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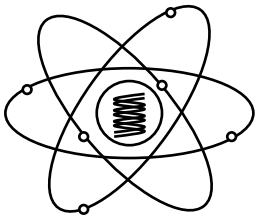

Biological consequences of Ionizing Radiation Exposure and the role of XRCC4 Protein Phosphorylation in DNA Double-strand Break Repair through Non-homologous End Joining

Doctoral Thesis

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CHAPTER 1

Introduction

1.1 Radiation and its biological effects

1.1.1 Radiation

Radiation can be defined as electromagnetic wave (ray) or matter (particles), which have enough energy for ionization atoms or molecules (substances). Radiation is the products of radioactive materials and it can be emitted not only through radioactive substances but also can be produced in nuclear reactors, accelerators and even by cosmic rays. **Table 1-1** shows the major types of Radiation.

Directly-ionizing radiation	**Charged particle**	**α-ray(helium nucleus)**	**Emitted from radionuclides**
		β^--ray(electron)	
		β^+-ray (positron)	
		protons	**Generated by accelerators**
		deuterons	
		electrons	
		Heavy ions. mesons	
Indirectly-ionizing radiation	**Neutral particle**	**Neutrons, neutrinos, mesons**	**Generated by reactors, accelerators and radionuclides**
	Electromagnetic wave	**X-ray -characteristics X-ray -Bremsstrahlung -Synchrotron radiation**	**Generated outside atomic nucleus or by accelerators**
		Y-ray	**Emitted from atomic nucleus**

Table 1-1. Major types of Radiation

Alpha particles have positive charge. An α-particle is a helium nucleus consisting 2 protons and 2 neutrons. They are emitted during decays of nuclear materials with heavy nucleus such as Uranium and Radium-226, Thorium, Radon-222. Heavy nucleus of this material consists of big numbers of protons and neutrons. They go under decay and emit two protons and two neutrons at very high speed particles; therefore after such emission the main nucleus has two protons and neutrons less the original nucleus such as nuclei of Helium. The phenomenon mentioned above is called alpha ray (decay).

In this phenomenon in the nucleus of a nuclear material (radioisotope) such as tritium and phosphorus-32, carbon-14, strontium-90 a neutron converts to an electron and a proton, and an electron is able to emit. This is called Beta Ray and includes of Beta particles (Protons and neutrons). Therefore, the discharge is particles of very small mass but with a negative or positive charge. Such as electrons and positrons (electron with positive electrical charge is known as positron). As a result of Beta decay or β (-) decay and after beta particle emission, the new nucleus has one proton more and one neutron less than the original nucleus. Positron decay or β^+ decay or β^+ ray, is another type of Beta radiation. In this situation conversion of a proton to neutron and positron (positive electron) in the nucleus will occur. The positrons have equal mass as electrons except their electrical charges. After β^+ decay and emission of one positive electron the remained nucleus, has one neutron more one proton less than its original nucleus before such emission. As a result of positron decay, the remained nucleus has one neutron more and one proton less than the original nucleus after decay.

The nature of gamma rays and X-rays both is electromagnetic wave; therefore, have similar features except the ways they are produced, however their production way is different. Nucleus after decay usually is in the most instability form, this can cause extra energy in the nucleus emitted in the shape of electromagnetic wave to reach to a stable state. This electromagnetic wave is called gamma radiation. X-ray was discovered by Wilhelm Conrad Rontgen in 1895 and named as an “unknown ray”. Production of X-rays is extranuclearly or man-made. By applying high voltage between cathode (in this case a heated filament) and anode (such as gold or tungsten) in a vacuum tube, the electrons that emitted from heated element (cathode) toward anode can be accelerated. Part of the kinetic or motion energy of

high speed electrons after strike with anode, change to X-rays. X-ray based on the way of production can be classified into in two groups:

1- Bremsstrahlung (Braking X-rays or Braking radiation). Emitted when a high speed electron is decelerated. The loss of motion (kinetic) energy resulted of very strong interaction between high speed electrons (negative electric charge emitted from filament) and nuclei of the atoms of the anode (positive electric charge). In this phenomenon X-rays is released as electromagnetic wave.

2- Characteristic X-rays. Characteristic X-rays also known as Auger electrons**.** This radiation emitted when an orbital electron belongs to anode, due to the strike of high speed electrons of heated filament(cathode) become excited and from their original orbit transit to a ground state (inner layer) that an electron vacancy exist. The energy corresponding to the difference between the two states (outer layer and inner layer) is released as electromagnetic wave.

Composition and approximate penetration of common types of ionizing radiation

Type of radiation	Constituent particles	Charge (per particle)	Mass (per particle)	Penetration (water or tissues) – varies wth energy
Alpha	Helium nucleus (2 protons + 2 neutrons)	+2	4	hundredths of a millimetre
Beta	Electrons	−1	(1/1840)	millimetres
	or Positrons	+1	(1/1840)	millimetres
Gamma	Photons	0	0	centimetres
Neutron	Neutrons	0	1	centimetres
X ray	Photons	0	0	centimetres

Table 1-2. (Cited from Nuclear Radiation: Risk and Benefits. Edward Pochin et al., 1983)

Ionizing radiation can be classified in two types:

A''- Directly Ionizing Radiation (Charged Radiations)

In this system, charged particles with sufficient motion energy (Kinetic Energy) that are able to directly disrupt the atomic structure of solute or target (for example DNA or RNA) through removing tightly bound electron from the orbit of its atom (target become ionized or excited) and causes chemical and biological change or damages, classified as Directly Ionizing Radiation. Therefore, all charged particle except neutrons in particulate radiation can be classified in this group.

B''-Indirectly Ionizing Radiation (Neutral Radiations)

Electromagnetic waves such as X-rays (characteristic X-ray, Bremsstrahlung, synchrotron radiation) and ϒ rays and Neutral particles such as Neutrons, that have no electrical charge, are capable of producing indirect chemical and biological damage and changes in DNA molecule. The biological effect of such type of radiation is indirect.

Dose of Radiation includes three types: **A-** Absorbed dose (Gray, Gy). **B-**Equivalent dose (Sievert, Sv) and **C-** Effective dose (Sievert, Sv) .

Absorbed dose can be defined as: One joule energy which is absorbed in one kilogram of the material. Absorb dose unit is Gray which is: 1Gy= 1 J/Kg

Equivalent dose it is a measurement of biological effects caused by radiation on tissues and organs in the body, and it is measured by Sievert unit (J/Kg)

Equivalent dose [Sv]= absorbed dose X radiation weighting factor (WR)

Radiation weighting (W_R)of X, gamma and beta rays is 1. In this case 1Gy = 1Sv.

Radiation weighting (W_R) of Alpha ray is 20. In this case 1Gy = 20Sv while Radiation weighting (WR) of Neutron not the same and changes between 2.5-20.

Effective dose is a measurement for total effects of radiation on tissues and organs of the entire body. The differences depend on the sensitivity of organs and tissues. It is measured by Sievert unit (J/Kg).

Effective dose= E (equivalent dose of tissues / organs X tissue weighting factor)

1.1.2 Action of radiation on biological systems

The process of biological action of DNA can be considered as 4 stages, i.e., **1-** The physical stage, **2-** Physio-chemical stage, **3-** Chemical stage and **4-** Biological stage.

1- **The physical stage**: In this stage ionization or excitation of molecule of water occurs. This stage is complete within 10^{-15} seconds. The kinetic energy from ionizing radiation to water molecule transfer in this such a short time and as a result excitation or ionization of the orbital electrons or nuclei of water happens. In case the energy is strong enough an electron will be removed from a water molecule. This action result of ionization of water. Otherwise there will be excitation of the orbital electrons of molecular water.

$H_2O \rightarrow H_20^+ + e^-$ $\qquad$ $H\text{-}O\text{-}H \rightarrow H^{\bullet} + 0H^{\bullet}$ **(Free radical)**

$H_20^+ + H_2O \rightarrow H_20^+ + OH^-$ **(Ionization)**

2- **Physico-chemical stage:** This stage occurs within 10^{-15} and 10^{-12} seconds after ionizing radiation. He ionized and excited molecule in physical stage is highly unstable and will dissipate energy to neighboring molecules or cause bond rapture. Hydroxyl radical ($0H^{\cdot}$) can be generated by proton transfer from an ionized water molecule to a neighboring water molecule. ($OH^{\cdot}$) hydroxyl radical is the most abundant among radiation –induced free radicals and are easy to interact with biological molecules in the cell as they are chemically reactive. The excited water molecules can dissipate energy by the splitting into hydrogen radical ($H^{\cdot}$), hydroxyl radical ($0H^{\cdot}$), H2 and $O^{\cdot}$ or $2H^{\cdot}$ radicals. It may also dissipate energy in the form of heat without causing any chemical bond breakage.

3- **Chemical stage:** This is the stage for free radical reaction. This stage occurs within 10^{12} and 10^{-6} seconds after ionizing radiation. Hydrogen radical ($H^{\cdot}$), hydroxyl radical ($0H^{\cdot}$),

H2 as well as H_2O_2 products created at this stage in a high concentration. In case the two radicals, hydrogen radical ($H^{\cdot}$) and hydroxyl radical ($0H^{\cdot}$) by chance collide to each other they will form water molecule again and dissipate heat. However, this is not always the case, if the free radicals meet biological molecules; the molecule will be activated and undergoes unusual chemical reactions, leading to loss of biological function. In this stage some molecule with abnormality in structures and function appears which is mostly harmful for the cell survival.

4- **Biological stage**: This stage is the final stage that forms all level of injuries from cellular structure to organism. This stage takes between second to years. After injury and damage, cells try to find a way to resolve their damages and injuries. For researchers some of the misbehavior in the cellular function can be examine through experiments. These experiments give valuable information regarding the biological effect of radiation. (Hall EJ, 2011)

1.1.3 Biological effects of radiation

Natural or artificial radiation has similar effect on living substances. Biological effects of radiation can be classified into two groups: somatic effects, that appears in the exposed person to radiation and hereditary effects that later on, appears in descendants of the exposed person.

Acute and Late effects are two sub-groups of Somatic effects that based on length of exposure to the radiation up to the time symptoms of exposure has seen in the person. For example, in case of acute effects the effects resulted of exposure to radiation appears in a short period of time after exposure. While in case of late effects symptoms like cancer and cataracts, appear much later or even decades.

Table 1-3 exhibits the symptoms of acute effects and dose delivered to the whole body by single exposure to gamma rays (or X-rays).

Dose (Gy)	Symptoms
0.25 or less	Almost no clinical symptoms
0.5	Temporary reduction of white blood cells (lymphocytes)
1	Nausea, vomiting, whole-body languor, substantial reduction of lymphocytes
1.5	Radiation sickness to 50%
2	Death to 5%
4	Death to 50% within 30 days
6	Death to 90% within 14 days
7	Death to 100%

Table 1-3. Acute effects induced by whole-body, single exposure to gamma rays (or X-rays). (Cited from Basic knowledge of Radiation and Radioisotopes, Japan Radioisotope Association.)

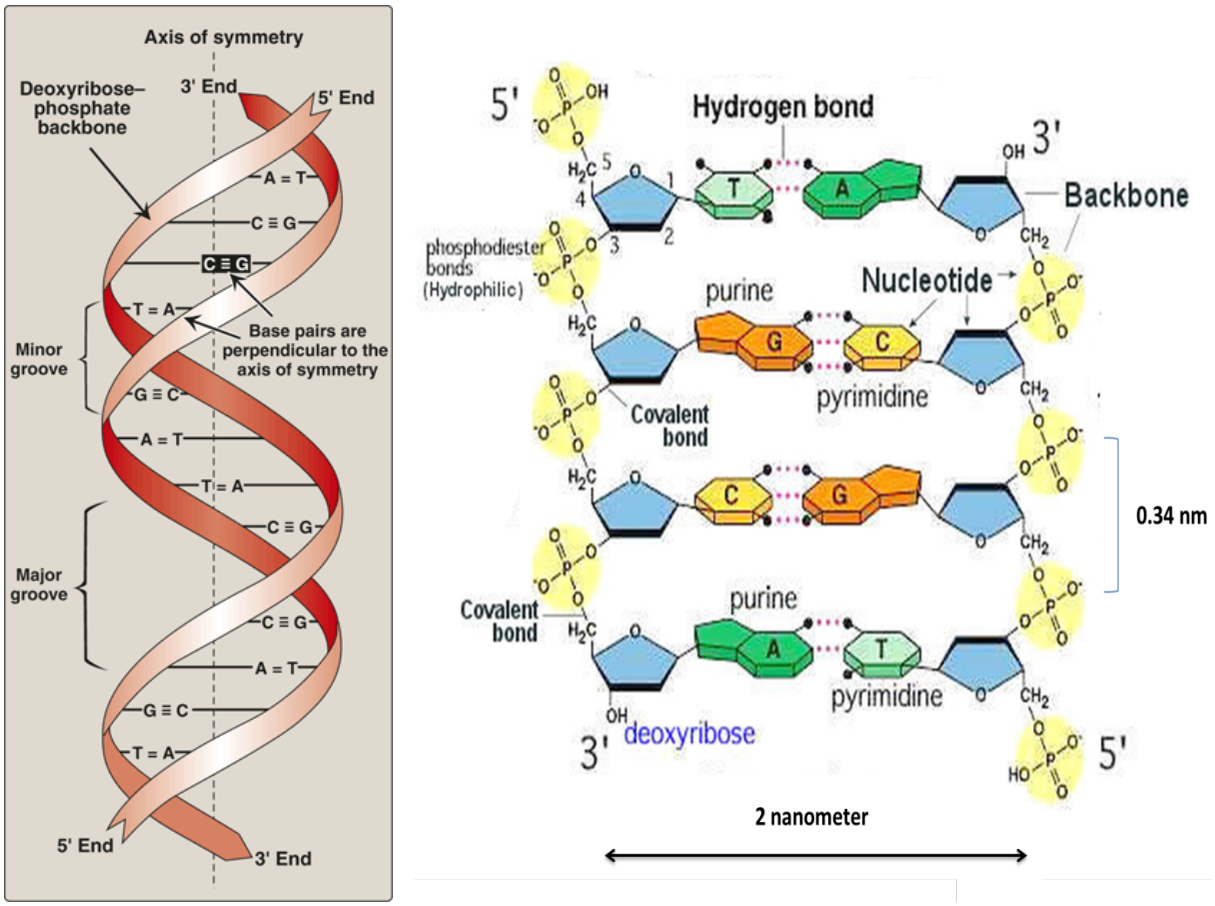
1.2 DNA

1.2.1 DNA structure

DNA, deoxyribonucleic acid, carries genetics information in human and almost all other living cells as well as some viruses, although there are a class of viruses, retroviruses, whose genetic information is carried by RNA, ribonucleic acid, instead of DNA. The information in DNA should be protected securely from damages and inherited from parents to their offspring during reproduction without any changes. DNA is the largest known molecule and the most important biological substance in the cell.

The structure of DNA molecules can be related to the shape of a long ladder double helix, due to the structural and chemical characteristics of its two polynucleotide strands (Fig. 1-2-1). Each single strand is made by polymerization of units or monomers known nucleotides, which is an ester of phosphoric acid with nucleotide. Each nucleotide consists of three parts: (1) one of four types of nitrogenous bases, (2) one 5-carbon sugar molecule or a pentose sugar called, 2-deoxyribose (or simply, deoxyribose hereafter) and (3) one phosphate group.

Nucleotides can have one, two or three phosphate groups and are designated as NMP's, NDP's, and NTP's respectively. In general, on the outside of the helix or the vertical sidepiece of DNA, repetitive sugar-phosphate groups are located and facing the surrounding water and considered the backbone of DNA molecules holding the bases together and giving directionality guide to molecules; one of the strands runs in the 5' to 3' direction whereas the other strand runs in the 3' to 5' direction. All the bases are located inside the double helix and are held together by hydrogen bonds in the opposite stands, forming the ladder-like rungs of the DNA molecule. The distance between two adjacent phosphates or nucleotides in each strand is 0.34 nm (3.4 Å). Each turn of DNA Molecules is equal with 3.6 nm (36 Å) and each turn includes 10.4 pairs of nucleotides. The thickness of two the strand is 2.0 nm (20 Å). The coiling of double strands around each other creates minor grooves and major grooves and the enzymes, histones and antibiotics based on their tendency to combine with DNA, will locate in one of these grooves (Fig. 1-2-1).



A **B**

Figure 1-2-1. Watson - Crick Model for the structure of DNA. (**A**) Cited from Lippincott's illustrated reviews, 5th edition.(**B**) Cited from http://www.discoveryandinnovation.com

In DNA, there are four types of nucleotides, which differ from each other in bases (Fig. 1-2-2). Two are the derivatives of pyrimidine, cytosine (4-amino2-oxopyrimidine, denoted as C) and thymine (5-methl-2,4-dioxypyrimidine, denoted as T). Other two are derivatives of purine, adenine (6-aminopurine, denoted as A) and guanine (2-amino-6-oxopurine, denoted as G). RNA also principally consists of four types of nucleotides but there are two major differences. It contains uracil (2,4-dioxypyrimidine, denoted as U) in replace of thymine and the sugar component is ribose, instead of 2-deoxyribose.

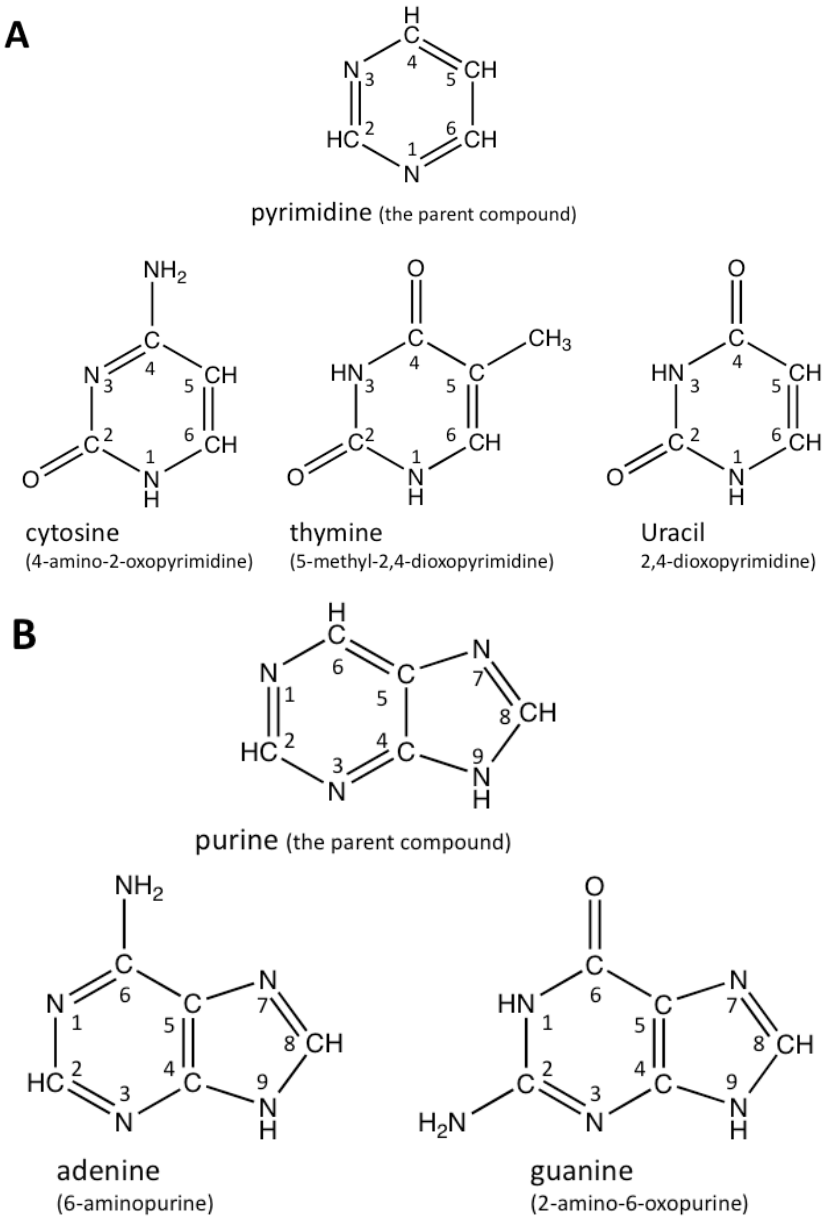


Figure 1-2-2. Structure of Purine (**A**) or Pyrimidine (**B**) bases in DNA .

