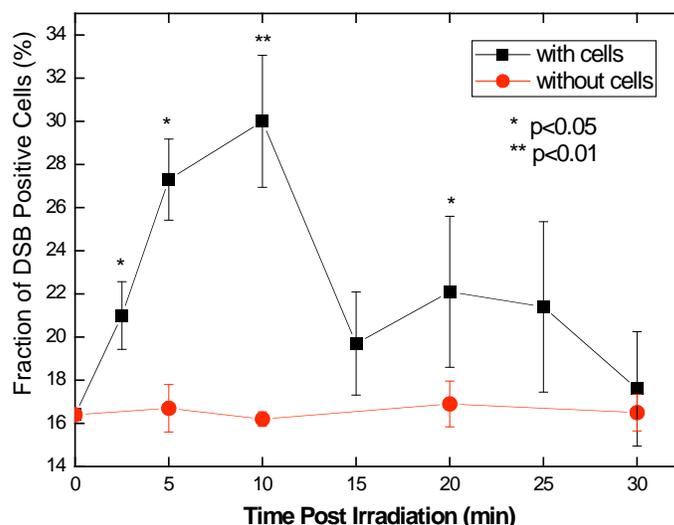
a dish were exposed to 2 or more alpha particles, the frequency of induced oncogenic transformation was indistinguishable from that observed when all the cells on the dish were exposed to the same number of alpha particles [148].

In another study, Shao *et al.* [150] suggested that direct targeting of nuclear DNA was not always required for expression of a radiation-induced bystander effect. A helium-ion ( $^3\text{He}^{2+}$ ) microbeam was employed to target individual cells within a population of radioresistant glioma cells cultured alone or in co-culture with primary human fibroblasts. Even when only a single cell was traversed with one  $^3\text{He}^{2+}$  ion through its cytoplasm, bystander responses were observed in the non-irradiated glioma or fibroblast cells. Specifically, the micronuclei yield was increased by 36% for the glioma population and 78% for the bystander fibroblast population. Furthermore, they did not depend on whether the cell cytoplasm or nucleus was targeted. This is very important in redefining the source of the bystander effect signal(s).

### **RIBE after Transfer of Conditioned Medium from Irradiated Cells**

As previously described, irradiated individuals release their plasma clastogenic factors that will induce chromosomal damages in cultured cells from non-irradiated donors. Although this phenomenon had been known for half a century, the underlying reasons have remained unknown. In the recent ten years, considerable evidence for the toxic effects of bystander signals has been gathered from studies where the culture media from irradiated cells have been transferred to cultures of non-irradiated cells [142,151,152]. The first study was carried out by Mothersill and Seymour's group. They demonstrated that irradiated epithelial cells, but not fibroblasts, secreted a toxic signal or the so-called bystander factor into the culture medium which, if transferred to non-irradiated cells, could significantly reduce the plating efficiency of the latter [153]. In relation to this phenomenon, further studies showed that a medium irradiated in the absence of cells had no toxic effects on survival when transferred to non-irradiated cells. The effect was dependent on the cell number at the time of irradiation, could be observed as soon as 30 min post-irradiation, and was still effective when medium transfer occurred 60 h after irradiation. On the other hand, the effect was independent of dose in the range from 0.5 to 5 Gy. The factor involved appeared to be a protein since it was heat-labile but stable if frozen, and did not require cell-to-cell contact to induce its effect in the recipient cells. The first detectable effect was a rapid (1-2 min) calcium pulse in the medium receptor cells, which was then followed (30 min to 2 h later) by changes in the mitochondrial membrane permeability and the induction of ROS. The critical role of mitochondrial metabolism was suggested by the lack of signal production by cells that did not have a functional glucose-6-phosphate dehydrogenase enzyme [154].

Held's group demonstrated that normal human fibroblast cells, when irradiated by broad field 250 kVp X-rays, could also release bystander factor(s) into the medium



**Fig. 8.** Timing of bystander factor(s) by normal human fibroblast cells AG1522 in the initiation of RIBE. The conditioned medium harvested from the irradiated culture induced a time-dependent DSB in the medium receptor cells. The irradiated medium without cells did not show distinct DSB-inducing activity [156].

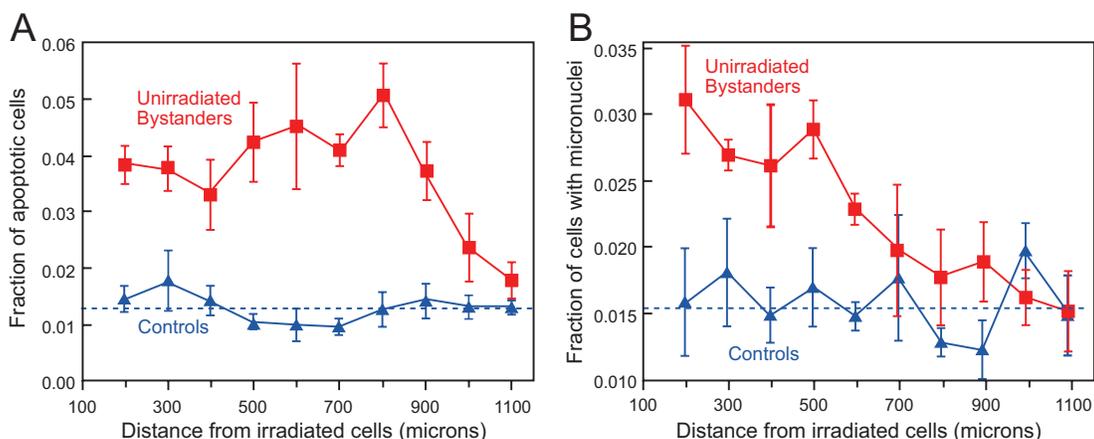
[155]. They used a transwell insert culture dish system to demonstrate the X-ray induced medium-mediated bystander effects in AG1522 normal human fibroblasts. The micronuclei yield in non-irradiated bystander cells was found to be approximately doubled that of the background in the dose range from 0.1 to 10 Gy. Induction of p21<sup>Waf1</sup> protein and foci of  $\gamma$ -H2AX in bystander cells were also independent of the dose delivered to the irradiated cells above 0.1 Gy. The ROS levels were increased persistently in irradiated cells up to 60 h after irradiation and in bystander cells up to 30 h. The clonogenic assay of bystander cells showed a decrease in their survival after receiving doses up to 0.5 Gy, but was then independent of the dose above 0.5 Gy.

