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DNA Damage Response/Repair in Cancer Stem Cells — Potential vs. Controversies

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1. Introduction

1.1. Genome integrity and DNA damage response

The genetic material of each cell maintains the information required for all cell processes including cell viability and proliferation. Preservation of genome integrity is essential for living cells, while being a prerequisite for survival of higher-order multicellular organisms. Thus, intact genetic information should pass to the forthcoming generations. On the other hand, genome is constantly subjected to endogenous and exogenous damages by a number of factors, such as reactive radicals, radiation, and genotoxins [1]. Despite the fact that in a few cases mutations may provide selective advantage in species evolution, a DNA damage response system to sense DNA damage, arrest cell cycle, repair DNA lesions, and/or induce programmed cell death is crucial for maintenance of genomic integrity and survival of the organism. Consequently, a coordinated cellular response to DNA damage is required for effective DNA repair, ensuring viability, and preventing disease.

Unfortunately, neither the chemical properties of the DNA molecule nor its interaction with environmental factors guarantee lifelong stability and proper functioning of the genome. Due to its chemical structure, DNA is particularly sensitive to spontaneous hydrolysis reactions that may create both abasic sites and base deamination. Furthermore, ongoing cellular metabolism generates reactive oxygen species and their highly reactive intermediate metabolites, which can create 8-oxoguanine lesions in DNA as well as a variety of base oxidations and DNA strand breaks that are all highly mutagenic. This phenomenon may also lead to genomic instability. DNA is also constantly assaulted by mutagens present in the external

environment. UV light from the sun, as well as various chemical reagents, can react with DNA and induce nucleotide chemical modifications. In addition, ionizing radiation generated by the cosmos, X-rays, and exposure to radioactive substances, as well as treatment with certain chemotherapeutic drugs, can induce base modifications, interstrand cross links, and DNA single - and double-strand breaks (DSBs), which can all lead to genomic instability. It is estimated that each cell is confronted with approximately 10^4–10^5 DNA lesions per day, indicating that clearance of genomic injuries constitutes the maintenance of proper genome function a demanding task [2].

Thus, maintenance of genomic stability through damage repair is essential for cell and organism longevity. Without genomic stability, replication errors and external stress as well as direct forms of DNA damage can induce mutations, which decrease cell survival, cause altered gene expression, and therefore can lead to cellular transformation.

In response to the wide diversity of potential DNA lesions, eukaryotic cells developed a number of highly conserved DNA repair mechanisms that can recognize and repair different types of DNA damage with varying fidelity and mutagenic consequences (Table 1). Irrespective of the type of lesion and the repair mechanism, DNA damage is rapidly sensed and activates evolutionarily conserved signaling pathways, known collectively as the DNA damage response (DDR). DDR components can be separated into four functional groups, extensively described in other chapters of the current edition: damage sensors, signal transducers, repair effectors, and arrest or death effectors. In brief, cells contain multiple DNA repair mechanisms including: base excision repair (BER) that removes damaged bases caused by small chemical alterations (base modifications), mismatch repair (MMR) that recognizes and removes mispaired base incorporation errors and base damage arising from replication errors, nucleotide excision repair (NER) that corrects bulky helix-distorting lesions caused by chemicals and ionizing radiations, and cross-link repair (ICL) that removes interstrand cross links. In addition, the most deleterious DNA lesions, breaks in the DNA backbone, (Double-Strand Breaks, DSBs) are mainly repaired via homologous recombination (HR) and nonhomologous end joining (NHEJ). These most challenging DSBs may be restored by a different degree of repair fidelity, related to the pathway chosen according to the phase of the cell cycle. While the the almost error-free HR repair dominates in dividing cells, the G1 phase acting NHEJ is error-prone, as genome has not yet undergone duplication; hence, a template for recombination used in HR is not yet available. These two pathways seem to repair the majority of chemotherapy- and radiotherapy-induced damage [2-7].

In a rapid overview, regarding the restoration processes of DSBs, HR allows cells to repair DNA damage in an error-free manner and can be performed only during S and G2 phases of the cell cycle due to the requirement for the undamaged sister chromatid as a template. HR starts with 5′-3′ resection of the DNA ends to create 3′ single-stranded DNA tails providing a substrate for assembly of RAD51 filaments which catalyze homology search and DNA strand invasion followed by repair synthesis and annealing with the second end of DSB. Each stage of this multistep pathway requires the sequential involvement of a number of distinct enzymes [8-11]. On the other hand, NHEJ is an error-prone DNA repair mechanism that utilizes the specialized DNA end-binding proteins Ku70/Ku80 and various DNA-specific enzymatic

proteins such as DNA-dependent protein kinase (DNA-PK), nucleases, polymerases, and ligases. NHEJ does not require DNA sequence homology and can take place throughout the cell cycle [10]. At the chromatin level, an initial event triggering DDR is the phosphorylation of H2A.X, which is called γ-H2A.X, forms nuclear foci on the sites of DNA damage and is necessary for the assembly of repair complexes [12,13].

DNA-DAMAGING CAUSES	- ROS - X-rays - Alkylating agents - Spontaneous reactions	- UV light - Polycyclic aromatic hydrocarbons	- Replication errors	- X-rays - Ionizing radiation - Antitumor agents
DNA LESIONS OBSERVED	- Oxidation (8-oxoG) - Uracil Abasic site - Single strand breaks	- Bulky adducts - Intrastrand cross links	- A->G mismatches - T->C mismatches - Insertions - Deletions	- Double-strand breaks - Interstrand cross links
DNA REPAIR PATHWAYS	- Base Excision Repair, BER	- Nucleotide Excision Repair, NER	- Mismatch Repair, MMR	- DSBs Repair: - Non-Homologous End Joining, NHEJ - Homologous Recombination, HR

Table 1. Common DNA-damaging causes and agents, examples of DNA lesions produced by these sources, and the relevant DNA repair mechanism responsible for their removal.

In normal conditions, under extensive damage or inability of repair, senescence or apoptosis may occur as distinct checkpoints block cell duplication and/or survival. Persistent DNA damage can entail mutagenesis such as base substitutions and small insertions/deletions, as well as gross chromosomal rearrangements. Such genome instability likely contributes to aging and age-related disease and it constitutes an essential step in the development of cancer [14,15].

