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Cell senescence and the DNA single-strand break damage repair pathway

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Abstract: Cellular senescence is a response to endogenous and exogenous stresses, including telomere dysfunction, oncogene activation and persistent DNA damage. In particular, radiation damage induces oxidative base damage and bond breaking in the DNA double-helix structure, which are treated by dedicated enzymatic repair pathways. In this review we discuss the correlation between senescence and the accumulation of non-repaired single-strand breaks, as it could occur during radiation therapy treatments. Recent experiments of cell irradiation *in vitro* by high-energy photons showed that single-strand breaks may be preferentially produced at the borders of the irradiated region, thereby inducing senescence, in competition with the apoptosis end-point typically induced by double-strand breaks. Such a peculiar response to radiation damage has been proposed as a possible source of radiation-induced second primary cancers, when such cells with accumulated, non-repaired single-strand breaks evade the senescent state at much later times. The peculiarities of strand-break repair pathways are highlighted, also in relation with the base-excision pathway that repairs several different DNA oxidation defects.

Keywords: Cell senescence; DNA damage; Radiotherapy; DNA repair pathways; Base-excision repair; Single-strand breaks; Radiation-induced stress

1. Introduction

Cellular senescence was defined about 60 years ago as the state reached by human diploid fetal lung fibroblasts after a finite number of cell doublings during *in vitro* culture [1,2]. As such, it indicated the arrest of cell divisions after a limiting number of doublings, in the range of about 50 iterations (what would become known as the "Hayflick limit", [1]). Between the end of the 70s and the end of the 80s, Elizabeth Blackburn and Carol Greider discovered that the telomeres play a major role in the process of senescence [3,4]. Telomeres are a set of repetitive DNA sequences at the end of eukaryotic chromosomes, which help to prevent incomplete replication and genomic instability. It was observed that after each cell division there is a gradual loss of a few nucleotides that results in progressive telomere shortening; as the telomeres reach a certain length, they become unable to bind to certain telomere-capping proteins, therefore leaving DNA ends exposed; this triggers the DNA repair pathway, and eventually leads to cell senescence or cell death [4,5]. Such a chain of events has been indicated as "replicative senescence" (RS).

Senescence, once thought to be but a flaw in tissue culture techniques, has since been recognized as a critical process in both physiological and pathological contexts [6,7]. It must be noted that, besides telomere shortening, cellular states identical or similar to RS can be reached by cells subjected to various developmental signals or external stresses that may include mitogenic signals, radiation, oxidative and genotoxic stress, etc [8,9]. A mechanism common to all these stress factors is that they induce cell senescence by ceasing the cells to proliferate further, while cells remain metabolically active and adopt a

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peculiar immunogenic phenotype. However, cellular senescence plays both beneficial and harmful roles. On the one hand, it has key physiological functions in normal development, tissue remodeling, wound healing, and limits tumor progression by preventing damaged cells from proliferating. On the other hand, the entire process of senescence is also one of the main reasons for cellular aging, as aging cells secrete inflammatory factors that lead to tissue deterioration, which in turn leads to aging and age-related diseases. The accumulation of senescent cells can impair tissue repair and regeneration, deplete stem and progenitor cell reserves, and trigger the release of the senescence-associated secretory phenotype (SASP) [9–11]. In particular, stress-induced premature senescence (SIPS) [12,13], as the name suggests, is a quick response of the cells to a stress induced by an external factors [14,15]. Notably, this mode is relevant to both normal cells and cancer cells, as it relies mainly on the DNA damaging effect of reactive oxygen species (ROS), which can preferentially affect telomeres, hence accelerating telomere shortening and RS. Notably, an important, related sub-class of senescent behavior, often dubbed as "therapy-induced" senescence (TIS, see below) occurs mainly as a consequence of the oxidative stress induced by different anticancer therapies, hence resembling SIPS.

Excellent reviews on the main mechanisms and different aspects of senescence were recently published (see e.g. [16–24]). The present, more concise and thematic review is mainly addressed at highlighting the correlation between stress-induced senescence and DNA damage, in particular the peculiar role played by single-strand breaks (SSB) with respect to double-strand breaks (DSB) produced in the DNA backbone by both endogenous sources and by exogenous attack, notably therapeutic irradiation with energetic photon and particle beams. One important motivation for this focus is the indication provided by some studies [25,26], according to which the ratio DSB/SSB is very important in the cell outcome determination: while normal or cancerous cells exposed to the main radiation field accumulate substantial fractions of both DSBs and SSBs and normally undergo apoptosis, cells receiving stray dose at the borders of the irradiation field appear to accumulate only SSBs, which rather favor cell senescence if not promptly repaired. The origins of such non-repaired damage accumulation are still to be elucidated, however they constitute an interesting and surprising evidence, possibly opening the way to novel therapeutic strategies to decrease the risk of secondary radiation-induced recurrent cancers.

The paper is organized as follows: section 2 provides a general description of the senescent cell phenotype; section 3 highlights the relationships between senescence and cancer; sections 4 and 5 give a synthetic review of DNA damage and repair pathways, respectively, with a focus on radiation-induced damage, and the interrelated base-excision and single-strand break repair pathways; finally, section 6 introduces the link between cell senescence and accumulation of single-strand breaks in irradiated cells; a brief discussion wraps up the review.

2. Morphological and functional characteristics of senescent cells

Senescence is a cellular state marked by a few, notable signatures: (i) an irreversible cell-cycle arrest, (ii) increased activity of senescence-associated β -galactosidase (SA- β -gal), and (iii) resistance to apoptotic stimuli [27]. The irreversible cell-cycle arrest persists even when there is an active mitogenic stimulus; this characteristic distinguishes the senescent cell from the simply quiescent one (that is, a reversible growth-arrest state) [27,28]. Over time, senescent cells undergo remarkable changes in their morphology and function. Morphologically, they become flattened and possess enlarged nucleus/multiple nuclei, cytoplasmic granularities, multiple vacuoles in the cytoplasm, and increase in cytoplasmic microfilaments (see Figure 1) [29,30]. These events could be the result of protein accumulation inside the cells due to lower proteasomal activity [31]. The second key signature of the senescent cell is SA- β -gal, a lysosomal enzyme found in large amounts, and also used as a senescent cell marker both *in vitro* or *in vivo*, since it highlights the cell with a bright blue color because of the increased β -gal activity.

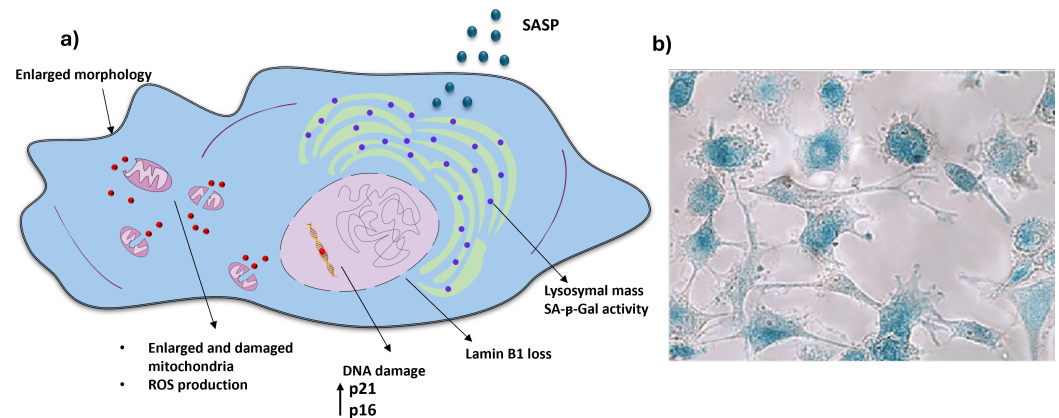


Figure 1. a) Morphological changes that happens within a senescent cell b) Senescent cells under Phase contrast microscopy. (From [36], w/ permission.)