These bases are paired in a defined way (Fig. 1-2-3). An adenine base on one strand is paired with paired with thymine base on the other strand and vice versa. A guanine base on one strand is paired with cytosine base on the other strand and vice versa. The formation of the pair between the bases is due to the shape and chemical structure of the purine (double-ring base) and pyrimidine (single-ring base) bases. These bases that can match with each other are called complementary bases; adenine and thymine are complementary bases to each other and guanine and cytosine are complementary bases to each other. DNA double strand held together by hydrogen bonds between the complementary bases. Therefore, in DNA molecules, the number or content of adenine is always equal to that of thymine and the number or content of guanine is equal to that of cytosine but the percentage of C+G does not necessarily equal the percentage of A+T. This was found as Chargaff's rule in 1950. There are two hydrogen bonds between adenine and thymine and three hydrogen bonds between two guanine and cytosine (Fig. 1-2-3). Therefore, the connection between guanine and cytosine is stronger than that between adenine and thymine.

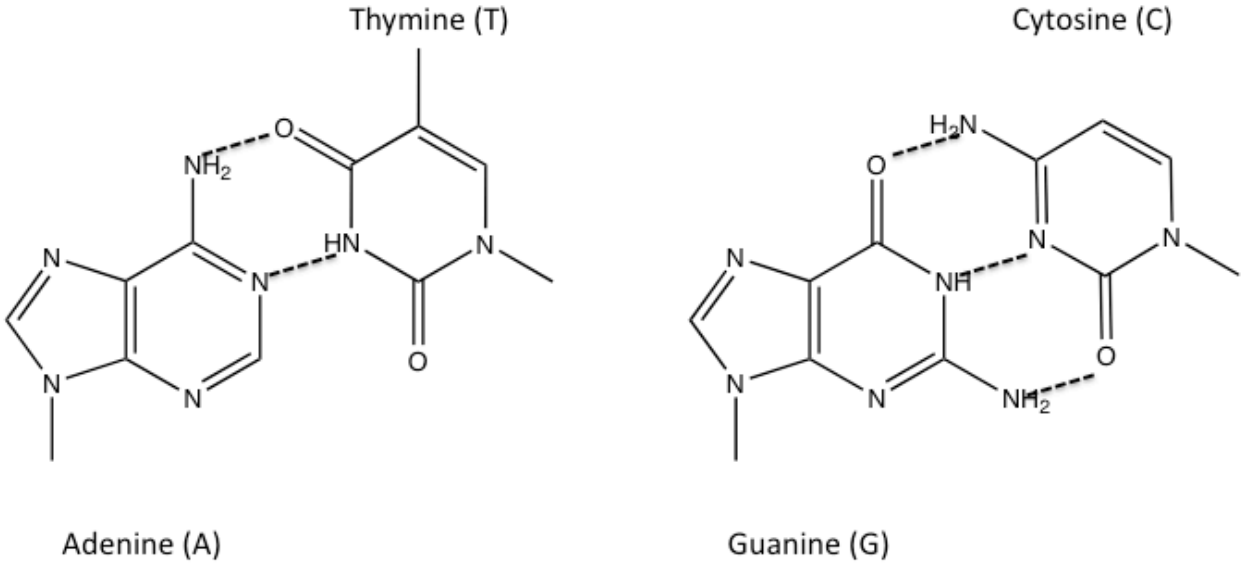


Figure 1-2-3. Association of adenine and thymine and of guanine and cytosine in DNA. Hydrogen bond is shown as dashed line.

1.2.2 Chromosome

In prokaryotes, nucleus practically does not exist, due to the lack of nuclear envelope, and DNA located directly inside the cellular cytoplasm. In eukaryotes, the major part of DNA is located within the nucleus in the shape of chromosomes, whereas mitochondria and chloroplast also carry small-sized DNA.

Nuclear DNA of human beings consists of about 6 billion pairs of nucleotides. The length of opened DNA molecule is approximately 2 meters but this macromolecules in a specific way coiled tightly that can accommodate themselves within a nucleus. Nuclear DNA exists as a complex with proteins, called chromatin. The unit structure, nucleosome, consists of 146 base pairs of DNA with an octameric protein called histone. Chromatin is extended, non-condensed in non-dividing cells and in cell cycle phase other than M phase. In M phase, chromatin is condensed and seen as a piece called chromosome, which is visible under light microscope (Fig. 1-2-4).

Each human somatic cell contains 46 chromosomes. There are 22 pairs of chromosomes, which are homologous to each other in shape and length. These chromosomes are called autosomes. In addition to autosomes, there is another pair of chromosomes, called sex chromosomes. Sex Chromosomes (XX In female and XY in male) contain genes which type of gender in human being depends on them. During the production of gametes, *i.e.*, sperms and eggs, there is a specialized process, called meiosis, where a chromosome from one parent and its homologous chromosome from the other parent join together and undergo crossing over for the homologous recombination to exchange genetic information and segregation so that each daughter cell carry either one of homologous chromosome. As the result, a sperm and an egg respectively carry 23 chromosomes and fuse together to produce a zygote, which carries 46 chromosomes. Through this process, the number of chromosomes in somatic cells in every specific species remains fixed.

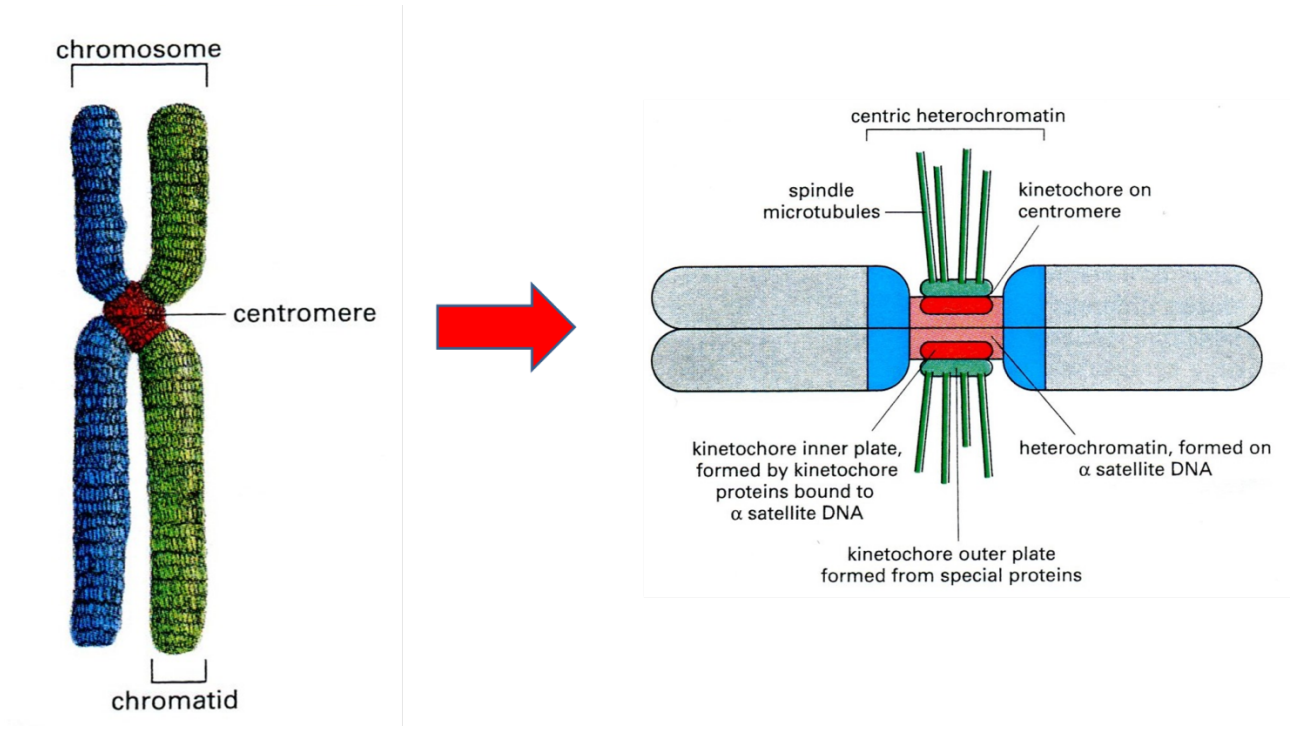


Figure 1-2-4. A typical mitotic chromosome at metaphase and the structure of a human centromere. Cited from Molecular biology of the Cell

1.3 DNA damage, DNA double-strand breaks and its repair

1.3.1 Types of ionizing radiation-induced DNA damage

DNA as macromolecules that carries genetic information is considered the main target of ionizing radiation and consequently important for biological effects such as mutation, cell killing and carcinogenesis. The genetic information damage is a result of replication error, chemical decay, and attack by reactive oxygen species or exposure to ionization radiation.

Ionizing radiation of 1Gy can generate over 500-1000 single-strand breaks, 1000-3000 base damages, 800-1600 sugar damages, 30 DNA-DNA crosslinks, 150 DNA-protein crosslinks, 200-300 Alkali-labile sites and 20-50 double strand breaks in normal human cells (Fig. 1-3-1) (Ward, 1990**).**

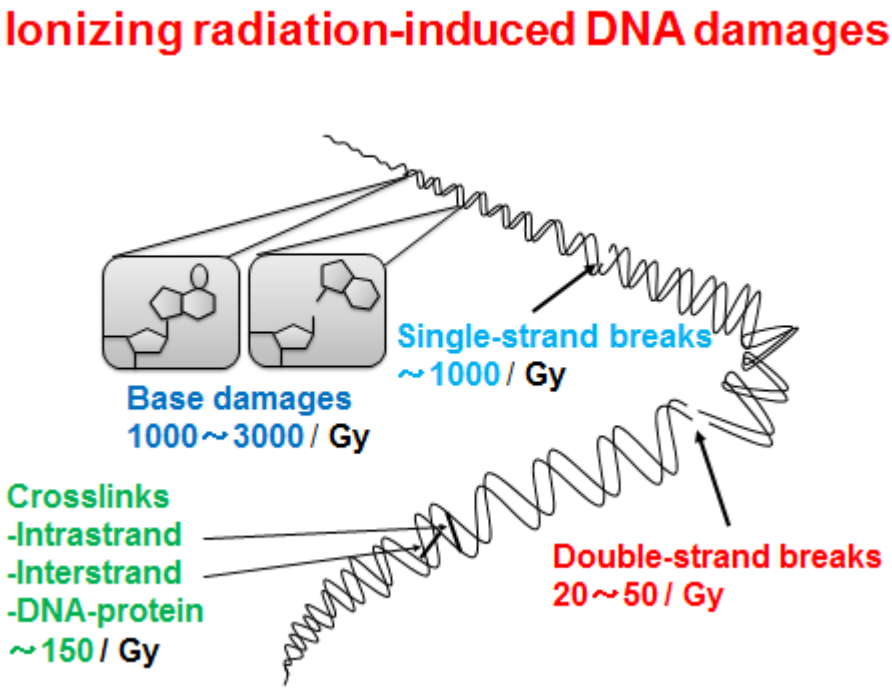


Figure 1-3-1. Types of DNA damages

1.3.2 DNA double-strand breaks

DNA double-stranded breaks (DSBs) are the most cytotoxic DNA lesion which compromises the integrity of the cell. It can lead to genomic instability such as chromosomal dislocation, loss of growth control and tumorigenesis due to defect and the inability to in DSB repair process (Davis et al., 2014; Walker et al., 2001).

DNA double-strand break can be cause by: **A-** exogenous DNA-damaging agents, such as ionizing radiation (IR) or reactive oxygen species **B-** *e*ndogenous cellular processes, such as recombination or replication. Because a DSB can lead to loss or rearrangement of genomic material, pathways for their repairs are critically important for genomic stability. Pathways that are involved in DSB repair are critically important for genomic stability (Dai et al., 2003).

1.3.3 Homologous recombination repair and non-homologous end joining

In a wide range of eukaryotic species, DSBs are repaired by two distinct pathways**:** homologous recombination repair (HRR) and non-homologous end joining (NHEJ) (Fig. 1-3-2).

HRR a mechanism that existence of an undamaged DNA strand such as homologous chromosomes/chromatid as a template is necessary for repairing the damage; therefore, homologous recombination repair is an error-free process. HRR can happen in the late S and G2 phases of the cell cycle if, an undamaged sister chromatid is available in these phases to be used as template.

On the other hand, NHEJ Is the direct re-ligation of the broken double helix chain in DNA without the requirement for sister chromatid or sequence homology. NHEJ is generally thought less accurate than HRR but can be used throughout the cell cycle and is of particular importance in G1 and G0. Also comparison between HRR and NHEJ pathway in DNA double-strand break in normal human cells shows that, the NHEJ pathway is much faster (about 30 minutes and 6 times more efficient) and more efficient compare to HRR pathway (about 7 hours) to the this very fact, 75% Of DNA double-helix break is repaired through NHEJ while the rest repair through HR joining (Mao et al., 2008). In mammalian cells, NHEJ is a major

pathway for the repair of DSBs resulted from DNA-damaging agents such as ionizing radiation. The NHEJ system is also responsible for programmed DSBs in V(D)J recombination and class switch recombination during development of immune diversity and plays an essential role for generating antibodies (van Gent et al., 2001; Walker et al, . 2001; Davis et al., 2014; Ahnesorg et al., 2005; Dahm K. et al., 2008; Hall and Giaccia, Radiobiology for the biologist, 6th Edition).

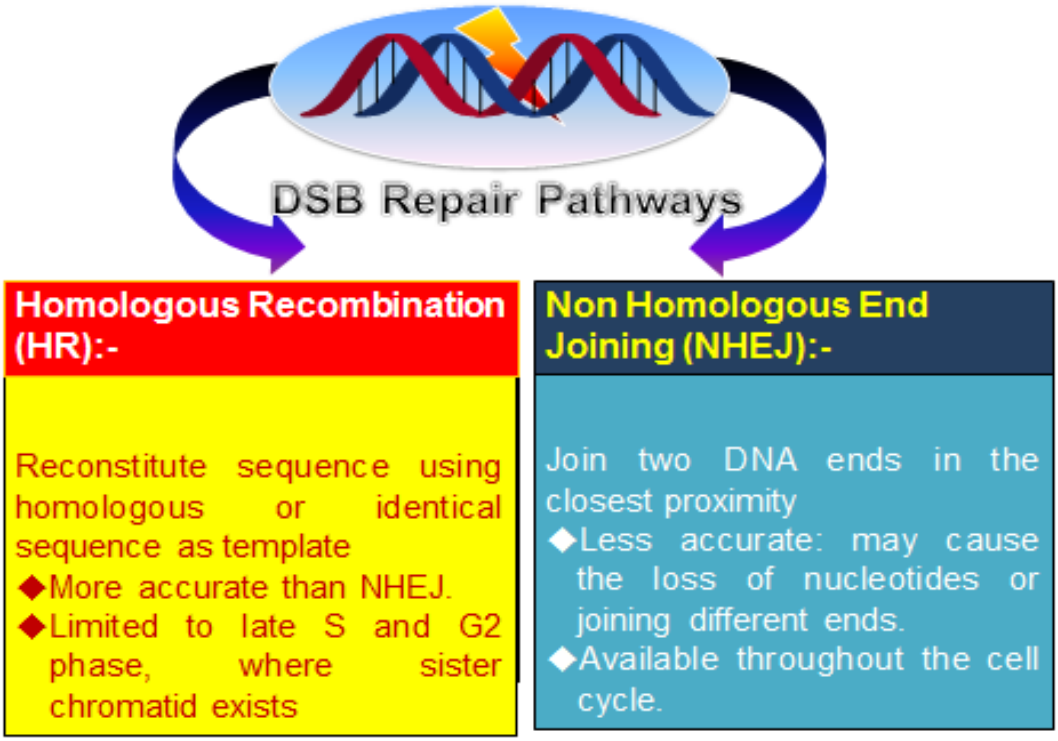


Figure 1-3-2. Comparison between NHEJ and HR pathways

1.3.4 Mechanism of NHEJ

The main NHEJ machinery units which join directly ligatable ends in NHEJ consist of Ku heterodimer, DNA-PKcs, XRCC4, DNA Ligase IV (LIG4) and XRCC4-like factor/Cernunnos (XLF/Cer). Recent studies added a new XRCC4 superfamily member, named PAXX (PAraloge of XRCC4and XLF, also called C9 or f14 as well as XLS for XRCC4-like small molecule), to this list.

NHEJ can be divided into 3 steps: 1. End recognition of broken ends, 2. Processing of DNA and 3. Ligation of products (Fig. 1-3-4).

The broken ends of DNA are recognized by Ku heterodimer, composed of Ku86 (also known as Ku80) and Ku70 subunits, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to the ends of the DNA double-strand break. PAXX interacts directly with Ku and is thought to stabilize the binding of this complex to the DNA end.

End processing occur when DNA is not readily ligatable. Artemis endonuclease cuts incompatible 5' and 3' DNA ends over-hangs. If there is a gap between 5'-end and 3'-end, fill-in synthesis is performed by DNA polymerase μ and DNA polymerase λ. PNKP removes 3'-phosphate group and add phosphate group to 5´-end, when it is missing.

In the final step of NHEJ, ligation of the two ends is accomplished by LIG4 and XRCC4 and XLF play essential regulatory role.

Next, main components of NHEJ are looked into more in detail.