1.2. Cancer stem cells

Cancer is broadly defined as a group of diseases characterized by uncontrolled growth and spread of abnormal cells bearing genetic or epigenetic alterations resulting in high morbidity and mortality. Since the declaration of "war on cancer" about 50 years ago, significant strides have been made in battling the disease thanks to the worldwide scientific community's concerted effort for a better understanding of cancer biology. As a result, an apparent target of antitumor therapy was proved to be the destruction of tumor genetic material. Tearing up the DNA of carcinoma cells, usually combined with tumor removal by surgery, is suggested as one of the most effective therapeutic schemes resulting in residual cancer cell death. Radiation therapy and common chemotherapeutics are DNA damaging agents targeting the genome of carcinoma cells and nowadays they represent conventional

treatment schemes. A common practice in cancer treatment includes a combination of surgery, chemotherapy, and radiotherapy, depending on the lesion type and the clinical picture of the patient. Nevertheless, cancer still plagues humanity as a largely incurable disease. Poor prognosis and low survival rate are even more prominent in cases where malignancy is detected at a late stage. Another challenge for oncologists arises from frequent metastasis and tumor recurrence, which further frustrates effective treatment protocols currently available. Despite "heavy" therapeutic schemes, a subpopulation of cancer cells seems to evade DNA-damage-induced cell death and retain the ability of tumor regrowth through metastatic spread capacity. This subpopulation was termed cancer stem cells (CSCs) or tumor initiating cells (TICs), and is considered to be a major cause of tumor relapse due to local and/or distant recurrence of carcinoma cells [16-22]. The underlying mechanisms are not clearly understood in detail but a lot of work is accumulating worldwide, aiming to elucidate CSCs resistance etiology to DNA-damaging agents [1,23].

CSCs seem to differentiate into a diverse panel of progeny cells that make up the tumor, and reproduce the original tumor after xenotransplantation. There are several theories regarding the origin of CSCs [24]. According to a widely accepted hypothesis, CSCs are considered to derive from malignant transformation of stem/progenitor cells, when encountering special genetic mutations or environmental alterations, instigating the tumorigenic process. This hypothesis indicates that only CSCs possess tumor-initiating potential whereas non-CSCs do not. CSCs can be distinguished from other cells within the tumor by symmetry of their cell division and alterations in their gene expression. CSCs possess stem-like properties, such as self-renewal, proliferation and differentiation abilities, expression of pluripotency factors (e.g., Sox2, Oct4, Nanog) and functional markers (e.g., ALDH1, CD133+, CD44+, CD24, CD38-), active signaling pathways (e.g., Notch, Hedgehog, Wnt), genetic and epigenetic profiles similar to stem cells, and the capacity to form form 3dimensional spheres *in vitro*. Cell surface markers such as CD44, CD24, CD133, epithelial specific antigen (ESA), and aldehyde dehydrogenase1 (ALDH1) have been used to isolate and enrich CSCs from different tumors. Markedly, CSCs express surface markers, which seem to be tissue-type-specific and in some cases tumor-subtype-specific. For example, breast CSCs are characterized as CD44+/CD24−/low and ALDH +, based on the presence or absence of the respective molecules. In analogy, CD133 expression is characteristic for colon, brain, and lung CSCs, while CD34 presence and CD8 absence (CD34+/CD8-) are characteristic for leukaemia. The list includes CD44+ for head and neck, CD90+ for liver, and CD44+/CD24+/ESA+ for pancreas CSCs [25-28]. Nevertheless, novel combinations of CSC markers are continuously being added to this group or combinations not previously defined are also described (Frangou et al., unpublished data). Therefore, expression of CSC surface markers can only be a manmade criterion to describe tumor stem cells and some CSCs may not fulfil these criteria. Moreover, CSCs have been found to exhibit similarities with normal stem/progenitor cells in cell surface marker expression, properties, phenotype, and function. For example, the mammary gland progenitor cells are characterized as a CD44+/CD24−/low cell population and resemble the CD44+CD24−/low cells identified as CSCs from breast cancer patients. Apart from the theory suggesting that CSCs derive from transformation of normal stem cells, an alternative theory about the origin of CSCs suggests that they may arise from transformation of normal somatic cells. According to this notion, somatic cells

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acquire stem-like characteristics and malignant behavior through genetic and/or heterotypic alterations. The epithelial to mesenchymal transition (EMT) of cancer cells during the metastatic process may provide a mechanism by which cancer cells may gain stem-like characteristics [29,30]. Further research is required to elucidate whether both theories may be correct about CSCs' origin but each explain distinct cases.

In addition, another role attributed to normal stem cells in the various tissues is their implication in the repair process of damaged tissue. In order to fulfill this task, normal stem cells have to overcome genotoxic insults and in turn proliferate to restore eradicated tissue cells. Since much evidence favors cancer originating from stem cells, it is not surprising that many of survival and proliferation pathways of stem cells have aberrant expression in cancer cells. In accordance to this hypothesis, the traditional pathways Notch, Hedgehog (Hh), and Wingless/Int (Wnt) have been proposed to also characterize CSCs [31].

Another aspect to consider is that tumorigenesis is followed by angiogenesis and by cancer cell invasion in other tissues (metastasis) as part of the disease progression. Not surprisingly, CSCs have been associated with the induction of tumor vascularization through the expression of vascular-related factors and by their contribution to metastasis through the induction of the Epithelial to Mesenchymal Transition (EMT) program [30,32].

1.3. Cancer stem cell resistance pathways

For all the aforementioned reasons it is obvious that CSCs' resistance to chemo- and radio therapies is clinically important as most anticancer agents target the tumor bulk but not the CSC population. As previously mentioned, mechanisms helping cells to escape cytostatic and cytotoxic effects after application of DNA-damaging treatment approaches are far from clearly understood despite intensive and extensive studies. Cancer stem cell (CSC) theories attempt to explain how CSCs overcome cell death caused by genotoxic treatment schemes. It is also suggested that CSCs possess specific intracellular molecular properties assisting them to avoid treatment-derived cytotoxicity [23]. Various mechanisms account for CSCs drug resistance. A comprehensive example comes from studies on breast cancer stem cells. Of all the CSCs identified in solid tumors, breast CSCs are one of the most commonly studied. They are defined as CD44$^+$/CD24$^-$ cells and have been linked to resistance to many forms of treatment, including radiation therapy. CD44$^+$/CD24$^-$ cells isolated from MCF-7 and MDA-MB-231 breast cancer cells were more radioresistant than non-CD44$^+$/CD24$^-$ cells [33]. The mechanisms involved possibly include decreased induction of reactive oxygen species and activation of the DNA damage checkpoint response [34]. Moreover, an *in vitro* study has documented that during a fractionated course of radiation, the number of CSCs with activation of Jagged-1 and Notch-1 increased, suggesting the possible induction of radiotherapy resistance via the Notch signaling pathway [33].