The other hallmark of senescent cells affecting their metabolic activity is known as the senescence-associated secretory phenotype (SASP). The proteins secreted in this process form a diverse group, including inflammatory cytokines, chemokines, matrix metalloproteinases, and growth factors, which have biological functions that vary depending on the context [32,33]; exosomes and ectosomes containing enzymes, microRNA, DNA fragments and other bioactive factors can also be part of the SASP [34,35]. SASP can perpetuate senescence through autocrine and paracrine signaling, and it also triggers immune responses that may result in the eventual elimination of senescent cells. The inflammatory component of SASP is primarily driven by the transcription factors $\text{NF-}\kappa\text{B}$ and $\text{C/EBP}\beta$, which are activated in response to DNA damage, along with other interconnected signaling pathways, such as GATA4, mTOR, and the p38 MAP kinase [28].

Senescent cells can also accumulate enlarged and dysfunctional mitochondria. This could be due to a decrease in the membrane potential, increased proton leak, and reduced rates of fusion and fission. Altogether, these events disturb the ability to regenerate ATP and increase the production of ROS within the cellular environment; the increase in oxidative stress, in turn, results in misfolded proteins and protein aggregation. To accommodate these mishaps, the cell tries to enlarge its organelles and triggers various cascade pathways to rectify it [28].

3. Cellular senescence and cancer

Cancer remains a significant concern among researchers and medical professionals due to its high mortality rates, complex pathophysiology, and profound impact on public health and quality of life [37]. Numerous techniques are employed to treat cancer, including chemotherapy, radiotherapy, targeted drug delivery, and immunotherapy [38]. Among these, the most widely used treatments in a clinical context are radiotherapy and chemotherapy, which can be combined or used individually depending on the type and severity of the cancer [39]. When cancer has not metastasized and is in its early stages, radiotherapy is often the preferred treatment method; depending on the clinical condition, it can also be combined with chemotherapy, or used before surgery (neo-adjuvant radiotherapy). Even though radiotherapy has proven to be a very effective mode of treatment, similarly to any other treatment it is always associated with other health-related risks, majorly that of developing secondary cancers within or at the margins of the irradiated field [40,41]. Several factors (e.g., lifestyle, environmental, genetic) may be implicated in the development of second malignancies in cancer survivors, besides the type and dose of radiation administered [42]. However, the detailed molecular origins of the events linking the cancer recurrence to the extra dose given to (initially healthy) tissues are not yet elucidated (see below, Sect.6).

Cellular senescence has long been known to have a link with cancer. On the one hand, cancer cells acquire the outstanding capability to proliferate uncontrollably; by contrast, senescence with its distinctive characteristic of cell-cycle arrest, acts as a limiting criterion for cancer progression [43]. Oncogene-induced cell senescence (OIS) is a phenomenon triggered by an aberrant activation of oncogenes, especially members of BRAF and RAS families [44]. Such mitogenic changes overwhelm the cellular division mechanisms, by starting cascade pathways and unscheduled DNA replication. In response, the cell tries to induce proliferation arrest, by taking advantage of the beneficial role of senescence, which tends to switch off the malignant nature of cancerous cells.

Notably, cells can go into a senescent state not just due to cancer development, but also due to cancer treatments. Such clinical procedures as chemotherapy, radiotherapy and targeted drug deliveries are known to be able to induce cell senescence, which in this case is often termed "therapy-induced" cell senescence (TIS). Among the possible sources of TIS we find the exposure to chemotherapy drugs including alkylating agents [45], e.g., cisplatin, cyclophosphamide, and temozolomide; topoisomerase inhibitors [45] like doxorubicin, etoposide, and camptothecin; microtubule inhibitors like paclitaxel [46]; to a lesser extent, vinca alkaloids like vincristine [47]; and of peculiar interest for this review, the role of irradiation by photon or charged particle external beams [44,48,49].

Sustained sub-lethal injury as produced by radiation therapy or chemotherapy induces a continued oxidative stress and chronic inflammation, which prompt entry into cellular senescence. Irrespective of the cancer treatment, however, almost all such cancer therapies chiefly lead to molecular-level DNA damage, that is considered a primary inducer of senescence in both normal and cancer cells [50,51]. However, it is important to note that the level of DNA damage required to induce senescence may differ between malignant and non-malignant cells, in a way similar to the varying thresholds for apoptosis. For instance, patients with breast cancer were found to have an increased number of T-cells expressing p16^{INK4A} when administered with different chemotherapeutic agents, hence indicating immuno-senescence as a bystander effect [44,52]. Similarly, radiation therapy was found to lead to the up-regulation of p16^{INK4A} in non-malignant cells [53,54].

Induction of cellular senescence occurs through distinct molecular pathways, for example when comparing TIS and OIS. In fact, TIS results from the triggering of DNA damage-repair pathways leading to the p53 and p21^{WAF1/CIP1} activation, whereas OIS results from the activation of either, or both of the p53/p21^{WAF1/CIP1} and p16^{INK4A}/pRB tumor suppressor pathways, along with the participation of DDR and the Ras-Raf-MEK-ERK, PI3K/AKT/mTOR and p38/MAPK signaling pathways (see Figure 2) [55,56]. These proteins are essential components of the senescent evolution, as they inhibit CDK2-cyclin E and CDK4/6-cyclin D complexes and prevent the phosphorylation of the Rb (retinoblastoma) protein, thereby blocking the entry in the S-phase and inducing senescence [55,56].

It is worth noting that, while the tumor suppressor proteins p53/p21^{WAF1/CIP1} and p16^{INK4A} are primarily involved in TIS and OIS, senescence can also occur independently of these pathways. Additionally, p21^{WAF1/CIP1} can be activated by pathways other than p53 [57,58]. Also, it appears that prolonged over-expression of any of these four factors – p53, pRB, p16^{INK4A}, or p21^{WAF1/CIP1} – is sufficient to induce senescence [56]. Deregulated expression, mutations, secondary alterations or complete loss of tumor suppressor proteins may result in inadequate senescence induction, or escape from senescence [53,56]. The cell could then enter apoptosis, or reenter in a proliferation stage, depending on whether the damaged DNA is being efficiently repaired or not. Regardless of the cancer type, the level of senescence (whether it is of OIS, or TIS origin) appears to significantly influence the outcome for cancer patients [56,59]: it is definitely a tumor suppressive process, both by preventing cancer cell proliferation and suppressing malignant progression from pre-malignant to malignant, and can act as effector mechanism of many types of anticancer therapies by stimulating an immune response; however, senescence can contribute to reduced patient resilience to cancer therapies and may provide a pathway for disease recurrence after cancer