Wu's group also revealed that the normal human fibroblast cells AG1522, when irradiated with low-dose (1 cGy) alpha particles, could release bystander factor(s) into the medium [156]. Medium transfer experiments showed that the conditioned medium harvested from the irradiated culture induced excessive DNA DSBs in the cells receiving the medium, and the capability of the medium of DSB induction was time-dependent (Figure 8). The results indicated that the bystander signaling molecule(s) had been generated very quickly (probably less than 2.5 min) after irradiation and persisted continuously up to 30 min although the production of signaling molecule(s) decreased after 10 min post irradiation. Their findings revealed an initiation and early process of bystander response induced by low dose alpha-

particle irradiation, and the findings were very important for understanding the mechanisms underlying the bystander response.

### RIBE in 3-Dimensional Tissue

The bystander effect experiments described above made use of cultured cell populations. It is also pertinent to study the effect *in vivo*. To simulate the bystander effects in a tissue, Belyakov and coworkers [157] used reconstructed normal human three-dimensional skin tissue systems. One of the systems used was a full thickness skin model corresponding to the epidermis and dermis of normal human skin. A charged-particle microbeam was used to irradiate cells at defined locations while guaranteeing that cells located more than a few micrometers away received no radiation exposure. The studied endpoints were the induction of micronucleated and apoptotic cells. As a result, significant effects were detected in non-irradiated cells up to 1 mm away from the irradiated cells, namely, an average increase of 1.7-fold for micronuclei and 2.8-fold for apoptosis (Figure 9).



**Fig. 9.** Induction of (A) apoptotic and (B) micronucleated cells in non-irradiated bystander cells at different distances from the plane of irradiated cells in a 3-D human epidermal skin model (EPI-200) [157]. (Copyright 2005 National Academy of Sciences, U.S.A.)

In their further study, DSBs were detected in the bystander cells in the 3-D tissue [158]. Occurrences of DSBs in irradiated cells and bystander cells are markedly different: the former reached a maximum 30 min after irradiation, while the latter reached a maximum 12 to 48 h after irradiation and gradually decreased over a 7-day time course. At the maxima, 40% to 60% of bystander cells were affected, i.e., a 4- to 6-fold increase over the controls. The rise in bystander DSB occurrences were followed by a larger number of apoptosis and micronucleus formation, by loss of nuclear DNA methylation, and by an increased fraction of senescent cells. These showed that DNA DSBs were involved in tissue bystander responses and that they were precursors to downstream effects in human tissues. Bystander cells which

showed genomic instability after irradiation were also found more likely to become cancerous than unaffected cells.

### **Bystander Effects in Mouse and Fish Models**

The existence of RIBE *in vitro* is well established and supported by solid experimental evidence. On the other hand, however, clear experimental demonstrations of bystander effects *in vivo* are limited. One of these came from a series of experiments performed by Brooks and coworkers [159]. Chinese hamsters were injected with different-sized particles internally deposited with alpha-particle emitting plutonium. The radioactive particles concentrated in the liver and caused chronic low-dose radiation exposure, causing the highest absorbed dose and dose rate to cells located closest to the largest particles. However, analysis of induced chromosome damages in these livers revealed increased cytogenetic damages but no changes in the aberration frequency as a function of the local dose. These indicated that all the cells in the liver had the same risk of induced chromosome damage despite the small fraction of liver cells that were irradiated.

In another study, Kovalchuk's group reported RIBE in mouse after X-ray irradiation of one-half of the mouse body. They showed that RIBE increased DNA strand breaks and Rad51 levels in the unexposed bystander tissue [160]. In terms of epigenetic changes, unilateral radiation suppressed global methylation in directly irradiated tissue, but not in bystander tissue at studied time points. They observed a significant reduction in the levels of the *de novo* DNA methyl transferases DNMT3a and 3b and a concurrent increase in the levels of the maintenance DNA methyl transferase DNMT1 in bystander tissues. Furthermore, the levels of two methyl-binding proteins known to be involved in transcriptional silencing, MeCP2 and MBD2, were also increased in bystander tissue. These findings illustrated radiation induced DNA damages in bystander tissue more than a centimeter away from directly irradiated tissues, and suggested that epigenetic transcriptional regulation might be involved in RIBE.

RIBE in nervous system was studied by Saran and co-workers [161], who regarded the neonatal mouse cerebellum as an accurate *in vivo* model to detect, quantify, and understand radiation-bystander responses. They conducted experiments using specially designed lead shields for protecting mouse heads and observed marked enhancement of medulloblastoma in mice irradiated with their brains shielded. They further analyzed DSBs in the shielded cerebella through the  $\gamma$ -H2AX foci, and the fractions of apoptotic cells. Besides these genetic events, they also reported bystander-related tumor induction in the cerebellum of radiosensitive *Patched-1* (*Ptch1*) heterozygous mice after X-ray irradiation of the remainder of the body. They showed that genetic damage was critical for *in vivo* oncogenic bystander responses,

and suggested the involvement of gap-junction intercellular communication (GJIC) in RIBE in the central nervous system.

Mothersill's Group reported RIBE in a cultured rainbow trout population [162]. They found that fish irradiated to 0.5 Gy X-ray (100 kVp) total body dose released factors into the water that could induce bystander effects in other unexposed fish. The unexposed fish were either arranged to share the same aquarium with the irradiated fish, or accommodated in the water previously used to hold the irradiated fish. Five organs were removed from each fish and tissue explants were cultured. The RIBE was expressed as increased cell deaths in a reporter system. The responses varied for different cell types, with the gill and fin showing the most pronounced responses. These results suggested that bystander signals involved chemical messengers secreted by an irradiated fish into the water, which could be passed to other fish.

### **POSSIBLE MECHANISMS**

Possible mechanisms of RIBE have been studied widely. Most experimental results broadly pointed to three types of mechanisms:

- (i) soluble transmissible factor(s) generated by irradiated cells;
- (ii) GJIC-mediated transmission of RIBE;
- (iii) oxidative metabolism-mediated transmission of RIBE.

These mechanisms are not independent of one another. In fact, in most cases, these mechanisms can co-exist. In the next part, these three mechanisms will be discussed in more details.

### **RIBE Mediated by Soluble Transmissible Factor(s) Secreted by Irradiated Cells**

In the previous sections, we introduced RIBE after medium transfer. The conditioned medium harvested from  $\gamma$ -irradiated keratinocytes and transferred to control keratinocytes or fibroblasts resulted in a toxic effect in the latter. Effects of transmissible factor(s) can also be observed in subconfluent cell cultures irradiated with alpha particles. Irradiation of the nuclei of a few mammalian cells in a subconfluent culture with alpha particles resulted in the induction of genetic damages (micronucleus formation and apoptosis) in a larger fraction of cells [163]. In these studies, targeting the alpha particles outside the cells did not have the effect, which suggested that RIBE was due to transmissible factor(s) released from the irradiated cells.

