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Review

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 can occur during radiation therapy treatments. Recent in vitro cell irradiation experiments using high-energy photons have shown that single-strand breaks may be preferentially produced at the borders of the irradiated region, inducing senescence in competition with the apoptosis end-point typically induced by double-strand breaks. Such a particular response to radiation damage has been proposed as a possible cause of radiation-induced second primary cancer, as cells with an accumulation of non-repaired single-strand breaks might evade the senescent state at much later times. In addition, we highlight the peculiarities of strand-break repair pathways in relation to 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

Citation: Sarma, P.A.; Abbadie, C.; de Launoit, Y; Cleri, F. Cell Senescence and the DNA Single-Strand Break Damage Repair Pathway. *DNA* **2024**, *4*, 530–552. https://doi.org/10.3390/dna4040036

Academic Editor: Karen M. Vasquez

Received: 8 November 2024
Revised: 27 November 2024
Accepted: 2 December 2024
Published: 9 December 2024

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 indicates the arrest of cell divisions after a limited number of doublings (in the range of about 50 iterations, which has become known as the "Hayflick limit" [1]). In the 1980s, Elizabeth Blackburn and Carol Greider discovered that 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 has been 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, leaving the DNA ends exposed. This triggers the DNA repair pathway, eventually leading to cell senescence or cell death [4,5]. This chain of events has been referred to as replicative senescence (RS).

Senescence was once thought to be just a flaw in tissue culture techniques, but has subsequently been recognized as a critical process in both physiological and pathological contexts [6,7]. It must be noted that cellular states identical or similar to RS can be reached by cells subjected to various signals or stresses independent of their telomere status. Signals can occur during embryonic development, in which case senescence is referred as developmental senescence. When senescence is induced by non-physiological stresses, it is referred as stress-induced premature senescence (SIPS). These stresses can be either endogenous or exogenous,

including abnormal mitogenic signals, radiation, oxidative and genotoxic stress, etc. [8,9]. A subtype of SIPS is oncogene-induced senescence (OIS) resulting from the activation of certain oncogenes, especially members of the RAS family (notably KRAS and BRAF). Another subtype of SIPS is therapy-induced senescence (TIS), a term generally restricted to senescence induced by anticancer therapies. A mechanism common to all these stress factors is that they induce cell senescence by stopping cells from proliferating in response to DNA damage, while these same cells remain metabolically active and adopt a specific immunogenic phenotype [10].

Cellular senescence plays both beneficial and harmful roles. On the one hand, it has key physiological functions in normal development, tissue remodeling, and wound healing; in addition, it limits tumor progression by preventing damaged cells from proliferating. On the other hand, the entire process of senescence is one of the main reasons for cellular aging, as senescent cells have a modified secretome, known as the senescence-associated secretory phenotype (SASP). The SASP is enriched in growth factors, inflammatory cytokines, and matrix-remodeling enzymes that lead to tissue deterioration, in turn leading to aging and age-related diseases. The accumulation of senescent cells can impair tissue repair and regeneration, and can also deplete stem and progenitor cell reserves [9,11–16]. Notably, SIPS is relevant to both normal cells and cancer cells, as it relies mainly on the DNA damaging effect of reactive oxygen species (ROS). ROS affect all cell components, including DNA, leading to a host of senescent characteristics. Notably, telomeres can be affected in SIPS contexts, as they are especially sensitive to oxidative damage. An important related subclass of senescence, often dubbed therapy-induced senescence (TIS) resembles SIPS, occuring mainly as a consequence of oxidative stress and direct DNA damage induced by different anticancer therapies.

Several excellent reviews on the main mechanisms and different aspects of senescence have been published recently; see, e.g., [17–24]. The present paper is focused on the correlation between SIPS and DNA damage. In particular, we highlight the peculiar role played by single-strand breaks (SSBs) with regard to double-strand breaks (DSBs) produced in the DNA backbone by both endogenous sources and exogenous attack, notably in the context of therapeutic irradiation with energetic photon and particle beams. One important motivation for this focus is the indication provided by previous studies [25,26], according to which the DSB/SSB ratio is very important in cell outcome determination. On the one hand, normal or cancerous cells exposed to the therapeutic dose delivered in the targeted tissue volume accumulate substantial fractions of both DSBs and SSBs, and usually undergo apoptosis; on the other hand, cells localized at the borders of the targeted volume and receiving the out-of-field dose appear to accumulate many more SSBs than DSBs, which favors cell senescence if some fraction of these SSBs are not promptly repaired. Although the origins of this non-repaired damage accumulation are yet to be elucidated, they constitute interesting and surprising evidence that could possibly open the way to novel therapeutic strategies that might decrease the risk of secondary radiation-induced neocancers.

The remainder of this review 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 respectively provide a review of DNA damage and repair pathways, with a focus on radiation-induced damage and the interrelated base-excision repair (BER) and single-strand break repair (SSBR) pathways; finally, Section 6 introduces the link between cell senescence and accumulation of SSBs in irradiated cells, while 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, of which the main are: (i) a stable cell-cycle arrest; (ii) an increased activity of senescence-associated β-galactosidase (SA-β-gal); (iii) a modified secretome, the SASP; and (iv) a resistance to apoptotic stimuli, making senescent cells long-lived [27]. Cell cycle arrest of senescent cells persists even when

there is an active mitogenic stimulus; this characteristic distinguishes the senescent cell state from the quiescent one, that is, a reversible growth-arrest state [27,28]. Over time, senescent cells undergo remarkable changes in their morphology and functions. With the exception of the development and wound healing context, they undergo DNA damage, some of which can remain unrepaired and accumulate, leading to a permanent activation of cell cycle arrest pathways. Senescent cells also undergo complex epigenetic modifications that can result in the formation of heterochromatin foci. Morphologically, they become enlarged and flattened, with an enlarged and distorted nucleus. Parts of senescent cells can even become polynucleated. Changes in cell shape are associated with both quantitative and qualitative reorganization of cytoskeletal elements (see Figure 1) [29,30]. The second key signature of a senescent cell is increased SA-β-gal activity. β-galactosidase is a lysosomal enzyme found in larger amounts in senescent cells, reflecting increases in lysosomal mass and autophagic activity. These events can result in part from the accumulation of altered proteins due to decreased proteasomal activity. Thus, increased SA-β-gal activity is very often used as a senescent cell marker both in vitro and in vivo, based on specific substrates that generate blue or fluorescent products [31].

The other important hallmark of senescent cells reflecting their multiple changes in metabolic activities is the SASP, an acronym referring to the quantitative and qualitative changes in the secretory activity of senescent cells compared to their proliferating counterparts. Proteins that are oversecreted during this process may include inflammatory cytokines, chemokines, matrix metalloproteinases, and growth factors, which have biological functions that vary depending on context [32,33], as well as exosomes and ectosomes containing enzymes, microRNA, DNA fragments, and other bioactive factors such as NO and ROS [34]. The SASP can perpetuate senescence through autocrine and paracrine signaling, and also triggers immune responses that may result in the eventual elimination of senescent cells. The expression of the inflammatory proteins is primarily transcriptionally controlled by the transcription factors NF-κB and C/EBPβ, which are activated in response to DNA damage, along with other interconnected signaling pathways such as GATA4, mTOR, and p38 MAP kinase [28].