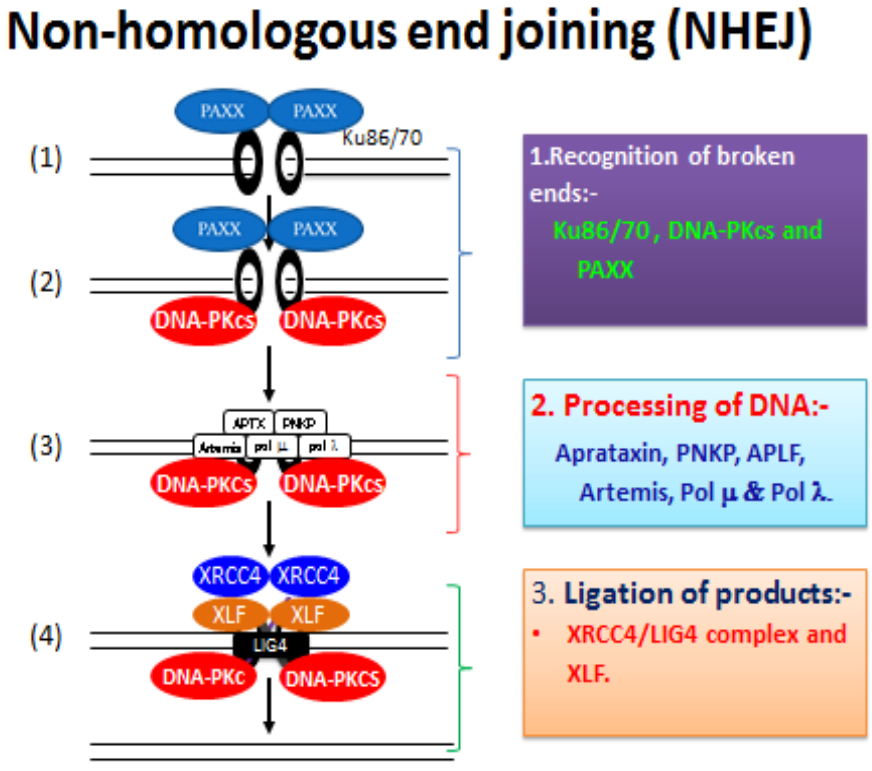


Figure 1-3-4. Non-homologous End Joining (NHEJ) Pathway

1.3.5 DNA-PKcs

DNA-PKcs is a large polypeptide with molecular weight of 469 kDa, which is for the first time purified from HeLa cells (Lees-Miller et al., 1990; Carter et al., 1990). DNA-PK is consisted of 4,128 amino acids, belonging to the phosphatidylinositol-3-OH kinase (PI3K)-related kinase (PIKKs) family (Hartley et al., 1995; Sipley et al., 1995). It is recruited to DSB through its interaction with the C-terminus of Ku80 (Gell and Jackson, 1999; Falck et al., 2005) and, together with Ku70/Ku80 heterodimer, forms the DNA-activated serine/threonine protein kinase, known as, DNA-dependent protein kinase (DNA-PK) complex (Gottlieb and Jackson, 1993).

There are lines of evidence indicating that the kinase activity but not body itself of DNA-PK is indeed required for NHEJ. Strongest support came from the demonstration that catalytically inactive DNA-PKcs, otherwise intact, can restore at most partial NHEJ activity to DNA-PK knockout cells (Kurimasa et al. 1999; Kienker et al. 2000). However, it has not been clear, what is the role of the phosphorylation in NHEJ and what protein should be phosphorylated. DNA-PK inhibitor (Wortmannin, NU7026, NU7441, etc.) sensitizes cells to radiation (Rosenweig et al., 1997; Veuger et al., 2003; Leahy et al., 2004; Tavecchio et al., 2012). Mutation in DNA-PKcs is found in radiosensitive or V(D)J recombination defective cell lines and animals, such as murine severe combined immunodeficiency scid (Biedermann et al., 1991; Blunt et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995).

1.3.6 Ku

Ku is a heterodimer of Ku70, which consists of 609 amino acids, and Ku86, consists of 732 amino acids, and exists 500,000 copies in each human cell (Mimori et al., 1986; Ma et al., 2005). Ku was first identified as an antigen of the auto-antibodies from patients of polymyositis-scleroderma overlap syndrome (Mimori et al., 1981, 1986) and subsequently found to be an essential component of DNA-dependent protein kinase (DNA-PK) (Gottlieb and Jackson, 1993). Ku70 and Ku86 share a common topology and form dyad-symmetrical molecule with a hollow ring-shaped that encircles and binds with duplex DNA in its pore (Gell and Jackson, 1999; Walker et al., 2001).

1.3.7 XRCC4

XRCC4 (X-ray repair cross-complementation group 4) was found as the human cDNA, which could complement the defective V(D)J recombination and radiosensitivity of XR-1 cells derived from Chinese hamster ovary cell (Li et al., 1995). Mice deficient in XRCC4 are associated with radio sensitivity and embryonic lethality (Junop et al., 2000). XRCC4 is likely to be the second NHEJ scaffold molecules and recruits other NHEJ factors to the DSB ends (Davis et al., 2013). XRCC4 is known to interact with other repair proteins such as PNKP (Koch et al., 2004), Aprataxin (Clements et al., 2004) and APLF, which stand for Aprataxin-and PNK-like factor, also as PALF, C2orfl13 or XIP1 (Iles et al., 2007; Kanno et al., 2007).

XRCC4 exist as a homodimer (Junop et al., 2000) (Fig. 1-3-7-1). Equilibrium mixture of XRCC4 in solution consists of dimer and tetramers, but only the dimer can complex with LigIV (Modesti et al., 2003). Conformation of XRCC4 dimer is based on disulfide cross-linking (Leber et al., 1998). A tail-to-tail interaction of the two dimers is shown through tetramer structure in XRCC4 (Junop et al., 2000).

XRCC4 consists of 336 amino acids with three distinct domains (Fig. 1-3-7-2): **A-** A globular N-terminal head domain consist of amino acids 1–115. Serine 53 site (S/T-Q motif) located in this domain**.** XLF binding with XRCC4, occur at this domain. **B**- A long coiled-coil C-terminal or stalk of the L subunit, started from amino acids 119–203. The stalk domain mediates interaction of XRCC4 with LIG4 between amino acids 173 to 195. Dimerization and tetramerization of XRCC4 occur between amino acid 119-137. **C-** C terminal domain consists of amino acid 204–336 (334). Ser260, which is one of the focus of this study, is located in the C-terminal region of XRCC4. It may be added that it is close to caspase-cleavage site (Asp265) (Sunatani et al., 2018) and nuclear localization signal (270-275) (Fukuchi et al., 2015). XECT domain (XRCC4 extremely C-terminal domain) located between amino acids 319-336 and highly conserved among vertebrates but absent in invertebrate, plants and yeast (Wanotayan et al., 2015). Ser320, which is another focus of this study, is located in XECT region.

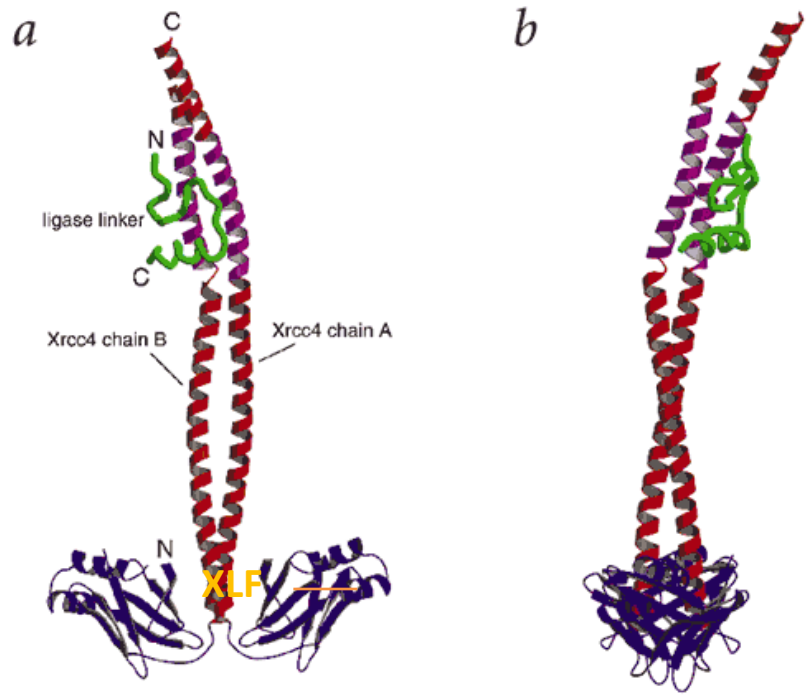


Figure 1-3-7-1. Overall architecture of the XRCC4 complex.

Cited from: Bancinyane L. ,2001.

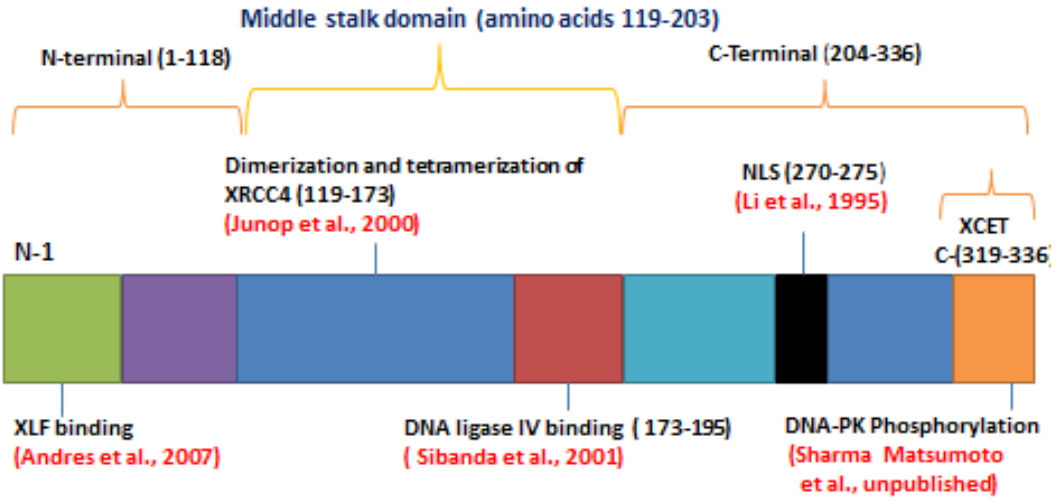


Diagram of full length XRCC4 with interacting domains

Figure 1-3-7-2. Schematic diagram of human XRCC4 structure.

Human cells contain two isoforms of XRCC4, that differ only in a small two amino acids insertion and a conservative change of lysine to arginine at amino acid 298 of hXRCC4 isoform (Fig. 1-3-7-3). Human XRCC4 (isoform 1) contain 334 amino acids while hXRCC4 (isoform 2) contains 336 amino acids (Yu et al., 2003). In this study, isoform 2 was used entirely, because it is the form initially isolated from human T cell leukemia MOLT-4 cells and shown to be functional (Kamdar and Matsumoto, 2010).

Figure 1-3-7-3. The acid sequence of human XRCC4 isoform 1 and 2.

1.3.8 DNA ligase IV (LIG4)

DNA ligase IV (LIG4) is one of three types of ATP-dependent DNA ligase enzymes found in mammals. LIG4 is exclusively required for the Ku-dependent NHEJ pathway of DSB repair and that other DNA ligase such as Ligase I and Ligase III do not substitute for this function (Adachi et al., 2001). LIG4 is initially found in the purification of DNA ligase III from HeLa cell nuclear extracts (Robins et al., 1996) and subsequently found to be associated with XRCC4 (Critchlow et al., 1997; Grawunder et al., 1997).

LIG4 consists of 911 amino acids and four conserved motifs (designated I, III, IV, and VI) have been identified among DNA ligases. Motif I, containing a critically important lysine residue, forms the active site loop of the enzyme and comprises part of the ATP binding pocket. A standard ligation by DNA ligase usually contains 3 irreversible steps. 1- adenylate group is transferred from ATP or NAD^+ to the active site of DNA ligase for adenylation. 2- This adenylated DNA ligase transfers the adenylate group to 5' end of broken DNA. 3 end to 5' D- Finally the unadenylated DNA ligase catalyzes the nucleophilic attack by 3' end to 5' DNA adenylated, resulting a release of AMP (Ellenberger et al., 2008). Structure of DNA ligase IV can be divided into two regions: **A**-catalytic and **B-**the interaction regions. The catalytic region of DNA ligases among human being, is conserved and contains three parts: 1- DNA binding domain, (DBD), 2-the nucleotidyl transferase domain (NTase) and 3- the OB-fold domain (OBD) (Tomkinson et al., 1998; Ochi et al., 2013) (Fig. 1-3-8-1).

Following the N-terminal core of DNA ligase IV is a large C-terminal region containing a tandem repeat of BRCT domains, a structural motif that is commonly found in proteins involved in DNA repair and the signaling of DNA damage (Fig. 1-3-8-2) and (Fig.1-3-8-3. It is this also C-terminal region that is responsible for binding to XRCC4 (Sibanda et al., 2001). This interaction occurs at a conserved binding site located within a short linker sequence of around 100 amino acids between the two BRCT domains (Fig. 1-3-8-2 and Fig. 1-3-8-3) (Martin et al., 2002).

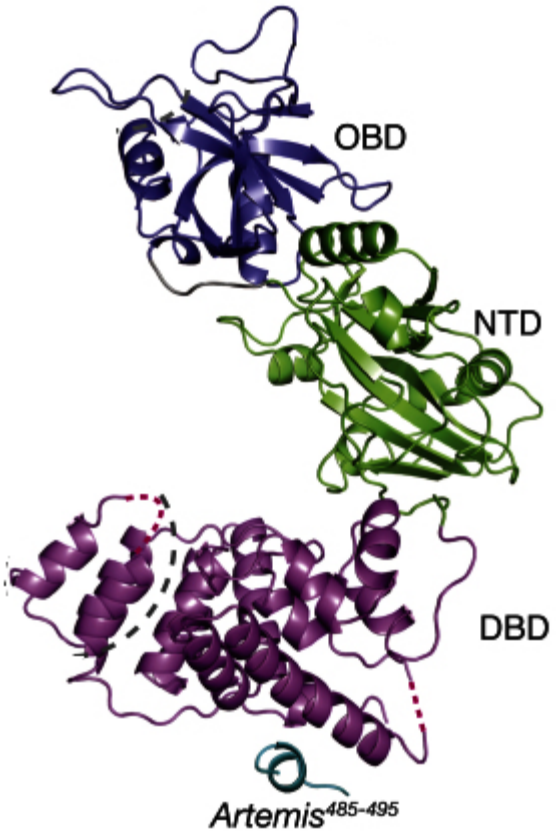


Figure 1-3-8-1. The catalytic region of LIG4 (Ochi et al., 2013).

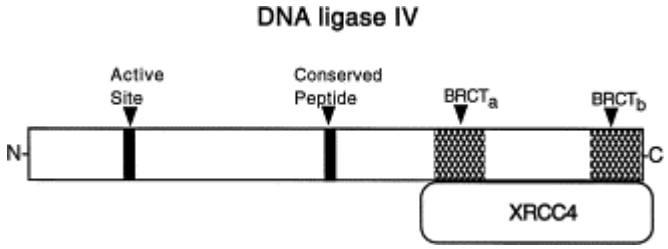


Figure 1-3-8-2. Schematic diagram of the DNA ligase IV–XRCC4 complex.

Cited from: Structure and function of mammalian DNA ligases.

BRCT domains stands for Breast Cancer Associated 1 C-terminal and have been found in many proteins involved in DNA damage response, such a 53BP1, Rad 9, XRCC1 and etc., participating in binding of phosphorylated and non-phosphorylated proteins with DNA in difference (Bork, 1997). BRCT sequence contains two highly conserved motifs. Motif-1 consists of a conserved Gly-Gly/Gly-Ala pair and Motif-2 consists of a highly conserved Trp-X-X-X-Cys/Ser motif (Huyton et al., 2000). A protein can have one BRCT domains, double or even multiple BRCT domains, and giving to the BRCT domains a diversity in structure and function (Leung et al., 2011).

It is considered that not BRCT domains in Ligase IV themselves but rather the linker between the domains that mediate the important interaction with XRCC4. However, our laboratory showed that BRCT domains are essential to drive XRCC4 to chromatin (Liu et al., 2013). There are some reports that of Ligase IV mediate the direct interaction with Ku (Constantine et al., 2007) and the BRCT2 domain is also necessary for stable binding to XRCC4 (Wu et al., 2009). The α2 helix of Ligase IV BRCT1 domain is responsible for targeted degradation by adenovirus infection (Gilson et al., 2012)

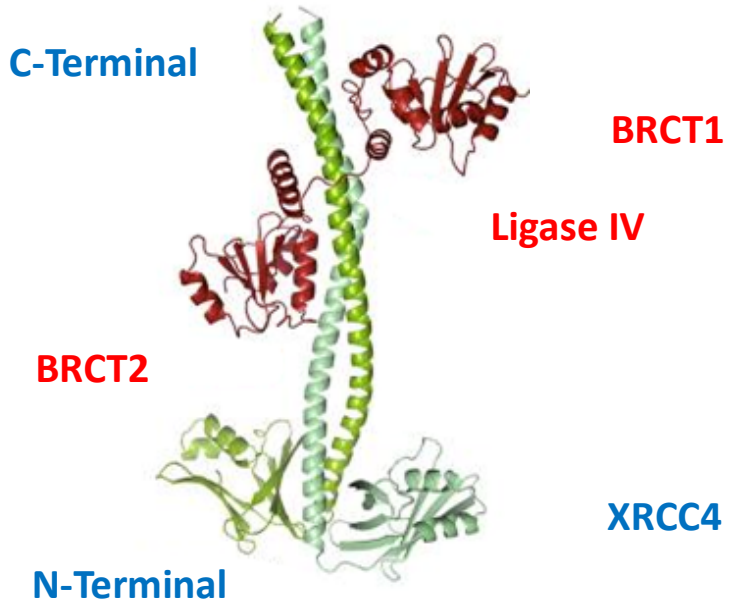


Figure 1-3-8-3. Structure of XRCC4 dimerization and its binding with Ligase IV and BRCT1 and BRCT2 (Wu et al., 2009).

1.3.9 XLF

XRCC4-**l**ike Factor (XLF), also known as Cernunnos, in human with a molecular weight of 33 kDa consists of 299 amino acids and is encoded by a gene located on chromosome 2q35 (Ahnesorg et al., 2006; Buck et al., 2006). Derivation name of XRCC4-like factor, is due to similarity in the tertiary structures with XRCC4, despite the rather low sequence homology between these proteins (Ahnesorg et al., 2006; Callebaut et al., 2006). Although in the most studies the term XLF are commonly used, however NHEJ1 is an official symbol for the human Cernunnos/XLF gene is registered as NHEJ1 (non-homologous end-joining factor 1) in GenBank. In vitro, purified XLF at the final ligation step in NHEJ, can interact and stimulate the purified XRCC4/DNA ligase IV complex in a direct assay for ligation activity (Lu et al., 2007; Yano et al., 2009).