As regards the nature of the soluble factor(s), studies showed that alpha-particle irradiation of cultured cells generated a factor(s) capable of inducing SCEs in the bystander cells, and the factor(s) survived freeze thawing and was heat labile [164]. In parallel experiments, the same group showed that alpha-particle irradiation of the culture medium devoid of cells also caused generation of SCE-inducing factor(s);

such factors however were short-lived. In both situations, the supernatant from irradiated cells or irradiated medium caused the induction of excessive SCEs in non-irradiated cells to the same extent observed with direct alpha-particle irradiated cell cultures. Interestingly, both the short lived medium- and cell-derived SCE-inducing activities were inhibited by the anti-oxidant enzyme superoxide dismutase (SOD), suggesting that reactive oxygen species (ROS) were involved.

Secreted transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) [165] or Interleukin-8 (IL-8) [166] in the medium of alpha-particle irradiated cultures were suggested to play a role in mediating the bystander response-induced cell proliferation. Shao's recent study suggested that secreted TGF- $\beta$ 1 played a role in mediating the micronuclei induction in the bystander cells [167]. In this study, a fraction of cells within a glioblastoma population were individually irradiated with microbeam alpha particles, and the micronuclei yield in the non-targeted cells was found to have increased. This RIBE was mitigated by treating the cells with either aminoguanidine, which was an inhibitor of inducible nitric oxide (NO) synthase, or anti-TGF- $\beta$ 1, indicating that NO and TGF- $\beta$ 1 were involved in the RIBE. Intracellular NO was detected in the bystander cells, and additional TGF- $\beta$ 1 was detected in the medium from irradiated T98G cells, but it was diminished by aminoguanidine. Conversely, treatment with diethylamine nitric oxide, which was an NO donor, induced TGF- $\beta$ 1 generation in T98G cells. In relation to these experiments, treatment with recombinant TGF- $\beta$ 1 also induced NO and micronuclei. Treatment with anti-TGF- $\beta$ 1 inhibited the NO production when only 1% of cells were irradiated, but not when 100% of cells were irradiated. In general, downstream of radiation-induced NO, TGF- $\beta$ 1 could be released from irradiated T98G cells and played a key role as a signaling factor in RIBE by further inducing free radicals and DNA damages in the bystander cells.

In another study, Han and coworkers investigated the possible nature of secreted soluble bystander signal(s) in the initiation and propagation of the early processes of bystander signaling induced by low-dose alpha-particle irradiation [168]. They focused on the 10 min time point after irradiation, when the secreted signal(s) reached the maximum. Their former studies showed that the secreted signal(s), which rapidly induced phosphorylated H2AX in non-irradiated cells that had received the conditioned medium, might be short-lived and was released in a time-dependent manner in the first 30 min after irradiation [156]. Using N<sup>G</sup>-methyl-L-arginine, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate and N<sup>o</sup>-nitro-L-arginine (L-NNA) treatment before exposure to 1 cGy  $\alpha$  particles, they showed that NO produced in the irradiated cells was important and necessary for the DNA DSB inducing activity (DIA) of conditioned medium, and the generation of NO in irradiated confluent AG1522 cells was in a time-dependent manner and that almost all NO was generated within 15 min post-irradiation. Concurrently, the kinetics of NO production in the medium of irradiated cells after irradiation was rapid and in a time dependent

manner as well, with a maximum yield observed at 10 min after irradiation, determined with electron spin resonance analysis. Furthermore, they also showed that 7-Nitroindazole and L-NNA, but not aminoguanidine hemisulfate, treatment before exposure to 1 cGy  $\alpha$  particles significantly decreased the DIA of the conditioned medium, and this suggested that constitutive NO from the irradiated cells possibly acted as an intercellular signaling molecule to initiate and activate the early process (<30 min) of bystander response after low-dose irradiation.

### **Role of GJIC in the Transmission of RIBE**

Little's group first studied the possible role of GJIC in RIBE. Direct evidence for the participation of GJIC in RIBE from alpha-particle irradiated to non-irradiated mammalian cells was obtained when GJIC proficient or deficient confluent cultures of human and rodent cells were exposed to very low fluences of alpha particles [144]. *In situ* immunofluorescence studies of p21<sup>Waf1</sup> expression in AG1522 cultures exposed to 0.3 cGy of alpha particles (a dose at which about 2% of the nuclei would be irradiated) showed that the typical aggregate pattern of induction of the protein disappeared in the presence of a chemical inhibitor lindane of GJIC. In irradiated cultures treated with lindane, p21<sup>Waf1</sup> was induced primarily only in single cells. The WB-F344 cells were GJIC competent as was demonstrated with the transfer of the Lucifer yellow dye among contiguous adjacent cells. The WM-aB1 cells were derived from WB-F344 cells and were deficient in GJIC function [144]. An increase in p21<sup>Waf1</sup> levels in confluent WB-F344 cultures was observed after exposure to mean doses as low as 0.3 cGy, and at this dose 1% or less of the cells would have their nuclei traversed by alpha particles. While small clusters of responding cells were observed in WB-F344 cells, only single isolated and presumably irradiated WM-aB1 cells showed up-regulation of p21<sup>Waf1</sup> after exposure of 0.3-1.0 cGy. The p21<sup>Waf1</sup> levels increased significantly only at mean doses of 5 cGy or higher in WM-aB1 cell cultures. These findings were confirmed in low passage mouse embryo fibroblasts from wild type and isogenic knockout embryos for connexin 43. Similar to WB-F344 and WM-aB1 cells, western blot analyses demonstrated lack of detectable increase in p21<sup>Waf1</sup> expression in connexin43<sup>-/-</sup> cells exposed to mean doses less than 10 cGy. Conversely, p21<sup>Waf1</sup> was induced in wild type cells after exposure to mean doses as low as 0.6 cGy. All these observations strongly supported the involvement of GJIC in the bystander gene expression response in confluent, density-inhibited cell cultures exposed to alpha particles. When GJIC was inhibited, micronuclei induction was also inhibited in bystander cells in confluent cultures exposed to alpha particles.