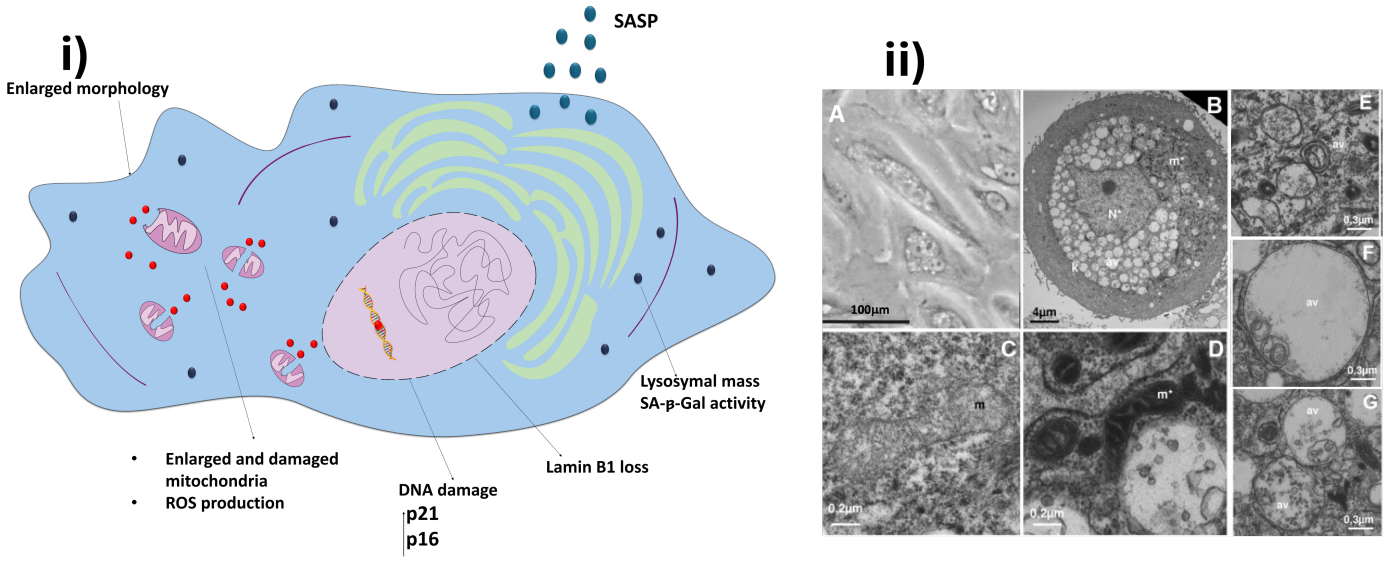

Figure 1. (**i**) Schematic representation of the main morphological changes that happen within a senescent cell. (**ii**) (**A**) Senescent keratinocytes under phase-contrast microscopy; note the granular and vesicular-like material around the nucleus and the polynucleation (from [35], w/permission). (**B**) Keratinocytes at the senescence plateau; N* is the deformed nucleus of a senescent cell and m* indicates mitochondria aggregated close to the nucleus. (**C**,**D**) Details of mitochondria morphology in the growth phase and at the senescence plateau, respectively. (**E**–**G**) Details of autophagic vesicules (av) in keratinocytes at the senescence plateau (from [36], w/permission).

Senescent cells can also accumulate enlarged and dysfunctional mitochondria. This could be due to a decrease in the mitochondrial membrane potential, increased proton leakage, 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; in turn, increased oxidative stress results in misfolded proteins and protein aggregation. To accommodate these defects, the cell tries to enlarge its organelles and triggers various cascade pathways to rectify the situation [28].

3. Cellular Senescence and Anti-Cancer Therapies

Cancer remains a significant concern among researchers and medical professionals due to its high mortality rates, complex physiopathology, and profound impact on public health and quality of life [37]. Numerous techniques are employed to treat cancer, including surgery, conventional chemotherapy, radiotherapy, targeted therapy, and immunotherapy [38]. Among these, the most widely used treatments in a clinical contexts are radiotherapy and conventional chemotherapy, which can be combined or used individually depending on the type and severity of the cancer [39]. When cancer is not invasive, has not metastasized, and is in its early stages, surgery and radiotherapy are often the preferred treatment method. Depending on the clinical condition, radiotherapy can also be combined with targeted or conventional chemotherapy or used before surgery (neo-adjuvant radiotherapy). Even though radiotherapy has proven to be a very effective mode of treatment, like any other treatment it is always associated with side effects. While rare, one harmful side effect of bad prognosis is the development of second primary cancers within or along the margins of the irradiated field [40,41]. These cancers are not a recurrence or metastasis of the primary cancer, but are rather newly-formed cancers originating from normal cells that were somewhat affected by the preceding irradiation. Several additional factors (e.g., lifestyle, environmental, genetic) may contribute to the development of second malignancies in cancer survivors other than the type and dose of administered radiation [42]. However, the detailed molecular origins of the events linking neocancer initiation to the extra dose given to (initially healthy) tissues have not yet been elucidated (see Section 6 below).

The relations between cellular senescence and cancer are complex and ambivalent. On the one hand, cellular senescence is viewed as a cell-autonomous mechanism protecting cells from cancerous transformation. On the other hand, accumulation of senescent cells in the tumor stroma can stimulate cancer progression and invasion as well as metastasis and relapse, mainly because of the inflammatory and matrix-remodeling properties of the SASP [43]. Compared to cancer cells that have acquired the outstanding capability to proliferate uncontrollably, the distinctive character of cell cycle arrest in senescent cells is viewed as limiting the evolution of damaged cells, which would risk cancerous evolution [44]. Notably, cells in OIS are often found in pre-neoplastic lesions, but are no longer present when the lesion evolves into a malignant cancer. The initial mitogenic stimulation induced by the oncogene can overwhelm cellular capacities, leading to DNA replication stress and contributing to increased oxidative stress. In response, the cell enters senescence, an adaption viewed as a protective mechanism against the tumorigenic potential of oncogene activation.

Importantly, not just tumor stromal cells but also cancer cells themselves can enter TIS in response to medical cancer treatments [45]). Among the possible inducers of TIS are conventional chemotherapy drugs such as alkylating agents [46], e.g., cisplatin, cyclophosphamide, and temozolomide; topoisomerase inhibitors [46], e.g., doxorubicin, etoposide, and camptothecin; microtubule inhibitors such as paclitaxel [47]; and to a lesser extent vinca alkaloids such as vincristine [48]. A number of targeted therapies are also able to induce cancer cells to enter TIS [49,50]. TIS is assumed to have a curative effect in the short term, as it halts the proliferation of cancer cells; however, several experiments have suggested that cancer cells induced into TIS could also evade growth arrest in the long term, leading to progeny daughter cells with newly acquired malignant traits such as cellular

plasticity, stem-like properties, tumorigenicity, and aggressive growth behavior [51]. This ability would indeed rely on two major characteristics of senescent cells, namely, their long life span and their reservoir of unrepaired DNA damage, which represents a potential source of mutations. Although this still needs to be further explored, such a mechanism is now being considered as a major contributor to cancer relapse. Therefore, given the dual-edged consequences of TIS, one proposed approach is to combine cancer therapies with so-called senolytic or senomorphic pharmaceutical drugs, i.e., molecules that are able to preferentially kill senescent cells or suppress the SASP, respectively [52–55].

At the global level of the organism, radiation therapy or chemotherapy produce a sustained sublethal injury associated with continued oxidative stress and chronic inflammation; at the cellular level, this prompts entry into senescence. In addition, irrespective of the cancer treatment, almost all such cancer therapies chiefly lead to molecular-level DNA damage, which is considered a primary inducer of senescence in both normal and cancer cells [56,57]. However, it is important to note that the level of DNA damage required to induce senescence may differ between malignant and nonmalignant cells in a way that is similar to the varying thresholds for apoptosis. For instance, patients with breast cancer have been found to have an increased number of T-cells expressing $p16^{INK4A}$ when administered with different chemotherapeutic agents, indicating immuno-senescence as a bystander effect [54,58]. Similarly, radiation therapy was found to lead to the upregulation of $p16^{INK4A}$ in nonmalignant cells [26,59].