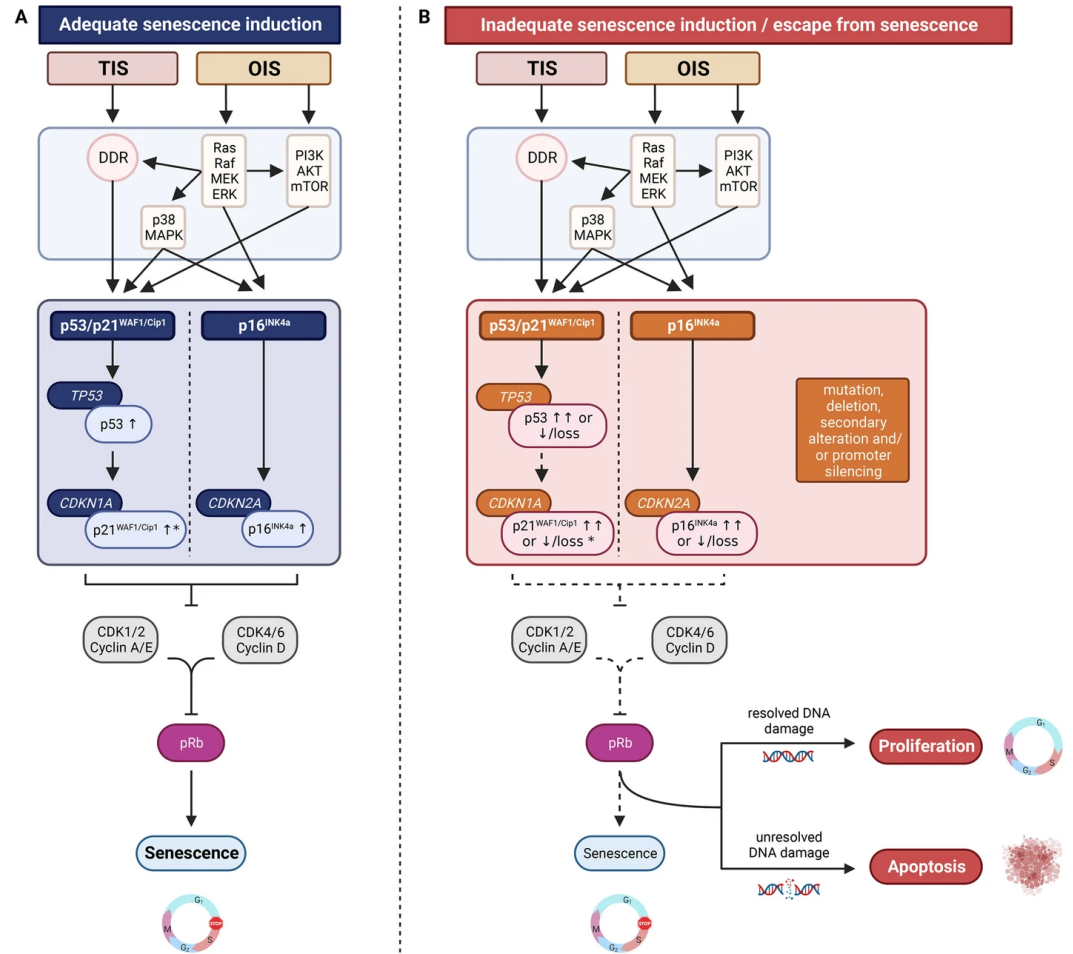


Figure 2. (A) Adequate senescence induction, and (B) Inadequate cell senescence pathway (From [56], w/ permission.)

therapy, to the point that specific *senolytic* drugs to suppress senescent cells are under active development [59–61].

4. DNA damage and Radiotherapy

During radiotherapy, ionizing radiation (IR) is targeted at cancerous cells by either an external beam of high-energy photons (x-rays, gamma-rays), electrons or heavier charged particles (protons, carbon ions); or by injecting radioactive species (alpha- or beta-emitters) complexed with molecular species aiming directly at the neighborhood of the tumor volume (internal radiotherapy). While photons are indirectly ionizing radiations, whose ionizing effect is carried by the secondary electrons set in motion during their interactions with the matter, charged particles are able to directly induce ionization in the target cells, which is the key to induce DNA damage, and ultimately lead to arrest or kill the cell [63,64]. IR inflicts damage on molecules, and most notably DNA, by imparting energy capable of causing electron ionization and excitation of molecular energy levels. The loss of an electron in ionization can be viewed as an oxidation process, which leaves behind a charged molecular species that can further evolve chemically. On the other hand, electronic excitation can lead to break up of the molecular species into neutral but highly reactive fragments, the free radicals (that is, molecular moieties with unpaired electrons).

Most of the energy transferred during radiation exposure is mediated by energetic electrons, set in motion by the interactions of photons or charged particles within the cell. Importantly, these ionization events are spatially confined along the tracks of the primary ionizing particles, or of the secondary electrons. An important difference between directly

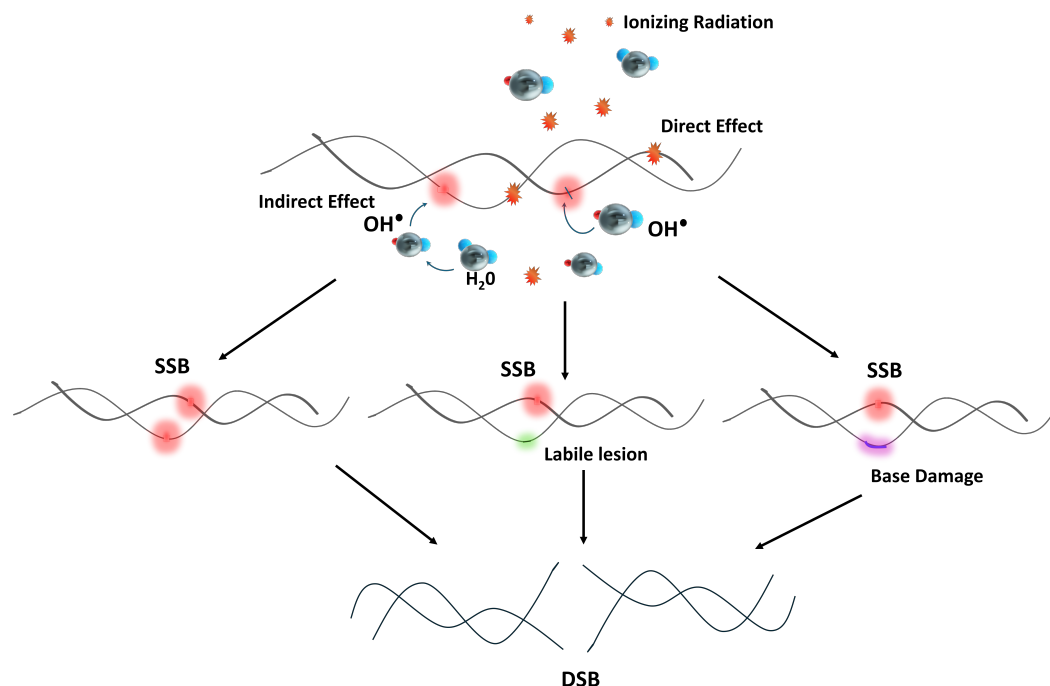


Figure 3. Ionizing radiation can directly attack the DNA or it can generate ROS via interacting with the water molecule (direct and indirect effect). These can in turn cause various types of damage in DNA. At the same time IR can also deposit a significant portion of its energy in the form of ionization clusters, which lead to clustered DNA damage. Double-strand breaks (DSBs) can form from these damage clusters either immediately, after chemical modifications of sugar lesions that initially don't disrupt the sugar-phosphate backbone, or following the enzymatic repair of base damage. (From [62], w/ permission.).

and indirectly ionizing radiation, is that neutral photons produce quite sparse ionization events (the linear energy transfer, LET, of a 1-MeV γ -ray in human tissue being about 0.2 keV/ μm , corresponding to about 5-6 ionization events per micrometer); sparse ionizations induce localized defects in the DNA molecule, such as abasic sites, single- or double-strand breaks (see below). On the other hand, heavy charged particles and low-energy electrons have a much higher ionization density (a 1-MeV proton has a LET of ~ 14 keV/ μm and produces about 400 ionizations per micron, that is several tens of thousands in a typical cell); moreover, charged particles create dense ionization clusters within a small volume near the ends of their tracks, called the "Bragg peak" [62,65–67]. Such dense ionization clusters produce more complex DNA defects ("clustered" damage), aside of localized defects (Figure 3).