The role of GJIC in mediating the alpha-particle induced bystander response was further confirmed by mutation studies using microbeam irradiation [169]. Microbeam facility was used to irradiate the nuclei of a precise fraction (either 100% or <20%) of the cells in a confluent population with exactly one alpha particle each. Irradiation of 10% of the cells resulted in a mutant yield similar to that from irradiation of 100% of

the cells. The effect was significantly eliminated when the cells were pretreated with a 1 mM of octanol which inhibited GJIC, or in cells carrying a dominant negative connexin 43 vector.

### **The Role of Oxidative Metabolism in Mediating the Radiation-Induced Bystander Response**

Radiation-induced ROS were known to participate in damages to various cellular components, and to produce DSBs in addition to base damages and single-strand breaks. Alpha-particle-induced metabolic ROS production was also involved in the activation of signaling pathways in bystander cells [145]. An induction of SCEs in bystander cells after exposure to very low fluences of alpha particles was inhibited by SOD, a superoxide radical scavenger [165]. Further studies directly showed that alpha particles (0.4-19 cGy) initiated the intracellular production of ROS (superoxide anions and hydrogen peroxide) in human cells through involvement of the plasma bound NADPH-oxidase [164]. These studies suggested that the ROS response did not require direct nuclear or even cellular transverse by alpha particles [164].

After exposing confluent cell cultures to very low fluences of alpha particles, the ROS formed were shown to induce stress-inducible proteins in the p53 and mitogen activated protein kinase pathways in bystander cells. Active SOD and catalase enzymes were capable of suppressing these effects and also inhibiting the activation of redox sensitive transcription factors in bystander cells [145]. Diphenyliodonium (DPI) is an inhibitor of flavin containing oxidase enzymes such as NAD(P)H-oxidase. NAD(P)H-oxidase enzymes are known to produce ROS in quantities capable of stimulating signaling pathways and these enzymes are rapidly activated by a variety of soluble mediators and engagement of cell-surface receptors. Treatment with DPI led to a significant reduction in the production of micronuclei by low doses of alpha particles. This suggested that activation of NAD(P)H-oxidase might induce the production of ROS in bystander cells.

The role of flavoprotein oxidases in the alpha-particle-induced bystander effect was extended when the enhanced accumulation of p53 and p21<sup>Waf1</sup> occurring after mean doses of 1 to 3 cGy was significantly reduced by the treatment with DPI [145]. These suggested that the increased numbers of SCEs in bystander cells from cultures exposed to low fluences of alpha particles involved membrane bound NAD(P)H-oxidase [165]. In general, these results supported the hypothesis that a DPI sensitive flavin containing oxidase activity represented a significant source of ROS production in human fibroblast cultures exposed to low fluences of alpha particles. They also suggested that ROS production from the activation of membrane bound NAD(P)H oxidase(s) might trigger the signaling pathway leading to the accumulation of p21<sup>Waf1</sup>

and p53 as well as induction of micronuclei and SCEs following radiation exposure to these doses.

Cell membrane involvement in the bystander response to low fluences of alpha particles was further confirmed by the complete suppression of SCEs and HPRT mutations in CHO cells exposed to very low fluences of alpha particles in the presence of Filipin, a drug that disrupted lipid rafts [143]. Importantly, at a dose of 10 cGy, when most effects occurred in directly irradiated cells, no suppressive effect of Filipin was observed. It was also interesting to note that gap-junctions were reported to partition in lipid rafts [170]. Of further interest is the finding that ROS-activated kinase(s) (e.g. member(s) of the mitogen-activated protein kinase (MAPK) superfamily) also have a role in activation of gap-junction proteins [171]. Furthermore, binding sites for the redox-sensitive AP-1 and NF- $\kappa$ B transcription factors, which are activated by low fluences of alpha particles have been shown to exist in the connexin 43 gene promoter region [172]. It is tempting to speculate that the mechanisms described above act in concert to promote the bystander effect.

There were some contradictory results regarding the involvement of oxidative metabolism in the induction of a bystander mutagenic effect. For example, in microbeam alpha-particle experiments with human-hamster hybrid cells, DMSO failed to suppress the induction of mutations in bystander cells, suggesting that ROS were not involved in the mutagenic bystander effect [149]. In contrast, induction of HPRT mutations in CHO bystander cells from cultures exposed to low fluences of broad beam alpha particles was consistent with the involvement of oxidative metabolism in the effect [173]. Further evidence of upregulation of oxidative metabolism in bystander cells was gathered from gene expression studies in human diploid fibroblast cultures exposed to very low fluences of alpha particles [145].

Oxidative metabolism was also implied in toxic bystander effects observed in media transfer experiments involving  $\gamma$ -radiation [174,175]. Treatment of the irradiated cultures with the antioxidants, L-lactate and L-deprenyl or with drugs that inhibited collapse of mitochondrial membrane potential prevented the cytotoxic effects from the irradiated cell conditioned medium. Similar observations were obtained in out-of-field *in vivo* experiments examining the genetic effects of partial organ irradiation. For example, DNA damages detected in the shielded apex region of rat lung when the lung base was irradiated was blocked by intravenously injected Cu-Zn SOD [176].

The involvement of oxidative metabolism in the early and initiation process was studied by Wu's group [156]. After irradiating half of the AG1522 cells cultured on a mylar dish with 1 cGy alpha particles, time-dependent DNA DSBs were induced shortly after irradiation in bystander AG1522 cells as observed through *in situ* detection. The induction of DSB in bystander cells was greatly reduced after treatment with DMSO. The capability of the conditioned medium to induce DSB can

also be inhibited by DMSO treatment. These results demonstrated the importance of oxidative metabolism in the early and initiation process of RIBE.

## **Other Pathways Involved in the Transduction of RIBE**

### **(I) Cyclooxygenase-2 as a central component of the bystander signaling scheme**

Experiments have been conducted in Hei's lab with normal human lung fibroblasts (NHLF) to identify genes that are expressed differently in directly irradiated and bystander cells [177]. Among the 96 genes represented on the platform, the abundance of one message, COX-2, was found to be consistently higher by more than three-fold, while the RNA level of insulin growth factor binding protein-3 was found to be consistently lower by more than seven-fold. Addition of the COX-2 inhibitor NS-398 (50 mM) suppressed COX-2 activity in NHLF cells and bystander mutagenesis at the HPRT locus. These results indicated that COX-2 expression was involved in the bystander effect.