Induction of cellular senescence occurs through distinct molecular pathways, for example when comparing TIS and OIS; in fact, TIS results from the the triggering of DNA damage-repair pathways, leading to 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) [60,61]. These proteins are essential components of senescence evolution, as they inhibit the CDK2-cyclin E and CDK CDK4/6-cyclin D complexes and prevent phosphorylation of the Rb (retinoblastoma) protein, thereby blocking the entry in the S-phase and inducing senescence [60,61].

It is worth noting that athough 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 [62,63]. Furthermore, it appears that prolonged overexpression of any of these four factors, i.e., p53, pRB, $p16^{INK4A}$, or $p21^{WAF1/CIP1}$, is sufficient to induce senescence [61]. Deregulated expression, mutations, secondary alterations, or complete loss of tumor suppressor proteins may result in inadequate senescence induction or escape from senescence [26,61]. The cell could then enter apoptosis or reenter a proliferative stage, depending on whether or not the damaged DNA is being efficiently repaired. Regardless of the cancer type, the level of senescence (i.e., whether it is of OIS or TIS origin) appears to significantly influence the outcome for cancer patients [45,61]. Senescence is definitely a tumor suppressive process, preventing cancer cell proliferation as well as suppressing malignant progression from premalignant to malignant, and can act as an effector mechanism of many types of anticancer therapies by stimulating an immune response; however, it can contribute to reduced patient resilience during cancer therapies, and may provide a pathway for disease recurrence after therapy, to the point that specific *senolytic* drugs to suppress senescent cells are under active development [45,64,65].

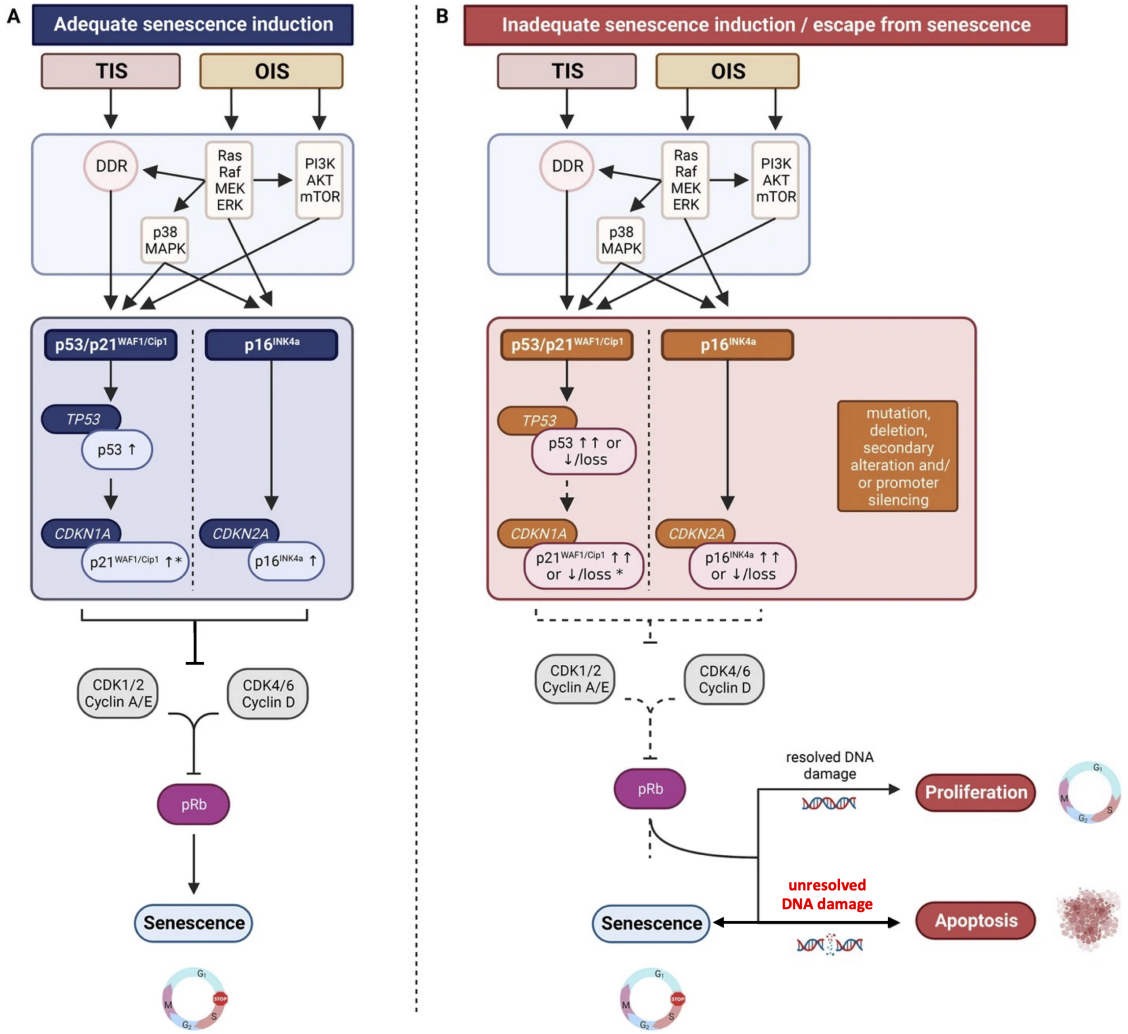

Figure 2. (**A**) Adequate senescence induction vs. (**B**) inadequate cell senescence pathway. Note the bifurcation between senescence and apoptosis for unresolved DNA damage in the latter; (*) indicates that p21$^{WAF1/Cip1}$ can also be activated by pathways that are independent of p53. (adapted from [61], w/permission).

4. DNA Damage and Radiotherapy

During radiotherapy, ionizing radiation (IR) is targeted at cancerous cells, either by an external beam of high-energy photons (X-rays, gamma rays), electrons, or heavier charged particles (protons, carbon ions), or by injecting radioactive (alpha- or beta-emitting) nuclides complexed to a bifunctional chelator, which is in turn conjugated to a biological targeting molecule (peptide, antibody, amino acid, or small molecule) aimed directly at the neighborhood of the tumor volume (internal radiotherapy). Photons provide indirectly ionizing radiation, the ionizing effect of which is carried by secondary electrons set in motion during their interactions with the matter; on the other hand, charged particles are able to directly induce ionization in the target cells, which is key to inducing the DNA damage that ultimately leads to the arrest or death of cancer cells [66,67]. IR inflicts damage on molecules, most notably DNA, by imparting energy that is 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 undergo further chemical evolution. Electronic excitation can lead to the breakup of molecular species into neutral but highly reactive fragments called 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 the secondary electrons. An important difference between directly and indirectly ionizing radiation (see, e.g., [68]) is that neutral photons produce quite sparse ionization events (the linear energy transfer (LET) of a 1-MeV γ ray in human tissue is about 0.2 keV/µm, corresponding to about 5–6 ionization events per micrometer); sparse ionization induces localized defects in the DNA molecule, such as abasic sites and 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 an LET of ∼14 keV/µm and produces about 400 ionizations per micron, which 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 [69–72]. Such dense ionization clusters produce more complex DNA defects ('clustered' damage) in addition to localized defects (Figure 3).