Radiation-induced molecular damage to DNA, notably by photons, can occur mainly through two mechanisms: (i) *direct effect* or (ii) *indirect effect* [68]. The former is a one-electron oxidation reaction: the direct absorption of a photon (with frequency ν and energy $E = h\nu$) results in a secondary electron being released by an atom, thereby creating a radical cation species (indicated by a \bullet) localized on the DNA molecule; such radical is transferred within the DNA, eventually leading to the breaking of a chemical bond either in the phosphate backbone, in the ribose, or in the base itself [68] (Figure 3). By contrast, the indirect effect results from the formation of free radicals due to the radiolysis of water (e.g., $\text{H}_2\text{O} + h\nu \rightarrow \text{OH}\bullet + \text{H}\bullet$, or $\text{H}_2\text{O} + h\nu \rightarrow \text{H}_2\text{O}^+ + e_{aq}^-$) or other molecular components within the cell; both the free radicals (called ROS in the case of water radiolysis, since it is an oxygenated species) and the liberated electron in aqueous solution (e_{aq}^-) diffuse, and can attack the nearby DNA molecule. The interaction of free radicals with DNA leads to a complex variety of outcomes depending on many chemical details (notably, availability of free oxygen O_2), the main result being as well the breaking of the backbone phosphodiester

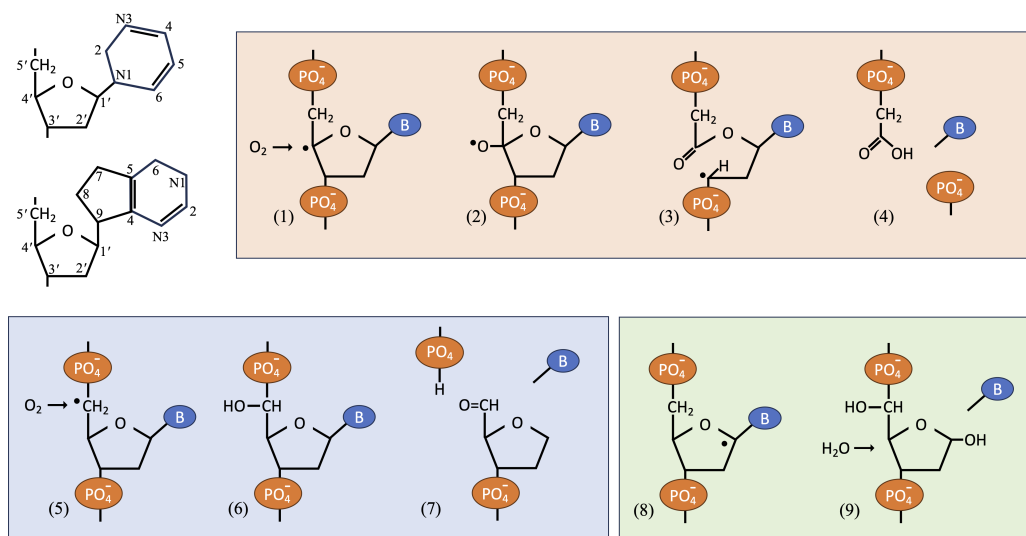


Figure 4. Top-left: numbering convention of the C and N atoms in the nucleotides. (1-4) Direct ionization at C4' in presence of O₂ leads to SSB with base release, and a phosphoglycolate (CH₂COOH) at the 3' terminal. (5-7) Direct ionization at C5' in presence of O₂ leads to SSB with base release, and an aldehyde (HC=O) at the 5' terminal. (8-9) Direct ionization at C1' followed by hydrolysis leads to base release and an AP-site.

bond, or the glycosidic bond with subsequent base release. In photon irradiation, the direct effect accounts for 30%-40% of the DNA lesions, while the indirect effect contributes 60%-70% [63]; by contrast, proton irradiation (as well as charged particles released in internal radiotherapy) relies much more on the direct effect, the indirect action being comparatively less relevant. Such chemical-molecular interactions create various types of DNA damage, each necessitating a different, specialized DNA repair pathway.

Breaks in the DNA backbone ("strand" breaks) can be classified based on the nature of the damage, irrespective of the specific chemistry (i.e., whether they are 5'/3' position). They are classified majorly as double-strand breaks (DSB), single-strand breaks (SSB), base damage (BD), and damage to the phosphodiester bond or ribose sugar in the DNA. A SSB is created when a random isolated break occurs only on one of the DNA complementary strands. This is commonly associated with oxidized/reduced base damage or base loss. When two SSBs occur in the opposite strands within less than 10bp apart (that is, one full DNA helix turn), the result is dubbed a DSB. The breaking of the N-glycosidic bond between the DNA and the ribose sugar results in the removal of the nitrogenous base, thereby creating an *abasic* (AP) site [69], the phosphate backbone remaining intact. It has been observed that during high-LET radiation, two or more ionizations within a few nm apart can be formed (ionization clusters), resulting in multiple lesions. Such multiple lesions can include more than 2 SSBs, AP-sites, or DSBs formed within the same, or opposite DNA strand from the same event of energy deposition. Such clustered lesions are rarely formed by endogenous stress, and are invariably associated with the toxicity of ionizing radiation (see Table 1) [62,70].

Free-radical attack on the ribose moiety of DNA can lead to the formation of both AP-sites and strand breaks. On the other hand, strand breaks in the phosphate backbone can occur mainly due to the formation and transfer of free radicals, either in the presence or absence of O₂ molecules. Usually, breaks are the result of an attack at the C4' or C5' (less frequently the C1') carbon atom sites [68], the radical being transferred from one site to another, until the condition is energetically favorable to break the phosphodiester bond (Figure 4). In either case, the strand break also results in the loss of the corresponding base, and can further evolve into more complex damage, notably a double-strand break [71]. In general, this attack leads to the formation of "clean" 3'-phosphate and 5'-OH terminals, and less likely to a 3'-OH and 5'-phosphate pair; it is also often observed, notably in the

presence of oxygen, that attack on 5' carbon can give rise to either 5'-aldehydes or 3'-phosphoglycolate (PPG) terminations, or other "dirty" ends [71,72]. It is worth noting that dirty ends are virtually unprocessable, as DNA-ligases cannot reseal a SSB or a DSB unless the ends are clean [73]. For example, tyrosyl-DNA phosphodiesterase can require several hours to remove PPG from 3' DNA ends [74].

Type of damage	Radioinduced damage per cell per Gy	Endogenous damage per cell per day
Single strand breaks	1000	> 10000
Base damage	2000	3200
Abasic sites	250	12600
Double strand breaks	40	40-50
DNA-protein cross-links	150	?
Non-DSB clustered lesions	122	?
Complex DSB	?	?

Table 1. DNA damage induced by ionizing radiation. (From [70].)