### **(II) Role of nuclear factor NF- $\kappa$ B in the bystander response**

Since NF- $\kappa$ B is an important transcription factor for many signaling genes, including COX-2, it is likely that NF- $\kappa$ B also participates in the bystander response. There was strong evidence that alpha-particle irradiation up-regulated NF- $\kappa$ B binding activity in both directly irradiated and bystander cells, while Bay 11-7082, an inhibitor of I $\kappa$ B kinase (IKK)/NF- $\kappa$ B, efficiently suppressed this up-regulation and also reduced levels to below the basal amount [178]. This inhibitor of NF- $\kappa$ B activity also efficiently down-regulated the expression of COX-2 and inducible NO synthase in both directly irradiated and bystander fibroblasts. Earlier studies using confluent human skin fibroblasts exposed to low fluences of alpha particles showed a rapid up-regulation of NF- $\kappa$ B, c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK) in the exposed population [145] and suggested activation of these stress-inducible signaling pathways in bystander cells. Furthermore, addition of the antioxidant SOD was found to suppress the induction. Since induction of NF- $\kappa$ B binding activity could be found in both directly irradiated and bystander cells, its role in the bystander response in this study is equivocal.

### **(III) Important Role of Mitochondria in RIBE**

Mitochondria play an important role in the generation of free radicals and in the regulation of apoptosis [179]. Ionizing radiation induced mitochondrial damages through increases of ROS production, depolarization of mitochondrial membrane potential, and release of cytochrome c in directly irradiated cells [180]. The involvement and the role of mitochondria in RIBE were studied by three independent labs almost simultaneously.

By using charged particle microbeams, Hei's group found that mitochondrial DNA depleted human skin fibroblasts ( $\rho^0$ ), when a fraction was irradiated with lethal doses, showed a higher bystander mutagenic response in confluent monolayers compared to their parental mitochondrial functional cells ( $\rho^+$ ) [178]. However, when mixed cultures of  $\rho^0$  and  $\rho^+$  cells were employed and only one population of cells were irradiated with a lethal dose of alpha particles, a decreased bystander mutagenesis was uniformly found in non-irradiated bystander cells of both cell types, which suggested that signals from one cell type could modulate expression of bystander response in another cell type. Furthermore, they found that Bay 11-7082, an inhibitor of NF- $\kappa$ B activation, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, a scavenger of NO, significantly decreased the mutation frequency in both bystander  $\rho^0$  and  $\rho^+$  cells. In addition, they found that the NF- $\kappa$ B activity and its dependent proteins, COX-2 and iNOS, were lower in bystander  $\rho^0$  cells when compared with their  $\rho^+$  counterparts. These results indicated that mitochondria played an important role in the regulation of RIBE and that mitochondria dependent NF- $\kappa$ B/iNOS/NO and NF- $\kappa$ B/COX-2/prostaglandin E2 signaling pathways were important to the process.

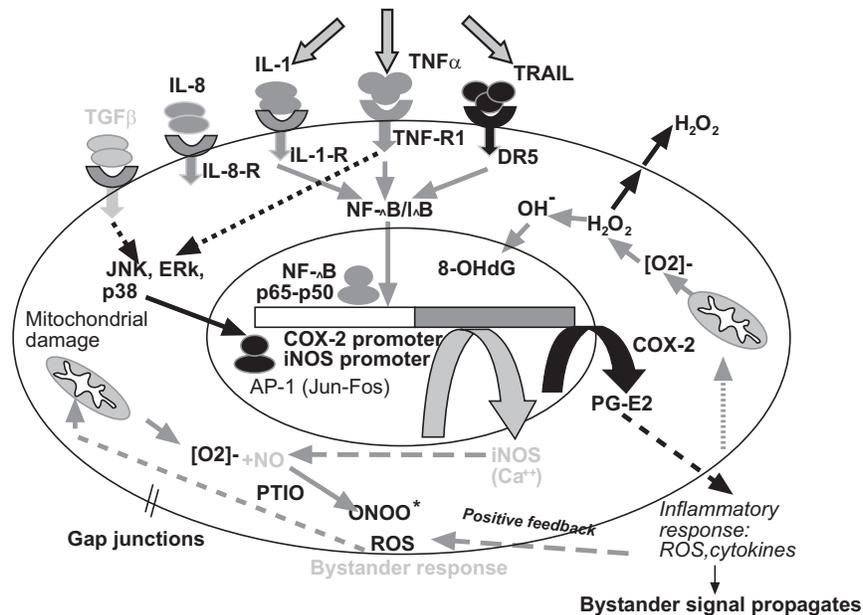
Prise's group found that mitochondria might be the source of damage signals of RIBE when the cytoplasm was irradiated with a microbeam facility [181]. The p53 binding protein 1 (53BP1) formed foci at DNA DSB sites and was an important sensor of DNA damages. They used the microbeam facility to irradiate specifically the nucleus or cytoplasm of a cell and quantified the response in irradiated and bystander cells by studying the 53BP1 foci. Their results showed that irradiating only the cytoplasm of a cell led to formation of 53BP1 foci in both hit and bystander cells, which did not depend on the dose or the number of cells targeted. The use of common inhibitors against ROS and reactive nitrogen species prevented the formation of 53BP1 foci in hit and bystander cells. On the other hand, treatment with Filipin to disrupt membrane-dependent signaling did not prevent the formation of 53BP1 foci in the irradiated cells, but did prevent signaling to bystander cells. Finally, active mitochondrial function was shown as a requirement for these responses because  $\rho^0$  cells could not produce a bystander signal, although they could respond to a signal from normal  $\rho^+$  cells.

The role of mitochondria in the early process of RIBE was investigated by Wu's group [182]. They used either  $\rho^0$  human-hamster hybrid  $A_L$  or  $\rho^+$   $A_L$  cells as irradiated donor cells and normal human skin fibroblast AG1522 cells as receptor cells in a series of medium transfer experiments to investigate the mitochondria-related signal process. The mitochondrial-DNA depleted cells or normal  $A_L$  cells treated with inhibitors of mitochondrial respiratory chain function had an attenuated  $\gamma$ -H2AX induction, which indicated that mitochondria played a functional role in bystander effects. Moreover, treatment of  $\rho^+$   $A_L$  donor cells with specific inhibitors of

NO synthase, or inhibitor of mitochondrial calcium uptake (ruthenium red) significantly decreased  $\gamma$ -H2AX induction and that radiation could stimulate cellular NO and superoxide anion production in irradiated  $\rho^+$  A<sub>L</sub> cells, but not in  $\rho^0$  A<sub>L</sub> cells. These results suggested that radiation-induced NO derived from mitochondria might be an intracellular bystander factor and calcium-dependent mitochondrial NO synthase might play an essential role in the process.