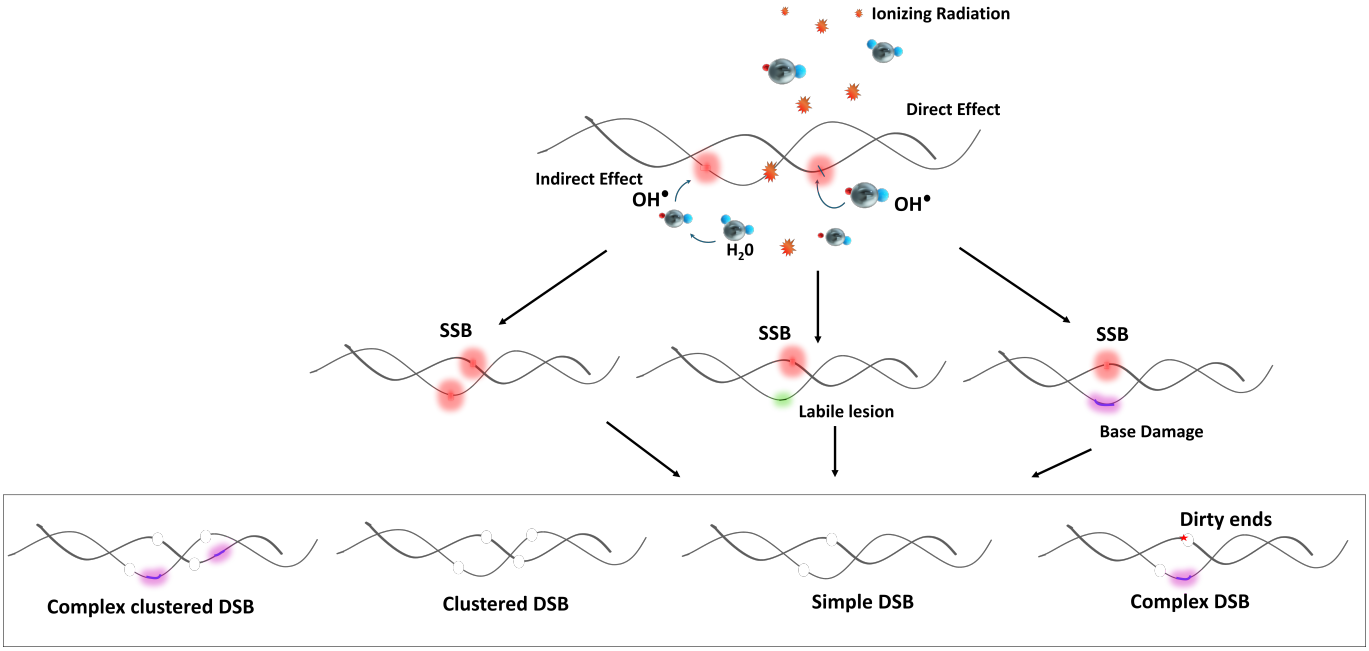

Figure 3. Ionizing radiation (IR) can directly attack DNA or can generate reactive oxygen species (ROS) by interacting with water molecules (direct vs. indirect effect). These ROS in turn cause various types of damage in DNA. At the same time, IR can deposit a significant portion of its energy in the form of ionization clusters, which lead to clustered DNA damage (including the combination of SSB+Base damage). New simple or clustered DSB lesions with dirty ends (see Figure 4) and base damage can form from these damage clusters, either immediately via chemical modifications of sugar lesions that do not initially disrupt the sugar–phosphate backbone, or following enzymatic repair of base damage.

Radiation-induced molecular damage to DNA, notably by photons, occurs mainly through two mechanisms: (i) the *direct effect*, or (ii) the *indirect effect* [73]. The former is a one-electron oxidation reaction; the direct absorption of a photon (with frequency ν and energy $E = h\nu$, with Planck's constant $h = 4.14 \times 10^{-15}$ eV·s) results in a secondary electron being released by an atom, thereby creating a radical cation species (indicated by a •) localized on the DNA molecule. This 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 [73] (Figure 4). By contrast, the indirect effect results from the formation of free radicals due to the radiolysis of water (e.g., $H_2O + h\nu \rightarrow OH^{\bullet} + H^{\bullet}$, or $H_2O + h\nu \rightarrow H_2O^{+} + e^{-}_{aq}$) or other molecular components within the cell; both the free radicals and the

liberated electron in aqueous solution (e^-_{aq}) are diffused, 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, the availability of free oxygen O_2), the main result being the breaking of the backbone phosphodiester bond or the glycosidic bond, with subsequent base release. In photon irradiation, the direct effect accounts for 30–40% of DNA lesions, while the indirect effect contributes 60–70% [66]; by contrast, proton irradiation (including 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.

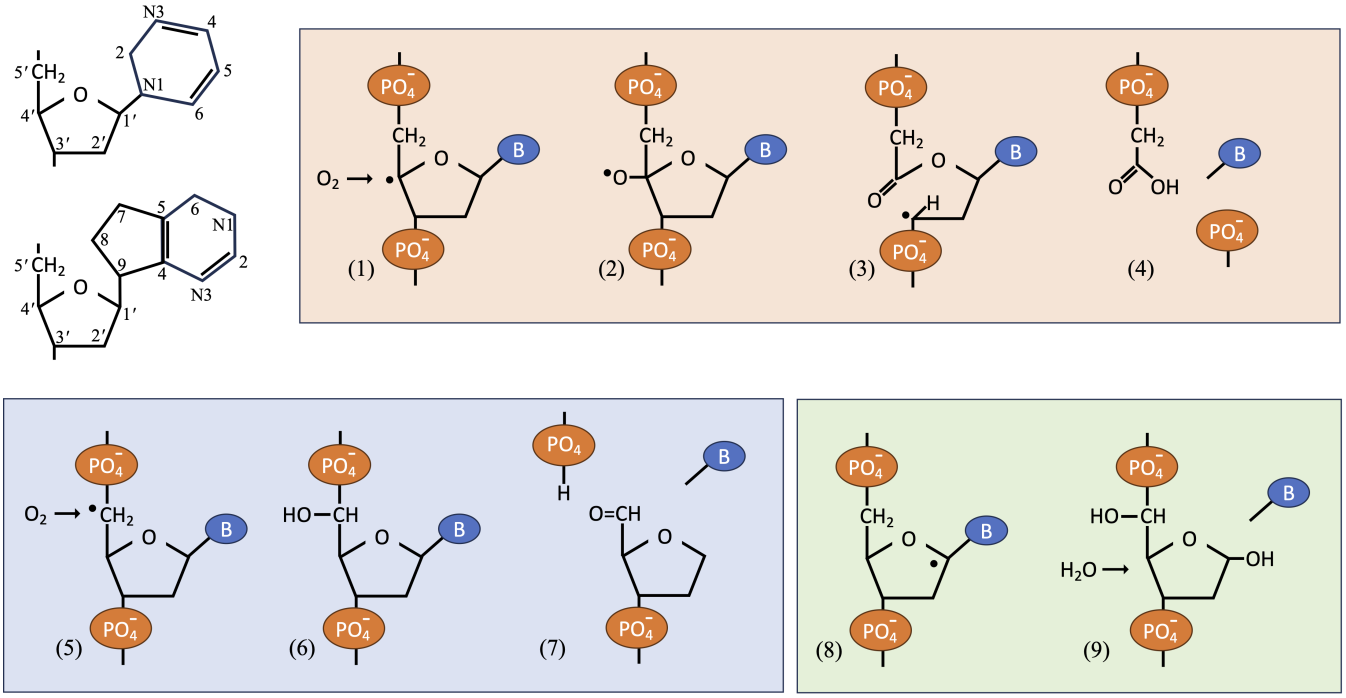

Figure 4. Top left: numbering convention of the C and N atoms in the nucleotides. (1–4) Direct ionization at C4′ in presence of O_2 may lead to SSB with base release and a phosphoglycolate (CH_2COOH) at the 3′ terminus; (5–7) direct ionization at C5′ in presence of O_2c may lead to SSB with base release and an aldehyde (HC=O) at the 5′ terminus; (8–9) direct ionization at C1′ followed by hydrolysis may lead to base release and an AP site.