5. DNA damage repair pathways

Irrespective of its origin, DNA damage triggers a specific chain of multi-enzyme reactions known as the DNA damage repair (DDR) pathway, which defends against genomic instability and accumulation of mutations. Cells have developed multiple complex DNA repair pathways, no less than seven different variants which enable them to identify the type of damage, and repair DNA to the correct sequence if possible [75,76]. Even though these pathways are complex, all of them follow a common workflow performed by certain specific molecular components. This can be summarized as follows [77]:

1. recognizing the DNA damage: this is performed by DNA damage sensor proteins (like Poly(ADP)Ribose-1 protein; Ku70/80, γ -H2AX etc. [78]);
2. recruiting repair proteins and excising the damaged segment: this is performed by transducer proteins, which typically include a repair protein bound to a scaffold protein forming a complex at the site of lesion; this complex thereby removes the damaged segment and restores the correct 3'-hydroxyl 5''-phosphate terminations;
3. re-synthesizing the missing parts of the DNA sequence: this is done by different effector proteins, which add new nucleotides;
4. finally, ligation of the clean broken ends.

Each of the distinct pathways is dedicated to repairing a different type of DNA damage. Among these, Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) are critical for the repair of double-strand breaks (DSBs), while Base Excision Repair (BER) and Single-Strand Break Repair (SSBR) address damage to individual bases and single-strand breaks, respectively.

It is estimated that a human cell suffers $10^4 - 10^5$ DNA lesions each day, from either endogenous processes or environmental sources, which are constantly repaired with high efficiency. Among these, SSBs are one of the most frequently occurring lesions [80,81]. It is well established that SSBs are, in principle, far less lethal than DSBs, and are repaired quickly [79,82], with a relatively small rate of non-repaired defects compared to DSBs (Figure ??). This is mainly due to the fact that SSB repair pathways dispose of the intact complementary sequence of the undamaged DNA strand to perform a correct repair, whereas that information is not available when both DNA strands are affected. However, even a small fraction of non-repaired SSBs can interfere with gene transcription [83,84]; the most likely consequence of non-repaired SSBs in proliferating cells is the blockage or collapse of DNA replication forks during the S-phase, possibly evolving into DSBs [84,85]; eventually these events can lead to apoptosis [84,86].

It is also important to realize that the relative amount of SSBs generated during the cell exposure to endogenous and exogenous insults plays a major role. For instance, during exposure to x-ray photons, around 50-100 SSBs are generated for each DSB, per Gy of dose delivered [87]; Monte-Carlo simulation studies of DNA damage due to proton irradiation showed that at high proton energy about twice as many SSBs are formed, compared to

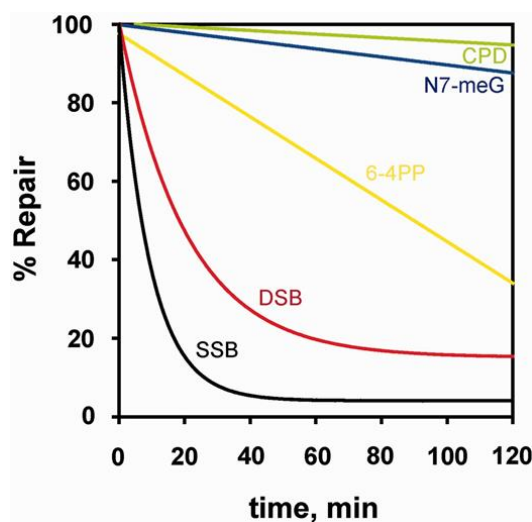


Figure 5. Kinetics of repair of different types of DNA lesions. Shown is the kinetics of removal from CHO-AA8 cells of SSBs, DSBs, 6–4 photoproducts (6–4PP), cyclobutane pyrimidine dimers (CPD) and, for human lymphocytes, of N7-meG. Note the two curves for SSB and DSB, which do not go to zero but rather saturate at some constant value at long times, signaling some fraction of non-repaired defects. (From [79], w/ permission.)

the fraction of DSBs [88]. In general, x-ray-induced DSBs, as detected by the formation of 53BP1 foci, are quickly and more dynamically resolved than those induced by protons and α -particle radiation, due to the high ionization density of the latter [89]. Traditionally, most studies focused the attention on DSBs because of the major danger they pose to the cell, also accompanied by more fault-prone repair pathways, and a higher rate of non-repaired defects for a given amount of initial damage. However, also the role of SSBs is increasingly coming under scrutiny (see, e.g., [53,90–94]). Despite a very quick and efficient SSB repair machinery, induction of a very large amount of SSBs can lead to saturation of the repair pathway and increase the fraction of non-repaired defects, resulting in DNA replication stress, transcriptional stalling [95,96] (especially for SSBs with dirty ends), and excessive PARP activation [97,98], leading to genome instability with an overall toxicity likely equivalent to more harmful types of damage.

Altogether, any SSB which is not repaired or "tolerated" during the entire repair pathway has a potential to change into a more lethal DSB. Considering the prevalence of endogenous SSBs, for example produced as intermediate steps during the base-excision repair (BER) of a damaged nucleotide, it is therefore understandable that cells have developed highly effective mechanisms to reduce their effects. To ensure that SSBs are entirely repaired before the start of DNA replication in mitosis, cells need to detect the SSB, along with inducing the repair pathway and delay the cell cycle progression, but there is no well-established evidence yet to support the existence of such a signaling pathway [99]. Hence, understanding how cells navigate this intricate repair process is not only crucial for grasping fundamental cellular biology but also for uncovering potential therapeutic strategies in disease management. To preventing genomic instability and ensuring cellular homeostasis, SSBs are repaired by a peculiar pathway (single-strand break repair, SSBR), which partly overlaps with the BER pathway [100]. The upcoming subsections will delve into the molecular intricacies of BER and SSBR to highlight their pivotal roles in safeguarding DNA integrity in the case of SSB damage.

5.1. Base Excision Repair Pathway

The BER pathway rectifies small base damage that does not significantly contribute to the distortion of the DNA helix. ROS species mainly induce these small lesions resulting in oxidized bases, such as the well-known 8-oxo-G, oxidative deamination and dealkylation,