### A Model of RIBE transmission

A unifying model proposed by Hei [183] summarizes the signaling pathways involved in the transduction of RIBE (Figure 10). Expression/secretion of the inflammatory cytokines are profoundly increased after exposure to ionizing radiation or oxidants. Secreted or membrane-associated forms of cytokines such as TNF- $\alpha$  activate I $\kappa$ B kinase (IKK)-mediated phosphorylation of I $\kappa$ B, which releases nuclear factor NF- $\kappa$ B. NF- $\kappa$ B enters the nucleus and acts as a transcription factor for COX-2 and inducible NO synthase genes. TNF- $\alpha$  also activates MAPK pathways (ERK, JNK and p38) that, via the activation protein AP-1 transcription factor, additionally up-regulate expression of COX-2 and inducible NO synthase, which stimulates production of NO. Activation of COX-2 provides a continuous supply of reactive radicals and cytokines for the propagation of bystander signals through either gap junctions or medium. Furthermore, mitochondrial damages lead to the production of hydrogen peroxide, which migrates freely across plasma membranes.



**Fig. 10.** A model of the signaling pathways involved in RIBE [183].

## LOW DOSE RADIATION-INDUCED ADAPTIVE RESPONSE

### OVERVIEW: LOW DOSE RADIATION-INDUCED ADAPTIVE RESPONSE

Adaptive responses are another response which has challenged traditional thinking in radiation effects besides the bystander effect. The adaptive response was first observed for chromosomal aberrations [184]. Pre-exposing cells to a low “priming” dose of radiation appeared to protect these cells from the effects of a second larger “challenging” dose typically given several hours later.

The earliest studies were performed in human lymphocytes. Those pretreated with a small priming dose would have decreases up to 50% in the frequency of aberrations induced by the challenging dose in these cells [185,186]. Adaptive responses in mice *in vivo* were also detected in terms of cancer induction [187]. Since the initial report appeared about 20 years ago, literally hundreds of reports have been published describing this phenomenon in various experimental systems and for various biological endpoints including micronucleus formation, mutations and neoplastic transformation. Adaptive response is evolutionarily conserved and has been observed in eukaryotes, human and other mammalian cells, and in humans and animals. This effect increases the rate of DNA repair [188], reduces the frequency of radiation-induced and spontaneous neoplastic transformation in rodent [189] and human cells [190], and has been shown to increase tumor latency in mice [191]. Adaptive response has been reported in response to both low-LET (X-rays,  $\gamma$ -rays,  $\beta$  particles) and to high-LET (neutrons,  $\alpha$  particles) radiation [192]. The effect is not consistently seen in all cell types, and there has been considerable donor variation in studies with human lymphocytes.

In earlier studies of the adaptive response regarding chromosomal aberrations in lymphocytes, low dose-rate exposure from tritiated thymidine was used as a priming dose, but acute exposure to X-rays was also subsequently shown to induce the effect [193]. Priming doses of 5-100 mGy were generally required to induce the protective effect [194], and these doses were high enough to produce significant damages in all cells irradiated. The adaptive effect occurred within 3-6 hours when the cells became resistant to the higher challenging dose. The magnitude of the effect depended on many factors including dose, dose-rate, cell and tissue type and the endpoint measured.

### SIGNIFICANCE OF ADAPTIVE RESPONSE IN RISK ASSESSMENT

Several studies investigated the adaptive response and neoplastic transformation [195]. The results showed that pre-exposure to low priming doses of radiation reduced the effectiveness of a subsequent challenging dose in inducing neoplastic transformation, and thus suggested the adaptation induced by the priming dose.

Adaptive responses in relation to radiation induced cancer and stimulatory effects on the immune system were also studied in the past decades. There was insufficient information on the role and mechanisms of adaptive responses to influence judgment on low dose cancer risk. Recent animal carcinogenesis studies relating to adaptive responses [196] suggested that adaptive-like responses might increase tumor latency whilst not affecting the life-time risk. These data are of scientific interest but the relevance to radiological protection has remained relatively uncertain. The phenomenon clearly appears to be a real one in many cellular systems. However, it will be important to determine the extent to which it is active for human exposures *in vivo* at relevant dose and dose-rate levels before it can be considered a factor in risk estimation. In the absence of molecular mechanisms, it is not straightforward to evaluate the potential significance of the adaptive response in the risk assessment of ionizing radiation exposure to human populations.

### **BYSTANDER EFFECT AND ADAPTIVE RESPONSE**

Both the bystander effect and the adaptive response have been measured for immediate or short-term effects, such as gene-expression alterations, apoptosis, DNA double strand breaks, and neoplastic transformation, etc. These two responses seem to have opposite effects on exposure to low dose radiation, namely, the bystander effect would increase the risk while the adaptive response would reduce the risk. However, evidence showed that bystander effect mechanisms might be involved in adaptive responses as well. The study made by Mothersill's group [197] demonstrated, through using 13 different human cell lines, a weak inverse relationship between the adaptive response and bystander effect. The cell lines showing the weakest bystander response and the strongest adaptive response were the least efficient at cell-to-cell communication, the most malignant and the most rapidly dividing among all the cell lines.

### **POSSIBLE MECHANISMS OF ADAPTIVE RESPONSE**

Adaptive responses vary significantly for different cell systems and end-points used. Despite the proposed involvement of repair processes and antioxidant activity, detailed molecular mechanisms remain unclear. Adaptive responses have also been linked to radiation hormesis which refers to beneficial radiation responses at low doses. Radiation hormesis is characterized with a threshold dose above which the risk increases, in contrast to the linear no-threshold hypothesis. In fact, there are not a lot of data in support of the linear no-threshold hypothesis, and it is common to estimate the risk at low dose through extrapolation from existing epidemiological data [198]. It is interesting that many adaptive responses were observed for very low priming doses (0.2 Gy), which could also induce bystander signaling pathways. Further studies on the interactions between these two processes appear to be pertinent [199]. One possibility is that non-targeted responses to low-dose irradiation are simply stress response mechanisms of biological systems. These are outlined in the following.

### **(I) P53 plays a key role in the adaptive response**

It is now known that low doses of radiation can modulate the expression of a variety of genes [200,201]. Sasaki *et al.* found that p53 played a key role in the adaptive response, and proposed adaptive response and apoptosis to constitute a complementary defense mechanism. In cultured cells *in vitro* and in the spleens of mice *in vivo*, through a priming low-dose irradiation, p53-dependent apoptosis after exposure to high-dose radiation could be suppressed [202]. Induction of heat shock proteins was also reported to be involved in the adaptive response [203,204].