Cernunnos/XLF (Fig. 1-3-9) has an N-terminal globular head domain followed by a coiled-coil region and C-terminal helices. 1- N-terminal globular head domain. XLF and XRCC4 interact through their globular head regions with each other (Deshpande and Wilson 2007). Quick respond is a characteristic of XRCC4-like factor/Cernunnos/XLF at an early stage of DSBs. After Ionizing radiation in living cells resulting the XRCC4-DNA Ligase IV complex accumulate enough amount of XLF/Cernunnos at DNA damage sites through interaction between the XRCC4 and XLF/Cernunnos head domains dependent on components of the DNA-PK complex Existence of Ku is essential for recruitment of XLF to DSBs because Ku-XLF interaction occurs on DNA, due to stimulation of XLF by Ku, causing XLF, binding to DNA. In another word, XLF is Ku-dependent but recruitment of in DSBs is XRCC4-independent (Dahm et al., 2008; Yano et al., 2008, 2009). N- terminal globular head domain contain four α-helices known as αA, αB, αC and αD. Two sets of antiparallel β-sheets known as β-mender. α A and α C are the binding site in the head region of XLF that can interact with the XRCC4 head region. 2- Stalk or coiled-coil region. This region provides a tight interaction between two monomeric Cernunnos/XLF. The coiled-coil (stalk) of The XLF in comparison to that of XRCC4 is much shorter and does not contain an equivalent region to the XRCC4-Ligase IV-binding site. 3- C-terminal region. From the reverse direction of coiled-coil region in XLF, a short distorted four helical bundle that is located between the coiled-coil and head domain of XLF originated. This part of XLF is known

as C-terminal helices region. Comparing C-terminal of Cernunnos/XLF with XRCC4 this region is the compact and folded structure but XRCC4 has extended C-terminal helices and serve as the Ligase IV-binding site. Existence of 10 amino acids located at the C-terminal of XLF protein plays an important role for interaction with Ku heterodimer and for recruitment to double-strand break (Yano et al., 2009).

Cernunnos/XRCC4-like factor/XLF, stimulates ligation of cohesive and non-cohesive broken DNA ends by XRCC4-DNA Ligase IV complex. The ligation activity of DNA ends involves the carboxy-terminal DNA binding region of XLF/Cer and can happens through different, non-exclusive ways such as**: A-** Better stability of the XRCC4-DNA Ligase IV complex on DNA ends by XLF/Cernunnos, **B-** Modulation of the efficiency and/or specificity of DNA Ligase IV by binding of XLF/Cer to the XRCC4-DNA Ligase IV complex**, C-** promotion of the alignment of blunt or other non-complementary DNA ends by XLF/Cer for ligation. XLF/Cer promotes the preservation of 3' overhangs, restricts nucleotide loss and thereby promotes accuracy of DSB joining by XRCC4-DNA Ligase IV during NHEJ and V(D)J recombination (Dahm et al., 2008). XLF not only stabilizes LigIV/XRCC4 in broken DNA ends, but also enhance the LigIV/XRCC4 end-joining process (Sibanda et al, 2001, 2010; Li et al., 2007; Brewerton et al., 2004).

The helical region of Cernunnos/XLF forms a folded structure that wraps back to the N-terminal globular head domain. In the XRCC4/ DNA ligase IV / XLF complex, the Ligase IV and XRCC4 can bind to unfolded C-terminal of XLF molecules. In this complex, there are two DNA ligase IV polypeptide molecules exist. DNA-Ligase IV BRCT- Linker polypeptide domain region is capable of binding to XRCC4 coiled-coil region, and then folded Cernunnos/XLF known as cone-shaped, can bind to the XRCC4 through its head domains region. Under physiological conditions, Cernunnos/XLF exists as a homodimer and does not form a heterodimer with XRCC4. Therefore XRCC4 and XLF by forming a homoodimer are able to bind to DNA ligase IV polypeptide in the coiled-coil region (Andres et al., 2007; Yi et al., 2007; Hentges et al., 2006; Li et al., 2008; Dahm et al., 2008).

DNA binding activity of purified XLF factor, dependence on the length of DNA molecules in regards with orientation of the C-terminal alpha helices parallel of XLF to the DNA helix (Lu

et al., 2007). XLF is thought to support LIG4 activity toward incompatible or mismatched DNA ends (Tsai et al., 2007; Gu et al., 2007).

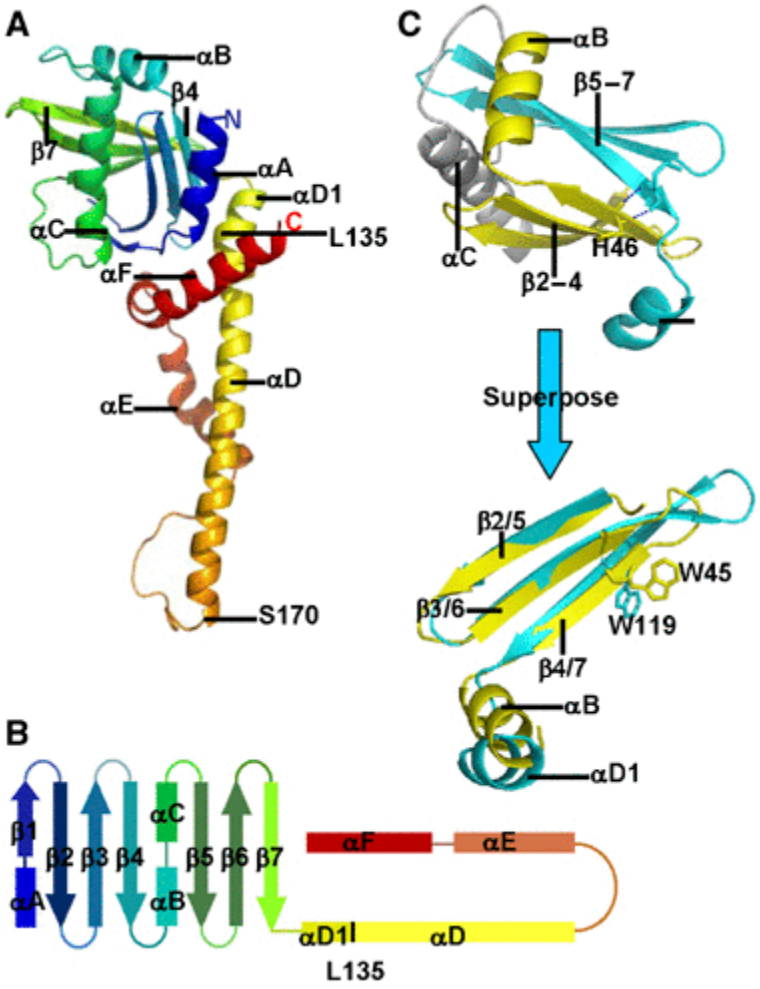


Figure 1-3-9. The XLF crystal structure. (**A**) The structure of the XLF protomer. (**B**) Topology diagram of XLF protomer, secondary structure elements are in the same color as (A). (**C**) Superposition of β2, β3, β4, αB (yellow) to β5, β6, β7, αD1 (cyan). β strands overlap well, and α-helices are in similar orientations. W45 and W119 are found at the topologically equivalent positions (Yi et al., 2008).

1.3.10 Phosphorylation of XRCC4 by DNA-PK

XRCC4 is phosphorylated by both DNA-PK and other protein kinases *in vitro* within carboxyl-terminla 130 amino acids (Leber et al., 1998; Modesti et al., 1999 and Critchlow et al., 1997). DNA-PK has been shown to phosphorylate XRCC4 also in living cells in response to treatment with ionizing radiation or a DSB-inducing agent in a manner dependent on DNA-PKcs (Matsumoto et al., 2000; Drouet et al., 2005).

In 2003 and 2004 two papers by (Yu et al., 2003; Lee et al., 2004) reporting the identification of Ser260 as well as Ser320 (termed Ser318, reflecting the alternatively spliced form) as major phosphorylation sites in XRCC4 by purified DNA-PK *in vitro* through mass spectrometry (Fig. 1-3-10). Our laboratory has identified four other phosphorylation sites on XRCC4 by DNA-PK, Ser53, Ser327, Ser328 and a threonine (Fig. 1-3-10). Our laboratory also showed that XRCC4 Ser320 phosphorylation is mainly mediated by DNA-PK in living cell (Sharma et al., 2016) .

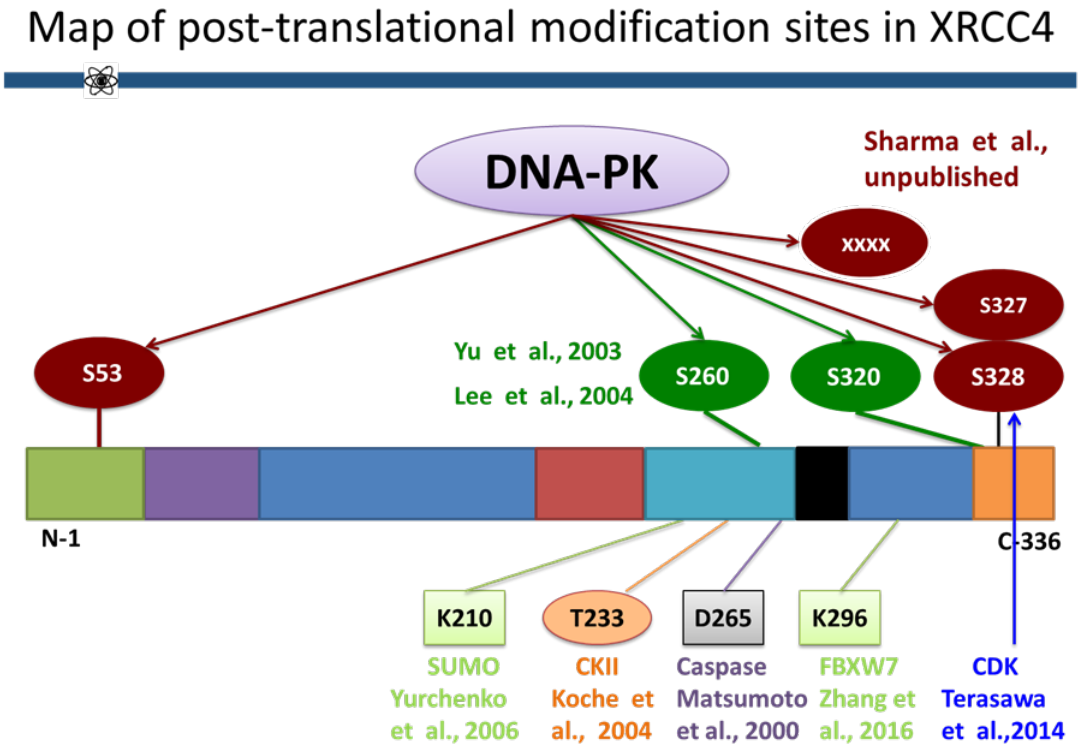


Figure 1-3-10. Map of post-translational modification sites in XRCC4.

1.4 Purpose of this study

Ionizing radiation is thought to exert various effects on biological systems, including human body, through generation of DNA damages. Among various DNA damage, which can be induced by ionizing radiation, DSB is thought the most critical type. In eukaryotic organisms, DSB is mainly repaired through two pathways: homologous recombination and non-homologous end joining (NHEJ). In NHEJ, Ku86, Ku70 and DNA-PKcs recognize DSB and DNA ligase IV ligates two DNA ends in cooperation with XRCC4 and XLF. There are several lines of evidence indicating that the protein phosphorylating activity of DNA-PKcs is essential for NHEJ, but the targets and significance of phosphorylation remain to be clarified.

XRCC4 is shown to undergo phosphorylation by DNA-PK in response to DNA damage by our group and others. Moreover, our group and other group have identified at least six phosphorylation sites in XRCC4 by DNA-PK. Ser260 and Ser320 were initially identified as the major phosphorylation sites but the biological significance was not clear. It was also unclear whether these sites were the sites for phosphorylation in response to DNA damage in cell. Our group recently generated a phosphorylation-specific antibody toward XRCC4 Ser320 and successfully demonstrated its phosphorylation by DNA-PK in response to DNA damage.

This study is aimed to clarify the regulation of XRCC4 through phosphorylation at Ser260 by DNA-PK. Toward this aim, an antibody, which specifically reacts with XRCC4 phosphorylated at Ser260, was generated and the phosphorylation status of this residue in cells with or without DNA damage was examined. In addition, cell lines, expressing mutant XRCC4, in which Ser260 was replaced by alanine, to disable phosphorylation, or by aspartic acid, to mimic phosphorylation, were generated and their DNA repair function was examined.

CHAPTER 2

Experimental procedures

2.1 Cell Culture

2.1.1 General Principles of Cell Culture

Cell culture refers to the cultivation and growth of cells in a favorable artificial environment after their removal from animals or plants (directly removed from tissues) or those cells that has already been established (cell line or cell strain).

Cell culture starts from isolating the cells tissue, under appropriate and suitable condition for growth and proliferation of that type of cell, up to the time, when they occupy entire available substrate. This stage of cultivation refers to primary culture. At this point, by transferring the cells to new plates containing new fresh growth medium, cells are able to continually grow. This process is called passage or subculture. The life span of cell line, that is originated from normal, *i.e.*, non-cancerous tissue, is usually limited, which is known as Hayflick's limit. On the other hand, immortal cell lines refer to group of cells that have capability to grow and divide infinitely and unlimitedly. They are derived from cancerous tissues as well as those cells, which are transformed *in vitro*. The consistency and reproducibility are the two major and important advantages of using cell lines.

The type of the tissue culture medium is one of the most important elements that have great influence on the growth of the cells. Therefore, the type of medium for different type of cells must be selected carefully. In adherent culture, when all the available substrate (surface) is occupied by the cells or when there is no room left for expansion cells or in suspension cultures when the number of the cells exceeds the capacity of the medium to support their further growth, the proliferation of the cells is greatly reduced or even completely stopped. Therefore, to avoid such effect and in order cells, continued growth, and remain at an optimal density, as well as to stimulate further proliferation, it is important the cell culture divide (subculture), either into the culture flasks or into the culture plates and they should supply with fresh medium.

Growth of cells in culture contains three major phases that need to be considered: (1) lag phase, (2) logarithmic phase and (3) stationary phase (also termed plateau phase or confluency). After the culture is seeded, the first phase of growth is the lag phase, where cells

are trying to adapt themselves with culture environment and preparing themselves for fast proliferation. Sometimes, cells require growth factors for proliferation, which they secrete by themselves, and, therefore, proliferation is slow when the cell density is low. Thus, during the lag phase, there is a slow proliferation of cells, if any. In logarithmic phase, cells proliferate exponentially and rapidly. As the number of the cells increases, the nutrients in the growth medium are gradually exhausted and the available substrate (surface) is fully occupied. Then, cells reach the stationary phase, where the proliferation of the cells is greatly reduced or completely stopped, or begin to die sometime after reaching the stationary phase.

2.1.2 Cell Lines Used in This Study

In this study, two types of cell lines were used. Human cervical carcinoma-derived HeLa cells, named after the patient Henrietta Lacks, is the first immortal cell line of human origin, that can be grown in the laboratory (Puck and Markus, 1956). HeLa cells were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% (v/v) of calf bovine serum (CBS, Hyclone; Logan, UT, USA), which had been heated at 37°C for 30 min, and 1% (v/v) of Penicillin-Streptomycin Mixed Solution (100 Unit/ml of penicillin and 100 μg/ml streptomycin; Nacalai Tesque) at 37°C in a humidified atmosphere controlled to contain 5% CO_2.

M059K and M059J were established from the same biopsy specimen of a glioma patient. However, M059J lacks DNA-PKcs expression, while M059K expresses normal DNA-PKcs (Lees-Miller et al., 1995). These cell lines provide a useful model system, that helps studying the role of DNA-PKcs. M059K and M059J were obtained from American Type Culture Collections (ATCC). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) /Ham's F-12 medium supplemented with 10% CBS, which had been heated at 37°C for 30 min, and 1% (v/v) of Penicillin-Streptomycin Mixed Solution (100 Unit/ml of penicillin and 100 □g/ml streptomycin; Nacalai Tesque) at 37°C in a humidified atmosphere controlled to contain 5% CO_2.

2.1.3 Cell count

To perform accurate quantity experiments, knowing the concentration of the cells in cell culture is critically important. I measured the concentration of cells by using automated cell counter, Scepter (Millipore; Billerica, MA, USA), or hemocytometer (ASONE, Tokyo, Japan). Cell counting using Scepter followed manufacturer's protocol. Disposable tips with aperture diameter of 60 μm were used. Cell size gate was set 10 - 25 μm for HeLa cells and 12-36 μm for M059K and M059J cells.

Hemocytometer is a thick glass slide with a grid with defined interval. There is also footplates so that putting a thick, even- surfaced, coverslip makes a defined depth of space between the surface of the glass slide and the covership. The dimension of hemocytometer used in this study is shown in Figure 2-1. The volume of smallest square is:

$$\textbf{height } 0.25 \text{ mm} \times \textbf{width } 0.25 \text{ mm} \times \textbf{depth } 0.2 \text{ mm} = 1.25 \times 10^{-2} \text{ mm}^3.$$

Therefore, cells in 16 squares surrounded by thick lines were usually counted and multiplied by 5,000 to obtain number of cells per ml.

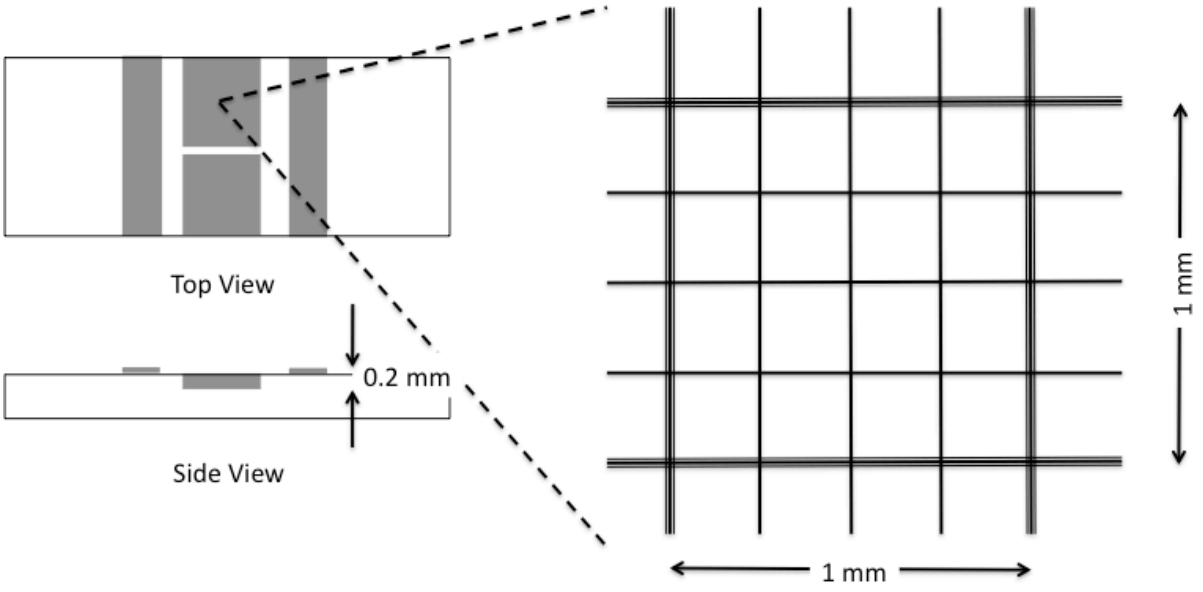


Figure 2-1. The dimension of hemocytometer.