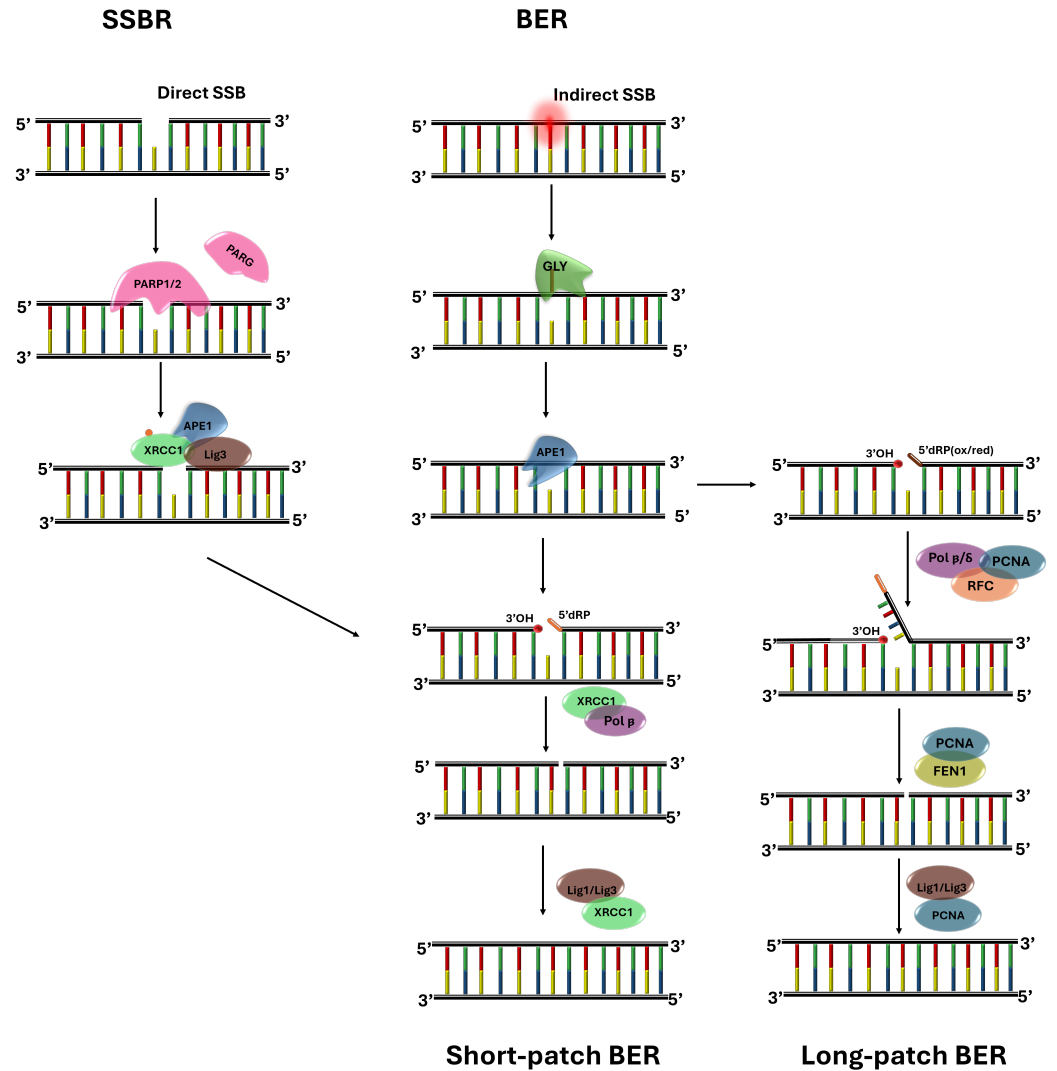


Figure 6. Model for BER and SSBR pathway: During base excision repair (BER), most abasic sites are processed by APE1, which cleaves the site and recruits Pol β . Pol β then inserts a single nucleotide and repairs the 5'-deoxyribose phosphate (dRP) left by APE1, creating a ligatable nick. This nick is sealed by XRCC1-Lig3 α . In cases where Pol β cannot remove the 5'-dRP (such as when it is oxidized or reduced), the repair is stalled. At these stalled sites, Replication Factor-C(RFC), Pol- δ/ϵ , PCNA are recruited to extend the gap by several nucleotides, and FEN1 cleaves the resulting flap. Ligation is then completed by Lig1. Direct single-strand breaks (SSBs) from sugar damage, and some SSBs arising during BER, are recognized by PARP-1 or PARP-2. PARP then recruits phosphorylated-XRCC1 and Lig3 α to form a scaffold for repair. APE1 or PNK processes the damaged termini into 5'-phosphate and 3'-hydroxyl groups, aided by XRCC1. Pol β fills the gap with a nucleotide, followed by ligation by Lig3 α .

and more than 20 other different oxidation products [101,102]. BER requires majorly 4 or 5 enzymes to carry out the DNA repair process (see Figure ??). It is initiated by removing the damaged base by one of the 11 mono- or bi-functional glycosylases [103], depending upon the type of defect. This process forms apurinic or apyrimidinic sites (AP) sites. The hanging AP-site is then cleaved by an AP-endonuclease enzyme, which generates a 3'-OH and 5'-deoxyribose phosphate (dRP) group at the break site. Notably, at this stage the BER has created a SSB as intermediate in the repair chain, which makes the overlap with the SSBR repair. The subsequent step of filling the single-nucleotide gap can follow either a long- or short-patch repair. Short-patch repair is considered more dominant; long-patch repair is observed in post-replicative BER initiated by UNG2 or NEIL1 glycosylases expressed during the S phase. Long-patch repair is also observed when Pol- β cannot remove the 5'-dRP terminus (e.g., if the dRP is oxidized or reduced).

In long-patch base excision repair, the nucleobase along with several adjacent nucleotides is replaced through the action of polymerase- δ (Pol- δ) and polymerase- ϵ (Pol- ϵ) in conjunction with proliferating cell nuclear antigen (PCNA). The displaced strand is excised by Flap-endonuclease 1 (FEN1), and the DNA backbone is subsequently sealed by DNA ligase-I (LigI). By contrast, the third step of short-patch repair does not involve the addition of several nucleotides, DNA polymerase- β (pol β) adds just a single nucleotide to the damaged site, leaving a nick between two adjacent nucleotides. Finally, the nick is ligated by the DNA ligase-III, along with the help of X-ray repair cross-complementing protein-1 (XRCC1), which serves as a scaffold for ligase-III.

The decision making process underlying the choice short-patch repair vs. long-patch repair pathway is still under research [100,104,105]. Long-patch repair pathway is frequently observed in proliferating cells and use of replicating proteins like Pol- ϵ/δ , FEN1, PCNA and Lig-I [106]. One of the hypothesis is that it depends on the ATP concentration near the AP site, which is controlled by the Lig-III and XRCC1, with some studies showing that the long-patch repair is preferred during higher ATP concentration [107]. A few other studies purport that the decision depends on the initiating glycosylase enzyme available, and the type of damage. For instance, if the 5'-dRP intermediate produced by the APE1 is successfully removed by the Pol- β , then short-patch BER is preferred, otherwise it will follow the long-patch repair pathway [100,108].

5.2. Single-strand break repair pathway

Typically, when a SSB is formed by direct or indirect radiation damage it is readily accompanied by the loss of a single nucleotide at the broken phosphodiester bond (see Fig. 4 above). Such a defect in the DNA strand is structurally very similar to the intermediate strand-break produced during BER, apart from the 5' and 3' terminations, which in the former case may be irregular. Irrespective of their origin, however, all the SSBs are sensed by poly(ADP)ribose polymerase (PARP) enzymes, which are catalytically activated. PARP proteins are particularly interesting because of their key role in identifying the broken DNA ends, notably in both SSB and DSB.

The PARP superfamily is a group of chromatin-associated proteins sharing several microscopic features and mechanisms of action. The well-studied PARP-1 consists of at least six functional domains: three DNA-binding Zinc-finger N-terminal domains named Zn1, Zn2 and Zn3; one BRCT domain; one WGR domain; and one catalytic C-terminal domain, including a helical subdomain (HD) [109]. PARP-1 binding to the SSB site activates a complex sequence of allosteric and cooperative effects between the different domains, which are not yet completely elucidated. The Zn1 and Zn2 are known to specifically recognize DNA breaks. Zn1 from one PARP-1 copy may also cooperate with Zn2 from another PARP-1 protein to form a dimeric module that specifically recognizes DNA breaks [110]. The detailed mechanism by which PARP-1 identifies the SSB is not entirely clear [111], in particular it is yet to be understood to what extent PARP-1 may be sensitive to dirty SSB ends occurring in radiation-induced radical attack. On the other hand, Zn3 mediates as an inter-domain contact and is required to confer with PARP-1, to regulate chromatin

structure [112]. The BRCT domain acts also as a DNA binding domain, but of lower affinity, and is able to bind only intact DNA without concomitant catalytic activation. The BRCT-DNA interaction mediates DNA intra-strand transfer of PARP-1 (the so-called "monkey-bar mechanism") that allows rapid movements of PARP-1 through the chromatin [113]. By analogy with the action of PARP-2, it is assumed that the WGR domain of the sister protein domain of PARP-1 can bridge two nucleosomes with the broken DNA ends aligned in a position suitable for ligation. Such bridging induces structural changes in PARP-1 that signal the recognition of a DNA break to the catalytic domain of PARP-1. This, in turn, promotes the recruitment of Histone PARylation factor-1 (HPF1) and subsequent activation of PARP-1, followed by licensing serine ADP-ribosylation of target proteins [114,115]. The HD subdomain prevents effective NAD⁺-binding in the absence of an activation signal; however, after binding to damaged DNA, the self-inhibition is relieved, HD unfolds, and PARP-1 becomes able to bind NAD⁺, thus starting PARylation [116,117].