Overall, CSCs display features different from the bulk of cancer cells, like high-level expression of ATP-binding cassette membrane transport proteins (ABC transporters), mainly including ABCG2/BCRP and ABCB1/MDR1; high capacity for DNA signaling and repair; reduced immunogenicity; inherent anti-apoptotic properties; and quiescence. Understanding the mechanisms of chemo- and radiation-resistance of CSCs may pave the way towards discov-

ering a set of signaling pathways unique to CSCs. Such mechanisms may be responsible for CSCs' capability to resist and survive the current chemoradiation therapeutic schemes. Targeting such set of pathways would ideally provide more effective and presumably personalized therapeutic potential towards complete tumor eradication. Despite difficulties and though such ideal pathway has not been found yet, elucidation of developmental pathways that control survival, proliferation, and differentiation of stem cells is under extensive investigation.

As summarized in Figure 1, the resistance ability of CSCs appears to be associated with their slow-cycling phenotype, and/or expression of efflux transporters, antiapoptotic proteins, altered profile of cell surface markers, DNA response and repair mechanisms, or presence of free radical scavengers [27,31,35-38].
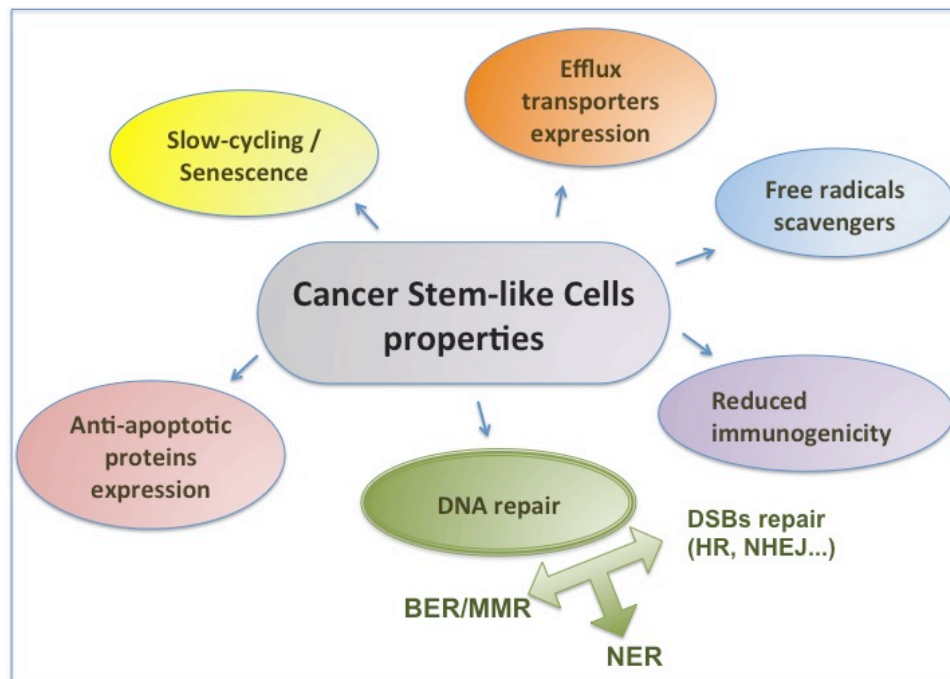
Figure 1. Schematic representation of CSCs properties related to chemo- and/or radioresistance (details in the text).

Among these characteristics, the multifaceted protection of genome integrity by a prompt activation of the DNA damage sensor and repair machinery is one of the key features rendering resistance to applied genotoxic insults. CSCs possess highly effective DNA repair systems mainly consisting of double-strand breaks (DSBs) repair, base excision repair (BER), transcription-coupled nucleotide excision repair (NER), and mismatch repair (MMR) pathways. These pathways seem to be distinctly regulated in CSCs, resulting in significant enhancement of DNA repair capability and finally radio- and chemoresistance [23,39-43].

Therefore, the aim of the current chapter is to highlight issues and discuss controversies of CSC genotoxic resistance through DNA Damage Response (DDR) and DNA repair pathways,

which seem to play a key role in evading genotoxicity-induced cell death. This is a highly evolving topic as the anticipated information about the basic mechanisms governing DNA repair processes is also expected to contribute in predicting and improving therapy responses and the clinical outcome in cancer patients treated with DNA-damaging agents.

2. DNA damage resistance of CSCs

Accumulating reports tend to indicate that there are CSCs in almost all tumor types (reviewed in [22]). For example, using the CD133 as the brain stem cell marker, Bao et al. at Duke University have described an increased proportion of brain CSCs, from about 2 to about 8% in control versus irradiated tumors, which was associated with tumor radioresistance [34]. A group at UCLA showed that breast-cancer-initiating cells displaying the marker of breast CSCs (CD24−/low/CD44+) are radioresistant and cells expressing these markers increase after short courses of fractional irradiation [33]. All of these findings shed new light on the mechanisms of an accelerated tumor cell proliferation with an increase in the percentage of radioresistant CSCs.

The use of radiation therapy and chemotherapeutic drugs aim to provoke DNA damage in cancer cells. If the damage is quite extensive and cannot be repaired, cell death is inevitable [44]. Research on radio- and chemoresistance of CSCs after exposure to DNA-damaging agents has been extensively conducted and there is great evidence demonstrating that this subpopulation in tumors protects itself from DNA-damaging treatment by multiple mechanisms. First of all, CSCs are considered to have an enhanced DNA repair capability and consequently they are protected more effectively than the rest of tumor cells. Numerous studies suggest that the resistance of cancer stem cells to therapy is mediated by more robust DNA damage response and repair pathways [45-48]. Special regulation and elongation of cell cycle is also regarded to be another protection mechanism incorporated, providing CSCs more available time to repair damaged DNA. Moreover, CSCs demonstrate great efficiency in scavenging of reactive oxygen species (ROS) and therefore eliminate the primary cause of DNA insults [49-51].