2.1.4 Passage (Subculture)

Passage, or subculture, of cells must be done before, or not a long time after, the cells reach to confluency. At this point, neglect and avoid in subculturing the confluent cells, has direct and severe effect, such as reduction in mitotic index and finally cells death. Detaching cells by treatment with trypsin (trypsinization) from the surface of primary culture plate, is the first main step in subculturing monolayers. Trypsin digests cell surface proteins, like integrins, which mediate cell-cell or cell-substrate adhesion. EDTA, a chelating agent of divalent cations, is used simultaneously because a subset of cell adhesion molecule, cadherin, requires calcium for adhesion. The second step is to subdivide the resultant cell suspension into fresh cultures and these secondary cultures should be constantly checked for the quality of their growth and fed. Since the medium usually contains phenol red as the pH indicator, the color of medium is red at the beginning and turns to orange and then to yellow, as the cell proliferates. Orange color is an indication of good metabolic growth and yellow color indicates the accumulation of lactate.

Figure 2-2. Incubator used cell culture in this study

The procedure of passage of HeLa cells is as follows. Prior to passage, culture medium (see above), Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) and 0.25% Trypsin-1 mM EDTA solution (Nacalai Tesque) were kept in water bath set at 37°C, as they are usually stored at 4°C. First, the culture medium was removed by aspiration using pipet. For a 10 cm-plate, 4 ml of HBSS was poured onto the dish and sucked immediately. Then 2 ml of Trypsin-EDTA solution was poured onto the plate and sucked immediately.

The plate was kept at 37°C for ~3 min Incubator (Fig. 2-2) until cell detached from the surface and looked round. Cells were collected by 2 to 4 ml of pre-warmed medium and transferred into a 15 ml-conical tube. Ten ml of culture medium and aliquot of cell suspension was poured onto each fresh plate. Typically, the cell number at the time of passage is $5X10^5$ to 10^6 per plate. As the doubling time of HeLa cell was 21-24 hours and the cell number at the confluency is $8X10^6$ to 10^7 per plate, passage was done every 3 to 4 days.

The procedure of passage of M059K and M059J cells is principally the same to above (Fig. 2-3). However, the doubling times of M059K and M059J cells were ~30 hours and the cell number at the confluency is $2X10^6$ to $3X10^6$ per plate.

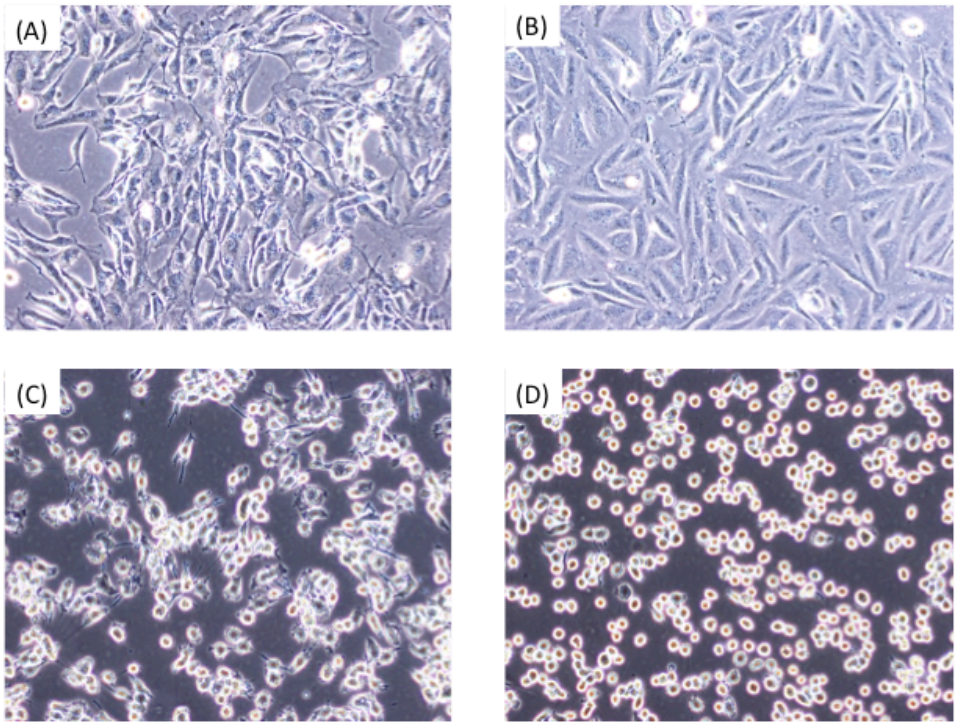


Figure 2-3. Observation of M059K and M059J cells by phase contrast microscopy. (A) and (B): M059K cells, (C) and (D): M059J cells. (A) and (B): before treatment with trypsin, (C) and (D) during treatment with trypsin.

2.1.5 Freeze Stock of Cells

Cell lines can be kept frozen for long time storage at -70^{o}C or below to avoid contamination and senescence or to minimize the effect of genetic changes. To protect cells from damage and death by avoiding formation of ice crystals in the them, temperature is gradually lowered and cryoprotective agents, such as DMSO (dimethylsufoxide), are used.

The procedure of preparing freeze stock of cells is as follows. Cells in the logarithmic phase are harvested from culture plates by treatment with Trypsin and EDTA and collected into a 15 ml-conical tube. The tube was centrifuged at 1,200 rpm (290 g) for 5 min and the supernatant was removed by aspiration. The cell pellet was resuspended at the concentration of 2-4X10^{6} cells/ml in freezing medium, which includes culture medium supplemented with 15% (v/v) of fetal bovine serum (FBS) and 10% DMSO. Alternatively, Cell Reserver ONE (Nacalai Tesque) was used as the freezing medium. Then 1 ml of cell suspension was dispensed into 1 ml

cryotube and stand on ice for 15-30 min. Cryotubes were transferred to -85°C freezer and next day or later to liquid nitrogen container.

Cryopreserved cells should be thawed as fast as possible and plated at high concentration to optimize the survival rate and recovery. The process of fast thawing would protect the cells from formation of ice crystals, which cause cell lysis that can lead to cell death.

The procedure of thawing freeze stock of cells is as follows. Prior to start cell thawing, culture medium were warmed in the water bath to 37°C. Cryotube was kept on dry ice for more than 30 min to avoid burst of the tubes due to abrupt evaporation of nitrogen. Then the cryotube was soaked into the water bath at 37°C to thaw it quickly, usually within a minute. Before opening the cryotube, the cover was sterilized with 70% alcohol and wiped. Then the content of the cryotube was transferred to a 15 ml-conical tube, into which 10 ml of warmed culture medium had been added. Tube was centrifuged at 1,200 rpm (290 g) for 5 min and the supernatant was removed by aspiration. Then the cell pellet was suspended in 5 or 10 ml of culture medium and poured onto 10 cm plate or 25 cm^2 flask.

2.2 γ-ray Irradiation and Treatment with Chemicals

2.2.1 γ-ray Irradiation

Cells were irradiated using ^{60}Co γ-ray source, which emits the γ-ray of 1.17 MeV and 1.33 MeV, with a half-life of 5.3 years. The tubes containing cells were stood in an acryl holder with a wall thickness of 1 cm. To obtain high doses, tubes were stood at the center of the source. The dose rate was measured using ionizing chamber–type exposure dose rate meter C-110 (Oyo Giken, Tokyo, Japan) and calculated as follows:

Dose rate (Gy/min) = Read of dose rate meter (Roentgen)
X 1.049 (Correction and conversion factor)
X (Temperature (K)/295) X (1013/Atmosphere (hPa)).

Furthermore, the delay time during the ascending and descending the irradiation source was corrected. Decay was corrected by calculation and also by actual measurement with interval of 1-2 months. The dose rate at the center was 1.91 Gy/sec and the delay time was 18 seconds at February 3, 2014, as an example.

Prior to irradiation, appropriate number of cells were recovered from plastic dishes and transferred into 15 ml-conical tubes. Note that the cells were in logarithmic phase. After irradiation, the test tubes were placed in 37°C water bath for 30 minutes and then transferred onto ice, to slow down the metabolism including repair. The tubes were centrifuged at 1,200 rpm (290 g) for 5 min at 4°C and the supernatant was aspirated. The cell pellet was suspended in 5 ml of ice-cold Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) and centrifuged at 1,200 rpm (290 g) for 5 min at 4°C. The supernatant was again aspirated. The pellet was suspended in 1 ml of PBS and transferred into 1.5 ml microtube. Then the tube was centrifuged at 3,300 rpm (1,000 g) for 5 min and supernatant was removed. The cell pellet was processed for SDS-PAGE or biochemical fractionation (see below) or kept at -85°C until use.

2.2.2 Zeocin

Zeocin (Figure 2-4) is a family member of structurally complex related antibiotics of bleomycin and phleomycin. Zeocin antibiotic can be isolated from culture of *Streptomyces verticillus* mutant. Zeocin and its family are soluble in the water giving a blue color to the solution because these antibiotics exist as the copper-chelated form.

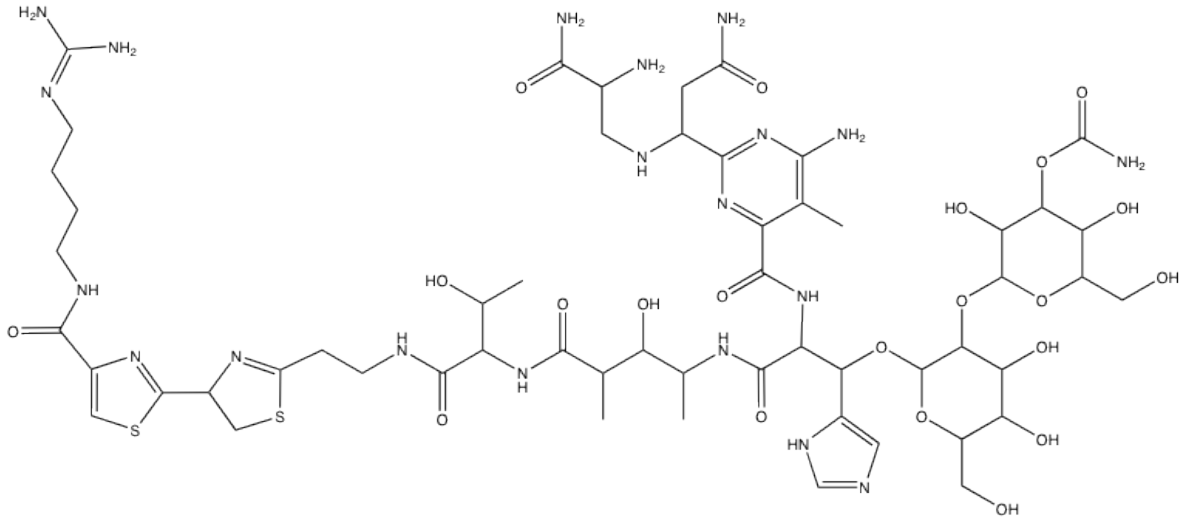


Figure 2-4. Structure of Zeocin. Formula is $C_{55}H_{86}N_{20}O_{21}S_2$. IUPAC name is "2-({2-[2-{[(6-Amino-2- {3-amino-1- [(2,3-diamino-3-oxopropyl)amino] -3-oxopropyl} -5-methyl-4-pyrimidinyl) carbonyl] amino} -3- [(5- {[1- ({2- [4- ({4- [(diaminomethylene) amino] butyl} carbamoyl)-4', 5'-dihydro-2, 4'-bi-1,3-thiazol-2'-yl] ethyl}amino) -3-hydroxy-1-oxo-2-butanyl] amino}-3-hydroxy-4-methyl-5-oxo-2-pentanyl) amino] -1-(1*H*-imidazol-5-yl) -3-oxopropoxy]-4, 5-dihydroxy-6- (hydroxymethyl) tetrahydro-2*H*-pyran-3-yl} oxy) -3, 5-dihydroxy-6-(hydroxy methyl)tetrahydro-2H-pyran-4-yl carbamate". Drawn using ChemBioDraw Ultra Ver 12.0.

Zeocin causes cell death through inducing DNA cleavage due to its ability to bind to DNA through its amino–terminal peptide and by intercalation of planar bithiazole-containing moiety between DNA bases. DNA cleavage is due to the formation of an active complex between the metal ion chelating portion of the molecule with iron II and molecular oxygen, and the activated complex generates free radicals that are responsible for cleavage of DNA chain. Plasma membrane of the cells also can be perturbed by Zeocin.

Cytotoxic effect of Zeocin can be counteracted by resistance genes, such as *ble* gene from Streptoalloteichus hindustanus. This gene encodes small proteins of 13.6 kDa that can bind to Zeocin and inhibit DNA cleavage. Thus, Zeocin is frequently used as a marker for selection in gene transfection experiments.

Zeocin can kill the majority of the aerobically growing cells in concentration range of 0.5 to 1,000 μg/ml. However, it is important to note that the sensitivity of cells is pH dependent. In mammalian cell lines, the working concentration of Zeocin can be as low as 20 μg/ml or as high as 1,000 μg/ml but in most cases it is between 50 to 400 μg/ml.

In this study, treatment with Zeocin was done as follows. Immediately before the addition of Zeocin, the culture medium was aspirated and replaced with fresh medium. Zeocin (InvivoGen, San Diego, CA, USA) was added at the final concentration of 30-300 μg/ml. Cells were harvested 18 hours after the addition of Zeocin. Procedure for harvesting cell is the same as that after γ-ray irradiation.

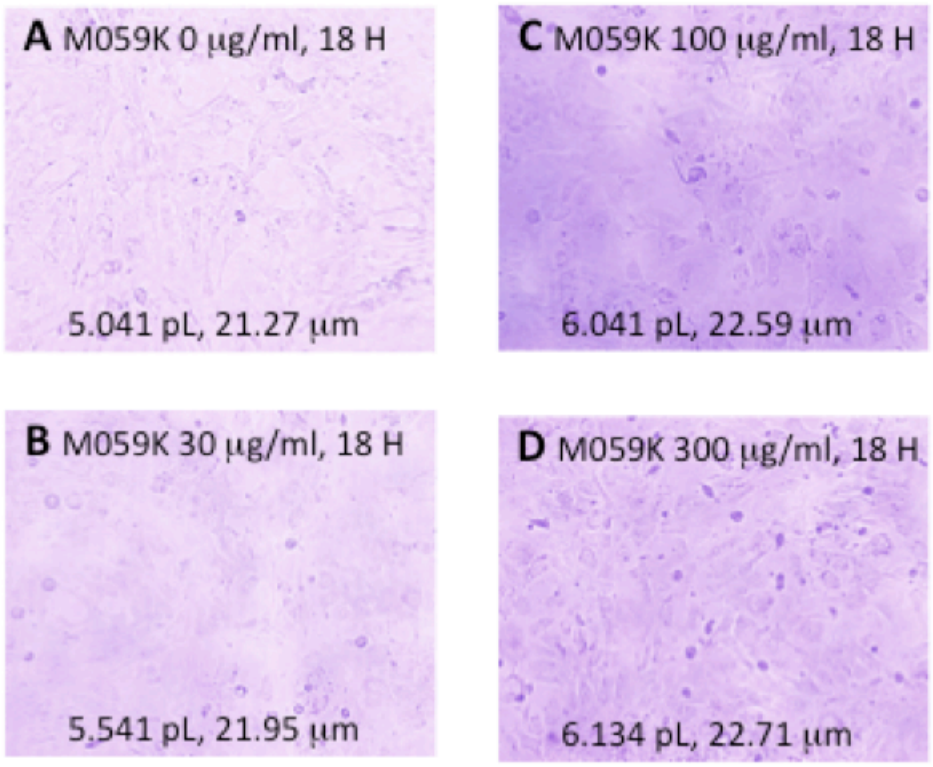


Figure 2-5. Phase contrast microscopic image of M059K after Zeocin treatment. Numbers at the bottom of each image is cell volume and diameter measured by Scepter.

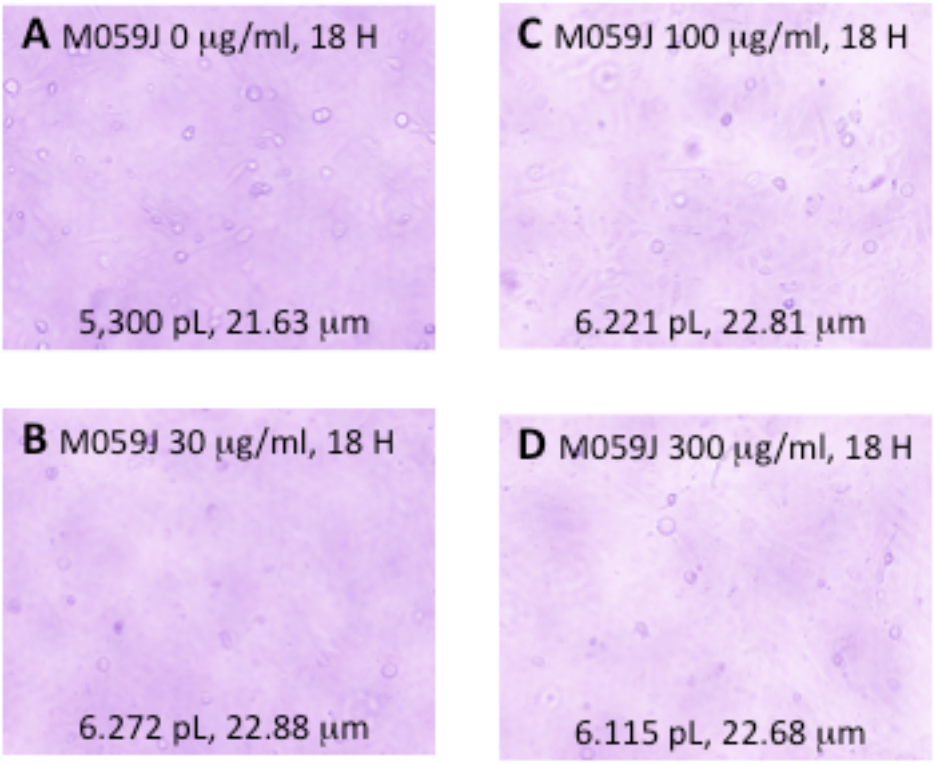


Figure 2-6. Phase contrast microsopic image of M059J after Zeocin treatment. Numbers at the bottom of each image is cell volume and diameter measured by Scepter.