In the PARylation process, PARP1 sequentially transfers a large number of ADP-ribose molecules onto itself or to other proteins, producing long chains of poly(ADP)ribose (PAR) units (see Figure ??). The accumulated PAR chains, in turn, favor the recruitment of XRCC1. It is also worth noting that these PAR chains are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG), and PARP1 can be recycled for subsequent detection of SSB.

Once XRCC1 is phosphorylated, it acts as a scaffold protein for recruiting enzymes required to repair SSBs. The key role of XRCC1 is indicated by the dramatic reduction of SSBR activity observed in cells lacking this protein [118]. Human XRCC1 is a molecular scaffold protein 633 amino acids in length [119], with an asymmetric, elongated shape (axial ratio of > 7 [120]), and three main domains: (1) its N-terminal domain (NTD) of about 160 amino acids interacts with Pol- β [121]; (2) the central BRCT domain of about 90 amino acids interacts with PARP-1 [121], PARP-2 [122], poly(ADP-ribose) [123], and DNA [124]; and (3) the about 100 amino acids of the other BRCT domain at the C-terminal binds DNA ligase-III [125]. These three main domains are connected by two linker domains: the first one contains a nuclear localization signal and phosphorylation-independent binding site for PNKP [126]; the second one includes a phosphorylation-dependent binding site for PNKP [127], APTX [128], and APLF [129]. XRCC1 reportedly also binds a number of additional proteins, the interactions of which are less well defined.

The XRCC1-initiated end-processing is the most diverse step of the SSBR repair, with an impressively large variety of enzymes available to deal with the many variants of "dirty" SSB ends. End-processing enzymes like polynucleotide kinase phosphatase (PNKP) and aprataxin (APTX) interact with phosphorylated XRCC1, DNA, Pol- β and DNA ligase-III. PNKP resolves special terminals like 3'-Pho /3'-PPG, while APE1 resolves 3'- α , β unsaturated aldehyde, all formed during irradiation. At the same time, Pol- β colocalize with XRCC1 to help with polymerase activity, notably in the case of oxidized 5'-dRP formed by indirect attack by ROS; the enzyme responsible for 5'-aldehyde treatment is still unknown [130].

Eventually, the entire process is terminated by nick sealing activity of ligase-III α [131]. Hence, it appears that the final steps of the SSBR pathway overlap the short-patch BER pathway, as two meticulously designed pathways crucial for repairing both the exogenous SSBs and oxidative damages in the DNA.

6. Radiation-induced single-strand breaks and cellular senescence

External-beam radiotherapy of cancer can impact various portions of tissues, through both direct and indirect mechanisms. The radiation beam is designed in such a way to maximize the dose (energy delivered per unit mass) to the tumor region as identified by the clinical treatment volume (CTV), while reducing ideally to zero the extra dose on the healthy organs-at-risk (OAR) surrounding the CTV [132]. Despite a great deal of effort in improving the irradiation geometry, and space and time fractioning of the radiation dose, however, some fraction of the beam will affect the region in the immediate vicinity of the CTV, also because of the error margin accounted for in the definition of the planning

treatment volume (PTV). The latter represents the zone actually being irradiated, and typically extends a few mm around the CTV, necessarily touching at the borders of some OAR. Therefore, clinical limits have been assessed in the literature for each organ of the human body, in order to maintain the damage to OARs within acceptable limits (see e.g. [133]).

Traditionally, it was believed that the DNA damage should be observed only in the irradiated area, due to the direct projection of the radiation field. However, this picture started to be questioned once chromosomal damage and changes (mutations, translocations) were observed in cells at nearby non-irradiated area, or even distant tissues [134,135]. Furthermore, even oxidative stress is not confined to the targeted irradiated area but is found to affect also neighboring, non-irradiated cells through inter cellular communication, a phenomenon known as the "bystander" effect [136]. Such effects, alone or in combination, may also increase the carcinogenic risk of the distant tissues, resulting in radiation-induced secondary malignancies (RISM) [40,137,138], also called second primary cancers (SPC). The latter definition is meant to stress the notion that such malignancies do not represent a recurrence of the primary cancer, instead they are newly transformed cancerous cells originating from the normal cells which were affected by the stray radiations. Although the causal relationship between the initial radiation therapy and the induction of the SPC is impossible to definitely prove, these second cancers are often observed to develop preferentially near the margins of the irradiated zone (the so-called "penumbra" segment where the dose profile drops continuously from 100% to zero), rather than within the PTV (where 100% of the irradiation dose is theoretically administered) [139].

The penumbra regions are exposed to scattered radiation from various sources, such as leakage from the machine jaws and multi-leaf collimators that shape the beam, the flattening filter that ensures uniform radiation dose field, and scattering that occurs inside the patient's body [140]. The internal scattering has been identified as the primary factor influencing the dose deposited in the closest margin around the target area [140]. This marginal radiation has three key traits: (i) the dose decreases exponentially as the distance from the target increases, (ii) the dose is roughly proportional to the size of the PTV, and (iii) the energy spectrum of the photons shifts toward lower energies compared to those within the PTV [140]. In addition to their spatial relationship with the PTV, another remarkable feature of SPCs is their extended latency period, which can range from a few years to as much as 40 years according to the initial cancer treatment [40]. This suggests that normal cells exposed to low-energy scattered radiation may remain dormant in the body while still retaining the potential for neoplastic transformation. However, the exact biological nature of this dormant state remains unknown. In a number of studies, we suggested it could be related to a form of TIS cell senescence [25,53,140–142].

It was observed that in keratinocytes undergoing spontaneous senescence, oxidative stress downregulates PARP-1 expression, thereby reducing the synthesis of PAR. This reduction impedes the recruitment of CK2 to the damage site, arresting the repair process at the step of XRCC1 recruitment [25]. Similarly, in fibroblasts exposed to radiation doses outside the PTV, PAR synthesis is also diminished, although the molecular mechanisms remain unclear [53]. Recent studies suggest that out-of-field IR may directly influence the expression of enzymes such as nicotinamide phosphoribosyl-transferase (NAMPT) and nicotinamide mononucleotide-adenylyltransferase-1 (NMNAT1), which are responsible for synthesizing NAD⁺, a substrate required by PARP-1 for PAR synthesis during DNA damage. Additionally, out-of-field IR could increase the production of poly(ADP-ribose) glycohydrolase (PARG), which degrades PAR. As a result, PAR formation declines significantly. Despite this reduction, research has shown that even low levels of PAR are sufficient to recruit unphosphorylated XRCC1. This unphosphorylated XRCC1 accumulates at the SSB sites and triggers the p38 mitogen-activated protein kinase (p38MAPK) pathway, leading to the upregulation of p16, a key factor in cell cycle arrest [141].