Irrespective of the specific chemistry, breaks in the DNA backbone ('strand breaks') can be classified based on the nature of the damage (i.e., whether it is at a 5′ or 3′ position). They are classified mainly as double-strand breaks (DSB), single-strand breaks (SSB), base damage (BD), and damage to the phosphodiester bond or ribose sugar in the DNA. An SSB is created when a random isolated break occurs on only one of the complementary DNA strands. This is commonly associated with oxidized/reduced base damage or base loss. When two SSBs occur in opposite strands within less than 10 bp distance (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* (a-purinic or a-pyrimidinic, AP) site [74], while the phosphate backbone remains intact. It has been observed that during high-LET radiation two or more ionizations (ionization clusters) can be formed within a distance of a few nm, resulting in multiple lesions. Such multiple ('clustered') lesions can include more than two SSBs, AP-sites, or DSBs formed within the same or opposite DNA strand from the same energy deposition event. Such clustered lesions are rarely formed by endogenous stress, and are invariably associated with the toxicity of ionizing radiation (see Table 1) [69,75].

Free-radical attacks 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 occur mainly due to the formation and transfer of free radicals, either in the presence or absence of O_2 molecules. Usually, such breaks are the result of an attack at the C4′ or C5′ (less frequently the C1′) carbon atom sites [73], the radical being transferred from one site to another until the local conditions energetically favor breaking of the phosphodiester bond (Figure 4). In either case, the strand break results in the loss of the corresponding base and can further evolve into more complex damage, notably a double-strand break [76]. In general, such attacks lead to the formation of 'clean' 3′-phosphate and 5′-OH termini, and less commonly to a 3′-OH and 5′-phosphate pair. However, it is often observed, notably in the presence of oxygen, that attack on 5′ carbon can give rise to either 5′-aldehydes or 3′-phosphoglycolate (PPG) termini, as well as to other more chemically complex ('dirty') ends [76,77]. It is worth noting that dirty ends have the property of being unprocessable, as DNA-ligases cannot reseal an SSB or a DSB unless the ends are clean [78]. For example, tyrosyl-DNA phosphodiesterase can require several hours to remove PPG from 3′ DNA ends [79].

Table 1. DNA damage induced by ionizing radiation (data adapted from [75] with permission).

Type of Damage	Radioinduced Damage per Cell per Gy	Endogenous Damage per Cell per Day
Single strand breaks	1000 [80]	>10,000 [81]
Base damage	2000	3200
Abasic sites	250	12,600
Double strand breaks	39 [80]	≈50 [82]
DNA-protein cross-links	150	?
Non-DSB clustered lesions	122	?
Complex DSB	?	?

5. DNA Damage Repair Pathways

Irrespective of its origin, DNA damage triggers specific chains of multi-enzyme reactions, globally known as DNA damage repair (DDR) pathways, which protect against genomic instability and accumulation of mutations. Cells have developed multiple complex DNA repair pathways. There are no less than seven different variants, which enable cells to identify the type of damage, and repair DNA to the correct sequence if possible [83,84]. Even though these pathways are complex, all of them follow a common workflow performed by certain specific molecular components, which can be summarized as follows [85]:

1. Recognizing DNA damage: This is performed by DNA damage sensor proteins such as Poly(ADP)Ribose-1 (PARP-1) protein, Ku70/80, etc. (see [86]).
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 to form a complex at the lesion site. This complex removes the damaged segment and restores the correct 3′-hydroxyl and 5′′-phosphate termini.
3. Re-synthesizing the missing parts of the DNA sequence: This is done by different effector synthetases, which add new nucleotides.
4. Finally, ligation of the clean broken ends by ligase enzymes.

Each one 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 repairing 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.

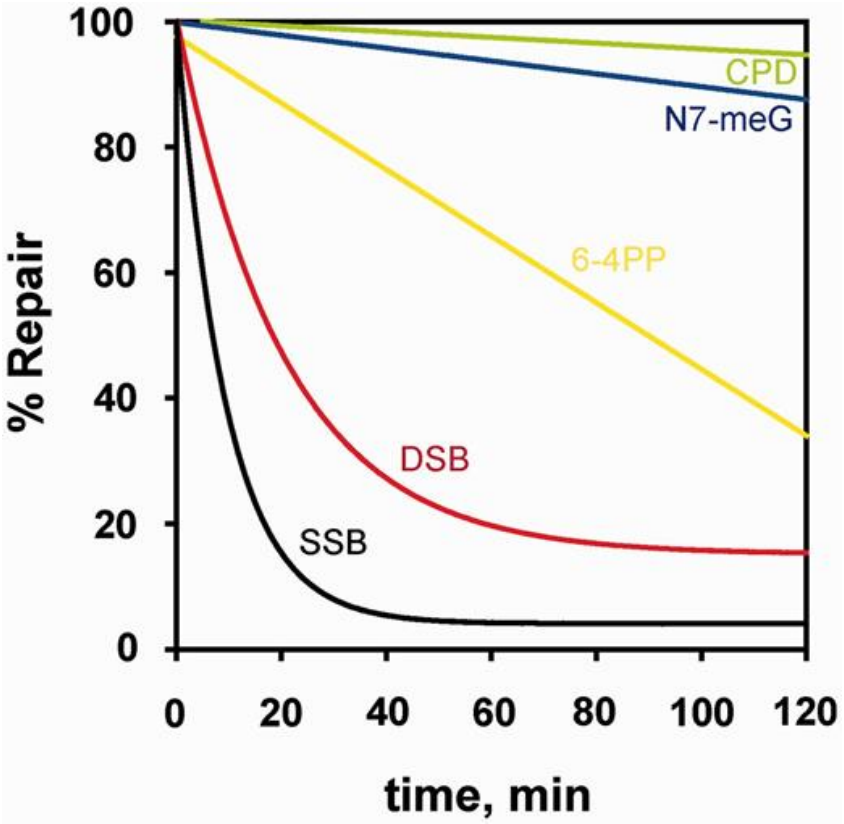

Figure 5. Repair kinetics of of different types of DNA lesions: removal kinetics of SSBs, DSBs, 6–4 photoproducts (6–4PP), and cyclobutane pyrimidine dimers (CPD) from CHO-AA8 cells as well as N7-meG from human lymphocytes. Note the two curves for SSBs and DSBs, which do not go to zero but rather saturate at some constant value at long times ($\gtrsim$60–120 min), signaling a residual fraction of non-repaired defects (from [87], w/permission.)

It is estimated that a human cell suffers 10^4–10^5 DNA lesions each day, either from endogenous processes or environmental sources; these are constantly repaired with high efficiency. Among these, SSBs are among the most frequently occurring lesions [88,89]. It is well established that SSBs are in principle far less lethal than DSBs, and are repaired quickly [87,90] and with a relatively small rate of non-repaired defects compared to DSBs (Figure 5). This is mainly due to the fact that SSB repair pathways have the intact complementary sequence of the undamaged DNA strand available to perform a correct repair, whereas that information is not available when both DNA strands are affected during the G1 phase of the cell cycle. However, even a small fraction of non-repaired SSBs can interfere with gene transcription and replication [82,91]; 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 special one-ended DSBs, for which repair is harder and necessarily leads to genomic rearrangement [91,92]. Eventually these events can lead to apoptosis or senescence [91,93]. Alternatively, the cell can adapt to a damage-tolerant pathway with the help of low-fidelity polymerases employed by error-prone trans-lesion synthesis (TLS) [94].