In the following paragraphs, information regarding CSCs' resistance to DNA damage through both genotoxicity inactivation and DNA Damage Response/Repair mechanisms will be presented. It is well documented that depending on the type and tumor stage, CSCs adopt distinct main and auxiliary mechanisms to protect their genome and overcome insults. The most prevalent intrinsic molecular determinants of radioresistance appear to be the protection from oxidative DNA damage by enhanced ROS scavenging as well as the enhanced DNA repair capability by post-translational modification of damage signalling factors (ATM and CHK1/CHK2 phosphorylation) and subsequent repair.

2.1. ROS scavenging

The indirect pathway of radiation-induced damage includes the generation of chemically reactive free radicals, including the product of oxygen metabolism called reactive oxygen species (ROS). These products play an important physiological role and participate in many

signaling events regulating cell proliferation, migration, angiogenesis, wound healing, and metabolism [52]. Both normal and cancer cells can control ROS level by balancing their production and elimination by ROS-scavenging molecules such as glutathione, peroxidase, catalase, superoxide dismutase, thioredoxin, etc. [53]. An excessive production of ROS in response to irradiation may lead to their interaction with critical cell macromolecules including DNA, lipids, and proteins, leading to cell death [54]. High resistance of CSC populations in breast and gastrointestinal carcinomas to genotoxic stress is related to a more efficient ROS scavenging system and lower levels of ROS production after irradiation as compared to non-CSC populations [49]. Genes involved in ROS scavenging, including superoxide dismutase, glutathione peroxidase, and catalase are upregulated in CD44+CD24– breast CSCs. The role of ROS scavenging in CSC radioresistance is supported by the observation that pharmacological depletion of ROS scavengers in tumor progenitors by treatment with buthionine sulfoximine (BSO), which inhibits glutamate-cysteine ligase, markedly decreased clonogenic properties and radioresistance of CSCs [49]. The functional link between stem cell markers and ROS metabolism was first demonstrated by Ishimoto and colleagues who showed that CD44 interacts with glutamate-cysteine transporter xCT and controls the intracellular level of ROS scavenger glutathione in gastrointestinal cancer cells [50]. These preclinical results are supported by recent clinical studies, which showed that high expression of CD44 in tumors was correlated with resistance to radiation therapy and associated with early recurrence in HNSCC patients [55-57].

The activity of aldehyde dehydrogenase (ALDH) enzymes is also highly correlated with the existence of cancer stem cells in tumors [58]. Furthermore, there is a relationship between poor clinical prognosis in breast and prostate cancer and increased expression of ALDH1 [59]. It has been proved that ALDH1 and ALDH3A1 play a key role in the cellular response to oxidative stress, since they contribute to the scavenging of radiation-induced free radicals and the production of the antioxidant NAD(P)H [60]. These observations suggest that ALDH activity can be crucial for regulation of cell radio-sensitivity. High ALDH1 activity is another characteristic of human breast and colonic cancer stem/progenitor cells [61]. As few as 500 ALDH1-positive cells (as documented by the ALDEFLOUR assay) can give rise to a new tumor in NOD/SCID mice [59]. Other studies have found overexpression of ALDH1 in cyclophosphamide-resistant leukemic and colonic cancer cells [62]. Thus, overexpression of the detoxification enzyme ALDH1 may also contribute to the resistance of CSCs to various cancer treatments, including chemo- and radiation therapy resistance.

Another characteristic example of ROS management is through the regulation of Ape1/Ref-1, also known as APEX1, a ~37kDa protein containing a redox activity and an endonuclease activity domain. Ape1/Ref-1 is involved in BER. During cell exposure to genotoxic agents its expression is increased in response to reactive oxygen radicals (ROS) production. Activation of Ref-1 redox domain decreases ROS levels resulting in the enhancement of carcinoma cell stemness and self-renewal. On the other hand, inhibition of the Ref-1 domain, which is responsible for ROS scavenging, results in increased intracellular ROS levels, activation of p53, and promotion of cancer cell differentiation and cell death. Permanent ROS production, possibly caused by microenvironmental factors in the CSC niche, results in overexpression of

Ape1/Ref-1, which may effectively protect CSCs from ROS genotoxic effects produced during treatment with ionizing radiation/DNA-damaging agents. Besides, redox modulation of p53 by this factor seems to contribute significantly to DNA repair in CSCs. Ape1/Ref-1 is also required for redox regulation of HIF-1α, thus regulating downstream controlled DNA repair genes. Likewise, Ape1/Ref-1 is also implicated in the regulation of Rac GTPase activity, a protein closely related with CSC formation, resistance to DNA-damaging agents (BER, G2-M checkpoints activation allowing repair of damaged DNA), and carcinoma cell motility and migration. On the other hand, BER allows mutation accumulation and further genome instability, occurring also due to Raf1, MEK1/2, and ERK1/2 pathway activation, which promotes enhancement of tumor aggressiveness, insensitivity to therapeutic approaches, and metastasis [23,63].

2.2. DNA damage response/signaling

Radiation-induced cell death may occur as a result of direct and indirect energy transfer to critical cellular structures including chromatin, plasma membrane and mitochondria.

Cell-cycle checkpoint components, such as Ataxia Telangiectasia Mutated (ATM) protein, Ataxia Telangiectasia/Rad3-Related kinase (ATR), and checkpoint kinases (Chk1 and Chk2), become engaged under replication stress or in response to DSBs. These cell-cycle arrest mechanisms allow the recruitment of either DNA repair effectors or in case of irreversible damage and repair failure, of proapoptotic molecules [65].

The observed resistance of CSCs to common chemo-/radiotherapy strategies is considered to partly occur through their extensive ability of repairing DNA damage that has been provoked through radiation or chemical drugs. This enhancement of DNA repair capacity can be either direct, through elevated DNA repair mechanisms, or indirect, through delayed cell-cycle progression.