In fibroblasts exposed to out-of-field IR, there is also an upregulation of p21, which contributes to cell cycle arrest (Figure ??). However, the exact mechanism driving this

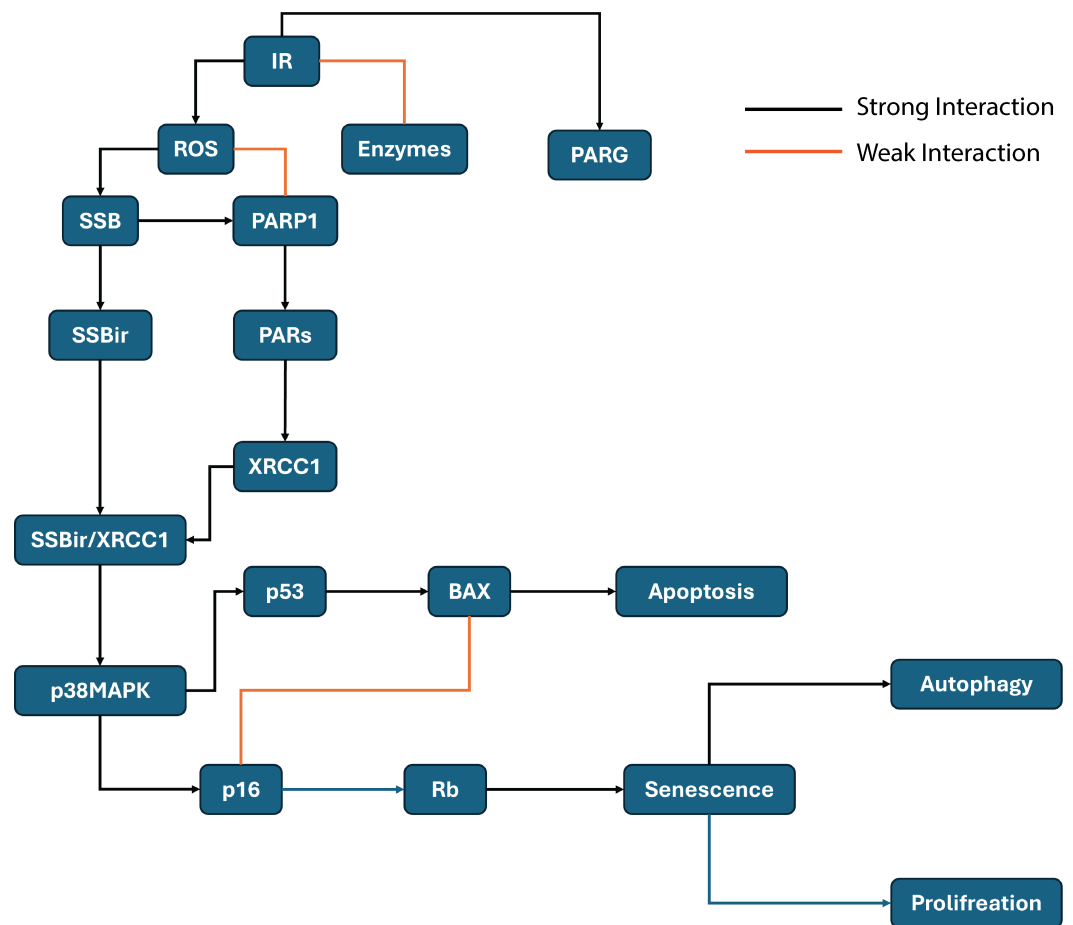


Figure 7. SSBR pathway followed by the keratinocytes in the margin of irradiated cell. SSBir indicates unrepaired SSB (From [25,53], w/ permission.).

upregulation remains unknown. Once the p38MAPK pathway is activated, it can either promote p16 or p53. While p53 often leads to apoptosis, p16 drives cells towards senescence. Experimental data indicate that cells preferentially enter senescence over apoptosis, possibly due to the inhibition of p16 on BAX, a pro-apoptotic factor [143]. This preference for senescence over apoptosis is particularly evident in conditions where SSBs are the prominent defects. Senescent cells are typically cleared by autophagy, a major lysosomal degradation pathway. However, in certain cases, such as irradiated fibroblasts located near the PTV irradiated region, cells may enter a state of premature senescence and later escape into a proliferative state which is also termed as inadequate senescence, or escape from senescence.

Generally, senescence can be established by an accumulation of DNA breaks. In [53] it was observed that fibroblasts irradiated in the penumbra region retain a rather large fraction of unrepaired SSBs and almost no DSBs, associated with an upregulation of p16; this same observation had been previously made also for keratinocytes [25]. The question why SSBs are not fully repaired in this radiotherapy context is not well understood. We established in [53] that the SSB repair capacity declines with the daily irradiations of a fractionated protocol, in correlation with a decline in the PARylation capacity.

On the other hand, it has been reported that an excessive accumulation of SSBs can induce cell death through a prolonged activation of PARP-1. This leads to depletion of NAD⁺ and ATP, with the release of apoptosis-inducing factor (AIF) from mitochondria [97]. This type of cell death is relevant to a number of pathological conditions involving oxidative stress, such as post-ischaemic brain damage from stroke [98].

7. Discussion

In this brief review we focussed the attention on the relationship between cell senescence and DNA damage accumulation, most notably in the context of radiotherapy-induced molecular damage by either direct or indirect effect. One main motivation was to introduce and rationalize in a broader context some recent findings, according to which accumulation of non-repaired single-strand breaks (SSBs), apparently originated by out-of-field (unwanted) irradiation of initially healthy tissues surrounding the primary treatment volume (PTV), can drive cells into a senescent state. Escape from such a state at very later times, even on the scale of many years after the initial radiotherapeutic treatment, could be at the origin of second primary cancers, that is, cancers located in or near the irradiated volume but carrying a different genotype with respect to the original one. To this end, we provided a synthetic description of the radiation-induced damage and the relative DNA damage repair pathway that are activated.

For the case of SSBs, we highlighted the partial overlap existing between the base-excision repair (BER), typically involved in curing the oxidative damage, and the SSB repair pathway (SSBR). Cells with impaired repair capability, or lacking some of the key proteins implicated in these two pathways, typically show a higher propensity for developing cancer and other diseases. The correlation between accumulation of non-repaired SSBs and senescence could be the first scenario that could explain at the same time the latency period of second primary cancers (SPC), favored by the long life of senescent cells, and the location of SPCs in the regions surrounding the PTV, underpinned by the accumulation of non-lethal but non-repaired SSBs. The atomistic origins of this phenomenon are far from being understood. That DSB population decays very quickly at the borders of the PTV is a readily expected finding, on the basis of the exponential dose profile; however, the persistence of SSB concentration at long distances (several mm) away from the irradiated zone, is something that escapes a mechanistic explanation purely based on irradiation geometry and radiation diffuse scattering. Other factors related to cell metabolic pathways must be at play in this context, e.g., some kind of interference induced by a "bystander" effect from molecules secreted by the cells in the PTV, which affects DNA repair pathways even at some far distance.

The attention of the DNA radiation damage community has been traditionally focused on the study of oxidative damage pathways and the production of DSBs, because of their dramatic impact on molecular structure and genomic stability. The studies reviewed in the present work draw the attention also on SSBs, typically assumed to be of no consequence because they are easily repaired and do not affect, at first stance, the genome stability. The intriguing relationship arising from recent experimental results suggests that cells escaping the lower threshold for DSB damage (which would actually drive them to apoptosis) can indeed adapt to some amount of non-repaired SSBs, and survive for a long time by adopting the senescent phenotype, thereby becoming a reservoir of potentially tumorigenic mutations.

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