It is also important to realize that the relative amount of SSBs generated during 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 gray (1 Gy = 1 Joule/kg) of dose delivered [95]. Monte Carlo simulation studies of DNA damage due to proton irradiation have shown that about twice as many SSBs are formed at high proton energies compared to the fraction of DSBs [96]. In general, X-ray-induced DSBs, as detected by the formation of 53BP1 foci, are resolved quickly and more dynamically than those induced by protons and α-particle radiation, which is due to the high ionization

density of the latter [97]. Traditionally, most studies have focused on DSBs because of the major danger they pose to cells, their more fault-prone repair pathways, and the higher rate of non-repaired defects for a given amount of initial damage. However, the role of SSBs is increasingly coming under scrutiny (see, e.g., [26,98–102]). Despite the very quick and efficient SSB repair machinery, induction of a very large amount of SSBs can lead to saturation of the repair pathway, increasing the fraction of non-repaired defects. This can result in DNA replication stress, transcriptional stalling [103,104] (especially for SSBs with dirty ends), and excessive PARP activation [105,106], which in turn leads to genome instability, with overall toxicity likely equivalent to more harmful types of damage.

Altogether, any SSB which is not repaired, or which is 'tolerated' during the entire repair pathway, has the potential to change into a more lethal (potentially mutagenic) DSB. Considering the prevalence of endogenous SSBs, for example those produced as intermediate steps during base-excision repair (BER) of a damaged nucleotide, cells have understandably developed highly effective mechanisms to reduce their effects. To ensure that SSBs are fully repaired before the start of DNA replication, cells need to detect the SSB and induce the appropriate repair pathway. It might be expected that cells would delay cell cycle progression until SSBs are repaired, as it happens during DSB repair; however, there is currently no well-established evidence to support the existence of such a signaling pathway [107]. 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 prevent genomic instability and ensure cellular homeostasis, SSBs are repaired by the specific SSBR pathway, which partly overlaps with the BER pathway [108]. The upcoming subsections examine the molecular intricacies of the BER and SSBR pathways 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 mainly induce these small lesions, resulting in oxidized bases such as the well-known 8-oxo-G, oxidative deamination and dealkylation, and more than twenty other different oxidation products [109,110]. BER typically requires four or five enzymes to perform the DNA repair process (see Figure 6). It is initiated by removing the damaged base by one of the eleven known mono- or bifunctional glycosylases [111], depending upon the type of defect. This process forms apurinic or apyrimidinic (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 pathway has created an SSB as an intermediate step in the repair chain, which constitutes an overlap with the SSBR pathway. The subsequent step of filling the single-nucleotide gap can take the form of either a long- or short-patch repair. Short-patch repair is considered more dominant, while long-patch repair is observed in postreplicative BER initiated by UNG2 or NEIL1 glycosylases expressed during the S-phase [112]. Long-patch repair is also observed when the 5′-dRP terminus is oxidized to different abasic lesions (e.g., dioxibutane [113], C4′- or C2′-AP [114]), in which cases Pol-β excises the damaged terminus with reduced efficiency.

In long-patch base excision repair [115], 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-1 (Lig1). 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, and the nick is finally ligated by DNA Lig3 with the help of X-ray repair cross-complementing protein-1 (XRCC1), which serves as a scaffold for Lig3.

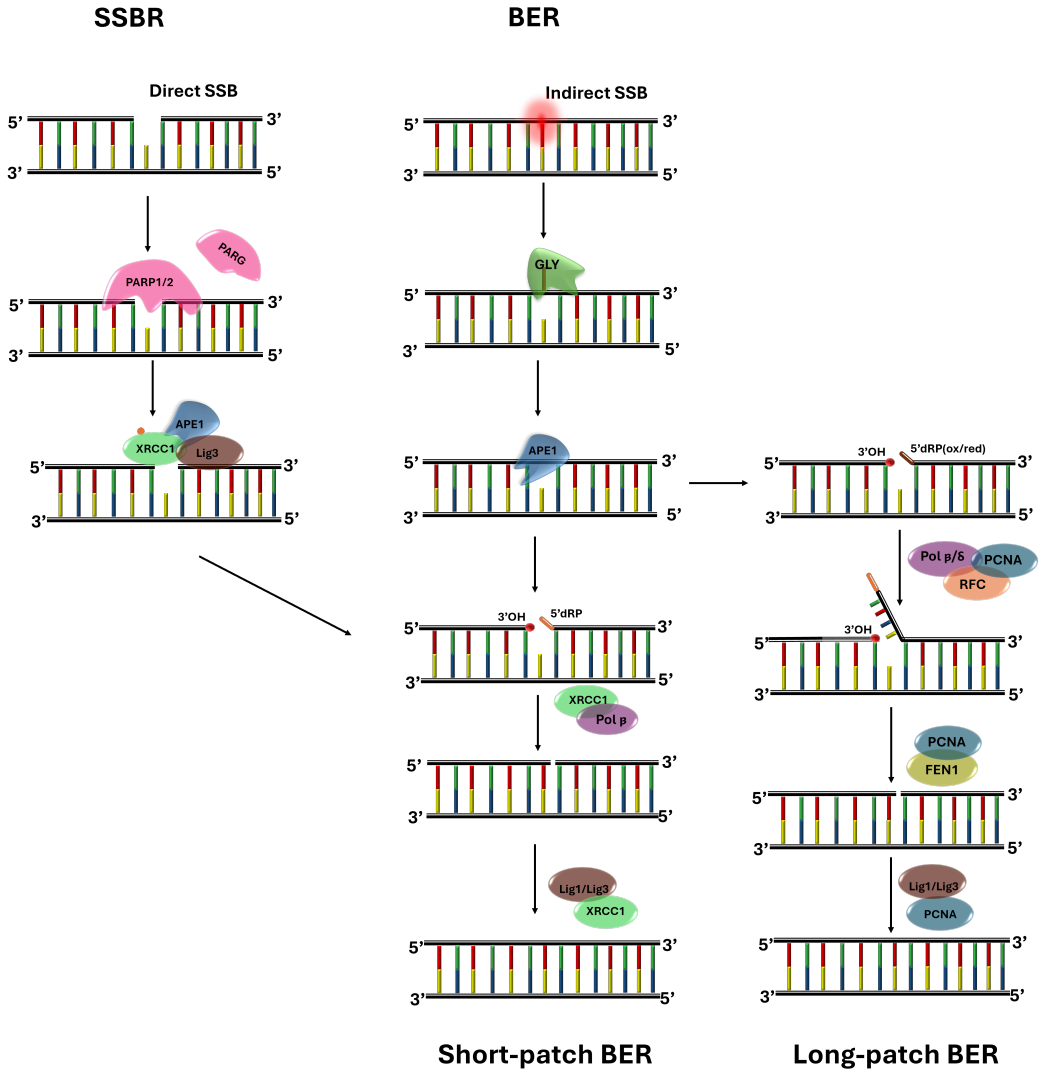

Figure 6. Model for BER and SSBR pathways: During base excision repair (BER), most abasic sites are processed by APE1, which cleaves the site and recruits Polβ. Polβ then inserts a single nucleotide and repairs the 5′-deoxyribose phosphate (dRP) left by APE1, creating a ligatable nick. This nick is sealed by XRCC1-Lig3α. In cases where Polβ 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, and PCNA are recruited to extend the gap by several nucleotides, forming an overhang at 5′ (a 'flap'), which is cleaved by flap-endonuclease-1 (FEN1). Ligation is then completed by Lig1. On the otherhand, direct single-strand breaks (SSBs) from ribose damage and some SSBs arising during BER are recognized by PARP-1 or PARP-2. PARP then recruits phosphorylated-XRCC1 and Lig3α to form a scaffold for repair. APE1 or PNK processes the damaged termini into 5′-phosphate and 3′-hydroxyl groups, aided by XRCC1. Polβ fills the gap with a nucleotide, followed by ligation by Lig3α. See the main text for data sources.