A principal player in DDR, in both normal and malignant cells, is the major sensor of DNA double-strand breaks termed MRN complex (a complex of MRE11, RAD50, and NBS1 proteins). MRN complex binds to and stabilizes broken DNA ends and is required for the activation of ATM. MRN complex functioning through BMl1 is also interconnected with CSC-related molecules like Notch1, ALDH1A1, CD44, and Sonic Hedgehog, together with telomere biology and upon deregulation, with aggressive tumor behavior and unfavorable disease prognosis [66]. Moreover, another mechanism rendering Glioblastoma Multiforme (GBM) stem cells relatively resistant to DNA damage through MRN-ATM-Chk2 network signaling involves L1CAM (CD171) interaction with NBS1. L1CAM intracellular domain (L1-ICD) nuclear translocation mediates NBS1 upregulation via c-Myc. Ectopic expression of NBS1 in GSCs rescues the decreased checkpoint activation and radioresistance caused by L1CAM knockdown. These data demonstrate that L1CAM augments DNA damage checkpoint activation and radioresistance of GSCs through the enhanced MRN-ATM-Chk2 signaling, resulting in GSCs displaying a preferential activation of DNA damage checkpoint and radioresistance [67].

The major sensor and signaling effector of DDR, ATM kinase, seems to also contribute to DNA damage resistance of CSCs. ATM comprises a key DNA damage sensor and downstream effector kinase playing central roles in DNA repair, cell-cycle control regulation, and development of senescence and/or apoptosis (refs). Several studies indicate that ATM activity may play a role in normal stem cell maintenance and proliferation. Two main roles are attributed to ATM: a role in stem cell survival and an implication, as part of DDR, in pathways classically linked to stem cell maintenance [68,69].

Regarding the first role, ATM seems to be implicated in regulation of neuronal stem cell survival (NSCs). More precisely, while ATM is abundantly expressed in NSCs, it is gradually reduced during cell differentiation. This observation suggests that ATM is vital for NSC survival and function [70]. ATM is required to maintain normal self-renewal and proliferation of NSCs due to its role in controlling the redox status. Loss of ATM renders NSCs defective for proliferation through oxidative-stress-dependent p38 MAPK signaling, suggesting that p38 is a central player in the defective proliferation of Atm/NSCs induced by oxidative stress [71, 72]. Moreover, it has been shown that ATM plays a central role in terminal differentiation of a human neural stem cell line model through its function in DDR [73].

In addition, ATM protein is a major player in signaling pathways classically implicated in stem cell maintenance. Lately, ATM was proposed to positively modulate the activity of ITCH E3-ubiquitin ligase. ITCH is a member of the NEDD4-like family of HECT-E3-ubiquitin ligases, a family of proteins that participates in several physiological signaling pathways, including the DNA damage response, tumor necrosis factor (TNFα), Notch, and Sonic-Hedgehog signaling [74]. The ATM-dependent activation of ITCH requires the single amino acid residue S161 of ITCH protein, which is part of an ATM S/T-Q consensus motif. Subsequent *in vitro* and *in vivo* genetic experiments provided evidence showing that ATM kinase enhances ITCH enzymatic activity and triggers ubiquitination/degradation of ITCH (itchy E3 ubiquitin protein ligase) substrates such as FLIP-L and JUN [75]. Possibly, ATM is also implicated in the regulation of other ITCH substrates including the transcription factor GLI-1. GLI-1 mediates Sonic-Hedgehog (SHH) signaling, resulting in regulation of tissue patterning and cell proliferation. These cellular functions are prerequisites ensuring the accurate developmental progress and homeostasis maintenance of adult tissues [76]. Interestingly, several lines of evidence suggest a putative cross talk between ATM and the SHH pathway. The first hint comes from the identification of GLI-1 as a substrate of ITCH [77,78] and from the observation that ATM modulates ITCH [75]. A second hint comes from the observation of the wild-type p53-induced phosphatase 1 (WIP1) function. WIP1 is a Ser/Thr phosphatase, which is aberrantly upregulated in cancer and modulates ATM activity by dephosphorylation [79]. Moreover, WIP1 is involved in the modulation of the SHH signaling [80]. Presumably, during tumorigenesis, overexpression of WIP1 may be implicated to increased GLI-1 activity resulting in both the proliferation and self-renewal of CSCs. This phenomenon may be responsible for CSCs expansion as the derived CSC progenitors retain the ability to sustain tumor growth [80]. In accordance to this hypothesis, it was recently shown that wild-type p53 downregulates GLl-1 function by sequestering the co-activator TATA Binding Protein Associated Factor 9 (TAF9), a process comprising an inhibitory loop controlling stem cell and tumor cell numbers

[81]. Furthermore, accumulated data suggest a possible bidirectional connection between GLI-1 and the DDR. It has been observed that there is a feedback loop in GLI-1 level regulation as abnormal increase of GLI-1 induces DDR, which in turn may decrease GLI-1 activity. The delicate control of GLI-1 activity may thus be part of the mechanisms controlling the precursors and stem cell numbers and preventing tumorigenesis [82,83]. Whether ATM kinase may directly modulate SHH signaling, therefore contributing to the maintenance of stem cell identity, remains to be elucidated.

Furthermore, DDR and ATM activation are also implicated in the regulation of CSCs' survival. In particular, upon therapy-induced DNA damage, temporal halt of proliferation through cell-cycle elongation may provide cancer stem cells with increased time for repair. Upon genome insults restoration, the replisome is reactivated for genome duplication. Through such pathways genotoxic resistance may be triggered in CSCs. Therefore, by specific inhibition of the DNA damage checkpoint response, a cell-cycle break may occur in CSCs, driving them again towards proliferation and thereby specifically sensitizing them to genotoxic insults caused by radiotherapy. In this context, ATM may serve as a useful candidate target for eliminating cancer stem cells in the tumor. This notion is further supported by recent studies showing that constitutive activation of a DDR started by ATM may promote radioresistance of CSCs. Utilization of this idea was pioneered by Bao and collaborators in glioblastoma multiforme (GBM). In GMB, cancer stem-like cells are characterized by expression of Promi-nin-1 (CD133). CD133 is a marker for both neural stem cells and brain cancer stem cells. CD133+ CSCs of GMB were shown to preferentially activate the DNA damage checkpoint in response to radiation accompanied by higher expression of activating phosphorylation of ATM, RAD17, CHK1, and CHK2 checkpoint proteins following IR treatment [34]. As a result, CD133+ cells exhibited preferential survival after irradiation, a phenomenon that may be reversed after treatment with CHK2 inhibitor. In consistency to these results, two distinct grade IV glioma cell lines, varying in CSC content (low and high, respectively), were preincubated with a nontoxic concentration of the ATM inhibitors KU-55933 and KU-60019 and then irradiated. The aim of this experiment was to investigate potential improvement of the therapeutic efficacy of radiation on glioma stem cells. Indeed, GSCs were sensitized to IR by ATM inhibitors, as revealed by a significant reduction in their survival. Quite interestingly, IR treatment following cell differentiation showed no sensitization, indicating that ATM inhibi-tors specifically sensitize GSCs [84]. Recently, a profound radiosensitization of GSCs was obtained by using a combination of PARP and ATR inhibitors which exceed the effect of ATM inhibition alone [85]. However, it is encouraging that similar results following ATM inhibition have been obtained when using CD44+/CD24−/low cells, a subpopulation enriched for CSCs, from two breast cancer cell lines and a primary breast cancer cell culture isolated from breast cancer patient. In these cases, the CSC-enriched subpopulations demonstrated enhanced expression of phosphorylated ATM after radiation, a results concomitant with increased radioresistance. These results were also reverted when the ATM inhibitor KU-55933 was used. In this case also the CD44+/CD24−/low subpopulation isolated both from the cell lines and from the primary culture exhibited significant decrease of radiation resistance [86]. Jointly, a crucial role for ATM signaling in survival capacity of CSCs in response to genotoxicity is supported by these findings, further suggesting that ATM inhibition may be exploited towards the development of novel therapeutic strategies against CSCs.