Figures 2-5 and 2-6 show M059K and M059J cells observed under phase contrast microscope after Zeocin treatment. At this time point, detached and floating cells are not significantly different between untreated and treated cells. Nevertheless, Zeocin-treated cells were slightly enlarged, as was also detectable by automatic cell counter, Scepter.

2.2.3 Inhibitors of DNA-PKcs and ATM

In order to clarify the roles of DNA-PKcs and ATM in phosphorylation, NU7441 (Tocris Bioscience; Bristol, UK) and KU55933 (EMD Biochemicals; San Diego, CA, USA), which are specific inhibitors of DNA-PKcs and ATM, respectively, were used in this study. Both of these compounds were derived from LY294002. LY294002 was initially identified as an inhibitor of phosphatidylinositol 3-kinase (PI3K). As DNA-PKcs and ATM are structurally related to PI3K,

LY294002 can inhibit kinase activity of DNA-PKcs and ATM. Structural modification of LY294002 lead to the development of NU7441, which inhibits DNA-PKcs but not ATM, and KU55933, which inhibits ATM but not DNA-PKcs. Figure 2-7 show the structure of NU7441 and KU55933 with LY294002.

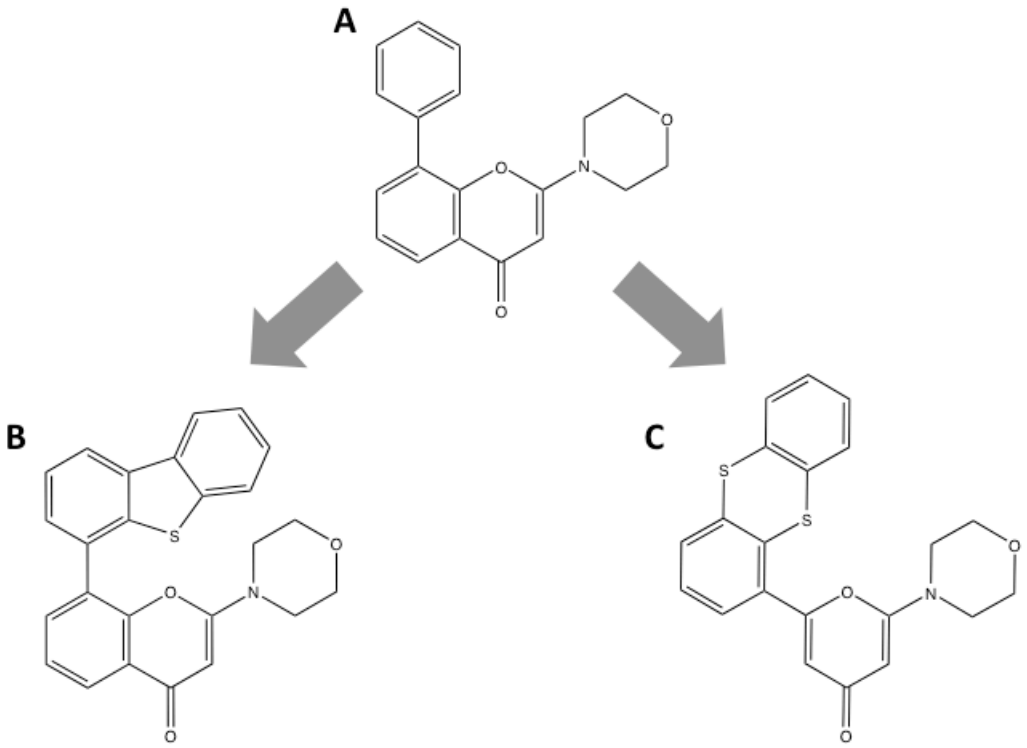


Figure 2-7. Structure of LY294002 (**A**), NU7441 (**B**) and KU55933 (**C**). IUPAC names of these compounds are as follows. LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; NU7441, 8-dibenzothiophen-4-yl-2-morpholin-4-yl-chromen-4-one; KU55933, 2-morpholin-4-yl-6-thialanthren-1-yl-pyran-4-one.

In this study, these compounds were dissolved in DMSO at the concentration of 5 mM and kept at -20 °C until use. In use, they were added to the culture medium at the final concentration of 10 μM 2 hours prior to irradiation or addition of Zeocin and were not removed until harvest.

2.3 Chromatin-binding Analysis by Biochemical Fractionation

In most of the experiments in this study, chromatin-bound fraction was isolated for Western blotting.Chromatin binding fraction obtained by sequential extraction with increase concentration of Nonidet P-40 (NP-40) following earlier publications of our group (Kamdar and Matsumoto, 2010; Liu et al., 2013). Fractionation buffer contain 50mM HEPES (Ph 7.5), 150 mM NaCl, 1mM EDTA, 0.2 or 0.5% (v/v) NP-40, supplemented with cocktails of protease inhibitors and phosphoatease inhibitors. Typically, 5 million cells (5×10^6) were suspended in 75 µl of 0.2% NP-40 buffer and, after keeping on ice for 5 minutes, centrifuged at 1,000 × g for 5 minutes of 4°C. The supernatant was collected as F-I and the remaining pellet P-I was suspended in 75 µl of 0.2% NP-40 buffer and centrifuge at 1,000 × g for 5 minutes. The supernatant was collected as F-II and pellet (P-II) was suspended in 75 µl of 0.5% NP-40 buffer and, after keeping on ice for 40 minutes, centrifuged at 16,000 × g for 5 minutes at 4°C. Supernatant was collected as F-III and the remaining pellet (P-III) was suspended in 75 μl of SDS-PAGE sample buffer (see below) and boiled for 10 minutes at 100°C. To obtain chromatin bind fraction and in order to precipitate, all precipitable substances, suspended cells were centrifuged at 15,000 × g for 5 minutes at room temperature and then cleared.

2.4 ELECTROPHORESIS, SDS-PAGE AND WESTERN BLOTTING

2.4.1 Electrophoretic separation technique (Electrophoresis)

It is a method that high separation efficiency can be achieved with this technique. Through electrophoresis, proteins can be separated by passing through an electric field. Charged molecules and particles under influence of an electric field can move to direction of opposite electrodes of their charges. One the most important characteristic of charge molecules is *electrophoretic mobility which can be defined as measure of migration velocity*. It is based on molecular weight, size of the charge molecules and electric charge. pk is the value of charge groups. Type of the buffer, pH and its concentration as well as strength of the electric field and the temperature has direct effect on electrophoretic mobility. Electrophoresis is useful for **A-** Analytical method and separation, **B-** Visualizing and **C-**The

estimating the molecular weight of the proteins. These mentioned three characteristics, can be considered as important advantages of electrophoresis technique. Electrophoresis of proteins generally carried out in gels made up the cross-linked polyacrylamide.The polyacrylamide gel act as sieve, slowing the migration of proteins approximately in proportion to their charge-to-mass ratio. Migration may also be affected by protein shape. The migration of a protein in a gel during electrophoresis is a function of its size and its shape (Fig 2-8). An electrophoretic method commonly employed for estimation of purity and molecular weight makes use of the detergent SDS. (Lehninger principal of biochemistry, Fourth edition, David L. Nelson & Michael M. Cox).

Band Number	Color	Molecular weight
1	Blue	251,000
2	Blue	124,000
3	Blue	90,000
4	Orange	69,000
5	Blue	54,000
6	Blue	41,000
7	Blue	34,000
8	Orange	29,000
9	Blue	17,000
10	Green	11,000

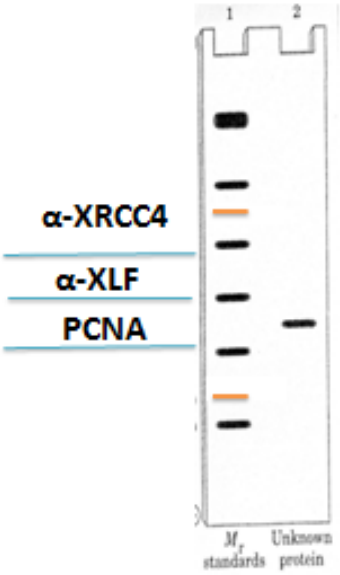


Fig 2-8. Estimating the molecular weight of a protein.

(a) Standard proteins of the known molecular weight shown on lane 1. (Estimate between molecular weight of unknown proteins (lane 2) with standard proteins of the known molecular weight (lane 1). **(b)** A plot of log M_r of the marker proteins versus relative migration during electrophoresis. Cited from: Lehninger Principles of Biochemistry.

2.4.2 SDS

SDS is abbreviation of **S**odium **D**odecyl **S**ulphate . It binds to most proteins in ratio of about, for two amino acids one molecule SDS. In the presence of SDS and on the basis of the molecular weight (mass), electrophoresis almost exclusively separates proteins from each other**.** Therefore the meaning of the different bands that appear in the gel can be evaluated and interpreted as below:

In electrophoresis method, when high voltage is applied, smaller molecules with lower molecular weight (due to the smaller polypeptide chains) with more negative charge will travel a longer distance and move faster through the gel toward the anode .In each reaction At the bottom of the gel ,there is a free labeled DNA that is not bound to any proteins . This free DNA, appears as a one lane bound. Since no proteins bound to this DNA, it can migrates furthest. Therefore having the lowest molecular weight, is the main reason for its fast movement toward down part of the gel. On the other hand, Complex of the DNA – protein is visible in different bands. They run more slowly on the gel in different lanes because they are not free DNA and bound to other proteins, this cause them to be heavier and move slower.

PAGE stands for **P**oly**a**crylamide **G**el **E**lectrophoresis of proteins. It can be used for Analytical PAGE of proteins, because it has faster separation, better defined bands, faster staining, better efficiency and higher sensitivity.

SDS-PAGE or Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis is a technique for separation of proteins based on their molecular weight due to their long polypeptide chains as well as their electrical charge. By adding specialized dye such as Coomassie blue proteins can be visualized. *This dye does not bind to the gel but only to the proteins itself.* SDS-PAGE component shown in Fig. 2-11.

2.4.3 Preparation of SDS separating gel solution

Tertiary structure of the proteins is due to disulfide linked. In order to non–disulfate this structures as well as denaturing the secondary structure of the proteins and to give negative charge to each protein, in the first step the sample should be mix with an anionic detergent such as SDS(**S**odium **D**odecyl**S**ulfate). The process and steps of SDS-PAGE preparation are as follow:

1- ***Prepration of SDS sample buffer:***

SDS sample buffer is mixture of 125 Mm Tris- hyroxymethyl amino methane known as TRIS with the Ph of 6.8 , 4 % w/v SDS ,

5% β -mercaptoetanol, 20% v/v glycerol, 0.01 % w/v crystal violet, 0.05 % w/v Bromophenol blue (BPB).

To simplify the above, sample buffer contain the following: 500 μl distilled water (MilliQ) ,

500 μl SDS-PAGE 2× sample buffer, 25 μl Bromophenol blue (BPB), 5 μL Crystal violet and finally 50 μl β –mercaptoetanol.

2- 50- 200 µl sample buffer should be added for every 10^6 cells then the sample cells must be heated with 100 ℃ tepmerture (Fig 2-9) for 10 minutes. This action helps all proteins to become denaturated and bind to SDS.

3- By using ethanol and clean tissue paper, made of glass SDS gel plate, should be cleaned and wiped perfectly. Rubber strip should be sterilized with ethanol and inserted between the gel glass plates.

Figure 2-9. Dry water bath device
Matsumoto Lab , Tokyo Institute of Technology
Nuclear Engineering Department

A- **SDS separating gel solution.** The chemical component of this gel solution is a mixture of 7.5 ml pure water (distilled water), 3.75 ml of 40% alkalymaid (acrylamide/ bis-acrylamide), 3.75 ml separating gel buffer (1.5M Tris-HCl , 0.4 % SDS sample buffer , Ph 8.8), 15 μl TEMED (tetramethylethylene diamine) and 45 μl of 10% APS

(Ammonium persulfate). APS initiate the process of phosphorylation and TEMED can facilitate the phosphorylation.

B - SDS stacking gel solution. Is another chemical component that is consisted of 5.2 ml pure water (distillated water) **,** 0.8 ml of 40% alkalymaid (acrylamide/ bis-acrylamide), 2 ml stacking gel buffer with volume of :

0.5 M Tris-HCl , 0.4 % SDS buffer with pH of 6.8. Adding, 8 µl TEMED , 60 µl of 10 % APS

2.4.4 General direction and materials for making SDS separating gel solution

For SDS separating gel solution, 10% acrylamide gels consist of: separating gel buffer (1.5M Tris-HCl , 0.4 % SDS sample buffer , Ph 8.8), mixed with 10% w/v of acrylamide in portion of 40:1 followed with 0.1 % v/v tetramethylethylene diamine. Then it was mixed with 0.03 % w/v APS (to initiate the process of phosphorylation). The APS was added immediately before use. After adding the APS, the cocktail should be mix very well with each other. The SDS separating gel solution should be poured out in between the gap of the two SDS glass plates. Leaving a space between 2.5 cm to maximum of 3 cm (SDS separating gel solution shrink slightly after solidification) empty from the top of the plates for SDS stacking gel solution.

A- 300 µl distilled water(Milli Q) added on the top of the SDS separating gel solution to protect the gel from direct contact with air. At the room temperature, solution left for about 60 minutes until the gel was solidified.

2.4.5 General direction for preparation of SDS stacking gel solution

Stacking gel solution (0.5 M Tris-HCl with pH of 8.8), followed by adding 0.4 % w/v SDS. Add 10% w/v acrylamide/ bis-acrylamide with of portion of 40:1, 0.1 % v/v of TEMED. At this step, before adding APS, the water was removed from the top of the solidified gel. 8 ml of this cocktail added on the top of the solidified gel. 0.075 % w/v APS (ammonium persulfate) added to the rest of the SDS stacking gel solution. APS should be added immediately before use.

A- To make loading well (Depressions) on the top of the stacking gel, comb must be inserted into solution on the top of the plates. Wait for about 30 minutes. The gel

after this time will be solidified at the room temperature. After gel solidification, by pouring distilled water (Milli Q) on the combs and wells, the comb was removed carefully. This pre-casted gel (known as hand- casted as well) with concentration of 5-20% of acrylamide, it can be use immediately or stored at the temperature of 4℃ before use for maximum of one day.

B-Run SDS-PAGE. The SDS-PAGE tank filled with SDS running buffer (¼ of the tank to cover the wire in the gel cassette). Then the hand- casted gel (pre-casted gel) placed into the gel cassette. The cassette along with pre-casted gel placed into the SDS-PAGE tank. Sample buffer and sample cells are loaded into the comb wells (Fig 2-12). As a general rule, using the hand –casted gel with 14 comb wells, loading with 4 types of sample cells started with: sample buffer, marker, sample No 1,2,3,4, and then sample buffer, marker, sample cells No 1,2,3,4, sample buffer and sample buffer. In case, the sample cells were less than of 4 types, the empty well comb must be filled with sample buffer.

After loading the wells comb with sample buffers, marker and sample cells, top of each well combs as well as the middle upper part of tank was filled with running buffer. This action helps that electrodes connect with each other's. SDS-PAGE electronic device was set and connected to the electricity. Setting for electrophoresis for one gel is: 20 mA. 40 mA for two gels, voltage in any case should remain fixed and equal with 200 V, the time length for running SDS-PAGE should be 85 min for any number of gel. After setting start button pushed to run the gel.