The decision-making process underlying the choice of short-patch repair vs. long-patch repair is still under investigation [108,116,117]. The long-patch repair pathway is frequently observed in proliferating cells, and makes use of replicating proteins such as Pol-ϵ/δ, FEN1, PCNA, and Lig1 [118]. One hypothesis is that the choice depends on the ATP concentration near the AP site, which is controlled by Lig3 and XRCC1, with some studies showing that long-patch repair is preferred under higher ATP concentrations [119]. A few other studies have suggested that the decision depends on the available initiating glycosylase enzyme and type of damage. For instance, if the 5′-dRP intermediate produced

by AP-endonuclease-1 (APE1) is successfully removed by Pol-β, then short-patch BER is preferred; otherwise, the long-patch repair pathway is chosen [108,120].

5.2. Single-Strand Break Repair Pathway

Typically, when an 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 Figure 4 above). Such defects in the DNA strand are structurally very similar to the intermediate strand-breaks produced during BER, apart from the 5′ and 3′ termini, which in the former case may be irregular. Irrespective of their origin, however, all SSBs are sensed by poly(ADP)ribose (PAR) 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. Their catalytic activity, called PARylation, consists of synthetizing long and branched chains of poly(ADP)ribose (PAR) from NAD+ used as substrate. The PAR chains can remain free or be covalently added to target proteins, including histones and PARP itself. PARP-1 is the most-used enzyme in the SSBR pathway, while PARP-2 is used to a lesser extent. The well studied PARP-1 enzyme consists of at least six functional domains: three DNA-binding Zinc-finger N-terminal domains, named Zn1, Zn2, and Zn3; one BRCT (BRCA1 gene C-terminus-1) domain; one WGR nucleic-acid binding domain (classically named after its central Trp-Gly-Arg motif); and one catalytic C-terminal domain, including a helical subdomain (HD) [121]. 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 motifs Zn1 and Zn2 are known to specifically recognize DNA breaks. Zn1 from one PARP-1 may also cooperate with Zn2 from another PARP-1 to form a dimeric module that specifically recognizes DNA breaks [122]. The detailed mechanism by which PARP-1 identifies the SSB is not entirely clear [123]; in particular, the extent to which PARP-1 may be sensitive to dirty SSB ends occurring in radiation-induced radical attacks it is not yet understood. On the other hand, Zn3 mediates inter-domain contact, and is required to confer with PARP-1 in order to regulate chromatin structure [124]. The BRCT domain also acts as a DNA binding domain, but of lower affinity, and is able to bind only to intact DNA without concomitant catalytic activation. The BRCT–DNA interaction mediates DNA intrastrand transfer of PARP-1 (the so-called 'monkey-bar mechanism') that allows rapid movements of PARP-1 through the chromatin [125]. 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 suitable position 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 serine ADP-ribosylation of the target proteins [126,127]. 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^+, initiating PARylation [128,129].

In the PARylation process, PARP-1 sequentially transfers a large number of ADP-ribose molecules onto itself or to other proteins, producing long chains of PAR that form a focus at the site of the break (see Figure 6). In turn, the accumulated PAR chains favor the recruitment of XRCC1. It is also worth noting that these PAR chains are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG) [130], with PARP-1 being recycled for subsequent detection of SSB.

After 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 in SSBR activity observed in cells that lack this protein [131]. Human XRCC1 is a molecular scaffold protein; it is 633 amino acids in length [132], with an asymmetrical elongated shape (axial ratio of >7 [133]) and three main domains: (1) an N-terminal domain (NTD) of about

160 amino acids, which interacts with Pol-β [134]; (2) a central BRCT domain of about 90 amino acids, which interacts with PARP-1 [134], PARP-2 [135], poly(ADP-ribose) [136], and DNA [137]; and (3) another BRCT domain of about 100 amino acids at the C-terminal, which binds DNA Lig3 [138]. These three main domains are connected by two linker domains; the first contains a nuclear localization signal and phosphorylation-independent binding site for polynucleotide-kinase (PNK)-phosphatase (PNKP) [139], while the second includes a phosphorylation-dependent binding site for PNKP [140], APTX [141], and APLF [142]. 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 such as polynucleotide kinase phosphatase (PNKP) and aprataxin (APTX) interact with phosphorylated XRCC1, DNA, Pol-β, and DNA Lig3. PNKP resolves special termini such as 3′-Pho /3′-PPG, while APE1 resolves 3′-α, β unsaturated aldehyde, all of which are formed during irradiation. At the same time, Pol-β colocalizes with XRCC1 to support polymerase activity, notably in the case of oxidized 5′-dRP formed by indirect attack by ROS. At present, the enzyme responsible for 5′-aldehyde treatment remains unknown [143].

Eventually, the entire process is terminated by the nick-sealing activity of Lig3α [144]. Hence, it appears that the final steps of the SSBR pathway overlap with the short-patch BER pathway, with both of these two meticulously designed pathways being crucial for repairing exogenous SSBs and oxidative damage in DNA.

6. Radiation-Induced Single-Strand Breaks and Cellular Senescence

External-beam radiotherapy for cancer treatment can impact various portions of tissues through both direct and indirect mechanisms. The radiation beam is designed in such a way as to maximize the dose (energy delivered per unit mass) to the tumor region, as identified by the clinical treatment volume (CTV), while ideally reducing to zero the extra dose applied to the healthy organs-at-risk (OAR) surrounding the CTV [145]. Despite a great deal of effort towards improving both the irradiation geometry and the space- and time-fractionation of the radiation dose, some rays of the beam inevitably affect the region in the immediate vicinity of the CTV. This is also affected by the error margin accounted for in defining the planning treatment volume (PTV), which represents the zone actually being irradiated, typically extending a few mm around the CTV and necessarily touching some OAR at the borders. Therefore, clinical limits have been assessed in the literature for each organ of the human body in order to maintain damage to OARs within acceptable limits (see, e.g., [146]).

Traditionally, it was believed that DNA damage should be observed only in the PTV, due to the direct projection of the radiation field; however, this picture started to be questioned after chromosomal damage and changes (mutations, translocations) were observed in cells within the nearby non-irradiated area, or even in distant tissues [147,148]. Furthermore, oxidative stress is not confined to the targeted irradiated area either, and has been found to affect neighboring non-irradiated cells through intercellular communication, a phenomenon known as the bystander effect [149]. Such effects, either alone or in combination, may also increase the carcinogenic risk to distant tissues, resulting in radiation-induced secondary malignancies (RISM) [40,41], 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, being instead newly transformed cancerous cells originating from normal cells that were affected by stray radiation. Although the causal relationship between the initial radiation therapy and the induction of the SPC is impossible to definitively prove, these second cancers are often observed to develop preferentially near the margins of the PTV (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) [150].