DNA damage induces checkpoint mechanisms including two distinct kinase signaling pathways, the ATM-Chk2 and ATR-Chk1 pathways, which are activated by DSBs and single-strand DNA breaks, respectively. DNA damage checkpoint signaling inhibits cell-cycle progression to allow DNA repair [87]. Recent findings of Bartucci et al. suggests that chemotherapy-induced activation of the DNA damage checkpoint-Chk1 signaling in a stem-cell-enriched cell subset within Non-Small-Cell Lung Carcinoma (NSCLC) led to cell-cycle arrest, more efficient DNA damage repair and a higher cell survival rate as compared to the differentiated tumor cell population. The use of Chk1 inhibitor AZD7762 in combination with chemotherapy significantly reduced survival of the stem cell population by inducing premature cell-cycle progression and subsequent mitotic catastrophe [88]. As aforementioned, studies of glioblastoma by Bao and co-workers demonstrated that ATR-Chk1 and ATM-Chk2 signaling pathways are preferentially activated in CD133+ progenitor cells, but not in CD133− cells in response to radiation-induced genotoxic stress, and CD133+ cells repair DNA more effectively than CD133− tumor cells [34]. Moreover, relative radioresistance of CD133+ glioblastoma progenitor cells can be reversed by pharmacological inhibition of the Chk1 and Chk2 kinases with debromohymenial-dine (DBH) [34]. Chk1 knockdown in CD133+/CD44+ prostate cancer tumor-initiating cells abrogated the radiation-induced cell-cycle arrest and conferred CSC radiosensitization [89]. In addition, ATR or Chk1 inhibition sensitizes colon cancer stem cells to cisplatin. Remarkably, treatment of human colon cancer cells with caffeine, an unspecific inhibitor of PIKK kinases, led to depletion of the CD133+ chemoresistant and tumor-initiating cell population, which may suggest an overlap of the signaling pathways regulating tumorigenic properties and DNA damage response [90].

In the case of glioma CSCs, high expression levels of checkpoint kinases CHK1 and CHK2 have been observed, indicating expansion of cell cycle. The delay of cell cycle offers more time for DNA repair 1 have been also monitored in non-small-cell lung CSCs. In leukemia, CSCs increased levels of p21, which inhibits cell-cycle progression, providing sufficient time to repair DNA damage.

Chk1 inhibition has been shown to inhibit both the DNA-damage-induced cell-cycle checkpoint response and homologous recombination repair [91,92] and many studies have also shown that cancer stem cells elicit a more robust Chk1-mediated DNA damage response than non-stem cells [34,47,90]. As an example, Chk1 inhibition was used to sensitize pancreatic cancer stem cells to gemcitabine. A combination of gemcitabine and AZD7762 significantly reduced the percentage of marker-positive cells (pChk1 (S345) as a pharmacodynamic biomarker of gemcitabine-AZD7762 activity [93]) and decreased the tumor-initiating capacity of cancer stem cells using a limiting dilution assay. The same study demonstrated that Chk1 inhibition displayed a heightened DNA damage response in stem cells (vs. non-stem cells) overall highlighting the potential efficacy of this approach to target pancreatic cancer stem cells [94]. The possibly underlying greater extent of DNA damage in the stem cells treated by this approach is consistent with their sensitization to gemcitabine by Chk1 inhibition. As inhibition of Chk1 produces more DNA damage that is marked by a more robust DNA damage response in cancer stem cells, an important future challenge remains to better understand the mechanisms contributing to selective cancer stem cell sensitization through the incorporation

of *in vitro* pancreatic cancer stem cell models. In parallel, other studies have shown that the AKT inhibitor, perifosine, sensitizes breast cancer stem cells to radiation [95], whereas targeting DR5, SHH, or mTOR in combination with gemcitabine reduces pancreatic cancer stem cells [96,97].

3. DNA repair capability

The most lethal form of DNA damage is considered to be the DNA double-strand breaks (DSBs), which can be repaired by either homology-directed recombination (HR) or nonhomologous end joining (NHEJ), depending on the phase of the cycle [98]. During HR, RAD51 filaments assemble to single-stranded DNA tails and catalyze homology search. One of the early and best characterized chromatin modification events in DNA DSB response is histone H2AX phosphorylation on serine 139 of its C-terminal tail by phosphatidylinositol 3-kinase-related kinases (PIKKs) DNA-PK, ATM, or ATR serine/threonine protein kinases, which are activated by DNA damage. A few independent studies demonstrated that CSCs have an activated DNA repair process. Recent finding suggests that human breast CSCs and murine mammary gland CSCs have significantly more RAD51 foci and less γ-H2AX foci after irradiation compared to non-CSC population that is reflective of more efficient DSB repair in these CSC populations [99]. A high DNA repair capability has been attributed to CSCs in a variety of tumor entities including glioma, nasopharyngeal carcinoma, lung, breast, and mouse mammary tumors [34, 100-103]. In a syngeneic p53 null mouse mammary gland tumor model, it was shown that this cell subset has an increased expression of genes involved in DNA damage response including Nek1, Brca1, Chek1, Hus1, Ung, Xrcc5, Sfpq, and Uhrf1 [104].