### **(II) Increased DNA repair ability**

While the phenomenon reflected that the induction of some types of DNA repair process required a certain level of damage in the cell, no such inducible DNA repair mechanism for DNA strand breaks had been clearly demonstrated in mammalian cells. Restriction enzymes that produced DSBs would induce adaptation in human lymphocytes [185], and the rate of repair was reported to be more efficient in adapted cells [205]. Some studies also gave evidence of involvement of DNA repair in the adaptive response in yeast [206]. Transcription and translation of genes related to DNA repair and in cell-cycle regulation were also found to be required for the adaptive response in human lymphocytes [207].

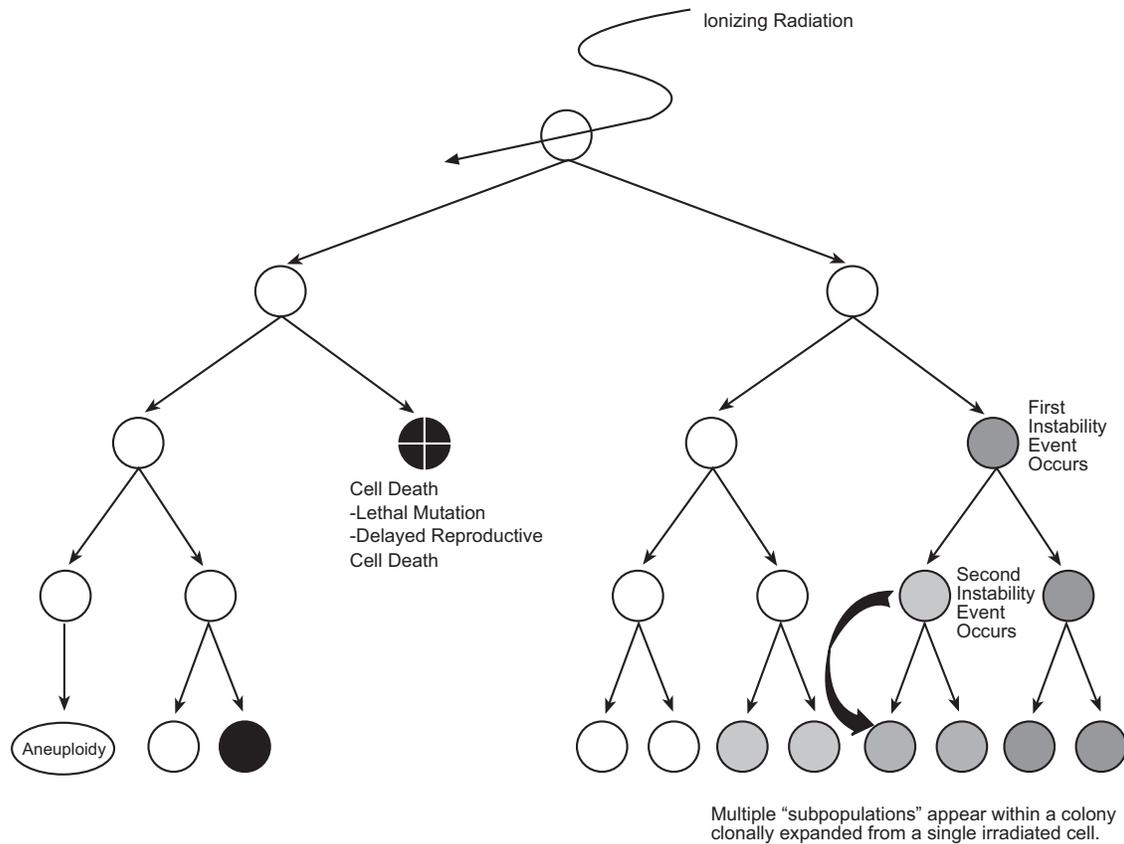
### **(III) Activation of protein kinase C**

Activation of protein kinase C was required for radiation-induced adaptive responses, and the intracellular signal transduction pathway induced by protein phosphorylation with protein kinase C was a key step in the signal transduction pathways induced by low-dose irradiation [208].

## **DELAYED EFFECT OF EXPOSURE TO IONIZING RADIATION**

### **RADIATION-INDUCED GENOMIC INSTABILITY: *IN VITRO/IN VIVO* STUDIES AND POSSIBLE MECHANISMS**

Genomic instability is an all-embracing concept to describe the increased rate of alterations in the genome. Radiation-induced genomic instability is observed in cells at a delayed time after irradiation and manifests in the progeny of the irradiated cells after several generations (Figure 11). Instability is measured with various biological endpoints such as gene mutations and amplifications, chromosomal alterations, changes in ploidy, micronucleus formation, microsatellite instabilities, and/or decreased plating efficiency [209]. Genomic instability is considered a hallmark in the process of carcinogenesis and most human tumors express instability as multiple, unbalanced chromosomal aberrations.



**Fig. 11.** A schematic presentation for radiation-induced genomic instability [203].

### DEMONSTRATION OF RADIATION-INDUCED GENOMIC INSTABILITY

The first demonstration of the existence of radiation-induced genomic instability was derived from a measurement of the kinetics of radiation-induced malignant transformation of cells *in vitro* [210-212]. These results suggested that the transformed foci did not arise from a single radiation-damaged cell. Instead, radiation actually induced a type of instability in 20-30% of the irradiated cell population and this instability enhanced the probability of occurrence of a second neoplastic transforming event. This transforming event occurred with a constant frequency per cell per generation, and had the characteristics of a mutagenic event [213]. Wright's group found that exposure to alpha particles (but not X-rays) produced a high frequency of non-clonal chromosomal aberrations in the clonal descendants, and this finding was compatible with alpha-particle-induced lesions in stem cells that resulted in the transmission of chromosomal instability to their progeny. These findings led to the hypothesis that radiation induced genomic instability would enhance the rate of malignant transformation, chromosomal aberrations, or other genetic events that would occur in the descendants of the irradiated cells after many generations of cell division.