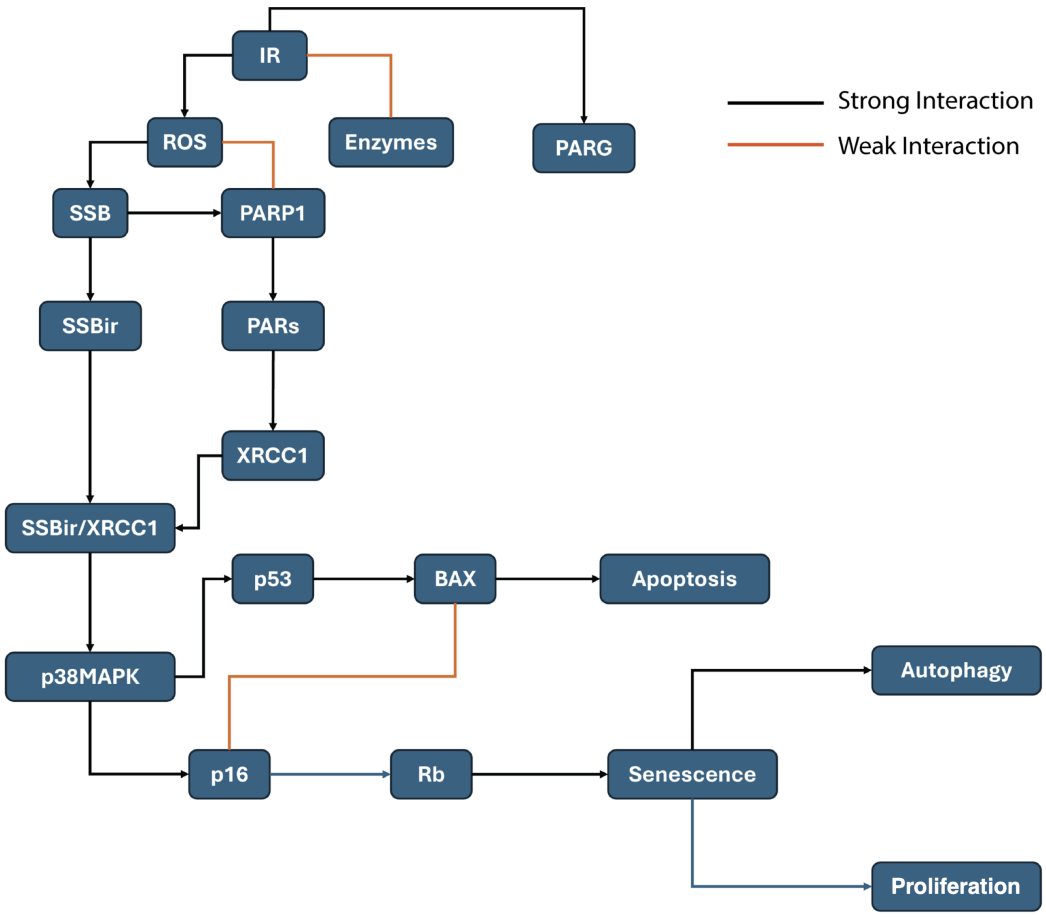

Figure 7. SSBR pathway followed by keratinocytes at the margin of irradiated cells; SSBir indicates unrepaired SSB (basic data from [25,26]).

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 a uniform radiation dose field, and scattering that occurs inside the patient's body [151]. Internal scattering has been identified as the primary factor influencing the dose deposited in the closest margin around the target area [151]. 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 [151]. 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 long as 40 years, depending on to the initial cancer treatment [40]. This suggests that normal cells exposed to low-energy scattered radiation could 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 previous studies, we have suggested tthat it could be related to a form of TIS [25,26,151–153].

Indeed, in [26] it was observed that fibroblasts irradiated in the penumbra region retain a rather large fraction of unrepaired SSBs and almost no DSBs. This is associated with upregulation of p21 and p16, leading to a cell cycle arrest and the acquisition of several senescence markers. The question of why SSBs should not be fully repaired in this radiotherapy context is not well understood. We established in [26] that the SSB repair capacity declines with the daily irradiations of a fractionated protocol, in correlation with a decline in PARylation capacity. However, we could not yet elucidate the molecular mechanisms responsible for the decreased PARylation activity or the underlying upregulation of p21 and p16. Notably, these mechanisms could be similar in part to those we highlighted in

keratinocytes undergoing spontaneous senescence, a form of SIPS induced by an endogenous oxidative stress; in this context, oxidative stress downregulates PARP-1 expression at the transcriptional level, thereby reducing the synthesis of PAR. This reduction impedes the recruitment of the CK2 kinase to the damage site, arresting the repair process at the step of XRCC1 recruitment, as XRCC1 cannot adequately recruit the downstream repair enzymes if these are not phosphorylated. Therefore, unphosphorylated XRCC1 accumulates at the break sites, leading to activation of p38MAPK. This in turn leads to downstream upregulation of p16, which is responsible for cell cycle arrest [25] (Figure 7).

Other studies have shown that when the p38MAPK pathway is activated, it can promote expression of either p16 or p53; while p53 more often leads to apoptosis, p16 predominantly drives cells towards senescence. Experimental data indicate that cells preferentially enter senescence over apoptosis, possibly due to inhibition of BAX, a pro-apoptotic factor [154]. This preference for senescence over apoptosis is particularly evident in conditions where SSBs are the prominent defects. However, it has been reported that excessive accumulation of SSBs of oxidative origin can induce cell death through prolonged activation of PARP-1. This leads to depletion of NAD^+ and ATP, with the consequent release of apoptosis-inducing factor (AIF) from mitochondria [105,106].

Senescent cells are typically cleared by the immune system or degraded by autophagy, a major lysosomal degradation pathway; however, senescent cells may skip autophagy and escape at later times into a proliferative state, which is sometimes termed 'inadequate senescence' or escape from senescence. When occurring in cells affected by the out-of-field radiation dose, this process could be one possible mechanism behind the origin of SPCs.

It may be worth noting that another phenomenon of remote activation of repair pathways, distinct from the bystander effect, may also occur during radiotherapy. In fact, it is sometimes observed that radiation not only reduces the targeted tumor, but can also induce regression of non-irradiated metastases at distant parts from the irradiation site. While the precise biological mechanisms underlying such 'abscopal' effects remain unclear, the immune system is believed to play a key role in this process. [155,156].

7. Discussion

In this brief review, we have focused 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 of our main motivations was to introduce and rationalize some recent findings in a broader context, according to which the 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 much 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 harboring anatomo-pathological features different from those of the primary cancer. To this end, we have provided a description synthesizing the processes of radiation-induced damage and the respective DNA damage repair pathways that are activated as a result.

In the case of SSBs, we highlight the partial overlap between base-excision repair (BER), typically involved in counteracting oxidative damage, and SSB repair (SSBR). Cells with inhibited repair capability, or that lack 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 to explain both 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 unrepaired SSBs. The molecular origins of this phenomenon are far from being understood. A swift decline in the number of DSBs at the borders of the PTV can be readily expected on the basis of the dose profile, which exponentially decreases from the border of the PTV; however, the persistence of SSBs

at long distances (several mm) from the border of the PTV currently escapes mechanistic explanations based purely on known irradiation geometry and radiation diffuse scattering. When considering individual patients, other possible supernumerary mechanisms related to cell metabolic pathways might be at play, e.g., a 'bystander' effect from molecules secreted by those cells affected by high doses in the PTV, which could diffuse and affect DNA repair pathways even at far distances.

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

Author Contributions: All authors contributed equally to conceptualization, methodology, investigation, resources, data curation, writing the original draft, review, and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Lille through the special i-SITE grant Programme for Early-stage Researchers in Lille (PEARL), project "Senesimex". C.A. is supported by The Institut National du Cancer, grant INCa-16078. F.C. is supported by the DYPROSOME Project, grant ANR-21-CE45-0032 of the French National Agency for Research.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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