A paradox seems to emerge in CD133+ glioblastoma stem cells which activate ATM and Chk1 more promptly than the CD133- counterpart [34]. This molecular response enabled CD133+ cells to survive ionizing radiation, as opposed to the CD133- population that underwent cell death. While radiosensitivity is restored by pharmacologic abrogation of Chk1 and Chk2, it seems that the glioblastoma stem cell pool does not possess enhanced DNA repair activity following exposure to ionizing radiation. In this case, radioresistance properties were linked to cell-cycle kinetics, as indicated by the significant increase in the population doubling time and enhanced basal activation of Chk1 and Chk2 [105]. This elongated cell cycle, therefore, theoretically provides more time for repairing DNA damage. To further intricate this picture, a direct comparison of radiosensitivity between glioblastoma stem cells and a panel of established glioma cell lines revealed that CD133+ cells exhibit reduced DSB repair ability [46]. Cell-cycle analysis revealed that although glioblastoma stem cells possessed an intact G2 checkpoint, they displayed deficient activation of the intra-S-phase checkpoint. Because the latter checkpoint is crucial for maintaining genome integrity, chemotherapy could paradoxically lead to the emersion of genetically unstable CSCs, thus explaining the pattern of disease progression during sequential chemotherapeutic regimens.

The genetic heterogeneity of CSCs could mirror a different DNA damage repair proficiency among subtypes, thus providing a possible explanation for the conflicting results discussed above.

High-grade primary brain tumors are also known to aberrantly activate the phosphoinositide 3-kinase (PI3K)/ Akt pathway [106], an oncogenic axis functionally interconnected with the DNA repair machinery, as highlighted by the ability of PI3K or Akt inhibitors to hamper the removal of radiation-induced DNA damage [107]. It is worth considering that the pharmacologic abrogation of Akt impaired glioblastoma stem cell fitness and abrogated neurosphere formation [111], thus allowing postulating that Akt inhibitors could be exploited as chemotherapy-enhancing agents.

Notch and Wnt signaling pathways, utilized in normal stem cell self-renewal, were also found to mediate radioresistance in glioblastoma and breast cancer [33, 108,109].

On the other hand, Facchino et al. have shown that the polycomb group protein BMI1 is enriched in CD133+ glioblastoma stem cells and operates as a recruitment platform for the double-strand break repair (DSB) response and nonhomologous end joining (NHEJ) proteins, resulting in increased cell radioresistance [110].

Based on the above mentioned studies, attempts for stratifying tumor types based on the DNA repair expression index were able to show statistically significant prognostic capability of the clinical outcome, paving the way towards personalized treatment schemes [112]. In addition, inhibitors of DNA damage response are increasingly utilized in order to increase DNA damage sensitivity [113]. Quite recently, an elegant approach developed a NOTCH tumor phenotype in C. elegans delineating the role of NOTCH receptors expression in relation to HR and radiosensitization [114]. Numerous ongoing studies are focusing on deciphering the detailed DDR /repair mechanisms governing CSCs resistance in solid tumors with expected encouraging results [115].

In the field of haematological malignancies, the development of solid and hematological tumors is strongly associated with the presence of small populations of cells known as tumor stem cells. In AML, these cells are referred to as leukemic stem cells (LSCs). LSCs, which are considered to originate from hematopoietic stem or progenitor cells due to defects in their self-renewal and differentiation processes, not only adopt the regulatory machinery operating in normal HSCs but also establish their own mechanisms against apoptosis and senescence. Therefore, hematopoietic stem cell transplantation along with combination of chemotherapy comprises one of the major therapeutic strategies for hematological malignancies, such as Chronic Lymphoid Leukemia (CLL) and Acute Lymphoid Leukemia (ALL) of lymphoid origin, Acute Myeloid Leukemia (AML) and Chronic Myeloid Leukemia (CML) of myeloid and plasma affecting multiple myelomas.

In a similar way to normal hematopoiesis, AML is arranged as a loose hierarchy in which a small population of self-renewing leukemic stem cells (LSCs) give rise to a large population of more mature leukemic blasts, which lack self-renewal capacity. This organization helps to explain the observed clinical scenario in AML whereby current chemotherapeutic regimes frequently induce remissions but often fatal relapses occur. A number of mechanisms have been suggested to explain the exquisite resistance of LSCs to chemotherapy like the expression of various ABC transporters that export drugs out of the cell, the efficient DNA repair mechanism and the protection provided by the bone marrow microenvironment, namely the

stem cell niche where the LSCs are located in a hypoxic extracellular matrix preventing the exposure of LSCs to chemotherapy [116,117]. Research on AML pathology over the past years has revealed that a variety of polymorphisms in DNA damage repair (DDR) genes are associated with increased risk of developing AML or lead to disease relapse. Moreover, epigenetic silencing of DDR genes affects leukemogenesis, while, on the other hand, elevated levels of DNA repair induces chemotherapy resistance, by allowing cells with severe damage to attempt repair and survive. Therefore, DNA repair mechanisms' status not only influences the genetic predisposition to leukemia but is also very important for refractoriness to treatment [118]. Several major pathways of DNA repair are known to be implicated in AML, including homologous recombination (HR), nonhomologous end joining (NHEJ), base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Different DNA repair processes overlap in their function. When the damage is moderate and the repair processes inadequate, the cells acquire mutations and genomic instability occurs, representing an initial step towards malignant transformation, while also, increased processes of DNA repair contribute to resistance to chemotherapy and radiation [118]. Myeloid malignancies, such as AML, are frequently characterized by defects in the DNA repair machinery. Defects of the HR machinery, as well as BER, NER, and MMR, have been mainly associated with therapy-related AML (t-AML) and are rarely seen in *de novo* AML. RAD51 is the central molecule of the HR pathway and its polymorphic variant RAD51-G135C correlates with increased risk of t-AML, a risk that increases when it is combined with XRCC3-Thr241Met variant [119,120], probably because it leads to RAD51 upregulation [121] that might have a dominant negative effect on HR initiation [122]. Although mutations involving NHEJ genes predisposition to leukemia have yet to be revealed, it is known that chromosomal instability in myeloid neoplasms often results from deregulated NHEJ and inadequate DSB repair. The rate of NHEJ in leukemic blasts is two- to sevenfold higher compared to normal cells and results in major mis-repair, accounting for increased genomic instability in AML cells [123] such as chromosomal translocations, mostly depletions [124]. BER mechanism concerns the removal of the bases changed by alkylation, oxidation, or ionizing radiation. BER also takes part in the single-strand breaks repair. Important components of BER pathway are the poly(ADP-ribose) polymerases (PARP) family containing 18 members which allow for access to DNA repair enzymes in the case of single-strand breaks. Cancer cells with defective HR processes switch to PARP-mediated BER mechanisms. Hence, PARP1 inhibitors are extremely active in tumors deficient in HR pathway. Moreover, AML1-ETO fusion gene is implicated in AML pathology [125] and acts by inhibiting differentiation and immortalizing the hematopoietic progenitors [126], through the repression of a variety of genes involved in DDR, in particular in BER (i.e., *OGG1*, *FEN1*, *MPG*, and *ATM*) [127]. The role of NER mechanism has also been investigated in AML. Common polymorphisms in XPD gene belonging to NER pathway are associated with the risk of AML development. XPD Lys 751 Gln variant is an independent prognostic marker for disease-free survival and overall survival in elderly AML patients [128].