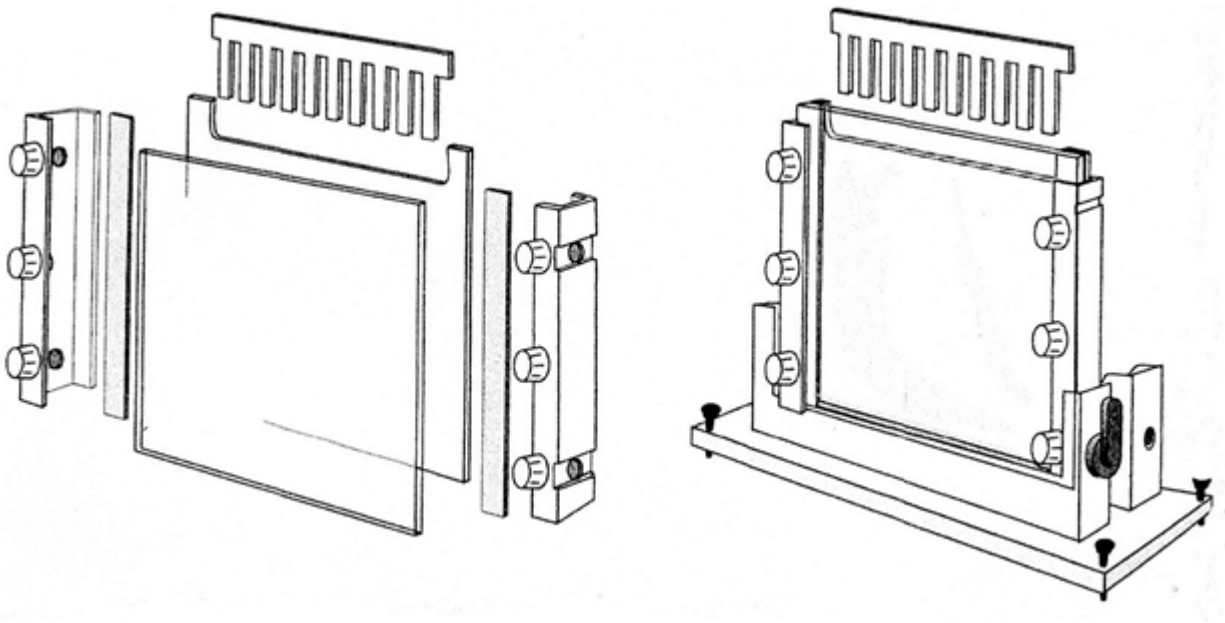


Figure 2-10. Preparation of the gel casting for one or two gel cassetters

Cited from: Reiner Westermeier, Electrophoresis in Practice, Fourth edition

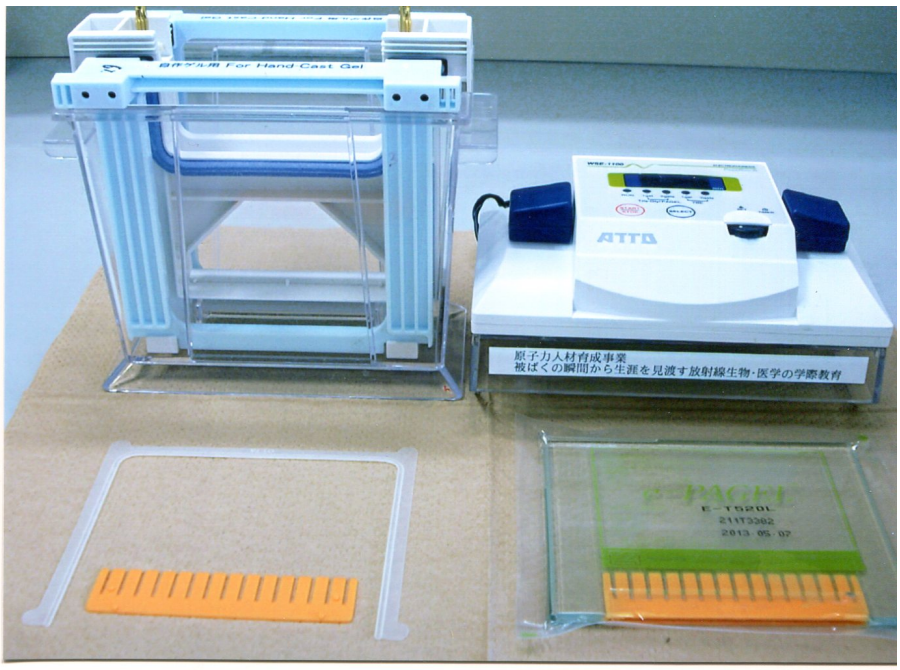


Figure 2-11. SDS-page component

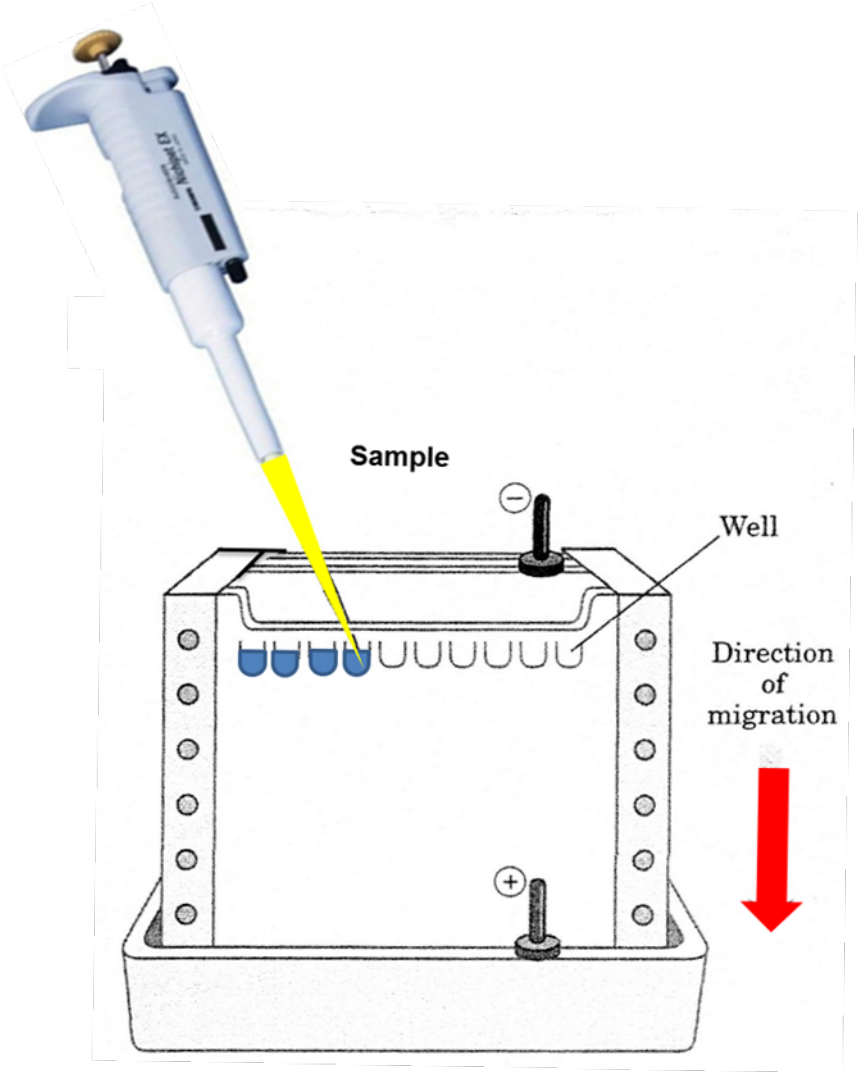


Figure 2-12. Loading Pre-casted gel

Cited from, Lehninger Principal of Biochemistry, fourth edition

2.4.6 Western blotting

Protein Immunoblotting or Western blotting is (technique of transferring proteins from a gel to a membrane) and is used to identify and determine the relative quantity and molecular weight of a protein within a mixture of proteins or other molecules. The mixture first subjected to analytical separation typically by SDS-PAGE, so that the final positions of

different proteins in the gel are a function of their molecular size. The array of separated proteins is then transferred from the separating polyacrylamide get to a support membrane by electrophoresis such that the membrane acquires replica of the array of separated macromolecules present in the gel. SDS is displaced from the protein during the transfer process, and native antigenic determinants are often regained as the protein refolds. The position of the protein antigen on the membrane can be detected by binding of an unlabeled antibody specific for that protein (the primary antibody) followed by a labeled second antibody that bind to the primary antibody. This approach provides information about antigen size and quantity. In general, second antibody probes are labeled with enzymes that generate chemiluminescent signals and leaves images on the photo photographic film, near infrared fluorophores (Fig 2-13). Cellular and molecular of immunology, Abul K. bbas, Andrew H. Lichtman, Shiv Pillai-7th edition)

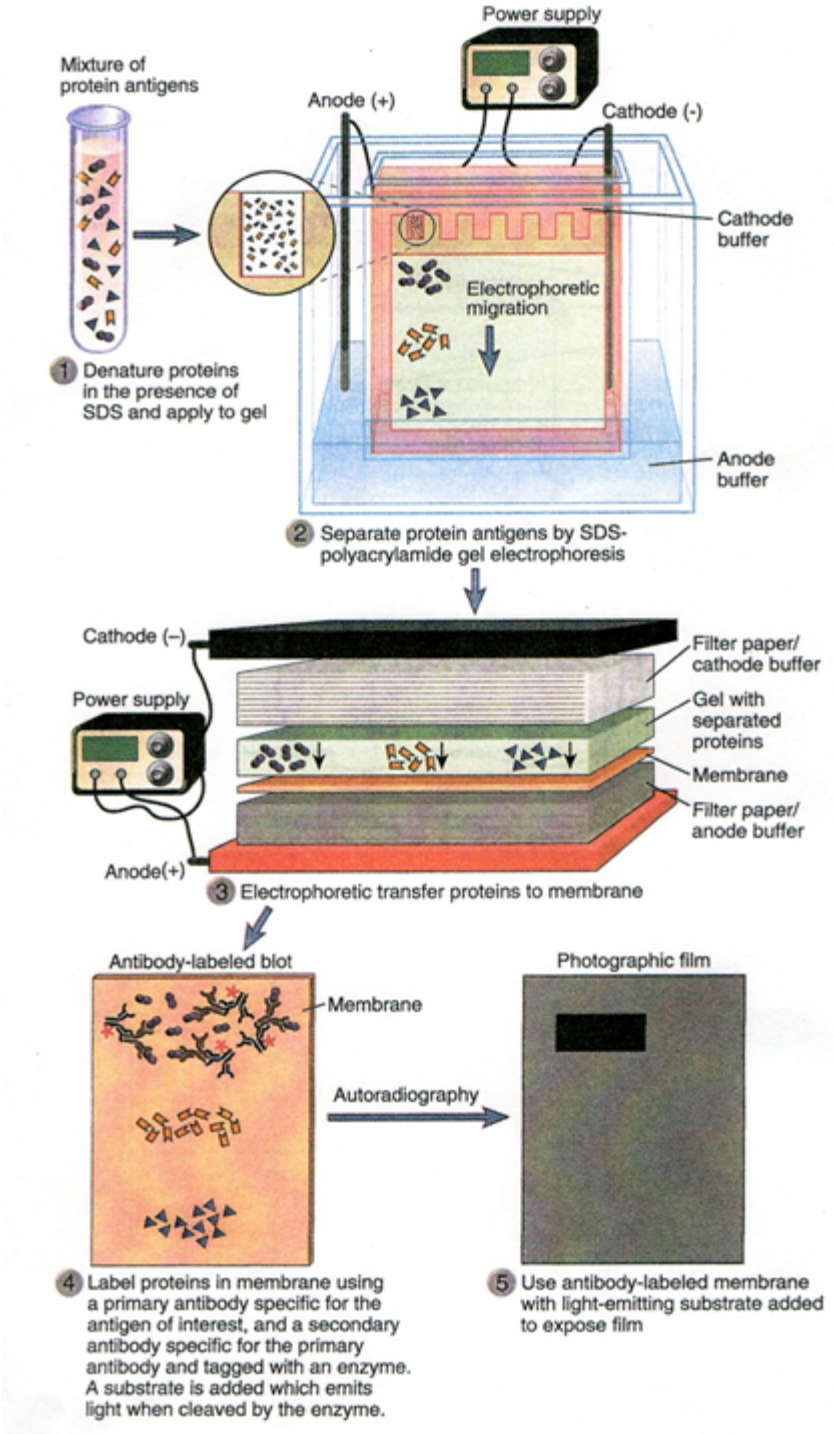


Figure 2-13. Characterization of antigens by Western blotting

Cited from: Cellular and Molecular Immunology, 7th edition

2.4.7 Preparation for membrane transfer

Since after completion of electrophoresis, the proteins, from acrylamide gel must electrophoretically transfer onto PVDF membrane (Immobilon Polyvinylidene Difluride) through a process known as Western Blotting Preparation of western blotting involves some steps as follow:

1- For each SDS gel, one PVDF membrane and six pieces of Chr paper (filter paper) with the size of 6.5 cm × 9 cm cut and prepared. The cut PVDF membrane should be treated and soaked completely into the methanol for one minute. After one minute, the PVDF , carried out into transfer buffer without SDS (100m M Tris , 192 Mm glycine , 5% methanol) and made sure that the membrane was submerged perfectly into it. Then it was placed on the swing machine to swing softly. The PVDF will remain into the transfer buffer without SDS until the electrophoresis process end. Transfer buffer without SDS poured on the stage of transfer apparatus (Fig 2-14) and then, three pieces of prepared and cut, Chr paper (filter paper) soaked into transfer buffer without SDS separately, and placed on the top of each other, on the transfer apparatus (The bubble in between each layer removed and made them smooth and flat). PVDF placed on the top of the Chr (filter) papers. The SDS gel softly and carefully transferred on the top of the PVDF membrane to completely cover it. Another 3 pieces of filter paper (Chr paper) soaked into transfer buffer without SDS with the same method and placed on the top of the SDS gel (The bubble in between each layer removed and made them smooth and flat).

2- ***Run membrane transfer :***

DC voltage should be applied to the transfer apparatus. Plug and connect the electric device into the DC voltage. Made sure that black and red cables are properly and firmly connected to the transfer apparatus (Figure 2-14). The apparatus transfer

should run by following set up : voltage is set to 100V . Watt of 20.0, time length of operation 60 minutes and more importantly, 110 mA for each gel (two gels 220 mA and three gels 330 mA). At the end of the western blotting, the proteins located on the SDS gel will transfer to the PVDF membrane.

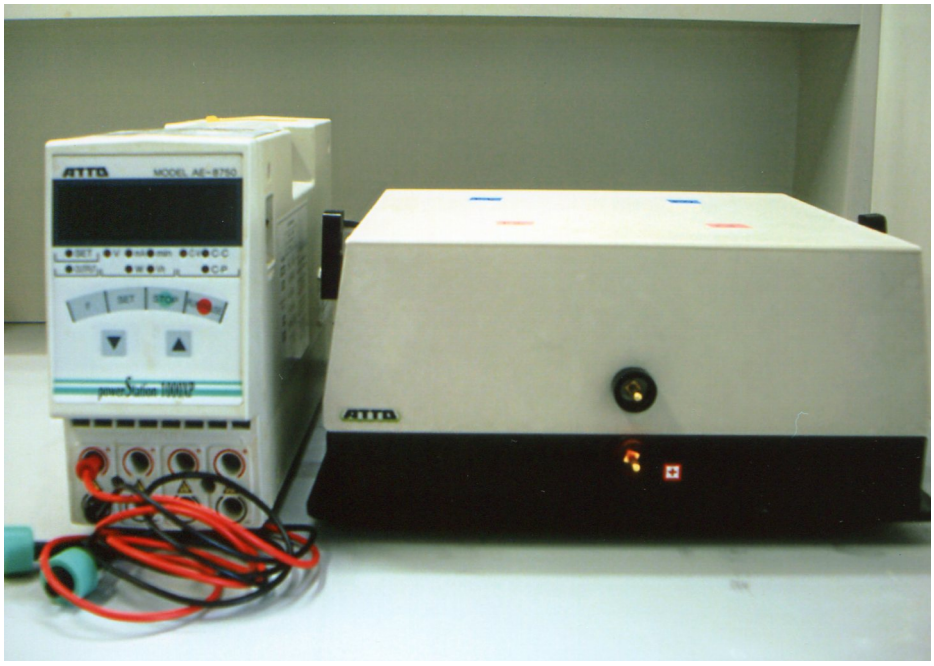


Figure 2-14. Apparatus machine used for transferring proteins from gel to PVDF membrane.

3- *Blocking solution :*

After completion of western blotting, the PVDF contain the proteins, should be immediately block by another solution known as blocking solution. This solution is a mixture of 1% - 2% skim milk diluted in TBST solution. TBST solution consist of 20 mM Tris with Ph of 7.6, 150 mM NaCl and 0.05 % v/v Tween 20. The blocking solution should be stir for at least 30 minutes before use. For purpose of blocking, The PVDF

membrane transferred into a box contains blocking solution and placed on the swing machine for minimum of 1 hour (preferably overnight at the refrigerator). Since the proteins in the skim milk will be attached to the PVDF membrane and for the purpose of reducing the noise in the result, the necessary following steps was taken to prevent the interaction between proteins on the membrane and antibody: First of all some of the western blotting is required two steps of antibody treatment known as Primary antibody reaction and secondary antibody reaction.

4- *Primary antibody reaction:*

After blocking process, the PVDF membrane transferred into a hybribag. The corresponded antibodies, XLF, XRCC4 and PCNA, diluted in blocking buffer(skim milk mix with TBST), for example: 1μl of XLF and XRCC5 in 500 μl of blocking buffer and 2.5 μl of PCNA in 500 μl blocking buffer. After adding the primary antibody solution, the air removed entirely from inside the hybribag and was sealed completely. The hybribag contain PVDF membrane, placed on the swing machine for at least 1.30 minutes (preferably overnight at the refrigerator) . After this period of time the seal of the PVDF membrane opened and the membranes washed for five times with TBST solution with specific time limits of 2, 2, 5, 5, 2 Minutes respectively, and at the end it washed once with TBS solution (20 m M Tris with Ph of 7.6, 150 m M NaCl) with specific time limit of 3 Minutes .In this experiments for equalize the cell concentration in each loading sample PCNA known as **P**roliferating **C**ell Nuclear **A**ntigen, was chosen and used. This antigen does not respond to irradiation and it is present in the cell nucleus.

In these experiments, PCNA with dilution of 500 × and XLF, XRCC4 antigen with dilution of 250 × as primary antigen were used.

5- *Secondary antibody reaction :*

Secondary antigen reaction is similar to the primary antigen reaction. In this reaction the swine anti-rabbit antigen was used as secondary antibody. In the first step, second antibody solution prepared (1.1 μl swine Anti –rabbit antigen, diluted in 500 μl of blocking solution for each membrane). For the next step, membranes placed inside the hybribag. The antibody solution added to the hybribag and sealed completely (the air inside the hybribag must be removed completely before sealing the bags). The hybribag left on the swing machine for at least an hour. After an hour and for the purpose of removing the entire unbinding antibody from the PVDF membrane, the membranes were removed from the hybribag and washed with TBST for five times accordingly 2, 2, 5, 5, 2 Minutes and at the end it was washed once with TBS for 2 minutes.

The main reason for using the primary antibody and secondary antibody is: The first antibody can recognizes the specific proteins that are under investigation however the second antibody can be used for the fluorescent detection, because it can bind to the first antibody.

6- *Fluorescent reaction:*

To generate luminescent signal against second antibody, membranes after probe with antibody should be treated with the ECL plus Western Blotting Detection Reagents.

After washing the PVDF for the second time, each of those membranes, were placed inside the hybribag. Then the solution A and the solution B are at ratio of 40 are mixed together and prepared. 500 μl of this mixture added to each hybribag. The air was removed from inside the hybribag before it was sealed. The bags were sealed for 4-5 minutes completely that reaction take place. The seals of the hybribag were opened and the abundant fluorescent mixture was removed from inside the seal bags

completely. After removing the air from inside the bags, the Hybribag resealed once more.

7- *Fluorescent checking :*

The hybribags were fixed inside the cassette of X-ray machine (Fig 2-15). A sensitive X-ray film placed on the top of the hybribags inside the cassette for certain time (for example one minute). The developed X-ray film removed from the cassette and a CCD camera (Gel documentator) used to record the image at the computer (Fig 2-16).

Figure 2-15. X-ray developing machine.

Figure 2-16. CCD camera (Gel documentator)

CHAPTER 3

Generation of phosphorylation-specific antibody for XRCC4 Ser260

3.1 Introduction

In response to treatment with ionizing radiation or DNA damaging agents, XRCC4 undergoes DNA-PK-dependent phosphorylation depending of radiation dose (Matsumoto et al., 2000). Furthermore, Ser260 and Ser320 (termed 'Ser318', reflecting the alternatively spliced form) of XRCC4 were identified as the major phosphorylation sites by purified DNA-PK in vitro through mass spectrometry by several groups (Yu et al., 2003; Lee et al., 2003; Wang et al., 2004). However, it has not been clear whether these sites are phosphorylated in vivo in response to DNA damage. Our laboratory recently found out, Ser320 in fact undergoes phosphorylation after irradiation in cellulo. Thus, we set out looking for S260 as another candidate for phosphorylation site in living cell.

Here, a rabbit polyclonal antibody that would react with XRCC4 phosphorylated at Ser260 was generated and *in cellulo* phosphorylation status of XRCC4 Ser260 was examined. In an earlier study of our laboratory (Sharma et al., 2015) generated an antibody (α-XRCC4 pS320) that reacts with XRCC4 phosphorylated at Ser320 and examined *in cellulo* phosphorylation status of XRCC4 Ser320. Sharma showed that, the phosphorylation of XRCC4 Ser320 was induced by γ-ray irradiation. This would help to investigate and understand, if the contribution of DNA-PK and ATM for XRCC4 Ser260 site could be the same as XRCC4 Ser320 or it is different.

The specificity and the titer of the antibody was tested by enzyme-linked immunosorbent assay (ELISA). Enzyme-Linked Immuno-Sorbent Assay (ELISA) is a sensitive immunoassay test that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein or to identify the substance, especially an antigen or antibody through color change. ELISA testing also is an excellent option to determine if an antibody program can be terminated or should be extended, or for the selection of the best responding animal. It is also of prior importance during the anti-peptide programs to determine the time point when the final bleed of the animals has to be carried out.