This hypothesis was confirmed subsequently in a number of experiment systems with the measurement of various genetic endpoints [214,215]. In terms of mutagenesis [216,217] approximately 10% of clonal populations showed a significant elevation in the frequency of spontaneous mutations when compared to that derived from non-irradiated cells, and this increased mutation rate persisted for approximately 30 generations post-irradiation, and then gradually subsided. These delayed mutations resembled spontaneous mutations in that the majority of them were point mutations. In this way, they were formed by a mechanism different from that involved in direct X-ray-induced mutations which primarily involved deletions. Enhancements of both mini-satellite [218] and micro-satellite [219] instabilities were also observed in the descendants of irradiated cells selected for mutations at the thymidine kinase locus. The instability measured by mini-satellite sequences in X-ray-transformed mouse 10T<sup>1/2</sup> cells was also significantly increased [220]. Nonclonal chromosome aberrations arising in the progeny of irradiated cells many generations after irradiation were revealed by a number of laboratories [151]. The genomic instability could be induced by high or low LET radiation in a variety of cell types. For low-dose irradiation, a dose-response relationship was not observed as the responses saturated at low doses. The instability-induced cytogenetic abnormalities resembled those that occurred spontaneously as a consequence of endogenous damaging processes. These unstable aberrations might result in apoptosis and thus accounted for a component of the delayed death phenotype in some cell systems. The most significant aberrations were gross chromosomal rearrangements, particularly chromosomal duplications, and partial trisomies which seemed to involve amplification and recombination of large chromosomal regions by a yet unknown mechanism.

Radiation-induced genomic instability was also observed in mouse models. After whole-body irradiation, Watson *et al.* [221] observed a constant fraction (10-13.4%) of cells with stable chromosomal aberrations up to 17.5 months and this increased to 49.8% at 24 months in samples from three CBA/H mice. However, Bouffler *et al.* [222] failed to find evidence of transmissible chromosomal instability 50 or 100 days after *in vivo* exposure of CBA/H mice to alpha particles from the bone-seeking radionuclide <sup>224</sup>Ra or X-rays. Similarly, chromosomal instability was also not detected in peripheral blood lymphocytes from C57BL/6 mice for up to 30 days [223] or up to 21 months [224] following whole-body  $\gamma$  irradiation.

Chromosomal instability was induced in human lymphocytes after irradiation and culture *in vitro* [225]. Using the same lymphocyte culture protocol, chromosomal instability was reported in blood samples from individuals exposed during the radiation accident in Estonia in 1994 [226]. Chromosome analysis of G-banded peripheral blood lymphocytes from two groups of plutonium workers with 20-50% and >50% maximum permissible body burdens of plutonium from the British Nuclear Fuels facility at Sellafield (in West Cumbria, United Kingdom) revealed a significant

difference in frequencies of symmetrical aberrations between plutonium workers, workers with similar histories of exposure to mainly external  $\gamma$  radiation but with little or no intake of plutonium, and controls with negligible exposure (<50 mSv) [227]. Frequencies of symmetrical aberrations increased significantly.

### **POSSIBLE MECHANISMS OF RADIATION-INDUCED GENOMIC INSTABILITY**

Until now, the molecular, biochemical and cellular events which are involved in the induction of genomic instability have remained unclear. Directly induced DNA damages such as induced DNA DSBs are probably not involved. In contrast, studies in the past decade revealed a possible role of ROS and mitochondria in the formation of genomic instability.

Morgan's group studied the role of oxidative stress in the human-hamster hybrid line GM10115, and its exposure to a variety of DNA damaging agents could lead to persistent destabilization of chromosomes [228]. All of the clones were derived from single progenitor cells surviving exposure to ionizing radiation or chemicals. Compared to their stable counterparts, unstable clones showed elevated ROS levels as demonstrated by their enhanced capability of oxidizing fluorogenic dyes. In particular, unstable clones had significantly higher mean fluorescence signals of ~2-fold and ~1.25-fold, as derived from the dyes 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate and dihydrorhodamine 123, respectively, determined through fluorescence automated cell sorting. Stable and unstable clones of cells were further analyzed for mitochondrial content, from which unstable clones were found to have an elevated number of dysfunctional mitochondria when compared with the stable clones.

In their further study [229], the dysfunction of mitochondria was investigated. Addition of free radical scavengers (e.g. DMSO, glycerol, and cationic thiol cysteamine) reduced the incidence of instability after irradiation, implying a role for ROS in the induction of genomic instability. Amplex Red fluorometry measurements showed that the relative contribution of uncoupler-sensitive mitochondrial hydrogen peroxide production to total cellular hydrogen peroxide generation was greater in unstable cells. Measurements of mitochondrial DNA levels and cell cytometric fluorescent measurements of Mitotracker Green FM showed that the different mitochondrial ROS production were not due to varying mitochondrial levels. However, mitochondrial respiration measured in digitonin-permeabilized cells was impaired in unstable clones. Furthermore, manganese SOD, a major mitochondrial antioxidant enzyme, showed increased immunoreactivity but decreased enzyme activity in unstable clones, which together with the decreased respiration rates might explain the increased levels of cellular ROS. These studies showed that mitochondria

in unstable cells were abnormal and they probably caused persistent oxidative stress in the unstable clones.

## TRANS-GENERATIONAL EFFECTS OF RADIATION EXPOSURE

Trans-generational effects are those effects observed in offspring born after one or both parents had been irradiated prior to conception [209]. Potential trans-generational effects might play a significant role in reproductive legacy of individuals living in radiation-contaminated areas.

Wiley and colleagues used a pre-implantation embryo chimera assay to demonstrate adverse effects in progeny embryos after acute whole-body paternal irradiation, and illustrated the trans-generational effects of radiation exposure. They measured the competitive cell-proliferation disadvantage of an embryo with a radiation history after challenge by direct cell-to-cell contact with a normal embryo in an aggregation chimera [230,231]. The F1 embryos conceived 6–7 weeks after paternal F0 irradiation were the most likely to display the phenotype, indicating that the type B spermatogonia were the most sensitive. Baulch *et al.* [232] evaluated F3 mouse offspring from F0 paternal mice exposed to 1 Gy  $^{137}\text{Cs}$   $\gamma$ -rays for gene products that could modulate cell proliferation rate including receptor tyrosine kinase, protein kinase C and MAP kinases and protein levels of nuclear p53 (Trp53) and p21<sup>Waf1</sup> (Cdkn1a). All three protein kinase activities were changed, and nuclear levels of Trp53 and Cdkn1a protein were higher in F3 offspring with a paternal F0 radiation history than in unirradiated littermates.

Fortunately, no radiation-induced genetic, i.e. hereditary, diseases have been demonstrated in a human population exposed to ionizing radiation until now. No excess in cancer incidence in children born to parents exposed by the atomic bombings in Japan [233,234] or in the offspring of cancer patients treated with radiotherapy [235] have been reported. Nevertheless, the risk of cancer in the offspring of humans irradiated prior to conception remains controversial.

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