Given the great implication of the DNA repair mechanisms in the pathology of AML, over the last years several new therapeutic strategies have been proposed for its treatment, based on DNA repair inhibitors. Therefore, RAD52 aptamers were proposed for BRCA-deficient AML [129], CHK1 inhibitors for AML patients who do not achieve remission with standard

chemotherapy [130], PARP inhibitors in combination with standard chemotherapy to reduce probable secondary leukemias [131] and HDAC inhibitors, which are believed to downregulate several genes of the DDR [132].

4. Ribonucleic acids and chromatin structure modifiers of CSC resistance

4.1. miRNAs

Another intriguing player in regulating CSCs' modified responses to genotoxic insults is gene expression regulation by microRNAs (miRNAs). The relatively recent discovery of miRNAs has added an entirely new dimension to our knowledge about the regulation of gene expression and the control of various cell functions, such as apoptosis, proliferation, differentiation, and therapy resistance [133]. Over the past decade, it has become progressively clearer that these tiny genetic regulators are linked to the development of cancer. The miRNA profiles have been shown to be highly informative, reflecting the developmental history and differentiation state of the tumors, and providing molecular links between cancer and normal stem cells. The fact that miRNAs expression may have adverse consequences for the functional properties of cancer cells has been recently highlighted for tumor radioresistance. Yan and coworkers for the first time demonstrated that miRNAs could be used to target the DNA repair machinery and thus sensitize tumor cells to radiation [134]. Since then, an accumulating body of research demonstrated that miRNAs can modulate tumor radioresistance [135-138]. *In vivo* experiments using xenograft models and clinical studies are needed to ascertain whether manipulation of miRNA expression can be a viable tool to augment current cancer therapies [139].

4.2. Chromatin structure and lncRNAs

Furthermore, CSCs display different epigenetic profiles, in comparison with their nontumorigenic progenies that result in changes of multiple signaling pathways [140]. These pathways may involve cell adaptation to microenvironmental stresses including inflammation, hypoxia, low pH, shortage in nutrients, and anti-cancer therapies as well. As a paradigm, the MGMT promoter methylation status is routinely assessed in patients diagnosed with glioblastoma multiforme. It is known that the MGMT pathway is adopted by glioblastoma cells to overcome temozolomide cytotoxicity and, to a similar extent, this enzyme protects glioblastoma stem cells from alkylating agents [141]. Notwithstanding, a comparative evaluation of the MGMT promoter methylation pattern between surgical samples and paired glioblastoma-derived neurospheres indicated that epigenetic silencing of MGMT is enriched in putative glioblastoma stem cells [142], thus shedding doubts on the biologic relevance of this pathway on survival of temozolomide-treated glioblastoma stem cells.

Another noteworthy example of prevention of mismatch DNA repair in human liver CSCs was revealed by the delineation of the mechanism of long non-coding (lnc) RNA HOTAIR function. HOTAIR, through downregulation of SETD2 gene expression, results indirectly in histone modifications and chromatin structure alterations leading to microsatellite instability and promoting tumorigenesis in liver CSCs [143,144]. In overall, chromatin structure seems to

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be another important player in the maintenance of genome integrity through a plethora of mechanisms and interactions with other nuclear components as the nuclear envelope [145-147]. Therefore, nuclear structure may be also significantly involved in CSC genotoxicity resistance.

5. Conclusion

In general, CSCs can successfully survive and initiate tumor regrowth largely due to mobilization of DNA response and repair mechanisms. Recent advances in high-throughput screening methods, like genomics, transcriptomics, proteomics, or epigenomics provide an enormous amount of data to the scientific community, contributing to the elucidation of the molecular pathways and networks underlying cellular DNA repair mechanisms.

Another interesting aspect is that normal stem cells retain the ability to repair tissue damage. The regenerative capacity of endogenous stem cells decreases with age, is impaired in degenerative diseases, and deregulated in cancer. Recent data reveal that "normal" stem cells detected in the neighborhood of a resected triple-negative breast tumor may be transformed to cancer stem cells and be also responsible for tumor recurrence. DNA repair capacity of these "neighboring" stem cells is under investigation and preliminary data from our group reveal possible deregulation of factors involved in BER and double-strand DNA breaks repair and a likely involvement of RecQ helicase family members in this process.

Despite ongoing progress, the regulation of DNA damage encountered in CSCs and the interrelation of genome surveillance pathways with cell cycle control, regeneration, and apoptosis possess many obscure sides and controversies. It is agreeable that an ideal CSC-specific therapeutic would target the CSC and bulk tumor cells with minimal adverse effects. Nevertheless, the phenotypical and functional properties of CSCs may be dynamically regulated during the course of genotoxic therapy. Understanding the complex mechanisms regulating the CSC population during the course of cancer treatment will turn CSCs into a powerful tool for therapeutic and diagnostics improvement. Integration of such information and further investigation are obviously required towards effectively targeting and eliminating CSCs from the malignant tumors, a feasible goal of the near future, especially in the rising era of genome editing and personalized therapeutic approaches.

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