In ELISA, a liquid sample is added onto a stationary solid phase with special binding properties and is followed by multiple liquid reagents that are sequentially added, incubated

and washed followed by some optical change (for example, color change by the product of an enzymatic reaction) in the final liquid in the well from which the quantity of the analyte is measured. The sensitivity of detection depends on amplification of the signal during the analytic reactions. Since enzyme reactions are very well-known amplification processes, the signal is generated by enzymes which are linked to the detection reagents in fixed proportions to allow accurate quantification – thus the name "enzyme linked".

The analyte is also called the ligand because it will specifically bind or ligate to a detection reagent, thus ELISA falls under the bigger category of ligand binding assays. The ligand-specific binding reagent is "immobilized", i.e., usually coated and dried onto the transparent bottom and sometimes also side wall of a well (the stationary "solid phase'/"solid substrate" here as opposed to solid microparticle/beads that can be washed away), which is usually constructed as a multiple-well plate known as the "ELISA plate". Conventionally, like other forms of immunoassays, the specificity of antigen-antibody type reaction is used because it is easy to raise an antibody specifically against an antigen in bulk as a reagent. Alternatively, if the analyte itself is an antibody, its target antigen can be used as the binding reagent.

There are five types of ELISA, thus, about ELISA protocol; a few differences exist amid indirect ELISA protocol, direct ELISA protocol, sandwich ELISA protocol, competitive ELISA protocol and ELISPOT protocol. However, the main ELISA principle and lots of procedures are the same. In this study, indirect ELISA (Fig.3-1) was used. Indirect ELISA is a two-step ELISA which involves two binding process of primary antibody and labeled secondary antibody. The primary antibody is incubated with the antigen followed by the incubation with the secondary antibody.

To further verify the specificity of the antibody, we also examined XRCC4 phosphorylated by DNA-PK in vitro.

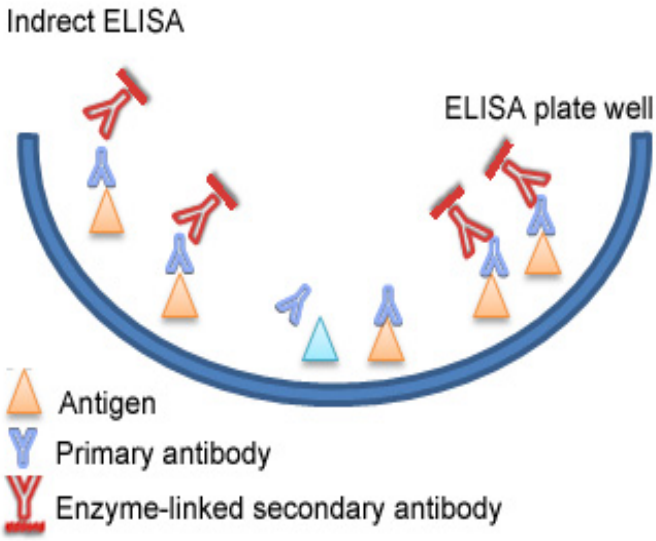


Figure 3-1. Indirect ELISA. Cited from; http://www.elisa-antibody.com

3.4 Conclusion

Here I generated a rabbit polyclonal antibody, α-XRCC4-pS260, which would react with Ser260 phosphorylated XRCC4. The specificity of this antibody along with α-XRCC4-pS320, which was generated in a preceding study (Sharma et al., 2016), was verified by ELISA assay using the synthetic peptides and by Western blotting analysis of XRCC4 with and without phosphorylation reaction with DNA-PK in vitro. These antibodies would be competent for analyses of phosphorylation status of XRCC4 in cellulo with or without DNA damage, as examined in the following chapters.

CHAPTER 4

Evaluation of *in cellulo* phosphorylation status of XRCC4 Ser260 and its role in radiation sensitivity

4.1 Introduction

In the previous chapter, I generated a phosphorylation-specific antibody, α-XRCC4-pS260. In this chapter, we first intended to examine the phosphorylation status of XRCC4 Ser260 *in cellulo* in response to DNA damage using this antibody.

Next, I intend to examine the functional significance of phosphorylation. Phosphorylation of XRCC4 molecule by DNA-PK has been reported but it has not been linked to any functional changes in vivo (reviewed in Kamdar and Matsumoto, 2011). Therefore, the sites and roles of XRCC4 phosphorylation by DNA-PK remain to be elucidated. To reach to this goal, Ser260 was mutated into alanine ($XRCC4^{S260A}$) to disable phosphorylation and into aspartate ($XRCC4^{S260D}$) to mimic phosphorylation. These mutants were introduced into M10 cell line, which lacks endogenous XRCC4, and the stable transformants, M10-$XRCC4^{S260A}$ and M10-$XRCC4^{S260D}$, respectively, were established. The radiosensitivity of these transformants was measured by colony formation assay and compared to that of wild-type XRCC4 and empty vector transformants, M10-$XRCC4^{WT}$ and M10-CMV, respectively.

In this chapter to search the role of XRCC4S260, in DNA DSB repair, XRCC4 S260 was mutated from Serine to Alanine (A), which is nonpolar, hydrophobic and neutral, and Aspartic acid (D), which is polar and negatively charged. Each amino acid, except for proline, has at least one amine and one acid functional group as the name implies. The different properties result from variations in the structures of different R groups. The R group is often referred to as the amino acid side chain.

1- Serine differs from Alanine in that one of the methylenic hydrogens is replaced by a hydroxyl group. Therefore Alanine is one of the structurally simpler amino acids and is the closest replacement to Serine. (To minimize the structural changes in amino acid).

2- Alanine is Non-polar, Neutral amino acid and due to lack of OH (hydroxyl group) can not be phosphorylated (Fig.4-1).

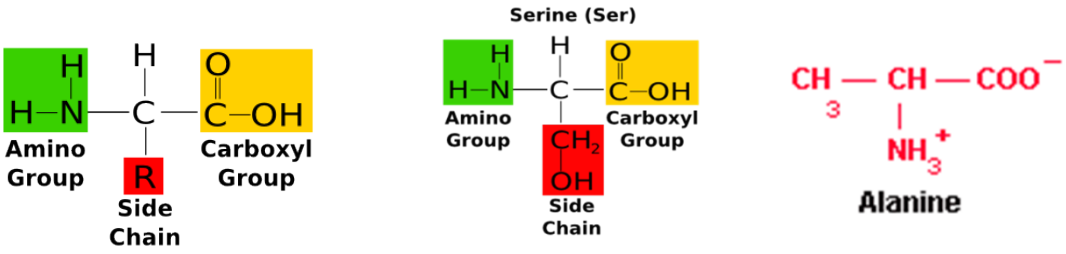


Figure 4-1. Amino acid structure of Alanine, Aspartic acid

3- Some non-phosphorylated amino acids appear chemically similar to phosphorylated amino acids. Therefore, by replacing an amino acid, the protein may maintain a higher level of activity. For example, aspartic acid is chemically similar to phospho-serine. Therefore, when an aspartic acid replaces a serine, it is a phosphomimetic of phospho-serine and can make the protein always in its phosphorylated form (Fig. 4-2).

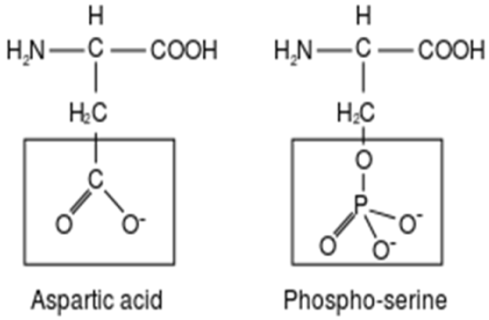


Figure 4-2. Structure of Asparatic acid and phosphor- serine

4.5 Conclusion

Here, I here demonstrated that XRCC4 Ser260 undergoes phosphorylation by DNA-PK in living cells constitutively and in response to radiation. I also showed that XRCC4 mutant lacking this phosphorylation site is mostly but not fully functional in DSB repair, in terms of cell survival after irradiation. The current results indicated a possibility that the phosphorylation of XRCC4 Ser260 by DNA-PK might play some role in DSB repair, which needs to be clarified in future studies.

CHAPTER 5

Analyses of phosphorylation of XRCC4 Ser320

5.1 Introduction

In the current study, I generated an antibody α-XRCC4-pS260, which reacts specifically with Ser260-phosphorylated XRCC4. I also used α-XRCC4-pS320, which had been established in our laboratory and shown to be an indicator for DNA-PK function in response to DNA damage. As they are polyclonal antibodies, I sought to generate a monoclonal antibody for Ser320-phosphorylated XRCC4.

Antibodies are the molecules circulating the blood, dedicated to the recognition of foreign organisms or substances. Upon infection by viruses, for example, our immune system raises the production of antibody molecules against them. Likewise, antibody molecules against some proteins of interest can be generated by injecting laboratory animals, such as mice, rabbits or goats with the whole protein or its part as the antigen. This process is termed the immunization of the animal. The immune system of the animals produces antibody molecules specifically reactive to the antigen and releases them into the serum of the blood, which is called the antiserum. Thus, the antibody molecules can be harvested by collecting the antiserum from animal following exposure to an antigen for a few weeks. The antibody molecules can be further purified on the basis of their affinity to the antigen.

Each antibody producing cell (B cell) produces only one type of antibody. A polyclonal antibody is a collection of antibody molecules from different B cells that recognize the same antigen. On the other hand, a monoclonal antibody represents an antibody from a single clone of B cells.

In general, the antiserum contains a mixture of multiple types of antibody molecules, each of which has different structures and recognizes different part of the antigen. Thus, the antibody purified from the antiserum is polyclonal antibody. Sometimes, the antiserum itself can be regarded and used as a polyclonal antibody.

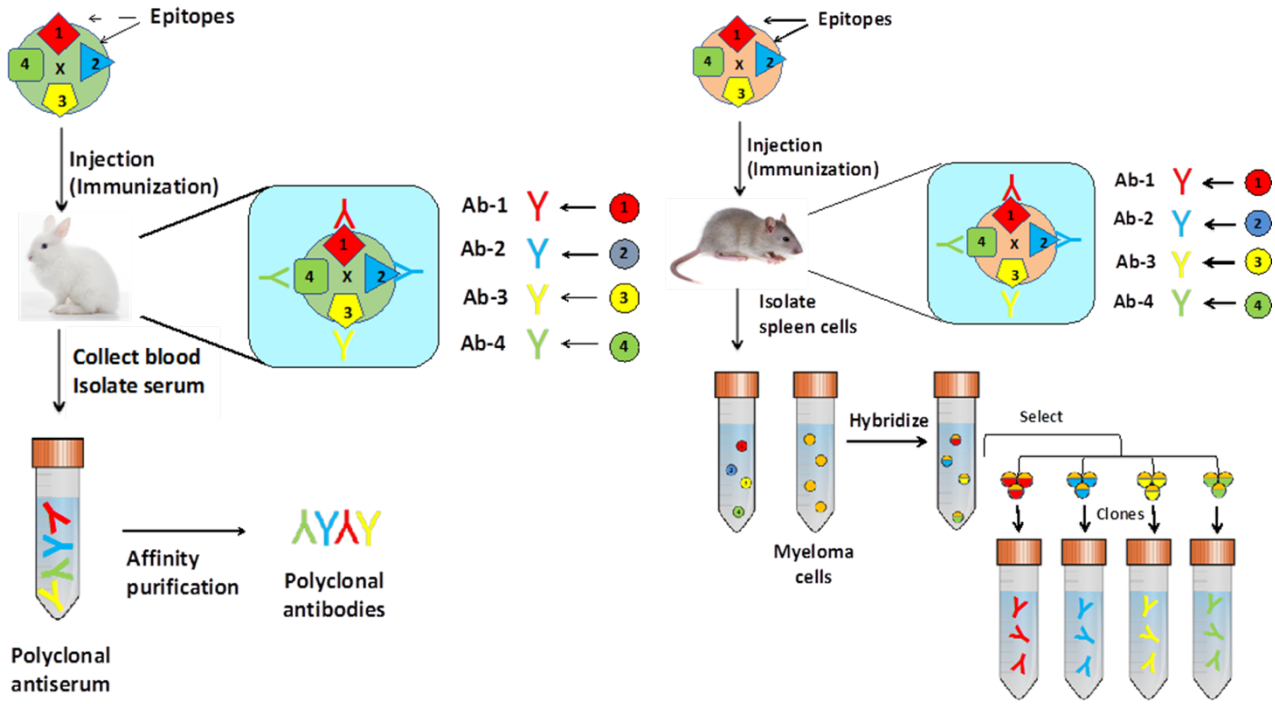


Figure 5-1. Comparison of the polyclonal antibodies (left) and monoclonal antibodies (right).

The procedure to generate a monoclonal antibody, is first established by Kohler and Milstein. Like in the case of generating polyclonal antibody, the antigen is injected into laboratory animals for immunization. When the concentration of antibody in blood serum is sufficiently increased, the spleen is excised to obtain B cells. Since normal B cells can undergo only a limited number of cell division, they are fused with immortal myeloma cells to yield a hybridoma, which have an ability to infinitely proliferate. The cells are transferred into a media, in which the original myeloma cells cannot grow. Thus, only the hybridomas can grow and are then examined for the production of the desired antibody.

Polyclonal antibodies and monoclonal antibodies have advantages and disadvantages to each other.

Polyclonal antibodies can be generated in shorter time and at lower cost than monoclonal antibodies, in general. Polyclonal antibodies can be obtained within a few months. On the other hand, in the process to generate monoclonal antibodies, the production and selection of hybridomas need several months. In addition, polyclonal antibodies exhibit higher overall affinity and robust detection for the antigens, as multiple antibody molecules can bind a single antigen molecule. Thus, polyclonal antibodies are suitable for experiments, such as immunoprecipitation or chromatin-immunoprecipitaiton, which require quick and efficient capture of protein. Polyclonal antibodies often show greater sensitivity to proteins, which exist at low amount.

On the other hand, monoclonal antibodies can be generated at large, or infinite, quantities with homogeneous properties, once appropriate hybridoma clones are obtained. In addition, except for the case of repeated sequence, one antibody molecule binds to one antigen molecule and, thus, is usually suitable for experiments, which requires accurate quantification of the antigen.

Therefore, the use of polyclonal antibody and monoclonal antibody, depending on the purpose, will greatly strengthen the usefulness of XRCC4 phosphorylation status, at Ser320, to capture DNA-PK activity and functionality in cells.

5.4 Conclusion

In this chapter, mouse monoclonal antibodies for Ser320-phosphorylated XRCC4 were generated. It was found that the monoclonal antibodies as well as polyclonal antibody for Ser320-phosphorylated XRCC4 could detect XRCC4 phosphorylation at Ser320 in response to DNA damage. Monoclonal antibodies several advantages compared to polyclonal antibodies. Most importantly, monoclonal antibodies can be generated at large, or infinite, quantities with homogeneous properties. The monoclonal antibodies generated here would be useful for basic research on the recognition and repair of DNA double-strand breaks and, in future, also for medical application, such as the prediction of radiosensitivity and cancer susceptibility.

CHAPTER 6

Conclusion

DNA double-strand break (DSB) is considered most critical type of DNA damage, among those generated by ionizing radiation. DSB is mainly repaired through two pathways: homologous recombination and non-homologous end joining (NHEJ). In NHEJ, Ku86, Ku70 and DNA-PKcs recognize DSB and DNA ligase IV ligates two DNA ends in cooperation with XRCC4 and XLF. There are several lines of evidence indicating that the protein phosphorylating activity of DNA-PKcs is essential for NHEJ, but the targets and significance of phosphorylation remain to be clarified. Earlier studies of our group and others demonstrated that XRCC4 is phosphorylated by DNA-PK in response to ionizing radiation and DSB-inducing chemicals. To date, at least six phosphorylation sites of XRCC4 by DNA-PK have been identified. Ser260 and Ser320 were initially identified as the major phosphorylation sites but the biological significance remained unclear. It was also unclear whether these sites undergo phosphorylation in response to DNA damage in cell. Our group recently generated a phosphorylation-specific antibody toward XRCC4 Ser320 and successfully demonstrated its phosphorylation by DNA-PK in response to DNA damage. This study is aimed to clarify the regulation of XRCC4 through phosphorylation at Ser260 by DNA-PK.

Toward this aim, in Chapter 3, I generated a rabbit polyclonal antibody, anti-XRCC4-pS260, which specifically reacts with XRCC4 phosphorylated at Ser260. The specificity of this antibody was verified by enzyme-linked immunosorbent assay (ELISA) and also by *in vitro* phosphorylation experiment employing purified XRCC4 and DNA-PK.

Next, in Chapter 4, using this antibody, I examined the phosphorylation status of this residue in cells with or without DNA damage and role of DNA-PK therein. Whereas the phosphorylation of Ser320 was induced by ionizing radiation up to 20 Gy, the phosphorylation of Ser260 was seen constitutively and found to increase by higher dose like 300 Gy. This increase was suppressed by DNA-PK inhibitor and the phosphorylation was absent in DNA-PKcs-deficient cells. Based on these results, I concluded that XRCC4 Ser260 undergoes constitutive and radiation-inducible phosphorylation, both of which are dependent on DNA-PK.

In Chapter 4, I also generated cell lines, which expresses mutant XRCC4, in which Ser260 is replaced by alanine ($XRCC4^{S260A}$), to disable phosphorylation, or by aspartic acid

(XRCC4^{S260D}), to mimic phosphorylation, and analyzed their radiosensitivity, as the measure of DNA repair function. XRCC4^{S260A} and XRCC4^{S260D} mutants could correct the high radiosensitivity of XRCC4-deficient M10 cells to a similar extent to wild-type XRCC4, indicating that these mutants were mostly functional. Nevertheless, cells expressing these mutants were slightly but significantly more radiosensitive than cells expressing wild-type XRCC4. Moreover, the cells expressing XRCC4^{S260A} exhibited higher number of γ-H2AX foci before and after irradiation than wild-type XRCC4 expressing cells, suggesting the existence of more unrepaired DSBs. Based on these results, I concluded that XRCC4^{S260A} and XRCC4^{S260D} are not fully functional in DSB repair, which means that XRCC4 Ser260 would play some essential role in DSB repair.

In Chapter 5, a mouse monoclonal antibody, which is specific to Ser320-phosphorylated XRCC4 was generated. Experiment using the lysate of HeLa cells, either unirradiated or after 20 Gy of γ-irradiation after knockdown the endogenous XRCC4, small interfering RNA (siRNA) shows that XRCC4 Ser320 phosphorylation can be detected using this monoclonal antibody α-XRCC4-pS320M.

As the summary, this study generated two antibodies to probe XRCC4 phosphorylation by DNA-PK, a rabbit polyclonal antibody for Ser260 phosphorylation and a mouse monoclonal antibody for Ser320 phosphorylation. These antibodies would be useful for studies to deepen our understanding of detailed molecular mechanism for the recognition and repair of DNA double-strand breaks and, in future, also for medical application, such as the prediction of radiosensitivity and cancer susceptibility. This study also examined the occurrence and role of XRCC4 Ser260 phosphorylation. XRCC4 Ser260 by DNA-PK might play some role in DSB repair, which needs to be clarified in future studies.

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